Original Article

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Efficient Generation of Bone Marrow Derived Murine Dendritic Cells: A Protocol Optimization Study

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ABSTRACT

Background: Dendritic cells (DCs) are the most potent antigen-presenting cells, playing a central role in the activation of T cells and the induction of B cell antibody production. Owing to their critical immunological functions, DCs have been extensively investigated for applications in cancer immunotherapy, dendritic cell–based vaccines, and therapeutic strategies aimed at overcoming the immunosuppressive tumor microenvironment.

Objects: To optimize key parameters influencing the generation of bone marrow-derived dendritic cells (BMDCs).

Methods: Variables including mouse strain, cytokine concentrations, culture plate type, duration of differentiation, incubation temperature, initial cell seeding density, and media replacement intervals were evaluated. CD11c expression was used as the primary marker for quantification of BMDCs, while CD80 and CD86 expression served as indicators of dendritic cell maturation status.

Results: The optimized protocol generated 60-70% CD11c$^+$ BMDCs with 70-80% CD80/CD86 expression within 5 days.

Conclusion: This robust and highly reproducible protocol provides a valuable platform for in vitro studies involving dendritic cells.

Keywords: Bone marrow-derived dendritic cells; Cell isolation protocols; CD11c; Cell differentiation; Granulocyte macrophage stimulating factor

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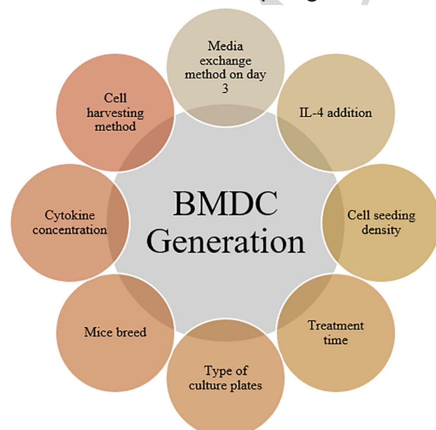

Graphical Abstract

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INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APCs) of the mononuclear phagocyte system, first described by Steinman and Cohn in 1973, and are essential for the initiation of adaptive immune responses through the activation of T and B cell (1, 2). Derived from hematopoietic stem cells (HSCs) in the bone marrow, they comprise conventional DCs (cDC), including cDC1 ($CD8α^+/CD103^+$) and cDC2 ($CD11b^+$), as well as non-conventional subsets such as monocyte-derived DCs (moDCs), plasmacytoid DCs (pDCs), and Langerhans cells (1, 2). Functionally, cDC2s are primarily involved in the activation of $CD4^+$ T cell and the polarization of Th2 and Th17 responses (3), whereas pDCs produce IFN-α in response to TLR7 and TLR9 signaling (4). Langerhans cells play a key role in the induction of cutaneous immune responses (5). Immature DCs characteristically express CD206, CD205, and CD209, while maturation is associated with the upregulation of co-stimulatory molecules including CD40, CD80, CD83, CD86, and the production of cytokines such as IL-12 (6). Although CD11c and MHC-II are commonly used as canonical DC markers, they can also be expressed by macrophages (2, 7, 8). Under the influence of cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4, monocytes can differentiate into DC-like cells in vitro (9, 10).

Since DCs are the most potent APCs, they have broad applications in immunotherapy, including tumor immunotherapy and dendritic cell-based vaccine development. DC-centered immunotherapies aim to enhance DC function, increase DC numbers, overcome immunosuppression within the tumor microenvironment, and modulate immune responses (11). In DC vaccines approaches, monocyte-derived DCs (moDCs) are typically generated from peripheral blood mononuclear cells and matured ex vivo using tumor lysates, antigens, or cytokine cocktails to induce robust tumor antigen–specific T cell responses. These vaccines are generally well tolerated; for example, Sipuleucel-T (Provenge), an FDA-approved DC vaccine for prostate cancer, involves culturing autologous peripheral blood mononuclear cells (PBMCs) with a prostatic acid phosphatase–GM-CSF fusion protein to promote antigen presentation and antigen-specific T cell activation (11, 12).

DC development is primarily regulated by Fms-like tyrosine kinase 3 (Flt3) ligand and GM-CSF (2). Flt3 signaling drives differentiation of hematopoietic progenitors into pDCs and cDCs, a pathway that is largely distinct from monocyte and granulocyte lineages (13). In contrast, GM-CSF promotes the generation of cDC from myeloid progenitors, particularly in peripheral non-lymphoid tissues (14, 15) and is widely employed in DC vaccine platforms as well as the in vitro production of BMDC (10, 16).

Despite the availability of multiple protocols for generating BMDCs, the growing demand for DC-based applications underscores the need for improved methodologies. Major challenges in BMDC production include cellular heterogeneity, inconsistent yields, variable antigen-presenting functionality (17-19), and relatively prolonged differentiation periods of approximately eight days. These limitations have driven efforts toward protocol optimization. For example, Wang et al. evaluated the combined use of GM-CSF and IL-4 and assessed subsequent T cell proliferation following BMDC generation (10), whereas Sauter et al. systematically examined diverse factors influencing BMDC production, including seeding density, culture duration, and cell harvesting methods (16). Nevertheless, further refinement of BMDC generation protocols remains essential to address these challenges and improve reproducibility and efficiency. In this study, multiple parameters were systematically evaluated to optimize and standardize BMDC production, including mouse strain, cytokine concentrations, type of culture plate, differentiation duration, cell seeding

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density, cell harvesting strategy, and media exchange procedures. This comprehensive approach incorporates all previously reported factors influencing BMDC generation to establish a coherent and optimized protocol. Key outcome measures included total bone marrow–derived cell yield, the proportion of $CD11c^+$ cell assessed by flow cytometry, CD80/CD86 expression within the $CD11c^+$ population, and cellular morphology.

MATERIALS AND METHODS

Chemicals and Materials

The PE anti-mouse CD11c antibody was purchased from BioLegend-USA. IL-4 and GM-CSF were obtained from PeproTech-USA. The FITC anti-mouse CD80 antibody and PE-Cy5 anti-mouse CD86 antibody were purchased from eBioscience-USA. RPMI-1640 media and Pen/Strep antibiotic solution were purchased from BioIdea-Iran. Fetal bovine serum (FBS) was obtained from ThermoFisherScientific-USA. 6- Non-pyrogenic cell culture plates (plasma treated and non-treated) were provided by Jet Biofil-China. 2-mercaptoethanol (2-ME), ammonium chloride, disodium EDTA, and potassium bicaronate were purchased from Merck-Germany. BALB/c and C57BL/6 mice were purchased from the animal facilities of Tarbiat Modares University and Tehran University, respectively. All animal procedures were conducted in accordance with the ethical standards and were approved by Research Ethics Committee of Tarbiat Modares University under approval number IR.MODARES.REC.1400.265.

Bone Marrow Flushing and Cell Isolation

The general procedure for BMDC preparation involved euthanizing female BALB/c or C57BL/6 mice (6 weeks old) using inhaled anesthetics. The femur and tibia were then cut from the hip joint to the paws. Muscles and tissues surrounding the femur/tibia were carefully removed (Fig. 1A) in a manners that preserved bones and epiphyses. The clean bones were first immersed in 70% ethanol, PBS, and RPMI-1640, each for two minutes.

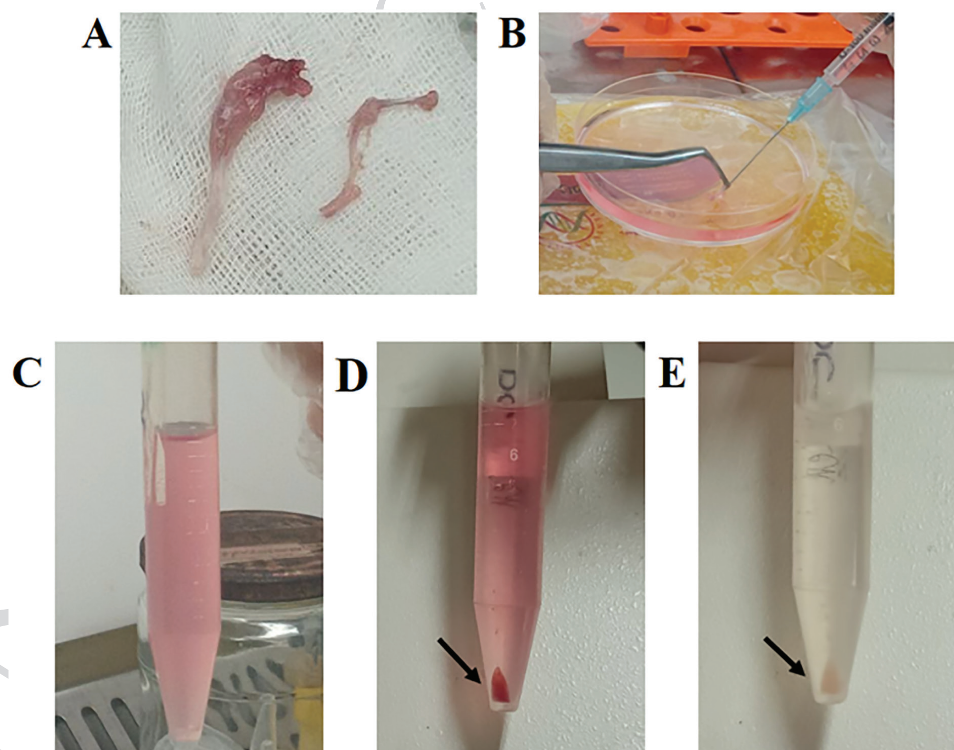

Fig. 1. General procedure for BMDC preparation: A) Tibia and femur before (left) and after (right) muscle removal; B) Bone marrow flushing; C) Suspended bone marrow cells; D) Same sample after centrifugation (pellet indicated by black arrow); E) Same sample following RBC lysis (white pellet indicated by black arrow).

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Next, the bones were placed under sterile condition, inside a petri dish containing RPMI-1640 with 10% FBS and 1% Pen/Strep. After dissecting of the femur and tibia from the knee joint, the epiphyses were removed from both ends, and the bone marrow was flushed out using RPMI-1640 medium with a 23-gauge insulin syringe (Fig. 1B). This process continued until the red color seen in the bone cavity turned white. The bone marrow obtained was pipetted gently to separate the cells from the clustered bone marrow. The cells were then transferred to a 15 mL falcon tube (Fig. 1C) and centrifuged (213×g, 7 min, 4 °C). The cell pellet was resuspended in 1 mL of ice-cold ACK lysis buffer. After a 1-minute incubation on ice, 5 mL of PBS was added. The suspension was then centrifuged at 213×g for 7 minutes at 4 °C. A visible change in pellet color from red to white indicated successful red blood cell (RBC) lysis. (Fig. 1D&E). The supernatant was then removed and the cells were suspended in 1 mL RPMI-1640 supplemented with 10% FBS, 1% Pen/Strep, and BMDC differentiating cytokines (GM-CSF and IL-4). Finally, the cells were counted using a Neubauer chamber and seeded for further processing.

Production of BMDCs from Bone Marrow Progenitors

The isolated cells were seeded in RPMI-1640 medium supplemented with 10% FBS, 1% Pen/Strep, 50 µM 2-ME, GM-CSF, and IL-4 (referred to as the complete medium). 2-ME should be added before use to reduce toxic levels of oxygen radicals (20) and improve murine immune responses (21). Cells were incubated at 37 °C and 5% CO_2 for 5-8 days. The medium was changed on the third day after seeding. Throughout the differentiation process, cells should be kept in a stable incubator without any harsh physical vibrations. The criteria and parameters studied were as follows:

A) Bone marrow preparation temperature:

The separation of the femur and tibia, bone marrow flushing, and removal of red blood cells (RBCs) were performed under two temperature conditions: ambient and on ice. To maintain a cold environment, media and buffers were stored on ice, and petri dishes were placed on sterile ice packs (22).

B) Procedure for media exchange on day 3:

BALB/c mouse bone marrow-derived cells were seeded at a density of $8×10^5$ cells/well in a 6-well non-treated plate. The cells were cultured in complete medium (2 mL/well) supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4. After three days of seeding, three different procedures for media exchange were tested:

i: Cells in each well were gently pipetted several times, then 1 mL of medium was transferred to a new well and 1 mL of complete media was added. The cells were then incubated for two more days (until day 5) (23).

ii: 1 mL of medium was removed from each well, centrifuged at 213×g for 7 min and the sedimented cells were suspended in 2 mL complete medium and returned to their original wells and incubated for 2 more days (24).

iii: 800 µL of medium was gently removed from the surface of each well without pipetting. Then, 2 mL of complete medium was added to each well and incubated for 2 more days (25).

C) IL-4 addition:

$8×10^5$ cells/well (from BALB/c mouse) were seeded in 6-well non-treated plates in complete medium (2 mL/well) supplemented with 20 ng/mL GM-CSF and the presence and absence of IL-4 were examined (0 and 10 ng/mL). After three days of incubation, cells in each well were gently pipetted several times, and 1 mL of the suspended cells was transferred to a new well. Then,1 mL of complete medium was added to this new well. The cells were then incubated for an additional two days (method i).

D) Cell seeding density:

Cells from BALB/c mice at different densities ($4×10^5$, $6×10^5$, and $8×10^5$) were seeded in 6-well non-treated plates in complete

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medium (2 mL/well) supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4. After three days, 1 mL of medium was removed from each well, centrifuged at 213×g for 7 min and the cells pellet was suspended in 2 mL of complete medium. The cell suspension was then returned to the original wells and incubated for an additional 2 days (method ii).

E) Treatment time:

4×10^5 cells/well (from BALB/c mice) were seeded in 6-well non-treated plates in complete medium (2 mL/well) supplemented with 20 ng/mL GM-CSF and10 ng/mL IL-4, and incubated for either 5 or 8 days:

1) 5-days test: After three days, 1 mL of medium was removed from each well, centrifuged at 213×g for 7 min and the sedimented cells were suspended in 2 mL completed medium, returned to the previous wells and incubated until day 5 (method ii).

2) 8-days test: On day 3, 1 mL of fresh complete medium was added to each well. On day 6, 1.5 mL of media was removed from each well, centrifuged at 213×g for 7 min and the sedimented cells were suspended in 1.5 mL complete medium. The cells were then returned to their respective wells and incubated until day 8.

F) Type of culture plates:

In this experiment, 4×10^5 cells/well obtained from BALB/c mice were seeded onto a 12-well non-treated plate and a 12-well treated plate in complete medium (1 mL/well) supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4. After three days, 400 µL of medium was gently removed from the surface without disturbing the cells. Then, 1 mL of complete medium was added to each well and incubated for 2 more days (method iii).

G) Mice breeds:

Two mice breeds (BALB/c and C57BL/6) were sacrificed and their bone marrow-derived cells were seeded onto 12-well treated plates (4×10^5 cells/well). The cells were cultured in complete medium (1 mL/well) supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4. On day 3, 400 µL of medium was gently removed from the surface of each well (without pipetting). Subsequently, 1 mL of complete medium was added to each well and incubated for an additional 2 days (method iii).

H) Cytokine concentrations:

Cells obtained from C57BL/6 mice were seeded in 12-well treated plates (4×10^5 cells/well). Cells were cultured in complete medium (1 mL/well) supplemented with two concentrations of cytokines (20 ng/mL GM-CSF and 10 ng/mL IL-4 or 40 ng/mL GM-CSF and 20 ng/mL IL-4). After three days, 400 µL of medium was gently removed from the surface of each well (without pipetting). Subsequently, 1 mL of complete medium was added to each well and incubated for an additional 2 days (method iii).

I) Cell harvesting method on day 5:

Cells obtained from C57BL/6 mice were seeded in 12-well treated plates (4×10^5 cells/well). Cells were cultured in complete medium (1 mL/well) supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4. On day 3, 400 µL of medium was gently removed from the surface of each well (without pipetting). Then, 1 mL of complete medium was added to each well and incubated for an additional 2 days (method iii). On day 5, one group of cells was collected by physical harvesting method (repeated pipetting) due to their semi-adherent nature, while the other group of cells was collected using the enzymatic harvesting method (by trypsin). In previous experiments, the physical harvesting method was used.

Evaluation and Characterization of BMDCs

After treating the cells with GM-CSF and IL-4, they were detached from the culture plate by gently pipetting several times. The cells were then separated by centrifugation at 213×g, 4 °C for 7 min, and washed twice with 500 µL of PBS. Next, the cells were suspended in 100 µL of PBS containing 3% FBS and 1.5 µL of each antibody (CD11c, CD80, and CD86 antibodies) and incubated at 4 °C in the dark. After 1 hour, the cells were centrifuged (213×g, 7 min, 4 °C) and washed twice with 500 µL of PBS. Finally, the cells

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were suspended in 100 μL of PBS containing 3% FBS and analyzed using a flow cytometer.

Flow Cytometry and Gating Strategy

Flow cytometry was performed using a BD FACSCalibur flow cytometer (BD Biosciences, USA), and data were analyzed with FlowJo v10 software. Monocyte populations were initially gated based on forward scatter (FSC) and side scatter (SSC) parameters (26). Cells within the monocyte gate were further analyzed using an SSC versus CD11c (PE, FL2-H) plot to identify differentiated BMDCs. The CD11c$^+$ population was subsequently assessed for the expression of CD80 (FITC, FL1-H) and CD86 (PE-Cy5, FL3-H) along with CD11c (FL2-H) to determine activation status. A minimum of 150,000 events were acquired per sample. The mean percentage of BMDCs across experimental replicates was reported, and representative flow cytometry plots were shown.

Statistical Analysis

All experiments in this study were conducted at least twice. The one-way ANOVA method was employed to determine the significance of various parameters ($P<0.05$: *, 0.01: **, and $P<0.001$: ***), followed by Tukey's post hoc test. Statistical analyses and data visualization were performed using FlowJo v10 and Origin Pro 2022 softwares.

RESULTS

Factors Influencing BMDCs Production

A) Bone marrow preparation temperature

Processing bone marrow under ambient conditions yielded approximately 5×10^6 to 7×10^6 cells per mouse, whereas preparation under cold conditions resulted in significantly higher yields (25×10^6 to 29×10^6 cells per mouse; $p<0.001$) (Fig. 4A). No significant difference bone marrow cell yield was observed between BALB/c and C57BL/6 mice of the same age. Based on these findings, all subsequent experiments were performed under cold conditions.

B) Media change conditions on day 3

As shown in Fig. 2, CD11c$^+$ BMDC yields

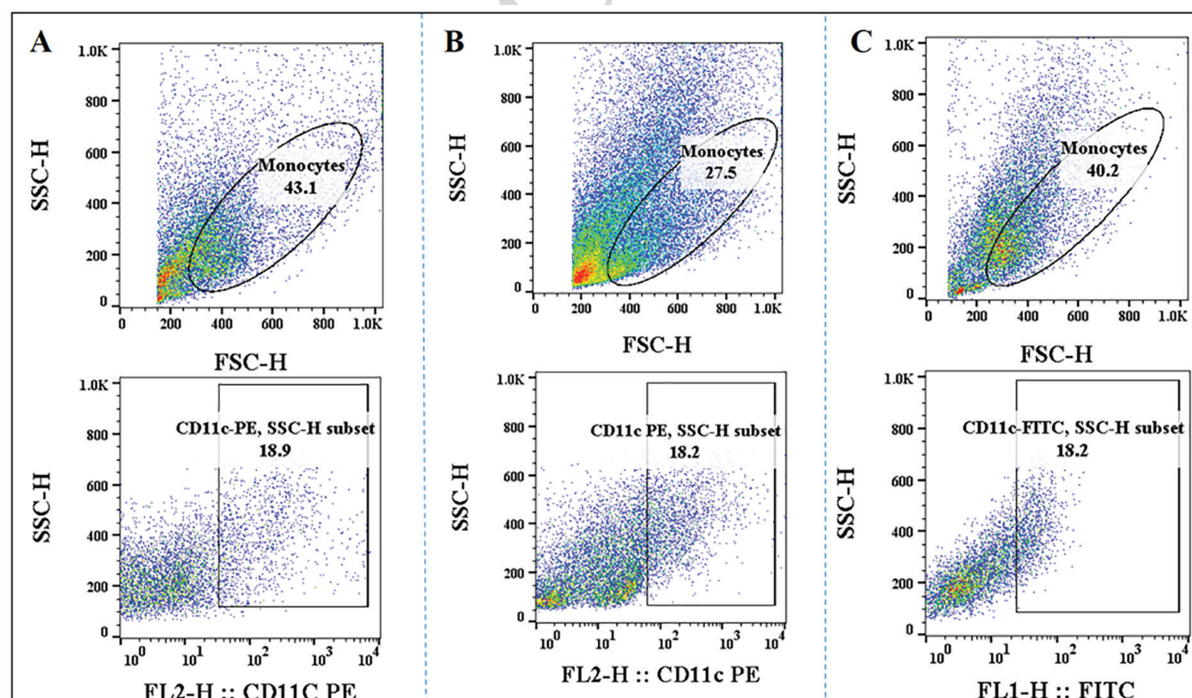

Fig. 2. CD11c$^+$ BMDC yield following different medium change strategies on day 3: A) Transfer of cells to a new well containing fresh medium B) Partial medium replacement with fresh medium C) Centrifugation followed by reintroduction of the cells into the original wells with fresh medium. A total of 8×10^5 cells/well were cultured in a 6-well non-treated plate in complete medium supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4

obtained using three different media change methods—(i) transferring cells to a new well with fresh medium, (ii) centrifugation followed by returning the cells to the original wells with fresh medium, and (iii) partial medium replacement—were 19.6%±0.7, 18.7%±0.5, and 14.35%±3.85, respectively (Fig. 4B). These results indicate that there were no statistically significant differences among the methods (P<0.05). Although the procedures were adapted from previous studies, certain modifications were introduced to improve both the efficiency and user-friendliness of the final protocol.

C) IL-4 addition

Showing in Fig. 3, the addition of IL-4 to the differentiating medium did not significantly affect the CD11c$^+$ BMDC production (P>0.05; 22.1%±0.5 vs. 19.6%±0.7) (Fig. 4C). For further optimization, subsequent experiments were performed using IL-4 in the culture medium.

D) Cell seeding density

As shown in Fig. 5, CD11c$^+$ BMDC yields obtained after seeding of 4×10^5, 6×10^5, and 8×10^5 cells per well in 6-well non-treated plates were 20.45%±0.15, 19.35%±0.15, and 18.7%±0.5, respectively (Fig. 8A). These results indicate that CD11c$^+$ BMDC yield was not significantly affected by cell density within this range (P<0.05). Therefore, subsequent experiments were performed using a seeding density of 4×10^5 cells/well.

E) Time of treatment

As shown in Fig. 6, the yield of CD11c$^+$ BMDC following treatment with GM-CSF and IL-4 for 5 and 8 days was 20.45%±0.15 and 48.25%±0.05, respectively, indicating that prolonged incubation significantly enhances CD11c$^+$ BMDC generation. One-way ANOVA revealed a statistically significant difference between these groups (P<0.001; Fig. 8B). Although extended incubation increased the CD11c$^+$ cell population, additional optimization experiments were conducted to achieve comparable yields within a shorter incubation period, consistent with the objectives of this study.

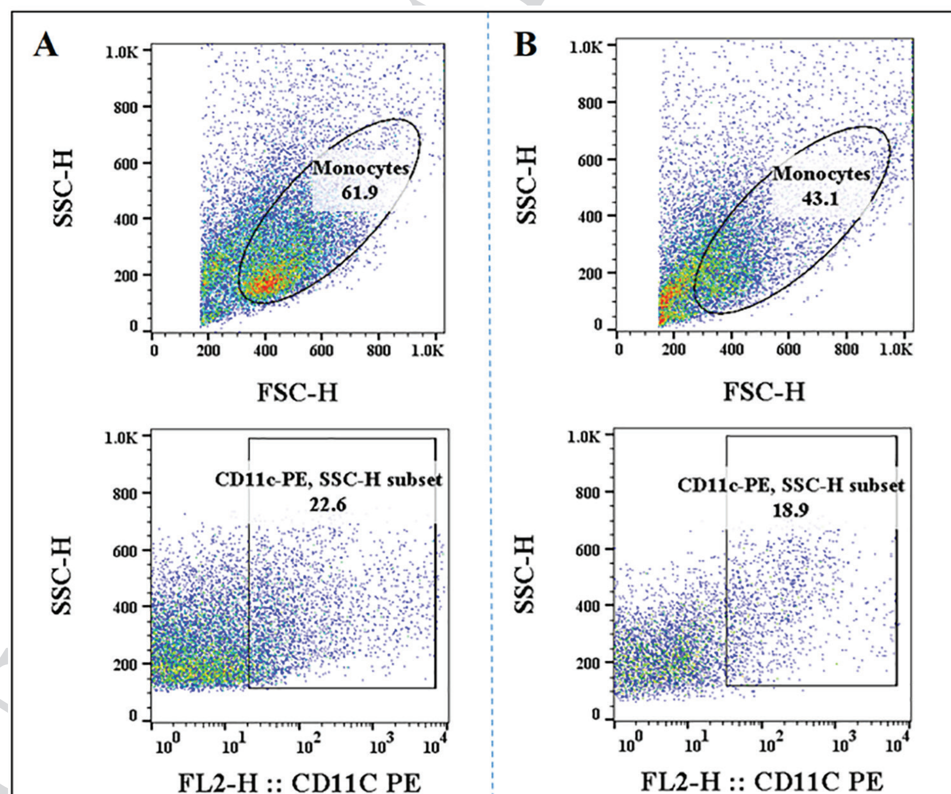

Fig. 3. CD11c$^+$ BMDC yields obtained using A) 0 ng/mL and B) 10 ng/mL IL-4 in the culturing medium. A total of 8×10^5 cells/well (derived from BALB/c mice) were seeded in 6-well non-treated plates containing complete medium supplemented with 20 ng/mL GM-CSF

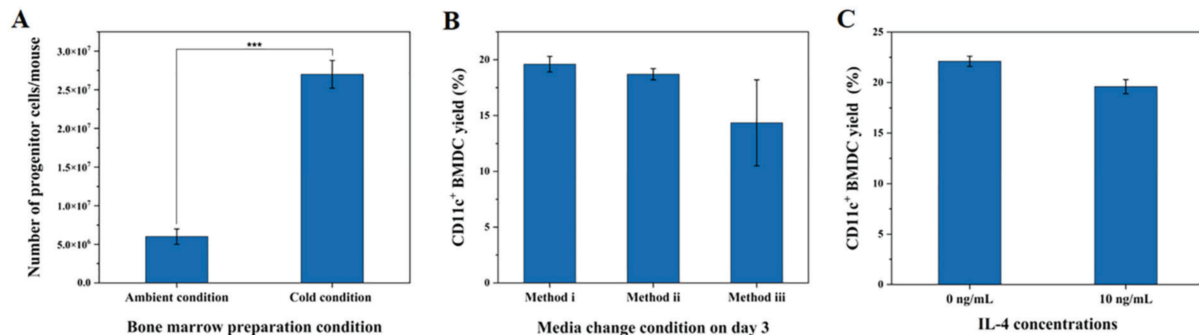

Fig. 4. The results of the optimization of A) bone marrow preparation conditions, B) medium change conditions on day 3, and **C)** IL-4 concentrations for the production of CD11c+ BMDCs.

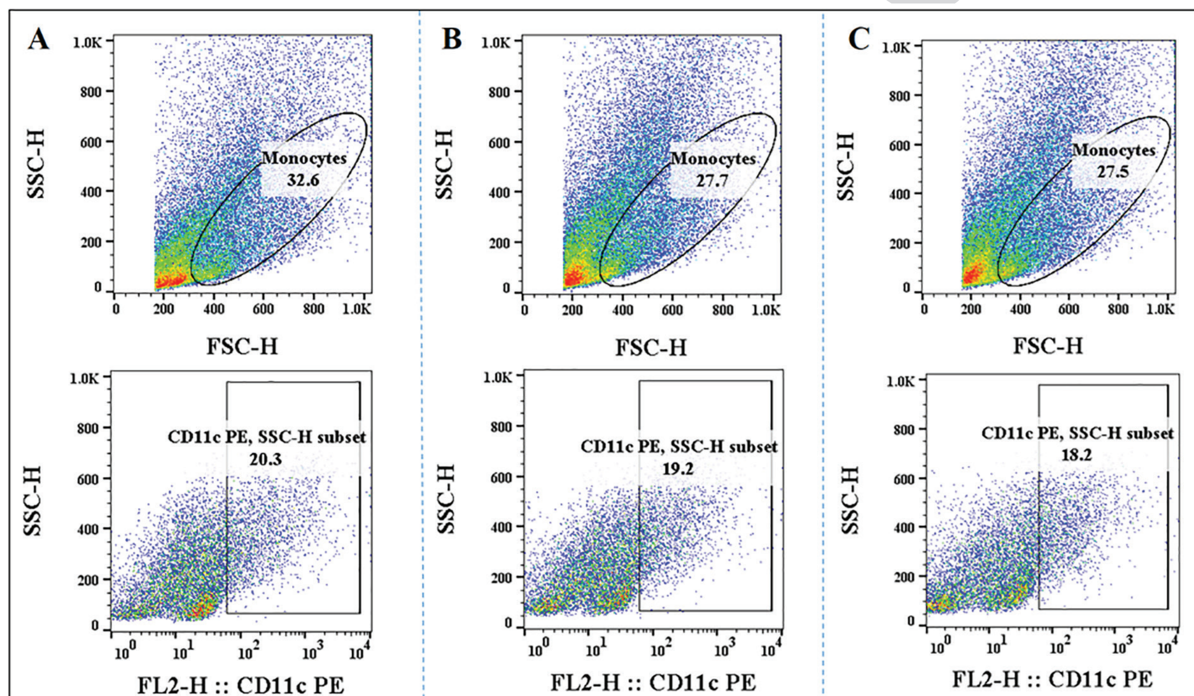

Fig. 5. CD11c+ BMDCs derived from BALB/c mice at different densities. Cells were seeded in 6-well non-treated plates (2 mL/well) containing complete medium supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4 at the following densities: A) $4×10^5$ cells/well B) $6×10^5$ cells/well C) $8×10^5$ cells/well.

F) Cell culture plates

Culturing BMDCs in treated versus non-treated plates (Fig. 7) showed a modest increase in the differentiation rate of CD11c+ BMDCs in treated plates (21.3%±5.3) compared with non-treated ones (14.35%±3.85). However, this difference was not statistically significant ($P>0.05$; Fig. 8C). Despite the lack of statistical significance, treated plates were preferred during washing and cell-harvesting steps, as they facilitated clearer discrimination between tightly adherent cells, typically macrophages, and loosely adherent BMDCs.

G) Mice breeds

Fig. 9 shows that mouse strain significantly influences CD11c+ BMDC production. The yield of CD11c+ BMDC from BALB/c mice was 21.3%±5.3, whereas C57BL/6 mice yielded 67.44%±6.2 a difference that was statistically significant ($P<0.001$; Fig. 12A). Consequently, all subsequent experiments were conducted using C57BL/6 mice.

H) Cytokine concentrations

Doubling the concentrations of GM-CSF and IL-4 significantly decreased the yield of CD11c+ BMDCs ($P<0.01$), from 67.44%±6.2 to 43%±4% (Fig. 10 & Fig. 12B). This indicates that lower cytokine concentrations (20 ng/mL GM-CSF and 10 ng/mL IL-4) are more favorable for BMDC generation.

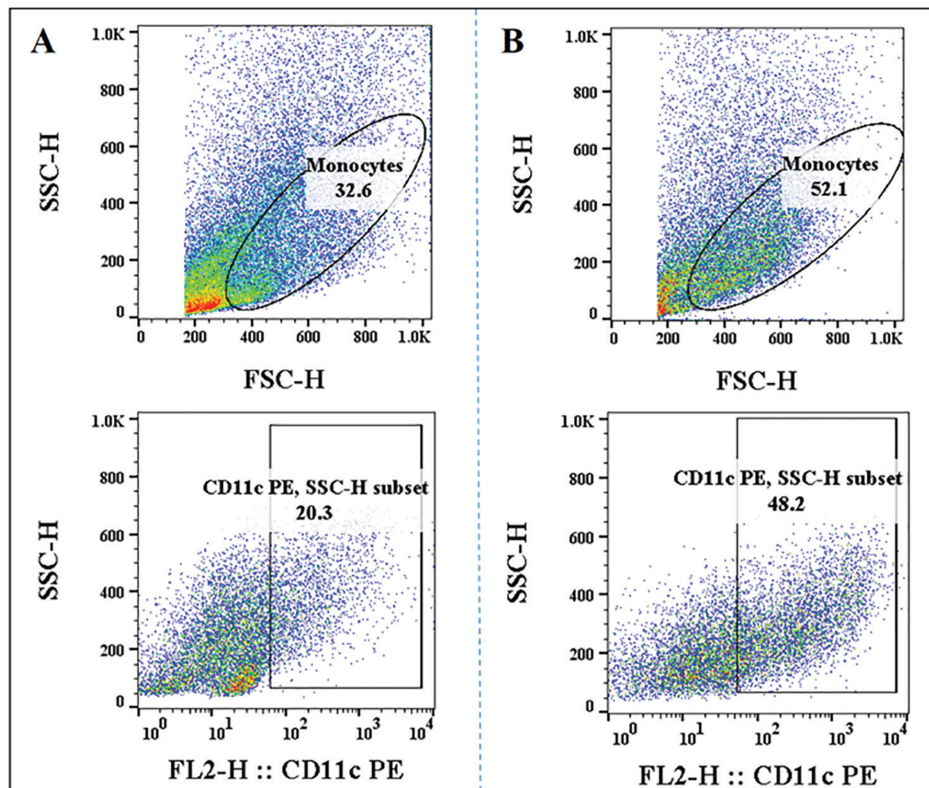

Fig. 6. CD11c+ BMDC yields after incubation for A) 5 days and B) 8 days in complete medium supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4. Bone marrow cells ($4×10^5$ cells/well) isolated from BALB/c mice were seeded in non-treated 6-well plates.

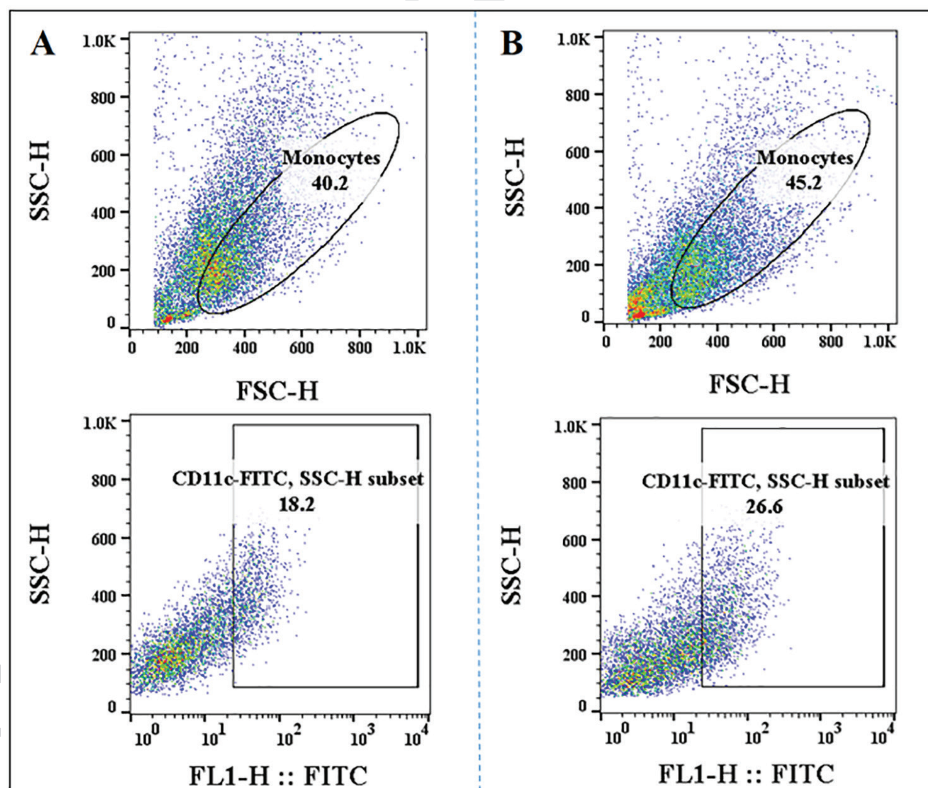

Fig. 7. CD11c+ BMDC yields obtained using A) non-treated polystyrene plates and B) tissue culture (TC)-treated polystyrene plates. Bone marrow cells ($4×10^5$ cells/well) isolated from BALB/c mice were seeded into 12-well non-treated plate or 12-well TC-treated (plasma-treated to enhance cell and tissue attachment) plates in complete medium (1 mL/well) supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4.

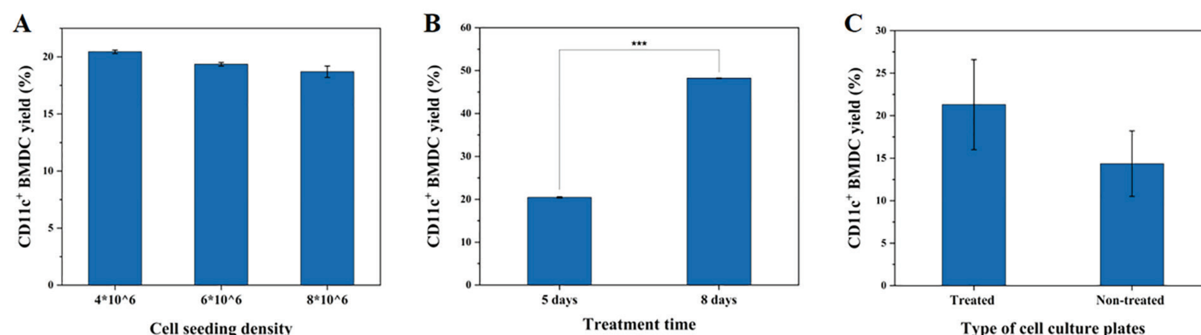

Fig. 8. Optimization of A) cell seeding density, B) treatment duration, and C) the type of cell culture plate on the production of CD11c+ BMDCs.

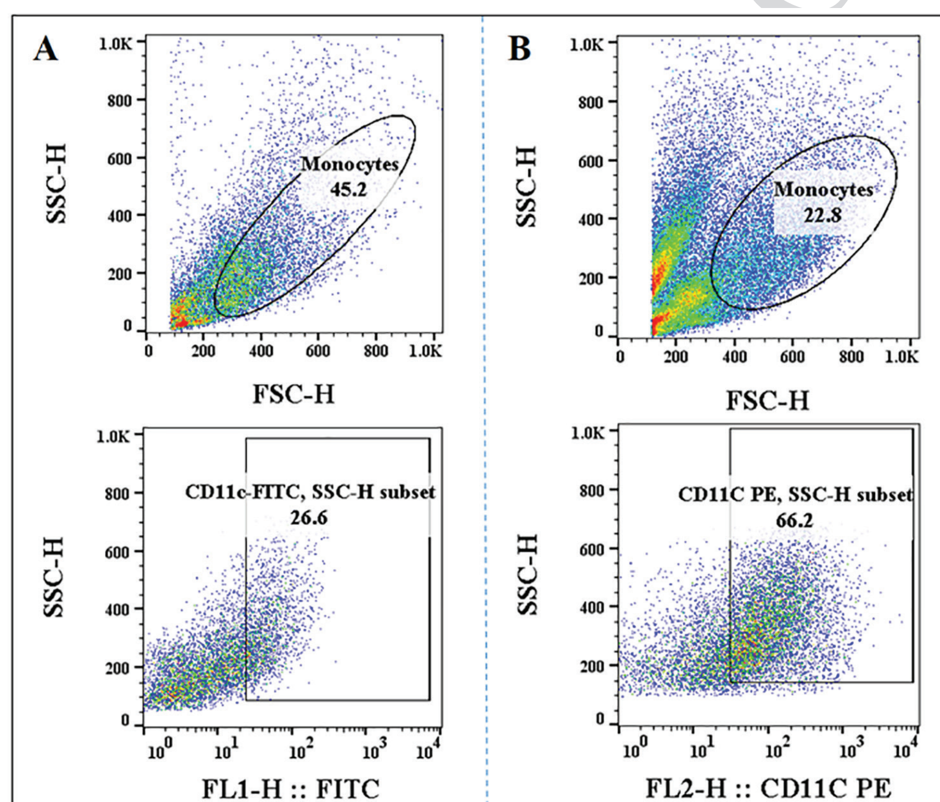

Fig. 9. CD11c+ BMDC yields obtained using the same procedure from A) BALB/c mice and B) C57BL/6 mice. Bone marrow cells (4×105 cells/well) were seeded in 12-well treated plates and cultured in complete medium supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4, using method (iii) on day 3.

I) Cell harvesting strategy

As shown in Fig. 11, detachment of cells using either an enzymatic method (trypsin) or a physical method (pipetting) yielded 69.75%±1.95 and 67.44%±6.2 CD11c+ BMDCs, respectively, indicating consistently high BMDC recovery (Fig. 12C). The difference between the two methods was not statistically significant (P>0.05). Subsequent experiments employed the pipetting method, which preferentially collects loosely adherent BMDCs, while leaving tightly adherent macrophages attached to plate.

Final Optimized Protocol for BMDC Production

The final optimized protocol involved euthanizing a 6-week-old C57BL/6 mouse, and flushing bone marrow under cold conditions. Red blood cells were lysed using ACK buffer, and 4×10^5 cells were seeded per well (1 mL in 12-well treated plates) in RPMI-1640 medium supplemented with 10% FBS, 1% Pen/Strep, 20 ng/mL GM-CSF, 10 ng/mL IL-4, and 50 µM freshly added 2-mercaptoethanol (2-ME).

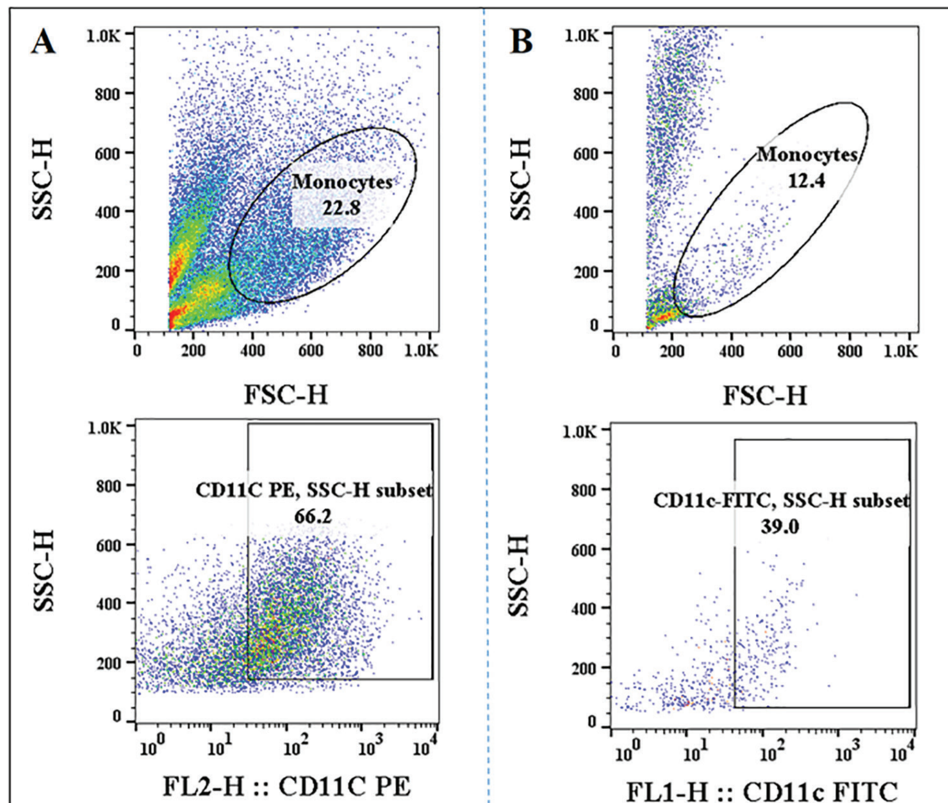

Fig. 10. CD11c⁺ BMDC yields cultured in complete medium supplemented with: A) 20 ng/mL GM-CSF and 10 ng/mL IL-4, or B) 40 ng/mL GM-CSF and 20 ng/mL IL-4. Cells were derived from C57BL/6 mice and seeded in 12-well treated plates at $4×10^5$ cells per well.

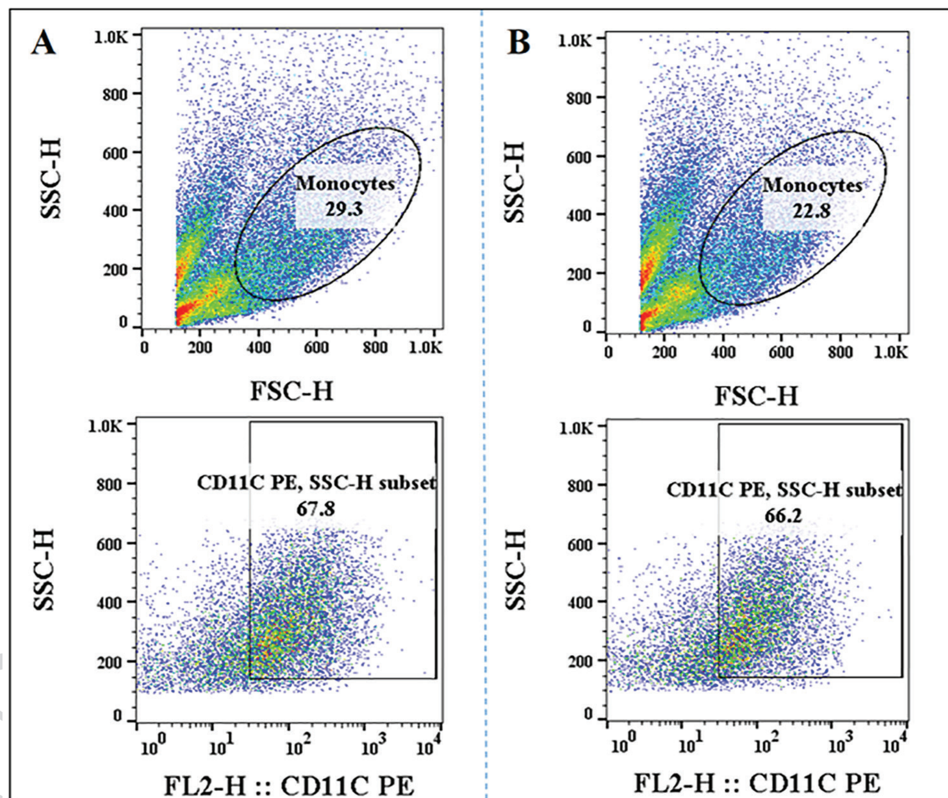

Fig. 11. CD11c⁺ BMDC yields after cell detachment using A) enzymatic harvesting (trypsin) or B) physical harvesting (pipetting). Cells were derived from C57BL/6 mice and seeded in 12-well treated plates ($4×10^5$ cells/well) in complete medium supplemented with 20 ng/mL GM-CSF and 10 ng/mL IL-4

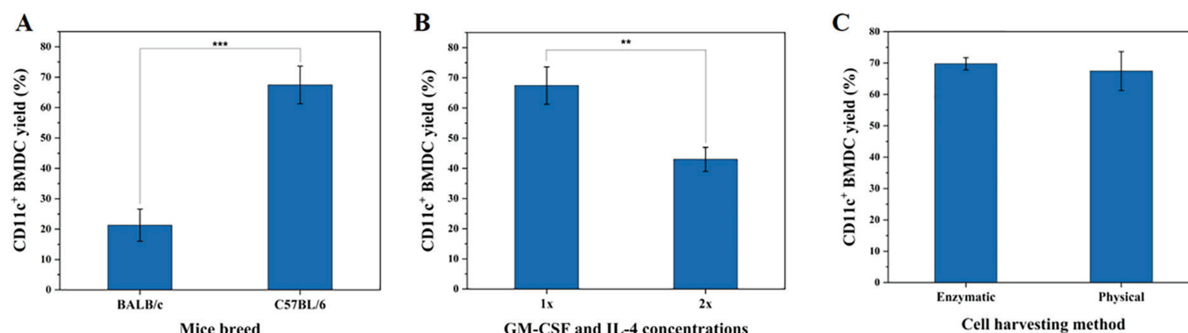

Fig. 12. Effects of optimization on CD11c$^+$ BMDC production: A) mouse strain, B) cytokine concentrations (x: 20 ng/mL GM-CSF and 10 ng/mL IL-4), and C) cell harvesting method

On day 3, 400 μL of medium was removed and replaced with 1 mL of fresh complete medium, followed by incubation for an additional two days (method iii). On day 5, cells were gently detached by pipetting and analyzed by flow cytometry (Fig. 13). CD11c expression was used to quantify BMDC yield, while CD80 and CD86 expression levels were measured to assess the maturation status of the generated BMDCs.

The final optimized protocol yielded 67.44%±6.2 CD11c$^+$ BMDCs within 5 days. As shown in Fig. 14A, mature BMDCs expressed high levels of CD80 and CD86 confirming their activation as APCs, with 73.7% CD11c$^+$/CD80$^+$ and 75.6% CD11c$^+$/CD86$^+$ populations. Fig. 14B shows that BMDCs exhibited the characteristic stellate morphology with loosely adherent filopodia, representing a heterogeneous population of

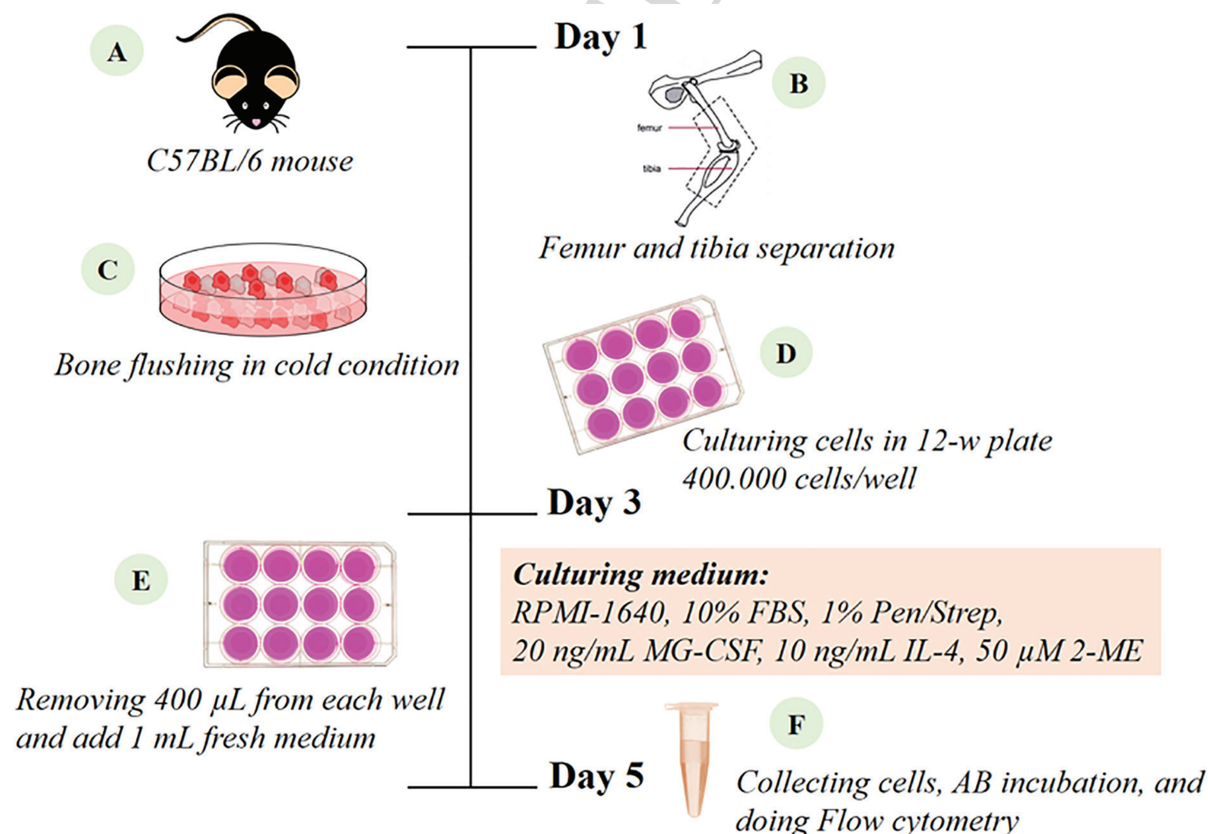

Fig. 13. Optimized BMDC production procedure: A) euthanasia of a 6-weeks old C57BL/6 mouse, B) isolation of femur and tibia, C) bone marrow flushing under cold conditions, D) seeding 4×10^5 cells per well in 12-well treated plates for three days, E) partial medium replacement (400 μL removed, 1 mL fresh medium added)followed by two more days of incubation, and F) cell detachment by pipetting on day 5 days and analysis by flow cytometry.

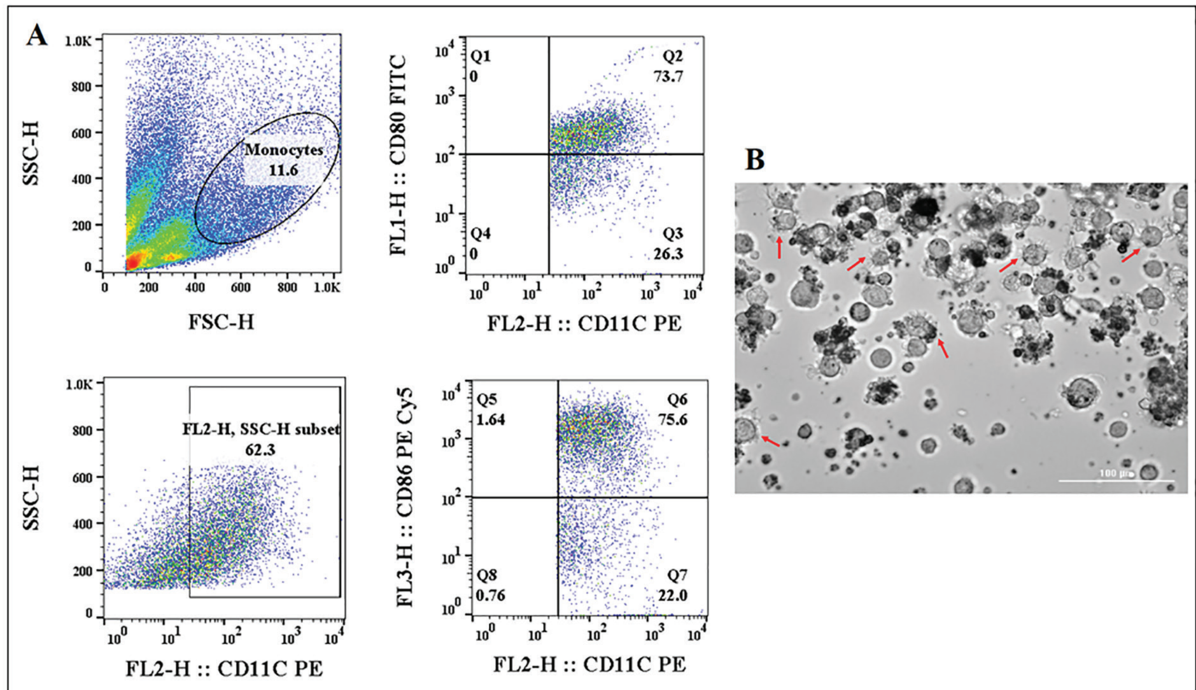

Fig. 14. A) Proportions of CD11c⁺/CD80⁺ and CD11c⁺/CD86⁺ in matured BMDCs generated using the optimized protocol, B) Stellate morphology of BMDCs after five days, indicated by red arrows (20x magnification).

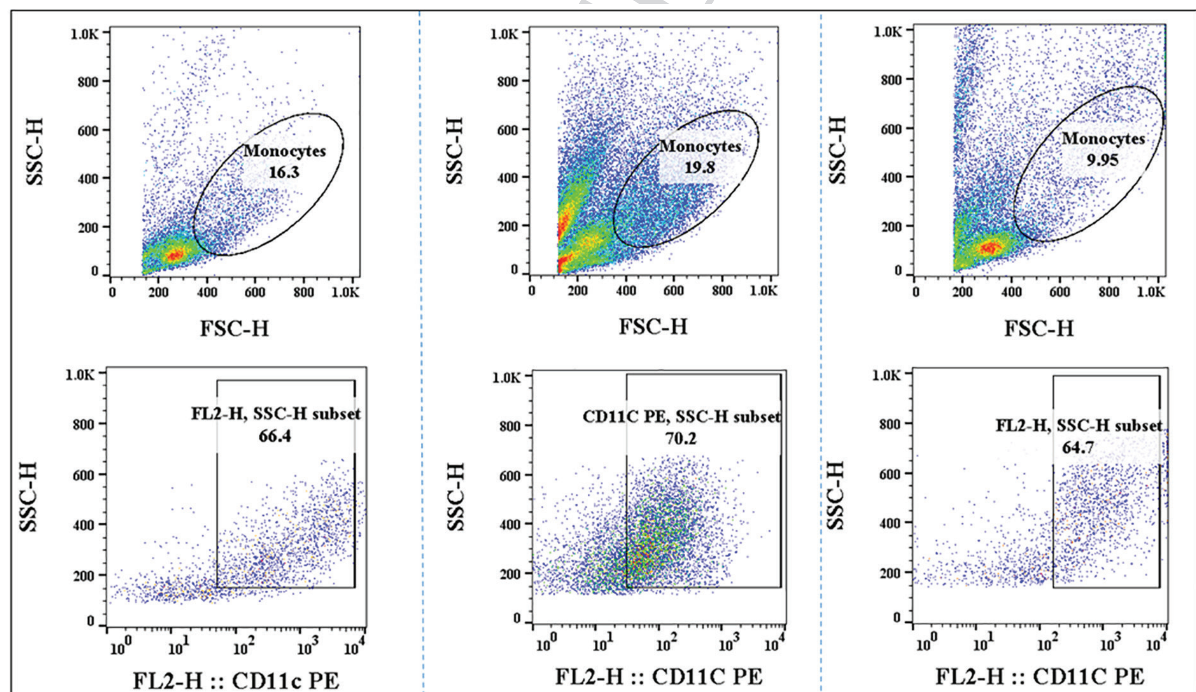

Fig. 15. The optimized BMDC production protocol consistently yielded 60–70% CD11c⁺ BMDCs. Representative data from three independent experiments (selected from over ten replicates) are shown to demonstrate reproducibility.

non-adherent and loosely adherent cells. This protocol demonstrated strong reproducibility (Fig. 15), and is suitable for in vitro DC-based studies. Notably, achieving 60–70% BMDCs in just 5 days represents a significant improvement over previous methods, which often required 10–12 days to reach similar yields. A summary of key parameters and corresponding BMDC yields is provided in Table.S1.

Future Directions

Despite the fact that most developed vaccines are based on neutralizing antibody production, cancer vaccines and vaccines against HIV, parasites, and tuberculosis require cytotoxic T cell (CTL) responses. DCs are the most important APCs that mediate CTL responses highlighting their critical role in future DC-based vaccine development (27). BMDCs can be used as the in vitro models for DC-based vaccine development (27), preparing neoantigen-reactive T cells (28), studying airway inflammatory cell infiltration (29) and conducting T cell priming studies (30). Additionally, BMDCs have been utilized in cell-based therapy for treating type 1 diabetes (T1D) (31). The development of reliable and efficient protocols for BMDC production is crucial for advancing both basic immunology and translational medicine. Given the pivotal role of DCs in orchestrating cytotoxic T cell responses, optimized BMDC generation methods are critical for developing next-generation vaccines against a range of currently incurable diseases. Standardized BMDC protocols also provide reproducible in vitro models for studying antigen presentation, T cell priming, and inflammatory cell infiltration, thereby facilitating mechanistic insights into immune regulation. Moreover, BMDCs can be efficiently generated under serum-free and xeno-free conditions using defined media supplemented with appropriate cytokine. These methods meet clinical standards, and pave the way for translational applications in DC-based vaccines and immunotherapies. However, BMDC production under serum-free or xeno-free conditions presents several challenges, including reduced cell yields, lower viability, and altered differentiation or maturation compared to serum-containing systems. Such cultures are highly sensitive to cytokine concentrations and media handling, making reproducibility more difficult, while the high cost of defined media limits scalability. Furthermore, most optimized serum-free/xeno-free protocols have been developed for human monocyte-derived DCs, with relatively few studies addressing murine BMDCs (32, 33).

DISCUSSION

DCs are the most critical APCs, owing to their unique ability to prime naïve T cells and stimulate antibody production by B cells. They play key roles in the differentiation and regulation of Th1, Th2, Th17, and Treg pathways. Serving as a central hub in the immune system, DCs form a crucial bridge between innate and adaptive immunity (1). Their capacity for targeted immune activation makes them valuable in cancer immunotherapy and vaccine development (34).

DC-based immunotherapies aim to enhance DC function, expand their numbers, and overcome the immunosuppressive tumor microenvironment (11). BMDCs are widely used in immune function assays (18), T cell priming studies (35), DC metabolism research (36), tumor ablation models (37), and in vitro DC maturation experiments (38). Despite the availability of various protocols for BMDC generation, the growing demand for dendritic cell applications highlights the need for more efficient and standardized production methods. Major challenges in current BMDC production protocols include population heterogeneity (17), inconsistent yields (19), variability in antigen-presenting capacity (18), and prolonged differentiation periods (10).

Culture conditions significantly influence BMDC functionality. For example, high-density cultures can impair T cell activation and attenuate Th1 responses due to lactic acid accumulation, which suppresses IL-12 and TNF-α while increasing IL-10 expression (39). Additionally, BMDC populations are inherently heterogeneous, exhibiting variability in maturation kinetics and cell adhesiveness, which complicates reproducibility and data interpretation (17, 40). Although BMDCs can be generated rapidly

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at low yields achieving high-yields typically requires prolonged culture periods. Extended culture period increases experimental turnaround time and reduces cost-effectiveness (41). Given that dendritic cells (DCs) constitute less than 1% of peripheral blood leukocytes in both humans and animals, derivation from bone marrow represents a more efficient source; however, this approach substantially limits direct applicability in human clinical studies. Despite these limitations s, murine BMDCs remain widely used in preclinical and in vivo investigations, particularly in the development of DC-based and anticancer vaccine therapeutics (10).

In the present study, multiple factors influencing BMDC production were systematically evaluated and optimized. The results showed that higher yields of bone marrow–derived progenitor cells was obtained under cold conditions compared with ambient temperatures. This improvement is likely due to reduced activation, differentiation, metabolic activity, and apoptosis of hematopoietic stem cells (HSCs) and progenitor cells at lower temperatures (22). In vitro BMDC generation typically relies on GM-CSF supplementation (2), and previous studies have demonstrated that the addition of IL-4 enhances antigen-presenting capacity and improves T cell activation (19). IL-4 has also been reported to increase BMDC viability by 10–15% compared s with GM-CSF alone (42). In the present study, inclusion of IL-4 did not significantly alter $CD11c^+$ BMDC yields; however, IL-4 was retained in the optimized protocol to support DC functionality. A seeding density of 4×10^5 cells per well was identified as optimal for BMDC generation, although variations in cell density did not substantially affect overall outcome. In a comparable study, Alotaibi et al. demonstrated that cytokine concentrations had a greater impact than cell density on BMDC quality, activation, and maturation under similar seeding conditions in 6-well plates (42). In the present study, BMDC yield after 8 days of culture was more than twice that obtained after 5 days. Consistently, Wang et al. reported $CD11c^+$ BMDC yields of 21.6% on day 4 and 63.7% on day 8, further highlighting the importance of extended culture duration for BMDC differentiation (10). In the optimized protocol developed in this study, tissue culture–treated plates were used during washing and cell harvesting steps to facilitate discrimination between tightly adherent cells, typically macrophages, and loosely adherent BMDCs (16). Furthermore, mouse strain was identified as a critical determinant of BMDC production, with C57BL/6 mice yielding higher BMDCs numbers than BALB/c mice. Previous studies have reported strain-dependent differences in immunological properties. For example, Koike et al. showed that BMDCs derived from C57BL/6 exhibited superior antigen-presenting capacity compared with those from BALB/c (43); while Pejawar et al. reported increased susceptibility to infection in C57BL/6-derived BMDCs relative to BALB/c-derived cells (44). Moreover, BMDCs derived from C57BL/6 mice have been suggested to promote stronger Th1-type immune response and to express higher levels of MHC class II, CD40, CD80, and CD86 than those derived from BALB/c mice (45).

Na et al. demonstrated that GM-CSF enhances phagocytic capacity and IL-10 secretion in BMDCs in a dose-dependent manner (46). Accordingly, optimizing GM-CSF concentration was essential in the present study to achieve a favorable balance between dendritic cell yield and macrophage contamination. Both physical and enzymatic harvesting methods were evaluated to maximize BMDC yield. Although harvesting method did not significantly affect overall yield, physical harvesting was preferred as it produced a more homogenous BMDC population. Consistent with these findings, Chiang et al., reported no significant differences between enzymatic harvesting (using TrypLE) and physical harvesting (using cold polystyrene) with respect to MHC-I, CD80, CD40, and CD11c expression or

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antigen-presenting capacity of BMDCs (47).

In summary, systematic optimization of key parameters influencing BMDC production, including temperature, mouse strain, cytokine concentrations, culture plate type, differentiation duration, cell seeding density, IL-4 supplementation, harvesting method, and medium exchange trategy, resulted in an optimized protocol yielding 60–70% CD11c+ BMDCs within five days, with 70–80% of mature cells expressing high levels of CD80 and CD86.

CONCLUSION

In the present study, systematic optimization of multiple parameters resulted in a highly reproducible, time- and cost- efficient protocol for murine BMDC production was established. BMDCs were generated from C57BL/6 mouse bone marrow under cold conditions by seeding $4×10^5$ cells/mL in RPMI-1640 medium supplemented with 10% FBS, Pen/Strep, GM-CSF, IL-4, and 2-ME). Medium was refreshed on day 3, and cells harvested on day 5, yielding 60–70% CD11c+ BMDCs. These BMDCs are broadly applicable in immune function assays, T cell priming studies, vaccine development studies, DC metabolism research, tumor ablation models, and cancer immunotherapy experiments.

ETHICAL APPROVAL

This study was conducted in accordance with the ethical approval code IR.MODARES.REC.1400.265 issued by the Research Ethics Committee of Tarbiat Modares University.

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AUTHORS CONTRIBUTION

M. Mousazadeh contributed to methodology development, data analysis, visualization, and manuscript writing and review. M. Nikkhah contributed to supervision, conceptualization, and manuscript writing and review. N. Seyed contributed to conceptualization and manuscript review. S. Rafati and S. Hosseinkhani contributed to project administration, and manuscript review.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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