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# The Effect of Age and Cell Culture Parameters on the Quantity and Function of Bone Marrow-derived Dendritic Cells

**Maryam Golara[1], Mohammad Mehdi Amiri[2], and Seyed Mohammad Moazzeni[1]**

*[1] Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
*[2] Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*

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## ABSTRACT

Dendritic cells (DCs) are a group of bone marrow-derived cells that play a crucial role in innate and acquired immune responses. Bone marrow-derived dendritic cells (BMDC) are used in many studies, so the efficiency and purity of the differentiated cells are essential. This study aimed to investigate the effect of several parameters, including the age of mice, cell culture medium, and swirling of the culture plate, to increase the efficiency of the induced cells, considering the standard protocols.

Bone marrow-derived dendritic cells were induced from both juvenile and adult mice bone marrow cells. Then, the purity of CD11c+ cells was compared between juvenile mice BMDCs and adult mice BMDCs. Cells were cultured in an enriched and non-enriched medium, and some wells were swirled when changing the medium on the 3rd day. Then the effect of enriched medium and swirling before medium replacement were evaluated based on the expression of the CD11c marker.

The efficiency of DCs differentiation ($CD11c^+$ cells) was higher when juvenile mouse bone marrow precursors were used compared to adult mice; using the enriched media with supplements and swirling the well before media replacement significantly affected the purity of immature $CD11c^+$ cells.

Due to our results, using juvenile mice, an enriched culture medium, and physical removal of granulocyte cells could significantly improve the purity and efficiency of $CD11c^+$ cells. Therefore, considering these three items in the production protocol of these cells can probably reduce the use of lymphocyte-removing antibodies and purification methods.

**Keywords:** Bone marrow cells; Cell differentiation; Dendritic cells; Primary cell culture

## INTRODUCTION

Dendritic cells are specialized cells in secondary lymphatic organs and most peripheral tissues plays a crucial role in initiating and directing immune responses.[1] Dendritic cell can be found in non-lymphoid organs such as skin, muscle, lung, kidney, intestine, or liver. These cells can uptake,

**Corresponding Author:** Seyed Mohammad Moazzeni, PhD; Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran Tel: (+98 21) 8288 3846, Fax: (+98 21) 8288 3846, E-mail: moazzeni@modares.ac.ir

process, and present antigens to T lymphocytes and stimulus signals, including membrane molecules and cytokines. After activation, dendritic cells undergo phenotypic and molecular changes and mature.[2] Numerous studies today try to regulate the function of this cell to prevent and vaccinate or treat some cancers[3,4] and diseases such as rheumatoid arthritis,[5,6] inflammatory arthritis,[5,7] autoimmunity,[8] and melanoma.[9-12] Therefore, the efficiency and purity of the induced dendritic cells are significant. The efficiency of differentiated dendritic cells is related to several factors, including the age of the mouse, the medium enriched with supplements and cytokines, purification methods, and the medium changing interval and even medium changing method. There are different protocols for dendritic cell isolation and differentiation.

In the primary methods of bone marrow cell extraction and dendritic cell induction, after removing lymphocytes using specific antibodies and rabbit complement, granulocyte-macrophage colony-stimulating factor (GM-CSF) with or without interleukin 4 is used in culture media for bone marrow precursors.[13-15] Then, to remove the granulocytes, after two days, three-quarters of the medium is replaced with a fresh culture medium,[13] and sometimes the culture time is extended to 9-11 days.[14] Finally, in some protocols, for further purification, the magnetic-activated cell sorting (MACS) method is used using the anti-CD11c antibody in mice attached to magnetic beads for positive selection[16] or using the Ficoll[17] and Percoll[18] gradients.

As mentioned above, in some dendritic cell isolation methods, usually after bone marrow extraction, the cells are treated with anti-lymphocyte markers antibodies and try to remove some cell impurities in the presence of rabbit complement.[13] Also, in some protocols, purification methods are used after dendritic cell induction to increase the purity of target cells in the final production.[16-18]

Antibiotic and purification methods are costly, time-consuming, and sometimes unavailable. In the present study, the effect of several factors (including the age of the mouse, the medium enriched with supplements, and the medium changing with swirling) on increasing the purity of dendritic cells was studied, which can increase the purity of target cells than what can be obtained without observing them.

## MATERIALS AND METHODS

### Mice

Female BALB/c juvenile mice (about 3-4 weeks old) and adult mice (10-12 weeks old) were purchased from the Laboratory Animal Center of Royan Institute. Mice were kept in cages under controlled light conditions (brightness - darkness 12:12) and temperature conditions of 22°C and had access to adequate amounts of water and food. All experiment stages were approved by the Tarbiat Modares University ethics committee (IR.MODARES.REC.1397.110).

### Bone Marrow Preparation

First, the mouse was morally sacrificed and placed in a container containing 70% alcohol. A small incision was made gently and carefully in the skin of the iliac region. This incision continued to the ankle area until the femur and tibiae became visible. Thigh and leg muscle tissue were removed entirely with scissors, and residual tissue was removed using sterile gauze. The bones were then put in 70% ethanol for 2 minutes for disinfection, then transferred to a container containing PBS. They were then placed in a petri dish containing RPMI (BioIdea, I.R. Iran). Bones were flushed by an insulin syringe containing 1 mL of RPMI medium. The cells were centrifuged (300 g for 5 minutes) and washed with PBS. Then about 2 mL of ammonium chloride lysis buffer was added to the cells and, after a maximum of two minutes, immediately neutralized with PBS and washed twice. The trypan blue vital dye evaluated the initial viability of cells.

### Mouse Bone Marrow Cells Culture

After bone marrow isolation from mice of different ages, cells were cultured in media with and without supplements to evaluate the effect of the culture medium. Then, the expression level of the CD11c marker was measured using flow cytometry.

After some investigations, based on the results, cultures of mouse bone marrow cells to induce dendritic cells were performed as follows. 500,000 cells in 0.5 mL of RPMI medium enriched with 10% FBS (Gibco, NY), 1 mM sodium pyruvate (Gibco, NY), 0.1 mM non-essential amino acids (Gibco, UK), and 50 μM 2-mercaptoethanol (Sigma, USA) in the presence of cytokine GM-CSF (Peprotech, Frankfurt, Germany) and

IL-4 (Peprotech, Frankfurt, Germany) was cultured in 24-well plates and incubated at 37°C and 5% CO2. The plate was swirled slowly on the third day, and then half of the supernatant was gently removed and discarded. Then 1 ml of fresh enriched medium containing the same amount of cytokine was added to the well. On the fifth day, the cells reached an immature stage, which was used for flow cytometric analysis of surface markers CD11c, CD40, CD86, and MHC class II.

**Evaluation of the Effect of Mice Age and Culture Media on the Efficiency of Bone Marrow-derived Dendritic Cells**

To investigate the effect of mouse age on dendritic cell differentiation efficiency and immature cell viability, juvenile and mature adult mice were used to extract bone marrow and differentiate it into dendritic cells. Then, to evaluate the effect of culture medium supplements and cell culture medium replacement method on the differentiation of dendritic cells, an enriched culture medium containing sodium pyruvate, non-essential amino acids, and 2-mercaptoethanol was used along with the medium without these supplements. On the third day, some wells were swirled before media replacement, and the medium of some was changed without mixing.

On the fifth day, the purity of immature dendritic cells was assessed using PE-conjugated anti-CD11c antibody (eBioscience, US) and flow cytometry.

**Induction of Dendritic Cells Maturation**

On the fifth day of dendritic cell culture, two-thirds of the surface culture medium was gently removed. The amount of fresh enriched medium containing cytokines was added to each well at half of the initial concentration. Then on the sixth day, LPS (Lipopolysaccharide) (Sigma, USA) was added to the medium as a maturation factor so that its concentration was equal to 0.5 μg/mL. It should be noted that the duration of exposure of cells to LPS should be a maximum of 14-16 hours. On the seventh day, cells were separated from the bottom of each well by stronger pipetting and collected in a falcon. Surface maturation markers were evaluated by flow cytometry.

**Flow Cytometry**

For phenotypic analysis of cell surface markers expression, cells were washed after collection with PBS containing 2% FBS. The cells were then incubated on ice with PE-conjugated anti-CD11c antibody (eBioscience, US) for 30 minutes. Cells were washed with PBS containing 2% FBS, then incubated with anti-CD40, CD86, or MHC-II antibodies conjugated with APC (eBioscience, US) in separate vials. Control mouse IgG1 isotype antibody was used in parallel as a control. The vials were incubated at 4°C in the dark for 45 minutes. The stained cells were washed twice and finally read with a FACS LYRIC flow cytometer. The results were processed using FlowJo V10 software.

In T cell stimulation assays, the extracted lymphocytes were labeled using Carboxyfluorescein Succinimidyl Ester (CFSE) (eBioscience, US) dye to evaluate the number of proliferative generations. After incubation under the desired conditions described below, the intensity of fluorescent light emitted from CFSE was investigated.

**Uptake Assay**

To determine the phagocytic ability, dendritic cells were incubated in a medium containing 10% FBS and FITC-dextran average molecular weight of 40 KDa (Sigma, USA) with a final concentration of 1 mg/mL at 37°C and 5% CO2 on the fifth day. The control well was incubated at 4°C. After four hours of incubation, they were washed twice using PBS containing 2% FBS. To evaluate the uptake of FITC-dextran by dendritic cells, CD11c was used as an indicator of dendritic cells, so after incubation, the cells were stained with PE-anti-CD11c antibody and analyzed by flow cytometry.

**Allogeneic Mixed Lymphocyte Reaction**

A mixed lymphocyte reaction test was used to assess the ability of the dendritic cells to induce an immune response. For this purpose, induced dendritic cells from BALB/c mice were co-cultured with lymph node cells (100,000 cells) extracted from C57BL/6 mice labeled with CFSE in DC: T ratios of 1:1, 1:5, 1:10, and 1:20. Cells were cultured in 200 μL of RPMI medium containing 10% FBS in a U-shaped well at 37°C and 5% CO2. Then, on the third, fourth, and fifth days, the number of proliferative generations was measured using fluorescent light emitted from CFSE of lymphocyte cells using flow cytometry.

**CFSE Cell Labeling Method**

Lymphocytes were labeled with CFSE dye, according to Parish's protocol.[19] After extracting and

washing the lymph node cells, a sample of cells was taken as an unlabeled sample. The desired number of cells, less than 100 million cells, were placed in 1 mL of RPMI medium containing 5% FBS at the end of a 15 mL falcon. The falcon was then placed horizontally, 110 µL of PBS containing 5% FBS was placed on top of it, and 1.1 µL of pre-prepared CFSE (5 M concentration) was added. To maintain the sterile condition, the falcon lid was closed horizontally, and the tube was taken to the vortex while preserving the state, and quickly placed vertically on the vortex from the falcon lid vortexed for 2-3 seconds. After incubation for 5 minutes at room temperature and in the dark, 10 mL of PBS containing 5% FBS was added and centrifuged. This washing step was performed twice. Labeled cells were examined alongside unlabeled cell samples using a flow cytometer. If the labeling process is done correctly, only one labeled peak should be visible in the labeled sample.

**Antigen Loading of Dendritic Cells**

Any protein or peptide antigen can be used to evaluate the ability of dendritic cells to induce a specific immune response against an antigen. Here, to investigate the mechanism of antigen processing by dendritic cells, a fusion protein consisted of three receptors tyrosine kinase-like orphan receptor 1 (ROR1) with IgG2a-Fc fragment and a C-fragment of tetanus toxin (TTC). To load the dendritic cells, on the sixth day and simultaneously with the addition of LPS maturation factor, antigen with a final concentration of 10 µg/mL of culture medium was added to the cells and incubated for 14-16 hours at 37°C and 5% CO2.

**Investigation of Specific Immune Response In vivo**

One million dendritic cells loaded with the antigen were injected into the mouse footpad. After five days, the popliteal lymph node of the foot was extracted, and after isolating the lymph node cells, they were labeled using CFSE dye. The zero peaks on day 0 indicate the accuracy of the cell labeling process. Labeled cells (300,000/ well of 96-well plate) were re-stimulated in DMEM medium (BioIdea, I.R. Iran) containing 0.5% of normal mouse serum in the presence of specific antigen (the same antigen used in dendritic cell loading as well as its components) with a concentration of 2 µg/mL. PHA (Sigma, USA) is used as a positive control. Then, on days 3, 4, and 5, the number of reproductive generations was examined using flow cytometry.

**Statistical Analysis**

Statistical differences were analyzed using the T-Test and Mann-Whitney test for parametric and nonparametric data, respectively. The Kolmogorov-Smirnov statistical test was used to examine the normality of data. The *p*-value equal to or less than 0.05 was considered statistically significant. All statistical studies were performed; using Microsoft Excel software and SPSS software.

## RESULTS

**Increased Efficiency and Viability of Dendritic Cells in Cells Extracted from Juvenile Mice**

All bone marrow precursor extraction and differentiation steps were performed in juvenile and adult mice. The flow cytometry results showed an increase in the purity of $CD11c^+$ cells. The viability test using trypan blue dye also showed an increase in the percentage of viable cells. The expression of the CD11c marker in cells obtained from juvenile and adult mice was 58% and 32% ($p<0.01$), respectively. Although not statistically significant, the cell viability averaged 92% and 83% on the fifth day, respectively (Figure 1).

**The Use of an Enriched Culture Medium has an Increasing Effect on the Efficiency of Dendritic Cell Differentiation**

To investigate the effect of an enriched culture medium on the percentage of differentiated dendritic cells, the culture medium containing sodium pyruvate, essential amino acids, and 2-mercaptoethanol was used along with the medium without these supplements. The differentiation efficiency of $CD11c^+$ cells was measured on the fifth day. The use of supplements in the culture medium of dendritic cell differentiation has a statistically significant effect on the production efficiency of these cells ($p<0.01$)(Figure 2).

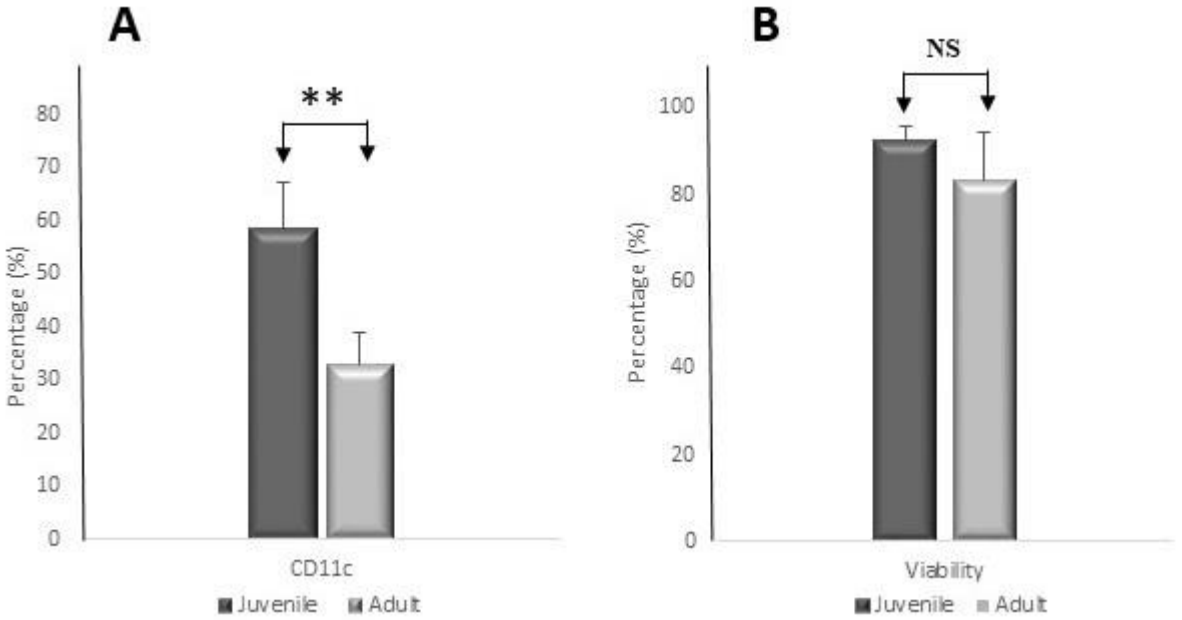


**Figure 1. The percentage of CD11c expression and viability of bone marrow-derived dendritic cells of juvenile and adult mice on the fifth day of culture**

**Bone marrow-derived dendritic cells obtained from juvenile and adult mice were cultured for five days in the presence of appropriate cytokines. The purity of immature dendritic cells was assessed using an anti-CD11c antibody and flow cytometry (A). The cell viability was also measured using trypan blue staining (B). The graphs show the mean ± SD of the values obtained (n=6). Statistically, significant differences are indicated with ** ($p$<0.01). NS: not significant**

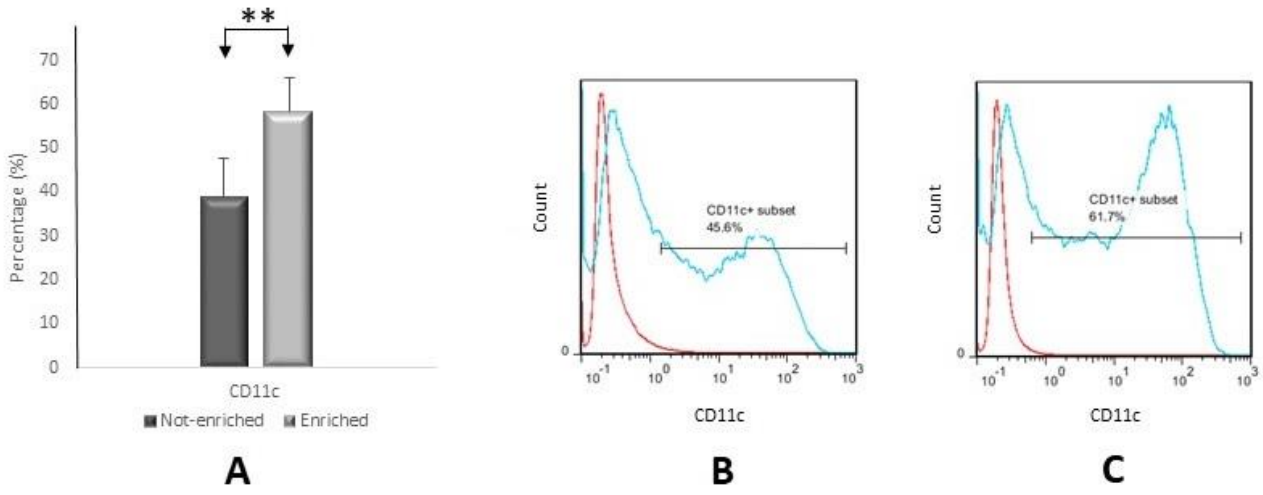


**Figure 2. The percentage of CD11c expression on immature dendritic cells in culture medium with and without enrichment supplements.**

**Dendritic cells were cultured separately in a culture medium enriched with and without medium supplements. The CD11c expression was measured as a surface marker of dendritic cells on the fifth day (A). The graph shows the mean ± SD of the obtained values (n=7). The representative flow cytometry results show the percentage of $CD11c^+$ cells in culture medium without enrichment supplements (B) and medium containing enrichment supplements (C). The red histograms show the unstained sample, and the blue ones show the sample stained with PE-anti-CD11c. Statistically, significant differences are indicated with ** ($p$<0.01).**

**Swirling of the Culture Medium Before Its Replacement has an Increasing Effect on the Efficiency of Bone Marrow-derived Dendritic Cells**

To remove cellular impurities, which are often non-adherent cells, we used physical swirling of wells when changing the media. At this stage, with the slow and soft movement of the cell culture plate, the granulocyte cells become more suspended and are removed better from the well during harvest. The results show a significant increase in the relative percentage of immature $CD11c^+$ cells on the fifth day (Figure 3).

**Induction and Maturation of Dendritic Cells with a Purity of Over 70%**

After extraction of bone marrow progenitor cells, they were cultured in the presence of cytokines GM-CSF (30 ng/mL) and IL-4 (15 ng/mL) for five days. Then, they were cultured in the presence of the LPS maturation factor on the sixth day. The flow cytometry results of the fifth day show that about 60-70% of the cells have differentiated into dendritic cells, which in the wells with and without LPS on the $7^{th}$ day reached 79 and 77%, respectively. (Figure 4A) The percentage of expression and MFI of maturation markers CD40, CD86, and MHC-class II on the surface of dendritic cells ($CD11c^+$ cells) increased significantly on the seventh day in the presence of LPS maturation factor (Figures 4A and 4B). The representative flow cytometry results comparing wells with and without the maturation factor are shown in Figures 4C and 4D.

**Induced Dendritic Cells Showed an Appropriate Efficiency in Antigen Uptake**

In optimizing the induction process of dendritic cells, the antigen uptake ability of the induced cells was investigated. The dendritic cells were incubated with FITC-dextran (molecular weight of 40 kDa) at 37°C. Endocytosis at 4°C does not occur well because of the decreased cell membrane fluidity. Therefore, the 4°C incubation was used as a control to remove a percentage of dextran that binds to the cell surface. After four hrs, cells were collected and stained with PE-anti-CD11c. Flow cytometry results showed that these cells have more than 75% efficiency in antigen uptake. The difference between 4°C and 37°C uptakes was statistically significant ($p$<0.001) (Figure 5)..

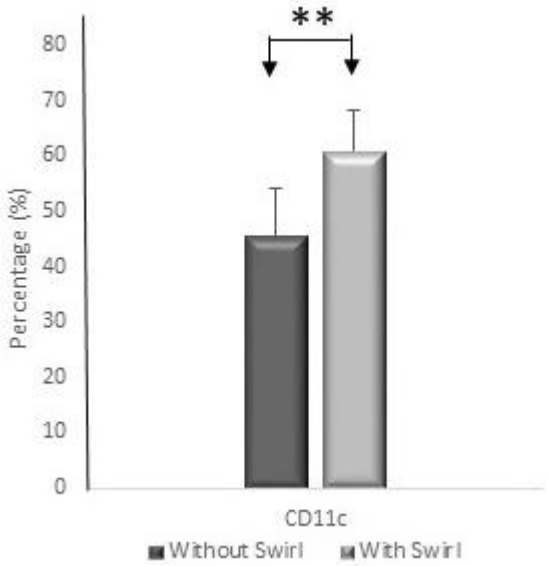


**Figure 3. The effect of swirling cell culture plate while changing the media on the percentage of immature dendritic cells**
**Bone marrow precursors were cultured in a culture medium. The cultural environment should be changed after three days. At this point, a gentle swirling of the cell culture plate results in better removal of cellular impurities and increases the relative percentage of immature $CD11c^+$ cells. The graph shows the mean ± SD of the obtained values (n=6). The difference between the percentage of $CD11c^+$ cells with and without swirling when changing the media is statistically significant ** ($p$<0.01).**

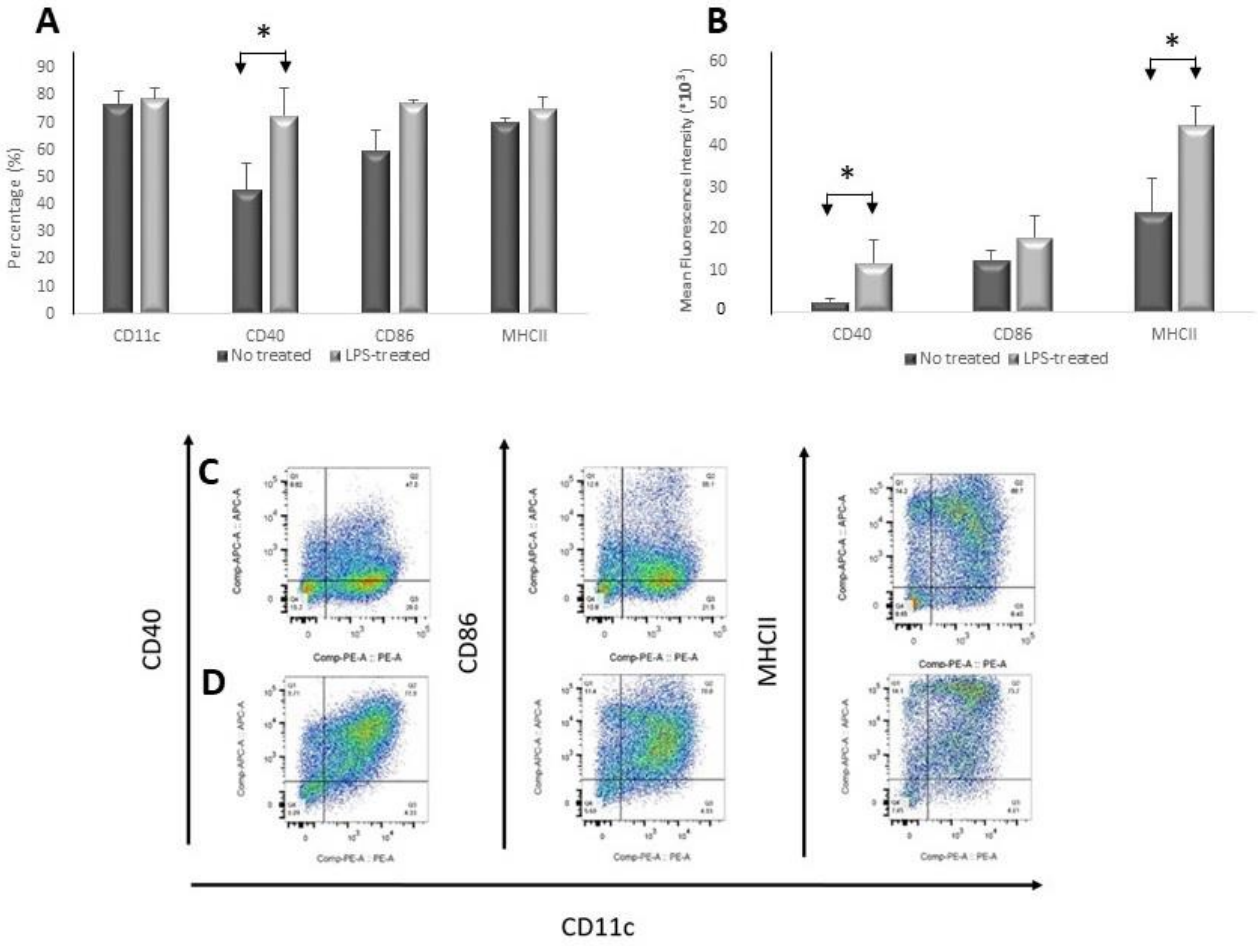


**Figure 4. Investigation of surface markers of mature dendritic cells on the seventh day in the presence and absence of LPS maturation factor**

**Dendritic cells were extracted from mouse bone marrow. The percentage of expression (A) and MFI (B) of their surface markers were measured on the seventh day in the presence of the maturation factor (LPS-treated) and its absence (non-treated). The graphs show the mean ± SD of the values (n=3). The representative flow cytometric results compare wells without maturation factor (C, upper part) and in the presence of maturation factor (D, lower position). Statistically, significant differences are shown with * ($p$<0.05). MFI: Mean Fluorescent intensity, LPS: Lipopolysaccharide**

**Induced Dendritic Cells Showed a Higher Ability to Provoke a Non-specific Immune Response in Culture with Lymphocytes at a Ratio of 1:10 for Five Days**

Induced dendritic cells from BALB/c mouse bone marrow were co-cultured with C57BL/6 mouse lymph node cells labeled with CFSE in DC: T ratios of 1: 1, 1: 5, 1:10, and 1:20 (Figure 6, A-D). The proliferative generations were examined on the third, fourth, and fifth days. The comparison between different ratios on the fifth day showed that the DC: T ratios of 1:5 and 1:10 induced a more appropriate immune response. Meanwhile, comparing the cell proliferation in three consecutive days indicates that more proliferative generations are shown on the fifth day (Figure 6, E-G).

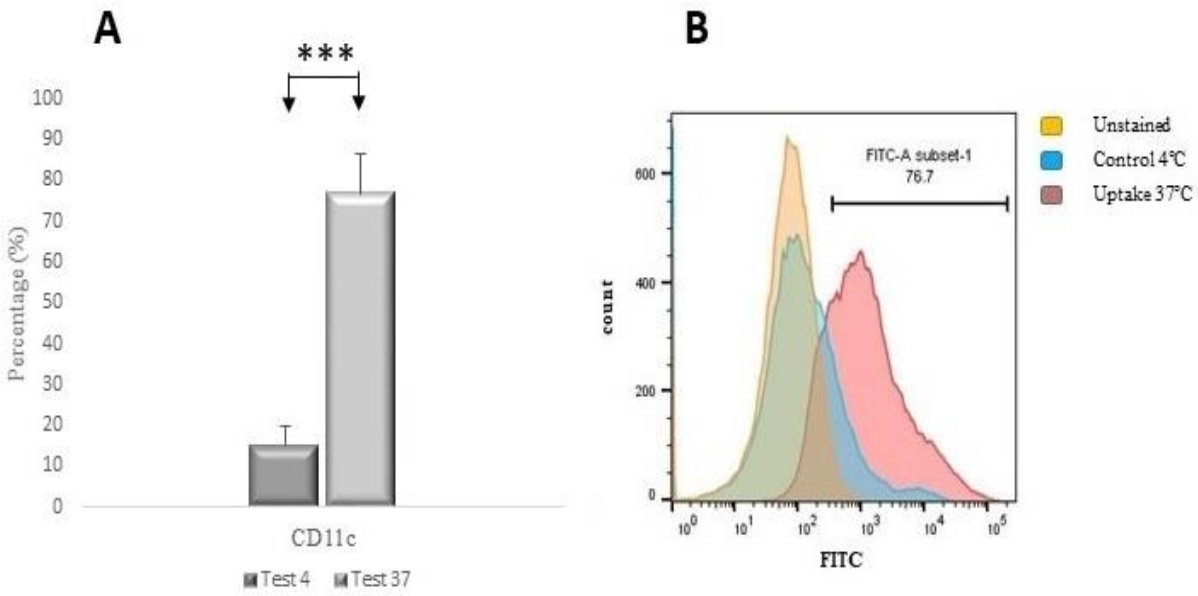


**Figure 5. Dendritic cells' antigen uptake efficiency**

**Immature dendritic cells were incubated with FITC-dextran at a molecular weight of 40 kDa at 37°C on the fifth day of culture. The 4°C incubation was used as a control. After 4 hours, cells were collected from the wells and stained with PE-anti-CD11c to evaluate the uptake of dextran conjugated with FITC in $CD11c^+$ cells (A). The graphs show the mean±SD of the FITC positive cells (n=4). The representative flow cytometric results show FITC-dextran uptake in wells incubated at 4°C (blue diagram) and 37°C (red graph). The orange graph shows a sample not incubated with FITC-dextran as an unstained sample (B). Statistically, significant differences are indicated with *** ($p$<0.001). FITC: Fluorescein isothiocyanate.**

### Antigen-loaded Dendritic Cells Have an Appropriate Efficiency in Inducing the Specific Immune Response In vivo

The desired fusion protein, consisting of three components, IgG2a-Fc, ROR1, and TTC, was loaded on induced dendritic cells to evaluate the specific function. Then, the loaded dendritic cells were injected into the mouse footpad; the popliteal lymph node was extracted five days later. Lymph node cells were CFSE-labeled and re-stimulated with the complete structure of the fusion protein and its components separately in a DMEM medium containing 0.5% of normal mouse serum. The cell proliferation rate on the third, fourth, and fifth days indicates the extent of a specific immune response against each component (Figure 7).

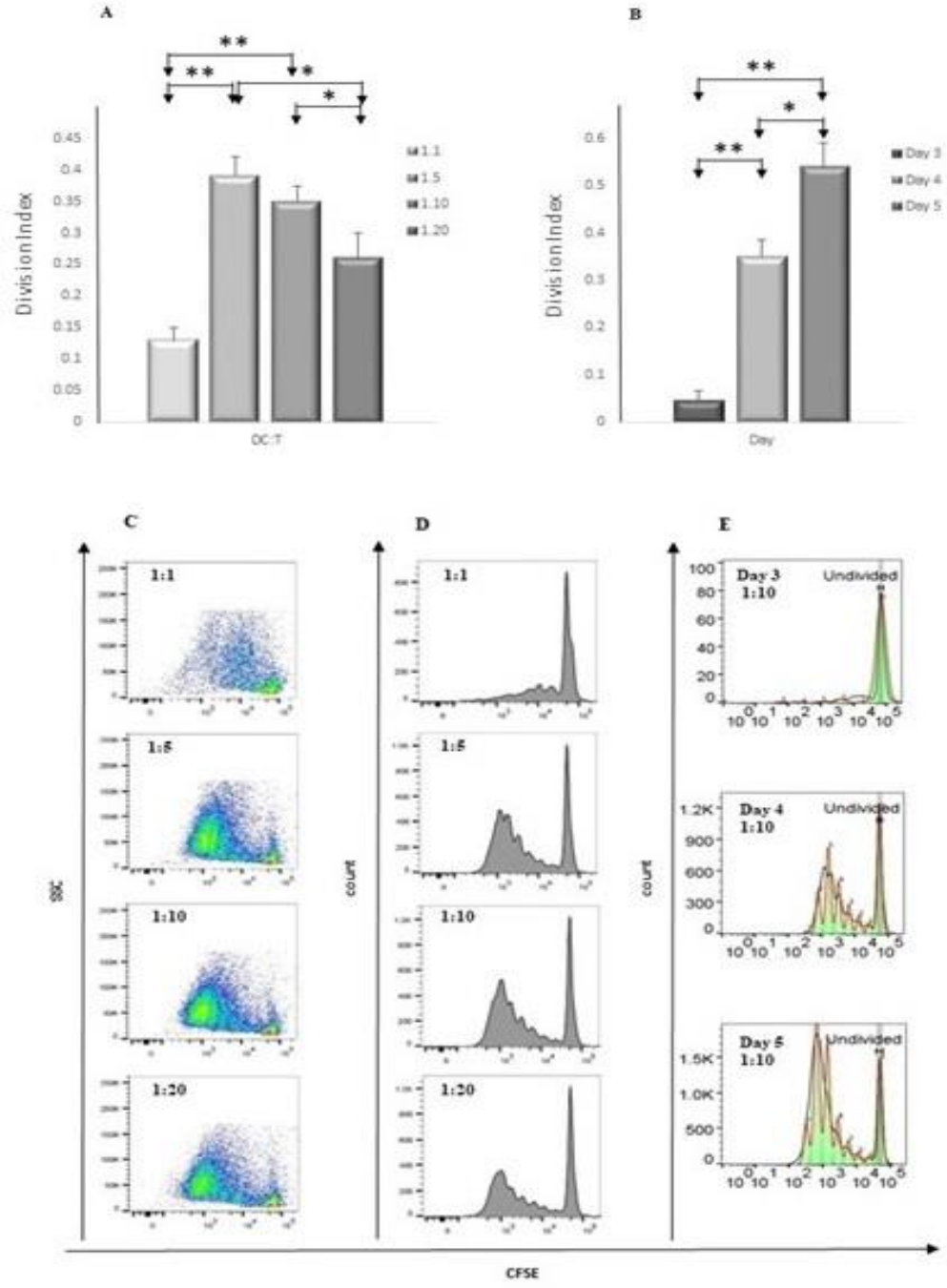


**Figure 6. Allogeneic MLR**

**Immature dendritic cells from the bone marrow of BALB/c mice were co-cultured with CFSE-labeled lymph node cells of C57BL/6 mice in different ratios. The results show that there is a significant difference in the ratio of 1:1 and 1:20 to 1:5 and 1:10 ($p$<0.05) (A). Also, the difference in division index on days 4 and 5 compared to day 3 is significant as well as between days 4 and 5 ($p$<0.05) (B). The graphs show the mean±SD of values (n=3). The dot plots (C) and histograms (D) show the representative flow cytometric results of DC: T ratios 1: 1, 1: 5, 1:10, and 1:20 on the fourth day, from top to bottom, respectively. The representative flow cytometry results from the co-culture of dendritic cells with allogeneic lymphocytes with a ratio of 1:10 on the third, fourth and fifth day are also shown (E). Statistically, significant differences are indicated with * ($p$<0.05). CFSE: Carboxyfluorescein Succinimidyl Ester**

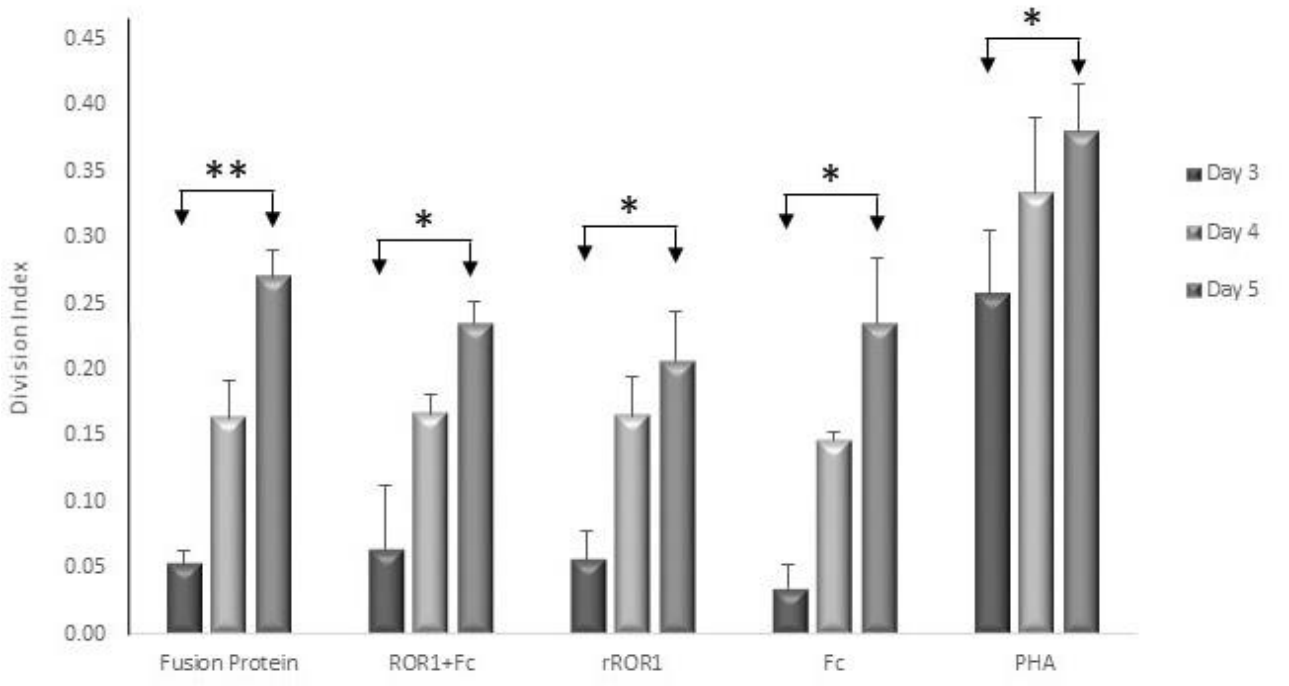


**Figure 7. Specific immune response by restimulation of lymph node cells for three consecutive days**

**Immature dendritic cells were loaded with the fusion protein containing three sections IgG2a-Fc, ROR1, and TTC on the fifth day. Then, one million loaded dendritic cells were injected into the mouse footpad, and after five days, the popliteal lymph node was extracted. Cells extracted from the lymph node were CFSE-labeled and re-stimulated in the presence of the complete structure and components of the fusion protein separately in a DMEM medium containing 0.5% of normal mouse serum. The graph shows the mean ± SD of the division indexes obtained from proliferative generations in flow cytometric results (n=3). Statistically, significant differences are indicated with * ($p$<0.05). Fc: Fragment c, ROR1: Receptor Tyrosine Kinase Like Orphan Receptor 1, TTC: Tetanus Toxin C-fragment.**

## DISCUSSION

Dendritic cells are a group of antigen-presenting cells that play an important role in initiating and regulating the immune response. Although there are many studies on the function of these cells and their usage in vaccination and treatment of various diseases, the study of these cells and their characteristics are still of interest to many researchers. One of the major challenges in studying these cells is the limited number of these cells in different tissues. Several methods exist to differentiate these cells from splenic and bone marrow precursors. Despite differences in bone marrow cell extraction and differentiation methods, dendritic cell production from mouse bone marrow is used as a known method to achieve a sufficient number of homologous dendritic cells for preclinical studies. In one of the preliminary methods of dendritic cell isolation, after separating the bones, the bone epiphyses are cut, and the bone is flushed using the RPMI culture medium. Antibodies, along with rabbit complement, are used to kill the leukocytes. The cells are then cultured in culture medium containing 5% FCS, 2ME (50 μM), gentamicin (20 μg/mL) and GM-CSF (500-1000 U/mL). Three-quarters of the medium is removed every two days, and the fresh medium is added. Finally, a 50% FCS-RPMI column is used to remove the granulocytes better.[13] Alternatively, a 100 mm petri dish is used instead of a 24-well plate to culture the bone marrow progenitor cells in culture media containing 10% FCS, 2ME, L-glutamine (2 mM), and GM-CSF (20 ng/mL). On the third day, only fresh medium is added to the cells; finally, on days 6 and 8, half of the medium is removed. After centrifugation, fresh medium is added to the cell pellet and returned to the petri dish.[14] Immature dendritic cell culture is

continued to reach maturity. Thus, in some methods, after bone marrow extraction, cells are treated with anti-lymphocyte antibodies and rabbit complement to remove some of the existing cellular impurities.[13] Also, in some methods, more purification is used after induction of dendritic cells using purification methods such as Percoll and Ficoll gradient and the MACS separation.[16-18] In addition to being costly, these methods need prolonged cell culturing and are sometimes unavailable.

In this study, after examining different methods of dendritic cell differentiation, we investigated the effect of three parameters: mouse age, the use of enriched culture medium, and swirling of the plate before changing the culture media on the purity and efficacy of the obtained DCs. All three parameters significantly affected the efficiency and purity of CD11c+ dendritic cells compared to cells that can be obtained without considering these parameters. Using juvenile mice instead of adult mice significantly increased the purity of immature dendritic cells on the fifth day of culture. Cells extracted and differentiated from the bone marrow of the juvenile mice have better efficiency and performance in differentiation into dendritic cells than cells obtained from the bone marrow of older mice.[20-24] On the other hand, the bone epiphysis of these mice is softer than older mice because the process of ossification of the epiphysis has not yet begun.[25] The softness of this part facilitates the needle's entry into the epiphysis and flushing of the bone marrow. In a study published by Liu et al, the number of cells extracted from the 4-week-old mice bone marrow was increased by 15% compared with the older mice.[26]

The media is wholly changed in conventional methods on the third day of culture. In our proposed method, half of the medium was replaced with twice its volume, but the critical point is the plate's smooth swirling before removing the cells' supernatant. Extreme movement or pipetting of the medium can cause dendritic cells to separate from the bottom of the well. While granulocytes, dead cells, and a large percentage of unwanted cells are suspended in the environment, the slow swirling of the plate makes them more suspended, and the gentle removal of the culture medium removes these cells. As a result, the relative percentage of target cells will increase on the fifth and seventh days.

Indeed, three parameters were discussed in the present study, among the productive parameters in increasing the differentiation efficiency of dendritic cells. It was shown that 1- Using juvenile mice (3-4 weeks old) instead of adult mice (10-12 weeks old) affects dendritic cell purity and viability. In addition, it facilitates the process of bone marrow progenitor cell extraction. 2- Using an enriched culture medium in dendritic cell culture is necessary. 3- Smooth swirling of the plate before removing the medium on the third day causes the granulocyte cells to be suspended in the supernatant and harvested and discarded during changing the environment without centrifugation and returning the cell pellet. This stage increases the relative percentage of target cells. In addition, another parameter, such as the preservation of the bone epiphyses, also affects the high efficiency of dendritic cell production. Previous studies have shown that the epiphysis has many bone marrow precursors,[27,28] so preserving these areas and flushing them is likely to increase the number of precursors obtained from the bone marrow, which requires further study.

Finally, the obtained dendritic cells had to be examined for their nonspecific and specific functions. Here, to evaluate the nonspecific function of the obtained dendritic cells, the antigen uptake ability and induction of T lymphocytes proliferation were assessed by the FITC labeled dextran ingestion and the allogeneic MLR, respectively. It was shown that the differentiated dendritic cells are pretty efficient in antigen uptake and stimulation of allogeneic T lymphocytes. To assess the specific T cell stimulation ability of the DCs, a three-component antigen was loaded on dendritic cells to simultaneously follow the fate of different antigen components during the antigen processing and presentation. During antigen processing within dendritic cells, the peptides of each component are presented separately to the T lymphocytes. After injection of antigen-loaded dendritic cells into mice and extracting the regional lymph node cells at the appropriate time, it is expected to re-stimulate lymph node cells with the target antigen components separately or all together as a fusion protein leads to cell proliferation. The results confirmed the proliferative response of primed lymphocytes in re-stimulating by the protein components individually or together. The proliferative response in the presence of all three antigenic components as fusion protein was more substantial, although the difference was insignificant.

It should be noted that, due to the presence of FBS in the loading phase of DCs, the medium without FBS should be used in the re-stimulation step, so the medium containing 0.5% of normal mouse serum was used. Comparing the proliferative generations of T lymphocytes in the mediums containing mouse serum and FBS, it was found that the proliferative generations of these cells in the medium containing mouse serum were less than the proliferative generations in the medium containing FBS (results not shown). This is related to the inhibitory effect of normal mouse serum on the process of lymphocyte proliferation, which has been studied in various investigations.[29-32]

Collectively, the results of our study show that paying attention to the described parameters increases the efficiency of dendritic cells more than what can be obtained without considering them. This can even help to increase the percentage of target cells when antibodies and purification methods are unavailable due to cost or unavailability.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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