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The Evaluation of the Effect of Tolerogenic Probiotics on the Maturation of Healthy Dendritic Cells versus Immature Dendritic Cells

Maryam Rastin[1, 2], Mahmoud Mahmoudi[1, 2], Nafiseh Tabasi[1], Nadia Kia[3], Jafar Hajavi[4], Seyed-Alireza Esmaeili[1, 2*]

[1]Immunology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran; [2]Immunology Department, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; [3]Skin Cancer Prevention Research Center, Torvergata University of Medical Sciences, Rome, Italy; [4]Department of Basic Sciences, Faculty of Medicine, Infectious Diseases Research Center, Gonabad University of Medical Science, Gonabad, Iran

ABSTRACT

Background: Dendritic cells, (DCs) as one of the important immune cell populations, are responsible for the initiation, development, and control of acquired immune responses. Myeloid dendritic cells can be used as a vaccine for several autoimmune diseases and cancers. Tolerogenic probiotics with regulatory properties can affect the maturation and development of immature dendritic cells (IDC) into mature DCs with certain immunomodulatory effects.

Objective: To assess the immunomodulatory effect of *Lactobacillus rhamnosus* and *Lactobacillus delbrueckii*, as two tolerogenic probiotics, in the differentiation and maturation of myeloid dendritic cells.

Methods: The IDCs were derived from the healthy donors in GM-CSF and IL 4 medium. Mature DCs (MDC) were produced with *L. delbrueckii*, *L. rhamnosus*, and LPS from IDCs. Real-Time PCR and flow cytometry were used to confirm the DC maturation and to determine DC markers as well as IDO, IL10, and IL12 expression levels, respectively.

Results: Probiotic-derived DCs showed a significant reduction in the level of HLA-DR ($P \leq 0.05$), CD86 ($P \leq 0.05$), CD80 ($P \leq 0.001$), CD83 ($P \leq 0.001$), and CD1a. Also, the expression of IDO ($P \leq 0.001$) and IL10 increased while IL12 expression decreased ($P \leq 0.001$).

Conclusion: Our findings revealed that tolerogenic probiotics could induce regulatory DCs by reducing co-stimulatory molecules along with increasing the expression of IDO and IL10 during the differentiation process. Therefore, the induced regulatory DCs probably can be used in the treatment of various inflammatory diseases.

Keywords: Immature dendritic cell, *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus*, Probiotics, Tolerogenic dendritic cell

**Corresponding author:*
Seyed-Alireza Esmaeili, Immunology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.
Tel: +98 51 38002379
Email: Esmaeiliar@mums.ac.ir

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INTRODUCTION

Dendritic cells (DCs) are important intrinsic immune cells responsible for the formation and safety of immune responses [1]. These antigen-presenting cells are effective in connecting innate to acquired immunity, and also in the development of inflammatory or inhibitory immune responses [2]. Regarding the poor expression of co-stimulators and surface receptors, immature DCs act as immunomodulators and by presenting the antigen to T-cells can drive these cells into an inhibitory phenotype [3]. This specific feature has enabled the use of immature DCs as a vaccine or a therapeutic agent in the treatment of various patients [4]. Inhibitory DCs can also induce regulatory immune cells by expressing less co-stimulatory and surface receptors. These cells provide a suitable microenvironment for the formation of inhibitory immune responses by secreting inhibitory cytokines [5-8]. CD80 and CD86 co-stimulatory molecules besides MHC class II molecules [9] are the most important surface markers for developing a powerful immune response, whereas DCs with an inhibitory phenotype have a reduced amount of these molecules [10]. As a marker of DC maturation, CD83 increases in mature tolerogenic and inflammatory DCs [11], whereas CD1a, as a surface receptor in DCs, contributes to the presentation of lipids and glycolipids to T-cells [12]. CD14 is the main marker of the monocyte category which decreases during the differentiation of immature and mature dendritic cells with a myeloid origin [13].

Many medications and herbal compounds have been introduced which can induce inhibitory phenotypes into dendritic cells [14, 15]. As an example, tolerogenic probiotics can affect the differentiation and maturation of dendritic cells through unknown mechanisms, the signaling pathway and downstream signaling of Proteins, and push them toward an inhibitory phenotype [16-18]. Many *lactobacilli* have anti-inflammatory and immunomodulatory properties. *Lactobacillus casei* and *Lactobacillus reuteri* induce anti-inflammatory effects and increase the production of IL10 through the engagement of the DC-SIGN receptor in DCs [19]. *Lactobacillus rhamnosus JB1* induces the production of Treg inhibitory cytokines through TLR2 [20]. *Lactobacillus rhamnosus (L. rhamnosus)* has shown anti-inflammatory effects on both animal models and *in vitro* studies by reducing inflammatory cytokines and cell proliferation, the induction of Treg cells, and the production of IL10 and TGF-β [21-24]. *Lactobacillus delbrueckii (L. delbrueckii)* has been shown to induce a regulatory systemic and mucosal immune response in IBD patients. It has also demonstrated regulatory function by increasing the differentiation of regulatory T-cells, blocking the NF-κB signaling, and reducing inflammatory cytokines [25, 26]. This study evaluated the immunomodulatory effect of *Lactobacillus rhamnosus* and *Lactobacillus delbrueckii,* as the two tolerogenic probiotics, in the differentiation and maturation of myeloid dendritic cells in comparison with the immature dendritic cells.

MATERIALS AND METHODS

Probiotics Culture and Dose Determination

Lyophilized *Lactobacillus delbrueckii subsp lactis (PTCC: 174) (Iranian Research Organization for Science and Technology (IROST)),* and *lactobacillus rhamnosus ((ATCC: 9595) (Pasteur Institute of Iran))* were initially grown in MRS broth (Biolife, Viale Monza, Milano-Italia (LOT: EB4301)) under microaerobic conditions for 1 hr at 37°C. Then, the probiotics were cultured on MRS agar (Biolife, Viale Monza, Milano-Italia (LOT: EB4302)) under microaerobic conditions for 24 hrs. At 37°C to reach adequate amounts of probiotic colonies. Finally, the number of probiotics in 1 ml of MRS broth was calculated by the formula "OD* 8* 10^8 "and the Glycerol stock colonies were stored at -70°C.

Monocytes Isolation and Culture

The Research Council Ethics Committee of Mashhad University of Medical Sciences (MUMS) approved the study protocol and all the donors provided informed consent. (Ethic code: 930895) A 15 ml whole blood sample was collected from each healthy individual in vacuum heparin tubes and by using Ficoll-Hypaque (Cedarlane, Toronto, Canada), the peripheral blood mononuclear cells (PBMCs) were obtained by density gradient method. To isolate the monocytes, PBMCs (1.5 * 10^6 / ml) were cultured on RPMI-1640 medium (BioSera, London, UK (and FBS (10%) (GIBCO) for 2 hrs at 37°C, and the monocytes (2* 10^5 / ml) were isolated through the plastic adherence. Afterward, the purity of the isolated monocytes was evaluated using anti-CD45 FITC/anti-CD14 R-PE (IQProducts, Groningen, The Netherlands) associated with the FITC/PE-isotype control antibody (IQProducts, Groningen, The Netherlands) by the flow cytometry (DB, FACScan). The monocytes were counted under the optical microscope, filed, and used for further evaluations.

Immature and Mature Dendritic Cell Culture

Immature dendritic cells (IDC) were produced during a five-day culture period. For this purpose, 0.2×10^6 monocytes/ wells were prepared and cultured in fresh complete RPMI supplemented with 100 IU/ ml penicillin (Sigma-Aldrich, USA), 100 µg/ml streptomycin (Sigma-Aldrich, USA), and 2mM L-glutamine (Sigma-Aldrich, USA) along with 10 ng/ml, GM-CSF (RD, Minneapolis, MN, USA) and 10 ng/ml IL-4 (RD, Minneapolis, MN, USA) on days 1 and 3. For the generation of mature DCs (MDCs) 10 ng/ml IL-4 and 10 ng/ml, GM-CSF was once again added on day five. The culture was subsequently incubated for 2 days in the presence of LPS (100 ng/ml) (Sigma-Aldrich, USA) (MDC-LPS), *lactobacillus delbrueckii* (MDC-Del), *lactobacillus rhamnosus* (MDC-Ram) and a mixture of both probiotics (MDC-Mix) (2×10^6 bacteria/well) in complete media to generate inflammatory and tolerogenic mature DCs.

Dendritic Cells Uptake Assay and Flow Cytometry Detection of Their Surface Markers

To evaluate the antigen uptake of immature DCs, FITC–dextran dye was used [24] and the amount of dye uptake was measured by the flow cytometry (DB, FACSCalibur). In brief, FITC–dextran dye (1 mg/ml) was incubated with 2×10^5 immature DCs for 2 hrs at 37°C, then washed (2X) with PBS , finally adjusted in 200 µl of PBS and quantified by the flow cytometry. The expression of surface markers in both the immature and mature DC populations was evaluated by the flow cytometry to confirm the process. Anti-CD14 R-PE, anti-CD1a R-PE, anti- HLA-DR-R-PE, anti-CD86 FITC, anti-CD80 FITC ,and anti-CD83 FITC (IQProducts, Groningen, The Netherlands) in the presence of FITC/ PE-isotype control antibody (IQProducts, Groningen, The Netherlands) were incubated for 45 min at 4°C. The cells were then washed (2X) with PBS and adjusted in 200 µl of PBS, next analyzed by the flow cytometry (BD, FACS Calibur).

RNA Extraction and Real-time PCR

The total RNA of 0.2×10^6 IDCs and MDCs were obtained by the Tripure isolation method (Roche Diagnostics GmbH, Mannheim, Germany) upon the manufacturer's instructions. In the next step, 1µg of total RNA along with Reverse Transcriptase enzyme (200 unit/ml) (Fermentas, Vilnius, Lithuania), random hexamer, and oligo-dT primers (100 mM) were used to cDNA synthesize upon the manufacturer's instructions. The expression level of IDO (Indoleamine 2, 3-dioxygenase), IL12 and , IL10 were determined using the SYBR Green (Takara, Shiga, Japan) Real-Time PCR method with specific primers by Rotor-Gene 6000 thermal cycler (QIAGEN, Hilden, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control and the Melting-

curve analysis was performed to ensure the specificity of each PCR. Primer sequences are shown in Table 1.

Data Analysis

SPSS 16 software program (IBM Corporation, NY, USA) and GraphPad Prism 5 Software (Graph Pad, Inc., San Diego, CA, USA) were used to analyze the data. $P \leq 0.05$ is considered a significance level. The one-way ANOVA and Tukey test as a post hoc test were used.

RESULTS

The Number and Unity of Isolated Monocytes

The cells were obtained from 5 healthy donors, and each test was repeated twice. The plastic adhesion ability of monocytes was used to isolate them from the PMBCs. After 2 hrs of culture on plastic plates, the monocytes were isolated and adjusted in 2×10^6 cells/ wells. Our results confirmed the purity of isolated monocytes in both PBMC and isolated monocyte populations. The flow cytometry results showed that 21.5% of the total PBMC cells were $CD45^+$, and $CD14^+$ cells which were considered monocytes. (Figure 1A) Moreover, the flow cytometry results on isolated monocytes confirmed the high purity of these cells and demonstrated that 86.4% of the isolated cells were monocytes ($CD45^+$, $CD14^+$cells) (Figure 1B), these isolated monocytes were then used to generate immature DCs.

Results of FITC-dextran Dye Uptake Through IDC

IDCs were differentiated from the monocytes and floated in the prepared culture media. According to the study design, IDCs were supposed to be able to uptake probiotics and differentiate into mature DCs. Therefore, we used the FITC-dextran uptake method to show the pickup ability of our generated immature DCs. The uptake flow cytometry results of generated IDCs showed that about

Table 1. Primers and primer sequences

Primers	Sense Primer	Anti-sense Primer
Human IDO	5′- TGTGGCAGCAACTATTATAAGATG-3′	5′- GGTACTCTTTACTGATTGTCCAG-3′
Human IL-12	5′- CGCAGCCTCCTCCTTGTG-3′	5′- GCAACTCTCATTCTTGGTTAATTCC-3′
Human 1L-10	5′- GGACTTTAAGGGTTACCTGG-3′	5′- GTCTGGGTCTTGGTTCTC-3′
Human GATA3	5-CCCAAGCGAAGGCTGTCT-3	5-GGCATTCCTCCTCCAGAGTG-3

IDO: Indoleamine 2, 3-dioxygenase

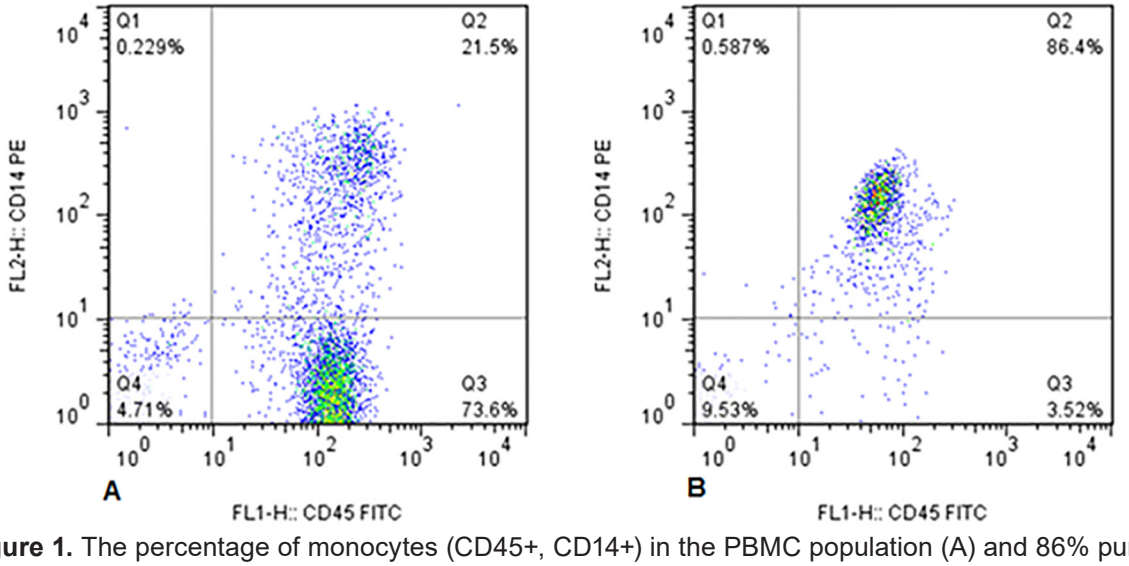

Figure 1. The percentage of monocytes (CD45+, CD14+) in the PBMC population (A) and 86% purity of isolated monocytes (CD45+, CD14+) (B)

95% of these cells could uptake the dye (FITC-dye) (Figure 2). The high uptake by IDCs approved the ability of these cells to pick up probiotics in co-culture for their maturation.

The Expression Results of CD86, CD80 and HLA-DR, CD1a, CD14 and CD83 on the IDC and MDC Surface

On days 5 and 7, the generated floating IDCs and MDCs were analyzed for CD86, CD80 HLA-DR, CD1a, CD14, and CD83 surface markers. As shown in Table 2, the expressions of HLA-DR and CD86 increased during the monocyte differentiation; the expression of both markers on the surface of IDCs was high and increased even further on the surface of the mature inflammatory DCs (MDC-LPS), whereas the expression on maturate modulatory DCs (MDC-Ram , MDC-Del , and MDC-Mix) was similar to that of immature DCs. Since HLA-DR and CD86 markers are involved in the antigen-presenting potency, therefore the expression of these markers on inflammatory DCs is rather greater than on the tolerogenic cells and IDCs. The expression of CD80 and CD83 as co-stimulatory and DC maturation markers on IDCs was low but increased during the maturation stage. These expressions were more prominent on inflammatory DCs (MDC-LPS) than on the modulatory DCs. The increased expression of CD80, similar to HLA-DR and CD86, is directly related to the maturation state and

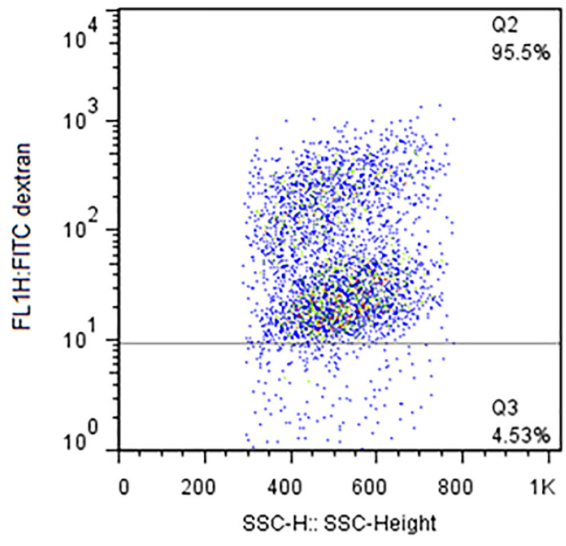

Figure 2. About 95.5% of immature DCs could uptake the dextran dye

also the inflammatory or inhibitory pattern of DCs. However, CD83 is a maturation marker for DCs and rarely found on IDCs, but it gradually increases on mature DCs, however, this expression was greater on inflammatory DCs compared with the tolerogenic phenotypes. CD1a, a DC-related lineage marker, was moderately expressed on IDCs but increased on inflammatory and modulatory DCs after maturation. The expression of the CD14 marker showed an impressive reduction during the DC differentiation. This reduction was significantly more prominent on MDCs than on the IDCs. Nevertheless, CD14 is a monocytic marker with high expression on monocyte lineage, which significantly decreases during DC maturation (Figure 3).

Table 2. Expression of HLA-DR, CD86, CD80, CD83, CD1a and CD14 on IDC and MDC cells following treatment with LPS (inflammatory DC), lactobacillus delbrueckii, lactobacillus rhamnosus and mixed probiotics (modulatory DCs)

CD marker%	IDC	MDC-LPS	MDC-DEL	MDC-RAM	MDC-MIX
HLA-DR	86±2	90±3	85.6±2	87.3±3	81.3±1.5
CD86	84.6±3	90.3±1.1	86.3±2.5	87.6±2.3	82.6±0.5
CD14	7.6±1.1	3±1	5.6±0.5	3.1±1	2.3±0.5
CD83	15.3±2	61±12.7	28.3± 5.7	22±3	25±5
CD1a	33±6	44.6±8	23.3±3.5	41±10.3	49±11.5
CD80	15.6±3.7	38.6±6.1	25.3±2	23.6±6.6	17.6±2.5

IDC: Immature Dendritic Cell; MDC: Mature Dendritic Cell; MDC-LPS: Mature Dendritic Cell with LPS; MDC- DEL: Mature Dendritic Cell with lactobacillus delbrueckii; MDC- RAM: Mature Dendritic Cell with lactobacillus rhamnosus; MDC- MIX: Mature Dendritic Cell with mixed probiotics

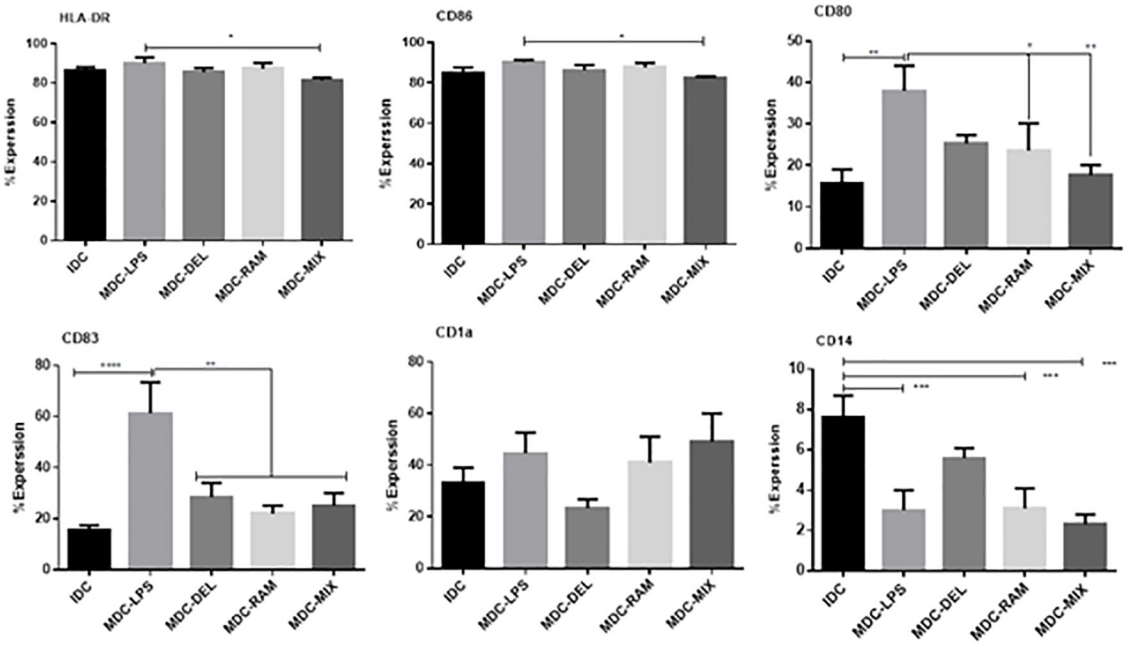

Figure 3. Expression of HLA-DR, CD86, CD80, CD83, CD1a and CD14 on immature and mature DCs following treatment with LPS (inflammatory DCs), *lactobacillus delbrueckii, lactobacillus rhamnosus* and mixed probiotics (modulatory DCs), respectively. # One-way ANOVA test was used for statistical analysis. *(P≤0.05), **(P≤0.005), *** (P≤0.001), IDC: Immature Dendritic Cell; MDC: Mature Dendritic Cell

The Gene Expression Results of IDO, IL10, and IL12 in IDCs and MDCs

The IDO gene expression in IDCs was significantly more than all the probiotics and LPS-treated ones. Probiotic-receiving cells (MDC-Ram, MDC-Del, and MDC-Mix) showed a higher level of IDO transcription compared with the MDC-LPS cells. The level of IDO is linked to the tolerogenic behavior of the immature and tolerogenic DCs; the reduction of IDO expression in inflammatory phenotypes confirmed this relation. Moreover, the expression level of IL12 in IDCs and mature modulatory DCs (MDC-Ram, MDC-Del, and MDC-Mix) were significantly less than that of MDC-LPS cells. The expression of IL10 gene in IDCs and modulatory DCs (MDC-Ram, MDC-Del, and MDC-Mix) increased compared with the inflammatory DCs (MDC-LPS), while not being statistically significant. The reduction in IL12 and the increase in IL10 and IDO expression in IDCs were almost similar to the tolerogenic DCs confirming the inhibitory properties of these cells. In contrast, the inflammatory DCs showed a high IL12 level and low expression of IL10 and IDO expression related to the inflammatory properties of these cells (Figure 4).

DISCUSSION

Dendritic cells are important immune regulating cells and due to their antigenic delivery nature, they express different surface receptors in the form of inflammatory or regulatory phenotypes responsible for the regulation or the activation of immune responses [1]. Immature dendritic cells express lower levels of surface receptors and are considered a vaccine candidate for different diseases [27]. These cells are also helpful in inducing tolerance and controlling the immune response in diseases with an overwhelming immune response such as autoimmune and allergic disorders [28]. Regulatory dendritic cells, due to their tolerogenic nature, modulate the immune system response and are applied in certain cases such as autoimmune diseases, whereas inflammatory DCs can be used in the treatment of cancer and infection in

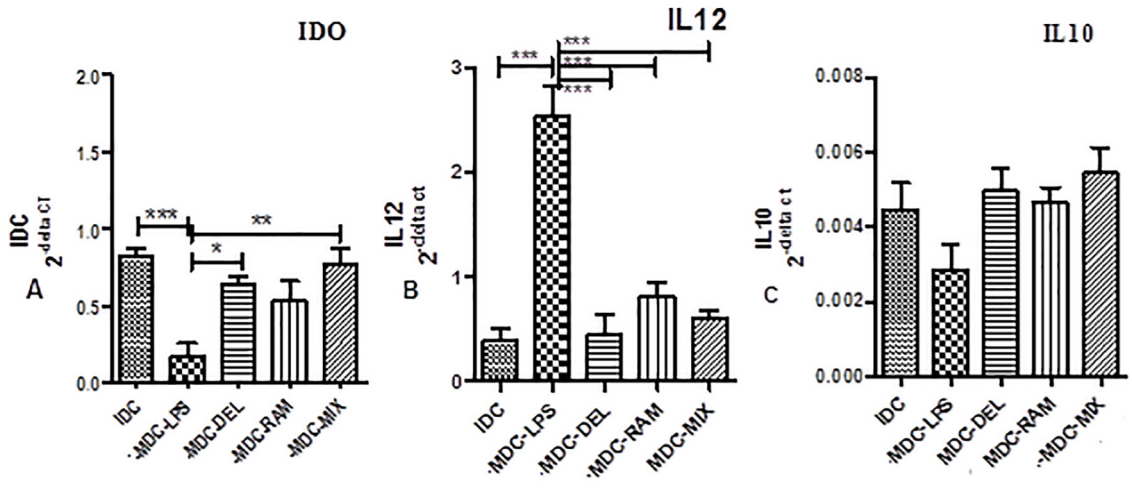

Figure 4. Gene expression analysis of IDO, IL12 and IL10 in IDCs and LPS /probiotic treated MDCs. The results were normalized through endogenous control and the relative gene expression was calculated (A). The relative gene expression of IDO in IDCs and inflammatory/modulatory MDCs (B). The relative gene expression of IL12 in IDCs and inflammatory/modulatory MDCs (C). The relative gene expression of IL10 in IDCs and inflammatory/modulatory MDCs. # One-way ANOVA test was used for statistical analysis *(P≤0.05), **(P≤0.005), ***(P≤0.001), IDC: Immature Dendritic Cell; MDC: Mature Dendritic Cell

which a strengthened immune response is essential [29-31].

Different studies have shown the role of probiotics in DC maturation, function, and cytokines-release modulation[32, 33]. Lactobacillus *rhamnosus Lcr35* showed that could induce regulatory phenotype of cells and increases anti-inflammatory cytokine in an *in vitro* study[34]. In our previous studies, the regulatory and safety effects of *L.delbrueckii* and *L.rhamnosus* were reported both *in vitro* [35] and *in vivo* [36, 37]. In this study, we were able to produce immature DCs from the healthy donors' monocytes in an *in vitro* environment. Subsequently, LPS and tolerogenic probiotics (*L.delbrueckii* and *L.rhamnosus*) were used for the maturation and generation of inflammatory and regulatory dendritic cells for comprehensive use in all types of diseases and not just a specific type. Therefore, we initially evaluated the immature dendritic cell phenotypes to confirm the accuracy of the differentiation process from monocytes. Differentiated immature DCs exhibited reduced monocytic CD14 markers. They also expressed low levels of CD83 and CD80, moderate levels of CD1a, more levels of HLA-DR and CD86. These results were consistent with other similar studies [38, 39]. The augmented expression of IDO and IL10, shown in this study, could signify that these cells may be suitable tools for controlling inflammatory diseases [40]. In the next step, we produced inflammatory dendritic cells by culturing immature DCs in the presence of LPS. Compared with the immature dendritic cells, these cells showed a high expression of co-stimulatory molecules HLA-DR, CD86, CD80 , and CD1a, as well as an increased expression level of CD83 as a dendritic maturation marker; this can be interpreted according to the LPS properties [41]. Unlike immature dendritic cells, IDO decreased and IL12 increased in the inflammatory phenotypes. These features may well explain the role of this phenotype in the immune system activation and the expansion of inflammation throughout the body [42].

The presence of the tolerogenic probiotics (*L.rhamnosus*, *L.delbrueckii,* and a mixture of both) induced the modulatory phenotype of mature DCs with a relative expression level of surface receptors resembling the immature dendritic cells, while being lower than in that of the inflammatory cells. In other words, HLA-DR, CD86, and CD1a were expressed similarly to immature DCs,

while CD80 was expressed at a level higher than the immature DCs but lower than the inflammatory phenotypes. CD83 also increased with dendritic maturity compared with the immature DCs. Modulatory DCs also showed increased expression of IDO and IL10 genes along with reduced levels of IL12; this was similar to the immature DCs and in contrast to the inflammatory phenotypes. In some studies, *L.rhamnosus* and *L.delbrueckii,* have been shown to reduce the inflammatory cytokines while increasing regulatory mediators [24-26, 43, 44]; in the current study, their impact on the production of DCs with inhibitory properties was presented. In future studies, we can investigate the role of generated tolerogenic DCs to induce regulatory T-cells in an *in vitro* study, also the regulatory role of these cells can be evaluated as a vaccine in a mice model.

CONCLUSION

The use of dendritic cells has shed new light on the prevention, control, and possibly the treatment of various diseases by the regulation of immune responses. IDCs have been used as tolerogenic cells to induce tolerance in autoimmune and inflammatory diseases. Our findings revealed that *L.rhamnosus* and *L.Delbrueckii* generated mature DCs with anti-inflammatory phenotypes similar to IDCs in both surface markers and gene expression. These generated tolerogenic DCs from the healthy donors can be widely applied as a therapeutic tool in the management of autoimmune or even allergic diseases in all MHC-matched patients.

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AUTHORS' CONTRIBUTION

Seyed Alireza Esmaeili participated in the performance of the experiment, data collection and drafted the manuscript. Jafar Hajavi and Nafiseh Tabasi were involved in the study performance. Nadia Kia participated as the grammatical editor. Mahmoud Mahmoudi and Maryam Rastin were involved in the grant providing, design, and management of the project. All authors have read and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The Research Council Ethics Committee of Mashhad University of Medical Sciences (MUMS) has approved all protocols and procedures according to the standard guidelines.

Conflict of Interest: None declared.

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