

## Eliciting Th1 Immune Response Using Casein (Alpha S1)-loaded Dendritic Cells

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### ABSTRACT

Allergen-specific immunotherapy (AIT) has been recently considered as an alternative approach to ameliorate the symptoms of allergen exposure and improve the patients' quality of life. Dendritic cells (DC) in the forms of tolerogenic or Th1-induced cells have been investigated in several studies as one of the promising approaches of AIT in allergic diseases.

The aim of this study was to evaluate the potency of casein-loaded DCs in eliciting the Th1 immune responses in Balb/c mice as a potential therapeutic approach in allergic condition. Immature bone marrow-derived DCs were loaded with casein (protein or mRNA) or green fluorescent protein (GFP) mRNA. DCs were evaluated based on the expression of specific markers and production of proinflammatory cytokines. Proliferation and cytokine production of lymph node lymphocytes and splenocytes were measured in DC-injected mice.

Expression of DC markers in all groups was significantly higher than immature DCs, but lower than LPS-activated DCs.

Despite an increase in TNF- $\alpha$  and IL-12, IL-6 was decreased in casein-DC treatments. Casein-loaded DCs could induce proliferation in lymphocytes and stimulate them to produce higher amounts of IFN- $\gamma$  and in some extent IL-10 and TGF- $\beta$ , while they could not stimulate IL-4 secretion. Casein-loaded DCs could partially elicit the Th1 responses; this would be a promising approach to use them as an allergic protective way for applying immune cell therapy in cow's milk allergy.

**Keywords:** Casein; Milk hypersensitivities; Dendritic cells

### INTRODUCTION

Cow's milk allergy (CMA) is known as one of the major causes of food hypersensitivity in children. The

clinical features of CMA manifest as immediate symptoms, ranging from mild local reactions to life-threatening anaphylaxis, which may involve the skin (eg, urticaria and eczema), respiratory tract (eg, asthma and rhinoconjunctivitis), gastrointestinal tract (eg, vomiting, diarrhea, and colic).<sup>1</sup> Among more than 25 various proteins in CMA, there are two main ones that can trigger an allergic reaction: whey proteins that are

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found in liquid part of milk and caseins that are found in solid part (curd) of milk.<sup>2</sup> It has been shown that one of the main cow's milk allergens named alpha 1 casein (CSN1S1) can be readily transferred to mother's milk and causes allergic reactions in predisposed breastfed infants.<sup>2,3</sup>

Allergen-specific immunotherapy (AIT) would be an alternative approach to ameliorate the symptoms subsequent to allergen exposure leading to fewer uses of rescuing anti-allergic drugs and improvement of the patients' quality of life. The benefits of AIT endure at least several years after finishing the treatment due to the features of clinical and immunological tolerance.<sup>4</sup> The considerable impacts of AIT have been indicated through the regulation of effector cells in both innate (dendritic cells, mast cells, basophiles) and adaptive (regulatory T and B cells) immune cells in order to switch the immune responses (from Th2 to Th1) and suppress the proliferation and function of allergen-specific T cells.<sup>5-7</sup> Dendritic cells are the key cells in initiating the allergen-specific immune responses by presenting the processed allergens to T cells in the presence of major histocompatibility complex (MHC) molecules.<sup>8</sup> Surprisingly, several studies have focused on the use of tolerogenic allergen-specific DCs in immunotherapy of allergic patients. For instance, Escobar et al reported tolerogenic latex-specific DCs could modulate allergen-specific T-cell responses and IgE production in natural rubber latex-allergic patients.<sup>9</sup> Frischmeyer-Guerrero et al have reported the original mechanism of sublingual (SLIT) and oral immunotherapy (OIT) as promising treatments in children with IgE-mediated CMA. Although myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) showed different functions in both protocols, they found that pDCs had the main role in dropping allergen-induced Th2 cytokine secretion by CD4+ T cells<sup>10</sup>. Overall, there is a controversy in relationship between DC phenotype/lineage and its role in immune regulation, in both humans and mice.<sup>11</sup>

Despite founding distinct function of splenic CD8 $\alpha$ + lymphoid DCs (to promote a Th1 response) and the CD8 $\alpha$ - myeloid DCs (to promote a Th2 response), recent studies indicated that DCs with distinct functional properties may emerge from the same precursors and present different roles. Therefore, the plasticity in function of DCs and their specific characterization is largely dependent on the expression of co-stimulatory markers and cytokines.<sup>12</sup> According

to the relevant evidences on steering the immune response from Th2 to Th1 following co-culture of autologous T cells with allergen-transfected DCs, using these specific DCs seems to be a promising approach in AIT of allergic patients.<sup>13-15</sup> Therefore, in this study we provided casein aS1 mRNA and protein-loaded DCs derived from bone marrow which can contain a mixture of DC types to evaluated their potency to regulate the immune responses.

## MATERIALS AND METHODS

### Animals and Materials

Balb/c mice (female, 6-8 weeks of age) were obtained from Pasteur Institute (Tehran, Iran). Casein protein and, lipopolysaccharides (LPS) of *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich (USA). Recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were provided from R&D (USA). phycoerythrin (PE)-conjugated monoclonal antibodies against CD11c, CD40, CD86 and MHC-II as well as mouse cytokine ELISA kits were obtained from eBiosciences (Freiburg, Germany). The isotype matched control mAbs were also used for flow cytometry analysis. RPMI-1640 and fetal bovine serum (FBS) were purchased from Invitrogen (USA).

### Bone Marrow Derived DCs Preparation

Dendritic cells were derived from bone marrow of BALB/c mice. Briefly, bone marrow cells were flushed from the bone shafts; RBCs were lysed using hemolysate and the cells were counted after washing. 1-1.5 $\times$ 10<sup>6</sup> cells/mL were cultured in complete RPMI-1640 (Gibco, USA) containing 10% FBS. Then, 20 ng/mL mGM-CSF plus 10 ng/ml mIL-4 were added to the medium on the first day of culture and cells were incubated in an atmosphere of 5% CO<sub>2</sub> at 37 °C. Fresh medium containing mGM-CSF (20 ng/mL) and mIL-4 (10 ng/mL) was added after three and six days of culture and immature DCs (iDCs) were harvested on day six.

### Casein Alpha S1 (CSN1S1) and EGFP-N1 in Vitro mRNA Transcription

CSN1S1 complete cDNA (GenBank: BC109618.1) was inserted in pCMV-Sport6 vector (Imagine, Lifebiosciences, The Netherlands). EGFP encoding region of EGFP-N1 (Invitrogen, USA) was subcloned

## Casein-loaded DCs in Eliciting Th1 Immune Responses

to the pCMV-Sport6 using Sall and NotI restriction sites. In pCMV-Sport6 vector a SP6 promoter upstream of each gene cDNA served as in vitro transcription promoter. CSN1S1 and EGFP-N1 mRNAs were generated by in vitro transcription using the mMESSAGING MACHINES SP6 kit (Ambion, Austin, TX, USA) according to the kit protocol. Quality and quantity of obtained mRNA were evaluated with spectrophotometry and gel electrophoresis. For transfection, one microliter of lipofectamine in 50  $\mu$ L Opti-MEM was mixed with 50  $\mu$ L of 100  $\mu$ g/mL mRNA mixture and incubated in room temperature for 30 minutes and then used for transfection of DCs.

### DCs Allergen Loadings

Immature DCs ( $1 \times 10^6$  cells/mL) were cultured in 24-well flat plates and loaded with casein protein (10  $\mu$ g/mL) or transfected with casein or green fluorescent protein (GFP) mRNA (using lipofectamine system). Intact DCs (DCs without antigen loading) and DCs + LPS (5  $\mu$ g/mL) served as controls. Provided DCs were cultured in one mL final volume of 10% FBS RPMI complete medium for 24 h in 37 °C and 5% CO<sub>2</sub> and then applied for next step evaluations.

### DCs Maturation Markers

After 24 h of manipulation, DCs were evaluated based on the expression of specific DC markers including CD40, CD86 and MHC-II and CD11c using fluorescence-activated cell sorting (FACS) analysis system (BD FACS calibur, USA). The isotype matched control monoclonal antibody (mAbs) were also used and the results were then analyzed using Flowjo software (version 7.6, USA).

### Cytokine Production by DCs

Supernatants of DCs' culture were collected for measurement of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, and interleukin (IL)-12 by mouse enzyme-linked immunosorbent assay (ELISA) kits (ebioscience, USA) according to the manufacture protocol. The limits of sensitivity of the kits were 3.7 pg/mL for TNF- $\alpha$ , 6.5 pg/mL for IL-6, and 10 pg/mL for IL-12.

### Lymphocyte Transformation Test (LTT)

To evaluate specific responses,  $5 \times 10^5$  of casein-loaded DCs and all other generated DCs in different groups were injected to flank region of inbred Balb/c

mice (5 mice in each group). After 7 days, draining lymph nodes were harvested and lymphocytes were passed through the nylon wool. Then,  $2 \times 10^5$  T cells and  $2 \times 10^4$  DCs in each group were co-cultured separately for 72 h. The T cell proliferation (LTT assay) was assessed using a proliferation assay kit I (Roche, Germany) and calculated as stimulation index (SI): (SI=optical density (OD) each test/OD control) and T cells cytokine secretion including IFN- $\gamma$ , IL-4, IL-10 and TGF- $\beta$  were measured according to the instruction of ELISA kits, respectively. The limits of sensitivity of the kits were 4.0 pg/mL for interferon (IFN)- $\gamma$ , 0.32 pg/mL for IL-4, 5.0 pg/mL for IL-10 and 12.0 pg/mL for transforming growth factor (TGF)- $\beta$ .

### In Vivo Systemic Splenocytes Responses

In order to assess induced in vivo systemic immune responses, treated mice were sacrificed on day 7 and the spleen was harvested and smashed. The obtained cell suspensions were cultured  $4 \times 10^5$  cells/well in 10% FBS complete RPMI-1640. After 48 h of incubation in 37 °C and 5% CO<sub>2</sub>, proliferation of splenocytes was measured as previously described for lymph node T cells (LTT assay) and the results were reported as SI. Additionally, the supernatant of each well was collected and measured for IFN- $\gamma$ , IL-4, IL-10, and TGF- $\beta$  by ELISA.

### Statistical Analysis

All in vitro experiments were done in triplicates. Data were analyzed using IBM SPSS software (SPSS Inc., version 15.0, Chicago, IL, USA). *p* values less than 0.05 were reported as statistically significant. Kruskal Wallis and Mann-Whitney U Test were used for within and between group statistical differences, respectively.

## RESULTS

### Maturation Markers of DCs

After loading or transfection of DCs, CD40, CD86, and MHC-II surface maturation markers were evaluated on CD11c+ gated cells (more than 80% positive cells as shown in previously published paper) using flowcytometry (Figure 1).<sup>16</sup> Results showed that DCs loaded with casein protein or transfected with CSN1S1 (or GFP) mRNA molecules expressed higher levels of all three examined markers on protein, mRNA or GFP loaded-DCs in comparison with intact

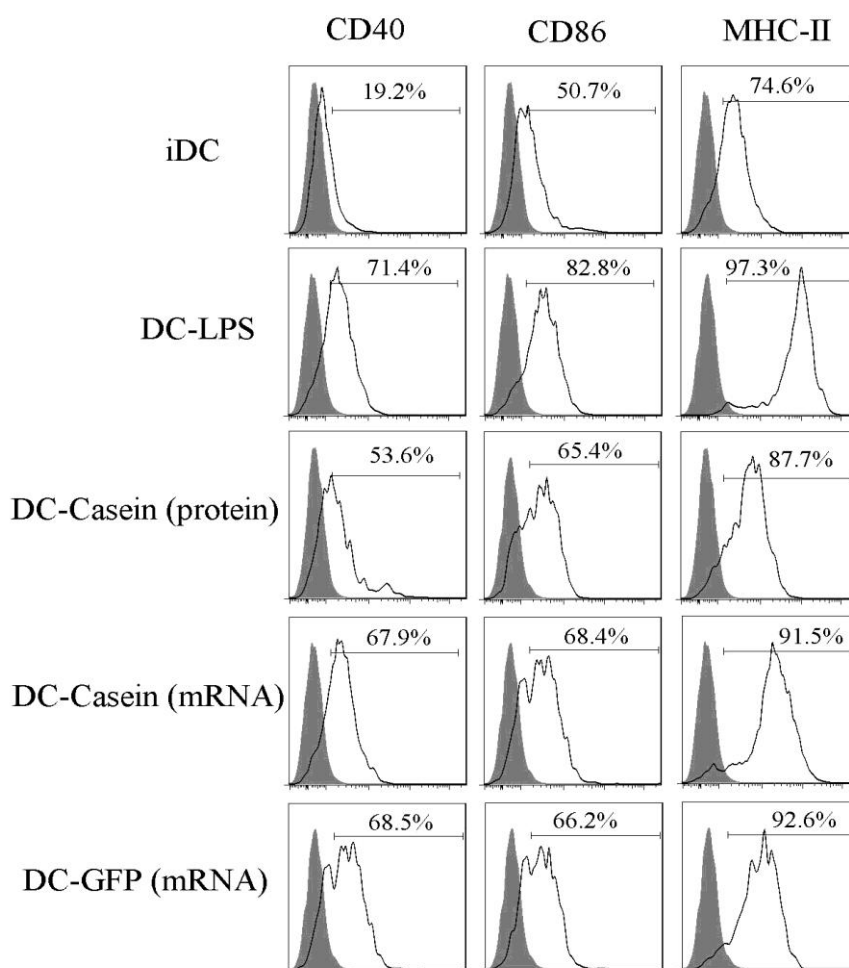
immature DCs but expression of these molecules were lower than those on LPS-activated DCs.

### Cytokine Production of Manipulated DCs

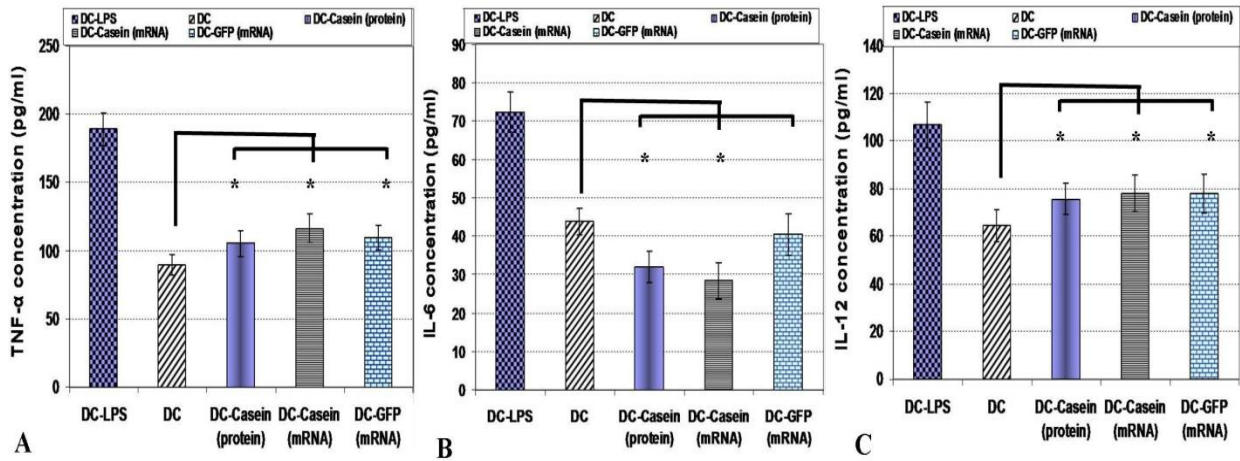
Following 24 h culture of different groups of DCs, pro-inflammatory cytokines like TNF- $\alpha$ , IL-6 and IL-12 were measured to evaluate the effect of pulsing casein protein or mRNA on cytokine production compared to the control groups (Figure 2). Our findings showed that casein protein-loaded or CSN1S1 (and GFP) mRNA-transfected DCs released higher levels of TNF- $\alpha$  and IL-12 in comparison to the intact DCs ( $p < 0.05$ ). On the other hands, casein protein or mRNA loading caused reduced amount of IL-6 production by DCs ( $p < 0.05$ ).

### Lymphocytes Proliferation and Cytokine Release in LTT Assay

Antigen specific proliferation of lymphocytes extracted from lymph nodes of manipulated DC-injected mice was evaluated following co-culture of lymphocytes with the same DCs generated in different groups (Figure 3). Cytokine production of T cells was examined to determine the dominant T cell immune responses induced by different groups of DCs (Figure 4). Enhancement of antigenspecific cell proliferation was observed for all three casein protein, casein mRNA and GFP mRNA loading of DCs compared to intact DCs ( $p < 0.05$ ). Despite higher SI of test groups than that of LPS-matured DCs (as a positive control), the differences were not statistically significant. All of

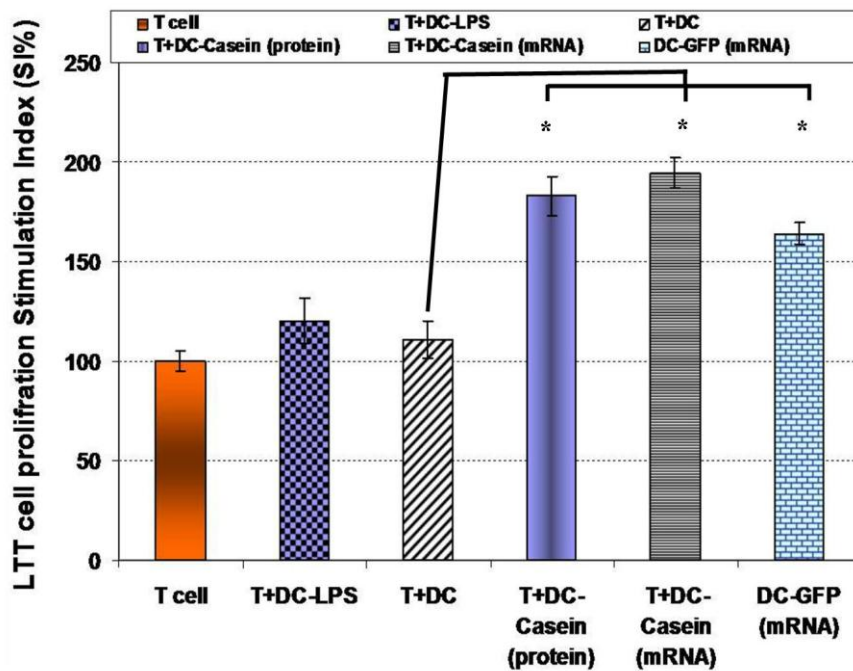


**Figure 1.** Dendritic cells (DCs) maturation markers on CD11c<sup>+</sup> gated cells in different groups are shown. DCs loaded with casein  $\alpha$ 1 protein or transfected with mRNA molecules expressed higher levels of maturation markers on loadings and manipulations compared to intact immature DCs while they were lower than those matured by lipopolysaccharide (LPS). Grey histogram represents isotype control. GFP: green fluorescent protein



**Figure 2.** Dendritic cells (DCs), TNF- $\alpha$ , IL-6, and IL-12 cytokine secretions are shown. In comparison to intact DCs, casein aS1 protein-loaded or mRNA-transfected DCs released higher levels of TNF- $\alpha$  and IL-12 but diminished production of IL-6. All data are represented as the mean value of experiments in triplicates. Mann-Whitney U Test was used for between group statistical differences. Statistically significant differences are shown with \* ( $p < 0.05$ ).

GFP: green fluorescent protein, LPS: lipopolysaccharide



**Figure 3.** Lymphocytes from collected lymph nodes of test and control DC-injected mice cultured with manipulated DCs in vitro and lymphocytes proliferation were assessed in a setting of lymphocyte transformation test (LTT). DCs loaded with casein aS1 (both protein and mRNA forms) induced specific proliferation of lymphocytes while specific responses to GFP protein was also observed. All data are represented as the mean value of experiments in triplicates. Mann-Whitney U Test was used for between group statistical differences. Statistically significant differences are shown with \* ( $p < 0.05$ ). GFP: green fluorescent protein, LPS: lipopolysaccharide

these groups also induced IFN- $\gamma$  release and suppressed production of IL-4 ( $p < 0.05$ ). Moreover, induction of IL-10 secretion was seen by casein (protein or mRNA) loadings DCs ( $p < 0.05$ ) while these cells could not suppress TGF- $\beta$  release ( $p > 0.05$ ).

### DC-treated Mice Splenocytes Proliferation and Cytokines Release

Systemic shift of the immune responses in DC-treated mice was evaluated by examination of splenocytes proliferation (Figure 5) and their IFN- $\gamma$ , IL-4, IL-10

and TGF- $\beta$  cytokine secretion patterns (Figure 6). There were not any significant differences in splenocytes proliferation between different DC-treated mice groups ( $p > 0.05$ ). Cell treatment of mice with casein- (both protein and mRNA form) loaded DCs caused a gentle but statistically significant increase of IFN- $\gamma$  secretion and suppression of IL-4 production ( $p < 0.05$ ). We could not find any significant differences between groups in terms of releasing IL-10 and TGF- $\beta$  after 48 h of cultures ( $p > 0.05$ ).

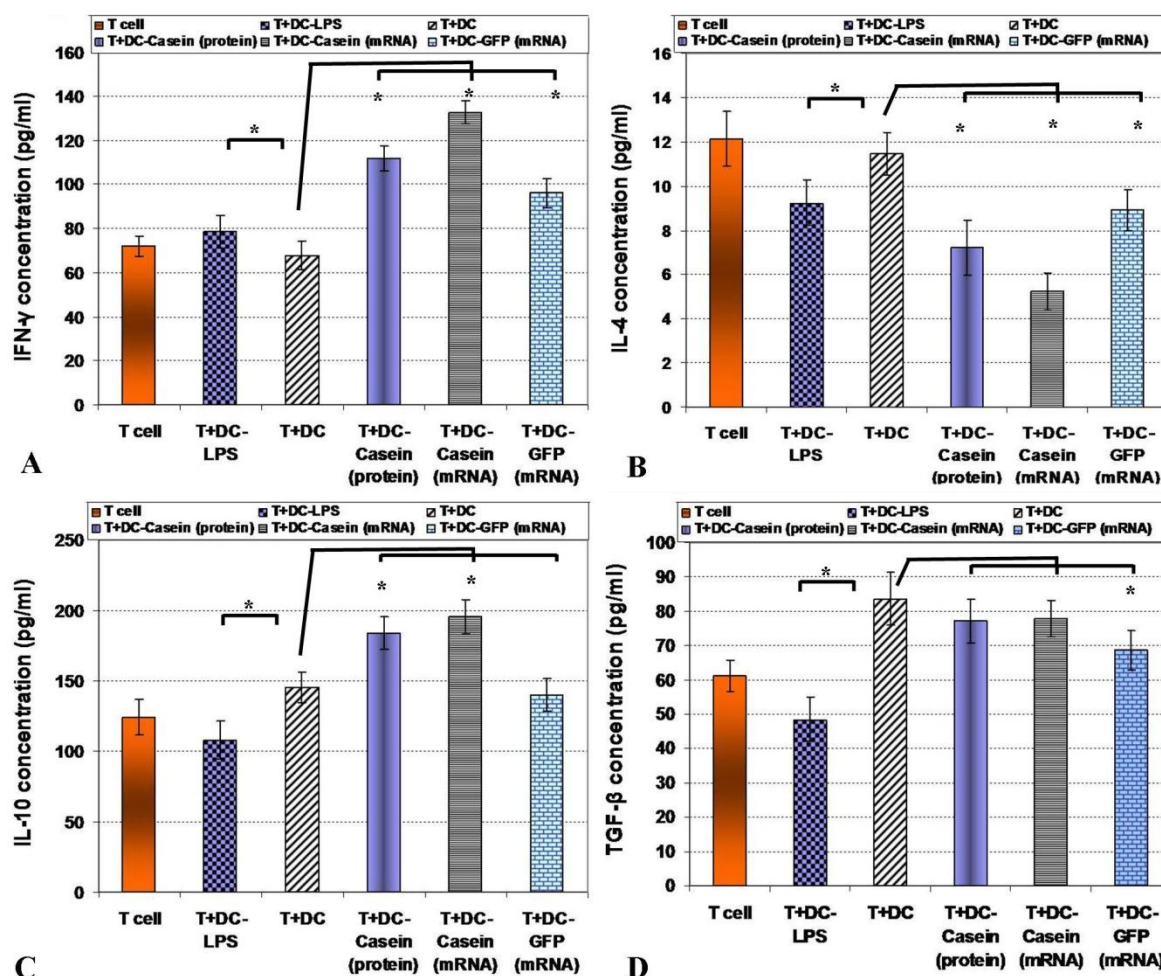


Figure 4. Lymphocytes from collected lymph nodes of test and control DC-injected mice cultured in the presence of manipulated DCs in vitro and cytokines (IFN- $\gamma$ , IL-4, IL-10 and TGF- $\beta$ ) were assessed. Both casein aS1- (both protein and mRNA forms) and GFP-loaded DCs induced IFN- $\gamma$  and suppressed IL-4 release but specifically casein aS1-loaded DCs induced secretion of IL-10, while it could not suppress TGF- $\beta$  release. All data are represented as the mean value of experiments in triplicates. Mann-Whitney U Test was used for between group statistical differences. Statistically significant differences are shown with \* ( $p < 0.05$ ).

GFP: green fluorescent protein, LPS: lipopolysaccharide

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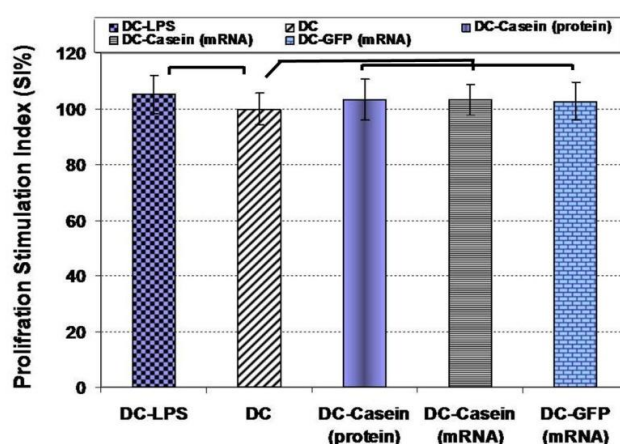


Figure 5. A systemic shift of the immune responses in DC-treated mice was evaluated by examination of splenocytes proliferation. All data are represented as the mean value of experiments in triplicates. Mann-Whitney U Test was used for between group statistical differences. There were not any significant differences in splenocytes proliferation between different groups of mice. GFP: green fluorescent protein, LPS: lipopolysaccharide

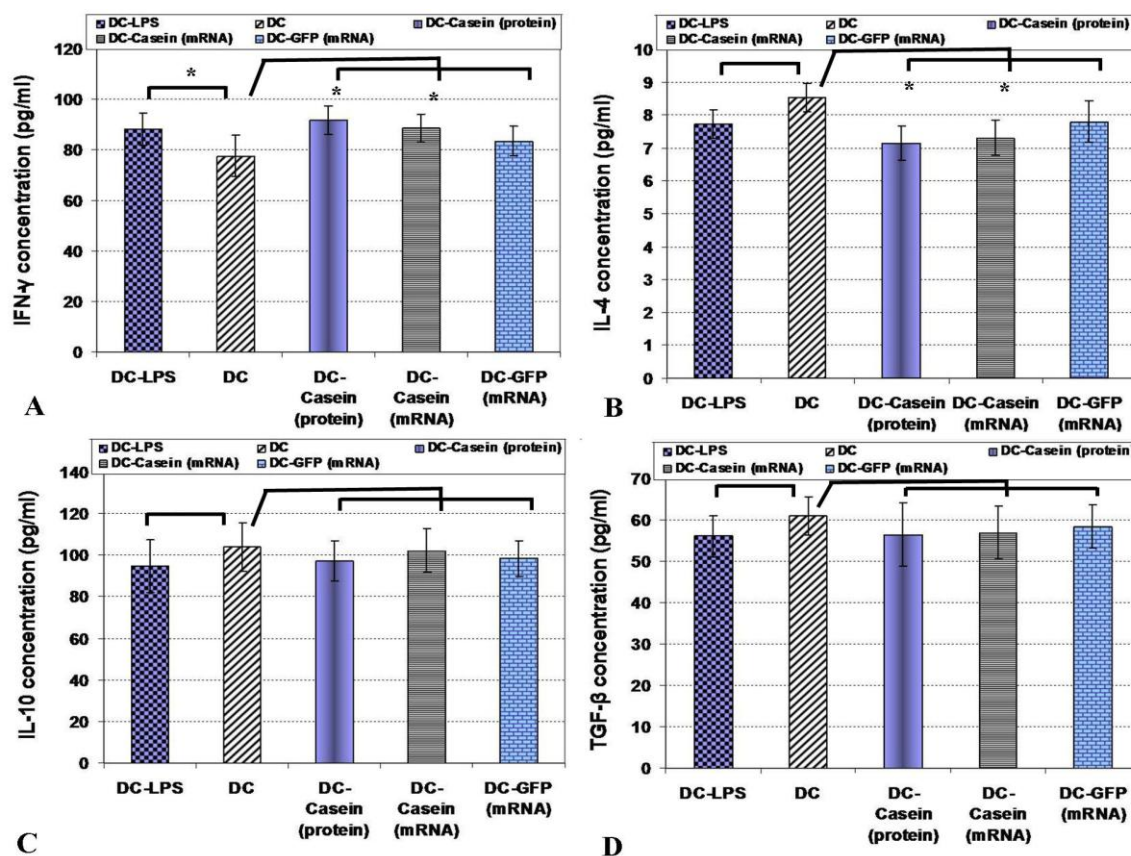


Figure 6. A systemic shift of the immune responses in DC-treated mice was evaluated by assessing the cytokine production of splenocytes including IFN- $\gamma$ , IL-4, IL-10 and TGF- $\beta$ . A mild but statistically significant increase of IFN- $\gamma$  secretion and suppression of IL-4 production were observed in mice treated with casein aS1 especially for mRNA transfected DCs. Splenocytes in evaluated groups did not show any differences in release of IL-10 and TGF- $\beta$ . All data are represented as the mean value of experiments in triplicates. Mann-Whitney U Test was used for between group statistical differences. Statistically significant differences with the p value of less than 0.05 are shown with \*. GFP: green fluorescent protein, LPS: lipopolysaccharide

## DISCUSSION

Despite the established clinical benefits of standard AIT performed by subcutaneous injection or sublingual application, most of the allergic patients find them as difficult and time-consuming procedures.<sup>6,7</sup> Although the beneficial effects of milk oral immunotherapy has been recently shown in several studies, the concomitant undesirable side effects and even unchanged levels of IgE have also been reported.<sup>17,18</sup> Considering pathophysiology of allergy and crucial roles of APCs, especially DCs in launching the immune responses, they direct scientists toward using DCs in immunotherapy procedures.<sup>19</sup>

Here, we evaluated the potency of casein-loaded DCs to shift the immune responses. Our results showed that casein- (protein or mRNA) loaded DCs had a potential capacity to elicit a Th1 instead of Th2 immune response. These DCs could express higher levels of DC specific markers (CD40, CD86 and MHC-II) than intact DCs, although the expression was not as much as LPS-activated DCs as a positive control. In addition, the potential activity of manipulated DCs in proliferating autologous T cells in a co-culture setting confirmed the ability of DCs in presenting allergen by MHC molecules in the presence of up-regulated co-stimulatory molecules (CD40 and CD86).

In a recent study, a fused molecule has been designed containing Der f 1 as an important asthma allergen of *Dermatophagoides farinae* and invariant chain (Ii)-segment as a basis to enhance the efficacy of vaccine to stimulate an immune response to asthma. The vaccine was designed to target the MHC class II pathway and could shift the cytokine profile by stronger secretion of IFN- $\gamma$  and IL-10, and a decreased production of IL-4 and IL-17.<sup>20</sup>

In contrast, Ashjaei et al indicated that allergen-pulsed monocyte-derived DCs of polysensitized allergic patients were capable of proliferating T cells and shifting them toward allergen-specific Th2 in cells stimulated with allergens while the patients were sensitized before and not for the other unexposed allergens.<sup>14</sup>

In the present study, although provided DCs could express molecules required for specifically presenting allergen to T cells, but the amount of these expressions were lower than fully activated DCs (LPS-activated group). This phenotype is appropriate for induction of

regulatory responses and modulating allergic reactions. For instance a study showed that diminished levels of CD40 using siRNA systems could induce allergy protective responses in allergy mice model.<sup>21</sup> Although transfection of DCs with protein or mRNA could activate cells for producing pro-inflammatory cytokines like TNF- $\alpha$  and IL-12, it inversely resulted in diminished capacity for IL-6 secretion in casein-loaded (protein or mRNA) DCs. Induction of TNF- $\alpha$  and IL-12 was accompanied with DCs activation and also subsequent Th1 derivations.

In allergic conditions, a change in the intestinal DC subsets from tolerogenic to inflammatory DCs makes them susceptible following recognition of allergens to polarize naïve T cells into Th2 cells in the presence of IL-4 (mostly produced by allergen-activated innate immune cells).<sup>25</sup> Therefore, steering the immune responses towards the Th1 or regulatory T cells would be valuable to control the Th2 immune responses.

Regarding suppression of IL-6 and parallel to our finding, a study also showed that oral and sublingual immunotherapy decreased IL-6 secretion by pDCs and mDCs, respectively in a TLR-activated manner.<sup>10</sup> In a study published by Vordenbaumen et al, the immunomodulatory role of CSN1S1 on in vitro differentiating of macrophage-like cells from monocytes has been reported. This effect might be a consequence of inducing pro-inflammatory cytokines (IL-6 or IL-1 $\beta$ ), which could be suppressed in the presence of JNK and p38 inhibitors.<sup>26</sup> In milk allergy immunotherapy, a study showed that intradermal administration of alpha s1-casein protein (similar to our protein) to mice induces substantial immunological tolerance in the antibody responses of IgG2a and IgG2b (Th1-induced subclasses) and of IgG1 (Th2-induced subclasses) against intact protein antigen.<sup>27</sup>

The potential activity of provided DCs to induce the immune response through priming T cells was evaluated in a co-culture of draining lymph node T cell and casein-loaded DCs in DC-injected mice. Antigen-specific responses including the considerable proliferation of T cells, higher production of IFN- $\gamma$  and lower secretion of IL-4 than control for all three sets of casein protein, casein mRNA and also GFP were observed. Noticeably, casein-loaded (protein or mRNA) DCs could release anti-inflammatory cytokines like IL-10 and TGF- $\beta$ , which might be as a result of launching the cytokine cascade signaling pathway and



modulating the immune responses toward regulatory responses.<sup>22</sup>

GFP is a small green fluorescent protein that is commonly utilized as a co-expressed marker for easily sorting the gene-transferred cells. Unexpectedly, the GFP-derived peptide can be presented by MHCs and trigger the GFP-specific T cells.<sup>23</sup> For that reason, although the GFP was used just as a marker to monitor the correct transferring of protein, an immune response was found against it. Similar studies have been conducted to evaluate the potential efficacy of OVA-loaded DCs to regulate the immune responses in OVA-induced allergic asthma.<sup>24</sup>

In order to find out the response of immune system, we assessed the proliferation capacity and cytokine production of splenocytes in DC-treated mice without second challenging. Mice splenocytes showed a mild enhancement of cell proliferation and IFN- $\gamma$  release and suppression of IL-4 secretion. We could not detect any significant differences between release of IL-10 or TGF- $\beta$  from splenocytes of treated and untreated mice groups.

Although producing IFN- $\gamma$  can be considered as one of the major signs of successful immunotherapy in allergic diseases, but more confirmation using the specific transcription factors (e.g. T-bet) or the other cytokines (like IL-2) is needed to be done in the future studies.

In this study we showed that DCs loaded with casein protein and also casein mRNA could elicit partially a Th1 immune response, which can be a potential way to protect the allergic responses. However, more evaluations in allergic animal models is obviously needed. Our manipulated DCs showed active form in terms of phenotype and cytokine secretions as well as lymphocytes responses toward Th1 cytokine pattern in mice.. Loaded DCs also caused systemic Th1 immune responses. Further studies may lead to direct cell therapy of allergies and more appropriate understandings of allergic diseases management.

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