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Experimental study of the mechanism of tolerance induction in dexamethasone-treated dendritic cells

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: The aim of this study was to investigate the mechanisms underlying tolerance induction of dexamethasone (Dex)-treated dendritic cells (DCs).

Material/Methods: Well-grown DC2.4 cells were randomly assigned to receive control, 50 μg/L, 100 μg/L, or 200 μg/L of dexamethasone and then were cultured for 6 days. The expressions of CD80, CD86, galectin-9, and PD-L1 on the surface of DC2.4 cells were analyzed with flow cytometry and the level of IL-12 secreted by DC2.4 cells was determined by ELISA. The stimulating activity of DC2.4 cells on allogeneic T cells was assessed with mixed lymphocyte reaction. Dexamethasone-treated DC2.4 cells were co-cultured with allogeneic splenic lymphocytes and the Foxp3 expression in naive T lymphocytes was determined with flow cytometry.

Results: Compared with the control group, the expressions of CD80, CD86, galectin-9, and PD-L1 on the surface of DC2.4 cells exposed to different doses of dexamethasone showed no significant changes; however, dexamethasone treatment significantly reduced IL-12 secretion and inhibited DC2.4's stimulation on the proliferation of allogeneic T lymphocytes. Moreover, dexamethasone-treated DC2.4 cells effectively promoted FOXP3 expression in naive T lymphocytes.

Conclusions: DC2.4 is a stable cell line with high expressions of CD80, CD86, and PD-L1. Dexamethasone does not significantly change the cell phenotype of DC2.4 cells, but inhibits the secretion of IL-12 cytokine and attenuates DC2.4's stimulation of the proliferation of allogeneic T cells. Dexamethasone-treated DC2.4 cells also effectively promote FOXP3 expression in naive T lymphocytes.

key words: DC2.4 • galectin-9 • PD-L1 • FOXP3 • dexamethasone • immune tolerance

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BACKGROUND

Dendritic cells (DCs) are involved in immune response against foreign antigens and play an important role in inducing immune tolerance. Previous studies have shown that DC-induced immune tolerance is mainly achieved through inducing T cell anergy and T cell clone deletion and promoting immunosuppression of regulatory T cells [1,2]. Galectin-9, a ligand of Tim3 (Tim-3L), plays an important role in suppressing graft rejection [3]. Programmed death ligand 1 (PD-L1), also known as B7-H1, belongs to the B7 family of costimulatory molecules. Studies have demonstrated that binding of PD-L1 to PD-1 markedly inhibits the function of T cells [4].

Dexamethasone, an immunosuppressant, is widely used for inhibiting organ-graft rejection in clinical practice. However, the long-term application of dexamethasone has obvious adverse effects on the human body. Thus, it has been a research focus in the field of transplantation immunology to seek new immunosuppressants or immunosuppressive therapies with high curative effects and lower or even no adverse effects. Induction of DC cells' tolerance is very important in inhibiting graft rejection. Studies have shown that dexamethasone exerts its effects by modulating the function of DCs in the early stages of immune response. Dexamethasone can promote the generation of tolerogenic dendritic cells that have immune regulatory function, and further induces immune tolerance in the body [5,6]. In this study, the phenotype and functional changes of DC2.4 cells after dexamethasone treatment were investigated in order to explore the underlying mechanisms of dexamethasone-treated DC cells in inducing immune tolerance and inhibiting immune response.

MATERIAL AND METHODS

Materials

The mouse DC2.4 cell line was provided by the Department of Molecular Immunology, Institute of Basic Medical Research, Academy of Military Medical Sciences. Male BALB/c mice weighing 18–22 g and raised under SPF condition were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences.

Antibodies used in the study included rmIL-2(PEPROTECH, USA), PE-labeled anti-mouse CD80 mAb, CD86 mAb, and matched isotype control (BD, USA), PE-labeled anti-mouse Galectin-9 (Tim-3L) mAb and matched isotype control (Biolegend, USA), PE-labeled anti-mouse PD-L1 (B7-H1) mAb and matched isotype control (Biolegend, USA), FITC-labeled anti-mouse CD4 mAb and matched isotype control (Biolegend, USA), and PE-labeled anti-mouse FOXP3 mAb and matched isotype control (eBioscience, USA). Other reagents used included fixation/permeabilization solution (eBioscience, USA), 10× permeabilization buffer (eBioscience, USA), mouse IL-12 ELISA kit (Dakewe, China), CCK-8 (Dojindo Laboratories, Japan), RPMI-1640 culture medium (Hyclone, USA), fetal bovine serum (Beijing Yuanhengjinma, China), dexamethasone sodium phosphate injection (Tianjin Kingyork, China), and 1% paraformaldehyde fixation solution.

DC2.4 cell culture and grouping

Well-grown DC2.4 cells were seeded onto 6-well culture plates at a density of 10^4 cells/well and maintained in RPMI-1640 complete medium supplemented with 10% fetal bovine serum. Each treatment was done in triplicate. The cells were randomized to 4 groups: group A, control group; group B, 50 μg/L dexamethasone; group C, 100 μg/L dexamethasone; and group D, 200 μg/L dexamethasone. The cells were grown in an incubator (37°C, 5%CO_2). Half of the medium was replaced every 2 days and the concentrations of dexamethasone were kept constant. The cells were cultured for a total of 6 days.

Changes in biological properties of dexamethasone-treated DC2.4 cells

Morphology

Morphological changes of cells were observed microscopically.

Determination of growth curve and doubling time

After 6 days of culture, well-grown DC2.4 cells treated with 0, 50 μg/L, 100 μg/L, or 200 μg/L of dexamethasone were seeded onto 12-well culture plates at a density of 10^4 cells/well. Each condition was run in triplicate. The cells were then randomly assigned to 4 groups: group A, control group; group B, 50 μg/L dexamethasone; group C, 100 μg/L dexamethasone; and group D, 200 μg/L dexamethasone. The cells were cultured as described above. Three wells were chosen each day to count cell numbers for 6 consecutive days. A growth curve was plotted by designating culture time as the abscissa axis and the number of cells as the ordinate axis. The doubling time of the cells in logarithmic growth was calculated by using Patterson's formula: Td=Tlg2/lg(Nt/N0), where Td is defined as the doubling time (h); T is defined as the time needed for the number of cells growing from N0 to Nt; and N denotes the number of cells.

Flow cytometry analysis for CD80, CD86, galectin-9, and PD-L1 expressions in DC2.4 cells

Cultured DC2.4 cells were collected at day 6 and washed twice with phosphate-buffered saline (PBS)., The cells were resuspended in PBS to a cell density of (1~2)×10^6/mL, and then 200 μL of cell suspensions was added to each test tube, followed by addition of 1 μL of PE-labeled anti-mouse CD80 mAb, 1 μL of CD86 mAb, 1.5 μL of Galectin-9mAb, 1 μL of PD-L1 mAb, or corresponding isotype controls. The mixtures were incubated in the dark at 4°C for 30 min before centrifugation at 2000 r/min for 5 min. Next, the cell pellets were washed twice with PBS, and 200 μL of 1% paraformaldehyde fixation solution was added. The mixtures were kept in the dark at 4°C until analysis.

ELISA for IL-12 levels in the culture supernatants

At day 6, cell culture supernatants were collected and centrifuged at 5000 r/min for 5 min. The levels of IL-12 in the supernatants of all groups were determined using a mouse IL-12 ELISA kit following the manufacturer's instructions.

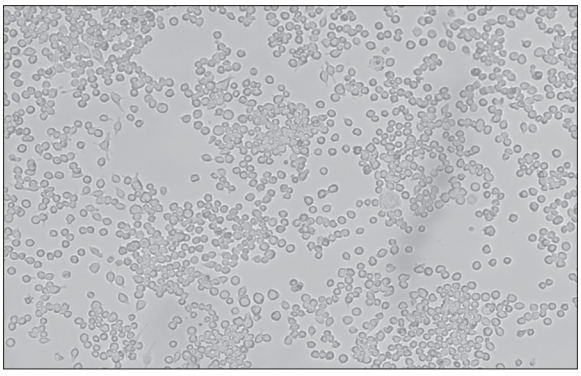

Figure 1. Adherent DC2.4 cells were adherent (100×).

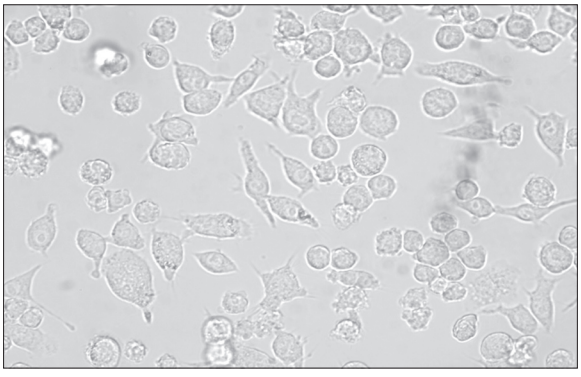

Figure 2. DC2.4 cells were dendritic, irregular or spindle-shaped (400×).

Mixed lymphocyte reaction

At day 6, DC2.4 cell suspensions (1×10^5/ml) were taken and mitomycin C was added to a final concentration of 30 μg/mL. The mixture was incubated in water wash at 37°C for 30 min before centrifugation at 2000 r/min for 5 min. The cell pellets were washed twice with RPMI-1640 medium and centrifuged at 2000 r/min for 5 min after each wash. The cell pellets were resuspended in the RPMI-1640 medium to a final concentration of 1×10^5/ml and these cells were used as stimulator cells. Meanwhile, single cell suspension was prepared by harvesting the spleens of BALB/c mice under sterile conditions; lymphocytes were isolated by use of lymphocyte separation medium and the cell density was adjusted to 1×10^6/ml. These cells were used as responding cells, 100 μL of stimulator cells or responding cells were added to each well on the 96-well plates and the responding cells were used as controls. Each sample was tested in triplicate. The cells were incubated in an incubator (37°C, 5% CO_2) for 3 days, and 20 μl of CCK-8 was added to each well 4 h before the end of culture. After culture, the optical absorbance values at 450 nm were recorded by a microplate reader. The stimulation index (SI) was calculated as optical absorbance values of the experimental group at 450 nm/optical absorbance values of control group at 450 nm. The results reported were the mean of 3 wells.

Dexamethasone-treated DC2.4 cells promote FOXP3 expression in naive T cells

Dexamethasone-treated DC2.4 cells at day 6 of culture were collected and adjusted to a cell density of 1×10^6/ml.

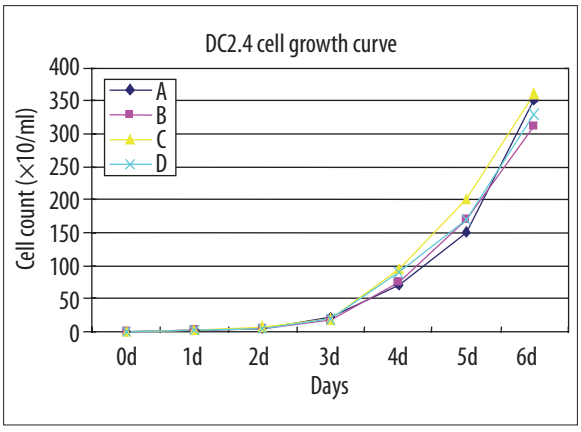

Figure 3. Growth curve of DC2.4 cells. (**A**) control group; (**B**) 50 μg/L Dex group; (**C**) 100 μg/L Dex group; (**D**) 200 μg/L Dex group.

Meanwhile, single cell suspension was prepared by harvesting the spleen of BALB/c mice under sterile conditions; lymphocytes were isolated by use of lymphocyte separation medium and the cell density was adjusted to 1×10^6/ml. T cells were added to 6-well plates at a density of 1×10^6/well. Subsequently, DC2.4 cells treated with 0 or 100 μg/L of dexamethasone were added to the plates at a ratio of 1:10 (T cells: DC2.4 cells) and 300U/ml IL-2 was added to each well. T cells with no addition of DC2.4 cells were used as controls. The cells were cultured in an incubator (37°C, 5%CO_2) for 5 days.

Flow cytometry analysis for CD4+FOXP3+ expressions of T cells

After 5 days of culture, T cell suspensions were washed twice with PBS and the cell pellets were collected. Meanwhile, control T cells were collected and 200 μL of PBS containing 2×10^5 T cells were added to each test tube, followed by addition of 1.5 μL of FITC-labeled anti-mouse CD4 mAb or an equal volume of isotype control. Thereafter, the test tubes were kept in the dark at 4°C for 30 min before centrifugation at 1000 r/min for 10 min to pellet the cells. The cell pellets were washed once with PBS and then treated with 400 μL of permeabilization buffer (1:3 dilution). The cells were placed in the dark overnight. After centrifugation, the cell pellets were washed once with PBS and once with 1× permeabilization buffer, followed by addition of 1× permeabilization buffer to resuspend the cells to a final volume of 200 μL. One μL of PE-labeled anti-mouse FOXP3 mAb or an equal amount of isotype control was added to the cells. The cells were then kept in the dark at 4°C for 30 min before centrifugation at 1000 r/min for 10 min. The resultant cell pellets were washed with 1× permeabilization buffer and subsequently fixed in 200 μL of 1% paraformaldehyde solution. The cells were kept at 4°C in the dark until analysis.

Statistical analyses

All statistical analyses were performed by using SPSS software version 11.5. Continuous variables are presented as mean ± standard deviation. Differences in the means of various groups were compared using one-way ANOVA. Multiple comparisons of the means of various groups were conducted using the LSD test. P values less than 0.05 were considered to be statistically significant.

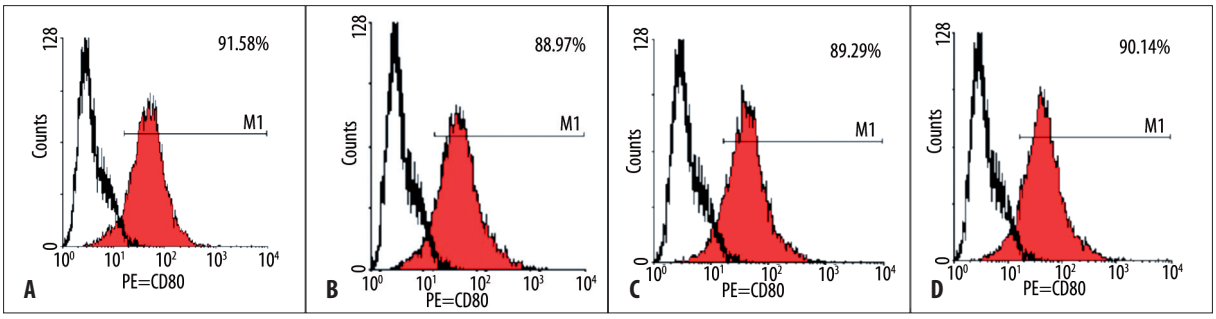

Figure 4. Flow cytometry showing the expression of CD80. (**A**) control group; (**B**) 50 μg/L Dex group; (**C**) 100 μg/L Dex group; (**D**) 200 μg/L Dex group.

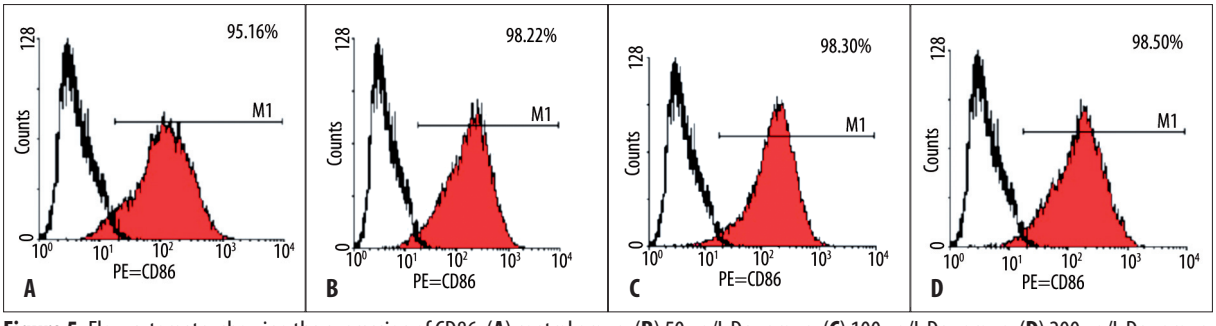

Figure 5. Flow cytometry showing the expression of CD86. (**A**) control group; (**B**) 50 μg/L Dex group; (**C**) 100 μg/L Dex group; (**D**) 200 μg/L Dex group.

Results

Changes in cell morphology

Microscopically, DC2.4 cells appeared to be dendritic, irregular or spindle-shaped and adherent. Dexamethasone treatment did not result in notable changes in the morphology of DC2.4 cells examined under a microscope (Figures 1, 2).

Growth curve and doubling time

The growth curve of DC2.4 cells was plotted according to the results of cell counting (Figure 3). The doubling times were 17.04 h, 17.40 h, 16.96 h, and 17.21 h for the control group, 50 μg/L dexamethasone group, 100 μg/L dexamethasone group, and 200 μg/L dexamethasone group, respectively, showing no significant difference in doubling time between dexamethasone-treated groups and the control group. In the first 3 days of culture, the number of cells did not markedly increase and the cells were in the latent phase. Thereafter, the number of cells began to increase markedly, indicating that the cells were in the exponential growth phase. Dexamethasone treatment caused no observable abnormality in the growth of DC2.4 cells. Microscopically, DC2.4 cells in various groups reached nearly full confluence at day 5 and completely covered the culture plates at day 6, when local cell clusters were noted.

Expressions of CD80, CD86, galectin-9, and PD-L1 in DC2.4 cells

The percentage of DC2.4 cells that expressed CD80 was 87.02±4.78% in the control group, 84.46±3.90% in the 50 μg/L dexamethasone group, 86.23±2.84% in the 100 μg/L dexamethasone group, and 86.45±3.28% in the 200 μg/L dexamethasone group (Figure 4).

The percentage of DC2.4 cells that expressed CD86 was 96.46±2.31% in the control group, 95.98±1.96% in the 50 μg/L dexamethasone group, 98.28±1.44% in the 100 μg/L dexamethasone group, and 96.88±1.45% in the 200 μg/L dexamethasone group (Figure 5).

The percentage of DC cells that expressed galectin-9 was 14.20±2.32% in the control group, 13.37±3.32% in the 50 μg/L dexamethasone group, 12.51±1.45% in the 100 μg/L dexamethasone group, and 13.24±2.31% in the 200 μg/L dexamethasone group (Figure 6).

The percentage of DC cells that expressed PD-L1 was 94.11±2.21% in the control group, 95.06±2.56% in the 50 μg/L dexamethasone group, 95.90±2.24% in the 100 μg/L dexamethasone group, and 93.65±1.90% in the 200 μg/L dexamethasone group (Figure 7).

No significant differences in the expression levels of CD80, CD86, galectin-9, and PD-L1 were noted among groups ($P>0.05$).

IL-12 contents in the culture supernatants

The standard curve was constructed by using concentrations of standards, yielding an equation: $y=275.927x–87.625$ ($R^2=0.953$). As shown in Table 1, the IL-12 level in the supernatant was (126.576±6.305)pg/ml in the control group, (42.548±12.157)pg/ml in the 50 μg/L dexamethasone group, (31.879±6.079) pg/ml in the 100 μg/L dexamethasone group, and (37.237±14.170) pg/ml in the 200 μg/L dexamethasone group ($F=56.855$, $P=0.000$). Compared with the control group, the IL-12 level was significantly lower in dexamethasone-treated groups ($P<0.05$) (Table 1).

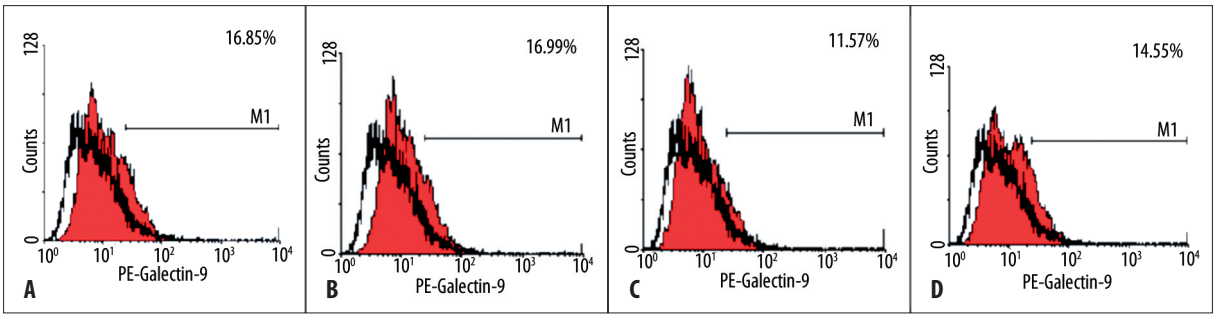

Figure 6. Flow cytometry showing the expression of galectin-9. (**A**) control group; (**B**) 50 μg/L Dex group; (**C**) 100 μg/L Dex group; (**D**) 200 μg/L Dex group.

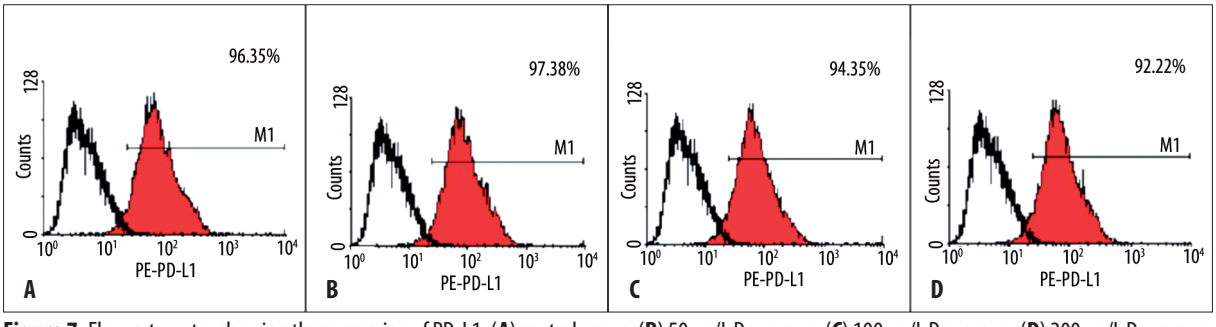

Figure 7. Flow cytometry showing the expression of PD-L1. (**A**) control group; (**B**) 50 μg/L Dex group; (**C**) 100 μg/L Dex group; (**D**) 200 μg/L Dex group.

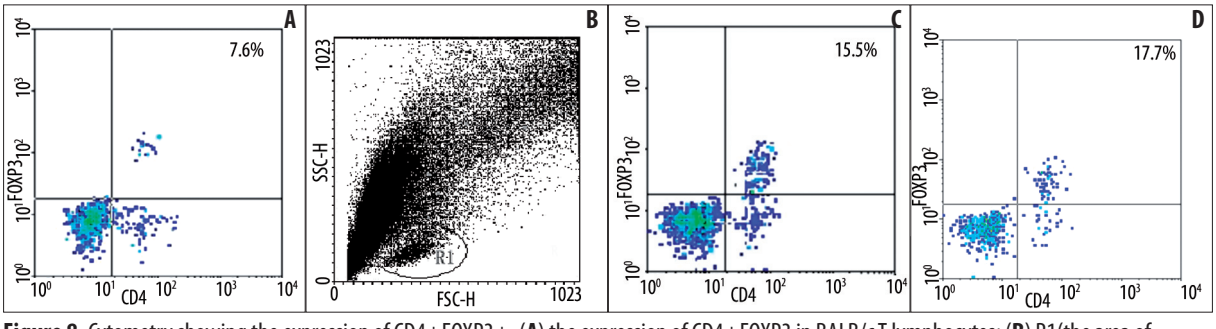

Figure 8. Cytometry showing the expression of CD4+FOXP3+. (**A**) the expression of CD4+FOXP3 in BALB/c T lymphocytes; (**B**) R1(the area of lymphocytes); (**C**) the expression of CD4+FOXP3 in BALB/c T lymphocytes co-cultured with DC2.4; (**D**) the expression of CD4+FOXP3 in BALB/c T lymphocytes co-cultured with Dex treated DC2.4 cells.

SI measured by mixed lymphocyte reaction

The SI was 3.986±0.119 in the control group, 1.739±0.043 in the 50 μg/L dexamethasone group, 1.480±0.064 in the 100 μg/L dexamethasone group, and 1.647±0.084 in the 200 μg/L dexamethasone group (F=623.129, P=0.000). Compared with the control group, the SI was significantly decreased in various dexamethasone-treated groups (P<0.05). Compared with the 50 μg/L and 200 μg/L dexamethasone groups, the 100 μg/L dexamethasone group had a dramatically lower SI (P<0.05).

Dexamethasone-treated DC2.4 cells promote FOXP3 expression in naive T cells

The CD4+FOXP3 expression was observed in 6.93±0.91% of control T cells, 15.17±1.04% of T cells stimulated by DC2.4, and 17.93±0.97% of T cells stimulated by dexamethasone-treated DC2.4 (F=103.391, P=0.000). Compared with the control group, DC2.4 effectively promoted the expression of CD4+FOXP3+ in naive T cells (P<0.05). Compared with the DC2.4 group, dexamethasone-treated DC2.4 effectively promoted the expression of CD4+FOXP3+ in naive T cells (P<0.05) (Figure 8).

Table1. Cell culture supernatant IL-12 levels and SI in different groups.

Group	IL-12 (pg/ml)	SI
Control	126.576±6.305	3.986±0.119
50 μg/L Dex	42.548±12.157*	1.739±0.043**,***
100 μg/L Dex	31.879±6.079*	1.480±0.064**
200 μg/L Dex	37.237±14.170*	1.647±0.084**,***
F	56.855	623.129
P	0.000	0.000

* *P*<0.05, compared with control group; ** *P*<0.05, compared with control group; *** *P*<0.05, compared with 100 μg/L Dex group.

Discussion

The unique characteristics of DCs determine their dual roles in eliciting immune responses and inducting immune tolerance. Hence, induction of immune tolerance by harvesting the characteristics of DCs has become an area of extensive study in transplant immunology. Studies have shown that pharmacologically-treated DCs significantly prolong the survival of transplanted organs and effectively induce immune tolerance to the transplanted organs [7,8]. The DC2.4 cell line is a mouse dendritic cell line established by transfecting C57BL/6 mouse bone marrow cells with GM-CSF, myc, and raf genes [9]. Our present study examined the effects of various doses of dexamethasone on the phenotypes and function of the DC2.4 cell line. Our results showed that dexamethasone treatment resulted in no significant changes in the expressions of CD80, CD86, galectin-9, or PD-L1 in DC2.4 cells. However, dexamethasone treatment significantly inhibited IL-12 secretion by DC2.4 cells and suppressed the proliferation of allogeneic T lymphocytes. Moreover, the expression of FOXP3 in naive T cells was effectively augmented in dexamethasone-treated DC2.4 cells.

Galectin-9 (Tim-3L) is widely expressed in the human and mouse spleen and lymphoid tissues. Galectin-9/Tim-3 (Tim-3 is a specific surface molecule of Th1 cells [10]) interaction can trigger negative regulatory signals, downregulate Th1 immune response, and promote the development of peripheral immune tolerance, thus playing an important role in autoimmune diseases and immune tolerance [11]. Programmed death 1 ligand (PD-L1, B7-H1) is expressed in resting cells and is upregulated in activated T cells, B cells and DCs. Deficiency in PD-L1 is known to accelerate the development of autoimmune diseases [12]. Programmed death-1 (PD-1), an important protein involved in negative immune regulation, is mainly expressed in activated T cells and B cells [13]. The PD-L1/PD-1 signaling pathway exerts negative regulation on the activation of lymphocytes [14,15]. Previous studies have demonstrated that regulatory T cells exert inhibitory effects on immune response by upregulating PD-L1 (molecular mechanism) via DCs (cytological effect) and that *in vivo* or *in vitro* blockade or inhibition of PD-L1 expression abolishes the inhibitory effect of DCs treated by regulatory T cells on effector T cells [16].

In this study, we found that dexamethasone neither increased the expression of galectin-9 in DCs nor reduced the expressions of CD80 or CD86, suggesting that the phenotypes of this cell line are relatively stable. Nevertheless, dosage and duration of dexamethasone treatment may also contribute to the observed stability of this cell line. In addition, our study revealed that dexamethasone suppressed the secretion of IL-12 in DC2.4 cells. IL-12 is an important cytokine involved in immune response. It can effectively induce proliferation and differentiation of T lymphocytes and elicit specific immune response in Th1 cells [17]. Dexamethasone may suppress T cell proliferation by inhibiting the production of IL-12, which is in agreement with the findings previously reported [18]. Our present study demonstrated that CD80 and CD86 were highly expressed in DC2.4 cells, characteristic of the phenotypes of mature DCs. Although dexamethasone did not reduce the expression of CD80 or CD86 in DC2.4 cells, it resulted in inhibition of IL-12 production and T cell proliferation.

The interplay between DCs and T cells has an important role in immune response and induction of immune tolerance [19]. Studies have shown [20,21] that dexamethasone-treated DC cells induced the production of regulatory T cells, inhibited the responses of effector T cells, and induced immune tolerance. By using RNA interference to specifically silence the expressions of CD40, CD80, and CD86 in DCs, effector T cell-elicited immune response was effectively suppressed and the development of autoimmune diseases was thus avoided [22,23]. Allogeneic DCs effectively augmented the proliferation of CD4+ regulatory T cells [24,25], which in turn inhibited DC-mediated T cell immune response by suppressing proliferation, phenotypic maturation, and IL-12 production of DCs [26,27]. Our understanding of which subset of DCs is capable of inducing immune tolerance has recently been advanced by the finding that mature DCs can also induce T cell tolerance in addition to immature DCs [28,29]. A study has shown that CD4 regulatory T cells induced by human autologous mature DCs markedly inhibited allogeneic mixed lymphocyte reaction [30]. High expressions of CD80 and CD86 on the surface of DCs are beneficial for proliferation of regulatory T cells [31,32].

Our study found that DC2.4 cells expressed high levels of CD80 and CD86 costimulatory molecules and that dexamethasone treatment did not cause a notable change in their expressions. Meanwhile, the results of mixed lymphocyte reaction showed that 100 μg/L dexamethasone was more effective in inhibiting allogeneic T cell proliferation than other doses of dexamethasone. To further investigate the underlying cause, we analyzed the effect of dexamethasone on the proliferation of regulatory T cells. The results demonstrated that DC2.4 cells co-cultured with allogeneic spleen lymphocytes, irrespective of dexamethasone treatment, could effectively promote FOXP3 expression in naive T cells and that dexamethasone-treated DC2.4 cells were more potent in promoting FOXP3 expression. Accordingly, an elevated FOXP3 expression stimulated by dexamethasone-treated DC2.4 further inhibited T cell immunity, and DC2.4-stimulated allogeneic lymphocyte proliferation was more effectively suppressed. Therefore, there is no strict division between the phenotypes of tolerogenic DC and those of reactive DC. Because of the complexity of the immune system and the conditionality of the regulatory network, a molecular phenotype of DCs may perform different immune functions depending on the stimuli.

Conclusions

Although dexamethasone treatment did not cause changes in the expressions of cellular phenotypes in DC2.4 cells, it indeed significantly decreased IL-12 production, promoted the production of regulatory T cells, and inhibited the proliferation of allogeneic T cell. Our present study provides an experimental basis for further study into the mechanism of DC-induced transplant tolerance.

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