

# Effect of immune tolerance induced by immature dendritic cells and CTLA4-Ig on systemic lupus erythematosus: An *in vivo* study

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**Abstract.** Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease in which tissue damage is caused by autoantibodies. The induction of specific immune tolerance, including the utilization of immune regulatory cells, may enhance the therapeutic effects of organ transplantation in patients with SLE. Furthermore, inhibiting immune responses has been reported to be an effective treatment for SLE. However, few studies have explored the association between an increased immune tolerance and a decreased immune response in SLE treatment. Dendritic cells (DCs), which are highly efficient antigen-presenting cells, are able to induce specific tolerance, while cytotoxic T lymphocyte antigen 4-immunoglobulin (CTLA4-Ig) inhibits the immune response. In the present study, interleukin (IL)-10-treated DCs and CTLA4-Ig were administered to mice with SLE alone or in combination and the therapeutic effects were investigated. IL-10 was added into the culture medium of bone marrow-derived DCs to prevent them from differentiating into mature cells. Low levels of major histocompatibility complex II, cluster of differentiation (CD)40, CD80 and CD86 were

detected, which indicated that the immature state of DCs was maintained. IL-10-treated DCs were subsequently injected into the caudal vein of B6.MRL-Fas<sup>lpr</sup>/J lupus mice, which are an established animal model of SLE. To amplify the tolerance effect, mice were simultaneously injected with CTLA4-Ig. Compared with the IL-10-treated DC and CTLA4-Ig groups, combined treatment with IL-10-treated DCs and CTLA4-Ig strongly induced immune tolerance in mice with SLE, as indicated by the significantly reduced levels of urine protein, anti-nuclear antibody, double-stranded DNA and IL-17A. A significant decrease in the proportion of T helper cells and an increase in the proportion of CD4<sup>+</sup> forkhead box protein P3<sup>+</sup> Treg cells was also observed, further confirming the induction of immune tolerance. These results suggest that combined treatment with IL-10-DCs and CTLA4-Ig may be a promising novel therapeutic strategy for the treatment of SLE.

## Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multi-organ involvement in which autoantibodies induce tissue damage (1). Immunosuppressive therapy with pharmacological agents, including mycophenolate mofetil (2) and cyclophosphamide (3), is widely used to improve the clinical outcomes of SLE. However, nonspecific suppression of the immune system and the associated side effects reduce patient quality of life (4). Several studies have aimed to induce specific tolerance to enhance the therapeutic effect of organ transplantation via utilizing immune regulatory cells, in particular antigen-presenting cells (APCs) (5,6) and regulatory T cells (Tregs) (7). However, few reports have focused on the use of tolerogenic APCs as a therapeutic strategy for the treatment of SLE.

Dendritic cells (DCs) are highly efficient APCs that have been studied in rodents and humans (8,9). DCs may be identified at immature and mature stages based on their phenotypic and functional characteristics (10). Mature DCs, which express high levels of major histocompatibility complex II (MHC II), cluster of differentiation (CD)80 and CD86 (11), secrete the T helper (Th) 1 cell-driving cytokine interleukin (IL)-12 (12). These molecules are essential for the T cell immunological response, which demonstrates that mature DCs serve an important role in the initiation of immune responses (13).

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**Abbreviations:** ANA, anti-nuclear antibody; APCs, antigen-presenting cells; DCs, dendritic cells, dsDNA, double-stranded DNA; IL, interleukin; MHC II, major histocompatibility complex II; PE, phycoerythrin; CTLA4-Ig, cytotoxic T lymphocyte antigen 4-immunoglobulin; rmGM-CSF, recombinant mouse granulocyte-macrophage colony-stimulating factor; SLE, systemic lupus erythematosus; Th, T helper cells; Treg, regulatory T cells

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Immature DCs express low levels of MHC II and co-stimulatory molecules, including CD40, CD80 and D86 (14), and have low T cell stimulatory ability. These cells are associated with T cell energy induction and Treg cell generation (15). In clinical practice, infusion with immature DCs, either alone or in combination with a co-stimulation blockade, regulates host T cell responses and effectively prolongs allograft survival (16-18). However, the maturity status of DCs is not stable as immature DCs differentiate into mature DCs when exposed to antigens and stimulation factors (19). Therefore, maintaining the status of immature DCs is key for the successful application of immature DCs in immunotherapy.

It has previously been reported that IL-10 is able to inhibit DC maturation (20), causing more IL-10 to be secreted by immature DCs; this feedback loop amplifies the tolerogenic effect of immature DCs. IL-10 therefore serves a pivotal role in the prevention of DC maturation and the induction of immune tolerance (21) during the progression of autoimmune disease or transplant rejection (22). In culture, DCs transduced with IL-10 have been demonstrated to have reduced levels of co-stimulatory molecules (CD80/CD86) and, furthermore, do not produce the potent allo-stimulatory cytokine IL-12 (23). A previous *in vivo* study revealed that animals which received IL-10-overexpressing DCs had a reduced incidence of skin graft rejection compared with animals that received DCs modified with a control virus, suggesting that there was reduced mononuclear cell infiltration and less dermo-epidermal junction destruction (24). In addition, IL-10-treated DCs inhibit antigen-specific immune responses in pre-activated immunocytes and these effects persist following repeated antigen restimulation (25). Immature DCs have been introduced as a therapy for SLE (26), and it has been reported that cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (CTLA4-Ig) is able to induce immune suppression in autoimmune diseases (27) and organ transplantation (28). It was therefore hypothesized that the combination of immature DCs and CTLA4-Ig may effectively induce immune tolerance.

The aim of the present study was to explore the effect of DC-induced immune tolerance in SLE. Immature DCs, which were prevented from maturing using IL-10, were injected into the caudal vein of lupus-prone B6.MRL-Fas<sup>lpr</sup>/J mice. The mice were also treated with CTLA4, which may serve to prevent the transmission of co-stimulatory signals and induce T cells to undergo apoptosis, become inactivated or anergic, thus inducing immune tolerance.

## Materials and methods

**Materials.** Recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF), rmIL-10, and rmIL-4 were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Mouse antibodies directed against CD40 (cat no. 11-0402-82), CD80 (cat no. 15-0801-82), CD86 (cat no. 12-0862-82), MHC II (cat no. 12-5321-82), IL-17A (cat no. 12-7177-81), IgG2a (cat no. 12-4321-80), CD4 (cat no. 11-0042-82) and forkhead box protein P3 (Foxp3; cat no. 12-4774-42) were purchased from eBioscience (Thermo Fisher Scientific, Inc., Waltham, MA, USA). rmCTLA4-Ig was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). RPMI-1640 medium and fetal bovine serum (FBS) was purchased from

Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). IL-17A (cat no. kt21287), anti-nuclear antibody (ANA; cat no. kt40119), and double-stranded (ds)DNA (cat no. kt21274) ELISA kits were purchased from MSK Biological Technology, Ltd. (Wuhan, Hubei, China).

B6.MRL-Fas<sup>lpr</sup>/J lupus mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). C57Bl/6J mice were purchased from Hunan SJA Laboratory Animals Co., Ltd. (Changsha, Hunan, China).

**Immature DC culture.** All experiments used in the present study were approved by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University (Nanning, China), and all experimental procedures were conducted in conformity with the institutional guidelines for the care and use of laboratory animals. All surgeries were performed under sodium pentobarbital (Merck KGaA, Darmstadt, Germany) anesthesia.

All mice were housed in an SPF level lab under controlled a temperature of 20-24°C and a relative humidity of 50-60% with a 12/12 h light-dark cycle. All of them had free access to formula feed and water.

A total of 10 female B6.MRL-Fas<sup>lpr</sup>/J lupus mice (2-months-old; weighing 18-20 g) were sacrificed, following which femurs and tibias were carefully harvested under aseptic conditions. Mouse bone marrow cells were collected by flushing the medullary cavity gently using RPMI-1640 medium supplemented with 10% FBS. The cells (1×10<sup>6</sup> cells/ml) were transferred into 6-well plates (2 ml/well) and incubated at 37°C in an humidified atmosphere containing 5% CO<sub>2</sub> for 4-6 h. Non-adherent cells were removed and 2 ml RPMI-1640 with 10% FBS, 20 ng/ml rmGM-CSF and 10 ng/ml rmIL-4 was added to each well. The medium was replaced every other day. At 5 days, IL-10 (10 ng/ml) was added to the medium, and cells were cultured for an additional 1 and 4 days until day 6 (6-IL-10-DC group) or day 9 (9-IL-10-DC group), respectively. Non-treated cells were also cultured for 6 days (D6-DC group) or 9 days (D9-DC group). Changes in the morphology and number of cells in the four groups were observed using an inverted microscope. A total of 10 high power fields of vision (magnification, x200) from each group were chosen randomly to count the cell numbers. The expression of CD80, CD86, MHC II and CD40 was examined using flow cytometry to identify the purity and maturity of the cells. Prior to detection, cells (1×10<sup>7</sup> cells/ml) were washed with PBS 3 times (5 min each time). Fc receptor-blocking pharmacon (1:50; eBioscience; cat no. MFCR00-4; Thermo Fisher Scientific, Inc) was added into the reaction tube and incubated for 15 min at 4°C. All primary antibodies, including CD80 (cat no. 15-0801-82), CD86 (cat no. 12-0862-82), MHC II (cat no. 12-5321-82) and CD40 (cat no. 11-0402-82; all Thermo Fisher Scientific, Inc.) were directly labeled with phycoerythrin or fluorescein isothiocyanate. Each antibody was incubated for 30 min at room temperature. Samples with the added antibody (CD80, 1:50; CD86, 1:50; MHC II, 1:200 and CD40, 1:200) were incubated at room temperature in the dark for 30 min. The cells were washed with PBS 3 times, cells were fixed in paraformaldehyde (1:50) at 4°C and analyzed using a FACS Calibur flow cytometer within 24 h. Data were quantified using FCS software (version no. 4.0; BD Biosciences, Franklin Lakes, NJ, USA).

**SLE mouse treatment.** A total of 24 4-month-old female B6.MRL-Fas<sup>lpr</sup>/J lupus mice weighing 18-20 g were randomly divided into the following groups (n=6/group): IL-10-DC group; CTLA4-Ig group; IL-10-DCs + CTLA4-Ig group; and PBS group. Mice were administered with 0.1 ml IL-10-treated DCs ( $1 \times 10^8$  cells/ml), 0.1 ml CTLA4-Ig (100  $\mu$ g/ml), 0.1 ml mixture of IL-10-treated DCs and CTLA4-Ig, or 0.1 ml PBS (1 M/l) via the tail vein. The normal group included 6 female C57Bl/6J mice weighing 24-27 g with no interventions. All interventions were repeated at 0, 2, 4 and 6 weeks. Housing conditions were as described above.

**Biochemical analysis.** Samples of urine and serum from SLE and normal mice were collected 2 days prior to the first intervention and 2 weeks following the last intervention. Mice were fasted for 12 h and transferred to metabolic cages prior to urine collection. During the 24 h collection period, urine was collected every 6 h via collecting tubes, the samples were centrifuged at 108 x g for 5 min at room temperature to remove impurities and the supernatant was collected and stored at 4°C for further experiments. Urine proteins (Shanghai Enzyme-linked Biotechnology Co., Ltd.; cat no. 1037585, Shanghai, China) were detected using an automatic biochemical analyzer according to the manufacturer's protocol. The concentrations of IL-17A (MSK, cat no. kt21287, Wuhan, Hubei, China), ANA (MSK, cat no. kt40119, Wuhan, Hubei, China) and dsDNA (MSK, cat no. kt21274, Wuhan, Hubei, China) antibodies in the serum were detected using ELISA kits according to the manufacturer's protocol.

**Lymphocyte separation.** Mice in each group were sacrificed at 2 weeks following the last intervention. Spleens were harvested and macerated in RPMI-1640 with 4% FBS. Spleen cells were filtered and lymphocytes were collected using density gradient centrifugation. Spleen cells suspension was added to the lymphocyte separation medium at a ratio of 1:1. The cell mixture was divided into 4 layers following centrifugation at 672 x g for 20 min at room temperature. The second layer solution was extracted (counting from top to bottom) by washing twice with PBS at 1:6 ratio and centrifuged at 168 x g for 10 min at room temperature. After discarding the PBS, spleen lymphocytes were collected and resuspended in RPMI-1640 at a density of  $2 \times 10^6$  cells/ml.

**Th17 cell detection.** The proportion of Th17 cells out of the lymphocytes derived from SLE mouse spleens was quantified. Briefly, cell suspensions ( $2 \times 10^6$  cells/ml) were added into centrifuge tubes (0.5 ml) with phorbol 12-myristate 13-acetate (1:40) (Shanghai Yisheng Biotechnology Co. Ltd, cat no. 50601ES02, Shanghai, China), ionomycin (1:50) and brefeldin A (1:50), and incubated for 4 h at 37°C. Cells were washed with PBS and centrifuged at 168 x g for 5 min at room temperature. Prior to incubation with antibodies, Fc receptor-blocking pharmacon (1:50) (eBioscience, cat no. MFCR00-4, Thermo Fisher Scientific, Inc.) was added and incubated for 15 min at 4°C. CD4 antibodies (1:200; eBioscience, cat no. 12-0041-82, Thermo Fisher Scientific, Inc.) were subsequently added to the cell suspensions and incubated at 4°C in the dark for 30 min. Cells were washed with permeabilization buffer (eBioscience, cat no. 00-8333-56,

Thermo Fisher Scientific, Inc.) and centrifuged at 168 x g for 5 min at 4°C. Subsequently, fixation/permeabilization solution (1:100; eBioscience, cat no. 00-5123-43, Thermo Fisher Scientific, Inc.) was added and the suspension was incubated for 45 min at 4°C in the dark. Cells were washed with permeabilization buffer (eBioscience, cat no 00-8333-56, Thermo Fisher Scientific, Inc.) and centrifuged at 168 x g for 5 min. Fc receptor-blocking pharmacon (1:50) was added secondly and incubated for 15 min. IL-17A (1:100; eBioscience, cat no. 12-7177-81, Thermo Fisher Scientific, Inc.) antibodies or an isotype control IgG2a (1:100; eBioscience, cat no. 12-4321-80, Thermo Fisher Scientific, Inc.) antibodies were added to each tube, which were incubated for 15 min at 4°C. Cells were subsequently washed with PBS and centrifuged at 168 x g for 5 min at 4°C. The supernatant was discarded, and the cells were resuspended with 0.2 ml paraformaldehyde (1:50) at 4°C. The cells were analyzed by flow cytometry within 24 h. Data were quantified using FCS software (version no. 4.0). CD4<sup>+</sup>IL-17A<sup>+</sup> cells were identified as the Th17 cell subset.

**Treg cell examination.** A low proportion of Th17 cells and high proportion of Treg cells is an indicator of immune tolerance induced *in vivo* (29). Therefore, the proportion of Treg cells out of the lymphocytes derived from the mouse spleens was examined and the Th17/Treg cell ratio was analyzed to investigate the tolerogenic effect of combined treatment with IL-10-treated DCs and CTLA4-Ig in SLE. All preparation was controlled at 4°C. Briefly, cell suspensions ( $2 \times 10^6$  cells/ml) were added to tubes ( $1 \times 10^6$  cells/tube) and then Fc receptor-blocking pharmacon (1:50) (cat no. MFCR00-4; Thermo Fisher Scientific, Inc) and CD4 (1:200) antibodies (eBioscience, cat no. 11-0042-82, Thermo Fisher Scientific, Inc.) were added to the reaction tubes and incubated for 30 min at 4°C. Cells were washed with permeabilization buffer (eBioscience, cat no. 00-8333-56, Thermo Fisher Scientific, Inc.) and centrifuged at 168 x g for 5 min at 4°C. After fixation and permeabilization with fixation/permeabilization solution (1:100; eBioscience, cat no. 00-5123-43, Thermo Fisher Scientific, Inc.) for 45 min, cells were washed with permeabilization buffer (cat no. 00-8333-56; eBioscience; Thermo Fisher Scientific, Inc.) and centrifuged at 168 x g for 5 min at 4°C. Fc receptor-blocking pharmacon was then added and incubated for 15 min at 4°C. Foxp3 (1:100) (eBioscience, cat no. 12-4774-42, Thermo Fisher Scientific, Inc.) or IgG2a (1:100) (eBioscience, cat no. 12-4321-80, Thermo Fisher Scientific, Inc.) were added and incubated for 45 min at 4°C. Cells were washed with permeabilization buffer and centrifuged at 168 x g for 5 min twice at 4°C. The cells were fixed with 0.2 ml paraformaldehyde (1:50) at 4°C and analyzed by flow cytometer within 24 h. CD4<sup>+</sup>Foxp3<sup>+</sup> cells were identified as the Treg cell subset, and IgG2a was used as an isotype control.

**Statistical analysis.** All experiments were performed a minimum of three times with six replicate samples for each group. All data are presented as the mean  $\pm$  standard deviation. The homogeneity of variances in the data was calculated using Levene's test. One-way ANOVA was used to analyse the data. Multiple comparisons were conducted using Games-Howell and Student-Newman-Keuls tests for unequal and equal variances, respectively. P<0.05 was considered to indicate a statistically significant difference.



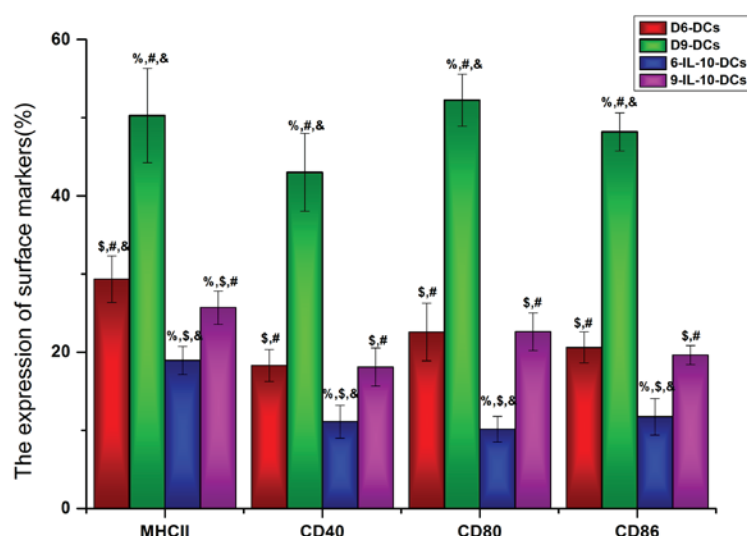


Figure 1. Immature DCs are induced by IL-10. The DC maturation state markers MHC II, CD40, CD86 and CD80 were detected using flow cytometry. DCs, dendritic cells; IL, interleukin; MHC II, major histocompatibility complex II; CD, cluster of differentiation; D6-DCs, DCs cultured until day 6; D9-DCs, DCs cultured until day 9; 6-IL-10-DCs, D6-DCs; treated with IL-10; 9-IL-10-DCs, D9-DCs treated with IL-10. <sup>#</sup>P<0.05 vs. the D6-DCs group, <sup>\$</sup>P<0.05 vs. the D9-DCs group, <sup>&</sup>P<0.05 vs. the 6-IL-10-DCs group, <sup>^</sup>P<0.05 vs. the 9-IL-10-DCs group.

## Results

**Immature DCs are induced by IL-10.** To identify the maturation state of DCs that were cultured with or without IL-10 *in vitro*, the DC maturation state markers MHC II, CD40, CD86 and CD80 were detected using flow cytometry. The results revealed that the expression of MHC II, CD40, CD86, and CD80 was significantly lower in IL-10-treated DCs compared with the non-treated DC groups ( $P<0.05$ ; Fig. 1). Furthermore, the expression of MHC II ( $P<0.05$ ), CD80 ( $P<0.05$ ) and CD86 ( $P<0.001$ ) was significantly lower in D6-DCs compared with D9-DCs (Fig. 1), indicating that, even with exposure to IL-10, DCs are able to differentiate into mature DCs during long-term *in vitro* culture. Based on these results, D6-DCs were used for the *in vivo* study.

**Immune tolerance is induced by IL-10-DCs and CTLA4-Ig.** Urine protein levels of the mice were assessed using an automatic biochemical analyzer, whereas IL-17A, ANA and anti-dsDNA levels were analyzed using ELISA kits. No significant differences were observed in urine protein levels among the SLE groups prior to treatment (Fig. 2A). However, urine protein levels were significantly higher in the IL-10-DCs, CTLA4-Ig, IL-10-DCs + CTLA4-Ig and PBS groups compared with the normal group ( $P<0.05$ ; Fig. 2A). Following treatment, urine protein levels in the IL-10-DCs, CTLA4-Ig and IL-10-DCs + CTLA4-Ig groups were significantly lower compared with pre-treatment values ( $P<0.05$ ) and the PBS group ( $P<0.05$ ; Fig. 2A). Post-intervention urine protein levels were significantly lower in the IL-10-DCs + CTLA4-Ig group compared with the IL-10-DCs and CTLA4-Ig groups ( $P<0.001$ ), and were close to those in the normal group ( $P>0.05$ ; Fig. 2A).

No significant difference was observed in IL-17A, ANA or dsDNA levels among the SLE groups prior to treatment (Fig. 2B-D); however, these levels were significantly higher in the IL-10-DCs, CTLA4-Ig, IL-10-DCs + CTLA4-Ig and PBS

groups compared with the normal mouse group (Fig. 2B-D). Levels of IL-17A, ANA and dsDNA were significantly lower following treatment compared with pre-treatment in the IL-10-DC, CTLA4-Ig and IL-10-DCs + CTLA4-Ig groups ( $P<0.05$ ; Fig. 2B-D). Post-intervention the relative levels of IL-17A, ANA and dsDNA were significantly lower in the IL-10-DCs + CTLA4-Ig group compared with the other treatment groups (Fig. 2B-D). These results indicate that SLE activity was most reduced in the IL-10-DCs + CTLA4-Ig group and that immune tolerance was induced.

**T cell responses.** The proportions of Th17 and Treg cells, identified by IL-17A and Foxp3 expression, respectively, were analyzed using flow cytometry (Fig. 3). The results revealed that the proportion of Th17 cells was significantly lower in the IL-10-DCs, CTLA4-Ig and IL-10-DCs + CTLA4-Ig groups compared with the PBS group ( $P<0.05$ ), whereas it was significantly higher in the treatment groups compared with the normal group ( $P<0.05$ ; Fig. 3B). Following treatment, the proportion of Th17 cells in the IL-10-DCs + CTLA4-Ig group was significantly lower compared with the IL-10-DC and CTLA4-Ig groups ( $P<0.05$ ; Fig. 3B).

In contrast, the proportion of Treg cells was significantly higher in the IL-10-DCs, CTLA4-Ig and IL-10-DCs + CTLA4-Ig groups compared with the PBS group ( $P<0.05$ ) and significantly lower compared with the normal group ( $P<0.05$ ; Fig. 3D). Following treatment, Treg cell numbers in the IL-10-DCs + CTLA4-Ig group were significantly higher compared with the IL-10-DCs and CTLA4-Ig groups (Fig. 3D).

Following intervention, the Th17/Treg cell ratio in the IL-10-DC, CTLA4-Ig and IL-10-DCs + CTLA4-Ig groups was significantly lower compared with the PBS group ( $P<0.05$ ) and significantly higher compared with the normal group ( $P<0.05$ ; Fig. 4). The Th17/Treg cell ratio in the IL-10-DCs + CTLA4-Ig group was much lower compared with the IL-10-DC and CTLA4-Ig groups (Fig. 4). These results suggest that immune

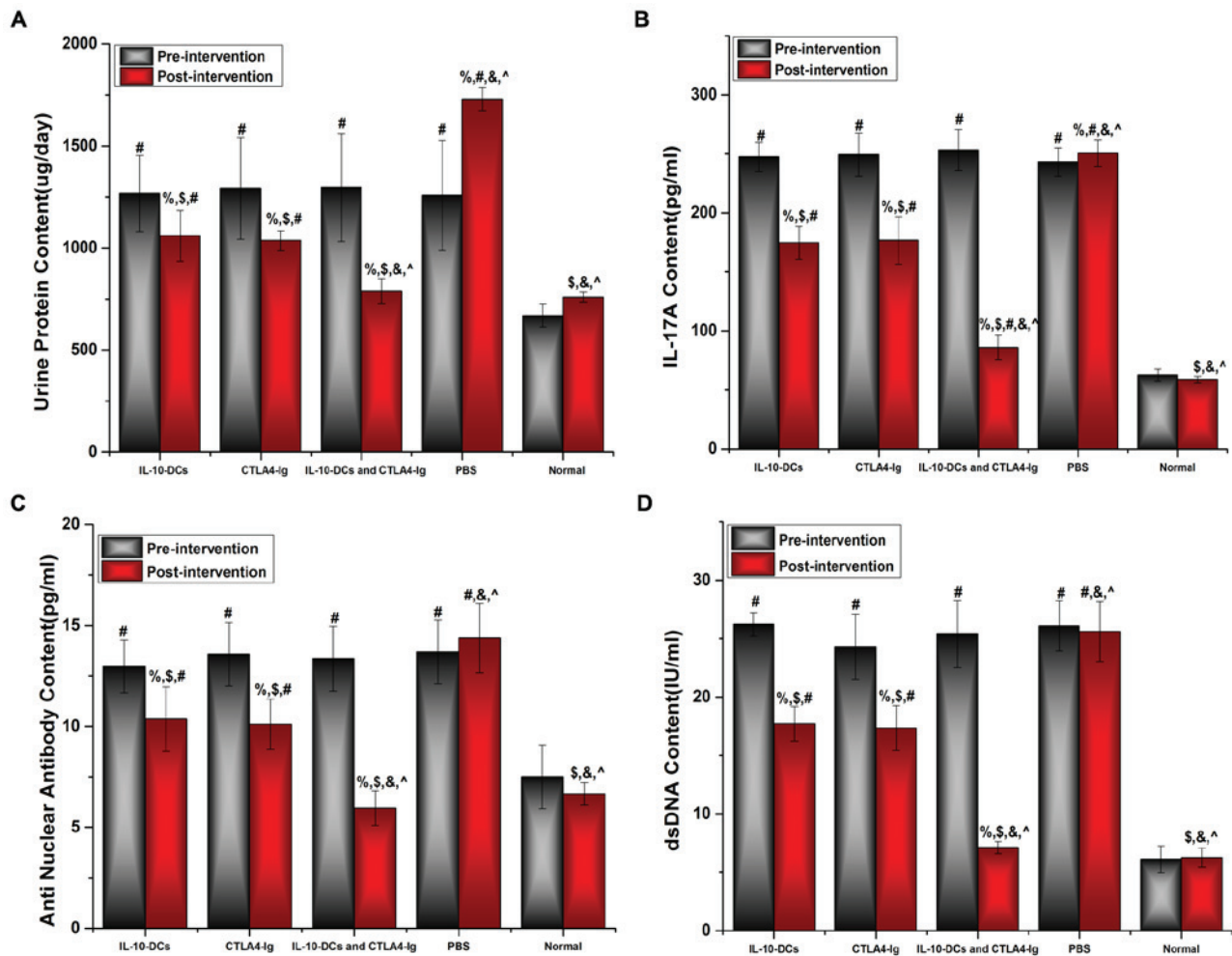


Figure 2. Immune tolerance is induced by IL-10-DCs and CTLA4-Ig. (A) Urine protein, (B) IL-17A, (C) ANA and (D) dsDNA levels in mice pre- and post-intervention. IL, interleukin; DCs, dendritic cells; CTLA4-Ig, cytotoxic T lymphocyte antigen 4-immunoglobulin; ANA, anti-nuclear antibody; ds, double stranded; Normal, untreated C57Bl/6J mice. <sup>#</sup>P<0.05 vs. the same group pre-intervention, <sup>%</sup>P<0.05 vs. the PBS group post-intervention, <sup>\$</sup>P<0.05 vs. the normal group, <sup>&</sup>P<0.05 vs. the IL-10-DCs group, <sup>^</sup>P<0.05 vs. the CTLA4-Ig group.

tolerance was induced by the administration of IL-10-DCs and CTLA4-Ig in mice with SLE.

## Discussion

The present study aimed to investigate DC-induced immune tolerance in mice with SLE, an effect that was amplified by the administration of CTLA4-Ig. *In vitro*, lower levels of MHC II, CD40, CD80, and CD86 were expressed by IL-10-treated DCs compared with non-treated cells, indicating that the process of maturation was prevented. In the *in vivo* study, administering mice with SLE with IL-10-treated DCs reduced the levels of urine protein, ANA, dsDNA and IL-17A. Furthermore, treatment with IL-10-treated DCs decreased the proportion of Th17 cells and increased the proportion of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, suggesting that immune tolerance was induced by immature DCs in mice with SLE. Co-administration with IL-10-DCs and CTLA4-Ig resulted in a greater tolerance-inducing effect compared with IL-10-DCs or CTLA4-Ig treatment alone.

To prevent DCs from maturing, IL-10 was added into the culture medium *in vitro*. DCs are APCs that are specialized to regulate T cell immunity, including activation of T cells and maintenance of peripheral tolerance (30), and

the function of DCs is dependent on their state of activation and differentiation (31). Mature DCs are able to induce the development of T effector cells (32), whereas immature DCs are associated with the maintenance of immunological tolerance (33). In an *in vitro* culture system, FBS acts as an antigen to facilitate DC maturation (34). For the D6-DCs group, the expression of surface markers, including the immune response molecules MHC II, CD40, CD86 and CD80, was evident in 25-30% of mature DCs. However, in the D9-DCs group this expression increased to 40% under the same conditions, indicating that DCs differentiate into mature cells when cultured for an extended period. As an immunomodulatory cytokine that inhibits DC function, IL-10 is a major factor that prevents the differentiation of DCs from monocytes (35-37). Furthermore, IL-10 is able to inhibit receptor-mediated macropinocytosis and endocytosis following exposure to a soluble immunogen (36), and serves an important role in immune tolerance (38). In addition to exogenous IL-10, autocrine IL-10 prevents spontaneous DC maturation *in vitro*, limits lipopolysaccharide- and CD40-mediated maturation, and increases IL-10 production by DCs (39). IL-10 secretion therefore assists in maintaining the immature state of DCs.

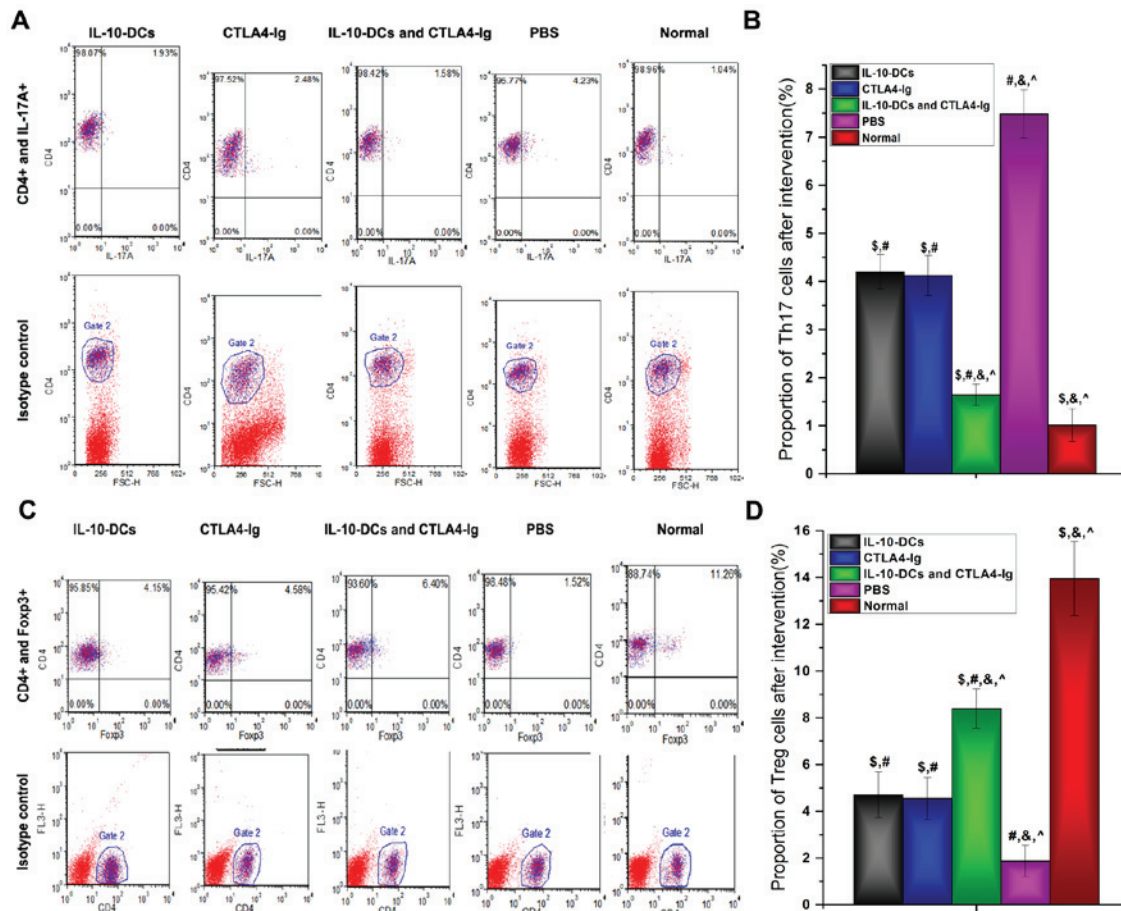


Figure 3. Proportion of Th17 and Treg cells as analyzed by flow cytometry. (A) CD4<sup>+</sup>IL-17A<sup>+</sup> cells were identified as the Th17 cell subset and (B) analyzed. (C) CD4<sup>+</sup>Foxp3<sup>+</sup> cells were identified as the Treg cell subset and (D) analyzed. Th, T helper cells; Treg, regulatory T cells; CD, cluster of differentiation; IL, interleukin; Foxp3, forkhead box protein P3; DCs, dendritic cells; CTLA4-Ig, cytotoxic T lymphocyte antigen 4-immunoglobulin; PE, phycoerythrin; Normal, untreated C57Bl/6J mice. <sup>§</sup>P<0.05 vs. the PBS group post-intervention, <sup>#</sup>P<0.05 vs. the normal group, <sup>^</sup>P<0.05 vs. the IL-10-DCs group, <sup>^</sup>P<0.05 vs. the CTLA4-Ig group.

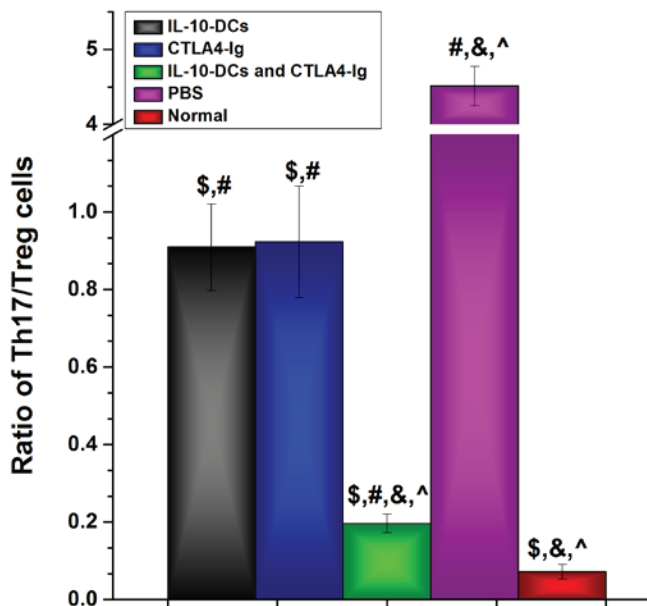


Figure 4. IL-10-DC and CTLA4-Ig intervention lowers the Th17/Treg cell ratio. Th, T helper cells; Treg, regulatory T cells; IL, interleukin; DCs, dendritic cells; CTLA4-Ig, cytotoxic T lymphocyte antigen 4-immunoglobulin. <sup>§</sup>P<0.05 vs. the PBS group post-intervention, <sup>#</sup>P<0.05 vs. the normal group, <sup>^</sup>P<0.05 vs. the IL-10-DCs group, <sup>^</sup>P<0.05 vs. the CTLA4-Ig group.

The capacity of immature DCs and CTLA4-Ig to induce immune tolerance was further explored in mice with SLE. The results demonstrated that immune tolerance was strongly induced, as evidenced by lower expression of urine protein, ANA, dsDNA and IL-17A, as well as a decrease in Th17 cells and an increase in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. Immature DCs are considered to be prototypic tolerogenic DCs due to their poor T cell stimulatory capacity (40). Immature DCs have therefore been utilized to induce immunosuppression in mammals with specific malignancies or autoimmune disease, as well as following transplantation (41–43). Several methods, including co-culture with marrow stromal cells (42), anti-vascular endothelial growth factor antibody (44) and IL-10 (45), have been used to maintain the immature state of DCs. IL-10-treated DCs induce alloantigen-specific T cell hyporesponsiveness and are also able to inhibit antigen-specific immunological responses (46,47), thereby prolonging liver allograft survival and enhancing immune tolerance (48). Furthermore, in the present study, CTLA4-Ig amplified the immune tolerance of IL-10-treated DCs *in vivo*. The interaction of CD80 and CD86 molecules expressed on DCs with CD28 molecules expressed on T cells is crucial for inducing T cell immune responses (49). CTLA4-Ig blocks CD28-mediated co-stimulatory signaling to T cells to induce tolerance (50) and maintain the tolerogenic



state of DCs (51). It has previously been demonstrated that the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg population in mouse joints and spleen is increased in CTLA4-Ig-treated collagen-induced arthritis model mice and that DCs are modified to become tolerogenic (52). However, there are few reports on the effects of co-injection with immature DCs and CTLA4-Ig in SLE. In the present study, the combination of immature DCs and CTLA4-Ig effectively induced immune tolerance in mice with SLE.

In summary, the results of the present study demonstrate that combined treatment with IL-10-treated immature DCs and CTLA4-Ig induces immune tolerance in mice with SLE. Co-injection of DCs and CTLA4-Ig may therefore have potential as a therapeutic strategy to promote immune tolerance, alleviate multiple organ dysfunction and improve the quality of life of patients with SLE.

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