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Original Research

Naringenin Impedes the Differentiation of Mouse Hematopoietic Stem Cells Derived from Bone Marrow into Mature Dendritic Cells, thereby Prolonging Allograft Survival

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Abstract

Background: The use of immature dendritic cells (imDCs) to induce donor-specific immunotolerance following *in vivo* stimulation is limited by their low rate of induction and their tendency to undergo maturation. We derived imDCs from bone marrow hematopoietic stem cells (HSCs-imDCs). We then tested the ability of naringenin (Nar) to impede the maturation of HSCs-imDCs for inducing transplantation immune tolerance. **Methods**: HSCs derived from bone marrow were collected and induced to differentiate into imDCs by treating with Nar (Nar-HSCs-imDCs). Flow cytometry was used to evaluate DC surface markers, apoptosis, and endocytic ability. The ability of DCs to influence the *in vitro* proliferation of T cells and of regulatory T cells (Tregs) was analyzed by mixed lymphocyte reaction assays. Enzyme-linked immunoassays were used to quantify cytokine levels in supernatants from co-cultured DCs and Tregs, as well as in the serum of experimental animals. The level of immunotolerance induced by Nar-HSCs-imDCs was evaluated by skin grafting in recipient Balb/c mice, while the Kaplan-Meier method was used to statistically evaluate graft survival. **Results**: Compared with HSC-imDCs, Nar-HSCs-imDCs showed higher expression of cluster of differentiation 11c (CD11c), but lower expression levels of CD80, CD86, and major histocompatibility complex class II. Nar-HSCs-imDCs also showed stronger inhibition of T cells and higher Treg cell proliferation. Interleukin 2 (IL-2) and interferon gamma levels were downregulated in Nar-HSCs-imDCs, whereas IL-4, IL-10, and transforming growth factor beta levels were upregulated. The rate of apoptosis and endocytic capacity of Nar-HSCs-DCs increased significantly after treatment with lipopolysaccharide. HSCs-imDCs or Nar-HSCs-imDCs were injected into Balb/c mice via the tail vein 7 days before skin grafting. Significantly reduced donor-specific $CD4^+$ T cells and induced proliferation of $CD4^+CD25^+FoxP3^+$ Treg cells were observed in the spleen of mice from the Nar-HSCs-imDCs group, especially at a dose of 10^6 Nar-HSCs-imDCs. The latter group also showed significantly prolonged survival of skin grafts. **Conclusions**: Nar-HSCs-imDCs markedly improved the acceptance of organ allografts, offering a potentially new strategy for inducing immune tolerance in transplantation.

Keywords: naringenin; hematopoietic stem cells; immature dendritic cells; immune tolerance; skin graft

1. Introduction

Organ transplantation is considered the most successful and effective treatment for end-stage organ failure. Advances in medical technology and immunosuppression regimens have to some extent improved the quality of patient life. However, acute and chronic rejection are still major factors that restrict long-term graft survival [1,2]. It is therefore important to seek new treatment strategies with minimal side effects and with high safety and efficacy for the induction of immune donor-specific hypo-responsiveness, or even tolerance to the transplant.

Dendritic cells (DCs) are specialized antigen-presenting cells that play an essential role in initiating and regulating the immune response to pathogenic microorganisms and to allograft rejection by balancing tolerance and immunity [3]. Immature DCs (imDCs) may prolong allograft survival by inhibiting specific T cells and by enhancing the proliferation of regulatory T cells (Tregs) [4–6]. However, the application of imDCs for immune tolerance is complicated by their limited induction rate and by their tendency to mature after stimulation.

Numerous studies have reported on the feasibility of deriving imDCs from stem cells, including the use of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 10 (IL-10) and transforming growth factor-β (TGF-β), as well as the use of immunosuppressants, gene transfection, and RNA interference technology. Although these methods strongly inhibit the maturation of DCs, they still have some drawbacks. For

example, imDCs tend to mature following the injection of cytokines or hormones into the body. Moreover, the efficiency of gene modification is generally low, with potential biosafety and immunogenicity problems [7].

Naringenin (Nar) (5,7,4'-trihydroxyflavanone) is a Chinese medicinal product and natural citrus flavonoid that has been proposed as a potential immunomodulator [8]. Nar demonstrates a wide range of anti-inflammatory and neuroprotective properties [9]. A previous study in a murine model of collagen-induced arthritis also confirmed that Nar had therapeutic effects by inhibiting the maturation of DCs [10]. Thus, Nar could potentially prevent the rejection of transplanted organs [11]. Nar also inhibits T cell proliferation in response to anti-cluster of differentiation 3 (CD3)/CD28 antibody-stimulated immune cells [12], enhances the effect of $CD4^+CD25^+$ regulatory T (Treg) cells [13], decreases the secretion of IL-2, IL-17, IL-6, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), and increases the secretion of IL-10 [14]. However, it remains to be determined whether Nar can prolong the immature stage of hematopoietic stem cell (HSC)-derived DCs, thereby increasing the immune tolerance to organ transplants.

In the present study we therefore investigated the ability of Nar to maintain HSC-derived imDCs (Nar-HSCs-imDCs) in an immature state, as well as the mechanism by which Nar-HSCs-imDCs can induce immune tolerance *in vitro*. The findings of this study could offer a new strategy to prevent rejection following organ transplantation.

2. Methods

2.1 Animals

Balb/c and C57BL/6 mice (6–8 weeks old, 19.9 $\pm$ 2.8 g) were purchased from the Laboratory Animal Center of Xi'an Jiaotong University (Shaanxi, China). All procedures were carried out in accordance with NIH Guidelines, and animal experiments were reviewed and approved by the Biomedical Ethics Committee of Xi'an Jiaotong University.

2.2 Sorting of HSCs, $CD3^+$ T Cells and $CD4^+$ T Cells

The induction and culture of imDCs derived from mouse bone marrow HSCs was performed as described previously [15]. Briefly, bone marrow was obtained from the femurs of C57BL/6 mice and the erythrocytes were lysed. Lin-$Sca1^+cKit^+$ HSCs were then sorted using a mouse lineage cocktail and fluorochrome-conjugated antibodies (Miltenyi, Cologne, Nordrhein-Westfalen, Germany). Magnetic cell sorting (MACS; Miltenyi, Cologne, Nordrhein-Westfalen, Germany) was also used to isolate $CD3^+$ T cells and $CD4^+$ T cells.

2.3 Optimal Concentration of Nar Screening Assay

HSCs were treated with different concentrations of Nar (purity >98%, Solarbio, Cat#SN8020, Beijing, China) (0, 50, 100, 200, 300, and 400 μM). To optimal concentration of Nar was determined using the Cell Counting Kit-8 (CCK-8) assay (MedChemExpress, Rocky Hill, NJ, USA) to measure cytotoxicity. The optimal concentration was defined as the maximum Nar concentration at which cell viability was >95%. Finally, HSCs were cultured for 3 days in RPMI-1640 with 10% fetal bovine serum (FBS, Cat#12664025, Gibco, Carlsbad, CA, USA) and 20 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF, Cat#31503, Perprotech, Rocky Hill, NJ, USA), 10 ng/mL IL-4 (Perprotech, USA) and 200 μM Nar (optimal concentration). Flow cytometry was used to detect the hematopoietic cell marker antigens Flk-1 and CD45, and of the monocyte antigen CD11b. This allowed analysis of the molecular phenotypic changes that occured during HSC differentiation into DCs. Following cell culture for a further 3–4 days, some cells were then treated for 48 h with 5 μg/mL Lipopolysaccharide (LPS, Cat#L5293, Sigma, Germany) for the subsequent experiment.

2.4 Flow Cytometry

Flow cytometry was also used to evaluate the phenotype of HSCs-imDCs, Nar-HSCs-imDCs, sorted CD3 and CD4 T cells, the effect of HSCs-imDCs and Nar-HSCs-DCs on Treg cells, apoptosis, and the phagocytic capacity of cells. HSCs-imDCs and Nar-HSCs-imDCs (1×10^5) were stained with FITC-CD80 (Cat# 104705), FITC-CD86 (Cat# 105005), FITC-MHC-II (Cat# 125507), and FITC-CD11c (Cat# 117305), (Biolegend, San Diego, CA, USA). Multicolor staining for Tregs was carried out with FITC-CD4 (Cat# 100405), PE-CD25 (Cat# 113703) and APC-FoxP3 (Cat# 420201) (Biolegend, San Diego, CA, USA). Apoptosis was detected using an Annexin V-FITC Apoptosis Detection Kit (C1062M; Beyotime, Beijing, China), and the data analyzed using FlowJo software (version 10.0, FlowJo LLC, Ashland, OR, USA).

2.5 Mixed Lymphocyte Reaction (MLR)

$CD4^+$ T cells were isolated from the spleen of Balb/c mouse using MACS immunomagnetic beads (Miltenyi, Germany). HSCs-imDCs and Nar-HSCs-imDCs were treated with 25 μg/mL mitomycin. RPMI-1640 medium containing 5 μg/mL LPS was used as a control. HSCs-imDCs, Nar-HSCs-imDCs, or controls were co-cultured for 72 h at varying proportions with $CD4^+$ T cells (DCs: T cells at 1:80, 1:40, 1:20, or 1:10). The inhibition of $CD4^+$ T cells was analyzed using the CCK-8 assay, with the extent of inhibition was calculated as follows: (1 – OD of the experimental group/OD of the control group) $\times$ 100%.

2.6 Enzyme-Linked Immunoassay

Enzyme-linked immunoassay (ELISA; Elabscience, Hubei, China) was used to quantify the levels of IL-2 (Cat#E-ELM0042c), IL-4 (Cat#E-EL-M0043c), IL-10 (Cat#E-ELM0046c), IFN-γ (Cat#E - EL - M0048c), and

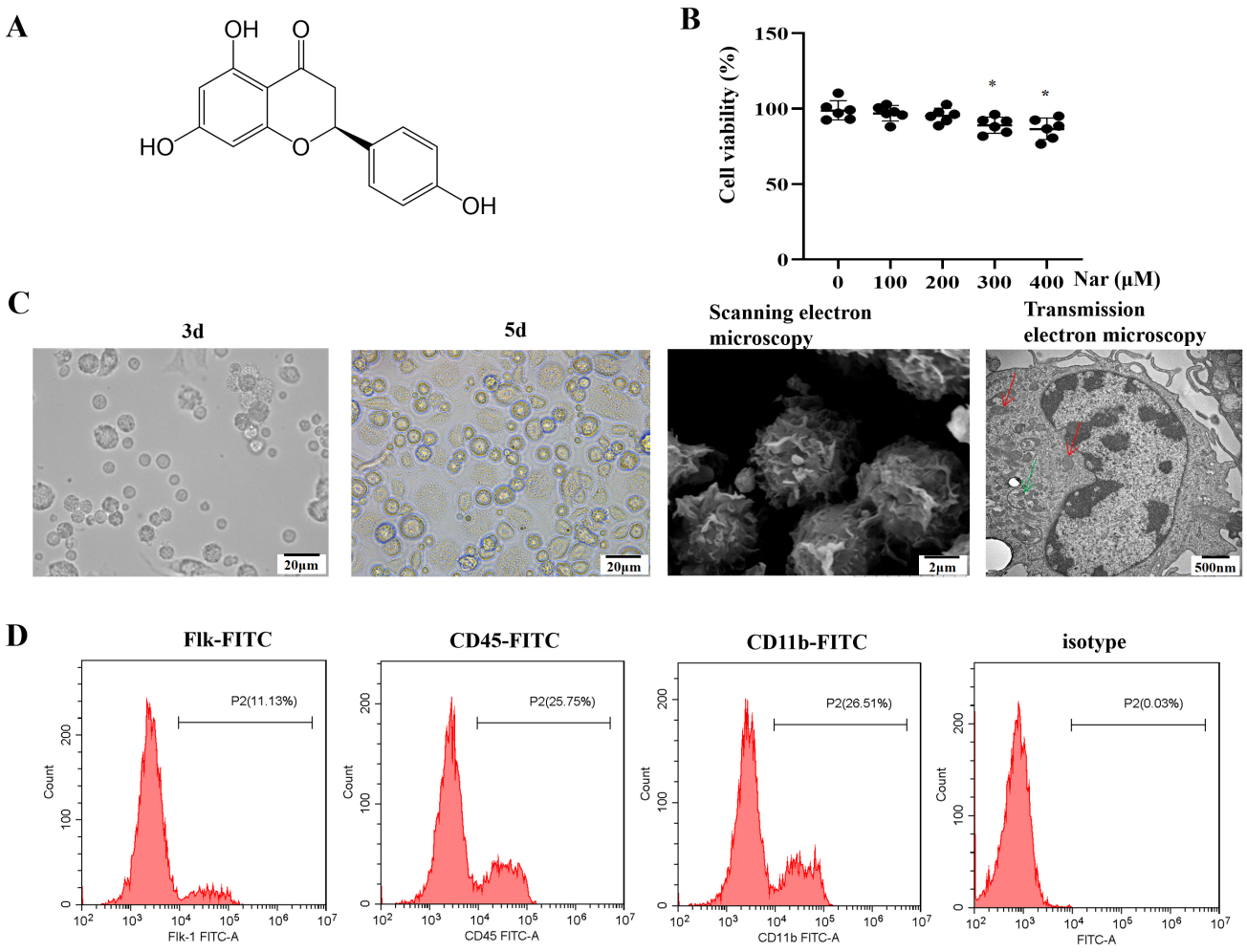

Fig. 1. Differentiation of HSCs into imDCs under the action of Nar. (A) Chemical structure of naringenin; molecular formula: $C_{15}H_{12}O_5$. (B) The optimal concentration of Nar (maximum concentration at which cell viability was >95%) was 200 μM. Compared with 0 μM, $^*p < 0.05$. (C) Phase contrast microscopy showing morphological changes in Nar-HSCs-imDCs after 3 d and 5 d of culture. Scanning electron microscopy and transmission electron microscopy of Nar-HSCs-DCs after 6 d of culture (red arrow = mitochondria; green arrow = lysosome). (D) Flow cytometry detection of Flk, CD45 and CD11b expression on the surface of Nar-HSCs-imDCs. Each experiment contained six biological replicates.

TGF-β (Cat#E-EL-M1191c) in the culture supernatant of HSCs-imDCs and Nar-HSCs-imDCs before and after 48 h of stimulation with LPS (5 μg/mL). ELISA was also used to analyze cytokine levels in the serum of graft recipients at 14 days post graft. The detection limits for IL-2, IL-4, IL-10, IFN-γ and TGF-β varied between 15.63 to 1000 pg/mL.

2.7 Evaluation of Endocytic Capacity

FITC-dextran was used to determine the endocytic ability of HSCs-imDCs and Nar-HSCs-imDCs before and after stimulation with 5 μg/mL LPS for 48 h at 37 °C. Post-stimulation HSCs-imDCs and HSCs-DCs were used as the control groups.

2.8 Tunel Staining

The Tunel Apoptosis Assay Kit (Cat#1086, Beyotime, Beijing, China) was used to evaluate the apoptosis of Nar-HSCs-imDCs and Nar-HSCs-DCs cells. Briefly, cells were treated with protein kinase K and 3% H_2O_2 and then incubated with Tunel detection solution and Streptavidin-HRP solution. DAB solution was added before observation and photography with the BX41 fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan; amplification: $\times$400). Cell apoptosis was analyzed using Image-Pro Plus analysis software 6.0 (Media Cybernetics, San Diego, CA, USA). The proportion of apoptotic cells was calculated as follows: number of positive cells/total number of cells $\times$ 100%.

2.9 Skin Grafting

C57BL/6 mice served as donors, while Balb/c mice served as the recipients. Balb/c mice were randomly divided into 8 groups, with 6 mice in each group. ① Sham group; allogeneic mouse skin graft model without treat-

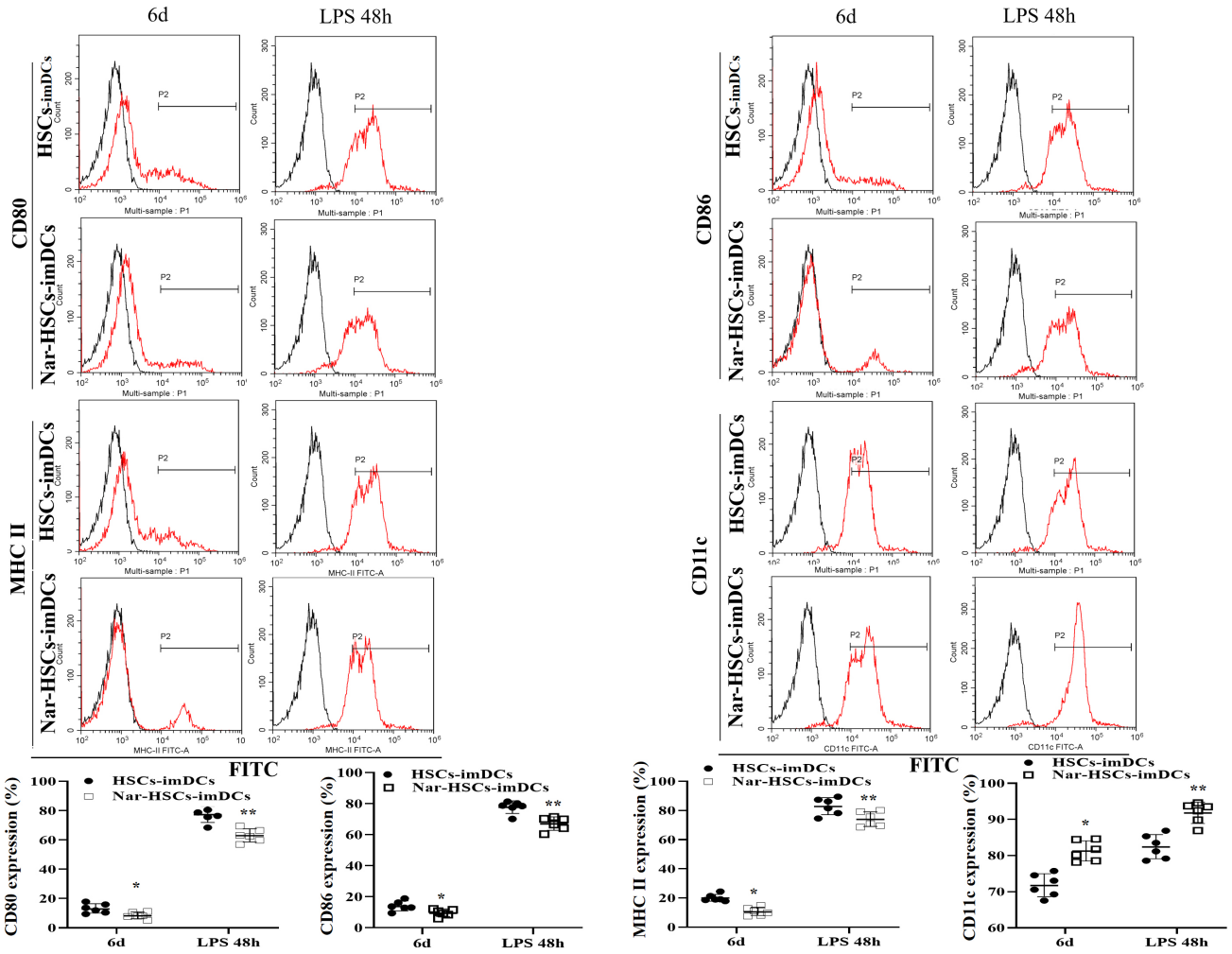

Fig. 2. DC surface phenotype in differentiation culture. The Nar-HSCs-imDCs group is compared with the HSCs-imDCs group. $^{*}p < 0.05$, $^{**}p < 0.01$. Each experiment contained six biological replicates.

ment; ② phosphate-buffered saline (PBS group); 0.3 mL PBS was infused intravenously into recipients 7 days before grafting; ③ 10^5 HSCs-imDCs cell group (10^5 HSCs-imDCs/mouse); ④ 10^6 HSCs-imDCs cell group (10^6 HSCs-imDCs/mouse); ⑤ 10^5 Nar-HSCs-imDCs cell group (10^5 Nar-HSCs-imDCs/mouse); ⑥ 10^6 Nar-HSCs-imDCs cell group (10^6 Nar-HSCs-imDCs/mouse): ③–⑥ group: corresponding cells for each group suspended in 0.3 mL PBS were infused intravenously into recipients 7 days before transplantation; ⑦ Nar group; ⑧ Cyclosporin A (CsA) group; ⑦–⑧ group: 30 mg/kg Nar or CsA were infused into recipients through the tail vein once a day for 3 d before grafting. Allogeneic skin grafting was performed as described previously [16]. Starting from 4 days post-transplant, skin grafts were assessed for rejection once daily for 24 days. Kaplan-Meier analysis was used to evaluate graft survival. The graft together with the recipient's spleen were dissected for analysis on day 14 after grafting.

2.10 Detection of Tregs in Animal Spleens

Spleen cells from each group were collected after mincing. The effect of Nar-HSCs-imDCs on Tregs in the spleen was evaluated by flow cytometry. Unrelated C3H mice were used to evaluate specific reactivity with lymphocytes.

2.11 Hematoxylin and Eosin Staining

Grafted skin sections (4-mm thickness) were stained with hematoxylin and eosin (H&E). Images were captured using a BX41 fluorescence microscope (amplification: 200×; Olympus Corporation, Tokyo, Japan).

2.12 Statistical Analyses

Quantitative data were shown as the mean ± standard error (SEM). Experiments were repeated six times. Intergroup deviations were analyzed using one-way analysis of variance (ANOVA) and GraphPad Prism 9.0 software (Dotmatics, Boston, MA, USA), with $p < 0.05$ considered to represent statistical significance.

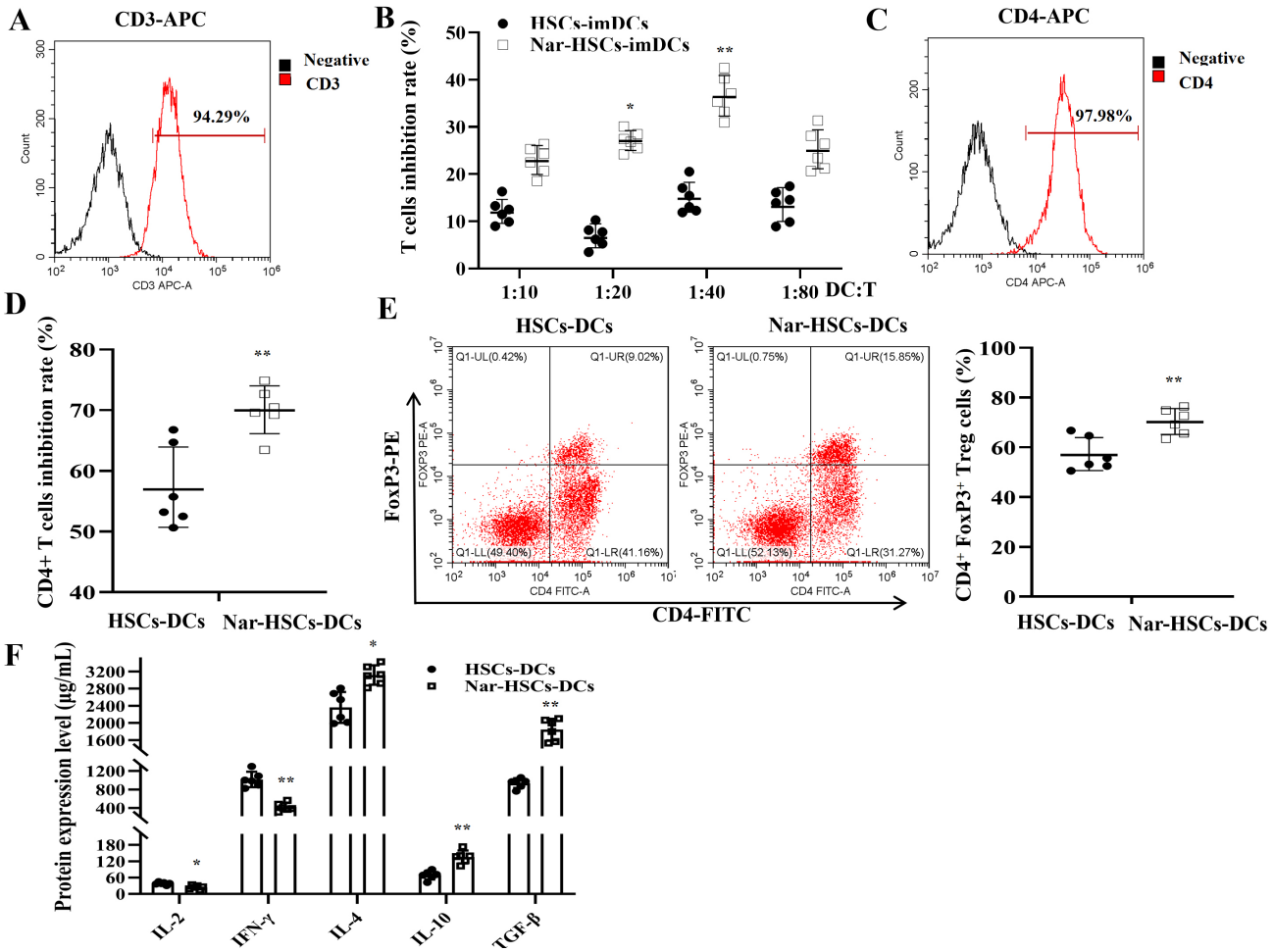

Fig. 3. Nar-HSCs-imDCs suppress T cells and enhance the proliferation of Treg cells. (A) Purity of $CD3^+$ T cells after sorting. (B) MLR with different ratios of imDCs to $CD3^+$ T cells. (C) Purity of $CD4^+$ T cells after sorting. (D) Mixed cultures of HSCs-DCs or Nar-HSCs-DCs with CD4+ T cells at a ratio of 1:40. (E) Effect of HSCs-DCs or Nar-HSCs-DCs on Treg cell proliferation. (F) Cytokine levels in supernatants from co-cultured DCs and Treg cells. Nar-HSCs-DCs are compared with the HSCs-DCs group. $^*p < 0.05$, $^{**}p < 0.01$. Each experiment contained six biological replicates.

3. Results

3.1 Nar Promotes the Differentiation of HSCs into imDCs

The chemical structure of naringenin is shown in Fig. 1A. Morphological changes in Nar-HSCs-imDCs were documented in order to assess the role of Nar in promoting HSC differentiation into imDC, while the cell phenotype during differentiation was examined by flow cytometry. The optimal concentration of Nar (maximum concentration at which cell viability was >95%) was determined to be 200 μM (Fig. 1B). Phase contrast microscopy was used to evaluate morphological changes in Nar-HSCs-imDCs after 3 d and 5 d of culture (Fig. 1C). Burr-like protrusions on the cell surface were observed by scanning electron microscopy. Mitochondria, vesicles, and lysosomes were observed by transmission electron microscopy (Fig. 1C). Elevated expression of the hematopoietic cell markers antigen Flk-1 and CD45 and of the monocyte marker antigen CD11b was observed on the Nar-HSCs-imDC surface (Fig. 1D).

3.2 Surface Phenotype of DCs Grown in Differentiation Culture

Cell surface phenotypes were analyzed by flow cytometry in order to determine the effect of Nar on the differentiation of HSCs into imDCs. The results showed significantly greater downregulation of CD80, CD86, and MHC-II expression on the surface of Nar-HSCs-imDCs than HSCs-imDCs ($p < 0.05$). In contrast, the expression of CD11c was significantly upregulated in Nar-HSCs-imDCs ($p < 0.01$) (Fig. 2). These results suggest that Nar inhibits the maturation of DCs derived from HSCs.

3.3 Nar-HSCs-imDCs Suppresses T Cells and Increases the Proliferation of Treg Cells

We next investigated the effects of HSCs-imDCs and Nar-HSCs-imDCs on T cells by isolating $CD3^+$ and $CD4^+$ T cells from the spleen of recipient mice using magnetic beads. The purity of $CD3^+$ T cells was 94.31 $\pm$ 2.57% (Fig. 3A). HSCs-imDCs and Nar-HSCs-imDCs were first

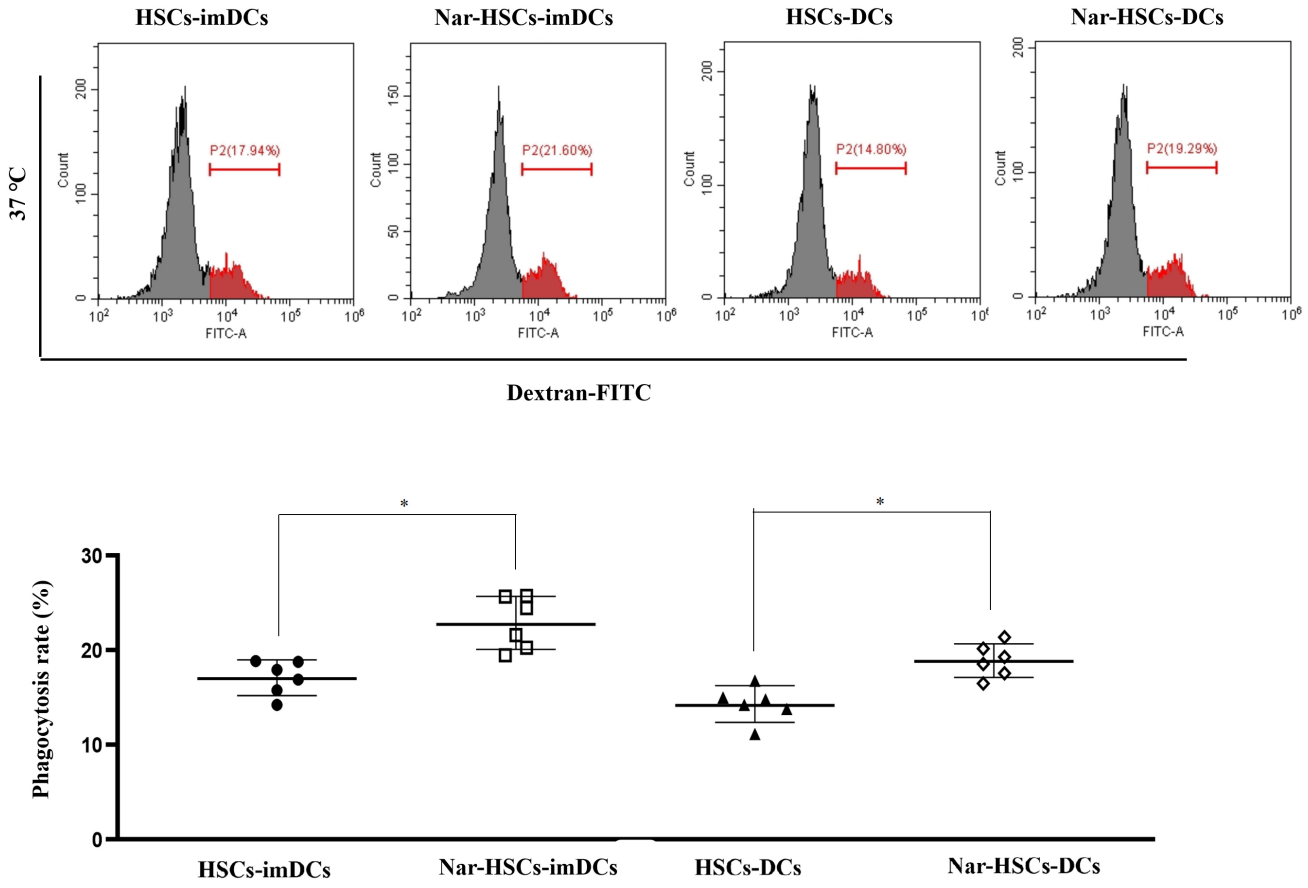

Fig. 4. Endocytic capacity of Nar-HSCs-imDCs and Nar-HSCs-DCs. The results are compared with the HSCs-imDCs and HSCs-DCs groups, respectively. $^{*}p < 0.05$. Each experiment contained six biological replicates.

stimulated with LPS for 48 h. MLR with sorted T cells was then performed for 72 h. DCs were co-cultured with T cells in varying proportions (1:10, 1:20, 1:40, and 1:80). The Nar-HSCs-DCs group inhibited T cell proliferation more than the HSCs-DCs group. The difference was most significant at a proportion of 1:40 (Fig. 3B) ($p < 0.01$). The purity of $CD4^+$ T cells was 97.95 $\pm$ 1.28% (Fig. 3C). HSCs-DCs and Nar-HSCs-DCs were co-cultured with $CD4^+$ T cells at a ratio of 1:40 for 72 h. Nar-HSCs-DCs again inhibited the proliferation of $CD4^+$ T cells significantly more than HSCs-DCs (Fig. 3D) ($p < 0.01$). We next sorted $CD4^+CD25^+$ Treg cells and co-cultured them with HSCs-DCs or Nar-HSCs-imDCs at a ratio of 40:1. Flow cytometry showed that $CD4^+CD25^+FoxP3^+$ Treg cell proliferation was promoted significantly more by Nar-HSCs-DCs than by HSCs-DCs (Fig. 3E) ($p < 0.01$). Furthermore, IL-2 and IFN-γ levels in culture medium from the Nar-HSCs-DCs group were significantly lower than in the Nar-HSCs-DCs group ($p < 0.05$), whereas the levels of IL-4, IL-10 and TGF-β were significantly higher (Fig. 3F) ($p < 0.01$). In summary, these results showed that Nar-HSCs-DCs can inhibit the proliferation of $CD4^+$ T cells, enhance the proliferation of $CD4^+CD25^+FoxP3^+$ Treg cells, suppress the secretion of IL-2 and IFN-γ, while increasing the secretion of IL-4, IL-10 and TGF-β.

3.4 Endocytic Capacity of HSCs-DCs and Nar-HSCs-DCs

imDCs have been reported to have more vital phagocytosis ability than DCs [6]. The present study found that Nar-HSCs-imDCs and Nar-HSCs-DCs had significantly higher endocytic capacity for FITC-dextran at 37 °C than HSCs-imDCs and HSCs-DCs, respectively (Fig. 4) ($p < 0.05$).

3.5 Apoptosis in Nar-HSCs-imDCs and Nar-HSCs-DCs

We hypothesized that LPS stimulation of Nar-HSCs-imDC resulted in apoptosis. To test this, flow cytometry was used to quantify the level of apoptosis in Nar-HSCs-DCs. The apoptosis rate of Nar-HSCs-DCs was significantly higher than that of HSCs-DCs (Fig. 5A). TUNEL staining showed similar results (Fig. 5B).

3.6 Immunization with Nar-HSCs-imDCs Prolongs the Survival of Allogeneic Skin Grafts and Increases the Number of Activated Treg Cells in Allografts

H&E staining showed that lymphocyte infiltration was reduced in the HSCs-imDCs and Nar-HSCs-imDCs groups compared to the control group, especially in the 10^6 Nar-HSCs-imDCs group (Fig. 6A). The graft rejection score

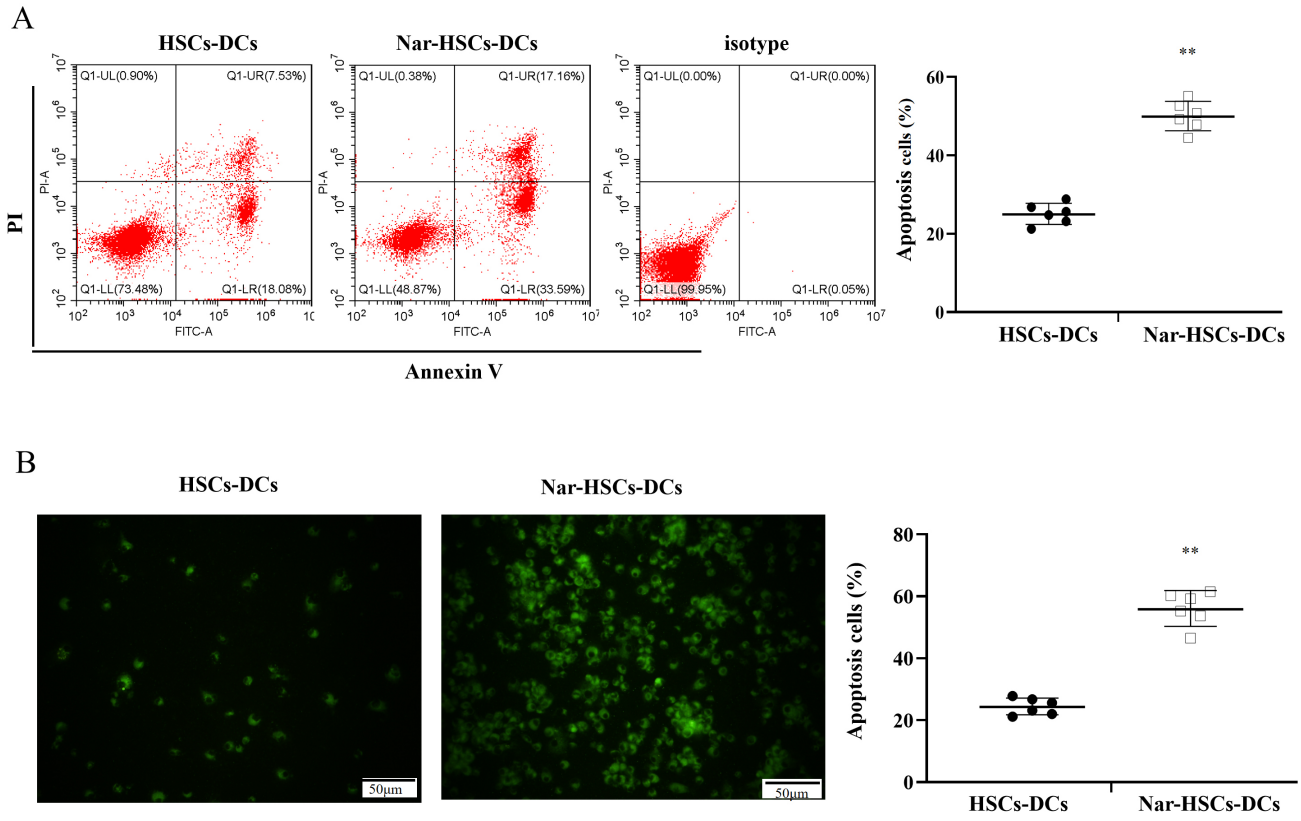

Fig. 5. Apoptosis in HSCs-DCs and Nar-HSCs-DCs. (A) Apoptosis in HSCs-DCs and Nar-HSCs-DCs was measured by flow cytometry. (B) Apoptosis calculated by TUNEL staining. Nar-HSCs-DCs was compared with the HSCs-DCs group. $^{**}p < 0.01$. Each experiment contained six biological replicates.

was lower in the Nar-HSCs-imDCs group compared to the HSCs-imDCs group, particularly in the 10^6 Nar-HSCs-imDCs group (Fig. 6B). Moreover, the allograft survival time was significantly prolonged in the 10^6 Nar-HSCs-imDCs group (Fig. 6C).

3.7 Immunization with Nar-HSCs-imDCs Suppresses T Cells and Promotes Activated Treg Cells in Allografts

The Nar-HSCs-imDCs group showed a higher percentage of $CD4^+CD25^+$ Treg/$CD4^+$ T cells than the HSCs-imDCs and Nar groups, while the percentage of $CD4^+CD25^+FoxP3^+$ Treg/$CD4^+CD25^+$ Treg showed the same trend. The percentage was also significantly higher in the 10^6 Nar-HSCs-imDCs group compared to the 10^5 Nar-HSCs-imDCs group (Fig. 7A,B) ($p < 0.05$). Cytokine levels in the serum of all groups were measured on day 7 post-transplant. IL-2 and IFN-γ levels were lower, whereas IL-4, IL-10, and TGF-β levels were higher in the serum from 10^6 Nar-HSCs-imDCs immunization recipients compared with 10^5 Nar-HSCs-imDCs and 10^6 HSCs-imDCs immunization recipients (Fig. 7C) ($p < 0.05$). On day 7 post-transplant, spleen lymphocytes were taken from donor mice and unrelated third-party C3H mice, treated with 20 μg/mL mitomycin (MMC), and then used as stimulatory cells. Lymphocytes from the spleen of recipient mice were used as reaction cells. The *in vitro* MLR model was used to test whether the effect was specific. The responsiveness of spleen lymphocytes was found to be significantly lower in donor mice than in C3H mice in both the HSC-imDCs and Nar-HSCs-imDCs groups, and was lowest in the 10^6 Nar-HSCs-imDCs group (Fig. 7D) ($p < 0.05$). This result demonstrates that the infusion of donor-derived Nar-HSCs-imDCs can induce donor-specific immune tolerance, especially in the 10^6 Nar-HSCs-imDCs group.

4. Discussion

Induction of transplantation tolerance could avoid the side effects of long-term use of immunosuppressants and reduce the risk of immune rejection [17]. With recent advances in cellular immunotherapy, various cellular solutions for the induction of immune tolerance have emerged in the transplantation field. imDCs are increasingly recognized as possible mediators of T cell tolerance [18]. Previous reports showed that CD4 T cell anergy could be induced by the injection of *in vitro*-generated imDCs [19]. These imDCs also induced T cells to differentiate into T helper 2 cells, inhibited the secretion of inflammatory factors (e.g., IL-2, IFN-γ), increased the secretion of anti-inflammatory factors (e.g., IL-4, IL-10, TGF-β), and eventually increased the proportion of Treg cells [20–22]. Increased levels of

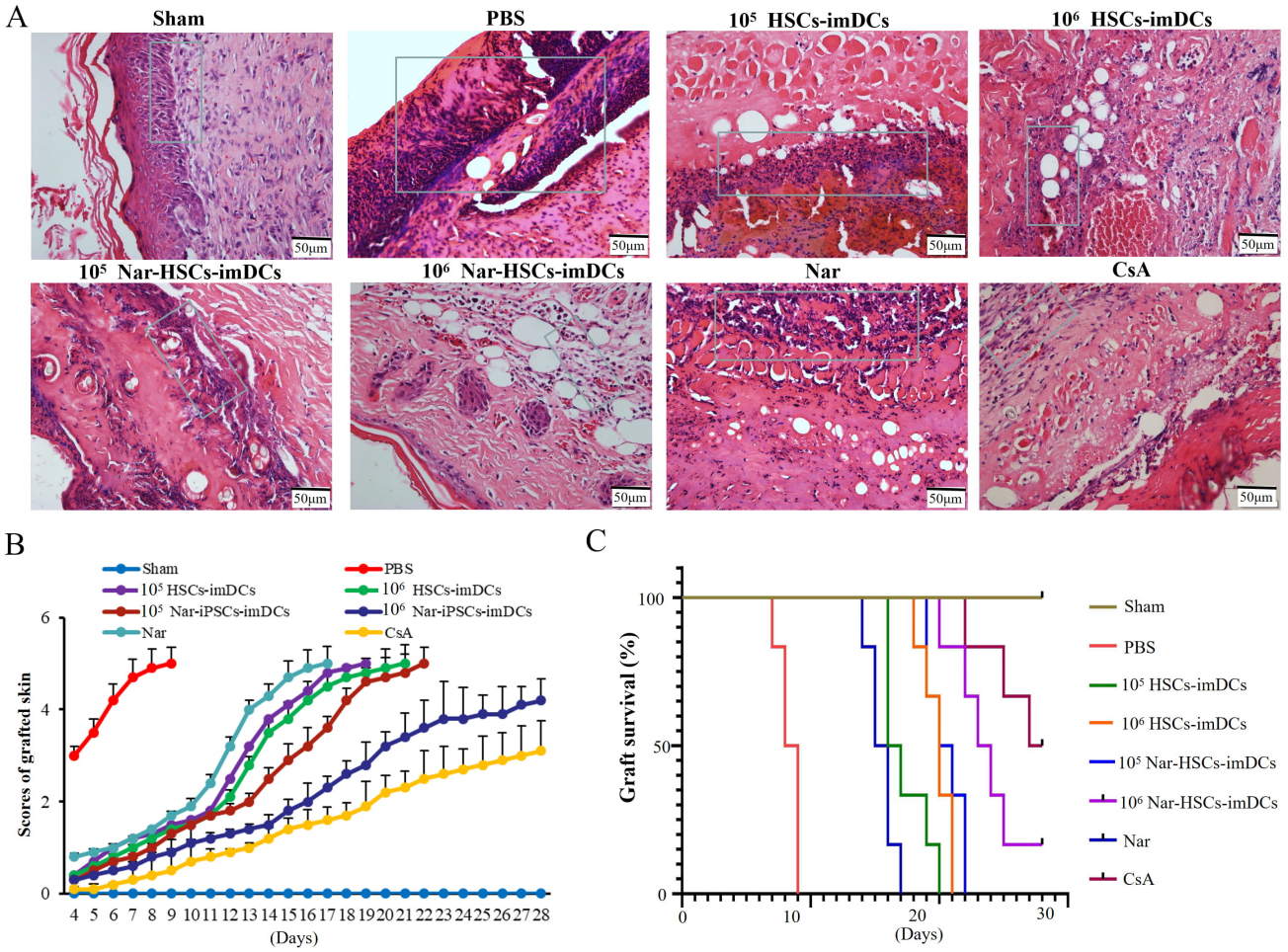

Fig. 6. Nar-HSCs-imDCs induce immune hypo-responsiveness in the mouse skin transplant model. (A) H&E staining. (B) Graft scores. (C) Graft survival curves. Each experiment contained six biological replicates.

Treg cells maintain the immature state of imDCs [23], thus forming a positive feedback loop that ultimately induces immunotolerance [24].

There are currently several ways to impede the maturation of imDCs. These include blocking the expression of imDC surface-specific molecules through gene modification [25–27], inhibiting the expression of imDCs co-stimulatory molecules with drugs [28], inhibiting imDCs maturation with immunomodulatory factors [29,30], and preventing the development of imDCs with immunosuppressants [31].

However, these methods have several drawbacks. First, gene modification has potential immunogenicity and biosafety problems, and the transfection efficiency is low. Second, the vast inter-individual differences make it difficult to control the concentration of imDCs in the body using drug intervention. Third, imDCs are prone to mature when stimulated by cytokines, pathogenic microorganisms, grafts, etc., in the body. Therefore, maintaining imDCs in an immature state poses a significant challenge.

In recent years, natural plant-derived ingredients have been widely used in the medical field due to their low toxicity and wide-ranging biological activities [32,33]. Nar is a major flavanone extracted from grapefruit. It has various pharmacological activities, including antioxidant, anti-tumor, anti-atherosclerotic, antibacterial, and neuroprotective effects, as well as high bioavailability and safety [34]. Niu *et al.* [35] reported that Nar can ameliorate experimental autoimmune encephalomyelitis by suppressing the initiation and proliferation of T lymphocytes and inhibiting production of the cytokines IL-6, IFN-γ, and IL-17. In addition, Nar inhibited T cells by arresting the T cell cycle and regulating the IL-2/IL-2 receptor pathway, thus preventing or alleviating autoimmune diseases. Interestingly, Nar was also shown to promote the induction of Treg cells and to enhance their suppressive function in an inflammatory model [36]. These findings suggest that Nar could induce hyporesponsiveness or tolerance. In the present study, we successfully obtained Nar-HSCs-imDCs and found that Nar could maintain the immature state of HSCs-DCs. We further confirmed that Nar can promote the apoptosis of HSCs-imDCs after stimulation with LPS. This is important because it prevents the occurrence of an immune storm caused by Nar-HSCs-imDCs after maturation *in vivo*.

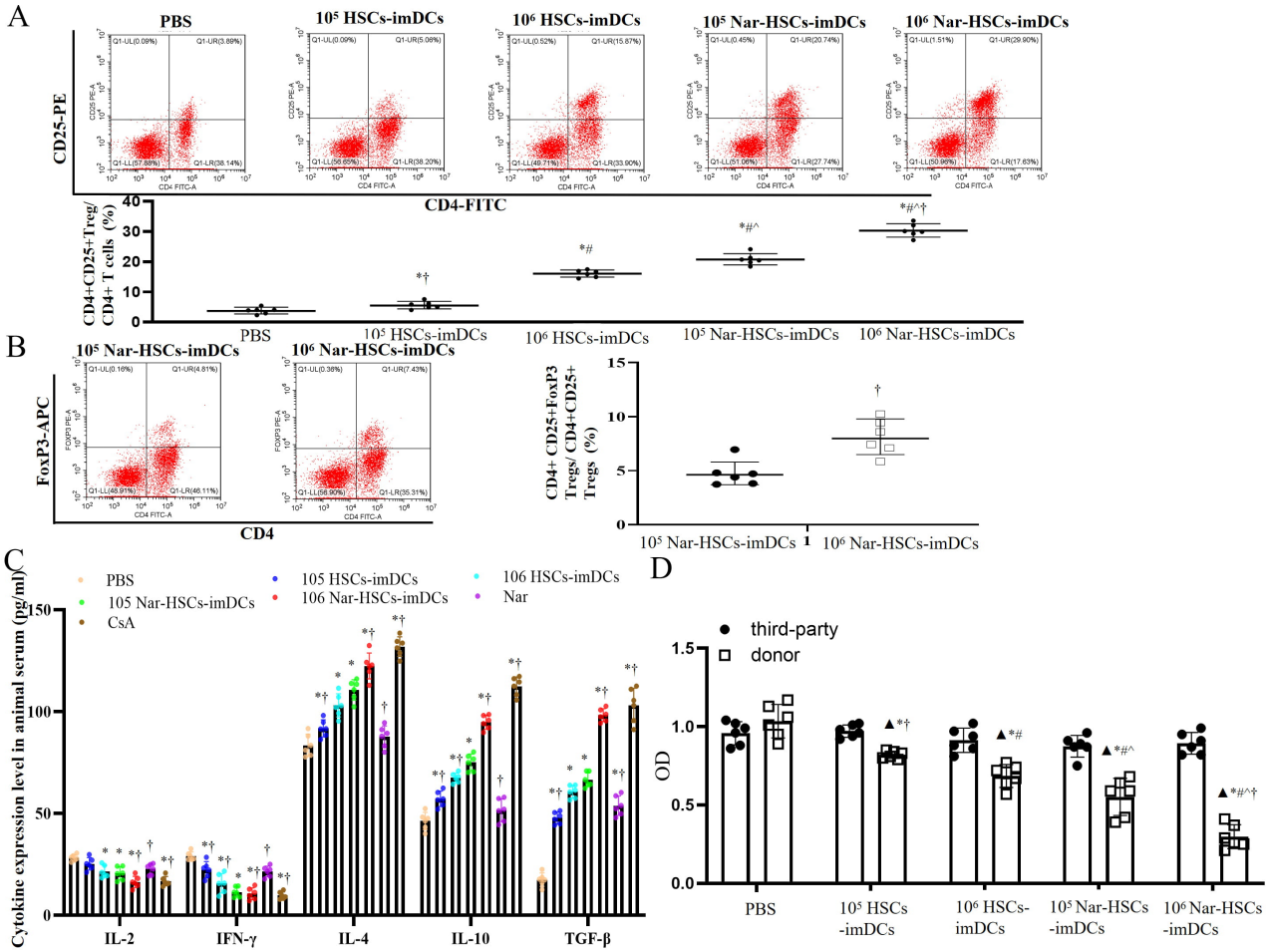

Fig. 7. Nar-HSCs-imDCs inhibited T cells and promoted Treg cells proliferation in allografts. (A) The ratios of $CD4^+CD25^+$Treg cells/$CD4^+$ T cells in the spleen in all groups. (B) $CD4^+CD25^+FoxP3^+$ Treg cells/$CD4^+CD25^+$ Treg cells in the spleen in allgroups. (C) Cytokine levels in the serum in all groups were measured on day 7 post-transplant. (D) Reactivity of recipient lymphocytesto the donor or unrelated third-party lymphocytes. Compared with the PBS group, $^*p < 0.05$. Compared with the 105 HSCs-imDCsgroup, $^\#p < 0.05$. Compared with the 106 HSCs-imDCs group, $^\wedge p < 0.05$. Compared with the 106 Nar-HSCs-imDCs group, $^\dagger p < 0.05$. Compared to third-party lymphocytes, $^\blacktriangle p < 0.05$. Each experiment contains six biological replicates.

Donor immune cell infusion therapy can increase negative immune regulation in recipients, which is the most effective approach for attenuating graft rejection [37,38]. In the present study we infused Nar-HSCs-imDCs with immunosuppressive properties from donor C57BL/6 mice into recipient Balb/c mice. This was done through the tail vein 7 d before skin grafting. We found that Nar-HSCs-imDCs could maintain immature characteristics for longer, increase Treg cells, and prolong the graft survival time in a donor-specific manner. This is consistent with previous research showing that DCs coordinate the growth and homeostasis of organ-specific Treg cells [39]. An explanation for these findings may be that donor-type Nar-HSCs-imDCs act as a primary vaccine, while the alloantigen acts as a secondary vaccine, thereby promoting the proliferation and sustained activation of donor-specific Treg cells.

We also found that the currently used CsA dose showed the strongest immune-suppressive effect. However, CsA can cause side effects such as increased opportunistic infections and tumorigenicity. In future studies we will therefore use Nar in combination with half or smaller doses of CsA in an attempt to achieve the same induction of immune tolerance as full dose CsA.

This study had several limitations. First, only two groups (high dose [10^6/mouse] and low dose [10^5/mouse]) were used to investigate Nar-HSCs-imDCs infusion into recipient mice. More detailed, efficient, and safe dosages should be explored in future work. Second, further research is required to reveal the migration pattern of Nar-HSC-imDCs *in vivo* and to identify the final distribution of Nar-HSC-imDCs in various immune organs. Third, the serum used for cell culture contains mediators that could impact the immune response. Moreover, the potential for disease transmission and the possibility of immune reaction to non-

self-proteins renders serum unsuitable for clinical applications. In follow up studies, we plan to culture stem cells under serum-free conditions. The long-term goal of our research is to investigate combinations of traditional Chinese medicine monomers (such as naringenin/sinomenine) and stem cells for their ability to induce immune tolerance following organ transplantation. We plan to continue screening traditional Chinese medicine monomers to identify those with the best effect on stem cells for inducing tolerance, while also clarifying their mechanism of action.

5. Conclusions

We have shown for the first time that Nar-treated HSCs-imDCs may be an effective therapeutic strategy for inducing immune tolerance in organ transplantation. This study presents a novel approach for the clinical implementation of stem cell biotechnology in combination with traditional Chinese medicine.

Availability of Data and Materials

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

Author Contributions

PT, XH, and CX, designed the research study. KZ, ZJ, YL, XZ, and BZ performed the research. PT, XZ, YF, QF, DL, and JW performed the transplant operations. PT, XZ, and XH analyzed the data. XH and CX wrote the manuscript. PT, XH, and KZ revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All procedures were carried out in accordance with the NIH Guidelines and ethics of animal use were reviewed and approved by the Biomedical Ethics Committee of Xi'an Jiaotong University (No. 2022-198). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2805091.

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