TRANSLATIONAL AND CLINICAL RESEARCH

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Mesenchymal stem cells regulate type 2 innate lymphoid cells via regulatory T cells through ICOS-ICOSL interaction

Xingliang Fan[1] | Zhi-Bin Xu[1] | Cheng-Lin Li[1,2] ᴵᴰ | Hong-Yu Zhang[1] |
Ya-Qi Peng[1] | Bi-Xin He[1] | Xiao-Qing Liu[1] | De-Hua Chen[1] | Dong Chen[1] |
Cezmi A. Akdis[3,4] | Qing-Ling Fu[1,5] ᴵᴰ

[1]Otorhinolaryngology Hospital, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, People's Republic of China

[2]Center for Clinical Medicine Innovation, ShenZhen Hospital of Southern Medical University, Guangdong, People's Republic of China

[3]Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland

[4]Christine Kühne—Center for Research and Education (CK-CARE), Davos, Switzerland

[5]Key Laboratory for Stem Cells and Tissue Engineering, Ministry of Education, Sun Yat-sen University, Guangzhou, Guangdong, People's Republic of China

Correspondence
Qing-Ling Fu, MD, PhD, Otorhinolaryngology Hospital, The First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan Road II, Guangzhou, 510080, People's Republic of China.
Email: fuqingl@mail.sysu.edu.cn

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Abstract

Group 2 innate lymphoid cells (ILC2s) are recognized as key controllers and effectors of type 2 inflammation. Mesenchymal stem cells (MSCs) have been shown to alleviate type 2 inflammation by modulating T lymphocyte subsets and decreasing T_H2 cytokine levels. However, the effects of MSCs on ILC2s have not been investigated. In this study, we investigated the potential immunomodulatory effects of MSCs on ILC2s in peripheral blood mononuclear cells (PBMCs) from allergic rhinitis patients and healthy subjects. We further investigated the mechanisms involved in the MSC modulation using isolated lineage negative (Lin⁻) cells. PBMCs and Lin⁻ cells were cocultured with induced pluripotent stem cell-derived MSCs (iPSC-MSCs) under the stimulation of epithelial cytokines IL-25 and IL-33. And the ILC2 levels and functions were examined and the possible mechanisms were investigated based on regulatory T (Treg) cells and ICOS-ICOSL pathway. iPSC-MSCs successfully decreased the high levels of IL-13, IL-9, and IL-5 in PBMCs in response to IL-25, IL-33, and the high percentages of IL-13⁺ILC2s and IL-9⁺ILC2s in response to epithelial cytokines were significantly reversed after the treatment of iPSC-MSCs. However, iPSC-MSCs were found directly to enhance ILC2 levels and functions via ICOS-ICOSL interaction in Lin⁻ cells and pure ILC2s. iPSC-MSCs exerted their inhibitory effects on ILC2s via activating Treg cells through ICOS-ICOSL interaction. The MSC-induced Treg cells then suppressed ILC2s by secreting IL-10 in the coculture system. This study revealed that human MSCs suppressed ILC2s via Treg cells through ICOS-ICOSL interaction, which provides further insight to regulate ILC2s in inflammatory disorders.

KEYWORDS
group 2 innate lymphoid cells, ICOS-ICOSL interaction, IL-10, immunomodulation, mesenchymal stem cells, regulatory T cells

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Xingliang Fan, Zhi-Bin Xu, and Cheng-Lin Li contributed equally to this study.

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1 | INTRODUCTION

Allergic airway inflammation, including allergic rhinitis (AR) and asthma, is an abnormally exacerbated reaction associated with conjunctival symptoms toward common environmental factors.[1,2] Since the discovery of CD4^{+} T-cell subpopulations, T_H2 cells have become the key player in the development of allergic inflammation disorders via secreting specific cytokines IL-4, IL-5, and IL-13. Besides, the contribution of IL-9 has also become into focus in the development of allergic immune reactions.[3] With regard to the mechanisms of allergic inflammation, there has been the development of a variety of novel therapeutic approaches interfering with the secretion of specific cytokines. In recent years, group 2 innate lymphoid cells (ILC2s) are increasingly recognized as a key controller of type 2 inflammation by their capability to secrete allergic cytokines IL-13, IL-5 and IL-9, and they are well known to be highly elevated and activated in a variety of human allergic disorders including AR and asthma.[4] ILC2s are demonstrated to be critical for the initiation and exacerbation of type 2 immune response.[5-9] This applies to allergic immune responses, in which in addition to CD4^{+} T-cell subpopulations, ILC2s are a crucial target in the treatment of allergic inflammation disorders.

It has been shown that mesenchymal stem cells (MSCs) have an immunomodulatory therapeutic function for inflammatory disorders.[10] We have previously demonstrated that induced pluripotent stem cell-derived MSCs (iPSC-MSCs) alleviate type 2 inflammation by modulating T lymphocyte subsets and decreasing T_H2 cytokine expression levels in patients with AR and animal models.[11,12] Besides, MSCs were found able to promote regulatory T (Treg) cell differentiation from CD4^{+} T cells via inducible costimulator (ICOS)-ICOS ligand (ICOSL) signaling through phosphoinositide 3-kinase (PI3K)-Akt pathway.[13] We also found that iPSC-MSCs promoted Treg cell expansion and activation, by which to inhibit effector T cell proliferation and induction in peripheral blood mononuclear cells (PBMCs) from AR patients.[14] However, the effects of MSCs on ILC2s have not been investigated. It is of great importance to illustrate the immunomodulatory role of MSCs on ILC2s for their possible clinical application in treating allergic diseases. Hence, this study aimed to elucidate the immunomodulatory effects of iPSC-MSCs on circulating ILC2s after epithelial cytokine induction, the possible mechanisms involved in the regulatory effects were also examined. We hypothesized that iPSC-MSCs may significantly inhibit ILC2 proliferation and activation, by which to suppress the development of allergy-specific inflammation.

We cocultured iPSC-MSCs with PBMCs from AR patients and healthy subjects under the stimulation of IL-25/33, by which to investigate the immunomodulatory role of iPSC-MSCs on ILC2s during the effector phase of allergic airway inflammation. Lineage negative (Lin⁻) cells and ILC2s were further isolated from PBMCs to investigate the possible mechanisms involved in the MSC modulation. Unexpectedly, iPSC-MSCs did not directly exhibit suppression effects on ILC2 activation, Treg cells were required in mediating the inhibitory effects. Notably, we found that ICOS-ICOSL interaction might be critical for

Significance statement

Group 2 innate lymphoid cells (ILC2s) are increasingly recognized for their ability in the initiation and acceleration of type 2 inflammation. Mesenchymal stem cells (MSCs) have been shown to alleviate type 2 inflammation by modulating T lymphocyte subsets and decreasing T_H2 cytokine expression levels in patients with allergic rhinitis and in animal models. However, the effects of MSCs on ILC2s have not been investigated. It is of great importance to illustrate the immunomodulatory role of MSCs on ILC2s for their possible clinical application in treating allergic diseases. The present study demonstrated for the first time that induced pluripotent stem cell-derived MSCs (iPSC-MSCs) are able to inhibit ILC2 functions via the assistance of regulatory T (Treg) cells. ICOSL induced Treg cells are critical for the inhibitory functions of iPSC-MSCs on ILC2s. These new findings are of possible clinical value and provide further insight to regulate ILC2s in inflammatory disorders.

iPSC-MSC modulation on ILCs, there was a competitive mechanism for iPSC-MSCs to interact with ILC2s and Treg cells. Thus, we conclude that the immunomodulatory function of MSCs on ILC2s is a complex network interaction.

2 | MATERIALS AND METHODS

2.1 | iPSC-MSC culture

Human iPSC-MSCs were generated from urine cell-derived-induced pluripotent stem cells (U-iPSCs) which donated by Guangzhou Institute of Biomedicine and Health, Chinese Academy of Science (Guangzhou, China) as described in our previous study.[15] In brief, U-iPSCs were cultured in MSC-inducing-culture media containing Minimum Essential Medium Eagle-α modified (α-MEM, Gibco, Gaithersburg, Maryland), 10% serum replacement (Stem Cell Technologies, Vancouver, Canada), penicillin/streptomycin, sodium pyruvate, 10 mM L-ascorbate-2-phosphate, L-glutamine and nonessential amino acids (Sigma-Aldrich, Inc, St. Louis, Missouri) for 2 weeks. The generated iPSC-MSCs were cultured in Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum (FBS), 1% penicillin-streptomycin (Gibco, Gaithersburg, Maryland), 5 ng/mL EGF, and 5 ng/mL β-FGF (PeproTech, Inc, Rocky Hill, New Jersey) at 37°C, 5% CO_2 and 95% humidity. They were positive for CD44, CD73, CD90, CD105, CD144, and CD146, but negative for CD14, CD34, and CD45. Their adipogenic, chondrogenic, and osteogenic differentiation capacity was also confirmed before usage.[15]

2.2 | PBMC isolation and the treatments

The study was approved by the Ethics Committee of The First Affiliated Hospital, Sun Yat-sen University. Written informed consents were obtained from all participants. PBMCs were isolated from healthy controls (n = 6) and AR patients (n = 8) by density centrifugation with Ficoll-Paque Plus (MP Biomedicals, Santa Ana, California). Control subjects did not report any symptoms of AR and did not have a history of allergic airway inflammation. The inclusion criteria for patients with AR were established according to the Initiative on Allergic Rhinitis and its Impact on Asthma (ARIA): (a) history of nasal symptoms by nose itching, obstruction, sneezing, and rhinorrhea; (b) positive specific immunoglobulin E (sIgE). Patient information can be found in Table S1.

The isolated PBMCs were cocultured with iPSC-MSCs in a ratio of 10:1 in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin. Recombinant human (rh)IL-2 (20 U/mL), rhIL-25 (10 ng/mL), and rhIL-33 (10 ng/mL; PeproTech, Inc, Rocky Hill, New Jersey) were added into the coculture system as a positive control following the previous study.[16] ELISA, quantitative PCR and flow cytometry were performed to analyze the ILC2 functions from the coculture system after 3 days.

2.3 | Lineage negative (Lin⁻) cell and ILC2 isolation, and the treatments

Lin⁻ cells were isolated from human blood buffy coats using Lineage Cell Depletion Kit, human (Miltenyi Biotec, Bergisch Gladbach, Germany). Human blood buffy coats from "anonymous donors" were obtained from Guangzhou Blood Centre; exemption of written informed consent was approved. The isolated Lin⁻ cells were cocultured with iPSC-MSCs in a ratio of 10:1 in the presence of rhIL-2 (20 U/mL), rhIL-25 (10 ng/mL), and rhIL-33 (10 ng/mL) for 3 days. The culture supernatants were collected for cytokine level analysis by ELISA. The Lin⁻ cells were analyzed for CRTH2⁺ cells, CD127⁺ cells, IL-9⁺ILC2s, and IL-13⁺ILC2s by flow cytometry. Furthermore, ILC2s were isolated from Lin⁻ cells using ILC2 Isolation Kit, human (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, Lin⁻ cells were incubated with CD294 (CRTH2)-phycoerythrin (PE) and with anti-PE MicroBeads, and they were isolated by positive selection over MS columns as ILC2s. The gating plots showing the collection of purified ILC2s were presented in Figures S1 and S2. The isolated ILC2s were cocultured with iPSC-MSCs in a ratio of 10:1. Then the culture supernatants were collected for cytokine level analysis by ELISA. For ICOS-ICOSL interaction blocking experiments, CD275 (B7-H2) Monoclonal Antibody (10 μg/mL, e-Bioscience, San Diego, California) was directly added into the coculture system. A total of six independent repeats were performed for each experiment unless otherwise specified.

2.4 | Regulatory T (Treg) cell isolation and the treatments

Treg cells were isolated from human blood buffy coats using human CD4⁺CD25⁺CD127^{dim/−} Regulatory T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). For the Treg cells pre-treated with iPSC-MSCs (MSC-Treg), PBMCs were cocultured with iPSC-MSCs for 3 days and then the Treg cells were isolated. The isolated Treg cells were cocultured with Lin⁻ cells and iPSC-MSCs in a ratio of 10:10:1 for 3 days. For ICOS-ICOSL interaction blocking experiments, Treg cells were cultured for 24 hours in the presence of CD275 (B7-H2) Monoclonal Antibody (10 μg/mL, e-Bioscience, San Diego, California) before coculture. For IL-10 blocking experiments, Anti-Human IL-10 monoclonal antibody (75 ng/mL, R&D Systems, Minneapolis, Minnesota) was directly added into the coculture system. The culture supernatants were collected for cytokine level analysis by ELISA. The floating cells were analyzed for CRTH2⁺ cells, IL-9⁺ILC2s, and IL-13⁺ILC2s by flow cytometry.

2.5 | Monocytes isolation

Monocytes were isolated from human blood buffy coats using human CD14 MicroBeads from Miltenyi Biotec (Bergisch Gladbach, Germany). The isolated monocytes were cocultured with Lin⁻ cells and iPSC-MSCs in a ratio of 10:10:1 for 3 days. The culture supernatants were collected for IL-13, IL-9, and IL-5 level analysis by ELISA. A total of three independent repeats were performed.

2.6 | Flow cytometry

The cocultured PBMCs or Lin⁻ cells were stained with the following specific monoclonal antibodies (mAbs): FITC-conjugated lineage cocktail: anti-CD2 (RPA-2.10), anti-CD3 (OKT3), anti-CD14 (61D3), anti-CD16 (CB16), anti-CD19 (HIB19), anti-CD56 (TULY56), anti-CD235a (HIR2); PE-conjugated anti-CRTH2 (BM16); PE-Cy7-conjugated anti-CD127 (eBioRDR5; all from eBioscience, San Diego, California) for ILC2 evaluation. The ICOS expression on Lin⁻ cells and Treg cells was examined using eFluor 450-conjugated anti-CD278 (ICOS) (ISA-3, eBioscience). And the ICOSL expression on Lin-cells and iPSC-MSCs was examined using APC-conjugated anti-CD275 (B7-H2, eBioscience). For intracellular cytokine detection, PBMCs or Lin⁻ cells were treated with cell stimulation cocktail (plus protein transport inhibitors) consisting of phorbol 12-myristate 13-acetate (PMA, 30 ng/mL), ionomycin (500 ng/mL), and GolgiStop (3 μg/mL, eBioscience, San Diego, California) for 5 hours in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin at 37°C before collection. The supernatants were collected for the determination of the cytokine levels using ELISA. Thereafter, the cells were stained for intracellular IL-13 (eFluor 450-conjugated anti-IL-13, eBio13A; eBioscience), IL-9 (Alexa Fluor 647-conjugated anti-IL-9, MH9A3; BD Pharmingen, Bergen, New Jersey).

2.7 | ELISA

The IL-5, IL-9, and IL-13 levels in the culture supernatant of PBMCs from AR patients and healthy subjects were measured using ELISA kit

from RayBiotech, Inc, Peachtree Corners, Georgia. The IL-5, IL-9, and IL-13 levels in the culture supernatant of Lin$^-$ cells and ILC2s, and the IL-10 levels in the culture supernatant of Treg cells were measured using ELISA kit from Invitrogen, Carlsbad, California. All procedures were carried out according to the manufacturer's instructions.

2.8 | Quantitative real-time PCR

Total RNA was isolated from the PBMCs using Trizol reagent (Invitrogen, Carlsbad, California). RNA samples were then reverse transcribed into first-strand cDNA using the PrimeScript (TaKaRa Bio, Otsu, Japan). Real-time quantitative PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California). The primer sequences of IL-5, IL-13, and β-actin (internal reference) are listed in Table S2.

2.9 | Western blot

The Lin$^-$ cells cocultured with iPSC-MSCs were collected and lysed in RIPA lysis buffer (CoWin Biosciences, Beijing, China) supplemented with phosphatase inhibitor PhosSTOP (Roche, Basel, Switzerland). The lysates were subjected to SDS-PAGE for detecting AKT (Akt Antibody #9272), phospho-AKT (Phospho-Akt (Ser473) (D9E) XP Rabbit mAb #4060, Cell Signaling Technology, Danvers, Massachusetts). β-Actin (IGX3831H, Abcam, Cambridge, UK) was detected as the internal control. Immunoreactive bands were captured on the G:BOX Chemi Imaging System (Syngene, India) and quantified with ImageJ software.

2.10 | Statistics

All data were analyzed using GraphPad Prism 6 software (GraphPad, San Diego, California) and expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey's test was performed for comparisons of the data with Gaussian distribution in three or more groups. A Kruskal-Wallis rank-sum test followed by a Mann-Whitney *U* test was performed for two-group comparisons of the data with abnormal distribution. *P* values of less than .05 indicated statistical significance.

3 | RESULTS

3.1 | MSCs suppressed ILC2 functions in PBMCs from patients with AR

We first cocultured iPSC-MSCs with PBMCs isolated from patients with AR and healthy subjects. Epithelial cytokines IL-25 and IL-33 plus IL-2 were used to activate ILC2s and as the positive control, and the ILC2 functions were examined using ELISA, quantitative real-time

PCR, and flow cytometry. Significant high protein levels of the IL-13, IL-9, and IL-5 were observed in response to epithelial cytokines in cultured PBMCs from healthy subjects and especially from patients, and iPSC-MSCs significantly reversed their high levels (Figure 1A). In equivalent, the high mRNA levels of IL-13, IL-9, and IL-5 induced by epithelial cytokines were also significantly decreased by iPSC-MSCs (Figure 1B). The intracellular cytokine levels were examined using flow cytometry in the gates of both Lin$^-$CRTH2$^+$ and Lin$^-$CD127$^+$CRTH2$^+$ (Figure S1A). We found that epithelial cytokine stimulation significantly increased the percentages of IL-13$^+$Lin$^-$CD127$^+$CRTH2$^+$ ILC2s and IL-9$^+$Lin$^-$CD127$^+$CRTH2$^+$ ILC2s in PBMCs, while the high percentages of IL-13$^+$ILC2s and IL-9$^+$ILC2s were significantly reversed after the treatment of iPSC-MSCs (Figure S1B). Recently, Lin$^-$CD127$^-$ cells have been shown to give rise to ILC2s,[17,18] and CRTH2$^+$ ILC2s were proven to express high levels of type 2 cytokines.[19] Thus, to increase the accuracy of true ILC2 frequency, we examined the percentages of IL-13$^+$Lin$^-$CRTH2$^+$ and IL-9$^+$Lin$^-$CRTH2$^+$ cells in the PBMCs, which showed same trends with those of conventional defined ILC2s (Figures 1C and S1A). Besides, the IL-13 and IL-9 MFI calculation of the ILC2s following iPSC-MSC treatment were significantly decreased, indicating that iPSC-MSCs exhibit inhibitory effects on ILC2s in PBMCs (Figure S1C). This demonstrated that iPSC-MSCs can inhibit ILC2 functions in PBMCs from both AR patients and healthy subjects.

3.2 | MSCs directly enhanced the function of ILC2s in purified Lin$^-$ or ILC2 system

It is hard to evaluate the direct effects of MSCs on ILC2s due to the complex compositions in PBMCs especially for the involvement of T cells and B cells. Then we further cocultured iPSC-MSCs with Lin$^-$ cells isolated from the buffy coat of human volunteers, by which to investigate the possible mechanisms involved in the immunomodulation of iPSC-MSCs on ILC2s. Surprisingly, the levels of IL-13$^+$Lin$^-$CRTH2$^+$ and IL-9$^+$Lin$^-$CRTH2$^+$ cells were significantly elevated by iPSC-MSCs rather than decreasing compared to the negative control and epithelial cytokine stimulation groups (Figures 2A and S2A). Comparably, the IL-13$^+$Lin$^-$CD127$^+$CRTH2$^+$ ILC2s and IL-9$^+$Lin$^-$CD127$^+$CRTH2$^+$ ILC2s, IL-13 and IL-9 MFI calculation of ILC2s in Lin$^-$ cells were also significantly elevated after cocultured with iPSC-MSCs, and their percentages were similar to those of Lin$^-$CRTH2$^+$ cells (Figure S2). Similarly, the levels of type 2 cytokines (IL-13 and IL-5) in the supernatant were dramatically increased by iPSC-MSCs compared to the control groups without iPSC-MSCs, the level of IL-9 in the supernatant also showed increasing trend after iPSC-MSC treatment (Figure 2B, *P* = .0517), demonstrating that iPSC-MSCs significantly enhanced ILC2 functions. We also found that iPSC-MSCs elevated the ratios of ILC2-defining markers of CRTH2 and CD127 no matter with or without the stimulation of epithelial cytokines after cocultured with Lin$^-$ cells (Figure 2C). Moreover, using isolated ILC2s, we confirmed the above findings that iPSC-MSC treatment upregulated IL-13, IL-9,
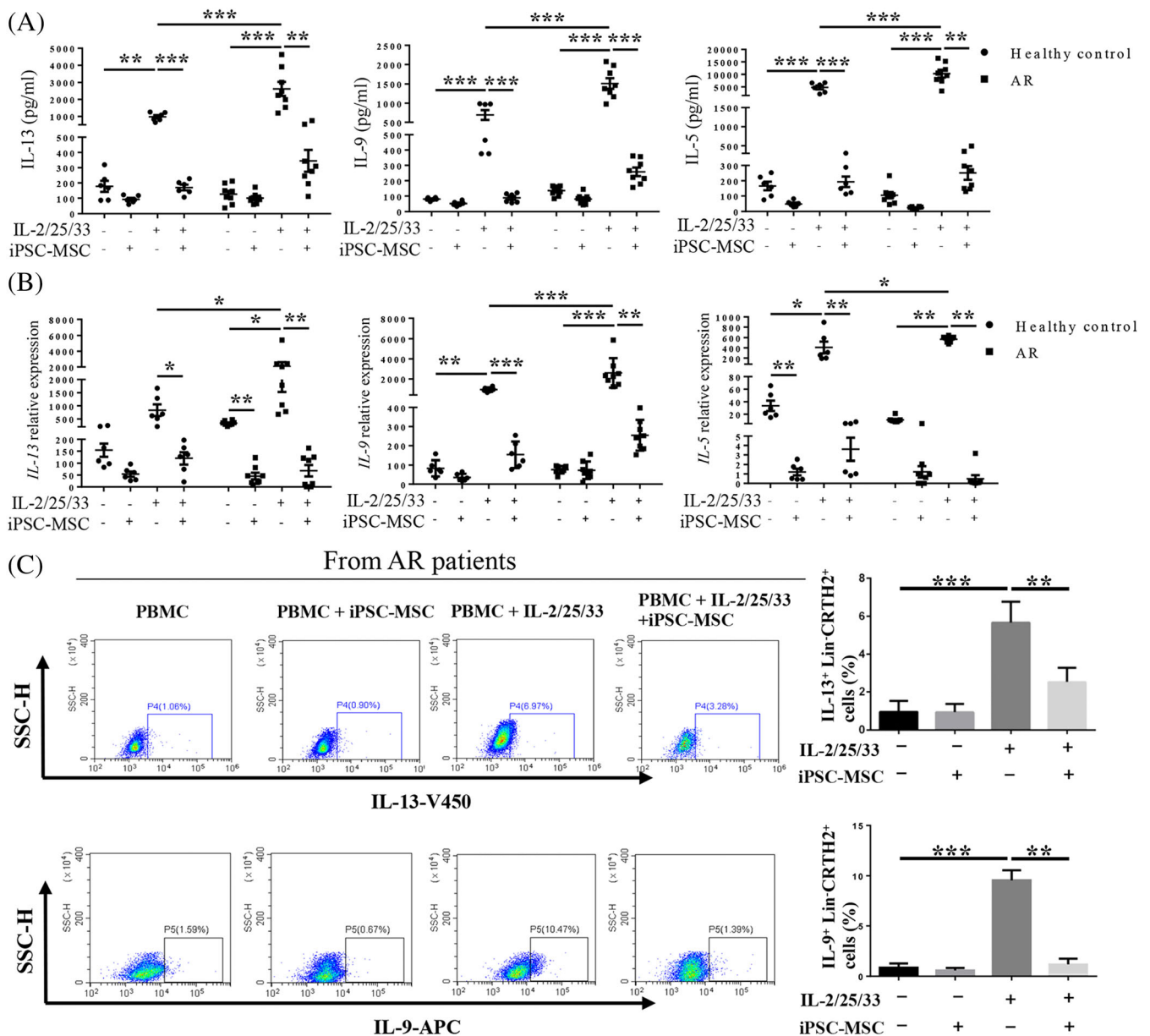
FIGURE 1 iPSC-MSCs inhibited ILC2 function in PBMCs from patients with allergic rhinitis. PBMCs isolated from AR patients and healthy subjects were cocultured with iPSC-MSCs. A, IL-13, IL-9, and IL-5 levels in the supernatant were analyzed by ELISA (n = 6 for HC, n = 8 for AR patients); B, *IL-13*, *IL-9*, and *IL-5* mRNA levels in PBMCs were analyzed by qPCR (n = 6 for HC patients, n = 8 for AR patients). C, The intracellular IL-13$^+$ and IL-9$^+$ Lin$^-$CRTH2$^+$ cell levels in PBMCs were analyzed by flow cytometry (n = 8). Data are shown as mean ± SEM. *P < .05, **P < .01, and ***P < .001. AR, allergic rhinitis; HC, healthy control; ILC2, group 2 innate lymphoid cell; iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; Lin$^-$, lineage negative; PBMCs, peripheral blood mononuclear cells

and IL-5 production by ILC2s (Figure 2D). The results suggested that iPSC-MSCs can directly activate ILC2s, which contradicted with what had been found in PBMCs.

3.3 | Cell-cell contact is necessary for MSCs in enhancing ILC2 function

MSCs were reported to exert their immunomodulatory effects mainly through paracrine functions and cell-cell contact.[20,21] Thus, transwell

was employed into the coculture system to separate iPSC-MSCs with Lin$^-$ cells. After separated by the transwell system, iPSC-MSCs could not contact with ILC2s directly. We found that the high ratios of IL-13$^+$Lin$^-$CRTH2$^+$ cells, IL-9$^+$Lin$^-$CRTH2$^+$ cells and Lin$^-$CRTH2$^+$ cells, enhanced by iPSC-MSCs were significantly reversed by transwell system (Figure 3A,B), indicating that iPSC-MSCs elevate ILC2 levels and function via direct cell-cell contact. Moreover, Western blot analysis found that the phospho-AKT expression, which can be activated by ICOSL signaling through the PI3K-Akt pathway, in Lin$^-$ cells was significantly increased when cocultured together with iPSC-MSCs
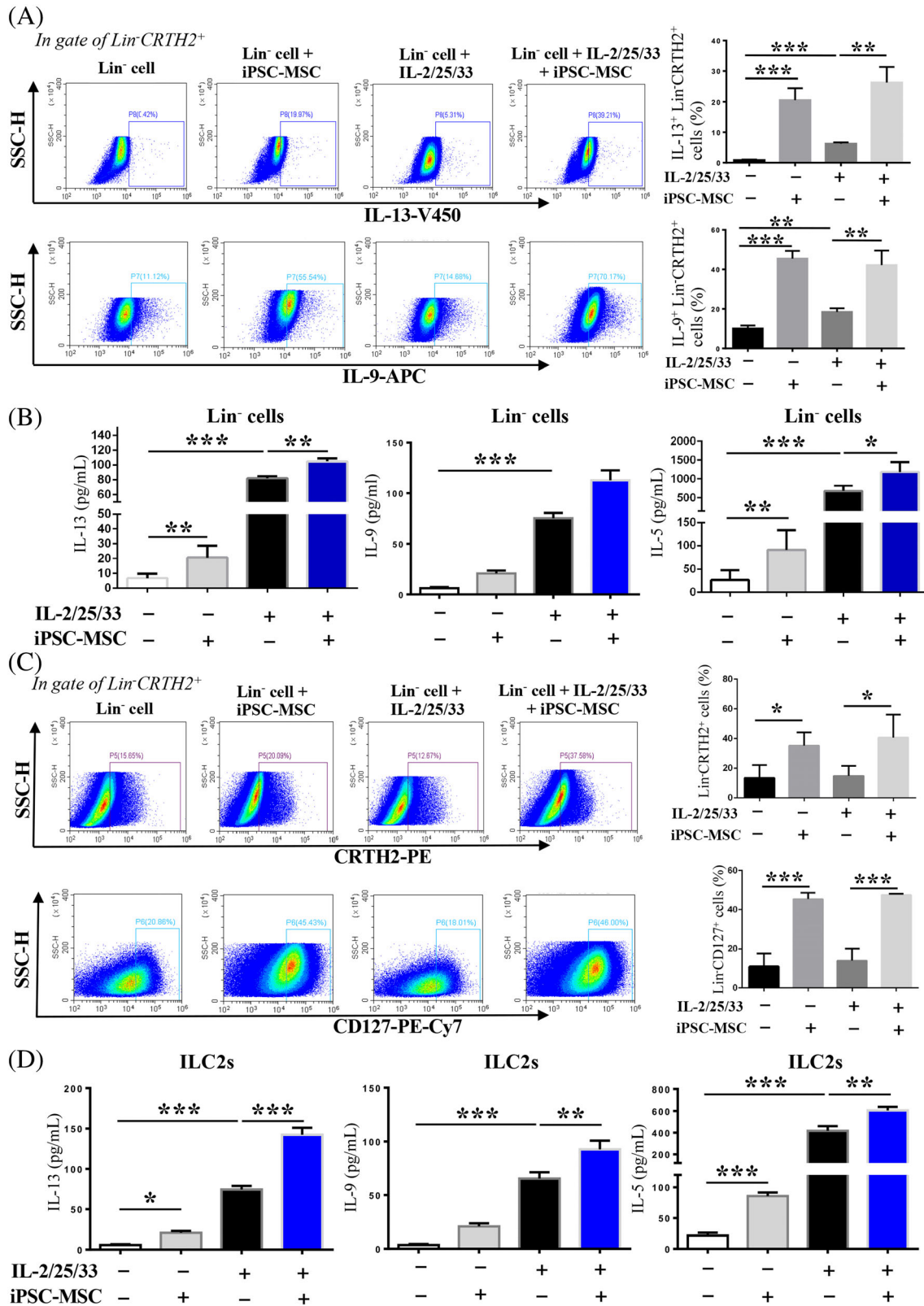
FIGURE 2   MSCs directly enhanced the levels and functions of purified ILC2s. Lin⁻ cells (A-C) or ILC2s (D) isolated from the buffy coat were cocultured with iPSC-MSCs. A, The intracellular IL-13⁺ and IL-9⁺ Lin⁻CRTH2⁺ cell levels were analyzed by flow cytometry (n = 6). B, IL-13, IL-9, and IL-5 levels in the supernatant were analyzed by ELISA (n = 6). C, The CRTH2⁺ Lin⁻ cell and CD127⁺ Lin⁻ cell levels were analyzed by flow cytometry (n = 6). Furthermore, sorted ILC2s were cocultured with iPSC-MSCs, IL-13, IL-9, and IL-5 levels (D, n = 6) in the supernatant were analyzed by ELISA. Data are shown as mean ± SEM. *P < .05, **P < .01, and ***P < .001. AR, allergic rhinitis; ILC2, group 2 innate lymphoid cell; iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; Lin⁻, lineage negative
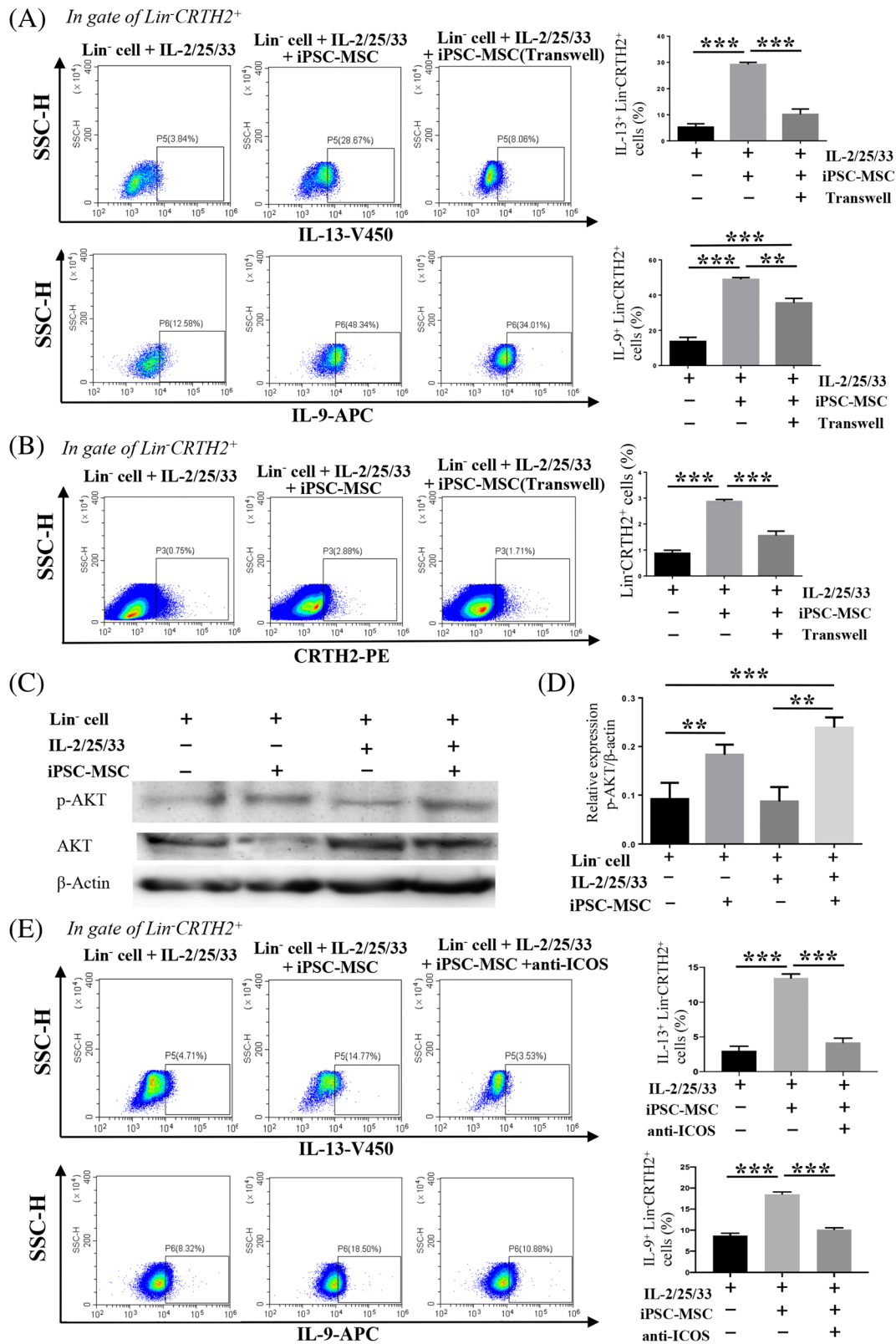
FIGURE 3　iPSC-MSCs enhanced ILC2 levels and function via direct cell-cell contact. Transwell culture system was employed to separate Lin⁻ cells and iPSC-MSCs during coculture. Intracellular IL-13$^+$ and IL-9$^+$ Lin⁻CRTH2$^+$ cell levels (A, n = 6), CRTH2$^+$ Lin⁻ cell levels (B, n = 6) were analyzed by flow cytometry. C, Protein levels of phospho-AKT and AKT in Lin⁻ cells after cocultured with iPSC-MSCs. D, Bars show phospho-AKT levels as determined by densitometry normalized to β-actin (n = 3). E, Intracellular IL-13$^+$ and IL-9$^+$ Lin⁻CRTH2$^+$ cell levels in Lin⁻ cells cocultured with iPSC-MSCs in the presence of anti-ICOS mAb were analyzed by flow cytometry (n = 6). Data are shown as mean ± SEM. **P < .01 and ***P < .001. ILC2, group 2 innate lymphoid cell; iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; Lin⁻, lineage negative; p-AKT, phospho-AKT

(Figure 3C,D). In order to verify the ICOS-ICOSL interaction between iPSC-MSCs and ILC2s, an anti-ICOS mAb was added into the coculture system to block ICOS in ILC2s. It was found that the high levels of IL-13^{+} and IL-9^{+} Lin[−]CRTH2^{+} cells enhanced by iPSC-MSCs were significantly decreased by the addition of the anti-ICOS mAb (Figure 3E). These results suggested that iPSC-MSCs enhance ILC2 functions in a contact-dependent manner. And this enhancement effect was achieved by ICOS-ICOSL interaction.

3.4 | MSC inhibited ILC2 function via Treg cells through ICOS-ICOSL interaction

It is very interesting to verify the mechanism about the effects of iPSC-MSCs to inhibit ILC2 function in the system of PBMCs but not in pure ILC2 or Lin[−] systems. Our above findings suggested that some cells or some factors in PBMCs may help or support MSCs to exert their inhibition on ILC2s. ICOSL expression in MSCs has been demonstrated to promote the induction of Treg cells. And Treg cells were reported to suppress ILC2 function both in vitro and in vivo.[22] Therefore, we hypothesized that iPSC-MSCs may inhibit ILC2 functions via Treg cells through ICOS-ICOSL interaction. Then we first added natural Treg (nTreg) cells into the MSC-Lin[−] coculture system. The administration of Treg cells significantly reversed the high levels of IL-13, IL-9, and IL-5 enhanced by iPSC-MSCs (Figures 4A and S3A). It was very interesting that nTreg alone did not inhibit ILC2 function, while nTreg cells pretreated by iPSC-MSCs (here were called MSC-Treg) or nTreg combined with iPSC-MSCs significantly decreased IL-13, IL-9, and IL-5 levels compared with those of positive control (Figures 4B and S3B).

Flow cytometry analysis found that the ICOS and ICOSL expressions on Lin^{−} cells were significantly elevated after coculture with iPSC-MSCs, while the increased ICOS and ICOSL expression on Lin^{−} cells were significantly decreased after the addition of Treg cells in the coculture system (Figures 4C and S4A). The overall expression levels of ICOS and ICOSL on Lin^{−} cells in the mixed coculture system were still higher than those on negative control. ICOS expression on Treg cells and ICOSL expression on iPSC-MSCs were significantly elevated and maintained at a constant level after coculture in different groups (Figures 4D,E and S4B,C). Furthermore, we identified that iPSC-MSCs combined nTreg significantly reversed the high levels of IL-13^{+}Lin[−]CRTH2^{+} and IL-9^{+}Lin[−]CRTH2^{+} cells, IL-13^{+}ILC2s and IL-9^{+}ILC2s, and IL-13 and IL-9 MFI calculation of ILC2s induced by iPSC-MSCs (Figures 4F and S5). Similarly, the level of CRTH2^{+}Lin^{−} cells exerted a similar phenomenon (Figure 4G). Taken together, it suggested that iPSC-MSCs can inhibit ILC2 function via activating Treg cells.

We next investigated the possible mechanism involved in via targeting ICOS-ICOSL interaction between MSCs and Treg. An anti-ICOS mAb was used to treat Treg cells for 24 h before their coculture in MSC-Lin[−] system. We found that the ratios of IL-13^{+}ILC2s, IL-9^{+}ILC2s, and CRTH2^{+} Lin[−]cells were significantly reversed to high levels after the Treg cells were pretreated with anti-ICOS mAb (Figure 4F,G). Similarly, the levels of IL-13, IL-9, and IL-5 were also significantly decreased with iPSC-MSCs cocultured with nTreg, and

increased again after nTreg were pretreated with anti-ICOS mAb (Figure 4H). It suggested that ICOS-ICOSL interaction is required for iPSC-MSCs in mediating Treg cells to inhibit ILC2s.

It is quite possible that monocytes in PBMCs, which suppress the proliferation of hematopoietic cells by producing myeloid-derived suppressor cells, also mediate some of the inhibitory effects of iPSC-MSCs on ILC2s. Therefore, we added isolated monocytes into the MSC-Lin[−] coculture system. According to ELISA, there was no significant difference of IL-13, IL-9, and IL-5 levels between the monocyte treatment group and positive control group (Figure S6), indicating that monocytes did not mediate the inhibitory effects of iPSC-MSCs on ILC2s.

3.5 | IL-10 is necessary for Treg cells in inhibiting ILC2s

As IL-10, alongside ICOS-ICOSL interaction, was reported an important mediator of Treg suppression on ILC2s.[22,23] Therefore, we tested the production of IL-10 by nTreg cells following interaction with iPSC-MSCs. The IL-10 levels in the supernatant of nTreg cells and Lin^{−} cells cultured with iPSC-MSCs maintained low. While the IL-10 level in the supernatant of nTreg cells following interaction with iPSC-MSCs was significantly increased (Figure 5A). The anti-IL-10 mAb was used to validate the role of Treg cells in the coculture system. We found that blocking of IL-10 significantly increased the ratios of IL-13^{+}Lin[−]CRTH2^{+} cells, IL-9^{+} Lin[−]CRTH2^{+} cells and CRTH2^{+} Lin^{−} cells (Figure 5B,C), and the levels of IL-13, IL-9, and IL-5 in the supernatant decreased by Treg cells with the combination of iPSC-MSCs were significantly increased by anti-IL-10 mAb (Figure 5D), similar to those of anti-ICOSL mAb. Taken together, these results demonstrated that MSCs suppress ILC2 function via inducing IL-10 secretion by Treg cells via ICOS-ICOSL interaction.

4 | DISCUSSION

In the current study, we found that iPSC-MSCs can inhibit the function of ILC2s in PBMCs from patients with AR, indicating iPSC-MSCs exert a regulating effect on diseases in the comprehensive systems. In contrast, iPSC-MSCs promoted the function of ILC2s through ICOS-ICOSL interaction on sorted ILC2s or in Lin^{−} cells. We further identified that there is a competitive mechanism for MSCs to interact with either ILC2s or Treg cells through ICOS-ICOSL interaction. iPSC-MSCs exerted inhibitory effects on ILC2s via activating Treg cells through ICOS-ICOSL interaction, the MSC-induced Treg cells then suppressed ILC2s by secreting IL-10 alongside ICOS-ICOSL interaction. The significance of this is that MSCs exhibit the suppressive role on ILC2s in an integrated system, which further confirms the plasticity of MSCs.

MSCs have been most frequently considered as the candidate for immunotherapy due to the beneficial effects of MSC-based therapies to treat different pathologies. The immunomodulatory role of MSCs is particularly interesting for clinical application since it modulates
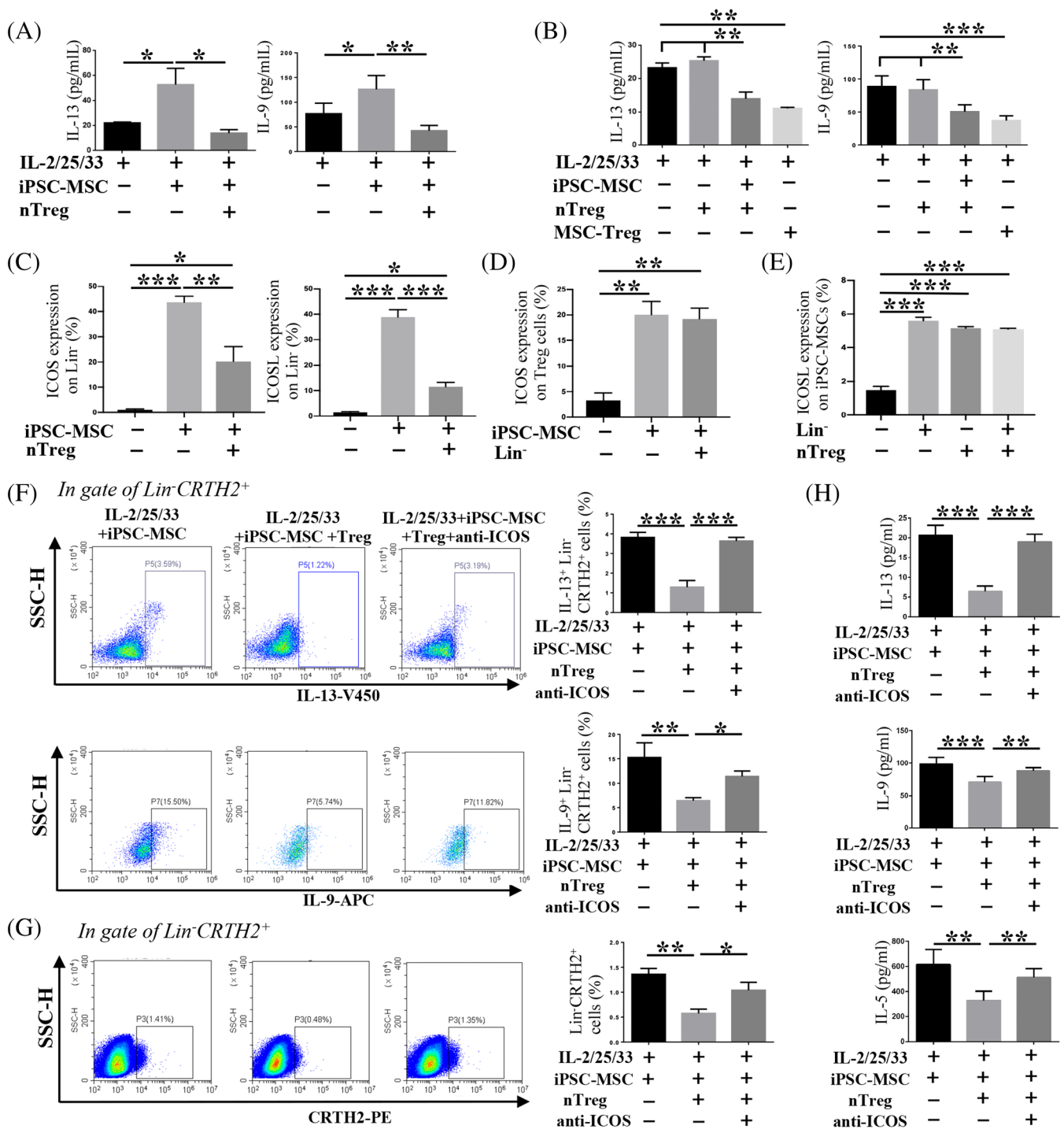
FIGURE 4 iPSC-MSCs suppressed ILC2 function via Treg cells through ICOS-ICOSL interaction. nTreg cells or MSC-Treg were cocultured together with Lin⁻ cells and/or iPSC-MSCs. IL-13 and IL-9 levels in the supernatant were analyzed by ELISA (A and B, n = 6). ICOS and ICOSL expression on Lin⁻ cells (C), ICOS expression on Treg cells (D), and ICOSL expression on iPSC-MSCs (E) were analyzed by flow cytometry prior to and after coculture (n = 6). After Treg cells were pretreated with anti-ICOS mAb, intracellular IL-13⁺ Lin⁻CRTH2⁺ cell and IL-9⁺ Lin⁻CRTH2⁺ cell levels (F, n = 6), CRTH2⁺ Lin⁻ cell levels (G, n = 6) were analyzed by flow cytometry. IL-13, IL-9, and IL-5 levels (H, n = 6) in the supernatant were analyzed by ELISA. Data are shown as mean ± SEM. *P < .05, **P < .01, and ***P < .001. ILC2, group 2 innate lymphoid cell; iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; Lin⁻, lineage negative; MSC-Treg, Treg cells were pretreated with iPSC-MSCs; nTreg, natural Treg cells; Treg, regulatory T cells

immune imbalance to treat inflammatory disorders.[24,25] MSCs have been demonstrated to modulate different immune cells including T cells,[26] B cells,[27] natural killer cells,[28] and dendritic cells,[15,29] and so forth. In particular, MSCs can regulate T cell proliferation, function, balance T_H1 and T_H2 activities, and upregulate Treg cell functions, by which to inhibit type 2 inflammation.[11,21,30] ILC2s are emerging as
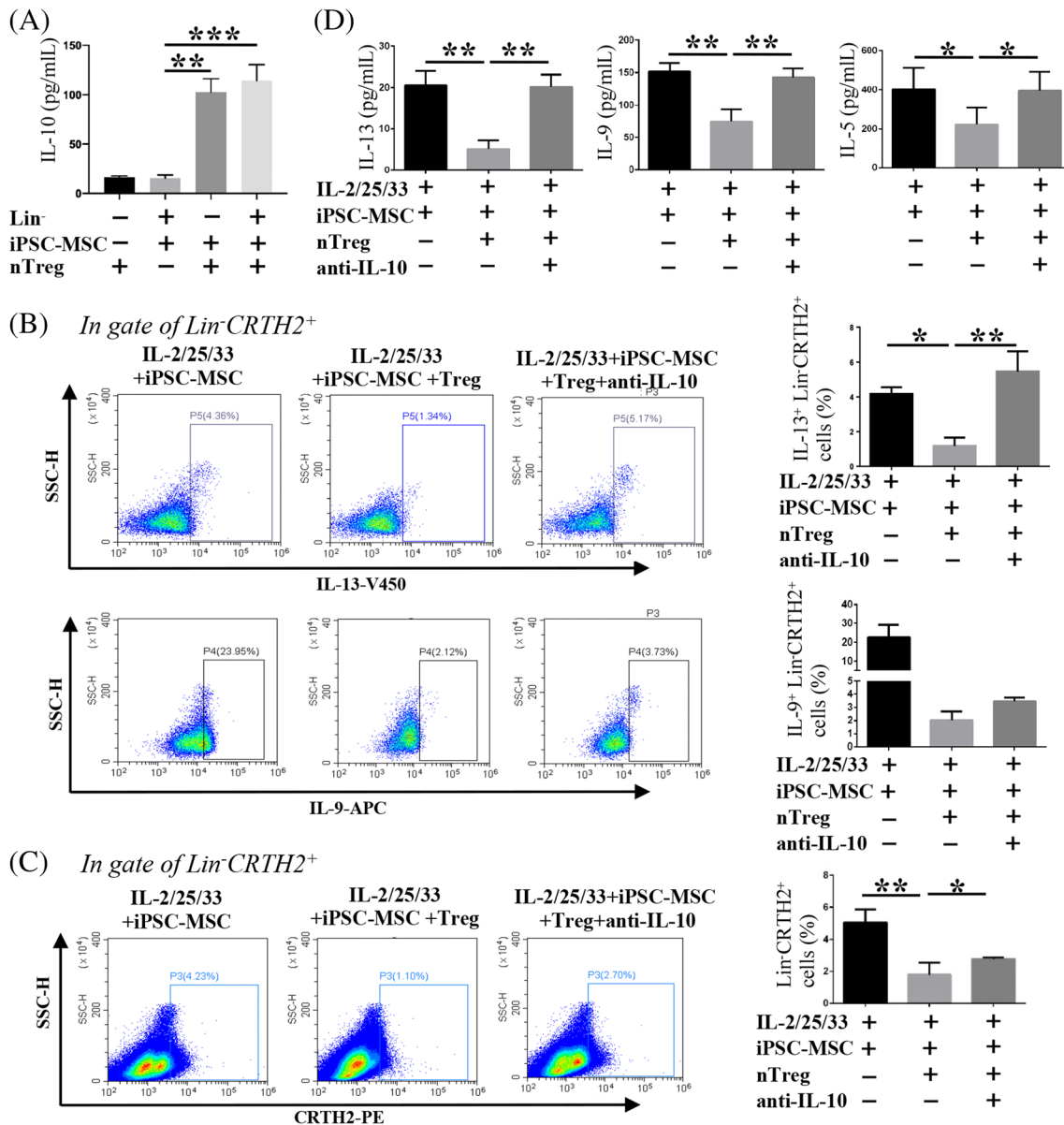
FIGURE 5 iPSC-MSCs inhibited ILC2 functions via IL-10 secreted by Treg cells. A, The production of IL-10 by Treg cells following interaction with iPSC-MSCs and Lin⁻ cells were analyzed by ELISA (n = 6). An anti-IL-10 mAb was added into the coculture system to block IL-10 function. Intracellular IL-13⁺ and IL-9⁺ Lin⁻CRTH2⁺ cell levels (B), CRTH2⁺ Lin⁻ cell levels (C) were analyzed by flow cytometry (n = 6). D, IL-13, IL-9, and IL-5 levels in the supernatant were analyzed by ELISA (n = 6). Data are shown as mean ± SEM. *P < .05, **P < .01, and ***P < .001. iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; Lin⁻, lineage negative; nTreg, natural Treg cells; Treg, regulatory T cells

key regulators and effectors in type 2 immune response, ILC2s promote T$_H$2 cells either by direct cross-talking with T cells or by recruiting dendritic cells that induce T$_H$2.[31] ILC2s are enriched in the blood and tissues of allergic individuals, and allergen exposure will readily recruit functional ILC2s for type 2 cytokine production.[32,33] The IL-5, IL-9, and IL-13 production of ILC2s will lead to tissue eosinophilia, mast cell accumulation, airway remodeling and hyperresponsiveness, resulting in type 2 inflammation.[34] ILC2s play roles both during the early induction of effector functions and by enhancing adaptive T$_H$2-driven immunity.[35] Here we demonstrated that MSCs inhibit circulating ILC2 function in PBMCs from both AR patients and

healthy subjects, this is consistent with what we have hypothesized, yet the mechanism driving this effect has remained unclear. Given the complex composition in PBMCs, we isolated Lin⁻ cell fraction to further investigate the involved mechanisms.

However, we found that in contrast to inhibit ILC2s, MSCs directly promote and activate ILC2s in a cell-contact dependent manner in ILC2 or Lin⁻ systems. MSCs were reported to promote ILC3 proliferation and IL-22 production in the previous study, the authors also found that MSCs promoted ILC2 proliferation in Lin⁻ systems.[36] Here in this study, we also demonstrated that ILC2 levels were significantly promoted by iPSC-MSCs as the ratios of ILC2 defining markers

of CRTH2 and CD127 were significantly increased in Lin⁻ cells. Besides, we found that the number of IL-13$^+$ or IL-9$^+$ cells was markedly increased when Lin⁻ cells were cultured with iPSC-MSC, whereas that number was moderately increased when Lin⁻ cells were cultured in the presence of IL-2/25/33. Does the treatment of PBMCs or Lin⁻ cells with iPSC-MSC stimulate ILC2 cell proliferation and an overall increase in cells that produce cytokines or is there an increase in the amount of cytokine produced by the ILC2s? We have also checked the MFI calculation of the ILC2s following iPSC-MSC treatment in key experiments in this study. The IL-13 and IL-9 MFI calculation of the ILC2s following stimulation and iPSC-MSC treatment showed the same trend with those of IL-13$^+$ and IL-9$^+$ ILC2/Lin⁻CRTH2$^+$ ratios, indicating that iPSC-MSC treatment not only promotes ILC2 cell proliferation in Lin⁻ cells, but also enhances the cells to produce type 2 cytokines. Here, we examined Lin⁻CRTH2$^+$ cells in comparison with conventional CRTH2$^+$CD127$^+$ ILC2s in order to increase the accuracy of true ILC2 frequency, for Lin⁻CD127⁻ cells have been shown to give rise to ILC2s. We found that both Lin⁻CRTH2$^+$ cells and conventional ILC2s showed the same trend when cultured with iPSC-MSCs under the stimulation of epithelial cytokines. Moreover, the levels of IL-13, IL-9, or IL-5 were markedly increased in the culture medium when Lin⁻ cells were cultured in the presence of IL-2/25/33, while those levels were moderately increased when Lin⁻ cells were cultured with iPSC-MSCs. When the iPSC-MSCs cocultured mature ILC2s were stimulated by the epithelial cytokines, more type 2 cytokines were secreted. Why the combination treatment induced much higher IL-13/9/5 production than IL-2/25/33 or iPSC-MSC alone? We next found that this enhancing effect is mediated by cell-cell contact. We further identified that ILC2s cocultured with iPSC-MSCs showed higher phospho-AKT expression, which was found activated by ICOSL signaling. Previous studies have demonstrated that ICOS signaling regulates ILC2 homeostasis by promoting proliferation and accumulation of mature ILC2s.[37,38] ICOS-ICOSL interaction among ILC2s themselves has also been reported to play an important role in the proliferation and proinflammation of ILC2s.[38] Thus, iPSC-MSCs may enhance ILC2 pool via direct cell-cell contact through ICOS-ICOSL interaction. Lin⁻ cells cocultured directly with iPSC-MSCs will tend to polarize into ILC2s. We have previously found that iPSC-MSCs promote T cell activation in quiescent-state PBMCs.[14] It suggests that MSCs might exert different effects, different strengths, and even opposing effects depending on different conditions.

In allergic patients, Treg cells count to reduce the disease responses mediated by T$_H$2 cytokine-producing cells.[39,40] Mice transferred with both ILC2s and Treg cells showed reduced airway hyper-responsiveness and lung eosinophil counts compared with mice given only human ILC2s.[34] We found that the addition of Treg cells abolished the enhancing effect of iPSC-MSC coculture on the production of IL-13, IL-9, and IL-5. Furthermore, the inhibitory effect of Treg cells on the production of IL-13, IL-9, or IL-5 from coculture of Lin⁻ cells and iPSC-MSC in the presence of IL-2/25/33 was canceled by the addition of anti-ICOS. In addition to promoting ILC2 activation, ICOSL expression in iPSC-MSCs played an important role in contact-dependent regulation of Treg cell induction.[13,41] However, in this

study, we found that both ICOS and ICOSL expression on Lin- cells were significantly downregulated after Treg cells were added into the coculture system of Lin⁻ and MSCs, suggesting weaker upregulation of MSCs on ILC2 function via the interaction of ICOS and ICOSL on them. Then the ICOS/ICOSL interaction between Treg and MSCs exhibited the dominance, and results in the next inhibitory effects of Treg on ILC2 function. Here we provided strong evidence that iPSC-MSCs exerted their inhibition on ILC2s via Treg cells through ICOS-ICOSL interaction. Treg cells exert their functions by secreting IL-10, TGF-β and cell-contact to maintain immune homeostasis.[42] Although Treg cells employ multiple mechanisms of suppression, IL-10 produced by Treg cells play an important role in the regulation of ILC2s to attenuate airway hyperactivity.[22,43,44] Importantly, IL-10 was shown to be effective in regulating IL-33 stimulated ILC2s.[45] We found that the addition of anti-IL-10 canceled the inhibitory effect of Treg cells, which further demonstrated the inhibitory role of MSC-induced Treg cells. It is quite possible that other cell types (especially monocytes) are also mediating some of the indirect effects of iPSC-MSC on ILC2 by serving as a source of induced IL-10. This is important for clinical relevance as monocytes are likely to be more numerous and accessible than Treg cells to MSC delivered intravascularly. However, by adding monocytes back into the Lin⁻ cells and iPSC-MSCs coculture system. We have not found any suppression of the type 2 cytokine levels in the supernatants. Therefore, monocytes are probably not involved in the regulatory effects of iPSC-MSCs on ILC2s.

Of course, the immunomodulatory function of MSCs on ILC2s is a complex network interaction between cells and cytokines, which may require further deep investigation. The secretome of MSCs could represent a valid alternative of their functional use.[46] Lipid mediators including PGD2 and cysteinyl leukotrienes were shown promoting ILC2 activation.[47] And mesenchymal sourced PGD2 were found acting on ILC2s through CRTH2.[48] However, in our study, we have not observed any significant elevation of CRTH2 in the coculture system separated by a transwell system. ILC2 activation in local tissue is one of the major mechanisms for increased T$_H$2 associated inflammation in allergic inflammation. Thus, the regulation of MSCs on local tissue ILC2s, in addition to circulating ILC2s, would be attractive.

There were still some limitations in this study. First, the gating strategy of Lin⁻CRTH2$^+$ cells used for the primary experiments does not very accurately exclude contributions from non-ILC2s. The Lin⁻CRTH2$^+$ population is likely to consist non-ILC2s and other cells (eg, CD4$^+$ T$_H$2 cells, CD8$^+$ cytotoxic T2 cells, and eosinophils) capable of producing type 2 cytokines that have not been completely excluded by the lineage cocktail.[49] Therefore, future replication of the results using additional approaches to accurately define or purified ILC2s might be employed to verify these findings. Also, further studies employing MSCs from other sources will be important to maximize the clinical relevance of the findings. Second, considering the ethical issues and to save samples, we collected the supernatants of PBMCs, Lin⁻ cells which were treated with PMA and Golgistop for cytometry analysis, and examined the cytokine levels using ELISA. We found that the administration of PMA and Golgistop only further increased the

intracellular levels but did not affect the levels in the supernatants (data not shown). More importantly, the experiments in our study were done in parallel under the same condition. Of course, it is better to examine the cytokines in the supernatants without the treatment of PMA and Golgistop.

5 | CONCLUSION

In summary, the present data show that iPSC-MSCs can inhibit ILC2 functions in patients with AR via the assistance of Treg. ICOSL induced Treg cells are critical for the inhibitory functions of iPSC-MSCs on ILC2s. Our findings provide a better understanding of the cellular and molecular mechanisms mediating stem cell therapy on ILC2s. These new findings are of possible clinical value and provide further insight to regulate ILC2s in inflammatory disorders.

CONFLICT OF INTEREST
The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
X.F.: collection and/or assembly of data, data analysis and manuscript writing; Z.-B.X. and C.-L.L.: collection and/or assembly of data, data analysis and interpretation; H.-Y.Z.: collection of data; Y.-Q.P., B.-X.H. and X.-Q.L.: preparation of PBMCs, Lin⁻ cells and ILC2s; D.-H.C. and D.C.: patient recruitment; C.A.A.: help the revision; Q.-L.F.: conception and design, manuscript writing, final approval of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Cheng-Lin Li https://orcid.org/0000-0002-8781-3789
Qing-Ling Fu https://orcid.org/0000-0002-5969-628X

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