Original Article

Enriched Peripheral Blood-Derived Mononuclear Cells for Treating Knee Osteoarthritis

Chang-Han Chuang[1,2,3], Chi-Chung Kuo[4,5],
Yueh-Feng Chiang[6], Pei-Yuan Lee[1], Fu-Hui Wang[7],
Chia-Ying Hsieh[7], Ching-I Shen[7], Yu-Hsuan Chung[1,2,3],
Kuan-Der Lee[8,9], Shih-Fang Wu[10,11],
Hong-Lin Su[12] [iD], and Chih-Lung Lin[13,14]

Abstract

Osteoarthritis (OA) is a common chronic skeletal disease in the elderly. There is no effective therapy to reverse disease severity and knee OA (KOA) progression, particularly at the late stage. This study aims to examine the effect of peripheral blood-derived mononuclear cells (PBMNCs) on pain and motor function rescue in patients with Kellgren–Lawrence (KL) grade II to IV KOA. Participants received one intra-articular (IA) injection of autologous PBMNCs. The mononuclear cells were isolated from peripheral blood, enriched by a specialized medium (MoFi medium), and separated by Ficoll-Paque solution. The isolated and enriched PBMNCs could differentiate into M1 and M2 macrophages *in vitro*. The *in vivo* anti-inflammatory effect of the PBMNCs was similar to that of bone marrow mesenchymal stem cells, evaluated by complete Freund's adjuvant-induced arthritis in rodents. A single-arm and open-label pilot study showed that patients' knee pain and motor dysfunction were significantly attenuated after the cell transplantation, assessed by visual analogue scale (VAS) and Knee injury and Osteoarthritis Outcome Score (KOOS) at 6 and 12 months post-treatment. Notably, the therapeutic effect of the PBMNCs treatment can be stably maintained for 24 months, as revealed by the KOOS scores. These preclinical and pilot clinical data suggest that IA injection of MoFi-PBMNCs might serve as a novel medical technology to control the pain and the progress of KOA.

[1] Department of Orthopedics, Show Chwan Memorial Hospital, Changhua, Taiwan
[2] PhD Program in Translational Medicine, National Chung Hsing University, Taichung, Taiwan
[3] Rong Hsing Research Center for Translational Medicine, National Chung Hsing University, Taichung, Taiwan
[4] Department of Neurology, Taichung Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taichung, Taiwan
[5] School of Post-Baccalaureate Chinese Medicine, Tzu Chi University, Hualien, Taiwan
[6] Department of Orthopedics, Taichung Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taichung
[7] DuoGenic StemCells Corporation, Taichung, Taiwan
[8] Department of Medical Research and Cell Therapy and Regenerative Medicine Center, Taichung Veterans General Hospital, Taichung, Taiwan
[9] Department of Medicine and Center for Cell Therapy and Regenerative Medicine, College of Medicine, Taipei Medical University, Taipei City, Taiwan
[10] The Joint Program of Tissue Engineering and Regenerative Medicine, National Chung Hsing University, Taichung, Taiwan
[11] National Health Research Institutes, Taichung, Taiwan
[12] Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan
[13] Department of Neurosurgery, Asia University Hospital, Taichung, Taiwan
[14] Department of Occupational Therapy, Asia University, Taichung, Taiwan

Corresponding Author:
Chih-Lung Lin, Department of Neurosurgery, Asia University Hospital, 222 Fuxin Rd., Wufeng Dist., Taichung 413.
Email: jefflin0529@gmail.com

Introduction

Osteoarthritis (OA) is the most common motor dysfunction disease in the elderly, characterized by gradual and irreversible damage to articular cartilage and joint tissues. Nonsurgical treatments for knee OA (KOA) are limited as there is no effective therapy to reverse the disease severity or delay the progression of KOA, particularly at the late stage[1,2]. People with late-stage KOA suffer from persistent pain and have difficulty performing daily activities, leading to substantial physical and psychological problems. It is urgent to find a practical solution to manage symptoms and disease progression for OA patients before receiving surgical arthroplasty.

For patients with KOA, intra-articular (IA) hyaluronic acid (HA) injection provides pain relief up to 6 months posttreatment. Nevertheless, IA-HA shows no benefit over controls in the late OA subgroup[3]. Several trials also delivered a weaker response to HA therapy when KOA was severe[4–6]. Moreover, these results also indicate that severe OA patients experienced more treatment-related adverse effects[3]. These data suggest that HA injections should only be considered for patients at the early KOA stage.

Bone marrow aspirate concentrate (BMAC) is an operative approach to provide a viable and sufficient autologous stem cell source with minimal cellular manipulation. $CD34^+$ hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) in the BMAC may contribute to the anti-inflammation and tissue remodeling in KOA patients[7–9]. In addition, monocytes in the BMAC are competent to differentiate into anti-inflammatory M2 macrophages after the migration and invasion into the destructive tissues[10,11]. Especially, a previous study demonstrated that the administration of autologous M2 cells from peripheral blood is safe and beneficial for neurological outcomes in stroke patients, suggesting that monocyte/M2 macrophages could be an alternative therapeutic cell source to control inflammatory diseases[12,13].

Here, we investigate the potential of monocyte-enriched peripheral blood-derived mononuclear cells (PBMNCs) as a novel cell therapy for controlling pain and motor dysfunction in KL-II to IV KOA patients. Before the practices of clinical trials, we also conducted the preclinical *in vitro* examinations of the isolated PBMNCs, the enriched ratios of monocytes and $CD34^+$ cells population, the competency of M2 polarization, and *in vivo* anti-inflammatory activities in rodents.

Materials and Methods

Clinical Trials

The pilot clinical trials were conducted between Mar 2020 and August 2022 in three hospitals in Taiwan. The protocols were approved by the Institutional Review Board and Ethic Committee of China Medical University (CMUH 109-REC 1-012), Show Chwan Memorial Hospital (1090803), and Taichung Tzu Chi Hospital (REC 109-45). These trials were single-arm, open-labeled pilot studies. Patients with knee pain on most days for at least 6 months and KL grade II to IV were enrolled. All pain medications were discontinued except the approved rescue analgesic drugs. The exclusion criteria for the participants are notifiable infectious diseases, tumor history, and severe anemia. This study did not include patients with rheumatoid arthritis, psoriatic arthritis, gout arthritis, and severe valgus knee deformity.

Participants with informed consent were aware of the offlabel use of the medical devices and the risks of medical treatment. They were followed up at 6, 12, and 24 months for efficacy and safety evaluations after a single IA injection. Pain Visual Analog Scale (pain-VAS) and Knee injury and Osteoarthritis Outcome Scores (KOOS) were applied to assess pain relief and functional motor recovery. The KOOS covers pain, symptoms, activities in daily living (ADL), function in sport and recreation (Sport/Rec), and knee-related quality of life (QOL). Each subscale was converted to a score ranging from 0 to 10. A lower score indicates more severe pain and physical function impairment.

Radiographs of the knee were obtained at the pre-treatment stage, assessed by a standing semiflexed anteroposterior radiograph of the affected knee, and scored by KL grade 0–4 standard.

Adverse events of the IA-PBMNCs treatment were closely monitored at each follow-up visit during the study. Any serious adverse event was recorded on a separate form and was notified within 24 h.

The Blood Sampling and the Preparation of the Mononuclear Cells

Peripheral blood samples were collected from donors' peripheral veins and stored in blood bags with CPDA (citrate phosphate dextrose adenine), carried out in the Asia University Hospital (CMUH 109-REC 1-012), Show Chwan Memorial Hospital (1090803), and Taichung Tzu Chi Hospital (REC 109-45). A 100 ml peripheral blood sample was mixed with 80 ml normal saline (Nang Kuang Pharmaceutical, Tainan, Taiwan) or good manufacturing practice (GMP)-grade MoFi medium (comprising a chemical-defined medium for ex vivo human cells; classified as a medical device; Duogenic StemCells Corporation, Taichung, Taiwan) for 30 min. The mixed blood was then carefully loaded in a set of cell isolation (CS. 900.2, Cytiva, Marlborough, MA, USA) with the Sepax II machine (Cytiva). PBMNCs were separated by densitygradient centrifugation with Ficoll-Paque premium (Cytiva). The isolated PBMNCs were automatically washed with normal saline three times to remove the residue of the MoFi medium and Ficoll-Paque before the cell transplantation. The final concentration of the PBMNCs was adjusted to be 3.5 to 4 ml for one knee/person, containing $7–12 \times 10^7$ PBMNCs from 100 ml blood. The cell numbers of engrafted cells were estimated by manual counting using a hemocytometer.

The Characterization of the PBMNCs

The percentage of monocytes in the PBMNCs was measured by flow cytometry (Accuri, Becton-Dickinson, USA) using FSC/SSC dot plot or anti-CD14 FITC conjugated antibody (BioLegend, USA). The isolated PBMNCs (about 2×10^6 cells) were incubated in 3 ml of Iscove's Modified Dulbecco's Medium (IMDM) containing 1% autologous plasma for 1 and 3 days culture at 37°C under a 5% CO_2 environment. The ratios of M1 or M2 macrophages were examined by measuring their specific surface markers with flow cytometry, including anti-CD14 FITC (a pan-monocyte and macrophage marker, BioLegend, clone M5E2, mouse IgG2a), anti-CD206 PE (an M2 marker, BD Pharmingen, clone 19.2, mouse IgG1), anti-CD86 APC (an M1 marker, BioLegend, clone BU63, mouse IgG1), and anti-HLA-DR PerCP (an M1 marker, BioLegend, clone L243, mouse IgG2a) antibodies (Abs)[14,15]. The immunocytostaining protocols followed the manufacturer's suggestions.

The Indoleamine 2,3-Dioxygenase (IDO-1) Assessment in the BM-MSCs and MoFi-Monocytes

We maintained both 5×10^5 BM-MSCs (on CellBind-coating plate; Corning, Glendale, AZ, USA) and MoFi-PBMNCs (in α-Plus low-binding dishes; Alpha Plus Scientific Corp, Taoyuan, Taiwan) with IMDM and 1.0% auto-plasma. The cells were treated with interferon-γ (IFN-γ; PeproTech, Rocky Hill, NJ, USA) at 0, 10, or 50 ng/ml for 72 h. Total cells were harvested for CD14 and IDO-1 staining and quantification. The monocytes in the PBMNC population were gated from the CD14 positive (BioLegend, M5E2 Ab, FITC conjugated) population. Total cells were fixed (BioLegend fixation buffer, 420801), permeabilized (BioLegend permeabilization buffer, 421002), and then stained by anti-IDO-1 Alexa 647 Abs (BioLegend, 2E2/IDO1 clone). The working protocols for both surface CD14 staining and intracellular IDO-1 staining followed the manufacturer's recommendations. The representative IDO-1 expression level was shown as the mean fluorescence intensity (MFI) of bound anti-IDO-1 Ab in the IFN-γ treated or non-treated cells.

Animal Experiments

The therapeutic evaluation of the isolated PBMNCs in arthritis rodents was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (NCHU) (NCHU 109-093) in Taichung, Taiwan, and carried out in the Department of Life Sciences NCHU, Taichung, Taiwan.

Adult male Sprague-Dawley (SD) rats weighing 200 to 250 g were housed in a room with constant temperature (24°C –26°C) and humidity (40%–60%) and had free access to food and water under a typical light cycle environment.

A 0.25 ml of complete Freund's adjuvant (CFA, Sigma-Aldrich, USA) was injected into the hind footpad of each rat to induce inflammatory arthritis. On day 6, the inflammation was boosted by injecting an additional 0.05 ml of CFA into the same sites. On day 7, when the arthritis was established, the footpads were treated with 0.2 ml of phosphate-buffered saline (PBS, control), 0.2 ml human platelet-rich lysates (hPL), 0.2 ml PBS containing 2×10^5 PBMNCs or 2×10^5 bone marrow-derived mesenchymal stem cells (BM-MSC) (PCS-500-012, ATCC, USA). Ingredients in the medium were cleared from collected cells by PBS-wash twice. The swelling footpads were measured using a vernier every 2 to 3 days for 2 weeks.

Statistic Analysis

We used one-way or two-way analysis of variance (ANOVA) with Tukey post hoc to determine the significance of differences between the experimental groups. This study's graphic creation and statistical analysis were conducted using Microsoft Excel (version 2019) or GraphPad Prism 9 (GraphPad, La Jolla, CA, USA).

Results

Enriched Monocytes and CD34$^+$ Cells in Peripheral Blood

We developed a method for preparing mononuclear cells (MNCs)-enriched concentrate from peripheral blood using a GMP-grade MoFi medium and a density-gradient separation solution. A 100 ml of peripheral blood was collected from the patients and mixed with MoFi medium for 30 min. A clinically authorized machine (Sepax II, Cytiva) was used to separate the peripheral blood mononuclear cells (PBMNCs) automatically with a single-use disposable centrifugation device. The sterility of this peripheral blood cell purification (PCP) method for PBMNCs was ensured by the closed tubing system, clinical-grade solutions, and sophisticated software control approved by the IRB at three medical institutes.

We first examined the subpopulation ratio of the processed PBMNCs. The populations of monocytes (CD14$^+$) and HSC (CD34$^+$) were analyzed by flow cytometry, and a representative result was shown (Fig. 1A) after the purification of PBMNCs from peripheral blood with normal saline (control) or with MoFi medium (MoFi). Compared with the control, the MoFi process consistently increased the ratio of monocytes from $10.43 \pm 0.91\%$ to $19.86 \pm 1.42\%$ (n = 20, $P < 0.01$) (Fig. 1B). The fold induction was 2.02 ± 0.13 ($P < 0.01$) (Fig. 1C). Because the cell density of monocytes and HSCs was similar, and both cells were harvested from the same fraction after Ficoll purification. We next explored whether the MoFi treatment could enrich the HSCs subpopulation. Interestingly, we found that the ratio of CD34$^+$ cells in isolated PBMNCs also increased from $0.095 \pm 0.01\%$
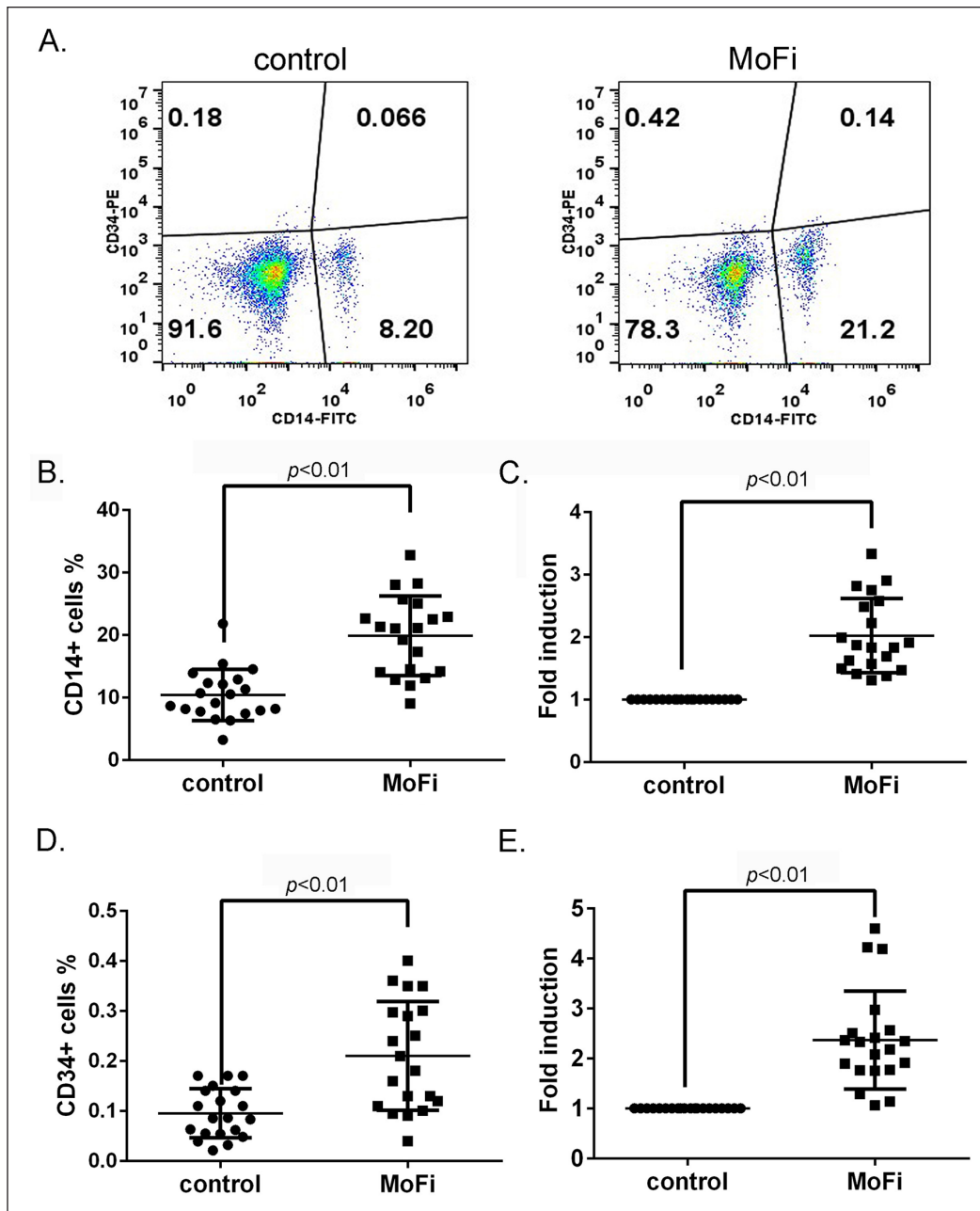
Figure 1. MoFi-enriched monocytes and HSCs in collected PBMNCs. (A) After the cytostaining with CD14 (X-axis) and CD34 (Y-axis), the normal saline (control)-treated and MoFi-treated PBMNCs were analyzed by flow cytometry to show the ratios of CD14$^+$ monocytes and CD34$^+$ HSC cells, respectively. The monocytes (B, C) and HSCs (D, E) percentages (B, D) in PBMNCs and the fold induction (C, E) for the cells were shown in control and MoFi-treated groups. The data were statistically analyzed with one-way ANOVA (n = 20). HSC: hematopoietic stem cells; PBMNC: peripheral blood-derived mononuclear cells; ANOVA: analysis of variance.

(control) to $0.21 \pm 0.02\%$ (MoFi) (n = 20, $P < 0.01$) (Fig. 1D). The fold induction was 2.37 ± 0.22 ($P < 0.01$) (Fig. 1E).

The Cell Properties of MoFi-Treated Monocytes

To examine the differentiation potency of the isolated monocytes, we cultured the PBMNCs in IMDM with 1.0% autoplasma for 3 days. We cultured the cells in low-binding culture dishes and determined the M1/M2 macrophage polarization by flow cytometry. CD206, a specific mature M2 macrophage marker, was barely detected in freshly harvested PBMNCs in both groups (less than 3% on day 0) (Fig. 2A). We showed that the monocytes (CD14$^+$ cells) in control and MoFi-processed PBMNCs could be polarized efficiently toward CD206$^+$ M2 macrophages at a ratio of $66.22 \pm 13.53\%$ and $68.73 \pm 15.00\%$ on day 1 (n = 6,
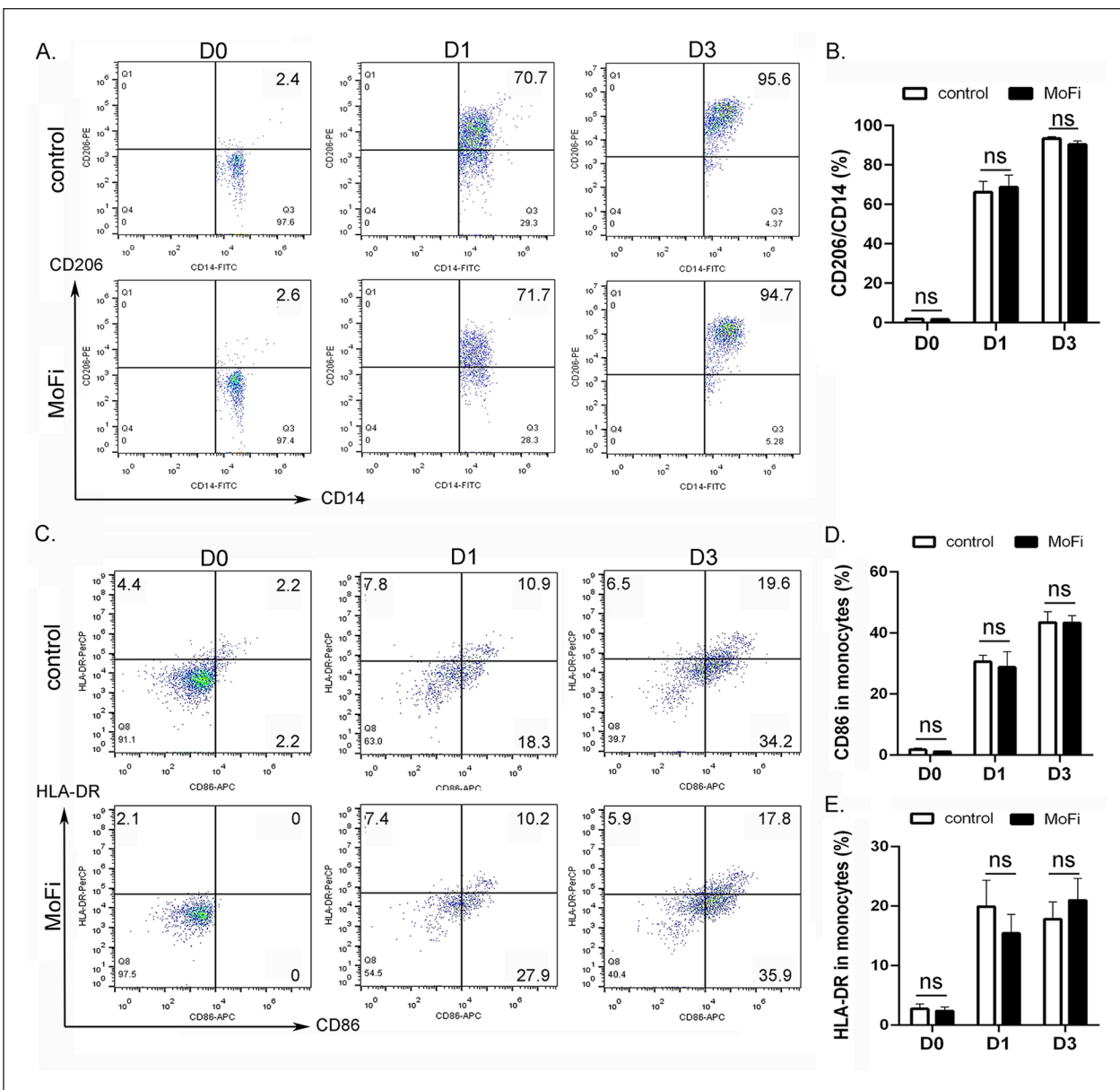
Figure 2. M1/M2 macrophage differentiation of the MoFi-treated PBMNCs. (A) Representative flow cytometry data showed the ratio of differentiating M2 macrophages (X-axis, CD14; Y-axis, CD206) on day 0 (D0), 1 (D1), and 3 (D3) in collected PBS (control) or MoFi-treated (MoFi) PBMNCs, cultured in IMDM with 1% auto-plasma. (B) The statistical ratios of M2/monocytes (CD206$^+$/CD14$^+$ cells) in control and MoFi-treated PBMNCs on indicated days were presented (n = 6) (ns, *P*>0.05). (C-E) The representative ratios of CD86$^+$ (X-axis) and HLA-DR$^+$ (Y-axis) M1 macrophages (C) in control and MoFi-treated PBMNCs were analyzed by flow cytometry. The statistical results of CD86 (D) and HLA-DR (E) positive cells in the monocytes were analyzed by one-way ANOVA (n = 6) (ns, *P*>0.05). ns: non-significant; PBMNC: peripheral blood-derived mononuclear cells; PBS: phosphate-buffered saline; IMDM: Iscove's Modified Dulbecco's Medium; ANOVA: analysis of variance.

P > 0.05), and 93.37 ± 2.33% and 90.38 ± 4.45% on day 3 (n = 6, *P* > 0.05), respectively (Fig. 2A, B).

We also evaluated the potency of M1 macrophage differentiation, determined by the CD86 and HLA-DR expressions. We detected 43.36 ± 8.82% and 43.25 ± 5.80% CD86$^+$ cells in control and MoFi-treated monocytes on day 3,

respectively (n = 6, *P*>0.05) (Fig. 2C, D). The ratios of HLA-DR expressions were 17.76 ± 7.05% and 20.93 ± 9.01% in control and MoFi-treated monocytes on day 3, respectively (n = 6, *P*>0.05) (Fig. 2C, E). These results demonstrated that MoFi treatment might not alter the intrinsic property of monocyte differentiation when the harvested
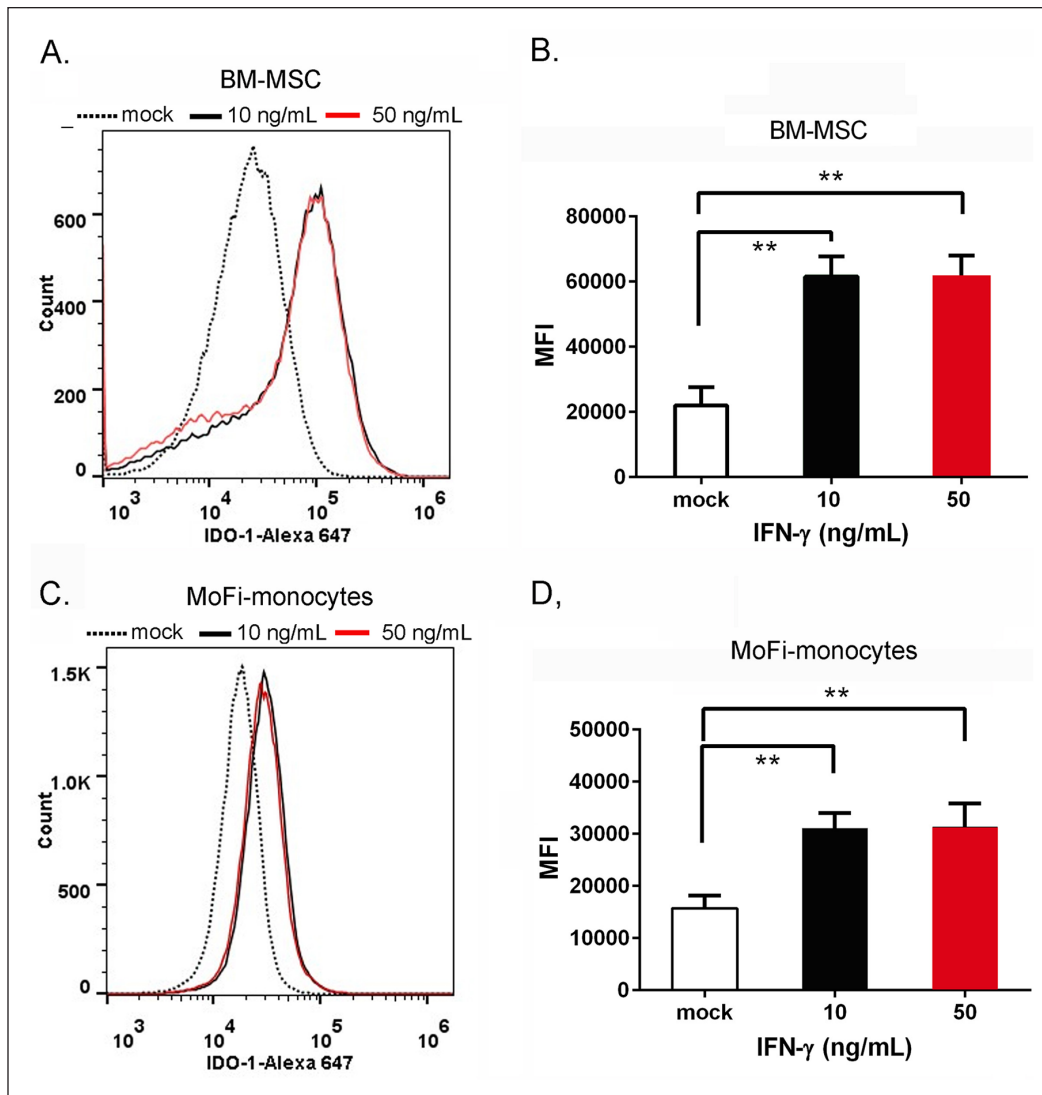
Figure 3. MoFi-treated monocytes expressed IDO-1 by IFN-γ induction. The BM-MSC (A, B) and CD14^{+} monocytes (C, D) were treated with PBS (mock), 10 ng/ml IFN-γ and 50 ng/ml IFN-γ for 3 days, and the intracellular IDO-1 protein expressions were analyzed by anti-IDO-1 antibody staining and flow cytometry. The mean fluorescent intensity (MFI) of the IDO-1 expression was determined by two-way ANOVA in mock, IFN-treated BM-MSCs (B) (n = 3) and MoFi-monocytes (D) (n = 3). IDO-1: indoleamine 2,3-dioxygenase; BM-MSC: bone marrow-derived mesenchymal stem cells; PBS: phosphate-buffered saline; IFN-γ: interferon-γ. **$P < 0.01$.

PBMNCs were cultured in the IMDM with auto-plasma culture condition.

The Anti-Inflammatory Activities of MoFi-Treated Monocytes *In Vitro and* In Vivo

Due to the enriched CD34^{+} cells and the differentiation potency of M2 macrophage, we next explored the anti-inflammatory activity of the MoFi-processed PBMNCs, especially the CD14^{+} monocytes, *in vitro* and *in vivo*[16,17]. The *in vitro* immuno-modulation activity of the cells was evaluated by the IDO-1 expression[18,19]. We used BM-MSC as a positive control. We treated the cells with 10 and 50 ng/ml IFN-γ and analyzed the IDO-1 expression by flow cytometry, a reliable quantitative method for IDO-1 detection[20]. After

3 days of treatment, the mean fluorescence intensity (MFI) was induced from 22,066 ± 5,478 (PBS, mock) to 61,433 ± 6,361 (10 ng/ml IFN-γ) ($P < 0.01$) and 61,333 ± 6,658 (50 ng/ml IFN-γ) ($P < 0.01$) (Fig. 3A, B). We next demonstrated that as the BM-MSC, the MoFi-treated CD14^{+} monocytes also strongly expressed IDO-1 protein after the 10 and 50 ng/ml IFN-γ on day 3 (Fig. 3C). The MFI of IDO-1 was elevated from 15,666 ± 2,516 (mock) to 31,133 ± 3,202 (10 ng/ml IFN-γ) ($P < 0.01$) and 31,466 ± 4,717 (50 ng/ml IFN-γ) ($P < 0.01$) (Fig. 3D). These results highlight the intrinsic anti-inflammatory ability of the MoFi-treated monocytes. The lower IDO-1 expression in MoFi-monocytes than that of BM-MSCs might cause by the suspension culture and immature differentiation, which might contribute to the weaker expression profiles of IFN-γ receptor
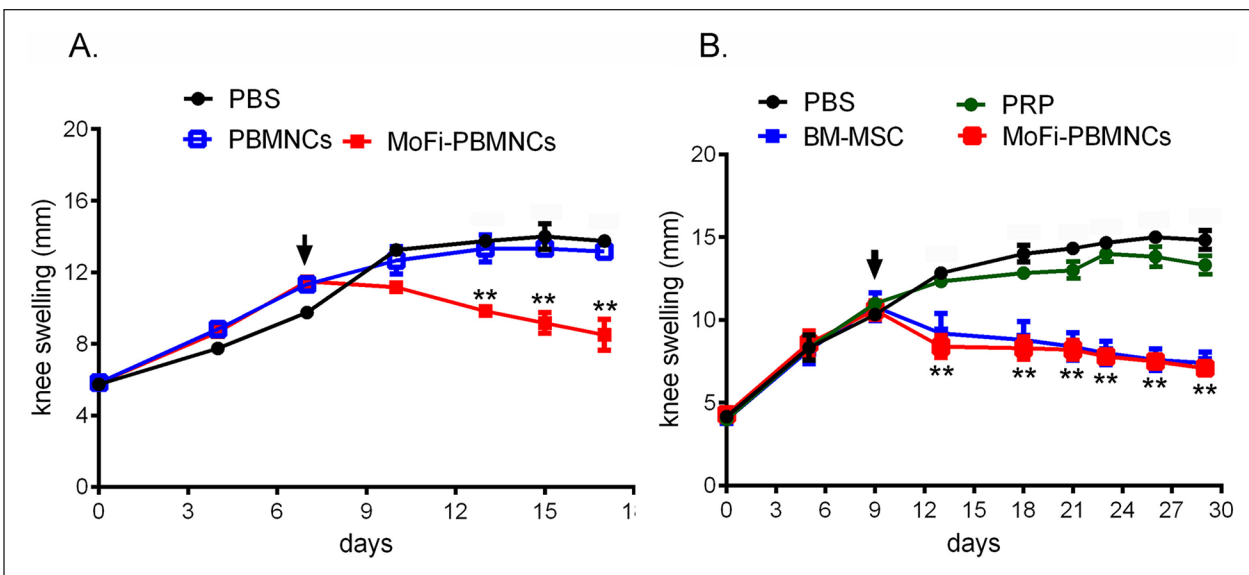
Figure 4. MoFi-treated PBMNCs attenuated acute inflammatory arthritis in rats. (A) MoFi-treated PBMNCs, but not mock (PBS) and control (normal saline-treated PBMNCs), attenuated the footpad swelling of CFA-induced arthritis in SD rats. MoFi vs control, $P < 0.01$ (**). (B) The tissue swelling degrees of the CFA-induced arthritis were attenuated by BM-MSCs and MoFi-treated PBMNCs, rather than mock (PBS) and PRP treatment. N = 6 for each treatment condition. MoFi vs PRP, $P < 0.01$ (**). Arrow indicates the day of cell engraftment. PBMNC: peripheral blood-derived mononuclear cells; PBS: phosphate-buffered saline; CFA: complete Freund's adjuvant; BM-MSC: bone marrow-derived mesenchymal stem cells; PRP: platelet-rich lysates.

and downstream signal molecules in the monocytes on day 3 culture.

An inflammatory arthritis model in rats was established by injecting complete Freund's adjuvant (CFA) into the hind footpad. Acute inflammation in the footpad was observed on day 3, and CFA was boosted again on day 6. We injected 2×10^5 cells PBMNCs or the MoFi-PBMNCs into the lesion on day 7. Interestingly, we found that only the MoFi-treated PBMNCs can significantly attenuate the inflammation and swelling, rather than the PBS (mock) or the normal saline-treated PBMNCs (control) ($P < 0.01$), after 5 days of engraftment (Fig. 4A).

We next injected the CFA-induced lesion with PBS, human platelet-rich plasma (hPL), 2×10^5 cells BM-MSCs or 2×10^5 cells MoFi-PBMNCs. We demonstrated that providing BM-MSCs attenuated the tissue swelling after 12 days post-transplantation but not the PBS negative control, validating the anti-inflammatory activity of the BM-MSCs ($P < 0.01$) (Fig. 4B). Notably, we discovered that administrating MoFi-treated PBMNCs, but not platelet-rich plasma (PRP), showed competitive potency as BM-MSCs to control the inflammatory progress after 12 days post-transplantation ($P < 0.01$) (Fig. 4B). In sum, these results support the anti-inflammatory activity of MoFi-PBMNCs *in vitro* and *in vivo*.

A Human Pilot Study of MoFi-Treated PBMNCs for Controlling KOA

Based on the immuno-modulation potency of the MoFi-processed PBMNCs, we organized investigator-initiated

Table 1. Baseline Characteristics of the Patients.

Characteristic	Total (n = 20)
Age (y)	68.2 ± 12.4
Male sex, *n* (%)	10 (50)
Weight (kg)	66.0 ± 15.2
Height (cm)	157.9 ± 9.6
BMI (kg/m^2)	26.4 ± 5.4
Kellgren–Lawrence grade, n (%)	
II	5 (25)
III	9 (45)
IV	6 (30)
VAS (cm)	4.99 ± 2.54
KOOS Pain (%)	50 ± 25
KOOS Symptoms (%)	51 ± 17
KOOS ADL (%)	55 ± 24
KOOS Sports (%)	29 ± 22
KOOS QOL (%)	39 ± 20

BMI: body mass index; VAS: visual analogue scale; KOOS: Knee injury and Osteoarthritis Outcome Score; ADL: activities of daily living; QOL: quality of life.

trials (IITs) in three medical institutes to evaluate the safety and efficacy of the cells in controlling KOA patients with KL II-IV.

The baseline profile of the recruited patients is summarized in Table 1. All recruited patients suffered knee pain for at least 6 months and were unsatisfied with the hyaluronic acid treatment. They have no plan for knee replacement or arthroscopy for 12 months before enrollment.
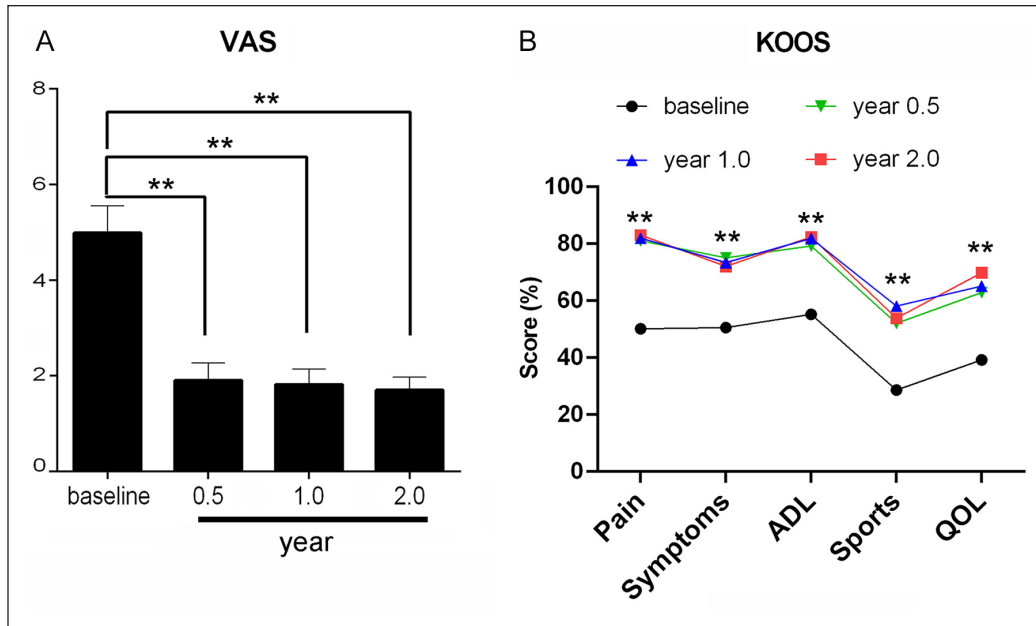
Figure 5. The pain-relief and the motor activities in treated KOA patients with MoFi-PBMNCs IA injection. KOA patients (n = 20) were IA injected with MoFi-treated PBMNCs once. The pain-VAS (A) and KOOS scores (B) were recorded at baseline, year 0.5, year 1.0, and year 2.0. Baseline vs treatments, **$P < 0.01$. KOA: knee Osteoarthritis; PBMNC: peripheral blood-derived mononuclear cells; IA: intra-articular; VAS: visual analogue scale; KOOS: Knee injury and Osteoarthritis Outcome Score; ADL: activities of daily living; QOL: quality of life.

Twenty patients were treated once with autologous PBMNCs (about 8×10^7 cells in 4 ml for one knee joint per person) from 100 ml peripheral blood and followed for 2 years. Compared with the baseline, the treated patients reported significant improvement in their knee pain and daily motor activities at 6 months, 1 year, and 2 years post-treatment (Fig. 5 and Table 2). Statistically, significant improvements in pain-VAS and KOOS scores were continuously observed for 2 years after the treatment ($P < 0.01$ for VAS and all KOOS subscores at year 0.5, 1.0, and 2.0). No patient took knee replacement or arthroscopy after enrollment. No severe adverse effect was observed during the trial period. The minor adverse effects were joint pain and swelling, lasting for 1 to 3 days after IA injection (n = 15, 75%). Persistent pain/swelling over 1 week, burning sensation, skin reddening, infection, or extra-care of hospitalization caused by the cell transplantation were not observed.

Discussion

Accumulative evidence demonstrates that CD34$^+$ HSCs and M2 macrophages are anti-inflammatory and may facilitate tissue regeneration after cell engraftment[16,17]. Both cells inhibited the production of T cells-triggered inflammatory cytokines, such as IL-6, IL-13, IFN-γ, and TNF-α[21,22]. Our animal studies have demonstrated that IA injection of the M2 macrophages effectively controlled the adjuvant-and collagen-induced arthritis (under revision). Here, we further

Table 2. Outcome Measures at Baseline and Years 0.5, 1, and 2.

Parameter (time)	Mean ± SD	95% CI
VAS, baseline	4.99 ± 2.54	3.87–6.10
VAS, year 0.5	1.90 ± 1.36	1.19–2.62
VAS, year 1	1.82 ± 1.43	1.19–2.45
VAS, year 2	1.70 ± 1.07	1.18–2.22
KOOS Pain, baseline	50 ± 25	39–61
KOOS Pain, year 0.5	81 ± 14	74–88
KOOS Pain, year 1	82 ± 14	76–88
KOOS Pain, year 2	83 ± 11	78–88
KOOS Symptoms, baseline	51 ± 17	43–58
KOOS Symptoms, year 0.5	75 ± 13	68–82
KOOS Symptoms, year 1	73 ± 17	66–80
KOOS Symptoms, year 2	72 ± 11	66–77
KOOS ADL, baseline	55 ± 24	45–65
KOOS ADL, year 0.5	79 ± 15	71–87
KOOS ADL, year 1	82 ± 18	74–90
KOOS ADL, year 2	82 ± 13	76–88
KOOS Sports, baseline	29 ± 22	19–38
KOOS Sports, year 0.5	52 ± 31	35–68
KOOS Sports, year 1	58 ± 23	48–68
KOOS Sports, year 2	54 ± 19	44–63
KOOS QOL, baseline	39 ± 20	30–48
KOOS QOL, year 0.5	63 ± 24	50–75
KOOS QOL, year 1	65 ± 20	56–74
KOOS QOL, year 2	70 ± 15	62–77

VAS: visual analogue scale; KOOS: Knee injury and Osteoarthritis Outcome Score; CI: confidence interval.

illustrate that the MoFi-processed PBMNCs showed anti-inflammatory activities as BM-MSCs in the IDO-1 expression and the potency for attenuating inflammatory arthritis.

In the pilot human clinical trial, we demonstrated that MoFi-processed PBMNCs effectively ameliorated pain and stiffness in OA patients. The administration of the PBMNCs might prohibit the inflammatory response of synovial tissues by suppressing IL-1, TNF-α, and C-reactive protein (CRP) expression and decreasing bone marrow lesions or edema (BML/BME) and synovitis even at the late stage of KOA[23,24]. The validation of the anti-inflammatory effects of PBMNCs injection in humans requires further magnetic resonance imaging (MRI) to examine the reduced knee synovitis and BML at 1 year and 2 years post-treatment[25,26]. Similar to the high tolerance of MSC injection, treating KOA with PBMNCs was safe and did not cause severe adverse effects after 2 years following. We will further examine whether multiple injections rather than single injection steer higher risks of side effects and measure MRI changes at baseline and follow-up visiting.

BMAC is an alternative approach for controlling the symptoms of KOA[7,27]. The containing HSCs and MSCs are supposed to be critical therapeutic factors[9,28]. However, surgical procedures are required for BMAC and could bring some high-risk surgical complications. In addition, the granulocytes are not effectively removed from the BMAC, and their pro-inflammatory activity may compensate for the HSC- and MSC-mediated immunosuppression. Moreover, the preparation of the BMAC is usually done in an open operating room. Both surgical and cell processings of BMAC require careful manipulation to prevent potential risks of microbial infections.

Peripheral blood stem cells (PBSCs) have also shown the safety and potential for controlling KOA progress and ameliorating KOA-related pain and syndromes.[29–34] Administrating multiple doses of PBSCs promotes tissue repair on cartilage defects and bone marrow lesions[30,31,34]. The concentrated PBSCs are generally isolated from granulocyte colony-stimulating factor (G-CSF) -stimulated patients by leukapheresis[29,32]. Compared with the PBSC processing, the advantages of the PCP are fast and easy preparations, point-of-care, and free of concerns of G-CSF adverse effects, such as fever, fatigue, and unsustainable pain in bones and muscles[29,32]. Moreover, in contrast to the apheresis of PBSCs, the Ficoll gradient centrifugation effectively separates the RBCs and granulocytes from harvested PBMNCs. This clearance of granulocytes might consequently potentiate the anti-inflammatory and regenerative effects of the grafted cells.

Tissue injury at an early stage triggers an inflammatory response to attract the first line of immune cells against microbial infection, such as neutrophils and natural killer cells. The neutrophil-released cytokines, such as G-CSF, polarize tissue-invading mononuclear cells into M1 macrophages to exaggerate the tissue destruction[22]. After clearing the invading pathogens at a later stage, circulating monocytes will be recruited and polarized into M2 macrophages for anti-inflammation and tissue reconstruction. Recent evidence also shows that the M1/M2 ratio in synovial fluid is critical for arthritis progress and could be a valuable biomarker in arthritis management[15,35,36].

The ratios of HSCs and monocytes in MoFi-treated PBMNCs were about two-fold than that of normal saline-treated PBMNCs after the density-gradient purification, respectively. We demonstrated that the monocytes collected from MoFi plus Ficoll separation are competent to differentiate into M2 macrophage lineages. It is interesting to find that the MoFi-treated PBMNCs significantly attenuated the footpad swelling of CFA-induced arthritis. The human pilot study further showed that intra-articular injection of MoFi-processed PBMNCs relieved pain symptoms and motor activities in KOA patients. It still requires a randomized controlled trial (RCT) at multi-centers and larger sample size to consolidate the conclusion. We will assess structural changes using MRI or arthroscopic examination for the treated KOA patients. It is also informative to determine the duration for waiving or delaying knee replacement surgery in patients with severe KOA after the PCP treatment.

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Author Contributions

Conceptualization: CHC, HLS, and CLL. *In vitro* and *in vivo* experiments and analysis: CHC, CCK, YFC, FHW, CYH, WSF, HLS, and CLL. Resources: CHC, CCK, YFC, PYL, JIS, YHC, KDL, HLS, and CLL. Writing the original draft: HLS and CLL. Writing, review & editing: CCK, HLS, and CLL.

Ethical Approval

Ethical issues of the study were approved by the Research Ethics Committee of China Medical University (CMUH 109-REC 1-012), Show Chwan Memorial Hospital (1090803), and Taichung Tzu Chi Hospital (REC 109-45).

Statement of Human and Animal Rights

All procedures involving human subjects were conducted in accordance with the clinical trials act and the tenets set down in the Declaration of Helsinki, and with the ethical guidelines for medical and health research involving human subjects of Taiwan policies, adopted by the Research Ethics Committee of China Medical University (CMUH 109-REC 1-012), Show Chwan Memorial Hospital (1090803), and Taichung Tzu Chi Hospital (REC 109-45). All procedures involving animal subjects were conducted in accordance with the animal protection policies of Taiwan, adopted by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (NCHU) (NCHU 109-093).

Statement of Informed Consent

Declaration of Conflicting Interest

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ORCID iD

Hong-Lin Su https://orcid.org/0000-0001-7734-3499

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