

# The hUCMSCs Exerts Excellent Anti-inflammation Effect which is Different from TCZ IL-6 Antagonist by Inducing High IL-6 Secretion

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## Research Article

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# Abstract

**BACKGROUND** The biologic and cellular IL-6-related therapies have been used to treat the autoimmune diseases (AID), which prompting us to furtherly explore the IL-6 role in human umbilical cord mesenchymal stem cells (hUCMSCs) therapy.

**MATERIALS & METHODS** Peripheral blood mononuclear cells (PBMCs) were responder co-cultured with hUCMSCs or exogenous IL-6. PBMC suppression assay was used to analyze the anti-inflammatory effects by using the MTT assay. The IL-6 concentration in supernatant was measured using ELISA. The correlation between anti-inflammation effect of hUCMSCs and IL-6 levels, and the relevant roles of IL-6, IL-6 mRNA expression was analyzed using the MetaCore functional network constructed from gene microarray data. The location of IL-6 and IL-6 receptor (IL-6R) expression was furtherly evaluated.

**RESULTS** Initially, hUCMSCs did not exert any inhibitory effect on PBMCs, however, a potent inhibitory effect on PBMCs was observed and the IL-6 concentration reached about 1000 ng/mL after 72 hours. Exogenous 1000 ng/mL IL-6 could inhibit PBMCs inflammations but less than that of hUCMSCs. The hUCMSCs exerts excellent anti-inflammation effect by inducing higher IL-6 level which is different from TCZ IL-6 antagonist

**CONCLUSIONS** High concentration IL-6 cytokine secretion plays an important role in the anti-inflammation effect of hUCMSCs cell therapy.

## Introduction

Autoimmune diseases (AID), such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), are highly complex inflammatory diseases. It has been reported that IL-6 production can be triggered by either by external stimuli or self-induced as part of a biological defense mechanism [1, 2]. However, dysregulation of IL-6-mediated inflammatory responses (excess levels/oversensitivity) may trigger a series of inflammatory responses that lead to the onset of AID [3, 2]. The severity of RA showed a positive linear correlation with IL-6 levels [4–6]. Currently, two types of biological agents are used to block IL-6 in clinical settings: (1) agents that directly block IL-6 production, such as siltuximab and (2) agents that prevent the binding of IL-6 to IL-6R, such as tocilizumab [3, 7, 8, 2]. A statistical review of research articles conducted in 2019 revealed that blocking of the IL-6R using tocilizumab comprehensively inhibited the binding of IL-6 to IL-6R, thereby inhibiting IL-6 production and effectively alleviating IL-6-induced inflammation in clinical treatments [2, 9]. Nevertheless, therapeutic approaches that involve inhibition of IL-6 production or blocking IL-6, IL-6R binding result in various frustrating and unsolvable phenomena of disease deterioration, indicating that there is still room for improvement in IL-6-targeted therapies [10, 9].

Several studies on MSCs and AID (SLE and RA) published by Professor Sun Lingyun [11, 12] from Nanjing Medical University showed that elevation of IL-6 suppressed inflammatory responses. We obtained similar results in our previous study [13]. There are two main therapeutic approaches to suppress inflammation via IL-6 pathways: (1) Reducing the elevation of IL-6 and (2) promoting IL-6

secretion or increasing the concentration of IL-6 to reach a certain threshold to suppress inflammation. Both therapeutic approaches involved IL-6 at both low and high concentrations exert similar anti-inflammatory effects, prompting us to investigate the mechanisms of action and the roles of IL-6 under these two conditions.

IL-6 is a cytokine with multiple biological activities [3, 14]. The synthesis of IL-6 is regulated at both the transcriptional and post-transcriptional levels. Upon infection or injury, the biosynthesis of IL-6 is induced immediately as an emergency signal to activate host defenses and an acute immune response [15]. After the environmental stress diminishes or disappears, the concentration of IL-6 decreases and returns to baseline [2]. IL-6 exerts its biological activities mainly through IL-6R, which exists in two forms, a membrane-bound form (mIL-6R) that exerts its actions through cell membranes, and a soluble form (sIL-6R) that is secreted into the extracellular space and in body fluids. The latter mainly binds to IL-6 and exerts its activity in extracellular environments [10, 15, 16]. Signals mediated by mIL-6R and sIL-6R are termed classical signaling and trans-signaling, respectively. mIL-6R is the predominant form of IL-6R in human [17, 18]. These different IL-6R form may be a key to the different anti-inflammatory pathways involving IL-6 [15].

Numerous previous studies [19, 20] have demonstrated that MSCs can suppress inflammatory responses. Our previous study [21, 22, 13] revealed that hUCMSCs exhibit a significant inhibitory effect against the inflammatory responses of PBMCs. We also uncovered eight immunomodulatory pathways that are closely associated with IL-6 in MSCs, one of which promotes inflammatory responses, while the remaining seven pathways are associated with anti-inflammatory responses, suggesting the presence of diverse IL-6-mediated inflammatory pathways [2]. Since it is known that hUCMSCs play an important role in cell therapy, the question arises as to whether the efficacy of stem-cell therapies aimed at immunomodulation is related to the presence of different IL-6-mediated inflammatory pathways.

Therefore, we aimed to observe the changes in IL-6 concentration and the inhibitory effect of IL-6 on inflammatory responses in co-cultures of MSCs and PBMCs to explain the correlation between the anti-inflammatory effect of MSCs and the concentration of IL-6. We also aimed to investigate the role of hUCMSCs in suppressing inflammatory responses.

## **Material And Methods**

### **Cell culture and cell line authentication**

PBMCs: Whole blood samples collected from research subjects were mixed thoroughly with an equal volume of Hank's balanced salt solution (HBSS) and then carefully and slowly added to an equal volume of Ficoll Hypaque. After centrifugation at  $1731 \times g$  for 15 minutes, mononuclear cells in the MNC layer were harvested using a drop pipette and mixed thoroughly with an equal volume of HBSS. For cell counting, 10  $\mu$ L of the cell suspension was mixed with 90  $\mu$ L of 10 $\times$  trypan blue, and the remaining cell

suspension was centrifuged at 623 ×g for 5 minutes. After discarding the supernatant, the resulting cell pellet was resuspended in Gibco™ AIM V™ Medium and incubated at 37°C with 5% CO<sub>2</sub> and 21% O<sub>2</sub> [13].

**hUCMSCs:** The identity of the MSCs isolated from Wharton's jelly of the umbilical cord was verified by characterizing their surface markers and functions. hUCMSCs were expanded and cultured prior to cryopreservation. Cells from the second passage were used for subsequent experiments. For culture, hUCMSCs were seeded at an initial density of 3000 cells/cm<sup>2</sup> in alpha Minimum Essential Medium (α-MEM; Invitrogen, Life Technologies Corporation, Gaithersburg, MD, USA) containing 5% UltraGRO (Helios Bioscience, AventaCell BioMedical Corporation, Atlanta, GA, USA) and 1% penicillin-streptomycin at 37°C in an incubator (Thermo Scientific, Waltham, MA, USA) with 5% CO<sub>2</sub> and 21% O<sub>2</sub>. TrypLE (Gibco, Life Technologies Corporation, Waltham, MA, USA) was used to harvest cells during subculture [13].

For authentication, MSCs were immunolabeled with mouse anti-human antibodies against the following antigens: CD34, CD45, CD29, CD31, CD44, CD90, HLA-A, HLA-B, HLA-C, HLA-DR (BD Biosciences, San Jose, CA, USA), CD105 (AbD Serotec, Oxford, UK), CD73, CD117, and CD184 (BD Pharmingen, San Diego, CA, USA)[22, 21]. The immunolabeled cells were incubated with anti-mouse fluorescein isothiocyanate (FITC)- or phycoerythrin-conjugated IgG as the secondary antibodies, followed by flow cytometry analysis (BD Biosciences).

## Evaluation of cell growth rate

Harvested cells were diluted in culture medium and mixed thoroughly with an equal volume of trypan blue (1:1) for cell counting.

## PBMC suppression assay

**Co-culture of hUCMSCs and PBMCs:** Before the experiment, the cells were cultured at 37°C with 5% CO<sub>2</sub>. The PBMCs were divided into the phytohemagglutinin (PHA) and control groups. PHA was added to the former at a final concentration of 5 µg/mL. For co-culture, the PBMCs were divided into two experimental groups, i.e., the PBMC control and hUCMSCs co-culture groups. PBMCs grown in suspension were harvested and co-cultured with stably growing, adherent hCMSCs in Transwell culture plates. Following co-culture, the PBMCs were isolated by centrifugation and subject to cell counting to calculate and compare the number of cells [22, 23].

In co-cultures of exogenous IL-6 and PBMCs, the PBMCs were exposed to different concentrations of exogenous IL-6 (2.5, 5, 25, 50, 100, 250, 500, and 1000 ng/ml), and cell counting was performed on days 0, 1, 2, 4, and 6.

## Enzyme-linked immunosorbent assay (ELISA)

IL-6 levels in cell culture supernatant were analyzed using the ELISA MAX™ Deluxe Set Human IL-6 kit (BioLegend, San Diego, CA, USA). After terminating the TMB reaction with stop reagents, the optical density of the reaction mixture was measured at 450 and 570 nm ( $OD_{450}$  and  $OD_{570}$ , respectively) within 15 minutes to obtain the experimental result [13].

## **Analysis of the effects of IL-6-neutralizing antibody on PBMCs**

PBMCs (PHA-stimulated cells): The PBMCs were divided into five groups: the PBMC-control group and four groups of PBMC-UCMSC co-cultures exposed to different concentrations (1000, 316, 100, and 31.6 ng/mL) of IL-6-neutralizing antibody. Adherent cultures of hUCMSCs, as well as cell harvesting and cell counting of PBMCs, were carried out as described above. Then, the PHA-stimulated PBMCs were co-cultured with hUCMSCs in Transwell chambers and exposed to different concentrations (1000, 316, 100, and 31.6 ng/mL) of IL-6 neutralizing antibody for 0, 1, 2, 3, and 4 days. The media in the wells and inserts were mixed and collected for cell counting to observe the growth of cells and determination the IL-6 concentration in the culture medium.

## **Determination of the effects of tocilizumab (TCZ) on PBMCs**

PBMC were divided into four groups. The two groups were treated as control groups without any pretreatment. One group was co-cultured with UCMSC, and the other group was not co-cultured with UCMSC. The commonly used clinical immunosuppressive drug TCZ was selected as the pretreatment of the experimental group. 100 µg tocilizumab (TCZ) was pretreated for 5 hours and 10 hours. At each time point, there is a group co-cultivating with UCMSC. The other group did not add UCMSC to the co-culture. The co-culture of PBMC and UCMSC was prepared and processed as described above, and after 72 hours of culture, the number of PBMC cells was counted.

## **RNA extraction**

Cells were lysed in TRI Reagent (Sigma) and shaken with 1-bromo-3-chloropropan (BCP) for 15 seconds prior to centrifugation. After centrifugation, the aqueous layer was mixed gently with isopropanol and allowed to stand. After centrifugation, the resulting RNA pellet was washed with 75% ethanol and centrifuged again. The washed RNA pellet was then air-dried or vacuum-dried and reconstituted with an appropriate amount of DEPC water or 0.5% SDS. The quality of the extracted RNA was assessed based on the  $A_{260}/A_{280}$  ratio, and its concentration was determined based on the  $A_{260}$  value [13].

# Reverse transcription-polymerase chain reaction (RT-PCR) and Polymerase chain reaction (PCR)

Total RNA (3  $\mu$ l containing  $>1 \mu$ g) was subject to RT-PCR using GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Then, the concentration of the reverse-transcribed cDNA was measured [24]. Primers (shown below) were designed according to the experimental requirements. The concentration of DNA template was adjusted accordingly and added to reaction mixtures containing the following pairs of forward and reverse primers:

IL-6\_forward (5'-CTGGATTCAATGAGGAGACTTGC-3') and IL-6\_reverse (5'-GGACAGGTTTCTGACCAGAAG-3'), IL-6R\_forward (5'-AAGGACCTCCAGCATCACTGTGTCA-3') and IL-6R\_reverse (5'-CCTTCAGAGCCCGCAGCTTCCACGT-3'), GAPDH\_forward (5'-ATCAAGAAGGTGGTGAAGCAGG-3') and GAPDH\_reverse (5'-GCAACTGTGAGGAGGGGAGATT-3'), along with DNA Taq polymerase, PCR buffer, and nucleotides.

The PCR cycling conditions consisted of 30 cycles (for IL-6 and GAPDH) or 35 cycles (for IL-6R) of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, to amplify the targeted DNA fragments [25].

## Statistical analysis

All experiments were repeated three times, and the data are presented as mean  $\pm$  standard error of the mean (SEM). The inhibitory effect of hUCMSCs is illustrated in graphs. Multiple comparisons were performed using the Kruskal-Wallis test with Bonferroni correction. Reductions in cell proliferation, i.e., the number of PBMCs between the control and experimental groups, were compared using the Wilcoxon rank-sum test. Differences with P values less than 0.05 were considered statistically significant.

## Results

### Authentication of hUCMSCs

MSCs isolated from the human umbilical cord were cultured and subject to cell line authentication according to the research project approved by Taoyuan General Hospital (MOHW) on human MSCs (IRB number: TYGH 104043).

### Relationship between human MSCs and IL-6 (IL-6 expression in MSCs)

Microarray analysis of 31,099 genes in MSCs revealed 260 upregulated genes (0.84%) and 699 downregulated genes (2.25%) [13].

Functional network analysis using MetaCore™ (Enrichment Analysis Workflow and analysis network, GeneGo, Inc. MI, USA) showed that eight different immune pathways. It can be found by observing changes in the eight immune pathways that IL-6 directly or indirectly plays key roles in facilitating immune responses, including pro-inflammatory pathways mediated by histamine signaling and anti-inflammatory pathways mediated by Jak-STAT pathway, IL-4 signaling, and TREM1 signaling [13].

Our previous study showed that hUCMSCs have a significant inhibitory effect on the inflammatory response of PBMCs. IL-6 may activate the mRNA expression of genes in the MAPK pathway, including leukemia inhibitory factor (LIF, or cholinergic differentiation factor, CDF), IL-6, insulin receptor substrate 2 (IRS2), and growth factor receptor-bound protein 14 (GRB14), suggesting that IL-6 activates its downstream pathways by binding to different receptors or stimulating relevant factors (supplementary Figure 1, table 1).

Table 1  
IL-6 may activate the mRNA expression of genes in the MAPK pathway

Name	Cell line 1	Cell line 2	Cell line 3	Cell line 4	Cell line 5
LIF Homo sapiens leukemia inhibitory factor (cholinergic differentiation factor) (LIF), mRNA [NM_002309]	0.860	2.367	2.485	2.540	2.157
LIF Homo sapiens leukemia inhibitory factor (cholinergic differentiation factor) (LIF), mRNA [NM_002309]	1.154	1.511	1.345	1.624	1.519
IL-6 Homo sapiens interleukin 6 (interferon, beta 2) (IL6), mRNA [NM_000600]	0.882	2.036	3.549	3.207	1.146
IL-6 Homo sapiens interleukin 6 (interferon, beta 2) (IL6), mRNA [NM_000600]	0.885	2.023	3.549	3.243	1.154
IL-6 Homo sapiens interleukin 6 (interferon, beta 2) (IL6), mRNA [NM_000600]	0.862	2.074	3.246	3.142	1.152
IRS2 Homo sapiens insulin receptor substrate 2 (IRS2), mRNA [NM_003749]	0.571	1.874	1.969	2.016	1.763
GRB14 Homo sapiens growth factor receptor-bound protein 14 (GRB14), mRNA [NM_004490]	1.658	3.702	2.948	0.804	0.855

## IL-6 expression in PBMCs and MSCs

To understand the cell proliferation and IL-6 secretion in PHA-stimulated PBMCs. After 72 hours, there were significantly higher numbers of PHA-stimulated PBMCs ( $375.78 \pm 2.78\%$ ) than non-stimulated PBMCs ( $256.67 \pm 3.71\%$ ) (Figure 1a,  $P < 0.001$ ). There was no significant difference in the IL-6 concentration between PHA-stimulated PBMCs ( $15.91 \pm 0.67$  ng/mL) and non-stimulated PBMCs ( $12.30 \pm 0.38$  ng/mL) (Figure 1b).

Continuing the previous experiments, we observed the cell viability and IL-6 levels in PBMC and UCMSC co-cultures at 72 hours. After co-culture with hUCMSCs for 72 hours, the cell counts of PHA-stimulated PBMCs ( $205.33 \pm 3.53\%$ ) were 45.5% lower than those in the control group ( $375.78 \pm 2.78\%$ ) (Figure 1a,  $P < 0.001$ ).

The initial concentration of IL-6 on day 0 was 50.70 ng/mL, which increased to 145.06 ng/mL after 24 hours, to 716.22 ng/mL after 48 hours, and to 996.81 ng/mL (about 1000 ng/mL) after 72 hours (Figure 1B). Our study showed that the concentration of IL-6 in co-cultures of PBMCs and hUCMSCs was significantly higher than that in PBMC control group, and it increased by more than 20 fold after 72 hours of co-culture.

## **Expression of IL-6 and IL-6R in co-cultures of hUCMSCs and PBMCs**

The results of PCR and RT-PCR assays revealed that IL-6 was mainly expressed by hUCMSCs, while PBMCs only expressed a small amount of IL-6. In contrast, IL-6R was highly expressed on PBMCs and was expressed at low levels on hUCMSCs. Thus, IL-6 was primarily expressed by hUCMSCs, and IL-6R was primarily expressed on PBMCs in co-cultures of hUCMSCs and PBMCs (Figure 2).

## **Inhibitory effects of hUCMSCs on inflammatory model PHA-stimulated PBMCs**

Without PHA stimulation, the control group, compared to day0, the increase of cell viability was 67.1%, 125.8%, 156.7% on day1, 2, and 3, respectively. In the co-cultured group, compared to day0, the increase of cell viability was 32%, 44.2%, 81.1% on day1, 2, and 3, respectively. Thus, co-culture with hUCMSCs inhibited PBMC proliferation by 21%, 36.1%, and 29.5% on day 1, 2, and 3 of co-culture, respectively ( $P < 0.001$ ). With PHA stimulation PBMC, the control group, compared to day0, the increase of cell viability was 89.6%, 164.9%, 275.8% on day1, 2, and 3, respectively. In the co-cultured group, compared to day0, the increase of cell viability was 52.2%, 66.9%, 105.3% on day1, 2, and 3, respectively. Thus, co-culture with hUCMSCs inhibited PBMC proliferation by 19.7%, 37.0%, and 45.4% on day 1, 2, and 3 of co-culture, respectively ( $P < 0.001$ ).

## **Inhibitory effects of exogenous synthetic IL-6 on PBMCs**

Analysis of the cell counts of PBMCs that were exposed to different concentrations of exogenous IL-6 (2.5, 5, 25, 50, 100, 250, 500, and 1000 ng/ml) for 0, 1, 2, 4, and 6 days showed that the exogenous IL-6 did not exert a dose-dependent inhibitory effect on the proliferation of PBMCs at concentrations below 500 ng/ml. However, after exposure to 1000 ng/ml of exogenous IL-6 for 6 days, the cell count of PHA-

stimulated PBMCs ( $389.02 \pm 13.31\%$ ) was 10.57% lower than that of the control ( $435.02 \pm 8.43\%$ ) (Figure 1a,  $P < 0.001$ ), suggesting a potent inhibitory effect on the proliferation of PBMCs (Figure 3a).

Comparison of the inhibitory effect of exogenous synthetic IL-6 and hUCMSCs on the inflammatory response of PBMCs is shown in Figure 3b. PBMCs displayed a significant anti-inflammatory response (10.57%) after being exposed to high concentrations of exogenous IL-6 for 6 days. For comparison, PBMCs co-cultured with hUCMSCs for 48 hours showed a significant anti-inflammatory response (reduction of 35.41% on Day 2, 57.69% on Day 3, and 70.96% on Day 4).

## The relationship between IL-6 and IL-6 receptors

The growth of PBMCs in co-cultures of hUCMSCs and PBMCs was observed after exposure to different concentrations of IL-6 neutralizing antibody (31.6, 100, 316, and 1000 ng/mL). After exposure to 1000 ng/mL of neutralizing antibody for 48, 72, and 96 hours, the cell counts of PHA-stimulated PBMCs ( $135.56 \pm 7.58\%$ ,  $199.11 \pm 3.36\%$ , and  $228.89 \pm 4.29\%$ , respectively) did not differ significantly from counts of the control group ( $143.11 \pm 2.04\%$ ,  $199.78 \pm 6.05\%$ , and  $235.11 \pm 4.68\%$ , respectively) (Figure 4A,  $P > 0.05$ ). The results showed that when treated with the IL-6 neutralizing antibody, the number of PBMCs increased over time, and the growth efficiency improved with increasing concentrations of IL-6 neutralizing antibody, forming a dose-dependent pattern. The PBMC suppression assay showed that the inhibitory effect of hUCMSCs disappeared the day after being exposed to the highest concentration (1000 ng/ml) of IL-6 neutralizing antibody (Figure 4a).

Determination of the inhibitory effect of hUCMSCs on the proliferation of PBMCs via IL-6 receptor blockage. PBMCs were divided into four groups. Two groups did not undergo any pretreatment as the control groups. One group was co-cultured with hUCMSCs and the other group was not co-cultured with hUCMSCs. After 72 hours of culture, the number of PBMC cells was counted. The results showed that PBMCs would be inhibited by hUCMSCs and slow down the proliferation of cells. Clinically, TCZ is used as an immunosuppressive drug. Tocilizumab (TCZ) 100  $\mu$ g is added to pre-treatment for 5 hours and 10 hours. After 72 hours of culture, the number of PBMC cells is counted. Compared with the control group, the cell proliferation is 98.79% and 99.34%; adding UCMSC Compared with the control group, the cell proliferation of the group was 96.37% and 100%, respectively. The results showed that the effect of hUCMSCs on inhibiting PBMC proliferation disappeared after TCZ pretreatment (Figure 4 b).

## Discussion

Our findings differ from those of previous studies in several ways. First, co-culture of UMSCs and PBMCs induced the production of different concentrations of IL-6, which have differential effects on PBMCs at different time points. At low concentrations ( $< 1000$  ng/mL), IL-6 did not exert any inhibitory effect on PBMCs, but it induced hUCMSCs to secrete more IL-6 [13]. However, at the highest concentration ( $\geq 1000$  ng/mL), IL-6 exerted a potent inhibitory effect on PBMCs after 72 hours of incubation. Second, IL-6R plays a key role in the application of hUCMSCs in cell therapy.

Cell therapy mainly refers to approaches that use healthy stem cells to enhance immunity against inflammation or cultured autologous stem cells to repair/replace damaged cells and tissues or to control disease [26]. AID refer to the immune dysregulation and disorders that trigger inflammatory responses and IL-6 production [3]. Under these conditions, the concentration of IL-6 continues to rise along with the expansion of inflamed areas. Previous studies have shown that IL-6 exhibits both pro- and anti-inflammatory properties [3, 27]. In this study, we found that hUCMSCs exerted a significant inhibitory effect on the inflammatory response of PBMCs. IL-6 may activate the mRNA expression of genes in the MAPK pathway, such as LIF/CDF, IL-6, IRS2, and GRB14, indicating that IL-6 activates its downstream pathways by binding to different receptors or stimulating relevant factors (supplementary Fig. 1, table 1) [13].

Our study on PHA-stimulated PBMCs showed that although treatment with PHA alone activated the inflammatory responses of PBMCs, the concentration of IL-6 only increased about 1.5 fold after 72 hours (Fig. 1b). In contrast, after co-culture with hUCMSCs for 72 hours, the concentration of IL-6 increased significantly, by about 20 fold (Fig. 1b). In addition, we also found that the proliferation of PBMCs decelerated with a statistically significant difference after co-culture with hUCMSCs for 72 hours (Fig. 1a). Hence, we deduced that the PHA-induced pro- or anti-inflammatory responses of PBMCs are closely associated with the concentration of IL-6.

Our previous study of the PHA-induced inflammatory responses of PBMCs revealed that hUCMSCs may inhibit the proliferation of PBMCs by activating IL-6 synthesis to secrete high levels of IL-6 [22]. Based on the central dogma of molecular biology, we explored the IL-6 and IL-6R mRNA levels to clarify the consistency between the mRNA expression analysis and ELISA results in PBMCs and hUCMSCs. We analyzed the IL-6 and IL-6R mRNA levels by RT-PCR. The results showed that hUCMSCs had higher IL-6 mRNA expression levels, while PBMCs had higher IL-6R mRNA expression levels; thus, IL-6 and IL-6R were primarily expressed in human MSCs and PBMCs, respectively (Fig. 2)

If the inhibitory effect of hUCMSCs on PBMCs is attributed to the interaction between IL-6 and IL-6R, the immunomodulatory activity of hUCMSCs should be halted by blocking either of these two proteins. To this end, we designed an experiment in which IL-6 or IL-6R was neutralized by a monoclonal antibody or blocked with a biological agent, respectively. We confirmed that the study group after adding IL-6 neutralizing monoclonal antibody did not inhibit the proliferation of PBMCs when compared to that of the control (Fig. 4A). Thus, we inferred that the proliferation of PBMCs is not inhibited when most of the IL-6 protein secreted by the hUCMSCs is neutralized by an antibody. In addition, the proliferation of the PBMCs in the TCZ pre-treatment groups was not inhibited when compared to the proliferation of the TCZ-free group. We inferred that TCZ blocks the inhibitory effect of hUCMSCs on the proliferation of PBMCs by blocking the binding of UCMSC-secreted IL-6 to IL-6R (Fig. 4b). Based on the above experimental results, we drew the following conclusions: IL-6 is mainly secreted by hUCMSCs, while IL-6R is mainly expressed on the cell membrane of PBMCs, and the absence of either IL-6 or IL-6R hinders activation of IL-6-related “anti-inflammatory responses”[15].

PBMC-UCMSC co-culture experiments with and without exogenous IL-6 demonstrated that IL-6 exhibits the anti-inflammatory effects of hUCMSCs when present at high concentrations. On the third day of co-culture, hUCMSCs produced a high concentration of IL-6 (~ 1000 ng/mL), which induced a significant anti-inflammatory response (Fig. 1b and 3b). In comparison, the highest concentration of exogenous IL-6 (1000 ng/mL) induced a significant anti-inflammatory response beginning on the sixth day of culture (Fig. 3a). This showed that hUCMSCs have the potential for potent therapeutic effects against many AID, mainly by producing high concentrations of IL-6. However, our results showed that elevation of IL-6 is a gradual and dynamic process, during which, other pathways or inflammatory factors (other than IL-6) may be involved, which require further investigation. hUCMSCs achieve therapeutic efficacy faster than exogenous IL-6, which takes twice as long as the former or even longer (Fig. 3b). The above experimental results led us to conclude that IL-6 is not the only immunomodulatory factor secreted by hUCMSCs, and their anti-inflammatory activity may also be attributable to other immunomodulatory factors, such as TGF- $\beta$  and PGE2, other interleukins, and/or other pathways. Nevertheless, we still believe that IL-6 plays an important role in immunotherapy.

Tanaka et.al [2] reported that IL-6 can cause severe inflammation. Most IL-6-targeted AID therapies are focused on inhibition of inflammatory responses by blocking the binding of IL-6 to TCZ-antagonized IL-6 receptor. However, this therapeutic approach has not yielded any major treatment breakthroughs. Worse still, only one out of the eight IL-6-related immune pathways promotes inflammatory responses, while the remaining seven pathways are related to anti-inflammatory responses. Therefore, blocking the binding of IL-6 to IL-6 receptor may result in a loss of the one pro-inflammatory pathway in PBMCs, but it may also greatly affect the repair mechanisms initiated in response to inflammation, and may even reduce the therapeutic efficacy of MSCs. Hence, the advantages and disadvantages of TCZ for the long-term treatment of AID require further investigation.

Previous study [14] showed that a pro-inflammatory response is induced via a trans-signaling pathway mediated by binding to sIL-6R, while an anti-inflammatory response is induced through the classical pathway mediated by mL-6R. Our results revealed that IL-6 did not exert any anti-inflammatory effect at low concentrations, but began to exert significant anti-inflammatory activity at 1000 ng/mL. Therefore, we conclude that IL-6 binds to different forms of IL-6R at different concentrations, thus activating different inflammatory pathways, with a threshold concentration of 1000 ng/mL.

## Conclusions

The proposed anti-inflammatory cell therapy using UCMSC is based on the high production of IL-6. The hUCMSCs exerts excellent anti-inflammation effect which is different from TCZ IL-6 antagonist. We believe that the combination of IL-6 secreted by hUCMSCs and macrophage IL-6R can stimulate the anti-inflammatory process faster and more efficiently.

## Abbreviations

MSCs: mesenchymal stem cells

hUCMSCs: human umbilical cord mesenchymal stem cells

PBMCs: peripheral blood mononuclear cell

IL-6: interleukin-6

IL-6R: interleukin-6 receptor

mIL-6R: membrane-bound form interleukin-6 receptor

sIL-6R: soluble form interleukin-6 receptor

AID: autoimmune diseases

SLE: systemic lupus erythematosus

RA: rheumatoid arthritis

HBSS: Hank's balanced salt solution

FITC: fluorescein isothiocyanate

PHA: phytohemagglutinin

ELISA: Enzyme-linked immunosorbent assay

TCZ: tocilizumab

BCP: 1-bromo-3-chloropropan

RT-PCR: Reverse transcription-polymerase chain reaction

PCR: polymerase chain reaction

SEM: standard error of the mean

LIF: leukemia inhibitory factor

CDF: cholinergic differentiation factor

IRS2: insulin receptor substrate 2

GRB14: growth factor receptor-bound protein 14

## **Declarations**

## Consent for publication

Not applicable.

## Availability of data and material

The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

This work was approved by the institutional review board of Taoyuan General Hospital, Ministry of Health and Welfare, Taoyuan, Taiwan (IRB Number: TYGH104043). The research was conducted in accordance with the Helsinki Declaration.

## Authors' contributions

Huei-Yu Lo and Chin-Kan Chan designed the research and wrote the article. Kuo-Ting Chang, Yu-Lung Chang, Chia-Jen Chang, and Chien-Hua Chiu performed the studies. Shun-Ping Cheng, Jing-Long Huang, Chien-Hsun Huang, Chin-Kan Chan, and Huei-Yu Lo provided the study materials and resources. Yui Whei Chen-Yang and Chin-Kan Chan participated as leaders of the study design.

## Competing Interests

The authors have no conflicts to disclose.

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Not applicable.

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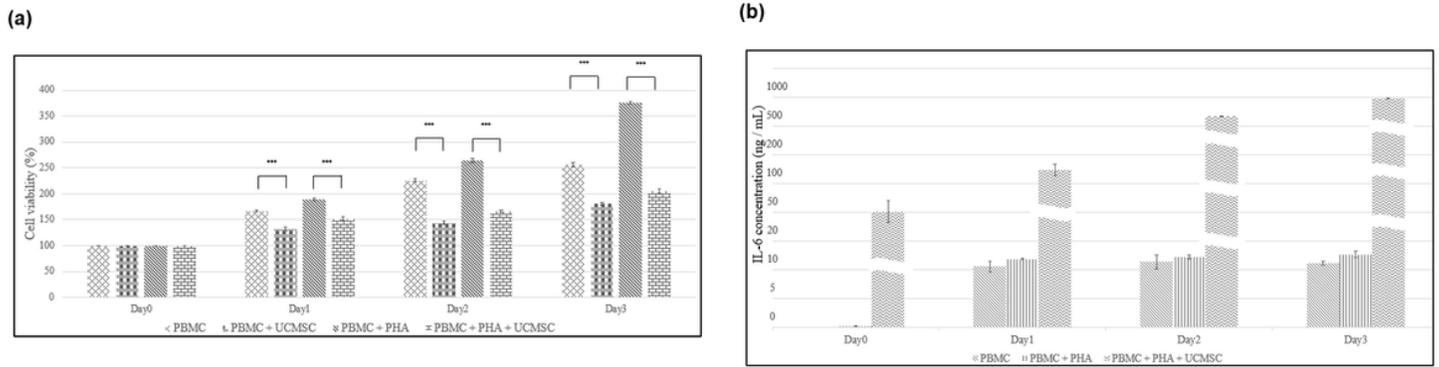
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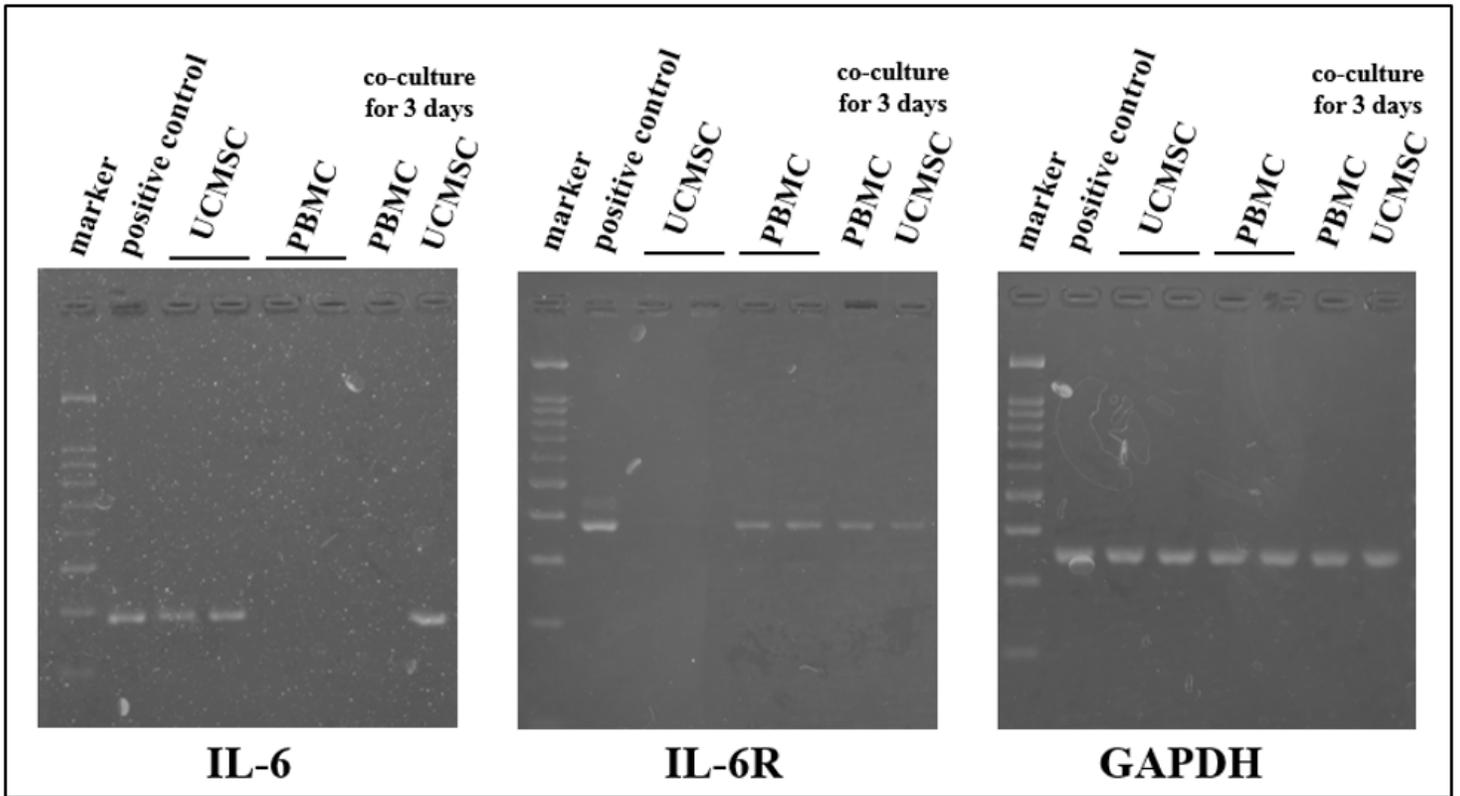
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## Figures



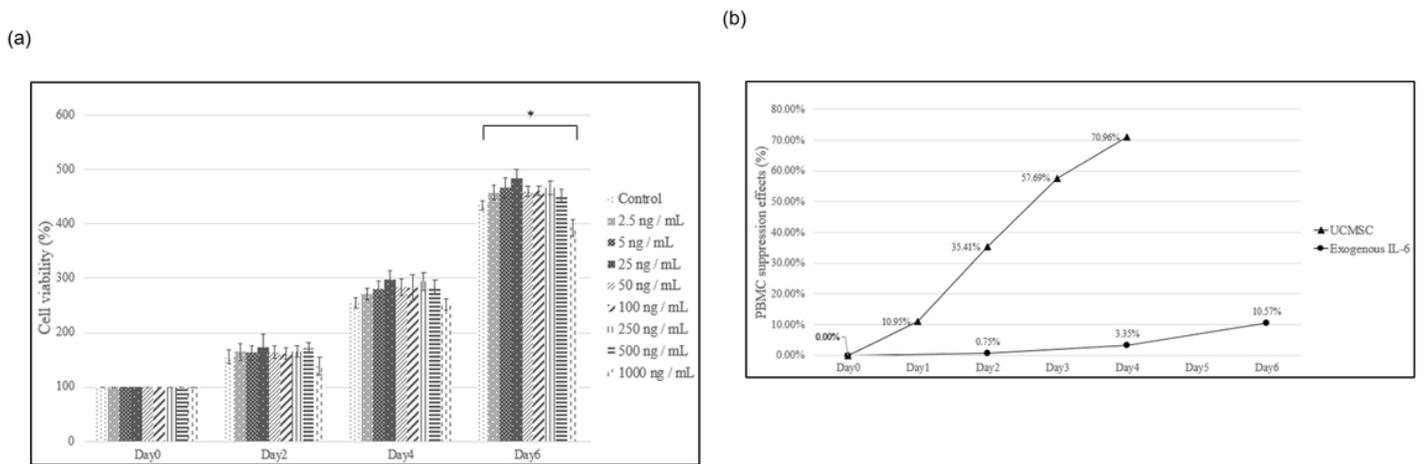
**Figure 1**

Cell viability and IL-6 levels of PBMCs suppression assay treated with/without PHA and co-cultured with/without hUCMSCs PBMCs were cultured alone or with hUCMSCs with and without PHA, and the effects on PBMC proliferation (a) and (b) were assessed. In Fig. 1a the increase of cell viability was 67.1%, 125.8%, 156.7% on day1, 2, 3 when PBMC cultured alone and 32%, 44.2%, 81.1% on day1, 2, 3 when PBMC cultured with hUCMSCs without PHA stimulation. Thus, co-culture with hUCMSCs inhibited PBMC proliferation by 21%, 36.1%, and 29.5% on day 1, 2, and 3 of co-culture, respectively ( $P < 0.001$ ). The increase of cell viability was 89.6%, 164.9%, 275.8% on day1, 2, 3 when PBMC cultured alone and 52.2%, 66.9%, 105.3% on day1, 2, 3 when PBMC cultured with hUCMSCs with PHA stimulation. Thus, co-culture with hUCMSCs inhibited PBMC proliferation by 19.7%, 37%, and 45.4% on day 1, 2, and 3 of co-culture, respectively ( $P < 0.001$ ). In Fig. 1b after 72 hours, the concentration of IL-6 increased from 0.008 ng/mL to 12.30 ng/mL, 0.02 ng/mL to 15.91 ng/mL, 50 ng/mL to 996.81 ng/mL in PBMC cultured without / with PHA, PHA-stimulated PBMC co-cultured with hUCMSCs, respectively.



**Figure 2**

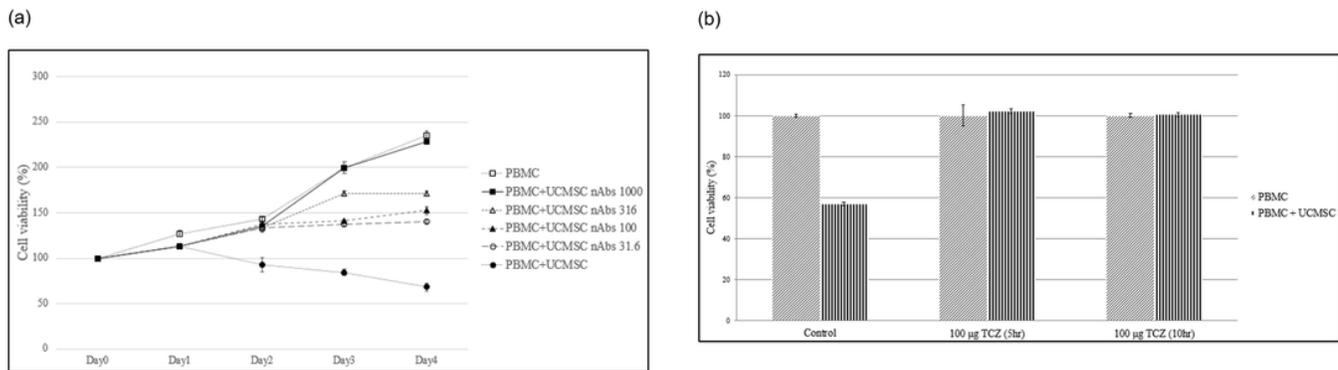
IL-6 and IL-6R expression in PBMC and hUCMSC co-culture RT-PCR assays showed that IL-6 was mainly expressed by hUCMSCs, while PBMCs only expressed a small amount of IL-6. IL-6R was highly expressed on PBMCs and expressed at low levels on hUCMSCs.



**Figure 3**

PBMC-suppressing effects of exogenous IL-6 and hUCMSCs. PBMC proliferation was suppressed by a high concentration of exogenous IL-6 and hUCMSCs, and the effects on (a) and (b) were assessed. In Fig. 3a PBMCs were exposed to different concentrations of exogenous IL-6 (2.5, 5, 25, 50, 100, 250, 500, and 1000 ng/ml), and then cells were counted on days 0, 1, 2, 4, and 6. In Fig. 3b a significant anti-

inflammatory effect on PBMCs (a 10.57% reduction in proliferation) was observed after exposure to high concentrations of exogenous IL-6 for 6 days.



**Figure 4**

Effect of IL-6 neutralizing antibodies and TCZ on PBMC suppression assay in co-culture with hUCMSCs. In Fig. 4a after exposing co-cultures of hUCMSCs and PBMCs to different concentrations (31.6, 100, 316, and 1000 ng/mL) of anti-IL-6 neutralizing antibody, the inhibitory effect of hUCMSCs on PBMCs disappeared on the next day. In Fig. 4b the results showed that the proliferation of PBMCs in the 5- and 10-hour TCZ pre-treatment groups was not inhibited, i.e., hUCMSCs could not inhibit the inflammatory response of TCZ-pretreated PBMCs.

## Supplementary Files

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