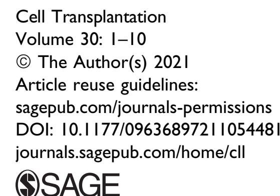

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High Induction of IL-6 Secretion From hUCMSCs Optimize the Potential of hUCMSCs and TCZ as Therapy for COVID-19-Related ARDS

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Abstract

Biological and cellular interleukin-6 (IL-6)-related therapies have been used to treat severe COVID-19 pneumonia with hyperinflammatory syndrome and acute respiratory failure, which prompted further exploration of the role of IL-6 in human umbilical cord mesenchymal stem cell (hUCMSC) therapy. Peripheral blood mononuclear cells (PBMCs) were responders cocultured with hUCMSCs or exogenous IL-6. A PBMC suppression assay was used to analyze the anti-inflammatory effects via MTT assay. The IL-6 concentration in the supernatant was measured using ELISA. The correlation between the anti-inflammatory effect of hUCMSCs and IL-6 levels and the relevant roles of IL-6 and 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 further evaluated. We reported that hUCMSCs did not initially exert any inhibitory effect on PHA-stimulated proliferation; however, a potent inhibitory effect on PHA-stimulated proliferation was observed, and the IL-6 concentration reached approximately 1000 ng/mL after 72 hours. Exogenous 1000 ng/mL IL-6 inhibited PHA-stimulated inflammation but less so than hUCMSCs. The inhibitory effects of hUCMSCs on PHA-stimulated PBMCs disappeared after adding an IL-6 neutralizing antibody or pretreatment with tocilizumab (TCZ), an IL-6R antagonist. hUCMSCs exert excellent anti-inflammatory effects by inducing higher IL-6 levels, which is different from TCZ. High concentration of IL-6 cytokine secretion plays an important role in the anti-inflammatory effect of hUCMSC therapy. Initial hUCMSC therapy, followed by TCZ, seems to optimize the therapeutic potential to treat COVID-19-related acute respiratory distress syndrome (ARDS).

Keywords

hUCMSC, COVID-19, tocilizumab, IL-6 neutralizing antibody, IL-6, IL-6 receptor

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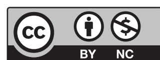

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Introduction

The COVID-19 global pandemic has caused substantial human catastrophes, including fatal mortality. The current treatments for COVID-19 include vaccines and antiviral and anti-inflammatory drugs[1]. COVID-19 can cause systemic inflammation, even leading to interleukin-6 (IL-6) cytokine storm, which can cause severe illness, including acute respiratory distress syndrome (ARDS)[1–3]. Because of the severe illness and high mortality rate of COVID-19, new treatments are still urgently needed. Some studies have reported that the IL-6 antagonist tocilizumab (TCZ) is clinically effective against COVID-19-related respiratory failure[4,5]. However, it has also been reported that there is no evidence of tocilizumab treatment efficacy for severe to critical SARS-CoV-2-infected patients[6,7]. In addition, some studies have reported that human umbilical cord mesenchymal stem cell (hUCMSC) infusions are safe and could be beneficial in treating patients with COVID-19 ARDS[8,9]. Therefore, there are still several ongoing clinical trials targeting TCZ and mesenchymal stem cells (MSCs) for COVID-19 infection[10,11]. Recently, one exciting article reported that TCZ combined with MSCs successfully treated severe COVID-19 infection[12]. However, studies investigating the dosage and order of administration between the two therapies is still lacking and warranted.

IL-6 is a cytokine with multiple biological activities[3,13–15]. The synthesis of IL-6 is regulated at both the transcriptional and posttranscriptional 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[16]. After 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[11,16,17]. 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[18,19]. These different IL-6R forms may be key to the different anti-inflammatory pathways involving IL-6[16].

Numerous previous studies[20,21] have demonstrated that MSCs can suppress inflammatory responses. Our previous study[14,22–24] revealed that hUCMSCs exhibit a significant inhibitory effect against the inflammatory responses of peripheral blood mononuclear cells (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[2,14]. 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 coculture 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

This study was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). Ethical approval to report this study was obtained from the Taoyuan General Hospital (MOHW) on human MSCs (IRB number: TYGH 104043). Written informed consent was obtained from the patient for publication of this invitro study.

Cell Culture and Cell Line Authentication

For the isolation of PBMCs, whole blood samples were collected from the patients, mixed thoroughly with an equal volume of Hank's balanced salt solution (HBSS) and added to an equal volume of Ficoll Hypaque. After centrifugation at 1731×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 μL of the cell suspension was mixed with 90 μL of 10× 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_2 and 21% O_2[14,22–24].

hUCMSCs were isolated from Wharton's jelly of the umbilical cord and identified 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^2 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 and incubated at 37°C, with 5% CO_2 and 21% O_2 (Thermo Scientific, Waltham, MA, USA). TrypLE Express enzyme (Gibco, Life Technologies Corporation, Waltham, MA, USA) was used to harvest cells during subculture[14,22–24].

For authentication, hUCMSCs 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)[22,23]. 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).

PBMC Suppression Assay

In the study of coculture of hUCMSCs and PBMCs, the cells were prepared and cultured at 37°C with 5% CO_2 before the experiment. The PBMCs were divided into phytohemagglutinin (PHA) and control groups. PHA was added to the former at a final concentration of 5 µg/mL. For coculture, the PBMCs were divided into two experimental groups, that is, the PBMC control and hUCMSC coculture groups. PBMCs grown in suspension were harvested and cocultured with stably growing, adherent hCMSCs in Transwell culture plates. Following coculture, PBMCs were isolated by centrifugation and subjected to cell counting to calculate and compare the number of cells[14,22–24].

In the study of coculture of exogenous IL-6 and PBMCs, 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.

For 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.

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[14,22–24].

Analysis of the Effects of IL-6-Neutralizing Antibody on PBMCs

The PHA-stimulated PBMCs were divided into five groups: the PBMC-control group and four groups of PBMC-UCMSC coculture 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, PHA-stimulated PBMCs were cocultured 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 determine the IL-6 concentration in the culture medium.

Determination of the Effects of Tocilizumab (TCZ) on PBMCs

The PHA-stimulated PBMCs were divided into four groups: two untreated control and two TCZ treatment One of the control groups was cocultured with hUCMSCs, while the other was not. The commonly used clinical immunosuppressive drug TCZ was selected as the pretreatment for the experimental groups. Treatment with 100 µg TCZ was performed for 5 hours and 10 hours. One of the TCZ-treated groups was cocultured with hUCMSCs while the other was not. The coculture of PBMCs and hUCMSCs was prepared and processed as described above, and after 72 hours of culture, the number of PBMCs was counted.

Experimental Design of IL-6 & IL-6R mRNA, Protein Expression After 72 hr PBMC and UMSC Coculture

$1.2*10^5$ UCMSCs were counted and seeded in a 6-well dish for overnight to make sure UCMSCs attached and grew well. In the next day, we then added equal amount of PBMCs ($1.2*10^5$) to the insert dish with 2 mL media for internal and external devices respectively. Three days later, the cocultured PBMCs and UCMSCs and the coculture supernatant were collected for use.

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[14,22–24].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction (PCR)

Total RNA (3 µl containing >1 µg) was subjected 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[25]. Primers (shown below) were designed according to the experimental requirements. The concentration of the 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′-AAGGACCTCCAGCATCA-CTGTGTCA-3′) and IL-6R_reverse (5′-CCTTCA-GAGCCCGCAGCTTCCACGT-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[26].

Protein Extraction

The proteins of the collected PBMC and UCMSC cells were extracted respectively. We also use Amicon® Ultra-4 Centrifugal Filter Units (UFC801024, Merck, NJ, USA) to concentrate the medium supernatant, and the ProteoSpin™ Abundant Serum Protein Depletion Kit (Catalog: 17300, Norgen Biotek Corp, Thorold, CA) was used to remove albumin for reducing interference. Finally, the protein expression of IL-6R in PBMC and UCMSC cells, and the soluble IL-6 level of the supernatant were analyses by using the Western blot.

Western Blot Analysis

Total protein (20 mg) was resolved by (IL-6R and β-actin: 8%; IL-6: 14%) SDS–polyacrylamide gel electrophoresis and subjected to western blot analysis using the most advanced chemiluminescent detection system MultiGel-21 (MGIS-21-C2-4 M, Topbio Bio Co., Taipei, Taiwan). Western blots were probed with a rabbit polyclonal antibody (ab128008; Abcam, Cambridge, UK) against IL-6R, a mouse monoclonal antibody (SC-28343; Santa Cruz Biotechnology, Dallas, USA) against IL-6, and a rabbit monoclonal antibody (MABT523; Merck Millipore, Burlington, USA) against β-actin, respectively. Anti-mouse IgG, HRP-linked antibody, and anti-rabbit IgG, HRP-linked antibody (7076 S; 7074 S; Cell signaling Technology, Inc., Boston, MA, USA) were our secondary antibodies. To ensure equal protein loading, membranes were stripped and subsequently probed with anti-GAPDH or anti-b-actin antibodies.

Statistical Analysis

All experiments were repeated three times, and the data are presented as the mean ± 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, that is, the number of PBMCs between the control and experimental groups, were compared using the Wilcoxon rank-sum test. Differences with P values <0.05 were considered statistically significant.

Results

Relationship Between hUCMSCs and IL-6 (IL-6 cDNA Expression in hUCMSCs)

Microarray analysis of 31,099 genes in MSCs revealed 260 upregulated genes (0.84%) and 699 downregulated genes (2.25%)[14].

Functional network analysis using MetaCore™ (Enrichment Analysis Workflow and analysis network, GeneGo, Inc. MI, USA) showed eight different immune pathways. It was observed, in the eight immune pathways, that IL-6 directly or indirectly plays key roles in facilitating immune responses, including proinflammatory pathways mediated by histamine signaling and anti-inflammatory pathways mediated by the Jak-STAT pathway, IL-4 signaling, and TREM1 signaling[14].

Our previous study showed that hUCMSCs have a significant inhibitory effect on the inflammatory response of PBMCs[14]. IL-6 may activate the mRNA expression of genes in Mitogen-Activated Protein Kinases (MAPK), 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 (Fig. 1 and Table 1).

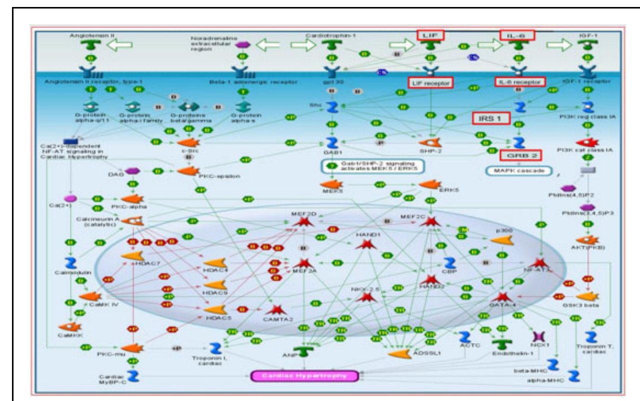

Figure 1. The possible downstream pathway by which IL-6 activates PBMC suppression assay in coculture with hUCMSCs. Microarray analysis and functional network analysis showed that IL-6 may activate the mRNA expression of genes in the MAPK pathway, including leukemia inhibitory factor (LIF), IL-6, insulin 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.

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 Protein Expression in the Coculture Medium of PBMCs and hUCMSCs

To understand cell proliferation and IL-6 secretion in PHA-stimulated PBMCs, after 72 hours, there were significantly higher numbers of PHA-stimulated PBMCs (375.78% ± 2.78%) than nonstimulated PBMCs (256.67 ± 3.71%) (Fig. 2A, $P < 0.001$). There was no significant difference in the IL-6 concentration between PHA-stimulated PBMCs (15.91 ± 0.67 ng/mL) and nonstimulated PBMCs (12.30 ± 0.38 ng/mL) (Fig. 2B).

We also observed the cell viability and IL-6 levels in PBMC and hUCMSC cocultures at 72 hours. After cocultured with hUCMSCs for 72 hours, the cell counts of PHA-stimulated PBMCs (205.33% ± 3.53%) were 45.5% lower than those in the control group (375.78 ± 2.78%) (Fig. 2A, $P < 0.001$).

The initial concentration of IL-6 on day 0 was 50.70 ng/mL, which increased to 145.06 ng/mL after 24 hours, 716.22 ng/mL after 48 hours and 996.81 ng/mL (approximately 1000 ng/mL) after 72 hours (Fig. 2B). Our study showed that the concentration of IL-6 in cocultures of PBMCs and hUCMSCs was significantly higher than that in the PBMC control group, and it increased by more than 20-fold after 72 hours of coculture.

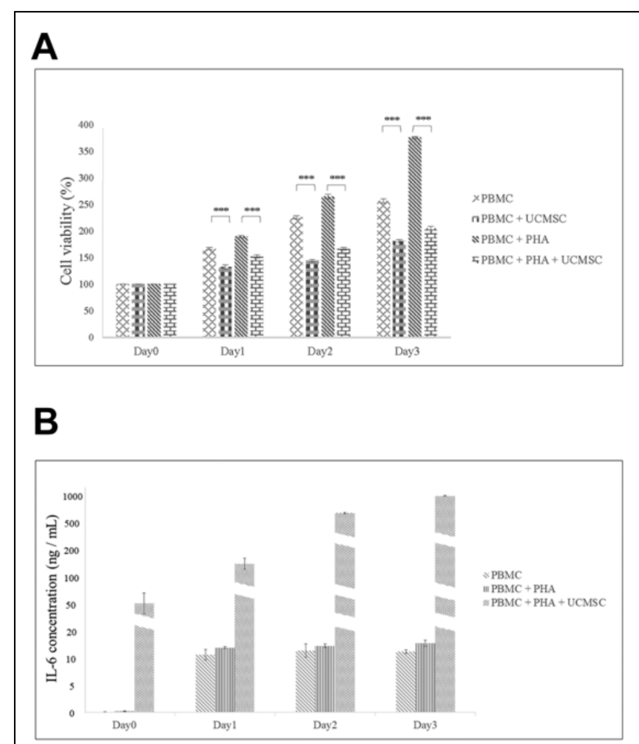

Figure 2. Cell viability and IL-6 levels of PBMC suppression assays treated with/without PHA and cocultured with/without hUCMSCs. PBMCs were cultured alone or with hUCMSCs with/without PHA, and the effects on PBMC proliferation were assessed. (A) The increase in cell viability was 67.1%, 125.8%, and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

The mRNA and Western Blot Protein Expression of IL-6 and IL-6R in Cocultures 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 coculture of hUCMSCs and PBMCs (Fig. 3A). The mRNA level of IL-6 and IL-6R were relative quantified to GAPDH in Fig. 3B ($P < 0.01$).

The protein levels of IL-6 and IL-6R have been analyzed by western blot and IL-6R level was quantified. IL-6

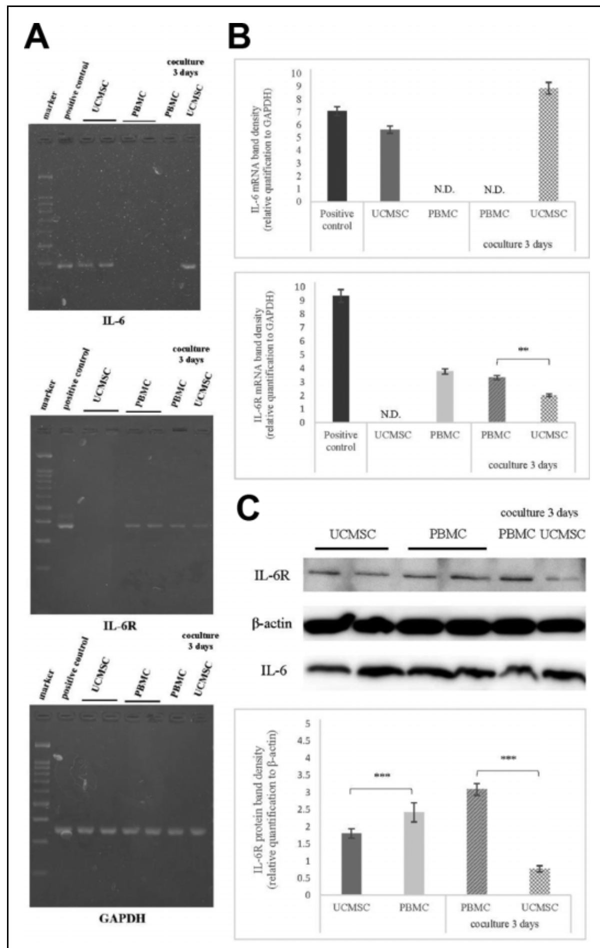

Figure 3. The mRNA and western blot protein expression of IL-6 and IL-6R in cocultures of hUCMSCs and PBMCs. (A, B) IL-6 mRNA was mainly expressed by hUCMSCs, while IL-6R mRNA was highly expressed on PBMCs. the mRNA level of IL-6 and IL-6R were relative quantified to GAPDH (B) ($P < 0.01$). (C) The protein levels of IL-6 and IL-6R have been analyzed by western blot and IL-6R was quantified ($P < 0.001$).

protein was primarily expressed by hUCMSCs, and IL-6R protein was primarily expressed on PBMCs ($P < 0.001$) (Fig. 3C).

Inhibitory Effects of hUCMSCs on PHA-Stimulated PBMCs

Without PHA stimulation, in the control group, compared to day 0, the increase in cell viability was 67.1%, 125.8%, and 156.7% on days 1, 2, and 3, respectively. In the coculture group, compared to day 0, the increase in cell viability was 32%, 44.2%, 81.1% on days 1, 2, and 3, respectively. Thus, coculture with hUCMSCs inhibited PBMC proliferation by 21%, 36.1%, and 29.5% on days 1, 2, and 3 of coculture, respectively ($P < 0.001$). With PHA stimulation of PBMCs, in the control group, compared to day 0, the increase in cell viability was 89.6%, 164.9%, 275.8% on days 1, 2, and 3, respectively. In the coculture group, compared to day 0, the increase in cell viability was 52.2%, 66.9%, 105.3% on days 1, 2, and 3, respectively. Thus, coculture with hUCMSCs inhibited PBMC proliferation by 19.7%, 37.0%, and 45.4% on days 1, 2, and 3 of coculture, respectively ($P < 0.001$)(Fig. 2A).

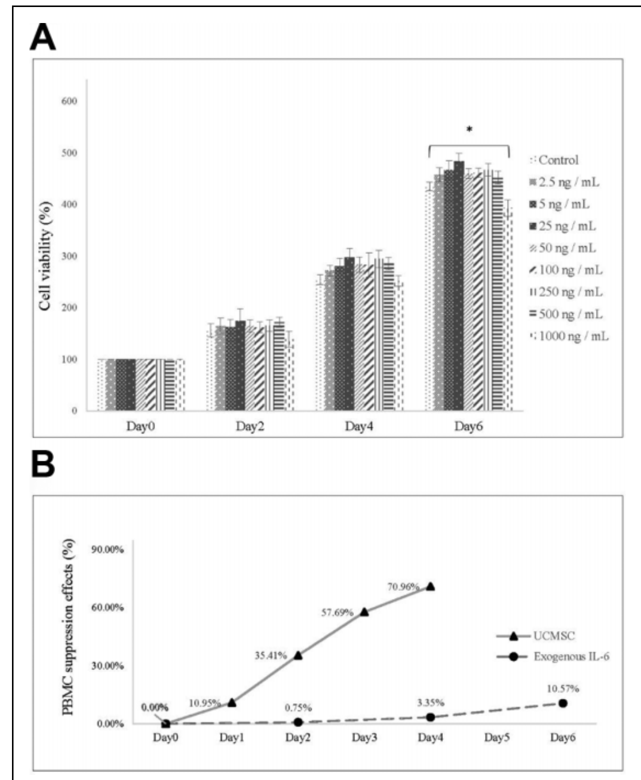

Figure 4. PBMC suppression effects of exogenous IL-6 and hUCMSCs. PBMC proliferation was suppressed by a high concentration of exogenous IL-6 and hUCMSCs, and the effects were assessed. (A) The PBMCs were exposed to different concentrations of exogenous IL-6 (2.5, 5, 25, 50, 100, 250, 500, and 1000 ng/ml), and then cells were counted on days 0, 1, 2, 4, and 6. (B) It showed 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.

Inhibitory Effects of Exogenous Synthetic IL-6 on PHA-Stimulated 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 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 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%) (Fig. 4A, $P < 0.05$), suggesting a potent inhibitory effect on the proliferation of PBMCs (Fig. 4A).

A comparison of the inhibitory effect of exogenous synthetic IL-6 and hUCMSCs on the inflammatory response of PBMCs is shown in Fig. 4B. 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 cocultured with hUCMSCs after 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).

Inhibitory Effects of hUCMSCs on PHA-Stimulated PBMCs After Adding IL-6 Neutralizing Antibody and IL-6R Antagonist

The growth of PBMCs in coculture 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 neutralizing antibody for 48, 72, and 96 hours, the cell counts of PHA-stimulated PBMCs (135.56% ± 7.58%, 199.11% ± 3.36%, and 228.89% ± 4.29%, respectively) did not differ significantly from the counts of the control group (143.11% ± 2.04%, 199.78% ± 6.05%, and 235.11% ± 4.68%, respectively) (Fig. 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 exposure to the highest concentration (1000 ng/ml) of IL-6 neutralizing antibody (Fig. 5A).

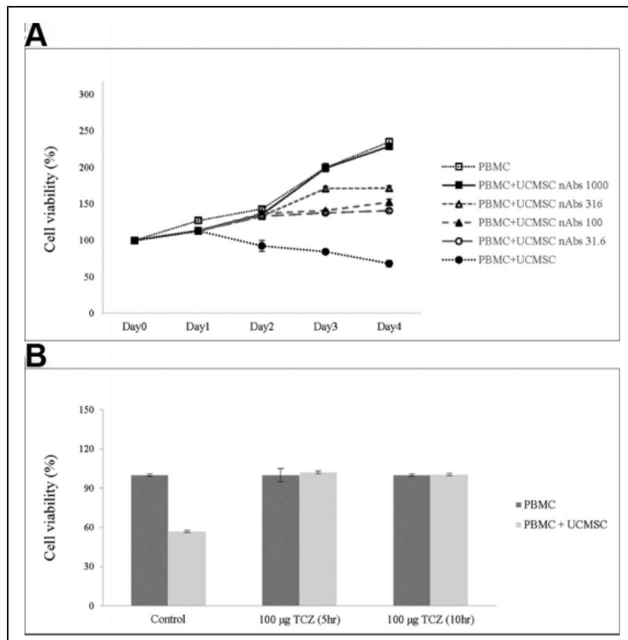

Figure 5. Effect of IL-6 neutralizing antibodies and TCZ on PBMC suppression assay in coculture with hUCMSCs. (A) After exposing coculture of hUCMSCs and PBMCs to different concentrations (31.6, 100, 316, and 1000 ng/mL) of anti-IL-6 neutralizing antibody, the inhibitory effect of hUCMSCs on PBMCs disappeared the next day. (B) The proliferation of PBMCs in the 5- and 10-hour TCZ pretreatment groups was not inhibited, i.e., hUCMSCs could not inhibit the inflammatory response of TCZ-pretreated PBMCs.

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 cocultured with hUCMSCs, and the other group was not cocultured with hUCMSCs. After 72 hours of culture, the number of PBMCs was counted. The results showed that PBMCs were inhibited by hUCMSCs and slowed the proliferation of cells. Clinically, TCZ is used as an immunosuppressive drug. Tocilizumab (TCZ) (100 μg) was added for pretreatment for 5 hours and 10 hours. After 72 hours of culture, the number of PBMCs was counted. Compared with the control group, the cell proliferation was 98.79% and 99.34%; adding hUCMSCs compared with the control group, the cell proliferation of the group was 96.37% and 100%, respectively. The results showed that the inhibitory effect of hUCMSCs on PBMC proliferation disappeared after TCZ pretreatment (Fig. 5B).

Discussion

There are some novel findings in our studies. First, we found that coculture of UMSCs and PBMCs induced the production of different concentrations of IL-6, which had 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[14]. However, at the highest concentration (≥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 applies either healthy stem cells to enhance immunity against inflammation or to repair/replace damaged cells and tissues to control disease[27]. Autoimmune disorder or severe COVID-19 pneumonia with hyperinflammatory syndrome and acute respiratory failure refer to 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. 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 of PBMCs (Fig. 1 and Table 1)[3,14,28].

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 approximately 1.5-fold after 72 hours (Fig. 2B).

In contrast, after cocultured with hUCMSCs for 72 hours, the concentration of IL-6 increased significantly, by approximately 20-fold (Fig. 2B). In addition, we also found that the proliferation of PBMCs decelerated with a statistically significant difference after cocultured with hUCMSCs for 72 hours (Fig. 2A). 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[23]. 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. 3).

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 an IL-6 neutralizing monoclonal antibody, did not inhibit the proliferation of PBMCs compared to that of the control (Fig. 5A). Thus, we inferred that the proliferation of PBMCs was not inhibited when most of the IL-6 protein secreted by the hUCMSCs was neutralized. In addition, the proliferation of PBMCs in the TCZ pretreatment groups was not inhibited compared to that in the TCZ-free group. We inferred that TCZ blocks the binding of IL-6 to mIL-6R (Fig. 5A). We therefore concluded that high levels of IL-6 and IL-6 receptors are essential for the inhibitory effect of hUCMSCs on PBMC proliferation[16].

PBMC-UCMSC coculture 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 coculture, hUCMSCs produced a high concentration of IL-6 ($\sim$1000 ng/mL), which induced a significant anti-inflammatory response (Figs. 2B and 4B). 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. 4A, B). This showed that hUCMSCs have the potential for potent therapeutic effects against COVID-19 infection, 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 to accelerate the anti-inflammatory effect, which requires further investigation. hUCMSCs achieve therapeutic efficacy faster than exogenous IL-6, which takes twice as long as the former or even longer (Fig. 4B).

Previous study[14] showed that a proinflammatory 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 mIL-6R. Our results revealed that IL-6 did not exert anti-inflammatory effects at low concentrations but began to exert significant anti-inflammatory activity at high concentrations of approximately 1000 ng/mL. Therefore, we conclude that IL-6 binds to different forms of IL-6R at different concentrations, thus activating pro- or anti-inflammatory pathways, with a threshold concentration of 1000 ng/mL.

Tanaka et al.[2] reported that IL-6 can cause severe inflammation. Most IL-6-targeted therapies are focused on the inhibition of inflammatory responses by nonselectively blocking the binding of IL-6 to the IL-6 receptor. However, this therapeutic approach has not always 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 the IL-6 receptor may result in a loss of one proinflammatory 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 MSC therapy. Hence, the infusion order of combined hUCMSU cell therapy with TCZ for the treatment of COVID-19 requires further investigation.

Conclusions

The proposed anti-inflammatory cell therapy using hUCMSCs is based on the high production of IL-6. hUCMSCs exert excellent anti-inflammatory effects that are different from those of IL-6 antagonists such as TCZ. We believe that the combination of IL-6 secreted by hUCMSCs and the subsequent macrophage IL-6R antagonist can stimulate the anti-inflammatory process faster and more efficiently.

Abbreviations

ARDS, acute respiratory distress syndrome; 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; 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; MAPK, Mitogen-Activated

Protein Kinases; LIF, leukemia inhibitory factor; CDF, cholinergic differentiation factor; IRS2, insulin receptor substrate 2; GRB14, growth factor receptor-bound protein 14

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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.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Ethical approval to report this study was obtained from the Ethics Committee in Human Research of Taoyuan General Hospital, Ministry of Health and Welfare, Taoyuan, Taiwan (IRB number: TYGH 104043).

Statement of Human and Animal Rights

The research was conducted in accordance with the Helsinki Declaration. All procedures in this study were conducted in accordance with the Taoyuan General Hospital, Ministry of Health and Welfare on human UCMSCs.

Statement of Informed Consent

Written informed consent was obtained from legally authorized representatives for anonymized patient information to be published in this article.

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