Aging, rather than Parkinson's disease, affects the responsiveness of PBMCs to the immunosuppression of bone marrow mesenchymal stem cells

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Abstract. Whether aging or Parkinson's disease (PD) affects the responses of peripheral blood mononuclear cells (PBMCs) to immunosuppression by bone marrow-derived mesenchymal stem cell (BM-MSCs) and which cytokines are more effective in inducing BM-MSCs to be immunosuppressive remains to be elucidated. PBMCs were isolated from healthy young (age 26-35), healthy middle-aged (age 56-60) and middle-aged PD-affected individuals. All the recruits were male. The mitogen-stimulated PBMCs and proinflammatory cytokine-pretreated BM-MSCs were co-cultured. The PBMC proliferation was measured using Cell Counting Kit-8, while the cytokine secretion was assayed by cytometric bead array technology. The immunosuppressive ability of BM-MSCs was confirmed in young healthy, middle-aged healthy and middle-aged PD-affected individuals. Among the three groups, the PBMC proliferation and cytokine secretion of the young healthy group were suppressed more significantly compared with those of the middle-aged healthy and middle-aged PD-affected group. No significant differences were identified in the PBMC proliferation and cytokine secretion between the patients with PD and the middle-aged healthy subjects. Interferon (IFN)-γ synergized with tumor necrosis factor (TNF)-α, interleukin (IL)-1α or IL-1β was more effective than either one alone, and the combinations of IFN-γ + IL-1α and IFN-γ + IL-1β were more effective than IFN-γ + TNF-α in inducing BM-MSCs to inhibit PBMC proliferation. The results of the present study suggested that aging, rather than PD, affects the response of PBMCs toward the suppression of BM-MSC, at least in middle-aged males. Patients with PD aged 56-60 remain eligible for anti-inflammatory BM-MSC-based therapy. Treatment of BM-MSCs with IFN-γ + IL-1α or IFN-γ + IL-1β prior to transplantation may result in improved immunosuppressive effects.

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Abbreviations: BM-MSC, bone marrow mesenchymal stem cell; CBA, cytometric bead array; HSCT, hematopoietic stem cell transplantation; MHC, major histocompatibility antigen complexes; NSAID, non-steroidal anti-inflammatory drug; PBMC, peripheral blood mononuclear cell; PD, Parkinson's disease

Key words: bone marrow, Parkinson's disease, mesenchymal stem cells, inflammation, transplantation

Introduction

Although inflammation has not yet been proven to be a direct cause of Parkinson's disease (PD), studies have identified its participation in PD progression and it is an important factor in the progressive deterioration of PD (1,2). The main supporting evidence includes microglia and T lymphocyte proliferation and activation in the substantia nigra-striatum system in patients with PD and PD animal models (3). In addition, epidemiological surveys have also demonstrated that the PD incidence in individuals that have used non-steroidal anti-inflammatory drugs (NSAIDs) for a period of time is significantly lower than that of the general population (1,2).

The activation of microglia is the main component of intracerebral inflammation. Microglia produce a variety of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α), IL-6, interferon-γ (IFN-γ) and nitric oxide. Additionally, microglia generate cytokines that inhibit inflammation, including IL-10 and IL-4. If microglial cells maintain an activated status for a long period and release inflammatory mediators continuously, they may damage the surrounding neurons (4-7).

It is widely accepted that the infiltration of lymphocytes into the brain is one of the key processes in cerebral inflammation. More cluster of differentiation (CD)8$^+$ and CD4$^+$ T cells were identified in the brain of patients with PD compared with healthy individuals. These CD8$^+$ and CD4$^+$ T cells were in close contact with blood vessels and near the dopaminergic neurons, suggesting that they migrated from the blood vessels and interacted with the dopaminergic neurons (8). This phenomenon was not identified in the red nucleus, which is not an area injured in PD (8).

In addition, substantial evidence has demonstrated that systemic inflammatory processes are closely associated with intracerebral inflammation and exacerbate neurodegeneration in PD animal models and patients (9). According to a recent study, under physiological conditions, the cerebral spinal fluid is drained from the central nervous system (CNS) into the lymphatic ducts in the meninges, which connect to the deep cervical venous system (10). Changes in blood-brain barrier function usually occur in the brain of patients with PD (11). Under abnormal inflammatory conditions, peripheral inflammation is involved in CNS damage via the following mechanisms: i) T and B lymphocytes, and dendritic cells in the venous system respond to the environmental variations and migrate from the central lymph ducts to attack the central neurons (12); ii) peripheral proinflammatory cytokines infiltrate into the brain and transform microglia from the 'resting' state into the 'active' state (7,9); and iii) macrophages pathologically diffuse from the blood to the CNS, transform into microglial cells and contribute to the development of intracranial degeneration (13).

As dopaminergic neurons are more vulnerable than other neurons or glial cells, one of the common consequences of chronic inflammation in the brain is the degeneration of neurons in the substantia nigra and, finally, PD. Therefore, in addition to treatment with levodopa, manipulating chronic inflammation may be a novel therapeutic direction for PD (14-16).

The role of mesenchymal stem cells (MSCs) in immunoregulation was initially identified during hematopoietic stem cell transplantation (HSCT). Intravenous transplantation of MSCs reduces the incidence and attenuates the severity of graft-versus-host disease significantly following HSCT (17,18). In recent years, it has been determined that MSCs can inhibit T cell proliferation, inhibit T cell-produced immune cytokines, inhibit B cell-synthesized antibodies and suppress natural killer cell function (19-22). In addition, MSCs express major histocompatibility complex class I (MHC I) molecules but do not express MHC II molecules; therefore, MSCs exhibit limited immunogenicity and can be applied in allogeneic transplantation without causing severe immunorejection. These properties potentially enable MSCs to participate in the clinical treatment of immune-associated diseases. Bone marrow-derived MSCs (BM-MSCs) can avoid the majority of ethical issues associated with stem cell transplantation. It has been demonstrated that BM-MSCs inhibit T lymphocyte proliferation and the production of proinflammatory cytokines (23).

These immunoregulatory results of MSCs have been predominantly derived from normal young animal models or healthy human subjects (23). However, the peripheral blood mononuclear cells (PBMCs) of patients with PD are not exactly the same as those of healthy people (24-26). For example, the studies of PD models and post-mortem PD human brains have suggested that at least two cellular functions are damaged; proteostasis and mitochondrial respiration (27). It is uncertain whether the lymphocytes or PBMCs of older people, particularly patients with PD, still exhibit the ability to respond to BM-MSC immunosuppression. The present study attempted to answer this question. Mouse and human MSCs require stimulation by proinflammatory cytokines to obtain immunosuppressive ability (28). IFN-γ is important in activating MSCs; however, the role of other cytokines remains to be elucidated (23,29). The current study aimed to determine which cytokines or cytokine combinations were more effective than others in stimulating the immunosuppressive potential of BM-MSCs.

Materials and methods

BM-MSC culture and identification. Spontaneously aborted male fetuses at 16, 17 and 25 weeks of pregnancy were provided by the Obstetrics and Gynecology Department of Xuanwu Hospital (Beijing, China) during the year 2012 with informed consent of the patients in addition to the approval of the Xuanwu Hospital Ethics Committee. The bone marrow was flushed out and plated in T75 culture bottles at a density of 10^6 cells/ml in medium consisting of α-Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 20 ng/ml epidermal growth factor (R&D Systems, Inc., Minneapolis, MN, USA), 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin and 2 mmol/l L-glutamine (all from Thermo Fisher Scientific, Inc.). The cells were incubated at 37˚C in 5% CO_2 for 4 days, then the floating cells were discarded. When the adherent cells grew to 80% confluence, they were digested using 0.25% trypsin-EDTA and were passaged at a ratio of 1:3.

BM-MSCs of passage 3-5 were identified by surface antigens using FACSCalibur flow cytometry (BD Biosciences, San Jose, CA, USA), and by differentiation into adipose, bone and cartilage using a human MSC differentiation kit (Lonza Group, Ltd., Basel, Switzerland). The differentiation of human MSC into adipose, bone and cartilage, and the corresponding Oil Red O staining, von Kossa staining and Safranin O staining, are all in accordance with the protocols of the human MSC differentiation kit.

For flow cytometry analysis, the Human MSC Analysis kit was used (BD Biosciences). In this kit, the MSC-positive cocktail includes: Fluorescein isothiocyanate (FITC)-CD90, phycoerythrin (PE)-CD44, peridinin chlorophyll protein complex (PerCP)-CD105 and allophycocyanin (APC)-CD73. The PE channel is used in combination with the negative MSC panel [PE-CD45, PE-CD34, PE-CD11b, PE-CD19 and PE-major histocompatibility complex, class II, DR (HLA-DR)]. The isotype controls for positive panel were 'mIgG1, κ FITC (clone X40)', 'mIgG1, κ APC (clone X40)', 'mIgG1, κ PerCP-Cy5.5' and 'mIgG2a, κ PE'. The isotype control for negative panel was 'PE Mouse IgG2b, κ Isotype'. Flow cytometric analysis was performed on 10,000 events, and data were analyzed with CellQuest (version 3.2.1; BD Biosciences) and FlowJo software (version 7.6.5; FlowJo LLC, Ashland, OR, USA).

Recruitment of healthy individuals and patients with PD. For recruitment, healthy people with no abnormalities in their blood and major organs were identified by the Medical Examination Department of Xuanwu Hospital and recruited. The recruitment period was between January and December 2012. Age 26-30 was regarded as young, and age 56-60 was considered middle-aged. Study subjects were divided into middle-aged PD, middle-aged healthy and young healthy. Each group contained 15, all of them male. Participant consent and ethical approval for the collection and use of the blood samples were obtained from the Ethics Committee of Xuanwu Hospital.

Patients with PD were diagnosed with primary PD in accordance with the UK Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria (30). In brief the inclusion/exclusion criteria were: Hoehn-Yahr score of the 'open period' was 2.5-4; diagnosed with PD for ≥3 years (30); treated with levodopa plus peripheral decarboxylase enzyme inhibitor or other anti-PD drug treatment and have reached a stable dose for ≥30 days; had responses to the drugs, which meant that the Unified Parkinson's Disease Rating Scale motor scoring of the 'open period' increased ≥33% compared with the 'closed period'. Daily total time of the 'closed period' was 2-6 h (morning stiffness was not included); magnetic resonance imaging examination revealed no significant brain lesions including atrophy (30); Hamilton Rating Scale for Depression score of patients was ≤12; without significant cognitive impairment (31), Mini-Mental State Exam scores of patients were >24 (for those whose education level was middle school or higher) or >22 (primary school or higher) (30). The clinical and hematological parameters of the three study groups are presented in Table I.

Culture of PBMCs. To isolate PBMCs, 700 μl hydroxyethyl starch (B. Braun Medical, Inc., Bethlehem, PA, USA) was added to 2 ml peripheral blood and allowed to incubate for 30 min with slight agitation. Subsequently, the transparent supernatant (~1.5 ml) was transferred to a new 15 ml centrifuge tube and mixed with 4.5 ml of erythrocyte lysis buffer (eBioscience; Thermo Fisher Scientific, Inc.). The mixture was centrifuged at 300 x g for 5 min. Following removal of the supernatant, the cells were washed twice in 5 ml PBS (Thermo Fisher Scientific, Inc.). The cells were counted and resuspended in complete medium [RPMI-1640 (Thermo Fisher Scientific, Inc.) + 10% FBS (Thermo Fisher Scientific, Inc.) + 200 pg/ml IL-2]. To induce PBMC proliferation *in vitro*, the cells ($1x10^6$/ml) were activated by 10 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h (19). The PHA was then removed and the cells were cultured with complete medium again.

Determination of cytokines effective in inducing immunosuppression in BM-MSCs. Cytokines that are effective for the activation of MSCs was determined by adding the recombinant proteins IFN-γ, TNF-α, IL-1α, and IL-1β, one by one or in combination, to the BM-MSC culture medium at a concentration of 20 ng/ml.

The cytokines were removed, and the BM-MSCs at $5x10^4$/cm^2 were incubated in 96-well plates with PBMCs at a density of 10^4 cells/well. The PBMCs were isolated from the healthy young, healthy middle-aged and the patients with PD groups. These PBMCs were incubated in complete medium (RPMI-1640+10% FBS+200 pg/ml IL-2) for 24-48 h and were pretreated with PHA for 24 h.

Prior to co-culture, PHA was washed off. The cytokine-treated BM-MSCs and PHA-treated PBMCs were co-cultured in the 96-well plate for 4 days in the PBMC medium (RPMI-1640+10% FBS+200 pg/ml IL-2).

Responsiveness of PBMCs to BM-MSC immunosuppression. The experimental protocol is schematically represented in Fig. 1A. The responsiveness of PBMCs toward the inhibitory effects of BM-MSC was compared among the three groups. The PBMCs from the three groups were isolated and cultured for 24-48 h in a 37°C incubator with 5% CO_2 and saturated humidity. In the experiments in which PHA-treated PBMCs were used, BM-MSCs were plated into 96-well culture plates at the density of $1x10^4$ cells per well. Subsequently, $2x10^5$ PBMCs were added to the BM-MSC culture. The co-cultures of BM-MSCs and PBMCs with or without PHA were incubated for 4 days. The relative proliferation rate, which was used as the basis for comparing the extent of suppression among the three groups, was the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) absorbance ratios of the co-cultures with or without PHA. The proliferation was assayed by the CCK-8 method according to the manufacturer's protocol.

In the experiments in which IFN-γ, TNF-α, IL-1α, and IL-1β were used to activate MSCs, the BM-MSCs were treated with these cytokines one by one or in combinations for 24 h. The cytokines were then washed off. The MSCs were digested and plated into 96-well culture plates at a density of $1x10^4$ cells per well. The PBMCs were co-cultured with PHA for 24 h to be induced to proliferate. Then, PHA was removed. The PHA-treated PBMCs were seeded at a density of $2x10^5$cells/well into the 96-well plate containing 10^4 BM-MSCs and incubated for 4 days.

To compare the immunosuppression among the three groups, the relative proliferation rate was determined as the CCK-8 absorbance ratios of the co-culture with cytokine-treated BM-MSCs and with untreated BM-MSCs.

Assessment of the BM-MSC suppression of PBMC proliferation. The proliferation was assayed by the CCK-8 method (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. To elucidate which type of cells in this co-culture, BM-MSCs or PBMCs, decreased their proliferative activity, the proliferation of BM-MSCs was inhibited by mitomycin C (10 μg/ml) treatment for 2 h. Following the treatment, the mitomycin C was removed. The MSCs were cultured for another 24 h and then were co-cultured with PBMCs. The effects of mitomycin C on BM-MSC proliferation were confirmed by Ki67 staining. In brief, $2x10^4$ treated BM-MSCs were fixed by 4% paraformaldehyde prior to the co-culture with PBMCs. The fixed cells were incubated in 0.3% Triton X-100 PBS for 1 h at room temperature, blocked with 2% donkey serum (Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA) at room temperature for 45 min, and incubated overnight with monoclonal antibodies for Ki67 (cat. no. sc-23900, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C. The next day, the cells were incubated with FITC-conjugated

Table I. Clinical and hematological parameters of the PD and the healthy groups.

Parameter	Middle-aged PD	Middle-aged healthy	Young healthy
Number	15	15	15
Sex	Male	Male	Male
Age (mean ± SD)	57.67±1.40	58.13±1.41	27.8±1.42
Age at symptoms onset (mean ± SD)	52.87±1.41	NA	NA
Symptom duration (mean ± SD)	4.80±1.33	NA	NA
Unilateral symptoms (number)	9	NA	NA
Bilateral symptoms (number)	7	NA	NA
Tremor (number)	3	NA	NA
Bradykinesia/rigidity (number)	4	NA	NA
Hoehn and Yahr (mean ± SD)	2.83±0.55	NA	NA
UPDRS motor score (mean ± SD)	15.31±0.59	NA	NA
MMSE score (mean ± SD)	25.07±1.84	NA	NA
White blood cells (10^9/l)	5.63±0.43	5.39±0.58	5.45±0.62
Red blood cells (10^9/l)	4.76±0.34	4.63±0.39	4.58±0.29
Neutrophils (%)	60.05±3.78	59.88±5.37	57.52±5.76
Lymphocytes (%)	32.03±4.78	30.97±4.71	28.61±4.78
Monocytes (%)	8.82±1.57	7.78±1.22	8.13±1.46
Hemoglobin (g/l)	143.25±2.56	140.35±1.47	142.38±2.49

Hoehn and Yahr, UPDRS motor score and MMSE score are measured in the open period. PD, Parkinson's disease; NA, not available; SD, standard deviation; UPDRS, Unified Parkinson's Disease Rating Scale; MMSE, Mini-Mental State Exam.

secondary antibodies (cat. no. 715-095-151,1:300; Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA) for 2 h at room temperature, followed by DAPI nucleus staining at 1 μg/ml at room temperature for 20 min, and observed under an inverted fluorescence microscope (magnification, x200; Leica DMI 4000B; Leica Microsystems GmbH, Wetzlar, Germany).

Detection of cytokines and chemokines in the co-culture. The suppression of PBMCs by BM-MSCs includes the suppression of cytokine production. A multiplex human cytokine assay was performed, which included IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α. The BM-MSCs were seeded at $1x10^4$ density per well in a 96-well culture plate. The concentration of the cytokines in the BM-MCS culture medium was used as a control. Since the BM-MSCs and PBMCs secrete certain cytokines physiologically, the BM-MSCs cultured in the PBMC medium with PHA and the PBMCs cultured in the presence or absence of PHA, were measured for the secretion of cytokines. For the co-cultures, $2x10^5$ PBMCs were cultured together with BM-MSCs for 96 h in the presence of PHA. The co-culture supernatant of the PBMCs was assayed for six different cytokines and chemokines with a Th1/Th2/Th17 bead array kit (cat. no. BD 560484, BD Biosciences, San Jose, CA, USA) using cytometric bead array (CBA) technology.

Statistical analysis. The data were analyzed with SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA). Quantitative data are expressed as the means ± standard deviation. Data were analyzed by one-way analysis of variance and least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Culture and identification of human BM-MSCs. The primary human BM-MSCs were cultured for 3-5 days and the cells were spindle-shaped and polygonal. Following the removal of non-adherent cells, the adherent cells further grew into a relatively uniform morphology of spindles. At days 10-14, the cultured cells reached 80% confluence and were digested by 0.25% trypsin-EDTA and passaged at a ratio of 1:3. Then, the cells were passaged every 3-5 days (Fig. 1B). To identify the BM-MSCs, differentiation and cytometry tests were applied. The BM-MSCs possessed the ability to differentiate into adipocytes, osteocytes and chondrocytes (Fig. 1C-E). The cytometry examination identified that the positive percentages of BM-MSCs at passages 3-5 for CD105, CD44, CD90 and CD73 were 99.98±0.02, 99.79±0.26, 99.94±0.06 and 99.99±0.01%, respectively, and negative for CD34, CD19, CD45 or CD11b. Furthermore, BM-MSCs did not express the MHC II molecule HLA-DR (Fig. 1F-K). The quantified flow cytometry results are summarized in Fig. 1L.

BM-MSCs suppress mitogen-induced PBMC proliferation. Initially, the co-culture system was used to examine whether PBMCs were required to be induced by mitogen to obtain the ability to stimulate BM-MSCs to exert their inhibitory effects. The PBMCs from the healthy young people were incubated in a 96-well plate at a density of $2x10^5$ cells/well with 10^4 cells/well BM-MSCs for 4 days (with or without PHA). Then, the effects

of BM-MSCs on the proliferation of PBMCs were tested by the CCK-8 method. The morphology of PBMCs from the young healthy group, cultured alone or with BM-MSCs, is shown in Fig. 2A-C.

The direct effects of PHA on the proliferation of PBMCs and BM-MSCs were determined. In the presence of PHA, the proliferation of PBMCs and BM-MSCs increased significantly (Fig. 2D). The effect of BM-MSCs on the proliferation of PHA-activated PBMCs was then examined. BM-MSCs were co-cultured with fresh PBMCs in the presence of PHA and it was identified that, when PBMCs were activated by PHA, their proliferation was markedly suppressed by BM-MSCs (Fig. 2D). These results indicated that BM-MSCs exert an inhibitory effect when co-cultured with activated PBMCs.

Following the mitomycin C treatment, the percentage of Ki67 positive MSCs was 7.56±4.35%, significantly lower than prior to the treatment (64.8±7.57%; Fig. 2E-I). These mitomycin C treated BM-MSCs were used in co-culture with PBMC and identified that even these MSCs still decreased the proliferation of PBMC to the same level as that of untreated BM-MSCs (Fig. 2D).

When the PBMCs were not stimulated by PHA, the influence of BM-MSCs on the proliferation of PBMCs was not suppressive. In fact, the measured proliferation value of co-cultured BM-MSCs and PBMCs increased compared with that of PBMCs cultured with PHA, but did not reach statistical significance (Fig. 2D). These results confirmed that the immunosuppressive ability of BM-MSCs is not innate but is induced by the PHA-treated PBMCs.

Responsiveness of PHA-treated PBMCs from the middle-aged healthy and PD groups toward BM-MSC suppression of proliferation. The PBMCs from the young healthy, the middle-aged healthy and the PD patient groups were seeded in a 96-well plate at a density of $2x10^5$ cells/well and were co-cultured with 10^4 cells/well BM-MSCs (with or without PHA-treatment). Without PHA treatment, no significant change was observed in the CCK-8 absorbance values among the three groups (Fig. 2K), demonstrating that there was no difference in the proliferation of PBMCs from the three groups co-cultured with BM-MSCs.

When PHA was added, compared with that of the PHA-untreated PBMCs, the proliferation of PBMCs co-cultured with BM-MSC was significantly decreased (Fig. 2L). This result was observed in the young healthy group, healthy middle-aged group and the PD group (P<0.05). The proliferation of PHA-treated PBMCs in the young group was lower compared with the middle-aged group and PD group (P<0.05). The PBMC proliferation of the middle-aged and PD group demonstrated no significant difference following the suppression by BM-MSCs (Fig. 2L).

Effect of cytokines on the activation of BM-MSCs. To detect which proinflammatory cytokines are required for BM-MSCs to exert inhibitory effects, the relative proliferation rates of PBMCs from young healthy males were compared following co-culture with BM-MSCs pre-treated with several cytokines (20 ng/ml).

All the proinflammatory cytokines tested, IL-1α, IL-1β, TNF-α and IFN-γ-treated BM-MSCs, demonstrated a suppressive effect on the proliferation of PBMCs, but none of them reached statistical significance, and no significant difference was observed among the inhibitory abilities of these proinflammatory cytokines (Fig. 3A).

The combination of proinflammatory cytokines was more effective than any single one in inhibiting PBMC proliferation. BM-MSCs pretreated with IFN-γ combined with IL-1α, IL-1β or TNF-α significantly inhibited the proliferation of PBMCs (P<0.05). The combinations of IFN-γ + IL-1α and IFN-γ + IL-1β-treated BM-MSCs had greater inhibitory effects on PBMCs than the combination of IFN-γ + TNF-α-treated BM-MSCs (P<0.05), and there was no significant difference between IFN-γ + IL-1α and IFN-γ + IL-1β-treated BM-MSCs (Fig. 3A).

Responsiveness of PBMCs from the middle-aged healthy and PD groups toward cytokine-pretreated BM-MSC suppression of proliferation. The responses of the PBMCs isolated from middle-aged healthy individuals and patients with PD to BM-MSCs that were activated by proinhibitory cytokine combinations were then tested.

Similar to the suppression experiment in the young healthy group, the combinations of IFN-γ + IL-1α, IFN-γ + IL-1β and IFN-γ + TNF-α-treated BM-MSCs significantly decreased PBMC proliferation (P<0.05) in middle-aged individuals and patients with PD compared with the co-culture of PBMCs and BM-MSCs without adding proinhibitory cytokines (control group in Fig. 3B).

The next question was which response was most prominent among the young healthy, middle-aged healthy and PD patient groups. The relative proliferation rate of PBMCs inhibited by IFN-γ + IL-1α- or IFN-γ + IL-1β-treated BM-MSCs in the healthy young people was significantly lower compared with middle-aged healthy people and PD patients (P<0.01; Fig. 3B). No significant difference was observed in the decrease in PBMC proliferation between the healthy middle-aged people and the patients with PD group.

The final question was which proinflammatory cytokine combination was most effective in inducing BM-MSCs to be inhibitory in the healthy middle-aged people and the patients with PD. Among the combinations of the three inflammatory cytokines, similar to the results in the healthy young people, BM-MSCs treated with IFN-γ + IL-1β had the most significant inhibitory effect on PBMCs compared with those treated with IFN-γ + TNF-α in the healthy middle-aged people (P<0.05), while no significant difference was observed between the effect of BM-MSCs treated with IFN-γ + IL-1α and those treated with IFN-γ + TNF-α (Fig. 3B).

BM-MSCs reduce the PBMC production of proinflammatory cytokines in young healthy males. To identify the immunosuppressive effects mediated by BM-MSCs on cytokine production in the young, middle-age and PD groups, the generation of cytokines during the incubation of the BM-MSCs and PHA-stimulated PBMCs (Fig. 4) was investigated.

The concentration of the cytokines in the culture medium was measured as a control. IL-2, IL-6, IL-10, IFN-γ, and TNF-α were analyzed (Fig. 4). Since BM-MSCs and PBMCs physiologically secrete certain cytokines, the BM-MSCs cultured in the PBMC medium with PHA and the PBMCs
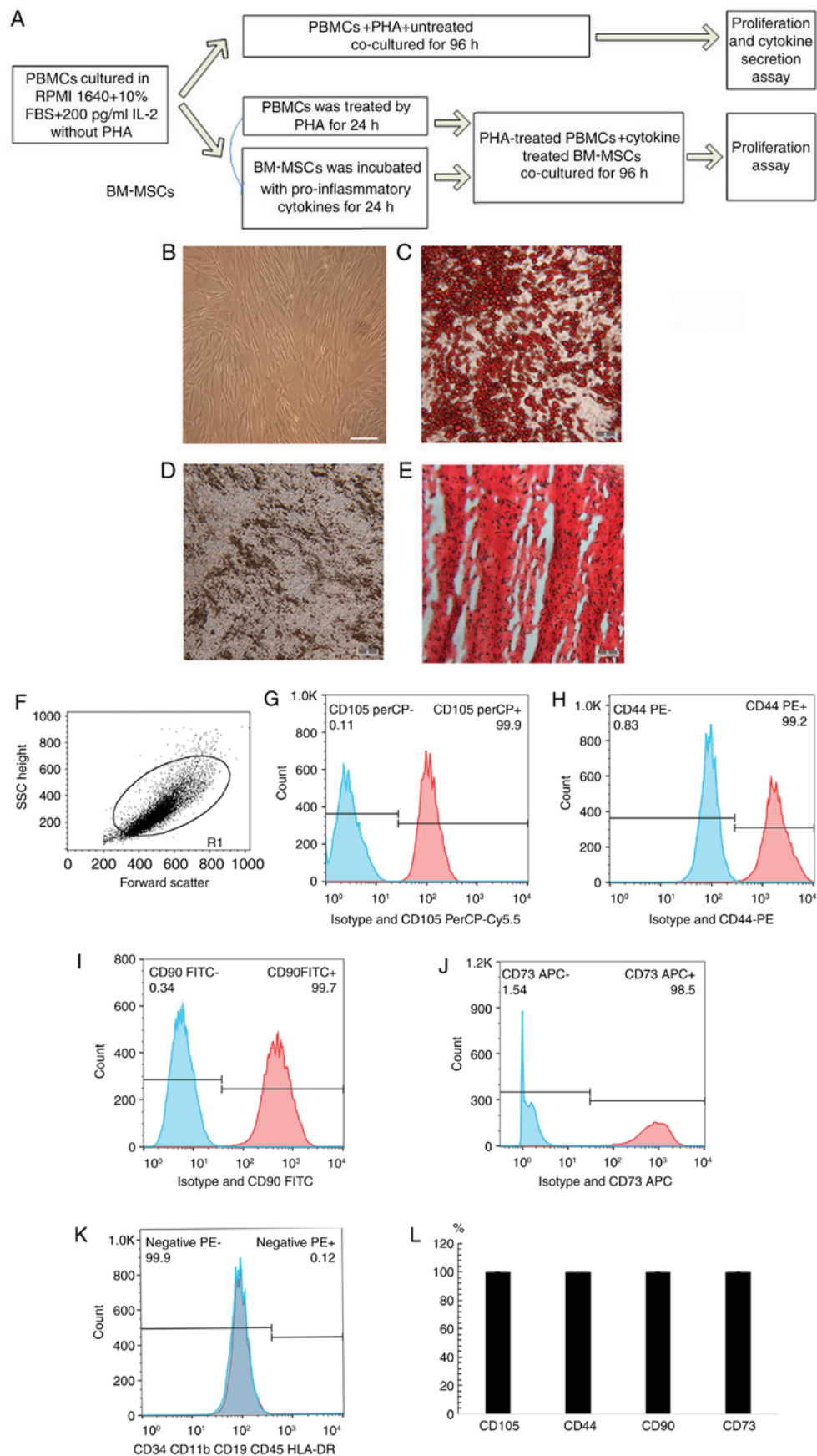
Figure 1. Differentiation and cytometry characterization of cultured human BM-MSCs. (A) Schematic paradigm of the co-culture experiments. (B) Following 3-5 passages, the BM-MSCs exhibited spindle-like shape. (C) Formation of lipid vacuoles was detected by Oil Red O staining. (D) Osteogenesis was tested by von Kossa staining of the mineralized matrix. (E) Chondrogenesis was indicated by Safranin O staining. Scale bar, 100 μm. (F) The cells gated for analysis. With isotype control, cytometry tests identified that BM-MSCs at passage 3-5 were positive for (G) CD105 (H) CD44, (I) CD90 and (J) CD73, and negative for (K) CD34, CD11b, CD19, CD45 and HLA-DR. (L) The summary of quantified flow cytometry results as percentages. n=3. PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; BM-MSCs, bone marrow-derived mesenchymal stem cells; CD, cluster of differentiation.
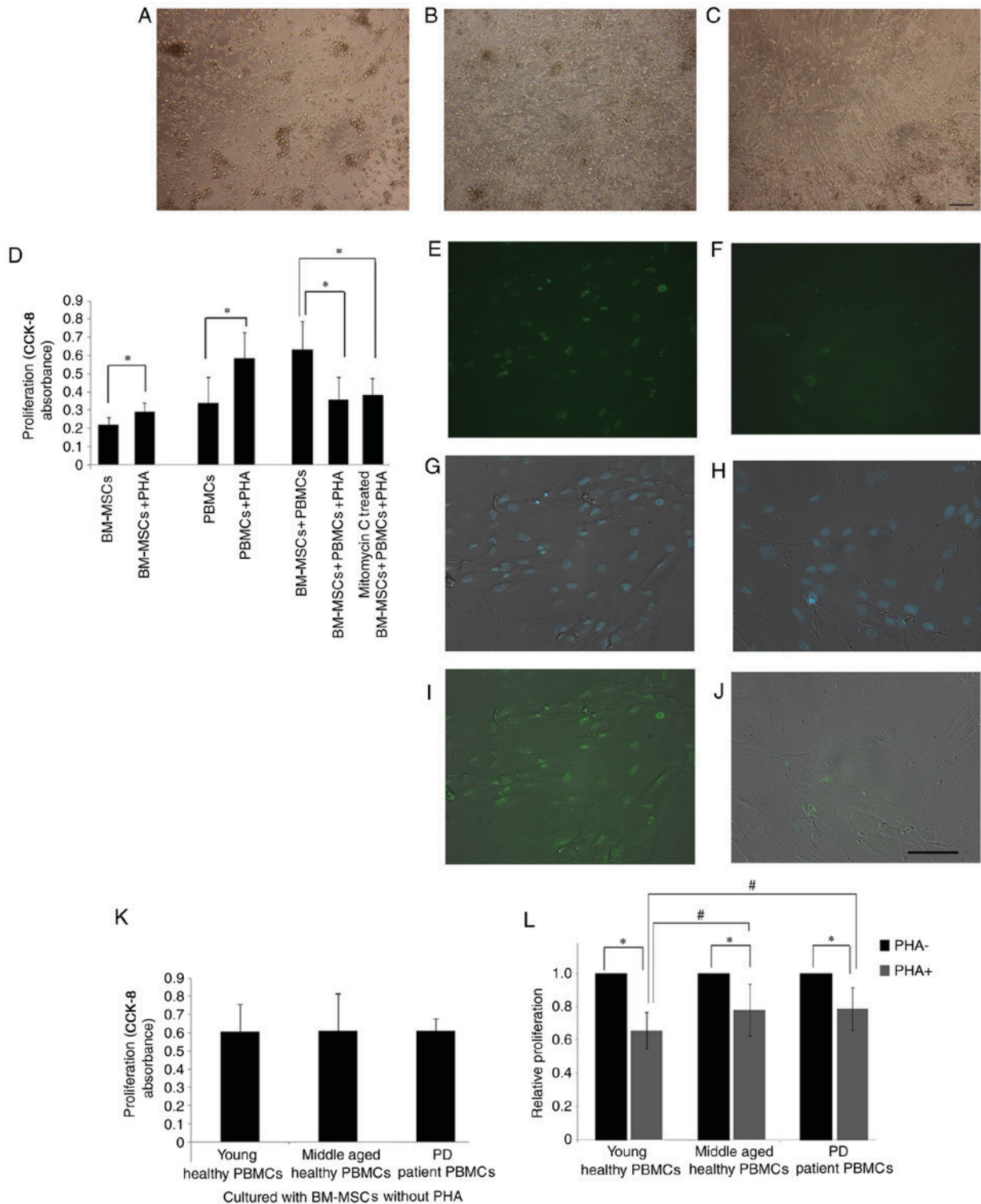
Figure 2. Proliferation suppression of PBMCs by PHA-treated BM-MSCs. (A) PBMCs isolated from young healthy individuals. (B) Co-culture of BM-MSCs and PBMCs without PHA at day 3. (C) Co-culture of PHA-activated PBMCs and BM-MSCs at day 3. (D) In the presence of PHA, the proliferation of PBMCs and BM-MSCs increased significantly (*P<0.05). When incubated with BM-MSCs and mitomycin C-treated BM-MSCs, PHA-activated PBMC proliferation decreased significantly. Ki67 staining of the MSCs prior to (E, G and I) and following mitomycin C treatment (F, H and J) were demonstrated. (E and F) Ki67 staining. (G and H) Merged image of DAPI nucleus staining and phase contrast. (I and J) Merged image of Ki67 and phase contrast. (K) Without PHA, the PBMC proliferation in the young healthy, the middle-aged healthy, and the middle-aged PD individuals was not significantly different. (L) Following treatment with PHA, the PBMC proliferation in the young healthy, the middle-aged healthy and the middle-aged PD groups decreased significantly (*P<0.05). The proliferation of PBMCs suppressed by BM-MSCs of the young healthy subjects was significantly lower than that of the middle-aged healthy and the PD individuals (#P<0.05). Scale bar, 50 μm, n=6. PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; BM-MSCs, bone marrow-derived mesenchymal stem cells; PD, Parkinson's disease.

cultured in the presence or absence of PHA were measured for the secretion of cytokines.

IL-2 is an essential factor for T cell proliferation *in vitro* and was added to the medium at a concentration of 200 pg/ml. The
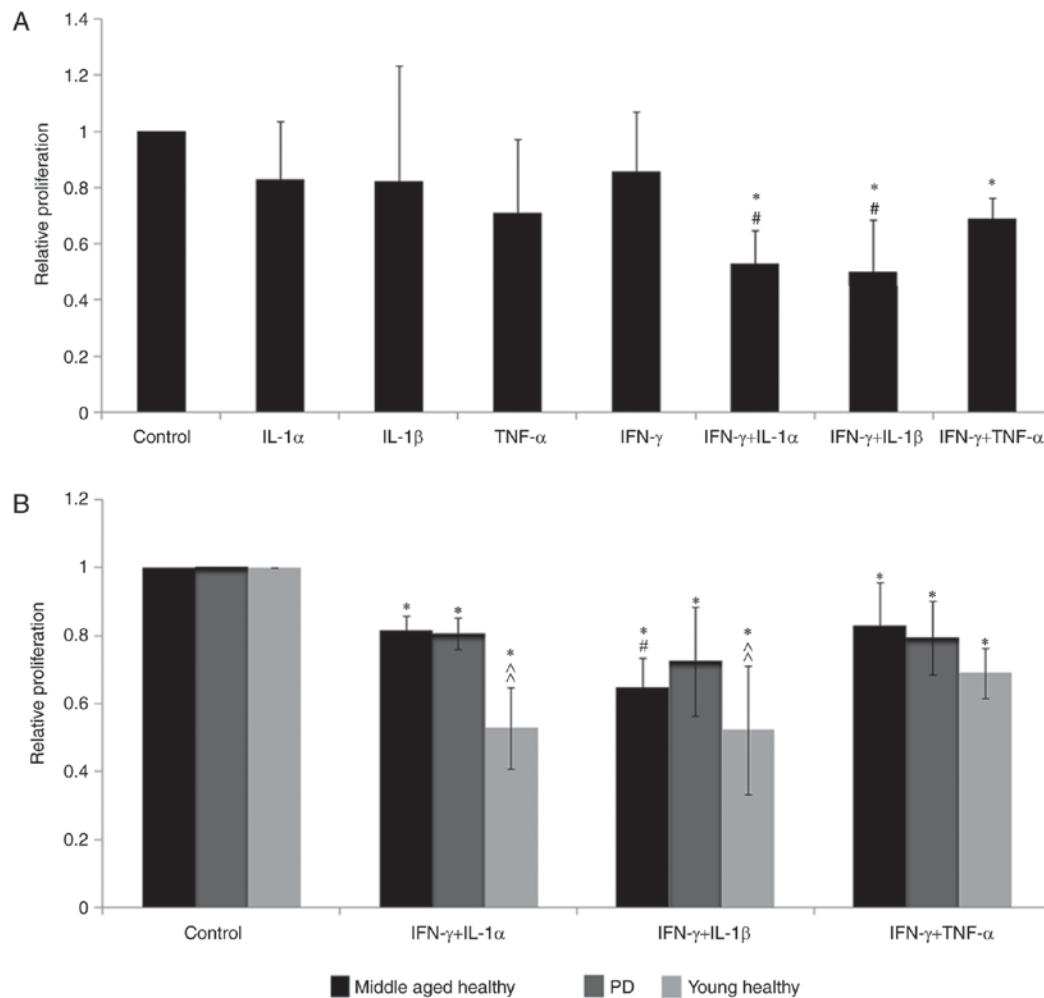
Figure 3. Proinflammatory cytokines are required by BM-MSCs to suppress the proliferation of PBMCs. (A) IL-1α, IL-1β, TNF-α or IFN-γ alone did not induce BM-MSCs to become immunosuppressive. Compared with the cytokine-untreated cells, BM-MSCs treated with the combinations of IFN-γ + IL-1α, IFN-γ + IL-1β and IFN-γ + TNF-α decreased the PBMC proliferation in young individuals significantly (*P<0.05). IFN-γ + IL-1α and IFN-γ + IL-1β were more powerful than IFN-γ + TNF-α in inducing BM-MSCs to suppress the PBMC proliferation in young individuals to lower levels (#P<0.05). (B) Proliferation of PBMCs treated with all three combinations in the young healthy, the middle-aged healthy and the PD groups decreased significantly compared with those in the control group (untreated BM-MSCs with PBMCs; *P<0.05). In the middle-aged healthy group, IFN-γ + IL-1β was more effective than IFN-γ + TNF-α in inducing BM-MSCs to be immunosuppressive (#P<0.05), while IFN-γ + IL-1α and IFN-γ + TNF-α did not show any significant difference. Following the treatment with IFN-γ + IL-1β or IFN-γ + IL-1α, the PBMC proliferation in young healthy subjects was significantly lower than that in the middle-aged healthy and the PD individuals (^^P<0.01). n=6. BM-MSCs, bone marrow-derived mesenchymal stem cells; PBMCs, peripheral blood mononuclear cells; IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ, PD, Parkinson's disease.

IL-2 concentration of the medium measured by the CBA method was 227.51±30.1 pg/ml. The PHA-treated BM-MSCs alone did not change the IL-2 concentration, which indicated that BM-MSCs did not produce IL-2 even with PHA stimulation. The culture of PBMCs consumed IL-2, and the activated PBMCs lowered the IL-2 concentration to 9.8±6.6 pg/ml. When the PBMCs were co-cultured with the BM-MSCs, the IL-2 concentration did not change significantly. This finding suggested that the BM-MSCs do not affect the production of IL-2 by PBMCs (Fig. 4A).

Regarding IL-6, BM-MSC together with PHA produced 624.87±138.06 pg/ml. PBMCs at the resting state secreted 322.82±100.27 pg/ml IL-6; when activated by PHA, PBMCs produced a significantly higher level of IL-6, which was 14,242.73±1,452.48 pg/ml. When cultured together, the IL-6 concentration did not increase significantly. This result suggested that the BM-MSCs and PBMCs are capable of secreting IL-6 and that the synergistic effect of BM-MSCs and PBMCs in producing IL-6 was not prominent (Fig. 4B).

The concentration of IL-10 in the BM-MSCs cultured with PHA demonstrated no difference compared with that in the medium with added PHA (without MSCs). This result suggested the BM-MSCs did not produce, or produced a very low level, of IL-10. PBMCs only produced 9.16±2.47 pg/ml of IL-10 without PHA. With PHA, however, the IL-10 concentration increased significantly to 562.34±186.78 pg/ml. The concentration of IL-10 was increased to 852.64±214.33 by co-culture with BM-MSCs. These results were evidence that BM-MSCs stimulate the secretion of IL-10 (Fig. 4C).

As demonstrated in Fig. 4D and E, IFN-γ and TNF-α were not present in the PBMC medium and not secreted by PHA-treated BM-MSCs. TNF-α was not produced by PHA-treated PBMCs (Fig. 4D), whereas the concentration of IFN-γ was as low as 31.91±7.8 pg/ml in the medium of PHA-treated PBMCs (Fig. 4E). When PBMCs were activated by PHA, the secretion of TNF-α and IFN-γ was significantly increased to 1,266.9±408.3 and 3,584.1±287.5 pg/ml,
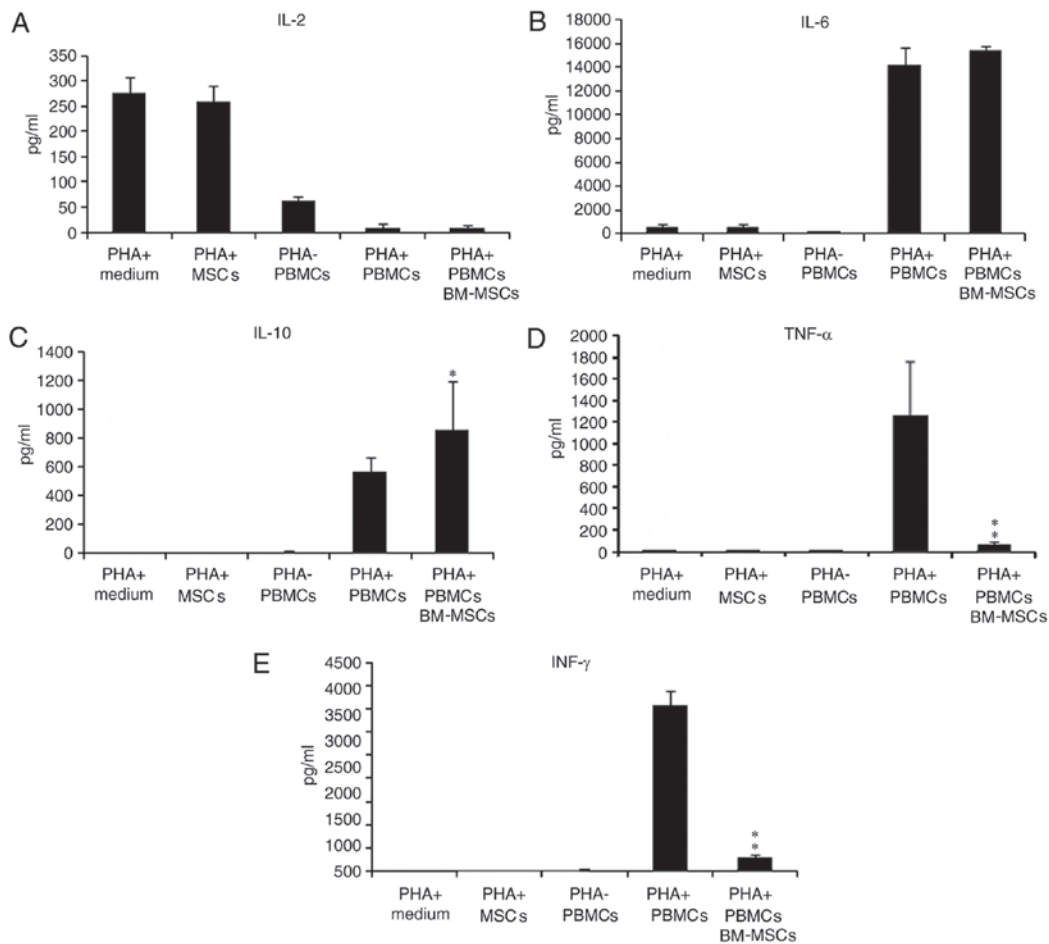
Figure 4. Influence of BM-MSCs on cytokine production in the co-culture of BM-MSCs and PBMCs. (A) BM-MSCs did not produce IL-2 even with PHA. The co-culture with PBMCs consumed IL-2, and the activated PBMCs lowered IL-2 levels to 9.8±6.6 pg/ml. (B) BM-MSCs and PBMCs are capable of secreting IL-6. With PHA, the IL-6 concentration increased significantly. The synergistic effect of BM-MSCs and PBMCs in producing IL-6 was not marked. (C) With PHA, the IL-10 concentration increased significantly to 562.34±186.78 pg/ml and was improved to 852.64±214.32 by co-culture with BM-MSCs (*$P<0.05$ vs. PHA-activated PBMCs alone). When PBMCs were activated by PHA, the secretion of (D) TNF-α and (E) IFN-γ was significantly inhibited by BM-MSCs (*$P<0.05$ and **$P<0.01$ vs. PHA-activated PBMCs alone;). BM-MSCs, bone marrow-derived mesenchymal stem cells; PBMCs, peripheral blood mononuclear cells; IL, interleukin; PHA, phytohemagglutinin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

respectively. When co-cultured with BM-MSCs, the concentrations of TNF-α and IFN-γ decreased to 110.7±14.4 and 368.9±105.7 pg/ml, respectively. These results demonstrated that BM-MSCs downregulated the production of inflammatory cytokines by PHA-activated PBMCs.

Responsiveness of PBMCs from the middle-aged healthy and PD groups toward BM-MSC suppression of cytokine production. TNF-α and IFN-γ were selected to examine the responsiveness of the PBMCs towards the BM-MSC suppression of cytokine production. The levels of TNF-α secreted by PBMCs in the young healthy group (3,584.1±287.5 pg/ml), the middle-aged healthy group (3,361.2±363.3 pg/ml) and the PD patient group (3,291.2±270.2 pg/ml) were of the same level and were all decreased markedly by BM-MSCs ($P<0.01$). The TNF-α production in the co-cultured PBMCs of the young healthy group (368.9±105.7 pg/ml) was significantly lower compared with the middle-aged healthy (733.8±126.9 pg/ml) and PD patient groups (812.4±145.5 pg/ml; $P<0.01$; Fig. 5A).

Similar phenomena were observed for IFN-γ. With the stimulation by PHA, no significant difference was observed in the IFN-γ production among the PBMCs isolated from

the young healthy (1,266.9±408.3 pg/ml), middle-aged healthy (1,096.8±225.0 pg/ml) and patients with PD groups (1,022.5±344.7 pg/ml). When BM-MSCs were added into the co-culture system, the IFN-γ secretion was markedly decreased in all three groups compared with that of the PBMC culture ($P<0.01$). Following BM-MSC suppression, the IFN-γ production in the young healthy group (110.7±14.4 pg/ml) was significantly lower than the middle-aged healthy (208.2±23.1 pg/ml) and patients with PD groups (243.5±43.2 pg/ml; $P<0.01$; Fig. 5B).

Discussion

The role of inflammation in the pathogenesis and progression of PD has gradually been recognized (14-16). The activation of glial cells, particularly microglia, is one of the typical signs of intracerebral inflammation (14-16). Activated microglia can secrete a large amount of inflammatory cytokines, which can trigger a sustained inflammatory reaction (14-16). McGeer *et al* (3) identified that an inflammatory reaction exists in the substantia nigra of patients with PD with localized microglial proliferation. Inhibiting microglia-mediated
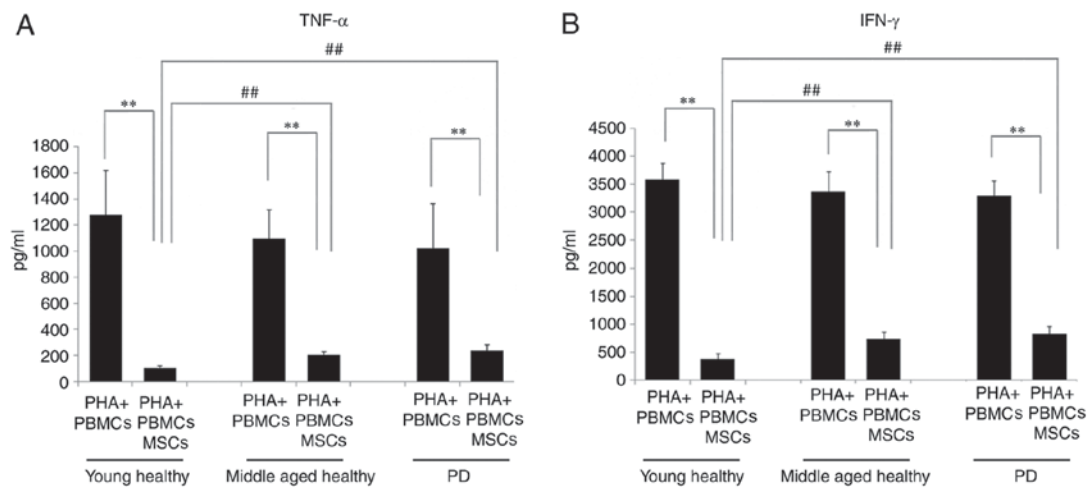
Figure 5. Responsiveness of PBMCs from the middle-aged healthy and the PD groups to BM-MSC suppression of cytokine production. Compared with PHA-activated PBMCs, BM-MSCs co-cultured with PBMCs decreased the secretion of (A) TNF-α and (B) IFN-γ in the young healthy, the middle-aged healthy and the PD patient groups ([**]P<0.01). Following BM-MSC suppression, the TNF-α and IFN-γ production in the young healthy group was significantly lower than that in the middle-aged healthy group and the PD patient group ([##]P<0.01). PBMCs, peripheral blood mononuclear cells; PD, Parkinson's disease; PHA, phytohemagglutinin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

inflammatory reaction has become a new research direction in PD treatment (14-16).

Recent studies have demonstrated that untreated mouse MSCs derived from bone marrow did not possess the ability to inhibit the immune responses, but that once they were stimulated by proinflammatory cytokines, MSCs obtained a strong immunosuppressive ability. These cytokines include TNF-α, IL-1α, IL-1β and IFN-γ. It is now clear that human BM-MSCs also require similar stimulation by inflammatory factors to serve an immunosuppressive role (28,32).

The serum levels of IL-2, IL-4, IL-6, IL-10 and TNF-α are significantly increased in patients with PD (9). It is known that peripheral inflammation is closely associated with neurodegenerative processes in the brain by triggering strong responses of microglia (9). Therefore, it is possible to delay the progression of PD by inhibiting the systemic inflammatory response. In recent years, numerous experiments have been performed in this research field. NSAIDs have been commonly used in clinical trials. Previous studies have confirmed that NSAIDs can inhibit and keep the inflammatory response in check to a certain extent and thereby can protect the dopaminergic neurons, but other studies have shown that the protective effect of NSAIDs is mild and cannot reduce the risk of Parkinson's disease. Furthermore, the long-term clinical application of NSAIDs has been proven to exhibit certain side effects (33,34).

MSCs exhibit immunosuppressive capacity and possess very limited immunogenicity. Using MSCs to regulate inflammation and the immune response will potentially become routine in clinical treatment (33,34). According to the results of mouse experiments, proinflammatory cytokines including IL-1α, IL-1β, TNF-α, and IFN-γ, whether released by mitogen-treated PBMCs *in vitro* or added to the MSC culture, activate MSCs, and then MSCs significantly inhibit PBMC proliferation and production of proinflammatory cytokines (28).

As the immunosuppressive capacity of MSCs depends on the preactivation of the proinflammatory cytokines, when the inflammation in the body gradually subsides, the immunosuppressive capacity of MSCs gradually diminishes. When the

inflammatory reaction recurs, the MSCs will be activated again and then possess an inhibitory effect. Therefore, the immune regulation by MSCs is a dynamic process, and it does not inhibit the regular immune function of patients. Compared with the effects of immunosuppressive drugs, the merits of MSCs include fewer or no side effects, long duration of action and regulation by feedback.

The main purpose of the present study was to verify the suppressive effects of human BM-MSCs on allogeneic PBMCs from patients with PD. In the experiments, the surface markers of the BM-MSCs were identical to those that are well accepted (21-23). The BM-MSCs possessed the ability to differentiate into adipose tissue, bone and cartilage. All these results confirmed that the isolated cells are indeed MSCs. The BM-MSCs isolated from young and middle-aged healthy participants, and patients with PD markedly inhibited PBMC proliferation and the proinflammatory cytokine production of PBMCs *in vitro*.

The proliferation and proinflammatory cytokine production of PBMCs from healthy young people were more markedly suppressed by BM-MSCs than the PBMCs from middle-aged people and the patients with PD, and no significant difference was observed in the suppression effect between the PBMCs from patients with PD and the age-matched middle-aged healthy participants. These results suggested that the weaker responses of PBMCs in the PD group toward the suppression of BM-MSCs are not necessarily due to PD but are more likely to be age-related.

In the present study, the PBMC proliferation and the extent of MSC suppression were not as marked as previously reported (28). This may be due to a difference in experimental design. Firstly, the cells that are suppressed by MSCs in PBMCs are not lymphocytes alone. Lymphocytes comprise ~20-50% of the PBMCs. That the present study used PBMCs marks a difference between this study and those studies that used CD4- or CD8-positive lymphocytes. Secondly, the PHA-treated PBMCs and BM-MSCs were only co-cultured for 4 days; more time is required for the cells to reach the exponential growth

phase. Thirdly, the suppressive capacity of MSCs was usually examined by adding the cells to lymphocytes at a ratio of 1:5, 1:10 and 1:20. Fewer MSCs mean a lower degree of suppression. In the present study, the 1:20 ratio was used.

In addition, the current study identified that the ability of BM-MSCs to inhibit PBMCs requires proinflammatory cytokines. BM-MSCs stimulated by IFN-γ combined with any one of IL-1α, IL-1β or TNF-α exerted an inhibitory effect on PBMCs isolated from the young healthy group, the middle-aged healthy group and the patients with PD. Among the combination of proinflammatory cytokines, in young healthy and middle-aged healthy people, BM-MSCs pretreated with IFN-γ + IL-1β and IFN-γ + IL-1α had stronger inhibitory effects on PBMCs compared with those treated with IFN-γ + TNF-α. The inhibitory effect of BM-MSCs induced by IFN-γ + IL-1β on PBMC proliferation appears stronger than that of IFN-γ + IL-1α, although without a significant difference.

The results of the present study suggest that although aging affects the response of PBMCs toward the suppression of BM-MSCs, male patients with PD in middle age still maintain responses toward MSC suppression. This finding is direct evidence that patients with PD aged 56-60 are still eligible for the anti-inflammatory cell therapy in which BM-MSCs are used. The results also suggested that the pretreatment of BM-MSCs with IFN-γ + IL-1β and IFN-γ + IL-1α prior to transplantation may result in more favorable immunosuppressive effects.

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Availability of data and materials

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

Authors' contributions

YQG, CSZ, XBL and YAZ designed the experiments. XBL, XMY, LLL, JLW, CSZ, HQZ and YQG performed the *in vitro* and *in vivo* experiments. XBL, YQG, HQZ and YAZ contributed to the data analysis. YQG, XBL and YAZ wrote the manuscript and YAZ gave final approval to the submitted version.

Ethics approval and consent to participate

Informed consent of the patients was obtained in addition to the participant consent and ethical approval obtained from the Ethics Committee of Xuanwu Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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