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Research article

GENE EXPRESSION PROFILES OF VARIOUS CYTOKINES IN MESENCHYMAL STEM CELLS DERIVED FROM UMBILICAL CORD TISSUE AND BONE MARROW FOLLOWING INFECTION WITH HUMAN CYTOMEGALOVIRUS

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Abstract: Mesenchymal stem cells (MSCs) have both multi-lineage differentiation potential and immunosuppressive properties, making them ideal candidates for regenerative medicine. However, their immunosuppressive properties potentially increase the risk of cancer progression and opportunistic infections. In this study, MSCs isolated from human umbilical cord blood (UCMSCs) and adult bone marrow (BMMSCs) were infected with human cytomegalovirus (HCMV). Cytopathic changes were observed 10 days post infection. PCR products amplified from genomic DNA and cDNA were used to confirm the HCMV infection of the UCMSCs and BMMSCs. Real-time PCR was conducted to quantify the expression of immunomodulatory molecules, including cytokines, chemokines, growth factors, adhesion molecules and

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Abbreviations used: BMMSC – bone marrow mesenchymal stem cells; HCMV – human cytomegalovirus; IFN – interferon; IL – interleukins; MCP – macrophage chemotaxis protein; MSC – mesenchymal stem cells; TCAM – testicular adhesion molecules; TGF – transforming growth factor; TNF – tumor necrosis factor; UCMSC – umbilical cord mesenchymal stem cells; VCAM – vascular adhesion molecules

cancer-related genes. Our results indicate high upregulation of the majority of these molecules, including many growth factors, tumor necrosis factor alpha, interleukin-8, interleukin-6 and interferon gamma. Adhesion molecules (VCAM-1, TCAM-1 and selectin-E) were downregulated in the infected UCMSCs and BMMSCs. Antibody chip array evaluation of cell culture media indicated that the growth factor secretion by UCMSCs and BMMSCs was greatly influenced ($p < 0.001$) by HCMV. The stimulation of MSCs with HCMV led to the activation of downstream signaling pathways, including pSTAT3 and Wnt2. Our results show that HCMV can significantly alter the functions of both UCMSCs and BMMSCs, although not in the same way or to the same extent. In both cases, there was an increase in the expression of proangiogenic factors in the microenvironment following HMCV infection. The discrepancy between the two cell types may be explained by their different developmental origin, although further analysis is necessary. Future studies should decipher the underlying mechanism by which HCMV controls MSCs, which may lead to the development of new therapeutic treatments.

Keywords: Mesenchymal stem cells, Bone marrow, Umbilical cord, Human cytomegalovirus, In vitro infection, Cytopathic change, Immunomodulatory molecules, Gene expression detection, Antibody chip, Kinase signal pathway

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells with the ability to differentiate into a variety of tissues [1]. A considerable body of work on MSCs has shown their ability to avoid allorecognition, modify T-cell and dendritic cell function, and through secretion of cytokines, generate immunosuppressive microenvironments [1]. MSCs circumvent alloreactive T-lymphocyte responses through low-level expression of both human leukocyte antigen and co-stimulatory molecules [2]. MSCs elicit their immunomodulatory functions on activated B cells and natural killer cells [3]. In addition, through downregulation of MHC class molecules and co-stimulatory molecules, MSCs are able to hamper the maturation of dendritic cells [4]. These immunomodulation activities are achieved through cell-cell contact and the release of soluble factors [5].

In order for MSCs to achieve their immunosuppressive functions, they need to be activated in a pro-inflammatory microenvironment in the presence of cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin 1-alpha (IL-1α) and interleukin 1-beta (IL-1β) [6]. Although MSCs impose their immunosuppressive activities on most of the lymphocyte cells, they mediate the expansion and induction of new regulatory T cells via interleukin-10 (IL-10) [7].

The importance of the immunosuppressive characteristics of MSCs has been demonstrated in some clinical studies where MSCs attenuated the treatments of graft-versus-host disease [8] and liver cirrhosis [9]. All of these unique MSC biological properties have generated a great interest in their potential for

application in regenerative medicine and immunosuppressive treatment. As of September 2013, 354 clinical trials using MSCs for a very wide range of therapeutic applications are in progress, most of them in phase I or II, with a small number in phase III (http://clinicaltrials.gov).

Although immunosuppression is an attractive feature of MSCs, it does mean a potentially increased risk of opportunistic pathogens. One such pathogen is human cytomegalovirus (HCMV), a member of the β-herpesviridae family, which is predominantly found in the peripheral blood mononuclear cells (PBMC) of normal individuals [10]. It is present in between 50% and 90% of the population, depending on the region of the world. Infection in healthy immunocompetent individuals is typically asymptomatic, albeit with latent infection [11]. However, in immunosuppressed groups, such as organ transplant recipients and persons with AIDS, HCMV may reactivate, leading to development of the disease [12]. HCMV oncomodulation has been documented in the past few years, with indications that HCMV may increase malignancy in tumor cells [13].

MSCs recruit to the tumor stroma, where through paracrine signaling, they create a microenvironment that is suitable for tumor growth, progression and metastasis [14]. Studies have shown that MSC secretion of IL-6 and chemokine (C-X-C motif) ligand 7 (CXCL7) stimulates increased sphere formation and supports accelerated tumor growth [15]. IL-8 plays an essential role in the potentiation of tumor progression by inducing epithelial–mesenchymal transition of epithelial tumor cells [16]. In some types of cancer, tumor cells are able to stimulate production of the chemokine RANTES in MSCs. This is then used in paracrine signaling to other cancer cells, enhancing their motility, invasion and metastasis [17].

Examples of cytokine and chemokine production that result in MSC signaling include stromal cell-derived factor 1 (SDF-1) and vascular endothelial growth factor (VEGF), which are the two most prominent chemotactic factors that have been characterized [14]. The SDF-1 secreted by MSCs plays an important role in the migration of MSCs to tumor cells [18], while VEGF promotes angiogenesis [19]. Moreover, chemokine macrophage chemotaxis protein-1 (MCP-1), which is secreted by breast cancer cells, promotes the cellular migration of MSCs to tumors [20]. Secretion of the tissue inhibitor of metalloproteinase-2 along with matrix metalloproteinase-2 seems to allow MSCs to pass through the basement membrane [21]. The interaction between MSCs and tumor cells involves numerous signaling molecules secreted by MSCs. These stimulate various signaling pathways, especially related to cell growth and the regulation of apoptosis in tumor cells [22].

In our previous study, HCMV was shown to infect and cause cytopathic changes in MSCs isolated from adult human bone marrow [23]. However, the study did not address the nuances of altered inflammatory cytokine gene expression during infection. In this study, in an effort to answer some of those questions, MSCs isolated from both human umbilical cord mesenchymal stem cells (UCMSCs)

and adult bone marrow mesenchymal stem cells (BMMSCs) were infected with HCMV. Cytopathic changes were observed in both groups 10 days post infection, as was differential expression of many inflammatory cytokines and chemokines, including adhesion molecules and tumor-related genes. Changes in protein expression were detected using antibody chip arrays and western blotting. The aim was to clearly identify which types of cytokine and chemokine were influenced by HCMV infection. The results showed that the expressions of many cytokines chemokines, adhesion molecules and growth factors were greatly influenced by HCMV infection. Most of these are known to be very important in immunomodulation, cell migration and tumor progression. The results from this study may provide target genes for future functional research into understanding how HMCV modulates MSC function.

MATERIALS AND METHODS

Isolation and culture of MSCs

UCMSCs were isolated from human umbilical cords following a previously published protocol [24]. Human umbilical cords were collected in PBS. After disinfection in 75% ethanol for 5 min, the mesenchymal tissue was diced into almost 0.5-cm^3 sections and centrifuged at 1200 rpm for 5 min. The pellet was treated with collagenase at 37ºC for 18 h, washed with PBS, and then digested with 2.5% trypsin. The dissociated MSCs were either maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin at 1 x 10^5 cells/well in a 6-well plate (Falcon) or stored in liquid nitrogen for later use.

BMMSCs were isolated from healthy volunteers at West China Hospital in Chengdu, China, according to a previously published protocol [25]. The whole marrow samples were subjected to fractionation on a density gradient, and the isolated cells were plated in a 6-well plate at cell density of 3 x 10^6 cells/well in 10% FBS DMEM at 5% CO_2 and 37ºC. Primary cultures were maintained for two weeks, during which time the non-adherent hematopoietic cell fraction was removed. The plastic adherent MSCs were then expanded in a large-scale culture for further research.

In vitro infection of MSCs

The HCMV strain Davis was purchased from ATCC (VR-807). HCMV was propagated using MRC-5 cells grown in DMEM medium at 5% CO_2 and 37ºC for five days. Cell-free supernatant containing HCMV was removed from cell debris by centrifugation at 3000 x g for 30 min. The virus was pelleted by ultracentrifugation (Beckman, Fullerton, CA, USA) at 54,000 x g for 3 h. All of the virus pellets were resuspended in 5 ml of DMEM. UCMSCs and BMMSCs were seeded into a 6-well plate at a concentration of 2 x 10^5 cells/well. The resuspended HCMV supernatant was added to the target cells for 2 h, followed

by washing with fresh DMEM two times before the complement medium was added. The target cells were collected 3, 5 and 10 days post infection for the preparation of DNA and RNA extraction. The UCMSCs used for the HCMV infection were from three donors and the BMMSCs from two donors. All of the infection experiments were repeated three times.

PCR for DNA and cDNA

Genomic DNA was extracted with a TIANamp Blood DNA Kit (Tiangen). The primers were HCMV-UL55 (forward: 5'-CGTGAGACCTGTAATCTGAAC-3', reverse: 5'-GAAGGTGAGCTGGCAGGTGAC-3') and UL83 (forward: 5'-GTCTACTACACGTCAGCGTTC-3', reverse: 5'-GATTATCATATTTTTGGGAC-3'). The PCR conditions were one cycle at 95ºC for 5 min, 35 cycles at 94ºC for 1 min, 57ºC for 1 min and 72ºC for 1 min, and one cycle at 72ºC for 10 min. Aliquots of 10 µl of each reaction were separated on 2% agarose gels containing ethidium bromide. The gels were then photographed.

Total RNA was isolated using RNeasy mini Kit (Qiagen). cDNA was synthesized using a ReverTra Ace qPCR RT kit (FSQ-101, TOYOBO), and the reverse transcription conditions were 65ºC for 5 min, followed by 37ºC for 15 min and 98ºC for 5 min.

Real-time PCR

The real-time PCR was performed using Tiangen Biotech RealMaster Mix (SYBR Green) (FP202). on an iCycler iQ Optical Module (Beckman) under the following conditions: one cycle at 95ºC for 30 sec, and 40 cycles at 95ºC for 30 sec, 58ºC for 30 sec and 72ºC for 30 sec, followed by a melt curve from 55 to 95ºC in 0.5ºC increments and 10 sec intervals. The primers are listed in Table 1. All tests were conducted three times. RNA isolated from normal human PBMC was used as a control.

Antibody array

Conditioned media from MSCs infected with HCMV were analyzed for protein expression using RayBio Human Antibody Array C Series 1000 (RayBiotech) according to the manufacturer's instructions. Blots were analyzed with ImageJ software (National Institutes of Health).

Western blot

HCMV-infected MSCs were washed twice with cold phosphate-buffered saline (PBS), and proteins were extracted using standard mammalian protein extraction reagent (Pierce) containing protease inhibitors (Roche Applied Science). Lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4ºC. The protein concentration was measured using a Micro BCA Protein Assay Kit (Pierce). Total protein (20 µg) was loaded on 4–12% SDS-polyacrylamide gels, electrophoresed for protein separation, and transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked with 5% nonfat dried milk in TBS containing 0.2% Tween-20 (TBST) for 1 h at room temperature, followed

Table 1. Oligonucleotides used for real-time PCR analysis of UCMSCs and BMMSCs infected with HCMV.

Gene	Forward primer	Reverse primer	GenBank number
IL-1β	ACGAATCTCCGACCACCACT	CCATGGCCACAACAACTGAC	M15330
IL-6	GACCCAACCACAAATGCCA	GTCATGTCCTGCAGCCACTG	M14584
IL-8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT	NM_000584
IL-10	GGTGATGCCCCAAGCTGA	TCCCCCAGGGAGTTCACA	U16720
IL-12	CGGTCATCTGCCGCAAA	CAAGATGAGCTATAGTAGCGGTCCT	M65272
IL-16	GACCCCACCAAAGCTGGAC	TCACAGTGCTGCTGTCTGCTG	M90391
IFN-α	GGTGCTCAGCTGCAAGTCAA	GCTACCCAGGCTGTGGGTT	J00207
IFN-β1	CAGCAATTTTCAGTGTCAGAAGCT	TCATCCTGTCCTTGAGGCAGT	M28622
IFN-γ	CCAACGCAAAGCAATACATGA	CGCTTCCCTGTTTTAGCTGC	J00219
TGF-β	TATCGACATGGAGCTGGTGAAG	CAGCTTGGACAGGATCTGGC	X02812
TNF	GGTGCTTGTTCCTCAGCCTC	CAGGCAGAAGAGCGTGGTG	M10988
iNOS	CCAACAATGGCAACATCAGG	TCGTGCTTGCCATCACTCC	L09210
IP-10	TGAAATTATTCCTGCAAGCCAA	CAGACATCTCTTCTCACCCTTCTTT	NM_001565
MCP-1	CTCTGCCGCCCTTCTGTG	TGCATCTGGCTGAGCGAG	NM_002982
CCL3	AGCTGACTACTTTGAGACGAGCAG	CGGCTTCGCTTGGTTAGGA	NM_002983
CCL4	CTGCTCTCCAGCGCTCTCA	GTAAGAAAAGCAGCAGGCGG	NM_002984
RANTES	GACACCACACCCTGCTGCT	TACTCCTTGATGTGGGCACG	NM_002985
MCP-3	AGCAGAGGCTGGAGAGCTACA	GGGTCAGCACAGATCTCCTTGT	NM_006273
P53	CTTGCATTCTGGGACAGCCAAG	CACGCAAATTTCCTTCCACTCGG	DQ892492
c-myc	CAAGACTCCAGCGCCTTCTC	GTTGAGTAACGAGCTGACCCC	AM393287
cdc2	CAGGTTATATCTCATCTTTGAG	GTTGAGTAACGAGCTGACCCC	AM393287
TCAM-1	CGAGCTTGGCTGTGGCCTCC	TCTCCGCCATCCCAGCCTCC	NR_002947
selectin-E	TTGTTCCTGCCAGCAGCTGCC	AGGGCCAGAGACCCGAGGAG	NM_000450
VCAM-1	AGGTGACGAATGAGGGGACCACA	CCAGCCTCCAGAGGGCCACT	NM_001078
CCL21	TGGTTCTGGCCTTTGGCA	AGGCAACAGTCCTGAGCCC	NM_002989
CCL24	AGCCTTCTGTTCCTTGGTGTCT	GGGAGAGGGTATGACCACAGAG	NM_002991
CCL26	CCAAGACCTGCTGCTTCCAA	GAATTCATAGCTTCGCACCCA	NM_006072
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	J04038

Table 2. Primary antibodies for western blotting.

Name	Company	Catalog number	Molecular weight
Wnt2	Abcam	3169-1	34 kDa
IRAK4	Abcam	1589-1	52 kDa
p38	Abcam	ab8805	38 kDa
AKT	Abcam	ab7960	56 kDa
pSTAT3	Abcam	2236-1	92 kDa
p21	Abcam	ab7960	18 kDa
GAPDH	Abcam	ab8245	37 kDa

by an overnight incubation at 4ºC with the primary antibodies (Table 2). The membranes were then washed in TBST (3 times, 60 min) and incubated with a secondary antibody conjugated to horseradish peroxidase (1:5000; Abcam) for 1 h at room temperature. Antigen–antibody complexes were visualized using x-ray film after exposure to enhanced chemiluminescence reagent (Amersham Biosciences).

Data analysis

The real-time PCR data were analyzed using Bio-Rad iQ5 software. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as an internal control. Results obtained using normal PBMC were used as the controls. Results were expressed as the mean values ± SEM using SPSS 16.0 (IBM SPSS Statistics). Values of $p < 0.05$ and $p < 0.001$ were considered significant as compared to the control group. The figures were drafted using GraphPad Prism5 (GraphPad Software, Inc.).

RESULTS AND DISCUSSION

Detection of HCMV infection in UCMSCs and BMMSCs

Immunosuppression enables MSCs to persist in patients during clinical stem cell therapy, but not without an increased risk of cancer development and opportunistic infection. Although gene expression profiles related to MSC differentiation and immunomodulation have been extensively described [26], little information is available about the variation in cytokine and chemokine expression in MSCs infected with pathogens. In this study, MSCs isolated from both umbilical cord blood (UCMSCs) and adult human bone marrow (BMMSCs) were infected with HCMV Davis strain as described previously in the Materials and Methods section.

Following extensive passaging in MRC-5 cells to release the virus into the medium, cell-free HCMV Davis virus particles were used to inoculate MSCs in 6-well plates cultured at 2 x 10^5 cells/well. To determine whether HCMV infection induced any pathological change in MSCs, cells were processed and analyzed 3, 5 and 10 days post infection. Consistent with earlier reports, infection with HCMV induced pathological changes in both UCMSCs and BMMSCs 10 days post infection, with more significant cytopathic effects in BMMSCs than in UCMSCs (Fig. 1A).

The presence of HCMV in the infected cells was determined using the primers UL55 and UL83 3, 5 and 10 days post infection. Total cellular genomic DNA was extracted and HCMV-specific sequences were amplified and analyzed 3, 5 and 10 days post infection. The results from 3 and 5 days post infection showed only minimal increase in HCMV replication compared to the control (Fig. 1B). Only 10 days post infection was the presence of cDNA detected using real-time PCR in both UCMSCs and BMMSCs (Fig. 1C).

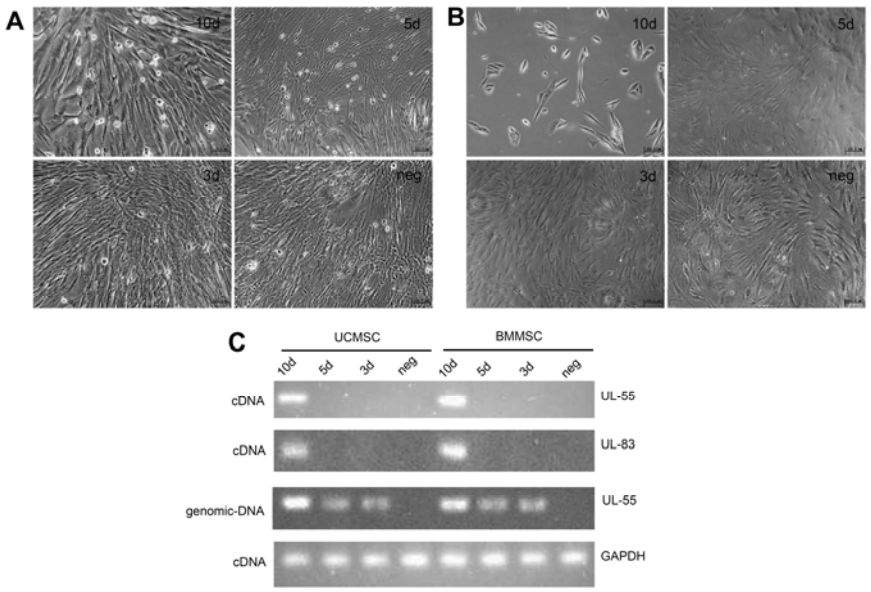

Fig. 1. Assessment of HCMV infection of UCMSCs and BMMSCs. A – UCMSCs infected with HCMV visualized 3, 5 and 10 days post infection. The cells were analyzed for the presence and the extent of the cytopathic effect following infection with HMCV. Non-infected UCMSCs were used as a negative control. B – BMMSCs infected with HCMV visualized 3, 5 and 10 days post infection. The cells were analyzed for the presence and the extent of cytopathic effects following infection with HMCV. Non-infected BMMSC cells were used as a negative control. C – Western blot analysis for the presence of HCMV DNA and cDNA in the infected UCMSCs and BMMSCs 3, 5 and 10 days post infection. GADPH was used as an internal control. All of the experiments were repeated three times. 10d – 10 days post infection; 5d – 5 days post infection; 3d – 3 days post infection; neg – uninfected UCMSCs or BMMSCs.

The expression profiles of IFN-α, IFN-β, IFN-γ, and chemokine (C-C motif) ligand 26 are influenced by HCMV

Variations in cytokine and chemokine expression were analyzed using real-time PCR in an effort to identify candidate genes responsible for HCMV-mediated functional changes in MSCs. In particular, alterations in the expression of genes implicated in the following biological processes were analyzed: 1) tumor-related genes; 2) immune and inflammatory responses; 3) defense responses; 4) regulation of cell proliferation; 5) regulation of cell migration; and 6) leukocyte chemotaxis. Changes in the expressions of these genes, either due to microenvironment or pathogen stimulation, could greatly influence the biological functions of MSCs.

We analyzed the expressions of three tumor-related genes, specifically p53, c-myc and cdc2, which are respectively a well-known tumor suppressor, an oncogene and a cell cycle gene, [27]. No detectable differences in the expression of p53 were found in the infected UCMSC groups. However, in the BMMSCs,

a 4-fold increase in the expression of p53 was detectable 10 days post infection ($p < 0.001$; Fig. 2A). The expression of cdc2 increased significantly ($p < 0.001$) in both UCMSCs and BMMSCs 10 days post-infection (Fig. 2A). On the other hand, expression of c-myc increased significantly in BMMSCs ($p < 0.001$) with no detectable expression in UCMSCs 10 days post infection (Fig. 2A). These results suggest that HCMV may play an important role in MSC malignancy.

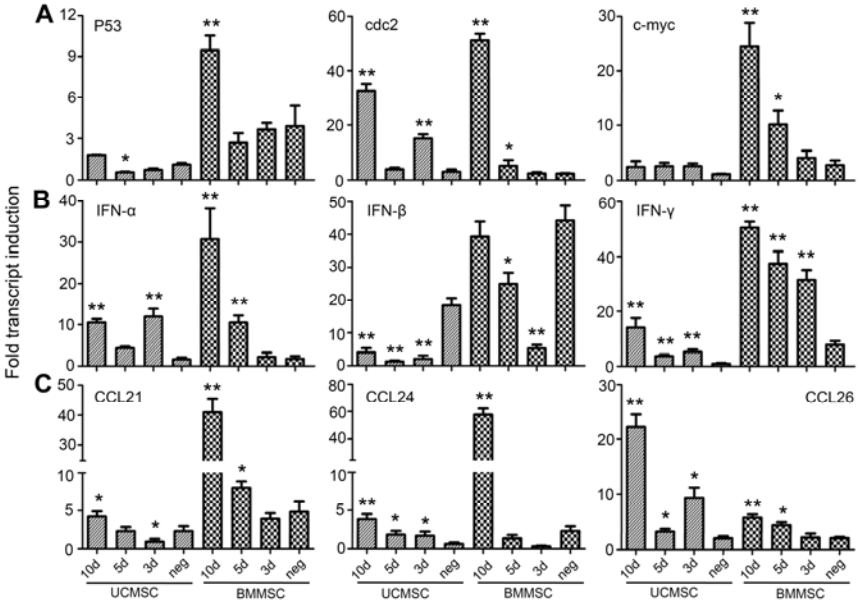

Fig. 2. Variation in the gene expression of cancer-related genes, interferons and chemokines in UCMSCs and BMMSCs following infection with HCMV. A – Real-time PCR analysis of the gene expressions of p53, cdc2 and c-myc in UCMSCs and BMMSCs 3, 5 and 10 days post infection. B – Real-time PCR analysis of IFN-α, IFN-β, and IFN-γ in UCMSCs and BMMSCs 3, 5 and 10 days post infection. C – Real-time PCR analysis of gene expression of CCL-21, CCL-24 and CCL-26 in UCMSCs and BMMSCs 3, 5 and 10 days post infection. Uninfected UCMSCs and BMMSCs were used as negative controls for each analysis. 10d – 10 days post infection; 5d – 5 days post infection; 3d – 3 days post infection; neg – uninfected UCMSCs or BMMSCs. Statistically significant differences are indicated: $^{**}p < 0.001$ or $^{*}p < 0.05$ versus the control group at each time point.

Interferon genes are key players used by host cells to fight viral infections. The changes in the expressions of three major interferons were measured: IFN-α, IFN-β and IFN-γ. An increase in IFN-γ expression in both UCMSCs and BMMSCs 10 d post infection ($p < 0.001$) was significant (Fig. 2B). IFN-γ expression levels increased slightly in UCMSCs and significantly in BMMSCs ($p < 0.001$) 10 days post infection (Fig. 2B). The expression of IFN-γ decreased 3 and 5 days post infection in both UCMSCs and BMMSCs, but recovered to pre-infection levels 10 days post infection (Fig. 2B). This occurrence may be

due to the ability of HCMV to initially suppress the expressions of IFN-γ, with host cells being able to recuperate by the 10th day post infection.
The expressions of three members of the chemokine (C-C motif) ligand family (CCL) were measured and analyzed: CCL26, CCL21 and CCL24. The expressions of CCL26 in both UCMSCs and BMMSCs dramatically increased ($p < 0.001$) 10 days post infection (Fig. 2C). The expressions of both CCL21 and CCL4 increased greatly in BMMSCs ($p < 0.001$), while the increase in UCSMCs was minor ($p < 0.05$; Fig. 2C). Differences in the expressions of these three proteins may be due to the ability of the HCMV to both increase immune tolerance and inhibit tumor development in the host cell (Fig. 2C).

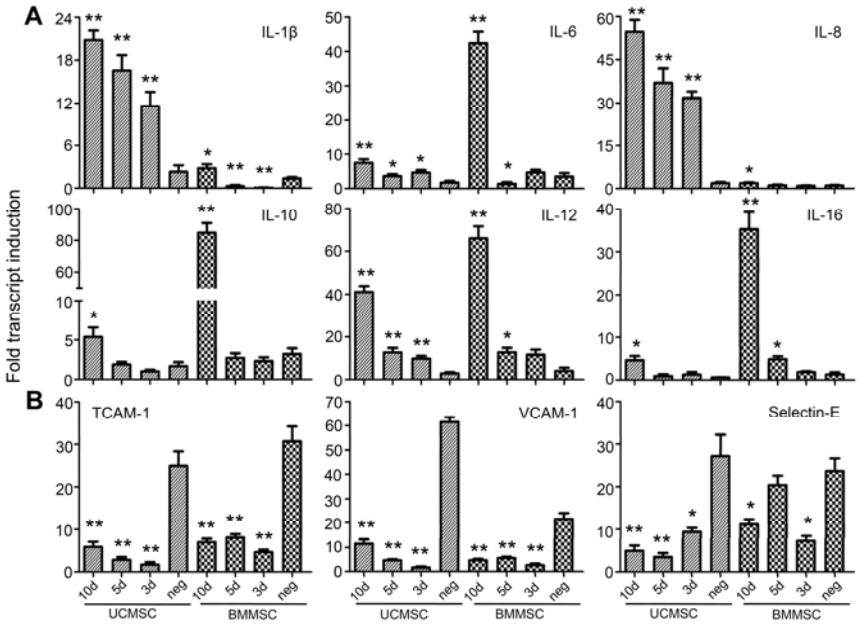

Fig. 3. Variation in the gene expression of interleukins and adhesion molecules. A – Real-time PCR analysis of the gene expressions of IL-1β, IL-6, IL-8, IL-10, IL-12 and IL-16 in UCMSCs and BMMSCs 3, 5 and 10 days post infection. B – Real-time PCR analysis of the gene expressions of TCAM-1, VCAM-1 and selectin-E in UCMSCs and BMMSCs 3, 5 and 10 days post infection. Uninfected UCMSCs and BMMSCs were used as negative controls for each analysis. 10d – 10 days post infection; 5d – 5 days post infection; 3d – 3 days post infection; neg – uninfected UCMSCs or BMMSCs. Statistically significant differences are indicated: $^{**}p < 0.001$ or $^{*}p < 0.05$ versus the negative control at each time point.

HCMV increased interleukin expression and inhibited the expression of adhesion molecules

Interleukins play important roles in both MSC immunosuppression and MSC–tumor interaction [28]. It has been previously shown that increased expression of IL-6, IL-10 and IL-12 leads to immune tolerance in MSCs [29–31]. IL-1β, IL-8 and

IL-16 [32] and CCLs were known to have tumor cell invasion inhibitory capacity against MSCs [14]. Our results showed that although all tested ILs (IL-1β, IL-6, IL-8, IL-10, IL-12 and IL-16) were upregulated by HCMV in both the UCMSC and BMMSC groups, there were still differences between the two infected groups (Fig. 3A). Of the six interleukins, IL-6, IL-10 and IL-12 play important roles in the MSC cancer signaling pathway, suggesting that HCMV may increase tumor signal pathways in MSCs [14].

An important characteristic of malignant cells is their enhanced migration ability [14]. MSC migration is dependent on multiple cell signals, including growth factors and chemokines, such as IL-10, transforming growth factor (TGF) and MCP-1, which are secreted by injured cells [33, 34]. In our study, the expressions of testicular adhesion molecule (TCAM), vascular adhesion molecule (VCAM) and selectin-E were investigated using real-time PCR. As shown in Fig. 6, both infected UCMSCs and BMMSCs had decreased expressions of TCAM-1, VCAM-1 and selectin-E ($p < 0.001$) compared to the control (Fig. 3B). These results suggest that HCMV may have enhanced migration of MSCs toward tumors.

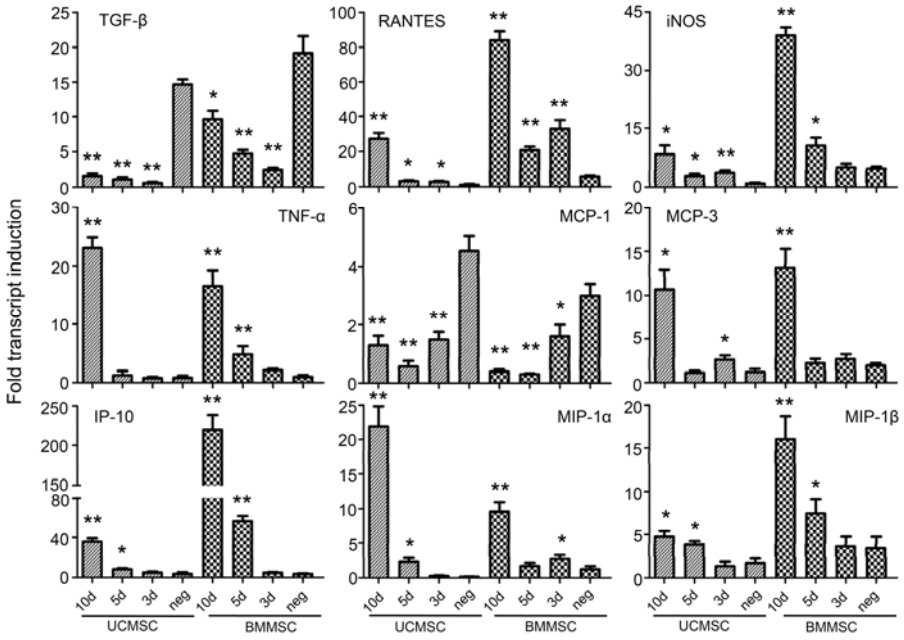

Fig. 4. Variation in the gene expressions of growth factors and macrophage chemotaxis proteins. Real-time PCR analysis of gene expression of TGF-β, RANTES, iNOS, TNF-α, MCP-1, MCP-3, IP-10, MIP-1α and MIP-1β in UCMSCs and BMMSCs 3, 5 and 10 days post infection. Uninfected UCMSCs and BMMSCs were used as negative controls for each analysis. 10d – 10 days post infection; 5d – 5 days post infection; 3d – 3 days post infection; neg – uninfected UCMSCs or BMMSCs. Statistically significant differences are indicated: **$p < 0.001$ or *$p < 0.05$ versus the negative control at each time point.

The gene expression of chemokines and growth factors due to HCMV infection of UCMSCs and BMMSCs

We analyzed certain growth factors, such as IFN-β and TNF-α, which are known to play important roles in the function of MSCs. In previously published research, it has been shown that downregulation of IFN-β inhibited T_{helper}-1 and T_{helper}-2 cells [31]. Reduced expression of TNF-α is important in promoting an effective cellular immunity by activating and differentiating T cells [31]. IFN-β, alone or together with TNF-α or IL-1, could stimulate the production of inducible nitric oxide synthase (iNOS), which inhibits T-cell proliferation [35, 36]. Our detection indicated that TGF-β and MCP-1 expression were greatly downregulated in both groups post infection ($p < 0.001$). The expressions of RANTES, iNOS, TNF-α, MCP-3, IP-10, MIP-1α and MIP-1β were significantly upregulated post infection ($p < 0.001$; Fig. 4). Based on this observation, we hypothesized that HCMV may regulate the anti-tumor immune response in infected MSCs.

Detection of cytokine and chemokine secretion in the culture medium

The supernatants from HCMV-treated MSC cultures were tested for the presence of secreted chemokines and cytokines using a RayBio antibody chip. A total of 20 molecules were chosen for detection (AFP, albumin, selectin-E, ICAM-1, IFN-α, IFN-γ, IL-10, IL-12, IL-18, IL-1β, IL-4, IL-5, IL-6, IL-8, MCP-1, MCP-3, MIP-1α, Notch-1, TGF-β and VEGF). The antibody chip assay confirmed that HCMV infection resulted in increased secretion of IL-1β, IL-6, IFN-γ and RANTES, and decreased secretion of MCP-1 in both UCMSCs and

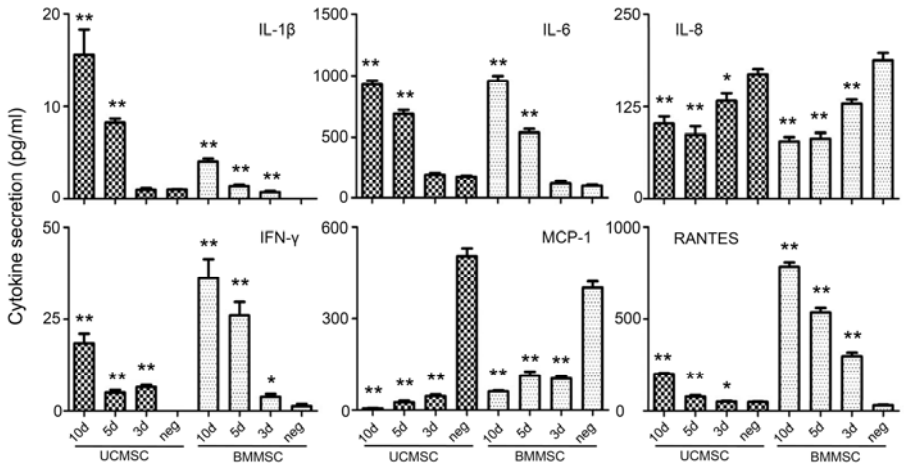

Fig. 5. The levels of cytokine and chemokine secretion in UCMSCs and BMMSCs following HCMV infection analyzed using an antibody chip array. 10d – 10 days post infection; 5d – 5 days post infection; 3d – 3 days post infection; neg – uninfected UCMSCs or BMMSCs. Statistically significant differences are indicated: $^{**}p < 0.001$ or $^{*}p < 0.05$ versus the control group at each time point.

BMMSCs, which were consistent with real-time PCR (Fig. 5). Not all of the interleukin gene expression resulted in the increased secretion of the protein. Such was the case for IL-8, where gene expression increased significantly ($p < 0.001$; Fig. 4) with only minimal detectable IL-8 in the medium (Fig. 5). The protein levels related to other target genes were either undetectable or the same.

HCMV infection of MSCs led to activation of downstream signaling molecules

Activation of downstream signaling molecules following HCMV infection of UCMSCs and BMMSCs was assessed using western blot analysis (Fig. 6). Both UCMSCs and BMMSCs displayed activated pSTAT3 and Wnt2 pathways 5 and 10 days post infection. Since pSTAT3 is mostly activated by IL-6 stimulation [37], this suggests that through increase in the expression of IL-6, HCMV can effectively regulate the growth and differentiation of MSCs. The Wnt protein family has been shown to play a role in oncogenesis and embryonic developmental processes, which can be activated by growth factors and other Wnt-regulated genes such as c-myc, AXIN2 and CycD1 [38]. An increase in Wnt2 expression in both UCMSCs and BMMSCs following HCMV infection may increase MSC oncogenic abilities and control of cell death through the regulation of growth factor- and cancer-related gene expression. Due to their important roles in the control of cell apoptosis, cell differentiation, migration and proliferation, the p21, p38 and IRAK4 levels were analyzed in the infected UCMSCs and BMMSCs 3, 5 and 10 days post infection [39–41]. All three were downregulated in both UCMSCs and BMMSCs, especially 10 days post infection (Fig. 6). These results suggest a complex regulation of MSCs by HCMV during infection.

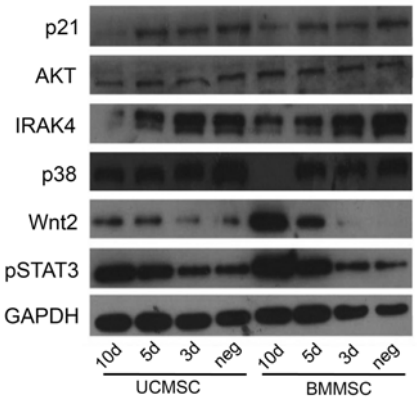

Fig. 6. The expression levels of various signaling components in UCMSCs and BMMSCs following infection with HCMV, as analyzed with western blot. GADPH mRNA expression was used as an internal control. 10d – 10 days post infection; 5d – 5 days post infection; 3d – 3 days post infection; neg – uninfected UCMSCs or BMMSCs.

Although BMMSCs are commonly used in clinical trials, the limitation in using these cells lies in the invasive procedure and limited cell numbers. Furthermore, BMMSCs can have lower self-renewal and proliferation abilities depending on the age of the patient [42]. Therefore, other sources of MSCs are needed for tissue regeneration.

MSCs isolated from umbilical cords are now considered an alternative candidate [43]. Clinical trials illustrate the safe and beneficial treatment results in graft-versus-host disease and systemic lupus erythematosus [44]. Compared to BMMSCs, UCMSCs express low levels of genes associated with teratoma formation [45]. An in vitro osteogenic study indicated that UCMSCs had a greater osteogenic potential than BMMSCs, although both formed sufficient bone matrix under osteogenic differentiation conditions [46].

In previous research, BMMSCs and UCMSCs shared high similarity in metabolic and functional processes, especially in the immune process, and in biological regulation, cell communication and other processes, such as migration and adhesion [47]. However, there were also some differences in gene expression levels between BMMSCs and UCMSCs. The predominant genes in UCMSCs include neurogenesis transcriptional factors such as SOX11 and PITX1, in addition to growth-related and blood vessel development genes such as FOXF1. Other genes that are upregulated in UCMSCs are heparin-binding epidermal growth factor, CXCL5, CXCL2, epidermal growth factor, fibroblast growth factor 9, midkine and nervous transcription factor 3. By contrast, the principal overexpressed genes in BMMSCs are associated with ossification and skeletal system development. BMMSCs also had a higher content of anti-apoptotic and angiogenic factors such as IL-6, VEGF and monocyte chemoattractant protein (MCP-1) [48].

While there were some differences in gene expression levels between BMMSCs and UCMSCs, there has not been any published research on the variation of expression upon stimulation due to pathogens. In this study, BMMSCs and UCMSCs were infected with HCMV, and the gene expression was analyzed. The observed significant differential expressions of cytokines and chemokines in HCMV-infected BMMSCs and UCMSCs provide novel insights into the functional prediction and importance of HCMV-mediated immunomodulation.

Some contradictory results were observed in our experiments, such as appearance of both anti-tumor (TNF-α) and accelerated-tumor effects (IL-6 and IL-12). One explanation for this phenomenon is that HCMV infection is a complex process, and the expression variation of mRNA did not necessarily lead to a change in protein levels. In addition, the differential expression of some markers (including p53, c-myc, CCL21, CCL24, RANTES and IL-10) between UCMSCs and BMMSCs may be explained by the difference in the developmental origin of these cells. Because the gene expression of these two types of cells may be at different background levels, their response to HCMV infection may differ as a result. Further investigation is needed to unveil the actual role of different cytokines and chemokines in the function of HCMV-infected MSCs as well as to provide a better

understanding of the underlying mechanisms that facilitate HCMV control of the microenvironment of MSCs. This will be required for the successful development of MSC therapeutics.

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