Cellular Immunology 278 (2012) 35–44

Contents lists available at SciVerse ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Immune characterization of mesenchymal stem cells in human umbilical cord Wharton's jelly and derived cartilage cells

Shuyun Liu, Mei Yuan, Kedong Hou, Li Zhang, Xifu Zheng, Bin Zhao, Xiang Sui, Wenjing Xu, Shibi Lu, Quanyi Guo *

Key Laboratory of the People's Liberation Army (PLA), Institute of Orthopedics, Chinese PLA General Hospital, No. 28 FuXing Road, Haidian District, Beijing 100853, China

ARTICLE INFO

Article history:
Received 30 June 2011
Accepted 27 June 2012
Available online 16 July 2012

Keywords:
WJMSCs
Immune character
Chondrogenic differenciation
Transplantation tolerance

ABSTRACT

Mesenchymal stem cells derived from human umbilical cord Wharton's jelly (hWJMSCs) became prospective seed cell candidate for tissue engineering and cell-based therapy because of its variety source, easy procurement, robust proliferation, and high purity compared with bone marrow- and adipose-derived MSCs. Such neonatal stem cells can be isolated from a variety of extraembryonic tissues and appear to be more primitive and have greater multi-potentiality than their adult counterparts. In this study, we investigated the immune characters of hWJMSCs and its derived cartilage cells (hWJMSC-Cs) by detecting the expression of major histocompatibility complex I/I(MHC-I/II), costimulatory molecules (CD40, CD80 and CD86) and immune inhibitors including human leukocyte antigen G (HLA-G), indoleamine-2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2). We found that hWJMSCs did not express MHC-II and costimulatory molecules, but moderately expressed MHC-I, and positively expressed immune inhibitors as HLA-G, IDO, PGE2, demonstrating their very low immunogenicity and potential to induce immune tolerance microenvironment in hosts. The results of chondrogenic differentiated hWJMSCs(hWJMSC-Cs) are similar to those of undifferentiated cells, except for the slightly elevated MHC-II and costimulators expression. Additionally, we detected cytokine profile of hWJMSCs through cytokine antibody array and verified by western blot the positive expression of immune suppression-related molecules, HGF, VEGF, TGF, and IL-10. Furthermore, to investigate the *in vivo* immune response of the cells, hWJMSCs-scaffold constructs were implanted into rabbits and rats, and the result showed that hWJMSCs did not elicit immune rejection in the animals. Their intermediate state between adult and embryonic stem cells makes them an ideal candidate for reprogramming to the pluripotent status. Additional studies are necessary to clarify the potential of hWJMSCs to be used in cartilage and other tissue regeneration and cell-based therapies.

1. Introduction

Mesenchymal stem cells (MSCs) are a rare population of multipotent precursors which can be isolated from many different tissue sources and also can differentiate into different lineages under appropriate induction conditions. MSCs represent an attractive cell source for therapeutic applications due to their potential of secreting bioactive molecules which are both trophic and immunomodulatory in nature [1]. Although human bone-marrow-derived MSCs (BM-MSCs) are extensively studied and most widely used, their harvest involve a highly invasive procedure, and the frequency, proliferation efficiency, differentiation potential of BM-MSCs decline with age [2,3].

As an alternative source of MSCs, fetal or neonatal MSCs appear to be more primitive and have greater multi-potentiality than their adult counterparts. Several studies have reported superior cell biological properties such as improved proliferative capacity, life span and differentiation potential of MSCs from birth-associated tissues over BM-MSCs. Their intermediate state between adult and embryonic stem cells also makes them an ideal candidate for reprogramming to the pluripotent status. They are very attractive for a wide range of regenerative medicine applications [4]. MSCs in Wharton's jelly (WJ) from umbilical cord possess desirable characteristics: firstly, the umbilical cord, which is discarded at birth, can provide an inexhaustible source of stem cells for therapy; secondly, the Wharton's jelly-derived MSCs (WJMSCs) from the umbilical cord, have been shown to have faster proliferation rates and greater expansion capability compared with adult MSCs, with wide multipotency and no induction of teratomas [5,6]; thirdly, they are believed to be more primitive than MSCs derived from other tissue sources [7]; additionally, the collection procedure is noninvasive and painless, and umbilical cord Wharton's jelly is ethically noncontroversial source of MSCs. However, for clinical application,

* Corresponding author.
E-mail address: guoquanyi301@gmail.com (Q. Guo).

0008-8749/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cellimm.2012.06.010

using WJMSCs as allogeneic donor cells inevitably raises the question as to whether these donor cells would be immunogenic, and if so, would they be rejected after transplantation. The immunogenicity of WJMSCs was firstly concerned but was not clear at present.

Two outstanding features of MSCs are relevant to immunity: (1) immunosuppression, through specific interactions with immune cells that participate in both innate and adoptive responses, and (2) the so called immunoprivilege. The mechanisms of immunoprivilege are largely unknown but are most probably due to low expression of MHC-I and MHC-II as well as the immunosuppressive functions, meaning that they do not challenge a response of allogeneic immune cells.

Immunosuppressive effects of BMSCs have been extensively studied [8,9], and tested for all species. The MSCs can reduce alloimmune responses and promote tolerance in allograft models, as in mice [10], rat [11], rabbit [12], dog, goat, swine and baboon. BMSCs were examined in skin [13], pancreas islet or heart transplantation, and most of them were shown to be effective in treatment when receiving immunosuppressive drugs, and the inferior repair results from transplantation of hMSCs in a xenogenic setting were achieved compared with autogenous MSC [12].

BMSCs could prevent graft versus host disease (GvHD) and graft rejection after hematopoietic cell transplantation (HCT), while Marco et al. [14] and Nauta AJ [15] reported the failed study in canine and mice. Marco et al. administered a combination of 3 different immortalized marrow-derived MSC lines ($15–30 \times 10^6$ MSCs/kg/day, 2–5 times/week) or third-party primary MSC (3 times/week) to canine recipients of dog-leukocyte antigen–haploidentical marrow grafts prepared with 9.2 Gy of total body irradiation. Among 14 dogs evaluable, 7 (50%) rejected their grafts and 7 engrafted with ensuing rapidly fatal acute GVHD (50%). Their data failed to demonstrate MSC-mediated protection against GVHD and allograft rejection, even the *in vitro* use showed the MSC products could suppress alloantigen-induced T cell proliferation in a dose-dependent, major histocompatibility complex–unrestricted fashion [14].

BMSCs have been used as allograft in combination with cell or organ transplants in clinical trials, and they may come from donor-derived (allogenic), or third party (allogeneic, derived from neither recipient nor donor) sources, focusing on MSCs to treat graft-versus-host disease (GvHD) and autoimmune diseases underway. Allogeneic umbilical cord blood-derived mesenchymal stem cell has been used in many clinical disease therapies [16,17]. Recently, a larger-scale placebo controlled phase III clinical trials utilize third-party BMSCs as a first and second line therapy to treat GvHD and steroid-resistant GvHD, and BMSCs treatment did correlate with a significant improvement in patients gastrointestinal GvHD [16].

In current analyses it suggests that MSCs will improve cell and solid organ transplantation by ameliorating rejection, while all transplant animals or patients still receive immunosuppressive drugs. To find more effective MSCs which can enhance the immunosuppression and eliminate the requirement for prolonged regimens of conventional immunosuppressive drugs is needed.

Neonatal and adult MSCs exhibit considerable differences in their functional abilities. But it is still unknown whether there are differences in the immunosuppressive capacity of MSCs from neonatal and adult tissues. The immune characterization of neonatal stem cells has been less reported compared with adult bone marrow stem cells [18]. Some previous studies have reported the immunosuppressive capability of cord blood stem cells (CB-SCs) and amniotic fluid MSCs, with tests in animal models [19,20]. They integrated human amniotic fluid stem cells (hAFSC) into murine lung and hAFSC can differentiate into pulmonary lineages after injury, differentiated to both bronchioalveolar and bronchiolar epithelial cell [21]. MSCs from fetal membranes of term placenta transplanted into infarcted rat heart can differentiated to vascular cells [22], and no immunorejection was found.

The immune characterization of WJMSCs has been reported by Weiss ML et al. [23]. A few previous studies have reported the comparison immunogenicity of human umbilical cord-derived MSCs (clMSCs) and adult bone marrow-derived MSCs (bmMSCs). clMSCs had significantly lower HLA-I expression, higher production of tolerogenic TGF-β and IL-10, significantly higher proliferation activity ($p = 0.01$), stronger *in vitro* activation of allogeneic lymphocytes, and delayed rejection *in vivo* [24,25]. Prasanna et al. reported different influence of IFN-γ and TNF-α on MSCs and WJMSCs [26]. Kyu Hyun Han et al. compared the immunosuppressive capacity of MSCs and ESCs, and found Granzyme B was only involved in immunosuppression by the ESCs in a perforin-independent manner [27]. But whether neonatal MSC has the similar immuno-characterization as ESC still needs to be clarified.

For tissue transplantation, MSCs were used as seed cells and usually differentiated into the linage cells for the respective needs. Do the features of immunity change during differentiation? Studies by Chen-Tao Liu et al. showed that (HLA) class I and class II molecules and the co-stimulatory protein CD80 were increased on the surface of MSCs in the course of neuronal differentiation. But neither of the co-stimulatory proteins, CD40 or CD86, was expressed, and the MSCs could suppress the proliferation of PBLs [28]. While Le Blanc K showed HLA-I was increased and HLA-II was negative when MSCs were differentiated to bone, adipose or cartilage cells [29]. Studies by Liu et al. showed that a significantly higher secretion of IL-10 by osteogenic cells differentiated from MSCs than that by undifferentiated MSCs [30]. Huang et al. reported that when MSCs were differentiated to myogenic and endothelial linages, both MHC-I and MHC-II were changed also [31]. Additionally, MSCs and differentiated MSCs pretreated with IFN-gamma expressed the different results [25–27]. However, the features of WJMSCs differentiation into cartilage lineage were not thoroughly clear.

Lower immunogenicity and stronger immunosuppressive capacity makes neonatal MSCs appear to be more viable for therapeutic approaches. Undoubtedly, there is much truth in the reports of MSC-mediated immune effects. Nevertheless, there also seems to be some conflicting data. Several challenges need to be addressed and refined, and require further study. Further work also needs to be completed to determine when to incorporate growth factors into the process to maximize their contribution to the regenerative process. Here we will systemically examine the immunosuppresion and immunoprivilege characters of WJMSCs and examine the features of immunity change during WJMSCs differentiated into cartilage cells.

2. Materials and methods

2.1. Materials, reagents and animals

Umbilical cord (from obstetrics of General Hospital with informed consent), Dulbecco modified Eagle medium (DMEM, Sigma), fetal bovine serum (Beijing Yuanheng Jinma), transforming growth factor-β1 (Pepro Tech Asia), fibroblast growth factor (Pepro Tech Asia), insulin-transferrin-Se (Sigma), dexamethasone (Sigma), agents for the histochemical staining including saffron"O", toluidine blue and alcin blue (Beijing chemical reagent company), antibodies for flow cytometry: (PE)anti-human CD29, (PE)anti-mouse/human CD44, (PE)anti-human CD71, (PE)anti-human CD105, (PE)anti-human CD80, (PE)anti-human CD86, (PE)anti-human HLA-ABC, (PE)anti-human CD45 (eBioscience), (PE)anti-human CD73, (PE)anti-human CD166, (FITC)anti-human HLA-DRDPDQ,

(PE)anti-human CD34 (BD Pharmingen), antibodies for immunohistochemical staining: anti-human CD40, anti-human CD80 (eBioscience), anti-human CD80, anti-human HLA-G(BD Pharmingen), anti-human Prostaglandin E2 (assay designs), anti-human indolamine 2,3-dioxygenase(AbD serotec), anti-human CD4, anti-human CD8(BD Pharmingen), human ribonucleoprotein antibody (Chemicon), PE-conjugated mouse anti-rabbit antibody (BD Pharmingen), Trizol Reagent (Invitrogen), chloroform, isopropyl alcohol and ethanol all from Beijing chemical reagent company, ribonuclease free water (Solarbio), M-MLV reverse transcriptase (Promega), RNase Inhibitor (TAKARA), Oligo dT(18) and primers for PCR are all synthesized by Invitrogen, Tap DNA polymerase and dNTP (TIANGEN), cytokine antibody microarray (RayBio™ Human Cytokine Array V, Angilen), antibodies for western blot: anti-human IL-1, anti-human TGF-β, anti-human VEGF, anti-human HGF (Santa Cruz Biotechnology).

2.2. Isolation and culture of hWJMSCs

In our experiments, a sum of 20 human umbilical cords has been aseptically collected from normal full-term births in maternity department of PLA General Hospital. Umbilical arteries and vein were removed, and the remaining tissues were diced into small fragments. The explants were transferred into culture flasks containing DMEM with 10% FBS and were left undisturbed for 5–7 days to allow migration of cells from the explants, when the media was replaced. Cells were re-fed and passaged as necessary. The sampling of human umbilical cords was approved by the ethics committee of PLA General Hospital and informed consent was obtained from the mothers before labor and delivery of infants.

2.3. Flow cytometry

Confluent cells were trypsinized and suspended in PBS (pH 7.4) at 5×10^6/ml, and a 100-μl sample was incubated with FITC/PE-conjugated mouse anti-human antibodies for 45 min at room temperature. Finally, they were washed twice with PBS, centrifuged and resuspended in 0.5 ml PBS. Control samples were incubated with PBS instead of antibody. A FACScan machine (Gilson, France) was used to analyze antibody binding.

2.4. Chondrogenic differentiation

Culture cells at passage 1 were incubated in chondrogenic differentiation medium (DMEM containing insulin-transferrin-selenium, TGF-β1, fibroblasts growth factor, dexamethasone, ascorbic acid, non-essential amino acids) for 14 days with the medium changed every 2–3 days. The cells, after completion of differentiation had been established by morphology, were subjected to the histochemical staining (safranine O, toluidine blue, alcin blue) and immunohistochemistry staining (collagenII) for chondrogenic induction result.

2.5. Immunocytochemistry

Staining was performed on fixed monolayers of cells grown on coverslips. The cells were washed 3 times with PBS, fixed with 95% ethanol for 10 min and then soaked in 3% hydrogen peroxide for 10 min to block the activity of endogenous peroxidase. Then the coverslips were washed 3 times with PBS and incubated with primary antibodies at 4 °C overnight and horseradish peroxidase(HRP)-conjugated rabbit anti-mouse antibody for 15 min at room temperature. Finally, the DAB coloration was carried out and the coverslips were mounted in resin and viewed by optical microscopy.

2.6. RNA extraction and RT-PCR

Total RNA was extracted from passage 2 hWJMSCs and chondrogenic differentiated cells, hWJMSC-Cs, with TRIZOL Reagent. A total RNA sample (2 μg) was reverse transcribed with M-MLV reverse transcriptase for 90 min at 42 °C in the presence of oligo-dT primer. PCR involved a mixture of 10X Taq DNA polymenrase buffer 2.5 μl, dNTP(2.5 mM each) 2 μl, cDNA 1 μl, forward and reverse primers(10 μM) 0.5 μl each, Taq DNA polymenrase 0.25 μl and deionized water to a final volume of 25 μl. cDNA was amplified in a cycler (TECHEN, TC-512). Primers for genes were as follow: CD40 (forward) 5′-TCCATCCAGAACCACCCACT-3′, (reverse) 5′-AAA GACCAGCACCAAGAGGAT-3′; CD80 (forward) 5′-ACGAGGGCACAT ACGAGTGT-3′, (reverse) 5′-AAAGCAGTAGGTCAGGCAGC-3′; CD86 (forward) 5′-CCAAAATGGATCCCCAGTGCA-3′, (reverse) 5′-TGTGAA GTCTCAGGGTCCAAC-3′; IDO (forward) 5′- CGCTGTTGGAAATAGC TTC-3′, (reverse) 5′- CAGGACGTCAAAGCACTGAA-3′; β-actin (forward) 5′-CGTGGACATCCGCAAAGAACC-3′, (reverse) 5′-ACATCTGCT GGAAGGTGGAC-3′. The PCR products were separated by electrophoresis with 1% agarose gel, stained, and photographed under ultraviolet light.

2.7. Cytokine antibody array analysis

Two cell lines of hWJMSCs, hWJMSC-1 and hWJMSC-2, and chondrogenicly differentiated cells, hWJMSC-C1 and hWJMSC-C2, were expanded to 10^7 cells and harvested at passage 2. Proteins were isolated from the cell lines in the presence of protease inhibitor and assayed for expression of a panel of 79 cytokines and growth factors with cytokine antibody array kit. The membrane was blocked with blocking buffer, incubated with the protein sample at room temperature for 1–2 h, and then washed with PBS and wash buffer. Diluted biotin-conjugated antibodies and HRP-conjugated streptavidin were sequentially added, incubated for 2 h and washed with wash buffer. After incubation with detection buffer, the membrane was exposed to X-ray films and signals were detected. Relative expression levels of cytokines were quantified by densitometry.

2.8. Western blot analysis

hWJMSC-1 and hWJMSC-2 cells were cultivated to 1×10^7 cells after 2 passages and total proteins were isolated in the presence of protease inhibitor. The proteins were separated by SDS–PAGE and transferred to PVDF filter for 2 h. Membranes were blocked with 5%BSA and incubated with antibodies against IL-10, TGF-β, HGF, VEGF and β-actin. Then they were washed and incubated with HRP-conjugated goat anti-mouse antibody, developed by KC™ chemiluminescence reagent kit (KangChen, KC-420) and visualized by autoradiography.

2.9. Cell – scaffold construction and subcutaneous implantation

Scaffolds derived from ECM of pig cartilage were prepared as previously described [32] and our experiment demonstrated that they did not cause immune response when implanted into rabbits [33]. The scaffolds were sterilized with Co60 irradiation and passage-2 hWJMSCs (1×10^7 cells/ml) were seeded onto each scaffold. The cell-scaffold constructs were cultured at 37 °C *in vitro* for 48 h before implantation to allow for complete adhesion of the cells to the scaffold. Three constructs each were implanted between the deep fascia and sarcolemma of the backs of 3 New Zealand rabbits (#1–3). The 3 implants of one rabbit were aligned in a line with a 3-cm distance between two implants. The three constructs in each rabbit were taken out respectively at 1-, 2- and 4-week after implantation for pathology and immunofluorescence analysis.

The cell-scaffold constructs were also subcutaneously implanted into the SD rat backs and cell survival and immune response were investigated by histochemistry and immunohistochemistry staining at time point of 1, 2 and 4 weeks after implantation.

3. Results

3.1. Characterization of the surface markers of cells derived from hWJ

After 5–7 days culture of umbilical cord tissue, cells began to migrate from the explants. Fig. 1a shows the phase contrast view of passage-1 hWJMSCs by optical microscopy, with the adherent cells in spindle shape. Flow cytometry revealed that the isolated cells were positive for MSC markers including CD29, CD44, CD71, CD73, CD105 and CD166 and negative for endothelial and hematopoietic markers CD34 and CD45, which indicates that the cells isolated were mesenchymal stem cells free of contamination of endothelial and hematopoetic cells (Fig. 1b) and was named hWJMSCs as reported previously [7].

3.2. Chondrogenic differentiation of hWJMSCs

After 14 days of chondrogenic induction, hWJMSCs displayed spindle, chondrocyte-like, shape. Histochemistry and immunohistochemistry shows that induced cells were strongly positive for GAG and type II collagen (Fig. 2). The image analysis software,

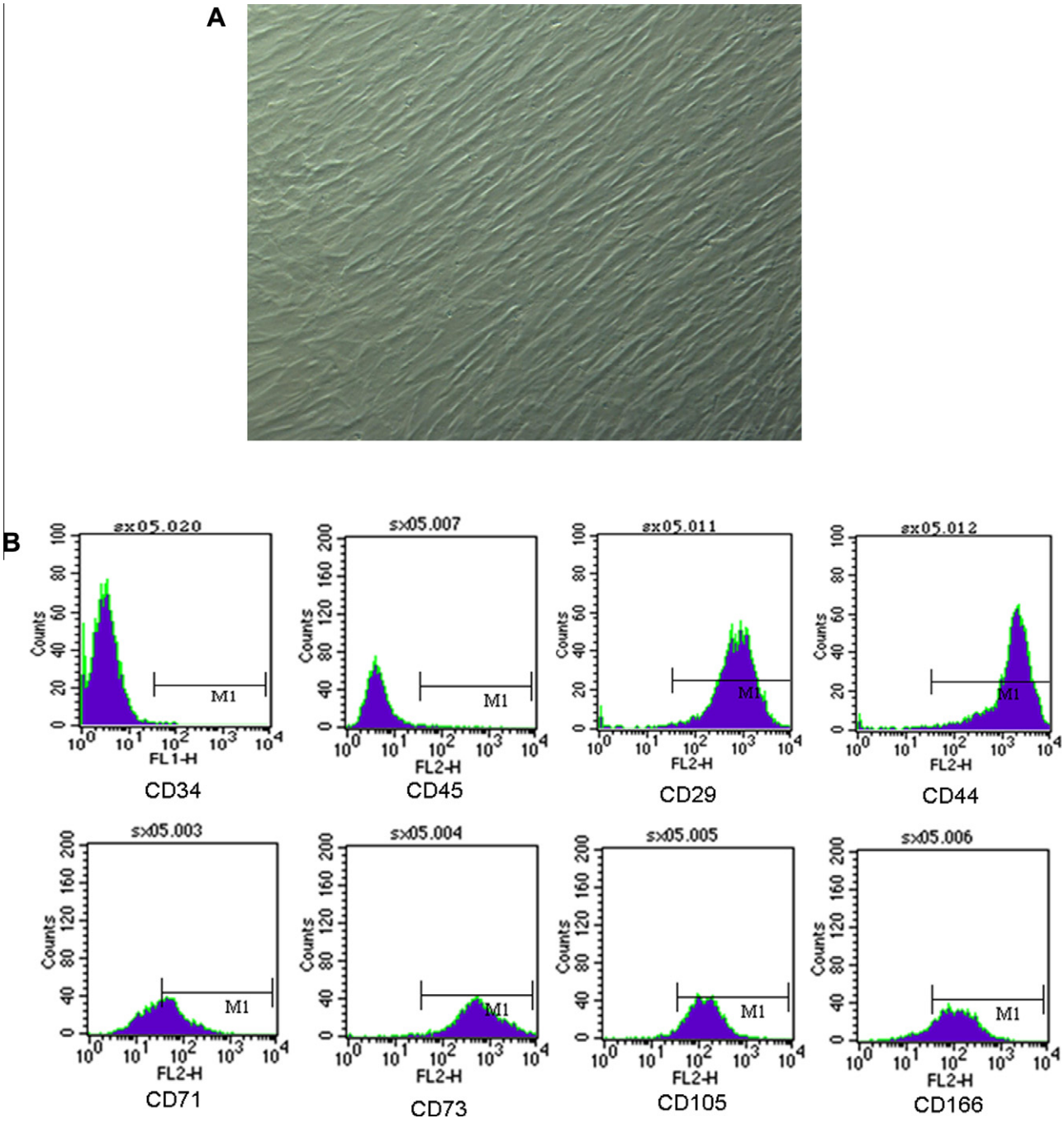

Fig. 1. Isolation and identification of Wharton's jelly derived MSCs. (A) Phase contrast view of passage 1 hWJMSCs by optical microscopy (20×); (B) Flow cytometry detection of MSC surface markers. hWJMSCs showed negative expression of hematopoietic markers CD34 and CD45, and positive expression of MSC markers CD29, CD44, CD71, CD73, CD105 and CD166.

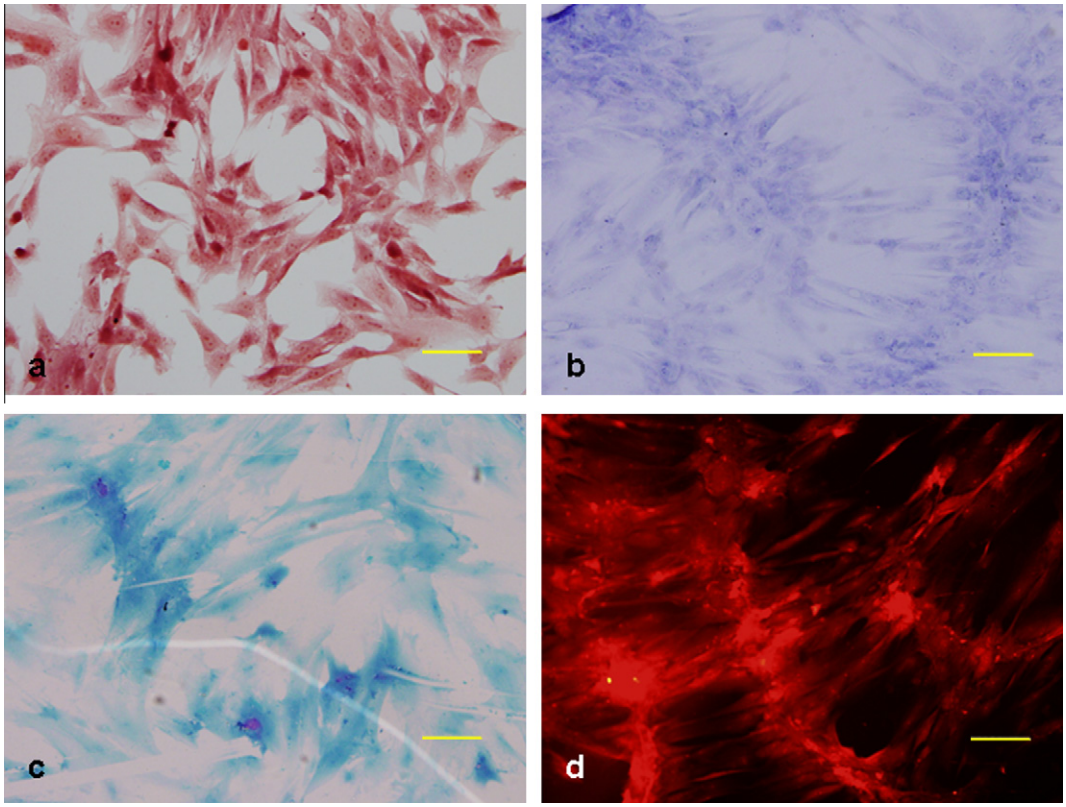

Fig. 2. Cytochemistry and immunocytochmistry of hWJMSC-derived cartilage cells (20×) Staining with (A) saffron"O", (B)toluidine blue, (C)alcin blue and (D)collagenII (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Image-Pro Plus 5.0, was used to calculate the positive staining cells, and the results of these staining revealed that the positive percentage would be more than 90%.

3.3. Expression of MHC and costimulators with hWJMSCs and hWJMSC-Cs in vitro

The results of flow cytometry showed HLA-ABC(MHC-I) was expressed in both hWJMSCs and hWJMSC-Cs (Fig. 3a). The expression of HLA-DPDQDR(MHC-II) was near negative in hWJMSCs(0.23%), and faintly positive in hWJMSC-Cs, with positive percentage up-regulated to 2.36%.

CD40, CD80(B7–1) and CD86(B7–2) are costimulatory molecules, which combine with CD28 of T cells and form the second signal necessary for activation of the T cells. The absence of these molecules causes T-cell anergy. CD80 and CD86 expression were negative in hWJMSCs and faintly positive in hWJMSC-Cs (Fig. 3a), with positive percentage from 0.44% and 0.45% to 1.19% and 2.43%, respectively. Immunocytochemistry showed CD40 faintly expressed in hWJMSC-Cs but not in hWJMSCs (Fig. 3b). RT-PCR also revealed CD80 mRNA faintly expressed in hWJMSC-Cs but not in hWJMSCs (Fig. 3c).

3.4. Production of Immunology Inhibitors by hWJMSCs and hWJMSC-Cs in vitro

HLA-G, IDO and PGE2 are molecules that can inhibit proliferation and differentiation of immune cells and induce immune tolerance. Immunocytochemistry revealed the 3 inhibitors were all positively expressed in hWJMSCs and hWJMSC-Cs, especially PGE2 (Fig. 4a). RT-PCR also verified IDO mRNA expression in both hWJMSCs and hWJMSC-Cs (Fig. 4b).

3.5. Elevated immune suppression factors in hWJMSCs

As microarray analysis showed, human WJMSCs synthesized substantial levels of all 78 factors except for IL-13 which were included in this array. The expression profile of WJMSCs-1 and WJMSCs-2 revealed the elevated level of immune suppression factors, which were TGF-β1, IL-10, VEGF, and HGF (Fig. 5a). The expression of these 4 factors in the two hWJMSC lines was verified by western blot analysis (Fig. 5b).

3.6. Reduced immune response to subcutaneous implantation with hWJMSCs loaded scaffold

To investigate the *in vivo* immune response of hWJMSCs, we seeded passage 2 cells onto the pig cartilage ECM-derived scaffold and implanted the constructs into the interspace between the deep fascia and sarcolemma of three New Zealand rabbits back. The scaffolds were studied in our previous research, and we did not found they evoke any immune response in rabbits [26]. So we applied them in our *in vivo* immune response of hWJMSCs study here. All three animals subjected to the test (#1–3) lived to the end of observation period, and also human nuclear ribonucleoprotein(hNRP) immunofluorescence positive staining cells consistently exist, which suggested no immune rejection against the implanted cells (Fig. 6). CD4 + or CD8 + T cells were found to only sparsely distribute in or around the implants during the first and second weeks after implantation, and disappeared at four weeks after implantation. HE staining results also showed cells in the scaffolds remained alive throughout the observation period. What's more, the implanted cells began to show multiple appearances after two weeks as HE staining results showed, with spindle-shape, round-shape, and epithelial-like cells, fibroblast-like cells, demonstrating

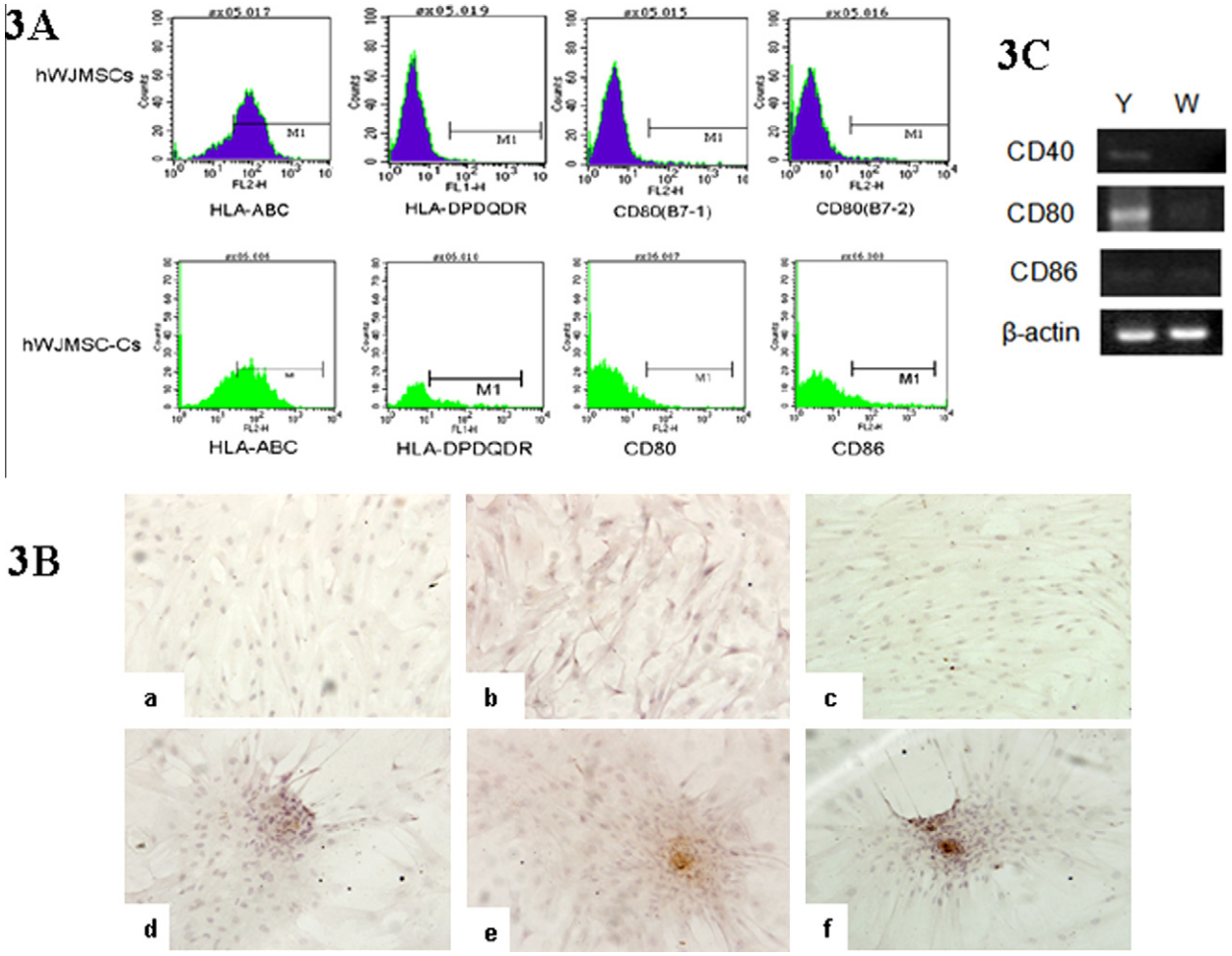

Fig. 3. (A) Flow cytometry of major histocompatibility complex (human leukocyte antigen, HLA) and costimulators of hWJMSCs and hWJMSC-Cs. The expression of HLA-DPDQDR in hWJMSCs is negative, and is faint in hWJMSC-Cs, with positive percentage up-regulated from 0.23% to 2.36%. CD80 and CD86 were negatively expressed in hWJMSCs and faintly expressed in hWJMSC-Cs, with positive percentage up-regulated from 0.44% and 0.45% to 1.19% and 2.43%, respectively. (B)Immunocytochemistry detection of costimulators in hWJMSCs and hWJMSC-Cs(20×). (a–c), hWJMSCs stained with antibodies against CD40, CD80 and CD86; (d–f), hWJMSC-Cs stained with the same antibodies. It showed CD40, CD80 and CD86 were negative in hWJMSCs, while very weakly expressed in a few hWJMSC-Cs. (C)RT-PCR detection of costimulators in hWJMSC-Cs and hWJMSCs (Y, hWJMSC-Cs; W, hWJMSCs;). Only CD80 and CD40 were expressed in hWJMSC-Cs.

multiple differentiation tendencies of hWJMSCs in the subcutaneous circumstance of rabbit backs. The scaffolds were found to degrade at two weeks post-implantation. From these above results, we can conclude that human WJMSCs do not or only faintly evoke immune rejection and can remain alive when implanted into rabbit backs, which means the cells may be a safe and promising cell candidate for cartilage or other tissue engineering.

The hWJMSCs implanted into rat backs did not induce immune response in the subcutaneous environment during the observation period. At time point of 1, 2 and 4 weeks postoperation, the implanted constructs were taken out and subjected to histobiochemistry and immunohistochemistry staining. The staining with antibody against human MHC-I antigen (Santa Cruz) showed the hWJMSCs remained alive till the last time point and constructs with chondrogenically differentiated hWJMSCs, hWJMSC-Cs, also did not evoke marked immune rejection and the cells remained alive (Fig. 7).

4. Discussion

Advances in stem cell research have provided important understanding of the cell biology and offered great promise for developing new strategies for tissue regeneration. Understanding the immune characters is essential for allotransplantation treatments in preclinical models and designing clinical protocols. In current analyses it suggests that MSCs will improve cell and solid organ transplantation by ameliorating rejection, while all transplant animals or patients still receive immunosuppressive drugs. To find more effective MSCs which can enhance the immunosuppression and eliminate the requirement for prolonged regimens of conventional immunosuppressive drugs is needed. But it is still unknown whether there are differences in the immunosuppressive capacity of MSCs from neonatal and adult tissues. In this study, we systematically investigated the immune-related molecules expression of hWJMSCs and its derived cartilage cells, hWJMSC-Cs, to reveal whether the immune properties change after the chondrogenical differentiation. Furthermore, we investigated the *in vivo* immune response of hWJMSCs in rabbits and rats, which as we know has not been reported in other literatures.

MHC is the most important mechanism that determines the matching of allografts with hosts. Our flow cytometry results showed that hWJMSCs expressed HLA-ABC (MHC-I) in a subpopulation of about 76%, but did not express HLA-DPDQDR (MHC-II) in all the cells (>99.7%). This result is similar to those with bone marrow- and adipose-derived MSCs [34,35]. The expression frequency showed no significant changes with cell passage or cryopreservation. This result differs from those with human umbilical cord perivascular (HUCPV) cells [36], as the MHC−/− phenotype of

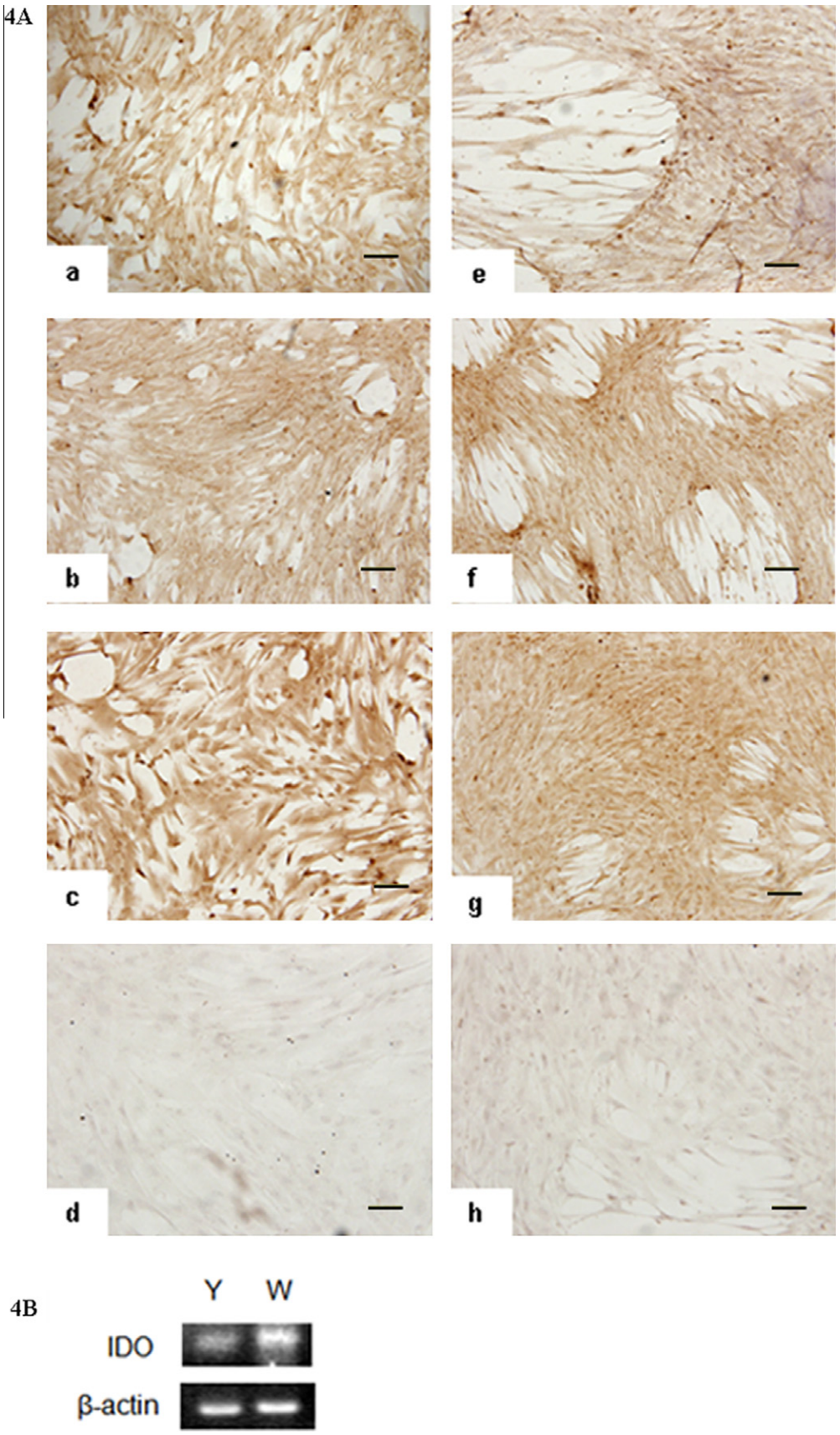

Fig. 4. (A) Immunocytochemistry detection of immunology inhibitors in hWJMSCs and hWJMSC-Cs (20×). (a–d) hWJMSC stained with antibodies of HLA-G, IDO, PGE2 and negative control; (e–g) hWJMSC-Cs stained with antibodies of HLA-G, IDO, PGE2 and negative control. It showed HLA-G, IDO and PGE2 are all positive in hWJMSCs and hWJMSC-Cs. B) RT-PCR detection of immunologic inhibitor IDO in hWJMSC-Cs and hWJMSCs (Y, hWJMSC-Cs; W, hWJMSCs;), IDO was expressed in both hWJMSC-Cs and hWJMSCs.

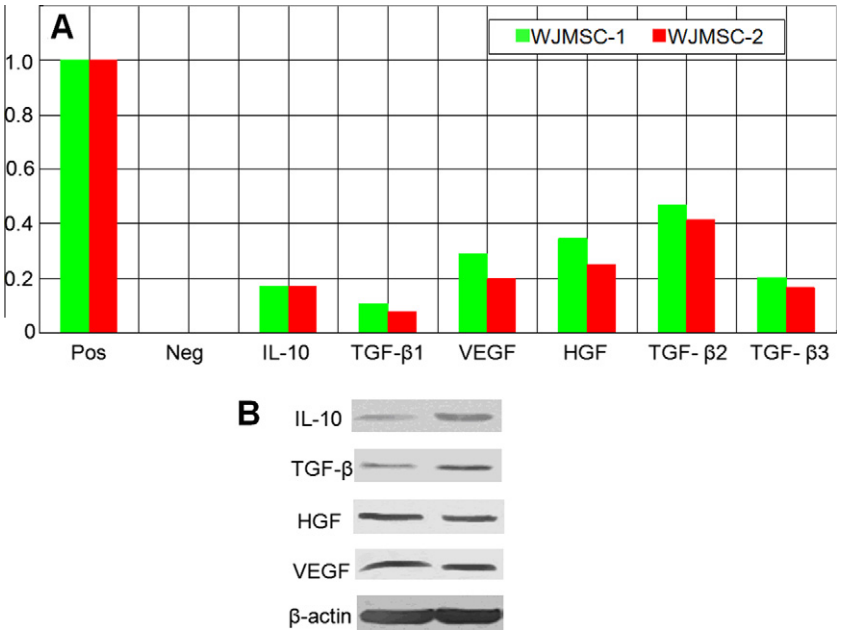

Fig. 5. Immune suppressive factors in hWJMSC cell lines, WJMSC-1 and WJMSC-2. (A) Cytokine antibody array assay shows positive expression of immune suppressive factors, IL-10, TGF-β1/2/3, VEGF and HGF. (B) Western blot verification of these 4 factors in the 2 hWJMSC cell lines.

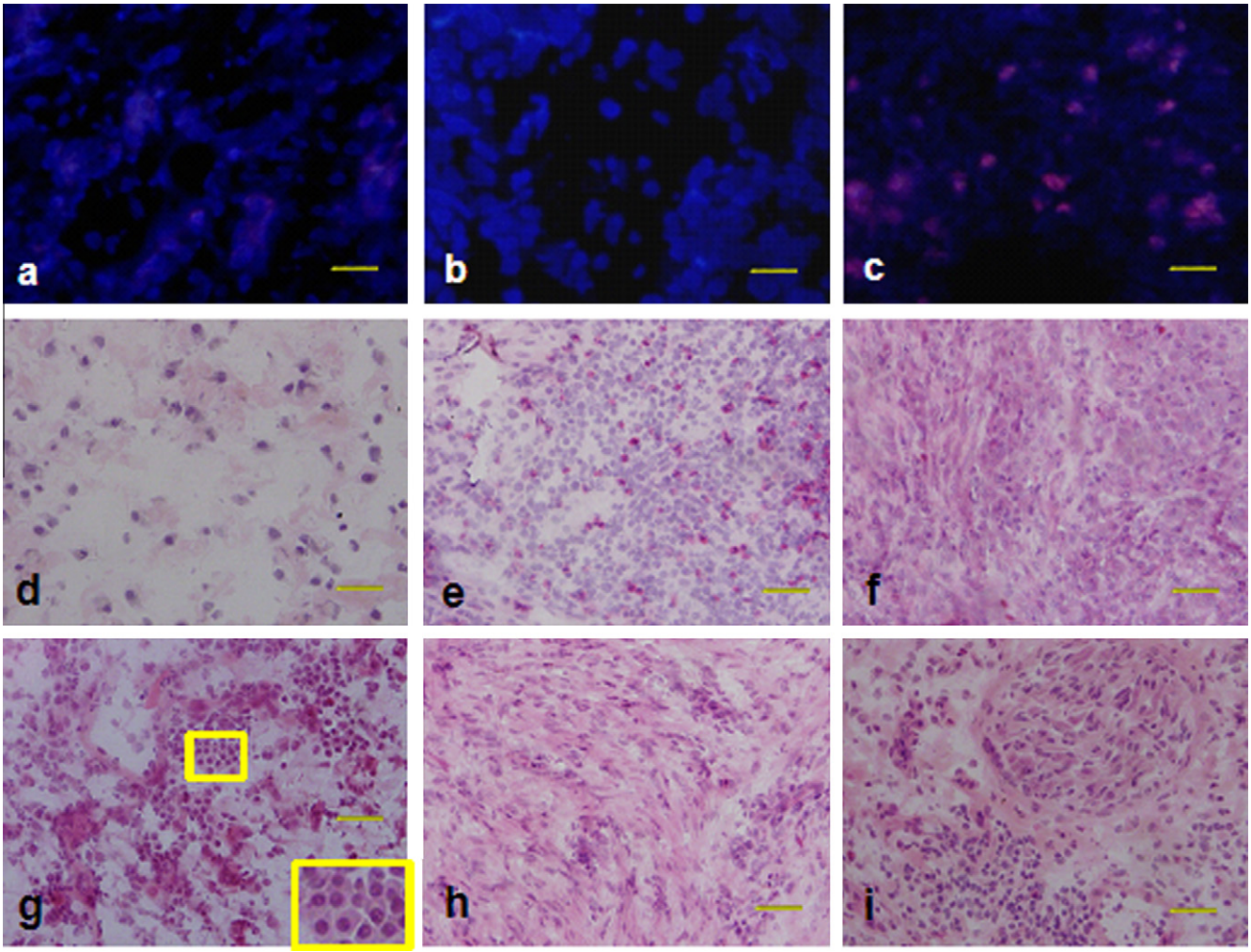

Fig. 6. Detection of the immune response against hWJMSC-scaffold constructs in rabbits with immunofluorescence and histochemistry staining. (a–c), samples from #3 rabbit (2 weeks), (a) CD4, (b) CD8, (c) human ribonucleoprotein (hRNP). It showed CD4 and CD8 lymphocyte occasionally appeared, and hWJMSC still existed; (d–f) HE staining of #3 rabbit samples at 1-, 2-, and 4-week after implantation, showing the implanted cells kept proliferating during all the observation period; (g–i) HE staining of samples at the second week, showing multiple morphologic appearance similar as epidermal or endothelial cells, fibroblasts, and fasciculus cells.

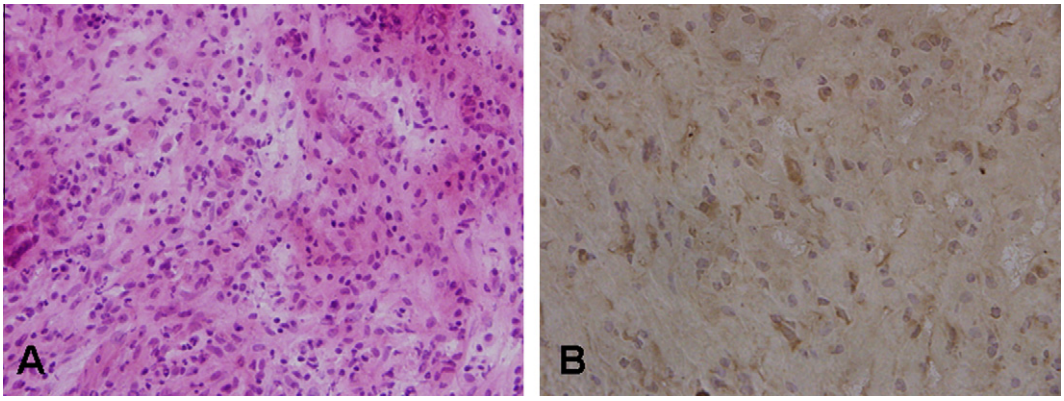

Fig. 7. Immune response to hWJMSCs and hWJMSC-Cs in rat. (A) HE staining of hWJMSC-scaffold construct (2 weeks), (B) human MHC-I antibody immunochemistry staining of hWJMSC-C-scaffold construct (4 weeks).

HUCPV cells after cryopreservation increased considerably to 65% at passage 0, 90% at passage 3, and 95% at passage 5. HUCPV cells are a morphologically homogeneous fibroblastic cell population derived from human umbilical cord perivascular section and express α-actin, desmin, vimentin, and 3G5 (a pericyte marker) in culture. And they are thought to be ancestors of adult multipotent stem cells [37,38], which may be more primitive than other mesenchymal stem cells. So this may be the reason for the increase of MHC−/− phenotype subpopulation after passage and cryopreservation.

Costimulation is another signal system, in addition to MHC, that is involved in and necessary for the activation of T cell. Our results showed negative expression of costimulators, CD40, CD80 and CD86, in undifferentiated hWJMSCs, which is consistent with the Weiss et al.'s study [13].

Several groups have demonstrated the immune inhibitors expression of WJMSCs, but the inhibitors detected were very limited [13], nor have they examined the effects of *in vivo* implantation on cellular proliferation, differentiation or long term tracking capability. In this research, we systemically detected immune inhibitors, which were most frequently studied in MSC immune moderation, such as HLA-G, IDO and PGE2 [39–46], and cytokines/growth factors with immune suppressive effect, such as IL-10, VEGF, TGF-β and HGF [47–52]. Immunohistochemistry and RT-PCR showed positive expression of HLA-G, IDO and PGE2. Antibody array assay and western blot verified expression of IL-10, VEGF, TGF-β and HGF in hWJMSCs. All these results indicated that hWJMSCs have the potential to suppress the immune rejection of the host to the allograft.

In addition to the *in vitro* study, we also fabricated constructs of hWJMSC–scaffold, with involvement of pig-cartilage-ECM, maintained in culture for 2 days and implanted into rabbits subcutaneously for 4 weeks to observe the *in vivo* immune response of the cells. Immunofluorescence results showed that CD4 + and CD8 + T were sparsely distributed in the constructs, and the implanted cells remained alive until the end of the observation period (4 weeks). Furthermore, the implanted cells showed multiple differentiation potencies in the subcutaneous circumstance of rabbit backs. The result showed hWJMSCs did not induce immune rejection in this xeno-transplatation model. Although MHC-I expression in hWJMSCs showed they were immunogenicity, they did not express co-stimulating factors and could not active T lymphocytes, in addition, the many immune inhibitors secreted may suppress the immune rejection to the cells. The *in vivo* implantation of hWJMSCs and hWJMSC-Cs into rat backs showed the same result, in which the implanted cells remained alive till the last time point (4 weeks) and there was no lymphocyte aggregating in the construct. P. Niemeyera et al. and other researchers have also reported the survival of implanted MSCs in the allogenic host environment [10–12].

Up to now, the immune characters of chondrocytes differentiated from hWJMSCs was not clear. We examined the expression of immunogen(MHC), costimulators and immune inhibitors and found that after chondrogenic differentiation, the expression of HLA-DPDQDR, CD40 and CD80 was slightly up-regulated in the cells. These results were similar to the report of Chen et al., in which they found chondrocytes differentiated from bone marrow-derived MSCs could promote the proliferation of peripheral blood derived dendritic cells, as compared with osteogenic- and adipogenic-differentiated and undifferentiated cells [53]. But the result of our *in vivo* implantation of hWJMSC-Cs into rats revealed that the chondrgenically differentiated cells did not evoke remarkable immune reaction in the host environment.

From our *in vitro* and *in vivo* studies, we considered that hWJMSCs could be a prospective kind of seed cells for cartilage and other tissue engineering for allograft with little or no immune response.

Acknowledgments

This study was funded by the National Science Foundation of China (30973047, 81000810). We thank Mr. Michael Shamtoub at University of California, Los Angeles for his great assistance in English editing of the manuscript.

References

[1] A.I. Caplan, Why are MSCs therapeutic? New data: new insight., J. Pathol. 217 (2008) 318–324.
[2] S. Sethe, A. Scutt, A. Stolzing, Aging of mesenchymal stem cells, Ageing Res. Rev. 5 (2005) 91–116.
[3] Gala K, Burdzinska A, Idziak M, Makula J, Paczek L, Characterization of bone marrow derived rat mesenchymal stem cells depending on donor age, Cell Biol. Int. 35 (10) (2011) 1055–1062.
[4] D. Baksh, R. Yao, R.S. Tuan, Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow, Stem Cells 25 (2007) 1384–1392.
[5] M.Y. Chen, P.C. Lie, Z.L. Li, et al., Endothelial differentiation of Wharton's jelly-derived mesenchymal stem cells in comparison with bone marrowderived mesenchymal stem cells, Exp. Hematol. 37 (2009) 629–640.
[6] C.Y. Fong, L.L. Chak, A. Biswas, et al., Human Wharton's jelly stem cells have unique transcriptome profiles compared to human embryonic stem cells and other mesenchymal stem cells, Stem Cell Rev. 7 (1) (2011) 1–16.
[7] D.L. Troyer, M.L. Weiss, Wharton's jelly-derived cells are a primitive stromal cell population, Stem Cells 26 (3) (2008) 591–599.
[8] W.E. Fibbe, A.J. Nauta, H. Roelofs, Modulation of Immune Responses by Mesenchymal Stem Cells, Ann. N.Y. Acad. Sci 1106 (2007) 272–278.
[9] Shyam.A. Patel, Lauren. Sherman, Jessian. Munoz, Pranela. Rameshwar, Immunological properties of mesenchymal stem cells and clinical implications, Arch. Immunol. Ther. Exp. 56 (2008) 1–8.
[10] W. Ge, J. Jiang, M.L. Baroja, J. Arp, R. Zassoko, W. Liu, A. Bartholomew, B. Garcia, H. Wang, Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance, Am. J. Transplant. 9 (2009) 1760–1772.

[11] F.C. Popp, E. Eggenhofer, P. Renner, P. Slowik, S.A. Lang, H. Kaspar, E.K. Geissler, P. Piso, H.J. Schlitt, M.H. Dahlke, Mesenchymal stem cells can induce longterm acceptance of solid organ allografts in synergy with low-dose mycophenolate, Transpl. Immunol. 20 (2008) 55–60.
[12] P. Niemeyera, K. Szalayb, R. Luginbühlc, N.P. Südkampa, P. Kasten, Transplantation of human mesenchymal stem cells in a non-autogenous setting for bone regeneration in a rabbit critical-size defect model, Acta Biomater. 6 (3) (2010) 900–908.
[13] A. Bartholomew, C. Sturgeon, M. Siatskas, K. Ferrer, K. McIntosh, S. Patil, W. Hardy, S. Devine, D. Ucker, R. Deans, A. Moseley, R. Hoffman, Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo, Exp. Hematol. 30 (2002) 42–48.
[14] Marco Mielcarek, Rainer Storb, George E. Georges, Ludmila Golubev, Alla Nikitine, Billanna Hwang, Richard A. Nash, Beverly Torok-Storb, Mesenchymal Stromal Cells Fail to Prevent Acute Graft-versus-Host Disease and Graft Rejection after Dog Leukocyte Antigen-Haploidentical. Bone Marrow Transplantation, Biol. Blood Marrow Transplant. 17 (2011) 214–225.
[15] A.J. Nauta, G.Westerhuis, A.B. Kruisselbrink, E.G. Lurvink, R. Willemze, W.E. Fibbe, Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting, Blood 15,108 (6) (2006) 2114–2120.
[16] C.R. Mills, Osiris Therapeutics Announces Preliminary Results for Prochymal Phase III GvHD, Trials 2009.
[17] M.L. Macmillan, B.R. Blazar, T.E. DeFor, et al., Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I-II clinical trial, Bone Marrow Transplant. 43 (6) (2009) 447–454.
[18] Ralf Hass, Cornelia Kasper, Stefanie Böhm, Roland. Jacobs, Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC, Cell Commun. Signal. 9 (2011) 12.
[19] Roberto Francese, Paolo Fiorina, Immunological and regenerative properties of cord blood stem cells, Clin. Immunol. 136 (3) (2010) 309–322.
[20] Meng Wang, Yuan Yang, Dongming Yang, Fei Luo, Wenjie Liang, Shuquan Guo, Xu Jianzhong, The immunomodulatory activity of human umbilical cord blood-derived mesenchymal stem cells *in vitro*, Immunology 126 (2) (2009) 220–232.
[21] G. Carraro, L. Perin, S. Sedrakyan, S. Giuliani, C. Tiozzo, J. Lee, G. Turcatel, S.P. De Langhe, B. Driscoll, S. Bellusci, P. Minoo, A. Atala, R.E. De Filippo, D. Warburton, Human amniotic fluid stem cells can integrate and differentiate into epithelial lung lineages, Stem Cells 26 (11) (2008) 2902–2911.
[22] C. Ventura, et al., Hyaluronan mixed esters of butyric and retinoic acid drive cardiac and endothelial fate in term placenta human mesenchymal stem cells and enhance cardiac repair in infarcted rat hearts, J. Biol. Chem. 282 (14) (2007) 243–252.
[23] M.L. Weiss, C. Anderson, S. Medicetty, K.B. Seshareddy, R.J. Weiss, I.V. Werff, D. Troyer, K.R. Mcintos, Immune properties of human umbilical cord Wharton's jelly-derived cells, Stem Cells 26 (2008) 2865–2874.
[24] T. Deuse, M. Stubbendorff, K. Tang-Quan, N. Phillips, M.A. Kay, T. Eiermann, T.T. Phan, H.D. Volk, H. Reichenspurner, R.C. Robbins, S. Schrepfer, Immunogenicity and immunomodulatory properties of umbilical cord lining mesenchymal stem cells, Cell Transplant. 20 (5) (2011) 655–667.
[25] M. Stubbendorff, T. Deuse, X. Hua, T. Gossler, C. Pahrmann, K.R. Tang-Quan, H. Reichenspurner, R.C. Robbins, S. Schrepfe, Immunogenicity and immunomodulatory properties of umbilical cord lining mesenchymal stem cells for regenerative therapies, J. Heart Lung Transplant. 30 (Supplement 1) (2011) 183.
[26] S. Jyothi Prasanna, Divya Gopalakrishnan, Shilpa Rani Shankar, Anoop Babu Vasandan, Pro-inflammatory cytokines, IFNγ and TNFα, influence immune properties of human bone marrow and wharton jelly mesenchymal stem cells differentially, PLoS One 5 (2) (2010) 9016.
[27] Kyu Hyun Han, Han Ro, Ju Ho Hong, Eun Mi Lee, Bumrae Cho, Hye Jung Yeom, Myung-Gyu Kim, Kook-Hwan Oh, Curie Ahn, Jaeseok Yang, Immunosuppressive mechanisms of embryonic stem cells and mesenchymal stem cells in alloimmune response, Transplant Immunol. 25 (1) (2011) 7–15.
[28] Chen-Tao Liu, Yu-Jia Yang, Fei Yin, Xia Wang, Yu Xiao-He, Qing-Hong Wang, Xiao-Li Wang, Min Xie, The immunobiological development of human bone marrow mesenchymal stem cells in the course of neuronal differentiation, Cell Immunol. 244 (2006) 19–32.
[29] K. Le Blanc, C. Tammik, K. Rosendahl, E. Zetterberg, O. Ringdén, HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells, Exp. Hematol. 31 (10) (2003) 890–896.
[30] H. Liu, D.M. Kemeny, B.C. Heng, H.W. Ouyang, A.J. Melendez, T. Cao, The immunogenicity and immunomodulatory function of osteogenic cells differentiated from mesenchymal stem cells, J. Immunol.176 (5) (2006) 2864–2871.
[31] X.P. Huang, Z. Sun, Y. Miyagi, H. McDonald Kinkaid, L. Zhang, R.D. Weisel, R.K. Li, Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair, Circulation 122 (2010) 2419–2429.
[32] Q. Yang, J. Peng, Q.Y. Guo, J.X. Huang, L. Zhang, J. Yao, F. Yang, S.G. Wang, W.J. Xu, A.Y. Wang, S.B. Lu, A cartilage ECM-derived 3-D porous acellular matrix scaffold for in vivo cartilage tissue engineering with PKH26-labeled chondrogenic bone marrow-derived mesenchymal stem cells, Biomaterials 29 (2008) 2378–2387.
[33] L. Lu, S.B. Lu, Q.Y. Guo, et al., Research of immunoreaction of heterogeneic articular cartilage extracellular matrix derived scaffold, Orthopedic J. China 01 (2010) 58–62.
[34] M.K. Majumdar, M. Keane-Moore, D. Buyaner, W.B. Hardy, M.A. Moorman, K.R. McIntosh, J.D. Mosca, Characterization and functionality of cell surface molecules on human mesenchymal stem cells, J. Biomed. Sci. 10 (2003) 228–241.
[35] P. Niemeyer, M. Kornacker, A. Mehlhorn, A. Seckinger, J. Vohrer, H. Schmal, P. Kasten, V. Eckstein, N.P. Südkamp, U. Krause, Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro, Tissue Eng. 13 (1) (2007) 111–121.
[36] R. Sarugaser, D. Lickorish, D. Baksh, M.M. Hosseini, J.E. Davies, Human Umbilical Cord Perivascular (HUCPV) Cells: A Source of Mesenchymal Progenitors, Stem Cells 23 (2005) 220–229.
[37] Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, and Pé ault B. A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. Cell Stem Cell 3 (2008) 301–313.
[38] M. Corselli, C.W. Chen, M. Crisan, L. Lazzari, B. Péault, Perivascular ancestors of adult multipotent stem cells, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 1104–1109.
[39] J.S. Hunt, D.K. Langat, R.H. McIntire, et al., The role of HLA-G in human pregnancy, Reprod. Biol. Endocrinol. 4 (Suppl 1) (2006) S10.
[40] J.S. Hunt, M.G. Petroff, R.H. McIntire, et al., HLA-G and immune tolerance in pregnancy, FASEB J. 19 (2005) 681–693.
[41] S.L. Rond, C. Azéma, I. Krawice-Radanne, et al., Evidence to Support the Role of HLA-G5 in Allograft Acceptance through Induction of Immunosuppressive/Regulatory T Cells, J. Immunol. 176 (2006) 3266–3276.
[42] A. Nasef, N. Mathieu, A. Chapel, J. Frick, S. François, C. Mazurier, A. Boutarfa, S. Bouchet, N.C. Gorin, D. Thierry, L. Fouillard, Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G, Transplantation 84 (2) (2007) 231–237.
[43] U. Grohmann, F. Fallarino, P. Puccetti, Tolerance, DCs and tryptophan: much ado about IDO, Trends Immunol. 24 (5) (2004) 242–248.
[44] P. Han, Z.L. Mi, X.T. Cao, Bone marrow-derived indoleamine-2,3-dioxygenase gene-modified dendritic cells induce transplantation immune tolerance and its mechanism, 2003, doctorial dissertation.
[45] J.W. Kang, K.S. Kang, H.C. Koo, et al., Soluble Factors–Mediated Immunomodulatory Effects of Canine Adipose Tissue–Derived Mesenchymal Stem Cells, Stem Cell Dev. 17 (2008) 681–694.
[46] S.G. Harris, J. Padilla, L. Koumas, et al., Prostaglandins as modulators of immunity, Trends Immunol. 23 (3) (2002) 144–150.
[47] M. Di Nicola, C. Carlo-Stella, M. Magni, M. Milanesi, C. Stucchi, L. Cleris, Human bone marrow stromal cells suppress T-lympjocyte proliferation induced by cellular or nonspecific mitogenic stimuli, Blood 99 (2002) 3838–3843.
[48] S. Sakaguchi, Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses, Annu. Rev. Immunol. 22 (2004) 531–562.
[49] H. Von Boehmer, Mechanisms of suppression by suppressor Tcells, Nat. Immunol. 6 (2005) 338–343.
[50] Y. Shi, G. Hu, J. Su, W. Li, Q. Chen, P. Shou, C. Xu, X. Chen, Y. Huang, Z. Zhu, X. Huang, X. Han, N. Xie, G. Ren, Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair, Cell Res. 20 (5) (2010) 510–518.
[51] Martin J Hoogduijn, Felix Popp, Richard Verbeek, Mojgan Masoodi, Anna Nicolaou, Carla Baan, Marc-H Dahlke, The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy, Int. Immunopharmacol. 10 (12) (2010) 1496–1500.
[52] Soufiane Ghannam, Carine Bouffi, Farida Djouad, Christian Jorgensen, Danièle Noël, Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications, Stem Cell Res. Ther. 1 (2010) 2, http://dx.doi.org/10.1186/scrt2.
[53] X. Chen, A. Mcclurg, G.Q. Zhou, M. Mccaigue, M.A. Armstrong, G. Li, Chondrogenic Differentiation Alters the Immunosuppressive Property of Bone Marrow-Derived Mesenchymal Stem Cells, and the Effect Is Partially due to the Upregulated Expression of B7 Molecules, Stem Cells 25 (2007) 364–370.