学位論文

Alloreactivity and immunosuppressive properties of articular chondrocytes from osteoarthritic cartilage

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Alloreactivity and immunosuppressive properties of articular chondrocytes from osteoarthritic cartilage

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ABSTRACT

Purpose. To determine whether articular chondrocytes derived from osteoarthritic knee joints could evoke alloreactive proliferation of peripheral blood mononuclear cells (PBMC) and inhibit mitogenic activity of polyclonally activated $CD4^+$ major histocompatibility complex (MHC) class II–restricted T cells *in vitro*.
Methods. Osteoarthritic cartilages of 17 patients aged 61 to 85 years were harvested during total knee arthroplasty. Chondrocytes were cultured for experiments. PBMCs, $CD4^+$ T cells, $CD8^+$ T cells, and $CD14^+$ monocytes from healthy subjects were also used. To investigate the allogeneic response and immunosuppressive properties of chondrocytes, assays for one-way mixed lymphocyte reaction (MLR), apoptosis, activated $CD4^+$ T-cell proliferation, and cytotoxic $CD8^+$ T-cells were performed. Chondrocyte cell-surface antigens were examined using flow cytometry.
Results. Chondrocytes failed to trigger an allogeneic PBMC reaction and did not induce apoptosis of allogeneic PBMCs in the MLR assay. Chondrocytes inhibited the proliferation of polyclonally activated $CD4^+$ T cells via cell-cell contact and escaped the allogeneic cytotoxic reactivity of $CD8^+$ T cells. Chondrocytes expressed MHC class I but not MHC class II molecules or B7-1/-2–positive co-stimulatory molecules.
Conclusion. Chondrocytes from osteoarthritic knees in older patients exhibited similar immunomodulatory properties *in vitro* to those in juveniles or adults.

Key words: *chondrocytes; immune system; osteoarthritis*

INTRODUCTION

Articular cartilage injury can progress to irreversible degradation and osteoarthritis if left untreated. Fresh-frozen osteochondral allografts can be used to repair large articular cartilage defects. Allogeneic or xenogeneic whole cartilage can remain viable for a long period but is eventually rejected.[1] This may be due to the lack of vessels and lymph ducts, as well as the thick extracellular matrix that prevent host immune recognition and contact with immunocompetent

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cells.[2–4] Allogeneic juvenile cartilage (DeNovo NT, Zimmer, Warsaw [IN], USA) and allogeneic juvenile 3-dimensional neocartilage (RavaFlex/DeNovo ET, ISTO Tec, St Louis [MO], USA) have been used for cartilage repair,[5–7] but their immune response remains unclear. The use of autologous chondrocyte implantation (ACI) is limited by the waiting period for cell culture and low cell number and density for large defects or subsequent surgeries. Allogeneic chondrocyte implantation can solve these problems, but the immunogenicity of chondrocytes remains controversial. Human articular chondrocytes suppress proliferation of activated peripheral blood mononuclear cells (PBMC),[8] and are non-alloreactive in an mixed lymphocyte reaction (MLR) assay under serum-free conditions, and exert immune modulatory functions to inhibit the proliferation of polyclonally activated $CD4^+$ T cells via cell-cell contact.[9–13] These chondrocytes express negligible levels of major histocompatibility complex (MHC) class II molecules and lack B7-1 and B7-2 antigens, which are positive co-stimulators of $CD4^+$ T-cell proliferation.[9–13] This suggests that the presence of negative co-stimulatory molecules is indicative of immune privilege in chondrocytes. Nonetheless, expression of MHC molecules by transplanted allogeneic chondrocytes is upregulated at an early stage, and the tissue-fluid environment *in vivo* affects their immunological properties.[14]

This study aimed to determine whether articular chondrocytes derived from osteoarthritic (OA) knee joints could evoke alloreactive proliferation of PBMCs and inhibit mitogenic activity of polyclonally activated $CD4^+$ MHC class II–restricted T cells *in vitro*.

MATERIALS AND METHODS

This study was approved by the ethics committee of our hospital (No.162-1400).

Chondrocyte culture

OA cartilages of 17 patients aged 61 to 85 years who had International Cartilage Repair Society grade 0 or 1 OA knees were harvested during total knee arthroplasty. The cartilage was minced and digested by sequential treatment with Pronase for 90 minutes, and then incubated overnight in HL-1 medium (BioWhittaker) with 0.5 mg/ml CLS4 collagenase (Worthington), 0.3 mg/ml hyaluronidase (type IV hyaluronidase; Sigma), and 50 μg/ml ascorbic acid. After enzymatic dissociation, the chondrocytes were cultured in Dulbecco's Modified Eagle's Medium containing 10% foetal bovine serum and used for experiments at passage 1 or 2.

Purification of $CD14^+$ monocytes and $CD4^+CD14^-$ T cells

PBMCs from healthy volunteers were isolated and enriched using Histopaque-1077 (Sigma). $CD4^+CD14^-$ T cells ($CD4^+$ T cells), $CD8^+$ T cells, and $CD14^+$ monocytes were isolated using the magnetic-activated cell sorting system, and microbeads conjugated to anti-human CD4, CD8, and CD14 mAbs, respectively.

Mixed lymphocyte reaction assay

Alloreactivity was examined using the MLR assay. Chondrocytes were stimulated by irradiation with 3000 rad. As a positive control for stimulation, $CD14^+$ monocytes were irradiated with 1500 rad, and then stimulated with 200 U/ml of interferon-γ and 1 μg/ml of lipopolysaccharide as described previously.[15] Next, 4×10^3, 2×10^4, or 1×10^5 stimulator cells and 5×10^5 PBMCs (as responder cells) were co-cultured in triplicate for 5 days in 96-well flat bottom plates with AIM-V serum-free media (BioWhittaker). [^{3}H] thymidine (1 μCi/well; Amersham) was added to the culture medium for the last 18 hours, and lymphocyte (responder cell) proliferation was measured based on radiolabel incorporation. Results were expressed as counts per minute, and a stimulator index of >10 was considered a positive response.

Cell-cycle analysis by flow cytometry

Cell-cycle analysis was performed by propidium iodide staining followed by flow cytometry. PBMCs were fixed with ice-cold 70% ethanol and resuspended in 300 μl of phosphate-buffered saline containing 200 μg/ml RNase A and 25 μg/ml propidium iodide staining. The mixture was incubated at room temperature in the dark for 30 minutes. Nuclear changes were detected by flow cytometry on the FACSCalibur (Becton Dickinson). Analysis was made using the CellQuest software. Cells that exhibited DNA fragmentation were considered to be dead.

Activated lymphocyte proliferation assay with or without cell-cell contact

To measure the immunosuppressive properties of chondrocytes, purified $CD4^+$ T cells were activated by simultaneous ligation of the T-cell receptor complex and co-stimulatory molecules with 10 ng/ml CD3 mAb and 5 μg/ml CD28 mAb as previously

described.[12] Irradiated chondrocytes were co-cultured with activated lymphocytes at responder/stimulator ratios ranging from 1/1 to 25/1. After 3 days of co-culture, proliferation of activated lymphocytes was measured using the MLR assay. To disrupt cell-cell contact, chondrocytes and activated $CD4^+$ T cells were co-cultured separately in the Transwell system for 3 days, after which proliferation of activated $CD4^+$ T cells was measured in a similar manner.

Cytotoxicity assay

Allogeneic PBMCs and $CD8^+$ T cells were prepared as effector cells. Chondrocytes were labelled with ^{51}Cr for 4 hours and used as target cells. Effector and target cells were incubated in triplicate cultures in the AIM-V medium at effector/target cell (E/T) ratios of 5/1 or 1/1. Briefly, 1×10^5 target chondrocytes were incubated with 5×10^5 PBMCs (E/T ratio, 5/1) or 1×10^5 $CD8^+$ T cells (E/T ratio, 1/1). After a 4-hour incubation, supernatants were collected, and the amount of ^{51}Cr released from targets destroyed by effectors was measured using a gamma counter. To measure the amount of ^{51}Cr that was spontaneously released from the targets, chondrocytes were incubated alone in medium. To determine the maximum isotope that could be released from the target cells, chondrocytes were incubated in medium containing 1% Triton X-100. In general, maximum release should be at least 3 times greater than spontaneous release.

Flow cytometry

To examine MHC class I and II expression on chondrocytes, cell-surface molecules were examined by immunofluorescence. FITC-conjugated mouse anti-human HLA-ABC mAb (IOTets), purified mouse anti-human HLA-DR, DP, and DQ mAbs (BD Pharmingen), and fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories) were used. To confirm expression of B7-1 (CD80) and B7-2 (CD86) antigens on chondrocytes, FITC-conjugated mouse anti-human CD-80 mAb (IOTets) or PE-conjugated mouse anti-human CD-86 mAb (IOTets) was used. Non-specific mouse IgG (R&D Systems) was used as an isotype-specific negative control. Quantitative flow-cytometric analyses were performed on the FACSCalibur. A total of 10 000 live cells were examined, and data were analysed using the CellQuest software.

Statistical analysis

Comparisons were made using the Student's *t*-test. A p value of <0.05 was considered statistically significant.

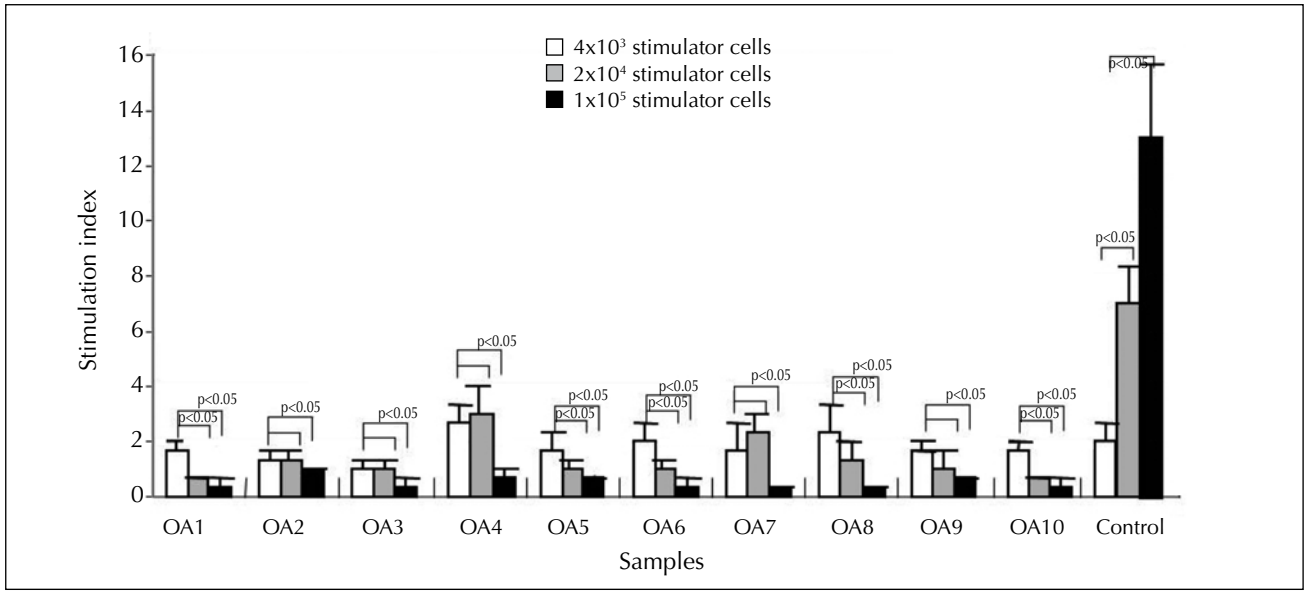

Figure 1 Mixed lymphocyte reaction assay: 5×10^5 allogeneic peripheral blood mononuclear cells (PBMCs) [responder cells] are cultured with 4×10^3, 2×10^4, or 1×10^5 chondrocytes or positive-control cells (stimulator cells) for 5 days. The responder/stimulator cell ratios range from 125/1 to 5/1. Proliferation of allogeneic PBMCs is calculated as the stimulation index of triplicates in 10 samples (OA 1–10). A stimulation index of >10 is defined as a positive allogeneic reaction.

RESULTS

Chondrocytes fail to elicit an allogeneic response

In contrast to the potent stimulatory capacity of $CD14^+$ cells, chondrocytes failed to elicit a dose-dependent positive reaction (Fig. 1). This indicated that chondrocytes could elude immune surveillance at a responder/stimulator ratio of 1/5.

Chondrocytes not induce apoptosis of allogeneic PBMCs

Based on their DNA content, cells were categorised as M1 (apoptotic), M2 (G0/G1 phase), or M3 (S to M phase). Their respective percentages were 5.9±0.1%, 84.3±0.4%, and 1.1±0.2% for PBMCs cultured alone, and 0.8±0.2%, 93.6±2.8%, and 0.7±0.5% when 5×10^5 PBMCs were co-cultured with 1×10^5 chondrocytes (Fig. 2). The percentages of PBMCs in respective categories did not differ significantly in cells cultured with or without chondrocytes, nor did the percentages of apoptotic cells. This indicated that OA chondrocytes did not induce apoptosis of allogeneic PBMCs in co-culture.

Chondrocytes inhibit proliferation of activated $CD4^+$ T cells via cell-cell contact

Juvenile and adult chondrocytes suppress immune responses in a dose-dependent manner *in vitro*.[12,15] Purified $CD4^+$ T cells were activated by cross-linking the T-cell receptor/CD3 complex with anti-CD3 mAb and the B7–1/B7–2 receptor (CD28) with anti-CD28 mAb. This combination of antibodies strongly activated $CD4^+$ T cells, which then proliferated. Its proliferation ratio (the rate of proliferation in the presence of chondrocytes divided by that without chondrocytes) was >1 (accelerated) when 1×10^5 activated $CD4^+$ T cells were cultured with a smaller number (4×10^3 or 2×10^4) of chondrocytes, and <1 (inhibited) when 1×10^5 activated $CD4^+$ T cells were cultured with an equal number of chondrocytes (Fig. 3). When there was no cell-cell contact, $CD4^+$ T cell proliferation was restored (proliferation ratio >1). This indicated that OA chondrocytes inhibited proliferation of activated $CD4^+$ T cells *in vitro* via a cell-cell contact mechanism. Chondrocytes might also secrete a soluble factor that promoted T-cell proliferation because of the proliferation ratio >1.

Chondrocytes fail to elicit allogeneic $CD8^+$ T cell reactivity

$CD8^+$ T cells are cytotoxic and directly destroy their target cells. When 1×10^5 chondrocytes were incubated with 5×10^5 PBMCs (E/T ratio, 5/1) or 1×10^5 $CD8^+$ T cells (E/T ratio, 1/1) for 4 hours, the rate of release of ^{51}Cr from chondrocytes was not significantly higher than the rate of spontaneous release from chondrocytes cultured alone (Fig. 4). This indicated that chondrocytes failed to elicit allogeneic $CD8^+$ T-cell reactions and eluded MHC class I–restricted immune surveillance.

Chondrocytes express MHC class I antigens

Flow-cytometric analysis of 10 samples demonstrated that 80.7±8.8% of OA chondrocytes expressed MHC class I antigens—HLA-ABC, whereas only 1.9±1.5% of

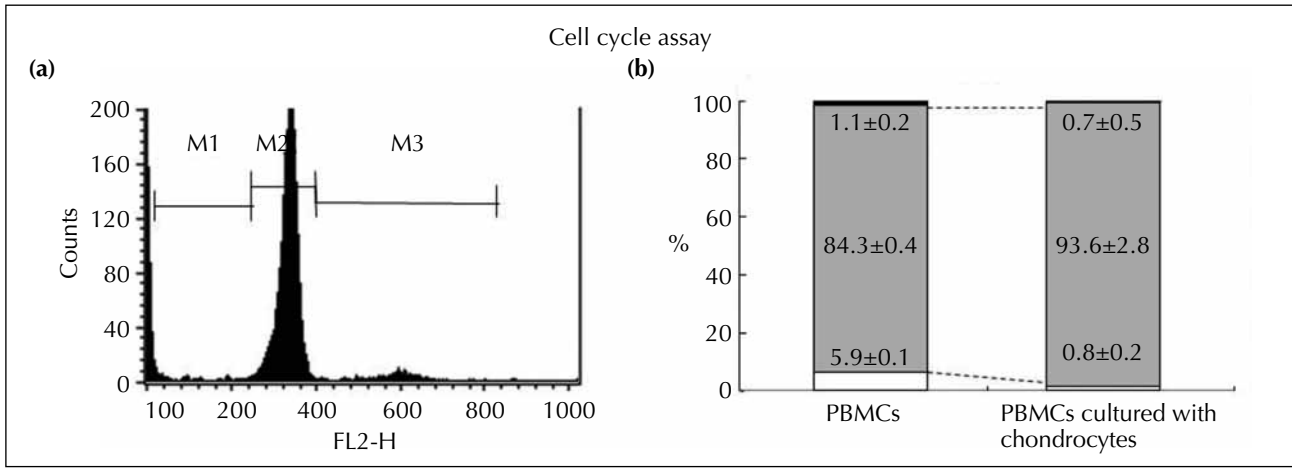

Figure 2 Apoptosis assay: **(a)** a representative histogram of peripheral blood mononuclear cells (PBMCs) DNA content showing M1 (apoptotic cells), M2 (G0/G1 phase), and M3 (S to M phase). **(b)** The respective percentages of the 2 groups are comparable; chondrocytes do not induce apoptosis of PBMCs.

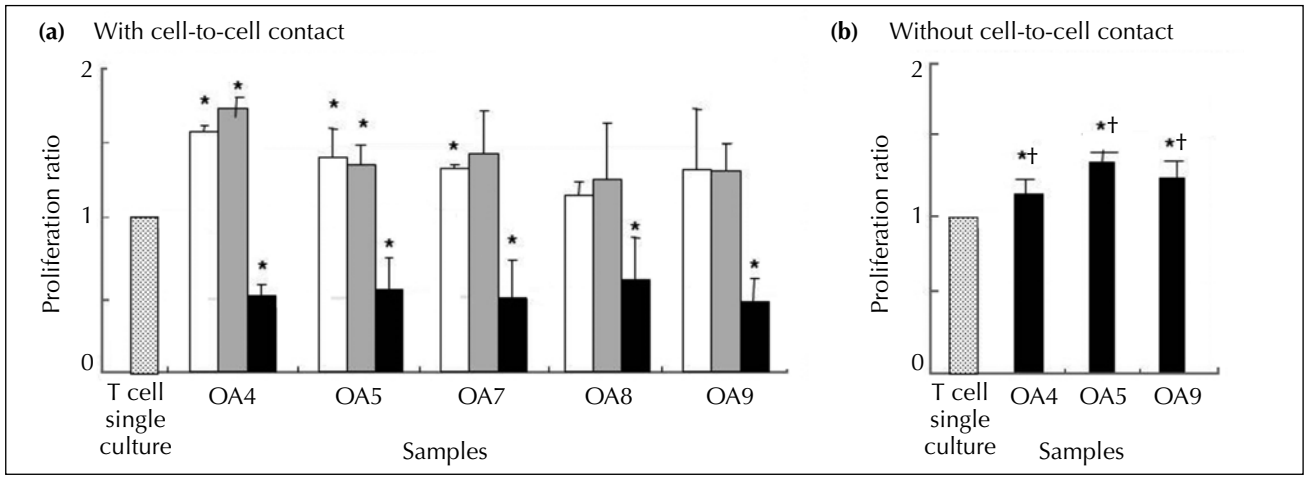

Figure 3 Activated CD4⁺ T cell proliferation assay **(a)** with or **(b)** without cell-cell contact: 1×10^5 activated allogeneic CD4⁺ T cells are cultured alone or with 4×10^3 (white bar), 2×10^4 (gray bar), or 1×10^5 (black bar) chondrocytes for 3 days in samples. The proliferation ratio of activated CD4⁺ T cells cultured alone is defined as one and compared with other samples.
* denotes $p<0.05$ vs. CD4⁺T cells cultured alone
† denotes $p<0.05$ vs. with cell-cell contact

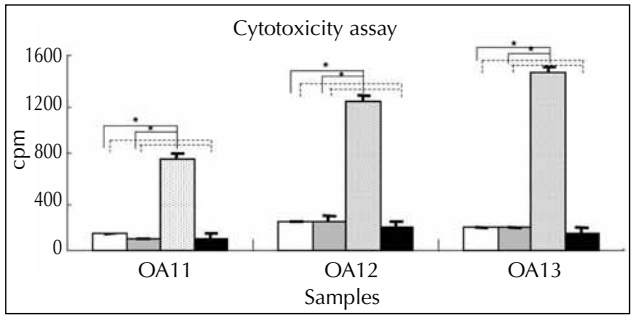

Figure 4 Cytotoxicity assay: 1×10^5 chondrocytes (target cells) are incubated with 5×10^5 peripheral blood mononuclear cells (PBMCs) [effector cells) for 4 hours (effector/target cell ratio, 5/1) or 1×10^5 CD8⁺ T cells (effector/target cell ratio, 1/1), and the amount of released ^{51}Cr is measured in 3 samples. White and gray bars indicate chondrocytes that were cultured with allogeneic PBMCs and CD8⁺ T cells, respectively. Black dots indicate the maximum release when all chondrocytes are lysed with detergent. Black bars indicate spontaneous release when chondrocytes are cultured alone. There is no significant difference in the amount of ^{51}Cr release between chondrocytes cultured with allogeneic PBMCs or CD8⁺ T cells and chondrocytes cultured alone.
* denotes $p<0.01$ vs. maximum release when chondrocytes are lysed

the chondrocytes expressed MHC class II antigens—HLA-DP, -DQ, and -DR, and only 0.4±0.7% and 0.3±0.5% of the chondrocytes expressed B7-1 (CD80) and B7-2 (CD86), respectively (Fig. 5).

DISCUSSION

Articular chondrocytes function as accessory cells for immune cells.[15,16] Alloreactivity of chondrocytes remains unclear.[17–19] Articular chondrocytes express molecules involved in heterotypic cell-cell interactions such as ICAM-1 and VCAM-1, MHC class I antigens, and MHC class II antigens under specific conditions,[20–23] suggesting that articular chondrocytes can function as accessory cells for immune cells. If articular chondrocytes function as accessory cells, xenogeneic antigens in the serum could affect the MLR assay that used medium containing xenogeneic bovine serum. To avoid these potential effects, serum-free conditions are recommended.[24] In our study, serum-free medium was used in MLR assays.

To investigate immunomodulatory function, activated lymphocyte proliferation assays were used to compare direct co-culture versus Transwell cultures. OA chondrocytes inhibited proliferation of activated CD4⁺ T cells via cell-cell contact. This finding is consistent with that for juvenile and adult healthy chondrocytes in previous studies.[9–13] In contrast to the situation with juvenile cells, a large number of OA chondrocytes (chondrocyte/T-cell ratio of 1/1) was required to inhibit the proliferation of active CD4⁺ T cells. The contact requirement indicated that the surface molecules of chondrocytes play an important role in immunomodulatory function. We propose 3 potential mechanisms: (1) the presence of an inhibitory cell-surface molecule, (2) extracellular matrix production by chondrocytes results in transduction of negative proliferation signals to CD4⁺ T cells, and (3) secretion of an extracellular matrix–binding protein by chondrocytes that negatively affects CD4⁺ T-cell proliferation (Fig. 6).

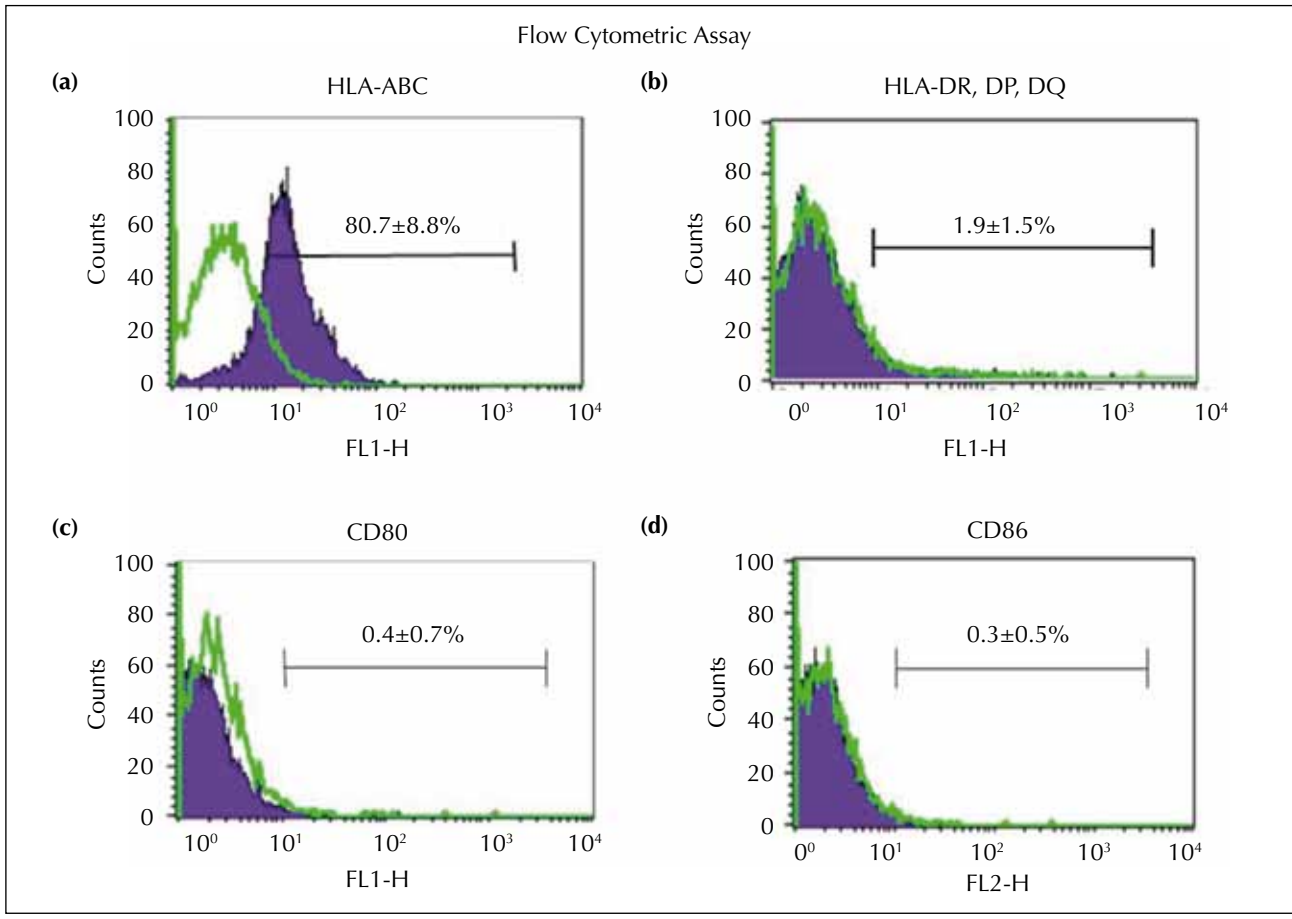

Figure 5 Flow-cytometric assay: filled bars represent **(a)** HLA-ABC; **(b)** HLA-DP, -DQ, and -DR; **(c)** CD80; and **(d)** CD86 staining. Open bars represent isotype control staining and the negative control.

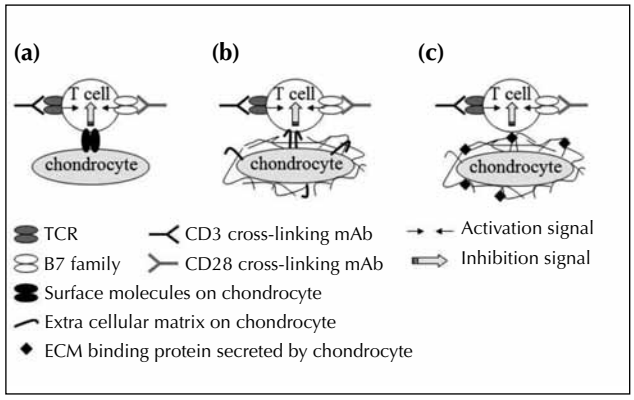

Figure 6 Potential mechanisms of inhibition by articular chondrocytes: **(a)** surface molecules on chondrocytes transduce signals to $CD4^+$ T cells that negatively influence proliferation; **(b)** extracellular matrix produced by chondrocytes transduces negative proliferation signals to $CD4^+$ T cells; and **(c)** extracellular matrix–binding protein secreted by chondrocytes negatively affects $CD4^+$ T-cells proliferation.

To distinguish these mechanisms, cell-surface molecules were examined by flow cytometry. $CD8^+$ and $CD4^+$ T cells are respectively restricted by MHC class I and MHC class II. The inhibition of proliferation of activated $CD4^+$ T cells could be due to their negligible expression of MHC class II and B7-1/-2. Even when MHC class II or B7-1 and B7-2 were transfected into mesenchymal stem cells (MSCs), they still failed to elicit an allogeneic T-cell reaction.[25,26] These results indicated that the lack of MHC class II and B7-1/-2 was not critical to the immunomodulatory mechanism. Immune-inhibitory B7 molecules such as B7-DC, B7-H1, and B7-H4 are expressed in both juvenile and adult chondrocytes.[9–13] Chondrocytes express other negative regulators of immune responses, including chondromodulin-I and indoleamine 2,3 dioxygenase (IDO).[9] In our study, the percentage of apoptotic lymphocytes did not increase, whereas the percentage of PBMCs in each category did not differ significantly ($p>0.05$) in cells cultured

in the presence or absence of chondrotyes. Therefore, the surface molecules that induce lymphocyte apoptosis and destruction, such as B7-DC and B7-H1, are not primary candidates for immunomodulation produced by chondrocytes.

Intramuscularly transplanted cartilage in syngeneic rats is rejected in sensitised recipient rats previously exposed to allogeneic chondrocytes.[27] Cartilage fragments generated from syngeneic rat chondrocytes and transplanted into the articular surface are rejected in rats sensitised with allogeneic chondrocytes.[27] The synovium around the rejected fragment contains $CD8^+$ lymphocytes and macrophages.[28] This indicates that chondrocyte-specific antigens and $CD8^+$ T cells may play a role in rejection. Nonetheless, in our study, OA chondrocytes failed to elicit allogeneic PBMC and $CD8^+$ T-cell reactivity (E/T ratios of 1/5 and 1/1). Chondrocytes could elude MHC class I–restricted immune surveillance. Natural killer cells may also exert cytotoxic functions.

Transplantation of isolated allogeneic chondrocytes evokes a specific humoral and cellular immunological response.[28–30] Infiltrating immune cells on the subchondral side of the transplant gradually destroy the reformed cartilage.[31,32] Species and the technical details of chondrocyte isolation might influence the immunological response of the host animals.[33,34] The immune privilege of articular cartilage depends on the donor source and transplant location.[35] Therefore, to assess the safety of allogeneic chondrocyte implantation, additional *in vivo* studies are needed.

MSCs have immunomodulatory properties, including the ability to reduce immune cell infiltration and modulate inflammation. Their immunosuppressive ability is attributed to the secretion of immunomodulatory molecules, including prostaglandin E2, nitric oxide, IDO, and TGF, that suppress the proliferation and activation of immune cells including $CD4^+$ T, $CD8^+$ T, natural killer, B, and antigen-presenting cells. Allogeneic mesenchymal stromal cells have been investigated in the context of cartilage repair.[36] Nonetheless, chondrogenic differentiation increases the immunogenicity of MSCs, and there is an increase in their expression of MHC class I, class II, and B7-1/-2.[37] Therefore, the safety of allogeneic MSC transplantation for cartilage repair remains in doubt.

This study had limitations. Traditional methods were used, and the error of the thymidine uptake assay is larger than that of the MTT assay, as cells may be lost during collection in the thymidine uptake assay.[38] Furthermore, blast cells fail to incorporate sufficient amounts of ^{51}Cr and/or spontaneously release high levels of ^{51}Cr. The lactate dehydrogenase–release assay should have been used as a cytotoxic assay as in a previous study.[39] Negative controls using autologous lymphocytes may have enabled a more straightforward conclusion if ethic approval was obtained.

CONCLUSION

Cultured OA chondrocytes (1) expressed MHC class I but not MHC class II or positive co-stimulatory molecules, (2) failed to evoke both MHC class I and II mismatch allogeneic lymphocyte responses, and (3) inhibited proliferation of activated $CD4^+$ T cells via cell-cell contact. Therefore, chondrocytes preserved their immunological characteristics *in vitro*, regardless of ageing or degenerative condition.

DISCLOSURE

No conflicts of interest were declared by the authors.

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