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Original Article

Immunogenicity of Bovine and Leporine Articular Chondrocytes and Meniscus Cells

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Immune rejection is a major concern for any allogeneic or xenogeneic graft. For *in vivo* investigations of cartilage tissue engineering strategies, small animal models such as the leporine model are commonly employed. Many studies report little to no immune rejection upon allogeneic or xenogeneic implantation of native articular and meniscal cartilages. This study investigated whether bovine and leporine articular chondrocytes (ACs) and meniscus cells (MCs) have immunoprivileged characteristics because of their ability to stimulate proliferation of leporine peripheral blood mononuclear cells (PBMCs) *in vitro*. After 6 days of co-culture, none of the cell types caused a proliferative response in the leporine PBMCs, indicating that these cells may not elicit immune rejection *in vivo*. Reverse transcriptase polymerase chain reaction analysis for major histocompatibility complex class (MHC) I and II and costimulation factors CD80 and CD86 revealed that all cell types produced messenger RNA for MHC I and II, but only some were CD80 or CD86 positive, and none were positive for both costimulation factors. Flow cytometry found that bovine MCs and ACs displayed MHC II (MCs: 32.5%, ACs: 14.4%), whereas only leporine ACs were MHC II positive (7.5%). Although present in isolated cells, MHC I and II were not observed in intact bovine or leporine hyaline cartilage or meniscus tissues. Despite some presence of MHC II and costimulation factors, none of the cell types studied were able to cause PBMC proliferation. These findings indicate that bovine and leporine MCs and ACs share a similar immunoprivileged profile, bolstering their use as allogeneic and xenogeneic cell sources for engineered cartilage.

Introduction

Because of their lack of vasculature and relative acellularity, articular cartilage lining the ends of long bones and the hyaline-like cartilage of the inner meniscus have little capacity to self-repair after injury.[1–8] Although tissue engineering strategies are being developed to address this problem, they often require the use of large numbers of primary cells.[9–11] Donor site morbidity and the lack of available tissue render autologous techniques for cartilage tissue engineering prohibitive, so focus has increasingly been on the development of allogeneic and xenogeneic approaches. A major concern with any allogeneic or xenogeneic implant is immune rejection, resulting in a breakdown of the implanted material over time.[12]

Typically, T cell sensitization triggers an immune response to implanted tissue, followed by activation. Sensitization occurs when T-cell receptors (TCRs) CD8 and CD4 recognize antigens present on donor cells, specifically major histocompatibility complex class (MHC) I and II, , respectively.[13,14] T-cells become activated when costimulatory binding of donor cell B7 antigens (CD80 or CD86) with T-cell receptor CD28 happens simultaneously, causing proliferation of the T-cell and initiation of an immune response to destroy the foreign material.[15–17] Therefore, the MHCs and B7 antigens present on donor cells are important constituents involved in immune rejection of implanted tissue engineered constructs.

Mounting evidence suggests that cartilaginous tissues are immunoprivileged, causing little to no immune response when implanted.[18–23] Although the precise reasons for the immunoprivileged nature of cartilage tissue are not well understood, cartilage cells and extracellular matrix (ECM) seem to play a role in inhibiting an immune reaction. Flow cytometry analysis of human and sheep articular chondrocytes (ACs) has shown that these cells present MHC I antigens but not MHC II, CD80, or CD86. In addition to lacking some important immunogenic surface markers, these cells are unable to promote allogeneic T-cell proliferation *in vitro*.[24–26] Moreover, studies testing allogeneic (human and leporine) and xenogeneic (porcine to leporine and leporine to caprine) implantation of ACs support these *in vitro* experiments, reporting that they produce little to no immune response.[27–31] A comparison of the literature results suggests that the degree of immune reaction to implanted cartilage

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material is inversely related to the amount of ECM it contains, indicating that, along with lacking critical surface markers involved with the induction of an immune response, cartilage ECM may shield immunogenic markers on chondrocytes from host T-cells, enhancing the reparative capacity of these therapies.[31]

Although little investigation has been made into the immunogenicity of meniscus cells (MCs), some evidence suggests that they may share an immunoprivileged profile similar to that of chondrocytes. When used to fill an articular cartilage defect in the leporine model, allogeneic and xenogeneic (bovine) meniscus tissue failed to elicit a measurable immune response.[30] Given these promising results, it is possible that the meniscus may provide an abundant cell source for allogeneic and xenogeneic tissue engineering strategies that avoids for the problem of immune rejection.

A variety of cartilage engineering strategies could benefit from using an abundant allogeneic or xenogeneic cell source. One promising strategy used in cartilage and meniscus tissue engineering is the self-assembly method, under which ACs are seeded alone or in cocultures with MCs to produce functional articular cartilage and fibrocartilage replacements.[32–36] Although these self-assembled constructs show great promise biochemically and biomechanically, the potential immunogenicity of these highly cellular constructs in an allogeneic or xenogeneic animal model is unknown. Therefore, this study investigates the immunogenicity of bovine and leporine ACs and MCs in a rabbit model. The ability of cartilaginous cells to induce proliferation of leporine peripheral blood mononuclear cells (PBMCs) in a mixed lymphocyte reaction (MLR) test determines immunogenicity. In addition, the presence of MHC II is assessed using flow cytometry, and reverse transcriptase polymerase chain reaction (RT-PCR) is performed to detect MHC I, MHC II, CD80, and CD86 messenger RNA (mRNA). Finally, immunohistochemistry is performed on intact bovine and leporine articular cartilage and meniscus tissues to detect MHC I and II. It is hypothesized that both of these cell types have an immunoprivileged profile, lacking the ability to induce PBMC proliferation and not expressing key factors for immune response initiation, MHC II, CD80, and CD86.

Methods

Isolation of cartilaginous cells

Cartilage and meniscus tissue was sterilely dissected from bovine and leporine knee joints and minced into small pieces. After 18 hours of digestion in 0.2% collagenase (Worthington, Lakewood, NJ), cells were isolated using sequential centrifugation and rinses with phosphate-buffered saline (PBS). Cells were then cryopreserved in medium containing 20% fetal bovine serum (Gemini Bio-Products test, Sacramento, CA) and 10% dimethyl sulfoxide (Sigma, Saint Louis, MO) until needed.

Previously, primary bovine ACs and MCs had been used to form self-assembled constructs, so no further steps were needed to prepare these cells, although when leporine cells are used to generate self-assembled constructs, they are expanded in monolayer culture. The leporine cells used for subsequent MLR assessment were expanded to test the immunogenicity of the population of leporine cells previously employed for self-assembly (unpublished data). Leporine ACs and MCs were separately expanded in a cell culture medium consisting of Dulbecco's modified Eagle medium with 4.5 g/L glucose; GlutaMAX (Invitrogen, Carlsbad, CA); 100nM dexamethasone; 1% Fungizone; 1% penicillin/streptomycin (BD Biosciences, Bedford, MA); 1% insulin, human transferrin, and selenious acid (ITS)+premix (BD Biosciences); 50 mg/mL ascorbate-2-phosphate; 40 mg/mL L-proline; 100 mg/mL sodium pyruvate (Fisher Scientific, Pittsburgh, PA); and 5 ng/mL basic fibroblastic growth factor. Cells were seeded at a density of 2.5×10^4 cells/cm^2 and allowed to grow until 4 days after confluence was reached. Expansion proceeded until passage 3 was reached.

Mixed lymphocyte reaction

The mixed lymphocyte reaction test was based on a protocol described previously.[37] Bovine and leporine ACs and MCs were treated with 25 μg/mL mitomycin-C (Sigma) for 45 minutes, and then mitomycin was removed using three medium rinses with centrifugation between each. Leporine PBMCs (Rockland Immunochemicals, Gilbertsville, PA) were mixed with the each of the four cartilaginous cell types to obtain two cell solutions each that contained10^3 cartilaginous cells plus 10^5 PBMCs and 10^4 cartilaginous cells plus 10^5 PBMCs per 100 μL. One hundred μL of each cell solution was dispensed into a well on a 96-well plate. In addition, control groups (cartilaginous cells at 10^3 or 10^4 cells per well) corresponding to each of the MLR groups were seeded. As a positive control, concanavalin A (Sigma) was added to 10^5 PBMCs at a concentration of 12.5 μg/mL and seeded into wells of a 96-well plate. Negative controls consisted of 10^5 PBMCs seeded with or without mitomycin pretreatment. In all, this generated eight groups of MLR assays, eight control groups without PBMCs corresponding to the MLR groups, a positive PBMC control (with concanavalin), and two negative PBMC controls (cells only and cells with mitomycin pretreatment). Five replicates were employed gor each of the 19 groups.

After 6 days of culture, plates were centrifuged to pellet all nonadherent cells, and trypsin ethylenediaminetetraacetic acid (Invitrogen) was applied for 15 minutes to ensure that all adherent cells entered into solution. After removal of trypsin using centrifugation and rinsing, plates containing the cell solution were subjected to three freeze thaw cycles to ensure cell lysis. Aliquots from each well were tested in triplicate for DNA content using the PicoGreen double strand DNA (dsDNA) reagent (Invitrogen) and dsDNA controls. The amount of DNA was converted to cell number using a conversion factor of 7.8 pg DNA/cell.

Reverse transcriptase polymerase chain reaction

RNA was extracted from bovine ACs and MCs, leporine passage 3 ACs and MCs, and bovine and leporine PBMCs (positive controls) using an RNaqueous Micro Kit (Ambion, Carlsbad, CA). The extracted RNA was then reverse transcribed to cDNA using Superscript III First Strand Synthesis System (18080051, Invitrogen). For bovine samples, 86.4 ng of mRNA was used for reverse transcription, and for leporine samples, 358.4 ng of mRNA was used. PCR was run on the resulting cDNA to determine whether MHC I, MHC II, CD80, and CD86 were present. Amplification of

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for bovine cells and β-actin for leporine cells served as housekeeping genes. All PCR was performed on a RotorGene 6000 (Corbette Life Sciences, Valencia, CA) using Platinum PCR Supermix (11306-016, Invitrogen) and run using the following thermal cycling protocol: 94°C for 2 minutes, 40× (94°C for 15 sec, annealing temperature for 30 seconds, 72°C for 60 seconds). Reactions were visualized using gel electrophoresis with gel green dye (41004, Biotium, Hayward, CA). The specific primers, target lengths, and annealing temperatures were determined from the literature[38–42] and are outlined in Table 1.

Flow cytometry

Bovine ACs and MCs, leporine passage 3 ACs and MCs, and bovine and leporine PBMCs (positive controls) were analyzed using flow cytometry to determine the presence of immunogenic antigens MHC I and MHC II. Cells were first blocked with 5% goat serum in PBS for 30 minutes and then incubated with mouse anti-MHC I or anti-MHC II primary antibodies (VMRD, Pullman, WA) or mouse anti-IgG2a isotype control (Invitrogen) for 30 minutes and washed with 1% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma) in PBS. The cells were then stained with GtxMs immunoglobulin (Ig)G phycoerythrin-conjugated secondary antibody (Abcam, Cambridge, MA) for 20 minutes, washed, and resuspended in 1% paraformaldehyde in PBS (Sigma). After incubation at 4°C for 24 hours, cells were analyzed for the presence of MHC I and II using a FACScan flow cytometer (BD Biosciences). Up to 10^5 cells were recorded for each group, and forward scatter (FSC), side scatter (SSC), and fluorescence channel 2 (FL2) were recorded using CellQuest software (BD Biosciences). Cyflogic software was used to analyze the generated flow cytometry files. The cell populations of interest were identified by gating FSC versus SSC plots; fluorescence histograms of the isotype control and MHC II stained cells were created using the cell population of interest. Positive staining for each marker was represented as the percentage of the curve exceeding the fluorescence value at 95% of the isotype control curve.

Immunohistochemistry

Bovine and leporine hypertrophic cartilage, articular cartilage, and meniscus tissue were stained for the presence of MHC I and MHC II using immunohistochemical techniques. Briefly, tissues were cryosectioned at 14 μm and fixed in acetone. After fixation, sections were blocked with serum and incubated for 1 hour with antibodies to rabbit and bovine MHC I and II (VMRD). Sections were then incubated with a peroxidase-conjugated anti-mouse secondary antibody, and staining was visualized using a 3,3′-diaminobenzidine substrate.

Statistics

After subtraction of control group cellularity from their corresponding MLR assay group, the number of PBMCs in each group was compared with the number of cells in the PBMC-only group using a t-test. Significant differences were defined as $p < 0.05$.

Table 1. Bovine and Leporine RT-PCR Primers

Species	*Gene*	*5′-3′ Forward Primer*	*5′-3′ Reverse Primer*	*Target Length (bp)*	*Annealing Temp. (°C)*	*Accession Number*	*Ref.*
Bovine	GAPDH	ACCCTCAAGATTGTCAGCAA	ACGATGCCCAAAGTGGTCA	86	60	U85042	39
	MHC I	GGCTCCCACTCCCTGAGGTATTTC	TCTCCAGGTATCTGCGGAGCC	534	60	X82672 & X82673	40
	MHC II	GGAAGAAGGAGACGGTGT	CAGGAAGACCGTCTGTGA	305	60	X78308	41
	CD80	TGTGGCCTGAATACAAGAACC	CAGGTGCTGATTAGCAGAAGG	488	60	Y09950	42
	CD86	GACCTTGAGACTCCACAACG	GTAGAGCTGCAATCCAGAGG	534	60	AJ291475	41
Leporine	β-actin	CGTGCGGGACATCAAGGA	AGGAAGGAGGGCTGGAACA	177	56	AF309819.1	43
	MHC I	CGACTACATCGCCCTGAACG	CCCAGAAGGCACCACCACA	394	56	K02441.1	43
	MHC II	GGAGCACTGGGGCCTGGAGA	GCACCACCTGAGCGCAGTCC	421	56	M15557.1	43
	CD80	TGCGCATATACTGGCAGAAG	TTCTCCATCTTCCATCCAGG	356	56	NM_001082663.1	43
	CD86	TGACCAGGAAAGTTGGAACC	ACACACAACGATCAGGGTGA	517	60	NM_001082208.1	43

RT-PCR, reverse transcriptase polymerase chain reaction

Results

Mixed lymphocyte reaction

The results of the cell number analysis of the MLR assay are shown in Figure 1. Total cell numbers from the control groups containing only mitomycin-treated cartilaginous cells were subtracted from the numbers in their corresponding group in which mitomycin-treated cartilaginous cells were cocultured with PBMCs. No statistically significant differences ($p < 0.05$) were observed between the coculture groups and the PBMC-only control. Significantly more cells were noted when the concanavalin-treated PBMCs were compared with the PBMC-only controls. Proliferation was found to be inhibited significantly more in the mitomycin-treated PBMCs than in the PBMC-only control group.

Reverse transcriptase polymerase chain reaction

RT-PCR analysis of bovine PBMCs, ACs, and MCs revealed positive expression of GAPDH, MHC I, and MHC II. Bovine PBMCs also expressed CD80 and CD86, whereas bovine ACs did not express either costimulatory molecule, and bovine MCs expressed only CD86 (Figure 2). Analysis of leporine PBMCs, ACs, and MCs was similar to that of the bovine cells in that all cell types showed positive expression of β-actin, MHC I, and MHC II, and leporine PBMCs also expressed CD80 and CD86. Leporine ACs and MCs showed positive expression of CD80 but not CD86.

Flow cytometry

Flow cytometric analysis of MHC II on bovine and leporine MCs and ACs revealed that this marker is present on all cells studied except for leporine MCs. As seen in Figure 3, both types of bovine cells stained positive for MHC II. Bovine ACs were 14.4% MHC II positive. Bovine MCs, when stained for MHC II, displayed a bimodal distribution of fluorescence. These cells displayed slightly higher expression for MHC II (32.5%) than bovine ACs. For leporine cells (Figure 3), ACs showed some positive staining for MHC II (7.5%), whereas leporine MCs showed no positive staining for MHC II. All leporine fluorescence histograms were normally distributed, and no bimodal phenomena were observed. Analysis of MHC I showed similarity to MHC II staining, with positive staining observed for bovine ACs (31.2%) and MCs (61.8%) and for leporine ACs (10.3%). Leporine MCs did not show positive staining for MHC I.

Immunohistochemistry

Immunostaining for the presence of MHC II was performed on bovine and leporine native cartilages (Figure 4). Positive staining for both markers was observed in bovine hypertrophic cartilage, but all other tissues (bovine and leporine articular cartilage and meniscus) did not show the presence of these markers. The same phenomena were observed for MHC I staining of native tissues (data not shown).

Discussion

Assessment of the potential for immune response is a critical step in selecting a cell source for tissue engineering. Although allogeneic sources have been commonly used for *in vivo* studies of cartilaginous tissue replacement, the use of xenogeneic sources is less common,[24,27–31] but the available data indicate that xenogeneic cell sources may be used in cartilage and meniscal tissue engineering because of the immunoprivileged nature of these cells.[24,27–31] The benefits of using a xenogeneic cell source for cartilage engineering are significant. Concerns regarding donor-site morbidity and difficulties associated with obtaining a sufficient number of cells required for tissue engineering efforts such as high-density scaffold-free cartilage formation can be mediated using a xenogeneic approach. Toward this end, this study sought to test *in vitro* whether allogeneic (leporine) or xenogeneic (bovine) MCs and ACs show nonimmunogenic characteristics when introduced to leporine lymphocytes. The major hypothesis of this study, that neither leporine nor bovine ACs or MCs would induce an *in vitro* immune response by leporine PBMCs, was proven, suggesting that the functionality of bovine cell based constructs can be assessed in a leporine *in vivo* model without concern regarding immune rejection.

In agreement with previous results assessing the immune reaction after allogeneic or xenogeneic implantation of articular or meniscal cartilage, none of the cartilaginous cells in this study induced proliferation of PBMCs.[30,31] Previous studies on the immunogenicity of cartilaginous cells have linked this lack of immunogenicity to the absence of cell surface markers required for promoting an immune response, including MHC II and costimulation factors of the B7 family.[24–26] The present study confirmed the presence of MHC II and costimulation factors CD80 and CD86 in bovine and leporine ACs and MCs using PCR and flow cytometry. All of the cell types studied showed the presence of mRNA for at least some of the immunogenic markers studied. MHC II

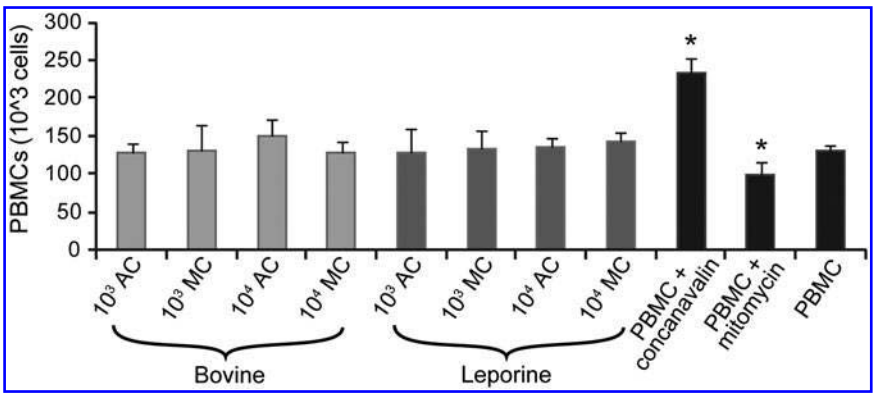

FIG. 1. Results of mixed lymphocyte reaction assay. After subtraction of the control cartilaginous cell-only group, the average number of peripheral blood mononuclear cells (PBMCs) present in each group is compared to the PBMC-only group. Significant differences are defined as $p < 0.05$ and denoted with an asterisk (*).

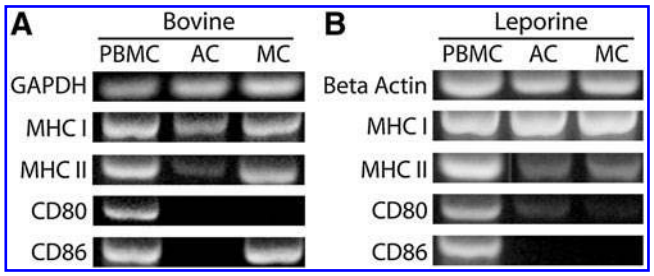

FIG. 2. Reverse transcriptase polymerase chain reaction analysis of bovine **(A)** and leporine **(B)** PBMCs (positive control), articular chondrocytes (ACs), and meniscus cells (MCs). All bovine and leporine cell types express major histocompatibility complex (MHC) I and MHC II messenger RNA, whereas CD80 is expressed only in bovine PBMCs and leporine PBMCs, ACs, and MCs. CD86 is expressed in bovine and leporine PBMCs but only in one other cell type bovine MCs.

mRNA was found in all cell types, CD80 mRNA was present in leporine cells, and CD86 mRNA was found only in bovine MCs. Translation of the MHC II mRNA was confirmed using flow cytometric analysis, which showed that bovine MCs and ACs, and leporine ACs express this marker to some degree.

Because MHC class II molecules are traditionally associated with professional antigen-presenting cells (APCs) such as macrophages, dendritic cells, and B-cells, the presence of MHC II in the cartilaginous cells examined in this study may appear counterintuitive. Although cartilage-derived cells are not typically thought of as APCs, researchers have discovered that chondrocytes possess the potential to obtain properties of APCs.[26,43,44] In particular, chondrocytes isolated from arthritic joints have been shown to possess higher amounts of MHC II molecules and are more immune-reactive when used in an MLR assay than chondrocytes from healthy joints.[43–46] Although the cells used in this study were not isolated from arthritic joints, it is possible that the insult due to enzymatic digestion of the cartilage matrix induced the cells to transition to a phenotype more amenable to antigen presentation. In support of this claim, other researchers have discovered that MHC II is present in isolated chondrocytes and absent in intact, healthy cartilage.[26,46–48] Immunostaining for MHC II in native bovine and leporine articular cartilage used in this study also showed that these markers were not present *in situ*. Overall, the results of this study are in accord with those of previous studies demonstrating the presence of MHC II on chondrocytes isolated from healthy cartilage.

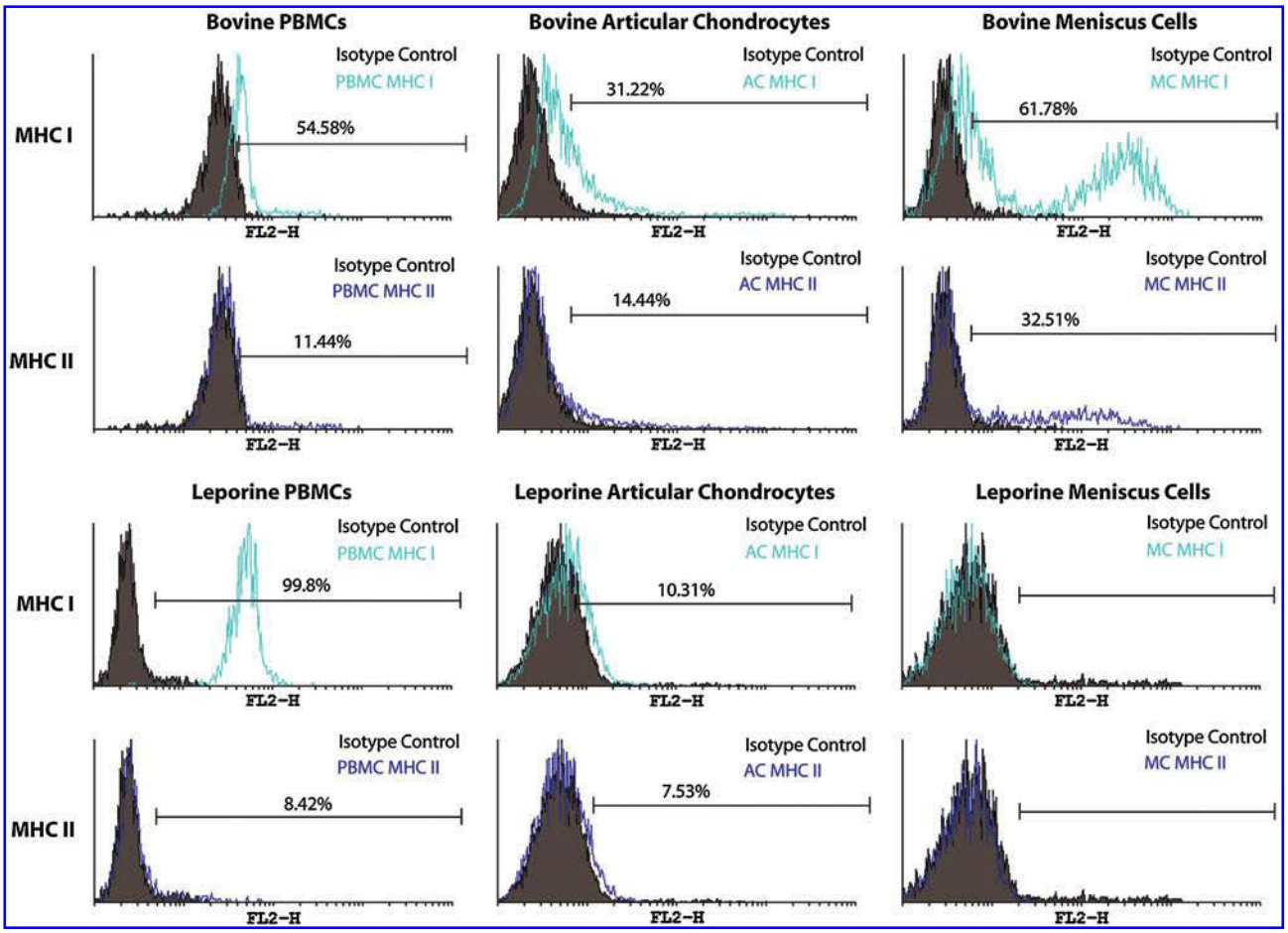

FIG. 3. Flow cytometry for MHC II. Histograms of relative cell count versus fluorescence for isotype control and anti-MHC II staining for leporine and bovine articular chondrocytes and meniscus cells. Percentages indicate the number of cells with fluorescence values greater than 95% of the isotype control. Color images available online at www.liebertonline.com/tea

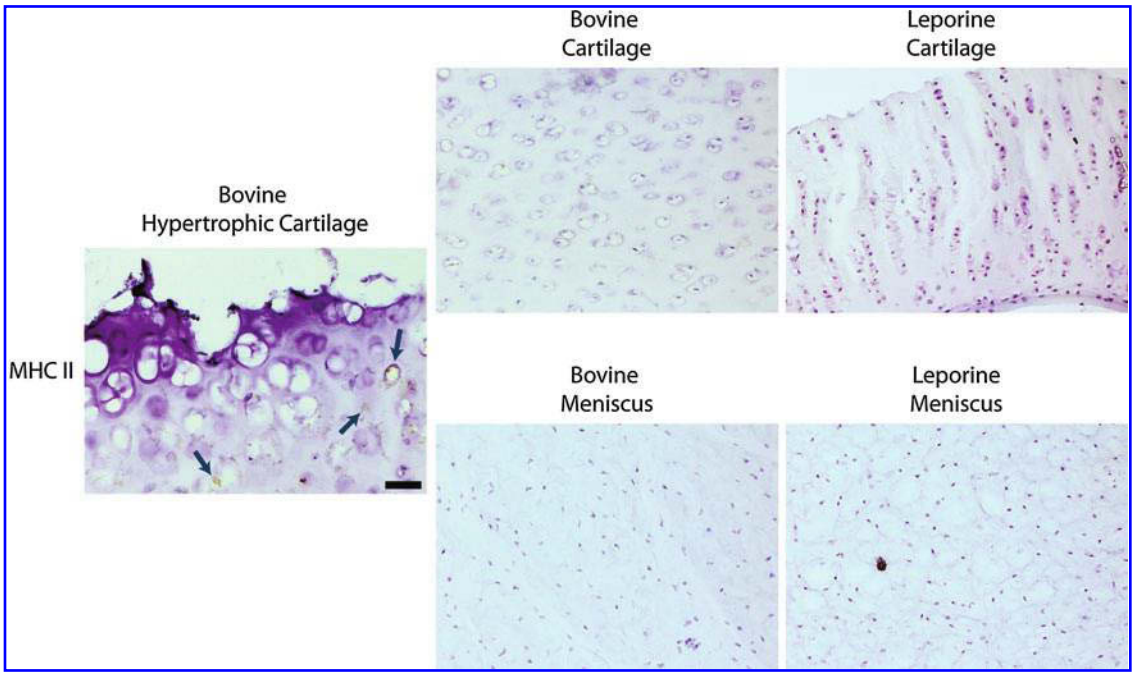

FIG. 4. Immunohistochemistry for MHC II. MHC II staining was performed on native bovine and leporine cartilages. Bovine hypertrophic cartilage showed positive staining for MHC II (arrows), whereas bovine and leporine articular cartilage and meniscal tissue did not show presence of this marker. Scale bar = 50 μm. Color images available online at www.liebertonline.com/tea

In contrast to ACs, few accounts exist regarding the presence of MHC II molecules on cells isolated from the meniscus. Before this investigation, the presence of MHC II had not been analyzed for isolated MCs, although researchers have stained native human and ovine meniscus tissue for MHC class II and found that MCs do not express MHC class II, whereas synovial and endothelial cells within the meniscus do.[47–51] These results were also confirmed through immunohistochemical staining of bovine and leporine meniscus tissues in this study. In agreement with native tissue investigations, the results from the present study suggest that meniscus tissue may be similar to articular cartilage when MHC II is not present on cells *in situ*, but upon isolation MHC II expression is enhanced.

Along with expressing MHC I and II, some of these cells also showed mRNA expression of B7 family costimulation factors, although none of the cell types showed expression of both CD80 and CD86. The presence of B7 mRNA indicates that some of the cell types studied have the molecular machinery to stimulate T-cells but fail to initiate a proliferative response when exposed to T-cells. This suggests that these cells are unable to induce an immune response because of a lack of B7 family cofactors required to activate T-cells or that a factor produced by the cartilage cells is actively quenching immune response. Costimulatory molecules CD80 and CD86 are known to be necessary for the effective priming and activation of T-cells and seem to act on the T-cells through distinct mechanisms.[52–58] Regarding cartilaginous cells, Adkisson et al.[24] showed the absence of costimulatory molecules on juvenile human ACs, which is in agreement with the present results for bovine ACs. The absence of costimulatory molecule transcripts in bovine ACs may explain their inability to stimulate xenogeneic T-cells, although bovine MCs and leporine ACs and MCs showed gene expression for CD80 or CD86 yet failed to elicit T-cell activation. One possible explanation for this phenomenon is that, although mRNA for these proteins is present, there may be some regulatory mechanism inside the cell inhibiting translation of these mRNAs into functional costimulatory proteins or that both of these molecules are necessary for immune response initiation.

It is also possible that these cells are actively producing factors that suppress an immune response. There are a number of molecules known to inhibit T-cell activation, such as negative costimulators in the B7 family and chondromodulin-I, an immunosuppressive factor that juvenile and adult ACs have been shown to produce.[24,59] The action of these factors may play a role in inhibiting the action of B7 molecules on the cell's surface or affect the way T-cells interact with cartilaginous cell types. Another factor that chondrocytes are known to produce is transforming growth factor beta, which has been shown to be a potent inhibitor of T-cell activation in studies using chondrocytes and mesenchymal stem cells.[24,26,60–62] The immunomodulatory factors that the cells produce may therefore have contributed to the lack of immune response seen in *in vitro* cocultures. Thus, it is possible that an absence of B7 cofactors due to lack of transcription or translation in the cell or by the production of an immune response inhibitor by the cells can explaine the lack of PBMC proliferation in coculture with leporine and bovine ACs and MCs.

Given that some of these cell types express costimulation factors, future studies are needed to investigate the soluble factors produced by cartilaginous cells that may play a role in inhibiting an immune response, but because the present results indicate that neither bovine nor leporine MCs or ACs stimulate leporine PBMC proliferation, it is likely that engineered cartilage constructs using these cells may be implanted into a leporine model without concern for immune rejection. Considering these promising results, it is possible that immune

evasion by these cells is widely applicable to scaffold-based and scaffold-free tissue engineering approaches, both of which require high cellularity to achieve functional properties.

Conclusions

Overall, this study shows that it is unlikely that the use of tissue engineered cartilaginous constructs formed from leporine or bovine ACs or MCs will elicit an immune response when implanted into the leporine knee. This opens the door for a plethora of cell types to be evaluated for *in vivo* assessments in animal models. It also suggests that xenogeneic transplantation into humans could be a possibility if the presence of alpha-galactosyl of tissues generated from animal cells can be eliminated.

Disclosure Statement

No competing financial interests exist.

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