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Cartilage immunoprivilege depends on donor source and lesion location

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ABSTRACT

The ability to repair damaged cartilage is a major goal of musculoskeletal tissue engineering. Allogeneic (same species, different individual) or xenogeneic (different species) sources can provide an attractive source of chondrocytes for cartilage tissue engineering, since autologous (same individual) cells are scarce. Immune rejection of non-autologous hyaline articular cartilage has seldom been considered due to the popular notion of "cartilage immunoprivilege". The objective of this study was to determine the suitability of allogeneic and xenogeneic engineered neocartilage tissue for cartilage repair. To address this, scaffold-free tissue engineered articular cartilage of syngeneic (same genetic background), allogeneic, and xenogeneic origin were implanted into two different locations of the rabbit knee ($n = 3$ per group/location). Xenogeneic engineered cartilage and control xenogeneic chondral explants provoked profound innate inflammatory and adaptive cellular responses, regardless of transplant location. Cytological quantification of immune cells showed that, while allogeneic neocartilage elicited an immune response in the patella, negligible responses were observed when implanted into the trochlea; instead the responses were comparable to microfracture-treated empty defect controls. Allogeneic neocartilage survived within the trochlea implant site and demonstrated graft integration into the underlying bone. In conclusion, the knee joint cartilage does not represent an immune privileged site, strongly rejecting xenogeneic but not allogeneic chondrocytes in a location-dependent fashion. This difference in location-dependent survival of allogeneic tissue may be associated with proximity to the synovium.

Statement of Significance: Through a series of *in vivo* studies this research demonstrates that articular cartilage is not fully immunoprivileged. In addition, we now show that anatomical location of the defect, even within the same joint compartment, strongly influences the degree of the resultant immune response. This is one of the first investigations to show that (1) immune tolerance to allogeneic tissue engineered cartilage and (2) subsequent implant survival are dependent on the implant location and proximity to the synovium.

1. Introduction

Lining the ends of long bones, articular cartilage allows for the movement of two bones against each other. Cartilage is distinct from other tissues due to its lack of innervation and vascularization and has minimal capacity for repair [1,2]. As a result, articular cartilage lesions may predispose to degenerative joint disease [3].

Numerous strategies have been employed to repair articular cartilage defects by filling them with an engineered biomimetic tissue that recapitulates the biomechanical and biochemical properties of native cartilage [1,2,4]. One of the challenging and intriguing questions for translation of tissue engineered cartilage for cartilage repair involves the immune response to the implanted tissue, which involves both the innate and adaptive immune responses. The innate immune response is an immediate generic response to the chemical properties of an antigen and involves various cell types such as macrophages, neutrophils, natural killer cells, basophils, and eosinophils. Unlike the innate, the adaptive or acquired immune response is rather delayed, highly specific, and a learned response to

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a presented non-self antigen. It involves the lymphocytes which can be divided into two categories: cell mediated (T cells) and antibody produced (B cells). Articular cartilage's avascular and dense extracellular matrix (ECM) has led to the assertion that the tissue is immunoprivileged, whereby the body's immune system is limited in its ability to detect and reject implanted cartilage [1,2,5–8]. However, that concept has been challenged by studies showing that chondrocytes and their associated ECM are antigenic and elicit varying degrees of immune reactions [2,9–16]. Moreover, the proteoglycans and collagens of the ECM from xenogeneic sources have antigenic properties that elicit immune responses.[17–20] Chondrocytes are susceptible to attack by natural killer cells [11,16] and they express major histocompatibility class (MHC) II antigens, which can activate CD4 T lymphocytes and provoke cell and antibody-mediated immune responses [13,21–23]. However, when the cartilage tissue is intact, the chondrocytes might be hidden from immune surveillance by the dense ECM, which could impart an immunoprivileged nature to the tissue [24].

There are limitations to the use of autologous, i.e., the patient's own tissues, such as minimal donor tissue availability [25]. Therefore, research efforts have turned to allogeneic (same species, different individual) and xenogeneic (different species) tissues as potential sources to replace or regenerate articular cartilage tissue [1,2,6,26,27]. These tissue sources would be preferable due to the need for only a single surgical procedure for implantation and the lack of donor site morbidity during autologous graft harvest. Although allogeneic studies using multiple species and techniques have produced results ranging from complete defect filling to partial coverage [5,8,15,28,29], they have been successful enough for allogeneic tissue sources to be clinically approved for human use, such as for example juvenile particulated cartilage/cells used in the USA in a Phase III clinical trial [30]. Other allogeneic applications involve the use of osteochondral implants despite issues related to graft viability, quality, infection/disease transmission, and immunogenicity [2]. Nonetheless, availability of such tissues is low. In contrast, animal source (xenogeneic) cells and tissues, while not clinically viable, are widely available and provide an intriguing possible source of tissue for cartilage repair in humans.

The goal of this study was to provide a thorough comparison of the outcomes achieved by implantation of allogeneic and xenogeneic tissue engineered constructs. Therefore, a full-thickness defect model within two locations of the rabbit knee was used. To accomplish this, we implanted scaffold-free tissue engineered cartilage constructs from either xenogeneic bovine or allogeneic leporine (rabbit) chondrocytes into defects in the patella or the trochlea. It was hypothesized that allogeneic but not xenogeneic engineered tissue would be suitable for cartilage repair.

2. Materials and methods

2.1. Study design

An evolving stepwise approach as described in Fig. 1 was utilized. This approach was based on a multidisciplinary analysis, described below, of the local and systemic reaction with regard to surgical site and cell source. The goal of this approach was to identify an ideal combination of surgical site and implant source resulting in an outcome of reduced inflammation and subsequent implant survival and integration. Forty-eight adult female New Zealand White rabbits (weight range 3.5–4.5 kg; age range 6–8 months) were used in the study. The rabbits were divided into the study groups as shown in Fig. 1. For the first three studies, marked in yellow, red, and blue in Fig. 1, a full-thickness defect model in the patella was used. All implants were placed into a 5 mm defect created in the articular patellar cartilage. There were six groups: (1) empty defect control, (2) autologous articular cartilage, (3) allogeneic articular cartilage, (4) xenogeneic articular cartilage, and (5) allogeneic and (6) xenogeneic tissue engineered cartilage constructs. Three rabbits per group were sacrificed at 3 and 6 weeks.

Following completion of the patellar study and analysis of the results, location-dependent factors within the patellofemoral compartment were further investigated. For the location-dependent study, a full-thickness defect model in the trochlea was utilized. Rabbits were divided into three groups consisting of four rabbits each (1) empty defect control, (2) allogeneic, or (3) xenogeneic tissue engineered cartilage construct; animals were sacrificed at 6 weeks.

2.2. Cartilage graft and construct production

Cell culture media components were purchased from Invitrogen (Carlsbad, CA) or Sigma–Aldrich (St. Louis, MO) unless otherwise noted. Transforming growth factor beta 1 (TGF-β1) was obtained from Peprotech Inc. (Rocky Hill, NJ). Chondroitinase-ABC (C-ABC) was obtained from Sigma–Aldrich. Self-assembled constructs were produced from both xenogeneic bovine and allogeneic leporine sources based on methods previously established by our group [31–34]. To isolate chondrocytes, articular cartilage from bovine stifle (knee) joints (Research 87, Boston, MA) or 9–12 month old New Zealand White rabbits (Heaton Rabbitry) was aseptically minced and digested with 0.2% collagenase P (Worthington, Lakewood, NJ) in culture medium containing 3% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) for 16 h as described previously [31]. Leporine cells were used at passage 3 due to lower cell numbers at the time of harvest and redifferentiated. Constructs were then produced as previously published by our group [34]. Bovine cells were used at passage 0 and constructs produced as previously published by our group [35]. In brief, self-assembled constructs were produced by seeding isolated chondrocytes into non-adherent 5 mm agarose wells. At no time were chondrocytes embedded into the agarose. Constructs were then treated with a regimen of TGF-β1, hydrostatic pressure, and C-ABC and maintained in a humidified incubator at 37 °C and 10% CO_2 until the day of surgery (42 days) [32,33]. Native tissue explants were harvested shortly after sacrifice using a 5 mm biopsy punch and trimmed to 0.4 mm in thickness in a custom cutting jig (retaining the articulating surface) to produce a cartilage explant. Culture manipulation was kept to a minimum to reduce extraneous variables. Specifically, due to the logistics of harvesting and the surgeries, the explants were kept overnight in DMEM lacking serum in a humidified incubator at 37 °C and 10% CO_2. This helped to ensure no bacterial contamination was present. All implanted materials were copiously irrigated with 0.9% sterile saline (medical grade) prior to implantation.

Compressive and tensile mechanical properties as well as gross morphology and histology of both xenogeneic and allogeneic constructs were characterized, as previously described [36–38]. Compressive aggregate modulus was determined via a creep indentation apparatus [39] using a 0.8 mm flat porous indenter tip applying a tare weight of 0.2 g and a test load of 0.7 g [40] with data modeled using the linear biphasic theory [41]. Tensile tests consisted of a uniaxial pull-apart test until failure at a displacement of 1% of the gauge length using a materials testing system (TestResources, Shakopee, MN), as previously described [42]. Cross-sectional area and gauge length were measured [31] and a load–displacement curve was used to calculate a stress–strain curve, with Young's modulus determined from the linear region.

2.3. Surgical procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee, University of California, Davis.

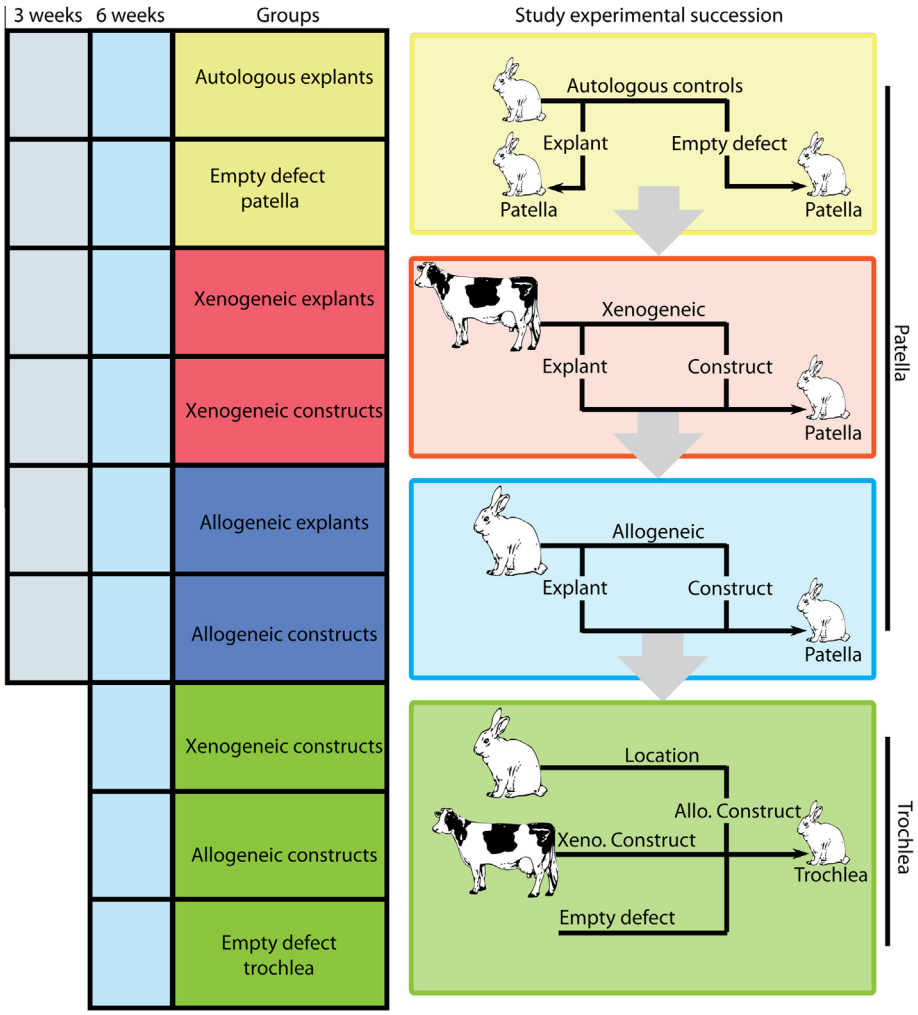

Fig. 1. Stepwise approach to experimental design and resulting study groups, $n = 6$ rabbits per group; $n = 3$ per timepoint, were used.

Adult female New Zealand White rabbits (weight range 3.5–4.5 kg; age range 6–9 months) were used in the study as a source of allogeneic chondrocyte tissue and for knee joint studies. Animals were housed at the Center for Laboratory Animal Science at the University of California, Davis.

Surgeries were performed with the rabbits under general anesthesia using a cocktail of ketamine (50 mg/kg), xylazine (5 mg/kg) and acepromazine (0.5 mg/kg). Following endotracheal intubation, anesthesia was maintained on isoflurane 1–3% accompanied with mechanical ventilation. The left knee was shaved and the skin was aseptically prepared for surgery. A cranio-medial parapatellar approach to the stifle joint was performed and the patella was everted. A 5 mm dermal punch was used to uniformly mark the center of the patellar articular cartilage or the opposing trochlea. A dental unit mounted with a sterile burr was used to create a 5 mm diameter full-thickness cartilage defect followed by microfracture using a sterile needle until the bleeding subchondral bone was encountered. All procedures were performed under continuous sterile 0.9% saline irrigation avoiding thermal insult. Implants were secured at four locations using a total of 1.0 µl of 2-ocyl cyanoacrylate tissue adhesive (Surgi-Lock 2oc, Meridian Animal Health, Omaha, NE), while avoiding placing the adhesive between the implant and the underlying bone [43,44]. Following copious irrigation of the joint with 0.9% sterile saline, the patella was carefully reapproximated, and the joint was closed with 4–0 polyglactin 910 (Vicryl, Ethicon Inc, Somerville, NJ). Skin was closed using 4–0 nylon (Ethilon, Ethicon Inc, Somerville, NJ). The operated leg was then bandaged in an Ehmer sling bandage for one week. Postoperative medication consisted of buprenorphine (0.03 mg/kg subcutaneously twice per day for 5 days), meloxicam (0.2 mg subcutaneously once per day for 5 days), and penicillin G (50000 IU/kg, subcutaneously every 48 h for a week). Following the removal of the sling, all animals were allowed to move freely. The rabbits were euthanized at 3 or 6 weeks via pentobarbital intravenous overdose. Gross morphology of the experimental surgery for both the patellar and trochlear defects is depicted in Fig. 2.

2.4. Clinicopathologic evaluation

Peripheral blood was collected from each rabbit for complete blood count (CBC) and full biochemistry panel analysis prior to surgery and immediately prior to sacrifice. Bloodwork consisted of hematology and chemistry panels. Hematology panels included: White blood cell count, absolute heterophil cells, absolute lymphocyte cells, absolute monocyte cells, absolute eosinophil cells, absolute basophil cells, percentage heterophils, lymphocytes, monocytes, eosinophils, basophils, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, platelets, mean platelet volume, and total protein. Chemistry panels included: sodium, potassium, chloride, calcium, phosphorus, total protein, albumin, glucose, blood urea

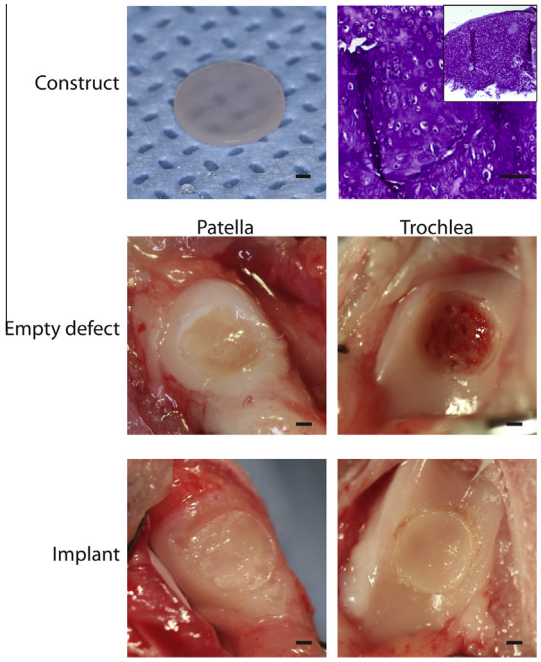

Fig. 2. Gross surgical morphology of empty defect and implanted tissue engineered construct in both the patella and trochlea. Construct gross appearance and H&E staining demonstrating chondrocytes residing in lacunae surrounded by a densely stained extracellular matrix rich in glycosaminoglycans (top panels), insert showing lower magnification histology of construct, scale bar is 1 mm and 50 μm, respectively. Scale bar is 1 mm for all other images.

nitrogen, creatinine, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and amylase. In addition, the rabbits were clinically examined daily for the first 2 weeks and every other day for the remainder of the study. In each animal, weight, appetite, overall attitude, ability to walk, and joint mobility were evaluated. Immediately after euthanasia, the skin over the knee was removed and the joint was measured for swelling in comparison with the intact contralateral knee. Synovial fluid was collected from the operated and intact knees and blinded analysis of the fluid was performed by an experienced veterinary clinical pathologist (DLB). The assessment consisted of the physical parameters of the fluid, total cell count, and nucleated cell differential.

2.5. Histology and immunohistochemistry

The stifle joint (synovial membrane, patella, and trochlea) was removed and fixed *en bloc* in 10% neutral buffered formalin for a minimum of 48 h, and hard tissues were decalcified in 10% formic acid. Specimens were paraffin-embedded and 5 μm sections were cut and stained with hematoxylin and eosin (H&E) according to standard protocols.

Immunohistochemistry was performed on 5 μm serial sections of the synovial membrane using a standard streptavidin biotin detection system (Biocare Medical, Concord CA). Briefly, the deparaffinized slides were hydrated to 70% ethanol and then immersed in 0.03% hydrogen peroxide methanol for 30 min in order to block endogenous peroxidase activity. After a PBS wash, all sections were antigen retrieved with steam heating in citrate buffer (S1699 Dako Corp) for 20 min at 98 °C and then cooled for 20 min, washed well in PBS and immersed in 10% normal horse serum for 20 min to block nonspecific antibody interactions. The primary antibodies, rat anti-CD3 epsilon 1:10 (gift of Dr. P.F. Moore, UC Davis) and mouse anti-CD79a 1:50 (clone HM57, Dako Corp.) to detect T and B cells respectively, were applied to the sections for 60 min at room temperature. Secondary biotinylated horse anti-mouse IgG or anti-rat (Biocare) and the streptavidin-horseradish peroxidase (HRP) label antibody were applied for 10 min each respectively. A PBS wash followed each step. Positive staining was visualized using 3-amino-9-ethylcarbazole (AEC Invitrogen, San Francisco, CA) as the chromogen. All sections were counter-stained with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, MO). Positive and negative control tissues were prepared for each experiment. Rabbit spleen or lymph node served as positive tissue controls. Negative controls were prepared by omitting the primary antibody and substituting a matched isotype control antibody.

The histologic sections were evaluated by an experienced veterinary pathologist (BM) for the presence and type of inflammatory reaction as well as the characterization of the pathologic process within the patella, trochlea, and the synovial membrane. The pathologist was blinded to treatment group status. In addition, evaluation and qualitative grading of the inflammatory cell infiltrate was performed by two blinded investigators (BA, GDD) on H&E stained tissue samples in five representative microscopic high-power fields (×200) [45]. The grade of inflammatory infiltrate [inflammatory score (IS)] was designed as follows: 0 – absence of inflammatory cells, 1 – mild (<25% of the stroma infiltrated), 2 – moderate (25–50% of the stroma infiltrated), and 3 – severe inflammatory infiltrate (>50% of the stroma infiltrated).

2.6. Statistical analysis

The 45 rabbits were divided among groups by location and cell source of the implant. Group-wise comparisons were done by a one-way ANOVA; the Tukey–Kramer post hoc test was conducted where appropriate using the statistical analysis software package JMP (SAS, Cary, NC) with $p < 0.05$ denoting statistical significance. All data are reported as mean ± standard deviation; different letters between groups indicate statistically significant differences.

3. Results

3.1. Clinical observations

Following surgery, most rabbits lost weight but regained this weight as the study progressed. Following removal of the sling, the rabbits regained use of the affected leg and no lameness was observed in any rabbit, independent of their treatment. Clinical signs of joint swelling and effusion were observed for all treatment groups with no significant differences between the groups. Of 48 rabbits assigned to surgical groups, three rabbits died during the study period for causes unrelated to the experiment; these rabbits were not replaced and their results were excluded from the study.

3.2. Transplantation of chondrocyte constructs did not result in systemic inflammatory responses

Construct implant properties used for this study were similar to those previously described [31–34]. For both xenogeneic and allogeneic groups construct gross dimensions, ~5 mm in diameter and 0.5 mm in thickness, and histological appearance were similar (Fig. 2) with the presence of both collagen and glycosaminoglycan. Compressive modulus for xenogeneic and allogeneic constructs was 159 ± 53.4 kPa and 225 ± 26.0 kPa, respectively, while Young's modulus was 2870 ± 981 kPa and 6360 ± 1820 kPa, respectively.

None of the rabbits demonstrated significant alterations in their blood cell count (data not shown). Furthermore, biochemical

analysis of serum showed no significant alterations. There were also no signs of systemic infection. Thus, independent of the type of treatment, systemic inflammatory responses were below the level of detection.

3.3. Tissue transplantation results in significant changes to synovial fluid

Cytological results of the synovial fluid analysis demonstrated that inflammatory reactions were observed for all treatment groups, but varied in degree and type of inflammatory infiltrate (Fig. 3A). At 6 weeks, synovial fluid from rabbits with an empty defect or an autologous construct was mostly normal, consisting primarily of large, nonreactive, mononuclear cells, regardless of implant location (patella or trochlea; Fig. 3A). Implantation of a xenogeneic construct (at 6 weeks) elicited a marked inflammatory and immune response including marked mononuclear reactivity (Fig. 3A), moderate to marked increases in lymphoid reactivity and plasma cell numbers (Fig. 3B), and variable heterophilic (equivalent to neutrophilic in humans) inflammation (Fig. 3C). The reaction to a xenogeneic implant was profound and significantly different (per Fig. 3, $p < 0.05$) from that observed with the allogeneic and autologous tissue transplants, or without tissue transfer, regardless of implant location (trochlea or patella).

Implantation of allogenic constructs into the trochlear grove elicited only a mild mononuclear response, cytologically similar to those in non-treated joints or in joints where the defect was left empty or implanted with an autologous implant. Implantation of an allogeneic construct in the patellar groove elicited a stronger inflammatory response with variable mononuclear reactivity and increases in total cell counts (Fig. 3A).

Synovial fluid analyses indicated strong innate and adaptive immune responses with heterophilic and lymphocytic inflammation for all xenogeneic tissues, producing a 3–234 fold increase in heterophil number and a 4–13 fold increase in lymphocyte number compared to control fluid. A milder response was generated toward allogeneic tissues with a 0–8 fold increase in heterophils and a 0.8–2.8 fold increase in lymphocytes.

3.4. Xeno- but not allo-transplantation induces tissue rejection

To further assess the extent of inflammation and/or healing of the defect, we conducted histology and immunohistochemistry of the lesion and surrounding joint tissues as well as of the synovia of all study animals. Synovial quantitative histologic grading and significant differences between groups ($p < 0.05$) are represented in Fig. 4. Recipients of xenogeneic explants showed severe inflammatory processes at both 3 and 6 weeks dominated by T and B cell infiltration with the appearance by 6 weeks of immune follicles within the synovial membrane. The defect was filled with necrotic debris with no sign of viable implant at both time points. Similarly, transplantation of a xenogeneic tissue engineered construct into the patella led to the appearance of immune follicles throughout the synovia at 6 weeks and necrotic tissue debris with no signs of viable construct (Figs. 5 and 6). This strong inflammatory reaction was a sign of tissue rejection of the xenograft, as rabbits which received no tissue or autologous explants showed only mild or mild/moderate inflammation at 3 weeks, which subsided by 6 weeks.

Interestingly, transplantation using allogeneic explants led to only moderate inflammation with large numbers of T-cells and moderate numbers of B-cells in immune follicles, which was less than observed after xenogeneic transplant. At 3 weeks the defect site retained portions of the degenerated implant, which was still present as partially attached and often mineralized degenerate to necrotic fragments at 6 weeks. Transfer of the allogeneic tissue engineered construct also caused mild inflammation at 3 weeks, which subsided by 6 weeks. The quality of the inflammatory infiltrate appeared different from that noted after explant transfer, as it consisted mainly of T cells with only rare B cells. Importantly, by 6 weeks a viable implant was still noted within the defect.

Analysis of tissues 6 weeks after tissue transplantation into the trochlea showed overall very similar effects. At this timepoint there was no apparent inflammation noted for empty defects, while the transfer of xenogeneic tissue engineered construct resulted in severe inflammation with immune follicles containing abundant T and B cells. In one of three defects degenerate xenograft implant was still present with pannus filing the defect, while the other two defects had full thickness loss of cartilage.

In contrast, 6 weeks after transfer of allogeneic tissue engineered construct into the trochlea the synovium was histologically normal with at most mild inflammatory responses (Fig. 6). In the defect site the construct was largely viable with graft integration into the subchondral bone without signs of inflammation. The junction of the construct and native tissue had a defined interface but without the presence of a gap.

4. Discussion

The most important finding(s) of the present study are that an experimentally induced cartilage defect in the patella and trochlea of NZW rabbits could be filled by the implantation of allogeneic tissue engineered constructs, while the transplantation of xenogeneic tissue or construct resulted in rapid tissue rejection and implant destruction. Our studies thereby suggest that the articular cartilage of the knee does not represent an immune privileged site, strongly rejecting xenogeneic but not necessarily all allogeneic materials. Indeed, large differences were observed in this study, depending on the type of tissue engrafted. In general, we observed innate inflammatory response with autologous graft and a mild adaptive response for the allogeneic implants in the patellar location. However, the xenogeneic implants in both locations provoked a strong innate and adaptive response with high influx of T and B cells and formation of immune follicles within the synovium. Foreign antigens, from allogeneic or xenogeneic tissues, initiate both innate and acquired immune response resulting in inflammation at minimum and more commonly rejection [46]. Even autologous cells when injured release intracellular molecules identified as “danger” or damage-associated signals (DAMPS) that trigger an inflammatory reaction that can be local or systemic in nature [47]. Despite the various degrees of local response between the cell sources used, no systemic response was noted, indicating the ability of the body to isolate the response to the joint. Importantly, these results show that articular cartilage exhibits a location-dependent immune tolerance to allogeneic materials, provided they are implanted in a location that is not physically adjacent to the synovium.

Creating a defect in articular cartilage, even in the absence of any implant, provoked an innate but not an adaptive immune response. Following surgical insult, a cascade of overlapping inflammatory phases ensues. The rate and patterns of these phases depend upon the host, local, systemic, and surgical factors [48]. Chemo-attractants released by injured tissue and platelets recruit inflammatory cells to the site of the surgical injury. This reaction is a function of the innate immune response in which influx of neutrophils (heterophils in rabbits) occurs within the initial few days and then is rapidly outnumbered by macrophages derived from mobilized monocytes. These macrophages regulate the formation of granular tissue. Thus, surgical insult to the joint is sufficient to induce synovial changes, inflammation, and recruitment of the innate immune cells.

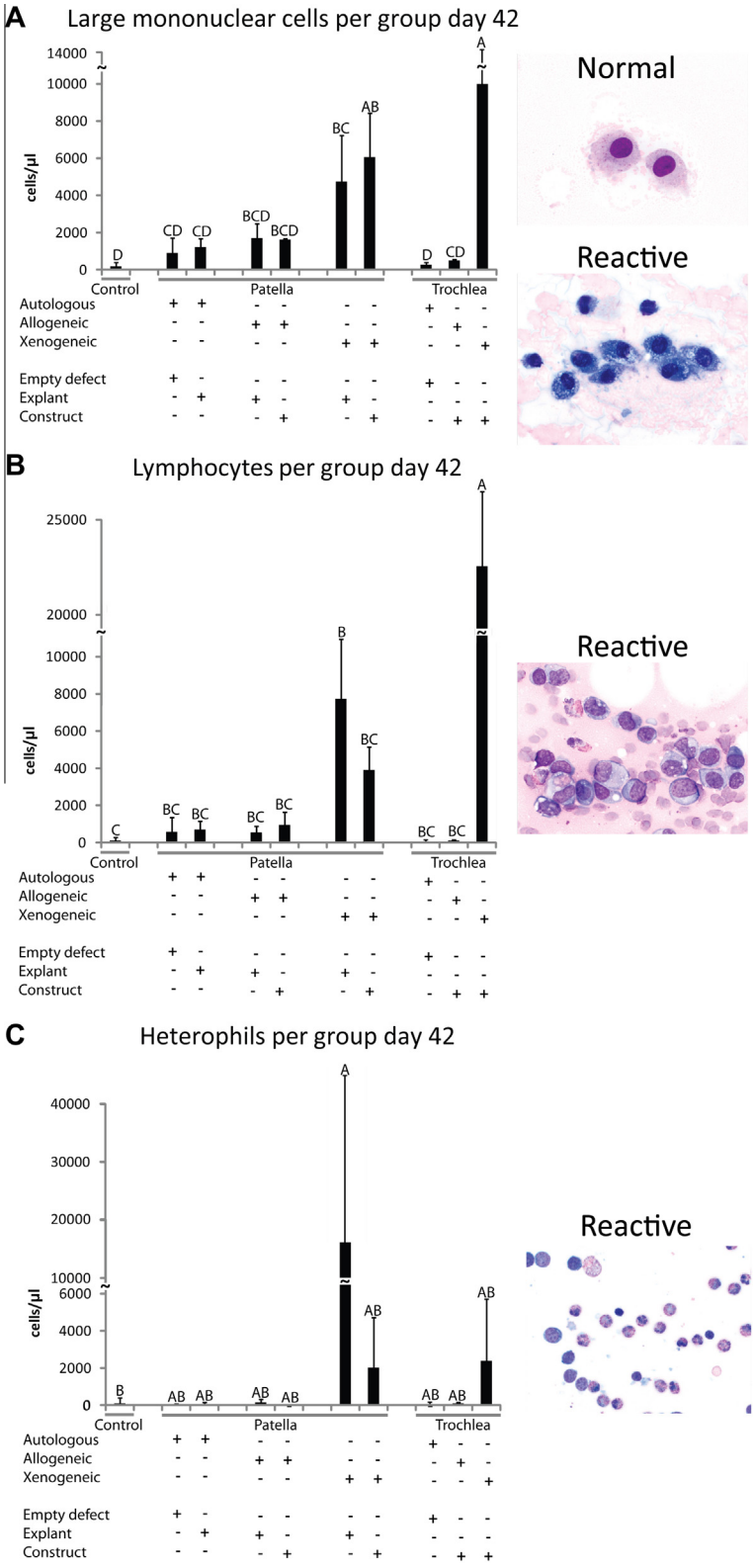

Fig. 3. Quantitative cytology and representative histology of the synovial fluid obtained at sacrifice day 42. (A) Large mononuclear cells, (B) lymphocytes, and (C) heterophils (equivalent to human neutrophils), while control represents the non-operated joint. Histological images (right) are representative images from the synovial fluid of the cells for each graph (left). Images from top, (A) normal and reactive large mononuclear cells, (B) reactive lymphocytes, (C) reactive heterophils. Groups are divided by anatomical location, patella or trochlea, and cell source, autologous, allogeneic, or xenogeneic. Groups per graph are as follows: control (non-operated knee), patella autologous empty defect and autologous explant, patella allogeneic explant and construct, patella xenogeneic explant and construct, trochlea autologous empty defect, allogeneic construct, and xenogeneic construct. Groups which are not connected by a common letter are significantly different ($p < 0.05$).

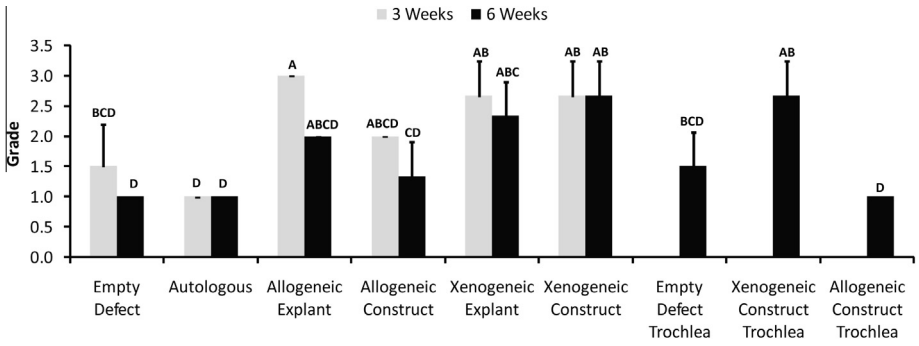

Fig. 4. Synovium was graded based on the presence of inflammatory cells by two blinded reviewers (BA, GD) and averaged per group. Numbers were as follows: 0 – absence of inflammatory cells, 1 – mild (<25% of the stroma affected), 2 – moderate (25–50% of the stroma affected), 3 – severe inflammatory infiltrate (>50% of the stroma affected). Groups which are not connected by a common letter are significantly different ($p < 0.05$).

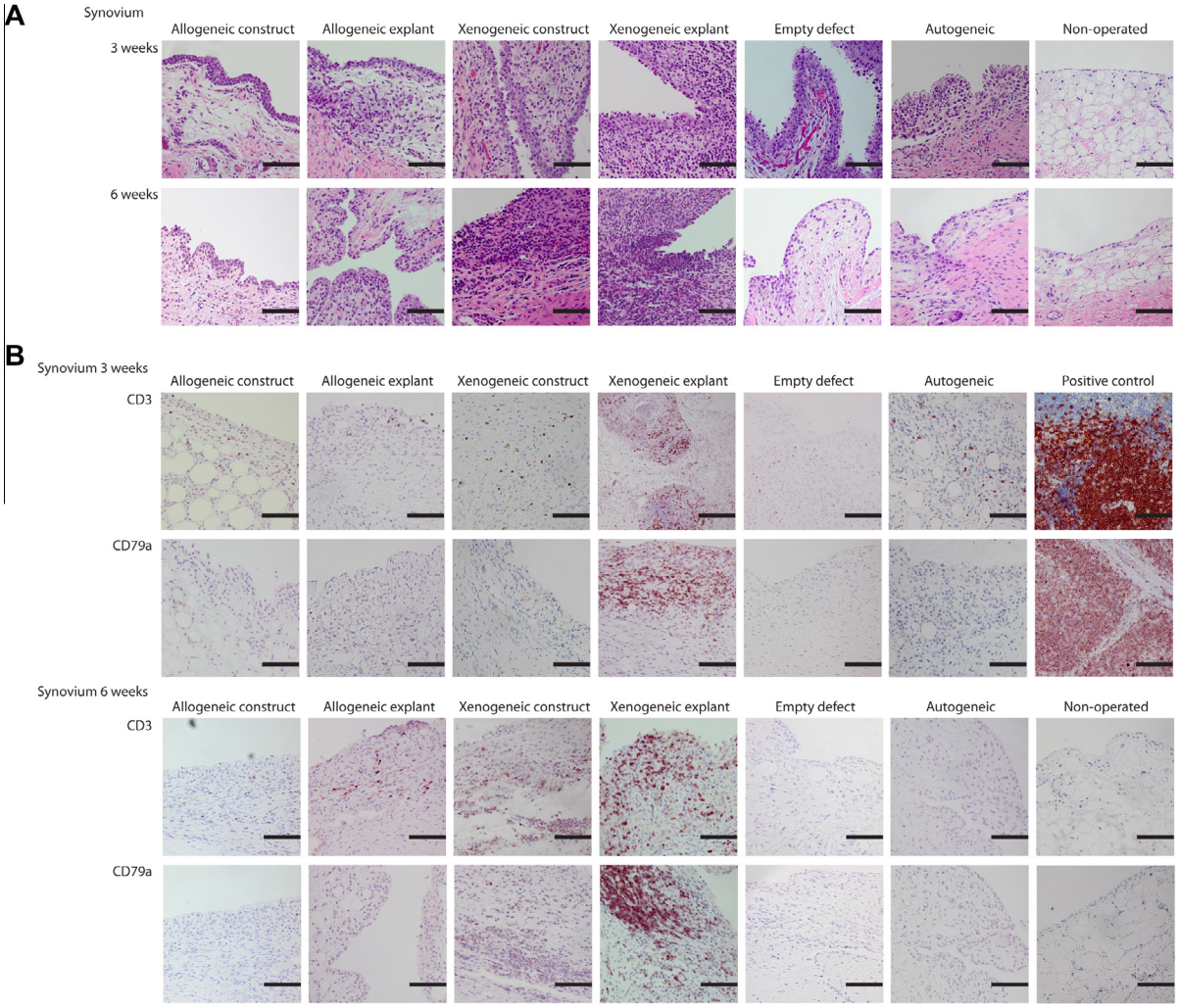

Fig. 5. Synovial histology and immunohistochemistry of 3 and 6 week patellar groups. (A) H&E of synovium. (B) IHC for CD3 (T cells) and CD79a (B cells) of synovium, positive controls consisted of either spleen or thymus tissues. All images are 20×. Scale bar is 100 μm.

The patellar surgical site resulted in low quality fill of the empty defects, an adaptive immune response to allogeneic implants, and poor overall outcome. The rationale for choosing the patella as one of the defect sites was due to two reasons: (1) to target regenerative treatments for chondromalacia patellae, and (2) the patella is easily accessible, relatively flat, and a site for previous cartilage regeneration efforts.[49–50] Patellar chondral lesions are extremely common, second only to lesions of the medial femoral condyle in patients younger than age 40 [51]. Treatments options for the management of chondromalacia of the patella are limited, ranging from conservative to surgical. Conservative measures include physical therapy to correct biomechanical imbalance of the joint as well as various injectable medications like corticosteroid, hyaluronic acid, or platelet rich plasma. Operative measures

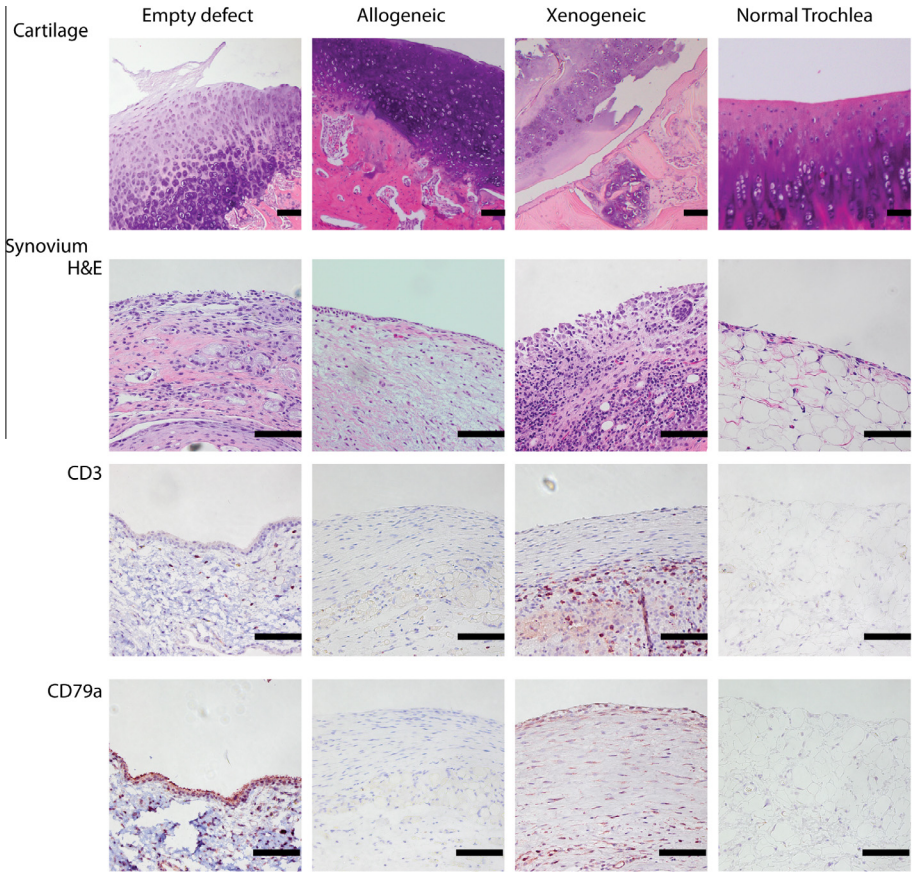

Fig. 6. Cartilage and synovial histology and immunohistochemistry of 6 week trochlear groups, all cartilage images are 10×, while synovial images are 20×. Scale bar is 100 μm.

range from arthroscopic thermal or mechanical debridement of chondral flaps to marrow stimulation techniques to allograft replacement (particular juvenile cartilage, osteohchondral allograft) to cell based therapy (autologous chondrocyte implantation, matrix assisted chondrocyte implantation). For severe cases of malalignment, osteotomies for restoration of mechanical axis are warranted. However, the patella is effectively embedded with the synovial tissue and adjacent fat pad. The synovial membrane is critical in mediating immune response within the joint [52,53]. Using the patella as a resurfacing model, an immune reaction was observed for allogeneic and xenogeneic treatments. Lack of integration and implant degradation was observed for all patella implant groups with synovial pannus formation, hyperplasia, and moderate to severe inflammatory cell infiltration, indicative of innate and adaptive immune responses. Recent work has demonstrated that synovial fibroblasts promote monocyte adhesion [54] and act as a bridge for inflammatory cell infiltration to the implant. This further emphasizes the importance of the synovium, its contact/adhesion, and its potential as an important moderator of immunity toward engineered tissues. Due to its proximity to the synovium, the patellar location exhibited inadequate healing due to adverse immune response.

The healing response was also inadequate in the patella likely due to lack of a robust blood supply. Ossification of the patella arises by blood vessels penetrating the proximal and medial border of the cartilage anlage via the quadriceps tendon. Unlike the distal femur, which has a rich blood supply, the patella lacks a primary nutrient foramen and is nourished by a few small vessels entering the bone [55]. Because of this relatively impoverished blood supply, it is perhaps not surprising that the patella seldom undergoes bony remodeling. Lack of integration and devitalization of the construct indicates cellular activity and a supportive blood supply is necessary for cartilage integration [56]. In addition, recruitment of multipotent stem cells requires access to the richly vascular subchondral bone; a necessary step for stimulating integration and healing [57]. Therefore, the patella's limited blood supply may be one of the underlying factors in creating a challenging environment for cartilage integration as compared to the highly vascular distal femur.

To determine if defect location was a factor for implant success, an alternative location within the patellofemoral compartment was also investigated. The trochlear groove opposes the patella but is isolated from the adjacent synovium and is supported by the rich blood supply of the distal femur. Since the allogeneic construct elicited minimal immune response and was still viable in the patella, this group was carried forward for the trochlear full-thickness defect, with a xenogeneic construct as negative control. Overall, the immune response to allogeneic implants was less than xenogeneic constructs or control and varied by location with a stronger response at the patella. Implant outcome was more favorable in the trochlea than the patella. Synovial fluid and tissue analysis indicated negligible immune response or hyperplasia for trochlear allogeneic constructs. Implanted allogeneic constructs exhibited strong integration with the underlying bone, remained intact, and filled the defect acting as a functional cartilage replacement. Xenogeneic constructs failed in both locations and provoked a strong immune response. The outcome of the immune response was similar between grafts of xenogeneic origin regardless of

location: no difference was noted between explants or constructs. However, the outcome of grafts of allogeneic origin was dependent on location.

Limitations of this study derive from the use of a relatively small sample number per timepoint and the use of the rabbit as a small animal model. While the number of animals used in this study can be considered low, we diligently tried to reduce the number of animals as much as possible assuming that statistical power is met. However, the results are clear and the overall larger number of rabbits (48), including multiple timepoints, is sufficient to deliver the clinically relevant conclusions of the study. A second major limitation can be found in the use of the rabbit as an animal model of the human immune response in the knee. As always with research of this nature, subsequent work needs to examine the similarities and differences that exist between species to determine the suitability of the rabbit as an appropriate model.

This study demonstrates that in articular cartilage an observed adaptive immune response to allogeneic implants is mediated by proximity to the synovium and not as much by intimate contact with the blood supply. The patella has close proximity to the synovium but minimal blood supply, in contrast to the trochlear groove which is isolated from the synovium but has good blood supply. Influx of inflammatory cells at the subchondral bone and marrow was not observed. Instead, synovial inflammation, characterized by synovial hypertrophy and hyperplasia, inflammatory cell infiltrates, and angiogenesis, was more pronounced in the patella than the trochlea. Furthermore, allogeneic constructs in the trochlear groove induced minimal immune response compared to the patellar location. Previous work has linked the importance of synovitis in osteoarthritis progression and cartilage destruction [52], and it appears that the synovium mediates the immune response to the implanted cartilage. A graft in the patella in close proximity to the synovium induced formation of a pannus, composed of actively proliferating fibroblasts, immune cells, and rich vascularity, which invaded the defect and implant. Previously, synovium-derived pannus was linked to cartilage degeneration [53]. Thus, location-dependent immune tolerance to allogeneic implants in articular cartilage appears to be explained by the location's proximity to the synovium.

5. Conclusions

This study showed that xenogeneic materials provoked a profound innate and adaptive immune response whereas allogeneic neocartilage created a milder immune response in the patella and negligible response within the trochlea. Furthermore, allogeneic neocartilage survived within the trochlea and demonstrated graft integration into the underlying bone. The immunological outcomes of allogeneic implants depend on the location of implantation, with respect to the proximity of the synovium. Inasmuch as (1) xenogeneic materials are strongly immunogenic in articular cartilage and (2) the immune response in articular cartilage depends on anatomical location and proximity to the synovium and less so on contact with the blood supply, the assumption that articular cartilage is immunoprivileged should be reconsidered.

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–3, 5, and 6, are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2015.05.025.

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