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Supplementary Information for

Mechanically Superior Matrices Promote Osteointegration and Regeneration of Anterior Cruciate Ligament Tissue in Rabbits

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Supplementary Information Text

Materials and Methods

Fabrication of Bioengineered ACL Matrices

Two types of bioengineered ACL matrices were fabricated for the surgical procedure. The constituent components of the braided constructs were 30 filament 75 denier PLLA fibers and 70 denier polyethylene terephthalate (PET) fibers (Teleflex Medical OEM, Coventry, CT). Sixteen PLLA fibers were plied together to form yarns of 1200 denier. Fourteen PET fibers were plied together to form yarns of 980 deniers. The first bioengineered ACL matrix, termed L-C Ligament, was fabricated by braiding 24 yarns of PLLA using a 4 x 4 braiding configuration. The second bioengineered ACL matrix, termed Tiger Graft, was fabricated by braiding 20 yarns of PLLA and 4 yarns of PET together with a 4 x 4 braiding configuration.

Both bioengineered ACL matrices were braided to have two distinct regions, a bony and intra-articular region (Fig. 1A). The bony region was braided at the height of 37 cm, and the intra-articular region was braided at the height of 67 cm. This was achieved by modulating the braiding height throughout the braiding process, such that after 1 cm of the bioengineered ACL matrix was braided at 37cm, the braiding process was halted, and the braiding height raised. Subsequently, 1 cm was braided, and so on. Each 1 cm region was tied off by a 14-ply yarn of PLLA to maintain the integrity of each region, and the final length of a bioengineered ACL matrix was 3 cm, and each region was 1 cm long.

Bioengineered ACL matrices were cleaned after fabrication by incubating in 70% ethanol for one hour to remove any lubricant from the extrusion process. Subsequently, the bioengineered ACL matrices were rinsed in ultrapure water three times for thirty minutes each. Finally, the bioengineered ACL matrices were lyophilized. The bioengineered ACL matrices were treated with oxygen plasma for 1 minute at 0.5 Torr and 100 Watts to increase their hydrophilicity. Finally, the bioengineered ACL matrices were double bagged in autoclave pouches, sterilized by ethylene oxide gas, and stored in a desiccator until surgery.

ACL Reconstruction Procedure

Pre-operative analgesic was administered (Buprenorphine). Anesthesia was administered with a cocktail of ketamine, xylazine, and atropine. Isoflurane was utilized for anesthesia maintenance. The left knee was operated on for all animals. A vertical midline longitudinal incision was made extending from the distal femur to the tibia. The skin and subcutaneous fascia were retracted to expose the patellar tendon. The dissection was continued between the quadriceps tendon and the vastus medialis muscle. The capsule and the synovial membrane were divided from the medial aspect of the patella and the patellar tendon. With the knee in extension, the patella was dislocated. Subsequently, the knee was flexed to expose the joint cavity. The fat pad was dissected with a midline incision, but left intact, to expose the ACL. The ACL was excised at its tibial attachment, and the stump was removed.

For BMAC therapy, bone marrow was harvested from the femur and tibia after ACL transection using an 18-gauge Jamshidi needle (Movie S1). A 5 mL syringe was flushed with heparin 1000 U/mL, and 2 mL of heparin was loaded. BMAC was biopsied so that the tunnel made by the Jamshidi needle was in line with the desired bone tunnel placement. After the Jamshidi needle punctured the bone, the stylet was removed, and a 5 mL syringe attached. Approximately 200 µL of heparin was injected into the bone, which was followed by applying negative pressure to the syringe. Typically, 3 mL of bone marrow and blood was collected in addition to the 2 mL of heparin in the syringe.

There are several surgical techniques for rabbit ACL reconstruction that are reported in the literature (1–11). The notable differences in these surgical techniques are the size of the bone tunnel and method of fixation. Bone tunnel sizes range from 1.5 to 3.5 mm, and grafts are

predominately fixed by suturing to the periosteum and soft tissue. Additionally, the degree of knee flexion varies from 0 to 90 ° at the time of graft fixation. In our previous studies, the bioengineered ACL matrix was fixed at 0 ° of flexion (12, 13). However, the rabbit knee is flexed ~140° at rest (14). Thus, we hypothesized that fixation at 0° likely caused excessive tension on the bioengineered ACL matrix and likely contributed to the high rupture rate observed. Accordingly, in this study we changed our surgical approach and fixed the bioengineered ACL matrices at 140° of knee flexion.

Bone tunnels were made with a 1.1 mm k-wire. Femoral and tibial bone tunnels were drilled through the anatomic footprint of the native ACL with the knee in hyperextension. After the desired bone tunnel placement was achieved, a 3.2 mm cannulated drill bit was used to dilate the bone tunnel to accommodate the graft. Due to the size of the joint space, an outside in-drilling technique was used when dilating the tunnel. Care was taken to not damage the PCL and meniscus during drilling. Finally, a lateral notchplasty was performed to widen the intercondylar notch (Fig. S8 A). This was achieved with a 1.4 mm burr. Finally, the bone tunnels were flushed with saline to remove debris.

L-C Ligaments and Tiger Grafts were whipstitched in the bony region of the braid. 2-0 fiberloop was used for whipstitching, and three throws were placed in the 5 mm region of the most distal end. A tensioning suture, 2-0 Vicryl, was placed on the tibial end of the bioengineered ACL matrices. With the bioengineered ACL matrices positioned in both bone tunnels, fibrin glue was implanted to lie just underneath the titanium suture button. Titanium suture buttons (3.5 mm) were used for femoral fixation, and titanium suture buttons (12 mm) were used for tibial fixation. The femoral side was fixed first. A 500-gram weight was attached to the tensioning suture and allowed to hang freely to provide 5 N of tension to the graft (Fig. S8 B). With the knee joint at 140 degrees of tension, the free ends of the fiberloop were tied down onto the titanium suture button (Fig. S8C, Movie S2). Care was taken not to supersede the tensioning force of the 500-gram weight during the knotting of the free ends of the fiberloop. The knee joint cavity was closed with 2-0 Vicryl sutures in a figure eight fashion. Postoperatively, the rabbits received daily administration of antibiotics (Baytril) for three days. Post-operative analgesics were given in the form of fentanyl patches and were removed after three days.

Preparation of Fibrin Glue for Growth Factor and Bone Marrow Aspirate Delivery
Bone tunnel and knee joint cavity were sealed with Tisseel fibrin sealant (Baxter Healthcare), by mixing the fibrin and thrombin components together. 100 µL of fibrin glue was inserted to the extra cortical exit of the bone tunnels (Movie S3), and 400 µL of fibrin glue was injected into the knee joint cavity (Movie S4). For BMAC therapy, BMAC was mixed with thrombin and subsequently mixed with the fibrinogen component to form fibrin glue. The ratio of BMAC: thrombin: fibrinogen was 1:1:2. Additionally, 1 mL of BMAC was injected into the knee cavity after suturing it closed (Movie S5). For growth factor therapy, 10 µg of BMP-2 (355-BM/CF, R&D Systems, Inc.) was mixed with 25 µg of heparan sulfate (rHS001, TEGA Therapeutics, Inc.) in sterile PBS and incubated for 10 min at 37ºC. Then, this solution was mixed with the thrombin component, followed by mixing with fibrinogen to form fibrin glue that was implanted in the extra-cortical exit of the bone tunnels. In the same manner, 100 ng of FGF-2 (233-FB/CF, R&D Systems, Inc.), 100 ng of FGF-8b (423-F8/CF, R&D Systems, Inc.), and 25 µg of heparan sulfate were prepared for the knee joint cavity. For the L-C Ligament and Tiger Graft groups, the same amount of sterile PBS was mixed with the thrombin component before forming fibrin glue.

Concentration of Bone Marrow Aspirate and Flow Cytometry Analysis
All tools and tubes were pre-rinsed with 1000 U/ml of heparin three times. Approximately 6 mL of bone marrow was aspirated and transferred to a new tube. It was then filtered by 500 µm filter to get rid of fatty tissue and aggregates. The bone marrow was then loaded into a dual syringe system (Arthrex ACP Double-syringe System) and was spun at 800 rpm for 10 minutes. The supernatant was transferred slowly by the inner syringe and 3 fractions were obtained.

Subsequently, flow cytometry was performed to assay cell viability and cell count for the obtained fractions. One hundred µL were sampled from each fraction. The live/dead assay (life technology) was used. Bone marrow before concentration, BMA, served as the control. The analysis was carried out on a MACSQuant® Analyzer 10 Flow Cytometer. The fraction with the highest viability and cell count was injected back into the rabbit.

Colony Forming Unit Assay
One hundred µLs of BMAC was cultured in 100 mm2 dishes in triplicate. Cultures were incubated in 10 mL of DMEM high glucose medium was used (Gibco™, Catalog # 11965) and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. After 48 hours of culture, the medium was aspirated and replenished. Culture dishes were not washed with PBS until day 7. Subsequently, the medium was changed every three days. At day 14, plates were fixed with ice cold methanol for 10 minutes. Cells were incubated in 0.5% (w/v) crystal violet dissolved in 25% methanol for 10 minutes and then subsequently washed until the culture dish was clear. Colonies that had a defined center and consisted of > 50 cells were counted.

Calcein and Xylenol Orange Labeling
Rabbits harvested 12 weeks post-operatively received an intraperitoneal injection of 10 mg/kg calcein 13 days prior to euthanasia and an intraperitoneal injection of 90 mg/kg xylenol orange 5 days prior to euthanasia. Both dyes were prepared in a 1% sodium bicarbonate (w/v) in PBS buffer, and the pH was adjusted to 7.4. All dyes were sterile filtered prior to injection. Rabbits harvested at 24 weeks received two calcein injections, at 84 and 5 days prior to euthanasia, and one xylenol orange injection 13 days prior to euthanasia.

Tissue Harvest
Both hind limbs of the rabbits were harvested at the end of the study. Synovial fluid was also collected. After the initial skin incision, a small incision was made in the medial aspect of the patellar tendon. The knee joint was then palpated to force the synovial fluid out of the joint. The synovial fluid was collected with an insulin syringe, dispensed in an Eppendorf tube, and kept on ice until freezing at -80ºC. Samples designated for mechanical testing were incubated in PBS directly after harvest and subsequently wrapped in gauze, vacuum sealed, and stored at -80ºC until mechanical testing. Samples designated for histology were immediately incubated in 10% neutral buffered formalin.

Processing of Tissue for Histology
Immediately after harvest, tissues were fixed in 10% neutral buffered formalin for 24 hours. At 24 hours, the samples were trimmed to allow the fixative to penetrate the bone tunnel area and placed in approximately 300 mL of 10% fresh formalin. Samples were placed on a rocker and fixed for 14 days, and formalin was replenished every three days. After 14 days of fixation, samples were placed under running tap water overnight to remove the fixative. Subsequently, the samples were dehydrated by 70% ethanol, 95% ethanol, 100% 2-propanol, and xylene. Subsequently, samples were infiltrated with methyl methacrylate as described by Erben (15).

Sectioning and Staining of Tissues
X- ray scans were utilized to determine the location of the bone tunnels, and samples were trimmed to allow for sagittal sections of the bone tunnel to be made. An automated microtome was used for sectioning. Japanese tape was applied to the block face to support the maintenance of fiber morphology. Femoral sections were cut at a thickness of 12 µm, and tibial sections were cut at a thickness of 8 µm. Sections were mounted on gelatin coated glass slides in a similar fashion as conducted by Erben (15). Unstained sections were used to visualize bone labels. Prior to staining sections, sections were deplasticized by 2-methoxy ethyl acetate solution and rehydrated. Sections were stained with Masson-Goldner's trichrome stain (Sigma-Aldrich, St. Louis, MO), 2% (w/v) Toluidine Blue O solution, or 0.1% (w/v) safranin O solution following the manufacturer's instructions.

Semi-Quantitative Histological Analysis
The area of metachromasia within the bone tunnel of toluidine blue stained samples was quantified using ImageJ. The area of the entire bone tunnel was measured. Then areas of metachromasia staining were measured (Fig. S1). Finally, areas in which no tissue was present, due to histological processing, were measured. The total bone tunnel area was defined as the area of the bone tunnel subtracted by the area of blank tissue area (i.e. adjusted bone tunnel area). The percentage of metachromasia was determined by dividing the area of metachromasia by the adjusted bone tunnel area. Analysis of mineral apposition rate was conducted with ImageJ. The distance between calcein orange and xylenol orange labels was measured and divided by the time in between labeling events.

Biomechanical Testing
Knee joints were thawed overnight prior to potting and biomechanical tests. All soft tissue, ligaments, and tendons were removed such that only the Femur-ACL-Tibia complex remained. The titanium suture buttons were maintained for mechanical testing. Knee joints were potted in acrylic resin (Bozworth Duz-All, Self-Cure, Acrylic Resin), and samples were kept moist by continually spraying with PBS. Samples were then mounted on a custom-made clamping system, and alignment of the ACL was ensured to be parallel with the line of force. The diameter of the ligaments was measured with digital calipers, and a 2% strain rate was used for all tests and was generally 0.2 mm/s. After bone tunnel pullout from both the femur and tibia, the graft alone was clamped again and loaded to failure. The Instron 5544 was used for all tests. A 500N load cell was used for all explants (Instron 2530-416). A 2kN load cell (Instron 2530-418) with a pneumatic action grips for cord and yarns (Instron 2714-040) was used for tensile tests of the bioengineered ACL matrices prior to implantation.

Synovial Fluid Analysis
Synovial fluid was collected after euthanasia. A small incision in the knee joint cavity was made, and the synovial fluid was extracted with an insulin syringe. Synovial fluid was then stored in an Eppendorf tube at -80 °C until analysis. An independent laboratory conducted the ELISA assays (RayBiotech Life, Peachtree Corners, GA). Samples were digested at 37 °C for 30 minutes with 20 units/mL of type IV hyaluronidase (Sigma-Aldrich, St. Louis, MO) (16). Subsequently, were loaded into a Spin-X column (Corning, Amsterdam, the Netherlands) and centrifuged for filtration. In some cases, the synovial fluid from multiple rabbits was combined to reach a sample volume adequate for the ELISA. Two multiplexed sandwich ELISAs were used for quantification of cytokine levels (QAL-CYT-1-1 & QAL-CYT-2-1, RayBiotech Life).

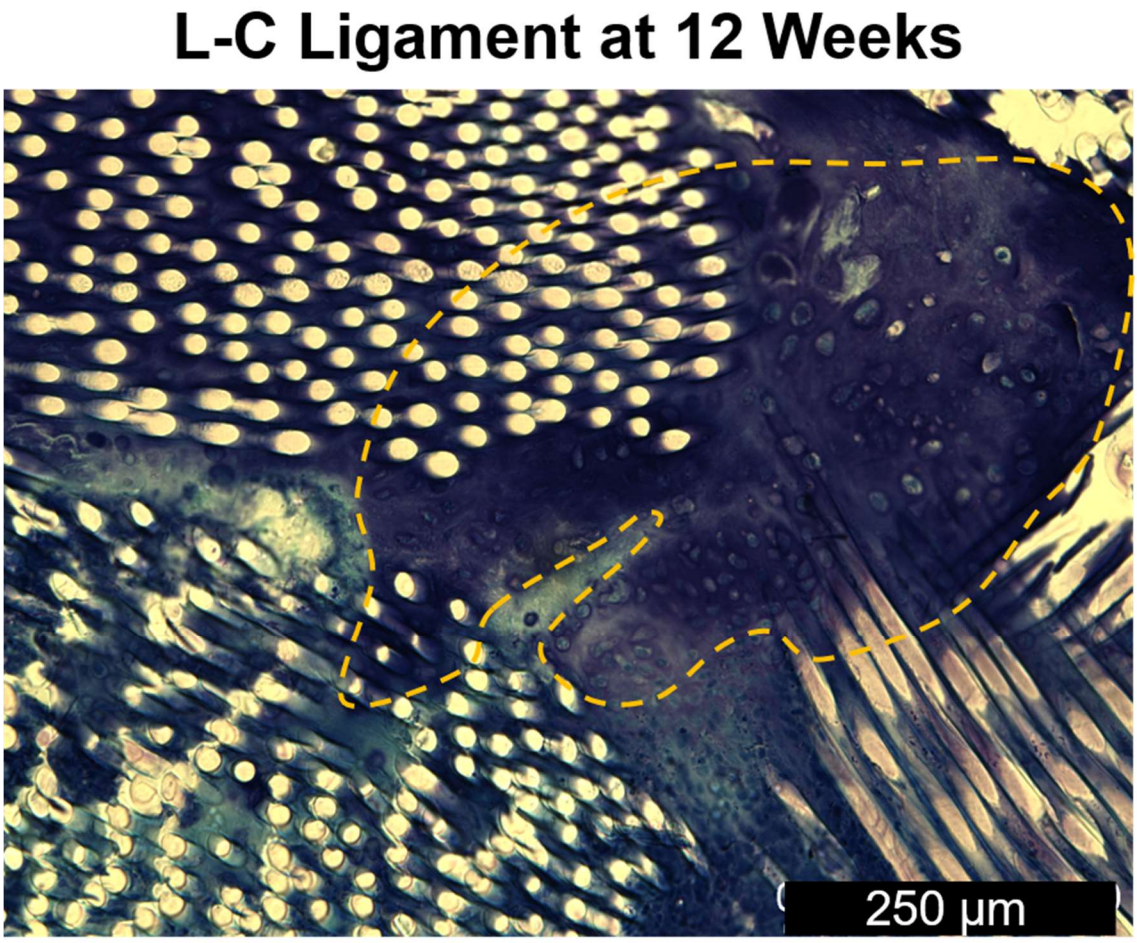

Fig. S1. Representative Toluidine Blue staining of the bioengineered ACL matrices. Yellow outlined area demonstrates region of metachromasia with cuboidal cells and is an example of the methods used for the quantification of metachromasia area (Fig. 5 *B* and *C*).

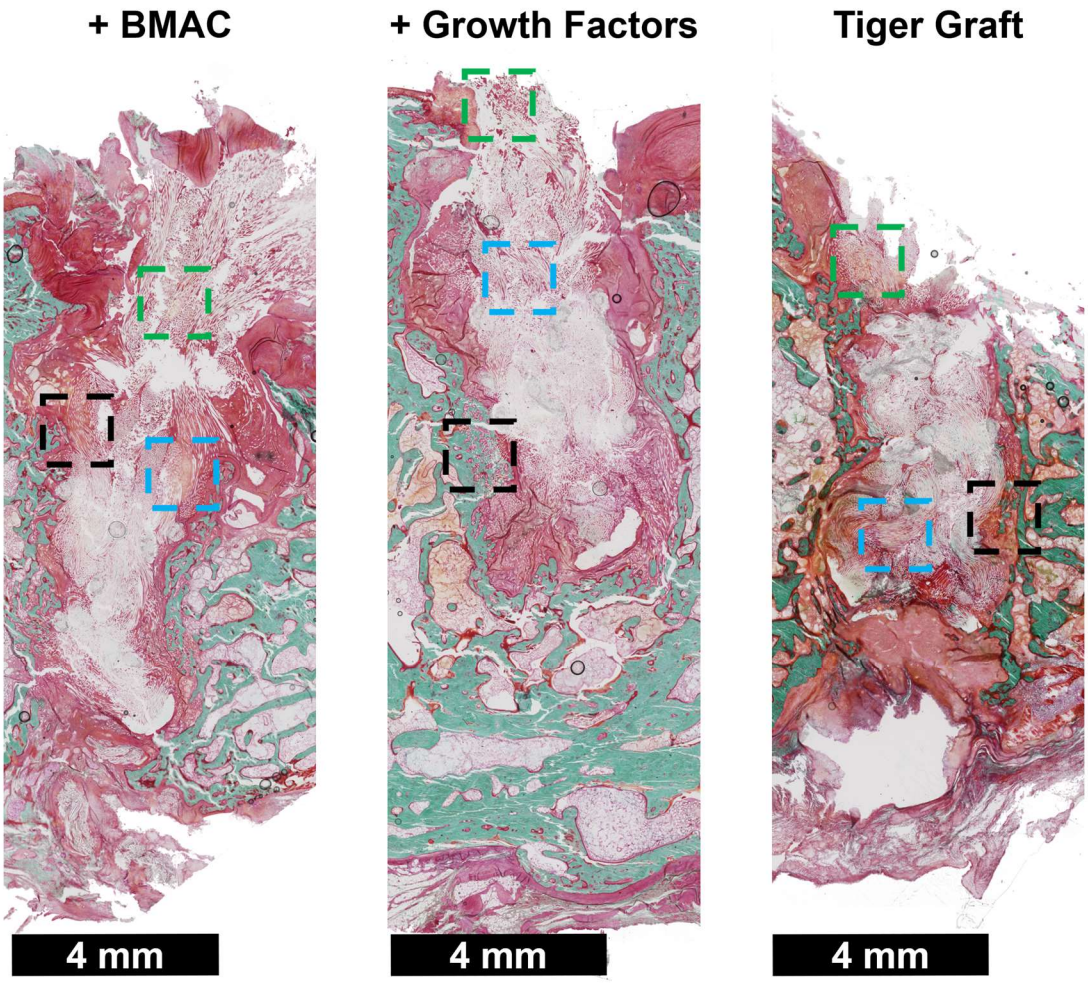

Fig. S2. Representative femoral bone tunnel area for the treatment groups. The bone tunnel area corresponds to Fig. 5. The outlined areas were imaged at higher magnification and correspond with Fig. 5 i – iii.

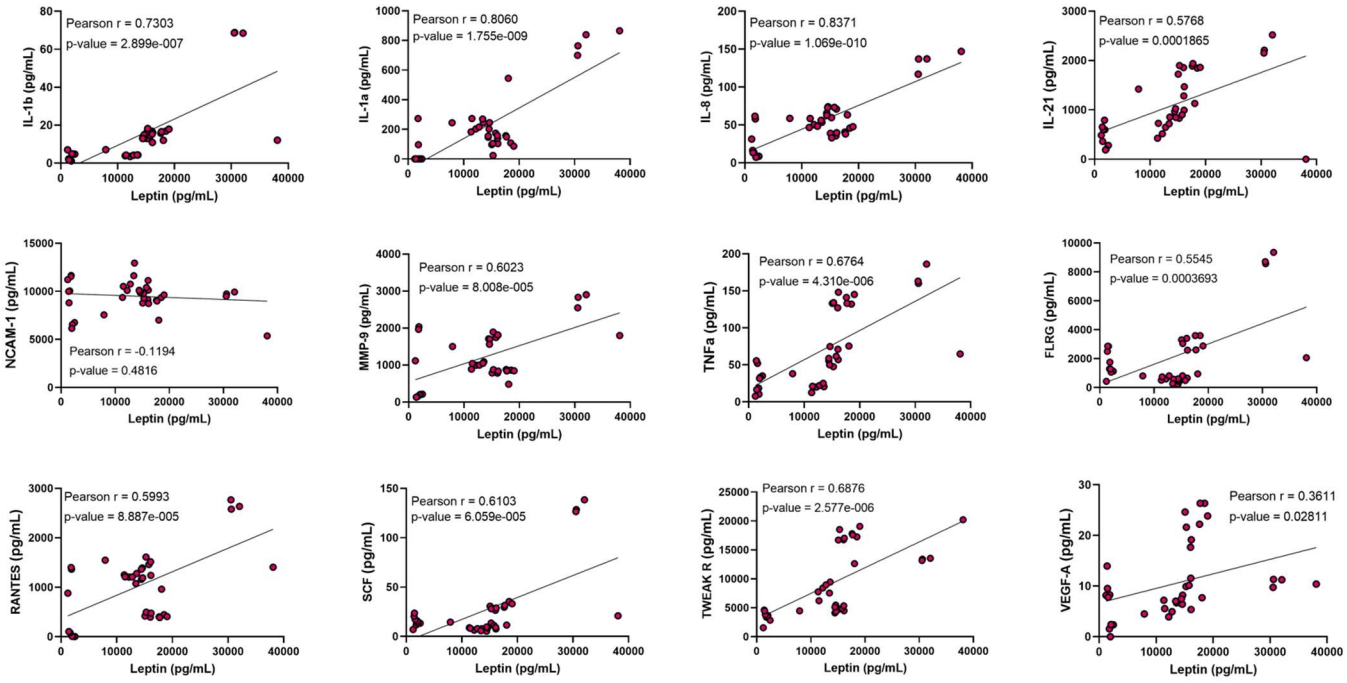

Fig. S3. Leptin positively correlates with pro-inflammatory cytokines. Correlation of leptin and cytokine levels from a multiplex ELISA assay. XY pairs = 37. Pearson correlation analysis.

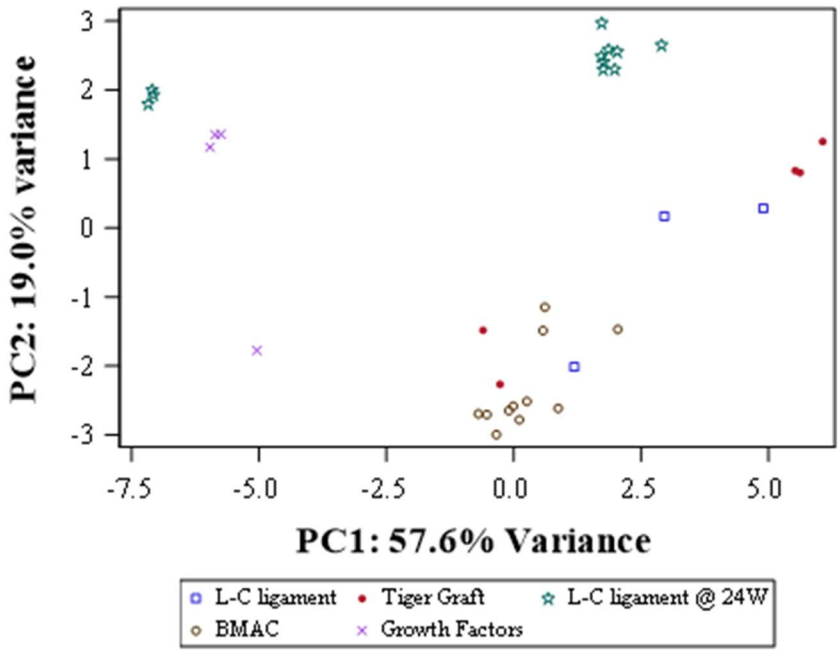

Fig. S4. Principle component analysis of cytokine data. The first and second principle component captured 76.6% of the variance.

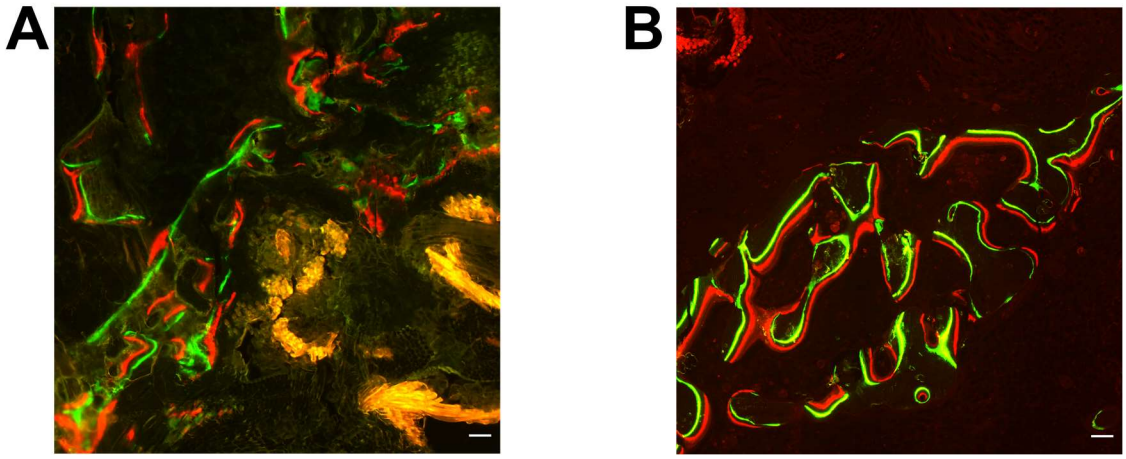

Fig. S5. Representative calcein and xylenol orange staining of the (A) femur and (B) tibia (n = 3, 12 weeks post-ACL reconstruction)

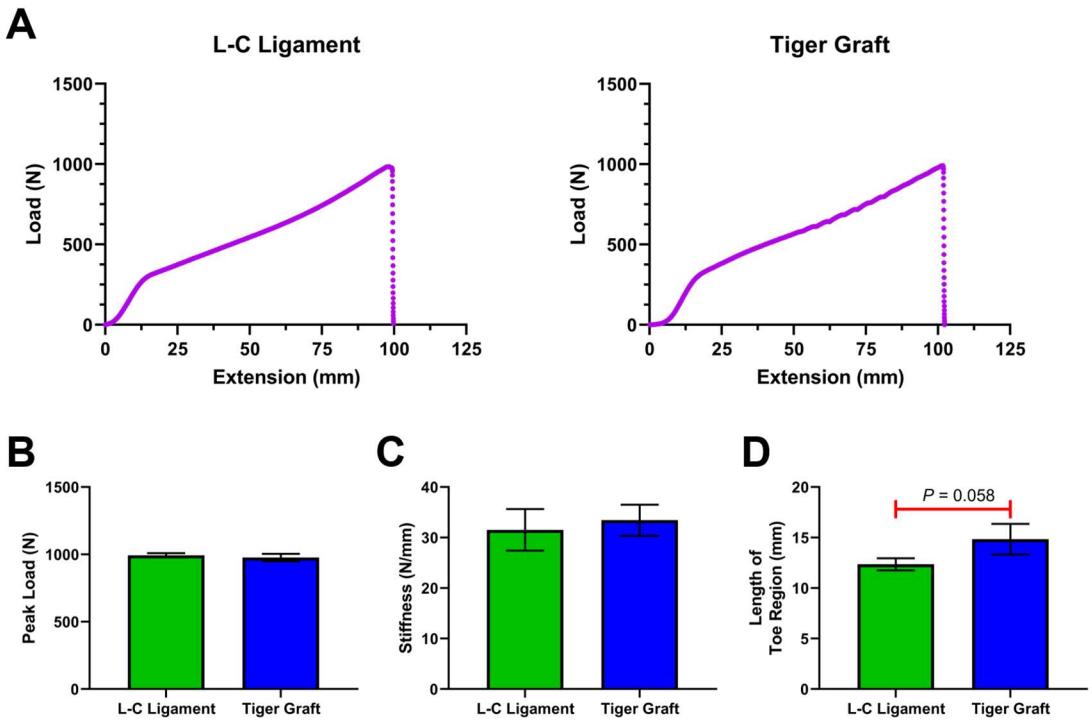

Fig. S6. L-C Ligament and Tiger Graft have nearly identical mechanical properties. (**A**) Representative extension versus load plot of the L-C Ligament and Tiger Graft from tensile testing. (**B**) Quantification of the peak load of the L-C ligament and Tiger Graft. Mean peak load ± SD (n = 3). No significant difference between groups, unpaired t test. (**C**) Quantification of the stiffness of the L-C ligament and Tiger Graft. Mean stiffness ± SD (n = 3). No significant difference between groups, unpaired t test. (**D**) Quantification of the length of the toe region of the L-C ligament and Tiger Graft. Mean stiffness ± SD (n = 3). No significant difference between groups, unpaired t test.

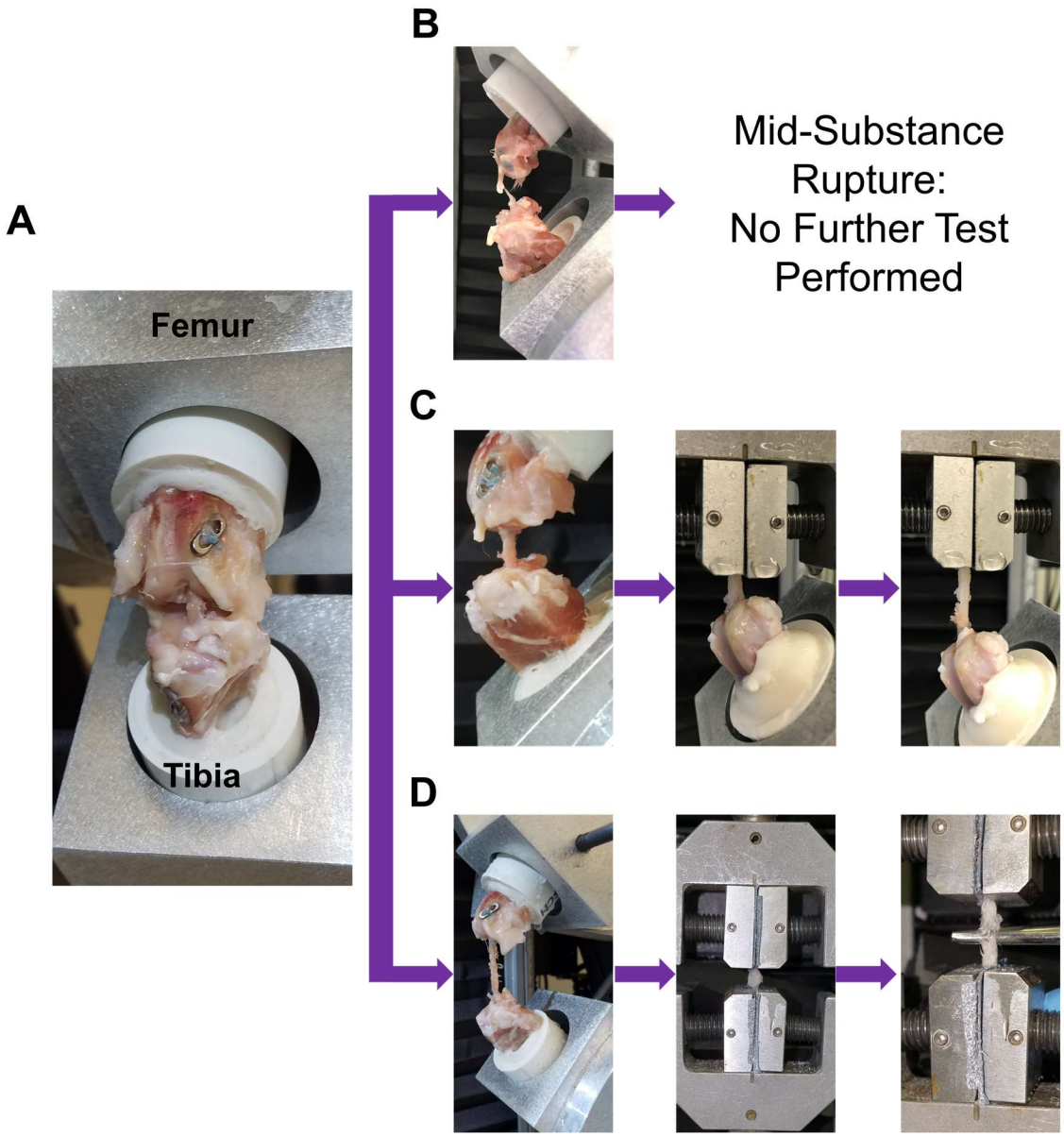

Fig. S7. Biomechanical testing schematic demonstrates three modes of failure. (**A**) Representative image of a potted femur-bioengineered ACL matrix- tibia complex. (**B-D**) Demonstration of the possible outcomes of the bioengineered ACL matrix after initial tensile test. (**B**) Representative image of a primary mid-substance rupture following tensile testing of the femur-bioengineered ACL matrix-tibia complex. (**C**) Representative flow chart for tensile tests to determine osteointegration strength of the bioengineered ACL matrix-bone interface. Suture is removed after bone tunnel pullout and the bioengineered ACL matrix is clamped and loaded to failure. (**D**) Representative flow chart of tensile tests after pullout from both the femoral and tibial bone tunnel. The bioengineered ACL matrix is clamped at both ends and loaded to failure.

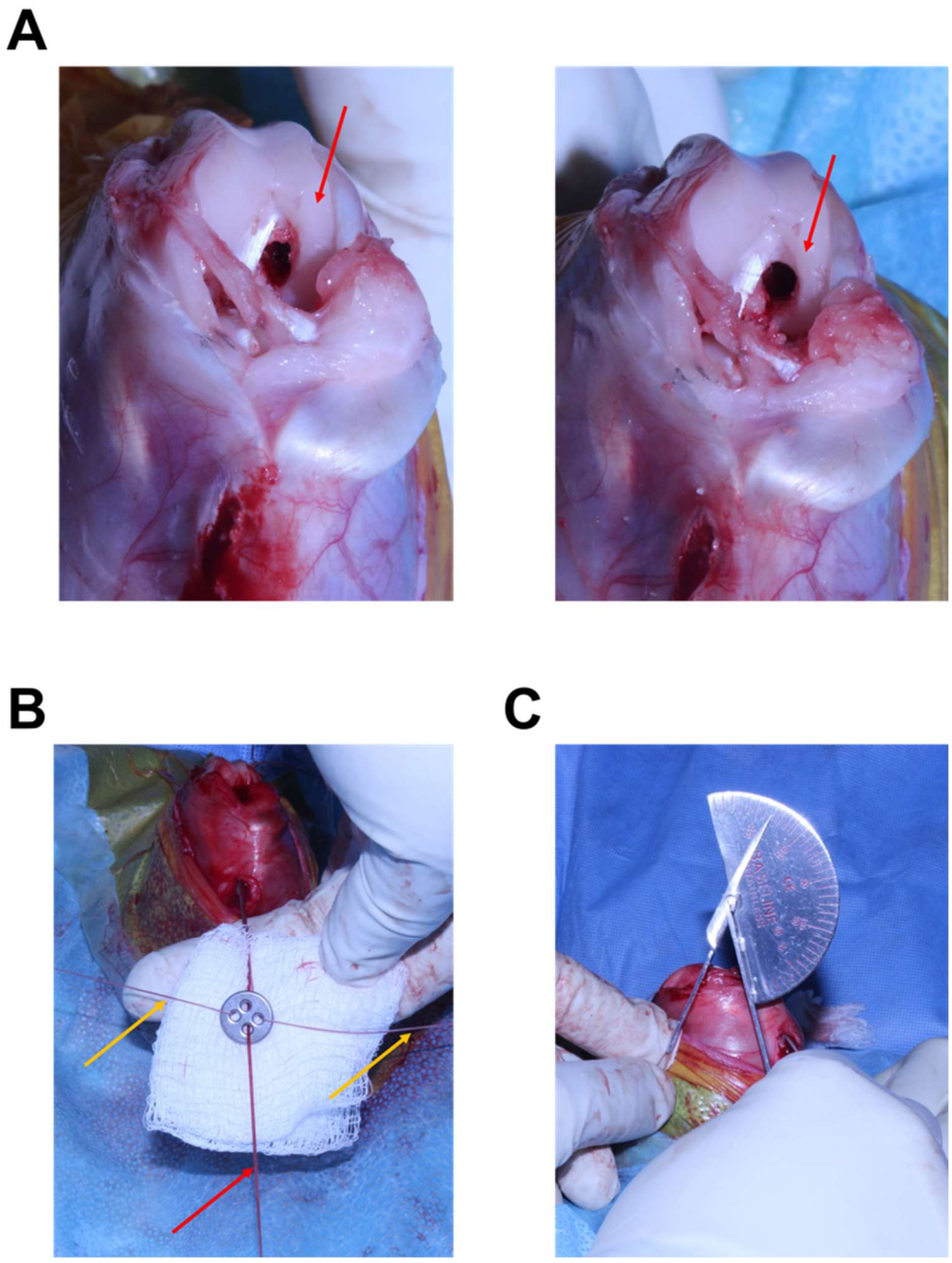

Fig. S8. Notchplasty and graft fixation at 140° are considered critical to the survival of the bioengineered ACL matrices in vivo. (**A**) Representative image of the femoral bone tunnel aperture before (left panel) and after (right panel) notchplasty. The red arrows point to the site where a notchplasty was performed. (**B**) Representative image of the tensioning suture (red arrow) used to apply 5N of tensile force to the graft while the fiberloop (yellow arrows) are tied. (**C**) Representative image of the positioning of the knee joint to 140° of flexion prior to graft fixation.

Movie S1. Bone marrow aspiration procedure.

Movie S2. Demonstration of suture fixation of the bioengineered ACL matrix.

Movie S3. Implantation of BMAC fibrin glue gel into the femoral bone tunnel.

Movie S4. Intra-articular application of BMAC complexed with fibrin glue.

Movie S5. Intra-articular injection of BMAC after joint closure.

Dataset S1. Excel file of data.

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