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TITLE:

Biological Compatibility Profile on Biomaterials for Bone Regeneration

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SUMMARY:

The number of novel biomaterials engineered for repairing large bone lesions is continuously expanding with the aim to enhance bone healing and overcome the complications associated with bone transplantation. Here, we present a multidisciplinary strategy for pre-clinical biocompatibility testing of biomaterials for bone repair.

ABSTRACT:

Large non-union bone fractures are a significant challenge in orthopedic surgery. Although auto- and allogeneic bone grafts are excellent for healing such lesions, there are potential complications with their use. Thus, material scientists are developing synthetic, biocompatible biomaterials to overcome these problems. In this study, we present a multidisciplinary platform for evaluating biomaterials for bone repair. We combined expertise from bone biology and immunology to develop a platform including *in vitro* osteoclast (OC) and osteoblast (OB) assays and *in vivo* mouse models of bone repair, immunogenicity, and allergenicity. We demonstrate how to perform the experiments, summarize the results, and report on biomaterial biocompatibility. In particular, we tested OB viability, differentiation, and mineralization and OC viability and differentiation in the context of β -tricalcium phosphate (β -TCP) disks. We also tested a β -TCP/Collagen (β -TCP/C) foam which is a commercially available material used clinically for bone repair in a critical-sized calvarial bone defect mouse model to determine the effects on the early phase of bone healing. In parallel experiments, we evaluated immune and allergic

responses in mice. Our approach generates a biological compatibility profile of a bone biomaterial with a range of parameters necessary for predicting the biocompatibility of biomaterials used for bone healing and repair in patients.

INTRODUCTION:

Bone repair is a complex process that begins with hematoma formation, inflammation, callus formation and then remodeling^{1,2}. However, bone regeneration potential is limited to the size of the bone fracture^{1,3}. For instance, large bone fractures caused by trauma, cancer or osteoporosis may not heal and are termed non-union bone fractures. These bone lesions often require treatment to promote healthy physiological bone repair and regeneration. Currently, autograft and allograft bone transplantation is the approach of choice⁴ with 2.2 million bone replacement procedures annually⁵. Though these procedures have a high success rate, there may be complications, for example, limited availability of bone, infection, donor site morbidity, and rejection⁴. New alternatives for bone tissue engineering are being sought to address these challenges.

The design of biomaterials based on natural or synthetic polymers, bioceramics or metals in combination with cells and bioactive molecules is on the rise⁶. Our current understanding of physiological bone healing and healing in the context of biomaterials depends on multiple factors such as mechanical properties and multiple local and systemic factors including cells from the circulation and fracture site⁷⁻⁹. Biomaterials for bone regeneration aim to promote osteogenicity and osseointegration¹⁰ and are ideally biocompatible, biodegradable, and porous (promoting cell migration, oxygen, and nutrients). They also need to be sufficiently strong to support the fracture site to relieve pain. Additionally, inflammatory factors are required to initiate the healing process. However, if the biomaterial induces excessive inflammation and allergic responses, this might limit or inhibit bone healing^{11,12}. Thus, an interdisciplinary approach is necessary to evaluate biomaterials developed for bone repair.

In this study, we present a pre-clinical evaluation of representative materials, 1) Orthovita Vitoss foam which is a commercially available cancellous bone graft substitute consisting of tricalcium phosphate composed of nanometer-sized pure β -tricalcium phosphate (β -TCP) particles and Type 1 bovine collagen (C) (β -TCP/C foam) and 2) β -TCP disks. Here, we illustrate biocompatibility testing of these biomaterials using primary osteoblast (OB) and osteoclast (OC) assays, an *in vivo* model of bone repair, an immunological assessment comprising *in vitro* T lymphocyte proliferation and cytokine production, and *in vivo* immunogenicity and allergenicity, as previously reported¹³.

PROTOCOL:

The procedures were done with BALB/c mice following all guidelines for the Care and Use of Laboratory Animals of the Austrian Ministry of Science and were approved by the Committee on the Ethics of the Austrian Ministry of Science.

1. Primary Mouse OB Culture

1.1. OB isolation from neonatal mouse calvaria using enzymatic digestion

1.1.1. Euthanize 1-2-day old neonatal pups (60 in total) by decapitation and place the heads in a Petri dish with sterile 1x phosphate-buffered saline (PBS).

1.1.2. Hold the head by the nape of the neck, and cut the skin away using sterile scissors. Incise the calvarium by piercing it (approximately 0.1-0.3 mm) with a pair of scissors that are then inserted underneath the calvarium in the back of the head at the base of the skull and cut along the calvarial edge laterally from back to front above the ears and then above the nasal bridge (Figure 1).

1.1.3. Incubate dissected calvaria with 8 mL of filter sterilized digestion solution (300 units/mL collagenase type IV and 2.14 units/mL dispase II dissolved in α -minimum essential medium (α -MEM)) in a 50 mL conical tube at 37 °C for 10 min in a shaking incubator at 200 shakes/min.

1.1.4. Discard the first supernatant and repeat the digestion procedure three times with 8 mL of digestion solution. Collect the supernatant containing the cells after each digestion step and add it to a 50 mL conical tube. Store the 50 mL conical tube with the cells in an ice water bath until the digestion procedure (approximately 40 min) is completed.

1.1.5. Centrifuge the cells at 300 x g for 5 min at 4 °C, aspirate the supernatant (with a Pasteur pipette attached to a vacuum pump) and resuspend in 40 mL of bone growth medium (BGM) containing α -MEM with 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin-streptomycin solution. Then add 10 mL of the cell suspension to 4 tissue culture plates (10 cm).

1.1.6. Culture the cells at 37 °C and 5% CO₂ until confluence (2-3 days).

1.1.7. At confluence, aspirate the old medium and wash the tissue culture plates once with 1x PBS (37 °C). Then aspirate the PBS and add 2 mL of 1x trypsin solution containing 0.5% ethylenediaminetetraacetic acid (EDTA) to each 10 cm tissue culture plate.

1.1.8. Incubate the 4 culture plates at 37 °C and 5% CO₂ for 5 min and then check the plates with an inverted light microscope to ensure that the cells are detached from the plastic. Then transfer all cell suspensions (total 8 mL) to a 50 mL conical tube containing 10 mL of fresh BGM. Wash each plate with 5 mL of fresh BGM and transfer to the same 50 mL conical tube.

1.1.9. Centrifuge the cells at 300 x g for 5 min at 4 °C, aspirate the supernatant and resuspend the cells in 16 mL of fresh BGM. Prepare 16 new 10 cm tissue culture plates (splitting 1:4) containing 9 mL of BGM. Add 1 mL of the cell suspension to each plate.

1.1.10. Repeat steps 1.1.6. to 1.1.8.

1.1.11. Centrifuge the cells at 300 x g for 5 min at 4 °C, aspirate the supernatant and resuspend in 10 mL of fresh BGM. Count the cells and calculate the cell concentration.

Note: This procedure generates approximately $7.5\text{--}8.3 \times 10^5$ cells/pup.

1.1.12. Centrifuge at $300 \times g$ for 5 min at 4°C , aspirate the supernatant and resuspend in freezing medium containing 10% dimethyl sulfoxide (DMSO), 40% α -MEM and 50% FBS. Transfer 1.5 mL of the cell suspension to 2 mL cryovials for a total of 2×10^6 cells per vial.

1.1.13. Flash freeze the vials in liquid nitrogen for long-term storage in a liquid nitrogen tank. Use the cells within one year to ensure full functionality. Use these primary OBs for evaluating proliferation (step 1.2), differentiation (ALP assays: step 1.3.4., 1.3.5.) and mineralization (step 1.3.6.) in the presence of the biomaterials. Use these cells for the maturation of bone-marrow-derived OC precursors in co-culture (step 2.3).

1.2. OB proliferation assay

1.2.1. Pre-incubate 14 mm diameter β -TCP disks in 1 mL of BGM in a 24-well suspension culture plate at 37°C and 5% CO_2 for 24 h before adding primary OBs. Use standard tissue culture plates for controls and suspension culture plates for the biomaterial samples to avoid cell attachment to the plastic.

1.2.2. Aspirate the pre-incubation medium and then resuspend primary mouse OBs in BGM. Add 4.4×10^4 OBs/ cm^2 onto the pre-incubated β -TCP disks in a 24-well suspension culture plate and for the control group, into a 24-well tissue culture plate (1 mL/well). OBs will attach to the tissue culture plate or the biomaterial.

1.2.3. Incubate for 14 days at 37°C and 5% CO_2 . During the incubation period, change the medium every 2-3 days and add 1 mL of fresh BGM to each well.

1.2.4. To assess OB proliferation, transfer the β -TCP disks on days 7 and 14 to new wells to exclude the signals from the cells grown on the suspension culture plate.

1.2.5. Aspirate the old medium, add 0.5 mL of BGM (37°C) and incubate the culture plates for 30 min at 37°C and 5% CO_2 . Then add 55 μL of 10x cell proliferation reagent (1:10 dilution) directly into the culture well. For blanks, prepare three wells containing 0.5 mL of BGM and 55 μL of the 10x cell proliferation reagent. Incubate all plates for 30 min at 37°C and 5% CO_2 .

1.2.6. Withdraw and transfer 150 μL of the supernatant from each well into a 96-well black plate and read fluorescence with excitation at 560 nm and emission at 590 nm. Subtract the blank readings from the sample readings.

1.3. OB differentiation and mineralization assays

1.3.1. Pre-incubate 14 mm diameter β -TCP disks in 1 mL of BGM in a 24-well suspension culture plate at 37°C and 5% CO_2 for 24 h before adding the OBs. Use standard tissue culture plates for

controls and suspension culture plates for the biomaterial samples to avoid cell attachment to the plastic.

1.3.2. Aspirate the pre-incubation medium and then resuspend primary mouse OBs in BGM. Add 8.8×10^4 OBs/cm² onto the pre-incubated β -TCP disks in a 24-well suspension culture plate and for the control group, into a 24-well tissue culture plate (1 mL/well). OBs will attach to the tissue culture plate or the biomaterial sample.

1.3.3. Replace the BGM with 1 mL of osteogenic mineralization medium (MM) containing BGM, 50 μ g/mL ascorbic acid and 5 mM β -glycerophosphate 24 h after the addition of the OBs and incubate for 14 days at 37 °C and 5% CO₂. Change the medium every 2-3 days with 1 mL of freshly prepared MM to each well.

1.3.4. OB differentiation assessed by alkaline phosphatase activity (ALP) from cell lysates

1.3.4.1. To measure ALP activity on day 7 after the addition of MM, aspirate the culture medium from the wells and then wash with 1 mL of sterile 1x PBS (37 °C). Carefully aspirate the PBS to allow the attached cells to remain on the tissue culture plastic or biomaterial and freeze the plates at -80 °C.

1.3.4.2. After 24 h (or up to 2 weeks), thaw the control tissue culture plate and biomaterial suspension culture plate at room temperature (RT) to prepare for OB lysis. For the biomaterial samples, transfer the β -TCP disks into a new suspension culture plate to exclude the cells attached to the plate. Add 75 μ L per well of the 1x cell lysis buffer to both plates. Shake the plates for 5 min on a shaker at 400 shakes/min.

1.3.4.3. Transfer the cell lysate into a 0.5 mL microcentrifuge tube and centrifuge at 300 x g for 6 min at RT to remove debris. Add 50 μ L of the sample cell lysate supernatant to a 96-well black plate and then add 50 μ L of a solution consisting of 200 μ M 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) fluorogenic substrate dissolved in 2x ALP buffer (pH 10) per well. Set up two blank wells with 100 μ L of a 1:1 solution containing 1x cell lysis buffer and 2x ALP buffer.

1.3.4.4. Prepare 8 reference standards with 100 μ L/well with the 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) ranging from 0.5 to 200 μ M dissolved in 1:1 solution containing 1x cell lysis buffer and 2x ALP buffer to generate a standard reference curve.

1.3.4.5. Incubate the black plate at 37 °C for 15 min and then read the fluorescence with the excitation at 358 nm and emission at 455 nm. Subtract the blank readings from the reference standards and sample readings.

1.3.4.6. Normalize the ALP enzyme activity from the cell lysates to the total amount of protein using a colorimetric assay for protein concentration. Prepare bovine serum albumin (BSA) protein reference standards at a range from 0.05-2.5 μ g/ μ L dissolved in 1x cell lysis buffer to generate a standard curve.

1.3.4.7. Prepare the sample cell lysates and BSA protein standards in duplicates. Add 5 μ L of the sample cell lysate or 5 μ L of BSA protein standard into a 96-well plate and then add 25 μ L of reagent A' and 200 μ L of reagent B. Set up two blank wells with 5 μ L of 1x cell lysis buffer. Incubate the plates at RT for 15 min and then read the absorbance at 690 nm. Subtract the blank readings from the reference standards and sample readings.

1.3.5. OB differentiation assessed by staining ALP in cell culture

1.3.5.1. Stain ALP in cultured OBs on day 7 after the addition of MM, on a control tissue culture plate and the biomaterial in a suspension culture plate.

1.3.5.2. Aspirate the culture medium from the wells and replace it with 0.5 mL of 1x PBS (37 °C). Aspirate the PBS and fix the cells by incubating them in 0.5 mL of 10% buffered formalin at RT for 1 min.

1.3.5.3. Aspirate the 10% buffered formalin with a single-use pipet and add 0.5 mL of wash buffer (0.05% Tween20 in 1x PBS).

1.3.5.4. Dissolve one 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate tablet in 10 mL of ultrapure water and vortex until completely dissolved. The solution must be protected from light and used within 2 h.

1.3.5.5. Aspirate the wash buffer and replace with 0.5 mL of BCIP/NBT substrate solution and incubate the plate at RT in the dark for 10 min. Aspirate the staining solution and replace with 0.5 mL of wash buffer.

1.3.5.6. Transfer the stained β -TCP disks into a new well and scan the ALP-stained plates with a conventional flatbed scanner to record ALP staining.

1.3.6. OB mineralization assessed by staining with Alizarin Red S (ARS)

1.3.6.1. Aspirate the culture medium from the wells containing primary OBs 14 days after the addition of MM and rinse twice with 0.5 mL of 1x PBS at RT. Aspirate the PBS and fix the cells by adding 0.5 mL of the 10% buffered formalin solution at RT for 10 min.

1.3.6.2. Aspirate the 10% buffered formalin with a single-use pipet and wash twice with 0.5 mL of ultrapure water.

1.3.6.3. To the fixed OBs, add 0.25 mL of 40 mM ARS staining solution (pH 4.2) dissolved in ultrapure water and incubate the plate at RT for 10 min on a shaker at 100 shakes/min.

1.3.6.4. Aspirate the staining solution with a single-use pipet and rinse with 1 mL of ultrapure water. Repeat this step 5-10 times to remove unspecific staining.

Note: The rinsing solution should be without color.

1.3.6.5. Aspirate the ultrapure water and add 1 mL of cold PBS. Incubate the plate at RT for 10 min on a shaker at 100 shakes/min.

1.3.6.6. Aspirate the PBS, transfer the stained β -TCP disks to a new well and scan the plates with a flatbed scanner to record mineralization.

1.3.6.7. Add 0.25 mL of 10% cetylpyridinium chloride solution and incubate the plate at RT for 15 min on a shaker at 100 shakes/min to extract the ARS dye.

1.3.6.8. Transfer the supernatants to a 1.5 mL tube and centrifuge at 17,000 x g for 5 min at RT.

1.3.6.9. Add 10% cetylpyridinium chloride solution to the extracts with a dilution ratio of 1:10-1:20 and add the samples (300 μ L) into a 96-well plate including two blank wells containing only 10% cetylpyridinium chloride.

1.3.6.10. Prepare 7 ARS reference standards ranging from 4 to 400 μ M by diluting the 40 mM ARS staining solution (pH 4.2) with 10% cetylpyridinium chloride solution to generate a standard curve.

1.3.6.11. Add 300 μ L of the reference standard in duplicates to the 96-well plate.

1.3.6.12. Read the absorbance of the samples, blanks, and reference standards at 520 nm.

1.3.6.13. Subtract the blank readings from the reference standards and sample readings.

2. Mouse OB-OC Co-culture to Derive Mature OCs

2.1. Preparation and sterilization of bovine cortical bone slices

2.1.1. Clean segments of bone from the surrounding tissue and remove the trabecular bone and bone marrow.

2.1.2. Dry the bone at 50 °C for 24 h.

2.1.3. Slice the bone into smaller chunks and cut it into slices (approximately 300-350 μ m thick) using a low-speed saw with a diamond blade.

2.1.4. Cut the 300-350 μ m slices into quadratic disks (1 cm²).

2.1.5. To clean the bone disks (1 cm^2), put them into a lockable glass vial and fill with distilled water. Transfer the filled glass vial into an ultrasonic bath and sonicate for 5 min. Repeat this step three times.

2.1.6. Pour off the distilled water, add 70% ethanol, and sonicate for 5 min.

2.1.7. Pour off the 70% ethanol and replace with 100% ethanol.

Note: Store the bone slices in 100% ethanol at $4\text{ }^{\circ}\text{C}$ for up to 4 weeks.

2.1.8. Irradiate the bone slices with UV light for 15 min on each side under sterile conditions. Store the sterilized bone slices in a sterile culture plate at RT until further use.

2.2. Isolation of bone marrow OC precursors

2.2.1. Euthanize naïve 8-12 week old BALB/c mice using an intraperitoneal (i.p.) injection of a solution of 200 mg/kg ketamine and 24 mg/kg xylazine.

2.2.2. Isolate the bone marrow OC precursors from mouse femurs and tibiae.

2.2.3. Remove the legs with sterile scissors from the closest point to the body at the hip joint, cut the limbs at the knee and ankle joints and remove the soft tissue with sterile scalpel and forceps. Cut off the epiphyses. Flush out and suspend the marrow with BGM into a 6 cm sterile Petri dish using a 27G needle to obtain a cell suspension.

2.2.4. Add the cell suspension to a 50 mL conical tube, wash the 6 cm Petri dish with 5 mL of BGM and transfer to the same 50 mL conical tube. Centrifuge at $350 \times g$ at $4\text{ }^{\circ}\text{C}$ for 5 min. Resuspend the cells in OC differentiation medium (DM) containing BGM, 1 nM $1,25\text{-(OH)}_2\text{-vitamin D}_3$ and $1\text{ }\mu\text{M}$ prostaglandin E_2 (1 mL/well).

Note: One BALB/c mouse provides sufficient numbers of OC precursors for one 24-well tissue culture plate.

2.3. Preparation of mouse OB-OC co-culture with biomaterial

2.3.1. Pre-incubate 14 mm diameter β -TCP disks or bovine bone slices (1 cm^2) in 1 mL of BGM in a 24-well suspension culture plate at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 for 24 h before adding the cells.

Note: Bovine bone slices are a physiologic substrate, control group for studying OC differentiation in the presence of biomaterials.

2.3.2. Add $8.8 \times 10^4\text{ cells/cm}^2$ of primary OBs suspended in BGM onto the biomaterials or the control bone in a 24-well suspension culture plate or to a 24-well tissue culture plate. Incubate at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 for 24 h.

Note: OBs attach to the plastic or the biomaterial or bovine bone.

2.3.3. Add bone marrow OC precursors to the 24 h-cultured primary OBs and culture at 37 °C and 5% CO₂ for 5 days. Replace the medium with freshly prepared OC DM every other day. Then stain OCs for tartrate-resistant acid phosphatase (TRAP) to evaluate OC differentiation.

2.4. OC differentiation assessed by TRAP staining

2.4.1. To characterize mature multinucleated OCs, obtained from step 2.3.3, aspirate the culture medium from the control tissue culture plate and the biomaterial suspension culture plate and add 1 mL of 1x PBS (37 °C). Aspirate the PBS and fix the cells by incubating them in 1 mL of 10% buffered formalin solution at RT for 10 min.

2.4.2. Aspirate the 10% buffered formalin solution with a single-use pipet and add 1 mL of 1x PBS at RT. Repeat this step three times, then aspirate the PBS, replace with 1 mL of TRAP buffer (pH 5) and incubate the plates at 37 °C for 30 min.

2.4.3. Aspirate the TRAP buffer and add 1 mL of 1:1 acetone and 100% ethanol. Incubate the plate at RT for 30 s. Quickly aspirate the acetone-ethanol solution with a single-use pipet and completely dry the plates at RT.

2.4.4. For the TRAP staining solution, prepare a 10 mg/mL naphthol-AS-MX phosphate stock solution in N,N-dimethylformamide, dissolve 0.6 mg/mL of fast red violet salt in TRAP buffer, and add 0.1 mL of naphthol-AS-MX phosphate stock solution to 10 mL of fast red violet salt in TRAP buffer.

2.4.5. Add 1 mL of TRAP staining solution and incubate the plate at 37 °C for 10 min, then aspirate the TRAP staining solution, and add 1 mL of ultrapure water to stop the reaction.

2.4.6. Transfer the β -TCP disks and bovine bone slices after staining into a new well, observe red stained OCs using a light microscope (magnification: 10X) and enumerate TRAP+ mature OCs with three or more nuclei.

3. Critical-sized Mouse Calvarial Defect Model

3.1. Inject 0.1 mg/kg buprenorphine subcutaneously (s.c.) into 8-week old BALB/c mice for analgesia.

3.2. Anesthetize the mice with a mixture of 100 mg/kg ketamine and 5 mg/kg xylazine i.p., add a gel or ointment to the eyes to keep them moist and place the mouse on a heating plate at 37 °C during the entire procedure to prevent hypothermia. Assess the anesthesia depth by toe and tail pinch.

3.3. Shave the fur on the top of the head between the ears and clean the surface with 7.5% povidone iodine solution and 70% ethanol.

3.4. Make a midline sagittal incision on the top of the skull between the ears with a sterile scalpel to expose the calvarium and remove the pericranium connective tissues above the right parietal bone by scraping it with a scalpel.

3.5. Create a critical-sized defect of approximately 4 mm in the right parietal bone using a sterile dental trephine (2000 rpm) under constant irrigation with normal saline solution to notch the right parietal bone and cut through the ectocortex and some endocortex.

3.6. To prevent damage to the dura mater, use a small periosteal elevator to break through the remaining endocortex, carefully lift the calvarial bone up and remove it with forceps. The resulting defect should be circular and approximately 3.5 mm in diameter.

3.7. Fill the calvarial defect with pre-soaked (sterile 1x PBS at RT) β -TCP/C foam using forceps.

3.8. Prepare the appropriate number of age-matched, sham negative controls with an empty defect.

3.9. To keep the biomaterial in place, put 0.5 μ L of tissue adhesive at the edge of the defect at two opposite points.

3.10. Close the skin with a non-absorbable suture.

3.11. Clean all surgical instruments with 70% ethanol to maintain sterile conditions before performing the next surgery on an anesthetized mouse.

3.12. For post-surgical treatment, place the animals on a dry and clean heating plate at 37 °C in the surgery area for appropriate monitoring during the recovery period. Monitor the respiratory rate, body temperature and color of mucous membranes and eyes every 10 min until they wake.

3.13. Transfer the operated conscious mice into a cage with food and water separated from the other animals until full recovery. Inject 0.1 mg/kg buprenorphine s.c. for post-surgical analgesic therapy twice per day for 72 h. Return fully recovered mice to their cages.

3.14. To determine the effectiveness of the biomaterial, euthanize the mice i.p. with a solution of 200 mg/kg ketamine and 24 mg/kg xylazine.

3.15. At 8-12 weeks post-implantation, decapitate the euthanized mouse with sharp scissors.

3.16. Fix the decapitated mouse head in 30 mL of 4.5% buffered formalin solution for 1-2 weeks to guarantee full penetration of the fixative into the tissue.

3.17. Transfer the heads into 70% ethanol for long-term storage at 4 °C for up to one year.

3.18. Use micro computed tomography (microCT) or standardized undecalcified histological techniques to evaluate bone regeneration. It is possible to use microCT scanning on postmortem samples at high resolution (90 kV, 4 min) in the 2D and 3D sagittal and frontal planes.

3.19. For undecalcified histology, dehydrate the heads in ascending grades of ethanol and embed in glycol methyl methacrylate.

3.20. Cut glycol methyl methacrylate-embedded blocks with a diamond saw and grind the sections to a thickness of approximately 80-100 µm.

3.21. Evaluate Levai Laczko stained bone sections to identify new bone formation¹⁴.

4. *In vitro* Immune Responses

4.1. Euthanize naïve 8-week old BALB/c mice i.p. with a solution of 200 mg/kg ketamine and 24 mg/kg xylazine.

4.2. Place the mouse on its right side, shave the fur on the left side and clean the skin over the left side of the abdomen with 70% ethanol.

4.3. Make a 10 mm long and 1 mm deep incision in the skin of the mouse on the left side posteriorly under and following the ribs over the stomach using sterile scissors.

4.4. Open the skin and then expose the peritoneum. Make a 10 mm long and less than 1 mm deep incision into the peritoneum using sterile scissors.

4.5. Push the stomach proximally to expose the spleen.

4.6. Hold the spleen gently with forceps and remove it by cutting the tissue and vessels attached below it.

4.7. Place the intact spleen into a 50 mL tube containing 10 mL of cold 1x PBS.

4.8. Prepare a single cell suspension by mincing the spleen using 2 forceps or 2 glass slides.

4.9. Remove debris by passing it through a sterile 40 µm cell strainer with cold 1x PBS using the plunger of a 1 mL syringe.

4.10. Centrifuge the single cell suspension at 300 x g for 10 min at 4 °C in a 50 mL conical tube, aspirate and discard the supernatant. Then resuspend the cell pellet in 5 mL of red blood cell (RBC) lysis buffer.

4.11. Incubate the cell suspension for 14 min at RT and stop the reaction by adding 45 mL of RPMI medium.

4.12. Centrifuge the cells after the cell lysis at 300 x g for 10 min at 4 °C and then discard the supernatant.

4.13. Resuspend splenocytes in Roswell Park Memorial Institute (RPMI) medium containing 10% heat-inactivated FBS, 1% penicillin-streptomycin solution, 0.1% gentamicin, 0.2% β -mercaptoethanol, and 1% non-essential amino acids.

4.14. Pre-incubate β -TCP/C foam in a 96-well sterile culture plate containing RPMI medium for 24 h at 37 °C and 5% CO₂ before cell seeding.

4.15. Add splenocytes at titrated numbers, *e.g.*, 1.25×10^5 , 2.5×10^5 and 5×10^5 /well to wells in a 96-well tissue culture plate containing RPMI medium and additives, with or without the pre-incubated β -TCP/C foam.

4.16. Add 10 μ g/mL concanavalin A (Con A) dissolved in medium for a total of 100 μ L/well to the appropriate wells and then incubate at 37 °C and 5% CO₂ for 72 h.

4.17. Measure cell proliferation with a bromodeoxyuridine (BrdU) assay. Add 10 μ L/well of BrdU labeling solution at 48 h of cell culture and then incubate the plate for an additional 24 h.

4.18. At 72 h, collect the supernatant and store at -20 °C until further use.

4.19. Add 200 μ L/well of the fixation solution to each well and then incubate for 30 min at RT. Aspirate the fixation solution. Add 100 μ L/well of the anti-BrdU antibody working solution and incubate for 90 min.

4.20. Aspirate the antibody solution, rinse the wells three times with 200-300 μ L/well of washing solution and remove the washing solution.

4.21. Add 100 μ L of substrate solution and incubate for 3-10 min at RT, then measure the absorbance at 450 nm.

4.22. Measure interleukin-1 β (IL-1 β), interleukin-2 (IL-2), interleukin-4 (IL-4) and interferon- γ (IFN- γ) in the collected supernatants using a standard ELISA.

5. High Throughput Intraperitoneal Model

5.1. Anesthetize female 6-8-week old BALB/c mice with a mixture of 100 mg/kg ketamine and 5 mg/kg xylazine i.p. and add a gel or ointment to the eyes to keep them moist and place the mouse on a heating plate at 37 °C during the entire procedure to prevent hypothermia. Assess the anesthesia depth by toe and tail pinch.

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527 5.2. Shave the abdominal fur and clean with 7.5% povidone iodine solution and 70% ethanol.
528

529 5.3. Make an 8 mm long midline incision through the skin along the linea alba. Make a small
530 incision into the peritoneum using fine surgical scissors.
531

532 5.4. Place sterile PBS soaked 2 mm³ β -TCP/C foam grafts into the peritoneal cavity or without
533 added materials for the age-matched sham control mice.
534

535 5.5. Suture the peritoneum and skin with absorbable and non-absorbable suture, respectively.
536

537 5.6. Clean all surgical instruments with 70% ethanol to maintain sterile conditions before
538 performing the next surgery on an anesthetized mouse.
539

540 5.7. For post-surgical treatment, place the animals on a dry and clean heating plate at 37 °C in
541 the surgery area for appropriate monitoring during the recovery period. Monitor the respiratory
542 rate, body temperature and color of mucous membranes and eyes every 10 min until they wake.
543

544 5.8. Transfer the operated conscious mice into a cage with food and water separated from the
545 other animals until full recovery. Return fully recovered mice to their cages.
546

547 Note: This procedure induces minimal pain. Post-surgical analgesic therapy is usually not
548 necessary. If the operated animals show signs of pain, inject 0.1 mg/kg buprenorphine s.c. or
549 another appropriate analgesic drug for pain relief.
550

551 5.9. Euthanize the mice 7 days post-implantation, with a solution of 200 mg/kg ketamine and 24
552 mg/kg xylazine i.p.
553

554 5.10. At 7 days post-implantation, lavage the peritoneal cavity using a 1 mL syringe with a 25G
555 needle with a total of 3 mL of cold PBS by holding the mouse head down and inserting the needle
556 in the left lower abdominal quadrant and aim proximally.
557

558 Note: The peritoneal fluid should be free of RBCs.
559

560 5.11. Centrifuge the peritoneal lavage fluid at 300 x g for 5 min at 4 °C and collect the peritoneal
561 fluid to store at -20 °C for ELISA measurements of IL-1 β , IL-2, and IL-4 cytokines.
562

563 5.12. Aspirate the supernatant, resuspend the cell pellet in 1 mL of PBS, and count the peritoneal
564 cells using trypan blue on a hemocytometer.
565

566 5.13. Cytoцентрифуге the cell suspension at 5 x 10⁵ cells on to glass slides, allow the slides to dry
567 and stain for a differential cell count.
568

5.14. Using a light microscope, count a minimum of 300 cells in total and differentiate between macrophages, eosinophils, neutrophils, and lymphocytes.

6. Subchronic Subcutaneous Model

6.1. Anesthetize female 6-8-week old BALB/c mice with a mixture of 100 mg/kg ketamine and 5 mg/kg xylazine i.p., add gel or ointment to the eyes to keep them moist and place the mouse on a heating plate at 37 °C during the entire procedure to prevent hypothermia. Assess the anesthesia depth by toe and tail pinch.

6.2. Place the mouse on its back, shave the abdominal fur, and clean the shaved surface with 7.5% povidone iodine solution and 70% ethanol.

6.3. Make an 8 mm long midline incision through the skin along the linea alba of the abdomen using fine surgical scissors and then implant PBS soaked biomaterial under the skin or add no materials for the age-matched sham control mice.

6.4. Suture the skin with a non-absorbable suture.

6.5. Clean all surgical instruments with 70% ethanol to maintain sterile conditions before performing the next surgery on an anesthetized mouse.

6.6. For post-surgical treatment, place the animals on a dry and clean heating plate at 37 °C in the surgery area for appropriate monitoring during the recovery period. Monitor the respiratory rate, body temperature and color of mucous membranes and eyes every 10 min until they wake.

6.7. Transfer the operated conscious mice into a cage with food and water separated from the other animals until full recovery. Return fully recovered mice to their cages.

Note: This procedure induces minimal pain. Post-surgical analgesic therapy is usually not necessary. If the operated animals show signs of pain, inject 0.1 mg/kg buprenorphine s.c. or another appropriate analgesic.

6.8. When ready to examine the implantation site, euthanize the mice i.p. with a solution of 200 mg/kg ketamine and 24 mg/kg xylazine.

6.9. Eight weeks post-implantation, excise the implantation site with surrounding tissue (1 cm²).

6.10. Fix the tissue in 4.5% buffered formalin solution overnight, embed in paraffin and cut into 4 µm sections.

6.11. Stain 4 µm paraffin-embedded tissue sections with Hematoxylin and Eosin (H&E) for inflammation or Masson's trichrome for collagen deposition and fibrosis.

REPRESENTATIVE RESULTS:

To assess β -TCP for its effectiveness as a biomaterial for bone repair, we used *in vitro* and *in vivo* screening methods. Firstly, we measured the OB responses to the β -TCP disks compared with baseline medium alone controls. **Figure 2** demonstrates the OB viability in response to β -TCP disks at 7 and 14 days of culture. Cell viability measured from metabolically active cells in the culture wells was the same for OBs with medium in tissue culture plastic as well as with β -TCP disks indicating that this biomaterial is neither enhancing nor suppressing OB proliferation.

To further evaluate the OBs, we measured ALP activity as a marker of differentiation using qualitative and quantitative approaches. **Figure 2** illustrates ALP enzyme activity in tissue culture wells after 7 days of culture. OBs in the wells with the medium alone had baseline ALP activity, while in optimal mineralization medium (MM) OBs had intense ALP staining, reflecting a high level of OB differentiation. In contrast, the OBs plated on β -TCP disks differentiated less than the OBs incubated in MM. In a quantitative assay, ALP concentration was 77% higher for the wells containing MM compared with the baseline medium alone, whereas ALP concentration was 40% lower in the cells cultured on β -TCP disks compared to MM controls. Although these results demonstrate that the cells grown on β -TCP disks differentiated less than those with optimal conditions of plastic with MM, they differentiated sufficiently on the biomaterials.

Another critical feature of OBs is their capacity to induce mineralization, which is an essential step in bone healing. We stained cultured OB cells with ARS after 14 days and found that mineralization was higher for MM controls compared to OBs cultured in medium alone and on β -TCP disks in tissue culture wells (**Figure 2**). When we measured the ARS concentration, we found that the MM controls were more than 45% higher than the β -TCP group. These data illustrate that OBs cultured on plastic in the presence of MM mature, differentiate and mineralize better than those with medium alone and on β -TCP disks.

To determine how OCs respond to β -TCP disks, we used a culturing technology in which OBs are co-cultured with bone marrow OC precursors followed by the examination of OC morphology. OB-OC co-cultures were observed at 5 days and differed substantially between the cells grown on plastic with OC DM and the cells grown on bone slices and β -TCP. On plastic, the OCs were large and widespread whereas the OCs on physiological substrates were smaller, less-spread out and irregularly-shaped (**Figure 3**). To quantitate the OCs, we enumerated TRAP+ OCs and found that there were higher numbers when incubated with β -TCP ($1755 \pm 21.41/\text{cm}^2$) compared to tissue culture controls ($1140 \pm 15.71/\text{cm}^2$) and bone slices ($709 \pm 59.69/\text{cm}^2$), suggesting enhanced OC differentiation on β -TCP disks (**Figure 3**).

To determine a commercially available β -TCP/C foam *in vivo*, we used a critical-sized calvarial defect model in mice. We show representative histological sections processed by an undecalcified histological technique with glycol methyl methacrylate embedding and Leiva Laczko staining. MicroCT and histology may be used to evaluate new bone formation within the defect area. Here, we show an example with histological sections in **Figure 4**. When the surgically-induced bone defect was left empty (sham), we observed a thin layer covering the entire defect, but no significant bone formation was present at 12 weeks post operation confirming the critical

size of the created bone fracture. In contrast, when the defect contained β -TCP/C foam, there were β -TCP/C foam remnants surrounded by dense fibrous tissue including some blood vessels and inflammatory cells were bridging the defect area without evidence of bone formation.

To evaluate the foreign body response, we assessed immunological and allergic reactions to the biomaterials, using an *in vitro* assay. **Figure 5** demonstrates that when naïve splenocytes were incubated with medium alone or with β -TCP/C foam, the naïve spleen cells did not respond by proliferating or producing IL-2, IL-4, and IFN- γ cytokines. In contrast, in ConA containing cultures, cell proliferation and cytokine production increased except for IL-1 β . Cell responses were unaffected when co-cultured with ConA and β -TCP/C foam compared with ConA alone, indicating that β -TCP/C foam neither increased nor decreased *in vitro* responses.

To determine whether β -TCP/C foam induced an *in vivo* immune response, we implanted it 1) intraperitoneally and measured inflammatory cell counts and cytokine concentrations in the peritoneal lavage fluid and 2) subcutaneously and evaluated inflammation and fibrosis on histological sections of the implantation site. In **Table 1**, the cell differential in the peritoneal lavage fluid reveals that the total number of inflammatory cells was significantly higher in the β -TCP/C foam implanted mice compared with the sham controls. Furthermore, there were increased numbers of all cell types. In **Figure 6A**, there are higher concentrations of IL-1 β , IL-2, and IL-4 cytokines in the β -TCP/C foam compared with the sham controls. In β -TCP/C foam s.c. implanted mice, we observed an inflammatory response with foreign body giant cells on H&E-stained sections (**Figure 6B**) and evidence of fibrosis on Trichrome Masson's-stained sections (**Figure 6C**) at 8 weeks. In contrast, the implantation site of the sham controls had minimal inflammation and no fibrosis (**Figure 6C**).

FIGURE & TABLE LEGENDS:

Figure 1: Calvaria removal for primary OB cell isolation diagram. The diagram illustrates how to remove the calvarium with 4 cuts (red dashed line) using curved scissors. The first cut is perpendicular to right (R) eye socket from X1 to X2, and the second is perpendicular to left (L) eye socket from X3 to X4. The third cut is to separate the calvarium at the front from X4 to X2, and the fourth cut is to separate the back from X3 to X1. The calvarium is then free to be removed.

Figure 2: β -TCP-induced *in vitro* OB differentiation and maturation. OB viability and proliferation on days 7 and 14 for the cells cultured in medium alone (open bars) or β -TCP (closed bars) (mean \pm SEM; n=3). ALP activity quantification of cell lysates and normalization to the protein content (μ M DIFMU/ μ g protein, mean \pm SEM, n = 3) with representative images illustrating ALP-stained culture wells from day 7. Mineralization quantified from ARS-stained cultures by a cetylpyridinium chloride extraction method shown as the concentration of ARS (μ M ARS, mean \pm SEM, n = 3) with representative ARS-stained culture wells on day 14. Groups include bone growth medium alone (BGM); Mineralization medium (MM); β -TCP.

Figure 3: β -TCP-induced *in vitro* OC differentiation. Representative photomicrographs show TRAP+ MNCs at day 5 after co-culturing mouse OBs and bone marrow OC precursors. Endpoint analysis of TRAP+ MNCs demonstrates the absolute count of TRAP+ MNCs (≥ 3 nuclei) per cm²

(mean \pm SEM, n = 3) *** p <0.001. Groups include OC differentiation medium alone (DM); Bone; and β -TCP.

Figure 4: *In vivo* evaluation of β -TCP/C foam bone grafts in a critical-sized calvarial defect model. Non-healing calvarial defects created in 8-week old female BALB/c (n = 3) mice using a 4-mm dental trephine. Treatment groups included sham control (empty defect) and defects treated with β -TCP/C foam. Representative histological sections prepared at 12 weeks post-implantation. Formalin-fixed tissue glycol methyl methacrylate-embedded sections (80-100 μ m) stained with Levai Laczko dye. Photomicrographs shown at low (left) and high (right) magnification. Black triangles indicate the bone defect. Black * denotes bone tissue and white * refers to β -TCP/C foam.

Figure 5: β -TCP/C foam-induced *in vitro* cell proliferation and cytokine production. Splenocytes from naïve BALB/c mice cultured in medium alone, with β -TCP/C foam or ConA. Supernatant cell proliferation (BrdU), and production of IL-1 β , IL-2, IL-4, and IFN- γ (medium alone ●, ConA ○, β -TCP/C foam ■, β -TCP/C foam and ConA ◐). Proliferation results presented as the mean of triplicate samples (O.D. \pm SEM) in the BrdU assay and the mean of duplicate samples (pg/mL \pm SEM) for cytokine concentration from two independent experiments. * p <0.05 is considered significant for biomaterial vs. medium and biomaterial and ConA vs. ConA alone.

Figure 6: *In vivo* immune response of β -TCP/C foam bone grafts in a rapid high throughput i.p. and subchronic mouse model. (A) Female BALB/c mice implanted i.p. with β -TCP/C foam or without added materials (sham). Seven days later, peritoneal lavage analyzed for cytokine concentrations (data presented as mean cytokine concentrations pg/mL \pm SEM). These data are representative of two independent experiments (n = 5). * p <0.05 is considered significant compared to sham. (B-C) Female BALB/c mice (n = 5) implanted s.c. with β -TCP/C foam or without added materials (sham). At 8 weeks after implantation, skin from the implantation sites stained with H&E (B) and Masson's Trichrome (C) to evaluate inflammation and fibrosis, respectively.

Table 1: *In vivo* immune response of β -TCP/C foam bone grafts in a rapid high throughput i.p. mouse model. Female BALB/c mice were implanted i.p. with β -TCP/C foam or without materials (sham). Seven days later, peritoneal lavage was obtained and analyzed for inflammatory cell number and differential cell counts (data presented as mean cell counts \pm SEM). These data are representative of two independent experiments (n = 5).

DISCUSSION:

Here, we show a multidisciplinary approach for the preclinical assessment of biocompatibility for representative biomaterials developed for bone regeneration and repair. We tested the responses of OBs, OCs, and the *in vivo* healing response in a critical bone defect model in mice as well as *in vitro* and *in vivo* immune responses. We aimed to demonstrate how the assays work and summarize the data and conclusions derived from the examination of the biomaterials. We show that our strategy generates a valuable profile of bone biomaterial biocompatibility.

Primary cell assays were used to evaluate OB and OC function. OBs are responsible for bone formation and physiological repair. They must remain viable, differentiate, and induce mineralization. In this study, we show how to perform assays for cell viability, ALP, and ARS as markers of physiologically differentiated cells with the capacity to mineralize. The controls for the assays included medium alone, which provides a baseline and osteogenic mineralization medium (MM), which optimized differentiation and mineralization of OBs on plastic. The latter control group was a reference standard for the biomaterial. For OC evaluation, we co-cultured OBs and bone marrow-derived OC precursors, differentiated the precursors into multinucleated cells, stained them with TRAP, an osteoclastic enzyme widely used to identify OCs *in vitro*¹⁵ and then enumerated the cells using light microscopy. These assays are state-of-the-art and did not require modifications. However, we noted a limitation related to the quality of isolated primary OBs to mineralize. Preserved OBs are stored in liquid nitrogen and used within one year for optimal results.

OB attachment and activity were higher on tissue culture plastic than on the β -TCP disks tested. When we assessed OCs, we observed the expected differences between the response to plastic and bone, which is the physiologic substrate¹⁶. In comparison, bone and β -TCP induced similar morphological changes. The TRAP assay enumeration of OCs in response to the β -TCP disks showed that the numbers were significantly different between bone slices and β -TCP. β -TCP induced higher OC differentiation than on bone slices. For OC differentiation, TRAP is a well-established assay. There were no significant modifications necessary in this method. However, to obtain the best results, it is essential not to incubate the cells for too long, or all cells of monocytic origin will become TRAP-positive.

To address the *in vivo* response, we used β -TCP/C foam as an exemplary biomaterial because it contains β -TCP, which was used in the *in vitro* assays and collagen and promotes bone healing¹³. Although β -TCP/C foam is commercially available and used clinically for bone repair^{17,18}, it is only one of many different types of materials that would be interesting to study, *e.g.*, biphasic calcium phosphate (hydroxyapatite/ β -TCP) as well as demineralized human bone in these assays to determine how biological responses differ between materials. For the *in vivo* response, we implanted β -TCP/C foam into a critical-sized calvarial bone defect in mice and 12 weeks later assessed histology and showed differences compared with sham controls. It is also possible to evaluate the responses with microCT which provides complimentary information¹⁹. The defect in the sham control mice had no significant bone formation, as expected, whereas β -TCP/C foam induced an inflammatory response, fibrosis, and angiogenesis, which is the evidence of the early phase of bone formation. This method has been demonstrated previously in JOVE²⁰. However, our approach differed in that we used a modified “elevator” technique with a periosteal elevator to reduce the risk of injuring the dura mater by the trephine. We reasoned that the dura mater plays a significant role in the healing process of calvarial defects by producing osteogenic cells and osteoinductive factors^{3,21-23}. Notably, the material implanted, the size of the defect, and method for creating the defect influences bone regeneration of calvarial defects. Another modification in the procedure involved the stabilization of the biomaterial in the calvarial defect with a biocompatible tissue glue that is routinely used clinically for wound closure. This

modification guaranteed that the material in the defect area would not be displaced during healing.

Inflammation regulates the early phase of bone healing, but too much inflammation or allergic responses may reduce repair¹¹. The ideal immune response to biomaterials is to initiate an inflammatory cascade that promotes bone formation. However, certain biomaterials might cause a foreign body response leading to an array of inflammatory signals that cause fibrosis or allergic sensitization. In our studies, we evaluated immune responses to β -TCP/C foam and found that it was not toxic to naïve spleen cells and did not interfere with T lymphocyte expansion or function (cytokine secretion) when added to cultures with ConA. In the intraperitoneal experiments, β -TCP/C foam induced inflammation, and there was some evidence of an increase in eosinophilia and macrophages with concomitant increases in Th1- and Th2-type cytokines. In the subcutaneous implantation experiments, β -TCP/C foam also induced inflammation, but there was no evidence for chronic, destructive inflammatory or allergic responses, which suggests that β -TCP/C foam is biocompatible. These models provide information on the immune response to biomaterials. Firstly, the *in vitro* model addresses the effect of the biomaterial on naïve immune cells in the presence of a mitogen to provide evidence that there is no suppression of the mitogenic response caused by the biomaterial. Secondly, the intraperitoneal model provides a fast 7 day readout of the type of immune response, *e.g.*, allergic and inflammation as observed by the type of inflammatory cell infiltration and the cytokine profile. Thirdly, the subcutaneous, subchronic model illustrates the tissue response over a longer period, allows for the evaluation of chronic inflammation, fibrosis, antibody titers, and can be used to test repeated implantation for immunological memory responses. These models have been previously published and are shown here without any modifications¹³. It is crucial that there are appropriate negative and positive controls for these models. We suggest performing all three models to avoid the limitations of each method. While the models shown are well established in other areas of immunology, the approach for testing biomaterials is recent.

In summary, bone and immune assays provide a biological compatibility profile on a biomaterial. For bone, OB and OC responses to the biomaterial provide preliminary data necessary before performing complicated and expensive animal experiments and to adhere to the 3Rs principle. Immunological *in vitro* assays provide data on antigen cross-reactivity and cytotoxicity, which may also preclude further animal experiments. The rapid high throughput experiments offer results on inflammatory and cytokine response (*e.g.*, type of T lymphocyte responses), while the subchronic model is useful because of data on the duration of inflammation and the potential for damaging fibrosis. This novel interdisciplinary approach which includes bone and immune responses to biomaterials offers an excellent pre-clinical assessment of biocompatibility for future applications in the materials field.

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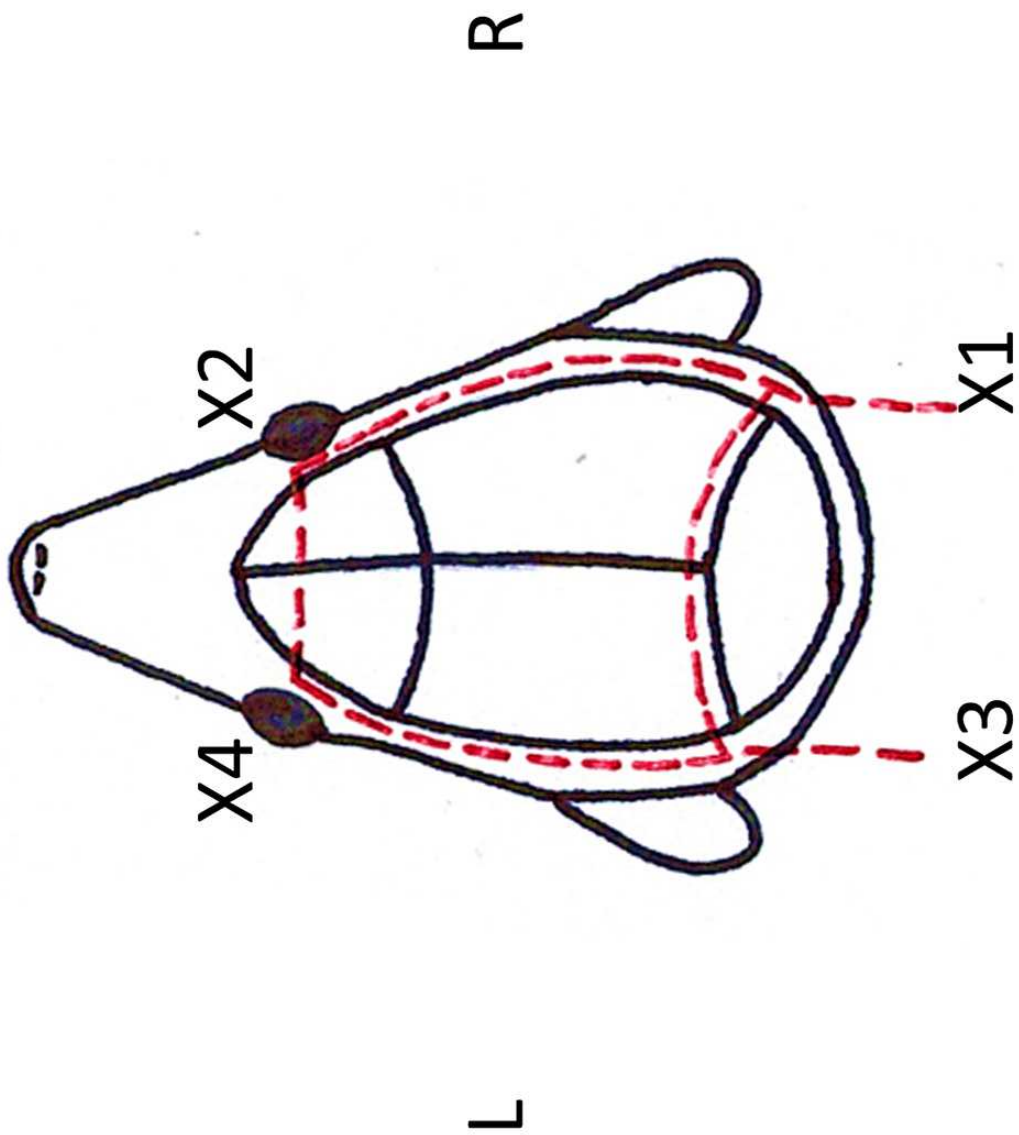
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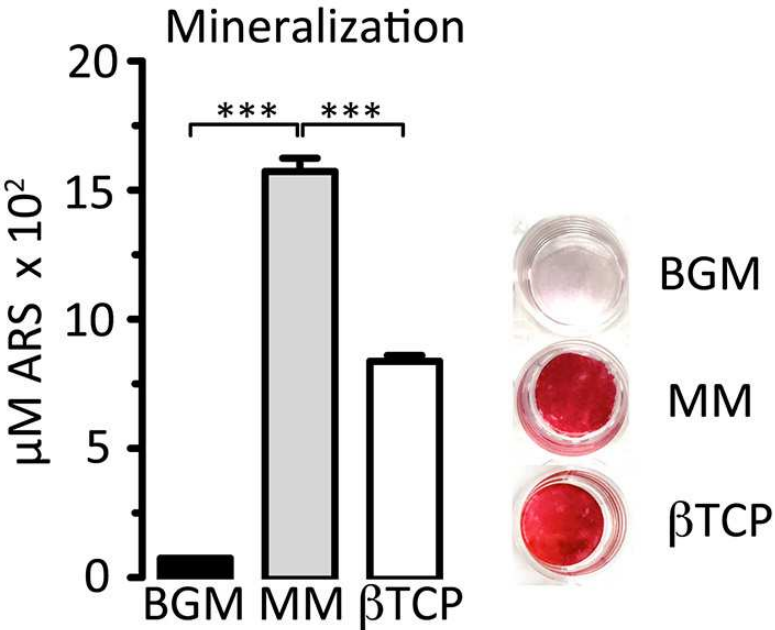
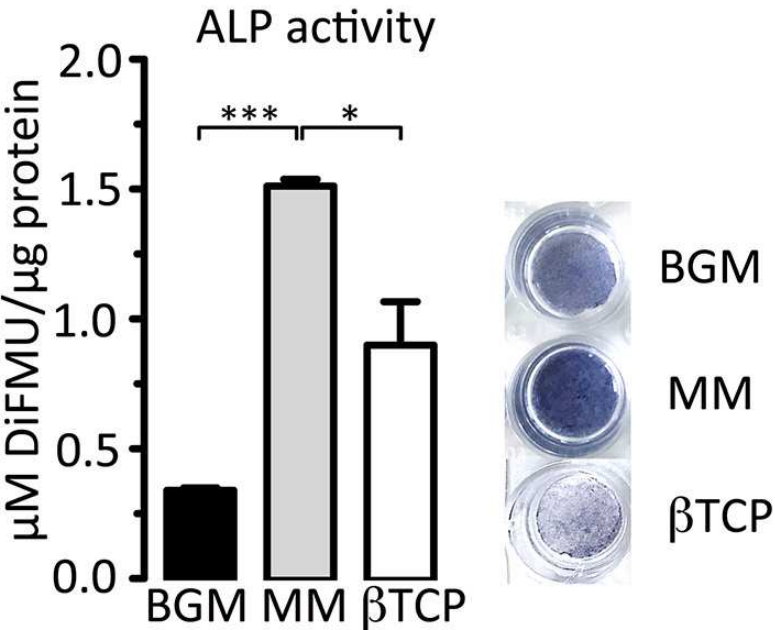
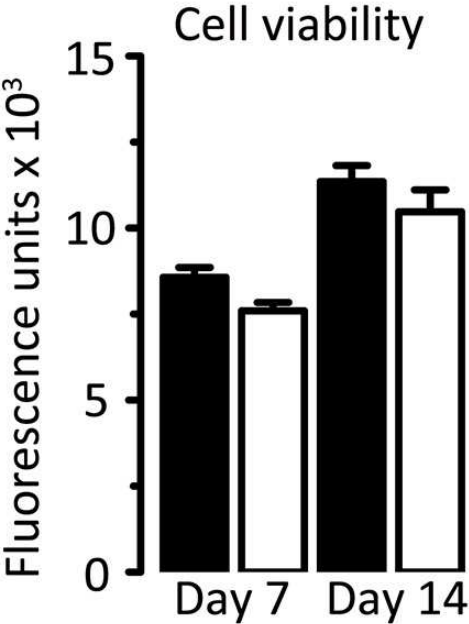
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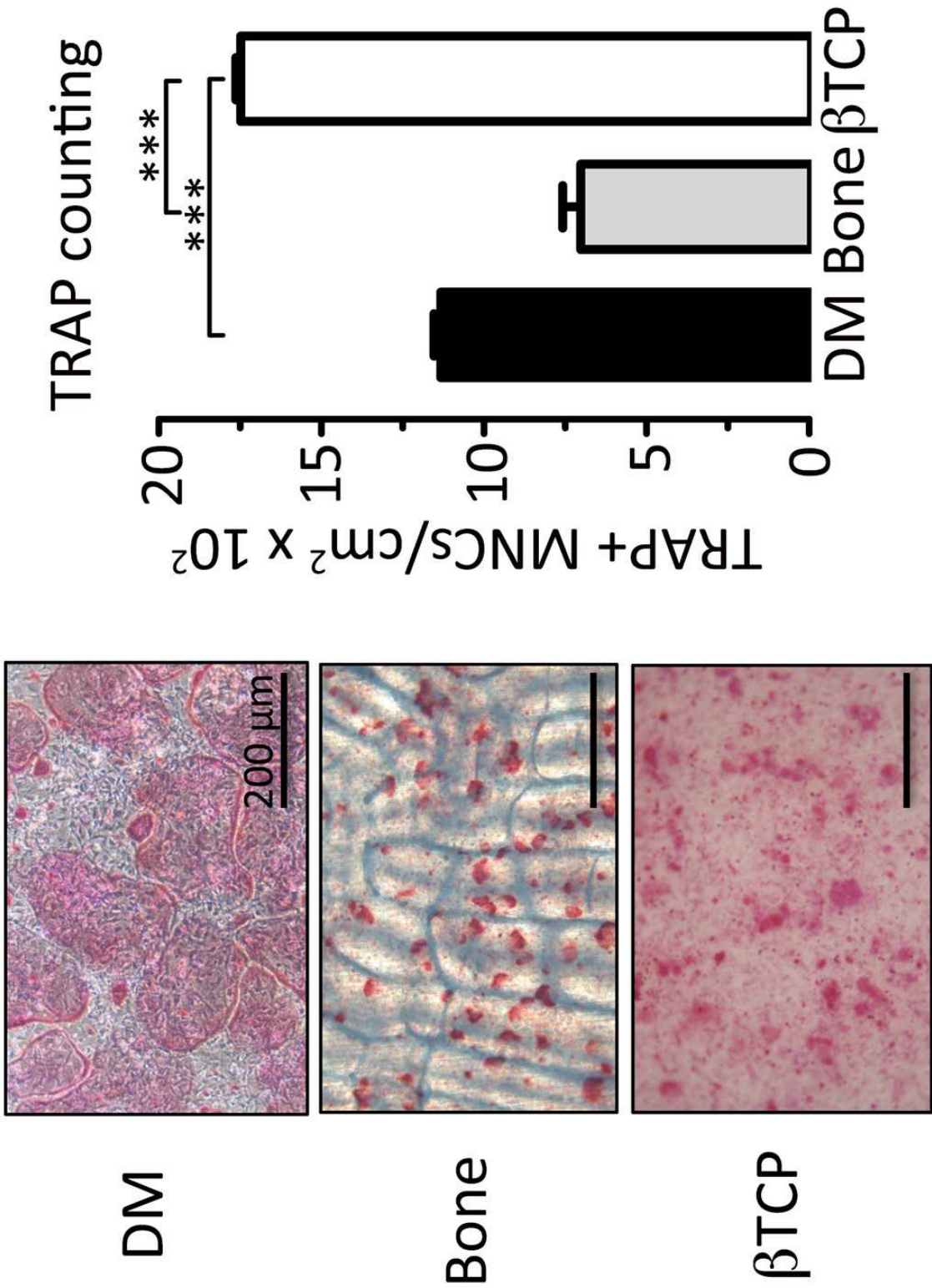
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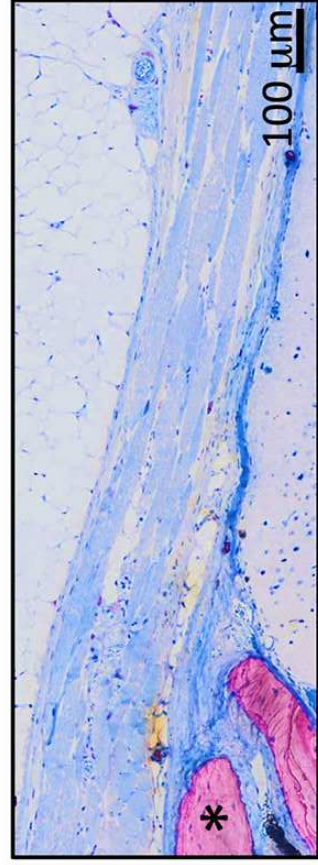
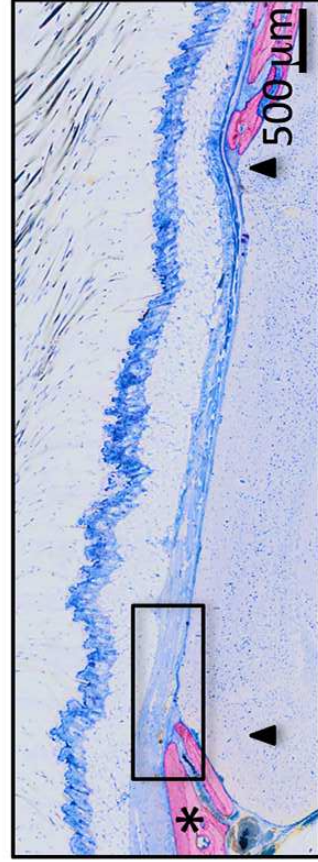




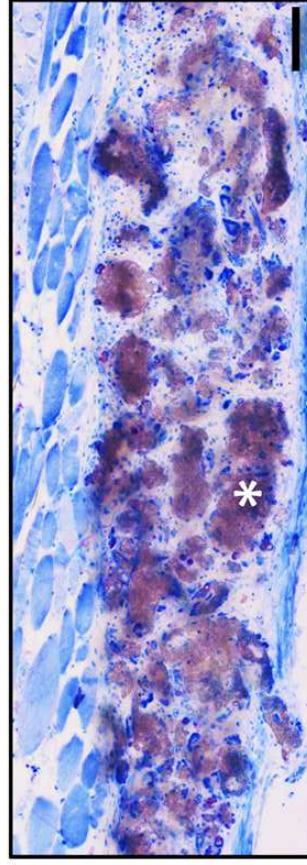
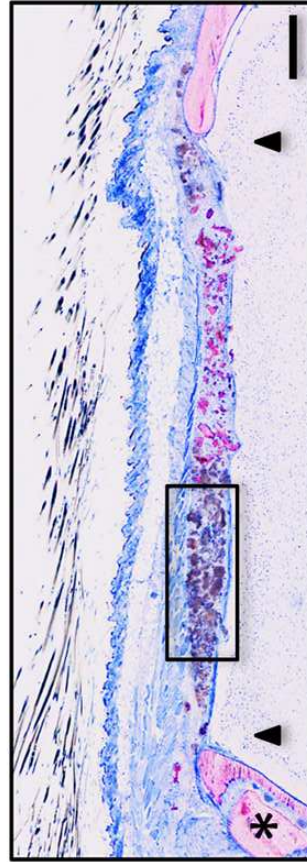


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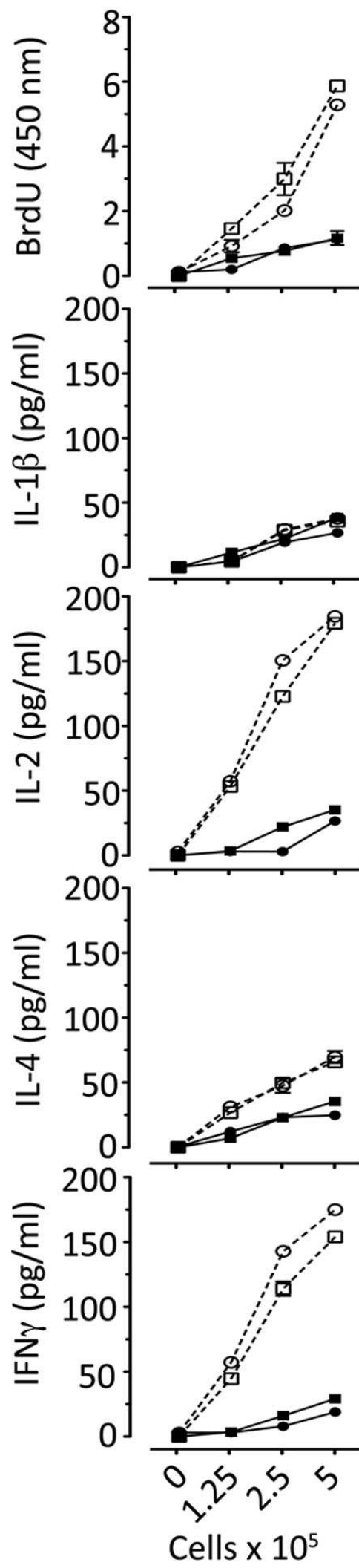
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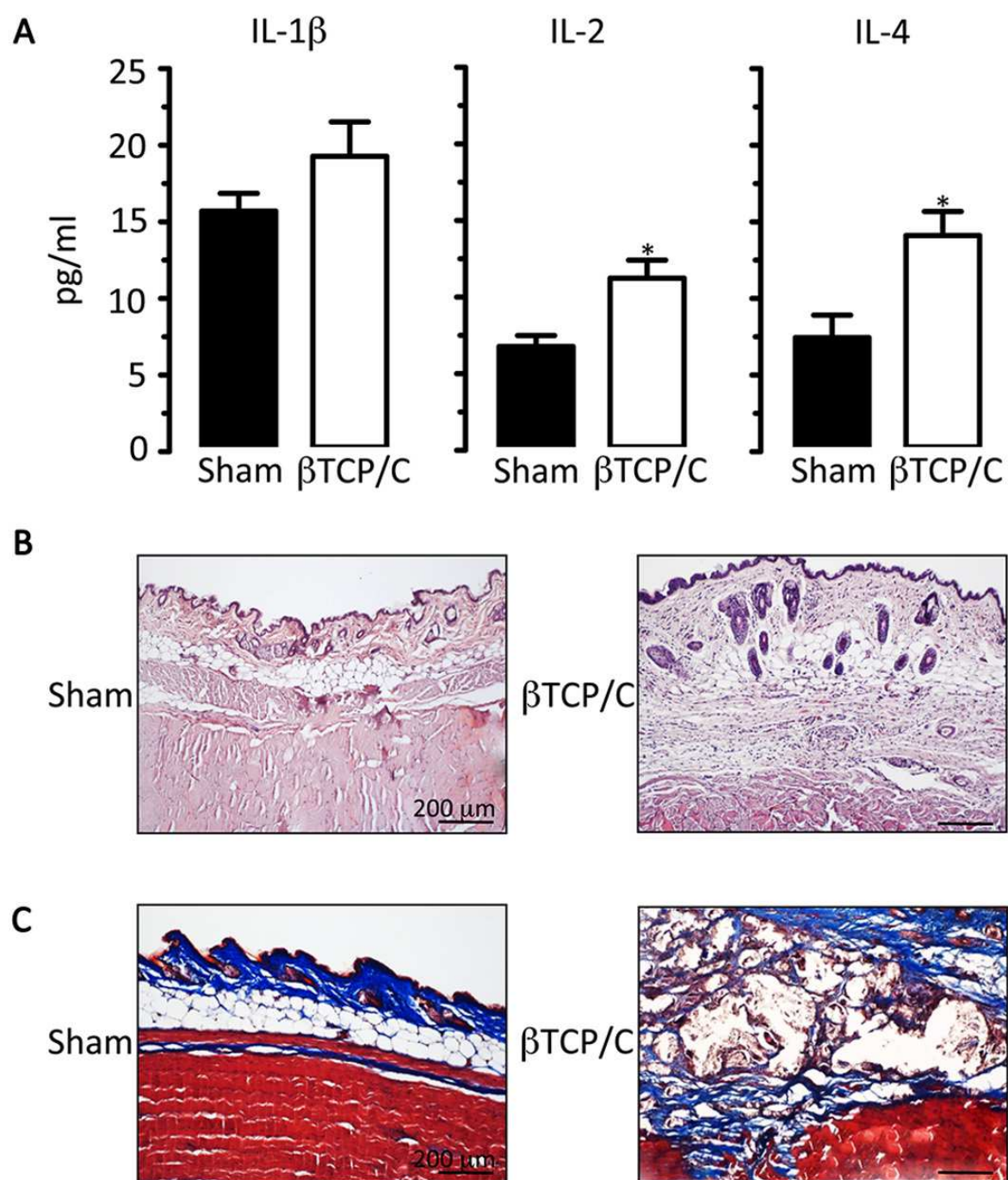


Sham



BTCP/C





	Sham	Vitoss	
	Cell counts x 10 ⁶ (% of total cells)		
Macrophages	1.240 ± 0.051 (88.1)	3.262 ± 0.380 (70.4)	p<0.001
Eosinophils	0.120 ± 0.011 (8.5)	1.181 ± 0.254 (25.5)	p<0.01
Neutrophils	0.002 ± 0.001 (0.2)	0.029 ± 0.011 (0.6)	ns
Lymphocytes	0.048 ± 0.020 (3.2)	0.148 ± 0.041 (3.5)	ns
Total cell count	1.410 ± 0.066	4.620 ± 0.452	p<0.001

Name of Reagent/ Equipment	Company	Catalog Number
Biomaterials		
β -tricalcium phosphate disks (β -TCP, 14 mm)		
Orthovita Vitoss foam (β -TCP/C foam)	Orthovita	2102-1405
Mouse osteoblast culture		
Alizarin Red S	Sigma-Aldrich	A5533
ALP assay buffer (2x) pH 10.4		
Bone growth medium (BM)		
Cell lysis buffer: CyQuant cell lysis buffer 20x concentrate	Thermo Scientific	C7027
Cell viability reagent: Presto Blue cell viability reagent	Invitrogen, Thermo Fisher Scientific	A13261
Collagenase type IV from clostridium histolyticum	Sigma-Aldrich	C5138
DC protein assay kit II	Bio Rad	5000112
Dimethylsulfoxide (DMSO)	Sigma-Aldrich	D8418
Dispase II (neutral protease, grade II)	Roche	4942078001
EnzCheck Phosphatase Assay Kit for alkaline phosphatase activity	Invitrogen, Thermo Fisher Scientific	E12020
Fetal bovine serum (FBS)	Sigma-Aldrich	F7524
Formalin solution neutral buffered 10%	Sigma-Aldrich	HT501128
Glycine	Sigma-Aldrich	G8898
Hexadecylpyrdinium (cetylpyridinium) chloride monohydrate	Sigma-Aldrich	C9002
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich	A8960
Magnesium chloride hexahydrate	Sigma-Aldrich	M2670
MEM alpha medium (α -MEM)	Gibco Life Technologies	11900-073
Osteogenic mineralization medium (MM)		
Penicillin-streptomycin	Sigma-Aldrich	P4333
Phosphate-buffered saline 1x (PBS) pH 7.4	Gibco, Thermo Fisher Scientific	10010023
Sigmafast BCIP/NBT	Sigma-Aldrich	B5655
Trypsin-EDTA (0.5%), no phenol red (10x)	Gibco, Thermo Fisher Scientific	15400054
Tween20	Sigma-Aldrich	P1379
Ultrapure water: Cell culture water pyrogen free	VWR	L0970-500
Wash buffer		
Zinc chloride	Sigma-Aldrich	1.08816
β -Glycerophosphate disodium salt hydrate	Sigma-Aldrich	G5422

24-well suspension culture plate	Greiner bio-one	662 102
24-well tissue culture plate	Greiner bio-one	662 160
50 mL conical tube	Greiner bio-one	227 261
96-well black plate	Greiner bio-one	655076
96-well transparent plate	Greiner bio-one	655001
Centrifuge: VWR Mega Star 600R	VWR	
CO2-Incubator: HeraCell 240	Thermo Scientific	
Cryovial 2 mL	Greiner bio-one	122263
Disposable filter unit FP30/0.2 CA-S	GE Healthcare Life Sciences Whatman	10462200
Flatbed scanner: Epson Perfection 1200 Photo	Epson	
Infinite M200 Pro microplate reader	Tecan	
Microcentrifuge tube: Eppendorf tube safe-lock 0.5 mL	VWR	20901-505
Microcentrifuge tube: Eppendorf tube safe-lock 1.5 mL	VWR	21008-959
Shaker Swip (orbital and horizontal)	Edmund Buehler	
Shaking incubator GFL3032	GFL	3032
Single-use pipet: serum pipet	Greiner bio-one	612301
Sterile instruments (scissors, tweezer, curved forceps)		
Tissue culture plate (10 cm)	Greiner bio-one	664 960
Mouse osteoblast-osteoclast co-culture		
1 α ,25-Dihydroxyvitamin D3	Sigma-Aldrich	17936
Acetone	VWR Chemicals	22065.327
Bone growth medium (BM)		
Distilled water	Carl Roth	3478.4
Ethanol (100% vol/vol)	VWR Chemicals	20821.365
Ethanol (70% vol/vol)	VWR Chemicals	93003.1006
Fast red violet LB salt	Sigma-Aldrich	F3381
Fetal bovine serum (FBS)	Sigma-Aldrich	F7524
Formalin solution neutral buffered 10%	Sigma-Aldrich	HT501128
MEM alpha Medium (α -MEM)	Gibco Life Technologies	11900-073
N,N-Dimethylformamide	Sigma-Aldrich	D4551
Naphthol AS-MX phosphate	Sigma-Aldrich	N4875
Osteoclast differentiation medium (DM)		

Penicillin-streptomycin	Sigma-Aldrich	P4333
Phosphate-buffered saline 1x (PBS) pH 7.4	Gibco, Thermo Fisher Scientific	10010023
Prostaglandin E2 (PGE2)	Cayman Chemical Company	9003016
Sodium acetate trihydrate	Sigma-Aldrich	S7670
Sodium tartrate dibasic dihydrate	Sigma-Aldrich	S8640
Sterile instruments (scissors, forceps, scalpel)		
TRAP buffer pH 5.0		
Ultrapure water: Cell culture water pyrogen free	VWR	L0970-500
24-well suspension culture plate	Greiner bio-one	662 102
24-well tissue culture plate	Greiner bio-one	662 160
50 mL conical tube	Greiner bio-one	227 261
Centrifuge: VWR Mega Star 600R	VWR	
CO2-Incubator: HeraCell 240	Thermo Scientific	
Diamond wafering blade (10.2 cm x 0.3 cm)	Buehler	
Isomet low-speed saw	Buehler	
Petri dish (6 cm)	Greiner bio-one	628,161
Screw cap glass bottle 20 mL	Carl Roth	EXY4.1
Single-use sterile syringe 1 mL	Henry Schein Animal Health	9003016
Single-use pipet: serum pipet	Greiner bio-one	612301
Sterile needle: Hypodermic needle RW 27 G x 3/4"	Henry Schein Animal Health	9003340
Mouse calvarial defect model		
Analgesia: Buprenorphine (Bupaq)	Richter Pharma AG	
Anesthesia: Ketamine (Ketamidor), Xylazine (Rompun)	Richter Pharma AG/Bayer HealthCare	
Ethanol (70% vol/vol)	VWR Chemicals	93003.1006
Eye ointment: VitA POS 5 g	Ursapharm	
Normal saline solution (0.9% sodium chloride solution)	Fresenius Kabi	
Phosphate-buffered saline 1x (PBS) pH 7.4	Gibco, Thermo Fisher Scientific	10010023
Povidone iodine solution: Braunol 1000 mL	B. Braun	3864154
Roti-Histofix 4.5% buffered formalin	Carl Roth	2213.6
Tissue adhesive: Histoacryl 5 x 0.5 mL	B. Braun Surgical	1050060

Personal protective equipment: surgical gloves, cap and mask, gown	Henry Schein Animal Health	1045073, 1026614, 9009062, 370406
Sterile instruments (scalpel, scissors, forceps, needle holder)		
Sterile needles: Hypodermic needles RW 27 G x 3/4" and 30 G x 1/2"	Henry Schein Animal Health	9003340, 9003630
Single-use sterile syringe 1 mL	Henry Schein Animal Health	9003016
Heating plate: Physitemp	Rothacher Medical	TCAT-2LV
Sterile gauze swabs	Henry Schein Animal Health	220192
Sterile disposable scalpel (Figur 20)	Henry Schein Animal Health	9008957
Surgical/dental drill: Implantmed SI-923	W&H Dentalwerk Bürmoos GmbH	16929000
Handpiece type S-II	W&H Dentalwerk Bürmoos GmbH	30056000
Trephine, diameter 4 mm	Hager&Meisinger GmbH	229040
Irrigation tubing set 2.2 m	W&H Dentalwerk Bürmoos GmbH	4363600
Periosteal elevator 2 mm / 3 mm	Henry Schein	472683
Non-resorbable suture: Polyester green, DS19, met. 1.5, USP 4/0 75 cm	Henry Schein	300715
<i>In vitro</i> immune responses		
Anesthesia for euthanasia: Ketamine (Ketamidor), Xylazine (Rompun)	Richter Pharma AG/Bayer HealthCare	
Cell Proliferation ELISA, BrdU (colorimetric) Assay Kit	Sigma-Aldrich	11647229001
Concanavalin A (ConA)	MP Biomedicals	150710
Culture medium splenocytes		
Ethanol (70% vol/vol)	VWR Chemicals	93003.1006
Fetal bovine serum (FBS)	Gibco, Thermo Fisher Scientific	10500064
Gentamicin	Gibco, Thermo Fisher Scientific	15750037
Mouse IL-1 β ELISA Ready-SET-Go!	Invitrogen, Thermo Fisher Scientific	88-7013-88
Mouse IL-2 ELISA MAX Standard	BioLegend	431001
Mouse IL-4 ELISA MAX Standard	BioLegend	431101
Mouse INF- γ ELISA MAX Standard	BioLegend	430801
Non-essential amino acids solution	Gibco, Thermo Fisher Scientific	11140050
Penicillin-streptomycin	Gibco, Thermo Fisher Scientific	15140122
Phosphate-buffered saline 1x (PBS) pH 7.4	Gibco, Thermo Fisher Scientific	10010023
Red blood cell (RBC) lysis buffer: BD Pharm Lyse	BD Bioscience	555899

RPMI 1640 medium, HEPES	Gibco, Thermo Fisher Scientific	52400025
β -Mercaptoethanol	Gibco, Thermo Fisher Scientific	31350010
50 mL conical tube	Greiner bio-one	227 261
96-well tissue culture plate	Greiner bio-one	655 180
Centrifuge: VWR Mega Star 600R	VWR	
Cell strainer 40 μ m	BD Bioscience	352340
Infinite M200 Pro microplate reader	Tecan	
Single-use sterile syringe 1 mL	Henry Schein Animal Health	9003016
Sterile surgical instruments (forceps, glass slides)		
High throughput intraperitoneal model		
Anesthesia: Ketamine (Ketamidor), Xylazine (Rompun)	Richter Pharma AG/Bayer HealthCare	
Ethanol (70% vol/vol)	VWR Chemicals	93003.1006
Eye ointment: Vita POS 5 g	Ursapharm	
Mouse IL-1 β ELISA Ready-SET-Go!	Invitrogen, Thermo Fisher Scientific	88-7013-88
Mouse IL-2 ELISA MAX Standard	BioLegend	431001
Mouse IL-4 ELISA MAX Standard	BioLegend	431101
Phosphate-buffered saline 1x (PBS) pH 7.4	Gibco, Thermo Fisher Scientific	10010023
Povidone iodine solution: Braunol 1000 mL	B. Braun	3864154
Shandon Rapid-Chrome Kwik-Diff Staining Kit	Thermo Scientific	9990700
Heating plate: Physitemp	Rothacher Medical	TCAT-2LV
Hemocytometer: Neubauer chamber	Carl Roth	PC72.1
Non-resorbable suture: Ethibond Excel 4-0	Ethicon	6683H
Resorbable suture: Polysorb	Covidien	SL-5628
Shandon centrifuge for cytopsin	Thermo Scientific	
Single-use sterile syringe 1 mL	Henry Schein Animal Health	9003016
Sterile gauze swabs	Henry Schein Animal Health	220192
Sterile needles: Hypodermic needles RW 27 G x 3/4" and 25 G	Henry Schein Animal Health	9003340, 420939

Sterile surgical instruments (scissors, forceps, needle holder)		
Trypan blue solution 0.4%	Gibco, Thermo Fisher Scientific	15250061
Subchronic subcutaneous model		
Anesthesia: Ketamine (Ketamidor), Xylazine (Rompun)	Richter Pharma AG/Bayer HealthCare	
Ethanol (70% vol/vol)	VWR Chemicals	93003.1006
Eye ointment: VitA POS 5 g	Ursapharm	
Phosphate-buffered saline 1x (PBS) pH 7.4	Gibco, Thermo Fisher Scientific	10010023
Povidone iodine solution: Braunol 1000 mL	B. Braun	3864154
Roti-Histofix 4.5% buffered formalin	Carl Roth	2213.6
Heating plate: Physitemp	Rothacher Medical	TCAT-2LV
Non-resorbable suture: Ethibond Excel 4-0	Ethicon	6683H
		1045073, 1026614,
Personal protective equipment: surgical gloves, cap and mask, gown	Henry Schein Animal Health	9009062, 370406
Single-use sterile syringe 1 mL	Henry Schein Animal Health	9003016
Sterile gauze swabs	Henry Schein Animal Health	220192
Sterile instruments (scissors, forceps, needle holder)		
Sterile needle: Hypodermic needles RW 27 G x 3/4"	Henry Schein Animal Health	9003340, 420939

Comments/Description

Disks were sintered in a muffle furnace at a temperature of 1150 °C. A detailed description of the production can be found in Zimmer et al (doi: 10.1002/jbm.a.34639). Samples were UV-irradiated (15 min for each side) before using in cell cultures.

Dissolve 200 mM glycine, 2 mM magnesium chloride, 2 mM zinc chloride in ultrapure water and adjust to pH 10.

α -MEM + 10% heat-inactivated FBS + 1% penicillin/streptomycin

Dilute the concentrated cell lysis buffer stock 20-fold in ultrapure water.

DC protein assay kit contains reagent A, reagent B, reagent S and BSA for the reference standard. For reagent A', add 20 μ L reagent S to 1000 Steril-filter DMSO before usage.

EnzCheck Phosphatase Assay Kit contains 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP), N,N-dimethylformamide and 6,8-Difluoro-7

α -MEM + 10% heat-inactivated FBS + 1% penicillin-streptomycin + 50 μ g / ml ascorbic acid + 5 mM β -glycerophosphate

Dilute the concentrated stock solution 10-fold in PBS.

Add 0.05% Tween20 to 1x PBS.

For sterile filtration of enzyme solution.


1000x concentrated stock (10⁻⁵ M in ethanol 100%)


α-MEM + 10% heat-inactivated FBS + 1% penicillin-streptomycin

α-MEM + 10% heat-inactivated FBS + 1% penicillin-streptomycin + 1 nM 1,25-(OH)₂-vitamin D₃ + 1 μM prostaglandin E₂

1000x concentrated stock (10⁻³ M in ethanol 100%)

Dissolve 40 mM sodium acetate, 10 mM sodium tartrate in ultrapure water and adjust to pH 5.





The kit contains BrdU labeling solution, fixation solution (FixDenat), Anti-BrdU antibody solution, washing buffer and substrate solution.

RPMI medium + 10% heat-inactivated FBS + 1% penicillin-streptomycin solution + 0.1% gentamicin + 0.2% β -mercaptoethanol +1% non-



For differential cell count.





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Author(s):

Carina Kampleitner, Jessika Obi, Nicola Vassilev, Michelle M. Epstein, Oskar Hoffmann

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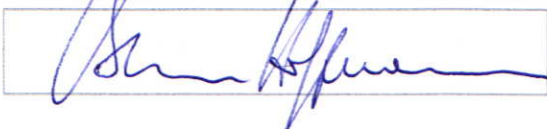
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Subject: JoVE58077R2 Manuscript revision

Vienna, 10.06.2018

Dear Dr. Bing Wu,

Please find our point-by-point response below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We revised the manuscript, checked the grammar and corrected mistakes.

2. The highlighted protocol steps are over 2.75 pages limit. Please highlight less than 2.75 pages of protocol steps for filming.

We reduced the highlighted text to under 3 pages by removing one method (high throughput i.p. method).

3. Please highlight complete sentences (not part of sentences) for filming.

The manuscript was revised to ensure that no partial sentences are highlighted.

Yours sincerely,



Oskar Hoffmann