A comparative study of cartilage engineered constructs in immunocompromised, humanized and immunocompetent mice

Journal Article

Author(s):
Cavalli, Emma; Fisch, Philipp; Formica, Florian A.; Gareus, Ralph; Linder, Thomas; Applegate, Lee Ann; Zenobi-Wong, Marcy

A comparative study of cartilage engineered constructs in immunocompromised, humanized and immunocompetent mice

Emma Cavalli[1, †], Philipp Fisch[1, †], Florian A. Formica[1], Ralph Gareus[2], Thomas Linder[3], Lee Ann Applegate[4], Marcy Zenobi-Wong[1,§]

[1] Tissue Engineering and Biofabrication, Institute for Biomechanics, Swiss Federal Institute of Technology Zürich (ETH Zürich), Otto-Stern-Weg 7, 8093 Zürich, Switzerland

[2] The Jackson laboratory, 600 Main St, Bar Harbor, ME 04609, USA

[3] Department of Otorhinolaryngology, Head- & Neck Surgery, Luzerner Kantonsspital, Spitalstrasse, 6000 Luzern, Switzerland

[4] Department of Musculoskeletal Medicine, Regenerative Therapy Unit, University Hospital of Lausanne (CHUV), EPCR/ch. Croisettes 22, 1066 Epalinges, Switzerland

[†]These authors contributed equally to this study

[§] Address for correspondence:

Prof. Marcy Zenobi-Wong

ETH Zürich

Tissue Engineering and Biofabrication

HPL J22

Otto-Stern-Weg 7

8093 Zürich, Switzerland

zmarcy@ethz.ch

Phone: +41 44 632 5089

Highlights

- Chondrogenesis of a tissue-engineered cartilage graft is feasible in immunocompetent small animals
- Immunocompetent and immunodeficient animals lead to analogous results in terms of chondrogenesis, as long as the implanted cells are shielded from the host by a biomaterial
- Subcutaneous implantation in small animals with a complete and human immune system could help to predict the outcome of engineered grafts for cartilage applications

Abstract

Choosing the best ectopic *in vivo* model for cartilage engineering studies remains challenging and there is no clear consensus on how different models compare to one another. The use of xenogenic cells can often limit the choice to immunocompromised animals only and thus prevents the understanding of how tissue-engineered grafts perform with potential active inflammatory and immunological responses. The aim of this study was to evaluate the chondrogenic potential of a recently developed hydrogel, hyaluronan transglutaminase (HA-TG), in four mouse strains with varying immune systems: NSG, nude, NSG-SGM3 humanized and C57BL/6. The hyaluronan-based hydrogel

44 was implanted subcutaneously for 4 weeks after an *in vitro* pre-culture time of 4 weeks.

45 Scaffolds were prepared without cell seeding as the control and in combination with

46 either human auricular chondrocytes (hAUR) or human fetal chondroprogenitor cells

47 (hCC). We have seen that constructs were able to maintain their volumes and resisted

48 vascularization as well as macrophage infiltration *in vivo.* Both hAUR and hCC maintained

49 and produced ECM *in vivo*, but hAUR showed higher levels of innate collagen 2 even

50 without mechanical stimulation. Collagen 1 and 2 deposition as well as mechanical

51 properties of the scaffolds were comparable in all mouse strains. The C57BL/6 mouse

52 model consistently displayed higher levels of C-reactive protein (CRP), serum amyloid A

53 (SAA), and serum amyloid protein (SAP) in serum as a reaction to the foreign material and

54 human cells. In addition, the number of CD68+ and CD163+ macrophages as well as CD3+

55 lymphocytes around the constructs in C57BL/6 mice was significantly higher than in

56 humanized and immunocompromised mouse models. The results show that it is possible

57 to engineer a cartilage-like graft subcutaneously not only in immunocompromised, but

58 also in immunocompetent and humanized mouse model.

59

1. Introduction

In vivo testing plays a key role in tissue engineering and in providing the pre-clinical data necessary to bring new therapies to the clinic [1]. Despite recent advances with *in vitro* techniques such as bioreactors, organ-on-a-chip and organoids, animal models remain critical, particularly for safety assessment of tissue engineering components. This is no exception in the cartilage repair field where animal models continue to be required for regulatory approval of biologics and devices [2]. Nevertheless, choosing the appropriate animal model for the assessment of new cellular-, drug- or biomaterial-based strategies for cartilage repair remains a major challenge. Animal type, size and physiological differences inter- and intra-species can have a significant influence on the outcome of a study. Additionally, the limited ability of cartilage to regenerate has driven the development of cell-based and tissue engineering techniques [3] which often involve the use of allogenic cells [4-6] and allografts [7-9]. The widespread use of immunocompromised mice for preclinical studies often prevents a full understanding of how tissue-engineered constructs perform in terms of chondrogenesis and inflammatory response when exposed to an active immune system.

Under current regulatory scrutiny, a number of aspects need to be kept in consideration when choosing the animal model for cartilage tissue engineering. Despite the value of large animal models, there are still ethical, economical and technical constraints that prevent their wide-spread utilization and acceptance [1]. Furthermore, despite the recent advances in gene-editing techniques like CRISPR-Cas9, the availability of transgenic models in large animals is still limited [10]. Rodents, and particularly mice, are affordable, easy to manage and can provide proof-of-concept data in a time-effective manner. They

84 can be genetically modified to insert and delete specific genes and they are commercially

85 available in a variety of athymic, transgenic and knockout strains. Therefore they are

86 usually a first choice in cartilage engineering studies for screening purposes and to bridge

87 the gap between *in vitro* experiments and large animal preclinical studies [11].

88 Chondrogenesis for cartilage engineering strategies has been extensively studied in

89 murine models by subcutaneous implantations. The subcutaneous implant model was

90 developed due to the limited joint size and cartilage thickness of mice [11, 12] and allows

91 the evaluation of tissue-engineered constructs to sustain and induce cartilage formation

92 in an ectopic site as well as the ability of the construct to resist cellular infiltration.

93 Athymic nude mice, which have a limited adaptive immune response, are the most

94 commonly used strain of mice in cartilage engineering [12]. They are hairless and their

95 lack of the thymus prevents them from having a cell-mediated immunity [13]. Indeed in

96 nude mice, the immune response to foreign and xenogeneic bodies

97 does not involve the activation of antigen-specific T lymphocytes and the consequent release

98 of cytokines. Another widely-used mouse strain is the NOD (non-obese diabetic) SCID

99 (severe combined immunodeficiency) gamma, or NSG. NSG mice are among the most

100 immunodeficient mouse strain described to date, lack mature T cells, B cells, and natural

101 killer (NK) cells and have a defective innate immune system [14].

102 In the attempt to reduce the gap between pre-clinical and clinical studies, mouse-human

103 chimeras, or humanized mice, have been developed [15]. Humanized mice are

104 immunodeficient mice engrafted with human hematopoietic cells or human peripheral-

105 blood mononuclear cells. Of these mice, the NSG™-SGM3 transgenic strain contains three

106 co-injected human transgenes, the Stem Cell Factor (SCF), the Granulocyte/Macrophage

107 colony factor 2 (GM-CSF) and the interleukin-3 (IL-3). Thanks to this triple transgenic

108 modification, NSG™-SGM3 mice constitutively express cell proliferation and survival

109 signals and support a stable engraftment of human hematopoietic cells. This strain is

110 gaining popularity in the immune-oncology field to understand the interactions between

111 human immune cells and specific patient-derived tumors due to its high count of human

112 immune cell populations (i.e. CD19+ B cells, CD3+ T cells, CD33+ myeloid cells) [16-18].

113 Additionally, they have proven to be a very valuable tool in the study of the human

114 immune system development and dysfunction as well as in the modelling of several

115 human diseases (e.g. infectious diseases such as Epstein Barr virus infection and HIV, and

116 autoimmune diseases such as diabetes and arthritis) with minor logistical and ethical

117 concerns [19]. Nevertheless, current humanized mice lack secondary lymphoid tissue and

118 can only partially mimic the complexity of a fully developed, human immune system,

119 which is the coordinated response of stromal, lymphoid, myeloid and secondary lymphoid

120 structures. Furthermore, the remaining murine immune system prevents the complete

121 human engraftment and murine cytokines do not fully support human myeloid and

122 lymphoid development [20, 21]. While effort are currently undergoing to overcome these

123 limitations, it is still an open question whether humanized mice could be used in the tissue

124 engineering field and represent a useful model in the preclinical study of tissue-

125 engineered graft transplantation.

126 Despite the widespread use of immunodeficient mice for the subcutaneous implantation

127 of engineered constructs for cartilage repair applications, there is no consensus as to

128 whether this should be the ideal choice for a mouse model. The recent development of

129 humanized mice has furthermore raised the question of whether these chimeras would

130 represent a suitable animal model for the field.

131 The aim of this study was to evaluate and compare the chondrogenic potential of a

132 recently developed tissue engineered scaffold for cartilage repair applications in four

different mouse strains, namely NSG, nude, NSG-SGM3 humanized and C57BL/6. The biomaterial used was a hyaluronan-based hydrogel [22, 23], produced by functionalizing the hyaluronan (HA) backbone with transglutaminase (TG) crosslinkable peptides, hereby addressed as HA-TG. The material was investigated alone and in combination with one of two cell types of different origins: human auricular chondrocytes (hAUR) and human fetal chodroprogenitor cells (hCCs) (*Figure 1*). We investigated how differences in the innate and adaptive immune systems of the mouse strain affect the quality and amount of extracellular matrix (ECM) produced and maintained by the cells. hCCs are a newly proposed cell source [24], which have previously shown to produce phenotypically stable cartilage in combination with HA-TG *in vitro* [22] and in different collagen scaffolds in an *in vivo* subcutaneous mouse model [25]. Allogeneic cells derived from fetal or juvenile tissue have the additional benefit of not only having a more stable phenotype and higher chondrogenic potential than adult articular chondrocytes [26], but also lower immunogenicity [27]. Fetal chondroprogenitors have high stability and therefore very large quantities of cells may be prepared from one, single organ donation allowing for the development of Master and Working Cell banks (MCB & WCB). These cell banks provide a long-term solution as the cells can be thawed just before use in transplantation. Alternatively, auricular chondrocytes are currently emerging as a potential autologous cell source for tissue engineering purposes due to their ease in harvesting from the patient, enhanced proliferation capacity, high and reproducible chondrogenic potential after *in vitro* expansion and the quality of the generated tissue [28]. Importantly, auricular chondrocytes can be obtained from a cartilage biopsy with minimal donor site morbidity compared to articular cartilage sites and therefore are suitable for autologous applications in a clinical setting.
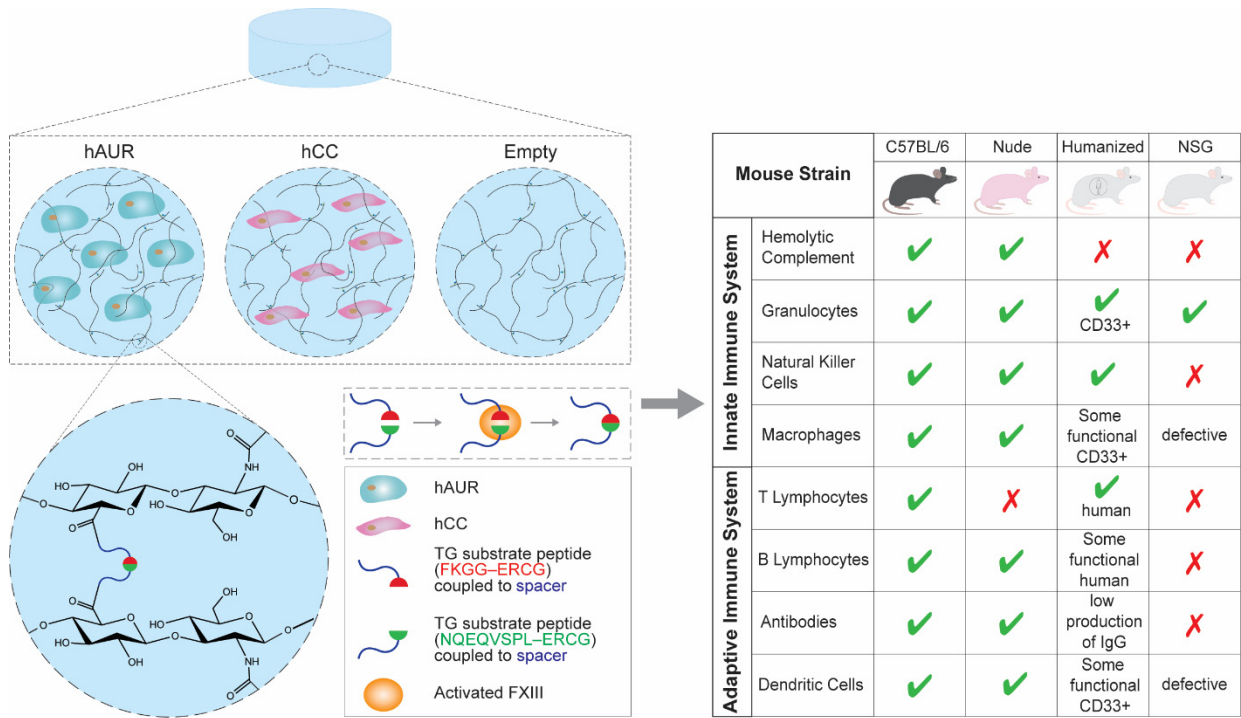
157

158 *Figure 1 Schematics of the study. Cell-laden (either human auricular chondrocytes, hAUR or human fetal*
159 *chondroprogenitors, hCCs) and acellular hyaluronic acid-transglutaminase (HA-TG) hydrogels were prepared. HA-TG*
160 *synthesis was achieved by functionalizing the side chains of a hyaluronan backbone with transglutaminase (TG) substrate*
161 *peptides coupled to a spacer. Gelation occurred via crosslinking of the two TG peptides and was triggered by addition of*
162 *activated factor XIII (FXIII) in presence of calcium. The constructs were subcutaneously implanted in four mouse strains*
163 *with varying immune systems: NSG, nude, NSG-SGM3and C57B6/6.*

164

Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

2.2 Cell sources

Human hCCs were isolated from the proximal ulnar epiphysis of a 14 week gestation organ donation (same donor as used in Darwiche *et al.* [24] and Studer *et al.* [25], Centre Hospitalier Universitaire Vaudois, Ethics Committee Protocol No. 62/07) and registered under the Federal Transplantation Program complying with the associated laws, Biobank procedures and regulations.

Human auricular chondrocytes (male, 17 years old) were isolated from auricular cartilage of otoplasty patients after having received informed consent (BASEC-Nr.2017-02101).

2.3 Cell isolation, culture and expansion

- hCC. The biopsy was collected and processed as previously described [24] except that Trypsin/EDTA (Thermo Fisher Scientific) was used during processing to prepare more uniform populations from the tissue and no antibiotics were used for cell culture to develop the MCB. A WCB was developed for the project that was derived from the MCB from the organ donation to provide equivalent cells throughout the current project. The cells were expanded to passage 4 in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% v/v FBS (Gibco), 2 mM L-glutamine (Gibco) and 10 µg/ml Gentamycin (Gibco).

185 • hAUR. The cartilage pieces were washed extensively with PBS containing 50 µg/ml

186 gentamicin and incubated in 0.5% w/v pronase solution for 1.5 hour. The cartilage

187 was then minced into pieces of 1-3 mm^3 and digested in 0.12% w/v collagenase

188 solution (DMEM, 12 mg/ml collagenase from Clostridium histolyticum, 10% v/v

189 FBS) overnight with gentle stirring at 30°C. The resulting cell suspension was

190 passed through a 40 µm cell strainer before collecting the cell pellet by

191 centrifugation (500 rcf for 10 minutes). The cells were plated at 10'000 cells/cm^2

192 and expanded to passage 2 in DMEM, 10% v/v FBS, 10 µg/ml gentamycin and 50

193 µg/ml L-ascorbate-2-phosphate at 37°C, 5% CO_2 and 95% humidity.

194 ## *2.4 HA-TG synthesis and hydrogel formation*

195 • HA-TG synthesis. HA-TG hydrogel precursors were synthesized as described

196 previously by Broguiere *et al.* [23]. Briefly, 400 mg of HA sodium salt (Lifecore

197 Biomedical, 1.01-1.8 MDa), and 23.8 mg of 3,3'-Dithiobis(propanoic dihydrazide)

198 (Frontier Scientific) were dissolved in 160ml of 150mM MES solution. Thereafter

199 38.4 mg of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (Fluka) was added

200 dropwise and left to react overnight. 143.33 mg of TCEP-HCl (Fluorochem) was

201 added, and the reduction left to proceed overnight. The product was dialyzed

202 against ultrapure water balanced to pH 4.5. The recovered solution was added

203 dropwise into a solution of 1 ml divinyl sulfone (DVS) in 40 ml of 300mM

204 triethanolamine (TEOA) buffer, pH 8.0. The reaction was left to proceed for 8h at

205 RT and then dialyzed against ultrapure water to yield vinyl sulfone-substituted HA

206 (HA-VS). The recovered HA-VS was split in two equal parts. One half was

207 functionalized with a substrate peptide that provided a reactive glutamine residue

208 (TG/Gln: NQEQVSPL-ERCG) and the other half with the peptide providing the

209   reactive lysine (TG/Lys: FKGG-ERCG). For conjugation, 10 ml of TEOA buffer 300

210   mM, pH 8.0 was added to each HA-VS portion and the peptides were added at 1.3

211   excess over the 10% DVS substitution. The reactions were allowed to proceed

212   overnight without stirring. Finally, the products were dialyzed against ultrapure

213   water, sterilized by 0.4 µm filtration, lyophilized and stored at -80°C until use.

214   • Cell encapsulation. Cells (either hCC or hAUR) were suspended at a concentration

215   of 15 million cells/ml in HA-TG. The gelation was triggered as described by

216   Broguiere & Cavalli *et al.* [22]. Briefly, the gel precursors were resuspended at 2%

217   w/v in sterile filtered TBS (NaCl 150mM, $CaCl_2$ 50 mM, TRIS 50 mM, balanced to

218   pH 7.6). The crosslinking was initiated by adding thrombin (Baxter) and factor XIII

219   (Fibrogammin, CSL Behring) to a final concentration of 12.5U/ml and 10U/ml

220   respectively. The gels were quickly cast in UV-sterilized PDMS cylindrical molds

221   (SYLGARD 184, Corning, diameter=4mm, height= 2mm) adhered to 10 mm glass

222   coverslips. The gels were allowed to crosslink for 15 minutes at 37°C before adding

223   chondrogenic medium, consisting of high glucose DMEM supplemented with 10

224   ng/ml transforming growth factor β3 (TGF-β3, Peprotech), 50 µg/ml L-ascorbate-

225   2-phosphate, 40 µg/ml L-proline, 0.5% penicillin-streptomycin (Gibco) and 1%

226   ITS+ Premix (Corning). The PDMS molds were then detached from the cover slip

227   and the gels left free floating.

228   Gels without encapsulated cells were prepared as well and used as controls. The

229   cultures were maintained for up to 8 weeks, replacing the medium 3 times a week.

2.5 Subcutaneous implantation

Animal studies were performed in compliance with the ethical license (Application No. ZH189/2014). Nude (Crl:NU(NCr)-Foxn1nu) and Black 6 (Crl:C57BL/6J, indicated in the figures as Bl6) animals were obtained from Charles River, while NOD scid gamma (NSG) and CD34+ humanized (NSG-SGM3, indicated in the figures as hu-NSG) mice were obtained from The Jackson Laboratory. All animals were female with an age of 2-3 months and a weight of 23 g ± 4g. NSG-SGM3 animals were engrafted using fetal CD34+ cord blood cells purchased from Lonza (as the Certificate of Analysis states "The cells were isolated from donated human tissue after obtaining permission for their use in research appplications by informed consent or legal authorization", Lot N. 0000625909). The animals were used 18 weeks post engraftment, when the highest number of myeloid cells was present (*Supplementary Table 1*). Animals were housed in groups of 4-5 and allowed to move without restrictions. Standard food and water were provided *ad libitum*.

Different conditions were investigated during the study: HA-TG alone (acellular), HA-TG with hCC and HA-TG with hAUR. Following randomization, six scaffolds of each condition were subcutaneously implanted after 4 weeks of preculture in four different mouse strains: C57BL/6, nude, NSG and NSG-SGM3. Six constructs per condition were further cultured *in vitro*. Three animals were used for each experimental condition, for a total of 9 mice per strain. Mice were anesthetized with 4.5% isofluorane and Meloxicam (Metacam, 2 mg/kg) was administered via subcutaneous injection before surgery. Eye cream was applied to prevent desiccation of the cornea and the anesthesia was continued with 1.5-3% isoflurane. Two incisions were made in the skin lateral to the dorsal midline at the level of the hip joint and constructs were placed subcutaneously. The incisions were closed with surgical staples (3M), which were removed after 1 week. Blood samples were

254 collected 10 days before surgery through puncture of the Vena Saphena and at the end of

255 all procedures and experimentation via heart puncture. After 4 weeks, the animals were

256 euthanized via CO_2 asphyxiation and the explants fixed for 2 hours in 4%

257 paraformaldehyde.

258 ## *2.6   Histology and immunohistochemistry*

259 After dehydration, samples were paraffin embedded and 5 µm sections were cut using a

260 microtome. Collagen 1 and 2 staining were performed after 30 minutes of 0.2% (w/v)

261 hyaluronidase digestion at 37 °C and 1 hour blocking with 5% normal goat serum (NGS)

262 with 1:1500 diluted rabbit anti-collagen 1 (Abcam ab138492) and 1:200 diluted rabbit

263 anti-collagen 2 (Rockland 600-401-104) antibodies. CD68, CD163 and CD3 stainings

264 were performed after heat-mediated epitope retrieval in sodium citrate buffer at pH 6 for

265 20 minutes, permeabilization in 0.3% v/v Triton-X for 15 minutes and 1 hour blocking

266 with 5% NGS with 1:200 diluted rabbit anti-CD68 (Abcam ab125212), 1:200 anti-CD163

267 (Abcam ab182422) and 1:100 anti-CD3 (Abcam ab5690) antibody respectively. Negative

268 controls were performed using a rabbit IgG isotype control (Novus Biologicals, NBP2-

269 24891) diluted 1:200.

270 All primary antibodies were diluted in 1% w/v NGS in PBS and incubated overnight at

271 4°C. Alexa Fluor 594 Goat Anti-Mouse IgG (Thermo Fisher Scientific, A11005) and Alexa

272 Fluor 488 Goat anti-rabbit Alexa 488 (Thermo Fisher Scientific, A11008) secondary

273 antibodies were used at 1:200 dilution in 1% NGS in PBS for 1 hour at RT. Finally, the

274 slides were incubated for 15 minutes with the nuclear stain DAPI (Molecular Probes) and

275 mounted with VectaMount AQ Mounting Medium (Vector Laboratories).

276 Safrainin O/Fast Green and hematoxylin and eosin (H&E) staining were performed using

277 standard protocols.

278 All of the images in the same figure were acquired using an automated digital slide scanner

279 (Panoramic 250 Flash III, 3D Histotech) using the same exposure time and light intensity.

280 For semi-quantitative analysis of collagen 1 and 2, the 8-bit integrated density of the

281 stainings were analyzed using ImageJ v1.51 software (National Institutes of Health). For

282 quantification analysis of CD68 and CD163, positively stained cells were counted in

283 randomly selected regions of interest in the fibrous capsule around the scaffolds and

284 expressed as the mean number of cells per region of interest (cells/ROI).

285 ## *2.7 Mechanical testing*

286 Scaffolds were tested under unconfined compression using a TA.XTplus Texture Analyser

287 (Stable Microsystems) with a 500 g load cell. After careful removal of the fibrous tissue

288 around the scaffolds, the compression probe was brought in close contact with the sample

289 and a slight preload applied to ensure proper contact of the probe with the surface of the

290 sample. Samples were compressed to a strain of 15% at a loading rate of 0.01 mm per

291 second. The compressive modulus E was calculated as the slope of the linear range of the

292 stress–strain curve. It has to noted that, despite being careful and as accurate as possible

293 in removing the fibrous tissue, the difference in fibrous capsule thickness might have

294 affected the results of the mechanical testing and in particular for the softer, acellular

295 scaffolds.

2.8 Ultrasound and photo-acoustic imaging

The Vevo LAZR (Visualsonics) system with a LZ550 transducer was used to perform high-frequency ultrasound and photoacoustic imaging for the visualization and quantification of scaffold volumes and oxygen saturation surrounding the implants. The images were acquired 1 week post-surgery and right before sacrifice via excitation of the tissues with high-frequency ultrasound waves for volume monitoring and with non-ionizing laser pulses for blood flow analysis.

Oxy- and deoxy-hemoglobin absorb near infrared light differently, therefore photoacoustic imaging can be used to generate a high-resolution parametric maps of the oxygen saturation of blood in real-time. Total hemoglobin content was visualized by exciting the tissue around the implant with laser pulses at 700nm while the oxygen saturation was assessed using the Oxyhemo mode, a software algorithm that uses a dual wavelength (750 and 850 nm) approach. The volumes of the scaffolds were calculated at each time point by scanning the implant using the 3D mode with a motorized scan stage at a step size of 0.2 mm. The scans were manually processed to identify the scaffold contours and used to reconstruct the scaffold volumes.

The imaging is a non-invasive, painless procedure conducted under anesthesia that lasts no longer than 30 minutes per animal assessments.

2.9 Serum inflammation marker analysis

Mouse blood serum was prepared by clotting the whole blood for 2 hours at RT followed by centrifugation at 3'000 rcf for 20 min at RT. The supernatant was then collected and frozen at -80°C until further analysis. Values were normalized to baseline levels of serum obtained 10 days before surgery.

320   •   Multiplex assay. The serum concentrations of the cytokines IL-1β, IL-4, IL-6, IL-10

321   and TNF-α as well as the C-reactive protein (CRP) were quantified using multiplex

322   immunoassay technology xMAP on a MAGPIX instrument (Luminex) according to

323   manufacturer's instructions. Mouse serum was diluted 1:2 with compatible

324   "Universal Assay Buffer" and measurements were performed with a custom-

325   designed multiplex assay kit (IL-1β - EPX01A-26002-901; IL-4 - EPX01A-20613-

326   901; IL-6 - EPX01A-20603-901; IL-10 - EPX01A-20614-901; TNF-α - EPX01A-

327   20607-901, CRP - EPX01A-26045-901 ProcartaPlex assays; ThermoFisher).

328   Protein levels were measured in technical duplicates for each of the 36 animals.

329   The calibration was performed using a serial dilution of the standard mix provided

330   with the multiplex kit. Data were fitted using a four-parameter logistic regression

331   model. The quantification of the lower limit of detection (LOD) using this system

332   was 2.2, 0.2, 0.2, 0.1, 1 and 2.7 pg/ml for IL-10, IL-1β, IL-4, IL-6 and TNF-α,

333   respectively.

334   •   Enzyme Linked Immunosorbent Assay (ELISA). Pentraxin 2 (PTX 2), also known

335   as Serum Amyloid P (SAP), and Serum Amyloid A (SAA) levels in mouse serum

336   were quantified using Quantikine® ELISA kits (MPTX20 and MSA00 respectively,

337   R&D Systems) according to manufacturer's instructions. For the SAA ELISA assay

338   mouse serum was diluted 1:200. For SAP analysis, serum was diluted 1:400 with

339   the exception of C57BL/6 serum that was diluted 1:100 due to different levels of

340   endogenous SAP.

2.10 Statistical analysis

342   All data are reported as mean ± standard deviation. Statistical analysis was performed

343   with Matlab (Matlab 2017b, MathWorks). Comparison of results was carried out by 2-way

344   analysis of variance (ANOVA) using Tukey's multiple comparison post hoc test for

345   significance. The different mouse strains were compared between each other with

346   Bonferroni post-hoc tests and their statistical significance indicated in the graphs with

347   asterisks (*p<0.05, **p<0.01, ***p<0.001). The F values and the p values of the ANOVAs

348   are reported in the respective figure captions. Significant differences between the cellular

349   conditions were similarly assessed and are reported in the respective result sections.

350

Results

2.1 Macroscopic appearance, volume retention and vascularization

of the scaffolds

354
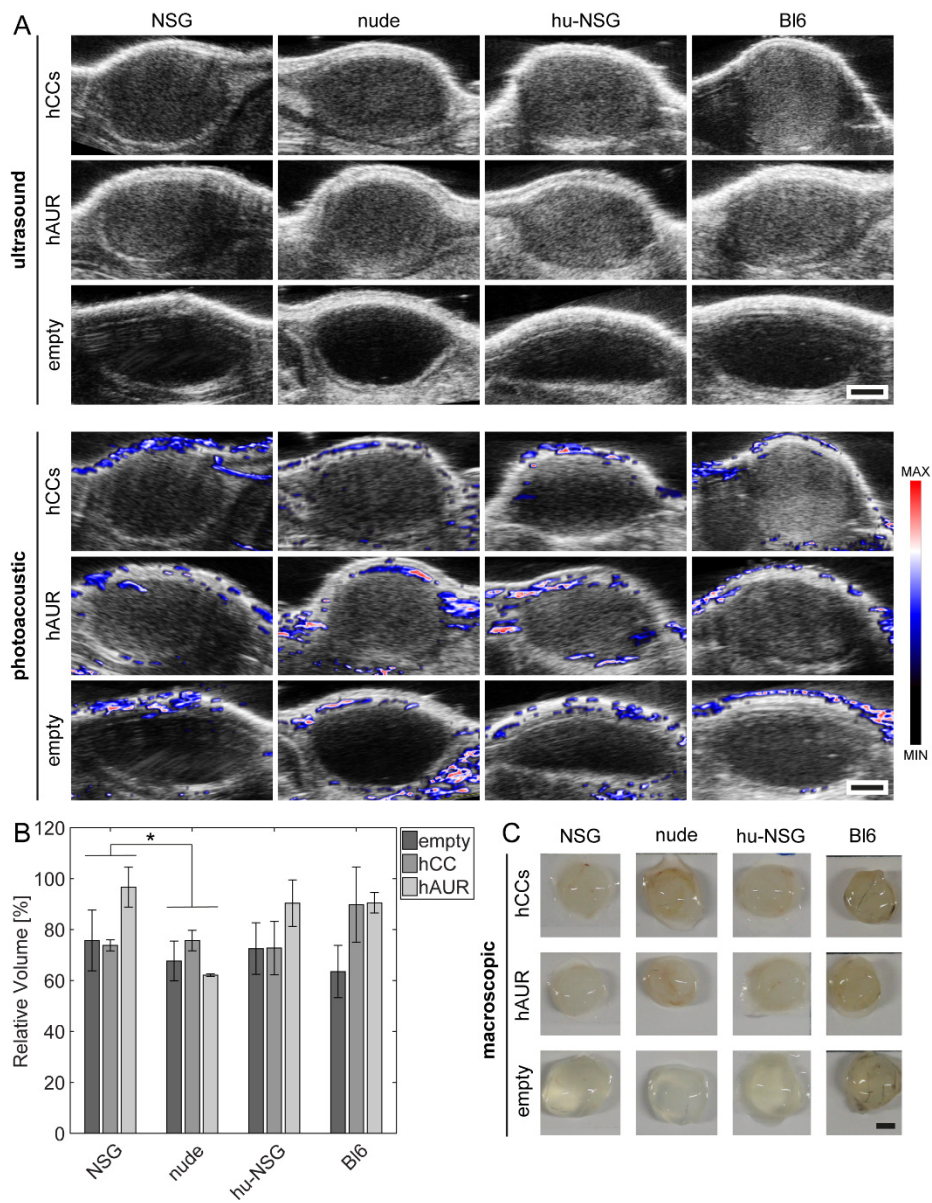
355 *Figure 2 A: Ultrasound and photoacoustic images of HA-TG constructs 4 weeks after implantation. Photoacoustic images*
356 *are depicted as overlay of ultrasound and heat map of oxygen saturation. Scale bar: 1mm. B: Relative volume*
357 *quantification (week 4/ week 1) of ultrasound images. Asterisk (*p<0.05) indicates the statistical differences between*
358 *mouse strains (F(3,24) = 4.576, p=0.011). C: Macroscopic images of HA-TG hydrogels after explantation. Scale bar: 2mm*

359 Ultrasound scans of cell-laden and acellular constructs (*Figure 2A*) revealed the

360 homogenous structure of the constructs with the cellular scaffolds being brighter than the

361 acellular ones, suggesting that those tissues are denser and with a lower fluid content,

362 likely due to their higher collagen content. The oxy-hemo photoacoustic images (*Figure*

363 *2B and Supplementary Figure 1*) of the constructs showed an absence of oxy-hemoglobin

364 inside the scaffolds. On the other hand, the tissue that surrounded them appeared to be

365 highly vascularized. Quantification of the ultrasound images acquired with the device's

366 3D mode showed that the scaffolds retained 60 to 95% of the initial volume. Acellular

367 scaffolds showed a reduction of volume compared to both hCC (*p<0.05) and hAUR

368 (***p<0.001) containing scaffolds. No statistically significant difference in volume

369 reduction was observed between the cellular conditions across mice while there was a

370 significant difference between NSG and nude mice (*p<0.05).

371 Shape retention and lack of vascularization could also be observed from the macroscopic

372 pictures (*Figure 2C*) of the constructs after explantation from mice. No adverse effects or

373 macroscopic signs of toxicity such as necrosis of nearby tissue, edema or hyperemia were

374 observed.

2.2   *Chondrogenesis of hCCs and hAURs in HA-TG hydrogels*

376 Both cellular conditions resulted in high amounts of extracellular matrix (ECM) produced

377 *in vitro* during pre-culture (*Supplementary Figure 3*). Yet, only hAUR were able to maintain

378 and/or produce collagen 2 consistently *in vivo* (*Figure 3*). In hAUR–seeded scaffolds

379 collagen 2 appeared homogeneously distributed throughout the constructs with a dense

380 ring in the outer border of the scaffolds implanted in nude animals. These results were

381   confirmed by semi-quantitative analysis of the staining intensities (hAUR vs. hCC,

382   ***$p < 0.001$).

383   Collagen 1 production was comparable in all mouse strains, while for hCC it was

384   homogeneously distributed through the cross section, for hAUR it was only present in the

385   outer border. The differences in collagen distributions could be due to a different cell

386   response to nutrients, growth factors and oxygen, although more studies will be required

387   to clarify it. The integrated density quantification showed no significant difference

388   between the mouse strains.

389
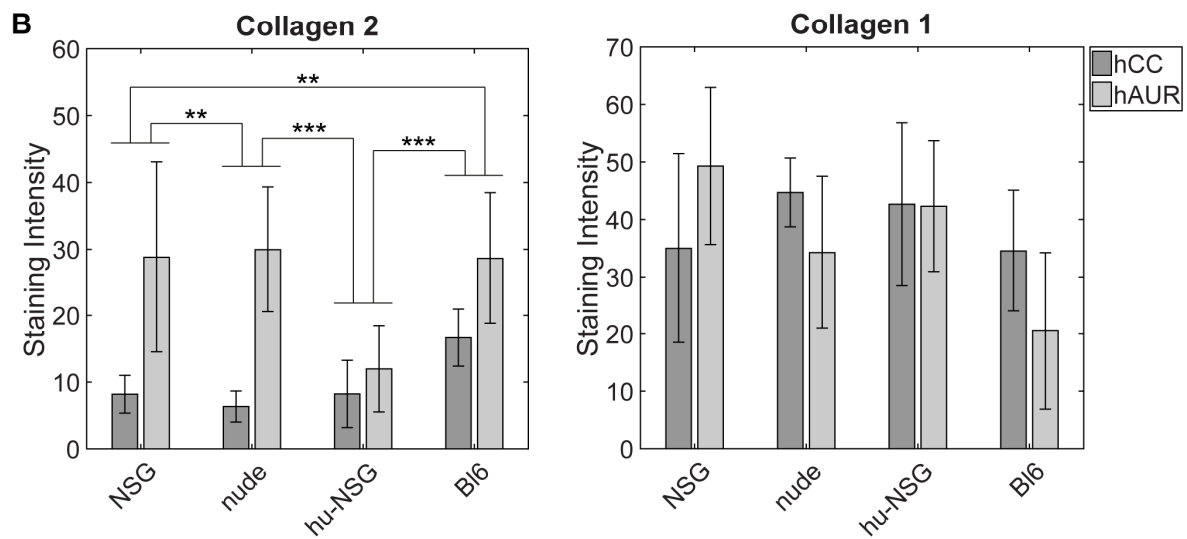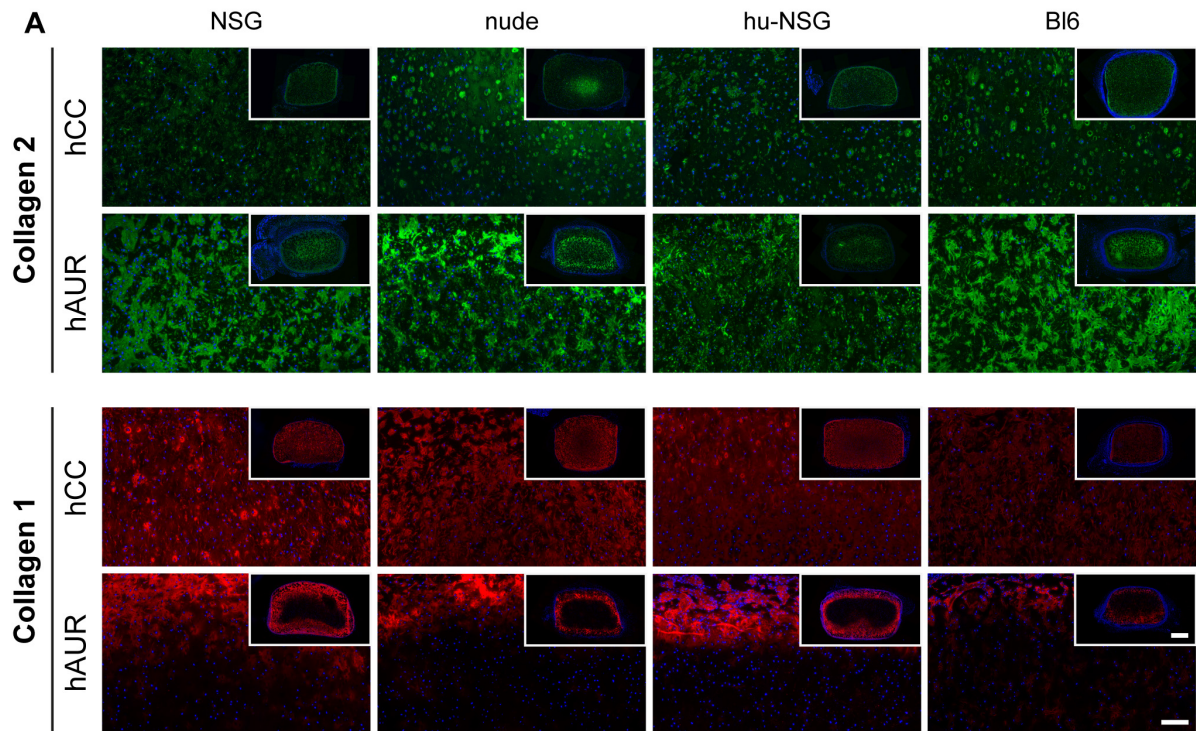
Figure 3 A: Collagen 2 and collagen 1 histological staining with DAPI counterstaining of cell-laden HA-TG hydrogels after
4 weeks of in vitro pre-culture and 4 additional weeks in vivo. Scale bar close up: 100µm, scale bar insert: 500µm. B:
Quantification of collagen stainings. The integrated density of the staining was used for semi-quantitative analysis of
collagen 2 and 1. Asterisks (**p<0.01, ***p<0.001) indicate the statistical differences between mouse strains ($F_{(3,60)}=9.71$,
$p<0.001$ for collagen 2 and $F_{(3,48)}=6.10$, $p=0.001$ for collagen 1).

21

2.3   Mechanical properties of HA-TG scaffolds
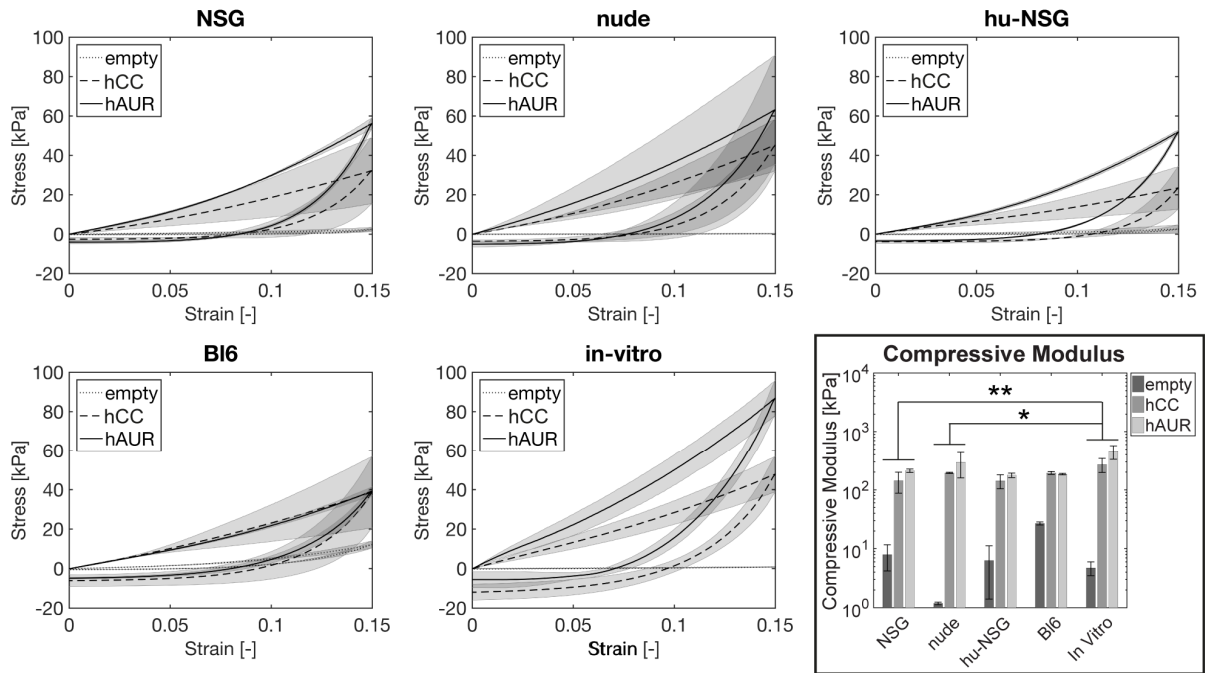
Figure 4 Stress strain curves of compression tests from in vivo samples after explantation and in vitro samples after 49 days. Compressive modulus of cellular and acellular samples corresponding to the depicted stress strain curves. Asterisks (*p<0.05, **p<0.01) indicate the statistical differences between mouse strains and/or in vitro controls (F(4,30)=8.38, p<0.001).

A significant increase in compressive modulus of the constructs compared to the acellular gels was observed as a result of matrix produced by hCCs and hAUR (*Figure 4*). Acellular scaffolds *in vitro* maintained their mechanical strength over 7 weeks (E = 4.7 ± 1.2 kPa) while acellular scaffolds *in vivo* displayed different behavior: samples implanted in NSG and hu-NSG mice maintained their strength (E = 8.4 ± 4.0 kPa, E = 7.0 ± 5.4 kPa, respectively), while samples implanted in nude mice decreased in strength compared to the time of implantation (E = 1.2 ± 0.1 kPa) and samples implanted in C57BL/6 increased in strength (E = 30.4 ± 1.6 kPa). Both cell types led to a significantly higher modulus compared to acellular scaffolds (***p<0.001) and hAUR performed significantly better than hCCs (***p<0.001). All mouse strains led to comparable compressive moduli, with

411   only NSG and nude mice resulting in significantly lower moduli than in vitro controls

412   (**p<0.01 and *p<0.05).

2.4   Inflammation markers

414
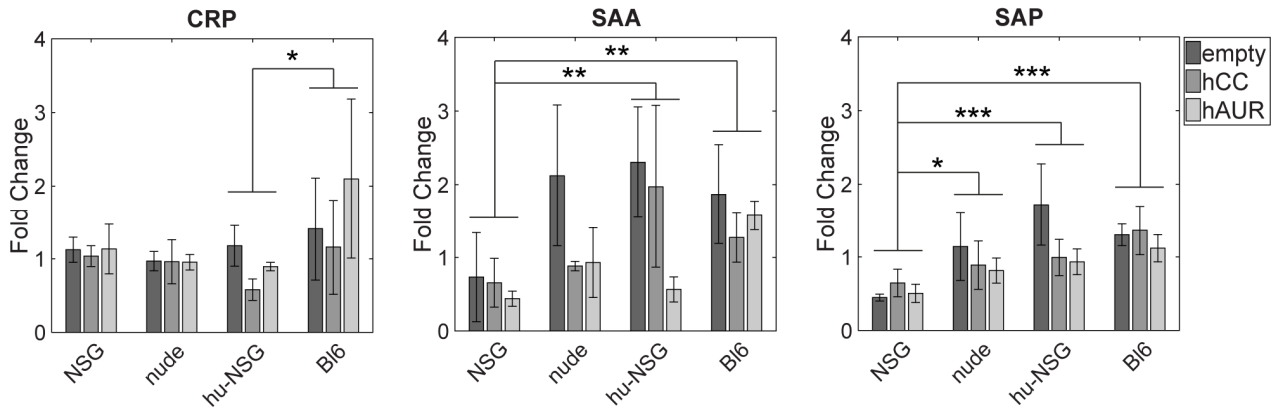
Figure 5 Acute phase inflammation markers CRP, SAA and SAP detected in mouse serum 4 weeks post-implantation and normalized to baseline values acquired 10 days before surgery. Asterisks (*p<0.05, **p<0.01, ***p<0.001) indicate the statistical differences between mouse strains (F(3,24)=3.96, p=0.020 for CRP, F(3,24)=5.73, p=0.004 for SAA, F(3,24)=12.42, p<0.001 for SAP).
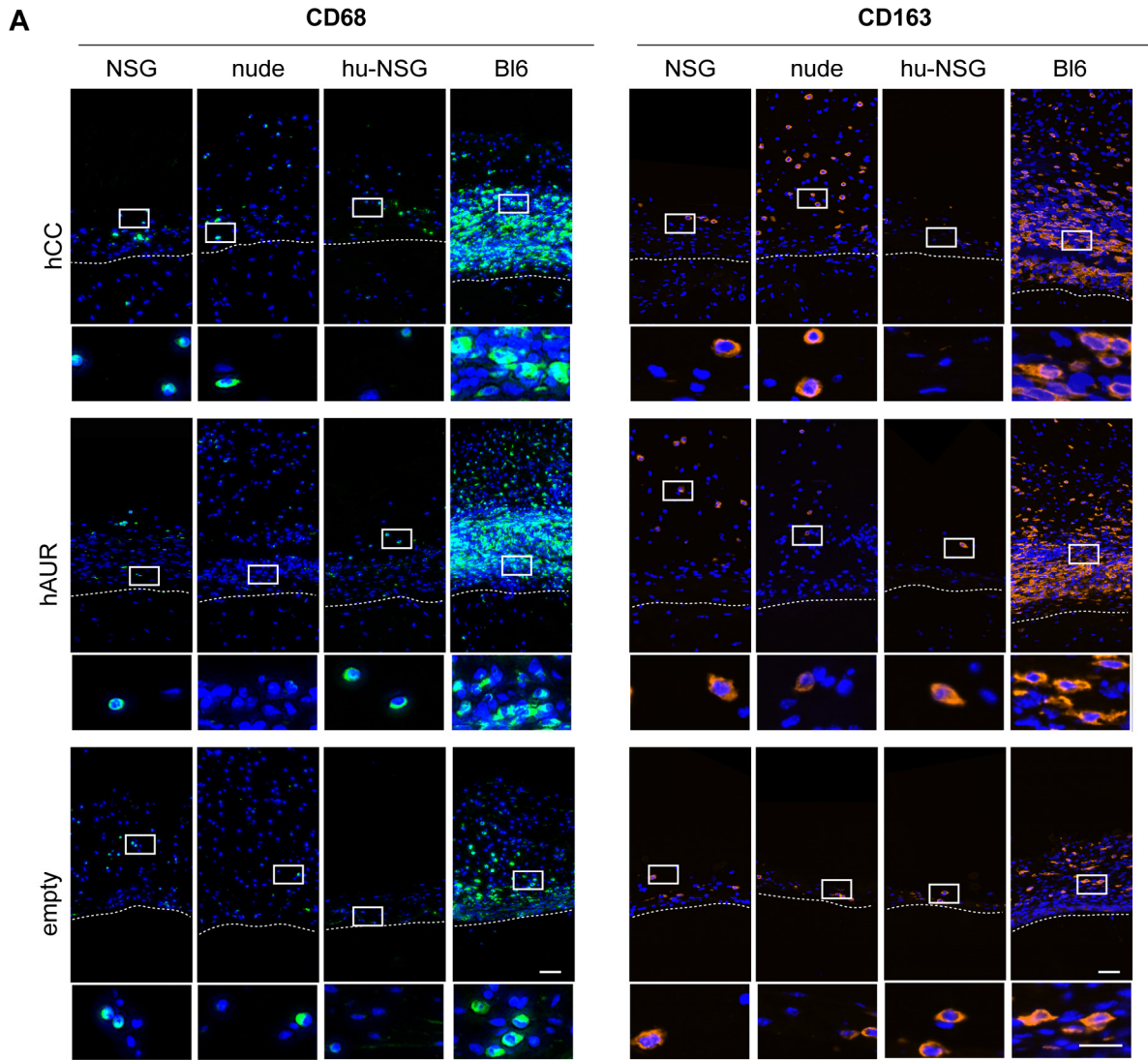
419   CRP, SAA and SAP are acute phase proteins (APP), primarily synthetized by hepatocytes as

420   part of the acute phase response [29]. Although not specific, APP upregulation is triggered by

421   different stimuli including trauma, infection, stress and inflammation with the goal of

422   reestablishing homoeostasis [30, 31]. In mice SAA and SAP are considered major acute

423   phase proteins, meaning that they can be increased 10- to 100-fold. CRP, on the other hand, is

424   a moderate acute phase protein and can be upregulated 2- to 10-fold following a stimuli [29,

425   32].

426   CRP levels in serum (*Figure 5*) at 4 weeks after surgery showed no increase to baseline

427   levels (*Supplementary Table 3*) in NSG, nude and humanized NSG-SGM3 mice. 2.0 ± 1.0

428   and 1.4 ± 0.7 fold increase were observed in C56BL/6 mice for hAUR and acellular

429   scaffolds respectively. No significant differences were observed between the cellular and

430　　acellular conditions across strains and, although hCC seeded scaffolds routinely showed

431　　lower CRP values, they were not significantly different.

432　　SAA levels in serum (*Figure 5*) were significantly higher (**$p<0.01$) in humanized NSG-

433　　SGM3 and C56BL/6 mice (2.2 ± 1.1 for hCC, 0.56 ± 0.2 for hAUR, 2.0 ± 0.8 for acellular and

434　　1.3 ± 0.3 for hCC, 1.6 ± 0.2 for hAUR, 1.8 ± 0.7 for acellular respectively) than NSG (0.7 ±

435　　0.5 for hCC, 0.4 ± 0.1 for hAUR, 0.7 ± 0.6 for acellular). Both cell-loaded scaffolds led to an

436　　overall downregulation of SAA levels compared to acellular scaffolds (with hAUR showing

437　　a significant difference to acellular, **$p<0.01$). SAP levels in serum (*Figure 5*) followed a

438　　similar trend, with the lowest values found in NSG mice (0.9 ± 0.3 for hCC, 0.7 ± 0.2 for

439　　hAUR, 1.3 ± 0.3 for acellular) and the highest in C56BL/6 mice (1.4 ± 0.3 for hCC, 1.1 ± 0.2

440　　for hAUR, 1.3 ± 0.1 for acellular). Similarly, hAUR and hCC led to an overall

441　　downregulation of SAP levels compared to acellular scaffolds (*$p<0.05$ for hAUR vs

442　　acellular) showing that cellular associations have a positive effect on the biomaterial
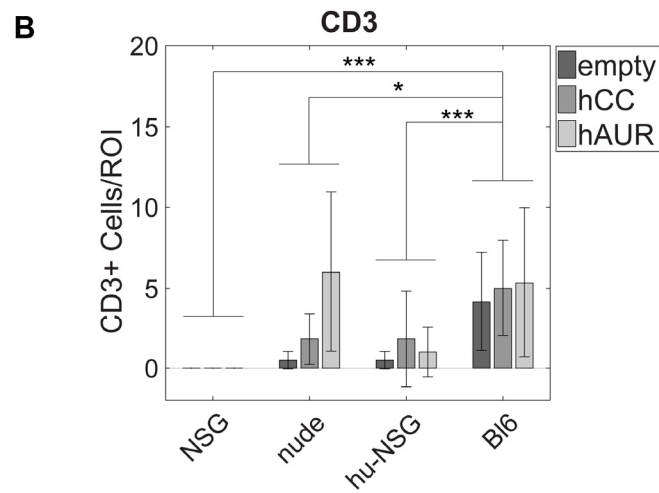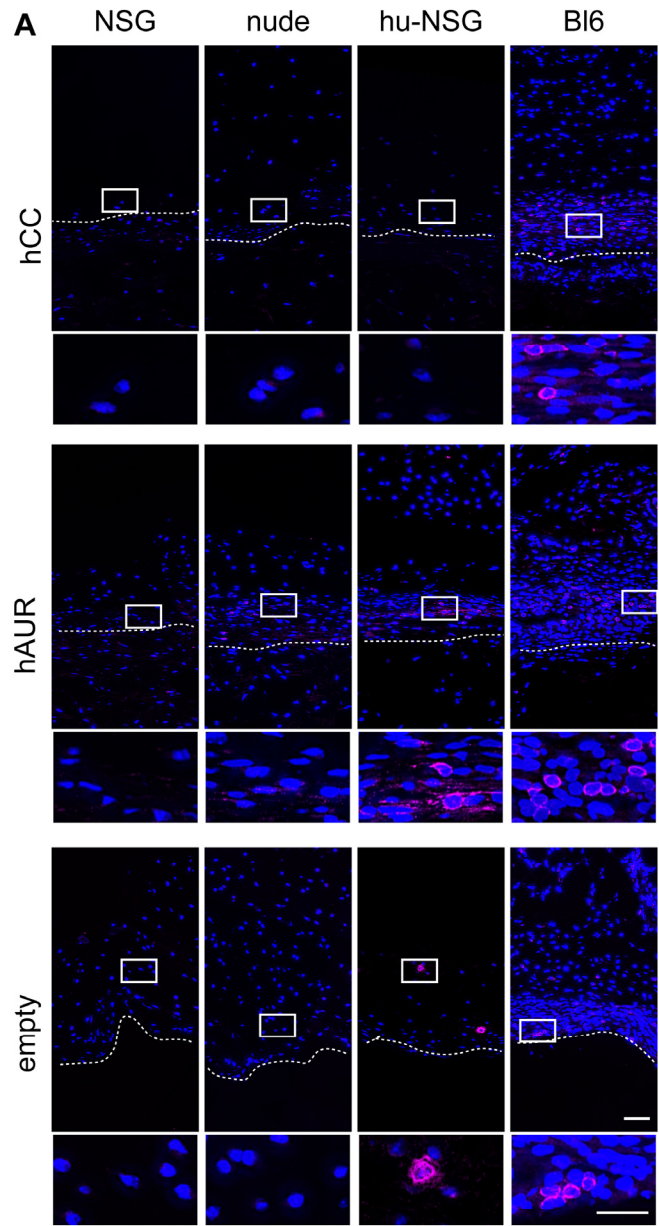
443　　implantation.

2.5   Macrophage presence and infiltration
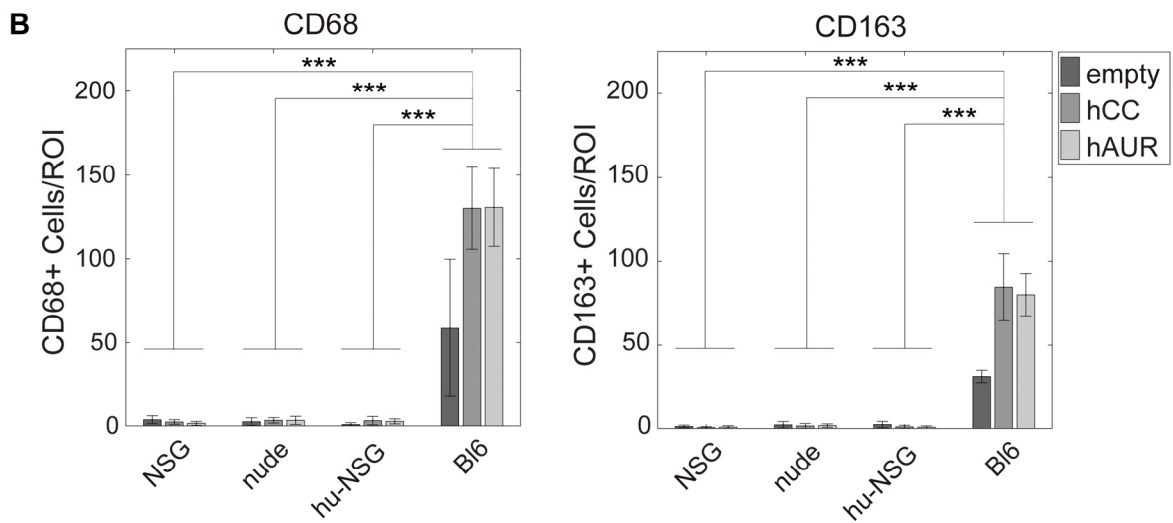
451 CD68 (Cluster of Differentiation 68) and CD163 (Cluster of Differentiation 163) are

452 transmembrane glycoproteins expressed by cells in the monocyte and macrophage

453 lineages [33, 34]. CD68 and CD163 are used to identify macrophages in tissue sections

454 with CD163 positive cells usually considered as M2 (anti-inflammatory) macrophages

455 [35]. A significantly higher (***$p<0.001$) number of both CD68+ and CD163+ cells was

456 observed in the fibrous capsule around the scaffolds implanted in C57BL/6 mice

457 compared to the other mouse strains (*Figure 6*). For both markers, a significantly lower

458 (***$p<0.001$) number of positive cells was found around the acellular scaffolds compared

459 to both hAUR and hCC scaffolds. No macrophage infiltration into samples was observed

460 in any of the conditions but a thicker fibrous capsule was observed in C57BL/6 mice

461 around the scaffolds.

2.6   Lymphocyte presence

The CD3 (Cluster of differentiation 3) is part if the T cell receptor complex of mature T lymphocytes and is used in immunology to identify both the cytotoxic T cells (CD8+) and the T helper cells (CD4+). As expected, no CD3+ cells could be found in the fibrous tissue surrounding the scaffolds implanted in NSG mice, however a limited amount of CD3+ cells could be found in the humanized mouse samples (*Figure 7*). Given that the humanized mice are developed from NSG animals and have relatively high human engraftment, the lymphocytes that were found in the histological sections from samples implanted in humanized animals can be considered from human origin. Despite their lack of thymus, some lymphocytes could be observed in the nude mouse samples. Extrathymic maturation of T cells has been documented in nude mice and an increasing number of CD3+ cells correlates with the mouse age [36]. Not surprisingly, the highest number of CD3+ cells could be identified in C57BL/6 mice (*p<0.05 to nude, *<0.001 to NSG and to hu-NSG).

3. Discussion

Despite the critical role that mouse studies play in providing cost and time effective proof-of-concept data, there is no unanimity on the optimal mouse strain for cartilage regeneration studies [37]. This could be attributed to several reasons. Firstly, there are no regulatory guidelines for conducting cartilage engineering studies in small animal models. Secondly, the use of human cells often limits the choice to immunodeficient animals [38]. Lastly, despite cartilage being considered immune-privileged [39, 40], there is a lack of understanding of how the host immune system affects chondrogenesis *in vivo* [41]. The recent advances in the development of humanized mice has further increased the range of possible mouse strains to choose from. The advancement of humanized mice raised the question of whether those could represent a suitable animal model to bridge the gap between mouse and human studies at least for pre-screening purposes. In this study, we evaluated the chondrogenic potential of xenogenic, tissue-engineered grafts by implanting it subcutaneously in mice that present varying immune systems.

The HA-TG hydrogel system has already proven to be biocompatible, mitogenic and adhesive to cartilage tissue. In addition, it induces cartilaginous matrix deposition by encapsulated human chondroprogenitor cells *in vitro* [22]. In this study, we were able to show that HA-TG supports human auricular chondrocyte survival and extracellular matrix deposition i*n vitro* and *in vivo*. Both cellular and acellular HA-TG hydrogels did not degrade and resisted vascular and cellular infiltration for up to 4 weeks *in vivo*. Importantly, as reflected by the increase in mechanical strength and collagen 2 content, HA-TG hydrogels supported ECM production by both adult (hAUR) and chondroprogenitor (hCC) chondrocytes independently of the mouse strains.

29

505 Furthermore, in hAUR-seeded scaffold, HA-TG induced the synthesis of a collagen 2-rich

506 ECM while keeping low expression levels of collagen 1.

507 Different *in vitro* results were seen with hCC seeded scaffolds as high levels of collagen 2

508 were not maintained *in vivo*. The differences of hCC to preserve the matrix that was

509 produced during *in vitro* preculture could be due to several factors. The collagen 2

510 produced *in vitro* might not be sufficiently mature and crosslinked [42] and therefore

511 diffuse out of the hydrogel once *in vivo,* however mechanical testing would suggest

512 otherwise. hCC have been shown to undergo a certain degree of spontaneous

513 differentiation (i.e. without stimulation by growth factors) towards the chondrogenic

514 lineage [24], however their chondrogenic potential can be impaired in the absence of TGF-

515 β growth factors (data not shown). On the other hand, hAURs were fully differentiated

516 before implantation and therefore had a more stable phenotype and did not required

517 exogenous supplementation of TGF-β. No obvious inflammatory infiltrates or foreign-body

518 reactions were observed at histological examination but host cytokines and the

519 surrounding subcutaneous environment could contribute to cell death and/or phenotype

520 instability [43]. Although it remains unclear whether the same cells would make durable

521 cartilage tissue when implanted into a cartilage-inducing/maintaining environment, such

522 as an articular cartilage defect, ex-vivo studies showed promising results [22]. Moreover,

523 there are differences for the two investigated cell types as they have different origins

524 during embryonic development [44]. While articular chondrocytes develop from the

525 mesoderm, auricular chondrocytes originate from the neural crest. Cells from the neural

526 crest have the capacity to generate various cell and tissue types even across germ layers

527 [45] and are known for their multipotency and ability to undergo chondrogenic

528 differentiation in heterotopic transplantation sites [46]. Therefore, the two cell types have

529   a different stability depending on the environment and mechanical/biochemical

530   stimulation that they will receive upon transplantation.

531   C57BL/6 mice have complete innate and adaptive immune systems. Not surprisingly,

532   systemic inflammation markers (CRP, SAA and SAP: *Figure 5;* IL6, IL10 and TNF-α:

533   *Supplementary Figure 6*) were consistently upregulated in C57BL/6 animals and a higher

534   number of macrophages and lymphocytes (*Figure 6* and *Figure 7* respectively), together

535   with a larger fibrous capsule, was found around the implants in comparison to

536   immunodeficient and humanized mice. In addition to their phagocytic properties,

537   macrophages actively regulate tissue repair by secreting various cytokines, growth

538   factors, ECM components and proteases [47]. HA-TG is synthetized from high molecular

539   weight hyaluronic acid (HA) which has been documented as antiangiogenic, antioxidant

540   as well as anti-inflammatory [48]. A CD163 macrophage-mediated reaction to high

541   molecular weight HA might have reduced inflammation and promoted chondrogenesis in

542   immunocompetent animals, as indicated by the higher collagen 2 production observed in

543   this strain [49]. Another factor that could have played a role is the thicker fibrous capsule

544   which might have produced a more hypoxic environment compared to the capsules

545   observed in the immunodeficient and humanized mice and therefore led to a stronger

546   chondrogenesis [50]. The stronger innate immune response observed in the C57BL/6

547   mice, compared to humanized mice, could also be due to the fact that the former is a

548   xenogeneic reaction, while the latter is allogeneic. Indeed, xenogeneic transplantation of

549   human chondrocytes have been reported to be rejected by minipigs [51] and rabbits [39] and

550   the transplanted cells failed to undergo chondrogenesis in vivo despite allogenic transplantation

551   could be successfully performed [39].

552 Previous studies reported mixed outcomes of ectopic cartilage reconstruction and bone

553 regeneration in immunocompetent small animals [38, 52-55]. Failures in chondrogenesis

554 and osteogenesis were attributed to immune reaction of the host to the allogenic

555 materials and cells, but also to vascular invasion that cause construct degradation and

556 host tissue ingrowth [56]. The absence of T-cells in nude animals was suspected to be the

557 reason for reduced cytotoxic mechanisms associated with macrophages and soluble

558 factors, including complements and antibodies [57]. HA-TG resistance to cellular and

559 vascular infiltration could explain why chondrogenesis of hAUR in immunocompetent

560 C57BL/6 animals was comparable to that of in immunodeficient mice. In addition, HA-TG

561 is a naturally derived material which is degraded by MMPs and hydrolytic enzymes in low

562 molecular weight hyaluronan molecules [58]. The non-toxicity of its degradation products

563 exclude another common reason of chondrogenic failure [59].

564 This study provides an insight on the role of the murine immune system in subcutaneous

565 implantation of cartilage engineering scaffolds. Overall, we observed a similar trend in the

566 development of the scaffolds in all four mouse strains in terms of mechanical

567 strengthening of the grafts and ECM expression. Our results suggest that despite being

568 subjected to a functional immune system, chondrogenesis still occurs in samples

569 implanted in C57BL/6 and that, despite being subjected to a humanized immune system

570 in NSG-SGM3, chondrogenesis still occurs albeit not as strongly as in the other mouse

571 strains. Nevertheless there are some limitations to our study. Even though our results

572 were reproducible among the biological replicates, studies with larger sample size would

573 be needed for validation. The inflammation markers were analyzed by multiplex assay on

574 serum whereby several cytokines were below the lower limit of detection and therefore

575 a subcutaneous tissue cage model [60] utilizing the analysis of exudate could be better

576 suited for this purpose. Finally, more research is required to develop reliable humanized

577 mice (i.e. with a stronger humanized innate immune response and in particular with an

578 increased number of macrophages and natural killer cells) and to systematically evaluate

579 how mouse studies translate into large animal models and humans.

4. Conclusions

581 To our knowledge, this is the first study that compared the chondrogenic potential of a

582 tissue-engineered construct for cartilage regeneration in several mouse strains. In

583 addition, this is the earliest attempt to use a humanized mouse model in the cartilage

584 engineering field. Collectively the results of this study suggest that chondrogenesis of a

585 tissue-engineered cartilage graft is feasible in immunocompetent small animals. We

586 suggest that immunocompetent and immunodeficient animals could lead to analogous

587 results in terms of chondrogenesis, as long as the implanted cells are shielded from the

588 host by a biomaterial. Preclinical investigation in immunocompetent animals is an

589 essential step in the clinical translation of orthopedic scaffolds [59, 61] and

590 immunodeficient rodent models are still the model of choice for early testing procedures

591 and screening. Subcutaneous implantation in small animals with a complete and human

592 immune system could help understanding how tissue-engineered constructs perform

593 with an active immune system.

5. Acknowledgments

6. Author contributions

604 This study was designed by E.C., P.F., R.G. and M.Z.W.; acquisition of all data was conducted

605 by E.C., P.F and. F.A.F.; animal experiments were performed by E.C., P.F. and M.Z.W.; data

606 analysis was performed by E.C., P.F and F.A.F.. All authors were involved in interpreting

607 the data, drafting the article, revising the manuscript for important intellectual content,

608 and approved the final version to be submitted.

7. Disclosure Statement

610 Marcy Zenobi-Wong serves as a consultant for Auregen S.A. . Ralph Gareus is an employee

611 of The Jackson Laboratory.

612

8. References

1. Piedrahita, J.A. and J.K. Williams, *Animal Models in Tissue Engineering. Part I.* Tissue Eng Part C Methods, 2017. **23**(11): p. 641-642.

2. Cook, J.L., et al., *Animal models of cartilage repair.* Bone Joint Res, 2014. **3**(4): p. 89-94.

3. Makris, E.A., et al., *Repair and tissue engineering techniques for articular cartilage.* Nature Reviews Rheumatology, 2015. **11**(1): p. 21-34.

4. Ha, C.W., et al., *Initial phase I safety of retrovirally transduced human chondrocytes expressing transforming growth factor-beta-1 in degenerative arthritis patients.* Cytotherapy, 2012. **14**(2): p. 247-256.

5. Lee, M.C., et al., *A placebo-controlled randomised trial to assess the effect of TGF-beta 1-expressing chondrocytes in patients with arthritis of the knee (vol 97, pg 924, 2015).* Bone & Joint Journal, 2015. **97b**(12): p. 1732-1732.

6. Cherian, J.J., et al., *Preliminary results of a phase II randomized study to determine the efficacy and safety of genetically engineered allogeneic human chondrocytes expressing TGF-beta 1 in patients with grade 3 chronic degenerative joint disease of the knee.* Osteoarthritis and Cartilage, 2015. **23**(12): p. 2109-2118.

7. Farr, J. and J.Q. Yao, *Chondral Defect Repair with Particulated Juvenile Cartilage Allograft.* Cartilage, 2011. **2**(4): p. 346-53.

8. Buckwalter, J.A., et al., *Clinical outcomes of patellar chondral lesions treated with juvenile particulated cartilage allografts.* Iowa Orthop J, 2014. **34**: p. 44-9.

9. Bugbee, W.D., et al., *Osteochondral allograft transplantation in cartilage repair: Graft storage paradigm, translational models, and clinical applications.* J Orthop Res, 2016. **34**(1): p. 31-8.

10. Whitelaw, C.B., et al., *Engineering large animal models of human disease.* J Pathol, 2016. **238**(2): p. 247-56.

11. Chu, C.R., M. Szczodry, and S. Bruno, *Animal models for cartilage regeneration and repair.* Tissue Eng Part B Rev, 2010. **16**(1): p. 105-15.

12. Reinholz, G.G., et al., *Animal models for cartilage reconstruction.* Biomaterials, 2004. **25**(9): p. 1511-21.

13. Segre, J.A., et al., *Positional cloning of the nude locus: genetic, physical, and transcription maps of the region and mutations in the mouse and rat.* Genomics, 1995. **28**(3): p. 549-59.

14. Shultz, L.D., et al., *Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells.* J Immunol, 2005. **174**(10): p. 6477-89.

15. Shultz, L.D., F. Ishikawa, and D.L. Greiner, *Humanized mice in translational biomedical research.* Nat Rev Immunol, 2007. **7**(2): p. 118-30.

16. Kitchen, S.G., et al., *In Vivo Suppression of HIV by Antigen Specific T Cells Derived from Engineered Hematopoietic Stem Cells.* Plos Pathogens, 2012. **8**(4).

653   17.   McDermott, S.P., et al., *Comparison of human cord blood engraftment between immunocompromised mouse strains.* Blood, 2010. **116**(2): p. 193-200.

655   18.   Billerbeck, E., et al., *Development of human CD4+FoxP3+ regulatory T cells in human stem cell factor-, granulocyte-macrophage colony-stimulating factor-, and interleukin-3-expressing NOD-SCID IL2Rgamma(null) humanized mice.* Blood, 2011. **117**(11): p. 3076-86.

659   19.   Walsh, N.C., et al., *Humanized Mouse Models of Clinical Disease.* Annual Review of Pathology: Mechanisms of Disease, Vol 12, 2017. **12**: p. 187-215.

661   20.   Ito, R., et al., *Current advances in humanized mouse models.* Cellular & Molecular Immunology, 2012. **9**(3): p. 208-214.

663   21.   Carrillo, M.A., A.J. Zhen, and S.G. Kitchen, *The Use of the Humanized Mouse Model in Gene Therapy and Immunotherapy for HIV and Cancer.* Frontiers in Immunology, 2018. **9**.

666   22.   Broguiere, N., et al., *Factor XIII Cross-Linked Hyaluronan Hydrogels for Cartilage Tissue Engineering.* ACS Biomaterials Science & Engineering, 2016. **2**(12): p. 2176-2184.

669   23.   Broguiere, N., L. Isenmann, and M. Zenobi-Wong, *Novel enzymatically cross-linked hyaluronan hydrogels support the formation of 3D neuronal networks.* Biomaterials, 2016. **99**: p. 47-55.

672   24.   Darwiche, S., et al., *Epiphyseal Chondroprogenitors Provide a Stable Cell Source for Cartilage Cell Therapy.* Cell Med, 2012. **4**(1): p. 23-32.

674   25.   Studer, D., et al., *Human chondroprogenitors in alginate-collagen hybrid scaffolds produce stable cartilage in vivo.* J Tissue Eng Regen Med, 2017. **11**(11): p. 3014-3026.

677   26.   Adkisson, H.D.t., et al., *The potential of human allogeneic juvenile chondrocytes for restoration of articular cartilage.* Am J Sports Med, 2010. **38**(7): p. 1324-33.

679   27.   Bell, S.P., et al., *Reduced Subendocardial Perfusion Correlates with Impaired Energy Supply-Demand Relations in Patients with Non-Ischemic Dilated Cardiomyopathy.* Circulation, 2010. **122**(21).

682   28.   Snider, T.N. and Y. Mishina, *Cranial neural crest cell contribution to craniofacial formation, pathology, and future directions in tissue engineering.* Birth Defects Res C Embryo Today, 2014. **102**(3): p. 324-32.

685   29.   Cray, C., J. Zaias, and N.H. Altman, *Acute phase response in animals: a review.* Comp Med, 2009. **59**(6): p. 517-26.

687   30.   Murata, H., N. Shimada, and M. Yoshioka, *Current research on acute phase proteins in veterinary diagnosis: an overview.* Vet J, 2004. **168**(1): p. 28-40.

689   31.   Petersen, H.H., J.P. Nielsen, and P.M. Heegaard, *Application of acute phase protein measurements in veterinary clinical chemistry.* Vet Res, 2004. **35**(2): p. 163-87.

691   32.   Ceron, J.J., P.D. Eckersall, and S. Martinez-Subiela, *Acute phase proteins in dogs and cats: current knowledge and future perspectives.* Veterinary Clinical Pathology, 2005. **34**(2): p. 85-99.

694 33. Holness, C.L. and D.L. Simmons, *Molecular cloning of CD68, a human macrophage*
695 *marker related to lysosomal glycoproteins.* Blood, 1993. **81**(6): p. 1607-13.

696 34. Lau, S.K., P.G. Chu, and L.M. Weiss, *CD163: a specific marker of macrophages in*
697 *paraffin-embedded tissue samples.* Am J Clin Pathol, 2004. **122**(5): p. 794-801.

698 35. Barros, M.H., et al., *Macrophage polarisation: an immunohistochemical approach for*
699 *identifying M1 and M2 macrophages.* PLoS One, 2013. **8**(11): p. e80908.

700 36. Kennedy, J.D., C.W. Pierce, and J.P. Lake, *Extrathymic T-Cell Maturation - Phenotypic*
701 *Analysis of T-Cell Subsets in Nude-Mice as a Function of Age.* Journal of Immunology,
702 1992. **148**(6): p. 1620-1629.

703 37. Moran, C.J., et al., *The benefits and limitations of animal models for translational*
704 *research in cartilage repair.* J Exp Orthop, 2016. **3**(1): p. 1.

705 38. Pomerantseva, I., et al., *Ear-Shaped Stable Auricular Cartilage Engineered from*
706 *Extensively Expanded Chondrocytes in an Immunocompetent Experimental Animal*
707 *Model.* Tissue Eng Part A, 2016. **22**(3-4): p. 197-207.

708 39. Arzi, B., et al., *Cartilage immunoprivilege depends on donor source and lesion*
709 *location.* Acta Biomater, 2015. **23**: p. 72-81.

710 40. Revell, C.M. and K.A. Athanasiou, *Success rates and immunologic responses of*
711 *autogenic, allogenic, and xenogenic treatments to repair articular cartilage defects.*
712 Tissue Eng Part B Rev, 2009. **15**(1): p. 1-15.

713 41. Bolano, L. and J.A. Kopta, *The Immunology of Bone and Cartilage Transplantation.*
714 Orthopedics, 1991. **14**(9): p. 987-996.

715 42. Athens, A.A., E.A. Makris, and J.C. Hu, *Induced collagen cross-links enhance cartilage*
716 *integration.* PLoS One, 2013. **8**(4): p. e60719.

717 43. De Bari, C., F. Dell'Accio, and F.P. Luyten, *Failure of in vitro-differentiated*
718 *mesenchymal stem cells from the synovial membrane to form ectopic stable cartilage*
719 *in vivo.* Arthritis Rheum, 2004. **50**(1): p. 142-50.

720 44. Pelttari, K., et al., *Nasal chondrocytes as a neural crest-derived cell source for*
721 *regenerative medicine.* Current Opinion in Biotechnology, 2017. **47**: p. 1-6.

722 45. Baggiolini, A., et al., *Premigratory and migratory neural crest cells are multipotent*
723 *in vivo.* Cell Stem Cell, 2015. **16**(3): p. 314-22.

724 46. Le Douarin, N.M., et al., *Neural crest cell plasticity and its limits.* Development, 2004.
725 **131**(19): p. 4637-50.

726 47. Wynn, T.A. and K.M. Vannella, *Macrophages in Tissue Repair, Regeneration, and*
727 *Fibrosis.* Immunity, 2016. **44**(3): p. 450-462.

728 48. Masuko, K., et al., *Anti-inflammatory effects of hyaluronan in arthritis therapy: Not*
729 *just for viscosity.* International Journal of General Medicine, 2009. **2**: p. 77-81.

730 49. Julier, Z., et al., *Promoting tissue regeneration by modulating the immune system.*
731 Acta Biomaterialia, 2017. **53**: p. 13-28.

732 50. Meretoja, V.V., et al., *The effect of hypoxia on the chondrogenic differentiation of co-*
733 *cultured articular chondrocytes and mesenchymal stem cells in scaffolds.*
734 Biomaterials, 2013. **34**(17): p. 4266-73.

735 51. Niemietz, T., et al., *Xenogeneic transplantation of articular chondrocytes into full-
736   thickness articular cartilage defects in minipigs: fate of cells and the role of
737   macrophages.* Cell Tissue Res, 2014. **358**(3): p. 749-61.

738 52. Britt, J.C. and S.S. Park, *Autogenous tissue-engineered cartilage - Evaluation as an
739   implant material.* Archives of Otolaryngology-Head & Neck Surgery, 1998. **124**(6):
740   p. 671-677.

741 53. Shieh, S.J., S. Terada, and J.P. Vacanti, *Tissue engineering auricular reconstruction:
742   in vitro and in vivo studies.* Biomaterials, 2004. **25**(9): p. 1545-1557.

743 54. Cao, Y.L., et al., *Comparative study of the use of poly(glycolic acid), calcium alginate
744   and pluronics in the engineering of autologous porcine cartilage.* Journal of
745   Biomaterials Science-Polymer Edition, 1998. **9**(5): p. 475-487.

746 55. Zhang, Y., et al., *Comparing immunocompetent and immunodeficient mice as animal
747   models for bone tissue engineering.* Oral Diseases, 2015. **21**(5): p. 583-592.

748 56. Zheng, L., et al., *In Vivo Cartilage Engineering with Collagen Hydrogel and
749   Allogenous Chondrocytes After Diffusion Chamber Implantation in
750   Immunocompetent Host.* Tissue Engineering Part A, 2009. **15**(8): p. 2145-2153.

751 57. Kanazawa, S., et al., *Tissue responses against tissue-engineered cartilage consisting
752   of chondrocytes encapsulated within non-absorbable hydrogel.* Journal of Tissue
753   Engineering and Regenerative Medicine, 2013. **7**(1): p. 1-9.

754 58. Necas, J., et al., *Hyaluronic acid (hyaluronan): a review.* Veterinarni Medicina, 2008.
755   **53**(8): p. 397-411.

756 59. Liu, W. and Y.L. Cao, *Application of scaffold materials in tissue reconstruction in
757   immunocompetent mammals: Our experience and future requirements.*
758   Biomaterials, 2007. **28**(34): p. 5078-5086.

759 60. Chisholm, G.D., *The tissue cage model in the distribution of antibacterial agents.*
760   Scand J Infect Dis Suppl, 1978(14): p. 118-24.

761 61. Liu, Y., G.D. Zhou, and Y.L. Cao, *Recent Progress in Cartilage Tissue Engineering-Our
762   Experience and Future Directions.* Engineering, 2017. **3**(1): p. 28-35.

763

764

Supplementary information

766 *Table 1 Flow cytometry quantification of human cell engraftment in NSG-SGM3. Data were obtained 12 weeks after birth*
767 *(8 weeks after engraftment).*

hCD45+ [total %]	hCD19+ (B cells) [% of hCD45]	hCD3+ (T cells) [% of hCD45]	hCD33+ (myeloid) [% of hCD45]	hCD45- [total %]
28 ±2.4	60.5±16.4	19.9±20.3	12.1±4.3	67.3±14.4

768

769 *Table 2 HLA typing of the human donor used to engraft the humanized mice in the study, of the human chondroprogenitor*
770 *cells (hCCs) and the human auricular chondrocytes (hAUR)*

Human engraftment donor for hu-NSG (female)					
A*01:01	A*30:02	B*08:01	B*08:01	C*07:01	C*07:01
DRB1*03:01	DRB1*03:01	DRB3*01:01	DRB3*02:02		
DQB1*02:01	DQB1*02:01	DQA1*05:01	DQA1*05:01		
DPB1*01:01	DPB1*130:01	DPA1*01:03	DPA1*02:01		

hCC (male)					
A*03	A*30	B*35	B*55	C*03	C*04
DRB1*12	DRB1*14	DRB3*02			
DQB1*03	DQB1*05	DQA1*01	DQA1*05		
DPB1*02	DPB1*04	DPA1*01	DPA1*01		

| hAUR (male)					
A*01	A*26	B*18	B*38	C*07	C*12
DRB1*04	DRB1*11	DRB3*02	DRB4*01		
DQB1*03	DQB1*03	DQA1*03	DQA1*05		
DPB1*04	DPB1*04	DPA1*01	DPA1*01		

771

772
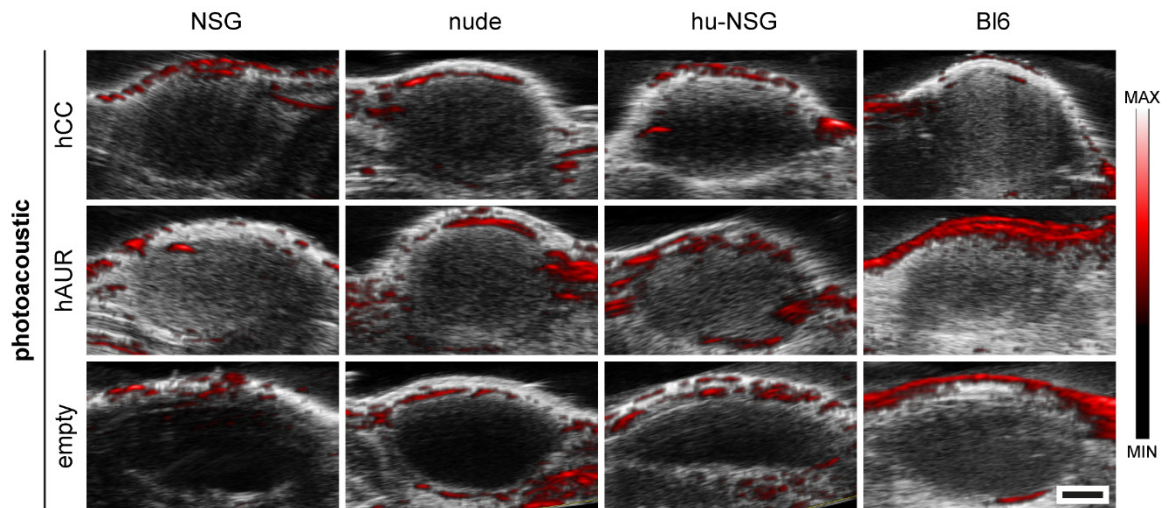
773

774 *Supplementary Figure 1 Photoacoustic images of HA-TG constructs at 4 weeks after implantation. Photoacoustic images*
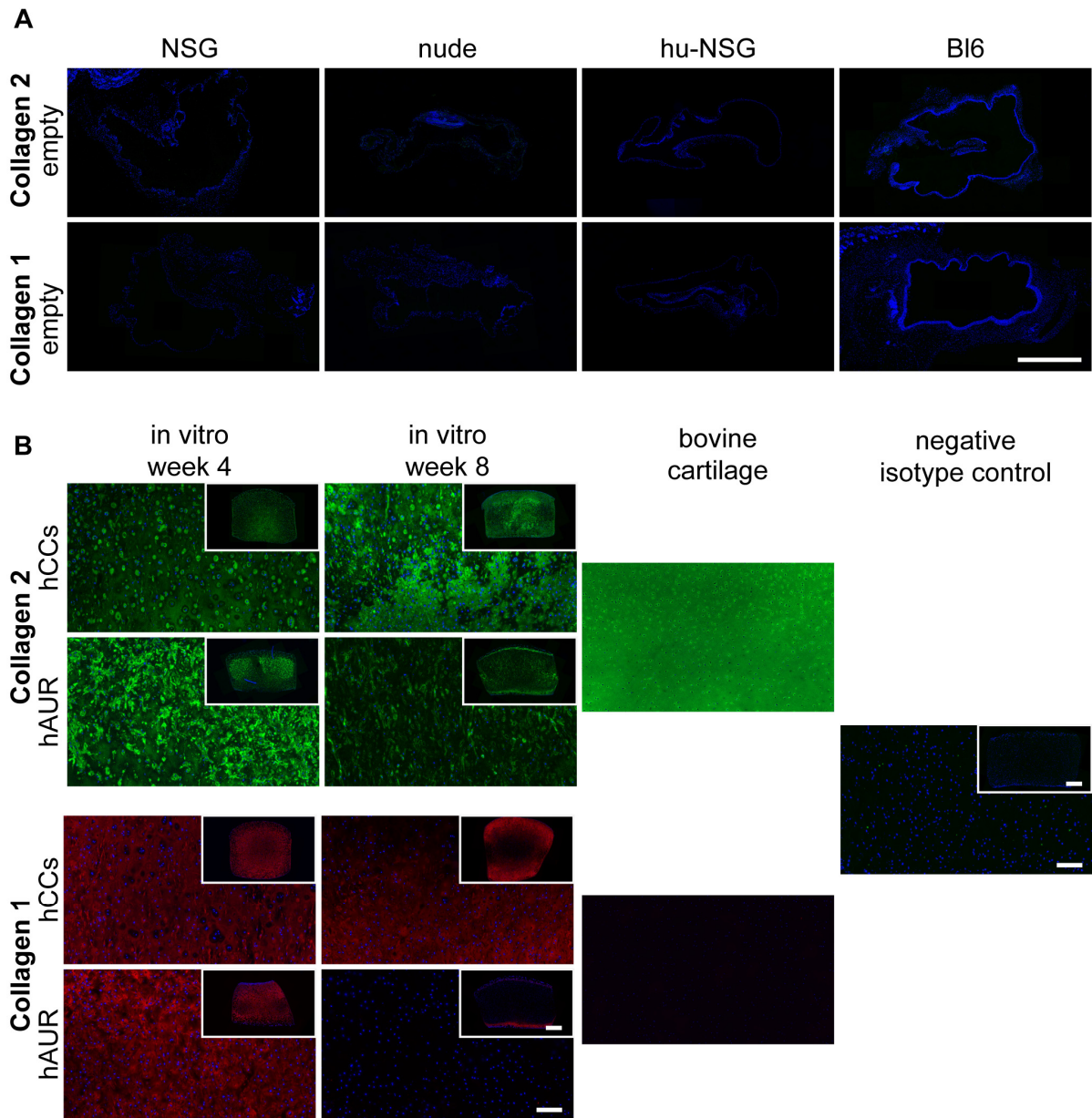775 *are depicted as overlay of ultrasound and heat map of oxyhemoglobin saturation (λ=700nm). Scale bar: 1mm.*

776

777
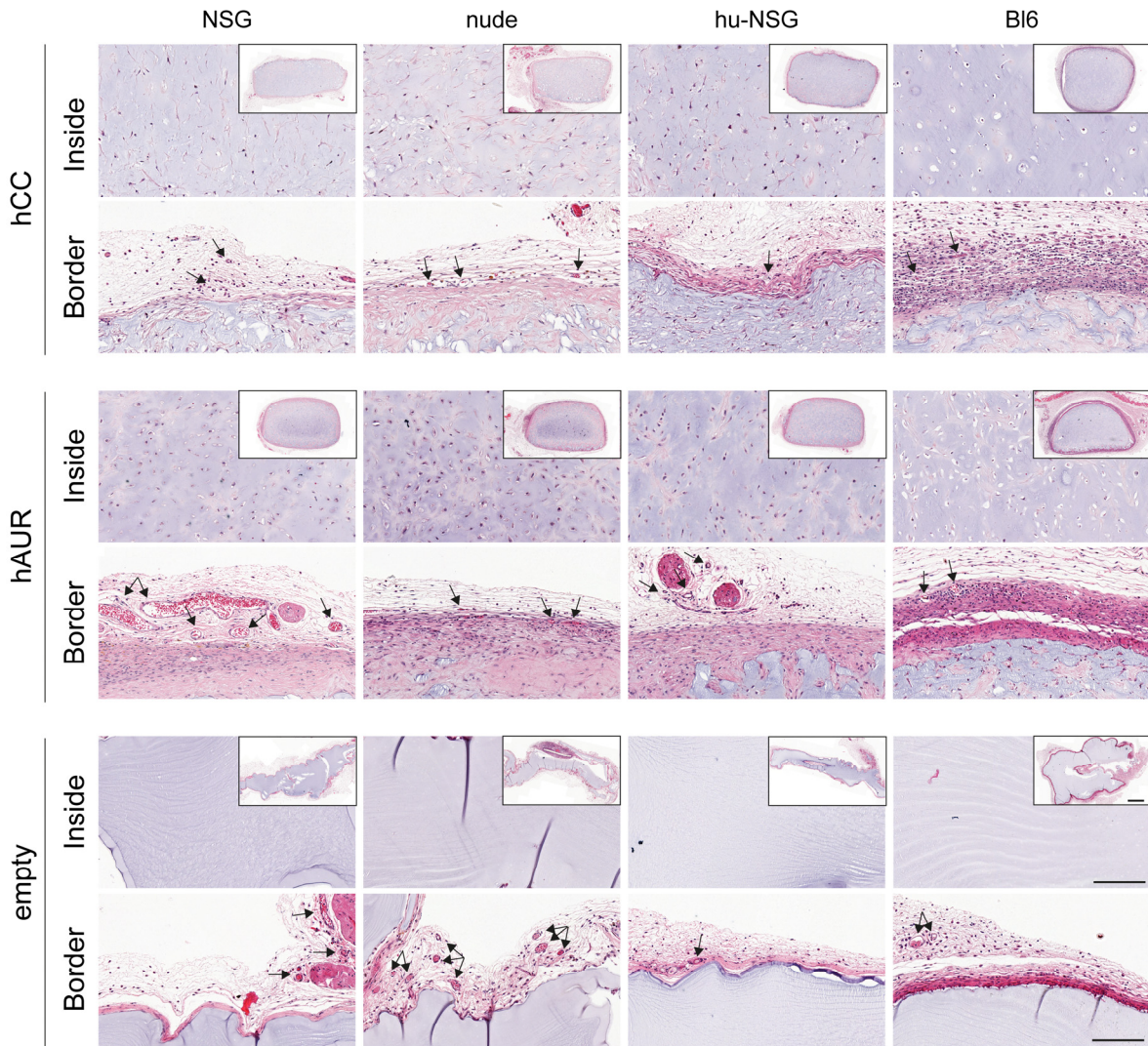
Supplementary Figure 2 Hematoxylin and eosin (H&E) staining of HA-TG hydrogels after 4 weeks of in vitro preculture and 4 additional weeks in vivo. Representative images of hydrogel inner region (inside) and outer border with fibrous capsule (border). Arrows point to visible blood vessels. Scale bar close up: 500µm, scale bar insert: 100µm.

778
779
780

781

A

	NSG	nude	hu-NSG	Bl6
Collagen 2 empty				
Collagen 1 empty				

B

|	in vitro week 4	in vitro week 8	bovine cartilage	negative isotype control
Collagen 2 hCCs				
Collagen 2 hAUR				
Collagen 1 hCCs				
Collagen 1 hAUR				

782

783 *Supplementary Figure 3 A: Collagen 2 and 1 immunohistochemical staining of acellular HA-TG hydrogels after 4 weeks of*
784 *in vitro preculture and 4 additional weeks in vivo. B: Collagen 2 and 1 staining of HA-TG hydrogels after 4 and 8 weeks in*
785 *vitro and HA-TG background staining. Bovine cartilage and IgG isotype antibody were used as controls. Scale bar close up:*
786 *500μm, scale bar insert: 100μm.*

787
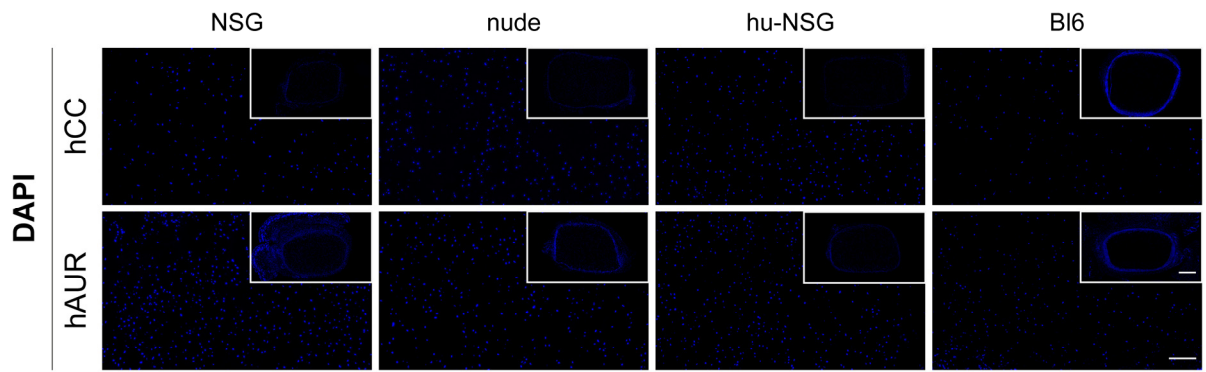
Supplementary Figure 4 DAPI staining of HA-TG hydrogels after 4 weeks of in vitro preculture and 4 additional weeks in vivo. Scale bar close up: 500µm, scale bar insert: 100µm.

790

791
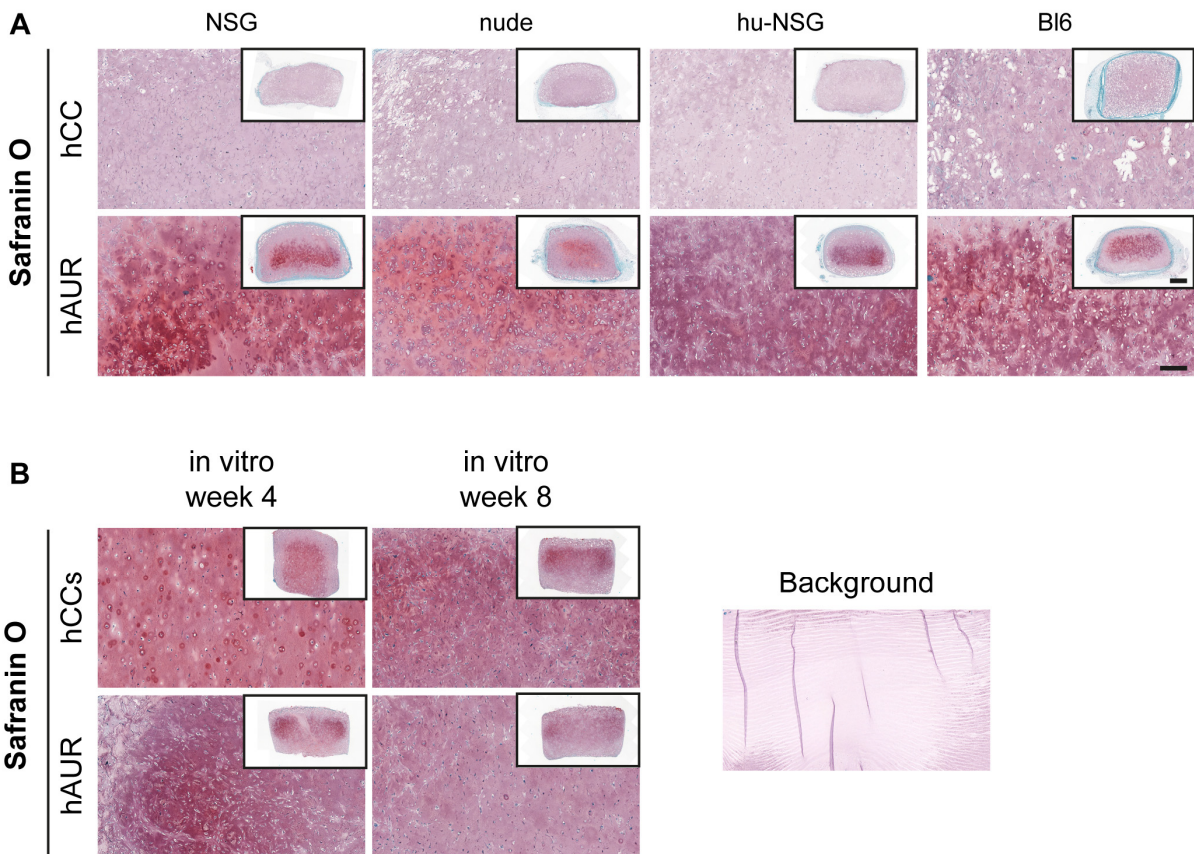
Supplementary Figure 5 A: Safranin O staining of HA-TG hydrogels after 4 weeks of in vitro preculture and 4 additional weeks in vivo. B: Safranin O staining of HA-TG hydrogels after 4 and 8 weeks in vitro and HA-TG background staining. Scale bar close up: 500µm, scale bar insert: 100µm.
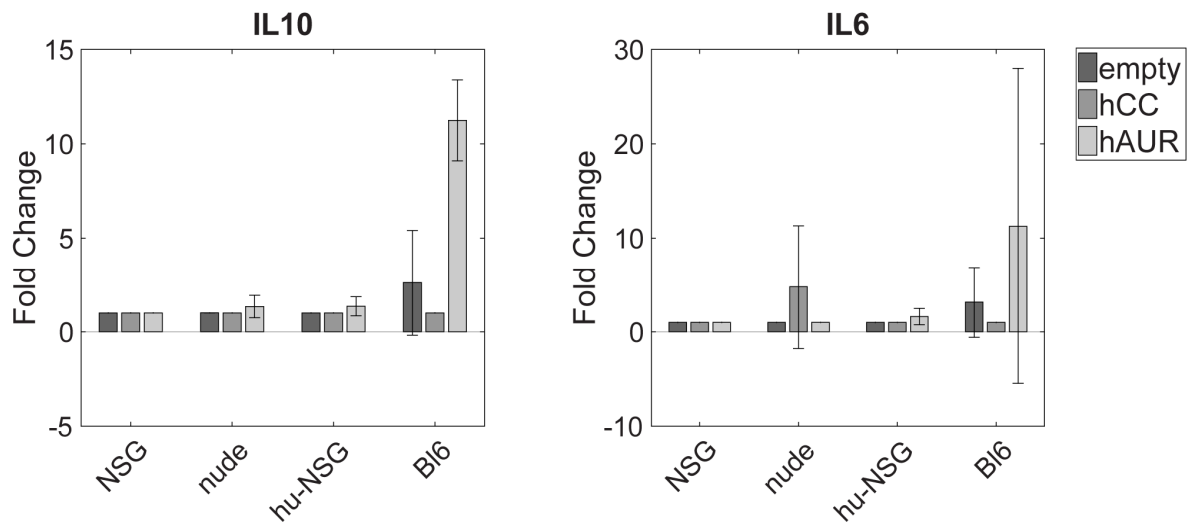
795

796 *Supplementary Figure 6 Inflammation markers IL6 and IL10 detected in mouse serum and normalized to baseline values*
797 *acquired 10 days before surgery. Values below the limit of detection were set to the corresponding lower limit of detection.*
798 *IL1-β, IL4 and TNF-α values were below the limit of detection of the assay.*

799

800 *Table 3 Baseline values of C-reactive protein (CRP), serum amyloid A (SAA) and serum amyloid protein (SAP) prior to*
801 *experiment*

	NSG	nude	hu-NSG	Bl6
CRP (pg/ml)	5104.8	11895.5	10451.6	6062.5
SAA (pg/ml)	961.0	5509.1	2217.4	881.2
SAP (pg/ml)	90286.5	71014.9	61907.2	3662.9

802 *.*

803