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Biocompatibility and immunogenicity of elastin-like recombinamer biomaterials in mouse models

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Abstract: Novel thermo-sensitive elastin-like recombinamers (ELRs) containing bioactive molecules were created for use as a biomimetic biomaterial for tissue regeneration. For effective use for *in vivo* applications, it is essential to ensure that they do not induce adverse inflammatory, immune, or allergic responses that inhibit tissue repair. Therefore, we sought to establish a pre-clinical approach to evaluate biocompatibility in experimental mice using ELRs as a prototype biomaterial. First, we measured *in vitro* proliferation and cytokine production from BALB/c and C57BL/6 mouse splenocytes incubated with ELRs. Second, we used a rapid, high throughput *in vivo* approach in which inflammatory cells and cytokines were measured following an intraperitoneal implantation. Lastly, a subchronic *in vivo* approach was used in which ELRs or positive controls were subcutaneously implanted and the implantation sites were assessed for inflammation and gene expression. We found that ELRs induced mild inflammation and minimal fibrosis compared to the intense response to Vitoss. Additionally, implantation increased antigen-specific antibody titers for both groups and gene expression profiling of the implantation sites revealed the upregulation of inflammation, fibrosis, and wound healing-related genes in ELR and positive control-implanted mice compared to sham controls. These data demonstrate that ELRs appear safe for use in tissue engineering. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2017.

Key Words: regenerative medicine, biomaterials, immunogenicity, foreign body reactions, elastin-like recombinamers

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INTRODUCTION

A promising approach in tissue repair, healing, and regeneration is the use of biomaterials created with bioactive components that amplify physiological mechanisms. However, a key feature of healing is the generation of an optimal milieu in which there is sufficient inflammatory signals initiating the response, but too much and/or chronic inflammation may delay and reduce tissue repair. Moreover, an allergic immune reaction to a biomaterial may generate inflammation that may also be detrimental to engraftment and repair. Therefore, for a biomaterial to enhance healing, it requires the capacity to generate inflammatory signals for healing without being immunogenic or allergenic.

Recently, elastin-like recombinamers (ELRs), which can be engineered to include bioactive peptides for use in tissue regeneration have drawn lots of attention due to their great potential.[1,2] These biomaterials can be designed to be water soluble at cold temperatures and form hydrogels at 37°C, making them particularly easy to work with.[1,3,4] Additionally, ELRs with specific bioactive molecules tagged may stimulate specific cellular responses.[5,6] For example, ELR biogels may mimic many aspects of the extracellular matrix (ECM), which plays an important role in regulating cell activity and tissue morphogenesis and repair.[7] For instance, when RGD (L-arginine-glycine-L-aspartic acid) motifs are added to the basic ELR, it stimulates cell attachment and increases the production of ECM proteins[6–8] and influences bone growth.[2] Newly designed ELRs possessing bioactive moieties may be useful in tissue regeneration, but the addition of new motifs may alter

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their immunogenicity and allergenicity and thus, require further investigation.

Inflammatory, immune, and fibrotic responses to foreign materials have been used extensively with established immunological tools developed for organ transplantation and joint replacement and extended to study foreign body responses of biomaterials.[9,10] The aim of this study was to combine existing *in vitro* and *in vivo* approaches to assess inflammation, immune, allergic, and fibrotic responses of bioactive ELRs.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the guidelines for the Care and Use of Laboratory Animals of the Austrian Ministry of Science. The protocol was approved by the Committee on the Ethics of the Austrian Ministry of Science (N°: BMWF-66.006/0012-II/3b/2012). All painful procedures were performed under anesthesia, and all efforts were made to minimize suffering.

Mice

Female 6–8 week old BALB/c and C57BL/6 (B6) mice (Charles River Laboratories, Sulzfeld, Germany) were used for all *in vitro* and *in vivo* experiments. The mice were housed in the mouse facility at the Department of Pharmacology, University of Vienna, Vienna, Austria. Mice were provided food (Ssniff Spezialdiäten GmbH, Soest, Germany) and autoclaved tap water ad libitum.

ELR synthesis

Elastin-like recombinamers were produced using genetically modified *E. coli*. Each ELR was uniquely encoded by a gene sequence built by recursive directional ligation using standard recombinant techniques with a modified pDrive cloning vector previously described in detail.[1,11] The structure of the ELRs is illustrated in Figure 1a. The building blocks of the amphiphilic thermo-responsive ELRs are as follows: E25 represents the hydrophilic block with the sequence $[(VPGVG)_2(VPGEG)(VPGVG)_2]_5$; I20, is the hydrophobic block composed of twenty repetitions of the pentapeptide (VPGIG); and EL refers to the elastase sensitive sequence which corresponds to three repetitions of the amino acid sequence (VGVAPG). The bioactive blocks include RGD, which is the arginine-glycine-aspartic acid cell adhesion motif embedded in a larger amino acid sequence that favors its exposure $(VPGIG)_{10}$—AVTGRGDSPASS—$(VPGIG)_{10}$, and the addition of sample proteins potentially useful for bone tissue regeneration, bone morphogenetic proteins BMP2 and BMP7, which are present in a truncated version of the complete BMP lacking the heparin binding site[12] and composed of the last 104 amino acids of the complete human protein. To produce ELRs, we used a transfected expression strain grown in a bioreactor supplemented with auto-induction growth media (Terrific Broth, Formedium, Norfolk, UK) under controlled conditions of stirring, pH, temperature, and oxygen pressure. ELRs were purified from the lysate by sequential inverse transition cycling (ITC), taking advantage of the thermoresponsive properties of ELRs.[11] Endotoxin removal was done using an adapted "salting-out" method combined with basic pH.[4,13] Endotoxin levels were below 5 EU/mg as measured using the FDA approved test (Endosafe®-PTS, Charles River).

In vitro evaluation of ELRs

For *in vitro* tests, biomaterials were incubated with BALB/c and B6 mouse splenocytes. Spleens were minced and passed through a sterile 40 μm cell strainer (Corning Life Sciences, Durham, USA) in cold sterile PBS. The cells were centrifuged and RBCs were lysed with lysis buffer (BD Pharm Lyse™, BD Bioscience, New Jersey, USA). Splenocytes were suspended in titrated doses in RPMI with 10% FBS, 1% penicillin/streptomycin, 0.1% gentamycin (Gibco, Thermo Fisher Scientific, Waltham, USA), 0.2% β-mercaptoethanol (50 mM, Gibco), and 1% non-essential amino acids (Gibco) and then incubated with a constant concentration of either ELRs (50 mg/ml), positive control Orthovita Vitoss™ Foam packs (50 mg/ml, Vitoss, Malvern, USA) containing bovine collagen, Concanavalin A (10 μg/ml, Con A, Amersham Pharmacia Biotech, Piscataway, USA) or a combination of Con A and biomaterials in a 96-well plate at 37°C and 5% CO_2 for 72 h. To prepare the Vitoss suspension, Vitoss was crushed using a tissue homogenizer in PBS. Cell proliferation was measured using a cell proliferation ELISA, BrdU kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) by adding BrdU 48 h after cell incubation and measuring O.D. at 450 nm at 72 h. In addition, supernatants were removed at 72 h and stored at −20°C until thawed for the ELISA measurements of IL-1β (eBioscience Inc., San Diego, USA), IL-2, IFN-γ, IL-4, and IL-5 (ELISA MAX™ Standard, Biolegend, San Diego, USA) cytokine concentration.

In vivo ELR evaluation

Sample preparation. To implant bioactive moiety-containing ELRs, we dissolved them in cold sterile PBS at a final concentration of 300 mg/ml and incubated them at 4°C overnight to ensure complete dissolution. Disk-shaped ELR hydrogels were prepared for *in vivo* implantation by introducing 50 μl of the ELR (15 mg total) solution into wells of a 96-well culture plate at 37°C for 10 min, resulting in hydrogel disks of 6 mm in diameter by 1 mm thick. Vitoss samples were cut into 6 mm diameter ×0.5–1 mm thick disks weighing 15 mg (600 mg/kg b.w.) and then soaked in 50 μl PBS before implantation. For implantation, mice were anesthetized with 100 mg/kg i.p. Ketanest (Pfizer Corporation Austria GmbH, Vienna, Austria) and 6 mg/kg Rompun (Bayer AG, Leverkusen, Germany).

Vitoss was used in all *in vivo* and *in vitro* experiments as a positive control. Vitoss is a porous ceramic material that includes type I bovine collagen, which is recognized as a foreign protein and generates an immune response in mice. Because ELRs contained BMPs for potential in bone healing, Vitoss as a bone graft substitute provides a standard for comparison of the ELRs in terms of the immune and inflammatory responses necessary for bone healing. Although Vitoss and ELR biogels differ structurally and biologically, we address the immune responses that they induce.

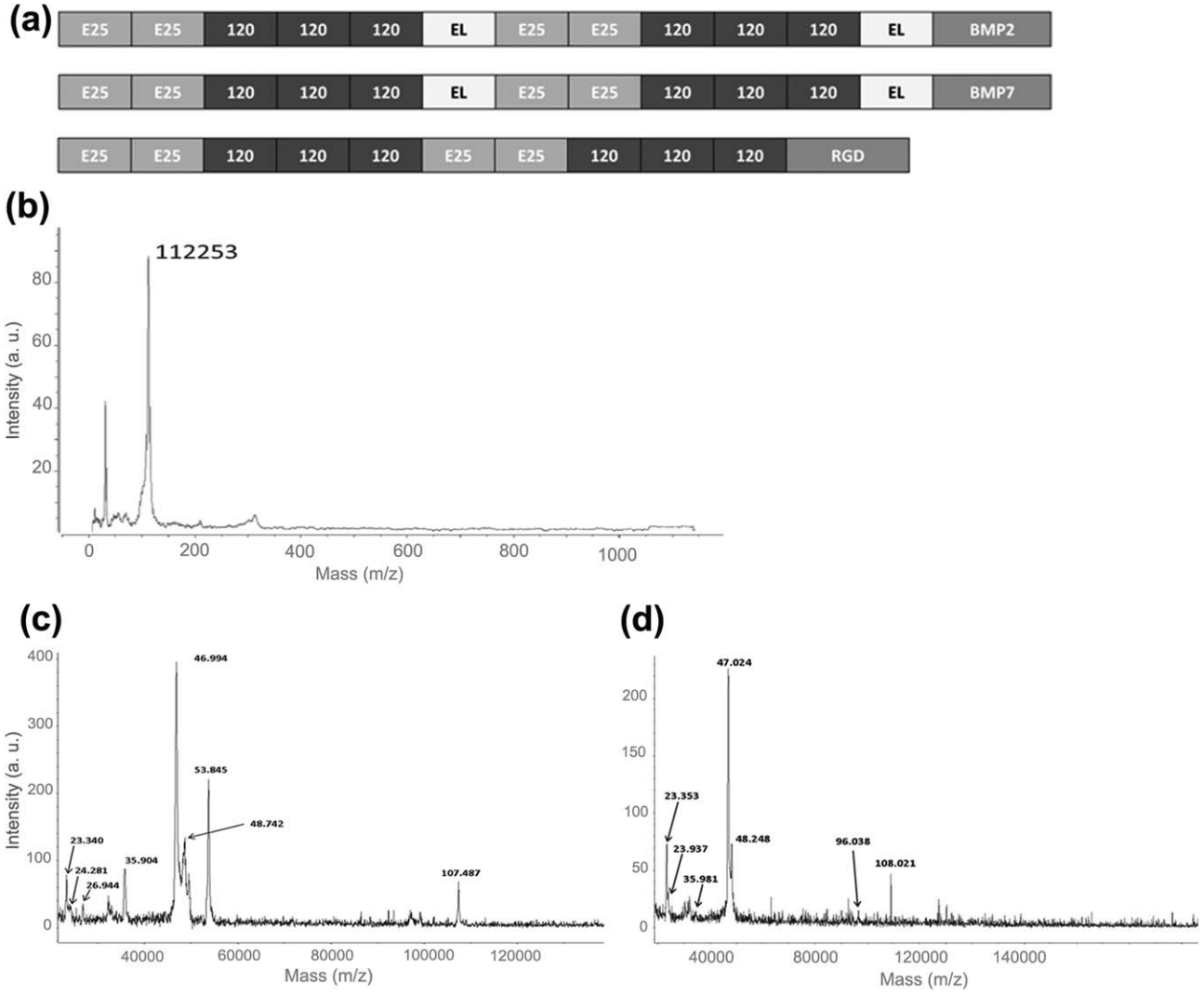


FIGURE 1. ELR structures and mass spectra. (a) The structures of the ELRs are shown with the hydrophilic/polar block (E25), the hydrophobic/apolar block (I20), elastase sensitive sequence (EL), and the bioactive blocks (RGD, BMP2, and BMP7) components. (b) Mass spectrum of ELR-RGD shows a main peak at 112,253 Da that corresponds to the single ionization of the molecule. Other peaks at lower molecular weights may correspond to ELR fragments produced by the ionization process during the test. (c, d) Mass spectra of ELR-BMP2 and -BMP7 are shown with peaks in the spectrum.

Rapid high throughput and subchronic mouse models. *Rapid high throughput mouse model*: For i.p. implantation, we used a modified implantation approach[10] in which the abdominal fur was shaved and the surface cleaned with 70% alcohol and 7.5% Betadine. Using aseptic techniques, an 8 mm long midline incision was made along the linea alba. ELRs or Vitoss were placed in the peritoneal cavity and pushed carefully under the loops of the intestine with a blunt forceps. The abdominal muscles and skin were sutured with absorbable 4–0 Vicryl suture (Ethicon Inc., Somerville, USA) and 4–0 nylon suture (Ethicon), respectively. The same incision and suturing was used without implanting biomaterials for sham controls. Seven days after implantation, the peritoneal cavity was lavaged with 3 mL PBS. Lavage fluid (3 ml) were harvested and used to count peritoneal cells and stored at $-20°C$ until thawed for the measurement of IL-2, IL-4, and IL-1β cytokines.

Subchronic mouse model: For s.c. implantation, we modified the implantation approach[9] in which an 8 mm midline abdominal incision was made under aseptic conditions. ELRs or Vitoss of the same size were then inserted under the skin and closed with 4–0 nylon suture. The same incision and suturing was used without implanting biomaterials for sham controls. The mice were monitored until recovery from anesthesia. The implantation site with surrounding tissue (1 cm × 1 cm) from each mouse was excised and fixed in 4% formaldehyde overnight and then embedded in paraffin. Tissue sections of 4 μm thickness were prepared and stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) stains for inflammation and fibrosis, respectively. Quantification of fibrosis was done by analyzing the density of the blue stained areas with Image J analysis software[14] on three or more random images per high power field for all Mason's Trichrome stained sections ($n = 30$) per sample group.

The high throughput and subchronic approaches were selected to study *in vivo* immune responses to foreign materials as an indication of immune responses in other tissue sites such as bone because they are economical, not technically challenging, quick to perform, and less invasive and traumatic to the animals compared with other sites.

RNA isolation and quantitative real-time PCR. Tissue fragments (36 mm^2) were obtained from cutting the implant area using skin punch and stored at −80°C until use. RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from an equal amount of total RNA from each sample using cDNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific). For quantitative real-time PCR, a reaction mix was prepared containing sample cDNA, forward and reverse primers (Microsynth AG, Balgach, Switzerland), and SYBR Green (Applied Biosystems, Thermo Fisher Scientific). Primer sequences are shown in Supporting Information Table S1. Data are presented as expression fold-change relative to the geometric mean of expression values for housekeeping genes (HPRT, β-Actin, and GAPDH).

Antigen-specific immunoglobulin. For the measurement of Vitoss- or ELR-specific IgG1 and IgE, sera were taken 21 and 56 days after s.c. implantation. ELISA plates were coated with bovine collagen type 1 or ELR at a concentration of 2 μg/ml and incubated at 4°C overnight. The plates were washed and blocked with 2% BSA in PBS with 0.05% Tween 20 for 2 h at RT. Titrated sera were then incubated for 24 h at 4°C. Plates were washed and then incubated for 2 h at 4°C with biotinylated anti-IgG1 or anti-IgE detection mAb (BD Biosciences, Franklin Lakes, USA), followed by incubation with streptavidin horseradish peroxidase (Southern Biotechnology Associates Inc., Birmingham, USA) for 1 h at RT. After washing, 100 μl 3.3′, 5.5′-tetrametylbenzidine (TMB, BD Bioscience) was added to each well and then incubated in the dark at RT for 10 min. Dye development was stopped by the addition of 100 μl of 0.18 M H_2SO_4 and the plates were measured for optical density (O.D.) in an ELISA reader (Thermo Fisher Scientific) at 450 nm.

Statistical analysis

Statistical analysis for *in vitro* and *ex vivo* cytokine secretion and cell proliferation was done using a one way repeated measures ANOVA. Statistical analyses for *in vivo* cell number and qPCR were done with one or two-way ANOVA (GraphPad Prism v 5.0, San Diego, USA). *p* values were considered significant at <0.05. Comparisons were made against the sham controls and between ELR groups.

RESULTS

ELR purification

ELRs were purified from a bacterial lysate by sequential ITC.[11] Mass spectrum MALDI-TOF confirmed the monodispersed character of ELR-RGD and purity and correlated with the theoretical and experimental molecular weight (Fig. 1b). Mass spectrometry of ELR-BMP2 and ELR-BMP7 showed some minor ELR fragments that arise from the presence of degradation sequences (EL in Fig. 1a). Fragments that lost BMP2 and BMP7 varied in size due to different possible degradation points within the same molecule (Fig. 1c,d, Table I, and Supporting Information Fig. S1). As observed in the MALDI-TOF spectra of the BMP-ELRs, some ELRs lack the bioactive domain. This break-down of the protein might be either a consequence of the purification process or an artefact related to high-energy ionization from the energy from the absorption of the green laser (337 nm wavelength) by the ELR causing heating and vaporization. Nevertheless, SDS-PAGE electrophoresis of purified ELRs illustrates that a large majority of the purified product consists of the complete sequence including the bioactive domain and only a small percentage lacking BMP motifs.

TABLE I. MALDI-TOF Peaks and Ionization States Corresponding to Different ELR-BMPs

ELR	Peak (Da)	Corresponding fragment	Ionization state
BMP2	107.487	ELR-BMP2	+1
	53.845	ELR-BMP2	+2
	48.742	ELR-BMP2 without BMP2	+2
	46.994	ELR-BMP2 without BMP2	+2
	35.904	ELR-BMP2	+3
	26.944	ELR-BMP2	+4
	24.281	ELR-BMP2 without BMP2	+3
	23.340	ELR-BMP2 without BMP2	+3
BMP7	108.021	ELR-BMP7	+1
	96.038	ELR-BMP7 without BMP7	+2
	48.248	ELR-BMP7 without BMP7	+2
	47.024	ELR-BMP7 without BMP7	+2
	35.981	ELR-BMP7	+3
	23.937	ELR-BMP7 without BMP7	+3
	23.353	ELR-BMP7 without BMP7	+3

In vitro analysis of immune responses to biomaterials

To evaluate the immune response to the ELRs *in vitro*, we measured IL-2, IL-1β, IFN-γ, IL-4, and IL-5 cytokine production and cell proliferation after incubating female BALB/c and B6 splenocytes with ELR-RGD, ELR-BMP2, ELR-BMP7, or Vitoss (Fig. 2). Neither ELRs nor Vitoss significantly induced BALB/c and B6 splenocytes to proliferate or secrete cytokines. As a positive control for T cell function and to determine whether ELRs had an inhibitory effect on proliferation or cytokine production, Con A was added to the cultures. We observed that BALB/c and B6 splenocytes were stimulated with Con A, but were not influenced by Vitoss or ELRs (Fig. 2).

High throughput *in vivo* model for rapid screening of immune responses

We implanted ELRs or Vitoss i.p. and compared immune responses with sham controls. All biomaterials were well tolerated by the animals. Seven days after implantation, we found that Vitoss implanted BALB/c and B6 mice had 565.4

$\times 10^4$ and 405.4 $\times 10^4$ cells/ml in the peritoneal lavage fluid, respectively, with an increase in lymphocytes, macrophages, and eosinophils, which is mostly likely attributed to the collagen moiety (Fig. 3a). In contrast, ELR-RGD implanted BALB/c and B6 mice had similar cell counts compared with sham controls with 197.8 $\times 10^4$ versus 167.0 $\times 10^4$ and 150.7 $\times 10^4$ versus 104.0 $\times 10^4$, respectively. Moreover, the addition of BMP moieties did not increase in the response to ELRs, showing that these bioactive molecules did not induce more of an inflammatory response. Peritoneal lavage fluid from Vitoss-implemented mice had more IL-1β (pro-inflammatory cytokine and foreign body response mediator) and IL-4 (Th2 marker and macrophage fusion induction) in both strains and IL-2 (only in B6 mice) compared with the sham controls. Whereas, BMP2- and BMP7-containing ELRs, but not ELR-RGD induced significant quantities of IL-4 compared with sham controls in BALB/c, but not B6 mice (Fig. 3b), suggesting some added effect based on these growth factors. BMPs are known to influence T cell responses[15] and it is thus, possible that BMP2 and BMP7 may preferentially increase IL-4 production which would be more prominent in the BALB/c compared with the B6 strain.

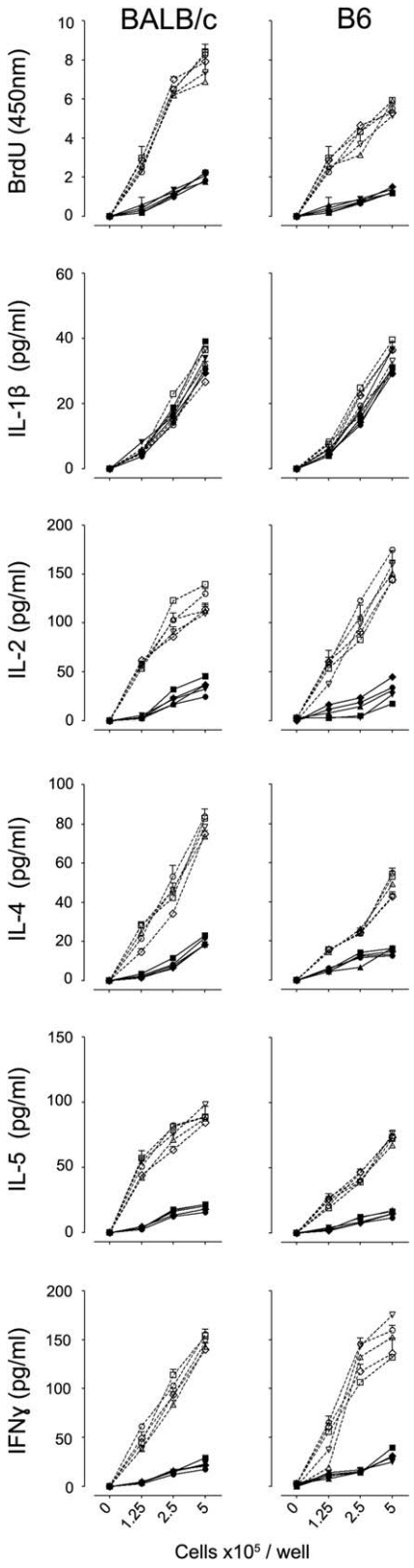


FIGURE 2. ELR-induced *in vitro* cytokine production and cell proliferation. BALB/c and B6 splenocytes were cultured in the presence of Vitoss or ELRs. Cell proliferation, IL-2, IL-1β, IFN-γ, IL-4, and IL-5 were measured upon incubation with either media alone ●, Con A ○, Vitoss ■, Vitoss + Con A □, ELR-RGD ◆, ELR-RGD + Con A ◇, ELR-BMP2 ▲, ELR-BMP2 + Con A △, ELR-BMP7 ▼, or ELR-BMP7 + Con A ▽ at 37°C for 72 h. Proliferation results are presented as the mean of triplicate wells (O.D. ± SEM) in the BrdU assay and the mean of duplicate wells (pg/ml ± SD) for cytokine concentrations from two independent experiments. *p was considered statistically significant at <0.05 for biomaterials versus media and biomaterials + Con A versus Con A alone.

Subchronic subcutaneous *in vivo* implantation

To identify subchronic reactions to ELR implantation, we implanted Vitoss and ELRs s.c. and examined the implantation site up to day 56. We observed that Vitoss induced moderate inflammation and foreign body giant cells by day 21, which subsided by day 56 (Fig. 4), whereas the sham controls and ELR-implants induced a similar inflammatory response with only few foreign body giant cells in the ELR implanted animals. We examined the site for evidence of fibrosis using the Masson's Trichrome stain, which specifically stains collagen fibers. The stained sections were evaluated by determining the percentage of blue-pixel areas in the photomicrographs 200 μm beneath the dermis to exclude normal collagen there (Fig. 5a,b). Vitoss induced thick, dispersed collagen fibers at day 21, which became denser and more localized by day 56. In contrast, ELR implantation sites were similar to the sham controls at both time points.

We then evaluated gene expression at the implantation sites to investigate genes important in wound healing and foreign body reactions. Using qPCR, RNA isolated from the implantation sites was measured at days 3, 7, 21, and 56 (Fig. 6a). Normal healing in the sham control mice was compared to Vitoss and ELR-RGD implanted mice. All pro-inflammatory genes (for example, IL-1β, TNFα, IL-6, IL-17, and iNOS)[16,17] were elevated above baseline for normal skin including sham controls, which demonstrates that these genes were increased

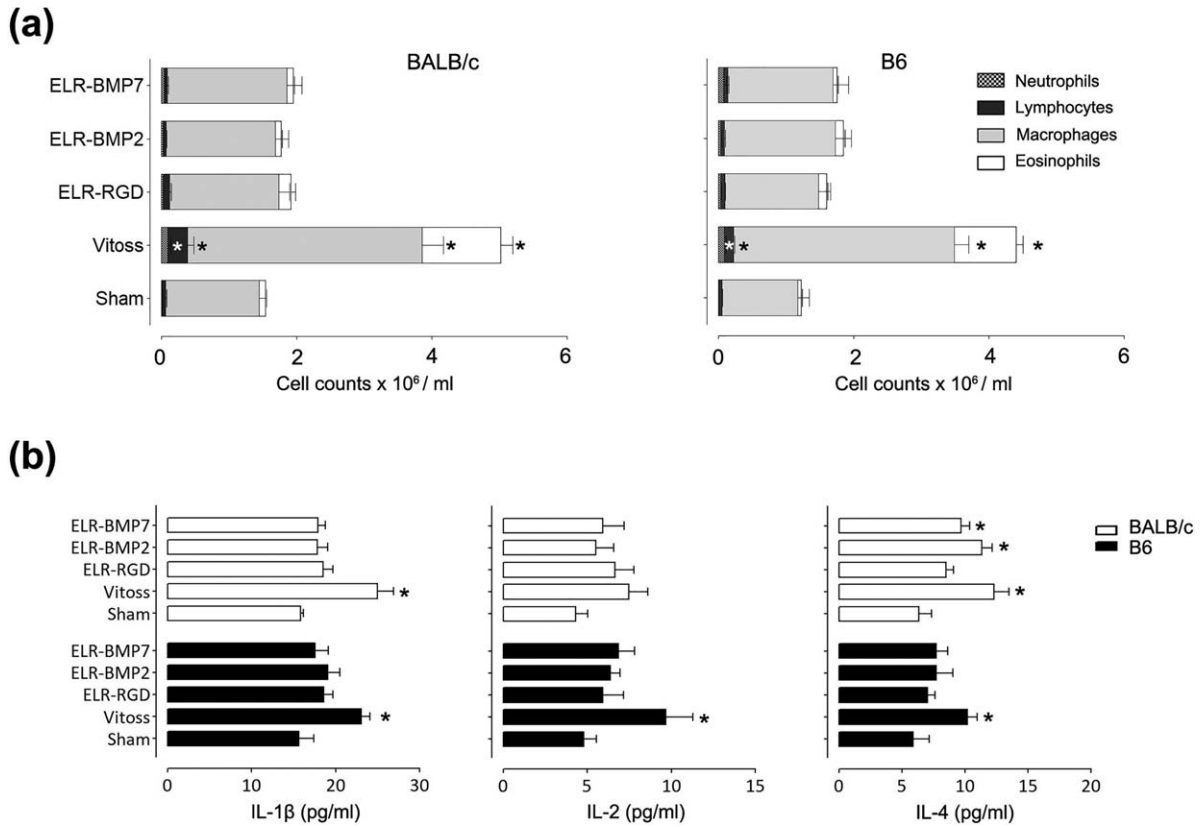


FIGURE 3. ELR evaluation in a rapid high throughput mouse model. Female BALB/c and B6 mice were implanted i.p. with either ELRs, Vitoss or no materials (sham). Seven days later, mice underwent peritoneal lavage. The lavage fluid was analyzed (a) for type and number of inflammatory cells; data are presented as mean absolute cell counts ± SEM, and (b) for cytokine concentrations; data are presented as mean cytokine concentrations pg/ml ± SEM. These data are combined from two identical experiments done on different days ($n = 10$). $^{*}p < 0.05$ and $^{\#}p < 0.05$ are considered significant compared to sham and between ELR groups, respectively. Statistically significant results were comparisons with the sham controls.

in normal wound healing. Vitoss induced higher levels of pro-inflammatory genes compared with ELR-RGD, and Vitoss and ELR implantation had higher expression than sham controls. The anti-inflammatory or immunoregulatory (arginase-1 [Arg-1], IL-4, and IL-10)[16,18] genes were expressed in all groups with Vitoss having higher gene expression compared with ELR-RGD and sham control mice, especially high for Arg1. We measured genes representative of wound healing and fibrosis including TGFβ, alpha-1 type I collagen (Col1A1), alpha-2 type I collagen (Col1A2), and matrix metalloproteinase-2 (MMP2). These genes were highly expressed in sham controls indicating that these genes are crucial for normal healing.

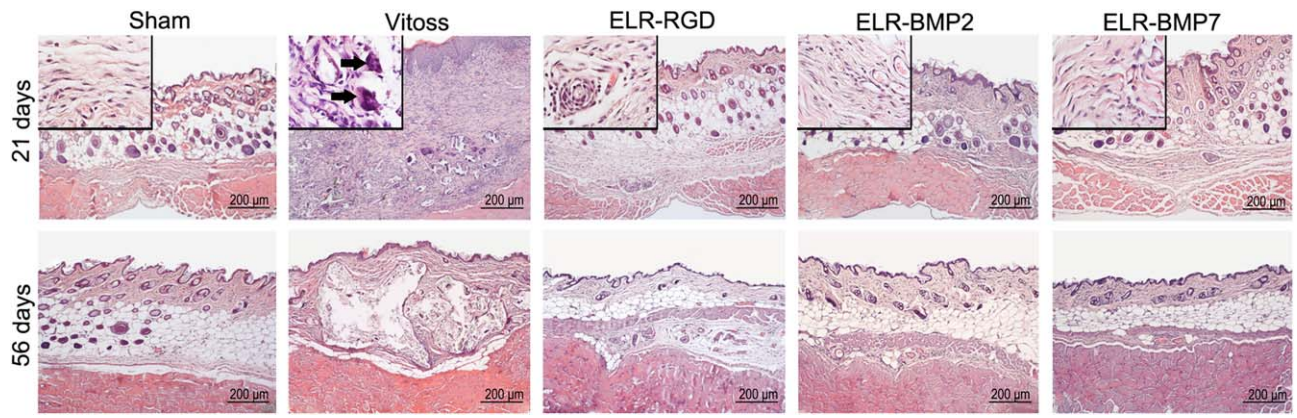


FIGURE 4. Evaluation of the inflammatory response to ELRs in a subchronic mouse model. Female BALB/c mice were implanted s.c. with either ELRs, Vitoss or no materials (sham). Photomicrographs of H&E-stained skin sections at days 21 and 56 after implantation at 10× and insets at 40× are shown and are representative of two independent experiments ($n = 10$). Arrows indicate foreign body giant cells around the implant.

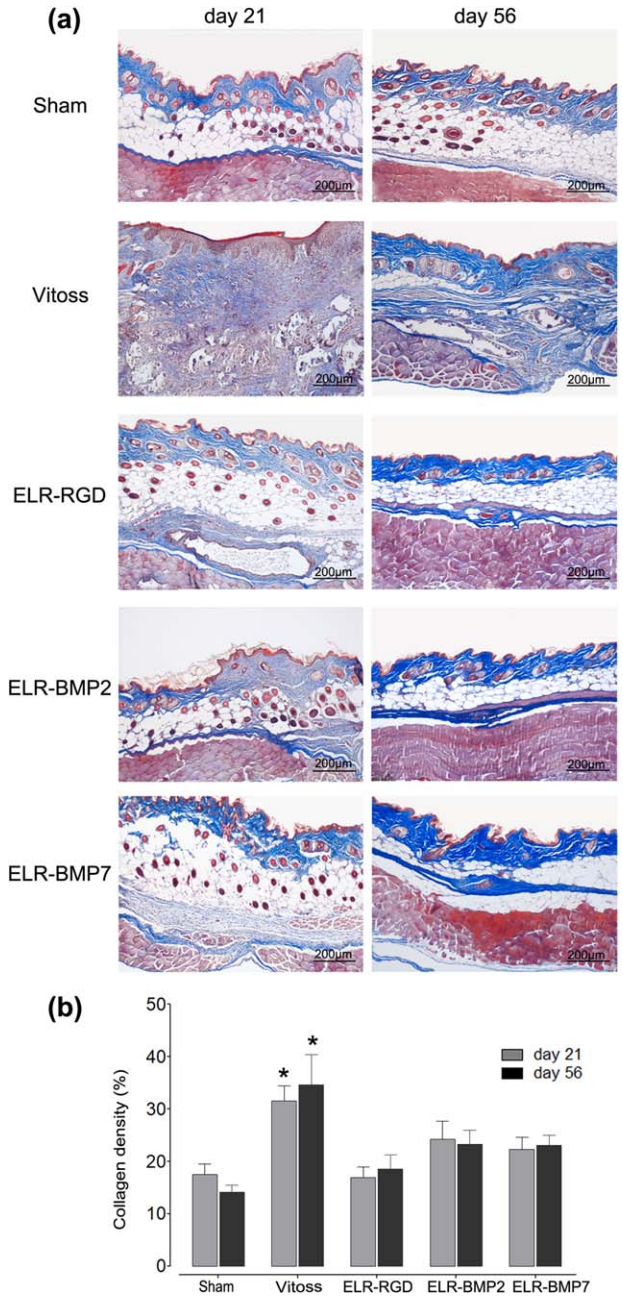


FIGURE 5. Evaluation of fibrosis related to ELR implantation in a subchronic mouse model. BALB/c female mice were implanted s.c. with ELRs, Vitoss or no materials (sham). (a) On days 21 and 56 after implantation, skin from the implantation sites was incised and stained with Masson's Trichrome to evaluate wound healing and fibrosis. Photomicrographs of Masson's Trichrome-stained skin sections at days 21 and 56 after implantation at 10× are shown and are representative of two independent experiments ($n = 10$). Blue structures represent collagen, red/pink structures are cell cytoplasm and black structures are cell nuclei. (b) Collagen density was quantified by measuring the percentage of blue pixels in three high power fields of each section and the results were averaged and compared with a one-way ANOVA. These data are combined from two identical experiments done on different days ($n = 10$). $^{*}p < 0.05$ and $^{\#}p < 0.05$ are considered significant compared to sham and between ELR groups, respectively. Statistically significant results were comparisons with the sham controls.

Moreover, Vitoss induced higher expression compared with ELR-RGD and sham controls.

To assess whether chimeric constructions of the ELRs with other natural proteins, ELR-BMP2 and -BMP7 influenced gene expression, we selected days 21 and 56 to measure gene expression and found that the sham controls had high expression of all genes at day 21, but reverted to baseline values by day 56 (Fig. 6b) indicating normal healing. Vitoss induced high expression of all measured genes at both time points, which shows more intense inflammation and an extended healing response. For ELR-RGD, we found that there was an increase in IL-10 and TGFβ above background at day 21 and fell to baseline by day 56. In contrast, the ELR-BMP2 and -BMP7 induced TGFβ, Col1A2, and IL-1β and ELR-BMP2 induced IL-10 at day 21, but not at day 56 suggesting that the chimera influences gene expression.

Systemic immune and allergic responses to the biomaterials were evaluated in two separate assays. First, we measured antigen-specific antibody titers in serum at days 21 and 56 (Fig. 7) and second, we evaluated *ex vivo* antigen-specific secondary splenocyte responses at month 3 after s.c. biomaterial implantation (Fig. 8). Serum collagen-specific IgG1 and IgE antibody titers were elevated in response to the bovine collagen in Vitoss. ELR-specific IgG1 and IgE titers were also elevated in ELR implanted mice compared to sham controls. The *ex vivo* antigen-specific proliferation of splenocytes revealed that Vitoss implantation leads to a secondary immune response with an increase in cell proliferation and the production of IL-2 and IFN-γ, whereas the *ex vivo* response to ELRs was similar to sham controls (Fig. 8). There was no IL-4 or IL-5 production from Vitoss or ELRs, suggesting an absence of allergic responses. Notably, the histological findings revealed that ELRs did not induce local inflammation and there was no secondary *ex vivo* response to the ELRs. Nevertheless, there were increased ELR-specific antibody titers, which indicate that the mice had developed an immune response to the ELRs.

DISCUSSION

This study established a stepwise *in vitro* and *in vivo* strategy for evaluating local and systemic inflammatory immune and allergic reactions of ELR-based bioactive biomaterials for tissue regeneration and repair. Our study was done with ELRs containing BMP2 and BMP7, which are growth factors essential for bone formation. Currently, BMP2 is used in clinical practice and both BMP2 and BMP7 are considered the most important factors for bone regeneration.[19] Our results show that RGD-, BMP2-, and BMP7-containing ELRs were less immunogenic compared to the currently used Vitoss bone graft substitute for clinical practice for bone regeneration. The ELRs induced a humoral immune response, caused minimal inflammation and fibrosis, which was less intense compared to Vitoss. Taken together, these data demonstrate that ELRs have low immunogenicity and allergenicity in preclinical mouse models.

Elastin-like recombinamers are genetically engineered protein polymers with repeating sequences found in elastin. For the multiblock amphiphilic molecular designs used here,

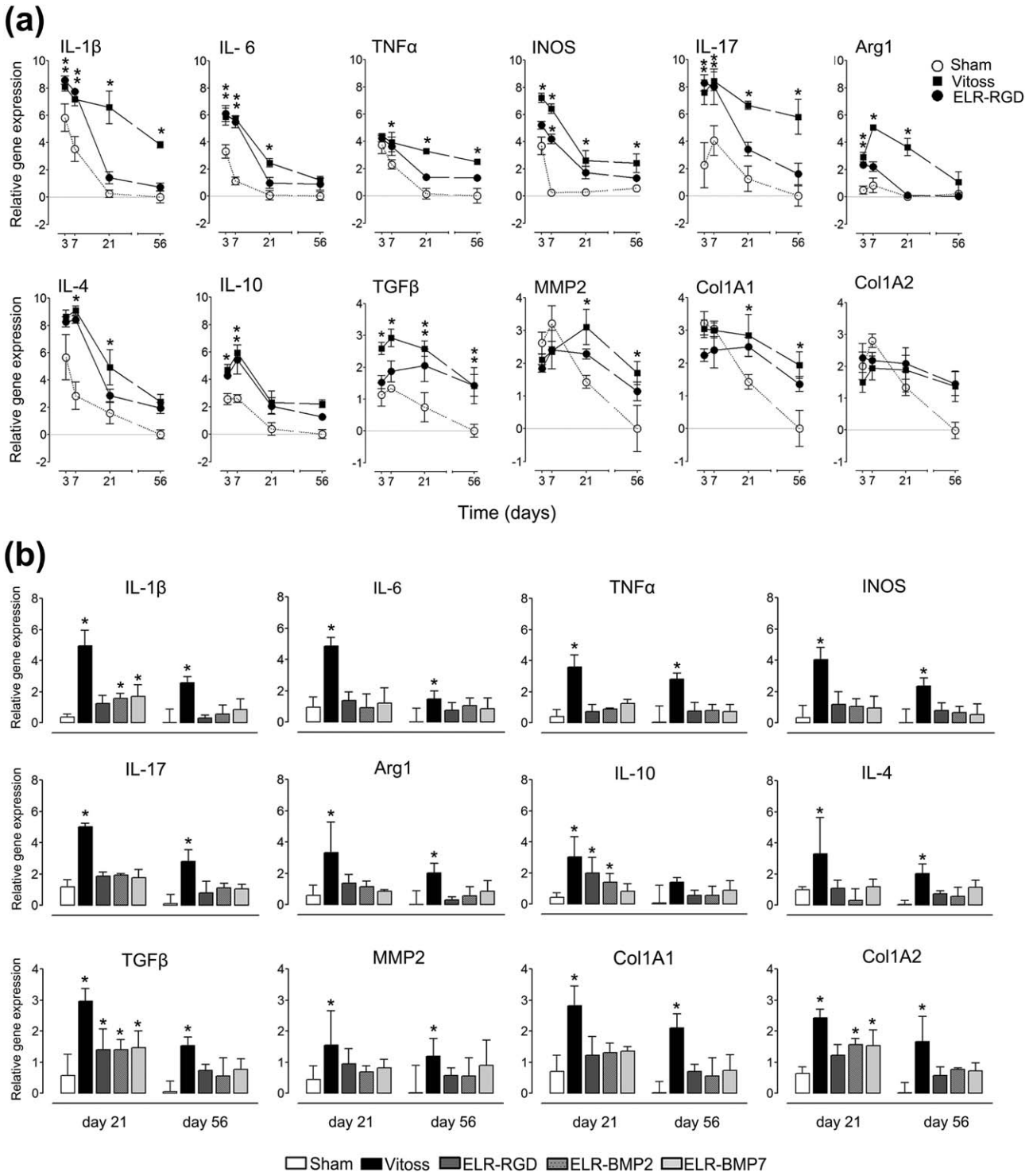


FIGURE 6. Inflammation- and fibrosis-related gene expression in implantation sites. BALB/c female mice were implanted s.c. with ELRs, Vitoss or no materials (sham). (a) On days 3, 7, 21, and 56 after ELR-RGD or Vitoss implantation, and (b) on days 21 and 56 after ELR-RGD, ELR-BMP2, ELR-BMP7, and Vitoss implantation, the tissue surrounding the implantation sites was incised, RNA extracted, and qPCR was done on selected inflammation- and fibrosis-related genes. Data are presented as mean $\log_2$ fold change ± SEM ($n = 5$). $^{*}p < 0.05$ and $^{\#}p < 0.05$ are considered significant compared to sham and between ELR groups, respectively. Statistically significant results were comparisons with the sham controls.

their aqueous solutions are a liquid at RT and a gel at 37°C.[1] The basic ELR structure has tremendous potential for tissue engineering, because ELRs can be tailor-made with specific bioactivities, are biodegradable, and in a few studies were found to perform well, due to the similarity to natural, endogenous elastin.[1,3] Additionally, in a previous study, a

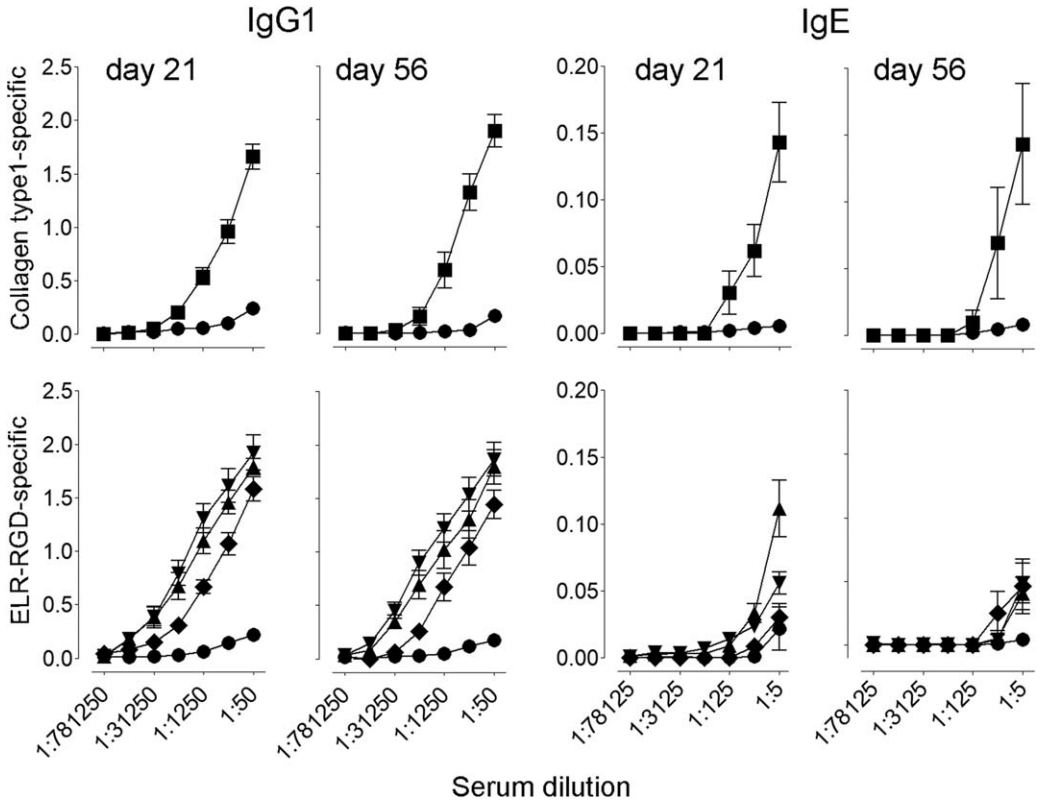


FIGURE 7. Serum antigen-specific IgG1 and IgE titers after s.c. implantation. Female BALB/c mice were implanted s.c. with either Vitoss ■, ELR-RGD ◆, ELR-BMP2 ▲, ELR-BMP7 ▼, or no material (sham) ●. On days 21 and 56, sera were taken from implanted mice and bovine collagen- and ELR-specific antibodies were measured with a standard sandwich ELISA. Data are presented as means of duplicate O.D. readings ± SEM. These data are combined from two identical experiments done on different days ($n = 10$).

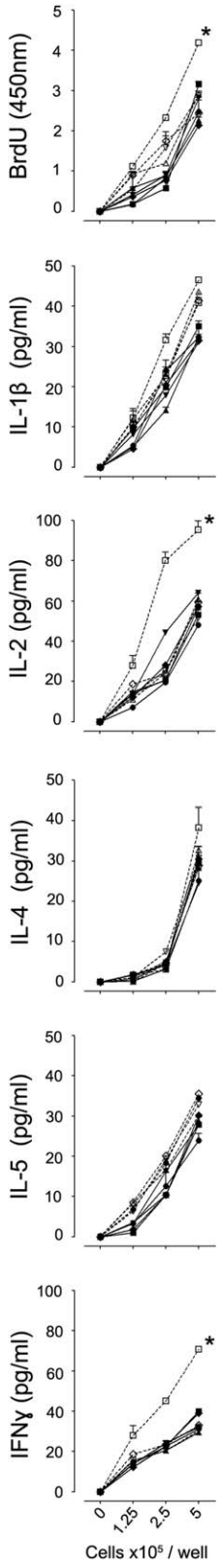
bioactive ELR membrane containing mesenchymal stem cell adhesion (RGD) and mineralization moieties enhanced mineralization, osteogenesis, and *in vivo* bone healing in a rat model.[2] Importantly, it is essential to determine whether the addition of bioactive proteins in chimeric constructions, for example, BMPs containing ELRs alters the immune response to the basic ELR.

The ELRs tested in our studies contained cell adhesion (RGD) and BMP2 and BMP7 bioactive molecules, which could potentially induce undesired immune or inflammatory responses. *In vitro* assays revealed that these ELRs were unable to stimulate or inhibit splenocytes upon primary and secondary (*ex vivo*) antigen stimulation. Furthermore, implantation of the ELRs in the peritoneum (high throughput model) or under the skin (subchronic model) induced little inflammation in the peritoneum and at the implantation sites, respectively. Nevertheless, gene expression at the implantation site demonstrated that inflammatory- and fibrosis-related genes were upregulated above normal healing levels observed in the sham controls. Additionally, we observed a humoral immune response generated by the ELRs, which demonstrates that ELR-specific B cells were primed in the subchronic model.

Bovine collagen in Vitoss induced immune and inflammatory responses in our experiments in mice and has also been reported to stimulate immune responses in humans.[20] Vitoss did not stimulate naïve mouse splenocytes in primary *in vitro* cultures, but stimulated them *ex vivo* in secondary antigen-specific re-stimulation cultures indicating that a primed T cell response is induced against the collagen within Vitoss. Vitoss induced inflammation and immune responses upon peritoneal and subcutaneous implantation. It upregulated inflammatory- and fibrosis-related genes and systemic antigen-specific antibody responses. In all assays, Vitoss was more immunogenic than ELRs. Possible reasons for Vitoss being more immunogenic compared to the ELRs might be related to Vitoss being a hard ceramic powder that releases ions and precipitates, having a higher accessible surface area, different biochemical surface motifs, or because it remains in the implantation site for a longer time compared with the ELRs.

Although Vitoss and ELRs are structurally, compositionally, and biologically distinct, Vitoss is an ideal control because (1) it is already in the clinics and is effective for the indication that we are ultimately aiming for, (2) it induces an immune and inflammatory response that is important for tissue repair, and (3) it acts as a control for validating the models. Even though, the materials differ considerably, the readout for these experiments are inflammation, immunity, and fibrosis. Arguably, the differences between the materials may result in major changes in the way that cells respond to them, but for

the purposes of this study, the Vitoss results provide a clinically useful standard for which to compare the ELRs, specifically for the BMP molecules.



Although the early phase of healing is enhanced with an inflammatory microenvironment, chronic and aggressive inflammation, and immune and allergic responses may lead to reduced regeneration and chronic problems.[21] For example, allergic and immune reactions to biomaterials could cause major problems for patients in the long-term, especially if repeated administration of the biomaterial is clinically indicated. Because of the similarity between ELRs and endogenous elastin, it is probable that allergic and immunologic reactions will not occur. However, there are reported cases of antibodies including IgE and cell-mediated immune responses against elastin in patients with the autoimmune disease, systemic sclerosis (scleroderma),[22] and in susceptible smokers with emphysema.[23] However, the link between anti-elastin antibodies and disease pathogenesis is not clear and allergic responses against elastin are not well documented in the literature and are not well understood. While the mouse is a good model for testing materials, the IL-4 secondary to i.p. implantation in BALB/c mice and IgE reaction secondary to s.c. implantation that we observed may be a result of an anti-ELR response. Alternatively, it is possible that there is an allergic antibody response against ELRs, but the lack of a secondary *ex vivo* anti-ELR response suggests an absence of allergic responses. Using mouse models to predict human responses to biomaterials may be limited because of xenogeneic differences. Further studies and validation of these models are necessary to ensure the predictability of reactions in humans.

CONCLUSION

Here, we show an established immunogenicity platform for evaluating the local and systemic inflammatory, immune, and allergic reactions of newly-designed ELR-based bioactive biomaterials for tissue regeneration and repair. We tested ELRs with three separate assays. The *in vitro* assays provide information on antigen cross reactivity and cytotoxicity. The i.p. response to biomaterials provides *in vivo* data on the inflammation and the cytokine milieu, while the s.c. reaction is long-term and provides information on the longevity of the inflammation and capacity to induce fibrosis. The *in vitro* assay demonstrated that there was neither a stimulatory nor inhibitory immune response against ELRs, whereas i.p. and s.c. administration of biomaterials including Vitoss demonstrated inflammatory reactions and upregulation of gene expression. Overall, these assays provide distinct information and should be considered for evaluating biomaterials.

FIGURE 8. Secondary antigen-specific *in vitro* responses to implanted biomaterials. BALB/c splenocytes from sham (closed symbols) or biomaterial implanted (open symbols) mice were cultured *in vitro* at 37°C for 72 h with Vitoss ■/□, ELR-RGD ◆/◇, ELR-BMP2 ▲/△, ELR-BMP7 ▼/▽, or media alone ● to measure cell proliferation, IL-2, IL-1β, IFN-γ, IL-4, and IL-5 cytokine production. Proliferation results are presented as the mean of triplicate samples (O.D. ± SEM) for the BrdU assay and mean of duplicate samples (pg/ml ± SEM) for cytokine concentration. These data are representative of two identical experiments done on different days ($n = 10$). $^{*}p < 0.05$ is considered significant compared to sham controls and $^{\#}p < 0.05$ is considered significant between ELR groups.

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ABBREVIATIONS

BMP	bone morphogenic protein
ELR	elastin-like recombinamer
RGD	arginine-glycine-aspartic acid
ECM	extracellular matrix
C57BL/6	B6
RBC	red blood cells
Vitoss	Orthovita Vitoss™ Foam packs
Con A	Concanavalin A
H&E	Hematoxylin and Eosin
O.D.	optical density
Th cell	T helper cell

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