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Extracellular matrix inclusion in immunoisolating alginate-based microcapsules promotes longevity, reduces fibrosis, and supports function of islet allografts *in vivo*

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ABSTRACT

Immunoisolation of pancreatic-islets in alginate-microcapsules is applied to treat diabetes. However, long-term islet function is limited, which might be due to damaged and lack of contact with pancreatic extracellular matrix (ECM) components. Herein we investigated the impact of collagen IV combined with laminin sequences, either RGD, LRE, or PDSGR, on graft-survival of microencapsulated bioluminescent islets *in vivo*. Collagen IV with RGD had the most pronounced effect. It enhanced after 8-week implantation in immune-incompetent mice the bioluminescence of allogeneic islets by 3.2-fold, oxygen consumption rate by 14.3-fold and glucose-induced insulin release by 9.6-fold. Transcriptomics demonstrated that ECM enhanced canonical pathways involving insulin-secretion and that it suppressed pathways related to inflammation and hypoxic stress. Also, 5.8-fold fewer capsules were affected by fibrosis. In a subsequent longevity study in immune-competent mice, microencapsulated allografts containing collagen IV and RGD had a 2.4-fold higher functionality in the first week after implantation and remained at least 2.1-fold higher during the study. Islets in microcapsules containing collagen IV and RGD survived 211 ± 24.1 days while controls survived 125 ± 19.7 days. Our findings provide *in vivo* evidence for the efficacy of supplementing immunoisolating devices with specific ECM components to enhance functionality and longevity of islet-grafts *in vivo*.

Statement of significance

Limitations in duration of survival of immunoisolated pancreatic islet grafts is a major obstacle for application of the technology to treat diabetes. Accumulating evidence supports that incorporation of extracellular matrix (ECM) molecules in the capsules enhances longevity of pancreatic islets. After selection of the most efficacious laminin sequence *in vitro*, we show *in vivo* that inclusion of collagen IV and RGD in alginate-based microcapsules enhances survival, insulin secretion function, and mitochondrial function. It also suppresses fibrosis by lowering proinflammatory cytokines secretion. Moreover, transcriptomic analysis shows that ECM-inclusion promotes insulin-secretion related pathways and attenuates inflammation and hypoxic stress related pathways in islets. We show that inclusion of ECM in immunoisolating devices is a promising strategy to promote long-term survival of islet-grafts.

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1. Introduction

Type 1 diabetes (T1D) is a metabolic disorder caused by autoimmune destruction of insulin-producing pancreatic β cells [1]. Exogenous insulin is currently the only therapeutic option but cannot regulate the glucose levels as precisely as pancreatic β cells. The fluctuation in glucose levels after chronic administration of insulin can lead to diabetic complications such as angiopathy and nephropathy [2]. To achieve strict glycemic control, transplantation of pancreatic islets is proposed [3] but its application is restricted as it requires lifelong administration of immunosuppressive drugs which as such are associated with severe side effects [4–6].

Chronic immunosuppression can be prevented by immunoisolation of islets by encapsulation in semipermeable membranes which isolate grafts from host immune cells and antibodies but allow diffusion of glucose, insulin and nutrients such as oxygen [7]. The feasibility of curing diabetes with encapsulated islet-grafts has been demonstrated in several studies but a major obstacle for application is that graft survival is usually limited to several months and never permanent [8,9].

An important factor in the limited duration of graft survival of transplanted pancreatic islets might be insufficient interactions with extracellular matrix (ECM) molecules [10]. During the isolation procedure of islets from the pancreas, enzymes are infused into the pancreas to disconnect the exocrine tissue from the endocrine tissue [11]. This breakdown of ECM connections between exocrine-endocrine tissue is not restricted to these islet-exocrine interface but also affects ECM molecules in the endocrine tissue [11,12]. Many ECM molecules that surround the islets and interconnect the endocrine cells have been reported to be damaged after islet isolation [11,13], impacting islet-function and survival [14,15].

Recently, we have shown that specific types of ECM molecules may benefit the fate of islets in immunoisolating microcapsules. Especially collagen IV and specific laminin sequences such as RGD, LRE, and PDSGR had a positive effect on glucose induced insulin release of islets *in vitro*. Other ECM molecules were ineffective or even damaging islets in certain concentrations [10,14–16]. These same beneficial ECM molecules were also effective in reducing cytokine-mediated cell death in islet-cells. All combinations of collagen IV with either RGD, LRE or PDSGR improved islet-cell survival and reduced necrosis and apoptosis after IL-1β, IFN-γ, and TNF-α exposure [17,18]. However, there were also laminin specific effects. Collagen IV + RGD and collagen IV + LRE reduced danger-associated molecular patterns (DAMPs) released from islets while PDSGR was ineffective. Moreover, oxygen consumption rate (OCR) of islets was only beneficially influenced by collagen IV + RGD and collagen IV + PDSGR and to a lesser extent by LRE inclusion [15,17]. These *in vitro* studies demonstrated that inclusion of ECM may benefit isolated islets, but the effect is specific for the type of included ECM-components. Whether the effects have any impact on islet function *in vivo* is unknown and is the subject of this study.

In our study we tested the effects of inclusion of collagen IV and either RGD, LRE or PDSGR on functional survival of allogenic mice-islet grafts in immunoisolating capsules *in vivo*. To study the fate of the islets in capsules supplemented with ECM in the same animal at several time points, we used islets isolated from transgenic mice expressing luciferase. Islets in capsules containing collagen IV and either RGD, LRE or PDSGR were implanted on the back of the mice to allow bioluminescence imaging. After 8 weeks of implantation, islet-grafts were retrieved for *ex vivo* evaluation of glucose induced insulin release, OCR and a transcriptomics study on differentially regulated genes in the islets was performed to determine the impact and specificity of the ECM supplementation on islet function. In a subsequent long term follow up study we determined the effect of ECM inclusion in the core of alginate microcapsules on longevity of islet allografts.

2. Methods

2.1. Animals

MIP-Luc-VU mice (Jackson Laboratory, ME, USA) served as islet donors. The mice were bred in our own facility. Male athymic nude mice were obtained from Charles River (Wilmington, NC, USA) and male C57BL/6 albino mice were purchased from Envigo (Cambridgeshire, UK). They were used as recipients. Animals were housed at the central animal facility (CDP) and maintained under 12 h light/dark cycles with *ad libitum* access to water and standard chow. All experiments were approved by both the local animal ethical committee of the University of Groningen and the national ethical commission for experimental animal use. All surgical procedures and bioluminescence imaging were performed under general anesthesia with isoflurane (1.5% in 98.5% O_2).

2.2. Genotyping

The MIP-Luc-VU line were genotyped on tissue obtaining by an ear punch. DNA extraction was performed using the prepGEM® Tissue kit (ZyGEM^{TM}, Southampton, UK). The genotype and copy number of the transgene were determined by PCR. The primer sequence for the luciferase gene were 5'-GAATGTCCGTTCGGTTGGC AGAAGC-3' and 5'-CCAAAACCGTGATGGAATGGAACAACA-3' and for the control, 5'-CAATGTTGCTTGTCTGGTG-3' and 5'-GTCAGTCGA GTGCACAGTT-3'. Female heterozygous mice were bred with wild-type siblings or with FVB/NJ.

2.3. Diabetes induction

Diabetes was induced in athymic nude mice and C57BL/6 albino mice (males, 8-10 weeks of age) by a single intraperitoneal injection of streptozotocin (STZ, 180 mg/kg, in 0.1 M citrate buffer, pH 4.5). Blood glucose measurements were obtained from tail vein blood measured with a Accu-check glucose meter (Ascensia Contour, Bayer, NJ, USA) and glucose test tapes (Contour, Bayer, Switzerland). If diabetes was not established within 1 week (defined as blood glucose levels $>$ 25 mM), a second dose of STZ of 220 mg/kg was administered. Mice were monitored at least once a week for their glycemic state and weight.

2.4. Islet isolation

The pancreas of MIP-Luc-VU mice were distended by injecting 2 mL collagenase solution (1 mg/mL) in Hanks' Balanced Salt solution containing 25 mM HEPES via the pancreatic duct. Islets were isolated by dissection of the splenic portion of the pancreas as previously described [19]. Islets were washed 5 times with RPMI 1640 (Lonza, Basal, Switzerland) supplemented with 10 % newborn calf serum (Gibco, DE, USA) and cultured in CMRL 1066 (Gibco, DE, USA) supplemented with 8.3 mM D-glucose, 1% penicillin/streptomycin (Gibco, DE, USA) and 10% fetal calf serum (FCS) (Gibco, USA) at 37°C, 5% CO_2 overnight.

2.5. Microencapsulation and incorporation of extracellular matrix

The applied laminin peptides were obtained from GenScript (NJ, USA). These were the peptide sequences 0.01 mM RGD, 1 mM LRE, 0.01 mM PDSGR. The laminin peptides were combined with 50 µg/ml collagen type IV (Sigma, the Netherlands) and mixed with 3.2-3.4% purified alginate (ISP Alginate, Girvan, UK) solution. The

composition of alginate was studied by nuclear magnetic resonance (NMR). It was composed of 44% G-chains, 56% M-chains, 23% GG-chains, 21% GM- chains, 37% MM-chains [20]. After the gelation of the alginate, the laminin and collagen fibers were entrapped within the alginate network as previously described [15,17]. The alginate applied was purified and tested for absence of endotoxins or pathogen associated molecular patterns as previously described [21,22]. Alginate without any ECM served as control. The alginate solution was converted into droplets with a droplet-generator [23,24]. Droplets were gelled in 100 mM $CaCl_2$ solution for 5 min to allow complete gelification. The droplets had a final diameter of 700 µm. All droplets were washed with Krebs-Ringers-Hepes (KRH) buffer containing 2.5 mM $CaCl_2$ for 2 min. After encapsulation, capsules were inspected under the microscope and capsules with imperfections or that were broken were discarded.

2.6. Islet transplantation

For the first study involving comparison of survival of islets in capsules containing different types of peptide sequences, four pockets were created on the back of nude mice by funneling with a blunt surgical probe underneath the skin via an incision of 3 mm. At least 2 cm space was kept in between the pockets to avoid floating of the capsules into neighboring pockets. Subsequently, the microcapsules suspended in 0.5 mL of KRH were gently injected via a syringe connected to a 16-G blunted cannula. The grafts containing at least 300 islets per group were: alginate (control), alginate containing collagen IV + 0.01 mM RGD, collagen IV + 1 mM LRE, and collagen IV + 1 mM PDSGR. Encapsulated islets were circulated with every experiment at the back to avoid that a specific group was always studied at the same location at the back of the mice. By using this strategy, each group was studied at least one time at the lower left, the lower right, the upper left, and the upper right pocket at the back. There were no differences in outcome per experimental group at the different locations. During the 8 weeks follow up, non-fasting blood glucose levels were measured at least once a week. After 8 weeks, mice were euthanized, and the capsule grafts were removed for further analysis.

To study longevity in a fully immunocompetent mouse, 1000 islets were transplanted in the peritoneal cavity of diabetic C57BL/6 mice in alginate capsules containing collagen IV with a selected ECM that was found to have benefits for islet survival in the first set of experiments. Capsules without ECM served as controls. For intraperitoneal implantation, a small incision was made in the skin and the abdominal muscle layer. The microcapsules suspended in 0.5 ml of KRH were gently injected via a syringe connected to a blunt cannula. The incision was closed with a suture. None of the recipients received immunosuppressive drugs before or after islet transplantation. Graft rejection was defined as 2 consecutive blood glucose measurements of above 20 mM. The day of the first measurement of blood glucose > 20 mM was considered the day of rejection. After graft failure, mice were terminated, and the capsule grafts were removed by peritoneal lavage by flushing the peritoneal cavity 3 times with 5 ml of KRH. Retrieval rate was always above 90% and processed for histological analysis.

2.7. Bioluminescence imaging

All bioluminescence imaging was performed using an IVIS 100 SSD camera (Xenogen, caliper Alameda, CA) or IVIS Spectrum *In Vivo* Imaging System (PerkinElmer Inc., Waltham, MA, USA) as previously described [25]. The substrate D-luciferin was injected subcutaneously or intraperitoneally at a saturating dose of 150 mg/kg body weight. Before starting the experiments, the settings were calibrated on full MIP-Luc-VU mice and on isolated islets. Before starting the experiments, we confirmed that 300 islets and 1000 islets were sufficient to study the fate of bioluminescence after implantation under the skin or in the peritoneal cavity, respectively. Below 30 islets we were not able to find any reliable signal under the skin.

Bioluminescence signals were measured from approximately 1 min after luciferin administration to 20 min post injection. Bioluminescence intensity was analyzed using Living Image 4.3 software (PerkinElmer Inc., Waltham, MA, USA). To quantify emitted light, regions of equal surface area were drawn around the region of interest (ROI), and average photons/sec/cm^2/steradian were determined as previously described [26].

2.8. Assessment of cellular overgrowth and histology

The degree of cellular overgrowth was quantified microscopically in bright fields and was defined as the percentage of capsules with overgrowth as percentage of the total number of harvested capsules. After that, harvested capsules were fixated in pre-cooled 2% paraformaldehyde. This was replaced by 6% sucrose in PBS (-), and embedded in glycol methacrylate (GMA, Technovit 8100, Germany) as previously described [27]. The GMA embedded capsules were sectioned at 2 µm and processed for staining and the sections were dried at 37°C. The sections were stained with Toluidine Blue Solution to visualize and quantify capsules with cellular adhesion.

For immunohistochemistry, the sections were incubated with 0.01% trypsin (6.8 mM 0.1% $CaCl_2$ and 0.1M Tris-HCl, pH 7.8) for 10 min at 37°C. Subsequently, they were incubated with an anti-mouse insulin antibody (Cell signaling 4590s, 1:100 in PBS + 1% BSA) for 2 h at 37°C. Nonspecific binding was blocked by a 5 min incubation with 10% normal goat serum. A rabbit anti-mouse alkaline phosphatase conjugated secondary antibody (Dako, Heverlee, Belgium; 1:100 in PBS + 1% BSA) was applied for 45 min. Alkaline phosphatase activity was demonstrated by incubating the sections for 10 min with SIGMAFAST Fast Red (Sigma-Aldrich, St. Louis, MD, USA). Hematoxylin was used as counterstain.

2.9. Ex vivo *glucose-induced insulin secretion*

After retrieval of the capsules from the implantation site, islets were tested for the capacity to secrete insulin upon a glucose challenge. Encapsulated islets (25 islets) were preincubated for 1.5 h in 2 ml KRH, gassed with 95% O_2 and 5% CO_2, containing 0.25% BSA, and 2.75 mM glucose. The incubations were performed in an incubator at a stirring rate of 120 cycles/min at 37°C. This is our conventional protocol. Stirring is needed to support diffusion of insulin out of the encapsulated islets. The quantitative insulin secretion was then assessed by three consecutive incubations in low glucose concentration solution in KRH (2.75 mM) for 1 h, high glucose concentration solution in KRH (16.5 mM) for 1 h, and low glucose concentration solution in KRH (2.75 mM) for another 1 h. At the end of each incubation, media was taken and frozen for insulin measurement via Enzyme-Linked Immunosorbent Assay (ELISA) (Mouse Insulin ELISA, Mercodia AB, Sweden) [23]. DNA content of islets was quantified with a fluorescent Quant-iT PicoGreen double-strand DNA (dsDNA) assay kit (Invitrogen, Carlsbad, CA, USA). The insulin secretory responses were expressed as nanogram of insulin/mL/µg (DNA)/hour.

2.10. Oxygen consumption rate measurement

OCR was measured in islets using the extracellular flux analyzer XF24 (Seahorse Bioscience, MA, USA), as previously described in detail [15,28]. This was done after removal of the alginate capsule around the islets by incubating the capsules for 20 min with 25 mM citrate solution at 37°C as alginate interferes with the measurements. Between 80 and 100 islets per condition were incu-

bated overnight in CMRL 1066 (Gibco, DE, USA) with 8.3 mM D-glucose, penicillin/streptomycin (1%) (Gibco, DE, USA), and 10% FCS (Gibco, DE, USA) at 37°C. After a washing step, islets were prepared for analysis and equilibrated in modified Seahorse XF assay medium (MA media; pH 7.4) at 37°C, supplemented with 3 mM glucose, and 1% FCS. Islets were subsequently plated by pipetting the islets into the wells together with 500 µL of MA media. Four wells were kept as blank, empty controls. To avoid bubble formation in the screen-net in the XF sensor cartridge, screens were prewetted with MA media. The plates were then incubated for 60 min at 37°C before it was loaded into the XF24 machine. The assay test-reagents were added at either 60 or 130 min. The test reagents were either glucose (16.7 mM final) or the mitochondrial inhibitor oligomycin (5 µM). All reagents were adjusted to pH 7.4. Baseline rates were measured at 37°C five times before sequentially injecting glucose (16.7 mM) or mitochondrial inhibitors-oligomycin (5 µM). After the addition of each reagent, five readings were taken.

2.11. RNA isolation and microarray processing of pancreatic islets

RNA was purified from pancreatic islets using TRIzol (Life Technologies, Bleiswijk, the Netherlands) followed by an additional round of purification with RNeasy Microkit columns (Qiagen, Venlo, the Netherlands). RNA quality was assessed using RNA 6000 nanochips on the Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). Total RNA (10 ng) was labelled using a GeneChip WT pico reagent kit and hybridized to whole genome Genechip Mouse Gene 2.1 ST arrays, (Life Technologies, Bleiswijk, the Netherlands). Sample labelling, hybridization to chips and image scanning were performed according to manufacturer's instructions.

Quality control of the microarray data was performed using Bioconductor packages [29] integrated in an online pipeline [30]. Expression values were calculated using the robust multichip average (RMA) method, which includes quantile normalization [31], probe sets were defined according to Dai et al. [32]. To identify differential gene expression induced by collagen IV + RGD, collagen IV + LRE, and collagen IV + PDSGR after 8 weeks exposure in the subcutaneous site of nude mice, we applied Intensity-Based Moderated T-statistics [33] (IBMT, treatment *versus* control *i.e.* islets in capsules without ECM) and genes with an IBMT *P*-value < 0.05 were selected for further data analyses.

To gain insights into the biological role of the genes which were differentially expressed in islets treated with ECM, we performed Gene Set Enrichment Analysis [34]. In addition, Ingenuity Pathway Analysis (Qiagen, The Netherlands) was used to identify differentially expresses canonical pathways and potential upstream regulators.

2.12. Intraperitoneal glucose tolerance test (IPGTT)

Recipient C57BL/6 albino mice underwent an IPGTT at 8 weeks after transplantation. After 4 h of fasting, the mice received a glucose solution (3 g glucose/kg body weight) intraperitoneally. Blood samples were collected from the tail vein at -5, 0, 5, 10, 20, 30, 60, 90, and 120 min after glucose administration and glucose levels were determined at each time point. All blood samples were kept on ice and centrifuged at 2000 g for 10 min at 4°C. The plasma was collected and stored at −80°C until determination of the plasma C-peptide concentration by Mouse C-peptide ELISA kit (Crystal Chem Inc., IL, USA).

2.13. Statistical analysis

Data were analyzed using GraphPad Prism (version 8.0; GraphPad Software, Inc., La Jolla, USA). Normal distribution of the data was confirmed using the Kolmogorov– Smirnov test. Comparisons between two groups were performed using student t-test for parametric distributed data, while nonparametric data were analyzed with the Mann-Whitney test. Comparisons among three or more groups were performed using one-way analysis of variance (ANOVA) for parametric data, while nonparametric data were analyzed with a Kruskal–Wallis test, followed with post hoc Dunnett's test. Bioluminescence intensity data were analyzed by two-way ANOVA and post hoc multiple comparisons tests. A log-rank test was used to compare graft survival between the experimental and the control group. $P < 0.05$ were considered statistically significant.

3. Results

3.1. ECM-peptides promotes functional performance of islets in microcapsules

MIP-Luc-VU mouse islets encapsulated in alginate-capsules supplemented either with collagen IV + 0.01 mM RGD, collagen IV + 1 mM LRE, collagen IV + 1 mM PDSGR were implanted under the skin of STZ-induced diabetic (STZ-) athymic nude (nude) mice ($n = 10$). Islets in alginate capsules without ECM served as controls. The pretransplant blood glucose of STZ-nude mice was 27.6 ± 3.0 mM which decreased to normoglycemic levels within 10.2 ± 4.5 days after subcutaneous implantation of the grafts (Fig. 1A).

After subcutaneous graft implantation into nude mice, luciferase bioluminescence intensity, which is related to islet graft functional survival, was studied at 1, 4 and 8 weeks after implantation. Before starting the implantation study, we confirmed that we could reliably measure bioluminescence of subcutaneous grafts in the range of 30 to 300 islets. Fig. 1B shows a representative picture of a nude mouse at day 1 and at 1 and 8 weeks after implantation. After luciferin injection, *in vivo* bioluminescence peaked at 5 to 20 min, and could be reassessed repeatedly for 8 weeks in the same animal.

Fig. 1C shows the luciferase activity in the grafts. We found a strong enhancing effect of RGD and LRE but not of PDSGR on luciferase activity in the grafts at 4 and 8 weeks. Supplementing the intracapsular environment with collagen IV + RGD enhanced luciferase activity 2.7-fold at 4 weeks ($P < 0.05$) and 3.2-fold at 8 weeks ($P < 0.01$) compared to the activity at day 1 after implantation. LRE did not enhance luciferase activity. Grafts supplemented with collagen VI + RGD had 6.5-fold ($P < 0.001$) enhanced luciferase activity compared to controls.

At the end of the bioluminescence experiments, animals were sacrificed, and the capsules were harvested. Both macroscopically and microscopically, we found no signs of inflammation or adhesion of fibroblasts or inflammatory cells on the capsule surfaces. Islets in the capsules were vital and contained insulin (Fig. 1D, E).

3.2. ECM incorporation in alginate-based microcapsules improves insulin release and islet-cell viability ex vivo

To study whether the addition of selected combinations of ECM molecules to the intracapsular environment impacted islet function, we subjected the retrieved islet-containing capsules from the subcutaneous sites to glucose-stimulated insulin secretion tests and OCR-measurements to confirm viability and to gain insight in ECM-specific promotion of function. Retrieved islets in alginate-capsules secreted insulin in response to glucose concentration changes at 8 weeks after implantation irrespective of the type of ECM applied (Fig. 2A). Stimulation index (stimulated divided by basal insulin release) of encapsulated grafts supplemented with collagen IV + RGD was significantly higher than that of controls.

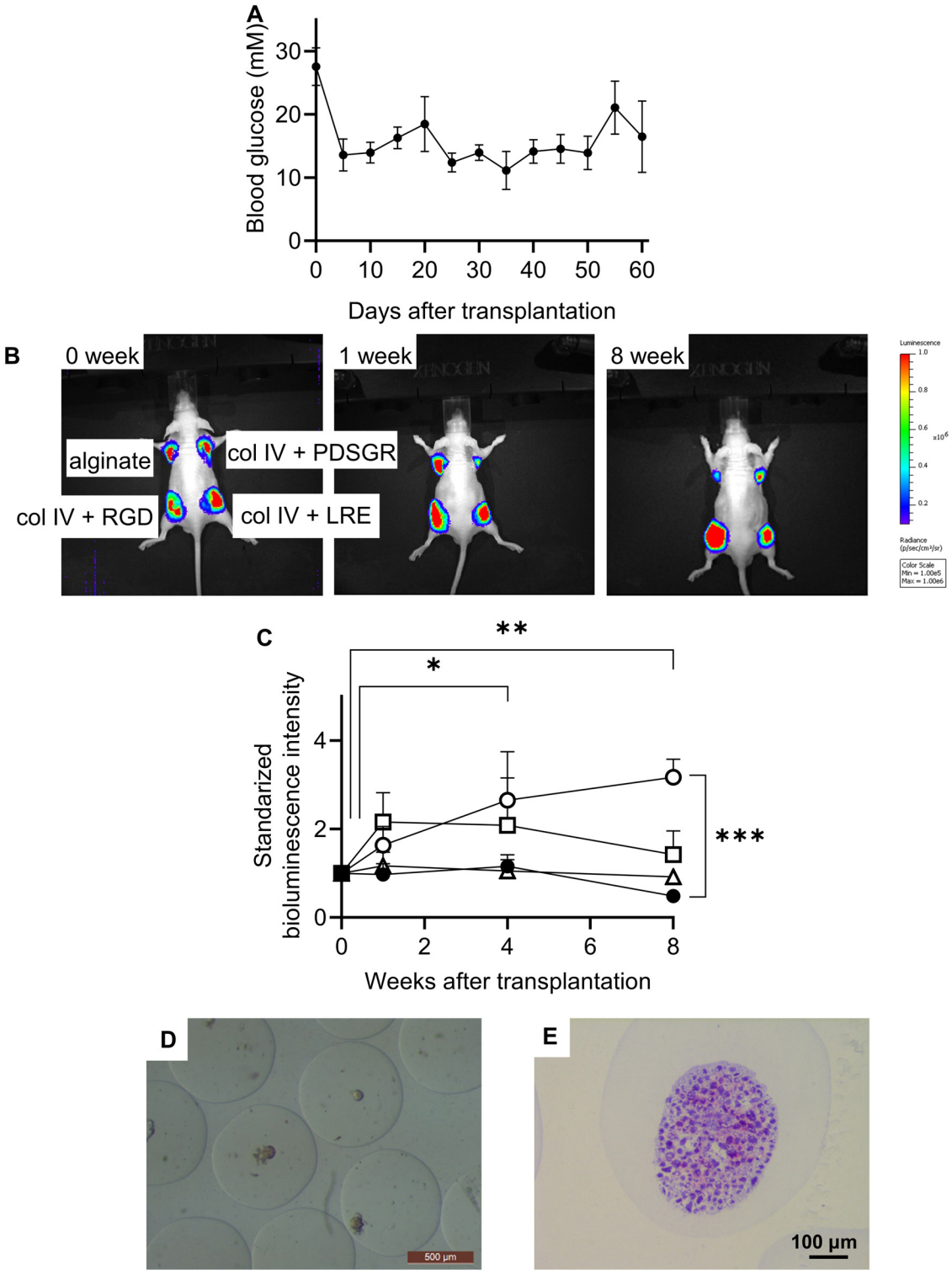

Fig. 1. Survival of bioluminescent MIP-Luc-VU mouse islets encapsulated in capsules with different types of extracellular matrix (ECM) under the skin of athymic nude mice. (A) Blood glucose levels of diabetic nude mice transplanted subcutaneously with 4 groups of alginate encapsulated islets (n=10). Data are expressed as mean ± SEM. (B) Representative IVIS-images of diabetic nude mice at 0, 1 and 8 weeks after implantation of encapsulated islet-grafts. Each image was adjusted for optimal color scale using Living Image software by applying the same photon ranges. (C) The standardized bioluminescence intensity of the encapsulated grafts under the skin (n = 5). The values were normalized to the signal on the day of implantation (set on 1). Closed circles are recipients of islets encapsulated in alginate-capsules without ECM (controls), open circles are islets in alginate-capsules supplemented with 50 µg/mL collagen type IV and 0.01 mM RGD, open squares are in alginate-capsules supplemented with 50 µg/mL collagen type IV and 0.1 mM LRE, and open triangles are in alginate-capsules supplemented with 50 µg/mL collagen type IV and 1mM PDSGR. Each data point represents mean ± SEM. Statistically significant differences were quantified using two-way ANOVA and post-hoc Dunnett's multiple comparisons test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (D) MIP-Luc-VU mouse islets encapsulated in alginate-based microcapsules supplemented with 50 µg/mL collagen type IV and 0.01 mM RGD after 8 weeks of implantation. (E) MIP-Luc-VU islets in alginate capsules containing 50 µg/mL collagen type IV and 0.01 mM LRE explanted 8 weeks after subcutaneous transplantation. Encapsulated islets in 2 µm glycol-methacrylate (GMA)-embedded sections were immunostained with rabbit anti-insulin antibody and counterstained with hematoxylin. Scale bar is 100 µm.

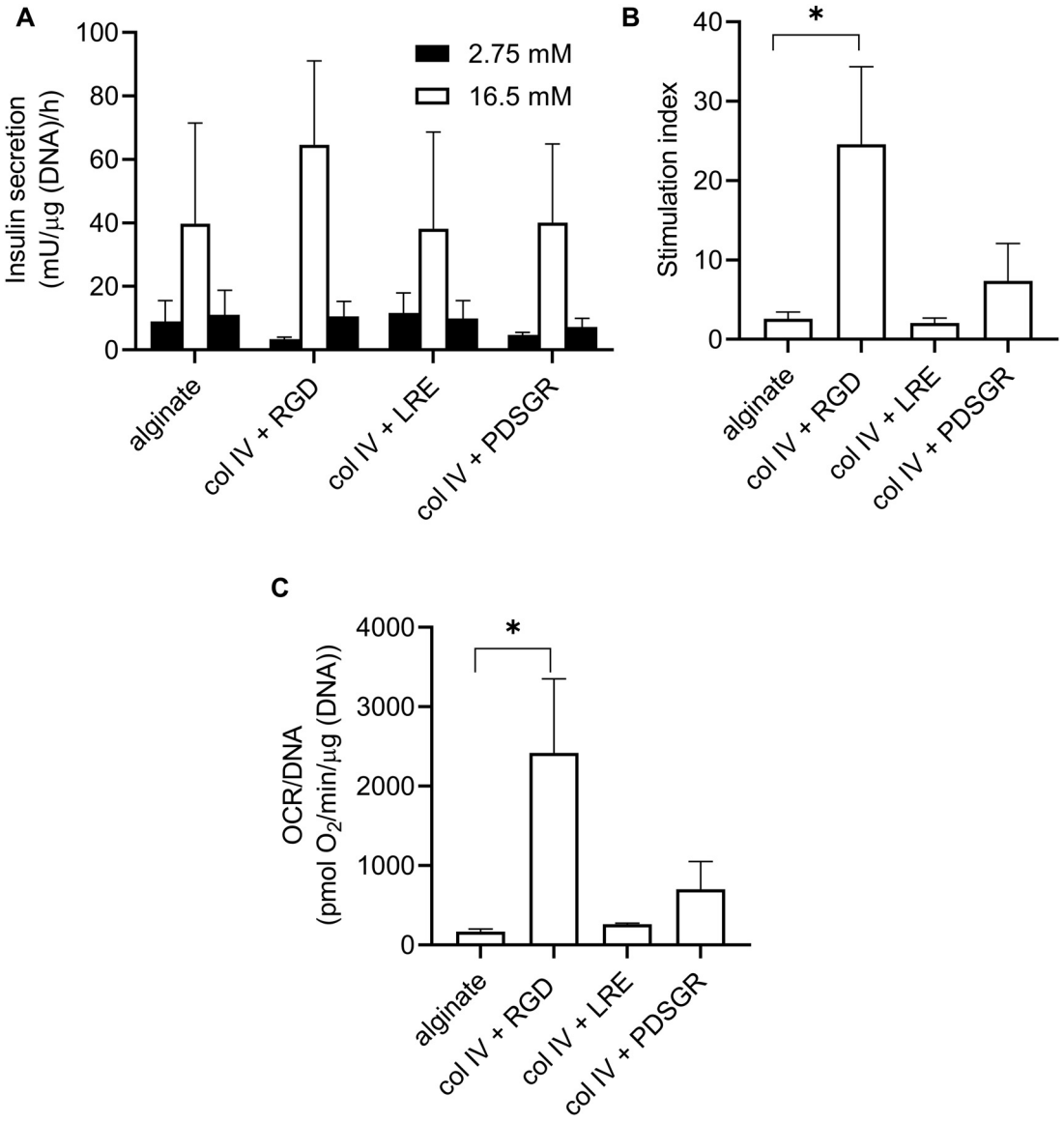

Fig. 2. Effect of ECM incorporation on functional survival of encapsulated islet grafts *ex vivo* after 8 weeks of subcutaneous implantation in mice. (A) Glucose-stimulated insulin secretion of explanted MIP-Luc-VU mouse islets encapsulated in alginate-based microcapsules supplemented with 50 µg/mL collagen type IV and either 0.01 mM RGD, 0.1 mM LRE, or 1mM PDSGR after 8 weeks of implantation. Control islets were encapsulated in an alginate microcapsule without ECM. Glucose stimulated insulin release was tested after graft explanation after 8 weeks of implantation. Insulin release was measured after exposure to low (2.75 mM), high (16.5 mM), and a second incubation in low glucose for 1 h. (B) Insulin secretion stimulation index of encapsulated islets (n = 5: alginate, LRE, and PDSGR, n = 6: RGD). (C) Oxygen consumption rates of explanted MIP-Luc-VU mouse islets encapsulated in alginate-based microcapsules supplemented with 50 µg/mL collagen type IV and either 0.01 mM RGD, 0.1 mM LRE, or 1mM PDSGR after 8 weeks of implantation. Control islets were encapsulated in an alginate microcapsule without ECM. Islets retrieved from microcapsules were tested in a Seahorse Bioscience XF24 extracellular flux analyzer (n = 3: alginate, n = 4: RGD, LRE, and PDSGR). Each data point represents mean ± SEM. Statistically significant differences were quantified using one-way ANOVA or Kruskal–Wallis test followed by post-hoc Dunnett's multiple comparisons test. $^{*}P < 0.05$.

It was 9.6-fold higher ($P < 0.05$, Fig. 2B). OCR was statistically enhanced in islets from capsules containing collagen IV + RGD but not in islets from capsules containing collagen IV + LRE nor collagen IV + PDSGR (Fig. 2C). The effects of collagen IV + RGD on OCR of the islets was strong and was 2418.2 ± 934.9 pmol O_2/min/µg DNA which was 14.3-fold higher than the OCR of the control group ($P < 0.05$).

3.3. Gene expression in encapsulated islet grafts exposed to ECMs

To get further insight in which pathways influenced function of encapsulated islets when exposed to specific laminin sequences and/or collagen IV a comprehensive gene analysis was performed.

Fig. 3A–D shows the number of differentially expressed genes compared to controls ($P < 0.05$, fold-change > 2 or < -2) in islets encapsulated in capsules containing collagen IV and either RGD (829 genes), LRE (1320 genes), or PDSGR (1383 genes). Independent of the type of laminin sequence 726 genes were differentially expressed compared to capsules composed of only alginate. Interestingly we found that the expression of *Il33*, which is a so-called alarmin and associated with inflammation [35,36], was significantly lower and the expression of *Pdx1*, which is known to be an essential factor involved in pancreatic development and adult β cell function [37], was significantly higher with all laminin treatments. In addition to that, only RGD enhanced some of gene expressions which are involved in the mitochondrial activity *e.g. SLC4A8* and *Slc25a22* (FC = 2.02 and 1.36, $P = 0.0044$ and 0.0133, respectively) against control.

Gene Set Enrichment Analysis (GSEA) was performed to determine which gene sets were impacted. There were 84, 188,

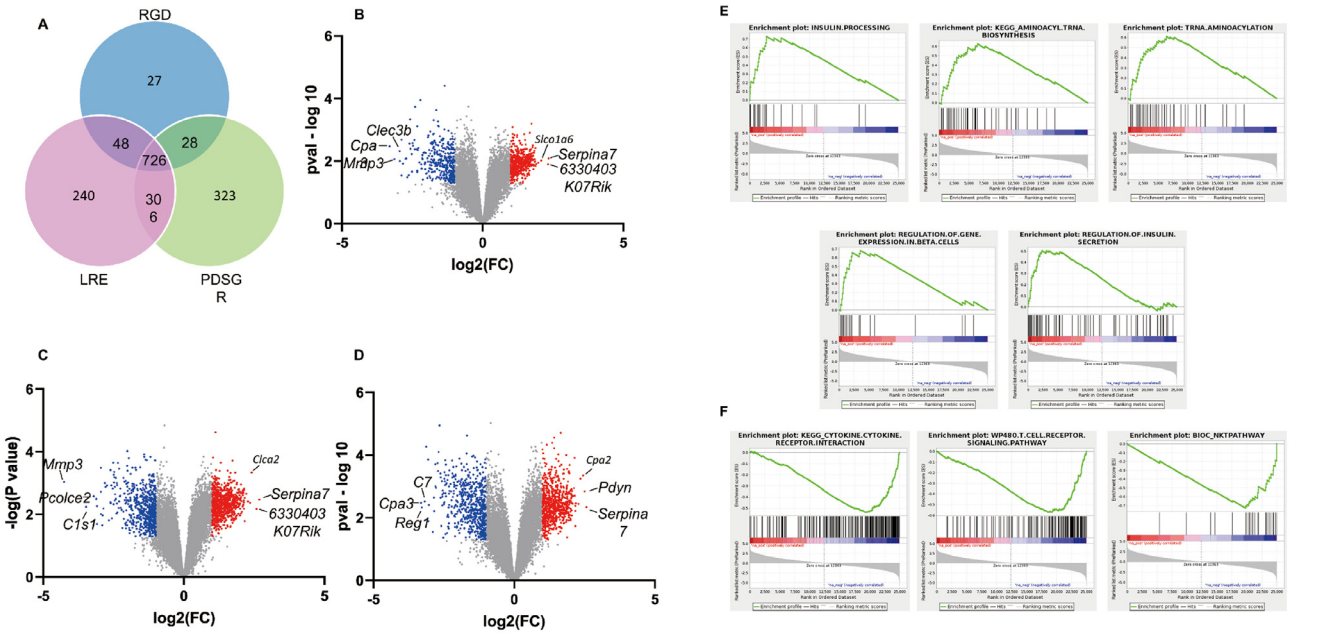

Fig. 3. Gene expression changes of encapsulated islets implanted subcutaneously and supplemented with different ECM combinations at 8 weeks after implantation. (A) A Venn diagram of differentially expressed genes compared between islet grafts in control alginate and islet grafts treated with collagen IV and either 0.01 mM RGD, 1 mM LRE, or 1 mM PDSGR for 8 weeks ($P < 0.05$ and fold changes > 2 or < -2). (B–D) Volcano plots on whole gene expression of islet grafts compared between islet grafts in control alginate and islet grafts treated by laminin sequences, collagen IV and either (B) 0.01 mM RGD, (C) 1 mM LRE, or (D) 1 mM PDSGR for 8 weeks. Red filled circles indicate genes that showed significantly higher expression in treated mice ($P < 0.05$, fold change > 2, n = 5). Blue filled circles indicate significantly lower gene expression in treated mice ($P < 0.05$, fold change < -2). The x axis shows the log2 of the fold change of gene expression in encapsulated grafts with each laminin treatment relative to their expression in control alginate. The y axis indicates the − log10 of the *P* value. Representative gene names are shown. (E, F) Enrichment plots on gene sets (E) show higher expression levels and (F) lower expression levels in grafts treated with collagen IV and RGD against control alginate. n = 5 per group.

55 upregulated gene sets, and 520, 625, 483, downregulated gene sets (False Discovery Rate < 0.25) in respectively RGD, LRE, and PDSGR when compared with control alginate (Table S1). Gene sets related to insulin secretion *e.g.,* insulin processing and regulation of insulin secretion, and mitochondrial activity *e.g.,* tRNA aminoacylation, showed higher expression levels (Fig. 3E) and gene sets involved in immune reactions and inflammation *e.g.*, T-cell receptor signaling pathways and cytokine-cytokine receptor interactions, showed lower expression levels in all three laminin inclusions (Figs. 3F, S1).

These gene expression changes were further analyzed with Ingenuity Pathway Analysis (IPA). We observed 15 canonical pathways that were significantly affected in all treatment groups compared with control ($P < 0.05$, z score > 2 or -2) (Table S2). Notably, the canonical pathways Insulin Secretion Signaling Pathway was identified as significantly upregulated by all three laminins (Fig. S2A). The pathways involved in inhibition of matrix metalloproteinases and LXR/RXR activation tended to be identified as potentially activated (z-score = 1.89 and 1.387, respectively). In contrast, acute phase response signaling, IL-8 signaling and complement system activation, which are involved in inflammation were predicted to be downregulated in all three groups, as well as HIF1α and eNOS signaling which are regulated by cells under hypoxic stress [38,39] (Fig. S2B–D).

As shown in Fig. 2, RGD had a somewhat better impact on insulin secretion and a profound effect on OCRs of islets that were retrieved. This stronger effects of RGD might be related to the 5 and 117 genes which were differently affected by RGD compared to LRE and PDSGR, respectively (Table S3). Upstream regulated gene analysis shows that compared to LRE, RGD treatment significantly downregulated *NUPR1*, which supports β cell proliferation [40,41] as well as *l-asparaginase*, which prevents reduction of pancreatic β cell mass [42]. This RGD effect might have been substantiated by the higher expression of gene sets related to adherens junctions, ECM organization, and ECM receptor interactions which are involved in regulation of insulin secretion and facilitate survival of pancreatic β cells [43,44] (Fig. S2E) as follows from GSEAs comparison of LRE versus RGD.

3.4. Long-term graft survival of islet grafts encapsulated in alginate with collagen IV + RGD

As we found that incorporation of collagen IV + RGD had benefits for islet grafts, we next investigated the impact of collagen IV + RGD on allograft survival of encapsulated islets. To again allow follow up of bioluminescence of the grafts after implantation, we implanted 1000 MIP-Luc VC mice islets intraperitoneally in fully immunocompetent STZ diabetic C57BL/6 albino mice.

Recipients of islets in control capsules became normoglycemic (< 10 mM) in 5.4 ± 2.5 days. This was somewhat shorter but not statistically significantly different in recipients receiving encapsulated islets with collagen IV + RGD which were normoglycemic in 10.7 ± 1.9 days after implantation (Fig. 4A). The control group showed 125 ± 19.7 days of mean survival time and none of recipients were normoglycemic after 200 days. This was different in recipients of islets in collagen IV + RGD containing capsules as visualized in the Kaplan Meijer plot (Fig. 4B). The mean survival time of collagen IV + RGD group is 211 ± 24.1 days which is significantly longer than in the controls ($P < 0.05$) and 4 of 6 recipients in the group demonstrated normoglycemia longer than 200 days. Fig. 4C shows retrieved encapsulated islets in collagen IV + RGD capsules at 299 days after implantation. There is no inflammation or overgrowth on the surfaces of capsules and we still can see viable islets in the capsules.

IPGTTs were performed at 8 weeks after transplantation. The blood glucose of the recipients reached a maximum glucose level in 30 min and then gradually returned to a normal range within 120 min after glucose challenge (Fig. 4D). The plasma C-peptide

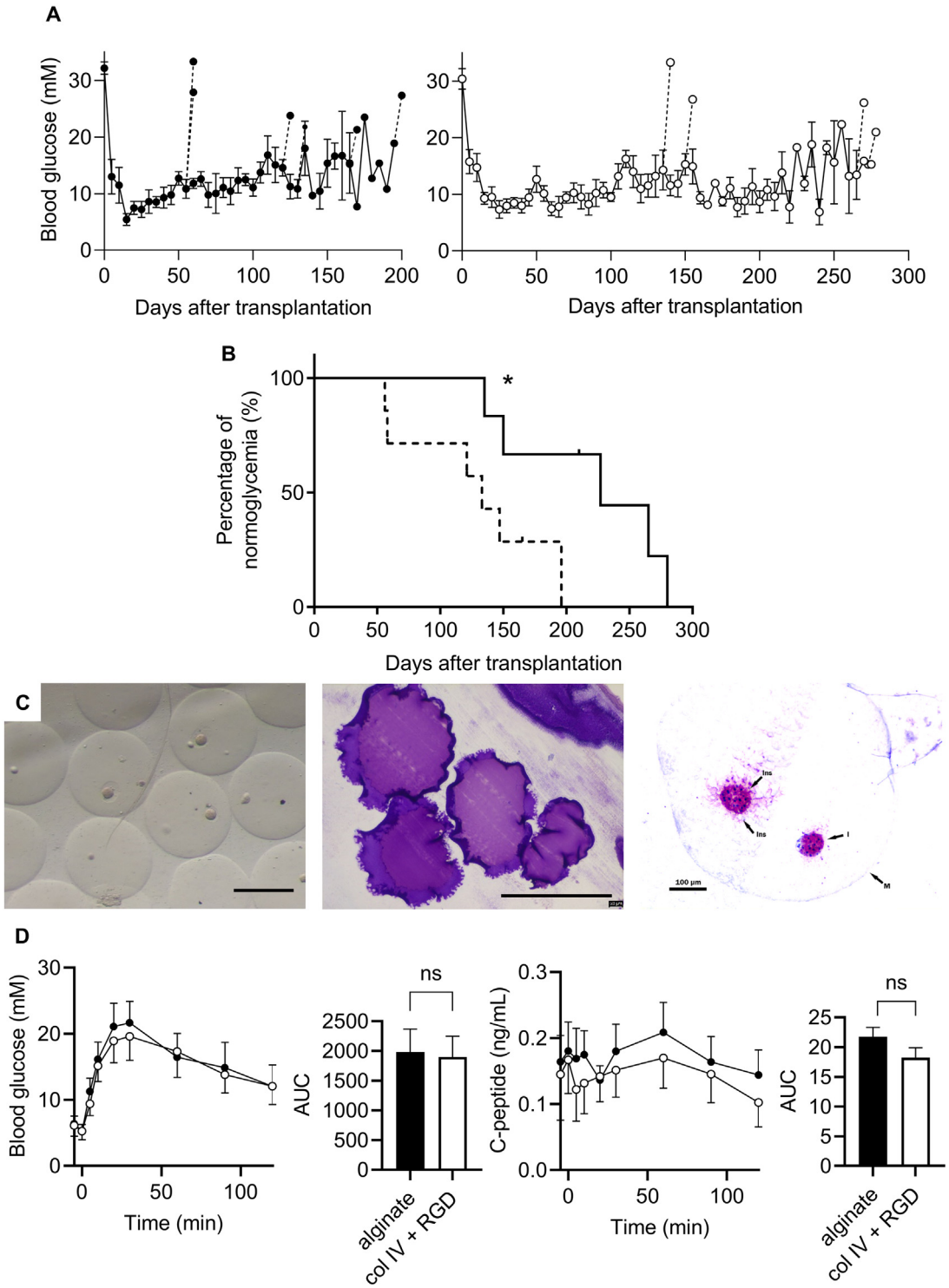

Fig. 4. Incorporation of collagen IV and RGD in alginate encapsulated islets prolongs longevity of islet allograft in absence of any immunosuppression. (A) Blood glucose levels of diabetic C57BL/6 albino mice after allotransplantation of 1000 MIP-Luc-VU mouse islets either in control alginate (closed circles, n = 7) and in alginate-based microcapsules supplemented with 50 µg/mL collagen type IV and 0.01 mM RGD (open circles, n = 6) into the peritoneal cavity. Values represent mean ± SEM. Dashed lines indicate graft failure. (B) Kaplan-Meier plot of graft survival. Dash line and solid line indicate recipients received MIP-Luc-VU mouse islets in control alginate and in alginate microcapsules with col IV + RGD, respectively. Statistically significant differences were quantified using log-rank test. $^{*}P < 0.05$. (C) Bright field images of encapsulated isles in alginate microcapsules with col IV + RGD harvested 299 days after implantation. Toluidine blue staining of harvested encapsulated islet in 2 µm GMA-embedded sections. Scale bars are 500 µm. Encapsulated islets in GMA-embedded sections were immunostained with rabbit anti-insulin antibody. Membrane of alginate capsule (M). There was no adhesion of inflammatory cells, islets (I), and insulin (Ins). Scale bar is 100 µm. (D) The levels of blood glucose and plasma C-peptide secretion during intraperitoneal glucose tolerance test at 8 weeks after implantation. Each data point represents mean ± SEM. Statistically significant differences were quantified using student t-test.

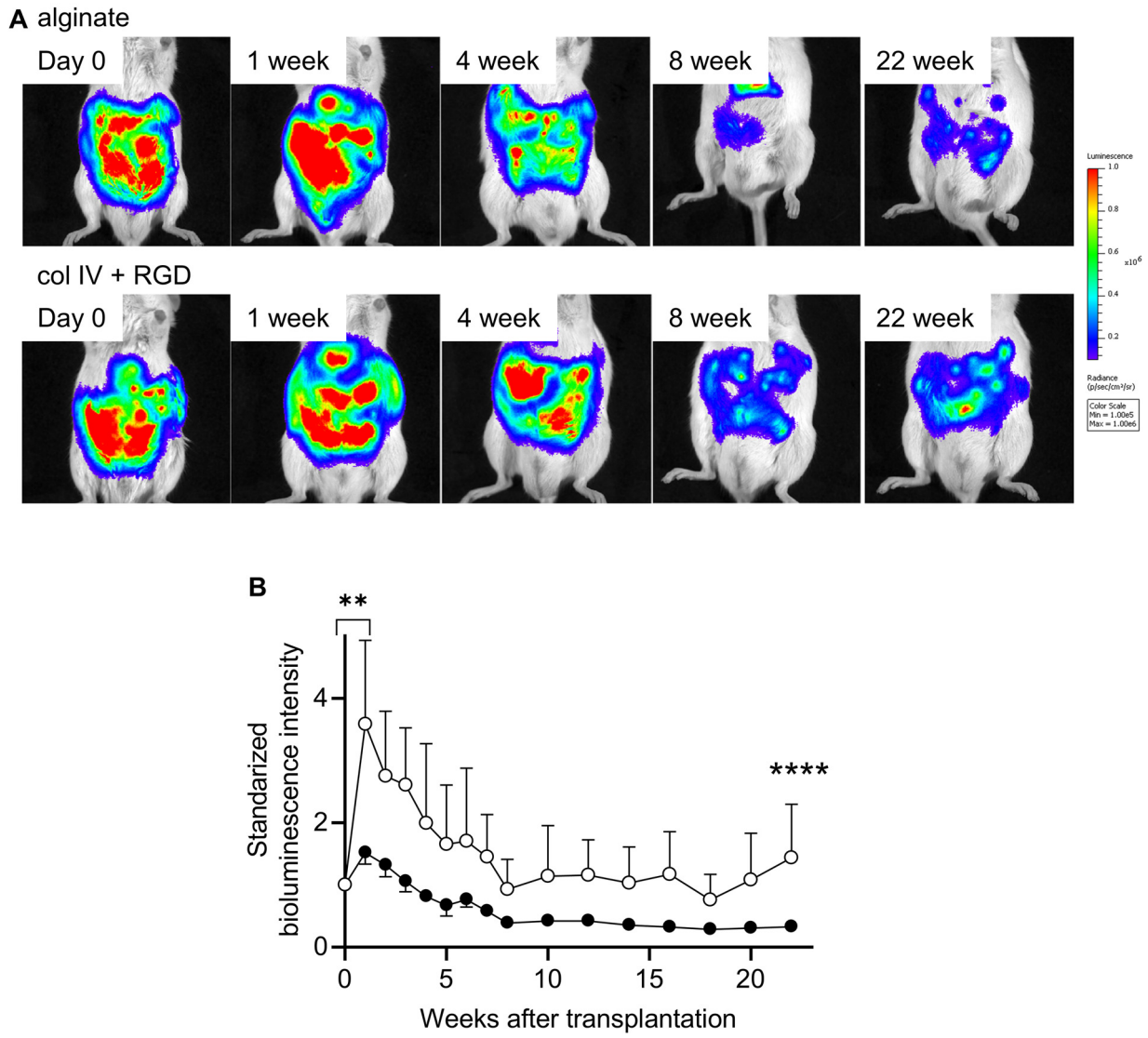

Fig. 5. Incorporation of collagen IV and RGD improves and maintains luciferase activity of MIP-Luc-VU mouse islets in alginate-based microcapsules. (A) Representative images of diabetic C57BL/6 albino mice at 0, 1, 4, 8, and 22 weeks after islet transplantation. Each image was optimally adjusted using Living Image software with the same photon scale. (B) The standardized bioluminescence intensity of alginate encapsulated MIP-Luc-VU mouse islets in the peritoneal cavity. (n = 3-7 for each time point). The values were normalized to the signal of the day of implantation (set on 1). Closed circles indicate recipients receiving islets encapsulated in control alginate capsules and open circles indicate recipients receiving islets encapsulated in alginate capsules containing col IV + RGD. Each data point represents mean ± SEM. Statistically significant differences were quantified using two-way ANOVA and post-hoc Sidak's multiple comparisons test. $^{**}P < 0.01$; $^{****}P < 0.0001$.

levels elevated in response to glucose changes. The areas under the curve for blood glucose changes and C-peptide response did not differ significantly between recipients of islets encapsulated in alginate containing collagen IV + RGD and islets in control alginate-capsules.

Bioluminescence was traced until 22 weeks after implantation. Fig. 5A shows representative pictures of mice receiving islets encapsulated in alginate containing collagen IV + RGD and in control alginate at respectively the day of implantation, 1, 4, 8, till 22 weeks after implantation. As shown the bioluminescent signal varies throughout the peritoneal cavity and is not stuck to one position such as the mesenterium, liver capsules or other organ illustrating the free-floating nature of the grafts. Fig. 5B demonstrates that the most pronounced effects of collagen IV + RGD in capsules is observed in the immediate period after transplantation where a statistically significant 3.6-fold higher luciferase signal than that of ECM-free controls was observed ($P < 0.01$). This signal declined gradually but remained at least 2.1-fold higher than in the control during the 22 weeks study period. The inclusion of collagen IV + RGD was demonstrated to have statistically significant effects on the luciferase activity during the 22 weeks compared with control ($P < 0.0001$, two-way ANOVA).

3.5. Lower degree of fibrotic overgrowth on the surface of capsules by the supplement of collagen IV + RGD

We harvested encapsulated grafts after failure to determine cellular responses on microcapsules. In general, only a very small portion of the capsules was affected by cellular overgrowth (Fig. 6 A, B). A portion of 2.9 ± 0.8% of the retrieved control alginate capsules were containing cellular overgrowth on the surface (Fig. 6C). In the collagen IV + RGD microcapsules grafted mice, only 0.5 ± 0.3% were affected by overgrowth which was lower than with control alginate capsules ($P < 0.05$).

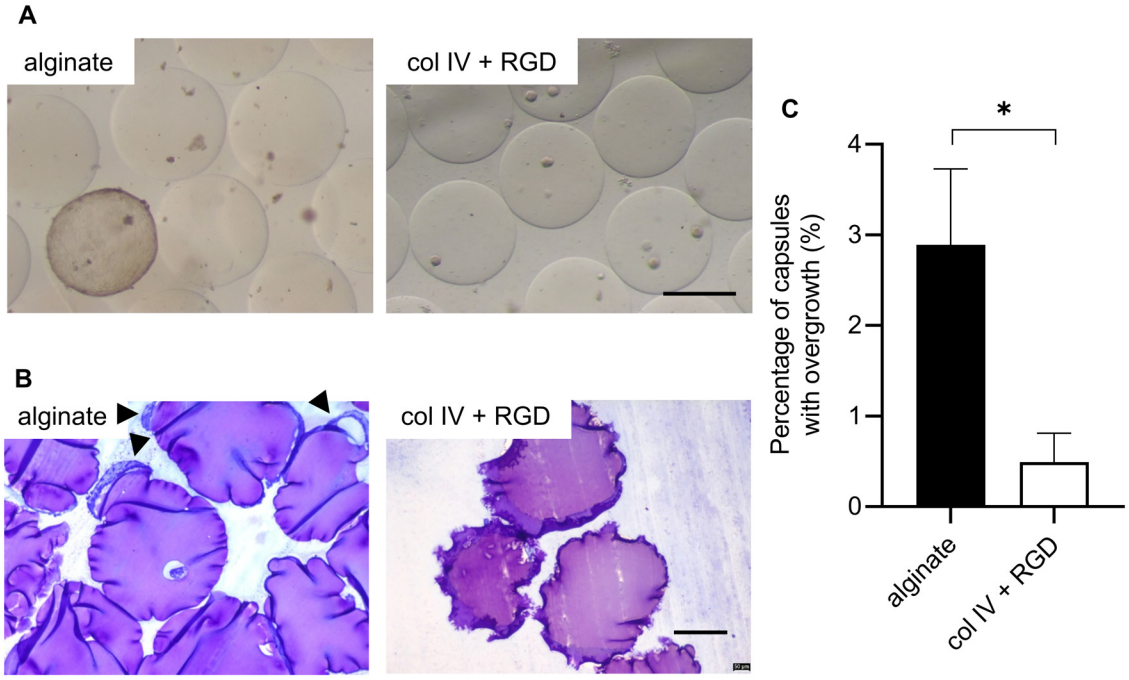

Fig. 6. Attenuation of foreign body reactions by incorporation ECM in alginate encapsulated islets. (A) Bright field images of overgrowth on the surface of encapsulated islets. Scale bar is 500 µm. (B) Toluidine blue staining of harvested encapsulated islet in 2 µm GMA-sections. Arrows indicate inflammatory cell adhesion on a small portion of capsules. Scale bar is 200 µm. (C) Percentage of encapsulated islets covered partly or totally by fibrotic or inflammatory cells. Each data point represents mean ± SEM. (n = 5: alginate, n = 6: RGD) The statistical difference between two groups was quantified using Mann-Whitney test. $^{*}P < 0.05$.

4. Discussion

We provide *in vivo* evidence that inclusion of specific ECM molecules impact function of pancreatic islets in an ECM-type dependent fashion. Based on previous studies we selected collagen IV and specific laminin sequences for this *in vivo* study as these were the only three combinations that had demonstrated efficacy in supporting islet function *in vitro* [15,16]. Other tested components were either ineffective or attenuated islet function *in vitro* [16,45]. *In vitro* we observed specific effects of the three laminin sequences. Here we report the first *in vivo* findings and demonstrate that even after 8 weeks of implantation beneficial effects of laminin inclusion in immunoisolating capsules can be observed on insulin secretion and OCR.

OCR *in vivo* was highly impacted by RGD but not by LRE and PDSGR which contrasts *in vitro* findings where LRE had the most potent OCR enhancing effect. This discrepancy between *in vitro* and *in vivo* should be explained by short versus long term exposure of islets to laminin sequences. RGD binds to many members of the integrin family, including $\alpha3\beta1$ $\alpha5\beta1$, $\alpha5\beta3$, $\alpha v\beta3$ and $\alpha v\beta5$ on pancreatic islets [46,47]. The laminin adhesive recognition sequence PDSGR present in the $\beta1$ chain [48], is known to accelerate cell-proliferation [49]. LRE has also been reported to guide cellular processes [50] but LRE, in contrast to RGD and PDSGR is lacking integrin binding capacity for subunits $\alpha3$ and $\alpha5$ and $\beta1$ [47,50]. In fact, absence of these subunits in LRE and especially the ligand for $\alpha5$ on adult mouse islets may therefore be the cause of the lesser beneficial effect of this laminin sequence [51]. PDSGR has been reported to stimulate $\alpha6\beta1$ integrins on mouse islets [52,53], while RGD peptides bind to $\alpha5\beta1$ integrins on islets [46]. This might explain the pronounced differences in *ex vivo* function and gene regulation observed in this study.

RGD was the only laminin sequence that enhanced OCR. This corresponded to enhanced expression of genes associated with oxygen consumption in RGD-exposed islets as illustrated by our transcriptomics analysis. Only RGD significantly enhanced the expression of *SLC4A8* (FC = 2.02, $P = 0.0044$) which is involved in mitochondrial activity and the positive-regulated *Slc25a22* (FC = 1.36, $P = 0.0133$) which is related with mitochondrial glutamate transportation and has a critical role in metabolite-transport into mitochondria in islets [54–56]. The finding of strong enhancing OCR effects by RGD is considered to be beneficial as it has been shown that higher OCR correlates with longer function of transplanted islets [15,57,58].

RGD also had a beneficial effect on glucose induced insulin secretion illustrating again the specificity of the function-promoting effects of ECM components. The tripeptide RGD is one of the most studied sequences [59] and known to induce cellular functions such as adhesion and spreading. This recognition sequence interacts as outlined above with many members of the integrin family, including $\alpha3\beta1$ $\alpha5\beta1$, $\alpha5\beta3$, $\alpha v\beta3$ and $\alpha v\beta5$ [46,47]. *In vitro* studies on encapsulated islet grafts containing RGD corroborate our findings on stimulating effects on graft viability [60], but to the best of our knowledge a systemic comparison of islets in capsules without RGD or with other ECM molecules *in vivo* has not been performed till now. The bioluminescence analysis demonstrates that RGD has a strong and significant enhancing effect on functional survival of encapsulated grafts at 4 and 8 weeks after implantation. We observed a twofold higher bioluminescent signal of islets in RGD incorporated capsules *in vivo* than in capsules without RGD.

Our transcriptomics study illustrates that not only RGD but all laminin sequences influence gene expression and several pathways in islets that might be responsible for the observed improved longevity. All laminin treatments showed lower expression of inflammation signals such as the alarm signal *i.e.* IL33 that cells release when encountering stress or cellular damage [61]. This finding corroborates our previous finding that ECM inclusion in islet grafts may downregulate release of DAMPs which we consider to be one of the essential mechanisms by which ECM incorporation

supports graft survival. IL33 attenuation might contribute to this as IL33 binds to the IL-1RL1/ST2 receptor on T-cells and mast cells, basophils, eosinophils and natural killer cells and may contribute to enhanced immunity and foreign body responses. Our data suggest that this DAMP and IL33 release is lowered by incorporation of ECM and is possibly an explanation for the lower fibrosis observed in the RGD-supplemented grafts in the longevity study. In addition to that, all laminin inclusions showed higher expression of genes related to the canonical pathway of insulin secretion, and lower expression of genes related to canonical pathways involved in acute proinflammatory responses. We also found an activation tendency of the LXR/RXR pathway in encapsulated islet grafts. This suggests enhanced control of insulin secretion and biosynthesis in pancreatic β cells [62]. Overall, these are all ECM dependent processes indicating beneficial effects on islet function at 8 weeks after implantation.

Additionally, our study shows some specific effects of RGD that might explain the higher impact on functional survival of islets. RGD regulated *Nupr1* and *L-asparaginase* which are involved in pancreatic β cell mass and proliferation, and gene sets related to adherens junctions, ECM organization, and ECM receptor interactions which are involved in regulation of insulin secretion and β cell survival [43,44]. This effect of RGD was stronger than after LRE exposure.

The inclusion of collagen IV + RGD significantly prolonged longevity of allografts in absence of any immunosuppression. This strong beneficial effect was most pronounced in the first week after implantation where the luciferase activity was 2.4-fold higher than in controls. This should be explained by the fact that RGD makes islets less susceptible for cytokine stress [17] and is therefore less vulnerable for the loss of islets in the inflammatory environment directly after implantation. This effect was long lasting as we found that the grafts with collagen IV and RGD were having a twofold higher viability than the controls during the study period of 22 weeks. It however did not lead to a better response to the IPGTT at 8 weeks after implantation. This should be explained by the fact that at that time point the metabolic capacity in the grafts with or without ECM was still adequate to respond to the glucose load.

In summary our study demonstrates that inclusion of ECM in the intracapsular environment of immunoisolating microcapsules promotes islet-cell survival and function in an ECM dependent fashion. Collagen IV in combination with RGD had the most pronounced effects as it enhanced OCR and the glucose induced insulin release. It was also demonstrated that collagen IV in combination with RGD inclusion has a profound effect on islet graft survival and suppresses fibrosis possibly by lowering secretion of proinflammatory molecules such as IL33 by islets. We show that grafts exposed to ECM components may survive for periods of up to a year *in vivo*. Our findings provide new insights in means to establish a clinically applicable encapsulation strategy that guarantees long term survival of grafts for the treatment of T1D.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2022.12.068.

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