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Full length article

Inclusion of extracellular matrix molecules and necrostatin-1 in the intracapsular environment of alginate-based microcapsules synergistically protects pancreatic β cells against cytokine-induced inflammatory stress

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A B S T R A C T

Immunoisolation of pancreatic islets in alginate-based microcapsules is a promising approach for grafting of islets in absence of immunosuppression. However, loss and damage to the extracellular matrix (ECM) during islet isolation enhance susceptibility of islets for inflammatory stress. In this study, a combined strategy was applied to reduce this stress by incorporating ECM components (collagen type IV/RGD) and necroptosis inhibitor, necrostatin-1 (Nec-1) in alginate-based microcapsules *in vitro*. To demonstrate efficacy, viability and function of MIN6 β-cells and human islets in capsules with collagen type IV/RGD and/or Nec-1 was investigated in presence and absence of IL-1β, IFN-γ and TNF-α. The combination of collagen type IV/RGD and Nec-1 had higher protective effects than the molecules alone. Presence of collagen type IV/RGD and Nec-1 in the intracapsular environment reduced cytokine-induced overproduction of free radical species and unfavorable shifts in mitochondrial dynamics. In addition, the ECM components collagen type IV/RGD prevented a cytokine induced suppression of the FAK/Akt pathway. Our data indicate that the inclusion of collagen type IV/RGD and Nec-1 in the intracapsular environment prevents islet-cell loss when exposed to inflammatory stress, which might contribute to higher survival of β-cells in the immediate period after transplantation. This approach of inclusion of stress reducing agents in the intracapsular environment of immunoisolating devices may be an effective way to enhance the longevity of encapsulated islet grafts.

Statement of significance

Islet-cells in immunoisolated alginate-based microcapsules are very susceptible to inflammatory stress which impacts long-term survival of islet grafts. Here we show that incorporation of ECM components (collagen type IV/RGD) and necrostatin-1 (Nec-1) in the intracapsular environment of alginate-based capsules attenuates this susceptibility and promotes islet-cell survival. This effect induced by collagen type IV/RGD and Nec-1 was probably due to lowering free radical production, preventing mitochondrial dysfunction and by maintaining ECM/integrin/FAK/Akt signaling and Nec-1/RIP1/RIP3 signaling. Our study provides an effective strategy to extend longevity of islet grafts which might be of great potential for future clinical application of immunoisolated cells.

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1. Introduction

Type 1 diabetes (T1D) is characterized by chronic hyperglycemia resulting from autoimmune destruction of insulin-producing β-cells [1]. Patients with T1D require lifelong insulin therapy. Currently, insulin administration via the subcutaneous site is the most common treatment but this therapy cannot prevent regular hyper- and hypoglycemic events [2]. Transplanting insulin-producing cells is currently one of the few therapies that offer a minute-to-minute regulation of glucose metabolism and is therefore a preferred therapy for T1D. Unfortunately, its widespread application is still limited by donor shortage and the need for lifelong administration of immunosuppressive drugs [3,4].

The need for immunosuppression can be avoided by immunoisolating pancreatic islets in microcapsules [5,6]. These capsules provide a physical barrier for the isolated islets and protect them from the host's immune system [6–9]. The capsules are semi-permeable and do allow ingress of essential survival factors and egress of secreted insulin [6,10]. Successful transplantation of microencapsulated islet grafts has been demonstrated in rodents, non-human primates and even in some humans [11–13] but despite these successes and demonstrated metabolic efficacy, graft function has been always limited and varies from several months to a year [14–16]. Consequently a key challenge for application of microencapsulated islets is to identify factors that contribute to prolonged longevity of the grafts [7].

Many factors have been mentioned as causative for the limited graft survival of encapsulated islets of which insufficient supply of essential nutrients such as oxygen and occurrence of a foreign body reaction (FBR) are the most well-studied [17]. However, even in small capsules where oxygen supply is of lesser essence for survival and in absence of a FBR, grafts still have a limited life span [7,11]. An important contributing factor might be insufficiencies in the islet-microenvironment in capsules. During the isolation procedure, the islets' microenvironment is destroyed, and associated with loss of the abundant interactions with the extracellular matrix (ECM) components. This impairs β-cell functioning and survival [18]. It was recently shown in pancreatic islets that ECM components are important for the viability and survival of islets and for normal insulin secretion and that ECM even protects against cytokine induced cytotoxicity [19–21]. The supplementation of ECM molecules, especially collagen type IV and laminins such as RGD, in immunoisolating capsules or polymeric scaffolds has been reported to preserve β-cell survival and function *in vitro* [21–23].

As the consequence of the disturbed interactions with ECM molecules and higher susceptibility for proinflammatory stress, isolated islets may suffer after isolation from loss of cells by necroptosis [21,24–26]. This might be prevented by necrostatin-1 (Nec-1). Nec-1 is a specific allosteric receptor-interacting protein-1 (RIP1) targeted inhibitor of necroptosis and its application for preventing β-cell injury has been demonstrated in recent years [27]. The protective role of Nec-1 for preserving pancreatic β-cell function has been demonstrated in several *in vitro* studies [28–30]. It is however currently unknown whether and how supplementation of Nec-1 or together with ECM components in the intracapsular environment can contribute to the survival of islets in microcapsules. Especially the immediate post-transplantation period is a vulnerable period as islets are exposed to a proinflammatory environment in which reportedly up to 60% of the tissue might be lost [31].

This study was designed to gain insight in how supplementation of the intracapsular environment with essential ECM components such as the abundantly present collagen type IV and RGD and Nec-1 contributes to the survival of pancreatic islets. This was done in the presence and absence of the proinflammatory cytokines interleukin 1-β (IL-1β), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). To this end, we investigated the impact of incorporation of ECM components (i.e., collagen type IV and RGD) and Nec-1, or a combination thereof in the intracapsular microenvironment on the survival and function of a mouse β-cell line MIN6 and human islets in the presence and absence of proinflammatory stress. As proinflammatory cytokines provoke cell death via mitochondria dysfunction and induce the generation of free radical species, we also studied mitochondrial fusion and fission gene expression. The underlying signaling pathways were also investigated.

2. Materials and methods

2.1. Cell culture

MIN6 β-cells (ATCC, Manassas, VA, USA) were cultured in DMEM high-glucose medium (Lonza, Basal, Switzerland), containing 15% fetal bovine serum, 50 μmol/L β-mercaptoethanol, 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured with 5% CO_2 at 37 °C in humidified air.

2.2. Human islets isolation and culture

Human pancreatic islets were isolated from cadaveric pancreata at the Leiden University Medical Center and the European Consortium for Islet Transplantation (provided through the JDRF award 31-2008-416, Islet for Basic Research program, Milan, Italy), as previously described [32,33]. The detailed information of islet and donor data are mentioned at Table 1. All the procedures were approved and carried out in accordance with the code of proper secondary use of human tissue in The Netherlands as formulated by the Dutch Federation of Medical Scientific Societies.

After shipment to the University Medical Center Groningen, islets were cultured in CMRL-1066 (GIBCO, Bleiswijk, the Netherlands), supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin, as previously described [33].

2.3. Microencapsulation and treatments

Intermediate α-L-guluronic acid (G) alginate (ISP Alginate, Girvan, UK) was applied as previously described [34,35]. The capsules were composed of 3.4% alginate, this concentration was shown in our previous studies to have a viscosity to form spherical and uniform capsules with optimal biomechanical performance [36,37], and was supplemented with ECM components (50 μg/ml collagen type IV, 10 μM RGD), 100 μM Nec-1 or a combination of both. Mouse and human derived collagen type IV (Cat# C7521 and Cat# C0543), laminin sequence RGD (Cat# A8052) and Nec-1 (Cat# N9037) were all purchased from Sigma-Aldrich. The applied concentration of collagen type IV and RGD came from a previous study [21,22] and the chosen concentration of Nec-1 was selected after a pilot study where three different doses (50, 100, 200 μM) of Nec-1 were compared for protecting cells against cytokine stress. In this study we found that 100 μM had the most pronounced protective effect and was therefore applied for the current set of experiments (Supplementary Fig. S1).

MIN6 β-cells were mixed with alginate at a concentration of 5×10^6 cells/ml while islets were mixed at 1000 islets/ml. Droplets were produced with a droplet generator as previously described [11,38]. The droplets were transformed into microcapsules by collecting droplets in a 100 mM $CaCl_2$ (10 mM HEPES, 2 mM KCl) solution for 10 min. All droplets were washed with KRH buffer containing 25 mM $CaCl_2$ for at least 3 times. Capsules were handpicked and used in further experiments. The capsules had a final diameter of 500–600 μm.

Table 1
Islet and donor information.

Age	Gender	BMI	Blood type	Islet isolation center	Cause of death	Estimated purity (%)	Estimated viability (%)
57	F	27	O	ECIT center[1]	cerebral bleeding	60	95
53	F	27	A	ECIT center	cerebral bleeding	75	90
29	M	28	A	LUMC[2]	non cardiac	25	>80
69	M	22	O	LUMC	non cardiac	25	>80
52	M	27	O	ECIT center	cerebral bleeding	40	90

1 ECIT: European Consortium for Islet Transplantation. 2 LUMC: Leiden University Medical Center. BMI: body mass index.

To determine the influence of the components of capsules under stress, the cells and islets were then exposed to proinflammatory cytokines IL-1β, IFN-γ, and TNF-α (all from ImmunoTools, Friesoythe, Germany) in culture medium. The mouse or human cytokine mixture was IL-1β (150 U/mL), IFN-γ (2000 U/mL) and TNF-α (2000 U/mL). Encapsulated MIN6 β-cells and islets were incubated at 37 °C with or without cytokine stimulation during the study until further experiments performed on day 1, 5 and 7 post-encapsulation.

2.4. Mechanical stability

The mechanical stability of microcapsules was quantified with a Texture Analyzer XT plus (Stable Micro Systems, Godalming, UK) equipped with a force transducer with a resolution of 1 mN as previously described [36]. Briefly, single, individual microcapsules were handpicked under a dissection microscope (Leica MZ75 microsystems, Heerbrugg, Switzerland) equipped with an ocular micrometer with an accuracy of 25 µm. The mechanical stability of beads was measured by compressing individual microcapsules to 60% using P/25P mobile probe with a pretest speed of 0.5 mm/sec, a test speed of 0.01 mm/sec, and a posttest speed of 2 mm/sec. The trigger force was set to 2 grams. Texture Exponent software version 6.0 was used for data analysis (Stable Micro Systems).

2.5. Cell viability assays

MIN6 β-cell or islet-cell viability was determined by applying LIVE/DEAD Cell Viability/Cytotoxicity Assay Kit (Thermo Scientific) and the cell proliferation reagent WST-1 (Roche, Indianapolis, IN, USA). For the WST-1 test, which is also a measure for mitochondrial activity, the encapsulated MIN6 β-cells (15 capsules/well) and human islets (15 islets/well) were seeded in 96-well plates. MIN6 β-cells and human islets were cultured with or without cytokine exposure. On day 1, 5 and 7 after incubation, 10 µl/well of WST-1 was added to each well. Wells without capsules were used as blank control. After 60 min incubation at 37 °C, the absorbance was measured at 450 nm (reference at 650 nm) using a Bio-Rad Benchmark Plus microplate spectrophotometer reader (Bio-Rad Laboratories B.V, Veenendaal, The Netherlands). Capsules without incorporation with ECM components or Nec-1 was used as control.

For LIVE/DEAD staining, on day 1, 5 and 7, encapsulated MIN6 β-cells and islets cultured with or without cytokine mixture were incubated in 25 mM KRH buffer containing Calcein AM (1 mM) and Ethidium homodimer (EthD) (2 mM) for 30 minutes in the dark under room temperature as previously described [21]. After this incubation period, capsules were washed 5 times with 25 mM KRH buffer prior to imaging. All images were taken with a Leica SP8 confocal microscope (Wetzlar, Germany). The percentages of viable cells were measured using ImageJ software (Version 1.52; National Institutes of Health, Bethesda, MD, USA).

2.6. *In vitro* glucose-stimulated insulin secretion (GSIS) test

MIN6 β-cells and human pancreatic islets were tested for GSIS on day 1, 5 and 7. Briefly, 25 capsules containing MIN6 β-cells or 25 encapsulated human islets were transferred to glass incubation tubes. The first incubation consisted of a low glucose concentration (2.75 mM) solution in KRH buffer containing 25 mM $CaCl_2$ for 1 hour, followed by a high glucose concentration (16.7 mM) buffer in KRH for 1 hour, and another 1-hour incubation in 2.75 mM glucose in KRH. At the end of each incubation, the supernatants were removed for further insulin measurements via Enzyme-Linked Immunosorbent Assay (ELISA) (Mercodia AB, Sweden) using a spectrophotometric plate reader according to the manufacturer's protocol. Finally, insulin concentrations were calculated through the interpolation of sample absorbance values from the standard curves. DNA content of islets was quantified with a fluorescent Quant-iT PicoGreen double-strand DNA assay kit (Invitrogen, USA). The insulin secretory responses were normalized to total DNA content and expressed as µg L^{-1} µg^{-1} DNA hour^{-1} (mouse) or mU L^{-1} µg^{-1} DNA hour^{-1} (human).

2.7. Oxidative stress assays

Intracellular ROS was detected using a DCFDA Cellular ROS Detection Assay Kit (Abcam, Cambridge, UK) according to the instructions of the kit manufacturer. The NO concentration in the supernatant of the encapsulated MIN6 β-cells and the human islets was measured with a Nitric Oxide Assay Kit (Invitrogen, Vienna, Austria) according to the manufacturer's instructions.

2.8. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

A qRT-PCR was performed to determine the gene expression of mitochondrial fission and fusion in MIN6 β-cells and human islets after encapsulation and incubation with or without cytokines as described above. Alginate was removed by an incubation step in 25 mM citrate solution in calcium free KRH for 20 min. After capsule dissolving, MIN6 β-cells and human islets were homogenized with TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Total RNA was isolated following the manufacturer's instructions, followed by obtaining reverse-transcribed cDNA using SuperScript II Reverse Transcriptase (Invitrogen, USA). qPCR was performed with a FastStart Universal SYBR-Green Master kit (Roche, Indianapolis, IN, USA) for the genes listed below (primer sequences are listed in Table 2). Reactions were carried out in 384-well PCR plates (Thermo Scientific) with a ViiA7 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Delta Ct values were calculated and normalized to the housekeeping gene β-actin. The $2^{-\Delta\Delta Ct}$ method was used for the comparative quantification of gene expression of the genes.

2.9. Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). The Kolmogorov–Smirnov test was performed to determine parametric distribution of the data. Statistical differences of parametric data were analyzed using one-way ANOVA, while nonparametric data were analyzed with a Kruskal–Wallis test for comparisons among three or more groups, followed with Dunnett's post hoc

Table 2
Primer sequences for qRT-PCR.

Gene	Species	Forward Sequence 5′-3′	Reverse Sequence 5′-3′
β-Actin	Mouse	ACGGCCAGGTCATCACTATTC	AGGAAGGCTGGAAAAGAGCC
Mfn1	Mouse	TCTCCAAGCCCAACATCTTCA	ACTCCGGCTCCGAAGCA
Mfn2	Mouse	ACAGCCTCAGCCGACAGCAT	TGCCGAAGGAGCAGACCTT
Opa1	Mouse	TGGGCTGCAGAGGATGGT	CCTGATGTCACGGTGTTGATG
Drp1	Mouse	GCGCTGATCCCGCGTCAT	CCGCACCCACTGTGTTGA
Fis1	Mouse	GCCCCTGCTACTGGACCAT	CCCTGAAAGCCTCACACTAAGG
Il-6	Mouse	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
Cxcl-15	Mouse	GTCCTTAACCTAGGCATCTTCG	TCTGTTGCAGTAAATGGTCTCG
Il-10	Mouse	AGCCGGGAAGACAATAACTG	GGAGTCGGTTAGCAGTATGTTG
Il-1rn	Mouse	GGGACCCTACAGTCACCTAA	TCCCAGATTCTGAAGGCTTG
β-ACTIN	Human	CGCGAGAAGATGACCCAGAT	AGCACAGCCTGGATAGCAAC
MFN1	Human	TGGCTAAGAAGGCGATTACTGC	TCTCCGAGATAGCACCTCACC
MFN2	Human	CTCTCGATGCAACTCTATCGTC	TCCTGTACGTGTCTTCAAGGAA
OPA1	Human	TGTGAGGTCTGCCAGTCTTTA	TGTCCTTAATTGGGGTCGTTG
DRP1	Human	CTGCCTCAAATCGTCGTAGTG	GAGGTCTCCGGGTGACAATTC
FIS1	Human	GTCCAAGAGCACGCAGTTTG	ATGCCTTTACGGATGTCATCATT
IL-6	Human	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
IL-8	Human	ACTCCAAACCTTTCCACCCC	TTCTCAGCCCTCTTCAAAAACTTC
IL-10	Human	TCAAGGCGCATGTGAACTCC	GATGTCAAACTCACTCATGGCT
IL-1Ra	Human	CAATGCTGACTCAAAGGAGACGA	TCCCTCCATGGATTCCCAAGA

test. The data were analyzed using GraphPad Prism (v. 8.00; GraphPad Software Inc, La Jolla, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Design and characterization of microcapsules

To investigate whether the addition of ECM molecules or Nec-1 in the intracapsular microenvironment can protect pancreatic β cells from cytokine-induced cytotoxicity, we incorporated into the alginate matrix either 50 µg/ml collagen type IV and 10 µM RGD (col IV/RGD), 100 µM necrostatin-1 (Nec-1), or a combination of both (col IV/RGD + Nec-1) (Fig. 1**a**). The effect of incorporation of these molecules had no effect on size of the capsules which always had a diameter of 500–600 µm (Fig. 1**b-d**). The effect of collagen type IV/RGD and Nec-1 incorporation on mechanical stability of microcapsules was also investigated by performing a texture analyzer analysis. Within this technology we determined the force required for compressing microcapsules to 60% deformation (Fig. 1**e, f**) [36]. The incorporation of collagen type IV/RGD and Nec-1 did not have any significant effect on the mechanical stability as the force required for compression was always the same.

3.2. Incorporation of collagen type IV/RGD and Nec-1 in MIN6-containing microcapsules supports survival of islet cells

To investigate the effect of the ECM components and Nec-1 on the viability and function of insulin-producing β-cells, we encapsulated mouse MIN6 β-cells and cultured the β-cell containing capsules for 1, 5 and 7 days. Viability was determined by performing a LIVE/DEAD staining, a glucose stimulated insulin secretion (GSIS) test and by measuring mitochondrial activity with a WST-1 assay (Fig. 2).

Culturing of alginate encapsulated MIN6 β-cells in the absence of a cytokine challenge was associated with a gradual decline in cell-viability as the percentage of surviving cells went from 82 ± 3% on day 1 to 63 ± 3% on day 5 and to 47 ± 11% on day 7. (Fig. 2**a,b**) The decrease in the percentage of viable cells is probably due to the gradual growth of the cell population and gradual enhanced use of nutrients in the culture medium. We did not change the culture medium during incubation to keep the cells in the environment with generated free radical species. This viability decline was not influenced by addition of either collagen type IV/RGD, Nec-1 or a combination thereof. The GSIS was not statistically significantly influenced by the addition of the molecules (Fig. 2**c**). Also, no statistical differences were found in the stimulation index (Supplementary Fig. S2a). However, the addition of these molecules did have a profound effect on mitochondrial activity (Fig. 2**d**). We found that addition of collagen type IV/RGD upregulated the mitochondrial activity by 34% on day 1 ($p < 0.05$), 43% on day 5 ($p < 0.001$) and 100% on day 7 ($p < 0.001$). For addition to the intracapsular environment of Nec-1 this beneficial effect on mitochondrial activity was 33% on day 1 ($p < 0.05$), 18% on day 5 ($p = 0.24$) and 109% on day 7 ($p < 0.001$). Combining collagen type IV/RGD and Nec-1 had a synergistic effect and elevated the mitochondrial activity to 67% on day 1 ($p < 0.001$), 64% on day 5 ($p < 0.001$) and 132% on day 7 ($p < 0.001$) compared to the control.

We also tested incorporation of collagen type IV/RGD and Nec-1 in microcapsules containing pseudo-islets formed from MIN6 β-cells to confirm that effects are similar on single β-cells or β-cells in clusters (see Supplementary methods). The obtained pseudo-islets had a similar size as human islets, which is around 100 µm (Supplementary Fig. S3a). The pseudo-islets were then encapsulated in alginate-based microcapsules as described before (Supplementary Fig. S3b), followed by incubation for 7 days to test effects of collagen type IV/RGD, Nec-1 or the combination of both on viability. However, just like with single MIN6 β-cells we did not observe a statistically significant effect on cell viability (Supplementary Fig. S3c). However, in accordance with the results with MIN6 β-cells in single cell suspension, the incorporation of collagen type IV/RGD and Nec-1 increased mitochondrial activity of encapsulated MIN6 β-cell pseudo-islets (Supplementary Fig. S3d). The addition of collagen type IV/RGD enhanced mitochondrial activity by 2.1 ± 0.3 folds ($p < 0.05$) on day 5. The addition of Nec-1 also induced a 2.0 ± 0.2-fold ($p < 0.05$) increase of mitochondrial activity on day 5. The combination of collagen type IV/RGD and Nec-1 had the most pronounced effect on the mitochondrial activity where a 2.2 ± 0.3-fold ($p < 0.01$), 2.1 ± 0.3-fold ($p < 0.05$) and 1.7 ± 0.2-fold ($p < 0.01$) increase was observed on day 1, 5 and 7 respectively.

3.3. Incorporation of collagen type IV/RGD and Nec-1 in human-islet containing microcapsules prevents cell-dysfunction

Human cadaveric pancreatic islets were also tested for viability after encapsulation and supplementation with ECM components and Nec-1 in capsules. We observed a gradual decrease of viable

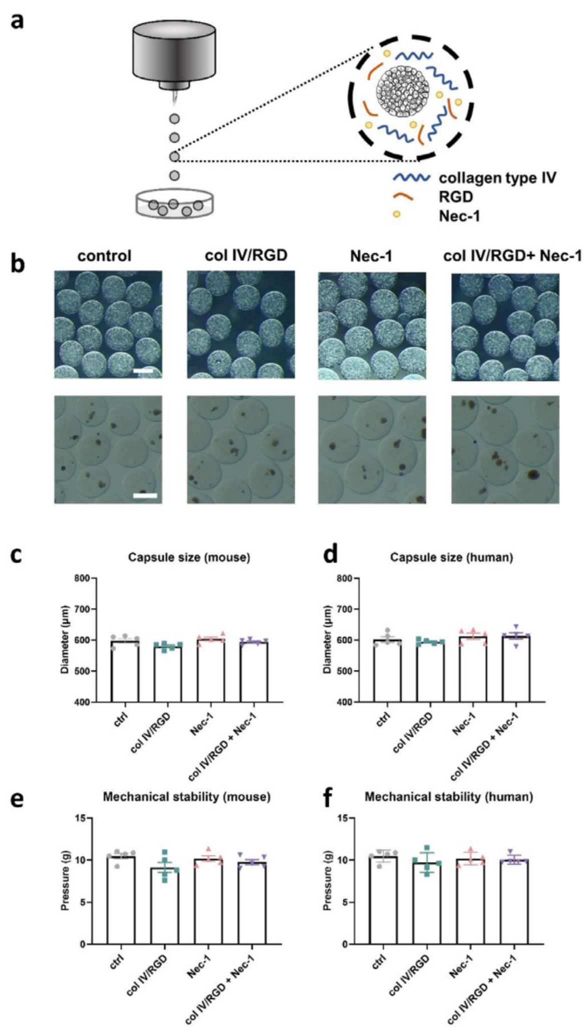

Fig. 1. Size and mechanical characteristics of alginate capsules, capsules containing 50 µg/ml collagen type IV and 10 µM RGD (col IV/RGD), 100 µM necrostatin-1 (Nec-1), or a combination of both (col IV/RGD + Nec-1). **(a)** Schematic representation of fabrication of alginate microcapsules. **(b)** Encapsulated MIN6 β-cells and human islets in different microcapsule types. Scale bar is 500 µm. **(c)** Capsule size data of encapsulated MIN6 β-cells and **(d)** human islets. **(e, f)** Mechanical stability data. Data are presented as mean ± SEM from 5 independent experiments (n = 5). The statistical differences were quantified using one-way ANOVA analysis with Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control group).

LIVE/DEAD stained islet cells when comparing day 1, 5 and 7 of culture (84%, 73% and 71% on day 1, 5 and 7, control group). The percentage of viable cells in human islets was higher than that in MIN6 β-cells. Also, we found no effects of the addition of collagen type IV/RGD and Nec-1 on cell viability (Fig. 3**a,b**). The GSIS also demonstrated no significant effect on the function of the islets (Fig. 3**c**, supplementary Fig. S2c).

However, as observed with MIN6 β-cells we did find a significant effect on mitochondrial activity. We found that addition of collagen type IV/RGD induced an increase in mitochondrial activity of 99% on day 1 ($p < 0.05$), but not on day 5 or day 7. For addition to the intracapsular environment of Nec-1 this was 121% on day 1 ($p < 0.01$), 26% on day 5 ($p < 0.05$) and 42% on day 7 ($p < 0.05$). Combining collagen type IV/RGD and Nec-1 had a synergistic effect and elevated the mitochondrial activity significantly to 154% on day 1 ($p < 0.001$), 41% on day 5 ($p < 0.001$) and 37% on day 7 ($p < 0.05$).

3.4. Collagen type IV/RGD and Nec-1 prevents β-cell viability loss during exposure to IL-1β, IFN-γ, and TNF-α

Next it was determined whether collagen type IV/RGD and/or Nec-1 can prevent loss of β-cells when exposed to the proinflammatory cytokine cocktail IL-1β, IFN-γ and TNF-α.

Exposure to these cytokines reduced the viability of the MIN6 β-cells in control group from 82 ± 3% on day 1 to 42 ± 2%, from 63 ± 3% to 32 ± 1% on day 5, and from 47 ± 11% to 28 ± 1% on day 7 ($p < 0.05$, Fig. 4**a,b**). This impact on cell-viability could be reduced by collagen type IV/RGD which showed an enhanced survival of 55 ± 9% on day 1, 54 ± 6% on day 5 and 45 ± 5% on day 7 but only reached statistical significance on day 5 ($p < 0.01$). Also, Nec-1 prevented loss of cells which reached statistical significance on day 5 (49 ± 3%, $p < 0.05$) while the combination of collagen type IV/RGD and Nec-1 prevented cell loss with survival of 69 ± 5% on day 1 ($p < 0.05$), 57 ± 3% on day 5 ($p < 0.01$), but not on day 7. To study the inflammatory status of encapsulated MIN6 β-cells, we also tested the impact of cytokines and incorporation of collagen type IV/RGD and Nec-1 on the expression of the proinflammatory cytokines interleukin 6 (*Il-6*) and C-X-C motif chemokine 15 (*Cxcl-15*, a homolog of human *IL-8*) and anti-inflammatory cytokines interleukin 10 (*Il-10*) and interleukin 1 receptor antagonist (*Il-1rn*, a homolog of human *IL-1Ra*) (Supplementary Fig. S5a). Exposure to the cytokine mixture upregulate the expression of *Il-6* and *Il-10* by 26 ± 8-fold ($p < 0.01$) and 137 ± 40-fold ($p < 0.001$). Incorporation of collagen type IV/RGD, Nec-1 or together inhibited the increase in expression of *Il-10* ($p < 0.001$) but not of *Il-6*. However, we did not observe any statistically significant effect of cytokines or collagen type IV/RGD and Nec-1 on *Cxcl-15*, while the expression of *Il-1rn* in MIN6 β-cells was not detectable.

The GSIS results also showed beneficial effects of collagen type IV/RGD and Nec-1. As shown in Fig. 4**c**, the exposure to cytokines reduced the stimulated high glucose induced insulin release profoundly from 38 ± 10 to 16 ± 6 µg L^{-1} μg^{-1} DNA $hour^{-1}$ on day 1 ($p = 0.07$), from 77 ± 13 µg L^{-1} μg^{-1} DNA $hour^{-1}$ to 19 ± 3 on day 5 ($p < 0.001$), and from 79 ± 14 to 13 ± 2 µg L^{-1} μg^{-1} DNA $hour^{-1}$ on day 7 ($p < 0.001$). The stimulation index was also decreased by exposure to cytokines on day 5 and day 7 (Supplementary Fig. S2b, $p < 0.05$) compared to controls. This could be rescued by collagen type IV/RGD that kept the high-glucose induced insulin secretion on 25 ± 5 µg L^{-1} μg^{-1} DNA $hour^{-1}$ but did not reach statistical significance on day 7. The addition of Nec-1 prevented this drop to 21 ± 5 µg L^{-1} μg^{-1} DNA $hour^{-1}$ on day 1, 47 ± 6 µg L^{-1} μg^{-1} DNA $hour^{-1}$ on day 5 and 35 ± 8 µg L^{-1} μg^{-1} DNA $hour^{-1}$ on day 7, but only on day 5 this beneficial effect was significant ($p < 0.05$). Combining collagen type IV/RGD and Nec-1 had a synergistic effect and attenuated the decrease of insulin level to 53 ± 8 µg L^{-1} μg^{-1} DNA $hour^{-1}$ on day 5 ($p < 0.05$) and 45 ± 10 µg L^{-1} μg^{-1} DNA $hour^{-1}$ on day 7 ($p < 0.05$). However, no statistical difference was found in stimulation index by incorporation of collagen type IV/RGD or Nec-1 (Supplementary Fig. S2b).

The mitochondrial activity was reduced by the cytokine cocktail by 33 ± 4% on day 1 ($p < 0.001$), 9 ± 1% on day 5 ($p < 0.001$) and 6 ± 1% ($p < 0.01$) on day 7 (Fig. 4**d**). However, the addition of collagen type IV/RGD and Nec-1 prevented this decline in mitochondrial activity. We observed that the incorporation of collagen type IV/RGD prevented loss of mitochondrial activity with 49% on day 1 ($p < 0.001$), 15% on day 5 ($p < 0.01$). Also, intracapsular addition of Nec-1 attenuated the cytokine-induced loss with 47% on day 1 ($p < 0.001$), 13% on day 5 ($p < 0.05$). Notably, the combination of collagen type IV/RGD and Nec-1 significantly prevented this decrease with 66% of control on day 1 ($p < 0.001$), 21% on day 5 ($p < 0.001$), and 14% on day 7 ($p < 0.05$).

Also, for this set of experiments we prepared pseudo-islets of MIN6 β-cells to determine whether cell-cell contact is involved

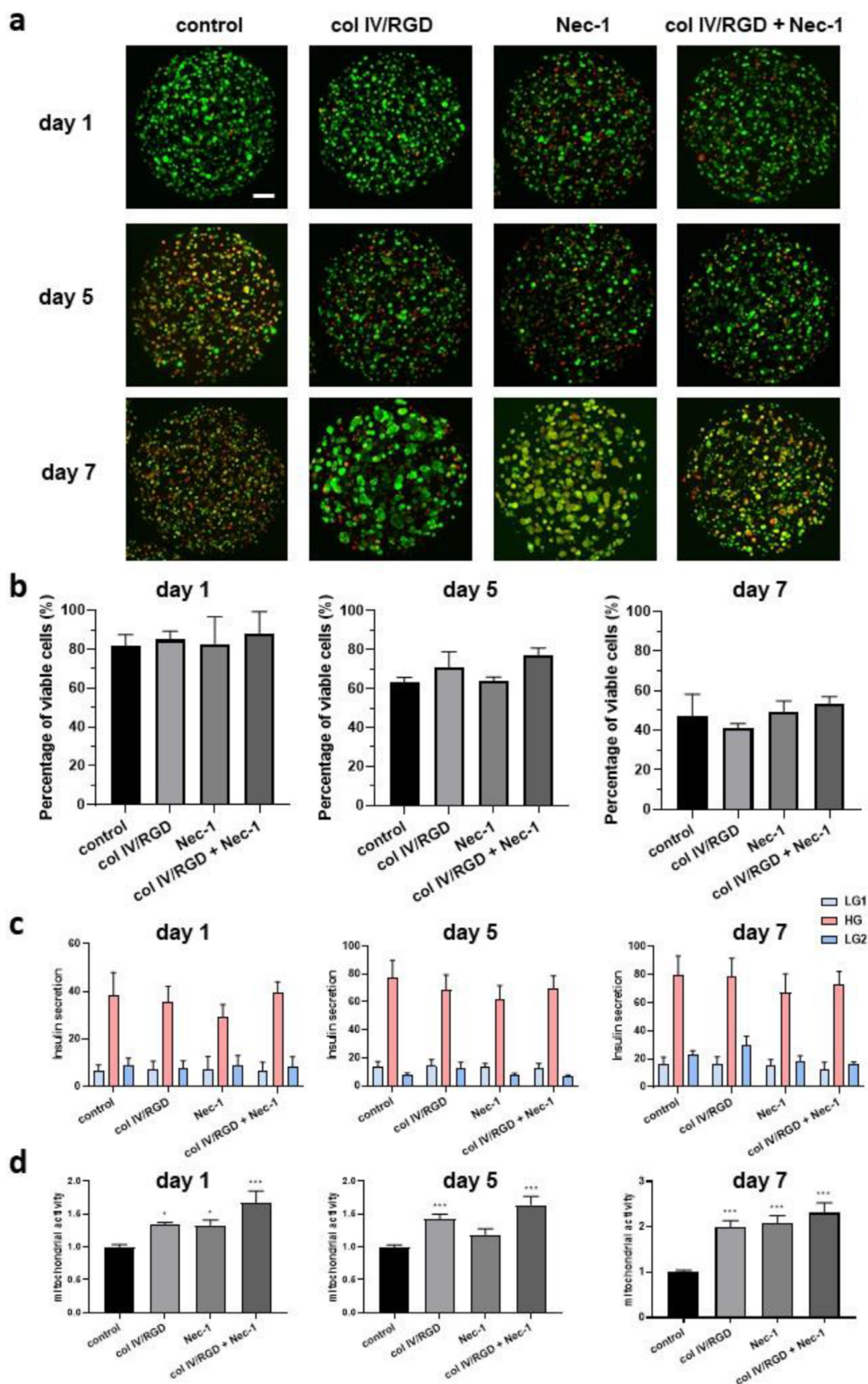

Fig. 2. Incorporation of collagen type IV/RGD and Nec-1 in MIN6 β-cells containing microcapsules supports survival of the β-cells. (**a**) MIN6 β-cell viability was determined by LIVE/DEAD staining. Scale bar is 100 µm. (**b**) LIVE/DEAD staining results were analyzed by using Image J. (**c**) Glucose induced insulin secretion (GSIS) of MIN6 β-cells encapsulated in alginate-based capsules. LG, low glucose, 2.75 mM; HG, high glucose, 16.7 mM. Insulin secretion is expressed as µg L^{-1} µg^{-1} DNA hour^{-1}. (**d**) MIN6 β-cell mitochondrial activity was determined by a WST-1 assay. Results represent mean ± SEM (n = 5). The statistical differences were quantified using one-way ANOVA analysis with Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control group).

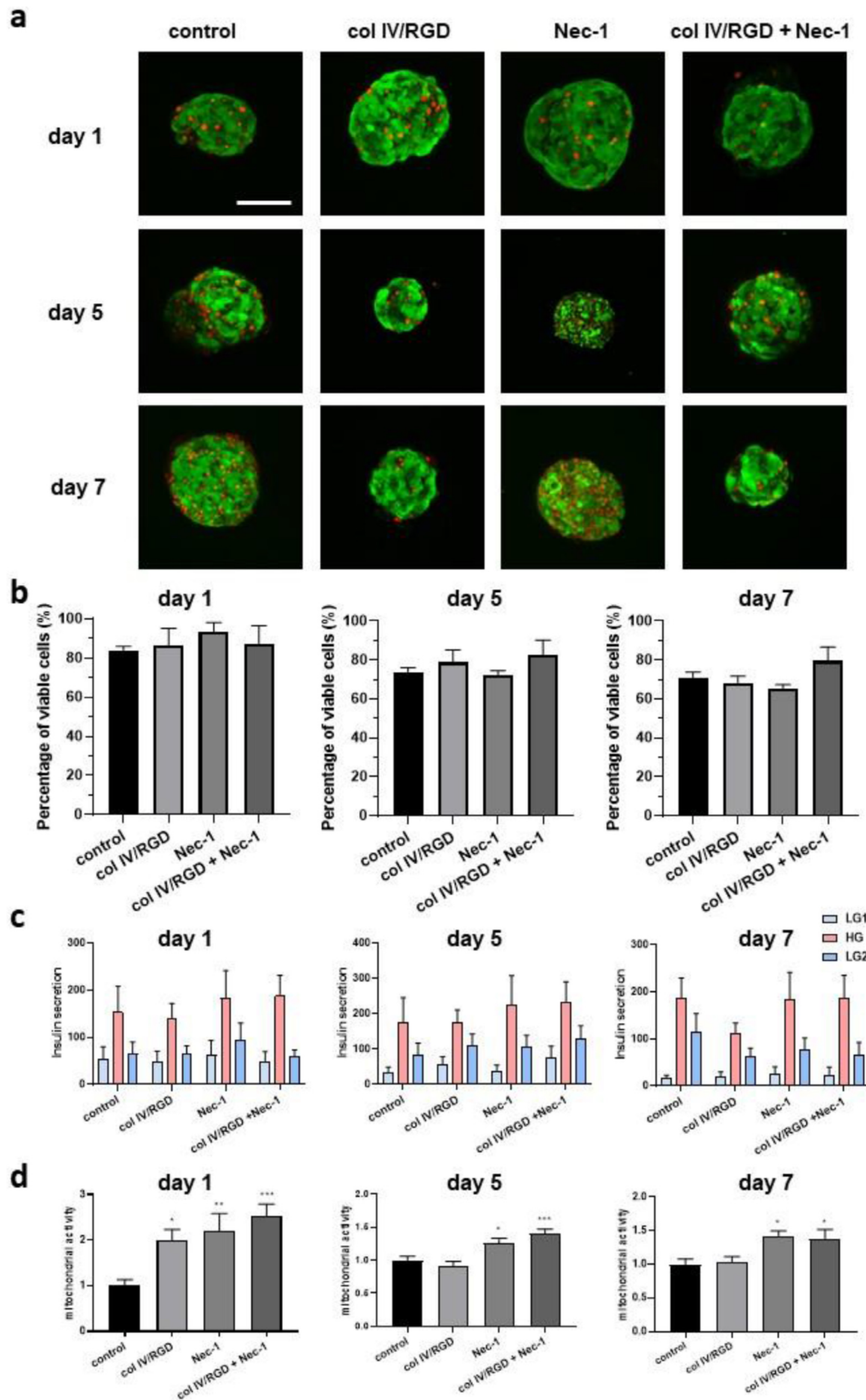

Fig. 3. Incorporation of collagen type IV/RGD and Nec-1 in human islet-containing microcapsules supports survival of islet cells. (**a**) Islet viability was determined by LIVE/DEAD staining. Scale bar is 100 μm. (**b**) LIVE/DEAD staining results were analyzed by using Image J. (**c**) Glucose induced insulin secretion (GSIS) of human islets encapsulated in alginate-based capsules. LG, low glucose, 2.75 mM; HG, high glucose, 16.7 mM. Insulin secretion is expressed as mU L^{-1} μg^{-1} DNA $hour^{-1}$. (**d**) Islet mitochondrial activity was determined with a WST-1 assay. Results represent mean ± SEM (n = 5). The statistical differences were quantified using one-way ANOVA analysis with Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control group).

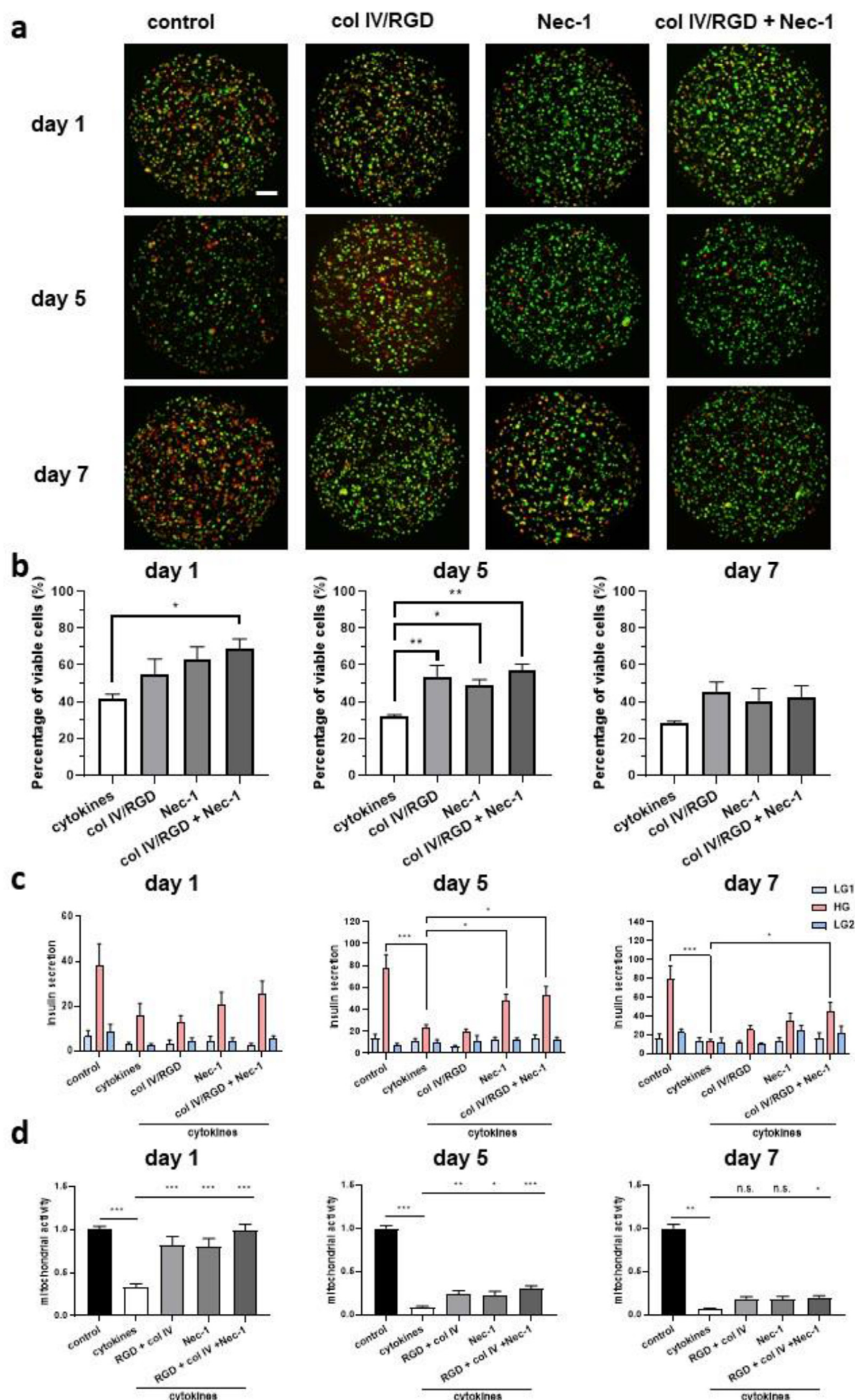

Fig. 4. Collagen type IV/RGD and Nec-1 prevent β-cell viability-loss during exposure to IL-1β, IFN-γ and TNF-α. (**a**) MIN6 β-cell viability was determined with LIVE/DEAD staining. Scale bar is 100 µm. (**b**) LIVE/DEAD staining results were analyzed by using Image J. (**c**) Glucose induced insulin secretion (GSIS) of MIN6 β-cells encapsulated in alginate-based capsules of different compositions. LG, low glucose, 2.75 mM; HG, high glucose, 16.7 mM. Insulin secretion is expressed as µg L^{-1} μg^{-1} DNA $hour^{-1}$. (**d**) MIN6 β-cell mitochondria activity was determined with a WST-1 assay. Results represent mean ± SEM ($n = 5$). The statistical differences were quantified using one-way ANOVA analysis with Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

in beneficial effects on cell-survival. In encapsulated MIN6 β-cell pseudo-islets, the cytokine mixture reduced the percentage of viable cells from 88 ± 1% on day 1 to 69 ± 2%, from 82 ± 4% to 67 ± 2% on day 5, and from 71 ± 3% to 57 ± 1% on day 7 ($p < 0.05$, Supplementary Fig. S4a). The incorporation of collagen type IV/RGD prevented the cytokine-induced viability decrease. Cells in collagen type IV/RGD capsules had an enhanced survival of 64 ± 2% ($p < 0.05$) on day 7. Nec-1 prevented the decrease in viability which had a survival rate of 87 ± 1% ($p < 0.05$) on day 5 and 65 ± 2% ($p < 0.05$) on day 7. Effects were more pronounced with the combination of collagen type IV/RGD and Nec-1 which showed a rescuing effect on survival of 83 ± 3% ($p < 0.01$) on day 1, 76 ± 1% on day 5 ($p < 0.05$) and 69 ± 2% on day 7 ($p < 0.01$). Cytokine exposure significantly decreased mitochondrial activity of encapsulated MIN6 β-cell pseudo-islets by 41 ± 10% on day 1 ($p < 0.05$), 20 ± 3% on day 5 ($p < 0.001$) and 14 ± 3% ($p < 0.01$) on day 7 (Supplementary Fig. S4b). This was prevented by addition of collagen type IV/RGD by 62% ($p < 0.05$) on day1 and 46% ($p < 0.01$) on day 7. Addition of Nec-1 also inhibited the cytokine induced decrease of mitochondrial activity by 54% ($p < 0.05$) on day 1 and 36% ($p < 0.05$) on day 5. The combination of collagen type IV/RGD and Nec-1 had the strongest effect and prevented the cytokine-induced mitochondrial activity decline by 107% ($p < 0.001$) on day 1, 43% ($p < 0.01$) on day 5 and 84% ($p < 0.01$) on day 7.

3.5. Collagen type IV/RGD and Nec-1 prevent loss of encapsulated human islet-cells when exposed to IL-1β, IFN-γ, and TNF-α

Encapsulated human islets were also exposed to the cytokine-cocktail IL-1β, IFN-γ and TNF-α and tested for possible rescuing effect by collagen type IV/RGD and/or Nec-1. As shown in Fig. 5**a, b**, exposure to the cytokine mix resulted in a drop in the percentage of viable cells from 84 ± 2% to 72 ± 3% on day 1, from 73 ± 3% to 68 ± 5% on day 5, and from 71 ± 3% to 65 ± 4% on day 7 ($p < 0.05$). This could be partially prevented by collagen type IV/RGD in the alginate-network that showed a higher viable cell number than the cytokine-exposed control group on day 1 (85 ± 1%, $p < 0.05$). Moreover, the combination of collagen type IV/RGD and Nec-1 had a synergistic effect on survival on day 7 (83 ± 3%, $p < 0.05$) compared to the cytokine-exposed control group. We also studied the impact of encapsulation, cytokine exposure and incorporation of collagen type IV/RGD and Nec-1 on the expression of proinflammatory cytokines *IL-6* and *IL-8* and anti-inflammatory cytokines *IL-10* and *IL-1Ra* to determine the inflammatory status of encapsulated human islets (Supplementary Fig. S5b). We did not observe any statistically significant effect of the encapsulation procedure on the inflammation-related cytokines expression compared to nonencapsulated fresh human islets. Also, we did not find an effect of incorporation of collagen type IV/RGD and Nec-1 on expression of these cytokines.

The GSIS data also showed rescuing effects. The exposure to cytokines reduced the high-glucose induced insulin level from 185 ± 44 to 61 ± 18 mU L^{-1} μg^{-1} DNA $hour^{-1}$ ($p = 0.03$) on day 7 (Fig. 5**d**). However, GSIS was not influenced by the treatment of inflammatory cytokines on day 1 or day 5. There was a trend toward higher GSIS-values for the Nec-1 containing capsules and the capsules with a combination of collagen type IV/RGD and Nec-1 at day 7 but the differences did not reach significance. The stimulation index of encapsulated human islets under exposure to cytokines did not show significant differences (Supplementary Fig. S2d).

As also observed for MIN6 β-cells, the mitochondrial activity of human islet-cells went down when exposed to the cytokine mixture. It was lowered by 48 ± 8% on day 1 ($p < 0.05$), by 31 ± 5% on day 5 ($p < 0.001$) and by 29 ± 4% on day 7 ($p < 0.001$). Also, mitochondrial function was rescued by collagen type IV/RGD that showed elevated mitochondrial activity of 93% on day 1 ($p < 0.001$) and 63% on day 5 ($p < 0.01$). Additionally, Nec-1 had a strong effect on mitochondrial activity and rescued the loss with 84% on day 1 ($p < 0.01$), 59% on day 5 ($p < 0.01$), and 35% on day 7 ($p < 0.05$). Notably, rescuing effects were strongest with the combination of collagen type IV/RGD and Nec-1 which significantly diminished cytokine-induced mitochondrial activity decline with 135% on day 1 ($p < 0.001$), 63% on day 5 ($p < 0.01$), and 69% on day 7 ($p < 0.001$).

3.6. Collagen type IV/RGD and Nec-1 attenuate cytokine-induced free radical species generation in MIN6 β-cells and human islets

As one of the most important mechanisms of proinflammatory cytokine-induced cell-death in β-cell is the induction of intracellular free radical species, we evaluated the production of ROS and NO in both encapsulated MIN6 β-cells and human islets after treatment with the cytokines in the presence and absence of collagen type IV/RGD and/or Nec-1. We only evaluated this on day 7 the assays required significant amounts of the scarce human islets.

In MIN6 β-cells, the cytokines increased the production of ROS by 9.8-fold ($p < 0.001$, Fig. 6**a**) compared to the controls. This increase of ROS was prevented by incorporation of collagen type IV/RGD as well as by Nec-1. In the encapsulated MIN6 β-cells containing collagen type IV/RGD we did not observe a decline in ROS-production (9.0-fold of the control group, $p = 0.93$). With Nec-1 we observed a statistically significant prevention of the cytokine-induced increase of ROS production which was 47% lower than of the cytokine-exposed control group ($p < 0.01$). The combination of collagen type IV/RGD and Nec-1 had a synergistic effect on ROS generation which was only 3.8-fold of the control group ($p < 0.001$).

The effects of collagen type IV/RGD and Nec-1 on ROS production in human islets were even more pronounced than observed in MIN6 β-cells (Fig. 6**b**). We found that exposure to the cytokine cocktail enhanced production of ROS with 2.8-fold ($p < 0.01$) which enhancement was completely prevented in the human islets in capsules with collagen type IV/RGD capsules ($p < 0.05$) and Nec-1 containing capsules ($p < 0.05$). In capsules containing the combination of collagen type IV/RGD and Nec-1 we even found a lower ROS production than in the controls. It was reduced 0.6-fold ($p < 0.01$) compared to the controls.

In addition, the cytokine-induced NO production was profoundly reduced by the tested molecules (Fig. 6**c**). The cytokine cocktail had a strong impact on NO synthesis in MIN6 β-cells, which was enhanced 1.5-fold ($p < 0.01$) compared to controls. Addition of Nec-1 in capsules prevented the elevated NO production with 78% ($p < 0.01$) while the combination of collagen type IV and Nec-1 also effectively inhibited the cytokine-induced NO production with 70% ($p < 0.01$).

In human islets, NO production was statistically significantly increased by 1.4-fold ($p < 0.05$) by the cytokine cocktail (Fig. 6**d**). Collagen type IV /RGD had no statistically significant effect on NO enhancement but Nec-1 prevented the increase by 44% ($p < 0.05$), as well as the combination of collagen type IV and Nec-1 which lowered NO with 35% ($p < 0.05$).

3.7. Collagen type IV/RGD and Nec-1 promote mitochondria function by modulating mitochondrial fusion and fission in MIN6 β-cells and human islets

As strong effects of intracapsular collagen type IV/RGD and Nec-1 were found on mitochondrial function, we studied expression of mitochondrial fission and fusion genes by qRT-PCR (Fig. 7). After exposure to the cytokine mixture, the fusion genes *Mfn2* and *Opa1* were found to be statistically significantly downregulated in MIN6 β-cells to 13 ± 3% ($p < 0.05$), 11 ± 5% ($p < 0.001$) respectively

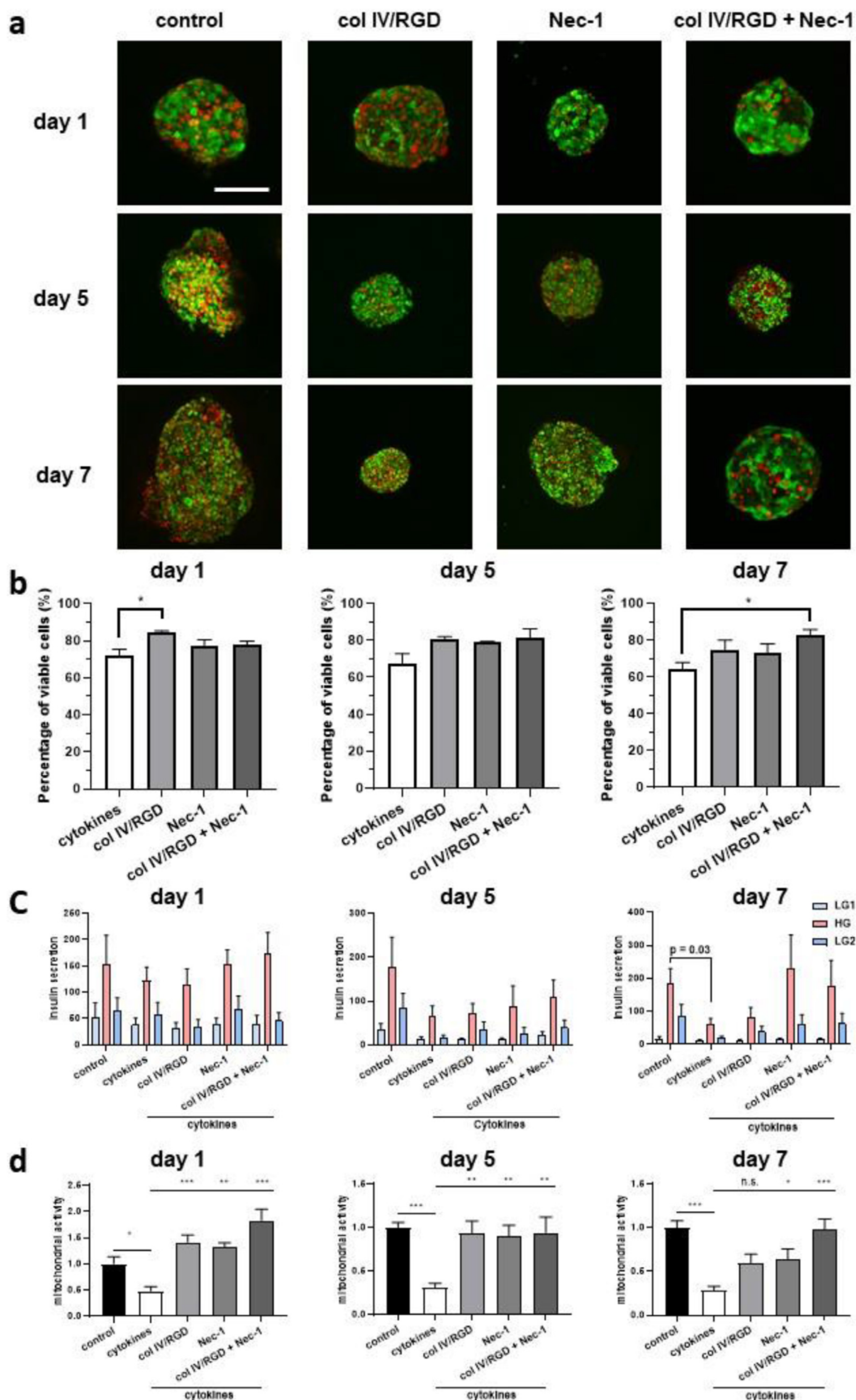

Fig. 5. Collagen type IV/RGD and Nec-1 prevent loss of encapsulated human islet-cells when exposed to IL-1β, IFN-γ and TNF-α. (**a**) Human islet-viability was determined with LIVE/DEAD staining. Scale bar is 100 µm. (**b**) LIVE/DEAD staining results were analyzed by using Image J. (**c**) Glucose induced insulin secretion (GSIS) of human islets encapsulated in alginate-based capsules of different compositions. LG, low glucose, 2.75 mM; HG, high glucose, 16.7 mM. Insulin secretion is expressed as mU L^{-1} μg^{-1} DNA $hour^{-1}$. (**d**) Islet mitochondrial activity was determined by using a WST-1 assay. Results represent mean ± SEM (n = 5). The statistical differences were quantified using one-way ANOVA analysis with Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

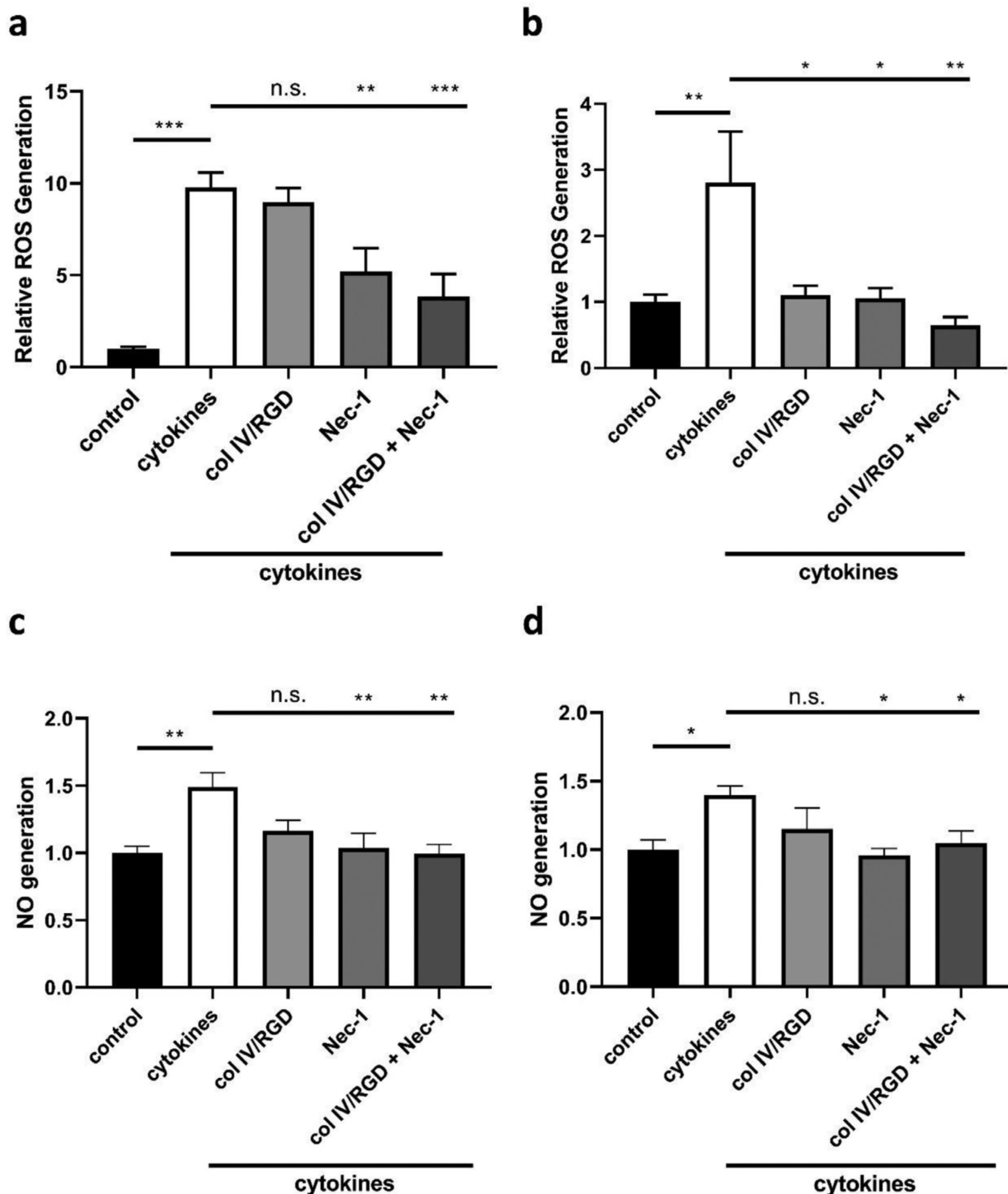

Fig. 6. Effects of collagen type IV/RGD and Nec-1 on cytokine-induced oxidative and nitrosative stress in MIN-6 β-cells and human islets on day 7 post-encapsulation. Intracellular reactive oxygen species (ROS) was measured with 2′,7′-dichlorofluorescin diacetate (DCFDA) Cellular ROS Detection Assay Kit (**a, b**). The fluorescence signal was monitored at Ex/Em = 485/535 nm. Nitric oxide (NO) was detected using a Nitric Oxide Assay Kit (**c, d**). Results represent mean ± SEM ($n = 5$). The statistical differences were quantified using one-way ANOVA analysis with Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

(Fig. 7**a**). However, cytokine stimulation did not affect the expression of *Mfn1*. Furthermore, exposure to the cytokine cocktail increased the expression of the fission genes *Drp1* and *Fis1* in encapsulated MIN6 β-cells by respectively 2.2 ± 0.7-fold ($p < 0.01$) and 2.5 ± 0.3-fold ($p < 0.001$). This result demonstrates that proinflammatory cytokines induce reduction in mitochondria fusion and elevation in fission in MIN6 β-cells. Under cytokine-induced mitochondrial stress, we found that collagen type IV/RGD prevented the elevation of *Drp1* gene by 0.8 ± 0.4-fold of the control group ($p < 0.01$), and the elevation of *Fis1* gene to 0.5 ± 0.2-fold of the control group ($p < 0.001$). Addition of Nec-1 prevented against the cytokine-induced reduction in fusion gene *Opa1* with 28% ($p < 0.01$). The elevation of fission genes *Drp1* and *Fis1* was also prevented by Nec-1 to 0.3 ± 0.1-fold ($p < 0.001$) and 0.5 ± 0.1-fold ($p < 0.001$) of control values, respectively. The combination of collagen type IV/RGD and Nec-1 had the most pronounced effect on pro-fusion and anti-fission genes as the cytokine-induced decrease of the fusion genes *Mfn2* and *Opa1* was prevented with 66% ($p < 0.05$) and 69% ($p < 0.001$), respectively. The increase of fission genes *Drp1* and *Fis1* provoked by cytokines was also prevented by 0.5 ± 0.2-fold ($p < 0.001$) and 0.5 ± 0.2-fold ($p < 0.001$) compared to the control group.

The same experiments were repeated with human islets as well (Fig. 7**b**). Also, in human islets the presence of the cytokine cock-

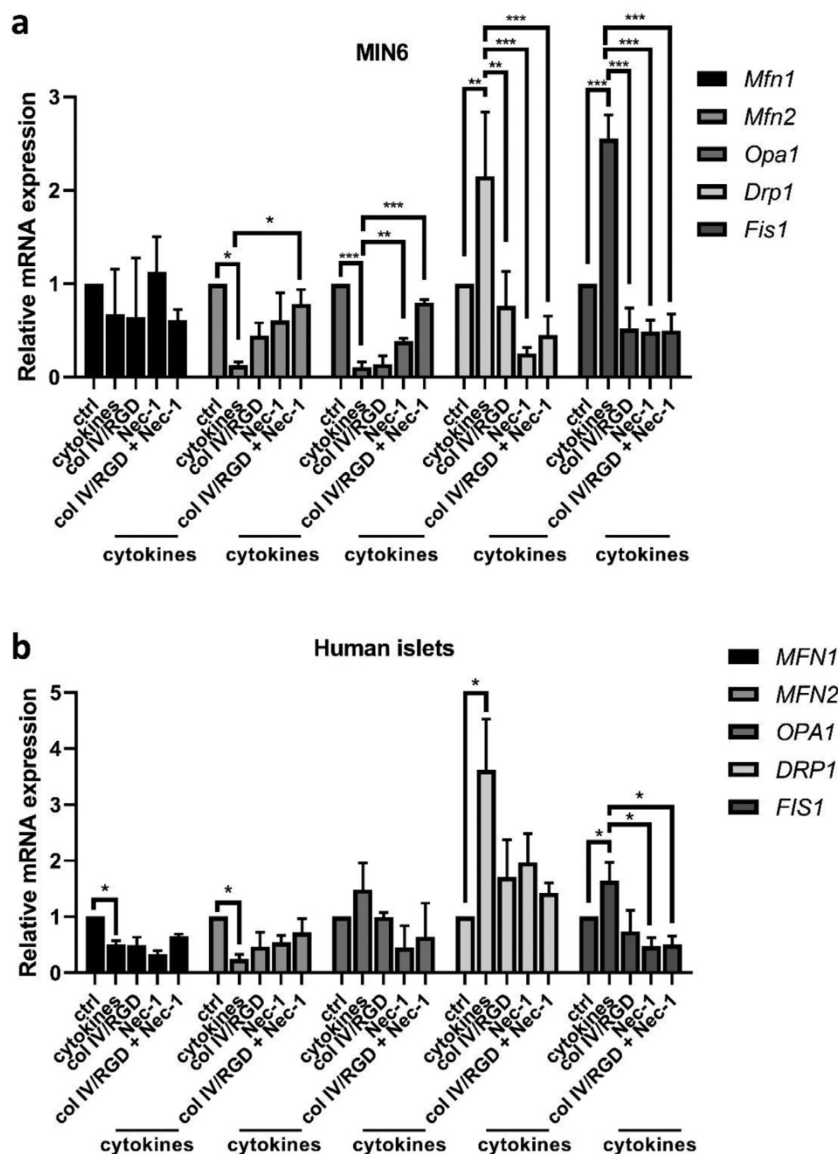

Fig. 7. Effects of collagen type IV/RGD and Nec-1 on mitochondrial fusion and fission gene expression in MIN6 β-cells (**a**) and human islets (**b**). The expression level of the mitochondrial fusion and fission genes were quantified with qRT-PCR analysis. Results are plotted as mean ± SEM (n = 5). The statistical differences were quantified using one-way ANOVA analysis with Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

tail induced an anti-fusion and pro-fission effect. The fusion genes *MFN1* and *MFN2* was found to be statistical significantly downregulated by the cytokine cocktail in human islets to 51 ± 7% ($p < 0.05$) and 24 ± 8% ($p < 0.05$) of untreated control (Fig. 7**b**). The expression of the fission genes *DRP1* and *FIS1* were also upregulated by 3.6 ± 0.9-fold ($p < 0.05$) and 1.6 ± 0.3-fold ($p < 0.05$) of the control. However, *OPA1* were not affected by the addition of cytokine cocktail. Incorporation of Nec-1 in microcapsules prevented the increase in the fission gene *FIS1* expression to 0.5 ± 0.2-fold of control ($p < 0.05$). The combination of collagen type IV/RGD and Nec-1 also prevented the increase of mitochondria fission gene *FIS1* by 0.5 ± 0.1-fold ($p < 0.05$) of the untreated control group.

Expression of mitochondrial fusion and fission genes were also determined on a protein level by Western blot using encapsulated MIN6 β-cells harvested from day 7 (Supplementary Fig. S6). The protein expression of fusion genes MFN2 and OPA1 were suppressed by cytokine exposure. Addition of the combination of collagen type IV/RGD and Nec-1 prevented the cytokine-induced reduction in MFN2 protein expression. The protein expression level of DRP1 was enhanced by cytokine exposure, which was prevented by addition of the combination of collagen type IV/RGD and Nec-1.

3.8. Effects of collagen type IV/RGD and Nec-1 supplementation on phosphorylation of signaling proteins FAK, Akt, and Erk

ECM components, including collagens and laminins, are known to be ligands for integrins. Integrins are the principal transmembrane receptors and play a vital role in signal transduction from ECM. Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase involved in integrin-mediated signal transduction. Activation of FAK by integrin leads to phosphorylation at Tyr397, which activates the downstream activation of PI3K/Akt (phosphatidylinositol 3–kinase/protein kinase B) pathway [39]. Akt is a major mediator of cell survival, proliferation, and metabolism. Activation of the Akt pathway enhances β-cell function and prevent β-cell

death [40]. Therefore, phosphorylation of Tyr397-FAK and Ser473-Akt was analyzed by Western blot (Supplementary Fig. S7). We observed a suppressed FAK and Akt phosphorylation by exposure to the cytokines. Addition of collagen type IV/RGD and the combination of collagen type IV/RGD and Nec-1 prevented the cytokine induced decrease of FAK and Akt phosphorylation, suggesting that collagen type IV/RGD exerts its rescuing effect via this pathway.

The FAK signaling complex is also known to mediate the activation of MAPK/Erk (mitogen-activated protein kinase/extracellular-regulated kinase) pathway, which is well-known for its pro-survival effect in several cell types [39,41]. The phosphorylation of Thr202-Erk1 and Tyr204-Erk2 were tested. However, we did not observe any statistically significant effect of the cytokines or addition of collagen type IV/RGD or Nec-1. Thus our data showed that incorporation of collagen type IV/RGD in MIN6 β-cell-containing capsules activate the ECM/integrin/FAK/Akt pathway.

4. Discussion

The application of an encapsulation device, which provides a physical barrier between islet grafts and the recipients immune system, has shown great efficacy in islet transplantation by eliminating the need for immunosuppression [6,7]. In the present study, we created a viability promoting microenvironment for β-cell by incorporating collagen type IV/RGD and Nec-1 into alginate-based microcapsules. We demonstrated an enhanced pancreatic β-cell survival and function under cytokine-induced stress. In our study, the cytokine mixture composed of IL-1β, IFN-γ and TNF-α was applied to create proinflammatory stress, which plays an important role in the pathogenesis of T1D as well as in the extensive loss of functional islets during the immediate posttransplant period after grafting [42]. We observed that microcapsules containing either collagen type IV/RGD or Nec-1 or a combination thereof could make β-cells more resistant to the applied cytokine stress and prevent loss of insulin production. This could be attributed to the fact that collagen type IV/RGD or Nec-1 prevented cytokine-induced damage in mitochondrial dynamics and lowered the generation of free radical species (i.e., ROS and NO). In addition, collagen type IV/RGD activated the ECM/integrin/FAK/Akt pathway and promoted survival of the encapsulated cells. The combination of collagen type IV/RGD and Nec-1 showed the most significant synergistic beneficial effect.

The protective effect of collagen type IV/RGD and Nec-1 for cytokine exposure was most pronounced on day 5 as more statistically differences were observed in viability, insulin secretion and mitochondrial activity in encapsulated MIN6 β-cells and human islets. A possible explanation is that on day 1, a recovery period is needed from the encapsulation procedure and adaptation to the culture medium which may result in a lesser protective effect. That we observed somewhat lesser protection on day 7 should be explained by the prolonged, long exposure to the cytokine mixture. This long-term exposure rarely happens *in vivo* but was used as a model to demonstrate the robust protection of collagen type IV/RGD and/or Nec-1 on cytokine-induced stress.

The human islets in our study demonstrated a higher survival rate of cells than MIN6 β-cells, as well as a stronger resistance to cytokine exposure as demonstrated by a lesser decrease of viability and mitochondrial activity [43,44]. This is interesting as MIN6 β-cells is a mouse insulinoma cell line that expectedly should survive a longer time in alginate capsules than human islets that were expected to face a gradual loss of viability as the consequence of central necrosis due to hypoxia [45] and deprivation of other nutrients. It may be suggested that this is due to the single cell nature of the MIN6 β-cells and/or clustering of cells in islets. To study this, we designed the MIN6 β-cell pseudo-islets experiments. These pseudo-islets have a similar islet-like structure as human islets. In these pseudo-islets we observed a higher survival rate of cells compared to the single cell studies with MIN6 β-cells, but a similar pattern of rescuing effects of collagen type IV/RGD and/or Nec-1 on cytokine-induced mitochondrial dysfunction. This suggests that the higher viability of human islets than MIN6 β-cells in our studies is due to cell-to-cell interactions in cell aggregates [46].

ECM is responsible for providing mechanical and physiological support for islets. Every single islet-cell in the interior of the native pancreas is in connection to the basement membrane, composed of collagen, laminin, fibronectin, and other ECM components, which are co-responsible for communication between cells in islets [24]. These ECM components promote β-cell proliferation and survival through interaction with cell-surface integrin receptors. This network is damaged during the process of digestion by collagenases which is needed to separate islets from exocrine tissue. When islets are encapsulated, they may not regain the same ECM structure as in the normal pancreas which may play an important role in the limited longevity and function of the grafts [26,47]. Addition of specific ECM components or ECM from decellularized tissue in alginate-based hydrogel is a promising strategy to enhance β-cell function as well as survival [48,49]. As there are numerous ECM components and interactions in islets it was somewhat surprising that only a supplement of collagen type IV and laminin sequence RGD has such an enhancing effect on cell viability and glucose induced insulin release of encapsulated human islets when exposed to cytokine stress [21,22]. The explanation is likely that both collagen type IV, the predominant constituent subtype surrounding islets, and RGD are in crosstalk with integrins which support downstream signaling pathways that are involved in protection against inflammatory stress as will be explained below [20,50].

Integrins are a large family of heterodimeric, transmembrane glycoprotein receptors for ECM components. They consist of one α- and one β-subunit that are non-covalently associated. In β-cells, integrins play a vital role in proliferation, cell survival and in insulin secretion [24,39]. The strong impact of collagen type IV and RGD could be explained by the strong effects on downstream signaling via binding to integrin subunits. Collagen type IV is formed from six homologous α-chains and has been shown to bind with several integrins, including $\alpha1\beta1$, $\alpha3\beta1$ and $\alpha6\beta1$ [51]. RGD also binds to the integrins $\alpha3\beta1$ but also to $\alpha v\beta3$ to transduce signals that promote cell survival in β-cells [20]. $\beta1$ integrin is one of the most studied integrin subunits in the pancreas. Notably, $\beta1$ integrin regulates β-cell survival and function by mediating the activation of focal adhesion kinase (FAK) and subsequent initiation of critical intracellular signaling including Akt and Erk [39]. In our study, the activation of ECM/integrin/FAK/Akt pathway by collagen type IV and RGD was confirmed to be involved, which could be the underlying mechanisms that contribute to the promotion of β-cell survival and function. Our data suggest that the prolonged viability of β-cells in collagen type IV and RGD supplemented capsules could be mediated by the enhanced Akt pathway.

Nec-1 is a potent inhibitor of necroptosis and ameliorates necroptotic cell death [27]. Application of Nec-1 provides protective effects in multiple disease models. Nec-1 has been demonstrated to attenuate cellular ROS production probably via RIP1-RIP3 signaling [52–54]. Recently, the protective role of Nec-1 in islets was found to exist in reducing islet cell death and attenuation of damage-associated molecular patterns (DAMP) release [25,55]. DAMPs are involved in immune response initiation and therewith Nec-1 also contributes to lowering of immune responses against implanted cells. The supplementation of Nec-1 has been reported to mitigate islet damage, improve β-cell development and matu-

ration, and promote insulin secretion in pre-weaned porcine islets [28,30]. Nec-1 has been reported to indirectly reduce RIP3 expression and phosphorylation, as well as RIP3 activity probably by diminishing the RIP1-RIP3 complex [27]. Reduction of RIP3 kinase activity by Nec-1 reduces the formation of the pyruvate dehydrogenase complex and subsequently reduces ROS generation [56,57]. This is probably the reason why we observed lower ROS generation and lower cell-death rates when Nec-1 was incorporated in the capsules.

Our observation of beneficial effects of collagen type IV/RGD and Nec-1 are not only applicable for microencapsulated grafts but most likely also for free islet transplantation. Currently, immune reactions after intrahepatic islet infusion, such as the instant blood mediated inflammatory reaction (IBMIR), have been reported to induce loss of up to 80% of the graft mass [58]. IBMIR together with adaptive immune responses plays an important role in the limited long-term survival of human-islet grafts which is only 20% by 5 years after transplantation [4,7]. Application of collagen type IV and RGD and/or Nec-1 might make the islets less vulnerable for the inflammatory stress coming from immune responses after implantation and contribute to a better engraftment of the islets [21,22,59].

Mitochondria dynamics plays a pivotal role in the regulation of β-cell insulin secretion [60,61]. Under basal conditions, where a substantial loss of β-cells could be observed throughout the culture period, the incorporation of collagen type IV/RGD and Nec-1 showed improved cell survival and mitochondrial activity. Furthermore, this protective effect on mitochondrial activity was more pronounced under cytokine stimulation. Therefore, to explore the potential mechanism, we measured free radical species generation and mitochondria fusion and fission gene expression. The applied cytokine exposure leads to an increase of free radicals which diminish mitochondrial function [62]. This could be attenuated by addition of collagen type IV/RGD and Nec-1. Mitochondria are dynamic organelles that continuously undergo fusion and fission events. Disruption of mitochondrial fusion and fission does compromise β-cell function and survival [63]. Favoring fusion seems to maintain mitochondrial function and to prevent apoptosis [63–66]. As shown here collagen type IV/RGD and Nec-1 enhanced the fusion genes (MFN2 and OPA1) and downregulated fission genes (DRP1 and FIS1) which might explain the beneficial effects of our approach.

Our findings suggest that the tested collagen type IV/RGD and Nec-1 inhibit cytokine-induced oxidative stress in islet β-cells by reducing ROS and NO levels. β-cells are susceptible to oxidative stress as they have an active oxidative metabolism combined with a low antioxidant enzyme content [62]. As a consequence, when exposed to immune stress such as directly after implantation [67], islets will activate pro-inflammatory pathways after which necroptosis is provoked with overgeneration of ROS and NO [32,68,69]. However, many studies have found that the ECM components may lower the generation of ROS and NO through interaction with integrins [21,70,71]. As Nec-1 is also known to attenuate ROS production via RIP1 and RIP3 [53,72], the tested collagen type IV/RGD and Nec-1 combinedly had a strong, synergistic effect on β-cell survival and function. The synergistic effect can be explained by a combined effect on lowering free radical species and attenuated detrimental shift of mitochondrial dynamics. The synergistic protective strategy can be applied to protect islets better than a single molecule approach. However, a limitation needs to be mentioned as the applied components, collagen type IV, laminin sequence RGD does not necessarily reflect the beneficial effects of the native ECM structure of the pancreas that is composed of a variety of ECM molecules. Therefore, a bioengineered scaffold with an ECM network closely resembling the ECM of the pancreas might lead to even further improved islet function. We do feel however that our study shows that ECM supplementation of the intracapsular environment is necessary for optimal function of islet-grafts.

5. Conclusions

In conclusion, our study demonstrates that incorporation of specific ECM components, collagen type IV and laminin sequence RGD, and Nec-1 in immunoisolated microcapsules can protect the β-cells against proinflammatory stress. Especially the combination enhances β-cell viability and function by reducing generation of free radical species, rescuing mitochondrial dysfunction, and maintaining ECM/integrin/FAK/Akt signaling. Our findings lay the groundwork for identifying effective combination strategies in the intracapsular space of immunoisolating devices to improve islet transplant outcomes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.04.042.

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