

## RESEARCH ARTICLE

# Laminin and collagen IV inclusion in immunoisolating microcapsules reduces cytokine-mediated cell death in human pancreatic islets

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**Abstract**

Extracellular matrix (ECM) molecules have several functions in pancreatic islets, including provision of mechanical support and prevention of cytotoxicity during inflammation. During islet isolation, ECM connections are damaged, and are not restored after encapsulation and transplantation. Inclusion of specific combinations of collagen type IV and laminins in immunoisolating capsules can enhance survival of pancreatic islets. Here we investigated whether ECM can also enhance survival and lower susceptibility of human islets to cytokine-mediated cytotoxicity. To this end, human islets were encapsulated in alginate with collagen IV and either RGD, LRE or PDSGR, i.e. laminin sequences. Islets in capsules without ECM served as control. The encapsulated islets were exposed to IL-1 $\beta$ , IFN- $\gamma$  and TNF- $\alpha$  for 24 and 72 h. All combinations of ECM improved the islet cell survival, and reduced necrosis and apoptosis after cytokine exposure ( $P < 0.01$ ). Collagen IV-RGD and collagen IV-LRE reduced danger-associated molecular patterns (DAMPs) release from islets ( $P < 0.05$ ). Moreover, collagen IV-RGD and collagen IV-PDSGR, but not collagen IV-LRE, reduced NO release from encapsulated human islets ( $P < 0.05$ ). This reduction correlated with a higher oxygen consumption rate (OCR) of islets in capsules containing collagen IV-RGD and collagen IV-PDSGR. Islets in capsules with collagen IV-LRE showed more dysfunction, and OCR was not different from islets in control capsules without ECM. Our study demonstrates that incorporation of specific ECM molecules such as collagen type IV with the laminin sequences RGD and PDSGR in immunoisolated islets can protect against cytokine toxicity.

**KEYWORDS**

alginate capsules, apoptosis, cytokines, extracellular matrix, islet, necrosis

## 1 | INTRODUCTION

Pancreatic islets have an extensive network of extracellular matrix (ECM) molecules of a specific composition. These ECM molecules are involved in the maintenance of function of islet tissue (Stendahl, Kaufman, & Stupp, 2009). Before islet transplantation, islets are isolated from the pancreas. The process of islet isolation requires administration of enzymes into the pancreas such as collagenases that damage the ECM molecules, which impacts several cellular functions in the islets including ATP generation and insulin secretion (Irving-Rodgers, et al., 2014; Llacua, de Haan, Smink, & de Vos, 2016). The most common and essential ECM structures in islets are collagen type I and IV, and laminins (Stendahl et al., 2009).

It has been shown that cytokines and ECM act synergistically, and combined can regulate fundamental processes during and after inflammation such as proliferation, differentiation and cell death processes (Schonherr & Hausser, 2000). The interactions with the immune system are regulated by cell surface receptors for matrix proteins, which can be integrins or non-integrin receptors. This can be mediated by changing the expression of ECM molecules by specific cytokines (Grande, Melder, & Zinsmeister, 1997). However, cytokines may also enhance their efficacy by using ECM molecules as co-receptors (Yayon, Klagsbrun, Esko, Leder, & Ornitz, 1991), or by influencing intracellular signal transduction pathways (Grande et al., 1997). Also, cytokines can bind to specific ECM constituents whereby their effects are localized to specific areas and/or they may be stored in the matrix for later release (Schonherr & Hausser, 2000).

An emerging field in which ECM molecules may be used to enhance survival and lower susceptibility cytokines is in immunoisolation of pancreatic islets (Farney, Sutherland, & Opara, 2016; Peloso et al., 2016). Immunoisolation is a technology in which islets are enveloped in semi-permeable membranes. It protects pancreatic islets from the hostile effects of the immune system (de Vos, Lazarjani, Poncelet, & Faas, 2014). The membranes are impermeable to large immune effector molecules such as immunoglobulins and complement factors, but they are permeable for smaller molecules such as glucose and insulin (de Vos, Faas, Strand, & Calafiore, 2006). Most cytokines are also able to pass the capsule membrane, and have been reported to be a contributing factor in the success and failure of islet grafts (Kulseng, Thu, Espevik, & Skjak-Braek, 1997). Cytokines such as TNF- $\alpha$ , IFN- $\gamma$  and IL-1 $\beta$ , known to be involved in islet cell death (Wang, Guan, & Yang, 2010), may pass the capsule membrane and induce cell death. These processes include cell death by necrosis and necroptosis (Aikin, Rosenberg, Paraskevas, & Maysinger, 2004), which is associated with the release of danger-associated molecular patterns (DAMPs) that are strong activators of immunity (Paredes-Juarez et al., 2015). This enhances immunity in the vicinity of the capsules and contributes to additional loss of islet cells. Preventing cell death is therefore of essential importance.

As ECM supplementation may change the impact of cytokines (Beenken-Rothkopf et al., 2013; Peloso et al., 2016), we investigated whether combinations of ECM molecules that we previously found to be beneficial for islet function (Llacua et al., 2016) also lower sensitivity for cytokines and contribute to the maintenance of islet function during cytokine exposure. To this end, we investigated the effect of supplementation of collagen type IV combined with the laminin sequences RGD, LRE or PDSGR on cell death and function of human pancreatic islets encapsulated in alginate-based microcapsules during exposure to TNF- $\alpha$ , IFN- $\gamma$  and IL-1 $\beta$  for 24 and 72 h.

## 2 | RESEARCH DESIGN AND METHODS

### 2.1 | Human pancreatic islet isolation

Human islets were obtained from cadaveric pancreata at Prodo Laboratories (Irvine, USA). Dithizone (Merck, USA) staining was performed before shipment to determine the purity. After shipment, islets were washed five times with CMRL 1066 (Gibco, USA) before culture. All the methods and experimental protocols 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.

### 2.2 | Islet encapsulation and ECM building

In-house purified intermediate-G alginate (ISP Alginate, UK) was applied as described before (Tam et al., 2009). All the solutions were sterilized by filtration (0.2  $\mu$ m). ECM components were added to human pancreatic islets enveloped in alginate-based microcapsules. The specific enhancing laminin sequence known to be present in islets in the native pancreas was obtained from GenScript (NJ, USA); 0.01 mM RGD, 1 mM LRE, 0.01 mM PDSGR. Those active laminin sequences were combined with 50  $\mu$ g/ml collagen type IV and mixed in the intracapsular environment of alginate

microcapsules. The concentrations mentioned are the final concentrations that are used to encapsulate the islets. The concentrations were based on a previous study from our group (Llacua et al., 2016). Highly purified alginate was mixed with the appropriate ECM components by physical entrapment within 3.4% purified alginate. The matrix was mixed with human pancreatic islets in a ratio of 1000 islets/ml alginate–ECM mixture. Alginate solutions without ECM components served as control. Subsequently, the solution was converted into droplets using an air-driven droplet generator as previously described (de Haan, Faas, & de Vos, 2003). The capsules had a final diameter of 500–600  $\mu$ m.

### 2.3 | Exposure to human cytokines

Encapsulated islets with or without ECM (control) in the intracapsular space, as previously described in detail (Llacua et al., 2016), were incubated in CMRL 1066 complete media containing the human cytokine mix; TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml; PeproTech, Germany), and were cultured for 24 and 72 h in 24-well non-treated plates (Costar<sup>®</sup>, NY, USA). Each well contained 25 encapsulated islets in 1 ml of CMRL 1066 medium, and was incubated in control and ECM mix combined with or without the cytokine mix.

### 2.4 | Confocal analysis

Encapsulated islets were stained with a LIVE/DEAD Cell Viability/Cytotoxicity assay Kit (Invitrogen, USA). To this end, a stock solution of calcein AM (4 mM) and ethidium homodimer (EthD; 2 mM) was added. Encapsulated islets were incubated at room temperature for 30 min in darkness, and then washed with Krebs–Ringer–Hepes (KRH), pH 7.4, prior to imaging. The percentage of viable cells was determined by green fluorescence, while dead cells were recognized by red fluorescence upon binding of EthD to exposed DNA (Ext/Abs 528/617 nm). Apoptosis was quantified using Annexin-V, Alexa Fluor (Invitrogen Life Technologies, NY USA) in combination with propidium iodide (PI) according to the standard protocol provided by the manufacturer. Annexin-V-positive cells stained green and were quantified as apoptotic cells by counting the green cells with loss of integrity of the plasma and decreased nuclear membranes. Necrotic cells stained in this procedure combined white and green, and were also counted. Fluorescent confocal microscopy was performed at an emission wavelength of 488 nm using a Leica TCS SP2 confocal microscope. Stained islet cells were manually counted in images ( $n \geq 3$ ) of each sample. Data were expressed as the percentages of apoptotic or necrotic cells relative to the total number of stained cells and analysed by Imaris x64 version 7.6.4 software and ImageJ 1.47.

### 2.5 | DAMPs

To quantify the DAMPs, supernatants of incubated islets were analysed by ELISA for the presence of either double-stranded DNA (dsDNA, BlueGene Biotech, Shanghai, China) or uric acid (Abcam, Cambridge, UK). ELISA was performed according to the manufacturer's directions.

### 2.6 | Nitrite determination

Nitrite production was determined using Griess Reagent System (Promega, USA) by mixing 50  $\mu$ l of culture media of each experimental

sample with 50  $\mu$ l of de Griess reagent according to the manufacturer's directions. Absorbance was measured at 550 nm, and nitrite concentrations were calculated from a nitrite standard curve (Bredt & Snyder, 1994).

## 2.7 | Islets oxygen consumption rate (OCR)

Cytokine-induced changes in OCR were measured in human pancreatic islets using the extracellular flux analyser XF24 (Seahorse Bioscience, USA), as previously described (Llacua et al., 2016). After 72 h of exposure to cytokines, alginate was removed by an incubation step in 25 mM citrate solution in KRH for 15 min at 37 °C. This was necessary as the capsule interfered with the measurements. Between 80 and 100 islets per condition were incubated overnight in CMRL 1066 (Gibco, USA) with 8.3 mM D-glucose, penicillin/streptomycin (1%; Gibco, USA) and 10% fetal calf serum (FCS; Gibco, 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  $\mu$ l 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 pre-wetted with MA media. The plates were then incubated for 60 min at 37 °C before they were loaded into the XF24 machine. The assay test reagents were added at either 59 or 130 min. The test reagents were either glucose (16.7 mM final) or the mitochondrial inhibitors-oligomycin (5  $\mu$ M). 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  $\mu$ M). After the addition of each reagent, five readings were taken. To adjust for the variation in islet numbers, the OCR of each individual well was normalized with basal conditions. The area under the curve (AUC) analyses were determined during high-glucose exposure by measuring the OCR enhancement over the full period of exposure. This was calculated by the Seahorse XF-24 software. Every point represents an average of four different wells.

## 2.8 | Statistical analysis

Values were expressed as mean  $\pm$  standard error of the mean (SEM). Normal distribution of the data sets was determined using the Kolmogorov-Smirnov test. Statistical comparisons between experimental conditions in each study were performed by one-way ANOVA to

compare outcomes of the non-parametric, unmatched treatments of controls and encapsulated islets in ECM mix, using GraphPad Prism 6.0. *P*-values < 0.05 were considered to be statistically significant.

## 3 | RESULTS

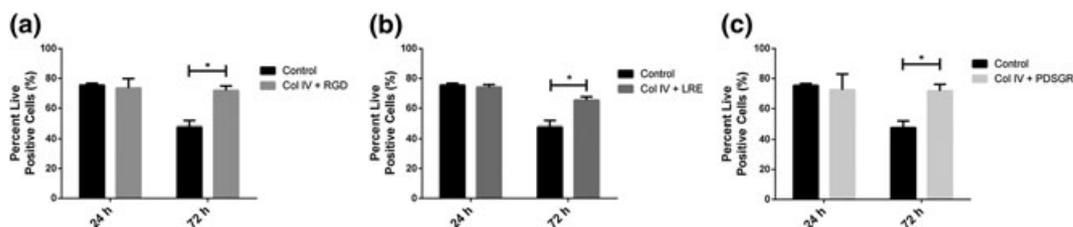
### 3.1 | Preventing cytokine-induced cell death in microencapsulated human islet cells by adding ECM molecules

To study whether the addition of selected combinations of ECM molecules to the intracapsular environment can protect human encapsulated islets from cytokine-induced cytotoxicity, we exposed human islets in capsules containing 50  $\mu$ g/ml collagen type IV with either laminin sequences 0.01 mM RGD, 1 mM LRE or 0.01 mM PDSGR to a cytotoxic mixture of TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml) for 24 h and 72 h. After incubation, the islets were stained with calcein AM and EthD.

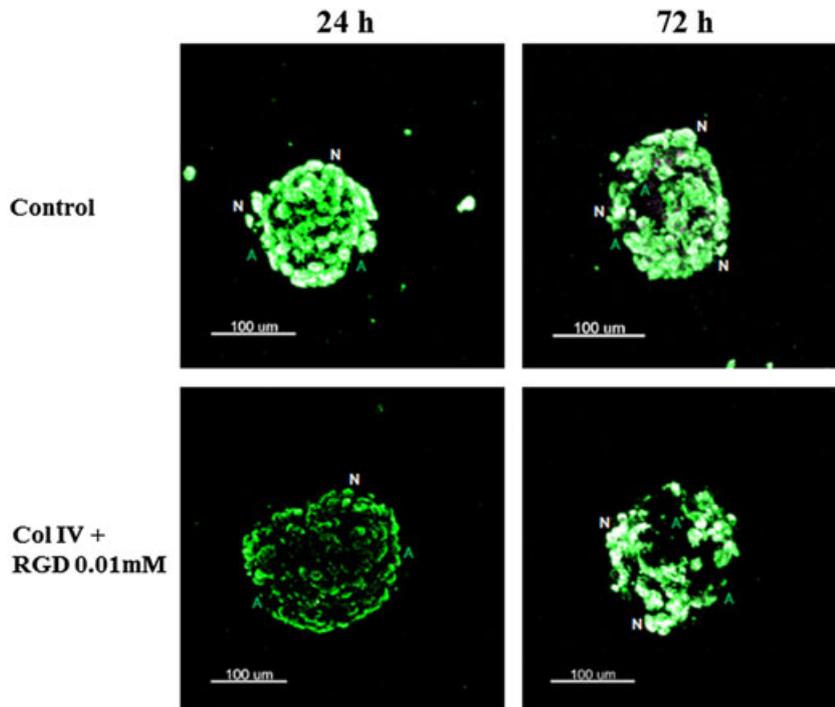
After 24 h, there were no differences in viability between controls and islets in capsules with either of the three ECM combinations. This was different at 72 h of exposure. The number of viable cells in the controls, without ECM, was decreased to 47.7  $\pm$  3.9%. This decrease was reduced by 50  $\mu$ g/ml collagen type IV with either laminin sequences 0.01 mM RGD (*P* < 0.005), 1 mM LRE (*P* < 0.05) or 0.01 mM PDSGR (*P* < 0.005; Figure 1).

To determine which cell death process was responsible for the loss of viability after cytokine exposure and to investigate whether ECM addition influences the type of death process of the cells, we performed dual staining with Annexin-V and PI staining. Apoptotic cells stain green and necrotic cells stain green with white nuclei (Figure 2).

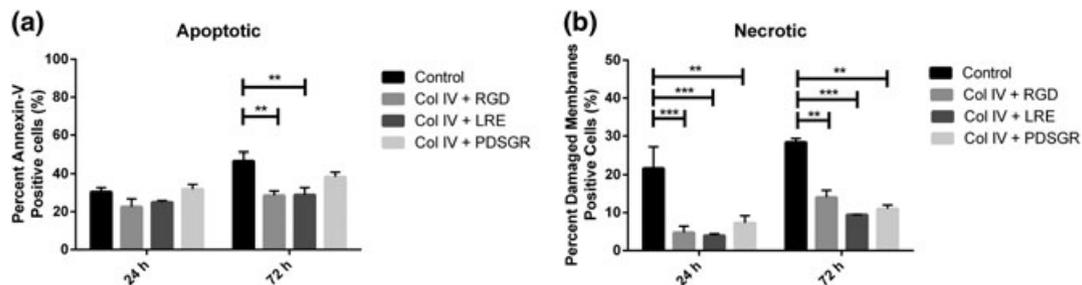
After 24 h, islet cells predominantly died by apoptosis in the control group that had no ECM in the capsules (30.5  $\pm$  3.9%). This was different in the ECM-containing capsules. As shown in Figure 3, the increase in numbers of apoptotic cells was prevented by 50  $\mu$ g/ml collagen type IV with either laminin sequences 0.01 mM RGD (*P* < 0.01), 1 mM LRE (*P* < 0.01) or 0.01 mM PDSGR (*P* = 0.06) after 72 h in culture (Figure 3). The beneficial effects of ECM components on necrosis were even more pronounced. Necrosis after cytokine exposure was more than two times lower in islets in capsules containing ECM molecules compared with controls (*P* < 0.005). Necrosis increased after 72 h exposure to cytokines, but remained reduced in islets encapsulated in capsules with ECM component (28.4  $\pm$  1.0%).



**FIGURE 1** Effect of cytokines on viability of islets encapsulated in alginate-based microcapsules containing 50  $\mu$ g/ml collagen type IV, and either (a) 0.01 mM RGD, (b) 1 mM LRE, or (c) 0.01 mM PDSGR. Encapsulated islets were treated for 24 h and 72 h with a mix of human cytokines [TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml)]. Results represent mean  $\pm$  SEM of four independent experiments. \* Indicates statistically significant differences (*P* < 0.05) compared with control islets. Col IV, collagen type IV



**FIGURE 2** Cytokines predominantly induced apoptosis and necrosis in encapsulated human islets. Representative illustration of islets in control conditions (in alginate capsule without ECM), and a capsule containing collagen IV with RGD 0.01 mM, stained with both Annexin-V and propidium iodide (PI) at 24 h and 72 h after exposure to human cytokines [TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml)]. A, apoptotic cells (green); N, necrotic cells (white and Green); ECM, extracellular matrix



**FIGURE 3** Addition of 50  $\mu$ g/ml collagen type IV, and either 0.01 mM RGD, 1 mM LRE or 0.01 mM PDSGR influence the mode of cell death in human encapsulated islets after exposure to a mixture of TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml). Encapsulated islets were stained with both Annexin-V and propidium iodide (PI), treated for 24 h and 72 h with the cytokine mixture. Apoptotic (a) and necrotic (b) cells identified by Annexin-V/PI staining were quantified by microscopy. Values represent the mean  $\pm$  SEM of four independent experiments.

\*\* , \*\*\*Indicate statistically significant differences ( $P < 0.01$  and  $P < 0.005$ ) when compared with control islets, respectively. Col IV, collagen type IV

### 3.2 | ECM components reduce release of DAMPs

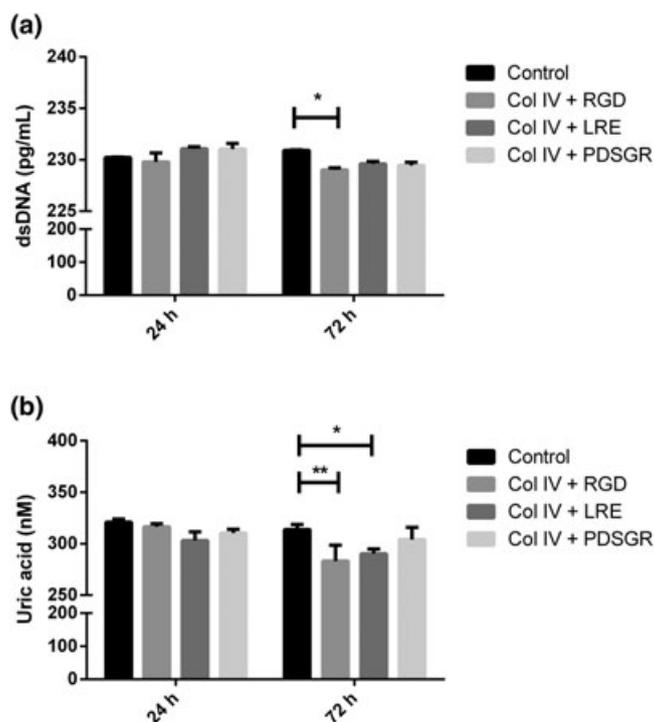
Necroptosis and necrosis are cell death processes responsible for the release of DAMPs and provocation of inflammatory responses. Double-stranded DNA (dsDNA) and uric acid are DAMPs known to be produced by human islets (Paredes-Juarez et al., 2015). At 24 h, there was no clear effect of ECM addition to the capsules on production of DAMPs. However, differences were observed after 72 h. As shown in Figure 4, the addition of ECM molecules to capsules can prevent the release of DAMPs after 72 h exposure to the cytokine mix of TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml).

Double-stranded DNA was present in all conditions. The dsDNA release of islets exposed to an ECM mix containing 50  $\mu$ g/ml collagen type IV with 0.01 mM RGD was significantly lower ( $P < 0.05$ ) than the dsDNA concentrations in controls (Figure 4a). Although some reduction of dsDNA was observed in islets exposed to mixtures of collagen IV and LRE and PDSGR, we observed no statistically significant differences. Moreover, uric acid concentrations were significantly lower in the medium of islets containing 50  $\mu$ g/ml collagen type IV with either

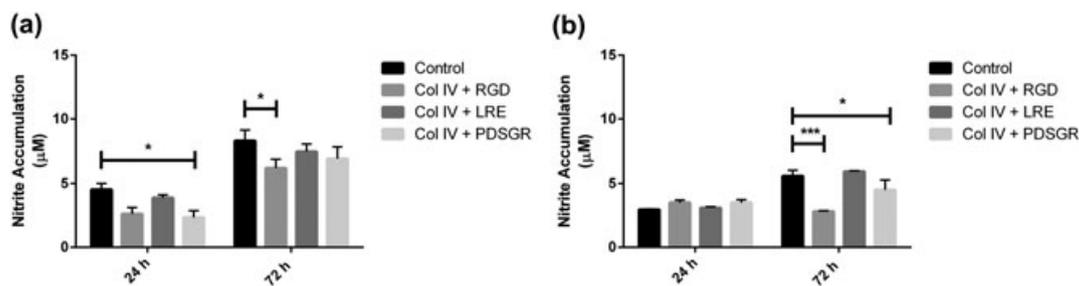
laminin sequences 0.01 mM RGD ( $P < 0.01$ ) or 1 mM LRE ( $P < 0.05$ ) after 72 h in culture. PDSGR also had a lowering effect, but this never reached statistical significance (Figure 4b).

### 3.3 | RGD and PDSGR reduce NO release from encapsulated human islets

NO is a primary sign and marker of cell damage after cytokine exposure. Therefore, NO release was studied in the supernatant of islets cultured for 24 h and 72 h with or without the cytokine mix (TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ ). After 24 h of exposure to the cytokine mix, NO was found in the culture medium, but only islets encapsulated in collagen type IV with 0.01 mM PDSGR ( $P < 0.05$ ) reduce significantly in comparison to controls (Figure 5a). At 72 h of exposure, clear effects of ECM incorporation in the capsules were found. NO in controls was nearly two-fold higher than at 24 hours. Collagen type IV with 0.01 mM RGD presented a lower NO release ( $P < 0.05$ ), when was culture with human cytokines. Moreover, NO values were statistically significantly lower in



**FIGURE 4** Danger-associated molecular patterns (DAMPs) release from human islets encapsulated in alginate-based capsules supplemented with combinations of extracellular matrix (ECM) molecules. Encapsulated islets were treated for 24 h and 72 h with human TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml). DAMPs found were double-stranded DNA (dsDNA) (a) and uric acid (b). The results represent the mean  $\pm$  SEM of four independent experiments. \* \*\*Indicate statistically significant differences ( $P < 0.05$  and  $P < 0.01$ ) when compared with control islets, respectively



**FIGURE 5** NO production by human islets in alginate capsules containing 50  $\mu$ g/ml collagen type IV, and either 0.01 mM RGD, 1 mM LRE or 0.01 mM PDSGR. Encapsulated islets were treated for 24 h and 72 h with (a) and without human cytokines mix [TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml)] (b). Supernatants were harvested, and nitrite production was determined by the Griess assay. The results represent the mean  $\pm$  SEM of four independent experiments. \* \*\*\*\*Indicate statistically significant differences ( $P < 0.05$  and  $P < 0.005$ ) when compared with control islets

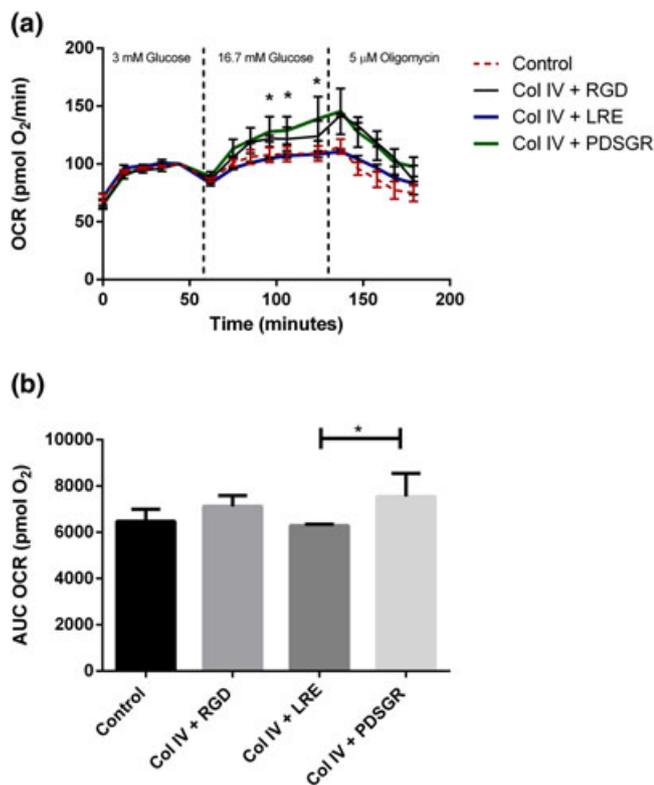
capsules containing 50  $\mu$ g/ml collagen type IV with either laminin sequences 0.01 mM RGD ( $P < 0.005$ ) or 0.01 mM PDSGR ( $P < 0.05$ ), when was culture without human cytokines (Figure 5b). There was an ECM-dependent effect as the combination of collagen IV and LRE had no reducing effect on NO.

### 3.4 | Specific ECM combinations protect energy metabolism of pancreatic islets after exposure to cytokines

Next, we determined the effect of ECM on OCR, which is an indicator of mitochondrial respiration in islet cells (Pike Winer & Wu, 2014). This was done in the presence and absence of TNF- $\alpha$  (2000 U/ml), IFN- $\gamma$  (2000 U/ml) and IL-1 $\beta$  (150 U/ml). A higher OCR is associated with a better long-term maintenance of islet function, and is correlated with

a higher success rate of islets after implantation (Papas et al., 2015). As high numbers of islets are required for the experiments, we only determined the OCR after 72 h of culture. First, we measured the OCR of islets exposed to human cytokines. To this end, islets were first incubated for 60 min at 3 mM glucose before the start of the test. Then, islets were exposed for 58 min to high glucose (16.7 mM), followed by an incubation of 53 min in 5  $\mu$ M oligomycin to determine whether the enhanced response to high glucose was indeed ATP-dependent and did not occur via other pathways (Wikstrom et al., 2012).

As shown in Figure 6a, there were no differences in OCR under low-glucose conditions. This was different for specific ECM combinations during high-glucose exposure. To quantify the differences, we did two comparisons. First, we compared with time point 58 min after



**FIGURE 6** The effects of cytokines on oxygen consumption rate (OCR) of human islets encapsulated with different types of extracellular matrix (ECM). Human islets in alginate capsules containing a combination of 50  $\mu\text{g}/\text{ml}$  collagen type IV, and either 0.01 mM RGD, 1 mM LRE or 0.01 mM PDSGR were treated for 72 h with human cytokines (150 U/ml IL-1 $\beta$ , 2000 U/ml IFN- $\gamma$  and 2000 U/ml TNF- $\alpha$ ). (a) Seahorse Bioscience XF24 extracellular flux analyser was used to measure OCR (pMoles/min), indicative of OXPHOS in encapsulated islets containing ECM and/or controls without ECM. After 68 min, islets were challenged with glucose (16.7 mM). Next, after incubation with high-glucose, islets were treated with the F1FO ATP synthase inhibitor oligomycin (5  $\mu\text{M}$ ). (b) OCR values upon glucose stimulation of encapsulated islets containing ECM after cytokine challenge. Each data point represents the mean  $\pm$  SEM of four independent experiments. \*Indicates statistically significant differences ( $P < 0.05$ ) when compared with control islets, respectively

16.7 mM glucose exposure and, second, we compared the AUC. At time point 137 min we found a statistically significantly higher OCR for islets in collagen IV with RGD ( $P < 0.05$ ) and collagen IV with PDSGR ( $P < 0.003$ ). Also, the AUC values were higher, but only reached statistically significant differences when comparing islets with 0.01 mM PDSGR and 1 mM LRE ( $P < 0.05$ ; Figure 4b). All responses to high glucose were ATP-dependent, as oligomycin administration decreased OCR almost instantly under all conditions.

#### 4 | DISCUSSION

In the present study, we show to the best of our knowledge, for the first time, that incorporation of collagen IV and specific laminin sequences, i.e. RGD, LRE or PDSGR, can contribute to the survival of encapsulated human pancreatic islets when the islets are exposed to

the inflammatory cytokine cocktail IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ . We choose this cocktail as it is accepted that islets are sensitive for IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ , and play a critical role in the pathogenesis of T1D (Wang et al., 2010). This cytokine combination inhibits insulin synthesis and secretion in pancreatic islets (Wang et al., 2010). Studies in isolated islets have shown that IL-1 $\beta$  is cytotoxic to both  $\alpha$ - and  $\beta$ -cells, but it selectively inhibits  $\beta$ -cell secretion of insulin and not glucagon secretion from  $\alpha$ -cells (Ling et al., 1993). As shown here, islet cell viability decline occurred within 72 h after exposure to the cytokine mix. In islets containing a supplement of laminin sequences, decline and cell death was prevented. The tested laminins contain specific regions in laminin  $\alpha 5$  and  $\beta 1$  (Otonkoski, Banerjee, Korsgren, Thornell, & Virtanen, 2008) that can decrease apoptosis by binding to integrins (Otonkoski et al., 2008). The effects are laminin-specific, which explains the differences in dynamics of the release of NO and DAMPs when the islets were exposed to the cytokine mix.

The cell death process can be further enhanced when islet cells are exposed to cytokines (Vincenz et al., 2011). We demonstrate that all applied incorporations of ECM molecules could avoid or prevent, to some extent, cell death in islet cells. This observation corroborates the findings of Zhao et al. (2010), who observed that islets contain more apoptotic or necrotic cells than those supplemented with ECM. This can probably be explained by the fact that the tested ECM components support integrin-ECM interactions by  $\alpha 3$  and  $\beta 1$ , which are critical for modulating cell survival and function (Krishnamurthy et al., 2011).

The beneficial effects of ECM on graft function go further than just preventing cell death. As shown here, the encapsulated islets supplemented with ECM also release less DAMPs. DAMPs can bind Toll-like receptors (TLRs) on cells of the immune system in the vicinity of the graft and activate both the innate and adaptive immune systems, with graft failure as the ultimate consequence (Braza, Brouard, Chadban, & Goldstein, 2016). Human islets are potent producers of DAMPs, such as dsDNA and uric acid (Paredes-Juarez et al., 2015), which are released in reduced amounts after supplementation of collagen type IV and the specific laminins sequence RGD, PDSGR and LRE. The dsDNA is an inflammation inducer when islet cells undergo necrosis or necroptosis, resulting in CD8+ T-cell responses (Piccinini & Midwood, 2010; Rocic, Vucic-Lovrencic, Poje, Poje, & Bertuzzi, 2005). Uric acid is more than an enhancer of immune responses via binding to TLR2 and 4 (Rocic et al., 2005). It also induces insulin resistance in peripheral tissues,  $\beta$ -cell dysfunction, and might be responsible for the enhanced NO production (Corbett and McDaniel, 1994). All these processes can be reduced by adding ECM to the intracapsular environment.

Proinflammatory cytokines can impair basic functions such as insulin release by interfering with  $\beta$ -cell production of ATP through mechanisms that are not well defined (Wang et al., 2010). To gain insight into the dynamics of impaired ATP production in the presence of cytokines, we used real-time metabolic flux analysis to monitor changes that acutely follow after exposure of human pancreatic islets to the proinflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ . This type of analysis involves measurement of, for example, OCR, which is a measure for glycolysis and mitochondrial respiration in the islets (Wikstrom et al., 2012). We observed a statistically significant higher OCR in islets in capsules containing collagen type IV with

0.01 mM RGD or 0.01 mM PDSGR at 60 min after exposure to glucose. The 1 mM LRE group was always lower in OCR and reached statistically significant differences when comparing the AUC with that of 0.01 mM PDSGR. Again, this is probably due to different interactions of LRE and PDSGR with islet cells with differences in responses to the cytokines as a consequence. One of these differences is that islets in capsules with LRE also had a higher NO production. NO is known to induce a nitric oxide-dependent inhibition of mitochondrial aconitase, resulting in a decrease in oxidative metabolism and ultimately insufficient generation of ATP and islet dysfunction (Welsh, Eizirik, Bendtzen, & Sandler, 1991). A mechanism by which NO generation by cytokines impairs islet function is by NO-induced acute disruption of the  $\beta$ -cell mitochondrial respiration, which subsequently causes inhibition of mitochondrial aconitase activity, resulting in a decrease in oxidative metabolism and ultimately insufficient generation of ATP and islet dysfunction (Welsh et al., 1991). This suggestion is corroborated by the findings of Corbett and McDaniel (1994), who observed a negative effect of NO on insulin secretion from nitrosylation of iron-containing enzymes in the mitochondria, most notably aconitase, that are necessary for ATP generation.

The islets containing RGD and PDSGR produced less NO and a lesser fall in OCR under cytokine exposure than the islets containing collagen IV and LRE. This should be explained by differences in subunits in the three laminin sequences. Laminins are heterotrimeric ( $\alpha/\beta/\gamma$ ) glycoproteins present in the basal laminae on the membrane cells. Multiple integrin-binding regions have been identified in laminins (Stendahl et al., 2009). RGD is one of the most studied adhesion sequences (Humphries, Byron, & Humphries, 2006), related to mediate cell functions such as adhesion and spreading. This recognition sequence interacts with many members of the integrin family, including  $\alpha 3\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 5\beta 3$  and  $\alpha v\beta 5$  (Takagi, 2004). The laminin adhesive peptide PDSGR is present in the  $\beta 1$  chain (Stendahl et al., 2009). It is known to facilitate the adhesion of epithelial cells such as islets cells (Ali, Saik, Gould, Dickinson, & West, 2013), and it has been reported to accelerate the proliferation of some cell types (Kumada and Zhang, 2010). LRE has also been reported to guide cellular processes (Hunter et al., 1991), but LRE, in contrast to RGD and PDSGR, are lacking integrin-binding subunits  $\alpha 3$ ,  $\alpha 5$  and  $\beta 1$  (Hunter et al., 1991; Takagi, 2004). These subunits might be important for islet function, as it has been shown that in the human pancreas  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha v$ ,  $\beta 1$ ,  $\beta 4$  and  $\beta 5$  integrin subunits are present and essential for function (Virtanen et al., 2008; Wang et al., 2005). The absence of these subunits in LRE may therefore be the cause of the lesser beneficial effect of this laminin sequence compared with RGD and PDSGR. A previous study in mice has shown that a lack of  $\beta 1$  integrin in insulin-producing cells results in a dramatic reduction of the number of  $\beta$ -cells (Diaferia et al., 2013). Additionally, it has been shown by Kaido et al. that human  $\beta$ -cells contain  $\alpha v\beta 1$ ,  $\alpha v\beta 5$  and  $\alpha 1\beta 1$  integrins throughout development and adulthood, and are important for normal development (Kaido, Perez, et al., 2004; Kaido, Yebra, Cirulli, & Montgomery, 2004). The lack of specific ligands in the sequences in LRE can explain the differences in NO and OCR between the islets containing RGD and PDSGR. The reduced NO production in islets exposed to RGD and PDSGR should be explained by interaction with the integrins present in human islets, such as  $\alpha v\beta 3$ , and  $\alpha v\beta 5$  reported by Cirulli et al. (2000), which can recognize an RGD motif within their ligands (Takagi, 2004) and are lacking in LRE.

In conclusion, our study presented here demonstrates that incorporation of specific ECM molecules such as collagen type IV with the laminin sequences RGD and PDSGR in immunisolated islets can protect against cytokine toxicity. The ECM supplementation prevented cell death and preserved islet cellular activity. The interaction between different integrins such as  $\alpha 3$ ,  $\alpha 5$  and  $\beta 1$  present in RGD and PDSGR, which are absent in LRE, might possibly explain the specificity of the observed protection and maintenance of the viability and functionality of encapsulated human islets exposed to cytokines. Our findings can be applied to improve function and protection of islets in immunisolating devices.

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## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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