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The role of pathogen-associated molecular patterns and islet-derived danger-associated molecular patterns in longevity of microencapsulated pancreatic islets
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Chapter 5

ROLE OF DAMPS ON GRAFT SURVIVAL OF MICROENCAPSULATED ISLETS IN A MyD88-DEFICIENT MOUSE MODEL
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Work in progress.

ABSTRACT

Despite the benefits of encapsulation, after transplantation encapsulated islets face low nutrition and hypoxia due to the lack of revascularization. These conditions are highly detrimental for the islets promoting necrosis and necroptosis. These factors contribute to islet loss and release of islet-derived factors from the capsules immediately after transplantation. These islet-derived factors are a family of molecules called danger-associated molecular patterns (DAMPs). DAMPs are recognized by the innate immune system and provoke an immune response when they bind to Toll-like receptors (TLRs) leading to cellular death and eventually graft failure. We found that microencapsulated islets release the DAMPs dsDNA and uric acid. To study the impact of these DAMPs on graft survival duration and function in vivo we used a MyD88 knockout (KO) mice model to compare the graft survival of mice with non-functional TLRs with that of wild type mice. After streptozotocin-induced diabetes, both MyD88 KO and wild type (BL6) mice received encapsulated rat islets. The MyD88 KO recipients became normoglycemic in only 4 days while the wild types required up to 11 days. Normoglycemia was maintained successfully in both strains for the duration of the experiments. Glucose tolerance test was performed after normoglycemia in MyD88 KO and in wild type mice as a measure of β-cell function, and demonstrated a faster return to homeostasis in MyD88 KO. After explantation, capsules from MyD88 KO mice had insulin producing islets and low or no cellular overgrowth around the capsules, contrary to BL6 mice. We show here that DAMPs released by encapsulated islets contribute to failure of the grafts.

Keywords: DAMP, MyD88 KO mouse, glucose, c-peptide, streptozotocin-induced diabetes.
INTRODUCTION

Microencapsulation of islets allows for transplantation of an endogenous insulin source in type 1 diabetes without the need for immunosuppression [1–3]. Microencapsulation surrounds islets in a semipermeable membrane that protects these cells from the immune system of the host, yet still permits diffusion of oxygen and nutrients, and the release of insulin. This strategy abrogates the need for immunosuppression and its undesired secondary effects [4–7], and permits the use of xenogeneic tissue [2,3,8–11].

Although a number of clinical trials are ongoing, many obstacles remain to be overcome before its use is a reality [9,12–14]. Among those obstacles, nearly 60% of transplanted islets does not survive the period immediate after transplantation [7]. During recent years many groups have identified factors that determine the success or failure of islet grafts, i.e. the instant blood mediated inflammatory reaction [2,6,15], alloreactivity [6], recurrent autoimmunity [6], NK-cell cytotoxicity [16], inadequacy of the transplantation site [17], and insufficient revascularization [7]. The focus of islet survival has been on these external host factors, however, islets are potent producers of numerous bioactive molecules that may contribute to their own destruction by enhancing inflammatory responses [7].

Among those islet-derived molecules are the so-called danger-associated molecular patterns (DAMPs). DAMPs are intracellular molecules that maintain homeostasis but when exposed to the extracellular space, they act as danger molecules that promote immune activation. DAMPs like uric acid and DNA-fragments [18], are recognized by the innate immune system and provoke an immune response when they bind to pattern recognition receptors (PRRs) such as well-studied toll-like receptors (TLRs) via coupling protein MyD88.

A current theory in transplantation research is that the release of DAMPs by islets plays a crucial role in the responses against grafts [18], which to the best of our knowledge, has not been studied yet. The release of DAMPs from cells exposed to ischemic conditions [19] as a consequence of cell necrosis and necroptosis [20–22] has been established in other models. It is known that islets undergo a relative long phase of ischemia in the period between implantation and revascularization and as a consequence, DAMPs are being released [23]. Therefore, in this study we investigated the role and contribution of DAMPs in islet-graft failure.

By using a MyD88 knock-out (KO) mouse model, we established the role of TRL-mediated DAMP signaling to the survival of islet grafts in vivo. We investigated the contribution of islet-derived factors on islet-loss and graft function post-transplantation. Understanding this mechanism of transplantation failure will outline new strategies to improve the survival of encapsulated pancreatic islets for clinical use in the treatment of type 1 diabetic patients.

MATERIALS AND METHODS

Animals

As recipients, male C57BL/6 (BL6, Harlan Laboratories Inc., IN, USA) and B6.129P2(SJL)-Myd88tm1.1Defr/J (MyD88 KO, a strain without TLR function) mice (Jackson Laboratory, ME, USA) were acquired. All the experiments and procedures were approved and performed in accordance with the institutional Animal Care Committee of the
University of Groningen. All animals received animal care in compliance with the Dutch Law on Experimental Animal Care.

**Diabetes induction**

Diabetes induction was achieved in the MyD88 KO mice with a single intraperitoneal (IP) injection of streptozotocin (STZ, Sigma-Aldrich, MO, USA) at a dose of 200 mg/kg of body weight dissolved in citrate buffer (pH 4.5). Diabetes in BL6 mice was induced with an IP injection of STZ at a dose of 180 mg/kg of body weight. If diabetes was not induced (blood glucose levels < 15 mM) within 1 week, a second dose of 200 mg/kg was given. Mice are monitored every two days for their glycemic state and weight loss due to diabetes induction. Animals were considered diabetic when two blood glucose measurements over a week were higher than 15 mM and were recipients for encapsulated islets. Glucose was monitored using a blood glucose meter (Ascensia Contour, Bayer, NJ, USA) and glucose test tapes (Contour®, Bayer, Switzerland). A graft was considered successful when mice had glucose levels below 11 mM. Grafts were considered to have failed when two blood glucose measurements over a week were higher than 20 mM. These animals were subsequently sacrificed.

**Islet isolation**

As islet donors we selected male Albino Oxford rats from our own breeding colony. Laparotomy was performed under general anesthesia using isoflurane and oxygen. Subsequently the part of the ductus, just above the pancreas, was cannulated. The pancreas was distended by 10 ml collagenase solution (1 mg/ml) in Krebs-Ringer-HEPES buffer (KRH) buffered with 25 mM HEPES and 10% (w/v) bovine serum albumin (BSA). After digestion of the pancreas with collagenase solution, approximately 1000 islets were manually selected by handpicking under a Leica DM IL inverted contrasting microscope with a S 90/0.23 condenser, free working distance of 90 mm and a numerical aperture of 0.23 (Leica microsystems, Wetzlar, Germany).

**Microencapsulation**

After isolation, islets were washed with CMRL 1066 (CMRL 1066 medium containing 10% FCS, 8.3 mM glucose, 10 mM HEPES, 2 mM L-glutamine and 1% Penicillin/Streptomycin) culture medium and then with calcium-free KRH. The islets were suspended in sterile filtered (0.2 μm pore size) 3.4% (w/v) intermediate-G alginate (ISP Alginates Ltd., Ayrshire, UK) and encapsulated as previously described [24]. Briefly, alginate was dissolved in KRH with an appropriate osmolarity, and the solution was converted into droplets using an air-driven droplet generator. The solution of 3.4% (w/v) of alginate is the minimum concentration that allows its filtration and provides mechanical strength and spherical capsules, minimizing the effect of swelling and shrinking [25]. Alginate droplets are transformed to alginate beads by gelling in a 100 mM CaCl2 (10 mM HEPES, 2 mM KCl) solution for at least 5 min. Subsequently, the calcium beads are suspended for 1 min in KRH buffer containing 2.5 mM CaCl2. A poly-L-lysine (PLL) layer is form by suspending the alginate beads in 0.1% (w/v) PLL solution dissolved in KRH for 10 min (poly-L-lysine-HCl, Mr: 22000, Sigma). The outer alginate-layer is subsequently applied by 5 min incubation in a 10 times diluted alginate solution. After encapsulation, capsules were inspected under the microscope and those with imperfections, broken or with
more than one islet per capsule were discarded. Capsules had a final diameter of 600-700 µm.

**Experimental culture conditions**
Microencapsulated pancreatic human islets were exposed to culture conditions to simulate what they may encounter in vivo. To simulate the relative low oxygen tensions after transplantation [17,26], islets were cultured under hypoxic conditions (1% O2, 5% CO2, and 94% N2). Insufficient revascularization after implantation of islets can also lead to low nutrition [27]. To simulate low nutrition, islets were cultured in CMRL 1066 medium (Life Technologies, NY, USA) without fetal calf serum (FCS). As control served CMRL 1066 medium with 10% FCS, under normoxic (20% O2, 5% CO2, and 75% N2) incubation conditions.

**Measurement of specific DAMPs and cell viability**
To quantify the danger-associated molecular patterns (DAMPs), ELISAs were performed on supernatant of incubated islets. ELISAs for detecting High-Mobility Group Box 1 (HMGB1, Qayee-Bio, Shanghai, China), Heat Shock Protein 70 (HSP70, Donglin Sci & Tech, Wuxi/Jiangsu, China), double stranded DNA (dsDNA, BlueGene Biotech, Shanghai, China), and uric acid (Abcam®, Cambridge, UK). All ELISAs were performed according to standard protocols provided by the manufacturer. Viability of encapsulated cells was assessed using a LIVE/DEAD Cell Viability/Cytotoxicity Assay Kit from InvitroGen, Life Technologies (New York, USA). Encapsulated cells were incubated for 30 min with Calcein AM (1 mM) and Ethidium Bromide (EB) (2 mM). After incubation, fluorescent confocal microscopy was measured at an emission wavelength of 517 nm (Calcein AM) and 617 nm (EB) using a Leica TCS SP2 AOBS confocal microscope (Wetzlar, Germany) equipped with an objective HC PL APO CS 10×/0,30, dry immersion, and working distance of 11 mm [28].

**Islets transplantation and explantation**
Transplantation was performed (under general anesthesia with isofluorane) by injecting the microcapsules suspended in 0.5 ml of KRH into the peritoneal cavity of the mice using a 16-G blunt cannula via a small incision (3 mm) through the linea alba. The transplants contained at least 1000 capsules. The capsules were retrieved by peritoneal lavage. Peritoneal lavage was performed by infusing 5 mL of KRH buffer into the peritoneal cavity and subsequent aspiration of the KRH containing the capsules.

Two experimental groups were assessed, 1) a MyD88 KO mice (STZ-induced diabetic mice) and 2) a BL6 mice (STZ-induced diabetic mice). Those groups allowed the comparison of the role of DAMPs in the survival of the encapsulated islets. Since DAMP signaling is mainly TLR mediated, a prolonged survival of the grafts in the MyD88 KO mice was expected when compared with BL6 mice.

**Oral glucose tolerance test**
Animals that were normoglycemic for at least four weeks were subjected to an oral glucose tolerance test (OGTT). Animals were fasted for 2 hrs and then trained to eat 1 gram of chow (20% protein, 9% fat, 10% glucose, 3% fiber, 6.5% ash, and 2.5% minerals) within 5 min. To avoid competition for food, each mouse is subjected to a meal challenge in separate cages. Blood samples were obtained from the tail and collected in Microvette®
Samples were taken 5 min before and time 0 of providing the pellet and at 10, 20, 30, 60, 90, and 120 min after start of the test to determine c-peptide and glucose.

**Histology and immunohistochemistry**

To analyze and detect physical imperfections and to assess the degree of overgrowth after implantation, we used immunohistochemistry as described elsewhere [28]. Briefly, half of the samples of adherent capsules and/or samples of non-adherent capsules were fixed in pre-cooled 2% paraformaldehyde, buffered with 0.05 M phosphate in saline (pH 7.4), and processed for glycol methacrylate (GMA) embedding. Sections were stained with hematoxilin to qualify the degree of cellular overgrowth. The other half were fixed in Bouin’s solution and processed for paraffin embedding and staining to determine insulin production. Mouse-anti-Rat-IgG1 (Sigma-Aldrich, MO, USA) was used as primary antibody (1:300) and goat-anti-mouse- IgG1-PO (Sigma-Aldrich, MO, USA) conjugate (1:100) as secondary antibody. Endogenous peroxidases were blocked with 0.1% H2O2 in phosphate buffered saline (PBS).

**C-peptide determination**

C-peptide concentrations were measured using a Rat C-Peptide ELISA Kit (Cristal Chem, IL, USA) following the protocol provided by the company. Briefly, blood samples were collected and centrifuged at 4°C for 20 min at 2000 G to obtain plasma. After centrifugation, 5 µl of sample or standard (6.4 ng/ml and half dilutions until 0.1 ng/ml) were added to the antibody-coated microplate containing 95 µl of sample diluent and incubated for 1 hr at room temperature. After washing the plate, 100 µl of anti C-Peptide Enzyme Conjugate were added and incubated for 1 hr. After a final wash, 100 µl of Enzyme Substrate Solution were added per well and allowed to react for 30 min avoiding direct exposure to light. Reaction was stopped by addition of 100 µl of Enzyme Reaction Stop Solution and absorbance (A450-A630) was measured within 30 min using a spectrophotometer. All incubations were performed at room temperature.

**Statistical analysis.**

Values are expressed as mean ± standard error of the mean (SEM). Normal distribution of the data was confirmed using the Kolmogorov–Smirnov test. Statistical comparisons were performed using t-test to compare between two groups. Values of p < 0.05 were considered to be statistically significant.

**RESULTS**

**Microencapsulated islets release the DAMPs dsDNA and Uric acid**

To confirm the release of DAMPs, microencapsulated human pancreatic islets were incubated under hypoxic and low nutrient conditions, simulating those that can be found after implantation. We measured a continuous release of dsDNA under normal nutrient conditions and normoxia, and under low nutrients and hypoxia (Figure 1a), with no statistical significant differences. Equally, we were able to measure uric acid with a statistically significant increase (p < 0.01) when incubated under hypoxia and low nutrients (Figure 1b). Interestingly, we could also find nucleic acids retained throughout the capsules (Figure 1c) stained with ethidium bromide.
Figure 1. Microencapsulated islets in culture release the DAMPs dsDNA (a) and uric acid (b) incubated for 7 days under control at 20% oxygen and low nutrients conditions at 1% oxygen. Data is presented as mean ± SEM (n = 4 separate batches of pancreatic islets) (**, p < 0.01). Immunoisolated pancreatic islet in alginate microcapsule (c) stained with Calcein AM (green, live cells) and Ethidium Bromide (red, dead cells). Red staining can be observed all over the capsule indicating the presence of nucleic acid retained by the capsule.

Oral glucose tolerance test, plasma glucose and c-peptide concentrations
Diabetic MyD88 KO mice became normoglycemic (glucose < 11 mM) within 4 days after transplantation, while the wild type required in average 7 days, and sometimes up to 11 days (Figure 2). Plasma glucose values during the OGTT showed that MyD88 KO mice have a faster glucose clearance (glucose < 6 mM) that occurred after 30 min, while in the BL6 it took up to 60 min. In our experiments, BL6 mice showed, during the OGTT, higher values of glucose when compared with the MyD88 KO (Figure 3a) up to 120 min after the beginning of the test.

Figure 2. Non-fasting blood glucose concentration of (a) STZ-diabetic MyD88 KO (•) (n=4) and STZ-diabetic BL6 (■) (n=4) mice. Baseline glucose values were measured one week before streptozotocin (STZ) induced diabetes and after transplantation with microencapsulated AO rat-islets. Data is presented as mean ± SEM.
Plasma concentrations of c-peptide were also measured during the OGTT (Figure 3b). C-peptide instead of insulin was quantified since the second is readily adsorbed in the peritoneal organs and undergo for hepatic metabolism [29]. C-peptide values start increasing after only 5 min after food consumption and reach its highest point (0.5537 ± 0.28 ng/ml) after 30 min in MyD88 KO mice. After that time point, c-peptide decayed slowly during the rest of the test. In BL6 mice, c-peptide values were already measurable before the beginning of the test (0.40 ± 0.001 ng/ml) while in MyD88 KO they were nearly absent. Higher levels of c-peptide were observed during the challenge in BL6 mice (Figure 3b). Interestingly, despite the high levels of c-peptide in BL6 mice, glucose clearance was slower than that in the MyD88 KO, expressed as a higher area under the curve (AUC, 605.1 ± 11.0 mM·min in MyD88 KO and 828.8 ± 10.75 mM·min in BL6 mice, Figure 3a). Analysis of the AUC for c-peptide responses showed that control BL6 mice values were higher approximately by 50% when compared with MyD88 KO mice (84.21 ± 0.81 ng·min/ml and 62.03 ± 6.2 ng·min/ml respectively). Both elevations, in glucose and c-peptide seem to indicate a low sensitivity of the BL6 mice to detect insulin.

The ratio between the c-peptide and glucose concentrations in plasma was also calculated as an estimation of the functionality of β-cells [30] (Figure 3c). A higher ratio was observed in the MyD88 KO indicating a better function of the encapsulated islets to metabolize plasmatic glucose when compared with BL6 mice with lower concentrations of c-peptide in plasma per minute (AUC 15.59 ± 0.17 ng·min/mM·ml for MyD88 KO and 13.21 ± 0.14 ng·min/mM·ml for BL6 mice).

Figure 3. Plasma glucose (a) and c-peptide (b) concentration during meal challenge of STZ-diabetic mice and the ratio of c-peptide/glucose (c). The test was performed after, at least, 4 weeks of normoglycemia (< 11 mM) in STZ-diabetic MyD88 KO (•) (n=2) and STZ-diabetic BL6 (■) (n=2) mice transplanted with microencapsulated AO rat-islets. Data is presented as mean ± SEM.
Reduced inflammatory responses in MyD88 KO mice compared to wild type mice

After 13 weeks of implantation in MyD88 KO, the microcapsules were retrieved by peritoneal lavage. Capsules retrieved from MyD88 KO mice had low or no cellular overgrowth of immune cells around the capsule and encapsulated islets were able to produce insulin demonstrated by immunohistochemistry (Figure 4a). In the non-adherent, free capsules retrieved from the BL6 mice, a percentage of approximately 70% capsules contained cellular overgrowth. The islets in these capsules stained negative to insulin (Figure 4b) when compared with those in MyD88 KO (Figure 4). In BL6 mice also small groups of capsules (< 20%) were free of cellular overgrowth and some capsules were found as groups adherent to fatty and connective tissue in the abdominal cavity (Figure 5). In one MyD88 KO mouse failure was observed after 8 weeks. This animal had attachment of cells surrounding all the retrieved capsules (Figure 6).

Figure 4. Non-adherent alginate-PLL-alginate (APA) capsules containing AO islets explanted from peritoneal cavity of STZ-diabetic MyD88 KO (A) and STZ-diabetic BL6 (B) mice. Encapsulated islets were stained for insulin (5 µm paraffin-embedded sections stained for insulin and counterstained with hematoxylin). Membrane of APA capsule (M), islet (I), cellular overgrowth (CO). Original magnification x20.

Figure 5. Alginate-PLL-alginate (APA) capsules adhered to fatty tissue retrieved from peritoneal cavity of STZ-diabetic BL6 (2 µm GMA-embedded sections stained with hematoxylin). Membrane of APA capsule (M), fatty tissue (F), cellular overgrowth (CO). Original magnification x10.
Figure 6. Alginate-PLL-alginate (APA) capsules retrieved from peritoneal cavity of STZ-diabetic MyD88 KO mice after graft failure. The capsules were surrounded by cellular overgrowth and the encapsulated islets stained negatively for insulin. (5 µm paraffin-embedded sections stained for insulin and counterstained with hematoxylin). Membrane of APA capsule (M), islet (I), cellular overgrowth (CO). Original magnification x40.

DISCUSSION

In the last year the group of Wada et al. [31] establish a relation between islet-derived danger molecules and failure of islet grafts in type 1 diabetes. In addition, our group previously described the release of danger associated molecular patterns (DAMPs) by islets [32] and pathogen associated molecular patterns (PAMPs) from the capsules [33] in vitro which were able to elicit an immune response via MyD88-mediated activation of toll-like receptors (TLRs) [33,34]. In this study we established DAMPs contribution to immune activation in vivo after islet transplantation. Use of the MyD88 KO model revealed the specific contribution of MyD88-dependent TLR signaling to immune activation and islet graft failure.

Other groups have reported that the primary factor in islet rejection is capsule size [35]. However, we found that the size of capsules for successful transplantation can be expanded to around 600-700 µm when alginate is purified prior to encapsulation [33]. We discovered that the implants were able to survive over 5 months, with functional islets and almost no cellular outgrowing around the capsule (Figure 4). We hypothesize that the small capsules reported by Veiseh et al. [35] have a relative high surface to volume ratio that allows the release of other immune activators including PAMPs such as lipopolysaccharides (LPS), lipoteichoic acid (LTA) and peptidoglycans (PG) [33]. When impurities are removed, the main factor that determines the success of islet transplantation using capsules is the release of DAMPs. Effects of PAMPs were excluded by using highly purified alginates with confirmed absence of PAMPs [33,34].

Encapsulated islets improved glycemic control in MyD88 KO mice indicating that DAMP-signaling play a role in maintaining functional islets. C-peptide and glucose levels in the blood, well-established parameters for β-cell function [30], were lower in MyD88 knock-out mice. We observed that MyD88 KO mice re-establish homeostasis better, returning to normoglycemic values after an OGTT faster when compared with the BL6 control mice. This difference may be due to higher loss of islets in the BL6 control mice due to activation of the innate immune response by DAMPs from encapsulated via TLR signaling, inducing the recruitment of immune cells around the capsule, and ultimately, the failure of the grafts [24].
To further evaluate the β-cell function, a relationship between the levels of insulin and the sensitivity of the body towards it must be taken into account [30]. The ratio between c-peptide and glucose reflect those aspects. We observed that MyD88 KO mice metabolized plasma glucose with more efficiently than BL6 mice with lower levels of c-peptide, this could be related with reduced inflammatory processes occurring due to the lack of functional TLRs and therefore a reduced cellular death DAMP mediated.

Capsules explanted from BL6 mice confirmed that hypothesis, since they were surrounded by fibrotic growth, and the encapsulated pancreatic islets were not able to produce insulin. In contrast, immune cells did not surround capsules in most MyD88 KO model mice and the encapsulated islets could produce insulin. However, as shown in supplementary Figure S2, the transplanted grafts in one MyD88 KO mouse failed after 8 weeks. This might be due to the fact that small deformations and imperfections in the capsules may serve as anchorage sites for immune cells to adhere [36]. Consequently, immune activation can occur after transplantation as described by de Vos, et al. [7].

The use of biomaterials for encapsulation and replacement of β-cells is a promising solution in the treatment and control of type 1 diabetes [20,37–39]. The safety and feasibility of microcapsules for islet transplantation has already been established [40]. However, their efficacy still needs to be improved and standardized for clinical translation.

CONCLUSION

This study demonstrates that DAMPs are directly involved in the recognition of islet-containing microcapsules by the immune system. In the absence of functional TLRs to recognize these alarm signals, the survival of the grafts was enhanced and the functionality of the encapsulated islets was improved. We also established MyD88 KO mice as a model to characterize the effect of PAMPs and DAMPs in vivo and developed a strategy to reduce their release and effects in the immediate period after transplantation.

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