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In Vitro Studies of Squalene-Gusperimus Nanoparticles in Islet-Containing Alginate Microcapsules to Regulate the Immune Response in the Immediate Posttransplant Period

Carlos E. Navarro Chica, Tian Qin, Bart J. de Haan, Marijke M. Faas, Alexandra M. Smink, Ligia Sierra, Betty L. López, and Paul de Vos*

Grafting of microencapsulated pancreatic islets has been proposed as an alternative to exogenous insulin for the treatment of type 1 diabetes mellitus. Microencapsulated islets are protected from direct contact with immune cells and larger immune-active molecules such as immunoglobulins. Unfortunately, many islet cells in the microcapsules are lost in the immediate period after transplantation due to an early host immune response limiting long-term function of the graft. Gusperimus has shown to reduce the inflammatory responses to grafted encapsulated islets, but it cannot be appropriately used because it is easily hydrolyzed leading to loss of activity. To temporarily modulate the inflammatory response directly after implantation and stabilize gusperimus, squalene-gusperimus nanoparticles (Sq-GusNPs) are developed and incorporated in human islets-containing alginate-based microcapsules. A prolonged and continuous release of gusperimus is achieved. This offers an anti-inflammatory microenvironment in the vicinity of the microcapsules and a reduction of cytokine secretion by lipopolysaccharides-activated human macrophages. Release of gusperimus from Sq-GusNPs does not affect the in vitro viability or function of human pancreatic islets. The data illustrate that incorporation of Sq-GusNPs in alginate microcapsules offers an opportunity to temporarily modulate the immediate immune response after the grafting procedure of encapsulated islets cells and reduce loss of islet cells.

1. Introduction

Type 1 diabetes mellitus (T1DM) is a disease caused by the autoimmune destruction of β cells in the pancreas, causing hyperglycemia.[1] Patients with T1DM require continuous monitoring of blood glucose levels and daily injections of insulin, which impacts quality of life.[2] Also, the therapy cannot regulate glucose levels precisely enough to avoid regular hyper- and hypoglycemic episodes.[3] Long-term insulin therapy and frequent hyperglycemia can as a consequence lead to several diabetic complications such as angiopathy, nephropathy, neuropathy, and cardiovascular diseases.[2,4] Nowadays also frequent hypoglycemia is considered to be a major threat as it may lead to hypoglycemic unawareness that contributes to disability to participate in society.[5] A therapy to regulate glycemia from minute to minute is urgently needed to avoid these complications. Transplantation of the insulin-producing cells, such as pancreatic islets, is such a therapy that allows for near-normal regulation of glucose metabolism. Unfortunately, clinical islet transplantation requires chronic immunosuppression, which has many side effects and is therefore not considered an alternative for insulin therapy.[6]

Islet transplantation might be possible without chronic immunosuppression by encapsulating islets in immunoprotective membranes.[7] Within this approach, islets are enveloped in a biocompatible and semipermeable membrane that allows the ingress of nutrients and glucose and the egress of therapeutic molecules, waste products, and insulin.[8] This membrane also protects the islets from direct contact with immune cells and larger deleterious biomolecules such as antibodies.[7] By shielding the islets from the effects of the immune system, it is possible to avoid the use of chronic immunosuppression, but it might also contribute to a solution for the worldwide shortage of donors as encapsulation allows application of xenogeneic islets or islet cells obtained from progenitor cell sources.[7]

Grafting of microencapsulated islets has shown some success in animal models and even in humans, but the duration of the graft function varies extremely, from 1 month to 1 year.[3,6,7] This

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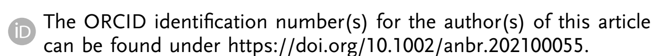
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variability is associated with variations in technologies between laboratories,[6] but also with an early host immune response directly after transplantation. After the grafting procedure of the microencapsulated islets, an immune response is triggered, which involves influx of cells into the transplantation site and a subsequent tissue repair response.[9] During this influx of immune cells, activation may occur either by the biomaterials[10,11] or by islet-derived danger-associated molecular patterns (DAMPs).[12] This may lead to release of inflammatory molecules[6] that can diffuse into the microcapsule and induce islet cell death.[8] We have shown that this immune response directly after implantation may lead to up to 60% loss of the islet functional mass in the first 2 weeks after grafting even in the absence of adhesion of cells to the surface of the microcapsules.[8,13]

The immediate immune response after implantation is difficult to manage by changing capsule characteristics as it starts with the mandatory surgery and dying cells in the intracapsular environment. A temporary attenuation of immune responses in the immediate environment of the graft has been proposed as a solution.[14] The incorporation of some anti-inflammatory agents into alginate microcapsules such as dexamethasone,[15] curcumin,[16] ketoprofen,[17] the incorporation of the Fas ligand with streptavidin (SA-FasL) on the surface of microparticles synthesized by reacting a biotin–poly(ethylene) glycol-thiol (biotin–PEG) thiol with maleimide-terminated four-arm poly(ethylene) glycol (PEG–4MAL) macromers,[18] and the coencapsulation of islets with erythrocytes in alginate microcapsules[19] are some of the approaches that have been used to reduce the immune response in the microenvironment of transplanted islets. This may be done with gusperimus, a proven potent inhibitor of macrophages, which are the driving force behind the foreign body responses.[20] Recently we developed a controlled release system in which gusperimus was nanoencapsulated as squalene-gusperimus nanoparticles (Sq-GusNPs)[21] to avoid stability issues and loss of activity associated with free gusperimus.[21–23] These nanoparticles (NPs) are shown to be stable, have a high drug-loading capacity, high uptake, and lack of toxicity, and exert an enhanced anti-inflammatory activity over time compared with free gusperimus.[21] Here we applied and tested this system when loaded into alginate microcapsules, which could be transplanted together with encapsulated islets to reduce immune responses in the immediate posttransplant period. We studied the effect of Sq-GusNPs on pancreatic islets and immune cells after their incorporation into alginate microcapsules and we determined the anti-inflammatory effect after triggering a proinflammatory response in macrophages in vitro.

2. Results

2.1. Incorporation of Sq-GusNPs into Alginate Microcapsules Allows a Controlled and Prolonged Anti-inflammatory Effect on Lipopolysaccharide (LPS)-Activated Human Macrophages

As about 60% of all islet cells undergo death in the immediate posttransplant period[24] as a consequence of the inflammatory reaction in the vicinity of the graft, we developed a system allowing for controlled release of the anti-inflammatory agent gusperimus. This system is designed to attenuate the immune response locally and temporarily in the transplantation site of the encapsulated islets. To facilitate sustained release of gusperimus, we previously encapsulated gusperimus by binding it to squalene to form Sq-GusNPs after nanoprecipitation.[21] By this, we avoid the stability issues associated with the free drug under physiological conditions, which cause loss of its immunosuppressive activity.[21–23] The Sq-GusNPs were then incorporated into alginate microcapsules by suspending them in an alginate solution followed by gelation in 100 mM $CaCl_2$ to immobilize the NPs. Subsequently, the immunomodulatory effects were determined by studying cytokine release from LPS-stimulated U-937 macrophages when cultured with alginate microcapsules or Sq-GusNP-containing alginate microcapsules. Tumor necrosis factor α (TNF-α) and IL-10 secretion was determined at 1, 2, and 3 days after starting the culture.

As shown in **Figure 1**A,B, LPS induced an increase in TNF-α and IL-10 secretion. TNF-α secretion could be reduced by 41.68% ($p < 0.0005$) at day 3 by the Sq-GusNP-containing alginate microcapsules. Surprisingly, when macrophages were cultured with alginate microcapsules a slight reduction of 6.08% ($p = 0.0005$) of TNF-α production was observed on day 2 and a reduction of 21.73% ($p < 0.05$) on day 3. Also, a tendency of reduced IL-10 secretion (15%) was observed for both alginate microcapsules and Sq-GusNP-containing alginate microcapsules, but this never reached statistical significance (Figure 1B). No decrease in viability was observed irrespective of the treatment or exposure time (Figure 1C). The fact that viability was higher than 100% on days 2 and 3 indicates that the treatments do not affect cell proliferation, as corroborated by the statistical analysis. No statistical differences were observed over time or at each time point with respect to the control or untreated macrophages. A tendency to decrease the proliferation rate was observed for both encapsulation systems on macrophages on days 2 and 3 but this was never under 100%, indicating that the inclusion of the nanoparticles had no negative effect on the cell number and the observed anti-inflammatory effects were not due to cell death.

Next, we determined whether the anti-inflammatory effect was also maintained over time. To study this, we cultured alginate microcapsules and Sq-GusNP-containing alginate microcapsules with macrophages for 24 h. Subsequently we stimulated the macrophages with LPS and after 24 h of stimulation, the secretion of TNF-α and IL-10 was evaluated at 2, 9, and 16 days (**Figure 2**A,B). On day 2 no reduction in cytokine secretion was observed for any of the evaluated cytokines. However, on days 9 and 16, TNF-α production by LPS-stimulated macrophages exposed to Sq-GusNP-containing alginate microcapsules was reduced by 45.24% ($p < 0.0001$) and 46.11% ($p < 0.05$), respectively. Also, a lower reduction for TNF-α secretion of 38.44% ($p < 0.0001$, day 9) and 42.98% ($p < 0.05$, day 16) was obtained for the U-937 macrophages cultured with alginate microcapsules. This confirms the anti-inflammatory effect found for alginate itself in the short-term release experiment.

Gusperimus has been shown to reduce not only TNF-α but also IL-10 secretion in macrophages.[20,21] IL-10 was reduced by 37.88% ($p < 0.05$) and 40.96% ($p < 0.0005$) at respectively days 9 and 16 when cultured with Sq-GusNP-containing alginate microcapsules. With alginate microcapsules, only a significant reduction of 34.55% ($p < 0.001$) for IL-10 was observed at day 16. Figure 2C,D shows the alginate microcapsules and Sq-GusNP-containing alginate microcapsules after 30 days of culture. No variations in size or shape were observed during

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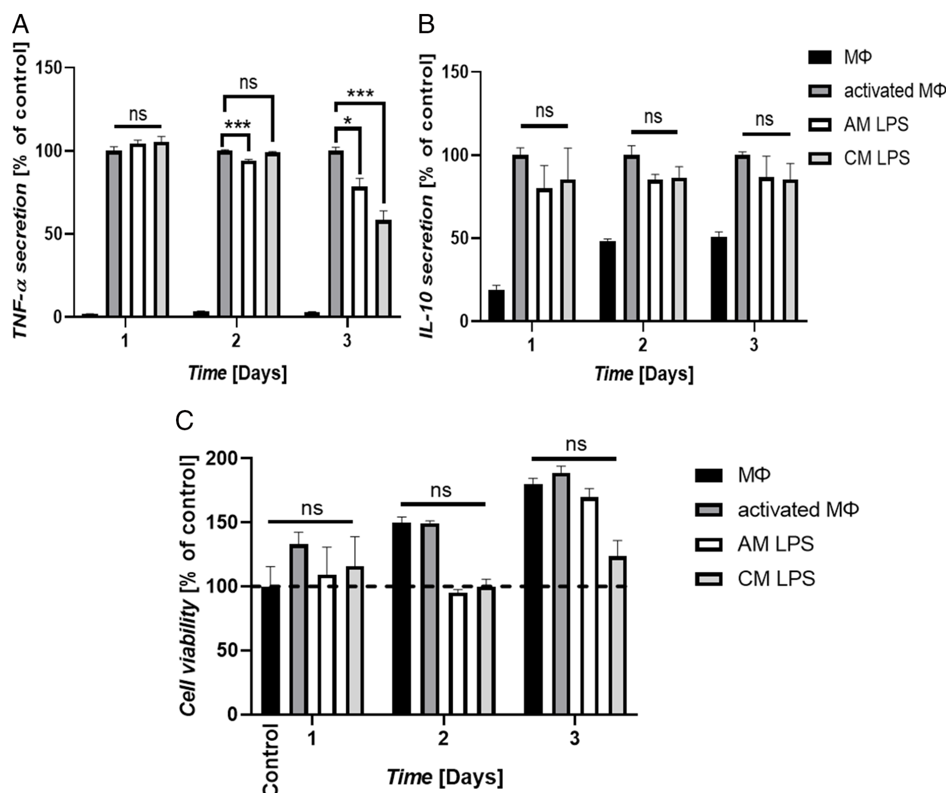

Figure 1. Cytokine secretion for U-937 macrophages cultured with alginate microcapsules or Sq-GusNP-containing microcapsules 1, 2, and 3 days after application of a LPS stimulus for A) TNF-α and B) IL-10. The anti-inflammatory effect exerted by gusperimus release was evident 3 days after the stimulus was applied. Comparisons were made using one-way ANOVA with Dunnett's multiple comparisons test. C) Cell viability for the evaluated treatments over time; none of the treatments were toxic for macrophages (Cs). No significant differences were founded between the control and the treatments over time, or between Cs and the treatments at 1, 2, or 3 days. Comparisons were made using two-way ANOVA with Tukey's multiple comparisons test. MΦ (U-937 macrophages without treatment); activated MΦ (U-937 macrophages stimulated with LPS); AM LPS (U-937 macrophages cultured with alginate microcapsules and stimulated with LPS); CM LPS (U-937 macrophages cultured with Sq-GusNP-containing microcapsules and stimulated with LPS). Data represent mean values ± SEM of three experiments. $p \leq 0.0005$ (***); $p < 0.05$ (*); ns (no significant difference).

the follow-up period, indicating no destabilization of the microcapsule structure due to Sq-GusNPs incorporation or because of the release of the Sq-Gus bioconjugate from microcapsules.

2.2. Sq-GusNPs Do Not Affect the Viability or Function of Pancreatic Islets

Pancreatic islets are sensitive to many immunosuppressant drugs, which have been shown to impair insulin release.[15,25–27] To determine whether Sq-GusNPs have any effect on the functional survival of pancreatic islets, we cultured unencapsulated human islets with Sq-GusNPs for 24 h and compared their viability and function with untreated controls. As shown in **Figure 3**, the Sq-GusNPs do not affect the viability or function of the islets. The viability of islets treated with Sq-GusNPs was not reduced compared to the untreated control (Figure 3A) and islets cultured with Sq-GusNPs maintained their capacity to respond properly to glucose stimulation during the glucose-stimulated insulin secretion (GSIS) test (Figure 3B). For Sq-GusNP-treated islets a fourfold increase in insulin secretion was observed after stimulation with a high-glucose solution ($p < 0.0001$). This was not statistically significantly different from the responses of untreated islets (Figure 3B). No statistical differences were found in the stimulation index between treated and nontreated islets, indicating no impairment of insulin secretion (Figure 3C).

2.3. Encapsulation of Pancreatic Islets in Sq-GusNP-Containing Alginate Microcapsules Maintains Their Viability and Function and Exerts an Anti-Inflammatory Effect over Time

Finally, we determined whether encapsulation of pancreatic human islets in Sq-GusNP-containing alginate microcapsules can protect islets against cytotoxicity from LPS-activated macrophages. To this end, we encapsulated pancreatic islets in alginate microcapsules with or without Sq-GusNPs, cocultured them with LPS-stimulated macrophages for 24 h, and determined cytokine secretion at days 1 and 5.

The protective effect of the encapsulation systems on islets was confirmed by determining viability and function over time. **Figure 4**A shows that viability was not affected by the encapsulation of islets in alginate microcapsules or Sq-GusNP-containing

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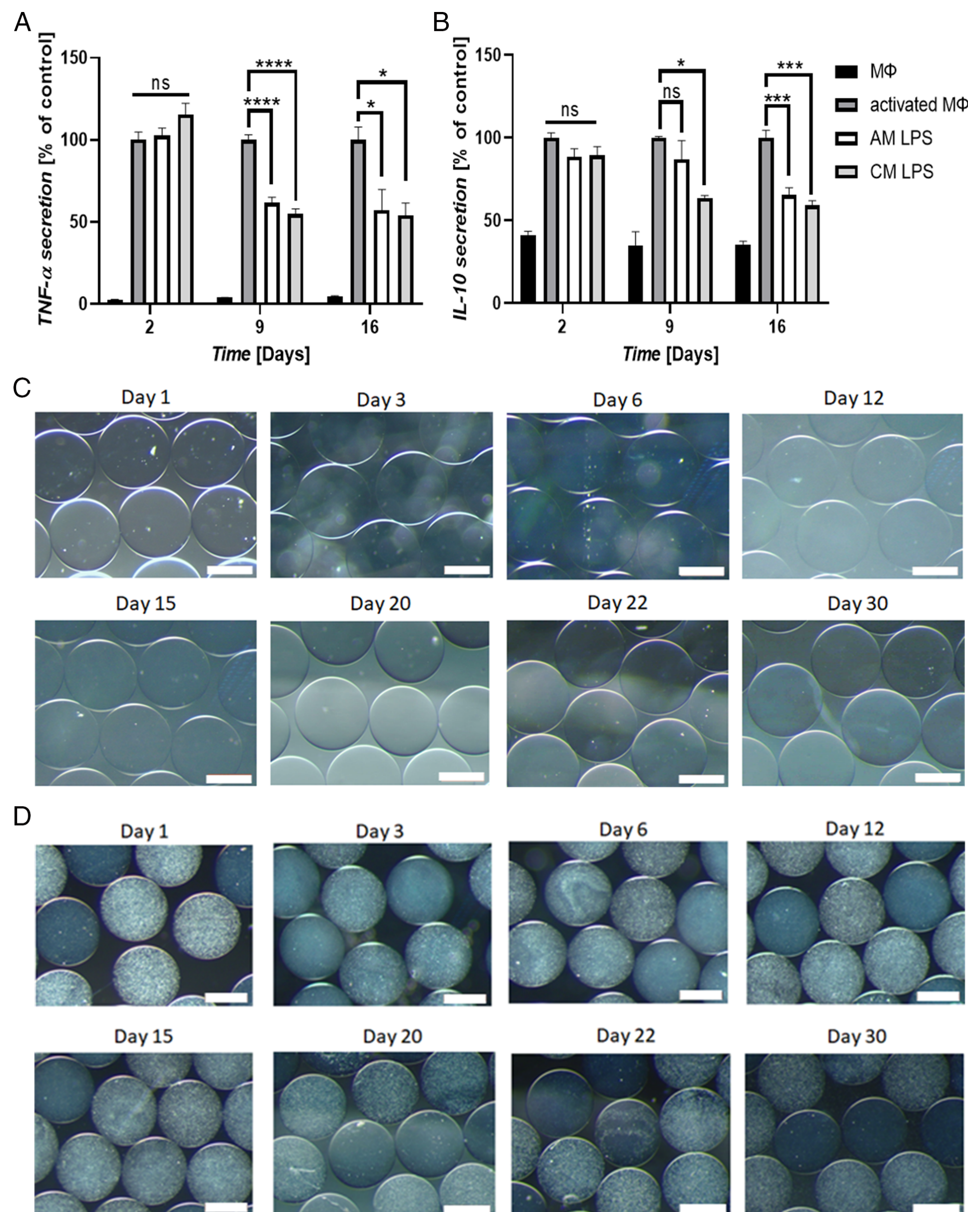

Figure 2. Anti-inflammatory effect exerted by gusperimus release from microcapsules containing Sq-GusNPs over time. A) TNF-α and B) IL-10 secretion at 2, 9, and 16 days for U-937 macrophages cultured with alginate microcapsules or Sq-GusNP-containing microcapsules after a 24 h stimulus with LPS. For both cytokines downregulation was observed at 9 and 16 days. Comparisons were made using one-way ANOVA with Dunnett's multiple comparisons test. C) Images of alginate microcapsules cultured for 30 days at 37 °C in Kreps–Ringer–Hepes (KRH) 25 mM. No differences in shape or size were observed during the follow-up period. D) Images of Sq-GusNP-containing microcapsules cultured for 30 days at 37 °C in KRH 25 mM. No differences in shape or size were observed during the follow-up period. Scale bar 500 µm. MΦ (U-937 macrophages without treatment); activated MΦ (U-937 macrophages stimulated with LPS); AM LPS (U-937 macrophages cultured with alginate microcapsules and stimulated with LPS); CM LPS (U-937 macrophages cultured with Sq-GusNP-containing microcapsules and stimulated with LPS). Data represent mean values ± SEM of three independent experiments. $p < 0.0001$ (****); $p < 0.001$ (***); $p < 0.05$ (*); ns (no significant difference).

alginate microcapsules as no differences in viability were observed at day 0, 1, or 5. An interesting finding was that after 5 days of culture of the encapsulated islets insulin secretion was considerably enhanced compared to days 0 and 1 for both encapsulation systems (Figure 4B). For islets encapsulated in Sq-GusNP-containing alginate microcapsules, a 4.7- ($p < 0.005$) and 3.3- ($p < 0.05$) fold increase of insulin secretion at day 5 was observed compared to days 0 and 1, respectively. The same was observed for the stimulation index for alginate microcapsules without Sq-GusNPs. Islet function was not affected by any of the encapsulation systems because the capacity of the islets to produce insulin after a high-glucose stimulus and recovering after a new stimulus with low glucose was maintained at 0, 1, and 5 days (Figure 4C).

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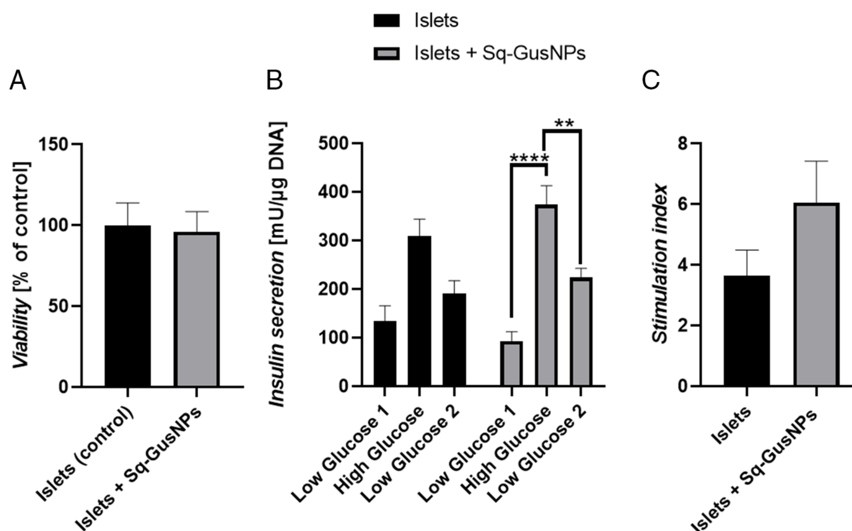

Figure 3. Effect of Sq-GusNPs on viability and function of nonencapsulated pancreatic islets. A) Viability of nonencapsulated pancreatic islets after 24 h treatment with Sq-GusNPs. Viability was not affected by treatment of islets with Sq-GusNPs. Comparison was made using a two-tailed, impaired *t*-test. B) Glucose-stimulated insulin secretion of pancreatic islets after 24 h treatment with Sq-GusNPs. Response to glucose stimulus was maintained after treatment of islets with Sq-GusNPs. Comparisons were made using two-way ANOVA with Tukey's multiple comparisons test. C) Stimulation index after 24 h treatment of islets with Sq-GusNPs. Nonstatistical differences were found in the stimulation index for the treated and nontreated groups. Comparison was made using a two-tailed, impaired *t*-test. Data represent mean values ± SEM of 9 independent experiments for 3 different donors. $p < 0.0001$ (****); $p < 0.01$ (**).

The anti-inflammatory effect of the encapsulation systems on islets and on immune cells in the vicinity was corroborated by attenuated levels of IL-6, TNF-α, IL-8, and IL-10, as shown in **Figure 5**A–D. The secretion of all the evaluated cytokines was highly increased when macrophages were cocultured with islets encapsulated in alginate microcapsules and stimulated with LPS compared to stimulated macrophages without encapsulated islets ($p < 0.0001$). A tendency to reduce the secretion of all cytokines was observed on day 1 of coculture for islets encapsulated in Sq-GusNP-containing alginate microcapsules. The anti-inflammatory effect was significant on day 5. For cocultures of macrophages and islets encapsulated in Sq-GusNP-containing alginate microcapsules, we observed a reduction in secretion of 62.08% for IL-6 ($p < 0.0005$), 64.94% for TNF-α ($p < 0.0005$), 69.75% for IL-8 ($p < 0.0001$), and 60.95% for IL-10 ($p < 0.05$). For islets encapsulated in alginate microcapsules, a lower but also significant reduction in cytokine secretion was observed, being 57.34% for IL-6 ($p < 0.005$), 55.31% for TNF-α ($p < 0.01$), 69.07% for IL-8 ($p < 0.0001$), and 60.68% for IL-10 ($p < 0.05$). Similarly to the previous results, these new results confirm two important findings obtained with the functional release experiments. The first is the anti-inflammatory effect due to the incorporation of Sq-GusNPs into the microcapsules and the second is the anti-inflammatory effect associated with the alginate used for microcapsule preparation.

3. Discussion

Grafting of encapsulated pancreatic islets is associated with severe inflammatory responses in the first weeks after implantation.[9] It may lead to loss of the islets' functional mass and limit long-term survival of the islet graft.[24] This response starts with the activation of the innate immune system directly after the surgical procedure required for implantation of the microcapsules in the peritoneal cavity.[9] To reduce this deleterious response, we designed an alginate-based microencapsulation system that incorporates the immunomodulatory agent gusperimus stabilized as NPs. To do this, we used purified intermediate-G alginate and incorporated Sq-GusNPs to later encapsulate human pancreatic islets.[11,12] To evaluate the anti-inflammatory and protective effects of the Sq-GusNP-containing alginate microcapsules on human islets, we followed the viability and function of the islets over time. We determined viability, islet function, and cytokine secretion during coculture of microencapsulated islets with innate immune cells (macrophages) in the presence and absence of an LPS-induced inflammatory response. Sq-GusNPs were protective and maintained viability and insulin-secretory capacity of the human islets.

Gusperimus was selected because it has been shown to be safe and can act on different arms of the immune system involved in the immediate innate immune responses.[20] Gusperimus has been shown to reduce the inflammatory responses to grafted encapsulated or nonencapsulated islets, reduce adherence of peritoneal macrophages to capsules, attenuate pericapsular cellular infiltration, prolong graft survival, and to have no toxicity for islets.[27–31] Unfortunately, it cannot be appropriately used as a temporary drug to reduce immediate immune responses without stabilization strategies because it is easily hydrolyzed at physiological conditions, leading to production of cytotoxic byproducts and losing its activity.[21–23] For this reason, we stabilized it as Sq-GusNPs, which were later incorporated into alginate microcapsules.[21] As shown in this study, Sq-GusNPs incorporated into the alginate microcapsules allow for a continuous and prolonged release of gusperimus and attenuate LPS-induced inflammation in its microenvironment without affecting human islet function.

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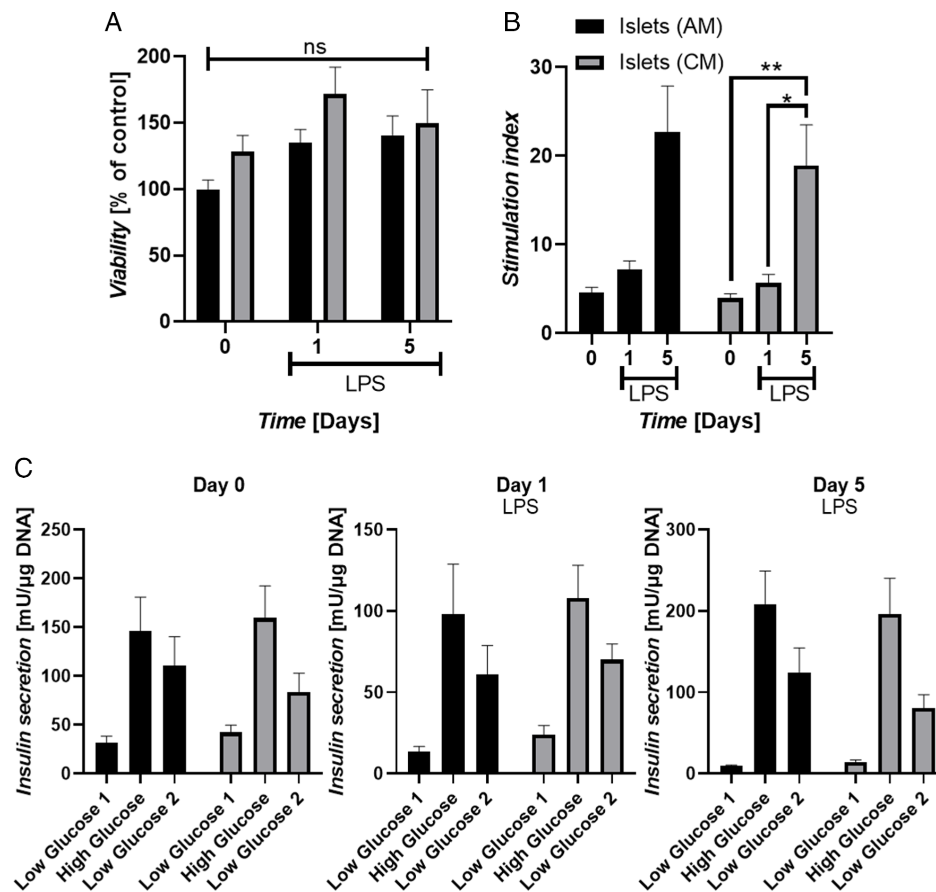

Figure 4. Viability and function of human islets after encapsulation in alginate microcapsules and Sq-GusNP-containing alginate microcapsules in presence of macrophages and after a 24 h stimulation with LPS. Encapsulated islets were cocultured with U-937 macrophages, stimulated with LPS, and 24 h later viability and function were determined at 1 and 5 days after encapsulation. Encapsulated islets on day 0 were not cocultured with macrophages or stimulated with LPS. Islets encapsulated in alginate microcapsules at day 0 were used as control group for viability and insulin secretion over time. A) Viability over time for islets encapsulated in alginate microcapsules with or without Sq-GusNPs. No differences on viability were observed due to the encapsulation systems over time, at 0, 1, or 5 days. B) Stimulation index (SI) over time for islets encapsulated in alginate microcapsules with or without Sq-GusNPs. A significant increase in insulin secretion was observed at 5 days for both encapsulation systems. No differences were found when the SI was compared between groups over time. C) Glucose-stimulated insulin secretion of islets encapsulated in alginate microcapsules with or without Sq-GusNPs at 0, 1, and 5 days. The capacity of the islets to produce insulin after a high-glucose stimulus and recovering after a new stimulus with low glucose was maintained over time for both encapsulation systems. Comparisons were made using two-way ANOVA with Tukey's multiple comparisons test. Islets (AM) (human islets encapsulated in alginate microcapsules); islets (CM) (human islets encapsulated in Sq-GusNP-containing alginate microcapsules); LPS (encapsulated islets were stimulated with LPS for the indicated days). Data represent mean values ± SEM of 9 experiments for 3 different donors. $p < 0.005$ (**); $p < 0.05$ (*); ns (no significant difference).

Gusperimus has been shown to have inhibitory effects on macrophage function[27] and downregulate the production of TNF-α and IL-10.[20,21,32] Using a functional release experiment in which we determined the cytokine secretion after provoking an inflammatory response in macrophages we showed the anti-inflammatory effects of Sq-GusNP-containing alginate microcapsules. This was concluded after observing a high reduction in TNF-α for Sq-GusNP-containing alginate microcapsule treated macrophages 3 days after stimulation with LPS. Surprisingly, a two times lower but significant anti-inflammatory effect was also observed when macrophages were cultured with alginate microcapsules without Sq-GusNPs, indicating an anti-inflammatory effect of alginate itself. This was corroborated when macrophages were cultured with empty alginate microcapsules downregulating cytokine secretion at 9 and 16 days. This finding can be explained by the presence of unbound chains of alginate oligosaccharides present in the alginate microcapsules, which can diffuse out of the microcapsules and have been shown to have anti-inflammatory effects.[33] Within a polymer sample such as the alginate we used with a high dispersity index ($Đ = 1.95$), there are chains of different lengths.[34] Between these different chains, oligomers with low molecular weight are present.[35] As alginate is a polymer composed of α-L-guluronate (G) and β-D-mannuronate (M) monomers,[13] both mannuronate and guluronate oligomers exist as a part of the different polymer chains in the alginate.[36] These oligomers have shown anti-inflammatory activity through the reduction of proinflammatory bioactive molecules such as

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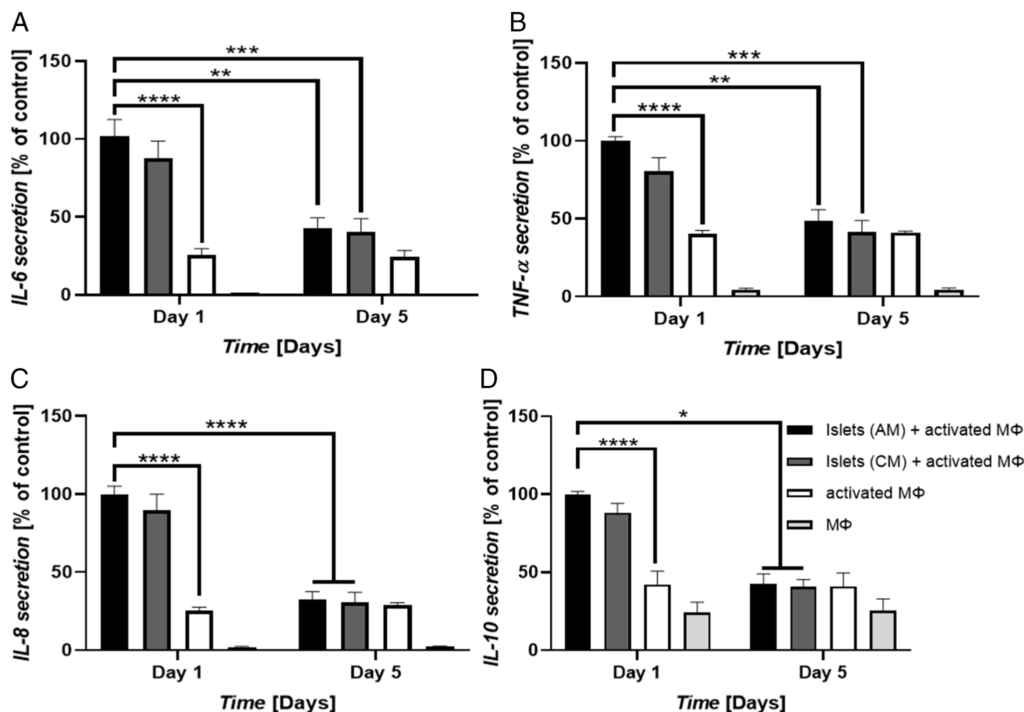

Figure 5. Anti-inflammatory effect observed for the system composed of encapsulated human islets in alginate microcapsules or Sq-GusNPs-containing alginate microcapsules in presence of macrophages and after 24 h stimulation with LPS. Cytokine secretion was determined at 1 and 5 days after encapsulation. A–D) Cytokine secretion profiles for IL-6, TNF-α, IL-8, and IL-10 respectively for islets encapsulated in alginate microcapsules with or without Sq-GusNPs. A tendency to reduction in secretion of all the evaluated cytokines was observed on day 1 when islets were encapsulated in Sq-GusNP-containing alginate microcapsules. A high reduction in cytokine secretion for all cytokines on day 5 was obtained for both encapsulation systems. Comparisons were made using two-way ANOVA with Tukey's multiple comparisons test. Transformation using the function $Y = \mathrm{Log}(Y)$ was applied to the data for analysis and to ensure normal distribution. MΦ (U-937 macrophages without treatment); activated MΦ (U-937 macrophages stimulated with LPS); islets (AM) + activated MΦ (human islets encapsulated in alginate microcapsules cocultured with macrophages and stimulated with LPS); islets (CM) + activated MΦ (human islets encapsulated in Sq-GusNP-containing alginate microcapsules cocultured with macrophages and stimulated with LPS). Data represent mean values ± SEM of 9 experiments for 3 different donors. $p < 0.0001$ (****); $p < 0.0005$ (***); $p < 0.01$ (**); $p < 0.05$ (*).

nitric oxide, prostaglandin E2, reactive oxygen species, and proinflammatory cytokines.[33] The reduction of the inflammatory responses by blocking the activation of the nuclear factor NF-κB and mitogen-activated protein kinases has also been observed for guluronate oligosaccharides.[37]

Downregulation in cytokine secretion was maintained over time and was observed up to 16 days after encapsulation and stimulation with LPS. The fact that no cytokine downregulation was observed before day 2 for the Sq-GusNP-containing alginate microcapsules indicates that the release of gusperimus from this system might be delayed due to diffusion mechanisms.[38] Gusperimus needs to be released from the Sq-Gus bioconjugate, which in turn needs to be released from the NPs and diffuse through the alginate microcapsule. Therefore, the release of gusperimus from Sq-GusNP-containing microcapsules is a time-dependent process, as proposed in **Figure 6**. Sq-GusNPs are formed by autoassembly of the Sq-Gus molecules or prodrug forming a micelle-like structure.[21] The constant ingress of water from the external medium into the Sq-GusNP-containing alginate microcapsules provokes the release of individual Sq-Gus bioconjugate molecules from the NPs in a time-dependent fashion. Due to their small size, the Sq-Gus molecules can diffuse through and outside of the microcapsules, where they can be internalized by the islets into the microcapsules or by the immune cells outside them. After internalization, the Sq-Gus prodrug is cleaved inside the cells, releasing the active principal gusperimus provoking the observed anti-inflammatory effect.[39] This process may take up to 2 days.

We proved that encapsulation of islets in Sq-GusNP-containing alginate microcapsules does not affect their viability or function under the harsh conditions elicited by coculturing them with LPS-stimulated macrophages at 1 and 5 days. By coculturing the encapsulated islets with LPS-activated macrophages, we mimicked the situation in vivo in which peritoneal macrophages may be activated directly after the implantation procedure. The surgical procedure might be associated with bleedings in the peritoneal cavity that introduce serum factors such as thrombin and fibronectin, which have chemotactic effects attracting inflammatory cells and triggering the inflammatory response against the microcapsules.[9] This response is enhanced due to the presence of islets inside the microcapsules, which can release bioactive factors such as DAMPs that activate the inflammatory cells.[7]

The high increase in cytokine secretion of the LPS-stimulated coculture of encapsulated islets and macrophages can be explained because LPS induces inflammatory responses in macrophages[10] and human islets, provoking secretion of

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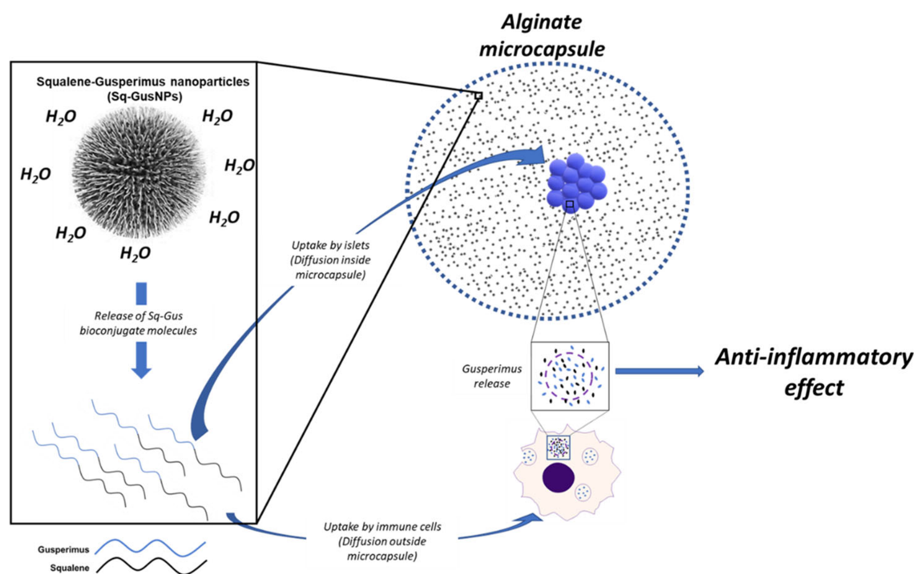

Figure 6. Proposed mechanism for gusperimus release from Sq-GusNP-containing alginate microcapsules. Sq-Gus bioconjugate molecules are released from the Sq-GusNPs contained in alginate microcapsules due to the constant ingress of water molecules that facilitate their diffusion to the islets and to the external immune cells. After the bioconjugate is internalized by the cells, it is cleaved and finally gusperimus is released exerting the observed anti-inflammatory effect.

inflammatory mediators such as cytokines and chemokines, such as IL-1β, IL-6, IL-8, TNF-α, and MCP-1.[40] These bioactive components together with DAMPs[12] from islets can cross the microcapsules[7] and overstimulate the macrophages already stimulated by LPS,[10] causing the considerable increase in cytokine secretion observed on day 1. These cytokines induce deleterious effects in pancreatic islets, resulting in β-cell dysfunction and apoptosis.[40,41] The fact that after 5 days of culture the cytokine secretion was the same as in stimulated controls without encapsulated islets under the same conditions suggests that the release of gusperimus from Sq-GusNPs containing alginate microcapsules avoided the LPS-induced dysfunction in pancreatic islets,[40] illustrating the anti-inflammatory effect of our encapsulation system that might directly protect the islets.

4. Conclusion

The present findings demonstrate that incorporation of Sq-GusNPs in alginate microcapsules allows a controlled release of gusperimus and a prolonged anti-inflammatory effect on LPS-activated human macrophages. Sq-GusNPs were effective in maintaining human islet function even when the islets were coincubated with LPS-activated macrophages. The Sq-GusNPs lacked toxicity and showed an enhanced anti-inflammatory effect after incorporation into alginate microcapsules. This is an important finding as many immunosuppressive drugs in islet transplantation such as cyclosporin, FK506, rapamycin, glucocorticoids, and corticosteroids have deleterious effects on islet function and survival.[17,27] Because of their effectivity and lack of toxicity, we consider the incorporation of Sq-GusNPs in alginate microcapsules a promising approach to control the immediate immune response directed to the encapsulated islets after the grafting procedure.

5. Experimental Section

5.1. Materials

The squalene gusperimus (Sq-Gus) bioconjugate was synthesized in the laboratory through squalene transformation until obtaining its carboxylic acid derivative and consequent reaction with gusperimus as previously reported.[21] Absolute ethanol (EtOH), calcium chloride ($CaCl_2$), Hepes, and potassium chloride (KCl) were purchased from Merck (Darmstadt, Germany). Intermediate-G alginate (42% G-chains, 58% M-chains, 23% GG-chains, 19% GM-chains, 38% MM-chains, $M_n = 73.9$ kDa, $M_w = 144.4$ kDa, dispersity index ($Đ$) = 1.95) was purchased from ISP Alginates Ltd. (Ayrshire, UK) and purified according to the protocol previously established in the laboratory[11] Monocytes of the U-937 cell line were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). RPMI 1640 supplemented with 25 mM Hepes and L-glutamine was purchased from LONZA (Verviers, Belgium). Fetal calf serum, glucose, phorbol 12-myristate 13-acetate (PMA), and LPSs from *Escherichia coli* O111:B4 purified by phenol extraction were purchased from Sigma-Aldrich Chemie N.V. (Zwijndrecht, The Netherlands). L-glutamine, sodium pyruvate, 2-mercaptoethanol, gentamycin, amphotericin B, CMRL-1066, GlutaMax, penicillin–streptomycin, and the alamarBlue reagent were purchased from Life Technologies Europe BV (Bleiswijk, The Netherlands). ELISA DuoSet for human IL-10, TNF-α, IL-6, and IL-8 were purchased from R&D systems (Abingdon, UK). The insulin ELISA kit Mercodia was purchased from Bio-Connect Diagnostics B.V. (Huissen, The Netherlands). The Invitrogen Quant-iT PicoGreen dsDNA reagent was purchased from Fisher Scientific (Landsmeer, The Netherlands).

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5.2. Nanoparticle Preparation

Sq-GusNPs were prepared through the nanoprecipitation method.[42] For this, the Sq-Gus bioconjugate was dissolved in EtOH at a concentration of 2 mg mL^{-1}. Later 380 µL of the solution was added drop by drop to 1 mL of deionized water under stirring (500 rpm) for 10 min, after which EtOH was evaporated using the concentrator SpeedVac SPD2010 (Thermo Fisher Scientific, Bleiswijk, The Netherlands), obtaining an aqueous suspension of pure NPs with a size of 193.1 ± 64.64 nm as measured by dynamic light scattering (DLS) with the particle size analyzer NICOMP 380 ZLS (Particle Sizing Systems, Inc., Santa Barbara, CA, USA).

5.3. Microcapsule Preparation

5.3.1. Alginate Microcapsules

Alginate was purified as previously described by Paredes-Juarez et al.[11] and dissolved in 220 mOsm calcium-free KRH buffer at a concentration of 3.4% w/v overnight under vortexing. After filtration using a 0.2 µm sterile syringe filter, the alginate solution was transformed into droplets using an air-driven droplet generator.[43] The droplets were collected in a 100 mM $CaCl_2$ solution (10 mM Hepes, 2 mM KCl), allowed to gel for 5 min, washed three times with 25 mM KRH buffer, and left in this solution until use. Microcapsules between 600 and 700 µm diameter were obtained.

5.3.2. Sq-GusNP-Containing Alginate Microcapsules

Sq-GusNPs were prepared as indicated in the nanoparticle preparation section (Section 5.2). Later 832 µL of the filtered 3.4% alginate solution was added to 1 mL of nanoparticle suspension (0.76 mg mL^{-1}) and vortexed for 1 h. Subsequently, water was evaporated until a final volume of 1 mL using the concentrator SpeedVac SPD2010. The final composite solution was transformed into droplets using an air-driven droplet generator and processed in the same way as the alginate microcapsules (section 5.3.1). Microcapsules between 600 and 700 µm diameter were obtained.

5.4. Cell Culture

U-937 cells were cultured in RPMI 1640 supplemented with 25 mM Hepes and L-glutamine, 10% fetal calf serum (FCS, deactivated phosphatases), L-glutamine (2 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (50 µM), gentamycin (60 µg mL^{-1}), and amphotericin B (1.1 ng mL^{-1}). Pancreatic islets were cultured in CMRL-1066 medium supplemented with 8.3 mM glucose, 20 mM Hepes, 10% FCS deactivated phosphatases, 2 mM GlutaMax, and 50 µg mL^{-1} penicillin/streptomycin as previously reported.[44] Cells and islets were cultured in an incubator at 37 °C and 5% CO_2.

5.5. Islet Isolation

Human pancreatic islets were isolated from cadaveric pancreata as previously described.[45] The islets were obtained from the Leiden University Medical Center or through the JDRF award 31-2008-416 (European Consortium for Islet Transplantation, Islet for Basic Research program, Milan, Italy). Islet preparation and donor characteristics are specified in **Table 1**. Islets were used for research when the quality and/or number were insufficient for clinical application according to national laws and when research consent was available. All the procedures were approved and carried out under 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 for 24 h as described in the cell culture section (Section 5.4) before starting the experiments.

5.6. Islet Microencapsulation

Islets were handpicked, washed three times with a 310 mOsm KRH buffer, and gently mixed with a 3.4% alginate solution with or without Sq-GusNPs prepared as indicated previously. Later the previous mixture was transformed into microcapsules using the droplet generator following the same procedure as that indicated in the microcapsule preparation section (Section 5.3).

5.7. Cell Viability with AlamarBlue

To determine cell viability with alamarBlue the reagent was diluted in culture medium (10% v/v). After treatment, cells or islets were washed with plain medium and incubated for 4 h with 0.5 mL of the diluted reagent. Later fluorescence was measured with the plate reader CLARIOstar^{Plus} (BMG LABTECH, Offenburg, Germany) Ex/Em 560/590 nm. For the islets, results were normalized to total DNA content as determined using the Quanti-iT PicoGreen dsDNA kit (Invitrogen). Fluorescence obtained from cells or islets without any treatment was used as reference and control. Results were expressed as percentage of the control.

Table 1. Donor information.

Donor	Age [years]	Gender	BMI [Kg/m^2]	Blood glucose [mg/dL]	Islet isolation center	Death cause	Purity [%]	Viability [%]
1	63	Male	21	Nondiabetic	LUMC[a)]	Noncardiac	60	>80
2	56	Female	19.4	90	ECIT[b)]	Cerebral bleeding	70	95
3	52	Female	22.7	122	ECIT	Trauma	80	90

[a)]LUMC: Leiden University Medical Center; [b)]ECIT: European Consortium for Islet Transplantation.

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5.8. ELISA

Sandwich ELISA (DuoSet ELISA R&D systems) for human IL-10, IL-6, IL-8, and TNF-α was performed according to the manufacturer's instructions using a microplate spectrophotometer Benchmark Plus BIO-RAD (Bio-Rad Laboratories B.V, Veenendaal, the Netherlands) at 450 nm with correction at 540 nm.

5.9. Functional Release Experiments

5.9.1. Short-Term Release Experiment

Release of gusperimus from composite microcapsules was tested using a functional experiment in which the secretion of the cytokines TNF-α and IL-10 was quantified after incubation of LPS-stimulated human U-937 macrophages with microcapsules. For this, human U-937 monocytes at a density of 5×10^5 cells/well in 24-well plates were differentiated into macrophages by adding PMA at a concentration of 200 nM.[46] After 24 h, cells were washed with PBS buffer and left to incubate in 0.5 mL of culture medium for 24 h. Later the macrophages were washed with PBS and cultured with 150 alginate microcapsules or 150 Sq-GusNP-containing alginate microcapsules in a total volume of 1 mL of culture medium. After 2 h of incubation, LPS (100 ng mL^{-1}) was added to stimulate the macrophages, and IL-10 and TNF-α secretion was quantified in the supernatant on days 1, 2, and 3. Macrophages stimulated with LPS were used as positive control. Nontreated macrophages served as 100% viability control on day 1 and results were normalized to this control.

5.9.2. Long-Term Release Experiment

U-937 macrophages differentiated from monocytes as described previously were seeded at a density of 5×10^5 cells/well in 24-well plates. Later the macrophages were washed with PBS and 150 alginate microcapsules or 150 Sq-GusNP-containing alginate microcapsules were added per well in a total volume of 1 mL of culture medium (day 0). After 24 h of incubation (day 1), LPS (100 ng mL^{-1}) was added to stimulate inflammatory responses in the macrophages, and IL-10 and TNF-α secretion was quantified in the supernatant 24 h after the stimulus was applied (day 2). Capsules from day 2 were collected, washed three times with 25 mM KRH buffer, and left on incubation in 1 mL of culture medium until day 7. On day 7, microcapsules were passed to a new plate with macrophages and processed in the same way as for days 0 and 1. On the ninth day, supernatants were collected for cytokine determination, microcapsules were washed, left on incubation in culture medium until day 14, and treated in the same way as indicated for day 7. Finally, supernatants were collected for cytokine determination on day 16.

5.10. Glucose-Stimulated Insulin Secretion (GSIS) Test

After treatment 25 handpicked encapsulated or nonencapsulated islets were washed two times with 2.75 mM glucose solution prepared in 25 mM KRH buffer (low glucose solution). After a 1.5 h preincubation period with a low-glucose solution, islets were incubated for 1 h with the low-glucose solution, followed by a 1 h incubation with a 16.5 mM glucose solution prepared in 25 mM KRH buffer (high-glucose solution), and finally incubated for one additional hour with the low-glucose solution. For each incubation step, supernatants were collected, and insulin secretion was determined using the insulin ELISA kit (Mercodia). Results were normalized to total DNA content as was determined using the Quanti-iT PicoGreen dsDNA kit (Invitrogen). The stimulation index (SI) was calculated by dividing the amount of insulin secreted after incubation with the high-glucose solution by the amount secreted after the first incubation with the low-glucose solution.

5.11. Effect of Sq-GusNPs on Viability and Function of Nonencapsulated Pancreatic Islets

To determine if Sq-GusNPs affect the viability or function of human islets, 40 handpicked nonencapsulated islets were cultured with the Sq-GusNPs in a concentration of 22.31 μg mL^{-1} in nontreated 24-well plates with a total volume of 1 mL culture medium. After 24 h of incubation, viability was determined with the alamarBlue reagent, and the function was tested through the GSIS test. Islets without treatment were used as the control group.

5.12. Evaluation of Viability, Function, and Anti-Inflammatory Effect on Pancreatic Islets Encapsulated in Microcapsules Containing Sq-GusNPs

To determine the viability, function, and anti-inflammatory capacity of islets encapsulated in microcapsules containing Sq-GusNPs, 40 handpicked islets encapsulated in alginate microcapsules or Sq-GusNP-containing alginate microcapsules were cocultured with U-937 macrophages at a density of 5×10^5 cells/well in 24-well plates. After 2 h incubation, LPS (100 ng mL^{-1}) was added to stimulate inflammatory responses, and 24 h later supernatants were collected to quantify TNF-α, IL-6, IL-8, and IL-10 secretion. The experiment was conducted at 1 and 5 days of culture. Afterward, viability and function were evaluated using the alamarBlue reagent and the GSIS test. For the experiment at 5 days, the encapsulated islets were cultured for 4 days, washed with 25 mM KRH buffer, passed to plates with macrophages, and stimulated as earlier. Results were expressed as percentage of the control. Viability on day 0 and cytokine secretion on day 1 for islets encapsulated in alginate microcapsules were used as controls.

5.13. Statistics

The experiments were performed at least three times and statistical analysis was conducted in GraphPad Prism, Version 8.2.0 (GraphPad Software Inc., USA). Normal distribution of data was confirmed using the D'Agostino-Pearson omnibus (K2) test. Where indicated, data transformation using the function $Y = \text{Log}(Y)$ was applied for data analysis. Comparisons between two groups were analyzed using two-tailed, unpaired *t*-tests. Comparisons for more than two groups were done using one-way ANOVA with Dunnett's post hoc test or two-way ANOVA

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with Tukey's post hoc test. A p-value < 0.05 was considered statistically significant. Results are expressed as mean $\pm$ standard error of the mean (SEM).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

alginate microcapsules, gusperimus controlled release, islet microencapsulation, local immunosuppression, squalene-gusperimus nanoparticles, type 1 diabetes mellitus

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