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Enhanced function of immuno-isolated islets in diabetes therapy by co-encapsulation with an anti-inflammatory drug

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Abstract

Immuno-isolation of islets has the potential to enable the replacement of pancreatic function in diabetic patients. However, host response to the encapsulated islets frequently leads to fibrotic overgrowth with subsequent impairment of the transplanted grafts. Here, we identified and incorporated anti-inflammatory agents into islet-containing microcapsules to address this challenge. *In vivo* subcutaneous screening of 16 small molecule anti-inflammatory drugs was performed to identify promising compounds that could minimize the formation of fibrotic cell layers. Using parallel non-invasive fluorescent and bioluminescent imaging, we identified dexamethasone and curcumin as the most effective drugs in inhibiting the activities of inflammatory proteases and reactive oxygen species in the host response to subcutaneously injected biomaterials. Next, we demonstrated that co-encapsulating curcumin with pancreatic rat islets in alginate microcapsules reduced fibrotic overgrowth and improved glycemic control in a mouse model of chemically-induced type I diabetes. These results showed that localized administration of anti-inflammatory drug can improve the longevity of encapsulated islets and may facilitate the translation of this technology towards a long-term cure for type I diabetes.

Keywords

Anti-inflammatory drugs; curcumin; encapsulated islets; diabetes; fibrotic overgrowth; host response

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1. INTRODUCTION

Immuno-isolation of therapeutic cells has potential to address medical challenges such as hormone deficiencies and neurodegenerative diseases [1]. In this approach, a semi-permeable hydrogel membrane is used to encapsulate non-autologous cells, thus preventing direct contact between the donor cells and host immune cells while allowing exchange of nutrients, oxygen, and secreted therapeutic molecules [2]. Microencapsulated pancreatic islets can produce insulin to restore normoglycemia in diabetic animal recipients without the need for exogenous insulin administration [2, 3]. However, despite encouraging results in various animal models, translation of preclinical results to clinical outcome for diabetes management free of exogenous insulin requirement has remained elusive [4, 5].

One factor implicated in the limited success of encapsulated islets is the host immune response. Though direct cell-to-cell contact is prevented by the presence of the isolating membrane, the host immune system can impair islet function due to the development of pericapsular overgrowth [6]. Recruitment of fibroblasts can result in the formation of fibrotic cell layers and collagen deposition on the surface of transplanted microcapsules [7]. This pericapsular overgrowth reduces oxygen and nutrient transport leading to necrosis of the islet cores and eventual failure of the transplanted grafts [7, 8].

Administration of anti-inflammatory drugs has been employed as a strategy to mitigate host response and improve the stability of implantable biomedical devices [9, 10]. Controlled-release formulations of glucocorticoids or anti-proliferative drugs have reduced fibroblast proliferation and collagen deposition on pacemaker leads [11] and biosensors [12]. However, similar attempts to utilize anti-inflammatory drugs in cell-based therapeutics have remained challenging. Short-term systemic delivery of steroids and antifibrotic drugs can transiently inhibit recruitment of inflammatory cells, and improve the protein secretion function of the immuno-isolated cellular grafts [13, 14]. Nonetheless, systemic administration of immunosuppressants also resulted in deleterious side effects such as increased insulin resistance, opportunistic infection, and nephrotoxicity [15, 16]. Several studies have suggested that temporary localized delivery of immunomodulating agents could reduce tissue response caused by the limited biocompatibility of the encapsulating hydrogel membrane [17–20]. Bunger *et al* reported that temporary release of encapsulated dexamethasone from islet-free alginate-poly-L-lysine microcapsules reduced fibrotic overgrowth four weeks after intraperitoneal transplantation in rats [17]. Other reports indicate that biodegradable microparticles containing ketoprofen may reduce pericapsular overgrowth on alginate-poly-L-ornithine-alginate microcapsules in mice [18, 19]. However, to date, all studies investigating the effects of locally released anti-inflammatory agents on fibrotic growth against islet-based therapeutics have been limited to the use of only a few drugs, with islet-free hydrogel microcapsules in non-diabetic animals [17–19].

To address these limitations, we sought to perform a comprehensive examination of anti-inflammatory molecules to identify promising drug candidates that can mitigate fibrotic growth and thereby support improved islet function. We employed non-invasive imaging techniques to perform *in vivo* subcutaneous screening of several classes of small molecule anti-inflammatory drugs in immunocompetent mice. We identified drug candidates that suppressed early inflammation markers such as reactive oxygen species and inflammatory proteases in the host response to subcutaneously injected biomaterial. Alginate hydrogel microcapsules were subsequently fabricated to co-encapsulate selected drugs and xenogeneic pancreatic islets for *in vivo* efficacy evaluation.

2. MATERIALS AND METHODS

2.1. Animal care and use

The animal protocol was approved by the local animal ethics committees at Massachusetts Institute of Technology (Committee on Animal Care) prior to initiation of the study. Male SKH-1E hairless immunocompetent mice, aged 8–12 weeks, were obtained from Charles River Laboratories (Wilmington, MA, USA). Male Sprague–Dawley rats, 200–250 g, also obtained from Charles River Laboratories, were used as islet donors. Diabetic male C57B6/J mice (Jackson Laboratory, Maine, USA) were the recipients of encapsulated islets. Diabetes was induced in C57B6/J mice via a research contract with Jackson Laboratory, Maine, USA. Briefly, male C57B6/J mice, aged 6–8 weeks, were subjected to multiple low-dose intraperitoneal injections of streptozotocin (STZ) (Sigma Aldrich, St. Louis, MO, USA) at a daily dose of 50 mg/kg. 200 μ l of STZ freshly dissolved in saline at a concentration of 5 mg/ml was administered to each mouse daily for a period of 5 consecutive days. Induction of diabetes was confirmed 10–14 days post STZ-administration by the presence of hyperglycemia when fed blood glucose levels of these mice rose above 300 mg/dL for two consecutive daily readings. Most animals reached this criterion by day 10 after STZ administration, and only those with stable hyperglycemia were used for subsequent transplantation. The mice received from Jackson laboratory were housed under standard conditions with a 12-hour light/dark cycle at the animal facilities of Massachusetts Institute of Technology, accredited by the American Association of Laboratory Animal Care. Both water and food were provided *ad libitum* except for the night before Intraperitoneal Glucose Tolerance Test (IPGTT).

2.2. Subcutaneous injection of PLGA microparticles in SKH-1E mice

Before subcutaneous injection of the PLGA microparticles, hairless immunocompetent SKH-1E mice were kept under inhaled anesthesia using 1–4% isoflurane in oxygen at a flow rate of 2.5 L/min. Lyophilized microparticles with or without encapsulated drug were suspended in sterile 0.9% (w/v) phosphate buffered saline at a concentration of 50 mg/mL. A volume of 100 μ L of this suspension was injected subcutaneously via a 23G needle at each of the six spots on the back of each hairless immunocompetent SKH-1E mouse. Each formulation of drug-loaded microparticles was injected in triplicate on the dorsal side of each mouse. Control particles without encapsulated drug were similarly administered at the three remaining sites on the same mouse[21].

2.3. Non-invasive fluorescent and bioluminescent imaging of SKH-1E mice

SKH-1E mice were started on a non-fluorescent alfalfa-free diet (Harlan Teklad, Madison, WI, USA) three days prior to subcutaneous injections of microparticles and maintained on this diet till the desired sacrifice time point for tissue harvesting. Cathepsin activity was detected by a fluorescent-activated probe (ProSense-680) whose signal correlates with the presence of neutrophils in the acute inflammatory response [22, 23]. ROS were detected by luminol which emits bioluminescent signal upon oxidation by ROS[22]. To monitor cathepsin activity, the imaging probe ProSense-680 (VisEn Medical, Woburn, MA, USA), at a concentration of 2 nmol in 150 ml of sterile phosphate buffered saline, was injected into the mice tail vein. After 24 h, *in vivo* fluorescence imaging was performed with an IVIS-Spectrum measurement system (Xenogen, Hopkinton, MA, USA). The animals were maintained under inhaled anesthesia using 1–4% isoflurane in oxygen at a flow rate of 2.5 L/min. Whole-animal near-infrared fluorescent images were captured at an excitation of 605 nm and emission of 720 nm and under optimized imaging configurations. To monitor reactive oxygen species, a volume of 200 μ l of Sodium Luminol (Sigma Aldrich, St. Louis, MO, USA) dissolved in PBS buffer at a concentration of 50 mg/ml was injected intraperitoneally to each mouse prior to imaging (dose of 500 mg/kg). Ten minutes after this

injection, the mice were imaged under bioluminescent setting in the IVIS system. Data were analyzed using the manufacturer's Living Image 3.1 software. Fluorescent images are presented in fluorescence efficiency which is defined as the ratio of the collected fluorescent intensity normalized against an internal reference to account for the variations in the distribution of incident light intensity. Regions of interest (ROIs) were determined around the site of injection. ROI signal intensities were calculated in total fluorescent efficiency for fluorescence images and in photons per second for bioluminescent images. Higher fluorescent signal signifies increased activity of cathepsin enzymes while higher bioluminescent signal indicates increased presence of reactive oxygen species.

2.4. Fabrication of microcapsules co-encapsulating drug and islets

Alginate with high guluronic acid content SLG20 (Novamatrix, FMC Polymer, Drammen, Norway) was dissolved in sterile 0.9% (w/v) NaCl to give a solution of 1.5% (w/v). To prepare hybrid drug-islet capsules, 1.5% (w/v) alginate was mixed with curcumin (Sigma Aldrich, St. Louis, MO, USA) at 1.0mg/ml or with dexamethasone (Sigma Aldrich, St. Louis, MO, USA) at 2mg/ml and stirred for 4 days to ensure that the drug was homogeneously dispersed. During this mixing period, the curcumin-alginate mixture was wrapped in aluminium foil to avoid light exposure, which might oxidize the drug. One day after islet isolation, islets were washed twice with Ca-free KREBS buffer (135mM NaCl, 4.7mM KCl, 25mM HEPES, 1.2mM KH_2PO_4 , 1.2mM MgSO_4) and mixed with the alginate suspension with or without dispersed drug. Microcapsules containing islets with or without drugs were produced using an electrostatic droplet generator by extrusion of the islet-alginate suspension through a 22G needle at a volume flow rate of 0.155ml/min and a voltage of 6kV into a cross-linking bath of 20 mM BaCl_2 solution. Encapsulated islets were then left to cross-link in this solution for 5 minutes before being collected into a 50ml Falcon tube. The capsules were subsequently washed four times with HEPES buffer (132mM NaCl, 4.7mM KCl, 25mM HEPES, 1.2mM MgCl_2) and two times with RPMI-1640 medium supplemented with 10% Fetal Bovine Serum and 100units/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin (Invitrogen, Grand Island, NY, USA). The final microcapsule diameter was in the range of 500–600 μm .

2.5. Assessment of fibrosis by DNA fluorescent staining

50ul of the capsules explanted from diabetic mice were transferred to a 24well Millicell® cell culture insert (Millipore, Billerica, MA, USA) using wide-orifice pipette tips (Fisher Scientific, Pittsburgh, PA, USA). The capsules were incubated at 37°C for 45minutes in 800ul of 0.001mg/ml Hoechst 33342 dye (Invitrogen, Grand Island, NY, USA) prepared from dye stock solution by dilution with HEPES buffer. Afterwards, these capsules were washed four times with HEPES buffer. The capsules were contained in the upper Millicell® insert which had a porous bottom membrane separating the capsules from the lower container well. The use of a porous insert helped to avoid the loss of capsules during washing steps as washing buffer could be removed by aspiration from the lower well or draining away from the upper insert by placing a Kimwipe below the porous membrane. All capsules were subsequently transferred in HEPES buffer into a black 96 well plate (Greiner BioOne, Monroe, NC, USA). Finally, fluorescent images of the stained capsules were obtained using an EVOS Fluorescent microscope.

2.6. qPCR analysis of fibrotic cells on microcapsules and surrounding fat pad

Isolation of total RNA from frozen cells (on retrieved microcapsules and surrounding fat pad tissues), reverse transcription, and qPCR analysis were carried out as described using primer sets shown in Supplemental Table S1. Primers were designed using Primer Express software (Applied Biosystems, Carlsbad, CA, USA) and evaluated using LaserGene software (DNASTar, Madison, WI, USA) to ensure mouse-specificity. Results were analyzed using

the comparative C_T ($\Delta\Delta C_T$) method and presented as relative RNA levels compared to the RNA levels in samples retrieved from mice transplanted with control capsules after normalization to the β -actin RNA content of each sample.

2.7. Statistical analysis

All values were averaged and expressed as the mean $\pm$ standard error of the mean. Fluorescent or bioluminescent signals from injection sites of drug-loaded microparticles and control microparticles were compared using the Student's two-tailed two-sample *t*-test. Comparison of the blood glucose levels was performed using one-way ANOVA analysis with Fisher's LSD post-hoc test. Comparison of the immunological markers from the qPCR study was performed using one-way ANOVA analysis with the Bonferroni multiple comparison. *P*-values less than 0.05 were considered significant.

3. RESULTS

3.1. *In vivo* subcutaneous screening of small molecule anti-inflammatory drugs

We sought to evaluate the effects of anti-inflammatory agents on the host response to implanted biomaterials by *in vivo* subcutaneous screening of 16 small molecule drugs (Table 1). These agents, whose efficacies in inhibiting inflammation have been reported, belong to several different classes including steroidal [24] and non-steroidal immunosuppressants [25], NSAIDs [26] and naturally occurring polyphenols [27]. The subcutaneous site was selected for screening of the drugs as it induces aggressive host response, hence providing a stringent threshold to identify effective drug candidates [28]. This administration site also facilitated rapid drug screening through non-invasive monitoring of the host response by imaging techniques which allows analysis of multiple particle formulations injected into the same mouse [21, 23]. Cathepsin enzymes and reactive oxygen species (ROS), two markers of inflammation secreted by early immune cells, were monitored using a combination of non-invasive fluorescent and bioluminescent imaging techniques [21–23].

The imaging data from the *in vivo* drug screening were summarized in Figs. 1 and S1. Dexamethasone and curcumin formulations were most effective in suppressing cathepsins and ROS activities respectively (Fig. 1). To further examine the effects of promising drug candidates, three representative drug formulations were studied with a higher number of injection replicates (Figs. 2 and S2). Dexamethasone 5wt% and Curcumin 15wt% represented the drug formulations most effective in decreasing cathepsin activities and reactive oxygen species respectively. Ketoprofen 15wt% represented the drug formulation with no effect on both types of inflammation markers.

Fig. 2A shows the injection pattern of PLGA microparticles with and without drugs. Figs. 2B–D shows bioluminescent images of representative mice at the peak of ROS activity on day 2. Quantification of the peak ROS signals (Fig. 2E) confirmed that curcumin caused a significant three-fold reduction ($p < 0.01$) in ROS activity at the implant sites. Dexamethasone also caused a slight decrease ($p < 0.05$) while ketoprofen did not affect ROS production in the host response to PLGA particles. Representative fluorescent images at the peak of cathepsin activity on day 9 are shown in Figs. 2F–H. Quantification of these fluorescent signals on day 9 (Fig. 2I) showed a significant ($p < 0.0001$) reduction in cathepsin activity with dexamethasone. Curcumin caused a less significant decrease ($p < 0.01$) while ketoprofen did not affect cathepsin activity.

To monitor the infiltration of immune cells at the site of microparticle injection, we performed histology analysis of excised tissues from SKH-1E mice sacrificed at different time points up to 28 days. Fig. 3 shows representative hematoxylin and eosin sections of tissues with control particles and particles containing dexamethasone, curcumin and

ketoprofen. In the control samples (Figs. 3A–E), cellular infiltration followed the typical time-course of the host response [29]. In the early phase, neutrophils occupied the spaces between the microparticles while at the later time points, extensive macrophage infiltration was observed throughout the control samples. In contrast, the samples containing dexamethasone-loaded particles (Figs. 3F–J) were almost free of immune cells for four weeks, except for a few cells present at the edges of the samples. Curcumin also minimized the host response; samples containing this drug remained free of cellular infiltration for up to 2 weeks (Figs. 3K–M). After this time point, macrophages gradually infiltrated the spaces between curcumin-loaded particles resulting in partial cellular coverage at day 15 to complete coverage on day 28 (Figs. 3N–O). Samples containing ketoprofen showed similar pattern of cellular infiltration as compared to the control samples (Figs. 3P–T), confirming that ketoprofen did not inhibit the host response. Furthermore, PLGA particles excised from SKH1E mice were also analyzed to confirm that all three drugs were still present in the sample after 28 days (Fig. S3). This *ex-vivo* analysis proved that the lack of inhibitory effects by ketoprofen and curcumin at the end of the experiments was not due to depletion of the encapsulated drugs.

3.2. Improved glycemic control by microcapsules co-encapsulating drug and islets in diabetic mice

Based on the results from the *in vivo* subcutaneous screening, we examined the potential of top performing drugs in reducing the host response to encapsulated pancreatic islets and improving their efficacy in diabetes therapy. Hybrid drug-islet microcapsules were fabricated using an electrostatic droplet generator to co-encapsulate curcumin or dexamethasone with pancreatic islets isolated from Sprague-Dawley rats. Drug molecules were homogeneously dispersed in 1.5wt% SLG20 alginate at a concentration of 1mg/ml or 2mg/ml for curcumin or dexamethasone respectively. These drug concentrations were determined as optimal from preliminary experiments with different drug loadings ranging from 0.3 to 3.0mg/ml. Due to each drug's hydrophobic nature, higher drug concentrations were avoided as these conditions often resulted in a large fraction of defective capsules with non-uniform sizes, non-spherical shapes and undesirable irregularities which can potentially lead to increased attachment of immune cells [6, 7]. Figs. 4A–C shows the hybrid drug-islet capsules with uniform spherical shape and diameters of 500–600µm. Dexamethasone-loaded capsules appeared white while curcumin capsules appeared yellow due to the intrinsic color of each drug.

To evaluate the *in vivo* efficacy of the hybrid drug-islet capsules, we utilized a xenogeneic C57B6/J mouse model of Streptozotocin (STZ)-induced type I diabetes with marginal islet mass transplantation [30]. Our objective was to determine whether the hybrid drug-islet capsules improved graft survival and function with a minimal amount of transplanted islets. The amount of encapsulated Sprague-Dawley rat islet tissue that would marginally cure the STZ-induced diabetic mouse recipients was determined in preliminary transplant experiments with different islet doses (Fig. S4). A marginal islet mass of 250 islet equivalent (IE) was chosen for evaluation of the hybrid drug-islet capsules. Two types of hybrid capsules, each containing curcumin or dexamethasone, and control capsules which contain only the islets were transplanted into STZ-induced diabetic mice for comparison (n=6–7). Over the two months post-transplantation, capsules with curcumin achieved better glycemic control compared to control and dexamethasone-loaded capsules as shown in Fig. 4D. During the time period of day 29 to day 60, the blood glucose level of mice with curcumin capsules were statistically lower than that of the control mice. However, the difference between blood glucose levels of the animals transplanted with dexamethasone-loaded capsules and control capsules was not significant. The average blood glucose level of the curcumin-loaded capsules rose above the normoglycemic level (200mg/dl) after day 30

compared to days 15 and 21 for the control capsules and dexamethasone-loaded capsules respectively.

In addition, intraperitoneal glucose tolerance tests (IPGTT) showed that the mice receiving islets co-encapsulated with curcumin were able to clear glucose most effectively (Fig. 4E). At each time point during the two-hour IPGTT, significantly lower blood glucose levels were observed in the mice that received curcumin-loaded capsules compared to the animal group with control islet microcapsules ($p < 0.01$ at all time points from 15–105 min and $p < 0.05$ at 120 min). Dexamethasone-loaded capsules did not significantly improve the glucose clearance compared to the control capsules.

3.3. Reduced fibrotic overgrowth on explanted hybrid drug-islet capsules

We also evaluated the effect of the anti-inflammatory drugs in reducing the fibrotic overgrowth on microcapsules encapsulating islets. We first performed qPCR expression analysis of early immunological and fibrosis markers in the fibrotic cell layers on the surface of alginate capsules and surrounding intraperitoneal tissues retrieved from diabetic mice one month post-transplantation (Fig. 5A). The levels of numerous immune cell markers—CD68 (macrophages), CD8 (cytotoxic T cells), CD74 (dendritic cells), and CD19 (B cells), inflammatory cytokines—TNF α and TGF β , and fibrosis markers—collagen 1A1 (Col1a1) and α smooth muscle actin (α SAct), were decreased by curcumin, as compared to dexamethasone-loaded and control capsules.

We also assessed later fibrotic overgrowth on encapsulated islets which were retrieved two months post-transplantation (Figs. 5B–G). Figs. 5B–D and 5E–G are the fluorescent and phase contrast images of the explanted microcapsules stained with Hoechst 33342 dye. In the fluorescent images, enhanced blue fluorescence was observed on all of the control (Fig. 5B) and dexamethasone-containing capsules (Fig. 5C) due to the binding of Hoeschst 33342 dye to the DNA of the fibrotic cell layers covering the capsule surface. Most of the curcumin-containing capsules (Fig. 5D) gave no fluorescence signal due to the absence of fibrosis. In the phase contrast images, control (Fig. 5E) and dexamethasone-containing capsules (Fig. 5F) appeared darker than curcumin-containing capsules (Fig. 5G) due to the presence of fibrotic cell layers obstructing light transmission. Though the majority of the curcumin-containing capsules were free of cellular overgrowth, a small fraction of these capsules was still fibrotic as represented by the single capsule emitting blue fluorescence in Fig. 5D and the same capsule appearing darker in Fig. 5G. Figs. 5H–J showed representative histological cross-sections of explanted capsules embedded in agar hydrogel and stained with hematoxylin and eosin. The histology data in Figs. 5H–J confirmed the presence of immune cellular layers on the surface of explanted control capsules and dexamethasone-loaded capsules. Several dark purple circular cross-sections of the alginate capsules have been lost during histological processing but the pericapsular overgrowth on fibrotic capsules remained as exterior rings comprised of single or multiple cell layers. In addition, inspection of the explanted capsules by optical microscopy suggested that residual curcumin remained in the capsules after two months (Fig. S5) correlating to *in vitro* observation (Fig. S6).

4. DISCUSSION

Anti-inflammatory drugs have the potential to mitigate immunological response to implanted medical devices [11, 12] or transplanted cell-based therapeutics [13, 14]. Here, we performed a comprehensive examination of existing anti-inflammatory molecules to identify drug candidates that can decrease the activities of cathepsins and ROS, which are produced by immune cells in the host response to biomaterials [21–23].

Steroidal glucocorticoids act through numerous genomic and non-genomic pathways to block the synthesis of metabolites which are important mediators for cytokine production, leukocyte recruitment and activation in early inflammation [24, 31]. In the subcutaneous screening (Fig. 1 and Fig. S1), several steroidal glucocorticoids with the higher glucocorticoidal potency (dexamethasone, flucortisone, and prednisolone) [31] were able to suppress cathepsin activities in the early host response to implanted PLGA microparticles. Most glucocorticoids, except for dexamethasone, did not have any inhibitory effects on ROS activity as showed by bioluminescent screening data.

Polyphenols are reported to be responsible for the blockage of the transcription factor NF- κ B which is required for transcription of genes involved in the inflammatory responses [27]. In our study, curcumin significantly inhibited ROS activity and cathepsin enzymes. Interestingly, resveratrol did not reduce ROS or cathepsin activities though it belongs to the same class of compounds as curcumin and has been assumed to have similar mechanisms of action [32].

Non-steroidal anti-inflammatory drugs (NSAIDs) bind to the hydrophobic active sites of cyclooxygenase (COX) enzymes to inhibit the COX-mediated generation of proinflammatory molecules [26]. Our study demonstrated that locally released NSAIDs, especially ketoprofen, were not effective in suppressing both cathepsin and ROS in the subcutaneous host response to implanted PLGA microparticles. This finding was surprising given numerous prior studies reporting the effectiveness of NSAIDs in alleviating inflammation when administered systemically [26, 31, 33]. There have also been a limited number of studies claiming the efficacy of locally administered NSAIDs in reducing fibrosis formation [18, 34]. These discrepancies can potentially be due to the difference in dosages or the pharmacodynamic properties of controlled-release NSAID formulations versus systemic administration of the same compounds.

Other non-steroidal immunosuppressant drugs such as tacrolimus and cyclosporine also did not effectively inhibit ROS or cathepsin enzymes. These findings are not surprising because these drugs primarily inhibit the activation of T and B lymphocytes [25] of the adaptive immune systems while the subcutaneous host response involves innate immune cells such as neutrophils and macrophages [29].

The most effective anti-inflammatory agents identified from the subcutaneous screening, dexamethasone and curcumin, were incorporated in hybrid drug-islet alginate capsules to mitigate the fibrotic response against transplanted microcapsules containing pancreatic rat islets. Fibrosis or pericapsular overgrowth has been implicated in the limited long-term survival of encapsulated islets in various animal models [8, 35]. Cellular and collagen deposition on the surface of transplanted microcapsules has been suggested to result in delayed insulin secretion in response to plasma glucose fluctuations, reduced nutrient transport and subsequent decrease in graft viability and function [35, 36]. With our current capsule design, curcumin reduced fibrotic overgrowth, and improved graft function while dexamethasone did not. Innate immune macrophages/monocytes produce and secrete immunostimulatory cytokines, such as TNF α , after the formation of inflammation intermediates via COX-2-mediated degradation of arachidonic acid [37]. This metabolic cascade is inhibited by curcumin at numerous steps both upstream and downstream of arachidonic acid production [38, 39]. Curcumin also decreases macrophage nitric oxide synthesis [40] and inhibits dendritic cell stimulation and cytokine production [41]. These potential mechanisms may be responsible for the herein observed inhibition of fibrosis markers (Col1a1 and α SMA) and decrease in CD68 (macrophages), CD74 (dendritic cells), TGF β , and TNF α levels by curcumin. At the level of adaptive immunity, curcumin decreases T cell proliferation [42], and may explain the observed decrease in CD8, a marker

for cytotoxic T cells. Though fibrosis is decreased by co-encapsulating curcumin with the xenogenic rat islets, a small percentage of curcumin-containing capsules still remained fibrotic. A similar observation has been reported for islet-free alginate capsules [7] and alginate capsules containing isografts or allografts transplanted into rats [43]. Though the reasons why these few fibrotic capsules persisted was not clear, we speculate that the capsules pooling near the abdominal surgical incision might have been exposed to more aggressive immunological attack during wound healing and were more susceptible to fibrotic overgrowth.

In addition to its anti-inflammatory effects, curcumin has been reported to have favorable effects on encapsulated islets. Several studies have demonstrated that curcumin could prevent cytokine-induced islet death *in vitro* and diabetogenesis *in vivo* as well as improve islet recovery after cryopreservation [44, 45]. These protective effects may also explain the ability of the hybrid curcumin-islet alginate microcapsules in maintaining better glycemic control.

With our current drug-loaded alginate capsule design, the anti-fibrotic effects of dexamethasone and curcumin on the encapsulated islets did not correlate directly with their impact on the subcutaneously injected PLGA microparticles, potentially due to the different drug release kinetics in the two drug-loaded particulate systems. In the subcutaneous study with PLGA microparticles, dexamethasone was either as or more potent than curcumin in inhibiting the activities of inflammation markers (Fig. 2) and cellular infiltration (Fig. 3). However, intraperitoneally transplanted islets co-encapsulated with curcumin were more effective in decreasing fibrosis (Fig. 5) and improving glycemic control (Fig. 4). While the hypothesis that decreased fibrosis leads to better islet graft function was supported by the data for curcumin, the same effect was not achieved with the dexamethasone-containing capsules due to the faster release kinetics of dexamethasone from the alginate capsules resulting in its rapid depletion (Fig S6).

Drug release mechanisms play a significant role in the better performance of curcumin in reducing fibrosis on the alginate capsules and improving the efficacy of encapsulated islets. Alginate is a non-degradable hydrophilic hydrogel [46] while PLGA is a biodegradable hydrophobic polymer [29]. The mechanism of drug release from the PLGA particles involves both passive drug diffusion out of the polymer pores and drug release due to the degradation of the polymer matrix [47]. In contrast, only diffusion influences the drug release kinetics from the non-degradable alginate capsules. In the subcutaneous study, both curcumin and dexamethasone remained in the injected PLGA particles at the end of the 28-day experiment as determined by *ex vivo* analysis of the excised particles. However, in the study with encapsulated islets, curcumin remained in the alginate hydrogel capsules for a longer period of time and thus prolonged its effectiveness in mitigating the fibrotic response. This was possibly due to the lower aqueous solubility of curcumin [48] compared to that of dexamethasone [49] resulting in the slower release of curcumin as demonstrated in the *in vitro* drug release study (Fig S6).

Several strategies could be explored in future studies to improve the efficacy of the hybrid drug-islet capsules. First, improved release kinetics of dexamethasone can potentially be achieved by covalent attachment of the drug to the alginate hydrogel via a degradable linking moiety such as an ester group or a hydrazide group [50]. Hydrolysis of the linking ester groups *in vivo* may release dexamethasone at a slower rate over an extended period of time. Second, a combination of both dexamethasone and curcumin or other combinations of anti-inflammatory agents might have the potential to act synergistically in suppressing immune responses against transplanted microcapsules. Third, a next generation of hybrid drug-islet capsules can be designed to have a core-shell structure in which the cells are

encapsulated in the inner core and the anti-inflammatory drugs are encapsulated within an external shell[51–53]. In such a design, the drug will be confined to the surface of the hydrogel capsules to facilitate outward drug diffusion, maximize drug interaction with immune cells, and minimize its interference with the therapeutic cells inside.

4. Conclusion

We performed *in vivo* screening of a variety of small molecule anti-inflammatory drugs utilizing both non-invasive imaging and end-point histology. Dexamethasone and curcumin were identified as the most effective drugs in inhibiting the activities of early inflammatory proteases and reactive oxygen species. They also minimize cellular infiltration in the subcutaneous host response to implanted PLGA microparticles. When co-encapsulated with rat islets in alginate microcapsules, curcumin improved glycemic control and mitigated the formation of pericapsular overgrowth on the microcapsules transplanted into STZ-induced diabetic mice. Our findings have potential applications in reducing fibrosis and improving the performance of a broad range of cell-based therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *In vivo* subcutaneous screening of anti-inflammatory drugs encapsulated in PLGA microparticles

SKH-1E mice were imaged three days after subcutaneous injection of microparticles. Data arranged in order of decreasing relative fluorescent or bioluminescent signals. Relative fluorescent or bioluminescent signal was calculated as a ratio of the signal from the drug-loaded microparticles to the signal from the control particles in the same mice. **A)** Activity of cathepsin enzymes was assessed using Prosense 680, a cathepsin-activated fluorescent imaging probe. **B)** Activity of reactive oxygen species was assessed using luminol which emits bioluminescence when oxidized by reactive oxygen species. Data: mean $\pm$ s.e.m (n=6 replicate injections). The dotted lines represent the position where the relative fluorescent or bioluminescent signal is equal to 1. The data points on the left of the lines (relative signal less than 1) correspond to drug formulations that were effective in decreasing the activity of the corresponding inflammation markers.

Fig. 2. Effects of selected drugs on the peak activities of cathepsin enzymes and ROS in the subcutaneous host response to PLGA microparticles

A) Injection pattern of the PLGA microparticles without (●) and with drugs (■) B–D) Bioluminescent images of representative mice on day 2 at the peak of ROS activity. E) Quantified bioluminescent signals on day 2. F–H) Fluorescent images of representative mice on day 9 at the peak of cathepsin activity. I) Quantified fluorescent signals on day 9. Data: mean ± s.e.m (n=15 replicate injections). *, **, *** denotes p<0.05, 0.01, 0.0001 respectively.

Fig. 3. Histology analysis of subcutaneously injected PLGA microparticles with and without drugs excised from SKH-1E mice at different time points

Scale bar represents 50μm for all images. Yellow arrows indicate areas with minimal infiltration of immune cells. **A–E)** Samples with the control microparticles showed the typical time-course of the subcutaneous host response. **F–J)** Samples containing dexamethasone showed minimal infiltration of immune cells throughout the 28 day duration. **K–O)** Samples containing curcumin remained free of immune cells during the first two weeks (K–M) but cellular infiltration was observed at later time points (N–O). **P–T)** Samples containing ketoprofen showed the similar pattern of cellular infiltration as the control particles.

Fig. 4. Effects of hybrid drug-islet capsules on glycemic control of STZ-induced diabetic mice with a marginal islet mass transplanted into the intraperitoneal space

A–C) Phase contrast images of alginate microcapsules without any drug (**A**), with dexamethasone (**E**) and curcumin (**F**). **D)** Daily non-fasting blood glucose level of STZ-induced diabetic C57B6/J mice transplanted with control islet capsules (n=7), capsules containing dexamethasone (n=7) and curcumin (n=6) co-encapsulated with islets isolated from Sprague-Dawley rats. **E)** Fasting blood glucose level of the same groups of mice during the IPGTT on day 60. Data :mean ± s.e.m (n=6 or 7).(*) and (#) represent p<0.01 and p<0.05 respectively.

Fig. 5. Characterization of fibrotic pericapsular overgrowth on microcapsules retrieved after transplantation into STZ-induced C57B6J diabetic mice

A) qPCR analysis of host (mouse) expression of immunological and fibrosis markers on alginate capsules and surrounding fat pad tissue retrieved one month post-transplantation. The markers were macrophage (Mφ) marker CD68, B cell marker CD19, dendritic cell marker CD74, cytotoxic T cell marker CD8, inflammatory cytokines TNFα and TGFβ, and fibrosis-associated activated-fibroblast marker α-smooth muscle actin (α SMAct) and collagen 1A1 (Col1a1). Data: mean ± s.e.m, (n=7). *, **, *** denotes $p < 0.05$, 0.01, 0.001 respectively. **B–D)** Fluorescent images of DNA-stained control microcapsules (**B**) and microcapsules with dexamethasone (**C**) or curcumin (**D**) retrieved two month post-transplantation. **E–F)** Phase contrast images of the same control microcapsules (**E**) and microcapsules with dexamethasone (**F**) or curcumin (**G**). **H–J)** Histology H&E sections of retrieved control microcapsules (**H**) and microcapsules with dexamethasone (**I**) or curcumin (**J**).

Table 1

Small molecule anti-inflammatory drugs investigated in the *in vivo* subcutaneous screening. Different classes of compounds including steroidal glucocorticoids, naturally derived polyphenols, NSAIDs and non-steroidal immunosuppressants were screened in hairless immunocompetent SKH-1E mice.

Compound name	Drug loading (wt%)	Drug classification
Dexamethasone	10, 5, 2	Steroidal glucocorticoid
Fludrocortisone	15, 10, 5	Steroidal glucocorticoid
Methylprednisolone	15, 10, 5	Steroidal glucocorticoid
Prednisolone	10, 5	Steroidal glucocorticoid
Prednisone	15, 10, 5	Steroidal glucocorticoid
Hydrocortisone	15, 10, 5	Steroidal glucocorticoid
Curcumin	15, 10, 5	Polyphenol
Resveratrol	15	Polyphenol
Ketoprofen	15	NSAIDs (COX-1 inhibitor)
Diclofenac	15	NSAIDs (COX-1 inhibitor)
Sunlindac	15	NSAIDs (COX-1 inhibitor)
Piroxicam	15	NSAIDs (COX-1 inhibitor)
Celecoxib	15, 10, 5	NSAIDs (COX-2 inhibitor)
Rapamycin	15, 10, 5	Non-steroid immunosuppressant
Tacrolimus	15, 10, 5	Non-steroid immunosuppressant
Cyclosporin	15, 10, 5	Non-steroid immunosuppressant