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Nanoparticle delivery of donor antigens for transplant tolerance in allogeneic islet transplantation

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Abstract

Human islet cell transplantation is a promising treatment for type 1 diabetes; however, long-term donor-specific tolerance to islet allografts remains a clinically unmet goal. We have previously shown that recipient infusions of apoptotic donor splenocytes chemically treated with 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (donor ECDI-SP) can mediate long-term acceptance of full major histocompatibility complex (MHC)-mismatched murine islet allografts without the use of immunosuppression. In this report, we investigated the use of poly(lactide-co-glycolide) (PLG) particles in lieu of donor ECDI-SP as a synthetic, cell-free carrier for delivery of donor antigens for the induction of transplant tolerance in full MHC-mismatched murine allogeneic islet transplantation. Infusions of donor antigen-coupled PLG particles (PLG-dAg) mediated tolerance in ~20% of recipient mice, and the distribution of cellular uptake of PLG-dAg within the spleen was similar to that of donor ECDI-SP. PLG-dAg mediated the contraction of indirectly activated T cells but did not modulate the direct pathway of allorecognition. Combination of PLG-dAg with a short course of low dose immunosuppressant rapamycin at the time of transplant significantly improved the tolerance efficacy to ~60%. Furthermore, altering the timing of PLG-dAg

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administration to a schedule that is more feasible for clinical transplantation resulted in equal tolerance efficacy. Thus, the combination therapy of PLG-dAg infusions with peritransplant rapamycin represents a clinically attractive, biomaterials-based and cell-free method for inducing long-term donor-specific tolerance for allogeneic cell transplantation, such as for allogeneic islet transplantation.

Keywords

Allogeneic cells; Islet; Transplantation; Tolerance; 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (ECDI); Poly(lactide-co-glycolide) (PLG)

1. Introduction

Cell therapies are currently a billion dollar market with significant growth expected [1,2]. Applications that are being developed commercially include cell immunotherapies, hematopoietic progenitor cells, chondrocytes for cartilage repair, and keratinocytes for wound healing [1,3]. While autologous cells are ideal to avoid immune rejection, allogeneic sources are attractive for diseases in which autologous cells are not readily available, such as type 1 diabetes (T1D) or trauma (e.g., spinal cord injury) [4]. Allogeneic islet cell transplantation is a promising treatment for type 1 diabetes and exemplifies the potential of the approach [4]. However, life-long immunosuppressive drugs are currently used for allogeneic islet cell transplantation to prevent rejection, which can be harmful to the transplanted islets and lead to the non-specific suppression of the entire host immune system [5]. The non-specific effects of these drugs result in enhanced patient susceptibility to opportunistic infections and/or an increased risk of neoplasia.

An alternative to systemic immunosuppression is the induction of donor-specific tolerance, for which successful regimens have been developed in small and large animal models, as well as in human clinical trials [6–8]. One type of tolerance approaches involves conditioning the recipient pre- and post-transplant with infusions of donor cells administered in combination with co-stimulation blockade [9] or apoptotic donor cells in the absence of additional treatment [10]. We have previously demonstrated that treatment of donor splenocytes (SP) with the chemical crosslinker 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (ECDI) induces apoptosis, and infusions of such donor ECDI-SP mediate long-term donor-specific allograft acceptance in full MHC-mismatched allogeneic islet transplantation models [10,11]. Mechanistic studies revealed that in the treated recipients, apoptotic ECDI-SP regulate both directly- and indirectly-activated T cells through anergy and deletion, respectively, accompanied by an induction of regulatory T cells (Treg) [11]. Despite the success of this method, several limitations challenge its direct translation to clinical applications. Inherent difficulties in adapting cell-based therapies to humans include complex *ex vivo* human cell manipulation, donor-to-donor variability, and stringent safety considerations for clinical applications.

A promising alternative to donor splenocytes for recipient preconditioning is to employ synthetic particles as the carrier of soluble donor antigens obtained from processed donor cells. This approach would allow for a storable form of donor antigens for the manufacturing

of therapeutic products for tolerance induction, hence significantly expanding the applicability of this approach to include deceased donor organ transplantation and facilitate repetitive tolerance boosters post-transplant if needed. Synthetic particles can also be manufactured reproducibly, further providing a platform upon which modifications can be made to enhance the therapeutic efficacy of this tolerance approach. Recent work by our lab and collaborators has demonstrated that biodegradable poly(lactide-co-glycolide) (PLG) particles, with an approximate diameter of 500 nm, are capable of effectively delivering peptide antigens to mediate tolerance to autoimmunity in both preventative and therapeutic models of relapsing-remitting experimental autoimmune encephalomyelitis (R-EAE) [12]. PLG has been shown to be a safe delivery system in preclinical models [13–15], and has also been approved by the FDA for a number of therapeutic applications [16–18].

In this report, we investigated the ability of PLG particles carrying allogeneic donor antigens for transplant tolerance induction in full MHC-mismatched allogeneic islet transplantation models. Based on our prior work showing the efficacy of donor ECDI-SP in inducing transplant tolerance in the same models, the current study investigated the efficacy of replacing splenocytes with soluble donor antigens coupled to PLG particles in the establishment and maintenance of long-term tolerance in allogeneic islet transplantation. Initial studies focused on optimizing donor antigen (dAg) coupling to the PLG particles (PLG-dAg). We subsequently investigated the long-term protection of transplanted islet allografts provided by the PLG-dAg and characterized the potential mechanisms of their protection. Our studies thus provide the basis for the future development of synthetic particles for transplant tolerance induction that will likely have a broader impact on cell therapies beyond that for allogeneic islet cell transplantation.

2. Materials and methods

2.1. Mice

Eight to ten week old male BALB/c (H-2^d), C57BL/6 (B6, H-2^b), and SJL/J (H-2^s) mice were purchased from the Jackson Laboratory and Harlan. 4C mice were provided by Dr. Qizhi Tang from the University of California at San Francisco. TCR75 mice were provided by Dr. Anita Chong from the University of Chicago. All mice were housed under specific pathogen free conditions at Northwestern University and protocols were approved by Northwestern University IACUC.

2.2. Islet transplantation

Mice were treated with streptozotocin (Sigma–Aldrich) at 170 mg/kg. Two consecutive glucose readings (1 day apart) greater than 250 mg/dl were used to determine diabetes. The protocol for islet isolation and transplantation has been described previously [19]. Approximately 500 islets were implanted under the kidney capsule of recipient mice. Graft rejection was determined by two consecutive blood glucose readings greater than 250 mg/dL.

2.3. Antibodies and FACS analysis

Cell phenotype was measured by flow cytometry. Isolated cells were stained with fluorochrome-conjugated antibodies for 30 min on ice, washed, read on the FACSCanto II (BD) and analysed using FlowJo v6.4.7 (TreeStar). The following antibodies (clones) were used: CD11c-APC (HL3), I-A^b-PE and CD90.1-PerCPCy5.5 (OX-70) from BD Biosciences, and CD11b-eFluor780 (M1/70), F4/80-PerCPCy5.5 (BM8), B220-PECy7 (RA3-6B2) and CD8α-eFluor450 (53-6.7) from eBiosciences.

2.4. Preparation of donor antigens from donor cells

Donor BALB/c splenocytes were processed into single cell suspensions and erythrocytes lysed. Up to 1×10^9 cells were sonicated twice in PBS at an amplitude of 30% for 20 s, followed by 30 s at 60% amplitude (Cole-Parmer). Total protein was quantified by the Coomassie Plus (Bradford) Protein assay (Thermo Fisher Scientific Inc.) prior to coupling to PLG particles.

2.5. PLG particle synthesis

Single emulsion poly(lactide-co-glycolide) (PLG) particles were synthesized with poly(ethylene-alt-maleic acid) (PEMA) as a surfactant as described in Ref. [20]. Briefly, PLG (50% D,L-lactide/50% glycolide) (Lactel Absorbable Polymers) was dissolved in dichloromethane to make a 20% (w/v) solution. This solution was sonicated (Cole-Parmer) at 16 W in 1% w/v PEMA (Polysciences, Inc.) to create particles. After overnight stirring, particles were collected by centrifugation, washed 3 times with 1 M Sodium Bicarbonate buffer, and lyophilized overnight with 4% w/v sucrose and 3% w/v D-mannitol.

2.6. Particle characterization

Particles were imaged with a scanning transmission electron microscope (Hitachi HD2300 Field Emission STEM) operating at 200 kV. Particles were drop casted on 400 mesh Cu/Rh grids containing a carbon membrane and negatively stained with 1% UA in ddH₂O. Particle size and surface ζ-potential distributions were obtained using dynamic light scattering on a Zetasizer Nano ZSP (Malvern Instruments Ltd).

2.7. Preparation of donor antigen-coupled particles (PLG-dAg) and ECDI-SP

PLG particles, 3.0 mg, were washed 3 times to remove sugars from lyophilization and incubated for 1 h with stirring with 30 mg/ml 1-Ethyl-3-(3' dimethylaminopropyl) carbodiimide, HCl (ECDI) (EMD Millipore Chemicals, Inc.) and 1200 µg lysate (from 2 donor spleens) per dose. Coupled particles were washed twice to remove excess ECDI and filtered through a 40 µm cell strainer (BD Falcon). Lysate coupling efficiency was determined by quantifying remaining protein in supernatants after the coupling reaction using the Coomassie Plus (Bradford) Protein assay (Thermo Fisher Scientific Inc.). Donor ECDI-SP were prepared as previously described [10]. Briefly, splenocytes were incubated with ECDI (Calbiochem, every 3.2×10^8 cells in 1 ml of DPBS (Life Technologies, Grand Island, NY) with a final concentration of 30 mg/ml of ECDI) on ice for 1 h with agitation on a shaker (Labline Instruments Inc., Melrose Park, IL) followed by washing.

2.8. Tolerance induction by PLG-dAg

PLG-dAg (3.0 mg) or control blank PLG particles (3.0 mg) were injected i.v. into recipient B6 mice on day -7 and day +1 with reference to islet transplantation (on day 0). Rapamycin (rapa) (Enzo Life Sciences, Inc.) was dissolved in 0.2% carboxymethyl cellulose solution and sonicated prior to each intraperitoneal injection of 0.1 mg/kg on days -1, 0, +1, and +2.

2.9. PKH67 labeling of ECDI-SP and PLG-dAg

For tracking studies, donor (BALB/c) ECDI-SP were labeled with 2 μ M PKH67 (Sigma-Aldrich) according to manufacturer's instructions. Briefly, 2×10^7 cells were resuspended in 1 ml Diluent C and mixed with 1 ml Diluent C containing 4 μ l PKH67 dye before washing 3 times in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (Gibco). To track PLG-dAg, donor SP were labeled with PKH67 as above, sonicated, and then coupled to PLG particles with ECDI as above. 1×10^8 donor ECDI-SP^{PKH67+} or 3.0 mg PLG-dAg^{PKH67+} were injected i.v. to B6 mice. After 18, 48 or 96 h, mice were sacrificed and organs were isolated, processed to single cell suspensions, and PKH67⁺ cells were analysed by flow cytometry (BD FACSCanto II).

2.10. Adoptive transfer of T cells

T cell receptor transgenic (TCR Tg) TCR75 (CD45.1⁺) and 4C (Thy1.1⁺) CD4 T cells were purified from spleens of the respective TCR Tg mice using CD4⁺ negative isolation kit (Miltenyi). TCR75 and 4C CD4 T cells were labeled with 5 μ M CFSE (Molecular Probes) and injected i.v. into CD45.2⁺Thy1.2⁺ B6 recipients. Mice were subsequently injected with BALB/c ECDI-SP or PLG-dAg prepared as above and analyzed on the indicated days. For *in vitro* restimulation of 4C cells, harvested cells were rested overnight in RPMI 1640 supplemented with 10% FCS, followed by stimulation with anti-CD3/28 dynabeads (Invitrogen) for 4 days. Further proliferation of the 4C cells was determined by CFSE dilution.

2.11. Statistics

Statistical analyses were performed using the statistical package Graphpad Prism (Graphpad, La Jolla, CA). Results are presented as mean $\pm$ standard error of the mean (SEM) in all figures. Student's *t*-test or one-way ANOVA were used to determine statistical significance of groups. The log-rank statistic was used for comparison of graft function in Kaplan Meier analysis between groups. A value of probability (*p*) less than 0.05 was considered statistically significant.

3. Results

3.1. PLG-dAg for donor antigen delivery for transplant tolerance induction

We first investigated whether donor cell lysate could be delivered by synthetic PLG particles for the induction of allogeneic tolerance. The single emulsion process produced spherical PLG particles (Fig 1A) with a mean diameter of 458.8 ± 14.8 nm and zeta potential of -72.5 ± 1.42 mV (Fig 1B). Donor cell lysate was prepared by sonication of donor spleen leukocytes and coupled to the PLG particles via an ECDI coupling reaction as detailed in

Materials and methods. Efficiency of protein coupling by ECDI conjugation was investigated by varying the ECDI amount and the reaction volume, neither of which significantly impacted the amount of protein coupled (Fig 1C). Hence, subsequent coupling reactions for *in vivo* studies were performed using 20 μ g ECDI and 700 μ l reaction volume per dose as previously used [10].

BALB/c cell lysate coupled to PLG particles (PLG-dAg) was then administered to diabetic B6 recipients intravenously (i.v.) pre- and post- islet transplantation on days -7 and +1 at a dose of 3 mg (total weight of PLG-dAg) per mouse per injection, with BALB/c islet transplantation performed on day 0. Mice receiving blank particles without BALB/c cell lysate coupling were used as controls. As shown in Fig 2, in the PLG-dAg treated group, ~20% of recipients achieved long-term graft function without rejection, whereas 100% of the blank particle treated recipients rejected the islet allograft by day 24 post transplantation ($p = 0.0099$). Neither increasing the input protein during the coupling reaction nor the number of doses post-transplant (additionally given on day +7 and day +14 post transplant) increased the efficacy of graft protection (data not shown). Therefore, injections of the PLG-dAg particles resulted in detectable protection of the transplanted islet allografts, although the level of protection was less robust compared with that provided by injections of donor ECDI-SP themselves as we reported previously [10].

3.2. Interactions between PLG-Ag and host splenic phagocytes

We next determined the biodistribution of the PLG-dAg after their injection into recipient B6 mice. Donor splenocytes were labeled with a fluorescent dye PKH67 (green fluorescence) prior to sonication and coupling to PLG particles. Eighteen hours after i.v. injection of PLG-dAg^{PKH67+}, recipient mice were sacrificed and the spleen, the heart, the liver, and the lungs were harvested and analyzed for PKH67⁺ cells by flow cytometry. Comparisons were made to the biodistribution of similarly PKH67 labeled donor ECDI-SP (ECDI-SP^{PKH67+}). As shown in Fig 3A, the percentages of PKH⁺ cells among all live cells in the spleen, the heart, the liver and the lungs were similar in mice injected with PLG-dAg^{PKH67+} and those injected with ECDI-SP^{PKH67+}, suggesting that the overall trafficking and retention of the injected PLG-dAg^{PKH67+} or the ECDI-SP^{PKH67+} are similar in the various organs examined. The spleen has been identified as a key component to tolerance induction [11,12]. We next examined the various subsets of recipient splenic antigen presenting cells (APCs) uptaking of PLG-dAg^{PKH67+}, and compared with those uptaking ECDI-SP^{PKH67+}. Spleen cells that were double positive for PKH67 and recipient class II (I-A^b) (i.e. I-A^bPKH67⁺ cells marking recipient splenic APCs that had internalized the injected PLG-dAg^{PKH67+} or ECDI-SP^{PKH67+}) were further phenotyped for markers of APC subsets, including F4/80, B220, CD11b, CD11c, and CD8 α . As shown in Fig 3B, the distribution of I-A^bPKH67⁺ cells among F4/80⁺ macrophages, B220⁺ B cells, and CD11c⁺CD11b⁺, CD11c⁺B220⁺, CD11c⁺CD8 α ⁺ dendritic cell subsets in the spleen was similar in recipients injected with PLG-dAg^{PKH67+} and recipients injected with ECDI-SP^{PKH67+}, again suggesting that the targeted APC populations within the spleen by PLG-dAg^{PKH67+} or ECDI-SP^{PKH67+} are similar. Additionally, similar analysis at 48 or 96 h post injection showed gradual loss of PKH67 positivity among recipient I-A^b cells (data not shown), due likely to efficient host APC processing of the internalized donor antigens.

Again, no difference was observed between hosts injected with PLG-dAg^{PKH67+} and those injected with ECDI-SP^{PKH67+} (data not shown).

3.3. Effect of PLG-dAg on donor-specific T cells with direct or indirect donor specificities

The ability of PLG-dAg to modulate recipient T cell activation was next investigated. Recipient T cells with direct donor-specificity respond directly to donor MHC molecules that are present on donor cells, whereas recipient T cells with indirect donor-specificity respond to donor MHC peptides processed by recipient APCs and presented by recipient MHC molecules. We have previously reported that in the treated recipients, donor ECDI-SP differentially modulate directly-activated vs. indirectly-activated T cells. Directly-activated T cells initially undergo limited proliferation followed by anergy, whereas indirectly-activated T cells initially undergo rigorous clonal expansion followed by clonal contraction and deletion [11]. To examine the effect of PLG-dAg on recipient T cells with direct or indirect specificities, CD4 T cells with transgenic (Tg) T cell receptors (TCR) which have either direct antigen specificity (4C TCR Tg T cells) or indirect specificity (TCR75 TCR Tg T cells) were labeled with an intracellular fluorescent dye CFSE and injected individually on the indicated days (Fig 4A) to recipient mice subsequently treated with PLG-dAg. Comparisons were made to TCR Tg T cells injected to recipients subsequently treated with ECDI-SP. The spleens were then harvested on the indicated days (Fig 4A) and examined for TCR Tg T cell proliferation. Each round of proliferation/division of the CFSE-labeled TCR Tg T cells would dilute the intracellular CFSE by ~50%, therefore reduce its fluorescence intensity by ~50%. Consequently, multiple rounds of proliferation/division would result in multiple peaks of lower CFSE intensities, each about 50% of the intensity of the previous peak.

As shown in Fig 4B left panel, in PLG-dAg treated recipients, 4C TCR Tg T cells showed minimal proliferation (black line) in response to the injected PLG-Ag. This result is in sharp contrast to that seen in recipients treated with ECDI-SP, in which several rounds of low level proliferation of the 4C TCR Tg T cells were seen in response to the injected ECDI-SP (shaded gray histogram). However, 4C T cells retrieved from PLG-dAg treated mice were readily responsive to further *ex vivo* stimulation by anti-CD3/CD28 within a short period of time (48 h), showing rigorous proliferation (Fig. 4B, right panel, gray line) compared with unstimulated cells (black line). This is again in sharp contrast to that previously observed of 4C TCR Tg T cells from ECDI-SP treated mice which exhibited a complete unresponsiveness to further *ex vivo* stimulation [11]. These data collectively indicate that in contrast to ECDI-SP, PLG-Ag do not anergize T cells with direct donor-specificity.

We next investigated indirectly-activated T cells by using TCR75 Tg T cells. As shown in Fig 4C left panel, three days after the injection, PLG-dAg treatment resulted in TCR75 proliferation and expansion (black line) intermediate to that seen in ECDI-SP treated recipients (rigorous proliferation, thin gray line) and that in blank PLG treated recipients (no proliferation, thick gray line). However, at seven days post injection of PLG-dAg (Fig 4C, right panel), the TCR75 Tg T cells also underwent significant contraction (black bars), similar to that seen in ECDI-SP treated recipients (white bars). These results collectively

indicate that, similar to ECDI-SP, PLG-dAg can modulate T cells with indirect donor-specificity through initial clonal expansion followed by clonal contraction.

3.4. Synergy between PLG-dAg and low dose peri-transplant rapamycin on islet allograft protection

Given that PLG-dAg do not modulate both pathways of T cell activation in a manner similar to ECDI-SP, we investigated if short-term treatment with a known inhibitor of T cell responses might provide additional control of T cell activation thereby increase graft protection by PLG-dAg. Based on its use in the clinic, rapamycin (rapa) was chosen and administered at a low dose (0.1 mg/kg) daily from day -1 to day +2, and mice were additionally treated with PLG-dAg on day -7 and day +1 and transplanted with donor islets on day 0. As shown in Fig 5, islet allograft survival was significantly increased in the PLG-dAg + rapa group compared with that in either treatment alone groups ($p = 0.0066$, compared with PLG-dAg alone; $p = 0.0011$, compared with rapa alone) (Fig 5). To confirm that this effect was donor-specific, lysate from SJL/J splenocytes was coupled to PLG particles as third party donor antigens and injected to B6 recipients subsequently transplanted with BALB/c islet grafts. As expected, protection of BALB/c islet grafts was not observed in recipients treated with SJL/J PLG-Ag (Fig. 5), confirming the donor-specificity of this therapy. Collectively, these data indicate that rapamycin can synergize with PLG-dAg to provide long-term donor-specific protection to the islet allograft.

3.5. Pre-transplant infusion of PLG-dAg on day -2 rather than day -7

The clinical translation of this tolerance strategy might be challenging if PLG-dAg particle infusion is required at day -7, particularly in settings of deceased donor tissues/organ transplantation. Thus, we sought to determine if the first infusion of PLG-dAg could be administered closer to the day of transplantation. Diabetic B6 recipients were treated with PLG-dAg + rapa as described in the previous section, with the exception that PLG-dAg were administered either on day -7 and day +1 as above, or on day -2 and day +1. As shown in Fig 6, no difference in graft outcome was observed whether the first infusion was given on day -7 or day -2 ($p > 0.05$). Consistent with previous data, all islet allografts were rejected by 40 days post transplantation if blank PLG particles were administered on day -2 (Fig 6). Collectively, these data suggest that the timing of the first dose of PLG-dAg can be moved closer to the day of transplantation, therefore improving the clinical feasibility of this potential tolerance approach.

4. Discussion

This study reports the development of biodegradable PLG particles as a synthetic carrier for donor antigen delivery for the induction of donor-specific transplant tolerance, resulting in long-term protection of the transplanted islet allografts in treated recipients when combined with low dose, short-term rapamycin administration. The development of particles for delivering donor antigens was based on our prior work in which fresh donor or recipient cells were used as antigen carriers, which has been effective in preclinical models of autoimmune and alloimmune tolerance. This approach has been recently employed in a clinical trial for tolerance induction for patients with multiple sclerosis [21]. The acquisition

of large numbers of donor cells and their manipulation prior to the tolerance therapy are viewed as challenging in part due to the timing between acquisition and therapy, as well as possible variability in processing. Alternative delivery methods based on synthetic materials may provide an opportunity for greater standardization. PLG was selected in part because of its established safety profile in humans [16] and relative ease of large scale production. Polystyrene and PLG particles have been shown to be highly efficient substitutes for apoptotic splenocytes in both the prevention and treatment of R-EAE, an autoimmune model of multiple sclerosis [12]. Unlike the R-EAE model where the antigens causing the disease have been precisely identified at the epitope level, our full MHC-mismatched transplant model involves a multitude of complex protein antigens with less well-defined epitopes. Thus, we employed donor cell lysates, which contain the entire spectrum of donor antigens, for coupling to the particles using carbodiimide chemistry. Particles were fabricated with a high density of surface carboxylate groups using PEMA as a surfactant to which amines within the lysate proteins can be coupled via ECDI.

Delivery of particles modified with solubilized donor antigens resulted in long-term allograft protection, demonstrating the potential of this therapy for inducing transplantation tolerance (Fig 2). However, the particles were not as effective as donor ECDI-SP at promoting allograft protection. Our data shows that PLG-dAg mediate clonal expansion and subsequent contraction of T cells with indirect donor specificity in a similar fashion to that seen with ECDI-SP, although to a lesser extent (Fig 4C). Alloantigens on transplanted tissues or organs can be processed and re-presented by recipient APCs in the context of recipient MHC. This would in turn activate T cells with indirect specificities and contribute to acute rejection [22]. Therefore, controlling T cells with indirect specificity by PLG-dAg similar to that seen with ECDI-SP is likely an important mechanism underlying the graft protection provided by this approach. Although few indirectly-activated T cells were observed after treatment with the blank PLG particles (Fig 4C), these T cells had not undergone a similar expansion/contraction process, likely leaving them completely naïve and fully competent to respond to the transplanted allografts later on as we have previously shown [11]. CD4⁺ T cells are established players in allograft rejection in part through their effects on CD8⁺ T cells [23]. However, clinical data has shown that post-transplant proliferation of CD4⁺ T cells themselves also correlate with decreased islet graft function [24]. This indicates the importance of CD4⁺ T cell modulation for tolerance induction, targeting both acute and chronic allograft rejection.

Finally, we note that the initial expansion of T cells induced by the PLG-dAg was less than that observed with ECDI-SP (Fig 4C). This difference in magnitude may be due to the difference in the dose of donor antigens delivered by these two methods. The PLG-dAg delivered ~85 µg of protein lysate per mg of particles, with 3 mgs particles administered per dose (i.e. ~255 µg of protein per dose). The delivery of 10⁸ ECDI-SP per dose of treatment corresponds to ~650 µg of protein, about 2.5 times greater than that delivered by PLG-dAg per dose of treatment. Lysate protein attachment to the particles was primarily due to ECDI conjugation, as substantially less protein was adsorbed in the absence of ECDI (data not shown). Previous reports with PLG particles formulated with PEMA had minimal adsorption of peptides to particles in the absence of crosslinking reagents [25]. It is possible that enhancing protein coupling to PLG particles or refining the lysate preparation technique

to improve protein yield might further enhance the tolerance efficacy by this method, an area that is currently under active investigation in our labs.

PLG-dAg particles were not effective at modulating the activation of T cells with direct donor specificity (Fig 4B). Donor passenger leukocytes stemming from the transplanted allografts are capable of priming T cells with direct donor specificity. Studies using cell depletion strategies have shown that activated CD4⁺ T cells with direct donor specificities can readily mediate islet graft rejection in the absence of other pathways of T cell activation [26], although a large number of T cells with such specificities may be required under this circumstance [27]. It is likely that both the direct and the indirect pathways of allorecognition contribute to allograft rejection. Their relative importance is likely dependent on the tissue/organ transplanted and the post-transplant time frame in which they are examined, with the direct pathway being the primary mode of T cell activation immediately after transplantation whereas the indirect pathway predominates at later times [28].

The incomplete modulation of T cell responses by the PLG-dAg particles led to the investigation of combining them with short-term rapamycin treatment. Rapamycin is an mTOR (mammalian target of rapamycin) inhibitor that is a widely used immunosuppressant in clinical transplantation. The addition of low dose peri-transplant rapamycin significantly increased the efficacy of PLG-dAg in allograft protection, an effect not observed with either PLG-dAg or rapamycin alone (Fig 5). Rapamycin inhibits proliferation of effector T cells through cell cycle arrest, and also selectively induces and expands Tregs [29–31]. These specific effects may account for the increased efficacy observed in our model whereby the initial action of directly-activated T cells is inhibited by rapamycin, while PLG-dAg mediate the deletion of indirectly-activated T cells and protect the allograft long-term. While reports have linked rapamycin to islet toxicity [32], studies reporting this effect used higher rapamycin dosage over longer periods of time (e.g. 0.2 mg/kg for 26 days [33] and 0.5–1.5 mg/kg for 14 days [34]) compared to our dosage of 0.1 mg/kg for 4 days only. This dose may not reach the threshold for islet damage, yet retain inhibitory effects on T cell activation. Additional potential benefits of rapamycin include inducing apoptosis in dendritic cells and monocytes [35,36], which may aid in reducing peri-transplant inflammation and improve overall graft function.

5. Conclusions

We demonstrate that PLG-dAg particles are a viable delivery mechanism for donor antigens capable of promoting long-term donor-specific allograft protection, and may provide a versatile platform for further functionalization and combination with different immunosuppressants and/or refined antigen source in the attempts to establish robust donor-specific transplant tolerance. Moreover, the pre-transplant infusion can be administered two days prior to transplant, which significantly improves the logistics of applying this therapy to human transplantation. The PLG-dAg particles are effective at modulating indirect T cell activation but less capable of modulating direct T cell activation. Furthermore, the addition of peri-transplant low dose rapamycin significantly improves the efficacy of PLG-dAg, and this combination therapy represents a clinically attractive method for inducing long-term,

donor-specific transplant tolerance for allogeneic islet cell transplantation. These particles have the potential to be functionalized, allowing them to serve as a platform to investigate mechanisms underlying tolerance induction or to facilitate personalized modifications based on the type of transplant and pathways that require modulation. This particle-based tolerance therapy would eliminate the need for life-long immunosuppression for current allogeneic cell and tissue transplantation, and could potentially enable the use of stem cell banks for numerous emerging allogeneic cell-based therapies.

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Fig. 1. PLG particle characterization and donor antigen coupling conditions. (A) Scanning Transmission Electron Micrograph (STEM) image of PLG particles prior to lysate coupling with ECDI. (B) PLG particle size (458.8 ± 14.8 nm) and surface ζ -potential (-72.5 ± 1.42 mV) distributions obtained using dynamic light scattering, $n = 3$. (C) The amount of donor cell lysate proteins coupled per mg of PLG particles was investigated by varying the amount of ECDI (10, 20, and 40 mg) and reaction volume (300 or 700 μl) in the coupling reaction, $n = 6$.

Fig. 2.

PLG-dAg infusions provide significant protection to the islet allograft in treated recipients. Diabetic B6 mice were injected with 3.0 mg BALB/c PLG-dAg or blank PLG on day -7 and day +1, and transplanted with BALB/c islets on day 0. Survival of the islet allografts was compared between PLG-dAg treated recipients and blank PLG treated recipients, $**p < 0.01$.

Fig. 3.

PLG-dAg and donor ECDI-SP have a similar distribution in injected recipients. (A) The distribution of PKH67⁺ cells in the spleen, heart, liver, and lung in recipients 18 h after the injection of PLG-dAg^{PKH67+} or donor ECDI-SP^{PKH67+}. (B) The distribution of I-A^bPKH67⁺ cells among F4/80⁺ macrophages, B220⁺ B cells, and CD11c⁺CD11b⁺, CD11c⁺B220⁺, CD11c⁺CD8a⁺ dendritic cell subsets in the spleen of the recipients injected with PLG-dAg^{PKH67+} or ECDI-SP^{PKH67+}. Data is average of three experiments, **p* < 0.05.

Fig. 4.

PLG-dAg modulate the indirect, but not direct, pathway of allorecognition. (A) Time line for 4C or TCR75 transgenic T cells adoptive transfers and subsequent spleen harvests for examination. 4C or TCR75 T cells were isolated by magnetic bead separation, labeled with CFSE, and injected into B6 mice on at 5×10^6 cells per mouse 1 day prior to the injection with either PLG-dAg or ECDI-SP. Mice were sacrificed 3 days or 7 days later, and the spleens were examined for proliferation (by CFSE dilution) of the 4C T cells (day 3) or TCR75 T cells (day 3 and day 7). (B) Left panel: 4C T cell proliferation in the spleen of mice 3 days after the injection of PLG-dAg or ECDI-SP. Right panel: *in vitro* anti-CD3/CD28 stimulated proliferation of 4C T cells retrieved from the spleen of mice injected with PLG-dAg. Unstimulated 4C T cells retrieved from the spleen of the same mice were used as control. (C) Left panel: TCR75 T cell proliferation in the spleen of mice 3 days after the injection of PLG-dAg or ECDI-SP. Mice injected with blank PLG particles were used as control. Right panel: TCR75 T cells demonstrated proliferation at day 3 followed by contraction at day 7 in response to the PLG-dAg or ECDI-SP injection. The y-axis shows the % of CD4⁺CD45.1⁺ TCR75 T cells among all live splenocytes. For the TCR75 studies, data is representative of one experiment (4 mice in each group). For the 4C studies, data is representative of three experiments (3 mice in each group).

Fig. 5.

A short course of low dose rapamycin synergizes with PLG-dAg to enhance tolerance efficacy. Mice receiving donor (BALB/c) PLG-dAg injections at days -7 and +1 in combination with a 4-day course (days -1, 0, +1, and +2) of low dose (0.1 mg/kg) rapamycin demonstrated significantly greater islet allograft survival ($n = 11$) compared with mice treated with rapamycin alone ($n = 9$), BALB/c PLG-dAg alone ($n = 17$), or PLG particles coupled with lysate proteins from a third party donor SJL/J ($n = 4$). $**p < 0.01$.

Fig. 6.

Pre-transplant PLG-dAg infusion can be administered on day -2 instead of day -7. Diabetic B6 recipients were treated with rapamycin on days -1, 0, +1, and +2 and the second dose of BALB/c PLG-dAg on day +1. The first dose of BALB/c PLG-dAg was administered either on day -7 or on day -2 ($n = 9$ for each group). BALB/c islet transplant was performed on day 0. Islet allograft survival was not statistically different between the day -7 group and the day -2 group, but significantly better than that of the recipients treated with blank PLG particles on day -2, $*p < 0.05$.