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Tolerance induction using nanoparticles bearing HY peptides in bone marrow transplantation

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Abstract

Allogeneic cell therapies have either proven effective or have great potential in numerous applications, though the required systemic, life-long immunosuppression presents significant health risks. Inducing tolerance to allogeneic cells offers the potential to reduce or eliminate chronic immunosuppression. Herein, we investigated antigen-loaded nanoparticles for their ability to promote transplant tolerance in the minor histocompatibility antigen sex-mismatched C57BL/6 model of bone marrow transplantation. In this model, the peptide antigens Dby and Uty mediate rejection of male bone marrow transplants by female CD4⁺ and CD8⁺ T cells, respectively, and we investigated the action of nanoparticles on these T cell subsets. Antigens were coupled to or encapsulated within poly(lactide-co-glycolide) (PLG) nanoparticles with an approximate diameter of 500 nm. Delivery of the CD4-encoded Dby epitope either coupled to or encapsulated within PLG particles prevented transplant rejection, promoted donor-host chimerism, and suppressed proliferative and IFN- γ responses in tolerized recipients. Nanoparticles modified with the Uty peptide did not induce tolerance. The dosing regimen was investigated with Dby coupled particles, and a single dose delivered the day after bone marrow transplant was sufficient for tolerance induction. The engraftment of cells was significantly affected by PD-1/PDL-1 costimulation, as

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blockade of PD-1 reduced engraftment by ~50%. In contrast, blockade of regulatory T cells did not impact the level of chimerism. The delivery of antigen on PLG nanoparticles promoted long-term engraftment of bone marrow in a model with a minor antigen mismatch in the absence of immunosuppression, and this represents a promising platform for developing a translatable, donor-specific tolerance strategy.

Keywords

tolerance; transplantation; PLG nanoparticles; bone marrow engraftment; PD-1 costimulation

Introduction

Specifically inactivating select immune cell populations is desirable for the development of therapies to prevent transplant rejection. Transplant rejection is mediated by a diverse array of donor antigens, many of which are major histocompatibility (MHC) antigens found on the surface of nucleated cells. In the clinic, transplant donor/recipient pairs are matched as closely as possible on MHC antigens in an attempt to delay transplant rejection. However, even among optimally matched pairs, rejection occurs due to minor histocompatibility (mH) antigens, such as proteins encoded on the male Y chromosome not recognized by females [1, 2]. Current approaches to prevent rejection in the clinic focus on systemic immunosuppression, putting patients at risk for opportunistic infections, malignancies, and other side effects of varying severity [3]. Chronic graft rejection and dysfunction are frequent even with long-term immunosuppressive therapies, with only 47–61% of grafts surviving past 10 years [4, 5].

Tolerance in allogeneic transplantation models offers the potential to avoid systemic immunosuppression and preserve function of the remainder of the immune system. Transplantation tolerance requires modulation of alloantigen-reactive T cells, and current strategies include the use of antibodies to block costimulatory pathways, expansion of regulatory T cells, and T or B cell depletion as well as other immunosuppressive drugs [6]. Peptides delivered intravenously have also been investigated for immune tolerance [7, 8]. More recently, we have demonstrated an antigen-specific tolerance approach in which apoptotic splenocytes or PLG nanoparticles are carriers for antigens (Ag) to induce long-term Ag-specific tolerance in models of autoimmune disease (R-EAE) and islet transplantation [9–11]. The association of peptide with the cells or particles can avoid complications associated with high concentrations of intravenous peptides and deliver the peptides to antigen presenting cells [12]. The involvement of splenic APCs, regulatory T cells, and PD1/PD-L1 costimulation have been implicated in particle-induced tolerance within autoimmune models, but the importance of these mechanisms are not clear in transplantation [9]. Additionally, transplant rejection typically involves both CD4+ T helper and CD8+ T effector cells, and a greater understanding of these subsets is needed towards delineating transplantation tolerance.

The induction of transplant tolerance and mechanisms of rejection were investigated using the well-characterized minor histocompatibility Ag sex-mismatched model of bone marrow

transplantation. In this model, transplanted C57BL/6 male bone marrow is recognized and rejected by female recipient mice in major MHC matched donor/recipient pairs. The peptides Dby and Uty have been identified in transplant rejection as mediated by CD4+ and CD8+ T cells, respectively, allowing the examination and separation of CD4+ and CD8+ T cell responses in transplant tolerance [13, 14]. These Hy peptide antigens were loaded onto or encapsulated within nanoparticles and investigated for their ability to promote engraftment of transplanted bone marrow. Furthermore, we investigated the efficacy of particles delivered at multiple time points relative to cell transplantation. In addition to determining the ability of peptide-loaded particles to tolerize CD4+ and CD8+ T cells, we investigated the relative role of PD-1/PD-L1 costimulation and regulatory T cells in the induction of tolerance.

Materials and Methods

PLG particle synthesis

Poly(lactide-co-glycolide) (PLG) particles were formed using a single emulsion technique with poly(ethylene-alt-maleic acid) (PEMA) as a surfactant as described in [10]. Briefly, a 20% w/v solution of PLG (50% D,L-lactide/50% glycolide) (Lactel Absorbable Polymers) dissolved in dichloromethane was sonicated (Cole-Parmer) in 1% w/v PEMA (Polysciences, Inc.) to produce particles. After overnight stirring, particles were collected by centrifugation, washed 3 times with water, and lyophilized overnight with 4% w/v sucrose and 3% w/v D-mannitol. PLG particles encapsulating peptide were fabricated using a double emulsion technique, where 50 mg/ml Dby, Uty, or OVA_{323–339} peptide was sonicated in 20% PLG dissolved in dichloromethane. This emulsion was then sonicated in 1% PEMA to produce particles that were collected as described above. The encapsulation efficiency and amount of peptide encapsulated was determined by quantifying protein from particles dissolved in 1 M NaOH using the BCA Protein assay (Thermo Scientific). Peptides Dby (NAGFNSNRANSSRSS), Uty (WMHHNMDLI), and OVA_{323–339} (ISQAVHAAHAEINEAGR) were obtained from Genemed Synthesis (San Antonio, TX).

Preparation of peptide-coupled particles

PLG particles (1.25 mg) were washed 3× in PBS prior to the coupling reaction. Particles were coupled to the CD4 epitope Dby, CD8 epitope Uty, or control OVA_{323–339} peptide in the presence of 20 mg/ml 1-Ethyl-3-(3' dimethylaminopropyl) carbodiimide, HCl (ECDI) (EMD Millipore Chemicals, Inc.) and 2 mg/ml peptide. After reacting for 1 hour, coupled particles were washed 3× in PBS to remove excess ECDI and resuspended in PBS. The coupling efficiency and amount of peptide coupled was determined by quantifying protein from particles dissolved in 1 M NaOH using the BCA Protein assay (Thermo Scientific).

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).

The amount of peptide released from peptide coupled or peptide encapsulated particles was investigated, with 10 mgs of PLG particles washed, resuspend in 1 ml PBS, and incubated at 37°C with agitation. At indicated time points, particles were centrifuged and peptide in the supernatant was measured using the micro BCA Protein assay (Thermo Scientific).

Tolerance induction

Peptide-coupled particles (Dby-PLG, Uty-PLG, or OVA₃₂₃₋₃₃₉-PLG) (1.25 mg) were administered i.v. 7 days before transplant (Day -7), 1 day after transplant (Day +1), or on both days relative to bone marrow transplant (Day 0). Particles containing encapsulated peptide — (Dby)PLG, (Uty)PLG, or (OVA₃₂₃₋₃₃₉)PLG — were administered i.v. (2.50 mg) on Day +1.

Mice

Six to eight week old CD45.1 male C57/BL6 mice, female CD45.1 C57/BL6, and female CD45.2 C57/BL6 mice were purchased from the Jackson Laboratory. All mice were housed under specific pathogen free conditions at Northwestern University and protocols were approved by Northwestern University IACUC.

Bone Marrow Transplantation

Female CD45.2 B6 recipients were treated with low-dose irradiation (200 cGy) one day before transplant (Day -1). Donor bone marrow from male or female CD45.1 B6 mice was flushed from the femur and tibia in RPMI media supplemented with 10% FBS and washed 3× in PBS. Recipients were administered 5×10^6 donor bone marrow cells i.v. in 0.2 mL PBS (Day 0). Mice were examined for engraftment by collecting tail peripheral blood samples for determination of donor engraftment as indicated by the presence of CD45.1+CD90.2+ lymphocytes. For blockade experiments, anti-PD-1 or control hamster IgG antibodies were administered i.p. on Days 0 (500 µg) and 2, 4, 6, 8, 10 (250 µg). Anti-CD25 or control rat IgG antibodies were administered i.p. on Days 0 and 2 (500 µg). Anti-PD-1 (J43), aCD25 (PC-61.5.3), and isotype control IgG antibodies were obtained from BioXCell.

Antibodies and FACS Analysis

Cell phenotype was measured by flow cytometry. Isolated cells were blocked using anti-mouse CD16/32 (eBioscience) for 30 min, stained with fluorochrome-conjugated antibodies for 30 min, and fixed with BD Cytfix/Cytoperm for 30 min. The following antibodies (clones) were used: CD45.1 PE-Cy7, CD90.2 (Thy1.2) APC, Foxp3 APC, CD4 FITC, CD25 PE, CD8a PE, CD4 PerCP, CD4 PE-Cy7, CD4 APC, CD4 eFluor 450, CD45.2 FITC. Live-dead discrimination was performed using LIVE/DEAD fixable staining reagents (Life Technologies) and intracellular staining for Foxp3 performed using Foxp3/Transcription Factor Staining Buffer Set (eBiosciences). Flow cytometry was performed using the FACSCanto II (BD Biosciences) with analyses by FlowJo v6.4.7 (TreeStar). All samples were gated on doublet, lymphocytes, and live cells.

T cell recall assays

Mice were sacrificed 10 days post transplantation and single cell suspensions from the spleen were cultured in 96-well plates and challenged with 1 μ M Dby or OVA_{323–339}. Anti-CD3 (2C11) stimulation was used as a control for proliferation and cytokine secretion. Samples were cultured in complete HL-1 media for 72 h. At 48 h, plates were pulsed with 1 μ Ci [³H]thymidine and harvested 24 h later for detection of uptake using a Topcount microplate scintillation counter. Culture supernatants were collected at 72 h and cytokine secretion was measured by Milliplex MAP multiplex assay (Millipore) and the results read on a Luminex Luminex microplate reader.

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. Students t-test or one way analysis of variance (ANOVA) with appropriate post hoc tests were used to determine statistical significance between groups. A value of probability (p) less than 0.05 was considered statistically significant.

Results

Coupling and encapsulation of Dby and Uty peptides to PLG particles

Initial studies characterized the PLG nanoparticles and the loading of male CD4 or CD8 encoded peptides, Dby and Uty respectively, onto or into the particles. Fabrication of particles using a single emulsion solvent evaporation method produced particles 447.2 ± 5.3 nm in size (Fig 1A–B) with a zeta potential of -44.7 ± 0.4 mV (n=3). Dby, Uty, or OVA_{323–339} (control) peptides were coupled to the particles using ECDI chemistry with coupling efficiencies ranging from 22–32% (Fig 1C). Peptide conjugation led to an increase in particle size relative to naked particles suggesting some particle aggregates developed, though no significant impact on the zeta potential was observed. The amount of peptide coupled was comparable between the three peptides and ranged from 9–13 μ g peptide coupled per mg of PLG particles (Fig 1C). *In vitro* release studies demonstrated low levels of peptide released at 2 hours for Dby and Uty coupled particles (2% and 25%, respectively), which increased by 48 hours (10% and 50%) (Fig 1D).

Peptide-encapsulated particles were similarly sized to unmodified particles, ranging from 400 to 500 nm (Fig 2A–C). Encapsulation efficiencies ranged from 2–8%, with lower amounts of peptide delivered per mg of particles (1 – 2.5 μ g peptide per mg of PLG particles) (Fig 1C). Approximately half of the loaded peptide was released from (Dby)PLG and (Uty)PLG by 2 hours *in vitro*, with most of the peptide released by 24 hours (Fig 2D).

Treatment with Dby-PLG promotes long-term survival of male bone marrow grafts

Subsequent studies investigated the potential of antigen-coupled particles to induce chimerism, or tolerance, when delivered on the surface of PLG particles. Dby coupled particles, denoted as Dby-PLG, were injected intravenously to female recipient mice on Days -7 and +1 pre- and post- male bone marrow transplantation. These days were chosen in reference to Luo *et al.*, who demonstrated that Day -7 and +1 doses of ECDI fixed apoptotic

splenocytes induced tolerance in allogeneic islet transplantation [15]. Mice received a female bone marrow transplant as a positive control or a male bone marrow transplant (with either PBS or OVA₃₂₃₋₃₃₉-PLG sham particle injections) as negative controls. Chimerism was evaluated through the percentage of donor T cells (CD45.1+CD90.2+) in peripheral blood samples of female (CD45.2+) recipients.

Dby-PLG recipients demonstrated tolerance to male grafts with approximately 40% chimerism by week 10 post transplant measured from peripheral blood samples (Fig 3A). These levels were consistent with our positive control (mice receiving female bone marrow, which should not be rejected by female recipients). OVA₃₂₃₋₃₃₉-PLG mice demonstrated low levels of chimerism at early time points post transplant, slowly diminishing to 1% engraftment by week 16 (Fig 3B). Therefore, Dby-PLG injections on Days -7 and +1 induced tolerance to male bone marrow grafts as shown by sustained levels of male donor CD90.2+ cells in peripheral blood samples.

In vitro recall responses of splenocytes from naive, Dby-PLG, OVA₃₂₃₋₃₃₉-PLG, and female bone marrow transplanted mice were assessed 10 days post transplantation. [³H]thymidine incorporation and IFN- γ secretion was measured 3 days after stimulation with Dby peptide, OVA₃₂₃₋₃₃₉ peptide (negative control), or anti-CD3 (positive control). Upon stimulation with the Dby peptide, proliferation was suppressed in Dby-PLG mice compared to OVA₃₂₃₋₃₃₉-PLG mice (Fig 3C). Secretion of IFN- γ was also markedly reduced in response to Dby stimulation in Dby-PLG treated mice compared to sham tolerized OVA₃₂₃₋₃₃₉-PLG mice (Fig 3D). These *in vitro* assays further demonstrate that Dby-PLG mice are tolerized to the Dby peptide, as evidenced by reduced proliferation and IFN- γ secretion upon challenge with the Dby peptide.

Optimal timing of Dby-PLG doses and required dosage

We next investigated the timing of doses (whether pre- or post-transplant) by injecting particles on Day -7 and +1 as previously described, Day -7 only, or Day +1 only. Mice receiving Dby-PLG on Day -7 only had reduced engraftment, with the level of chimerism peaking at 20% at week 12 and declining slightly by week 20 (Fig 4A). Conversely, mice receiving Dby-PLG on Days -7 and +1 or Day +1 alone demonstrated higher levels of engraftment, increasing to greater than 40% by week 20. The Day +1 dose, whether alone or in combination with a dose on Day -7, conferred similar levels of chimerism in recipient mice. Interestingly, the Day -7 dose alone reduced the level of engraftment; this may be due to the sublethal dose of irradiation on Day -1, which could remove immune cells responsible for tolerance induction before transplant. OVA₃₂₃₋₃₃₉-PLG mice did not show significant chimerism regardless of the number and timing of doses as expected. In subsequent studies, only a Day +1 dose was given.

A titration of particles was used to evaluate the amount of peptide necessary for tolerance induction. Dby-PLG were administered on Day +1 at doses of 1.25 mg (1 $\times$), 0.125 mg (0.1 $\times$), and 0.0125 mg (0.01 $\times$), which were tested alongside a control of OVA₃₂₃₋₃₃₉-PLG (1 $\times$). Based on coupling efficiencies (Fig 1C) for Dby-PLG, the 1 $\times$, 0.1 $\times$, and 0.01 $\times$ doses correspond to delivery of approximately 15, 1.5, and 0.15 μ g of Dby peptide, and the 1 $\times$ dose of OVA₃₂₃₋₃₃₉-PLG corresponds to delivery of approximately 16 μ g of OVA₃₂₃₋₃₃₉.

At week 16 post transplant, Dby-PLG 1× mice showed ~45% engraftment, which was significantly greater than the ~25% engraftment seen with Dby-PLG 0.1× mice and 0% engraftment in Dby-PLG 0.01× mice (Fig 4B). Therefore, delivering ~1.5 µg of Dby on Day +1 is sufficient to induce significant engraftment in recipients.

Treatment with (Dby)PLG promotes long-term survival of male bone marrow grafts

The method of antigen loading into the particle was subsequently investigated through encapsulation of Dby into the PLG particles, as encapsulation may more easily allow the use of extracts of donor histocompatibility antigens to be employed for tolerance induction. Dby encapsulated particles, denoted as (Dby)PLG, were injected intravenously to female recipient mice on Day +1. Note that due to the lower payload of peptide encapsulated compared to surface coupled, mice receiving double emulsion particles were administered 2.5 mg PLG particles as opposed to 1.25 mg of coupled particles, which corresponds to a dose of 6.3 µg of peptide. A female bone marrow transplant again served as a positive control, and a male bone marrow transplant treated with (OVA₃₂₃₋₃₃₉)PLG served as a negative control. Similar to peptide coupled particles, (Dby)PLG induced robust tolerance at levels similar to the female positive control group, where the level of donor cells was 49% by Week 20 (Fig 5).

PD-1 blockade reduces the tolerogenic effects of Dby-PLG

Peptide coupled particles were more efficient at inducing tolerance (i.e., required less peptide) than particles encapsulating peptide; thus, subsequent studies investigating tolerance mechanisms were performed using Dby-PLG. The involvement of the PD1/PD-L1 negative costimulatory pathway was assessed in Dby-PLG tolerized mice by administering an anti PD-1 antibody or control hamster IgG antibody. At week 7 post transplant, Dby-PLG mice receiving the IgG antibody displayed ~23% engraftment, whereas Dby-PLG mice receiving the PD-1 blockade displayed only ~11% engraftment (Fig 6). This significant reduction in the level of chimerism indicates that the PD1/PD-L1 costimulatory pathway is involved in the induction of tolerance by Dby-PLG particles, yet alternative pathways must also be involved given the presence of engrafted cells.

Natural Tregs (nTregs) are not necessary for tolerance induced by Dby-PLG

The requirement for regulatory T cells was also investigated in tolerance induction. The numbers of Tregs within tolerized heart allografts has been shown to increase from day 7 to day 30 [16], thus, the numbers of nTregs both within the bone marrow graft and spleen were determined in Dby-PLG tolerized mice and appropriate controls at Day 34. No difference in the percentage of CD4+CD25+Foxp3+ nTregs was found between groups in either tissue (Fig 7). To evaluate the necessity of CD4+CD25+Foxp3+ Tregs in the initiation of tolerance, Dby-PLG tolerized mice were given either an anti-CD25 antibody (PC61) to deplete/inactivate Tregs or control rat IgG antibody one day before and one day after (Days 0 and 2) the administration of particles on Day +1, and the level of engraftment was assessed at week 7 post transplant. This dose and regimen (injections before and after tolerance induction) has been shown to significantly reduce Tregs in the spleen, peripheral lymph nodes, and draining lymph nodes of the transplanted tissue [15]. No significant differences were observed between the Dby-PLG mice with functional or impaired Tregs, suggesting

that conventional CD4+CD25+Foxp3+ Tregs are not involved in the initiation of tolerance in this sex mismatched transplant model (Fig 7B–C).

Uty-PLG or (Uty)PLG does not promote tolerance

The CD8 T cell-dependent Uty peptide was investigated for its ability to induce tolerance to bone marrow grafts when delivered either surface coupled or encapsulated within PLG particles. Neither delivery on the surface or encapsulated resulted in engraftment of male bone marrow grafts (Fig 8A–B). Additionally, to evaluate whether these peptides could act synergistically towards inducing tolerance, one group received 1.25 mg (Dby)PLG and 1.25 mg (Uty)PLG combined. Mice receiving half (Dby)PLG and half (Uty)PLG displayed slightly lower levels of engraftment at 21% at Week 12 compared to a full dose of (Dby)PLG, which allowed 30% engraftment. Because (Uty)PLG alone did not support engraftment, engraftment in the combined group is likely due to the half dose of (Dby)PLG.

Discussion

In this report, we demonstrated that intravenous injection of the CD4 epitope Dby delivered either surface coupled or encapsulated within PLG particles induced antigen-specific tolerance to male bone marrow grafts (Fig 3,5). Both pre- and post-transplant doses of Dby-PLG (alone or combined) induced robust tolerance, with mice exhibiting higher levels of engraftment when receiving Dby-PLG on Day +1 post transplant (Fig 4A). In a clinical setting, a single dose near the time of transplant would be a favorable alternative to a multiple dose, longer regimen.

We demonstrated a minimal peptide dose equal to 1.5 µg to promote engraftment, which was delivered by intravenous administration of surface coupled Dby peptide (Fig 4B). This dose is substantially lower than the 100 µg of soluble Dby delivered on 3 consecutive days intranasally for a total of 300 µg in Hya mismatched bone marrow transplantation [17], indicating that the particle platform has a significantly greater efficiency. Encapsulated Dby at a dose of 2.5 mg, corresponding to 6.3 µg peptide, also induced long-term tolerance (Fig 5A). Though a greater amount of attached Dby was released *in vitro* by encapsulated Dby compared to coupled Dby, both modifications induced tolerance (Fig 1D, 2D). PLG particles injected intravenously accumulated most significantly within the liver, and to a lesser extent the spleen and the lung (Fig S1). Although conjugating peptide to the particles led to some particle aggregates, our results [11] and other literature [18] suggests that these particles would distribute similarly to the liver and spleen. Administering high levels of soluble peptide has successfully induced tolerance in transplant and autoimmune disease models [17, 19, 20], however, variable side effects such as exacerbation of disease and fatal anaphylaxis were occasionally observed [12, 21]. Due to these safety concerns, coupling peptides to nanoparticles or splenocytes, or encapsulation within nanoparticles is an attractive strategy to avoid high concentrations of peptide in the vasculature. Antigen-coupled splenocytes have been used for tolerance in models of autoimmune disease and allogeneic cell transplantation; however, the use of cells as carriers for antigen may be challenging to translate due to the relative expense and variability in cell handling. Synthetic particles offer a platform for antigen delivery that is relatively easy to produce at low cost.

The negative costimulatory PD-1/PD-L1 pathway between T cells and APCs was implicated in tolerance using peptide-loaded nanoparticles in this bone marrow model. The PD-1/PD-L1 pathway inhibits T cell responses and plays an important role for tolerance induction and maintenance in a number of transplant and autoimmune models [22–26]. In full MHC mismatched heart transplantation, PD-1 and PD-L1 blockade accelerates rejection of cardiac allografts [24, 27] and early blockade of PD-L1 has been shown to prevent tolerance induction [27]. Blockade of PD-1 or PD-L1 has also been shown to accelerate skin and liver rejection [23, 25, 28]. We have previously demonstrated that PD1/PD-L1 interactions are essential for tolerance induction by antigen-coupled splenocytes in R-EAE [9, 22]. Interestingly, in this minor Ag mismatched model of BMT, blockade of PD-1/PD-L1 interactions reduced, but did not eliminate, the extent of chimerism (Fig 6A–B). This regulatory pathway is clearly involved in tolerance, yet other pathways likely contribute to the induction of tolerance, such as ligation of the negative regulator CTLA-4 or the generation of tolerogenic DCs with reduced activation markers.

Natural CD4+CD25+Foxp3+ Tregs have been associated with tolerance in both autoimmune and transplant models [15, 16, 29], though blocking their activity did not significantly impact the extent of chimerism in this bone marrow model. In allogeneic skin grafts, Tregs have the capacity to prevent transplant rejection and act at the graft itself as well as the spleen [29]. In minor antigen sex mismatched transplants, Chai *et al.* suggests that a population of CD4+ regulatory cells is involved with the maintenance of long-term tolerance, shown by the presence of functional antigen specific effector CD8+ T cells in tolerant recipients [17]. Similarly, tolerance induced by ECDI-fixed splenocytes to allogeneic islet grafts was partially blocked when nTregs were impaired using anti-CD25 at the time of tolerance induction, with Foxp3+ Tregs present in the graft and spleen of tolerized recipients [15]. These studies collectively indicate the involvement of Tregs in the initiation of tolerance to allogeneic grafts in these models. Herein, blocking nTregs did not have an effect on chimerism and the level of Tregs present in tolerized recipients was similar between experimental groups and rejecting controls (Fig 7A–C).

A possible explanation could be that the dominant tolerance mechanism, rather than the induction of Tregs, is the induction of anergy secondary to the generation of tolerogenic DCs that uptake Dby-PLG and fail to prime an effector response to initiate graft rejection. Similarly, Chai *et al.* found that in minor Ag sex mismatched skin grafts, Dby-specific CD4+ T cells from tolerant mice failed to produce IL-2, suggesting that these cells were not activated by DCs to reject the grafts and display an anergic phenotype [17]. It is also possible that other Foxp3- regulatory T cells, *e.g.* IL-10-producing Type 1 regulatory (Tr1) T cells [30], are involved in minor Ag tolerance induced by particles encapsulating antigen, which is consistent with our recent finding that IL-10-producing Tr1 cells are induced by PLG particles encapsulating myelin peptides in the R-EAE model of MS (unpublished).

Though tolerizing with the CD4 encoded Dby peptide was able to promote chimerism, particles loaded with the CD8 encoded Uty peptide did not promote engraftment (Fig 8). Both CD4+ T helper cells and CD8+ T effector cells are capable of recognizing alloantigens and play a role in the rejection process. Traditionally, CD4+ cells are accepted as coordinating the adaptive immune response by activating cytolytic T cells and secreting

cytokines while CD8+ T cells induce apoptosis and cytolysis dependently or independently of CD4+ T cell help [31–34]. However, overlap or redundancy was observed between these roles depending on the particular transplant model, such as instances where CD4+ T cells assume effector roles in mediating rejection [35]. Using split thickness tail grafts, we previously reported that splenocytes coupled to the CD4 epitope Dby resulted in graft protection, while splenocytes coupled to the CD8 epitope Uty did not confer graft survival [13]. Similarly, Chai *et al.* induced tolerance with Dby administered intranasally to both male bone marrow and skin grafts, implying that CD4 T cells are the primary mediators of rejection [17].

Paradoxically, several studies have shown tolerance induced with the CD8 epitope Uty in skin transplantation, and in fact show rapid graft rejection when using Dby [17, 36]. These differences between the effectiveness when using the Dby and Uty peptides may arise from the delivery route of the peptide, such as intranasal or intravenous, as the peptide likely encounters distinct subsets of immune cells in the mucosa (intranasal delivery) as opposed to liver or spleen (intravenous delivery). Also, whether the peptide is administered as a soluble peptide or delivered through a vehicle (*i.e.* PLG particles) may involve varying cellular uptake and processing pathways. We show that delivery of the CD4 Dby epitope via two methods, surface coupled and encapsulated to PLG particles, induced tolerance in bone marrow transplantation, which is consistent with previous work in autoimmune R-EAE models delivering CD4 epitopes on PLG particles for tolerance induction [9, 10].

Conclusions

We demonstrate the induction of antigen specific tolerance by a single dose of the CD4 epitope Dby surface coupled to or encapsulated within PLG particles delivered one day after a minor antigen sex mismatched bone marrow transplant, whereas delivery of the CD8 epitope Uty did not promote engraftment. Delivering 1.5 µg of the Dby peptide intravenously was sufficient to induce tolerance, which is 200 times less than the amount of intranasal soluble Dby previously reported for tolerance induction. It is likely that this strategy invokes multiple, redundant tolerance mechanisms, as PD-1 blockade lowered the level of chimerism but did not completely abrogate tolerance. Contrary to many transplantation models, impairment of CD25+Foxp3+ nTregs did not block tolerance, indicating that these cells are not required for tolerance in this model. Using PLG particles as a platform for inducing tolerance in minor HY antigen mismatched bone marrow transplantation is a step towards developing a translatable, donor-specific tolerance strategy for modern transplantation procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Coupling of peptides to PLG nanoparticles

(A) Scanning transmission electron micrograph of a PLG nanoparticle demonstrating 500 nm diameter. (B) Size distribution of single emulsion particles measured by dynamic light scattering. (C) Coupling efficiencies and amount of Dby, Uty, and OVA₃₂₃₋₃₃₉ peptides conjugated to the surface of PLG nanoparticles as calculated by the BCA Protein Assay, $n=3$ per peptide. (D) Release of peptide from coupled particles as a percentage of total loading, $n=3$ per peptide.

Figure 2. Encapsulation of peptides within PLG nanoparticles

Size distribution of double emulsion particles encapsulation Dby (**A**) or Uty (**B**) measured by dynamic light scattering. (**C**) Encapsulation efficiencies, amount of peptide encapsulated within PLG nanoparticles, and size and zeta potential of double emulsion particles, $n=3$ per peptide. (**D**) Release of peptide from encapsulated particles as a percentage of total loading, $n=3$ per peptide.

Figure 3. Dby-PLG induces tolerance to male bone marrow grafts

(A) Injection of Dby-PLG on Days -7 and +1 relative to Day 0 bone marrow transplant induces tolerance to donor cells in recipient mice compared to PBS or OVA₃₂₃₋₃₃₉-PLG injection, n=5 per condition. (B) At Week 16 post transplant, mice receiving Dby-PLG on Days -7 and +1 demonstrate chimerism compared to mice receiving PBS or OVA₃₂₃₋₃₃₉-PLG, n=5 per condition. (C) *In vitro* recall responses of splenic T cells from naive and Dby-PLG tolerized mice were determined 10 days post transplantation upon stimulation with anti-CD3 (clone 2C11, positive control), OVA₃₂₃₋₃₃₉ (negative control), and Dby peptides by [³H]thymidine incorporation and IFN-γ secretion (D). Proliferative and IFN-γ responses were suppressed in Dby-PLG tolerized mice upon challenge with the Dby peptide, n=3 per condition, pooled samples.

Figure 4. Administration of Dby-PLG on Day +1 only induces tolerance

(A) Injection of Dby-PLG on Days -7 and +1 or Day +1 alone relative to Day 0 bone marrow transplant induces tolerance to donor cells in recipient mice compared to PBS or OVA₃₂₃₋₃₃₉-PLG injection. Mice tolerized with Dby-PLG on Day -7 alone demonstrated lower levels of engraftment, n=5 per condition. (B) At Week 16 post transplant, mice receiving the full 1× dose of Dby-PLG on Day +1 (1.25 mg) or 0.1× dose (0.125 mg) demonstrate chimerism compared to mice receiving OVA₃₂₃₋₃₃₉-PLG, n=5 per condition. Mice receiving a female bone marrow transplant serve as a positive control (n=10), * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 5. Dby encapsulated within PLG nanoparticles induces tolerance

(A) Injection of (Dby)PLG on Day +1 relative to Day 0 bone marrow transplant induces tolerance to donor cells in recipient mice compared to (OVA₃₂₃₋₃₃₉)PLG injection, n=5 per condition. Mice receiving a female bone marrow transplant serve as a positive control, n=10.

(B) At Week 20 post transplant, mice receiving (Dby)PLG demonstrate chimerism compared to mice (OVA₃₂₃₋₃₃₉)PLG, n=5 per condition.

Figure 6. PD-1 blockade reduces engraftment and the tolerogenic effect of Dby-PLG (A–B) At Week 7 post-transplant, Day +1 injection of Dby-PLG + anti PD-1 Ab (n=6) reduces the level of chimerism compared to Day +1 injection of Dby-PLG + control hamster IgG (n=6), * p < 0.05. Mice received 500 ug antibody treatment on Day 0 and 250 ug on Days 2, 4, 6, and 8 relative to Day 0 bone marrow transplant. Positive controls received female bone marrow on Day 0 (n=5) and OVA₃₂₃₋₃₃₉-PLG mice received male bone marrow on Day 0 and particles on Day +1 (n=5).

Figure 7. Natural regulatory T cells are not necessary for tolerance

(A) The percentage of CD4+CD25+Foxp3+ Tregs were similar between female positive controls, PBS negative controls, Dby-PLG tolerized mice, and OVA₃₂₃₋₃₃₉-PLG mice in both bone marrow grafts and spleens taken at 5 weeks post-transplant, n=5. (B–C) At Week 7 post-transplant, Day +1 injection of Dby-PLG + anti CD25 Ab (n=5) does not impact the level of chimerism compared to Day +1 injection of Dby-PLG + control rat IgG (n=5). Mice received 500 ug antibody treatment on Days 0 and 2 relative to Day 0 bone marrow transplant. Positive controls received female bone marrow on Day 0 (n=5) and OVA₃₂₃₋₃₃₉-PLG mice received male bone marrow on Day 0 and particles on Day +1 (n=5).

Figure 8. Delivery of Uty by PLG nanoparticles does not induce tolerance to bone marrow grafts (A–B) At Week 12 post transplant, mice receiving Uty-PLG or (Uty)PLG do not demonstrate chimerism compared to mice receiving (Dby)PLG, n=5 per condition, ** p < 0.01.