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Tolerogenic Ag-PLG nanoparticles induce tregs to suppress activated diabetogenic CD4 and CD8 T cells

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ABSTRACT

Type 1 diabetes (T1D) is mediated by destruction of pancreatic β cells by autoantigen-specific $CD4^+$ and $CD8^+$ T cells, thus the ideal solution for T1D is the restoration of immune tolerance to β cell antigens. We demonstrate the ability of carboxylated 500 nm biodegradable poly(lactide-*co*-glycolide) (PLG) nanoparticles PLG nanoparticles (either surface coupled with or encapsulating the cognate diabetogenic peptides) to rapidly and efficiently restore tolerance in NOD.SCID recipients of both activated diabetogenic $CD4^+$ BDC2.5 chromagranin A-specific and $CD8^+$ NY8.3 islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific TCR transgenic T cells in an antigen-specific manner. Further, initiation and maintenance of Ag-PLG tolerance operates via several overlapping, but independent, pathways including regulation via negative-co-stimulatory molecules (CTLA-4 and PD-1) and the systemic induction of peptide-specific Tregs which were critical for long-term maintenance of tolerance by controlling both trafficking of effector T cells to, and their release of pro-inflammatory cytokines within the pancreas, concomitant with selective retention of effector cells in the spleens of recipient mice.

1. Introduction

Type 1 diabetes (T1D), the most frequently observed chronic disorder found in children, is an autoimmune disease that develops as a consequence of failed peripheral immune tolerance, resulting in the specific destruction of insulin producing pancreatic β cells by autoreactive $CD4^+$ and $CD8^+$ T cells [1]. The loss of pancreatic β cells renders the host incapable of regulating normal glucose metabolism, which if left unchecked, culminates in death [1]. Current treatment therefore, focuses on the life saving provision of insulin, however insulin therapy is laborious, requiring constant patient blood glucose monitoring, life-long insulin administration, and is associated with serious complications. As such there is a need for more elegant therapeutic approaches in T1D.

The ideal solution to treat T1D is the restoration of immune tolerance, prior to significant β cell loss. Treatments employing broad-spectrum immune suppression have shown only moderate efficacy in preventing/treating T1D in animal models and human clinical trials [2,3]. In addition, these approaches lack long-term efficacy and are associated with numerous deleterious events including cytokine release syndrome, and increased incidence of infections and neoplasias. To date, therapeutic attempts to induce Ag-specific tolerance to overcome side effects associated with broad-based immune suppression, including the administration of soluble islet Ags such as insulin, glutamate decarboxylase 65 (GAD65), or heat shock protein 60 (HSP60) by various routes have

Abbreviations: Ag-PLG, antigen-coupled PLG nanoparticles; Ag-SP, antigen-coupled apoptotic splenocytes; APCs, antigen presenting cells; ChgA, chromogranin A; IGRP, islet specific glucose 6 phosphatase catalytic subunit related protein; PEMA, 2-ethyl-2-phenylmalonamide monohydrate; PLG, poly(lactide-*co*-glycolide) nanoparticles; PLG(Ag), antigen-encapsulating PLG nanoparticles; TCR, T cell receptor; T1D, type 1 diabetes; TIMP, tolerogenic immune-modifying particles; Treg, regulatory T cells.

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failed to show efficacy in large clinical trials [3]. A complete understanding of why these therapies failed is unclear. However, it is significant that we and others have shown that the subcutaneous route of Ag administration primarily employed in T1D tolerance trials is not adequately armed to induce immune tolerance without add on immunotherapy. We previously demonstrated the ability of i.v., but not s.c., administered Ag-conjugated apoptotic leukocytes (Ag-SP) to induce protective tolerance for the prevention *and* treatment of established of murine Th1/Th17-mediated autoimmune disease models of MS [4] and T1D [5], as well as Th2-mediated murine models of allergic airway inflammation and food allergy [6]. Antigen-conjugated autologous leukocytes which induce specific tolerance by two synergistic mechanisms, T cell intrinsic anergy and the activation of Tregs [4], have interestingly shown initial promise in clinical trials of MS patients [7]. However, the need for autologous cells to be isolated and manipulated prior to each treatment is costly, complex, and therefore limits the broad clinical application this tolerogenic treatment especially for treating adolescents at risk for T1D.

The development of shelf-stable, tolerance-inducing Ag carriers manufactured under GMP conditions represents the next generation of Ag-specific medicine. To this end, we have recently pioneered the use of i.v. infusion of Ag-associated carboxylated biodegradable poly(lactide-*co*-glycolide) nanoparticles (Ag-PLG), also termed tolerogenic immune-modifying particles (TIMP) for the safe and efficient induction of tolerance to prevent and treat Th1/Th17 EAE and Th2 allergic airway disease [8–11]. Mechanistic studies have shown that Ag-PLG are internalized by splenic marginal zone macrophages and liver phagocytic cells via scavenger receptors, such as macrophage receptor with collagenous structure (MARCO) [8]. While we have determined that ligation of these scavenger receptors triggers particle uptake and tolerogenic representation of the cargo antigen resulting in the upregulation of negative-co-stimulation pathways, release of regulatory cytokines and induction of Ag-specific regulatory T cells (Tregs), the precise contribution of each factor to tolerance induction remains to be fully defined.

Our previous report employing tolerance induced by Ag-SP in the spontaneously arising NOD mouse model of T1D [5], showed that T cell responses to the insulin $B_{9\text{-}23}$ epitope were dominant in young mice, but that epitope spreading to additional epitopes occurred as mice progressed to overt hyperglycemia. Given that development and progression of T1D in NOD mice and humans has been attributed to immune responses to numerous CD4 and CD8 T cell epitopes expressed on insulin, proinsulin, GAD65/67, islet specific glucose 6 phosphatase catalytic subunit related protein (IGRP), Islet antigen-2 (IA-2), phogrin (IA-2β), chromogranin A (ChgA), zinc transporter 8 (ZnT8), and vasostatin-1 [12–15], as well as hybrid β cell protein epitopes [16], it is critical to determine the efficacy of Ag-PLG treatment to restore tolerance in activated effector diabetogenic $CD4^+$ and $CD8^+$ T cells and to define the precise effector mechanisms responsible for tolerance induction and maintenance. Employing adoptive transfer models of T1D induced by the transfer of activated diabetogenic $CD4^+$ BDC2.5 chromagranin A-specific [17] and $CD8^+$ NY8.3 IGRP-specific [18] TCR transgenic T cells, we demonstrate the ability of PLG nanoparticles (either surface coupled with or encapsulating the cognate diabetogenic peptides) to rapidly and efficiently restore tolerance in NOD.SCID recipients of both activated $CD4^+$ and/or $CD8^+$ T cells in an antigen-specific manner. Further, Ag-PLG-induced peripheral tolerance initiation and maintenance were demonstrated to operate via several overlapping, but independent pathways including regulation via negative-co-stimulatory molecules (namely CTLA-4 and PD-1) and the systemic induction of peptide-specific Tregs. The net result of the tolerance therapy was inhibition of both trafficking of effector diabetogenic cells to the pancreas, as well as the release of pro-inflammatory cytokines via selective retention of effector cells within the spleens of recipient mice. These results clearly demonstrate the unique ability of Ag-PLG-induced tolerance to halt/reverse β cell destruction in mice with high numbers of activated effector diabetogenic $CD4^+$ and $CD8^+$ T cells.

2. Materials and methods

2.1. Mouse strains

Female NOD/MrkTac mice were purchased from Taconic Farms (Germantown, MD). BDC2.5 TCR transgenic and NOD.SCID mice were purchased from Jackson laboratories (Bar Harbor, ME). NY8.3 TCR transgenic mice were obtained from Dr. Pere Santamaria (Univ. of Calgary) and bred in the Northwestern University Center for Comparative Medicine. All mice were housed under SPF conditions and maintained according to protocols approved by the Northwestern University Animal Care and Use Committee.

2.2. Antibodies

Monoclonal antibodies anti-CD25 (PC61.5), anti-PDL-1 (10F.9G2), anti-CTLA-4 (CD152) and anti-ICOS (7E.17G9) and their respective isotype control antibodies were purchased from Bio X Cell (West Lebanon, NH). The following antibodies along with their respective isotope controls were purchased from BD Biosciences, eBiosciences and/or Biolegend: V500 conjugated anti-CD4, PEcy7 conjugated anti-CD90.2 (53-2-1), PE conjugated anti-ICOS (C398.4A), Brilliant Violet 421 conjugated anti-PD-1 (J43), APC conjugated anti- Foxp3 (FJK-1ba), Brilliant Violet 421 conjugated anti-TNF-α (MP6-XT22), AlexaFluor700 conjugated anti-IFN-γ (XMG1.2), PE conjugated anti-IL-2 (JES6-5H2), FITC conjugated anti- GM-CSF (MP1-22E9) and efluor 450 conjugated anti-Ki67 (SolA15).

2.3. Peptides

BDC2.5 mimetope 1040-31 (p31) (YVRPLWVRME), NY8.3 mimetope NRPA7 (KYNKANAFL), and the linked p31-NRPA7-$InsB_{9\text{-}23}$ (YVRPLWVRMEGAVVRGAKYNKANAFLGAVVRGASHLVEA-LYLVCGERG) peptides were purchased from Anaspec (Fremont, CA). $MOG_{35\text{-}55}$ (MEVGWYRSPSRVVHLYRNGK) was purchased from Genemed Synthesis (San Francisco, CA).

2.4. Activation and adoptive transfer of BDC2.5 and NY8.3 T cells

BDC2.5 and NY8.3 TCR Tg lymphocytes were harvested and pooled from spleen and brachial, axillary, mesenteric and pancreatic lymph nodes. The cells were activated *in vitro* with 0.5 μM of p31 or NRPA7 peptide in complete RPMI (Gibco) containing 5×10^{-5} M β-2-ME (Gibco), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (Gibco), 0.1 M nonessential amino acids (Gibco), and 10% fetal bovine serum (FBS) at a final concentration of 1×10^6 cells/ml in 96 well, round bottom plates. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and harvested after 96 h and washed, and $5–10 \times 10^6$ viable T cells were transferred i.v. to 8–12 week old NOD.SCID recipients.

2.5. Preparation and tolerance induction with Ag ECDI-fixed splenocytes, Ag-PLG and PLG(Ag) nanoparticles

For Ag-SP tolerance, single cell suspensions of erythrocyte-free splenocytes harvested from donor NOD mice in PBS were coupled with peptide (1 mg/ml) using ECDI (150 mg/ml) (Calbiochem) on

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