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Generation of antigen-specific Foxp3 + regulatory T-cells *in vivo* following administration of diabetes-reversing tolerogenic microspheres does not require provision of antigen in the formulation

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Abstract We have developed novel antisense oligonucleotide-formulated microspheres that can reverse hyperglycemia in newly-onset diabetic mice. Dendritic cells taking up the microspheres adopt a restrained co-stimulation ability and migrate to the pancreatic lymph nodes when injected into an abdominal region that is drained by those lymph nodes. Furthermore, we demonstrate that the absolute numbers of antigen-specific Foxp3+ T regulatory cells are increased only in the lymph nodes draining the site of administration and that these T-cells proliferate independently of antigen supply in the microspheres. Taken together, our data add to the emerging model where antigen supply may not be a requirement in "vaccines" for autoimmune disease, but the site of administration – subserved by lymph nodes draining the target organ – is in fact critical to foster the generation of antigen-specific regulatory cells. The implications of these observations on "vaccine" design for autoimmunity are discussed and summarized.
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1. Introduction

Type 1 diabetes mellitus (T1D) is an autoimmune disease where a heterogeneous population of leukocytes, mainly T-cells, progressively impairs and eventually destroys a

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critical mass of pancreatic endocrine insulin-producing beta cells [1,2]. Most of the mechanistic studies that underpin a significant aspect of etiopathogenesis as well as preventive and active "intent-to-treat" new-onset disease have been conducted in the non-obese diabetic (NOD) mouse strain which spontaneously develops diabetes of an autoimmune nature, even though there is a respectful gravity of data highlighting critical differences in the disease course and possible therapeutic targets between the NOD strain and humans [3]. While prophylaxis from disease has long-been considered an attractive option to decrease the incidence T1D in subjects at very high risk (concordance of high-risk genetics, presence of T1D autoantibodies, impaired glucose tolerance, first-degree relation to existing patients) [4,5], the practicality for this approach remains in doubt since not even combinations of high-risk biomarkers are predictive to a level where confidence can be instilled for a population scale primary intervention [5–8]. Instead, almost all of the therapeutic strategies in the past decade have aimed to preserve residual beta cell function and mass as early as possible following the onset of disease [8]. Despite some success in prolongation of the insulin-free period, all the approaches eventually required a return to pharmacologic insulin replacement and more importantly, a variety of adverse events associated with almost all of these approaches, largely biologics, decreases patient enthusiasm to enroll and be retained in these clinical trials [7,9,10].

Among all the prophylactic and therapeutic approaches, administration of putative T1D-associated intact autoantigens or derivative natural/synthetic peptides has shown to be largely safe with few significant adverse events during the relevant clinical trials [6,11–13]. Even though some bioactivity is reliant on the ability of the peptides/autoantigens to trigger energy in a population of rare antigen-specific activated T-cells [11], the majority of mechanistic studies concur that the effect of these peptide/autoantigen approaches is through antigen-presenting cell intercessors, mainly dendritic cells (DC) [6,12]. Provision of these peptides or autoantigen-derived peptides by DC to a range of immune cells inside the lymph nodes draining the site of administration results in the generation of antigen-specific T- and B-cells from naïve and/or memory precursors [6,10,12]. This approach has largely-defined the space of antigen-specific immunotherapy over the last twenty years, even though there is little evidence that it can prevent, delay, or reverse new-onset T1D in humans [7,10,13,14] in spite of overwhelming success in the NOD strain [3]. A number of investigators have questioned whether enforced presentation of specific antigens to an immune system that has moved past the point of single-antigen reactivity and into the realm of a spread of antigens is actually better than simply allowing the natural antigen-presenting cells of the patient to migrate into the lymph nodes draining the target of the autoimmunity and acquiring all potential antigens that naturally drain into it [15–46]. Indeed, it is now very clear that DC stabilized into a state that promotes immunosuppression (directly or indirectly) can prevent and reverse a number of organ-specific autoimmune diseases in the relevant animal models, including T1D [47–53]. In fact, we first demonstrated that autologous DC generated in the presence of antisense oligonucleotides (AS-ODN) targeting the CD40, CD80 and CD86 costimulation protein primary transcripts could prevent and reverse T1D in the NOD strain via upregulation of Foxp3+ Tregs and novel IL-10+ B-regulatory cells (Bregs) [54–57] and that human embodiments of these cell products were safe and potentially of some benefit in humans, in a phase I clinical trial [58].

In spite of these promising approaches, a strategy more attractive than *ex vivo* DC manipulation to stabilize a tolerogenic state can be the *in vivo* targeting of DC with biologics or chemical drugs that facilitate and confer this stabilized state. Given the labile nature of biologics and most of the immunosuppressive chemicals, as well as their potential for systemic spread, biodegradable microparticles have evolved that mitigate these unwanted effects. Additionally, microparticles can be formulated in a manner that can result in the co-delivery of immunosuppressive agents along with disease-relevant antigens [59]. Furthermore, microparticles can be engineered to be multifunctional and modular. For example, they can be coated with chemoattractants (e.g. CCL19, CCL20, CCL21) that specifically stimulate DC accumulation to the area of administration *in vivo* [60,61], and once taken up, the microparticle can release its tolerogenic payload with or without the provision of antigens. Finally, microparticles can also be programmed to release their various contents at different times after *in vivo* injection. We have shown, in NOD mice, that microsphere formulations of the AS-ODN combination are phagocytosed by DC and confer to them a T1D-suppressive phenotype [62]. When injected, these microspheres mobilized endogenous DC to the injection site and within 3 hours the loaded DC moved to the closest lymph nodes. Microsphere-administered mice remained diabetes-free when injected prior to disease and at least 40% exhibited reversal of new onset disease. In diabetes-free mice, we also showed augmented $Foxp3^+$ $CD25^+$ $CD4^+$ Treg cell frequency as well as hypo-responsiveness to beta cell antigens, without compromising global immune response to alloantigens. Additionally, T-cells from successfully treated mice suppressed adoptive transfer of disease by diabetogenic splenocytes into secondary immunodeficient NOD-SCID recipients. Finally, a fraction of the microspheres was found to be accumulated within the pancreas and the spleen indicating an uptake and the migration by phagocytes. Among the questions raised by these studies was whether success of T1D "reversal" in newly-diagnosed NOD could be improved if the microspheres were rendered antigen-specific by providing T1D-relevant autoantigen(s) and/or autoantigen-derived peptide(s) in the formulation. To address this question, we ascertained the efficacy of T1D-relevant autoantigen-formulated microspheres in the generation of Foxp3+ Tregs inside the pancreatic lymph nodes as well as other lymph nodes of mice transgenic for T-cell receptors that are antigen-specific. Herein, we show that antigen-formulated microspheres promote an increased absolute number of pancreatic lymph node Foxp3+ antigen-specific Tregs, but not significantly more than control microsphere formulations. The expansion of the antigen-specific Tregs occurred exclusively inside the pancreatic lymph nodes which also drained the site of microsphere administration, but not in distant lymph nodes. Even though antigen-specific Foxp3+ Tregs expanded in response to antigen-formulated microspheres, these cells were not proliferating. Instead, proliferating Tregs were observed only in response to control microspheres. These data suggest that antigen provision in a "vaccine" microsphere formulation is not a *conditio sine qua*

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