Targeted, immunosuppressive microparticles modify immune cell behavior for the prevention of autoimmune diabetes in mice.

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Introduction: Recent research has implicated the cause of auto-immune diseases, particularly type 1 diabetes, to be a reduction in number and function of regulatory T cells (Tregs) which suppress low levels of physiologic auto-reactive cells. Dendritic cells (DCs), the body's 'natural adjuvants', also play a critical role in the maintenance of peripheral tolerance. In this context, cellular vaccines have been investigated using exogenously-generated DCs attempting to induce Tregs and curtail auto-immune responses. However, problems such as the plasticity, donor specificity, ex-vivo stability, antigen specificity and cost abrogate the benefits of this therapeutic approach. In order to address these issues, we are developing synthetic microparticle-encapsulated vaccine that can be easily administered with simultaneous delivery of both prime & boost doses using time-release materials (poly lactide-co-glycolide). This flexible approach addresses issues that plague alternative vaccines strategies such as ex-vivo stability and antigen specificity. Furthermore, these microparticle-based vaccines are being targeted to DCs, and provide both intracellular and extracellular delivery of immunomodulatory agents. Our *long-term goal* is to develop a novel synthetic microparticle vaccine capable reversal of the onset of type 1 diabetes. To date, we have investigated (i) the ability of surface-modified PLGA microparticles (MPs) to specifically target DCs for phagocytosis, (ii) induction of tolerogenic DCs (tDCs) and Tregs using drug-loaded MPs (iii) antigen presentation of peptide-loaded MPs by DCs and (iv) the efficacy of our particle vaccine system to prevent diabetes onset in non-obese diabetic (NOD) mice.

Materials and Method: A 50:50 polymer composition of poly(d,l lactide-co-glycolide) (PLGA) (Lactel, AL, USA) was used to generate microparticles. Microparticles were formed using a standard oil-water solvent evaporation technique and sized using standard DLS equipment.

Microparticle uptake was determined by measuring total fluorescence of fluorescent dye-loaded microparticles prior to and after exposure to DCs for 1 hr using either a plate reader or flow cytometer. These assays were done as either single or co-cultures of DC and MΦ to compare how specific our particles are for DCs compared to MΦs. Cell culture supernatants were collected after 24 hours of cell culture with various surface-modified MPs. The IL-12 cytokine subunit, IL-12p40, and IL-10 cytokine production was analyzed using sandwich enzyme-linked immunosorbant assay (ELISA) kits (Becton Dickinson) according to manufacturer's directions.

To determine the level of expression of Treg-inducing indoleamine deoxygenase (IDO), DCS were collected and stained with fluorescently-tagged anti-IDO antibodies following manufacturer's instructions and analyzed as using flow cytometry. Additionally, T cell suppression and Treg induction was analyzed using standard allogenic MLC procedures flowed by immuno-staining and flow cytometry.

Finally, we studied the ability of our particle vaccine approach to prevent diabetes in a cohort of NOD mice given injections of our formulation at 4 and 5 weeks of age. The blood glucose levels of mice were then monitored once weekly for the next 32 weeks. Once the blood glucose level is over 250 mg/dl for two consecutive days, diabetes was diagnosed and the mouse sacrificed.

Results:

Surface-modified DC-targeting Microparticles

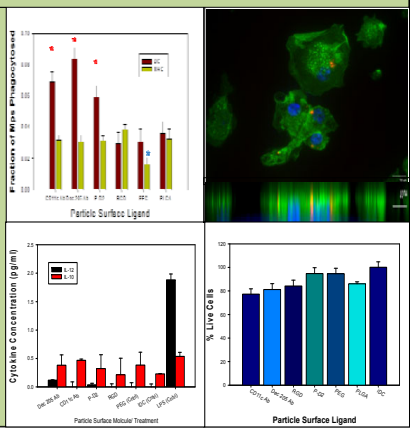


Figure 1. Particles are engineered for DC-targeting and improved uptake, while remaining minimally activating. **Top left panel.** DC uptake of MPs modified with tethered DC-targeting Abs anti-CD11c and anti-Dec 205, and P-D2 peptide increase uptake by DCs (blue bars) compared to unmodified MPs (Blank MPs) while not affecting uptake by macrophages (red bars). MPs with tethered RGD peptide and PEG are included for comparison. **Top right panel.** Confocal microscopy image confirms phagocytosis of fluorophore-loaded microparticles. **Bottom left panel.** P-D2 modified MPs do not activate DCs compared to blank MPs, determined by cytokine staining for IL-10 and IL-12. Immature DCs (iDCs) and LPS-matured DCs (LPS) are included for comparison. **Bottom right panel.** Surface modified MPs have neglible toxic effects on dendritic cells.

Maintenance of Immature DC state/ Induction of 'Tolerogenic' DC

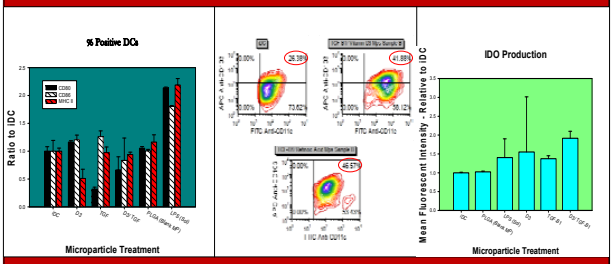


Figure 2. MP formulations maintain low DC activation state, and promote indoleamine 2,3 dioxygenase (IDO) and CD103 production ('tolerogenic' DC markers) **Left Panel.** DCs were incubated 72 hr with MP formulations: unmodified PLGA MPs ("Blank MP") or loaded with Vitamin D3 ("D3"), and/ or Transforming growth factor beta-1 ("TGF-B1"). Immature DCs ("iDC") and addition of soluble liposaccharide groups were included for comparison. Cells were immunostained for **MHC-II, CD86, and CD80.** The percent positively stained cells were quantified by flow cytometry. Results show low levels of DC activation for the D3/TGF group. **Centre Panel.** Similarly, DCs were incubated 72hr with MP formulations: loaded with Vitamin D3 ("D3") and Transforming growth factor beta-1 ("TGF-B1"), Retinoic Acid ("RA") and Transforming growth factor beta-1 ("TGF-B1"). Cells were then immuno-stained for **CD103.** Flow cytometry results show CD103 levels of expression for the D3/TGF group is higher than iDC and comparable to RA/TGF-B1 (positive control). **Right Panel.** DCs were incubated 72 hr with MP formulations (encapsulated with vitamin D3 and/ or TGF-β), fixed, permeablized and immuno-stained for intracellular **IDO.** Shown is flow cytometry data - forward scatter versus IDO. Controls of iDCs and blank PLGA MPS included for comparison. Results show increased IDO expression in DCs incubated with MPs loaded with both D3 and TGF-β1.

Induction of Regulatory T Cells by MP-treated DCs

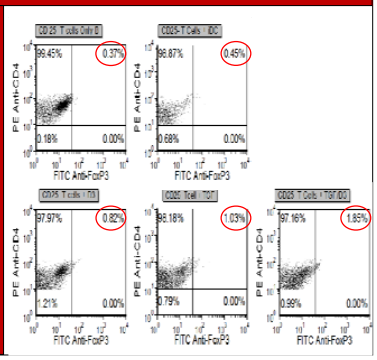
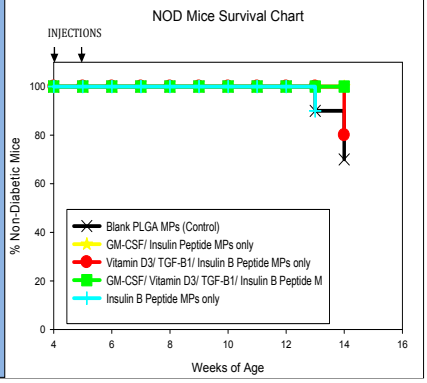


Figure 3. DCs treated with D3/TGF-B1 MP formulations become tolerogenized and further induce FoxP3+ regulatory T Cells. After pre-incubating DCs for 72 hr with MP formulations, syngenic purified CD4+ CD25- T cells were added to culture wells (ratio of 1:5, DC: T cell) and this mixed lymphocyte coupling was cultured for 3 d. Cultures were then pulsed with monensin for 5h, fixed, permeabilized, and immunostained for FoxP3 (Treg marker) and CD4 (T cells). Flow cytometric analysis shows DCs treated with formulations of MPs loaded with TGF-β1 and vitamin D3 are best capable of increasing FoxP3-expressing T cells.

Early Prevention of T1D in NOD Mice

Figure 4. To study prevention of diabetes in vivo, a cohort of NOD mice were given injections of control and experimental microparticle formulations at 4 and 5 weeks of age. The blood glucose levels of mice were then monitored once weekly for the next 32 weeks. Once the blood glucose level is over 250 mg/dl for two consecutive days, diabetes was diagnosed and the mouse sacrificed. Though this study is still at a nascent stage, early results show indications that microparticle formulations with loaded Vitamin D3, GM-CSF and TGF-B1 may be capable of delaying the onset of type 1 diabetes in mice.



Conclusions: These preliminary studies demonstrate engineered microparticle vaccine formulations that: (a) target DCs in vitro for phagocytosis (b) induce DCs with 'tolerogenic' phenotype (i.e. low MHC and co-stimulatory molecules, and production of suppressive agents (indoleamine deoxygenase [IDO]/ CD103) and (c) reduce T-cell proliferation and induce Treg diffrentiation. Studies to determine prevention of T1D in mice are ongoing. The potential impact of optimized vaccines for the prevention and reversal of auto-immune diseases worldwide is quite significant.

References: (1) Kabelitz et al, Trends Immunol. (2008); (2) Lo et al, N. Y. Acad. Sci. (2006); (3) Steinman et al, Annl. Rev. Immunology (2003); (4) Morelli et al, Seminars in Immunology (2001); (5) Tamber et al, Advanced Drug Delivery Reviews (2005).

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