Dual-sized microparticle system for generating suppressive dendritic cells prevents and reverses type 1 diabetes in the non-obese diabetic mouse model

Lewis, Jamal S. [†, 1, 4, 5], Stewart, Joshua M. [†, 1], Marshall, Gregory P. [4], Carstens Matthew R.[1], Zhang Ying[1], Dolgova Natalia V.[1], Xia, Changqing.[2], Brusko, Todd M.[2], Wasserfall, Clive H.[2], Clare-Salzler, Michael J.[2], Atkinson, Mark A.[2, 3], Keselowsky Benjamin G.[1,2*]

[1]J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, 1275 Center Dr., Gainesville, FL 32611

[2]Department of Pathology, Immunology and Laboratory Medicine, University of Florida, 1600 SW Archer Rd., Gainesville, FL 32611

[3]Department of Pediatrics, University of Florida, Gainesville, 1600 SW Archer Rd., FL 32611

[4]OneVax, LLC, 12085 Research Drive, Alachua FL 32615

[5]Department of Biomedical Engineering, University of California - Davis, One Shields Ave., Davis, CA 95616

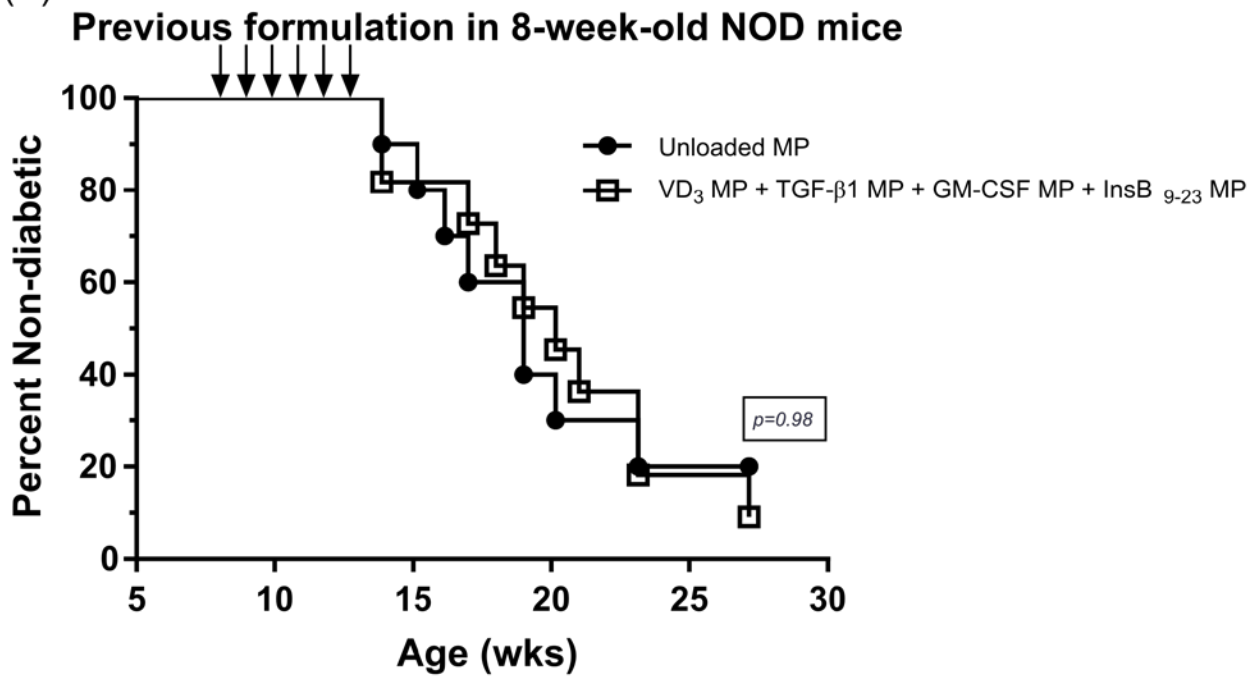
Supporting information:

Eleven supplementary pages

Eleven supplementary figures

Supplemental Figures

(A)



(B) Encapsulated Agent	Mass Loaded/PLGA (μg/100 mg)	Encapsulation Efficiency ± SD (%)	Mass Delivered per Injection ± SD (ng)
Insulin B_{9-23}	1000	50 ± 0.1	12,500 ± 250
Vitamin D_3	5	80 ± 7	100 ± 7.5
TGF-β1	1	62 ± 5	15 ± 2.5
GM-CSF	2.8	72 ± 6	50 ± 2.5

Figure S1. Previously published dMP formulation (see reference [31]), with smaller amounts of immunomodulatory factors and insulin peptide antigen, did not prevent T1D incidence in 8-week-old NOD mice. (A) A cohort of 8-week-old NOD mice (*n* = 10/group) were injected weekly over seven weeks (8-14 weeks of age) at a subcutaneous site anatomically proximal to the pancreas with unloaded MPs or an initial formulation of the dMP with Insulin B_{9-23} peptide as antigen (VD_3/TGF-β1/GM-CSF/$InsB_{9-23}$ MPs). Blood glucose was monitored weekly until week 28 and mice were considered diabetic when blood glucose levels were ≥ 240 mg/dL on two consecutive days. Survival data is fit using the Kaplan–Meier non-parametric survival analysis model and statistical analysis performed via log-rank test (Mantel-Cox method). (B) Particle loading characterization of the previous dMP formulation using insulin B_{9-23} peptide as antigen and lower doses of chemotactic and tolerogenic factors.

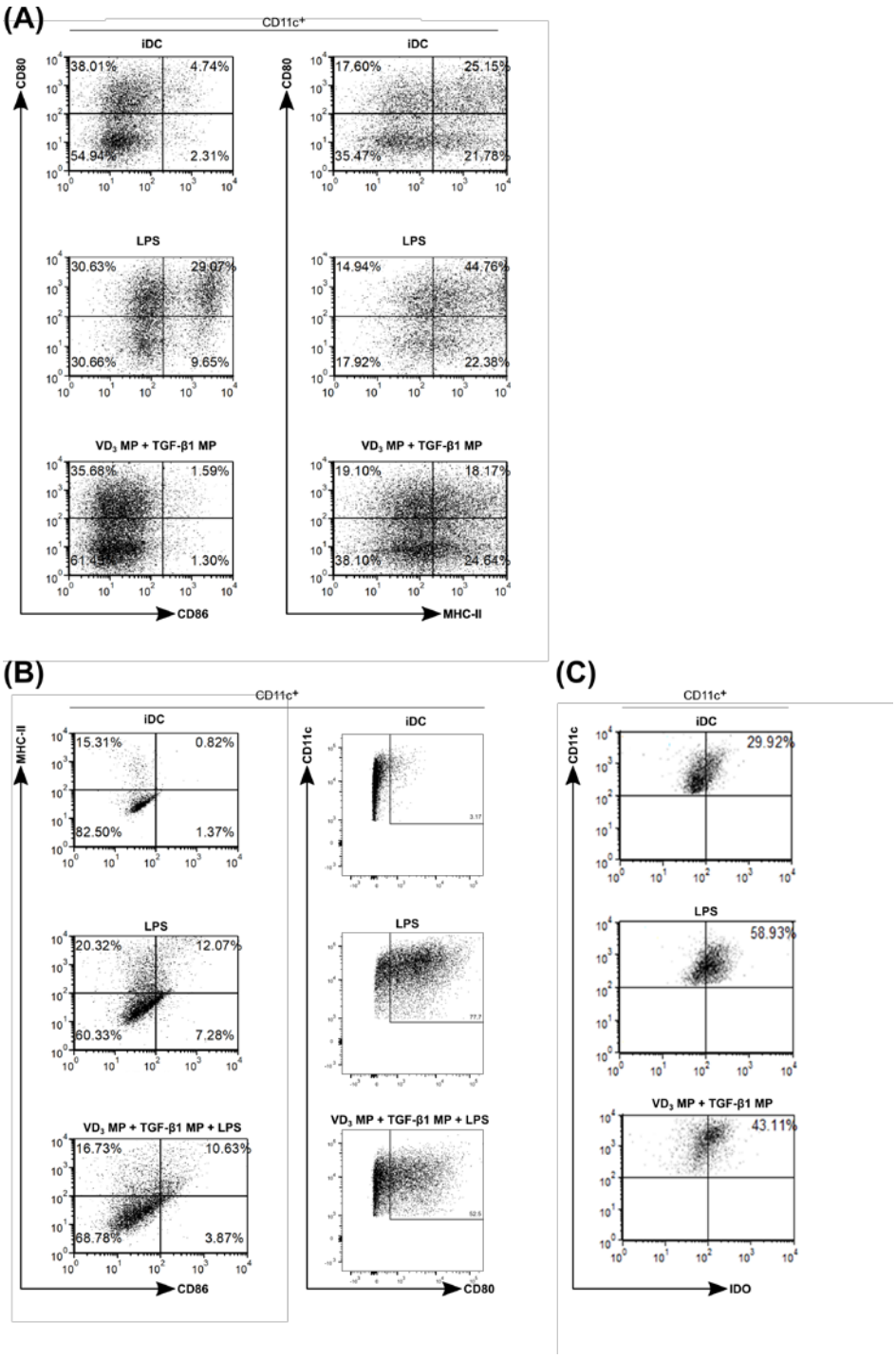


Figure S2. Representative flow cytometry analysis of MP-treated DCs. (A) Surface expression of CD80, CD86, and MHC-II of untreated, immature DCs (iDC), LPS stimulated DCs, and VD_3 MP and TGF-β1 MP-treated DCs. Gated on $CD11c^+$ live cells. (B) Surface expression of CD80, CD86, and MHC-II of iDC, LPS stimulated DCs, and LPS + VD_3 MP and TGF-β1 MP-treated DCs. Gated on $CD11c^+$ live cells. (C) Intracellular immune-staining of IDO in iDC, LPS stimulated DCs, and VD_3 MP and TGF-β1 MP-treated DCs. Gated on $CD11c^+$ live cells.

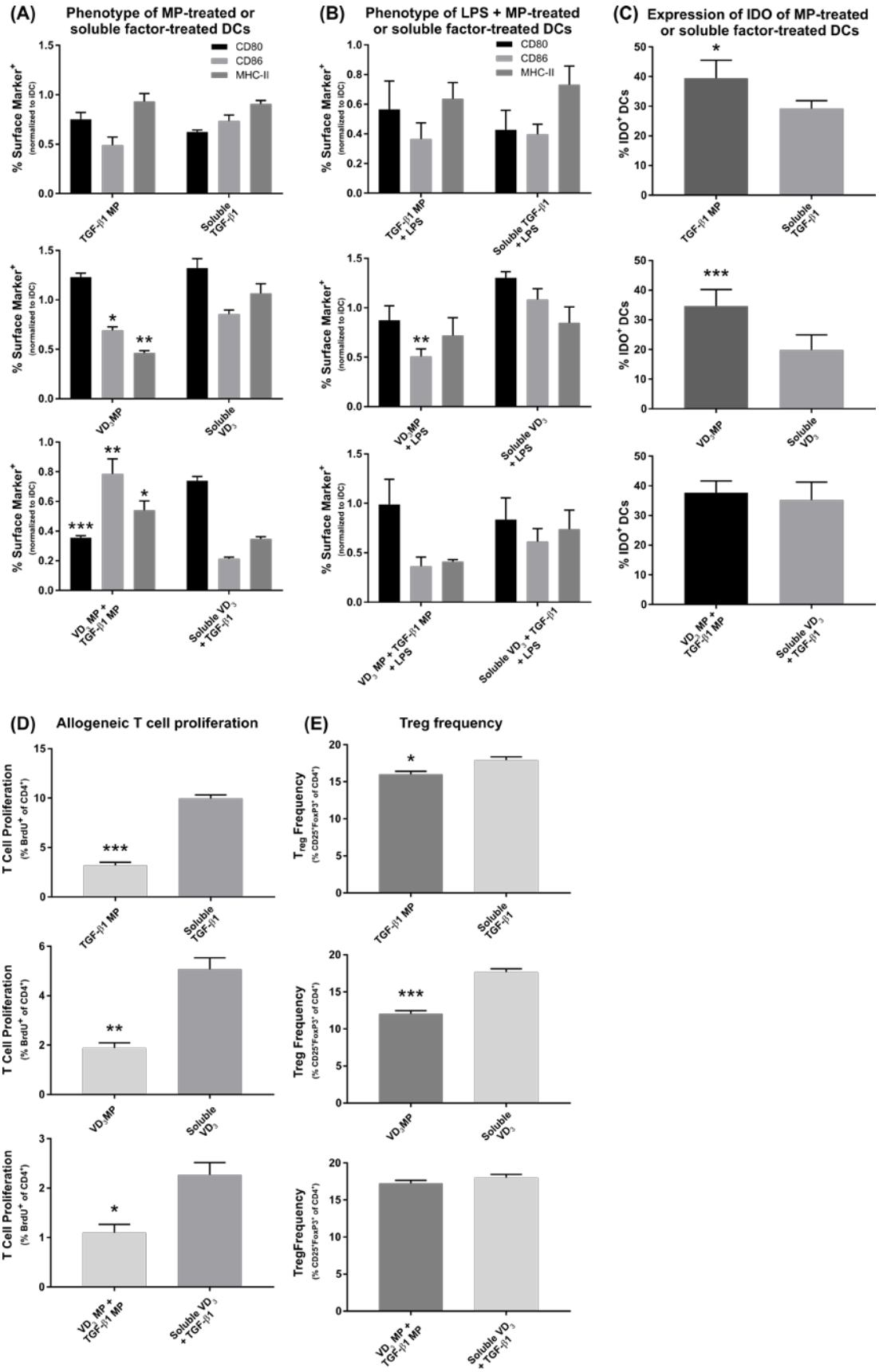

(A)
Phenotype of MP-treated or soluble factor-treated DCs
CD80
CD86
MHC-II
% Surface Marker+ (normalized to iDC)
TGF-β1 MP
Soluble TGF-β1
VD3MP
Soluble VD3
VD3 MP + TGF-β1 MP
Soluble VD3 + TGF-β1
(B)
Phenotype of LPS + MP-treated or soluble factor-treated DCs
TGF-β1 MP + LPS
Soluble TGF-β1 + LPS
VD3 MP + LPS
Soluble VD3 + LPS
VD3 MP + TGF-β1 MP + LPS
Soluble VD3 + TGF-β1 + LPS
(C)
Expression of IDO of MP-treated or soluble factor-treated DCs
% IDO+ DCs
(D)
Allogeneic T cell proliferation
T Cell Proliferation (% BrdU+ of CD4+)
(E)
Treg frequency
Treg Frequency (% CD25+FoxP3+ of CD4+)
TregFrequency (% CD25+FoxP3+ of CD4+)

Figure S3. Microparticle encapsulation of tolerogenic agents VD_3 and TGF-β1 influences the suppressive phenotype of DCs *in vitro.* Dendritic cells were incubated with 10 mg of non-phagocytosable TGF-β1 MPs and phagocytosable VD_3 MPs were added at a 10:1 MP to DC ratio. Microparticles were incubated with DCs for 48 h and subsequently washed with PBS to remove MPs. Equivalent doses of soluble VD_3 (0.2 ng/mL) and TGF-β1 were calculated by loading and release kinetics of respective MPs and supplemented in media. Cellular responses were characterized by flow cytometry. (A) Expression of maturation markers CD80, CD86, and MHC-II between MPs and equivalent soluble doses was characterized and expression normalized to iDCs (*n* = 3). (B) Expression of maturation markers CD80, CD86, and MHC-II between MPs and equivalent soluble doses was characterized following LPS stimulation of MP-treated or soluble factor-treated DCs (*n* = 3). Surface expression is normalized to LPS stimulated DCs. (C) Dendritic cell expression of the immunosuppressive enzyme IDO was quantified in response to MP or soluble treatment (*n* = 3). (D-E) Separately, Balb/c splenic $CD4^+$ T cells were co-incubated with MP-treated or soluble factor-treated C57Bl/6 bone marrow-derived DCs at a 150,000:25,000 ratio. After 72 h, flow cytometry was used to assess T cell proliferation via BrdU incorporation (D) and $CD25^+FoxP3^+$ Treg frequency (E) (*n* = 3). P-values (* = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001) were obtained by two-tailed unpaired Student's t-tests. Data is represented by mean ± SEM.

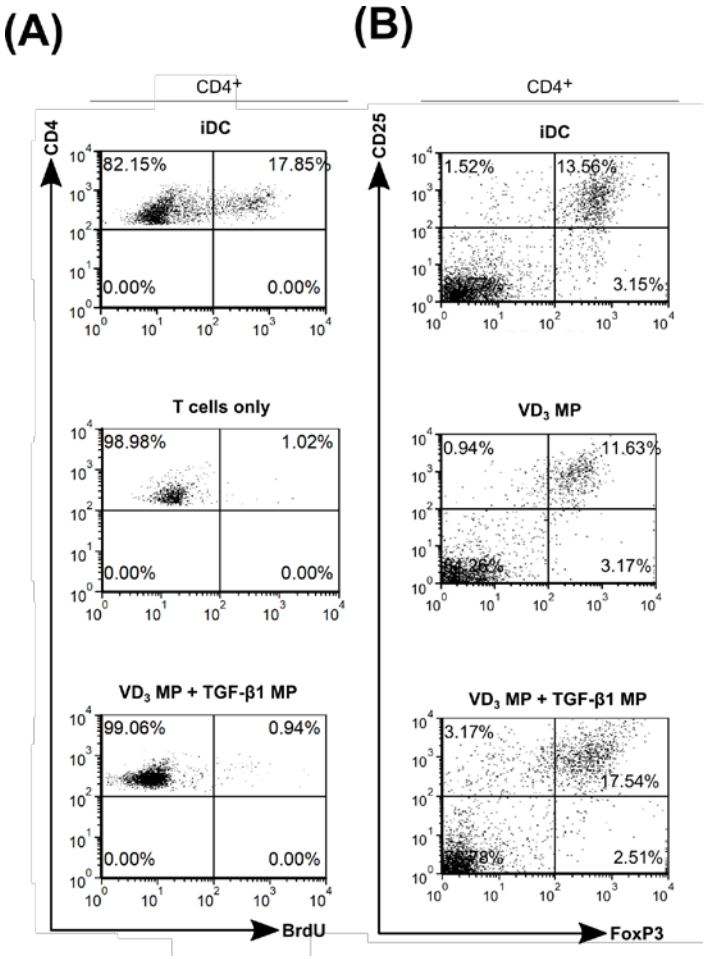


Figure S4. Representative flow cytometry analysis of T cell proliferation and Treg differentiation in response to MP-treated DCs. (A) Frequency of BrdU incorporation in $CD4^+$ T cells as a measure of T cell proliferation. Gated on $CD4^+$ live cells. (B) Frequency of $CD25^+FoxP3^+$ Tregs as a percent of total $CD4^+$ T cells. Gated on $CD4^+$ live cells.

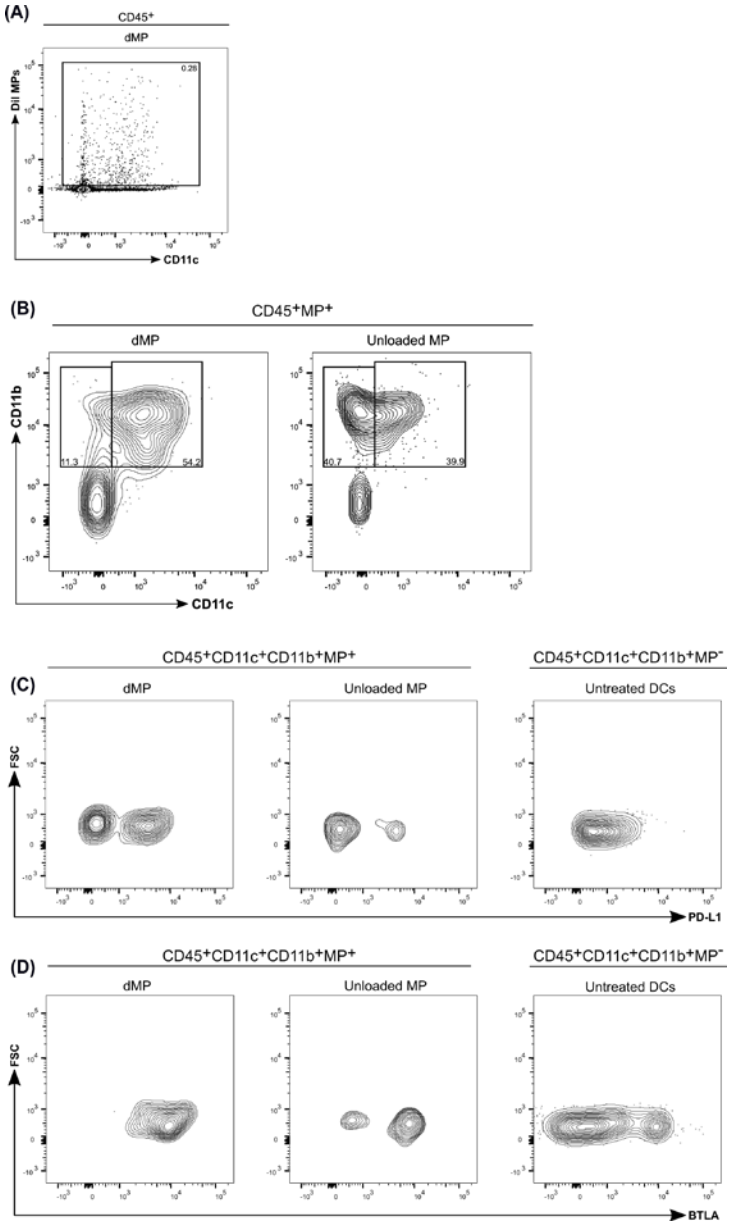


Figure S5. Representative flow cytometry analysis of MP trafficking. (A) Frequency of MP^+ cells in the live $CD45^+$ population. Inclusion of CD45 gate was to eliminate the incidence of free MPs unassociated with leukocytes. (B) Frequency of MP^+ events in DC ($CD11b^+CD11c^+$) or MΦ ($CD11b^+CD11c^-$) subpopulations in axillary LNs of dMP-treated or unloaded MP-treated mice as a percentage of $CD45^+MP^+$ cells. (C-D) Surface expression of PD-L1 (C) and BTLA (D) of dMP^+ DCs, unloaded MP^+ DCs, and DCs from untreated mice.

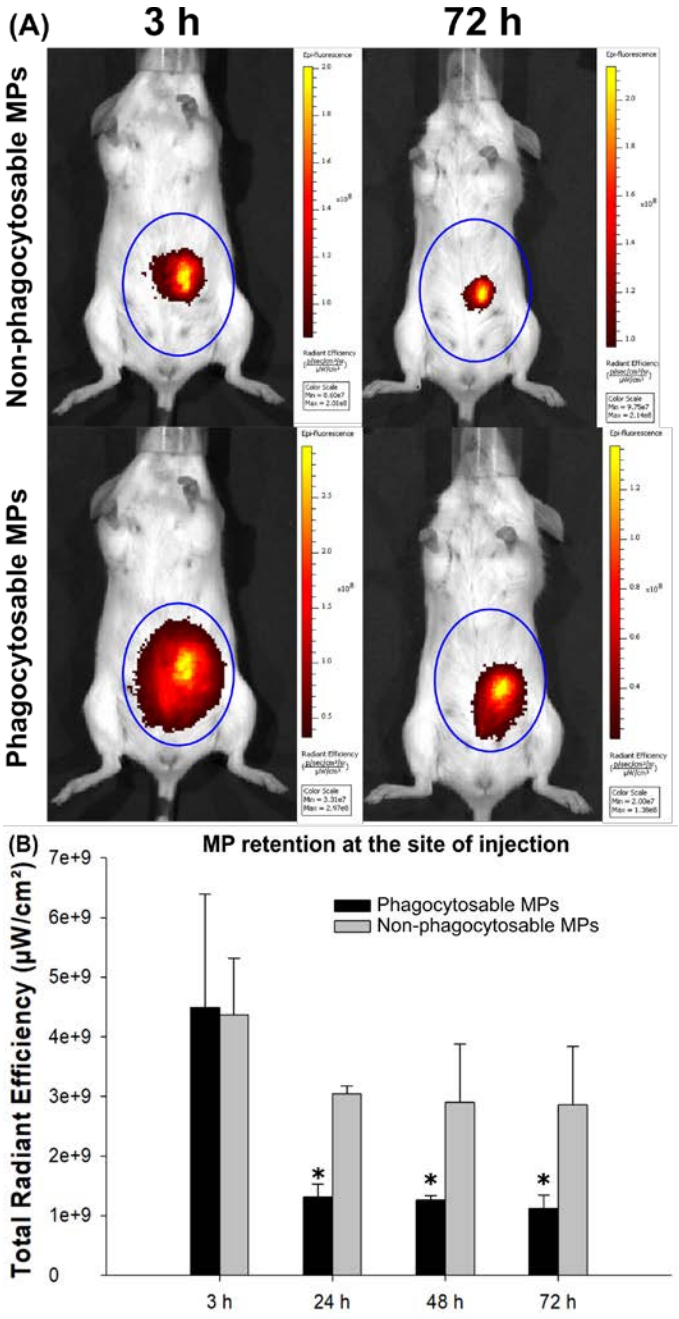


Figure S6. Non-phagocytosable MPs persist to a greater extent at the site of injection compared to phagocytosable MPs. Balb/c mice were subcutaneously injected in the abdominal region with MPs encapsulating the four immunomodulatory agents previously described, as well as infrared dyes (IRDye 800RS in phagocytosable MPs and IRDye 700DX in non-phagocytosable MPs) (n = 3). *In vivo* imaging of mice was performed at 3 h, 24 h, 48 h and 72 h after injection and total radiant efficiency quantified based on a defined region of interest (blue circle). (A) Representative images of the fluorescent intensity of phagocytosable MPs and non-phagocytosable MPs at 3 h and 72 h post injection (note the change in the scale of fluorescent intensity per image). (B) The total radiant efficiency for two particle size classes at the site of injection was quantified at different time points and plotted. P-values (* = ≤ 0.05) were obtained by one-way ANOVA with Dunnett's multiple comparisons test against the 3 h time point. Data is represented by mean ± SEM.

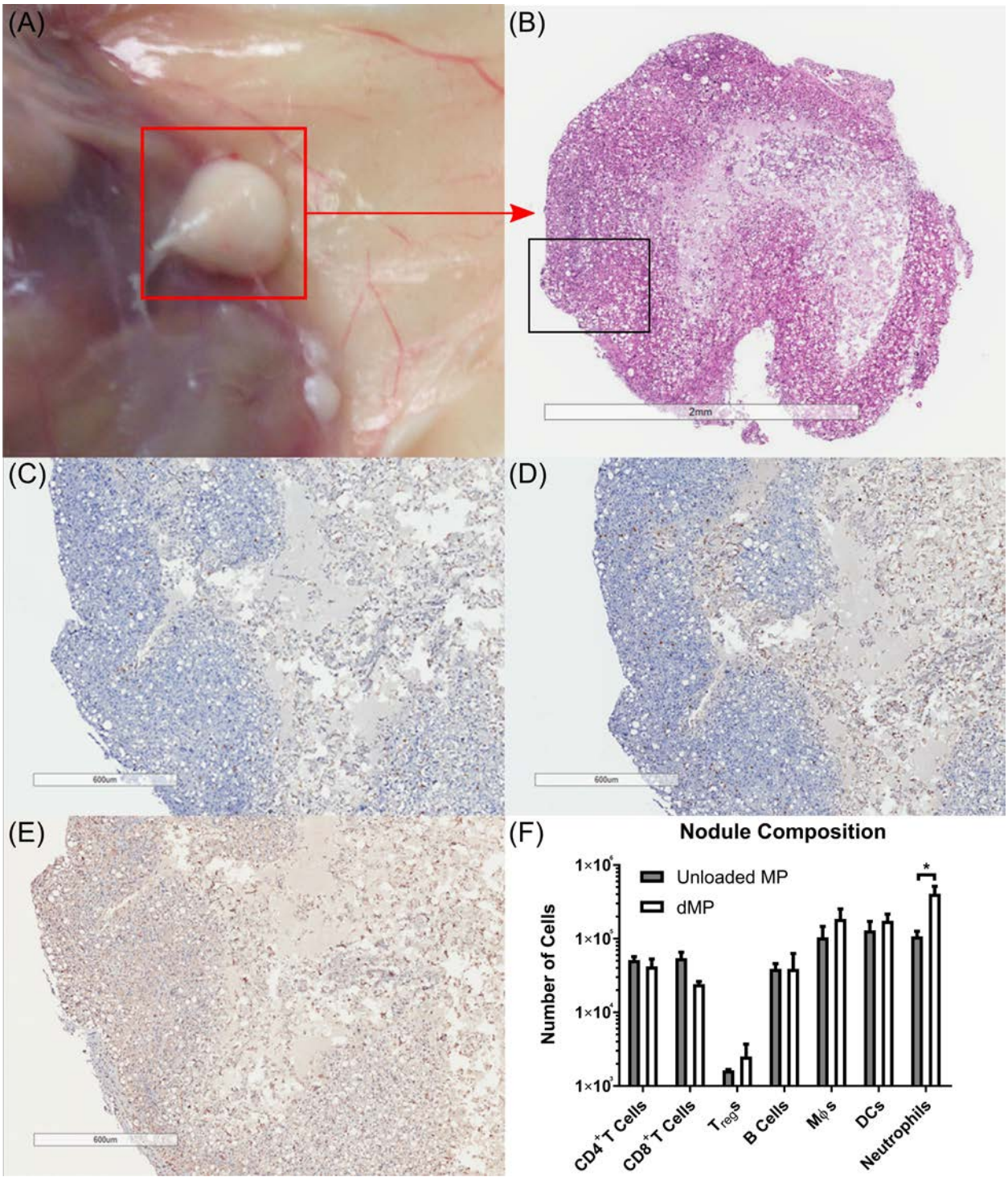


Figure S7. Subcutaneous dMP depot recruits significant numbers of immune cells. 8-week-old NOD mice were subcutaneously injected in the abdomen with three MP injections (days 1, 4, 7). Two weeks after the initial injection (day 15), mice were sacrificed and the nodule formed at the injection site (A) was excised, sectioned and stained with (B) hematoxylin and eosin (H&E), (C) B220 (B cell marker), (D) CD3 (T cell marker), and (E) LYVE-1 (lymphatic endothelial marker). (F) Flow cytometry analysis determined the total cell numbers in MP nodules. P-values (* = ≤ 0.05) were obtained by Student's T test (*n* = 3-8). Data is represented by mean ± SEM.

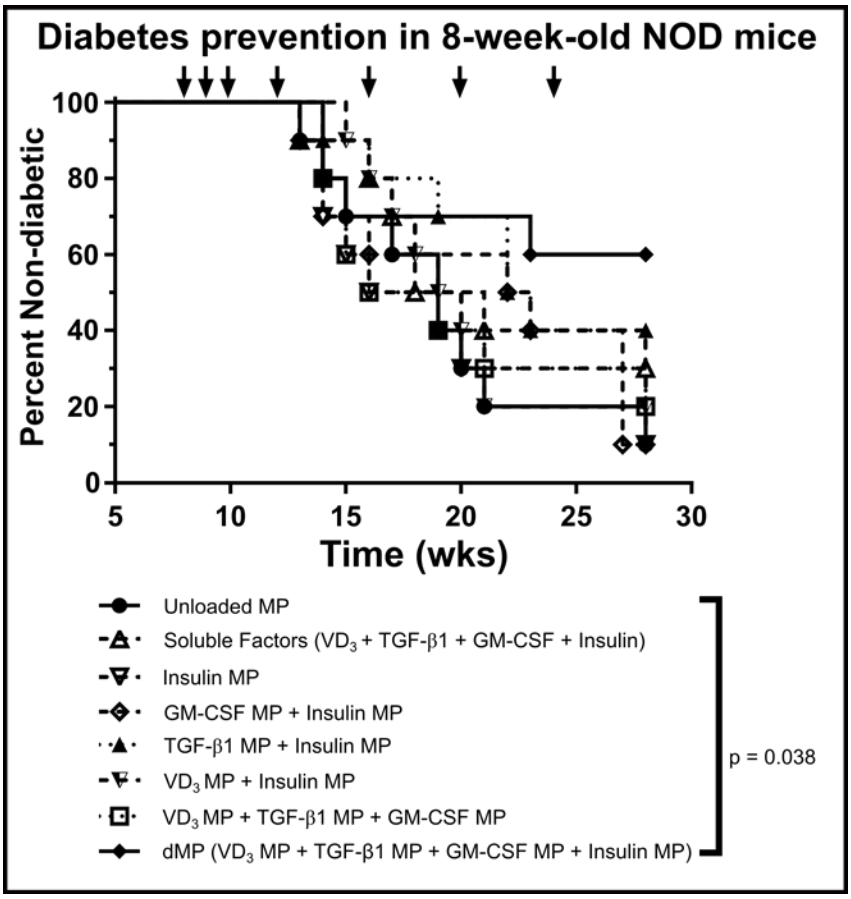


Figure S8. dMP administration prevents diabetes onset in NOD mice. A cohort of 8-week-old NOD mice (*n* = 10/group) were injected at a subcutaneous site anatomically proximal to the pancreas with the described MP formulations over 16 weeks. Animals received MP injections (arrows) once a week for the first three weeks (8, 9 and 10 weeks of age) and a booster injection once monthly thereafter for four months (12, 16, 20, and 24 weeks of age). Unloaded MPs, a soluble bolus of factors without MPs, and omission of factors were investigated for a total of eight treatment groups. When a factor-loaded MP was omitted, unloaded MPs were delivered to ensure delivery of an equivalent PLGA mass. Animals were monitored weekly until week 28 and mice were considered diabetic when blood glucose levels were ≥ 240 mg/dL on two consecutive days. Survival data is fit using the Kaplan–Meier non-parametric survival analysis model and statistical analysis performed via log-rank test (Mantel-Cox method). Statistical significance was not realized when accounting for multiple comparisons via Bonferroni correction, as the study was not powered to resolve this large number of groups. However, pairwise comparison between survival curves of mice that received the dMP and mice that received unloaded MPs resulted in a p-value < 0.05, suggesting a difference between treatments.

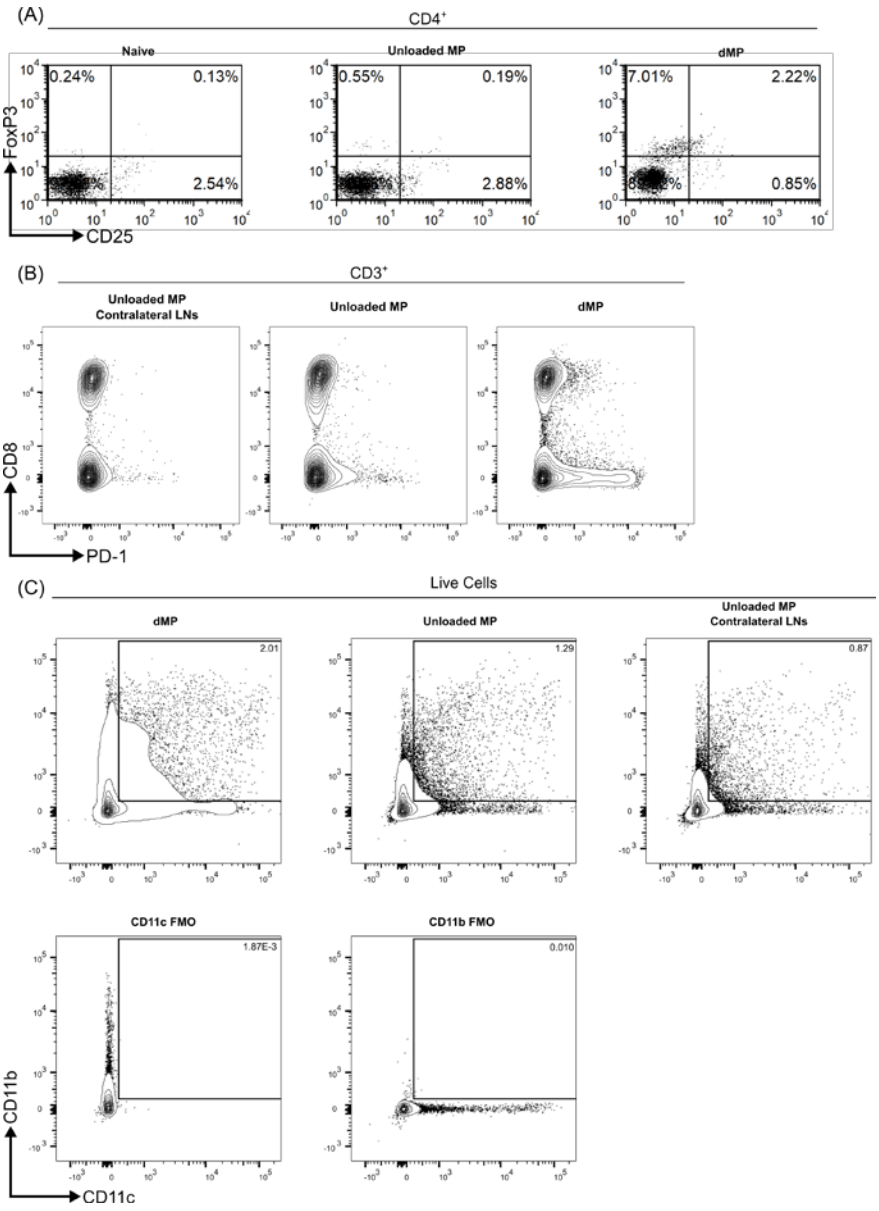


Figure S9. Representative flow cytometry analysis from diabetes prevention study. (A) Frequency of FoxP3$^+$CD4$^+$ T cells isolated from spleen of 12-week-old untreated naïve, unloaded MP-treated, and dMP-treated mice of total CD4$^+$ T cells. Gated on CD4$^+$ live cells. (B) PD-1 expression of CD4$^+$ and CD8$^+$ T cells from draining lymph nodes (combined axillary and inguinal LNs) of dMP-treated or unloaded MP-treated animals euthanized at 12 weeks of age. Gated on CD3$^+$ live cells. (C) Frequency of DCs in dLNs of dMP- or unloaded MP-treated animals euthanized at 12 weeks of age. Full minus one (FMO) analyses, wherein the full panel of antibodies except the marker of interest is stained for, for CD11c and CD11b staining highlighting how gating was defined. Gated on live cells.

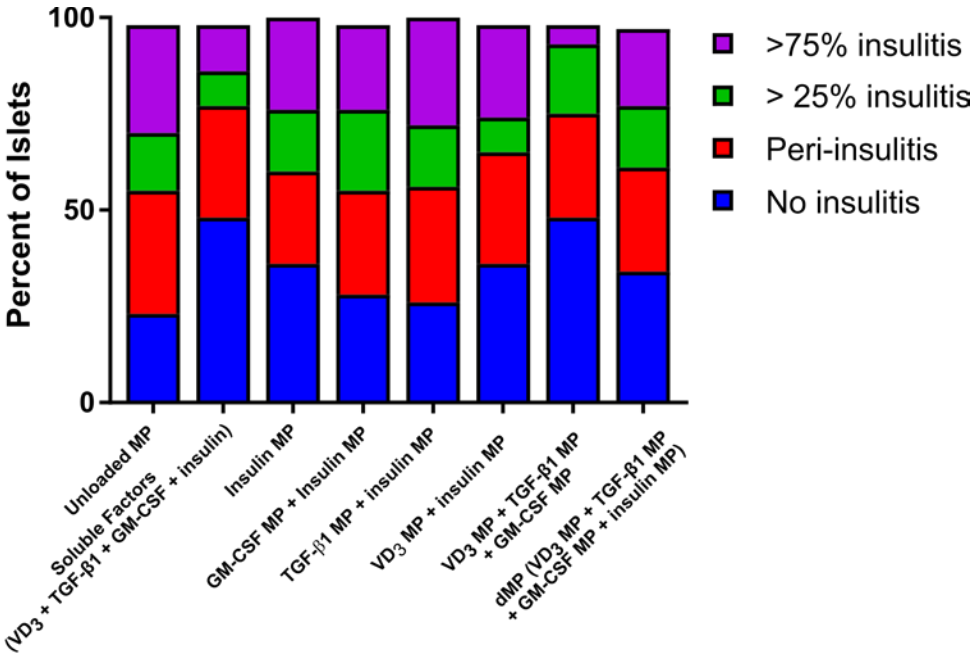


Figure S10. Insulitis scoring of NOD mice pancreata at midpoint from prevention study. A cohort of female, NOD mice were enrolled at 8 weeks of age in the prevention study and at 12 weeks of age mice were sacrificed, pancreata excised, processed, sectioned and stained with H&E (*n* = 3). Sections were examined and scored for insulitis in a blinded fashion (0 = no insulitis; 1 = peri-insulitis; 2 = 25–75% insulitis; 3 = >75% insulitis). The percent of islets assigned to the different grades of insulitis for each treatment is displayed. Data were analyzed by Pearson's chi-squared test.

Test Material	Rabbit Weight (kg)	Dose Volume (ml)	Temperature (°C)						
			Pre-injection	1 h	1.5 h	2 h	2.5 h	3 h	Max. Rise
dMP (VD_3 MP + TGF-β1 MP + GM-CSF MP + insulin MP)	1.6305	4.9	39.6	39.4	39.5	39.6	39.6	39.6	0.0
	1.7004	5.1	39.5	39.5	39.5	39.5	39.4	39.4	0.0
	1.8502	5.6	39.5	39.1	39.2	39.2	39.5	39.6	0.1

Figure S11. Rabbit pyrogenicity test of dMP vaccine. A rabbit pyrogen test result indicates dMP vaccine does not induce significant rise in body temperature following administration (*n* = 3). Data were analyzed by repeated measures ANOVA with Tukey's significance test.