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Combinatorial delivery of immunosuppressive factors to dendritic cells using dual-sized microspheres†

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Microparticulate systems are beginning to show promise for delivery of modulatory agents for immunotherapeutic applications which modulate dendritic cell (DC) functions. Co-administration of multiple factors is an emerging theme in immune modulation which may prove beneficial in this setting. Herein, we demonstrate that localized, controlled delivery of multiple factors can be accomplished through poly (lactic-*co*-glycolic acid) (PLGA) microparticle systems fabricated in two size classes of phagocytosable and unphagocytosable microparticles (MPs). The immunosuppressive ability of combinatorial multi-factor dual MP systems was evaluated by investigating effects on DC maturation, DC resistance to LPS-mediated maturation and proliferation of allogeneic T cells in a mixed lymphocyte reaction. Phagocytosable MPs (~2 μm) were fabricated encapsulating either rapamycin (RAPA) or all-trans retinoic acid (RA), and unphagocytosable MPs (~30 μm) were fabricated encapsulating either transforming growth factor beta-1 (TGF-β1) or interleukin-10 (IL-10). Combinations of these MP classes reduced expression of stimulatory/costimulatory molecules (MHC-II, CD80 and CD86) in comparison to iDC and soluble controls, but not necessarily to single factor MPs. Dual MP-treated DCs resisted LPS-mediated activation, in a manner driven by the single factor phagocytosable MPs used. Dendritic cells treated with dual MP systems suppressed allogeneic T cell proliferation, generally demonstrating greater suppression by combination MPs than single factor formulations, particularly for the RA/IL-10 MPs. This work demonstrates feasibility of simultaneous targeted delivery of immunomodulatory factors to cell surface receptors and intracellular locations, and indicates that a combinatorial approach can boost immunoregulatory responses for therapeutic application in autoimmunity and transplantation.

Introduction

Dendritic Cells (DCs) are specialized phagocytic cells that are influential in the control of the innate and adaptive immune responses.[1] Circulating through most tissues, DCs possess qualities that allow them to rapidly recognize foreign from self-entities, efficiently intercept and process material, and then display antigen in the context of the major histocompatibility complex (MHC) to the T-cell receptor on T-cells.[1,2] While much attention has been paid to the role of DCs as strong, "natural adjuvants",[3] their function in the initiation of suppressive networks required for maintenance of peripheral tolerance is more recently being uncovered.[4] Moreover, modulating dendritic cells to have regulatory functions, or "tolerogenic DCs" (tDCs) is emerging as an immunotherapeutic goal.[5]

Of particular interest is the application of tDCs for the abrogation of autoimmune diseases and transplant rejection. Tolerogenic dendritic cells initiate a number of modalities that can lead to hyporesponsiveness by effector immune cells. These include anergic pathways, regulatory T cell generation (Treg), as well as effector T-cell deletion.[1] So far, therapeutic approaches have primarily entailed the use of exogenously manipulated DCs conditioned with tolerance-inducing factors (*e.g.* oligonucleotides, corticosteroids, cytokines) to produce tDCs that are re-introduced to the body.[6–8] A number of pharmacological and biological agents have been investigated to generate tDCs. For instance, studies have shown that administration of either biological agents like the cytokines, interleukin-10 (IL-10) and transforming growth factor beta-1 (TGF-β1), or pharmacological drugs such as rapamycin (RAPA), and all-*trans* retinoic acid (RA) have been explored for this application.[9–13]

Interleukin-10 is a pleiotropic immunomodulatory cytokine expressed and secreted by helper T cells and antigen presenting cells (APCs).[14] This immuno-suppressive cytokine initiates its

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effects *via* binding the extracellular region of the transmembrane IL-10 receptor protein.[14] IL-10-treated DCs show reduced cytokine production (including IL-1, IL-10 itself, IL-12, TNF), and reduced MHCII and co-stimulatory molecule (*e.g.* CD80, 86) which correlated with the ability of these DCs to inhibit activation when coupled with allogeneic T cells.[15,16] Another pleiotropic, anti-inflammatory cytokine of interest is TGF-β1. Transforming growth factor is produced and secreted in a latent form by an array of lymphoid cells, particularly DCs and T cells, and can exhibit paracrine effects *via* binding cell surface membrane-bound receptors.[17,18] These cells are not only sources for TGF-β1 but also targets of action for this immunosuppressive cytokine. The scope of impact of TGF-β1 on DCs is still being discovered, but it has been demonstrated TGF-β1 immuno-modulatory effects are inhibitory in nature and lead to a tolerogenic DC phenotype that is capable of inducing antigen-specific $CD25^+$ $FoxP3^+$ T cells (induced Tregs) from CD^+4 naïve T cell population.[19,20] Notably, TGF-β1-conditioned DCs produce indoleamine 2,3 deoxgenase (IDO), an enzyme involved in tryptophan catabolism and responsible for the generation of kynurenines. Kynurenines are thought to be a key factor in the spread of 'infectious tolerance', a state where tolerance is thought to be spread from one immune cell population to another.[21]

The pharmacologic drug, rapamycin (RAPA) is a macrolide antibiotic derived from the filamentous bacterium, *Streptomyces hygroscopicus*.[22] Rapamycin exerts potent immunosuppressive action, *via* binding the intracellular membrane-bound mammalian target of rapamycin (mTOR) complex,[22] on immune cells including T cells and DCs. In DCs, rapamycin has been implicated for inhibition of cytokine-mediated signal transduction. It has been demonstrated that exposure of DCs to RAPA results in reduced expression of MHCII and co-stimulatory molecules, LPS resistance as well as inability to stimulate allogenic T cells *in vitro*.[23] *In vivo*, it has been suggested that RAPA-treated DCs are capable of generating $CD4^+CD25^+FoxP3^+$ regulatory T cells in addition to inducing apoptosis of effector T cells resulting in transplant acceptance in various mouse models.[23,24] Lastly, all-*trans* retinoic acid (RA), a metabolite of vitamin A, is the final immunosuppressive agent presently considered. All-*trans* retinoic acid can act through the nuclear receptor, retinoic acid receptor, to modulate both innate and adaptive immune cellular elements.[25,26] In APCs, RA treatment results in diminished production of inflammatory cytokines such as IFN-γ.[27,28] Further, supplementation of vitamin A and its metabolites in mice have been reported to mitigate autoimmune diseases including type 1 diabetes and encephalomyelitis.[29–31]

An emerging theme is that co-administration of immunomodulatory agents can produce cumulative, sometimes synergistic effects on DC populations that boost robust immune modulation. For example, RA acts a co-factor to TGF-β1 on $CD103^+$ DCs derived from gut associated lymphatic tissue, enhancing their ability to generate $FoxP3^+$ Tregs.[32] In this work, we explore combinatorial co-delivery of these factors in a targeted, localized controlled release manner, achieved through polymeric microparticulate delivery systems. We hypothesize such multi-factor particulate systems could benefit autoimmune and transplant applications, offering reduced doses and yielding immunoregulatory effects not achievable by delivering a single factor. Targeted, local, controlled delivery of these factors can be accomplished through poly (lactic-*co*-glycolic acid) (PLGA) microparticles (MPs).

Poly (lactic-*co*-glycolic acid) particulate systems have been widely established as a delivery system for a plethora of biomolecules, and pharmacological agents.[33–37] Key qualities include biocompatibility, biodegradability and tunable release kinetics. Additionally, PLGA MPs can be fabricated to specific micron size ranges, which is a key consideration for phagocytic cell targeting.[34,37] Particles below the ~1 μm size range can be taken up by pinocytosis, a process not limited to only APCs. In contrast, particles larger than ~7 μm are not readily ingested by APCs, but release their contents to the extracellular environment. Microparticles in the phagocytosable, 1–7 μm size are readily phagocytosed by APCs, capable of delivering their encapsulated payload to intracellular targets.[38] Delivery of immunomodulatory factors to their relevant targets is achieved by exploiting this feature of phagocytosis. Either of the small hydrophobic molecule, RAPA or RA, is encapsulated in phagocytosable MPs (~2 μm diameter) for targeting intracellularly, and either of the soluble proteins TGF-β1 or IL-10, is encapsulated in unphagocytosable MPs (~30 μm diameter) for targeted release to cell surface receptors. Combinatorial pairings of loaded MPs (one phagocytosable and one unphagocytosable) are assessed for their ability to modulate murine DC immune function. The goal of this approach is to not only boost immunosuppressive effects over those obtainable by single factor administration but also to deliver factors at a lower dosage, in a clinically translatable fashion for autoimmune and transplant applications.

Materials and methods

2.1 Microparticle preparation

A 50 : 50 polymer composition of poly(D,L-lactide-*co*-glycolide) (PLGA) ($M_w \sim 44\,000$ g mol^{-1}) in methylene chloride (MC) (Purac) was used to generate MPs. Polyvinyl alcohol (PVA; 87% hydrolyzed) ($M_w \sim 100\,000$ g mol^{-1}) was purchased from Fisher Scientific (NJ, USA) and was used as an emulsion stabilizer. Distilled water (DiH_2O) was used as the aqueous phase to form the emulsions while methylene chloride (Fisher Scientific, NJ, USA) was used as the organic solvent to dissolve the PLGA polymer. Microparticles were formed using a standard oil–water solvent evaporation technique.

To make phagocytosable MPs, 100 mg of PLGA polymer was dissolved in methylene chloride at 5% w/v ratio. Either Rapamycin (RAPA) (LC Laboratories) or All-*trans* Retinoic acid (RA) (Acros Organics) in DMSO was loaded into 2 ml of 5% PLGA solution. This solution was added to 2 ml of 5% PVA solution in DiH_2O and homogenized at 35 000 rpm for 180 s using a tissuemiser homogenizer (Fisher Scientific, NJ, USA) to form a primary emulsion. This was added to 30 ml of 1% PVA solution. The particles thus formed were agitated using a magnetic stirrer (Fisher Scientific, NJ, USA) for 24 h to evaporate residual methylene chloride. The remaining solution was centrifuged at

10 000 × *g* for 10 min to collect MPs which were subsequently washed three times with DiH_2O. The water was aspirated from the centrifuged MPs, which were then flash-frozen in liquid nitrogen and kept under vacuum in dry ice overnight. The MPs were stored at −20 °C until used. Unphagocytosable MPs (TGF-β1- and IL-10-loaded) (BD Pharmingen) were fabricated using by a double emulsion solvent evaporation technique similar to that described above but with the addition of a second emulsification step and using a vortexter (Fisher Scientific) instead of a homogenizer. The TGF-β1 solution was reconstituted in 10 mM Citric Acid and 2 mg ml^{-1} bovine serum albumin in PBS to a final concentration of 100 μg ml^{-1}. For IL-10, after a quick spin the lyophilized powder was reconstituted in DiH_2O to a concentration of 100 μg ml^{-1}.

2.2 Microparticle characterization – sizing, loading, release kinetics

The size distribution of the MP was measured by the Microtrac Nanotrac Dynamic Light Scattering Particle Analyser (Microtrac, Montgomery, PA). Particle sizes are reported as the average diameter ± standard deviation (S.D.).

The loading efficiency of the phagocytosable MPs was measured by dissolving 100 mg of MPs into 2 ml MC and re-precipitating the PLGA with a known volume of methanol (Acros Organics). The suspension was centrifuged and the supernatant removed to a new tube. Following evaporation, residue remaining in the tube is concentrated in a known, small quantity of DMSO and the solution concentration measured by spectrophotometer. For the second particle type (unphagocytosable), loading efficiency was measured using a solvent evaporation technique followed by spectrophotometric analysis.

The *in vitro* release kinetics from MPs was determined as described. Briefly, a known mass of MPs was re-suspended in a known volume of phosphate buffer saline (PBS) (Hyclone, UT, USA) containing tween-20 (2% w/v; Acros Organics). These samples were vortexed and placed in a shaking incubator at 37 °C. At regular intervals MPs were pelleted, the supernatant collected and stored at −20 °C, and MPs re-suspended in an equal, fresh volume of release media. Drug content of phagocytosable MPs releasate was determined through spectrophotometric detection. The amount of biological content released by the respective MPs at the studied time points was analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (Becton Dickinson, NJ) for TGF-β1 and GM-CSF, according to manufacturer's directions.

2.3 Dendritic cell culture and microparticle incubation

Dendritic cells were obtained from 8–12 weeks old, female, C57BL6/j mice in accordance with guidelines approved by University of Florida using a modified 10 days protocol.[39] For DC culture, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation and tibias and femurs were harvested for isolating marrow cells. The marrow cells were obtained by flushing the shaft of the bones with a 25 g needle using RPMI medium (MP Biomedicals, OH, USA) containing 1% fetal bovine serum (Lonza, Walkersville, MD) and 1% penicillin-streptomycin (Hyclone) and mixed to make a homogenous suspension. The suspension was then strained using 70 μm cell strainers (Becton Dickinson, NJ, USA) and cells were collected at 1300 rpm for 7 min. The red blood cells (RBCs) were removed by lysing with ACK lysis buffer (Lonza, Walkersville, MD) followed by centrifugation at 1500 rpm for 5 min to recover leucocytes. Leucocytes were then re-suspended in DMEM/F-12 with L-glutamine (Cellgro, Herndon, VA), 10% fetal bovine serum, 1% sodium pyruvate (Lonza, Walkersville, MD), 1% non-essential amino acids (Lonza, Walkersville, MD), 1% penicillin-streptomycin (Hyclone) and 20 ng ml^{-1} GM-CSF (R&D systems, MN, USA) (DC media) and plate on tissue culture flasks for 2 days in order to remove adherent cells. At 2 days the floating cells were transferred to low attachment plates and cultured in fresh DC media for expansion of DC precursor cells. At 7 days, cells were transferred to tissue culture plates to allow for DC adhesion and proliferation. At 10 days, they were lifted from tissue culture plates and used for various studies. For these studies, MPs were incubated at 37 °C for a period of 48 h prior to analysis or addition of T cells. Phagocytosable MPs (RAPA MP, RA MP) were added at a 10 : 1 MP to cell ratio, while unphagocytosable MPs (TGF-β1 MP, IL-10 MP) were incubated at a mass that encapsulated the effective concentration of that respective drug for the incubation media volume. Unloaded MPs and the soluble equivalent of released drugs doses (RAPA – 30 ng ml^{-1}; RA – 22 ng ml^{-1}; TGF-β1 – 11 ng ml^{-1}; IL-10 – 5 ng ml^{-1}) were included as controls.

2.4 Dendritic cell phenotype – maturation and tolerogenic markers

Dendritic cell maturation and tolerogenic molecule expression were quantified by measuring cell surface marker and intracellular cytokine levels by flow cytometry. Following MP incubation, DCs were lifted by incubating with a 5 mM Na_2EDTA in PBS solution at 37 °C for 20 min. Dendritic cells were then washed with 1% fetal bovine serum in PBS and incubated with antibodies against CD16/CD32 (Fcγ III/II Receptor) (clone 2.4G2, IgG2b, κ); (BD Pharmingen, CA, USA) for 15 min at 4 °C to block Fcγ receptors on DCs. Cells were washed and then stained with antibodies against CD80 (clone 16-10A1, IgG2, κ), CD86 (clone GL1, IgG2a, κ), I-A/I-E (clone M5/114.15.2, IgG2b, κ), CD11c (clone HL3, IgG1, λ2) (BD Pharmingen) and Ilt3 (Millipore) for 30 min at 4 °C. For intracellular molecules, cells were fixed, permeabilized and stained with antibodies against IDO (clone 10.1, IgG3) (eBiosciences), IL-10 (JES5-16E3, IgG2b) IL-12 (clone C15.6, IgG1) and IFN-γ (BD Pharmingen). Appropriate isotypes were used for each antibody species as negative controls. Data acquisition was performed using (FACScalibur, Becton Dickinson, NJ, USA) flow cytometry and the geometric fluorescent intensities as well as percent of positively stained cells determined. More than 10 000 events were acquired for each sample and data analysis was performed using FCS Express version 3 (De Novo Software, Los Angeles, CA).

Cell culture supernatants were collected after 48 h of MP incubation and after mixed lymphocyte culture, centrifuged to remove any cell debris and stored at −20 °C until analysis. The

IFN-γ, TGF-β1, IL-4, IL-10 and IL-12 cytokine production were analyzed using sandwich enzyme-linked immunosorbant assay (ELISA) kits (Becton Dickinson, NJ, USA) according to manufacturer's directions.

2.5 $CD4^+$ T cell and Treg isolation

Mouse $CD4^+$ T cells were purified from splenocyte suspensions by negative selection using Miltenyi $CD4^+$ T cell isolation kit II following the manufacturer's instructions. The purity of $CD4^+$ T cells as determined by flow cytometry was >90%.

2.6 Mixed lymphocyte coupling (MLC) – T cell suppression and Treg generation

For suppression studies, C57Bl6 DCs (2.5×10^4 per well) were co-incubated with the different combinations of MPs, as well as the relevant control treatments, in a 96 well tissue culture plate for 72 h at 37 °C in culture media consisting of RPMI 1640 with 10% FBS and 1% penicillin-streptomycin (Hyclone). After thoroughly washing away all unphagoctyosed and unbound MPs, Balb/cbyj $CD4^+$ T cells (1.25×10^5 per well) were added to each well and incubated at 37 °C for 3 days. Bromodeoxyuridine (BrdU) (kit from Beckton Dickinson) was pulsed into the culture media for the last 4 h. T cells were then immunofluorescently stained for BrdU according to manufacturer's specifications. Flow cytometry was then used to quantify T cell proliferation for the different treatments.

A similar method was employed for Treg generation studies with purified $CD4^+$ Balb/cbyj T cells. However, following DC – T cell co-culture for 72 h, T cells were immunofluorescently stained using anti-CD4-PECY7 (clone RM4-5), anti-CD25-APC (clone 7D4) and anti-Foxp3-PE (FJK-16s) antibodies (BD Pharmingen). Cells were analyzed using the FCS express V3 software (De Novo Software, Los Angeles, CA).

2.7 Statistical analysis

Statistical analyses were performed using general linear model ANOVA, followed by posthoc assessment using Tukey test to make pair-wise comparisons. Differences were considered significant when $p \leq 0.05$ using Systat (Version 12, Systat Software, Inc., San Jose, CA).

Results

3.1 Microparticle characterization

Microparticles were fabricated using a single emulsion, solvent evaporation technique for phagocytosable MPs, and by double emulsion, solvent extraction method for the larger unphagocytosable MPs. Microparticles were characterized by determining their size distributions, loading efficiencies and *in vitro* release kinetics. The size range of the phagocytosable MPs was determined by dynamic light scattering, calculated by volume, to be 1–5 μm, with RAPA MPs and RA MPs having average diameters of 2.3 μm and 1.6 μm, respectively. The average diameter for TGF-β1 MPs and IL-10 MPs was 29.9 μm and 30.5 μm, correspondingly. Representative dynamic light scattering plots are displayed (Fig. 1). The physical characteristics of the fabricated MPs are provided (Table 1), including the encapsulation efficiencies and the amount of factor encapsulated in the MPs on a per mass basis. Encapsulation efficiencies for RAPA and RA in MPs were 72 ± 3% and 62 ± 6%, respectively. The loading efficiencies for MPs prepared by the double emulsion technique, TGF-β1 and IL-10 MPs, were slightly lower at 61 ± 5% and 52 ± 5%, respectively. These loading values are consistent with values reported in previous studies. For instance, Lu *et al.* reported an entrapment efficiency of 84% for TGF-β1 in PLGA MPs using a double emulsion solvent evaporation technique.[40] Similarly, IL-2 (similar in mass to IL-10)-loaded MPs had a loading efficiency up to 60% in a study by Thomas *et al.*[41] Differences in encapsulation efficiencies reflect differences in encapsulate properties, differences in MP preparation techniques between phagocytosable and unphagocytosable MPs, and also differences in initial loading concentrations and solution composition.

The *in vitro* release kinetics for each MP was examined at pH 7.4 in a 2% Tween20-PBS solution. Fig. 2 shows the release profiles of RAPA MPs (Fig. 2A), RA MPs (Fig. 2B), TGF-β1 MPs (Fig. 2C) and IL-10 MPs (Fig. 2D). After 28 days, the cumulative release of RAPA and RA reached 95% and 100%, respectively. In contrast, cumulative release for the unphagocytosable MPs was less, reaching only 61% for TGF-β1 MPs and 74% for IL-10 MPs. The release profiles generally provided an initial burst release within the first week followed by a more gradual release for the

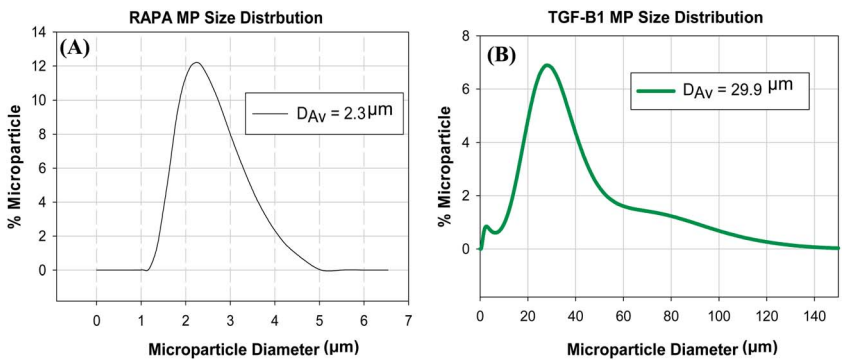

Fig. 1 Microparticle size determined using dynamic light scattering techniques for (A) Phagocytosable MPs (average diameter ~2.3 μm) and, (B) Un-phagocytosable (average diameter ~30 μm).

Table 1 Size and encapsulation efficiency of MPs loaded with pharmacological and biological agents

Biological/pharmacologic agent	Average diameter (μm)	Amount used/PLGA (μg 100 mg^{-1})	Encapsulation efficiency ± SD (%)	Loading ± SD (ng mg^{-1})
Rapamycin	2.3	250	72 ± 3	1800 ± 80
Retinoic acid	1.6	100	62 ± 6	620 ± 60
TGF-β1	29.9	6.25	61 ± 5	38 ± 3
IL-10	30.5	5	52 ± 5	26 ± 3

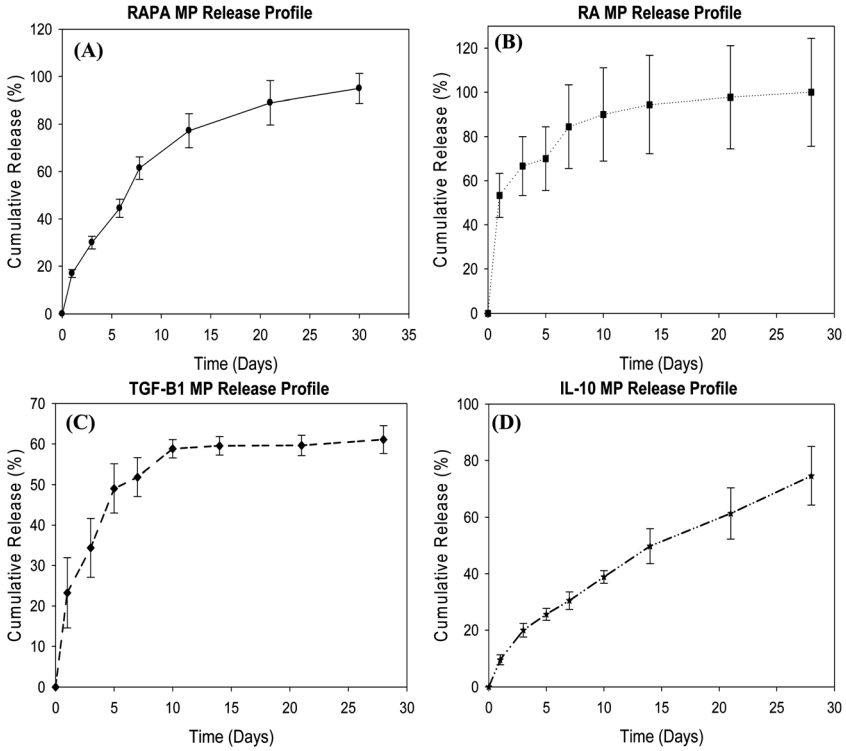

Fig. 2 *In vitro* release profiles of factors from phagocytosable microparticles: (A) Rapamycin, (B) Retinoic acid; and unphagocytosable microparticles: (C) TGF-β1 and (D) IL-10 in 2% Tween-20 PBS solution. Error bars on graphs represent standard deviation based on $n = 3$ for each MP type.

following three weeks. Differences in release profiles reflect differences in encapsulate properties, and differences in MP preparation methods. For example, the release profile of IL-10 MPs is more linear compared to TGF-β1 MPs. In this case, while the MPs were fabricated similarly by a double emulsion-solvent evaporation process, and the encapsulated proteins only slightly differ in molecular weights, solubility and isoelectric points, the initial loading solutions were different. The TGF-β1 was loaded with the excipients – citric acid and bovine serum albumin in a phosphate buffered solution, whereas the IL-10 was reconstituted in only DiH_2O. Additionally, the drug loading of IL-10 by weight percentage is less than that for TGF-β1 MP loading. Rothstein *et al.* reported that excipient addition and manipulation of drug loading may modify the "initial burst" typical observed in release of hydrophilic biologicals from PLGA particulate systems.[42] Excipient addition may influence osmotic pressure during fabrication resulting in increased burst magnitude. Initial burst magnitude of MP protein release could also be intensified by an increase of the protein initial loading concentration.[42] We postulate that these factors are influential in the notably different release profiles observed for TGF-β1 MPs and IL-10 MPs. Further, the acidity of micro-environments in the TGF-β1 MP likely accelerates the degradation rate of the polymer and therefore, the initial release burst.

3.2 Maturation status of MP-treated DCs

To assess the immunosuppressive ability of the combinatorial multi-factor dual MP systems, we investigated their effects on DC maturation. Specifically, expression of the markers of activation – stimulatory (MHC II) and costimulatory molecules (CD80, CD86), were quantified. These membrane-bound cell surface molecules bind cognate receptors on the surface of T cells. Formation of this APC-T cell immune synapse, along with DC production of cytokines, stimulates clonal expansion of specific T cell populations. Dual MP formulations were

co-cultured with DCs for 48 h, and control groups were included: untreated DCs (iDCs), unloaded MPs, the soluble equivalent doses and single factor MP controls. To simplify the comparison of DC activation states, the percent cells expressing a given marker, normalized by the expression of the control, immature DC (iDC) was found. Additionally, we devised a simple "composite maturation index", which is an equally-weighted average of the three activation markers measured (MHC II, CD80, CD86) in order to succinctly depict the comparisons.

The effect of combined RAPA MPs and TGF-β1 MPs on DC maturation was determined (ESI, Fig. S2A†). MHC II remained unchanged for all groups. CD80 expression decreased for the RAPA MP, TGF-β1 MP and the combined RAPA/TGF-β1 MP groups while CD86 levels decreased dramatically for RAPA MP, Sol RAPA, TGF-β1 MP, the combined RAPA/TGF-β1 MPs and Sol RAPA/TGF-β1 groups. Comparing the composite maturation index highlights these changes in maturation state for the RAPA/TGF-β1 groups (Fig. 3A). In particular, the single factor MP groups (RAPA MP and TGF-β1 MP) and the Sol RAPA group is considerably lower than the iDC and Unloaded MPs groups. The Sol TGF-β1 group did not suppress activation markers below that of iDC. The RAPA/TGF-β1 dual MP system, on the other hand provided lower expression of activation markers compared to iDC, Unloaded MPs, and Sol RAPA groups, and even lower expression than the single factor RAPA MP (but not the TGF-β1 MP). This result indicates that dual the RAPA/TGF-β1 MPs can substantially suppress DCs *via* reduction of stimulatory/costimulatory molecules. Notably, MP formulations performed as well or better than equivalent soluble doses of the factors, individually, or combined.

Next, RAPA/IL-10 MPs were investigated. MHC II expression levels varied only slightly among groups. Compared to iDC, significant reductions in CD80 were again found for the RAPA MP group, as well as the RAPA/IL-10 MP group, however this combination group did not provide further reduction than the RAPA MP alone. Significant reductions compare to iDC were also found in CD86 for the RAPA MP, Sol RAPA and RAPA/IL-10 MPs groups (ESI, Fig. S2B†). In this case, the composite maturation index comparisons confirm that this combination treatment, the RAPA/IL-10 MP, significantly reduces maturation markers but not to a greater extent than the single factor RAPA MP and Sol RAPA groups (Fig. 3B).

Combined TGF-β1 MPs and RA MPs were next investigated. MHC II levels were unchanged for all treatments (ESI, Fig. S2C†). Application of the single factor MP groups, TGF-β1

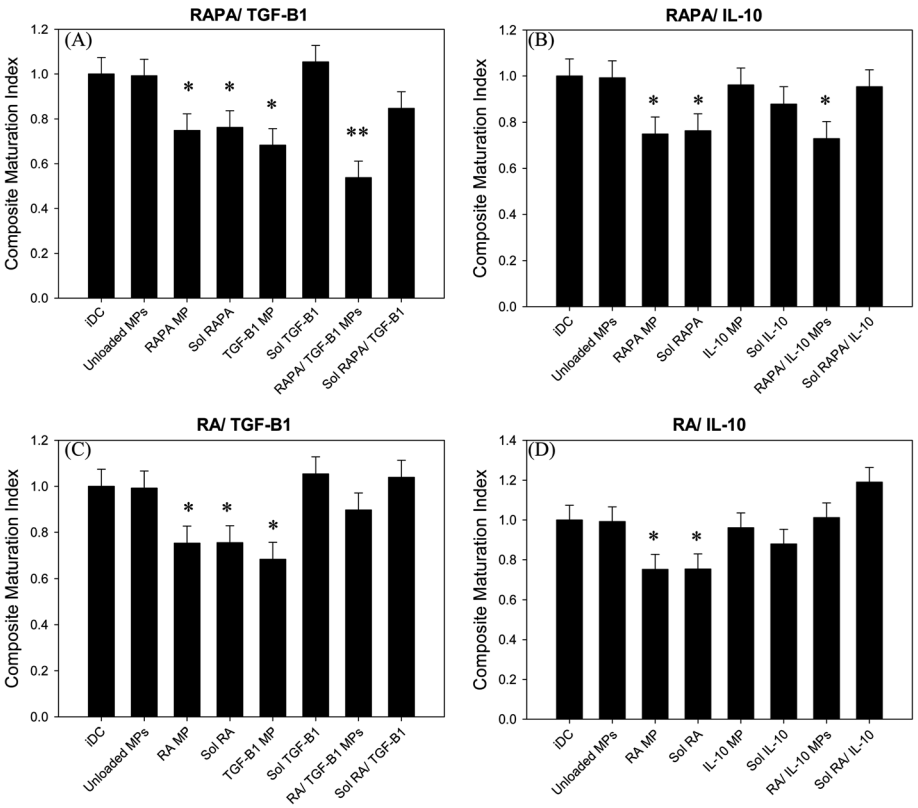

Fig. 3 Dual microparticle (MP) systems result in reduced levels of surface expression of MHC II and costimulatory markers (CD80, CD86) relative to immature dendritic cells (iDCs). Combinations of immunomodulatory phagocytosable MPs (RAPA MP, RA MP) and un-phagocytosable MPs (TGF-β1 MP, IL-10 MP) were investigated. Unloaded MPs and soluble equivalent doses were included as controls. The composite maturation index (Panels A – D) represents an unweighted average of the percent positive cells for activation markers in a treatment group, normalized to the iDC group. Pair-wise significant differences from: iDC and Unloaded MP groups are denoted by the * symbol; iDC, Unloaded MP and either one of the single factor MP groups are denoted by the ** symbol; iDC, Unloaded MP and both single factor MP groups are denoted by the ¥ symbol ($p \leq 0.05$).

MPs and RA MPs, significantly lowered expression of CD80 below that of iDC and unloaded MPs groups, but neither the soluble or combination groups had this effect. Similarly, for CD86 the single factor MP groups, as well as the Sol RA group, reduced expression levels. The composite maturation index comparisons (Fig. 3C) for these treatments indicate that overall, the single factor MP groups, TGF-β1 MP and RA MP, as well as the soluble RA reduce DC activation below that of iDC, with the combination MP and other treatments being comparable to the iDC group.

Lastly, combined RA MP and IL-10 MP treatment was investigated. Expression levels of MHC II were relatively unaffected compared to those seen for the iDC group (ESI, Fig. S2D†). The RA MP group was the only group that significantly reduced CD80 expression levels in comparison to the iDC group. The RA MP, as well as the Sol RA group also lowered CD86 surface expression considerably. The overall effect summarized by the composite maturation index (Fig. 3D), was that stimulatory/costimulatory molecule expression was only reduced for RA MP and Sol RA groups, where the combination MP treatment was no different from the iDC group.

Overall, we conclude that MP treatment either maintained a maturation profile equivalent to that of iDCs, or significantly reduced expression of activation markers. The lack of DC activation is one characteristic of tDCs, and these results do not rule out any of the treatments investigated. However, those treatments which suppressed activation marker levels below that of iDC, could indicate promise for durable suppression, requiring further investigation. It was also observed that MP treatments had a greater effect on DC activation than their respective soluble factor doses, illustrating the benefit of localized and direct delivery of immunosuppressive agents to cells by MP formulations.

In addition to expression of surface activation markers, DC cytokine secretion was measured (Fig. 4). Activated DCs characteristically express and secrete pro-inflammatory cytokines such as IL-12.[1] The control groups which included the addition of soluble lipopolysaccharide (Sol LPS, Unloaded MPs + Sol LPS) increased secretion of IL-12 dramatically (Fig. 4A), while the iDC and Unloaded MP groups produced low to negligible amounts. Interestingly, the RAPA/IL-10 dual MP treatment as well as the single MP treatments, RAPA MP, TGF-β1 MP and IL-10 MP, also induced higher amounts of IL-12 compared to iDCs. In contrast, the RA MP, RAPA/TGF-β1 MPs and RA/TGF-β1 MPs groups demonstrated negligible IL-12 levels, illustrating that different combinations of factor-loaded MPs can work together to suppress inflammatory cytokine section. In particular, single factor RAPA MP and TGF-β1 MP groups each induced IL-12 production, while the combination RAPA/TGF-β1 MPs suppressed it. Additionally, RA MPs, singly or in combination with other MPs, almost completely suppressed IL-12.

3.3 Maturation resistance of MP-treated DCs

To determine the extent to which DCs treated 48 h with immunomodulatory MPs were resistant to stimulating conditions, cells were subsequently exposed to lipopolysaccharide

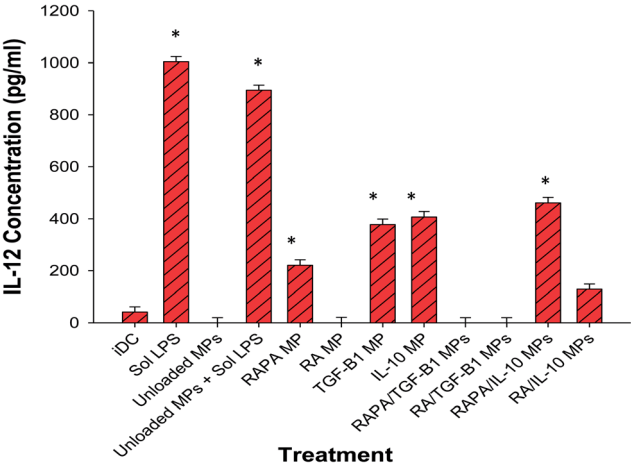

Fig. 4 Dual MP systems modulate levels of IL-12 secretion by dendritic cells (DCs). Combinations of immunomodulatory phagocytosable MPs (RAPA MP, RA MP) and un-phagocytosable MPs (TGF-β1 MP, IL-10 MP) were investigated. Unloaded MPs, unloaded MPs + Sol LPS, iDC and Sol LPS conditions were included as controls. Supernatants were by ELISA. Data shown represent mean cytokine concentration ± standard error ($n \geq 7$). Pair-wise significant differences from iDC and Unloaded MP groups are denoted by the * symbol ($p \leq 0.05$).

(LPS) for 24 h, and expression of MHC II, CD80 and CD86 was again quantified. The results are summarized using a composite maturation index (Fig. 5), as before, providing an average of the normalized (to iDC) percentages of cells expressing these activation markers. Broadly, all of the combined MP treatments provided resistance to 24 h LPS activation, maintaining equivalent expression levels of activation markers as iDCs, and at levels lower than the controls, Sol LPS and Unloaded MPs + Sol LPS. In detail, the composite maturation indexes for the dual MP systems were significantly different from the levels observed for Sol LPS, Unloaded MPs + Sol LPS, single factor unphagocytosable MP, single soluble factors, and dual soluble factors groups (Fig. 5A–D). Notably, the phagocytosable single factor MPs, RAPA MPs and RA MPs, drove the maturation resistance effect, indicating their ability to counteract external maturation stimuli. Comparing the Sol LPS and Unloaded MPs + Sol LPS groups, reveals the surprising ability of unloaded MPs to confer resistance to maturation, indicating that 50 : 50 PLGA itself can have biological, immunosuppressive function.

3.4 Expression of Ilt3 on DCs exposed to MP formulations

In addition to down-regulating stimulatory markers, one mechanism tDCs can resist activation is through surface expression of inhibitory molecules such as immunoglobulin-like transcript 3 (Ilt3), and high expression of Ilt3 can be a feature of some regulatory DCs.[43,44] However, none of the treatments investigated here, either in soluble or MP form, increased Ilt3 expression compared to iDC (ESI, Fig. S3†). In contrast, the Sol LPS control increased Ilt3 expression to a small extent. In fact, RAPA and RA treatments decreased Ilt3 levels

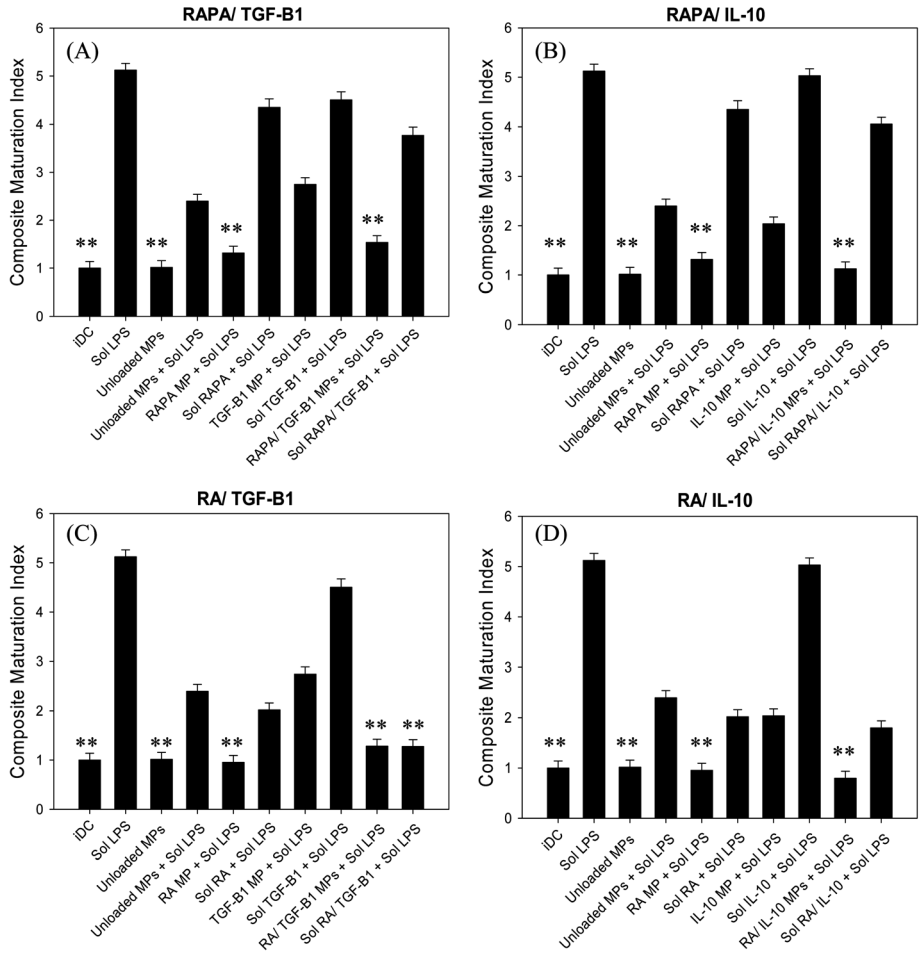

Fig. 5 Dendritic cells treated with dual microparticle (MP) systems resist LPS stimulation. Combinations of immunomodulatory phagocytosable MPs (RAPA MP, RA MP) and un-phagocytosable MPs (TGF-β1 MP, IL-10 MP) were investigated. Unloaded MPs, soluble equivalent doses and addition of soluble LPS were included as controls. MPs or soluble treatments were incubated with DCs for 48 h then washed to remove unbound MPs and soluble factors. Subsequently, soluble LPS was added to culture wells at a concentration of 1 μg ml^{-1}. After 24 h incubation period, DCs were lifted and surface markers of activation – MHC II, CD80, CD86 were quantified by flow cytometry. The composite maturation index represents an average of the activation markers. Pair-wise significant differences from Sol LPS, Unloaded MPs + Sol LPS and either one of the single factor MP + Sol LPS groups are denoted by the ** symbol ($p \leq 0.05$).

below iDC, and this result with rapamycin is consistent with others.[45,46] The only MP formulation that maintained Ilt3 levels was the IL-10 MP treatment, which is not inconsistent with other reports that IL-10 exposure induces upregulation of Ilt4, a closely related inhibitory receptor.[47,48]

3.5 Suppression of allogenic T-cell proliferation by MP-treated DCs

To determine the suppressive effect of MP-treated DCs (from C57Bl6 mice) on allogenic effector T cell response, we quantified proliferation of CD4$^+$ T cells (from Balb/cbyj mice) when cocultured in a mixed lymphocyte reaction. Dendritic cells were treated for 2 days and then washed to remove unbound MPs and soluble factors. Allogeneic splenic CD4$^+$ T cells were then co-cultured for 3 days. The proliferating T cells were quantified and expressed as the percent proliferating in the treatment group, normalized by the percent in the iDC group (Fig. 6). Dual RAPA/TGF-β1 MP treatment inhibited T cell proliferation drastically beyond the levels observed for both the iDC and Unloaded MPs groups, to a lower level of proliferation than the TGF-β1 MP group, but equivalent to the RAPA MP formulation (Fig. 6A). The RAPA/IL-10 MPs combination, as well as the single factor MP groups, RAPA MP and IL-10 MP, resulted in significant reduction of T cell proliferation compared to both iDCs and Unloaded MP-treated DCs. However, no additive effect was produced for this combination (Fig. 6B). The RA/TGF-β1 dual MP system inhibited T cell proliferation to levels well below either the iDC and Unloaded MPs groups, and to a lower proliferation level than the TGF-β1 MP, but equivalent to the RA MP formulation (Fig. 6C). Finally, the dual RA/IL-10 MPs inhibited T cell proliferation to the lowest levels across the combination formulations, well below either the iDC or Unloaded MPs groups (Fig. 6D). This was the only MP combination which suppressed T cell proliferation to levels significantly lower than both of its single factor MPs. Lastly, a word

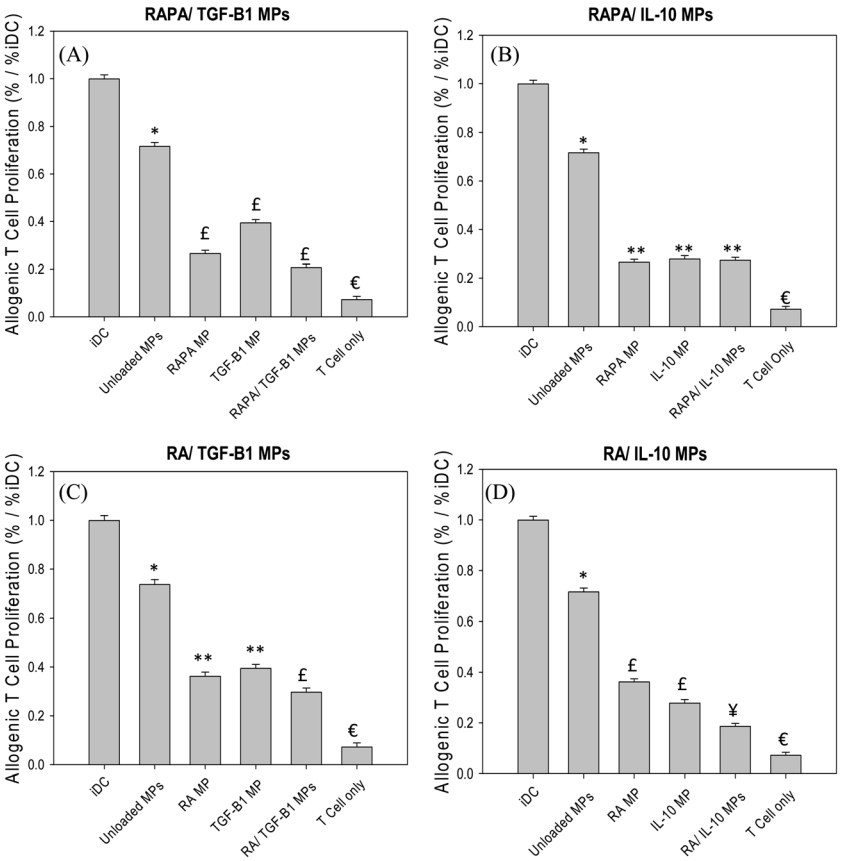

Fig. 6 Dendritic cells treated with dual MP systems differentially suppress T cell activation. Briefly, MPs were incubated with C57Bl6 DCs for 2 days followed by washing to remove unbound MPs. Subsequently, freshly-isolated Balbc CD4+ T cells were added to culture wells and co-cultured in a mixed lymphocyte reaction. Allogeneic T cell proliferation was then measured. Data shown represent the mean ± standard error ($n \geq 5$). Pair-wise significant differences are denoted by: the * symbol for comparisons to the iDC group; the ** symbol for comparisons to the iDC and Unloaded MPs groups; the £ symbol for comparisons to the iDC, Unloaded MPs and either one of the single factor groups; the ¥ symbol for comparisons to the iDC, Unloaded MPs and both of the single factor groups; and the € symbol for comparisons to the all other groups ($p \leq 0.05$).

regarding control groups – without allogeneic DC stimulus, T cell proliferation is minimal as shown in the T cell only group. More interestingly, it is noted that DCs treated with unloaded MPs have a significant effect on allogeneic T cell proliferation, consistent with the prior result above showing the ability of 50 : 50 PLGA MPs to confer DC resistance to maturation stimulus.

3.6 Generation of Tregs by MP-treated DCs

After determining the highly suppressive nature of our dual MP-treated DCs, we investigated if MP-treated DCs could increase $CD4^+CD25^+FoxP3^+$ regulatory T cell numbers. The same experimental setup as the above mixed lymphocyte reaction was used and cells were then immuno-stained against CD4, CD25 and FoxP3. Comparing the controls, iDC and T Cells only, the dramatic difference between the percent of Tregs illustrates that in this allogeneic coupling, DCs are required for the induction of Tregs. While all the MP systems supported Treg numbers equivalent to iDC and Unloaded MPs, with the exception of the combination RA/TGF-β1 MPs, none of the formulations resulted in DCs capable of increasing $CD4^+CD25^+FoxP3^+$ regulatory T cell numbers over the iDC control group (ESI, Fig. S4†). This indicates a role for regulatory mechanisms other than the upregulation of Tregs. Finally, again using the mixed lymphocyte reaction, the production of cytokines typified as Th1 (IFN-γ), Th2 (IL-4), and regulatory (TGF-β1), was quantified in supernatants. Microparticle formulations did not induce altered production of IFN-γ (Fig. 7A) or IL-4 (Fig. 7B). On the other hand, TGF-β1 MPs in all formulations, the single TGF-β1 MP formulation, the RAPA/TGF-β1 MPs and the RA/TGF-β1 MPs, all induced increased production of TGF-β1 in mixed lymphocyte cultures (Fig. 7C).

Discussion

Over the past decade, exploration of PLGA particulate systems for delivery of a wide range of drugs in therapeutic applications has increased exponentially[34–37] Moreover, researchers have recognized that the versatility of polymeric particulate systems could be adopted for DC-based immunotherapy. Dendritic cells, the most potent of APCs, play a critical role in the initiation and guidance of adaptive immunity, as well as maintenance of suppressive networks that prevent autoimmunity.[1,4] DC-based

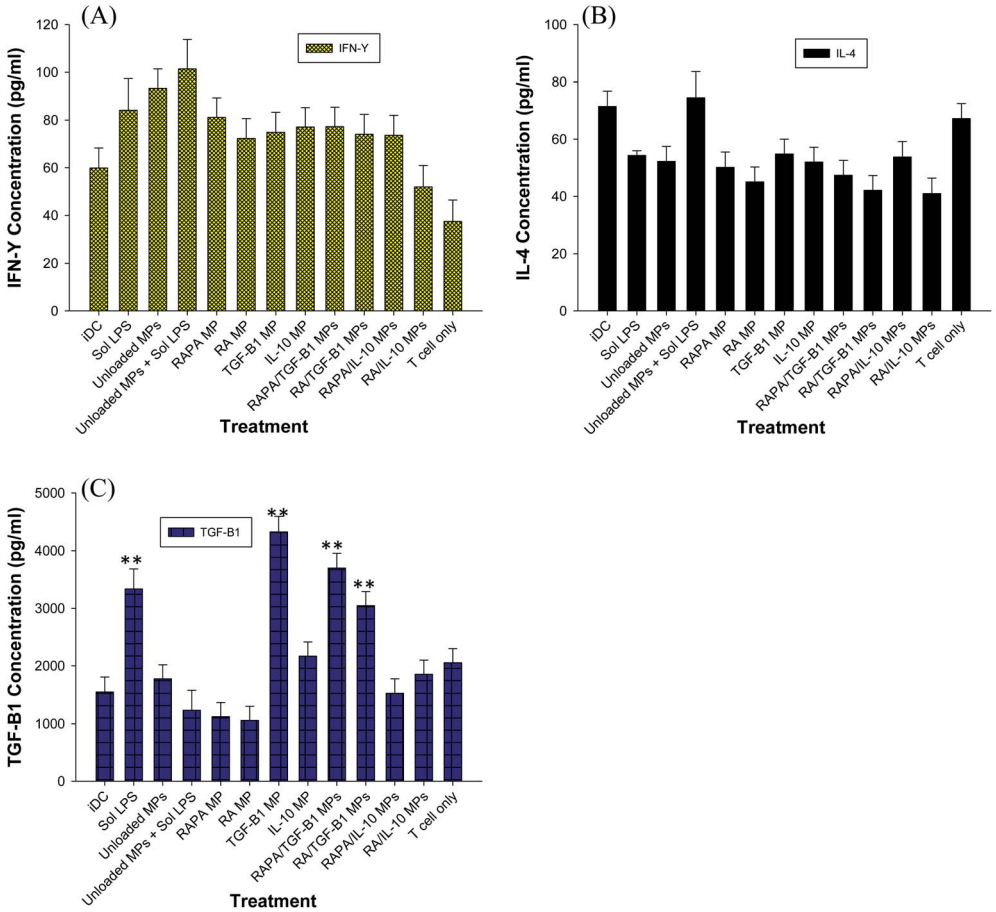

Fig. 7 Dendritic cells treated with dual MP systems do not produce IFN-γ or IL-4, Th1/Th2 skewing cytokines, but do induce anti-inflammatory cytokine TGF-β1 secretion in a mixed lymphocyte setting. Briefly, MPs were incubated with C57Bl6 DCs for 2 days followed by washing to remove unbound MPs. Subsequently, freshly-isolated Balbc CD4+ T cells were added to culture wells and co-cultured in a mixed lymphocyte reaction (MLR). Following the MLR, supernatants were collected and analyzed for (A) IFN-γ, (B) IL-4, and (C) TGF-β1 cytokines by ELISA. Data shown represent mean cytokine concentration ± standard error ($n \geq 7$). Pair-wise significant differences are denoted by: the * symbol for comparisons to the iDC group and the ** symbol for comparisons to the iDC and Unloaded MPs groups ($p \leq 0.05$).

immunotherapy attempts to exploit this by programming DCs to a set functionality that provokes advantageous downstream immune responses which may not be possible *in vivo* under diseased conditions. For instance, in patients with autoimmune conditions, *ex vivo* DCs are manipulated with tolerance-inducing factors (*e.g.* oligonucleotides, corticosteroids, UV radiation) to produce tolerogenic DCs (tDCs) that can be reintroduced to the patient's body.[3,49] In a study by Pedersen *et al.*, human monocyte-derived DCs exposed to a combination of dexamethasone and 1α,25-dihydroxyvitamin D induced DCs that secreted IL-10 and, to which allogeneic T cells were non-responsive.[50] Until recently, DCs have been exclusively manipulated *ex vivo* for this purpose, but scientists have recently begun to realize the advantages of *in vivo* modulation by targeting DC populations *in vivo*.[38,51] These approaches include the use of PLGA particulate systems, which can be tailored to be multi-functional and actively targeted. For instance, Jhunjhunwala *et al.* demonstrated that PLGA MPs encapsulating immunomodulatory factors supported regulatory cell phenotypes.[52,53]

Our interest in the use of PLGA delivery systems for modulation of DCs lies in the realm of protective autoimmunity, particularly for type 1 diabetes. As demonstrated by Phillips and associates, there is huge potential for type 1 diabetes therapy through *in vivo* delivery of drug-loaded MPs to DCs and other APCs.[54] We were also cognizant that immunomodulatory effects on immune cells often occur when a plurality of agents are acting in concert.[20,32,44,53] Therefore, we hypothesized that PLGA MPs could be used for targeted, local, and simultaneous delivery of multiple factors to DCs. Further, we hypothesized that combinatorial delivery of immunosuppressive factors may result in cumulative effects and ultimately the generation of a DC with superior regulatory features. We tested these suppositions by fabricating PLGA MPs that delivered combinations of either RAPA or RA intracellularly, and either TGF-β1 or IL-10 extracellularly to murine bone marrow-derived DCs. The aim was to target delivery to the respective receptors for these agents.

To accomplish this, we prepared two classes of MPs: (i) phagocytosable MPs that deposit their payload intracellularly and (ii) MPs that are not be readily taken up and release agents into the extracellular milieu. We fabricated phagocytosable MPs with an average diameter between 1.5 and 2.5 μm. At this size,

MPs are efficiently taken up by DCs. In contrast, our unphagocytosable MPs at approximately 30 μm in diameter are beyond the size an APC can phagocytose particulate matter.[55] Prepared MPs were loaded with desired immunosuppressive agents as verified using well established pharmaceutical drug and protein detection methods.[40,52,56] The amounts of agent loaded in PLGA MPs are comparable to that observed in previously published studies. For instance, Jhunjhuwala *et al.* and Hadaddi *et al.* used particulate systems with rapamycin weight loadings of 0.37% and 0.05% respectively to effectively influence dendritic cell phenotype.[52,57] At a weight loading of 0.18%, it was expected that our rapamycin-loaded MPs would induce responses similar to these studies. In a different study, Jhunjhunwala *et al.* fabricated TGF-β1 MPs using an analogous method to that described above. However, their 25% loading efficiency[53] was much lower than our 61%, an inconsistency which can be accounted for in differences in the composition of loading solutions. In our MP formulations, the release of agents followed general solute release behavior as described by Fick's second law of diffusion.[58] The general profile follows an initial burst release within the first week followed by steady incremental emission. Release kinetics of our fabricated MPs varied across MP formulation, these profiles could be manipulated through modification of parameters such as polymer molecular weight, lactide to glycolide ratio as well as particle size.[37]

Tolerogenic DCs are typically characterized by reduced levels of expression of stimulatory/costimulatory molecules (*e.g.*, MHC II, CD40, CD80, CD86) and expression of inhibitory markers (*e.g.*, Ilt3).[44] For this reason, we determined the expression levels of CD80, CD86 and MHC II on DCs exposed to our combination MPs. Salient points to be highlighted from this analysis – (i) all formulations including the unloaded MP group at least maintained an iDC maturation profile, (ii) MPs with encapsulated agents generally effected greater modulation than their soluble equivalent controls and, (iii) combination of single factor MPs does not always lead to cumulative dampening of DC activation markers. These results suggest that microparticles fabricated with 50 : 50 PLGA are well suited for non-activating applications,[39] that targeted, localized release of factors can be more effective than a soluble dose,[59] and also that various factor combinations should be explored to optimize for DC function, for example, *via* high throughput platforms.[33,60]

With regards to maturation of DCs treated with unloaded MPs, a number of authors have reported on the stimulatory nature of 75 : 25 PLGA particulates and further, its use as an adjuvant to boost immunity against targeted antigens.[61,62] On the contrary, other reports have described PLGA particles as being immune-inert systems that function only as vehicles.[63] Our findings support the latter case, but the differences in PLGA formulations used should be pointed out. The more hydrophobic nature of the 75 : 25 PLGA polymer compared to 50 : 50 PLGA may explain this discrepancy, as the more hydrophobic polymer will persist longer, and the composition and conformation of surface adsorbed proteins are expected to be different. Interestingly, there are reports that the degradation products of PLGA into it constituent monomers, particularly lactic acid, can down-regulate stimulatory molecules on DCs following exposure.[64,65] Particularly striking are our results on the resistance LPS maturation of DCs when treated with unloaded PLGA MPs, which is consistent with this finding.

Under diseased conditions, DCs are exposed to external stimuli that dictate an activated phenotype.[4] For intervention of autoimmunity in such an activating environment, DCs with the capacity to resist maturation may be advantageous. In addition to the carrier PLGA itself acting as an immunomodulatory, factor-loaded MPs further modulated DCs. Single and dual factor MPs generally resisted LPS maturation, where the single factor phagocytosable MP groups, seemed to drive this effect. This suggests that ingested MPs act as an intracellular controlled release depot of immunomodulatory agent that prevents upregulation of positive stimulatory molecules on DCs, which is consistent with observations by other groups as well.[63]

Although some factors disappointingly reduced levels of the inhibitory molecule, Ilt3, in fact there are numerous mechanisms by which DCs are capable of regulatory action, and MP-treated DCs were still able to dramatically suppress T cell activation. Some dual MP systems improved suppression compared to single factor MPs, particularly for the RA/IL-10 MPs, and the RA/TGF-β1 MPs to a lesser extent.

Mechanisms capable of suppressing activation of T cells are multiple and complex. DC modalities that retard T cell propagation include T cell anergy, generation of regulatory T cells (*e.g.*, $FoxP3^+$ Tregs, Tr1 cells, TGF-β-secreting T cells), and DC-mediated T cell apoptosis.[66–68] Given that we observe low constitutive expression of activation molecules due to dual MP and single MP treatment, it is plausible to attribute suppression to T cell anergy. However, the levels of suppression do not always correlate well with the maturation status for our treatments. For instance, RAPA/IL10 MPs combination treatment show higher levels of activation markers on DCs than the RAPA MP treatment but still result in greater T cell suppression. This indicates either the possibility for some unknown threshold effect regarding activation marker levels, or that other modes of suppression may be engaged. To explore this, we quantified DC-mediated generation of $CD25^+CD4^+FoxP3^+$ Tregs. This class of regulatory T cells has been implicated for maintenance of local immune suppression through induction of tDCs, effector T cell inactivation and apoptosis.[69] Our results demonstrate that, with the exception of the RA/TGF MP group, MP treatments, while not increasing Treg numbers, at least were able to support levels equivalent to iDC. It is possible there could be MP treatment-specific functional differences in the Treg populations which may not be reflected in numbers. Moreover, there are indications that other immunosuppressive mechanisms are involved. For instance, the mixed lymphocyte reaction cytokine secretion data, in particular for TGF-β1, indicates that DCs treated with TGF-β1 MP formulations induce a TGF-β1-secreting cell population. Based on these observations as a whole, we can conclude that the high levels of suppression we observe for MP formulations are likely due to DC hypo-stimulatory state and T cell anergy. However, other uninvestigated mechanisms like DC-mediated T cell apoptosis could also be involved. While synergism was not observed, these results demonstrate that

combinations of immunomodulatory agents can have cumulative effects on DCs that may be beneficial to the treatment of conditions caused by excessive immune activation. Screening combinatorial particle formulations with *in vitro* cell populations for biomarker expression is useful for advancing potential formulation candidates. This approach is being explored with increasing throughput[33,60] and could aid in the search for definitive *in vitro* correlates of *in vivo* efficacy.

Conclusion

The ability of combinatorial pairings of immunosuppressive factor-loaded MPs to modulate DC function was examined. Two classes of MPs (one phagocytosable and one unphagocytosable) encapsulating immunosuppressive agents were fabricated and our results demonstrate feasibility of this controlled release scheme to target factors to relevant cell surface and intracellular locations. Treated DCs were immature, resisted maturation, and capable of dramatic suppression of T cell activation. The greatest suppression resulted from a combination MP formulation, which was more effective than the single factor MP formulations. Altogether, these results demonstrate that combinatorial formulations of immunosuppressive factors using dual-sized microspheres can boost immunosuppressive effects over those obtainable by single factor MP and soluble bolus administration. This study suggests that polymeric microparticulate systems are a viable approach for delivery of tolerance-inducing vaccines for autoimmune and transplantation applications.

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