



In vivo induction of regulatory T cells promotes allergen tolerance and suppresses allergic contact dermatitis



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ABSTRACT

Allergic contact dermatitis (ACD) is a common T-cell mediated inflammatory skin condition, characterized by an intensely pruritic rash at the site of contact with allergens like poison ivy or nickel. Current clinical treatments use topical corticosteroids, which broadly and transiently suppress inflammation and symptoms of ACD, but fail to address the underlying immune dysfunction. Here, we present an alternative therapeutic approach that teaches the immune system to tolerate contact allergens by expanding populations of naturally suppressive allergen-specific regulatory T cells (Tregs). Specifically, biodegradable poly(ethylene glycol)-poly(lactic-co-glycolic acid) (PEG-PLGA) microparticles were engineered to release TGF- β 1, Rapamycin, and IL-2, to locally sustain a microenvironment that promotes Treg differentiation. By expanding allergen-specific Tregs and reducing pro-inflammatory effector T cells, these microparticles inhibited destructive hypersensitivity responses to subsequent allergen exposure in an allergen-specific manner, effectively preventing or reversing ACD in previously sensitized mice. Ultimately, this approach to *in vivo* Treg induction could also enable novel therapies for transplant rejection and autoimmune diseases.

1. Introduction

Allergic contact dermatitis (ACD) is a common inflammatory skin condition that affects an estimated 15–20% of the general population [1], with annual direct medical costs in excess of \$1.6 billion in the U.S [2]. ACD typically presents as an intensely pruritic rash at the site of contact with one of > 4350 potential chemical allergens, including urushiol oil (poison ivy), metals (e.g. nickel), fragrances, topical antibiotics, and industrial chemicals [3]. Mechanistically, ACD is an antigen-specific, T-cell-mediated delayed-type hypersensitivity (DTH). During the sensitization phase, or first contact, chemical allergens, known as haptens, bind to endogenous epidermal proteins. Cutaneous dendritic cells (DCs) educated in the skin carry the resulting hapten-protein conjugates (or foreign protein) to skin-draining lymph nodes

(DLN), and present them to T cells in a pro-inflammatory context. Naïve T cells that recognize these specific antigens proliferate and differentiate into effector and memory T cells. Upon subsequent exposure to the same hapten (or foreign protein), primed effector T cells are recruited to the skin and cause destructive cutaneous inflammation in sensitized individuals (elicitation phase) [4,5].

Whenever possible, identifying and avoiding contact with offending allergens is the best way to manage ACD. In the event of incidental contact with an allergen, topical or systemic corticosteroids are typically used to suppress the resulting inflammation [6]. Corticosteroids exhibit broad anti-inflammatory effects on innate and adaptive immune cells, as well as keratinocytes; however, adverse effects are associated with the prolonged use of moderate to high potency corticosteroids, which is often required for treatment of ACD [7–9]. Even with

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treatment, persistent dermatitis occurs in > 1 in 3 individuals [10]. Furthermore, current therapies do not address the underlying allergen-specific adaptive immune responses or prevent future allergic reactions. Thus, novel therapeutic approaches to modulate T-cell-mediated immune responses to contact allergens may improve the treatment of ACD.

One such approach involves increasing the presence of populations of suppressive T cells, known as regulatory T cells (Tregs). $CD4^+ FoxP3^+$ Tregs suppress inflammation through a combination of secreted and surface bound factors, and promote peripheral tolerance, or hypo-responsiveness, to self and foreign antigens [11]. While IFN- γ producing $CD8^+$ T-bet $^+$ cytotoxic T cells (Tc1) and $CD4^+$ T-bet $^+$ helper T cells (Th1) are predominant effectors of cutaneous inflammation in ACD, prior studies have identified a pivotal role for Tregs in the resolution of DTH responses [4,12]. Specifically, endogenous Tregs have been shown to control sensitization and contribute to the resolution of inflammation in the later stage of the elicitation phase [4,13]. Notably, depletion of Tregs exacerbates and prolongs DTH responses [13–15], while systemic infusion of *ex vivo* expanded Tregs significantly reduces skin inflammation [16]. To date, the primary clinical method for increasing Treg populations involves isolation, *ex vivo* expansion, and reinfusion of Tregs; however, drawbacks with such an approach include difficulty isolating and expanding pure populations of Tregs, the requirement for GMP facilities, and the need for multiple clinic visits for cell isolation and reinfusion [17,18].

To develop alternative methods to expand Treg *in vivo*, we considered the natural mechanism by which tolerogenic DCs induce differentiation of naïve $CD4^+$ T cells to Tregs, including their secretion of the cytokines IL-2 and TGF- β 1 [19]. Furthermore, rapamycin, a natural macrolide with immunosuppressant properties, is known to preferentially suppress proliferation of effector T cells and promote expansion of Treg populations [20]. To partially mimic the Treg-inducing function of tolerogenic DCs, we developed biodegradable polymeric microparticles (MPs) that controllably release TGF- β 1, rapamycin, and IL-2, and previously showed that they promote *in vitro* differentiation of naïve $CD4^+$ T cells to functionally suppressive $CD4^+ FoxP3^+$ Tregs [21]. Recognizing the importance of Tregs in suppressing inflammation associated with DTH responses, we hypothesized that TReg-Inducing “TRI” MPs could be used to expand Tregs *in vivo* and suppress ACD. Here we present new TRI MP formulations engineered to provide short-term (~ 1 week) sustained release of TGF- β 1, Rapamycin, and IL-2, and demonstrate that they expand Treg populations and reduce effector T-cell populations in hapten- and protein-mediated murine models of ACD. Furthermore, *in vivo* Treg-induction with TRI MP effectively suppress DTH responses and protect skin from subsequent allergen exposures. Ultimately, this therapeutic approach may have the potential to ameliorate or protect against destructive inflammation in a variety of T-cell-mediated conditions, including allograft rejection, autoimmune diseases, and chronic inflammatory diseases.

2. Materials and methods

2.1. Mice

Female C57BL/6 and congenic CD45.1 B6 (B6-SJL-*Ptpcr^aPepe^b*/BoyJ) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 8–12 weeks of age. OVA TCR-transgenic B6 Rag1 $^{-/-}$ OT-I (B6.129S7-Rag1 ^{tm1Mom} Tg(TcraTcrb)1100Mjb) and B6 Rag2 $^{-/-}$ OT-II (B6.129S6-Rag2 ^{tm1Fwa} Tg(TcraTcrb)425Cbn) mice were purchased from Taconic (Rensselaer, NY). All mice were maintained under specific pathogen-free conditions at the University of Pittsburgh, and experiments were conducted in accordance with IACUC guidelines.

2.2. Microparticle fabrication

Poly(ethylene glycol)-poly(lactic-co-glycolic acid) (PEG-PLGA)

microparticles (MPs) were fabricated using an emulsion-solvent evaporation method [21]. A 2.5% (wt/vol) polymer solution was prepared by dissolving 40 mg mPEG-PLGA (5 kDa PEG:20 kDa PLGA; Poly-SciTech, West Lafayette, IN) and 160 mg ester-capped PLGA (14 kDa for IL-2 MP and Rapa MP or 40 kDa for TGF- β 1 MP, 50:50 LA:GA; Sigma Aldrich, St. Louis, MO) in 8 mL dichloromethane. For IL-2 and TGF- β 1 MPs, 5 μ g of recombinant protein (rmIL-2 from R & D Systems, Minneapolis, MN; or rhTGF- β 1 from PeproTech, Rocky Hill, NJ) was dissolved in 200 μ L deionized water (diH_2O), added to the organic polymer phase, and sonicated at 25% amplitude for 10 s (Vibra-Cell, Newton, CT). For Rapa MPs, 1.5 mg rapamycin (Alfa Aesar, Ward Hill, MA) was dissolved in 150 μ L DMSO and added to the polymer phase without sonication. The resulting primary emulsion (solution for rapamycin) was transferred to 60 mL of 2% (wt/vol) poly(vinyl alcohol) (PVA, MW ~ 25 kDa, 98% hydrolyzed; Polysciences, Warrington, PA) in diH_2O and homogenized (L4RT-1; Silverson, East Longmeadow, MA) on ice at 10,000 rpm for 1 min. The resulting double or single emulsion was then added to 80 mL of 1% PVA, and stirred (600 rpm) for 3 h on ice to allow the dichloromethane to evaporate. Subsequently, MPs were centrifuged (3000g, 8 min, 4 $^{\circ}C$), washed 4 times in diH_2O to remove residual PVA, re-suspended in 10 mL diH_2O , flash frozen, and lyophilized for 72 h (Virtis Benchtop K freeze dryer, Gardiner, NY).

2.3. Microparticle characterization

Surface characterization of MPs was conducted using a scanning electron microscope (JSM-6330F; JEOL, Peabody, MA), and particle size distributions were determined by volume impedance measurements using a Multisizer-3 (Beckman Coulter, Brea, CA). For IL-2 or TGF- β 1 release assays, 5 mg MPs were suspended in 1 mL PBS with 1% bovine serum albumin (BSA), and incubated at 37 $^{\circ}C$ with end-over-end rotation. Supernatant release media was sampled and replaced daily, and IL-2 or TGF- β 1 quantified by ELISAs (R & D Systems). For rapamycin release assays, 5 mg MPs were suspended in 1 mL PBS with 0.2% Tween80 (to maintain sink conditions [22]), and rapamycin concentrations in supernatant were determined by spectrophotometry (absorbance at 278 nm). Total loading of IL-2 and TGF- β 1 was determined using a two-phase extraction method with surfactant [23]. Briefly, 5 mg MPs were dissolved in 0.5 mL dichloromethane and cytokines extracted three times into 0.25 mL volumes of PBS + 0.1% sodium dodecyl sulfate (SDS; Sigma). Cytokine concentrations in the pooled aqueous phases were determined by ELISAs, and used to calculate total encapsulation. Rapamycin loading was determined by dissolving 5 mg Rapa MPs in acetonitrile and measuring absorbance (278 nm) of the resulting solution. Acetonitrile spiked with rapamycin was used to generate a standard curve. Encapsulation efficiencies are expressed as ratios of actual to theoretical loading.

Bioactivity of encapsulated IL-2 and TGF- β 1 was assessed by IL-2-induced proliferation of HT-2 cells, or TGF- β 1-mediated inhibition of IL-4-induced HT-2 proliferation [24]. HT-2 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA), 10 mM Hepes (Lonza, Walkersville, MD), 2 mM L-glutamine (Gibco by Life Technologies, Thermo Fisher), 1 mM sodium pyruvate (Sigma), 1 $\times$ antibiotic-antimycotic solution (Sigma), 1 $\times$ non-essential amino acids (NEAA; Lonza), and 55 μ M 2-mercaptoethanol (Gibco). For HT-2 expansion, media also contained 10 ng/mL rmIL-2. To assay encapsulated IL-2 and TGF- β 1, 10 mg MPs were incubated at 37 $^{\circ}C$ in 1 mL supplemented RPMI, and release samples taken at 24 and 48 h. IL-2 and TGF- β 1 concentrations in the release samples were determined by ELISAs. Unencapsulated, stock cytokines and release samples were serially diluted in supplemented RPMI, and added to 96-well flat-bottom plates (100 μ L/well). HT-2 cells in the log-phase of growth (2 days after last sub-culture) were washed three times, re-suspended in supplemented media without IL-2, and added to each well (100 μ L, 2×10^4 cells/well). For the TGF- β 1 assay, all wells also contained 7.5 ng/mL rmIL-4 (PeproTech). HT-2 cells were cultured for

48 h at 37 °C and 5% CO₂, and cell proliferation measured with a colorimetric MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI).

2.4. Hapten-mediated DTH model

Two chemical haptens, 2,4-dinitrofluorobenzene (DNFB) and 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, OXA) were purchased from Sigma Aldrich and dissolved in acetone and olive oil (4:1 v/v). C57BL/6 mice were sensitized by topical application of 20 µL of 0.5% DNFB (or 1.0% OXA) to the dorsal size of both ears. Alternatively, 50 µL of 0.5% DNFB was applied to the shaved abdomen for sensitization. To elicit a DTH response, mice were challenged 10 days post-sensitization (5 days if sensitized on abdomen) with 20 µL of 0.5% DNFB (or 1.0% OXA) applied to both ears. For the re-challenge experiment, mice were challenged with DNFB a second time 10 days after the first challenge. Ear thickness was measured immediately prior to challenge (or re-challenge) and 24, 48, 72, and 96 h post-challenge using an engineer's spring-loaded micrometer (Mitutoyo, Aurora, IL). Increases in ear thickness (*i.e.* ear swelling), relative to the baseline measurements, were indicative of inflammation.

2.5. Protein-mediated (OVA) antigen-specific DTH model

OVA-specific CD8⁺ and CD4⁺ T cells were isolated from spleens of OT-I and OT-II mice (CD45.2⁺) and adoptively transferred to congenic CD45.1 B6 mice by tail vein injection (5×10^6 OT-I and 5×10^6 OT-II cells per mouse). One day later, the mice were sensitized to ovalbumin (OVA) by transdermal application of dissolvable microneedle arrays (MNAs), each containing 100 µg OVA (Grade V, Sigma Aldrich), to both ears. The carboxymethyl cellulose-based MNAs were fabricated according to the spin-casting method described in [25]. To elicit a DTH response, mice were challenged 5 days post-sensitization by applying an OVA (100 µg) MNA to the right ear. A blank (empty) MNA was applied to the left ear to control for any swelling caused by MNA application itself. Ear thickness was measured prior to the OVA challenge, and at 24, 48, 72, and 96 h post-challenge, and data presented as differences between OVA MNA-treated and contralateral Blank MNA-treated ears.

2.6. Suppression of skin DTH with TRI MPs

Mice received subcutaneous MP injections at the base of each ear two days before hapten sensitization, or immediately prior to OVA sensitization. For treatment of previously sensitized mice, TRI MP were injected at the base of each ear immediately prior to DNFB challenge. For some experiments, MPs were injected intradermally at the abdomen. Each injection (2 per mouse) contained a total of 8 mg “Blank” (empty) or TRI MPs in 150 µL sterile PBS. TRI MPs included a mix of 2.2 mg IL-2 MPs, 4.3 mg TGF-β1 MPs, and 1.5 mg Rapa MPs. For the OVA-specific DTH model, some mice were treated with TRI MPs supplemented with soluble (un-encapsulated) Treg-inducing factors (50 ng IL-2, 75 ng TGF-β1, and 10 µg rapamycin per injection; 2 per mouse). Additional controls for some experiments included soluble TRI, as well as individual TRI MP formulations or combinations of two TRI MPs (*e.g.* IL-2 MP + TGF-β1 MP). For these treatments, the total mass of MPs was kept constant by supplementing with Blank MPs.

2.7. Inhibiting Treg-mediated suppression with anti-GITR

To inhibit effects of Treg-mediated suppression on effector T cells, a monoclonal antibody specific for glucocorticoid-induced TNFR-related protein (GITR, clone DTA-1; BioXcell, West Lebanon, NH) was injected intraperitoneally (0.5 mg per mouse) 3 days prior to DNFB challenge.

2.8. Phenotypic analysis of T-cell populations in skin draining lymph nodes by flow cytometry

Four days after sensitization with DNFB or OVA, ear-draining cervical lymph nodes (or non-draining inguinal lymph nodes) were harvested, passed through 70 µm filters to create single cell suspensions, stained for T-cell markers, and analyzed with a flow cytometer (LSR-II; BD Biosciences, San Jose, CA). Lymphocytes were also counted with a hemocytometer to determine total cells per lymph node. Cells were stained with fluorescently labeled antibodies purchased from BD Biosciences, eBioscience (San Diego, CA), or BioLegend (San Diego, CA). To identify Treg, Th1, and Tc1 populations, lymphocytes were blocked with anti-CD16/32 (2.4G2; BD) and stained for CD4 (RM4-5; eBio), CD8b (H35-17.2; eBio), CD25 (PC61; BD), FoxP3 (FJK-16s; eBio), and T-bet (O4-46; BD). Adoptively transferred OVA-specific T cells were identified by staining for CD45.2 (104; BD). For further Treg phenotypic analysis, cells were also stained for CTLA-4 (CD152, UC10-4B9; eBio), LAP (TGF-β1, TW7-16B4; BioLegend), CD39 (Duha59; BioLegend), and GITR (DTA-1; eBio). FlowJo (Tree Star, Ashland, OR) software was used for analysis, and population gates were set based on isotype, single-stain, and fluorescence minus one controls.

2.9. In vitro suppression assay

OT-II cells were adoptively transferred to CD45.1 B6 mice (5×10^6 cells/recipient) one day prior to sensitization of ears with OVA MNA and local injection of TRI MP and soluble factors. Four days post-sensitization, DLN from five mice were harvested under sterile conditions, passed through 70 µm filters to create a single cell suspension, and stained with fixable viability dye (eBioscience) and antibodies for CD45.2, CD4, and CD25. Live CD45.2⁺ CD4⁺ CD25⁺ cells (CD25⁺ OT-II) were isolated by FACS sorting (FACSaria; BD Biosciences). Conventional T cells (Tconv; CD4⁺ CD25[−]) were isolated from the spleen of a naïve CD45.1 B6 mouse using a CD4⁺ CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec, San Diego, CA), and labeled with 5 µM CFSE (Vybrant CFDA SE Cell Tracer Kit; Invitrogen, Thermo Fisher). Suppression assays were performed in 96-well round-bottom plates in 200 µL supplemented RPMI per well. Tconv (5×10^4 cells/well) were cultured with anti-CD3/CD28-coated beads for stimulation (5×10^4 beads/well; Dynabeads Mouse T-Activator; Life Technologies, Thermo Fisher) and different quantities of CD25⁺ OT-II cells. After 3 days, cells were stained with fixable viability dye and antibodies for CD45.2 and CD4. Proliferation of Tconv (live CD45.2[−] CD4⁺ cells), as indicated by CFSE-dilution, was analyzed by flow cytometry (LSR Fortessa; BD Biosciences).

2.10. Cutaneous histology and immunohistochemistry

Ears from mice sacrificed 4 days after DNFB (or OVA) challenge or re-challenge were excised and flash frozen in OCT compound. Skin cross-sections (7 µm thick) were stained with hematoxylin and eosin and imaged with a Nikon Eclipse E400 microscope. For fluorescent immunohistochemistry (IHC), 10 µm thick sections were fixed with 96% ethanol, blocked with PBS containing 5% donkey serum and 1% Tween20, and treated with a streptavidin/biotin blocking kit (Vector Labs, Burlingame, CA). Blocked sections were incubated overnight at 4 °C with primary antibodies: biotin-FoxP3 (FJK-16s; eBio), biotin-CD8a (53–6.7, eBio), or CD3 (SP7, monoclonal rabbit IgG; Thermo Scientific, Waltham, MA). Sections were then incubated with Cy3-streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) or Alexa Fluor 555 donkey anti-rabbit IgG (Thermo Scientific) for 1 h at room temperature, counterstained with DAPI, and fixed with 2% paraformaldehyde. Slides were imaged with an epifluorescence microscope (Olympus Provis AX-70; Center Valley, PA). For histology and IHC, ears from naïve mice were used as controls.

2.11. Cutaneous cytokine expression by qRT-PCR

Two days after DNFB challenge, total RNA was extracted from excised ear tissue using TRI-reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions, and quantified using a NanoDrop 2000 (Thermo Scientific). For each reverse transcriptase assay, 2 µg RNA was converted to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Quantitative real-time PCR was then performed using VeriQuest Probe qPCR Mastermix (Affymetrix, Santa Clara, CA), according to the manufacturer's instructions, with 5' nuclease PrimeTime qPCR assays (Integrated DNA Technologies, Coralville, IA) specific for IFN γ , IL-1 β , TNF, and β -glucuronidase (GUSB, endogenous control). Duplex reactions (target gene + GUSB) were run and analyzed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Relative fold changes of IFN γ , IL-1 β , and TNF expression were calculated and normalized based on the $2^{-\Delta\Delta C_t}$ method, with naïve ear skin as the untreated control.

2.12. Statistical analyses

Statistical analyses were performed with GraphPad Prism v6 (San Diego, CA). For cytokine bioactivity assays, ED₅₀ values were determined by nonlinear 4- or 5-parameter logistic regression. Data from experiments with multiple treatment groups were analyzed by one-way ANOVA, followed by Tukey's post-hoc testing. For experiments with only two groups, two-tailed independent t-tests were used. Ear thickness measurements from multiple time points were analyzed by two-way mixed ANOVA, followed by post-hoc testing of treatment effect with a Sidak correction. Differences were considered significant if $p < 0.05$. Data represent mean $\pm$ SD, except for ear thickness measurements, which are mean $\pm$ SEM.

3. Results

3.1. TRI MPs release TGF- β 1, rapamycin, and IL-2 for about 1 week

We previously reported encapsulation and sustained release of Treg-inducing factors from biodegradable PLGA MPs [21]. For the current study, particle formulations were re-engineered to achieve faster release kinetics (*i.e.* greater release within the first week), which is more suitable for short-term immunomodulation during acute immune responses, such as allergen sensitization. Accordingly, we encapsulated the Treg-inducing factors in MPs composed of a blend of ester-terminated poly(lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG)-PLGA diblock copolymer. New formulations contained PEG (4 wt %, MW $\sim$ 5 kDa), which helped to augment early release by enhancing the hydration rate and matrix swelling [26], and ester-terminated PLGA, which served to reduce electrostatic interactions with positively charged amino acid residues [27].

Surface morphology and particle size distributions were consistent for all formulations, regardless of whether they were fabricated using a single emulsion (Rapa MP) or double emulsion protocol (IL-2 MP and TGF- β 1 MP). A representative scanning electron micrograph (Fig. 1A) shows spherical particles with somewhat irregular surface morphology, consistent with that reported for other PEG-coated MPs [28]. Particle size distributions (Fig. 1B) are also consistent across all formulations, with volume-average diameters of 1.73 ± 0.90 µm (IL-2 MP), 1.78 ± 0.92 µm (TGF- β 1 MP), and 1.77 ± 0.89 µm (Rapa MP). Total loading of TGF- β 1, rapamycin, and IL-2 were 12.64 ± 0.97 ng, 5.53 ± 0.04 µg, and 16.92 ± 1.11 ng per mg MP, respectively. Given theoretical loading of 25 ng TGF- β 1, 7.5 µg rapamycin, or 25 ng IL-2 per mg MP, encapsulation efficiencies for TGF- β 1, rapamycin, and IL-2 were $50.6 \pm 3.9\%$, $73.8 \pm 0.5\%$, and $67.7 \pm 4.4\%$, respectively. PEG-PLGA MPs provide sustained release of each factor over a period of approximately one week (Fig. 1C). For Rapa MP, $95.7 \pm 3.8\%$ of total

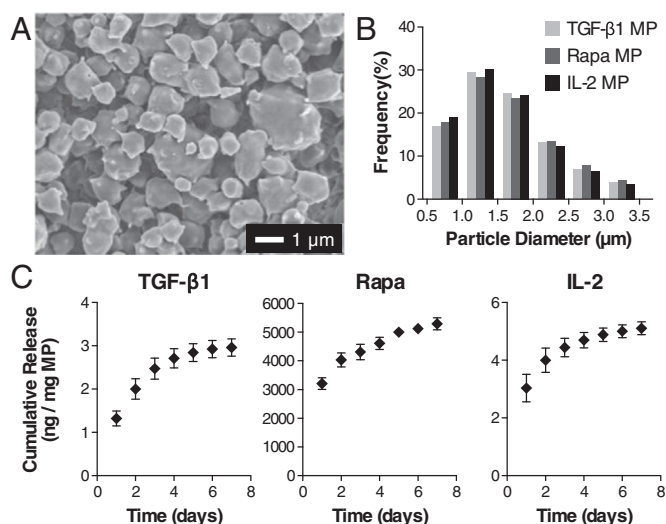


Fig. 1. Microparticle characterization. (A) Representative scanning electron micrograph showing surface morphology of PEG-PLGA MPs (5000 $\times$ magnification). (B) Number-weighted size distributions for IL-2, TGF- β 1, and Rapamycin (Rapa) MPs, determined by volume impedance measurements of 50,000 particles. (C) Cumulative release profiles for IL-2, TGF- β 1, and Rapa formulations (N = 5–8). *In vitro* release was measured for MPs incubating at 37 °C in PBS with 1% BSA for IL-2 and TGF- β 1, or PBS with 0.2% Tween-80 for Rapa.

encapsulated drug was released within 7 days; however, for TGF- β 1 and IL-2 MPs, only $23.5 \pm 3.6\%$ and $30.2 \pm 3.7\%$ of total encapsulated cytokines were released in the first week. Release assays for IL-2 and TGF- β 1 MPs were carried out to 22 days, and no cytokine release was detected between days 8 and 22 (not shown), consistent with a lag phase. Finally, bioactivity of IL-2 and TGF- β 1 released from MPs and detected by ELISAs was comparable to that of unencapsulated cytokines, as determined by HT-2 cell-based bioassays (Fig. S1).

3.2. Prophylactic treatment with TRI MPs during hapten sensitization increases the Treg/Teff ratio in skin draining lymph nodes

We previously demonstrated that TRI MP induce differentiation of naïve CD4⁺ T cells to CD4⁺ FoxP3⁺ regulatory T cells (Tregs) *in vitro* in the presence of T-cell activating microbeads [21]. To determine whether our new TRI MP formulations could promote Treg differentiation and enhance Treg populations *in vivo*, TRI MP were injected subcutaneously at the base of the ears two days prior to sensitization of the ears with 2,4-dinitrofluorobenzene (DNFB), a small molecule hapten and model contact allergen. Naïve mice, sensitized but untreated mice (DNFB only), and sensitized mice treated with Blank MP served as controls. Flow cytometry analysis on DLN from four days post-sensitization revealed significant effects of DNFB and TRI MPs on T-cell populations. Relative to naïve mice, sensitized mice (w/ or w/o Blank MP) had significantly greater Treg, Th1, and Tc1 frequencies and numbers in the DLN (Figs. 2A, S2). Notably, TRI MP treatment dramatically enhanced the frequency and absolute number of CD4⁺ FoxP3⁺ Tregs (including both CD25⁺ and CD25[−] Treg subsets), relative to sensitized controls (Figs. 2A, S2). Tregs from TRI MP-treated mice expressed several characteristic markers important for suppressive function, including CD25 (IL-2R α), CTLA-4 (Cytotoxic T-Lymphocyte Antigen-4), GITR (Glucocorticoid-Induced TNFR-Related protein), LAP (Latency Associated Peptide, pro-TGF- β 1), and CD39 (ectonucleotidase that generates anti-inflammatory adenosine) [11]. Further, expression of CTLA-4, GITR, and LAP was greater on Tregs from TRI MP-treated mice, relative to naïve Tregs (Fig. 2D). In addition to expanding Treg populations, TRI MP treatment led to decreases in frequencies and numbers of proinflammatory CD4⁺ T-bet⁺ Th1 and CD8⁺ T-bet⁺ Tc1 effector T cells (Teff) (Figs. 2A, S2B). Concurrent enhancement of Treg

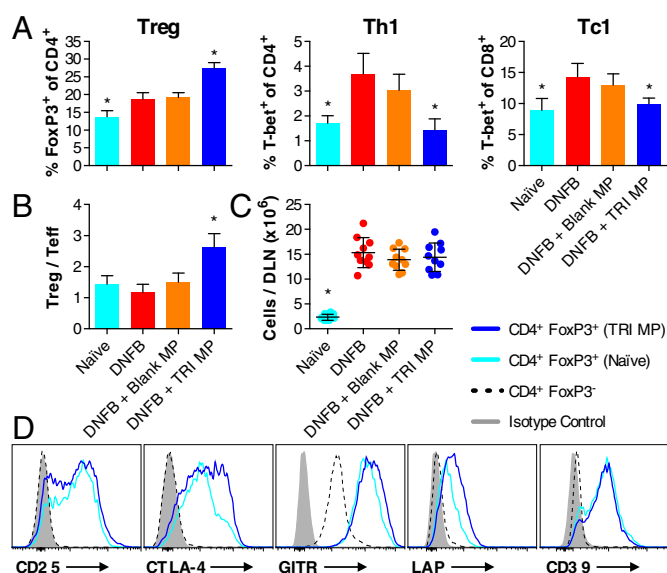


Fig. 2. Treatment with TRI MP during DNFB sensitization enhances Treg populations and reduces effector T-cell populations (Teff, Th1 and Tc1) in skin draining lymph nodes (DLN). Sustained release TRI MP were injected 2 days prior to sensitization and DLN were isolated and analyzed by flow cytometry four days post-sensitization. Naïve (unsensitized) mice were used as a control. (A) Relative frequencies of Treg (CD4⁺ FoxP3⁺), Th1 (CD4⁺ T-bet⁺), and Tc1 (CD8⁺ T-bet⁺) populations in DLN. (B) Ratios of anti-inflammatory Treg to pro-inflammatory Teff in DLN. (C) Total cells per DLN as counted with a hemocytometer. (D) Representative histograms showing expression of characteristic Treg markers by CD4⁺ FoxP3⁺ Tregs from DLN of TRI MP treated or naïve mice. CD4⁺ FoxP3⁻ T cells and isotype controls are also shown. Significant differences, relative to DNFB sensitized control, are indicated by *p < 0.001 (N = 8–20).

populations and reduction of Teff populations contributed to a dramatic increase in the Treg/Teff ratio (Fig. 2B) in TRI MP-treated mice, relative to naïve and sensitized controls (Fig. 2B). Despite shifts in T-cell subpopulations, total numbers of lymphocytes in DLN were consistent across all sensitized groups, and significantly greater than in DLN from naïve mice (Fig. 2C). Finally, we observed similar trends in T-cell populations using a second model hapten, oxazolone (OXA). Specifically, treatment with TRI MPs prior to topical application of OXA, nearly doubled the Treg/Teff ratio in skin DLN by expanding Treg and suppressing Th1 and Tc1 populations (Fig. S4). These data suggest that our approach to *in vivo* modulation of T cell responses during allergen sensitization is not restricted to a particular hapten, and could potentially be extended to a variety of other allergens.

To investigate the importance of sustained delivery of Treg-inducing factors during the sensitization phase, some mice were treated with a single local bolus of soluble TRI (TGF- β 1, Rapamycin, and IL-2 without MPs). Unlike treatment with sustained release TRI MPs, soluble TRI injected one or two days before sensitization (as with MP treatments) did not affect Treg or effector T-cell populations (Fig. S3A). Interestingly, local injection of soluble TRI at the time of sensitization (day 0), or 1 to 3 days after sensitization, reduced Th1 and Tc1 frequencies, but failed to expand Treg populations (Fig. S3A). This suggests that these mediators must be present at the time of antigen exposure, and that Treg induction requires their sustained presence throughout antigen presentation.

Finally, while IL-2 and TGF- β 1 are essential for Treg induction [29], and addition of rapamycin significantly enhanced Treg-induction efficiency *in vitro* [21], we wanted to determine whether delivery of all three factors was essential to enhance Treg populations and suppress Th1 and Tc1 populations *in vivo*. Treatment with different combinations of TRI MP revealed that all three factors were in fact required to significantly enhance Treg frequencies and reduce Th1 and Tc1 frequencies (Fig. S3C). The combination of TGF- β 1 MP and Rapa MP also increased Treg frequencies and reduced Th1 frequencies, but did not

have a significant effect on Tc1 frequencies (Fig. S3C). Together, these data suggest that sustained delivery of all three Treg-inducing factors is capable of enhancing Treg populations and reducing Th1 and Tc1 populations.

3.3. Prophylactic treatment with TRI MPs during hapten sensitization suppresses DTH responses to multiple subsequent exposures

Since TRI MPs expanded suppressive Treg populations and reduced pro-inflammatory Th1 and Tc1 populations after sensitization, we hypothesized that TRI MP treatment would suppress DTH responses to subsequent allergen exposure (challenge). To test this hypothesis, mice treated with TRI MP and sensitized to DNFB were challenged by re-painting the ears with DNFB 10 days after sensitization (see experimental timeline in Fig. 3A). DNFB sensitized mice (with or without Blank MP) served as controls. Ear swelling responses, indicative of cutaneous inflammation, were measured after DNFB challenge. Notably, TRI MP treatment significantly reduced ear swelling compared to sensitized controls (Fig. 3B). Furthermore, tolerance induced by TRI MP treatment was persistent enough to suppress the hypersensitivity response to a second allergen challenge (“re-challenge”) 10 days after the first (Fig. 3C). Importantly, this effect was generalized, as TRI MP treatment was also able to suppress DTH responses to a second model hapten, OXA, in OXA-sensitized mice (Fig. S4B), suggesting that *in vivo* expansion of Treg populations with TRI MP may be a viable therapeutic approach for a variety of contact allergens.

In addition to macroscopic differences in ear swelling, histological evaluation of ear skin from four days after DNFB challenge or re-challenge revealed remarkable differences in skin morphology from TRI MP treated mice, compared to sensitized controls (Fig. 3D,E). After DNFB challenge or re-challenge, skin from sensitized controls showed acanthosis (epidermal hyperplasia), hyperkeratosis (thickening of the stratum corneum), some spongiosis (intercellular edema between keratinocytes), and lymphocyte infiltrates in the epidermis and dermis (Fig. 3D,E). These features are consistent with subacute allergic dermatitis [30]. Notably, TRI MP treatment reduced acanthosis, hyperkeratosis, spongiosis, and cellular infiltrates (Fig. 3D,E), resulting in skin that looked more like naïve skin histologically (Fig. 3F). Quantification of epidermal thickness also confirmed that TRI MP treatment significantly reduced epidermal hyperplasia associated with repeated allergen exposure (Fig. 3G). To better characterize cutaneous T-cell infiltrates, skin sections were stained for CD3, CD8, or FoxP3 and evaluated by immunofluorescence (Fig. S5). Quantification of cells in imaged fields revealed considerable CD3⁺ T-cell infiltrates in skin from sensitized controls after DNFB challenge, the majority of which were CD8⁺ effector T cells (Fig. 3H). In contrast, skin from TRI MP treated mice contained significantly fewer total T cells, including fewer total FoxP3⁺ Tregs; however, the ratio of FoxP3⁺/CD8⁺ T cells was roughly twofold greater than in sensitized and naïve controls (Fig. 3H).

To determine whether treatment with TRI MP suppressed cutaneous production of inflammatory cytokines in response to DNFB challenge, skin tissue was harvested 48 h post-challenge and mRNA isolated for qRT-PCR analysis. Specifically, we examined expression of IL-1 β and TNF, which are predominantly produced by keratinocytes and neutrophils [4], as well as IFN- γ , which is produced by Th1 and Tc1 cells, in response to allergen challenge [31]. As expected, expression of IL-1 β , TNF, and IFN- γ was considerably enhanced in DNFB challenged skin from DNFB sensitized controls, relative to naïve tissue (Fig. 3I). TRI MP treatment significantly suppressed expression of these three pro-inflammatory cytokines, though expression levels were still greater than in naïve tissue (Fig. 3I).

Finally, to confirm that suppression of hypersensitivity responses was mediated by expanded Treg populations, we used a GITR agonist to inhibit the suppressive function of Tregs [32]. Anti-GITR (DTA-1, 0.5 mg/mouse) was injected intraperitoneally 3 days before DNFB challenge of TRI MP treated mice (Fig. 3A). These mice developed a

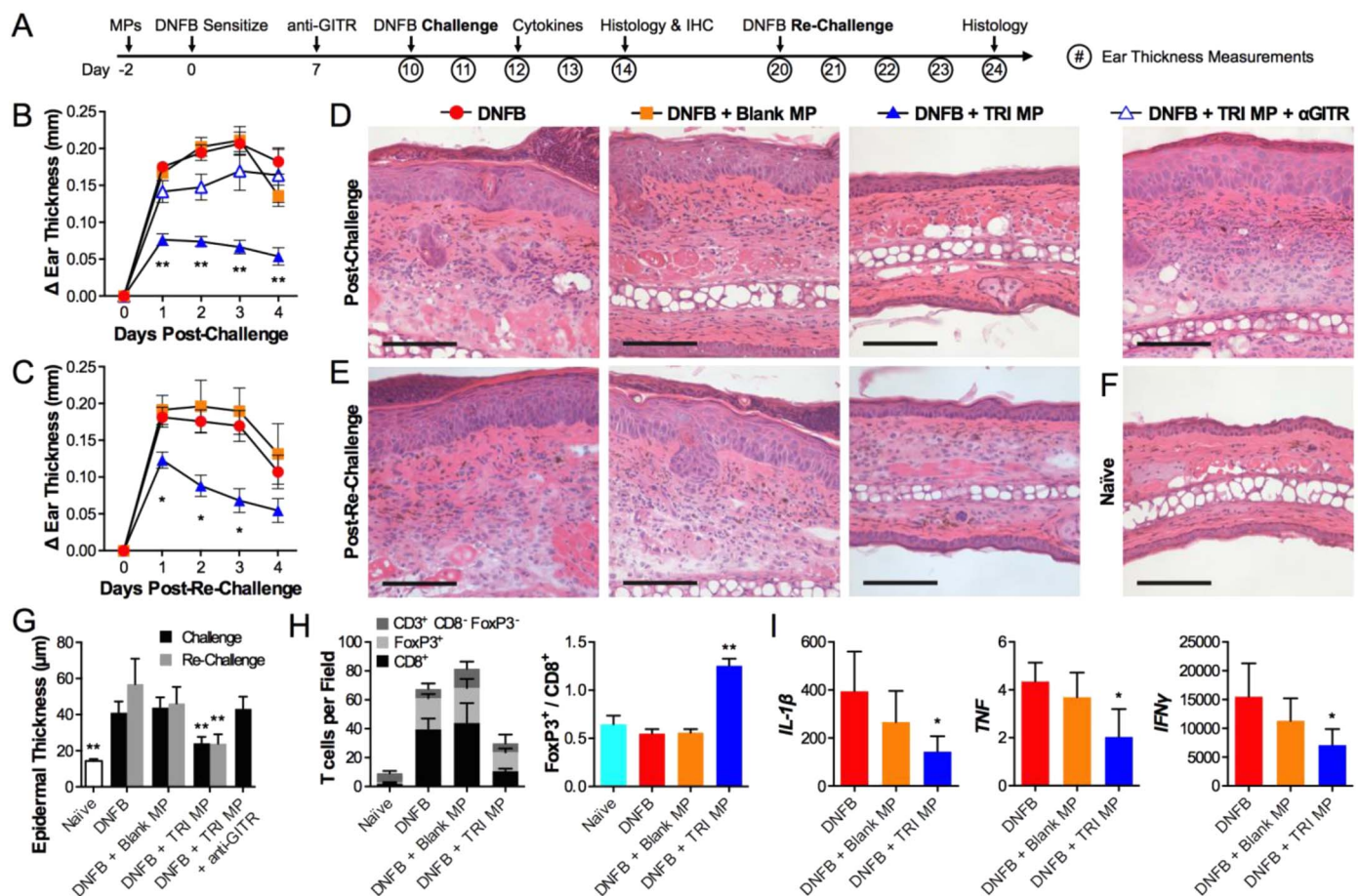


Fig. 3. Prophylactic treatment with TRI MP (prior to sensitization) protects skin and suppresses DTH responses to repeated challenges with DNFB. (A) Experimental timeline. (B–C) Increases in ear thickness (swelling) after (B) DNFB challenge ($N = 20$, $N = 5$ for anti-GITR) or (C) re-challenge ($N = 6–7$), relative to pre-challenge or pre-re-challenge thickness. (D–F) Representative ear skin histology (H & E) from 4 days post-challenge (D) or re-challenge (E), or from naïve ears not exposed to DNFB (F). DNFB was applied to the dorsal side of ears (top of images). Scale bars are 100 μm . (G) Average epidermal thickness as measured from histology images as in (D–F) ($N = 5–8$). (H) T-cell counts in skin sections from 4 days post-challenge. Representative immunofluorescence images (fields) used for cell counts are presented in Fig. S5 ($N = 5$). A high FoxP3⁺/CD8⁺ T-cell ratio is indicative of a more tolerogenic microenvironment. (I) Expression of pro-inflammatory cytokines in skin tissue 48 h post-challenge ($N = 5$). mRNA was quantified by qRT-PCR, and expression is relative to naïve skin ($2^{-\Delta\Delta C_t}$). Significant differences, relative to DNFB alone, are indicated by * $p < 0.05$ or ** $p < 0.001$.

robust DTH response to DNFB challenge, comparable to sensitized controls without TRI MP, as indicated by ear swelling (Fig. 3B) and histological features of skin tissue 4 days post-challenge (Fig. 3D,G). Together, these data suggest that blocking Treg function abrogates the suppressive effects of Treg-induction therapy with TRI MPs.

3.4. Treg-inducing factors must be delivered locally (near the sensitization site), but generate systemic and specific tolerance to contact allergens

To determine whether local treatment (at the same site as hapten challenge) was necessary, we injected TRI MPs at the abdomen, a distal site that doesn't drain to the cervical LN, and examined T-cell populations in ear DLN four days after sensitizing ears with DNFB. Interestingly, treatment at a distal site had no effect on Th1 and Tc1 populations in ear DLN, and a significantly diminished effect on Treg frequencies (Fig. S3B). This led to Treg/Teff ratios similar to those seen in DNFB sensitized mice without treatment, and more than two-fold less than Treg/Teff ratios in DLN of mice treated with TRI MP near the sensitization site (Fig. 4A). Since we observed significantly greater Treg/Teff ratios in non-draining inguinal LN (NDLN) of mice treated with TRI MP near the sensitization site, compared to sensitized mice (Fig. 4A), we next wanted to see whether local treatment could generate systemic hyporesponsiveness to subsequent allergen exposure. Accordingly, we injected Blank MP or TRI MP at the abdomens of mice prior to sensitizing abdomens with DNFB, and then challenging ears

(Fig. 4B). Notably, TRI MP treatment at the sensitization site effectively suppressed the DTH response to challenge at a distal site (ears), as evidenced by a significant reduction in ear swelling (Fig. 4C).

Finally, to demonstrate allergen-specificity of TRI MP-mediated DTH suppression, mice were treated with Blank MP or TRI MP near the site of sensitization to one allergen (OXA), and then sensitized to a second allergen (DNFB) at a different site and time (see timeline in Fig. 4D). As with prior hapten-mediated ACD experiments (Figs. 3 and S4), ears were challenged 12 days after treatment with MPs. Ear-swelling responses to DNFB challenge were comparable for both TRI MP- and Blank MP-treated mice (Fig. 4E). In contrast, TRI MP-treatment near the site of DNFB or OXA sensitization suppressed DTH responses to subsequent challenge with the same allergen (Figs. 3B and S4B). Collectively, these results suggest that TRI MP treatment suppresses DTH responses in an allergen-specific manner, rather than by acting as a non-specific anti-inflammatory or immunosuppressant.

3.5. In previously sensitized mice, TRI MPs administered at the time of allergen challenge suppress subsequent DTH responses

To determine whether TRI MPs could generate allergen tolerance in previously sensitized individuals, mice were exposed to DNFB twice at the abdomen prior to administration of TRI MP and challenge of left ears with DNFB. Mice were later re-challenged at the opposite ears according to the experimental timeline in Fig. 5A. As with prophylactic

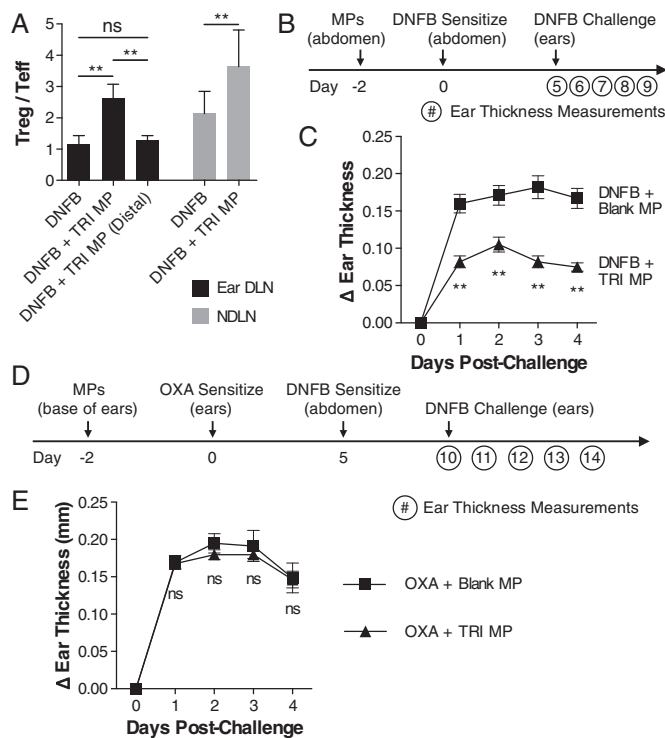


Fig. 4. TRI MP injected near sites of skin sensitization can suppress DTH responses to challenge at distal sites. (A) Four days after sensitization of ears with DNFB, ear-draining cervical LN (DLN, black) or non-draining inguinal LN (NDLN, gray) were isolated and T-cell populations analyzed by flow cytometry ($N \geq 7$ for DLN, $N \geq 20$ for NDLN). TRI MP were injected either at the base of the ears or at the abdomen, a site “distal” to sensitization. Treg/Teff represents the ratio of $CD4^+ FoxP3^+ Treg$ to $CD4^+ T-bet^+ Th1$ and $CD8^+ T-bet^+ Tc1$ effector T cells (Teff). DNFB and DNFB + TRI MP DLN data are also presented in Fig. 2B. (B) Experimental timeline for allergen challenge at a distal site to sensitization and treatment. (C) Increases in ear thickness (ear swelling) after DNFB challenge, relative to naïve (pre-challenge) thickness ($N = 10$). (D) Experimental timeline to demonstrate allergen-specific DTH suppression. (E) Ear swelling after DNFB challenge ($N = 7-8$) of mice sensitized to OXA and DNFB at different sites, according to the timeline in (D). Mice were treated with Blank MP or TRI MP near the site of OXA sensitization only. Significant differences are indicated by * $p < 0.05$ or ** $p < 0.001$.

treatment at the time of sensitization, TRI MP treatment of pre-sensitized mice at the time of allergen challenge significantly inhibited DTH responses, as indicated by a significant reduction in ear swelling relative to untreated mice (Fig. 5B). Notably, TRI MP-induced tolerance persisted, resulting in suppressed DTH responses to a subsequent re-challenge (Fig. 5C). TRI MP treatment remarkably improved ear skin histology post-challenge (Fig. 5D) and re-challenge (not shown). As with prophylactic treatment, treatment at the time of allergen challenge reduced acanthosis, hyperkeratosis, spongiosis, and cellular infiltrates (Fig. 5E). Skin DLN from TRI MP treated mice also contained enhanced Treg populations and decreased Th1 and Tc1 populations, relative to untreated mice, contributing to an increase in Treg / Teff ($FoxP3^+ / T-bet^+$) ratios that is consistent with a more tolerogenic immune profile (Fig. 5F). Finally, TRI MP treatment significantly reduced the influx of T cells into the skin after allergen challenge and re-challenge, as indicated by reductions in total $CD3^+$ T cells in skin sections (Fig. 5F,G).

3.6. TRI MPs induce protein-specific Tregs and suppress protein-mediated ACD

While ACD typically involves polyclonal T-cell responses to hapten-autologous protein complexes, DTH responses to foreign protein antigens can develop when sensitizing exposure occurs through skin with barrier defects (e.g. from pre-existing dermatitis, physical damage, or chemical damage by detergents). To investigate whether TRI MP could generate protein antigen-specific Tregs and suppress protein DTH

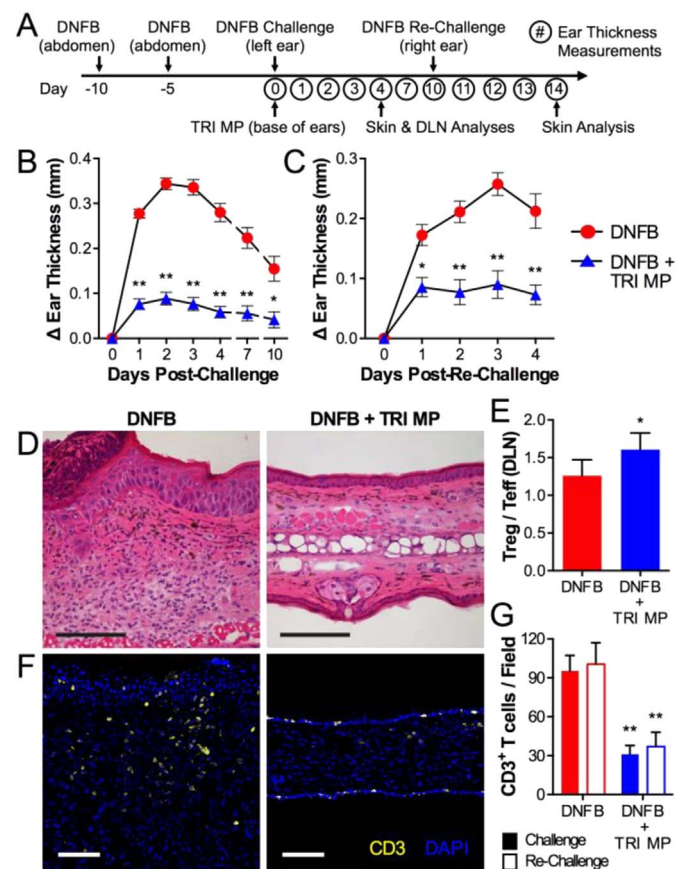


Fig. 5. Treatment of previously sensitized mice with TRI MP at the time of allergen challenge reverses ongoing ACD and suppresses DTH responses to subsequent exposures. (A) Experimental timeline. (B–C) Increases in ear thickness (swelling) after (B) DNFB challenge and treatment ($N = 15$) or (C) re-challenge ($N = 8$), relative to naïve ear thickness. (D) Representative ear skin histology (H & E) from 4 days post-challenge. DNFB was applied to the dorsal side of ears (top of images). Scale bars are 100 μm. (E) Ratio of $FoxP3^+ Treg$ to $T-bet^+ Th1$ and $Tc1$ effector T cells in DLN 4 days post-challenge. (F) Representative immunofluorescence images of ear skin from 4 days post-challenge. (G) Quantification of T-cell infiltrates in skin 4 days after challenge or re-challenge, based on immunofluorescence ($N = 7-8$). Significant differences are indicated by * $p < 0.05$ or ** $p < 0.001$, relative to DNFB alone.

responses, we used dissolvable microneedle arrays (MNAs) to deliver ovalbumin (OVA, model antigen) through the stratum corneum, into the epidermis and dermis [25]. In this application, MNAs serve as an alternative to tape-stripping and topical application of protein or intradermal injections of proteins, as others have reported for protein sensitization [33]. Adoptive transfer of $CD45.2^+$ OVA-specific T cells (from OT-I and OT-II T-cell receptor transgenic mice) to congenic $CD45.1$ B6 mice enabled identification of OVA-specific T-cell responses to sensitization with OVA MNA and treatment with TRI MP (Fig. 6A). OVA-sensitized mice (with Blank MP) generated strong DTH responses when challenged with OVA MNA, as indicated by ear swelling that peaked 48 h post-challenge (Fig. 6B). While TRI MPs alone were not able to suppress the ear swelling response (data not shown), likely due to the artificially high frequency of adoptively transferred OVA-specific T cells, treatment with TRI MP plus extra soluble factors (IL-2, TGF- β 1, and rapamycin at the same time as TRI MP administration) significantly suppressed the OVA DTH response (Fig. 6B). Immunofluorescence stained ear tissue from 4 days post-challenge revealed a significant reduction in cutaneous $CD3^+$ T-cell infiltrates (Fig. 6C,D) with treatment, and a significantly greater proportion of $FoxP3^+$ Tregs (Fig. 6D). Finally, analysis of T-cell populations in ear skin DLN at 4 days post-sensitization revealed that Treg-inducing treatment significantly reduced OVA-specific T-cell expansion, as indicated by fewer $CD45.2^+$

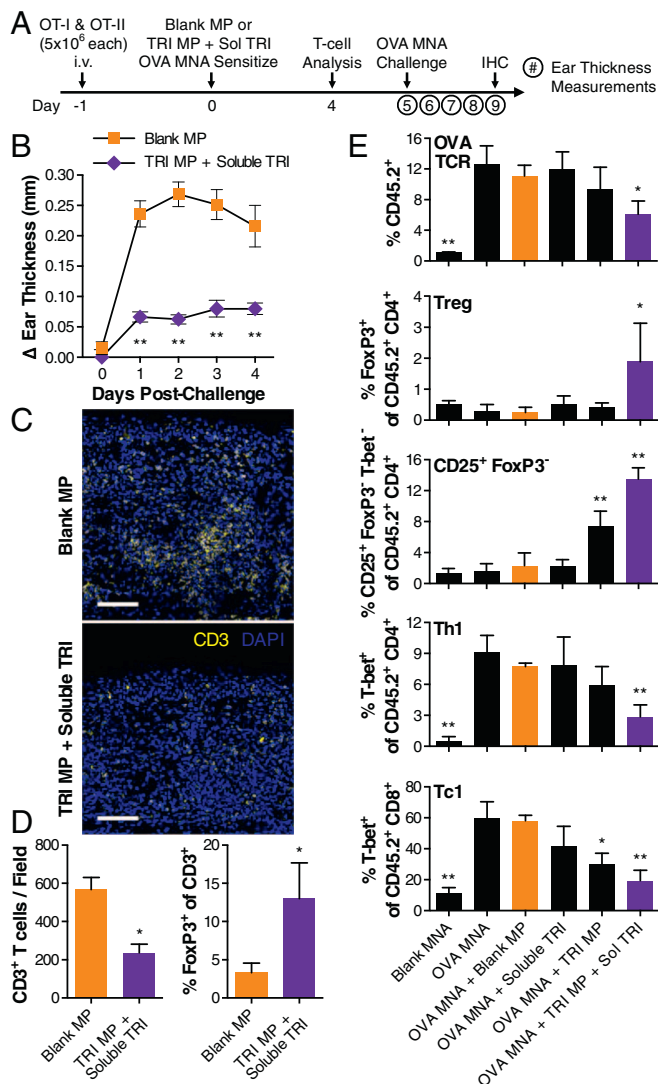


Fig. 6. Treatment with TRI MP prior to OVA sensitization suppresses a protein-specific contact hypersensitivity response. (A) Experimental timeline. OT-I & OT-II cells (CD45.2⁺) were transferred to congenic CD45.1⁺ B6 recipient mice. (B) Ear swelling after OVA challenge, represented as the difference in thickness between OVA MNA treated ears and contralateral Blank MNA treated ears (N = 8). (C) Representative ear skin sections from 4 days post-challenge, stained for T cells (CD3, yellow) and counter-stained with DAPI. Scale bars are 100 μ m. (D) Quantification of cutaneous T cells per imaged field (as in C) and % Treg, based on similar IHC images with FoxP3 staining (N = 5). (E) Frequencies of various OVA-specific (CD45.2⁺) T-cell subsets in skin DLN at 4 days post-sensitization (N = 5). Significant differences, relative to OVA MNA + Blank MP, are indicated by *p < 0.05 or **p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells (Fig. 6E). Notably, increased frequencies of OVA-specific FoxP3⁺ Tregs were accompanied by significantly lower frequencies of OVA-specific T-bet⁺ Th1 and Tc1 effector T-cell populations (Fig. 6E). Interestingly, we observed a substantial increase in a population of OVA-specific CD4⁺ CD25⁺ FoxP3⁺ T-bet⁺ T cells with potential suppressive capacity in an *in vitro* suppressive assay (Figs. 6E, S6). As TRI MP treatment alone was unable to suppress the OVA DTH response, it was also unable to enhance Treg populations or reduce Th1 and Tc1 populations as well (Fig. 6E). Notably, soluble factors alone had no significant effects on OVA-specific Treg, Th1, or Tc1 populations (Fig. 6E), suggesting sustained delivery of the Treg-inducing factors is important. Collectively, these data demonstrate that TRI MP (along with extra soluble factors as a “burst” at the time of administration) can induce protein antigen-specific Tregs *in vivo* and suppress protein DTH

responses.

4. Discussion

As an alternative to the current symptomatic treatments for ACD, which use non-specific anti-inflammatories (e.g. corticosteroids), we developed a microparticle-based system called “TRI MPs” to promote tolerance to contact allergens by expanding Treg populations *in vivo*. Since Tregs are known to suppress aberrant immune responses in a variety of inflammatory and autoimmune diseases [11], there is considerable interest in therapeutic approaches to expand Treg populations. In addition to infusion of *ex vivo* expanded Tregs, which faces a number of barriers to clinical translation [17,18], several novel strategies have been investigated recently (reviewed in [34,35]). Systemic administration of cytokines (e.g. IL-2), antibodies (e.g. non-mitogenic anti-CD3), or combinations of factors (e.g. IL-2/anti-IL-2 [36] or IL-2 plus rapamycin [37]) has been used to expand polyclonal non-antigen-specific Tregs. Additionally, antigen-specific Treg populations have been expanded by systemic administration of nanoparticles coated with specific antigens (e.g. peptide-MHCII [38], peptide plus ITE [39], or peptide alone [40]), or nanoparticles encapsulating peptide or protein antigens and immunomodulatory agents (e.g. antigen plus rapamycin [41]), which promote development of tolerogenic DCs that induce Tregs. A recent study also demonstrated antigen-specific tolerance in rodent and non-human primate models, following co-administration of unencapsulated therapeutic proteins and rapamycin nanoparticles *via* intravenous or subcutaneous routes [42]. While most of these nanoparticle formulations were injected systemically, microparticles containing rapamycin and peptide antigen have been injected directly into lymph nodes to reprogram the microenvironment in which T-cell polarization occurs [43]. Finally, allergen-specific immunotherapy is currently used in the clinic to desensitize patients by generating allergen-specific Tregs, but it requires long-term treatment, over a period of months to years, with repeated subcutaneous or oral administration of low doses of allergen [44].

In the present study, we demonstrated that sustained local release of TGF- β 1, rapamycin, and IL-2 from TRI MPs injected near the site of cutaneous sensitization or challenge expanded allergen-specific Treg populations and suppressed pro-inflammatory Th1 and Tc1 effector T-cell populations in skin DLN (Figs. 2, S4A, 5E, 6E). Although an injection of un-encapsulated TRI factors 0 to 3 days after sensitization suppressed effector T cells, expansion of Treg populations required sustained release from TRI MPs (Fig. S3A). This could be due to the fact that rapamycin, which can suppress effector T-cell proliferation, has a relatively long half-life of at least 6 h in mice [45]. In contrast, IL-2 and TGF- β 1, which are essential for Treg differentiation and proliferation [29], have very short *in vivo* half-lives of a few minutes [36,46]. TRI MPs extend the presence of these immunomodulatory cytokines, presumably providing a tolerogenic microenvironment in which antigen presentation by skin-emigrating DCs occurs during the sensitization phase. Importantly, MP formulations directed toward conditioning the local antigen presentation microenvironment significantly reduce therapeutic doses of TRI factors. For example, TRI MP treatment translates to < 100 ng/kg/day of cytokines and 0.15 mg/kg/day of rapamycin, doses which are orders of magnitude less than those typically used for systemic immunomodulation [47,48]. These dose-sparing effects associated with controlled release formulations are important because of adverse effects associated with high-dose cytokine and immunosuppressant therapies [36,37]. Notably, while TRI MP do not appear to release sufficient amounts of factors to affect T-cell differentiation at distal sites (Fig. 4A), local effects on Treg and effector T-cell populations can generate systemic tolerance and suppress DTH responses to subsequent allergen exposure at distal sites (Fig. 4B), presumably due to circulation of locally expanded Tregs and fewer circulating effector T cells.

In addition to increasing the ratio of anti-inflammatory, tissue-

protective Tregs to pro-inflammatory, tissue-destructive effector T cells, TRI MP treatment during sensitization suppressed DTH responses to subsequent allergen exposures (Figs. 3, 6, S4). Hyporesponsiveness persisted through at least two allergen challenges 10 and 20 days after sensitization (Fig. 3), and TRI MP reduced ear swelling to levels seen in un-sensitized mice when first exposed to DNFB (data not shown). In other words, ear swelling in TRI MP-treated mice was comparable to that caused by the innate immune response to a hapten without T-cell involvement [4]. Furthermore, TRI MPs appeared to suppress DTH responses in an allergen-specific manner, with minimal non-specific or off-target immunosuppression. While TRI MPs may potentially expand some non-allergen-specific Tregs, the increased populations of OXA-specific Tregs and/or non-specific Tregs (Fig. S4A) failed to inhibit DTH responses to challenge with a second allergen (DNFB), for which sensitization occurred at a distal site and time, relative to TRI MP treatment (Fig. 4E). This allergen-specific suppression of ACD distinguishes TRI MP-treatment from traditional therapies involving anti-inflammatories (e.g. corticosteroids) applied topically after allergen challenge to reduce inflammation non-specifically, regardless of the allergen. Finally, TRI MPs were also able to reverse established allergen-specific immune responses in previously sensitized mice and promote allergen tolerance, as evidenced by inhibited DTH responses to allergen challenge and re-challenge (Fig. 5).

Suppression of DTH responses appear to be mediated by the expanded Treg populations, as impairing Treg suppressive function via anti-GITR administration [32] reversed the beneficial effects of treatment with Treg-inducing MPs (Fig. 3B). Previously, circulating Tregs were shown to suppress DTH responses by blocking influx of effector T cells into inflamed tissue. This process is mediated by contact-independent mechanisms, including production of cytokines and adenosine (by CD39), which inhibit effector T-cell adherence to vascular endothelial cells and subsequent extravasation [49,50]. Immunohistochemical analysis of skin from TRI MP treated mice revealed a significant reduction in total T-cell infiltrates (Figs. 3H, 5F–G), and especially CD8⁺ effector T cells (Fig. 3H). Interestingly, although there were fewer total FoxP3⁺ Tregs in skin tissue after TRI MP treatment, there was at least a two-fold increase in the ratio of FoxP3⁺ to CD8⁺ T cells (Fig. 3H). We observed a similar trend in the protein-mediated OVA DTH model, where total T-cell infiltrates were significantly reduced in TRI MP plus Soluble TRI treated skin, and the frequency of FoxP3⁺ Tregs was enhanced (Fig. 6C,D). In that model, we also observed increases in a population of CD25⁺ FoxP3⁺ OT-II cells, which, given their apparent suppressive function (Fig. S6) and lack of T-bet expression (Fig. 6E), may be an unstable population or a type of unconventional FoxP3⁺ Treg reported previously in the setting of allergic disease [51,52]. Future studies will be needed to delineate the contributions of skin-resident and circulating Tregs toward suppressing the DTH responses, and to identify allergen-specific Tregs with resident-memory or central memory phenotypes at extended time-points. Additionally, future studies will be needed to determine the maximum duration of tolerance induced by TRI MP under various conditions, including repeated allergen exposure or challenge more than three weeks after treatment. In the event that repeated allergen exposure eventually breaks tolerance induced by TRI MPs, a booster treatment and/or greater initial dose of TRI factors may be necessary. These longer-term studies will require the IL-2 and TGF- β 1 release assays to be extended beyond three weeks, and if bioactive cytokines are detected at later time points, formulations may be modified to restrict release to one week. This could involve increasing the overall PEG:PLGA ratio or blending the PEG-PLGA co-polymer with a lower molecular weight PLGA [53].

In addition to demonstrating efficacy of Treg-inducing MPs in models of hapten-mediated ACD, which involve polyclonal allergen-specific T-cell responses, we were also able to induce monoclonal protein-specific Tregs in a well-defined TCR transgenic adoptive transfer model. The fact that TRI MP alone did not expand OVA-specific FoxP3⁺

Tregs or suppress DTH responses in this model emphasizes that different amounts of Treg-inducing factors may be needed depending on the nature of the acute immune response to be modulated. In this particular case, adoptive transfer of a pool of clonal OVA-specific T cells from OT-II Rag2^{-/-} mice significantly alters both the frequency and range of T-cell receptor binding affinities of available T-cell precursors. Consistent with this notion, difficulty in inducing OVA-specific Tregs also may be related to the dose of antigen delivered (up to 100 μ g OVA per MNA). Dose-dependent responses to antigen have been reported, with maximal Treg proliferation observed at low antigen doses, and Th1 proliferation favored at high antigen doses *in vitro* [54]. Accordingly, protein-specific Treg induction *in vivo* may be further enhanced by optimizing the dose and concentration gradient of protein antigens delivered with MNAs. For previously sensitized individuals, application of MHC-II-restricted peptides (e.g. OVA_{323–339}, a CD4⁺ T cell epitope) instead of whole proteins, at the time of TRI MP treatment could prevent further expansion of class I restricted peptide-specific CD8⁺ effector T cells, while still allowing TRI MPs to induce and expand class II restricted peptide-specific Treg populations [33]. Ultimately, such considerations would be relevant to both inducing tolerance to protein allergens, as well as treating autoimmune diseases by loading MNAs with auto-antigenic peptides or proteins.

The current study focused on two distinct strategies for tolerance induction. The first involved administration of TRI MPs at the time of allergen sensitization, a prophylactic approach that would be clinically relevant for common and potent contact allergens, such as urushiol in poison ivy, which sensitizes an estimated 85% of the population. Alternatively, for less common and/or weakly sensitizing contact allergens, limiting treatment to patients with established ACD is more feasible. In this case, TRI MP treatment delivered in the context of a patch test defined allergen would need to expand Treg populations sufficiently to subdue the primed memory T-cell response. This scenario may be more difficult than inducing tolerance with treatment at the time of sensitization, as memory T cells can be more resistant to Treg-mediated suppression than naïve T cells under some conditions [55]. Encouragingly, in the hapten-mediated ACD model, we found that treatment of previously sensitized mice with the same dose of TRI MPs at the time of allergen challenge was able to suppress the ensuing DTH response and prevent a subsequent DTH response to a re-challenge (Fig. 5). Optimizing the dosing and/or ratios of TRI factors for prophylactic and curative therapies may enhance therapeutic efficacy even further. Further investigation will be necessary to determine whether TRI MPs can permanently reverse the allergen-specific memory T-cell response in previously sensitized subjects and induce persistent tolerance to protect against chronic, repeated allergen exposure.

Finally, unlike other experimental approaches to Treg expansion, treatment with TRI MPs enables expansion of allergen-specific Tregs, regardless of whether the antigens are specific known proteins (e.g. OVA), or a broad array of unknown haptened epidermal proteins. This is especially important for treatment of hapten-mediated ACD, since a single defined allergenic protein or peptide is unlikely to be available for therapy, and immunogenicity of the extensive, heterogeneous repertoire of hapten-protein conjugates differs among individuals. This approach may also be suitable for suppressing allograft rejection and graft vs. host disease, which involve T-cell responses against multiple unknown graft-associated or self-antigens. Ultimately, sustained local delivery of Treg-inducing factors from TRI MP may also be used to generate tolerance and halt the destructive inflammation responsible for a variety of other autoimmune diseases. In these scenarios, the patient's tissue or allograft would serve as a source of unidentified antigens, and TRI MP would provide a Treg-inducing micro-environment for antigen presentation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2017.07.006>.

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***In Vivo* Induction of Regulatory T Cells Promotes Allergen Tolerance and Suppresses Allergic Contact Dermatitis**

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Supplementary Figures:

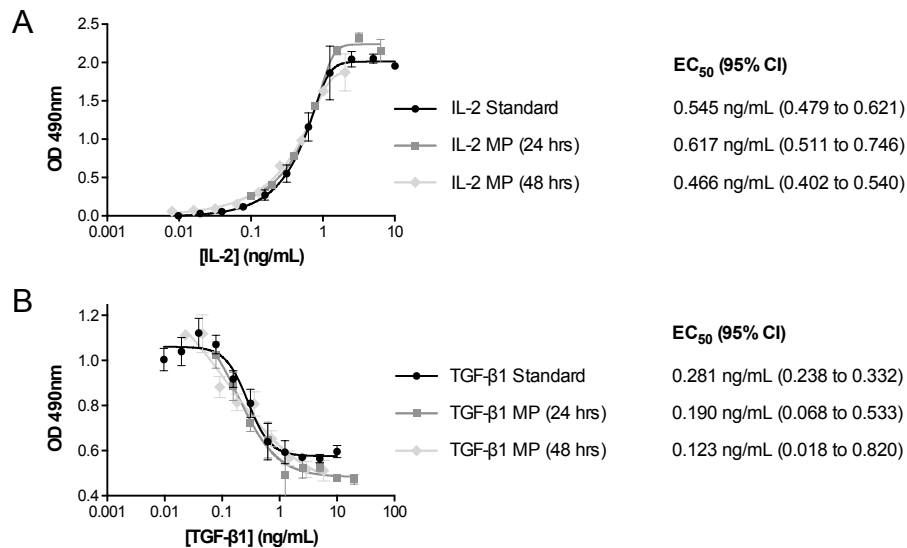


Fig. S1. IL-2 and TGF-β1 released from MPs and detected by ELISAs are bioactive. **(A)** IL-2 released from MPs induces proliferation of HT-2 cells with potency and efficacy comparable to unencapsulated IL-2 standard. **(B)** TGF-β1 released from MPs inhibits IL-4-induced proliferation of HT-2 cells with potency and efficacy comparable to unencapsulated TGF-β1 standard. For both bioassays, MPs were incubated in complete RPMI media, release samples taken at 24 and 48 hours, and cytokine concentrations determined by ELISA. Total proliferation of HT-2 cells after 48 hours of culture with cytokines was measured by MTS assay, and half-maximal effective concentrations (EC₅₀) determined by nonlinear regression on dose-response curves. Data are representative of release samples from two different batches of MPs per cytokine, and HT-2 cultures were performed in triplicate.

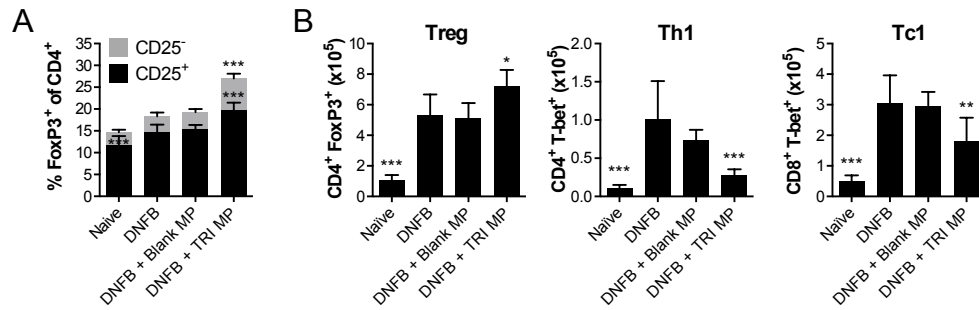


Fig. S2. (A) Treatment with TRI MP during DNFB sensitization expands both CD25⁺ (black) and CD25⁻ (grey) populations of CD4⁺ FoxP3⁺ Tregs in skin DLN, four days post-sensitization. Overall Treg frequencies (% FoxP3⁺ CD25^{+/+} of CD4⁺) are also presented in Fig. 2A. **(B)** TRI MP treatment also increases absolute numbers of Tregs and decreases absolute numbers of Th1 and Tc1 cells in skin DLN. Numbers are cells per DLN. Significant differences, relative to DNFB alone, are indicated by * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ ($N \geq 14$ for % Treg; $N = 5-10$ for absolute numbers).

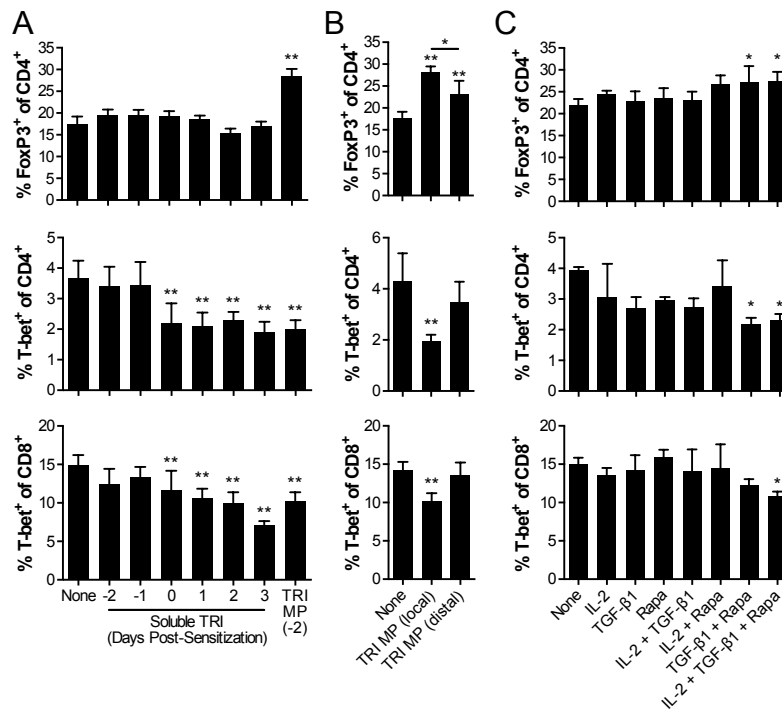


Fig. S3. Effects of sustained local delivery and different combinations of TRI MP on Treg (FoxP3⁺ CD4⁺), Th1 (T-bet⁺ CD4⁺), and Tc1 (T-bet⁺ CD8⁺) populations in DLN four days after sensitizing ears with DNFB. **(A)** Two days prior to sensitization, TRI MP were injected near the sensitization site (base of ears). Alternatively, a bolus of soluble TRI (TGF- β 1, Rapa, and IL-2 w/o MPs) was injected at the base of the ears at the indicated time point with respect to sensitization ($N \geq 5$). **(B)** TRI MP were injected at the base of the ears (local) or at the abdomen (distal) two days prior to sensitization ($N = 7$). **(C)** Mice were treated with different combinations of TRI MP two days prior to DNFB sensitization ($N = 3$). Total mass of injected particles was kept constant by supplementing single or double particle combinations with Blank MP. * $p < 0.05$ or ** $p < 0.001$ vs. DNFB alone (Treatment: "None").

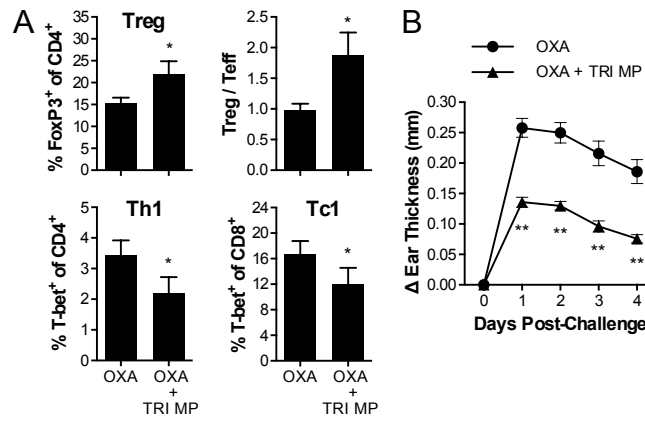


Fig. S4. TRI MPs modulate T-cell responses to OXA and suppress inflammatory response to OXA challenge. **(A)** Frequencies of T-cell subsets in skin DLN at 4 days post-sensitization with OXA. Treg / Teff is the ratio of Treg to Th1 and Tc1 populations. **(B)** Increases in ear thickness (swelling) relative to baseline (pre-challenge) measurements. OXA challenge occurred 10 days after sensitization of ears, and 12 days after treatment with TRI MP at the base of the ears. Significant differences indicated by * $p < 0.05$ or ** $p < 0.001$ (N=5).

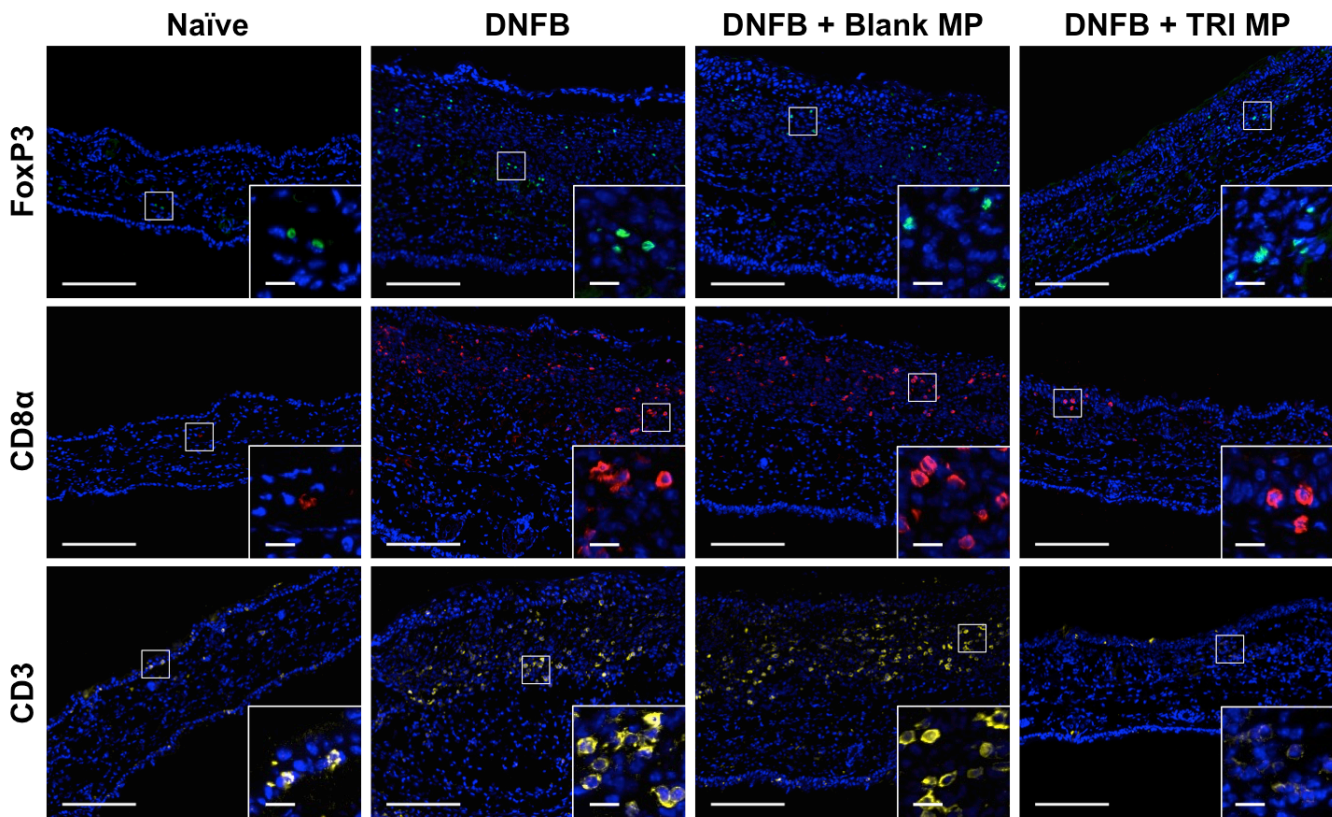


Fig. S5. Representative immunofluorescence images of ear sections used for T-cell counts in Fig. 4I. Sections of naïve ears or ears from four days post-challenge were stained for FoxP3 (Tregs), CD8α (cytotoxic T cells), or CD3 (total T cells), and counterstained with DAPI. False-colored immunofluorescence images were taken using a 20X objective. Large scale bars are 150 μm . Scale bars in magnified insets are 15 μm .

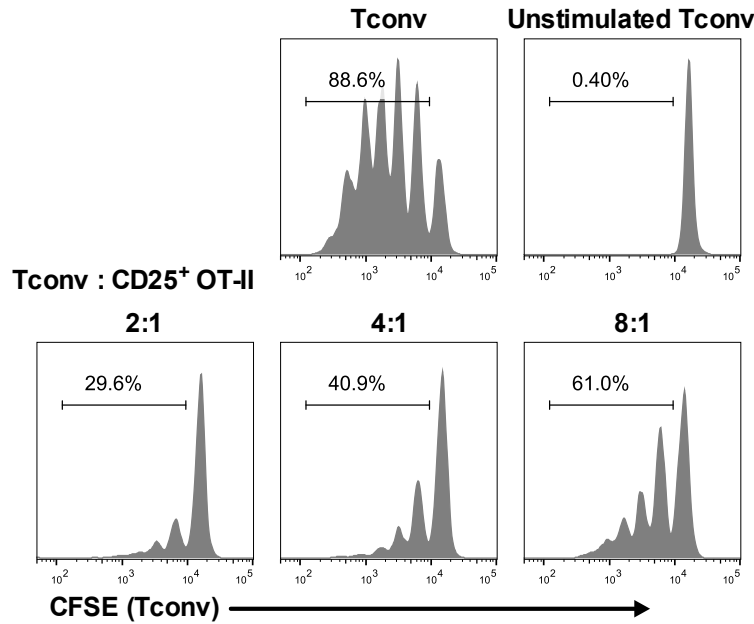


Fig. S6. TRI MP-induced CD25⁺ OT-II T cells suppress proliferation of naïve CD4⁺ CD25⁻ conventional T cells (Tconv). Live CD45.2⁺ CD4⁺ CD25⁺ cells (CD25⁺ OT-II) were FACS sorted from DLN of CD45.1 B6 mice five days after adoptive transfer of 5×10^6 CD45.2⁺ OT-II cells, and four days after OVA sensitization and TRI MP treatment. CFSE-labeled CD45.1⁺ Tconv (5×10^4 / well) were cultured with anti-CD3/anti-CD28 beads (5×10^4 / well) and CD25⁺ OT-II cells at the indicated ratios. Unstimulated Tconv were cultured without anti-CD3/anti-CD28 beads. Based on flow cytometry analysis (Fig. 6E), the CD25⁺ OT-II population contains less than 12.5% FoxP3⁺ Tregs; therefore, a Tconv : CD25⁺ OT-II ratio of 8:1 corresponds to a Tconv : FoxP3⁺ OT-II ratio of at least 64:1. Representative CFSE-dilution histograms depict proliferation of Tconv, and gates indicate percent of cells proliferating after 72 hours. Data are representative of two co-cultures, and CD25⁺ OT-II T cells were pooled from five mice.