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-β1, 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

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-β1 [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-β1, 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 **TR**eg-**I**nducing "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 **T**GF-β1, **R**apamycin, and **I**L-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-*Ptprc^aPepc^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-β1 MP, 50:50 LA:GA; Sigma Aldrich, St. Louis, MO) in 8 mL dichloromethane. For IL-2 and TGF-β1 MPs, 5 μg of recombinant protein (rmIL-2 from R & D Systems, Minneapolis, MN; or rhTGF-β1 from PeproTech, Rocky Hill, NJ) was dissolved in 200 μL deionized water (diH₂O), 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 μL 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 diH₂O 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 (3000*g*, 8 min, 4 °C), washed 4 times in diH₂O to remove residual PVA, re-suspended in 10 mL diH₂O, 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-β1 release assays, 5 mg MPs were suspended in 1 mL PBS with 1% bovine serum albumin (BSA), and incubated at 37 °C with end-over-end rotation. Supernatant release media was sampled and replaced daily, and IL-2 or TGF-β1 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-β1 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-β1 was assessed by IL-2-induced proliferation of HT-2 cells, or TGF-β1-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 × antibiotic-antimycotic solution (Sigma), 1 × non-essential amino acids (NEAA; Lonza), and 55 μM 2-mercaptoethanol (Gibco). For HT-2 expansion, media also contained 10 ng/mL rmIL-2. To assay encapsulated IL-2 and TGF-β1, 10 mg MPs were incubated at 37 °C in 1 mL supplemented RPMI, and release samples taken at 24 and 48 h. IL-2 and TGF-β1 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 μL/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 μL, 2×10^4 cells/well). For the TGF-β1 assay, all wells also contained 7.5 ng/mL rmIL-4 (PeproTech). HT-2 cells were cultured for