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Development of a nanoparticulate formulation of retinoic acid that suppresses Th17 cells and upregulates regulatory T cells

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Abbreviations: RA, retinoic acid; Th17, T helper 17 cell; Treg, regulatory T cell; PGLA- poly(lactic-*co*-glycolic acid); PVA, poly(vinyl alcohol)

Retinoic acid (RA) is a small molecule capable of shunting developing T cells away from the Th17 lineage and towards the Treg phenotype, making it a potentially useful therapeutic for autoimmune and inflammatory diseases. However, therapy can be complicated by systemic toxicity and unpredictable bioavailability, making a targeted drug delivery vehicle for local therapy desirable. A promising approach is the use of nanoparticles, which have been demonstrated to increase potency and decrease toxicity of therapies in a variety of disease models including Th17 mediated diseases. Nanoparticles can also be targeted to specific cell types via surface modification, further increasing the potential specificity of this approach. We therefore constructed a nanoparticulate drug delivery platform from poly(lactic-*co*-glycolic acid) (PLGA) capable of encapsulating and releasing RA. Here we report the fabrication, characterization, and in vitro bioactivity of this platform. We demonstrate that RA containing PLGA nanoparticles suppress IL-17 production and ROR-γ(t) expression in T cells polarized towards the Th17 phenotype in vitro with similar potency to that of free drug. Furthermore, we show that these particles enhance TGF-β dependent Foxp3 expression and IL-10 production of T cells in vitro with similar potency to free RA. Finally, we demonstrate that T cells polarized towards the Th17 phenotype in the presence of free and nanoparticulate RA have similarly suppressed ability to induce IL-6 production by fibroblasts. Our findings demonstrate the feasibility of RA delivery via biodegradable nanoparticles and represent an exciting technology for the treatment of autoimmune and inflammatory diseases.

Introduction

A unique subset of interleukin-17A (IL-17) producing CD4$^+$ cells, termed Th17 cells, is implicated in a variety of autoimmune diseases.[1,2] Th17 cells are potent inducers of inflammation and play key roles in the development of autoimmunity in animal models[3,4] as well as inflammatory human diseases such as rheumatoid arthritis,[5] multiple sclerosis[6] and inflammatory bowel disease.[7] Th17 differentiation occurs when naïve T cells are activated under the influence of TGFβ and IL-6 in mice.[8] Similarly, regulatory T cells (Tregs) also require TGFβ signaling[9] yet have a divergent phenotype that promotes immune tolerance.

The common requirement of TGFβ signaling for the production of two distinct subsets of CD4$^+$ cells with such diverse roles has prompted the investigation into additional regulators of Th17 and Treg cell differentiation. In particular, Mucida et al. demonstrated that retinoic acid (RA) can modulate T cell differentiation between the Th17 and Treg lineage by promoting the development of Treg cells and away from Th17 differentiation (**Fig. 1**).[10] Given the respective and largely antithetical roles of Th17 and Treg cells in autoimmune inflammation, it has been speculated that RA could be used as an effective immunotherapeutic agent for autoimmune conditions.[11-13] To that end, it has recently been demonstrated that RA is effective in decreasing disease burden in an animal model of colitis as well as decreasing IL-17 production in cells from humans with ulcerative colitis.[14,15]

RA, a derivative of vitamin A, is extensively used for the treatment of acute promyelocytic leukemia[16] and dermatological conditions.[17] Studies involving RA use in these conditions have shown that high doses of RA can induce the potentially fatal reaction Retinoic Acid Syndrome[18-20] as well as complications

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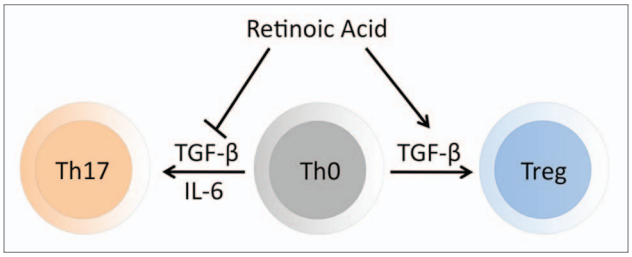

Figure 1. Role of retinoic acid in T cell differentiation. TGFβ is required for the development of both regulatory T cells (Treg) and inflammatory Th17 cells from naïve CD4+ T cells (Th0). Differentiation of Th0 cells into Th17 cells also requires IL-6. Retinoic acid regulates the differentiation of Th0 cells by promoting Treg and suppressing Th17 development.

such as myositis,[21] ascites[22] and hypervitaminosis A, leading to hepatotoxicity, bone abnormalities and birth defects.[19,23] The incidence and severity of these effects are directly related to serum concentration; however, it has been demonstrated that oral bioavailability of RA is extremely variable and unpredictable between patients.[24] A vehicle for targeted localized delivery of RA to immune cells is therefore highly desirable for the treatment of autoimmune disease.

One such potential drug delivery platform is drug loaded biodegradable nanoparticles. Such particles are composed of a biodegradable matrix and release drug into their local environment over time. Nanoparticles can be fabricated from biodegradable polymeric materials, such as poly(lactic-*co*-glycolic acid) (PLGA), that are non-toxic and FDA approved for use in humans and have been used extensively for localized drug delivery.[25] Nanoparticles have been demonstrated to increase potency and bioavailability of immunotherapeutics as well as minimize systemic toxicity in vitro and in vivo.[26-29] In addition, these systems can be targeted to specific tissues or cell types via surface modification by a variety of methods.[26,30-32] Orally delivered nanoparticles have been demonstrated to adhere to inflamed intestinal mucosa[33] and are effective in the treatment of experimental colitis.[34,35]

Here we describe the fabrication, characterization, and in vitro testing of a biodegradable nanoparticulate drug delivery vehicle for retinoic acid composed of PLGA. We determined fabrication conditions and characterized the drug release profile and physical dimensions of our drug delivery vehicle. We also ascertained bioactivity of RA released from our particles on the development of Th17 and Treg cells relative to free retinoic acid. These initial studies demonstrate the promise of this system for modulation of the regulatory/inflammatory axis important in many disease states.

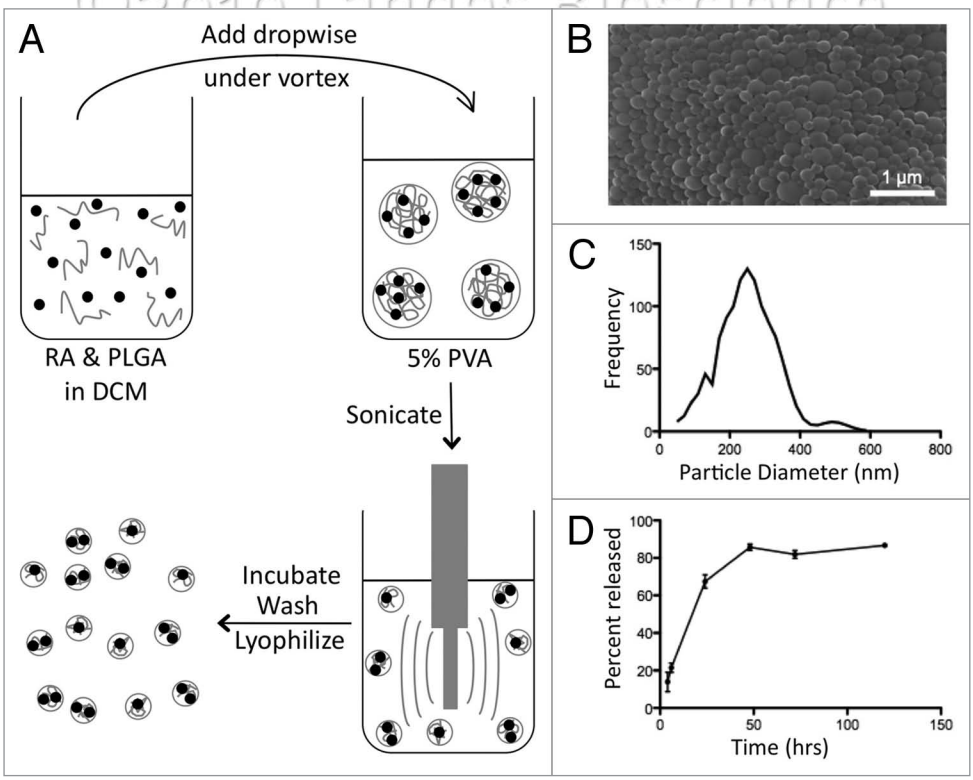

Figure 2. Fabrication and characterization of RA-loaded PLGA nanoparticles. (A) Schematic representation of single emulsion nanoparticle fabrication. RA (black circles) and PLGA (gray lines) are dissolved in dichloromethane (DCM) and added dropwise to a vortexing solution of 5% PVA to form an oil-in-water emulsion. Probe sonication produces nano-sized droplets of DCM containing dissolved PLGA and RA. Evaporation of organic solvent, washing and lyophilization yields spherical PLGA particles with RA molecules entrapped in the polymer matrix. (B) SEM reveals similarly sized spherical particles with smooth surface morphology. (C) Hydrodynamic particle size distribution reveals a monodisperse distribution with average particle diameter of 252 nm. (D) Control release curve of RA from PLGA particles at 37°C in PBS reveals biphasic release kinetics with an initial burst release for 24 hours followed by gradual release. Results shown are for triplicate samples.

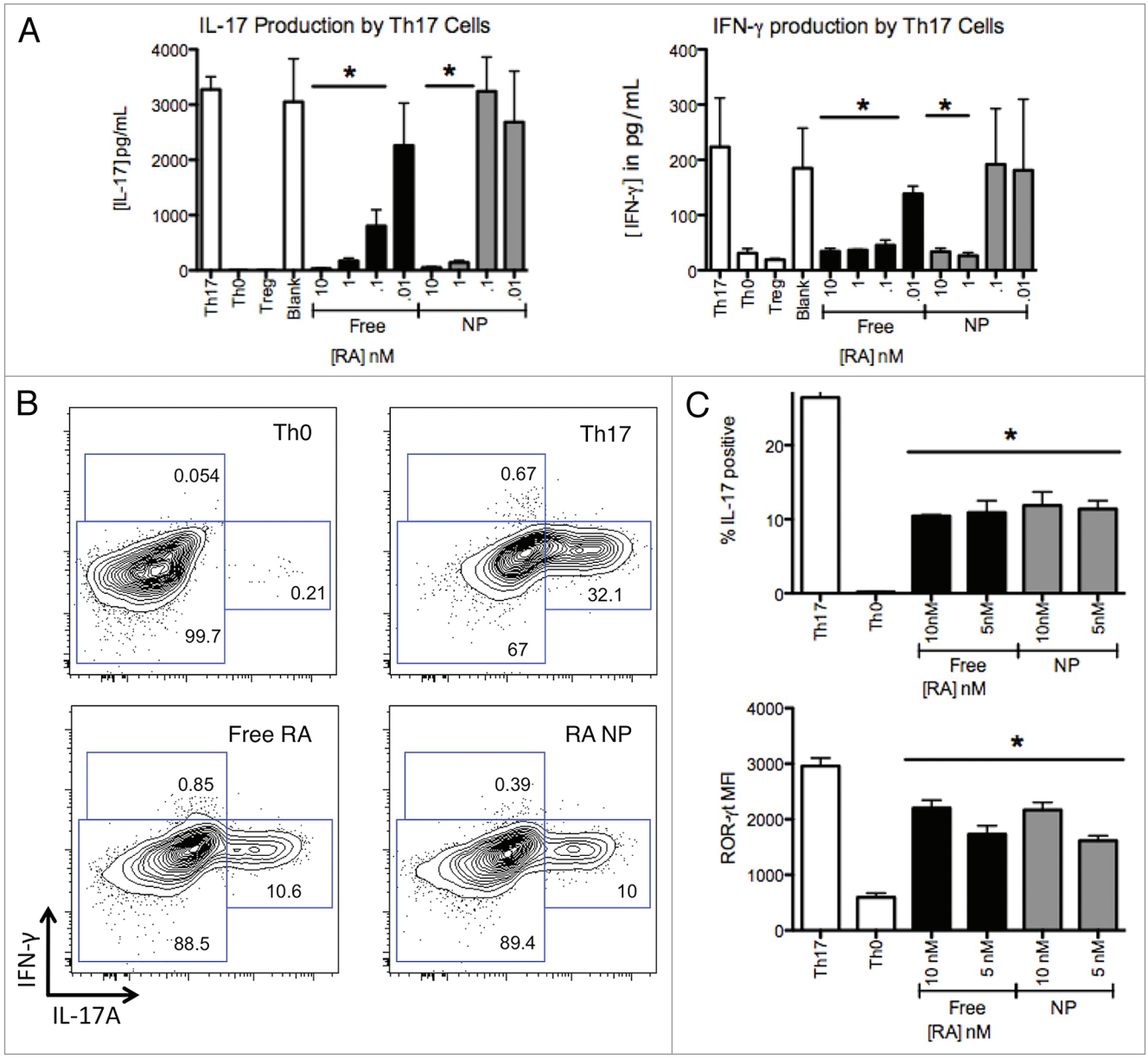

Figure 3. RA treatment decreases Th17 differentiation. CD4⁺ cells were polarized towards the Th17 phenotype and treated with soluble RA or RA-loaded PLGA nanoparticles for 5 days. (A) Cells treated with RA had decreased production of IL-17 and IFNγ. (B and C) Flow cytometric analysis revealed that RA treatment decreased intracellular expression of IL-17 and the key Th17 transcription factor RORγ(t). Flow cytometry plots are for [RA] = 10 nM. *$p < 0.05$, comparing treated to untreated Th17 cells.

Results and Discussion

Particle characterization. A schematic representation of nanoparticle fabrication using a single emulsion oil-in-water technique is depicted in **Figure 2A**. Observation of particles via scanning electron microscopy (SEM) revealed similarly sized spherical particles with a smooth surface morphology all of roughly similar dimension (**Fig. 2B**). The hydrodynamic particle size distribution was measured by nanoparticle tracking analysis using a Nanosight LM-10HS and the average particle diameter was 252 nm (**Fig. 2C**). The control release curve of RA-loaded nanoparticles is typical for PLGA fabrication, revealing a biphasic profile with an initial burst of RA release during the first 24–48 hours, followed by its slow gradual release in a plateau phase (**Fig. 2D**). Loading of particles was 3.15–2.29 μg RA/mg PLGA and loading efficiency was 72.5 ± 11.5%. Observed morphology via SEM, particle size distribution, control release characteristics and loading efficiency were similar for all batches of particles used in experiments.

RA-loaded nanoparticles inhibit Th17 differentiation. In order to assess the bioactivity of RA released from PLGA nanoparticles relative to free compound, we cultured CD4⁺ cells under Th17 polarizing conditions[36] in the presence of RA dissolved freely in solution or loaded in nanoparticles. We found

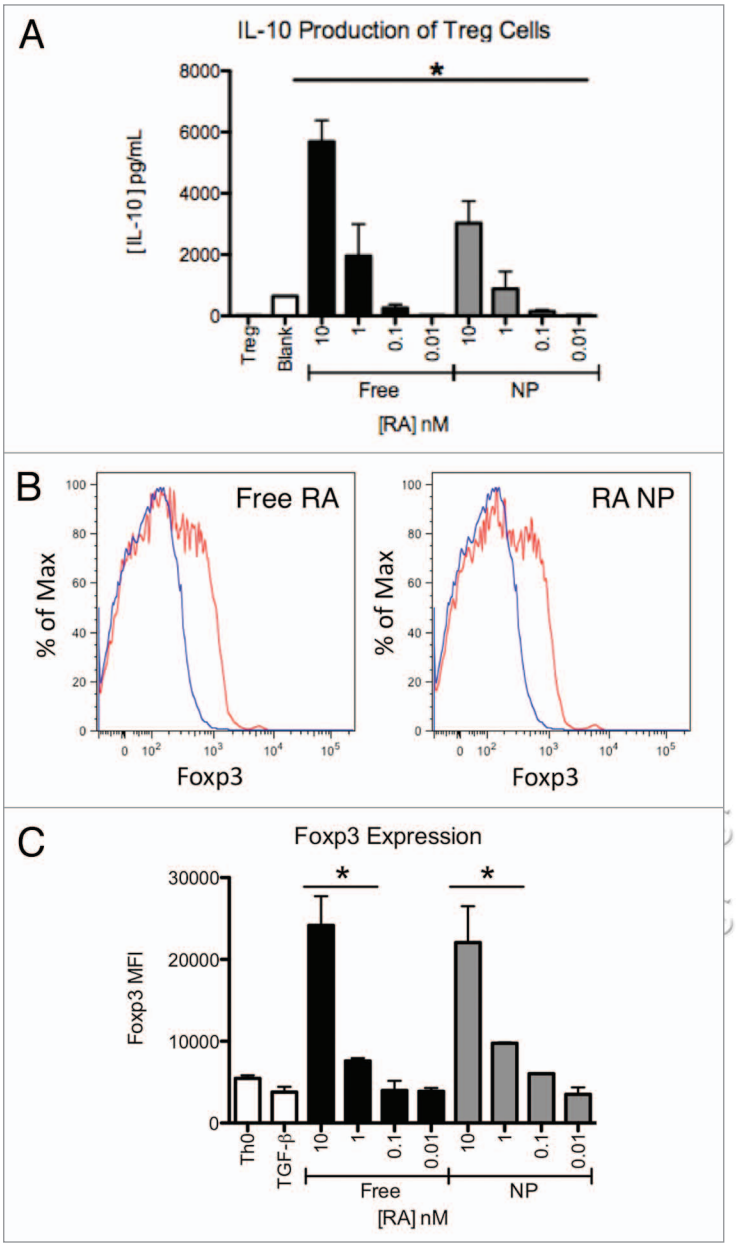

Figure 4. RA treatment enhances the Treg phenotype. CD4⁺ cells were treated with RA and TGFβ for 5 days in order to quantify the effect of RA on the Treg phenotype. (A) RA treatment increases the production of IL-10. (B and C) Flow cytometry reveals that cells treated with RA have an increased expression of Foxp3. Histograms are shown for cells treated with 10 nM RA. Blue depicts TGFβ only, Red depicts TGFβ and RA. *p < 0.05, comparing treated to untreated cells.

that both free and particulate-encapsulated RA decreased the secretion of IL-17 and IFNγ in a dose dependent manner (**Fig. 3A**). RA potency was similar between free and particulate drug for concentrations of 10 nM, 1 nM and 0.01 nM. Free drug was more potent than nanoparticles at 0.1 nM. In order to more closely investigate the phenotype of cells treated with RA, we examined cells for Th17 specific markers via flow cytometry. We observed that CD4⁺TCRβ⁺ cells treated with free or encapsulated RA similarly expressed lower levels of intracellular IL-17 and RORγ(t), the key transcription factor of the Th17 lineage (**Fig. 3B and C**).[37]

RA nanoparticles enhance the Treg phenotype. Since RA can enhance the development of Treg cells,[10] next we investigated the effect of RA nanoparticles on cells cultured under conditions that promote T regulatory cell differentiation. Free and PLGA-encapsulated RA increased the IL-10 expression of Treg cells in a dose responsive manner (**Fig. 4A**); however, free RA treatment resulted in greater increases in IL-10 production than did nanoparticles. Unlike IL-10 production, free RA and RA-containing nanoparticles resulted in similar increases in Foxp3 expression, as measured by flow cytometry (**Fig. 4B and C**).

RA nanoparticles suppress IL-17 dependent IL-6 production by fibroblasts. Th17 cells promote inflammation and autoimmunity in part by inducing the production of IL-6 in neighboring fibroblasts via IL-17, thereby establishing a positive feedback cycle favoring the development of more Th17 cells.[38] We therefore tested the ability of T cells treated with free and nanoparticulate RA to trigger the production of IL-6 by fibroblasts in vitro (**Fig. 5**). Whereas fibroblasts treated with IL-17 alone or co-cultured with Th17 cells produced significant amounts of IL-6, those co-cultured with T cells polarized towards the Th17 phenotype in the presence of free or nanoparticulate RA did not, with a similar magnitude of suppression.

In this work, we demonstrated a methodology for delivering bioactive RA in a biodegradable, FDA-approved polymer-nanoparticle format that can potentially address issues related to delivery of this drug. We showed that RA-loaded PLGA nanoparticles reduce IL-17 production and RORγ(t) expression by CD4⁺ T cells exposed to Th17 polarizing conditions in vitro. Furthermore, RA nanoparticles are able to enhance the phenotype of CD4⁺ cells polarized towards the Treg phenotype in vitro. These findings are promising for the treatment of those autoimmune and inflammatory diseases thought to be driven by an excess of inflammatory Th17 cells and a relative deficiency of the anti-inflammatory action of Treg cells.

Although the potency of our RA nanoparticles is largely equivalent to that of free drug in vitro, it should be noted that concentrations of RA delivered to cells within particles was determined according to particle loading and not the amount of RA released from particles. Therefore, cells treated with RA nanoparticles received only approximately 80% as much RA as those cells treated with free drug at a given concentration. In addition, our experiments were designed to evaluate the feasibility of nanoparticulate encapsulation and delivery of RA, not to demonstrate an advantage of nanoparticulate drug delivery over free drug per se. For example, our particles were not functionalized with T cell targeting ligands. In addition, the advantage of local drug delivery via nanoparticles versus systemically administered free drug cannot be determined in vitro. Therefore, our findings present a promising line of inquiry into the usage of this novel formulation for autoimmune and inflammatory disease therapy.

Materials and Methods

Particle fabrication. PLGA nanoparticles containing all-trans retinoic acid (RA) (Sigma-Aldrich, R2625) were fabricated using a single emulsion oil-in-water technique. Briefly, 200 mg of PLGA 50/50 with an average molecular weight of 80 kD (Durect Corporation B6010-2P) and 0.751 mg of RA were dissolved in 2 ml of dichloromethane. This was added to 5% aqueous poly(vinyl alcohol) (PVA) (Sigma-Aldrich P1836) and sonicated three times for 10 seconds at 38% amplitude (TEKMAR VCW 400W) on ice. The emulsion was incubated for 1 hour in 100 ml of 0.2% aqueous PVA and stirred to allow for evaporation of the dichloromethane and hardening of the nanoparticles. Particles were collected via centrifugation at 12,000 rpm at 4°C and washed three times with de-ionized water. Particles were then lyophilized and stored at -20°C in an opaque container to protect RA from ambient light until use.

Particle characterization. Particle size was determined using the nanoparticle tracking analysis instrument Nanosight LM-10 with specialized software (Nanosight LTD). Particle morphology was observed with scanning electron microscopy. Loading was determined by dissolving a predetermined mass of particle in dimethyl sulfoxide and quantifying the amount of RA in the fully dissolved sample spectrophotometrically using absorbance spectroscopy at 360 nm. Loading efficiency was calculated by dividing the mass of RA present in particles by the amount of RA initially added in the particle formulation and multiplying by 100%. Controlled released characteristics for RA PLGA nanoparticles was determined via dialysis in PBS at 37°C.

Cell culture and in vitro T cell stimulation. Six-week-old C57BL/6 mice were purchased from Charles River. Mice were housed under specific pathogen-free conditions and used between 7–12 weeks of age. $CD4^+$ T cells were isolated from the spleen and lymph nodes of mice using an Easy Sep $CD4^+$ T cell enrichment kit (Stemcell Technologies, 19752) according to the manufacturer's instructions. $CD4^+$ T cells were cultured in Click's Media (Irvine Scientific, 9195) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol for 5 days. Cell stimulation conditions were adapted from previously described protocols.[10,36] Cells were plated at a density of 1 x 10^6 cells/ml and volume of 500 μL/well in 24 well plates or 250 μL/well in 48 well plates. Th17 cells were generated by culturing cells with 1 μg/ml anti-CD3ε, (BD Bioscience, 553058), 1 μg/ml anti-CD28 (BD Bioscience 553295), 20 ng/ml IL-6 (Peprotech 216-16) and 5 ng/ml TGFβ(Peprotech 100-21). Treg cells were generated by stimulating with immobilized anti-CD3ε (250 μL of 10 μg/ml), immobilized anti-CD28 (250 μL of 2 μg/ml) and 5 ng/ml TGFβ. Media for Treg generation was identical to that used for Th17 generation. RA or RA PLGA nanoparticles were added at day 0 at the appropriate concentration. Cytokine production was quantified by ELISA for IL-17 (BD Bioscience 555068 and 555067) and IFNγ (BD Biosciences 551309 and 551506) for Th17 cells and IL-10 (eBioscience 88-7104-77) for Treg cells. Th0 cells were used as negative controls and were generated by stimulating with soluble anti-CD3ε (250 μL of 10 μg/ml) and immobilized anti-CD28 (250 μL of 2μg/ml) only. All other stimulation conditions for control cells were kept constant. $CD4^+$ cells treated with PLGA nanoparticles not containing RA (blank) were also used as negative controls. All animal care and experimentation were consistent with NIH guidelines and approved by the Yale University Institutional Animal Care and Use Committee.

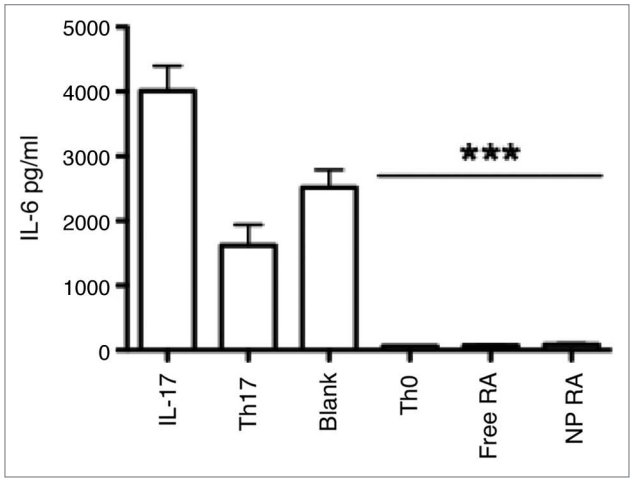

Figure 5. RA suppresses IL-17 dependent IL-6 production by fibroblasts. $CD4^+$ T cells were polarized towards the Th17 phenotype and treated with soluble RA or RA-loaded PLGA nanoparticles for 5 days and subsequently co-cultured with fibroblasts for 24 hours. IL-6 was then quantified in the supernatant by ELISA. Fibroblasts treated with IL-17 or co-cultured with Th17 cells had higher production of IL-6 than those co-cultured with $CD4^+$ cells polarized towards the Th17 phenotype that were also treated with RA. ***$p < 0.001$, for comparison of any of the three rightmost columns with any of the three leftmost columns.

Flow cytometry analysis. On day 5 of culture, $CD4^+$ cells were washed with Click's media, resuspended in media containing 20 ng/ml PMA, 2,000 ng/ml ionomycin and 1 μL/ml Golgi Plug (BDBioscience 555028) and incubated for 6 hrs. Cells were washed and resuspended in staining buffer containing 1x PBS and 2% BSA. Anti-CD4 conjugated to Pacific Blue (BD Bioscience 558107) and anti-TCRβ conjugated to APC780 (eBioscience 47-5961-80) were added and cells were incubated for 30 minutes on ice. Cells were permeabilized using Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BDBioscience 555028) according to the manufacturer's instructions and stained with anti-IL-17A conjugated to Alexa647 (eBioscience 51-7177-80), anti-RORγ(t) conjugate to PE (eBioscience 12–6988) and anti-Foxp3 conjugated to Alexa700 (eBioscience 56-5773-80). Analysis was performed the same day using a LSRII Flow Cytometer (Becton-Dickinson and Company).

Fibroblast/Th17 co-culture. NIH 3T3 fibroblasts were cultured in high-glucose DMEM media supplemented with 10% heat inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and allowed to adhere to the plate surface overnight. Cells were plated at a density 2 x 10^5 cells/mL and a volume of 1 mL. T cells were polarized towards the Th17 phenotype and treated with 10 nM free RA or RA nanoparticles for 5 days as described above. Cells were subsequently washed, restimulated with soluble 1 μg/ml anti-CD3ε (BD Bioscience, 553058) and

1 μg/ml anti-CD28 (BD Bioscience 553295) and added to the fibroblasts at a density of 5 x 10^5 cells/well. T cells and fibroblasts were co-cultured for 24 hrs, after which time supernatant was collected and analyzed for IL-6 via ELISA (BD Biosciences 550950).

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