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Development Of A Nanoparticulate Drug Delivery Vehicle For Retinoic Acid

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Abstract

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. 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 and IFN-γ 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 RA 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.

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List of Abbreviations

AICD- activation induced cell death

AIRE- autoimmune regulator

APECED- autoimmune polyendorinopathy-candidiasis-ectodermal dystrophy

BCR- B cell receptor

BSA- bovine serum albumin

CD- cluster differentiation

CIA- collagen induced arthritis

D- diffusion doefficient

DCM- dichloromethane

DMEM- Dulbecco's modified eagle medium

dsDNA- double stranded DNA

EAE- experimental allergic encephalitis

ELISA- enzyme-linked immunosorbent assay

EPR- enhanced permeability and retention

FBS- fetal bovine serum

G-CSF- granulocyte colony-stimulating factor

GM-CSF- granulocyte-macrophage colony-stimulating factor

IBD- inflammatory bowel disease

IFN-γ- interferon gamma

IL- interleukin

MFI- mean fluorescence intensity

MWCO- molecular weight cutoff

NP- nanoparticle

o/w- oil in water

PBS- phosphate buffered saline

PLGA- poly(lactic-co-glycolic acid)

PVA- poly (vinyl alcohol)

RA- retinoic acid

SEM- scanning electron microscopy

SLE- systemic lupus erythematosis

TCR- T cell receptor

TGF-β- transforming growth factor beta

Th- T helper

TLR- toll-like receptor

TNF-α- tumor necrosis factor alpha

Treg- regulatory T cell

w/o/w- water in oil in water

Chapter 1

Introduction

1.1- Targeted Therapy

The concept of targeted therapy is an old one. It was first formally proposed in 1906 by the eminent physician Paul Ehrlich in which he imagined a drug that selectively affected a specific diseased structure or pathological process to the exclusion of all else[1]. As an early developer of chemotherapy for cancer treatment, the appeal of a "magic-bullet" that could cure disease without causing side effects must have been immense. The concept of targeted therapy has broadened since Ehrlich's time to include therapies not only for cancer but any pharmacologic treatment that has side effects.

1.1.1- Therapeutic Targeting Strategies

Vast improvements have been made in the development of targeted therapeutics since the concept was first proposed over 100 years ago. Two strategies have been identified to achieve targeted drug delivery- pharmacologically targeted therapies

and local drug delivery[2,3]. Pharmacological targeting refers to the use of a therapeutic that selectively affects a specific pathological process implicated in the disease being treated with the hope that normal biological processes remain unaffected. Local drug delivery achieves targeting by applying or delivering a drug to the physical location of disease, thereby leaving normal structures free of potential side effects.

Modern medicine is rife with examples of pharmacologically targeted therapies. Many antibiotics and chemotherapy agents selectively affect processes or molecules exclusive to invasive microorganisms or tumor cells respectively. A few examples include penicillin class antibiotics that interfere with bacterial cell wall synthesis, tyrosine kinase inhibitors that disrupt replication of tumor cells, and monoclonal antibodies directed against specific cell types such as the cell surface receptor HER2/neu expressed in breast cancer, CD-20 in B cell lyphomas, and the hematopoetic marker CD33 for treatment of AML[4]. Pharmacologically targeted therapies have recently revolutionized the treatment of autoimmune and inflammatory diseases as well. Examples include monoclonal antibodies against molecules implicated in the pathogenesis of these diseases such as anti-TNF therapy for rheumatoid arthritis[5] and inflammatory bowel disease[6] as well as the monoclonal antibody natalizumab against alpha-4 integrin for the treatment of multiple sclerosis[7] and Crohn's disease[8].

Local drug delivery, although less frequently employed, is also used to increase the specificity of pharmacological treatments. Due to ease of application, local drug delivery is most frequently used for treatment of skin diseases. The respiratory tract is another convenient site for local drug delivery. Common examples include nasal delivery of anti-inflammatory agents and vaccines as well as inhaled drugs for the treatment of pulmonary diseases such as asthma.

Despite the success of using local drug delivery to achieve targeting, it has been difficult to implement in cases where the disease does not involve easily accessible areas of the body such as the skin or respiratory tract. Several orally administered therapies use the fact that they are not absorbed across the gut epithelium to their advantage and are used to treat gastrointestinal illnesses. Examples of this strategy include anti-inflammatory formulations for the treatment of inflammatory bowel disease[9] and antibiotics such as rifaxamin, nyastatin, and vancomycin against pathological organisms in the gastrointestinal tract[10,11].

It has proven difficult to access other sites of the body for local drug delivery, however. Methods for achieving local drug delivery to other sites are invasive and undertaken only during the treatment of serious illness when the toxicity of treatment makes it absolutely necessary to minimize side effects. For example, neurosyphilis is often treated with intrathecally-administered penicillin. Prostate cancer can be treated by surgically implanting radioactive seeds within the tumor. In 2003, the FDA approved a therapy that incorporated the chemotherapeutic

carmustine into a biodegradable wafer that is surgically implanted into the brain for treatment of malignant gliomas[12]. This final example of local drug delivery has the further advantage of utilizing a drug delivery vehicle that gradually releases drug into the environment over time, called controlled release, resulting in steadier drug levels and increased therapeutic efficacy [13-15].

1.1.2- Nanoparticles as Drug Delivery Vehicles

Nanoparticles refer to a class of engineered colloidal systems with physical dimensions measured on the nanometer scale, typically less than 1μm. There are five major classes of nanoparticles; particles with polymeric matrices, particles with inorganic matrices such as gold or silica, liposomal nanoparticles, viral nanoparticles, and particles composed of carbon networks such as nanotubes[16,17]. Each class of nanoparticle differs significantly with respect to physical characteristics, biological activities, applications, and fabrication methods. Although this thesis will focus exclusively on polymeric nanoparticles, many of the concepts described below are applicable to most or all of the nanoparticle classes[16].

A nanoparticulate drug delivery system is created by fabricating particles that incorporate drug into the interior of the particle, a process called encapsulation or drug loading[17]. The matrix of the particle is chosen such that it degrades in a time dependent manner, often via hydrolytic cleavage with water molecules so that drug release occurs when particles are in an aqueous environment, releasing drug from its core and into the vicinity (see Figure 1)[18].

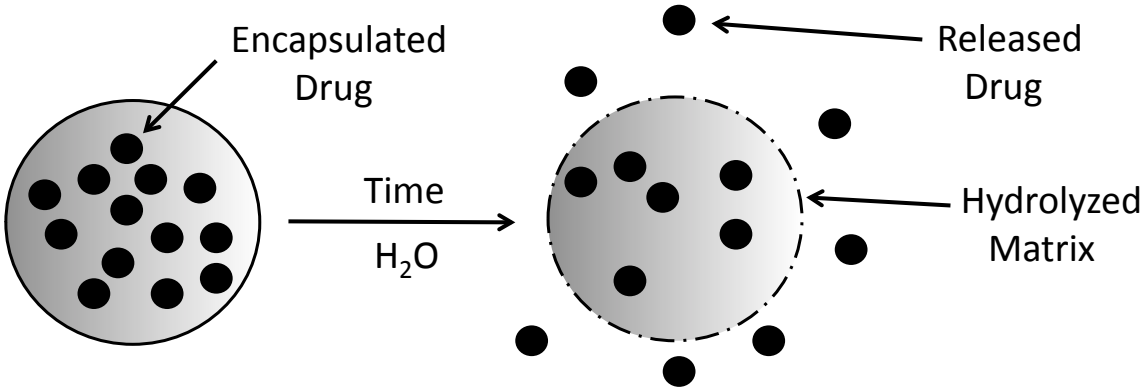

Figure 1- Schematic representation of drug loaded nanoparticle. Encapsulated drug is released upon degradation of particle matrix, in this case via hydrolysis.

Nanoparticles have been under investigation for several decades and many properties that make them ideal for local drug delivery have been characterized. One advantage is the ability to fabricate particles of various sizes, affecting biodistribution[19], bioavailability[20], excretion[21], and trafficking across biological barriers[22,23]. Another advantage is the ability to adjust the composition of particle matrices in order to alter the kinetics of drug release[24,25]. Particles can also be loaded with a variety of therapeutic and imaging agents simultaneously, allowing for a multimodal diagnostic and treatment approach[26]. Finally, particles can be fabricated from a wide variety of polymers, many of which are non-toxic and have already been approved for clinical use in humans by the FDA[27,28].

Perhaps the most intriguing property of nanoparticles is their ability to deliver drug to specific sites or cell types within the body, thereby being useful in achieving local drug delivery[29]. As previously discussed, drug loaded nanoparticles release the encapusulated drug contained within their core and into the local environment in a

time dependent manner. Particles can be fabricated to encapsulate a high density of drug within the core, making it possible to achieve high local concentrations of drug in the immediate vicinity of the particle as it is releasing its contents[29]. *In vivo,* drug-loaded nanoparticles within close proximity to a site of disease would deliver a high local concentration and low systemic concentration of drug, maximizing treatment efficacy and minimizing systemic side effects.

Physical localization of particles to sites of disease or to specific cell types can be achieved through a variety of mechanisms. The simplest of these mechanisms is called "passive targeting", in which particles localize to sites of disease purely by virtue of their small size[30]. The best-characterized type of passive targeting is called the Enhanced Permeability and Retention Effect (EPR). EPR occurs in areas with leaky vasculature and impaired lymphatic drainage associated with solid tumors and sites of inflammation, allowing for the preferential accumulation of nanoparticles in these sites[31,32].

Passive targeting also occurs to the intestinal mucosa for orally delivered particles. Nanoparticles have been shown to be retained within the gastrointestinal tract in a manner inversely proportional to their size[33,34]. Furthermore, it has been demonstrated that nanoparticles localize specifically to areas of inflammation in animals models of colitis[35,36]. Authors have postulated that the mechanism of this selective retention is increased mucous production and greater number of phagocytic cells in areas of inflammation, specifically macrophages[35,36]. When

loaded with therapy, orally delivered nanoparticles demonstrate increased therapeutic efficacy and decreased systemic side effects over free drug in the treatment of animal models of colitis[37,38].

Localization of particles to organs or cell types that may not be conducive to passive targeting can be achieved via active targeting in which the particle surface is functionalized with targeting ligands[39]. Equipped with these targeting moieties, particles may act as "homing devices" to specific sites of disease[29]. Typically, targeting ligands are chosen that have a high binding affinity to surface receptors expressed by specific cell types such that the particle will preferentially bind to individual targeted cells to the exclusion of others[39].

Various strategies exist for active targeting, the most common of which utilize lectin-glycoprotein, ligand-receptor, and antibody-antigen interactions. Lectin targeting (carbohydrates on particle surface targeted to cell lectins) and reverse lectin targeting (lectins on particle surface targeted to cell glycoproteins) represent some of the earliest strategies for active targeting of nanoparticles[40]. A high degree of cell specificity has been achieved by coating particles with artificially synthesized polysaccharides that are tailored to bind lectins expressed on certain types of cells[40]. Lectin-glycoprotein targeting strategies have been shown to enhance efficacy of nanoparticulate formulations of chemotherapy for colon and liver cancer[41,42], antibotics for H. pylori eradication[43], and have even been evaluated *in vitro* for colonic delivery of ondansetron for treatment of Irritable Bowel

Syndrome[44]. In ligand-receptor targeting, particles are functionalized with ligands that bind to receptors overexpressed specifically by diseased tissue. The most studied example is folate functionalized particles targeted against folate-receptor overexpressing tumors[45-47]. Other examples of successful ligand-receptor particle targeting include the functionalization of antigen containing particles with TLR agonists such as LPS and flagellin. Such particles have been shown to enhance antigen delivery and enhance vaccination efficacy *in vivo*[48,49]. The final method of active targeting, antibody-antigen targeting, has been widely studied for applications to cancer. Nanoparticles can be made to bind to certain cells with high selectivity by decorating the particle surface with an antibody against a specific cell surface marker. Examples of successful application of this strategy include studies that target B cell lymphoma with particles conjugated to anti-CD19 and anti-CD20[50], breast cancer cells with anti-HER2 functionalized particles[51,52], and neuroblastoma cells with anti-GD2 functionalized particles[53]. Antigen-antibody targeting has also been employed to shape immune responses by functionalizing IL-2 containing nanoparticles with a stimulatory anti-CD3 antibody alongside peptide-loaded MHCII for stimulation of naïve T cells[54].

1.1.3- Nanoparticle Fabrication

Nanoparticles have been manufactured for over two decades using a variety of techniques[55]. Considerations when choosing between fabrication techniques include the particle material, desired size, toxicity, ability to carry therapeutics, and ability to add targeting ligands to the surface of the particle. The most commonly

utilized methods for the fabrication of drug loaded particles include emulsion chemistry[56], spray drying[57], and nanoprecipitation[58].

Emulsion techniques are especially conducive to drug encapsulation and allows for high degrees of control over particle size and encapsulation efficiency while remaining flexible enough to utilize a variety of particle materials and encapsulants[59]. An emulsion refers to a mixture of two immiscible liquids in which one liquid is dispersed as tiny droplets within the other. In this technique, particles are formed within these small droplets of dispersed solvent. Emulsion techniques take advantage of the fact that small droplets of predictable size are produced upon the addition of mechanical energy to a mixture of two immiscible liquids. Solid particles can be obtained from an emulsion when the particle matrix material has differential solubilities between the two emulsion components.

Typically, polymeric particles are produced by first dissolving the chosen polymer in a small volume of volatile organic solvent. This organic solvent is then slowly added to a larger volume of an aqueous solution of an amphiphilic stabilizer. Mechanical energy is added to the system through stirring or sonication which disperses the organic solvent containing the dissolved polymer matrix into tiny droplets, forming an oil in water emulsion (denoted o/w, in which the smaller volume solution, and hence that which is forming droplets, is written first). The viscosity of both the organic and aqueous phase as well as the magnitude of mechanical energy put into the system affect droplet size. As the droplets are produced, the amphiphilic

stabilizer molecules associate with the surface of the particle and prevent reassociation of droplets. The solution is then incubated to allow for evaporation of the volatile solvent, and solid polymeric particles are left behind[27].

Particles can be fabricated to incorporate an encapsulant within the interior of the particle[27,60]. Encapsulants with a similar solubility profile to that of the particle matrix can be incorporated simply through addition to the initial particle matrix solution. In the case of polymeric particles described above, hydrophobic encapsulants can be dissolved along with the polymer in the organic phase. The encapsulant will remain in the organic phase throughout particle fabrication along with the polymer and will become incorporated within the particle matrix itself. This is called a single emulsion (see Figure 2).

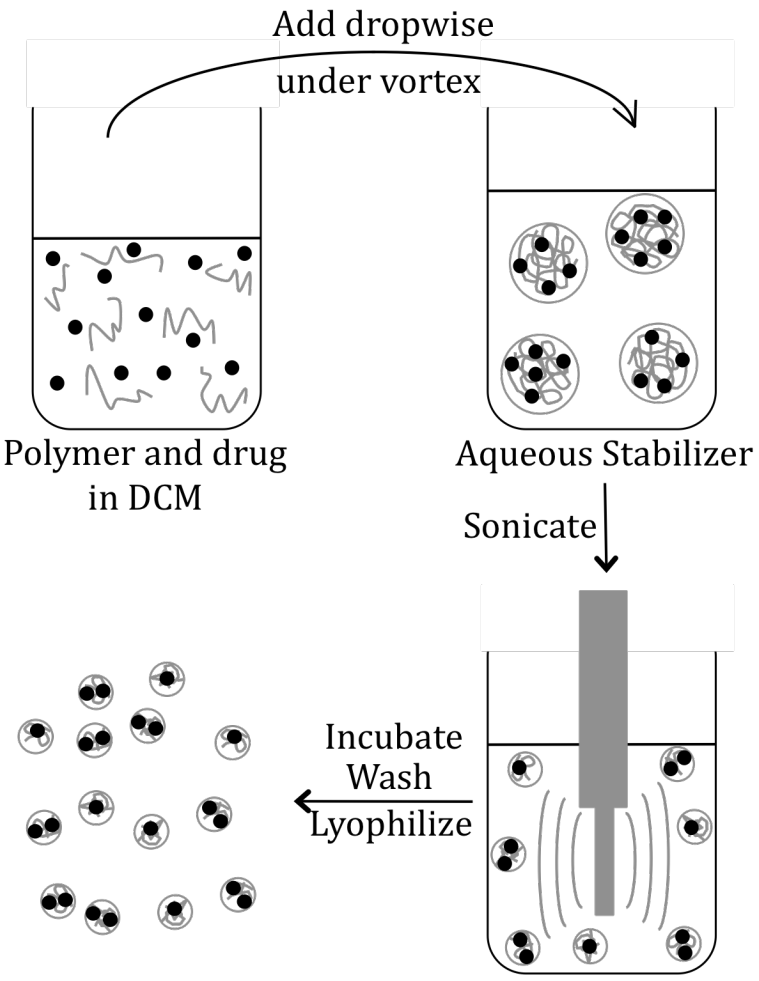

Figure 2- Schematic representation of single emulsion nanoparticle fabrication where ∿ represents a polymer and ● represents a drug. The drug and polymer are dissolved in an organic solvent and added dropwise to a vortexing solution of aqueous stablizer to form an oil-in-water emulsion. Probe sonication produces nano-sized droplets of organic solvent containing dissolved drug and polymer. Evaporation of the organic solvent, washing, and lyophilization yields spherical drug loaded polymeric nanoparticles.

Encapsulants with a dissimilar solubility profile to that of the particle matrix can be incorporated using a double emulsion technique. This technique is called double emulsion because the steps outlined above are repeated twice, essentially forming an emulsion of an emulsion. In this technique, a solution of the encapsulant that is immiscible with that of the particle matrix is prepared first. This solution is added

to the polymer matrix solution, mechanical energy is added, and an emulsion is produced. This emulsion is then added to a larger volume of solution containing a stabilizer as in the single emulsion technique. This is especially useful when producing polymeric particles that contain proteins. Proteins are particularly difficult to incorporate in polymeric particles because they are soluble only in aqueous solutions and easily denature when they contact organic solvents. Protein containing polymeric particles are fabricated by first adding an aqueous protein solution to a solution of polymer in volatile organic solvent and forming a water-in-oil emulsion (w/o). This w/o emulsion is then added to a large volume of a stabilizer containing aqueous solution, mechanical energy is added, and a second emulsion is produced called a water-in-oil-in-water double emulsion (denoted w/o/w emulsion). As above, the mixture is incubated to allow for solvent evaporation and solid particles with aqueous protein containing pockets are produced.

1.2- Autoimmunity

In order to defend against a wide array of pathogens, the immune system has developed potent mechanisms to identify and destroy invaders. However, these mechanisms have the potential to damage the host and it therefore becomes critical that the immune system be able to discriminate between self and non-self[61]. The immune system's natural aversion to self destruction was originally termed *horror autotoxicus* in 1897[62], literally meaning "the horror of self-toxicity". The concept of

horror autotoxicus is represented today in the multiple mechanisms employed by the immune system to discriminate self from pathogens, a process collectively called self-tolerance. Occasionally however, the mechanisms of tolerance can break down, in which case the immune system becomes activated against self-antigens, and autoimmune disease ensues[61].

1.2.1- Mechanisms of Autoimmunity

The induction of self-tolerance begins early in the life of a lymphocyte. Immature lymphocytes develop in the thymus or bone marrow, a place in the body that should be pathogen free. Furthermore, the transcription factor AIRE is responsible for the expression of many peripheral proteins in the thymus[63]. Therefore, antigens recognized by such developing lymphocytes are likely to be self antigen. Such antigen recognition in immature lymphocytes leads to a negative signal and cell death, a mechanism known as central tolerance[64]. Another mechanism by which self-tolerance occurs is that lymphocytes constantly recognizing high and unchanging levels of antigen become tolerized. This occurs because levels of pathogenic antigens should increased sharply following invasion and the levels of antigen should fluctuate given the state of infection; antigens present at constant high levels are likely to be self-antigens. These self-reactive cells are deleted from the T cell repertoire via a process known as activation induced cell death (AICD), process that is mediated by Fas/Fas-ligand interactions[65]. A third mechanism of tolerance is called peripheral tolerance and this acts on mature lymphocytes that are circulating in the periphery. In order for a lymphocyte to become activated, it

must receive co-stimulatory signals from an antigen-presenting cell. Uninfected peripheral tissues do not express co-stimulatory molecules, and therefore lymphocytes that have escaped central tolerance and recognize an antigen in the absence of a co-stimulatory signal are likely autoreactive. This lack of co-stimulation leads to the cell becoming anergic and eventually cell death[66].

The process of maintaining self-tolerance can be thought of as a series of checkpoints that begin with the central deletion of self-reactive immature lymphocytes. None of these multiple checkpoints are 100% efficient, however the cumulative result is a system that can respond to pathogen while also maintaining tolerance to self. In approximately 5% of people however, these mechanisms are insufficient and the immune system becomes activated against self-antigens resulting in autoimmune disease[67].

In rare cases, the autoimmunity is purely the result of genetic defects. For example, mutations in Fas or Fas-ligand that impair activation induced cell death results in Canale-Smith syndrome, an autoimmune syndrome in which patients suffer from massive accumulation of T cells in lymphoid organs and express multiple autoantibodies[68]. Another example of genetically determined autoimmunity is that AIRE defects can lead to APECED (autoimmune polyendorinopath-candidiasis-ectodermal dystrophy) in which patients have autoimmune destruction of multiple endocrine organs[69].

Instances of pure genetic autoimmunity are rare however. It is now generally accepted that autoimmunity most commonly develops as a result of genetic predisposition and failure of intrinsic tolerance mechanisms in the setting of environmental triggers (see Figure 3)[70]. Examples of genetic predispositions to autoimmunity include HLA-B27 and the associated high risk of ankylosing spodylitis[71], HLA-DR3 and HLA-DR4 that confer a 20 fold higher risk of developing Type 1 diabetes[72], and NOD2 mutations that increase the risk of IBD[73]. Although these genetic variances increase risk, they are not fully penetrant and thus not everyone that has them will develop autoimmune disease. Another requirement is the presence of lymphocytes capable of recognizing a self-antigen. Since TCRs and BCRs are produced through random recombination, the development of autoreactive receptor specificity is largely due to chance[70].

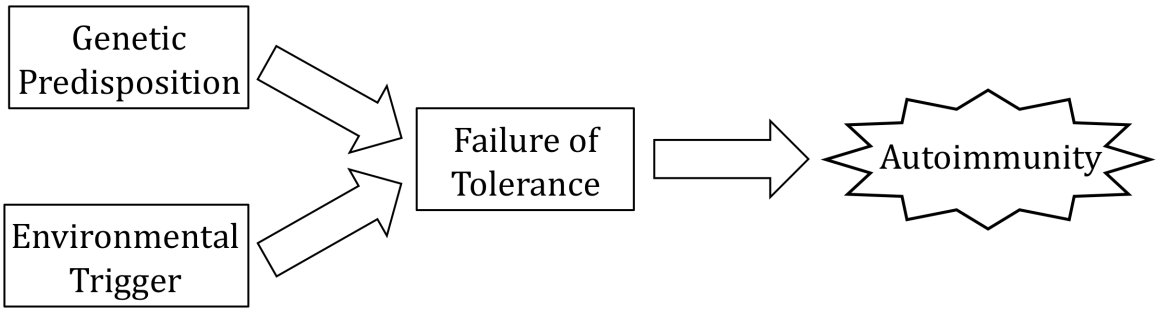

Figure 3- Autoimmunity is often the result of a combination of factors. Genetically predisposed individuals can develop autoimmune disease following an environmental trigger and the failure of the intrinsic mechanisms of tolerance. Adapted from [70].

Finally, development of autoimmunity can follow one or more environmental triggers, most often an infection or some other inflammatory insult. Several hypotheses exist as to how such triggers induce autoimmunity, although most involve the activation of previously naïve lymphocytes with self-reactive potential in

the new inflammatory milieu. The theory of molecular mimicry postulates some lymphocyte receptors that have high affinity for a pathogenic antigen may also be weakly cross-reactive with a self-antigen that resembles the pathogenic antigen[74]. During an infection, cells that express these receptors and become activated against a pathogen can then go on to recognize and orchestrate a response against the self-antigen. For example, it is believed that rheumatic heart disease following streptococcal infection results when activated T cells recognizing streptococcal antigen cross react with antigens on heart valves, leading to tissue destruction[75]. Another mechanism by which infection and inflammation can trigger autoimmunity is by making intracellular antigens available to lymphocytes in the extracellular environment. High rates of cell death during inflammation and large-scale tissue destruction such as myocardial infarction release intracellular antigens that can lead to autoimmunity[76].

Once initiated, an autoimmune inflammatory reaction is often self-sustaining given the continuous presence of the antigen[77]. In fact, a phenomenon called epitope spreading can occur in which the immune system becomes reactive to more and more self-antigens over time. It is thought that the disease begins with an initial autoimmune reaction against a single inciting antigen but that the subsequent recruitment of other inflammatory cells and production of pro-inflammatory cytokines can lead to increased availability and higher rates of activation against additional self antigens[78]. An example of antigen spreading can be seen in the disease systemic lupus erythematosis (SLE)[79]. Patients with SLE can express

antibodies to multiple self-antigens including anti-dsDNA, anti-Smith, anti-Ro, anti-La, and anti-nuclear antibodies. It has been observed that these antibodies develop as the disease progresses and increases in severity[80,81].

The pattern of autoantibody production is often correlated with disease specificity and symptomology. In general, autoimmune disease can be classified in two categories: organ specific, and systemic. Organ specific autoimmune diseases affect a single organ or cell type and are usually associated with individual autoantibodies that are not widely expressed. For example, beta islet cell destruction in type 1 diabetes is associated with antibodies directed against components of beta cells[82], Goodpasture's syndrome is associated with anti-glomerular basement membrane antibodies[83], and myasthenia gravis is associated with anti-acetylcholine receptor antibodies[84]. In contrast, systemic autoimmune diseases are associated with ubiquitously expressed antigens such as DNA and RNA in the case of SLE[85], IgG in rheumatoid arthritis, and ribonuclear proteins in Sjogrens Syndrome[86].

1.2.2- Autoimmune Regulation by Tregs

Autoreactive lymphocytes may avoid the negative selection process of tolerance but still not cause overt disease. One mechanism of control over these cells is through the action of a population of anti-inflammatory regulatory T cells (Tregs). These cells can be identified by their expression of the IL-2 receptor α-chain CD25 on their surface and the transcription factor Foxp3[87]. Two populations of Tregs have been identified, natural Tregs (nTregs) and inducible Tregs (iTregs). nTregs are

generated in the thymus where they begin expressing CD25 and Foxp3[88]. iTregs are generated in the periphery when naïve T cells are activated in the presence of the innate cytokine TGF-β[87].

There are various mechanisms by which Tregs mediate their regulatory function. Although they express the conventional antigen specific α:β T cell receptor themselves, they are able to regulate the actions of T cells that bind a variety of antigens as long as these cells are interacting with the same antigen presenting cell (see Figure 4)[89]. Tregs suppress inflammatory functions through both contact dependent mechanisms involving membrane bound TGF-β and the surface marker CTLA-4, as well as through the production of the anti-inflammatory cytokines IL-10 and TGF-β, both of which serve to directly suppress the proliferation of T cells by inhibiting the production of IL-2, TNF-α, and IL-5[89]. IL-10 also serves a regulatory function by inhibiting antigen presentation and IL-12 production by dendritic cells, thereby decreasing T cell activation and their subsequent differentiation into inflammatory Th1 cells[89].

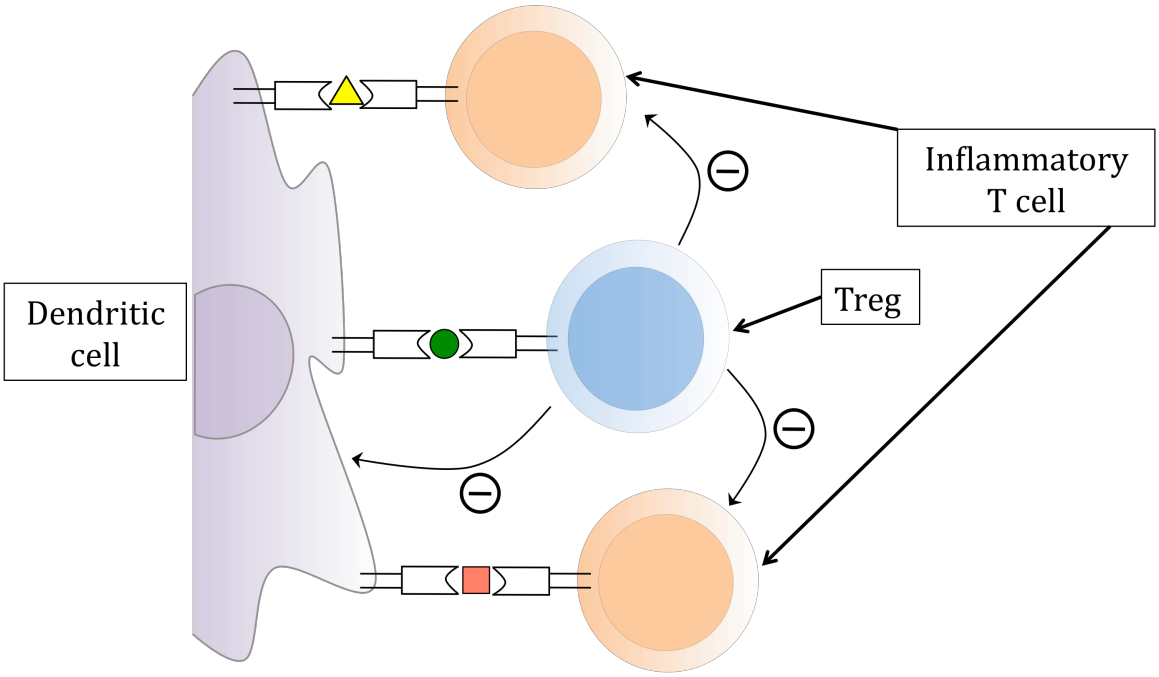

Figure 4- Schematic of Treg actions. Treg cells can exert a regulatory function on T cells that bind a variety of antigens. Tregs also inhibit the inflammatory actions of antigen presenting cells through the production of IL-10. Adapted from[70]

It is now widely accepted that these actions of Tregs are central to the regulation of inflammation and the suppression of autoimmune disease. Evidence of their importance comes from both experimental animal models as well as from human diseases. Transfer of Tregs has been shown to serve a protective role in the CD4CD45RB^{hi} T cell transfer model of colitis[90], the Experimental Allergic Encephalitis (EAE) model of multiple sclerosis[91], and several animal models of autoimmune diabetes[92]. In addition, depletion of Tregs has been demonstrated to exacerbate existing disease and can even lead to fatal autoimmunity in animals[93]. Studies of human type 1 diabetes have shown that Tregs from these patients are ineffective at modulating the production of the cytokines TNF-α and IFN-γ by inflammatory cells[94]. Defects in the regulatory activity of Tregs have also been found in patients with rheumatoid arthritis[95], multiple sclerosis[96], and autoimmune

polyglandular syndrome type 2[97]. Together, these findings all indicate that Tregs play an important role in preventing the immune system from becoming activated against self antigen and suggest interesting potential therapeutic targets and modalities for the treatment of autoimmune disease.

1.2.3- The Th17 Subset

The differentiation of naïve T cells into distinct subsets is determined by the cytokine milieu at the time of activation. These cytokines dictate the cell's subsequent actions since each subset has a unique effector phenotype. One of the subsets produced early in the course of an infection is the Th17 class of T cells[98]. Naïve T cells become Th17 cells when they are activated in the presence of TGF-β and IL-6 and express the master Th17 transcription factor ROR-γt[99]. The class gets its name because, once differentiated, Th17 cells produce high levels of IL-17 class cytokines, namely IL-17A (also simply called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F[98]. At rest, dendritic cells do not produce appreciable amounts of IL-6. However, in early infection, dendritic cells may increase IL-6 production and also produce TGF-β causing naïve T cells to differentiate towards the Th17 phenotype. This class of T cells is highly inflammatory and serves to coordinate the inflammatory response once differentiated. They travel to the site of infection where they produce IL-17 and IL-22, which induce the production of inflammatory cytokines by fibroblasts, epithelial cells, and keratinocytes. These cells in turn produce IL-6, granulocyte colony-stimulating factor (G-CSF) and

granulocyte-macrophage colony-stimulating factor (GM-CSF) which increase neutrophil and macrophage production in the marrow, and CXCL8 and CXCL2 which serve as neutrophil chemoattractants[100,101].

While these actions of Th17 cells serve a physiological role in the defense against pathogens, these highly inflammatory cells have been implicated in the pathogenesis of a variety of autoimmune and inflammatory diseases[102,103]. The first evidence of the importance of Th17 cells in autoimmune pathogenesis came from the study of the animal model of multiple sclerosis, EAE. At the time, it was believed that EAE was largely a Th1 mediated disease, however it was found that disease was not ameliorated when the principle Th1 cytokine IFN-γ was removed via anti-IFN-γ antibodies or genetic knockout[104-106]. These findings suggested that another cell type was at least partially responsible for the disease phenotype and further investigation led to the subsequent identification of Th17 cells as playing a central role in EAE inflammation[107,108]. Additional support for the importance of Th17 in EAE pathogenesis came with the findings that animals treated with antibodies against IL-23, a cytokine that serves to expand previously differentiated Th17 cells[102], and IL-17 deficient animals develop EAE with delayed onset and decreased severity[109,110].

Further investigations have implicated the role of Th17 cells in a variety of other autoimmune and inflammatory diseases in both animal models and human patients. Th17 cells have been found to be critical in the development and maintenance of

many of these diseases such as rheumatoid arthritis [111], multiple sclerosis [112,113], inflammatory bowel disease[102,114], SLE[115], Sjögren's Syndrome[116], scleroderma[117], diabetes[118], and even asthma[119]. These recent findings have generated considerable interest in exploiting Th17 cells and Th17 cell associated cytokines for therapy of these diseases.

1.2.4- Retinoic Acid

While other subsets of T cells undoubtedly play an important role in autoimmunity, an imbalance between Th17 cells and Tregs has been implicated in the pathogenesis of many autoimmune and inflammatory diseases. An excessive inflammatory reaction driven by Th17 cells without proper regulation by Tregs is a major driving factor in many of these conditions. Given that these cells types play such divergent roles both in the healthy immune system and in autoimmunity, it is interesting to note that development of each cell type is TGF-β dependent.

The common requirement of TGF-β signaling for the production of two distinct subsets of $CD4^+$ cells with such diverse roles prompted the investigation into additional regulators of Th17 and Treg cell differentiation. In particular, Mucida et al. demonstrated that retinoic acid (RA) modulates T cell differentiation between the Th17 and Treg lineages by promoting the development of Treg cells and away from Th17 differentiation (see Figure 5)[120]. 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 pharmacologically targeted

immunotherapeutic agent for autoimmune conditions[121-123]. Furthermore, RA expressing DCs from the lamina propria of the gut have been shown to promote Treg development and promote tolerance[124] while vitamin A[125] deficiency can predispose to inflammation. 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[126,127].

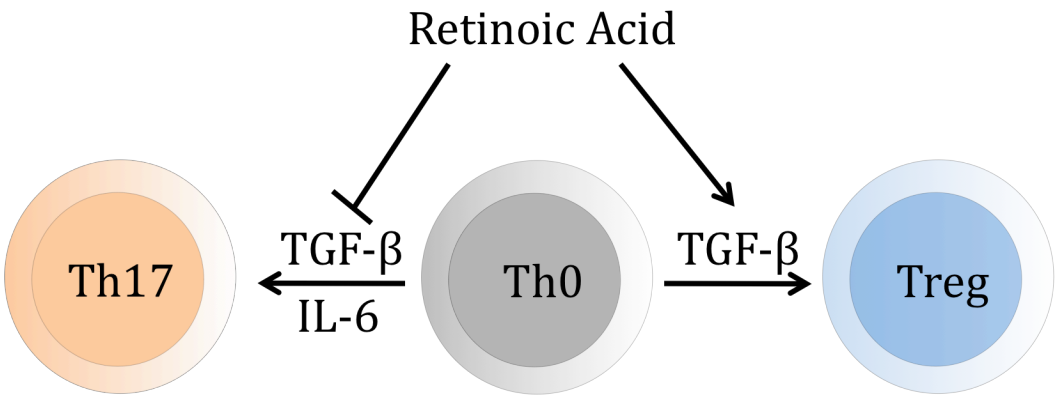

Figure 5- Role of retinoic acid in T cell differentiation. TGF-β is required for the development of both regulatory T cells (Treg) and inflammatory Th17 cells from naïve $CD4^+$ T cells (Th0). Differentiation of Th0 cells into Th17 cells also requires IL-6. Retinoic acid regulates the differentiation of Th0 cells by promoting Treg and suppressing Th17 development.

RA, a derivative of vitamin A, is extensively used for the treatment of acute promyelocytic leukemia[128] and dermatological conditions[129]. Studies involving RA use in these conditions have shown that high doses of RA can induce the potentially fatal reaction Retinoic Acid Syndrome[130-132] as well as complications such as myositis[133], ascites[134], and hypervitaminosis A, leading to hepatotoxicity, bone abnormalities, and birth defects[131,135]. 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[136].

1.3- Statement of Hypothesis and Specific Aims

RA is a promising pharmacologically targeted treatment for autoimmune and inflammatory diseases. However, experience with the use of this drug in the clinic has revealed significant shortcomings and difficulties with RA therapy. Therefore, a vehicle for targeted localized delivery of RA to immune cells is highly desirable.

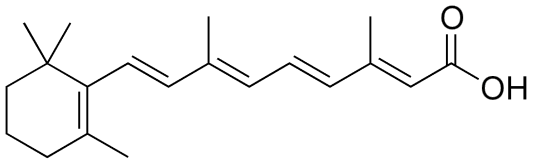

Figure 6- Molecular structure of retinoic acid.

As described in Section 1.1.2, polymeric biodegradable nanoparticles are effective drug delivery vehicles for achieving targeted local drug delivery. Examination of RA structure reveals its hydrophobic nature (see Figure 6) and RA does in fact dissolve readily in organic solvent and has low water solubility[137], making it an ideal encapsulant for nanoparticles fabricated via the single emulsion o/w technique. The polymer poly(lactic-co-glycolic acid) (PLGA) has a similar hydrophobicity profile and degrades via hydrolytic cleavage, making it an appropriate choice of particle matrix material for RA encapsulation. In addition, PLGA is non-toxic and is approved by the FDA for use in humans[27].

We propose to evaluate the feasibility of nanoparticulate drug delivery of retinoic acid for the treatment of autoimmune and inflammatory diseases by creating RA loaded PLGA nanoparticles. We hypothesize that RA delivered via PLGA nanoparticles will be as effective or more effective at modulating immune responses than soluble RA. We will evaluate this hypothesis according to the following specific aims:

I- Fabricate RA loaded PLGA nanoparticles using a single emulsion o/w technique, and characterize particle dimensions and pharmacokinetics.

II- Assess particle ability to modulate Th17 and Treg differentiation relative to soluble RA *in vitro.*

Chapter 2

Materials and Methods

2.1- Relative Contributions

The entirety of the experiments described below were performed by the author with the following exceptions; flow cytometry measurements of intracellular IL-17 and ROR-γ(t) in Th17 cells were performed with Michael Look and Heba Nowyhed (Figures 10B & 10C), the SEM image of PLGA nanoparticles was obtained by Dr. Ragy Ragheb (Figure 7B).

2.2- Vehicle Fabrication and Characterization

2.2.1- Particle Fabrication

PLGA nanoparticles containing RA (Sigma-Aldrich, R2625) were fabricated using a single emulsion o/w technique. Briefly, 200mg PLGA 50/50 with an average molecular weight of 80kD (Durect Corporation B6010-2P) and 0.751mg of RA were dissolved in 2ml of the volatile organic solvent dichloromethane (DCM). This was added to 4mL of a 5% aqueous solution of the amphiphilic stabilizer poly(vinyl

alcohol) (PVA) (Sigma-Aldrich P1836) and sonicated three times for 10 seconds at 38% amplitude (TEKMAR VCW 400W) on ice forming the o/w emulsion. The mixture was incubated for 1 hour in 100ml 0.2% aqueous PVA and stirred to allow for evaporation of the DCM and hardening of the nanoparticles. Particles were collected via centrifugation at 12000 rpm at 4°C and washed three times with de-ionized water to remove excess PVA. Particles were then lyophilized and stored at -20°C in an opaque container to protect RA from ambient light until use.

2.2.2- Particle Characterization

Particle size was determined using the nanoparticle tracking analysis instrument Nanosight LM-10 (Nanosight LTD). The technique has been recently developed and therefore will be described here. The instrument visualizes particles by illuminating them in aqueous suspension using a laser light and a computer captures real-time video of particle motion. The particles are small enough such that their movement is affected by the random motion of individual molecules on a microscopic scale. This phenomenon is called translational diffusion or Brownian motion. Specialized computer software tracks the motion of individual particles and generates a number called a Diffusion Coefficient (D) which is directly proportional to the square of the displacement of a particle per unit time. Observation of D allows the calculation of particle size according to the Stokes-Einstein equation;

$$D = \frac{k_B T}{6\pi\eta R}$$

where k_B is the Boltzman constant, T is the temperature, η is the viscosity, and R is the particle radius. Concurrent measurement of the temperature determines T and η, therefore allowing the particle radius R to be calculated.

Particle morphology was observed with scanning electron microscopy. Loading was determined by dissolving a predetermined mass of particle in dimethyl sulfoxide and the amount of RA was quantified in the fully dissolved sample using absorbance spectroscopy at 360nm. 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%.

Pharmacokinetics of RA release was determined by adding an aqueous suspension of RA loaded PLGA nanoparticles to dialysis cassettes (Pierce 69590) placed in 2L of PBS on a rotary stirrer at 37°C. Dialysis membranes with a MWCO=20,000kD were used such that released RA molecules could freely diffuse out of the cassette but the PLGA particles themselves along with any encapsulated RA were trapped within the cassette. At various time points, the particle suspension within a cassette was removed, particles were isolated via centrifugation, dissolved in dimethylsulfoxide, and RA content determined spectrophotometrically using absorbance spectroscopy at 360nm. Amount of RA released as a function of time was determined by calculating the difference between measured RA content and predicted RA content based on particle loading. The dialysate was changed daily and each time point was measured in triplicate.

2.3- In Vitro Bioactivity

2.3.1- Cell Culture and 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. The spleen and axillary, cervical, and inguinal lymph nodes were removed. Lymphocytes were isolated by combining organs and crushing them through a 20μm filter into sterile PBS. Cells were centrifuged, counted, and re-suspended at a concentration of $10X10^9$ cells/mL. $CD4^+$ T cells were isolated using an Easy Sep $CD4^+$ T cell enrichment kit (Stemcell Technologies, 19752) according to the manufacturer's instructions.

Once isolated, $CD4^+$ T cells were cultured in Click's Media (Irvine Scientific, 9195) supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, and 50μM β-mercaptoethanol using cell stimulation conditions adapted from previously described protocols[120,138]. Cells were plated at a density of $1X10^6$ cells/ml and volume of 500μL/well in 24 well plates or 250μL/well in 48 well plates. Th17 cells were generated by culturing cells with 1μg/ml anti-CD3ε (BD Bioscience, 553058), 1μg/ml anti-CD28 (BD Bioscience 553295), 20ng/ml IL-6 (Peprotech 216-16), and 5ng/ml TGF-β(Peprotech 100-21). Treg cells were generated by stimulating with immobilized anti-CD3ε (250 μL of 10μg/ml), immobilized anti-CD28 (250 μL of 2μg/ml), and 5ng/ml TGF-β. Media for Treg generation was identical to that used for Th17 generation. RA or RA loaded

PLGA nanoparticles were added at day 0 at the appropriate concentration. Cells were cultured for 5 days in an incubator that maintained 37°C at 5% CO_2.

On day 5, the cell suspension was collected and centrifuged in order to separate the supernatant from cells. Cytokine content of the supernatant was quantified using enzyme linked immunosorbant assay (ELISA). IL-17 (BD Bioscience 555068 and 555067) and IFN-γ (BD Biosciences 551309 and 551506) were measured for Th17 cells, and IL-10 (eBioscience 88-7104-77) for Treg cells. The supernatant was not diluted for the measurement of any cytokines.

Th0 cells were used as negative controls and were generated by stimulating with soluble anti-CD3ε (250 μL of 10μg/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.

2.3.2- Flow Cytometry Analysis

On day 5 of culture, $CD4^+$ cells were washed with Click's media, resuspended in media containing 20ng/ml PMA, 2000ng/ml ionomycin, and 1μL/ml Golgi Plug (BDBioscience 555028), and incubated for 6 hrs. Cells were washed and

resuspended in staining buffer containing 1XPBS 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 for surface staining. Cells were permeabilized using Cytofix/Cytoperm Plus Fixation-Permeabilization Kit (BDBioscience 555028) according to the manufacturer's instructions and stained with anti-IL-17 conjugated to Alexa647 (eBioscience 51-7177-80), anti-ROR-γ(t) conjugated to PE (eBioscience 12-6988), and anti-Foxp3 conjugated to Alexa700 (eBioscience 56-5773-80) for intracellular staining. Analysis was performed the same day using a LSRII Flow Cytometer (Becton-Dickinson and Company).

2.3.3- Fibroblast/Th17 co-culture

NIH 3T3 fibroblasts were cultured in hi-glucose DMEM media supplemented with 10% heat inactivated FBS, 100U/ml penicillin, and 100μg/ml streptomycin and allowed to adhere to the plate surface overnight. Cells were plated at a density $2X10^5$ cells/mL and a volume of 1mL. T cells were polarized towards the Th17 phenotype and treated with 10nM free RA or RA nanoparticles for 5 days as described in Section 2.3.1. Cells were subsequently washed, restimulated with soluble 1μg/ml anti-CD3ε (BD Bioscience, 553058) and 1μg/ml anti-CD28 (BD Bioscience 553295), and added to the fibroblasts at a density of $5X10^5$ 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).

Chapter 3

Results

3.1- Particle Characterization

Particle sizing via the Nanosight LM-10HS revealed an average particle diameter of 252nm with a mondisperse size distribution (see Figure 7A). Observation of particles via SEM revealed similarly sized spherical particles with a smooth surface morphology all of roughly similar dimension (see Figure 7B). Although not quantitatively measured, the particle size distribution determined using the Nanosight LM-10HS correlates well to the size of particles visualized via SEM.

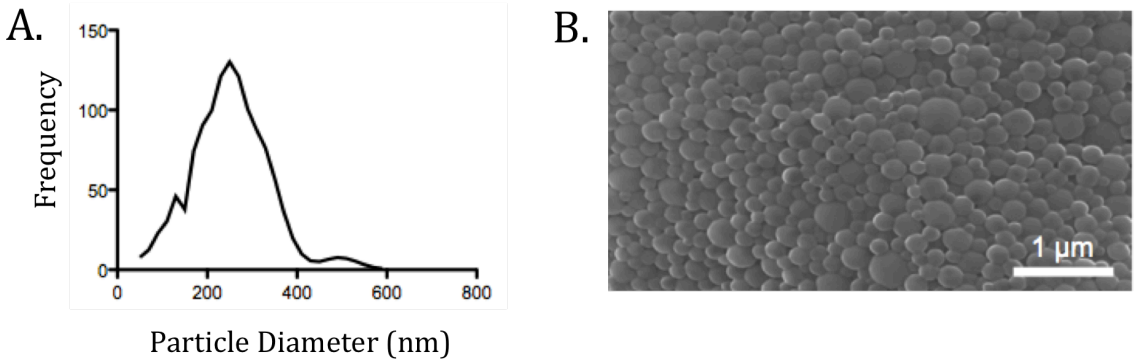

Figure 7- Nanoparticle characterization. (A) Hydrodynamic particle size distribution as measured with Nanosight LM-10HS reveals a monodisperse distribution with average particle diameter=252nm. (B) SEM reveals similarly sized spherical particles with smooth surface morphology.

The control release curve of RA-loaded nanoparticles determined using dialysis reveals a biphasic release profile with an initial burst of RA release during the first 24-48 hours, followed by its slow gradual release in a plateau phase (see Figure 8). This type of biphasic release is a typical release profile observed in biodegradable nanoparticulate systems. It has been speculated that the initial burst release is due to release of surface associated encapsulant and the longer plateau phase is the release of encapsulant truly incorporated within the particle matrix itself[139].

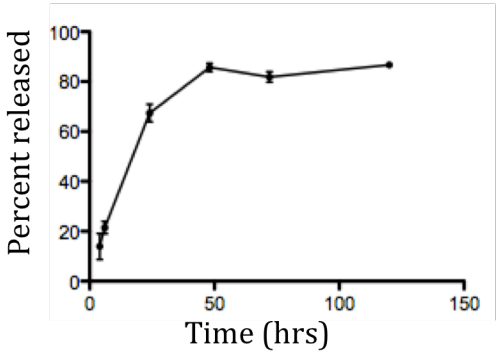

Figure 8- Drug release kinetics. Control release curve of RA from PLGA particles at 37°C in PBS reveals biphasic release kinetics with an initial burst release for 24 hours followed by gradual release. Results shown are for triplicate samples and error bars represent SEM.

Loading of particles was 2.29-3.15 μg RA/mg PLGA and loading efficiency was 72.5 ± 11.5%. Such a high loading efficiency is expected when encapsulants possess similar solubility characteristics as the particle matrix, in this case hydrophobic RA and PLGA. Observed morphology via SEM, particle size distribution, control release characteristics, and loading efficiency were similar for all batches of particles used in experiments.

3.2- In Vitro Bioactivity

3.2.1- RA 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[138] in the presence of RA dissolved freely in solution or loaded in nanoparticles. We found that both free RA and particulate-encapsulated RA decreased the secretion of IL-17 and IFN-γ in a dose dependent manner (see Figure 9). RA potency was similar between free and particulate drug for concentrations of 10nM, 1nM, and 0.01nM. Free drug was more potent than nanoparticles at 0.1nM.

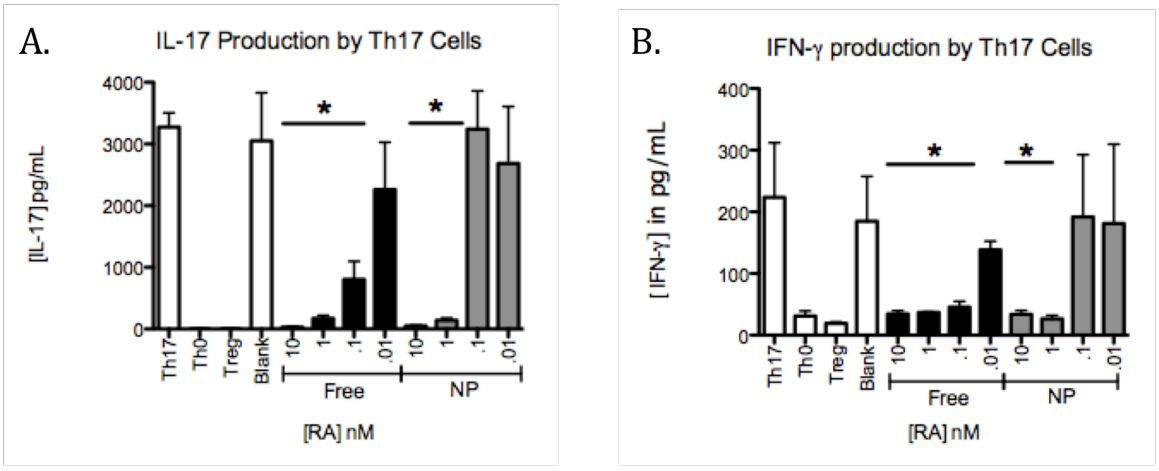

Figure 9- Cytokine production of Th17 cells. $CD4^+$ cells polarized towards the Th17 phenotype and treated for 5 days with soluble RA (free) and RA loaded nanoparticles (NP) showed decreased production of IL-17 (A) and IFN-γ (B). * = $p<0.05$, separately comparing Th17 cells treated with RA at the each indicated concentration to Th17 cells not treated with RA. The data represent 5 replicates and the experiment was performed independently three times.

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 $CD4^{+}TCR\text{-}\beta^{+}$ cells treated with free or encapsulated RA similarly expressed lower levels of intracellular IL-17 and ROR-γ(t), the key transcription factor of the Th17 lineage (see Figure 10)[140].

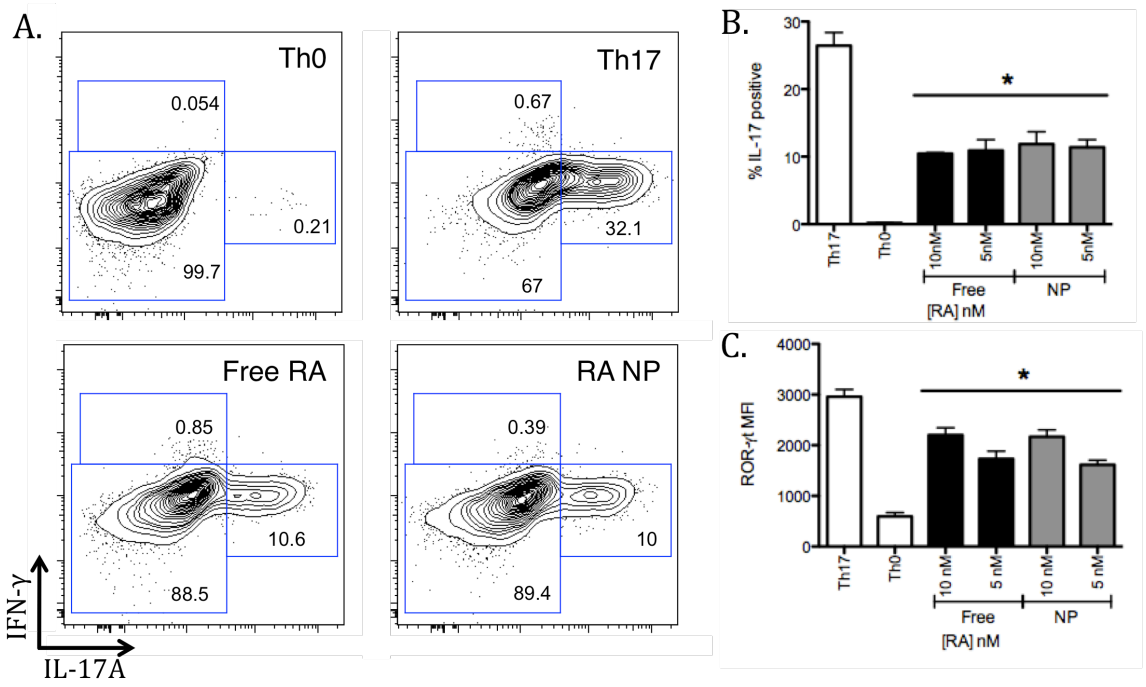

Figure 10- FACS analysis of Th17 cells. Flow cytometry revealed that RA treatment decreased intracellular expression of IL-17 (A & B) and the key Th17 transcription factor ROR-γ(t) (C). Flow cytometry plots are for [RA]=10nM * = $p<0.05$, separately comparing Th17 cells treated with RA at the each indicated concentration to Th17 cells not treated with RA. The data represent 5 replicates and the experiment was performed independently three times.

3.2.2- RA nanoparticles enhance the Treg phenotype

Since RA can enhance the development of Treg cells[120], 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 (see Figure 11); however, free RA treatment resulted in greater increases in IL-10 production than did nanoparticles.

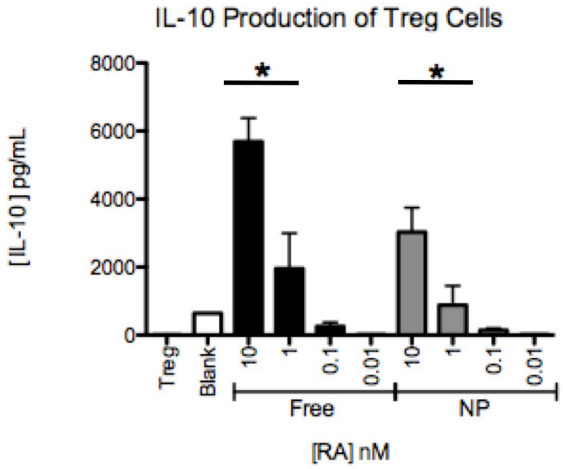

Figure 11- IL-10 production by Treg cells. $CD4^+$ cells treated with TGF-β and treated for 5 days with soluble RA (free) and RA loaded nanoparticles (NP) showed increased production of IL-10. * = $p<0.05$, separately comparing Treg cells treated with RA at the each indicated concentration to cells not treated with RA. The error bar on the blank condition is small and therefore not visible. The data represent 5 replicates and the experiment was performed independently two times.

Unlike IL-10 production, free RA and RA containing nanoparticles resulted in similar increases in Foxp3 expression, as measured by flow cytometry (see Figure 12).

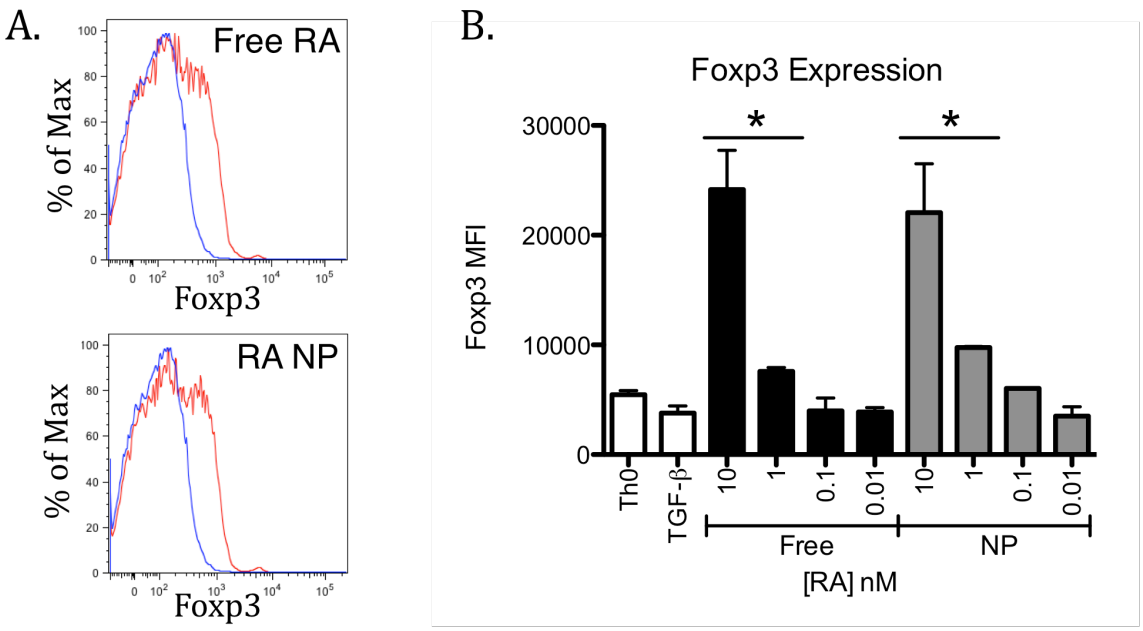

Figure 12- FACS analysis of Treg cells. Flow cytometry reveals that cells treated with RA have an increased expression of Foxp3. Histograms are shown for cells treated with 10nM RA. Blue=TGF-β only, Red= TGF-β and RA. *=p<0.05, comparing treated to untreated cells.

3.2.3- RA nanoparticles inhibit IL-6 production in 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[101]. 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* (see Figure 13). 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 RA or nanoparticulate RA did not, with a similar magnitude of suppression with both of these RA administration modalities.

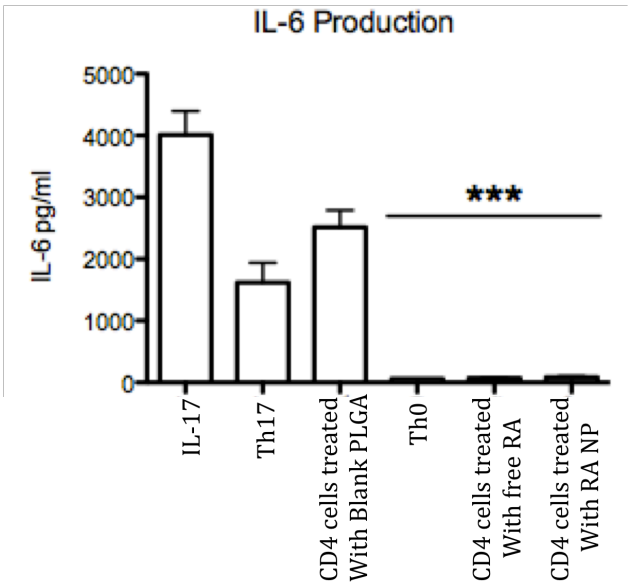

Figure 13- IL-6 production by fibroblasts. $CD4^+$ T cells were polarized towards the Th17 phenotype and treated with soluble RA or RA loaded PLGA nanoparticles for 5 days, washed to remove excess RA or RA loaded NPs, and subsequently co-cultured with fibroblasts for 24 hours. IL-6 was then quantified 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. The data represent 3 replicates.

Chapter 4

Discussion

In this work, we demonstrated a methodology for delivering bioactive RA in a biodegradable, FDA approved polymeric nanoparticle format that can potentially address issues related to delivery of this drug. RA released from our drug delivery system is capable of reducing the production of Th17 cells and increasing the Treg phenotype of developing naïve T cells. Specifically, we showed that RA loaded PLGA nanoparticles reduce IL-17 and IFN-γ production and ROR-γ(t) expression by $CD4^+$ T cells exposed to IL-6 and TGF-β *in vitro* as well as increase IL-10 production and Foxp3 expression by T cells treated with TGF-β alone. We also demonstrate that $CD4^+$ cells polarized towards the Th17 phenotype have a dramatically reduced ability to induce IL-6 production in fibroblasts when treated with RA loaded PLGA nanoparticles. These findings are promising for the treatment of autoimmune and inflammatory diseases driven by an excess of inflammatory Th17 cells and a relative deficiency of the anti-inflammatory action of Treg cells.

Our results show that the potency of RA delivered via PLGA nanoparticles is largely equivalent to that of free drug *in vitro*. It should be noted that in our experiments, concentrations of RA delivered to cells within particles was determined according to particle loading and not the amount of RA released from the particles. Considering the control release curve of RA from particles, cells treated with RA loaded nanoparticles received only approximately 80% as much RA as those cells treated with free drug at a given concentration, demonstrating a moderate increase in RA potency delivered via nanoparticle over free drug.

In fact, increased bioactivity is commonly seen in molecules delivered via particulate delivery systems relative to free drug both *in vitro* and *in vivo*[29]. In the case of phagocytic cells such as macrophages and dendritic cells, a mechanism for enhanced drug potency is that phagocytosed particles deliver high doses of drug when degraded within the phagolysosome[16]. Such a mechanism is not evident in non-phagocytic cells such as T cells, however. It has been speculated that since drug is being released from point sources rather than freely dissolved, it is possible to achieve high local drug concentrations in the vicinity of the particle in a manner similar to paracrine delivery[54]. Therefore in these experiments, T cells may receive a relatively higher dose of RA when in close proximity to nanoparticles, possibly explaining the observed moderate increase in drug potency.

The experiments presented here have been 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. The many potential advantages that targeted local drug delivery has over systemic administration unfortunately cannot be easily reproduced *in vitro*. It is therefore necessary to evaluate the efficacy of RA loaded nanoparticles relative to soluble RA in an animal model of Th17 mediated autoimmune disease in order to demonstrate the full potential of our drug delivery platform.

Models for testing of our platform include acute and chronic models of colitis, rheumatoid arthritis, and multiple sclerosis. T cell models of arthritis that would be conducive for testing advantages of RA loaded nanoparticles include the Collagen Induced Arthritis (CIA) and SKG models[141,142]. These models are mediated by self-reactive Th17 cells and represent an imbalance between inflammatory Th17 and regulatory T cell responses. Another animal model in which to test RA loaded nanoparticles treatment is the EAE model of multiple sclerosis. Like the models of arthritis, EAE is driven by Th17 cells and would therefore be conducive to RA loaded nanoparticulate treatment. Evaluation of our platform in these models could be done by comparing therapeutic efficiency of drug-loaded nanoparticles to that of free RA administered systemically. Potential advantages of particles relative to free drug in these models include higher drug residence time, more stable drug levels due to controlled release of RA from particles, and localization of particles to areas of inflammation secondary to leaky vasculature and inflammation via the EPR effect. However, functionalization of the particle surface with T cell targeting ligands such

as anti-CD4 antibodies could increase specificity of the particulate therapy even further.

Ease of particle targeting makes treatment of colitis with RA loaded nanoparticles particularly intriguing. Currently, biologic therapy for IBD can only be administered via injection[143], however RA loaded nanoparticles would be orally administrable, presenting a substantial improvement in patient comfort and convenience. Furthermore, studies have shown that orally administered nanoparticles selectively adhere to the intestinal epithelium and specifically localize to areas of inflammation, allowing for high intestinal concentrations of drug that are difficult to achieve with systemic therapy[35,36]. It has been demonstrated that adherent nanoparticles are selectively retained within the GI tract, increasing their utility in IBD which is associated with diarrhea and decreased residence time of orally administered tablets[35,36]. This natural adherence and localization to inflamed intestinal mucosa abrogates the need of any form of particle targeting via surface functionalization, further increasing the appeal and simplicity of this approach. Specific T cell mediated animal models of colitis include the acute colitis model TNBS colitis and the chronic colitis model $CD4^{+}CD45RB^{hi}$ T cell transfer model[144].

In summary, we have developed a novel biodegradable nanoparticulate vehicle for the localized delivery of RA. We have demonstrated our ability to reproducibly fabricate RA loaded PLGA nanoparticles with predictable time dependent drug release kinetics. We have shown that the RA released from nanoparticles is

bioactive and capable of altering the phenotype of developing T cells *in vitro* similarly to free soluble RA. Our findings present a promising line of inquiry into the usage of this novel formulation for autoimmune and inflammatory disease therapy and represent the first step in the development of a therapeutic for clinical use.

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