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ALLERGEN SENSITIZATION AND IMMUNOMODULATION BY SYNTHETIC LIGANDS, PUL-042, IN AN ALLERGEN-INDUCED ASTHMA MURINE MODEL

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A

THESIS

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by

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Dedication

I dedicate this thesis to my husband Dale of fifteen years, and my three beautiful children, Brandon, Jonathan, and Serena. Thank you for you unfailing love, patience, and confidence in me, which without, I would not have been able to accomplish all that I have today. You are truly the best part of me, and I love you so much.

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Allergen-induced asthma is the leading form of asthma and a chronic condition worldwide. Common allergens are known to contribute to the pathogenesis of this disease. Murine models of allergic asthma have mostly used an intraperitoneal route of sensitization (not airway) to study this disease. Allergic asthma pathophysiology involves the activation of T_H2-specific cells, which triggers production of IgE antibodies, the up-regulation of T_H2-specific cytokines (*i.e.* IL-4, IL-5, IL-9 and IL-13), increased airway eosinophilia, and mucin hypersecretion. Although there are several therapeutics currently treating asthmatic patients, some of these treatments can result in drug tolerance and may be linked to increased mortality. CpG oligodeoxynucleotides (ODNs) is a synthetic ligand that targets Toll-like Receptor (TLR) 9. It has been evaluated as a therapeutic agent for the treatment of cancer, infectious diseases, and for treating allergy and asthma. PUL-042 is a combination of two synthetic TLR ligands and is composed of two agonists, one against TLR2/6 heterodimer, and the other against TLR9. Previous studies have evaluated PUL-042 for its ability to confer protection against bacterial and viral lung infection. These findings, combined with studies performed using CpG ODNs, led to speculation that PUL-042 dampens the immune response in allergen-induced asthma. My thesis research investigated airway route sensitization and airway delivery of PUL-042 to evaluate its effects in reducing an allergen-induced asthma phenotype in a murine model. The results of this study contribute to the foundation for future investigations to evaluate the efficacy of PUL-042 as a novel therapy in allergic-asthma disease.

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Chapter 1: Introduction and Background

Asthma

Prevalence of Asthma

Asthma is a leading cause of lower respiratory disease. It is a chronic disease, and according to the National Center for Health Statistics it is the third leading cause of death affecting over 300 million people worldwide [1]. In the United States, the incidence of asthma has almost tripled between 1980 and 2009 currently affects almost 25 million people (8.2% of the population) [2, 3]. According to the Centers for Disease Control and Prevention (CDC) and independent researchers, the risk of developing asthma is higher for individuals with a family history of asthma; African American adults and children; male versus female children; and for individuals living in poverty [3-6].

Characteristics and Pathogenicity of Asthma

In general, asthma is categorized as a disease that affects the respiratory system and is characterized by persistent inflammation in the airways [1, 7, 8]. Classical clinical symptoms of asthma include shortness of breath, sporadic periods of wheezing, coughing, and tightness in the chest due to constriction of the muscles that surround the airways [9]. Asthma is multifaceted and causes include both intrinsic and extrinsic factors [10, 11]. Intrinsic asthma refers to asthma conditions resulting from innate or external factors that are not linked to allergens [11]. The onset of this form of asthma usually occurs during adulthood, and is triggered by various factors such as exercise, infections and stress (Fig. 1) [11]. Extrinsic asthma accounts for 90% of asthma cases in children and is caused by inhalation of common environmental allergens (*i.e.* dust mites, animal dander and pollen) [11]. In many cases; however, people with asthma often have a combination of two or more

factors that work synergistically to contribute to the development of the disease (Fig. 1) [1, 12].

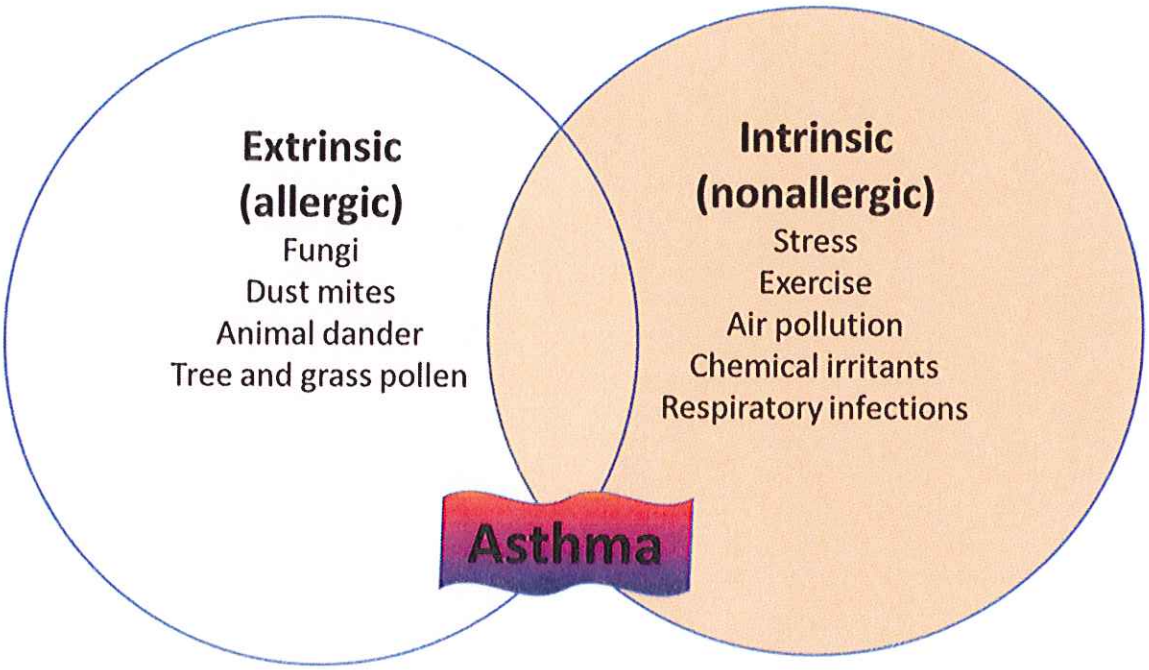


Figure 1: Extrinsic and Intrinsic Factors that Cause Asthma Disease. Extrinsic factors include common allergens (beige circle) while intrinsic factors include non-allergens (darker beige circle). The disease pathways are complex and most likely include both allergen and non-allergen induced factors (modified figure, [1]).

According to the Asthma and Allergy Foundation for America (AAFA), extrinsic factors play a larger role in the development of asthma than intrinsic factors [13]. Both the American Academy of Allergy, Asthma and Immunology (AAAAI) and the AAFA reported that allergen-induced asthma is the most common form of asthma, accounting for at least 50% of all cases [13, 14]. Previous studies have investigated the various mechanisms involved in the development of asthma and have primarily focused on the innate immune system, such as Toll-like receptors (TLRs) and white blood cells (WBCs) [1, 14-17]. Additional studies have

reported activation of the T-helper 2 (T_H2) pathway in the progression of asthma [15, 18, 19]. These particular studies concentrated on specific cells, chemical mediators, and cellular receptors that are involved in the T_H2 response [1, 10, 19, 20].

Other mechanisms that play a role in the recruitment of specific types of WBCs into the pulmonary airways and up-regulate inflammatory mediators involved in allergic asthma need to be further evaluated [21, 22]. According to Holgate *et al*, future research should also address the adaptive immune response in order to reduce or eliminate the inflammatory response characteristically seen in asthma and to provide insight into new potential therapies [21-24]. For this reason, the focus of my project is to further investigate factors that are involved in the T_H2 allergic pathway and evaluate the immune response that occurs when allergens are introduced into the airways and lead to asthma disease.

Allergen-Induced Asthma

Pathophysiology

Statistical reports from the AAAAI indicate that 70% of asthma sufferers have atopy [14]. Atopy occurs when an individual has an inherited susceptibility to commonly encountered allergens that cause over-reactive immune responses, which result in allergic asthma [13]. Allergen-induced asthma is characterized by many of the same immunopathological features seen in other forms of the disease [1]. The key features of allergen-induced asthma as illustrated in Figure 2 are inflammation of the pulmonary airways, airway hyper-reactivity or hyper-responsiveness (AHR) and bronchial constriction [1, 7, 8, 25-27].

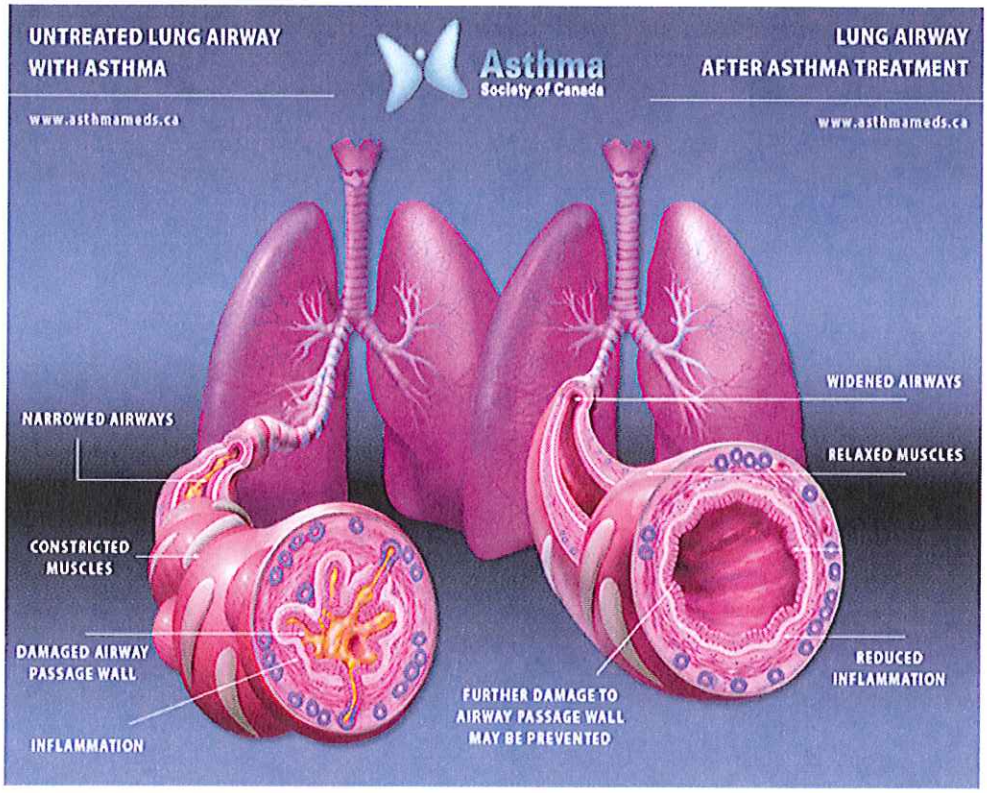


Figure 2: Key Features of a Lung Airway with Active Asthma Disease and a Lung Airway after Asthma Treatment. The physical features of the pulmonary airways during an asthma attack are shown to the left and include swelling and narrowing of the airways. The lung illustrated on the right shows reduced swelling and relaxation of bronchial muscles after treatment [28].

AHR is described as the excessive constriction and narrowing of the airways in response to a variety of nonspecific stimuli [25, 29, 30]. Bronchial constriction is a narrowing and obstruction of the airways of the lungs due to the tightening of the smooth muscles that surround the bronchi [25, 29, 30]. In addition to these features, there is proliferation or hyperplasia of specific cells in the airways called goblet cells [1, 27]. These goblet cells are specialized cells that produce mucus. Increased production of these cells leads to the hypersecretion of mucus into the airways [1]. All of the above conditions lead to reduced airflow in the lungs [1, 29].

T_H2 Immune Response in Allergen-Induced Asthma

Allergen-induced asthma is primarily a T_H2 driven immune response marked by infiltration of T_H2-specific lymphocytes and eosinophils into the airways and induction of T_H2-specific inflammatory cytokines [16, 27]. The process of allergen-induced asthma first involves sensitization to an inhaled allergen [31]. Once inhaled, the allergen comes into contact with moisture from the airways. This solubilizes the antigens from the allergen so that they are then able to enter the submucosa. In the submucosa, these antigens interact with professional antigen presenting cells (APCs), such as dendritic cells (DCs), and are further processed for recognition by the immune system [31, 32]. DCs are major APCs of the host immune system that are located in the host's peripheral tissues, including those that line the nose, lungs and skin [31, 33, 34]. These APCs present processed antigens on their surfaces for specific antigen recognition by T-lymphocytes, including the T_H2 lymphocytes. This, in turn triggers, the activation and differentiation of these T_H2 cells and adaptive immunity [31, 34].

Cellular Response in Allergen-Induced Asthma

An adaptive immune response induces the activation of B-lymphocytes (B-cells) and T-lymphocytes (T-cells) [35]. B-cells are the only lymphocytes that produce antibodies against specific antigens. T-cells express receptors on their surfaces that recognize specific fragments of antigens [34, 36]. T-cells, once activated, are further differentiated into specific types of effector T-cells, which are either cytotoxic T-cells ($CD8^+$) or helper T-cells (T_H cells, $CD4^+$) [34].

There are four main subsets of T_H-cells: T_H1, T_H2, T_H17 and T_{reg} [33, 34, 37] (Fig. 3). T_H2 cells are the subpopulation involved in allergen-induced asthma [34]. This subpopulation evolves when naïve T-cells interact with antigens from inhaled allergens, and are then processed by DCs in the mucosal tissues, which then become differentiated into T_H2 cells [15]. Once activated, these T_H2 cells produce and secrete specific proteins called cytokines [18, 19].

Inflammatory Proteins and Mediators in Allergen-Induced Asthma

A cytokine is a soluble protein secreted by cells that affects cellular activity and controls inflammation in an immune response [34, 38]. There are several key cytokines that are involved in the allergic asthma inflammatory response. Activated T_H2 cells produce and secrete four major cytokines: Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interleukin 9 (IL-9) and Interleukin 13 (IL-13) [1, 34]. There are several key cytokines that are involved in the inflammatory response against people with allergic asthma. These cytokines are key factors in determining disease exacerbations. IL-4 promotes the proliferation and differentiation of T_H2 cells and can also stimulate B-cells [15, 18, 30, 34]. IL-5 stimulates the infiltration of eosinophils into the airways and growth and terminal differentiation of eosinophils in the bone marrow [1, 27, 34, 39]. IL-13 is important for activation of B-cells and their production of immunoglobulin E (IgE), and it also mediates AHR [1, 20, 34, 40-42].

IgE Production in Allergen-Induced Asthma

IgE is the predominant immunoglobulin produced in allergen-induced asthma [27, 34, 43]. IgE are specific to and react with allergens encountered in the airways [15, 27]. This antibody isotype contains high-affinity receptors that allow it to bind to

the surfaces of mast cells in the pulmonary airways [44, 45]. IgE activation of mast cells leads to the up-regulation of other inflammatory mediators and T_H2-specific cells that further contribute to the characteristic features seen in an allergic asthma phenotype [27].

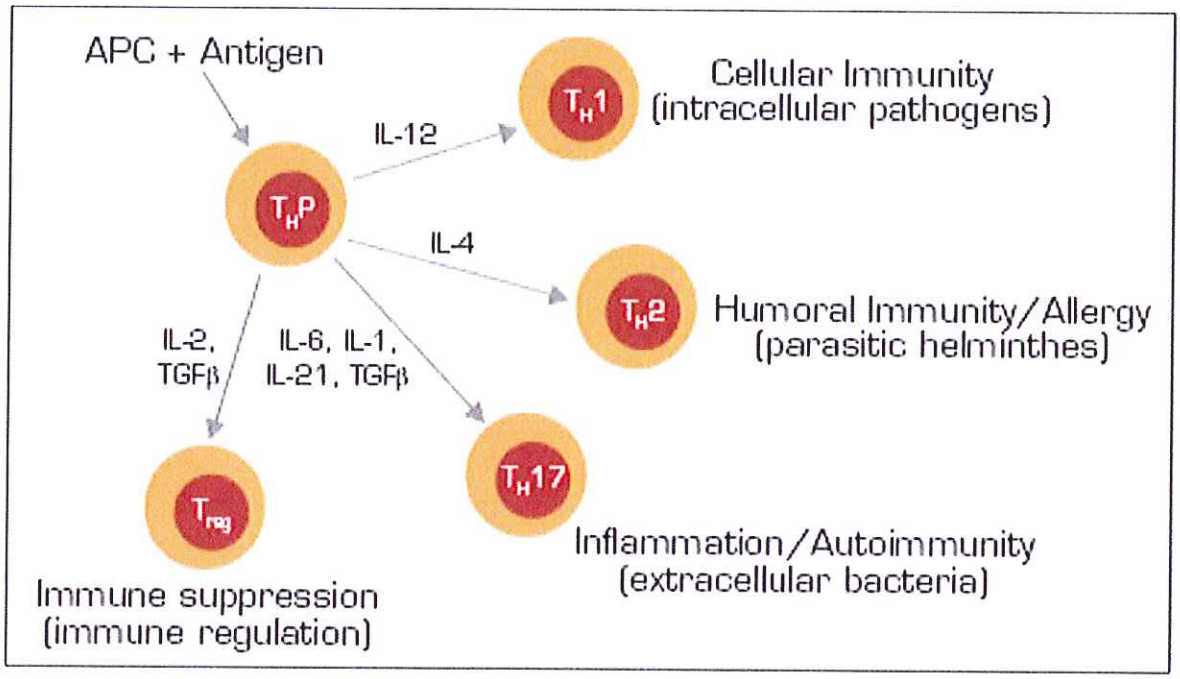


Figure 3: T_H Subsets that Result from Interaction with Antigens expressed on APCs Presented to Naïve Progenitor T-cells (T_HP). Naïve $CD4^+$ T_H cells are differentiated into one of four T_H cells: T_H1, T_H2, T_H17, and Treg. Each subset of T_H cell has a specific cytokine that facilitates its terminal differentiation and each differentiated T_H cells have specific effector functions as indicated in parentheseses [37].

Granulocytes Involved in Allergen-Induced Asthma

There are three types of granulocytic cells involved in allergic asthma disease [46]. Mast cells, neutrophils and eosinophils [30]. Each of these cells has granules containing substances capable of producing lung airway inflammation in response to an allergen [44-49]. Eosinophils are the major subgroup of WBCs found

in the airways of people with allergic asthma [1, 34, 46]. An elevated amount of eosinophils can be measured in patients with allergen-induced asthma compared to healthy individuals [46, 50-52].

Management Strategies

Asthma Therapies

The current treatments for asthma focus on inflammation reduction and bronchial muscle relaxation [21, 53]. Inhaled corticosteroids are a class of drugs categorized as anti-inflammatories, and works by reducing the swelling and production of mucus in patients with asthma [28]. Beta-2 adrenergic receptors (β_2-AR) agonists are the class of drugs that activate β_2-ARs. This class of receptors belongs to the family of G protein-coupled receptors that transfer signals from outside of cells within the host's system to inside the cell [54]. Specifically, β_2-ARs increase cyclic adenosine monophosphate (cAMP) by activating adenylate cyclase [54]. The two main classes of treatments used for muscle relaxation are short-acting and long-acting β_2-AR agonists [21, 53]. Short-acting β_2-AR agonists are most often used to relieve acute asthma symptoms [55]. Long-acting β_2-AR agonists are usually prescribed along with inhaled corticosteroids as a maintenance therapy for moderate to severe asthma [55]. However, these therapies are unable to alter the underlying disease physiology and exacerbations of allergic asthma [21, 53]. In addition, current treatments have been linked to disease tolerance and loss of disease control, which increases the risk of death for asthma sufferers [55, 56]. The increasing incidence of allergic asthma compounded with inefficient therapies necessitates the development of a therapy that provides a continual protective

effect against the allergic asthma phenotype [21, 53, 57-59]. Evans, *et al* investigated the potential of using synthetic ligands to specific TLRs as novel therapeutic agents for various respiratory diseases with the potential for use in treatments for allergic asthma [50-52, 60].

TLR Ligands as Therapeutic Agents for Asthma

Toll-Like Receptors

Toll receptors were first discovered in *Drosophila* species. Shortly after this discovery, researchers identified receptors (TLRs) in mammals that are structurally similar to the *Drosophila* Toll [34, 53]. There are eleven identified TLRs in humans and thirteen in mice [34, 61, 62]. Toll receptors and TLRs are pattern-recognition receptors [34, 63]. These receptors belong to a group of proteins that recognize specific, conserved molecular patterns of pathogens invading the host [63]. TLRs form homo- and heterodimers that confer specificity for ligand recognition [34, 61, 62]. Activation of TLRs produces both primary and secondary immune responses involving the innate and adaptive immune system [63].

Evans *et al*, showed that specific combinations of TLR ligands have the ability to work together to produce synergistic effects and confer protection against microbial infections [60, 64, 65]. Furthermore, their results provided evidence that one particular combination of TLR ligands (ligands to TLR2/6 heterodimer and TLR9) provided the most protection against bacterial and viral microorganisms used in their study [60, 64]. The TLR2/6 heterodimer is a membrane protein expressed on the surface of certain innate immune cells (*i.e.* macrophages and DCs) [62, 66, 67]. It recognizes diacylated lipoproteins found on various types of pathogens [66-

69]. TLR9 is found in the endosome and recognizes unmethylated CpG deoxyribonucleic acid (DNA), short single-stranded DNA molecules that consist of a cytosine followed by a guanine [60, 65, 69]. These unmethylated CpG oligodeoxynucleotides (ODNs) are found in numerous bacterial pathogens, but not in mammalian hosts, which allows the host to recognize this DNA as non-self [69].

TLR Ligands

Additional studies have demonstrated that synthetic ODNs have the potential to be used in a variety of therapeutic strategies for the treatment of cancers, infectious diseases and asthma [17, 70, 71]. In addition, CpG ODNs were investigated as anti-allergens due to their ability to stimulate T_H1-specific and T_{reg}-specific cytokine responses [17, 53, 71]. These cytokine responses demonstrated a decrease in the T_H2 response associated with allergen-induced asthma [17, 53, 71]. We speculated whether theses synthetic ligands could dampen an allergen-induced asthma phenotype. Evans *et al* developed a compound containing two synthetic molecular peptides, a lipopeptide (Pam2CSK4) and an ODN (M362), which is referred to as PUL-042 [60, 64]. This compound acts as an agonist to both TLR2/6 heterodimer and to TLR9. Results showed that mice treated with PUL-042 were protected against bacterial and viral challenges in a pneumonia model [50, 60, 64, 65]. Since pneumonia and asthma are both respiratory conditions, we sought to test the effects of PUL-042 in an allergen-induced asthma model. Therefore, the goal of my project is to investigate the protective phenomenon of PUL-042 in treating allergic asthma and analyze whether PUL-042 confers protection in an allergen-induced asthma model.

Animal Models for Testing Allergen-Induced Asthma

The most widely used asthma animal models are mice, rats and guinea pigs [72]. Mouse models have been used to study both acute and chronic allergen-induced asthma phenotypes. With these models, researchers have studied mechanisms involved in the initiation, development and progression of the disease (Table 1 and 2) [73]. Mouse asthma models involve sensitization by administering the allergen via an intraperitoneal injection along with an adjuvant [73]. Only a

Gender/strain	Allergen	Sensitisation	Exposure/challenge	Responses to challenge	References
Female BALB/c	OVA	OVA/alum (i.p.) on days 0 and 12	OVA aerosol on days 18-23	From day 24: AHR, increased eosinophils in BAL and lung tissue. Remodelling and Th2 cytokine induction	Lloyd et al., 2000; McMillan et al., 2002; McMillan et al., 2005
Female BALB/c	OVA	OVA + $AlOH_3$ (i.p.) on days 0 and 14	OVA aerosol on days 28-30	From day 32: AHR, increased eosinophils in BAL and lung tissue. Remodelling and Th2 cytokine induction	Tomkinson et al., 2001; Hamelmann et al., 1999a
Male BALB/c	OVA	OVA (i.p.) on 7 alternate days	OVA aerosol for 8 consecutive days (starting on day 27)	24 hours post final OVA challenge: AHR, increased eosinophils in BAL and lung tissue. Th2 cytokine induction	Hessel et al., 1995; Janssen et al., 2000
Female BALB/c	OVA	OVA + alum (i.p.) on days 0 and 14	OVA (i.n) on days 14, 25, 26 and 27	From day 28: AHR and increased eosinophils in BAL and lung tissue. Remodelling	Henderson et al., 1996
Male BALB/c	OVA	OVA (i.p.) on 7 alternate days	OVA (i.t.) on day 42 for 3 days, each 3 days apart	24 hours post final OVA challenge: increased eosinophils in BAL and lung tissue	Blyth et al., 2000
Male A/J	Bla g 2 and Der f 1	OVA + $AlOH_3$ (i.p.) on days 0 and 7	Allergen (oro-tracheal) on day 14	From day 17: AHR (only to Bla g 2) and increased eosinophils in BAL and lung tissue	Sarpong et al., 2003
Male C57BL/6	HDM	Der p 1 + $AlOH_3$ (i.p.) on day 0	HDM (aerosol) on day 14 for 7 consecutive days	AHR and BAL and lung tissue inflammation at 24 hours post final challenge	Tournoy et al., 2000
Female BALB/c	OVA	OVA + alum (i.p.) on days 0 and 14	OVA aerosol on days 28-30 and 72 days after last challenge	AHR and BAL eosinophilia on days 32, 37, 44 and 74	Kanehiro et al., 2001
Male BALB/c	OVA	OVA + $AlOH_3$ (i.p.) on days 0 and 5	2×OVA inhalations, each 4 hours apart on day 17	Following OVA challenge: AHR, EAR and LAR observed	Fernandez-Rodriguez et al., 2008
Male BALB/c	OVA	OVA + $AlOH_3$ (i.p.) on day 0 and OVA i.p. on day 10	OVA aerosol on days 17 and 24	Following OVA challenge: EAR and LAR. Increased inflammatory cells in BAL and lung tissue	Choi et al., 2005

Table 1: Mouse Models of Acute Allergen-Induced Asthma. Common mouse strains, allergens, sensitization and challenge methods used to develop allergic asthma phenotype [73]

Mouse gender/ strain	Allergen	Sensitisation	Exposure/challenge	Responses post final allergen challenge	Reference
Female BALB/c	OVA	OVA + alum (i.p.) on days -7 and -21	OVA for 6/8 weeks (3 days/week)	Intraepithelial eosinophilia, infiltration of lamina propria by mononuclear cells, remodelling, Th2 cytokine induction and AHR	Temelkovski et al., 1998
Female BALB/c	OVA	OVA + alum (i.p.) on days 0 and 14	OVA (i.n.) on days 14, 27, 28, 47, 61 and 73-75	Eosinophilic and mononuclear cell inflammation; goblet cell hyperplasia and mucus occlusion of airways; widespread deposition of subepithelial collagen	Henderson et al., 2002
Female BALB/c	OVA	OVA + aluminium potassium sulphate (i.p.) on days 1 and 11	OVA (i.n.) on days 11, 19, 20, 33, 34, 47, 48, 61, 62, 75, 76, 89 and 90	Increased eosinophilia, remodelling, Th2 cytokine induction and AHR	Leigh et al., 2002a
Female BALB/c	OVA	OVA + alum (i.p.) on days 0 and 12	OVA aerosol on days 18-23 and then 3 days/week for 5/8 weeks starting on day 26	Inflammation, remodelling, Th2 cytokine induction and AHR; TGF-β induction	McMillan et al., 2005
Female BALB/c	HDM extract	-	HDM (i.n.) 5 days/week for up to 7 weeks	Eosinophilic inflammation, remodelling, Th2 cytokine induction and AHR	Johnson et al., 2004
Female BALB/c	HDM extract	-	HDM (i.n.) 5 days/week for up to 5 weeks	Eosinophilic inflammation, lung tissue inflammatory gene expression	Ulrich et al., 2008
Male BALB/c	OVA	OVA + $AlOH_3$ on days 0 and 5	OVA aerosol, starting day 17, 3 days/week for 6 weeks	Eosinophilic inflammation, AHR, and early and late asthmatic responses	Fernandez-Rodriguez et al., 2008
Female BALB/c	OVA	OVA and alum (s.c.) on days 0, 7, 14 and 21	OVA (i.n.) on days 27, 29 and 31, and then twice a week for 3 months	Eosinophilic inflammation, remodelling and TGF-β induction	Lee et al., 2008

Table 2: Mouse Models of Chronic Allergen-Induced Asthma. Common mouse strains, allergens, sensitization and challenge schedule for induction of allergic asthma phenotype [73]

limited number of studies have been performed using airway route sensitization. Airway route sensitization is important since it is the route of allergen exposure. Therefore, we investigated aerosolized sensitization to an allergen using a murine model. In addition, we tested if PUL-042 diminishes an allergen-induced phenotype by measuring biomarkers such as IgE levels, eosinophil airway infiltration, and airway mucin density. Overall, we hypothesized that PUL-042 dampens the immune response elicited in an airway route allergen-induced asthma model. These studies may provide insight for PUL-042 as a preventative drug therapy for allergen-induced asthma.

Chapter 2: Materials and Methods

Animals

Aseptic BALB/c mice were obtained from Harlan Laboratories. Mice were bred and maintained as per Institutional Animal Care Use Committee guidelines following transfer to the Texas A&M Health Science Center Institute of Bioscience and Technology Animal Facility. Five to seven week old female BALB/c mice fed an ovalbumin (OVA) free diet were used to carry out all experiments. All experimental protocols were approved by the Texas A&M Health Science Center Institute of Bioscience and Technology's Institutional Animal Care Use Committee standards.

Allergens, Antibodies and other Reagents

A lyophilized powder of albumin from chicken egg (OVA, grade V, Sigma-Aldrich Co. LLC) was either mixed in solution of aluminum phosphate in sterile saline for intraperitoneal injections (*i.p*) or just sterile saline for aerosolized treatments. Lyophilized allergenic fungal proteinase (FAP), which is derived from *Aspergillus oryzae* (Sigma) was prepared as a 1 mg/ml solution using sterile phosphate-buffered saline (PBS) and stored at -20°C. Synthetic ligands to Toll-like Receptor (TLR) 2/6 and TLR9, Pam2 and ODN (PUL-042, a synthetic diacylated lipopeptide and oligodeoxynucleotides) in a 4 μM:1 μM ratio were prepared in deionized H_2O and stored at -20°C. Polyclonal goat anti-mouse IgE (STAR 166, AbD Serotec); purified mouse anti-OVA IgE (MCA2259, AbD Serotec); purified mouse IgE κ isotype control (BD Biosciences); biotin rat anti-mouse IgE (BD Biosciences); OVA labeled with biotin using EZ-Link Sulfo NHS-LC-Biotin (21335, Thermo Scientific); streptavidin HRP (Amersham Pharmacia Biotech), and Sigma

Fast OPD peroxidase substrate (P9187-50, Sigma-Aldrich) were used for enzyme linked immunosorbent assays.

OVA Aerosol Treatments

Mice were given a 2.5% weight to volume OVA (Sigma-Aldrich Co. LLC) in 0.9% saline at ambient air (5% CO_2 blended in) via a compressed air nebulizer. Airway route challenges were carried out utilizing 30 minute exposure to OVA 3 times a week during the first 2 weeks, with a final OVA challenge given during week 5.

FAP Treatments

Mice sedated using isoflurane inhalant anesthesia in open-drop exposure procedure were given 25μL of a 1μg/μl concentration of FAP intranasally (*i.n.*) 3 times a week for 2 weeks.

Synthetic TLR Ligand Aerosol Treatments

PUL-042 was administered by aerosol using a disposable Aero Tech II nebulizer connected to polyethylene constructed aerosol chamber by polyethylene tubing and delivered under compressed air (5% CO_2 blended in) vented under BSL2 safety cabinet. Treatments were given for 20 minutes 3 times a week sequentially at the end of treatment with allergens.

OVA *I.p.* Sensitization

Specified groups of mice were given 100μL once a week for 4 weeks of aluminum and potassium sulfate (22.5 mg/ml in 0.9% sterile saline with 0.2 mg/ml OVA added to mixture) in addition to aerosol or *i.n.* treatments with the allergen, TLR ligands or a combination of both. At the beginning of the fifth week mice either

received an OVA aerosol challenge as described above or no challenge. Blood was collected by cardiac puncture under anesthesia (Avertin, 16 µl/g). Bronchoalveolar lavage (BAL) fluid was collected from one side of the mouse lungs. The lung was lavaged 2 to 3 times. Whole lung tissue samples were removed and stored in formalin for use in mucin density studies.

Total and Differential White Blood Cell (WBC) Counts

The mouse BAL fluid was used to count total WBCs using a hemacytometer. To determine differential WBCs counts, samples were applied to positively charged slides (67-762-14, Thermo Scientific), 150-200µL was placed into a cytofunnel sample chamber. Slides were centrifuged using a Cytospin* 4 Cytocentrifuge (Thermo Scientific) at 2000 rpm for 5 minutes. Slides were stained by submerging them into modified Wright-Giemsa stain (Accustain WG-16, Sigma-Aldrich) for 30 seconds in a coplin jar, followed by 2 minutes in phosphate buffer reconstituted in ultrapure deionized water (P3288-12VL, Sigma-Aldrich). Slides were then rinsed in running deionized water, air dried and then cover-slipped using cytoseal 60 (23-244257, Thermo Scientific). Cell morphology was evaluated using a 40X objective on a light microscope to differentiate cell types (300 cells were analyzed per slide).

Total and *Anti*-OVA serum IgE Antibody Levels

Total and *anti-OVA* IgE levels were measured by Enzyme Linked Immunosorbent Assay (ELISA). All antibody ELISAs were carried out using Immulon 4HBX 96-well microtiter plates (Immulon 4HBX, 3855, Thermo Scientific). Each microwell was coated with 50µL of capture antibody diluted in Tris buffered saline (TBS) to a 4 µg/mL concentration. This volume was used for all antibodies

and standards unless otherwise specified. Plates were sealed and incubated overnight at 4°C. Contents of wells were voided and plates blotted on dry absorbent material. A multichannel pipette was used to deliver 200μL per well of wash buffer (filtered and autoclaved TBS with 0.1% Tween-20, TBST) and plates incubated at room temperature on an orbiter for 5 minutes. Washes were repeated 3 times each. After each wash, plates were inverted and blotted on absorbent paper to remove any residual buffer.

Next, plates were blocked by adding 200μL per well of prepared assay diluent stored between 2-8°C (50 mM TBS with 1% Bovine Serum Albumin and 0.05% Tween-20) and incubated at 37°C for 60 minutes. Following blocking, plates were washed as described above; then standards and sample dilutions (primary antibody) prepared using assay diluent were aspirated into duplicate wells and incubated for 60 minutes at 37°C. The bottom row of each plate was not coated with standards or primary antibody and was used to subtract out background signal. Plates were washed again followed by the addition of a secondary antibody; either biotin-conjugated rat anti-mouse IgE (2 μg/μL diluted in assay diluent) or biotin-labeled OVA (10 μg/μL diluted in assay diluent). Plates were sealed and re-incubated at 37°C under the same conditions described above. Plates were washed again followed by the addition of enzyme-conjugated streptavidin (1:5000 dilution using assay diluent), resealed, and incubated for 30 minutes as above. Plates were then washed three times using TBST followed by 2 washes using 1X filtered and autoclaved TBS. Absorbance of samples were measured using a microplate reader at 450nm.

Histochemistry and Fluorescence Microscopy

The lungs of anesthetized and tracheostomized mice were perfused with saline via the right cardiac ventricle to clear blood from the pulmonary tissues. Lungs were fixed *in situ* for 30 minutes by instilling 4% paraformaldehyde in PBS (pH 7.0) intratracheally at 10-15 cm pressure. Lungs were removed from the thorax and fixed overnight at 4°C. Following overnight fixation, lungs were rinsed in 0.1M phosphate buffer (pH 7.0) to remove excess fixative, embedded in paraffin and dissected into 5 micrometer sections. Tissue sections were de-waxed, rehydrated and oxidized in freshly prepared 1% period acid (Sigma, electrophoresis grade) for 10 minutes at room temperature.

Next, sections were rinsed 3 times for 5 minutes in distilled water and treated with acriflavine fluorescent Schiff's reagent (0.5% acriflavine HCl w/v, 1% sodium metabisulfite w/v, 0.01 N HCl) for 20 minutes (Sodium metabisulfite, 255556, Sigma-Aldrich). Sections were rinsed again as described above followed by rinsing twice for 5 minutes in acid alcohol (0.1 N HCl in 70% ethanol) and allowed to air dry in the dark. Tissue sections were cover slipped with Canada balsam mounting medium (50% Canada balsam resin, 50% methyl salicylate; Fisher Chemicals). Slides were stained with Periodic acid fluorescent Schiff's (PAFS) to determine intracellular mucin content. Slides were examined under a 40x objective using MagnaFire 2.1 Optronics CCD camera to produce images. Ten fields of a minimum of three airway sections were imaged blindly (ImagePro Plus).

Chapter 3: Results

Airway route sensitization with experimental allergens results in an allergic asthma phenotype in mice

The established murine model for allergen-induced asthma first involves sensitizing mice using a mixture of ovalbumin (OVA) and a known adjuvant such as aluminum hydroxide (alum) via a series of intraperitoneal (*i.p.*) injections [73, 74]. After sensitization, mice are challenged with the allergen of choice to induce an allergic asthma phenotype [73, 74]. Environmental allergens are typically introduced into our lungs by inhalation; therefore, I investigated "airway" sensitization by an aerosol or intranasal (*i.n.*) delivery of common experimental allergens. Using this model, the allergic asthma phenotype was measured by two biomarkers; an increase in serum IgE levels, and the infiltration of white blood cells (WBCs), most specifically eosinophils into the lungs.

To test if allergen administration by aerosol or *i.n.* leads to an allergen-induced asthma phenotype, mice were treated with two known experimental allergens, OVA and fungal associated allergenic proteinase (FAP). Saline was administered to mice as a negative control, and *i.p.* injections of OVA/alum were administered to mice to serve as a positive control (Fig. 4). Mice receiving aerosol or *i.n.* administration of OVA and FAP were treated over a 12-day period and then sacrificed on Day 15. Mice that received *i.p.* injections were treated over a 24-day period followed by an OVA challenge via aerosol on Day 29. On Day 32, mice were sacrificed, and the blood and bronchoalveolar lavage (BAL) fluid samples were collected from each mouse and analyzed for total IgE, OVA-specific IgE, and the number of WBCs.

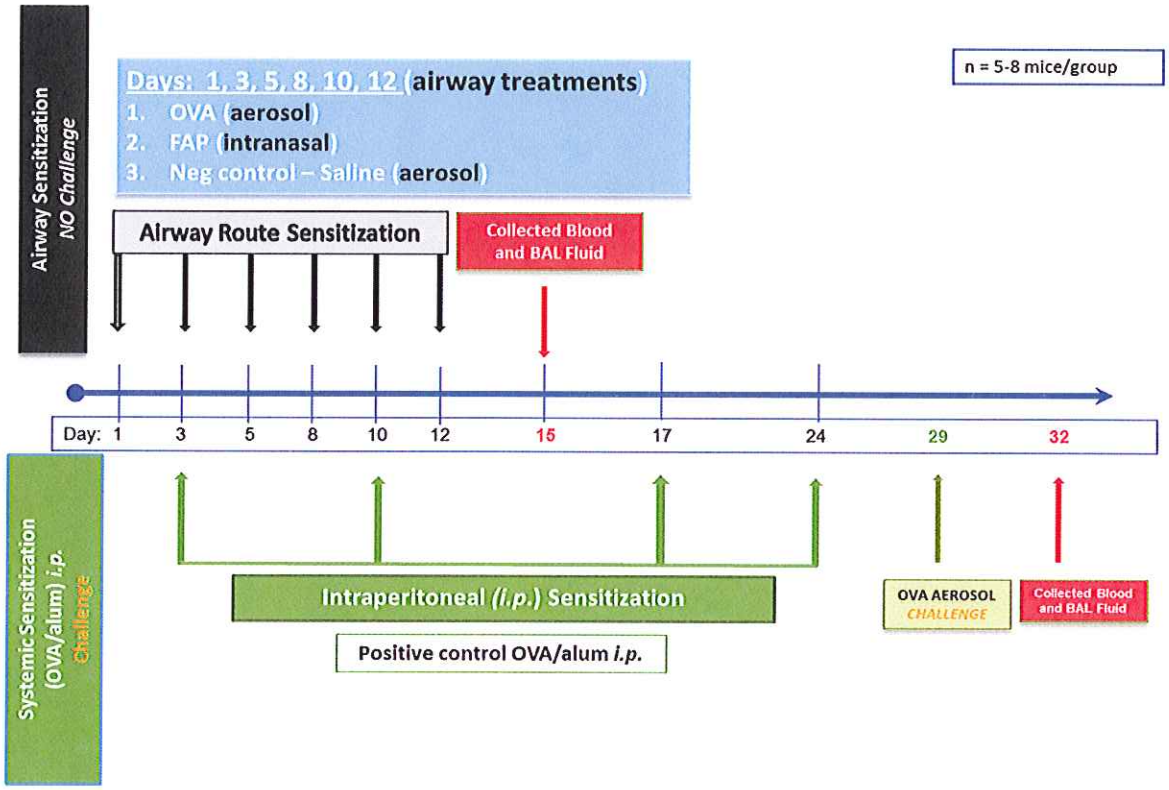
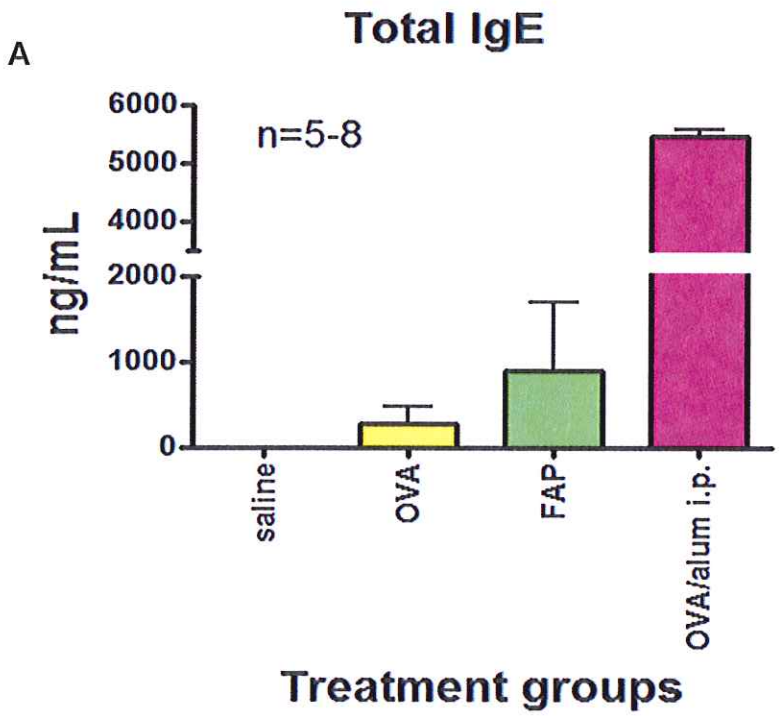


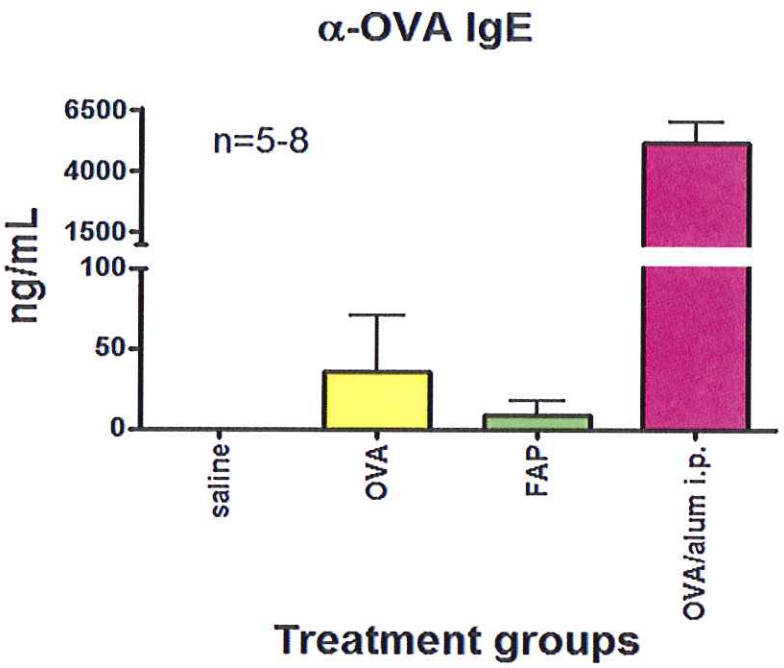
Figure 4: Experimental scheme of airway and *i.p.* routes of sensitization to induce an allergic asthma phenotype in mice. Mice were treated with saline (negative control) or OVA by aerosol on days 1, 3, 5, 8, 10 and 12. Mice that received FAP were treated on the same days using *i.n.* instillation. Mice treated with saline or an experimental allergen were sacrificed on Day 15 and BAL fluid and blood samples were collected. The positive control group received an *i.p.* injection of an OVA/alum mixture on days 3, 10, 17 and 24. On Day 29 these mice were challenged with OVA via aerosol and subsequently sacrificed on Day 32 and samples were collected.

Mice treated with both OVA and FAP showed an increase in total serum IgE levels compared to mice treated with saline (Fig. 5A). As expected, mice that received systemic administration of an OVA/alum mixture showed a robust response in the levels of total IgE (5454 ± 111 ng/ml) compared to the levels detected from mice treated with OVA or FAP (269 ± 196 ng/ml, 889 ± 809 ng/ml, respectively). Additionally, a partial amount of the levels of total IgE detected from mice treated with OVA were antigen-specific as seen in Figure 5B. Next, we

measured the total number of WBCs in the BAL fluid. The total number of WBCs in mice treated with OVA was increased compared to saline (5.4 $x10^6$ ± 1.3 $x10^6$ cells/ml versus 2.9 $x10^6$ ± 0.54 $x10^6$ cells/ml). Moreover, the total number of WBCs observed after mice were treated with FAP (12.7 $x10^6$ ± 1.6 $x10^6$ cells/ml) was greater than the number of WBCs observed in mice treated with saline and OVA (Fig. 5C). The most robust response was observed in mice sensitized systemically using OVA/alum (20.3 $x10^6$ ± 3.4 $x10^6$ cells/ml) (Fig. 5C). These results demonstrate that aerosol sensitization using OVA elicited a modest response compared to *i.p.* sensitization as measured by total serum IgE levels, ova-specific IgE levels, and total WBCs.



B


α-OVA IgE
n=5-8
ng/mL
6500
4000
1500
100
50
0
saline
OVA
FAP
OVA/alum i.p.
Treatment groups

C

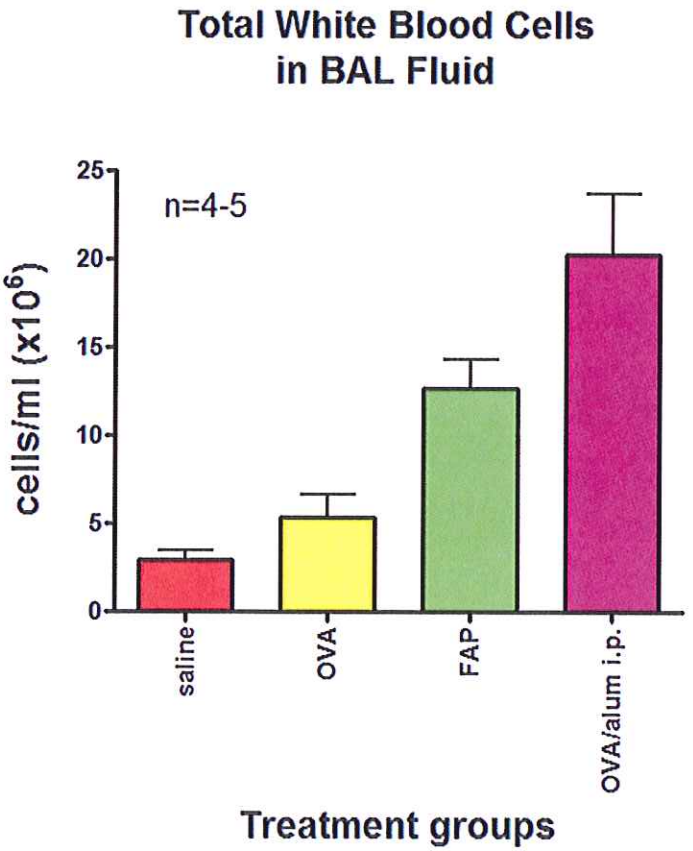

Total White Blood Cells
in BAL Fluid
n=4-5
cells/ml (x10^6)
25
20
15
10
5
0
saline
OVA
FAP
OVA/alum i.p.
Treatment groups

Figure 5: Total IgE and OVA specific IgE levels and WBCs measured from airway route and systemic sensitization in mice. (**A**) Serum total IgE levels were measured from the blood collected by cardiac puncture of mice that received saline (red bar), OVA (yellow bar), FAP (green bar) and OVA/alum (purple bar) either via an aerosol, *i.n.* or *i.p.* administration, respectively (**B**) OVA-specific IgE levels were measured from the serum of mice in all groups (**C**) Total WBCs were counted from the BAL fluid of mice in all groups. Results were performed in duplicates and expressed as the mean of each sample.

Allergen-induced asthma in mice with the addition of PUL-042

In the first set of experiments, the mice that were treated with OVA by aerosolization were not given an OVA challenge. We decided to investigate if an OVA challenge after OVA aerosol sensitization would result in an increased response when compared to the positive control. Therefore, my next experiments incorporated the OVA challenge using the same biomarkers to measure the immune response as in the initial experiments.

The total serum IgE and OVA-specific IgE levels were increased in mice that were treated with OVA aerosolization and subsequently challenged via aerosolization with OVA (1291 ± 766 ng/ml and 61 ± 9 ng/ml, respectively) (Fig. 7A and B) versus OVA aerosol treated mice that received no challenge (269 ± 195 ng/ml and 36 ± 36 ng/ml, respectively) (Fig. 5A and B). These results suggested that an OVA challenge is required after sensitization to elicit an immune response as measured by IgE levels. Therefore, I continued with this experimental design to determine if PUL-042 dampens the immune response.

In these experiments, mice in test groups were treated with OVA, PUL-042, or a combination of OVA plus PUL-042 (OVA/PUL-042) via an aerosol route during the first 12 days. On Day 29, all groups of mice were OVA challenged and on Day

32, mice were sacrificed and the blood, BAL fluid and lung tissue samples were collected (Fig. 6). The data showed that mice treated with PUL-042 had decreased levels of total serum IgE when compared to mice treated with OVA/PUL-042 (*p=0.03) (Fig. 7A). The data also showed that mice treated with PUL-042 had decreased levels of OVA-specific serum IgE when compared to mice treated with OVA/PUL-042 (***p=0.0005) (Fig. 7B). The total number of WBCs in the BAL fluid were similar for mice treated with OVA or PUL-042 (3.68 $x10^6$ ± 0.61, 4.51 $x10^6$ ± 0.50 cells/ml, respectively) compared to control mice 4.36 $x10^6$ ± 0.76 cells/ml) (Fig. 7C). On the other hand, mice treated with OVA/PUL-042 (9.74 $x10^6$ ± 2.5 cells/ml) (Fig. 7C) showed an increase in total WBCs compared to all other groups of mice. I also counted the number of eosinophils in the BAL fluid of mice because eosinophils are the major type of WBCs elevated in the lungs during an allergen-induced response. Mice treated with OVA showed a significant increase in the number of eosinophils in the BAL fluid compared to the negative control, PUL-042 and OVA/PUL-042 (*p=0.04) (Fig. 7D). Similarly, mice treated with OVA had a significant increase in airway mucin production compared to the negative control (*p=0.04) (Fig 7E), PUL-042 and OVA/PUL-042 (*p=0.02) (Fig. 7E).

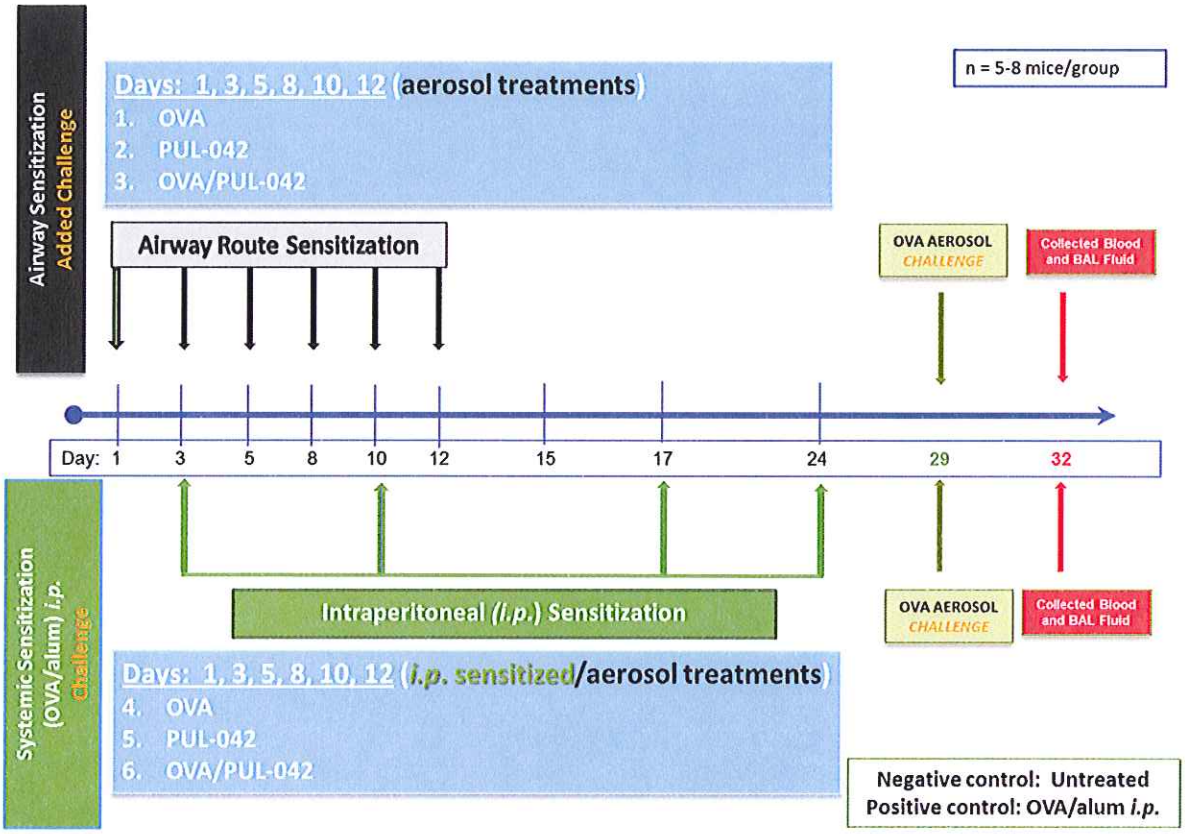
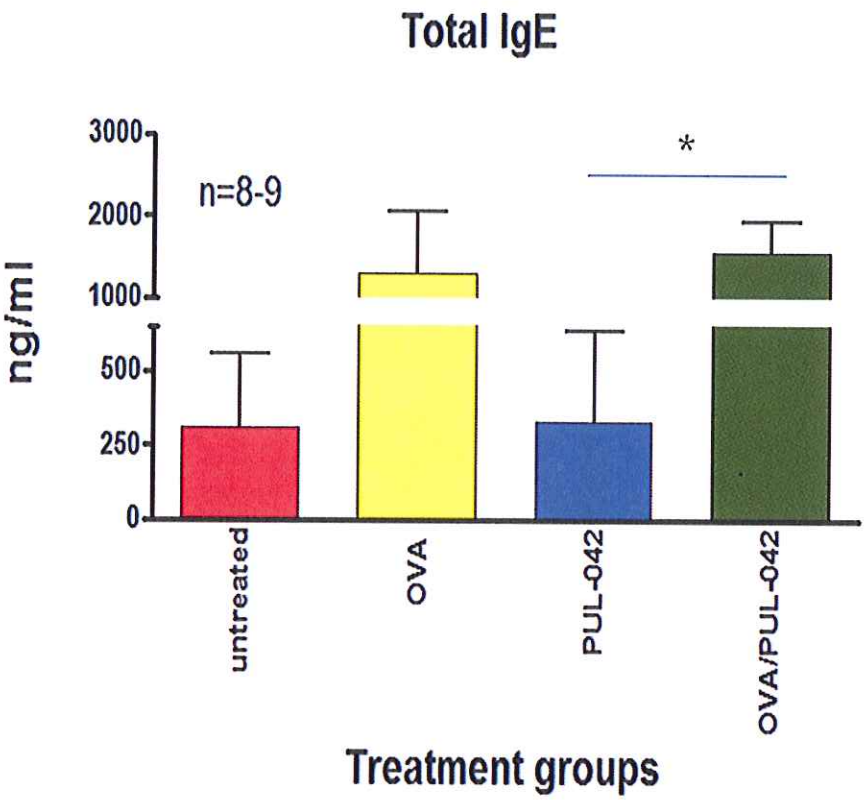
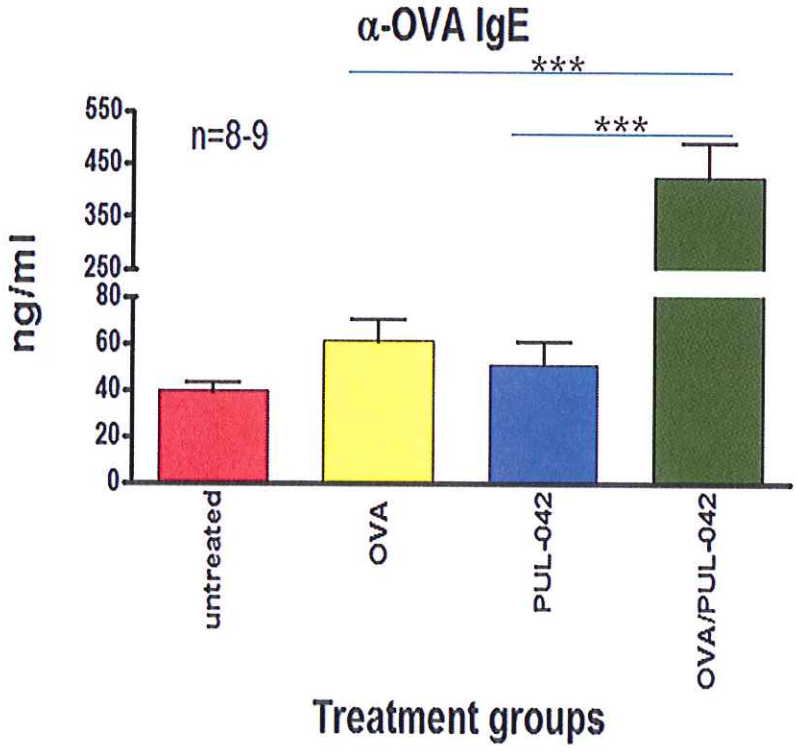


Figure 6: Experimental scheme for airway route and systemic sensitization in addition with PUL-042 treatment. Mice were treated with OVA, PUL-042 or OVA/PUL-042 by aerosol on days 1, 3, 5, 8, 10 and 12. At the end of 12 days no aerosol treatments were given. Mice were then challenged with OVA on Day 29. The negative control mice received no treatments during the course of the experiment. Another group of mice were given weekly *i.p.* injections of OVA/alum in parallel with aerosol treatments on days 1, 8, 15 and 22. On Day 29 mice were challenged with OVA. On Day 32, all mice were sacrificed and the blood, BAL fluid, and lung tissue samples were collected.

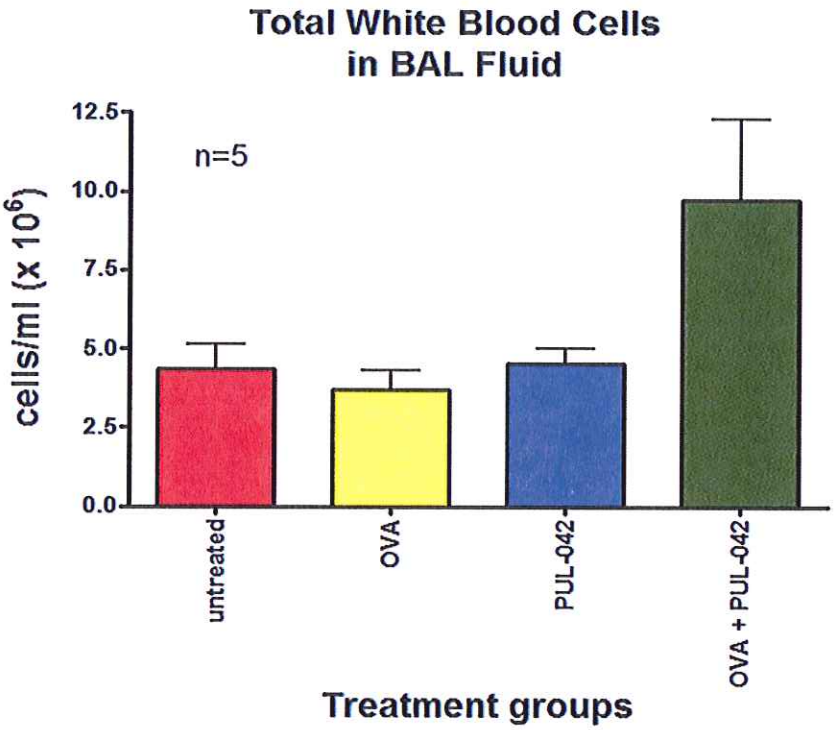
A


Total IgE
3000
2000
1000
500
250
0
n=8-9
*
ng/ml
untreated
OVA
PUL-042
OVA/PUL-042
Treatment groups

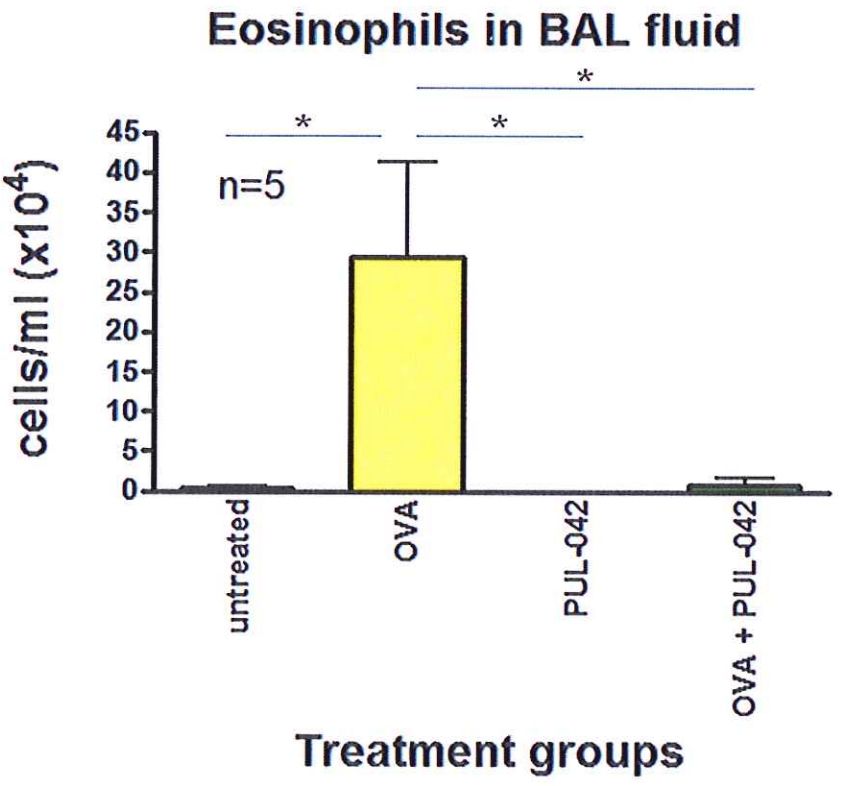
B


α-OVA IgE
***
***
550
450
350
250
80
60
40
20
0
n=8-9
ng/ml
untreated
OVA
PUL-042
OVA/PUL-042
Treatment groups

C


Total White Blood Cells
in BAL Fluid
n=5
cells/ml (x 10^6)
12.5
10.0
7.5
5.0
2.5
0.0
untreated
OVA
PUL-042
OVA + PUL-042
Treatment groups

D


Eosinophils in BAL fluid
*
*
*
n=5
cells/ml (x10^4)
45
40
35
30
25
20
15
10
5
0
untreated
OVA
PUL-042
OVA + PUL-042
Treatment groups

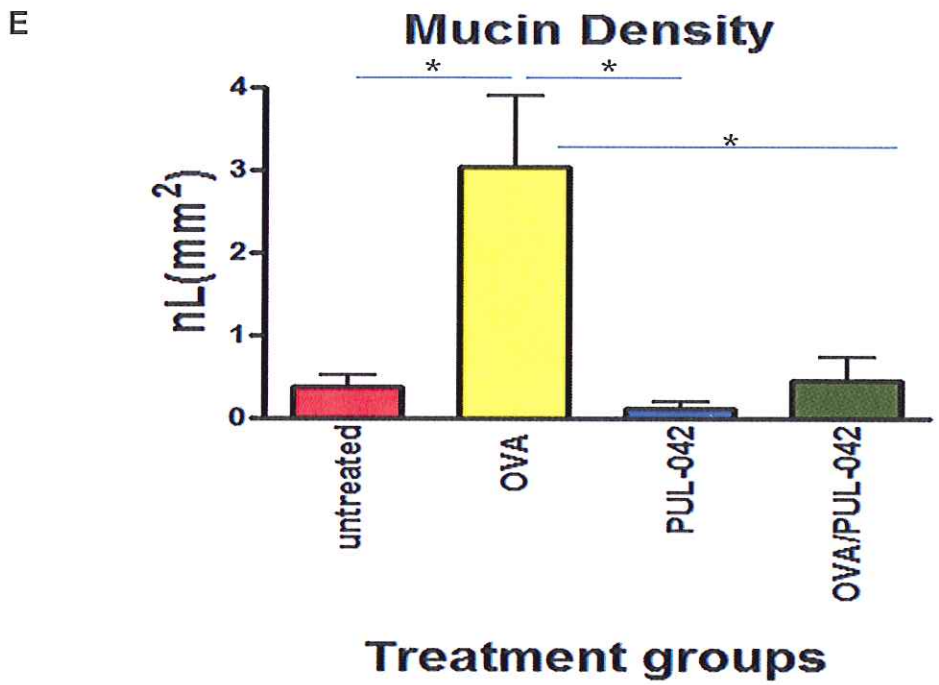
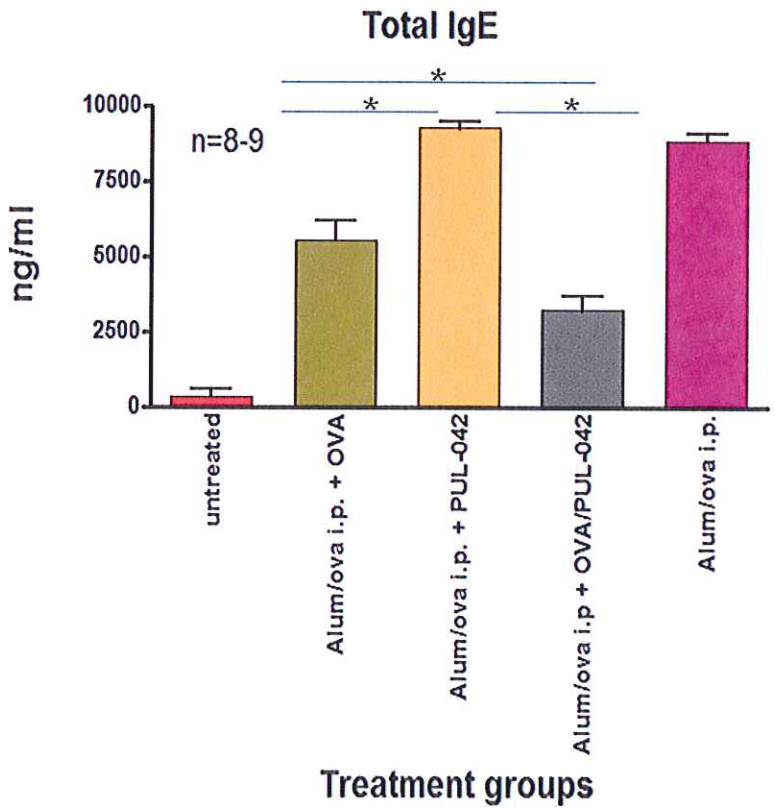


Figure 7: Biomarkers evaluated for airway sensitized mice in addition to PUL-042 after challenge with OVA. (**A**) Total IgE levels detected in blood from mice treated with OVA (yellow bar), PUL-042 (blue bar), and OVA/PUL042 (purple bar) or untreated (red bar). (*p=0.03, PUL-042 versus OVA or OVA/PUL-042). (**B**) OVA-specific IgE levels detected in blood for each group. (**C**) Total WBCs in BAL fluid collected from mouse lungs. (**D**) Eosinophil infiltration into the lungs determined by differential cell count of BAL fluid (*p=0.04, OVA versus untreated or OVA/PUL-042). Data were performed in duplicates and expressed as mean of samples. Statistical significance was measured by the student's *t* test with Welch's correction.

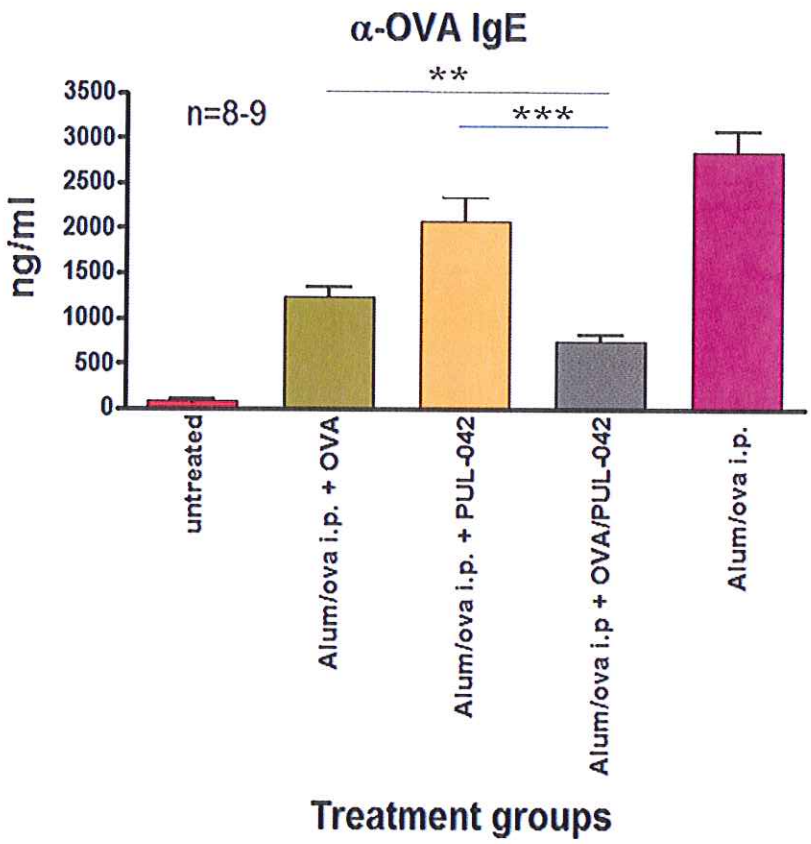
In parallel, mice were given *i.p.* injections of OVA/alum in combination with an aerosolization of either OVA, PUL-042, or OVA/PUL-042 for comparison with groups receiving aerosolization only (Fig. 6). The total serum and OVA-specific IgE levels, total WBC counts, eosinophil counts, and mucin densities were analyzed. The data revealed that *i.p.* sensitized mice showed an increase in all biomarkers when compared to mice receiving no *i.p.* treatment (Fig. 8A–E). Within the *i.p.* sensitized groups of mice, it was noteworthy that mice treated with OVA/PUL-042 demonstrated a decrease in all biomarkers measured in comparison to groups that

received either OVA only or PUL-042 only. The total serum levels of IgE (***p=0.0001, OVA/PUL-042 versus OVA and ***p<0.0001, OVA/PUL-042 versus PUL-042), OVA-specific IgE (**p=0.003; ***p=0.0007, respectively), eosinophils (*p=0.01 for both groups), and mucin densities (*p=0.04; ***p=0.0002, respectively) were significant (Fig. 8A–E). The number of WBCs did not show a statistically significant change. This data suggests that aerosol sensitization that includes *i.p.* administration of OVA/alum provides a robust immune response over aerosol sensitization alone. In addition, it appears that an OVA/PUL-042 combination diminishes the allergic asthma phenotype in *i.p.* sensitized mice for all biomarkers evaluated.

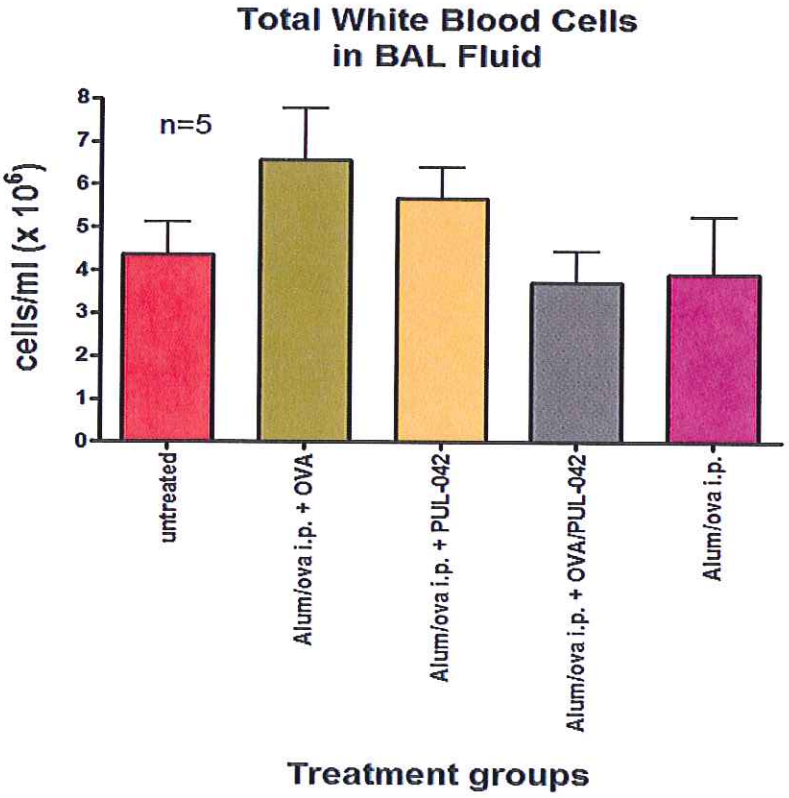
A



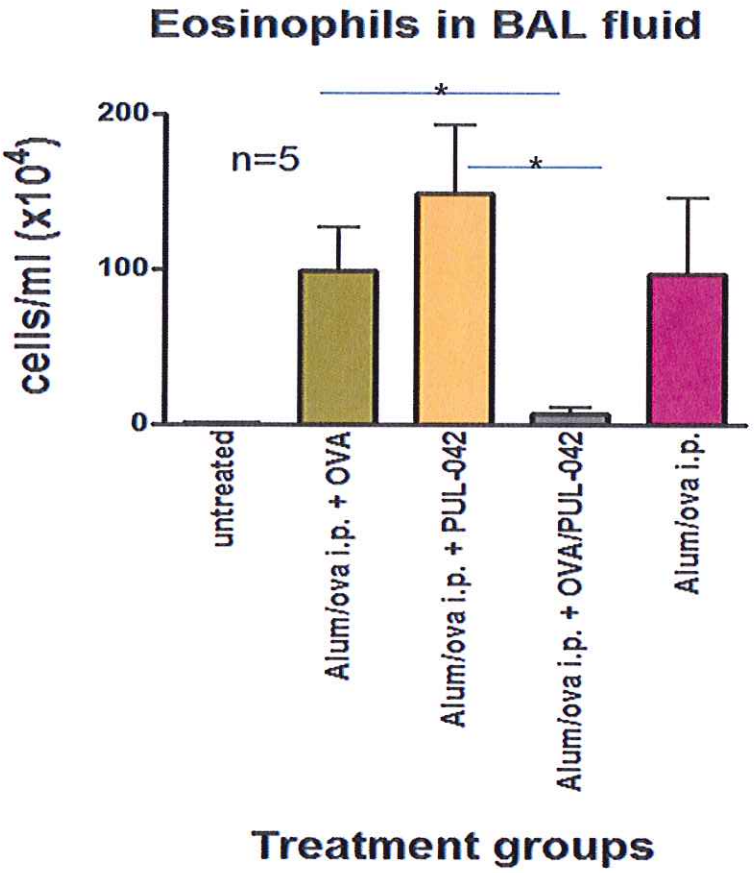
B


α-OVA IgE
**
***
n=8-9
ng/ml
3500
3000
2500
2000
1500
1000
500
0
untreated
Alum/ova i.p. + OVA
Alum/ova i.p. + PUL-042
Alum/ova i.p + OVA/PUL-042
Alum/ova i.p.
Treatment groups

C


Total White Blood Cells
in BAL Fluid
n=5
cells/ml (x 10^6)
8
7
6
5
4
3
2
1
0
untreated
Alum/ova i.p. + OVA
Alum/ova i.p. + PUL-042
Alum/ova i.p. + OVA/PUL-042
Alum/ova i.p.
Treatment groups

D


Eosinophils in BAL fluid
cells/ml (x10^4)
200
100
0
n=5
*
*
untreated
Alum/ova i.p. + OVA
Alum/ova i.p. + PUL-042
Alum/ova i.p. + OVA/PUL-042
Alum/ova i.p.
Treatment groups

E

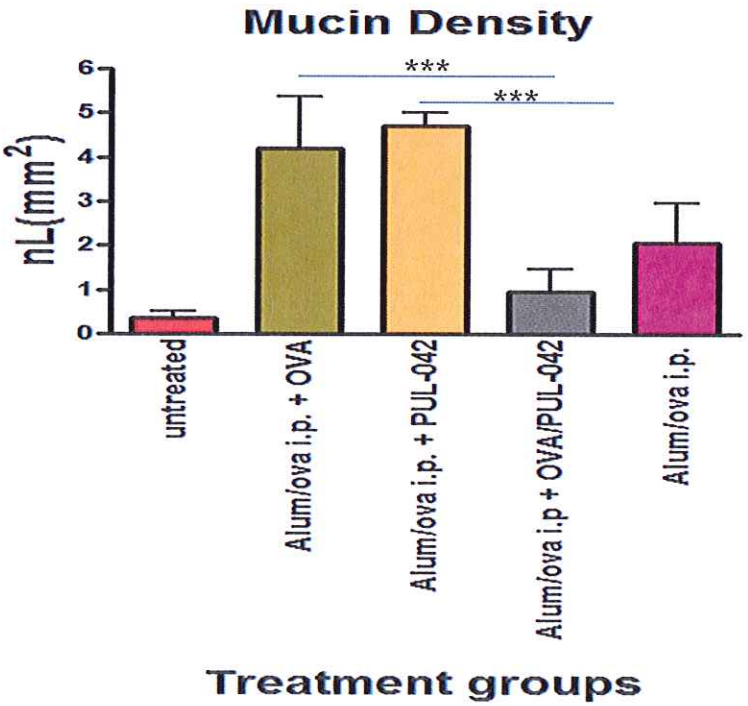

Mucin Density
nL(mm^2)
6
5
4
3
2
1
0
***
***
untreated
Alum/ova i.p. + OVA
Alum/ova i.p. + PUL-042
Alum/ova i.p + OVA/PUL-042
Alum/ova i.p.
Treatment groups

Figure 8: PUL-042 dampens the immune response of allergen-induced asthma in *i.p.* sensitized mice. (**A**) Total serum IgE levels in blood measured from mice treated with OVA (olive green bar), PUL-042 (peach bar), OVA/PUL-042 (grey bar), *i.p* sensitization with OVA/alum (purple bar) and untreated (red bar). (***p=0.0001, OVA/alum *i.p.* + OVA aerosol/PUL-042 versus OVA/alum *i.p* + OVA or versus OVA/alum *i.p* + PUL-042; ***p< 0.0001). (**B**) OVA-specific IgE levels measured from mice in all groups. (**p= 0.003, OVA/alum *i.p* + OVA aerosol/PUL-042 versus OVA or OVA/alum *i.p* + PUL-042, ***p=0.0007) (**C**) Total WBC counts in BAL fluid for each treated group. (**D**) Eosinophil infiltration into lungs (*p=0.01, OVA/alum *i.p.* + OVA aerosol/PUL-042 versus OVA/alum *i.p* + OVA or versus OVA/alum *i.p* + PUL-042) (**E**) Mucin density in lung tissue sections for each treated group (*p=0.04, OVA/alum *i.p.* + OVA aerosol/PUL-042 versus OVA/alum *i.p* + OVA or versus OVA/alum *i.p* + PUL-042; ***p=0.0002). Data were performed in duplicates and expressed as mean of samples. Statistical significance was measured by the student's *t* test with Welch's correction.

The dampened allergic asthma phenotype depends on the number of PUL-042 treatments in *i.p.* sensitized mice

Mice were treated using the same experimental regimen with the exception that PUL-042 was administered either one, three, or six times within the 12-day treatment regimen (Fig. 9). These results showed no significant difference in total IgE levels and OVA-specific IgE levels among mice that received OVA by aerosol along with 1, 3, or 6 PUL-042 treatments (Fig. 10A). For *i.p.* sensitized mice given OVA by aerosol, results showed no significant difference between 1 and 3 PUL-042 treatments; however, there was a 0.5-fold significant decrease in total IgE levels between mice that received 1 versus 6 PUL-042 treatments (*p= 0.026; Fig. 10A). Similarly, OVA-specific IgE levels showed a significant decrease in mice that received 1 versus 6 PUL-042 treatments (**p=0.003, Fig. 10B).

Additionally, there was no significant difference in WBC numbers between aerosol groups that received 1, 3 or 6 PUL-042 treatments or amongst *i.p.* sensitized groups that received the same number of PUL-042 treatments. (Fig.

10C). Next, I counted the number of eosinophils for each group. The number of PUL-042 treatments did not demonstrate significant differences in eosinophil counts for groups that received OVA by aerosol only. However, for *i.p.* sensitized mice given OVA by aerosol, there was a treatment-dependent trend observed. In these mice, 6 treatments (85 $x10^4$ ± 22 cells/ml) (Fig. 10D) resulted in a lower number of eosinophils detected in the lungs when compared to 1 and 3 PUL-042 treatments, respectively (311 $x10^4$ ± 49 cells/ml, 159 $x10^4$ ± 54 cells/ml, respectively) (Fig. 10D). These results suggests that at least 6 treatments of PUL-042 administered by aerosol in *i.p.* sensitized mice are required to statistically reduce the number of eosinophil infiltration in the lungs compared to only 1 or 3 treatments (Fig. 10D). Also, this reduction in eosinophil infiltration is only seen if OVA is included in the aerosol mixture with PUL-042 for *i.p* sensitized mice. The same trend follows for the levels of total IgE and OVA-specific IgE detected in the serum for these groups of mice (Fig. 10A and B).

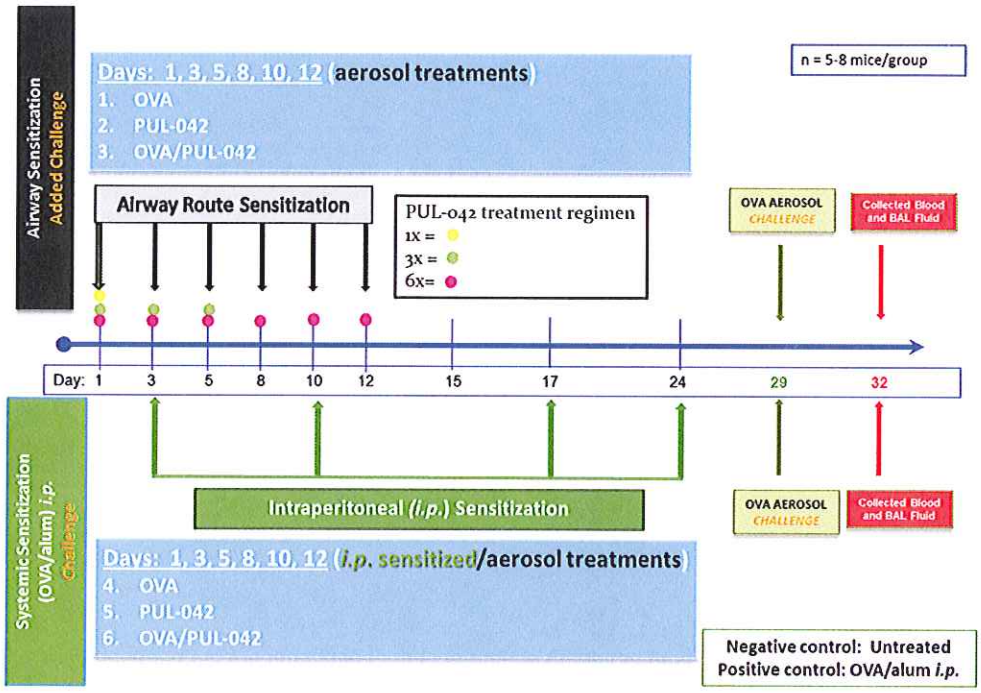
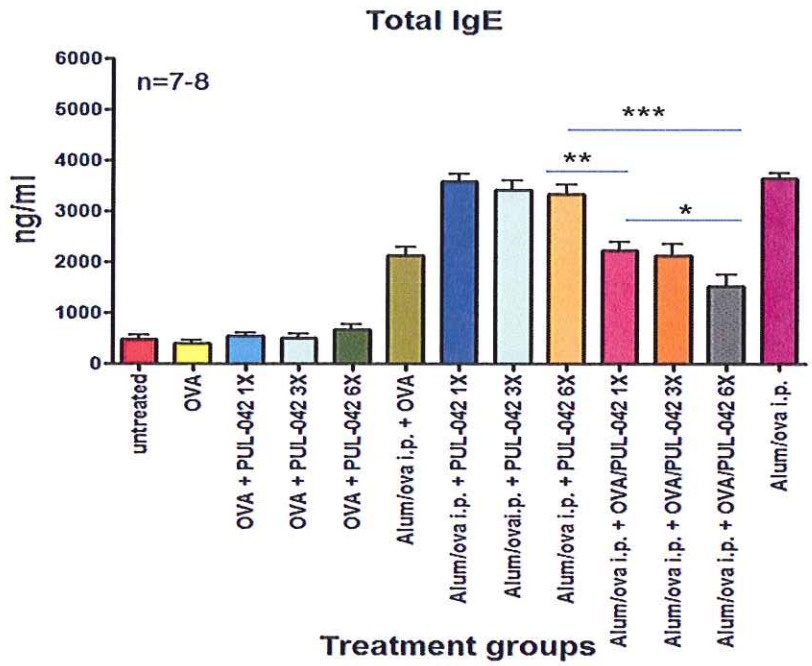
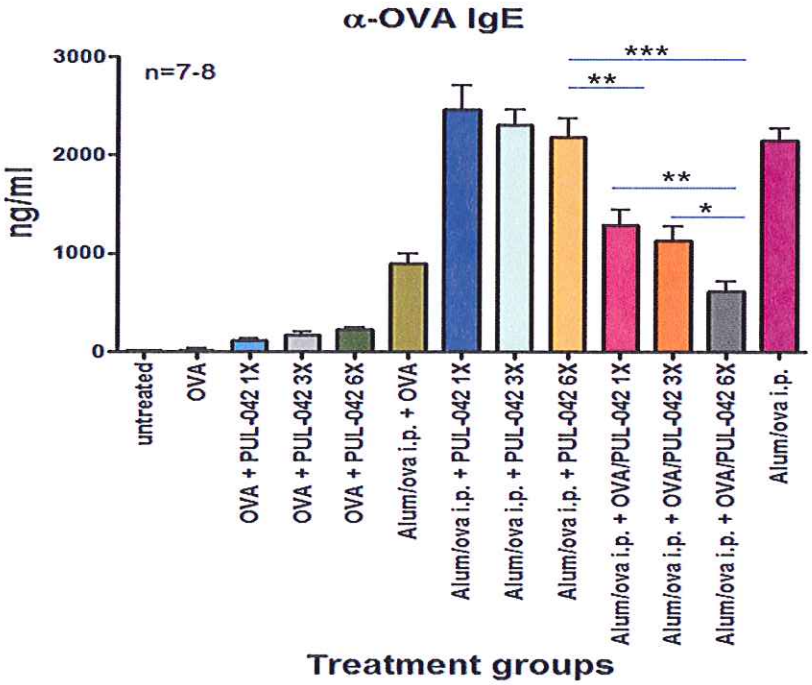


Figure 9: Experimental Scheme for airway and systemic sensitization of mice with the addition of specific number of PUL-042 treatments. PUL-042 was administered 1, 3, or 6 times in the first 12 days. Mice were subsequently administered an aerosol of OVA/PUL-042 or administered a series of *i.p.* injections of OVA/alum with an aerosol of OVA/PUL-042. Mice were OVA challenged on Day 29 and on Day 32 mice were sacrificed and samples were collected.

A


Total IgE
n=7-8
ng/ml
6000
5000
4000
3000
2000
1000
0
***
**
*
untreated
OVA
OVA + PUL-042 1X
OVA + PUL-042 3X
OVA + PUL-042 6X
Alum/ova i.p. + OVA
Alum/ova i.p. + PUL-042 1X
Alum/ovai.p. + PUL-042 3X
Alum/ova i.p. + PUL-042 6X
Alum/ova i.p. + OVA/PUL-042 1X
Alum/ova i.p. + OVA/PUL-042 3X
Alum/ova i.p. + OVA/PUL-042 6X
Alum/ova i.p.
Treatment groups

B


α-OVA IgE
n=7-8
ng/ml
3000
2000
1000
0
***
**
**
*
untreated
OVA
OVA + PUL-042 1X
OVA + PUL-042 3X
OVA + PUL-042 6X
Alum/ova i.p. + OVA
Alum/ova i.p. + PUL-042 1X
Alum/ova i.p. + PUL-042 3X
Alum/ova i.p. + PUL-042 6X
Alum/ova i.p. + OVA/PUL-042 1X
Alum/ova i.p. + OVA/PUL-042 3X
Alum/ova i.p. + OVA/PUL-042 6X
Alum/ova i.p.
Treatment groups

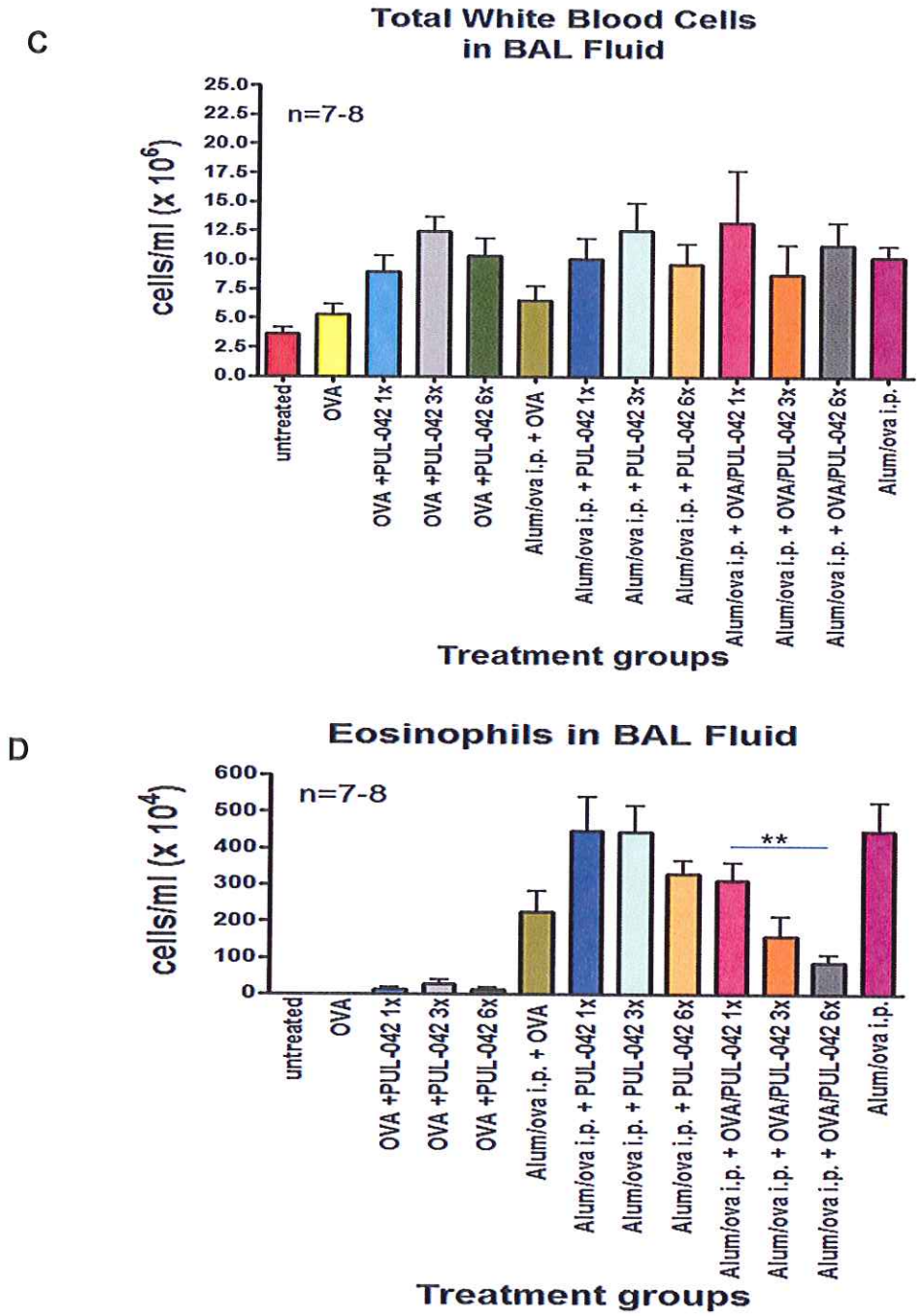


Figure 10: Specific number of PUL-042 treatments diminishes allergic asthma phenotype. (**A**) Total serum IgE levels measured from mouse blood. (OVA/alum *i.p.* + OVA/PUL-042 aerosol (1X) versus (6X) was significant, **p*=0.026) (**B**) OVA-specific IgE serum levels (OVA/alum *i.p.* + OVA/PUL-042 aerosol (1X) versus (6X) was significant, ***p*=0.003) (**C**) Total WBC counts in BAL fluid (**D**) Eosinophil infiltration into lungs. All data analysis were performed using GraphPad Prism 4.0 and statistical significance was measured by the student's *t* test with Welch's correction.

Chapter 4: Discussion

Allergen-induced asthma accounts for over 50% of asthma cases. Therapies used to treat allergen-induced asthma have limited efficacy and can result in detrimental effects in asthma sufferers, especially those with severe disease. For this reason, additional therapies for the treatment of allergic asthma need to be further investigated. This study has focused on two aspects of allergen-induced asthma: an allergen aerosolization for delivery via the airway route to induce an allergic phenotype in mice and the administration of PUL-042 as a treatment to diminish the allergen-induced phenotype in mice.

Airway sensitization by OVA demonstrated a modest response compared to systemic sensitization by OVA/alum, a mouse model that is known to induce a robust allergic phenotype in mice. Although airway sensitization by OVA demonstrated a response, these results are limited in that we tested only one concentration of OVA. We hoped to see a response comparable to the systemic model; and therefore, it is necessary to test different concentrations of OVA via the aerosol route.

Further, we expected to see reduced levels of total and OVA-specific serum IgE levels with treatment of PUL-042. Surprisingly, for mice that were given a systemic sensitization, we observed that PUL-042 only dampened the immune response when given concomitantly with OVA. In addition, a reduction in the allergen-induced immune response seen in these mice appears to be dependent on the number of treatments of PUL-042.

Additional biomarker testing may be necessary to fully investigate a reduction in the allergen-induced immune response mediated by PUL-042. These biomarkers

could include analysis of additional immunoglobulin isotypes such as IgG_1 and IgG_{2a}; could include quantification of T_H2-specific lymphocytes and allergen-specific T_H2 cytokines such as IL-4, IL-5, IL-9 and IL-13.

Overall, we did not observe a response in airway sensitization at the levels of systemic sensitization. However, results with treatments of PUL-042 were encouraging in that we did detect a reduced allergic asthma phenotype in mice. Therefore, future studies need to incorporate experimental optimization that includes concentrations of OVA, kinetic studies of immunoglobulin levels and cytokine expression, number of both OVA and PUL-042 treatments, and detection limits for biomarker assays.

Chapter 5: References

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Vita

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