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Fullerene C70 derivatives dampen anaphylaxis and allergic asthma pathogenesis in mice

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Fullerene C_{70} derivatives dampen anaphylaxis and allergic asthma pathogenesis in mice

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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DEDICATION

This dissertation is dedicated to my family for all their encouragement and support throughout his process. My parents, Ronald Kennedy and Penny Kennedy have supported me at every stage of my education and strongly encouraged me to attend graduate school. All my life they have made sacrifices to ensure I was given every opportunity to succeed, and have done everything possible to make this journey easier and celebrated my accomplishments with me. To my aunt, Patricia Petro who sparked my interest in science at a young age and encouraged my studies throughout this journey. To my mother-in-law, Lorraine Norton who has a kind heart and a listening ear and helps me keep everything in perspective. I must dedicate this dissertation to my wonderfully loving husband, Michael Norton, who has been with me every step of the way, through trials and triumphs, who helps pick me up when I fall. And finally, to my two beautiful daughters, Kendall Riley and Lindsay Claire Norton for giving me the motivation to succeed.

ACKNOWLEDGEMENTS

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List of Abbreviations

ALT   Alanine Transaminase

Alum   Aluminum hydroxide

AST   Aspartate Transaminase

BAL   Bronchoalveolar lavage

C57BL/6   Inbred mouse strain

C70-IR800 C_{70} Fullerene with 70 carbon atoms attached bound to an IR800 dye molecule

C70-TR C_{70} Fullerene bound to a Texas Red dye molecule

COX   Cyclooxygenase

Cyp1B1   Cytochrome P450 1B1

DNP   Dinitrophenol

EET   Epoxyeicosatrienoic acid

Gd   Gadolinium

HETE   Hydroxyeicosatetraenoic acid

H&E   Hematoxylin and Eosin stain

IgE   Immunoglobulin E

ICAM-1   Intercellular adhesion molecule-1

IL-4   Interleukin-4

IL-5   Interleukin-5

IL-13   Interleukin-13

i.n.   Intranasal

i.p.   Intraperitoneal

KO	Knockout
LOX	Lipoxygenase
LTC_4	Leukotriene C4
LTD_4	Leukotriene D4
OVA	Ovalbumin
PAS	Periodic Acid-Schiff
PBS	Phosphate Buffered Saline
PGE_2	Prostaglandin E2
PGF2α	Prostaglandin Factor 2 alpha
PI	Propidium Iodide
R_L	Airway Resistance
ROS	Reactive oxygen species
C_{70}-TGA	C_{70}-Tetraglycolate
Th2	T helper cell type 2
TXA_2	Thromboxane A2
VCAM-1	Vascular adhesion molecule-1
WT	Wild-type

Abstract

FULLERENE C$_{70}$ DERIVATIVES DAMPEN ALLERGIC AIRWAY INFLAMMATION, BRONCHOCONSTRICTION, AND SYSTEMIC ANAPHYLAXIS

By Sarah B. Norton, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2012

Director: Daniel H. Conrad, Professor, Department of Microbiology and Immunology

Fullerenes are carbon nanospheres that can be solublized by the addition of polar chemical groups to the carbon cage, forming fullerene derivatives. One specifically derivatized fullerene compound, termed C$_{70}$-Tetragylocolate (C$_{70}$-TGA), has been shown to stabilize mast cell responses in vitro thus we hypothesized it may have an effect on mast cell-driven diseases such as asthma and systemic anaphylaxis. To observe the effects of C$_{70}$-TGA on systemic anaphylaxis, mice were subjected to a model of passive systemic anaphylaxis. In this model, mice were injected with DNP-specific IgE 16 hours prior to challenge, then treated with C$_{70}$-TGA. Immediately prior to DNP challenge, mice were subjected to a second injection of C$_{70}$-TGA. Following DNP challenge, body temperature was recorded and blood was collected for quantitation of histamine levels. Treatment with C$_{70}$-TGA significantly reduced body temperature drop associated with systemic anaphylaxis and serum histamine levels. To observe the effects of C$_{70}$-TGA on chronic features of asthma in vivo, we utilized a heavily MC influenced model of asthma pathogenesis. Mice were sensitized by intraperitoneal (i.p.) injection of ovalbumin (OVA) in saline, challenged intranasally (i.n.) with OVA, and one

of two treatment strategies was pursued. In one, C_{70}-TGA was given i.n. throughout disease development. In the other, C_{70}-TGA was given following an initial set of challenges to allow disease to develop prior to treatment; mice were then re-challenged with OVA to assess the effect on established disease. We found that C_{70}-TGA treatment significantly reduced airway inflammation and eosinophilia and dramatically reduced bronchoconstriction in either model. Cytokines IL-4 and IL-5 and serum IgE levels are significantly reduced in C_{70}-TGA treated animals. Interestingly, we also saw an increase in the anti-inflammatory eicosanoid 11, 12-epoxyeicosatreinoic acid (11,12-EET) in the BAL fluid, suggesting the involvement of this mediator in C_{70}-TGA inhibition. Further experiments utilizing an inhibitor of 11,12-EET formation (6-(2-Propargyloxyphenyl)hexanoic acid) and a structural analog of 14,15-EET (14,15-EE-5(Z)-E) in vivo indicate that these mediators are closely associated with C_{70}-TGA mediated inhibition as their inhibition reverses the anti-inflammatory effects of C_{70}-TGA. Importantly, mice did not exhibit any acute toxicity following C_{70}-TGA treatment and liver and kidney function were normal. Collectively, these results show that the fullerene C_{70} derivative C_{70}-TGA is capable of dampening severe allergic responses including systemic anaphylaxis, airway inflammation, and bronchoconstriction. The mechanism of inhibition is through the upregulation of the anti-inflammatory EETs, which may dampen mast cell degranulation in vivo, thus contributing to the inhibitory effect of C_{70}-TGA on allergic disease

Introduction

I. Nanoparticles

Nanoparticles include natural and manufactured molecules measuring less than 100 nanometers that have a variety of uses in manufacturing, household products, and more recently, in medicine. Nanomedicine is a rapidly developing field with new uses for nanoparticles emerging in multiple disciplines. Both nanotubes and fullerenes (**Figure 1**) are subsets of nanoparticles that have been particularly well studied due to their diverse applications in medicine. These small molecules are useful in diagnostics such as magnetic resonance imaging and x-ray imaging [1-3], and are being investigated for use in improving drug delivery and as anti-inflammatories and radiation countermeasures[1]. Fullerenes, in particular, are exceptional antioxidants and have been proposed as therapeutics for the treatment of a wide range of neurodegenerative and inflammatory diseases [4-8]. Oxidative stress, created when reactive oxygen species (ROS) outnumber antioxidants, contributes substantially to these diseases. Therefore the antioxidant properties of fullerenes combined with the ability to add cell-type targeting derivatives to the fullerene core gives these molecules a novel therapeutic advantage in the treatment of such diseases [9-13]. Because ROS play a role in both the initiation and progression of inflammatory diseases the ability to quench these highly reactive molecules could lead to better therapeutic alternatives for neurodegenerative and inflammatory diseases that are currently quite difficult to treat.

Fullerenes are hollow spheres measuring roughly one nanometer in diameter. They are composed entirely of carbon atoms and are extremely stable owing to their aromatic structure which incorporates a large number of carbon-carbon double bonds[14].

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A.

B.

C_{60}

C_{70}
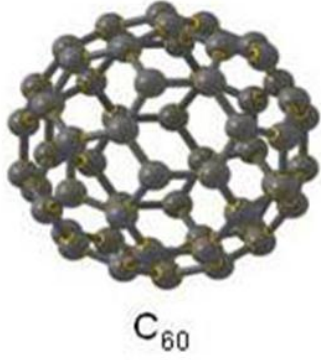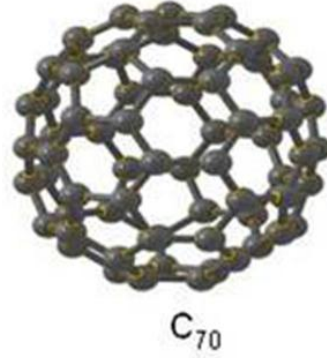
Figure 1. Native fullerene C_{60}, containing 60 carbon atoms (a) and C_{70}, containing 70 carbon atoms (b). The C_{60} fullerene is the most common in nature.

Due to their naturally electron deficient state fullerenes are easily attacked by oxygen free radicals, making them extremely efficient antioxidants [15]. This is how they are thought to derive their anti-inflammatory and neuroprotective functions, preventing the binding of free radicals to lipids, DNA, proteins, and other macromolecules and thus reducing cellular damage commonly induced by these highly reactive molecules [16,17].

Fullerenes were discovered in 1985, when it was found that vaporization of carbon species from the surface of graphite yielded a stable cluster consisting of 60 carbon atoms when analyzed with a mass spectrometer [18]. Fullerenes consisting of 70, 80, or more carbon atoms also exist, but are much less frequent in nature. Initially, fullerenes were of interest for their potential use in chemical processes. More recently there has been a growing interest in their application in nanomedicine due to their small size, stable nature, and natural antioxidant capabilities.

In their native state fullerenes are virtually insoluble in water but solubility can be increased by the addition of polar chemical groups to the carbon cage [19,20]. Thus far, all studies concerning fullerene distribution in the lungs have been performed using native fullerenes, which are hydrophobic and insoluble in aqueous solution. Distribution of fullerenes derivatized to increase solubility in aqueous solution has yet to be explored. Further, as the specific moieties added to the carbon cage can greatly affect tissue distribution, cellular targeting and toxicity, each compound will need to be examined separately. Specific chemical moieties also affect biological function, therefore a specific functional analysis will need to be made for each unique compound. Current knowledge of *in vivo* fullerene trafficking makes it difficult to answer even rudimentary

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questions, thus further studies will need to be performed before these compounds are considered safe for medical purposes.

Previous studies with native fullerenes do give us some insight into fullerene distribution, accumulation, and elimination. When fullerenes are inhaled, their deposition in the lung is dependent on their size, where smaller molecules are deposited lower in the airway. While fullerene derivatives can vary in size, the majority of these compounds are small enough that they should settle in the tracheobronchial and alveolar regions. Here fullerene compounds can access the bloodstream or lymphatics, from which they are distributed to internal organs [21].

Initial research with polyhydroxylated fullerene compounds shows inhibition of mast cell responses including degranulation and inflammatory cytokine production. These non-specific compounds did not affect IgE binding to the mast cell surface, but instead were localized within the cell in vitro and were shown to reduce ROS generation in mast cells treated overnight with fullerene compounds [22]. More recent work identifies the differential effects of fullerene compounds on mast cells are dependent on the specific moieties added to the carbon cage [23]. It is important to identify how the specific moieties added to the fullerene core affect mast cells and other inflammatory cells in addition to characterizing trafficking of these compounds through the body. This will allow the generation of safe and efficient compounds for use in the treatment of human disease.

Mast cells may not be the only cells affected by fullerenes as several inflammatory cell types are activated and reside in the lung throughout the course of allergic disease [24]. Once inhaled, fullerenes likely come into immediate contact with

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epithelial cells lining the airways. In asthmatic patients airway epithelial cells are activated and produce many of the chemokines that contribute to inflammatory cell recruitment, including the eotaxins that recruit eosinophils and thymus- and activation-regulated chemokine (TARC), a potent chemoattractant for Th2 lymphocytes [25,26]. Fullerenes may also come into contact with Th2 cells or eosinophils that are recruited to the airways, inhibiting critical cytokine and chemokine production by these cells, and thus reducing airway inflammation.

II. Asthma and Allergic Disease

Asthma is a chronic inflammatory disease of the lungs characterized by eosinophil infiltration into the airways, increased levels of inflammatory cytokines and chemokines in the lungs, and airway hyperresponsiveness to otherwise innocuous substances [27-30]. Bronchial hyperresponsiveness leads to bronchospasm which reduces airflow into the lungs, causing the patient to become hypoxic and hypercapnic. If airflow restriction is not relieved metabolic alterations and exhaustion will cause respiratory arrest and death. Each year over four thousand individuals die as a direct result of asthma, and it is the one of the leading causes of cardiac arrest in young patients [31,32] Thus, the search for promising new therapeutics is ongoing. Oxidative stress has been shown to contribute to asthma and related lung diseases, hence derivatized fullerenes may represent an effective alternative to current treatments [33,34]. Further, the biological actions of fullerenes have not been fully elucidated as of yet and thus fullerenes may prove efficacious against less treatable subsets of asthma, such as steroid resistant asthma, filling a major void in the treatment of asthma. Patients at the greatest risk of

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cardiac arrest are those who are unable to control disease with current therapeutics, thus these individuals stand to gain enormously from the development of novel therapeutics with unique mechanisms of action. Additional research is necessary to determine the therapeutic value of derivatized fullerenes in the treatment of asthma and other allergic diseases.

III. Allergic Sensitization, Inflammation, and Current treatments

Allergic sensitization begins the first time an allergen enters the body, where it is processed by antigen presenting cells and peptides are presented to allergen specific naïve T cells, driving them towards a T_H2 phenotype. Activated T_H2 cells produce a variety of cytokines which perform a wide range of functions to promote the initiation and progression of disease. Interleukin-4 and IL-13 induce B cell class switching to IgE in humans, while in mice IL-4 exclusively performs this function. Once B cells have been activated and stimulated to class switch they will begin producing large quantities of IgE. This immunoglobulin is released into the circulation and eventually binds its high affinity receptor FcεRI on the surface of mast cells and basophils, sensitizing them to future encounters with this allergen. Production of IL-9 by T_H2 cells, in addition to IL-4 and IL-13, further mediates recruitment of mast cells to the lung and stimulates their proliferation; basophil maturation is induced by IL-3 and IL-4 production. In addition to its role in B cell activation, IL-13 also contributes to goblet cell metaplasia, mucus overproduction, and airway remodeling, processes that combine to increase airway obstruction and hyperresponsiveness to stimuli [30,35].

If exposure to allergen is infrequent, inflammation will resolve and symptoms will subside. However, when allergen exposure is repetitive, chronic inflammation develops. Immune cells including eosinophils, basophils, T cells, and B cells are recruited to the lungs and remain there indefinitely. Mast cell numbers increase in the airways and these cells are increasingly activated, producing cytokines and other inflammatory mediators such as histamine and leukotrienes that maintain inflammation in the lung tissue. Airway inflammation persists chronically and symptoms of disease including bronchoconstriction are exacerbated[36].

Eosinophil infiltration of the airways is one of the defining characteristics of asthmatic disease. The recruitment and stimulation of eosinophils is mediated by IL-5, initially produced by T_H2 cells. Once activated, eosinophils themselves produce IL-5 and other inflammatory cytokines including IL-4 and IL-13, which induce B cell class switching and thus feed back to exacerbate the inflammatory process [37,38]. While the specific role of eosinophils in asthma pathogenesis is largely unknown, they are considered to be important mediators of disease through their production of inflammatory cytokines and their role in airway remodeling [39,40]. The extent of eosinophil infiltration into the airways has been shown to correlate with disease severity[41].

Neutralization of the major cytokines involved in asthma has been attempted therapeutically but has met with little success. Treatment with a soluble human IL-4 receptor appeared efficacious in Phase II trials involving patients with mild to moderate asthma [42,43], but Phase III trials failed to confirm efficacy. Antibodies targeting IL-13 or the IL-13 receptor show promise but are still in clinical trials [44]. The therapeutic use of

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anti-IL-5 antibodies was highly efficacious in rodents and therefore was widely anticipated to be a novel and extremely beneficial treatment for asthma [45]. While anti-IL-5 largely reduced the number of eosinophils found in the bone marrow, blood, and sputum, tissue eosinophils were found to lack IL-5 receptor α and thus persisted despite treatment. Ultimately, neutralization of IL-5 was unable to improve lung function [46-48].

The IgE specific antibody omalizumab, or xolair, is one of the only approved therapeutics that attempts to directly target mast cell activation. Omalizumab is a humanized IgE specific IgG1 antibody that can bind free IgE in the body and thus reduce its binding to FcεRI [49]. In response to reduced IgE binding mast cells and basophils will reduce FcεRI expression. Thus the amount of IgE bound to the mast cell or basophil surface is dramatically decreased, so that upon allergen stimulation mediator release is largely reduced. Over time the reduction of allergen induced mediator release alleviates asthma symptoms as well as the underlying airway inflammation associated with asthmatic disease [50]. However, recent concerns over the prevalence of xolair-induced anaphylaxis in long-term users have prompted an FDA investigation into the safety of this drug. Additionally, xolair can cost over two thousand dollars each month depending on the dose required, making it too costly for many asthma sufferers [51]. Finally, symptoms are not alleviated immediately, and in fact once treatments begin it may take as long as sixteen weeks for optimal improvement of clinical symptoms to be observed [52]. Therefore, while omalizumab supports the theory that mast cell activation is an appropriate target for the treatment of asthma and allergic disease, there are still many challenges to its widespread use.

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Mast cells perform a complex function in asthmatic disease as their immediate mediator release can induce symptoms such as bronchoconstriction and mucus secretion while long-term cytokine production maintains chronic airway inflammation associated with disease [53]. Most current treatments attempt to block mast cell mediators subsequent to their release, only anti-IgE therapy with Xolair or mast cell stabilization with cromolyn-based therapeutics (i.e. Intal) are designed to prevent the release of inflammatory mediators altogether [54,55]. We have shown that the selective addition of derivatives allow fullerene compounds to specifically inhibit either mast cell degranulation or cytokine production, and in some cases both, thus allowing for the targeted inhibition of mast cell diseases. Careful manipulation of these compounds may allow for more precise mast cell targeting and reduced toxicity, allowing us to determine their efficacy in the treatment of human disease [23,56].

IV. Role of Eicosanoids

Eicosanoids are derivatives of arachidonic acid that perform a variety of functions. These lipid signaling molecules are formed from arachidonic acid by one of three distinct metabolic pathways: the cyclooxygenase (COX) pathway, lipoxygenase (LOX) pathway, or cytochrome P-450 monooxygenase pathway [57,58]. Products of the COX pathway include the prostaglandins and thromboxanes, while the LOX pathway produces the cysteinyl leukotrienes, lipoxins, and some midchain hydroxyeicosatetraenoic acids (HETEs). These two pathways are well studied, and both prostaglandins and leukotrienes have been shown to play a role in asthma as they can induce bronchoconstriction. Specific inhibitors have been discovered and used

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therapeutically to counteract these molecules. Non-steroidal anti-inflammatory drugs, or NSAIDs, specifically inhibit the COX pathway and inhibit any downstream effects of the prostaglandins and thromboxanes [57]. The lipoxygenase pathway has multiple points of inhibition as Zileuton blocks the activity of 5-lipoxygenase, thus blocking leukotriene formation, while montelukast blocks the receptor for the cysteinyl leukotrienes, $CysLT_1$[59]. Comparatively, products of the cytochrome P-450 pathway, which include the HETEs and cis-epoxyeicosatrienoic acids (EETs) (**Figure 2**), are not as well studied. The HETEs and EETs function in several organ systems and both pro- and anti-inflammatory activities have been described[58]. Inhibitors of the P-450 pathway do exist, but are not well characterized in vivo. They include compounds that inhibit the formation of EETs, such as 6-(2-Propargyloxyphenyl) hexanoic acid (PPOH) and structural analogs such as 14,15-EE-5(Z)E that diminish their function [60]. There are currently no therapeutics that target this pathway.

Eicosanoids including LTC_4, LTD_4, PGF2α, and TXA_2 worsen asthma pathogenesis by inducing bronchoconstriction. However, not all eicosanoids are purely pro-inflammatory. For example, PGE_2 is a bronchodilator and can perform anti-inflammatory functions in the lung [61,62]. Similarly, EETs have been shown to have anti-inflammatory activity in the airways. Specifically, they have been shown to relax histamine pre-contracted guinea pig bronchi[63] and decrease levels of cell adhesion molecules including vascular adhesion molecules-1 (VCAM-1), E-selectin, and intercellular adhesion molecule-1 (ICAM-1) after cytokine upregulation [63-65]. This implies the EETs may play a role in dampening airway inflammation and bronchoconstriction, although this function has never been explored in vivo. Thus, the

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EETs may be previously unrecognized modulators of airway inflammation and bronchoconstriction in vivo.

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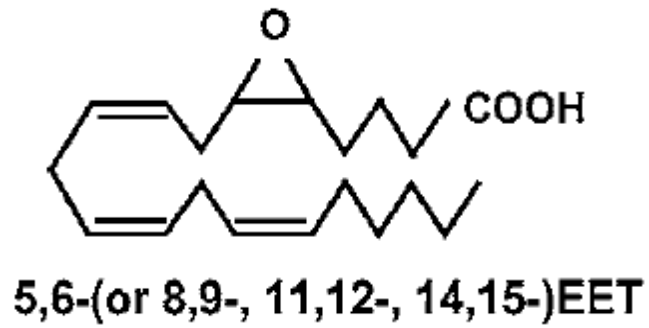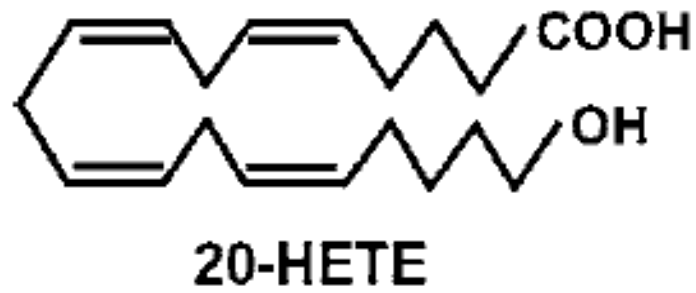
Figure 2. Structure of HETE and EET, products of the cytochrome P-450 metabolic pathway.

V. Dissertation Objective

While native fullerenes are impractical therapeutic options due to their insolubility in aqueous solutions, their derivitization can produce beneficial biological activities. Initial studies showed specifically derivatized C_{70} compounds capable of inhibiting mast cell degranulation and cytokine production. It was our hypothesis that these compounds would inhibit mast cell driven disease by a mast cell dependent mechanism. Thus, the objective of this dissertation was to characterize fullerene action in vivo, primarily observing their action in mast cell driven disease. In order to examine the biological action of derivatized fullerene C_{70} compounds in vivo, models of allergic asthma and anaphylaxis were developed and asthma parameters including airway inflammation and bronchoconstriction were assessed.

As derivatized fullerene compounds are a new advancement, much controversy surrounds their safety and usefulness in the clinical setting. Thus, once the efficacy of these compounds was established, a variety of studies examining their safety and mechanism of action were undertaken both in vivo and in vitro.

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Materials and Methods

I. Mice and Reagents

Chicken egg ovalbumin (OVA), decamethonium bromide, and acetyl-β-methylcholine chloride (methacholine) were purchased from Sigma-Aldrich (St. Louis, MO). Aluminum hydroxide (alum) was purchased from Pierce (Rockford, IL). Ketamine was purchased from Butler Schein (Dublin, OH) and xylazine was purchased from the department of animal resources (DAR) at VCU. Fullerene C_{70}-TGA was synthesized and tested at Luna Innovations Incorporated as described previously[66]. LC/MS grade acetonitrile (A955-4), water (W6-4), iso-propanol (A461-4), formic acid (A117-50) were purchased from Fisher Scientific (Pittsburgh, PA). All deuterated standards were obtained from Cayman Chemicals (Ann Arbor, MI). The HPLC is a Shimadzu Prominance 20A series purchased from Shimadzu (Columbia, MD). The mass spectrometer was a 4000 QTRAP from ABSciex (Foster City, CA). C57BL/6 and Balb/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). Female mice aged 8-12 weeks were used in all experiments. All mouse protocols were approved by the VCU Institutional Care and Use Committee.

II. Mouse B cell purification and culture

Murine B cells were purified from spleens by negative selection. Single cell suspensions were obtained by pressing spleens between frosted glass slides. T Cells were depleted by labeling with anti-CD5 (Ly-1), anti-CD8 (Ly-2), and anti-Thy1.1 (TiB99) followed by incubation with guinea pig complement (Harlan, Indianapolis, IN) and Mar 18.5 (mouse anti-rat kappa) added to enhance complement activation. Ly-1 and Ly-2

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producing hybridomas were originally obtained from William Paul (NIH, Bethesda, MD). Remaining spleen cells were layered over a discontinuous percoll gradient using 50, 60, 66, and 70% percoll (GE Healthcare) to separate B cells by density. Resting B cells were recovered from the 66/70% interface. The resting population was 80-90% B220+ as determined by flow cytometry. B cells were cultured in complete culture medium. To examine IgE production, resting B cells were cultured in 96 well plates (Corning Life Sciences, Lowell, MA) at varying concentrations (750 -50,000 cells/well) in 200 ul cRPMI containing 10,000 U/ml recombinant mouse IL-4 (kindly provided by William Paul), 25 ng/ml mouse CD40LT, and 100ng/ml M15 (anti-leucine zipper); fullerenes were added at varying concentrations (0.1, 1, 10 ug/mL) to treated wells. Both CD40 and M15 were generous gifts from Amgen, Seattle, WA. Fullerenes were obtained from Luna Innovations, Incorporated (Danville, VA). B cells were incubated for 8 days, at which time supernatants were harvested and analyzed by ELISA.

III. Human B cell purification

Human tonsils were obtained from routine tonsillectomies at Henrico Doctors Hospital (Richmond, VA) or the VCU TDAAC per approved IRB protocol. Tonsils were placed in RPMI-1640 and mechanically disrupted using a Seward Stomacher 80 Biomaster Lab Blender (Brinkmann, Westbury, NY) at normal speed for 60 seconds. To obtain a single cell suspension, the resulting product was underlayed with Ficoll–Hypaque (Amersham Pharmacia Biotech Inc., Piscataway, NJ) (30 ml diluted cells on 13 ml Ficoll–Hypaque solution). The tubes were centrifuged for 20 min at 400 × g. The cells were removed under the laminar flow hood, transferred to new tubes and washed in sterile PBS.

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Assessment for total cell yield was done using Gentian Violet staining and viability using Trypan blue exclusion. To isolate B cells, the tonsilar preparation was incubated with an anti-human IgD conjugated with fluorescein isothiocyanate (FITC) (BD Pharmingen San Diego, CA) at a 1:1 ratio cells to antibody ($1x10^6$ cells/1µl Ab), and 1:9 ratio cells to buffer ($1x10^6$ cells/9µl buffer) for 30 minutes on ice in separation buffer (PBS with 0.25% BSA and 0.19% EDTA). Cells were then washed one time in full volume separation buffer to remove unbound antibody and then re-suspended at a 1:9 ratio cells to buffer ($1x10^6$ cells/9µl buffer), and 1:1 ratio ($1x10^6$ cells/1µl Ab) cells to anti-FITC Microbeads (Miltenyi Biotec Auburn, CA) and incubated again on ice for 30 minutes. Post secondary incubation, cells were re-washed in full volume separation buffer and isolated via the Miltenyi MACS cell separation protocol to obtain a >95% pure IgD+ B cell population.

IV. IR800 Study

To assess tissue distribution of fullerene compounds, Luna Innovations Inc. produced a fullerene C_{70} sphere coupled to IR-800 dye (C70-IR800). Naïve mice were given 10 µg C70-IR800 i.n. and sacrificed at 4 hours, 24 hours, 96 hours, and 1 week following i.n. instillation. Lung tissue was collected and the presence of IR-800 dye in whole lung tissue was measured using the Odyssey Infrared Imaging Sytem.

V. Microarray analysis for CYP1B1

Mast cells (1×10^7 cells per condition, each condition performed in triplicate) were incubated with or without fullerene derivatives at a concentration of 10 µg/ml for 16 hours. Cells were then incubated with or without anti-FcεRI antibodies for 10 minutes,

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after which supernatants were removed (to remove preformed mediators) and fresh warm medium containing anti-FcεRI antibodies (1 µg/mL) added for 2 hours. Cells were centrifuged and the supernatant and pellet were immediately frozen. RNA was isolated using the Ambion MessageAmp aRNA kit (Applied Biosystems/Ambion, Austin, TX, USA); all samples passed the internal quality control checks. Microarray was performed using the Human Whole Genome OneArrayTM gene expression profiling service (Phalanx Biotech Group, Palo Alto, CA, USA).

VI. Passive Systemic Anaphylaxis Induction

Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) aged 10-12 weeks were utilized. In order to develop a mast cell dependent model of anaphylaxis, mice were passively sensitized by i.p. injection of 75 µg IgE-dinitrophenol (DNP) diluted in PBS. Two hours later mice were injected i.p. with C_{70}-TGA or Inos (100 ng/200 µL in PBS), or 200 µL PBS alone as a vehicle control. After 16 hours, mice were challenged i.p. with 100 µg DNP-BSA (Sigma-Aldrich, St. Louis, MO) in 100 µL PBS. Body temperature measurements were recorded with a digital rectal thermometer every 10 minutes for a total of 50 minutes. Peripheral blood was harvested by cardiac puncture and mixed with 10 µl EDTA to prevent coagulation 50 minutes after antigen challenge. Plasma histamine measurements determined by ELISA.

VII. Murine Asthma Induction and tissue collection

In the acute inflammatory model, mice were sensitized by injection of 0.5 mg OVA and 4mg alum in PBS on days 0 and 13. Ovalbumin and alum were injected i.p., 200µl per

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injection. On days 19, 22, and 27 Mice were challenged with 1% OVA in PBS aerosolized with an ultrasonic nebulizer for 45 minutes. Mice were sacrificed on day 29, at which time bronchoalveolar lavage (BAL) fluid was collected by flushing the lungs with 1 mL PBS. The BAL fluid was centrifuged and supernatant was saved for cytokine analysis. Pelleted cells were resuspended in 100 µl PBS and spun onto slides, then stained with Hema 3 stain set (Fisher diagnostics, Middletown, VA). Percentages were determined by counting at least 100 leukocyte cells per cytospin. Lung tissue was collected and fixed with 10% buffered formalin phosphate (fisher) and embedded in paraffin at the VCU pathology core. Five µM sections were cut onto microscope slides and stained with hematoxylin and eosin (H&E). A Nikon eclipse with a SPOT Flex Shifting Pixel Color Mosaic (Diagnostic Instrumental Inc., Sterling Heights, MI) camera was used to photograph lung sections.

To induce non-allergic airway inflammation, mice were sensitized by injection of 0.1 mg OVA and 2 mg alum in PBS on days 1 and 8. On days 14 to 20, Mice were challenged each day with 1% OVA in PBS aerosolized with an ultrasonic nebulizer for 45 minutes. Mice were sacrificed on day 21, BAL fluid and lung tissues were collected as above.

To induce MC dependent asthma, mice were sensitized by injection of 10 µg OVA in saline every other day for 13 days. Ovalbumin was injected i.p. in 100 µl saline solution. On days 40, 43, and 46 after the initial sensitization mice were challenged i.n. with 200 µg OVA in 20 µl saline. On day 47 mice were sacrificed, BAL fluid and lung tissues were collected. A modified version of this protocol that induces milder inflammation was

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utilized to assess bronchoconstriction. In this model, mice were sensitized by injection of 50 µg OVA on days 1, 3, 5, and 7 and then challenged on days 22, 25, and 28 with 200 µg OVA in saline. Mice were sacrificed on day 29 and AHR was assessed using the Flexivent system (Scireq, Montreal, QC, Canada). Mice were anesthetized by i.p. injection of approximately 200 mg/kg ketamine and 40 mg/kg xylazine. Once unresponsive a 19 gauge blunt end cannula was inserted into the trachea and ventilation began immediately. Following cannulation mice were paralyzed by i.p. injection of 0.5 mg decamethonium bromide. Mice were ventilated at a frequency of 2 breaths per second and a tidal volume of 2×10^7 m^3 and positive end-expiratory pressure of 3 cmH$_2$0. Lung function was assessed once it was determined that breathing was completely by mechanical ventilation. Responsiveness to methacholine was determined by exposing mice aerosolized PBS and then to increasing doses (10, 25, 50, 100 mg/ml) of methacholine. Each aerosol was delivered for 10 sec and resistance was measured every 6 seconds following aerosol challenge for a total of 3 minutes. The maximum airway resistance in response to each methacholine dose was determined by averaging the three highest values.

VIII. Established asthma induction

To induce established asthma, mice were sensitized by injection of 10 µg OVA in saline every other day for 13 days and challenged i.n. on days 40, 43, and 46 with 200 µg OVA in 20 µl saline. Treatment with 20 µg C$_{70}$-TGA began on day 46 and continued every three days following. Mice were challenged again with 200 µg OVA i.n. on days 66, 69, 72, and 75. On day 76 mice were sacrificed and BAL fluid was collected. Lung

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tissues were fixed in 10% buffered formalin phosphate for 24 hours, then sectioned and stained with H&E.

IX. Quantitative analysis of eicosanoids via HPLC ESI-MS/MS

BAL fluid was clarified by centrifugation at 15,000 g for 10 minutes. To 300 µl of the clarified fluid 300 µl of ethanol is added together with 10 ng each deuterated PGE_2, PGD_2, 6ketoPGF$_1\alpha$, 5HETE, 15HETE, 14,15EET, LTB4, LTC4, LTD4, LTE4, arachidonic acid and eicosapentaenoic acid as internal standards. 10 µl of this mixture was resolved in a 30 minute reversed-phase HPLC method. A Kinetex C18 column (100 x 2.1mm, 2.6µ) was used to separate the eicosanoids at a flow rate of 200 µl/min at 50°C. Prior to sample injection, the column was equilibrated with 100% Solvent A [acetonitrile:water:formic acid (40:60:0.02, v/v/v)]. 100% Solvent A was used for the first minute of elution. Solvent B [acetonitrile:isopropanol (50:50, v/v)] was increased following a linear gradient to 25% Solvent B by 3 minutes, to 45% between 3 and 11 minutes, to 60% between 11 and 13 minutes, to 75% between 13 and 18 minutes, and to 100% between 18 and 20 minutes. The gradient was maintained at 100% Solvent B from 20 to 25 minutes, and was then decreased to 0% by 26 minutes, and held at 0% until 30 minutes. The eluting eicosanoids were analyzed using an inline hybrid linear ion trap triple quadrapole tandem mass spectrometer (ABI 4000 Q-Trap®, Applied Biosystems) equipped with an electrospray ionization source operating in negative ion multiple-reaction monitoring mode. Eicosanoids were monitored using relevant precursor → product MRM pairs. Additional mass spectrometric parameters used are as follows: Curtain Gas: 30; CAD: High; Ion Spray Voltage: -3500V; Temperature:

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500°C; Gas 1: 40; Gas 2: 60; Declustering Potential, Collision Energy, and Cell Exit Potential vary per transition.

X. ELISA and multiplex cytokine assay

Muc5AC protein was measured by ELISA, as described by Takeyama et. al.[67] Bronchoalveolar lavage fluid was diluted to several concentrations with PBS, and 75 µl of each sample was incubated with bicarbonate-carbonate buffer (75µl) at 40° in a 96 well plate (Nunc) for a total volume/well of 150µl until dry. Plates were washed three times with PBS and blocked with 2% BSA (Sigma) for 1 hour at room temperature. Plates were again washed three times with PBS, then incubated with 50µl mouse monoclonal Muc5AC antibody(Pierce) (1:100), diluted with PBS containing 0.05% Tween 20, for one hour. Plates were washed three times with PBS and 100µl horseradish peroxidase-goat anti-mouse IgG conjugate (1:10,000) diluted in blocking solution was added to each well. Following one hour incubation, plates were then washed three times with PBS. Color reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase solution (BD Bioscience) and stopped with 0.18M H_2SO_4. Abosrbance was read at 450 nm. Cytokine levels in the BAL fluid were measured using a multiplex cytokine assay (Biorad, Hercules, CA) according to the manufacturer's instructions.

Total mouse IgE was measured as described previously[68]. Briefly, IgE levels in mouse serum were determined by coating ELISA plates with 10 ug/ml B1E3, a rat anti-mouse IgE mAb. Standard curves were generated with mouse IgE anti-DNP beginning at a concentration of 1000 ng/ml and serially diluted across the plate. IgE levels were

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detected by incubation with R1E4, a rat anti-mouse IgE mAb, followed by streptavidin-AP. Plates were developed using pNPP substrate tablets diluted in substrate buffer and read at a dual wavelength of 405-650 nm.

The liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured as described previously [69]. Serum creatinine levels were measured according to manufacturer's instructions (Arbor Assays, Ann Arbor, MI).

XI. Statistical Analysis

P-values were calculated using unpaired two-tailed Student's t-tests for data following a Gaussian distribution or an unpaired two-tailed Mann-Whitney test for data that did not follow a Gaussian distribution. A one-way analysis of variance (ANOVA) test was used when three or more groups were compared. Error bars represent standard error of the mean between samples. * indicates $p<0.05$ and ** indicates $p<.01$.

Results

I. Fullerene C$_{70}$ Derivatives inhibit mast cell and B cell responses in vitro

While native fullerenes have been studied extensively in vitro, their derivatized counterparts have varying levels of biological activity and toxic potential and thus each compound must be characterized independently. Before progressing to lengthy in vivo studies, fullerene derivatives were examined in vitro for their ability to inhibit the actions of mast cells and B cells, which are of interest for their role in allergic disease.

A. Effect of Fullerene C$_{70}$ derivatives on mast cell degranulation and cytokine production. In order to examine the biological activity of specifically derivatized fullerene compounds, a panel of fullerene derivatives were developed and characterized by Luna Innovations, Incorporated (Danville, VA). Each of these compounds consisted of a fullerene core (C$_{60}$ or C$_{70}$) with differing chemical moieties attached, making them compatible with aqueous systems. These compounds were then analyzed in vitro to assess biological activity by examining their ability to inhibit degranulation (**Figure 3A)** and cytokine production (**Figure 3B)** by human skin mast cells. Beta-hexosaminidase is released when mast cells degranulate, and this mediator can be measured in the supernatants from cultured cells in order to quantitate the extent of mast cell degranulation. Of the fullerene compounds examined, several were able to dampen beta hexosasaminidase release. One of the best inhibitors was the compound C$_{70}$-tetraglycolate, or C$_{70}$-TGA, which inhibited beta-hexosaminidase release by greater then 40%. Additionally, C$_{70}$-TGA pretreatment reduced TNF-α production by approximately 40% and GM-CSF production by 60% (**Figure 3B**). As C$_{70}$-TGA was

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A.

B.
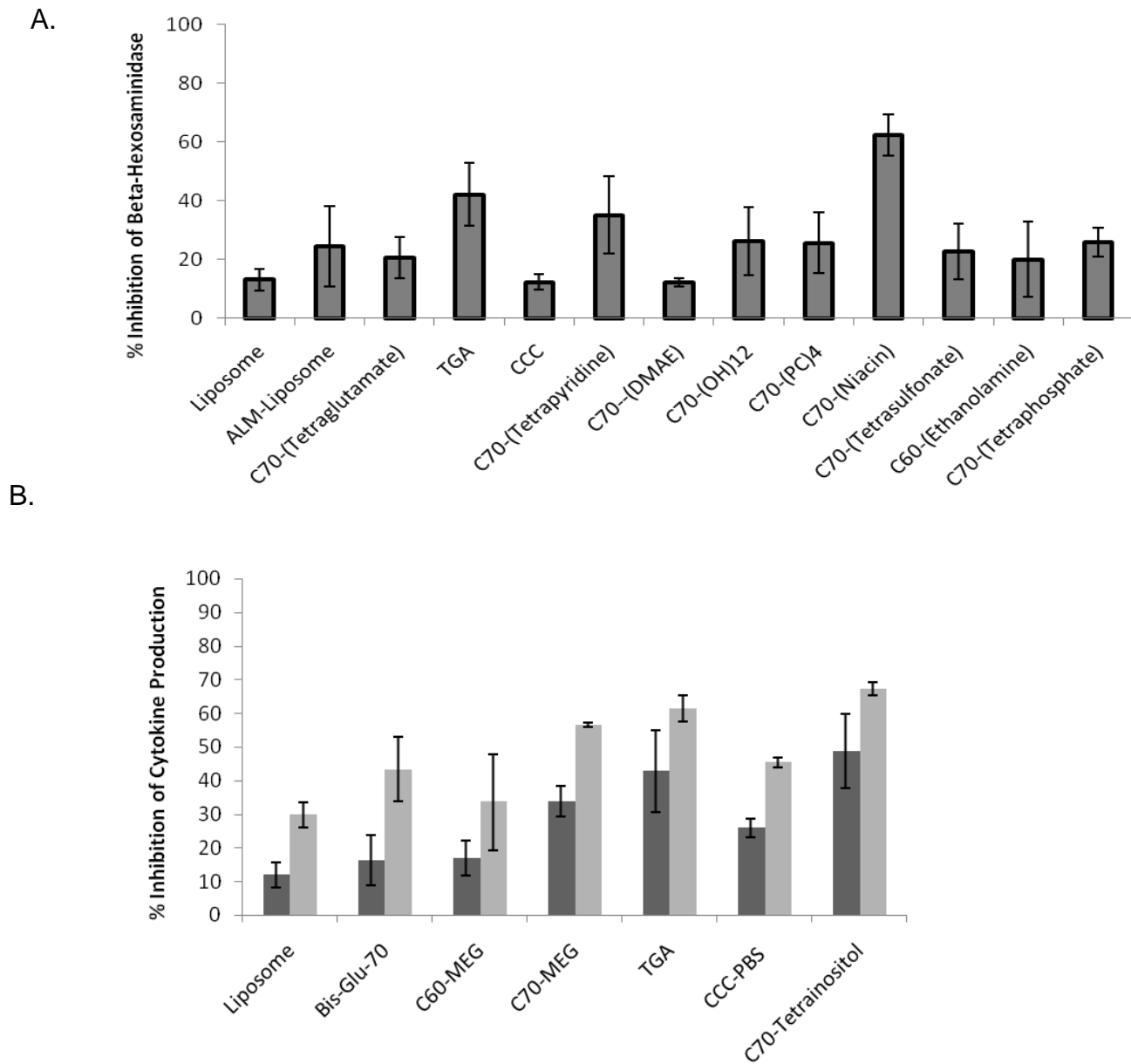
Figure 3. In a panel of fullerene derivatives, C_{70}-TGA is one of the best inhibitors of mast cell function. Human skin mast cells were incubated overnight with or without fullerenes, then stimulated with an anti-FcεRI antibody, 3B4. (A) Supernatants were collected for Beta-hexosaminadase 30 minutes later. (B) Supernatants were collected for cytokine analysis 24 hours after 3B4 stimlation TNF-α (dark gray bars) and GM-CSF (light gray bars).

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best able to inhibit all aspects of the mast cell response in vitro, this compound was chosen for further in vitro and in vivo studies to assess biological activity and its effect on mast cell driven disease. Shown in **Figure 4,** C_{70}-Tetraglycolate was created by the addition of four glycolic acids to the fullerene C_{70} core. This compound was used in the majority of the studies discussed here.

      B. Effect of C_{70}-Tetraglycolate and murine B cells in vitro. Because B cells also play a role in mast cell mediated diseases such as asthma and other allergic diseases, the effect of C_{70}-TGA on B cells was also assessed in vitro prior to attempting in vivo studies (**Figure 5)**. Specifically, B cells function in allergic responses by producing large amounts of allergen specific antibodies, with IgE being most commonly associated with allergic disease. Murine B cells isolated from the spleen were pre-treated with or without C_{70}-TGA, then activated with IL-4 and CD40 ligand. In the presence of as little as 1µg/mL C_{70}-TGA, B cell IgE production was moderately reduced (**Figure 5**). B cells activated in the presence of 10 µg/mL C_{70}-TGA almost completely eliminated production of IgE (**Figure 5**). To be sure that this effect was not merely the result of fullerene toxicity, viability of B cells treated with C_{70}-TGA was assessed (**Figure 6**) using propidium iodide staining.   Fullerene treated B cells were compared to those left untreated and to B cells treated with a non-toxic antioxidant, ascorbic acid. Viability was assessed for the first five days following B cell isolation because after this time viability is largely reduced regardless of treatment. A dose of 1 µg/mL C_{70}-TGA had no effect on B cell viability, as these cells were just as viable as untreated or ascorbic acid treated B cells (**Figure 6A**). However, the higher 10 µg/mL dose of C_{70}-TGA greatly reduced B cell viability starting at day 4, as indicated by the large increase in PI+

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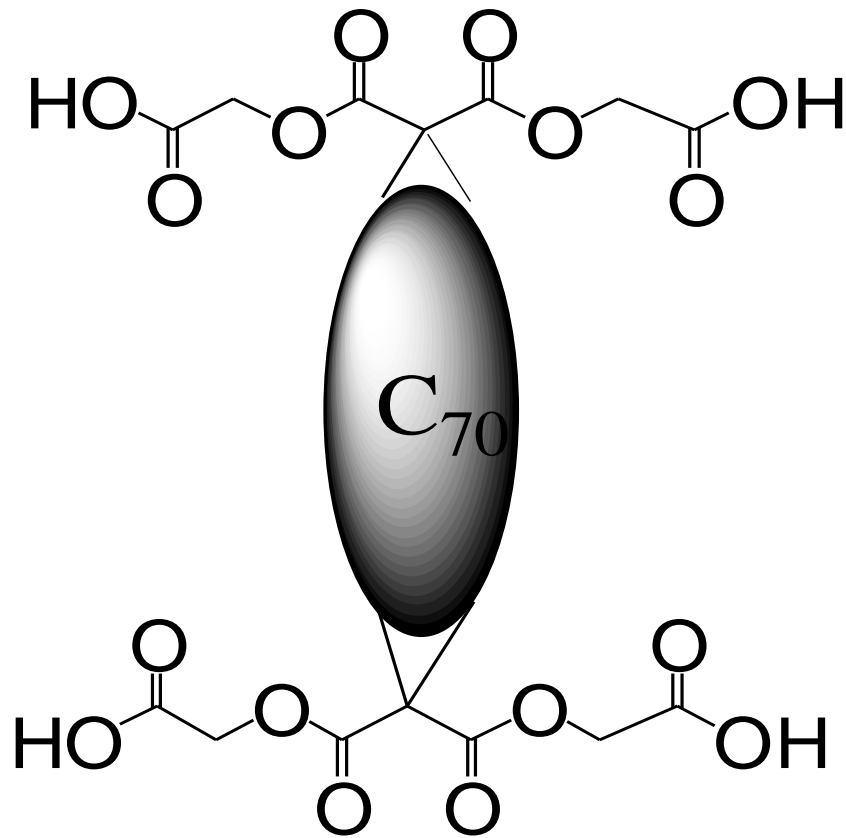
Figure 4. C$_{70}$-Tetraglycolate (C$_{70}$-TGA). Formed by the addition of four glycolic acids to the fullerene C$_{70}$ core, this compound was used in all studies unless otherwise stated.
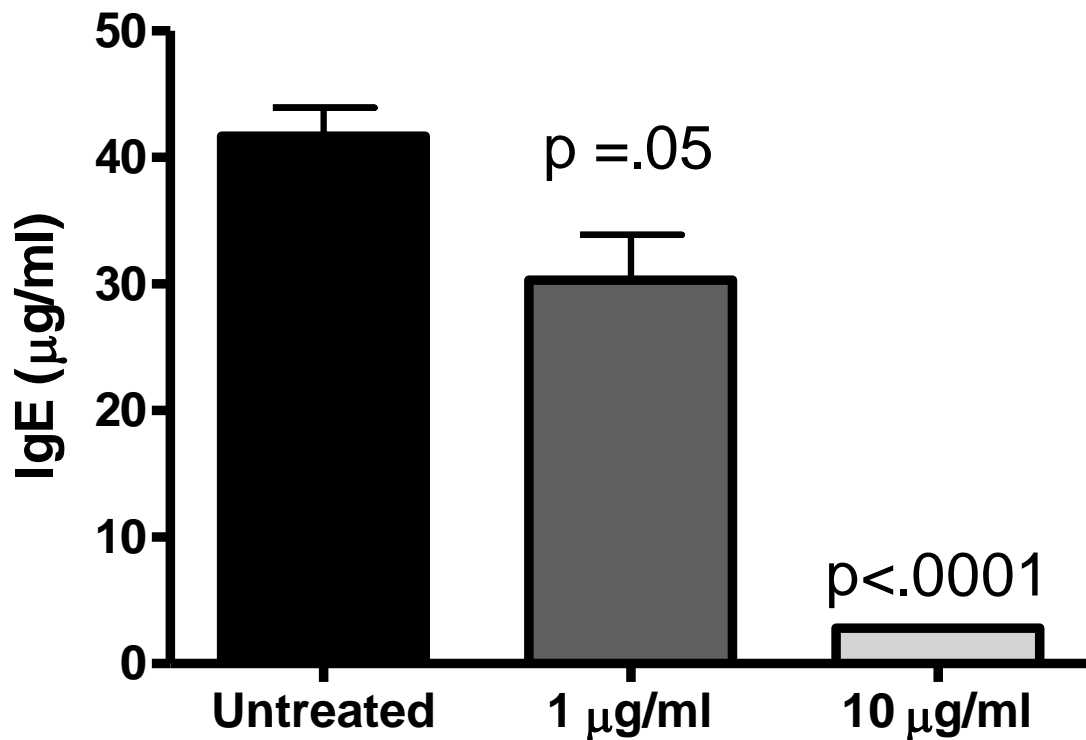
Figure 5. C_{70}-TGA reduces IgE production by B cells in vitro. Murine B cells were isolated from the spleen. B cells were activated with CD40 ligand and IL-4 in the presence or absence of C_{70}-TGA. Cells were incubated for 8 days in order to allow for B cell proliferation and class switching. At the end of this time, supernatants were collected and IgE ELISA was performed. P-values were determined using an unpaired two-tailed student t-test.
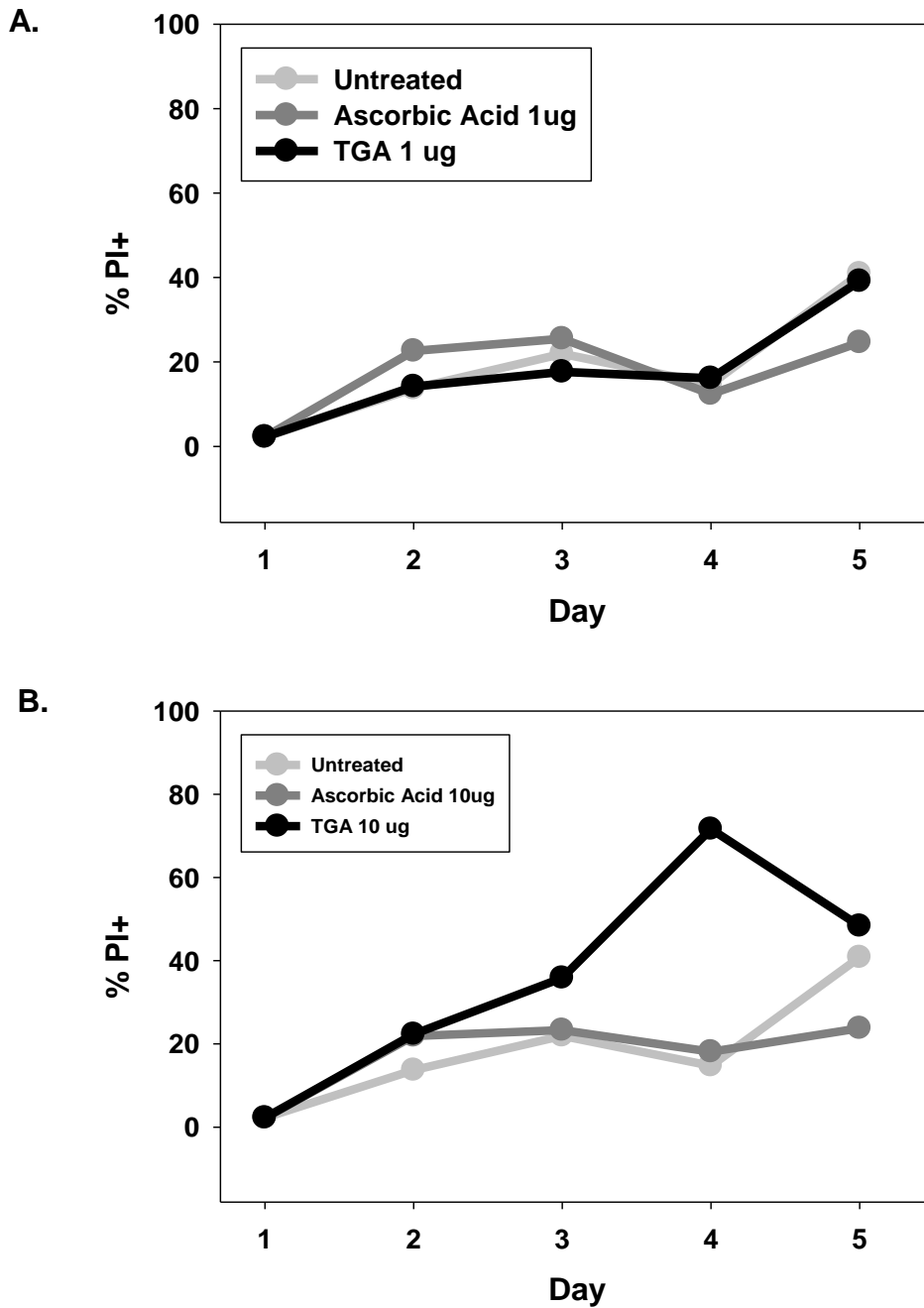
Figure 6. C_{70}-TGA reduces B cell viability at high doses in vitro. Murine B cell viability was assessed every day for 5 days of incubation with C_{70}-TGA. Each day, cultured B cells were washed and stained with propidium iodide. The percentage of PI+ cells was determined using a FACS cytometer.

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stained cells (**Figure 6B**). Therefore, the significant reduction in IgE production seen in Figure 5 following treatment with 10µg C_{70}-TGA was most likely due to B cell death rather than an inhibitory mechanism of the C_{70}-TGA. Still, these results were promising as even a low dose of C_{70}-TGA that is incapable of affecting B cell viability was able to moderately reduce IgE production, an effect that could potentially modulate and even reduce disease pathogenesis in vivo. Results were similar in human B cells isolated from tonsils, where C_{70}-TGA was able to significantly, but not completely, reduce IgE production (**Figure 7**). Data shown are representative as there is much more variability in human samples, but displays the trend that was seen in the majority of human tonsillar B cell samples. Because C_{70}-TGA significantly inhibits mast cell and B cell function in vitro, we decided to examine the effect of C_{70}-TGA on mast cell driven allergic disease utilizing murine models of anaphylaxis and asthma.

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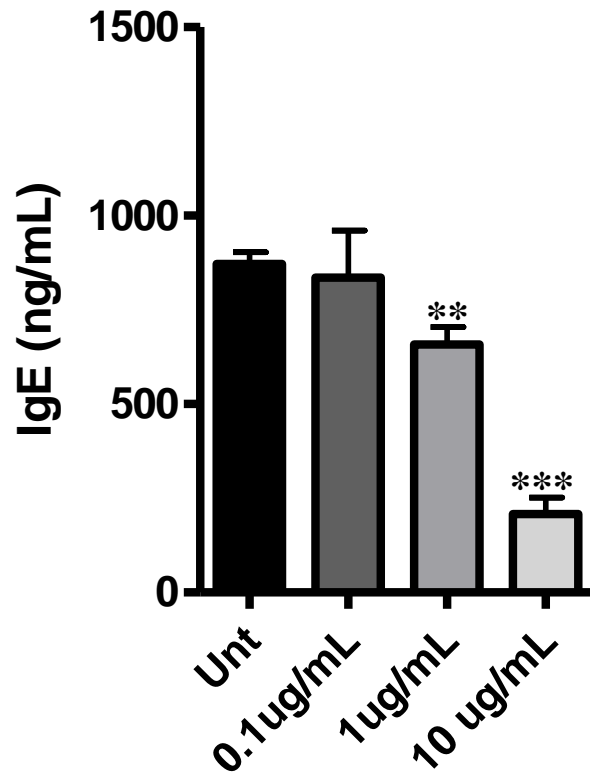
Figure 7. C$_{70}$-TGA reduces IgE production by human tonsillar B cells. Human B cells were isolated from tonsils obtained from routine tonsillectomies. B cells were incubated with 0.1, 1, or 10 µg/mL C$_{70}$-TGA then activated with anti-CD40 and IL-4 for 8 days. Supernatants were collected and IgE levels were measured by ELISA. Data shown are representative as variability between patients is high. ** = $p<.01$ and *** = $p<.001$ using unpaired two tailed student t-test.

II. C_{70}-Tetraglycolate, a specifically derivatized fullerene compound, reduces pathogenesis of allergic asthma and anaphylaxis in vivo

Previous work showed that non-specific fullerene C_{60} compounds can inhibit mast cells in vitro by entering the cell and directly affecting expression of signaling molecules [70]. Specifically, fullerenes were shown to dramatically reduce phosphorylation of Syk, a kinase associated with FcεRI receptor signaling. Further, C_{70}-TGA was shown to inhibit the release of intracellular calcium stores, which is induced upon FcεRI crosslinking[66]. This could explain, at least in part, why degranulation is reduced in the presence of C_{70}-TGA. Because fullerenes, and particularly C_{70}-TGA, seem to have mast cell targeted effects in vitro, we anticipated this compound would have the greatest in vivo effect in heavily mast cell influenced models of disease.

Using a passive systemic anaphylaxis model we were able to directly examine the effect of C_{70}-TGA on the mast cell response in vivo as passive sensitization focuses this response around mast cell function, bypassing other immune cells involved in allergic response. We further characterized the in vivo activity of C_{70}-TGA using various models of asthmatic disease. These models incorporate active sensitization, allowing us to view the overall immune response to C_{70}-TGA.

A. Effect of C_{70}-TGA in a passive systemic anaphylaxis model. A passive systemic anaphylaxis model was used to examine the in vivo effect of C_{70}-TGA on the mast cell response. Anaphylaxis is not generally dependent solely on mast cell function as it is an allergic response to allergen and thus many immune cells are involved. Injection of allergen specific IgE passively sensitizes mice, immediately preparing mast cells for a reaction against allergen. Passive sensitization involved injecting mice with

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DNP-specific IgE followed by i.p. injection with C_{70}-TGA two hours later so as not to interrupt binding of IgE to its receptor, FcεRI. Mice were then challenged with DNP-albumin 16 hours after IgE sensitization, causing a systemic anaphylactic response in which sensitized mast cells released mediators such as histamine and leukotrienes and caused the animal's body temperature to drop. Severity of this response was measured by recording the change in body temperature (**Figure 8**) and quantitating plasma histamine levels (**Figure 9**). By 30 minutes post-challenge the body temperature drop commonly associated with anaphylaxis was drastically reduced in C_{70}-TGA pre-treated animals. Significant improvement in body temperature was seen at 40 and 50 minutes, compared to animals that had not been given C_{70}-TGA. Mice were sacrificed 50 minutes post-challenge and blood was collected for quantitation of plasma histamine levels. Corresponding to body temperature reduction, plasma histamine levels were also significantly reduced in C_{70}-TGA treated animals. This reduction was seen only in animals given C_{70}-TGA, which specifically inhibited mast cell degranulation and cytokine production in vitro (Figure 9, light gray middle bar), and not in animals treated with non-specific fullerene compounds (Figure 9, dark gray right bar).

B.   Effect of C_{70}-TGA in a model of allergic asthma. In order to further examine the inhibitory capabilities of C_{70}-TGA in vivo, its therapeutic efficacy was examined in a murine model of allergic asthma. Because prior experimentation suggested that C_{70}-TGA may exert its inhibitory effects primarily on mast cells, a heavily mast cell-influenced

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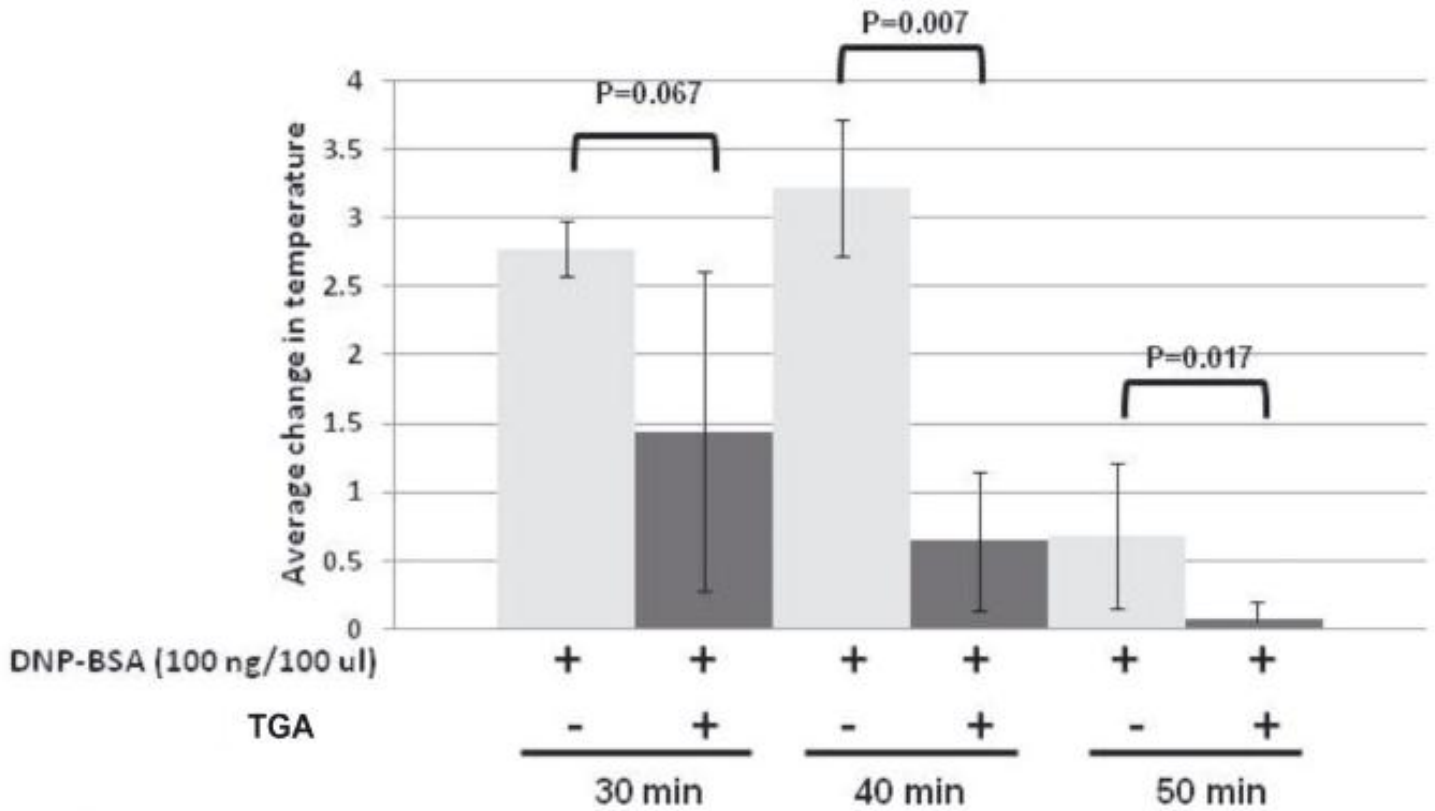
Figure 8. C$_{70}$-TGA prevents the drop in body temperature associated with systemic anaphylaxis. Mice were passively sensitized with 75 µg DNP-IgE i.p., then injected i.p. with 2 µg C$_{70}$-TGA in PBS or 100 µl PBS alone two hours following IgE injection. Mice were challenged with DNP-BSA 16 hours later and body temperature was recorded every 10 minutes. P-values were determined using an unpaired two-tailed student t-test. N= 5 mice per group.
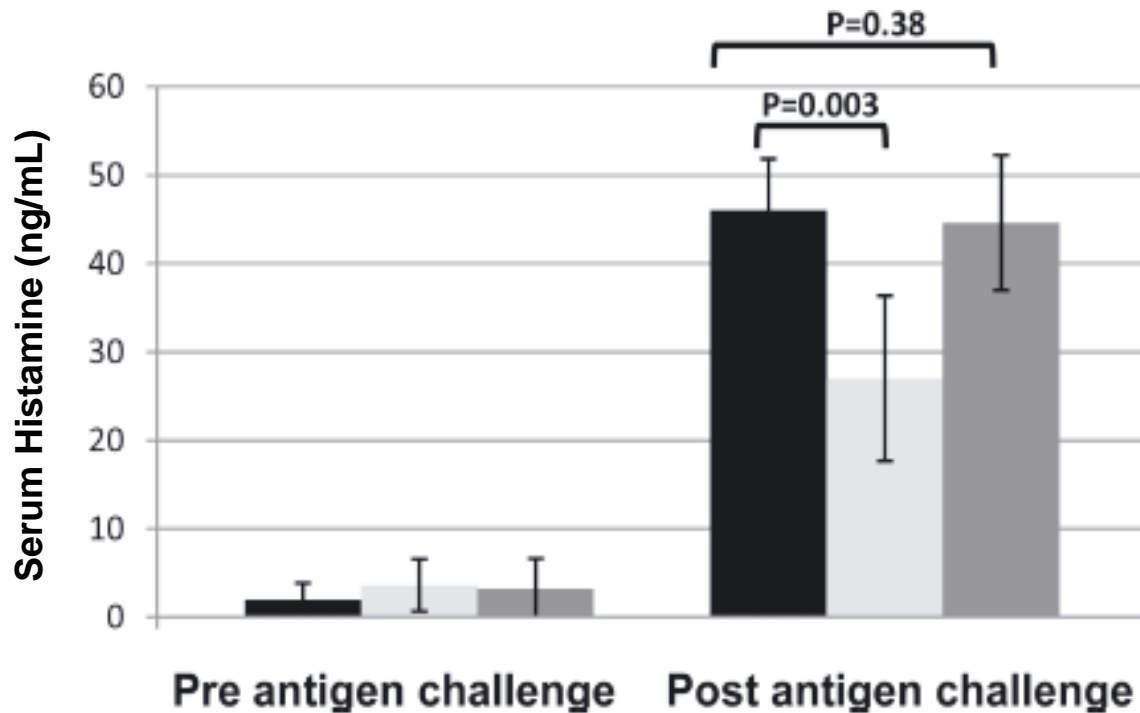
Figure 9. C$_{70}$-TGA significantly reduced serum histamine levels following induction of passive systemic anaphylaxis. Mice were treated as described in Figure 8. Fifty minutes after antigen challenge, mice were sacrificed and blood was collected via cardiac puncture. Plasma histamine levels were quantitated by ELISA, as described in materials and methods. Black bar represents mice treated with vehicle control (PBS), dark gray bar represents mice treated with a non-inhibitory control fullerene, and light gray bar represents mice treated with C$_{70}$-TGA. P-values were determined using an unpaired two-tailed student t-test. N = 5 mice per group.

model was selected. This model (**Figure 10**) was developed and tested by Dr. Stephen Galli's laboratory, and a previous publication characterizing it finds mast cell deficient mice are much less susceptible to disease, exhibiting significantly reduced levels of airway inflammation and bronchoconstriction following antigen challenge[71]. This is due, at least in part, to the elimination of adjuvant from the sensitization process. Treatment with C_{70}-TGA began one day prior to the initial sensitization injection, and continued every three days throughout the course of the experiment. This dosing regimen was decided based on localization studies using a dye conjugated C_{70}-TGA compound. Specifically, C_{70}-TGA was conjugated to an IR-800 dye molecule (IR800-C_{70}-TGA) which can be visualized with the Odyssey infrared imaging system. Using this method, whole organs can be excised from an animal and imaged to observe localization of the dye compound. When C_{70}-TGA localization was observed by this method, the compound had a strong presence in the lung 4 hours after i.n. instillation, but began to dissipate out by 24 hours (**Figure 11**). Ninety-six hours after i.n. instillation, C_{70}-TGA was completely removed from the lung. While we wanted C_{70}-TGA to be present in the lung throughout asthma induction, we also wanted to avoid toxic build-up of the compound that could have other effects on the mice. Thus, we chose to dose the mice every three days, allowing enough time for the compound to be mostly removed from the lung but still present long enough to assess the effect on asthma pathogenesis.

Mice were sensitized and challenged with OVA in saline and on day 47, mice were sacrificed and airway hyperresponsiveness was assessed, and bronchoalveolar lavage (BAL) fluid and lung tissue were collected for further analysis. The BAL fluid was collected by flushing the lungs with 1 mL PBS, allowing us to collect a sampling of cell

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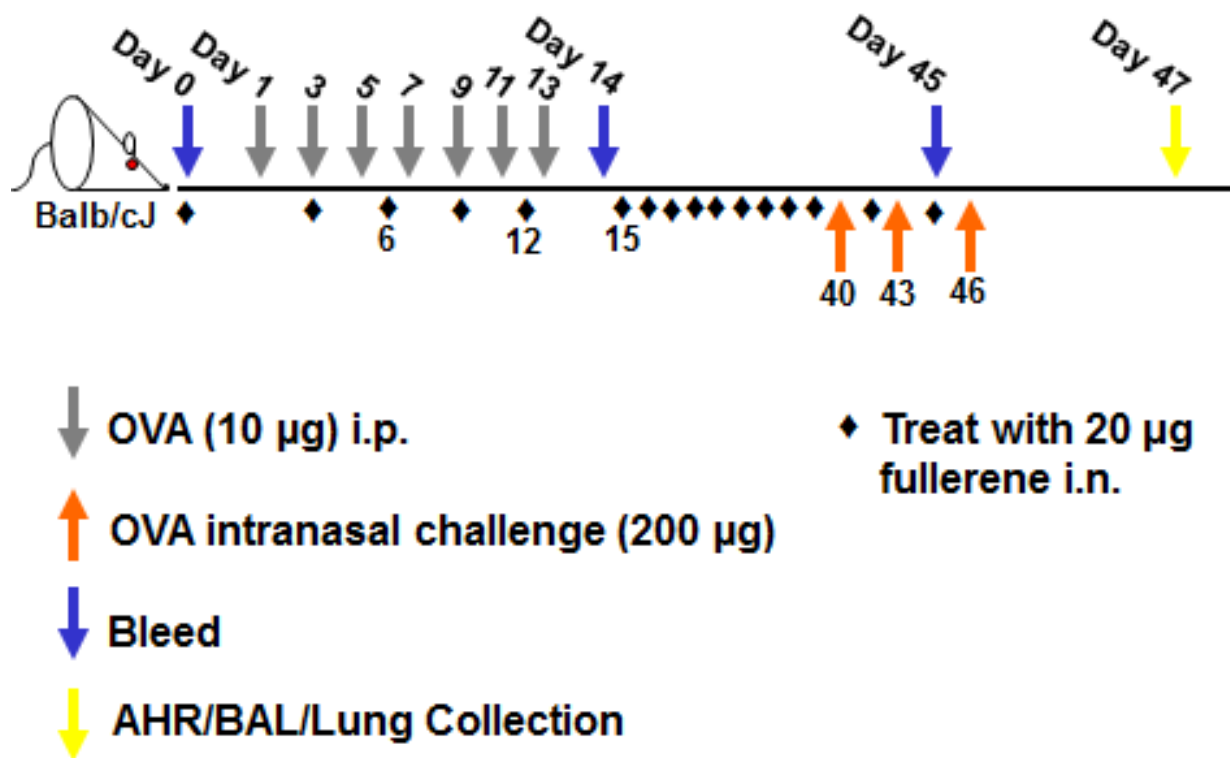
Figure 10. **Mast cell influenced model of allergic asthma**. In this model, mice are sensitized by i.p. injection of 10 μg OVA in saline every other day for two weeks, then challenged with 200 μg OVA in saline at 40, 43, and 46 days after the initial injection. On day 47, mice are sacrificed and airway hyperresponsiveness (AHR) is assessed, bronchoalveolar lavage (BAL) and lung tissue are collected for further analysis. Treatment with C_{70}-TGA began one day prior to the initial sensitization injection and continued every three days throughout the experiment. Each mouse was given 20 μg C_{70}-TGA i.n. on each treatment day. Mice were also bled prior to initiating sensitization, after sensitization, and once again two days prior to sacrifice.

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Lung Tissue

| No C70-IR800 | 4 hours | 24 hours | 96 hours | 1 week |
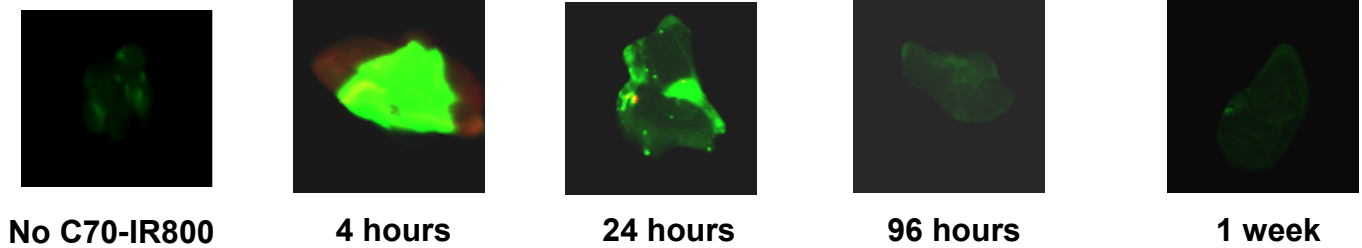
Figure 11. Localization of IR800-C$_{70}$-TGA in lung tissue. IR-800 dye conjugated C$_{70}$-tetraglycolate was delivered to the lung i.n. and allowed to disseminate for various amounts of time. At each time point, a mouse was sacrificed and lungs were excised and staining visualized using the Odyssey infrared imaging system. A control mouse given no C70-IR800 represents background staining.

types and cytokines present in the lung. Total cells in the BAL fluid were counted, spun onto slides and stained with Hema 3 stain, a combination of eosin and methylene blue dyes. This allowed for the differentiation of eosinophils from other cell types as these cells take up the eosin dye and are thus dyed a pinkish-blue color (**Figure 12**). As shown in these representative photographs, eosinophils and other immune cells are relatively abundant in mice given PBS i.n. while these cells are virtually absent in animals that received i.n. C_{70}-TGA treatment.

Immune cells in the BAL were distinguishable based on staining and morphology. Therefore, we were able to count the number of eosinophils in the BAL as a percentage of total immune cells. Doing this, we found the percentage of eosinophils is significantly reduced in C_{70}-TGA treated mice when compared to those given PBS treatment (**Figure 13**). Using this percentage and total cell counts that were done prior to slide preparation (**Figure 14A**), the total number of eosinophils in each BAL sample was extrapolated. Not surprisingly, total eosinophil numbers are also significantly reduced in C_{70}-TGA treated animals (**Figure 14B**). In fact, total eosinophil numbers in C_{70}-TGA treated animals are quite similar to those seen in our non-sensitized controls, suggesting that disease pathogenesis is significantly altered in these animals. Total cellular infiltration of the lung is also significantly reduced, as indicated in Figure 14A by the significant reduction in total BAL cell counts seen in C_{70}-TGA treated mice. Taken together, these data indicate that local administration of C_{70}-TGA significantly dampens lung inflammation associated with allergic asthma in vivo.

To further assess lung inflammation, whole lungs were sectioned and stained with hematoxylin and eosin (H&E stain). This staining reveals cellular infiltration of the

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PBS

TGA
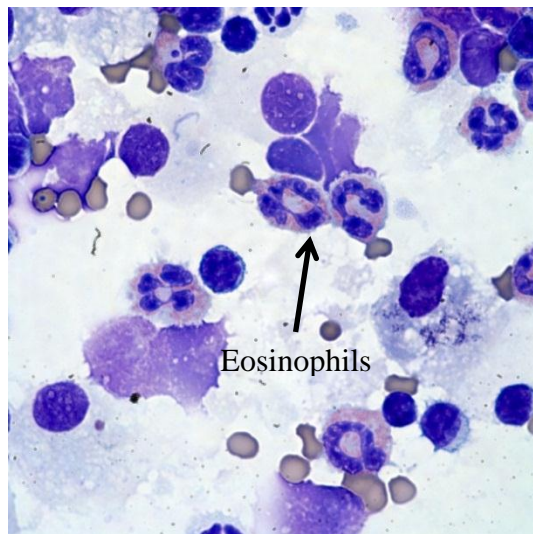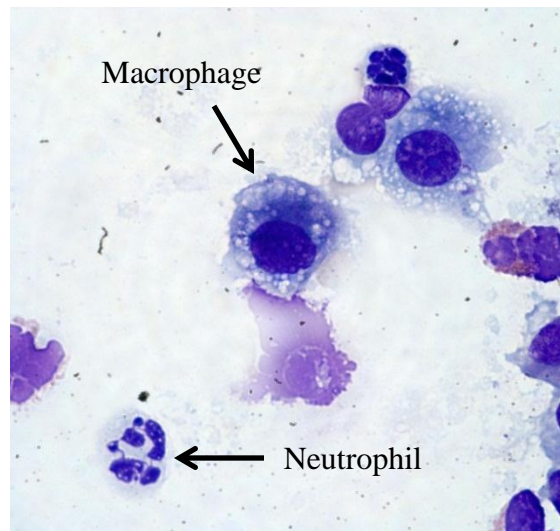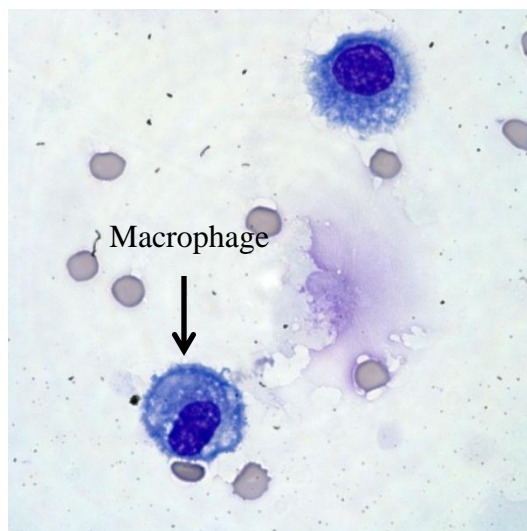
OVA Sensitized

Non-Sensitized Control

Figure 12. Cell types found in the BAL fluid. Bronchoalveolar lavage cells were spun onto slides and stained with Hema 3 stain kit. Immune cells can be distinguished based on staining and morphology. Representative photographs are shown.

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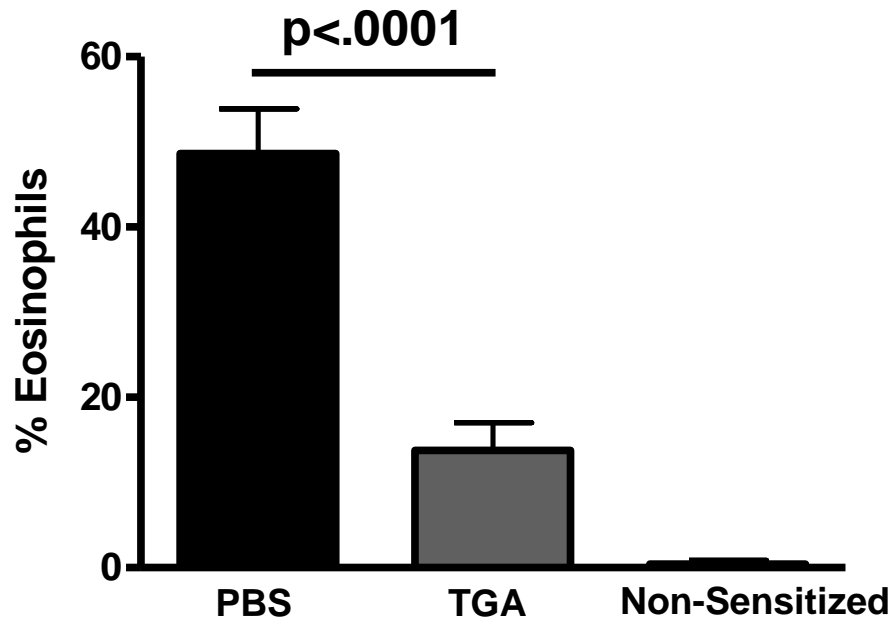
Figure 13. **C$_{70}$-TGA significantly inhibits the percentage of eosinophils observed in the BAL fluid.** Mice were treated using the model shown in figure 10. On day 47, animals were sacrificed and BAL fluid was collected by flushing the lungs with 1 mL PBS. Cells in the BAL were spun onto slides and stained with Hema 3 stain set. Inflammatory cells found in the BAL fluid were counted and eosinophils are represented as a percentage of total inflammatory cells. Results are significant using one-way analysis of variance and student t-test test. N = 6-10 animals per group.
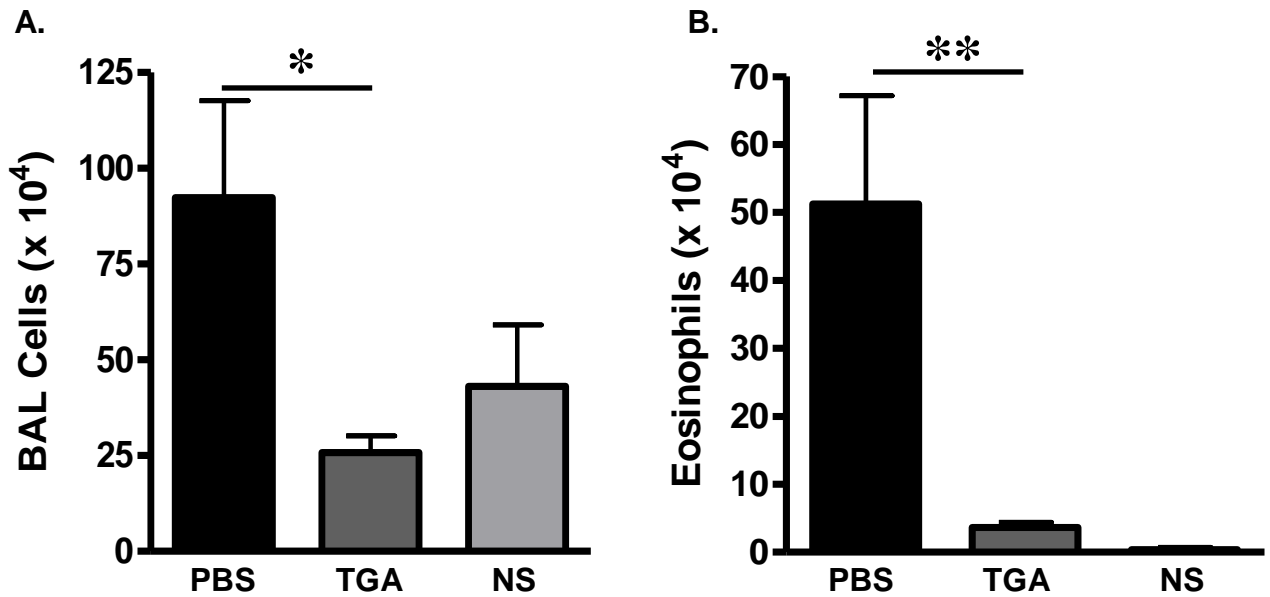
Figure 14. C$_{70}$-TGA significantly inhibits the total number of cells infiltrating the airways. Mice were treated using the model shown in Figure 10. Cells in the BAL fluid were quantitated by trypan blue exclusion. Total eosinophil numbers were extrapolated using percentages observed in Figure 13. Non-sensitized (NS) controls are shown for reference. n = 6-10 animals per group. * = p<.05 or ** = p<0.001 as determined by student t-test and one-way analysis of variance.

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lung tissue and allows us to identify areas of inflammation. In mice treated with PBS, several large areas of inflammation are seen, as indicated by arrows (**Figure 15**). In contrast, only small areas of mild inflammation can be seen in mice treated with C_{70}-TGA. In fact, lungs of C_{70}-TGA treated animals look similar to non-sensitized mice, with minimal inflammation and airway thickening. Moreover, within areas of inflammation PBS treated mice contain more eosinophils then are found in C_{70}-TGA treated animals (**Figure 16**). Together, these data indicate that C_{70}-TGA strongly reduces cellular, and specifically eosinophil, infiltration into the lung following allergic airway challenge.

Because mucus overproduction is a major contributor to death in cases of fatal asthma[72], lung sections were also stained with periodic acid-Schiff (PAS) to assess mucus production in the airways of OVA-challenged mice. This stain reacts with glucose and other sugars, creating a purple-magenta color. It is commonly used to identify mucus production in tissues. Periodic acid-Schiff staining is reduced in C_{70}-TGA treated animals compared to those given PBS alone (**Figure 17**), although no difference was seen in Muc5AC protein in the lung tissue (**Figure 18**).

Currently, therapeutics are available to treat either airway inflammation or bronchoconstriction, but none have been successful at treating both symptoms of disease. Therefore, combination therapies including both corticosteroids (to treat inflammation) and a bronchodilator (to treat bronchoconstriction) are generally given. Developing a therapeutic capable of inhibiting both airway inflammation and bronchoconstriction would be very unique, so we examined whether C_{70}-TGA was capable of such inhibition. To examine bronchoconstriction in this model, the flexivent system was utilized. The flexivent measures airway constriction through an invasive

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PBS   **TGA**
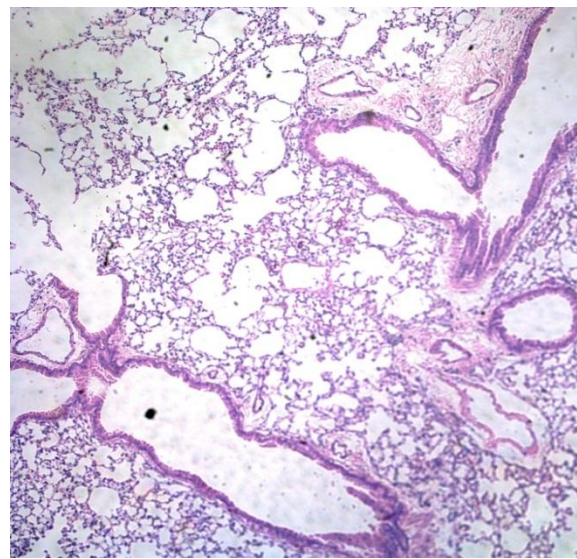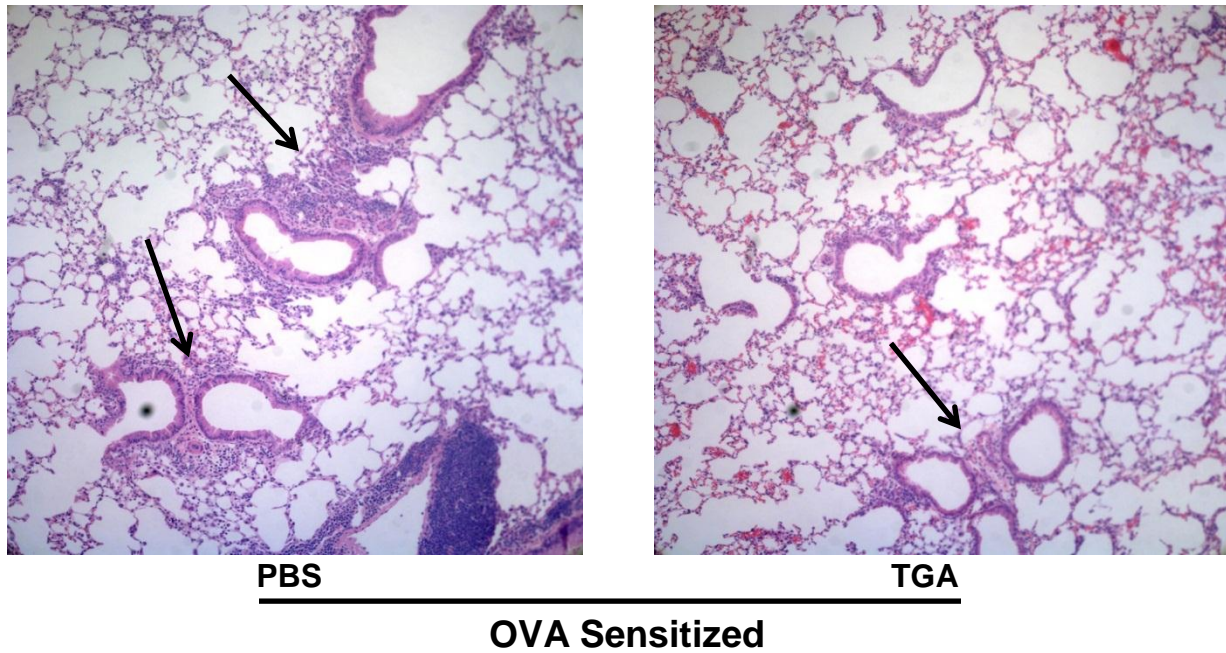
OVA Sensitized

Non-Sensitized Control

Figure 15. Lung sections show cellular infiltration of airways. Lung tissue was fixed in 10% formalin, sectioned, and stained with H&E. Representative photographs are shown, taken at 10X magnification. Arrows indicate areas of inflammation.

PBS   **TGA**

OVA Sensitized

Non-Sensitized Control
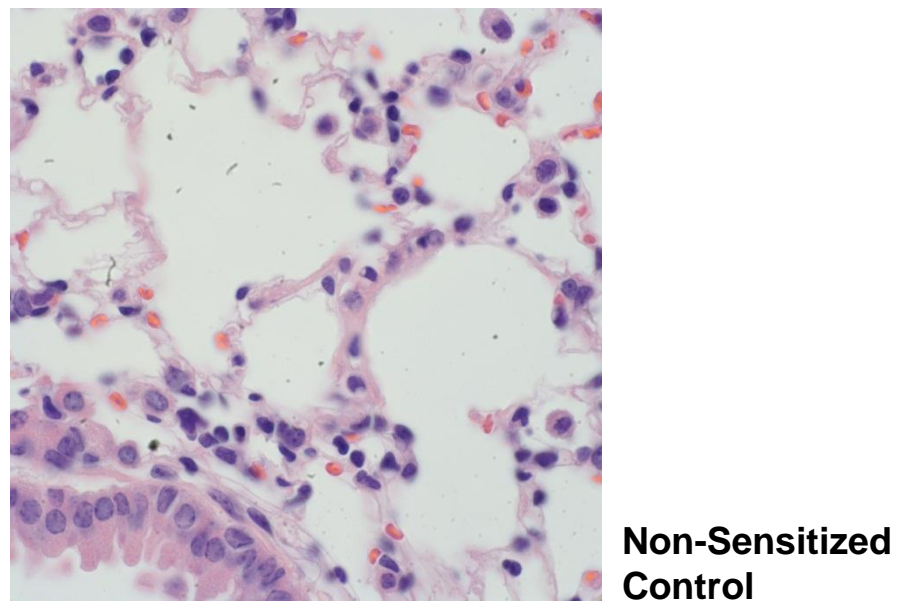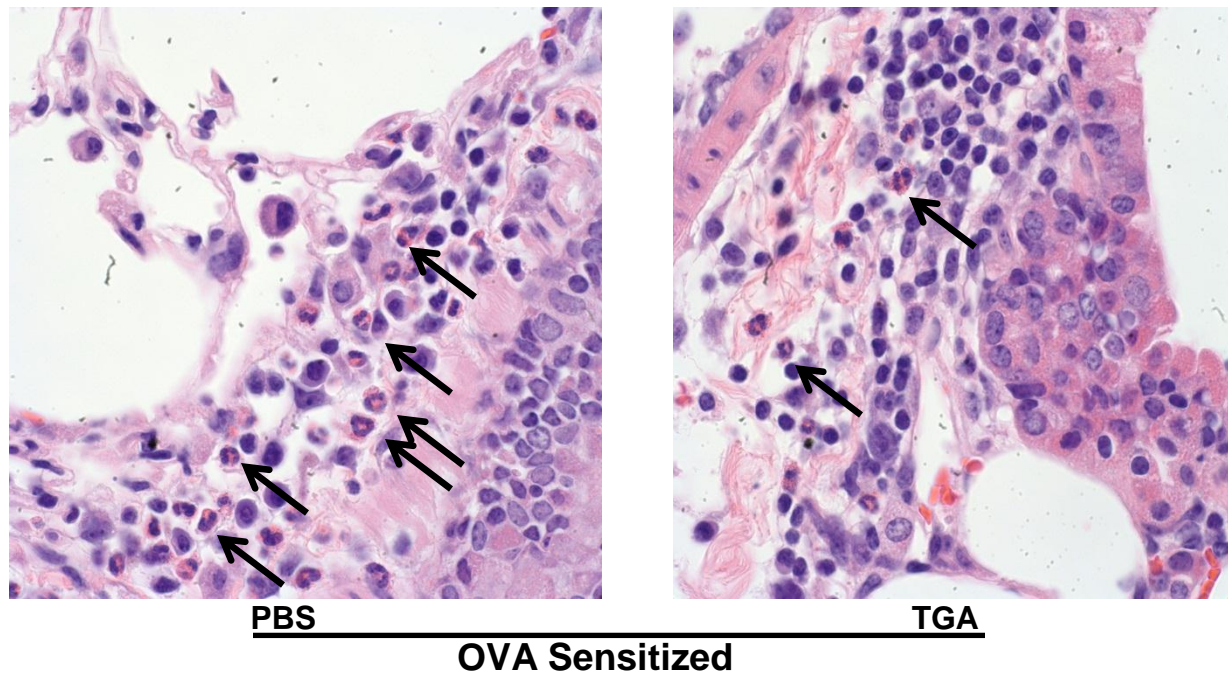
Figure 16. **Lung sections show eosinophils infiltrating airways.** Lung tissue was fixed in 10% formalin, sectioned, and stained with H&E. Representative photographs are shown, taken at 100X magnification. Arrows indicate eosinophils.
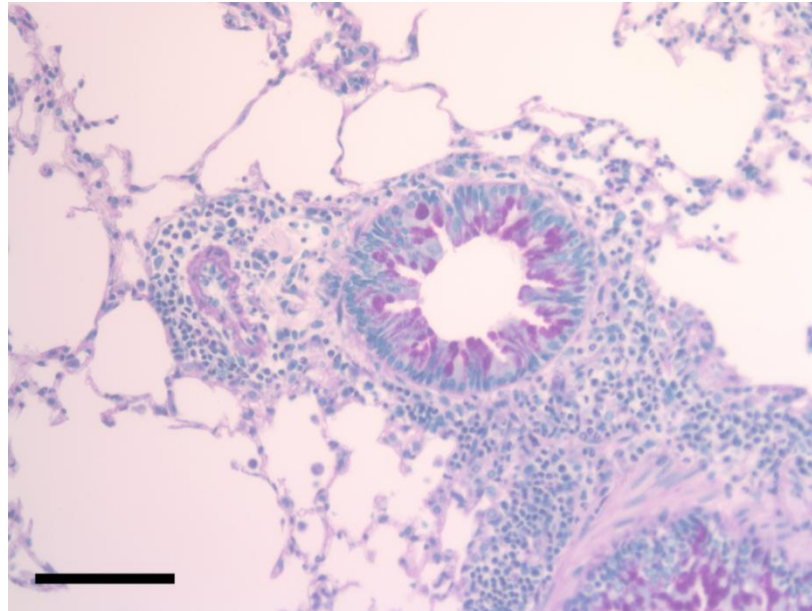
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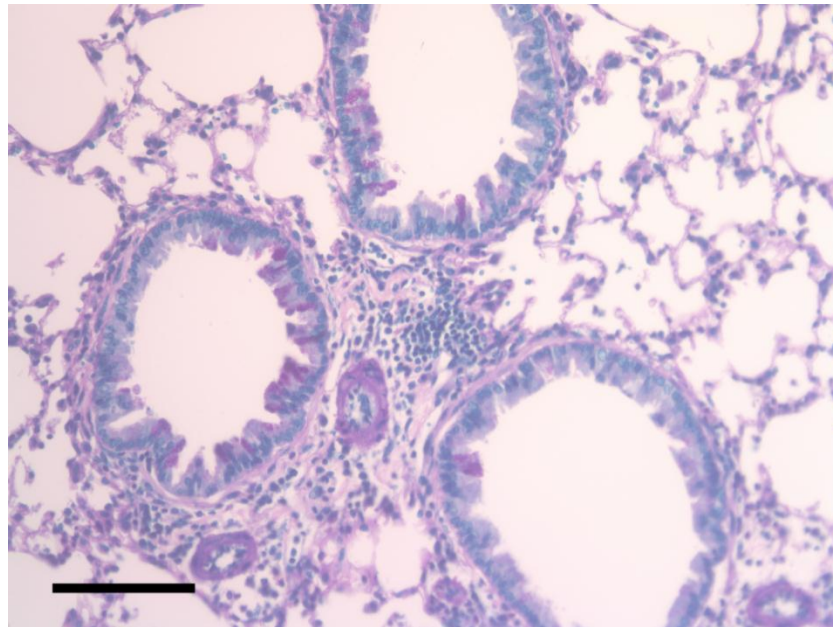
TGA

Figure 17.   PAS staining in C$_{70}$-TGA treated mice shows reduced mucus production. Lung tissue was fixed in 10% formalin, sectioned, and stained with PAS. Representative photographs are shown, taken at 20X magnification.   Bar measures 100 μm.

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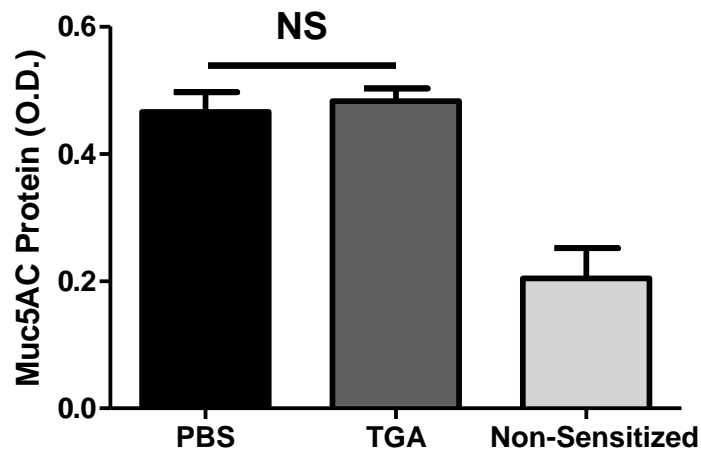
Figure 18. C$_{70}$-TGA does not alter Muc5AC protein levels in bronchoalveolar lavage fluid. Mice were treated as indicated in Figure 10. Bronchoalveolar lavage fluid was collected on day 47 and Muc5AC was measured by ELISA. N= 6 mice per group. Student t-test and analysis of variance were used to test for significance.

method, where tracheotomy is performed and a cannula inserted directly into the trachea in order to measure levels of constriction. Airway response to increasing doses of methacholine was measured, and what we found was rather intriguing. Tetraglycolate was able to significantly inhibit bronchoconstriction in mice (**Figure 19**), suggesting that the mechanism of C_{70}-TGA inhibition is different than other therapeutics currently available. Many of the PBS treated animals died after receiving higher doses of methacholine due to severe airway constriction. In contrast, C_{70}-TGA treated animals seemed to fare much better (**Figure 20**). Several of the C_{70}-TGA treated animals survived to the highest dose of methacholine, just like their non-sensitized counterparts. This suggests that the reduction in airway constriction induced by C_{70}-TGA has functional relevance, and opens up a new set of questions regarding the action of C_{70}-TGA.

In human patients, asthma is diagnosed only after symptoms become evident, and therefore therapeutics are given after disease has been established. While preliminary studies established efficacy of C_{70}-TGA in the treatment of asthma, experiments where C_{70}-TGA was given throughout sensitization and challenge are not the best models for human disease. Therefore a model of established disease was developed, where airway inflammation and bronchoconstriction develop prior to C_{70}-TGA treatment. In this model (**Figure 21**), mice were sensitized and challenged as in the previous model. Because this model had been performed several times, we know the animals have severe airway inflammation and bronchoconstriction following this first set of allergen challenges. Following this initial set of challenges, i.n. C_{70}-TGA treatments were given over a 20 day period. As before, animals were given 20 μg C_{70}-

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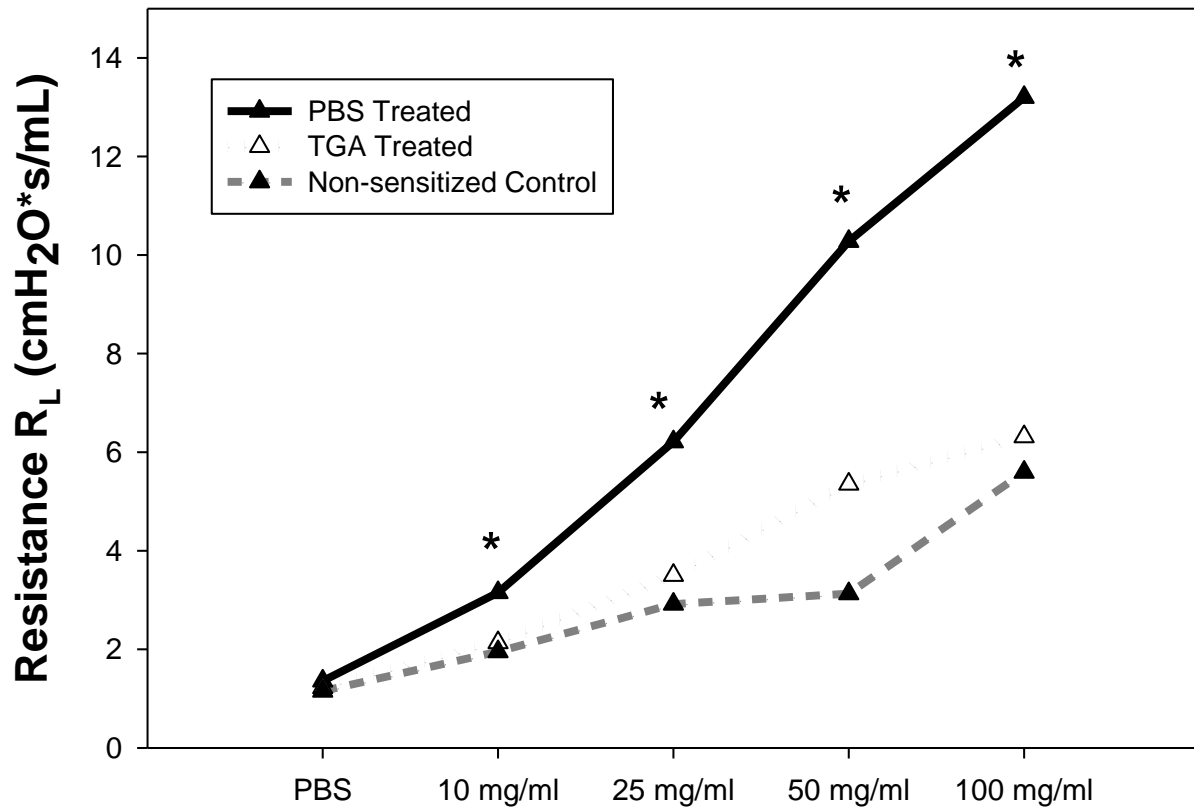
Figure 19. C$_{70}$-TGA treatment significantly reduces bronchoconstriction. Mice were treated as indicated in Figure 10. On day 47, a cannula was placed in the trachea and airway resistance was measured in response to increasing doses of methacholine using the flexivent system. N = 6-10 animals per group. $*$ = p<.05 using unpaired two tailed student t-test.

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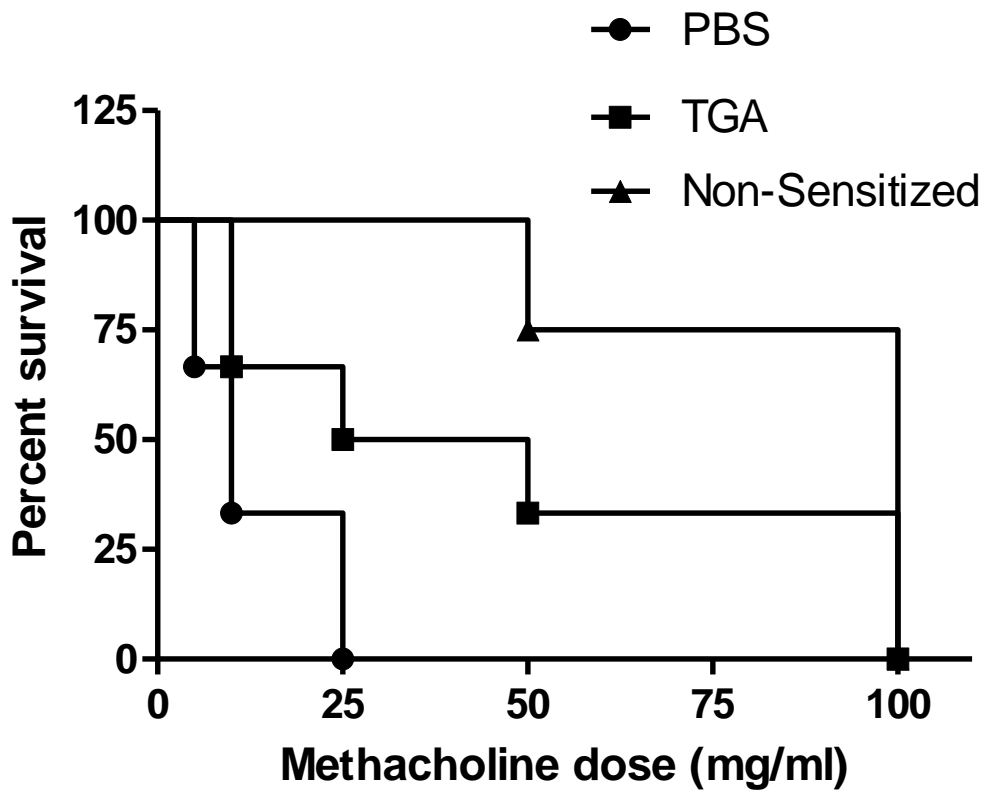
Figure 20. C$_{70}$-TGA rescues mice from methacholine induced death. Mice were treated as indicated in figure 10. On day 47, airway resistance was measured in response to methacholine. When airway constriction was severe enough to cause death, the dose at which death occurred was recorded and survival is plotted. N = 6 mice per group.

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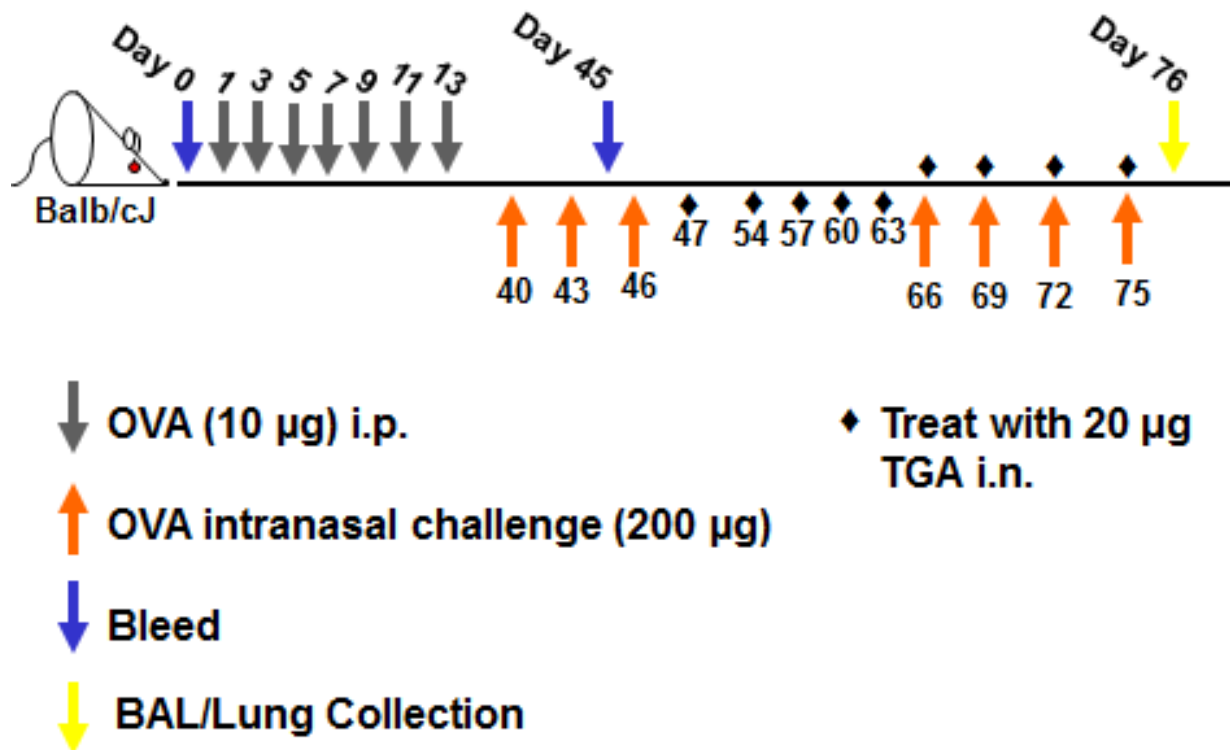
Figure 21. Model of established asthma in mice. Mice are sensitized and challenged as described in Figure 10. C_{70}-TGA treatment begins on day 47, following the initial allergen challenge. Treatments continue for a twenty day period, 20 µg per mouse is given i.n. every three days. A second set of allergen challenges begins on day 66, where 200 µg OVA is given i.n. every three days. Following this second set of challenges, mice are sacrificed on day 76 and airway hyperresponsiveness is assessed, BAL fluid and lung tissues were collected for analysis.

TGA every three days during this time. Another four allergen challenges were given after the 20 day treatment period in order to re-initiate asthma pathogenesis. Mice were sacrificed after this secondary set of allergen challenges, and again airway inflammation and bronchoconstriction were assessed.

Immune cells in the BAL fluid were counted and the percentage of each cell type was recorded (**Figure 22**). Even when airway inflammation was already established in mice, C_{70}-TGA treatment was able to significantly reduce eosinophil infiltration into the airways. Lymphocyte infiltration was increased in C_{70}-TGA treated mice over what was seen in the controls, but the percentage lymphocytes in C_{70}-TGA treated animals was not significantly different from PBS treated animals, and so probably represents variability

between mice. Lung tissues sectioned and stained with H&E showed a vast reduction in cellular infiltration into the lungs of C_{70}-TGA treated animals (**Figure 23**). Thus, C_{70}-TGA is capable of significantly reducing existing allergic airway inflammation in the lung.

While inhibition of airway inflammation is very important, the ability to inhibit both airway inflammation and bronchoconstriction would make C_{70}-TGA a very unique compound. However, assessing bronchoconstriction in the established model of asthma was complicated as the secondary challenge caused severe airway constriction in these mice, sometimes leading to death after minimal methacholine stimulation. In those mice that survived to higher doses of methacholine, C_{70}-TGA treated animals tended to have lower airway resistance compared to those treated with PBS (**Figure 24**). While this data did not reach significance, it suggests that C_{70}-TGA may at least have some effect on bronchoconstriction in mice treated after disease establishment.

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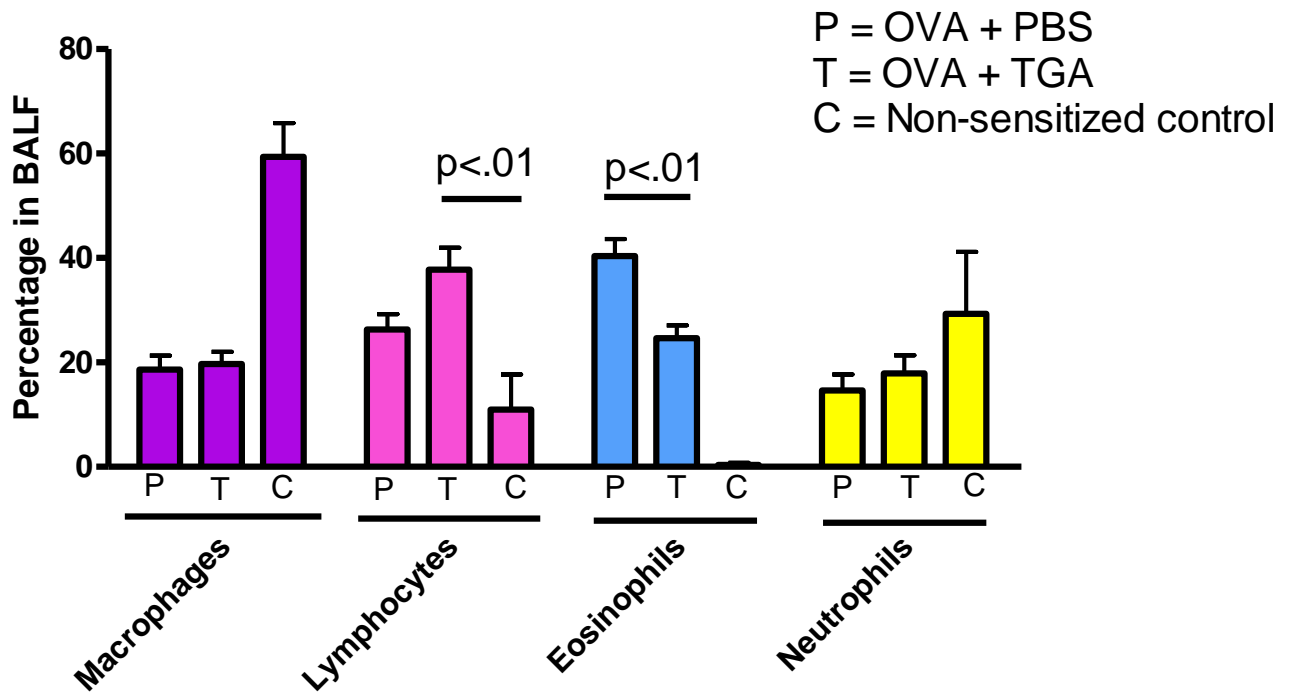
Figure 22. C_{70}-TGA significantly reduces eosinophilia when given after initial OVA challenge. Asthma was induced as shown in Figure 21. Cells in the BAL fluid were stained with Hema 3 stain set. Inflammatory cells in the BAL were counted and macrophages, lymphocytes, eosinophils and neutrophils are represented as a percentage of total inflammatory cells. P-values were determined by unpaired two-tailed student t-test. N = 11-12 animals per OVA sensitized group, 3 non-sensitized controls.

PBS   TGA

OVA Sensitized

Non-sensitized
Control
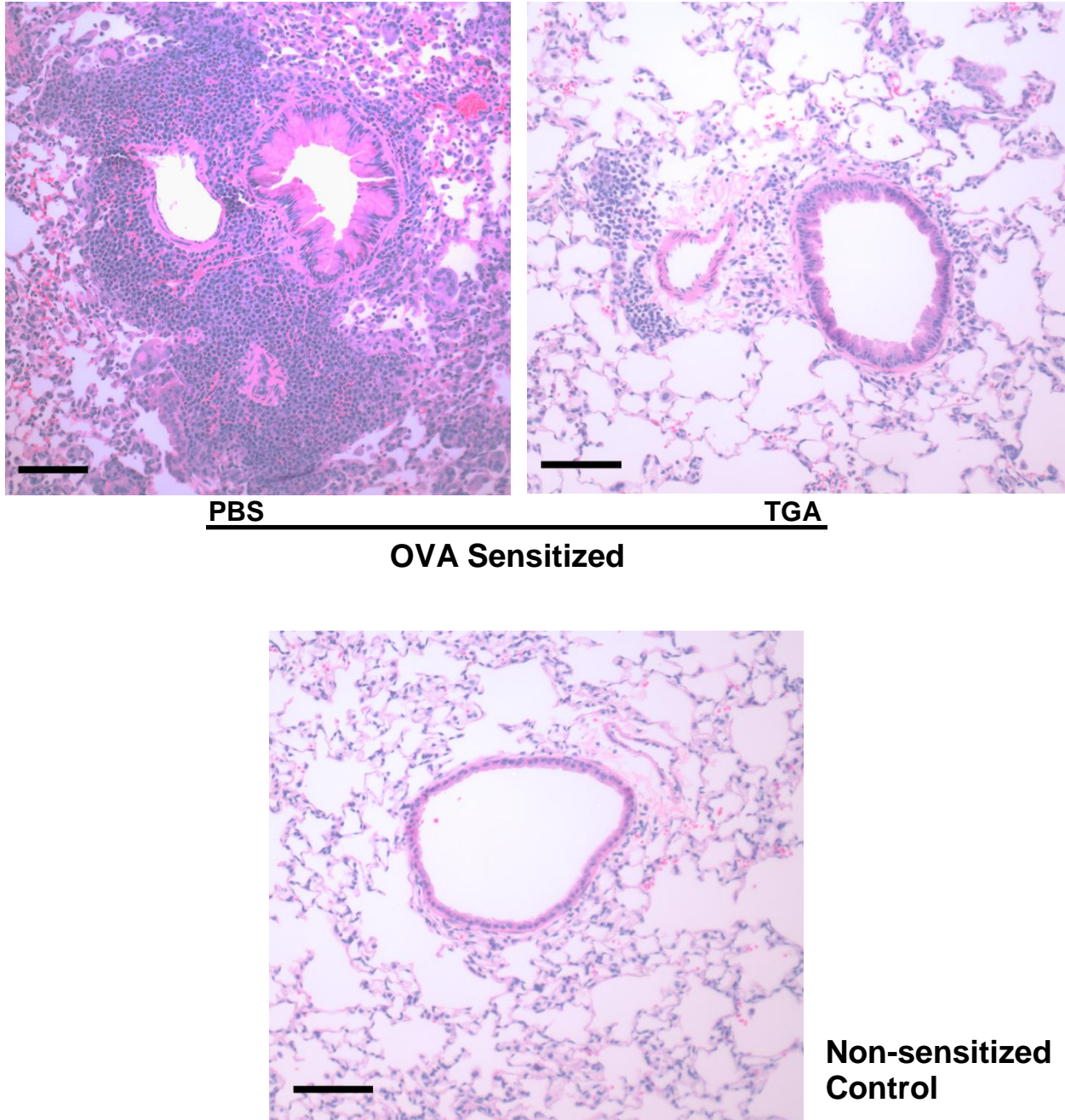
Figure 23. C$_{70}$-TGA severely dampens lung tissue inflammation in an established model of asthma. Lung tissue was fixed in 10% formalin, sectioned, and stained with H&E. Bar measures 100 μm. Representative photographs are shown.
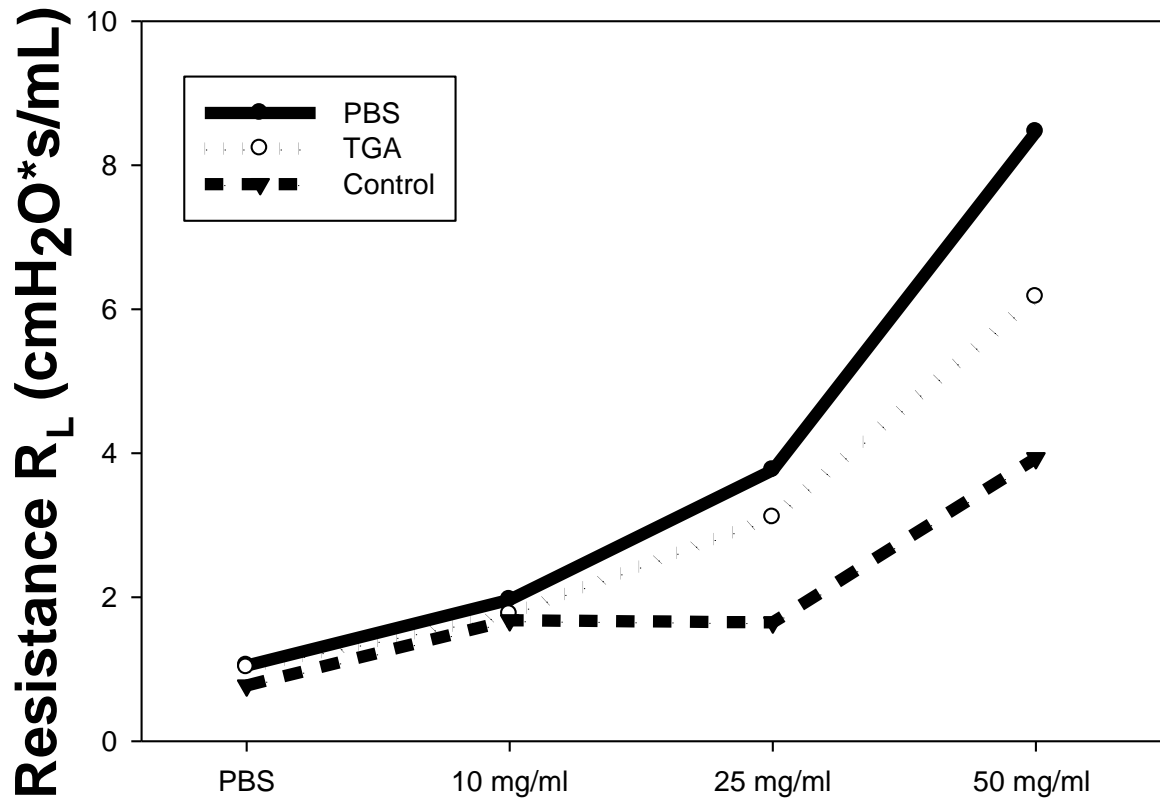
Figure 24. C$_{70}$-TGA dampens bronchoconstriction when given after initial OVA challenge. Asthma was induced as shown in Figure 21. Airway resistance in response to increasing doses of methacholine was measured using the flexivent system. N= 4-6 mice per group.

C. Effect of C$_{70}$-TGA on non-allergic airway inflammation. All experiments described thus far utilized a model in which the development of airway inflammation and bronchoconstriction depended on mast cell function. This is generally the best model for human disease, as mast cells are known to play a role in asthma pathogenesis and are abundant in the airways of asthmatic patients [72,73]. However, not all asthma is allergic in nature, although eosinophil infiltration is the cornerstone of both allergic and non-allergic disease[74]. Non-allergic asthma tends to be more severe and is often corticosteroid refractory. While these patients do have high levels of circulating IgE, it is not allergen specific, and patients have negative skin-prick tests[75].

In order to further characterize C$_{70}$-TGA function, its efficacy was examined in two models of non-allergic disease in which severe inflammation and bronchoconstriction is observed. In these models, mice are sensitized with ovalbumin and the adjuvant aluminum hydroxide, inducing a more severe immune response. The first of these models employs two i.p. OVA sensitizations followed by a week-long break, then three airway challenges with 1% aerosolized ovalbumin (**Figure 25**). Previous research in our laboratory has found this model is IgE dependent, as eosinophilia is not induced in IgE knockout mice[76,77], but mast cell independent, as eosinophilia is induced in mast cell deficient W/Wsh mice[76] (data not shown).

In order to assess C$_{70}$-TGA efficacy in this model of asthmatic disease, mice were given i.n. C$_{70}$-TGA treatments throughout sensitization and challenge. On day 29, mice were sacrificed and eosinophilia was assessed. In C$_{70}$-TGA treated animals, eosinophilia was significantly reduced compared to those given PBS alone (**Figure 26**).

Figure 25. **Asthma model of IgE dependent, mast cell independent disease.** Mice were sensitized by OVA/Alum i.p. injection on days 0 and 14. Aerosol challenge with 1% OVA took place on days 19, 22, and 27. On day 29 mice were sacrificed and BAL fluid was collected. In these experiments, a liposome mixture was used to solubilize C_{70}-TGA, thus a vehicle control, where mice are given liposome alone, is shown. Mice were treated i.n. with C_{70}-TGA or liposome throughout the experiment.

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Figure 26. C$_{70}$-TGA dampens eosinophilia in a model of IgE dependent, mast cell independent asthma. Mice were treated as indicated in Figure 25. Cells in the BAL fluid were stained with Hema 3 stain set. Inflammatory cells in the BAL were counted and eosinophils are represented as a percentage of total inflammatory cells. N = 4 mice per group. ** = p<.01 using unpaired two tailed student t-test.

The vehicle control represents animals treated with liposomal medium because C_{70}-TGA was solubilized in this medium. However, because eosinophilia is slightly reduced in the vehicle controls this medium was replaced with PBS for all other studies, and thus a vehicle control is unnecessary.

In addition to eosinophilia, cytokine levels were also assessed in the BAL fluid to determine the extent of C_{70}-TGA inhibition. Both IL-4 and IL-5 were significantly reduced following C_{70}-TGA treatment (**Figure 27**). This may explain, at least in part, the reduction in eosinophilia in this IgE dependent model.

A separate model of non-allergic lung inflammation that is independent of both mast cell function and IgE was utilized to examine both the efficacy and specificity of C_{70}-TGA (**Figure 28**). Individuals with non-allergic asthma generally have high levels of circulating IgE, but are non-responsive to skin prick tests [78]. It is generally thought that IgE in these individuals is not specific to allergen, although it is possible that an unidentified allergen is involved. This model involves a period of strong stimulation with allergen which prompts the development of non-specific lung inflammation that is independent of typical immune mechanisms. As we have found C_{70}-TGA consistently inhibiting asthma pathogenesis in models that closely represent human disease and seen its ability to dampen mast cell and B cell function in vitro, we hypothesized it would have very little effect in this model of non-specific lung inflammation.

In this model of non-allergic lung inflammation shown in Figure 28, animals were sensitized with OVA in alum similar to the model described in Figure 25. Lung challenge with OVA was much more severe, with seven daily challenges occurring from day 14 to day 20. This caused the development of severe lung inflammation

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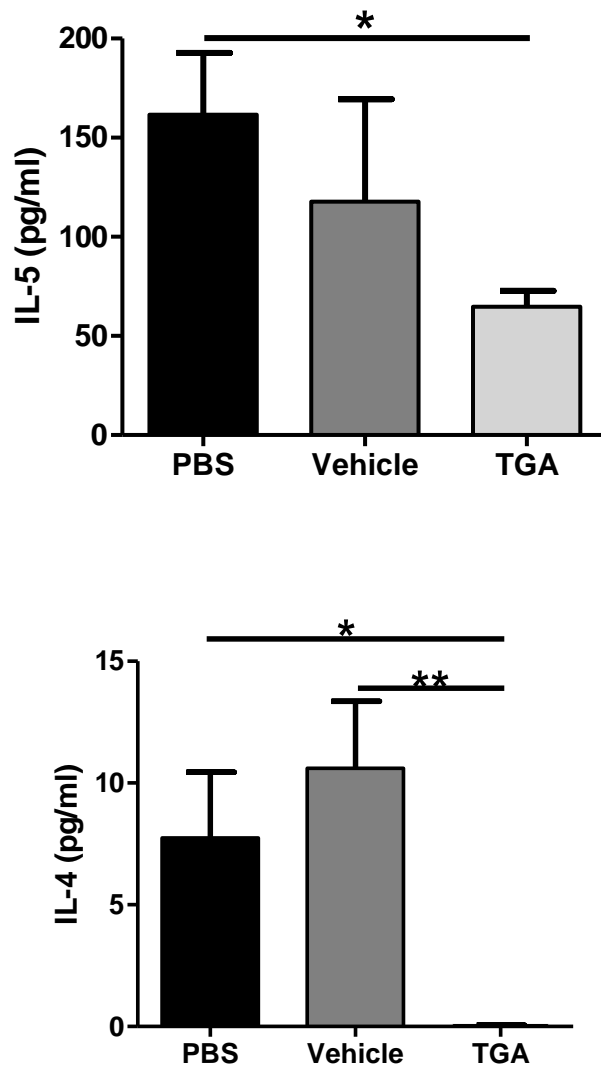
Figure 27. C$_{70}$-TGA reduces cytokine levels in a mast cell independent model of IgE dependent asthma. Mice were treated as shown in Figure 25. On day 29, BAL fluid was collected and cytokines were quantitated using a multiplex cytokine assay. N = 4 mice per group. * = p<.05 and ** = p<.01 using unpaired two tailed student t-test.
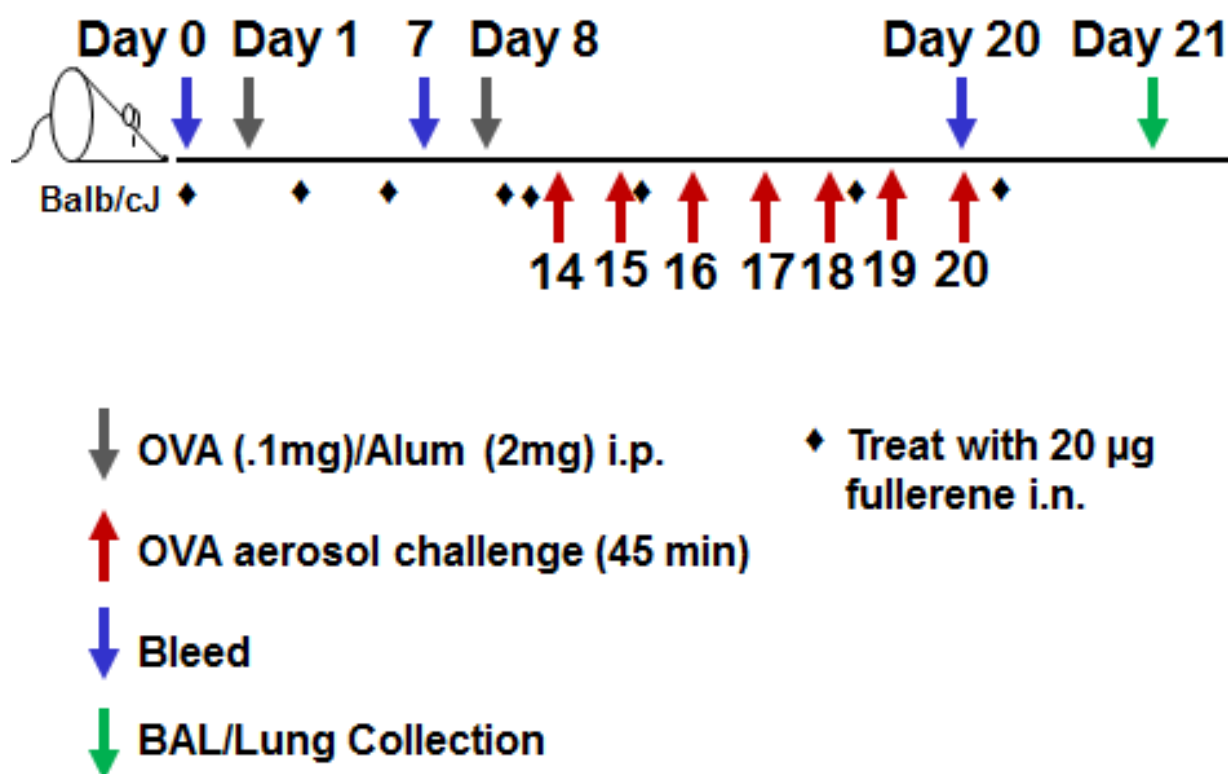
Figure 28. Non-allergic inflammatory model. Mice were sensitized with OVA in alum on days 1 and 8. Each day from day 14 to day 20, mice were challenged with aerosolized 1% OVA. On day 21, mice were sacrificed and BAL fluid and lung tissues were collected.

independent of mast cells and IgE, as both mast cell deficient Wsh mice and IgE knockout mice develop inflammation similar to that seen in wild-type mice[76]. Mice were sacrificed on day 21, and the bronchoalveolar lavage fluid was collected and analyzed for the presence of immune cells. As expected, eosinophils were abundant in both C_{70}-TGA treated and PBS treated animals **(Figure 29)** and no significant difference was seen in cellular infiltration. Cytokine levels in the BAL were also assessed, and again no difference was observed between C_{70}-TGA and PBS treated animals **(Figure 30)**. This data demonstrates the specificity of C_{70}-TGA's inhibitory effects on allergic responses.

Taken together, data presented in this section support the efficacy of C_{70}-TGA for the treatment of asthma and allergic disease. Next we attempt to define the mechanism by which C_{70}-TGA functions in vivo.

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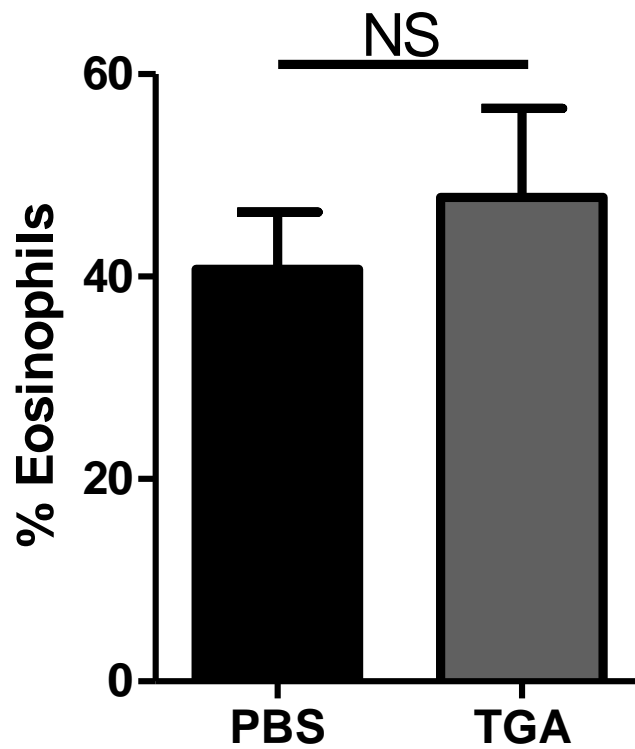
Figure 29. C$_{70}$-TGA has no effect on non-allergic lung inflammation. Mice were treated as indicated in Figure 28. Cells in the BAL fluid were stained with Hema 3 stain set. Inflammatory cells in the BAL were counted and eosinophils are represented as a percentage of total inflammatory cells. N = 4-5 mice per group. Results were not significant (NS) using an unpaired two-tailed student t-test.
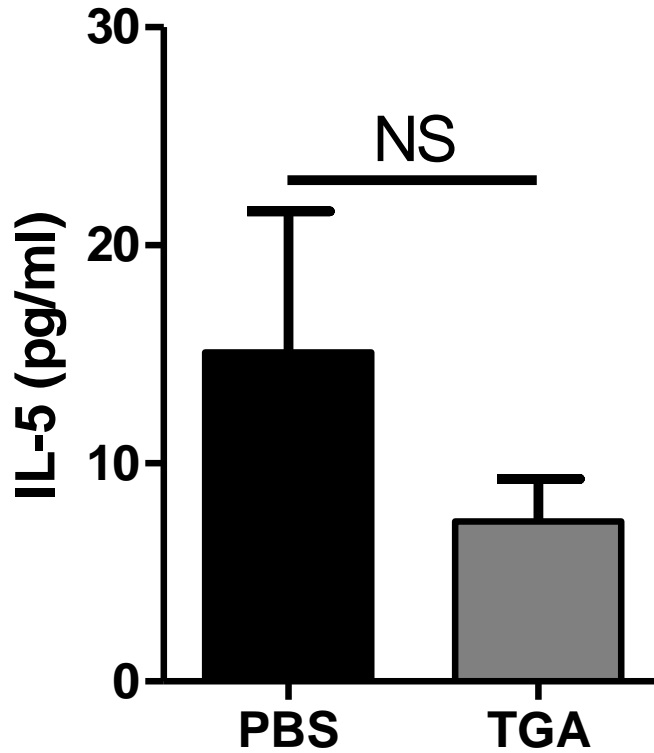
Figure 30. C$_{70}$-TGA does not inhibit BAL cytokines in a model of non-allergic lung inflammation. Mice were treated as shown in Figure 28. On day 21, BAL fluid was collected and cytokines were quantitated using a multiplex cytokine assay. N = 4-5 mice per group. Results are not significant (NS) using an unpaired two-tailed student t-test.

III. Proposed mechanism of C$_{70}$-TGA action

Asthma and allergic diseases are complex and involve the actions of several immune cells. While we have shown that C$_{70}$-TGA is capable of inhibiting mast cell and B cell function in vitro, it could have drastically different effects in vivo. It is important to clarify the mechanism of action of this compound in order to prevent off-target and possibly toxic effects in the body. Knowing the mechanism will also help define the usefulness of this compound for the treatment of asthmatic and other allergic diseases, and could assist in the identification of other disease targeting therapeutics in the future. The mechanism was assessed both by observing in vivo localization and by quantitating cellular mediators of disease in the serum and BAL fluid and relating this back to specific cell type involvement.

To determine in vivo localization of fullerenes, a C$_{70}$ compound was coupled to texas red (C70-TR) so that it could be visualized by immunohistochemistry. Because C$_{70}$-TGA has a profound effect on mast cells in vitro and on mast cell driven disease in vivo, we wanted to determine whether this compound is specifically localized to the mast cell in vivo or whether it has an indirect effect on mast cell function. Naïve mice were given C70-TR intranasally and sacrificed 24 hours later. Lungs were sectioned and stained with Anti-c-kit FITC in order to observe co-localization of mast cells (c-kit+) and the C70-TR compound. Interestingly, in these naïve mice the compound appeared to specifically co-localize with c-kit+ mast cells in the lung (**Figure 31**). While not all mast cells took up the C70-TR compound, it was only found localized within c-kit+ cells, and not in other unstained cells.

64

Anti-c-kit FITC   **Fullerene C$_{70}$ Texas Red**   **Overlay**
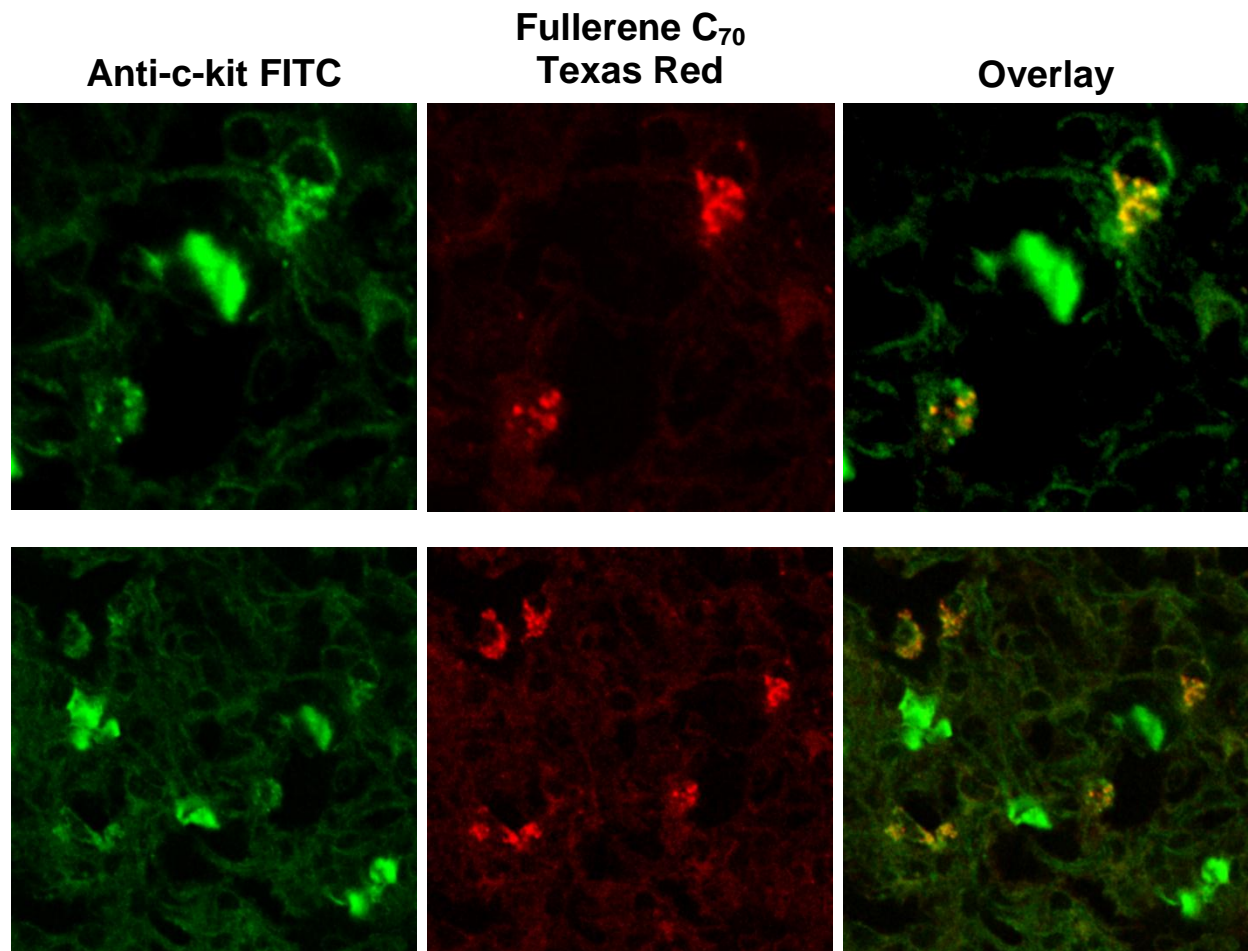
Photos courtesy of Dr. Mohey El-Shikh

Figure 31. Fullerene C70-TR compound is found co-localized with c-kit+ mast cells in naïve mice. Naïve mice were given 20 µg C70-Texas Red i.n. and sacrificed 24 hours later. Lungs were collected, sectioned, and stained with Anti-c-kit FITC. Overlay shows co-localization of C70-TR and c-kit+ mast cells.

This co-localization was quite intriguing, but we wanted to be sure that the C70-TR compound was also co-localized with mast cells in sensitized mice, when mast cells are recruited to the airways in large numbers. Mice were sensitized and challenged as indicated in Figure 10, and animals were treated every three days with 20 μg C70-TR instead of C_{70}-TGA. Following OVA challenge mice were sacrificed and lungs were sectioned and stained with Anti-c-kit FITC. Surprisingly, C70-TR was not taken up by mast cells which lined the airways in large quantities (**Figure 32**). While there are a few mast cells that co-localize with the fullerene compound, the vast majority do not (**Figure 33**). In an attempt to explain this outcome, cells in the BAL fluid were quantitated and percentage eosinophils measured relative to other immune cells. The percentage of eosinophils was not altered by C70-TR (data not shown), suggesting that its mechanism of action differs from that of C_{70}-TGA. Another possible explanation lies in the fact that macrophages weakly express c-kit, and thus the c-kit+ cells we see in figure 31 may actually be macrophages, not mast cells. While C_{70}-TGA may be interacting with mast cells in vivo, these studies could not support such a theory, most likely due to the structural differences between the two compounds.

Immune mediators are produced by many cell types and play an important role in immunological diseases. Cytokines are one form of immune mediator that function to initiate and maintain tissue inflammation. Immune cells secrete many different cytokines that modulate immune responses. Because allergic asthma is generally a Th2 response, we analyzed the BAL fluid for the presence of a panel of Th2 associated cytokines to determine if C_{70}-TGA influences asthma pathogenesis by altering cytokine levels in the lung. We found that after undergoing treatment as shown in the model in

66

Fullerene C$_{70}$ Texas Red   **Anti-c-kit FITC**

Overlay
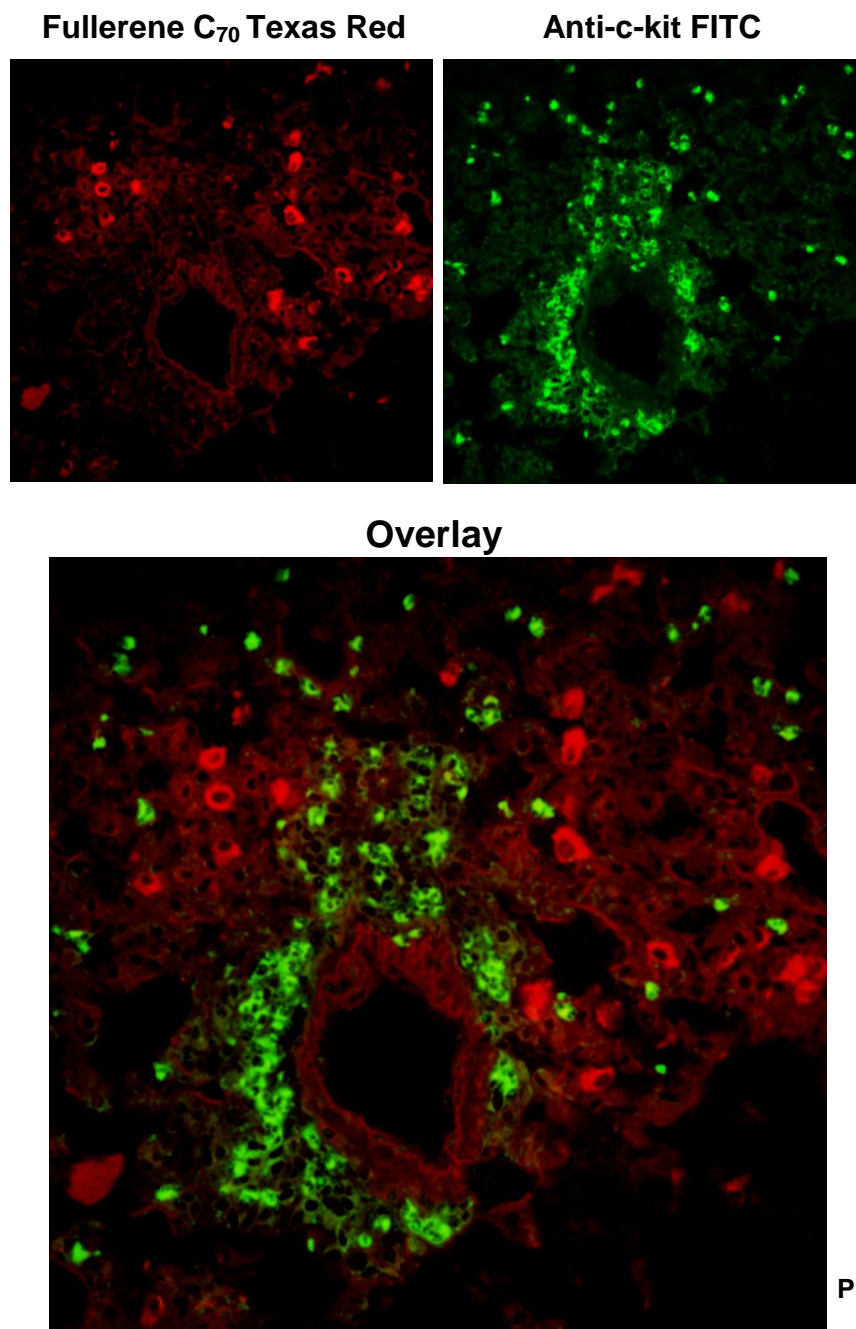
Figure 32. In OVA sensitized mice C70-TR is not taken up by mast cells surrounding the airways. Mice were sensitized and challenged as shown in Figure 7 except C70-TR was given in place of C$_{70}$-TGA. Following OVA challenge mice were sacrificed and lungs were collected, sectioned, and stained with c-kit-FITC. Overlay shows co-localization of C70-TR with c-kit+ mast cells.

67

Fullerene C$_{70}$ Texas Red   **Anti-c-kit FITC**

Overlay
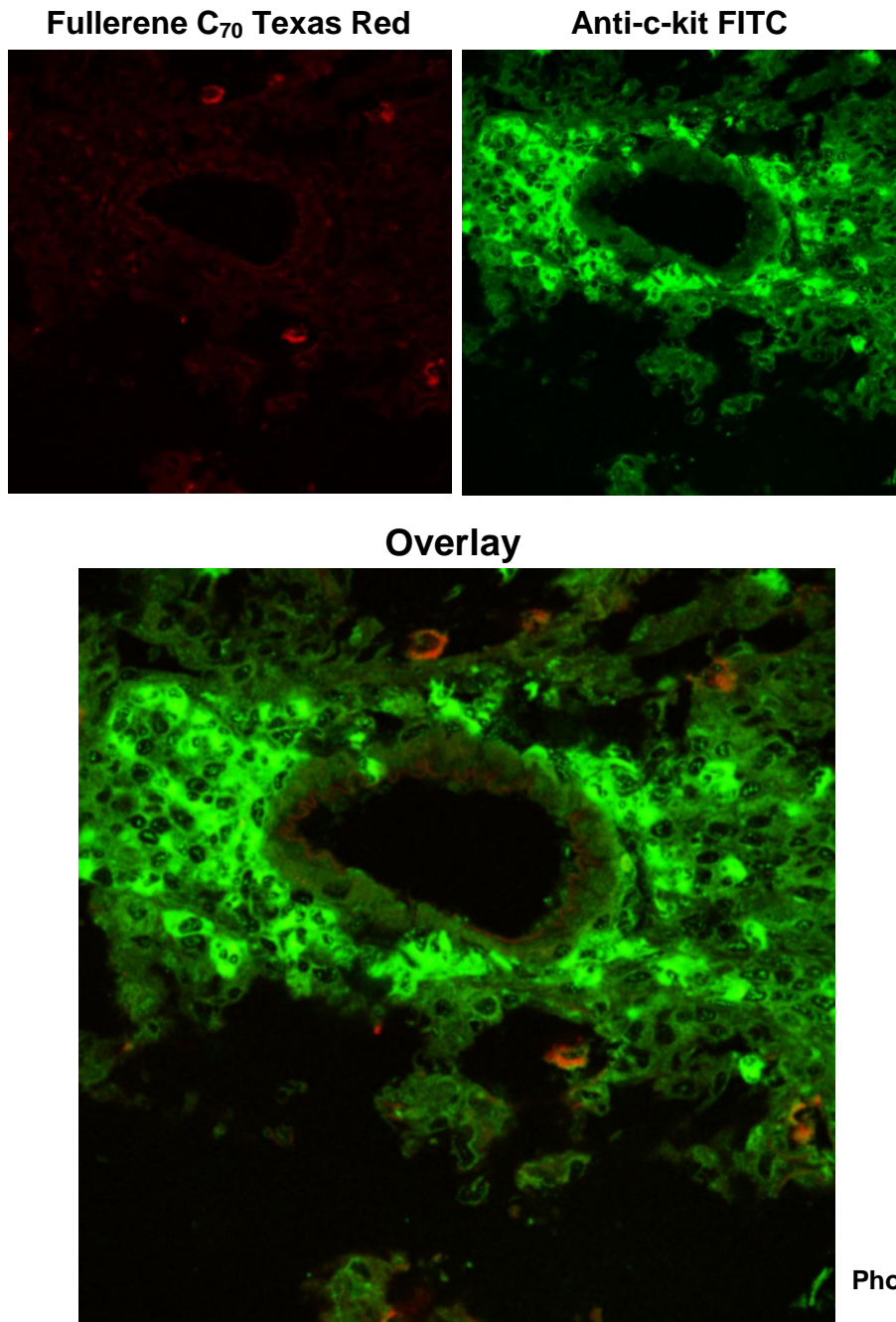
Figure 33. The majority of mast cells do not take up C70-TR in OVA-sensitized mice. Mice were sensitized and challenged as shown in Figure 10 except C70-TR was given in place of C$_{70}$-TGA. Following OVA challenge mice were sacrificed and lungs were collected, sectioned, and stained with c-kit-FITC. Overlay shows co-localization of C70-TR with c-kit+ mast cells.

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figure 10, both IL-5 and IL-4 levels were significantly reduced in C_{70}-TGA treated animals compared to those treated with PBS (**Figure 34**). This was consistent in all modelsstudied, particularly the established disease model (**Figure 35**), which is significant as this model best depicts therapeutic intervention in human disease. This suggests that C_{70}-TGA is altering more than just mast cell and B cell function in vivo as these cytokines are primarily produced by Th2 cells, and points to a multipotent inhibitory approach.

Tetraglycolate reduced B cell IgE production in vitro, so we wanted to know how this inhibition translated in vivo. Mice were pre-bled before initiation of each experiment in order to obtain baseline IgE levels and to ensure that the animals did not already have high IgE levels prompted by infection. Animals were bled again two days before sacrifice and serum IgE levels were quantitated by ELISA. In PBS treated animals, IgE was strongly induced following OVA challenge. Tetraglycolate treated animals had some induction of IgE, but only about half as much was produced in PBS treated animals **(Figure 36)**. This may not have a dramatic effect on disease pathogenesis as IgE levels in C_{70}-TGA treated mice are still greatly increased over non-sensitized animals, but it is clear that C_{70}-TGA is capable of dampening B cell function to some degree in vivo.

Other mediators of interest are the eicosanoids, whose functions vary. Eicosanoids such as the leukotrienes and prostaglandins are known to have pro-inflammatory activities that contribute to asthma pathogenesis. Current therapeutics such as Singulair target the leukotrienes and have had mixed success treating asthmatic disease [79]. These and other eicosanoids can be quantitated by mass

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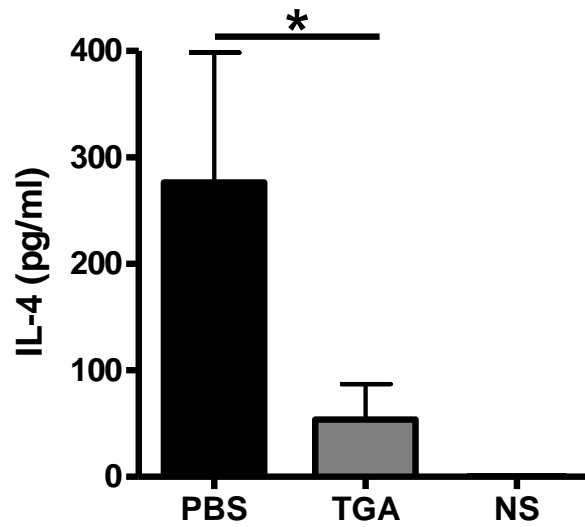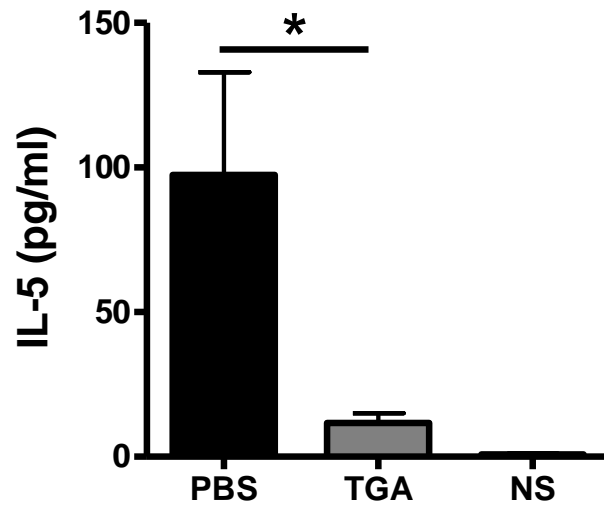
Figure 34. C$_{70}$-TGA treatment significantly reduces IL-4 and IL-5 levels in the BAL when given throughout sensitization and challenge. Mice were treated as indicated in figure 10. Bronchoalveolar lavage fluid was collected on day 47. Cytokine levels in BAL fluid were measured using a multiplex cytokine assay. N = 14 mice per group. * = p<.05 using unpaired two tailed student t-test
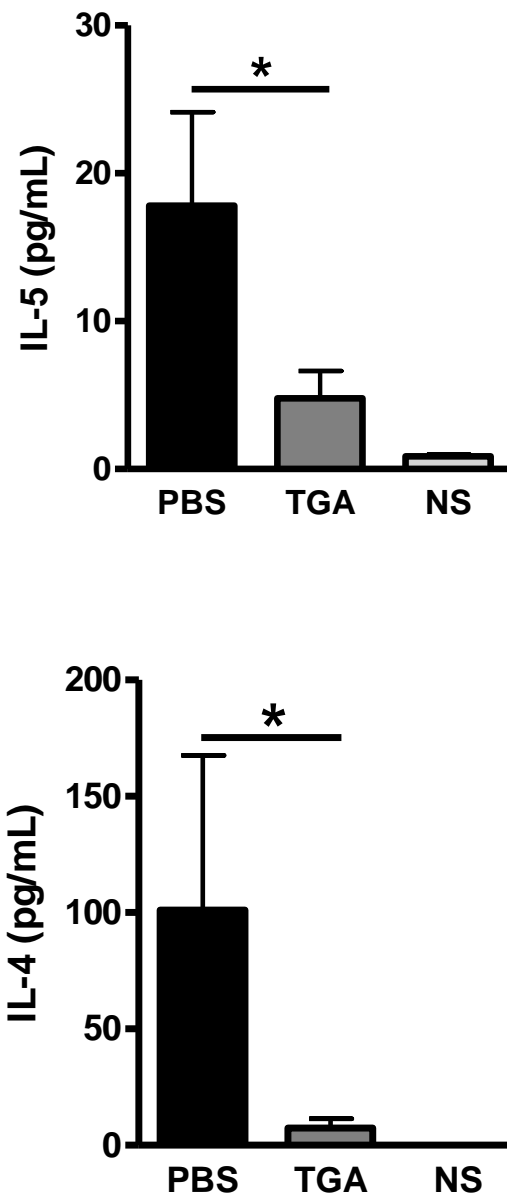
Figure 35. C$_{70}$-TGA treatment significantly reduces IL-5 and IL-4 levels in BAL fluid in an established model of asthma. Mice were treated as indicated in figure 21. Bronchoalveolar lavage fluid was collected on day 76. Cytokine levels in BAL fluid were measured using a multiplex cytokine assay. N = 6 mice per group. ***** = p<.05 using unpaired two tailed student t-test.

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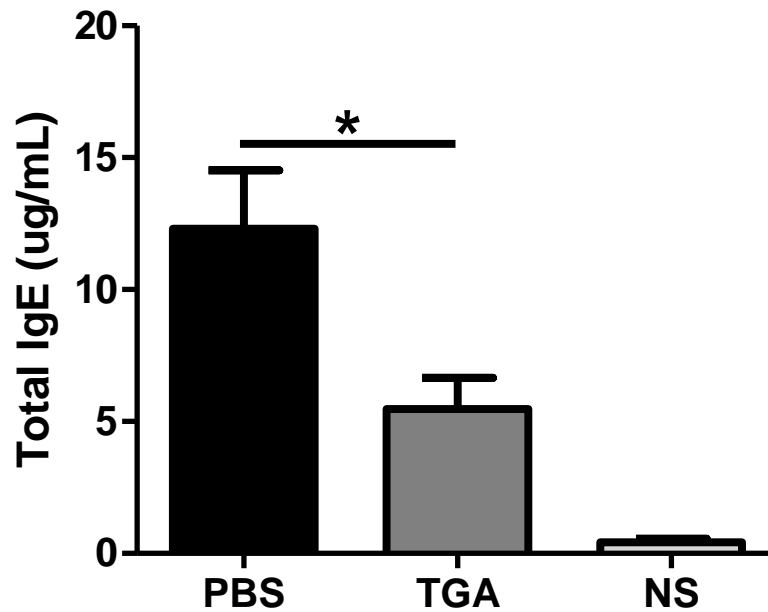
Figure 36. C$_{70}$-TGA significantly reduced serum IgE levels in vivo. Mice were treated as indicated in Figure 10. Serum was collected following OVA i.n. challenge and assayed by ELISA for the presence of IgE. N = 9 mice per group. * = p<.05 using unpaired two tailed student t-test.

spectrometry, which we attempted to do in the BAL fluid. Unfortunately we found that many prostaglandins and leukotrienes are too unstable to be measured in BAL fluid collected from our diseased mice, but were able to measure several of the more stable eicosanoids such as cis-epoxyeicosatreinoic acid (EET) and hydroxyeicosatetraenoic acid (HETE). Interestingly, C_{70}-TGA upregulates 11,12-EET, an anti-inflammatory eicosanoid consistently associated with relaxation of the bronchi and other anti-inflammatory actions in vivo [63-65] (**Figure 37**). Other eicosanoids were largely unaffected by C_{70}-TGA (**Table 1**). Experiments using purified human lung MC demonstrated that both 11,12 EET and 14,15 EET significantly stabilized MC challenged through FcεRI inhibiting both degranulation and cytokine production compared to non-EET treated cells (**Figure 38**). The 8,9 EET did not demonstrate any inhibition of MC mediator release (not shown).

The EETs are produced by cytochrome P450 proteins, whose RNA expression can be measured and correlates with EET production. Microarray was performed on human mast cells treated with or without C_{70}-TGA in order to identify genes that are affected by C_{70}-TGA. A significant increase in RNA levels of the cytochrome P450 protein CYP1B1 was seen in these cells (**Figure 39**), suggesting that this mechanism may be translatable to human disease.

In order to show a direct association between C_{70}-TGA upregulation of EET's and the improved asthma pathogenesis we see in C_{70}-TGA treated mice, an EET inhibitor was employed. This compound, 6-(2-Propargyloxyphenyl) hexanoic acid (PPOH), inhibits the formation of 11,12-EET by inhibiting epoxide formation by CYP4A2 and CYP4A3[80]. To examine the effect of EET inhibition on the severity of asthma

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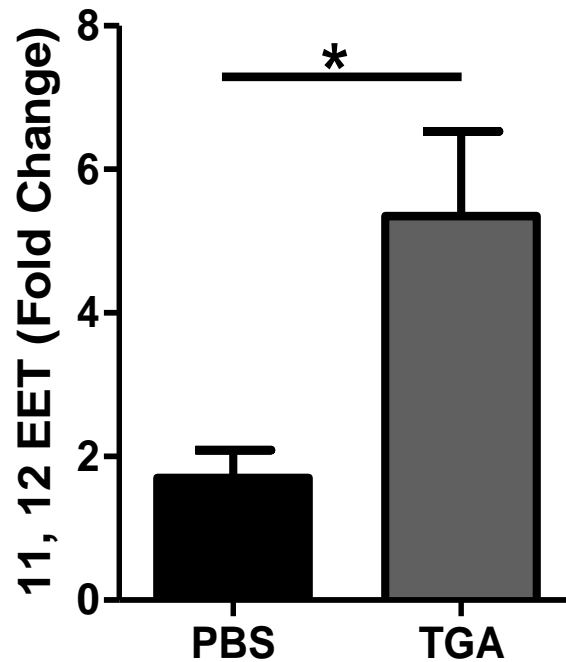
Figure 37. C$_{70}$-TGA upregulates 11, 12 EET production in BAL fluid. Mice were treated as shown in Figure 10. Bronchoalveolar lavage fluid was collected following OVA i.n. challenge and assayed by mass spectrometry for the presence of EETs. Fold change over non-sensitized animals is plotted. N = 6 mice per group. * = p<.05 using unpaired two tailed student t-test.
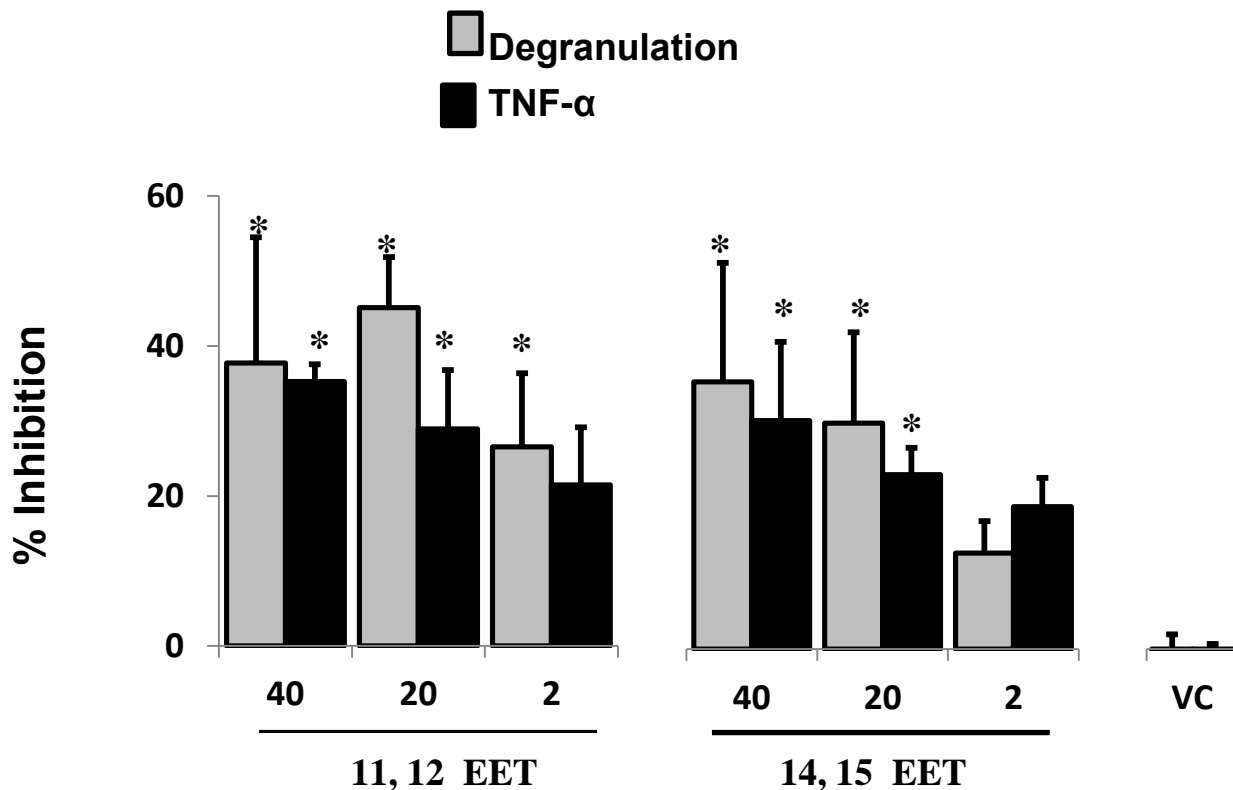
Figure courtesy of Anthony Dellinger

Figure 38. Epoxygenases reduce degranulation and cytokine production from human lung mast cells after anti-FcεRI activation. Cells were cultured with fixed concentrations of the indicated EET or Vehicle Control (VC: Ethanol 40 μM), washed and stimulated for 20 min with optimal concentrations of anti-FcεRI Abs (3B4; 1mg/ml). Cells were centrifuged and β-hexosaminidase release and cytokine production determined. The data shown are the average of two separate experiments performed in triplicate with means ± SE shown. The asterisk indicated statistical significance compared to VC, where *= p<.05 using unpaired two tailed student t-test.

Mean fold change (+/- SD) relative to non-sensitized		
Eicosanoid	PBS	TGA
8 HETE	4.0 +/- 2.9	6.14 +/- 4.13
12 HETE	2.58 +/- 1.85	2.19 +/- 1.62
15 HETE	4.37 +/- 3.15	4.76 +/- 3.23
PGE_2	1.78 +/- 0.99	1.55 +/- 0.32
PGF2α	1.34 +/- 0.18	1.35 +/-0.22
6-keto-PGF1α	1.22 +/- 0.23	1.43 +/- 0.41
Arachidonic Acid	1.66 +/- 0.66	1.77 +/- 1.04

Table 1. C$_{70}$-TGA has no effect on these eicosanoids. Mass spectrometry was performed on BAL fluid from mice treated as indicated in Figure 10. Eicosanoid levels were quantitated.
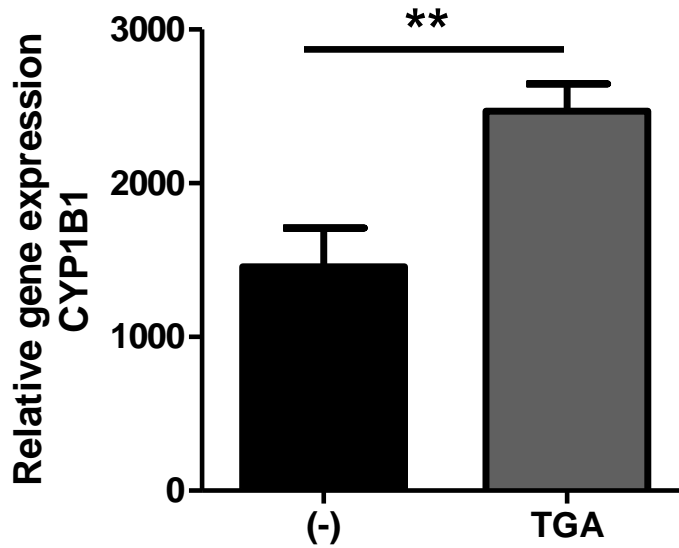
Figure 39. C$_{70}$-TGA upregulates CYP1B1 expression in human mast cells. Human skin mast cells were pre-treated with or without 10 μg/mL C$_{70}$-TGA overnight at 37°. The next day cells were washed and challenged with or without anti- FcεRI (1 μg/mL) for 2 hours. Cell pellets were used for RNA isolation and gene microarray. Relative gene expression of CYP1B1 is shown. Each condition was performed in triplicate. ****** = p<.01 using unpaired two tailed student t-test.

pathogenesis, asthma was induced as indicated in Figure 10 and mice were treated with PBS, C_{70}-TGA, or C_{70}-TGA and PPOH. Just as we hypothesized, the inhibition seen in C_{70}-TGA treated animals is completely reversed in those given C_{70}-TGA and PPOH. Mice treated with PPOH and C_{70}-TGA have significantly more eosinophils in the BAL fluid then those given C_{70}-TGA alone. Eosinophil levels in PPOH treated animals are similar to those seen in PBS treated animals, and are significantly greater than C_{70}-TGA treated and non-sensitized controls (**Figure 40**). Bronchoconstriction was also measured in these animals, and correlated with our findings on eosinophil infiltration. While C_{70}-TGA inhibited bronchoconstriction as shown previously, PPOH completely reversed this inhibition (**Figure 41**). Mice treated with PPOH had significantly higher airway constriction compared to C_{70}-TGA treated animals, and values were similar to those seen in PBS treated mice. When the mice were sacrificed, lungs were collected then sectioned and stained with H&E. These sections show dramatic cellular infiltration in animals treated with PPOH and C_{70}-TGA, similar to what is seen in PBS treated animals (**Figure 42**). Conversely, mice treated with C_{70}-TGA alone have minimal cellular infiltration and lung sections appear similar to non-sensitized mice.

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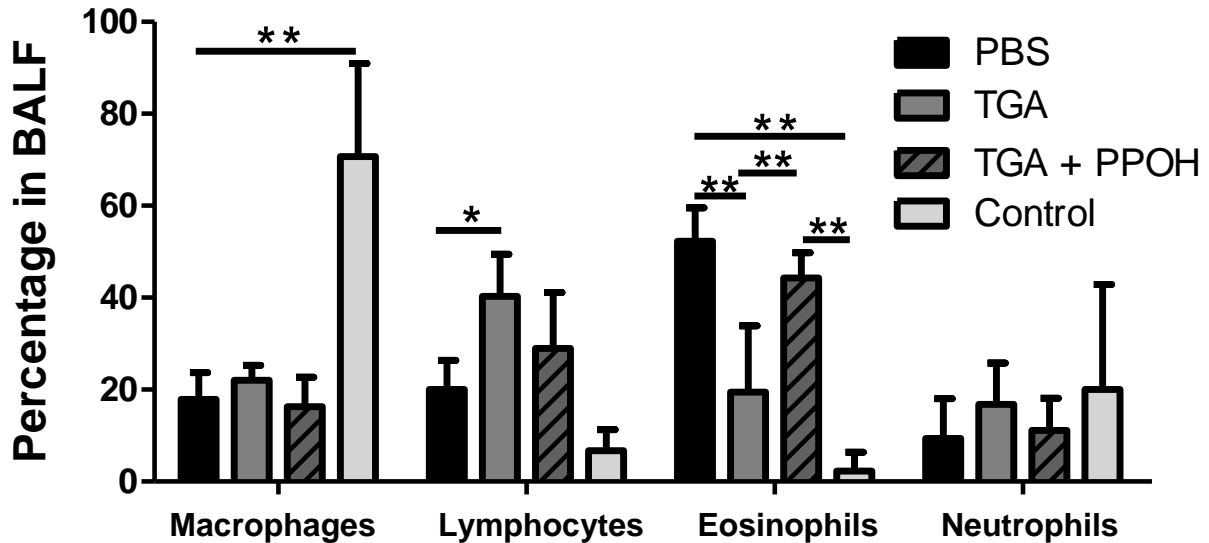
Figure 40. PPOH reverses inhibition of eosinophilia in C_{70}-TGA treated mice. Mice were treated as indicated in Figure 10. The EET inhibitor PPOH was given i.n. 15 minutes prior to and again 2 hours following C_{70}-TGA treatment. Cells in the BAL fluid were stained with Hema 3 stain set. Inflammatory cells in the BAL were counted and eosinophils are represented as a percentage of total inflammatory cells. n=6 mice per group. * = p<.05 and ** = p<.01 using unpaired two tailed student t-test.
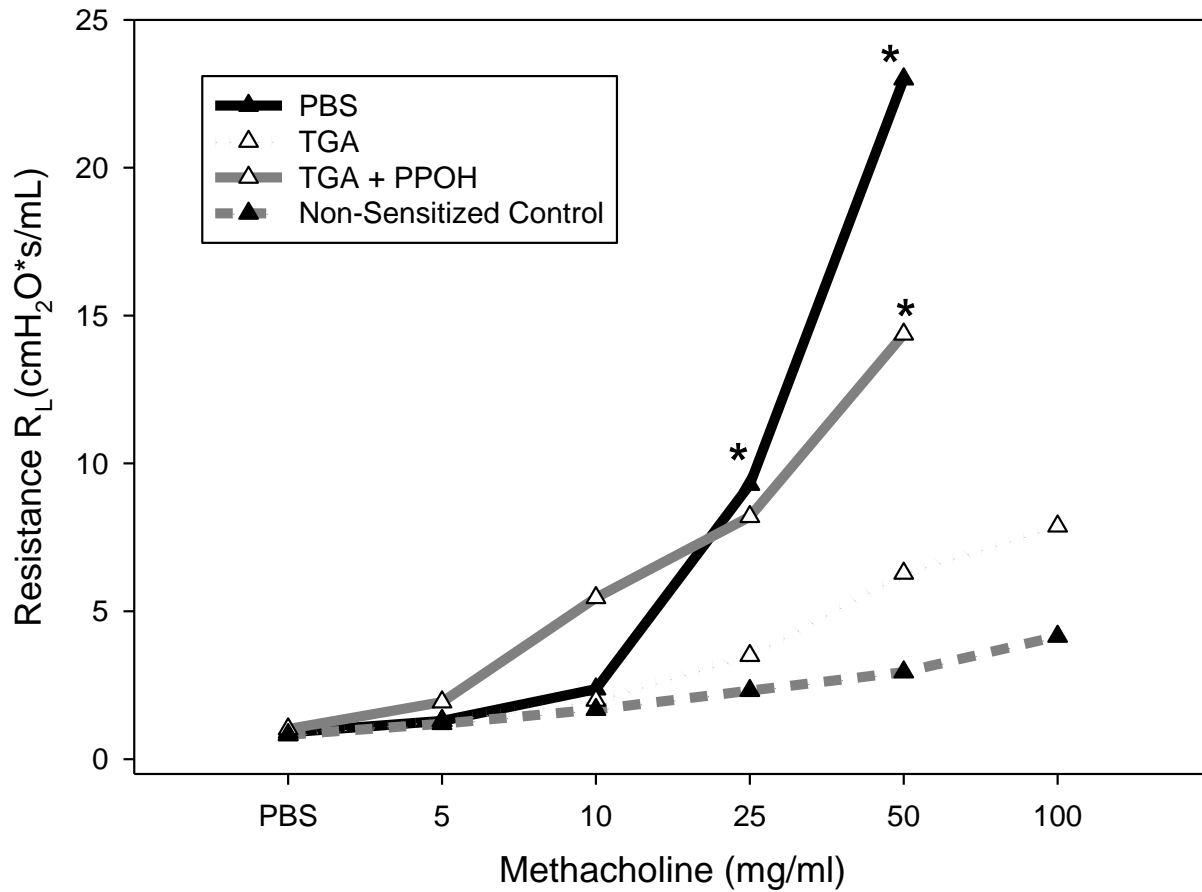
Figure 41. PPOH reverses inhibitory effect of C_{70}-TGA on bronchoconstriction. Mice were treated as indicated in Figure 10. The EET inhibitor PPOH was given i.n. 15 minute prior to and again 2 hours following C_{70}-TGA treatment. Maximum airway resistance in response to methacholine is plotted. n = 6 mice per group. * = p<.05 using unpaired two tailed student t-test.

PBS

TGA

TGA + PPOH

NS Control
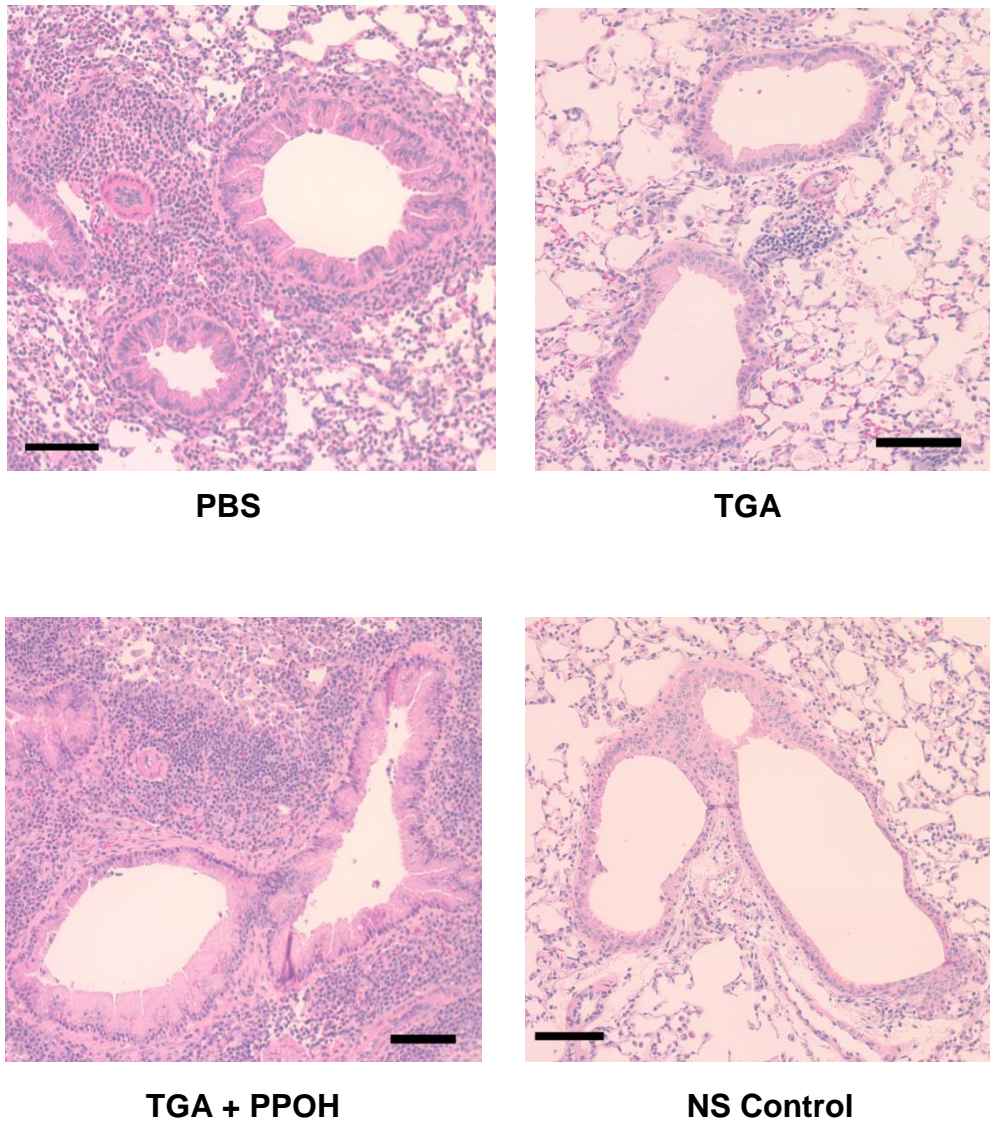
Figure 42. Lung sections show reversal of C$_{70}$-TGA-induced inhibition of cellular infiltration by PPOH. Lung tissue was fixed in 10% formalin, sectioned, and stained with H&E. Bar measures 100 µm. Representative photographs are shown.

IV. Toxic potential of C_{70}-TGA

The C_{70}-TGA compound was selected for study due to its specific inhibition of mast cell function, but its toxic potential remains unknown. Because each derivatized fullerene compound contains a unique chemical formulation that determines the biological function of the molecule, toxicity must be determined separately. The investigations discussed here are preliminary in nature, but suggest that C_{70}-TGA has no overt toxic effects in vivo.

A. Effect of C_{70}-TGA on liver and kidney function. The toxicity of FD is still widely debated and varies based on the specific moieties added to the fullerene core [81]. Therefore, in vivo toxicity of C_{70}-TGA was assessed via several methods. To assess liver function, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by ELISA using serum collected on the final treatment day using the model in Figure 10. These enzymes are present at low levels in healthy individuals and large increases would suggest liver toxicity [82]. No significant differences were seen between treated, untreated, and non-sensitized control animals (**Figure 43**). Additionally, serum creatinine levels were measured in order to assess kidney toxicity, as creatinine levels rise in the serum if the kidney is not properly filtering the blood [83]. No significant differences were seen between treated, untreated, and non-sensitized animals (**Figure 44**). This suggests that short-term use of C_{70}-TGA is not toxic to major organs, although further testing is necessary to confirm this finding in human studies.

B. Levels of C_{70}-TGA in major organs following i.n. treatment. To assess toxic aggregation and build up in the body, a C_{70}-TGA molecule containing gadolinium

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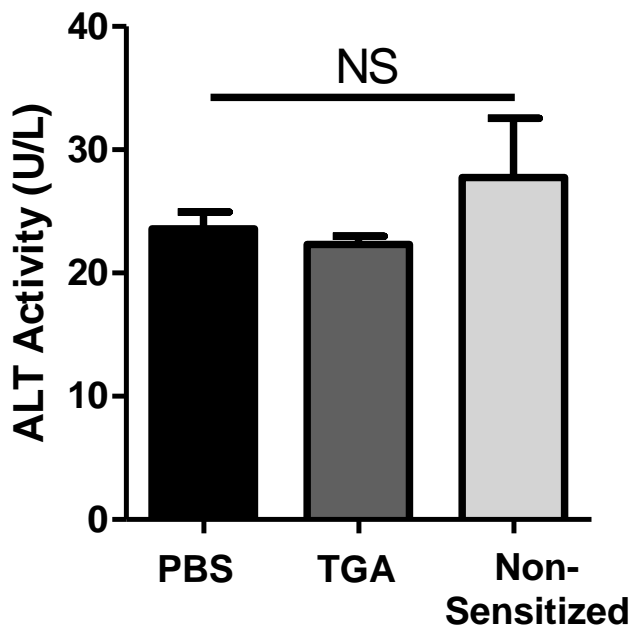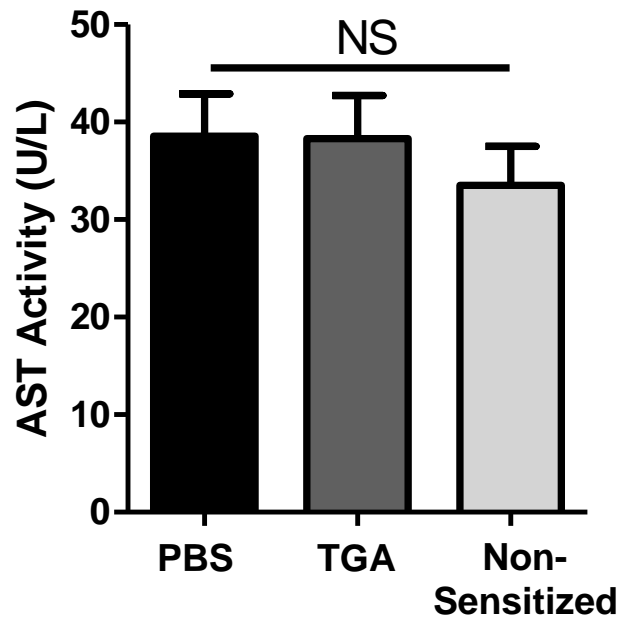
Figure 43. C$_{70}$-TGA has no effect on liver enzymes AST and ALT. Serum was collected from C$_{70}$-TGA treated mice on the final day of treatment (Figure 10). Liver enzymes AST and ALT were measured by ELISA. N = 8 mice per group. Data is not significant (NS) using unpaired two-tailed student t-test.

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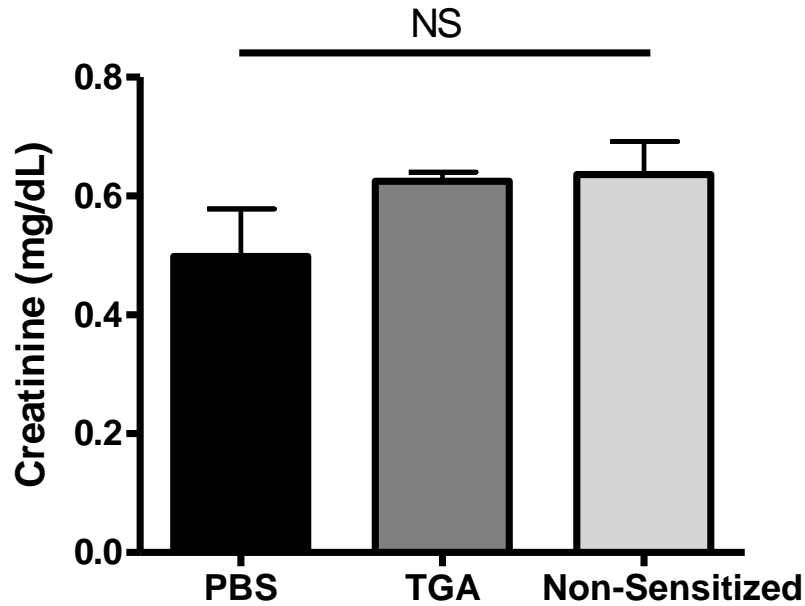
Figure 44. C$_{70}$-TGA has no effect on serum creatinine levels, a measure of kidney toxicity. Serum was collected from C$_{70}$-TGA treated mice on the final day of treatment (Figure 10). Creatinine levels were measured by ELISA. N = 6 mice per group. Data is not significant using an unpaired two-tailed student t-test.

(Gd) within the fullerene sphere [84] was administered i.n. to mice every three days for 30 days. Twenty-four hours after the last dosage gadolinium levels were measured in serum, lung, spleen, liver, kidney, and brain tissue. Gadolinium was detected only in the lung tissue, where less than 10 percent of that injected remained in the tissue. No Gd was detected in any other tissue examined (data not shown).

While further testing is necessary, these initial studies suggest that C_{70}-TGA does not accumulate within the body and is not acutely toxic to the liver or kidney.

Discussion

I. Fullerene C$_{70}$ Derivatives inhibit mast cell and B cell responses in vitro

Fullerenes are unique compounds whose therapeutic potential is only beginning to be studied. These molecules are natural antioxidants with an ability to react with and neutralize reactive oxygen species (ROS), making them natural candidates for study in diseases caused by the overabundance of ROS. Their effectiveness as MRI contrast agents and inhibitors of neurodegenerative disease has been shown previously[85], however very little research has been done to determine efficacy in other inflammatory diseases.

While fullerene derivatives may have the ability to dampen pathogenesis of several inflammatory disorders, our focus was on asthma and allergic diseases. Fullerene derivatives developed by Luna Innovations, Incorporated were tested for their ability to inhibit mast cells in vitro. Because mast cells are important mediators of allergic disease, and C$_{70}$-TGA is a potent inhibitor of mast cell degranulation and cytokine production, we hypothesized that this compound may be capable of reducing symptoms in mast cell driven disease. In vitro studies revealed C$_{70}$-TGA's ability to reduce B cell IgE production, which focused our studies more intently on asthmatic disease. There is a need for novel therapeutics to treat asthmatic disease as approximately fifty-five percent of patients with moderate to severe asthma have uncontrolled symptoms, despite receiving treatment [86]. While patients with mild to moderate asthma are able to control disease using non-specific anti-inflammatories such as inhaled corticosteroids, or in some cases by inhibiting mast cell mediators using anti-histamines or leukotriene inhibitors, these therapies are not always effective in

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severe asthmatics [87]. Even when non-specific inhibitors such as the inhaled corticosteroids are effective, they can have other potentially toxic effects on the body [88]. The data presented here show C_{70}-TGA has specific immune targeting inhibitory effects as well as the ability to dampen both aspects of disease pathogenesis, airway inflammation and bronchoconstriction, something most asthma therapeutics are incapable of.

One therapeutic capable of limiting both airway inflammation and bronchoconstriction is omalizumab (Xolair), which has been successful at controlling asthma by limiting MC and basophil activation [89]. This humanized IgE specific IgG1 monoclonal antibody controls asthma by limiting the amount of free IgE available to bind FcϵRI on the surface of MC and basophils. These cells then reduce expression of the high affinity receptor limiting antigen crosslinking, activation and degranulation. In patients with moderate to severe asthma omalizumab has been highly successful in controlling airway inflammation and reducing symptoms which can sometimes be severe enough to lead to hospitalization or even death. While omalizumab has been successful due to its novel mechanism of action, its distribution is limited due to cost and method of administration (injection). Another mast cell stabilizer, Cromolyn, has been effectively used to treat asthma and allergic rhinitis for many years. Due to an inconvenient dosing regimen and lack of efficient drug delivery it is no longer widely used in the United States, although many other countries continue to use cromolyn because of its known efficacy and favorable safety record [90]. Tetraglycolate is intended to similarly stabilize mast cells thus reducing the presence and exacerbation of asthma symptoms. Due to the remarkable stability of the fullerene carbon cage and our ability

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to introduce these small molecules directly to sites of inflammation, C_{70}-TGA may be an effective yet safe and easy to administer alternative to current therapeutics.

In the development of C_{70}-TGA, thought was given to both efficacy and safety. The compound was created using C_{70} as the core instead of C_{60} for two reasons. First, because C_{70} has ten more carbon atoms than C_{60}, it is a slightly more effective free-radical scavenger, which may improve its therapeutic benefit. Secondly, the C_{70} molecule tends to have an oval shape, and chemical moieties are more likely to react at the poles then throughout the molecule. From the drug development point of view, this is an advantage because the number of isomers is reduced. Historically, it has been seen that each chemical isomer can have a different and in some cases toxic effect, as was the case with thalidomide in the mid-1900's [91]. Compounds using the C_{70} core may have an easier time gaining approval for clinical use, so even preliminary experiments such as these should be carried out using fullerene compounds that resemble a possible therapeutic option.

II. C_{70}-Tetraglycolate, a specifically derivatized fullerene compound, reduces pathogenesis of allergic asthma and anaphylaxis in vivo

Anaphylaxis is a severe, life threatening reaction to allergen defined as the IgE mediated release of mast cell and basophil mediators following antigen crosslinking [92]. It is not well anticipated, and thus prevention and treatment can be difficult [93]. At least 1500 deaths each year are attributed to anaphylaxis, although it is likely that this number is an under-representation due to the lack of a proper diagnosis [92]. Because mast cells are a driving force in anaphylactic reactions, a model of passive systemic

anaphylaxis was utilized to quickly assess the efficacy of C_{70}-TGA against mast cell driven disease in vivo. Pathogenesis is due solely to mast cell activation in this model because IgE is injected, not produced by the animal's own B cells. As we hypothesized, C_{70}-TGA was able to significantly reduce the drop in body temperature associated with anaphylaxis in part through the inhibition of the mast cell mediator histamine. This prompted further investigation using models with active sensitization, where allergen is injected into the body and the immune system is allowed to react. The majority of our studies focused on the interaction between C_{70}-TGA and the immune system in this more natural model of immune sensitization.

In order to properly characterize C_{70}-TGA function, several models of asthma pathogenesis were utilized.   These models varied in their methods of allergen sensitization and challenge. Models in which allergen is delivered in saline rather than in an adjuvant such as alum are thought to be more closely related to human disease as they rely heavily on the influence of mast cells.   Initially, C_{70}-TGA was given i.n. throughout sensitization and challenge to determine in vivo efficacy in this mast cell influenced model.   Airway inflammation and bronchoconstriction were significantly reduced by C_{70}-TGA compared to untreated animals. In fact, total inflammation and bronchoconstriction in C_{70}-TGA treated animals is not only significantly reduced, but is similar to that seen in non-sensitized controls. Further, mice that were sensitized to OVA but untreated had a high mortality rate in response to methacholine. Those that were given C_{70}-TGA were much less susceptible to airway constriction and death upon exposure to methacholine. Tetragolycolate treatment practically alleviated symptoms of

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disease in these animals, and dramatically reduced allergen induced bronchoconstriction and death.

While useful in evaluating the efficacy of this compound, these initial experiments do not address the issue that asthma is well established before therapeutic intervention begins. For this reason, a model of established disease was developed in which C_{70}-TGA treatments begin only after asthma pathogenesis is induced. The efficacy of C_{70}-TGA was then evaluated in this more clinically relevant model. Consistent with the previous model in which mice were treated throughout disease development, we found C_{70}-TGA dampens eosinophilia and cytokine levels significantly in the BAL fluid. Lung sections show massive cellular infiltration in untreated animals, while those receiving C_{70}-TGA have minimal cellular infiltration surrounding the airways. Airway hyperresponsiveness was reduced in C_{70}-TGA treated animals, although due to high variability between mice significant differences were not observed. Thus, C_{70}-TGA may be useful in a clinical setting to reverse asthma pathogenesis and limit exacerbation of symptoms.

Other models of asthma pathogenesis in which sensitization was boosted by the use of the adjuvant alum were studied as well. In these models, pathogenesis develops independent of mast cell function as the use of alum alters sensitization. The first of these is an acute inflammatory model that, while shown to be mast cell independent, is dependent on IgE, as IgE KO animals do not develop eosinophilia[76,77]. Pathogenesis is most likely mediated by basophils in this model. Treatment with C_{70}-TGA throughout allergen sensitization and challenge significantly reduced eosinophilia and cytokine levels in the BAL fluid, suggesting that C_{70}-TGA is capable of inhibiting basophils in

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addition to the effects on mast cell responses in vivo. This is intriguing, as basophils are thought to play a role in human disease pathogenesis as these cells are recruited to the lungs in cases of fatal asthma [94].

A similar model using alum to enhance sensitization was examined to determine the specificity of C_{70}-TGA for allergic immune responses. This model involved seven daily allergen challenges with aerosolized OVA, causing a severe non-allergic form of asthma pathogenesis. In addition to being independent of mast cell function, pathogenesis developed independent of IgE as well, as IgE KO animals develop eosinophilia similar to wild-type[76,77]. When given throughout allergen sensitization and challenge C_{70}-TGA had no effect on eosinophilia or BAL cytokine levels in this model, suggesting that C_{70}-TGA inhibits specific immune cells and allergic responses.

III. Proposed mechanism of C_{70}-TGA action

In each of the models presented, inhibition of airway inflammation and bronchoconstriction corresponds with significant reductions in Th2 cytokines IL-4 and IL-5 in the BAL fluid. This partially explains the mechanism of C_{70}-TGA action. Interleukin-5 recruits and activates eosinophils, so it is not surprising that the significant reductions seen in eosinophil infiltration into the lung correspond with a significant reduction of this cytokine. In response to IL-4 B cells undergo class switching to IgE, so inhibition of this cytokine may help explain the in vivo IgE reductions seen in C_{70}-TGA treated animals. However, in vitro B cell inhibition in the absence of T cells is also observed, and thus IL-4 inhibition alone may not fully explain the inhibitory effect of C_{70}-

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TGA on B cell IgE production as this implies a more direct inhibition of B cell function by C_{70}-TGA.

The efficacy of omalizumab emphasizes the importance of IgE in allergic responses. Inhibition of serum IgE levels in sensitized mice may be an important factor in the inhibitory mechanism of C_{70}-TGA. However, due to the high affinity of IgE for its receptor, FcεRI, relatively small amounts of IgE are needed to trigger degranulation in mast cells. As an example, omalizumab, the only anti-IgE therapeutic currently available, reduces serum IgE levels by 84-99% [95] to induce efficacy. Because serum IgE levels in C_{70}-TGA treated animals are still greatly increased over non-sensitized mice, it is likely that IgE reduction only partially explains the inhibitory effects of C_{70}-TGA.

Eicosanoids are generally thought to be pro-inflammatory molecules that play an important role in asthma pathogenesis. These small molecules are derivatives of arachadonic acid, many of which are rapidly produced by activated mast cells. There are three pathways commonly associated with eicosanoid production: the lipoxygenase pathway, COX pathway, and cytochrome P450 pathway. While derivatives of the COX and lipoxygenase pathways proved too unstable to measure in BAL fluid, several eicosanoids derived from the cytochrome P450 pathway are relatively stable and thus we measured these molecules in BAL fluid samples using mass spectrometry. Intriguingly, 11, 12 EET was consistently upregulated in BAL fluid from C_{70}-TGA treated mice and was found capable of inhibiting degranulation and cytokine production by human mast cells *in vitro*. The EETs are consistently associated with relaxation of the bronchi and other anti-inflammatory actions *in vivo*, [63-65] leading us to question the role

of these mediators in C_{70}-TGA inhibition. Further in vivo studies demonstrated that the EET's play a major role in dampening the asthma phenotype as inhibitors of the EET's prevented the C_{70}-TGA-induced modulation of the OVA-induced asthma model. We also find that C_{70}-TGA upregulates expression of human lung mast cell CYP1B1, a gene involved in the production of EET's that could be the source of 11, 12 EET. Lung epithelial and endothelial cells may also contribute as they are thought to be the main source of EET production [65,96]. The EETs have been shown to relax histamine pre-contracted guinea pig bronchi[63] and can inhibit the upregulation of VCAM-1, E-selectin, and ICAM-1, thus potentially limiting cellular infiltration of the lung [65]. Consequently, 11, 12 EET upregulation is playing a significant role in dampening airway inflammation and bronchoconstriction in these models. These results implicate the EET's as a heretofore undiscovered mechanism for controlling asthma and suggest that strategies that induce the production of EET's may be a viable therapeutic strategy for treating asthmatics.

Originally we hypothesized that C_{70}-TGA exerted its inhibitory effects primarily through mast cells. But the data suggests that mast cells are just one of the many cell types inhibited by C_{70}-TGA in vivo. This may be of benefit as clinical disease is manifested differently in each individual patient, so the multipotent inhibition induced by C_{70}-TGA may be capable of greater efficacy.

The localization of C_{70}-TGA in vivo is a big concern, as this affects both the toxicity and the inhibitory actions of the compound. Several studies were carried out in an attempt to determine in vivo localization. It is clear from the IR-800 studies that C_{70}-TGA is immediately present in the lung, but drains out completely sometime between 24 hours and 4 days following i.n. instillation. By giving C_{70}-TGA i.n. every three days, we

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hoped to maximize the inhibitory activity of the compound while reducing toxic build-up in the body. But we were also interested in determining which cell types in the lung take up the compound, so we initiated the C70-TR studies. Unfortunately C_{70}-TGA itself cannot be bound to texas red as it quenches the dye, so a similarly derivatized C_{70} molecule was used instead. When the C70-TR compound was given to naïve mice 24 hours prior to sacrifice, it appears the compound is localized within c-kit+ mast cells. However, when C70-TR is given in place of C_{70}-TGA throughout allergen sensitization and challenge it is no longer found localized within mast cells, but appears to be taken up by macrophages instead. This finding is probably explained by the fact that C70-TR does not inhibit airway inflammation and eosinophilia as C_{70}-TGA does, so the two compounds are most likely using different mechanisms of action. Most likely it is the size of the texas red dye molecule that alters the function of the C70-TR compound. Further studies attempting to show a direct interaction between mast cells and C_{70}-TGA were unsuccessful as mast cells proved quite difficult to isolate from the lungs. Given this, we can conclude that C_{70}-TGA is localized to the lung for greater then 24 hours, and can inhibit B cell IgE production and inflammatory mediators released by T cells and mast cells. But showing the direct interaction between these cells and C_{70}-TGA has proved more challenging.

IV. Toxic potential of C_{70}-TGA

Levels of liver enzymes AST and ALT, which are indicative of liver toxicity when present at high levels in the serum, were not different between treated and untreated animals. Tetraglycolate treated animals had creatinine levels [97] similar to those seen in

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untreated animals, and all were within the normal range. Thus, liver and kidney function appear to be unaffected by short-term local administration of C_{70}-TGA. In addition, a C_{70}-TGA-like compound containing Gd within the fullerene core was developed so that its presence in tissue could be detected using ICP neutron bombardment. After one month of intranasal inhalations, only lung tissue contained detectable amounts of Gd. Thus C_{70}-TGA does not appear to build up in the body tissues, limiting the possibility of toxicity. Further testing will be necessary to determine the safety of these compounds in humans.

V. Conclusions and Significance

The goal of this research was to determine the efficacy of a specifically derivatized fullerene, C_{70}-TGA, for the treatment of asthmatic disease and to provide its mechanism of inhibition. Murine asthma models provide convincing evidence that C_{70}-TGA significantly reduces asthma pathogenesis to levels similar to that seen in non-sensitized controls, which were never exposed to allergen. Further, C_{70}-TGA inhibited both aspects of disease, airway inflammation and bronchoconstriction, which most therapeutics are incapable of. This was achieved through the inhibition of multiple immune cells. In response to C_{70}-TGA, mast cell degranulation was reduced, B cells produced less IgE, T cell cytokine production was inhibited, and the production of anti-inflammatory mediators was induced in lung epithelial and endothelial cells. The combination of these actions led to the in vivo inhibition of pathogenesis we observed. Further, no toxicity was observed in mice which supports the use of fullerene derivatives as pharmacological agents in the treatment of inflammatory diseases.

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These studies also uncovered a novel mechanism of asthma inhibition through the actions of 11, 12 EET. While previous work found 11, 12 EET capable of inhibiting human bronchi and adhesion molecules in vivo, its function in vivo had not been explored. This research represents the first time a role has been shown for the EET's in controlling asthma, which could help to guide future research into the development of more targeted asthma therapeutics.

Derivatized fullerene compounds reduce airway inflammation and hyperresponsiveness in murine models of asthmatic disease, and we propose that once adequate modifications have been performed these molecules will have a role in the treatment of human asthmatic disease. As specific manipulations to the fullerene core can direct the actions of these compounds, derivatized fullerenes may represent a targeted, non-toxic alternative to the corticosteroids. While currently the most commonly used therapeutic for the treatment of asthma, inhaled corticosteroids are non-specific anti-inflammatory compounds and can be toxic at high doses [98]. Further, inhaled corticosteroids are not effective for the treatment of severe asthma. While only 10 percent of asthmatics suffer from severe asthma, this population acquires over 50 percent of the total costs associated with disease [99,100]. Thus, there is a need for alternative therapies. This data suggests that derivatized fullerenes may provide dual functions, inhibiting both short term mediator release by immune cells as well as long term cytokine release and underlying inflammation. Hence these unique compounds could potentially have greater efficacy than current treatments.

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Vita

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