IMMUNOMODULATORS IN FELINE ASTHMA

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

IMMUNOMODULATORS IN FELINE ASTHMA

presented by Jason M. Eberhardt a candidate for the degree of master of Science, and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

I would like to dedicate this thesis to my Lord and Savior, Jesus Christ. Without Your grace and mercy I would truly be a lost soul forever. Even when I have been faithless, You have been faithful. I am living proof that we walk by faith not by sight.

ACKNOWLEDGEMENTS

My first and most important thanks go to my wife and son. Gayle, your support, understanding, and personal sacrifices have not gone unrecognized. God has truly blessed me with the most loving, caring, and faithful best friend. Words cannot express how much I love you and owe you! Joshua, you erupted into this world in blaze of glory and a smile on your face. You brought balance to my life during a time that I needed it most.

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I would like to also thank my other faculty members. Amy DeClue, thank you for teaching me the compliment sandwich and for always pushing me to ask, why? Leah Cohn, people always say you are a great veterinarian. I see you as a great person who happens to also be a great veterinarian. I hope you both are as proud that you helped train me as I am that I was trained by you.

Last, but not least, I want to thank my friends who I get the privilege to work with and call my "resident mates". In particular, a few deserve individual acknowledgement. To Tekla Lee-Fowler, I agree with you, we are lost siblings. Christine Cocayne and Meredith Thoen, thanks for always helping me keep life in prospective. John Punke, we are living proof that internists and surgeons really can be friends.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
ASIT	allergen-specific immunotherapy
BALF	Bronchoalveolar lavage fluid
BGA	Bermuda grass antigen
COX	Cyclooxygenase
CpG	Unmethylated cytosine-phospodiester-guanine dinucleotides
CXC	Chemokine ligand
DHA	Dososahexanoic acid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
feG	feG-COOH
HDMA	House dust mite allergen
ICAM	Intracellular adhesion molecule
IDST	Intradermal skin testing
Ig	Immunoglobulin
IL	Interleukin
IFN-γ	Interferon gamma
NK	Natural killer
ODN	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell

PUFA	Polyunsaturated fatty acids
RIT	Rush immunotherapy
SC	Subcutaneous
TGF	Transforming growth factor
Th0	T-helper 0
Th1	T-helper 1
Th2	T-helper 2
TLR	Toll-like receptor
TNCC	Total nucleated cell count
TNF	Tumor necrosis factor
Tregs	T-regulatory

ACADEMIC ABSTRACT

IMMUNOMODULATORS IN FELINE ASTHMA

Introduction: Feline allergic asthma is a common lower airway disease that has a complex underlying immunopathogenesis. Current therapeutic mainstays (glucocorticoids, bronchodilators) do not address the underlying immune regulatory dysfunction. Several new immunomodulators have been recently investigated in experimental models and clinical studies of allergic disease. The aim of this thesis was to review the immunomodulators that have been previously evaluated in feline asthma and to specifically evaluate the immunomodulator, feG-COOH (feG), in an experimental model of feline asthma.

Our laboratory previously evaluated the effect of a single dose of feG on eosinophilic airway inflammation when given prior to an allergen challenge in experimentally asthmatic cats. While this single dose partially blunted airway inflammation compared with placebo, this therapy needed to be tested with more chronic use, given that exposure to allergen in pet cats is an unpredictable event. We hypothesized that a chronic (2 week) course of feG in experimentally asthmatic cats would decrease airway inflammation and clinical signs of asthma.

Methods: Experimental asthma was induced in 10 cats using Bermuda grass allergen (BGA) and cats were randomly selected to receive either feG (1 mg/kg, PO) or saline for 2 weeks, followed by a 2 week washout period. Cats then received the alternate treatment. Cytologic examination of bronchoalveolar lavage fluid (BALF) was used to document the development of an asthmatic phenotype prior to enrollment into the study

(this disqualified one cat from the study). Aerosol challenge with BGA was performed weekly throughout the study. A clinical scoring system to evaluate clinical signs associated with the asthmatic phenotype, was employed prior to and after each 2 week treatment. Similarly, BALF and blood were collected prior to and after each of the 2 week treatment periods. Cytology and cytokine analysis were performed on BALF samples and in vitro cytokine restimulation was performed on peripheral blood mononuclear cells (PBMCs).

Results: There was no significant difference between the treatment groups in BALF total nucleated cell counts or eosinophil percentages. Greater than 40% of the BALF supernatant samples had IL-1, IL-4, IL-6, CXCL-8 (formerly IL-8) and IFN-γ concentrations below the lower limit of detection of the assay regardless of time point or treatment administered. Interleukin-4 and IFN-γ concentrations in the cell culture supernatant from stimulated PBMCs were below the lower limit of detection for all samples. Due to the low number of samples that had detectable concentrations of IL-1, IL-4, IL-6, CXCL-8 and IFN-γ, statistical analyses of this data was not meaningful. There was no significant difference in BALF or plasma TNF activity or clinical scores between treatment groups.

Conclusions: In cats with experimental asthma, daily use of feG (2 weeks) during chronic aeroallergen exposure did not dampen eosinophilic airway inflammation, alter cytokine profiles in the plasma or BALF, or decrease clinical signs associated with allergen challenge. These results support that feG at this dosage can not be recommended as monotherapy for the chronic treatment of allergic asthma in cats.

Whether feG has a role in the acute management of asthmatic attacks or in combination with other treatments in cats has yet to be determined.

Chapter 1

Immunomodulators in allergic asthma: potential treatments for feline asthma?

Allergic asthma is a common lower airway disease in cats and is estimated to affect 1-5% of the domestic cat population. While the entire mechanism is still not fully elucidated, as in humans, it is believed to be type I hypersensitivity response to inhaled allergens. The pathogenesis involves the uptake of these allergens by antigen presenting cells (APCs) and presentation on MHC II molecules to naïve T-helper (Th0) cells. These cells subsequently become polarized to differentiate into CD4+ effector Th2 cells, the major cell population orchestrating the asthmatic inflammatory cascade. The activation of Th2 cells lead to the production of inflammatory cytokines such as IL-4, IL-5 and IL-13, among others. By releasing cytokines, Th2 cells are responsible for increased mucous production, activation of endothelial cells, eosinophil chemotaxis and B-cell immunoglobulin class switching to IgE. Furthermore, when allergen binds to IgE anchored to high affinity FcεRI receptors on mast cells and basophils, their subsequent degraulation further perpetuates the inflammatory cascade. Eventually the patient develops hallmark features of asthma: eosinophilic airway inflammation, airway hyperresponsiveness and airway remodeling.

While corticosteroids are typically successful in controlling underlying airway inflammation, they have a myriad of unpleasant side effects that may limit long-term usage especially at high doses. Additionally, corticosteroids only blunt the inflammatory response long after it has been initiated and do nothing to reverse the underlying aberrant immune response. Recently, there has been interest in other therapeutics, including

immunomodulating agents, for the treatment of asthma. This chapter provides a brief review of some of the immunomodulating agents that may be beneficial for the management of feline allergic asthma.

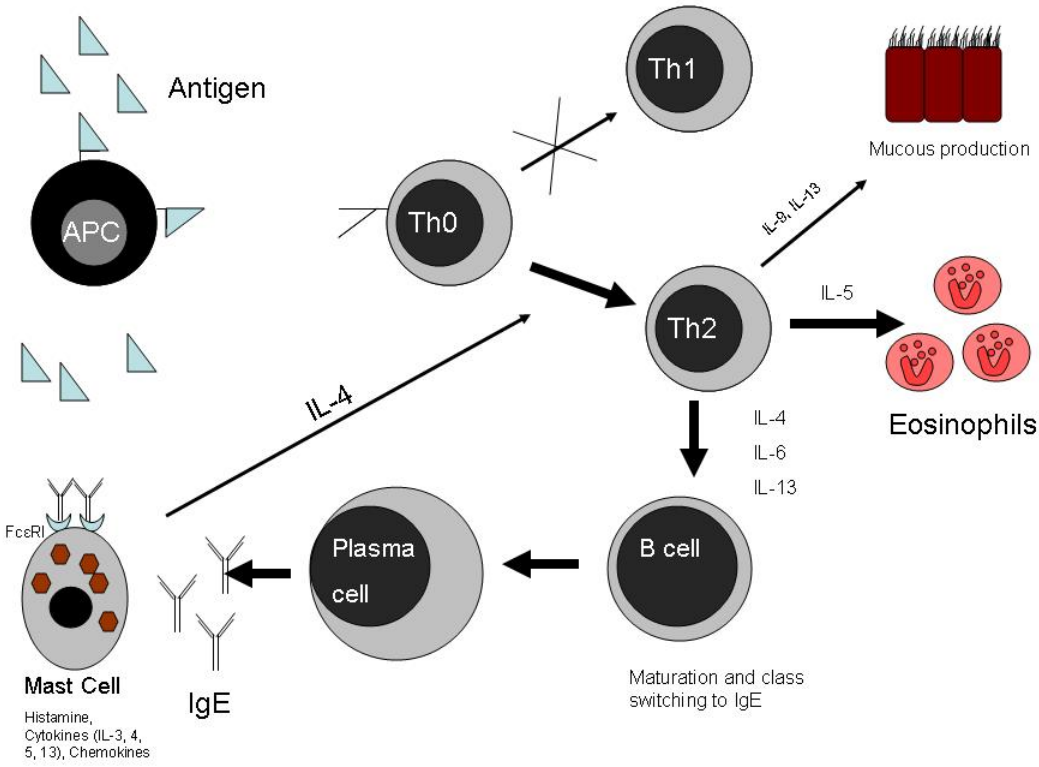

Figure 1-1. Schematic diagram of activation and consequence of allergic inflammation. The polarization towards Th2 inflammation eventually leads to increased mucous production, eosinophilic inflammation and immunoglobulin class switching to IgE by B-cells.

Unmethylated cytosine-phosphodiester-guanine oligodeoxynucleotides (CpG-ODN)

In recent years, investigators have been interested in the involvement of the innate immune system in the development of allergic inflammation, as this may be an early target for interventions to prevent or treat asthma. One of the main interactions that have been focused upon is "pathogen-associated molecular patterns" (PAMPs). These are molecules, expressed or released from microbes, which have a specific interaction with

pattern recognition receptors on target cells (often an effecter cell of the immune system). One example of a PAMP that has had a notable amount of attention in asthma is prokaryotic DNA.

Over a decade ago, it was established that bacterial DNA had unique immunostimulatory effects on B-cells from mammals (Krieg et al., 1995). Unmethylated cytosine-guanine dinucleotides (CpG) motifs, which are found in a much higher frequency in prokaryotic DNA, seem to be the distinctive base sequence pattern responsible for this immunostimulatory property. When cytosine-guanine sequences are found in eukaryotic DNA they are commonly highly methylated. The amount of methylation of these sequence patterns has been directly linked to the strength of their immunomodulatory effects (Fonseca and Kline, 2009). These effects are broad and CpG-ODNs will not only activate B-cells and direct their cytokine release, but also alter activity of NK cells, dendritic cells and CD4+ T-lymphocytes (Fonseca and Kline, 2009; Klinman et al., 1996; Krieg et al., 1995).

It is hypothesized that the effects of CpG-ODNs on effector cells involves the alteration of intra-nuclear signaling pathways. CpG-ODN initially binds to the surface receptor, CpG binding receptor, to enter the cell so it can bind to toll-like receptor (TLR)-9 (Fonseca and Kline, 2009). The intracellular location of TLR-9, allows for rapid and precise distinction of bacterial DNA and guards against misidentification of "self" DNA (Krieg and Vollmer, 2007). These initial events will eventually lead to the intra-nuclear activation of the NF-kB pathway; inducing cytokine and chemokine production from the cell (Fonseca and Kline, 2009).

There is a preferential induction of a Th1 inflammatory response by CpG motifs which is characterized by IFN-γ secretion. The exposure to CpG-ODNs also induces IL-10 and IL-12 production from dendritic cells and IL-10 and TGF-β production from T-regulatory cells (Tregs) (Chu et al., 1997; Jarnicki et al., 2008). There is tremendous appeal to exploit these effects in the treatment of asthmatics since classically a Th2 inflammatory state predominates in these patients. However, the effects that CpG-ODNs have on the immune response and allergic inflammation are neither simple nor fully elucidated. These effects as it is currently understood in humans is depicted in Fig. 1-2 (Akdis and Akdis, 2007; Fonseca and Kline, 2009).

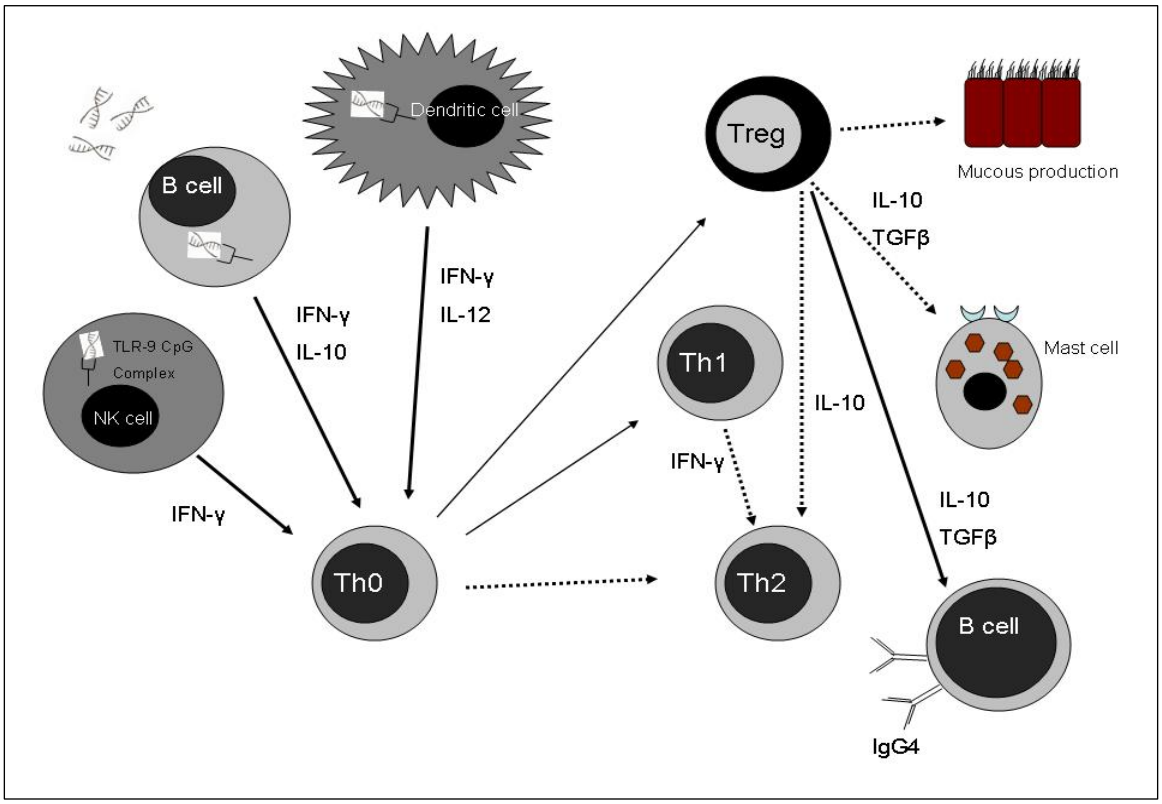

Figure 1-2. Schematic diagram of CpG-ODNs immunomodulating effects. CpG-ODNs have direct effects on dendritic cells, B-cells and NK cells. This leads to differentiation of naïve Th0 cells into Th1 and to Tregs. Both of these cell types can suppress Th2 cells. Tregs use multiple factors to regulate the immune response, including IL-10 and TGF-β. These two cytokines have many regulatory functions such as suppression of mast cells, basophils and eosinophils, suppression of IgE production, promoting isotype switching to the non-inflammatory IgG4 (in humans) and inhibiting Th2 activation.

In a murine asthma model, mice that received CpG-ODN prior to allergen challenge had significantly decreased eosinophilic lung inflammation and decrease in the number of allergen-specific IgE producing cells (Kline et al., 1998; Sur et al., 1999). Post-methacholine challenge airway hyperreactivity was also significantly less in mice treated with CpG-ODN compared to a placebo (Kline et al., 1998). Surprisingly, these protective effects lasted at least 6 weeks in these mice (Sur et al., 1999). In this same study, CpG administration did not confer any benefit in asthmatic IFN-γ knockout mice, supporting a linkage between CpG-ODNs and production of IFN-γ. This finding is particularly important since IFN-γ inhibits the Th2 cell pathway preventing development of eosinophilic inflammation. However, the immune pathways involved in allergy are complex and counter-intuitively IFN-γ may contribute to allergic airway inflammation in some situations (Koch et al., 2006).

Airway remodeling is an important adverse consequence of chronic asthma. In humans, airway remodeling can be associated with progressive airway hyperresponsiveness, goblet cell hyperplasia and epithelial collagen deposition. In a murine model of chronic asthma, a single dose of CpG-ODN given intraperitoneally, reduced chronic airway remodeling, decreased goblet cell hyperplasia, decreased subepithelial fibrosis and decreased airway hyperreactivity (Jain et al., 2002). These subjects additionally had decreased acute eosinophilic inflammation. The protective effects of CpG-ODN on airway inflammation and remodeling have also been investigated in a non-human primate model of asthma. In this study, rhesus monkeys sensitized to house dust mite allergen (HDMA) had significant reduction in eosinophilic airway

inflammation, airway hyperresponsiveness and markers of airway remodeling (Fanucchi et al., 2004).

Results from studies using CpG-ODN in humans with natural occurring allergic disease has been somewhat variable. In a clinical trial involving 40 mild human asthmatics, the treatment group received 4 weekly doses of neubulized CpG-ODN. While humans in the CpG group did have increased IFN-γ and IFN-γ inducible genes, they did not have improvement in forced expiratory volume in 1 second or in sputum eosinophils when compared to placebo (Gauvreau et al., 2006). The authors did raise the possibility that the duration of the trial was insufficient. More recently, an open-label, phase I/IIa clinical trial was completed using CpG-ODN as an adjuvant in subcutaneous (SC) allergen-specific immunotherapy (ASIT) in humans diagnosed with either allergic rhinitis or allergic asthma to house dust mite allergy (HDMA). After the 10 week protocol was completed, patients had reduced reactivity to HDMA on skin testing and were nearly symptom for at least 38 weeks post-treatment (Senti et al., 2009). There is enough evidence to support the development of larger, masked, placebo-controlled clinical trials using ASIT adjuvanted with CpG-ODNs in humans.

The effects of CpG motif administration have been evaluated in experimental feline asthma with the hope of finding a new therapy to treat pet cats with naturally developing asthma. Using a model of allergic asthma where cats were sensitized to Bermuda grass allergen, CpG motifs were administered by a variety of different routes, doses and timing of administration (with respect to allergen challenge) and only modest positive effects were observed (CR Norris, PhD Dissertation, UC Davis, 2004). In particular, while CpG motifs partially dampened eosinophilic airway inflammation, they

did not appear to diminish clinical signs associated with bronchoconstriction after allergen challenge nor did they decrease serum allergen-specific IgE. However, at the time of this study, use of CpG motifs as an adjuvant with ASIT was also being investigated in animal models and showed promise. Subsequently, CpG motifs were administered as an adjuvant with abbreviated ASIT known as rush immunotherapy (RIT) (Reinero et al., 2008). The cats treated with adjuvanted RIT had a significant reduction in eosinophilic airway inflammation and fewer adverse effects than an earlier report of RIT alone (Reinero et al., 2006). While these results in experimental asthmatic cats show promise, evaluation of these protocols in naturally affected feline asthmatics warrants further assessment.

Allergen-specific immunotherapy (ASIT)

The only known curative therapy for allergic disease is ASIT. There are many different protocols involving differences in the duration of therapy (conventional versus rush immunotherapy) and differences in routes of administration (SC injections versus mucosal delivery). In humans, ASIT is efficacious in the treatment of allergic rhinitis and venom anaphylaxis. However, there has been increasing interest and proof of efficacy in asthmatic people (Bousquet et al., 2001; Calamita et al., 2006; Kohno et al., 1998). Allergen-specific immunotherapy has been most commonly used in the treatment of atopic dermatitis in dogs and cats (Griffin and Hillier, 2001; Trimmer et al., 2005) but has recently been evaluated in experimentally asthmatic cats with some promising results (Lee-Fowler et al., 2009; Reinero et al., 2006; Reinero et al., 2008). While all the underlying immunological mechanisms of ASIT are still not fully understood, they can

be divided into categories based upon the time it takes for them to occur: early, intermediate and late effects.

Early immunologic effects of ASIT are thought to be secondary to desensitization, decreased inflammatory mediator release and reduction of tissue numbers of mast cells and basophils (Akdis and Akdis, 2007). These early changes are important in decreasing the development of systemic anaphylaxis either to allergen or during therapy. However, the risk of systemic anaphylaxis can fluctuate during therapy and asthma appears to be a risk factor for the development of this severe, life-threatening reaction during treatment (Lockey et al., 2001). Intermediate immunologic changes of ASIT primarily occur in allergen-specific Th2 cells and the development of Tregs. T-regulatory cells are especially important in improving the Th1:Th2 imbalance that occurs in allergic disease, damping the aberrant immune response (through IL-10 and TGF-β production) and potentially even lessening mucosal mucus production (Akdis and Akdis, 2007).

The increased production of the anti-inflammatory cytokines IL-10 and TGF-β by Tregs, are thought to play an important role in late immunologic effects of ASIT on B-cells. Late immunologic changes occur in B-cells as they decrease production of IgE and increase production of "blocking antibodies" such as IgG4 (in humans) and IgA (Akdis and Akdis, 2007; Reisinger et al., 2005). Protective effects of IgG4 are likely due to the binding of the allergen to IgG4; preventing crosslinkage of the allergen and IgE on mast cells and decreased IgE mediated Th2 activation (Durham and Till, 1998; van Neerven et al., 1999). These late immunologic changes are likely responsible for the potential cure some patients experience with ASIT.

There has been some debate on what is the most efficacious and safest route of administration of ASIT in asthma. While infrequent, adverse affects from SC ASIT do occur. This has lead to the investigation of mucosal therapy as a possible safer alternative. In humans, the clinical efficacy of mucosal therapy has been debated and data suggests that the efficacy compared to SC therapy is decreased, especially in children with allergic rhinitis (Wilson et al., 2005). Recently, there has been evidence that both SC and mucosal therapy is efficacious in cats with experimentally induced asthma (Lee-Fowler et al., 2009; Reinero et al., 2006). However, there is better control of clinical signs with SC therapy and thus it may be preferred in cats with asthma (Lee-Fowler et al., 2009).

While conventional ASIT can takes months to years for an effect, RIT allows for rapid loading of allergen over 2-3 days with subsequent weekly "maintenance" therapy, which may be useful in some situations. The rapid results of rush immunotherapy allows avoidance of confounding environmental changes and drug interactions (specifically corticosteroids). Rush immunotherapy has been shown to improve clinical signs, reduce airway eosinophilic inflammation and alter molecular evidence of allergic inflammation in experimental feline allergic asthma (Lee-Fowler et al., 2009; Reinero et al., 2006). Additionally, RIT adjuvanted with CpG-ODN was shown to be highly efficacious in reducing eosinophilic airway inflammation (Reinero et al., 2008). These studies in experimental feline asthma models are exciting and evaluation of these therapies in naturally occurring disease is important in the future.

Polyunsaturated fatty acids (PUFAs)

The eicosanoid mediators (prostaglandins, thromboxanes, leukotrienes, etc.) have key regulatory inflammatory effects in the body and are formed from 20 carbon polyunsaturated fatty acids (PUFAs). The predominant PUFA in most inflammatory cells is n-6 PUFA arachidonic acid and is the predominant substrate used in eicosanoid synthesis. While blockade of selected parts of this inflammatory cascade has long been exploited therapeutically with the usage of non-steroid anti-inflammatory drugs (COX inhibitors), not all eicosanoids are pro-inflammatory in nature (Calder, 2009). For example lipoxins have recently been recognized as anti-inflammatory and diminished biosynthesis of these molecules has been reported in severe asthmatics (Calder, 2009; Levy et al., 2005). Based on the particular PUFA, the rate and type (ie pro- or anti-inflammatory) of eicosanoid production varies (Calder, 2009).

Many cold water oily fish contain high concentrations of n-3 PUFAs eicosapentaenoic acid (EPA) and dososahexanoic acid (DHA). Both EPA and DHA have been shown to have important anti-inflammatory properties such as blunting of cytokine production, inflammatory cell function and eosinophil chemotaxis (Endres et al., 1993; Kikuchi et al., 1998; Lee et al., 1985). In humans, n-3 PUFAs have been studied in both young and old asthmatic patients with varying results (Wong, 2005). In a prospective clinical trial in children, dietary supplementation of either n-3 or n-6 PUFAs was given for a 6 month period. There was no difference in clinical parameters between the children that received n-3 PUFAs compared to n-6 PUFAs (Hodge et al., 1998). Interestingly, children that received n-3 PUFAs had reduction in TNF production, from PBMCs, when compared to baseline.

In humans, there has been conflicting results about the benefits of n-3 PUFAs in asthmatic adults. In a clinical trial, patients receiving Lyprinol (a n-3 PUFA lipid extract from New Zealand green-lipped mussels, *Perna canaliculus*) had reduced daytime wheezing and morning peak expiratory flow. However, symptoms in the evening and overall usage of short-acting bronchodilators did not significantly differ from controls (Emelyanov et al., 2002). The authors of this study did not directly investigate other measures of airway inflammation. Another study in adult asthmatics revealed that dietary supplementation of n-3 PUFA resulted in improved markers of airway inflammation; most notably reduction in exhaled nitric oxide and decreased serum eosinophilia (Schubert et al., 2009). However, patients in this study did not have decreased sputum eosinophils or blunted clinical symptoms when challenged with allergy.

In experimental feline asthma, n-3 PUFAs when given with luteolin (an antioxidant) improved some markers of airway inflammation and airway hyperresponsiveness (Leemans et al., 2009). Specifically, the cats treated with n-3 PUFA/luteolin had increased BALF lipoxin A4 post allergen challenge (REF). Lipoxin A4 is an anti-inflammatory mediator that has been shown to be diminished in severe human asthmatics (Levy, 2005; Levy et al., 2005). Additionally, cats treated with n-3 PUFAs had a significant reduction in airway hyperresponsiveness (measured using barometric whole body plethysmography) 48 and 72 hours after allergen exposure. Importantly, the cats in the treatment group did not have a significant reduction in the percentage of eosinophils in their BALF, making dietary change alone unsuitable as the sole treatment for allergic asthma. Thus, while it has been shown that experimentally

asthmatic cats may have reduction of airway hyperreactivity with increases in dietary 3-n PUFAs, their usage appears to be more appropriate for adjunctive treatment along with other traditional therapies.

Summary:

As current therapies for feline allergic asthma are only palliative, there is a need for other treatments which might alter the aberrant immunologic response driving asthma. There are a growing number of exciting immunomodulators that may have significant benefits in asthmatic patients. Some of these immunomodulators that have been studied specifically in cats are mentioned in this chapter. The most promising appears to be allergen-specific immunotherapy potentially in conjunction with an adjuvant like CpG motifs which has been demonstrated to reduce eosinophilic airway inflammation and reduce clinical signs of bronchoconstriction.

Future directions with the novel immunomodulator feG-COOH:

Another immunomodulator that has shown promise in rodent and sheep models of allergic disease is the salivary tripeptide feG-COOH (feG). In an experimental model of feline asthma, a proof of concept study illustrated that a single oral dose of feG significantly decreased eosinophilic airway inflammation when given prior to allergen challenge (DeClue et al., 2009). However, these cats were only exposed to a single allergen challenge and also did not have complete resolution of there eosinophilic airway inflammation. Therefore, the effect of feG in experimental asthmatic cats when administered chronically with repeated allergen challenge deserved further evaluation. Chapter 2 of this thesis will discuss the results of a prospective, randomized, masked,

cross-over design study to evaluate the efficacy of feG in an experimental model of feline asthma.

Chapter 2

Chronic Use of the Immunomodulating Tripeptide feG-COOH in Experimental Feline Asthma

Introduction:

Feline asthma is thought to be an allergic response to inhaled allergens (Norris Reinero et al., 2004; Reinero et al., 2009a). These aeroallergens activate CD4+ T helper 2 (Th2) cells resulting in elaboration of cytokines which help orchestrate the resultant inflammatory airway disease. Although an oversimplification, naïve Th cells can either be polarized towards a Th2 phenotype in which case interleukin-4 (IL-4) is the key cytokine or towards a Th1 phenotype where IFN-γ is the hallmark cytokine. While each of these cytokines has counter regulatory effects on one another, both lead to inflammation. Numerous inflammatory mediators have been implicated in the production, chemoattraction and biological effects of inflammatory cells that infiltrate the airways. One example is TNF-α, a chemoattractant for both eosinophils and neutrophils, which is increased in the bronchoalveolar lavage fluid (BALF) of asthmatics (Brightling et al., 2008; Howarth et al., 2005). Activation of inflammatory pathways, cytokine release and recruitment of inflammatory cells in asthmatics leads to the classic findings of airway eosinophilic inflammation, hyperreactivity and remodeling (Norris Reinero et al., 2004; Padrid et al., 1995).

Glucocorticoids and bronchodilators are routinely administered to diminish airway inflammation and temporarily reverse the bronchoconstriction seen in asthmatic patients. However, undesired adverse effects and/or certain concurrent health conditions

limit the use of glucocorticoids and bronchodilators in some patients. Thus, there is a need for other therapeutics to address airway inflammation and airway smooth muscle constriction in cats with asthma.

The cervical sympathetic trunk-submandibular gland axis provides communication between the immune, nervous and endocrine systems (Dery et al., 2001; Sternberg, 2001). The submandibular salivary glands produce multiple peptides that have a wide variety of local and systemic physiologic properties outside the gastrointestinal tract. There is evidence that one of these salivary peptides, feG-COOH (feG), has the potential to ameliorate eosinophilic cellular infiltration, neutrophilic cellular infiltration, and airway hyperreactivity; all important aspects of the pathogenesis of asthma (Dery et al., 2001; Dery et al., 2004; Mathison R, 2004). However, these studies have primarily focused on effects of feG on cell adhesion molecules and reactive oxygen species production, and have not evaluated cytokines involved in inflammatory pathways.

We have previously investigated the effect of single dose of feG on eosinophilic airway inflammation immediately prior to allergen challenge in our experimental model of feline asthma (DeClue et al., 2009). While airway inflammation was attenuated, only one of the seven cats treated with feG had a post-allergen challenge BALF eosinophil percentage in the normal range and the cats were only exposed to a single allergen challenge. Therefore, the effect of feG in experimentally asthmatic cats when administered chronically (2 weeks) with repeated allergen challenges deserves further study. The purpose of the current study was to determine if feG altered the clinical response, BALF cellular composition and BALF inflammatory mediator composition

when administered chronically to cats with experimental asthma. Our hypothesis was that a 2 week treatment period of oral feG would decrease airway inflammation, inflammatory cytokine profiles and clinical signs of airflow limitation compared with placebo.

Materials and Methods:

Animals - Ten healthy, 16 week old, mixed breed cats (5 sexually intact females and 5 sexually intact males) bred from a high-responder asthmatic cat research colony (University of Missouri, Columbia, MO) were used in this study. Cats were cared for according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals and all procedures were approved by the University of Missouri Animal Care and Use Committee. Cats were fed a commercially available kitten food and water ad libitum. Upon entry to the study cats weighed between 2.16-3.4 kg (mean 2.78 kg).

Allergen sensitization and aerosol challenge - Prior to induction of an asthmatic phenotype, all cats were tested to confirm they had not previously been sensitized to Bermuda grass antigen (BGA) by performance of intradermal skin testing (IDST). Additionally, they were screened to ensure they did not have preexisting eosinophilic airway inflammation by analysis of bronchoalveolar lavage fluid (BALF). The mean±SE BALF % eosinophils at baseline was 2.7% ± 1.6%. Cats were sensitized and challenged as previously described with minor modifications (Reinero et al., 2008). Briefly, on day 0, cats received a SC injection of 12 μg of BGA in 10 mg of alum and to induce IgE antibody isotype switching an intramuscular injection of 10^7 *Bordatella pertussis* organisms; on day 14, 75 μg BGA in 0.2 ml of PBS intranasally; and on day 21, another

SC injection of 12 μg of BGA in 10 mg of alum. On day 28, IDST was repeated to confirm sensitization to BGA. Although wheals to BGA were seen in all cats, six cats were boostered with a third SC injection of 12 μg of BGA in 10 mg of alum due to marginally positive reactions.

After parenteral sensitization, aerosol allergen challenge exposure was conducted on awake, spontaneously breathing cats in a sealed chamber. An air compressor attached to a nebulizer was used to aerosolize the allergen solution (200 μg of BGA dissolved in phosphate buffered saline solution delivered for 10-15 minutes/treatment). Allergen aerosol challenges were performed 7 times in 2 weeks. The asthmatic phenotype was confirmed prior to initiation of treatments by collecting BALF. We defined an asthmatic phenotype as a post-allergen challenge BALF eosinophil percent >16%; the mean±SE BALF % eosinophils after allergen sensitization and challenge was 47.8 % ± 4.0%. One cat was removed from the study for not having a BALF eosinophil percentage >16% prior to therapy.

Treatments - This study was conducted as a prospective, randomized, blinded, cross-over design with each cat acting as its own control. Asthmatic cats were randomized to receive either feG (1 mg/kg diluted into 1 ml 0.9% NaCl, PO) (CS Bio Company, Menlo Park, CA) or placebo (1 ml 0.9% NaCl, PO) as a single dose once daily for 2 weeks. Treatments were administered by an investigator (JME) blinded to the animal's treatment group assignment. There was a 2 week washout period before crossover to the other treatment. Each cat received an aerosol BGA challenge (200 μg) weekly throughout the study; aerosol challenges were coordinated so they occurred 24 hours prior to collection of blood and BALF.

Clinical Scoring - As a clinical correlate for severity of allergen-induced airway hyperreactivity/bronchoconstriction, a clinical scoring system was employed to grade the clinical signs the cats displayed during and immediately after delivery of allergen via aerosol (Table 1). The clinical scoring system was performed by the same investigator (JME) and was performed at the time of BGA challenge by aerosol that was done 24 hours prior to sample collection. The clinical scoring system is shown in Figure 2-1.

Figure 2-1. Respiratory scoring system (0-9 points)

Respiratory rate changes
0 points--<50% increase in baseline RR
0.5 points -->50% increase in baseline RR
1 point -->100% increase in baseline RR

Auscultation (pre-chamber)
0 points—no wheezes
1 point—wheezes

Auscultation (post-chamber)
0 points—no wheezes
1 point—wheezes

Inducible cough (pre-chamber)
0 points—no
1 point—yes

Inducible cough (post-chamber: 30 min later)
0 points—no
1 point—yes

Clinical signs (post-chamber)
0 points—no signs
0.5 points—mild to moderate increase in respiratory effort but not enough to affect activity or mentation; spontaneous cough
1.0 point—moderate signs of respiratory difficulty (expiratory push or rapid and shallow breathing)
1.5 points—moderate to severe signs of respiratory difficulty (nostrils flaring, impaired activity, obvious distress)
2.0 points—severe signs (open mouth breathing, lateral collapse, obtunded)

Time in chamber
0 points—10 minutes
0.5 points—5 to <10 minutes
1 point—2 to <5 minutes
1.5 points—1 to <2 minutes
2 points--<1 minute

Sample collection - Samples were collected prior to starting each treatment ("baseline") and after each 2 week treatment period. Whole blood was collected by jugular venipuncture into lithium heparin tubes for analysis of TNF bioactivity and EDTA tubes for in vitro cytokine restimulation assay (see below). Collection of BALF was performed using a blind technique. Briefly, the cats were anesthetized with ketamine (5-10 mg/kg, IV to effect) (Ketaset, Fort Dodge Animal Health, Ft. Dodge, IA) and intubated. A sterile 8 Fr, open ended red rubber catheter was advanced through the endotracheal tube and lodged in a bronchus. Warmed sterile saline (20 ml) was infused through the red rubber catheter and aspirated back with gentle manual suction. The BALF was then placed on ice for transport to the laboratory where total cell counts and differential counts were performed within 1 hour. Additionally, BALF supernatant was harvested after centrifugation (300 x g for 6 minutes) and banked in aliquots at -20^0 C until analysis.

Bronchoalveolar lavage fluid cellular composition - An investigator blinded to the treatments performed cell counts and differentials (JME). Total nucleated cell count (TNCC) of BALF was determined using a Coulter counter (Z1 particle counter, Beckman Coulter, Fullerton, CA). Cytological evaluation and differential cell counts were performed on samples prepared by cytocentrifugation (Shandon Cytospin 4, ThermoElectron Corporation, Waltham, MA). Differential cell counts were performed by evaluating 200 cells on a Wright's stained preparation and expressing the relative ratio of each cell type as a percentage.

In vitro cytokine restimulation assay - Using whole blood collected into EDTA tubes, peripheral blood mononuclear cells were isolated using Histopaque density centrifugation and were resuspended in complete RPMI (RPMI with FBS, Hepes, beta-mercaptoethanol and penicillin-streptomycin-glutamine (Gibco, Invitrogen Corporation, Grand Island, NY) to a concentration of 5 x 10^6 cells/well (Reinero et al., 2008). Cells were then cultured at 37^o C in humidified 5% CO_2/95% air (Hera Cell 150, Kendro Laboratory Products, Langenselbold, Germany) for 24 hours in a 24-well flat bottomed plate (Multiwell 353047, Becton Dickinson Labware, Franklin Lakes, NJ). Two wells per cat were plated in a final volume of 800 μl: one well contained cells with cRPMI only and the second well contained cells incubated with 20 μg BGA in cRPMI. Supernatant was harvested after 24 hours and banked at -20^{o}C.

Cytokine analysis - Bronchoalveolar lavage fluid supernatant was used for determination of IL-1, IL-4, IL-6, CXCL-8 and IFN-γ concentrations. Peripheral blood mononuclear cell (PBMC) culture supernatant was used for determination of IL-4 and IFN-γ concentrations. Tumor necrosis factor (TNF) bioactivity was measured in BALF supernatant and plasma. Interleukin-1, IL-4, IL-6, CXCL-8 and IFN-γ concentrations were determined using commercially available feline-specific cytokine ELISA kits (RnD Systems, Minneapolis, MN). The range of detection for IL-1and IL-6 ELISAs is 32.5-2000 pg/ml. The range of detection for IL-4, CXCL-8 and IFN-γ ELISAsis 62.5-4000 pg/ml. Samples of BALF supernatant and PBMC culture supernatant were run in triplicate.

Tumor necrosis factor bioactivity was evaluated using a previously described cell killing bioassay (Baarsch et al., 1991; DeClue et al., 2008). Briefly, 3.0 x 10^5 cells from

mouse fibroblast cell line (L929) were cultured on 96 well plates for 12 hours. Fifty microliters of BALF and plasma samples were then added to the wells in triplicate. After a 20 hour incubation with MEM (Gibco, Invitrogen Corporation, Auckland, NZ) plus horse serum (1%) (Gibco, Invitrogen Corporation, Auckland, NZ) and actinomycin D (3ug/ml) (Sigma-Aldrich, St. Louis, MO), 3-[4,5-dimethylthiazol-2-yl]-2,5,-di-phenyl tetrazolium bromide (i.e., MTT) (Sigma-Aldrich, St. Louis, MO) was added and the cells incubated for an additional 2.5 hours. Formazen crystals were solubilized in dimethylformanide (50%) (Fischer Scientific International, Pittsburgh, PA) and SDS (20%) (Fischer Scientific International, Pittsburgh, PA). Color development was measured at 630 nm. Feline recombinant TNF (Fischer Scientific International, Pittsburgh, PA) was used to construct a standard curve to quantify the TNF activity in the test wells. The range of detection of this assay is 0.25-25 ng/ml.

Data analysis - Statistical analyses were performed using commercially available software (SigmaStat, Systat Software Inc., Chicago, IL). The Kolmogorov-Smirnov test was used to determine if data were normally distributed. Results of each of the parameters were compared between treatments using a repeated measures ANOVA and Fisher least significant difference method was used for post-hoc comparisons. Data were reported as mean±SD unless otherwise indicated. A p-value of <0.05 was considered statistically significant.

Results:

Bronchoalveolar lavage fluid (BALF) analysis - At baseline, there was no significant difference in the BALF TNCC (placebo, 1.4±0.4 $x10^6$ cells/ul: feG, 3.0±2.3 $x10^6$ cells/ul;

p=0.14), absolute eosinophil count (placebo, 6.2±2.5 x10^5 cells/ul: feG, 13.2±9.9 x10^5 cells/ul; p=0.057) or eosinophil percentage (placebo, 45±10%: feG, 45±16%; p=0.965). There was a significant difference in BALF neutrophil percentage (placebo, 10±8%: feG, 2±2 %; p<0.01) but not the absolute neutrophil count (placebo, 1.3±1.0 x10^5 cells/ul: feG 0.5±0.6 x10^5 cells/ul; p=0.052) between the two treatments at baseline. After two weeks of treatment, there was no significant difference in the BALF TNCC (placebo, 1.21±0.23 x10^6 cells/ul: feG 1.23±1.10 x10^6 cells/ul; p=0.14), eosinophil % or neutrophil % between feG and placebo treatments (Figure 2-2).

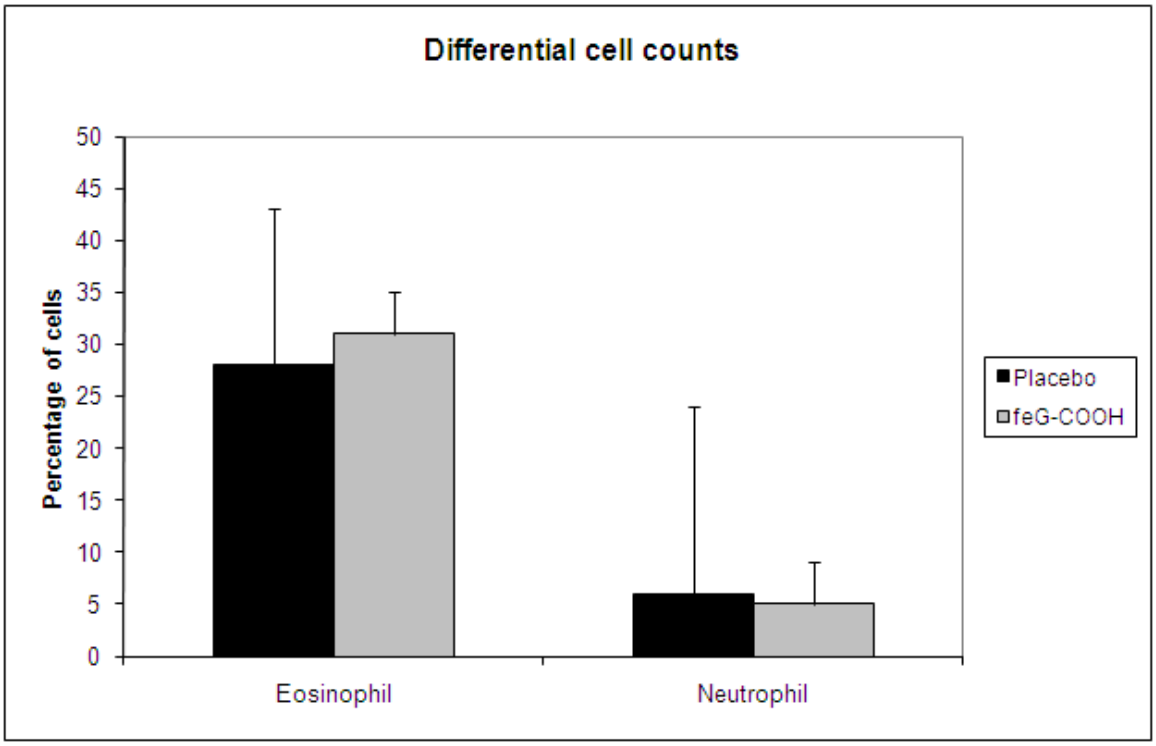

Figure 2-2. Bronchoalveolar lavage fluid (BALF) eosinophil and neutrophil differential cell counts from cats with experimental asthma after 2 weeks of treatment with feG-COOH (1mg/kg/day) or placebo. Administration of feG did not significantly blunt airway inflammation compared with placebo as evidenced by lack of a difference in BALF post-treatment eosinophil percent (p=0.479) or neutrophil percent (p=0.504).

Cytokine analysis (BALF supernatant, in vitro restimulated PBMCs, plasma) -

Greater than 40% of the BALF supernatant samples had IL-1, IL-4, IL-6 and IFN-γ

concentrations below the lower limit of detection regardless of time point or treatment administered. Bronchoalveolar lavage fluid CXCL-8 was below the lower limit of detection for 2/9 placebo and 3/9 feG treatment samples. Interleukin-4 and IFN-γ concentrations in the cell culture supernatant from stimulated PBMCs were below the lower limit of detection for all samples. Due to the low number of samples that had detectable concentrations of IL-1, IL-4, IL-6, CXCL-8 and IFN-γ, statistical analyses of these data were not meaningful. Tumor necrosis factor was detectable in the BALF and plasma of all cats. However, there was no significant difference in BALF or plasma TNF at baseline or between treatments (Figure 2-3).

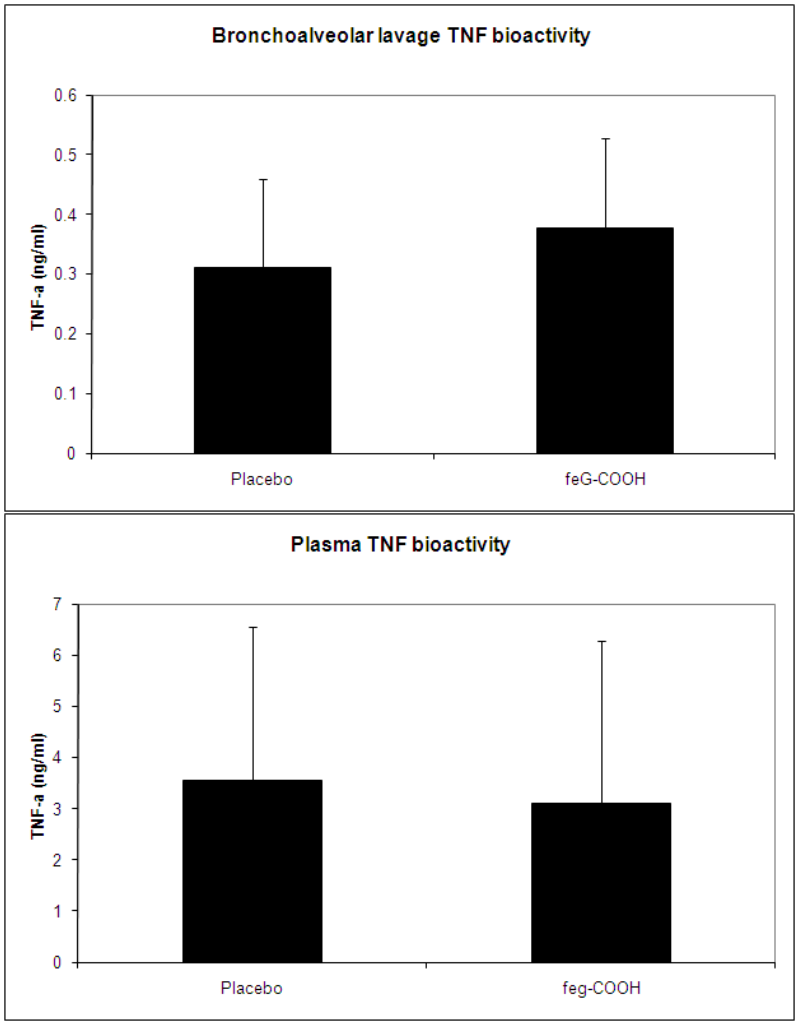

Figure 2-3. Bronchoalveolar lavage fluid and plasma TNF bioactivity from cats with experimental asthma after 2 weeks of treatment with feG-COOH (1mg/kg/day) or placebo. There was no significant difference in BALF or plasma TNF between treatments (p=0.251 and p=0.333, respectively).

Respiratory scores - There was no significant difference in the baseline clinical scores between the two treatments (placebo, 0.7±1.0, feG, 1.0±1.5, p=0.362). Additionally, there was no effect of placebo versus feG in post-treatment clinical scores (placebo, 1.2±1.1, feG, 1.0±1.1, p=0.386).

Discussion:

Regular (2 week) use of feG-COOH in experimentally asthmatic cats did not significantly diminish eosinophilic airway inflammation or improve clinical signs of bronchoconstriction after allergen challenge. As one of the hallmarks of asthma, eosinophilic airway inflammation is a vital target for therapy because it significantly contributes to both airway hyperreactivity and remodeling (Cohn et al., 2004). Previously we demonstrated that a single dose of the salivary tripeptide feG at 1 mg/kg administered prior to allergen challenge partially blunted eosinophilic airway inflammation evaluated 24 hours later (DeClue et al., 2009). However only 1/7 cats had complete resolution of airway eosinophilia. Since a single dose of feG failed to completely control airway inflammation, we wanted to investigate if chronic administration of feG would be more efficacious. Additionally, prediction of allergen exposure is not generally feasible in pet cats so it was important to evaluate the anti-inflammatory properties of feG after sequential allergen challenges.

The important role of salivary peptides in allergic disease was first identified by Ramaswamy et al. and Mathison et al. who discovered that denervation of the salivary gland (i.e., up regulation of salivary peptides) results in significant reduction of allergen-induced airway inflammation in rats and concurrent sialadenectomy nullified these effects (Mathison et al., 1992; Ramaswamy et al., 1990). More recently, the salivary peptide, feG was shown to blunt allergen-induced eosinophilic airway inflammation in rats (Dery et al., 2001; Dery et al., 2004). While these studies are important for proof of concept, for clinical use it is critical to evaluate whether a therapy will be effective when given on a consistent basis when timing of aeroallergen exposure is unknown. Based on the results of this study, regular administration of feG can not be recommended as monotherapy for the treatment of feline asthma as it did not effectively blunt eosinophilic inflammation.

The protective effects of feG during allergen-induced inflammation are due, at least in part, to down regulation of eosinophil intracellular adhesion molecule-1 (ICAM-1), a surface receptor necessary for eosinophil transmigration from the blood into the airway (Dery et al., 2004; Mathison et al., 2003). Although there is evidence that salivary peptides play a role in immunoregulation (Kemp et al., 1985), other mechanisms by which the salivary tripeptide feG controls eosinophilic airway inflammation have not been elucidated. The lack of difference between placebo and feG treatment in this study was not altogether surprising given that there are multiple redundant and overlapping inflammatory pathways in allergic asthma. Simply blunting one pathway of inflammation, like ICAM-1 expression, could easily be overridden by up-regulation of other pathways with chronic aeroallergen exposure. Other explanations for the lack of

efficacy may relate to feG's biological half-life, absorption, and sequence homology to the feline salivary peptides.

As there are no clear cut criteria to definitively discriminate cats with chronic allergic asthma from those with chronic bronchitis, the role of the neutrophil in feline asthma is still highly controversial. Eosinophils are still considered the hallmark cell infiltrating airways in allergic asthma, but neutrophilic inflammation can also contribute to allergic airway inflammation in humans (Douwes et al., 2002; Gibson et al., 2001) and neutrophils have been recognized in a different cat model of allergic airway inflammation (Kirschvink et al., 2007). In rodent models of allergic airway inflammation, a single dose of feG given 30 minutes prior to allergen challenge and given up to 6 hours post allergen challenge, blunted neutrophilic airway inflammation (Dery et al., 2001; Dery et al., 2004). Our model, admittedly, is a poor test for neutrophilic inflammation as cats generally displayed very little BALF neutrophilia, therefore feG's effects on dampening a neutrophilic component of allergic inflammation have not been adequately tested making it unclear as to whether feG might have a therapeutic role in conjunction with other medications in select cases of feline asthma.

While there are numerous cytokines and chemokines that help orchestrate the inflammatory response in asthma, use of a feline model to study pathophysiology and response to therapy is limited by available feline-specific reagents. The cytokines and chemokines evaluated in this study (i.e., IL-1, IL-4, IL-6, CXCL-8, IFN-γ and TNF-α) were primarily selected because they play a role in feline or human asthma (Bureau et al., 2000; Gibson et al., 2001; Kumar et al., 2006; Norris Reinero et al., 2004; Ohkawara et al., 1997) and there are feline specific assays available to measure them. It was

disappointing that many of the samples had cytokine/chemokine concentrations below the lower limit of detection of the assays used. However, the authors felt it was important to include this information so that future investigators are aware of the limitations of these methodologies. Most human and rat ELISA kits are available that can detect inflammatory mediators <10 pg/ml while the lowest limit of detection of the ELISA kits used in this study ranged from 32.5-125 pg/ml. Techniques such as the use of a bench top concentrator (Speed-Vac, Thermo Fisher Scientific, Waltham MA) or low molecular weight cutoff filters to concentrate BALF have been attempted in our lab but have yielded inconsistent results using spike and recovery experiments (unpublished data). The development of more sensitive feline specific assays or modalities to reliably concentrate samples is needed and may have been useful in detecting a significant difference between the feG and placebo treatments.

Tumor necrosis factor is a cytokine with a myriad of functions; relevant to asthma, it is an important chemoattractant for eosinophils and also enhances expression of ICAM-1 on epithelial cells facilitating eosinophil chemotaxis (Lassalle et al., 1991; Lukacs et al., 1995). A prior study using feG documented reduction in BALF TNF activity when feG was administered prior to allergen challenge, it also demonstrated a significant reduction in BALF eosinophilia (DeClue et al., 2009; Dery et al., 2004). In this study we did not document a significant difference in BALF or plasma TNF activity between treatments, nor did we demonstrate a reduction in airway eosinophilia.

Airway hyperresponsiveness, defined as an increased bronchoconstrictor response to a nonspecific stimulus, is a hallmark feature in patients with asthma and has been linked to the perception of clinical signs in humans with asthma (Bijl-Hofland et al.,

1999; Koh et al., 2001). In a sheep asthmatic model, two oral doses of feG (given 24 hours and 30 minutes prior) or a single inhaled dose (given 20 minutes prior) to allergen challenge, significantly reduced bronchoconstriction and airway hyperresponsiveness the latter determined after carbachol challenge (Mathison R, 2004). Methods that have been previously used to determine airflow limitation in cats (e.g., using bronchoprovocants with direct measures of airway resistance under anesthesia or with indirect measures that are loosely correlated with airway resistance such as barometric whole body plethysmography) require special equipment unavailable to us during this study (Kirschvink et al., 2007; Norris Reinero et al., 2004). In adult humans with asthma, questionnaires that include clinical signs are important tools for predicting the likelihood a patient has allergic airway disease (Hirsch et al., 2004; Price et al., 2006). Clinical scoring systems have been previously used in the evaluation of cats with experimental asthma (Reinero et al., 2009b). The clinical scoring system developed for this study took into account important clinical markers that are suggestive of bronchoconstriction. In this study, there was no significant difference in the clinical scores after allergen challenge between the feG treatment and placebo treatment. While this would support lack of efficacy with chronic therapy on clinical signs exhibited in cats, the study did not address whether intermittent dosing of feG might be beneficial for cat presenting in an acute crisis ("status asthmaticus") to help alleviate increases in airway resistance.

In conclusion, in cats with experimental asthma, daily use of feG-COOH for 2 weeks during chronic aeroallergen exposure did not dampen eosinophilic or neutrophilic airway inflammation, reduce TNF-α bioactivity in the plasma or BALF, or decrease clinical signs associated with allergen challenge. The results of this study support that

feG at this dosage can not be recommended as therapy for the chronic treatment of allergic asthma in cats. Whether feG has a role in the acute management of asthmatic attacks in cats has yet to be determined.

Chapter 3

Conclusions and Future Directions

Asthma is a common respiratory disease in cats that is believed to be driven by an immune response against what should be benign aeroallergens. The mechanisms of this disorder in cats have not been completely elucidated. Current therapies are only palliative and do not provide correction of the underlying immunopathogensis and have no curative potential. However, exciting experimental and clinical research has shown promising results from new immunomodulators. The first chapter of this thesis was a brief review of three immunomodulators: CpG-ODNs, ASIT and dietary fatty acids.

In experimental models, CpG-ODNs have been shown to reduce allergic eosinophilic airway inflammation, airway hyperreactivity and airway remodeling. Likewise, there is growing data to support the benefit of ASIT in the control, and even cure, of allergic disease. However, both these therapies have shortcomings and have been slightly less impressive when used in large animal models of allergic asthma (e.g., the cat), underscoring the need for better pre-clinical models then current rodent models. Because of the complexity of the immune system with redundant and overlapping pathways, altering one aspect of the allergic inflammatory cascade may not result in expected clinical benefits. Combining ASIT with CpG-ODNs (i.e., an adjuvanted ASIT protocol) has promise in experimental feline asthma. Future studies need to be performed to compare adjuvanted ASIT directly with conventional ASIT to see if the combination therapy is safer, more efficacious, and has a shorter treatment timeline. The importance of this last advantage should not be underrated since this will improve patient/owner

compliance and reduce overall therapy cost; both of which are important considerations in human and veterinary medicine. Also, much like rush immunotherapy, there is benefit with shorter protocols in reducing the influence of drugs and environmental changes in individual patients.

While there is some evidence of immunomodulation with n-3 PUFA supplementation in allergic disease, there is currently not enough evidence to justify their usage or investigation as monotherapy. Yet, this does not negate a possible role as part of a combined treatment plan for an individual patient. Refractory asthma is still a problem in both humans and cats and adjunctive therapy with n-3 PUFAs may have potential to benefit a subset of these patients while having minimal side effects. Additional studies are needed in the future.

The second chapter of this thesis described the evaluation of the daily use of a novel immunomodulator, feG-COOH, in an experimental model of feline asthma. Previous experimental evidence supported that feG can ameliorate eosinophilic cellular infiltration, neutrophilic cellular infiltration, and airway hyperreactivity. (Dery et al., 2001; Dery et al., 2004; Mathison R, 2004). Additionally, our laboratory showed that a single dose of feG, given prior to a single allergen challenge, could reduce eosinophilic airway inflammation in our experimental model of feline asthma (DeClue et al., 2009). These results warranted further investigation of feG in experimentally asthmatic cats. Specifically, chronic administration with repeated allergen challenge was important to evaluate to determine if feG had potential applications in cats with naturally occurring asthma.

With "chronic" (2 week) administration of feG in our experimental feline asthma model, our results indicate that while therapy was safe and well tolerated, there was no benefit in terms of reduction of eosinophilic airway inflammation, airway or systemic markers of inflammation or clinical signs. Since inflammation is a driving force to induce further airway hyperreactivity and airway remodeling, it is critical that airway inflammation is effectively blunted if the treatment is to be used as monotherapy. Our study provided evidence that feG, alone, is insufficient as a sole treatment for feline asthma.

While our study does not support the usage of feG as a monotherapy for control of feline asthma, investigation should still be done to determine if it has benefit in other aspects of management of feline asthma. For example, feG may be useful as an adjunct to more traditional therapies (i.e. glucocorticoids) and allow reductions in either dosage or frequency of administration, reducing the undesirable side effects of these medications. Likewise, feG has also been shown to reduce airway hyperreactivity in an experimental sheep model (Mathison R, 2004). Airway hyperreactivity is an important factor to control since it significantly predisposes a patient to acute clinical signs through increased risk of bronchoconstriction. Studies should be designed to evaluate pulmonary mechanics using previously described methods in cats such as barometric whole body plethysmography (Kirschvink et al., 2007) or in using newer technology such as a mechanical ventilator that has a built in heated pnemotachograph capable of directly reporting airway resistance and lung compliance.

Our study also emphasized the need for additional feline-specific immunologic assays (to measure concentrations or activity of inflammatory markers) and for

improvement of the sensitivity of the current assays. Immunologic markers are not only important in understanding more about the pathogenesis of disease, but also for diagnosis, monitoring exacerbations or improvement in response to therapy, and potentially to provide prognostic information. Many key cytokines in asthma (e.g., IL-5, IL-13) do not have commercially available feline-specific assays for their detection. One major limitation of the currently available feline specific ELISAs for IL-1, IL-4, IL-6, CXCL-8 and IFN-γ was poor assay sensitivity. This is a particular problem with dilute samples like bronchoalveolar lavage fluid. Attempts by our laboratory to concentrate samples either by a Speed-Vac technique or by selective filtration did not lead to consistent and repeatable results for the cytokines assayed. Ultimately, having more accurate and sensitive assays available will improve our ability to determine if and how an investigated therapy alters immunologic pathways.

Our laboratory is actively evaluating what immunomodulators are beneficial in an experimental feline asthma model. As we continue to develop experimental protocols that show safety and promise in experimental feline asthma these techniques will become available in naturally occurring feline asthmatics and potentially in humans. It will be important that these be prospective studies that compare novel therapies to traditional therapies (i.e. glucocorticoids, bronchodilators) and evaluate eosinophilic airway inflammation, immunomarkers of airway inflammation, airway hyperresponsiveness and clinical signs of asthmatic control.

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