THE INDUCTION OF ANTIGEN-SPECIFIC IMMUNOLOGICAL TOLERANCE AND ATTENUATION OF INFLAMMATION VIA CYTOKINE ANTIGEN FUSION PROTEIN THERAPY IN A MOUSE MODEL OF PULMONARY INFLAMMATION

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Department of Physiology, December, 2013.

Allergic asthma is a significant medical issue, affecting more than 300 million individuals and causing approximately 250,000 deaths each year. Current asthma therapies temporarily minimize discomfort and manage symptoms, but there are no effective long-term, preventative, or curative agents available. Recent studies have indicated that targeting immunogens to specific immune cells via fusion with cytokines increases the effectiveness of immunotherapy in animal models for asthma as well as other immune-mediated disease models such as experimental autoimmune encephalomyelitis. This study tested the hypothesis that fusion proteins comprised of granulocyte macrophage colony stimulating factor (GM-CSF) and OVA 323-339 epitopes potentiate antigen-specific immunological tolerance and attenuate development of pulmonary inflammation. Fusion products were generated through combining OVA 323-339 and GM-CSF sequences, and biological activity of fusion protein cytokine and antigenic domains was assessed. A successful model of ovalbumin-induced inflammation in mice was then established for verification of cytokine fusion protein vaccine efficacy. In the model, pre-treatment with the cytokine fusion protein vaccine was tested for ability to attenuate OVA-induced increases in inflammation. GMCSF-OVA 323-339-treated mice

showed significantly decreased numbers of pro-inflammatory eosinophils, neutrophils, and lymphocytes, and cellular infiltration in perivascular, peribronchial, peribronchiolar, and parenchymal spaces were visibly lessened. In addition, small airway mucus deposition was reduced in mice given the fusion protein. In this study, it was found that pre-treatment of mice with GMCSF-OVA 323-339 fusion proteins reduced pulmonary inflammation when compared to mice given saline treatment, demonstrating that GMCSF-OVA 323-339 fusion proteins have the capability of influencing immune responses within a murine model of pulmonary inflammation. Data from this study suggests potential for cytokine-fusion proteins as a therapeutic tool in pathological pulmonary inflammation as well as in other fields involving immune system-mediated pathologies.

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To my family,

whose love and support make anything possible

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[3H]thymidine	Tritiated thymidine
AB-PAS	Alcian blue periodic acid-Schiff [stain]
AcGFP1	*Aequorea coerulescens* green fluorescent protein 1
AHR	Airway hyperresponsiveness
APC	Antigen presenting cell
APC	Allophycocyanin [fluorochrome]
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BMCs	Bone marrow cells
CCL17	Chemokine (C-C motif) ligand 17
CCL22	Chemokine (C-C motif) ligand 22
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CTLL-2	Cytotoxic T-lymphocyte Line
CysLTR-2	Cysteinyl leukotriene receptor
DC	Dendritic cell
DO11.10	OVA-specific, MHC-II-restricted, α β TCR transgenic mouse (BALB/c mouse background)
EAE	Experimental autoimmune encephalomyelitis
EAR	Early allergic response
EM	Expression medium

FcεRI	High affinity IgE receptor
FcγRI	High affinity IgG receptor
FEV_1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate [fluorochrome]
FVC	Forced vital capacity
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hanks' balanced salt solution
HDM	House dust mite
H&E	Hematoxylin and eosin [stain]
HEK	Human embryonic kidney [cells]
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
I.p.	Intraperitoneal
iNKT	Invariant natural killer T-lymphocytes
ITAM	Immunoreceptor tyrosine-based activation motif
LAR	Late allergic response
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I

MHC-II	Major histocompatibility complex class II
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHP	Non-human primate
NK	Natural killer [cells]
NKT	Natural killer T cells
OPN	Osteopontin
OVA	Ovalbumin
OVA 323-339	Ovalbumin peptide 323-339
OT-II.2	OVA-specific, MHC-II-restricted, α β TCR transgenic mouse (C57BL/6 mouse background)
OX40L	OX40 ligand
P.a.	Pharyngeal aspiration
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin [fluorochrome]
PerCP	Peridinin chlorophyll protein [fluorochrome]
PIN-1	Peptidyl-prolyl isomerase NIMA-interacting 1
pIRES2-AcGFP1	Mammalian vector containing internal ribosomal entry site, *Aequorea coerulescens* green fluorescent protein, and a Kanamycin/neomycin resistance gene

pVAX	Vector with a kanamycin resistance gene for selection in *E. coli*, designed for DNA vaccine development
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RPMI	Roswell park memorial institute [medium]
SIRPα	Signal regulatory protein-α
sST2	Soluble ST2 receptor
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{EM}	Effector memory T cells
TGF-β	Transforming growth factor-β
T_H	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
T_{Reg}	T regulatory Cell
TSLP	Thymic stromal lymphopoietin
VCAM	Vascular cell adhesion molecule

CHAPTER 1: INTRODUCTION

Allergic Asthma

Allergic asthma, also known as atopic asthma, is a significant medical problem, affecting more than 300 million individuals (over 20 million US) and causing approximately 250,000 deaths (~3,500 US) every year (Global surveillance, prevention and control of chronic respiratory diseases: A comprehensive approach. 2007; Heron et al., 2009). Allergic asthma is the most prevalent form of asthma, accounting for more than 50% of those afflicted with the condition (Asthma at a glance. 1999). Asthma is an immune-mediated chronic disease marked by airway inflammation, obstruction, and hyperresponsiveness to any of a variety of normally innocuous stimuli, leading to respiratory difficulty and possible complications for allergic asthma patients. Current asthma therapies temporarily minimize discomfort and manage symptoms, but there are no effective long-term, preventative, or curative agents available. New methods or compounds for treating allergic asthma are a necessity.

Asthma Pathogenesis

Asthma pathogenesis is the result of an inappropriate immune response to a typically innocuous immunogen. Inhaled aeroallergens are processed via the major histocompatibility complex (MHC) class II pathway, and are internalized by antigen presenting cells (APCs) via endocytosis. Vesicles containing the aeroallergen are then transported to lysosomes for degradation, and as vesicles approach the lysosome, vesicle pH lowers, aiding in degradation of the antigen. In the endosomal compartment, MHC class II (MHC-II) molecules come into contact with degraded antigen and bind

fragments 13-18 amino acids in length, transporting them to the cell surface. MHC-II bound to antigen at the cell surface can then be recognized by CD4+ T cells, that, in the event of recognition and subsequent activation, will activate T helper (T_H) 2 cells (Janeway and Travers, 1997). T_H2 cells will then differentiate into IL-4, IL-5, and IL-13 secretory cells, leading to increases in B cell IgE synthesis, mast cell activation via cross-linking of cell surface FcεRI by IgE and antigen, and the subsequent release of histamine, prostaglandins, and leukotrienes (Busse and Lemanske R.F., 2007). Upon release, these factors have the capacity to interact directly with bronchial smooth muscle, giving rise to an acute, early phase allergic response (EAR) characterized by bronchoconstriction, airway edema, and mucus hypersecretion, resulting in wheezing, airway hyperresponsiveness, and reduced forced expiratory volume in one second (FEV_1). The EAR may be present for 30 minutes to 2 hours, although in rare acute episodes, anaphylaxis, a severe and potentially life-threatening reaction, may occur within seconds or minutes. For nearly 60% of asthmatics, EAR is followed approximately 2 hours later by a late phase allergic response (LAR) that may persist for days. The LAR is due the migration and accumulation of inflammatory immune cells in the airways and the further release of inflammatory mediators. Accumulation of neutrophils and lymphocytes are highest within 24 hours of contact with the antigen, and accumulation of eosinophils is increased by 48 hours (Lommatzsch et al., 2006). LAR is characterized by bronchoconstriction and further wheezing due to airway hyperresponsiveness and reactivity to the released inflammatory mediators (Berend et al., 2008).

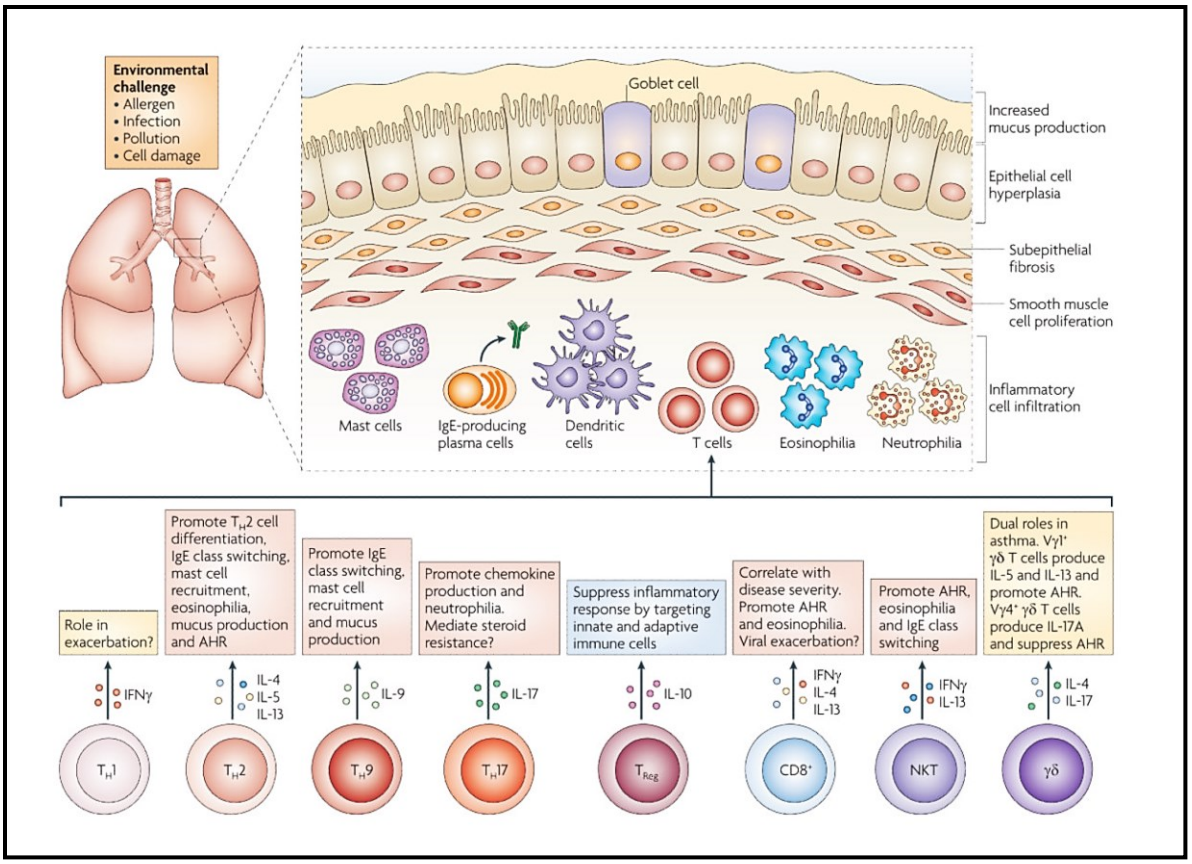
It is generally thought that allergic asthma results from imbalanced immune system T_H1/T_H2 responses, leading to a system that favors T_H2 interactions. The concept that such an imbalance could lead to asthma pathogenesis has roots in the T_H2 hypothesis, which suggests that in balanced systems, CD3+ CD4+ lymphocytes differentiate into either T_H1 or T_H2 cells, and that T_H1 and T_H2 cells reciprocally inhibit the differentiation of one another through IFN-γ and IL-4, respectively (Coffman, 2006). This is not to say, however, that T_H1 cells and cytokines do not contribute to allergic asthma. There is extensive overlap and redundancy inherent in the complex cell and cytokine networks contributing to asthmatic immunopathology, and the intricacies of these networks are central to the understanding of asthma pathogenesis and potential future therapeutic targets.

Cell Network

Various cell types participate in allergic asthma pathogenesis. Some of the key participants in the generation of the response generally include dendritic cells, T helper lymphocytes, Cytotoxic T-lymphocytes, Memory lymphocytes, Type 1 Natural Killer T-lymphocytes, Natural Killer cells, and γδ T cells, while T regulatory lymphocytes (T_{Regs}) aid in the balance and suppression of the response. These cell types can then exert effects on smooth muscle cells, eosinophils, goblet cells, mast cells, B cells, and neutrophils, cell types that are responsible for the mucus production, AHR (airway hyperresponsiveness), and inflammation seen in asthma. The actions of each particular cell type and the interactions between them within the asthmatic cell network underlie the inflammation and pathogenesis observed in allergic asthma (figure 1.1).

Figure 1.1 Overview of cell types involved in asthma pathogenesis

Multiple cell types participate in allergen-mediated pathogenesis. Some of the key participants in the initiation of the response include dendritic cells and various T cell subsets. Cytokines and other mediators from these cells can, in turn, act upon various other cell types, resulting in the further release of pro-inflammatory mediators. Interactions between these cells and their mediators contribute to the mucus production, AHR, and inflammation seen in asthma.



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Dendritic Cells

DCs are considered one of the few “professional” antigen presenting cells due to high efficiency in the up-take and presentation of antigen. Typically, DCs found in peripheral tissues are in an immature state, characterized by fewer T-cell activating costimulatory molecules as well as less efficient antigen presentation capabilities. Immature DCs undergo maturation upon activation, and the activation signals dictate DC responses and subsequent polarization toward T_H1, T_H2, T_H9, T_H17, or T_{Reg} responses. This process begins with extracellular peptides presented on the cell surface of DCs within the peptide binding groove of MHC-II molecules and as a unit, may lead to recognition by T cell receptors. Upon recognition, T cells synthesize and express CD40 ligand, connecting with CD40 on the surface of DCs, resulting in the DC synthesis of B7 costimulatory molecules, ligands for T cell CD28 receptors. Proper costimulatory signaling induces the production of IL-2 and, in allergic asthma, T_H2 cell proliferation. T_H2 cells may then secrete a number of cytokines such as IL-4, IL-5, and IL-13 to activate other immune cell types, resulting in an inflammatory cascade. Secretion of IL-4 contributes to immunoglobulin class switching from IgM to IgE in B cells, and the production of high-affinity IgE antibody to the antigen. IgE then complexes with FcεRI receptors on mast cells, inducing histamine release when crosslinked by antigen. IL-5 secretion increases eosinophil migration, activation, and survival and IL-13 secretion contributes to mucus secretion and airway hypersensitivity (Steinman and Hemmi, 2006; Chung and Barnes, 1999). While DCs are clearly one of the cell types involved in the initial stages of an allergic reaction, it should be noted that not all DC subsets are proinflammatory. Some subsets induce tolerance to antigen and decrease inflammatory

immune responses. Conventional DCs (cDC) and inflammatory DCs likely play a role in the asthmatic allergic responses while plasmacytoid (pDC) are capable of anti-inflammatory roles. cDCs in the conducting airways can project dendrites through bronchial epithelial cell tight junctions for the detection of inspired antigen, and within the lamina propria expression of SIRPα and production of chemokines such as CCL17 and CCL22 can aid in DC migration and recruitment (Hammad and Lambrecht, 2011; Hammad et al., 2010). pDCs, found in alveolar septa and large conducting airways on the other hand, may mediate anti-inflammatory responses through the expression of programmed death ligand 1 (PD-L1), which, when highly expressed on pDCs, has been correlated with elevated T_{Reg} numbers (Hammad and Lambrecht, 2011; Kool et al., 2009). DC phenotype can be determined by a number of factors, including the cytokine milieu. Fms-like tyrosine kinase receptor 3-ligand (Flt3L) has been shown to contribute to CD8a+ and CD8a- DCs and a skewed T cell response toward a T_H1 reaction while GM-CSF has been shown to contribute toward a pro-inflammatory phenotype that interestingly, does not counteract resolving inflammation, as well as a tolerogenic CD8a- phenotype that has been implicated in tolerance (Cheatem et al., 2009; Blyszczuk et al., 2013).

T-helper lymphocytes

Exposure to antigen can induce naïve CD4+ T-cell differentiation into effector cell T_H1, T_H2, T_H9, T_H17, or T_{Reg} phenotypes, some undergoing further differentiation into memory cells that reactivate rapidly upon antigen re-exposure. While T_H2 effector cells have been heavily implicated in asthma pathogenesis, other T_H subsets contribute as well and depending on the level of involvement, can give rise to various asthma

phenotypes (such as neutrophilic versus eosinophilic asthmatic inflammation, for example) (Lees and Farber, 2010; Thorburn and Hansbro, 2010; Lloyd and Saglani, 2013).

While T_H1 and T_H2 cells both develop from naïve CD4+ T cells, each subset reciprocally inhibits the growth of the other due to the release of opposing cytokine factors, particularly IFN-γ and IL-4, respectively (Coffman, 2006). In studies with mice lacking T-bet (T-box expressed in T cells), a transcription factor central to IFN-γ expression, mice spontaneously developed classic asthma symptoms. Mice lacking GATA-3, a transcription factor important to T_H2 differentiation, did not develop inflammation or AHR in an asthma model (Robinson, 2010). There is overlap and redundancy in the system, however, and although T_H2 cells have been implicated as the major players in the asthmatic response, there are studies that suggest a role for their T_H1 counterparts as well. It has been found, for instance, that adoptive transfer of T_H1 cells has caused mucosal eosinophilia in Brown Norway rats and increased neutrophils in mouse BAL (Huang et al., 2001).

T_H9 cells have been shown to develop from CD4+ T cells in the presence of IL-4 and TGF-β. Following house dust mite exposure in murine studies, differentiation and proliferation of IL-9-secreting T cells occurred quickly, even more so than for T_H2 cells. In addition, T_H9 cell adoptive transfer caused inflammatory alterations, specifically with regard to increased mast cell recruitment and activation. In human studies, T_H9 cells were elevated in the peripheral blood of asthmatic patients, and T_H9 elevation showed a positive correlation with patient IgE (Thorburn and Hansbro, 2010). An anti-IL-9

antibody, MEDI-528, had modest results in early clinical trials, and further study with the drug continues (Antoniu, 2010a).

T_H17 cells can develop from naïve CD4+ T cells in the presence of IL-23. They may also develop in the presence of IL-6 and TGF-β, although in the absence of IL-6, T_{Regs} are generated from the precursor cells instead of T_H17 cells (Thorburn and Hansbro, 2010; Robinson, 2010). The combination of IL-6 and TGF-β acts through transcription factors Smad2/3, STAT3, and NF-κB to induce IL-17 expression and T_H17 cell development (Lloyd and Saglani, 2013). T_H17 cells have been shown to secrete key asthma cytokines IL-4, IL-5, and IL-13 following exposure to high IL-4 levels, and in mouse studies, have been shown to induce neutrophilic (rather than eosinophilic) airway inflammation, AHR, and enhanced T_H2 responses (Lloyd and Saglani, 2013; Robinson, 2010; Cosmi et al., 2010). T_H17 cells were further linked to AHR in a study in mice with a defective Dendritic Cell αvβ8 integrin; mice with the defective integrin had no T_H17 cells in the lungs after antigen challenge, and a lack of airway narrowing and AHR. In addition, T_H17 cytokines have the capacity to act directly on airway smooth muscle (Lloyd and Saglani, 2013). While these studies suggest that blockade of IL-17 may be protective during sensitization phases, other studies have shown that treatment with IL-17A during antigen re-challenge has a protective effect, and that IL-17-secreting γδ T cells are capable of decreasing inflammation and remodeling in murine models. Together, these studies suggest that the timing of IL-17 secretion with respect to disease progression is an important consideration (Lloyd and Saglani, 2013).

T-regulatory lymphocytes

CD4+ CD25+ T-regulatory lymphocytes, or T_{Regs}, are immunosuppressive and may develop naturally in the thymus following exposure to self-antigens (natural T_{Regs}), or may be induced from CD4+ cells in the presence of IL-2 and TGF-β (induced T_{Regs}) (Thorburn and Hansbro, 2010). In addition to having the potential of being induced from CD4+ cells, recent observations have shown that T_{Regs} have the ability to then differentiate into other effector cell subsets, suggesting that T_{Regs} have great plasticity. Both natural and induced phenotypes are important in maintaining immune system balance, though FoxP3 (implicated in T_{Reg} suppressive activity, stability, and survival), which is present on most natural regulatory T cells, may or may not be expressed in induced T_{Regs} as both FoxP3+ and FoxP3- populations have been observed (Thorburn and Hansbro, 2010).

T_{Regs} exert immunosuppressive effects in various ways, including the release of anti-inflammatory cytokines such as IL-10 and TGF-β, through cell-contact mediated mechanisms leading to T effector cell cycle arrest or apoptosis or APC immaturity, as well as through competing for T effector cell growth factors such as IL-2. Human asthmatics have been shown to have fewer T_{Regs} in the blood and in BAL fluid, and for the T_{Regs} that are present, the response to chemokines regulating migration to the airways is mitigated (Vock et al., 2010). A few studies have shown an increase in T_{Regs} in severe asthmatics, although these increases may be due to induced T_{Regs} generated in an unsuccessful effort to combat overwhelming inflammation (Thorburn and Hansbro, 2010).

Cytotoxic T-lymphocytes

CD8+ cytotoxic T-lymphocytes respond to antigen presented on MHC Class I and are classified by subsets resembling those for CD4+ cells: Tc1, Tc2, Tc17, FoxP3+, γδ+, and effector and memory subsets (Betts and Kemeny, 2009). Each subset has the capacity to produce different cytokines, some T_H1-oriented and others T_H2-oriented, and thus may be pro-inflammatory or anti-inflammatory depending on the dominant subset. Some murine studies have observed that virus-activated CD8+ cells, when in the presence of IL-4, can produce IL-4, IL-5, and IL-10, as well as provide B-cell support, showing a means by which viral infection may potentiate asthmatic episodes (Coyle et al., 1995). Other studies have shown that memory CD8+ T cells may undergo nonspecific activation when exposed to IL-12 and IL-18, causing proliferation and production of IFN-γ and TNF-α, which may regulate or dampen T_H2 responses (Robinson, 2010).

Memory lymphocytes

Memory lymphocytes, key components of the acquired immune response, are antigen-specific and are developed after exposure and recognition of a particular antigen. They may be T or B cell-derived and are long-lived to provide rapid protection upon re-exposure to the antigen due to enhanced function and lower activation threshold. Memory T cells may be central memory (T_{CM}) or effector memory (T_{EM}), depending on CD62L and CCR7 expression, receptors allowing migration into high endothelial venules and so, the lymph nodes. Memory cells may also be CD4+ or CD8+ (Kaech et al., 2002; Sallusto et al., 2004; Zielinski et al., 2011).

T_{CM} express high levels of CD62L and CCR7, tend to be found in the lymph nodes and tonsils, and have higher proliferative ability than the CD62L- and CCR7-low T_{EM}, which tend to be found in the lungs, gut, and liver and have more potent effector function (Sallusto et al., 2004). Studies have shown that the T_{CM} and T_{EM} phenotypes may be flexible, however, as each phenotype has been observed to convert to the other; some T_{CM} resulting from T_{CM} proliferation and expansion have been shown to convert to T_{EM} cell types, and T_{EM} have been shown to convert to T_{CM} following CD4 or CD8 T cell activation (Lees and Farber, 2010). Both T_{CM} and T_{EM} may be either CD4+ or CD8+, although the T_{CM} phenotype is more common in the CD4+ subset while the T_{EM} phenotype is more common in the CD8+ subset (Sallusto et al., 2004).

γδ T-lymphocytes

γδ T-lymphocytes have T cell receptors composed of γ and δ chains rather than the α and β chains found in most CD4+ T cell receptors, and interact with lipid antigens such as pollen-derived phospholipids through receptor CD1d. T_H2-cytokine-secreting γδ T-lymphocytes have been observed in higher numbers in the airways and bronchoalveolar lavage fluid of asthmatic patients compared to normal controls, suggesting a role for γδ T-cells in asthma (Lloyd and Saglani, 2013; Robinson, 2010; Afshar et al., 2008). The role of γδ T-lymphocytes may be two-fold, however, depending on the dominant subtype present in the airways (Lloyd and Hessel, 2010; Spinozzi and Porcelli, 2007). Vγ1+ γδ T-lymphocytes secrete IL-5 and IL-13, contributing to eosinophilia and AHR while Vγ4+ γδ T-lymphocytes secrete IL-17A, which may inhibit inflammation if present during an antigen re-challenge (Lloyd and Saglani, 2013; Afshar et al., 2008; Murdoch and Lloyd, 2010).

Type 1 Natural Killer T-lymphocytes

Type 1, or invariant, Natural Killer T-lymphocytes (iNKT) account for 2 - 10% of the T-lymphocyte population (0.2 - 1% of the total white cells) in asthmatic airways. iNKT cells express a highly-restricted αβ T-cell repertoire with an invariant α chain that is only capable of interacting with CD1d bound to antigen. CD1d presents glycolipid antigen, and can promote secretion of IL-4, IL-5, IL-9, IL-13 and IFN-γ by iNKT cells (Meyer et al., 2008; Iwamura and Nakayama, 2010). iNKT cells also respond and become activated in the presence of IL-25 and IL-33, cytokines released by epithelial cells due to cell injury (Bartemes and Kita, 2012; Watarai et al., 2012).

IL-13 secretion due to iNKT cell activation stimulates macrophage production of IL-13, resulting in further autocrine-enhanced IL-13 production (Holtzman et al., 2009). Several studies have shown the presence of iNKT cells as a necessity for AHR induction. CD1d-/- mice, which lack iNKT cells, could not develop AHR after sensitization and challenge, nor could Jα18-/- mice, which lack the Type 1 iNKT cell receptor invariant α chain. In addition, Jα18-/- mice had fewer eosinophils post sensitization and challenge. Following adoptive transfer of functional wild-type iNKT into Jα18-/- mice, however, allowed for induction of AHR. It is thought that perhaps, under inflammatory conditions elicited by asthma pathogenesis, ensuing cellular alterations allow for the presentation of endogenous glycolipids and subsequent iNKT cell activation (Meyer et al., 2008).

Unlike most T_H2 cells, iNKT cells are resistant to corticosteroids, a common means of treating asthmatic inflammation. This is important in light of the fact that 10-30% of asthmatics have corticosteroid-resistant asthma. Also of note is that the majority

of patients with severe asthma are corticosteroid-resistant asthma cases, and that corticosteroid-resistant asthma contributes to a large number of asthma-related deaths (Meyer et al., 2008).

Natural Killer (NK) cells

NK T cells account for approximately 10% of lymphocytes in circulation. They are a glycolipid-responsive T cell subset that can process and present antigen to T_H2 cells via MHC Class II (Hanna et al., 2004). NK T cells heavily secrete T_H2 cytokines, although they may secrete T_H1 cytokines as well (Thorburn and Hansbro, 2010; Moretta et al., 2006). These cells have been seen in higher numbers in the sinus mucosa and sputum of asthmatic patients, and studies in laboratory animals suggest a necessity for NK T cells in AHR development. Noteworthy is that the cytotoxicity of NK T cells extends to T_{Regs} in asthmatic individuals, a phenomenon which is not observed in healthy controls, perhaps partly explaining the drop in T_{Reg} numbers in asthmatic individuals (Thorburn and Hansbro, 2010).

In a study examining the role of NK cells in mouse models of allergic asthma, NK cells were found to augment eosinophil numbers, while in another, eosinophilic inflammation, AHR, and goblet cell proliferation was attenuated through NK cell effects (Korsgren et al., 1999; Matsubara et al., 2007). Yet another study showed that NK cells played a role in the resolution of inflammation in a murine asthma model (Haworth et al., 2011). It is possible that the different roles of the NK cell in these studies were due to differences in the NK subsets.

NK cells may be $CD56^{bright}$ or $CD56^{dim,}$ and of the $CD56^{bright}$ cells (approximately 10% of NK cells), may be NK1 or NK2 depending on the cytokines produced (T_H1

cytokines or T_H2 cytokines, respectively). NK1 cells are $CD56^{bright}$ and $CD16^{dim}$, and tend to produce higher levels of IFN-γ. NK2 cells are $CD56^{bright}$ and CD16+, tend to produce IL-4 and IL-13, and higher numbers of these cells have been observed in asthmatic patients. (Wei et al., 2005; Wingett and Nielson, 2003; Scordamaglia et al., 2008; Deniz et al., 2002). The majority of NK cells, however, (80-90%) are $CD56^{dim}$ and CD16+, are more cytotoxic than the $CD56^{bright}$ subset, and are found circulating until signaled to migrate toward inflammatory tissue (Bellora et al., 2010; Agaugue et al., 2008; Timmons and Cieslak, 2008).

Cytokine Network

Cytokines, as central components of the immunological signaling network, are key mediators of pulmonary allergic responses. Cytokines possess the potential to affect specific antigen presenting cell populations (Mannie et al., 2007b; Blanchfield and Mannie, 2010), upregulate T_{Regs}, bias the immune system toward T_H1, T_H2, or T_H17 responses (Kim et al., 1997; Maecker et al., 2001), affect immunological tolerance to antigen (Mannie et al., 2007b; Blanchfield and Mannie, 2010), and recruit and activate various immune cell types. Cytokines involved in pulmonary allergy pathogenesis include general cytokine growth factors and acute response cytokines such as IL-2, a growth factor for lymphocytes; granulocyte-macrophage colony-stimulating factor, a growth factor for neutrophils, eosinophils, and basophils; and Tumor Necrosis Factor α, a key initiator of the allergic acute phase response (Smith, 1988; Ganesh et al., 2009). Also involved in pulmonary allergy pathogenesis are the canonical T_H2-specific cytokines (Interleukin (IL)-4, IL-5, and IL-13); T_H1 and innate immunity cytokines (IFN-γ, IL-12); T_H17, T_H22, and lesser known, but prominently expressed cytokines (IL-6, IL-9,

TSLP/Ox40L pathway, IL-22, IL-25, IL-33); as well as cytokines involved in the immunosuppressive T_{Reg} network (IL-10, Osteopontin, TGF-β) (Chung and Barnes, 1999; Konno et al., 2011; Barnes, 2008). This complex network is responsible for driving, in large part, the immune system reaction to antigen in asthma pathogenesis.

General Growth and Acute Response Cytokines

IL-2

IL-2 is a general growth factor for lymphocytes. The primary source of IL-2 is activated T cells, and activation of the TCR induces both secretion of IL-2 and expression of IL-2R. Activation of the IL-2R induces differentiation and clonal expansion of antigen-specific CD4+ and CD8+ T cells, as well as growth and differentiation of B cells, NK cells, macrophages, and T_{Reg} cells (Chung and Barnes, 1999; Stern and Smith, 1986; Beadling and Smith, 2002; Beadling et al., 1993; Letourneau et al., 2009).

Consistent with its function as a general growth factor, IL-2 has been implicated in allergic responses, cytotoxicity, and peripheral tolerance (Smith, 1988; Letourneau et al., 2009; Klebb et al., 1996; Fehervari et al., 2006; Thornton et al., 2004; Renauld, 2001). Lack of IL-2 is associated with lymphoproliferative disorder and autoimmunity (Smith, 1988; Thornton et al., 2004; Sakaguchi et al., 1995; Thornton and Shevach, 1998). Thus, in the context of allergic disorders, IL-2 appears to play a supportive role opposed to a direct pathogenic role.

Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF is a general growth factor for neutrophils, eosinophils, basophils, in addition to myeloid APCs such as macrophages and dendritic cells (Shin et al., 2009).

GM-CSF is primarily produced by T cells, as well as diverse cell types found in the airway mucosa including epithelial cells, smooth muscle cells, macrophages, fibroblasts, endothelial cells, and eosinophils (Chung and Barnes, 1999). GM-CSF signals through the GM-CSF receptor, found in low levels on hematopoietic cells. The GM-CSF receptor is comprised of a CD116 α chain and a common β chain, through which other cytokines such as IL-3 and IL-5 may interact. The β chain, the primary subunit involved in signal transduction, signals through JAK2, in turn activating STAT, MAPK, and P13K pathways (Shi et al., 2006; Broughton et al.).

The importance of GM-CSF in macrophage function is illustrated in GM-CSF knockout mice, which develop alveolar proteinosis, a state characterized by impaired macrophage function, an accumulation of surfactant in the airways, and inefficient gas exchange (Hamilton and Anderson, 2004). Alveolar proteinosis can also develop due to defects in the β chain of the GM-CSF receptor, causing interference with GM-CSF signaling (Hamilton, 2002). GM-CSF overexpression in human populations, caused by certain malignancies such as myeloid leukemia, results in the differentiation of cancerous cells as well as the generation of GM-CSF-neutralizing auto-antibody and subsequent alveolar proteinosis (Shi et al., 2006; Hamilton, 2002). Neutropenia caused by cancer or resulting chemotherapy treatment may be treated with GM-CSF or with recombinant GM-CSF therapies such as Sargramostim (Leukine) to mobilize stem cells. Bone marrow transplant recipients may also receive GM-CSF therapy to aid in myeloid reconstitution. Patients receiving GM-CSF therapy that have also been diagnosed with rheumatoid arthritis, however, experience a worsening of arthritis symptoms as a side-effect, indicating a pro-inflammatory role for GM-CSF (Shi et al., 2006).

Further supporting a pro-inflammatory role for GM-CSF, GM-CSF has been observed to contribute to T_H17-induced inflammatory responses, and transgenics expressing GM-CSF in the stomach have been shown to develop autoimmune gastritis (Shi et al., 2006; McGeachy, 2011). In studies with GM-CSF knockout mice, mice are resistant to development of EAE and type I diabetes in the respective disease models (Hamilton, 2002; Enzler et al., 2007). In asthma models, treatment with anti-GM-CSF antibody attenuates sensitization to aeroallergens, allergen-induced inflammation, and AHR (Hamilton and Anderson, 2004). Consistent with the mucosal sources of GM-CSF, GM-CSF mRNA is increased in the airways of human asthmatics (Chung and Barnes, 1999) where it promotes maturation, activation, and growth of monocytes and DCs resulting in increased antigen presentation and activation of antigen-specific B and T cells (Blanchfield and Mannie, 2010; Ganesh et al., 2009; Hamilton and Anderson, 2004; Ritz et al., 2002). Clinical application of GM-CSF inhibition has been seen with Mavrilmumab, a rheumatoid arthritis treatment targeting GM-CSFRα, and with MOR 103, an anti-GMCSF treatment used in multiple sclerosis (Nair et al., 2012; Deiß et al., 2013).

GM-CSF has also been shown to exert anti-inflammatory roles, however. GM-CSF treatment in rodent models of experimental autoimmune myasthenia gravis inhibited disease progression through the induction of immunosuppressive T_{Regs}, and likewise inhibited experimental autoimmune thyroiditis and type 1 diabetes in rodent models through dendritic cell interaction and T_{Reg} induction (Cheatem et al., 2009; Ganesh et al., 2009; Sheng et al., 2006). Clinical application of GM-CSF has been observed with Sipleucel-T (Provenge), an FDA-approved cancer vaccine. In this

treatment, a patient's peripheral blood mononuclear cells (including dendritic cell APC) are collected and incubated with GMCSF-PAP, a fusion protein comprised of GM-CSF and PAP (prostatic acid phosphatase), a protein found on the majority of prostate cancer cells. The cultured cells are then re-introduced into the patient, altering tumor antigen presentation and aiding in the tumor immune response (Shore et al., 2013). Due to the opposing effects seen for GM-CSF in the rodent type 1 diabetes model, as well as opposing pro- and anti-inflammatory effects observed in other models and therapies, it is likely that the timing of GM-CSF introduction or the balance of GM-CSF determines the nature of its effect.

Tumor Necrosis Factor-α (TNF-α)

TNF-α is an initiator of the acute phase response that is produced primarily by macrophages, but is also expressed in T lymphocytes, mast cells, neutrophils, eosinophils, natural killer cells, and epithelial cells (Costa et al., 1993; Devalia et al., 1993). TNF-α secretion by monocytes and macrophages is amplified by other cytokines such as IL-1, GM-CSF and IFN-α; and IgE has been shown to induce TNF-α expression in lung epithelial cells via FcεRI (Ohno et al., 1990; Hirshman et al., 1980; Ohkawara et al., 1992). TNF-α receptors, TNF-R55 and TNF-R75, are found on most cells with the exception of red blood cells and unactivated T cells. Expression of TNF-R75 is particularly high on hematopoietic cells (Chung and Barnes, 1999).

TNF-α augments allergic reactions by up-regulating MHC-II expression and IL-1 secretion by APCs (Chung and Barnes, 1999; Kips et al., 1993; Shah et al., 1995) and by acting as a costimulatory molecule for activated T cells, allowing for increased T cell proliferation and IL-2 receptor expression (Chung and Barnes, 1999). TNF-α induces

chemotaxis, degranulation, and respiratory burst activity in neutrophils (Chung and Barnes, 1999); and stimulates production of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin that mediate migration of granulocytes and mononuclear cells at sites of inflammation (Commins et al., 2010).

TNF-α expression is elevated in bronchoalveolar lavage fluid (Broide et al., 1992) and bronchial biopsies from human asthmatics (Ying et al., 1991), and airway responsiveness correlates with TNF-α levels in humans (Berry et al., 2007) and Brown-Norway rats (Kips et al., 1992). The effects of TNF-α in the airways may be secondary to increased production of GM-CSF, IL-8, and RANTES that promote growth and recruitment of inflammatory cells (Chung and Barnes, 1999). Inhalation of TNF-α by normal human subjects, causes neutrophil-induced AHR (Barnes, 2008). Blockade of TNF-α with etanercept (Embrel) and infliximab (Remicade) reduces AHR in patients with refractory and moderate asthma, respectively (Barnes, 2008).

Canonical T_H2 Cytokines

IL-4

IL-4 was originally described as a B cell growth factor, but is now known to also play a critical role in T cell differentiation. IL-4 is produced by T_H0 and T_H2 T lymphocytes, NK T cells, eosinophils, basophils, and mast cells (Commins et al., 2010). IL-4 synthesis is enhanced via costimulatory signals generated by cross linking of CD40 ligand on human CD4+ T cells, activation of TCR, and crosslinking of FcεRI on basophil and mast cells (Chung and Barnes, 1999).

IL-4 promotes differentiation of naive T_H0 into T_H2 cells, and inhibits apoptotic pathways within T_H2 cells (Commins et al., 2010). IL-4 is required for immunoglobulin

isotype switching to IgE in B cells and stimulates expression of IgE receptors on mast cells (Renauld, 2001). IL-4 also promotes antigen presentation by increasing MHC-II expression on APCs and increasing expression of B7 (CD80/CD86), CD40, low-affinity IgE receptor (CD23), and surface IgM on B cells. Transgenic mice overexpressing IL-4 develop symptoms of allergic disease including lymphocytic and eosinophilic inflammation and elevated IgE concentrations (Renauld, 2001). In humans, the severity of asthma has been associated with a gain of function mutation in the IL-4 receptor protein (IL-4Rα) (Hershey et al., 1997). Conversely, knock-out of the IL-4 gene in mice attenuates ovalbumin-induced eosinophilia and production of allergen-specific IgE, but has little effect on AHR (Chung and Barnes, 1999; Renauld, 2001). Yet, studies utilizing anti-IL-4 receptor (IL-4R) antibodies or mice deficient in the IL-4R subunit IL-4Rα revealed reductions in AHR. A potential explanation for this discrepancy is that IL-4Rα can also bind IL-13, providing a parallel pathway for activation of IL-4Rα and induction of AHR (Renauld, 2001). The overlap in IL-4 and IL-13 signaling through IL-4Rα has clinical significance, resulting in reduced effectiveness of therapies targeting IL-4 or IL-13 alone compared to therapies targeting IL-4Ra (Corren et al., 2010; Slager et al., 2012).

IL-5

IL-5 is a primary growth factor for eosinophils and is produced by T_H2 T lymphocytes, eosinophils, and mast cells. As the major cytokine in eosinophil development, IL-5 induces eosinophil migration, activation, and survival (Chung and Barnes, 1999). The primary source of IL-5 is T_H2 lymphocytes, but mast cells are also a source in the airways. IL-5 is increased in bronchial biopsies and BAL fluid and serum

from symptomatic asthmatic patients, knock-out of IL-5 in mice eliminates AHR and eosinophilia, and overexpression or treatment with rIL-5 results in asthma-like lung histopathology (Chung and Barnes, 1999; Barnes, 2008; Commins et al., 2010). Blockade of IL-5 in humans with Mepolizumab, an anti-IL-5 antibody, decreases eosinophils in the sputum and general circulation, and in asthma patients exhibiting hypereosinophilia and resistance to steroid therapy, Mepolizumab reduces exacerbations (Busse et al., 2010).

IL-9

IL-9 is secreted by CD4+ cells, typically T_H2 cells. Originally described as a T cell growth factor, IL-9 induces not only T cell proliferation, but also proliferation and differentiation of mast cells and hematopoietic progenitor cells, as well as B cell immunoglobulin synthesis. IL-9 has been shown to increase mast cell IgE receptor expression, upregulate IgE production, and promote eosinophil growth (Commins et al., 2010). In mice, mast cell proliferation and stimulation of B cell immunoglobulin synthesis appear to be mediated by IL-9 directly, whereas IL-9 effects on eosinophilia and mucus production are through IL-13 (Barnes, 2008).

Both IL-9 and the IL-9R are increased in the airways of asthmatic patients, and the expression IL-9 mRNA is correlated with increased airway responses to methacholine (Renauld, 2001). Transgenic mice with increased lung expression of IL-9 exhibit increased numbers of eosinophils and mast cells in the airways, excess mucus, airway remodeling, AHR, and elevated IgE. Conversely, antagonism of IL-9 suppresses allergen-induced airway eosinophilia, mucus production, and AHR (Chung and Barnes, 1999; Barnes, 2008). The ability of IL-9 overexpression to elicit symptoms of airway

disease may be due to IL-9-induced upregulation of IL-5 and IL-13 secretion (Finkelman et al., 2010). Clinical trials with a therapeutic antibody against IL-9, MEDI-528, have shown modest efficacy in asthma (Antoniu, 2010a; Parker et al., 2011).

IL-13

IL-13 is produced by activated CD8+ T cells as well as CD4+ T_H1, T_H2, and T_H0 cells in response to antigenic stimuli (Chung and Barnes, 1999). The IL-13 receptor shares a common subunit with the IL-4 receptor, IL-4Rα, and the two cytokines exhibit some functional overlap. The other component of the heterodimeric IL-13 receptor complex, IL-13Rα, is prominently expressed on smooth muscle cells. Unlike IL-4, IL-13 has no effect on T cell activation due to a lack of IL-13 receptors on T cells, although both IL-13 and IL-4 have been shown to increase IgE secretion and promote remodeling of the airway wall. IL-13 increases mucus secretion and airway hyperresponsiveness through STAT6 activation in the airway epithelium, and affects eosinophil activation and survival through CD69 (Chung and Barnes, 1999; Barnes, 2008).

IL-13 is elevated in the airways of human asthmatics and correlates with the severity of allergen-induced eosinophilia. In mice, IL-13 causes airway eosinophilia and AHR, and neutralization attenuates mucus production, airway eosinophilia, and AHR (Chung and Barnes, 1999; Barnes, 2008). Pitrakinra, an IL-4 mutein that blocks binding of both IL-13 and IL-4 to IL-4Rα decreases late response to inhaled allergen in humans (Antoniu, 2010b), and efficacy is increased in asthmatics with specific polymorphisms IL-4Rα (Slager et al., 2012).

T_H1 Cytokines

Interferon-γ (IFN-γ)

IFN-γ inhibits T_H2 cell responses, allergic inflammation, and IgE production; and promotes cell-mediated cytotoxicity. IFN-γ production is limited to CD4+ and CD8+ T lymphocytes and NK cells, but its receptors are found on diverse cell types including T and B cells, DCs, granulocytes, monocytes and macrophages, epithelial and endothelial cells, and platelets (Chung and Barnes, 1999). IFN-γ is a potent inhibitor of IL-4-induced IgE production by B cells and IL-10 synthesis by monocytes, which results in increased production of TNF-α IL-1, platelet activating factor (PAF), and production of ROI. IFN-γ also augments TNF-α effects on airway smooth muscle cells, upregulating RANTES synthesis.

The role of IFN-γ is asthma is not clearly understood. Consistent with its role as an inhibitor of T_H2 responses, production of IFN-γ is reported to be low in mild asthmatics (Truyen et al., 2006). Similarly, inhaled recombinant IFN-γ decreases the number of eosinophils in BAL fluid from asthmatic patients. In mice exposed to allergen, treatment with IFN-γ attenuates eosinophil infiltration in the airways and AHR, and knock-out of the IFN-γ receptor increases airway eosinophil infiltration (Chung and Barnes, 1999). However, IFN-γ is not entirely protective and has been shown to stimulate the expression of cysteinyl leukotriene receptor CysLTR-2 on eosinophils, which can contribute to cysteinyl leukotriene-mediated bronchoconstrictive and inflammation (Fujii et al., 2005). In addition, sputum IFN-γ levels are reported to be higher in severe asthma than in mild asthma (Truyen et al., 2006).

IL-12

IL-12 is primarily produced by monocytes and macrophages, and to a lesser degree by B cells and dendritic cells. IL-12 receptors are located mainly on T cells and NK cells, and stimulate T_H1 and NK cell growth, while inhibiting T_H2 cell responses. IL-12 augments cytotoxicity by T lymphocytes and NK cells, at least in part through the enhancement of IFN-γ and TNF-α secretion, and inhibition of IL-4 production. Consequently, IL-12 favors T_H1-type responses over T_H2-type and IgE inflammation, and may serve as a regulator of IgE-related inflammation in pulmonary allergic disease (Chung and Barnes, 1999).

In patients with allergic asthma, levels of IL-12 in whole blood are reduced, and IL-12 stimulated IFN-γ production is less than in healthy controls. IL-12 mRNA is reduced in airway biopsies from allergic asthma patients, and the levels are increased by administration of corticosteroids. In mice, IL-12 treatment reduces eosinophilia, IgE release, and AHR, but only if administered during active sensitization. IL-12 administered post-sensitization shows little or no difference from controls (Chung and Barnes, 1999). Thus, the effects of IL-12 are dependent upon the state of the allergic response.

Noncanonical Cytokines

IL-6

Originally described as a B cell growth factor, IL-6 is now recognized as a pleiotropic cytokine synthesized by diverse cell types, including T and B cells, monocytes and macrophages, bone marrow stromal cells, keratinocytes, fibroblasts, eosinophils, endothelial cells, and epithelial cells. Production of IL-6 is augmented by IL-

1, TNF-α, platelet-derived growth factor, and LPS. IL-6 signals through a receptor complex comprised of gp130 and IL-6R, which is found primarily on leukocytes and hepatocytes (Dienz and Rincon, 2009).

IL-6 plays a role in the differentiation, growth, and activation of T and B lymphocytes, and stimulates production of IgG, IgA, and IgM (Akdis et al., 2011). IL-6 is crucial to IL-4-induced IgE production, enhances IL-2 production and CD4 T cell survival, and promotes T_H2 cell differentiation (Chung and Barnes, 1999; Akdis et al., 2011). In addition, IL-6 can potentially exert anti-inflammatory activity through the inhibition of IL-1 and TNF synthesis by macrophages (Chung and Barnes, 1999).

In asthmatic patients, IL-6 secretion is elevated both at rest and after allergen challenge. *In vitro*, monocytes and macrophages release IL-6 in response to IgE. In transgenic mice expressing IL-6, mice show decreased AHR and increased numbers of airway lymphocytes (Chung and Barnes, 1999). A therapeutic antibody against IL-6, siltuximab, is in clinical trials for cancer, but its application to asthma has not yet been reported (Hudes et al., 2012).

Thymic Stromal Lymphopoietin (TSLP)

TSLP is a key initiator of allergic inflammation and considered to be one of the two major cytokines involved in mucosal T_H2 immune deviation along with IL-33. The cytokine is increased in asthmatic airways and mast cells (Barnes, 2008; Bird, 2005), and in the lungs is produced mainly by airway epithelial cells. Inflammation in allergic lung disease may be exacerbated by the ability of TSLP to react synergistically with IL-10 and TNF-α to stimulate mast cell growth and release of T_H2 cytokines. TSLP may also positively influence T_H2 cytokine release from CD4+ T cells. In addition, TSLP

stimulates the production of T_H2 chemoattractants CCL17 and CCL22 from airway DCs, leading to T_H2 airway infiltration (Barnes, 2008).

In TSLP receptor deficient mice, T_H2 cells, CCL11, and CCL17 in BAL were decreased and airway inflammation was reduced compared to wild-type mice. Adoptive transfer of wild-type CD4+ cells with normal TSLP receptors into the receptor deficient mice restored airway inflammation, indicating the TSLP was involved in T cell activation (Bird, 2005). Overexpression of the Tslp transgene in lung epithelial cells results in airway infiltration by eosinophils and T_H2 cells, and development of AHR. Blocking OX40 ligand (OX40L), an important mediator of TSLP signaling, inhibits allergic inflammation in mice and non-human primates (Barnes, 2008). The findings with TSLP and OX40 ligand have not yet been translated into human therapy for asthma.

IL-22

In humans, IL-22 is primarily produced by T_H1 and T_H22 helper T cells, but can also be produced by T_H17 cells, mast cells, and NK cells. In mice, however, IL-22 is predominately produced by T_H17 cells. IL-22 receptors are heterodimers composed of IL-22R1/IL-10R2 chains. IL22R1 is not expressed on immune cells, and the major role appears to be stimulation of hepatocyte production of acute phase proteins and stimulation of non-hematopoietic cell genes implicated in innate immunity, inflammation, chemotaxis, and cell proliferation (Sonnenberg et al., 2011).

IL-22 can exhibit proinflammatory or anti-inflammatory activity depending on the tissue and the cytokine milieu (Besnard et al., 2011). In a study by Besnard et al., investigators noted increased IL-22 protein concentrations in serum from asthmatic patients, and increased IL22 transcript and IL-22 protein in the lungs in an ovalbumin-

induced murine model of asthma. Within the murine asthma model, it was found that IL-22-KO mice exhibited significantly less airway inflammation accompanied by decreased IL-4, IL-13, IL-33, CCL11, and CCL17, as well as increased IL-17A compared to normal WT controls. Similarly, normal WT controls exhibited less inflammation if treated with an IL-22-neutralizing antibody during the sensitization phase, suggesting that IL-22 plays a pro-inflammatory role in the induction of airway inflammation. Once inflammation was established, however, administration of IL-22-neutralizing antibody amplified IL-17A and T_H2 cytokine production, and cellular infiltration, while administration of exogenous IL-22 during this phase reduced IL-17A and intracellular infiltration. This suggests a protective, anti-inflammatory activity for IL-22 in established inflammation. In addition, there were indications of a reciprocal regulation between IL-22 and IL-17A, with IL-22 being required for IL-17A-induced inflammation (Besnard et al., 2011).

IL-25

IL-25, or IL-17E, is produced by T_H2 cells, epithelial cells, activated mast cells, alveolar macrophages, activated eosinophils and basophils (Barnes, 2008; Pappu et al., 2008). The receptor for IL-25 is IL-17BR (also known as EVI27), and activation results in airway inflammation mediated via CD4+ T cells and the STAT6 pathway. *In vitro*, IL-25 enhances the release of T_H2 cytokines from T_H2 cells both during and after differentiation. In mice, IL-25 increases expression of IL-4, -5, and -13; leading to eosinophilic inflammation, elevation of IgE, mucus and epithelial cell hyperplasia, and AHR. Overexpression in the lung induces allergic T_H2 responses characterized by increased numbers of macrophages and eosinophils, and mucus secretion. In contrast,

IL-25 blockade attenuates allergen-induced AHR, lung eosinophilia, and antigen-specific T_H2 cells (Pappu et al., 2008).

In asthma patients, eosinophils and basophils are described as the major source of IL-25. IL-25 RNA is elevated in the lungs of chronic asthmatics, and IL-25 positive cells in the bronchial submucosa of these patients are reported to have eosinophil-like characteristics (Monteleone et al., 2010). IL-25 and TSLP stimulate human T_H2 memory cells in the presence of DCs to produce IL-4, IL-5, and IL-13 (Pappu et al., 2008). In addition to enhancing T_H2-related responses, IL-25 limits T_H1- and T_H17-related inflammatory responses associated with diseases such as Multiple Sclerosis, diabetes, and inflammatory bowel disease. Thus, while anti-IL-25 therapies have the potential to alleviate asthma and allergic inflammation, the approach might increase the risk for autoimmune-related activity (Monteleone et al., 2010).

IL-33

IL-33 is considered to be one of major cytokines involved in T_H2 immune deviation. It is produced primarily by bronchial epithelial cells, but can also be produced by smooth muscle cells, macrophages, DCs, and fibroblasts (Barnes, 2008; Commins et al., 2010). ST2, the IL-33 receptor, is located on T_H2 cells, mast cells, basophils, eosinophils, NK cells, and NKT cells. ST2 acts through an IL-1 receptor-related signaling cascade. The soluble form of the receptor, sST2, is produced in response to anti-inflammatory signals and acts to bind and reduce IL-33 activity. IL-33 is released during inflammation and tissue damage, and is therefore classified as an “alarmin” (Smith, 2010).

IL-33 exerts both nuclear and extracellular effects. IL-33 aids in T_H2 cell differentiation by translocating to the nucleus, and altering chromatin structure and

transcription. Extracellular effects of IL-33 include its role as a T_H2 activator and chemoattractant, and as a promoter of T_H2 cell production of IL-5 and IL-13. IL-33 is not limited to enhancement of T_H2 cytokine production from T_H2 cells, but can also induce IFN-γ production from T_H2 cells. The ability of IL-33 to enhance production of both IL-13 and IFN-γ provides a potential explanation for why some chronic asthma patients exhibit mixed T_H1/T_H2 cytokine profiles.

In addition to exerting effects on T_H2 T cell function, IL-33 aids in promoting eosinophil survival and adhesion to proteins in the extracellular matrix. IL-33 has also been shown to enhance mast cell survival and activation, as well as mast cell production of IL-6 and IL-13. These effects are further enhanced when IL-33 is accompanied by TSLP or IgE crosslinking (Smith, 2010).

In human asthmatics, there is a significant elevation in the concentration of sST2, possibly as a compensatory response to elevated IL-33 levels (Smith, 2010). In mice, treatment with IL-33 receptor antagonists downregulates T_H2 cytokines and allergen-induced inflammation, while treatment with IL-33 produces a T_H2 deviation, an escalation in T_H2 cytokines and IgE, and lung eosinophilia (Commins et al., 2010). Elucidation of IL-33's role in pulmonary allergic responses is an active area of research at this time.

T_{Reg} Cytokines

IL-10

IL-10, originally described as "cytokine synthesis inhibitor factor", is produced by T_H0, T_H1, T_H2, T_{Reg}, cytotoxic T cells, mast cells, and activated monocytes. IL-10 receptors are located on lymphoid, myeloid, and NK cells. IL-10 decreases T_H2

signaling by downregulating MHC-II, B7.1/B7.2 and CD23 expression (Chung and Barnes, 1999). IL-10 suppresses monocyte and macrophage activity, including release of ROI, and downregulates secretion of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-8, and MIP-1α. In T_H1 cells, IFN-γ and IL-2 synthesis are inhibited, as is synthesis of IL-4 and IL-5 by T_H2 cells. In contrast, IL-10 can promote growth of mast cells, T cells, and B cells. However, even these responses can be bimodal, with inhibition observed at high concentrations of IL-10.

IL-10 mRNA and protein are reduced in alveolar macrophages from asthmatic patients, but the number of IL-10 expressing T cells and macrophages may be increased. In Brown Norway rats, IL-10 is reported to attenuate late-phase responses and eosinophilia post-challenge. In mice, treatment with IL-10 at the time of sensitization decreases eosinophilia (Barnes, 2008). Studies involving antibodies against IL-10 and IL-10R, as well as IL-10 knockout have revealed attenuation of AHR and eosinophilia. This apparent inconsistency in the typical T_H1 functioning of IL-10 might be explained by the finding that IL-10 can inhibit IL-13Rα2, a protein responsible for binding and inhibiting IL-13, thereby potentially increasing IL-13 effects. It has been noted that mice lacking both IL-10 and IL-13Rα2 exhibit a more severe manifestation of pulmonary allergic disease than mice deficient in IL-10 or IL-13Rα2 alone (Finkelman et al., 2010).

Osteopontin (OPN)

Osteopontin is a T_H1 cytokine implicated as a potential regulator of allergic responses. OPN is produced by monocytes and monocyte-derived DCs and acts as a chemoattractant for monocytes, neutrophils, and lymphocytes (Konno et al., 2011). *In*

vivo studies with asthmatic patients have shown a significant correlation between OPN expression and eosinophilia in bronchoalveolar lavage fluid. OPN may also contribute to airway remodeling through the regulation of cellular interactions with extracellular matrix as well as the expression of matrix metalloproteinase (MMP). In mice, OPN is positively correlated with airway remodeling and stimulates collagen deposition in cell cultures. In addition, ovalbumin sensitization and challenge of Osteopontin-deficient mice results in an accentuated allergen-specific-IgE response. It has also been observed, however, that treatment with an anti-OPN antibody downregulates Ag-specific IgE and upregulates pDCs (Xanthou et al., 2007). This discrepancy in observations may have been due to the use of antibodies that recognized different domains on OPN, as it has been shown that the function of OPN can be dependent upon the ligated domain (Konno et al., 2011). In studies by Koguchi et al., the effects of OPN on IL-12 and IFN-γ production by PBMCs could be attenuated with a C-terminal-specific anti-OPN Ab, though not with an integrin-binding-domain anti-OPN Ab (Koguchi et al., 2003). It would seem, then, that effects exerted by OPN may change depending on the functional domain involved in the response. While OPN may play a role in the allergic response pathway, more studies are needed to elucidate the diverse nature of these roles.

Transforming Growth Factor-β (TGF-β)

TGF-β is a pro-fibrotic growth factor produced by activated monocytes, pulmonary macrophages, eosinophils, neutrophils, airway epithelial cells, smooth muscle cells, mast cells, lung fibroblasts, T helper and T_{Reg} lymphocytes (Chung and Barnes, 1999; Commins et al., 2010). In chronic allergic inflammation, eosinophils are a primary source of TGF-β (Commins et al., 2010; Akdis and Akdis, 2009). TGF-β receptors

include high-affinity (type I and type II) and low-affinity (type III) isoforms (Chung and Barnes, 1999). TGF-β is a strong stimulating factor for epithelial cell differentiation and fibroblast secretion of extracellular matrix proteins, contributing to the airway remodeling observed in chronic pulmonary allergic disease pathology. In addition, TGF-β is also a powerful chemoattractant for monocytes, mast cells, and fibroblasts.

TGF-β has contrasting roles in allergic disease pathology. Depending on the cytokine milieu, it can either stimulate or inhibit proliferation of airway smooth muscle cells and fibroblasts (Chung and Barnes, 1999; Commins et al., 2010). Likewise, TGF-β can be pro-inflammatory by augmenting differentiation of T_H17 and T_H9/IL-9-producing-T_H2 cells, or anti-inflammatory by inhibiting IgE production, mast cell proliferation, T cell, B cell, NK cell, and cytotoxic lymphocyte functions (Akdis and Akdis, 2009). One of the factors affecting the role played by TGF-β is peptidyl-prolyl isomerase Pin1 (protein NIMA-interacting 1). Pin1, shown to regulate eosinophil GM-CSF production, has also been shown to regulate eosinophil TGF-β production. Compared with normal control animals, inhibition of Pin1 in rat models of allergic disease caused decreased expression of TGF-β1 and type I collagen, decreased eosinophilia in bronchoalveolar lavage fluid, and a decline in fibrotic deposits in the lungs (Shen et al., 2008). In human asthma patients, increases in TGF-β have been observed in bronchoalveolar lavage fluid following allergen challenge, and TGF-β expression seems to have a positive correlation with increased basement membrane thickness, subepithelial fibrosis, and severity of asthma symptoms (Renauld, 2001).

Models of Allergic Asthma

Respiratory allergies and asthma are considered human diseases and with few exceptions, are not naturally-occurring in laboratory animals. Allergic reactions to aeroallergens in humans develop over the lifetime and change with environmental factors; and no animal model fully recapitulates the chronic human disease. Even so, animal models have proved useful in investigating the pathways underlying pulmonary allergic responses, developing mechanistic hypotheses to explain pathogenesis, and testing novel therapeutics. Models of allergic asthma have been implemented in mice, rats, guinea pigs, rabbits, dogs, sheep, horses, and non-human primates; each with distinct advantages and disadvantages.

Mice

The murine model of asthma has been the most widely-used in recent years due to the amount known about murine immunology, high availability of commercial species-specific reagents, ease of genetic manipulation leading to wide-availability of transgenic and knock-out strains, low cost of husbandry, and relative low level of social sensitivity and federal regulatory oversight. Mice can be sensitized to a wide array of allergens such as ovalbumin (OVA), and house dust mite (HDM), Aspergillus fumigatus, ragweed, and cockroach antigens (Zosky and Sly, 2007). Mouse models incorporating AHR, bronchopulmonary inflammation, and increases in IgE and mucus production have been described, and some chronic models have shown airway remodeling - all key features of human asthma. Inflammation and AHR vary across murine models of asthma, due in some part to strain-dependent differences in airway smooth muscle and predisposition to T_H1 and T_H2 mediated inflammation (Safholm et al., 2011).

Disadvantages of the mouse model include distinct anatomical and immunological differences between mice and humans (Wenzel and Holgate, 2006). Mouse lungs have fewer airway generations, and most airways lack the smooth muscle bundles found in human airways, which may contribute to differences in bronchoconstriction and AHR. Second, mice can be manipulated to accentuate T_H1 or T_H2 phenotypes, murine models of asthma are predominately T_H2 in nature. But there is a greater amount of overlap of these pathways in humans, and some human asthmatics have been observed to have increased levels of IL-12 and IFN-γ suggesting increased T_H1 involvement in these subjects (Wenzel and Holgate, 2006). Thirdly, while AHR has been observed in murine models of asthma, it is not clear whether or not mouse models reliably demonstrate late-phase responses as would be observed in humans (Safholm et al., 2011). In addition, many murine models of asthma lack chronicity in post-sensitization allergen responses. Following repeated allergen challenge, tolerance to allergen and diminished immune responses have been observed in many of these models. Careful titration of allergen exposure is required to induce long-term hyperresponsiveness, airway remodeling, and eosinophilia in the mouse (Kumar and Foster, 2002).

Immunological pathways shown to be important in mouse models have not easily translated to new treatments for human disease. Early clinical trials with anti-IL-4 and anti-IL-5 antibodies yielded disappointing results, and lack of efficacy in preventing asthma symptoms is often cited as lack of fidelity between mouse models and human asthma (Wenzel and Holgate, 2006). However, more recent studies with altered experimental design, modified exclusion criteria, use of biomarkers to identify

responsive asthmatic subjects, refinement of pharmacodynamic measures, and optimization of dosing has yielded encouraging results. For example, Pitrakinra exhibits greater efficacy in asthmatics with specific single nucleotide polymorphisms in IL-4Rα, (Slager et al., 2012), and Mepolizumab is somewhat more effective in steroid-refractory patients with hypereosinophilia than in the general population of mild and moderate asthmatics (Nair et al., 2009; Haldar et al., 2009). This controversy aside, the mouse remains an important tool for elucidating pathways involved in immune responses and identifying potential novel targets, and is often used in research due to the availability of a multitude of commercial species-specific reagents and wide-availability of inbred, transgenic, and knock-out strains.

Rats

Rats are small and inexpensive, but large enough to obtain ample serum and bronchoalveolar lavage fluid for analysis. In addition, they are large enough to be stable under anesthesia, which facilitates measurements of pulmonary resistance, compliance, and AHR (Zosky and Sly, 2007). Rats can be sensitized to a wide array of allergens including OVA, HDM, and *Ascaris* antigens resulting in a T_H2 eosinophilic inflammation and IgE responses, although the strength of the responses can be strain-specific. Compared with mice, however, rats have fewer transgenic and knock-out strains as well as a lack of species-specific reagents, ultimately limiting their usefulness in asthma research.

Guinea Pigs

Guinea pigs, utilized in some of the earliest models of allergic airway responses, present early and late phase responses with concurrent AHR and eosinophilia, much

like human manifestations of the disease. Following sensitization, the response of guinea pigs to methacholine, histamine, and allergen are similar to responses observed in human airways, but serotonin also induces marked bronchoconstriction in guinea pigs that is not seen in humans. In addition, differences are observed in response to leukotrienes, indicating an underlying mechanistic distinction between humans and guinea pigs. Guinea pigs also have elevated numbers of eosinophils prior to sensitization and challenge, and in chemical irritant studies, the immunological response of guinea pigs is often acute and sometimes fatal, requiring pre-treatment with antihistamines to dampen the severity of the immune response. As with rats, however, few inbred strains and species-specific reagents are available (Shin et al., 2009).

Rabbits

Rabbit asthma models exhibit early and late phase responses, and as with humans, the rabbit lung is the primary site of IgE-mediated anaphylactic responses (Shin et al., 2009; Zosky and Sly, 2007; Karol, 1994). An early phase response to allergen characterized by increased lung resistance and decreased dynamic compliance is observed within 15 minutes of challenge in this model, followed by a late phase response starting within 2 hours. In this model, neonatal immunization is needed to facilitate development of the late phase response.

Dogs

Dogs, unlike rodents and rabbits, may become naturally sensitized to antigens that also affect the human population (Zosky and Sly, 2007). Such naturally-occurring allergic reactions in dogs do not typically induce asthma-like symptoms despite an elevation in airway eosinophilia, however. Instead, the responses present as dermatitis

or conjunctivitis (Zosky and Sly, 2007). In contrast to the lack of an airway response in naturally occurring allergic reactions, there are canine models of asthma that show airway constriction and AHR in addition to increased IgE and eosinophilia. The Basenji Greyhound model, for example, shows AHR as well as responsiveness to methacholine, though baseline AHR is elevated in this model and AHR does not always mark clinical disease (Karol, 1994). Although canine models have the potential for evaluating chronic responses to allergen, they are relatively expensive, require additional time for breeding and sensitization, and, as with other larger-animal models, there are fewer species-specific reagents.

Sheep

Sheep present early and late phase responses that involve AHR and eosinophilia (Karol, 1994). As seen with some dogs, sheep can become naturally sensitized to *Ascaris suum*, though these responses vary from animal to animal (Zosky and Sly, 2007). Some sheep respond with an early phase response only, while 30-50% respond with both early and late phase responses, mimicking the variability found in the human population. AHR is observed in sheep exhibiting a late phase response, within 24 hours post-challenge. The allergic response in sheep and the timing of mediators observed during allergic pathogenesis, particularly LTE_4, shares many similarities with human asthma patients (Zosky and Sly, 2007). As in humans, cromolyn and corticosteroids are effective in sheep. Dissimilar to humans, however, is that allergic responses in sheep lungs may be ameliorated through the use of drugs targeting platelet activating factors (Zosky and Sly, 2007). Despite some similarities to aspects of human asthma

pathogenesis, sheep, like dogs, are larger and much more costly to maintain than smaller species, and fewer species-specific reagents are available.

Horses

Horses, naturally sensitized to a “barn environment,” develop AHR and recurrent episodes of disease (Shin et al., 2009). Following exposure to hay or barn dust, horses show elevated breathing rates and decreased tidal volume during an acute response (Karol, 1994). Increased lung resistance and decreased dynamic compliance have also been observed. In addition, sensitized horses show serum antibodies to allergen, goblet cell metaplasia, increases in airway smooth muscle, and inflammatory cell infiltration. Despite the similarities to aspects of human asthma pathogenesis especially the spontaneous recurrence of disease, horses, as with other larger animal species, are far more costly and fewer species-specific reagents are available.

Non-Human Primates

Non-human primates (NHPs) arguably share the most similarity with humans and have been used in asthma studies for over 40 years. While some NHPs are naturally sensitive to *Ascaris* antigen, most protocols involve active sensitization with antigens implicated in human asthma (Karol, 1994; Coffman and Hessel, 2005). Humans and NHPs share many genetic, immunological, and physiological similarities, and human reagents can often be used in NHPs (Coffman and Hessel, 2005). NHP and human lungs are similar in size, anatomy, and ultrastructure; and pulmonary function measurements that can be made, the instruments that are used, and the techniques are similar as well. Many of the characteristics central to human asthma have been reported in monkeys, including allergen-specific IgE cutaneous and airway responses, elevated

T_H2 cells in the airways, eosinophilia, goblet cell hyperplasia, airway remodeling, and histamine and methacholine sensitivity (Hyde et al., 2006). Only cynomolgus macaques and baboons could generate responses to leukotrienes, however, perhaps indicating a closer connection between airway response mechanisms for human subjects and these two species than other NHP species. While NHPs offer many advantages in modeling human pathophysiology, in addition to cost and social sensitivities, their social environment is complex, and responses to social stress can be very different from humans, which can affect their responses to corticosteroids.

Available Therapeutics

Extensive progress has been made in defining the immunological processes involved in allergic lung diseases, many owing to basic science experimentation with various animal models, but further complexities in development and regulation of immune processes, as well as complexities in the interaction of the immune system with cellular and molecular pathways controlling development and function of the pulmonary system, have thwarted development of novel efficacious therapeutics. Two factors that could enhance drug development in this area are: 1) emerging appreciation for distinct genotypes and phenotypes in asthma, and 2) elucidation of innate immune pathways that parallel, duplicate, enhance, and oppose adaptive immune pathways.

To date, the management of asthma symptoms has largely been through the use of bronchodilators, inhaled steroids, and corticosteroids. Leukotriene inhibitors and anti-IgE therapies have also been prescribed. Bronchodilators may be short- or long-acting, and generally contain one or a mixture of β2 adrenergic agonists (short- or long-acting), anti-cholinergics (paired with short- or long-acting agents), and/or theophylline (long-

acting) (Spencer and Krieger, 2013). Short-acting bronchodilators can be used to reverse symptoms once they have appeared or as an emergency measure, but do not manage the underlying inflammation. Long-acting bronchodilators do not provide the relief of short-acting bronchodilators, but are typically used together with inhaled steroids for added prevention of acute attacks. Inhaled steroids and corticosteroids are used to lessen airway inflammation in an effort to reduce the chances of the onset of an acute asthma attack. They must be taken 1-2 times daily or as prescribed to help control inflammation, but are not useful once symptoms are present. Leukotriene inhibitors have been used due to their anti-inflammatory effects, but are not as effective as corticosteroids and are therefore used for mild or moderate asthma cases or in conjunction with corticosteroids (Spencer and Krieger, 2013).

Specific Immunotherapy (SIT) has also been used in an attempt to reduce symptoms through building tolerance. With this form of therapy, very small amounts of the antigen are injected subcutaneously in an effort to build tolerance to the antigen due to re-exposure over time. At the start, patients receive small doses once or twice a week over a period of several months, then the dose is increased and given every two to four weeks for approximately four or five months, and finally, doses are given around once a month for a period of years. Due to the rare chance that anaphylactic shock may occur after injection with the allergen, therapy is performed under careful observation. Noteworthy is that most of the serious adverse reactions and fatalities have been due to SIT for asthma rather than for other conditions such as allergic rhinitis and venom hypersensitivity, suggesting that SIT should not be used for patients with severe asthma (Frew, 2010). While immunotherapy may be a worthwhile investment for some, it is

costly with regard to both time and monetary expense, and may or may not be efficacious, depending on the individual and sensitivity to the allergen.

Definition of phenotype-specific pathogenic factors has shown some promise in establishing effectiveness of agents targeting the IL-4 and IL-13 pathways. Elucidation of the role of innate immunity in asthma is leading to better understanding of how lipids and viruses can induce exacerbations in allergic asthmatics, even when their allergies are otherwise well controlled. However, other than some encouraging results from animal studies with agents that interrupt inflammation induced via the OX40 pathway (Kaur and Brightling, 2012), and modest effects of Pitrakinra (recombinant protein targeting IL-4Rα, attenuating IL-4 and IL-13 signaling) (Antoniu, 2010b), MEDI-528 (anti-IL-9 antibody) (Antoniu, 2010a; Parker et al., 2011), Mepolizumab (anti-IL-5 antibody), and Omalizumab (anti-IgE Fc domain antibody) (Busse et al., 2010; Nair et al., 2009; Haldar et al., 2009; Wenzel, 2009; Belliveau, 2005), little has emerged in terms of translating the basic observations into effective and affordable treatments. It is important, therefore, to continue investigations into new, novel therapeutics.

Tolerogenic Therapeutic Vaccines

Cytokines, as a central component of the immunological signaling network, have long been investigated as potential therapeutics for asthma. While methods for simply enhancing or inhibiting the biological activity of specific cytokines have not yet yielded a suitable treatment (Simon, 2006), the fusion of cytokines to immunogen has shown promise in applied animal models for asthma as well as other immune-mediated disease models such as experimental autoimmune encephalitis (EAE) (Mannie et al., 2007b; Kim et al., 1997; Maecker et al., 2001). In this system, the fusion of cytokine

DNA to immunogen DNA yields a single polypeptide following translation that retains the functionality of each original domain. These cytokine fusion proteins have demonstrated the ability to bias the immune system toward Th_1 responses (Kim et al., 1997; Maecker et al., 2001), target antigen to specific antigen presenting cell populations, as well as induce immunological tolerance to antigen (Mannie et al., 2007b; Blanchfield and Mannie, 2010). Fusion proteins incorporating GM-CSF, in particular, have demonstrated potential to target antigen to APC and induce immunological tolerance (Mannie et al., 2007b; Blanchfield and Mannie, 2010).

Goal of Research and Statement of Hypothesis

Recent studies have indicated that targeting immunogens to specific immune cells via fusion with cytokines increases the effectiveness of immunotherapy in animal models for asthma as well as other immune-mediated disease models such as experimental autoimmune encephalitis (Mannie et al., 2007b; Blanchfield and Mannie, 2010; Kim et al., 1997; Maecker et al., 2001; Maecker et al., 2001; Mannie and Abbott, 2007; Mannie et al., 2007a). Cytokine fusion proteins target antigen to specific antigen presenting cell populations (Mannie et al., 2007b; Blanchfield and Mannie, 2010), upregulate T Regulatory cells (Mannie et al., 2007b), bias the immune system toward T_H1 responses (Kim et al., 1997; Maecker et al., 2001), and induce immunological tolerance to antigen (Mannie et al., 2007b; Blanchfield and Mannie, 2010). Cytokines involved in asthma pathogenesis include the T_H2-specific cytokines - IL-4, IL-5, and IL-13 - as well as general cytokine growth factors such as IL-2, a growth factor for lymphocytes, and GM-CSF, a growth factor for neutrophils, eosinophils, and basophils (Mannie et al., 2007b; Blanchfield and Mannie, 2010). While IL-4, IL-5, and IL-13 are

recognized as key cytokines in asthma processes and may warrant future cytokine fusion projects, GM-CSF is equally involved. Upon exposure of DC and macrophage APC cytokine receptors to specific signals such GM-CSF, APC are more inclined to develop a tolerogenic state, leading to the deletion or anergization of reactive T cells (Kapsenberg, 2003) or the induction of suppressive T_{Regs} (Ganesh et al., 2009; Dhodapkar et al., 2001). In addition, GM-CSF has been implicated in the targeting and differentiation of dendritic cell and macrophage antigen presenting cells and has demonstrated the potential to induce immunological tolerance (Mannie et al., 2007b; Blanchfield and Mannie, 2010). As many murine asthma models use ovalbumin to induce allergic inflammation, OVA 323-339, the primary antigenic domain of ovalbumin, could thus be targeted to DC and macrophage APC populations in an effort to tolerize the immune system and reverse asthma symptoms. The objective of this study was to test the hypothesis that fusion proteins comprised of GM-CSF and OVA 323-339 epitopes potentiate antigen-specific immunological tolerance and attenuate development of asthma. The hypothesis was tested using OVA-sensitive mice and GMCSF-OVA 323-339 fusion proteins. The following specific aims will be pursued:

Specific Aims

Aim 1: Establish the efficacy of the GMCSF-OVA 323-339 cytokine-fusion protein *in vitro*

Fusion products were generated through the melding of OVA 323-339 to GM-CSF sequences using PCR with appropriate templates and primers. The biological activity of fusion protein cytokine domains were assessed by incubating respective supernatants with rat bone marrow cells pulsed with tritiated thymidine ([3H]thymidine).

Cell growth and proliferation of mouse bone marrow cells due to the presence of GM-CSF was measured via [3H]thymidine incorporation. The biological activity of fusion protein antigenic domains was validated by addition of protein to splenic cell cultures derived from C57BL/6-Tg(TcraTcrb) (OT-II.2) mice, containing OVA-reactive T Cell repertoires, incubated with [3H]thymidine. As with the cytokine domain bioassay, T Cell clonal expansion due to OVA 323-339 was measured via [3H]thymidine incorporation in samples.

Aim 2: Develop a murine asthma model for testing of the cytokine-fusion protein

A successful model of ovalbumin-induced inflammation in mice was essential for verifying the efficacy of the cytokine fusion protein vaccine. OVA-sensitized and challenged animal groups were tested for airway and lung inflammation, IL-4, 5, and 13, and IgE, IgG_1 compared to saline controls.

Aim 3: Verify the efficacy of the GMCSF-OVA 323-339 cytokine-fusion protein *in vivo*

Using a model of ovalbumin-induced inflammation in mice, the cytokine fusion protein vaccine was tested for ability to attenuate OVA-induced increases in airway and lung inflammation, increases in IL-4, 5, and 13, and increases in levels of IgE and IgG_1.

Study Justification

The approach of the study was novel with respect to fusion-protein-induced tolerance in an asthma model, but was a refinement of an existing model and research conducted by Mannie et al. using an experimental autoimmune encephalitis (EAE) model for Multiple Sclerosis (Mannie et al., 2007b; Blanchfield and Mannie, 2010; Mannie and Abbott, 2007; Mannie et al., 2007a). Multiple Sclerosis is an immune-

system-mediated disease involving an initial error within the antigen presentation pathway. As immunological tolerance to myelin basic protein was induced in EAE models through administration of cytokine fusion proteins (Mannie et al., 2007b; Blanchfield and Mannie, 2010; Mannie and Abbott, 2007; Mannie et al., 2007a), immunological tolerance to OVA 323-339 in the mouse model of asthma may be likewise induced through use of fusion proteins. Research by Umetsu et al., has demonstrated the ability for cytokine fusion protein and fusion DNA to precipitate a shift from a T_H2 to a T_H1 immunophenotype in mice (Kim et al., 1997; Maecker et al., 2001; Maecker et al., 1997). While the focus of this study was to prevent asthma-like inflammation rather than to skew the T_H response, documentation that such fusion proteins have the capacity to effect immunological change in an asthma model provides support that proposed immunological tolerance may be induced in this study. The demonstrated ability of fusion proteins to alter immune system balance in mice provides evidence that this approach may be effective in returning the immune system to homeostasis within an asthma model.

With regard to the choice of species for the asthma model, the mouse was deemed the best candidate for the study. Firstly, all research must begin with a lower-order model as a basis, and due to the fact that this study is proof-of-concept in nature, the mouse is a suitable vehicle for experimentation. In addition, many investigators have used murine models to study asthma and pulmonary allergic inflammation. Finally, the use of mouse model has distinct advantages including ease of manipulation, availability of transgenics for immunomodulation, as well as the multitude of commercially available murine-specific reagents necessary for experimentation. For this study, particularly due

to the availability of OVA 323-339-reactive transgenics, mice were chosen as the vehicle in which to test the GMCSF-OVA 323-339 fusion protein.

CHAPTER 2: EFFICACY OF THE GMCSF-OVA 323-339 CYTOKINE FUSION PROTEIN *IN VITRO*

Introduction

The production of the cytokine-antigen fusion proteins and assurance of their functionality *in vitro* is a critical initial step toward investigating their use as a therapeutic tool within an animal model. One of the challenges in the production of cytokine fusion proteins is the size of the proteins themselves. The fusion of an entire protein such as ovalbumin to a cytokine domain, for example, if even possible, would be a difficult undertaking. It is for this reason that OVA 323-339, the most antigenic epitope of ovalbumin, was chosen to create the fusion proteins and thus chosen for animal sensitization and challenge in the model. Another challenge is to ensure that fusion proteins retain bioactivity for both the cytokine and the antigenic domain, and that bioactivity is neither lost nor altered due to the fusion of the two original proteins. The bioactivity can be measured through specific assays for each individual domain, and in this way, protein functionality is ensured. For Specific Aim 1 of this project, cytokine-OVA 323-339 fusion proteins were generated and tested using a variety of *in vitro* systems.

Methods

Fusion DNA design

Two GM-CSF-based fusion proteins (GMCSF-OVA 323-339 and GM-CSF) were used in this study. The GMCSF-OVA 323-339 fusion protein contained an N-terminal mature mouse GM-CSF domain linked to a 17 amino acid C-terminal domain comprised

of the predominant antigenic domain of OVA (I-S-Q-A-V-H-A-A-H-A-E-I-N-E-A-G-R) coupled to six H residues for protein purification. We also expressed a GM-CSF fusion protein without the antigenic OVA peptide and directly coupled to the six C-terminal H residues. The mouse GM-CSF domain of both fusion proteins was based on a complete mouse mRNA sequence and encoded the mature mouse GM-CSF cytokine.

The mouse GM-CSF sequence was inserted by standard PCR techniques to allow for proper translation and secretion of the fusion proteins. In the first round of PCR, pVAX GM-CSF was used with forward and reverse primers to amplify the cytokine sequence from the pVAX vector. In the second round of PCR, GM-CSF-specific forward primer and OVA 323-339 reverse primer were added to the PCR round 1 GMCSF-OVA 323-339 product. GM-CSF-specific forward and reverse primers contained added overlaps for insertion into parental plasmid added to PCR round 1 GMCSF-only product. In PCR round 3, GMCSF- and OVA 323-339-specific forward and reverse primers containing added overlaps for insertion into parental plasmid were added to the PCR round 2 GMCSF-OVA 323-339 product. In the PCR round 4, the GM-CSF-only product from PCR round 2 and GMCSF-OVA 323-339 product from PCR round 3 were inserted into a pIRES2-AcGFP1 parental plasmid, a mammalian vector containing an internal ribosomal entry site, *Aequorea coerulescens* green fluorescent protein, and a kanamycin/neomycin resistance gene. Products generated were designated as follows: GM-CSF.18, GMCSF-OVA 323-339.21. Plasmid DNA was then washed to remove salts with the OriGene® PowerPrep™ HP Plasmid Midiprep Kit with Prefilters. In PCR round 5, methylated pIRES2-AcGFP1 plasmids (original, non-insert-containing plasmids) were

digested with restriction endonucleases. Plasmids were washed a second time, and gel electrophoresis confirmed that constructs were the correct length.

Fusion DNA expression and purification

GMCSF-OVA 323-339 and GM-CSF DNA constructs made via PCR were transformed into Top10 *E. Coli* bacteria using a BioRad MicroPulser™. Construct-containing bacteria were streaked onto Luria Bertani (10 g tryptone, 10 g NaCl, 5 g yeast extract, 950 mL deionized H_2O) agar plates containing 50 µg/mL kanamycin (Gemini Bio-Products, West Sacramento, CA). Nine individual colonies were selected and grown for each construct. Colonies were designated a-i as follows: GM-CSF.18 a-i, and GMCSF-OVA 323-339.21 a-i. Individual colonies were harvested and expanded in Luria Bertani broth supplemented with kanamycin. Glycerol stocks generated from each expanded colony (GM-CSF.18 a-i, and GMCSF-OVA 323-339.21 a-i) were prepared and frozen at -80°C. Fusion DNA plasmids were extracted from transformed Top10 *E. Coli* bacterial cultures and purified using the OriGene® PowerPrep™ HP Plasmid Midiprep Kit with Prefilters. Gel electrophoresis confirmed the presence and length of fusion DNA constructs. DNA for select constructs (GM-CSF.18b and c, and GMCSF-OVA 323-339.21b and c) was sequenced. All sequences were perfect or likely-perfect with the exception of GM-CSF.18c.

Fusion protein expression, purification, and quantitation

DNA constructs were transfected into a healthy 293F Human Embryonic Kidney (HEK) cell line using TurboFect cationic polymer (Fermentas, catalog # RO531). A stable line was generated from successfully transfected cells, and transfection success and stable line efficiency was confirmed via flow cytometry. Protein secreted in

supernatant from spinner flasks containing the GMCSF-OVA 323-339-expressing 293F HEK cell line was collected and frozen at -20°C. Supernatants were then pooled and concentrated by Amicon® Stirred Ultrafiltration Cells and YM3 membranes (3 kDa exclusion; Millipore, Billerica, MA, USA). Concentrated protein was purified by use of nickel-columns (Ni-NTA Superflow Nickel beads, Qiagen GmbH - Hilden, Germany; Poly-Prep Chromatography Columns, BioRad - Hercules, CA, USA) to bind the GMCSF-OVA 323-339 C-terminal histidine tail. Nickel-column elution fractions were subjected to SDS-PAGE, then silver-stained to confirm protein molecular weight (GM-CSF, ~17.4 kDa; GMCSF-OVA 323-339, ~19.1 kDa). Elution buffer was exchanged for PBS using an Amicon® Ultra-15 Centrifugal Filter Device (3 kDa), and protein was quantitated by use of a NanoDrop® ND-1000 Spectrophotometer.

Measurement of fusion protein bioactivity

To measure fusion protein cytokine domain bioactivity, fresh bone marrow cells from SJL mice (100,000 cells/well) were cultured with complete RPMI and 100 nM GMCSF-OVA 323-339 or GM-CSF fusion protein. Cultures were pulsed with 1 µCi [3H]thymidine (6.7 Ci/mmol, New England Nuclear, Perkin Elmer, Waltham, MA, USA) on day 3, and harvested onto filter-mats on day four with a Tomtec Mach III harvester (Hamden, CT, USA). [3H]Thymidine incorporation into DNA was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter (Perkin Elmer). Figure error bars represent the standard error of triplicate sample wells.

To measure fusion protein antigenic domain bioactivity, OVA 323-339-specific T cell responders (25,000 cells/well) were cultured with irradiated (3000 rads) splenocytes (500,000 cells/well) from wild-type C57BL/6J mice in complete RPMI and 1 µM GMCSF-

OVA 323-339 or GM-CSF fusion protein, or OVA 323-339 peptide. Cultures were pulsed with [3H]thymidine and harvested as described for cytokine domain bioactivity assays above.

FACS analysis

To determine transfection and stable line efficiency, FACS analysis was performed on cell samples. The pIRES2-AcGFP1 vectors containing the inserted fusion DNA contain a reporter AcGFP1 sequence downstream of the GMCSF-OVA 323-339 sequence, allowing successfully transfected 293F HEK cells to fluoresce in the FITC window. Data were acquired with a Becton Dickinson FACScan flow cytometer and were analyzed with the FloJo software program. Dead cells were excluded from analysis by gating forward versus side scatter plots.

Animals

OT-II.2 (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice (Jackson Labs, Bar Harbor, ME, USA), C57BL/6J mice, and SJL mice were bred and housed at the East Carolina University Brody School of Medicine (Greenville, NC, USA). Animal care and use were performed in accordance with animal use protocols and institutional guidelines approved by the East Carolina University Institutional Animal Care and Use Committee.

Cell lines and culture conditions

The OT-II.2 memory T cell line was an IL-2-dependent line derived from OT-II.2 mouse splenocytes incubated with 1 μM OVA 323-339 peptide at 37°C, 5% CO_2. This T cell line was cultured in complete RPMI medium [10% heat-inactivated FBS, 2 mM glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin (Whittaker Bioproducts,

Walkersville, MD, USA)] supplemented with rat rIL-2 (0.4% v/v Sf9 supernatant). The cell line was maintained by periodically stimulating proliferation and line expansion by incubating the OVA-specific T cells with 1 μM OVA 323-339 and irradiated mouse splenocytes to serve as antigen presenting cells in the culture. The OVA 323-339-specific T cell line was maintained at 37°C, 5% CO_2.

The 293F HEK cell line was cultured in Freestyle™ 293 Expression Medium (Gibco®, Grand Island, NY, USA) at 37°C, 5% CO_2. The GMCSF-OVA 323-339-expressing 293F HEK cell line was derived from 293F HEK cells stably transfected with GMCSF-OVA 323-339. The 293F HEK-derived line was cultured in Freestyle™ 293 Expression Medium and 400 μg/mL Geneticin (Gemini Bio-Products, West Sacramento, CA) to select for genetically manipulated cells possessing Geneticin resistance conferred by the introduced plasmid. Culture was maintained at 37°C, 5% CO_2.

Results and Discussion

The most important result of this study was the successful generation of GMCSF-OVA 323-339 cytokine fusion constructs. Although IL-2, IL2-OVA 323-339, IFN-β, IFNβ-OVA 323-339, and GM-CSF constructs were also generated, as the project focused on the GMCSF-OVA 323-339 fusion protein, the GMCSF-OVA 323-339 fusion was carefully assessed. The cytokine domain of GMCSF-OVA 323-339 purified protein was shown to have biological activity similar to that of the GM-CSF positive control, and that antigenic domain bioactivity was intact as well. Taken together, the GMCSF-OVA 323-339 may be described as a fully functional protein with neither domain affected by fusion to the other. It was concluded that GMCSF-OVA 323-339 fusion proteins met functionality criteria for further experimentation *in vivo*.

An overview of the fusion construct DNA generation is shown in figure 2.1, and a detailed representation of the GMCSF-OVA 323-339 vector is shown in figure 2.2. IL-2, IL2-OVA 323-339, IFN-β, IFNβ-OVA 323-339, GM-CSF, and GMCSF-OVA 323-339 cytokine fusion constructs were generated via PCR and run on gel electrophoresis to ensure constructs matched the expected size as compared to Invitrogen TrackIt 100 bp DNA Ladder (figure 2.4). Of samples IL-2.16 a-i, IFNβ.17 a-i, GM-CSF.18 a-i, IL2-OVA 323-339.19 a-i, IFNβ-OVA 323-339.20 a-i, and GMCSF-OVA 323-339.21 a-i, all were the correct size with the exception of IL-2.16a, IFNβ.17d, GM-CSF.18f, GM-CSF.18i, IFNβ-OVA 323-339.20h, GMCSF-OVA 323-339.21a, and GMCSF-OVA 323-339.21g. Constructs of the correct size were transformed into Top10 *E. Coli* and frozen as glycerol stocks for use in transfections for protein expression (figure 2.3).

Small-scale transfections with select IL-2, IL2-OVA 323-339, IFN-β, IFNβ-OVA 323-339, GM-CSF, and GMCSF-OVA 323-339 constructs were performed to determine the presence of functional cytokine domain biological activity in transfection supernatants (figure 2.5). 293 F HEK cells (75,000 cells per well) were transfected with IL-2 (16b, c, d, e), IL2-OVA 323-339 (19a, b, c, d), IFNβ (17a, b, c, e), IFNβ-OVA 323-339 (20a, b, c, d), GM-CSF (18a, b, c, d), and GMCSF-OVA 323-339 (21b, c, d, e) in 96-well plates, and supernatants were collected after 72 hours. Supernatants were added in half-log dilutions to cell lines in 96-well plates: IL-2 and IL2-OVA 323-339 supernatants were incubated with CTLL-2 cells (IL-2 dependent cells), IFN-β and IFNβ-OVA 323-339 supernatants were incubated with BW5147 cells (T-lymphocytes; cells die with increased concentrations of IFN-β), and GM-CSF and GMCSF-OVA 323-339 supernatants were incubated with SJL BMCs (cells proliferate in the presence of GM-CSF), 10,000 cells per well, each. Plates were pulsed with 1 μCi [3H]thymidine on day 3, and harvested onto filter-mats on day 4 with a Tomtec Mach III harvester. [3H]Thymidine incorporation into DNA was measured as counts per minute (CPM) using a Wallac 1450 Microbeta Plus liquid scintillation counter. Increases in CPM were indicative of cell proliferation.

Of the samples tested, IL2.16c and b, IL2-OVA 323-339.19b and c, IFNβ.17c and e, IFNβ-OVA 323-339.20c and a, GMCSF.18c and b, and GMCSF-OVA 323-339.21b and c, had the most active cytokine domains. The presence of cytokine domain biological activity suggested that cytokine fusion constructs were likely functional and could be used for large-scale transfections and protein production.

Large-scale transfections using 293 HEK cells and plasmids from appropriate GMCSF-OVA 323-339 constructs generated a stable GMCSF-OVA 323-339-producing cell line. To evaluate the success of the transfection and the protein-producing capabilities of the cell line, cellular AcGFP1 expression was evaluated via flow cytometry (figure 2.6). 1 x 10^6 cells were sampled from the 293F HEK + GMCSF-OVA 323-339 stably transfected cell line and placed into tubes for flow-cytometric analysis. AcGFP1 expression (denoted by FITC-A) data were acquired with a Becton Dickinson FACScan flow cytometer. The majority of dead cells were excluded from analysis by gating forward versus side scatter plots. Elevated numbers of FITC-A-bright cells suggested a successfully transfected 293F HEK + GMCSF-OVA 323-339 stable line and a good probability for high protein yield.

To determine the protein-production ability of the stable GMCSF-OVA 323-339-producing cell line, spinner flasks were heavily-seeded with 293F HEK + GMCSF-OVA 323-339 cells in EM medium. Supernatants were collected for 5 sequential days, and all cells were re-seeded into flasks with fresh EM medium and 400 µg/mL Geneticin daily. Flasks contained ~30 x 10^6 cells/mL on supernatant collection day 1; cells were allowed to grow, unrestricted, through day 5. Flasks were discarded after the day 5 supernatant collection.

Supernatants were concentrated using an Amicon Stirred Ultrafiltration Cell and a YM3 membrane, purified using a Nickel column, run on SDS-PAGE gel, then silver-stained to determine protein purity (see figure 2.7). Bands visible in lanes 9 and 10 occur due to protein glycosylation sites. Protein was quantified via NanoDrop ND-1000 Spectrophotometer following buffer exchange (250 mM Imidazole exchanged for PBS)

using an Amicon Ultra-15 Centrifugal Filter Device with 3K membrane. Protein yields were as high as 936 µg for the expression system when using four spinner flasks.

In order to determine GMCSF-OVA 323-339 antigenic domain bioactivity and responsiveness compared to OVA 323-339, a memory T cell line was derived from OT-II.2 mouse splenocytes (figure 2.8). Naive OT-II.2 mouse splenocytes (5 x 10^6 cells/10 mL cRPMI, no IL-2) were cultured with 1 µM OVA 323-339 for 3 days. Cells were supplemented with 0.3162% IL-2 (v/v) in complete RPMI. Flasks were observed and passaged on day 6. On day 10, cell line was centrifuged and washed with HBSS to remove IL-2 from culture, then cells were plated (25,000 cells/well) in 96-well plates with irradiated (3,000 rads) C57BL/6J splenocytes to serve as APC (250,000 cells/well) and 1-log dilutions of OVA 323-339 from 10 µM to 10 pM. Plates were pulsed with 1 µCi [3H]thymidine on day 13, and harvested onto filter-mats on day 14 with a Tomtec Mach III harvester. [3H]Thymidine incorporation into DNA was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter. The OT-II.2 memory T cell line was active and responsive to OVA 323-339 as evidenced by the increased CPM, and so, OT-II.2 memory T cell proliferation, corresponding with increasing concentrations of OVA 323-339 in figure 2.8.

GMCSF-OVA 323-339 antigenic domain bioactivity was evaluated in comparison to OVA 323-339 bioactivity with OT-II.2 mouse splenocytes (figure 2.9A) and with OT-II.2 mouse-derived memory T cell line (figure 2.9B). In 2.9A, OT-II.2 mouse splenocytes were harvested and plated (230,000 cells per well) in a 96-well plate with 1-log dilutions of whole OVA, OVA 323-339, GMCSF-OVA 323-339, or GM-CSF (0.5 µM to 0.5 pM) in complete RPMI. In 2.9B, OVA 323-339-specific T cell responders (25,000 cells/well)

were cultured with irradiated (3000 rads) splenocytes (500,000 cells/well) from wild-type C57BL/6J mice in a 96-well plate with 1-log dilutions of GMCSF-OVA 323-339 peptide, GM-CSF fusion protein, or OVA 323-339 peptide (1 μM to 1 pM) in complete RPMI. Cultures were pulsed with 1 μCi [3H]thymidine on day 3, and harvested onto filter-mats on day 4 with a Tomtec Mach III harvester. [3H]Thymidine incorporation into DNA was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter.

The antigenic domain of the GMCSF-OVA 323-339 purified protein was shown to be biologically active. GMCSF-OVA 323-339 cultured with naïve splenocytes from OT-II.2 mice attenuated T cell proliferation compared to naïve splenocytes cultured with OVA 323-339, while whole OVA and GM-CSF had no effect on T cell proliferation. In contrast, GMCSF-OVA 323-339 cultured with cells from the memory OT-II.2 T cell line caused antigenic domain bioactivity to be left-shifted compared with the OVA 323-339 positive control, suggesting targeting of antigen to T cell responders.

GMCSF-OVA 323-339 cytokine domain bioactivity was evaluated in comparison to GM-CSF bioactivity using SJL mouse bone marrow cells (figure 2.10). In figure 2.10, SJL bone marrow cells (cell line; 10,000 cells per well) were cultured in a 96-well plate with 0.5-log dilutions of GMCSF-OVA 323-339, rat GM-CSF, mouse GM-CSF, or GMCSF-MOG fusion protein (1 nM to 100 fM) in complete RPMI. The culture was pulsed with 1 μCi [3H]thymidine on day 3, and harvested onto filter-mats on day 4 with a Tomtec Mach III harvester. [3H]Thymidine incorporation into DNA was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter. The cytokine domain of GMCSF-OVA 323-339 purified protein was shown to have biological activity similar to that of the GM-CSF positive control. Taken together with the antigenic domain

bioactivity data, the GMCSF-OVA 323-339 may be described as a fully functional protein with neither domain affected by fusion to the other. Given that the cytokine fusion protein was successfully generated and shown to have appropriate bioactivity in both the cytokine and antigenic domains, the fusion protein is suitable for testing in an animal model to determine its potential for altering the immune response to the OVA 323-339 antigen.

Tables and Figures

Figure 2.1. Overview of GMCSF-OVA 323-339 fusion DNA production

Two GM-CSF-based fusion proteins (GMCSF-OVA 323-339 and GM-CSF) were used in this study. The mouse GM-CSF sequence was inserted by standard PCR techniques to allow for proper translation and secretion of the fusion proteins, and gel electrophoresis confirmed that constructs were the correct length. GMCSF-OVA 323-339 and GM-CSF DNA constructs made via PCR were transformed into Top10 *E. Coli* bacteria. Construct-containing bacteria were streaked onto Luria Bertani agar plates containing 50 µg/mL kanamycin, and individual colonies were selected and grown for each construct. Colonies were harvested and expanded in Luria Bertani broth supplemented with kanamycin. Glycerol stocks generated from each expanded colony were prepared and frozen at -80°C. Fusion DNA plasmids were extracted from transformed Top10 *E. Coli* bacterial cultures and purified using the OriGene® PowerPrep™ HP Plasmid Midiprep Kit with Prefilters. Gel electrophoresis confirmed the presence and length of fusion DNA constructs, and DNA for select constructs was sequenced.

Generation of Cytokine and Cytokine-OVA 323-339 Plasmid DNA and Top10 E. Coli Glycerol Stocks

Event	Event Details
pVAX GM-CSF	
↓	
Overlap Extension PCR	Overlap extension PCR performed for GM-CSF with and without OVA 323-339. Resulting vector was inserted into pIRES AcGFPI plasmids via directional restriction site-free insertion.
↓	
GMCSF/GMCSF-OVA 323-339 in pIRES AcGFPI	
↓	
Wash plasmid DNA	
↓	
Digest plasmid DNA	Methylated pIRES2 AcGFPI plasmids digested with restriction endonucleases.
↓	
Wash plasmid DNA	
↓	
Verify constructs	Plasmid DNAs run on agarose gel to ensure constructs were the correct length.
↓	
Transform completed plasmid DNA constructs into E. Coli	Constructs transformed into Top10 E. Coli and glycerol stocks generated, allowing for ease of storage and generation of more plasmid-containing bacteria.
↓	
Plate transformed bacteria, then select individual colonies to be grown in culture	9 colonies were chosen and grown for each construct. Colonies were designated a-i as follows: GMCSF 18 a-i, and GMCSF-OVA 21 a-i.
↓	
Run all plasmid DNAs on an agarose gel to verify constructs.	All constructs appeared to be the correct length with the exception of GMCSF 18 f, i and GMCSF-OVA 21 a, g.
↓	
Sequence plasmid DNA	DNA for select constructs (GMCSF 18 b, c and GMCSF-OVA 21 b, c) was sequenced. All sequences were perfect or likely-perfect with the exception of GMCSF 18c.
↓	
Create glycerol stocks of plasmid-containing bacteria	Glycerol stocks of Top10 E. Coli containing GMCSF 18 a-i and GMCSF-OVA 21 a-i were made and stored at -80°C. EXTRA stocks were made for GMCSF 18b and GMCSF-OVA 21b, which were determined to have perfect sequences.

Figure 2.2. General design of the cytokine-OVA 323-339 fusion construct

An overview of the GMCSF-OVA 323-339 fusion construct design. Major components include the murine GM-CSF cytokine domain followed immediately by the OVA 323-339 antigenic domain, and the downstream AcGFP1 sequence and histidine tail.

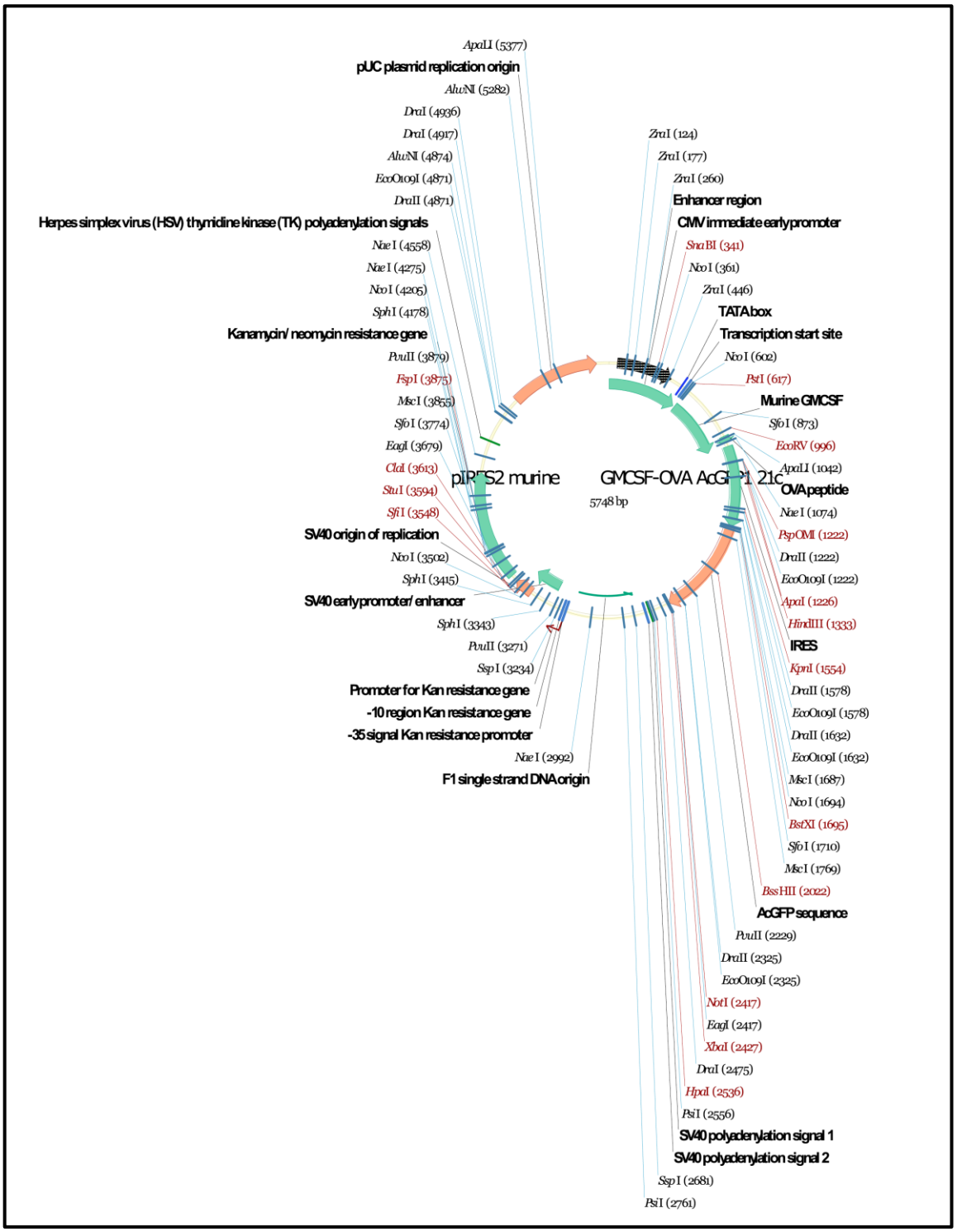

ApaLI (5377)
pUC plasmid replication origin
AluNI (5282)
DraI (4936)
DraI (4917)
AluNI (4874)
Eco0109I (4871)
DraII (4871)
Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signals
NaeI (4558)
NaeI (4275)
NcoI (4205)
SphI (4178)
Kanamycin/ neomycin resistance gene
PvuII (3879)
FspI (3875)
MscI (3855)
SfoI (3774)
EagI (3679)
ClaI (3613)
StuI (3594)
SfiI (3548)
SV40 origin of replication
NcoI (3502)
SphI (3415)
SV40 early promoter/ enhancer
SphI (3343)
PvuII (3271)
SspI (3234)
Promoter for Kan resistance gene
-10 region Kan resistance gene
-35 signal Kan resistance promoter
NaeI (2992)
F1 single strand DNA origin
ZraI (124)
ZraI (177)
ZraI (260)
Enhancer region
CMV immediate early promoter
SnaBI (341)
NcoI (361)
ZraI (446)
TATA box
Transcription start site
NcoI (602)
PstI (617)
Murine GMCSF
SfoI (873)
EcoRV (996)
ApaLI (1042)
OVA peptide
NaeI (1074)
PspOMI (1222)
DraII (1222)
Eco0109I (1222)
ApaI (1226)
HindIII (1333)
IRES
KpnI (1554)
DraII (1578)
Eco0109I (1578)
DraII (1632)
Eco0109I (1632)
MscI (1687)
NcoI (1694)
BstXI (1695)
SfoI (1710)
MscI (1769)
BssHII (2022)
AcGFP sequence
PvuII (2229)
DraII (2325)
Eco0109I (2325)
NotI (2417)
EagI (2417)
XbaI (2427)
DraI (2475)
HpaI (2536)
PsiI (2556)
SV40 polyadenylation signal 1
SV40 polyadenylation signal 2
SspI (2681)
PsiI (2761)
pIRES2 murine
GMCSF-OVA AcGFP1 21c
5748 bp

Figure 2.3. Overview of GMCSF-OVA 323-339 fusion protein production

DNA constructs were transfected into a healthy 293F Human Embryonic Kidney (HEK) cell line. Transfection success and efficiency was confirmed via flow cytometry. Protein secreted in supernatant from spinner flasks containing the GMCSF-OVA 323-339-expressing 293F HEK cell line. Supernatants were then pooled and concentrated using Ultrafiltration Cells. Concentrated protein was purified by use of nickel-columns to bind the GMCSF-OVA 323-339 C-terminal histidine tail. Nickel-column elution fractions were subjected to SDS-PAGE, then silver-stained to confirm protein molecular weight. Elution buffer was exchanged for PBS, and protein was quantitated via Spectrophotometer. Fusion protein cytokine and antigenic domain bioactivity was then assessed using bioassays.

Generation of Cytokine and Cytokine-OVA 323-339 Fusion Proteins

Event	Event Details
Streak agar plates with bacterial stocks containing constructs	LB + Kanamycin agar plates streaked with with Top10 E. Coli bacteria stocks containing GMCSF or GMCSF-OVA constructs.
Grow cultures of individual colonies in LB + Kanamycin	Individual colonies selected from a given agar plate and transfer into 14 mL polystyrene tubes containing 5 mL LB + Kanamycin. Plates incubated in warm room, shaking, for 2 - 8 hours.
Scale-up cultures	5 mL cultures transferred into 100 mL LB + Kanamycin-containing flasks. Cultures incubated in warm room, shaking, for 6 - 12 hours.
Lyse bacteria and purify plasmid DNAs	Bacteria lysed and plasmid DNAs purified via OriGene PowerPrep TM HP Plasmid Midiprep Kit.
Verify constructs	Plasmid DNAs run on an agarose gel to verify constructs.
Transfect 293F HEK Cells with constructs	Transfect 4 - 6 spinner flasks, each containing 30 x 10^6 cells, per construct.
Collect transfection day 2 supernatants, scale up flasks	Supernatant collected, and fresh medium and antibiotic added (G418, 400 µg/mL). Flasks re-seeded with cells and additional flasks seeded with excess cells.
Collect transfection day 3 supernatants	
Collect transfection day 4 supernatants, transfer cells to T75 flask to generate stable line	Supernatant collected as above. Cells transferred to a T75 flask for stable line generation.
Perform cytokine activity bioassay	Cytokine activity bioassay performed with supernatant aliquots to determine whether or not protein is present (if present, proceed to protein concentration and purification steps).
Concentrate supernatant proteins	Concentrate protein via Amicon Ultrafiltration Stir Cells to allow for quicker, easier purification.
Purify proteins	Protein purified via Nickel Chromatography Column (proteins contain a histidine tail).
Ensure protein is present	Proteins run on an SDS-PAGE gel and stain to ensure protein is present.
Quantify protein present in samples	
Verify cytokine domain activity	GMCSF and GMCSF-OVA proteins titrated and incubated with bone marrow cells (will proliferate in the presence of GMCSF), Cells pulsed with tritiated thymidine to measure cellular proliferation to determine cytokine domain activity.
Verify antigenic domain activity	GMCSF and GMCSF-OVA proteins incubated with splenic T Cells from OT.II mice. Cells pulsed with tritiated thymidine to measure cellular proliferation to determine antigenic domain activity.
Test proteins *in vivo*	Test fusion proteins *in vivo*.

Figure 2.4. Generation of IL-2, IL2-OVA 323-339, IFN-β, IFNβ-OVA 323-339, GM-CSF, and GMCSF-OVA 323-339 cytokine fusion constructs

IL-2, IL2-OVA, IFN-β, IFNβ-OVA, GM-CSF, and GMCSF-OVA 323-339 cytokine fusion constructs were generated via PCR and run on gel electrophoresis to ensure constructs matched the expected size (IL-2: 666 bp, IL2-OVA: 703 bp, IFN-β: 726 bp, IFNβ-OVA: 763 bp, GM-CSF: 582 bp, and GMCSF-OVA 323-339: 619 bp) as compared to Invitrogen TrackIt 100 bp DNA Ladder (*). Of samples IL-2.16 a-i, IFNβ.17 a-i, GM-CSF.18 a-i, IL2-OVA 323-339.19 a-i, IFNβ-OVA 323-339.20 a-i, and GMCSF-OVA 323-339.21 a-i, all were the correct size with the exception of IL-2.16a, IFNβ.17d, GM-CSF.18f, GM-CSF.18i, IFNβ-OVA 323-339.20h, GMCSF-OVA 323-339.21a, and GMCSF-OVA 323-339.21g.

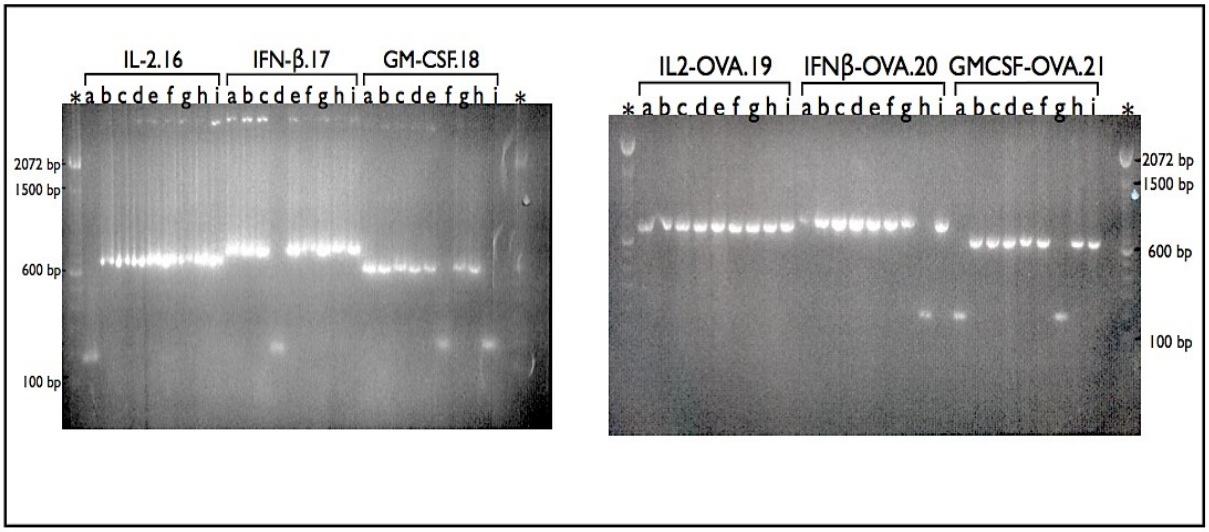

IL-2.16
IFN-β.17
GM-CSF.18
*a b c d e f g h i a b c d e f g h i a b c d e f g h i *
2072 bp
1500 bp
600 bp
100 bp
IL2-OVA.19
IFNβ-OVA.20
GMCSF-OVA.21
*a b c d e f g h i a b c d e f g h i a b c d e f g h i *
2072 bp
1500 bp
600 bp
100 bp

Figure 2.5. Cytokine domain bioactivity of IL-2, IL2-OVA 323-339, IFN-β, IFNβ-OVA 323-339, GM-CSF, and GMCSF-OVA 323-339 cytokine fusion supernatants

293 F HEK cells were transfected with IL-2 (16 b, c, d, e), IL2-OVA 323-339 (19 a, b, c, d), IFN-β (17 a, b, c, e), IFNβ-OVA 323-339 (20 a, b, c, d), GM-CSF (18 a, b, c, d), and GMCSF-OVA 323-339 (21 b, c, d, e) samples, and supernatants collected after 72 hours. Supernatants were added in half-log dilutions to cell lines in 96-well plates: IL-2 and IL2-OVA 323-339 supernatants were incubated with CTLL-2 cells, IFN-β and IFNβ-OVA 323-339 supernatants were incubated with BW5147 cells, and GM-CSF and GMCSF-OVA 323-339 supernatants were incubated with SJL BMCs. Plates were pulsed with 1 μCi [3H]thymidine on day 3, and harvested onto filter-mats on day 4 with a Tomtec Mach III harvester. [3H]Thymidine incorporation into DNA was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter. Error bars represent the standard error for values obtained in replicate wells in the assay.

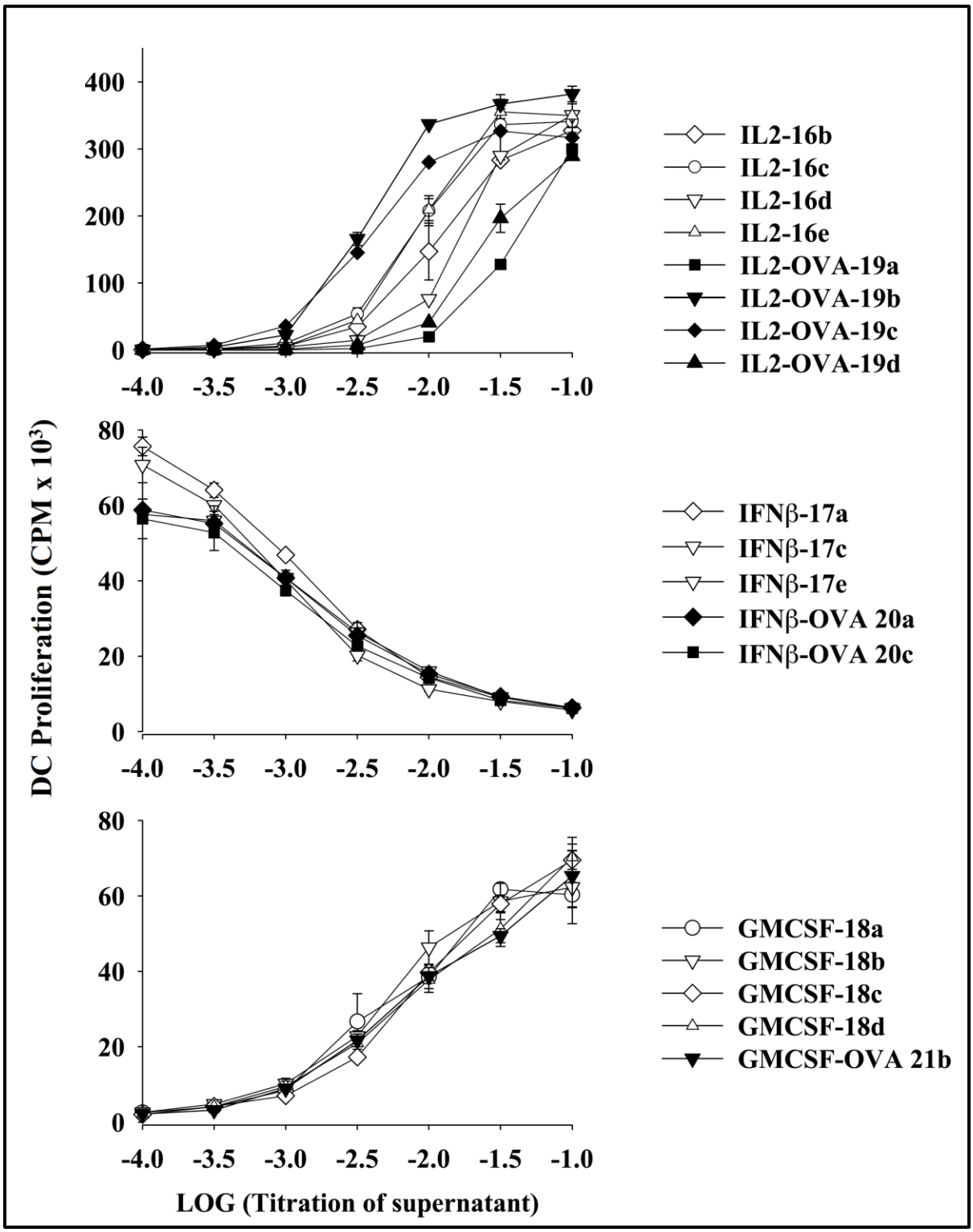
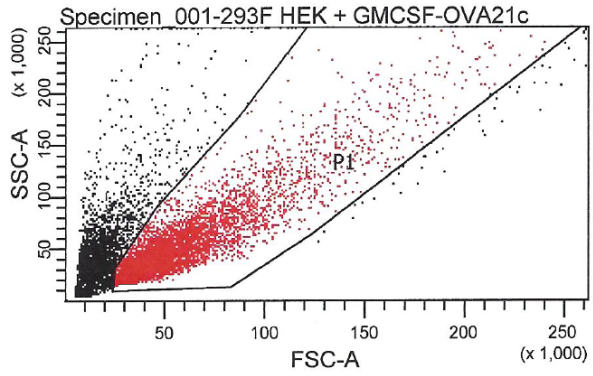

DC Proliferation (CPM x 10³)
400
300
200
100
0
-4.0
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
IL2-16b
IL2-16c
IL2-16d
IL2-16e
IL2-OVA-19a
IL2-OVA-19b
IL2-OVA-19c
IL2-OVA-19d
80
60
40
20
0
IFNβ-17a
IFNβ-17c
IFNβ-17e
IFNβ-OVA 20a
IFNβ-OVA 20c
GMCSF-18a
GMCSF-18b
GMCSF-18c
GMCSF-18d
GMCSF-OVA 21b
LOG (Titration of supernatant)

Figure 2.6. A flow-cytometric assessment of AcGFP1 expression in the 293F HEK + GMCSF-OVA 323-339 stably-transfected cell line

1 x 10^6 cells were sampled from the 293F HEK + GMCSF-OVA 323-339 stably transfected cell line for flow-cytometry. Cellular AcGFP1 expression is denoted by the FITC-A stain. When expressed, AcGFP1, located downstream of GMCSF-OVA 323-339 in the fusion construct, serves as an indicator of GMCSF-OVA 323-339 production and expression and thus, efficiency of the stably transfected cell line. Flow cytometry data and graph was gated on live cells (P1). P2 represents the GFP+ population within P1.

Population	#Events	%Parent	FSC-A Mean	SSC-A Mean
All Events	10,000	####	47,672	51,663
P1	6,543	65.4	58,076	51,412
P2	6,281	96.0	58,746	51,669



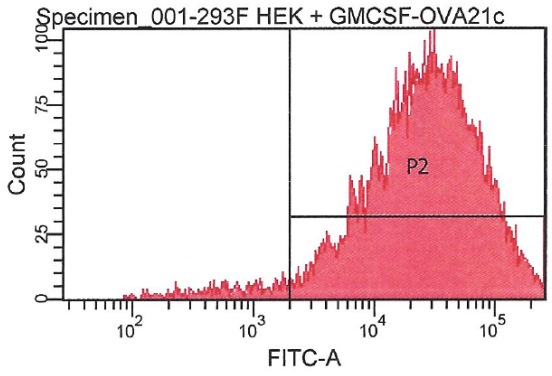


Figure 2.7. Determination of GMCSF-OVA 323-339 protein purity

Supernatants collected from the stably-transfected 293F HEK + GMCSF-OVA 323-339 cell line were concentrated and purified, run on SDS-PAGE gel, then silver-stained to determine protein purity (GMCSF-OVA 323-339 = 19.1 kDa). Lane 1: Fermentas SpectraTM Multicolor Broad Range Protein Ladder; lanes 2-8: flow-through solutions collected during protein concentration and purification; lane 9: Nickel Column GMCSF-OVA 323-339 elution #1/4; lane 10: Nickel Column GMCSF-OVA 323-339 elution #2/4. Bands visible in lanes 9 and 10 occur due to GMCSF-OVA 323-339 glycosylation sites.

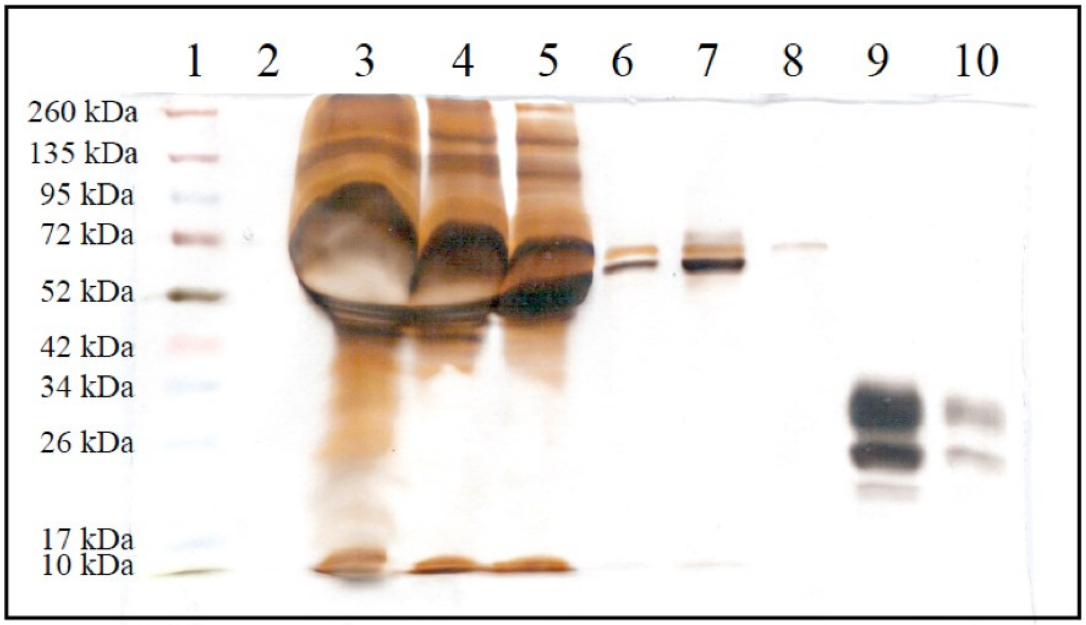

1 2 3 4 5 6 7 8 9 10
260 kDa
135 kDa
95 kDa
72 kDa
52 kDa
42 kDa
34 kDa
26 kDa
17 kDa
10 kDa

Figure 2.8. OT-II.2 mouse-derived memory T cell line efficacy

Memory OT-II.2 T Cell Line was derived by culturing naive OT-II.2 mouse splenocytes with 1 μM OVA 323-339 for 3 days. Cells were then supplemented with 0.3162% IL-2 (v/v) in complete RPMI. Cells were washed to remove IL-2 from medium, then plated in 96-well plates with irradiated C57BL/6J splenocytes and 1-log dilutions of OVA 323-339 from 10 μM to 10 pM. Plates were pulsed with 1 μCi [3H]thymidine on day 13, and harvested onto filter-mats on day 14 with a Tomtec Mach III harvester. [3H]Thymidine incorporation into DNA was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter. Error bars represent the standard error for values obtained in replicate wells in the assay.

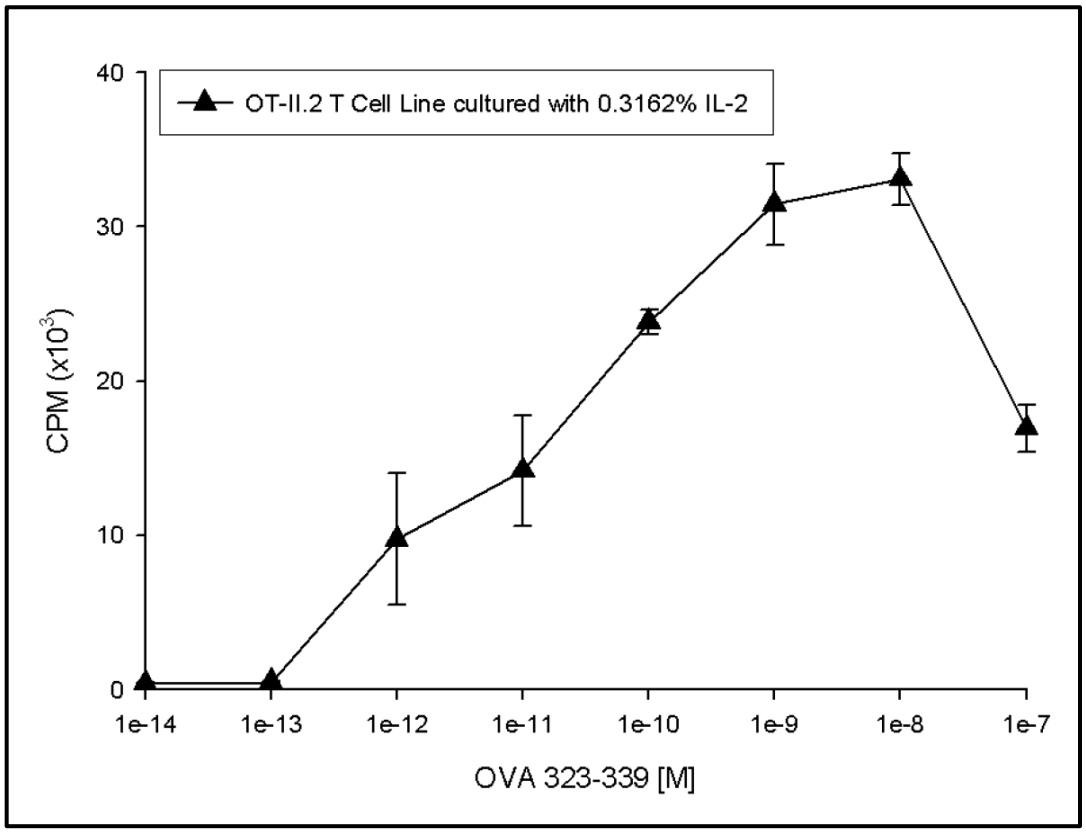
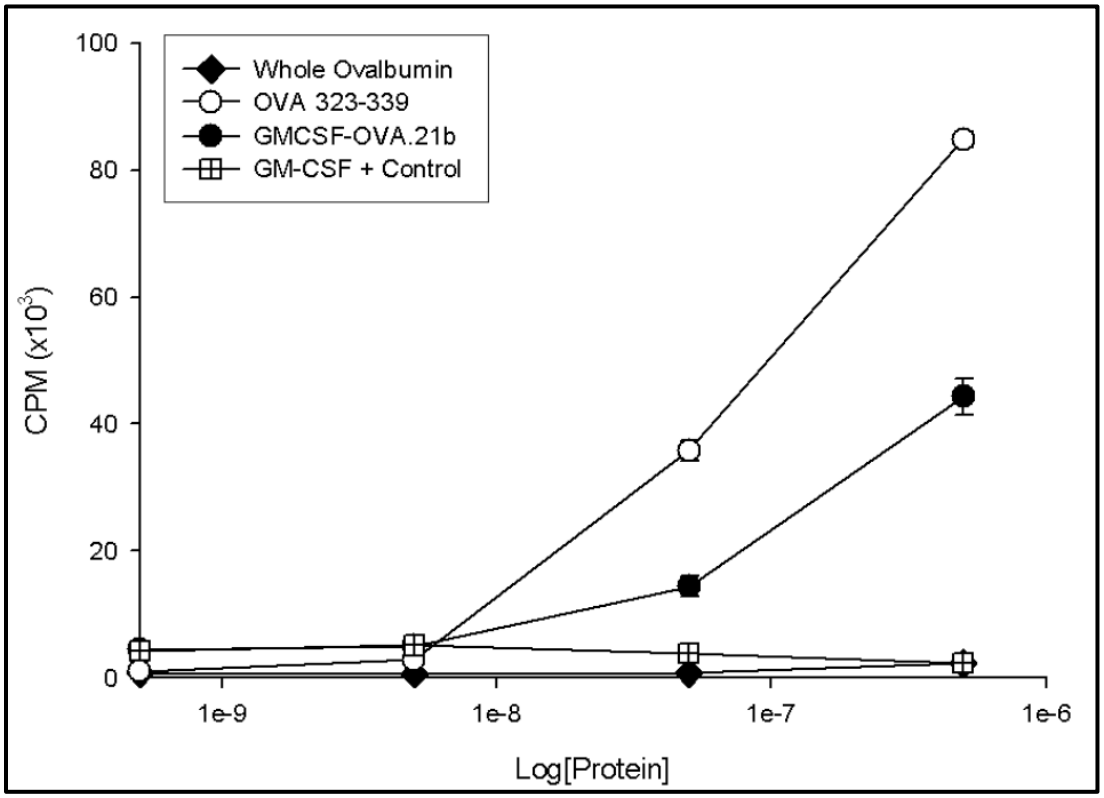

OT-II.2 T Cell Line cultured with 0.3162% IL-2
CPM (x10^3)
40
30
20
10
0
1e-14
1e-13
1e-12
1e-11
1e-10
1e-9
1e-8
1e-7
OVA 323-339 [M]

Figure 2.9. OT-II.2 T Cell proliferative response to GMCSF-OVA 323-339 compared with OVA 323-339 and GM-CSF

In 2.9A, OT-II.2 mouse splenocytes were harvested and plated with 1-log dilutions of whole OVA, OVA 323-339, GMCSF-OVA 323-339, or GM-CSF (0.5 μM to 0.5 pM) in complete RPMI. In 2.9B, cells from the Memory OT-II.2 T Cell Line were cultured with irradiated splenocytes from wild-type C57BL/6J mice and 1-log dilutions of GMCSF-OVA 323-339 peptide, GM-CSF fusion protein, or OVA 323-339 peptide (1 μM to 1 pM) in complete RPMI. For both 2.9A and 2.9B, cultures were pulsed with 1 μCi [3H]thymidine on day 3, and harvested onto filter-mats on day 4 with a Tomtec Mach III harvester. [3H]Thymidine incorporation into DNA was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter. Error bars represent the standard error for values obtained in replicate wells in the assay.

A


Whole Ovalbumin
OVA 323-339
GMCSF-OVA.21b
GM-CSF + Control
CPM (x10^3)
100
80
60
40
20
0
1e-9
1e-8
1e-7
1e-6
Log[Protein]

B

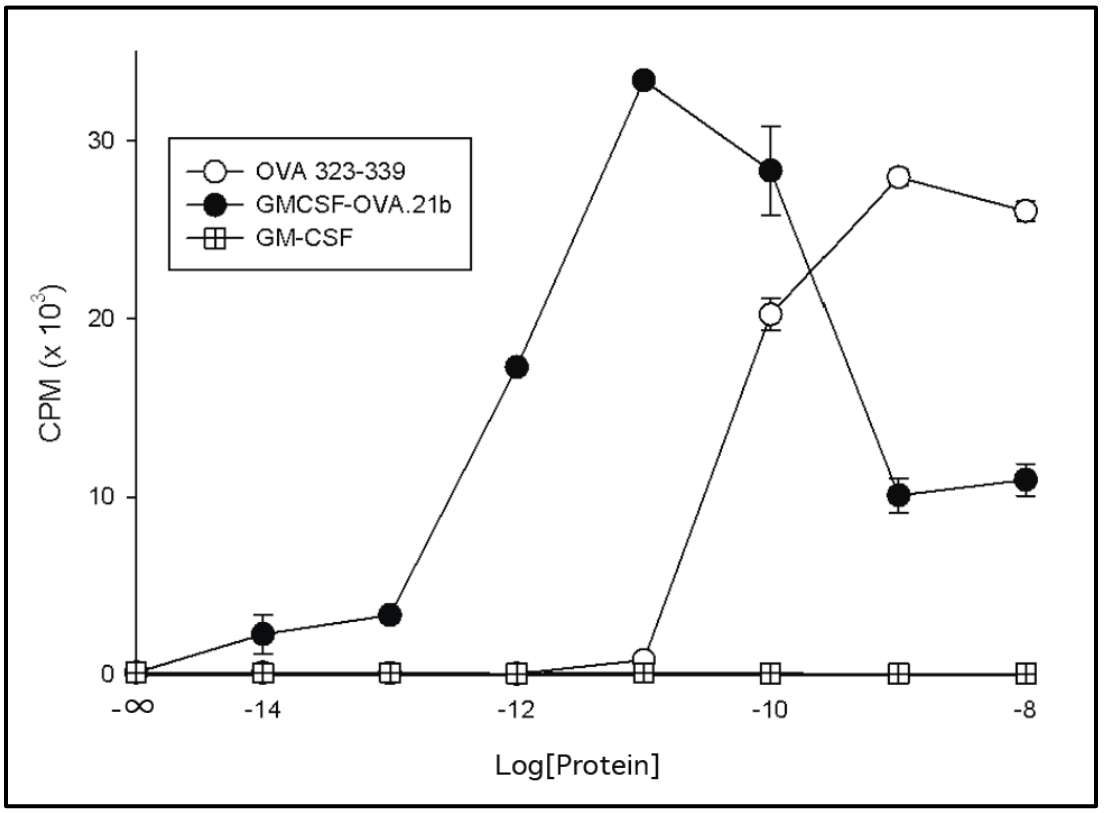
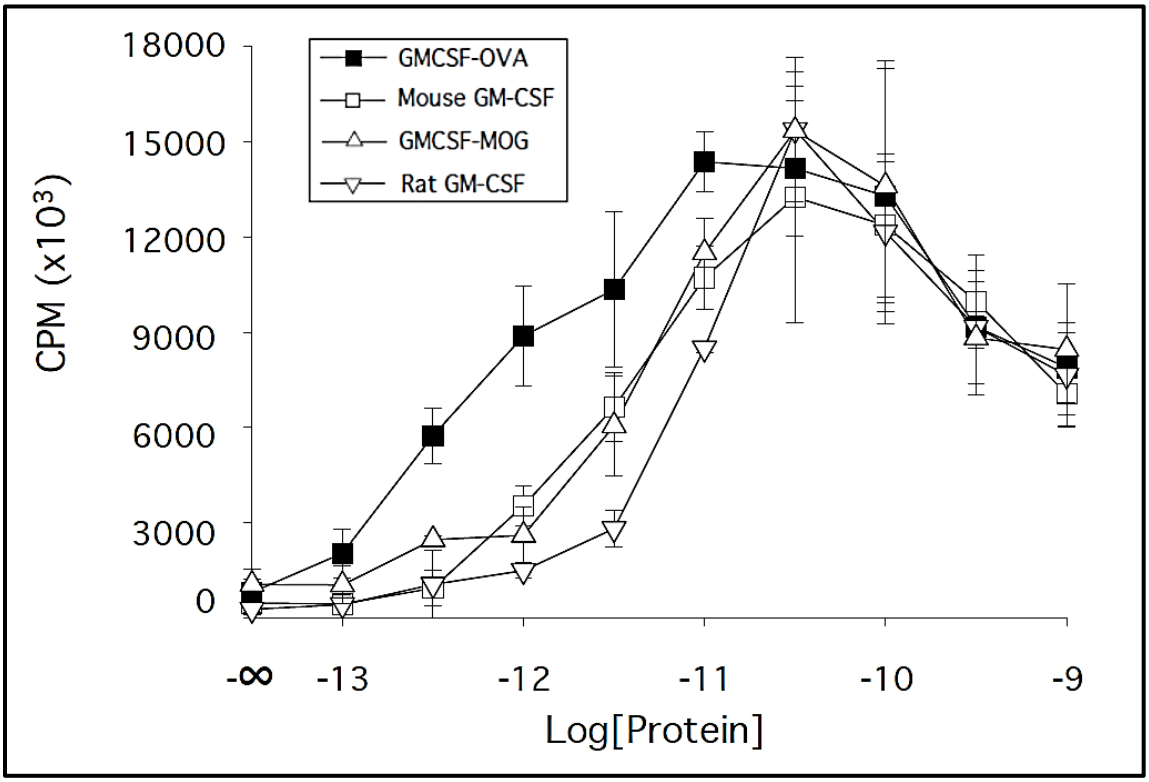

OVA 323-339
GMCSF-OVA.21b
GM-CSF
CPM (x 10^3)
30
20
10
0
-∞
-14
-12
-10
-8
Log[Protein]

Figure 2.10. SJL bone marrow cell proliferative response to GMCSF-OVA 323-339 cytokine domain compared with GM-CSF

SJL BMCs (cell line; 10,000 cells per well) were cultured in a 96-well plate with 0.5-log dilutions of GMCSF-OVA 323-339, rat GM-CSF, mouse GM-CSF, or GMCSF-MOG fusion protein (1 nM to 100 fM) in complete RPMI. The culture was pulsed with 1 μCi [3H]thymidine on day 3, and harvested onto filter-mats on day 4 with a Tomtec Mach III harvester. [3H]Thymidine incorporation into DNA was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter. Error bars represent the standard error for values obtained in replicate wells in the assay.


18000
15000
12000
9000
6000
3000
0
CPM (x10³)
GMCSF-OVA
Mouse GM-CSF
GMCSF-MOG
Rat GM-CSF
-∞
-13
-12
-11
-10
-9
Log[Protein]

Conclusion

IL-2, IL2-OVA 323-339, IFN-β, IFNβ-OVA 323-339, GM-CSF, and GMCSF-OVA 323-339 cytokine fusion constructs were successfully generated, and small-scale transfections were performed with these constructs. Functional cytokine domain biological activity assessed, and of the samples tested, IL2.16c and b, IL2-OVA 323-339.19b and c, IFNβ.17c and e, IFNβ-OVA 323-339.20c and a, GMCSF.18c and b, and GMCSF-OVA 323-339.21b and c, had the most active cytokine domains. The presence of cytokine domain biological activity suggested that cytokine fusion constructs were likely functional and could be used for large-scale transfections and protein production. Large-scale transfections were then initiated on AcGFP1-positive populations, as AcGFP1 production was indicative of fusion protein production. Transfection supernatants were concentrated, purified, run on SDS-PAGE gel, then silver-stained to ensure protein purity. Protein was quantified via NanoDrop ND-1000 Spectrophotometer following buffer exchange, with protein yields were as high as 936 µg for the expression system when using four spinner flasks.

Focusing on the GMCSF-OVA 323-339 fusion protein, antigenic domain bioactivity was assessed and shown to be biologically active. The cytokine domain of GMCSF-OVA 323-339 purified protein was shown to have biological activity similar to that of the GM-CSF positive control. Taken together with the antigenic domain bioactivity data, the GMCSF-OVA 323-339 may be described as a fully functional protein with neither domain affected by fusion to the other. Altogether, the fusion products were shown to be the correct sequence and the proteins were shown to be fully functional, even though an *in vitro* effect on OT-II.2 T cells could not be seen with

FACs analysis. It was concluded that GMCSF-OVA 323-339 fusion proteins should be tested in an *in vivo* system.

CHAPTER 3: CONFIRMATION OF A MURINE MODEL OF ALLERGEN-INDUCED AIRWAY INFLAMMATION

Introduction

In order to test fusion protein efficacy, a suitable animal model is imperative, and a murine model of allergen-induced airway inflammation is an ideal option for a preliminary vehicle in which to test the fusion protein. Most murine models are ovalbumin-based (although house dust mite and other antigens have been reported for use in murine models), and while most ovalbumin-based models use whole ovalbumin for sensitization and challenge, GMCSF-OVA 323-339 fusion protein design made it necessary for OVA 323-339 to be used for both sensitization and challenge. Due to the fact that very few models use OVA 323-339 for both sensitization and challenge, it was necessary to pursue different protocols comparing the advantages of chronic vs. acute models, the use of a C57BL/6 vs. BALB/c mouse strains, the use of various adjuvants, sensitization routes, and challenge routes, as well as varying OVA 323-339 doses, and numbers of sensitizations and challenges. For Specific Aim 2 of this project, animal models of allergic inflammation were tested, and the success or failure of each protocol was evaluated based on its ability to produce pulmonary inflammation, particularly eosinophilic inflammation.

Methods

Animals

BALB/c and C57BL/6 mice (Jackson Labs, Bar Harbor, ME, USA), were housed at the East Carolina University Brody School of Medicine (Greenville, NC, USA). Animal

care and use were performed in accordance with animal use protocols and institutional guidelines approved by the East Carolina University Institutional Animal Care and Use Committee.

Animal model of pulmonary inflammation chosen for study

Active sensitization of mice with OVA 323-339 in Incomplete Freund's Adjuvant (IFA) was performed by subcutaneous injection of 200 µg OVA 323-339 in 200 µL IFA. Prior to sensitization injections, mice were anesthetized using isoflurane in a vented anesthetic chamber and injection sites were cleaned with povidone-iodine followed by an alcohol rinse. Sensitizations were performed on days 0, 7, and 14, and injection sites were monitored for signs of inflammation. Mice were challenged via pharyngeal aspiration (p.a.) on days 21, 22 and 23. The p.a. solution was 60 µL of 1% OVA-323-339 in sterile saline, and was administered while mice were anesthetized with isoflurane in a vented anesthetic chamber. Control mice were given saline instead of the 1% OVA 323-339 solution. Mice were harvested on day 26.

Animal model of chronic pulmonary inflammation with pharyngeal aspiration sensitization and aerosol challenge

Mice were anesthetized with isoflurane and sensitized with either 75 µL phosphate buffered saline (PBS) or ovalbumin (OVA) + lipopolysaccharide (LPS) (100 µg OVA + 0.1 µg LPS/75 µL) via oropharyngeal aspiration on days 0 and 6. Starting on day 13 mice were be exposed to a nose-only aerosol of either PBS or 2% OVA (no LPS) for 30 minutes for 3 consecutive days. Briefly, mice were placed in vented restraining tubes that were then placed with just the nose exposed to the inside of the aerosol chamber. PBS or OVA aerosols of respirable droplet size (1-10 µm) were

generated with a Devilbiss nebulizer and delivered to the chamber at 1 L/min for 30 minutes. Three days of aerosol exposures were given at one-month intervals for three months. Three days after the final exposure, animals were harvested.

Animal model of chronic pulmonary inflammation with intraperitoneal sensitization and aerosol challenge

Mice were sensitized on days 0 and 12 with 10 μg OVA 323-339 in 200 μL aluminum hydroxide (alum). Six days after the second sensitization, mice underwent six consecutive days of aerosol challenge (5% whole OVA in PBS or PBS alone) on days 18-23 followed by two days of rest, then three added consecutive days (days 26-28) of aerosol challenge. During aerosol challenge, mice were placed in vented restraining tubes that were then placed with just the nose exposed to the inside of the aerosol chamber. PBS or OVA aerosols of respirable droplet size (1-10 μm) were generated with a Devilbiss nebulizer and delivered to the chamber at 1 L/min for 30 minutes. One-third of the mice were sacrificed 24 hours after the day 28 aerosol challenge. Remaining mice were subjected to three consecutive days of aerosol challenge the following week on days 33-35, and another one-third of the mice were sacrificed 24 hours after the day 35 aerosol challenge. The last one-third of the mice were given three aerosols per week for three weeks (days 40-42, 47-49, and 54-56), then sacrificed 24 hours after the day 56 aerosol challenge.

Animal model of acute pulmonary inflammation with intraperitoneal sensitization with OVA 323-339 in aluminum hydroxide and a single pharyngeal aspiration challenge

Mice were sensitized on days 0 and 12 with 10 μg OVA 323-339 in 200 μL alum via intraperitoneal injection. One week following the second sensitization, mice were

challenged with a single dose of 600 µg OVA 323-339 in 50 µL saline on day 19. Mice were sacrificed on day 21.

Animal model of acute pulmonary inflammation with intraperitoneal sensitization with OVA 323-339 in aluminum hydroxide and a single pharyngeal aspiration challenge

Mice were sensitized on days 0, 7, and 14 with 200 µg OVA 323-339 in 200 µL alum via intraperitoneal injection. One week following the second sensitization, mice were challenged with 60 µL of a single dose of a 1% OVA 323-339 solution on days 21-23. Mice were sacrificed on day 26.

Pulmonary function testing

On the day of harvest, mice were anesthetized with tribromoethanol (250 mg/kg) and underwent tracheostomy. The tracheostomy was performed on anesthetized animals by making an incision through outer skin on the neck to create a 1 cm flap of skin exposing a small area of the throat, allowing the thyroid lobes to be divided bluntly at the isthmus and pulled aside. The muscle overlying the trachea was then removed with sterile scissors. Next, a small incision was made in the trachea just below the larynx, and a tracheal cannula (18g) was inserted and secured to the trachea with 3-0 silk suture thread.

Following cannula insertion, vecuronium bromide (0.2 mg/kg, i.p.) was administered to prevent interference of breathing patterns during lung function measurements. Lung function measurements were made at baseline and at increasing concentrations of methacholine (MCh) aerosol: 0, 6.25, 12.5, 25, and 50 mg/ml MCh, to determine changes in airway resistance (R). Mice were euthanized via cardiac stick terminal bleed and pneumothorax immediately after lung function measurements,

bronchoalveolar lavage was performed to obtain lavage fluid, and tissues were collected for histological analysis.

Serum collection

Terminal bleeds were performed via intracardiac puncture as a secondary means to ensure euthanasia as well as to collect serum samples. Whole blood collected from terminal bleeds was placed in serum separator tubes and centrifuged at 1,500 x g for 10 minutes at 4˚C to obtain serum. Serum was collected for assay of cytokines and antibody titers, and to obtain cells for *in vitro* experimentation. Serum samples were stored at -80°C until assayed.

Bronchoalveolar lavage (BAL)

Shortly following euthanasia, the chest cavity was opened and the left lung clamped for later removal for use in histology. Mice were then tracheostomized and cannulated, and BALs were performed on the right lung to obtain lavage fluid containing lung cells for differential cell counts. BALs were performed by introducing HBSS (26 mL/kg body weight) into the right lung via a 1cc syringe, then withdrawing fluid through the cannula a total of four times in order to collect lung cells.

Cytospins

BAL samples were centrifuged at 500 x g for 10 minutes at 4˚C. The cell pellet was resuspended in 0.5 mL HBSS, and cell counts performed. Volumes were adjusted and added for cytospin to allow for 20,000 cells per slide. The cytospin apparatus was run at 100 x g for 5 minutes, then slides were removed and allowed to dry. Once dry, slides were stained with H&E stain so that differential cell counts could be performed.

Differential cell counts

Differential cell counts were performed on stained cytospin slides from BAL samples to provide data on the cellular profile of the lung from which the sample was obtained. Three hundred cells per slide were identified and counted. Common cell types included monocytes, macrophages, neutrophils, eosinophils, lymphocytes, and epithelial cells. Elevations in certain types of cells (particularly eosinophils, neutrophils and lymphocytes) denoted inflammation. Statistical differences in group differential cell counts were determined with GraphPad Prism version 6.

Cytokine analysis

Cytokine analysis was performed on serum samples with custom multiplex mouse cytokine ELISA kit, plate reader, and software from Meso Scale Discovery. ELISA was performed as instructed by the kit. Statistical differences in group cytokine expressions were determined with GraphPad Prism version 6.

Histology

Following BAL collection from the right lung, the left lung was unclamped, inflated with 10% formalin, and stored in 10% formalin for 24-72 hours. Following fixation, the left lung was sectioned into thirds and placed in 70% ethanol for at least 24 hours. The left lung was then embedded in paraffin, cut into 5μm sections, mounted onto slides, and stained with Hematoxylin and Eosin (H&E).

Results and Discussion

The key important finding from this study was the confirmation of a suitable animal model in which to test the GMCSF-OVA 323-339 fusion protein. Although pulmonary inflammation was not as prominent in this model as in others that use whole OVA for sensitization and/or challenge, inflammation was primarily eosinophilic, measurable, consistent, and significant ($p<0.05$, see figure 3.4). While this model cannot be classified as a true model of asthma due to the lack of airway response and the low-grade inflammation, likely a limitation of utilizing the 17-amino acid OVA peptide for both sensitization and challenge, it is a model of allergen-induced airway inflammation and is appropriate for the determination of GMCSF-OVA 323-339 anti-inflammatory capabilities.

As discussed previously with regard to the positive and negative aspects of the various animal models of asthma, it might be argued that the use of a murine model of asthma could have limited potential for real-world significance. While it is true that many asthma research related findings in mouse models have not translated into human clinical trials, all research must begin with a lower-order model as a basis, and due to the fact that this study is proof-of-concept in nature, the mouse is a suitable vehicle for experimentation. In addition, the use of a mouse model has distinct advantages including ease of manipulation, availability of transgenics for immunomodulation, as well as the multitude of commercially available murine-specific reagents necessary for experimentation. For this study, particularly due to the availability of OVA 323-339-reactive transgenics, the mouse is an acceptable vehicle in which to test the GMCSF-OVA 323-339 fusion protein.

The model development process presented difficulties due to the fact that various protocols attempted with OVA 323-339 for sensitization and challenge resulted in little or no inflammation. Protocol attempts included 1) chronic vs. acute models, 2) the use of a C57BL/6 strain vs. a BALB/c strain, 3) alum as an adjuvant vs. IFA, 4) pharyngeal aspiration vs. subcutaneous vs. intraperitoneal sensitization routes, 5) aerosol vs. pharyngeal aspiration challenge routes, as well as 6) varying OVA 323-339 doses and numbers of sensitizations and challenges (table 3.1). Early protocols attempts showed inflammation, but whole OVA had been used instead of OVA 323-339 for challenge (table 3.1, figure 3.2). Other model variations showed inflammation, but the inflammation was neutrophilic rather than eosinophilic in nature, and therefore not suitable as a model of typical allergic inflammation. Others yet resulted in no detectable inflammation whatsoever. The reasons underlying the difficulties encountered during model development may be explained by several factors.

One factor that may have limited the development of a robust inflammatory response may have been the use of the OVA peptide for sensitization and challenge. Due to the fact the antigenic portion of the fusion protein was comprised of OVA 323-339, it was decided that a model using OVA 323-339 for sensitization and challenge would be a best system in which to test the fusion proteins. The ability of the 17-amino acid peptide to elicit a robust response, however, might have contributed to the limited inflammatory responses observed in some of the model protocols. It is possible that the OVA peptide alone is not antigenically potent enough to induce an eosinophilic response equal to that seen with whole OVA in the literature.

Another factor that may have affected model development was the use of chronic aerosol exposure to recapitulate a more natural induction of pulmonary inflammation to parallel exposure routes in asthmatics. It is possible that a lack of inflammatory response in the attempted chronic aerosol models was due to underexposure, leading to a subpar inflammatory response, or overexposure, leading to a tolerance that has been observed in mouse asthma models due to the delivery of higher concentration of antigen or repeated exposures. Since it was not possible to determine the actual concentration of the aerosol in the exposure chamber or the ability of the nebulizer to evenly distribute the aerosol, aerosol delivery may have been a contributing factor. In addition, the animal restraints may have stressed the animals during the 30-minute exposures, which may have altered the immune response to antigen. Due to difficulties experienced with the chronic models, an acute model was chosen.

Another issue that potentially affected model development was related to pharyngeal aspiration, as variability in antigen distribution can be introduced due to animal body position during dosing or due to smaller aspiration volumes, leading to variability in results or small localized areas of inflammation rather than evenly-distributed general inflammation. Mice are anesthetized with isoflurane prior to pharyngeal aspiration, but must be dosed quickly due to the short-acting nature of the anesthetic. If mice are not completely vertical during dosing, the solution, when inhaled, may not be evenly distributed in both lungs. If too small a volume is used for aspiration, as may have been the case in earlier attempts based on other models, the distribution of solution containing antigen in the lungs may be uneven. Pharyngeal aspiration was preferred to inhalation as the delivery method due to the issues encountered with

aerosol exposures in the chronic models, though higher volumes (at least 60 µL) were chosen to allow for appropriate distribution of antigen and thus a more uniform response, thus aiding in the detection of inflammation in histological sections and differential cell counts.

An additional factor that influenced the development of inflammation was mouse strain. Specifically, the development of eosinophilic rather than neutrophilic inflammation would have been affected by the strain of mouse used for a particular protocol. While C57BL/6 mice have been used successfully in pulmonary inflammation models in the literature, these mice tend to have a T_H1-skewed immune response and therefore would not be as likely to develop the T_H2-skewed inflammation sought after for this model. Data illustrating differences between BALB/c and C57BL/6 mouse pulmonary resistance, inflammation, and cytokine measurements are shown in figures 3.1, 3.2, and 3.3, respectively. In figure 3.1, pulmonary resistance measured via FlexiVent showed no real differences between the two strains as neither achieved reliable increases in sensitivity to Methacholine challenge due to OVA 323-339 sensitization and challenge. This may have been due in part to difficulties measuring resistance in mice caused by lesser airway smooth muscle and resulting bronchoconstriction, or due in part to the use of the OVA peptide instead of whole OVA, or perhaps a combination of these factors. In figure 3.2, a clear increase in inflammation in H&E-stained lung sections can be seen for OVA 323-339 sensitized and challenged BALB/c mice versus C57BL/6 mice. As BALB/c mice have been shown to have a greater propensity for developing inflammation in asthma models, this increase in inflammation was not unexpected. In figure 3.3, IL-13 and IL-5 were measured for each

strain. IL-4 was assayed as well, though amounts present were unmeasurable (data not shown). While IL-13 measurements did not show differences between PBS- and OVA 323-339-treated mice, IL-5 measurements showed consistent increases in OVA 323-339-treated mice for both strains. Due to the increased inflammation present in H&E sections along with the differential cell count eosinophilia for BALB/c mice however, it was determined that BALB/c mice would be the preferred strain to use in the model.

Based on various protocols attempted for the generation of a pulmonary allergic model, it was concluded that for sensitization and challenge with the OVA peptide, higher levels of inflammation could be achieved in an acute model with BALB/c mice given subcutaneous injections of IFA and peptide for sensitizations, and at given least 60 µL of peptide (to allow for better dispersion in the lungs) via pharyngeal aspiration for challenges. The design of the animal model (table 3.2) that generated sufficient inflammation for study (denoted by eosinophil and neutrophil differential cell counts from bronchoalveolar lavage fluid, figure 3.4) was in BALB/c mice, and began with OVA 323-339 in IFA sensitizations on days 0, 7, and 14 via subcutaneous injection followed by challenge with a 1% OVA 323-339 solution on days 21, 22, and 23 via pharyngeal aspiration, then harvest on day 26. Unlike for BALB/c mice, differential cell count data for C57BL/6 mice did not show significant eosinophilic inflammation (data not shown).

Tables and Figures

Table 3.1. Attempted models of allergen-induced airway inflammation

Some of the protocols explored during model development are outlined. Various models attempted for induction of pulmonary inflammation included the use of different animal strains, sensitization routes, acute and chronic timelines, different adjuvants, as well as varying OVA 323-339 doses and numbers of sensitizations and challenges.

Attempted Protocols for Allergen-induced Airway Inflammation		
Chronic*	**vs**	**Acute***
Sensitization: Pharyngeal aspiration of 100 µg OVA 323-339 + 0.1 µg LPS/75 µL (days 0 and 6)		Sensitization: Intraperitoneal injection of 10 µg OVA 323-339 in 200 µL alum (days 0 and 12)
Challenge: Nose-only aerosol exposure with 2% OVA for 30 minutes (days 13-15, 41-43, and 69-71)		Challenge: Pharyngeal aspiration of 600 µg OVA 323-339 in 50 µL saline (day 19)
Harvest: One-third of mice on day 16, one-third of mice on day 44, and one-third of mice on day 72		Harvest: Day 21
IFA Adjuvant**	**vs**	**Alum Adjuvant***
Sensitization: Subcutaneous injection of 200 µg OVA 323-339 in 200 µL IFA (days 0, 7, and 14)		Sensitization: Intraperitoneal injection of 200 µg OVA 323-339 in 200 µL alum (days 0, 7, and 14)
Challenge: Pharyngeal aspiration of 1% OVA 323-339 in 60 µL saline (days 21-23)		Challenge: Pharyngeal aspiration of 1% OVA 323-339 in 60 µL saline (days 21-23)
Harvest: Day 26		Harvest: Day 26
Aerosol Challenge*	**vs**	**Pharyngeal Aspiration Challenge***
Sensitization: Intraperitoneal injection of 10 µg OVA 323-339 in 200 µL alum (days 0 and 12)		Sensitization: Subcutaneous injection of 200 µg OVA 323-339 in 200 µL IFA (days 0, 7, and 14)
Challenge: Nose-only aerosol exposure with 5% OVA for 30 minutes (days 18-23, 26-28, 33-35, 40-42, 47-49, and 54-56		Challenge: Pharyngeal aspiration of 1% OVA 323-339 in 60 µL saline (days 21-23)
Harvest: One-third of mice on day 29, one-third of mice on day 36, and one-third of mice on day 57		Harvest: Day 26

*BALB/c and C57BL/6 mice strains used
**BALB/c mice only

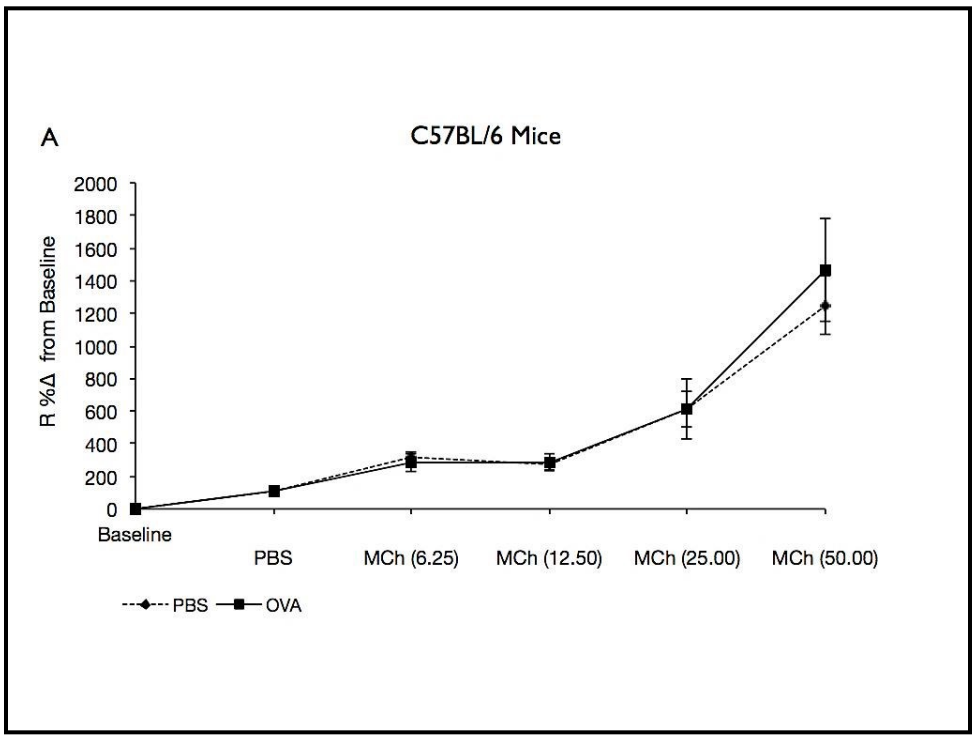
Table 3.2. Allergen-induced airway inflammation model timeline

In the animal model ultimately chosen for study, animals were sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection, then challenged with a 1% OVA 323-339 solution on days 21, 22, and 23 via pharyngeal aspiration. Animals were harvested on day 26.

	Protocol for Allergen-induced Airway Inflammation
Day 0	Sensitization #1
Day 7	Sensitization #2
Day 14	Sensitization #3
Day 21	Challenge #1
Day 22	Challenge #2
Day 23	Challenge #3
Day 26	Time-point

Figure 3.1. Comparison of Resistance (R) percent change from baseline in PBS- vs. OVA-challenged C57BL/6 and BALB/c mice

C57BL/6 (A) and BALB/c (B) mice (n = 4 per group, one experiment) were anesthetized with isoflurane and sensitized with either 75 µL phosphate buffered saline (PBS) or ovalbumin (OVA) + lipopolysaccharide (LPS) (100 µg OVA + 0.1 µg LPS/75 µL) via oropharyngeal aspiration on days 0 and 6. Starting on day 13 mice were be exposed to a nose-only aerosol of either PBS or 2% OVA (no LPS) for 30 minutes for 3 consecutive days. Three days of aerosol exposures were given at one-month intervals for three months. Three days after the final exposure, animals were subjected to increasing doses of methacholine (MCh) to determine changes in airway resistance (R) from baseline as measured via FlexiVent. Error bars represent the standard error of the mean for each group value.


A
C57BL/6 Mice
R %Δ from Baseline
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Baseline
PBS
MCh (6.25)
MCh (12.50)
MCh (25.00)
MCh (50.00)
PBS
OVA

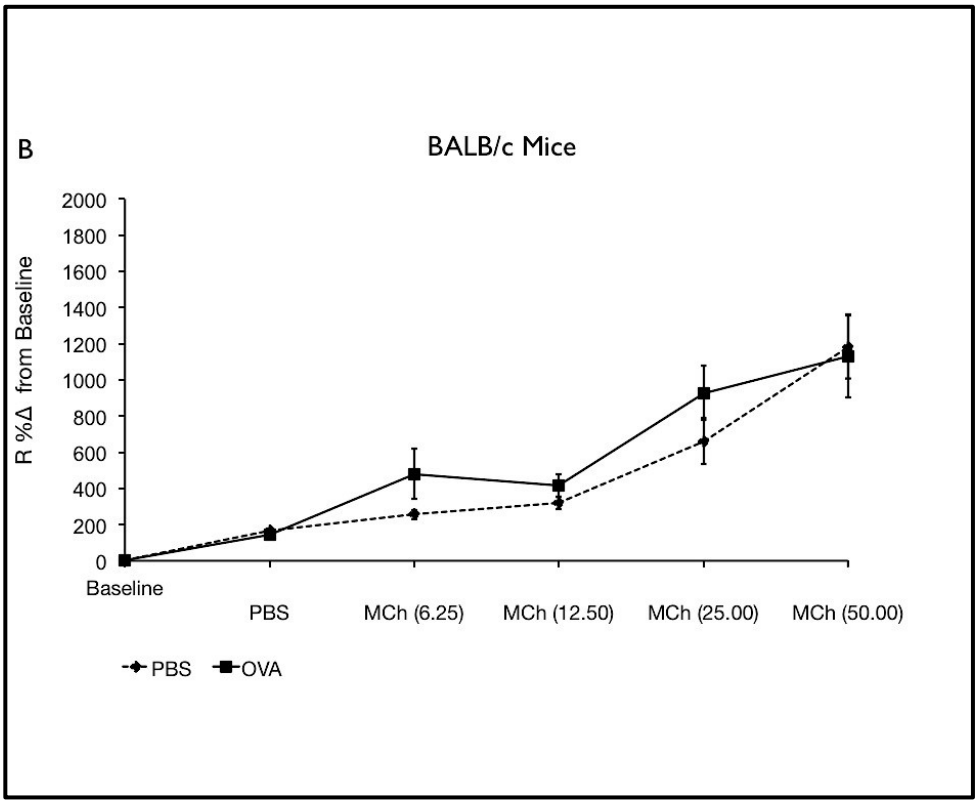
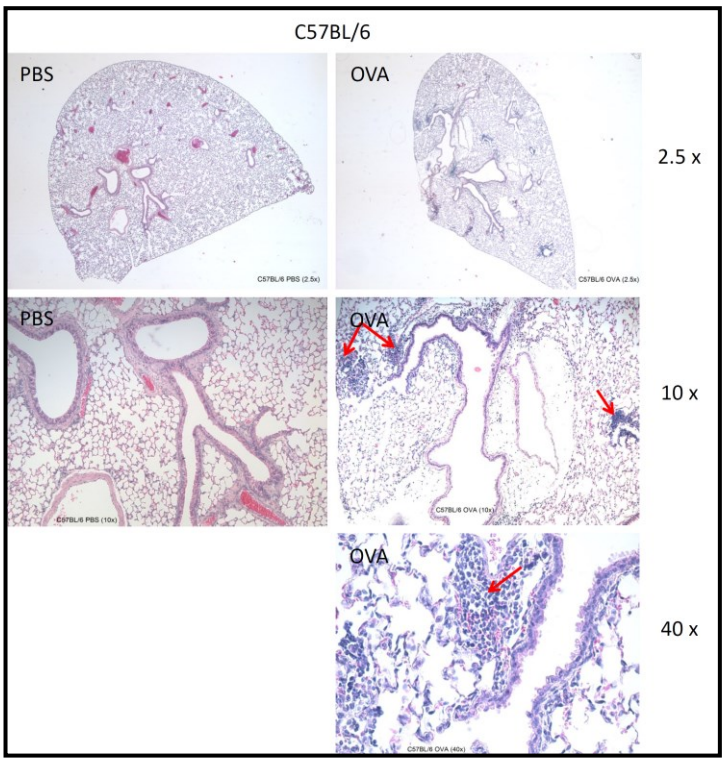

B
BALB/c Mice
R %Δ from Baseline
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Baseline
PBS
MCh (6.25)
MCh (12.50)
MCh (25.00)
MCh (50.00)
PBS
OVA

Figure 3.2. Comparison of cellular infiltration in lung tissue in PBS- vs. OVA-challenged C57BL/6 and BALB/c mice

C57BL/6 and BALB/c mice (n = 4 per group, one experiment) were anesthetized with isoflurane and sensitized with either 75 µL phosphate buffered saline (PBS) or ovalbumin (OVA) + lipopolysaccharide (LPS) (100 µg OVA + 0.1 µg LPS/75 µL) via oropharyngeal aspiration on days 0 and 6. Starting on day 13 mice were be exposed to a nose-only aerosol of either PBS or 2% OVA (no LPS) for 30 minutes for 3 consecutive days. Three days of aerosol exposures were given at one-month intervals for three months. H&E-stained lung sections are shown at 2.5x, 10x, and 40x for each animal, respectively. Red arrows denote areas of inflammatory cell infiltration.


C57BL/6
PBS
OVA
2.5 x
PBS
OVA
10 x
OVA
40 x

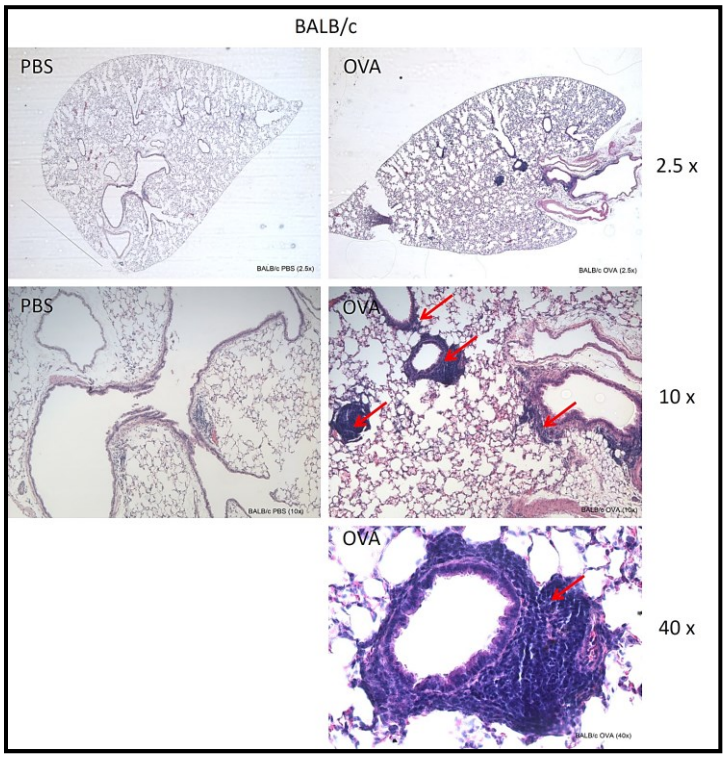

BALB/c
PBS
OVA
2.5 x
PBS
OVA
10 x
OVA
40 x

Figure 3.3. Comparison of serum IL-5 and IL-13 in PBS- vs. OVA-challenged C57BL/6 and BALB/c mice

Mice (n = 2-4 per group per timepoint, one experiment)[a] were sensitized on days 0 and 12 with OVA 323-339 and aluminum hydroxide. Six days after the second sensitization, mice underwent six consecutive days of aerosol challenge (5% whole OVA in PBS or PBS alone) on days 18-23 followed by two days of rest, then three added consecutive days (days 26-28) of aerosol challenge. One-third of the mice were sacrificed 24 hours after the day 28 aerosol challenge. Remaining mice were subjected to three consecutive days of aerosol challenge the following week on days 33-35, and another one-third of the mice were sacrificed 24 hours after the day 35 aerosol challenge. The last one-third of the mice were given three aerosols per week for three weeks (days 40-42, 47-49, and 54-56), then sacrificed 24 hours after the day 56 aerosol challenge. Statistics were run as an unpaired t-test between mice given PBS and those given OVA 323-339 at each timepoint. Error bars represent the standard error of the mean for each group value. (*) Denotes significant difference ($p<0.05$) versus the PBS group.

[a] *Some of the mice had died in the restraints during aerosol exposures, unintentionally reducing the group size (n) for some groups. "N" for BALB/c mice are as follows: day 29 PBS group = 2, day 29 OVA group = 2, day 36 PBS group = 4, day 36 OVA group = 3, day 57 PBS group = 3, day 57 OVA group = 4. "N" for C57BL/6 mice are as follows: day 29 PBS group = 4, day 29 OVA group = 4, day 36 PBS group = 3, day 36 OVA group = 4, day 57 PBS group = 3, day 57 OVA group = 3.*

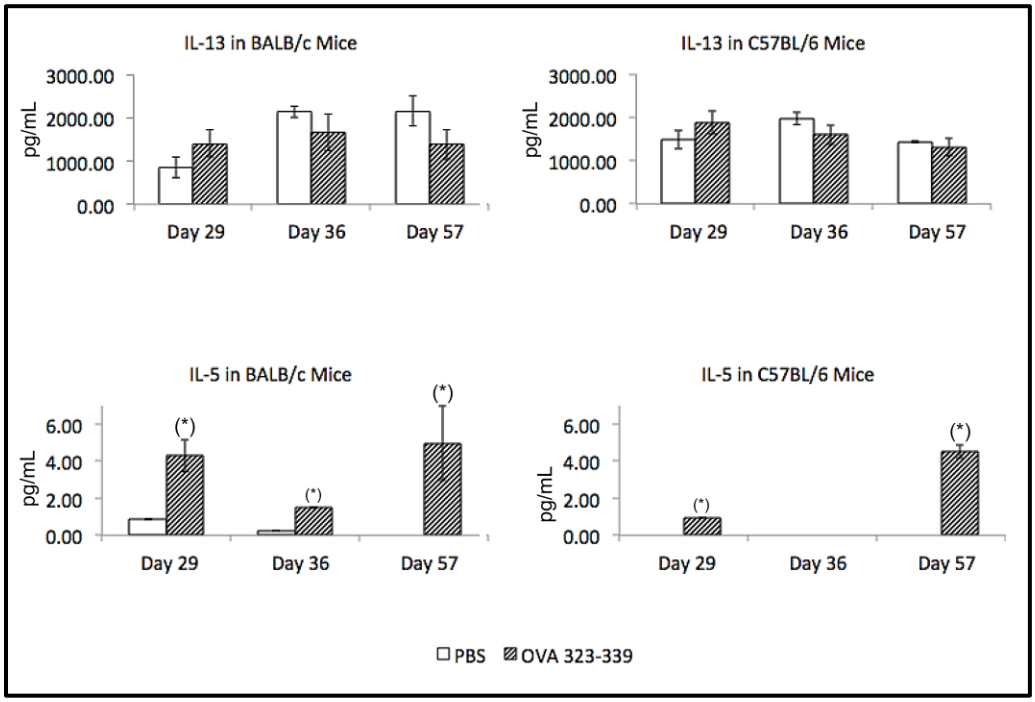
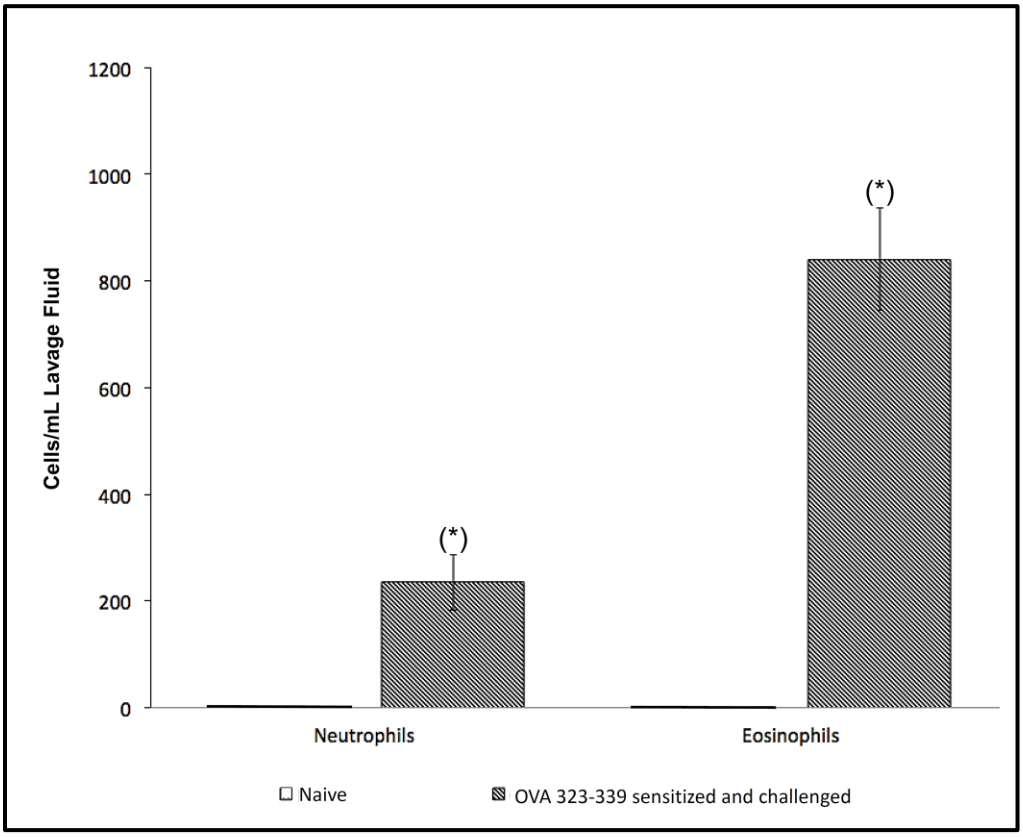

IL-13 in BALB/c Mice
3000.00
2000.00
1000.00
0.00
pg/mL
Day 29
Day 36
Day 57
IL-13 in C57BL/6 Mice
3000.00
2000.00
1000.00
0.00
pg/mL
Day 29
Day 36
Day 57
IL-5 in BALB/c Mice
6.00
4.00
2.00
0.00
pg/mL
(*)
(*)
(*)
Day 29
Day 36
Day 57
IL-5 in C57BL/6 Mice
6.00
4.00
2.00
0.00
pg/mL
(*)
(*)
Day 29
Day 36
Day 57
PBS
OVA 323-339

Figure 3.4. Neutrophil and eosinophil cell counts in PBS- vs. OVA-challenged BALB/c mice

Mice were sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection, then challenged with a 1% OVA 323-339 solution on days 21, 22, and 23 via pharyngeal aspiration (n = 8, one experiment). Naïve mice (n = 2, one experiment) did not receive any treatment or manipulation. Animals were harvested on day 26. Eosinophils and neutrophils were counted to determine the presence of inflammation in BALF. Statistics were run as an unpaired t-test. Error bars represent the standard error of the mean for each group value. (*) Denotes significant difference ($p<0.05$) versus the Naive group.


1200
1000
800
600
400
200
0
Cells/mL Lavage Fluid
(*)
(*)
Neutrophils
Eosinophils
Naive
OVA 323-339 sensitized and challenged

Conclusion

In the murine model of allergen-induced airway inflammation used for the study, eosinophilic inflammation was observed for mice sensitized and challenged with OVA 323-339, though eosinophilia was lower than expected compared to models that use whole OVA for sensitization and/or challenge. Because eosinophilic inflammation was measurable and consistent, however, this model should still provide insight into whether or not the GMCSF-OVA 323-339 fusion protein has potential as a therapeutic within the system.

CHAPTER 4: EFFICACY OF THE GMCSF-OVA 323-339 CYTOKINE FUSION PROTEIN *IN VIVO*

Introduction

Once the animal model of allergen-induced airway inflammation using OVA 323-339 for sensitization and challenge was established, the vehicle for testing the GMCSF-OVA 323-339 fusion protein treatment was available to put into motion. Simply testing the fusion protein *in vitro* would not yield insight into the full potential of the fusion protein; it is important to observe how a treatment performs in an intact system. To test the GMCSF-OVA 323-339 fusion protein within the animal model used for this study, animals were pre-treated with GMCSF-OVA 323-339, then subjected to a model of pulmonary inflammation described in Chapter 3. Differences in inflammation due to fusion-protein treatment or lack thereof could then be measured via differential cell counts from BALF, lung histology, anti-OVA IgG1, and cytokine profiles for the different treatment groups and controls.

For Specific Aim 3 of this study, we hypothesized that the GMCSF-OVA 323-339 fusion protein would target the OVA peptide antigenic epitope to dendritic cell APCs, overloading the APCs with antigen and providing GM-CSF to encourage the development of a tolerogenic state. A tolerogenic state induced by cytokine antigen fusion protein pre-treatment could arise due to the deletion or anergization of reactive T cells, thereby decreasing the likelihood of T cell activation and instead, promoting tolerance during APC-T cell interaction. Such a state could also be induced through an upregulation in T_{Regs}, leading to the suppression of reactive T cells. Tolerance to antigen due to GMCSF-OVA 323-339 pre-treatment would lead to a decrease in inflammatory

cells in differential cell counts, reduced pathology in Hematoxylin and Eosin stained lung sections, decreased mucus production in Alcian Blue Periodic Acid-Schiff stained lung sections, and decreased IgG1 and inflammatory cytokines.

Methods

Animals

BALB/c mice (Jackson Labs, Bar Harbor, ME, USA), were housed at Burleson Research Technologies, Inc. (Morrisville, NC, USA). Animal care and use were performed in accordance with animal use protocols and institutional guidelines approved by the Burleson Research Technologies, Inc. Institutional Animal Care and Use Committee.

GMCSF-OVA 323-339 (or saline) pre-treatment

GMCSF-OVA 323-339 fusion protein was solubilized in saline and injected subcutaneously into two injection sites in the hind flank (0.1 mL per injection site), for administration of 2 nM in 0.2 mL per mouse. Prior to injections, mice were anesthetized using isoflurane in a vented anesthetic chamber, and injection sites were cleaned with povidone-iodine followed by an alcohol rinse. Pre-treatment injections were administered on days -21, -14, and -7 and were monitored for signs of inflammation, though inflammation was unexpected. Control mice were given saline instead of GMCSF-OVA 323-339.

Sensitization

Active sensitization of mice with OVA 323-339 in IFA was performed by subcutaneous injection of 200 µg OVA 323-339 in 200 µL IFA. As with fusion protein

pre-treatment injections, prior to sensitization injections, mice were anesthetized using isoflurane in a vented anesthetic chamber and injection sites were cleaned with povidone-iodine followed by an alcohol rinse. Sensitizations were performed on days 0, 7, and 14, and injection sites were monitored for signs of inflammation.

Challenge

Mice were challenged via pharyngeal aspiration (p.a.) challenge on days 21, 22 and 23. The p.a. solution was 60 µL of 1% OVA-323-339 in sterile saline, and was administered while mice were anesthetized with isoflurane in a vented anesthetic chamber. Control mice did not receive 1% OVA 323-339 solution and instead were given 60 µL saline.

Serum collection

On day 26, mice were asphyxiated with $C0_2$ and terminal bleeds were performed via intracardiac puncture. Whole blood collected from terminal bleeds was placed in serum separator tubes and centrifuged at 1,500 x g for 10 minutes at 4˚C to obtain serum. Serum was collected for assay of antibody titers, and to obtain cells for *in vitro* experimentation. Serum samples were stored at -80°C until assayed.

Bronchoalveolar lavage (BAL)

Shortly following euthanasia, the chest cavity was opened and the left lung was clamped for later removal for use in histology. Mice were then tracheostomized and cannulated, and BALs were performed on the right lung to obtain lavage fluid containing localized lung cytokines for cytokine analysis and lung cells for differential cell counts. BALs were performed by introducing HBSS (26 mL/kg body weight) into the right lung

via a 1cc syringe, then withdrawing fluid through the cannula a total of four times in order to collect lung cells. The first fluid withdrawal was collected separately for cytokine analysis, and the remaining three withdrawals were pooled for cytospins and differential cell counting. Primary lavage samples were stored at -80°C until assayed.

Cytospins

BAL samples were centrifuged at 500 x g for 10 minutes at 4˚C. The cell pellet was resuspended in 0.5 mL HBSS, and cell counts performed. Volumes were adjusted and added for cytospin to allow for 20,000 cells per slide. The cytospin apparatus was run at 100 x g for 5 minutes, then slides were removed and allowed to dry. Once dry, slides were stained with H&E stain so that differential cell counts could be performed.

Differential cell counts

Differential cell counts were performed on stained cytospin slides from BAL samples to provide data on the cellular profile of the lung from which the sample was obtained. Three hundred cells per slide were identified and counted. Common cell types observed and counted for each sample included monocytes, macrophages, neutrophils, eosinophils, lymphocytes, and epithelial cells. Statistical differences in group differential cell counts were determined with GraphPad Prism version 6.

Anti-OVA IgG_1 ELISA

An anti-OVA IgG1 ELISA was performed using the Anti-Ovalbumin IgG_1 (mouse) EIA Kit from Cayman Chemical Company (cat# 500830). The anti-OVA IgG1 assay was performed on mouse serum samples according to instructions provided with kit, and the assay plate was read with Softmax Pro software. Statistical differences in group IgG1

levels were determined with GraphPad Prism version 6.

Cytokine analysis

Cytokine analysis was performed on bronchoalveolar lavage samples with custom multiplex mouse cytokine assay kit, plate reader, and software from Meso Scale Discovery. Assay was performed as instructed by the kit. Lavage fluid was tested for IL-4, IL-5, IL-10, and IFN-γ. Statistical differences in group cytokine levels were determined with GraphPad Prism version 6.

Histology

Following BAL collection, the left lung was unclamped, inflated with 10% formalin, and stored in 10% formalin for 24-72 hours. Following fixation, the left lung was sectioned into thirds and placed in 70% ethanol for at least 24 hours. The left lung was then embedded in paraffin, cut into 5μm sections, mounted onto slides, and stained with Hematoxylin and Eosin (H&E) and Alcian Blue Periodic Acid-Schiff (AB-PAS) stains. H&E- and AB-PAS-stained lung sections were evaluated by Dr. Rodney Miller from EPL in Durham, NC (see full report and raw data in Appendix C). Sections were graded based on the degree of perivascular infiltration, peribronchial and peribronchiolar infiltration, epithelial damage, and parenchymal infiltration, and were assigned scores of "-", denoting no effect, "X", denoting no remarkable effect, "+1", denoting minimal effect, "+2", denoting slight effect, or "+3", denoting moderate effect. No sections received grades higher than +3.

Results and Discussion

The key finding from this study was that GMCSF-OVA 323-339 pre-treatment in the pulmonary inflammatory model (table 4.1) attenuated the development of inflammation. Mice given saline prior to sensitization and challenge with OVA 323-339 showed consistent, measurable, significant increases in pulmonary inflammation compared to mice that received GMCSF-OVA 323-339 pre-treatment prior to sensitization and challenge with the OVA peptide and controls that did not undergo challenge with the OVA peptide. Mice given saline prior to sensitization and challenge showed eosinophilia in cytospin slides for differential cell counts (figure 4.1), cellular infiltration and cuffing of the airways in H&E-stained lung sections (figure 4.2), and a light mucus response in the AB-PAS-stained lung sections (figure 4.3), while mice given GMCSF-OVA 323-339 pre-treatment showed a marked reduction in eosinophils, absent or only slight cellular infiltration in lung sections, and no mucus secretion. To validate the use of histological sections in the evaluation of the GMCSF-OVA 323-339 pre-treatment, histological sections were sent for scoring by an outside party. The findings of Dr. Rodney Miller from EPL, Inc. in Durham, NC (figure 4.4; see full report and raw data in Appendix C) support a protective effect due to GMCSF-OVA 323-339 pre-treatment in the model. In agreement with differential cell count data and histological findings, serum analysis indicated a significant elevation in IgG_1, an indicator of T_H2 activity in murine models, for mice given saline prior to sensitization and challenge compared to those pre-treated with GMCSF-OVA 323-339 (figure 4.6). In addition, cytokine analysis of bronchoalveolar lavage fluid showed a significant increase in IFN-γ for mice given saline prior to sensitization and challenge compared to those pre-treated

with GMCSF-OVA 323-339 (figure 4.5). Studies have shown that IFN-γ localized in the lung tissue can result in increases in pro-inflammatory IL-5 and IL-13, as well as increased numbers of eosinophils in lavage fluid (Koch et al., 2006). While no increase in IL-5 was detected in the cytokine analysis, this was likely due to the timing of sample collection at three days post-challenge. Other studies have indicated post-challenge peak IL-5 concentrations at 12 hours in serum and 24 hours in bronchoalveolar lavage fluid, explaining the lack of IL-5 seen in this set of experiments. IL-4 was likely missed due to the timing of sample collection as well, with post-challenge peak concentrations reported at 3 hours in the serum and 24 hours in lavage fluid (Ohkawara et al., 1997).

Due to the successes of cytokine-antigen fusion proteins in other experimental systems such as Experimental Autoimmune Encephalomyelitis (EAE) with Mannie et al., it was thought that there would be potential for similar treatments within an animal model of pulmonary inflammation. While the pathogenesis of each disorder is quite different, each begins with the immune system engaging in an inappropriate response to typically innocuous antigen. Therefore, the underlying idea of targeting antigen to specific antigen presenting cell populations in an effort to tolerize the immune system in each disorder is quite similar. It was unsure, however, how GM-CSF would perform as the cytokine chosen for linkage to antigen within a model of pulmonary inflammation. While GMCSF linked to antigen, for example, has been used in EAE experiments by Mannie et al., EAE is rooted in T_H1-dominated responses while asthma is typically T_H2 in nature, and it was uncertain whether or not the same cytokine domain would behave similarly in oppositely polarized systems. This data suggests that despite the differences in T_H polarity related to immunopathology for murine EAE and pulmonary inflammation

models, GM-CSF performs well as the cytokine domain portion of therapeutic fusion proteins.

Tables and Figures

Table 4.1. Allergen-induced airway Inflammation model with GMCSF-OVA 323-339 pre-treatment

Animals were pre-treated with either GMCSF-OVA 323-339 or saline on days -21, -14, and -7 via subcutaneous injection. Animals were then sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection, and challenged with either a 1% OVA 323-339 solution or saline on days 21, 22, and 23 via pharyngeal aspiration. Animals were euthanized on day 26.

	Pre-Treatment Protocol			
	Pre-treatment with GMCSF-OVA 323-339		Pre-treatment with saline	
	Saline Group*	OVA323-339 Group*	Saline Group*	OVA 323-339 Group*
Day -21	Pre-treatment #1		Pre-treatment #1	
Day -14	Pre-treatment #2		Pre-treatment #2	
Day -7	Pre-treatment #3		Pre-treatment #3	
Day 0	Sensitization #1		Sensitization #1	
Day 7	Sensitization #2		Sensitization #2	
Day 14	Sensitization #3		Sensitization #3	
Day 21	Challenge #1		Challenge #1	
Day 22	Challenge #2		Challenge #2	
Day 23	Challenge #3		Challenge #3	
Day 26	Time-point		Time-point	

*n = 10 per group

Figure 4.1. Differential cell counts in saline- vs. GMCSF-OVA 323-339- pretreated BALB/c mice

Mice were pre-treated with either saline or GMCSF-OVA 323-339 on days -21, -14, and -7; sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection; and challenged with either a 1% OVA 323-339 solution or saline on days 21, 22, and 23 via pharyngeal aspiration (n=10 per group for each of four groups, one experiment). Animals were harvested on day 26. Eosinophils and neutrophils were counted along with macrophages, monocytes, lymphocytes, and epithelial cells to determine the presence of inflammation in BALF. Statistics were run as a one-way ANOVA with Dunnett's multiple comparisons test and a single pooled variance. (*) Denotes significant difference ($p<0.05$) versus the GMCSF-OVA pre-treated, sensitized and challenged group. Error bars represent the standard error of the mean for each group value.

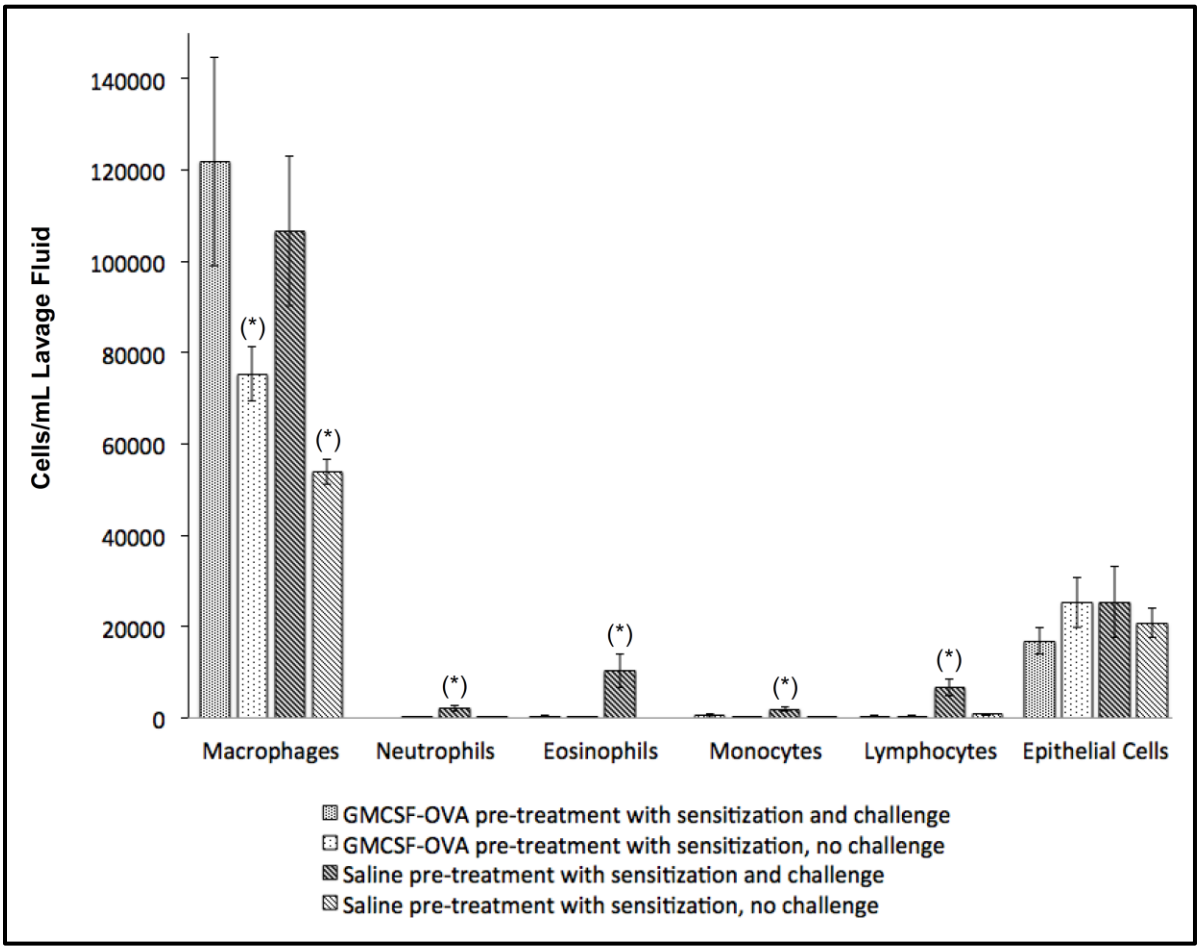

Cells/mL Lavage Fluid
140000
120000
100000
80000
60000
40000
20000
0
(*)
(*)
(*)
(*)
(*)
Macrophages
Neutrophils
Eosinophils
Monocytes
Lymphocytes
Epithelial Cells
GMCSF-OVA pre-treatment with sensitization and challenge
GMCSF-OVA pre-treatment with sensitization, no challenge
Saline pre-treatment with sensitization and challenge
Saline pre-treatment with sensitization, no challenge

Figure 4.2. H&E-stained lung sections for saline- vs. GMCSF-OVA 323-339-pretreated BALB/c mice

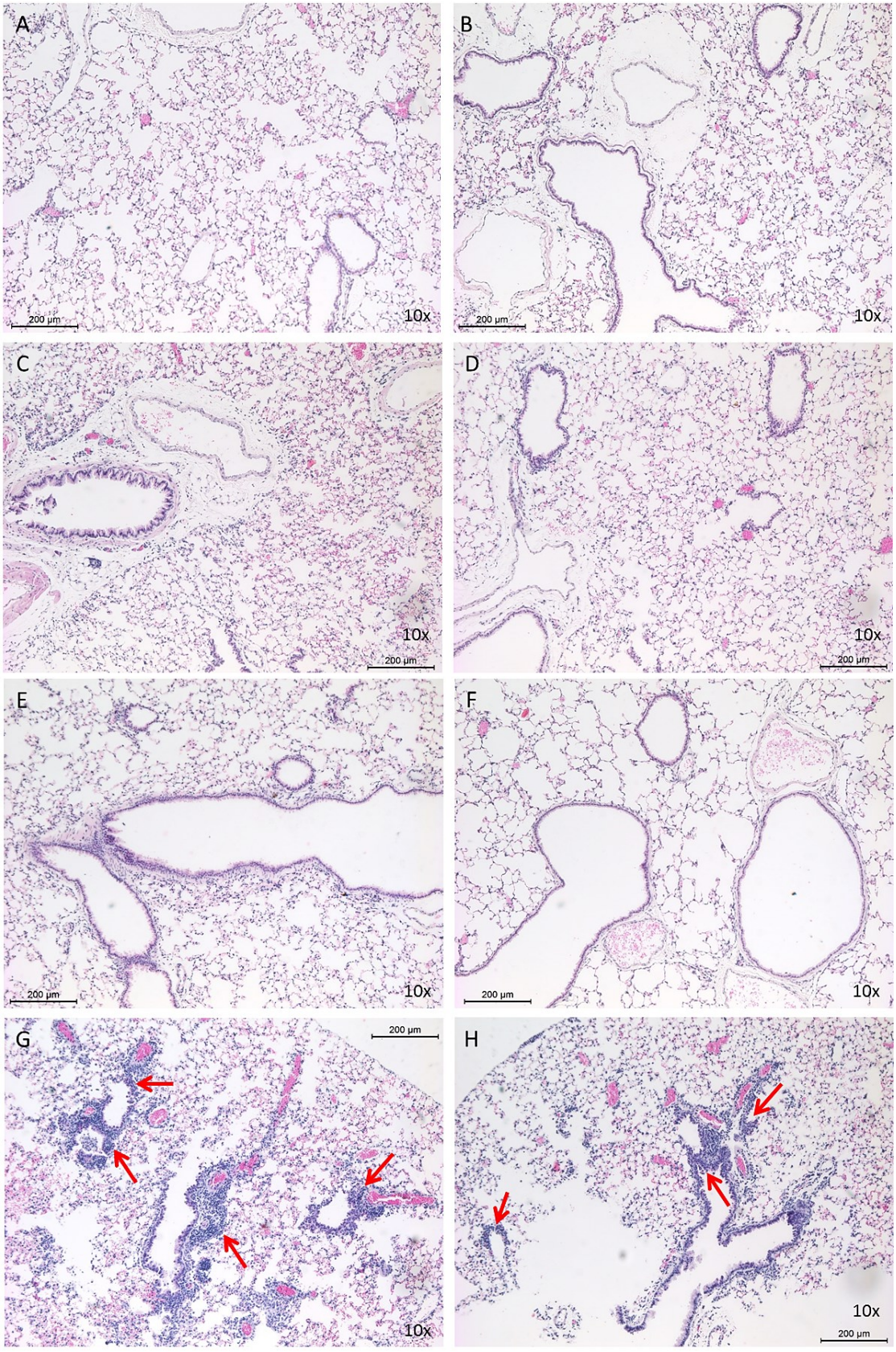
Mice were pre-treated with either saline or GMCSF-OVA 323-339 on days -21, -14, and -7; sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection; and challenged with either a 1% OVA 323-339 solution or saline on days 21, 22, and 23 via pharyngeal aspiration (n=10 per group for each of four groups, one experiment). Animals were harvested on day 26. Lung sections from the left lung of each mouse were stained with H&E to visualize perivascular infiltration, peribronchial and peribronchiolar infiltration, epithelial damage, and parenchymal infiltration (or lack thereof). Red arrows in G-H denote areas of inflammatory cell infiltration.

A-B: Negative control mice given GMCSF-OVA 323-339 pre-treatment followed by sensitization only.

C-D: Mice given GMCSF-OVA 323-339 pretreatment followed by sensitization and challenge.

E-F: Negative control mice given saline pre-treatment followed by sensitization only.

G-H: Positive control mice given saline pretreatment followed by sensitization and challenge.


A
200 µm
10x
B
200 µm
10x
C
10x
200 µm
D
10x
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E
200 µm
10x
F
200 µm
10x
G
200 µm
10x
H
10x
200 µm

Figure 4.3. Alcian Blue Periodic Acid-Schiff-stained lung sections for saline- vs. GMCSF-OVA 323-339- pretreated BALB/c mice

Mice were pre-treated with either saline or GMCSF-OVA 323-339 on days -21, -14, and -7; sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection; and challenged with either a 1% OVA 323-339 solution or saline on days 21, 22, and 23 via pharyngeal aspiration (n=10 per group for each of four groups, one experiment). Animals were harvested on day 26. Lung sections from the left lung of each mouse were stained with Alcian Blue Periodic Acid-Schiff to visualize mucus production (or lack thereof). Mucus present in the sections stained deep purple and blue hues. Red arrows in G-H denote areas of mucus staining.

A-B: Negative control mice given GMCSF-OVA 323-339 pre-treatment followed by sensitization only.

C-D: Mice given GMCSF-OVA 323-339 pretreatment followed by sensitization and challenge.

E-F: Negative control mice given saline pre-treatment followed by sensitization only.

G-H: Positive control mice given saline pretreatment followed by sensitization and challenge.

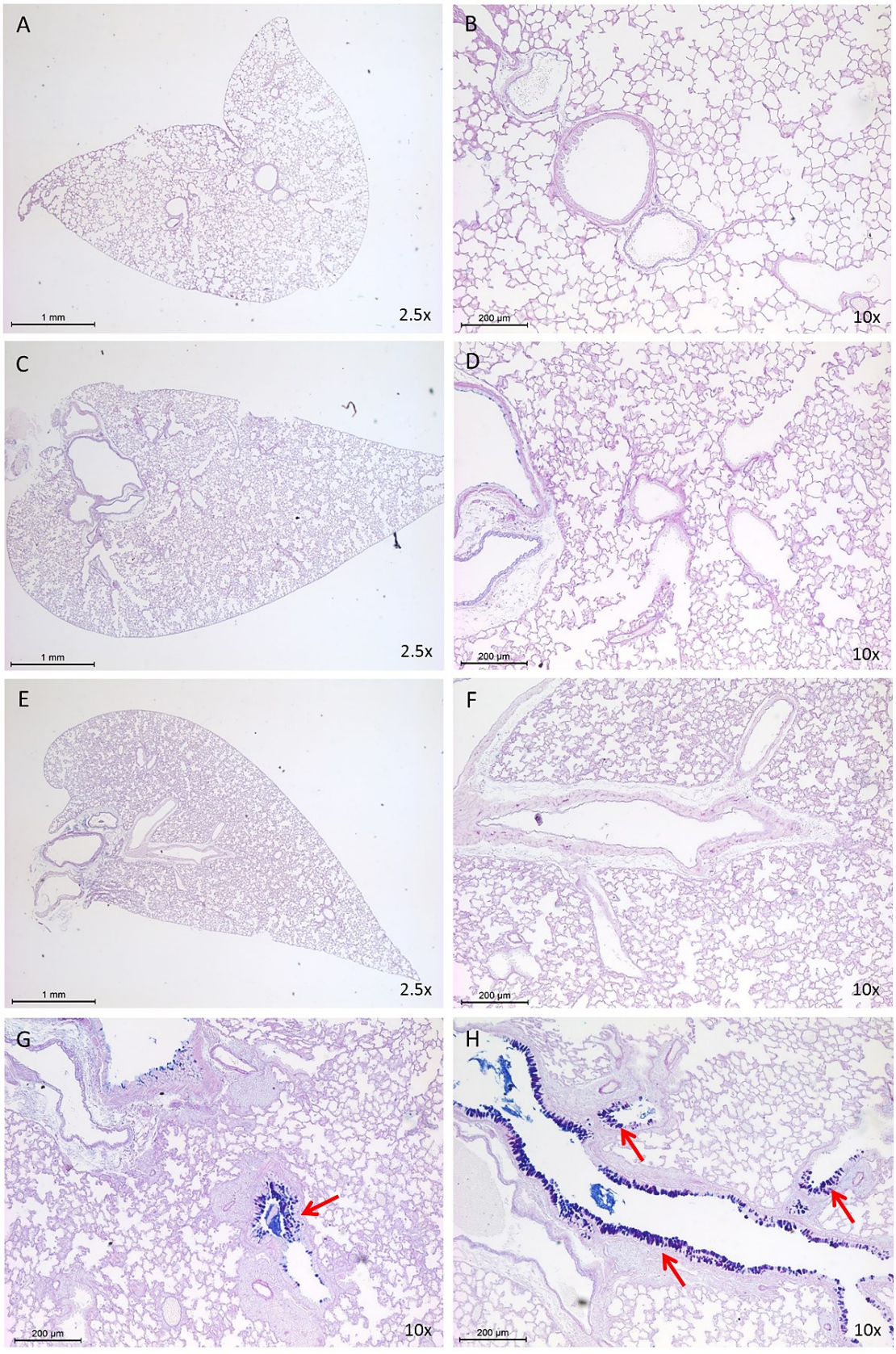

A
1 mm
2.5x
B
200 µm
10x
C
1 mm
2.5x
D
200 µm
10x
E
1 mm
2.5x
F
200 µm
10x
G
200 µm
10x
H
200 µm
10x

Figure 4.4. Scoring of histological sections

H&E- and Alcian Blue Periodic Acid-Schiff-stained lung sections were evaluated by Dr. Rodney Miller from EPL, Inc. in Durham, NC (see full report and raw data in Appendix C). Sections were graded based on the degree of perivascular infiltration, peribronchial and peribronchiolar infiltration, epithelial damage, parenchymal infiltration, and large and small airway mucus deposition. For each characteristic, sections were assigned scores of “-”, denoting no effect, “X”, denoting no remarkable effect, “+1”, denoting minimal effect, “+2”, denoting slight effect, or “+3”, denoting moderate effect. No sections received grades higher than +3.

Group	Animal ID	Perivascular Infiltration	Peribronchiolar infiltration	Epithelial Damage	Parenchymal Infiltration	Large Airway Mucus	Small airway Mucus	Combined Score	Group Average
GMCSF-OVA 323-339 pre-treatment followed by sensitization, no challenge	901	X	X	X	X	+1	-	1	**0.5**
	902	X	X	X	X	-	-	0	
	903	X	X	X	X	+1	-	1	
	904	X	X	X	X	-	-	0	
	905	X	X	X	X	+1	-	1	
	906	X	X	X	X	-	-	0	
	907	X	X	X	X	-	-	0	
	908	X	X	X	X	+1	-	1	
	909	X	X	X	X	-	-	0	
	910	X	X	X	X	+1	-	1	
GMCSF-OVA 323-339 pre-treatment followed by sensitization and challenge	911	X	X	X	X	+1	-	1	**1.2**
	912	X	X	X	X	+1	-	1	
	913	X	X	X	X	+1	-	1	
	914	X	X	X	+1	+1	-	2	
	915	X	X	X	X	+1	-	1	
	916	X	X	X	X	+1	-	1	
	917	X	X	X	X	+1	-	1	
	918	X	X	X	X	+2	-	2	
	919	X	X	X	X	+1	-	1	
	920	X	X	X	X	+1	-	1	
Saline pre-treatment followed by sensitization, no challenge	921	X	X	X	X	+1	-	1	**1.5**
	922	X	X	X	X	+1	-	1	
	923	X	X	X	X	+1	-	1	
	924	X	X	X	X	+1	-	1	
	925	+2	+2	X	+1	+1	-	6	
	926	X	X	X	X	+1	-	1	
	927	X	X	X	X	-	-	0	
	928	X	+1	X	+1	-	-	2	
	929	X	X	X	X	+1	-	1	
	930	X	X	X	X	+1	-	1	
Saline pre-treatment followed by sensitization and challenge	931	X	+1	X	X	+1	-	2	**7.2**
	932	+2	+2	X	X	+2	-	6	
	933	+3	+3	X	+2	+3	+1	12	
	934	+2	+2	X	+1	+2	+1	8	
	935	+1	+1	X	X	+1	-	3	
	936	+3	+3	X	+1	+2	-	9	
	937	+3	+3	X	+1	+2	-	9	
	938	+2	+2	X	+1	+1	-	6	
	939	+3	+3	X	+2	+3	+1	12	
	940	+2	+2	X	X	+1	-	5	

Figure 4.5. IL-4, IL-5, IL-10, and IFN-γ comparison for saline- vs. GMCSF-OVA 323-339- pretreated BALB/c mice

Mice were pre-treated with either saline or GMCSF-OVA 323-339 on days -21, -14, and -7; sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection; and challenged with either a 1% OVA 323-339 solution or saline on days 21, 22, and 23 via pharyngeal aspiration (n=10 per group for each of four groups, one experiment). Animals were harvested on day 26, at which time, bronchoalveolar lavage samples were obtained. Lavage fluid was then tested for IL-4, IL-5, IL-10, and IFN-γ. Statistics were run as a one-way ANOVA with Dunnett's multiple comparisons test and a single pooled variance. (*) Denotes significant difference ($p<0.05$) versus the GMCSF-OVA 323-339 pre-treated, sensitized and challenged group. Error bars represent the standard error of the mean for each group value.

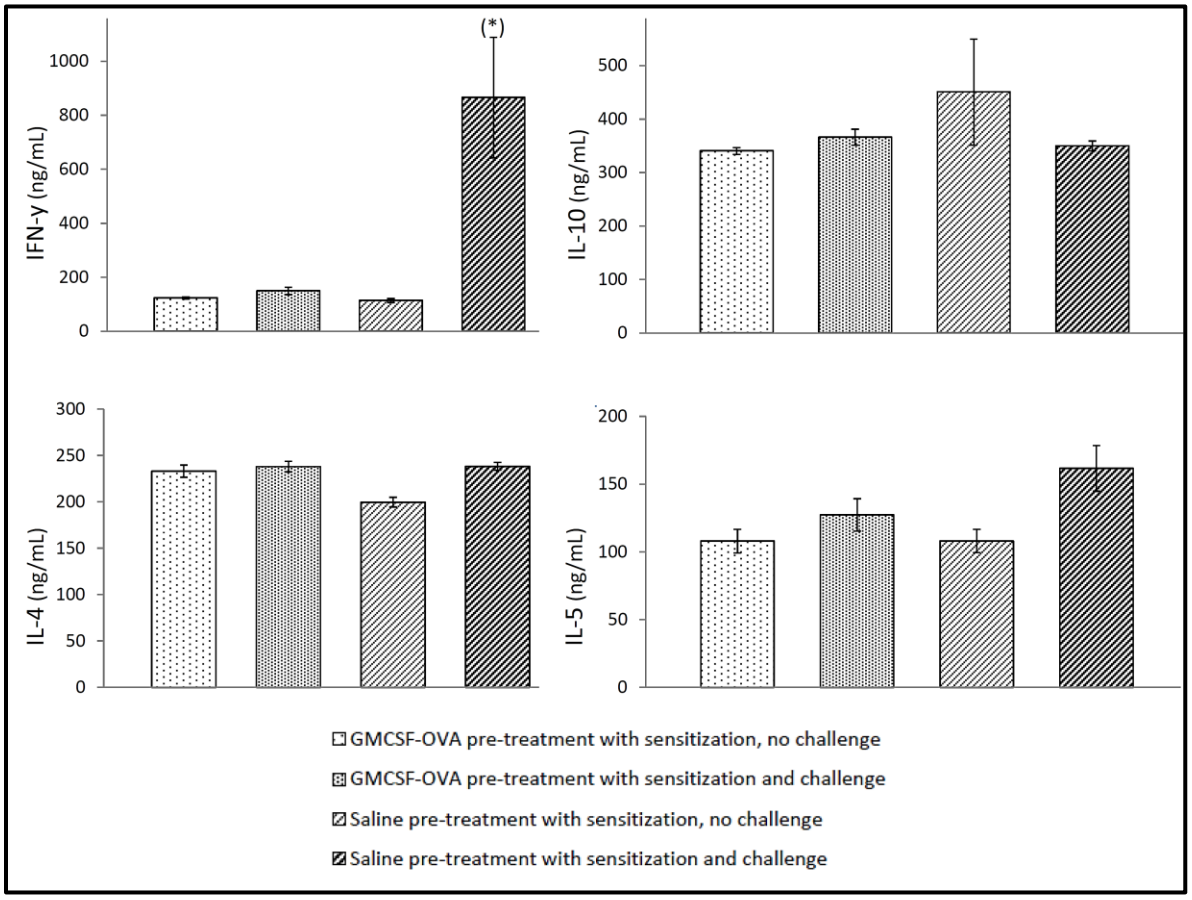
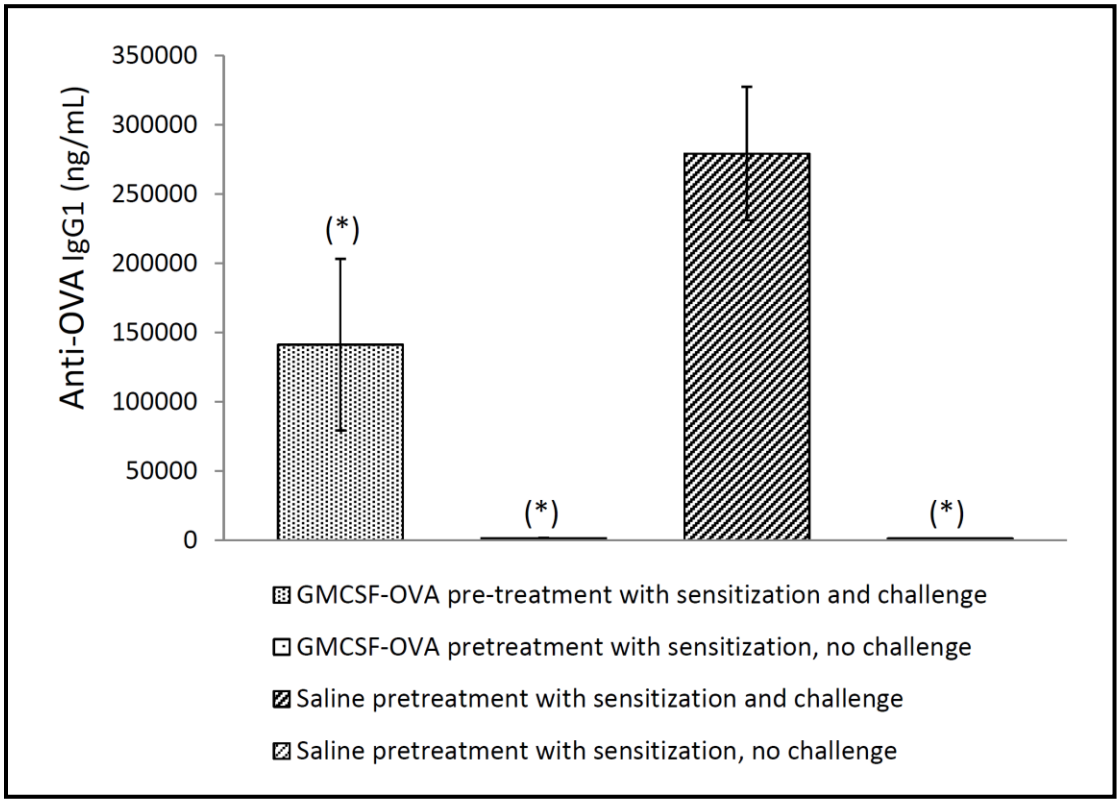

IFN-γ (ng/mL)
1000
800
600
400
200
0
(*)
IL-10 (ng/mL)
500
400
300
200
100
0
IL-4 (ng/mL)
300
250
200
150
100
50
0
IL-5 (ng/mL)
200
150
100
50
0
GMCSF-OVA pre-treatment with sensitization, no challenge
GMCSF-OVA pre-treatment with sensitization and challenge
Saline pre-treatment with sensitization, no challenge
Saline pre-treatment with sensitization and challenge

Figure 4.6. Serum IgG_1 comparison for saline- vs. GMCSF-OVA 323-339-pretreated BALB/c mice

Mice were pre-treated with either saline or GMCSF-OVA 323-339 on days -21, -14, and -7; sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection; and challenged with either a 1% OVA 323-339 solution or saline on days 21, 22, and 23 via pharyngeal aspiration (n=10 per group for each of four groups, one experiment). Animals were harvested on day 26, at which time, terminal blood collections were obtained. Serum was then tested for OVA-specific IgG_1. Statistics were run as a one-way ANOVA with Dunnett's multiple comparisons test and a single pooled variance. (*) Denotes significant difference ($p<0.05$) versus the Saline pre-treated, sensitized and challenged group. Error bars represent the standard error of the mean for each group value.


350000
300000
250000
200000
150000
100000
50000
0
Anti-OVA IgG1 (ng/mL)
(*)
(*)
(*)
GMCSF-OVA pre-treatment with sensitization and challenge
GMCSF-OVA pretreatment with sensitization, no challenge
Saline pretreatment with sensitization and challenge
Saline pretreatment with sensitization, no challenge

Conclusion

The GMCSF-OVA 323-339 fusion protein attenuated much of the pulmonary inflammation associated with the model used in this study. When OT-II.2 T cell responses to the fusion protein were analyzed via flow cytometry, however, data did not suggest an effect on CD3, CD4, TCRVβ5, or TCRVα2 populations, perhaps indicating a role for immunosuppressive T_{Reg} mechanisms unrelated to apoptosis of effector cells. The data shown suggests that fusion proteins such as GMCSF-OVA 323-339 have potential as immunomodulators within a model of pulmonary inflammation. Further study to determine dose, timing of treatment, and other cytokine-antigen fusion protein combinations within the model are warranted.

CHAPTER 5: GENERAL DISCUSSION

Study Summary

Allergic asthma is a significant medical problem, affecting more than 300 million individuals and causing approximately 250,000 deaths every year (Global surveillance, prevention and control of chronic respiratory diseases: A comprehensive approach. 2007; Heron et al., 2009). Current asthma therapies temporarily minimize discomfort and manage symptoms, but there are no effective long-term, preventative, or curative agents available. Complexities in development and regulation of immune processes, as well as complexities in the interaction of the immune system with cellular and molecular pathways controlling development and function of the pulmonary system, have thwarted development of novel efficacious therapeutics. To date, the management of asthma symptoms has largely been through the use of bronchodilators and inhaled steroids and corticosteroids. The prescription of leukotriene inhibitors and anti-IgE therapies has also aided in management of symptoms. Bronchodilators may be short- or long-acting, and are typically β2 agonists (short-acting), anti-cholinergics (paired with short- or long-acting agents), and/or theophylline (long-acting) (Spencer and Krieger, 2013). Short-acting bronchodilators can be used to reverse symptoms once they have appeared or as an emergency measure, but do not manage the underlying inflammation. Long-acting bronchodilators do not provide immediate relief, but are typically used together with inhaled steroids for added prevention of acute attacks. Inhaled steroids and corticosteroids are used to lessen airway inflammation in an effort to reduce the chances of the onset of an acute asthma attack. They must be taken at least once daily

or as prescribed to help control inflammation, but are not useful once symptoms are present. Leukotriene inhibitors have been used due to their anti-inflammatory effects, but are not as effective as corticosteroids and are therefore used for mild or moderate asthma cases or in conjunction with corticosteroids. Anti-IgE therapy has been prescribed to patients with severe and persistent asthma, although anaphylaxis is a potential side effect of the treatment (Spencer and Krieger, 2013).

Definition of phenotype-specific pathogenic factors has shown some promise in establishing effectiveness of agents targeting the IL-4 and IL-13 pathways. Elucidation of the role of innate immunity in asthma is leading to better understanding of how lipids and viruses can induce exacerbations in allergic asthmatics, even when their allergies are otherwise well controlled. However, other than some encouraging results from animal studies with agents that interrupt inflammation induced via the OX40 pathway (Kaur and Brightling, 2012), and modest effects of Pitrakinra (recombinant protein targeting IL-4Rα, attenuating IL-4 and IL-13 signaling) (Antoniu, 2010b), MEDI-528 (anti-IL-9 antibody) (Antoniu, 2010a; Parker et al., 2011), and Mepolizumab (anti-IL-5 antibody) (Busse et al., 2010; Nair et al., 2009; Haldar et al., 2009; Wenzel, 2009), little has emerged in terms of translating the basic observations into effective and affordable treatments. It is important, therefore, to continue investigations into new, novel therapeutics.

Recent studies have indicated that targeting immunogens to specific immune cells via fusion with cytokines increases the effectiveness of immunotherapy in animal models for asthma as well as other immune-mediated disease models such as experimental autoimmune encephalitis (Mannie et al., 2007b; Blanchfield and Mannie,

2010; Kim et al., 1997; Maecker et al., 2001; Maecker et al., 2001; Mannie and Abbott, 2007; Mannie et al., 2007a). These cytokine-antigen fusion proteins target antigen to specific antigen presenting cell populations (Mannie et al., 2007b; Blanchfield and Mannie, 2010), upregulate T Regulatory cells (Mannie et al., 2007b), bias the immune system toward T_H1 responses (Kim et al., 1997; Maecker et al., 2001), and induce immunological tolerance to antigen (Mannie et al., 2007b; Blanchfield and Mannie, 2010). Cytokines involved in asthma pathogenesis include the T_H2-specific cytokines - IL-4, IL-5, and IL-13 - as well as general cytokine growth factors such as IL-2, a growth factor for lymphocytes, and GM-CSF, a growth factor for neutrophils, eosinophils, and basophils (Mannie et al., 2007b; Blanchfield and Mannie, 2010). Upon exposure of DC and macrophage APC cytokine receptors to specific signals such GM-CSF, APC are more inclined to develop a tolerogenic state, leading to the deletion or anergization of reactive T cells (Kapsenberg, 2003) or the induction of suppressive T_{Regs} (Ganesh et al., 2009; Dhodapkar et al., 2001). As many murine asthma models use ovalbumin to induce allergic inflammation, OVA 323-339, the primary antigenic domain of ovalbumin, could thus be targeted to DC and macrophage APC populations in an effort to tolerize the immune system and reverse asthma symptoms.

The objective of this study was to test the hypothesis that fusion proteins comprised of GM-CSF and OVA 323-339 epitopes potentiate antigen-specific immunological tolerance and attenuate development of asthma. The objective was achieved through three Specific Aims: 1) the establishment of the efficacy of the GMCSF-OVA 323-339 cytokine-fusion protein *in vitro*, 2) the development of a murine

asthma model for testing of the cytokine-fusion protein, and 3) the verification of the efficacy of GMCSF-OVA 323-339 cytokine-fusion proteins *in vivo.*

In Specific Aim 1, to establish of the efficacy of the GMCSF-OVA 323-339 cytokine-fusion protein *in vitro*, GMCSF-OVA 323-339 cytokine fusion construct DNA was generated via PCR, transformed into Top10 *E. Coli*, expanded, and purified. DNA constructs were transfected into 293F Human Embryonic Kidney (HEK) cells, a stable line was generated, and protein secreted in supernatant was collected, pooled, concentrated, and purified. The bioactivity of cytokine and antigenic domains was confirmed individually with [3H]thymidine incorporation bioassays. GMCSF-OVA 323-339 cultured with cells from the Memory OT-II.2 T Cell Line caused antigenic domain bioactivity to be left-shifted compared with the OVA 323-339 positive control, suggesting targeting of antigen to T Cell responders. The cytokine domain of GMCSF-OVA 323-339 purified protein was shown to have biological activity similar to that of the GM-CSF positive control. Taken together with the antigenic domain bioactivity data, the GMCSF-OVA 323-339 was considered to be a fully functional protein with neither domain affected by fusion to the other.

In Specific Aim 2, in order to develop a murine model of allergen-induced airway inflammation for testing of the cytokine-fusion protein, many different protocols and avenues were pursued. This was made necessary by the fact that very few models use OVA 323-339 for both sensitization and challenge, and to that effect, many of the models attempted did not yield a robust inflammatory response. Options explored during model development included chronic vs. acute models, the use of a C57BL/6 vs. BALB/c mouse strains, the use of various adjuvants, sensitization routes, and challenge

routes, as well as varying OVA 323-339 doses, and numbers of sensitizations and challenges; the success or failure of each protocol was evaluated based on its ability to produce pulmonary inflammation, particularly eosinophilic inflammation. Based on the various protocols attempted for the generation of a pulmonary allergic model, it was decided that for sensitization and challenge with the OVA peptide, higher levels of inflammation could be achieved in an acute model with BALB/c mice given subcutaneous injections of IFA and peptide for sensitizations, and given at least 60 µL of peptide (to allow for better dispersion in the lungs) via pharyngeal aspiration for challenges. The animal model ultimately chosen for the study was as follows: sensitization with 200 µg OVA 323-339 in 200 µL IFA on days 0, 7, and 14 via subcutaneous injection; challenge with 60 µL of either a 1% OVA 323-339 solution or saline on days 21, 22, and 23 via pharyngeal aspiration; and harvest on day 26. Although pulmonary inflammation did not reach levels comparable to that of models with whole OVA for sensitization and/or challenge, inflammation was primarily eosinophilic, measurable, and consistent. Due to the measurable, consistent effects, it was decided that this model would be appropriate to determine whether or not GMCSF-OVA 323-339 exhibits immunomodulatory potential within a defined system.

In Specific Aim 3, to verify the efficacy of the GMCSF-OVA 323-339 cytokine-fusion protein *in vivo*, mice were pre-treated with either GMCSF-OVA 323-339 or saline on days -21, -14, and -7 via subcutaneous injection. Animals were then sensitized with OVA 323-339 in IFA on days 0, 7, and 14 via subcutaneous injection, challenged with either a 1% OVA 323-339 solution or saline on days 21, 22, and 23 via pharyngeal aspiration, and harvested on day 26. Pre-treatment of mice with GMCSF-OVA 323-339

fusion proteins reduced pulmonary inflammation within the model compared to that of mice given saline treatment. The numbers of eosinophils present in the BAL fluid of mice, as determined by differential cell counts, were significantly fewer for mice that received the fusion protein prior to sensitization and challenge with OVA 323-339. Histological scoring of H&E- and AB-PAS-stained sections agreed with the differential cell count data, showing pulmonary cellular infiltration and cuffing of the airways, along with a mild mucus response, in stained lung sections for animals treated with saline versus little-to-no cellular infiltration or mucus production for mice treated with fusion protein. Supporting the differential cell count data and histological findings, serum analysis indicated a significant increase in IgG_1, an indicator of T_H2 activity in murine models, for mice given saline compared to those pre-treated with GMCSF-OVA 323-339 prior to sensitization and challenge. In addition, cytokine analysis of bronchoalveolar lavage fluid showed a significant increase in IFN-γ for mice given saline prior to sensitization and challenge compared to those pre-treated with GMCSF-OVA 323-339. In a study by Koch et al., it was shown that IFN-γ localized in the lung tissue can result in increased IL-5 and IL-13, as well as elevated eosinophils numbers in lavage fluid (Koch et al., 2006).

Overall, the observed reduction in pulmonary inflammation and mucus demonstrates that GMCSF-OVA 323-339 fusion proteins have the capability of influencing immune responses within a murine model of allergen-induced airway inflammation, and suggests that cytokine-fusion therapies may have potential as asthma therapeutics. Yet, there were uncertainties regarding how GM-CSF would perform as the cytokine chosen for fusion within a model of pulmonary inflammation,

given that GM-CSF has been shown to exert both anti-inflammatory as well as pro-inflammatory effects under different circumstances. The importance of GM-CSF in macrophage function is illustrated in GM-CSF knockout mice, which develop alveolar proteinosis, a state characterized by impaired macrophage function, an accumulation of surfactant in the airways, and inefficient gas exchange (Hamilton and Anderson, 2004). Alveolar proteinosis can also develop due to defects in the β chain of the GM-CSF receptor, causing interference with GM-CSF signaling (Hamilton, 2002). GM-CSF overexpression in human populations, caused by certain malignancies such as myeloid leukemia, results in the differentiation of cancerous cells as well as the generation of GM-CSF-neutralizing auto-antibody and subsequent alveolar proteinosis (Shi et al., 2006; Hamilton, 2002). Neutropenia caused by cancer or resulting chemotherapy treatment may be treated with GM-CSF or with recombinant GM-CSF therapies such as Sargramostim (Leukine) to mobilize stem cells. Bone marrow transplant recipients may also receive GM-CSF therapy to aid in myeloid reconstitution. Patients receiving GM-CSF therapy that have also been diagnosed with rheumatoid arthritis, however, experience a worsening of arthritis symptoms as a side-effect, indicating a pro-inflammatory role for GM-CSF (Shi et al., 2006).

Further supporting a pro-inflammatory role for GM-CSF, GM-CSF has been observed to contribute to T_H17-induced inflammatory responses, and transgenics expressing GM-CSF in the stomach have been shown to develop autoimmune gastritis (Shi et al., 2006; McGeachy, 2011). In studies with GM-CSF knockout mice, mice are resistant to development of EAE and type I diabetes in the respective disease models (Hamilton, 2002; Enzler et al., 2007). In asthma models, treatment with anti-GM-CSF

antibody attenuates sensitization to aeroallergens, allergen-induced inflammation, and AHR (Hamilton and Anderson, 2004). Consistent with the mucosal sources of GM-CSF, GM-CSF mRNA is increased in the airways of human asthmatics (Chung and Barnes, 1999) where it promotes maturation, activation, and growth of monocytes and DCs resulting in increased antigen presentation and activation of antigen-specific B and T cells (Blanchfield and Mannie, 2010; Ganesh et al., 2009; Hamilton and Anderson, 2004; Ritz et al., 2002). Clinical application of GM-CSF inhibition has been seen with Mavrilmumab, a rheumatoid arthritis treatment targeting GM-CSFRα, and with MOR 103, an anti-GMCSF treatment used in multiple sclerosis (Nair et al., 2012; Deiß et al., 2013).

GM-CSF has also been shown to exert anti-inflammatory roles, however. GM-CSF treatment in rodent models of experimental autoimmune myasthenia gravis inhibited disease progression through the induction of immunosuppressive T_{Regs}, and likewise inhibited experimental autoimmune thyroiditis and type 1 diabetes in rodent models through dendritic cell interaction and T_{Reg} induction (Cheatem et al., 2009; Ganesh et al., 2009; Sheng et al., 2006). Clinical application of GM-CSF has been observed with Sipleucel-T (Provenge), an FDA-approved cancer vaccine. In this treatment, a patient's peripheral blood mononuclear cells (including dendritic cell APC) are collected and incubated with GMCSF-PAP, a fusion protein comprised of GM-CSF and PAP (prostatic acid phosphatase), a protein found on the majority of prostate cancer cells. The cultured cells are then re-introduced into the patient, altering tumor antigen presentation and aiding in the tumor immune response (Shore et al., 2013). GMCSF linked to antigen in EAE experiments by Mannie et al., has also resulted in an

altered immune response leading to attenuation of symptoms. Due to the opposing effects seen for GM-CSF in the rodent type 1 diabetes model, as well as opposing pro- and anti-inflammatory effects observed in other models and therapies, it is likely that the timing of GM-CSF administration or the balance of GM-CSF determines the nature of its effect.

Due to the successes of cytokine-antigen fusion proteins in other experimental systems such as Experimental Autoimmune Encephalomyelitis (EAE) with Mannie et al., it is not surprising to have found similar potential for related cytokine-antigen fusion treatments within an animal model of airway inflammation. Despite a differing pathogenesis in each disorder, each shares a commonality: that the immune system engages in an inappropriate response to innocuous antigen. Therefore, the underlying concept of targeting antigen to specific antigen presenting cell populations in an effort to tolerize the immune system in each disorder is quite similar. Antigenic targeting is achieved by increasing antigen concentration and subsequent uptake and presentation on the surface of dendritic cells via linkage of the GM-CSF domain of the fusion protein to GM-CSF receptors on the surface of dendritic cells. Tolerance to the antigen may then develop due to the effects of GM-CSF on dendritic cells or due to an upregulation in immunosuppressive T_{Regs} acting through a variety of mechanisms, including the release of granzymes causing effector T cell apoptosis, the release of anti-inflammatory cytokines such as IL-10 and TGF-β, sequestration of IL-2 leading to T effector cell death due to cytokine deprivation, cell-contact mediated mechanisms leading to T effector cell cycle arrest or APC immaturity, as well as through competing for T effector cell growth factors such as IL-2 (Vock et al., 2010). Indeed, in experiments by Ganesh et al., GM-

SCF-treated DCs adopted a semi-mature phenotype, and the GM-CSF-treated DCs induced T_{Reg} expansion and attenuation of autoimmune thyroiditis in a murine model (Ganesh et al., 2009). In other experiments by Gaudreau et al., mice treated with GM-CSF showed increased numbers of immature and semi-mature DCs, and that these DCs were critical in maintaining the immunosuppressive effects of T_{Regs} in a model of autoimmune diabetes (Gaudreau et al., 2007). Furthermore, studies by Kared et al. showed that GM-CSF interacted with T_{Regs} through the GM-CSF receptor α-chain CD116, and that this interaction increased T_{Reg} suppressive potency (Kared et al., 2008). (Cheatem et al., 2009; Ganesh et al., 2009; Sheng et al., 2006). In addition, clinical application of GM-CSF has been observed with Sipleucel-T (Provenge), an FDA-approved cancer vaccine in which a patient's peripheral blood mononuclear cells (including dendritic cell APC) are collected and incubated with GMCSF-PAP, a fusion protein comprised of GM-CSF and PAP (prostatic acid phosphatase), a protein common to prostate cancer cells. Following incubation, cells are re-introduced into the patient, aiding in the tumor immune response via altered tumor antigen presentation (Shore et al., 2013). GMCSF-OVA 323-339 fusion proteins, then, may target antigen to DC APC and induce an immature or semi-mature phenotype, leading to less effective antigen presentation and support for T_{Reg} activity. This data suggests that despite its potential to increase inflammation, GM-CSF performs well as the cytokine domain portion of therapeutic fusion proteins.

Future Directions and Study Alternatives

Animal Models

It was recognized from the start of the study that the mouse model of allergen-induced airway inflammation would need to show consistent and significant differences between OVA 323-339- and PBS-challenged animal groups, because if not, it would be ill-suited to demonstrating vaccine efficacy. While chronic models are understood to be a relevant model system as they more closely mimic some of the human asthma symptoms, it proved difficult to obtain reliable results with this model system in experiments in this project. Even acute mouse models, although well-characterized and often used in the literature, were problematic in that they did not induce high levels of eosinophilic inflammation in our system. While the acute model protocol utilized for this study was consistent and significant, it was weaker than many of the other pulmonary inflammation models used in the literature. Perhaps this was due to the fact that most ovalbumin-based models use whole ovalbumin for sensitization and challenge, and the 17-amino acid OVA 323-339 was not antigenic enough to illicit a robust inflammatory response when used for both sensitization and challenge. An alternative approach that could be pursued would be that of an adoptive transfer-induced mouse model of pulmonary inflammation. In an adoptive transfer model, T cells could be harvested from DO11.10 mice (BALB/c transgenic mice with an OVA 323-339-responsive T cell repertoire), then cultured with OVA 323-339. Cultured cells could then be adoptively transferred into wild-type BALB/c mice. Finally, mice would undergo a challenge with OVA 323-339, which might drive a more potent eosinophilic inflammation than we have observed in this study. While a stronger, more potent inflammatory model would be

potentially more difficult for fusion protein treatments to overcome, it could provide added information on the potency of fusion protein treatments.

Dose and Pharmacokinetic Analysis of the GMCSF-OVA 323-339 Fusion Protein

The GMCSF-OVA 323-339 fusion protein used in this study was not subjected to pharmacokinetic analysis. Pharmacokinetic analysis of the fusion protein could provide valuable insight into treatment efficacy through examining the relationship between the treatment concentrations administered to study animals, the time required for the bioavailability of given treatment concentrations to diminish, and the time required for the production of biomarkers indicating treatment effect. Because fusion proteins are expected to be targeted to dendritic cell and macrophage APC populations, systemic bioavailability would be expected to increase initially, and then decrease as the treatment reached its targets. As systemic bioavailability decreased, biomarkers indicating fusion protein activity would then be expected to increase.

Since cytokine domain and antigenic domain bioassays have been developed to test GMCSF-OVA 323-339 bioactivity (described previously), and since these bioassays could also be used to test for GMCSF-OVA 323-339 systemic bioavailability in the serum, the next step in the process would be to determine appropriate biomarkers for fusion protein activity. Biomarkers could be selected with a set of *in vitro* assays combining mouse splenocytes containing dendritic cells and macrophages with GMCSF-OVA 323-339, and detecting increases in various cytokines via ELISA or Multiplex assay. Any cytokines significantly increased by the interaction of GMCSF-OVA 323-339 with mouse APC *in vitro* could potentially be used as biomarkers of fusion protein activity *in vivo*. The pharmacokinetic analysis would be conducted by

administering various doses of treatment, then sampling serum from animals at regular time-points post-administration to test for the presence of GMCSF-OVA 323-339 bioactivity as well as the presence of biomarkers indicative of GMCSF-OVA 323-339 efficacy. The results of the analysis could provide information used to optimize dose concentration and timing with respect to the induction of the asthma model. It would also be interesting to explore the use of the cytokine-fusion protein as a treatment (after the sensitization and/or challenge within the asthma model) instead of as a pre-treatment (as was done in this study).

Additional Cytokine-Antigen Fusion Proteins

While GMCSF-OVA 323-339 fusion protein treatments were chosen for this project, other cytokine-antigen products could be considered. IL-16 and IFNβ, for example, would be potential candidates for the cytokine domain of a tolerogenic fusion protein. IL-16, a chemoattractant for CD4+ immune cells including T Cells, eosinophils, dendritic cells, and monocytes, has been implicated in allergic asthma-related IgE upregulation and airway hyperresponsiveness (Cruikshank et al., 2000; Hessel et al., 1998). IFNβ production is downregulated in the asthmatic epithelium and has been marketed for use in the treatment of severe asthma symptoms (Holgate et al., 2009). In addition, both IL-16 and IFNβ have been shown to be effectually tolerogenic when incorporated into fusion proteins in a rat EAE model (Mannie and Abbott, 2007).

There are potential candidates for the antigenic domain as well. Of course, if the antigenic domain is replaced, then an appropriate model test system that includes sensitization to the antigen of choice would have to be utilized. Taking this into account, possibilities for antigenic domains within the scope of available murine models would

include epitopes from house dust mite or recombinant *Blatella germanica* 2, a cockroach allergen. Due to the size or conformation of the epitopes, successful fusion to a cytokine domain may present a challenge, though such epitopes would be particularly interesting to incorporate into an antigenic domain for testing in a murine model due to their relevance to human allergy. Many other cytokine-antigen fusion combinations could be examined in addition to the possibilities listed above, and further exploration into various combinations is certainly warranted.

Fusion Protein Therapy in the Context of Age-related Changes in Asthma

Future directions and alternatives with regard to the project may also take into consideration the impact of aging on therapeutic efficacy, and that certain cytokine-antigen fusion combinations or doses may affect the aging population differently. Due to many age-related changes ranging from cell and tissue function to responsiveness to therapeutics, dose and formulation of the cytokine fusion proteins used for treatment may not translate for aging populations. Both lung function and immune responses decline in older adults, resulting in age-related changes in the manifestation of pulmonary diseases (Brand et al., 2011). During the first two decades of life, the lungs physically grow, and as lung capacity increases, maximum forced expiratory flow (FEV_1) and volume (FVC) increase. From birth to adolescence, the lung tissue becomes stiffer and elastic recoil increases. Thereafter, lung elastic recoil decreases, and the lungs become more flaccid as part of the normal aging process. In addition, with age, alveolar ventilation becomes more heterogenous, physicochemical transfer of inspired gas to the blood (diffusing capacity) declines, and FEV_1 and FVC gradually decline. A variety of exogenous factors impact lung function throughout life, causing gradual and cumulative

effects that are often not manifest until later life. These factors include exposure to environmental and industrial agents, and residual effects of disease.

The normal decline in lung function is less well tolerated in asthmatic individuals who have a lower level of basal function due to chronic disease (Sharma and Goodwin, 2006; Colebatch et al., 1979; Wahba, 1983). In addition, responsiveness to pharmacologic agents changes, altering efficacy of therapeutic agents in the elderly. For example, bronchodilator efficacy decreases with age in some individuals as reversible bronchoconstriction transitions towards fixed obstruction. Also, Zafirlukast, an oral leukotriene receptor antagonist, is less effective in improving lung function with age (Hanania et al., 2011).

Asthma-related inflammation becomes more severe with age, and the inflammatory cell profile is altered compared to the younger population (Busse and Mathur, 2010). Several factors contribute to this trend, including reduced capacity for mucociliary clearance of inhaled pathogens. Ciliary beating and particle clearance decreases from adolescence to old age, while production of mucins increases. The change in balance between mucus production and clearance increases the propensity for airway obstruction. Decreased mucus clearance also increases risk of infection (Busse and Mathur, 2010; Lee et al., 2012).

Immunosenescence that accompanies aging contributes to changes in the characteristics of pulmonary inflammation (Vignola et al., 2003). Immunosenescence results from somatic mutations and other forms of molecular damage coupled with decreased efficiency of DNA repair. Molecular damage affects cytokine expression and growth and function of diverse cell types, including epithelial cells, lymphocytes,

eosinophils, neutrophils, natural killer cells, macrophages and monocytes (Hanania et al., 2011; Busse and Mathur, 2010; Lee et al., 2012; Jones et al., 2011). Neutrophils are found in greater abundance in the airways of aging adults, and comparisons of sputum reveals elevated neutrophil numbers in older asthmatic subjects, as well as increased mediators associated with neutrophilic inflammation including IL-8, neutrophil elastase, and matrix metalloproteinase 9. Increased neutrophil elastase in the elderly may contribute to diminished recoil observed in the aging lung. Neutrophils from older adults also exhibit reduced chemotaxis, phagocytosis and respiratory burst activity, which can impact killing and clearance of pathogens from the airways (Busse and Mathur, 2010).

The contribution of eosinophils to asthma in the elderly is unclear. Eosinophils from older human asthmatics have been reported to exhibit decreased superoxide anion production. One study with aged mice exposed to antigenic sensitization and challenge reported a greater number of eosinophils in bronchoalveolar lavage fluid than in young mice, yet the aged mice experienced reduced airway responsiveness. In another study, the number of eosinophils in peripheral blood was elevated in aged animals, and the elevation correlated with airway hyperresponsiveness to methacholine. These discrepancies have not been fully explained at this time (Busse and Mathur, 2010).

T cell function also is impacted by age. The thymus, the site of early T cell development, undergoes involution and atrophies with age. It is currently unknown whether thymic involution causes age-dependent losses in cell-mediated immunity (Busse and Mathur, 2010; Vignola et al., 2003). The ratio of memory T cells compared to naïve T cells also increases as individuals grow older, most likely due to cumulative antigenic exposures. Age-dependent losses in T cell function concur with reduced

clonotypic diversity of the naïve T cell repertoire, and in some cases, with oligoclonal expansions of memory T cells in the elderly. Potential reductions in clonotypic diversity may be a reason why certain infectious diseases are much more severe in adulthood.

Numbers of B cell precursors in bone marrow decrease with age in humans (Lee et al., 2012; Vignola et al., 2003). Studies in mice indicate that antibody production is unaffected by age, but affinity and avidity of antibodies produced during a primary humoral immune response are decreased. It has been proposed that this may be due to limited somatic hypermutation, which plays a role in increasing antibody-antigen binding. Some of the age-related effects on B cells may be related to age-related changes in T cell function, since adoptive transfer of aged T-cells into younger recipient mice resulted in reduced B cell activation, expansion, and IgG production (Busse and Mathur, 2010).

Reports have indicated that the numbers of NK cells are increased in older adults, whereas the proliferative response of NKT cells is decreases with age, but both NK and NKT cells are reported to exhibit lower of cytotoxic capacity with age (Busse and Mathur, 2010). NK and NKT cell types are implicated in viral clearance, and impaired cytotoxicity and proliferative responses in the case of NKT cells may contribute to episodes of asthma induced by viral infection in elderly patients.

Monocyte and macrophage phagocytosis, chemotaxis, adherence, and antigen presentation show little or no difference with age (Busse and Mathur, 2010). TLR expression and signaling, and ROI production are reduced, however. Monocytes from aged adults, when stimulated through TLR 1/2 heterodimer, exhibit decreased production of IL-6 and TNF-α (Vignola et al., 2003). Monocytes from older adults, when

differentiated into macrophages *in vitro*, exhibit decreased TLR3 expression and response to virus, as well as less ROI and RNI production upon stimulation with LPS (Busse and Mathur, 2010). Given the many age-related changes that take place in asthma pathogenesis, perhaps it should be considered that while one cytokine-antigen fusion combination or dosing regimen may be effective in younger subjects, the same may not yield comparable results in an aging population.

Conclusion

In these studies, biologically active GMCSF-OVA 323-339 cytokine fusion constructs were successfully generated, the development of a suitable animal model of pulmonary inflammation was completed, and the anti-inflammatory properties of GMCSF-OVA 323-339 were confirmed *in vivo*. When tested in the animal model, mice given saline prior to sensitization and challenge with OVA 323-339 showed consistent, measurable, significant increases in pulmonary inflammation compared to mice that received GMCSF-OVA 323-339 pre-treatment prior to sensitization and challenge with the OVA peptide and controls that did not undergo challenge with the OVA peptide. Mice given saline prior to sensitization and challenge showed eosinophilia in cytospin slides for differential cell counts, cellular infiltration and cuffing of the airways in H&E-stained lung sections, and a light mucus response in the AB-PAS-stained lung sections, while mice given GMCSF-OVA 323-339 pre-treatment showed a marked reduction in eosinophils, absent or only slight cellular infiltration in lung sections, and no mucus secretion. In agreement with differential cell count data and histological findings, mice given saline prior to sensitization and challenge showed a significant elevation in IgG_1, an indicator of T_H2 activity in murine models, as well as a significant increase in BAL

IFN-γ, which correlated with increases in pro-inflammatory IL-5 and IL-13 as well as increased numbers of eosinophils, compared with mice pre-treated with GMCSF-OVA 323-339.

Given the anti-inflammatory nature of the GMCSF-OVA 323-339 fusion protein observed in these experiments, studies with fusion proteins should be continued in the asthma field as well as in other fields involving immune system-based pathologies. Cytokine-fusion proteins hold tremendous potential as therapeutic tools, and the many cytokine-fusion variations that could be produced and the outcomes of those various fusions may be keys to unlock and reverse many immunopathologies. I am hopeful that future exploration of the specific targeting and manipulation of existing immunological pathways using fusion protein technology will continue to provide insight into these processes.

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APPENDIX A: ANIMAL CARE AND USE COMMITTEE PROTOCOL APPROVAL

The Brody School of Medicine
Office of Prospective Health
East Carolina University
188 Warren Life Sciences Building Greenville, NC 27834
252-744-2070 office 252-744-2417 fax

Occupational Medicine
Employee Health
Radiation Safety
Infection Control
Biological Safety

TO: Dr. Dianne Walters
Department of Physiology

FROM: Eddie Johnson/John Williams
Biological Safety Officers

RE: Registration Final Approval

Date: January 22, 2010

Your Biological Safety Protocol Walters, 10-02, *"Generation of a chronic model of asthma in mice"* has received **final approval** based on your registration revisions submitted. This approval is effective for a period of 3 years and may be renewed with an updated registration if needed. Please notify the Animal Care staff before or if you begin work with Biohazard agents in animals. Also please keep in mind all individuals who will be exposed to or handle biohazardous agents in your work will be due for Blood Borne Pathogens refresher training annually.

Please do not hesitate to contact Biological Safety at 744-2070 if you have any questions, concerns, or need any additional information. Best wishes on your research.

cc: Dr. Jeff Smith, Chair, Biosafety Committee
Dr. Robert Lust, Chair
Janine Davenport, IACUC
Dr. Robert Carroll, IACUC
Dale Aycock, IACUC

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 13, 2011

Mark Mannie, Ph.D.
Department of Micro/Immuno
Brody 5E-106
ECU Brody School of Medicine

Dear Dr. Mannie:

Your Animal Use Protocol entitled, "Tolerogenic Therapeutic Vaccines in TCR Transgenic OT-II Mice" (AUP #K160) was reviewed by this institution's Animal Care and Use Committee on 12/13/11. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure

East Carolina University

Animal Care and
Use Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

August 16, 2012

Mark Mannie, Ph.D.
Department of Micro/Immuno
Brody 5E-106
ECU Brody School of Medicine

Dear Dr. Mannie:

Your Animal Use Protocol entitled, "Tolerogenic Therapeutic Vaccines in a Murine Model of Asthma" (AUP #K164) was reviewed by this institution's Animal Care and Use Committee on 8/16/12. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

SBMcRae

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure

BRT-BURLESON RESEARCH TECHNOLOGIES, INC.
APPLICATION FOR VERTEBRATE ANIMAL USE

Title of project: IACUC #BIC 2013-01

Tolerogenic therapeutic vaccines in a murine model of asthma

APPROVAL IS RENEWABLE EVERY 3 YEARS

ANY CHANGES TO THIS APPROVED PROTOCOL REQUIRE THE SUBMISSION OF A PROTOCOL AMENDMENT FORM. CHANGES SHOULD NOT BE IMPLEMENTED PRIOR TO IACUC REVIEW AND APPROVAL.

Date of Final Review and Approval: 7 June 2013 **Expiration Date**: 6 June 2016

Richard E. Fish Digitally signed by Richard E. Fish
DN: cn=Richard E. Fish, o=NC State, ou=CVM, email=rick_fish@ncsu.edu, c=US
Date: 2013.06.07 09:16:46 -04'00'

Chairman, Institutional Animal Care and Use Committee

APPENDIX B: PERMISSION LETTER

Permission to use a figure from “Functions of T cells in asthma: more than just T_H2 cells” by C.M. Lloyd, C.M. and E.H. Hessel, Nature Reviews Immunology, 2010. 10, 838-848 was granted online via Rightslink and Nature Publishing Group. The figure requested for use was figure 1, T cells involved in the induction of the allergic phenotype. See information regarding permission below.

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APPENDIX C: HISTOLOGY REPORT PROVIDED BY DR. RODNEY MILLER FOR THE DETERMINATION OF THE EFFICACY OF THE GMCSF-OVA 323-339 CYTOKINE FUSION PROTEIN *IN VIVO*

For Stefanie Burleson

Histopathologic Evaluation of Mouse Lungs from GMCSF-OVA 323-339 Fusion Protein Pretreatments in a Model of Pulmonary Inflammation.

Approximately 9 sections of lung per mouse, stained with H&E, were evaluated for 40 mice. An equal number of slides stained with AB/PAS were also evaluated for the presence of mucus substance.

Attached to this narrative are the Histopathology Individual Animal Data sheets. On these sheets are this pathologist's opinions on degrees of perivascular infiltration, peribronchial (and peribronchiolar) infiltration, epithelial damage and parenchymal infiltration somewhat following histopathology evaluation terminology of Silvia Naus, et al. (Am J Respir Crit Care Med. Vol 181. PP 1318-1328, 2010. Additionally, relative degrees of alcian blue PAS positive material (mucus) were tabulated for large and small airways.

Mice 901-910 – There was no indication of any inflammatory cell infiltrate in the lungs of these mice.
Half of the mice had +1 (minimal) evidence of mucus substance in the largest airway (bronchus) characterized by sparsely scattered epithelial cells that were associated with apical caps of blue/red staining material.

Mice 911-920 – Only one mouse had a small focus of inflammatory cell infiltration (pulmonary alveolar macrophages and some lymphocytes) in an alveolar location (parenchyma).
Nine of these mice had some (minimal) identifiable AB/PAS positive material in the largest airways. One mouse had AB/PAS positive material which was more easily seen and present in more cells and it was graded +2 (slight).

Mice 921-930 – Only two mice in this group had evidence of inflammatory cell infiltrates. They had infiltrates of inflammatory cells causing perivascular and peribronchiolar cuffing. Scattered inflammatory cells were present in alveolar (parenchymal) areas. The infiltrating cells were mainly lymphocytes. There were a few polymorphonuclear leukocytes (neutrophils and maybe some eosinophils which were very pale and difficult to identify with certainty).
Eight mice had minimal amounts of AB/PAS positive material in the larger airways.

Mice 931-940 – This group of mice were clearly different from the other three groups. All mice had some degree of peribronchiolar (and peribronchial), perivascular and parenchymal inflammatory cell infiltration. In four of these mice the infiltrates were prominent and categorized as moderate. Cuffs of inflammatory cells were prominent around both larger airways and the smaller bronchioles. Parenchymal infiltration was less prominent but present in over half of the mice. Mucus had accumulated in the airways in two animals (933 and 939). Again, the inflammatory cells were primarily lymphocytes and some neutrophils and macrophages. A few faint staining eosinophils appeared to be present.
Mucus substance was noted in larger airways in all rats with a couple being quite prominent (+3, moderate). In one rat (933), graded +3, the mucus in cells extended into smaller airways but not down to the terminal bronchioles.

Rodney A. Miller 11-19-13

Rodney A. Miller, D.V.M., Ph.D., DACVP

HISTOPATHOLOGY INDIVIDUAL ANIMAL DATA

EPL STUDY NO.:
CLIENT STUDY NO.:
GROUP ID: 901-910
SEX:
PATHOLOGIST: DATE:

TISSUE – DIAGNOSIS	ANIMAL NUMBER 901	902	903	904	905	906	907	908	909	910
Perivascular infiltration	X	X	X	X	X	X	X	X	X	X
Peribronchiolar infiltration	X	X	X	X	X	X	X	X	X	X
Epithelial damage	X	X	X	X	X	X	X	X	X	X
Parenchymal infiltration	X	X	X	X	X	X	X	X	X	X
AB/PAS – large airway (bronchus)	+1	–	+1	–	+1	–	[illegible]	+1	–	+1
AB/PAS – small airway (Bronchiole)	–	–	[illegible]	–	–	–	–	–	–	–

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Experimental Pathology Laboratories, Inc.

Key: P = Present; 1 = Minimal; 5 = Severe/High; N = No Section; I = Incomplete Section; A = Autolysis; 2 = Slight; 3 = Moderate; X = Not Remarkable; 4 = Moderately Severe/High; – = not present

HISTOPATHOLOGY INDIVIDUAL ANIMAL DATA

EPL STUDY NO.:
CLIENT STUDY NO.:
GROUP ID: 911-920
SEX:
PATHOLOGIST: DATE:

TISSUE – DIAGNOSIS	ANIMAL NUMBER 911	912	913	914	915	916	917	918	919	920
Perivascular infiltration	X	X	X	X	X	X	X	X	X	X
Peribronchiolar infiltration	X	X	X	X	X	X	X	X	X	X
Epithelial damage	X	X	X	X	X	X	X	X	X	X
Parenchymal infiltration	X	X	X	1	X	X	X	X	X	X
AB/PAS - Large airway (Bronchus)	+1	+1	+1	+1	+1	+1	+1	+2	+1	+1
AB/PAS - small airway (Bronchioles)	–	–	–	–	–	–	–	–	–	–

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Key: P = Present; N = No Section; A = Autolysis; X = Not Remarkable
1 = Minimal; 2 = Slight; 3 = Moderate; 4 = Moderately Severe/High
5 = Severe/High; I = Incomplete Section; – = not present

HISTOPATHOLOGY INDIVIDUAL ANIMAL DATA

EPL STUDY NO.:
CLIENT STUDY NO.:
GROUP ID: 921-930
SEX:
PATHOLOGIST: DATE:

TISSUE – DIAGNOSIS	ANIMAL NUMBER 921	922	923	924	925	926	927	928	929	930
Perivascular infiltration	X	X	X	X	2	X	X	X	X	X
Peribronchiolar infiltration	X	X	X	X	2	X	X	+1	X	X
Epithelial damage	X	X	X	X	X	X	X	X	X	X
Parenchymal infiltration	X	X	X	X	1	X	X	+1	X	X
AB/PAS - large airway (Bronchus)	+1	+1	+1	+1	+1	+1	–	–	+1	+1
AB/PAS - small airway (Bronchiole)	–	–	–	–	–	–	–	–	–	–

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HISTOPATHOLOGY INDIVIDUAL ANIMAL DATA

EPL STUDY NO.:
CLIENT STUDY NO.:
GROUP ID: 931–940
SEX:
PATHOLOGIST: DATE:

TISSUE – DIAGNOSIS	ANIMAL NUMBER 931	932	933	934	935	936	937	938	939	940
Perivascular infiltration	X	+2	+3	+2	+1	+3	+3	+2	+3	+2
Peribronchiolar infiltration (bronchiole)	+1	+2	+3	+2	+1	+3	+3	+2	+3	+2
Epithelial damage	X	X	X	X	X	X	X	X	X	X
Parenchymal infiltration	X	X	+2	+1	X	+1	+1	+1	+2	X
AB/PAS - large airway (Bronchus)	+1	+2	+3	+2	+1	+2	+2	+1	+3	+1
AB/PAS - small airway (Bronchioles)	–	–	+1	+1	–	–	–	–	+1	–

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Key: P = Present; 1 = Minimal; 5 = Severe/High; N = No Section; I = Incomplete Section; A = Autolysis; 2 = Slight; 3 = Moderate; X = Not Remarkable; 4 = Moderately Severe/High; – = not present