

**Investigation of the Immunomodulatory Properties of a Novel Allergen  
Variant That Targets Human Dendritic Cells**

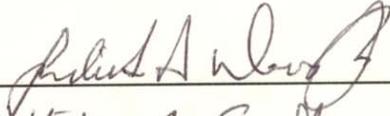
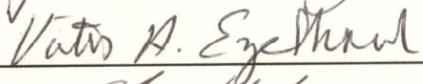
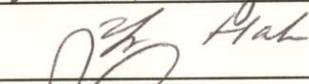
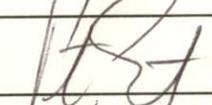
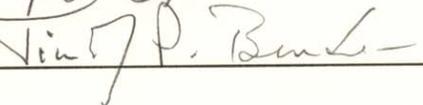
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## Abstract

The allergen variant H22-Fel d 1 targets cat allergen to the high affinity IgG receptor Fc $\gamma$ RI on DCs. This molecule was developed as a possible treatment for cat-allergic patients. The objective of this dissertation was to investigate the immunomodulatory properties of H22-Fel d 1 using an *in vitro* human-based system. H22-Fel d 1 induced a semi-mature phenotype in monocyte-derived dendritic cells (moDCs), characterized by enhanced production of Th1-associated cytokines with no change in expression of co-stimulatory molecules. Furthermore, H22-Fel d 1-primed moDCs induced a selective increase in Th2 and IL-10-expressing regulatory T cell types pointing to qualitative changes in the T cell cytokine repertoire. Importantly, this effect was restricted to cat-allergic subjects. Blockade of IL-10 selectively enhanced the Th2 component of the T cell response to H22-Fel d 1, suggesting that this arm of the response was regulated by IL-10. The clinical efficacy of H22-Fel d 1 will likely depend on its capacity to induce a protective T cell cytokine repertoire in a “pro-allergic” cytokine milieu *in vivo*. Therefore, we investigated whether the Th2-promoting cytokine, thymic stromal lymphopoietin (TSLP), which acts directly on DCs and is expressed in the skin and lungs of patients with allergic disease, mitigates the immunomodulatory capacity of H22-Fel d 1. Surprisingly, TSLP was a weak inducer of Th2 responses *in vitro* when, irrespective of atopic status; however, when moDCs were co-primed with TSLP and H22-Fel d 1 Th2 responses were amplified in a synergistic manner in highly atopic subjects. This effect was OX40 ligand-independent and not associated with secretion of the

Th2-attracting chemokine, CCL17, pointing to an unconventional TSLP-mediated pathway. Expression of TSLP receptor was markedly enhanced on atopic moDCs after priming with H22-Fel d 1 through a pathway regulated by PI3K and PKC. Moreover, inhibition of these molecules selectively abolished TSLP-mediated Th2 responses triggered by H22-Fel d 1. Discovery of a novel pathway linking Fc $\gamma$ RI to TSLP receptor upregulation and consequent TSLP-mediated effects questions the validity of allergen vaccines which engage surface receptors on DCs, but serendipitously identifies DC signaling components which could serve as new therapeutic targets for Th2-driven diseases.

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There are several people whose guidance and support were critical to my success during my doctoral studies. First and foremost, I would like to thank my mentor Dr. Judith Woodfolk for allowing me to complete my graduate work under her mentorship. Her guidance has been invaluable to me both scientifically and personally, and I know she has prepared me for the years ahead in academic research. I would also like to thank my co-mentor Dr. Victor Engelhard, as well as the rest of my thesis committee Dr. Timothy Bender, Dr. Young Hahn, Dr. Peter Ernst and Dr. Klaus Ley for their advice and guidance.

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Finally, I cannot thank my husband David enough for his enduring love, support and encouragement of my goals. My family has also provided much needed love and support.

## List of Abbreviations

Ab	Antibody
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
APC	Allophycocyanin
APC	Antigen presenting cell
aTreg	Adaptive regulatory T cell
Bet v	<i>Betulla verrucosa</i>
CD	Cluster of differentiation
CDR	Complementarity-determining region
ChIP	Chromatin immunoprecipitation
CI	Confidence interval
CLA	Cutaneous lymphocyte-associated antigen
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
DAG	Diacylglycerol
DC	Dendritic cell
Der p	<i>Dermatophagoides pteronyssinus</i>
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electromobility shift assay
EU	Endotoxin unit
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcR	Fc receptor
Fel d	<i>Felis domesticus</i>
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Gram
$\gamma_c$	IL-2 receptor common gamma chain
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IDEC	Inflammatory dendritic epithelial cells
IL	Interleukin
IL-7r	Interleukin 7 receptor
IP-10	Interferon-inducible protein 10
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IU	International unit
Jak	Janus kinase
kDa	Kilodalton
LC	Langerhans cells

LPS	Lippopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MCP	monocyte chemoattractant protein
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
moDC	Monocyte-derived dendritic cell
$\mu$ Ci	Microcurie
$\mu$ g	Microgram
mg	Milligram
ml	Milliliter
NF	Nuclear factor
ng	Nanogram
NS	No stimulus (unstimulated)
nTreg	Natural regulatory T cell
OVA	Ovalbumin
OX40L	OX40 ligand
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
pg	Picogram
PHA	Phytohemagglutinin
PI3K	Phosphoinositol-3 kinase
PIP	Phosphoinositol phosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMA	phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
RANTES	Regulated upon activation, normal T cell expressed and secreted
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
sFv	single-chain antibody fragment variable region
SH2	Src-homology 2
siRNA	Small interfering ribonucleic acid
SIT	Specific immunotherapy
SOCS	Suppressor of cytokine signaling
SRTK	Src-related tyrosine kinase
SSC	Side scatter

Stat	Signal transducer and activator of transcription
TARC	Thymus and activation-related chemokine
Tcm	Central memory T cell
Tem	Effector memory T cell
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Tr1	Type 1 regulatory T cell
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopietin
TSLPr	TSLP receptor
VEGF	Vascular endothelial growth factor
VH	Heavy chain variable region
VL	Light chain variable region

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## Chapter I – Introduction

Allergic sensitization is defined by the presence of IgE Ab to normally innocuous substances (i.e. allergens). CD4<sup>+</sup> T cells are pivotal not only in the induction of IgE, but also in the allergic inflammatory response. The main objective of this dissertation is to investigate the immunomodulatory properties of a novel allergen variant, H22-Fel d 1, at the T cell and dendritic cell level. This introductory chapter will begin with a review of some of the different types of allergic diseases, which include atopic dermatitis, asthma, and allergic rhinitis. We will then cover the role of type 2 CD4<sup>+</sup> T cells (Th2) in the initiation and maintenance of the allergic inflammatory response. We will also discuss what constitutes a protective T cell response in relation to allergy as well as some of the current strategies for inducing protective responses in allergic individuals. Finally we will discuss the features of H22-Fel d 1, which was designed to modulate T cell responses, and how an important Th2-promoting cytokine, TSLP, may influence these responses *in vivo*.

### Clinical Manifestations of Allergic Disease

Allergic disorders include atopic dermatitis (eczema), asthma, and allergic rhinitis. The clinical signs of atopic dermatitis generally manifest in early life before the development of asthma and allergic rhinitis. In some individuals there is a sequential clinical progression of different types of allergic disease which has been referred to as the “atopic march”. While atopic dermatitis may improve or dissipate with age, asthma or allergic rhinitis may subsequently develop and

persist into adulthood (1). Several longitudinal studies from around the world have provided evidence for the atopic march (2-8). These studies have demonstrated that early allergic sensitization to foods or aeroallergens is a major risk factor for the development of asthma as an adult (2), and that the severity of atopic dermatitis in childhood correlates with the development of asthma later in life (4). It has been postulated that epicutaneous sensitization to allergens may activate cutaneous antigen presenting cells that then migrate to regional lymph nodes and prime naïve Th2 cells. These activated Th2 cells can then migrate throughout the body and subsequently reach other sites of allergen exposure such as the nasal and lung mucosa where they orchestrate allergic rhinitis and asthma respectively upon allergen encounter at those sites (1). A clearer understanding of the immune mechanisms involved in the generation of allergic T cell responses, and how these responses may be modulated is pivotal to the design of new therapies.

### *Atopic Dermatitis*

Atopic dermatitis is a chronic inflammatory skin disease associated with allergic sensitization to common environmental triggers (9), and is thought to represent a broad-based defect in immune regulation (10). This disease is diagnosed based on clinical criteria which include a characteristic distribution of age-specific itchy skin lesions (1, 8). Atopic dermatitis usually presents in early infancy and childhood, with a worldwide prevalence of 10-20%, but can develop later in life and is present in 1-3% of adults (11). In children under two years of

age, atopic dermatitis usually manifests as itchy, erythematous papules and vesicles on the cheeks, forehead or scalp. In children aged two years to puberty, skin lesions become lichenified owing to hyperkeratosis, and this is a common sign of chronic disease. Some patients present with an “acute-on-chronic” phenotype where new lesions arise on chronically affected skin. Typical areas of skin involvement in older patients include hands, feet, wrists, ankles, and the flexural folds of the elbow and knee. In adults, skin lesions are often dry and scaly, and can include large lichenified plaques, and in severe disease, the whole body may be involved. The distinguishing features of atopic dermatitis versus other allergic diseases are the markedly elevated levels of serum IgE and sensitization to a broad array of allergens (12).

The dermis of acute atopic dermatitis skin lesions is characterized by an infiltration of activated CD4<sup>+</sup> memory T cells that express the skin homing marker cutaneous lymphocyte-associated antigen (CLA), and increased numbers of IL-4-, IL-5, and IL-13-expressing cells compared to normal skin from healthy subjects (11). Antigen presenting cells in acute lesions, which include Langerhans cells (LC) and inflammatory dendritic epithelial cells (IDEC), have surface bound IgE. This facilitates capture of allergens and antigen presentation either in the skin or in regional nodes (13, 14). Chronic atopic dermatitis lesions are characterized by tissue remodeling, which results from a chronic inflammatory process. These lesions contain increased numbers of IgE<sup>+</sup> LCs and IDECs as well as eosinophils, and reduced numbers of T cells compared to acute lesions. Interestingly, chronic lesions have diminished IL-4- and IL-13-expressing cells,

but enhanced levels of IL-5-, GM-CSF-, IL-12-, and IFN- $\gamma$ -expressing cells, which is consistent with a role for Th1 cells in chronic disease (15). In both acute and chronic lesions, skin barrier function is disrupted, and this has been proposed to allow entry of allergens and microbial products that can trigger or exacerbate the allergic response.

### *Asthma*

Asthma is a highly heterogeneous disease in which reversible airway obstruction is a pathognomonic feature. Manifestations of this disease range from a mild wheeze and breathlessness to a chronic corticosteroid-dependent phenotype that includes profound airway narrowing (16). Several cell types contribute to inflammatory responses in the asthmatic lung. These include CD4<sup>+</sup> T cells, eosinophils, neutrophils, and mast cells. Inflammation is generally restricted to the conducting airways, but can spread to the small airways and adjacent alveoli as the disease becomes more chronic and severe (17, 18). Clinical diagnosis of asthma is based on the patient's medical history, physical exam, assessment of airway obstruction reversibility and exclusion of other diseases which may mimic the asthma phenotype (19). In many patients asthma begins in childhood, and is the third leading cause of hospitalization for people under 18 in the United States (19). Asthma also develops during puberty or into adulthood, although many cases of adult onset asthma are due to a reappearance of symptoms in patients who were transiently symptom-free during adolescence.

In patients with allergic asthma, inhalation of common allergens leads to bronchial constriction and increased mucus secretion that impairs breathing (airway hyperresponsiveness (AHR)). Over time, these responses can lead to chronic disease and airway remodeling. This remodeling is characterized by airway smooth muscle thickening and development of fibrosis. It has been reported that in adult asthma the lung epithelium is chronically injured and cannot repair itself properly (20, 21). This results in reduced barrier function in the asthmatic lung, similar to what is seen in the skin of patients with atopic dermatitis. As a result, inhaled allergens, pollutants, and microbes can penetrate airway tissue (17). A dense network of subepithelial dendritic cells lines the respiratory tract. These cells are thought to be pivotal to priming T cell responses to allergens locally or in regional nodes.

### *Allergic Rhinitis*

Allergic rhinitis is characterized by inflammation of the nasal mucosa associated with itching, sneezing and mucus secretion occurring upon exposure to allergen. Clinical diagnosis is based on the patient's medical history, positive immediate hypersensitivity skin test reactions to allergens and the presence of serum allergen-specific IgE ab, as well as anatomical assessments of the nasal cavity (22). Associated inflammatory cells include CD4<sup>+</sup> T cells, eosinophils, mast cells and basophils. Allergic rhinitis is considered to be the most prevalent chronic respiratory disease, and as such, it is a worldwide public health issue (22). Depending on the frequency and intensity of symptoms, this disease can

be classified as either intermittent or persistent and as either mild or moderate/severe.

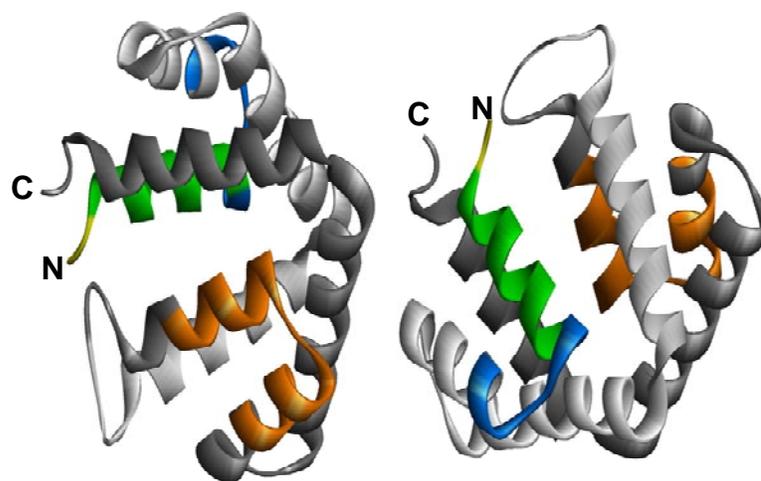
Allergic rhinitis is a significant risk factor for the development of asthma (23, 24) and there is a close epidemiological relationship between allergic rhinitis and asthma. It has been suggested that the upper (nasal) and lower (lung) respiratory airways are a single anatomical and functional unit (25). As such, the nose functions as a barrier which filters and conditions air traveling to the lungs. When this barrier function is disrupted by allergic rhinitis, allergens not only have increased access to the nasal mucosa, but also to the lung (26). This is in keeping with the feature of impaired barrier function leading to allergic pathology in atopic dermatitis and asthma.

### **Fel d 1: The Major Allergen From Cat**

This thesis focuses on the immunomodulatory properties of a molecule, which incorporates the major cat allergen Fel d 1, developed with a view to treating cat-allergic subjects. The domestic cat (*Felis domesticus*) is a common household pet as well as an important source of indoor allergens. The four known cat allergens include Fel d 1, Fel d 2 (cat albumin), Fel d 3 (cat cystatin), and Fel d 4 (27-30). Of these, Fel d 1 is the major allergen, and it accounts for up to 95% of the cat-specific IgE found in cat-allergic subjects (27). Fel d 1 is a 35kDa tetrameric glycoprotein composed of two heterodimer subunits (Figure 1.1)(31). Each heterodimer consists of two polypeptide chains which are

**Figure 1.1 Three-Dimensional Structure of Fel d 1 Indicating the Location of IL-10- and IFN- $\gamma$ -Inducing Epitopes.**

Polypeptide chains 1 and 2 are shown in dark and light gray, respectively. The immunodominant region from chain 1 (P1:2) is shown in orange, and the chain 2 epitopes P2:1 and P2:2 are shown in yellow and blue, respectively. Overlapping residues from P2:1 and P2:2 are shown in green. Figure provided courtesy of A. Pomes.



encoded by separate genes. Three disulfide bonds are formed between these two chains and are required for their linkage.

While the role of Fel d 1 in the cat is unknown, its structural similarities to proteins in the secretoglobin superfamily may provide insight to its function (32). Uteroglobin is one member of this family and is a steroid-inducible cytokine-like molecule that has potent anti-inflammatory and immunomodulatory properties (32-35). The protein Clara Cell 16 (CC16), another member of this family, is found in human lungs, and provides a protective effect against pathogens while also playing a role in controlling inflammation in this organ (32-34, 36, 37).

Development of IgE antibodies to Fel d 1 results in symptoms ranging from mild rhinitis to severe asthmatic reactions. It is estimated that the prevalence of IgE-mediated sensitization to cat in the United States is as high as 17% (38). This high prevalence of sensitization coupled with the continuing increase in the prevalence of other allergic diseases has led to a significant burden on the health care systems of many nations.

Understanding the nature of T cell responses to Fel d 1 is important for developing better therapeutic strategies. Unfortunately, Fel d 1 is a weak T cell stimulator *in vitro* and this has made evaluation of T cell responses to this allergen difficult in human-based systems. The next sections outline the role that T cells play in the allergic response and review our existing knowledge on t cell responses to Fel d 1.

## The Role of Th2 Cells in Allergic Inflammation

Chronic inflammation is a cardinal feature of allergic diseases. Allergen-specific type 2 CD4<sup>+</sup> T lymphocytes (Th2 cells) are central to the initiation and maintenance of the allergic inflammatory response through the cytokines they secrete (IL-4, IL-5, and IL-13)(39). IL-4 mediates antibody isotype switching to IgE. IgE binds the high affinity receptor, FcεRI, on the surface of mast cells, basophils, and eosinophils. Allergen-induced cross-linking of surface IgE on these cells leads to the release of inflammatory mediators including histamine and leukotrienes that perpetuate the allergic cycle. These mediators cause smooth muscle contraction and mucus production in the respiratory tract, or a wheal and flare reaction in the skin. The Th2-derived cytokines IL-5 and IL-13 are pivotal to other pathognomonic features of allergic disease such as eosinophil activation and migration as well as AHR. In addition, IL-4 and IL-13 are autocrine factors that stimulate Th2 differentiation and thus, amplify the allergic inflammatory response.

The pivotal role of Th2 cells in allergic disease has been established using murine models. For example, it has been shown that IL-4 knockout mice are resistant to allergen sensitization and do not develop eosinophilic lung infiltrates after subsequent allergen challenges (40). Similarly, IL-5 knockout mice show reduced eosinophilic lung infiltrates in response to allergen challenge and do not develop AHR (41). Moreover, several groups have demonstrated that passive transfer of Th2 cells from allergen-sensitized mice confers allergen-specific AHR in non-sensitized recipients (42-46). However, understanding the role of T cells

in allergic disease in humans is challenging. Due to practical considerations, it is difficult to study the evolution of Th2 responses in early life. Established allergic responses are associated with a “mixed” T cell infiltrate that includes Th1, Th2, Th0 and regulatory T cells (Treg), and represent only a snapshot in time of the progression of disease. In addition, it is not known why allergens preferentially promote Th2 responses in predisposed individuals.

Little is known about how Th2 responses develop in early life. Some have argued that transplacental priming to environmental allergens occurs in both low-risk and high-risk infants, as evidenced by induction of proliferation in cord blood mononuclear cells derived from both groups after allergen challenge (47, 48). Furthermore, several epidemiologic studies have demonstrated that production of allergen-specific IgE antibodies begins as early as the first two years of life, but the immunological mechanisms responsible for this are unclear (39). It has been suggested that there is a universal propensity for Th2 skewing in neonates independent of allergen exposure arising from production of Th2 cytokines by the placenta, which protects the allogeneic fetus from the toxic effects of IFN- $\gamma$  and maternal Th1-mediated immunity (47). It has been demonstrated that PBMC from low-risk infants produced enhanced Th2 cytokine responses to allergen shortly after birth, but these responses declined with age (48). In contrast to this, infants at high-risk for developing atopy had decreased cytokine responses (both Th1 and Th2) to allergens and a non-specific T cell stimulus (PHA) shortly after birth, but their Th2 cytokine responses increased over time (as early as one year after birth). This may reflect defective control of Th2 responses in atopic children

during a period when they are exposed to new allergens, although the genetic and/or environmental factors that provide the basis for their “predisposition” to atopy are still unclear.

The development of IgE-mediated sensitization and allergic disease in children across a wide age range suggests that memory Th2 responses continue to evolve over a prolonged time (6, 49, 50). While it may be difficult to intervene with the initiation of Th2 skewing events that occur *in utero* or shortly after birth before the manifestation of allergic disease, strategies that modulate established Th2 responses in allergic individuals are a rational approach to treatment. In support of this, it is known that an established Th2 response is not a fixed state, but can be altered by therapeutic (immunotherapy) or environmental (change in allergen exposure) interventions (39).

### **What Constitutes a Protective T Cell Response?**

This thesis focuses on the immunomodulatory properties of a molecule (H22-Fel d 1) developed with a view to treating cat-allergic subjects. In order to assess whether H22-Fel d 1 has therapeutic potential in cat-allergic subjects, it is important to consider what constitutes a protective T cell cytokine repertoire. Classical T cell paradigms hypothesize that Th1 and Th2 cells counter balance each other through the mutually inhibitory effects of the cytokines they secrete (i.e. IFN- $\gamma$  and IL-4 respectively). In the 1990's it was thought that treatments that induced Th1-skewing would promote the development of non-allergic responses. The recent consideration of Tregs has shifted this viewpoint. It is

now generally accepted that induction of IL-10, which had originally been considered a Th2 cytokine, may suppress Th2 responses to allergens and thus be a more appropriate objective of therapy. Despite all that we have learned about T-cell responses to allergens, including the role of Tregs, what defines a protective T-cell response to allergen still remains unclear.

The current T cell paradigm suggests that Tregs suppress Th2 responses to allergen (39). However, there are conflicting data on the nature of the Tregs which suppress allergic inflammation. Much of the work on Tregs within the immunology literature comes from murine models of disease. Many of these studies have focused on what have been defined as natural Tregs (nTregs). These cells are characterized by high level expression of the IL-2 receptor  $\alpha$  chain, CD25, and the transcription factor Foxp3 (51). Natural Tregs develop in the thymus, and exert their regulatory effects in a cell contact-dependent manner. Another Treg population which also expresses Foxp3 and high levels of CD25 has been described. Unlike nTregs, these cells, termed adaptive Tregs (aTregs), are induced in the periphery and generally suppress through the action of secreted IL-10 or TGF- $\beta$  (51). The T regulatory 1 cell (Tr1) is another subset of Tregs which is induced in the periphery and acts through IL-10, but does not express Foxp3. However, differences in the properties of murine and human Tregs make it difficult to accurately define a role for different Treg subsets in allergic disease. While Foxp3 expression appears to be a specific marker for Tregs in mice, this transcription factor is expressed in both nTregs and in activated effector T cells in humans (51). CD25 expression is also upregulated

on activated effector T cells. Thus, Treg populations isolated from humans based on CD25 and Foxp3 expression may be contaminated with effector populations. In addition, because some subsets of Tregs, such as the Tr1 cells, do not have defined distinct surface phenotypes, additional markers of regulatory function are needed to isolate these populations for analysis. Recently, it has been demonstrated that expression of the IL-7 receptor  $\alpha$  chain, CD127, is inversely correlated with both Foxp3 and *in vitro* suppressive function of human CD4<sup>+</sup> Tregs (52-54). This finding could provide us with a more effective strategy for the isolation and functional analysis of purified Treg populations that may be induced by H22-Fel d 1.

#### *Evidence of "Tolerance" to Inhaled Allergens*

In 2001, Platts-Mills et al. reported a novel type of immune response (modified Th2 response) in children living with a cat who were exposed to high levels of the major cat allergen Fel d 1 (>4.4  $\mu$ g Fel d 1/g of dust) (55). This response, which was characterized by the presence of anti-Fel d 1 IgG (including increased IgG<sub>4</sub>) antibodies in the serum without IgE and without allergic symptoms, was proposed to represent a form of high dose respiratory tolerance. This was a significant finding since the view at the time was that increasing levels of allergen exposure were associated with increasing prevalence of allergic sensitization and asthma (50). The modified Th2 response to Fel d 1 indicated that for some allergens, this linear dose-response relationship did not hold true at high levels of allergen exposure. Since that time, several studies have confirmed

these observations suggesting that Fel d 1 has unique tolerogenic properties (56-59).

Subsequent T-cell studies performed in subjects with a modified Th2 response suggested a role for enhanced recognition of an immunodominant region within polypeptide chain 2 of Fel d 1 in the development of tolerance. This region contained T-cell epitopes that preferentially induced IL-10 and IFN- $\gamma$  (Figure 1.1) (60). T cells from subjects with a modified Th2 response who were HLA-DR7<sup>+</sup> secreted higher levels of IL-10 in response to peptides spanning this region compared with both cat-allergic subjects and non-allergic controls (60). Moreover, T cells from patients receiving conventional immunotherapy using cat extract showed marked increases in IL-10 and IFN- $\gamma$  secretion after *in vitro* stimulation with Fel d 1 chain 2 peptides within four weeks of starting treatment (60). Collectively, these findings supported an association between IL-10 and IFN- $\gamma$  and protective responses to Fel d 1.

#### *Immunomodulatory Effects of Immunotherapy*

Allergen-specific immunotherapy (SIT) is a common therapeutic option for allergic patients. This treatment involves repeated subcutaneous injections, or else use sublingual application of extract containing allergens to which the patient is sensitized. Though extract is administered in an updosing regimen to minimize adverse effects, there are drawbacks. These include: a risk of IgE-mediated adverse events; prolonged treatment regimens that require considerable patient compliance; and lack of standardization of allergen extracts

that make it difficult to discern how much of the requisite immunogen is administered with each injection. Yet, in some settings, SIT has proven clinically efficacious for the treatment of allergic disease (61), and has been shown to improve quality of life by reducing symptoms and the need for medication (62). There is mounting evidence to suggest that the long term protective effects of immunotherapy are exerted at the T-cell level through induction of Tregs, which secrete IL-10 and/or Th1 cells, which secrete IFN- $\gamma$  (39, 63-65).

Multiple studies have reported that SIT modifies responses of several effector cell types involved in allergic inflammation, including Th2 cells (66-69). It is known that CD4<sup>+</sup> T cells from both allergic and healthy donors recognize the same allergen-specific T-cell epitopes (70-72). It has been suggested that it is the balance between allergen-induced IL-10-, IL-4-, and IFN- $\gamma$ -secreting T cells that determines the development of a healthy versus allergic immune response (73). Several studies in humans that analyzed T cell responses directly *ex vivo* after SIT treatment suggest that SIT influences this balance by augmenting both allergen-specific Tr1-like and CD4<sup>+</sup>CD25<sup>+</sup> nTreg subsets (74-76). These regulatory T cells can then modify Th2 cell responses to allergen by inducing anergy (77) or suppressing effector responses (76, 78), and enhancing production of regulatory cytokines (IL-10 and TGF- $\beta$ ) (63, 73, 74, 78, 79).

#### *Current Approaches to T Cell Modulation*

Due to adverse side effects involved with SIT, considerable effort has been devoted to the development of allergen-specific dendritic cell-based or T-

cell-based vaccines, which may confer long-term protection without IgE-mediated side effects. Allergen variants and allergen-derived peptides have been generated that lack conformational epitopes but retain the T-cell antigenic determinants necessary to activate T cells (80-85). In relation to peptide vaccines, peptides derived from allergens have been designed to retain linear T cell epitopes, without conformational B cell epitopes. Because of this, these peptides have a reduced capacity to cross-link allergen-specific IgE molecules, but can still stimulate allergen-specific T cells and induce protective responses (86). Initial T-cell epitope mapping studies of Fel d 1 revealed an immunodominant region of the molecule within polypeptide chain 1 (87). These studies led to the development of a peptide vaccine composed of two overlapping peptides (27 amino acids each) which spanned this region (88). While some improvement in lung and nasal symptom scores was seen in patients who received the highest doses of the vaccine, numerous adverse events (mainly late asthmatic reactions) were also reported, which may have been due to retained B cell epitopes within the peptides based on their large size (86, 88). A similar study which used a mixture of shorter peptides from chain 1 (~14-17 amino acids) also induced late asthmatic reactions which appeared to be T-cell-dependent but IgE-independent (89). Despite this, decreased production of IL-4 or IL-5 and increased IL-10 secretion, along with decreased proliferation in allergen- and peptide-stimulated PBMC cultures, have been reported after peptide immunotherapy (76, 85, 90-92). These data indicate there may be a clinical benefit associated with this approach.

In work performed in Europe, allergen variants have been developed which exhibit reduced IgE binding, while retaining critical T-cell epitopes. Clinical trials using modified derivatives of the major birch pollen allergen, Bet v 1 (two recombinant Bet v 1 fragments and a Bet v 1 trimer), were performed by Valenta and colleagues (80). Treatment induced Bet v 1-specific IgG responses which could bind wild-type allergen and prevent its binding to IgE and induction of allergic symptoms (80, 93). In addition, treated patients noted an improvement of symptoms, and seasonally-induced Bet v 1-specific IgE was inhibited in these patients (93). While more trials are needed to confirm these results, it appears that immunotherapy with modified allergens holds promise as an alternate therapeutic option which may minimize the adverse side effects of conventional immunotherapy.

### **Features of a Novel Allergen Variant Targeted to Dendritic Cells**

Allergen-specific CD4<sup>+</sup> T cells recognize allergen-derived peptides (epitopes) presented in the context of major histocompatibility complex (MHC) class II molecules by APCs. As such, dendritic cells play a pivotal role in the initiation and maintenance of T-cell responses to allergens, both in health and in disease. Specifically, the type of T-cell response generated can be influenced by the maturational state of dendritic cells. Engagement of a variety of surface receptors on dendritic cells can induce maturation, which is characterized by increased expression of an array of surface markers which are costimulatory for T cells (CD40, CD80, CD86, OX40L), as well as secretion of proinflammatory

cytokines. Whereas mature dendritic cells generally induce effector T-cell responses (i.e. Th1 or Th2), immature or semi-mature dendritic cells are tolerogenic based on their ability to induce Tregs (94, 95). However, the situation is likely more complex as evidenced by recent reports that both mature and immature dendritic cells can induce Tregs (96).

Work performed in this thesis focuses on a variant of the major cat allergen Fel d 1 that is targeted to the high affinity receptor for IgG, Fc $\gamma$ RI (CD64), which is expressed on dendritic cells. As demonstrated by the effects of high dose exposure to Fel d 1 and the development of a modified Th2 response, the nature of the T-cell response generated in response to any antigen is highly dependent on the dose of antigen administered. With this in mind, approaches that increase antigen delivery to the APC have the potential not only to enhance immunogenicity, but also to alter the quality of the T-cell response.

Dendritic cells express an array of Fc receptors that bind IgG. These include Fc $\gamma$ RI, Fc $\gamma$ RII and Fc $\gamma$ RIII. Whereas Fc $\gamma$ RI and Fc $\gamma$ RIII constitute activating receptors that contain ITAMs, Fc $\gamma$ RII exists in activating (Fc $\gamma$ RIIa) and inhibitory (Fc $\gamma$ RIIb) forms. However, because this thesis focuses on the effects of targeting allergen to Fc $\gamma$ RI, the next section will focus on the features of this particular receptor.

### *Targeting Antigens to Fc $\gamma$ RI*

The high affinity IgG receptor, Fc $\gamma$ RI (CD64), is found exclusively on cells of the myeloid lineage. This receptor comprises an IgG-binding  $\alpha$ -chain

complexed with an ITAM-containing  $\gamma$ -chain dimer. The ITAM within the  $\gamma$ -chain is critical to an array of Fc $\gamma$ RI-mediated effector functions, including phagocytosis, cytokine production and antibody-dependent cell-mediated cytotoxicity. In addition to these functions, Fc $\gamma$ RI also facilitates antigen presentation. However, though integrity of the  $\gamma$ -chain ITAM appears to be important for antigen presentation mediated by other Fc activating receptors, this may not be the case for Fc $\gamma$ RI. Specifically, Fc $\gamma$ RI has been reported to promote antigen presentation independently of a functional  $\gamma$ -chain ITAM (97). On the other hand, the  $\alpha$ -chain appears to be critical not only for antigen uptake, but also for antigen presentation through targeting of receptor-ligand complexes to the appropriate antigen processing compartments within the APC (97).

In the early 1990s, the effects of targeting antigen to Fc $\gamma$ RI were studied by conjugating antigens to the anti-human Fc $\gamma$ RI monoclonal antibody (mAb) 22.2. This mAb had been shown to bind to Fc $\gamma$ RI via its Fab region outside the Fc binding domain, making it feasible to target antigens to the receptor despite occupancy by its normal ligand, IgG. Targeting antigens to human Fc $\gamma$ RI using mAb 22 was shown to enhance T-cell responses *in vitro* in human-based systems and augment humoral responses *in vivo* in a transgenic mouse model (98, 99). Collectively, these findings pointed to enhanced immunogenicity mediated by Fc $\gamma$ RI targeting using mAb 22. A humanized version of anti-CD64 mAb 22 (H22) was developed, stimulated receptor internalization, an important first step in antigen processing (Figure 1.2) (100). This antibody cross-links

Fc $\gamma$ RI through binding by its Fc end as well as its Fab ends (101).

Interestingly, targeting antigenic peptides using just the monovalent form of H22 (Fab) enhanced both antigen specific CD4<sup>+</sup> T cell proliferation and cytokine production *in vitro* (102). Using a similar approach to target prostate specific antigen to Fc $\gamma$ RI in the human monocytic cell line THP-1 led to enhanced killing of those cells by antigen-specific cytotoxic T lymphocytes (103). Thus, in addition to facilitating presentation of antigen on MHC class II molecules to CD4<sup>+</sup> T cells, exogenous antigens targeted to Fc $\gamma$ RI using H22 can also be presented to CD8<sup>+</sup> T cells in the context of MHC class I. Moreover, a monovalent form of H22 is sufficient to mediate these effects.

#### *The Novel Allergen Variant H22-Fel d 1*

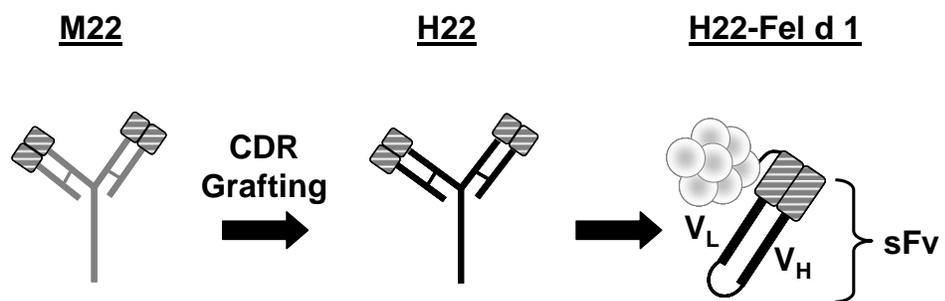
In 2002, Vailes et al. (104) developed a recombinant fusion protein, which linked Fel d 1 to a single-chain antibody fragment variable region (sFv) of H22 (H22-Fel d 1) (Figure 1.2). The sFv version of H22 (sFv22) binds monovalently and thus, does not crosslink Fc $\gamma$ RI, but nevertheless leads to receptor internalization. Interestingly, this effect requires IgG suggesting that occupancy of the ligand binding domain of Fc $\gamma$ RI is necessary for receptor uptake mediated by sFv22 (105). Subsequently, H22-Fel d 1 was shown to bind to Fc $\gamma$ RI on monocytes. Moreover, H22-Fel d 1 retained the ability to bind Fel d 1-specific

#### **Figure 1.2 Schematic of H22-Fel d 1.**

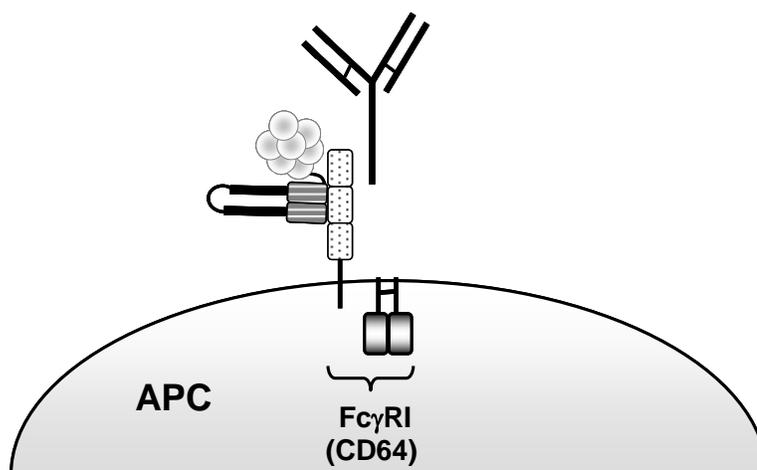
**A.** The humanized anti-CD64 monoclonal antibody, H22, was generated by complementarity-determining region (CDR) grafting from the mouse

monoclonal antibody (mAb) M22 (100). The humanized mAb contains 5-10% of the original mouse sequence. The sFv of H22 was linked to Fel d 1 by cloning and H22-Fel d 1 was produced in *Baculovirus* and *Pichia pastoris* expression systems (104, 106). The sFv comprises the variable regions of heavy (VH) and light (VL) chains of H22 produced by joining VH and VL DNAs together with a linker to obtain a single DNA fragment for cloning and expression. **B.** H22-Fel d 1 binds to Fc $\gamma$ RI outside the Fc binding region of the receptor.

A



B



IgE antibody, suggesting that fusion of Fel d 1 to sFv22 did not influence allergen folding (104). This also raises the possibility that H22-Fel d 1 could cross-link IgE and IgG receptors, and play an important role in altering the signaling cascades induced in cells which express both types of these receptors. H22-Fel d 1 provides us with a novel molecular tool for studying the effects of targeting allergen to Fc $\gamma$ RI at the level of both the dendritic cell and the T cell in cat-allergic subjects. In addition to exploring its immunomodulatory properties, this thesis describes Fc $\gamma$ RI-mediated events triggered by H22-Fel d 1 in dendritic cells. Prior to this work, the bulk of the work investigating the signaling events triggered by Fc $\gamma$ RI has been performed in cells other than dendritic cells; these include primary human monocytes or macrophages, and an array of human and murine cell lines. Because of this, little is known about the mechanisms involved in Fc $\gamma$ RI signaling in dendritic cells.

### **Thymic Stromal Lymphopoietin – A Potential Immunomodulator In Vivo?**

Allergen variants that target dendritic cells *in vitro* do not act in isolation. Since dendritic cells are subject to the cytokine milieu *in vivo*, the nature of the T cell response induced by allergen variants may differ substantially from that observed *in vitro*. However, these microenvironmental effects, which are highly clinically relevant, are rarely addressed in *in vitro* culture systems. The cytokine thymic stromal lymphopoietin (TSLP) is expressed at high levels in the skin of patients with atopic dermatitis and in the asthmatic lung. This cytokine is a

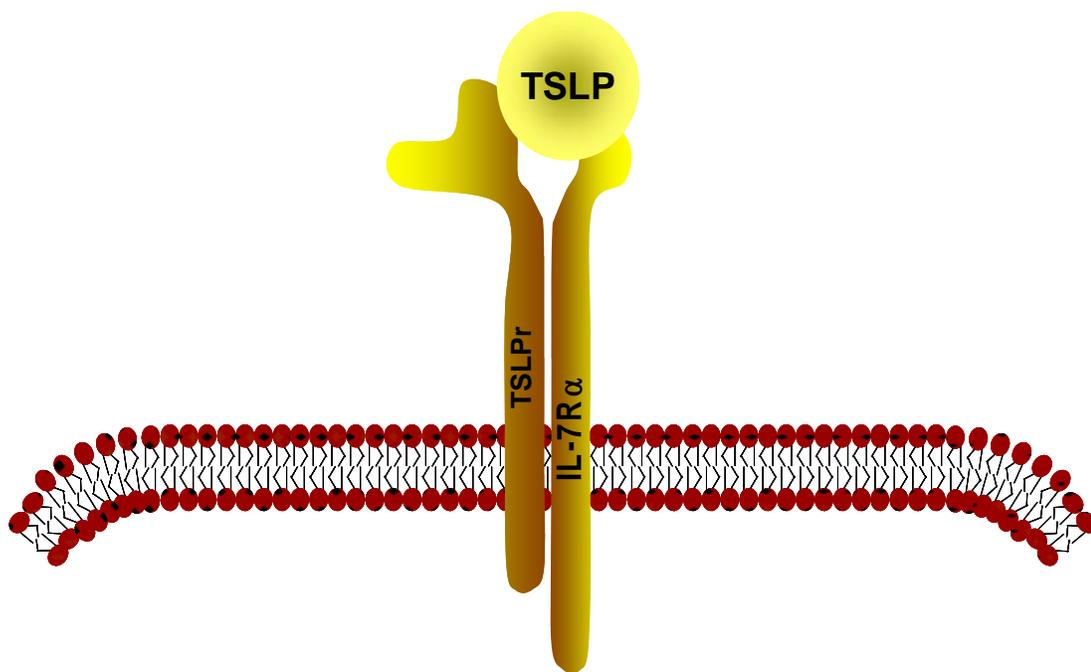
strong candidate for altering T cell responses induced by H22-Fel d 1 based on its Th2-polarizing properties and anatomical localization.

TSLP was originally characterized from the supernatant of a mouse thymic stromal cell line and subsequently identified as a four-helix bundle cytokine (107, 108). Human TSLP was discovered using database search methods, and had low homology (43%) to murine TSLP (109, 110). The high affinity receptor for TSLP is a heterodimer consisting of the IL7-R $\alpha$  and TSLP receptor (TSLPr) (Figure 1.3). Binding of TSLP to this high affinity receptor on T cells and dendritic cells results in Stat5 activation and cellular proliferation (109, 111, 112). TSLP is expressed by epithelial cells within the thymic medulla and mucosal-associated lymphoid tissues, as well as by keratinocytes of skin lesions from patients with atopic dermatitis and airway epithelial cells from asthmatic patients (109). This suggests that TSLP plays an important role both in T cell homeostasis and allergic inflammation, depending on where it is expressed (113).

Evidence that TSLP is involved in the generation of Th2 responses in mice has come from knock-out or overexpression models. Studies have demonstrated that TSLPr-deficient mice have attenuated Th2 responses, but normal Th1 responses (114). Moreover, mice with increased systemic TSLP expression have an increased number of IL-4-producing cells in addition to elevated levels of circulating IgE (115, 116). These mice also spontaneously develop a condition similar to atopic dermatitis. It has also been proven that TSLP is both necessary and sufficient to induce airway inflammation in mice (117). In models of allergen-

**Figure 1.3 Schematic of the High Affinity TSLP Receptor.**

The TSLP receptor (TSLPr) is a heterodimer composed of the IL-7R alpha chain and the TSLP receptor chain. The TSLP receptor chain is homologous to the common gamma chain. TSLPr is expressed on antigen presenting cells and a subset of activated Th2 cells in humans.



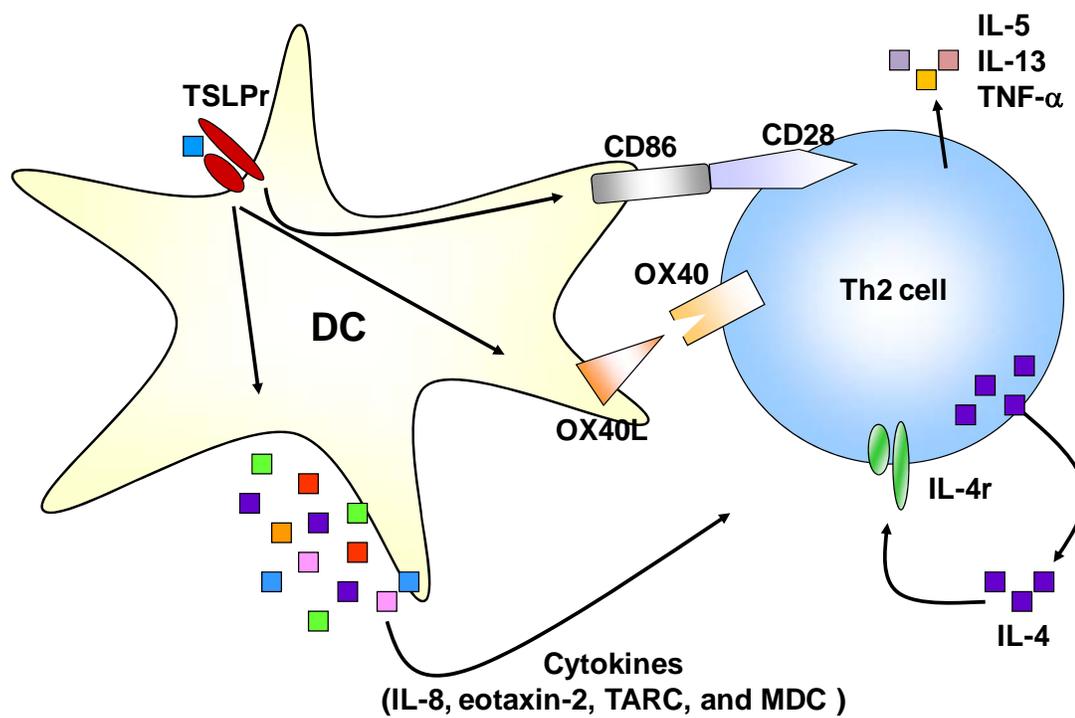
induced lung inflammation, TSLP<sup>r</sup> knock-out mice have significantly reduced disease after antigen challenge (114, 116). Conversely, increased TSLP expression in the lungs results in an airway-specific inflammatory disease which mimics human asthma (116). These murine models have provided important evidence that TSLP can induce inflammatory diseases with features similar to those found in human conditions, and may provide relevant models for studying these diseases.

Recent work has demonstrated that TSLP strongly enhances expression of MHC class II and co-stimulatory molecules on human dendritic cells (Figure 1.4). Unlike other conventional DC maturation stimuli, such as TLR ligands, TSLP-pulsed DCs do not express the Th1-polarizing cytokine IL-12. Instead, these DCs express the Th2-associated chemokines CXCL8 (IL-8), CCL24 (eotaxin-2), CCL17 (TARC), and CCL22 (MDC) (118). Furthermore, stimulation of naïve allogeneic CD4<sup>+</sup> T cells by TSLP-pulsed DCs induces a unique Th2 cell that produces IL-4, IL-5, and IL-13 as well as TNF- $\alpha$ , but not the regulatory cytokine IL-10, which is referred to as an inflammatory Th2 cell (118). Subsequently, it was shown that Th2 responses induced by TSLP-pulsed DCs occurred via an OX40L-dependent mechanism (119, 120). Since TSLP is expressed at the host/environment interface, it has been suggested that TSLP represents a “master allergic switch” *in vivo*. However, whether TSLP expression is triggered by environmental factors, including allergen, remains unclear.

In addition to its capacity to induce Th2 cells from naïve precursors, TSLP has been reported to play a role in the induction and maintenance of allergen-

**Figure 1.4 Effects of TSLP on Human Dendritic Cells.**

TSLP strongly enhances expression of MHC class II and co-stimulatory molecules on human dendritic cells. TSLP-pulsed DCs do not express the Th1-polarizing cytokine IL-12. Instead, these DCs express the Th2-associated chemokines IL-8, eotaxin-2, TARC, and MDC. TSLP-pulsed DCs induce a unique Th2 cell that produces IL-4, IL-5, and IL-13 as well as TNF- $\alpha$ , but no IL-10, which is referred to as an inflammatory Th2 cell.



specific memory Th2 cells. In humans, memory CD4<sup>+</sup> T cells can be divided into two distinct subsets based on their surface phenotype, homing capacity and effector functions. Central memory cells (T<sub>cm</sub>) are CD45RO<sup>+</sup>CCR7<sup>+</sup>CD62L<sup>+</sup>, can traffic to draining lymph nodes and undergo homeostatic proliferation. Effector memory cells (T<sub>em</sub>) are CD45RO<sup>+</sup>CCR7<sup>-</sup>CD62L<sup>+/-</sup>, are found in peripheral sites, and can rapidly gain effector function (121-123). These subsets can be further divided based on their propensity to produce Th1- or Th2-associated cytokines, and differential expression of homing receptors (124-126). Recently, Liu and colleagues demonstrated that TSLP-pulsed dendritic cells maintain a distinct subset of T<sub>cm</sub> which express the Th2-associated marker chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (124). In addition, they showed that this maintenance was dependent on OX40/OX40L interactions at the T cell/dendritic cell interface. Importantly, CRTH2<sup>+</sup> T cells were localized to skin lesions of patients with atopic dermatitis, but not lesions from patients with psoriasis (a Th1-associated disease) or non-lesional skin from atopic dermatitis patients (124). It was further demonstrated that TSLP could enhance the Th2 properties of CRTH2<sup>+</sup> T cells by leading to the upregulation of IL-17RB, the receptor for IL-25 (IL-17E) (127).

With these properties in mind, a major objective of this thesis was to investigate the capacity for TSLP to modulate T cell responses induced by H22-Fel d 1. An important aspect of our studies was to examine whether TSLP exerted a differential effect depending on allergic status. This is pertinent, since previous work that reported the Th2-promoting properties of TSLP using human-

based *in vitro* systems did not take into account the allergic status of donors used for isolating dendritic cells (119). Our findings provide evidence of a differential role for TSLP depending on allergic status and establish a link between molecular events triggered by H22-Fel d 1 and Th2 responses mediated by the TSLP pathway in human dendritic cells.

## **Goals of the Dissertation**

### **Aim 1: Analysis of the Immunomodulatory Effects of H22-Fel d 1.**

***Hypothesis: H22-Fel d 1 induces a T cell cytokine repertoire with protective features.***

A major aim of allergen immunotherapy is to induce allergen-specific protective responses at the T cell level. Exposure to high dose cat allergen (Fel d 1), either through natural exposure in the environment or through conventional immunotherapy, is associated with development of protective T cell responses. We theorized that enhancing delivery of cat allergen to the antigen presenting cell by targeting to Fc $\gamma$ RI could skew towards a protective T cell response, possibly by mimicking high dose natural allergen exposure. There is evidence that targeting antigens by this approach enhances antigen delivery to the antigen presenting cell and augments T cell responses. However, nothing is known about the immunomodulatory effects of targeting allergens to Fc $\gamma$ RI. Many of the previous *in vitro* studies that have investigated the T cell modulatory effects of conventional immunotherapy, or else of allergen variants or peptide vaccines, used bulk culture systems that do not yield information regarding qualitative

changes that occur at the single cell level. The first goal of this dissertation is to examine the immunomodulatory effects of targeting cat allergen to Fc $\gamma$ RI on dendritic cells, by analyzing the T cell cytokine repertoire at the single cell level. Initial studies will focus on investigating whether H22-Fel d 1 induces a protective phenotype in moDCs from cat-allergic subjects as judged by cytokine production and expression of surface co-stimulatory molecules. In order to investigate the effects of H22-Fel d 1 at the T cell level, co-cultures of H22-Fel d 1-stimulated moDCs and CD4<sup>+</sup> T cells will be established. Intracellular cytokine staining with flow cytometry analysis will then be used to determine whether H22-Fel d 1 induces qualitative changes in the T cell cytokine repertoire which incorporate elements of a protective response. Cell cultures will also be analyzed from non-allergic subjects (modified Th2 responders and serum antibody negative controls) in order to determine whether there is a differential effect of H22-Fel d 1 on T cell responses based on allergic status. The T cell cytokine repertoire induced by H22-Fel d 1 will also be compared to the repertoire induced by non-receptor targeted allergens, including Fel d 1 and the prototypic dust mite allergen Der p 1.

**Aim 2: Analysis of the Effects of TSLP on T Cell Responses Induced by**

**H22-Fel d 1.**

***Hypothesis: H22-Fel d 1 and TSLP coordinate to enhance Th2 responses in atopic subjects.***

In the *in vivo* setting, dendritic cells targeted by H22-Fel d 1 will be subject to the influence of the cytokine milieu. Thus, the nature of the T cell response induced by H22-Fel d 1 *in vivo* may differ substantially from that observed *ex vivo*. Such microenvironmental effects, which are clinically relevant, are rarely addressed in *in vitro* culture systems. We postulate that the clinical efficacy of H22-Fel d 1 will depend on its capacity to induce a protective T cell cytokine repertoire in a “pro-allergic” cytokine milieu. The Th2-promoting cytokine, TSLP, acts directly on dendritic cells and is pivotal to the induction of Th2 responses. This cytokine is expressed at sites of allergic inflammation, including sites commonly used to administer immunotherapy (eg. skin). Thus, in allergic subjects, exposure of dendritic cells to TSLP could mitigate the protective T cell elements induced by H22-Fel d 1. With this in mind, we will test whether H22-Fel d 1 and TSLP coordinate to enhance Th2 responses in cat-allergic subjects. An important aspect of these studies will be to examine whether there are differential effects of TSLP depending on atopic status, since this is not known. Initially, we will examine whether TSLP alone induces Th2 responses in atopic and non-atopic subjects. Next, the T cell cytokine repertoire induced by H22-Fel d 1 in the presence of TSLP will be compared to that induced by H22-Fel d 1 or TSLP alone. Secreted cytokines will be measured to confirm intracellular cytokine patterns observed by flow cytometry.

**Aim 3: Elucidation of the Molecular Events Induced by H22-Fel d 1 That Promote the TSLP Pathway.**

***Hypothesis: Targeting Fel d 1 to Fc $\gamma$ RI on dendritic cells triggers intracellular signaling events which enhance the TSLP pathway.***

The final aim of this dissertation is to elucidate the molecular mechanisms which mediate amplification of Th2 responses in atopic dendritic cells co-stimulated with H22-Fel d 1 and TSLP. Studies are designed to identify differences in atopic and non-atopic dendritic cells that could account for Th2 amplification. Little is known about the TSLP signaling pathway in human dendritic cells. However, because Th2 responses induced by TSLP were previously reported to be OX40L-dependent, we will first investigate whether this pathway is required for Th2 amplification. To do this, we will examine whether surface expression of OX40L is increased on dendritic cells by stimulating with H22-Fel d 1 in the presence or absence of TSLP. In conjunction, the effects of OX40L blockade on T cell responses will be analyzed. We will also investigate whether other factors, such as secreted cytokines or surface molecules are involved in Th2 amplification. Finally, we will examine whether targeting H22-Fel d 1 to Fc $\gamma$ RI amplifies the TSLP pathway through upregulation of TSLP receptor. It is well established that cytokine pathways can modulate expression of Fc receptors. However, discovery of a pathway linking Fc $\gamma$ RI to TSLPr would provide a novel finding. Initially we will compare the ability for H22-Fel d 1 to upregulate TSLPr expression in dendritic cells from atopic and non-atopic subjects. The requirement for Fc $\gamma$ RI-mediated signaling events in the expression of TSLPr will be investigated using kinase inhibitors. Finally, the capacity for these inhibitors to inhibit TSLP-driven Th2 responses triggered by H22-Fel d 1

will be examined in order to confirm a role for Fc $\gamma$ RI-mediated signaling events in Th2 amplification.

## Chapter II – Targeting Fel d 1 to Fc $\gamma$ RI Induces a Novel Variation of the Th2 Response in Subjects with Cat Allergy

### Introduction

Conventional immunotherapy, which involves repeated injection of allergen extracts, remains the mainstay of treatment for allergy to a variety of inhalant allergens. Although there is considerable evidence for its efficacy, this approach has several drawbacks, including a risk for anaphylaxis and prolonged treatment regimens. Although the immune mechanisms which underlie the protective effects of immunotherapy remain ill defined, there is evidence that induction of CD4<sup>+</sup> T cells, which secrete IL-10 or IFN- $\gamma$ , is a prerequisite. This suggests the involvement of T cells that are either regulatory or Th1-like (60, 74, 79, 128). Consequently, recombinant allergens or allergen variants, which may provide a more tailored approach to immunotherapy, have been engineered with a view to activating specific T-cell subsets preferentially (74, 129, 130). Unfortunately, many allergens induce weak T-cell responses *in vitro*, which may reflect the low precursor frequency of allergen-specific T cells in the T-cell repertoire. As a result, defining T-cell mechanisms that underlie the protective effects of immunotherapy has proved to be a challenge. Moreover, *in vivo* presentation of allergen to T cells during conventional immunotherapy is likely to be inefficient.

The approach described here uses a fusion protein designed to target allergen to the surface of antigen-presenting cells (APCs) as a method of

improving allergen presentation to T cells. This protein (designated H22-Fel d 1) is composed of the major cat allergen, Fel d 1, linked to a single chain fragment of the variable region (sFv) of the humanized anti-CD64 mAb, H22. This molecule has been shown to bind to the high-affinity IgG receptor, FcγRI (CD64), on APCs (104). Targeting to CD64 has been demonstrated to increase uptake of diverse antigens by APCs and to increase T-cell proliferative responses (98, 102, 104, 131).

A unique feature of the major cat allergen, Fel d 1, is its ability to induce a modified Th2 response (IgG<sup>pos</sup>IgE<sup>neg</sup>) in a subset of individuals with high-level environmental exposure (55). This protective response is associated with increased IL-10 production in PBMC cultures stimulated with major T-cell epitopes of Fel d 1 (60). Because targeting Fel d 1 to CD64 on APCs may enhance presentation of major T-cell epitopes, we hypothesized that H22–Fel d 1 would promote induction of IL-10-producing CD4<sup>+</sup> T cells. These studies were designed to examine the immunomodulatory effects of H22-Fel d 1 at both the APC and the T-cell level. To this end, we analyzed patterns of cytokine secretion by dendritic cells and performed rigorous single-cell analyses of cytokine production by CD4<sup>+</sup> T cells. Our findings provide evidence of a new approach to immunotherapy and present a new paradigm for variations of a Th2 response.

## Materials and Methods

### *Classification of Subjects*

Patients were recruited from the University of Virginia Allergic Diseases Clinic or by advertisement. Subjects with cat allergy were classified on the basis of high-titer serum IgE antibodies to cat extract (CAP > 0.7 IU/mL) and anti-Fel d 1 IgE antibodies measured by streptavidin CAP assay (132). Subjects with a modified Th2 response to cat allergen were identified on the basis of high-titer anti-Fel d 1 IgG antibodies measured by antigen binding radioimmunoassay (>500 U/mL) without anti-Fel d 1 IgE antibodies (55, 133). Control subjects had no measurable serum IgG or IgE antibodies to Fel d 1. Serum antibody profiles are shown in Table 2.1. All studies were approved by the University of Virginia Human Investigations Committee.

### *Recombinant Allergens and LPS*

Recombinant Fel d 1 and Fel d 1 targeted to the high-affinity IgG receptor, Fc $\gamma$ RI (H22-Fel d 1) (104), were expressed in *Pichia pastoris* and purified by multistep chromatography. The purity was >90% as determined by SDS-PAGE. The endotoxin content of Fel d 1 and H22–Fel d 1 was comparable (145 EU/mL and 153 EU/mL, respectively). LoTox Der p 1 (Indoor Biotechnologies Inc, Charlottesville, Va) (endotoxin content < 0.05 EU/mg) was purified from mite culture by affinity chromatography (>90% purity by SDS-PAGE) and endotoxin removed by proprietary techniques. LPS was obtained from Sigma-Aldrich (St Louis, Mo).

**Table 2.1 Serum Antibody Profiles in Subjects With Distinct Immune Responses to Cat**

Group	Total IgE <sup>1</sup> (IU/ml)	IgE to Cat <sup>1</sup> (IU/ml)	Fel d 1	
			IgE <sup>2</sup> (IU/ml)	IgG <sup>3</sup> (U/ml)
Allergic (n=11)	119 (60-235)	13 (7-25)	7 (0.5-99)	3168 (1392-7213)
Modified Th2 (n=9)	22 (10-46)	<0.35	<0.35	4917 (1923-12573)
Control (n=5)	14 (4-45)	<0.35	<0.35	<125

Values represent geometric means (95% CI)

<sup>1</sup>Measured by CAP assay; <sup>2</sup>Measured by Streptavidin CAP assay; <sup>3</sup>Measured by radioimmunoprecipitation assay

### *Proliferation Assays*

PBMCs were cultured for 7 days ( $2 \times 10^5$  cells/well in 96-well plates) in the presence of Fel d 1 or H22–Fel d 1 (0.1, 1, or 10  $\mu\text{g}/\text{mL}$ ;  $\geq 6$  replicate wells per condition). Cells were pulsed with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine/well during the final 8 h of culture before harvesting and counting (Topcount NXT; Packard Instrument, Meriden, CT).

### *Induction of cytokines in monocyte-derived dendritic cells*

CD14<sup>+</sup> monocytes were purified by magnetic-activated cell sorting (Miltenyi Biotec, Auburn, Calif) and cultured for 6 days in RPMI-1640 with 10% heat-inactivated FBS, L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% sodium pyruvate, 100 ng/mL recombinant human GM-CSF, and 100 ng/mL recombinant human IL-4 (BioSource International, Inc, Carlsbad, Calif) to generate monocyte-derived dendritic cells (moDC) (134). Half of the media in each well was changed every 2 to 3 days. moDCs were stimulated for 24 to 48 hours in medium containing 10% autologous human serum with allergen (10  $\mu\text{g}/\text{mL}$ ) or LPS (1  $\mu\text{g}/\text{mL}$ ). Cytokines were measured by cytometric bead assay (human 27-plex kit; BioRad, Hercules, CA) or ELISA (DuoSet Human CCL17/TARC; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

### *CD4<sup>+</sup> T-cell/moDC co-cultures*

CD4<sup>+</sup> T cells were purified by negative selection using magnetic-activated cell sorting (Miltenyi Biotec, Auburn, Calif), and purity was assessed by flow

cytometry (>95%). moDCs were pulsed with allergen (10  $\mu\text{g}/\text{mL}$ ) for 48 hours before coculture ( $2 \times 10^5$ ) with purified  $\text{CD4}^+$  T cells ( $8 \times 10^5$ ) for 10 days in 24-well plates. Cultures were supplemented with recombinant human IL-2 (12 U/mL; BioSource International, Carlsbad, Calif) on day 5, and intracellular cytokines were measured on day 10. In some experiments, a blocking anti-IL-10 receptor mAb (clone 3F9; BioLegend, San Diego, Calif) or isotype control (clone RTK2758; BioLegend) was added (20  $\mu\text{g}/\text{mL}$ ) on day 0 of coculture.

### *Flow Cytometry*

All flow cytometry was performed on a Becton Dickinson FACSCalibur machine equipped with CellQuest software version 5.2 (BD Biosciences, Mountain View, Calif) and upgraded with a 635-nm red diode laser run by Rainbow software version 1.2b3 (Cytex Development, Gordonsville, Va). All experiments were analyzed by using FlowJo version 6.4.1 (Tree Star Inc, Ashland, Ore).

Unless otherwise stated, all fluorescently conjugated mAbs were purchased from BD Biosciences. moDC surface mAbs included phycoerythrin-anti-CD1a (HI149), peridinin-chlorophyll-protein-anti-CD14 (MuP9), APC-anti-CD11c (B-ly6), biotin conjugated anti-CD64 (10.1) then avidin-APC-Cy7, and fluorescein isothiocyanate-anti-HLA-DR (L2234), anti-CD40 (5C3), anti-CD80 (L307.4), or anti-CD86 (2331). T-cell surface mAbs included peridinin-chlorophyll-protein-anti-CD8 (SK1) and APC-Cy7-anti-CD3 (SK7), and

intracellular mAbs included fluorescein isothiocyanate-anti-IL-5 (R&D Systems Inc, 22026, Minneapolis, Minn), phycoerythrin-anti-IL-10 (JES3-9D7), and APC-anti-IFN- $\gamma$  (25723.11). For all multicolor analyses, compensation controls (single stains, 1 for each fluorochrome) and gating controls (cells stained with all reagents minus 1) were included (135).

#### *moDC Phenotyping*

Monocyte-derived dendritic cells were collected immediately after generation or allergen pulsing (24-48 hours) and washed in cold FACS buffer (0.5% BSA, 2 mmol/L EDTA in PBS). Cells were stained for surface markers, washed, and analyzed by flow cytometry as described above.

#### *Intracellular cytokine staining*

CD4<sup>+</sup> T-cell/moDC cocultures were restimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (2  $\mu$ g/mL; Sigma-Aldrich) for 5 hours, and Brefeldin A (BD Biosciences, San Jose, Calif) was added during the final 4 hours. Cells were stained for surface and intracellular cytokines according to the manufacturer's instructions (Caltag International Inc, Carlsbad, Calif) and analyzed by flow cytometry as described above.

#### *Statistical analysis*

The Student t test (SPSS 14.0, SPSS Inc, Chicago, Ill) was used to compare proliferation, cytokines, and mean fluorescence intensity levels. The percentages of cytokine-positive CD4<sup>+</sup> T cells induced by allergen were analyzed

by general linear mixed-effects ANOVA models after transforming data to the natural logarithmic scale. Each ANOVA model was specified as a hierarchical model with respect to the model factors. For each ANOVA, the stimulus and cytokine type represented the highest and lowest level factor of the 2-level hierarchical model, respectively. Data from each subject represented an independent random block of correlated information within the model. ANOVA model parameters were estimated via restricted maximum likelihood, and the variance components were modeled in the compound symmetry variance-covariance matrix form. Linear contrasts of the ANOVA least-squares means were used to analyze the log-transformed data. All hypothesis tests were 2-sided, and the Tukey multiple comparison type I error rate adjustment was used so that the cumulative type I error rate ( $\alpha$ ) was  $\leq 0.1$  for the entire set of hypothesis tests. This value was selected on the basis of the conservative nature of the Tukey adjustment. The models were fit using PROC MIXED in SAS, version 9.1 (SAS Institute Inc, Cary, NC).

## **Results**

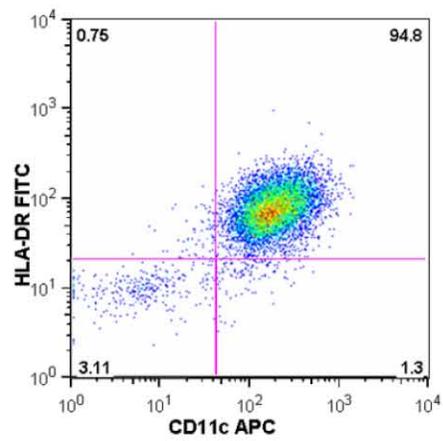
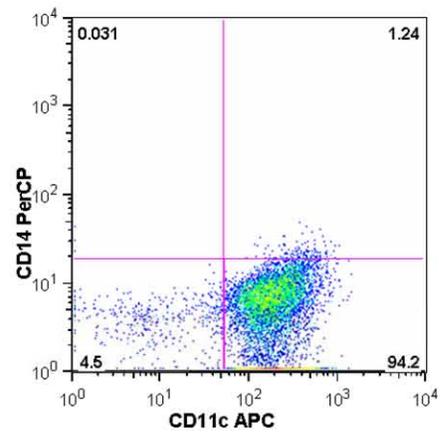
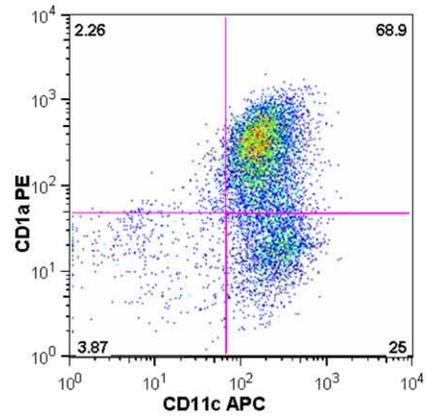
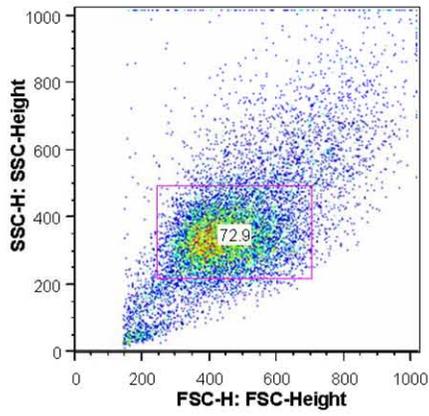
### *H22-Fel d 1 induces production of inflammatory cytokines by dendritic cells without increasing surface markers of maturation*

Dendritic cells are essential for generating T-cell responses to allergens. These responses may be influenced by the degree of maturation of the dendritic cell as well as by the cytokines these cells secrete (124, 129, 136). We investigated whether binding of H22-Fel d 1 to Fc $\gamma$ RI on dendritic cells alters the

properties of these cells. Flow-cytometry analysis showed that moDCs obtained from subjects with cat allergy expressed a phenotype characteristic of myeloid dendritic cells including high-level expression of CD11c, CD1a, and HLA-DR and lack of expression of CD14 (Figure 2.1). Importantly, these cells also expressed CD64 (Figure 2.2). As expected, stimulation with LPS led to increased expression of the costimulatory molecules CD40, CD80, and CD86 on moDCs, consistent with maturation, whereas expression of CD64 was decreased ( $p \leq .01$ ). However, stimulation with H22-Fel d 1 or with Fel d 1 had no significant effect on these markers (Figure 2.3). By contrast, H22-Fel d 1 induced increased secretion of Th1-promoting and inflammatory cytokines (IL-12, RANTES, IL-1 $\beta$ , monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 $\beta$ , MIP-1 $\alpha$ ) by moDCs compared with Fel d 1 ( $p < .05$ ). Moreover, levels for selected inflammatory cytokines (MCP-1, MIP-1 $\beta$ ) were comparable to those induced by LPS (Figure 2.4). H22-Fel d 1 also induced increased IL-10 compared with Fel d 1 ( $p = .04$ ), whereas no change in the levels of Th2-attracting chemokines (thymus and activation-regulated chemokine (TARC) and eotaxin) was observed for any stimuli (Figure 2.4, and data not shown). Induction of inflammatory cytokines in dendritic cells by H22-Fel d 1, without increased expression of maturation markers, indicates a semimature phenotype (137).

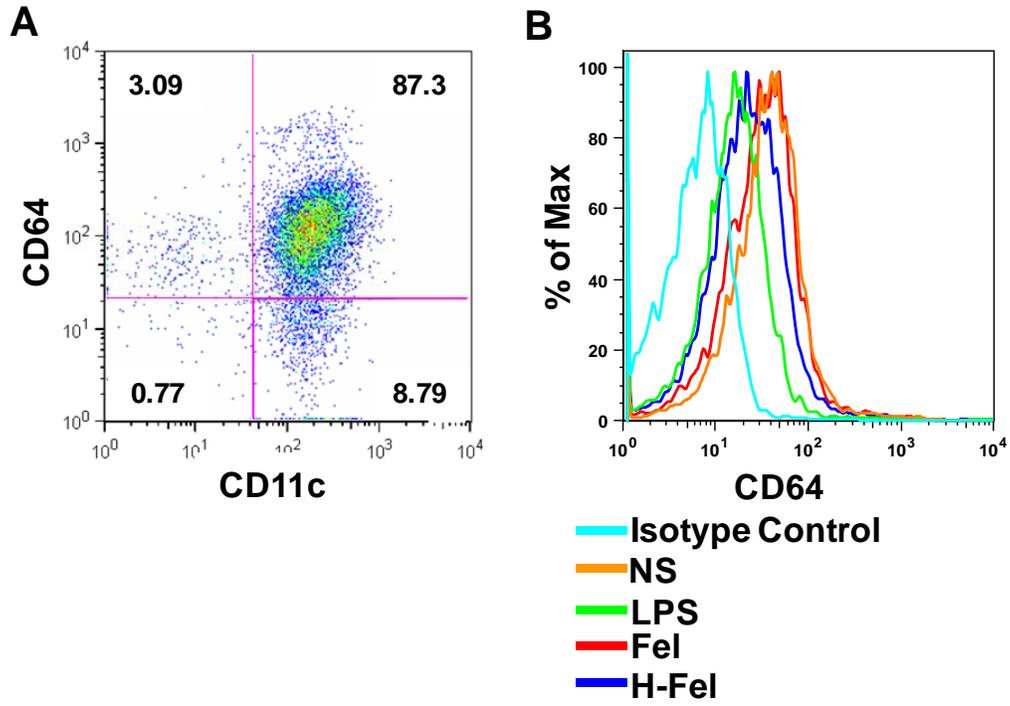
**Figure 2.1 MoDC phenotype.**

MoDCs were generated from CD14<sup>+</sup> T cells by culturing in the presence of IL-4 and GM-CSF for 6 days. Representative flow cytometry plots from 1 of 6 allergic subjects demonstrating the CD11c<sup>+</sup>, CD14<sup>neg</sup> phenotype of moDCs after 6 days in culture with IL-4 and GM-CSF.



**Figure 2.2 CD64 is Expressed on MoDCs.**

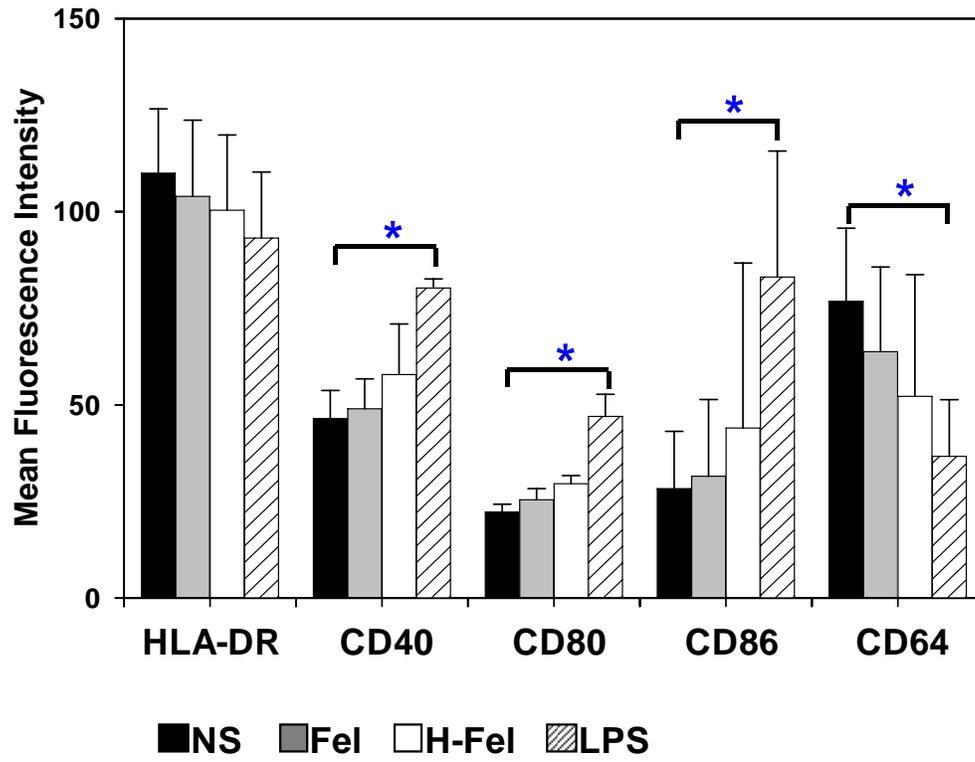
MoDCs from subjects with cat allergy (n=6) were pulsed with antigen (24 hours) and analyzed for expression CD64 by flow cytometry. **A.** Representative data showing expression of CD64 after 6 day generation of moDCs. **B.** Representative data showing expression of CD64 after 24 hour antigen pulse.



**Figure 2.3 H22-Fel d 1 Does Not Alter Expression of Co-Stimulatory Molecules.**

MoDCs from subjects with cat allergy (n=6) were pulsed with antigen (24 hours) and analyzed for expression of surface antigens by flow cytometry.

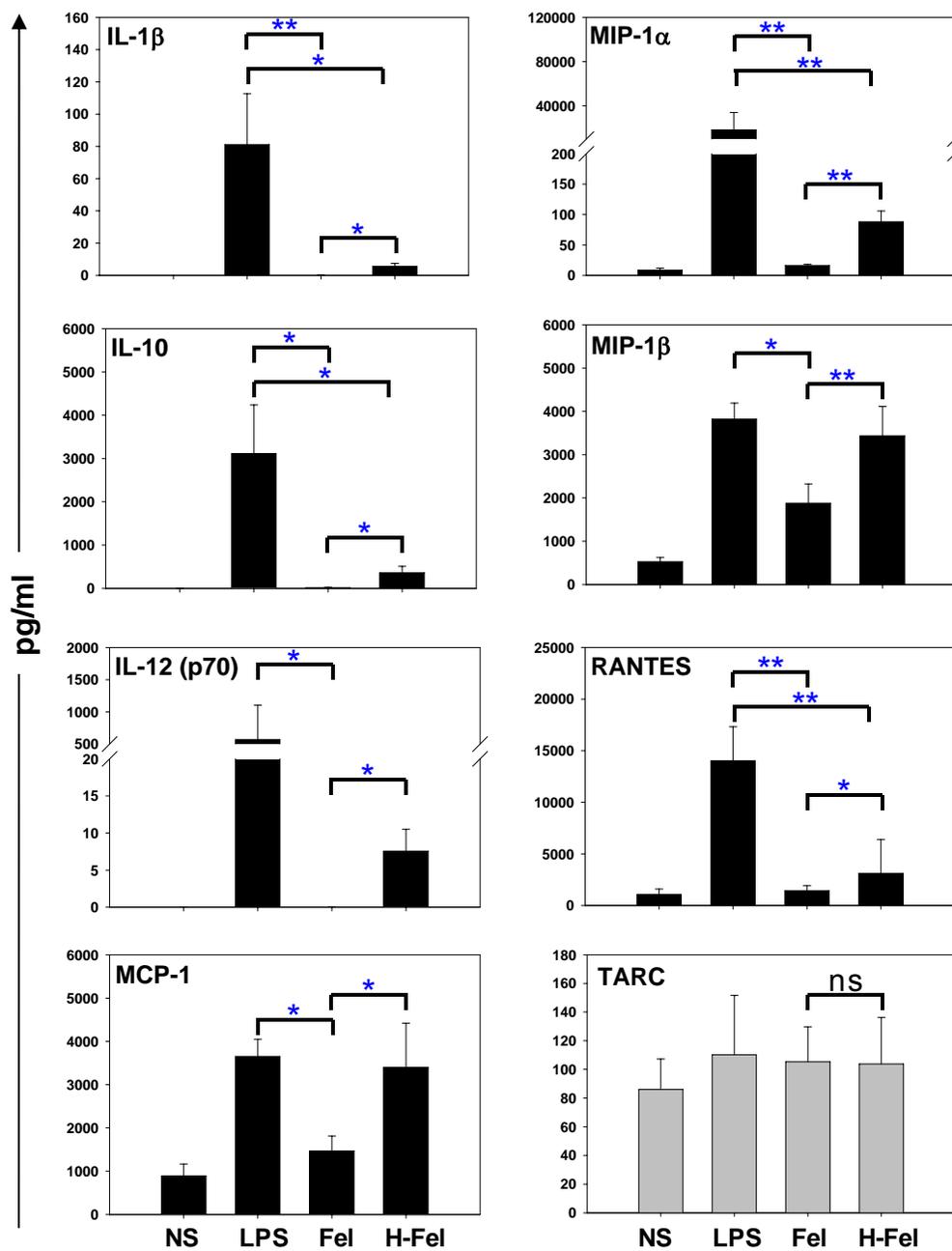
Effects of different stimuli on maturation markers and CD64. \*p < 0.01. Values represent the means + SDs.



**Figure 2.4 H22-Fel d 1 Enhances Production of Th1-Associated Cytokines and IL-10.**

MoDCs from subjects with cat allergy (n=6) were pulsed with antigen (24 hours) and analyzed for cytokine secretion by cytometric bead assay. Effects of different stimuli on cytokine secretion. Values represent the mean pg/ml + SDs.

\*p <0.05; \*\*p <0.01.



### *H22-Fel d 1 induces increased proliferation in PBMC cultures*

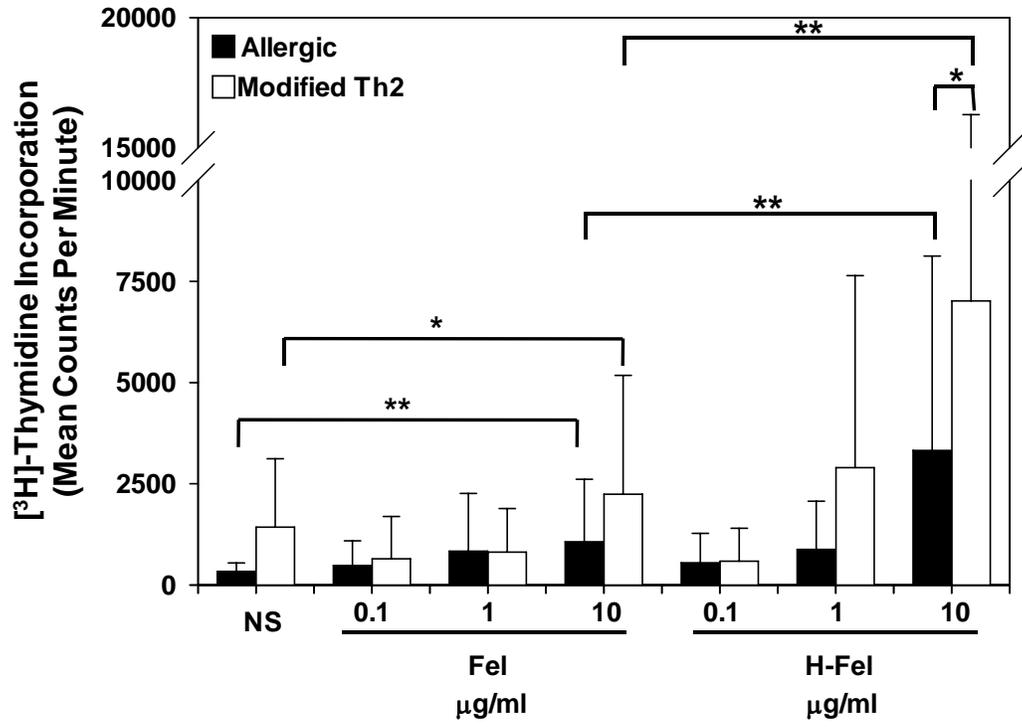
Targeting antigens to the high-affinity IgG receptor, Fc $\gamma$ RI (CD64), using the mAb H22 has been reported to enhance T-cell proliferative responses *in vitro* (102). We compared proliferation to receptor-targeted Fel d 1 (H22-Fel d 1) with Fel d 1 in PBMC cultures established from cat-allergic subjects and nonallergic subjects with a modified Th2 response to cat (n = 7 per group). Stimulation with H22-Fel d 1 resulted in increased proliferation compared with Fel d 1 at a dose of 10  $\mu$ g/mL in both subject groups (p < .001), and this effect was strongest for cultures from subjects with a modified Th2 response (p < .01; Figure 2.5).

### *H22-Fel d 1 amplifies IL-5- and IL-10-expressing CD4<sup>+</sup> T cells and this effect is restricted to cat-allergic subjects*

Next we examined whether changes induced by H22-Fel d 1 at the APC level were reflected at the T-cell level. Single-cell analysis of cytokine-producing CD4<sup>+</sup> T cells was performed by using flow cytometry after culture of purified CD4<sup>+</sup> T cells with allergen-primed moDCs for 10 days. Experiments were performed in 3 groups of subjects with distinct immune responses to cat allergen: (1) cat allergy, (2) modified Th2, and (3) serum antibody-negative controls (Table 2.1). Our objectives were to evaluate changes in the frequency of cytokine-positive CD4<sup>+</sup> T cells and to assess the relevance of these changes to allergic status. We performed a rigorous multiple comparison statistical test to evaluate these changes to provide strong statistical power. H22-Fel d 1 induced increased IL-10<sup>+</sup> and IL-5<sup>+</sup> CD4<sup>+</sup> T cells compared with Fel d 1, and this effect

**Figure 2.5 Proliferation of PBMCs is Significantly Increased by****H22-Fel d 1.**

PBMCs were stimulated with allergen and proliferation measured on day 7 (n=7 per group) by [<sup>3</sup>H]-thymidine incorporation. Values correspond to mean counts per minute + SDs. \*p < 0.01; \*\*p < 0.001.



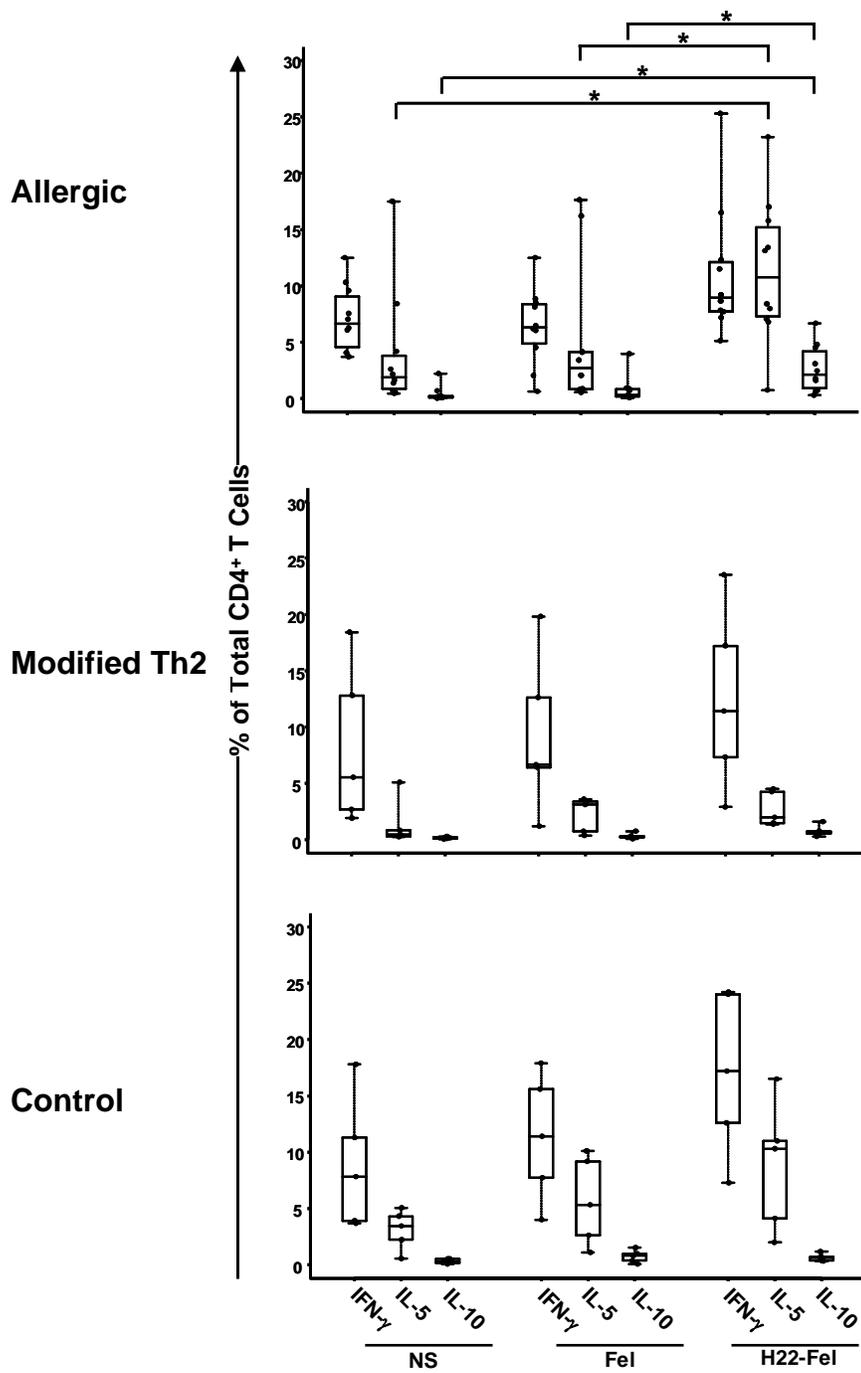
was restricted to subjects with allergy ( $p \leq 0.05$ ; Figure 2.6). Interestingly, although H22-Fel d 1 induced an increased frequency of IFN- $\gamma$ + cells compared with Fel d 1, this effect was not statistically significant for any group (Figure 2.6).

*H22-Fel d 1 induces cytokine-secreting subtypes consistent with Th0 and regulatory Th1 cells*

Single-cell analysis of CD4<sup>+</sup> T cells from subjects with allergy revealed that a significant proportion of cytokine-positive cells induced by H22-Fel d 1 expressed more than 1 cytokine (Figure 2.7). To assess further the nature of these cells, we analyzed the following 6 subtypes: (1) IFN- $\gamma$ + only, (2) IL-5+ only, (3) IFN- $\gamma$ +IL-5+, (4) IL-10+ only, (5) IL-10+IFN- $\gamma$ +, and (6) IL-10+IL-5+. Before adjusting for multiple comparisons, H22-Fel d 1 induced significant increases in all subtypes compared with Fel d 1 ( $p \leq 0.02$ ) with the exception of CD4<sup>+</sup> T cells expressing IFN- $\gamma$  only ( $p = 0.25$ ). After adjusting for multiple comparisons, changes in 3 subtypes remained significant (IFN- $\gamma$ +IL-5+, IL-10+ only, and IL-10+IFN- $\gamma$ +; Figure 2.8 and 2.9). Thus, although CD4<sup>+</sup> T cells expressing IFN- $\gamma$  alone were not significantly increased, cells expressing IFN- $\gamma$  together with IL-5 or IL-10 were increased, pointing to the production of IFN- $\gamma$  by Th0 (IL-5+IFN- $\gamma$ +) and regulatory Th1 (IL-10+IFN- $\gamma$ +) cells. Figure 2.9 is a color graphic summarizing geometric mean data for all cytokine-positive CD4<sup>+</sup> T-cell subtypes in each of the 3 groups of subjects. This figure shows that the T-cell repertoire induced by H22-Fel d 1 was distinct from that induced by Fel d 1 in subjects with a modified Th2 response (compare third bar, upper panel, with second bar,

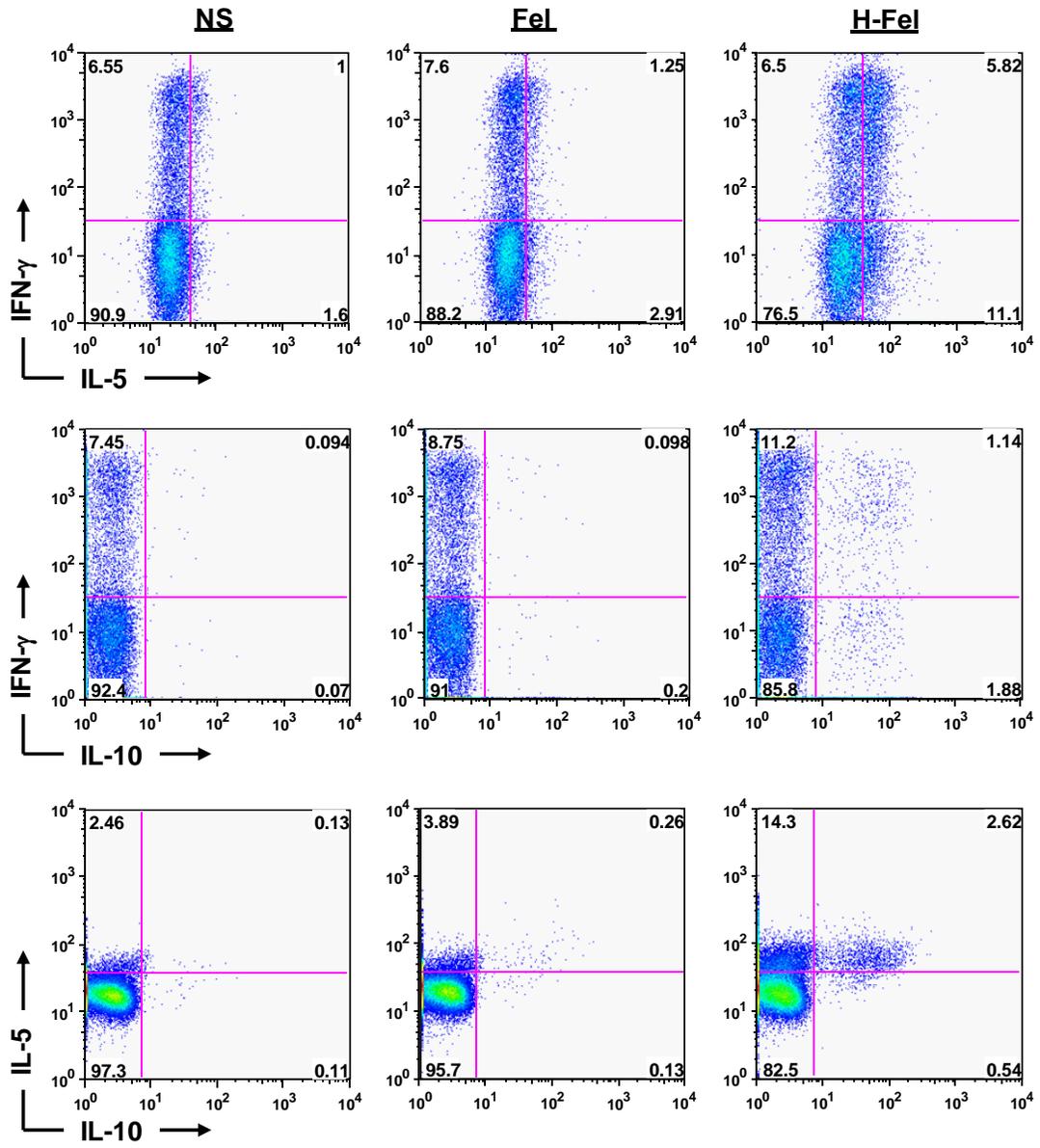
**Figure 2.6 H22-Fel d 1 Enhances the Frequency of IL-5+ and IL-10+ T Cells.**

CD4<sup>+</sup> T cells were cocultured with antigen-pulsed moDCs for 10 days, and the percentage of total CD4<sup>+</sup> T cells that were IFN- $\gamma$ +, IL-5+, and IL-10+ was analyzed by intracellular cytokine staining with flow cytometry. Experiments were performed in subjects with cat allergy (n=10), modified Th2 (n=5), and control (n=5) subjects. Boxes represent medians and interquartile ranges. \*Significant after adjusting for multiple comparisons.



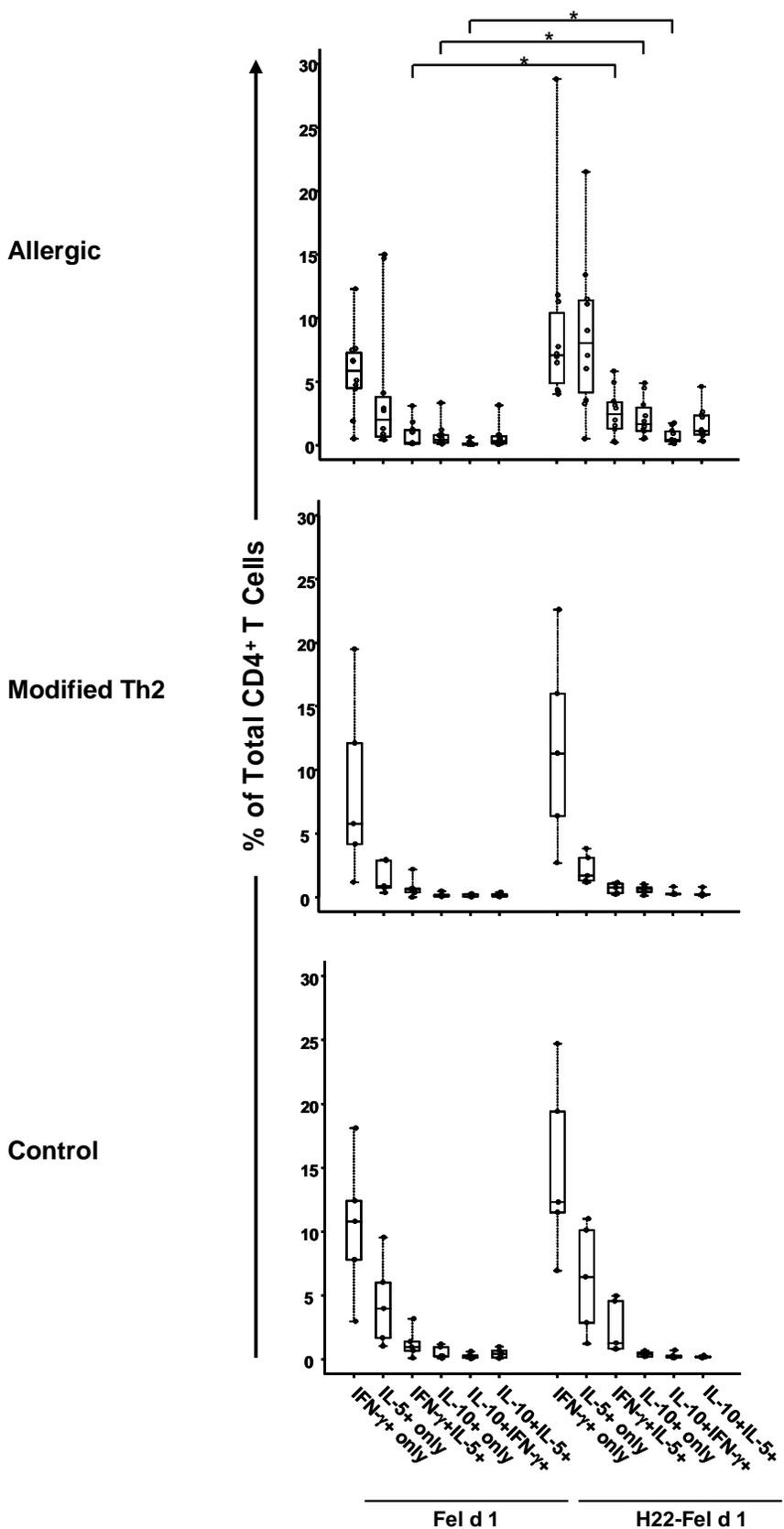
**Figure 2.7 Changes in Cytokine-Positive T cells Induced by H22–Fel d 1.**

CD4<sup>+</sup> T cells were cocultured with antigen-pulsed moDCs for 10 days, and the percentage of total CD4<sup>+</sup> T cells that were IFN- $\gamma$ <sup>+</sup>, IL-5<sup>+</sup>, and IL-10<sup>+</sup> was analyzed by intracellular cytokine staining with flow cytometry. Flow cytometry dot plots are shown for 1 of 10 patients with allergy.



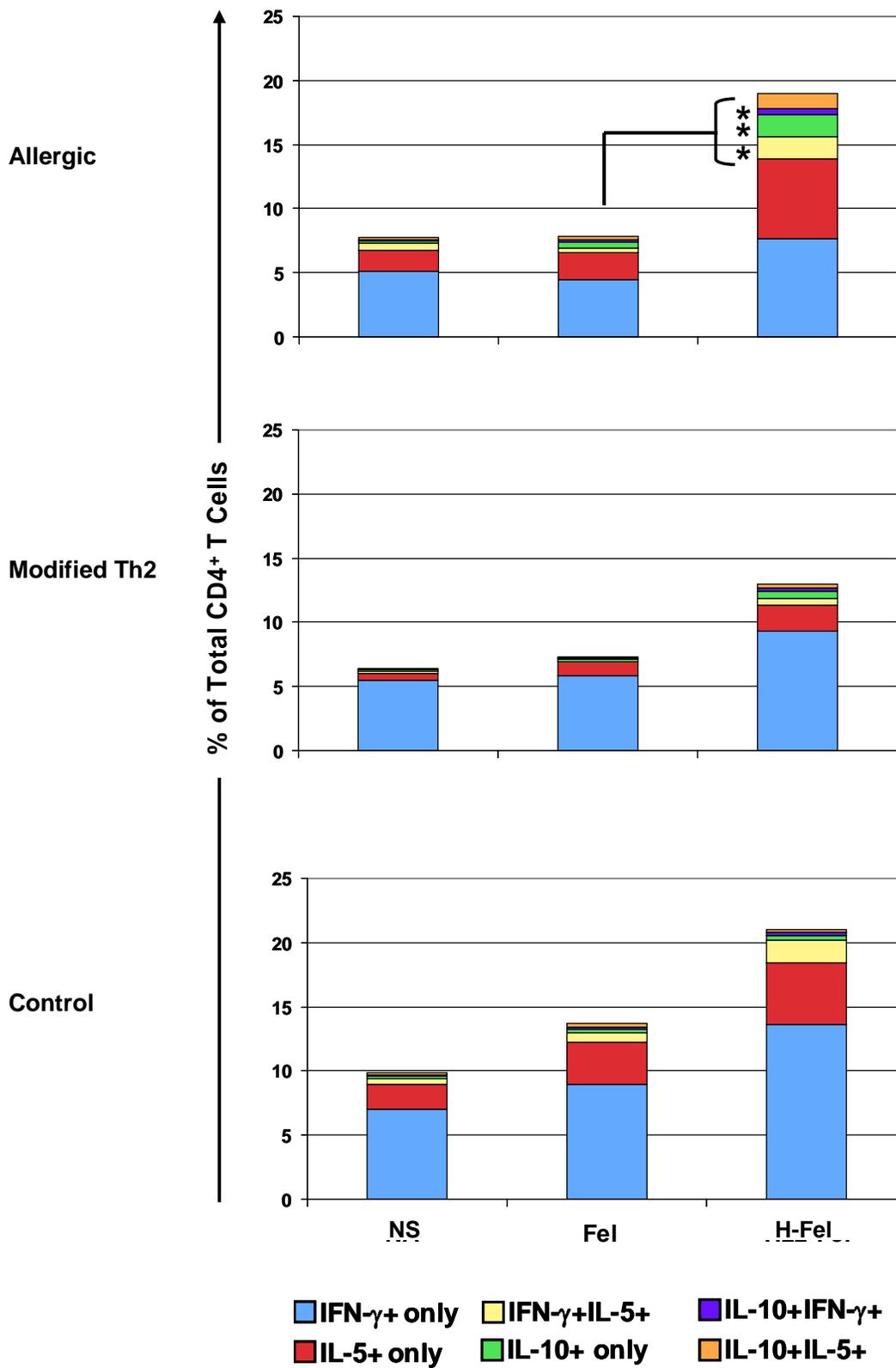
**Figure 2.8 H22-Fel d 1 Induces a Qualitative Change in the T Cell Repertoire.**

CD4<sup>+</sup> T cells were cocultured with moDCs pulsed with Fel d 1 or H22–Fel d 1 for 10 days, and the percentage of the following 6 CD4<sup>+</sup> T-cell subtypes was analyzed by intracellular cytokine staining with flow cytometry: (1) IFN- $\gamma$ + only; (2) IL-5+ only; (3) IFN- $\gamma$ +IL-5+; (4) IL-10+ only; (5) IL-10+IFN- $\gamma$ +; and (6) IL-10+IL-5+. Comparison of 6 CD4<sup>+</sup> T-cell subtypes induced by Fel d 1 and H22–Fel d 1 in subjects with cat allergy (n=10), modified Th2 (n=5), and control (n=5) subjects. Boxes represent median and interquartile ranges of the percentage of total CD4<sup>+</sup> T cells for each subtype. \*Significant after adjusting for multiple comparisons.



**Figure 2.9 Cytokine-Positive T Cell Subtypes Induced by H22-Fel d 1.**

CD4<sup>+</sup> T cells were cocultured with moDCs pulsed with Fel d 1 or H22-Fel d 1 for 10 days, and the percentage of 6 CD4<sup>+</sup> T-cell subtypes was analyzed by intracellular cytokine staining with flow cytometry: Comparison of 6 CD4<sup>+</sup> T-cell subtypes induced by Fel d 1 and H22–Fel d 1 in subjects with cat allergy (n=10), modified Th2 (n=5), and control (n=5) subjects. Data represents geometric mean. \*Significant after adjusting for multiple comparisons.



middle panel, Figure 2.9). In summary, H22-Fel d 1 induced a selective increase in Th0 cells and IL-10–expressing T-cell subtypes in CD4<sup>+</sup> T cells from subjects with allergy.

*H22-Fel d 1 does not induce a “pro-allergic” repertoire*

Given that H22-Fel d 1 stimulated strong T-cell proliferation and induced an increased frequency of IL-5<sup>+</sup> CD4<sup>+</sup> T cells (Figures 2.3 and 2.4), we investigated whether the repertoire of cytokine secreting T cells induced by this molecule was similar to that induced by the prototypic allergen, Der p 1. Five patients were selected for study who were cosensitized to cat and dust mite allergen, and cytokine expression was analyzed in CD4<sup>+</sup> T cells after stimulation with H22–Fel d 1 or Der p 1. After adjusting for multiple comparisons, H22-Fel d 1 induced significant increases in Th0 cells (IFN- $\gamma$ +IL-5<sup>+</sup>) and all 3 IL-10-expressing subtypes (IL-10<sup>+</sup> only, IL-10+IFN- $\gamma$ +, and IL-10+IL-5<sup>+</sup>) compared with Der p 1 (Fig 2.10). Thus, the T-cell repertoire induced by H22-Fel d 1 is distinct from a proallergic repertoire, comprising a higher proportion of Th0 cells and diverse IL-10-expressing subtypes.

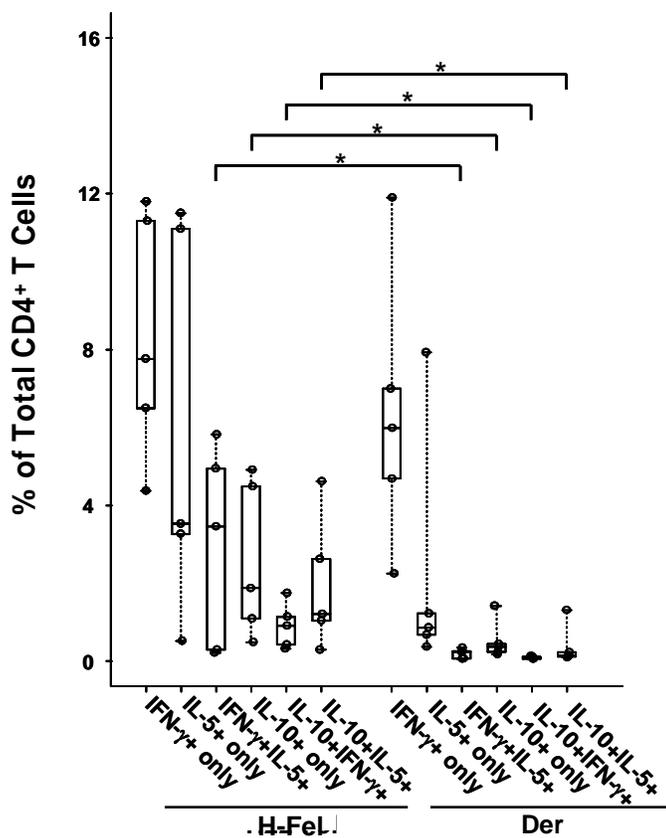
*IL-10 induced by H22-Fel d 1 selectively controls Th2 cells*

Our previous work suggested a role for IL-10 in the development of nonallergic protective responses (modified Th2) to cat allergen (60). Although IL-10<sup>+</sup> CD4<sup>+</sup> T cells were increased after stimulation with H22-Fel d 1 in cultures from subjects with allergy, IL-10-expressing T cells represented only a small fraction of total cytokine-positive T cells. This prompted us to question whether

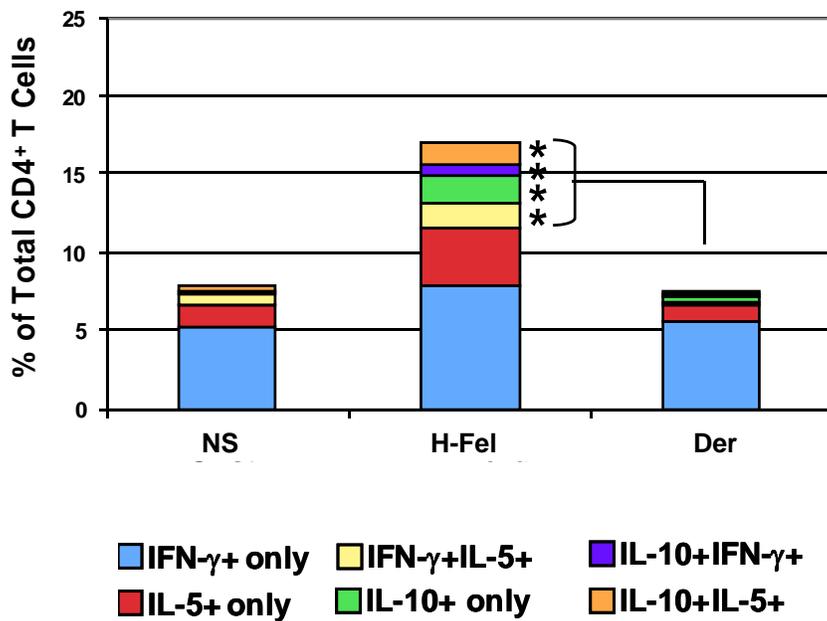
**Figure 2.10 The T Cell Repertoire Induced by H22-Fel d 1 is Distinct from a “Pro-Allergic” Repertoire.**

CD4<sup>+</sup> T cells were cocultured with moDCs pulsed with Der p 1 or H22–Fel d 1 for 10 days, and the percentage of 6 CD4<sup>+</sup> T-cell subtypes was analyzed by intracellular cytokine staining with flow cytometry: Comparison of 6 CD4<sup>+</sup> T-cell subtypes induced by Der p 1 and H22–Fel d 1 in subjects co-sensitized to dust mite and cat (n=5). **A.** Boxes represent the median and interquartile ranges of the percentage of total CD4<sup>+</sup> T cells for each subtype. \*Significant after adjusting for multiple comparisons. **B.** Data from A represented as the geometric mean for each subtype.

A



B



T-cell responses induced by H22-Fel d 1 were controlled. Thus, we examined the effects of IL-10 blockade on the frequency of IL-5<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells. In the presence of antibody to the IL-10 receptor, stimulation with H22-Fel d 1 resulted in an increased frequency of IL-5<sup>+</sup> CD4<sup>+</sup> T cells in cultures from 4 of 5 subjects with allergy. By contrast, this antibody had little or no effect on IFN- $\gamma$ <sup>+</sup> or IL-10<sup>+</sup> CD4<sup>+</sup> T cells (Figure 2.11). The increase in IL-5<sup>+</sup> CD4<sup>+</sup> T cells observed in the presence of anti-IL-10 receptor antibody suggests that the response to H22-Fel d 1 reflects a controlled Th2 response.

## Discussion

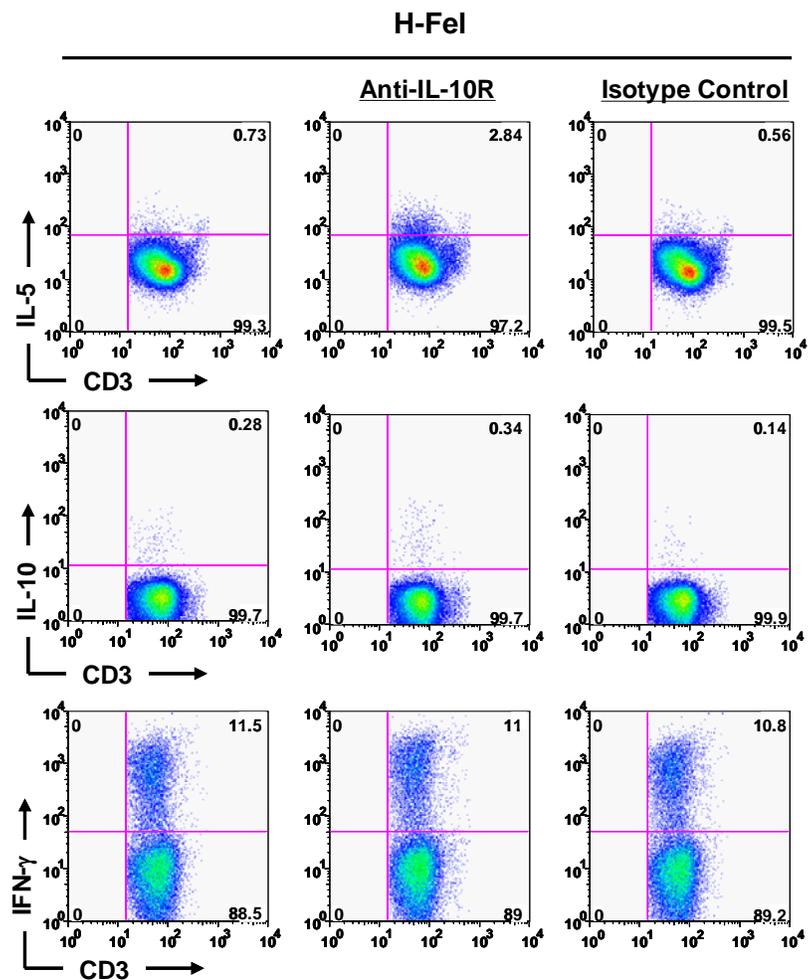
Defining the effects of H22-Fel d 1 on the allergic T-cell response may provide a new approach for treatment of patients who have allergy to cats. We have demonstrated that targeting Fel d 1 to Fc $\gamma$ RI alters the quality of the CD4<sup>+</sup> T-cell repertoire induced by Fel d 1 in subjects with cat allergy. Our findings showed a preferential increase in CD4<sup>+</sup> T cells expressing the Th2 cytokine, IL-5, which included Th0 (IL-5+IFN- $\gamma$ +) cells. These changes occurred in concert with increased IL-10-expressing T-cell subtypes. Moreover, we found evidence that IL-5<sup>+</sup> CD4<sup>+</sup> T cells induced by H22-Fel d 1 were controlled by IL-10. These observations point to complex changes in the T-cell repertoire at the single-cell level and provide a new variation on existing paradigms related to T-cell modulation.

Other studies have described a fusion protein of Fel d 1 that targets allergen to the inhibitory receptor, Fc $\gamma$ RIIb, on mast cells and basophils (138);

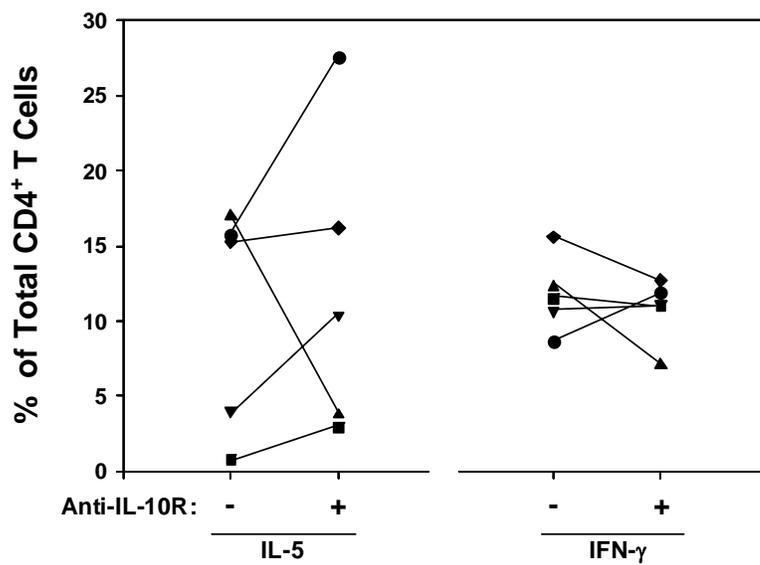
**Figure 2.11 IL-10 Blockade Selectively Enhances Th2 Responses.**

CD4<sup>+</sup> T cells were cocultured with moDCs pulsed with H22-Fel d 1 in the presence of a blocking anti-IL-10 receptor mAb or isotype control (n=5 subjects with cat allergy). **A.** Representative flow cytometry dot plots. **B.** Frequency of IL-5<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells in the presence or absence of anti-IL-10 receptor (IL-10R) mAb for each subject.

A



B



however, the effects of that molecule on T cells were not investigated. In the current study, H22-Fel d 1 selectively enhanced total IL-5<sup>+</sup> and IL-10<sup>+</sup> CD4<sup>+</sup> T cells, as well as the proportion of these cells coexpressing IFN- $\gamma$ . However, no change in CD4<sup>+</sup> T cells which secreted IFN- $\gamma$  alone was observed. Thus, H22-Fel d 1 induced an altered Th2 response that did not fit with typical Th1 skewing. Furthermore, although IL-5<sup>+</sup> CD4<sup>+</sup> T cells were a prominent feature of T-cell responses to H22-Fel d 1 in subjects with allergy, these cells were suppressed by IL-10 on the basis of IL-10 blocking experiments. The capacity for H22-Fel d 1 preferentially to increase IL-5<sup>+</sup> T cells (and different T-cell subtypes) in cultures from subjects with cat allergy may reflect more efficient antigen uptake and presentation to T cells coupled with a higher precursor frequency of Fel d 1-specific T cells in subjects with allergy compared with subjects without allergy (139).

If the T-cell response to H22-Fel d 1 does not signify Th1 skewing per se, then what does it represent? Despite the presence of IL-5<sup>+</sup> T cells, the T-cell repertoire induced by this molecule differed from that induced by the prototypic allergen, Der p 1.

Moreover, the repertoire did not resemble the Fel d 1-specific repertoire observed in subjects with a modified Th2 response or subjects without allergy (serum antibody-negative; Figure 2.9). For all comparisons, the prevailing difference was induction of IL-10<sup>+</sup> CD4<sup>+</sup> T cells. The population of IL-10-expressing cells induced by H22-Fel d 1 included cells that expressed IL-10 alone, or with IFN- $\gamma$  or IL-5. Such coexpression of IFN- $\gamma$  or IL-5 is consistent with

a regulatory Th1 or regulatory Th2 subtype, respectively. Similar types of IL-10-secreting cells have been previously described by Umetsu's group (140, 141). In those studies, induction of regulatory Th1 and Th2 cells was mediated by distinct types of dendritic cells, and both types of regulatory T cells were shown to inhibit the development of Th2-driven airway hyperreactivity and inflammation in mice. Specifically, regulatory Th1 cells were induced by dendritic cells producing both IL-10 and IL-12 (141). In the current study, H22-Fel d 1 induced increased production of IL-10 and IL-12 by dendritic cells from subjects with allergy in conjunction with increased IFN- $\gamma$ +IL-10+ CD4<sup>+</sup> T cells. Thus, H22-Fel d 1 may condition dendritic cells to prime regulatory Th1 cells in individuals with allergy.

Mature dendritic cells are very efficient at T-cell priming. Such dendritic cells are characterized by high expression of costimulatory molecules (CD40, CD80, and CD86) and production of inflammatory cytokines. Dendritic cells with an immature or semimature phenotype are characterized by lower degrees of expression of costimulatory molecules and/or cytokine production (137). H22-Fel d 1 induced production of inflammatory cytokines by dendritic cells without increased expression of costimulatory molecules, consistent with a semimature phenotype. Such cells are known to exhibit a potent ability to induce regulatory T cells (137). Interestingly, H22-Fel d 1 did not induce increased secretion of Th2-promoting cytokines (TARC and eotaxin) by dendritic cells. Thus, these factors are unlikely to promote the generation of IL-5+ CD4<sup>+</sup> T cells that we observed. Involvement of receptors expressed on dendritic cells (eg, OX40 ligand) provides

an alternate mechanism and warrants further investigation (119). Taken together, our findings suggest that H22-Fel d 1 induces a dendritic cell phenotype that is intermediate to that induced by Fel d 1 and LPS. This semimature phenotype may explain the selective enhancement of IL-5+ CD4<sup>+</sup> T cells, including Th0 cells, in parallel with IL-10-expressing subtypes.

Although H22-Fel d 1 induced enhanced proliferative responses in PBMC cultures from subjects with allergy compared with Fel d 1, proliferation was even higher for cultures from subjects with a modified Th2 response. Despite this, the lowest increase in cytokine-positive T cells was observed for the modified Th2 group. Thus, cytokine production appeared to be dissociated from proliferation. This could arise from altered signaling to T cells mediated at the APC/T-cell interface, which results in uncoupling of distinct signaling pathways within the T cell that are required for cell division and cytokine production (142). Because the modified Th2 response to Fel d 1 may be controlled at the T-cell level (60), it is possible that targeting H22-Fel d 1 to Fc $\gamma$ RI is not sufficient to overcome those regulatory mechanisms. Interestingly, <3% of total cytokine-secreting CD4<sup>+</sup> T cells were IL-10+ in cultures from subjects with a modified Th2 response. Thus, low frequencies of IL-10+ T cells may be effective in controlling T-cell responses to allergen.

In summary, we describe a novel variation of a Th2 response to Fel d 1 in subjects with cat allergy. This response, which is induced by targeting Fel d 1 to Fc $\gamma$ RI on dendritic cells, incorporates major elements of a protective T-cell

response. We propose that targeting allergen to the high-affinity IgG receptor on APCs provides a new approach to T-cell-based vaccines for allergic disease.

## Chapter III - Targeting Allergen to Fc $\gamma$ RI Reveals a Novel Pathway Which Regulates Th2 Responses in Atopic Individuals

### Introduction

Allergic diseases such as asthma and atopic dermatitis (eczema) are a manifestation of inflammatory processes driven by Th2 lymphocytes. Recently, the IL-7-like cytokine, thymic stromal lymphopoietin (TSLP), has been proposed to act as a major “switch” factor in the allergic response. This premise is based primarily on its capacity to differentiate pro-inflammatory Th2 cells from naïve CD4<sup>+</sup> T cell precursors. Such cells secrete high levels of IL-4, IL-5, and IL-13, in conjunction with TNF- $\alpha$ , but only low levels of IFN- $\gamma$  and IL-10 (118, 119). Induction of these cells has been reported to be mediated by dendritic cells activated by TSLP through the OX40 ligand pathway (119, 143). In an extension of these studies, TSLP has been shown to maintain and polarize circulating Th2 central memory cells, including allergen-specific T cells, suggesting an important role for this cytokine in driving Th2 responses associated with established allergic disease (124). More direct evidence of a role for TSLP in the manifestation of distinct allergic diseases is provided in mice which lack the TSLP gene or its receptor. Such animals fail to develop asthma, or else show attenuated disease (114, 116). Conversely, mice expressing an inducible TSLP transgene in the skin develop eczematous lesions (115).

In humans, high expression of TSLP is a feature of keratinocytes in the skin lesions of patients with atopic dermatitis (AD) and TSLP is also expressed

by bronchial epithelial cells derived from the asthmatic lung (118, 119, 124, 144, 145). The interactions of human dendritic cells with TSLP-expressing epithelial cells within the respiratory tract or skin are likely important not only for the generation of Th2 responses associated with allergic disease, but also for recruitment of T cells during ongoing inflammation. For example, human dendritic cells activated by TSLP secrete the chemokine, thymus and activation-regulated chemokine (TARC/CCL17). This factor is a key mediator of Th2 cell recruitment to sites of allergic inflammation and TARC is a serum marker of disease severity in AD (12, 118, 119, 124, 146-154). Thus, TSLP may act during both the initiation and the effector phases of the allergic response.

Allergen variants which target the dendritic cell could be a useful approach to treat allergic disease based on their ability to modulate APC function, and thus, alter the quality of allergen-specific T cell responses. Consistent with this view, we recently showed that targeting the major cat allergen, Fel d 1, to the high affinity IgG receptor, Fc $\gamma$ RI, on dendritic cells using the novel allergen variant, H22-Fel d 1, selectively enhanced the frequency of IL-5- and IL-10-expressing T cells *in vitro* (106). This phenomenon was restricted to T cells isolated from cat-allergic subjects. Interestingly, though the frequency of IL-10-expressing T cells was low in our system ( $\leq$  5% of total cytokine-expressing cells), IL-10 blockade resulted in a selective enhancement in Th2-like cells induced by H22-Fel d 1, suggesting that T cell responses were regulated by IL-10. Thus, H22-Fel d 1 induced a T cell repertoire which incorporated elements of a protective T cell response.

In order to assess whether dendritic cell-based therapies could be efficacious in the clinical setting, it is important to consider the effects of mediators which operate *in vivo* to modulate APCs in allergic individuals. High expression of TSLP at inflamed sites coupled with the presence of TSLP-primed APCs in regional lymph nodes could subvert the induction of a protective T cell repertoire to H22-Fel d 1 when this molecule is administered to allergic patients via subcutaneous or sublingual routes, or else by instillation into the respiratory tract. Consistent with this view, we report that Th2 responses to H22-Fel d 1 are amplified in the presence of TSLP and this effect is most marked in atopic subjects. Here, by dissecting the mechanism involved in this process we reveal a novel regulatory pathway linking Fc $\gamma$ R signaling to TSLP-driven events. Our findings have important clinical implications for the treatment of Th2-driven inflammatory disorders.

## **Materials and Methods**

### *Human Subjects*

Subjects were recruited from the University of Virginia Dermatology Clinic or the University of Virginia Allergic Diseases Clinic. Patients with atopic dermatitis reported physician-diagnosed eczema and the presence of itchy rash was confirmed upon physical exam. All atopic dermatitis patients had moderate-to-severe disease based on SCORAD index (8, 155), elevated serum titers of total IgE (>250 IU/ml) and high titer IgE ab to cat extract (CAP  $\geq$ 0.7 IU/ml). Cat-allergic subjects without atopic dermatitis were selected based on high titer IgE

ab to cat extract (CAP >0.7 IU/ml)(Table 3.1). The presence of Fel d 1-specific IgE ab was confirmed in atopic dermatitis patients and cat-allergic patients by Streptavidin CAP assay (132). All atopic dermatitis patients and cat-allergic subjects also had high titer IgE ab to dust mite allergen and were generally sensitized to multiple allergens (Table 3.1). Control subjects had no measurable serum IgG or IgE antibodies to common allergens, including Fel d 1 (Table 3.1). All studies were conducted according to Declaration of Helsinki principles and approved by the University of Virginia Human Investigations Committee.

#### *Cells and Reagents*

**Recombinant Allergens:** Recombinant Fel d 1 and Fel d 1 targeted to the high affinity IgG receptor, Fc $\gamma$ RI, (H22-Fel d 1) were expressed in *Pichia pastoris* and purified by multi-step chromatography. The purity was >90% as determined by SDS-PAGE. The endotoxin content of Fel d 1 and H22-Fel d 1 was comparable (145 EU/ml and 153 EU/ml respectively).

**Cells:** MoDCs were generated from CD14<sup>+</sup> monocytes isolated from fresh PBMCs as described previously (106). These cells were highly homogeneous based on expression of CD64/Fc $\gamma$ RI (>90%) and CD11c (>99%). Enriched myeloid dendritic cells were isolated from PBMCs by magnetic-activated cell sorting according to manufacturer's instructions (Blood Dendritic Cell Isolation Kit II, Miltenyi Biotec). CD4<sup>+</sup> T cells were purified from fresh PBMCs by negative selection using magnetic-activated cell sorting (Miltenyi Biotec) and purity was



assessed by flow cytometry (>95%). The THP-1 cell line was generously provided by Kodi Ravichandran.

**Flow Cytometry Antibodies and Reagents:** Unless otherwise stated, all fluorescently conjugated monoclonal antibodies were purchased from BD Biosciences. Monoclonal antibodies used for surface staining using standard flow cytometry were as follows: PerCP-anti-CD8 (clone SK1); APC-Cy7-anti-CD3 (SK7); APC-anti-TSLP receptor (1B4; Biolegend); PE-anti-OX40 ligand (Ik-1); FITC-anti-HLA-DR (L243); FITC-anti-CD40 (5C3); FITC-anti-CD80 (L607.4); and FITC-anti-CD86 (2331). Monoclonal antibodies used for intracellular staining were FITC-conjugated anti-IL-4 (MP4-25D2; R&D Systems); PE-anti-IL-10 (JES3-9D7); and APC-anti-IFN- $\gamma$  (25723.11). Monoclonal antibodies used for image-based flow cytometry were as follows: PE-anti-TSLP receptor (1A6; EBiosciences); PE-Cy5-anti-CD25 (M-A251); and FITC-anti-CD14 (M5E2). DNA binding dyes used were DRAQ5 (Biostatus Limited) and 7-AAD (BD Biosciences).

**Other Antibodies:** Anti-OX40 ligand mAb (Ik-1) was purchased from R&D Systems.

**Inhibitors:** The Syk kinase inhibitor piceatannol, the PI3-kinase inhibitor LY294002, and the protein kinase C inhibitor staurosporine were purchased from Sigma and used at a concentration of 10 $\mu$ M, 25 $\mu$ M and 1 $\mu$ M respectively. The Src-related tyrosine kinase inhibitor PP2 was obtained from Calbiochem and used at a concentration of 10 $\mu$ M.

### *CD4<sup>+</sup> T Cell Cultures*

MoDCs were pulsed with allergen alone (10 $\mu$ g/ml), TSLP alone (15ng/ml), or both for 48 hours in the presence of complete medium containing 10% autologous serum as previously described (106). After washing, moDCs were co-cultured (2x10<sup>5</sup>) in 24-well plates with autologous CD4<sup>+</sup> T cells (8x10<sup>5</sup>). Cultures were supplemented with recombinant human IL-2 (12U/ml)(BioSource International) on day 5, and then restimulated with PMA (50ng/ml) and ionomycin (2 $\mu$ g/ml)(Sigma-Aldrich) for 5 hours on day 10. Brefeldin A (BD Biosciences) was added during the final 4 hours of culture. Cells were stained for surface markers and analyzed by flow cytometry or by image-based flow cytometry. Because restimulation with PMA and ionomycin downregulates surface expression of CD4, cytokine positive CD4<sup>+</sup> T cells were identified by gating on CD3<sup>+</sup> cells which were CD8<sup>neg</sup>. Secreted cytokines were measured in culture supernatants harvested on day 10. In some experiments, cells were cultured in the presence of anti-OX40 ligand monoclonal antibody (20 $\mu$ g/ml). For inhibition studies, moDCs were incubated with Ly294002 or staurosporine for 1 hour (37<sup>o</sup>C) prior to pulsing with allergen or TSLP (48 hours). MoDCs were then washed and co-cultured with T cells (7 days).

### *APC Phenotyping*

THP-1 cells or moDCs were pulsed for 24 or 48 hours with allergen alone (10 $\mu$ g/ml), TSLP alone (15ng/ml), or both, in medium containing 10% autologous serum. Cells were then washed, stained for surface and intracellular markers,

and analyzed by standard flow cytometry or by image-based flow cytometry.

In time-course experiments for OX40 ligand and TSLP receptor expression, allergen and TSLP were removed at 48 hours by washing. In some experiments, APCs were incubated for 1 hour (37<sup>0</sup>C) with kinase inhibitors prior to stimulation with allergen (24 hours) and then analyzed for TSLP receptor expression.

### *Flow Cytometry Analysis*

Standard multi-color flow cytometry was performed on a Becton Dickinson FACSCalibur machine equipped with CellQuest software version 5.2 (BD Biosciences) and upgraded with a 635nm red diode laser run by Rainbow software version 1.2b3 (Cytek Development, Gordonsville, VA). Data was analyzed using FlowJo version 6.4.1 (Tree Star Inc., Ashland, OR). For all multi-color analyses compensation controls (single stains - one for each fluorochrome) and gating controls (cells stained with all reagents minus one) were included (156). Image-based flow cytometry was performed on an Imagestream100 (Amnis Corporation) using the manufacturer's collection program, IDEAS. Briefly, debris and cell aggregates were excluded and individual cells were identified by gating on cell area and aspect ratio as confirmed by brightfield (BF) microscopy. Identification of TSLP<sup>r</sup> cells was based on selection of distinct cell populations above background fluorescence and confirmed visually by the appearance of a circumferential fluorescence signal.

### *Cytokine Assays*

Cytokines were measured in culture supernatants harvested from DCs (48 hours) by cytometric bead assay (human 27-plex kit; BioRad, Hercules, CA), or by ELISA (Human CCL17/TARC ELISA (R&D Systems)). Cytokines in day 10 CD4<sup>+</sup> T cell cultures were assayed by cytometric bead assay (Th1/Th2 panel; BioRad). Serum TSLP was measured by monoclonal antibody-based ELISA.

### *Statistical Analysis*

Student's *t* test was used to compare cytokine levels and mean fluorescence intensity levels in dendritic cell cultures. Analyses were performed using SPSS 14.0 software. In order to account for within-group variability in T cell responses, a rigorous analysis was performed. Briefly, the frequency of cytokine-positive CD4<sup>+</sup> T cell types stimulated under different conditions was compared by log transforming the percentage of cytokine-positive cells for each condition to derive the geometric mean (gm) value. A T cell ratio was then calculated by dividing the gm value for condition A by the gm value for condition B and data displayed as the ratio of these gm values (*normalized index*) with 95% confidence intervals. The significance of observed changes was analyzed by general linear mixed effects ANOVA models as previously described (106). The Tukey's multiple comparison type I error rate adjustment was used so that the cumulative type I error rate ( $\alpha$ ) was  $\leq 0.1$ , based on the conservative nature of the Tukey's adjustment. The models were fit using PROC MIXED in SAS, version 9.1 (SAS Institute Inc, Cary, NC).

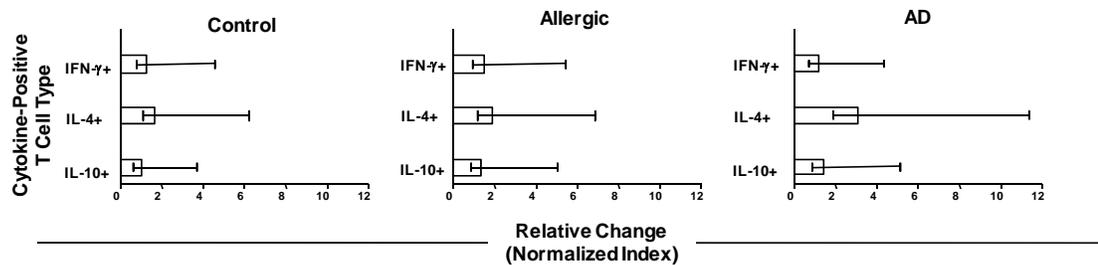
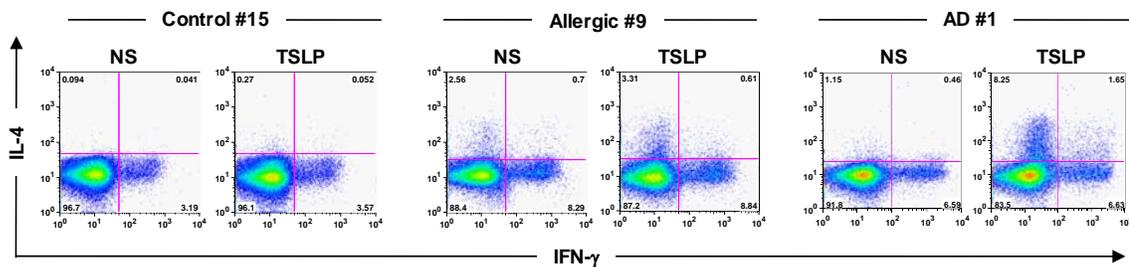
## Results

### *The Th2-Promoting Effects of TSLP are Increased in Atopic Subjects.*

Despite the capacity for TSLP-primed CD11c<sup>+</sup> dendritic cells to induce a robust Th2 response (118, 119, 143), the effects of TSLP have not been examined using T cells isolated from subjects with distinct allergic phenotypes. Experiments were performed in the following groups; (1) cat-allergic subjects with atopic dermatitis who were sensitized to multiple allergens [highly atopic]; (2) cat-allergic subjects without atopic dermatitis who were sensitized to at least 1 other allergen [atopic]; and (3) healthy non-atopic controls (Table 3.1). For T cell studies, a rigorous statistical approach was used to analyze cytokine-positive CD4<sup>+</sup> T cells induced by each stimulus in order to account for low frequency events and within-group variability (106). Our results showed that stimulation of T cells with TSLP-primed monocyte-derived dendritic cells (moDCs) preferentially enhanced IL-4<sup>+</sup> T cells and this effect was most marked in atopic dermatitis patients (normalized index=3.0 [1.1-8.3] versus 1.9 [0.7-5] for allergics and 1.7 [0.6-4.5] for controls)(Figure 3.1A and B). However, increases in the frequency of IL-4<sup>+</sup> T cells were not significant for any group after adjusting for multiple comparisons. TSLP preferentially induced the secretion of Th2 cytokines with highest levels in cat-allergic subjects with and without atopic dermatitis (Figure 3.2). Thus, the Th2-promoting effects of TSLP were modest irrespective of allergic status, but increased in atopic subjects.

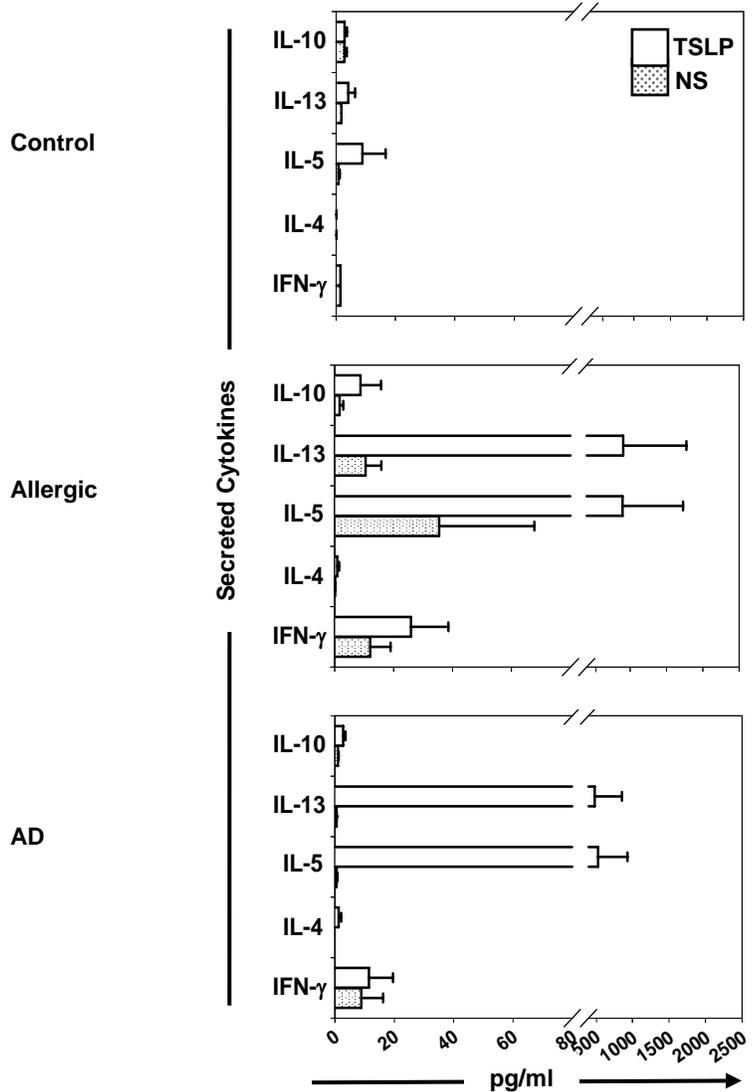
**Figure 3.1 The Th2-Promoting Effects of TSLP are Increased in Atopic Subjects.**

Enriched CD4<sup>+</sup> T cells were co-cultured with moDCs which had been pulsed for 48 hours with TSLP. Cells were stained for intracellular cytokines (day 10) and analyzed by flow cytometry, gating on CD4<sup>+</sup> T cells. **A.** Relative change in cytokine-positive T cell types as compared with non-stimulated cells was expressed as a normalized index value for each cell type within each group of subjects (n=5). Bars represent geometric means  $\pm$  95% confidence intervals. **B.** Representative flow cytometry dot plots showing the phenotype of gated CD4<sup>+</sup> T cells from unstimulated (NS) and TSLP-stimulated cultures from 3 subjects with distinct allergic phenotypes. Percentages of cells in each quadrant are shown. Data is representative of 5 subjects in each group.

**A****B**

**Figure 3.2 Secretion of Th2-Promoting Cytokines is Increased in Atopic Subjects in Response to TSLP.**

Enriched CD4<sup>+</sup> T cells were co-cultured with moDCs which had been pulsed for 48 hours with TSLP. Levels of secreted cytokines in supernatants harvested from unstimulated (NS) and TSLP-stimulated cultures (day 10) for each group (n=5). Bars represent the mean + SEM.



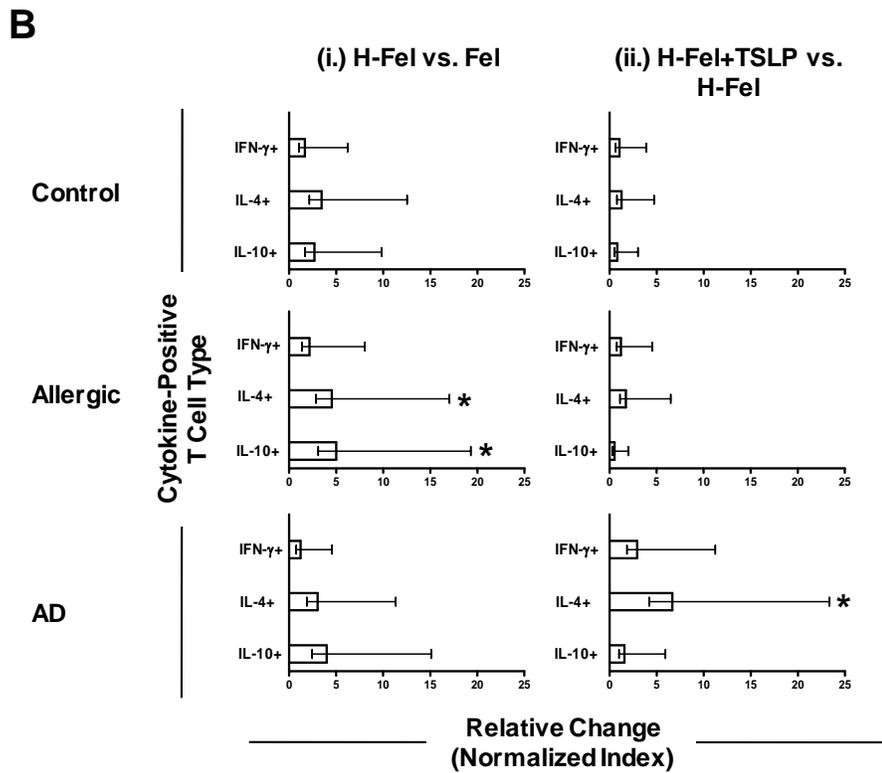
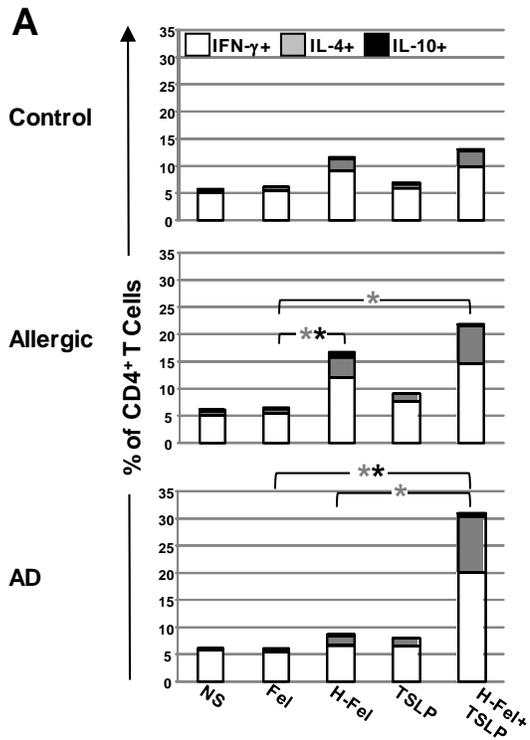
*Dendritic Cells Primed with H22-Fel d 1 and TSLP Provide a Potent Th2*

*Stimulus in Atopic Subjects.*

Consistent with our previous findings (106), stimulation of T cells with H22-Fel d 1-primed moDCs selectively increased IL-4<sup>+</sup> and IL-10<sup>+</sup> CD4<sup>+</sup> T cells in cat-allergic subjects compared with non-receptor-targeted allergen (Fel d 1)( $p < 0.05$ )(Figure 3.3A and B). We theorized that TSLP would selectively augment Th2 responses induced by H22-Fel d 1 in atopic subjects. However, when moDCs from cat-allergic subjects were primed with H22-Fel d 1 in the presence of TSLP there was no further increase in IL-4<sup>+</sup> T cells (Figure 3.3B). Surprisingly, in atopic dermatitis patients, stimulation with H22-Fel d 1 did not increase either IL-4<sup>+</sup> or IL-10<sup>+</sup> T cells as compared with non-receptor-targeted allergen (Figure 3.3B)( $p > 0.1$ ). However, when moDCs were primed with H22-Fel d 1+TSLP, IL-4<sup>+</sup> T cells were selectively enhanced compared with H22-Fel d 1 (normalized index=6.7 [2.4-16.7],  $p < 0.05$ ) and this effect was synergistic compared with either stimulus alone (Figures 3.3B and 3.4). Moreover, in contrast to allergics, total cytokine-positive T cells were markedly amplified (Figure 3.3A). Measurement of secreted cytokines showed that moDCs primed with H22-Fel d 1+TSLP selectively enhanced IL-5 and IL-13 in both atopic groups in a synergistic manner (Figure 3.5). Thus, moDCs primed with H22-Fel d 1+TSLP provided a potent Th2 skewing stimulus in cat-allergic subjects and this effect was enhanced in AD patients. Moreover, our findings showed that T cells from atopic dermatitis patients were refractory to the effects of H22-Fel d 1, but this was overcome in the presence of TSLP.

**Figure 3.3 Dendritic Cells Primed with H22-Fel d 1 and TSLP Provide a Potent Th2 Stimulus in Atopic Subjects.**

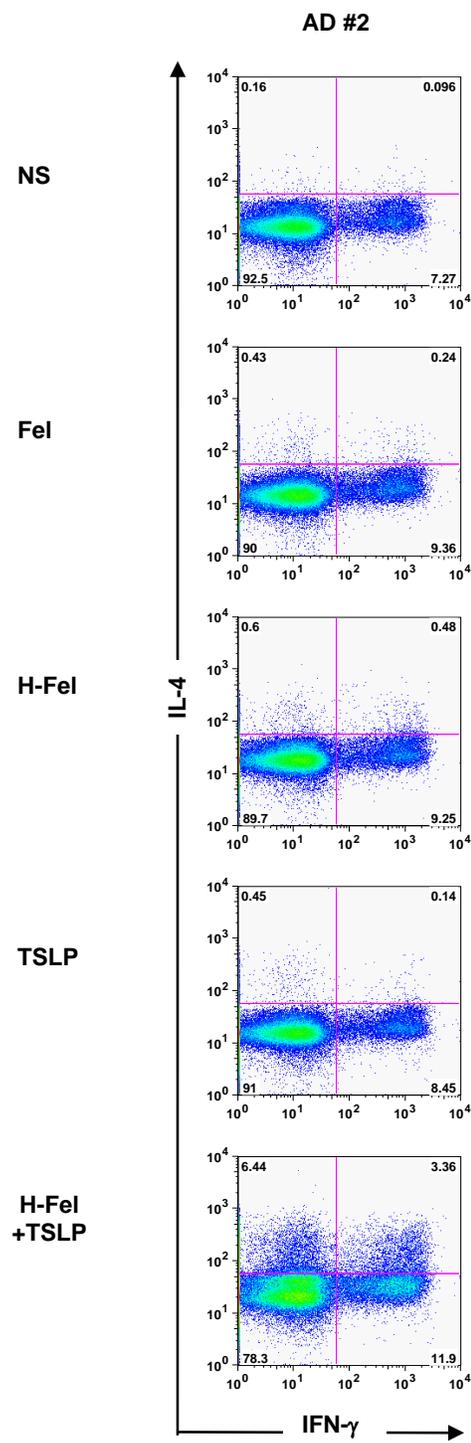
CD4<sup>+</sup> T cells were co-cultured with moDCs which had been pulsed for 48 hours with Fel d 1 (Fel), H22-Fel d 1 (H-Fel), TSLP, or H22-Fel d 1+TSLP (H-Fel+TSLP). Cytokine-positive T cells were analyzed on day 10. **A.** Summary of cytokine-positive T cell types induced by different stimuli (5 subjects per group). Bars represent the geometric mean of the percentage of total CD4<sup>+</sup> T cells for each cell type. \*Significant after adjusting for multiple comparisons. **B.** Relative change in T cell types for (i) H22-Fel d 1 versus Fel d 1, and (ii) H22-Fel d 1+TSLP versus H22-Fel d 1 (n=5 per group). \*Significant after adjusting for multiple comparisons.



**Figure 3.4 Dendritic Cells Primed with H22-Fel d 1 and TSLP Provide a Potent Th2 Stimulus in Atopic Subjects.**

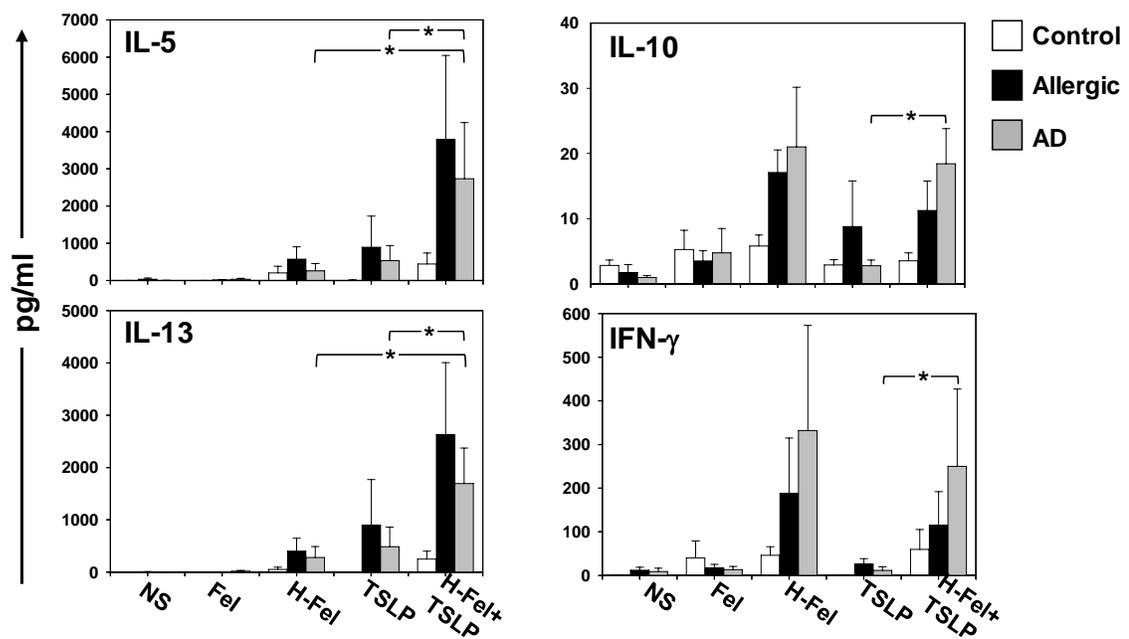
CD4<sup>+</sup> T cells were co-cultured with moDCs which had been pulsed for 48 hours with Fel d 1 (Fel), H22-Fel d 1 (H-Fel), TSLP, or H22-Fel d 1+TSLP.

Cytokine-positive T cells were analyzed on day 10. Representative dot plot from an AD patient (1 of 5) showing the phenotype of gated CD4<sup>+</sup> T cells induced by different stimuli.



**Figure 3.5 T Cells Co-Cultured with Dendritic Cells Primed with H22-Fel d 1 and TSLP Secrete Increased Levels of Th2, but not Th1 Cytokines.**

Enriched CD4<sup>+</sup> T cells were co-cultured with moDCs which had been pulsed for 48 hours with different antigens. Levels of secreted cytokines in supernatants harvested from cultures (day 10) for each group (n=5). Bars represent the mean  $\pm$  SEM.

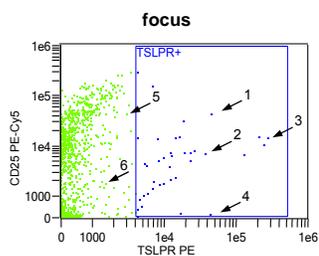


### *The Effects of TSLP are APC-Mediated*

It has recently been reported that TSLP can act directly on activated T cells owing to upregulation of TSLP receptor (TSLPr) after stimulation via the T cell receptor (157). In order to exclude this possibility, TSLP-primed moDCs were washed prior to co-culture with T cells in our system. Nevertheless, cells were cultured in complete medium containing 10% autologous serum which could, in theory contain trace levels of TSLP. Measurement of TSLP in serum samples isolated from atopic and non-atopic controls using a monoclonal antibody-based ELISA confirmed that levels were very low or undetectable (<10pg/ml). Thus, TSLP levels in our culture system were <1pg/ml, which is more than 1,000-fold less than the working concentration of TSLP used by us and other investigators. Thus, we concluded that the effects of TSLP in our system are APC-mediated. Nevertheless, since moDCs primed with H22-Fel d 1+TSLP provided a strong T cell activating stimulus, we tested whether this was sufficient to enhance TSLPr expression on T cells. This could provide a mechanism to arm T cells to respond directly to TSLP *in vivo*. In order to exclude false positives, image-based flow cytometry was performed to visually confirm each TSLPr<sup>+</sup> T cell based on a peripheral zone of staining. By this approach, TSLPr was found to be expressed on <0.1% of activated CD4<sup>+</sup> T cells (Figure 3.6). Thus, activation with a potent Th2 stimulus induces only low level expression of TSLPr on T cells.

**Figure 3.6 TSLP Receptor is Expressed on a Small Percentage of CD4<sup>+</sup> T Cells.**

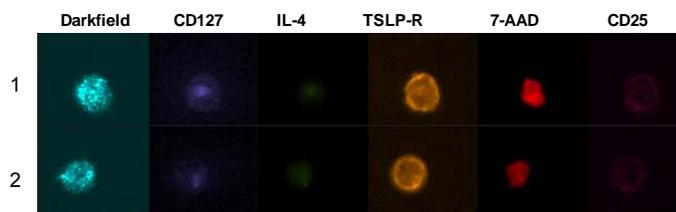
Bulk CD4<sup>+</sup> were co-cultured with autologous blood dendritic cells and stimulated with allergen + TSLP for 5 days. Cells were stained with surface and intracellular antibodies as indicated and analyzed using the image-based flow cytometry. CD4<sup>+</sup> cells were gated on CD25 and TSLPr. Representative true positive, false positive and true negative cells are shown; arrows indicate location of representative cells.



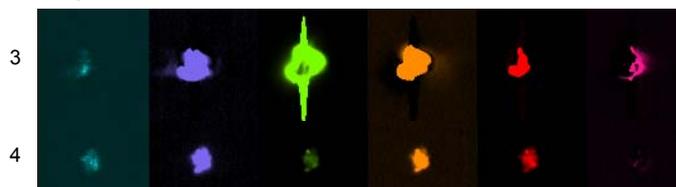
Population Statistics

Population	Count	%Gated
focus & singlets	9718	100
TSLPR+ & focus & singlets	38	0.39

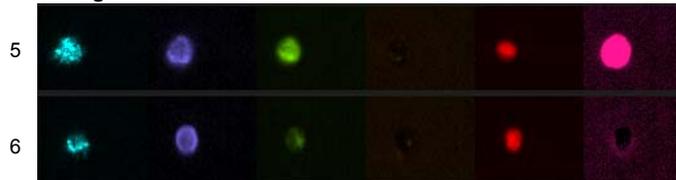
True positives



False positives



True negatives



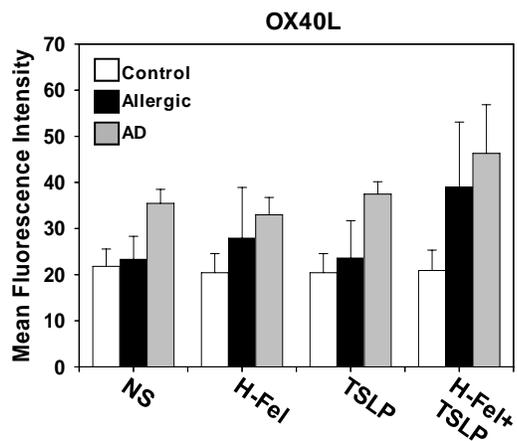
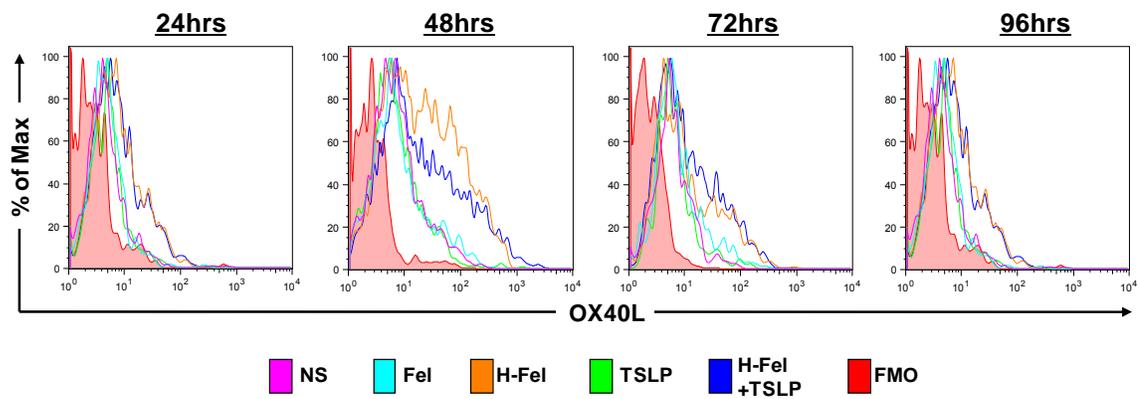
*The Th2-Promoting Effect of TSLP on H22-Fel d 1-primed moDCs is OX40 Ligand-Independent.*

It was previously reported that TSLP-primed CD11c<sup>+</sup> dendritic cells induced increased expression of OX40 ligand (OX40L) and that induction of Th2 responses was OX40 ligand-dependent (119). Thus, we investigated whether the Th2-promoting effect of TSLP was mediated via the OX40/OX40L pathway. Surprisingly, TSLP alone did not increase expression of OX40L on moDCs irrespective of allergic status and this was confirmed in time course studies (Figure 3.7A and B). In addition, H22-Fel d 1 had no effect on OX40L expression, though there was a trend towards increased expression of OX40L on H22-Fel d 1-primed moDCs in the presence of TSLP (Figure 3.7A). However, OX40L blockade did not inhibit the induction of IL-4<sup>+</sup> T cells by moDCs primed with TSLP alone, H22-Fel d 1 alone, or H22-Fel d 1 + TSLP (Figure 3.8A and B). In order to exclude a role for other factors in the Th2-promoting effects of TSLP, expression of co-stimulatory molecules (HLA-DR, CD40, CD80 and CD86) and a broad array of secreted cytokines (IL-1 $\beta$ , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, FGF basic, GM-CSF, IFN- $\gamma$ , IP-10, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , PDGFbb, RANTES, TNF- $\alpha$ , VEGF) was analyzed in moDCs. TSLP did not amplify any of these factors in H22-Fel d 1-primed moDCs (Figure 3.9A and B).

It was previously reported that TSLP-primed dendritic cells produced high levels of the Th2-attracting chemokine, CCL17/TARC (118, 119). However, TSLP alone did not induce secretion of CCL17 in moDCs and levels of CCL17

**Figure 3.7 The Th2-Promoting Effects of TSLP on H22-Fel d 1-primed moDCs are OX40 Ligand-Independent.**

**A.** MoDCs were pulsed with H22-Fel d 1 (H-Fel), TSLP, or H22-Fel d 1+TSLP and expression of OX40 ligand expression was measured by flow cytometry at 48 hours (n=5 per group). **B.** Representative time course data from one allergic subject. Antigen was washed out after 48 hours. OX40 ligand expression was measured by flow cytometry at the indicated time points. Data is shown for 1 of 2 allergic subjects and 1 of 3 AD subjects.

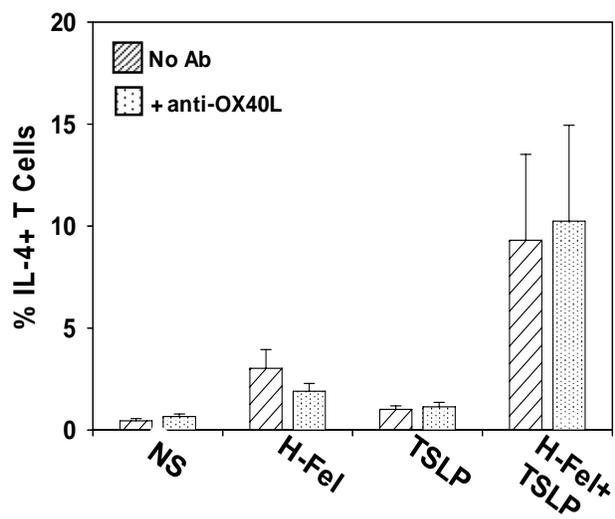
**A****B**

**Figure 3.8 OX40L Blockade Does Not Inhibit Induction of IL-4+ T Cells.**

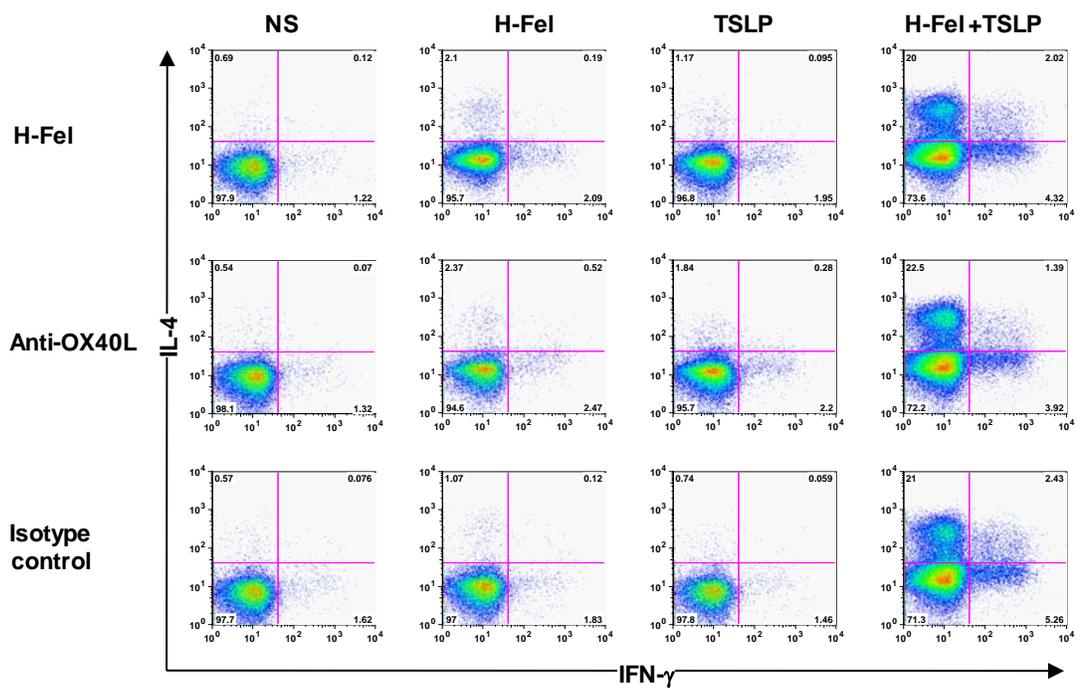
CD4<sup>+</sup> T cells were co-cultured in the presence or absence of anti-OX40L mAb with moDCs which had been pulsed for 48 hours with different stimuli. A.

Percentage of IL-4<sup>+</sup> T cells in the presence or absence of anti-OX40L mAb (n=4 atopic subjects). Bars represent the mean  $\pm$  SEM. B. Representative dot plot from an atopic subject (#8) showing the phenotype of gated CD4<sup>+</sup> T cells induced by different stimuli.

A

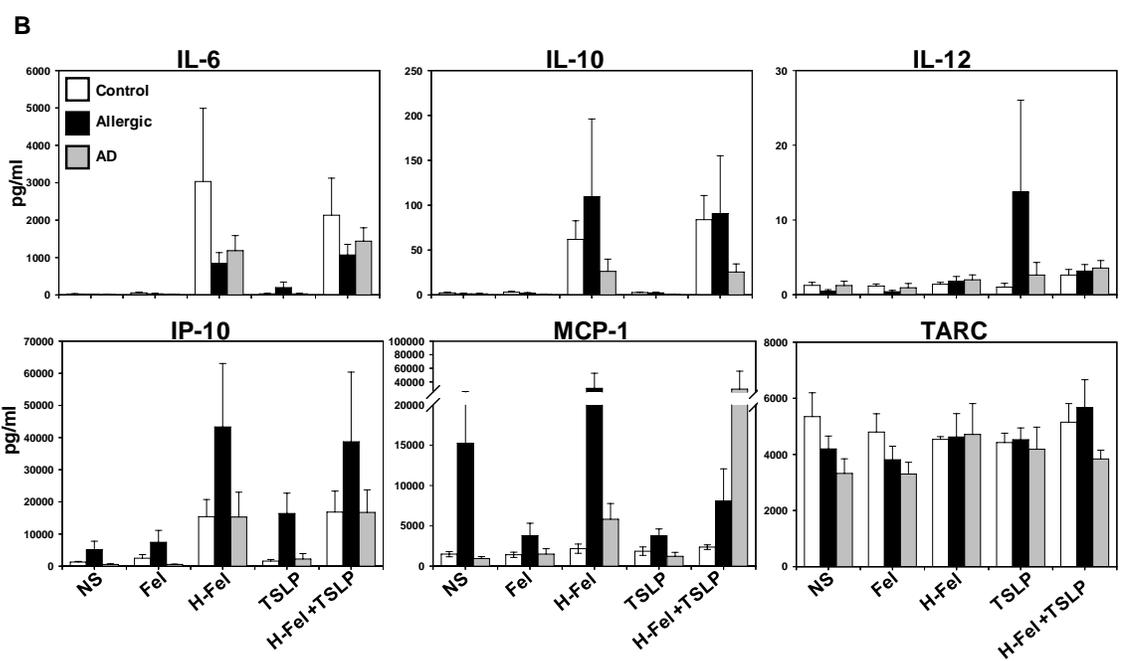
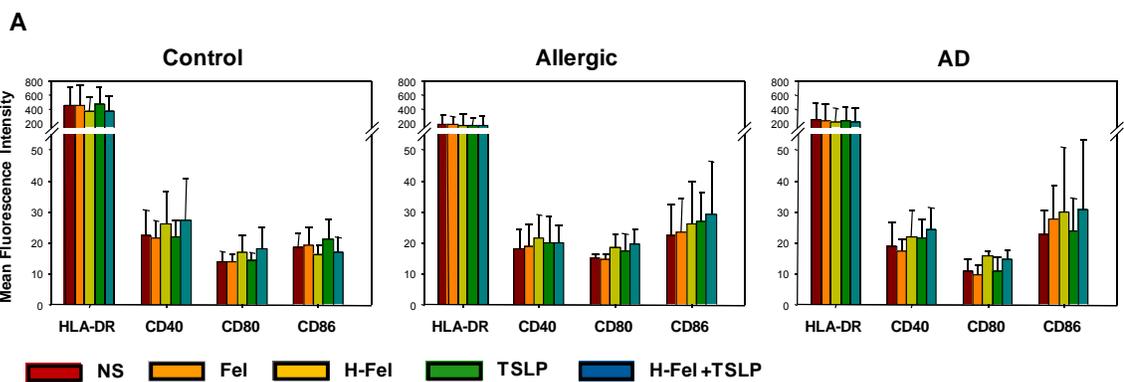


B



**Figure 3.9 TSLP Has No Effect on Co-Stimulatory Molecule Expression or Cytokine Secretion Induced by H22-Fel d 1.**

MoDCs were pulsed for 48hrs with different stimuli. **A.** Surface expression of co-stimulatory molecules was assessed by flow cytometry. Bars represent mean +SEM (n=5 per group). **B.** Cytokines were measured from culture supernatants. Representative cytokines shown of 27 analyzed by cytometric bead assay, and TARC was measured by ELISA. Bars represent mean +SEM (n=5 per group).



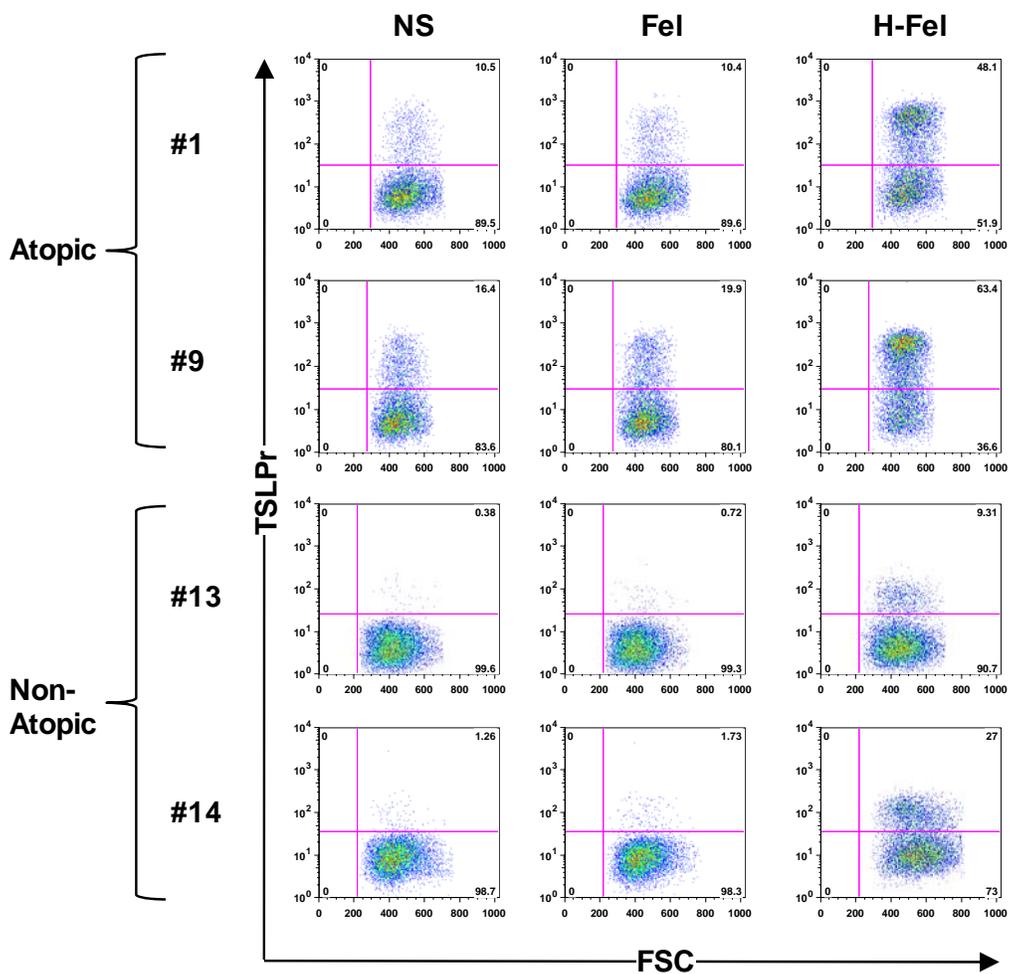
produced by H22-Fel d 1-primed moDCs in the presence of TSLP were comparable to unstimulated cells (Figure 3.9B). Collectively, our findings suggest that the Th2-promoting effects of TSLP on T cells stimulated with H22-Fel d 1-primed moDCs are mediated through a novel pathway which is OX40L-independent and not related to TARC production.

*H22-Fel d 1 Enhances TSLP Receptor Expression in Atopic MoDCs Through a Pathway Which is Regulated by PI3K and PKC.*

The high affinity IgG receptor comprises an IgG binding  $\alpha$ -chain complexed with a  $\gamma$ -chain dimer which contains a signaling motif (immune receptor tyrosine-based activation motif (ITAM)). This motif is critical to an array of Fc $\gamma$ RI-mediated effector functions. Thus, we postulated that targeting allergen to Fc $\gamma$ RI influenced TSLP-mediated pathways in atopic moDCs (ie cells from atopic dermatitis or allergic subjects) through intracellular signaling events. Initially, we examined whether H22-Fel d 1 had the capacity to induce TSLP receptor expression. TSLP receptor was constitutively expressed on atopic moDCs (Figure 3.10). Pulsing with H22-Fel d 1 markedly enhanced TSLPr expression in atopic moDCs with maximal levels expressed at 24 hours (Figure 3.11A). The presence of TSLP had no effect on TSLPr expression at early time points, while at later time points it enhanced TSLPr expression induced by H22-Fel d 1 (Figure 3.11A). In contrast to atopic cells, non-atopic moDCs did not constitutively express TSLPr; however, pulsing with H22-Fel d 1 enhanced receptor expression, though to a lesser degree compared with atopic cells

**Figure 3.10 TSLP Receptor is Constitutively Expressed on Atopic MoDCs and Upregulated by H22-Fel d 1.**

MoDCs were left unstimulated (NS) or pulsed with Fel d 1 or H22-Fel d 1 before analyzing TSLP receptor expression (24 hours) by flow cytometry. Data is representative of moDCs generated from 4 atopic subjects and 3 non-atopic controls.

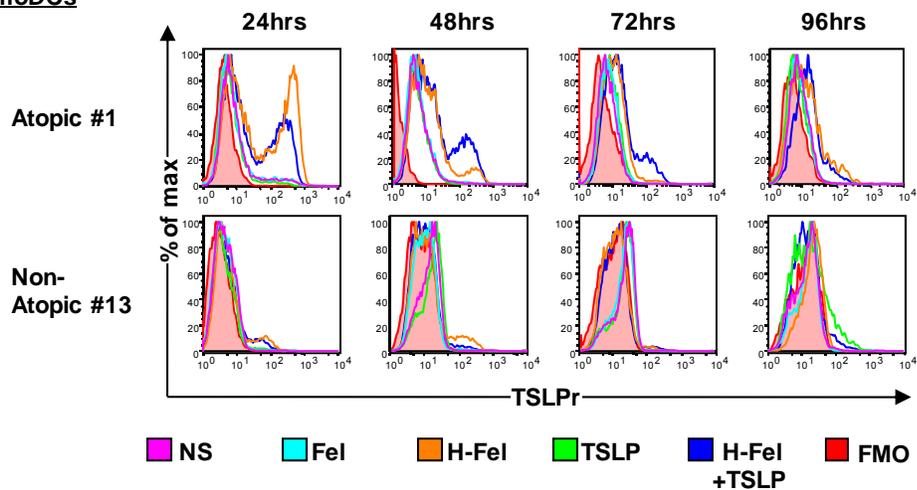


**Figure 3.11 H22-Fel d 1-Induced TSLPr Expression Peaks at 24 Hours.**

**A.** MoDCs were primed with the indicated stimuli and TSLP receptor expression was analyzed by flow cytometry. Data is shown for 1 of 3 atopic subjects and 1 of 2 non-atopic subjects. **B.** Enriched myeloid DCs were isolated from peripheral blood, pulsed with different stimuli, and analyzed at 24 hours for TSLP receptor expression. Data is shown for 1 of 2 atopic subjects.

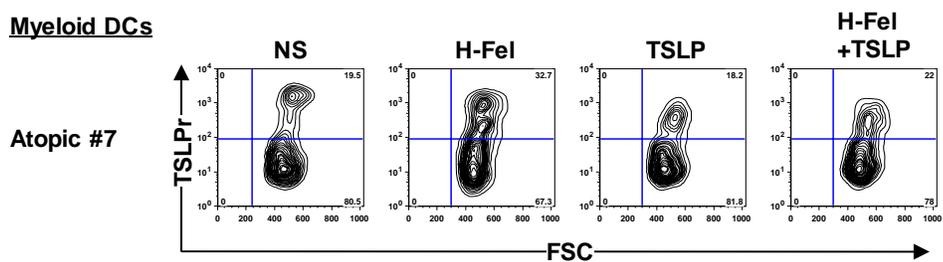
**A**

moDCs



**B**

Myeloid DCs



(Figures 3.10 and 3.11A). In contrast to H22-Fel d 1, non-receptor-targeted allergen only weakly induced TSLPr expression and this effect was variable and restricted to atopic subjects (Figures 3.10 and 3.11A).

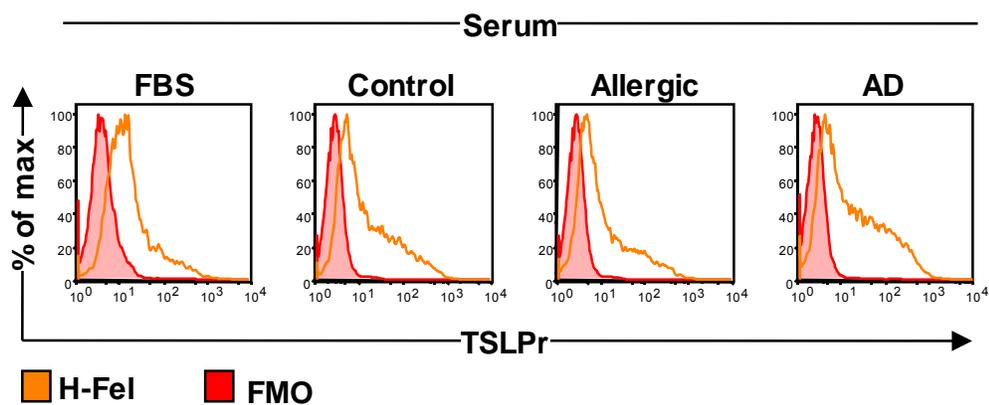
Since the properties of moDCs may differ from myeloid dendritic cells, we next examined whether myeloid dendritic cells freshly isolated from atopic subjects also upregulated TSLPr in response to H22-Fel d 1. Similar to atopic moDCs, cells enriched for myeloid dendritic cells constitutively expressed TSLPr and receptor expression was further enhanced after pulsing with H22-Fel d 1 (Figure 3.11B).

The H22 sFv domain of the H22-Fel d 1 molecule binds outside the Fc binding pocket and is monovalent. As such, it does not cross-link Fc $\gamma$ RI. Though cross-linking is not necessarily required for Fc $\gamma$ RI-mediated endocytosis, IgG antibodies are required for internalization of H22 sFv bound to Fc $\gamma$ RI (105, 158). Thus, we tested whether serum from subjects with distinct allergic phenotypes influenced TSLPr expression. To do this, we used THP-1 monocytes which have been used extensively to characterize cellular events triggered by ligation of Fc $\gamma$ RI. Interestingly, H22-Fel d 1-mediated upregulation of TSLPr was enhanced in serum from AD subjects as compared with fetal bovine serum which does not bind to human Fc receptors; however, TSLPr expression was only modestly increased in atopic dermatitis serum as compared with control serum (Figure 3.12). These findings suggested that, while serum antibodies may facilitate endocytosis, the difference in the capacity to upregulate TSLPr expression between atopic and non-atopic moDCs was primarily intrinsic to these cells.

**Figure 3.12 Serum from Atopic or Non-Atopic Subjects Does Not Affect TSLPr Expression.**

THP-1 monocytes were pulsed with H22-Fel d 1 in the presence of FBS or serum from atopic and non-atopic subjects and TSLP receptor was measured by flow cytometry at 24 hours. Data is representative of 8 experiments.

THP-1



Next, we examined whether signaling was required for H22-Fel d 1-induced TSLPr upregulation. Pulsing atopic moDCs with H22-Fel d 1, but not non-receptor-targeted Fel d 1, led to the release of intracellular calcium supporting a role for signaling (Figure 3.13A). The tyrosine kinase, Syk and the src-related tyrosine kinases (SRTK) associate with the FcR  $\gamma$  chain ITAM domain (159-161). These kinases can be inhibited by piceatannol and PP2 respectively. In the presence of these inhibitors there was a paradoxical increase in H22-Fel d 1-induced TSLPr expression in atopic moDCs and this was most evident when SRTKs were inhibited suggesting a negative regulatory role for these molecules (Figure 3.13B). To further investigate Fc $\gamma$ RI signaling we tested the effects of inhibiting downstream components of Fc $\gamma$ RI signaling. Inhibition of PI3K or PKC markedly reduced TSLPr expression in atopic moDCs in a dose-dependent manner (Figure 3.13B and C). In non-atopic moDCs, inhibition of SRTKs and PI3K had similar effects on H22-Fel d 1-induced TSLPr expression as in atopic cells. By contrast, inhibition of PKC markedly enhanced TSLPr expression. Moreover, inhibition of Syk had no effect or else reduced TSLPr expression (Figure 3.13C). Thus, PI3K positively regulates H22-Fel d 1-induced TSLPr upregulation in both atopic and non-atopic cells; however, there is a differential regulatory role for PKC and Syk kinase depending on atopic status.

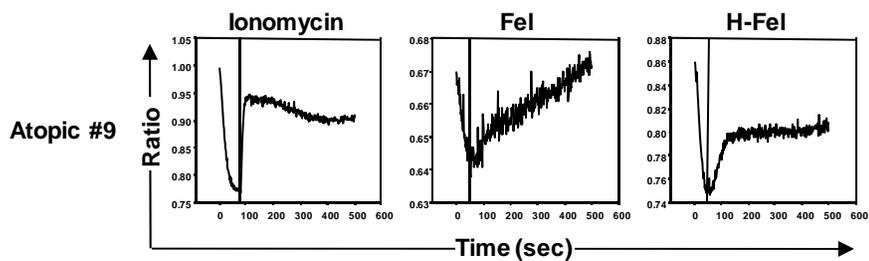
#### *TSLP Receptor is Weakly Induced by Low Dose LPS.*

Several studies support the view that LPS can act as a Th2 adjuvant in allergic models (162-165). Thus, we examined whether LPS contributed to

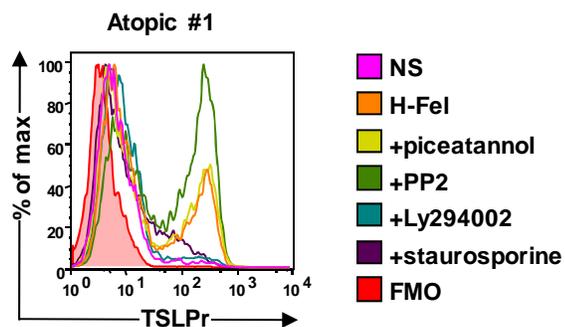
**Figure 3.13 H22-Fel d 1 Induces TSLP Receptor in Atopic moDCs Through a Pathway Which is Regulated by PI3K and PKC.**

**A.** Release of intracellular calcium was assessed by loading cells with Indo-1 and measuring the ratio of free to bound calcium after addition of antigen (indicated by vertical line). Data is representative of 4 atopic subjects. **B.** MoDCs were incubated with inhibitors for 1 hour before pulsing with H22-Fel d 1 and analyzing by flow cytometry (24 hours). Inhibitors tested were piceatannol (Syk kinase inhibitor); PP2 (src-related tyrosine kinase inhibitor); Ly294002 (PI3K inhibitor); staurosporine (PKC inhibitor). Data is representative of 4 atopic subjects. **C.** Differential effects of kinase inhibitors on H22-Fel d 1-induced TSLPr expression in atopic and non-atopic moDCs. Data is representative of 4 subjects.

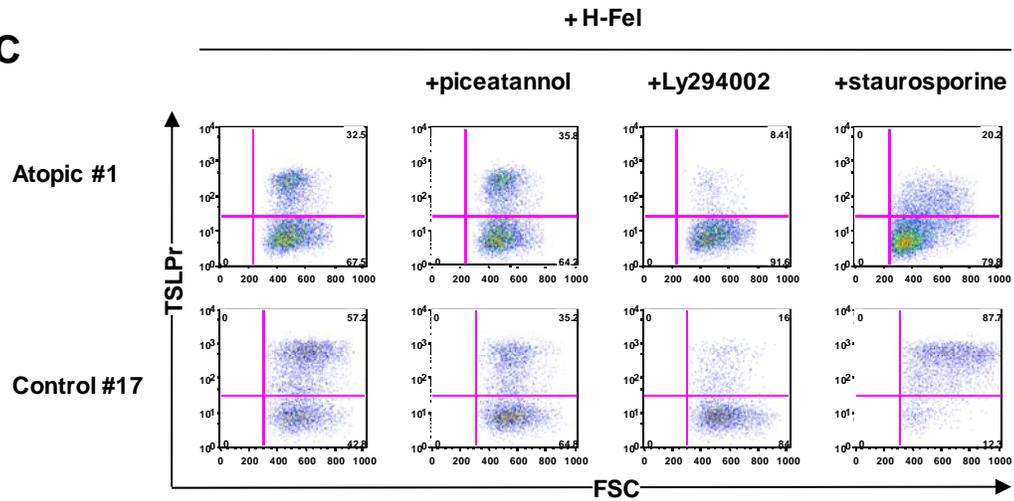
A



B



C



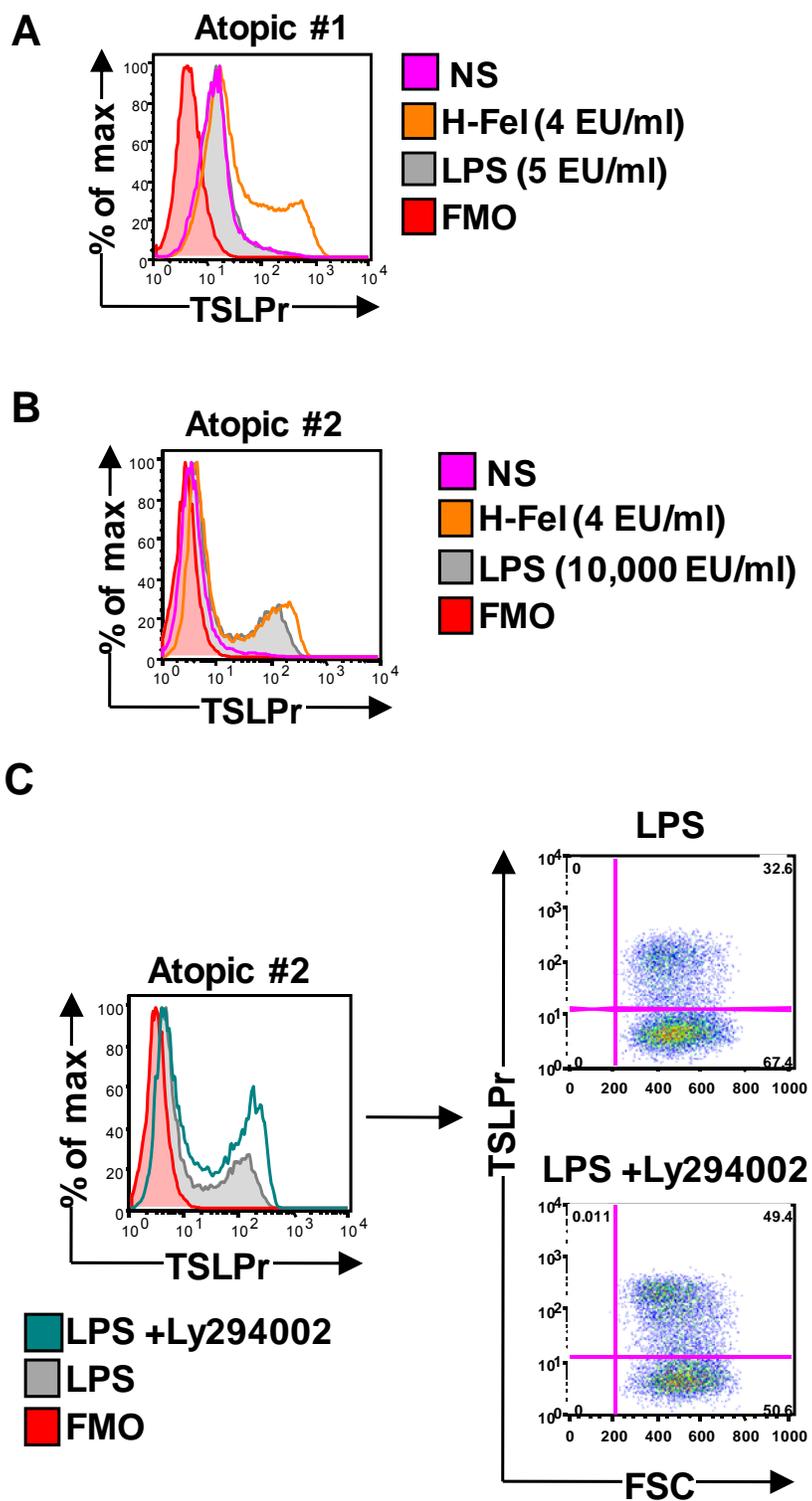
TSLPr upregulation mediated by H22-Fel d 1. The preparation of H22-Fel d 1 used contained low levels of endotoxin (~4 EU/ml working concentration). Equivalent levels of LPS (5 EU/ml) weakly induced TSLPr expression as compared with H22-Fel d 1 in atopic moDCs (Figure 3.14A). Moreover, high dose LPS (10,000 EU/ml) failed to upregulate TSLPr to levels comparable with H22-Fel d 1 in atopic moDCs (Figure 3.14B). Since LPS binds to TLR4, we examined whether signaling through this pathway was involved in LPS-induced TSLPr upregulation in atopic moDCs. Inhibition of PI3K, a negative regulator of TLR-mediated responses, enhanced LPS-induced TSLPr expression in these cells (Figure 3.14C). Thus, while LPS has the capacity to induce TSLPr expression, it mediates its effects through a distinct pathway to H22-Fel d 1.

*Inhibition of PI3K and PKC in Atopic MoDCs Abolishes the Th2-Promoting Effect of TSLP.*

Since inhibition of PI3K and PKC reduced TSLPr expression in atopic moDCs, we postulated that inhibiting these molecules in atopic moDCs would block the Th2-promoting effect of TSLP in cultures stimulated with H22-Fel d 1. Inhibition of PI3K or PKC selectively abolished IL-4<sup>+</sup> T cells in cultures stimulated with atopic moDCs which had been primed with H22-Fel d 1 in the presence of TSLP. This included those IL-4<sup>+</sup> T cells which co-expressed IFN- $\gamma$  (Figure 3.15). Inhibition of PI3K or PKC also markedly reduced IL-4-expressing cells induced by H22-Fel d 1 alone but had no effect on IL-4-expressing cells induced by TSLP

**Figure 3.14 TSLP Receptor is Weakly Induced by Low Dose LPS.**

**A.** Effect of low dose LPS and H22-Fel d 1 on TSLP receptor expression in moDCs. Cells were pulsed with H22-Fel d 1 or LPS (5 EU/ml) and TSLP receptor was analyzed at 24 hours by flow cytometry. Data is representative of 2 experiments. **B.** Comparison of TSLP receptor expression induced by high dose LPS (10,000 EU/ml) and H22-Fel d 1 on atopic moDCs. Cells were prepared and analyzed as for A. Data is representative of 4 AD subjects. **C.** Effect of inhibiting PI3K on TSLP receptor expression induced by high dose LPS. Cells were incubated with inhibitor for 1 hour prior to pulsing with LPS and analyzing as for A. Data is representative of 2 experiments.



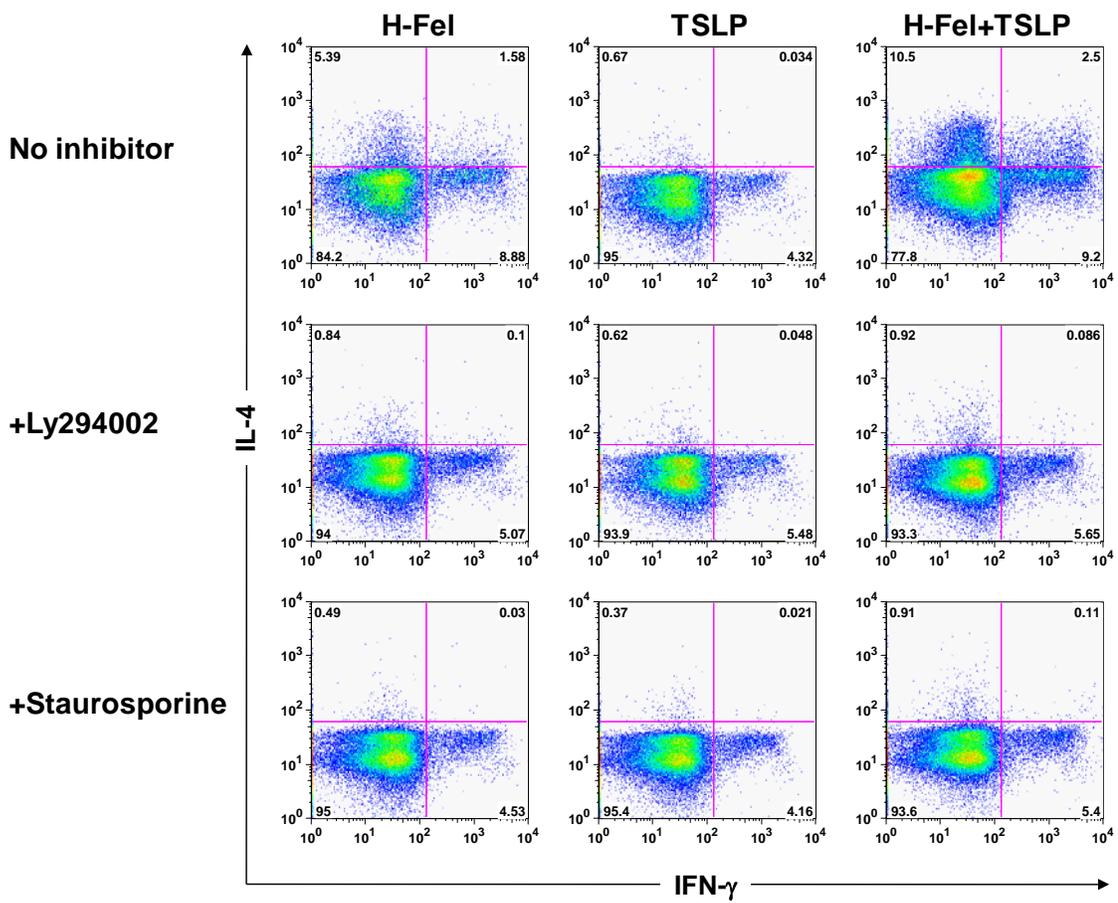
(Figure 3.15). These findings underscore the pivotal role of PI3K and PKC in mediating the Th2-promoting effects of TSLP triggered by H22-Fel d 1.

## **Discussion**

We have identified a novel immune pathway which amplifies Th2 responses in humans. Specifically, we have demonstrated that targeting cat allergen to Fc $\gamma$ RI upregulates expression of TSLPr in atopic moDCs. Upregulation of TSLPr was most pronounced in moDCs from atopic patients and Th2 responses triggered by H22-Fel d 1 were markedly amplified in the presence of TSLP in these individuals. These findings indicated that receptor-targeted allergen potentiates the Th2-promoting effect of TSLP through TSLPr upregulation. In support of this theory, we further showed that TSLPr upregulation was positively regulated by PI3K and PKC in atopic moDCs and inhibition of these molecules abolished the Th2-promoting effect of TSLP elicited by H22-Fel d 1. Our study is significant for several reasons: First, we have established differential effects of TSLP depending on allergic status; Second, we have shown that TSLP can exert Th2-promoting properties in an unconventional OX40L-independent manner; Finally, we have provided the first evidence of a link between Fc $\gamma$ R-mediated events and regulation of cytokine receptor expression in dendritic cells. It is well established that a variety of cytokines can modulate Fc $\gamma$ R expression, through cytokine-mediated signaling pathways (166-168). Our findings now show that the pathway linking Fc $\gamma$  receptors to cytokine-mediated pathways is bi-directional.

**Figure 3.15 Inhibition of PI3K and PKC Abolishes the Th2-Promoting Effect of TSLP.**

Atopic moDCs were incubated with Ly294002 or staurosporine prior to pulsing with different stimuli. Cells were then washed and cultured with CD4<sup>+</sup> T cells for 7 days. Intracellular cytokine expression was analyzed by flow cytometry. Dot plots show the phenotype of gated CD4<sup>+</sup> T cells.



Atopic moDCs are equipped to rapidly upregulate TSLPr in response to H22-Fel d 1 and this process is positively regulated by PI3K and PKC. PI3K has been shown to act downstream of Syk kinase in Fc $\gamma$ RI signaling (169). Thus, we expected that inhibition of Syk kinase would reduce TSLPr expression in a similar manner to that observed when PI3K was inhibited. However, while inhibition of Syk resulted in a variable decrease in TSLPr expression in non-atopic moDCs, expression of TSLPr was actually enhanced in atopic cells. These findings suggest that PI3K regulates TSLPr expression independently of Syk in atopic moDCs. This could occur through perturbed Fc $\gamma$ RI signaling or else through activation of an alternate Fc $\gamma$ RI-mediated pathway. Consistent with the latter scenario, it was recently shown that Fc $\gamma$  receptor signaling can occur independently of Syk through residues outside the ITAM domain (169).

Similar to Syk kinase, PKC exerted a differential regulatory role on H22-Fel d 1-induced TSLPr expression depending on atopic status. Specifically, PKC acted as a negative regulator in non-atopic moDCs, but as a positive regulator in atopic cells. A negative regulatory role for PKC in H22-Fel d 1-induced TSLPr expression is also observed in THP-1 monocytes (Hulse and Woodfolk, unpublished observations). While it is well established that PKC plays a key role in internalization of Fc $\gamma$ RI in monocytes (170), little is known about how this may translate to dendritic cells. It is tempting to speculate that the differential regulatory role of PKC on TSLPr expression in atopic versus non-atopic cells relates to enhanced receptor internalization in atopic cells owing to increased surface density of Fc receptors. Alternatively, dysregulated signaling in atopic

cells may subvert the negative regulatory role of PKC that operates in non-atopic cells.

LPS only weakly induced TSLPr in atopic moDCs; however, several lines of evidence indicate that our observations do not simply reflect the effects of endotoxin acting alone: (1) Non-receptor-targeted allergen which contains equivalent levels of endotoxin to H22-Fel d 1 is much less efficient at inducing TSLPr expression in atopic moDCs; (2) High dose LPS (10,000 EU/ml) fails to induce levels of TSLPr expression comparable to H22-Fel d 1 in atopic moDCs; (3) Blocking PI3K enhances TSLPr expression induced by LPS, but not by H22-Fel d 1. Since PI3K is a negative regulator of TLR-mediated pathways, this latter point supports the view that LPS-, but not H22-Fel d 1-induced upregulation of TSLPr, acts through the TLR4 pathway. Thus, if LPS is acting as an adjuvant in our system, it acts in a different manner.

Collectively, our data show that negative regulatory mechanisms operate to control TSLPr expression mediated by both Fc $\gamma$ R (via PKC or SRTKs) and TLRs (via PI3K). From an evolutionary standpoint, both of these receptor types evolved to eliminate pathogens. Thus, negative regulation of TSLPr by these receptors fits with a model designed to suppress non-protective Th2 responses to microorganisms. It is well established that AD patients are susceptible to skin infections and this has been attributed to cutaneous deficiencies in specific components of innate immunity. However, we propose an alternate paradigm whereby pathogens could trigger inappropriate Th2 responses in these patients through ligation of innate signaling receptors on DCs.

Interestingly, TSLPr expression can be upregulated by non-receptor-targeted allergen; however, this process is highly variable, much less efficient than for receptor-targeted allergen, and restricted to cells from atopic subjects. We postulate that this effect is mediated through ligation of FcRs expressed at high density on atopic moDCs by IgE- or IgG-bound allergen. It has been shown that moDCs from atopic patients express enhanced levels of Fc $\epsilon$ RI, and this is driven by enhanced expression of the  $\gamma$  chains of the receptor (171). Notably, these  $\gamma$  chains can be shared among Fc $\epsilon$ RI and Fc $\gamma$ RI. Moreover, high expression of Fc $\epsilon$ RI on dendritic cells from atopic subjects is pivotal to allergen uptake and T cell activation (172). Thus, dendritic cells from atopic individuals may be better equipped to upregulate TSLPr upon allergen encounter and drive Th2 responses in the presence of TSLP. Collectively, our findings provide insight into the nature of T cell responses to allergens *in vivo*. Specifically, it is widely observed that purified allergens are relatively weak inducers of Th2 responses in human-based *in vitro* systems, despite potent Th2-inducing properties *in vivo*. By examining *in vitro* T cell responses to H22-Fel d 1 in the presence of TSLP, thereby mimicking the cytokine milieu present at sites of allergic inflammation, the potent Th2-inducing effects of allergen are revealed in atopic subjects. Consistent with this, we have recently confirmed that moDCs from atopic dermatitis patients primed with non-receptor-targeted dust mite allergen (Der p 1) in the presence of TSLP, prime a robust Th2 response (unpublished observations). Thus, allergen binding to FcRs on DCs may provide the essential trigger for TSLP to act as a “master switch” for Th2 responses *in vivo*.

Interestingly, the TSLP-mediated amplification of Th2 responses triggered through Fc $\gamma$ RI was distinct from that previously described (118, 119). Notably, it did not involve the OX40L/OX40 pathway and was not associated with production of the Th2-attracting chemokine, TARC/CCL17. It is possible that these observations relate to the use of moDCs in our system, rather than CD11c<sup>+</sup> dendritic cells which have been used in previous studies (118, 119). Arguing against this, we showed that H22-Fel d 1 also enhanced TSLPr expression in freshly isolated myeloid dendritic cells. Regardless, this thesis establishes that TSLP can induce Th2 responses through a novel OX40L-independent pathway. Little is known about TSLP-mediated signaling events in human dendritic cells. While it is known that TSLP activates Stat5 in myeloid cells, other signaling molecules in this pathway are unknown (109, 173). Moreover, it appears that signaling events downstream of the TSLPr are complex with distinct pathways governing proliferation and Stat5 phosphorylation (173). From a clinical standpoint, Stat5 deficiency in humans leads to a profound eczematous phenotype (174). This raises the intriguing possibility that the effects of TSLP in atopic dermatitis do not involve Stat5.

Our findings have several major clinical implications. First, this report questions whether dendritic cell-targeted allergens will be clinically efficacious in atopic individuals. The route of vaccine administration will no doubt be a key factor to consider if TSLP-mediated effects are to be avoided. Second, thinking beyond the allergy field, it will be important to examine whether non-allergens targeted to Fc $\gamma$  receptors have the capacity to upregulate TSLPr and thus,

provide a new approach to treating Th1-driven disorders. Third, the identification of a novel pathway linking Fc $\gamma$ RI to an OX40L-independent Th2-promoting pathway paves the way to identify new TSLP-activated molecular targets for treating allergic disease. Finally and most importantly, our results strongly support the view that targeting PI3K and PKC could provide a new strategy for treating allergic disease.

## Chapter IV- Conclusions and Future Directions

### Synopsis

The work outlined in this thesis describes the immunomodulatory properties of a novel allergen variant, H22-Fel d 1, at the T cell and dendritic cell level. Initially, we examined the effects of H22-Fel d 1 on moDCs from cat-allergic and non-allergic subjects (Chapter II). Stimulation of moDCs with H22-Fel d 1 induced a semi-mature phenotype characterized by no change in expression of co-stimulatory molecules, but enhanced production of Th1-associated cytokines and chemokines with no change in Th2-promoting factors. Subsequently, we showed that H22-Fel d 1-primed moDCs induced an increased frequency of IL-5<sup>+</sup> and IL-10<sup>+</sup> CD4<sup>+</sup> T cells, and these included subtypes characteristic of regulatory T cells. Interestingly, the effects of H22-Fel d 1 on T cell responses were restricted to cat-allergic subjects. The repertoire of CD4<sup>+</sup> T cells induced by H22-Fel d 1 was distinct from that induced by Fel d 1 in modified Th2 responders and included more IL-10<sup>+</sup> subtypes than that induced by the prototypic dust mite allergen, Der p 1. Collectively, these data suggested that H22-Fel d 1 may provide a novel therapeutic molecule for treating cat-allergic subjects.

Next, we investigated whether H22-Fel d 1 had the potential to induce protective T cell subsets in the presence of a cytokine milieu akin to that encountered *in vivo* in allergic subjects. To do this, we examined the effects of H22-Fel d 1 on the T cell repertoire induced in the presence of TSLP (chapter III).

In contrast to previous reports, TSLP was a relatively weak inducer of IL-4<sup>+</sup> T cells irrespective of allergic status, though there was a trend towards increased Th2 induction in highly atopic individuals. However, moDCs stimulated with both H22-Fel d 1 and TSLP induced a robust Th2 response, and this effect was most marked in subjects with atopic dermatitis. After verifying that TSLP was acting on moDCs in our system, we examined the mechanisms involved in driving Th2 amplification. Analysis of expression of surface co-stimulatory molecules and secreted cytokines by moDCs stimulated with H22-Fel d 1 either with or without TSLP failed to identify candidate molecules which may play a role in this process. Given the magnitude of the Th2 response generated, we postulated that H22-Fel d 1 promoted the effects of TSLP by acting on the TSLP pathway. It was previously shown that TSLP induced Th2 responses in an OX40L-dependent manner (119). However, there was no effect of TSLP on the levels of expression of OX40L induced by H22-Fel d 1, and Th2 responses were not inhibited by OX40L blockade. Interestingly, atopic moDCs constitutively expressed TSLPr and H22-Fel d 1 enhanced expression of this receptor, thereby providing a mechanism for amplification of the TSLP pathway. Extending this observation, we incorporated selective kinase inhibitors to investigate whether signaling through Fc $\gamma$ RI was involved in TSLPr upregulation, and whether this played a role in the augmentation of Th2 responses. The bulk of previous work investigating signaling events triggered by Fc $\gamma$ RI was performed in cells other than dendritic cells; these include primary human monocytes or macrophages, and an array of human and murine cell lines. Because of this, existing

knowledge regarding mechanisms involved in Fc $\gamma$ RI signaling in dendritic cells is scant. Nevertheless, we demonstrated that upregulation of TSLPr induced by H22-Fel d 1 on moDCs from atopic subjects was positively regulated by both PI3K and PKC. By contrast, PKC negatively regulated TSLPr expression in moDCs from non-atopic subjects. Extending these results to the T cell level, inhibition of PI3K or PKC abolished IL-4+ T cells induced by H22-Fel d 1 in the presence of TSLP. Collectively, these results strongly support the view that H22-Fel d 1 potentiates the Th2-promoting effects of TSLP through Fc $\gamma$ RI signaling.

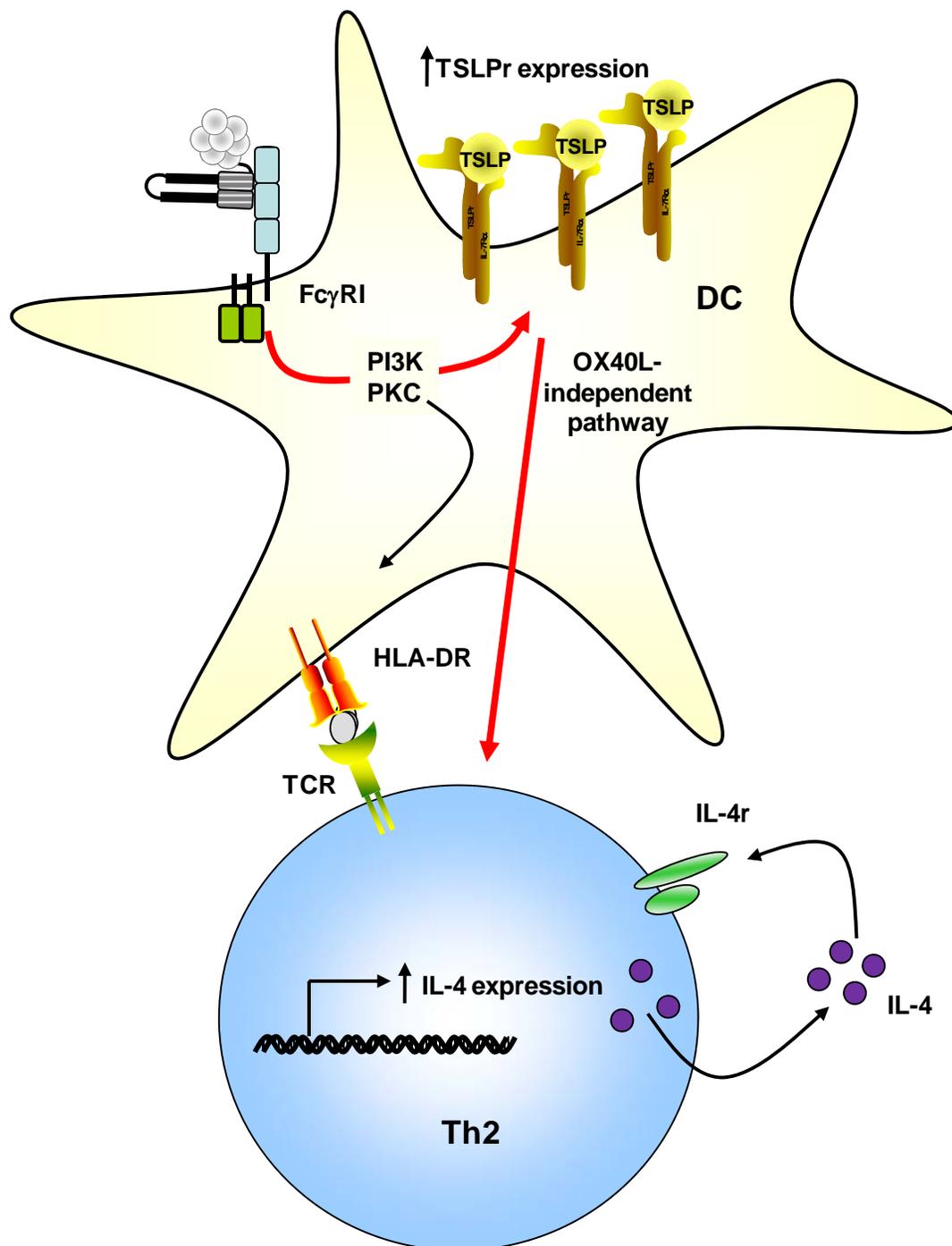
Our findings suggest that TSLPr upregulation involves components of different signaling pathways. Characterization of a novel Th2 regulatory pathway linking Fc $\gamma$ RI to TSLPr and consequent TSLP-driven events has important clinical implications for dendritic cell-based vaccines related to allergy. In addition, it identifies dendritic cell signaling components which could provide therapeutic targets for allergic disease.

#### *Model*

Based on our results, we propose a model whereby engagement of H22-Fel d 1 triggers signaling through Fc $\gamma$ RI on dendritic cells in atopic subjects. This leads to upregulation of TSLPr surface expression that primes these cells to respond to TSLP (Figure 4.1). Signaling pathways mediated by Fc $\gamma$ RI in dendritic cells include PI3K and PKC. These pathways may play a role in the uptake and processing of H22-Fel d 1, but also directly upregulate TSLPr expression. Consequently, TSLP-mediated signaling enhances the Th2-inducing

**Figure 4.1 Model of H22-Fel d 1-Induced TSLPr Expression and Th2 Augmentation in Atopic Subjects.**

Engagement of H22-Fel d 1 triggers signaling through Fc $\gamma$ RI on dendritic cells in atopic subjects. This leads to upregulation of TSLPr surface expression which primes cells to respond to TSLP. Signaling pathways mediated by Fc $\gamma$ RI in this system include PI3K and PKC, which could act through distinct pathways. These pathways may play a role in the uptake and processing of H22-Fel d 1, but also directly upregulate TSLPr expression. Consequently, TSLP-mediated signaling enhances the Th2-inducing properties of the antigen presenting cell in an OX40L-independent manner, which results in increased T cell expression of IL-4 and amplification of Th2 responses.



properties of the antigen presenting cell. In contrast to this, H22-Fel d 1-induced signaling in moDCs from non-atopic subjects leads to a more modest upregulation of TSLPr, and is negatively regulated by PKC. This suggests that negative regulatory mechanisms operate in non-atopic moDCs to inhibit TSLPr expression in response to Fc $\gamma$ RI-mediated signals (Figure 4.2). Future investigations should focus on elucidating epigenetic variations between moDCs from atopic and non-atopic subjects that could account for the observed differences in their ability to upregulate TSLPr expression and selectively amplify Th2 responses. These are discussed in detail below.

### **Future Directions**

*Examine accessibility of the *tslpr* gene locus in moDCs from atopic and non-atopic donors.*

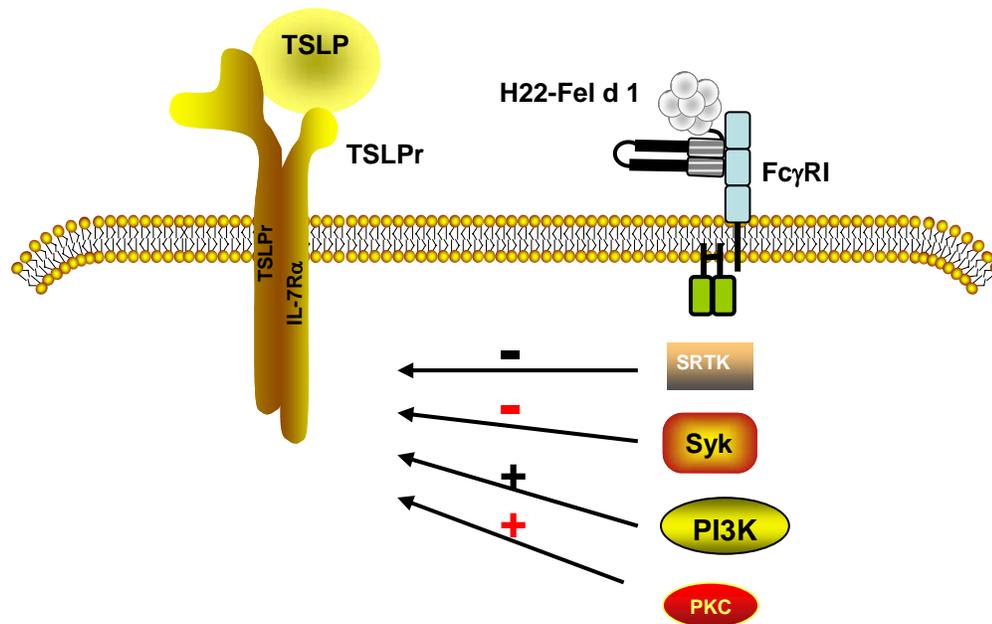
Work performed in this thesis identified positive and negative regulatory elements with evidence of differential roles for specific signaling components. Yet, the mechanisms that underly the ability for PKC to positively or negatively regulate expression of TSLPr in atopic and non-atopic cells are unclear. We expect that these signaling molecules activate transcription factors involved in TSLPr expression. However, the key difference between atopic and non-atopic moDCs may lie in the ability of transcription factors to bind to and activate the *tslpr* locus. Chromatin accessibility is a key factor in dictating whether a particular gene will be expressed and is determined by chemical modifications of

the histone proteins that are required for proper chromatin structure. These epigenetic changes involve methylation, acetylation, ubiquitination and

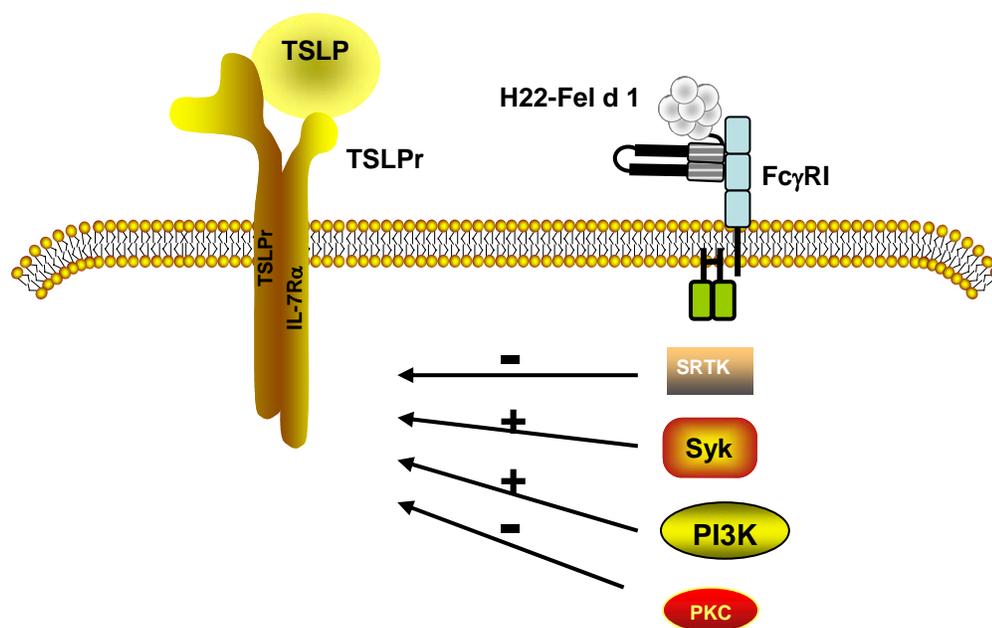
**Figure 4.2 Differential Effects of Selective Kinase Inhibitors on H22-Fel d 1-Induced TSLPr Expression in Atopic and Non-Atopic moDCs.**

SRTKs have a negative regulatory effect and PI3K has a positive regulatory effect on H22-Fel d 1-induced TSLPr expression, regardless of atopic status. However, in moDCs from non-atopic donors, Syk has a positive regulatory effect and PKC has a negative regulatory effect. The effects of Syk and PKC are reversed in moDCs from atopic subjects suggesting dysregulated control of H22-Fel d 1-TSLPr in atopic subjects.

## Atopic moDC



## Non-atopic moDC



phosphorylation of amino acids within the histone tails or amino-terminal ends of histone proteins. For example, acetylation of histones H3 and H4 is associated with uncoupling of the DNA-histone interaction, which results in accessibility of that region. Similarly, methylation of lysine 4 of histone H3 is associated with active gene expression; however, methylation of lysine 9 of histone H3 is linked to gene silencing.

We hypothesize that the *tslpr* locus is more accessible in cells from atopic subjects compared with non-atopics. The relative availability of the *tslpr* locus in cells from atopic and non-atopic subjects can be assessed using chromatin immunoprecipitation (ChIP) and quantitative RT-PCR techniques. Our data suggests that TSLPr is constitutively expressed on moDCs from atopic but not non-atopic subjects. Initial experiments could examine the baseline accessibility of the *tslpr* locus by using unstimulated cells. Quantitation of the *tslpr* gene by RT-PCR after pull-down with anti-acetylated histone H3 Abs will allow comparison of the baseline methylation state of that locus in atopic and non-atopic cells. These results could then be extended by priming atopic and non-atopic moDCs with H22-Fel d 1 and reassessing the relative levels of *tslpr* associated with acetylated histones after stimulation.

Based on our results we postulate that at baseline, the *tslpr* locus is more accessible in cells from atopic donors, and that this availability is enhanced in the presence of H22-Fel d 1 compared with cells from non-atopics. However, it is possible that even if the baseline accessibility is higher in cells from atopic subjects, stimulation with H22-Fel d 1 has no additional epigenetic effect, but

instead enhances activation of a transcription factor required for TSLPr expression in a PI3K- or PKC-dependent manner. If this is the case, we would expect to see no change in the accessibility of the *ts/pr* locus in response to H22-Fel d 1. CHIP analysis which incorporates Abs for methylated histones could also be performed to confirm results.

*Investigation of the transcription factors involved in ts/pr gene expression.*

Epigenetic modifications alter the accessibility of genes to transcription factors that are required for the expression of the gene. Once the role of epigenetic modifications of the *ts/pr* locus in regulating *ts/pr* expression has been determined, it will be important to characterize which transcription factors bind to this promoter region and induce expression. Promoter regions of genes contain many regulatory elements that are critical for gene expression. Investigation of the promoter region of the *ts/pr* gene could yield valuable information regarding putative binding sites for known transcription factors. Since, differences in TSLPr expression between atopic and non-atopic cells may be due to epigenetics, we would not expect to find a difference in the requirement for specific transcription factors in cells based on their atopic status. However, elucidation of which transcription factors are required for *ts/pr* expression will allow further understanding of the regulation of TSLPr expression in general.

To date, the promoter region of the human *ts/pr* gene has not been characterized. However, as mentioned in chapter I, the human TSLPr is 24% homologous to the IL-2 receptor common  $\gamma$  chain ( $\gamma_c$ ), and this could provide

insight into putative transcription factors that may also play a role in *ts/pr* gene expression (109). It has been reported that the 5' promoter region of the  $\gamma_c$  contains consensus Ets binding sites (EBS)(CCCCTTCG) that associate with the transcription factors GABP and Elf-1 (175, 176). We postulate that the promoter of the *ts/pr* gene also contains EBS that could activate transcription. Computer prediction algorithms could be used to scan the promoter region of the *ts/pr* locus in order to identify putative EBS motifs. If any EBS are identified, electrophoretic mobility shift assay (EMSA) could then be performed to confirm binding of known EBS transcription factors, such as GABP or Elf-1. Finally, the transcriptional activity of these proteins could be confirmed by luciferase assay in THP-1 cells (177).

Due to the sequence homology between the  $\gamma_c$  and *ts/pr* genes, we would predict that these genes are regulated by similar mechanisms, which operate through EBS domains. However, GABP and Elf-1 are only two examples of many possible transcription factors which are known to bind to these sites. It may be necessary to investigate alternate EBS binding factors in order to identify a specific binding partner for the *ts/pr* promoter region. Moreover, it is also reasonable to assume that the regulation of the *ts/pr* gene is distinct from, or involves more regulatory sites than the  $\gamma_c$ . If no putative EBS regions are identified, or regions identified do not account for the transcriptional activity of the locus, further characterization of the *ts/pr* promoter in order to identify other putative transcription factor binding sites will be necessary.

*Investigate the role of PI3K and PKC in activation of transcription factors required for *tslpr* expression.*

Our data suggest that both PI3K and PKC play a role in the regulation of TSLPr expression, and we hypothesize that they are responsible for the activation of a critical transcription factor. Once we have identified a transcription factor required for TSLPr expression, we would next investigate whether its activation is controlled by PI3K or PKC. We would not expect to find a differential role for PKC in transcription factor activation between atopic and non-atopic cells, since this may be controlled at the epigenetic level.

Activation of the EBS related transcription factors GABP and Elf-1 occurs via phosphorylation (178, 179). Initially, we would investigate the basal phosphorylation state of these proteins in unstimulated moDCs by immunoprecipitation and western blotting. These results could then be extended by incorporating Ly294002 or staurosporine to inhibit PI3K or PKC activity respectively. We would predict that inhibition of PI3K or PKC would decrease basal activation of GABP and Elf-1. Because H22-Fel d 1-primed moDCs upregulated expression of TSLPr, we could also investigate the effects of H22-Fel d 1 on the phosphorylation state of GABP and Elf-1 in the presence or absence of Ly294002 or staurosporine. We would expect H22-Fel d 1 stimulation to enhance the activation of transcription factors required for *tslpr* expression, and this would be decreased in the presence of Ly294002 or staurosporine. Alternatively, it is reasonable to assume that expression of the *tslpr* gene is controlled by both positive and negative regulators of transcription.

As such, we could also explore the complement of activating and repressing factors present, and how they may work together to ultimately determine gene transcription.

In summary, identification of the epigenetic and transcriptional machinery required for expression of the *ts/pr* gene could provide novel insights into the key differences between moDCs from atopic and non-atopic subject that ultimately result in amplification of Th2 responses. Furthermore, elucidation of these pathways will provide new strategies for treating allergic disease.

### **Final Conclusions and Clinical Implications**

We have demonstrated that targeting the major cat allergen to Fc $\gamma$ RI using the novel allergen variant, H22-Fel d 1, induces a T cell cytokine repertoire in cat-allergic subjects that incorporates protective elements. However, the magnitude and quality of the T cell response triggered by H22-Fel d 1 are altered in the presence of the “pro-allergic” cytokine, TSLP. Specifically, Th2 responses are selectively increased through Fc $\gamma$ RI-mediated events which enhance the TSLP pathway through upregulation of TSLP receptor. A key issue which remains is whether targeting allergens to Fc $\gamma$ RI is an appropriate strategy for treating allergic disease. This will depend on several factors including the dosing regimen and the capacity for TSLP and receptor-targeted allergen to exert a coordinated effect *in vivo*. In relation to the first point, numerous studies have established the clinical efficacy of conventional immunotherapy which involves injection of allergen extract in an updosing regimen (62, 64, 79, 86). Moving

forward, it will be important to determine how the dosing regimen *in vivo* influences the quality of the T cell response induced by H22-Fel d 1. In relation to the second point, TSLP is expressed in the skin lesions of patients with atopic dermatitis, and it has been reported to be constitutively expressed by skin keratinocytes (118). Thus, receptor-targeted allergen injected into the skin of patients with atopic dermatitis could, in theory, encounter dendritic cells which are actively responding to TSLP. Migration of these cells from the skin to regional lymph nodes could generate a robust Th2 response and homing of effector T cells back to the skin could then exacerbate inflammation. This process would obviously depend on the ability for dendritic cells to rapidly upregulate TSLPr in the skin in response to H22-Fel d 1 where TSLP is expressed. However, it is also possible that injection of receptor-targeted allergen into healthy skin where levels of TSLP are low would preclude these effects.

It should be pointed out that while moDCs stimulated with both H22-Fel d 1 and TSLP induced a robust Th2 response, the Th1 cytokine IFN- $\gamma$ , was also a feature of the T cell response generated. Specifically, a proportion of IL-4<sup>+</sup> T cells (roughly a third) co-expressed IFN- $\gamma$ , and the total frequency of IFN- $\gamma$ <sup>+</sup> T cells was increased compared with either stimulus alone, though this did not reach significance. Since Th1 skewing has been associated with a favorable treatment outcome in clinical trials of immunotherapy, it is difficult to predict whether the quality of the Th2 response induced by co-stimulated moDCs would translate to a protective or non-protective response *in vivo*. Unfortunately, small

animal models will likely not be useful for addressing this question in light of the disappointing discrepancies between mice and men which have now been reported for a variety of potential therapeutics for allergic disease. Thus, it is likely that definitive answers may only be attained in large animal models or else in clinical trials.

We have characterized a novel pathway linking Fc $\gamma$ RI signaling to upregulation of TSLPr expression and consequent TSLP-driven events. Inhibition of signaling components required for TSLPr upregulation essentially abolished Th2 responses. This finding is of major importance since it suggests that targeting FcR signaling components on dendritic cells may be a rational objective for treating Th2-driven inflammatory disorders. Conversely, targeting antigens to FcRs which modulate TSLPr expression could also have important implications for Th1-mediated diseases where enhanced Th2 responses would be favorable. Indeed, it has recently been shown in a mouse model of autoimmune diabetes that injection of TSLP-primed dendritic cells into pre-diabetic NOD mice was able to prevent induction of diabetes (180). Finally, elucidation of the mechanisms responsible for the differential upregulation of TSLPr expression in atopic cells could provide new molecular targets for the treatment of allergic diseases.

In conclusion, studies on H22-Fel d 1 described in this thesis reveal a novel pathway which regulates Th2 responses in atopic individuals. This work provides a springboard for studying new immune pathways which may be exploited in the treatment of allergic disease.

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