IL-8+ T CELLS IN ALLERGIES

IDENTIFYING THE FREQUENCY AND PHENOTYPE OF IL-8+ T CELLS IN CAT ALLERGIC AND NON-CAT ALLERGIC SUBJECTS

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

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McMaster University MASTER OF SCIENCE (2019) Hamilton, Ontario (Medical Sciences, Infection and Immunity)

TITLE: Identifying the frequency and phenotype of IL-8+ T cells in cat allergic and non-cat allergic subjects

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NUMBER OF PAGES: xiii, 130

LAY ABSTRACT

Allergies occur when immune cells in our body make an exaggerated response to a harmless foreign substance like a pollen protein, which is referred to as an “allergen”, because it causes an allergic response. We believe that part of this reaction may be happening due to a signalling molecule, called IL-8, that is released into the body after encountering the allergen and that may play a role in causing allergic symptoms. In this study, we looked to see if an immune cell, called a T cell, can produce IL-8 and if these cells are present in higher quantities in allergic people and not present in non-allergic people. We found that the number of these T cells were not different between allergic and non-allergics.

ABSTRACT

Advances in immunotherapy have led to the development of peptide immunotherapy (PIT) which has shown clinical efficacy in a reducing allergic symptoms and providing long-lasting benefits. In mechanistic studies, we identified that PIT reduced the transcription of IL-8 in allergen-specific T cells coinciding with no increase in blood neutrophil counts after a nasal allergen challenge. IL-8 is a chemoattractant that elicits the migration and activation of neutrophils and basophils, causing the release of histamines and leukotrienes which are effector molecules implicated in allergic diseases. We hypothesized that in allergic individuals, a novel T cell subset, 'Th8', may exist and are primed to produce and release IL-8 upon allergen exposure. In this study, we determined the frequency and phenotype of allergen-specific IL-8+ T cells through flow cytometry and ELISpot assays in a clinical study with 12 cat allergic and 13 non-cat allergic subjects. In cat allergics, the frequency of IL-8+CD4+ T cells was 2.14±1.02% while non-cat allergics had a frequency of 1.99±0.97% at baseline. Stimulation with cat allergens resulted in a small increase in the frequency of IL-8+ T cells in both groups. In the population of IL-8+ T cells, only a minor subpopulation were allergen-specific as <2% expressed CD154. Allergen-specificity of these cells were also assessed through MHC Class II tetramer staining, which yielded a similar result as 99.7±153.8 TET+IL-8+ T cells/million CD4 were detected in cat allergics compared to 50.9±43.5/million CD4 in non-cat allergics. Phenotyping of IL-8+ T cells suggests that these cells may have similar homing characteristics as Th2 cells due to the expression of CCR4. The presence of IL-8+ T cells detected in the study suggests that there may be a small population of allergen-

specific T cells that produce IL-8 upon allergen exposure that contributes to the chemotaxis/activation of neutrophils and basophils, leading to allergic symptoms.

ACKNOWLEDGEMENTS

This research is supported by CIHR. I thank my supervisor, Dr. Mark Larché for his continual guidance and expertise throughout my Master's degree; my supervisory committee members, Dr. Matthew Miller and Dr. Carl Richards, for their support and guidance; and to current and former members of the Larché lab for their support and assistance in my research, Dr. Elena Tonti, Dr. Varun Anipindi, Dr. Tom Mu, Cheryl Kipling, Lesley Wiltshire, Deanna French and Tracy Rerecich.

Lastly, thank you to my friends and family who supported me through all these years of school and to all the participants who joined the study for the good of science.

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ABBREVIATIONS

Acute generalized exanthematous pustolosis……...AGEP
Alexa Fluor……...…….AF
Alkaline phosphatase…….....ALP
Allergen-presenting cell…….APC
Allergen immunotherapy…….AIT
Allergic rhinitis…….AR
Amino acid……...AA
Brefeldin A…….BFA
Brilliant Violet……..BV
CD4+CD25+ regulatory T cell……...Treg
Cell preparation tube……...CPT
Central memory T cell……T_{CM}
Chemoattractant receptor-homologous molecule expressed on Th2 cells……....CRTH2
Cluster of differentiation…….……..CD
Complete blood count……...….CBC
Dendritic cell…….……DC
Early phase response…….…..EPR
Effector memory re-expressing CD45RA……....T_{EMRA}
Effector memory T cell…….……...T_{EM}
Endoplasmic reticulum…….…….ER
Enzyme-linked immune absorbent spot…….……...ELISpot
Enzyme-linked immunosorbent assay……ELISA
Ethylenediaminetetraacetic acid……..EDTA
Fluorescence minus one…….FMO
Forward scatter…….…...FSC
House dust mite…….…HDM
IL-10 secreting regulatory T cell..…….……...Tr1
Immunoglobulin…….……Ig
Interferon…….……IFN
Interleukin…….……..IL
Ionomycin…….I
Late phase response…….……...LPR
Leukotriene E4…….……..LTE_4
Lipopolysaccharide…….……LPS
Major Histocompatibility Complex……..MHC
Median fluorescence intensity…….MFI
Naïve T cell……T_N
Paraformaldehyde……...PFA
Peptide immunotherapy……....PIT
Peripheral blood mononuclear cell……..PBMC
Phorbol-Myristate-Acetate……PMA
Phytohemagglutnin……PHA

Polyinosinic-polycytidylic acid……..poly (I:C)
Prostaglandin D2……...PGD_2
Protein kinase C……..PKC
Recent thymic emigrant……..RTE
Red blood cell……RBC
Resiquimod…….……...R848
Ribonucleic acid…….…RNA
Side scatter……..……SSC
Subcutaneous allergen immunotherapy…….SCIT
Sublingual allergen immunotherapy……..SLIT
Synthetic peptide immune-regulatory epitopes……...SPIRE
T cell receptor…….…TCR
Tetramer……..TET
Toll-like receptor…….……...TLR
Tumor necrosis factor……TNF
Type 1 helper T cell…….……Th1
Type 2 helper T cell……Th2
United States……...U.S.

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Maria Nguyen, performed all experiments described in this document with assistance from Dr. Tom Mu, Lesley Wiltshire, Caitlin Obminski, Deanna French and Tracy Rerecich, in collecting blood samples from study participants. All sections in this document were written by me and reviewed by Dr. Mark Larché.

INTRODUCTION

Hundreds of millions of people worldwide are affected by one or more allergic disease which includes asthma, rhinitis, conjunctivitis, urticaria, eczema, and food, drug and insect allergies (Pawankar *et al.*, 2011; Pawankar, 2014). Allergic rhinitis (AR) is one of the more predominant diseases as it affects between 10-30% of the world's population (Pawankar *et al.*, 2011). AR presents with symptoms of nasal congestion, runny nose, and itching of the nose, eyes and throat. There are two types of allergic rhinitis, seasonal and perennial. Seasonal AR, also known as hay fever, occurs during spring, summer or fall, where levels of pollen from trees, grass and weeds, as well as airborne mold, are the highest. Meanwhile, perennial AR exhibits symptoms year-round and is caused by allergies to house dust mite (HDM) and animal danders (Skoner, 2001). The socioeconomic burden of AR is substanial as it can lead to loss of productivity in school and work and high medical costs to treat problems associated with AR, such as asthma and sinusitis (Skoner, 2001).

Overall, allergic diseases are a global health concern as the prevalence of many allergies, including AR, is projected to increase in both developed and developing countries, with a large proportion of this increase occurring in children and young adults (Pawankar *et al.*, 2011; Haahtela *et al.*, 2013; Pawankar, 2014). This increasing prevalence is mainly attributed to changes in the environment, such as changes in ambient temperature, climate and air pollution, which can alter pollen counts and the presence of insects or molds associated with allergic disease (Pawankar *et al.*, 2011; Haahtela *et al.*, 2013). Despite the steady increase of patients with allergic diseases, treatment and

services directed towards them are not readily available, resulting in increased morbidity and mortality rates, decreased quality of life, and considerable costs to manage symptoms (Pawankar *et al.*,, 2011).

Allergies and the immune system

Allergies are regarded as a hypersensitivity reaction of the immune system towards an allergen, which are common substances that elicit a robust inflammatory immune response despite being harmless, due to a process known as sensitization (Larché *et al.*, 2006; Averbeck *et al.*, 2007). Sensitization of the immune system towards the allergen occurs when antigen-presenting cells (APCs), such as dendritic cells (DCs), process and present the allergen to naïve CD4+ T cells. Upon antigen presentation, naïve T cells differentiate to CD4+ type 2 helper T cells (Th2 cells) and undergo clonal expansion to generate a population of allergen-specific memory Th2 cells. Stimulation of Th2 cells release cytokines such as interleukin (IL-4), IL-5 and IL-13. IL-4 and IL-13 stimulate class switching of the ε immunoglobulin heavy chain in naïve B cells, resulting in the production of allergen-specific IgE antibodies. IgE is a key driver of allergic diseases as allergens bind to IgE and cross-links FcεRI, a high affinity receptor for IgE, found on mast cells and basophils (Larché *et al.*, 2006). The cross-linking of FcεRI receptors causes degranulation and subsequent release of histamine, prostaglandins, cysteinyl leukotrienes, chemokines and other cytokines that ultimately cause allergic symptoms (Larché *et al.*, 2006). The generation of allergen-specific memory B cells and IgE antibodies which bind high-affinity FcεRI receptors on mast cells and basophils is known as sensitization and results in allergens being quickly recognized upon subsequent

exposure, causing an allergic immune response within minutes (Larché *et al.*, 2006; Averbeck *et al.*, 2007). This constitutes the immediate phase, or early phase response (EPR) of the allergic reaction, and is characterised by symptoms such as anaphylaxis, rhinoconjunctivitis, urticaria, and decreased respiratory volumes in cases of asthma (Galli *et al.*, 2008).

Meanwhile, DCs take up, process and present the allergen to allergen-specific Th2 cells which are drivers of the late phase response (LPR) in allergic reactions that peaks 2-6h after allergen exposure (Galli *et al.*, 2008). Production of IL-4, IL-5, IL-9 and IL-13 from allergen-specific Th2 cells results in increased eosinophil recruitment and activation, mast cell differentiation, and mucus secretion leading to airway hyperresponsiveness (Larché *et al.*, 2006). Type 1 helper (Th1) T cells also contribute to the pathogenesis of allergic diseases by secreting interferon-(IFN)γ and tumor necrosis factor (TNF) which leads to the apoptosis of lung and skin epithelial cells. The disruption of the epithelial cells compromises barrier function, leading to increased allergen exposure to the immune system which propagates the allergic response (Larché *et al.*, 2006). Overall, sensitization towards an allergen leads to an inflammatory allergic immune response that results in the adverse symptoms associated with allergic diseases to occur, all of which are discomforting to individuals with allergies.

Current treatments for allergic diseases

Treatment for allergies include allergen avoidance, pharmacotherapy and immunotherapy (Pawankar *et al.*, 2011). Allergen avoidance can be effective for food allergens but hard to achieve with airborne allergens. Pharmacotherapies include

antihistamines, leukotriene inhibitors, corticosteroids, decongestants, bronchodilators and epinephrine which provide quick and moderately effective relief of symptoms but are not curative (Pawankar *et al.*, 2011). Despite the available pharmacotherapies, there are still a significant number of patients who are unable to manage their symptoms, such as individuals with steroid-resistant allergies. Treatment for allergies have led to the development of whole allergen immunotherapy (AIT) which involves administration of whole allergen proteins over a course of 3-5 years with intent to alter the immune system's response towards the allergen. There are two types of AIT available, subcutaneous (SCIT) and sublingual (SLIT) immunotherapy. SCIT involves monthly subcutaneous injections of allergens which must be administered in a supervised medical facility with proper equipment and personnel due to the high risk of adverse effects which includes systemic allergic reactions, anaphylaxis and death. SLIT involves taking daily sublingual pills and has a better safety profile in comparison to SCIT allowing it to be self-administered, but still requires a multi-year treatment course (Pawankar *et al.*, 2011; Cox *et al.*, 2013; Tonti and Larché, 2016).

In several clinical trials and in practice, AIT has shown to decrease sensitivity to allergens, alleviate allergic symptoms and provide long-lasting effects upon completing the treatment regimen (Pawankar *et al.*, 2011; Cox *et al.*, 2013). This prolonged efficacy suggests that AIT modifies the pathophysiology of allergic diseases to prevent disease progression and subsequent allergic reactions, which does not occur with pharmacotherapies (Larché, 2014). It is currently available in the United States (U.S.), Canada, Europe, India and Asia for the treatment of allergic rhinitis, asthma,

Hymetoptera-induced anaphylaxis and respiratory allergies (Cox *et al.*, 2013; Jutel *et al.*, 2015). However, only an estimated 5% of the allergic population in the U.S. and Europe consider AIT, possibly due to the long treatment course and risk of adverse effects (Cox *et al.*, 2013).

Studies have shown that AIT alters the immune response towards the allergen. During AIT, peripheral tolerance is induced by the generation of regulatory T cells which is contributed to the context in which APCs present the allergens to naïve T cells. APCs, mainly DCs, induce peripheral tolerance or immunity against the antigens being presented through interpretation of environmental signals. In AIT, allergens are administered in the absence of pro-inflammatory signals, which signals to DCs to not evoke an inflammatory immune response against the allergen. Instead, tolerance is induced due to the partial maturation of the DCs and presence of autocrine IL-10 and TGF-β, generating allergen-specific regulatory T cells. With AIT, two broad classes of regulatory T cells increase: IL-10 secreting regulatory T cells (Tr1) and inducible CD4+CD25+ regulatory T cells (Treg) (Larché *et al.*, 2006). These regulatory T cells produce inhibitory cytokines such as IL-10 and TGF-β which modulate the activity of immune cells associated with allergic reactions. These effects include suppressing the generation of allergen-specific Th2 and Th1 cells (Akdis and Akdis, 2007), inhibiting IgE-dependent mast cell and basophil activation and release of histamine (Schandené *et al.*, 1994), inhibiting production of granulocyte colony-stimulating factor and expression of CD40 by activated eosinophils, and increasing the death of eosinophils (Varney *et al.*, 1993; Durham *et al.*, 1996; Ohkawara *et al.*, 1996; Tarzi *et al.*, 2006). In addition, AIT resulted in decreased IL-4, IL-

5, IL-9 and IL-13 and increased IFNγ in response to allergen exposure, which was indicative of immune deviation from a Th2 to Th1 immune response (Secrist *et al.*, 1993; Varney *et al.*, 1993; Schandené *et al.*, 1994; Ebner *et al.*, 1997). Furthermore, studies have shown that AIT resulted in the deletion of allergen-specific Th2 cells due to the administration of high doses of allergen for an extended period of time (Gardner *et al.*, 2004; Wambre *et al.*, 2012, 2014). From these studies, immune deviation, in addition to the deletion of allergen-specific Th2 cells, have been shown to attribute to the decrease in allergic immune response during subsequent allergen exposure after receiving AIT.

Changes in antibody production by B cells were also detected after treatment with AIT. Gradual decreases in allergen-specific IgE antibodies were detected, although the decrease occurred over several months or years of treatment (Akdis and Akdis, 2015). Clinical efficacy of AIT was associated with the generation of allergen-specific IgG, particularly IgG4, following treatment (Loveless, 1940; Lichtenstein *et al.*, 1968). IgG4 is known as a blocking antibody as it competes with IgE, preventing allergen-IgE complexes from forming and cross-linking FcεrI, therefore inhibiting the activation and degranulation of mast cells and basophils (Mothes *et al.*, 2003). IgG antibodies also inhibit IgE-mediated allergen presentation by DCs, which may prevent further activation of allergen-specific Th2 cells (Fujita *et al.*, 2012). These changes in allergen-specific antibodies may be due to the presence of IL-10 produced by regulatory T cells which has shown to be a potent suppressor of IgE while stimulating an increase in IgG4 production (Akdis and Akdis, 2007). Overall, the clinical efficacy of AIT is attributed to changes in the immunological response towards allergens, which included induction of regulatory T

cells and peripheral tolerance that led to decreases in Th2 response due to immune deviation and deletion of allergen-specific Th2 cells, and induction of IgG4 antibodies.

Peptide immunotherapy and its mechanism of action

Although generally effective, AIT has low uptake and adherence rates due to the length of treatment required and the associated risks of adverse side effects. These side effects occur due to the cross-linking of allergen-specific IgE on mast cells and basophils that lead to the activation of these cells during treatment (Cox *et al.*, 2013). To improve the safety and efficacy of immunotherapy, alternative approaches to the administration of the allergen have been explored, such as modifying the allergen being administered. One of these approaches is peptide immunotherapy (PIT) which uses short synthetic peptides derived from whole allergens as the administered agent to target allergen-specific T cells. These peptides are immunodominant T cell epitopes that are capable of inducing T cell proliferation without cross-linking allergen-specific IgE (Tonti and Larché, 2016). T cell epitopes used for peptide immunotherapy are selected based on *in silico* analysis for high affinity and good solubility. These peptides are then tested *in vitro* to verify that they have moderate to high affinity to various MHC class II molecules that are representative of the allergic population and that they do not activate basophils from allergic individuals (Tonti and Larché, 2016). Selected peptides are further validated with *ex vivo* T cell activation assays to identify their proliferative ability on allergen-specific T cells (Tonti and Larché, 2016).

Various peptides have been tested during the development of PIT. In early clinical trials for treatment of cat allergies, two 27 amino acid (AA) peptides from Fel d 1 were

administered and resulted in adverse effects, suggesting that the peptide length was still long enough to cross-link IgE (Norman *et al.*, 1996; Tonti and Larché, 2016). In treatment of bee venom and cat allergies, reducing the length of the peptide to 11-18 AAs for bee venom, or 13-17 AAs for cat, has shown to maintain clinical efficacy with reduction of severe adverse events during treatment (Müller *et al.*, 1998; Tonti and Larché, 2016). With lower risk of adverse events, higher doses of peptides can be administered in comparison to AIT, which shortens the treatment period of PIT to 4 intradermal injections over 3 months, compared to SCIT which requires monthly injections or SLIT with daily pills for 3-5 years (Tonti and Larché, 2016). Currently, PIT has undergone clinical trials for the treatment of cat, house dust mite, ragweed, grass, and peanut allergies.

Peptide immunotherapy resulted in changes in cellular and humoral immunity against the allergen, with some similarities to whole allergen immunotherapy (Larché, 2014; Tonti and Larché, 2016). However, the mechanism of action of peptide immunotherapy is not fully understood. In early studies using long peptides of greater than 40 amino acids from bee venom phospholipase A2 or birch pollen Bet v 1, administration of these peptides induced production of allergen-specific IgG4 antibodies but also caused adverse effects due to the peptides containing B cell epitopes and cross-linking IgE (Fellrath *et al.*, 2003; Spertini *et al.*, 2014). Studies using shorter peptides had mixed results on the generation of allergen-specific IgG antibodies. In studies for the treatment of grass pollen allergies, no significant differences in allergen-specific IgG production was detected before and after treatment (Ellis *et al.*, 2006), while in studies for the treatment of bee venom allergy, small increases in IgG4 occurred following but not

during peptide immunotherapy (Müller *et al.*, 1998; Tarzi *et al.*, 2006). This suggests that the administration of peptides itself during treatment may not induce IgG production, which occurred with whole allergen immunotherapy. Instead, subsequent exposure to the whole allergen post-treatment may cause allergen-specific IgG to be produced, which may contribute to the prolonged efficacy of PIT observed after completion of the treatment regimen (Tonti and Larché, 2016).

PIT also induced changes in T cell responses resulting in a reduction in the expression of Th2 cytokines with an increase in IL-10 production, and the induction of CD4+ T cells with suppressive functions (Cox *et al.*, 2013; Tonti and Larché, 2016). However, these changes in cytokines were not caused by immune deviation from Th2 to Th1 responses following allergen challenge, nor due to the deletion of allergen-specific Th2 cells, which was seen in whole allergen immunotherapy (Rudulier *et al.*, 2019). In addition, inter- and intra-molecular tolerance was observed with PIT. Inter-molecular tolerance, also known as bystander tolerance, refers to the induction of tolerance towards other antigens that were not the intended treatment antigen, due to the secretion of suppressive cytokines that act non-specifically during stimulation (Karlsson *et al.*, 2000). In a study conducted by Moldaver *et al.* (2014), bystander tolerance was generated against ovalbumin in mice after treatment with house dust mite peptides, and was characterized by an increase in IL-10 and an increase in the number of FoxP3+ cells producing IL-10. Intra-molecular tolerance, also known as linked epitope suppression, occurs when tolerance is induced towards non-treatment peptides of the same allergen. This phenomenon describes the observation that tolerance to the administered peptides in

PIT can be extended to other peptide sequences within the whole allergen. This was observed in a study conducted by Campbell *et al.* (2009), where intra-molecular tolerance was induced with the treatment of cat allergies with epitopes of Fel d 1. The induction of inter- and intra-molecular tolerance may improve the clinical efficacy of PIT as the effects induced by the administered peptides may extend to other allergy-associated antigens.

From these studies, the mechanism of action of peptide immunotherapy has some overlap with AIT as it can influence the proliferation of IL-10 secreting cells and secretion of IgG4 in subsequent exposures to allergen instead of during treatment. It is evident that the full extent of how peptide immunotherapy provides long-lasting clinical benefits is not yet known. We explored the mechanism of action of PIT for cat allergies in our lab. In our studies, we conducted RNA sequencing on allergen-specific T cells before and after therapy. Several genes were differentially regulated, however, one of the most affected genes was IL-8. IL-8 transcription in allergen-specific T cells was essentially shut-down after PIT with a 98.5% reduction in transcript levels. Concurrently, no increase in neutrophil counts were detected in peripheral blood following a nasal allergen challenge post-PIT in comparison to pre-PIT. IL-8 is a known chemoattractant for neutrophils and basophils causing their migration and subsequent activation. Activation of neutrophils and basophils leads to the release of allergic mediators, such as histamine and leukotrienes, that cause allergic symptoms. Our hypothesis is that IL-8 drives the pathogenesis of allergic diseases through a novel subset of T cells, called ‘Th8’ cells, which we believe exists in the allergic population and causes the recruitment and

activation of neutrophils and basophils upon allergen exposure. These granulocytes then contribute to the pathophysiology of allergic disease in an IgE-independent, T cell-dependent fashion. With peptide immunotherapy, the production of IL-8 may be downregulated in allergen-specific T cells, preventing subsequent neutrophil and basophil activation and presentation of allergic symptoms.

IL-8 and its role in the immune system

During an inflammatory immune response, immune cells such as mast cells, monocytes, macrophages and endothelial cells release IL-8, also known as CXCL8, upon stimulation with lipopolysaccharide (LPS), IL-1 and TNF-α (Fujishima *et al.*, 1996; Grützkau *et al.*, 1997; Viola and Luster, 2008). IL-8 is a known chemoattractant for neutrophils, causing the migration of neutrophils to the site of inflammation and subsequent activation/degranulation for pathogenic clearance (Nuzzi *et al.*, 2007; Viola and Luster, 2008). It has also been discovered that IL-8, along with IL-3, may act as a chemoattractant and activation factor for basophils (Krieger *et al.*, 1992).

A few populations of T cells that produce IL-8 have been identified in the literature, but have not been fully characterized as a novel subset of Th8 cells. The predominant population of IL-8+ T cells that have been studied were recent thymic emigrants (RTEs), or naïve CD4+ T cells, which have shown to produce IL-8 in both neonates and adults (Das *et al.*, 2017; Pekalski *et al.*, 2017). In neonates, the production of IL-8 was significant as it can offer proinflammatory properties that neonatal T cells lack as no IFNγ was produced (Das *et al.*, 2017). In adults, the number of IL-8+ CD4+ T cells was substantially lower than in babies. Multiple studies have identified that these IL-

8+ CD4+ T cells were precursors for IFNγ-producing T cells and that the conversion from IL-8+ to IFNγ+ cells occurs after TCR activation with its cognate antigen (Akhade and Qadri, 2015; Das *et al.*, 2017; Pekalski *et al.*, 2017). This suggests that IL-8+ T cells may be an innate population, with the ability to respond to stimuli through other receptors other than the TCR. The existence of these naïve IL-8+ CD4+ T cells was also supported by Wong *et al.* (2016) who conducted a high-dimensional atlas of human T cell diversity according to tissue type. In this study, an unique population of IL-8+ T cells was found in cord blood.

In relation to allergic diseases, drug-specific IL-8+ CD4+ T cells have been identified in patients with acute generalized exanthematous pustolosis (AGEP) which consists of a drug-induced hypersensitivity reaction that is highly neutrophilic (Schaerli *et al.*, 2004). These IL-8+ T cells co-expressed Th1-associated cytokines, IFNγ and GM-CSF with no expression of Th2-associated cytokines, IL-4 and IL-5. In addition, drug-specific IL-8+ T cells were capable of inducing neutrophil chemotaxis and enhanced the survival of neutrophils, possibly due to the production of IFNγ and GM-CSF (Schaerli *et al.*, 2004).

From these studies, it is evident that there are populations of IL-8+ T cells that exist in the immune system though in allergic diseases such as allergic rhinitis, allergen-specific T cells that produce IL-8 has not yet been studied. The identification of allergen-specific IL-8-producing T cells will provide insight into the pathology of allergic diseases and therefore was studied in this project.

PROJECT RATIONALE

Peptide immunotherapy was developed to lower the risk of adverse effects and increase efficacy when treating allergic diseases with immunotherapy. Our lab conducted clinical trials for PIT for cat allergies that led to reduction in symptoms and provided clinical benefits upon cessation of treatment. Our studies of peptide immunotherapy have led to the discovery of a potentially novel, unrecognized pathway for allergic diseases. Through mechanistic studies, we identified that there was a shutdown of IL-8 in allergen-specific T cells following peptide immunotherapy, bringing IL-8 into focus to determine its role in the pathogenesis of allergic diseases. More specifically, we suspect that there may be a novel T-cell dependent pathway for allergic diseases where allergen-specific T cells are primed to produce IL-8 upon subsequent exposure to allergens. The release of IL-8 then recruits and activates neutrophils and basophils, causing the release of histamines and leukotrienes that lead to allergic symptoms.

We hypothesize that (a) a population of IL-8-producing allergen-specific T cells exists in allergic subjects, and are a novel subset of T cells, called Th8 cells, and (b) these T cells cause allergic inflammation through recruitment and activation of neutrophils and basophils, leading to symptoms associated with late-phase reactions. In this study, we focused on identifying and phenotyping IL-8+ T cells in cat allergic and non-cat allergic participants to explore the existence of ‘Th8’ cells.

OBJECTIVES OF THE PROJECT

Aim 1. Identifying and phenotyping IL-8-producing (IL-8+) allergen-specific T cells in cat allergic and non-cat allergic subjects

The intent of this aim was to investigate the existence of a novel subset of 'Th8' cells that may contribute to the pathogenesis of allergic diseases. In this aim, we (1) optimized the protocol to detect IL-8+ T cells in peripheral blood, (2) established the flow cytometry parameters to phenotype IL-8+ T cells, and (3) determined if allergen-specific T cells produce IL-8 upon stimulation in allergic and non-allergic subjects. To remain consistent with our previous studies, we assessed allergen-specific IL-8+ T cells by recruiting 12 cat allergic and 12 non-cat allergic subjects in a clinical study for Aim 1. We aimed to characterize these allergen-specific 'Th8' cells to determine if a subset of IL-8-producing CD4+ T cells exists by using flow cytometry and enzyme-linked immunosorbent spot (ELISpot) assays.

Aim 1.1 Optimization of protocol to detect IL-8+ T cells in peripheral blood

In our preliminary experiments, we were able to detect IL-8+ T cells in unstimulated and PMA/ionomycin (PMA/I) stimulated peripheral blood mononuclear cells (PBMCs) through flow cytometry by intracellularly staining for IL-8. We noticed that the percentage of IL-8+ T cells at baseline differed depending on the isolation method of the PBMCs. Therefore, before recruiting subjects for Aim 1, we used blood samples from healthy volunteers to determine the more suitable isolation method to reduce the background of IL-8+ T cells in the unstimulated controls. The tested methods included using red blood cell lysis on whole blood and various density gradient

centrifugation media from different manufacturers. We also assessed two protein transport inhibitors that are widely used in detecting intracellular markers and cytokines in flow cytometry, brefeldin A and monensin, in the detection of IL-8+ T cells. Lastly, the stimulation time required to detect IL-8+ T cells was optimized by stimulating cells for up to 24h and collecting samples at 0, 2, 6, 12, 18 and 24h, and assessing the percentage of IL-8+ T cells in flow cytometry.

The frequency of IL-8+ T cells was also assessed using IL-8 ELISpot assays. The protocol for IL-8 ELISpots required pre-stimulation of PBMCs with cat allergens, PMA/I and αCD3/28, prior to CD4+ T cell isolation to allow for activation of allergen-specific T cells through APCs present in PBMCs. CD4+ T cell isolation was conducted to remove any other IL-8-producing cells present in PBMCs (ex. monocytes) as the ELISpot cannot distinguish between cell types. The time required for pre-stimulation of PBMCs (6h vs 24h) and incubating CD4+ T cells in the ELISpot (24h vs 48h) were optimized to determine the parameters that allow for detection of IL-8+ T cells with low backgrounds in the negative controls.

Aim 1.2 Establishment of flow cytometry parameters to phenotype IL-8+ T cells in cat allergic and non-cat allergic subjects

Prior to phenotyping IL-8+ T cells, the detection of IL-8+ T cells in flow cytometry was tested to ensure that IL-8+ T cells were captured in flow cytometry. CD4+ T cells were stimulated with PMA/I and αCD3/28 for 6h and stained with two IL-8 antibodies of the same clone (E8N1), but with different fluorescence molecules. As these

antibodies may stain differently, we observed whether the use of certain antibodies resulted in different percentages of IL-8+ T cells being detected in flow cytometry.

The phenotype of IL-8+ T cells in cat allergic and non-cat allergic subjects was determined by surface markers and/or cytokine profiles measured through flow cytometry. In preliminary experiments, the flow cytometry panel consisted of antibodies for CD3, CD4, CRTH2, CCR4, CD137, CD154, IL-8, IL-4, IL-5, IL-10, IL-13 and IFNγ. Upon analysis of these markers, the panel was optimized to include more surface markers to detect a wider range of T cell subtypes, including CD45RA, CCR7, CXCR3, HLA-DR, and CD38. In our flow cytometry analysis, CD3 and CD4 was used to gate for CD4+ T cells. CD45RA, CCR7, CXCR3, CRTH2, CCR4, CD137 and CD154 were used to phenotype IL-8+ CD4+ T cells. CD45RA, a marker for naïve T cells, in addition with CCR7, a lymph node homing marker, were used to identify the differentiation state of T cells as naïve (T_N; CD45RA+CCR7+), central memory (T_{CM}; CD45RA-CCR7+), effector memory (T_{EM}; CD45RA-CCR7-) and effector memory re-expressing CD45RA (T_{EMRA}; CD45RA+CCR7-) (Sallusto *et al.*, 1999, 2004). CXCR3, CRTH2 and CCR4 were used to determine if the IL-8+ T cells were Th1 (CXCR3+), Th2 (CRTH2+CCR4+), or to indicate the existence of a novel subset of T cells if no expression of CXCR3, CCR4 or CRTH2 was detected (Cosmi *et al.*, 2000; Wakugawa *et al.*, 2001; Yang *et al.*, 2004; Banfield *et al.*, 2010). Two activation markers were also included in the flow cytometry panel, CD137 and CD154. CD137 is selective of Treg cells (Bacher *et al.*, 2016) while CD154 is expressed on all activated CD4+ T cells (Bacher and Scheffold, 2015). The expression of CD154 upon stimulation was used to identify allergen-specific T cells in

PBMCs as CD154 is transiently expressed in T cells that have been activated through the TCR, which inferred allergen-specificity when stimulated by cat allergens (Frentsch *et al.*, 2005; Smith *et al.*, 2013). The cytokine profiles also aided in identifying the population of IL-8+ T cells as IL-4, IL-5 and IL-13 are Th2-associated cytokines, IFNγ is Th1-associated, and IL-10 is regulatory T cells (Akdis *et al.*, 2004). These markers, mainly IL-5 and IFNγ, were used in combination with the surface markers to determine if a novel subset of IL-8+ T cells exists in cat allergic and non-cat allergic subjects.

Aim 1.3 Determining if allergen-specific T cells, from cat allergic and non-cat allergic subjects, produce IL-8 upon stimulation with cat allergen

Our hypothesis suggests that there may be a difference between cat allergic and non-cat allergic subjects in the responsiveness of T cells to produce IL-8 upon stimulation with cat allergen. We assessed if this difference was attributed to allergen-specific T cells through two staining methods: CD154 staining and MHC Class II tetramer staining. As mentioned in Aim 1.2, allergen-specific T cells were identified by CD154 expression, and were characterized as CD4+ CD154+ T cells. Through flow cytometry, we assessed the IL-8+ CD4+ T cell population for CD154+ expression, which inferred allergen-specificity. Therefore, we observed allergen-specific T cells by recruiting cat and non-cat allergic subjects and stimulating PBMCs with a major cat allergen, Fel d 1, and whole cat dander, in addition to PMA/I and αCD3/CD28 as positive controls, and any production of IL-8 was attributed to allergen-specific T cells through flow cytometry gating for CD154. Initially, further isolation of CD4+ CD154+ T cells from PBMCs and re-stimulation with αCD3/CD28 was to be conducted but preliminary experiments suggested that these cells

did not produce a detectable level of IL-8+ T cells, if any, in flow cytometry. We also detected allergen-specific T cells through MHC Class II tetramer staining. Tetramers are a tetravalent complex composed of peptide-MHC class II molecules, allowing the tetramer to bind to antigen-specific TCRs and mimic the interaction that would occur naturally between APCs and the T cell (Nepom, 2012). Using both approaches, we analyzed the population of CD4+ T cells and determined if IL-8 production was characteristic of allergen-specific T cells (ie. CD154+ or Tetramer+ cells) and if differences in IL-8+ T cells were observed between the allergic and non-allergic populations. We further analyzed these IL-8+ T cells by phenotyping the cells with the flow cytometry protocol established in sub-aim 1.2, to determine if there is a novel subset of 'Th8' cells. We assessed the population of allergen-specific IL-8+ T cells in 12 cat allergic and 12 non-cat allergic subjects, through tetramer staining, in addition to CD154 staining.

Furthermore, we assessed the differences in IL-8+ T cells from cat allergic and non-cat allergic subjects through IL-8 ELISpots. ELISpots detect the production of cytokines from individual cells upon antigen stimulation and have a higher sensitivity compared to flow cytometry in detecting rare antigen-specific cells (McCutcheon *et al.*, 1997). Upon stimulation of PBMCs with cat allergens, CD4+ T cells were isolated and placed in the ELISpot for up to 24h. Any IL-8-producing T cells formed spots on the ELISpot plate and differences in the number of IL-8+ T cells between conditions and between allergics and non-allergics were measured. This was conducted concurrently with the flow cytometry analysis on 12 cat allergic and 12 non-cat allergic subjects. With

both flow cytometry and ELISpot data, we aimed to identify a novel allergen-specific 'Th8' subset of T cells in the cat allergic population, as well as to observe any differential T cell production of IL-8 between allergics and non-allergics.

Aim 2. Determining the stimulatory molecules that elicit IL-8 production in CD4+ T cells

The positive controls used in our study, PMA/I and αCD3/CD28, produced differential levels of IL-8 in CD4+ T cells. PMA/I induced IL-8 production in a larger percentage of CD4+ T cells in comparison to αCD3/CD28 in every subject, which was evident in flow cytometry, IL-8 ELISpots and ELISAs. To further understand the stimuli that control IL-8 production in CD4+ T cells, we looked into various stimulatory molecules used to activate T cells, such as other allergens, toll-like receptor (TLR) agonists, and other co-stimulatory molecules required for T cell activation.

There are various methods for stimulating T cells, in addition to PMA/I and αCD3/CD28, that may elicit the production of IL-8. To determine if any other allergen, aside from cat dander, could increase the percentage of IL-8+ T cells, we stimulated PBMCs with whole allergen extracts of house dust mite and ragweed, and their associated allergens. In the literature, it is evident that T cells also express toll-like receptors (TLRs), allowing for direct stimulation of the T cells by pathogens. There are 10 TLRs identified thus far, with TLR1-5, 7 and 9 expressed at low levels on the T cell surface (Caron *et al.*, 2005; Macleod and Wetzler, 2007). To determine if cat allergens or other stimuli have an effect on T cells through TLRs, we isolated and cultured CD4+ T cells with TLR agonists

for 6 and 24h. The percentage of IL-8+ T cells was measured in flow cytometry and concentration of IL-8 produced was measured by ELISA.

During T cell stimulation, proper activation of the cell requires co-stimulatory signals in addition to the recognition of antigens presented by APCs to the TCR (Chen and Flies, 2013). There are many co-stimulatory interactions that can occur on the T cell surface, with CD28 being the main stimulatory signal. Another stimulatory signal is CD2 which has been shown to induce IL-8 in T cells (Spinozzi *et al.*, 1996). The role of CD2 co-stimulation in the production of IL-8 was investigated using anti-CD2 (αCD2) antibodies with anti-CD3 (αCD3) and/or anti-CD28 (αCD28) to stimulate purified CD4+ T cells. In addition, the CD2 co-stimulatory pathway can be observed through stimulation with phytohemagglutinin (PHA) (Flynn *et al.*, 1986). Although these methods of stimulating T cells has been shown to induce IL-8 production, the detection of the IL-8+ T cells in flow cytometry has not been well documented. Using these stimulatory pathways, we aimed to gain an understanding of the signalling pathway that elicits IL-8 production in CD4+ T cells.

METHODS

PBMC and CD4+ T cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation on Ficoll-Paque (GE Healthcare, Chicago, Illinois), Lymphoprep (StemCell Technologies, Vancouver, Canada) and Histopaque-1077 (Sigma-Aldrich, St. Louis, Missouri), or through red blood cell (RBC) lysis. Whole blood was lysed twice for 10 mins each with commercial RBC Lysis Buffer (Biolegend, San

Diego, California) or homemade RBC lysis buffer containing 0.1 mM EDTA, 10 mM $KHCO_3$, 0.155 M NH_4Cl dissolved in RO water, and adjusted to pH 7.2 to 7.3. After isolation, the cells were washed, counted and resuspended in RPMI 1640 (Gibco Life Technologies, Burlington, Canada) containing 6% autologous plasma. Autologous plasma was obtained by spinning 10 ml of blood collected in Sodium Heparin BD Vacutainer at 1500xg for 15 mins, heat inactivated at 56°C for 30 mins, and spun again at 1000xg for 10 mins. For cultures with purified CD4+ T cells, the CD4+ T cells were isolated from PBMCs following the manufacturer's instructions of the EasySep Human CD4+ T cell Isolation Kit (Catalog #19052, StemCell Technologies).

PBMC and whole blood stimulation

Up to $5x10^6$ PBMCs were stimulated with allergens, PMA (25-50ng/ml) and ionomycin (0.5-1 μg/ml; PMA/I), and plate bound αCD3 (5 μg/ml) with soluble CD28 (2 μg/ml; αCD3/CD28) for up to 24h at 37°C, 5% CO_2 in a 24-well plates. These allergens included Fel d 1 (10 μg/ml), Amb a 1 (10 μg/ml), Der p 1 (10 μg/ml), which were obtained from Indoor Biotechnologies (Charlottesville, Virginia), cat dander (50 μg/ml), ragweed (50 μg/ml) and house dust mite (50 μg/ml), obtained from Stallergenes Greer (Lenoir, North Carolina). Cat synthetic peptide (CAT-SPIRE; 40 μM), *Candida albicans* (1 μg/ml) and LPS (1 EU/ml) were also used as a stimulants. Immune cells were also stimulated as whole blood samples. One ml of whole blood was stimulated with Fel d 1 and cat dander for 6h at 37°C, 5% CO_2. Brefeldin A (3 μg/ml) or monensin (2 μM) was added to culture in the last 4h of incubation.

CD4+CD154-/+ T cell stimulation

CD4+ T cells were isolated from PBMCs after stimulation with Fel d 1 and cat dander for 6h at 37°C, 5% CO_2. Extracted CD4+ T cells were washed, counted and resuspended in 40 µl PBS containing 0.5% bovine serum albumin and 2 mM EDTA (MACS) buffer for CD154+ isolation as per the manufacturer's instructions for the Human CD154 MicroBead Kit (Catalog #130-092-658, Miltenyi Biotec, Germany). CD4+ CD154+ T cells were washed, counted and resuspended in 200 µl RPMI + 6% autologous plasma and plated with αCD3/CD28 for 20h at 37°C, 5% CO_2. The negative fraction from the CD154+ isolation, ie. CD154- T cells, was also stimulated with αCD3/CD28 for 20h. In the last 4h of incubation, brefeldin A (3 µg/ml) was added.

CD4+ T cell stimulation

CD4+ T cells were purified from PBMCs isolated by Ficoll-Paque, using EasySep Human CD4+ T cell Isolation Kit (StemCell Technologies) and resuspended in RPMI + 6% autologous plasma. For TLR stimulation, CD4+ T cells were incubated with 1 µg/ml Pam_3CSK_4 (TLR2; Invivogen), 10 µg/ml short and long polyinosinic-polycytidylic acid (TLR3; poly (I:C) (generous gift of Dr. Karen Mossman, McMaster University)), 20 ng/ml LPS (TLR4), 5 µg/ml recombinant flagellin (TLR5; Cedarlane), 5 µg/ml resiquimod (TLR7/8; R848 (generous gift of Dr. Matthew Miller, McMaster University)), 2.5 µM ODN2395 (TLR9; Invivogen), 10 µg/ml Fel d 1 and 50 µg/ml cat dander for 6 and 24h. To test CD2/CD3/CD28 co-stimulatory pathways, CD4+ T cells were stimulated with plate bound αCD3 ± soluble αCD2 ± soluble αCD28, each at 5 µg/ml (ThermoFisher), or with phytohaemagglutnin P (PHA-P) and PHA-M (Sigma; 5 µg/ml) for 6h at 37°C, 5%CO_2. Brefeldin A (3 µg/ml) was added to culture in the last 4h of

incubation. After incubation, the cells were collected for flow cytometry and the supernatants were collected to measure the IL-8 concentration by ELISA (Invitrogen) following the manufacturer's instructions.

IL-8 ELISpots

To detect the number of IL-8-producing CD4+ T cells, 96-well filter plates with 0.45 μm pore size PVDF membrane (Cat#MSIPS4510, EMD Millipore, Darmstadt, Germany) were activated with 35% ethanol for 1 min and coated with 15 μg/ml IL-8 antibodies (MT8H6/8F19) overnight at 4°C, as outlined in the manufacturer's protocol (Cat#3560-2A, MabTech). Frozen PBMCs, or PBMCs isolated from RBC lysis of whole blood, from cat allergic and non-cat allergic subjects were cultured in RPMI + 6% autologous plasma, or human AB (hAB) serum if autologous plasma was not collected, with Fel d 1 (10 μg/ml; Indoor Biotechnologies), purified cat dander extract (50 μg/ml; generous gift of Professor Marianne van Hage (Karolinska Institut, Stockholm, Sweden)), PMA/I and αCD3/CD28 for 6h or 24h. CD4+ T cells were isolated from PBMC cultures after stimulation. Extracted CD4+ T cells were washed, counted and resuspended to $2.5x10^6$ cells/ml with CTL-Test Plus media (ImmunoSpot) supplemented with L-glutamine. The ELISpot plate was washed with sterile PBS and blocked with 200 μl of CTL-Test Plus media for 30 minutes. After blocking, the medium was removed and the purified CD4+ T cells were added and incubated in the ELISpot for up to 48h at 37°C, 5%CO_2. The supernatants of the ELISpot were collected for IL-5 ELISA (Invitrogen). For the detection of IL-8-producing spots, 0.5 μg/ml detection antibody (26E5-biotin) diluted in PBS + 0.5% FBS was added and incubated for 2h at room temperature, and washed with

PBS. Streptavidin-Alkaline Phosphatase (ALP) was added and incubated for 1h at room temperature, and washed with PBS. To develop the spots, BCIP/NBT solution (Cat#3650-10; MabTech), was added for 15 minutes. Spot development was stopped by washing extensively with RO water. The plate was left to dry overnight before enumeration of the spots using BioReader 6000-Fβ.

Participants and clinical study design

Participants were selected for the study following a screening visit that assessed the participants' cat allergy status and history, medical profile and tissue type. During the screening visit, a medical questionnaire was completed detailing the participants' cat allergy history and symptoms, frequency of exposure to cats, medical and surgical history, concomitant medication profile, and vaccination history (Appendix S1, Appendix S2). A blood sample was also collected for tissue typing, Fel d 1 IgE and cat dander IgE testing to confirm cat allergy status, and a complete blood count (CBC). Twenty-five participants were selected based on the inclusion and exclusion criteria outlined in Table 1 and were recruited into the clinical study which involved two study visits. Participants who had no history of cat allergies and an IgE of <0.35IU/ml for both Fel d 1 and cat dander were categorized as non-cat allergics. Participants with a minimum of one year history of allergic rhinoconjunctivitis to cats and IgE of >0.7IU/ml for Fel d 1 and cat dander were categorized as cat allergics. Of the 25 participants, twelve were cat allergic (3 males, 9 females; mean age 26.6 years old) and thirteen were non-cat allergic (4 males, 9 females; mean age 27.8 years old). Thirteen non-cat allergic subjects were recruited as one of the 12 originally recruited for the entire study was unable to continue for the

second study visit. These participants had one or more of the following tissue types: HLA-DRB1*07:01, HLA-DRB1*03:01, HLA-DRB1*01:01, or HLA-DRB5*01:01 which was required for Fel d 1-specific tetramer staining as these HLA alleles had tetramers available from the Benaroya Research Institute (Seattle, Washington; Table 2). A summary of subjects' information is outlined in Table 3.

Protocol for clinical samples

Blood samples (120 ml) were taken from the participants, with at least 30 days in between each blood draw, in two study visits. In the first study visit (Visit A1), PBMCs were isolated using RBC lysis. Whole blood was lysed twice for 10 minutes with homemade RBC lysis buffer containing 0.1 mM EDTA, 10 mM $KHCO_3$, 0.155 M NH_4Cl dissolved in RO water, and adjusted to pH 7.2 to 7.3. After isolation, the cells were washed, counted and resuspended in RPMI 1640 containing 6% autologous plasma. Five million PBMCs were stimulated with media, Fel d 1 (10 μg/ml; Indoor Biotechnologies), purified cat dander extract (50 μg/ml; generous gift of Professor Marianne van Hage (Karolinska Institut, Stockholm, Sweden)), PMA (50 ng/ml)/ionomycin (1 μg/ml) and plate bound αCD3 (5 μg/ml) with soluble CD28 (2 μg/ml; αCD3/CD28) for 6h and 24h at 37°C, 5%CO_2, with brefeldin A (3 μg/ml) added in the last 4h of incubation. Cells were collected and stained for flow cytometry.

Another $20x10^6$ PBMCs were plated and incubated with media, Fel d 1, cat dander, PMA (12.5 ng/ml)/ionomycin (0.25 μg/ml), αCD3/28 for 24h at 37°C, 5%CO_2 for IL-8 ELISpots. After incubation, PBMCs were collected for CD4+ T cell isolation using the negative selection kit from StemCell (#19052) following the manufacturer's

instructions. The number of CD4+ T cells was counted and 2.5-5x10^5 T cells were plated per well, in triplicates, in the ELISpot plate, prepared as mentioned above. CD4+ T cells were incubated in the plate for 20h at 37°C, 5%CO_2 prior to development.

In the second visit (Visit A2), PBMCs were isolated using density gradient centrifugation on Ficoll-Paque. CD4+ T cells were isolated by negative selection using the RoboSep (StemCell Technologies), counted, and incubated with protein kinase inhibitor, dasatinib (50 nM; Selleck Chemicals LLC, Houston, Texas), in RPMI+5% autologous plasma for 30 minutes at 37°C, 5%CO_2. To detect allergen-specific T cells, the CD4+ T cells were resuspended to 150x10^6 cells/ml and stained with MHC Class II tetramers bound to PE molecules at 10 μg/ml per tetramer and incubated for 100 mins at 37°C, 5%CO_2. The tetramers used are listed in Table 2. After tetramer staining, PE+ T cells were enriched using EasySep PE Positive Selection Kit II (StemCell Technologies) following the manufacturer's instructions and were counted. PE+ cells were stimulated with αCD3/28 Dynabeads (Invitrogen) for 24h at 37°C, 5%CO_2 with brefeldin A (3 μg/ml) added in the last 4h. PE- cells were also collected and stimulated with media, αCD3/28 beads and PMA/I for 24h as controls. Cells were collected and stained for flow cytometry.

The number of TET+ cells per million CD4+ T cells was calculated using the following equation: % of TET+ events in PE+ enriched fraction/100 x (PE+ cell count after enrichment/CD4+ cell count prior to enrichment) x 1,000,000. For TET+cytokine+ frequencies, the same calculation was used with the replacement of the % of TET+ events to % of TET+cytokine+ events in PE+ enriched fraction. Similarly, the number of

CD154+IL-8+ cells per million CD4+ was calculated as: % of CD154+IL-8+ T cells/100 x 1,000,000, where the % of CD154+IL-8+ T cells was determined by gating CD154 vs IL-8 in the CD4+ T cell population in FlowJo V10.

Flow cytometry staining

After incubation, cells were collected and washed with PBS for flow cytometry staining. Cells were stained with dead cell marker, APC-Cy7 (Biolegend), for 15 mins at room temperature in the dark. Following dead cell staining, the cells were washed with PBS containing 1% FBS and 0.1% sodium azide (FACS) buffer, stained with combination of (a) PerCP CD3, BV605 CRTH2, BV510 CCR4, BV650 CD137 (Biolegend), and PE-CF594 CD4 (BD), (b) AF488 CD3, AF700 CD4, BV785 CD45RA, PerCP/Cy5.5 CCR7, BV650 CXCR3, BV605 CRTH2, PE/Dazzle594 HLA-DR, Pe/Cy7 CD38 and BV510 CCR4 (Biolegend), (c) BV421 CD3, BV650 CD4, PerCP CD8, BV510 CD14, AF700 CD19, PE-Cy7 CD25, and FITC CD97, or (d) AF488 CD3, AF700 CD4, BV785 CD45RA, PerCP/Cy5.5 CCR7, PE/Dazzle594 CD137 and BV510 CCR4 (Biolegend), for 30 mins at 4°C. For tetramer-stained cells, AF488 CD3, AF700 CD4, PE/Dazzle594 CD4, BV510 CD14, BV510 CD19, BV785 CD45RA and PerCP-Cy5.5 CCR7 were used for surface staining. Cells were fixed with 4% paraformaldehyde (PFA) for 5 mins, vortexing at 1, 3 and 5 mins, for CD154-stained cells or with IC Fixation Buffer (eBioscience) for 20 mins for tetramer-stained cells and permeabilized with Permeablization Buffer (eBioscience) for intracellular staining. Cells were stained intracellularly with a combination of APC IL-8, PE CD154, BV650 or PE/Cy7 IFNγ, BV421 IL-5, PE-Cy7 IL-13, AF488 IL-10, AF700 IL-2 (Biolegend) and BV786 IL-4

(BD) antibodies, for 40 min at 4°C. After two washes, the cells were resuspended in FACS buffer and filtered into FACS tubes. For flow cytometry analysis, fluorescence minus one (FMO) samples were used as controls for gating. A list of antibodies and their clones are listed in Table 4. The samples were acquired on BD LSR II and data was analyzed with FlowJo V10.

Statistical analysis

Data was analyzed and graphed using GraphPad Prism 7.0 (San Diego, USA). To determine normality of data, Shapiro-Wilk normality test was used. Statistical comparisons between cat allergics and non-cat allergics were made using unpaired t-tests or Mann-Whitney tests. For comparisons within a group, a two-way mixed ANOVA followed by Tukey's multiple comparisons test or Friedman's tests were used. For non-parametric analysis of paired data, Wilcoxon's test was used. $p<0.05$ was used to determine significance.

RESULTS

OPTIMIZATION OF PROTOCOL TO DETECT IL-8+ T CELLS IN PERIPHERAL BLOOD

PBMC isolation method increased percentage of IL-8+ T cells detected in flow cytometry

In our previous studies, we isolated peripheral blood mononuclear cells (PBMCs) from whole blood using density gradient centrifugation with Ficoll-Paque. To determine if Ficoll was a suitable method for detecting IL-8+ T cells in peripheral blood for our current study, we stimulated whole blood, red blood cell (RBC)-lysed blood, and PBMCs

isolated with Ficoll-Paque with Fel d 1 (10 μg/ml) and cat dander (50 μg/ml) for 6h (Figure 1A). Overall, around a 3-fold increase in IL-8+ T cells were detected in PBMCs in comparison to RBC-lysed blood, with whole blood cultures having the lowest percentage of IL-8+ T cells (Figure 1A). Fel d 1 and cat dander did not elicit a change in the percentage of IL-8+ T cells in PBMCs and RBC-lysed blood, but Fel d 1 increased the percentage in whole blood cultures (Figure 1A). Two other density gradient media were also tested in comparison to RBC lysis: Lymphoprep (StemCell) and Histopaque-1077 (Sigma-Aldrich) (Figure 1B, C). RBC lysis was conducted using a commercial buffer (Biolegend) or a homemade buffer. The percentage of IL-8+ T cells were compared between isolation methods at 0h (Figure 1B) and 6h after incubation in RPMI + 6% autologous plasma (Figure 1C). For each density gradient media, there was large variability between subjects, resulting in an overall higher percentage of IL-8+ T cells at both 0h and 6h. In contrast, cells isolated by RBC lysis had the lowest IL-8+ T cells at both time points ($p>0.05$) and was selected as the method for PBMC isolation in the clinical study. The homemade RBC lysis buffer performed similarly to the commercial RBC lysis buffer as the background percentage of IL-8+ T cells remained low, with 1.55±1.35% of T cells expressing IL-8 with the homemade buffer, and 1.26±0.71% with the commercial buffer after 6h incubation (Figure 1C). This suggests that the homemade buffer can be used instead of the commercial buffer to process our blood samples as a large volume of RBC lysis buffer was required.

Comparison of monensin and brefeldin A in the detection of IL-8+ T cells

The detection of IL-8+ T cells in flow cytometry is dependent on protein transport inhibitors, such as monensin and brefeldin A, to prevent secretion of IL-8 for intracellular staining. To determine if monensin or brefeldin A affected the detection of IL-8+ T cells, PBMCs were cultured with media**,** cat dander (50 μg/ml), PMA/I or αCD3/CD28 for 6h, and monensin or brefeldin A was added in the last 4h of incubation. Both monensin and brefeldin A retained IL-8 within CD4+ T cells as stimulation with PMA/I and αCD3/CD28 resulted in the detection of IL-8+ T cells in flow cytometry with both inhibitors (Figure 2). A lower percentage of IL-8+ T cells were detected with monensin in media (1.05±1.03% compared to 1.88±1.47%; $p<0.05$) and tended to be lower with αCD3/CD28 (2.34±1.06% compared to 3.79±1.99%), while brefeldin A had slightly lower percentages in cat dander (2.68±1.54% compared to 3.91±4.57%). Despite the lower background observed with monensin, cells cultured with monensin had lower viability and lower cell recovery after incubation due to cell clumping (data not shown), and therefore in subsequent experiments, brefeldin A was used to inhibit IL-8 secretion.

Time-dependent expression of T cell markers and cytokines

To determine the optimal time to capture the IL-8+ T cell population after allergen stimulation, PBMCs were collected at 0, 2, 6, 12, 18 and 24h after stimulation with cat dander (50 μg/ml), PMA/I and αCD3/CD28 in two cat allergic subjects. The expression of various T cell markers and cytokines were also observed in the CD4+ T cell population to determine if the peak expression of the markers coincided with the peak of IL-8+ T cells. In the first subject, PBMCs were isolated using Ficoll-Paque density gradient centrifugation, resulting in a high percentage (8%) of IL-8+ T cells at 0h in the media

control which decreased over time (Figure 3A). The percentages of IL-8+ T cells between conditions remained relatively the same, except for PMA/I that had higher percentages of IL-8+ T cells at all time points (Figure 3A). IL-8+ T cells peaked at 12h, corresponding with the peak of CD154 when stimulated with cat dander and αCD3/CD28, respectively (Figure 3C). For other stimulants, CD154+ T cells are more abundant around 2h for media control and remained high after 2h of stimulation with PMA/I. CRTH2 expression fluctuated but tended to decrease overtime with stimulation with cat dander. CCR4 expression remained relatively constant for all time points and stimuli. CD137 expression peaked at 18h in PMA/I and αCD3/CD28 stimulated cells. The IL-8+ T cell peak at 12h also corresponded with peaks of IL-2 for all stimulants, and IFNγ, IL-4, and IL-10 for αCD3/CD28 (Figure 3C). For other stimulants, IFNγ remained relatively constant at all time points. IL-4 fluctuated between time points, with most IL-4 being expressed around 18h with cat dander, and 6h with PMA/I. Lastly, IL-10 along with IL-5 peaked after 24h of stimulation.

In the second subject, cells were isolated by RBC lysis of whole blood (Figure 3B,D). These cells were stained with a larger number of T cell markers instead of cytokines to better phenotype IL-8+ T cells, and due to the lower percentage of cytokines measured in the previous subject. In this subject, the percentage of IL-8+ T cells increased over time during stimulation with cat dander, PMA/I and αCD3/CD28 and PMA/I induced the highest percentage of IL-8+ T cells (Figure 3B). The peak of IL-8+ T cells differed from the other subject as the IL-8+ T cell population peaked at 18h for cat dander, 12h for PMA/I, and 24h for αCD3/CD28 (Figure 3B). The expression of T cell

markers in the CD4+ T cell population remained relatively constant overtime (Figure 3D, media), with the exception of CCR4, CD45RA and CD154. CCR4 increased between 18-24h in all conditions. The expression of CD45RA increased overtime with cat dander and αCD3/CD28 stimulation, peaking at 18h and 24h, respectively, and decreased with PMA/I (Figure 3D). CD154 increased with PMA/I and αCD3/CD28 stimulation peaking at 24h and 12h, respectively. With cat dander, the peak of CD154 occurred at 12h, which was before the peak of IL-8 at 18h. IL-5 production increased overtime with peaks at 18h for cat dander and PMA/I, and 24h for αCD3/CD28 stimulation.

Development of IL-8 ELISpot assay for detecting IL-8+ CD4+ T cells

To determine the optimal time to pre-stimulate PBMCs prior to CD4+ T cell isolation for detection of IL-8+ T cells in ELISpot assays, PBMCs from cat allergic subjects were stimulated for 6h or 24h with media, Fel d 1, cat dander, PMA/I and αCD3/28. After stimulation, CD4+ T cells were purified and incubated for two nights (approximately 40h) in the ELISpot plate. The mean background level of IL-8+ T cells for 6h pre-stimulation was 480.3±324.7 spots/million while 24h had 430.5±197.8 spots/million (Figure 4). For both 6h and 24h pre-stimulation, the number of IL-8+ T cells tended to increase similarly with each stimuli (Figure 4A). Although there was no significant difference between the two pre-stimulation times, the 24h time point was chosen for subsequent experiments to allow for adequate stimulation of CD4+ T cells prior to ELISpot plating.

The development of the ELISpot assay required incubation of the cells in the plate for 12 to 48h. To determine the ideal incubation time of CD4+ T cells in the ELISpot,

plates were developed 24h or 48h after plating the pre-stimulated CD4+ T cells. The number of IL-8+ T cells was slightly higher after 48h of incubation, compared to 24h (Figure 4B). After 48h of incubation, the number of IL-8+ T cells increased significantly with cat dander and PMA/I, compared to 24h where only PMA/I increased significantly (Figure 4B). Despite this, the incubation time was selected to be 24h, to minimize the background levels of IL-8+ T cells in the negative control.

OPTIMIZATION OF FLOW CYTOMETRY PANEL

Detection of IL-8+ T cells in peripheral blood through flow cytometry

In the optimization of the protocol, IL-8+ T cells were detected in peripheral blood by following the gating strategy for flow cytometry outlined in Figure 5. We first gated for all immune cells using side scatter (SSC) and forward scatter (FSC), which is further gated for single and live cells (Figure 5A). Upon selection of live cells, lymphocytes were selected and gated for CD3 and CD4. These CD4+ T cells were further analyzed for IL-8 production following stimulation with Fel d 1, cat dander, PMA/I and αCD3/CD28 for 6h (Figure 5B). In this subject, IL-8+ T cells were detected in all conditions, including a small percentage (0.5%) in the media control. PMA/I induced the most IL-8+ T cells in comparison to the other stimuli. Furthermore, the stimulated cells were also stained for markers for monocytes (CD14), Tregs (CD25), activated T cells (CD97), and B cells (CD19) to identify if the gating of IL-8+ CD4+ cells resulted in a clean population of T cells (Figure 5B, pie charts). Approximately 5% of the IL-8+ CD4+ cells were attributed to CD14 when monocytes were not gated out of the selected lymphocyte population. Inclusion of a CD14 and CD19 gate to remove CD14+ and

CD19+ cells from the lymphocyte population (Figure 5C) resulted in a similar percentage of IL-8+ CD4+ cells (Figure 5D). This suggests that, minimally, CD3 and CD4 were required in the flow cytometry panel to identify CD4+ T cells.

To ensure the detection of intracellular IL-8 was optimal, media, PMA/I and αCD3/28-stimulated CD4+ T cells were stained with two antibodies (APC and AF488) of the same clone (E8N1) against IL-8. The percentage of IL-8+ CD4+ T cells did not differ greatly with single stains in each stimuli, but was slightly lower when the cells were double stained (Figure 6). This suggests that the single antibody stain was adequate in detecting IL-8+ T cells.

Preliminary phenotyping of IL-8+ CD4+ T cells

We conducted a preliminary study on five subjects, with unconfirmed allergic status, to determine the phenotype of IL-8+ CD4+ T cells. For 4 of the subjects, PBMCs were isolated using Ficoll-Paque (Figure 7A,C,D) while the 5th subject was isolated using RBC lysis (Figure 7B,E,F). The PBMCs were cultured with cat dander, PMA/I and αCD3/CD28 for 6h (Figure 7A) or 18h (Figure 7B). In the PBMCs isolated via Ficoll-Paque, the highest percentage of IL-8+ T cells was detected in PMA/I stimulation for both cat allergic and non-cat allergic subjects (Figure 7A). Slight increases in the IL-8+ T cells were detected when stimulated with cat dander, but no significant difference was detected between allergics and non-allergics. Further analysis of the IL-8+ T cells showed that a low percentage (<3%) expressed CD154, a marker for allergen-specific T cells, in cat dander (Figure 7C). PMA/I and αCD3/CD28 stimulation significantly increased CD154 expression in comparison to the other stimuli. Furthermore, these cells did not

express CRTH2 or CD137, but around 30-40% expressed CCR4 in all conditions (Figure 7C). When stimulated with PMA/I, CCR4 significantly decreased in comparison to media. In our initial experiment for establishing the gating strategy, between 6-16% of IL-8+ CD4+ T cells expressed CD25, and less than 6% expressed CD97 (Figure 5D). In addition to producing IL-8, around 5% of these T cells also produced IFNγ, IL-5 and IL-10. Of the IL-8+ IL-5+ cells, a small percentage also expressed CD154 (<7%) (Figure 7D). Little to no IL-2, IL-4 or IL-13 was detected when stimulated with allergens (Figure 7D). However, a high percentage of IL-2-producing cells (approx. 25%) was detected when stimulated with PMA/I. No significant changes in T cell markers or cytokine production were detected between media control and stimulation with cat dander.

In the fifth subject, we established a new protocol for isolating PBMCs by using RBC lysis of whole blood that was used during recruitment of cat allergic and non-cat allergic subjects for our clinical study. This subject, who is cat allergic, displayed an increase in IL-8+ T cells during stimulation with cat dander, PMA/I and αCD3/CD28 for 18h (Figure 7B). The IL-8+ T cells were further phenotyped (Figure 7E) with a panel consisting of a larger number of T cell markers compared to the previous subjects, as the cytokines were not highly expressed as seen in Figure 7D. Again, a low percentage of IL-8+ T cells expressed CD154 in cat dander (2.56%) and media (5.53%) while a high percentage was observed in PMA/I (67.1%) and αCD3/CD28 (48%). These IL-8+ T cells also expressed CCR4 (21.2-44.7%), CD38 (16.4-32.3%), and HLA-DR (7.44-22.4%) (Figure 7E). Less than 1% of IL-8+ T cells from cat dander stimulation expressed CXCR3 and CRTH2, while for PMA/I and αCD3/CD28, around 5-8% of the cells were

CXCR3+ or CRTH2+. Analysis of CD45RA and CCR7 expression indicated that a majority of the IL-8+ T cells were CCR7-CD45RA- (Figure 7F). Lastly, no IL-5+, including CD154+IL-5+, cells were detected in any condition (Figure 7E).

OPTIMIZATION OF PROTOCOL TO DETERMINE ALLERGEN-SPECIFICITY OF T CELL RESPONSE

CD154 expression on IL-8+ T cells

CD154 expression was used to identify allergen-specific T cells upon stimulation with specific allergens. In the IL-8+ T cell population, few cells co-expressed CD154 when stimulated with cat dander as the percentage of IL-8+ CD154+ ranged from 2.12% to 3.41%, in cat allergic and non-cat allergic subjects, respectively (Figure 7C). Further isolation of CD154+ T cells after stimulation with cat allergens showed that <1.1% of purified CD4+ CD154+ T cells produced IL-8 upon re-stimulation with αCD3/CD28 for 20h (Figure 8A). In addition, CD4+ CD154- T cells also had a low percentage of IL-8+ T cells (<0.6%; Figure 8B), showing that re-stimulation of cells does not elicit IL-8 production in T cells.

MHC Class II tetramer staining of CD4+ T cells

Another method to detect allergen-specific T cells in flow cytometry is through MHC Class II tetramer staining. In a preliminary experiment, CD4+ T cells were isolated from a cat allergic subject and stained with tetramers. A small population of TET+ T cells were detected in the subject (Figure 9A) and upon stimulation with PMA/I for 6h, an increase in IL-8+ T cells in the CD4+ T cell population was observed (0.18% to 3.12%; Figure 9B). A small number of TET+IL-8+ T cells were detected in media and PMA/I but

no change in the number of TET+IL-8+ T cells was observed with PMA/I stimulation in this one subject (Figure 9A,B). Further testing of the tetramer protocol involved isolation of the TET+ cells by PE enrichment. Upon isolation of TET+ cells and stimulation with PMA/I, a small population of TET+IL8+ T cells was evident in two subjects as 0.13% and 0.22% of the PE+ enriched cells were TET+ and IL-8+ (Figure 9C,D).

CLINICAL STUDY RESULTS

Frequency of IL-8+ T cells in CD4+ detected by flow cytometry

Upon optimization of the protocol to detect IL-8+ T cells in flow cytometry and IL-8 ELISpots, 12 cat allergic and 13 non-cat allergic subjects were recruited to determine the frequency of IL-8+ CD4+ T cells in each group. PBMCs were stimulated with cat allergens, PMA/I and αCD3/28 for 6h and 24h and analyzed in flow cytometry. At baseline, the percentage of IL-8+ T cells detected in the CD4+ T cell population were similar between non-cat allergics and cat allergics, where 1.62±1.28% (6h) and 1.51±0.61% (24h) of CD4+ T cells expressed IL-8 in non-cat allergics, compared to 1.99±0.97% (6h) and 2.14±1.02% (24h) in cat allergics (Figure 10A,B).

The percentage of IL-8+ T cells were relatively similar between the two groups, irrespective of the stimuli used, as both groups portrayed the same trends upon stimulation. With Fel d 1, the percentage of IL-8+ T cells increased by approximately 0.2% in non-cat allergics and 0.4% in cat allergics at 6h, and 1% for non-cat allergics and 0.2% in cat allergics at 24h (Figure 10A,B). The number of IL-8+ T cells also increased with 6h and 24h cat dander stimulation in both allergics and non-allergics. This increase was significant with 6h stimulation in the cat allergics as the percentage increased almost

3-fold in comparison to media, and non-significant in the non-cat allergics (Figure 10A). At 24h, cat dander significantly increased the percentage of IL-8+ T cells by 3.7-fold in non-cat allergics compared to media (Figure 10B). The largest increase in IL-8+ T cells occurred with 6h and 24h PMA/I stimulation for both groups where the percentage increased up to 8-19% (Figure 10A,B; $p<0.05$). In contrast, the percentage of IL-8+ T cells did not significantly change with αCD3/28 stimulation at either 6h or 24h for both groups.

In comparison to IL-5+ and IFNγ+ CD4+ T cells, the percentages of IL-8+ T cells were predominantly higher with the stimulation of Fel d 1 and cat dander for 6h and 24h, as the percentages of IL-5+ and IFNγ+ cells were <1% and did not increase with cat allergen stimulation (Figure 10). At 24h, the production of IL-5 increased significantly with PMA/I and αCD3/28 in both cat allergics and non-cat allergics, increasing by 10 to 13-fold in non-cat allergics and 4-fold in cat allergics (Figure 10D). Similarly, the percentage of IFNγ+ cells increased with PMA/I at both 6h and 24h for both groups, and αCD3/28 at 24h for cat allergics (Figure 10E,F). Again, there was no significant difference between the two populations in IL-5+ and IFNγ+ CD4+ T cells.

Frequency of IL-8+ T cells detected in IL-8 ELISpot

Similar to the frequency of IL-8+ T cells detected in flow cytometry, the baseline number of IL-8+ T cells did not differ between non-cat allergics (44.2 ± 52.3 spots/10^6) and cat allergics (34.8 ± 42.1 spots/10^6; Figure 11). In the non-cat allergic subjects, the number of IL-8+ T cells increased with Fel d 1 stimulation by 1.6-fold and with cat dander by 2-fold and significantly increased with αCD3/28 by 3.1-fold (Figure 11).

Similarly in the cat allergic subjects, the number of IL-8+ T cells increased with Fel d 1 by 1.3-fold, cat dander by 1.3-fold and significantly increased with αCD3/28 by 4-fold (Figure 11). There was no significant difference detected between the cat allergic and non-cat allergic populations for any of the stimuli.

Frequency of allergen-specific IL-8+ T cells characterized as CD154+ or CD137+IL-8+ T cells

The detection of IL-8+ T cells in flow cytometry allowed for the analysis of various markers that were included in the panel. To determine the allergen-specificity of the IL-8+ T cells, PBMCs were stained for T cell activation marker CD154 after stimulation with cat allergens (Figure 12). The percentage of IL-8+ T cells attributed to the allergen itself (ie. CD154+) did not differ between non-cat allergic and cat allergic subjects at both 6h and 24h of stimulation (Figure 12A,B). The percentage of IL-8+ T cells expressing CD154 increased during 6h stimulation in cat allergic subjects with Fel d 1 (0.96±0.95%) and cat dander (2.02±1.34%; $p<0.05$) in comparison to media (0.61±0.29%; Figure 12A). In non-cat allergics, the percentage increased with cat dander stimulation from 0.57±0.29% to 0.73±0.7% ($p<0.05$). At 24h stimulation, the baseline percentage of IL-8+ CD154+ T cells was higher compared to 6h and did not increase with any cat allergen stimulation for either cat allergics or non-cat allergics (Figure 12B). In the context of CD4+ T cell population, the frequency of double IL-8+CD154+ T cells ranged from 0.008% to 0.08% in non-cat allergics and 0.01% to 0.1% of CD4+ T cells in cat allergics with cat allergen stimulation (Figure 12C,D).

Another activation marker that may be upregulated during T cell activation is CD137. The expression of CD137 in IL-8+ T cells was lower than the expression of CD154 when stimulated with cat allergens as approximately 0.5% of IL-8+ T cells expressed CD137 at both 6h and 24h (Figure 13A,B). In addition, the population of CD137+IL-8+ T cells comprised <0.01% of CD4+ T cells, in both non-cat allergics and cat allergics (Figure 13C,D).

Frequency of allergen-specific IL-8+ T cells characterized as TET+IL-8+ T cells

The frequency of allergen-specific T cells were also identified through the staining of CD4+ T cells with Fel d 1-specific MHC Class II tetramers in the second study visit. All subjects from the first study visit were included in the second visit, with the exception of one non-cat allergic subject who was replaced due to the withdrawal of subject from the study. Fel d 1-specific tetramers contained PE molecules that can be used to isolate tetramer-specific cells through PE enrichment with magnetic beads conjugated to anti-PE monoclonal antibodies, followed by identification by flow cytometry as PE+ cells. PE+ T cells were assessed in flow cytometry following the gating strategy outlined in Figure 14. PE+ enriched cells were gated for the lymphocyte population to disregard any debris left from the enrichment protocol. CD14-CD19- cells were selected and gated for CD3 and CD4. In the PE- cell fraction, CD3+ and CD4+ populations were evident (Figure 14B), while in the PE+ fraction, these populations merged with the CD3- and CD4- cells. As the cells prior to tetramer staining were purified CD4+ T cells, these cells were assumed to be CD3+CD4+ and were gated as so.

Further gating of the PE+ cells included the cytokine of interest, such as IL-8, IL-5 and IFNγ.

In cat allergics, the overall number of Fel d 1-specific tetramer-positive (TET+) detected was 328.2±369.5 per million CD4+ T cells, while in non-cat allergics, 166.8±107.1 per million CD4+ T cells was detected (Figure 15A; p=0.11). Of these TET+ cells, a large proportion expressed IL-8 in cat allergics (mean 99.7±153.8/million CD4) in comparison to IL-5 (9.5±15.8/million CD4) and IFNγ (4.8±3.1/million CD4). In non-cat allergics, the number of TET+IL8+ cells was lower (mean 50.9±43.5/million CD4), but the difference between the groups failed to achieve statistical significance. Similarly, TET+IFNγ+ cells and TET+IL-5+ were much lower in non-cat allergics (2.2±2.1 and 2.3±1.6/million CD4, respectively; Figure 15). The frequency of TET+IL8+ was similar to the frequency of CD154+IL8+ T cells detected after 6h Fel d 1 stimulation in non-cat allergics and cat allergics (Figure 15B).

Frequency of TET+IL-8+/IL-5+/IFNγ+ with and without dasatinib

To determine if the frequency of IL-8+, IL-5+ and IFNγ+ tetramer-positive cells were affected by the presence of dasatinib, cells from non-cat allergics and cat allergics were stained with tetramers without dasatinib added. The frequency of IL-8+TET+ was lower when no dasatinib was added, while IL-5+TET+ and IFNγ+TET+ increased (Figure 16). In TET- cells, the effects of dasatinib were more prominent, as there was a decrease in IL-5+TET- in non-cat allergics and IFNγ+TET- cells in cat allergics and non-cat allergics. IL-8+TET- cells also had higher frequencies in the presence of dasatinib, similar to the IL-8+TET+ cells.

Production of IL-8 measured by MFI and density of IL-8+ spots in ELISpot

To determine if the IL-8+ T cells differed in the production of IL-8, the median fluorescence intensity (MFI) of CD154+IL8+ and TET+IL8+ T cells were measured. In CD154+IL8+ T cells, the MFI increased with PMA/I stimulation in comparison to media at 6h (Figure 17A). At 24h, the MFI significantly decreased with cat dander and αCD3/28 in both cat allergics and non-cat allergics (Figure 17B). CD154+IL8+ T cells from cat allergics produced more IL-8 in comparison to non-cat allergics with 24h PMA/I stimulation, although the levels did not differ from media (Figure 17B). In TET+IL-8+ T cells, the MFI was higher in non-cat allergics (2342±1834 compared to 1113±507.8; Figure 17C).

In ELISpot assays, the amount of IL-8 produced per cell may be inferred by the area or density of the spots. The average area per spot was similar between non-cat allergics and cat allergics (Figure 17D) and did not differ between media, Fel d 1 and cat dander. The spot size increased by approximately 2-fold with αCD3/28 stimulation in both groups.

Phenotype of CD154+IL-8+ T cells

Stimulation with Fel d 1 and cat dander for 6h resulted in an increase in the number of CD154+IL-8+ T cells in both cat allergics and non-cat allergics that were further phenotyped. In the CD4+ T cell population, the majority of cells were naïve (52.9±14.8% in non-cat; 52.9±9.7% in cat; Figure 18A) and consisted of mainly CCR4-CRTH2- cells (79±6.2% in non-cat; 79.1±6.7% in cat) with <1% of CCR4-CRTH2- cells

expressing CXCR3, and CXCR3-CCR4- cells (78.9±6.1% in non-cat; 78.9±6.5% in cat; Figure 18B).

The phenotype of the CD154+IL8+ T cells were similar in both cat allergics and non-cat allergics. Upon stimulation with Fel d 1, the cells that produced IL-8 were either naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+) or effector memory (CD45RA-CCR7-) T cells. In non-cat allergics, more naïve CD154+IL-8+ T cells were detected in comparison to cat allergics (34.1±26.9% compared to 23.7±16.2%) while the frequency of central memory (33.9±18.4% in non-cat; 33.9±13.3% in cat) and effector memory T cell populations (28.4±21.1% in non-cat; 34.7±15.9% in cat) were similar in both groups (Figure 18C).

A majority of Fel d 1-stimulated CD154+IL-8+ T cells were CCR4+CRTH2- (73.2±8.1% in non-cat; 73.1±10.9% in cat), followed by CCR4-CRTH2- (16.7±11.6% in non-cat; 17.3±9.7% in cat) (Figure 18D). If the cells did not express CCR4 and CRTH2, 3.3±7.9% in non-cat allergics and 12.2±22.4% in cat allergics expressed CXCR3. Fel d 1-stimulated CD154+IL-8+ T cells were also characterized by CXCR3 in combination with CCR4. Most of these cells were CXCR3-CCR4+ (44.2±19.5% in non-cat; 45.9±25.4% in cat) and CXCR3+CCR4+ (38.4±26.2% in non-cat; 32.7±23.9% in cat). Of the CCR4- cells, 16.5±11.2% in non-cat allergics and 15.9±9.3% in cat allergics were also CXCR3-. In cat allergics, there was a higher percentage of CXCR3+CCR4- cells compared to non-cat allergics (5.4±7.9% vs 0.9±1.7%) (Figure 18D). In addition, Fel d 1-stimulated CD154+IL-8+ T cells did not co-produce IFNγ, but a small population co-produced IL-5 in both non-cat allergics (15.3%±13.2%) and cat allergics (8.9%±7.1%; Figure 18E).

With cat dander stimulation, the CD154+IL-8+ T cells were mostly effector memory cells (37.2±15.6% in non-cat; 44.8±11.5% in cat), followed by central memory (32.8±14.6% in non-cat; 31.1±7.5% in cat) and naïve cells (17.2±10.9% in non-cat; 18.9±8.3% in cat) (Figure 18F). Cat dander-stimulated CD154+IL-8+ T cells were also CCR4+CRTH2- (58.1±21.1% in non-cat allergics; 56.1±7.9% in cat allergics) or CCR4-CRTH2- (27.2±17.6% in non-cat allergics; 39.6±9.0% in cat allergics; $p<0.05$) (Figure 18G). Of the CCR4-CRTH2-, twice as many cells expressed CXCR3+ in cat allergics compared to non-cat allergics (7.3±4.9% vs 3.4±6.2%; $p<0.05$). A majority of cat dander-stimulated cells were CXCR3-CCR4+ (44.9±20.4% in non-cat; 43.5±9.3% in cat) followed by CXCR3-CCR4- (26.5±16.9% in non-cat; 37.5±10.6% in cat) and CXCR3+CCR4+ (18.1±19.9% in non-cat; 15.5±11.1% in cat). A small population of these cells were CXCR3+CCR4- (2.1±4.3% in non-cat; 3.4±3.3% in cat; Figure 18G). Similar to Fel d 1, cat dander stimulated IL-5 production in a small population of the CD154+IL-8+ T cells (6.1±8.1% in non-cat allergics and 5.4±4.3% in cat allergics), with little to no IFNγ (Figure 18H).

Phenotype of TET+IL-8+ T cells

The phenotype of TET+IL-8+ T cells differed from CD154+IL-8+ T cells in terms of the memory status of the cells. TET+IL-8+ T cells were predominantly effector memory T cells as 79±29.2% in non-cat allergics and 83.9±28.2% in cat allergics were CCR7-CD45RA- (Figure 19A). In non-cat allergics, the remaining cells were mostly CCR7+CD45RA- (15.4±30.3%) compared to cat allergics which were CCR7+CD45RA+ (10.2±28.4%) (Figure 19A). TET+IL-8+ T cells co-produced IL-5 (3.5±5.8%) and IFNγ

(3.1±7.1%) in non-cat allergics, while only IL-5 (2.2±2.6%) was detected in cat allergics (Figure 19B). Most of the TET+IL-8+ did not co-produce IL-5 or IFNγ (93.4±8.5% in non-cat; 97.1±2.8% in cat; Figure 19B).

Changes to CRTH2 staining

The percentage of CRTH2+ cells in the CD4+ T cell population was low (<0.2%; Figure 18). To test if the staining of CRTH2 was due to the antibody used, CRTH2 clone BM16 with two different fluorescence molecules, FITC and BV605, were compared on cat allergic samples. BV605 did not stain as well as FITC for CRTH2 (Figure 20A). To determine if fixation affected the staining of CRTH2 with BV605, samples were stained with BV605 CRTH2 and were not fixed (Figure 20B). In these samples, BV605 CRTH2 staining was increased as a higher percentage of cells were characterized as CRTH2+ in comparison to fixed cells (Figure 20).

With FITC CRTH2, a subset of cells from allergic and non-cat allergic subjects were stained with tetramer and CRTH2 and CCR4 antibodies after stimulation with αCD3/28 beads for 24h. TET+IL-8+ T cells expressed CRTH2 as 38.9±9.6% in non-cat allergics and 53.6±31.9% in cat allergics were CCR4+CRTH2+ (Figure 21), which was not evident in the phenotyping of CD154+IL-8+ T cells (Figure 18). The reminder of the cells were CCR4+CRTH2- (18.5±16.9% in non-cat; 12.7±11.1% in cat) or CCR4-CRTH2- (38.9±9.6% in non-cat; 26.5±27.3% in cat; Figure 21).

STIMULATORY MOLECULES THAT ELICIT IL-8 PRODUCTION IN T CELLS

Allergen stimulation

As IL-8+ T cells were present in all subjects, albeit with a low expression of CD154, we stimulated PBMCs for 6h with various allergens and other stimuli to determine if changes in the percentage of IL-8+ T cells were allergen specific. We tested cat dander, Fel d 1, ragweed (50 μg/ml) and its associated allergen, Amb a 1 (10 μg/ml), house dust mite (HDM; 50 μg/ml) and its allergen, Der p 1 (10 μg/ml), LPS (1 EU/ml), *Candida albicans* (1 μg/ml) and Cat-SPIRE (40 μM). Cat-SPIRE consisted of recombinant peptides of Fel d 1 and was used in clinical trials for PIT conducted by our lab. A high percentage of IL-8+ T cells were detected at 0h (4.0±1.8%) which decreased after the 6h incubation (2.3±1.3%; Figure 22). A slight, non-significant, increase in the percentage of IL-8+ T cells were detected with the allergens and various stimuli, except for PMA/I that caused a significant increase in IL-8+ T cells (10.4±6.8%; Figure 22).

TLR Stimulation of CD4+ T cells

To determine the signalling pathway of IL-8 production in T cells, purified CD4+ T cells were stimulated with toll-like receptor (TLR) agonists for 6 and 24h and supernatants were collected for IL-8 ELISA (Figure 23). The percentage of IL-8+ T cells did not significantly change when stimulated with TLR agonists (Figure 23A,B). Slight increases in IL-8+ T cells were detected with ODN2395 at 6h (Figure 23A), and with short and long poly (I:C), and αCD3/CD28 at 24h (Figure 23B). In contrast, the concentration of IL-8 produced by CD4+ T cells tended to increase after 6 and 24h of stimulation with cat dander (25.4±19.2 pg/ml at 6h; 852.5±745.4 pg/ml at 24h), LPS (16.9±4.3 pg/ml at 6h; 258.5±199.5 pg/ml at 24h) and R848 (103.9±95.1 pg/ml at 6h; 455.3±148.7 at 24h) (Figure 23C,D). Increases in IL-8 concentration also occurred with

Pam_3CSK_4 (107.8±11.3 pg/ml) and short poly (I:C) (32.4±17.5 pg/ml) stimulation compared to media at 24h (Figure 23D). The highest concentration of IL-8 was detected with PMA/I stimulation at both 6 and 24h, ranging from approximately 500 pg/ml to 3000 pg/ml.

Co-stimulatory T cell activation pathway

Activation of CD4+ T cells requires secondary signals after the initial activation from antigen-presenting cells. To explore the co-stimulatory signals that elicit IL-8 production in T cells, CD4+ T cells were stimulated with αCD2, αCD3, and αCD28 antibodies to explore co-stimulation by CD2 and CD28. Another method to assess CD2 co-stimulation is through stimulation with PHA-P (Flynn *et al.*, 1986). In one subject, the percentage of IL-8+ T cells did not change with any combination of αCD2/3/28 (Figure 24). However, with PHA-P and PHA-M, the percentage of IL-8+ T cells increased to 1.52% and 3.88%, respectively, which is indicative of CD2 co-stimulation for PHA-P and CD3 co-stimulation for PHA-M. The highest increase in IL-8+ T cells was observed with PMA/I as the percentage increased to 9.73% (Figure 24).

DISCUSSION

Optimization of protocol

Our mechanistic study of peptide immunotherapy for cat allergies resulted in a decrease in IL-8 production in T cells following treatment, suggesting that IL-8 may have an important role in the pathogenesis of allergic diseases. Our primary aim was to identify and phenotype IL-8+ CD4+ T cells to determine if a novel subset of 'Th8' cells exists in the allergic population. Several factors were optimized in our protocol for phenotyping

IL-8+ CD4+ T cells, before we recruited cat and non-cat allergic subjects, such as our PBMC isolation method, stimulation time, and flow cytometry parameters to detect and phenotype IL-8+ T cells.

In a preliminary experiment, we stimulated whole blood, RBC-lysed blood and PBMCs isolated by density gradient centrifugation with Ficoll-Paque, which is a well-used method in our laboratory, to determine if our PBMC isolation method affected the background percentage of IL-8+ T cells in 0h and 6h media controls. We observed that PBMCs isolated by Ficoll-Paque had a high background of IL-8+ T cells, in comparison to RBC-lysed blood and whole blood (Figure 1A). To further investigate if the methodology caused the higher background, i.e., density gradient centrifugation itself and not the Ficoll-Paque, two other density gradient media were tested, Lymphoprep and Histopaque-1077 (Figure 1B,C). In addition, we compared homemade RBC lysis buffer to a commercial buffer to determine if they functioned similarly. The density gradient centrifugation method resulted in a higher percentage of IL-8+ T cells of approximately 4% at 0h, and 2-5% at 6h compared to RBC lysis which was below 2% at 0h and 6h for both commercial and homemade buffers (Figure 1B,C). In the literature, it was evident that different isolation methods may result in differences in cell viability, recovery and functionality based on methodology itself (Bain and Pshyk, 1973; Berger and Edelson, 1979; Grievink *et al.*, 2016). In a study conducted by Grievink *et al*. (2016), three isolation methods were compared as PBMCs were isolated using density gradient centrifugation with Ficoll-Paque, cell preparation tubes (CPTs), or SepMate tubes with Lymphoprep. This study observed that IL-8 release increased in unstimulated PBMCs

isolated with CPTs in comparison to Ficoll and SepMate-isolated PBMCs, providing evidence that the isolation method can affect IL-8 production in PBMCs. Although RBC-lysed blood was not included in their study, our data suggests that RBC lysis does not increase IL-8 release in comparison to density gradient centrifugation.

The lower percentage in IL-8+ T cells detected in RBC-lysed blood at baseline and in the media controls may also be attributed to the immune cell types present in each isolation method. With density gradient centrifugation, larger, denser cells, such as granulocytes are removed, while with RBC lysis all immune cells remain present as only RBCs are removed (Dagur and McCoy Jr, 2016). The presence of granulocytes in RBC-lysed blood may have an inhibitory effect on lymphocytes, contributing to the lower IL-8+ T cell percentages at baseline. Sabbione *et al.* (2014) demonstrated that neutrophils had a suppressive effect on γδ T cells, preventing any spontaneous activation from occurring in unstimulated cultures. This phenomenon was also observed by Bain and Pshyk (1973) where lymphocytes isolated by Ficoll-Paque had enhanced stimulation in mixed lymphocyte reactions compared to cells in leukocyte-rich plasma, which was attributed to the removal of neutrophils during Ficoll-Paque isolation. This suggests that in RBC-lysed blood, the presence of granulocytes such as neutrophils, may be preventing T cells from producing IL-8 without the presence of a stimuli, resulting in the decreased IL-8+ T cell background observed with RBC lysis (Figure 1). During the clinical study for Aim 1, blood was processed using RBC lysis in the first study visit as this method resulted in a lower background of IL-8+ T cells in the unstimulated controls. However, the background percentage of IL-8+ T cells was not completely diminished with RBC

lysis, as between 1-2% of cells still expressed IL-8 (Figure 1). It may be possible that this baseline level of IL-8+ T cells is an innate population within the immune system. This theory is supported by a study conducted by Schaerli *et al.* (2014), where around 2.4% IL-8+ CD4+ T cells were detected in peripheral blood collected from healthy individuals. Further investigation of the possibility of an innate population of IL-8+ T cells, and whether it differs in allergic and non-allergic population, was conducted during the clinical study.

To detect IL-8+ T cells in flow cytometry, PBMC cultures required the addition of a protein transport inhibitor, such as monensin or brefeldin A (BFA), in the last 4h of incubation. Both are widely used for intracellular staining for flow cytometry and have different mechanisms of action (Fujiwara *et al.*, 1988; Mollenhauer *et al.*, 1990; Nylander and Kalies, 1999). Monensin is a Na+ ionophore that disrupts Na+ and H+ gradients in the Golgi apparatus, preventing trans-Golgi protein secretion (Mollenhauer *et al.*, 1990) while brefeldin A is a lactone that inhibits the transport of proteins from the ER to the Golgi, causing proteins to accumulate in the ER (Fujiwara *et al.*, 1988). Although monensin and BFA have different mechanisms of action, both inhibited the secretion of IL-8 in CD4+ T cells to a similar extent (Figure 2). There was no significant difference in the percentage of IL-8+ CD4+ T cells detected in unstimulated and stimulated samples. However, cell viability and cell recovery following 6h incubation were much lower with monensin compared to brefeldin A, as the cell cultures tended to clump during collection which was indicative of cell death (data not shown). In other studies, it was also evident that monensin caused more cell death and decreased viability in comparison to brefeldin

A in lymphocytes and monocytes (Dinter and Berger, 1998; Nylander and Kalies, 1999; Schuerwegh *et al.*, 2001). In addition, several studies showed that brefeldin A accumulated more cytokines in comparison to monensin, such as IL-1β, IL-6 and TNF-α in monocytes (Schuerwegh *et al.*, 2001), IL-4 in peripheral T cells (Miguel *et al.*, 2012), and IL-8 in $CD33^{intermediate}$ DCs (Bueno *et al.*, 2001). The results from our data and the literature suggests that brefeldin A is better suited for our study, and therefore experiments for the clinical study in Aim 1 was completed with brefeldin A.

The stimulation time required to detect IL-8+ T cells in flow cytometry was optimized. In two different cat allergic subjects, the peak of IL-8+ T cells with stimulation occurred between 12-18h (Figure 3A,B). Although the peak of IL-8+ T cells occurred with 12-18h stimulation times, we collected cells for flow cytometry in the clinical study after 6h and 24h of incubation. At 6h, preliminary data showed that this time point was adequate for T cells to produce IL-8 and to detect expression of T cell markers and other cytokines before any downregulation can occur (Figure 3B,C). At 24h, there may be a higher percentage of IL-8+ T cells as detected in one subject during the optimization of the method (Figure 3B). In the clinical study, cells were collected at 6h and 24h to ensure we were capturing a high frequency of IL-8+ T cells for further analysis.

The frequency of IL-8+ T cells was also measured by using an IL-8 ELISpot assay. Normally, ELISpots detect the number of cytokine-secreting cells by stimulating cells of interest directly in the plate. As we wanted to observe the frequency of allergen-specific T cells that produce IL-8, the parameters of the assay were optimized by using a

pre-stimulation method of PBMCs prior to plating isolated CD4+ T cells in the ELISpot plate. To determine allergen-specificity, PBMCs were stimulated with cat dander or Fel d 1 which requires APCs, such as DCs and monocytes, to activate cat allergen-specific CD4+ T cells. The pre-stimulation of PBMCs and CD4+ T cell incubation time in the ELISpot plate were both selected to be 24h. In allergic reactions, there are two phases of reactions that occur in the immune system – early and late phase reactions (Hansen *et al.*, 2004). Early phase reactions occur rapidly, within minutes of allergen exposure, due to the binding of IgE to basophils and mast cells that causes the release of histamines and other inflammatory molecules. Late phase reactions occur several hours (4-8h) after exposure and are mediated by other immune cells, e.g. T cells that are recruited to the site of allergen exposure. It is likely that *in vitro*, observing the effects of allergen on CD4+ T cells may take 6h or longer as it requires the processing of the antigen by APCs and subsequent activation of CD4+ T cells to produce and release cytokines. Therefore, a longer pre-stimulation time was selected for the ELISpot to ensure that allergen-specific CD4+ T cells were activated prior to plating in the ELISpot.

In our preliminary run-through of Aim 1.3, we were able to identify and phenotype IL-8-producing CD4+ T cells in five subjects, three of which were cat allergic. We adequately identified IL-8+ T cells with CD3+CD4+ gates without the removal of CD14+ and CD19+ cells from the lymphocyte gate (Figure 5). This suggests that it was possible to exclude CD14 and CD19 in our flow cytometry panel in subsequent experiments without compromising the purity of the IL-8+ CD4+ T cell population, which provided more space in our panel for T cell markers and cytokines. In four of the

five subjects, our preliminary panel included markers for CD154, CRTH2, CD137, CCR4, IFNγ, IL-10, IL-5, IL-4, IL-13 and IL-2. In these subjects, there was no significant difference in the percentage of IL-8+ T cells between cat allergic and non-cat allergic subjects for all stimuli (Figure 7A), though it should be noted that the allergic status of the subjects were not clinically tested. With PMA/I stimulation, a larger percentage of IL-8+ T cells was detected in the cat allergic population. During the optimization of the protocol for the clinical study, preliminary phenotyping of IL-8+ T cells was conducted. Approximately 30-40% of IL-8+ T cells expressed CCR4 and less than 5% co-produced IFNγ, IL-4, IL-5 or IL-10 (Figure 7C,D). CCR4 is a T cell marker associated with Th2 cells in allergic diseases such as atopic dermatitis and allergic rhinitis (Wakugawa *et al.*, 2001; Banfield *et al.*, 2010), and in autoimmune diseases (Yang *et al.*, 2004). However, CRTH2, a known surface marker for Th2 cells (Cosmi *et al.*, 2000), was not detected on these IL-8+ T cells which may be attributed to staining issues with fixation and the fluorophore used, BV605. With changes to the CRTH2 antibody, CRTH2 expression was detected on 40-54% of allergen-specific IL-8+ T cells (Figure 21). Some of the identified IL-8+ T cells may be part of the Th2 subset (ie. <5% of IL-8+ T cells) due to the expression of IL-4 and/or IL-5, which are known Th2-associated cytokines (Akdis *et al.*, 2004). IFNγ, a Th1-associated cytokine, and IL-10, a regulatory T cell cytokine, were also detected in preliminary experiments. In the study conducted by Yang *et al.* (2004), IFNγ and IL-10 were detected in CD4+ CCR4+ T cells from patients with rheumatoid arthritis, systemic lupus erythematosus and ankylosing spondylitis, which they speculated were Th2 cells. This suggests that IFNγ+ and IL-10+ IL-8+ T cells may also fall under

the Th2 subset due to the similarities with the subset of cells identified by Yang *et al.* (2004). Another possibility is that our population of IL-8+ T cells is a Treg population. In one subject, CD25, a marker for Tregs, was also expressed on 6-16% of IL-8+ CD4+ T cells in addition to CCR4 (Iellem *et al.*, 2001; Akdis *et al.*, 2004). CCR4+ CD25+ Tregs have been identified in the literature and have shown to produce IL-10 (Iellem *et al.*, 2001). However, as a low percentage of IL-8+ T cells expressed other cytokines and surface markers associated with Th1, Th2 or Treg cells, our IL-8+ T cells may constitute a novel Th8 subset that have similar characteristics to Th2 cells due to the expression of CCR4, and possibly CRTH2. We further explored the phenotype of allergen-specific IL-8+ T cells in the clinical study.

A few populations of IL-8+ T cells have been characterized in the literature. One of the predominant subsets of IL-8+ T cells were recent thymic emigrants (RTEs) which consisted of naïve T cells that were CD45RA+ and CD25- (Das *et al.*, 2017; Pekalski *et al.*, 2017). These IL-8+ T cells were found predominantly in neonates in comparison to adults (Das *et al.*, 2017), and were also identified in cord blood (Wong *et al.*, 2016). Schaerli *et al.* (2004) studied IL-8+ T cells in patients with AGEP, a neutrophilic drug-induced immune reaction. This population of T cells were present at the site of inflammation and produced Th1-associated cytokines, IFNγ and GM-CSF, and expressed CCR5, CXCR6 and CCR4 in <20% of the cells, characterizing the cells as Th1 effector memory T cells. As seen in these studies, there may be some important markers that we have not included in our first preliminary panel during optimization, including CD45RA, CCR5, CXCR6 and cytokines such as GM-CSF. In a fifth subject, we isolated immune

cells using RBC lysis of whole blood and stained stimulated cells with a panel consisting of CD45RA, CCR7, CXCR3, CRTH2, CCR4, HLA-DR, CD38, CD154, IL-8 and IL-5. The inclusion of these T cell markers provided more insight on the phenotype of IL-8+ T cells, in comparison to cytokines in the previous panel. As seen with the previous four subjects, around 20-40% of IL-8+ T cells expressed CCR4. In addition, we detected the expression of CD38 in 20-30% of IL-8+ T cells and HLA-DR in <20% (Figure 7E). CD38 and HLA-DR are activation markers of T cells, though CD38 expression is selective to memory T cells. In the population of IL-8+ T cells, little to no expression of CXCR3 or CRTH2 was detected, which are markers for Th1 and Th2 cells, respectively. Again, the low expression of CRTH2 may be attributed to the staining protocol which was not optimal for the fluorophore used for CRTH2 (BV605), which we determined during the clinical study, and resolved by using a different fluorophore. In a subsequent experiment, CRTH2 was detected on 40-54% of allergen-specific TET+IL-8+ T cells (Figure 21). In addition, the majority of IL-8+ T cells were CD45RA-CCR7-, indicative of effector memory T cells. This data suggests that IL-8+ T cells are a novel subset of T cells that are effector memory with no expression of markers for Th1 or Th2 T cell subsets, which was similar with the preliminary data from the previous four subjects. However, there are other T cell subsets we have not included markers for such as Th17 (CCR6+CCR4+, IL-17+ (Bonnevier *et al.*, 2013; Dunay *et al.*, 2016)), Th9 (IL-9+ (Kaplan, 2013)), Th22 (IL-22+ (Eyerich *et al.*, 2009)) and Tregs (CD25+, FoxP3+ (Chen and Oppenheim, 2011)). Also, the addition of CD45RA in the panel showed that the population of IL-8+ T cells we observed was different from IL-8 producing RTEs

previously described in the literature, as the IL-8+ T cells were CD45RA-. We further investigated the phenotype of these IL-8+ T cells in cat allergic and non-cat allergic subjects, using the panel described for the fifth subject, to determine if there is a differential expression of T cell markers and IL-8 that is dependent on allergic status, as discussed later on.

The contribution of these IL-8+ T cells in allergic diseases relies on the hypothesis that these cells are allergen-specific and produce IL-8 upon allergen exposure. In various studies, allergen-specific T cells have been identified by CD154 expression following *ex vivo* stimulation with allergens (Campbell *et al.*, 2010; Smith *et al.*, 2013). Despite detecting a population of IL-8+ T cells in our preliminary results, these cells may not be allergen specific due to the low expression of CD154 (Figure 7C,E). As we used volunteers from our lab for our preliminary study, the low expression of CD154 may be attributed to the allergic status of our subjects as we did not clinically measure their cat allergic status, as we did during recruitment. However, Smith *et al.* (2013) characterized CD154+ T cells within the cat allergic population and had found that upon stimulation with cat dander, only 0.74% of CD154+ T cells were detected. Therefore, it is possible that our detection of 1-5% of IL-8+ T cells expressing CD154 is sufficient in identifying allergen-specific T cells. Some allergen specificity was seen in our results (Figure 22) as certain allergens increased the number of IL-8+ T cells, though the allergy status of the subjects was not known so the relationship between the subjects' allergies to changes in IL-8+ T cells cannot be inferred. In addition, as whole allergen extracts were used, IL-8 production may occur irrespective of the allergen itself due to the composition of the

extracts. It is also evident that this response is not due to LPS, which is indicative of endotoxins present in our allergens, as it elicited the lowest percentage of IL-8+ T cells (Figure 22, Figure 23), therefore attributing the response to the allergens or other endotoxins. It may also be possible that our IL-8+ T cells are allergen-specific but did not express CD154 and may instead express other activation markers such as CD137 which is expressed on activated Treg cells (Bacher *et al.*, 2016), both of which were analyzed in the clinical study.

We needed to further investigate if these IL-8+ T cells we were detecting were allergen-specific which was done through recruiting known cat allergics and non-cat allergics as well as through different techniques, such as identifying allergen-specific T cells by using MHC class II tetramers, which our lab has previously used, and IL-8 ELISpots. Initially, we aimed to characterize IL-8+ T cells through detection of CD154 expression, though after preliminary experiments, it was evident that the IL-8+ T cells may not express CD154. To further determine if the IL-8+ T cells are allergen-specific, we conducted MHC Class II tetramer staining on recruited cat allergic and non-cat allergic subjects. Tetramers directly stain allergen-specific T cells, though they will not capture the entire repertoire of cat-allergen reactive T cells as the tetramers display certain epitopes of the cat allergen. Initially, these tetramer-stained T cells were to be stimulated with PMA/I to maximize the production of IL-8 as PMA/I induced the highest percentage of IL-8+ T cells in every subject. However, preliminary run-throughs of this experiment in cat allergic subjects had low numbers of TET+IL-8+ T cells (Figure 9), so

in the clinical study, TET+ cells were stimulated with αCD3/28 beads, similar to the protocol used in the mechanistic study on the effects of PIT in cat allergic subjects.

In addition, we observed differences in the number of IL-8+ T cells between cat allergic and non-cat allergic subjects through ELISpot assays. ELISpots detect cytokine producing cells at a single cell level by generating a single spot on a solid membrane for each antigen-specific cell (Cox *et al.*, 2006). This increases sensitivity, in comparison to flow cytometry, in detecting IL-8+ T cells as ELISpots can detect rare antigen-specific T cells in less than half a million cells (McCutcheon *et al.*, 1997). In a study conducted by Karlsson *et al.* (2003), ELISpot assays were more likely to detect low-level responses in comparison to flow cytometry, which may have occurred in our study in terms of the IL-8 response in T cells via stimulation with cat allergens. In our experiments, it was evident that there may have been IL-8+ T cells that were not captured by flow cytometry through intracellular staining. As seen in Figure 23, stimulation with various TLR agonists on purified CD4+ T cells did not elicit a change in the percentage of IL-8+ T cells but measurement of IL-8 production by ELISA showed that some TLR agonists elicited an increase in IL-8, although non-significant. However, this may be attributed to the purity of the isolated CD4+ T cells as the purity was approximately 95-97%. Some monocyte and neutrophil contamination were detected in the CD4+ T cells, albeit at a low percentage (<1%). The presence of monocytes and neutrophils may contribute to the production of IL-8 detected by ELISA as these cell types produce IL-8, especially in the presence of LPS, which is also in cat dander, and R848 (Merck *et al.*, 2006). Although we were able to detect IL-8+ T cells in flow cytometry, we may not have observed the

minute changes that occur during stimulation with cat allergens. Therefore, the addition of the IL-8 ELISpot, which has higher sensitivity in detecting antigen-specific cells, was expected to aid in observing the differences in IL-8+ T cells between the allergic and non-allergic population.

Frequency of allergen-specific T cells detected in the clinical study

Upon optimization of the protocol to detect IL-8+ T cells in flow cytometry and IL-8 ELISpot, 12 cat allergic and 13 non-cat allergic subjects were recruited into our clinical study to determine the frequency and phenotype of IL-8+ T cells. We hypothesized that if the population of IL-8+ T cells was inherent to the allergic population, there would be a higher frequency of IL-8+ T cells in cat allergic subjects. However, we did not detect a significant difference in the frequency of IL-8+ T cells between cat allergics and non-cat allergics, despite the higher percentage of IL-8+ T cells in cat allergics (Figure 10), which suggests that the our study may be insufficiently powered to detect small differences between the groups. Although non-significant, there may be more IL-8+ T cells in cat allergics at baseline that may contribute to the pathogenesis of allergic diseases.

In addition, the frequency of IL-8+ T cells increased with 6h stimulation with cat dander but not at 24h in cat allergic subjects. This suggests that the IL-8+ T cells may be allergen-specific and respond to cat allergens quickly, similar to the time a late phase reaction occurs *in vivo* (Hansen *et al.*, 2004). However, the percentage of IL-8+ T cells increased with cat dander in non-cat allergic subjects as well which is likely attributed to the presence of cat allergen-specific T cells in non-cat allergics and the composition of cat

dander. As identified in the literature, both cat allergics and non-cat allergics have cat allergen-specific T cells that respond to Fel d 1, although resulting in differential production of cytokines such as IL-5 and IFNγ (Haselden *et al.*, 2001; Carneiro *et al.*, 2004; Platts-Mills and Woodfolk, 2011). In our study, the frequency of IL-8-producing CD4+ T cells was higher in comparison to IFNγ- and IL-5-producing cells, which may suggest that IL-8 production may be upregulated in various T cell subsets, if not only from a novel Th8 subset. In addition, the composition of cat dander extract contains more than cat allergens (ex. endotoxins) that may activate CD4+ T cells to produce IL-8 in both cat allergics and non-cat allergics, rather than just the allergens itself. This was further supported by the stimulation of PBMCs with purified, endotoxin-free, Fel d 1, which elicited a smaller increase in IL-8+ T cells in comparison to cat dander, suggesting that there was more than the major cat allergen, Fel d 1, present in cat dander that elicited IL-8 production in CD4+ T cells (Figure 10). Again, as observed in preliminary experiments, in both cat allergics and non-cat allergics, stimulation with PMA/I induced the largest percentage of IL-8+ T cells at 6h and 24h (Figure 10), suggesting that CD4+ T cells have the capability to produce IL-8, but only a subset of them are responsive to other stimuli or through TCR stimulation.

The production of IL-5, indicative of Th2 cells, and IFNγ, indicative of Th1 cells, were used as controls as allergen-specific T cells from allergic subjects produced Th2 cytokines and non-cat allergics produced Th1 cytokines when stimulated (Haselden *et al.*, 2001; Kwok *et al.*, 2010). Despite the known difference between cat allergics and non-cat allergics, IL-5 production was not significantly higher in cat allergics nor was IFNγ

higher in non-cat allergics. This may be due to the small sample size and large variability observed in our study, suggesting that our study may not be powered sufficiently to detect significant differences between allergics and non-allergics. However, it should be considered that the protocols between our study and previous studies differ. In previous studies, IL-5 and IFNγ were measured after long incubation periods (ex. for up to 6 days) and by using ELISAs, not flow cytometry (Haselden *et al.*, 2001; Jenmalm *et al.*, 2001). In addition, when flow cytometry was used, the protocol involved stimulation with PMA/I on allergen-specific T cells for a shorter period of time (approx. 4h) (Kwok *et al.*, 2010; Uchtenhagen *et al.*, 2016). Although PMA/I stimulation was used in our study, the stimulation was conducted on PBMCs and not purified allergen-specific T cells, therefore, we were not able to determine the frequency of IL-5 and IFNγ cells that were attributed to cat allergen-specific T cells even though both cytokines were upregulated with PMA/I stimulation. Another factor that may affect the percentages of IL-5, in particular, may be that the asthmatic status of the participants were not controlled or tested for, so both groups may have had participants who were asthmatics. Levels of IL-5 have been found to be higher in people with asthma, even in compared to non-asthmatic allergics (Greenfeder *et al.*, 2001; Haselden *et al.*, 2001), and therefore may be a confounding factor that masks the differences between cat allergics and non-cat allergics.

Similar results were observed in the IL-8 ELISpot assay. The number of IL-8+ T cells did not differ between cat allergics and non-cat allergics, irrespective of the stimuli used (Figure 11), as both groups displayed the same trends in the increases of IL-8+ T cells. In contrast to flow cytometry, the number of IL-8+ T cells was significantly higher

with αCD3/28 stimulation in both groups. This suggests that both cat allergics and non-cat allergics have similar frequency of CD4+ T cells that were capable of producing IL-8 when stimulated by the TCR. Although the IL-8 ELISpot had a higher sensitivity compared to flow cytometry, minute differences in the frequency of IL-8+ T cells were not detected. This may be due to the high background detected in the media control wells as well as the variability between subjects. The high background may be attributed to the purity of CD4+ T cells after isolation from PBMCs. In previous experiments, the purity of CD4+ T cells isolated by negative selection was assessed in flow cytometry and was shown to be approximately 95-97% pure of CD4+ T cells. Some of the contaminating cells included monocytes and neutrophils, both of which can produce IL-8 and contribute to the high background in the media control.

The allergen-specificity of IL-8+ T cells was identified through the expression of CD154. In cat allergic subjects, the frequency of IL-8+ T cells expressing CD154 was higher in comparison to non-cat allergic subjects (Figure 12). Despite the higher frequency, these cells were a small subfraction of the IL-8+ T cells which suggests that although CD4+ T cells were responding to cat allergens to produce IL-8, only a small subset of these cells were allergen-specific T cells (ie. recognize cat allergens through the TCR). The other IL-8+ T cells that did not express CD154 may have been activated through bystander effect, or other stimulatory pathways, and may not upregulate CD154. The frequency of IL-8+ T cells that were allergen-specific (ie. CD154+) was approximately 2% in cat allergics and 1.5% in non-cat allergics with 6h cat dander stimulation (Figure 12A). As mentioned previously, the percentage may have been high

in cat allergics and non-cat allergics due to the composition of cat dander. The frequency of allergen-specific T cells that we detected were higher in comparison to previous studies in the literature. In a study conducted by Smith *et al.* (2013), 0.74% of T cells expressed CD154 after cat dander stimulation in cat allergic subjects, while other studies identified the frequency of Fel d 1-specific T cells to be approximately 1 in 7,000 to 1 in 300,000 in cat allergics and substantially lower in non-cat allergics (Kwok *et al.*, 2010; Ueno-Yamanouchi *et al.*, 2011). In contrast to 6h stimulation, at 24h stimulation, the IL-8+ T cells did not express CD154 which may be due to the downregulation of CD154 after stimulation which was evident in the timeline experiments where earlier stimulation times had higher expression of CD154 in comparison to 24h (Figure 3). This is further supported by a study conducted by Bacher and Scheffold (2013), who identified that CD154 expression was best captured at 6h.

We further investigated the existence of allergen-specific IL-8+ T cells through MHC Class II tetramer staining and stimulation with αCD3/28 beads. Tetramer-stained cells demonstrated a difference in CD3 and CD4 staining in comparison to unstained cells (Figure 14). Despite being a purified CD4+ population, the staining indicated that the tetramer-stained cells were "CD3-CD4-" cells. This may be due to the tetramer complex itself binding to the TCR and preventing the binding of CD3 and CD4 antibodies. In addition, we used αCD3/28 beads for stimulation which may also complex with the tetramers and TCR, further hindering the binding of CD3 and CD4. Another reason these tetramer-stained cells were "CD3-CD4-" may be the downregulation of the TCR complex

due to stimulation by the tetramer and αCD3/28. In further analysis of the TET+ cells by backgating, these cells were in the "CD3-" fraction (data not shown).

The mean frequency of Fel d 1-specific (TET+) CD4+ T cells was higher in cat allergics (328.2±369.5/million CD4) in comparison to non-cat allergics (166.8±107.1/million CD4), however, the difference was not statistically significant, possibly due to the limited sample size and heterogeneous data. The frequencies detected in our study were higher than the frequencies identified by Kwok *et al.* (2010). At most, Kwok *et al*. detected a frequency of 1 Fel d 1-specific T cell in 7000 CD4+ T cells, corresponding to roughly 142/million CD4, and virtually no Fel d 1-specific T cells in non-cat allergics. Our frequencies were twice as high compared to Kwok *et al.*, which may be due to the inclusion/exclusion criteria for our study as we did not control for cat exposure, or measure the strength of allergic reactions through skin prick tests or nasal allergen challenges during our selection of allergics and non-allergics. Differences in flow cytometry gating strategies may have also contributed to these differences.

Of the Fel d 1-specific T cells, less than half produced IL-8 when stimulated with αCD3/28 beads and a higher number of these TET+IL-8+ T cells were observed in the allergic population (Figure 15). This suggests that there may be more allergen-specific T cells that are capable of producing IL-8 in allergic subjects in comparison to non-allergics. However, this increase in TET+IL-8+ T cells was non-significant, and further studies with a larger number of subjects is required to determine if there are significantly higher frequencies of these cells in allergics compared to non-allergics. In comparison to CD154, the number of CD154+IL-8+ T cells per million CD4+ was higher than the

number of TET+IL-8+ T cells detected (Figure 15B). This is attributed to the stimuli used to assess CD154 expression. As CD154+IL-8+ T cells were detected in PBMCs stimulated with whole cat allergens, APCs can take up the allergens and process a wide range of epitopes that can be presented to a large number of T cells. In addition, cells that may be indirectly activated through the bystander effect may also express CD154 (Bonvalet *et al.*, 2011). In contrast, tetramer staining only targets a small subset of allergen-specific T cells due to the composition of the tetramer molecules. MHC Class II tetramers are difficult to synthesize and are limited to the HLA tissue type of the participants and only have 2-3 peptides available per HLA allele, in terms of Fel d 1-specific tetramers (Vollers and Stern, 2008; Nepom, 2012; see Table 3). Therefore, CD154+IL-8+ T cells represented a wider repertoire of allergen-specific T cells in comparison to TET+IL-8+ T cells, and may be more representative of the response that occurs when in contact with allergens, but may be more susceptible to bystander artifacts.

The frequency of TET+cytokine+ T cells may be affected by dasatinib, a protein tyrosine kinase inhibitor used to prevent the downregulation of the TCR and enhance the detection of tetramers in flow cytometry (Lissina *et al.*, 2009). In our study, IL-8 was not affected by the presence of dasatinib, while IL-5 and IFNγ were, as the number of TET+IL-5+ and TET+IFNγ+ cells was higher when no dasatinib was used (Figure 16). This was also observed in the phenotyping of allergen-specific IL-8+ T cells as approximately 15% of CD154+IL-8+ T cells and <3.5% of TET+IL-8+ T cells produced IL-5. CD154+IL-8+ T cells were not incubated with dasatinib while TET+IL-8+ T cells were, which suggests that dasatinib may have inhibited IL-5 in allergen-specific TET+ T

cells. Further studies on dasatinib have demonstrated an inhibitory effect on T cell activation, including the inhibition of proinflammatory cytokines such as IFNγ, TNFα, IL-2, IL-6 and IL-17 (Blake *et al.*, 2008; Schade *et al.*, 2008; Weichsel *et al.*, 2008), while the effects of dasatinib on IL-5 production has not been explored in the literature. However, Schade *et al.* (2008) demonstrated that the effects of dasatinib were reversible by using a stimulation method that bypasses the TCR, such as PMA/I. In many studies involving the assessment of TET+ cells for the production of Th1 or Th2 cytokines, PMA/I was predominantly used. Therefore, in our study, the use of αCD3/28 beads on the tetramer-stained cells would not be able to reverse any inhibitory effects of dasatinib, affecting the frequency of cells detected and possibly accounting for the low number of TET+ IL-5+ or IFNγ+ T cells.

Another factor that may contribute to immunological differences between allergics and non-allergics may be the amount of IL-8 produced by these IL-8+ T cells. In studies conducted by Xue *et al.*, they observed that increased Th2 cytokines were due to increased production of these cytokines per cell, instead of an increase in the number of Th2 cells (Xue *et al.*, 2012, 2014). In our study, the amount of IL-8 produced was inferred through MFI in flow cytometry and IL-8 spot area in ELISpots (Figure 17). Both of these measures displayed no significant difference between the two groups, suggesting that there was no differential production of IL-8 in these cells. As the number of IL-8+ allergen-specific T cells and the amount of IL-8 produced by these T cells did not differ between cat allergics and non-cat allergics in this study, the immunological significance

of these cells may be due to interactions of allergen-specific IL-8+ T cells with the immune system as a whole.

In allergic individuals, the immune system is primed or sensitized to react to allergens to generate Th2 cell-mediated responses. Sensitization occurs by priming allergen-specific CD4+ Th2 cells to release IL-4, IL-5 and IL-13, which cause B cells to produce IgE that binds to mast cells and basophils (Larché *et al.*, 2006). Subsequent exposure to allergen leads to the cross-linking of IgE on mast cells and basophils and causes degranulation and release of mediators that cause allergic symptoms (ex. histamines, leukotrienes). The allergens also bind to specific IgE that are on dendritic cells and monocytes which increases their uptake of the allergen and presentation of epitopes to Th2 cells to further propagate an allergic reaction (Larché *et al.*, 2006). In contrast, non-allergic individuals are not sensitized to the allergens as no allergen-specific IgE or Th2 cells are detected. Instead, non-allergics mount a Th1 response, producing IFNγ during allergen stimulation (Haselden *et al.*, 2001).

Although allergen-specific IL-8+ T cells were present in both cat allergics and non-cat allergics, the immunological significance of these cells may be due to the sensitization state of allergics compared to non-allergics. The effects of IL-8+ T cells may be synergistic to IgE antibodies, mast cells and Th2 cells found in the allergic population. In the literature, the activation of mast cells causes release of prostaglandin D2 (PGD_2) and leukotriene E4 (LTE_4), which are mediators known to enhance the production of cytokines in Th2 cells, as well as causing allergic symptoms such as affecting airway smooth muscles (Xue *et al.*, 2012, 2014). PGD_2 can also cause the migration of Th2 cells

to the site of allergen exposure as it binds to CRTH2 expressed on Th2 cells. In addition, Xue *et al.* (2014) identified that the synergy of PGD_2 and LTE_4 led to the production of non-classical cytokines in Th2 cells such as IL-8, IL-22 and GM-CSF. Therefore, it is possible that the effects of IL-8 are two-fold, where activation of mast cells through cross-linking of IgE causes production of IL-8 in Th2 cells while allergen-specific IL-8+ T cells produce IL-8 through stimulation with allergens itself, as identified in our study. This production of IL-8, especially in allergic asthma, may contribute to disease pathogenesis by causing the chemotaxis and activation of neutrophils. In addition, mast cells and Th2 cells produce IL-3, which along with IL-8, may also induce basophil chemotaxis and activation. Therefore, IL-8+ T cells may work synergistically with mast cells and Th2 cells to cause the migration of neutrophils and basophils to the site of allergen encounter and subsequent activation and release of histamines and leukotrienes that lead to stronger allergic responses. As non-allergics do not have Th2 cells present, the presence of IL-8+ T cells alone cannot propagate allergic responses.

The interplay between allergen-specific IL-8+ T cells with immune cells involved in allergic reactions needs to be addressed in future studies. First, the frequency of allergen-specific IL-8+ T cells needs to be addressed in a larger cohort of participants to determine if there is a significantly higher population of allergen-specific IL-8+ T cells in allergics compared to non-allergics. In addition, the downstream effects of IL-8 produced by allergen-specific IL-8+ T cells will need to be explored by conducting chemotaxis and activation assays on neutrophils and basophils when cultured with IL-8+ T cells to determine if there is differential activity in response to IL-8 produced by allergen-specific

T cells from allergics compared to non-allergics. We also need to explore whether the presence of mast cells and Th2 cells work synergistically with allergen-specific IL-8+ T cells, which can be assessed through co-culture experiments (Th2 ± Th8 with neutrophils and basophils) to see which cell types are required for the chemotaxis and activation of neutrophils and basophils. These experiments will further our understanding of the role of IL-8+ T cells in the context of allergic inflammation.

Phenotype of IL-8+ T cells detected in clinical study

The surface markers identified on CD154+IL-8+ T cells suggest that these cells may have similar homing characteristics to Th2 cells as a majority of the cells, both in cat allergics and non-cat allergics, expressed CCR4 (approx. 73%). The expression of CRTH2, the most reliable marker for Th2 cells (Cosmi *et al.*, 2000), was not detected which may have been due to the staining and fixation protocol used to detect CD154 expression. CRTH2 BV605 was unable to withstand fixation with 4% PFA and constant vortexing, and may have lost its fluorescence in the time period between staining and acquiring samples (approx. 40h). In comparison to a study conducted by Wambre *et al.* (2017), CRTH2 was detected on allergen-specific T cells when the same CRTH2 clone BM16 was used but with a different fluorophore, AF647, and fixation method, Cytofix/Cytoperm buffer. In a side experiment, CRTH2 FITC staining on TET+ cells showed that 38.9% to 53.6% of TET+IL-8+ T cells expressed CRTH2 along with CCR4, similar to the frequencies identified in a study conducted by Kwok *et al.* (2010) where approximately 95% of Fel d 1-specific T cells expressed CCR4 and a range of cells (17-88%) expressed CRTH2. Our percentages were also similar to the observations in peanut

allergy, where 15-20% of peanut-stimulated CD154+CD4+ T cells expressed CCR4, and 30-60% expressed CRTH2 (Blom *et al.*, 2018).

If the staining for CRTH2 was truly indicative of a low level of CRTH2 expression on IL-8+ T cells rather than insufficient staining, it may be possible that these cells have a Th2 phenotype with the expression of just CCR4. This has been observed in a subset of Ara h 1-specific T cells in peanut allergy, where 25% of cells expressed CCR4 and <8% expressed CRTH2, which DeLong *et al.* suggested was a modified Th2 cell type that may be functionally different from CRTH2+ Th2 cells (DeLong *et al.*, 2011). However, the cytokine profile of IL-8+ T cells indicates that they may not be Th2 cells as no IL-5 was detected. Other T cell subsets also express CCR4 with no CRTH2, such as CD25+ Tregs which produce IL-10 (Iellem *et al.*, 2001). However, due to limitations of flow cytometry, we were unable to assess the expression of CD25 or IL-10 and therefore unable to exclude whether these IL-8+ T cells expressed Treg markers. CD154+IL-8+ T cells also expressed CXCR3, as a large proportion of cells were CXCR3+CCR4+ cells. In a study conducted by Wambre *et al.* (2014), a subset of allergen-reactive T cells were identified as CXCR3+ with low expression of CRTH2 and CCR4 that also produced IL-10 and IFNγ, which was attributed to the allergen-epitope stimulating the cell. Therefore, it is possible that these IL-8+ T cells (CXCR3+CCR4+) differed from the cells that were CCR4+CRTH2- and may be reactive to different epitopes of cat allergens.

The differentiation state of the allergen-specific IL-8+ T cells was predominantly memory T cells in both CD154+ and TET+ cells. CD154+IL-8+ T cells were divided into effector memory (CD45RA-CCR7-) and central memory (CD45RA-CCR7+) while

TET+IL-8+ consisted of more effector memory cells. This was also observed by Kwok *et al.*, (2010) where Fel d 1-specific CD4+ T cells had a memory phenotype, characterized as CD45RO+CD28+CD26L+, and were a mixed population of central and effector memory cells. The memory phenotype of the allergen-specific IL-8+ T cells suggests that these cells were primed to produce IL-8 in response to allergen. Less than a quarter of the allergen-specific CD154+ and TET+IL-8+ T cells were naïve (CD45RA+CCR7+). These cells may have had allergen-specific TCRs that were stimulated by their cognate antigen when in the presence of Fel d 1 and cat dander, or were stimulated through other receptors, such as TLRs. The former is most likely the response observed with Fel d 1-tetramer staining, while the latter may be occurring in CD154+IL-8+ T cells. As a majority of the allergen-specific IL-8+ T cells were memory T cell subsets, these IL-8+ T cells differed from the IL-8+ T cells observed in the literature thus far. As mentioned previously, IL-8+ T cells have been observed to be recent thymic emigrants (ie. naïve cells) and have shown to produce IL-8 through activation by TLRs (Wong *et al.*, 2016; Das *et al.*, 2017).

We hypothesized that a novel subset of IL-8-producing T cells exists, called ‘Th8’ cells, and are primed during sensitization to produce IL-8 in response to allergens. We identified that allergen-specific IL-8+ T cells expressed CCR4, and possibly CRTH2, which are markers associated with a Th2 phenotype. However, many of these cells did not co-produce IL-5 (<15% in CD154+IL-8+ T cells and <3.5% in TET+IL-8+ T cells) which is a classical cytokine for identifying Th2 cells. It may be possible that some of these allergen-specific IL-8+ T cells were Th2 cells (i.e. the ones that produce IL-5), like

the ones identified by Xue *et al.* (2014), but the majority of the cells may be a novel Th8 subset due to the cytokine profile we observed. We believe that the allergen-specific IL-8+ T cells detected in our study may be a novel Th8 subset of cells that have similar homing capabilities as Th2 cells, due to the expression of CCR4 and CRTH2, that allow them to migrate and interact with Th2 cells at the site of allergen encounter. This fits with our hypothesis in which we believe the effects of IL-8+ T cells require the presence of Th2 cells, in addition to mast cells, to cause neutrophil and basophil activation and subsequent release of mediators associated with allergic symptoms, and therefore potentiate the allergic response.

Further phenotyping of allergen-specific IL-8+ T cells is needed to ensure that this population of IL-8+ T cells differ from known Th2 populations and if they constitute their own subset of Th8 cells. Various subsets of Th2 cells that have been characterized based on the expression of certain surface markers and cytokines. In phenotyping Th2 cells, cytokines such as IL-4, IL-5, and IL-13 are widely use as they are specific to Th2 cells. However, Th2 cells have shown to produce a lot of other cytokines such as IL-2, IL-3, IL-9, IL-10, GM-CSF, and TNF (Prussin *et al.*, 2010). Some of the populations of Th2 cells identified include regulatory Th2 cells that produce IL-10 and IL-5, IL-4- Th2 cells, IL-5+ or IL-5- Th2 cells, and Th2 follicular helper T cells (Prussin and Metcalfe, 1995; Upadhyaya *et al.*, 2011). Another subset of Th2 cells was recently discovered in the allergic population called pro-allergic Th2 cells (Th2A cells), which were shown to produce Th2 cytokines at higher quantities and exist predominantly in allergic subjects than non-allergics (Wambre *et al*., 2017). The production of IL-8 in these Th2 subsets

was not explored, and therefore allergen-specific IL-8+ T cells may correspond with an existing Th2 subset, or may be a novel subset. Further phenotypic analysis is required, such as including other cytokines (ex. IL-4, IL-13, IL-9, IL-10, IL-17) and surface markers (ex. CCR6, CD25) to exclude Th9/Th17/Th22 cells/Tregs subsets (Eyerich *et al.*, 2009; Bonnevier *et al.*, 2013; Kaplan, 2013; Mahnke *et al.*, 2013; Roesner *et al.*, 2015; Dunay *et al.*, 2016).

Stimulatory molecules that elicit IL-8-production in CD4+ T cells

In conjunction to identifying and phenotyping IL-8+ T cells, the mechanism of action that induces IL-8 production in T cells was also explored. Stimulation of CD4+ T cells can occur through multiple methods such as with PMA/I, αCD3 with co-stimulatory signals like CD28, TLR agonists, etc. To determine if other allergens may elicit a stronger IL-8 signal compared to cat allergens, various allergens were tested in volunteers (Figure 22), though their allergic status was not known. There were no significant differences in the number of IL-8+ CD4+ T cells between the allergens. However, we observed that PMA/I induced IL-8 production in a substantially larger percentage of T cells in comparison to αCD3/CD28, which suggests that certain signalling pathways are more involved in IL-8 production in T cells. We explored the role of TLR signalling in IL-8 production as IL-8+ T cells may have an innate function and CD4+ T cells were shown to express TLR1-5, 7 and 9 (Caron *et al.*, 2005; Kumar *et al.*, 2006; Tincati *et al.*, 2012; Akhade and Qadri, 2015). It is evident that the percentage of IL-8+ T cells did not significantly change when purified CD4+ T cells were stimulated with TLR agonists (Figure 23). However, this may be due to the sensitivity of detecting changes in IL-8+ T

cells in flow cytometry. In the literature, IL-8 production has been shown to be induced through TLR signalling in T cells by ELISAs. Pam_3CSK_4, a TLR2 agonist, flagellin (TLR5) and R848 (TLR7/8) upregulated proliferation and IL-8 production in CD4+ T cells (Caron *et al.*, 2005; Akhade and Qadri, 2015), which was not observed in our system with flow cytometry. In a subset of experiments conducted in Jurkat T cells, we observed increases in IL-8 measured through ELISA with flagellin (data not shown), again suggesting that flow cytometry may not be sensitive enough to pick up IL-8-producing T cells.

Full activation of T cells requires TCR and co-stimulatory signalling, in which the latter modulates the response elicited by the TCR signalling (Chen and Flies, 2013). Various co-stimulatory, and co-inhibitory, signalling pathways have been identified in the literature (Chen and Flies, 2013). One of these co-stimulatory pathways include CD2, a receptor expressed on T cells, that interacts with CD58 (ie. LFA-3) on APCs (Chen and Flies, 2013). We explored whether or not CD2 co-stimulation induces IL-8 production in CD4+ T cells by stimulating purified CD4+ T cells with αCD2 antibodies in addition to αCD3 and αCD28. The addition of αCD2 did not increase the percentage of IL-8+ T cells (Figure 24). This may be attributed to the use of an inhibitory CD2 antibody clone (ie. RPA-2.10) instead of a stimulatory antibody (ex. T11.2 and T11.3; (Wesselborg *et al.*, 1991)). Another method for assessing the role of CD2 co-stimulation is through the use of PHA. There are many types of PHA, depending on the stage of purification, two of which are used to distinguish between CD2 co-stimulation (PHA-P) or non-CD2 stimulation (PHA-M) (Flynn *et al.*, 1986). The percentage of IL-8+ T cells increased with both PHA-

P (1.52%) and PHA-M (3.88%; Fig. 22), suggesting that stimulation through CD2 may activate IL-8 production in a subset of CD4+ T cells. However, it is evident that stimulation through CD2 was not as potent as with PMA/I which elicited the highest percentage of IL-8+ T cells (Figure 24). This may mean that a large proportion of CD4+ T cells are capable of producing IL-8, as shown through PMA/I which non-specifically activates cells. Despite all T cell lymphocytes expressing CD2 (Bullens *et al.*, 2001), only a small proportion of T cells were able to produce IL-8 with CD2 co-stimulation, as shown with PHA-P. However, it should be noted that the dose of PHA used may not have been optimal which may account for the low frequency of IL-8-producing T cells. Whether this proportion of cells are the same as the PMA/I-inducible cells is not known. In addition, previous studies have shown that CD2 co-stimulation elicits the production of IL-8 in CD4+ T cells as detected through cytokine production (Spinozzi *et al.*, 1996), though the proportion of CD4+ T cells that are capable of producing IL-8 has not been explored. Further investigation of these signalling pathways and their involvement in IL-8 production in T cells is needed to understand the responses required to elicit IL-8 in relation to allergies.

CONCLUSIONS AND FUTURE DIRECTIONS

Thus far, the results of the clinical study suggests that IL-8+ T cells may be a novel subset of cells that can react to cat allergens in a similar manner between cat allergics and non-cat allergics. As the frequencies of allergen-specific IL-8+ T cells did not differ significantly, even though there was a higher number of cat allergics, nor did the cells differ in the amount of IL-8 produced, these cells may differ in their role in terms

of allergic responses. It may be that the sensitized immune system of allergics (e.g. presence of IgE, mast cells, and Th2 cells) works synergistically with IL-8+ T cells. We hypothesize that the production of IL-8 from allergen-specific IL-8+ T cells, and other cytokines such as IL-3 from mast cells and Th2 cells, lead to the chemotaxis and activation of neutrophils and basophils and subsequent release of allergic mediators such as histamines and leukotrienes that potentiate allergic responses. In non-allergics, this may not occur, despite the presence of allergen-specific IL-8+ T cells, due to the lack of specific IgE, mast cells and Th2 cells. Therefore, the reduction of IL-8 in allergen-specific IL-8+ T cells from PIT may prevent the downstream chemotaxis and activation of neutrophils and basophils, attributing to the clinical efficacy observed after receiving PIT.

Further insight into the phenotype and mechanisms of action of allergen-specific IL-8+ T cells in the context of allergies is required. For phenotyping, more markers and cytokines need to be assessed to determine if these cells differ from known Th2 subsets or constitute a novel subset of Th8 cells. In our study, the use of flow cytometry was limited by the number of markers that could be assessed, as around 13-15 markers could be observed simultaneously. Novel methods in flow cytometry include CyTOF, which uses isotopes to look at more than 40 markers at time. In addition, the phenotyping of IL-8+ T cells may also be conducted through gene transcription profiles (ex. RNA sequencing). The effects of IL-8 produced by allergen-specific T cells also needs to be assessed to determine its downstream effects on the chemotaxis and activation of neutrophils and basophils. Further understanding of the signalling pathways involved in IL-8 production

will be conducted by using CRISPR/Cas9 to knockdown T cell signalling genes that were also downregulated during peptide immunotherapy. By studying IL-8+ T cells and their effects on allergy-associated cells, we can develop a better understanding of these cells in the context of allergic diseases which may provide a potential novel target for the treatment of allergies.

TABLES

Table 1. Inclusion and exclusion criteria for clinical study.

INCLUSION CRITERIA
• Subject must be able to understand and provide informed consent • Male or female, age 18-65 years. • For cat-allergic subjects, a minimum 1-year history of allergic rhinoconjunctivitis on exposure to cats and cat specific IgE blood concentrations greater than or equal to 0.7IU/ml. • For non-allergic subjects, no history of allergic rhinoconjunctivitis on exposure to cats and cat specific IgE blood concentrations lower than 0.35IU/ml. • Subjects who express one or more of the Human Leukocyte Antigens (HLA) HLA-DRB1*0101, HLA-DRB1*0301, HLA-DRB1*0401, HLA-DRB1*0405, HLA-DRB1*0701, HLA-DRB1*1101, HLA-DRB1*1301 or HLA- DRB5*0101 • Willing and able to comply with the study requirements.
EXCLUSION CRITERIA
• Inability or unwillingness of a participant to give written informed consent or comply with study protocol. • Significant history of alcohol or drug abuse. • Subjects taking oral short-acting antihistamines for 48 hours prior to the visit, oral long-acting antihistamines for 5 days prior to the visit, oral corticosteroids for 2 months prior to the visit, or leukotriene inhibitors for 5 days prior to the visit. Visits can be rescheduled at the discretion of the Investigator. • Vaccination/ inoculation within the previous 6 weeks of any of the Assessment visits. Visits can be rescheduled at the discretion of the Investigator. • Allergen-immunotherapy for cat within the last 5 years. • Pregnant or could be pregnant • A history of any significant disease or disorder (e.g. autoimmune, cardiovascular, pulmonary, gastrointestinal, liver, renal, neurological, musculoskeletal, endocrine, metabolic, neoplastic/malignant, psychiatric, major physical impairment, severe atopic dermatitis) which, in the opinion of the Investigator, may either put the subject at risk because of participation in the study, or influence the results of the study, or the subject's ability to participate in the study. • Clinically abnormal CBC data, as decided to be clinically relevant by the Investigator. • Subjects who have taken an investigational drug within the last 3 months prior to any of the visits.

Table 2. Fel d 1-specific tetramers used in clinical study.

HLA-DR	Allergen	Peptide Fragment	Sequence
1*0101	Fel d 1	P2	IFYDVFFAVANGNELLLDLS
1*0101	Fel d 1	P14	VAQYKALPVVLENARILKNC
1*0301	Fel d 1	P3	VANGNELLLDLSLTKVNNATE
1*0301	Fel d 1	P10	CMGEAVQNTVEDLKLNTLGR
1*0301	Fel d 1	P11	EICPAVKRDVDLFLTGTPDE
1*0701	Fel d 1	P2	IFYDVFFAVANGNELLLDLS
1*0701	Fel d 1	P13	TPDEYVEQVAQYKALPVVLE
5*0101	Fel d 1	P2	IFYDVFFAVANGNELLLDLS
5*0101	Fel d 1	P13	TPDEYVEQVAQYKALPVVLE

Table 3. Summary of subject characteristics. Information pertaining to symptoms, other allergies and asthma were self-reported.

Subject	Sex	Age	Cat owner	Frequency exposure to cats	Symptoms	Total IgE (IU/mL)	Cat dander IgE (IUm/L)	Fel d 1 IgE (IU/mL)	Tissue Type (DRB)	Other Allergies	Asthma
CAT ALLERGICS											
1	M	21	No	None	Sneezing, runny nose, itchiness	1790	10.89	13.49	5*01:01	Peanuts, tree nuts, wheat, milk and eggs	Yes
2	F	28	No	None	Sneezing, runny nose, red eyes	65	2.11	2.17	1*03:01, 5*01:01	Plants, trees, grass, horses, dogs, rabbits, mold, dust mites and pollen	No
3	F	21	No	None	Runny nose, itchiness, red eyes	2210	>100	>100	1*07:01	Cats, dogs, dust, pollen, hay, mold, grass	No
4	F	35	No	None	Runny nose, itchiness, shortness of breath, chest tightness, wheezing	24	6.6	2.61	1*07:01	Pollen, penicillin	No
5	M	24	No	Once a month	Sneezing, runny nose, itchiness, red eyes	299	48.44	27.92	1*07:01	Seasonal allergies	No
6	F	20	No	1-2h/day	Sneezing, runny nose, red eyes	156	66.68	72.09	5*01:01	None	No
7	F	18	Yes	Once a month	Sneezing, runny nose, itchiness	193	59.74	60.38	1*03:01	Horses	Yes
8	F	50	Yes	Everyday	Sneezing,	121	2.78	1	1*07:01	Ragweed, nickel	No

					runny nose						
9	F	21	Yes	None	Sneezing, runny nose, watery eyes	718	26.76	27.44	5*01:01	None	No
10	F	30	No	None	Sneezing, runny nose, itchiness, red eyes	32	5.75	1.65	1*07:01, 5*01:01	Sulfa, codeine, pollen	No
11	F	29	No	Once a month	Sneezing, runny nose, itchiness	130	6.65	7.47	1*03:01	Penicillin	Yes
12	M	22	No	None	Sneezing, runny nose, itchiness, red eyes	599	5.97	6.74	1*07:01	Cats, dogs, kiwi, pollen	No
NON-CAT ALLERGICS											
13	F	20	No	None		26	<0.1	<0.1	5*01:01	No	No
14	M	25	Yes	Weekly		123	<0.1	<0.1	5*01:01	No	No
15	F	35	No	None		99	<0.1	<0.1	5*01:01	No	No
16	F	19	No	None		39	<0.1	<0.1	1*03:01, 1*07:01	No	No
17	M	20	No	None		74	<0.1	<0.1	1*03:01	Pecans	No
18	F	46	Yes	Everyday		3	<0.1	<0.1	1*03:01	Morphine, tetracycline, nutmeg, seasonal allergies	
19	M	21	Yes	1h/week		11	<0.1	<0.1	1*03:01	Seasonal allergies	No
20	F	18	No	No		50	<0.1	<0.1	5*01:01	Pollen	No
21	F	27	Yes	Everyday		2	<0.1	<0.1	1*04:01, 5*01:01	No	No
22	F	19	No	No		72	<0.1	<0.1	1*03:01, 1*07:01	No	No
23	M	34	Yes	Everyday		2	<0.1	<0.1	1*03:01	No	No
24*	F	27	No	2 days/week		1	<0.1	<0.1	1*03:01	Ragweed	Yes
25**	F	50	Yes	Everyday		17	<0.1	<0.1	1*04:01, 1*07:01	No	No

*Visit A1 only
**Visit A2 only

Table 4. **Antibodies and clones used in flow cytometry panels.** AF, Alexa Fluor; BV, Brilliant Violet; Cy, Cyanine

Marker	Isotype	Antibody	Clone	Company	Catalogue #
Live/dead*,**		Zombie NIR (APC-Cy7)		Biolegend	423105
CCR4*	Mouse	BV510	L291H4	Biolegend	359416
CCR7*,**	Mouse	PerCP/Cy5.5	G043H7	Biolegend	353220
CD137	Mouse	BV650	4B4-1	Biolegend	309828
CD137*	Mouse	PE/Dazzle594	4B4-1	Biolegend	309826
CD14**	Mouse	BV510	MφP9	BD Biosciences	563079
CD154*	Mouse	PE	24-31	Biolegend	310806
CD19**	Mouse	BV510	HIB19	Biolegend	302241
CD3	Mouse	PerCP	OKT3	Biolegend	317338
CD3*,**	Mouse	AF488	HIT3a	Biolegend	300320
CD38	Mouse	PE/Cy7	HIT2	Biolegend	303516
CD4	Mouse	PE-CF594	RPA-T4	BD Biosciences	562316
CD4**	Mouse	PE/Dazzle594	RPA-T4	Biolegend	300548
CD4*,**	Mouse	AF700	SK3	Biolegend	344622
CD45RA*,**	Mouse	BV785	HI100	Biolegend	304140
CRTH2	Rat	FITC	BM16	Biolegend	350108
CRTH2*	Rat	BV605	BM16	Biolegend	350122
CXCR3*	Mouse	BV650	G025H7	Biolegend	353730
HLA-DR	Mouse	APC/Fire750	L243	Biolegend	307658
IFNγ	Mouse	PerCP/Cy5.5	4S.B3	Biolegend	502526
IFNγ*	Mouse	PE/Cy7	4S.B3	Biolegend	502528
IFNγ**	Mouse	BV650	4S.B3	Biolegend	502538
IL-10	Rat	AF488	JES3-9D7	Biolegend	501411
IL-13	Rat	PE/Cy7	JES10-5A2	Biolegend	501914
IL-2	Rat	AF700	MQ1-17H12	Biolegend	500320
IL-4	Rat	BV786	MP4-25D2	BD Biosciences	564113
IL-5*,**	Rat	BV421	TRFK5	Biolegend	504311
IL-8*,**	Mouse	APC	E8N1	Biolegend	511410

***Used in Visit A1
**Used in Visit A2

FIGURES

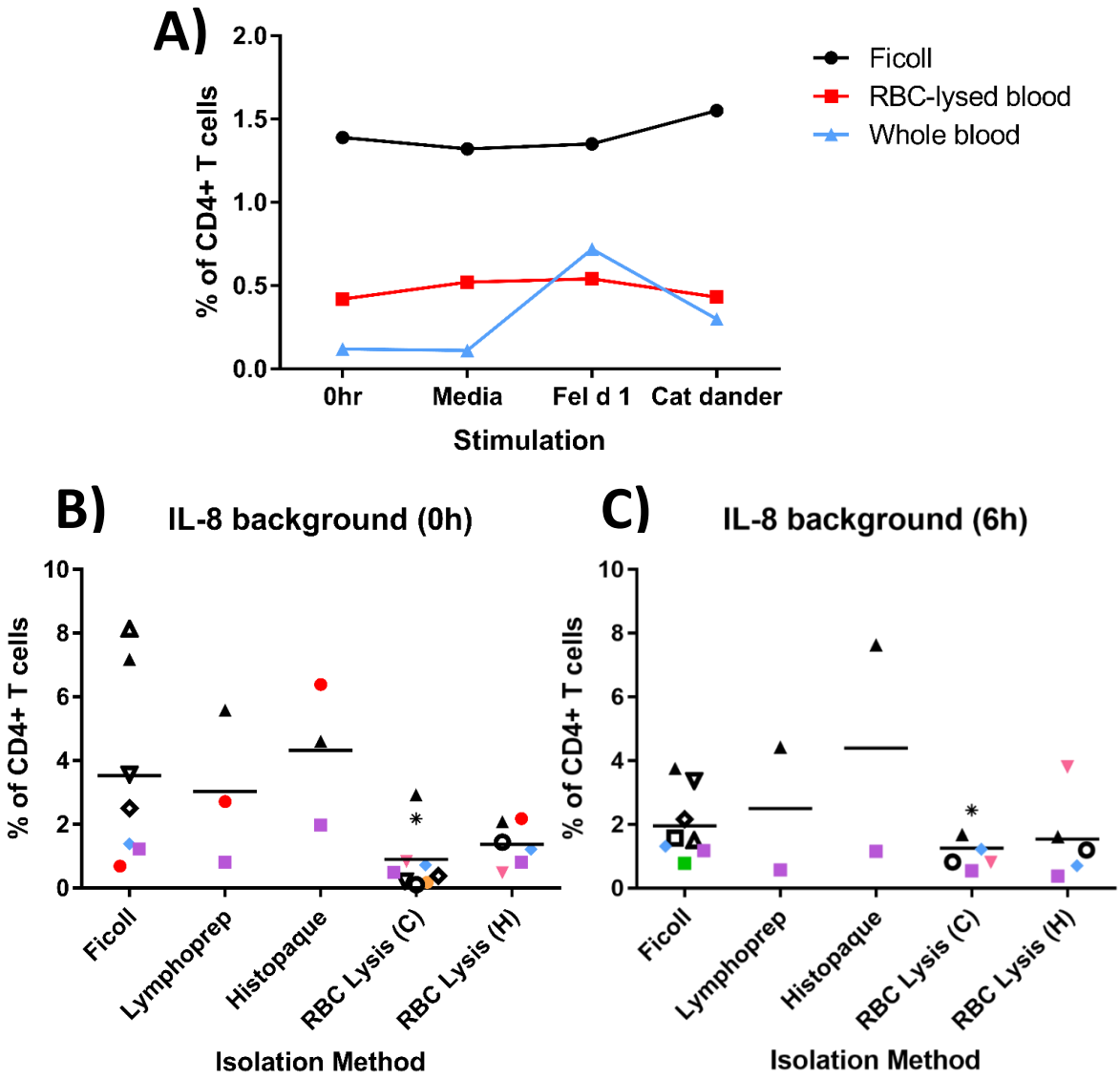


Figure 1. High background of IL-8+ T cells detected in controls due to blood processing methods. A) Whole blood, red blood cell (RBC)-lysed blood and PBMCs isolated with Ficoll-Paque density centrifugation were cultured with RPMI+6% autologous plasma, Fel d 1 (10 ug/ml) and cat dander (50 ug/ml) for 6h. Samples from each set of cells were also taken at 0h before stimulation and stained for flow cytometry. The percentage of CD4+ T cells expressing IL-8 (y-axis) was higher with Ficoll isolation method (n=1). **B, C)** Cells isolated from RBC-lysed blood were also compared to other density centrifugation media, Lymphoprep and Histopaque-1077, in addition to Ficoll-Paque. RBC lysis was performed with a commercial buffer (RBC Lysis (C)) or with a homemade buffer (RBC Lysis (H)). The percentage of CD4+ T cells expressing IL-8 (y-

axis) were measured at 0h **(B)** and 6h **(C)** after incubation in RPMI+6% autologous plasma through flow cytometry. RBC-lysed blood had the lowest percentage of IL-8+ T cells at both 0h and 6h. Each symbol is representative of one subject (total n=11).

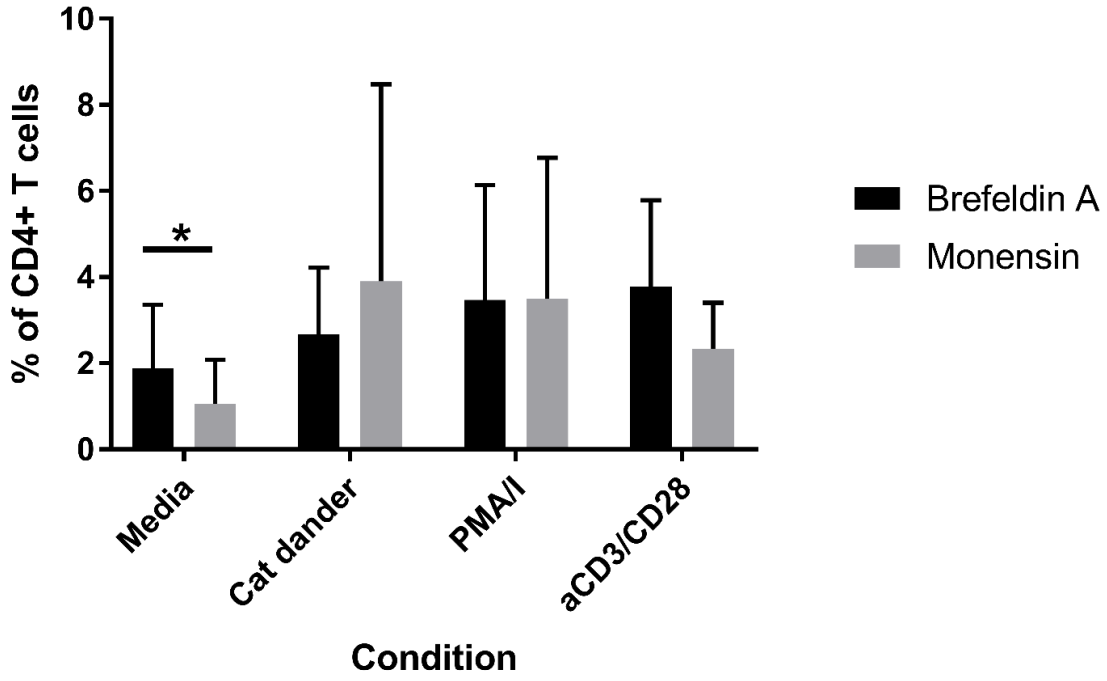


Figure 2. Detection of IL-8+ T cells with monensin and brefeldin A. PBMCs were cultured with RPMI + 6% autologous plasma, cat dander (50 μg/ml), PMA/I and aCD3/CD28 for 6h and monensin or brefeldin A was added into culture during the last 4h of incubation. The percentage of CD4+ T cells expressing IL-8 (y-axis) was measured through flow cytometry. Monesin and brefeldin A inhibited secretion of IL-8, though at different levels. Monensin resulted in a lower IL-8+ T cell background in media and during aCD3/CD28 stimulation, and brefeldin A was lower for cat dander stimulation. Data shown as mean±SD (n=7 for media, n=2 for cat dander and αCD3/CD28, n=4 for PMA/I; *$p<0.05$).

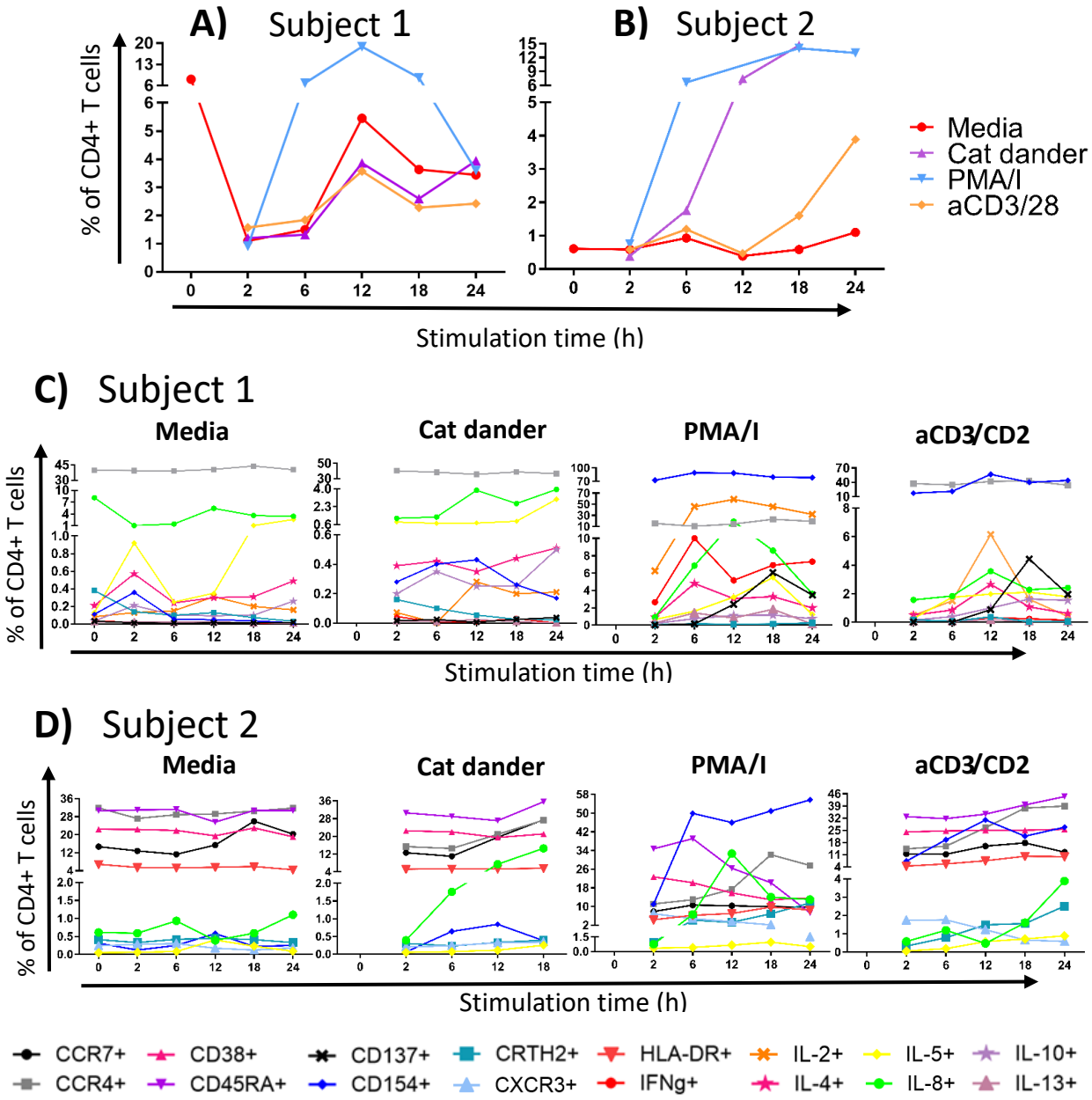


Figure 3. Time course of IL-8 production in CD4+ T cells in comparison to T cell markers and other cytokine expression. PBMCs were isolated using Ficoll-Paque (Subject 1) or RBC lysis of whole blood (Subject 2). PBMCs were cultured with cat dander, PMA/I and αCD3/28 for up to 24h at 37°C, 5% CO2 and stained for various T cell markers and cytokines at the indicated time points (x-axis). **A, B)** The percentage of CD4+ T cells expressing IL-8 (y-axis) over time. **C, D)** T cell markers and cytokines were analyzed from CD4+ T cells and expressed as a percentage of these cells (y-axis).

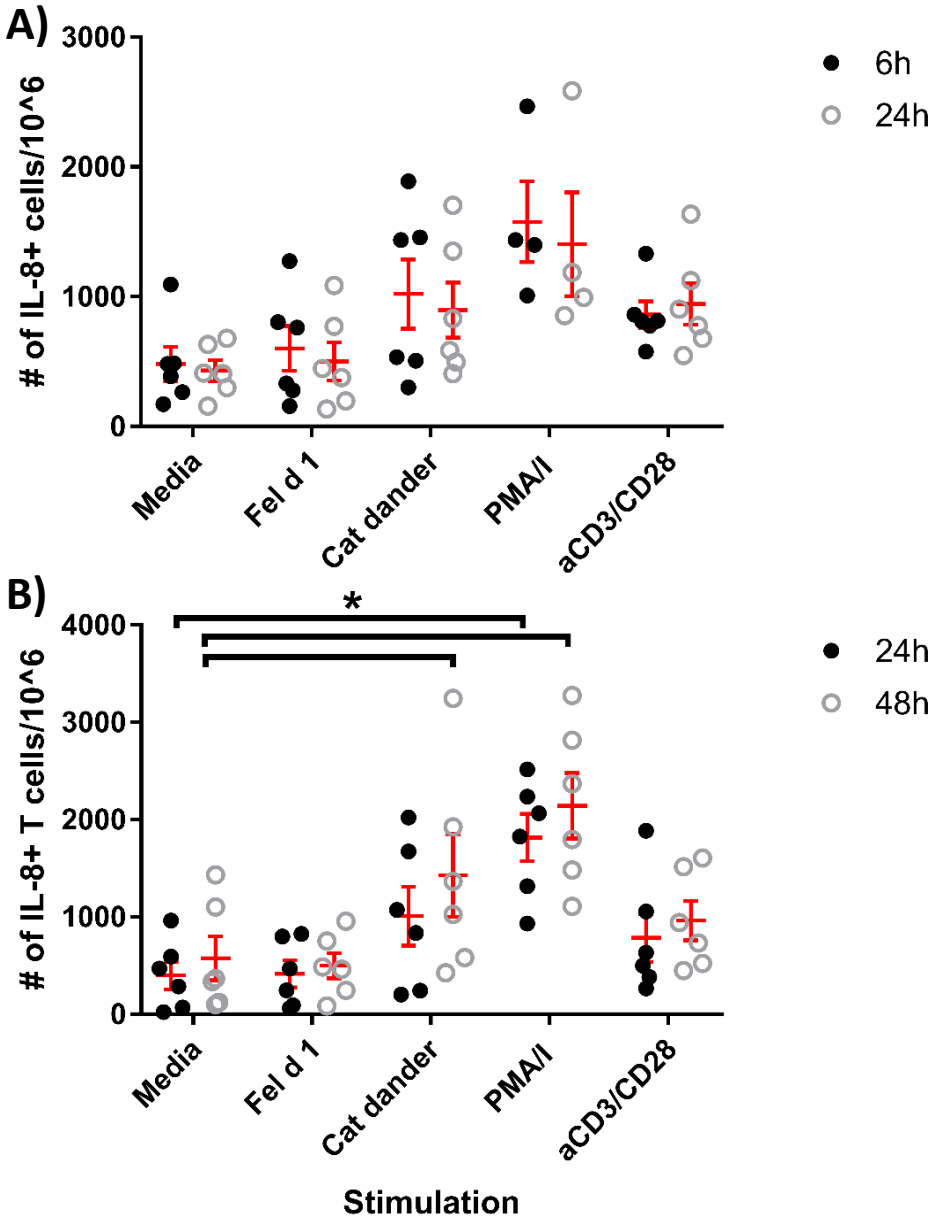
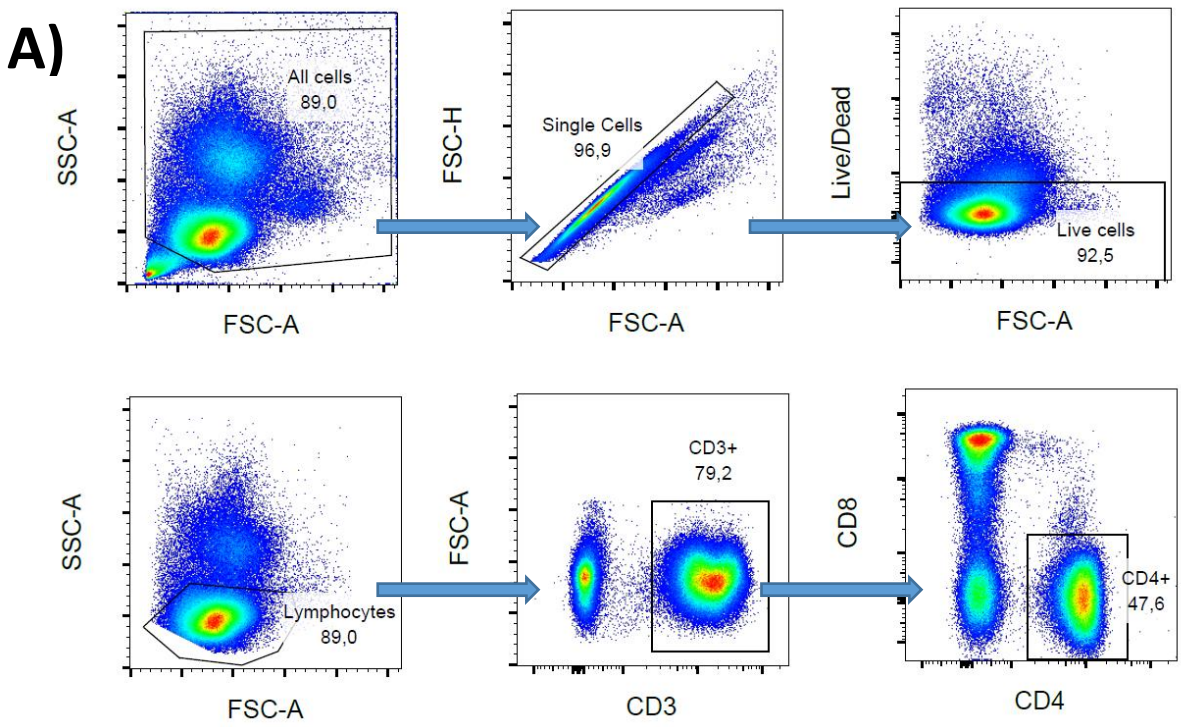
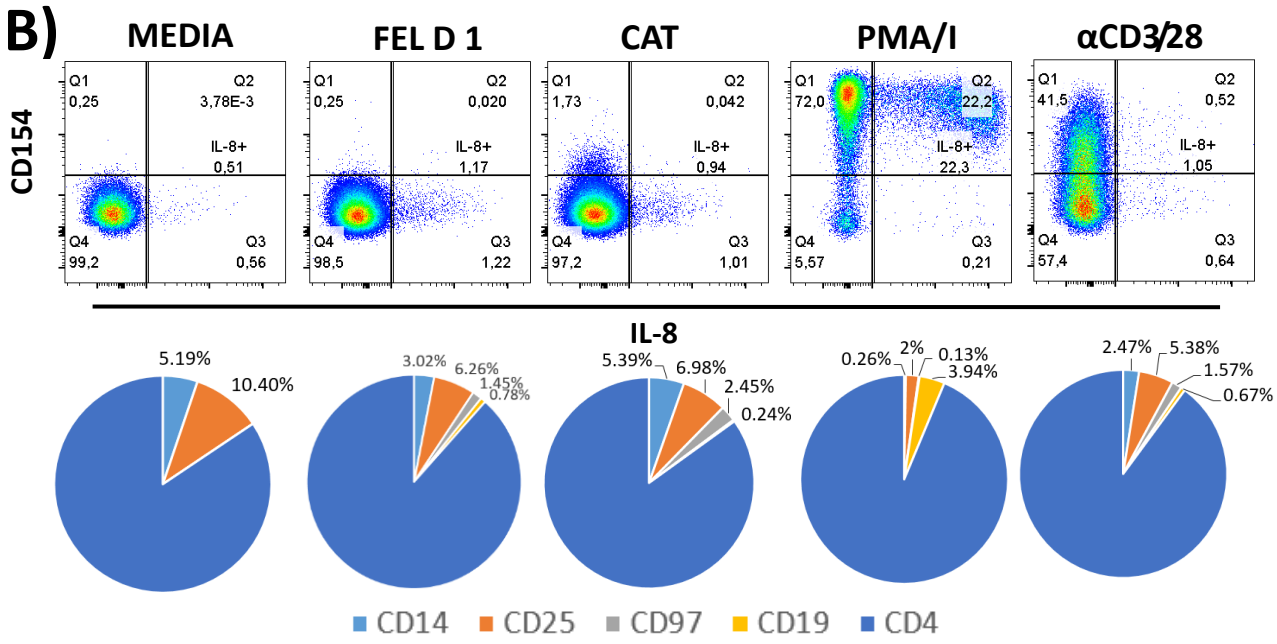


Figure 4. Optimization of IL-8 ELISpot parameters. A) Stimulation of PBMCs for 6h or 24h prior to CD4+ T cell isolation. PBMCs were stimulated with media, Fel d 1 (10 µg/ml), cat dander (50 ng/ml), PMA/I and αCD3/28 for 6h or 24h. CD4+ T cells were isolated by negative selection after PBMC stimulation and plated into the ELISpot plate for 48h. **B) CD4+ T cell incubation in ELISpot plate for 24h vs 48h.** PBMCs were stimulated for 24h prior to CD4+ T cell isolation. The purified CD4+ T cells were incubated in the IL-8 ELISpot plate for 24h or 48h before developing the plate. The number of IL-8-producing CD4+ T cells (spots) were counted and expressed as per million cells (y-axis). Data bar shown as mean ± SEM (n=6; *$p<0.05$)


A)
SSC-A
All cells
89,0
FSC-A
FSC-H
Single Cells
96,9
FSC-A
Live/Dead
Live cells
92,5
FSC-A
SSC-A
Lymphocytes
89,0
FSC-A
FSC-A
CD3+
79,2
CD3
CD8
CD4+
47,6
CD4


B)
MEDIA
FEL D 1
CAT
PMA/I
αCD3/28
CD154
Q1 0,25
Q2 3,78E-3
IL-8+ 0,51
Q4 99,2
Q3 0,56
Q1 0,25
Q2 0,020
IL-8+ 1,17
Q4 98,5
Q3 1,22
Q1 1,73
Q2 0,042
IL-8+ 0,94
Q4 97,2
Q3 1,01
Q1 72,0
Q2 22,2
IL-8+ 22,3
Q4 5,57
Q3 0,21
Q1 41,5
Q2 0,52
IL-8+ 1,05
Q4 57,4
Q3 0,64
IL-8
5.19%
10.40%
3.02%
6.26%
1.45%
0.78%
5.39%
6.98%
2.45%
0.24%
0.26%
2%
0.13%
3.94%
2.47%
5.38%
1.57%
0.67%
CD14
CD25
CD97
CD19
CD4

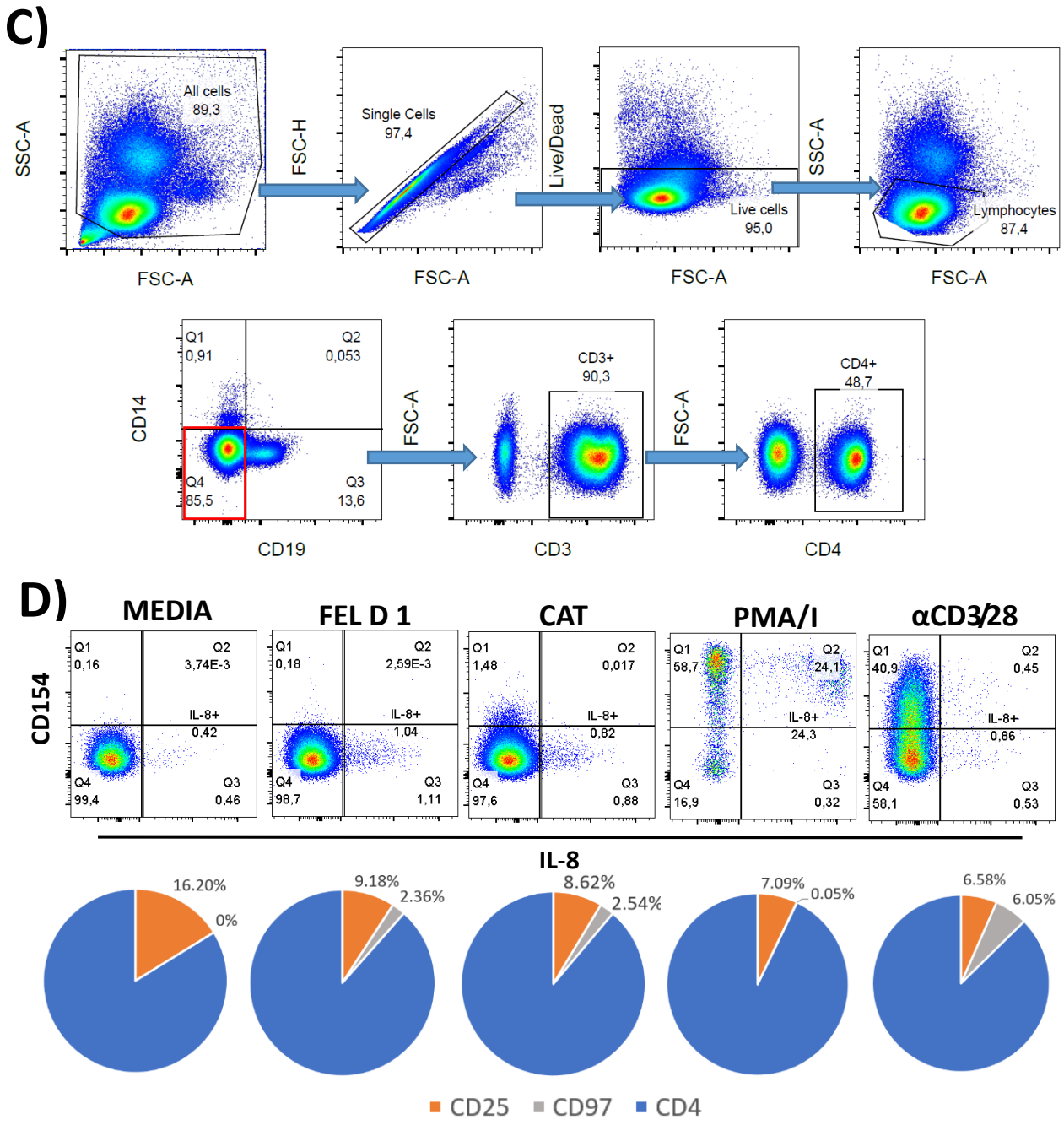


Figure 5. Gating strategies to identify IL-8+ CD4+ T cells. A) Gating strategy for CD4+ T cells. PBMCs were selected and gated for single and live cells. From this population, lymphocytes were gated according to SSC and FSC. Within the lymphocyte population, CD3+ cells were selected and gated according to CD8 and CD4. CD4+ T cells are therefore CD3+, CD8- and CD4+. The CD4+ T cells are then further analyzed for IL-8, and other cytokines and surface markers (see B and Fig. 2,3). **B) IL-8+ CD4+ T**

cells detected in PBMCs after 6h allergen stimulation. With the gating strategy shown in (A), a small population of IL-8+ CD4+ T cells were detected. From these IL-8+ cells, a small proportion contained markers for monocytes (CD14), B cells (CD19) and Treg (CD25), as shown in the pie charts**. C) Gating strategy for CD4+ T cells with CD14 and CD19 gate.** Lymphocytes were selected following the gating strategy as in (A). The lymphocytes were plotted for CD14 and CD19 and the CD14-CD19- population was gated. From this, CD3+ and CD4+ populations were gated**. D) IL-8+ CD4+ T cells detected in PBMCs after 6h allergen stimulation with removal of monocytes and B cells.** With the gating strategy shown in (C), a small population of IL-8+ CD4+ T cells were detected. From these IL-8+ cells, a small proportion expressed Treg marker, CD25, and little to no cells expressed early T cell activation marker, CD97, as shown in the pie charts. SSC, side scatter; FSC, forward scatter. (n=1)

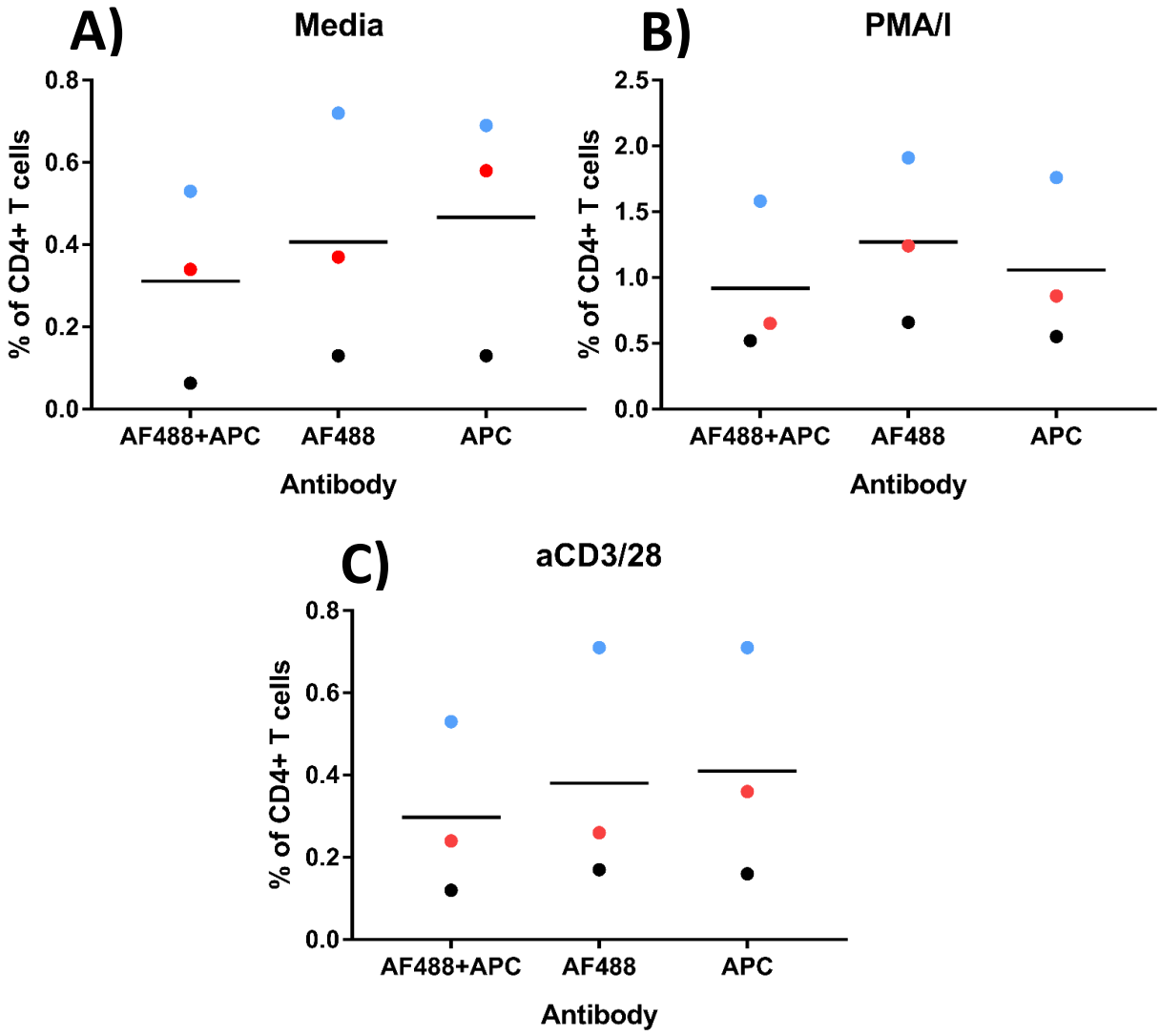
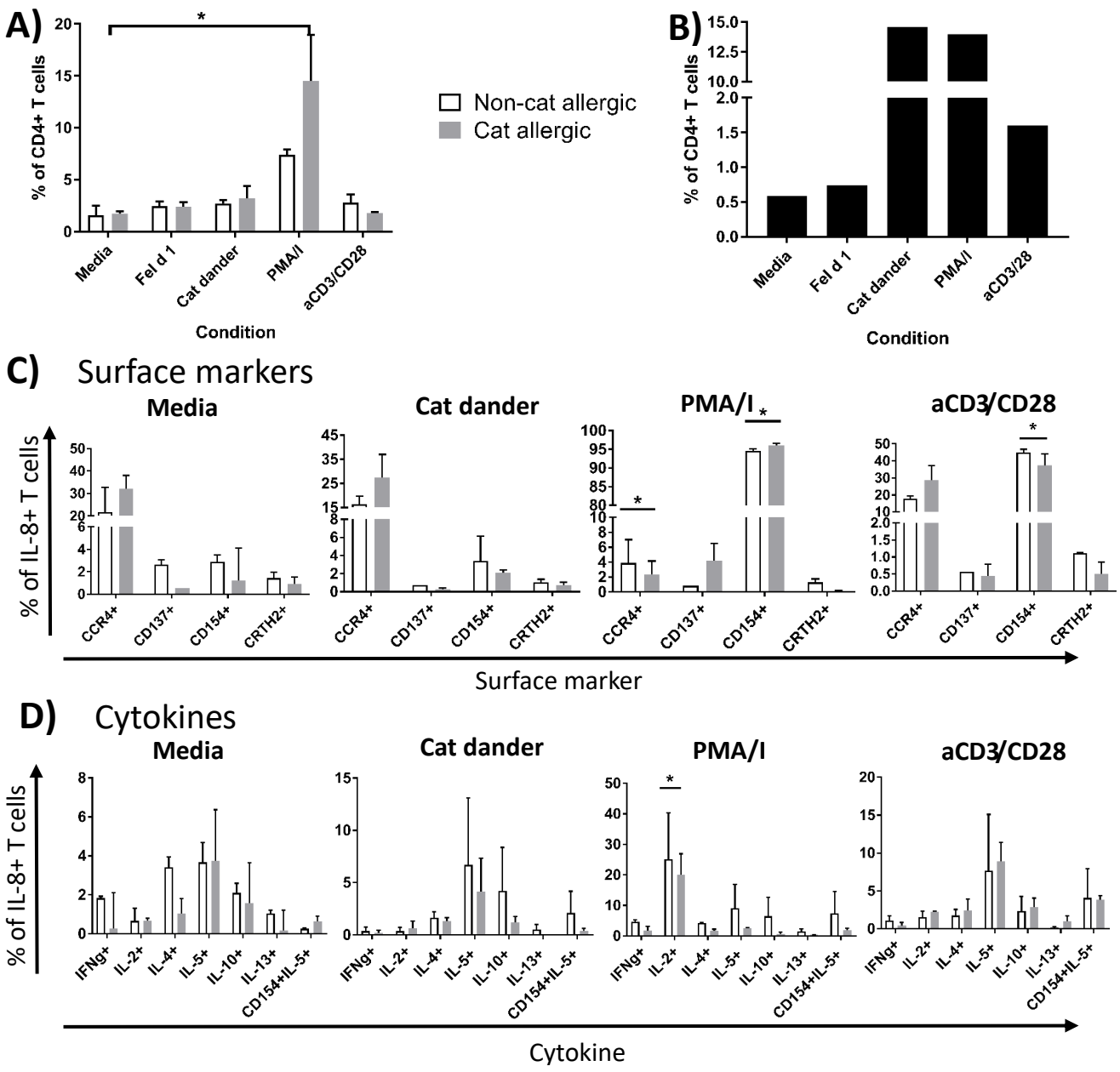


Figure 6. Percentage of IL-8+ T cells detected with double staining for IL-8. IL-8+ T cells were gated for AF488 IL-8 and/or APC IL-8 antibodies, clone E8N1, in PBMCs stimulated with **(A)** media, **(B)** PMA/I and **(C)** αCD3/28 for 6h. The frequency of IL-8+ T cells is expressed as a percentage of CD4+ T cells (y-axis; n=3).


A)
% of CD4+ T cells
Media
Fel d 1
Cat dander
PMA/I
aCD3/CD28
Condition
Non-cat allergic
Cat allergic
B)
% of CD4+ T cells
Media
Fel d 1
Cat dander
PMA/I
aCD3/28
Condition
C) Surface markers
Media
Cat dander
PMA/I
aCD3/CD28
% of IL-8+ T cells
CCR4+
CD137+
CD154+
CRTH2+
Surface marker
D) Cytokines
Media
Cat dander
PMA/I
aCD3/CD28
% of IL-8+ T cells
IFNg+
IL-2+
IL-4+
IL-5+
IL-10+
IL-13+
CD154+IL-5+
Cytokine

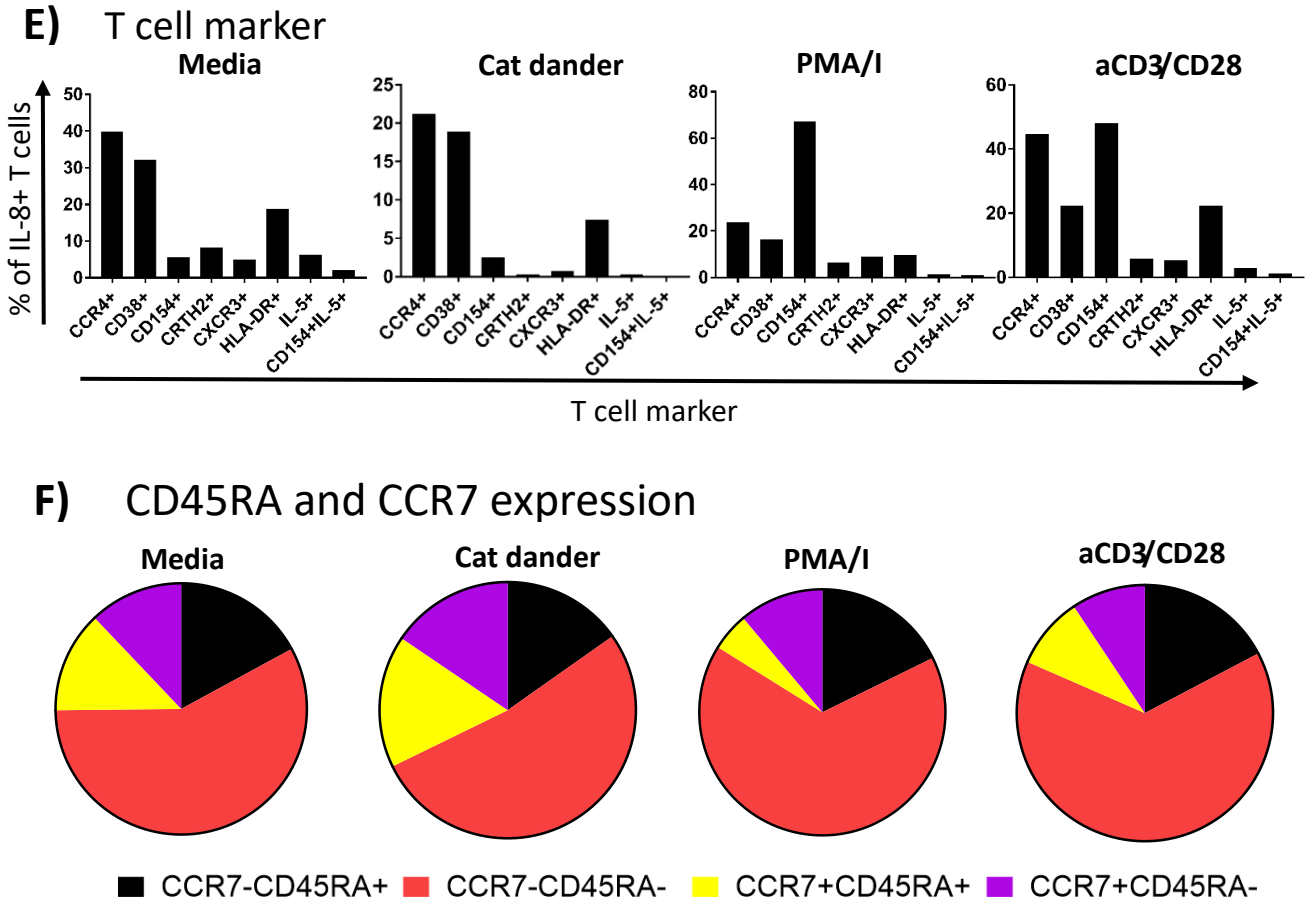


Figure 7. T cell markers and cytokine profiles of IL-8+ CD4+ T cells from PBMCs in preliminary studies. A) Percentage of IL-8+ T cells with 6h stimulation. PBMCs were isolated using Ficoll-Paque and incubated with cat dander, PMA/I and αCD3/CD28 for 6h in cat allergic and non-cat allergic subjects (n=4). IL-8+ T cells were further characterized in these subjects for **(C)** T cell surface markers and **(D)** T cell cytokines. **B) Percentage of IL-8+ T cells with 18h stimulation.** In one cat allergic subject, PBMCs were isolated using RBC lysis of whole blood and incubated with cat dander, PMA/I and αCD3/CD28 for 18h. IL-8+ T cells were further characterized by **(E)** T cell markers and **(F)** expression of CD45RA and CCR7. Data shown as mean ± SEM (*$p<0.05$ in comparison to media.)

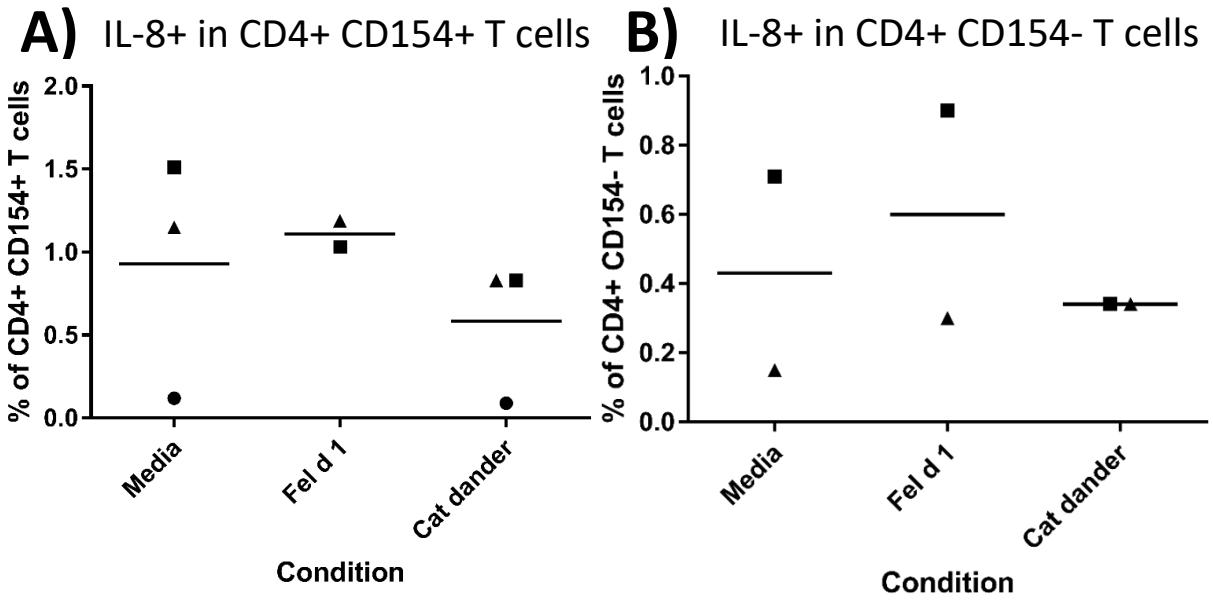


Figure 8. Expression of IL-8+ T cells in CD4+CD154+ T cell cultures. CD4+CD154+ T cells were isolated from PBMCs stimulated with media, Fel d 1 and cat dander for 6h. Both CD4+ CD154+ and CD154- cells were re-stimulated with αCD3/28 for 20h. The percentage of IL-8+ cells detected from **(A)** stimulated CD4+ CD154+ T cells (n=3) and **(B)** stimulated CD4+ CD154- T cells are shown (n=2). Little to no IL-8+ T cells were observed in CD4+ CD154+ or CD154- T cells.

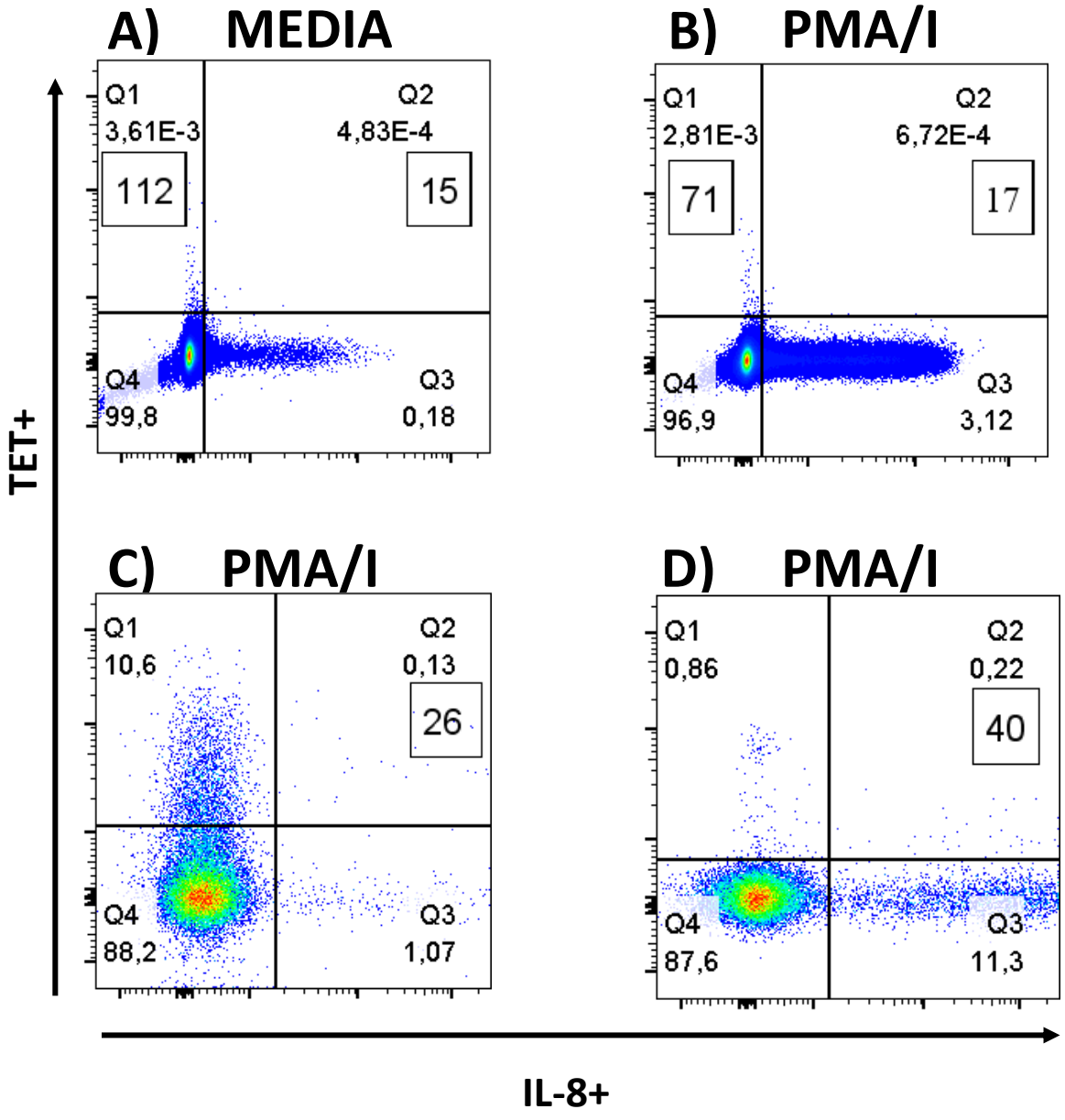


Figure 9. Detection of tetramer-positive CD4+ T cells in cat allergic subjects. A,B) Purified CD4+ T cells were stained with tetramers directed against cat allergen-specific T cells and stimulated with **(A)** media or **(B)** PMA/I for 6h in one cat allergic subject. **C,D) Enriched tetramer-positive (TET+) cells stimulated with PMA/I.** Tetramer-stained T cells were isolated using PE enrichment as the tetramers contained PE molecules. PE+ enriched cells were stimulated with PMA/I for 6h in two cat allergic subjects. TET+ cells (y-axis) were detected in both media and PMA/I and an increase in IL-8+ T cells (x-axis) was detected in PMA/I stimulated cells. A small number of TET+ IL-8+ T cells (top right quadrant) were observed in PMA/I stimulated cells. Number in box represents number of cells in the quadrant (n=3).

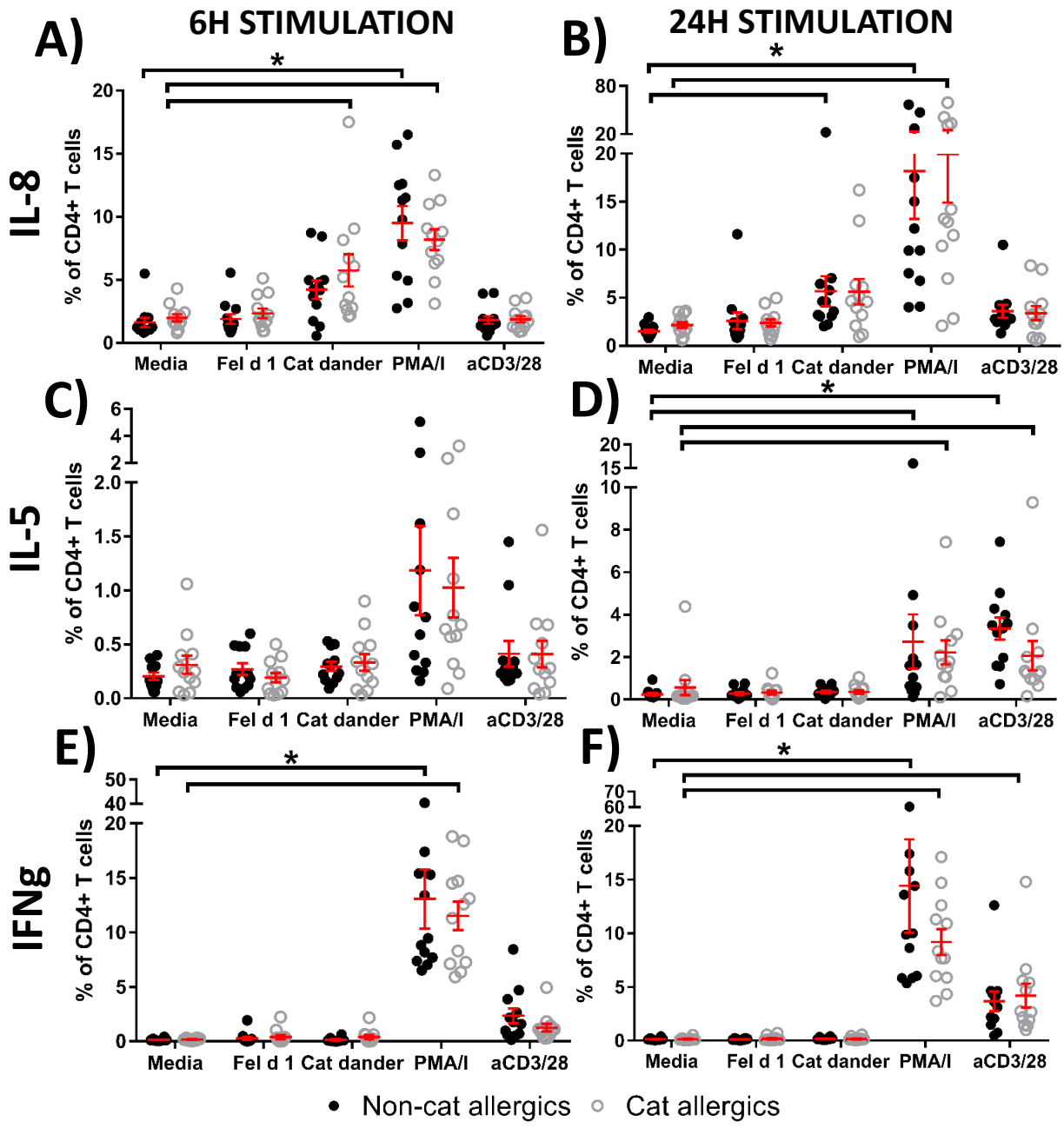


Figure 10. Frequency of IL-8+ CD4+ T cells compared to IL-5+ and IFNγ+ CD4+ T cells detected in flow cytometry. PBMCs isolated by RBC lysis of whole blood collected from cat allergics and non-cat allergics were stimulated for 6h **(left)** or 24h **(right)** and stained for flow cytometry. The percentage of CD4+ T cells (y-axis) that expressed **(A,B) IL-8, (C,D) IL-5 and (E,F) IFNγ** was measured. IL-8 increased with cat dander at 6h for cat allergics and 24h for non-cat allergics, and PMA/I for both cat allergics and non-cat allergics at 6h and 24h. In contrast, IL-5 and IFNγ increased with

24h PMA/I and αCD3/28 stimulation in both groups. Data bar shown as mean±SEM (n=12 for each group; *p<0.05).

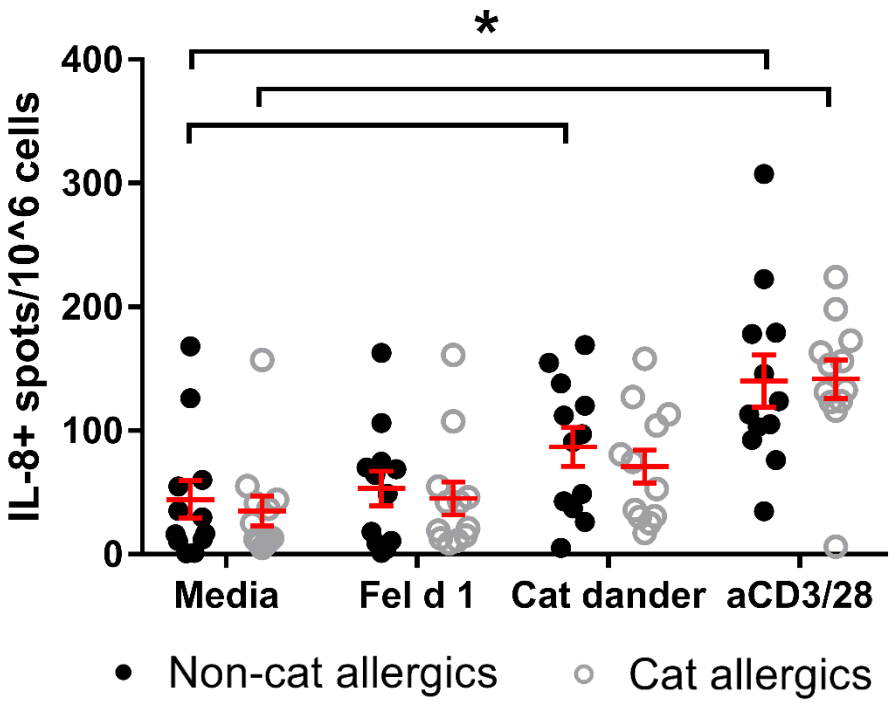


Figure 11. Frequency of IL-8+ T cells detected in ELISpot assay. PBMCs were stimulated for 24h prior to CD4+ T cell isolation. After stimulation, CD4+ T cells isolated and incubated in IL-8 ELISpot plates for 24h. The number of IL-8-producing T cells is expressed as number of spots per million cells (y-axis). Data bar shown as mean±SEM. (n=12 per group, *p<0.05).

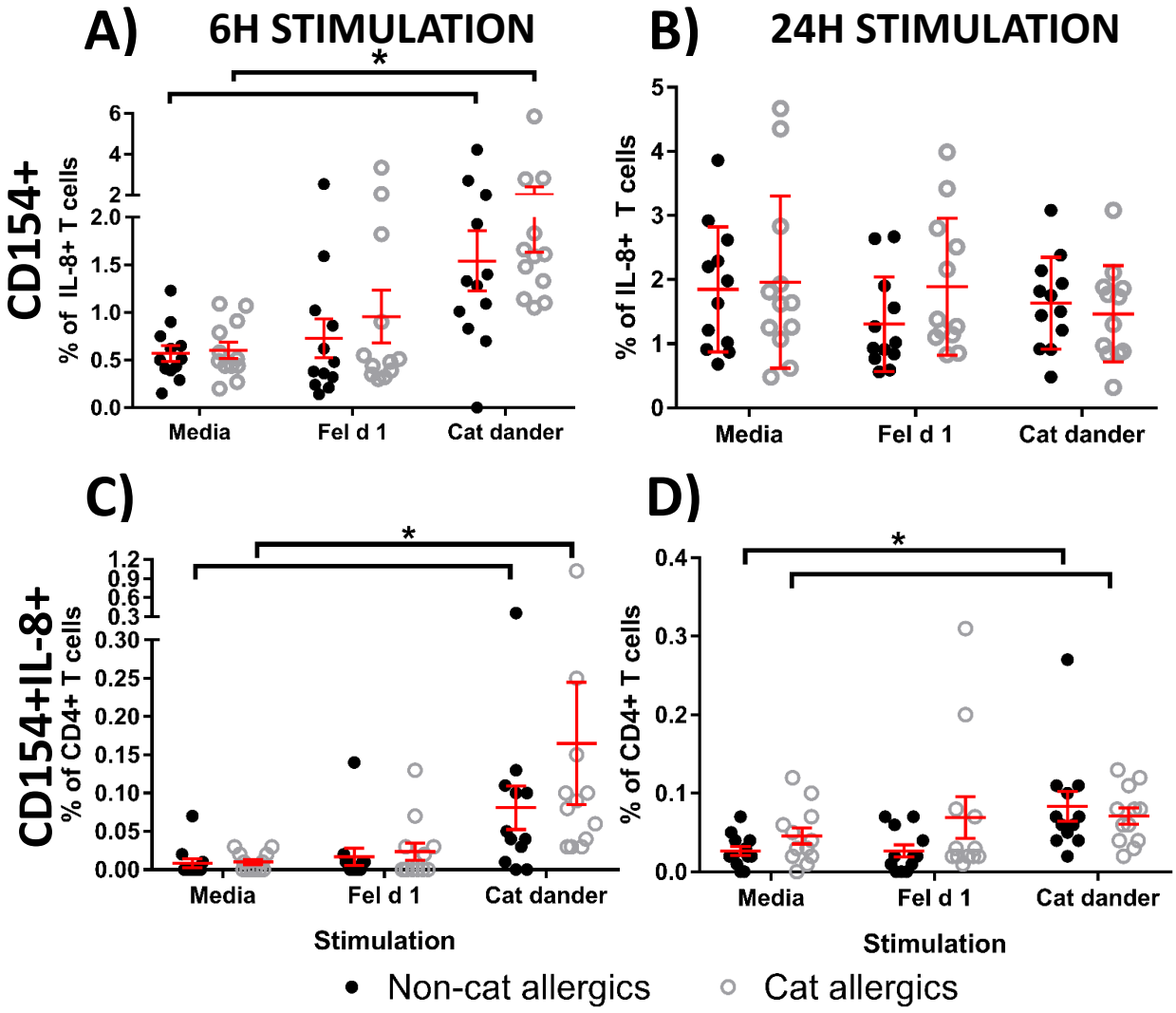


Figure 12. Frequency of IL-8+ T cells expressing CD154. PBMCs were stimulated for 6h **(left)** and 24h **(right)** with media, Fel d 1, and cat dander and run through flow cytometry. **A,B) Frequency of CD154+ in IL-8+ T cell population.** IL-8+ CD4+ T cells were gated for CD154 expression and the percentage of CD154+ cells was plotted as a frequency of IL-8+ T cells (y-axis). **C,D) Frequency of CD154+IL-8+ T cells in CD4+ T cell population.** The percentage of double positive CD154+IL-8+ T cells is expressed as a percentage of the CD4+ T cell population (y-axis). The number of CD154+IL-8+ T cells increased with 6h cat dander stimulation in comparison to media in both groups. Data bar shown as mean ±SEM (n=12 per group, *p<0.05).

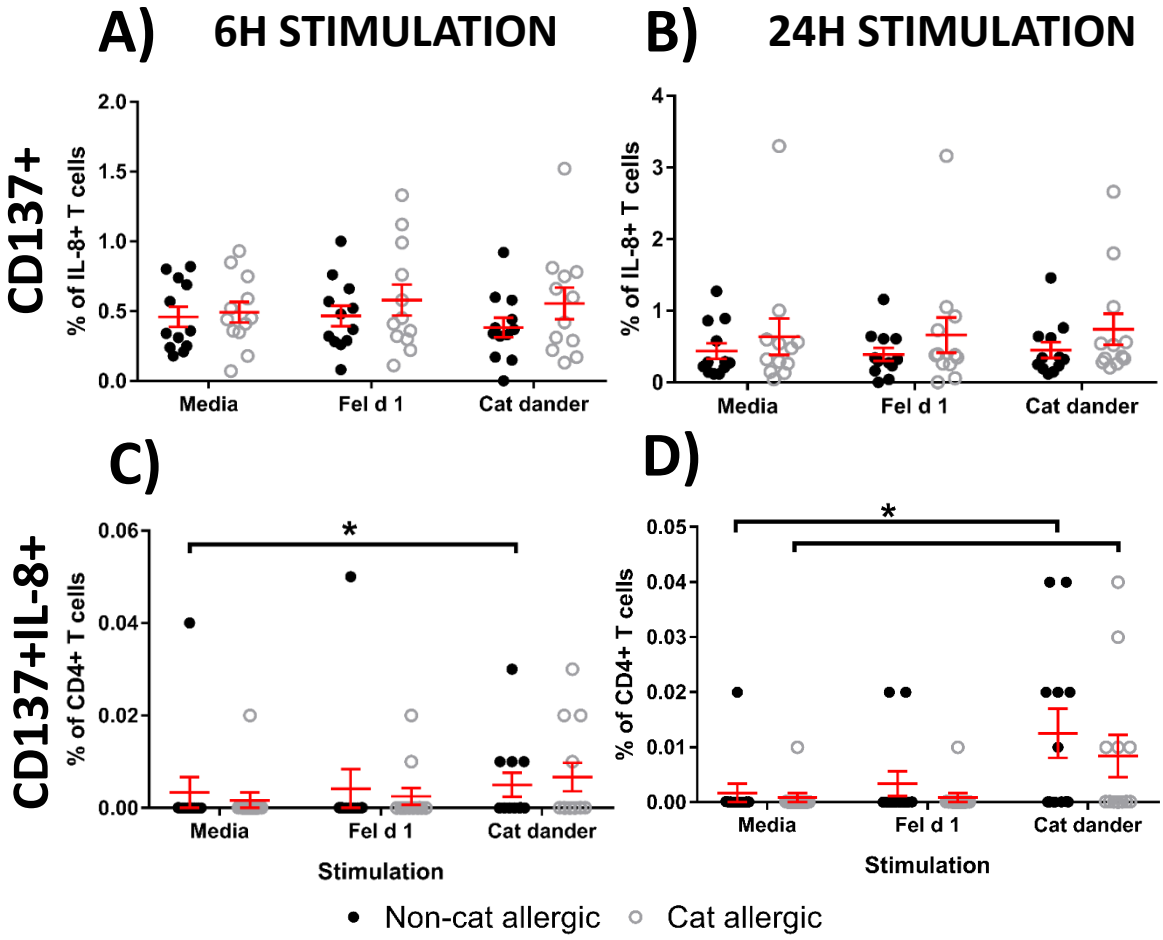


Figure 13. Frequency of IL-8+ T cells expressing CD137. PBMCs were stimulated for 6h **(left)** and 24h **(right)** with media, Fel d 1, and cat dander and run through flow cytometry. **A,B) Frequency of CD137+ in IL-8+ T cell population.** IL-8+ CD4+ T cells were gated for CD137 expression and expressed as a percentage of IL-8+ T cells (y-axis). **C,D) Frequency of CD137+IL-8+ T cells in CD4+ population.** The frequency of double positive CD137+IL-8+ T cells are expressed as a percentage of CD4+ T cells (y-axis). Data bar shown as mean±SEM (n=12 per group, *p<0.05).

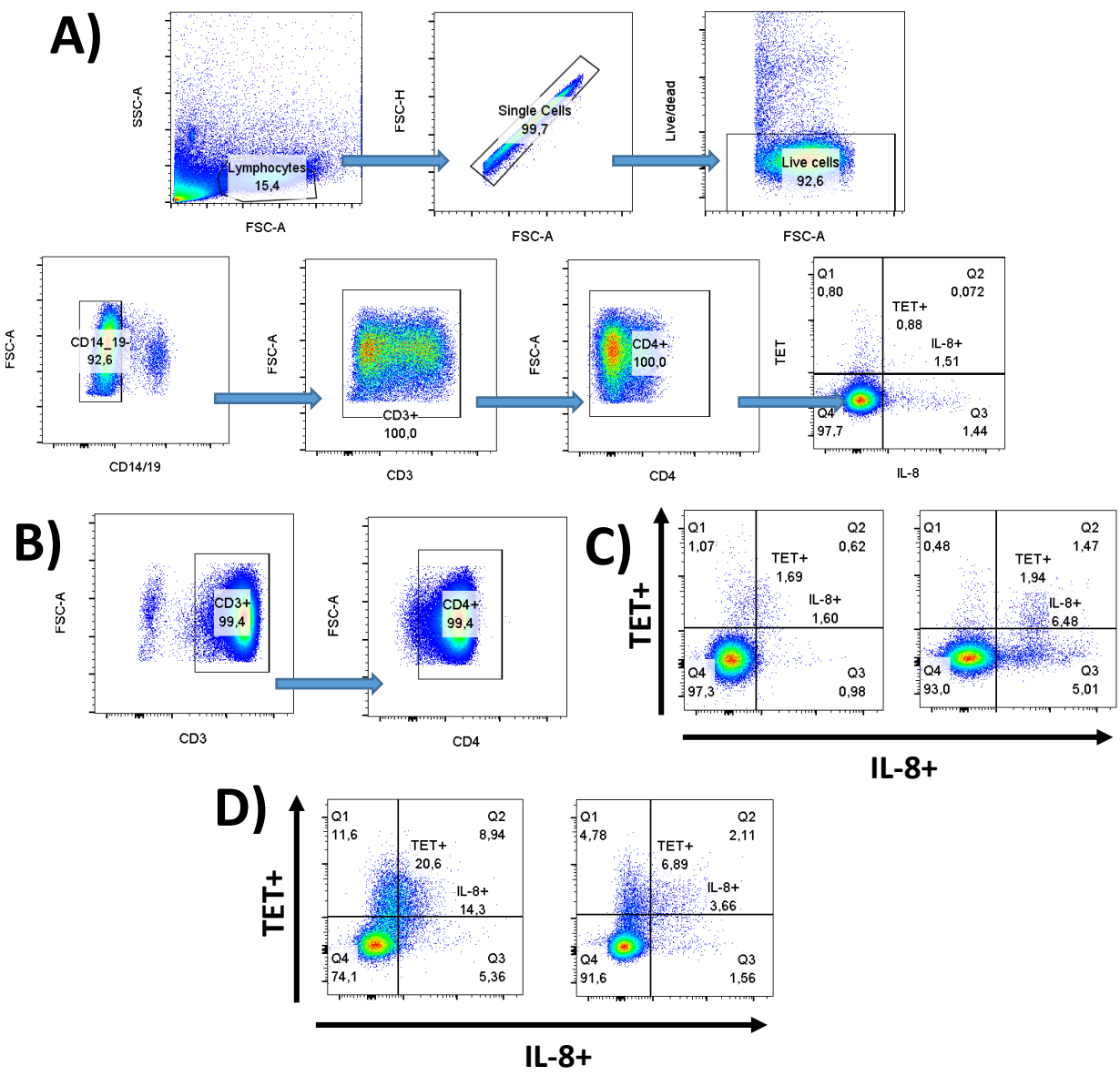


Figure 14. Gating strategy for tetramer-stained CD4+ T cells. A) Gating for PE enriched cells. Lymphocytes were selected from the PE enriched fraction according to SSC and FSC, and gated for single and live cells. Within the live population, CD14-CD19- cells were selected and gated for CD3 and CD4. CD4+ T cells were then further analyzed for IL-8, IL-5 and IFNγ. **B) Gate for CD3 and CD4 for PE- cells.** PE- cells were gated in the same manner as PE+ cells, but the CD3 and CD4 gates differed as CD3+ and CD4+ cells were evident, while in PE+ cells the CD3+ and CD4+ gates merged with CD3- and CD4- cells. **C,D) Flow plots demonstrating the gates for TET+ and IL-8+ T cells.** Example of the flow plots of TET+ vs IL-8+ T cells in two representative flow plots from **(C) non-cat allergics** and **(D) cat allergics.** SSC, side scatter; FSC, forward scatter.

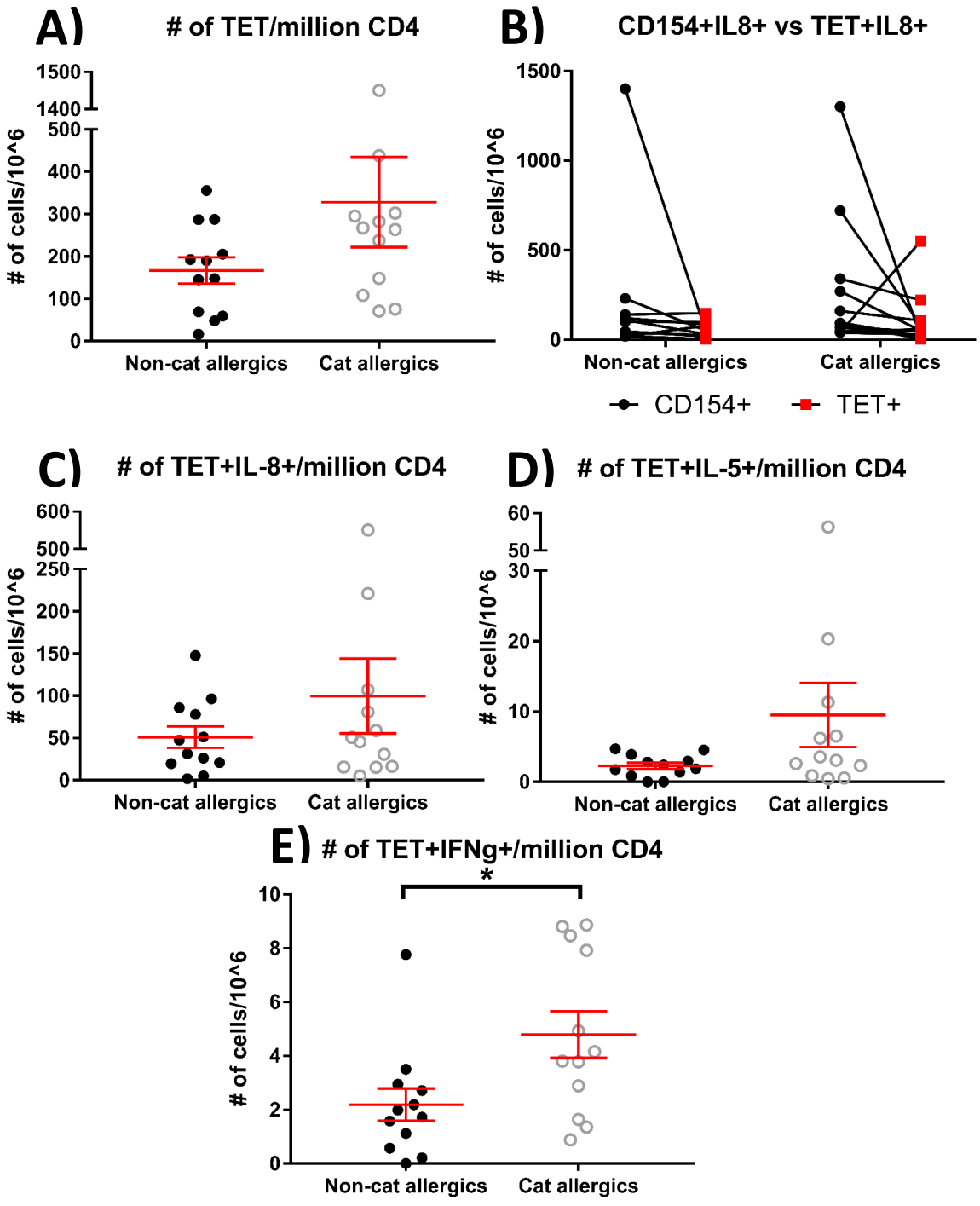


Figure 15. Number of TET+ cells expressing IL-8, IL-5 and IFNγ per million CD4+ T cells. Enriched Fel d 1-specific (TET+) CD4+ T cells were stimulated with αCD3/28 beads for 24h and stained for IL-8, IL-5 and IFNγ. **A)** The number of TET+ cells were calculated and expressed as per million CD4+ cells (y-axis) and compared to the number

of CD154+IL-8+ T cells/million CD4+ detected from PBMCs stimulated with Fel d 1 for 6h **(B)**. The number of TET+ T cells expressing **(C)** IL-8, **(D)** IL-5 and **(E)** IFNγ were also calculated as per million CD4+ T cells (y-axis). Data bar shown as mean±SEM (n=12 per group, *p<0.05).

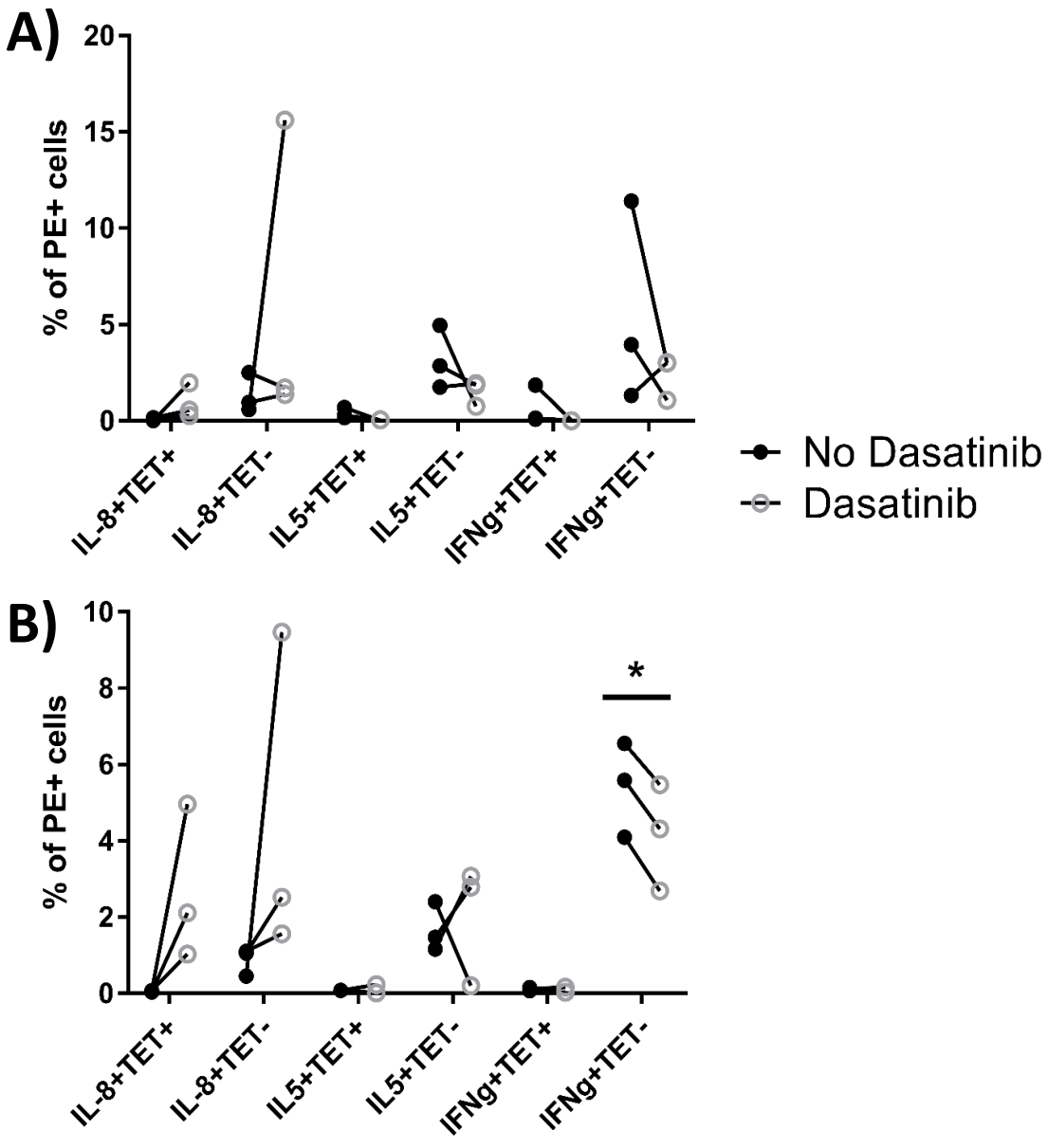


Figure 16. Effects of dasatinib on the production of IL-8, IL-5 and IFNγ in tetramer-stained CD4+ T cells. CD4+ T cells were stained with Fel d 1-specific tetramers with or without dasatinib and stimulated with αCD3/28 beads for 24h in **(A) non-cat allergics**

and **(B) cat allergics**. CD4+ T cells that were TET- were also stimulated and shown to be affected by dasatinib. Both TET+ and TET- cells were stained for IL-8, IL-5 and IFNγ expression in flow cytometry and the percentage of TET+/- cells expressing these cytokines were observed as a percentage of PE-enriched (PE+) T cells (y-axis; n=3 per group; *p<0.05).

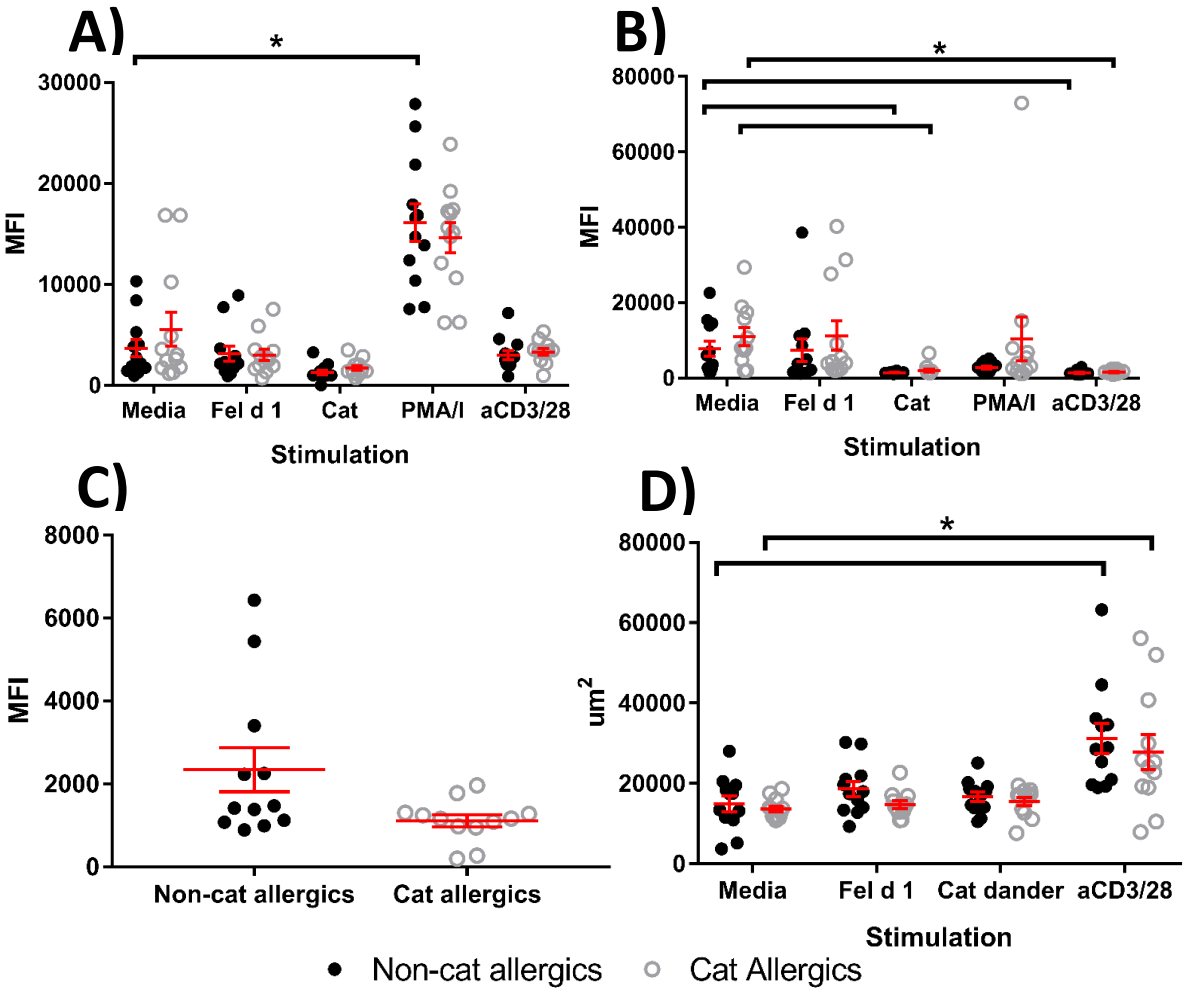
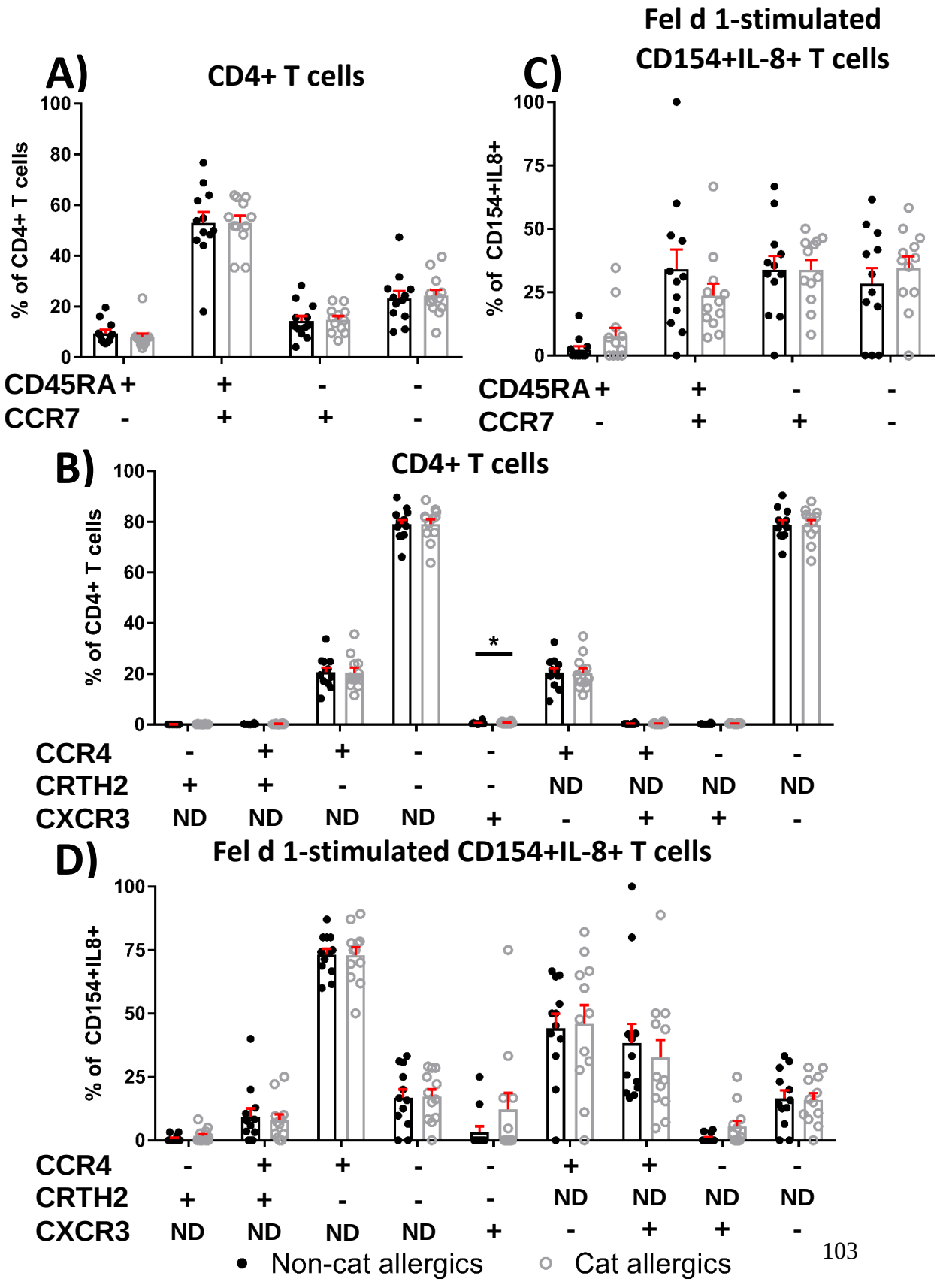
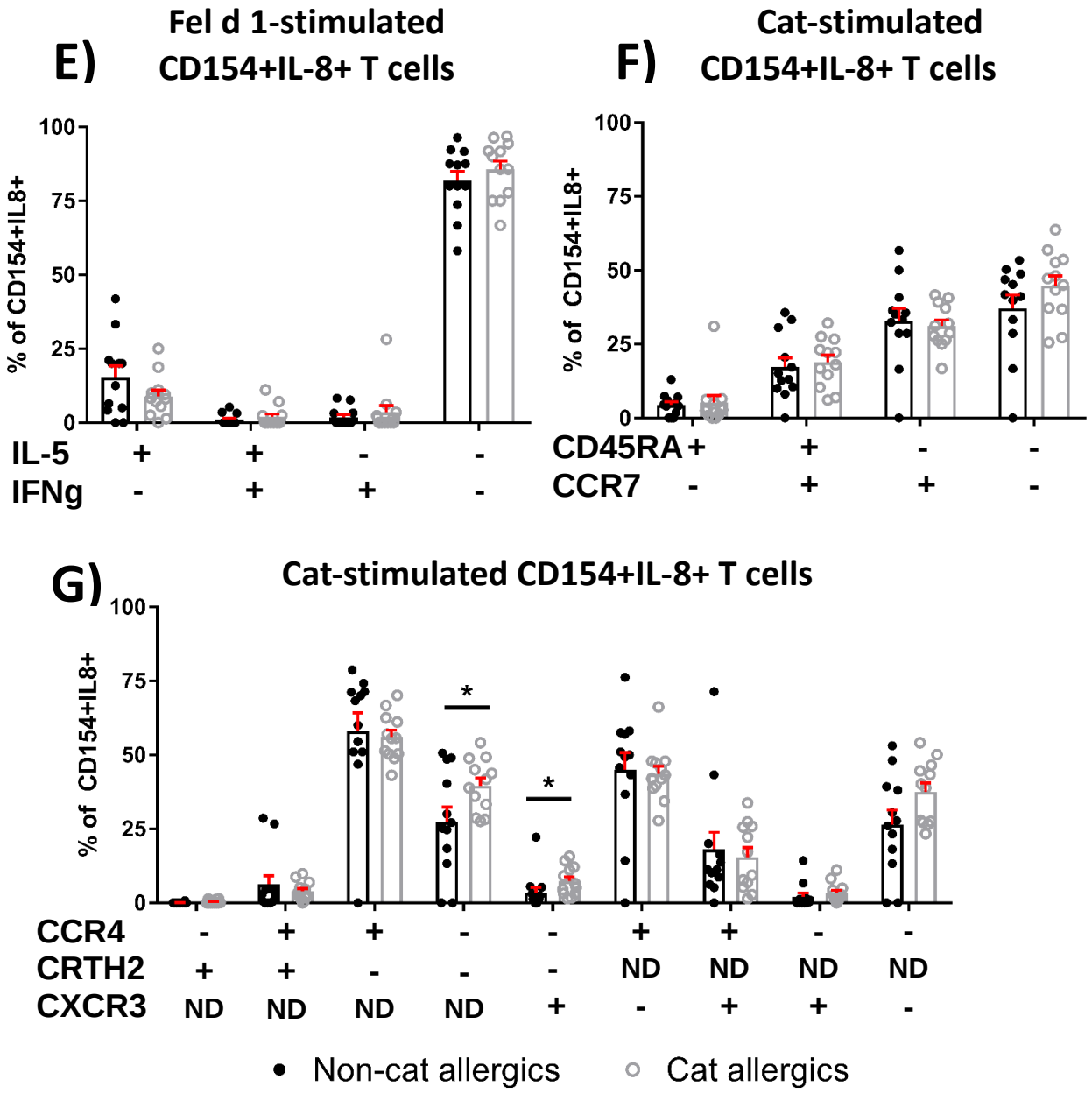


Figure 17. Level of IL-8 cytokine production in allergen-specific IL-8+ T cells. A,B) MFI of CD154+IL-8+ T cells. The median fluorescence intensity (MFI) of CD154+IL-8+ T cells detected in media, Fel d 1, cat dander, PMA/I and αCD3/28 stimulated cells was measured at **(A)** 6h and **(B)** 24h. **C) MFI of TET+IL-8+ T cells.** CD4+ T cells were stained with Fel d 1-specific tetramers and stimulated with αCD3/28 beads for 24h. TET+IL-8+ T cells detected after stimulation were gated and the MFI was measured. **D) Average spot size (μm^2) of IL-8+ T cells.** IL-8+ T cells were detected in IL-8 ELISpot assays after stimulation with cat allergens for 24h. The size of IL-8+ spots was measured and averaged over duplicates or triplicates per subject. Data bar shown as mean±SEM (n=12 per group, *p<0.05).


A)
CD4+ T cells
% of CD4+ T cells
CD45RA + + - -
CCR7 - + + -
C)
Fel d 1-stimulated
CD154+IL-8+ T cells
% of CD154+IL8+
CD45RA + + - -
CCR7 - + + -
B)
CD4+ T cells
% of CD4+ T cells
*
CCR4 - + + - - + + - -
CRTH2 + + - - - ND ND ND ND
CXCR3 ND ND ND ND + - + + -
D)
Fel d 1-stimulated CD154+IL-8+ T cells
% of CD154+IL8+
CCR4 - + + - - + + - -
CRTH2 + + - - - ND ND ND ND
CXCR3 ND ND ND ND + - + + -
Non-cat allergics
Cat allergics


E)
Fel d 1-stimulated
CD154+IL-8+ T cells
% of CD154+IL8+
100
75
50
25
0
IL-5 + + - -
IFNg - + + -
F)
Cat-stimulated
CD154+IL-8+ T cells
% of CD154+IL8+
100
75
50
25
0
CD45RA + + - -
CCR7 - + + -
G)
Cat-stimulated CD154+IL-8+ T cells
% of CD154+IL8+
100
75
50
25
0
*
*
CCR4 - + + - - + + - -
CRTH2 + + - - - ND ND ND ND
CXCR3 ND ND ND ND + - + + -
Non-cat allergics
Cat allergics

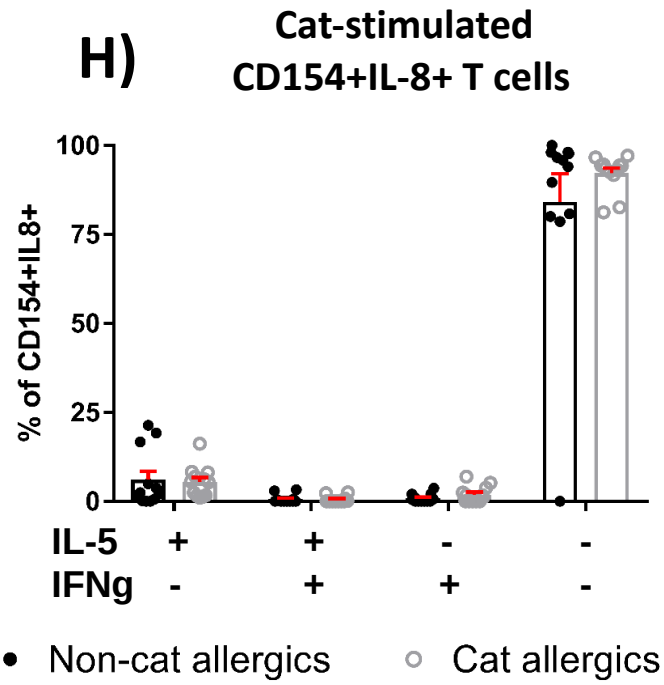


Figure 18. Phenotype of CD154+IL-8+ T cells detected in clinical study. CD4+ T cells were assessed for **(A) CD45RA and CCR7** and **(B) CRTH2, CCR4 and CXCR3** surface markers. In PBMCs stimulated with 6h Fel d 1 and cat dander, CD154+IL-8+ T cells were detected and gated in FlowJo to determine the percentage of surface markers and cytokines expressed. CD154+IL-8+ T cells from 6h Fel d 1 were assessed for **(C)** CD45RA and CCR7, **(D)** CRTH2, CCR4 and CXCR3 and **(E)** IL-5 and IFNγ. CD154+IL-8+ T cells from 6h cat dander were also phenotyped with **(F)** CD45RA and CCR7, **(G)** CRTH2, CCR4 and CXCR3 and **(H)** IL-5 and IFNγ. ND, not determined as marker was not including in gating. Data bar shown as mean±SEM (n=12 per group, *$p<0.05$).

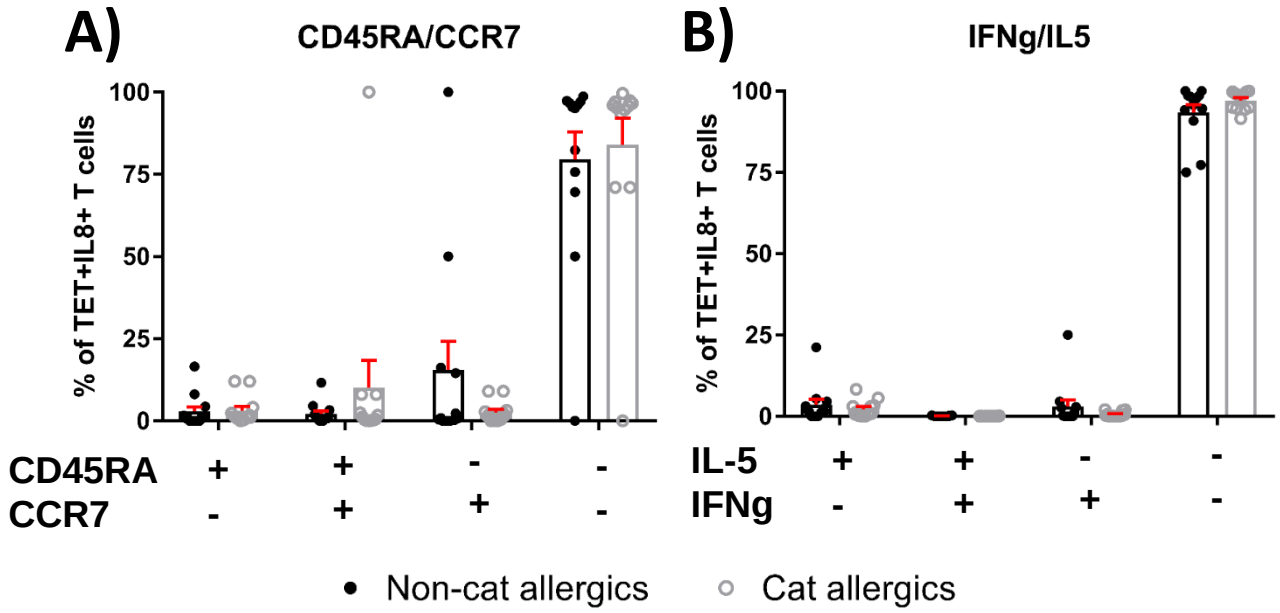


Figure 19. Phenotype of TET+IL-8+ T cells detected in clinical study. TET+IL-8+ T cells were detected through FlowJo after 24h stimulation with αCD3/28 beads and were assessed for **(A) CD45RA and CCR7 expression** and **B) IL-5 and IFNγ expression.** Data bar shown as mean±SEM (n=12 per group; p*<0.05)

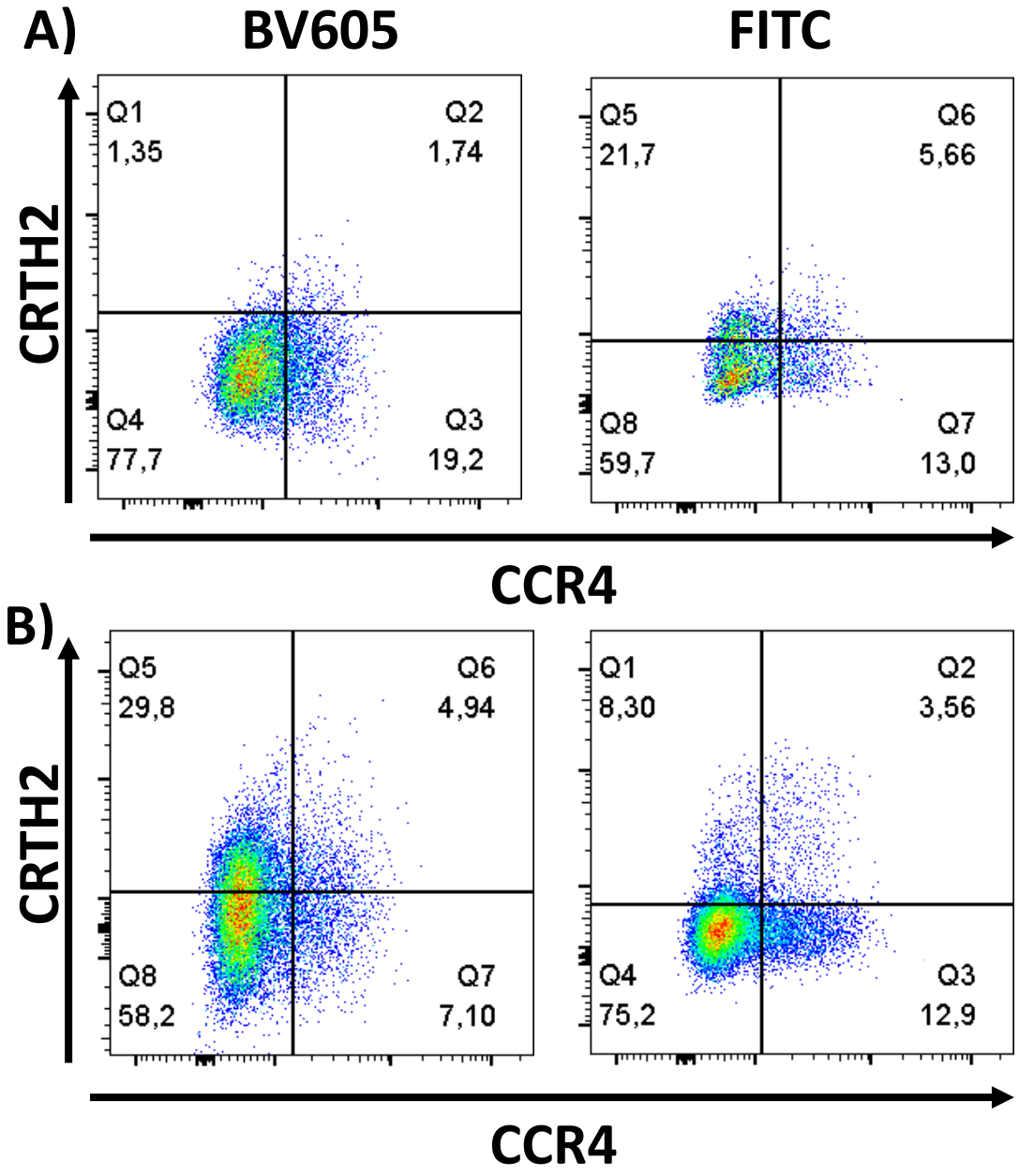


Figure 20. Staining of CRTH2 affected by antibody and staining protocol. A) CRTH2 and CCR4 expression on CD4+ T cells. PBMCs were stained with CRTH2 clone BM16 on two fluorescence molecules, BV605 and FITC (y-axis), and CCR4 (x-axis). Cells were fixed after staining with 4% PFA and constant vortexing. CRTH2 BV605 (left) did not stain cells as well as CRTH2 FITC (right). Data shown is representative of one of three subjects. **B) Effects of fixation on CRTH2 staining.** A subset of cells were stained with CRTH2 BV605 (left) or FITC (right) and ran through flow cytometry without fixation after staining. Without fixation, CRTH2 BV605 was similar to CRTH2 FITC. Data shown is representative of one of two subjects.

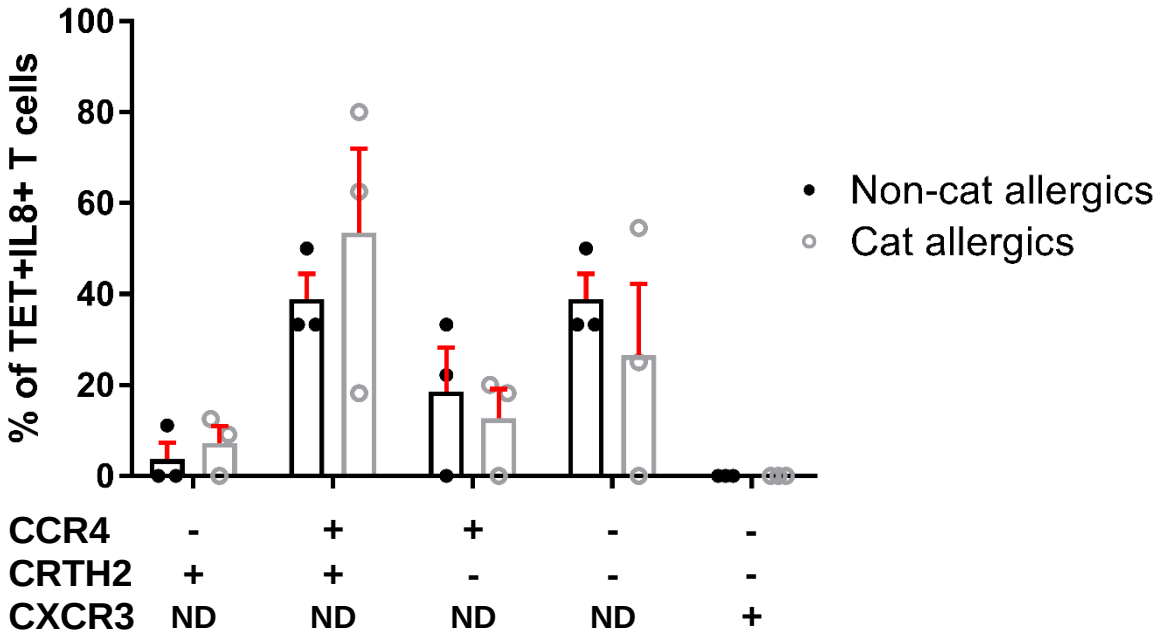


Figure 21. Expression of CRTH2 and CCR4 on Fel d 1-specific IL-8+ T cells. Fel d 1-specific (TET+) CD4+ T cells were isolated after tetramer staining and stimulated with αCD3/28 beads for 24h. TET+IL-8+ T cells were gated for CRTH2, CCR4 and CXCR3 expression using CRTH2 FITC antibody. The percentage of TET+IL-8+ T cells expressing the markers (y-axis) is shown. ND, not determined as marker was not including in gating. Data bar shown as mean±SEM (n=3 per group).

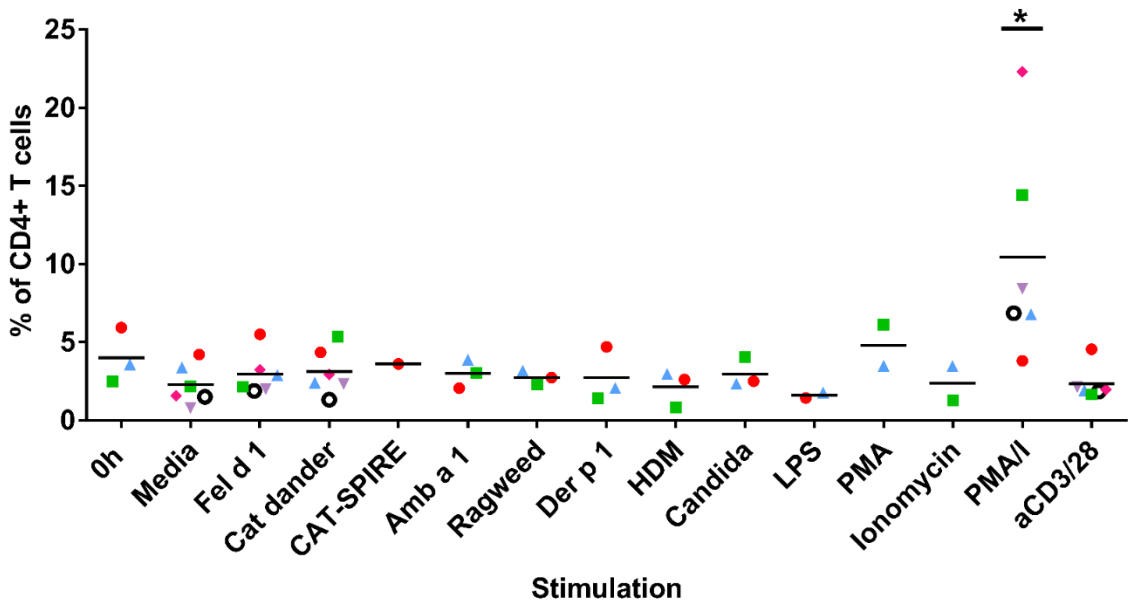


Figure 22. Allergen specificity of IL-8 production in CD4+ T cells. PBMCs were stimulated with whole allergen extracts: cat dander, house dust mite (HDM), ragweed at 50 ug/ml, recombinant allergens: Fel d 1, Der p 1 and Amb a 1 at 10 ug/ml, and recombinant peptides, Cat-SPIRE (40 uM) and other immunogenic stimuli, *Candida albicans* (1 ug/ml), and lipopolysaccharides (LPS; 1 EU/ml). The percentage of CD4+ T cells expressing IL-8 (y-axis) was measured through flow cytometry. (n=6; *$p<0.05$ in comparison to media)

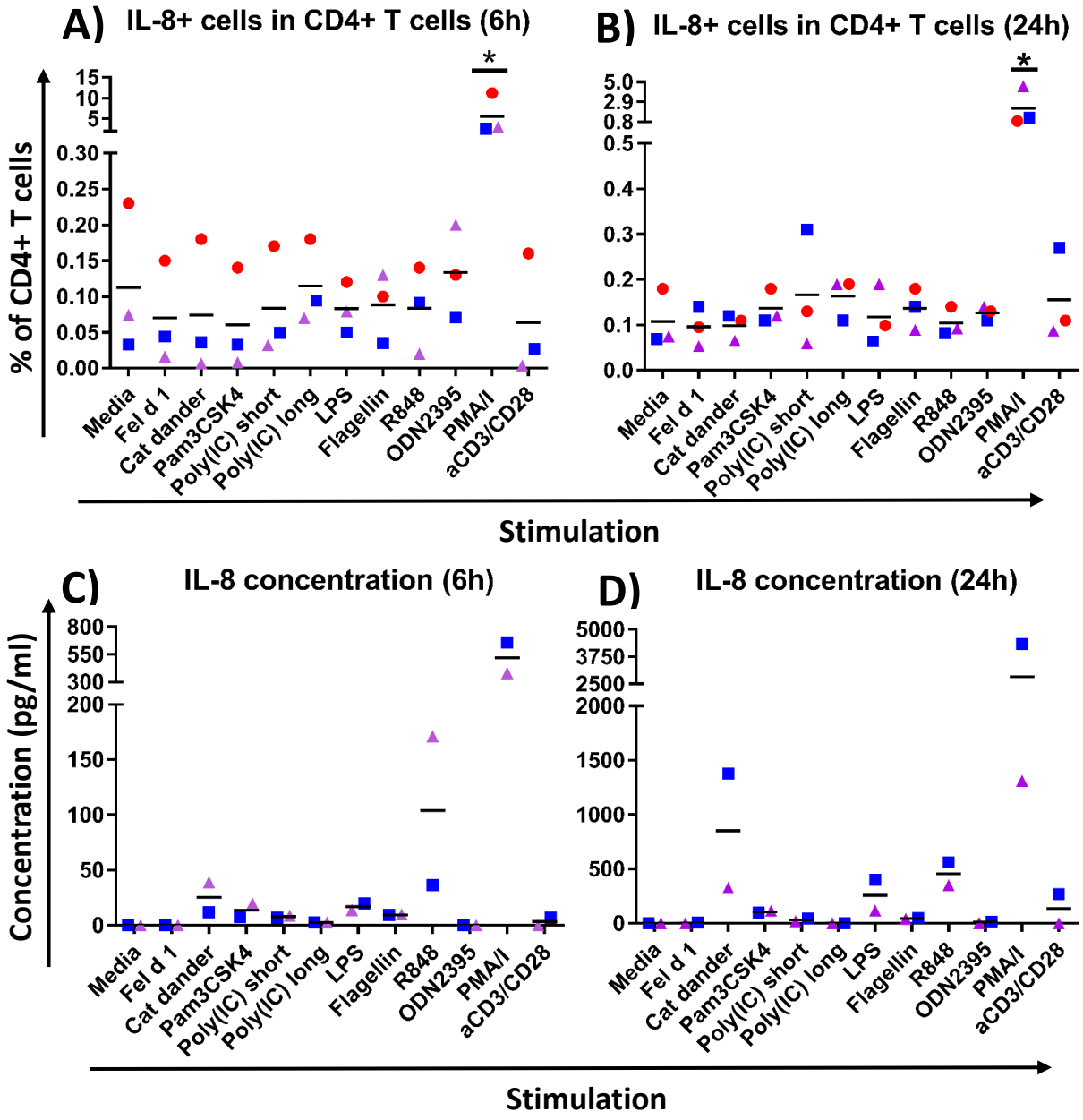


Figure 23. Stimulation of CD4+ T cells through toll-like receptors. A,B) Percentage of IL-8+ T cells in CD4+ T cells. Purified T cells were stimulated with toll-like receptor (TLR) agonists for 6h **(A)** and 24h **(B).** The percentage of CD4+ T cells expressing IL-8 (y-axis) was measured through flow cytometry. **C,D) Concentration of IL-8 produced with TLR stimulation.** Supernatants from stimulated CD4+ T cells were collected and the concentration of IL-8 was measured with by ELISA. Each dot is representative of one subject. (n=3; *p<0.05 in comparison to media)

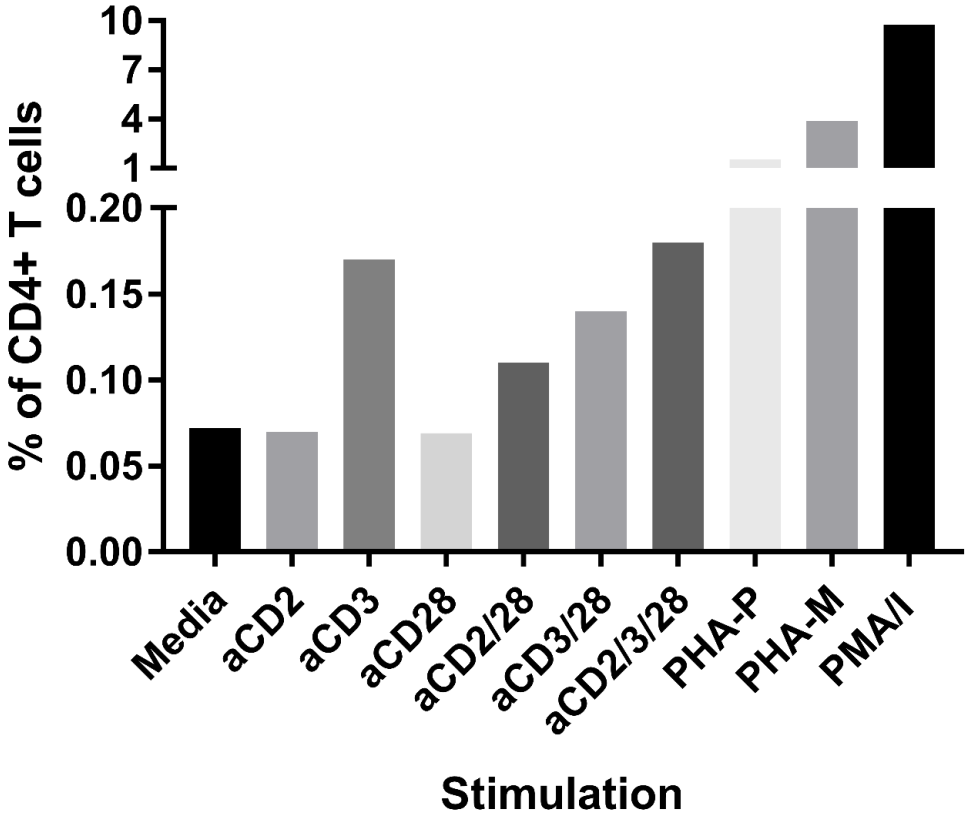


Figure 24. Co-stimulation of CD4+ T cells with αCD2/CD3/CD28 and PHA. Purified CD4+ T cells were stimulated with a combination of αCD2±CD3±CD28 antibodies, PHA-P, PHA-M, and PMA/I for 6h. The percentage of IL-8+ T cells (y-axis) was measured through flow cytometry. (n=1)

APPENDIX

Appendix S1. Source document used for screening visit.

Visit Date	____/ _____/ ____ DD MMM YYYY

Informed Consent		
Was the ICF obtained prior to starting any study procedures? ICF Version _____ Dated: _____ Date ICF signed: _____	❒ Yes	❒ No
The subject was given adequate time to read the ICF before signing it.	❒ Yes	❒ No
All study-related questions were answered to the subject's satisfaction. Please indicate significant protocol related questions: ❒ N/A _____ _____	❒ Yes	❒ No
The subject voluntarily signed the study ICF and agreed to amended information?	❒ Yes	❒ No
Was a copy of the ICF provided to the subject?	❒ Yes	❒ No
Name of Person Obtaining Consent:		

Inclusion Criteria (must be YES to be included in study)		
Is the subject between 18-65 years of age?	❒ Yes	❒ No (Exclude)
For allergic subjects, does the subject have a minimum of 1-year history of allergic rhinoconjunctivitis on exposure to cats?	❒ Yes ❒ N/A	❒ No (Exclude)
For allergic subjects, does the subject have a cat specific IgE blood concentration greater than or equal to 0.7IU/ml?	❒ Yes ❒ N/A	❒ No (Exclude)
For non-allergic subjects, does the subject have no history of allergic rhinoconjunctivitis on exposure to cats?	❒ Yes ❒ N/A	❒ No (Exclude)
For non-allergic subjects, does the subject have a cat specific IgE blood concentration less than 0.35IU/ml?	❒ Yes ❒ N/A	❒ No (Exclude)
Is the subject willing and able to provide written informed consent?	❒ Yes	❒ No (Exclude)

Exclusion Criteria (must be NO to be included in study)		
Does the subject have a cat specific IgE blood concentration between 0.35IU/ml and 0.7IU/ml?	❒ Yes (Exclude)	❒ No
Does the subject have a history of drug or alcohol abuse?	❒ Yes (Exclude)	❒ No
Has the subject used any oral short-acting antihistamines 48h prior to the visit?	❒ Yes (Exclude)	❒ No

Has the subject used any oral long-acting antihistamines and/or leukotriene inhibitors 5 days prior to the visit?	❒ Yes (Exclude)	❒ No
Has the subject used any oral corticosteroids 2 months prior to the visit?	❒ Yes (Exclude)	❒ No
Is the subject pregnant or could the subject be pregnant?	❒ Yes (Exclude)	❒ No
Has the subject received any allergen-immunotherapy for cat within the last 5 years prior to the visit?	❒ Yes (Exclude)	❒ No
Has the subject taken any investigational drugs within the last 3 months prior to the visit?	❒ Yes (Exclude)	❒ No
Does the subject have a significant disease or disorder which, at the discretion of the LPI, may put the subject at risk because of participation in the study or influence the results of the study or the subject's ability to participate?	❒ Yes (Exclude)	❒ No

Demographics

Date of Birth (dd/mmm/yyyy):		Age (years): (18-65 years)	
Gender:	☐ Male ☐ Female		
Height (cm):	___ ___ ___ . ___ cm	Weight (kg):	___ ___ ___ . ___ kg
Race: (Check all that apply)	☐ Aboriginal (Inuit, Metis, North American Indian) ☐ Arab/West Asian (eg. Armenian, Egyptian, Iranian, Lebanese, Moroccan) ☐ Black/African American (eg. Haitian, Jamaican, Somali) ☐ Chinese ☐ Latin American ☐ Filipino ☐ South Asian ☐ Japanese ☐ South East Asian ☐ Korean ☐ White (Caucasian) ☐ Other – Specify _____		
Vaccination History:	Have you had any vaccinations in the past 6 weeks? ☐ Yes ☐ No If yes, when? _____		
Cat Allergy History:	Have you experienced allergic symptoms when in contact with cats within the last year? ☐ Yes ☐ No → If no, do you have any history of cat allergies? ☐ Yes ☐ No If yes, when? _____ If yes, which symptoms? ☐ Sneezing ☐ Itchiness ☐ Runny or stuffy nose ☐ Red eyes ☐ Other. Please specify: _____		

	Do you own a cat? ☐ Yes ☐ No Do you have regular exposure to cats? ☐ Yes ☐ No If yes, define regular exposure: Average number of hours per week: _____ Have you ever done a skin prick test to determine if you are allergic to cats? ☐ Yes ☐ No If yes, when? _____ Do you have a record of the results to provide us? ☐ Yes ☐ No

Medical and Surgical History

System	None	Condition / Comment	Onset Date (DD/MMM/YYYY)	End Date / (DD/MMM/YYYY) Ongoing (O)	Investigator's Judgment	
					NCS	CS
Head, Eyes, Ears, Nose, Throat	❒				❒	❒
					❒	❒
Nervous System	❒				❒	❒
					❒	❒
Cardiovascular	❒				❒	❒
					❒	❒
Respiratory	❒				❒	❒
					❒	❒
GI and Hepatic	❒				❒	❒
					❒	❒
Genitourinary	❒				❒	❒
					❒	❒
Endocrine / Metabolic	❒				❒	❒
					❒	❒
Psychological / Psychiatric	❒				❒	❒
					❒	❒
Blood / Lymphatic Disorders	❒				❒	❒
					❒	❒
Cancer / Immunological Disease	❒				❒	❒
					❒	❒
Musculoskeletal	❒				❒	❒
					❒	❒
Dermatological	❒				❒	❒
					❒	❒
Infectious Disease	❒				❒	❒
					❒	❒

Other Medical	❒				❒	❒
					❒	❒
Surgery	❒				❒	❒
					❒	❒

Comments:

Completed by: (initial & date)		[1]**Investigator/NP:** (initial & date)	

[1] Investigator judgment is only required if a condition is documented or the Investigator conducted the exam.

Concomitant Medication Profile

Note: use of oral corticosteroids within 2 months of study visit, long-acting oral antihistamine within 5 days, short-acting oral antihistamine within 48 hours and leukotriene inhibitors within 5 days of study visit are not permitted.
(attach additional sheet if >6 concomitant medications)

Brand Name	Dose	Units (eg. mg)	Frequency	Route	Start Date dd-mmm-yy	Stop Date: dd-mm-yy or ongoing	Medical conditions (can be multiple reasons)

Blood Sample Collection (subject should be in a seated position)

Cat dander and Fel d 1 IgE	Sample Collected: ❒ Yes ❒ No	3 x 4 ml Serum tube (red top)	Time of collection:
Haematology and ESR	Sample Collected: ❒ Yes ❒ No	EDTA tube (purple top)	____:____ HH : MM
Tissue Typing	Sample Collected: ❒ Yes ❒ No	EDTA tube (purple top)	

End of Visit		
Did the subject complete all scheduled procedures?	❒ Yes	❒ No[2]
Has the subject had any change in health since the ICF was signed?	❒ Yes[1]	❒ No

[1] initiate an Adverse Event Form
[2] provide comment:

_____

_____

_____

_____

_____

_____

Inclusion Criteria Blood Sample Results

Allergen Skin Prick Test

Does subject have historical skin prick test results? ❒ Yes ❒ No

If yes, please attach scanned copy of results to file.

Date of skin prick test: dd / mmm / yy

Cat Dander and Fel d1 Specific IgE

Date Results Obtained: dd / mmm / yy

Fel d 1 specific IgE: _____ IU/mL ❒ Positive ❒ Negative (Must be greater than or equal to 0.7 IU/mL)

Cat Dander IgE: _____ IU/mL ❒ Positive ❒ Negative (Must be greater than or equal to 0.7 IU/mL)

Place a copy of results in subject's study binder

Study staff member documenting IgE results (initial): _____

Subject is deemed: ❒ Cat allergic ❒ Non-cat allergic (control)

HLA Tissue Typing

Date Results Obtained: dd / mmm / yy

HLA Tissue Typing: HLA-DRB ❒ Match ❒ No Match

__ * __ __ __ __; __ * __ __ __ __; __ * __ __ __ __; __ * __ __ __ __; __ * __ __ __ __: __ * __ __ __ __

__ * __ __ __ __; __ * __ __ __ __; __ * __ __ __ __; __ * __ __ __ __; __ * __ __ __ __; __ * __ __ __ __

Study staff member documenting HLA Typing Results (initial) _____

Inform subject when IgE and Tissue Typing results are received the coordinator will contact them to
schedule the next Visit or to inform them they did not meet all screening criteria.

Is subject eligible to continue in study at this time?

❒ Yes ❒ No → Subject is removed from study. Reason:

_____

↓

Contact subject to schedule Visit 1

Has the Subject's next visit been scheduled?

❒ No (TBD) ❒ Yes

_____

Investigator's Assessment of Subject's Eligibility

Upon review of the following:

- Medical/Surgical & Allergy History of the subject
- Concomitant Medications

I assess this subject to be:

❒ **Eligible** to continue in this study
❒ **Ineligible** to continue in this study

Reason: _____

Investigator Signature: _____ Date: |__|__|/ |__|__|__|/ |__|__|__|__|
DD MMM YYYY

Appendix S2. Source document for clinical study visits.

Visit Date	____/ _____/ ____ DD MMM YYYY

Informed Consent		
Since the screening visit, has an approved Revised ICF version been implemented?	❒ Yes	❒ No
IF YES:		
Was the ICF obtained prior to starting any study procedures? ICF Version _____ Dated: _____ Date ICF signed: _____	❒ Yes	❒ No
The subject was given adequate time to read the ICF before signing it.	❒ Yes	❒ No
All study-related questions were answered to the subject's satisfaction. Please indicate significant protocol related questions: ❒ N/A _____ _____	❒ Yes	❒ No
The subject voluntarily signed the study ICF and agreed to amended information?	❒ Yes	❒ No
Was a copy of the ICF provided to the subject?	❒ Yes	❒ No
Name of Person Obtaining Consent:		

Inclusion Criteria (must be YES to be included in study)		
Is the subject between 18-65 years of age?	❒ Yes	❒ No (Exclude)
Is the subject willing and able to provide written informed consent?	❒ Yes	❒ No (Exclude)
Exclusion Criteria (must be NO to be included in study)		
Does the subject have a history of drug or alcohol abuse?	❒ Yes (Exclude)	❒ No
Has the subject used any oral short-acting antihistamines 48h prior to the visit?	❒ Yes (Exclude)	❒ No
Has the subject used any oral long-acting antihistamines and/or leukotriene inhibitors 5 days prior to the visit?	❒ Yes (Exclude)	❒ No
Has the subject used any oral corticosteroids 2 months prior to the visit?	❒ Yes (Exclude)	❒ No
Is the subject pregnant or could the subject be pregnant?	❒ Yes (Exclude)	❒ No
Has the subject received any vaccinations within the last 6 weeks prior to the visit? If yes, when? _____	❒ Yes (Exclude)	❒ No
Has the subject received any allergen-immunotherapy for cat within the last 5 years prior to the visit?	❒ Yes (Exclude)	❒ No
Has the subject taken any investigational drugs within the last 3 months prior to the visit?	❒ Yes (Exclude)	❒ No

Does the subject have a significant disease or disorder which, at the discretion of the LPI, may put the subject at risk because of participation in the study or influence the results of the study or the subject's ability to participate?	❒ Yes (Exclude)	❒ No

Changes to screening visit profile	
Cat Allergy History:	Have you experienced any allergic symptoms when in contact with cats since the last visit? ☐ Yes ☐ No If yes, when? _____ If yes, which symptoms? ☐ Sneezing ☐ Runny or stuffy nose ☐ Itchiness ☐ Red eyes ☐ Other. Please specify: _____ Have you been exposed to cats since the last visit? ☐ Yes ☐ No If yes, when? _____
Medical and Surgical History:	Has there been any changes to your medical and surgical history since the last visit? ☐ Yes ☐ No If yes, what has changed? _____ _____
Concomitant Medication Profile:	Has there been any changes to the medications you are taking since the last visit? ☐ Yes ☐ No If yes, what has changed? _____ _____
Note: use of oral corticosteroids within 2 months of study visit, long-acting oral antihistamine within 5 days, short-acting oral antihistamine within 48 hours and leukotriene inhibitors within 5 days of study visit are not permitted.	

Blood Sample Collection (subject should be in a seated position)			
PBMC Isolation	Sample Collected: ❒ Yes ❒ No	12 x 10 ml Sodium Heparin tube (green top)	Time of collection: ____ : ____ HH : MM

End of Visit

Did the subject complete all scheduled procedures?	❒ Yes	❒ No[2]
Has the subject had any change in health since the ICF was signed?	❒ Yes[1]	❒ No

[1] initiate an Adverse Event Form
[2] provide comment: _____

_____

_____

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