ALLERGEN SPECIFIC B CELLS IN MURINE MODELS

DETECTING ALLERGEN-SPECIFIC B CELLS IN MURINE MODELS OF NOVEL PEPTIDE IMMUNOTHERAPY

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TITLE: Detecting Allergen-Specific B Cells in Murine Models of Novel Peptide Immunotherapy

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Lay Abstract

The prevalence of cat allergies is increasing globally. Symptoms of cat allergies vary in severity but may be life-threatening. Currently, there are approved treatments however, they target the symptoms and not the disease. A solution may lie in peptide immunotherapy, which uses synthetic fragments of the allergen to reprogram allergic responses. mRNA peptide immunotherapy vaccines for cat allergen, Fel d 1, were produced and tested in mouse models. This project aimed to determine the efficacy of these vaccines and detect any potential changes to allergen-specific B cells, a key component of the adaptive immune system. Unique tools, known as tetramers, to detect allergen-specific B cells were produced and utilized on samples extracted from the mice. Tetramers were shown to be antigen-specific and sensitive to Fel d 1 sensitized samples. Antigen-specific B cell responses were not detectable when mice were given vaccines with no allergen exposure. In allergic mice, the vaccines were not effective at reducing the allergic response.

Abstract

The prevalence of allergic disease is increasing, with 10-20% of adults globally classified as allergic. Domestic cats are a common source of allergic sensitization, with over 90% of cat allergic individuals being immunoglobulin (Ig) E sensitized to the *Felis domesticus* (Fel d) 1 protein. During an allergic reaction, affected individuals can experience a wide range of symptoms some of which are life-threatening. Current treatments for cat allergies fail to address the underlying causes of the disease. Peptide immunotherapy using T cell epitopes has demonstrated a strong safety profile and resulted in the development of long-lasting clinical tolerance in studies involving allergic human subjects. However, stability issues, varying responses and manufacturing cost were downsides to the therapy. mRNA peptide immunotherapy vaccines, which encoded the T cell epitopes were a proposed solution. This project aimed to produce and validate Fel d 1 B cell tetramers and to evaluate B cell responses in both naïve and sensitized mice immunized with two prototype vaccines (VXL01, VXL02). Fel d 1 B cell tetramers were produced, tested to ensure specificity and optimized for use in flow cytometry. Splenocytes from mice immunized with vaccine only, immunized with vaccine and given whole-allergen exposure, or allergically sensitized with Fel d 1 and immunized with vaccine were stained and analyzed. Fel d 1 specific B cells were not detected in the vaccine immunized only groups. Neither prototype peptide immunotherapy mRNA vaccine reduced allergic responses in Fel d 1 sensitized mice. There were no statistically significant changes in BAL cellularity, serum IgG and IgE when compared to control at study-endpoint. Fel d 1 sensitized mice immunized with VXL02 had a significantly

higher percentage of Fel d 1 tetramer+ B cells than the vehicle alone group, suggesting the vaccine may be capable of activating an established memory B cell pool. In conclusion, performing tetramer analysis on splenocyte populations was both sensitive and antigen-specific. Immunization with prototype vaccines alone did not activate a naïve Fel d 1 specific B cell response but appeared to expand Fel d 1 specific memory B cells. Fel d 1 specific B cells could be detected in mice sensitized to Fel d 1 prior to immunization with the mRNA vaccines with no changes in markers of allergic disease between vaccinated and control groups, indicating the vaccines were not effective at reducing the Th2 allergic disease phenotype.

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Figures and Tables

Abbreviations

ABBREVIATION	TERM
ALUM	Aluminium Hydroxide
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
AREB	Animal Research Ethics Board
BAL	Bronchoalveolar Lavage
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
CCAC	Canadian Council on Animal Care
CDE	Cat Dander Extract
COVID-19	Coronavirus Disease of 2019
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-linked Immunosorbant Assay
EQ	Epicutaneous
FBS	Fetal Bovine Serum
FC	Fragment Crystallizable
FEL D 1	Felis domesticus 1
FMO	Fluorescence Minus One
FSC	Forward Scatter
GC	Germinal Centre
GFP	Green Fluorescent Protein
HRP	Horseradish Peroxidase
IFN	Interferon
IG	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IMAC	immobilized metal affinity chromatography
IN	Intranasal
IP	Intraperitoneal
LNP	Lipid Nanoparticle
LPS	Lipopolysaccharide
MBC	Memory B Cell
MHC	Major Histocompatibility Complex
MRNA	Messenger Ribonucleic Acid
OD	Optical Density
OVA	Ovalbumin
PBS	Phosphate-buffered Saline
PEN/STREP	Penicillin-Streptomycin
RBD	Receptor Binding Domain
RO	Retro-orbital
RPMI	Roswell Park Memorial Institute
SA-PE	streptavidin-phycoerythrin

SARS-COV-2	Severe Acute Respiratory Syndrome Coronavirus 2
TBS	Tris-buffered Saline
TCR	T Cell Receptor
TH CELLS	T-helper Cells

Declaration of Academic Achievements

I, Mackenzie Thorpe, performed all experiments described in this thesis. Dr. Tom Mu produced the RBD of SARS-CoV-2 Spike protein and endotoxin-high Fel d 1 for B cell tetramer production. Dr. Mu also produced the majority of endotoxin-low Fel d 1 and provided direction on the remaining batch produced by me. Rangana Talpe Guruge provided guidance and support during B cell tetramer production. She additionally produced some batches of B Cell tetramers. Drs. Allyssa Phelps and Joshua Koenig performed the sensitization and flow staining of the IgE Reporter Mice. Study design and mouse work was completed in collaboration with Sudeshna Dhar. Assistance with tissue harvesting and processing was provided by Sudeshna Dhar, Ashwin Mathews and Irene Zhou. Some ELISAs were run in collaboration with or by Sudeshna Dhar. Sudeshna Dhar ran all ELISpots. All sections of this document were written my myself and reviewed by Dr. Mark Larché.

Introduction

The prevalence of allergic disease is increasing, with 10-20% of adults globally classified as allergic. Domestic cats are a common source of allergic sensitization, with over 90% of cat allergic individuals being immunoglobulin (Ig) E sensitized to the *Felis domesticus* (Fel d) 1 protein (Fujita et al., 2012; Sparkes, 2022). Symptoms of cat allergies can vary in severity but may be life threatening. During an allergic reaction, affected individuals can experience sneezing, a runny nose, itchy eyes, skin rashes, facial pain, coughing, chest tightness and anaphylaxis (Bonnet et al., 2018; Chan and Leung, 2018; Sparkes, 2022). Factors regulating the development of allergic responses (including allergen dose, route of exposure, influence of co-factors like LPS) remain poorly understood, particularly for the B cell compartment (Allen, 2022; Herre et al., 2013). Currently, there are approved treatments for cat allergies, however, the majority of them target the symptoms and not the disease. Avoidance is the first line of defence; however, this is not always possible especially with an aeroallergen (Bonnet et al., 2018; Chan and Leung, 2018; Sparkes, 2022). Allergic individuals may come in contact with Fel d 1 without seeing the physical cat, as dander containing Fel d 1 can be carried on another individual's clothing for example. Other therapies can include antihistamines, epinephrine (in the case of anaphylaxis), inhaled corticosteroids and nasal spray corticosteroids (Bonnet et al., 2018; Chan and Leung, 2018; Sparkes, 2022). Many of the attempted curative therapies, such as whole-allergen immunotherapy, have promoted desensitization to the allergen rather than long-lasting tolerance. Variability in immune responses can also reduce the effects of the treatment resulting in a modest clinical efficacy. (Bohle, 2024; Larché, 2014; Tamas and Ciurariu, 2025). In addition, these approaches are contraindicated in patients with uncontrolled or severe asthma or other significant co-morbid diseases such as cardiovascular disease and have a high risk of

severe allergic side-effects (Moote et al., 2018). Patient compliance is also low due to the rigorous administration protocol, often involving a course of injections. In cases of allergic rhinitis due to the aeroallergens found in grass pollen, at least three years of allergen-specific immunotherapy was required to provide beneficial long-lasting effects, two years was not sufficient (Durham et al., 1999; Eng et al., 2006; Moote et al., 2008; Scadding et al., 2017). The risk of adverse events is also a contributing factor in patient compliance and therapy success (Larché, 2014; Tamas and Ciurariu, 2025). In a study evaluating the use of allergen immunotherapy for cat allergies, increases in allergen specific IgG4, which are thought to be protective, were observed, however, there was an increase in both local and systemic reactions in the treated groups compared to the placebo groups, with the allergen immunotherapy only group having the highest rates of serious adverse events (Corren et al., 2022).

What are Allergies?

The study of allergic disease has been around for many years with the term “allergy” first coined in 1906 by Clemens von Pirquet. Evidence of allergic disease stems even further back with depictions of allergy in ancient Chinese, Egyptian, Roman, and Greek literature (Ring, 2022). Allergy can be classified as a type I hypersensitivity reaction due to the immediate inflammatory immune response characterized by excessive production of immunoglobulin (Ig) E (Dhanapala et al., 2015). Any individual may develop allergies over their lifetime, with childhood sensitization being the most prevalent (Fujita et al., 2012). Allergic disorders are characterized by an immune hyperresponsiveness to non-infectious exogenous antigens, known as allergens, which are harmless to the majority of the population in most instances (Yoshido & Naoki, 2023). Common sources of allergens include food, pollen, molds, animal dander, and mites (Goodman et al., 2020). Ovalbumin (OVA) is one of four major proteins found in chicken egg whites and serves as a common model allergen due to its high availability, low cost and well

characterized immune profile (Basto et al., 2015; Dhanapala et al., 2015; Zuurveld et al., 2023). Individuals can come into contact with these allergens via the skin, digestive tract or respiratory system. This can lead to many allergic disorders such as atopic dermatitis, hives, allergic rhinitis, allergic asthma and anaphylaxis (Galli et al., 2008), with individuals experiencing some of the symptoms mentioned above (Bonnet et al., 2018; Chan and Leung, 2018; Sparkes, 2022). The onset of allergic asthma can often occur later in life affecting predominately females. A history of hay fever and seasonal exacerbation of asthma have been associated with an allergic asthma phenotype (Romanet-Manent et al., 2002). Individuals with allergic asthma do not always respond to conventional treatments and have been shown to have higher levels of IL-4 (Romanet-Manent et al., 2002).

Allergic responses are thought to have evolved from the immune responses intended to defend the host against helminth infections. Helminth infections and allergies share many features such as a Th2 predominant cytokine response, with helminth infections even showing a protective effect against allergies. Moreover, in urban areas where helminths and other microbes are found in lower levels, there is a higher incidence of allergic disease (Santiago and Nutman, 2016; Schröder et al., 2015). The popular “Hygiene Hypothesis” that states that infections, especially during childhood, protect against inflammation-associated disorders since they modulate immune-mediated effector responses (Santiago and Nutman, 2016). This hypothesis may explain why allergic disease is more prevalent in developing countries with more urban areas.

Why Study Cat Allergies?

Cat allergies are the second most common cause of indoor respiratory allergy, with 20–30% of patients with respiratory allergy being allergic to cats (Sparkes, 2022). Cats have become

a common companion animal in recent years with their popularity exceeding that of dogs in many countries. With their increase in popularity, there is also an increase in the amount of exposure an individual may have. As previously mentioned, allergic individuals may encounter Fel d 1 without seeing a physical cat as dander containing Fel d 1 can be carried on another individual's clothing for example (Sparkes, 2022). While it may seem as easy as avoiding household pets, this is difficult for many and may affect one's health in other ways. Many studies have found positive associations between pet ownership and improved physical and mental health in owners (Sparkes, 2022). Additionally, in studies comparing socioeconomic and other differences between pet owners and non-pet owners where researchers have explored the reasons people choose not to own pets, the occurrence of allergies in humans in the household was shown to be an important factor (Sparkes, 2022). Conditions such as allergic rhinoconjunctivitis and allergic asthma often exacerbated by pet dander have been associated with individual morbidity and loss of productivity (Chan and Leung, 2018).

Allergic Sensitization

Briefly, allergic sensitization occurs through the differentiation of naive CD4+T cells into T helper (h) 2 cells and the production of IgE by allergen-specific B cells in a T cell-dependent manner, producing a type 2 inflammatory response upon re-exposure to the allergen (Fujita et al., 2012; Paul and Zhu, 2010). Upon initial allergen exposure, resident tissue antigen presenting cells (APCs) encounter and phagocytose allergen antigens. These APCs, such as dendritic cells, are sensitive to the tissue microenvironment and can be activated by danger signals and epithelial derived cytokines. The activated APCs then migrate to the draining lymph nodes and present the antigen to T cells via major histocompatibility type 2 (MHC II) molecules (Fujita et al., 2012; Paul and Zhu, 2010). In the presence of costimulatory molecules during T cell priming, there is production of allergen-specific Th2 cells that secrete inflammatory cytokines such as IL-4, IL-5,

IL-9 and IL-13. Without this stimulation, T cells become polarized to a tolerogenic phenotype (Paul and Zhu, 2010). These allergen specific Th2 cells then drive a type 2 allergic response through induction of secretion of allergen specific IgE by plasma cells, and recruitment of eosinophils and mast cells (Paul and Zhu, 2010). During first exposure to cognate allergen, naive IgM+ IgD+ B cells capture antigens with the BCR, are activated and differentiate into antibody secreting cells (such as plasma cells), memory B cells (MBCs), and germinal center (GCs) B cells (Koenig et al., 2021). When a sensitized individual is then re-exposed to the allergen, crosslinking occurs when the antigens bind to IgE bound to Fc receptors on mast cells and granulocytes. This results in rapid degranulation and mediator release, such as histamine, platelet activating factor, serotonin, and prostaglandin, resulting in the production of allergic disease symptoms (Paul and Zhu, 2010).

Sensitization in the Context of Cat Allergies

There are eight different classified feline allergens, Fel d 1 to Fel d 8, however, reactivity to Fel d 1 is recognised as the most important, with 90-96% of individuals with cat allergies responding to Fel d 1. Additionally, it is observed that Fel d 1 is responsible for up to 90% of the total reactivity seen in allergic individuals (Sparkes, 2022).

Allergic sensitization to Fel d 1 occurs through the differentiation of naive CD4+T cells into T helper (h) 2 cells and the production of IgE by Fel d 1-specific B cells in a T cell-dependent manner (Fujita et al., 2012). Repeated application of Fel d 1 to barrier-disrupted skin in murine models is thought to mimic one route of human sensitization, especially in children, and results in qualitatively different immunity to Fel d 1 depending on the dose applied (Alvarez et al., 2007; Jiménez-Saiz et al., 2019; Joubert et al., 2020; Laouini et al., 2003). While this method of sensitization is relevant for studying asthma models in relation to human disease, it is

slow, labour-intensive and shows considerable variability throughout the model (Eisenbarth, 2008). Intraperitoneal (IP) sensitization provides a more robust model of allergic sensitization when working with mice. Aluminum hydroxide (alum) is a type 2 adjuvant commonly used to investigate the role of cells, cytokines and signalling pathways of interest in asthma, allergic airway disease and food-hypersensitivities. After a boosting dose, a systemic type 2 immune response indicative of established allergic disease is demonstrated with increased IgG1, IgE and differentiated Th2 helper cells (Eisenbarth, 2008). While alum-based sensitization is reproducible and effective, it does not elicit a comparable priming event to human disease (Eisenbarth, 2008). Subsequent inhaled challenge of a fixed dose of Fel d 1 via the airways allows characterization of the quality of the immune response induced initially at the site of sensitization (Joubert et al., 2020). Low doses of Fel d 1 induce Th2 responses that are also dependent on lipopolysaccharide (LPS) (Campbell et al., 2009; Herre et al., 2013). Using unique staining reagents to track Fel d 1-specific T cells (Dileepan et al., 2021), previous work in the Larché lab observed recruitment of these cells to the airways at low, but not high, skin sensitization doses. The dose dependence of other routes of Fel d 1 sensitization, such as IP as well as the recruitment of Fel d 1-specific B cells, specifically in the airways, is unknown. The phenotype, IgE production, and trafficking of 1-specific B cells following Fel d 1 sensitization (via any route) and airway challenge is also unclear. IgE memory B cells (MBCs) are rare and difficult to detect, and IgE responses have been shown to be derived from IgG1 memory B cells in particular the CD73+CD80+ subset (Allen, 2022; Bruton et al., 2021). Additionally, IgE B cell populations can be very difficult to isolate and evaluate (Brooks et al., 2018). Recently a newly discovered population of type 2 polarized MBC, “MBC2s”, were found to be enriched in IgG1 and IgG4-expressing cells, while still additionally expressing germline transcripts for IgE (Koenig et al., 2024). Since these

MBC2s produced allergen specific IgE during immunotherapy treatment of allergic individuals, where allergen re-exposure occurs, it was concluded that these cells are the primary reservoir of IgE (Koenig et al., 2024).

Tetramers

Tetramers are proteins that consist of four subunit proteins, that are bound to a fluorophore core and are used to bind and label lymphocytes for flow cytometry (Rahe et al., 2018). T cell tetramers consist of four MHC-peptide complexes that are able to recognize a cognate specific T cell receptor, while B cell tetramers consist of specific antigens connected to a B cell's BCR (Nepom, 2012; Rahe et al., 2018). Because tetramers are in both cases antigen specific, tetramer staining gives a very clear picture of the behaviour of antigen specific cells, which quickly becomes an invaluable asset in tracking the movement and behaviour of these cells in different experimental outcomes – for example, being able to track changes in the population of antigen specific cells in different compartments upon an allergen challenge (Dileepan et al., 2021; Nepom, 2012; Rahe et al., 2018). The use of antigen specific B cell tetramers can replace or supplement the historical approach of analyzing the B cell compartment using bulk B cell analyses. With the rarity of allergen specific B cells even at peak immune response, the phenotype frequency of these cells can differ from the other B cells (Phelps et al., 2024; Pape et al., 2011). Antigen specific tetramers allow for the detection and characterization of these rare cells and can provide a more comprehensive understanding of the allergen specific B-lineage cells, which can be useful in the development of novel treatments (Phelps et al., 2024).

This project aimed to use B cell tetramers produced in-house following the Phelps et al., 2024 protocol to detect and characterize Fel d 1 specific B cells in murine models immunized with prototype peptide immunotherapy mRNA vaccines.

Peptide Immunotherapy and mRNA Vaccines

Peptide immunotherapy is a novel approach to allergen-specific immunotherapy, which involves the administration of short, synthetic peptide fragments that represent major T cell epitopes of the allergen (Larché, 2007). The safety and efficacy of peptide immunotherapy have previously been evaluated for cat, grass, and house dust mite allergies, which have demonstrated positive Phase IIb safety and efficacy data and have produced lasting allergy tolerance resulting from as few as 4 doses of the therapy (Simms et al., 2015). Additionally, some safety and efficacy of a peptide immunotherapy for peanut has also been demonstrated in pre-clinical work (Dhar and Larché, 2019). The peptides in this therapy are too short to cause IgE crosslinking, however, they are still able to be recognized by T cells, preventing the type 2 cascade that usually ensues upon allergen re-exposure. Developing peptide immunotherapy formulations includes evaluating the regions of major allergens that stimulate T cells and selecting candidate peptides based on affinity trends for common MHC II receptor molecules within the targeted population (Larché, 2007).

Traditional vaccine approaches such as the subunit peptide immunotherapy mentioned above have been the conventional approach to many treatments. Even though there was success in the vaccinology field prior to the introduction of mRNA vaccines, there are many obstacles in these conventional models especially in the application to non-infectious diseases (Pardi et al., 2018). Nucleic acid therapeutics first emerged as a promising therapeutic in the 1990s, however, there were barriers in mRNA therapeutics due to their instability, innate immunogenicity and inefficient methods of delivery *in vivo* (Barbier et al., 2022; Pardi et al., 2018). Recent technological advancements, particularly in nano technology, and strides made during the COVID-19 pandemic, allowed these mRNA vaccines to come to market (Pardi et al., 2018; Gote et al., 2023). mRNA vaccines for SARS-CoV-2 have been shown to have high potency, a good

safety and efficacy profile, capacity for rapid clinical development as well as a good potential for rapid, low-cost manufacturing (Gote et al., 2023).

In this project, prototype peptide immunotherapy mRNA vaccines were designed to encode previously established T cell epitopes of Fel d 1. Vaccines were manufactured by 2 companies using their proprietary lipid nanoparticles for encapsulation.

Aims & Hypotheses

Aim 1 of this project was to produce and optimize novel reagents to detect and characterize allergen-specific B cells. The already established B cell tetramer production protocol (Phelps et al., 2024) was used with in-house produced Fel d 1 and SARS-COV-2 Spike protein receptor binding domain (RBD) to detect allergen specific B cells without the need for enrichment.

Aim 2 of this project looked to evaluate the B cell response after immunization with a prototype peptide immunotherapy mRNA vaccine. These vaccines encoded previously selected T cell epitopes. *It was hypothesized that Fel d 1 mRNA peptide immunotherapy vaccine alone will not be sufficient to induce a Fel d 1 specific B cell response.* The peptides produced in this therapy are too short to cause antibody/B cell receptor crosslinking (Larché, 2007).

The third aim of this project was to evaluate the B cell response after immunization with the mRNA peptide immunotherapy vaccines in mice sensitized to Fel d 1. *It was hypothesized that vaccination with an mRNA vaccine encoding T cell epitopes of Fel d 1 in the context of a Th1-inducing lipid nanoparticle would modulate an existing allergen-specific Th2 response and reduce outcomes associated with allergic airways disease in mice.* The skewed Th1 response will

lead to a reduction in allergic sensitization and improvement of surrogate markers of disease (Campbell et al., 2009).

Methods

Production

Endotoxin Low Fel d 1 Production (Supplementary Figure 1)

Endotoxin-low Fel d 1 was produced following an adapted protocol from "Gibco's Expi293 Expression System User guide" and "Life Technologies' Maxiprep Procedure".

A human optimized code Fel d 1 gene in pcDNA3.1 vector was synthesized by GenScript Biotech (Piscataway, New Jersey, USA). The plasmid was then transformed in a DH5a E. coli strain and stored at -80˚C. Next the plasmid was prepared for transfection using a PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Waltham, Massachusetts, USA). E. coli containing the Fel d 1 pc DNA3.1 plasmid was grown in 600 ml LB medium containing ampicillin (Gibco, Waltham, Massachusetts, USA) on a bacterial shaker at 10 x g at 37˚C overnight. Following incubation, maxiprep plasmid DNA was isolated. In order to isolate the plasmid, the filtration cartridge was first equilibrated in the maxi column by adding 30 ml of equilibration buffer and allowed to drain by gravity flow into a 1 L Erlenmeyer flask. Cells from the LB culture were isolated via centrifugation at 4000 x g for 10 minutes and the medium was aspirated. Cells were resuspended in 10 ml of lysis buffer. The solution was mixed using a serological pipette and gentle swirling during incubation at room temperature for 5 minutes. Ten ml of precipitation buffer was added to the lysed cells and immediately mixed via inversion. The precipitated lysate was transferred to the prepared column and allowed to filter through the column by gravity flow. Following filtration, the inner filter was removed, and 10 ml of wash buffer was added to the column to rinse any excess solution. Fifteen ml of elution buffer was added to the column and

allowed to drain by gravity flow into a sterile 50 ml conical collection tube. The eluate was then precipitated using a PureLink™ HiPure Precipitator Module. Ten and a half ml of isopropanol was well mixed into the eluate and incubated for 2 minutes at room temperature. The plunger from a 30 ml syringe was removed and the PureLink™ HiPure precipitator was attached through the luer lock inlet to the syringe nozzle. The precipitated DNA solution was then added to the syringe. Using a slow consistent force, the plunger was used to push the DNA mixture through the precipitator into a 50 ml conical collection tube. The precipitator-syringe system was washed by disassembling the syringe and passing 5 ml of 70% ethanol through. A second push through of air was completed to ensure all ethanol was removed. The precipitator was blotted dry on a clean paper towel and removed from the 30 ml syringe. The precipitator was attached to a 5 ml syringe in the same manner as the 30 ml syringe detailed above. Seven hundred and fifty µl TE buffer was added into the 5 ml syringe and the plunger was used to push the buffer through the precipitator into a sterile microcentrifuge tube. The eluate was removed from the microcentrifuge tube and re-passed through the precipitator system into a new microcentrifuge tube. The A260 of the DNA containing eluate was measured on a nanodrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) to ensure enough plasmid was extracted for future transfection of Expi293 cells (Gibco). A small volume of sample was sent to the Mobix lab at McMaster for sequencing.

Expi293 cells were cultured following Gibco's Expi293 Expression System User guide and incubated in a CO_2 incubator shaker (New Brunswick S41i, Eppendorf, Hamburg Germany). The cells were inoculated in 60 ml of Expi293 medium in 250 ml flasks at 125 RPM at 37˚C with 8% CO_2 until a density of 1-3 x 10^6 cells/ml was reached. Cells were suspended in Expi293 medium and seeded at a density of 4-6 x 10^5 viable cells/ml in a 250 ml shaker flask. The cells

were passaged and split every 48-72 hours for a total of 3 passages. Expi293 cells were then seeded to a final density of 2.5–3 × 10^6 viable cells/ml and grown overnight in the conditions stated above. Using cells with a viability of greater than 95 % and density of 4.5-5.5 x 10^6 cells/ml, transfection with the Fel d 1 plasmid could proceed. Cells were diluted in pre-warmed Expi293 expression medium to a density of 3 x 10^6 viable cells/ml and re-seed in a 250 ml shaker flask. The transfection reagents were prepared by diluting the plasmid DNA with Opti-MEM™ I Medium to a final concentration of 1 µg/ml and gently inverting. The ExpiFectamine™ 293 Reagent was also mixed via inversion and 160 µl was diluted in 3 ml of Opti-MEM™ I Medium and incubated at room temperature for 5 minutes. The diluted ExpiFectamine™ 293 Reagent to the diluted plasmid DNA were combined, mixed via inversion and incubated for 10-20 minutes at room temperature. The complexes were slowly transferred with a serological pipette to the cells while gently swirling the culture flask. Cells were incubated at the settings described above for 18-22 hours. Three hundred µl of ExpiFectamine™ 293 Transfection Enhancer 1 and 3 ml of ExpiFectamine™ 293 Transfection Enhancer 2 were added to the transfection flask and gently swirled. The flask was immediately returned to the incubator at the previous settings. Five days post transfection, the cells were harvested. The contents of the transfected flasks were transferred to 50 ml conical tubes and centrifuged at room temperature at 400 x g for 5 minutes. The supernatant containing the produced protein was aspirated and spun at 4000 x g for 30 minutes at 8˚C to clarify the solution. The cells in pellet were resuspended in Expi293 medium and counted for re-culturing. Cells were seeded at 2-3 x 10^6 and cultured for an additional 5-6 days using the previous settings. The clarified supernatant was then collected and stored at -20˚C until further processing could occur. After 5-6 days the harvesting of the supernatant was repeated, this time disposing of the cells.

The stored supernatant containing the Fel d 1 protein was thawed for isolation and purification. Thawed samples were spun at 5000 x g for 30 minutes at 4˚C. The supernatant was then aspirated and passed through a 0.22 µm filter. Following filtration, the Fel d 1 was purified using immobilized metal affinity chromatography (IMAC). Two dilutions of imidazole (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS (McMaster Immunology Research Centre (MIRC) Tissue Culture and Media Facility, Hamilton, Ontario, Canada) were prepared: a 10 mM and a 250 mM solution. The filtered protein solution underwent a buffer change to loading buffer via centrifugation using a 3 KDa ultra centrifugal filter tube (Millipore Sigma, Burlington, Massachusetts, USA). Culture medium was first exchanged to a 1:10 dilution of loading buffer followed by an additional exchange to a 1:5 dilution of loading buffer in 10 mM imidazole. A Ni-NTA column (Sigma-Aldrich) was balanced and loaded with the prepared sample. The Fel d 1 was then eluted from the column using the 250 mM imidazole. The eluate was diluted with PBS to an imidazole concentration of less than 100 mM and a buffer exchange to PBS with a detectable imidazole concentration of 1 mM or less was completed via centrifugation using a 3 KDa ultra centrifugal filter tube. The eluate was then loaded through an ion exchange cation column (Sigma-Aldrich), followed by a 20 ml PBS wash, collected in the same 50 ml conical tube. The Fel d 1 protein was then measured using A280 on a nanodrop spectrophotometer for concentration measurement and an SDS PAGE gel (Invitrogen) was run to confirm purity. Fel d 1 was diluted in PBS to the desired concentration, aliquoted and stored in -80˚C until future use.

B Cell Tetramer Production

Four different tetramers were produced for the experiments carried out: two PE-antigen tetramers and two PE-CF594-decoy Tetramers (Table I).

Table I: B Cell Tetramer Flow Antibodies and Tetramers

Marker	Colour	Product ID	Clone
Viability	APC-Cy7	eBioscience Fixable Viability Dye eFlour 780 Cat No:65-0865-14	N/A
CD138	BV605	BD Horizon BV605 Rat Anti-Mouse CD138 Cat No:563147	281-2
B220	AF700	Alexa Flour 700 anti-mouse/human CD45R/B220 Cedarlane Product Code: 103232 Supplier: Biolegend	RA3-6B2
Lin- (CD3 + F4/80)	BV711	Brilliant Violet 711 anti-mouse CD3 Cedarlane Product Code: 100241 Supplier: Biolegend	17A2
		Brilliant Violet 711 anti-mouse F4/80 Cedarlane Product Code: 123147 (BLG) Supplier: Biolegend	BM8
GL7	PerCp Cy5.5	PerCP/Cyanine5.5 anti-MU/HU GL7 Antigen (T/B Cell Act. Marker) Cedarlane Product Code: 144610 Supplier: Biologend	GL7
CD38	PE/Cy7	PE/Cyanine7 anti-mouse CD3 Cedarlane Product code:102718 Supplier: Biolegend	90
IgD	BV510	Brilliant Violet 510™ anti-mouse IgD Cedarlane Product Code: 405723 Supplier: Biolegend	11-26c.2a
IgM	BV786	BD OptiBuild BV786 Rat Anti-Mouse IgM Cat No:743328	II/41
Fel d 1 B Cell Tet	PE	N/A	N/A
OVA B Cell Tet	PE	N/A	N/A
RBD Decoy Tet	PE-CF594	N/A	N/A
OVA Decoy Tet	PE-CF594	N/A	N/A

Tetramer production was completed following Phelps et al., 2024. Briefly, Fel d 1, OVA and the receptor binding domain of SARS-CoV 2 Spike protein (hereby referred to as RBD), were biotinylated and quantified via western blot. Next the biotinylated Fel d 1 and OVA were separately incubated with streptavidin-phycoerythrin (SA-PE) (Agilent, cat. no. PJRS25, Santa Clara, California, USA) at a 6:1 ratio to tetramerize the antigen before purification via centrifugation and quantification on a nanodrop spectrophotometer. Tetramer was diluted in 50% glycerol and stored in the dark at –20 °C until use. To produce the decoy tetramers, the control tetramer backbone was first produced using SA-PE and DyLight 594 NHS ester (Thermo Scientific, cat. no. 46413). RBD and OVA decoy tetramers were then made by incubating the antigen with the control tetramer back bone at a 6:1 ratio. Purification was not completed for the decoy tetramers, and they were diluted in 50% glycerol and stored in the dark at –20 °C until use.

Optimization

All procedures carried out were approved by the Animal Research Ethics Board at McMaster University. Mice in these experiments were housed in conventional cages in ultraclean room L306-6, or in vented caged in ultraclean room L306-5 located at St. Joseph's Hospital, Hamilton. These housing rooms were on a 12-hour light/12-hour dark cycle. BALB/c mice were purchased at 6-8 weeks (Charles River, strain code# 028, Wilmington, Massachusetts, USA) and acclimated for a minimum of 5 days prior to the start of each experiment. Mice in conventional cages were housed in groups of no more than 4 and never singly housed. Mice in vented cages were housed in groups of no more than 5 and never singly housed. Mice were given food and water *ad libitum* and were given enrichment and housing standards that were inline with the Canadian Council on Animal Care (CCAC) guidelines.

Murine Models for Optimization of B Cell Tetramers

To optimize the tetramer staining protocol for our use, 8–10-week-old wildtype female BALB/c mice from Charles River were sensitized to Fel d 1 or OVA (Figure 1).

Table II: Sensitization and IN Challenge Regimen for Optimization Experiments

IP Sensitization x 2	IN Challenge x 2	n
0.3µg Fel d 1 (1:1 alum)	10µg Fel d 1	3
0.5µg Fel d 1 (1:1 alum)	10µg Fel d 1	3
1µg Fel d 1 (1:1 alum)	10µg Fel d 1	3
1µg Fel d 1 (3:1 alum)	10µg Fel d 1	3
250µg OVA (1:1 alum)	25µg OVA	2

Following one week of acclimatization, the first sensitizing dose of allergen was prepared (Table II). The allergen (Fel d 1 (produced in-house) or OVA (Sigma-Aldrich)) was dissolved in PBS and mixed with aluminum hydroxide (alum) (Au-Gel-S; Serva Electrophoresis, Heidelberg, Germany) at a final volume of 200 µl for 2-3 hours on a nutator. Mice were anesthetized using gaseous isoflurane (3% in 1 L/min oxygen) (Fresenius Kabi, Bad Homburg, Germany) until surgical plane was achieved and manually restrained prior to IP injection. Thirteen-14 days later, a boosting IP dose was administered in the same manner.

Five to six days following the final sensitizing dose administration, mice were bled retro-orbitally (RO). To perform the RO bleed, mice were anesthetized with gaseous isoflurane (3% in 1 L/min oxygen) until they reached surgical plane. Blood was collected from the medial canthus using a microhematocrit capillary tube (Fisher Scientific, Waltham, Massachusetts, USA) positioned at the inner corner of the eye beside the orbit. The mouse was held by the scruff and the tube was gently pushed in a rotating motion a few millimetres into the inner corner of the eye

at a 30–45-degree angle from the nose. Blood was collected into a sterile microcentrifuge tube via drops from the capillary tube. After approximately 90 µl of blood was collected, the capillary tube was removed from the eye, and the restraint on the mouse was relaxed to stop the blood flow. Gentle pressure was applied to the eye by gently pressing on the closed eye lid with clean gauze until the bleeding had stopped. Once a clot had formed, ophthalmic gel (Aventix OptixCare, Burlington, Ontario, Canada) was applied to both eyes. Following this, fluid replacement was given via a 0.5 ml subcutaneous injection of sterile saline. Mice were returned to their cage and monitored until recovery.

Seventy-two and 48 hours prior to euthanasia, mice were challenged intranasally (IN) with 10 µg of Fel d 1 or 25 µg OVA in 25 µl of PBS (Figure 1, Table II). Mice were lightly anesthetized with isoflurane (3% in 1 L/min oxygen) and allergen was administered to the nares as previously optimized (Southam et al., 2002). Mice were monitored for qualitative signs of allergic symptoms, such as redness and swelling at each dose administration.

Forty-eight hours following the final intranasal allergen challenge, mice were given an anesthetic overdose with an IP injection of sodium pentobarbital (Bimeda-MTC, Cambridge, Ontario, Canada) at 120 mg/kg. Once the mouse reached surgical plane a tracheotomy was performed, with a cannula being inserted and secured with surgical suture. The bronchoalveolar lavage (BAL) was then collected to characterize cell populations at the site of allergen challenge. This was done by lavaging the airways with 250 µl of PBS twice using a 1 ml syringe and gently massaging the chest as fluids were recovered. The harvested fluids were then stored in a microcentrifuge tube on ice for processing. Following BAL collection, the chest was opened to exsanguinate the mouse, completing euthanasia. The blood collected via a cardiac bleed was used to assess serum levels of allergen specific antibody post allergen challenge. This procedure

was performed by inserting a 25-gauge needle attached to a 1 ml syringe (Becton Dickinson and Company (BD), Franklin Lakes, New Jersey, USA) into the ventricle of the heart and gently pulling back on the plunger of the syringe. Once the syringe had been filled, the needle was removed from the syringe and the collected blood was dispensed into a sterilized microcentrifuge tube. Additional attempts could be made if there was still a blood volume to collect from the mouse. Next the spleen was collected using forceps to gently remove the organ from the surrounding fat tissue. Spleens were stored in cold RPMI (Gibco or MIRC Tissue Culture and Media Facility) containing 1% Penicillin/Streptomycin (Pen/Strep) (Gibco) and 10% Fetal Bovine Serum (FBS) (Gibco), hereby referred to as cRPMI, on ice until processing could proceed. Following this, the lymph nodes were collected in a similar manner to the spleen and stored separately in cold cRPMI on ice for processing. Lymph nodes collected included the cervical and mediastinal (pooled together) and the inguinal lymph nodes. The lungs were also excised by gently cutting away the connective tissue and pulling the tissue out by the cannula secured during the tracheotomy. The lobed lung was tied of with suture and removed into cold cRPMI on ice for tissue processing, while the whole lung was filled with 10% formalin and fixed for 48 hours.

BAL processing

BAL fluids were centrifuged at 300 x g for 15 minutes, and the supernatants were stored at -20˚ C for potential future analysis of cytokines. BAL cell pellets were resuspended in 350 µl PBS. Total cells enumerated by performing a cell count with trypan blue (Invitrogen) using a Countess II Cell Counter (LifeTechnologies, Carlsbad, California, USA). The isolated cells were diluted to ensure each of the three cyto-spin slides had 1-5 x 10^5 cells. Cytocentrifugation (Epredia Cytospia Cytocentrifuge, Fisher Scientific) was completed to transfer the BAL cells to slides by first pre-wetting the filter paper and slides (VWR Avantor, Radnor Township,

Pennsylvania, USA) with PBS and centrifuging at 40 x g for 3 minutes. Next, 100 µl of sample was added and spun 115 x g for 5 minutes. Cells were then stained using a modified Wright-Giemsa stain (Sigma-Aldrich) and manual cell differential counts were performed to analyze eosinophil, neutrophil, macrophage, and lymphocytes percentages. Two to three drops of methanol (Sigma-Aldrich) to cover the pellet on the slide were added and allowed air dry in order to remove moisture from the slide. Once all slides were dry, 2 drops of Wright-Giemsa stain were added to each slide. After two minutes had passed, and an equivalent amount of distilled tap water was added to each slide and incubated for 3.5 minutes. Following incubation, slides were washed the slides with distilled water until precipitate was removed from the slide. Slides were left to dry and were cover slipped using permount media (Fisher Scientific). Two slides of 100 cells per slide were counted on a light microscope by a blinded investigator, and the relative proportions of each cell type were determined and multiplied by the total number of BAL cells obtained to determine absolute cell counts.

Spleen Processing

Spleens were processed under sterile conditions in sterile disposable petri dishes. The spleens were decanted into these dishes with 5 ml of cRPMI. Splenocytes were extracted from the membrane by perfusing the spleen with media by injecting an insulin syringe attached to a needle (VWR Avantor). Once the spleen was perfused, a 40 µm cell filter (Corning Falcon, Corning, New York, USA) was added to the dish and the cells were released by pushing the cells through the strainer with the plunger of a 1 ml syringe. A sterile transfer pipette was used to filter and wash the cells through the same cell strainer and return the cell suspension to a 50 ml conical tube. The strainer and plate were then washed with 5 ml of cRPMI, which was also added to the conical tube. Cells were centrifuged 435 x g at 4°C for 10 minutes and resuspended in a fresh 5 ml of cRPMI. If there were any persistent clumps of cells remaining, they were passed through

another pre-wetted 40 µm cell strainer. Samples were re-centrifuged at the previous settings and resuspended in 5 ml of 1x RBC Lysis Buffer (BioLegend, San Diego, California, USA) and incubated on ice for 5-6 minutes with occasional shaking. Following the incubation, the reaction was stopped with 20-30 ml of cRPMI. Cells were spun again at the previous setting and resuspended in 10 ml of cRPMI. A 1:10 dilution in cRPMI of the splenocytes in was performed prior to enumerating the cells. 10 µl of the diluted cells were mixed with 10 µl of trypan blue and added to the Countess Automated Cell Counter slide (Invitrogen) to be counted on the cell counter. Samples were re-centrifuged at the previous settings and resuspended in freezing media (RPMI containing 40% FBS and 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)) at a concentration of 1 x 10^7 cells/ml. Samples were stored in cryovials (Nunc, Thermo Fisher) at a 1 ml volume. Cells were slowly frozen by placing the filled cryovials in a room temperature Mr. Frosty (Thermo Scientific Cat. No. 5100-0001), which was placed in a -80˚C freezer for 24 hours prior to cells being moved to liquid nitrogen for long term storage.

Lymph Node Processing

First, cells were extracted from the separate lymph nodes under sterile conditions by adding the tissue with 5 ml or cRPMI to a 40 µm cell filter in a disposable petri dish. Cells were released through the strainer by using the plunger of a 1 ml syringe. A sterile transfer pipette was used to filter and wash the cells through the same cell strainer and return the cell suspension to a 15 ml conical tube. The strainer and plate were then washed with 5 ml of cRPMI, which was also added to the conical tube. Cells were centrifuged 325 x g at 4°C for 10 minutes and resuspended in a fresh 5 ml of cRPMI. If there were any persistent clumps of cells remaining, they were passed through another pre- wetted 40 µm cell strainer. Samples were re-centrifuged at the previous settings and resuspended in 10 ml of cRPMI. A cell count using the Countess Automated Cell Counter was completed as described above. Cells were spun at the previous

settings and resuspended in 2 ml of freezing media. Cells were frozen slowly in cryovials as described above, to be thawed when required for analysis.

Lung Processing-Digested Lobes

Lungs were processed under sterile conditions in a 10 cm disposable culture dish. The lungs were decanted into the dish with 2.5 ml of cRPMI. The lungs were minced into 2 mm sections using scissors and forceps. A sterile transfer pipette was used to transfer the media and tissue into a 50 ml conical tube. Each sample was topped up with 2.5 ml pre-warmed (37°C) digest media, containing DNase (Thermo Scientific) and collagenase (Worthington Biochemical, Lakewood, New Jersey, USA). Samples were incubated in a plate shaker at 5-10 x g for 90 minutes at 37°C. Following digestion, the minced lung solution was decanted into a pre-wetted 40 μm cell-strainer inside a 10 cm disposable dish. The remaining lung fragments were pushed through the cell strainer using the plunger of a 1 mL syringe. A sterile transfer pipette was used to filter and wash the cells through the same cell strainer and return the cell suspension to a 15 ml conical tube. The strainer and plate were then washed with 5 ml of cRPMI, which was also added to the conical tube. Cells were centrifuged 325 x g at 4°C for 10 minutes and resuspended in a fresh 5 ml of cRPMI. If there were any persistent clumps of cells remaining, they were passed through another pre- wetted 40 μm cell strainer. Samples were re-centrifuged at the previous settings and resuspended in 5 ml of complete media. A 1:10 dilution in cRPMI of the samples was performed prior to enumerating the cells. A cell count using the Countess Automated Cell Counter was completed as described above. Cells were spun at the previous settings and resuspended in 2-4 ml of freezing media. Cells were frozen slowly in cryovials as described above, to be thawed when required for analysis.

Lung Processing-Fixed Lobe

After 48 hours of formalin fixation, the heart and trachea were carefully cut away. A straight cut below the primary airway and another in the middle of the lower portion of the lung was made. The primary airway portion was placed in a cassette and stored in 70% ethanol at 4˚C for the Core Histology Facility at McMaster to embed at a later date for downstream staining to occur.

Serum Processing

Whole blood was left at room temperature to clot for a minimum of one hour. Samples were then centrifuged at 3000 x g for 16 minutes at 4˚C. Serum was aspirated off of each sample and stored at -20˚C in three separate sterile microcentrifuge tubes for analysis at a later date.

IgG ELISAs

Flat bottom 96-well plates were coated with Fel d 1 at a concentration of 5 µg/ml in 0.1M carbonate-bicarbonate buffer at a volume of 100 µl per well. Plates were incubated overnight for a minimum of 8 hours at 4˚C. Following the overnight incubation, plates were washed by aspirating and rinsing 4 times with 300 µl per well of ELISA wash buffer (0.05% PBS-Tween20). Plates were blotted to dry on absorbent paper towels. Wells were then blocked with 200 µl blocking buffer (0.05% PBS-Tween20 (Sigma-Aldrich) including 1% Bovine Serum Albumin (BSA) (Rockland Immunochemicals, Pottstown, Pennsylvania, USA)) and incubated at room temperature for 2.5 hours. After incubation, plates were washed 3 times with ELISA wash buffer as in the previous washing step. Sample dilutions were prepared in blocking buffer at a range of 1:100-1:100,000 in a master plate. One hundred µl of the lowest dilution level was prepared and a serial dilution was performed. Using a multi-channel pipette, prepared dilutions were moved into the washed ELISA plates. Blank wells were filled with 100 µl of blocking buffer. Samples were incubated at room temperature for 2 hours. ELISA plates were washed 4

times as above. One hundred µl of detection antibody (HRP-conjugated Anti-mouse IgG (H+L), Invitrogen, Cat. No. 61-6520) diluted 1:5000 in blocking buffer was added to each well. Plates were incubated at room temperature for 1 hour. Plates were washed 4 times as above. One hundred µl of TMB substrate (Life Technologies) was added to each well and incubated for 10-20 minutes in the dark. The reaction was stopped by adding 50 µl of ELISA stop solution (Invitrogen, Cat. No. SS04) in a fume hood. Plates were read using a microplate reader at 450 nm within 10 minutes of the stop solution being added. A secondary wavelength correction of 570 nm was also read for some ELISAs.

IgE ELISAs

IgE Mouse Uncoated ELISA Kits with Plates (Invitrogen, Cat. No. 88-50460) were used for IgE ELISAs with a modified protocol. Plates were coated with Fel d 1 at a concentration of 5 µg/ml in 0.1M carbonate-bicarbonate buffer at a volume of 100 µl per well. Overnight incubation for a minimum of 8 hours at 4˚C was completed. Following the overnight incubation, plates were washed 4 times with 300 µl per well of ELISA wash buffer, containing 0.05% PBS-Tween20. Two hundred and fifty µl blocking buffer made from a 1:10 dilution of Assay Buffer A in deionized water was used to block wells. Plates were incubated for 2 hours at room temperature. Plates were washed twice with ELISA wash buffer as described above. In a separate master plate, 1:25- 1:5000 serum dilutions were prepared in 1x Assay Buffer A. Fifty µl of 1x Assay Buffer A was added to each sample well and 100 µl of the same buffer was added to all blank wells. Fifty µl of the prepared serum dilutions in the master plate were transferred to the corresponding wells in the ELISA plate. Plates were incubated at room temperature for 2 hours. Plates were washed 3 times as previously detailed. An aliquot of 1:250 dilution in 1x coating buffer of detection antibody was added at 100 µl/well and incubated for 1 hour at room temperature. Three washes of the plate were completed as described above. Streptavidin-HRP

was diluted 1:400 in 1x Assay Buffer A. One hundred µl/well of the diluted Streptavidin-HRP was added to all wells. Plates were incubated at room temperature for 30 minutes. Following incubation, the plates were washed for a total of 4 times as describe above. One hundred µl of TMB substrate was added to each well and incubated in the dark for 15 minutes. The reaction was stopped using 100 µl of stop solution per well and plates were read at 450 nm on a microplate reader within 10 minutes of the addition. A secondary wavelength correction of 570 nm was also read for some ELISAs.

Thawing Cells

cRMPI was pre-warmed in a 37˚C water bath for a minimum of 30 minutes prior to the thawing of cells. Cryovials containing the desired samples for analysis were removed from liquid nitrogen and stored on dry ice before thawing. Two at a time, cryovials were gently swirled in the water bath until the sample is almost completely melted. One ml of warmed media was slowly added to the thawed cells using a p1000 pipette, drop by drop. The cell suspension was then transferred into a 15 ml conical tube containing 8 ml of pre-warmed media. Samples were centrifuged at 325 x g for 10 minutes at room temperature. Cells were resuspended in 5 ml of cRPMI. If clumps persisted samples were filtered through a 40 µm cell strainer. Samples were stored in an incubator until cell counts could be performed.

Unenriched Tetramer Flow Staining

Fc block was prepared using 25 µl of cell staining buffer and 0.5 µl of Mouse BD Fc block per sample. Antibody staining cocktail was prepared by adding appropriate dilutions of antibodies in 25 µl of cell staining buffer (BioLegend) per sample. The antibodies used in this flow cytometry panel are outlined in Table I. Fluorescence minus one (FMO) were prepared in the same was as the antibody cocktail, using BD Horizon PE-CF594 Rat Anti-Mouse CD4 (Cat.

No. 562314) and BD Pharmingen PE Rat Anti-Mouse CD19 (Cat. No. 561736) in addition to the dyes listed in Table I.

One to three million cells per sample well were stained for each experiment. Cells were seeded in a round U-bottom 96-well plate at 200 µl volume. The plate was centrifuged at 380x g for 7 minutes at 4˚C. The plate was then decanted and each cell containing well was resuspended in 25 µl of prepared Fc block. 0.5 µl of decoy tetramer was added to each sample well, this excluded the unstained and viability controls as well as any FMOs. The plate was then incubated covered from light on ice for 15 minutes. After the incubation, 25 µl of antibody cocktail containing 0.5 µl of antigen specific tetramer was added to each sample well and incubated on ice covered from light for an additional 30 minutes. Following incubation, 200 µl of cell staining buffer was added, without resuspending, to each well. Samples were spun in the plate at 380 x g for 6 minutes at 4˚C. Cells were washed with 200 µl of cell staining buffer and re-centrifuged at the previous spin settings. Samples were then resuspended in 200 µl of cell staining buffer and stored on ice until ready to be run on the flow cytometer. All samples were filtered directly before being placed on the flow cytometer.

Flow cytometry was run on the BD LSRFortessa and samples were analyzed using FlowJo software version 10.10.

PE-Enrichment Protocol

The enrichment staining protocol was completed following Phelps et al., 2024. For this thesis, enrichment was performed on two Verigem (IgE reporter) mice (Jordana/Koenig Laboratories, McMaster University, Hamilton, Ontario, Canada) were sensitized with 200 µg Fel d 1 IP with alum intraperitoneally and euthanized for spleen and lymph node harvest at day 7 and two naïve mice.

Isolated cells were centrifuged at 300 x g for 10 minutes at 4ºC and resuspended in 1 mL of cell staining buffer for transfer and pooling in a new 15 ml conical tube. Cells were re-spun at 300 x g for 5 minutes at 4ºC. Supernatants were aspirated and 1 µl of decoy tetramer was mixed with 0.4 µL of normal rat serum (Sigma-Aldrich) in 18.6 µL of cell staining buffer, per sample and added to the cells for incubation at room temperature for 5 minutes, in the dark. Following the incubation, 1 µl of Fel d 1 PE tetramer was added to the cell mixture and incubated in the dark on ice for 25 minutes. Once the staining had been completed, tubes were filled to 15 ml with cell staining buffer and the samples were mixed with a serological pipette. Samples were centrifuged at 300 x g for 5 minutes at 4ºC and supernatants were aspirated. 25 µl of anti-PE magnetic beads (Anti-PE microbeads UltraPure, Miltenyi Biotech, Cat. No. 130-105-639, Bergisch Gladbach, Germany) were added to each sample and resuspended before being incubation on ice for 30 minutes. LS Columns (Miltenyi Biotech) were loaded into the MACS cell separator (Miltenyi Biotech) and cleaned with 70% ethanol. Air bubbles were removed using the plunger and the ethanol was allowed to drip through the columns. 5 ml of cell staining buffer was passed through a 40 µm cell strainer into the column and allowed to drip through. Once the pre-wetting step was completed, 15 ml conical tubes were placed below the columns to collect the flow-through fraction. After the 30-minute incubation, samples were resuspended in 5 ml of cell staining buffer and passed through the cell strainer into the magnetic column. The tube was washed with an additional 5 ml of cell staining buffer, which was also passed through the strainer and column. After the column stopped dripping, PE-specific B cells were collected by removing the column from the magnet and placing the column over a new 15 ml conical tube. The plunger was used to flush 5 ml of cell staining buffer through the column. This flushing step was then repeated. Samples were then centrifuged, including the separated flow-through fractions, at 300

x g for 5 minutes at 4ºC. The supernatants were aspirated, and samples were resuspended in cell staining buffer for cell staining. Cells were stained using the same cell staining method as above in the unenriched protocols, however, no additional tetramer was added.

Flow experiments for optimization

For many of the optimization experiments, samples from sensitized mice were pooled. Following the unenriched cell staining protocol outlined in the section above, samples from Fel d 1 sensitized, OVA sensitized, and naïve mice were stained separately with a PE-Fel d 1 or PE-OVA tetramer. The Fel d 1 Tetramer was tested on splenocytes, cervical and inguinal lymph nodes as well as lung tissue. The OVA tetramer was tested on splenocytes and lymph nodes.

The first test compared pooled splenocytes from Fel d 1 sensitized mice to splenocytes from naïve discards using stains from previously tested flow staining panels. Half a microlitre of RBD decoy tetramer and Fel d 1 tetramer produced were used. Test two also compared pooled splenocytes from Fel d 1 sensitized mice to splenocytes from naïve discards. Samples were stained with equal amounts of RBD decoy and Fel d 1 tetramer at 0.25 µl, 0.5 µl or 1 µl volumes to determine the optimal volume of tetramer to use during flow staining. OVA decoy tetramer was also used on a Fel d 1. A third test aimed to validate the specificity of the Fel d 1 and OVA tetramers which had performed the best in previous tests. Splenocytes from Fel d 1, OVA sensitized, or naïve mice were stained with Fel d 1 or OVA tetramers, allowing us to determine the specificity of the tetramers. OVA decoy tetramer was also tested on splenocytes from Fel d 1 sensitized mice to compare to those stained with RBD decoy. In the fourth test, samples were not pooled. Splenocytes, cervical and inguinal lymph nodes from mice sensitized to Fel d 1 or OVA were stained with the corresponding tetramer, with naïve samples as a control, to determine if allergen specific B cells could be detected in each of these cell types. Lastly, a fifth test was

conducted in which lung samples from mice sensitized to Fel d 1 or naïve controls were stained with Fel d 1 tetramer and RBD decoy, in order to determine if allergen specific B cells could be detected in lung tissue.

mRNA Experiments

Various mRNA peptide immunotherapy vaccines were tested in the murine models described below (Table III).

Table III: Outline of mRNA Fel d 1 Peptide Immunotherapy Vaccinations

Name	Peptides Encoded	LNP (Low vs High Immunogenicity)	Company (A or B)
VXL00	Empty nanoparticle	Low	A
VXL01	11 T-cell epitopes + tag	Low	A
VXL02	3 T-cell epitopes + tag	Low	A
VXL03	11 T-cell epitopes + tag	High	B

mRNA Peptide Immunotherapy-Vaccination Only Model

The objective of this experiment was to determine the effects of the different mRNA peptide immunotherapy vaccines and the varying doses of each used (Table IV).

Table IV: mRNA Fel d 1 Peptide Immunotherapy Vaccine Regime for Vaccination Only Model

IM mRNA Peptide Immunotherapy x2	n
VXL01 10µg	4
VXL01 1µg	4
VXL01 0.1µg	4
VXL02 10µg	4
VXL02 1µg	4
VXL02 0.1µg	4

VXL03 10µg	4
VXL00 (LNP only)	4

Following the acclimation period, mice were given a 50 µl intramuscular (IM) injection in containing a predetermined vaccine regimen in the right hind limb on days 1 and 15 (Figure 2, Table IV). mRNA peptide immunotherapy vaccines were stored at -80˚C. To prepare the immunizations, vials of the vaccines and vehicle (TBS + Sucrose) were thawed on ice immediately prior to each injection. An undiluted vaccine contained 10 µg of VXL. Any required dilutions were preformed under sterile conditions using a sterile needle and syringe to mix vehicle and vaccine together in a sterile microcentrifuge tube. Vaccines were loaded into sterile 1 ml syringes with 25-gauge needles. Mice were anesthetized using gaseous isoflurane (3% in 1 L/min oxygen) until reaching surgical plane. Mice were restrained and the hind limb was stabilized across the body using the non-dominant hand. The needle was inserted at a 45° angle into the thigh muscle just past the bevel of the needle, as to avoid sciatic nerve damage. Vaccine was dispensed slowly before removing the needle.

RO bleeds were performed on the immunized mice on day 14, as previously described in this thesis, to assess the serum antibody levels following the first dose of vaccine. One week following the boosting dose of vaccine, mice were euthanized and their spleen, axillary and inguinal lymph nodes were collected and processed following the same protocol as above for B cell isolation and characterization via flow cytometry. Additionally, serum was collected to via a cardiac bleed, as described above, to assess allergen specific antibody levels after two doses of vaccine via ELISA.

mRNA Peptide Immunotherapy-Vaccination and Intranasal Allergen Challenge Model

The objective of this experiment was to determine the effects of the different mRNA peptide immunotherapy vaccines with subsequent intranasal whole allergen exposure (Table V).

Table V: mRNA Fel d 1 Peptide Immunotherapy Vaccine and IN Challenge Regime for Vaccination and Allergen Challenge Model

IM mRNA Peptide Immunotherapy x2	IN Challenge Fel d 1 x2	n
VXL01 5µg	1µg	4
VXL01 5µg	10µg	3*
TBS	10µg	4

*All groups began with n=4, one mouse was euthanized early and excluded.

Following the acclimation period, mice were given an intramuscular (IM) injection, as described in the previous study, containing 50 µl of VXL01 or TBS in the right hind limb on days 1 and 15 (Figure 3, Table V). Blood was collected to assess allergen specific antibody levels after one dose of vaccine via an RO bleed on day 14, as described in the optimization section. Mice were then given an intranasal challenge of 1 or 10 µg of Fel d 1 in 25 µl of PBS, as previously detailed, on days 22 and 29 (Figure 3, Table V). Following the immunization and challenge protocol, mice were euthanized on day 30 and their spleen, cervical, axillary and inguinal lymph nodes and lungs were collected for B cell isolation and characterization via flow cytometry as described above. The BAL was also collected and processed in order to characterize cell populations at the site of allergen challenge, as previously detailed in this work. Blood was collected via a cardiac bleed at euthanasia and processed, as previously described, to assess serum levels of antibodies following two doses of vaccine and IN challenge via ELISA.

mRNA Peptide Immunotherapy-Efficacy Study

The objective of this experiment was to determine the efficacy of the two optimal mRNA peptide immunotherapy vaccines compared to controls in sensitized mice (Table VI). Additionally, any changes made to the immune response following immunization with the novel vaccines in sensitized animals were important to observe.

Table VI: Fel d 1 Sensitization, IN Challenge and mRNA Fel d 1 Peptide Immunotherapy Vaccine Regime for Efficacy Model

IP Fel d 1 Sensitization x2	Post-Sensitization IN Challenge Fel d 1 x3	IM mRNA Peptide Immunotherapy x2	Outcome IN Challenge Fel d 1	Timeline	n
1µg (1:1 alum)	10µg	VXL01 5µg	2x 0.1µg , 2x 10µg	81-days	4
1µg (1:1 alum)	10µg	VXL00 (1:2 dilution in TBS)	2x 0.1µg , 2x 10µg	81-days	4
1µg (1:1 alum)	10µg	TBS + Sucrose	3x 10µg	88-days	4
1µg (1:1 alum)	10µg	VXL02 10µg	3x10µg	88-days	4

Following acclimation, mice were sensitized to Fel d 1 via an IP injection containing alum on days 1 and 15 (Figure 4, Table VI). The sensitizing doses were prepared in the same manner as described above in these methods. RO bleeds and serum processing were completed, as previously detailed, at various timepoints across these studies including post-sensitization, post-vaccine, and pre-outcome challenge (for 81-day timeline only) to assess serum levels of

allergen specific antibodies. A series of IN challenges of Fel d 1 were administered 7 days after the second sensitizing dose as well as prior to study endpoint (Figure 4, Table VI). Following sensitization, treatment and allergen challenge (Figure 4, Table VI), mice were euthanized and their spleen, lungs, cervical, axillary, brachial and inguinal lymph nodes were collected for B cell isolation and characterization via flow cytometry as previously detailed in these methods. The BAL was also collected and processed, as described above, in order to characterize cell populations at the site of allergen challenge. Additionally, blood was collected and processed via a cardiac bleed, as previously outlined, to assess allergen specific antibody levels after sensitization, immunization and challenge via ELISA.

Results

Optimization

As observed in IgE reporter mice, PE-enrichment provided a clearer picture of Fel d 1 tetramer positive cells compared to unenriched (Figure 5). In the enriched B and plasma cell population of the pooled splenocytes from sensitized mice, 58.6% of cells were Fel d 1 tetramer positive, whereas in the unenriched sample 3.93% of cells were Fel d 1 tetramer positive. While enrichment has been shown to provide sufficient resolution to study naïve and IgE-expressing cells and can allow for detection of antigen-specific B cells as rare as 1 in 100 million cells (Phelps et al., 2024), a significant difference can still be detected between naïve and sensitized mice in an unenriched sample (Figure 5 & 8), with the average of Fel d 1 tetramer positive cells being 0.85% in the spleens of sensitized animals versus 0.195% in the naïve splenocytes (0.6550% Difference Between Means ± 0.06185% SEM) (Figure 8). Additionally, a similar frequency of Fel d 1 tetramer positive B and plasma cells was observed in both enriched and

unenriched sample populations. Of the Fel d 1 tetramer positive cells, 97.3% were B cells in the enriched splenocyte sample and 95.5% were B cells in the comparable unenriched sample (Figure 6). Downstream B cell differentiation frequencies were also consistent across PE-enriched and unenriched samples, including germinal centre (GC) B Cells (68.1% in enriched sample; 63.9% in unenriched sample) vs combined memory (MBC) and naïve B cells (21.6% in both staining preparations) and class switched GC B cells (65.5% in enriched sample; 68.1% in unenriched sample) (Figure 6). This provided confidence in the accuracy of future unenriched experiments even though the previous standard protocol was to enrich when tetramer staining (Phelps et al., 2024).

The Fel d 1 B Cell tetramer also demonstrated good antigen specificity. In mice sensitized to Fel d 1, a significant increase in the total percentage of Fel d 1 tetramer positive cells was observed (0.6550% Difference Between Means ± 0.06185% SEM), as well as the percentage Fel d 1 tetramer positive germinal centre B cells (13.70 % Difference Between Means ± 1.927% SEM) and Fel d 1 tetramer positive class switched germinal centre B cells (94.45% Difference Between Means ± 0.5500% SEM) compared to a naïve control in the spleen (Figure 8). This was not observed in mice sensitized to an irrelevant allergen where there were no Fel d 1 tetramer positive class switched GC cells (Figure 7B). Observations of Fel d 1 tetramer specific GC B Cells (12.5% of Fel d 1 tetramer positive B cells) were only made when Fel d 1 tetramer staining was completed on Fel d 1 sensitized animals (Figure 7A). In mice sensitized to Fel d 1, but stained with an irrelevant OVA tetramer, there were no tetramer positive GC B cells (Figure 7C). A higher level of MBCs in mice sensitized to Fel d 1 and stained with Fel d 1 tetramer compared to the irrelevant antigen controls were also detected (9.13% vs 7.14%) (Figure 7). In conclusion, performing tetramer analysis using in-house produced Fel d 1 B cell tetramer on

unenriched populations was both sensitive and antigen-specific and was therefore used in all future experiments.

mRNA Experiments

mRNA Peptide Immunotherapy-Vaccination Only Model

BALB/c mice were given two doses of protype peptide immunotherapy mRNA vaccine or control 14 days apart and euthanized 7 days following the boosting dose. Mice immunized with prototype peptide immunotherapy mRNA vaccine but not sensitized to Fel d 1, had no detectable Fel d 1 specific IgG in their serum after one or two doses of vaccine (Figure 9A & B). Overall, Fel d 1 tetramer positive cells were not detectable, with the percentage of Fel d 1 tetramer positive cells not significantly different from the control in all vaccine groups tested (Figure 9C). In conclusion, our findings aligned with the hypothesis that Fel d 1 immunization with mRNA peptide immunotherapy vaccine alone would not be sufficient to induce a Fel d 1 specific B cell response as the encoded peptides were selected primarily to be T cell epitopes.

mRNA Peptide Immunotherapy-Vaccination and Intranasal Allergen Challenge Model

BALB/c mice were given two doses of protype peptide immunotherapy mRNA vaccine or control 14 days apart, followed by an IN challenge of Fel d 1 one and two weeks after the final vaccine and euthanasia occurring 24 hours following the final challenge. No difference in the total number of cells (Figure 10A), percentage or number of neutrophils (Figure 10B & C) or percentage or number of macrophages (Figure 10H & I) were observed across any treatment group. In mice immunized with VXL01 and given a 10 µg whole allergen exposure, a significant increase in the total BAL eosinophils (3.810 x 10^3 cells Difference Between Means ± 0.5885 x 10^3 cells SEM, compared to TBS immunized and 10 µg IN challenged control group (Figure

10F). No significance in the total BAL eosinophils was observed in the mice immunized with VXL01 and given a 1 µg Fel d 1 challenge when compared to the TBS immunized and 10 µg IN challenged control group (Figure 10F). In mice immunized with VXL01 and given a 1 µg whole allergen challenge, a statistically significant increase in the percentage (7.750% Difference Between Means ± 1.194% SEM) and total number of lymphocytes (5.362 x 103 cells Difference Between Means ± 1.378 x 103 cells SEM) was observed compared to the TBS control group (Figure 10D & E). While not statistically significant, there was also an increase in the lymphocyte levels (mean of 2.764 x 103 cells and 2.58%) in the VXL01 treated and 10 µg IN challenge group compared to the controls where no lymphocytes were detected (Figure 10D & E). The class of lymphocytes could not be determined using the manual cell differential count.

Fel d 1 specific serum IgG was significantly increased in mice immunized with VXL01 and given a 10 µg whole allergen exposure compared to the naïve control (0.7410 OD Difference Between Means ± 0.09064 OD SEM) (Figure 11A). No statistical significance was achieved in the other treatment groups (Figure 11A). Fel d 1 specific serum IgE was not statistically significant compared to the naïve control in any treatment group (Figure 11B).

The percentage and total number of Fel d 1 tetramer positive GC B cells (Figure 12C &G), as well as Fel d 1 tetramer positive class switched GC B cells (Figure 12D & H), appeared to be higher in the immunized and 10 µg IN challenged group, but not in the 1 µg IN challenged group; however, the increase failed to reach statistical significance . These increases appeared to be driven by one out of three mice in the group (Mean % Fel d 1 tetramer positive GC B cells 2.63% ± 2.84 SD ; Mean # of Fel d 1 tetramer positive GC B cells 1375.07 cells ± 1787.63 SD; Mean % Fel d 1 tetramer positive class switched GC B Cells 73.93% ± 20.89 SD; Mean # Fel d 1 tetramer positive class switched GC B cells 1072.40 cells ± 1700.55 SD) (Figure 12). No

differences were observed in the percentage or number of Fel d 1 tetramer positive cells (Figure 12A & E) or the percentage or number of Fel d 1 tetramer B cells that were memory B cells (Figure 12B & F) in either of the VXL01 treated and Fel d 1 challenged groups compared to the TBS treated and Fel d 1 challenged group.

Since the same observations were not made in the TBS immunized and 10ug Fel d 1 challenged group it could be concluded that the boosted eosinophil, lymphocyte and B cell responses were unique to the immunized group. While the increases observed in BAL eosinophils were statistically significant following vaccination, the degree of eosinophilia was not comparable to allergen- sensitized mice in which the increase in eosinophilia was typically much greater (up to 65%) than that observed in this model (up to 5%) (Supplementary Figure 4A & B; Figure 10G). Combining these findings with the those of the previous vaccine alone study, it was concluded that immunization alone was not enough to elicit a naïve B cell response, however, immunization boosted by a whole allergen challenge was able to elicit a small memory B cell response.

mRNA Peptide Immunotherapy-Efficacy Models

BALB/c mice were sensitized to Fel d 1, given three IN challenges of Fel d 1 to localize the response to the lungs, then treated with two doses of protype peptide immunotherapy mRNA vaccine or control 14 days apart, prior to additional IN challenges of Fel d 1 and euthanasia. There were no statistically significant changes in BAL cellularity between VXL01 treated mice and VXL00 treated controls (Figure 13). Fel d 1 specific serum IgG was elevated compared to the naïve control in both VXL01 and VXL00 immunized (Figure 14). Post-sensitization, post-vaccination and at harvest, there were no detectable differences in Fel d 1 specific serum IgG

between VXL01 and VXL00 treated mice (Figure 14A, 14B & 14D). At the pre-outcome challenge timepoint on day 76, mice treated with VXL01 had statistically significantly higher Fel d 1 serum IgG (0.2526 OD Difference Between Means ± 0.06510 OD SEM) (Figure 14C). Fel d 1 specific serum IgG levels remained consistent throughout the study in VXL01 treated mice (2.245 Mean OD post-sensitization, 2.181 Mean OD post-vaccination, 2.353 Mean OD pre-outcome challenge, and 2.353 Mean OD at Harvest) (Figure 14). Fel d 1 specific serum IgE was not different between VXL01 and VXL00 treated mice (Figure 14). Serum IgE was highest following sensitization compared to the other timepoints tested in VXL01 treated mice (2.823 Mean OD post-sensitization, 0.8824 Mean OD post-vaccination, 0.8579 Mean OD pre-outcome challenge, and 0.9285 Mean OD at Harvest) (Figure 14). There was also no detectable change in the percentage of Fel d 1 tetramer positive cells (Figure 15A), percentage of Fel d 1 tetramer B cells that were memory B cells (Figure 15B), percentage of Fel d 1 tetramer positive B cells that were germinal centre (GC) B cells (Figure 15C), percentage of Fel d 1 tetramer positive class switched GC B cells (Figure 15D), number of Fel d 1 tetramer positive cells (Figure 15E), number of Fel d 1 tetramer B cells that were memory B cells (Figure 15F), number of tetramer positive B cells that were GC B cells (Figure 15G), and number of Fel d 1 tetramer positive class switched GC B cells Fel d 1 (Figure 15H) in the sensitized mice treated with VXL01 or VXL00 (negative control vaccine).

There were no statistically significant changes in BAL cellularity between VXL02 treated mice and TBS vehicle alone controls (Figure 16). Post-sensitization and at harvest, there were no detectable differences in Fel d 1 specific serum IgG between VXL02 and TBS vehicle alone treated mice (Figure 17A & 17C). At the pre-outcome challenge timepoint on day 71, mice treated with VXL02 had statistically significantly higher Fel d 1 serum IgG (0.2216 OD

Difference Between Means ± 0.06051 OD SEM) (Figure 17B). Fel d 1 specific serum IgG levels remained consistent throughout the study in VXL02 treated mice (2.126 Mean OD post-sensitization, 2.133 Mean OD at pre-outcome challenge, and 2.245 Mean OD at Harvest) (Figure 17). Fel d 1 specific serum IgE was not different between VXL02 and TBS vehicle alone treated mice (Figure 17). Serum IgE was highest following sensitization compared to the other timepoints tested in VXL02 treated mice (2.526 Mean OD post-sensitization, 0.6202 Mean OD at pre-outcome challenge, and 0.6380 Mean OD at Harvest) (Figure 17).

Immunization with VXL02 in sensitized mice resulted in a significant increase in the percentage of Fel d 1 tetramer positive B cells in the spleen, compared to mice sensitized and immunized with vehicle alone (0.2525% Difference Between Means ± 0.1024% SEM) (Figure 18A). Immunization with VXL02 also appeared to increase the total number of Fel d 1 tetramer positive cells in the spleens of mice sensitized to Fel d 1 (Figure 18E) though this did not achieve statistical significance (1.20452 x 10^5 cells Difference Between Means ± 0.83262 x 10^5 cells SEM). Vehicle treated mice displayed a significantly higher percentage of class switched GC B cells in the spleen compared to the VXL02 treated mice (33.60% Difference Between Means ± 9.459% SEM) (Figure 18D). No differences were observed in the tetramer positive percentage of memory B cells (Figure 18B), percentage of GC cells (Figure 18C), number of memory B cells (Figure 18F), number of GC cells (Figure 18G) or number of class switched GC cells (Figure 18H).

Discussion

Tetramer Optimization

There are many established techniques to evaluate antigen-specific B cells in the context of disease, including include ELISpot, flow cytometry, mass cytometry, and fluorescence microscopy (Boonyaratanakornkit and Taylor, 2019). ELISpots are a common and sensitive way to quantify antigen-specific B cells, however, downstream analysis such as phenotyping cannot be completed. Additionally, this assay relies on antibody production to determine the presence of allergen-specific B cells, and not all B cells are antibody secreting (Boonyaratanakornkit and Taylor, 2019; Harriman et al., 2009; Phelps et al., 2024).

In recent years, fluorophore-conjugated ligands, including fluorochrome-conjugated antigen tetramers, have become the standard for detecting antigen-specific lymphocytes via flow cytometry (Fitzpatrick et al., 2023). Flow cytometry with tetrameric staining allows for detection of B cells expressing BCRs that could not be detected in monomeric form, characterization and downstream analysis of cells and improved sensitivity with pre-enrichment (Boonyaratanakornkit and Taylor, 2019). Previous work with B cell tetramers has demonstrated antigen specific results, allowing for analysis of B cells in different models of disease to be assessed (Aranda et al., 2023; Koenig et al., 2023; Phelps et al., 2024; Taylor et al., 2012). When using an appropriate control decoy tetramer, any B cells specific to the tetramer backbone or components that are not specific to the antigen, such as a “his-tag” in the case of the in-house produced Fel d 1, are excluded from the antigen specific population (Phelps et al., 2024). Initial experiments, in this thesis, using samples from IgE reporter mice, compared enriched vs unenriched staining and utilized the model antigen OVA as the decoy tetramer. In all subsequent experiments in the unenriched staining populations, an in-house produced SARS-CoV-2 spike protein RBD decoy was employed, as this antigen also had a his-tag from production. Recombinant proteins produced using metal-chelate affinity chromatography are commonly

engineered with an additional “tag” sequence such as a histidine “his-tag” (a sequence of six or more histidine residues) which have affinity for nickel ions immobilized on chromatography resins (Petty, 2001).

In comparing the sensitivity and specificity of the Fel d 1 tetramers, it was found that analysis on unenriched populations was both sensitive and antigen specific. While enrichment has been shown to provide sufficient resolution to study naïve and IgE-expressing cells and can allow for detection of antigen-specific B cells as rare as 1 in 100 million cells (Phelps et al., 2024), frequencies of tetramer positive B cells in unenriched splenocytes were still differentiable between naïve and Fel d 1 sensitized mice (enrichment was not required) (Figure 5 & 8). While PE-enrichment led to a greater frequency of Fel d 1 tetramer positive cells, it did not appear to change the relative proportions of cell subsets detected downstream. Pre-enrichment of samples for flow cytometry works to eliminate the non-antigen specific population of cells before running samples on the flow cytometer and is especially useful when looking at rare cell populations. By pre-enriching samples, the time spent running samples through the flow cytometer is greatly reduced (Cossarizza et al., 2017; Phelps et al., 2024). Not only does pre-enrichment save time running samples on the cytometer, in some cases it allows for the sorting of cells of low abundancy that may not be able to be detected otherwise due to the long running time and large number of events that would need to be recorded (Cossarizza et al., 2019; Nielson et al., 2024). Enrichment can be a useful tool; however, it does require manipulation of the collected sample that could introduce bias. A limitation of the PE-enrichment used in this model is the expression of mouse IgG_1 antibody (the anti-PE antibody) on the enrichment beads, resulting in all tetramer positive cells staining positively for IgG_1 (since beads cannot easily be removed). This can be addressed by utilizing an extracellular antibody block during staining or using an intracellular

stain to detect true IgG_1 (Supplementary Figure 2) if this is a marker of interest, however, this again requires more manipulation of the sample (Phelps et al., 2024). Since the population of interest is kept in both enriched and un-enriched samples, it is expected that these frequencies would remain the same. However, in some cases the gating quality is improved in enriched samples as potential fluorescence overlap is removed with the excess cell populations (Cossarizza et al., 2017). In this project, a similar relative proportion of Fel d 1 tetramer positive B vs plasma cells was observed in both enriched and unenriched sample populations. Downstream B cell differentiation frequencies were also consistent across PE-enriched and unenriched samples, including germinal centre (GC) B Cells vs combined memory (MBC) and naïve B cells, and class switched GC B cells (Figure 6). This provided confidence in the accuracy of future unenriched experiments, even though the previous standard protocol has been to enrich when tetramer staining (Phelps et al., 2024).

Mesenteric lymph nodes from mice sensitized with model antigen OVA and alum were stained with a PE-OVA tetramer and were shown to have 3.83% of OVA tetramer positive cells in an unenriched sample and 46.2% of OVA tetramer positive cells in an enriched sample (Phelps et al., 2024). These frequencies are similar to the ones observed in this study where 58.6% of cells were Fel d 1 tetramer positive, whereas in the unenriched sample 3.93% of cells were Fel d 1 tetramer positive in the spleens of mice sensitized to Fel d 1 and alum (Figure 5). However, while both samples were run in lymphoid tissue, the results are not directly comparable due to the different organs used. Phelps et al., 2024 also tested allergen specific tetramers for Alt a 1 and Ara h 1, where mice were the splenocytes from mice sensitized IP and alum to the relevant antigen and stained with the appropriate tetramers. In the Alt a 1 sensitized mice there were 3.67% tetramer positive cells in the unenriched population compared to 46.7% in the enriched

population. In the Ara h 1 sensitized mice there were 7.53% tetramer positive cells in the unenriched sample compared to 23.7% in the enriched sample. The findings from the Alt a 1 aeroallergen are comparable to the Fel d 1 aeroallergen tested in this thesis, however, the food allergen Ara h 1 demonstrated a larger population of cells in the unenriched sample and a smaller population in the enriched sample compared to the Fel d 1 sensitized samples in this study. In other areas of study enrichment is required for flow cytometry investigating antigen specific B cells especially if antigens may be polyclonal (Franz et al., 2011; Nielson et al., 2024; Snijder et al., 2018; Taylor et al., 2012).

The Fel d 1 B cell tetramer also demonstrated good antigen specificity. In mice sensitized to Fel d 1, we observed a significant increase in the total percentage of Fel d 1 tetramer positive cells, as well as the percentage of Fel d 1 tetramer positive germinal centre B cells and percentage of Fel d 1 tetramer positive class switched germinal centre B cells in splenocytes, compared to naïve control splenocytes (Figure 8). This was not observed in mice sensitized to an irrelevant allergen or mice sensitized to Fel d 1 and stained with an irrelevant tetramer (Figure 7B & C). An observation of Fel d 1 tetramer specific GC B Cells was only made when Fel d 1 tetramer staining was completed on Fel d 1 sensitized animals (Figure 7A). Higher levels of MBCs in mice sensitized to Fel d 1 and stained with Fel d 1 tetramer compared to the irrelevant antigen controls were also observed (Figure 7). Direct assessment of antigen binding has been considered the highest level of evidence for tetramer specificity (Phelps et al., 2024). Outside of the irrelevant antigen tests performed in this experiment, an induced GC B cell culture system could be implemented to ensure all the tested cells were Fel d 1 specific cells (Haniuda and Kitamura, 2019). In this kind of analysis, antibodies are produced by sorting tetramer-binding single cells onto a feeder line and culturing until secreted antibodies can be detected in the

culture medium and measured via ELISA (Haniuda and Kitamura, 2019; Phelps et al., 2024). Further analysis could be completed using adoptive cell transfer studies in mice to assess localization and development of Fel d 1 specific B cells (Boonyaratanakornkit and Taylor, 2019).

Previous work in the field has shown variability in the frequency of antigen specific tetramer positive cells as well as the efficiency of tetramer staining overtime between different batches of the same tetramer type. In a study looking at SARS-CoV-2 RBD using an APC B cell tetramer, the frequency of enriched cells decreased from an average of 9% to 0.3% when using the same set of pooled cells with a different tetramer batch (Fitzpatrick et al., 2023). For future work using the prototype peptide immunotherapy mRNA vaccines, the optimization and tetramer specificity studies should be repeated over-time and when any new batch of tetramer is produced. Additional assays with commercially available bead able to bind antibodies specific for the ligand of interest could be utilized to further validate any tetramers produced (Fitzpatrick et al., 2023; Reed et al., 2015; Takata et al., 2019).

Fel d 1 tetramer positive cells were also detected in the tissue of lymph nodes and lungs collected from sensitized mice (Supplementary Figure 5). A clear difference could be detected in the sensitized mice compared the naïve control, with the cervical lymph nodes having the largest percentage of Fel d 1 specific tetramer cells compared to the inguinal lymph nodes and lungs. This was likely due to the recent allergen challenge, within 48 hours of euthanasia, recruiting cells to the draining lymph nodes near the site of allergen challenge (Shi et al., 2000). A limitation of this experiment was the small sample size with only 1 naïve mouse and 1 mouse from each Fel d 1 condition being tested. Therefore, no statistical analysis could be performed. Future studies to validate these results with a larger sample size are required.

Phenotyping was used to determine heavy chain isotype expression in tetramer positive cells. IgG_1 and IgE tetramer positive GC B cells were detected in both PE-enriched and unenriched samples in the spleens from IgE reporter mice sensitized to Fel d 1 (Supplementary Figure 2). While unenriched samples demonstrated a similar frequency of IgE and IgG1 tetramer positive cells to that of the enriched sample, detection of IgE+ cells can be challenging. IgE is the least abundant antibody isotype in mammals and IgE producing cells can be difficult to detect. This is in part due to the fact that other immune cells are also coated in IgE through binding to CD23 (FcεRII) and FcεRI. Particularly, mast cells, basophils, monocytes, dendritic cells and other APCs constitutively express FcεRI and capture IgE in its monomeric form with high affinity (Nagata and Suzuki, 2022; Shin and Greer, 2015; Yang et al., 2019). IgE+ B cells are short-lived and can be very rare (Fernandes-Braga and Curotto de Lafaille, 2024; Koenig et al., 2024; Xiong et al., 2012). In the case of IgE MBCs, they are extremely rare. Instead, the IgE memory is carried by IgG_1 memory cells (He et al., 2017). In particular the memory is carried by MBC2s, which are enriched in IgG_1- and IgG_4-expressing cells which express germline transcripts for IgE (Koenig et al., 2024). Therefore, enrichment would likely be required to see these memory cells, as demonstrated by Koenig et al., 2024. Additionally, these experiments were carried out in IgE reporter mice, in which B cells expressing the membrane IgE BCR in homozygous Verigem reporter mice are labeled by Venus, a bright yellow fluorescent protein (Yang et al., 2019). This additional fluorescence could be useful in confirming the tetramer results via flow cytometry. The study would need to be carried out in a wild-type strain to ensure widespread use of both staining preparations are possible.

<u>mRNA Experiments</u>

mRNA Peptide Immunotherapy-Vaccination Only Model

The prototype peptide immunotherapy mRNA vaccines utilized in these studies were designed around T cell epitopes. The binding affinities of Fel d 1 peptides for 10 commonly expressed HLA-DR molecules were previously determined. Many MHC-binding sequences were identified throughout the Fel d 1 protein, with some regions contained multiple overlapping T cell epitopes able to bind multiple MHC molecules. Sequences exhibiting immunodominance on the basis of cytokine responses and proliferation were identified for use in peptide immunotherapy treatments (Worm et al., 2011). The selected peptides did not induce histamine release and a single administration of the peptides was found to be safe and well tolerated (Worm et al., 2011). Epitope mapping of Fel d 1 using human serum antibodies and mouse monoclonal antibodies demonstrated that B cells can recognize linear peptide sequences in Fel d 1, however, B cells frequently recognize conformational determinants that cannot be made from linear sequences, therefore the prototype peptide immunotherapy mRNA vaccines encoding linear T cell epitopes were unlikely to stimulate B cells and in particular, naïve B cells (Vailes et al., 1994; van't Hof et al., 1993; van Milligen et al., 1994). Additionally, the encoded peptides of the prototype vaccines are translated as monomers *in vivo*, which should preclude crosslinking of adjacent B cell receptors on naïve B cells making the vaccine unlikely to stimulate a naïve B cell response. When testing the prototype mRNA vaccines in naïve mice, no vaccine was able to elicit a primary Fel d 1 specific B cell response. The Fel d 1 specific serum IgG levels were comparable to that of a naïve mouse and the percentages of Fel d 1 tetramer positive cells were no different in the control vaccine compared to the vaccines encoding Fel d 1 peptides.

While no primary Fel d 1 specific B cell responses were detected, T cell responses were detected. ELISpot data revealed Interferon (IFN)-γ producing splenocytes for 2 of the 3 peptides tested in both of the 10 μg of VXL01 and VXL02 vaccine immunized groups, with VXL02

having the stronger response. The VXL03 immunization did not yield an IFN- γ response (Supplementary Figure 6). The increase in IFN- γ indicative of a Th1 response which is to be expected from immunization with the prototype vaccine. In a study analyzing traditional peptide immunotherapy in cat dander extract sensitized mice, increases in IFN- γ production and a similar deviation to the Th1 response were observed after treatment with physical Fel d 1 peptides (Moldaver et al., 2019). Previous studies with mRNA vaccines for infectious disease research have also shown strong innate immune responses, which include a wave of IFN responses, within a day after mRNA vaccine injection in the draining lymph nodes of mice (Kim et al., 2024).

VXL01 and VXL02 shared similar safety and tolerability profiles, with mice losing an average of less than 2% of their initial body weight after vaccination in contrast to mice immunized with VXL03 that lost an average of 8% of their initial body weight (Supplementary Figure 3A). In addition to weight loss as a measurement of safety and tolerability, the change (Δ) in thigh thickness at the injection site following immunization was measured in mm. For the first week after a single priming dose, mice immunized with VXL01 or VXL02 had an average Δ thigh thickness of less than 1 mm, where mice immunized with VXL03 had an average Δ thigh thickness of 1.5-2 mm. All swelling was resolved and measurements returned to baseline after 7 days. Following the boosting injection on day 15, a large spike in Δ thigh thickness was observed for 2 days, with VXL02 exhibiting the largest Δ at approximately 3mm. VXL01 and VXL03 groups had a Δ of approximately 1.5mm (Supplementary Figure 3B).

While VXL01 and VXL02 were produced by the same manufacturer and contained identical components, outside the additional 8 encoded peptides in VXL01, the shorter sequence of the VXL02 mRNA may have led to more efficient peptide production and thus, an increased

peptide response. VXL02, the 3x peptide vaccine, was created specifically for mouse experiments as the 3 peptides encoded are the only peptides that bind MHC class II in BALB/c mice. The findings of the ELISpots along with the safety profile of the vaccines (Supplementary Figure 3), provided the rationale for using VXL01 and VXL02 in future experiments.

mRNA Peptide Immunotherapy-Vaccination and Intranasal Allergen Challenge Model

Since a Fel d 1 specific T cell response, but not a primary B cell response, was seen in the vaccine immunized mice, it was thought that a whole allergen exposure (containing all B cell epitopes of the antigen) following immunization could lead to modulation of the allergen-induced Fel d 1 B cell response. The primed T cells from immunization may lead to the activation of the B cells in a T cell dependent manner.

A 10 µg IN challenge of Fel d 1 in mice immunized with VXL01, did lead to a Fel d 1 specific B cell response which was not seen in control mice immunized with TBS and subjected to IN challenge. The 10ug IN challenge also increased BAL eosinophils and serum IgG levels. The increases observed in BAL eosinophils (approx. 4-5% eosinophils) were not comparable to mice initially sensitized with Fel d 1 in alum in which the increase in eosinophilia was much greater (approx. 40% eosinophils) than that observed in this model (Supplementary Figure 4A & B). However, the results clearly demonstrated that vaccination primed the immune system to generate stronger Fel d 1 specific B cell responses and inflammatory infiltrates in the airways following intranasal allergen challenge.

Interestingly, the mice immunized with VXL01 that received an IN challenge of of 1 µg Fel d 1, had a significant increase in percentage and total BAL lymphocytes compared to both other groups. Other murine models of allergy have demonstrated lymphocytosis in the BAL at a

lesser extent than eosinophilia following inhaled allergen challenge (Jones et al., 2017; Ohkawara et al., 1997). In a model of inhaled house dust mite sensitization, intermittent exposure to allergen was shown to increase BAL lymphocytosis and eosinophilia compared to continuous exposure. Additionally, both a low and high dose challenge in the intermittently sensitized mice was able to induce lymphocytosis in the BAL (Jones et al., 2017). This aligns with the findings of this study, where lymphocytosis was observed at a lessor degree to eosinophilia and 1 μg of Fel d 1 IN challenge was able to increase the observed lymphocytes.

The class of lymphocytes could not be determined using the manual cell differential count, so it was unclear if this increase was in the T or B cell compartment. Based on the serum levels of IgG and IgE in this group being similar to the TBS control group, it could be inferred that the lymphocytes observed were T cells. These results are supported by the literature, in which it has been shown that lymphocytes increase in the BAL of asthmatics, with the increase being confined to the T cell sub population (Kelly et al., 1989). Additionally, evaluation of the splenocytes demonstrated low levels of Fel d 1 tetramer positive B cells, especially GC cells. ELISpots completed on these spleen samples also demonstrated a very slight increase in IFN-γ producing cells compared to the 10 μg group (Supplementary Figure 7). While possible, the trends observed in the splenocytes may not correlate with what is observed in the lung tissue. Tetramer flow staining of the cryo-preserved lung tissue could provide clarity.

mRNA Peptide Immunotherapy-Efficacy Models

Peptide immunotherapy utilizing physical synthetic peptides has been shown to induce clinical tolerance in allergic disease via immune regulation and/or immune deviation (Th2 to Th1

shift). A reduction in late phase allergic reactions was observed in cat allergic individuals treated with allergen specific peptide immunotherapy (Alexander et al., 2005; Oldfield et al., 2002). Using skin biopsies in cat allergic asthmatics given repeated peptide immunotherapy intradermal shots, Fel d 1 peptides were shown to increase the cutaneous Th 1 cells (CD4+/IFN-γ+ and CD4+/CD25+ cells) at the site of allergen challenge following treatment. Regulatory T cells were not shown to increase at the site of the skin. Therefore, it was determined that peptide immunotherapy in cat-allergic asthmatics recruited Th1 cells to the skin, but not regulatory T cells, inhibiting the late phase cutaneous reaction typically observed in allergic individuals (Alexander et al., 2005). In a second study evaluating late asthmatic reactions following treatment with peptide immunotherapy in cat allergic individuals, participants in the peptide group but not the placebo group had a significant reduction in the size of their late reaction to whole cat dander at follow-up with measures of IL-4 and IL-13 significantly decreasing and IL-10 significantly increasing (Oldfield et al., 2002). These findings support the role of regulatory T cells in clinical tolerance induced by peptide immunotherapy in cat allergic individuals (Oldfield et al., 2002). An additional study supporting the role of regulatory T cells in peptide immunotherapy in cat allergic asthmatic individuals found increases in IL-10 and decreases in IL-5 production in peripheral blood mononuclear cells (Verhoef et al., 2005). Isolated CD4+ T cells showed a reduction in proliferative activity after peptide immunotherapy supporting the hypothesis that peptide immunotherapy would suppress Th2 responses (Verhoef et al., 2005). Prototype peptide immunotherapy mRNA vaccines would be expected to induce a Th1 response, as demonstrated by IFN-γ ELISpot (Supplementary Figure 6), as well as regulatory T cells which could be measured via an IL-10 ELISpot or Luminex assay.

In historical studies where Fel d 1 peptides were given to mice sensitized to cat dander extract (CDE) and OVA, a protective effect against allergic inflammation following IN challenge with CDE was observed (Moldaver et al., 2019). The suppression of allergic responses to cat allergen challenge were shown to be caused by significant increases in numbers of regulatory T cells, Th1 cytokine-secreting cells, and B cells. The response to CDE was associated with a marked reduction in numbers of Th2 cytokine–secreting T cells (Moldaver et al., 2019). In a study looking at mRNA peptide immunotherapy vaccines targeting the liver with intravenous administration in peanut allergy, they were able to demonstrate an increase in IL-10-producing T regs in the spleen and a reduction in serum IgE in sensitized animals (Xu et al., 2023). In the Fel d 1 mRNA peptide immunotherapy vaccines, this same reduction of Th2 cytokine-secreting and increase in Th1-cytokine secreting cells was expected. A clinical level of tolerance was expected to be induced in mice sensitized to Fel d 1 and treated with VXL01 or VXL02 when compared to the controls.

Both the VXL01 and VXL02 mRNA peptide immunotherapy were ineffective at reducing allergic responses in Fel d 1 sensitized mice. The BAL cellularity and Fel d 1 specific serum IgG and IgE was comparable in the vaccinated animals to the control animals. Additionally, immunization with VXL02 in Fel d 1 sensitized mice resulted in a significant increase in the percentage of Fel d 1 tetramer positive B Cells, compared to mice sensitized and immunized with TBS vehicle alone in the 88-day timeline. This difference was not observed in the 81-day timeline between VXL01 and the empty nanoparticle control vaccine, VXL00. This may be due in part because empty nanoparticles have an immunogenic profile on their own (Lee et al., 2023). A study in BALB/c mice found that using empty nanoparticles as an adjuvant along side influenza hemagglutinin or SARS-CoV-2 Spike RBD proteins, had more immunogenic effects

compared to an MF57-like yeast adjuvant with the same viral protein. The empty LNP group showed higher neutralizing antibody and T-cell responses (Alameh et al., 2021). Prior to SARS-CoV-2 research, empty LNPs were shown to have good adjuvanticity when used with sub-unit vaccines containing hepatitis B virus protein antigens (Swaminathan et al., 2016). This immune response is prevalent in ionizable LNPs and occurs via the uptake by APCs of LNPs which induces both local and systemic inflammation. The empty LNP induced inflammation has been characterized by the release of pro-inflammatory cytokines such as IL-1β and IL-6 (Verbeke et al., 2022). Empty LNPs do not function the same way that mRNA-LNPs do. While both empty and mRNA-loaded LNPs have been shown to elicit the production of both chemokines and cytokines (Alameh et al., 2021; Fernandes et al., 2025), they have different characteristics such as size with empty LNPs typically being smaller than cargo loaded LNPs. LNPs size can dictate *in vivo* responses (Schober et al., 2024). Nanoparticle size has been shown to alter absorption and half-life during their uses in other pharmacological applications. Smaller particle size allowed the molecules to more efficiently reach target cells and induce an immune response (Hoshyar et al., 2016; Technov et al., 2021). While the timelines of the two efficacy studies are not directly comparable (81 vs 88 days), mice immunized with VXL00 had more Fel d 1 tetramer positive cells (average of 1.03234 x 10^5 cells/spleen) in the 81-day timeline compared to mice given vehicle alone (average of 0.66500 x 10^5 cells/spleen) in the 88-day timeline. This was not an artifact of the different timelines as VXL02 immunized mice had a larger number Fel d 1 tetramer positive cells (average of 1.86952 x 10^5 cells/spleen) in the 88-day timeline compared to VXL01 immunized mice (average of 0.59895 x 10^5 cells/spleen) in the 81-day timeline.

Since LNPs could be a potential factor for the inflammation seen in the 81-day model, TBS + Sucrose vehicle was chosen to be the control in the second efficacy model which was 88-

days. As mentioned previously there was still no difference between VXL02, or vehicle immunized mice in the context of BAL cellularity or Fel d 1 specific serum antibody levels at harvest. The increase in IgG following immunization with prototype peptide immunotherapy mRNA vaccines at the pre-outcome challenge timepoints in both the VXL01 and VXL02 immunized mice is likely due to the protective initial increase in Fel d 1 specific IgG_1, which acts as a blocking antibody. IgG_1 out competes IgE for allergen and therefore has been shown to reduce allergic reactions (Bohle, 2024). In allergen-specific immunotherapy a protective increase in allergen-specific IgG_1 followed by an increase in IgG_4 and delayed decrease in allergen-specific IgE antibodies for success to be considered (Bohle, 2024; Cady et al., 2009). Based on the high levels of serum IgE in both the VXL01 and VXL02 treated groups, the hypothesis that vaccination with an mRNA vaccine encoding T cell epitopes of Fel d 1 in the context of a Th1-inducing lipid nanoparticle would modulate an existing allergen-specific Th2 response and reduce outcomes associated with allergic airways disease in mice, failed to be demonstrated.

Changes were detected in the percentages of Fel d 1 tetramer positive cells. Even though, the percentage of total tetramer positive cells was significantly higher in the VXL02 treated group compared to the control, the percentage of class switched GC B cells was significantly lower. Following repeated allergen exposure, it is expected to see GC expansion and class switching. Since there was a significantly higher class switched percentage in the GC cells of the sensitized and TBS treated mice, as well as higher Fel d 1 specific serum antibody levels after sensitization compared to the later timepoint samples, the Fel d 1 response observed was likely driven by the sensitization with Fel d 1 and not significantly altered by the prototype vaccines. During first time exposure of naïve IgD/IgM double positive B cells to their cognate antigen, the BCR is used to capture the antigen and leads to activation (T cell dependent or independent) and

differentiation, which includes an expansion of GC cells (Koenig et al., 2021; Phelps et al., 2024). The class/isotype of B cells is determined by the heavy chain and is important for determining the role of the subsequently produced antibodies due to their different affinities for different receptor proteins (Stavnezer et al., 2008). Class switching occurs via an intrachromosomal deletional recombination event in the heavy chain locus. The change in switch regions of the heavy chain led to a change from IgM and IgD naïve B cells to the expression of a single downstream isotype, i.e, IgG, IgA or IgE (Stavnezer et al., 2008). Future experiments should look to test VXL01 and VXL02 vaccinations on mice with a lower degree of sensitization. The highly reproducible IP with alum sensitization elicits a large allergic response (Eisenbarth, 2008). Perhaps lowering the allergic sensitization with a more translational model such as EQ sensitization would allow for an inducible Th1 response after vaccination, as seen in studies with physical peptides (Moldaver et al., 2019).

Detection of allergen specific B cells in pre-clinical vaccine studies have demonstrated future uses of allergen specific B cell tetramers. The same tetramers can be utilized on human samples (Phelps et al., 2024), meaning clinical studies assessing therapeutics for cat allergies in human participants can be assessed with this novel method. The presence of Fel d 1 tetramer positive cells, as well as the phenotype of these detected cells, could be assessed before and after treatment is given to assist in determining the efficacy of the studied treatment. Allergen specific B cell tetramers may also be useful in diagnosis of allergic disease in clinic, as tetramers have been effective in developing diagnostic tools (Boonyaratanakornkit and Taylor, 2019).

Conclusion

Fel d 1 B cell tetramers produced in these studies were sensitive and allergen-specific, allowing for their use in characterization of B cell responses in murine models with prototype

peptide immunotherapy mRNA vaccines. The prototype vaccinations did not appear to alter B cell responses in an impactful way for clinical use and in animals systemically sensitized to Fel d 1 using an IP and alum model, vaccination was unable to ameliorate allergic airway inflammation. Prototype peptide immunotherapy vaccine VXL02 may be capable of activating an established memory B cell pool, but not capable of activating a naïve B cell pool.

Figures

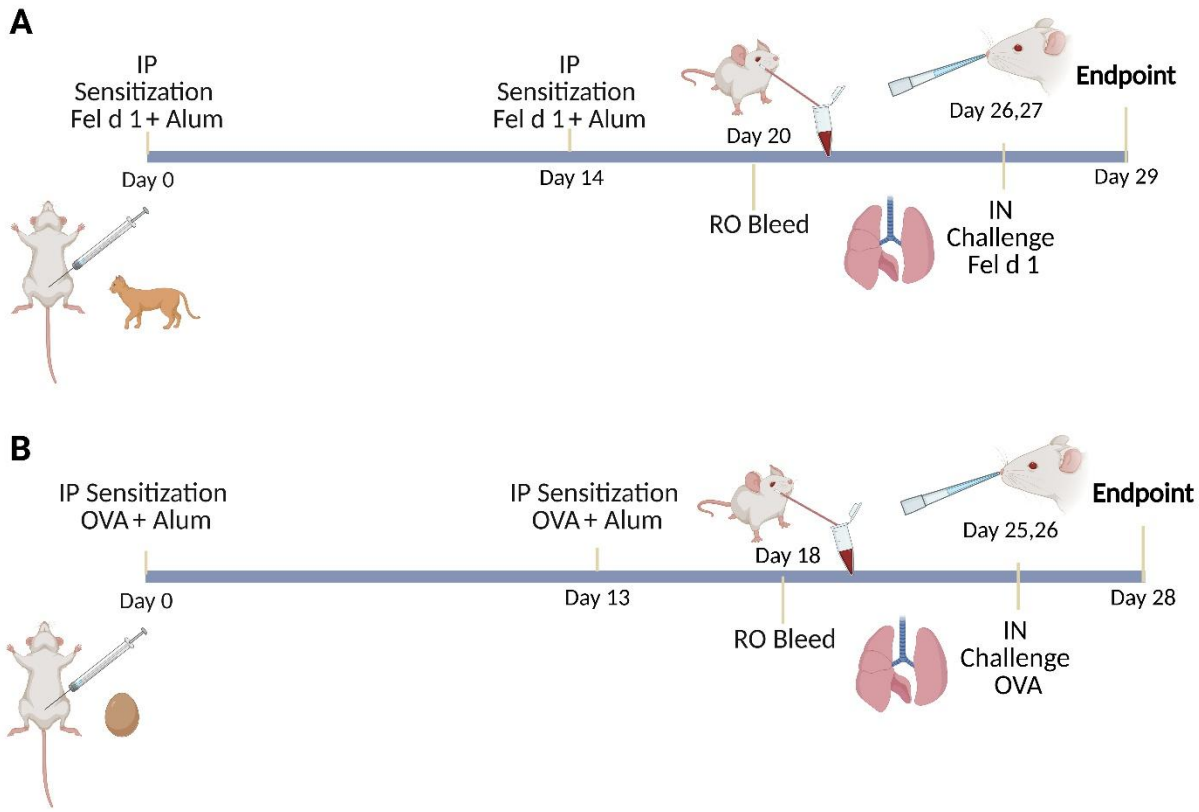


Figure 1: Mouse Model of A) Fel d 1 or B) OVA Allergen Sensitization and Challenge for Optimization Experiments Timelines.

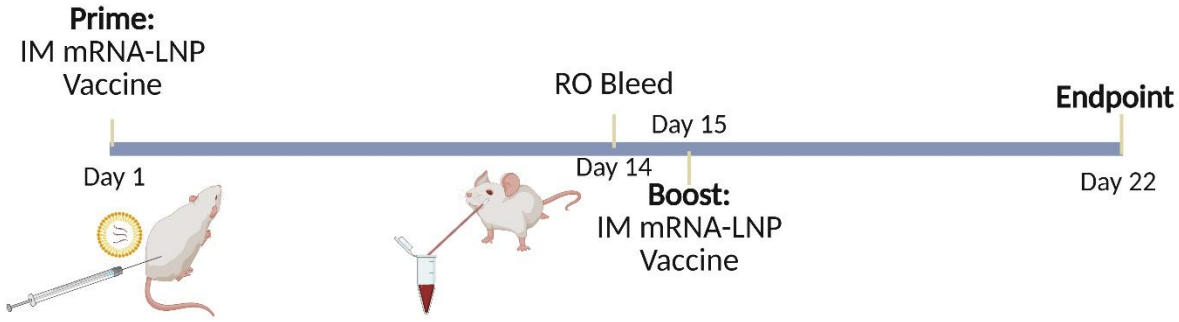


Figure 2: Experimental Timeline used to evaluate mRNA Fel d 1 Peptide Immunotherapy Vaccination in non-sensitized mice.

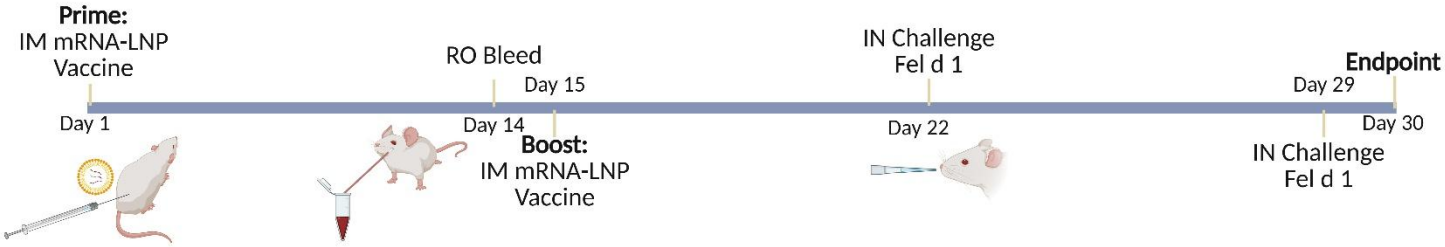


Figure 3: Experimental Timeline used to evaluate mRNA Fel d 1 Peptide Immunotherapy Vaccination with whole allergen exposure in non-sensitized mice.

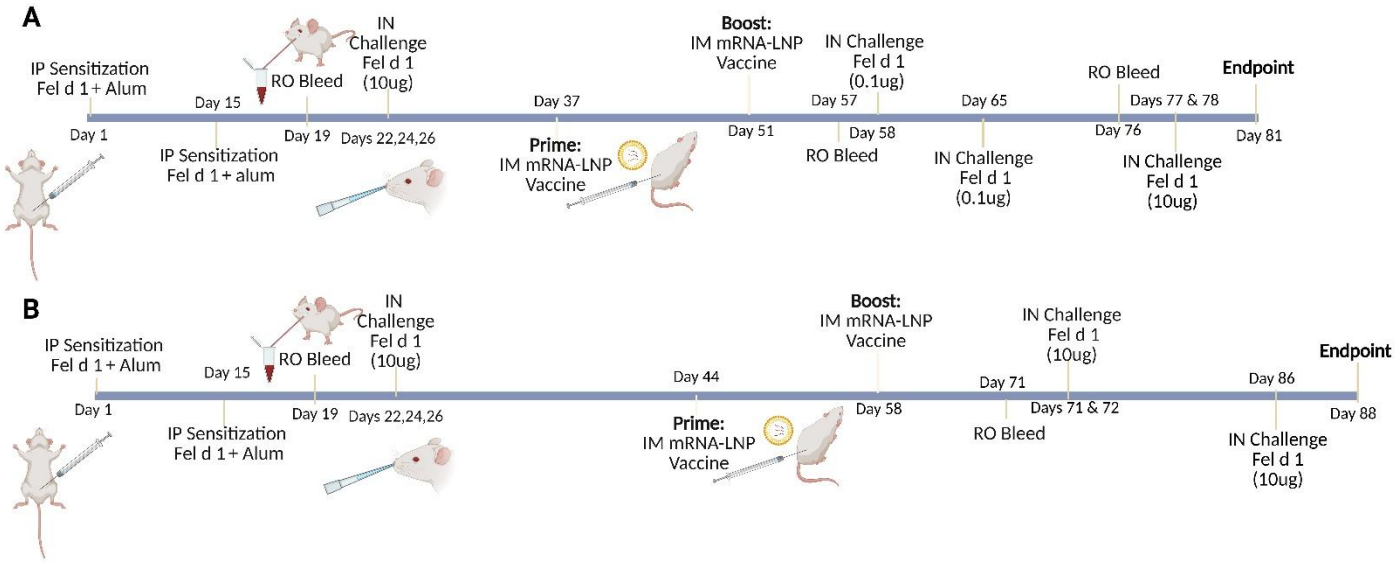


Figure 4: Experimental Timelines used to evaluate mRNA Fel d 1 Peptide Immunotherapy Vaccination in sensitized mice (IP + alum). A) 81-day timeline used in the first efficacy study. *B)* 88-day timeline used in the second efficacy study.

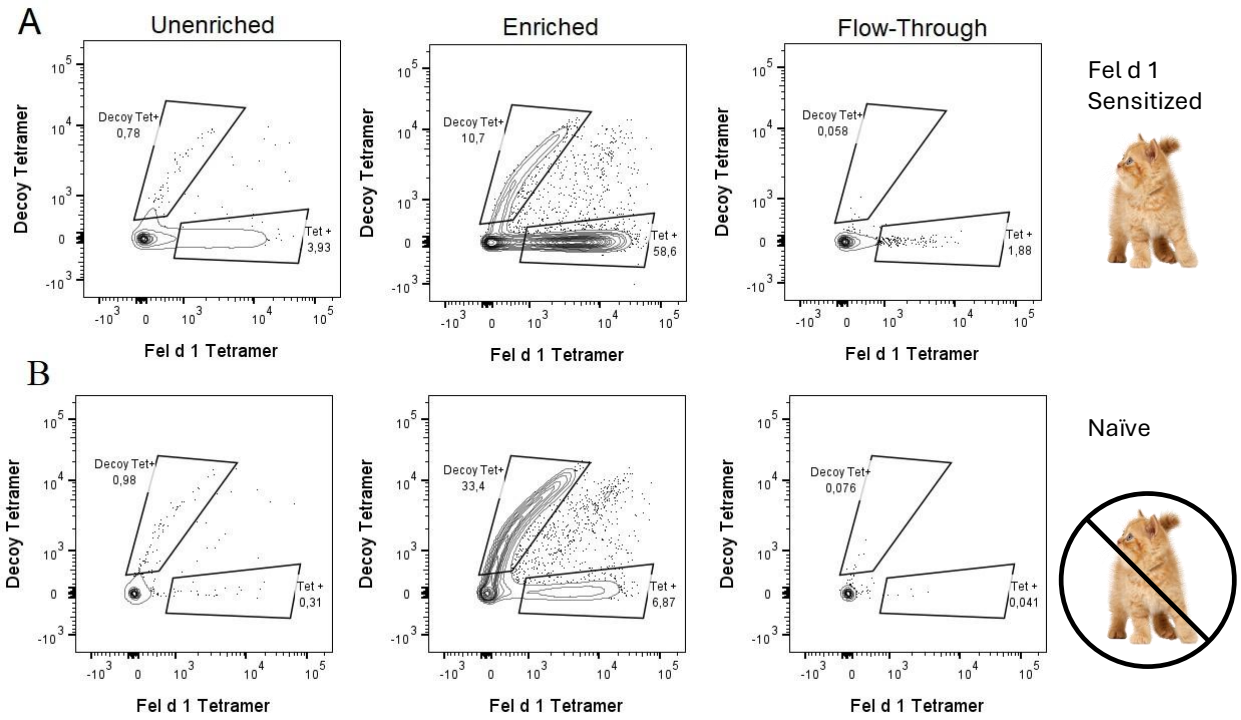


Figure 5: Fel d 1 Tetramer vs Decoy (OVA) Tetramer Positive B Cells in Unenriched, PE-enriched and Flow-through Samples. A) Two Verigem (IgE reporter) mice were sensitized with 200 µg Fel d 1 IP with alum intraperitoneally and euthanized for spleen and lymph node harvest at day 7. Cells were pooled and stained and split into two groups following the staining protocol for enriched or unenriched cells. Flow-through from the enrichment protocol was also stained. B) Splenocytes and lymph nodes were also collected from two naïve mice and stained in the same manner as the isolated cells from the sensitized animals.

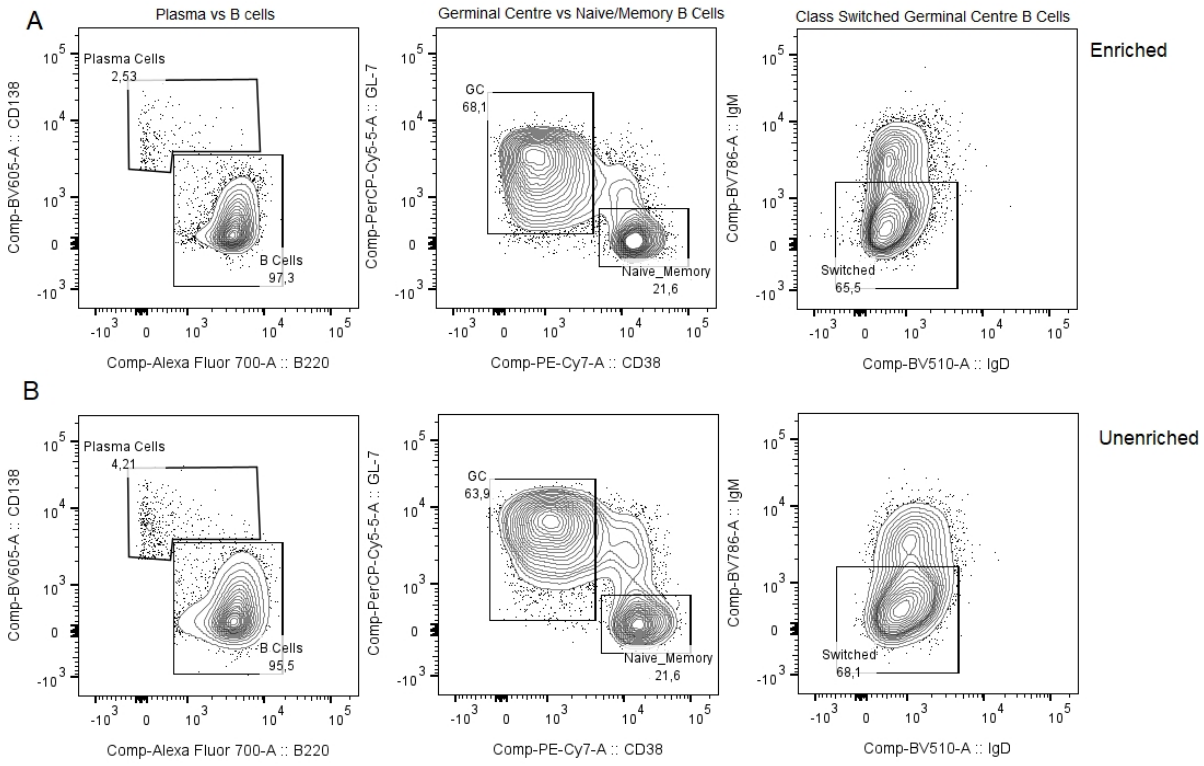


Figure 6: Fel d 1 Tetramer Positive B Cell Frequencies in A) Enriched vs B) Unenriched Samples. Two Verigem (IgE reporter) mice were sensitized with 200 µg Fel d 1 IP with alum intraperitoneally and euthanized for spleen and lymph node harvest at day 7. Cells were pooled and stained and split into two groups following the staining protocol for enriched or unenriched cells. Frequency of Fel d 1 specific plasma cells, B cells, germinal centre B cells, combined naïve and memory B cells as well as class switched germinal centre B cells were observed.

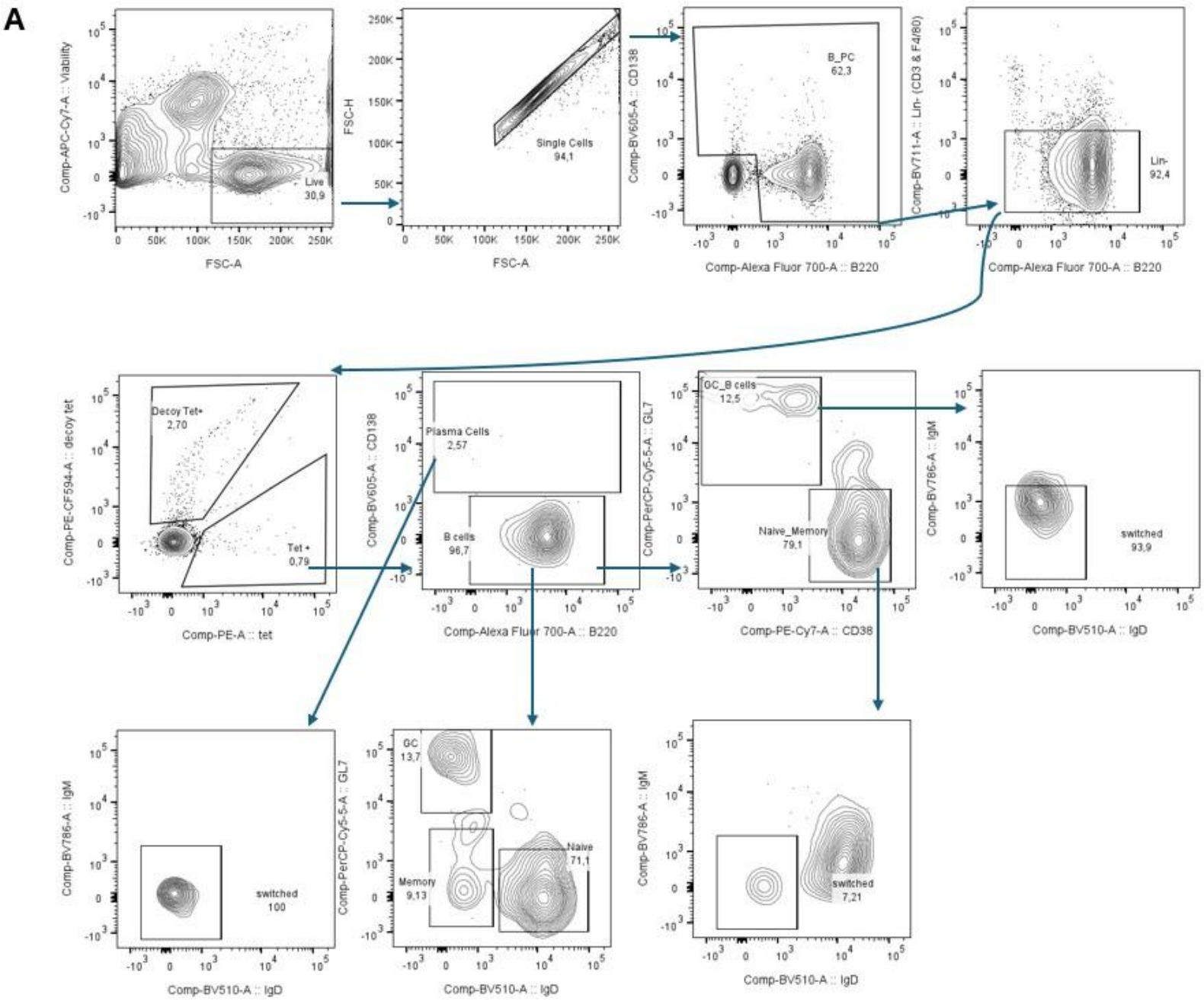


Figure 7: Sample Gating Strategy used in the B cell tetramer Flow Cytometry Panel. A) Splenocytes from mice sensitized to Fel d 1 stained with Fel d 1 Tetramer. B) Splenocytes from mice sensitized to OVA and stained with Fel d 1 Tetramer. C) Splenocytes from mice sensitized to Fel d 1 stained with OVA Tetramer.

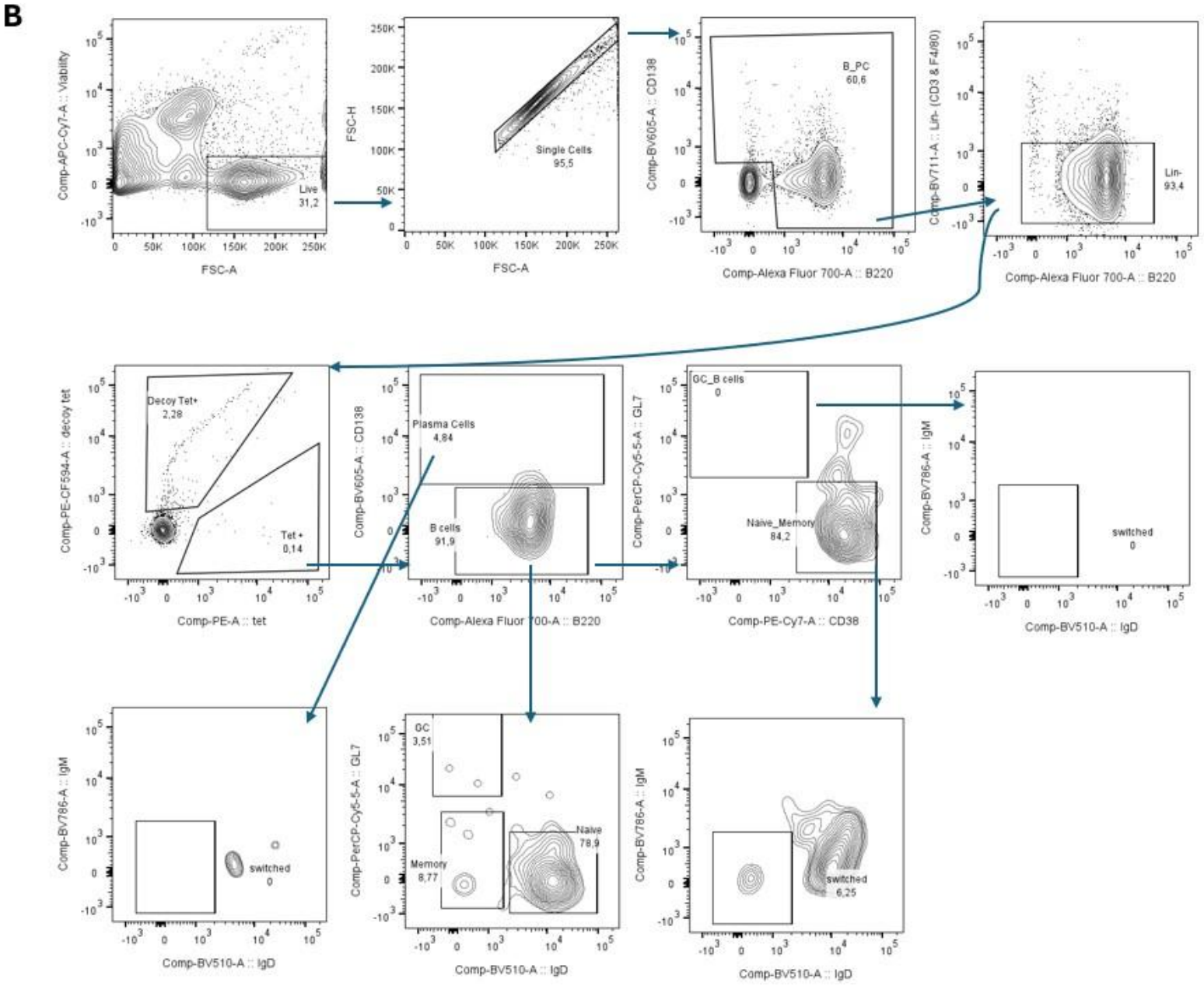


Figure 7: Sample Gating Strategy used in the B cell tetramer Flow Cytometry Panel. A) Splenocytes from mice sensitized to Fel d 1 stained with Fel d 1 Tetramer. B) Splenocytes from mice sensitized to OVA and stained with Fel d 1 Tetramer. C) Splenocytes from mice sensitized to Fel d 1 stained with OVA Tetramer.

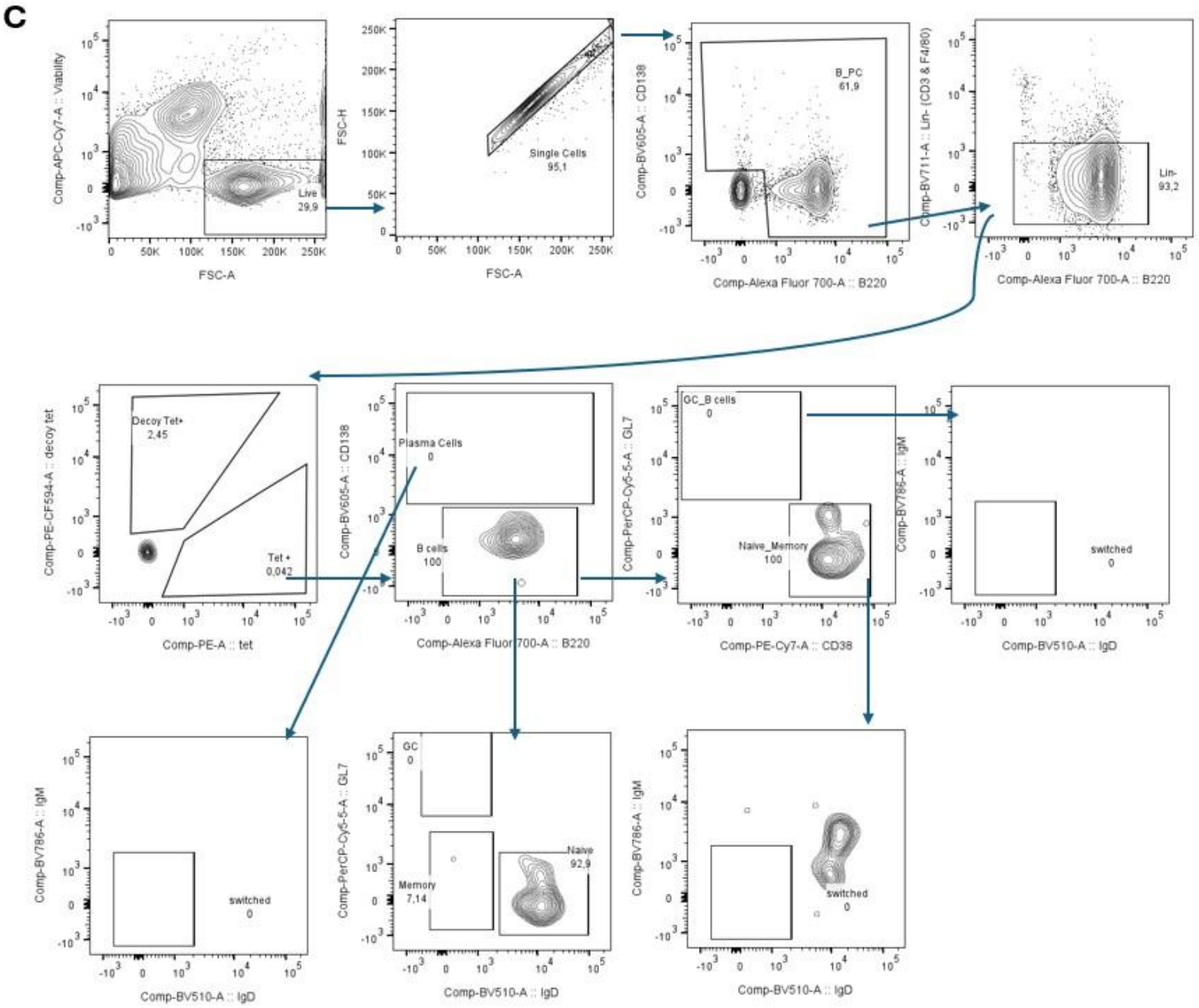


Figure 7: Sample Gating Strategy used in the B cell tetramer Flow Cytometry Panel. A) Splenocytes from mice sensitized to Fel d 1 stained with Fel d 1 Tetramer. B) Splenocytes from mice sensitized to OVA and stained with Fel d 1 Tetramer. C) Splenocytes from mice sensitized to Fel d 1 stained with OVA Tetramer.

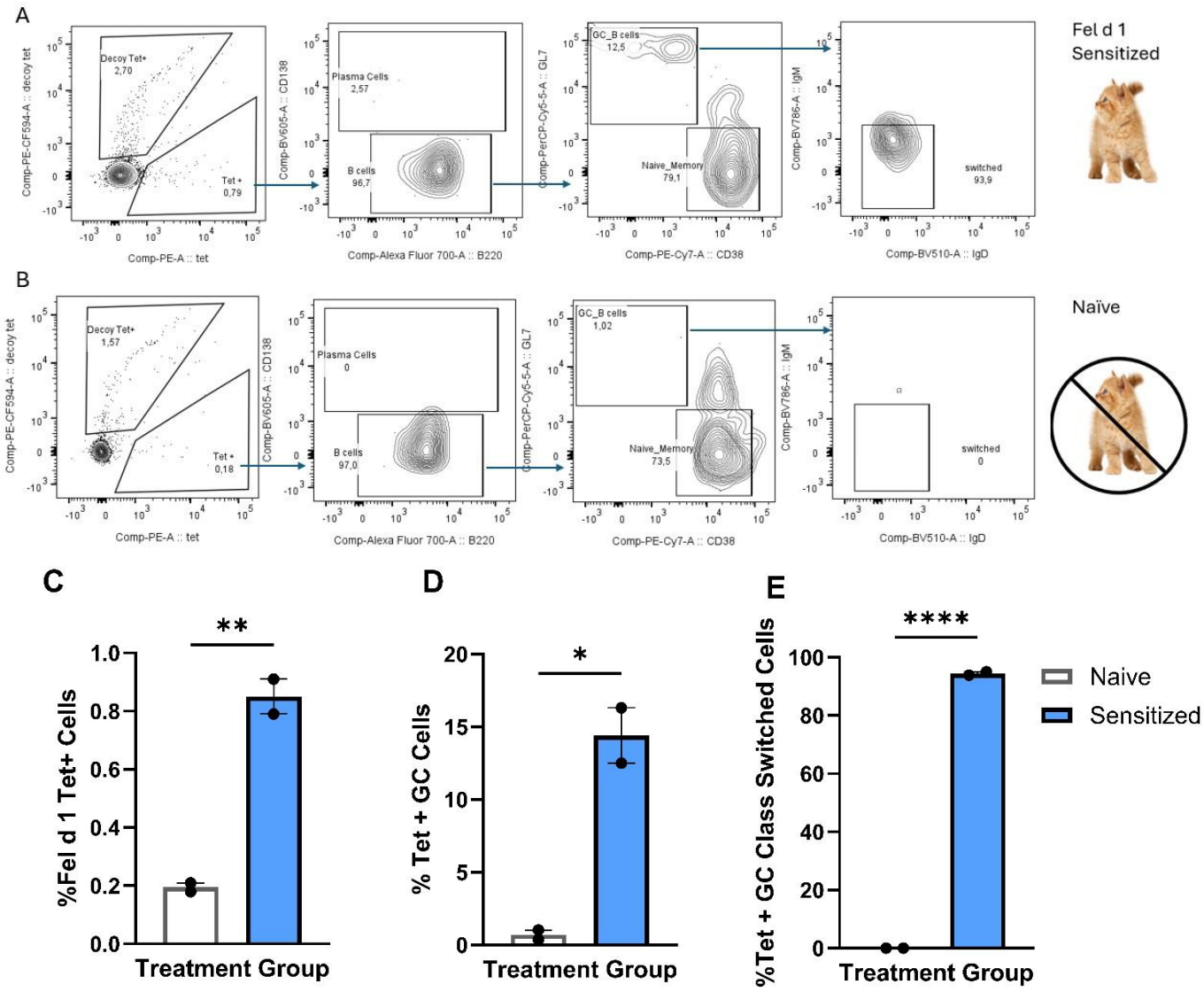


Figure 8: Unenriched B Cell Tetramer Staining in Naïve vs Sensitized Mice. Splenocytes from BALB/c mice sensitized as described in Figure 1A were pooled and tetramer flow staining was completed on 1 million cells. A) Fel d 1 Sensitized sample gating strategy. B) Naïve Sample gating strategy. C) % of Fel d 1 tetramer positive cells. D) % of Fel d 1 tetramer positive B cells that are germinal centre (GC) B cells. E) % of Fel d 1 tetramer positive class switched germinal centre B cells. Results were analyzed using an un-paired T-test (* $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$; n=2 per group). Error bars represent SEM.

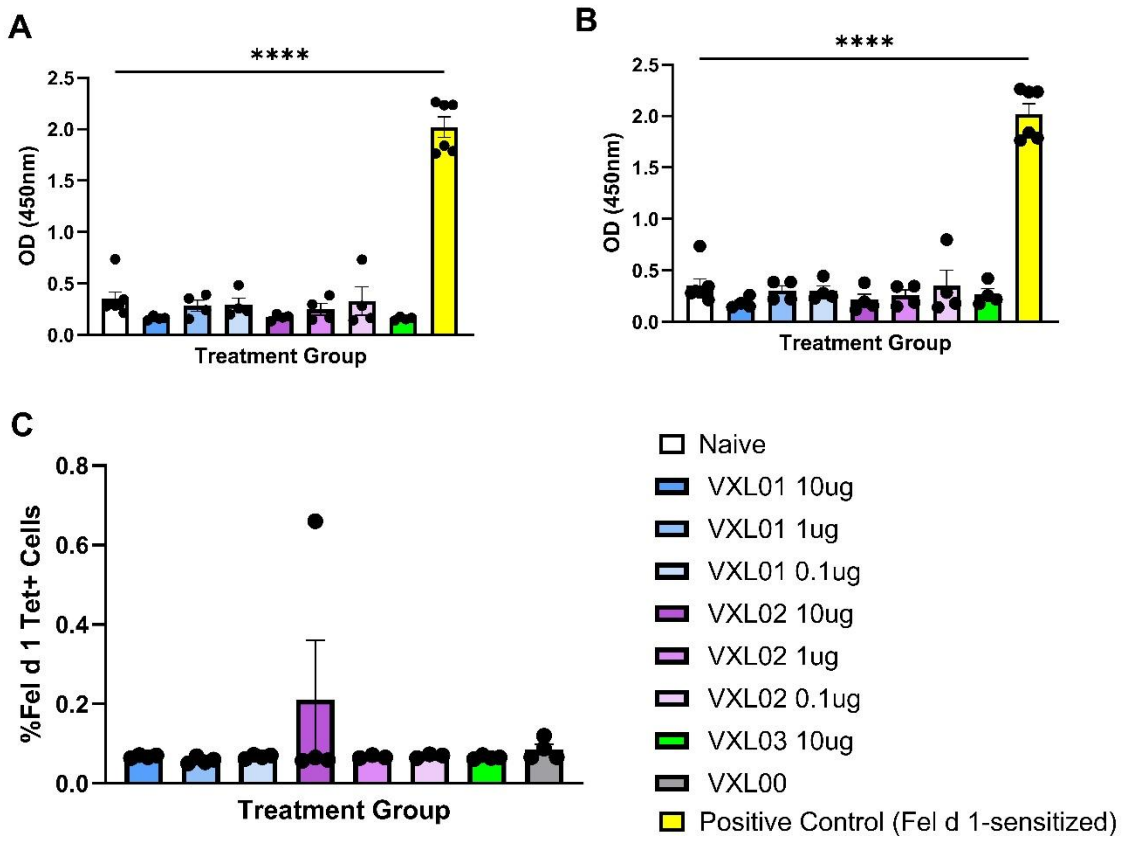


Figure 9: Fel d 1-Specific Serum Antibody Levels Across mRNA Peptide Immunotherapy Immunized Groups (1:100 Serum dilution). Mice were immunized IM with varying doses of different Fel d 1 mRNA peptide immunotherapy on days 1 and 15. Serum was collected via a RO bleed on day 14. Mice were euthanized on day 22, serum was collected via cardiac bleed and splenocytes were harvested. An ELISA was run to measure the Fel d 1 -specific serum IgG levels in serum collected on day A) 14 or B) 22. C) B cell tetramer staining was performed on the collected splenocytes and % of Fel d 1 tetramer+ cells were observed. Results were analyzed using a one -way ANOVA with Bonferroni's multiple comparison test. (****$P \leq 0.0001$; n=4 per group). Error bars represent SEM.

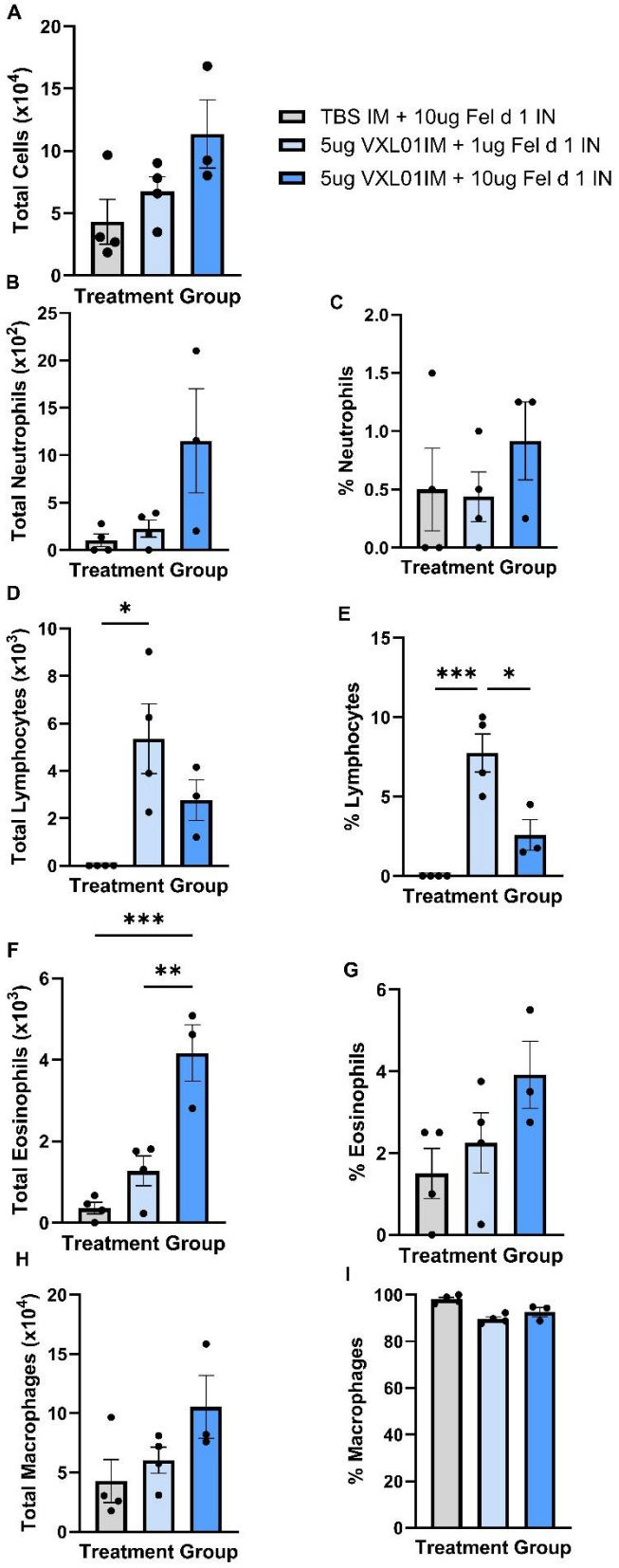


Figure 10: Cell Differential of Bronchoalveolar Lavage Fluid in Mice Immunized with mRNA Peptide Immunotherapy and Subsequently Challenged with Fel d 1. Mice were immunized IM with varying doses of different Fel d 1 mRNA peptide immunotherapy or TBS control on days 1 and 15. Following immunization mice were intranasally challenged with 1 or 10 µg of Fel d 1 on days 22 and 29. BAL fluid was collected, and a Wright-Giemsa stain was completed before manual cell differential counts were performed to analyze A) total cells, B) total neutrophils, C) percentage neutrophils, D) total lymphocytes, E) percentage lymphocytes , F) total eosinophils, G) percentage eosinophils, H) total macrophages, and I) percentage macrophages. Results were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test. (*p≤0.05, **P≤0.01, ***p≤0.001; n=3-4 per group). Error bars represent SEM.

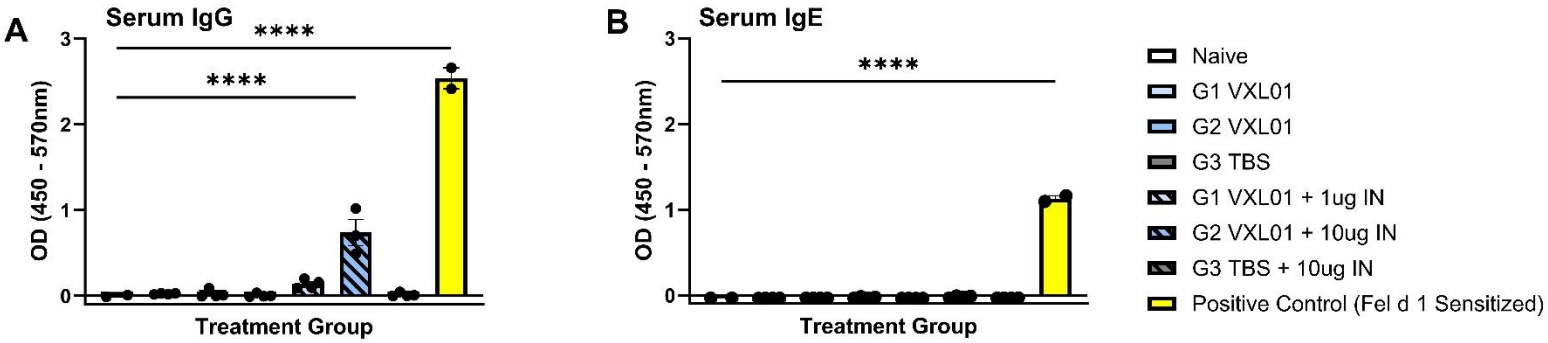


Figure 11: Fel d 1-Specific Serum Antibody Levels Across mRNA Peptide Immunotherapy Immunized and Allergen Challenged Groups (1:100 Serum dilution). Mice were immunized IM with 5 µg VXL01 Fel d 1 mRNA peptide immunotherapy (G1 & G2) or TBS control (G3) on days 1 and 15. Following immunization mice were intranasally challenged with 1 (G1) or 10 µg (G2 & G3) of Fel d 1 on days 22 and 29. Mice were euthanized on day 30 and serum was collected via cardiac bleed. An ELISA was run to measure the Fel d 1 -specific serum A) IgG or B) IgE. Results were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test. (****P ≤0.0001; n=3-4 per group). Error bars represent SEM.

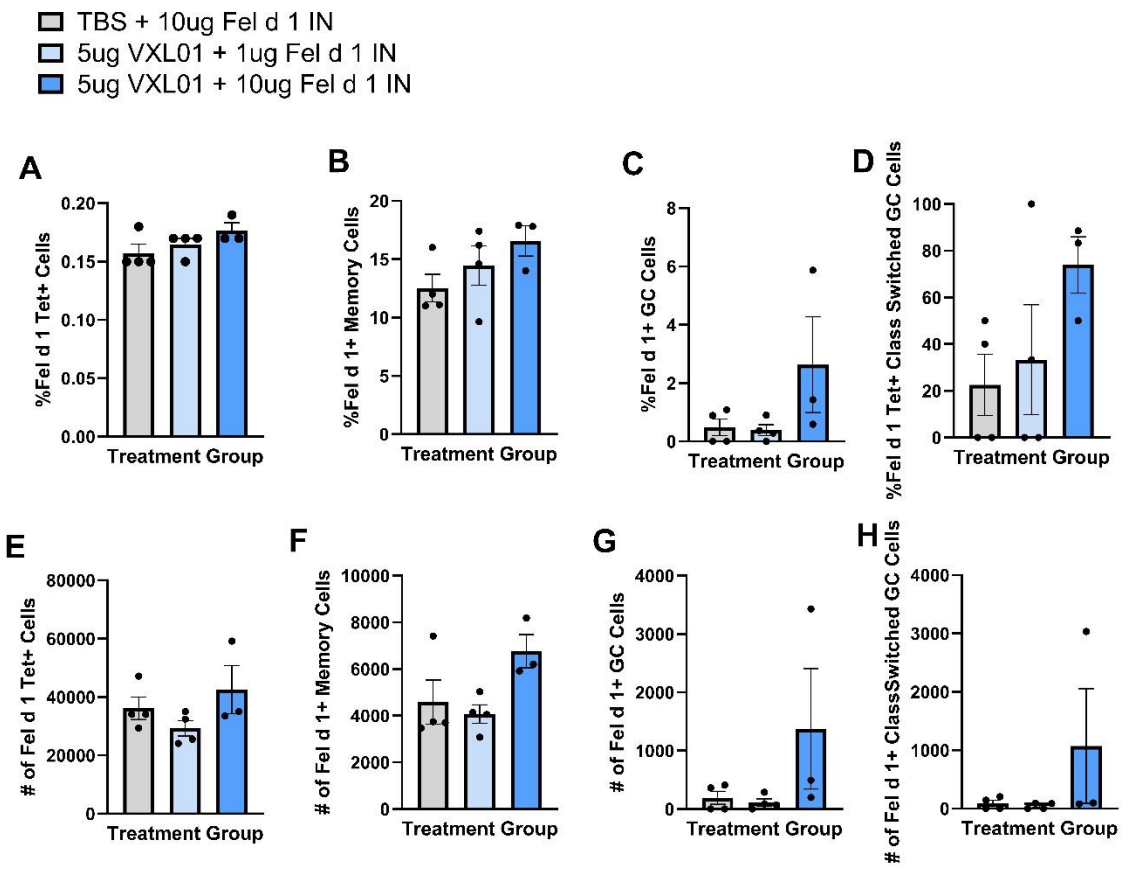


Figure 12: Fel d 1+ B Cells Across mRNA Peptide Immunotherapy Immunized and Allergen Challenged Groups. Mice were immunized IM with VXL01 Fel d 1 mRNA peptide immunotherapy or TBS control on days 1 and 15. Following immunization mice were intranasally challenged with 1 or 10 µg of Fel d 1 on days 22 and 29. Mice were euthanized on day 30 and splenocytes were harvested and B cell tetramer staining was performed. A) % of Fel d 1 tetramer positive cells, B) % of Fel d 1 tetramer B cells that were memory B cells, C) % of Fel d 1 tetramer positive B cells that were germinal centre (GC) B cells D) % of Fel d 1 tetramer positive class switched germinal centre B cells, E) number of Fel d 1 tetramer positive cells, F) number of Fel d 1 tetramer B cells that were memory B cells, G) number of tetramer positive B cells that were germinal centre (GC) B cells and H) number of Fel d 1 tetramer positive class switched germinal centre B cells were observed. Results were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test. No statistically significant differences were observed between groups. n=3-4 per group. Error bars represent SEM.

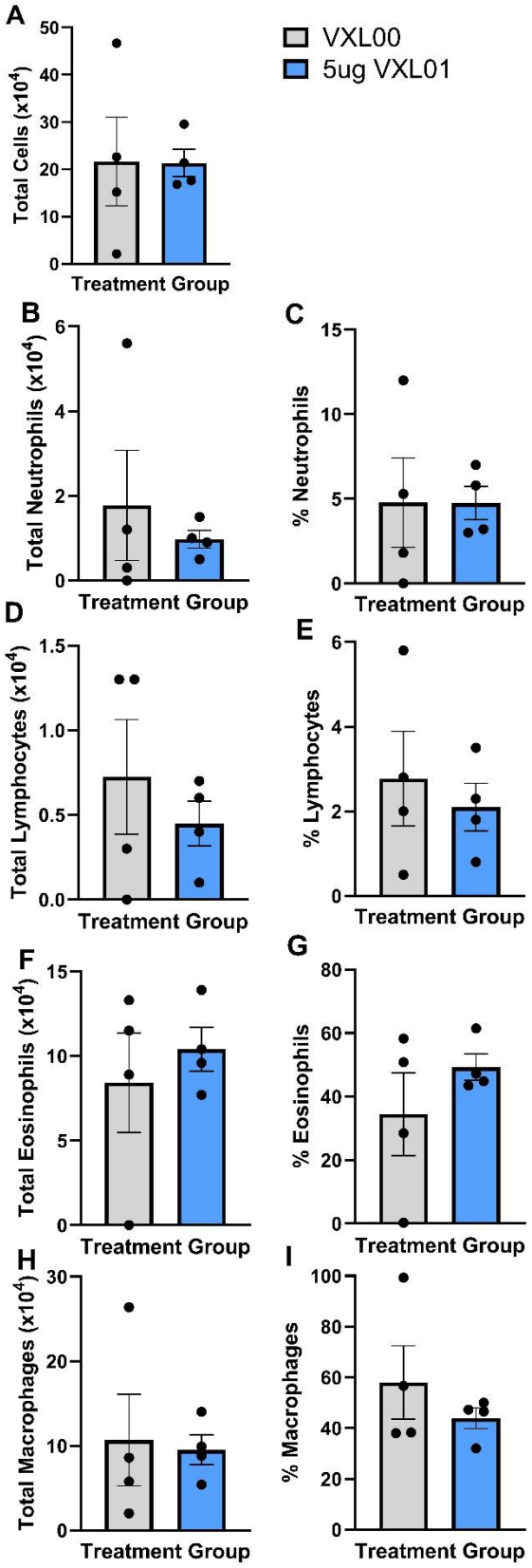


Figure 13: Cell Differential of Bronchoalveolar Lavage Fluid in the mRNA Peptide Immunotherapy 81-day Efficacy Model Assessing VXL01 vs VXL00 (negative control vaccine). Mice were sensitized and challenged with Fel d 1 prior to immunization with an mRNA peptide immunotherapy vaccine or control vaccine. Prior to euthanasia mice were given additional IN challenges of Fel d 1. BAL fluid was collected, and a Wright-Giemsa stain was completed before manual cell differential counts were performed to analyze A) total cells, B) total neutrophils, C) percentage neutrophils, D) total lymphocytes, E) percentage lymphocytes, F) total eosinophils, G) percentage eosinophils, H) total macrophages, and I) percentage macrophages. Results were analyzed using an unpaired T-test. No statistically significant differences were observed between groups was observed. n=4 per group. Error bars represent SEM.

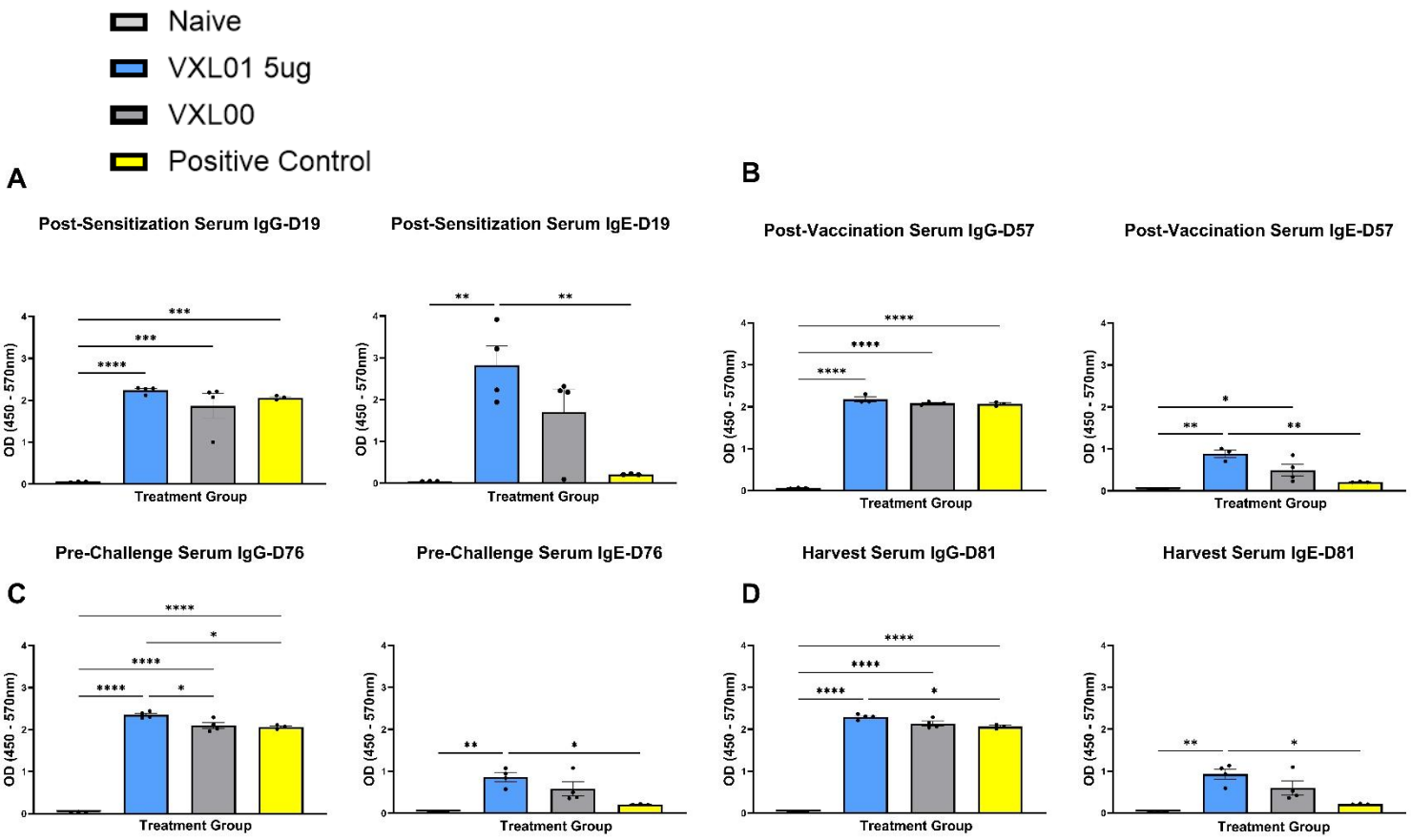


Figure 14: Fel d 1-Specific Serum Antibody Levels Across mRNA Peptide Immunotherapy 81-day Efficacy Study Assessing VXL01 vs VXL00 (negative control vaccine) (1:100 Serum dilution). Mice were sensitized and challenged with Fel d 1 prior to immunization with an mRNA peptide immunotherapy vaccine or control. Prior to euthanasia mice were given additional IN challenges of Fel d 1. Blood was collected via an RO or cardiac bleed (harvest only) and serum was analyzed for IgG and IgE levels A) post-sensitization, B) Post-vaccination, C) pre-harvest challenge and D) at harvest (euthanasia). Results were analyzed using a one -way ANOVA with Bonferroni's multiple comparison test. (*p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001; n=4 per group). Error bars represent SEM.

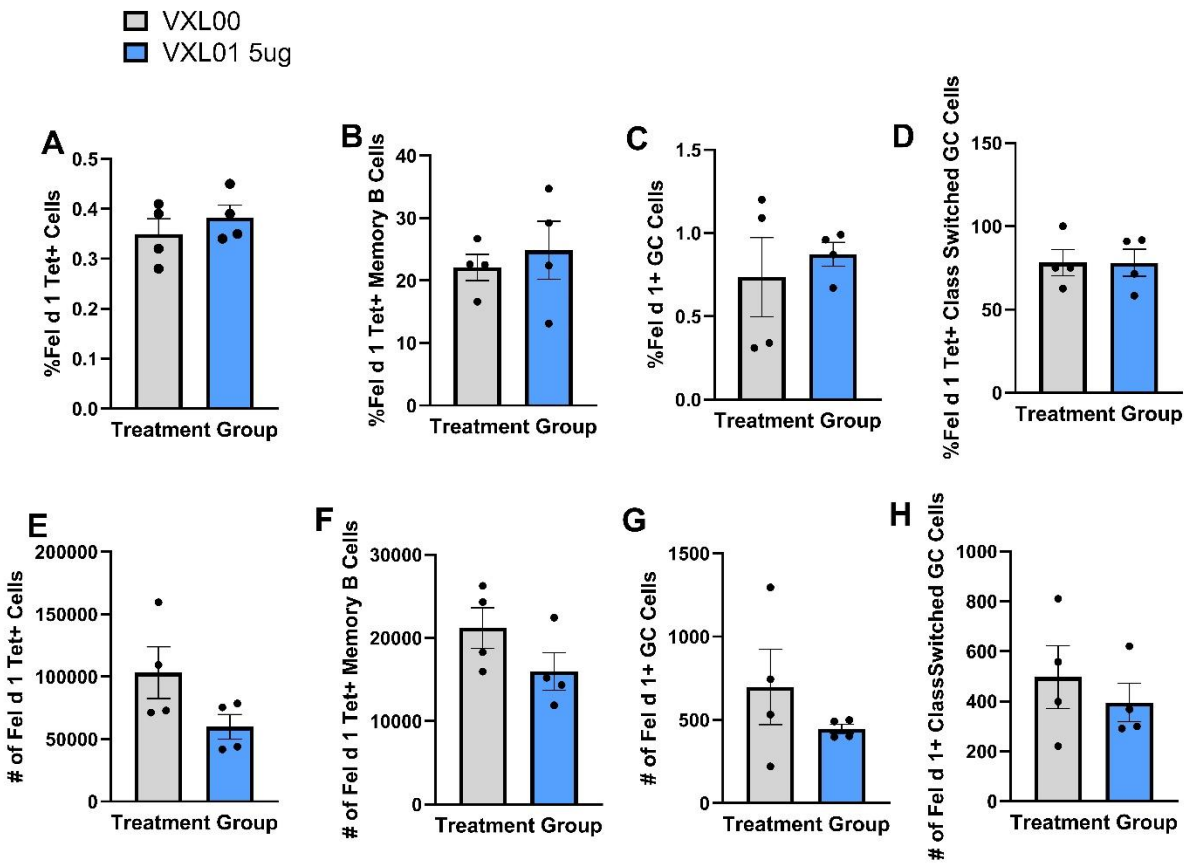


Figure 15: Fel d 1+ B Cells Across mRNA Peptide Immunotherapy 81-day Efficacy Study Assessing VXL01 vs VXL00 (negative control vaccine). Mice were sensitized and challenged with Fel d 1 prior to immunization with an mRNA peptide immunotherapy vaccine or control. Prior to euthanasia mice were given additional IN challenges of Fel d 1. Splenocytes were harvested and B cell tetramer staining was performed and A) % of Fel d 1 tetramer positive cells, B) % of Fel d 1 tetramer B cells that were memory B cells, C) % of Fel d 1 tetramer positive B cells that were germinal centre (GC) B cells, D) % of Fel d 1 tetramer positive class switched germinal centre B cells, E) number of Fel d 1 tetramer positive cells, F) number of Fel d 1 tetramer B cells that were memory B cells, G) number of tetramer positive B cells that were germinal centre (GC) B cells and H) number of Fel d 1 tetramer positive class switched germinal centre B cells were observed. Results were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test. No statistically significant differences were observed between groups. n=4 per group. Error bars represent SEM.

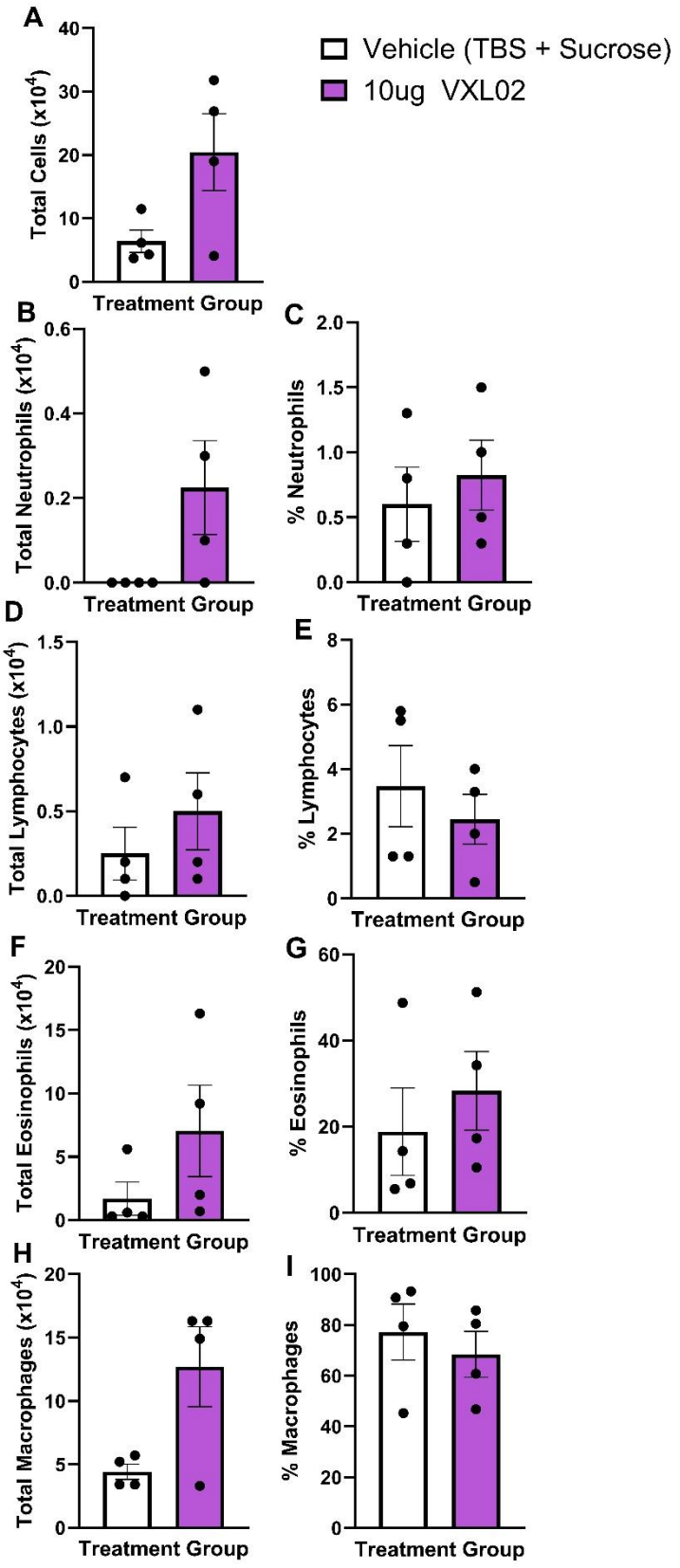


Figure 16: Cell Differential of Bronchoalveolar Lavage Fluid in the mRNA Peptide Immunotherapy 88-day Efficacy Model Assessing Vehicle vs VXL02. Mice were sensitized and challenged with Fel d 1 prior to immunization with VXL02 mRNA peptide immunotherapy vaccine or vehicle alone. Prior to euthanasia mice were given additional IN challenges of Fel d 1. BAL fluid was collected, and a Wright- Giemsa stain was completed before manual cell differential counts were performed to analyze A) total cells, B) total neutrophils, C) percentage neutrophils, D) total lymphocytes, E) percentage lymphocytes, F) total eosinophils, G) percentage eosinophils, H) total macrophages, and I) percentage macrophages. Results were analyzed using an unpaired T-test. No statistically significant differences were observed between groups. n=4 per group. Error bars represent SEM.

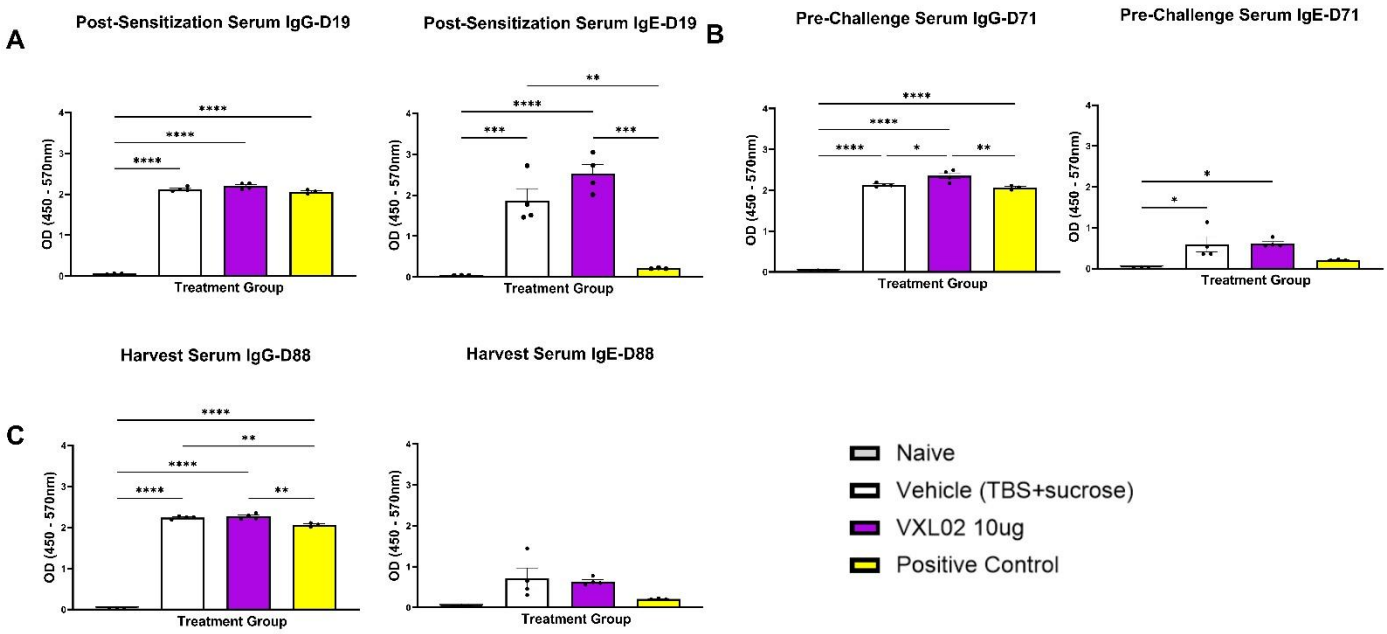


Figure 17: Fel d 1-Specific Serum Antibody Levels Across mRNA Peptide Immunotherapy 88-day Efficacy Study Assessing Vehicle vs VXL02 (1:100 Serum dilution). Mice were sensitized and challenged with Fel d 1 prior to immunization with an mRNA peptide immunotherapy vaccine or vehicle. Prior to euthanasia mice were given additional IN challenges of Fel d 1. Blood was collected via an RO or cardiac bleed (harvest only) and serum was analyzed for IgG and IgE levels A) post-sensitization, C) pre-harvest challenge and D) at harvest (euthanasia). Results were analyzed using a one -way ANOVA with Bonferroni's multiple comparison test (*p≤0.05, **P≤0.01, ***p≤0.001; ****p≤0.0001; n=4 per group). Error bars represent SEM.

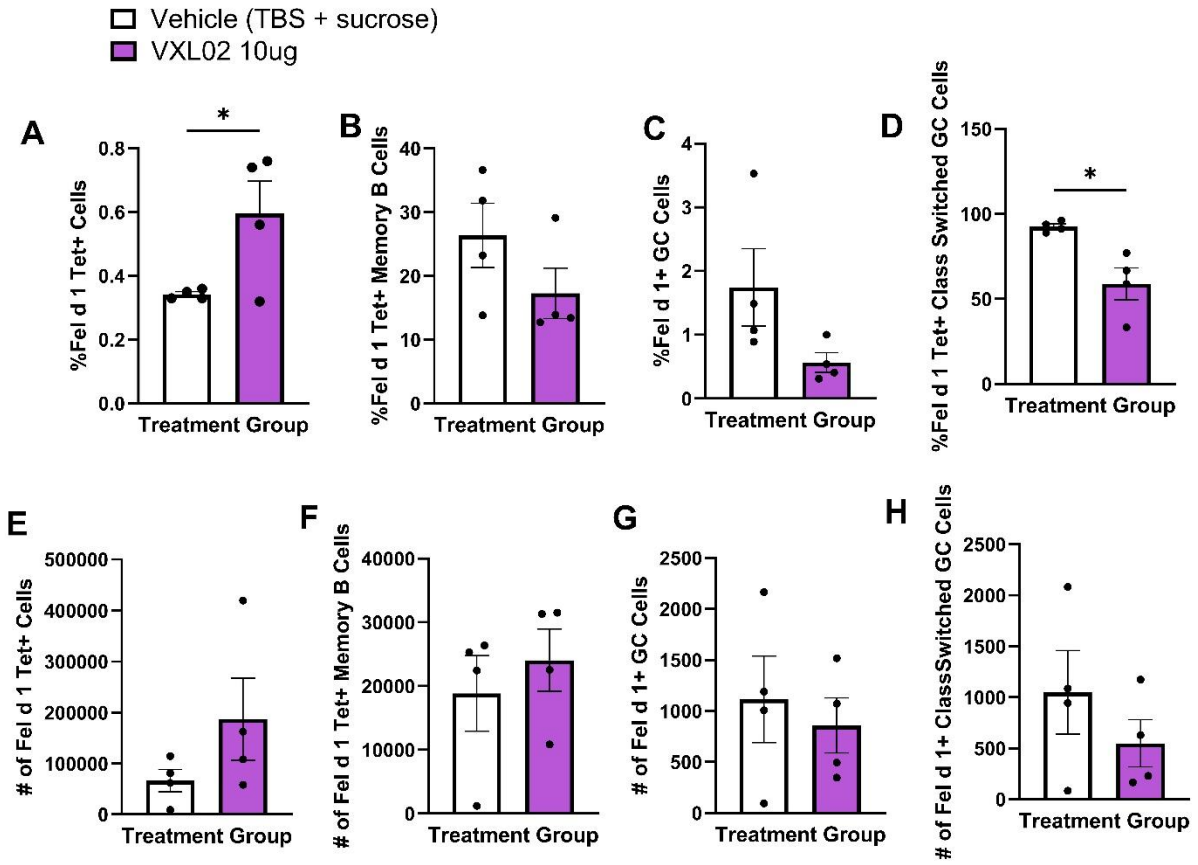
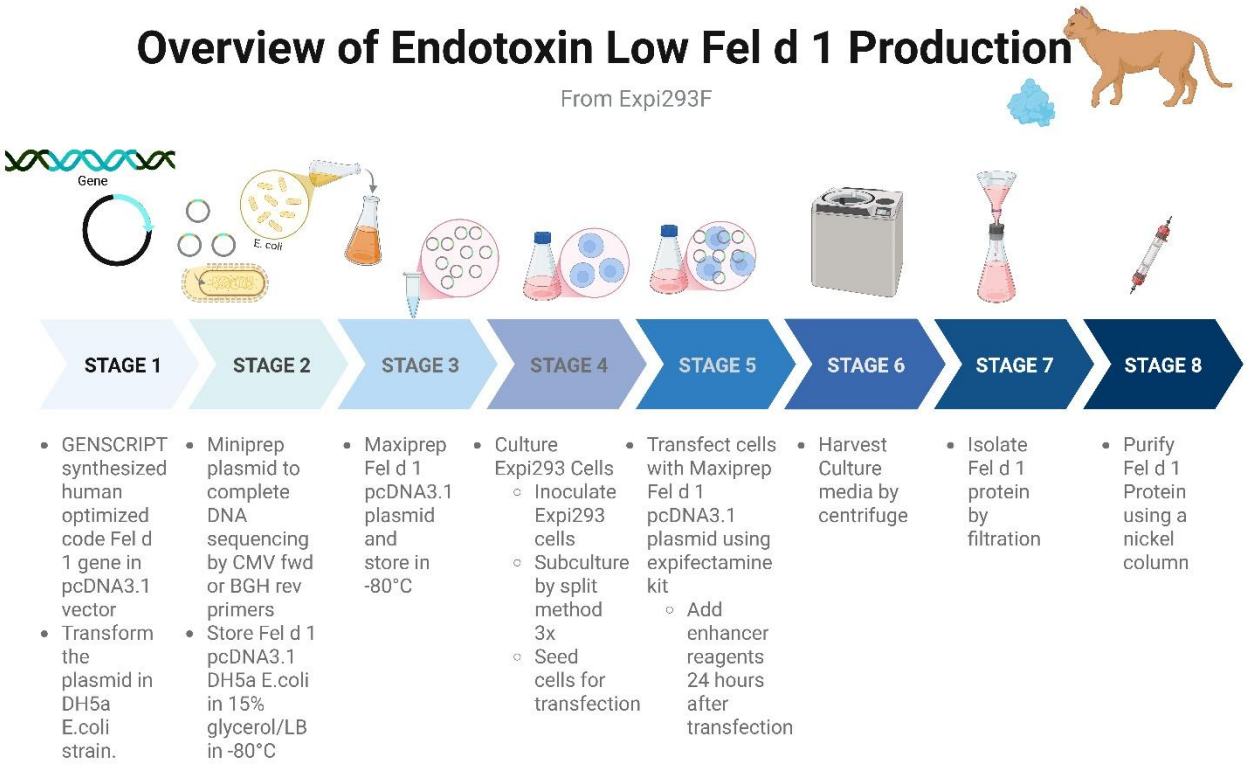
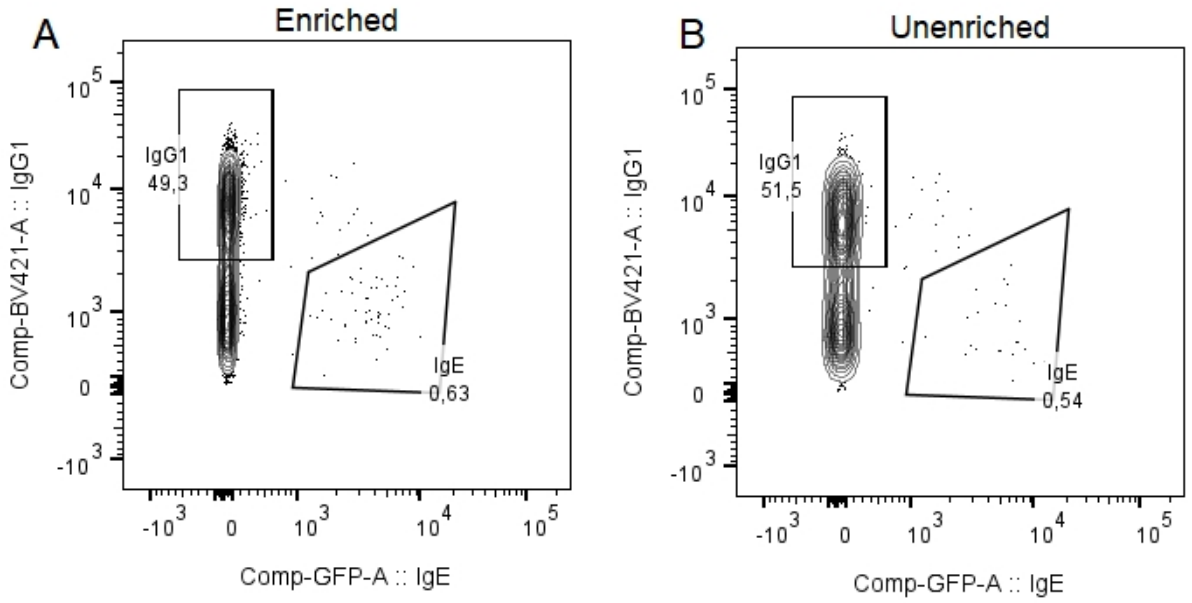


Figure 18: Fel d 1+ B Cells Across mRNA Peptide Immunotherapy 88-day Efficacy Study Assessing Vehicle vs VXL02. Mice were sensitized and challenged with Fel d 1 prior to immunization with an mRNA peptide immunotherapy vaccine or vehicle. Prior to euthanasia mice were given additional IN challenges of Fel d 1. Splenocytes were harvested and B cell tetramer staining was performed and A) % of Fel d 1 tetramer positive cells, B) % of Fel d 1 tetramer B cells that were memory B cells, C) % of Fel d 1 tetramer positive B cells that were germinal centre (GC) B cells, D) % of Fel d 1 tetramer positive class switched germinal centre B cells, E) number of Fel d 1 tetramer+ cells, F) number of Fel d 1 tetramer B cells that were memory B cells, G) number of tetramer positive B cells that were germinal centre (GC) B cells and H) number of Fel d 1 tetramer positive class switched germinal centre B cells were observed. Results were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test (*$p \leq 0.05$; n=4 per group). Error bars represent SEM.

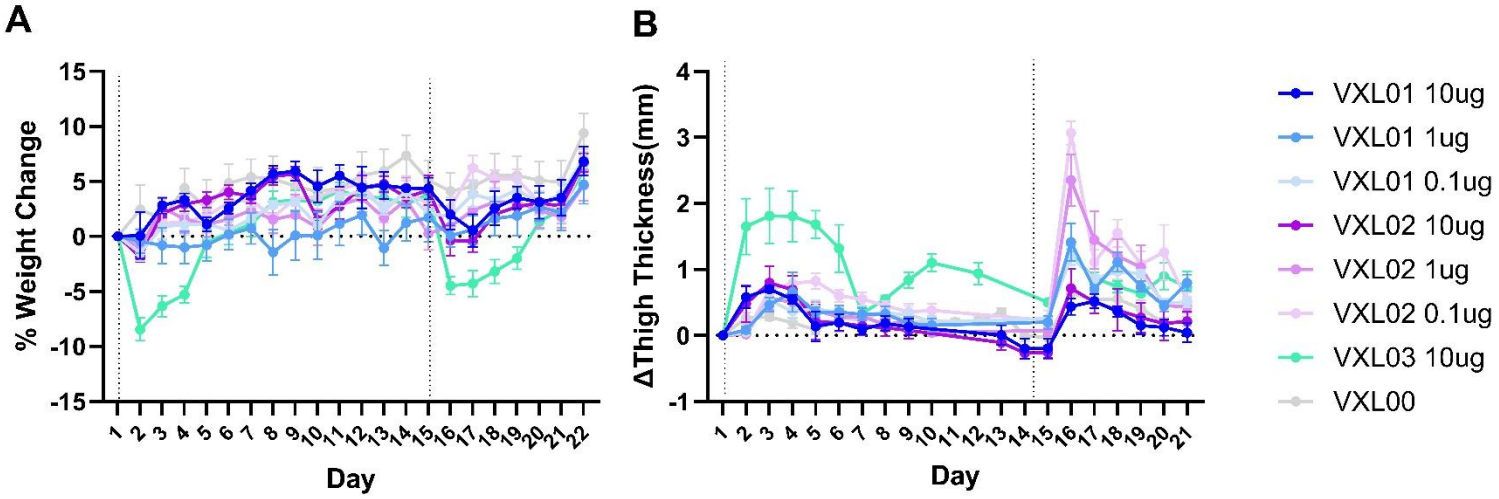
Supplementary Figures



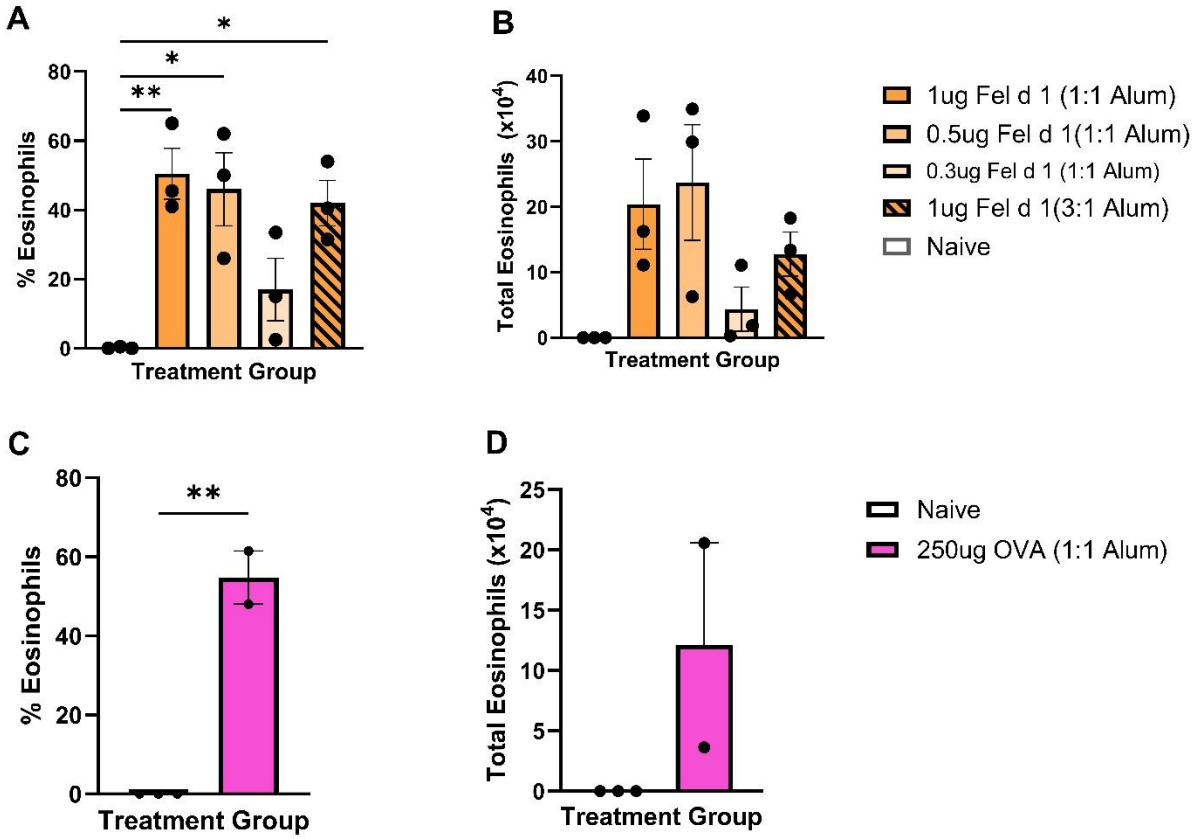
Supplementary Figure 1: Overview of Fel d 1 Production in an Endotoxin-low Environment.



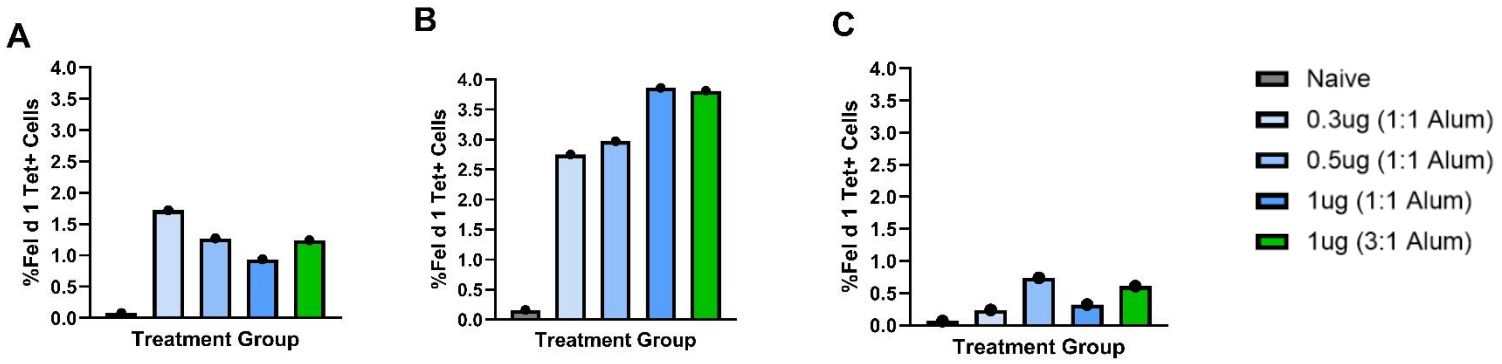
Supplementary Figure 2: Phenotyping of Fel d 1 Tetramer Positive Class Switched Germinal Centre B Cells in Sensitized mice in A) Enriched vs B) Unenriched samples. Two Verigem (IgE reporter) mice were sensitized with 200 µg Fel d 1 IP with alum intraperitoneally and euthanized for spleen and lymph node harvest at day 7. Cells were pooled and stained with tetramer and split into two groups following the staining protocol for enriched or unenriched cells.



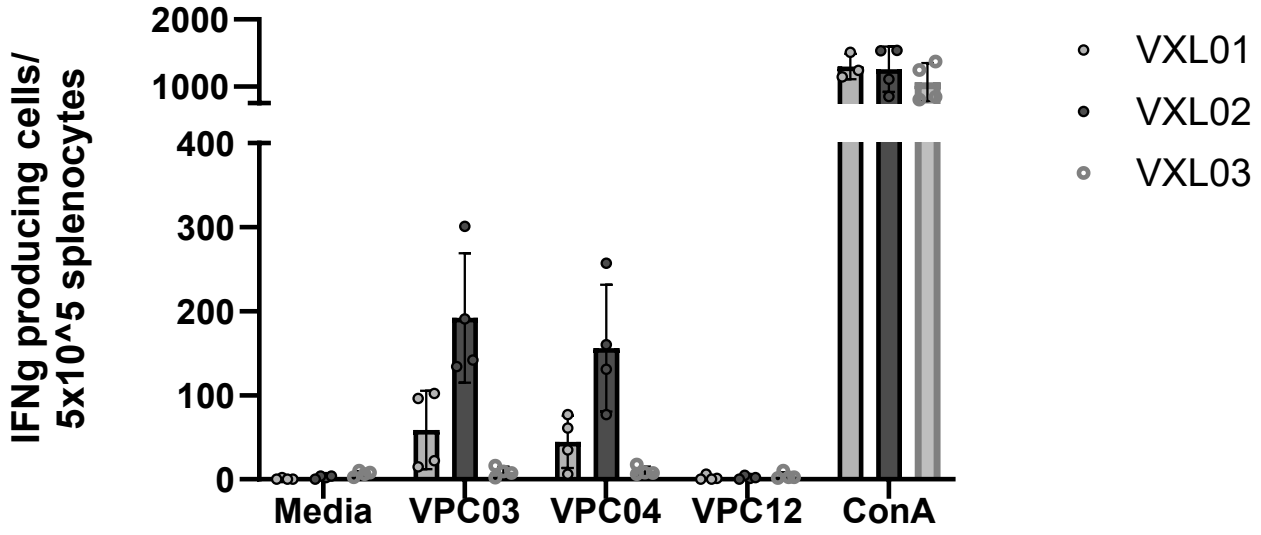
Supplementary Figure 3: Mouse Welfare Monitoring Data Across mRNA Peptide Immunotherapy Immunized Groups. Mice were immunized IM with varying doses of different Fel d 1 mRNA peptide immunotherapy on days 1 and 15. Weight and measurement of thigh thickness and the site of injection was measures. These were used A) percentage of weight loss and B) the delta thigh thickness from baseline were calculated.



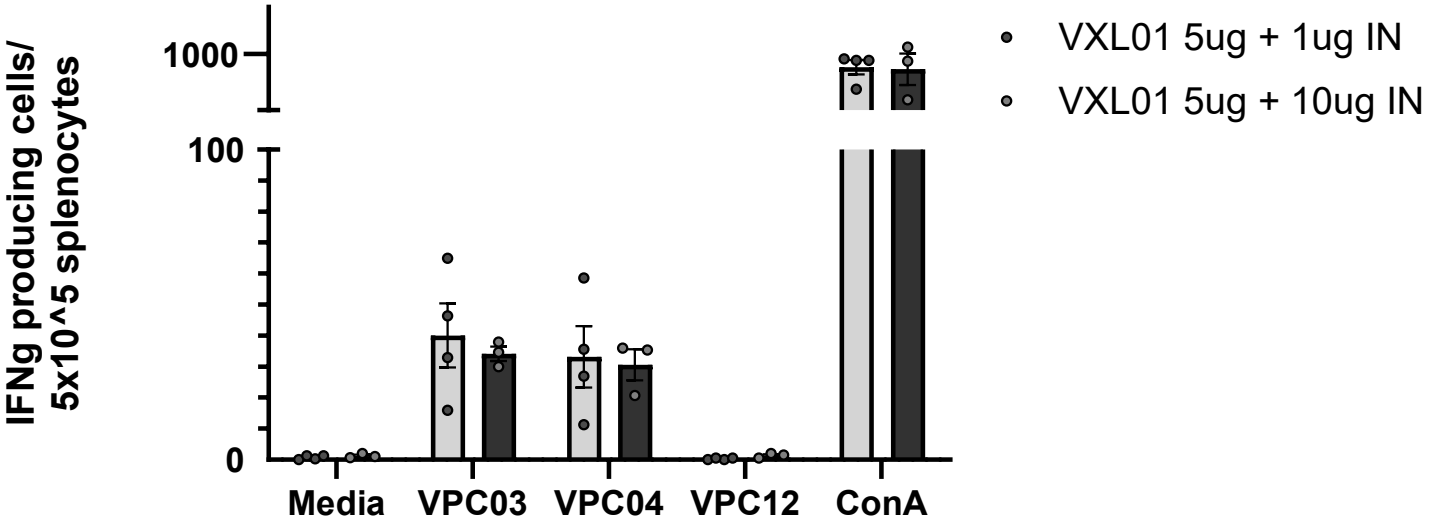
Supplementary Figure 4: Eosinophilia of Bronchoalveolar Lavage Fluid in Sensitized Mice. BAL fluid was collected, and a Wright- Giemsa stain was completed before manual cell differential counts were performed to analyze A) percentage eosinophils in Fel d 1 sensitized mice, B) total eosinophils in Fel d 1 sensitized mice, C) percentage eosinophils in OVA sensitized mice and D) total eosinophils in OVA sensitized mice. For panels A and B, results were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test (*p≤0.05, **p≤0.01; n=4 per group). For panels C and D, results were analyzed using an unpaired T-test **p≤0.01; n=2 per group). Error bars represent SEM.



Supplementary Figure 5: Unenriched B Cell Tetramer Staining in Naïve vs Sensitized Mice. BALB/c mice were sensitized IP to various doses of Fel d 1 in Alum on days 0 and 14, followed by 10 µg IN challenges of Fel d 1 on days 26 and 27. Mice were euthanized on day 29 and lymph nodes and lungs from were collected for tetramer flow staining to be completed on 1 million cells. Percentage of Fel d 1 Tetramer Positive Cells were observed in A) inguinal lymph nodes, B) cervical lymph nodes and C) lungs. n=1.



Supplementary Figure 6: Fel d 1 Peptide IFN- γ ELISpot in Different Prototype Peptide Immunotherapy Vaccines Evaluating Three Encoded Peptides. Mice were immunized IM with 10 µg of three different Fel d 1 mRNA peptide immunotherapy (VXL01, VXL02 or VXL03) on days 1 and 15. Mice were euthanized on day 22 and splenocytes were harvested. IFN-γ ELISpots were run testing responses against three of the encoded Fel d 1 peptides (VPC03, VPC04 and VPC12), with ConA as the positive control and media alone as the negative control conditions. Error bars represent SEM.



Supplementary Figure 7: Fel d 1 Peptide IFN- γ ELISpot Evaluating Three Encoded Peptides Following Different IN Challenge Doses. Mice were immunized IM with 5 µg Fel d 1 mRNA peptide immunotherapy (VXL01) on days 1 and 15. Following immunization mice were intranasally challenged with 1 or 10 µg of Fel d 1 on days 22 and 29. Mice were euthanized on day 30 and splenocytes were harvested. IFN-γ ELISpots were run testing responses against three of the encoded Fel d 1 peptides (VPC03, VPC04 and VPC12), with ConA as the positive control and media alone as the negative control conditions. Error bars represent SEM.

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