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# Metabolic regulation of allergic inflammation

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*ABSTRACT*

In the United States, allergic disease affects approximately 60 million people and impacts more people every year. While prevalence of allergic disease has steadily increased, there has concurrently been an increase in rates of metabolic syndrome—characterized by increased abdominal girth, decreased sensitivity to insulin, and higher levels of circulating blood glucose. These patients often experience chronic low-level inflammation, deemed meta-inflammation. Interestingly, patients with metabolic syndrome and related metabolic diseases also have increased rates of allergic disease and are over represented in the allergic population. Furthermore, patients with metabolic syndrome have increased severity of allergic inflammation, accounting for a disproportionate amount of healthcare spending and increased usage of sick time. While clinical work has been done to quantify these patient populations, there is a lack of understanding of the systemic metabolic processes that link allergic and metabolic diseases. This body of work examines the systemic connections between allergic lung inflammation and systemic metabolic tissues. We discover that 1) high glucose levels act as an adjuvant facilitate sensitization to OVA antigen, 2) mice sensitized under hyperglycemic conditions exhibit exacerbated lung inflammation, and 3) adipose tissue mounts a parallel Th2 inflammatory response during lung inflammation, characterized by infiltration of eosinophils and ILC2s and increased expression of *Il4, Il5, Il13*, and *Il33*. While Th2 immune cells are present in adipose tissue during homeostasis, this is the first report of increased Th2 activity in adipose tissue during inflammation. Furthermore, we discovered evidence of an antigen-specific effector response in adipose tissue, with adipose B cells producing OVA-specific IgE and adipose T cells proliferating in response to OVA restimulation. OVA-sensitized mice expressed a remodeling and development response in the adipose tissue and maintained glucose tolerance compared to saline mice.

Taken together, our work suggests that the Th2 response in adipose tissue is mounted to maintain metabolic homeostasis during lung inflammatory events. Future complimentary studies should examine the necessity of healthy adipose tissue in resolving lung inflammation.

*ACKNOWLEDGEMENTS*

This document is dedicated to my entire tribe. To my husband, who moved across the country to support me in this journey, and to my family who pushed me to keep going every time I wanted to quit. And to my sisters, who are my endless source of inspiration and push me to keep striving for more.

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# *CHAPTER 1: INTRODUCTION*

## *GENERAL INTRODUCTION*

### *Allergic Inflammation and Atopic Disease*

Allergic diseases are characterized by immunogenic responses to harmless environmental antigens to which healthy patients would be tolerant. Allergy is increasing in prevalence, particularly in the United States and other industrialized nations [1] , and is especially common in children. Allergic disorders include food allergy, atopic dermatitis, allergic rhinitis and allergic asthma. Asthmatic episodes can be triggered or exacerbated by allergen exposure [2]. Canonical inception steps of allergic immune response typically include allergen-induced release of innate alarmin cytokines IL-33 and TSLP by the epithelium, which activate dendritic cells to present antigen at mucosal sites and consequently induce type 2 immune response. The type 2 effector response includes production of IL-4, IL-5, IL-13, which activate and recruit eosinophils, basophils, and mast cells [3-6]. IL-4 also activates B cells to produce IgE specific to the antigen. Downstream, these specific IgE receptors are expressed on basophils and mast cells, which respond immediately via degranulation upon antigen exposure. Additionally, innate lymphoid-like cell 2 (ILC2s) can help orchestrate the type 2 response by producing IL-5 to recruit eosinophils, the key effector immune cells associated with allergy.

While allergy research has traditionally focused on exogenous induction of immune response by antigens, typically through disruption of airway epithelium by allergen proteases or environmental injury, there has been little focus on endogenous factors that can predispose to immune reactivity at mucosal sites. A study by Loffredo et al. showed that epithelium in human asthma is characterized by loss of differentiation programming and loss of barrier function and integrity [7]. The asthmatic signature in this study included downregulation of insulin receptor signaling pathway and changes in metabolic and mitochondrial genes. Limited research has

been conducted on the influence of metabolic factors on the development of allergic inflammation. Although asthma strongly associates with obesity and metabolic syndrome [8], the mechanisms by which metabolic factors may predispose individuals to development of asthma and allergic inflammation remain unknown.

*The NLRP3 inflammasome has roles in glycemic response and allergic development*

The NLRP3 inflammasome is a complex of proteins including nod-like receptor protein 3 (NLRP3), apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), and caspase-1 [9]. The inflammasome serves as a pattern recognition receptor that recognizes intracellular reactive oxygen species (ROS) and a number of markers of cellular stress [10, 11]. The NLRP3 inflammasome plays roles in both glycemic response and allergic development: multiple papers have demonstrated that the NLRP3 inflammasome is activated by hyperglycemia and produces active IL-1β and caspase-1 [12, 13]. Additionally, it has been demonstrated that NLRP3-/- mice cannot develop allergic airway inflammation, demonstrating that NLRP3 is critical in the induction of inflammation [14]. The NLRP3 inflammasome remains unexplored as a mechanistic link between metabolic and allergic processes.

*Heterogeneous immune responses in asthma correlate with metabolism*

Immune responses in allergic and asthmatic inflammation are not homogenous. Asthma severity occurs on a spectrum, with patient phenotypes that can be mild, moderate, or severe. Humans with mild and moderate asthma (MMA) are able to control their symptoms with low dose inhaled corticosteroids, while patients with severe asthma (SA) remain unresponsive [15]. The underlying reasons for the heterogeneity in therapeutic response remain unknown. Studies have demonstrated that MMA is orchestrated by type 2 inflammation and often exacerbated by allergic triggers, while SA patients exhibit type 1 and type 17 inflammation marked by high IFN-γ and IL-13/IL-17 [16]. Interestingly, the severe phenotype is commonly seen among obese,

middle aged women [17]. It is unknown whether metabolism influences the development of these divergent phenotypes.

## *METABOLIC DYSREGULATION AND ATOPIC DISEASE*

### *Metabolic syndrome and obesity correlate with increased asthma prevalence and severity*

Metabolic syndrome (MetS) is a complex disease, characterized by a generalized dysregulation of metabolites, glucose, and lipids. Clinical definitions of metabolic syndrome include a number of factors including increased waist circumference, elevated fasting blood glucose, increased high cholesterol, and high triglycerides [18]. While only roughly 20% of U.S. adults meet all of the requirements for MetS, over 56% have an elevated waist circumference, which may have metabolic consequences even in the absence of metabolic syndrome. Multiple studies have demonstrated that patients with MetS have increased incidence of asthma and decreased lung function [19, 20]. Other studies concluded that obesity associates with allergic sensitization and allergic airway inflammation [21, 22]. Further studies have demonstrated that obesity is an even better predictor of asthma development, with overweight women having the closest correlation between obesity and asthma [22-24]. A Nigerian cross-sectional study showed a high prevalence of metabolic syndrome among asthmatics (17.7%) and poorer asthma control in asthmatic patients with metabolic syndrome [25]. A Norwegian prospective study demonstrated that MetS was a risk factor for incident asthma (OR: 1.57). Two components of MetS remained associated with incident asthma after mutual adjustment for the other metabolic components: high waist circumference (OR: 1.62) and elevated glucose or diabetes (OR: 1.43) [19]. Another meta-analysis study showed that overweight and obesity are associated with a dose-dependent increase in the odds of incident asthma, both in men and women [26]. A retrospective Korean study also showed that obesity is significantly associated with self-reported severe asthma (OR: 1.61) [27]. Moreover, asthmatics with metabolic disease and obesity are often resistant to

traditional treatment with inhaled corticosteroids [28, 29]. The difficulty in treating these patients makes them a costly portion of the asthmatic population, accounting for over half of healthcare costs in asthma [30].

However, the relationship between MetS, obesity and allergic disease extends beyond asthma. A cross-sectional retrospective study of 116,816 patients diagnosed with atopic dermatitis (AD) between 1998 and 2016 concluded that severely affected patients with AD may have one or more undiagnosed components of metabolic syndrome [31]. Another cross-sectional population study of 8,217 US adults found significant association of atopic dermatitis with obesity, diabetes, and cardiovascular comorbidities [32]. A study of young adult males in Singapore found a significant association of metabolic and atopic conditions with moderate-to-severe AD [33]. A case-control study performed in pediatric dermatology practices in the United States found that moderate-to-severe pediatric AD may be associated with central obesity and high blood pressure [34]. A study by Agòm-Banzo et al. has shown that pediatric AD patients have higher body mass index than healthy controls [35]. A systematic literature review study found that central obesity is associated with AD, which was stronger for women than men [36]. Surprisingly, no studies report connections between metabolic disorders and food allergy, although it was reported that obese individuals with and without Type 2 diabetes have significant changes in function and composition of gut microbiota [37]. Obesity and disease severity also magnify disturbed microbiome-immune interactions in asthma patients [38]. Importantly, such connections between metabolic and allergic conditions may develop *in utero* or in early life. For example, a study of 15,145 mother child pairs in China found a significant association of maternal pre-pregnancy weight and gestational weight gain with children's allergic diseases [39].

*Insulin resistance and diabetes are associated with asthma severity, airway hyperresponsiveness, and airway function*

Multiple studies have brought to attention that insulin resistance correlates with asthma prevalence in both children and adults [20, 40]. Moreover, it appears that insulin resistance, prediabetes and diabetes are linked to exacerbation-prone asthma and increase in exacerbation frequency [41-43]. A pediatric study in an obese population at the Children's Hospital of Wisconsin found that higher insulin resistance was associated with asthma [44]. In addition, the authors showed that morbidly obese asthma patients have a higher degree of insulin resistance compared to morbidly obese non-asthmatic patients [44]. Another pediatric cross-sectional study linked asthma with abnormal glucose and lipid metabolism independent of body mass index [45]. Kozyrskyj et al. showed that, independent of puberty stage and progression, non-atopic asthma was 6-fold higher in adolescent girls who had insulin resistance but no central obesity two years earlier. Non-atopic asthma was 13 times more likely among girls with persistently high Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) [46]. A pediatric study in Spain showed that HOMA-IR insulin resistance values were significantly associated with positive skin tests and allergic asthma diagnosis [40]. Another cross-sectional study demonstrated that insulin resistance and metabolic syndrome are associated with worsened lung function in overweight/obese adolescents [20]. A pediatric study by Karampatakis et al. [47] showed that obese asthmatic children with confirmed insulin resistance have increased airway hyperreponsiveness compared to obese asthmatic children without insulin resistance. Authors suggest that obesity *per se* does not correlate to airway hyperresponsiveness unless it is accompanied by glucose intolerance and insulin resistance [47]. A study in Brazilian adolescents found that hyperinsulinemia, obesity, and metabolic syndrome associate with severe asthma [48]. A Swedish birth cohort study of 1,284,748 children found evidence for co-occurrence, sequential appearance and familial coaggregation of

asthma and type 1 diabetes [49]. An Indonesian cohort study found that females but not males diagnosed with pediatric asthma had significantly higher risk of diabetes [50]. A retrospective birth cohort study of 97,554 children from a single integrated health care system in the US found that maternal type 2 diabetes during pregnancy was a risk factor for development of childhood asthma in offspring [51].

In adult patients, British cross-sectional study in a female population found that FEV1 and FVC respiratory parameters were inversely associated with insulin resistance and prevalence of type 2 diabetes [52]. A Danish cross-sectional population-based study showed that insulin resistance was associated with aeroallergen sensitization and allergic asthma, but not non-allergic asthma [53]. A study in Turkey found that patients with asthma had higher insulin resistance (HOMA-IR), impaired fasting glucose, and impaired glucose tolerance when compared to the control group [54]. Danish population-based observational study found that insulin resistance was associated with incident wheezing (OR: 1.87) and asthma-like symptoms (OR: 1.61). The effect of insulin resistance was stronger than that of obesity and was independent of sex [55]. A South Korean study with patients that visited the hospital for a routine health check-up reported that subjects with airway hyperresponsiveness had higher insulin resistance when compared to those without AHR [56]. An adult cross-sectional study from Elliot Israel's group at the Brigham and Women's hospital published that insulin resistance differentiates itself from other component of the metabolic syndrome in that it is the only component that potentiates the obesity-asthma association [23]. Another article suggested that insulin resistance (a hallmark feature of polycystic ovary syndrome) might be the link between asthma and PCOS [57]. A very recent study by Yang et al. [58] of a cross-sectional cohort of 47,606 adult individuals with asthma but without physician-diagnosed diabetes found a positive association between glycated hemoglobin ($HbA_{1c}$) and lifetime odds of an asthma hospitalization, and an inverse association

between $HbA_{1c}$ and pulmonary function measured by $FEV_1$. $HbA_{1c}$ relates to an individual's blood glucose concentration and is a marker of metabolic health, used to both diagnose prediabetes and diabetes. This was an important finding because it demonstrated that metabolic dysfunction may be a feature of allergic disease even in absence of endocrine diagnosis, which warrants additional investigation.

Although an abundance of evidence points to a positive association between insulin resistance, diabetes and asthma in multiple age groups, association of diabetes and glycemic control with atopic dermatitis and other allergic diseases remains unclear. Despite positive associations between AD, MetS and obesity reviewed above, there are conflicting reports in regard to the relationship between AD and diabetes. A retrospective cohort study of 3386 patients with type 1 diabetes and 12,725 controls found that childhood type 1 diabetes may increase the risk of AD [59]. However, a study by Schmitt et al. [60] found an inverse relationship between these diseases. Other studies found normal insulin sensitivity, glucose tolerance, gut incretin and pancreatic hormone responses in adults with atopic dermatitis [61]. A US study by Silverberg et al. showed a higher prevalence of type 2 diabetes in adult patients with AD than in the general population [62]. However, a Danish study cohort of 30,079 adult patients with AD showed that patients with AD do not have an increased risk of new-onset type 2 diabetes compared with matched controls [63]. A cross-sectional analysis 259,119 participants between ages 30-74 from the Canadian Partnership for Tomorrow Project also found no evidence of a positive association between AD and type 2 diabetes, but found an inverse relationship [64].

*Heterogeneous immune responses in asthma correlate with metabolism*

Severity of asthma occurs on a spectrum, with patient phenotypes mild, moderate, and severe. Humans with mild and moderate asthma (MMA) are able to control their symptoms with low

dose inhaled corticosteroids, while patients with severe asthma (SA) remain unresponsive [15]. Studies have demonstrated that MMA is orchestrated by type 2 inflammation, while SA patients exhibit mixed type 1/17 inflammation marked by high IFN-γ and mixed eosinophil and neutrophil inflammation [16]. The SA phenotype is commonly seen among obese, middle aged women [17]. It is unknown whether metabolism influences the development of these divergent phenotypes.

*Adipose tissue and associated immune responses*

Immune cells are known to play a role in fat homeostatic processes. In the process of fat beiging, a process by which white adipocytes reprogram their metabolic activity to produce body heat, white adipocytes secrete fibroblast growth factor 21 (FGF21). FGF21 signals through autocrine and paracrine FGFR1 on adipocytes to trigger the release of eotaxin-1 [65]. Recruited eosinophils in fat then release IL-4, which polarizes macrophages to an M2 phenotype to mediate fat beiging [65]. This process is perhaps the most notable interaction of immune cells associated with Type 2 response in metabolic regulation.

Asthmatics have been shown to have increased FGF21 in serum [66]. The necessity of FGF signaling and eosinophils in homeostasis of airway and adipose tissue suggests that crosstalk may occur between lung and fat tissues during allergic inflammation. Therefore, changes in adipose tissue may inadvertently have consequences for plasticity of systemic immune response. Additionally, changes in inflammation in the airway may have systemic affects in adipose and other tissues. The work in this thesis will present the discovery of how allergic airway inflammation triggers changes in adipose tissue with concurrent eosinophil response at both sites.

*Mechanisms of glucose-induced inflammation in hyperglycemic conditions converge with mechanisms that promote allergic airway inflammation*

Damage-associated molecular patterns (DAMPs) are specific patterns that alarm systems to imminent danger and initiate and regulate inflammatory response. Within the context of allergy and asthma, DAMPs such as IL-33, uric acid (UA), and ATP are known inducers of early inflammatory processes [67-70]. In human asthmatics, as well as mouse models of experimentally induced asthma, levels of ATP in bronchoalveolar lavage fluid (BALF) are increased [67]. This increase in extracellular ATP triggers the activation of the NLRP3 inflammasome, which triggers the release reactive oxygen species (ROS), mitochondrial DNA (mtDNA), and IL-1β [71]. Following ATP-induced IL-1β production, dendritic cells become activated and induce the production of IL-33 [72], a key orchestrator and initiator of type 2 inflammation [6, 73]. Clinical studies show that the disruption of purinergic signaling by mutation in P2X7, an ATP receptor, protects against exacerbations of asthma [74, 75]. In allergy specifically, exposure to house dust mite was demonstrated to induce uric acid production in airway epithelium, which led to the production of IL-33, a key pro-Th2 alarmin cytokine [76].

Interestingly, glucose has been demonstrated to induce inflammatory pathways similar to those downstream of extracellular ATP. In hyperglycemic conditions, cells produce ROS and release IL-1β through activation of the NLRP3 inflammasome [12, 77-79]. Exactly how glucose stimulates the NLRP3 inflammasome remains unknown. It is known that the TCA cycle products and intermediates can induce inflammation, both through production of mitochondrial ROS [80] and production of succinate, which can induce IL-1β production [81]. It has been hypothesized that hyperglycemic conditions increase levels of ROS and IL-1β production by causing a dysregulation in the TCA cycle and increasing levels of intermediate succinate [81]. In both the allergy/asthma and hyperglycemic context, metabolites trigger the NLRP3 inflammasome and

the release of IL-1β. Glucose levels are elevated in lavages of subjects with the upper airway allergic disease. It remains unknown whether metabolites produced as a result of metabolic dysregulation can endogenously promote allergic inflammation.

*HYPOTHESIS AND SIGNIFICANCE*

Given the overwhelming evidence that metabolic dysregulation is linked to the presence, phenotype, and severity of allergic inflammation, this study set out to investigate the mechanistic links between allergic airway inflammation and systemic and local metabolic processes. To pursue these studies, we had two central hypotheses: 1) hyperglycemia-induced innate immune activation is a key mechanistic link that contributes to the exacerbated allergic response observed in hyperglycemic conditions and 2) allergic airway inflammation has far-reaching effects on systemic metabolism and adipose tissue beyond the airway. These lines of inquiry are significant due to the ever-increasing population of afflicted patients, and the lack of mechanistic understanding of connection between metabolic dysfunction and allergic inflammation. Furthermore, studies in allergic airway inflammation have focused largely on the airway tissue, in spite of systemically circulating cytokines and hormones that may induce an inflammatory signature in other tissues. In order to find therapeutic solutions for patients, it is imperative to study the systemic nature of inflammation taking into account the various metabolic baselines of the 21$^{st}$ century.

## *CHAPTER 2: MATERIALS AND METHODS*

### *Mice*

Wild-type age-matched male and female BALB/c or C57BL6 mice were purchased from Jackson Laboratories. All mice were 6–12 weeks old. All animal experiments were approved by Northwestern University's Institutional Animal Care and Use Committee (IACUC). All methods involving mice were performed in accordance with relevant guidelines and regulations.

### *Murine kinetic model of lung allergic inflammation*

Eight mice per group received intraperitoneal injections (200 uL) of chicken egg ovalbumin (OVA) grade V (50 ug) (Sigma) with 10 ug alum or saline/alum on days 0 and 7. On days 14, 16, 19, 21, and 23, mice received intranasal challenge with OVA grade V (50 ug) in saline or saline alone. By day 29, inflammation in mice had resolved. Experiments were conducted in a kinetic fashion with tissue harvests at different time points in a model with "Bas" indicating baseline, "Res" indicating resolution, "Ch1" indicating challenge 1, "Ch3" indicating challenge 3, *et cetera*. 24 hours after each challenge, 8 female and 8 male mice were harvested for analysis. Visceral adipose tissue, lung, bronchoalveolar lavage (BAL), spleen, and serum were collected from each mouse. Visceral adipose tissue from baseline, challenges 1, 3, 5, and resolution was processed for RNA-seq analysis.

### *Mouse model of allergic lung inflammation*

All experiments were performed on 6-12 week old female BALBc mice (Jackson Labs, Bar Harbor, ME). The mice were sensitized by an intraperitoneal injection (200 μL) of ovalbumin (OVA) grade VI 10 μg/alum or saline/alum on days 0 and 7 and then challenged intranasally on days 15, 16, and 17 with OVA grade VI or saline alone. Tissues were harvested and processed for analysis at 24 hours after the last OVA challenge.

*Preparation of glucose solution*

All glucose concentrations of media were made using glucose-free RPMI as base (Thermo-Fisher, Waltham, MA). Solid L-glucose (Sigma Aldrich) was added to glucose-free RPMI and sterile filtered to create concentrations of 5.5 mM, 10 mM, 15 mM, 20 mM, and 25 mM glucose.

*Intraperitoneal administration of glucose*

Mice were injected injected with 46 mg dextrose (200 uL) and their blood glucose was read at 30 minutes and 1 hour with True Mextrix Glucose Meter and test strips (Walgreens, Deerfield, IL). Tissues were harvested and processed for analysis one hour after glucose injection.

*Mouse model of induced antigen tolerance*

All experiments were performed on 6-12 week old female C57BL6/J mice (Jackson Labs, Bar Harbor, ME). On days 0-3, mice were intranasally administered 46 mg dextrose/50 µg ovalbumin (OVA), or 46 mg dextrose in 0.9% saline. On day 7, mice were given intraperitoneal injection (200 µL) of ovalbumin grade VI 10 µg/alum and then challenged intranasally on days 17-20 with OVA grade VI alone. Tissues were harvested and processed for analysis at 24 hours after the last OVA challenge.

*Flow cytometry*

Prior to harvest and homogenization, visceral fat pads were collected from each mouse and weighed. Visceral adipose tissue (VAT) was dissociated in 0.2 mg/ml DNAse I (Roche) and 2 mg/ml Collagenase D (Roche) for 1 hour. The cells were then filtered into a single cell suspension using a sterile mesh. VAT samples were centrifuged at 250 g for 8 minutes to separate adipose (containing adipocytes) and stromal vascular fraction (SVF) (containing immune cells). SVF (1-2 million cells/sample) was used for flow cytometry staining. Prior to antibody staining, cells were incubated with Zombie Live/Dead Aqua (Biolegend) dye followed by CD16/32 FC Block (BD Pharmingen). We used the following antibody cocktail to assess

leukocyte populations in adipose tissue: (1) Alexa Fluor-conjugated CD45 (clone 30-F11, Biolegend); (2) PerCP-Cy5.5-conjugated CD19 (clone eBio1D3, Invitrogen); (3) Alexa Fluor 647-conjugated Siglec-F (clone E50-2440, BD); and (4) FITC-conjugated F4/80 (clone BM8, Biolegend). Cells were then fixed in 2% paraformaldehyde and analyzed on an LSRII flow cytometer (BD). Compensation was set up using single color control fluorescent beads (OneComp, eBioscience). Negative gate boundaries were identified using fluorescence-minus-one (FMO) controls. FlowJo software (Treestar) was used for compensation and data analysis. Eosinophils were gated as CD45(+)Siglec-F(+)F4/80(+). Macrophages were gated as CD45(+)Siglec-F(-)F4/80(+).

*Cytospin differential staining and counts*

Cells cytospun from VAT immune cell fraction were stained with initial fixation/staining in Wright Giemsa solution (EMD Millipore, Burlington, Mass) for 2 minutes, incubated in Eosin Xanthene dye for 2 minutes, and dipped twice in Hematoxylin/Blue/Azure (Electron Microscopy Science). Nuclear morphology and eosin granule staining were visualized at 40× on Olympus DSU microscope (Olympus, Tokyo, Japan). Mature eosinophil counts were performed in a blinded fashion on 4 different 40× fields per slide.

*Beta hexaminodase assay*

The degree of mast cell degranulation was measured by release of β-hexosaminidase. BMMCs ($0.3 \times 10^6$ cells/ml) were preincubated overnight with anti-DNP IgE (100 ng/ml) in medium. Sensitized cells were stimulated for 30 min with indicated concentrations of DNP-HSA in Tyrode's buffer, nonsensitized cells were stimulated for 30 min with indicated concentrations of ionomycin, NaCl, or glucose solution. Cells were washed and centrifuged, and the cell pellets were solubilized with 0.5% Triton X-100 in Tyrode's buffer. The enzymatic activity of β-hexosaminidase in the supernatants and cell pellets was measured using *p*-nitrophenyl-*N*-

acetyl-β-d-glucosaminide (Sigma-Aldrich) in 0.1 M sodium citrate (pH 4.5) as a substrate. The reaction was allowed to run for 30 min at 37°C and then stopped by adding 0.1 M carbonate buffer (pH 9.0). Release of the product, *p*-nitrophenol, was detected based on the absorbance at 405 nm. The percentage β-hexosaminidase release was determined by dividing the measurements detected in the supernatant by the total measurements detected in the supernatant plus those from the cell pellet.

*Mast Cell Culture*

All experiments were performed with 6-12 week old female C57BL6/J mice (Jackson Labs). Mast cells were cultured following the established murine bone marrow culture protocol [82, 83]. Cells were used upon maturity in weeks 4-6 of culture. Cell purity was assessed by $Fc\varepsilon RI^{+}/CD117^{+}$ by flow cytometry.

*Preparation of peritoneum elicited macrophages*

Peritoneal macrophages were elicited from the peritoneum of wild type mice using 4% thioglycolate, and isolated according to the standard protocol [84]. After elicitation of macrophages from peritoneum, cells were plated in tissue culture plates for 2 hours at 37℃ and nonadeherent cells were washed away with glucose-free RPMI. Adherent cells were treated with experimental conditions for 24h before downstream analysis.

*Quantitative PCR (qPCR)*

The VAT for gene expression analysis was harvested from the same mice that were assessed for lung allergic inflammation. Tissue was stored in RNALater (Qiagen) stabilizing solution prior to RNA extraction. Approximately 10 mg of tissue was homogenized using a rotor homogenizer and RNA was extracted with the RNeasy Mini Kit (Qiagen). 500 ng of total RNA was used in a cDNA synthesis reactions, using a cDNA Synthesis Kit (Quanta). qPCR reactions utilized probe-based qPCR Master Mix (IDT), and commercially designed primer/probe gene expression

assays (IDT) for the following genes (targeted exons indicated in parentheses): Serpinb2 (exon 3-4), Postn (exon 2-3), Ccl2 (exon 1-3), Ccl11 (exon 1–2), Cxcl1 (exon 2-4), Cxcl13 (exon 2-4), (exon 2–3), Cxcl16 (exon 2-4), Plat (exon 9-10), Plau (exon 10-11), Rbpj (exon 10-11), Apobec1 (exon 5-6), Alox5ap (exon 3-4), Cybb (exon 8-9), and Cyba (exon 4-6). qPCR reactions were run using a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene expression was calculated relative to expression of TBP (housekeeping gene) and was reported as true copies of the gene of interest per $10^4$ copies of TBP as previously described [85].

*RNA-seq*

All cells were lysed in RLT buffer and RNA was immediately extracted using the RNeasy Plus Mini Extraction Kit (Qiagen). RNA quality was assessed using an Agilent High Sensitivity RNA ScreenTape System (Agilent Technologies). Only samples with RNA Integrity Number (RIN) scores > 7 were used in RNA-seq analysis. The NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs) was utilized for full-length cDNA synthesis and library preparation. The sequencing of cDNA libraries was done on an Illumina NextSeq 500 instrument (Illumina) at a target read depth of approximately 10 million aligned reads per sample. Sequenced reads were demultiplexed using bcl2fastq (v 2.17.1.14). Quality control was performed using FastQC. Low quality reads were discarded using trimmomatic (v 0.33). Reads were aligned using HISAT2 to mouse reference genome mm10. Read counts were generated using htseq-count. Differential expression analysis was done using DESeq2 (R/Bioconductor package). All the computational analysis was performed on genomic nodes of Quest, Northwestern's High Performance Computing Cluster.

*Serum hormone measurements*

Serum was analyzed using MagPlex mouse metabolic hormone panels (EMD Millipore). Following manufacturer's recommendations, 10 uL of undiluted mouse serum was incubated

with magnetic beads specific for metabolic hormone panel, including reported GLP-1, PP, PYY, Resistin, and MCP-1. Samples were analyzed using Luminex technology.

*Bioinformatics, statistical analysis, and data visualization*

Gene set enrichment analysis was performed using PANTHER software (v14) to access GO Biological Processes represented in gene expression signatures from RNA-Seq analysis. Venn diagram analysis was used to identify the intersections of gene signatures from multiple studies, using the Bioinformatics & Evolutionary Genomics Venn diagram tool (VIB/UGent, Belgium). Heatmap generation and hierarchical clustering were done using Morpheus (Broad Institute). Similarity matrices were created using Morpheus software (Broad Institute) using Pearson correlation across rows. Principal components analysis was performed using the multivariate statistical analysis software PAST, Version 4.03. Network analysis was performed using ClueGO analysis tool implemented in Cytoscape 3.4.0. interface. Statistical tests utilized in different experiments are identified in the captions of corresponding figures and tables. All graphs and statistical analyses were carried out in GraphPad Prism (Graphpad 8.2.0).

# *CHAPTER 3: ALLERGIC INFLAMMATION IMPACTS SYSTEMIC METABOLISM AND PROMOTES TYPE 2 RESPONSE AND EOSINOPHILS IN ADIPOSE TISSUE*

## *INTRODUCTION*

Allergic lung inflammation is characterized by the induction of Type 2 immune response, which involves the recruitment and accumulation of eosinophils in the airways, along with increased expression of eosinophil-recruiting eotaxin chemokines, innate alarmin cytokine IL-33 and Type 2 cytokines IL-4, IL-5, and IL-13. In antigen sensitization driven murine models of asthma, dendritic cells present antigen to naïve T cells and promote skewing to a Th2 phenotype in draining lymph nodes, while effector T cell responses triggered by local antigen challenge are thought to be restricted to the airway [86].

It has only more recently been appreciated that the cells and cytokines involved in Type 2 inflammation also play a homeostatic role in adipose tissue browning and homeostasis. Adipose tissue contains a mixture of white adipose tissue (WAT), brown adipose tissue (BAT), and beige adipose tissue, also known as induced brown adipose tissue (iBAT). During times of energetic need, WAT produces eotaxin CCL11, which recruits eosinophils to adipose tissue to induce beiging, or the conversion of white adipose tissue to brown adipose tissue [65]. Brown and beige adipose tissue are responsible for the majority of heat production and thermogenic capacity in mammals [87, 88]. The fat thermogenic cycle involves production of IL-33 by stromal cells, which activate group 2 innate lymphoid cells (ILC2s) to produce IL-5 and IL-13 [89-91]. The presence of IL-5 and IL-13 induces accumulation of IL-4 producing eosinophils and alternatively activated macrophages (AAMs), which induce the factors needed for adipose tissue browning [92]. Intriguingly, a recent study demonstrated that allergic lung inflammation alters the adipose micro RNA (miRNA) profile in rats [93], but no studies have analyzed the impact of allergic lung inflammation on the adipose transcriptome and immune function.

Although Type 2 immune cells and cytokines play a significant role in adipose tissue homeostasis and thermogenesis, the relationship between allergic Type 2 inflammation in the airway and adipose tissue responses remains largely unknown. This report fills this gap by demonstrating that (1) the induction of Th2 immune response in the visceral adipose tissue parallels lung allergic inflammation in a murine model of asthma, which includes eosinophil recruitment and adipose expression of "Th2 high" gene markers, (2) airway allergic inflammation promotes adipose tissue gene expression of markers of inflammation, stromal remodeling, and arachidonate metabolism; (3) significant sexual dimorphism exists in adipose tissue responses, and (4) allergic lung inflammation increases levels of circulating gut hormones with roles in systemic regulation of metabolism.

## *RESULTS*

### *Airway antigen challenge promotes Type 2 inflammation and eosinophil recruitment in visceral adipose tissue*

We induced airway inflammation in a chicken egg ovalbumin sensitization model in both male and female BALBc mice (**Fig. 1**). Both sexes exhibited increased spleen weights (peaking at challenges 3 and 4) in response to antigen challenge (**Fig. 2**). VAT weight has also changed during inflammation (**Fig. 2**). Changes in both spleen and adipose tissue weight were significantly different between sexes, with females showing greater tendency to lose adipose mass during inflammation (**Fig. 14A**). As expected, qPCR analysis of lung tissue in response to inhaled ovalbumin (challenge 3) showed significantly increased expression of Type 2 cytokines and chemokines *Il4*, *Il5*, *Il33* and *Ccl11*, as well as "Th2 high" markers *Serpinb2* and *Postn* (**Fig. 6**). Intriguingly, VAT qPCR analysis demonstrated equivalent induction of Th2 inflammation in adipose tissue measured by expression of the same markers in lung-VAT paired samples (**Fig.**

**3**). Consistent with induction of Type 2 response, we found increased numbers of eosinophils in VAT stromal vascular fraction (SVF) cytospin preparations after three antigen challenges (**Fig. 5**). Flow cytometry analysis of VAT harvested following challenge 3 confirmed a significant increase in Siglec-F(+)F4/80(+) eosinophils in the SVF compartment of OVA challenged mice compared to saline controls (**Fig. 4**). RNA-seq analysis of tissue samples showed convergence in genes changed in the lung and adipose tissue following allergic challenge (**Fig. 7**), with 376 out of 639 differentially expressed genes (DEGs) in VAT also represented in lung tissue response (59%). Taken together, these results show that lung allergic inflammation in mouse models of asthma is accompanied by induction of Type 2 inflammatory response in the adipose tissue.

*Adipose tissue gene expression promoted by airway inflammation is greater in females and represents diverse biological processes*

RNA-seq analysis of VAT from male and female mice exhibited changes in gene expression following antigen challenges and resolution. After each antigen challenge, female mice had a higher number of significantly differentially expressed genes than male mice, with the highest number of genes induced after challenge 1 (**Fig. 8**). Although the number of DEGs was different between male and female mice, there was convergence in gene expression signatures and biological processes represented by DEGs (**Fig. 8**). Male-female convergent DEGs (induced during challenges 1-5) represented a large number of diverse biological processes, including immune system process, metabolic process, developmental process and cellular adhesion and proliferation (**Fig. 9**). In summary, while biological processes represented by VAT gene profiles were similar across both sexes, female mice exhibited greater adipose response during allergic inflammation. Subsequent sequencing results will focus on biological processes induced in female visceral adipose tissue.

*The adipose immune response is marked by expression of immunoglobulin genes*

Of all of the adipose genes significantly differentially expressed in at least one of three challenges measured by RNA-seq, 78 were immunoglobulin (Ig) variants. Principal component analysis (PCA) revealed significant overall changes in immunoglobulin gene expression profiles during the course of allergic inflammation (**Fig. 10**). The table in **Fig. 10** lists the top five differentially expressed immunoglobulin genes (ranked by FDR adjusted p-value) in each challenge. Immunoglobulin gene analysis revealed some degree of convergence across challenges (for genes *Igj* (J chain of IgM and IgA antibodies), *Igkc* (immunoglobulin kappa light chain constant domain), *Igkv* (immunoglobulin kappa variable domain), but also suggested challenge-specific differences in immunoglobulin production. For example, gene *Ighm* (marker of IgM antibody production) showed greater expression in challenge 3, while expression of Igha (marker of IgA antibody production) was more prominent during resolution. Overall, this suggests adipose tissue as a significant contributor to antibody production and systemic immune response during allergic airway inflammation.

*Adipose inflammatory response is accompanied by expression of markers of development and tissue remodeling*

One group of VAT genes identified during OVA challenges was highly represented by matrix metalloproteases (MMPs) and tissue plasminogen system genes, which play a significant role in degradation of the extracellular matrix (**Fig. 11**). We validated increased expression of plasminogen system genes *Plat* (tissue plasminogen activator) and *Plau* (urokinase-type plasminogen activator) by qPCR in an independent group of samples (females, OVA challenge 3), confirming tissue remodeling activity in the adipose compartment in response to airway inflammation (**Fig. 11**). A variety of developmental genes with roles in immune system and tissue development, such as *Rbpj* (recombination signal binding protein for immunoglobulin

kappa J region), *Sox12* (SRY-box transcription factor 12), and *Ikzf1* (Ikaros family zinc finger protein 1), were also elevated over the course of inflammation in male and female mice (**Fig. 12**), with qPCR validation of *Rbpj* showing a significant increase in VAT expression in females following OVA challenge 3 (**Fig. 12**). Together, the increased expression of these genes suggest tissue remodeling and *in situ* hematopoiesis processes in the VAT in response to allergic lung inflammation.

*Changes in adipogenesis and lipid metabolism during allergic inflammation*

We found a specific loss of visceral adipose tissue weight in female mice during challenge phase of the asthma model (**Fig. 14**). Loss of adipose tissue was consistent with significant reduction in expression of markers of adipogenesis by RNA-seq (measured in female VAT, OVA challenge 3), which included decrease in expression of *Adig* (adipogenin), *Arxes1* and *Arxes2* (adipocyte-related X-chromosome expressed sequence 1 and 2) (**Fig. 14**). Over 200 significantly differentially expressed VAT genes during allergic inflammation were classified as part of the metabolic process based on gene ontology analysis . Network analysis of these genes highlighted increased activity in lipoxygenase pathway (phospholipases *Pla2g3* and *Pla2g7*) and arachidonic and fatty acid metabolism (arachidonate lipoxygenases Alox5, Alox15, Alox5ap and Alox12e), associated with increase in prostaglandin, leukotriene and thromboxane biosynthetic process (prostaglandin synthase *Ptgs1*, thromboxane synthase *Tbxas1*). At the same time, the analysis showed a decrease in triglyceride biosynthetic process and changes in regulation of peroxisome proliferator activated receptor signaling pathway (Lpin1 (lipin-1), Plin5 (perilipin-5)) (**Fig. 13**). We validated increased expression of *Apobec1* (apolipoprotein B mRNA editing enzyme) and *Alox5ap* (arachidonate 5-lipoxygenase-activating protein) by VAT qPCR analysis (females, OVA challenge 3) (**Fig. 15**). No other genes were tested. Collectively, these results suggest that loss of adipogenesis and lipolysis are linked to changes in lipid metabolism

to sustain production of prostaglandins and cysteinyl leukotrienes, thus contributing to overall inflammatory process.

*Allergic airway inflammation triggers a systemic hormonal response*

Since changes in adipose tissue immune and metabolic regulation implicate a systemic level process during allergic airway inflammation, we measured levels of serum hormones with roles in systemic regulation of metabolism. We found that serum concentration of hormones GLP-1 (glucagon-like peptide-1), PP (pancreatic polypeptide), PYY (peptide tyrosine tyrosine) was significantly elevated after each antigen challenge relative to saline baseline (**Fig. 16**). Increase in concentration of PP was sustained during resolution phase of the model, while other hormones were increased only during active antigen-driven inflammation (**Fig. 16**). Serum concentration of hormone resistin (also known as adipose tissue-specific secretory factor (ADSF)) was specifically elevated after the first antigen challenge (**Fig. 16**). Levels of C-peptide, insulin, glucagon, leptin, amylin, ghrelin and gastric inhibitory polypeptide (GIP) were not found to be significantly different between control and OVA-challenged animals (data not shown). These results highlight systemic level regulation of metabolism and communication occurring between tissues during allergic inflammation, with gut hormones acting as likely systemic messengers between airway and adipose tissue sites (**Fig. 17**).

*DISCUSSION*

Adipose tissue is structurally and functionally diverse harboring multiple cell types, including adipocytes, stromal cells, and cells of the immune system. This highly dynamic tissue serves as a depot of stored energy to meet systemic energetic demands and as an endocrine site capable of release of multiple systemic mediators; it also plays key roles in regulation of systemic metabolic homeostasis and stress response to cold and inflammation [94, 95]. Adipose tissue is contained in multiple body locations, including visceral (VAT) and subcutaneous (SAT)

compartments [96]. Visceral adipose tissue is one of the largest deposits and is contained within the peritoneal cavity. Our work demonstrates that lung allergic inflammation invokes a systemic inflammatory and metabolic response with potential communication between airway and visceral adipose tissue.

It is well appreciated that adipose tissue is a significant contributor to systemic inflammation. White adipose tissue is the major source of obesity-related inflammation and a site of systemic cytokine production [97]. In turn, adipose inflammation leads to insulin resistance and metabolic disease [98]. Our results show that allergic lung inflammation induces a Type 2 immune response in the visceral adipose tissue in wild type, metabolically healthy mice. Remarkably, the Th2 high inflammatory genotype of the VAT exactly parallels all hallmark features of the “Th2 high” phenotype of the allergic airway, which demonstrates that allergic inflammation necessitates a systemic response. Our RNA-seq analysis of the VAT in antigen-challenged mice revealed significantly increased expression of a striking number of diverse cytokines and chemokines. The increased expression of Ccl2, Ccl8, Ccl11, Cxcl1, Cxcl13, Cxcl16, and dozens of other chemoattractants and their receptors suggests continuous immune cell recruitment to the VAT during the course of inflammation. This is well supported by our demonstration of Ccl11 expression and eosinophil recruitment to the VAT. Multiple reports have shown that eosinophil-derived IL-4 and Th2 cytokines such as Il-5, IL-13, and IL-33 play a pivotal role in the browning of white adipose tissue [89, 90]. Whether similar process takes place in the VAT during allergic inflammation, and whether eosinophils are the source of significantly increased Il-4 expression we found in the VAT remains to be investigated.

The high number of immunoglobulin genes (78 unique genes found to be significantly differentially expressed after at least one antigen challenge) induced in adipose tissue during inflammation is intriguing. This suggests activity of B cells in the VAT during allergic inflammation and the potential of adipose tissue to be a significant source of antibodies. Indeed,

the production of immunoglobulins and pro-inflammatory cytokines by B and T cells, along with self-antigen presentation, is thought to contribute to the adipose inflammation and the establishment of metabolic disease [99, 100]. A study by Frasca et al. [101, 102] found that B cells in the human subcutaneous adipose tissue secrete autoimmune fat-specific IgG antibodies and that the SAT expresses RNA for cytokines known to promote germinal center formation, isotype class switch, and plasma cell differentiation. Another study found that VAT B cells secrete IgG2c antibodies, function of which was impaired in old mice [103]. Moreover, aging induces an Nlrp3 inflammasome-dependent expansion of adipose B cells that impairs metabolic homeostasis [104]. A recent study by Chudakov et al. [105] demonstrated that low dose subcutaneous injection of ovalbumin antigen to withers fat pad resulted in B cell antibody isotype switching to IgE in adipose tissue. It remains to be investigated whether IgE isotype switching has the potential to take place in the VAT in murine models of asthma, although our RNA-seq analysis provided evidence for production of IgM and IgA immunoglobulins. Interestingly, IgM antibodies are thought to have a protective role in obesity-associated inflammation, glucose intolerance, and insulin resistance [106]. Perivascular adipose tissue was also found to contain IgM-producing B cells with atheroprotective roles [107]. Likewise, high fat diet-fed IgA-deficient mice have dysfunctional glucose metabolism and obese mice have fewer IgA+ immune cells and less secretory IgA [108]. This suggests that adaptive immunity associated with adipose responses plays key roles in regulation of systemic homeostasis.

Our VAT RNA-seq analysis also suggests significant changes in adipogenesis and remodeling in adipose tissue compartment during antigen challenge phase of the model. This is evidenced by differential expression of developmental/tissue remodeling markers, including matrix metalloproteases, tissue plasminogen system genes, *Sox* genes (SRY-box transcription factors), *Dach1* (Dachshund family transcription factor), and *Rbpj* (Notch pathway marker). It is unclear whether the developmental signature arose from adipocytes or immune and stromal

cells contained in the adipose tissue. Further resolution via single-cell RNA sequencing will be required to resolve nature of these gene signals. Adipose remodeling, and decrease in expression of adipogenin and other markers of adipogenesis are consistent with overall reduction in adipose tissue mass during allergic inflammation. At the same time, analysis of metabolic pathways revealed an increase in lipoxygenase pathway activity, arachidonate metabolism and fatty acid biosynthesis. Such changes in lipid metabolism pathways may be indicative of lipolytic activity in the adipose tissue in response to inflammation. In particular, inflammatory cytokines decrease the lipid storage by inhibiting the differentiation of pre-adipocytes and increasing lipolysis, which leads to an increase in free fatty acid concentration in serum and abnormal fat accumulation in other tissues [109]. Increase in lipolysis and free fatty acids serum concentrations has also been documented during acute endotoxin-induced inflammation in a human randomized crossover trial [110]. Studies suggest that the release of fatty acids is a major energy source during insulin-induced hypoglycemia and that lipolysis may be an important component of the counter-regulatory response [111]. Arachidonic acid and its metabolism deserve further attention, as this pathway plays diverse roles in biosynthesis of inflammatory mediators (leukotrienes, prostaglandins, thromboxanes) [112], regulation of systemic metabolism and maintenance of body weight in mice [113, 114], and adipocyte differentiation [115].

We found significant sex differences in behavior of the adipose tissue during allergic inflammation, including differences in loss of adipose mass and number of genes induced by inflammation. In support, studies report sex differences not only in the amount and distribution of adipose tissue, but also in adipose metabolic capacity and functions between males and females [116, 117]. Inflammatory responses to adipose tissue lipolysis in diet-induced obesity are also known to be sexually dimorphic [118]. Contribution of sex and sex hormones to adipose responses during allergic inflammation are subject to further investigation by our group, given

strong clinical associations between asthma and obesity [119]. Asthma in obese females represents a particularly severe disease endotype [120]. The increase in levels of non-sex hormones we detected in serum in response to allergic inflammation is also intriguing. Glucagon-like peptide-1 (GLP-1) is known to regulate eosinophil activation and accumulation [121] and has known anti-inflammatory properties in allergy and obesity related asthma [122, 123]. While the primary role of GLP-1 is in satiety and nutrient intake regulation, the inherent roles in inflammation reinforce the systemic nature of allergic effector responses. Pancreatic polypeptide (PP) and peptide YY (PYY) are known for nutrient intake regulation and their circulating levels are known to be elevated in inflammatory and infectious disorders [124, 125]. Resistin, also known as adipose tissue-specific secretory factor (primarily secreted by adipose tissue in rodents), has known roles in promotion of inflammation [126, 127], in part via stimulating transcription of inflammatory cytokines TNFα, IL-1, IL-6, and IL-12 [128]. Moreover, resistin mitigates stemness and metabolic profile of human adipose-derived mesenchymal stem cells [129]. It has been linked extensively to atopic dermatitis, pulmonary function, asthma, and allergic rhinitis [130-135]. The release of pancreas, gut and adipose-derived hormones GLP-1, PP, PYY, and resistin suggests systemic communication between the inflamed lung, visceral organs and visceral adipose tissue, with potential activation of gut-brain regulatory axis.

In summary, our data show that allergic lung inflammation promotes a strong parallel Type 2 immune response and metabolic change in the visceral adipose tissue. It remains to be investigated whether adipose inflammation directly contributes to airway immune responses via production of antibodies and systemic inflammatory mediators. On the other hand, changes in adipose metabolism and metabolite release may be necessary to maintain systemic energy homeostasis and provide sufficient resources to sustain energy-expensive airway inflammation and resolution/repair processes. Another implication of our findings is that chronic airway

inflammation in asthma may promote adipose dysfunction, which has important consequences for understanding metabolic comorbidities and the systemic aspects of chronic disease.

# *CHAPTER 4: DYSREGULATED GLUCOSE METABOLISM ALTERS THE PHENOTYPE OF ALLERGIC INFLAMMATION AND PROMOTES ACTIVATION OF KEY IMMUNE CELLS*

## *INTRODUCTION*

Allergic inflammation is a prevalent disorder in which subjects become sensitized to ubiquitous compounds and mount an inflammatory response, typically characterized by the increased presence of type 2 cytokines and eosinophils [136, 137]. This diverse family of disease includes allergic asthma, allergic rhinitis, atopic dermatitis, and chronic rhinosinusitis, and impacts millions of adults and children each year. The mechanisms that underlie the development of allergic inflammation have been well studied, and mouse models have been developed to mimic the sensitization process in vivo.

While the exogenous factors that contribute to sensitization (e.g. environment, pollution) have been investigated, the endogenous mechanisms that contribute to sensitization remain poorly understood [138, 139]. Clinical studies have shown that allergic and asthmatic patients have increased rates of obesity, type 2 diabetes, hyperglycemia, and metabolic syndrome [22, 140-142]. However, the mechanisms that link allergic inflammation and metabolic syndromes remain poorly understood.

In vitro studies have demonstrated that hyperglycemic conditions can initiate inflammation by activating the NLRP3 inflammasome and promoting the release of IL-18 and IL-1b in macrophages and adipocytes [78, 143]. It is also well known that aluminum potassium sulfate (alum), a commonly used adjuvant, initiates inflammation by the same mechanism [144, 145]. Alum is often used as an adjuvant in allergic models due to its capacity to initiate an immune response. This study sought to understand whether hyperglycemic conditions could also promote an immune response to common allergic antigen. In this study we report that hyperglycemic conditions are able to promote allergic inflammation in a mouse model of allergic

sensitization. We hypothesized that exposing mice to chicken ovalbumin antigen (OVA) in hyperglycemic conditions would cause allergic sensitization comparable to the airway inflammation induced when mice are exposed to OVA with alum adjuvant (**Fig. 18**).

*RESULTS*

*Dextrose promotes inflammation and allergic inflammation in vivo*

Wild type mice were sensitized to chicken egg ovalbumin (OVA) using established protocol (**Fig. 19**) using dextrose or alum as adjuvant. Following the final challenge, animals were harvested and tissues analyzed for immune response. Mice sensitized to OVA in the presence of glucose had significantly higher numbers of cells in bronchoalveolar lavage (BAL) compared to controls (**Fig. 20**), indicating significant airway inflammation. Flow cytometry analysis of BAL showed a significant decrease in BAL neutrophils in dextrose/OVA sensitized mice as a % of total CD45+ cells (**Fig. 21**). Interestingly, flow cytometry of lung homogenates showed a significant increase in lung neutrophils while eosinophils, T cells, and B cells were unchanged (**Fig. 21**). Mice sensitized with dextrose/OVA showed a slight but nonsignificant increase in OVA-specific IgE following OVA challenge compared to control mice (**Fig. 21**). However, lung qPCR analysis showed increased levels of *Il4*, *Il33*, and *Il13* in dextrose/OVA sensitized mice (**Fig. 22**). These data suggest that mice sensitized to OVA in the presence of dextrose mount a mixed Th1/Th2 allergic response.

*Intraperitoneal injection of glucose induces inflammation in the peritoneal tissue and accumulation of antigen presenting cells in the peritoneum*

Mice were injected intraperitoneally with dextrose 1 hour prior to harvest and analysis (**Fig. 23**). After injection, mice exhibited increased blood glucose levels at 30 and 60 minutes compared to saline controls (**Fig. 23**). Following injection, dextrose treated mice exhibited an increased

number of cells within the peritoneal lavage and increased expression of *Siglec5* in the peritoneal tissue (**Fig. 24**).

To ensure that inflammation was not due to hypertonic effect causing degranulation of mast cells, bone marrow derived mast cells (BMMCs) were cultured to maturity (**Fig. 25**). Beta-hexaminodase release assay showed that exposure to hypertonic glucose solution did not cause mast cell degranulation (**Fig. 25**). The results of both dextrose sensitization and acute hyperglycemic challenge suggest that hyperglycemic conditions create a pro-inflammatory environment that increases the chances of antigen sensitization and airway inflammation. The working hypothesis is detailed in **Fig. 26**.

*Blocked NLRP3 assembly impairs establishment of tolerance to common OVA antigen*

A mouse model of induced antigen tolerance was used to determine whether impairment of the NLRP3 inflammasome signaling impairs establishment of tolerance. In the tolerance model **(Fig. 27**), mice were injected with MCC950 during the tolerance phase to determine whether tolerance to OVA could be established when NLRP3 assembly is blocked.

Following antigen challenges, OVA-specific IgE was detected in OVA tolerized mice, but was produced at significantly lower levels in mice tolerized to OVA in the presence of MCC950 (**Fig. 28**). Both groups of OVA tolerized mice (with and without MCC950) had an increased number of cells in the bronchoalveolar lavage following challenge (**Fig. 28**). qPCR analysis demonstrated that OVA tolerized mice had basal levels of *Il4*, *Il5*, and *Il13* with and without high dextrose exposure (**Fig. 29**). Flow cytometric analysis revealed that mice tolerized to OVA in the presence of MCC950 had a significantly lower number of $CD45^{+}/SigF^{+}$ eosinophils compared to non-MCC950 OVA tolerized mice (**Fig. 30**). Interestingly, OVA/MCC950 mice had a significantly higher amount of eosinophils present in BAL (**Fig. 30**). These results suggest that MCC950

exposed mice are not able to develop traditional antigen specific antibody or increased BAL count, but do show evidence of inflammation following allergic challenge.

*DISCUSSION*

In this study, we tested the ability of high glucose conditions to serve as an adjuvant in sensitization to common antigen. Because alum, a well-studied adjuvant, facilitates sensitization through activation of the same inflammasome that glucose is able to activate [144, 145] , we hypothesized that glucose may have similar adjuvant properties. High glucose concentrations are able to induce the release of IL-1b and IL-18, and promote the release of mitochondrial ROS [13, 146]. Due to the induction of these pro-inflammatory cytokines and molecules, we hypothesized that high glucose concentrations would create a pro-inflammatory environment that is capable of inducing sensitization to antigen. This is relevant because human patients with allergic diseases have increased rates of metabolic syndrome, obesity, and diabetes [22, 140-142]. It is unknown whether the presence of metabolic syndrome and related disorders make patients more likely to develop allergic disorder, or the inverse. This study demonstrates that the hyperglycemic condition creates a pro-inflammatory environment within the allergic context.

Studying the effect of glucose dysregulation specifically is vital to understanding the mechanisms that link allergic disease with metabolic disorders. Multiple reviews have hypothesized that impaired glucose metabolism is the mechanistic link between obesity and asthma [47, 147]. A recent study by Park et al. showed that insulin resistance, which leads to hyperglycemia, mediates airway hyperresponsiveness in a mouse model of pulmonary fibrosis [148]. Because metabolic disorders can cause dysregulation of metabolism of a number of substrates including glucose, lipids, and fatty acids, it is important to study each of them to understand how they impact allergic sensitization.

One particularly striking result of this study was the increase in lung neutrophils in dextrose/OVA sensitized mice and the decrease in neutrophils in the bronchoalveolar lavage (BAL). Other cell types including eosinophils, B cells, and T cells were relatively unchanged based on flow cytometry analysis. The increase in lung neutrophils and decrease in BAL neutrophils suggests that neutrophil egress from the lung was restricted in dextrose/OVA sensitized mice. The mechanisms that link these phenomenon are poorly understood. A recent study showed that UDP-glucose promotes neutrophil recruitment to the lung in a mouse model of cystic fibrosis [149]. UDP conjugated sugars are released commonly from glycosylation activity, and are known to activate purinergic receptors on a wide variety of cell types. UDP also has roles in airway hyperresponsiveness and is known to exacerbate airway hyperresponsiveness in mouse models of allergic asthma [150]. Taken together, these research suggest that restriction of neutrophil egress could be due to UDP recruitment of neutrophils specifically to the lung during airway inflammation. Another mechanism that could potentially influence neutrophil activity in the allergic sensitization model of is resistin. Resistin is canonically known as a gut-derived peptide that induces feelings of satiety after eating. However, studies have demonstrated that resistin promotes proinflammatory neutrophil activation and neutrophil extracellular trap formation in mouse models of acute lung injury [151]. My studies have demonstrated that resistin is upregulated following allergic challenge in the airway sensitization model. The role of systemic purinergic signaling and gut hormones should not be understated in studies of allergic airway inflammation, as this body of work demonstrates the truly systemic nature of inflammation and diverse number of tissues involved.

While mice sensitized to OVA using dextrose as an adjuvant did not significantly increase their production of OVA-specific IgE, they did produce increased cells in the BAL, neutrophils in the lung, and type 2 cytokines in the lung. Production of specific IgE is associated with a Th2 high

response, but since the induced response was mixed Th1/Th2, it is possible that antigen specific antibodies belong to another antibody isotype such as IgG. The increased lung inflammation is especially striking due to the fact that dextrose was injected only into the peritoneum, and not the lung or airway. Though hyperglycemia was never induced directly in the airway, systemic elevation of blood glucose was evidently able to induce lung-specific exacerbation of inflammation. The monitoring of blood glucose following showed that blood glucose ranged from 100-150 mg/dL following injection, which is comparable to a diabetic blood glucose range before meals [152]. Blood glucose returned to normal within 2-3 hours of injection as expected, but likely induced downstream effectors that induced inflammation over the longer term. This further supports the notion that hyperglycemia has far-reaching, systemic effects on diverse tissues.

Multiple studies have demonstrated that glucose causes inflammation through activation of the NLRP3 inflammasome, and have shown that diverse cell types such as macrophages and adipocytes are activated by hyperglycemic conditions [153, 154]. It remains unknown which cell types are activated to promote sensitization in our hyperglycemic model of sensitization. Literature suggests that not only immune cells, but also structural cells such as the epithelium and stromal cells contain NLRP3 inflammasomes that are capable of being activated to induce inflammation. Future studies will be needed to determine the mechanism underlying hyperglycemic sensitization.

## *CHAPTER 5: KEY EPITHELIAL METABOLIC PATHWAYS WITH RELEVANCE TO ALLERGIC DISEASE*

Balanced metabolism is key to normal barrier function of the epithelium. The airway epithelium is the first defense barrier encountered by an inhaled allergen (1). Disruption of the epithelial barrier is thought to make it more susceptible to allergic sensitization (2, 3). Epithelial barrier dysfunction is now thought of as a central event in the initiation and progression of allergic diseases (2, 4). Despite the growing appreciation of critical role for metabolism in homeostasis and chronic disease, there are surprisingly few studies of its contribution to allergic disease pathogenesis. The clinical evidence reviewed above illustrates the complexity of relationships between allergic and metabolic conditions, likely complicated by clinical endophenotypes of disease and multi-faceted and context-dependent nature of systemic metabolic dysfunction. While clinical evidence points to clear associations between various metabolic factors and atopic disease, there is still a very limited understanding of the mechanisms that link the two. In this part of the review, we are going to highlight several metabolic pathways in epithelial cells with relevance to allergy that are linked to processes of barrier deficiency, inflammation and allergic sensitization.

### *Insulin signaling and glucose metabolism*

Epithelial cells express insulin receptor and depend on insulin signaling for proliferation, differentiation and upstream regulation of glucose transport mechanisms and bioenergetics pathways (summarized in **Figure 34**). The critical role for insulin in maintenance of epithelial homeostasis has long been appreciated. The first efforts to culture bronchial epithelial cells from human lungs, dating back to the 1980s, demonstrated that insulin is essential for epithelial culture [155-157]. Today, insulin is a necessary supplement in all commercial growth and differentiation media for primary epithelial cultures. Multiple studies show that signaling defects

in insulin receptor or insulin receptor substrate molecules impair the differentiation and function of the epithelium and other cell types [158-160]. This may directly translate to the human condition, as insulin resistance is linked to multiple skin diseases [62, 161]. Insulin resistance in diabetes is directly linked to deficiencies in wound healing and epithelial dysfunction [162].

In mouse models, systemic hyperinsulinemia promotes airway hyperresponsiveness, collagen deposition and airway remodeling, which may be due to decreased pathogen clearance across the strengthened epithelial barrier [163]. Increased insulin in cultures of primary human nasal epithelial cells results in increased expression of MUC5AC at the mRNA and protein level [164]. MUC5AC is the mucin responsible for the viscoelasticity of mucus, which is produced to protect the airway epithelium from pathogens and dehydration. Decreasing insulin supplementation in epithelial culture (which aligns with cellular effects of insulin resistance) has been shown to increase expression of PAR2 on human airway epithelial cells, which is a proteinase activated receptor linked to allergic sensitization [165, 166]. We previously published a comparative study of epithelial gene expression patterns convergent across several transcriptomic studies of human adult asthma [167]. This computational study revealed that the downregulation of insulin signaling in epithelial cells represented a surprisingly conserved and most consistent feature across multiple studies of mild, moderate, and severe asthma. This was overrepresented by the downregulation of insulin target genes INSR, IRS2, FGFR1, and FGFR2, which progressed with asthma severity [167]. Our validation of data mining results in primary human bronchial epithelial cells grown in submerged and air-liquid interface conditions revealed that gene expression of healthy epithelial cells grown without insulin closely matched gene signatures of asthmatic epithelial cells at normoinsulinemic baseline [167]. Our further interrogation of insulin pathway genes in asthma revealed that this pattern of dysregulation is more specific to the Th2 high molecular endotype of asthma previously described by Woodruff et al. [168]. Moreover,

both insulin receptor (INSR) and insulin receptor substrate 2 (IRS2) showed significant negative association with markers of Th2 high inflammation (POSTN, SERPINB2) (unpublished data). These results indicate that the downregulation of insulin receptor and its proximal signaling targets is a conserved feature in asthmatic epithelium closely associated with Th2 high inflammation.

Insulin has several important interacting partners at the level of cellular membrane and downstream signaling (**Figure 34**). Among them, glucose transport proteins are differentially regulated by insulin, which determines rates of uptake and utilization of glucose in cellular biosynthetic and bioenergetics pathways. There are 14 glucose transporters (GLUT or SLC2A) encoded by the human genome, which are membrane proteins that facilitate the transport of glucose across the plasma membrane. Class I transporters (GLUT1-4) are best characterized with GLUT4 traditionally studied as an insulin-dependent transporter [169]. It is currently unknown whether glucose transporter biology is altered in allergic disease. At the level of downstream signaling, Molina et al. demonstrated that insulin regulates glucose uptake and barrier function in human airway epithelial cells via PI3K/Akt signaling pathway [170]. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase signaling pathway that is responsive to changes in PI3K/Akt pathway and controls insulin signaling [171]. mTOR controls a wide range of cellular processes, including cell growth, differentiation, and glucose and lipid metabolism [172, 173]. Dysregulation of mTOR leads to a number of metabolic pathological conditions, including obesity and type 2 diabetes [172]. Moreover, mTOR proteins are regulatory for epithelial morphogenesis and regulate epithelial barrier formation [174].

With immediate relevance to allergic inflammation, it is well documented that Th2 cytokines IL-4 and IL-13 signal through insulin receptor substrates IRS1 and IRS2 for activation of PI3K and other downstream pathways [175]. Originally discovered by Dr. William E. Paul in 1990s,

signaling interactions between IL-4/IL-13 receptors and insulin receptor substrates have been studied for over 25 years [176]. Although many facets of this interaction have been described in terms of cellular proliferation and survival, it is still not clear whether IRS2 is a positive or negative regulator of IL-4 and IL-13-induced responses, the potential of which is hardly explored in allergic diseases. Why Th2 cytokine signaling is so closely and proximally intertwined with key mediators of insulin signaling and metabolism remains an enigma.

*Insulin-like growth factor signaling*

Insulin-like growth factors (IGFs) are signaling hormones that share signaling with the insulin receptor signaling pathway. IGFs are predominately produced in the liver but can also be produced by many types of peripheral cells and has autocrine and paracrine functions. IGF-1, the primary IGF implicated in allergic airway inflammation, binds to insulin-like growth factor 1 receptor (IGF-1R) [177]. IGF-1 shares considerable homology with insulin, and can bind to insulin receptor [178]. IGF-1 binds to insulin-like growth factor binding protein 3 (IGBP3), and forms a complex that transports IGF-1 through the blood to target tissues. When bound to IGBP3, IGF-1 is not bioavailable and cannot activate signaling mechanism downstream of its receptor. Interestingly, IGF-1, IGF-1R, and IGFBP3 have multiple roles in the promotion and exacerbation of allergic inflammation. In mice sensitized to chicken-egg ovalbumin (OVA), treatment with an IGF-1 neutralizing antibody inhibited elevation of airway resistance, inflammation, and airway wall thickening in response to OVA challenge [179]. A study in human asthmatics demonstrated a correlation between IGF-1 expression and collagen thickening in bronchial biopsies [180]. Treatment with inhaled corticosteroid beclomethasome dipropionate decreases epithelial IGF-1 expression in bronchial biopsies of asthmatic patients [180]. Expression of IGF-1 and IGF-1R can also be regulated by Th17 cytokines [181] and eosinophils

[182]. Lee et al. [183] provide an excellent review on the diverse roles of IGF-1 and IGFBP3 in allergic airway disease and discuss the potential for therapeutic targeting of IGF-1 signaling.

*Mitochondria*

It is well established that chronic inflammation accompanies insulin resistance, in part due to increases in oxidative stress in insulin-resistant cells [184-186]. Allergic asthma is also characterized by increased oxidative state [186], although mechanistic links between oxidative stress and asthma remains poorly explored. Recent work by our group and others suggests that changes in mitochondrial function may play a significant roles in the pathogenesis and exacerbation of asthma (summarized in **Figure 35**). Electron microscopy has revealed ultrastructural changes in airway epithelial mitochondria in mouse models of asthma or biopsies from human subjects with asthma, associated with biochemical features of mitochondrial dysfunction such as increased oxidative damage or activation of apoptotic pathways [187, 188]. Xu et al. [187] have found that mitochondria in asthmatic bronchial epithelial cells have increased expression of iNOS, ARG2, and mitochondrial respiratory complexes III and IV. The study also reports a marked increase in oxygen consumption rate (OCR) and glycolysis measured by extracellular acidification rate (ECAR) in asthmatic epithelium [187]. A study by Winnica et al. [189] also found an increase in OCR parameters in cells from both lean and obese donors, and increase in ECAR in cells from obese asthmatic donors. Moreover, these increases were documented not only in epithelial cells but also platelets from asthmatic donors, which suggests a systemic-level metabolic dysfunction in these patients [189]. A key consequence of increase in oxidative phosphorylation is the generation of ROS. The primal ROS made by the mitochondrial electron transport chains is O–·, which is catalyzed by superoxide dismutase (SOD) to yield $H_2O_2$, the ROS most relevant to cell signaling [190]. A large body of evidence indicates that ROS plays a vital role in airway signaling and inflammation

by modulating cytokine transcription [190, 191]. Moreover, increased mitochondrial respiratory and glycolytic activity could explain the increased extracellular ATP commonly found in asthmatic exhaled breath condensate [192].

Mitochondrial products such as reactive oxygen species (ROS), excessive ATP, and mitochondrial DNA (mtDNA) are capable of initiating inflammation via potent activation of the nod-like receptor 3 (NLRP3) inflammasome, a group of proteins responsible for promoting an IL-1 mediated response [13, 193, 194]. In human tracheal epithelial cells, inhibition of mitochondrial ROS has been shown to downregulate expression and activation of NLRP3, its effector enzyme caspase-1, and pro-inflammatory cytokine IL-1β [11]. NLRP3 activation plays important roles in both type 1 and type 2 inflammation. Mouse models have demonstrated that activation of NLRP3 is necessary for allergic sensitization and establishment of allergic airway inflammation [11]. A mouse model of *Aspergillus fumigatus* induced airway inflammation demonstrated that inhibition of mitochondrial ROS in mouse epithelium through calcium signaling decreased AHR, eosinophil recruitment, NLRP3 activation, and NF-kB activation [195]. Another mouse study similarly demonstrated that epithelium mitochondrial inhibition through ubiquinol-cytochrome c reductase II core protein (UQCRC2) deficiency increased airway eosinophil infiltration, mucin, and AHR in response to ragweed challenge in sensitized mice [196]. In human airway epithelial cells, inhibition of mitochondrial $Ca^{2+}$ uniporter (MCU) protected cells against IL-13 induced apoptosis, and maintained mitochondrial membrane potential [197]. These human and mouse mechanistic studies demonstrate both that mitochondria in epithelial cells regulate the type 2 immune response, and suggests that mitochondrial dysregulation in epithelial may underlie and exacerbate type 2 inflammation. Mitochondria also play a key role in the airway remodeling [197, 198]. For example, mitochondrial ROS in airway epithelium have been shown to regulate collagen production [199].

Mitochondrial ROS in airway epithelium are necessary activators of TGF-β, which drives airway remodeling and collagen deposition commonly seen in asthma and allergic airway disease [198]. The importance of mitochondria in lung disease is also supported by genetics, for example, by associations of the mitochondrial haplogroup U with increased IgE and asthma [200, 201].

*Arginine and nitric oxide metabolism*

Several recent studies linked altered mitochondrial function to enhanced arginine metabolism as well as changes in nitric oxide bioavailability in the asthmatic airway. Studies of both lean and obese asthmatics demonstrated increased metabolism of arginine to ornithine due to the upregulation of arginase [187, 202, 203]. Increased mitochondrial respiration in asthmatics [187, 204] (described in the section above) has been specifically attributed to enhanced arginine metabolism to ornithine by upregulated arginase, which leads to generation of glutamate through catalysis by ornithine amino transferase and drives the production of substrates for the tricarboxylic acid (TCA) cycle [187]. Airway inflammation is also characterized by increased expression of inducible nitric oxide synthase (iNOS) [205, 206], which results in elevated production of fractional exhaled nitric oxide (NO) (FENO) in allergic asthmatics [205, 207]. Nitric oxide is metabolized from arginine and is produced constitutively in the airways of healthy individuals. In patients with allergic airway inflammation, elevated FENO is a biomarker for the presence and severity of asthma [205, 206, 208]. Increased epithelial iNOS activity and expression is commonly reported in type 2 allergic disease [187, 205, 206, 209, 210]. Expression and activity of iNOS can be induced by a number of inflammatory mediators and cytokines, including LPS, IFN-$\gamma$, and IL-13 [209, 211, 212]. Increased NOS activity can lead to an uncoupling from its substrate, arginine, which leads to the generation of superoxides and reactive nitrogen species (RNOS). iNOS uncoupling and subsequent RNOS induction in the

epithelium have been shown to promote downstream inflammation, and may be a contributing factor in allergic airway inflammation.

Notably, there are qualitative differences between arginine metabolism in epithelium from lean and obese asthmatics [213].The airways of obese asthmatics have been shown to be NO deficient, which contributes to airway dysfunction and reduced response to inhaled corticosteroids. FENO has been observed to be much lower in such asthmatic phenotypes despite iNOS upregulation, and this is thought to be due to deficiency of L-arginine, a critical substrate for nitric oxide synthase (NOS), as well as accumulation of asymmetric dimethylarginine (ADMA). This leads to decreased iNOS activity and uncoupling of the enzyme, which prevents NO synthesis [202, 203, 214]. In cultured human bronchial epithelial cells, L-citrulline, a precursor of L-arginine and NO formation, has been shown to prevent ADMA-mediated NO synthase (NOS2) uncoupling, restoring NO and reducing oxidative stress [213, 215]. In a short-term pilot clinical study, L-citrulline treatment improved asthma control and FeNO levels in obese asthmatics with low or normal FeNO [213]. Publications by Winnica et al. [189] and Holguin [216] further detail differences in this pathway between lean and obese asthmatics.

Although we are reaching greater mechanistic understanding of pathways and biomarkers downstream of arginine metabolism, increased mitochondrial respiration and alternative fueling of TCA cycle by amino acid metabolism suggest significant changes in upstream processes controlling epithelial bioenergetics in asthma. Although the upstream mechanisms are not yet known, changes in insulin and glucose metabolism in asthmatic epithelial cells (detailed above) represent one plausible candidate.

Although we are reaching greater mechanistic understanding of pathways and biomarkers downstream of arginine metabolism in asthma, increased mitochondrial respiration and

alternative fueling of TCA cycle by amino acid metabolism suggest compensation due to significant changes in upstream bioenergetic processes. Such upstream mechanisms are not yet known; however, changes in insulin and glucose metabolism in asthmatic epithelial cells (detailed above) represent one plausible candidate. Arginine metabolism, glucose metabolism and insulin signaling are intimately connected. It has been shown that in type 2 diabetes plasma arginase activity correlates with the degree of hyperglycemia, and is reduced by physiologic elevation in insulin levels [217]. Using [U-$^{13}$C]glucose metabolic flux analysis, which allows us to trace metabolism of glucose in downstream metabolic pathways, we found that glucose conversion to glycolytic intermediates for energy production is significantly decreased in asthmatic bronchial epithelial cells past the glucose 6-phosphate step in glycolysis (unpublished data). Rather, we found higher utilization of glucose in lipid, hexosamine and amino acid biosynthetic reactions upstream of arginine metabolism. **Figure 35** summarizes key metabolic pathways and highlights some of our recent findings in regard to metabolic changes in asthmatic epithelium. In lieu of connection of asthma to insulin resistance and obesity, utilization of glucose in bioenergetics vs. biosynthetic reactions and its upstream impact on arginine and nitric oxide metabolism warrants further investigation.

*PPAR-γ*

Peroxisome proliferator-activated receptor gamma (PPAR-γ) belongs to a family of nuclear hormone receptors that regulate lipid, fatty acid and glucose metabolism. In its canonical signaling role, activated PPAR-γ sequesters transcription factor NF-κB, which prevents the transcription of NF-κB and therefore prevents inflammation [218]. PPAR-γ is constitutively expressed in airway epithelial cells, and expression can be further induced by IL-4 administration [219]. Expression of PPAR-γ is elevated in the airway epithelium and airway smooth muscle of asthmatics [220], as well as in the nasal polyps of patients with seasonal

rhinitis [221]. The function of PPAR-γ in airway epithelia specifically remains a point of contention. While PPAR-γ is increased in asthmatic epithelium and correlates with decreased lung function [220], mouse studies suggest a protective role of PPAR-γ in the pathogenesis of allergic airway inflammation. Multiple studies have used PPAR-γ agonists to demonstrate that activation of PPAR-γ in mice ameliorates allergic symptoms including IgE, lung IL-4, IL-5, IL-13, mucus, AHR, and lung eosinophils [222-225]. A mouse study in which PPAR-γ was deleted specifically in airway epithelial cells *in vivo* showed a significant worsening of allergic airway inflammation and also demonstrated that PPAR-γ regulated MUC5AC and inflammatory cytokine production in cultured human bronchial epithelial cells *in vitro* [225]. Studies suggest that PPAR-γ is likely protective during the pathogenesis of allergic airway inflammation, and a less effective regulator of established inflammation [223]. PPAR-γ can also be regulated by a number of confounding factors including corticosteroid use and inhaled particulate matter [220, 226]. Further studies in airway epithelial cells are needed to determine the role of epithelial PPAR-γ at various stages of allergic airway inflammation. For further information, Banno et al. [227] have written an excellent review of PPAR-γ biology and its role in airway inflammation.

*Arachidonate metabolism*

Arachadonic acid (AA) is a fatty acid that can be metabolized into a number of lipid mediators called eicosanoids through the arachidonate metabolic pathway. Arachidonate enzymes and eicosanoids form a large, diverse family of mediators such as prostaglandins and cysteinyl leukotrienes that have both pro- and anti-inflammatory roles in atopic disease [228, 229]. Within the human airway, epithelial cells are the major source of airway eicosanoids with some contribution from airway infiltrating eosinophils [230-232]. Arachidonate 15-lipoxygenase (15-LO1 or ALOX15) and AA metabolite 15(S)-hydroxyeicosatetraenoic acid (15-HETE) are prominent in human airways and are increased in asthma [231, 233]. A landmark study by Zhao

et al. demonstrated that 15-lipoxygenase-1 (15-LO1) and 15-HETE in human airway epithelial cells induced production of MUC5AC, a major mucous protein overproduced during airway inflammation [230]. Multiple studies have also implicated AA metabolites in aspirin sensitivity disorders such as aspirin sensitive asthma/rhinosinusitis (ASARS) and aspirin exacerbated respiratory disease (AERD) [234, 235]. A number of other AA metabolites and enzymes have been associated with asthma including secreted phospholipoase A2 group X (sPLA2-X) and15-Oxo-ETE [235, 236]. Upregulation of these AA metabolites and enzymes has been largely associated with airway inflammation. However, some epithelial-derived AA metabolites, such as prostaglandin E2 (PGE2), have roles in wound healing and airway regulation [237]. The rich study of eicosanoids in airway inflammation has revealed a number of therapeutic targets, including inhibition of cyclooxygenase 1 and 2 (COX1 and COX2) by dexamethasone [237, 238].

*Summary and future directions*

Mechanistic and clinical study of epithelial metabolism in allergy represents a new frontier in the field that comes with multiple challenges. Studies of the epithelium in allergic diseases traditionally focus on physical and immune causes of barrier dysfunction, such as direct exogenous insult by allergens, proteases, injury, and inflammation [239]. Metabolic pathways reviewed here represent an entire suite of less appreciated but potent endogenous disruptors of epithelial homeostasis with key relevance to disease [240]. Several key questions remain to be answered in this regard. Complex interactions between systemic and cellular effects of obesity, metabolic syndrome, insulin resistance and inflammation in different disease contexts illustrate necessity to control for different aspects of systemic metabolic dysfunction in clinical and translational studies. Understanding metabolic underpinnings of clinical endophenotypes may bring us one step closer to personalized medicine and a systems biology level understanding of

allergic disease. More studies are necessary to bridge metabolic biomarkers and systemic metabolic dysfunction measured in clinical studies with mechanisms at the cellular level, as illustrated by studies connecting FENO biomarker levels with changes in arginine metabolic pathway. In particular, metabolic processes upstream of mitochondrial dysfunction and significance of insulin pathway in allergic asthma are still in their infancy. Although strongly supported by an ever growing number of mechanistic murine and *in vitro* studies, causality of metabolic dysfunction in promoting allergic inflammation is not yet understood. Longitudinal studies of maternal, in utero and postnatal metabolism hold great promise in elucidating gene-environment interactions, epigenetic reprogramming and early life susceptibility to allergic disease. Importantly, understanding early life factors may lead to new intervention and prevention strategies with upstream potential to target the development of allergy. Whether drugs used to treat metabolic disease can be repurposed to target allergic inflammation also remains to be investigated. Collectively, studies of metabolism have a great potential to reach different level of understanding of pathogenesis of allergic diseases and a new class of therapeutic approaches to target them.

*CHAPTER 6: OVERALL SUMMARY AND CONCLUSIONS*

Metabolic dysregulation is by no means a new phenomenon, but its presence is only increasing with the rise of the Western diet and sedentary way of life. This increased presence of metabolic dysregulation will continue to impact the way all human disease is studied, including allergic inflammation. Until recently, allergic studies focused almost entirely on the impact of exogenous antigen and environmental factors that influence antigen sensitization and inflammation. However, it has become increasingly apparent through clinical data that endogenous factors such as metabolism, glucose tolerance, and glucose metabolism do impact allergic airway inflammation. In our work we revealed that inflammation initiated in the airway has impacts on adipose tissue inflammation and the release of gut hormones. Our work demonstrated that adipose tissue is an additional site of type 2 high inflammation during allergic airway inflammation, and that adipose tissue is metabolized, likely to support the energetic needs of inflammation. It is also possible that there is direct immune communication between lung and adipose tissue, with immune cells traveling between the tissues in response to systemic cytokines and peptides. There is still much to be uncovered about precise nature of the immune relationship between lung and adipose tissue.

Mouse models of hyperglycemia revealed that high glucose conditions create a pro-inflammatory environment in the affected tissue and exacerbate inflammation in mouse models of allergic airway inflammation. We also revealed that inhibition of the NLRP3 inflammasome, the protein complex that glucose activates to cause inflammation, decreases production of antigen specific IgE, eosinophilia, and neutrophil infiltration in the lung following allergic challenge. These findings exemplify both the systemic nature of metabolic dysregulation and the central role of glucose metabolism in airway inflammation. However, continued investigation is

needed to further reveal the mechanisms that underlie the relationship between metabolic dysregulation and airway inflammation.

## *FUTURE DIRECTIONS*

### *Identifying cellular sources of inflammatory gene signature in VAT through scRNA-seq and cell sorting*

RNA-sequencing experiments revealed a type 2 inflammatory gene signature in visceral adipose following antigen sensitization in the airway. The gene signature included expression of type 2 cytokines, chemokines, and markers for B cells and eosinophils. What is not known, however, is which cells within the VAT and stromal vascular fraction actually produce these gene signatures. The VAT compartment contains diverse populations consisting of adipocytes, stromal cells, structural cells, and immune cells. Since cytokines such as IL-33, a Th2 skewing cytokine, can be produced by non-immune cells [241, 242], single cell RNA-sequencing should be used to determine which cell types are producing the observed inflammatory signatures.

### *Investigation of how lung inflammation activates VAT B cells*

Investigation of the VAT revealed a strong immunoglobulin mRNA presence associated with the activation of a B cell response. Previous papers have shown that B cells are present in homeostatic adipose tissue and help regulate adipose tissue function [100], so it is likely that B cells in the inflammatory VAT are becoming activated ad producing immunoglobulin in response to antigen challenge. Previous experiments to isolate and identify VAT B cells have been unsuccessful, and should be continued to analyze VAT B cells following allergic antigen challenge. Due to the low number of B cells within the VAT, both ELISA and ELISPOT should be used to analyze both antibody production and the frequency of antibody-producing cells in VAT. Additionally, single cell RNA-sequencing of the VAT will allow for further deconvolution of

the B cell immunoglobulin genotype by analyzing cells with high levels of CD19. Further experimentation should also investigate whether B cells taken on a protective, or “Breg” signature [243] vs. an inflammatory signature during allergic airway inflammation. Current evidence suggests that airway inflammation induces an inflammatory B cell signature.

*Understanding why adipogenesis genes in VAT are downregulated during lung inflammation*

Strikingly, analysis of the visceral adipose tissue revealed two related findings: 1) total mass of visceral adipose tissue dips following some allergic challenges in our model of allergic airway inflammation and 2) genes that regulate adipogenesis (the formation of adipocytes from stem cells) are downregulated. Further experimentation will be needed to confirm why this happens. It is likely that adipose tissue is being “browned”, a common process by which the body generates usable energy. This is likely happening to support the energetic demands of inflammation. Some of the major downregulated genes involved in adipogenesis were *Arxes1* and *Arxes2,* and rescue experiments could be performed to identify whether increased expression of *Arxes1* and *Arxes2* in VAT decrease the extent of inflammation observed in the airway.

*Determining whether hyperglycemia disrupts previously established tolerance and identifying immune cells involved*

Previous experiments in this work have determined how dysregulated glucose impacts the development of antigen tolerance in conditions of hyperglycemia. However, for many patients, metabolic dysregulation occurs later in life and thus has the potential to disrupt previously established tolerance to common antigen. Future experiments should investigate whether previously tolerized mice lose antigen tolerance following prolonged periods of hyperglycemia, high-fat diet, and other induced metabolic dysregulation.

*Analysis of how hyperglycemia impacts macrophages to initiate inflammatory cytokine production*

Of the extensive body of work studying the NLRP3 inflammasome, a lot of work has been done specifically in macrophages and dendritic cells examining how high glucose activates the inflammasome in macrophages. As a central player in the early innate immune response, it is especially important to investigate how hyperglycemia-driven inflammation in macrophages can potentiate an allergic airway response. The question to be answered regarding macrophages is two-fold: 1) answering whether hypergylcemic conditions simply activate macrophages and dendritic cells to produce inflammatory cytokines and cause an inflammatory signaling cascade and 2) determining whether hyperglycemic conditions alter the innate metabolism of these innate cells, causing alternative activation and antigen presentation to downstream cells.

*Use of NLRP3 inhibition and deletion to determine whether hyperglycemia-induced inflammation is NLRP3 dependent*

Since our experiments have demonstrated that hyperglycemic conditions exacerbate allergic airway inflammation, further studies should focus on determining the major mechanistic pathways for hyperglycemia-induced inflammation in vivo and in vitro. An existing mouse model with global NLRP3 deletion will help to solve multiple questions about hyperglycemia's potential to induce inflammation through the NLRP3 inflammasome. Individual cell types can be sorted and assayed in vitro and ex vivo as well. One major caveat to the model is that NLRP3KO mice cannot be sensitized to antigen using any known methods. Intact NLRP3 signaling is necessary for establishment of the allergic phenotype. The use of a conditional depletion or targeted, cell specific knockout model of NLRP3 deletion in eosinophils and macrophages should be explored for further mechanistic understanding.

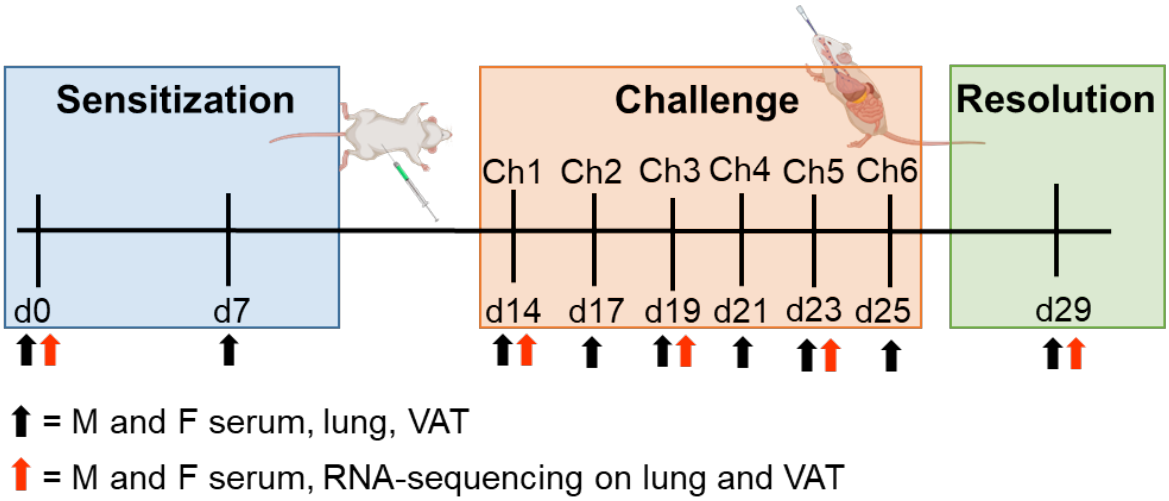



**Figure 1: Mouse kinetic model of allergic airway inflammation.**

Male and female mice were subjected to a kinetic model of allergic airway inflammation in a sensitization, challenge, and airway phase. N=8 female and n=8 male mice were harvested at each timepoint for analysis including RNA-sequencing.

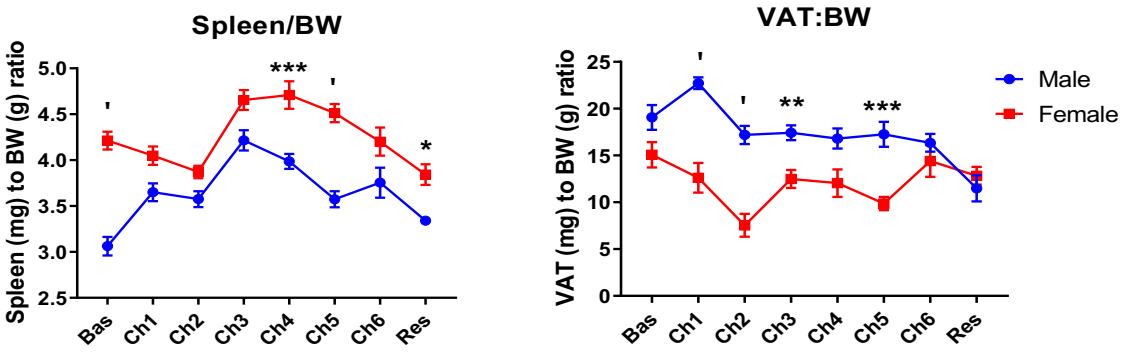



**Figure 2: Spleen and fat measurements in male and female mice at each harvest of kinetic model.**

Weight of spleens (left) and visceral adipose tissue (right) over the course of allergic model shown as a ratio of body weight *p < 0.05, **p < 0.01, ***p<0.001, ‡p < 0.0001, multiple t-tests with Benjamini, Krieger, and Yekuteli FDR correction.

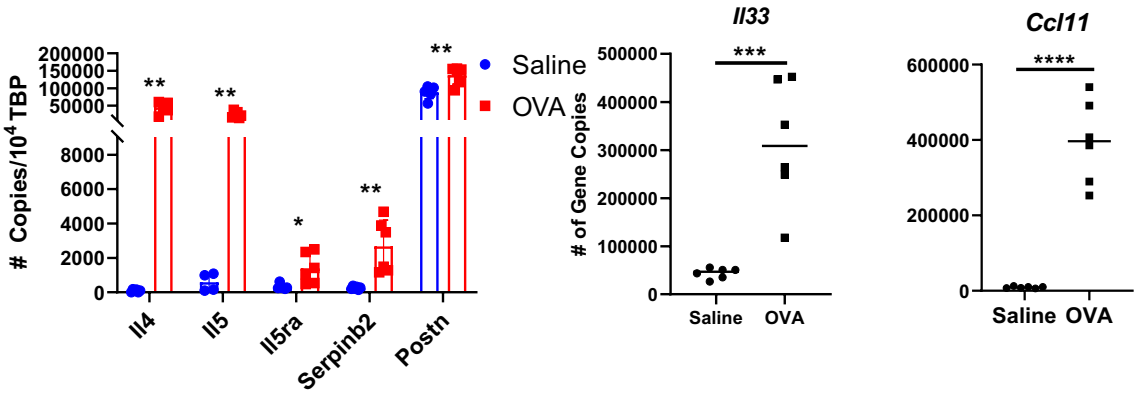



**Figure 3: Expression of type 2 inflammatory markers in the lung following challenge 3 of kinetic model.**

Graphs of lung cytokines and Th2 markers at gene level in female mice following challenge 3 *p < 0.05, **p < 0.01, ***p<0.001, ****p < 0.0001, (left) multiple t-tests with Benjamini, Krieger, and Yekuteli FDR correction and (right) t-test.

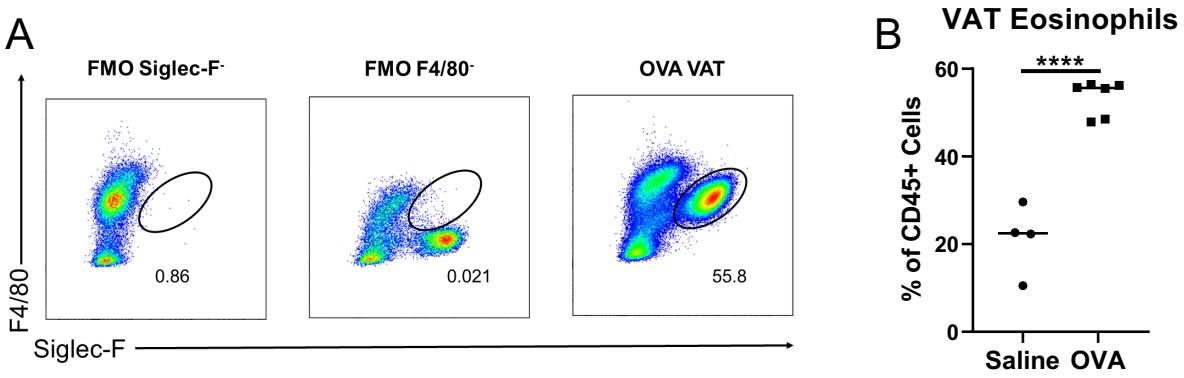



**Figure 4: Infiltration of eosinophils in the visceral adipose tissue following challenge 3 of kinetic model**

**(A)** Flow cytometry plots of Siglec-F$^-$ control (left), F4/80$^-$ control (center), and inflamed mouse sample (right) from independent validation of results following challenge 3. Representative samples are shown. **(B)** Quantification of eosinophils in VAT at baseline vs. challenge 3. n=4-6 mice per group, ****p<0.0001.

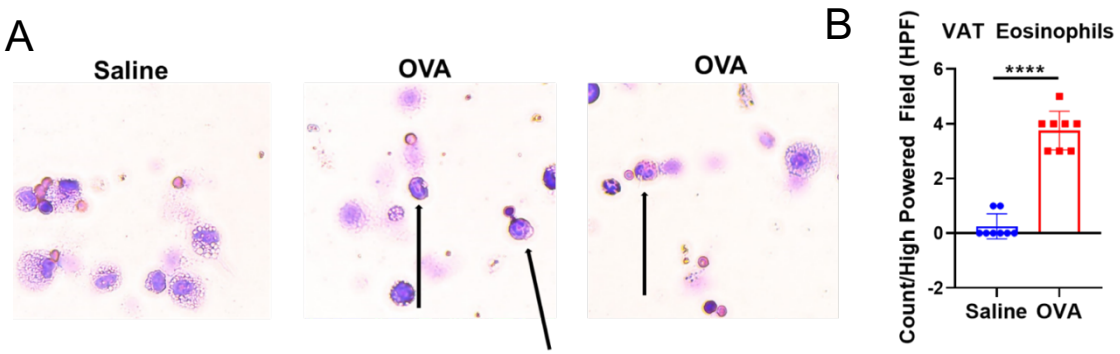



**Figure 5: Allergic lung inflammation induces eosinophil accumulation in VAT.**

**(A)** Images of immune cells isolated from VAT of control (left) and OVA sensitized (center, right) mice taken at 40x magnification. **(B)** Count of eosinophils per high-power field (HPF) in cytospins obtained from VAT. N=8 hpf counts from both saline and OVA mice following challenge 3. unpaired t-test, ****p<0.0001.

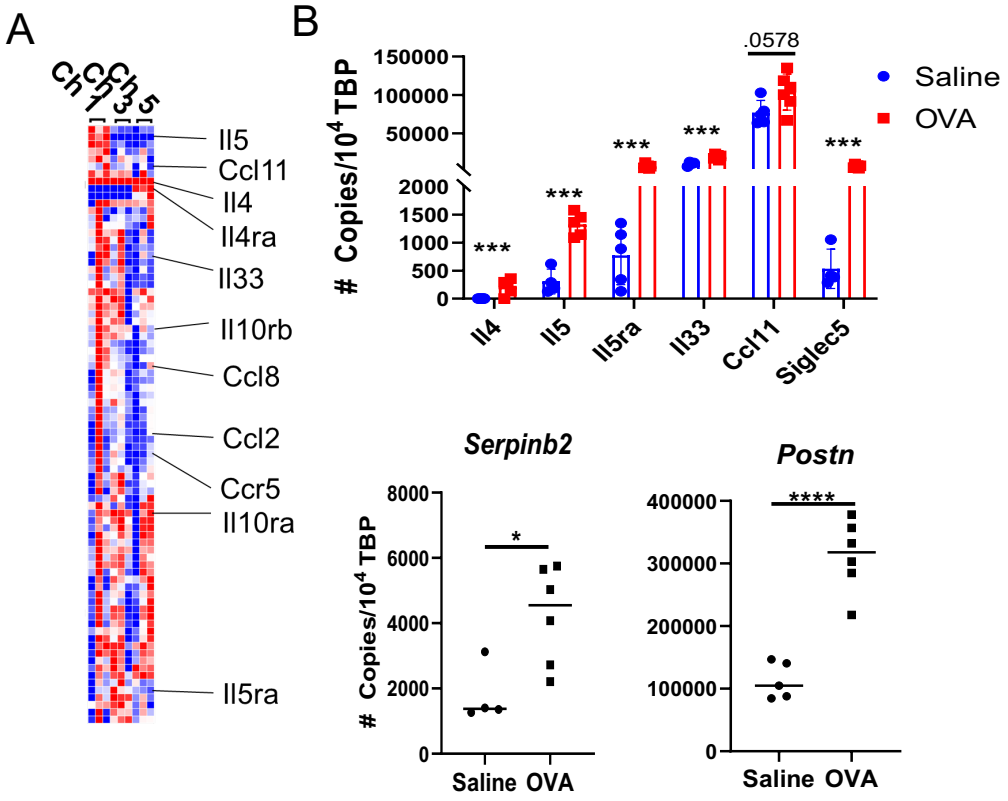



**Figure 6: Expression of type 2 inflammatory markers in adipose tissue following challenge 3 of kinetic model.**

**(A)** Heatmap genes identified as immune process related. **(B)** qPCR validation og type 2 cytokines and cell markers. T-test, n=4-6 mice per group, ***p<0.0001. (C) qPCR validation of type 2 high markers. T-test, n=4-6 mice per group, *p<0.05, ****p<0.0001.

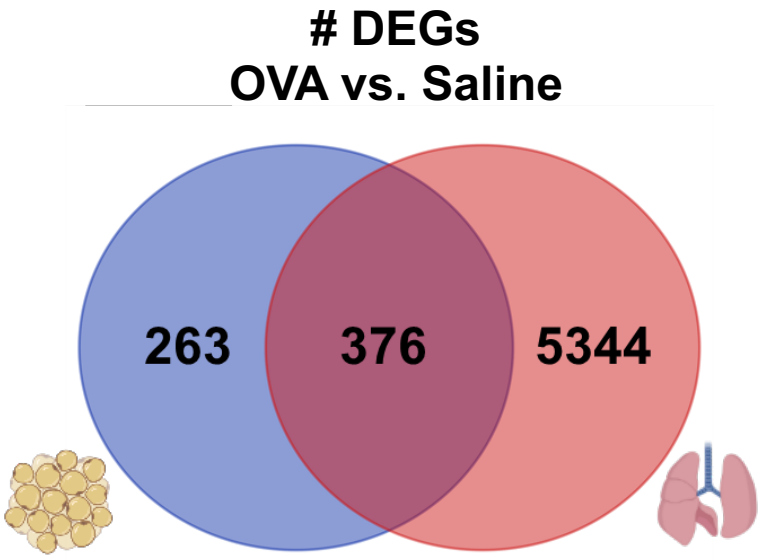
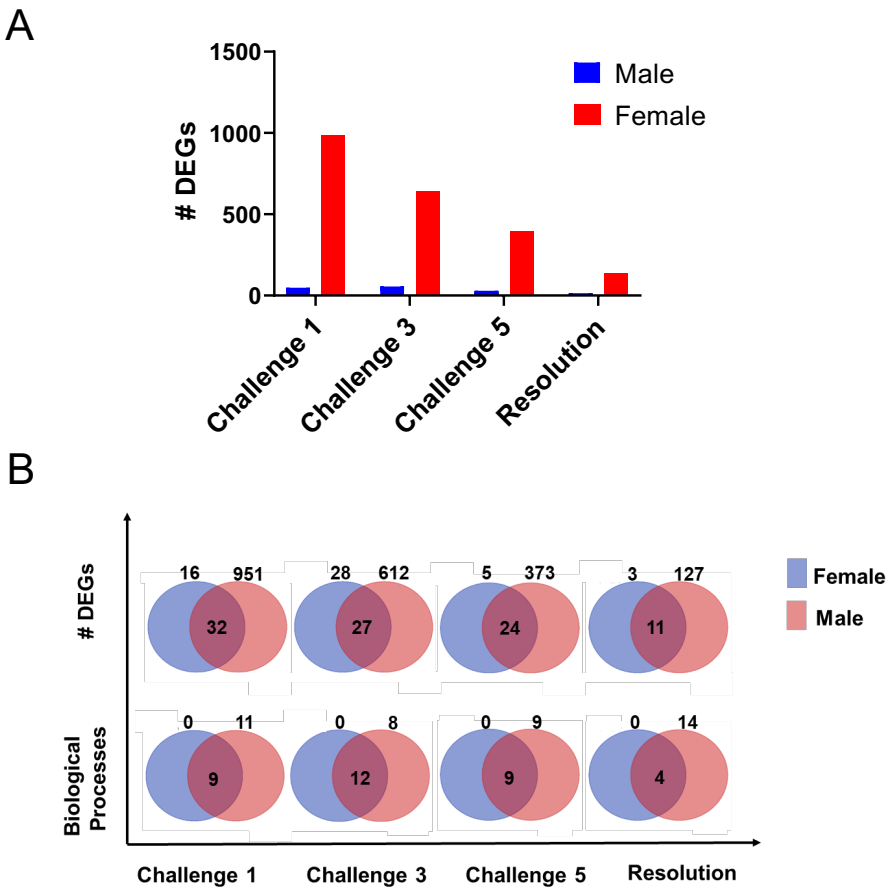



**Figure 7: Venn diagram of significantly changed genes in lung vs. visceral adipose tissue identified by RNA-sequencing.**

Left: VAT. Right: lung. DEGs shown as different expression in challenge 3 relative to baseline.




**'Figure 8: Total changed genes in adipose tissue over the course of the kinetic model in male and female mice.**

**(A)** Total # DEGs in male vs female VAT following challenge, n=3 male and n=3 female mice per group. **(B)** Sex-based comparison of significantly changed genes and biological processes represented following each allergic challenge.

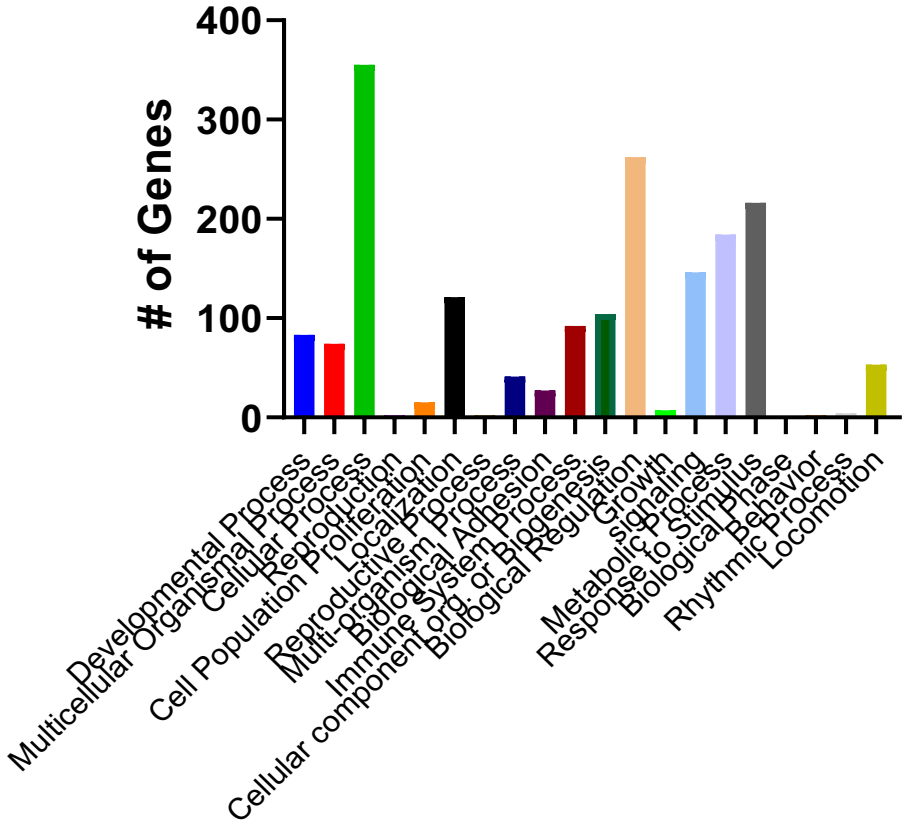
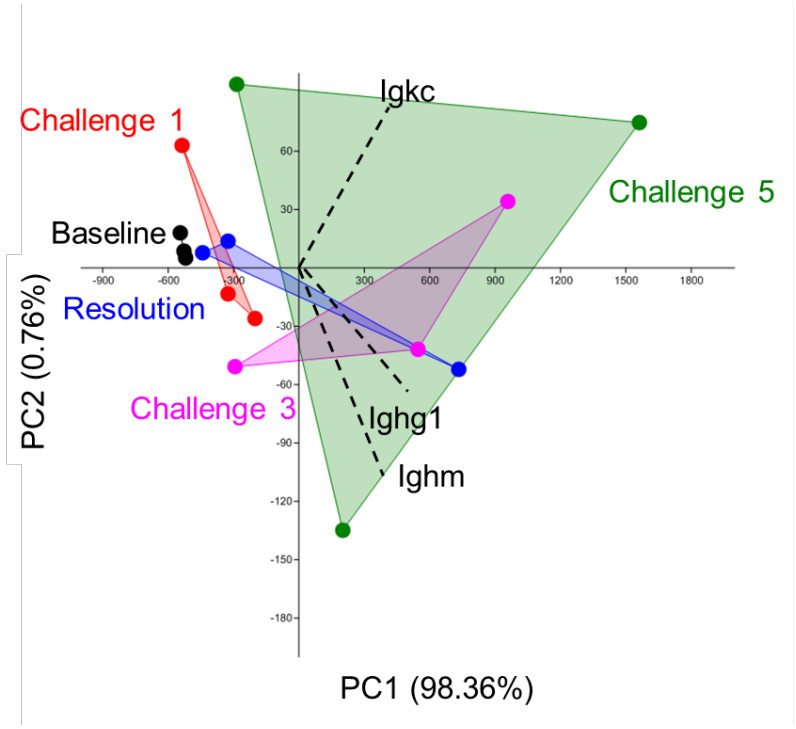



**Figure 9: Overview of biological processes changed significantly in visceral adipose tissue over the course of the kinetic model.**

Representation of biological processes represented in changed genes from RNA-seq on VAT. Number of genes represents total genes differentially expressed that are part of each biological process.

A




B

| Challenge 1 | Challenge 3 | Challenge 5 | Resolution |
|---|---|---|---|
| Iglv1 | Igkc | Ighg1 | Igkc |
| Igkc | Ighg1 | Igkc | Igkv14-126 |
| Igj | Igj | Igj | Igj |
| Igkv14-126 | Ighm | Iglv1 | Igha |
| Igkv3-7 | Igkv3-7 | Igkv14-126 | Igkv3-7 |

**Figure 10: Principal components analysis of significantly changed immunoglobulin genes in visceral adipose tissue following antigen challenge.**

**(A)** Principal components analysis of Ig related genes significantly altered following allergic challenge. **(B)** Table of top 5 significantly changed immunoglobulin (Ig) genes in each challenge compared to baseline.

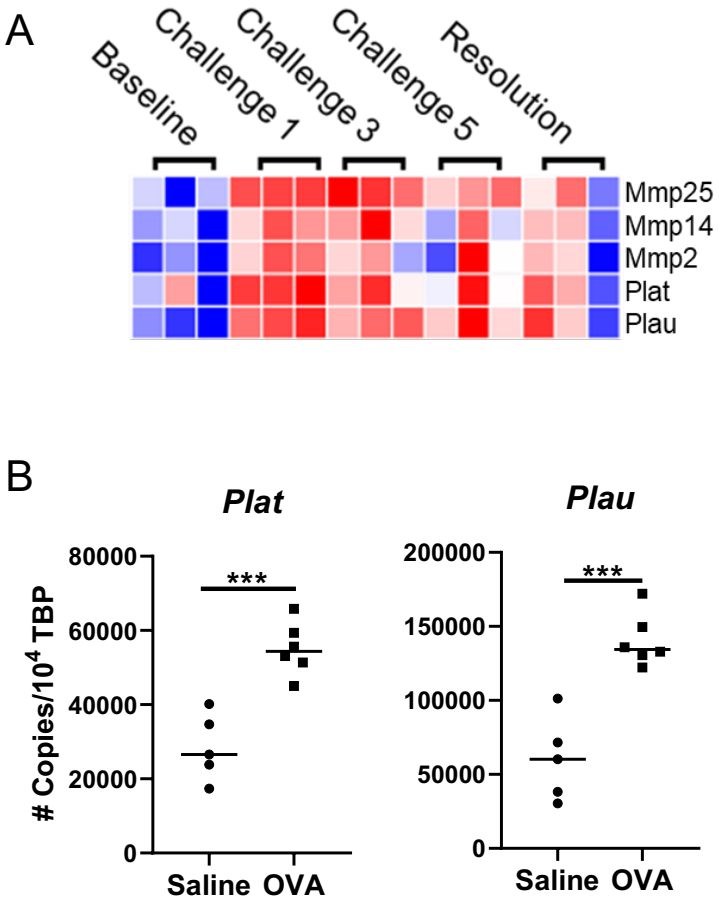



**Figure 11: Heatmap of significantly changed genes related to tissue remodeling in adipose tissue following antigen challenge, along with qPCR validation in lung and adipose tissue.**

**(A)** Heatmap of differentially expressed genes related to extracellular matrix and remodeling in female mice. **(B)** Independent qPCR validation of gene expression following challenge 3 of allergic model, n=4-8 female mice per group. ***p < 0.001, unpaired t-test.

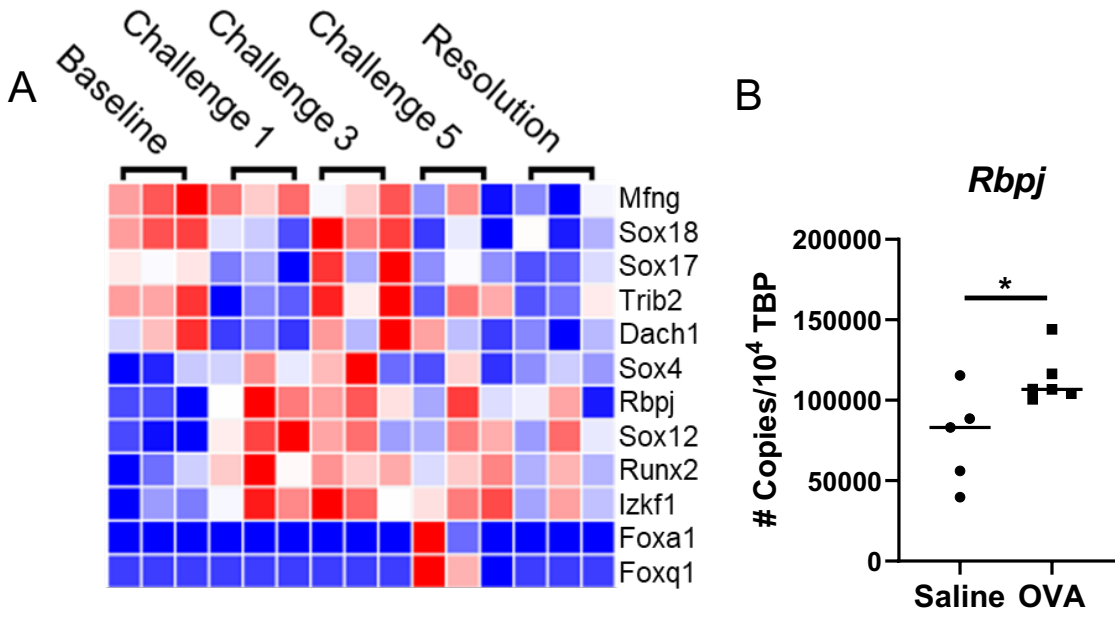



**Figure 12: Heatmap of significantly changed genes related to development in adipose tissue following antigen challenge, along with qPCR validation in adipose tissue.**

**(A)** Heatmap of differentially expressed development related genes in female mice. **(B)** Independent qPCR validation of gene expression following challenge 3 of allergic model, n=4-8 female mice per group. ***p < 0.001, unpaired t-test.

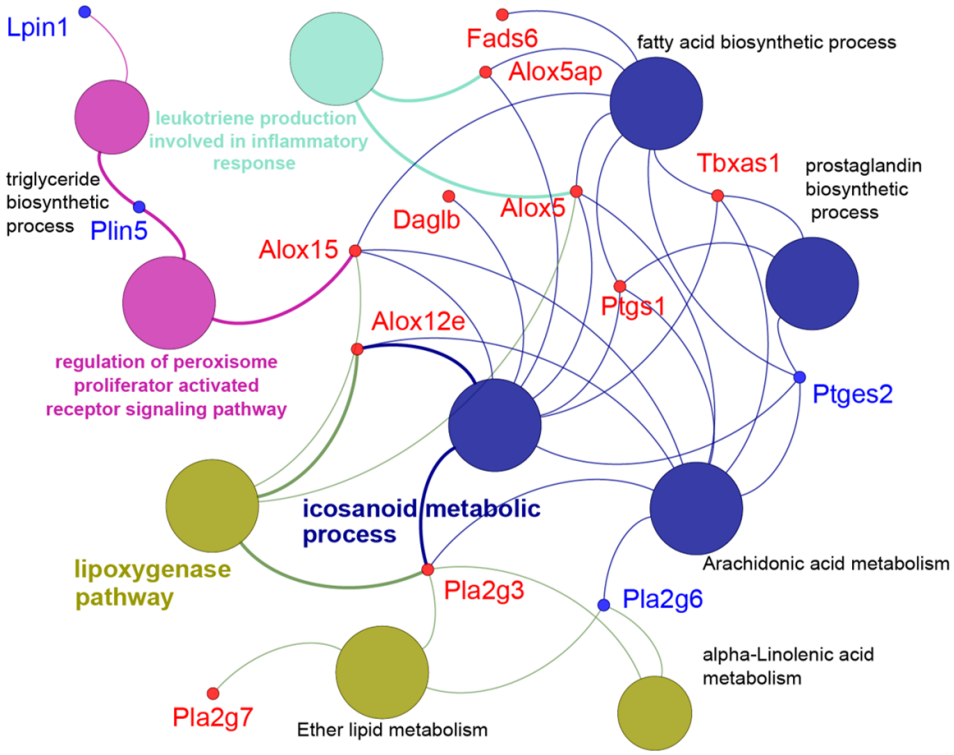



**Figure 13: Network analysis of metabolic genes significantly changed in visceral adipose tissue following antigen challenge.**

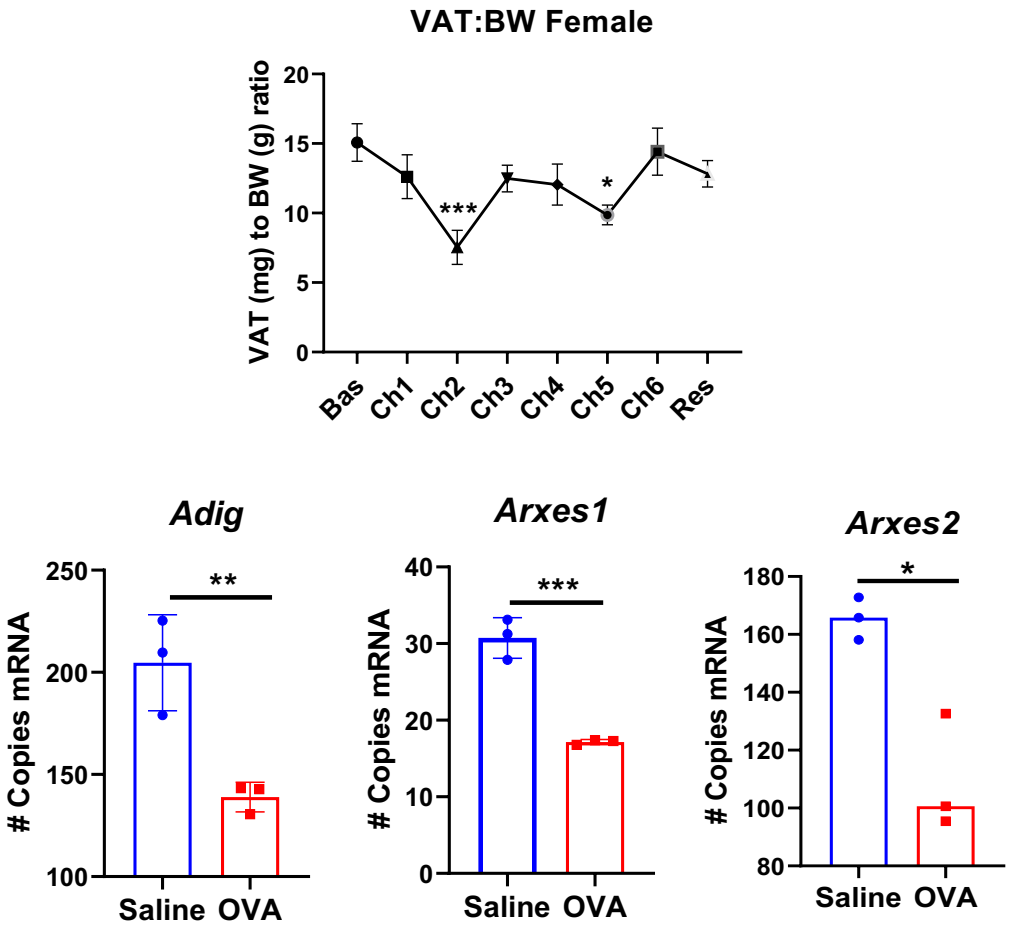



**Figure 14: Altered metabolic signature in VAT is induced by lung inflammation.**

**(A)** Female VAT significantly decreases following 2nd challenge of kinetic model n=4-8 mice/group. Multiple t-tests with FDR correction. **(B)** Decreased expression of genes related to adipocyte differentiation in RNA-seq following challenge 3, n=3 mice/group, t-test. *p<0.05, **p<0.01, ***p<0.001.

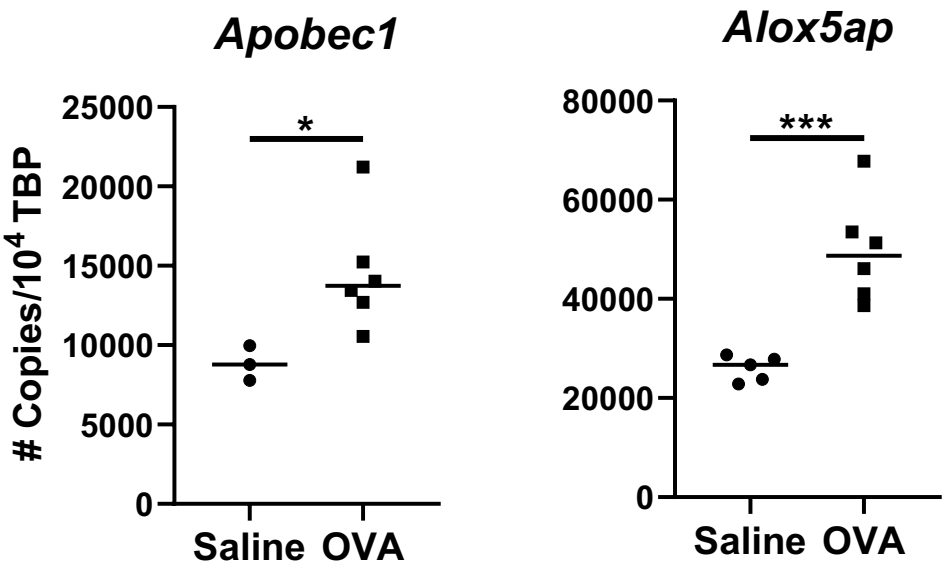



**Figure 15: Expression of genes related to lipid metabolism are increased in VAT during allergic lung inflammation.**

Independent qPCR validation of genes related to lipid metabolism n=4-6 mice/group, t-test. *p<0.05, **p<0.01, ***p<0.001.

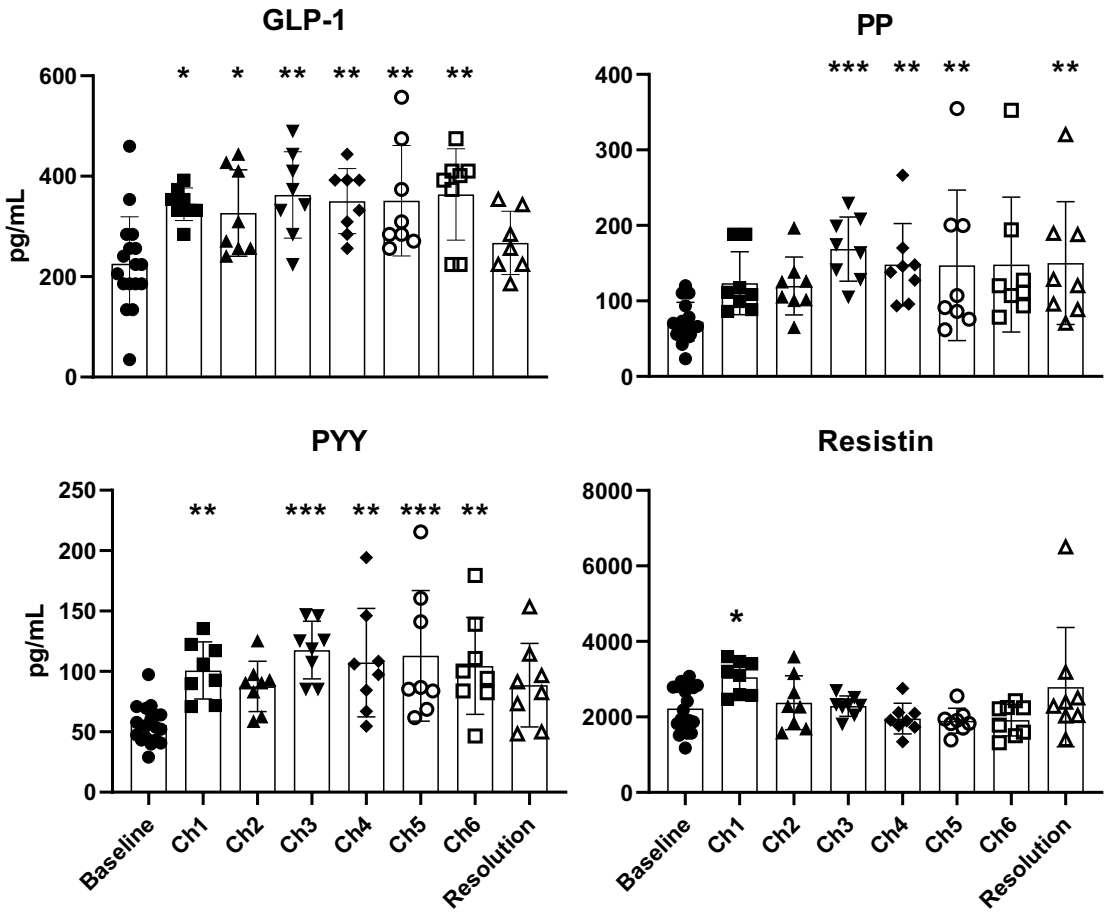



**Figure 16: Increased production of gut-derived metabolic peptides in serum following each antigen challenge of kinetic model.**

Luminex analysis of metabolic peptides in serum following each step of allergic model. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, ANOVA followed by multiple comparisons to baseline.

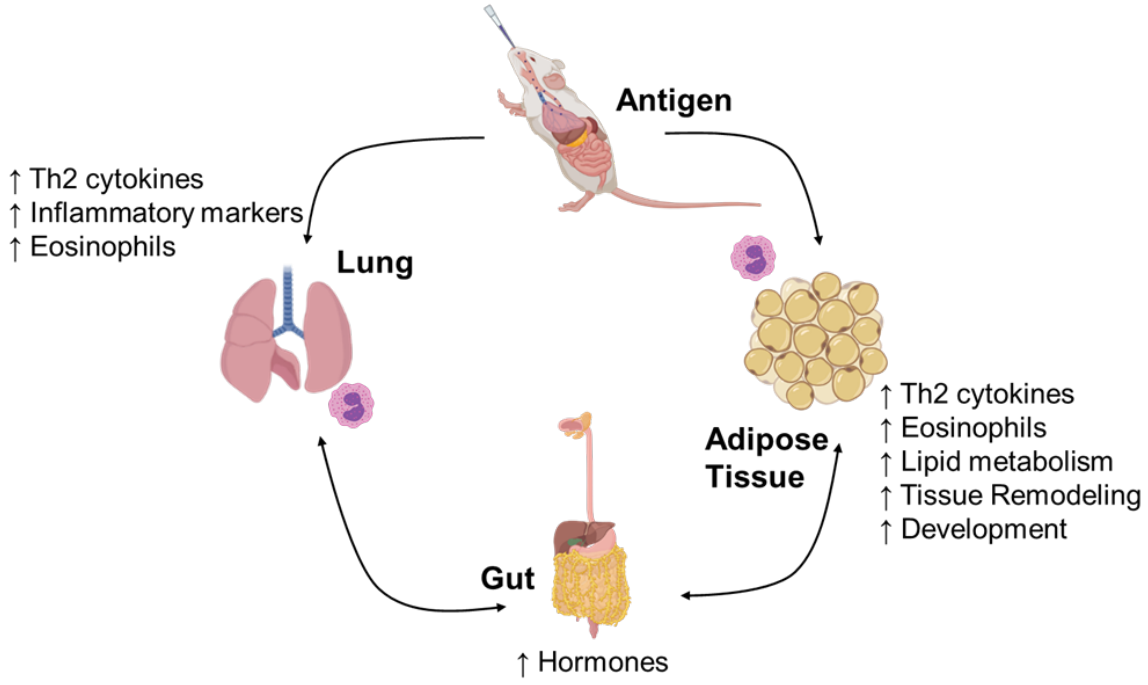



**Figure 17: Proposed model of communication among systemic tissues in response to allergic lung inflammation.**

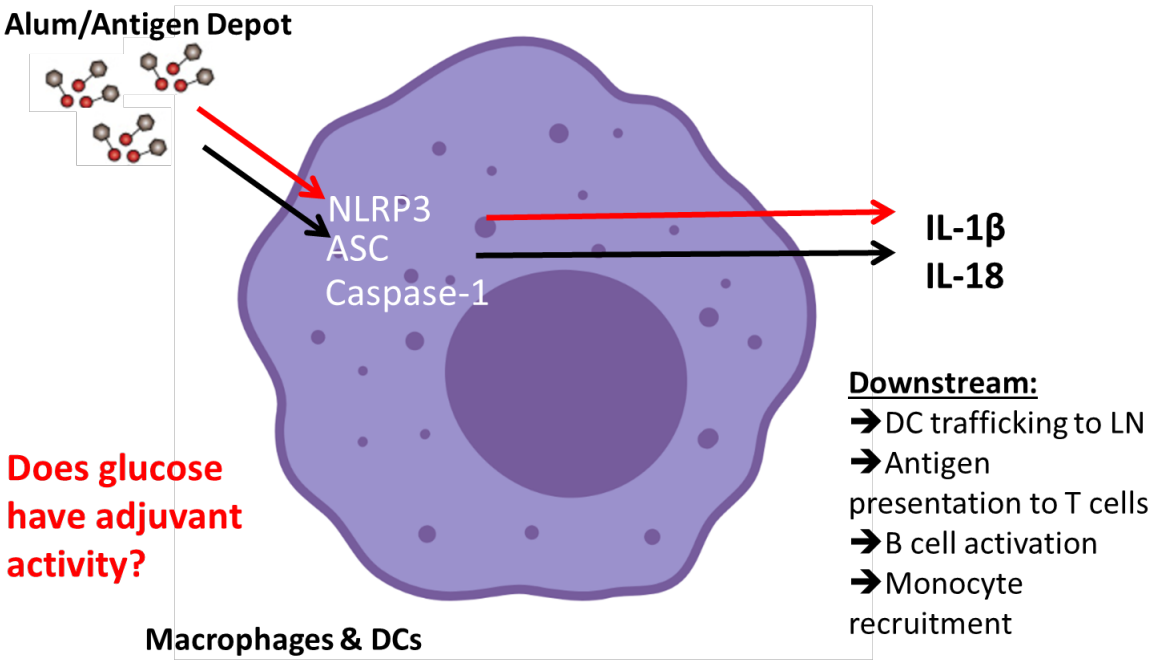



**Figure 18: Proposed mechanism of glucose adjuvant activity compared to alum.**

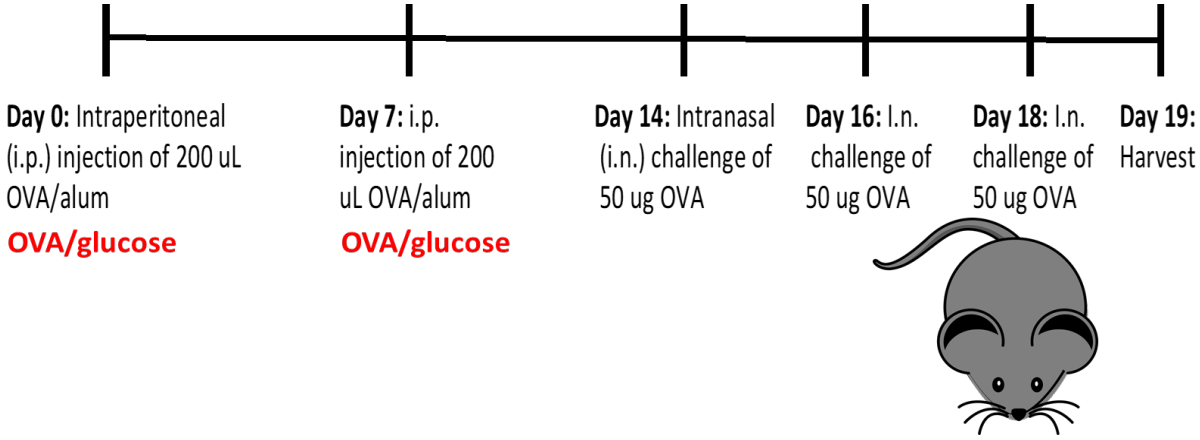



**Figure 19: Model of airway allergic inflammation using glucose and alum as adjuvants.**

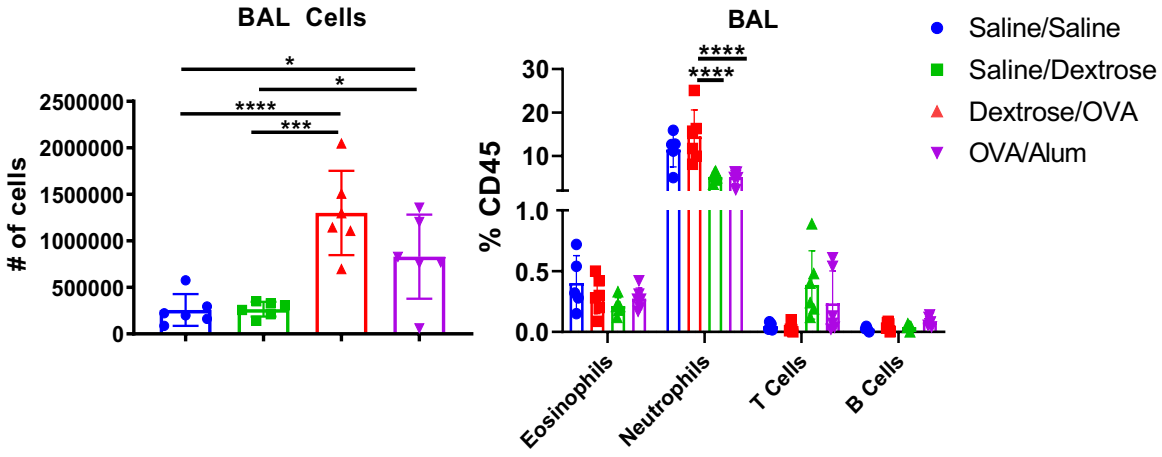



**Figure 20: Lung infiltrating immune cells in bronchoalveolar lavage following allergic sensitization and challenge**

BALBc female mice were sensitized with OVA/alum on day 0 and 7, then challenged with OVA on day 14, 16, and 18. Mice were euthanized and harvested on day 19 and flow cytometry on bronchoalveolar lavage was used to quantify lung infiltrates (left) and immune cell populations (right). ANOVA, n=6 mice per group, *p<0.05, **p<0.01.

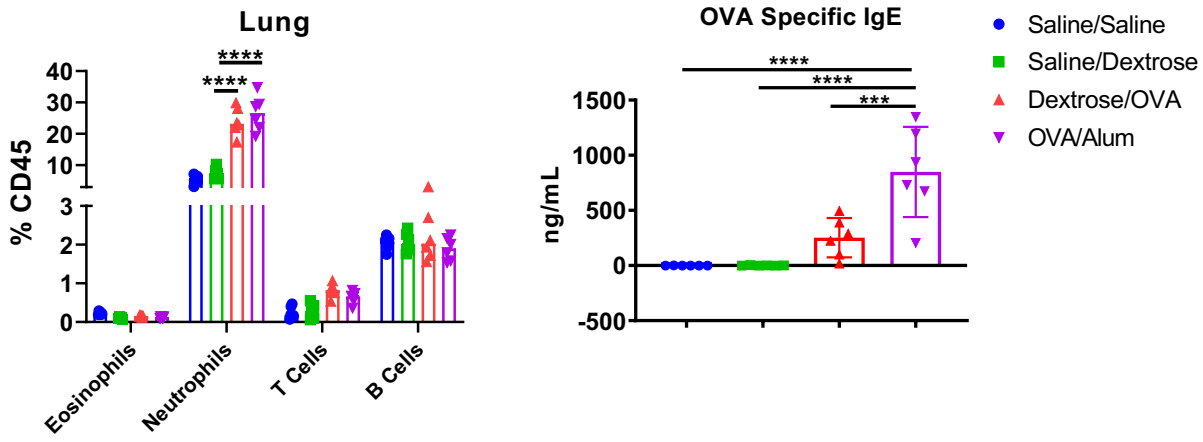



**Figure 21: Lung immune cells and antigen-specific IgE following allergic sensitization and challenge.**

Lung homogenates were stained for flow cytometry and analyzed for immune cell populations and serum was analyzed for levels of OVA-specific IgE to assess antigen sensitization and inflammation following antigen challenge. ANOVA, n=6 mice/group, ***p<0.001, ****p<0.0001.

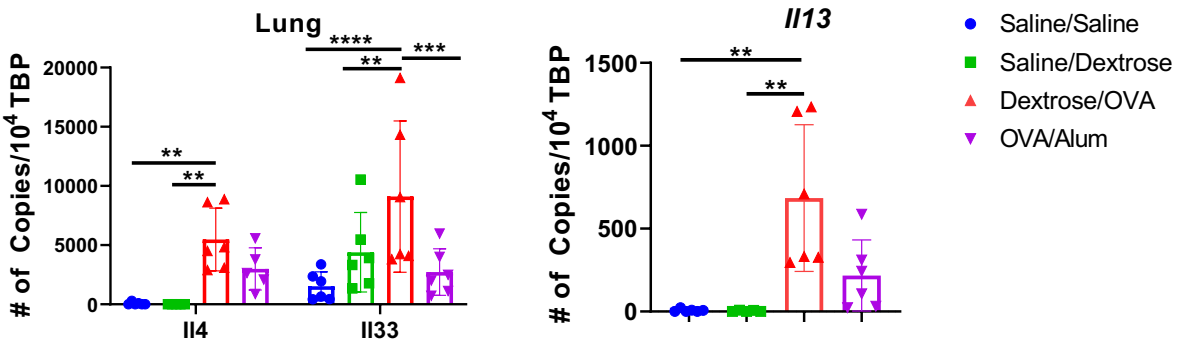



**Figure 22: Lung type 2 cytokines in lung tissue following allergic sensitization and challenge**

BALBc female mice were sensitized with OVA/alum on day 0 and 7, then challenged with OVA on day 14, 16, and 18. Mice were euthanized and harvested on day 19 and qPCR was used to measure *Il4*, *Il13*, and *Il33*. ANOVA followed by multiple comparisons with FDR correction. n=6 mice per group, *p<0.05, **p<0.01.

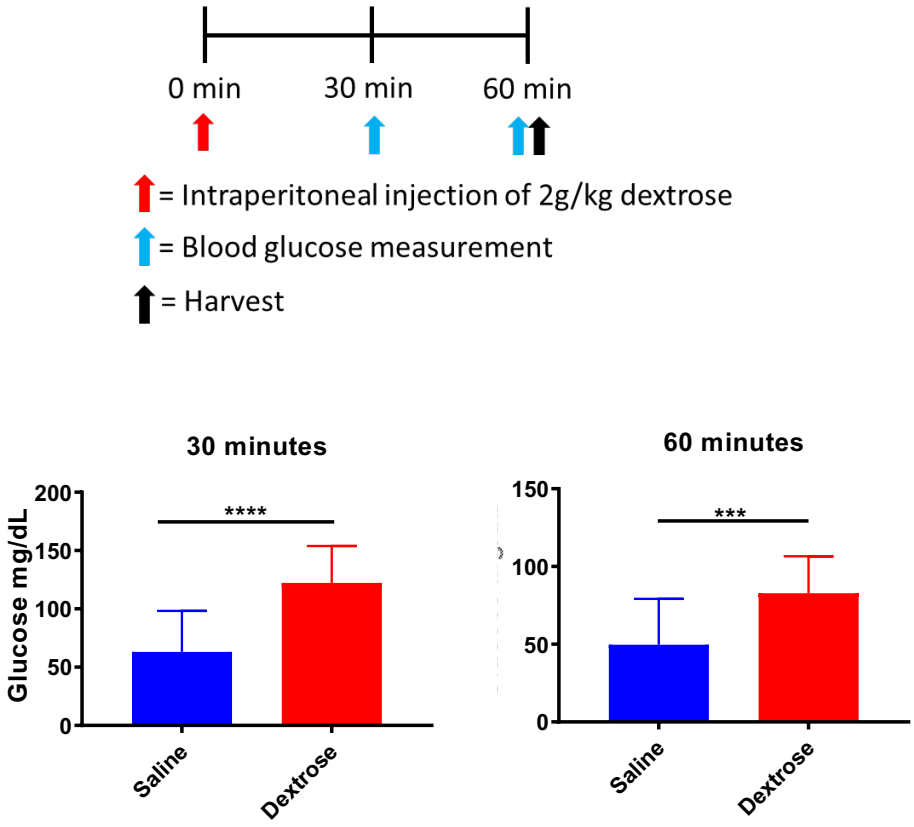



**Figure 23: Acute hyperglycemia mouse model and blood glucose measurements.**

Model of acute hyperglycemia (top) and mouse blood glucose measurements at 30 and 60 minutes post injection (bottom).

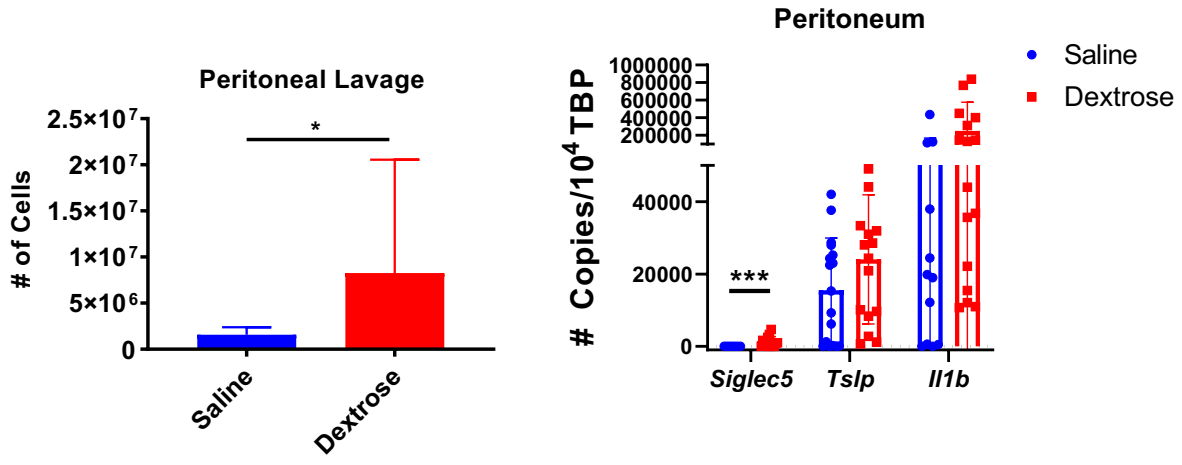



**Figure 24: Number of cells in peritoneal lavage following dextrose injection and qPCR analysis on peritoneal tissue.**

Peritoneal cells were collected in PBS 1 hour post dextrose injection and counted (left). qPCR analysis on peritoneal tissue at 1 hour post injection (right). T-test. N=6 mice/group, *p<0.05, ***p<0.001.

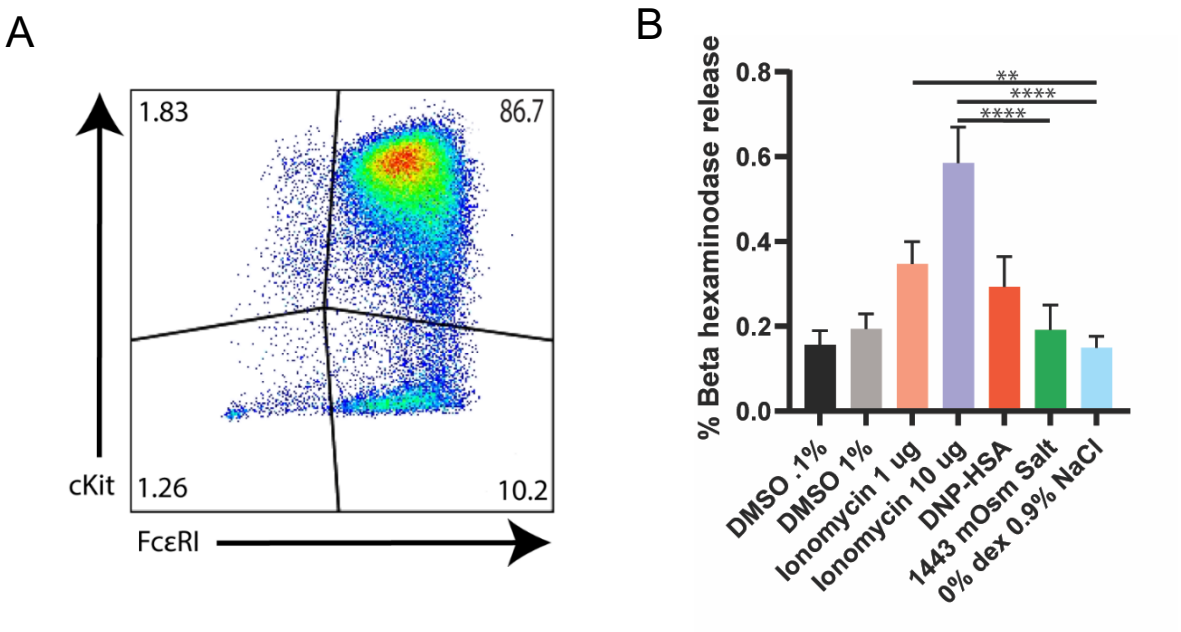



**Figure 25: Mast cell culture and β-hexaminodase assay reveal no degranulation in hyperosmotic glucose solution.**

**(A)** Purity of BMMC culture determined by expression of c-Kit and FcεRI. **(B)** % β-hexamiinodase release in hyperosmotic solutions.

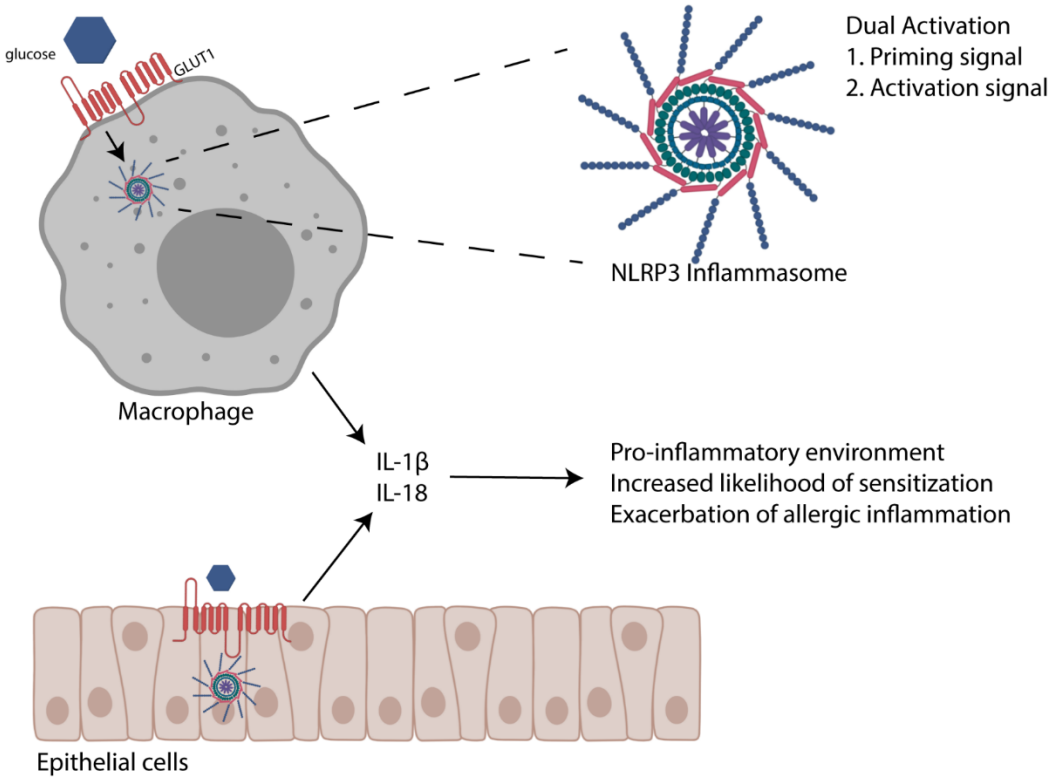



**Figure 26: Hypothesis of NLRP3-mediated glucose-induced inflammation.**

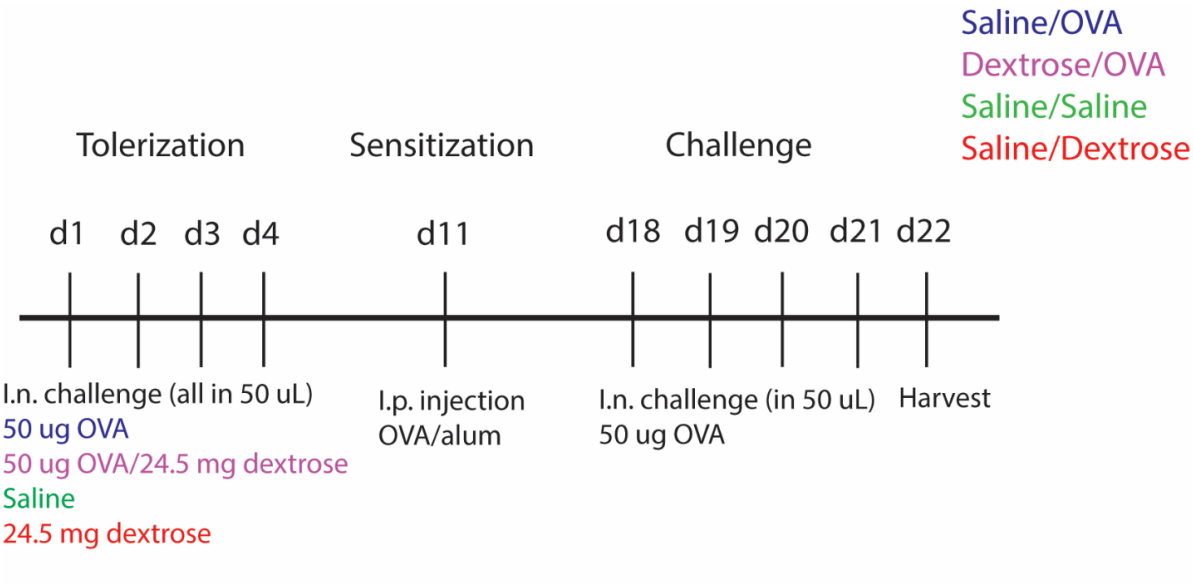



**Figure 27: Model of induced antigen tolerance in mice.**

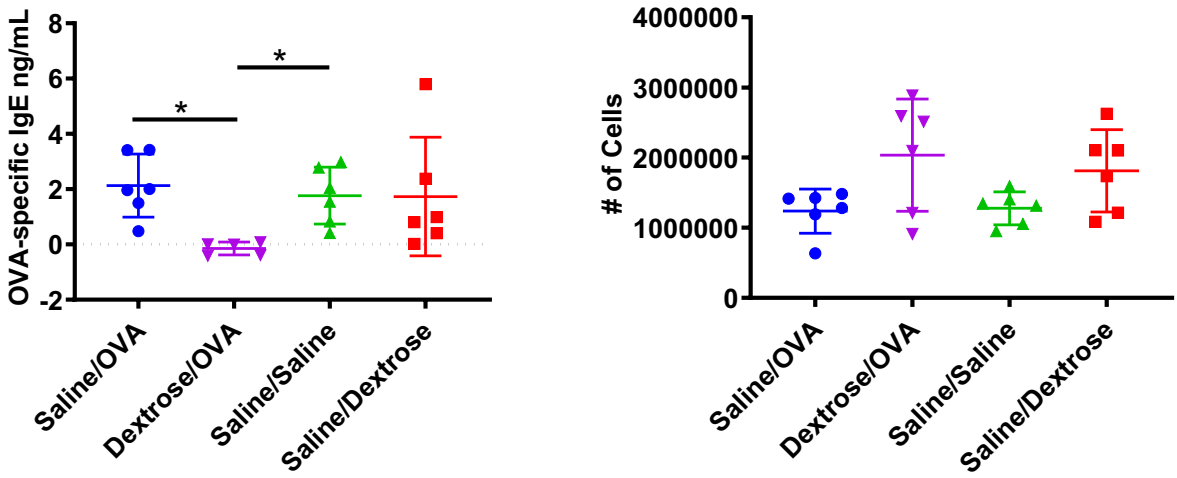



**Figure 28: Th2 response in dextrose-exposed mice measured by IgE and total cells in BAL.**

ELISA of OVA-specific IgE in serum (left) and total number of cells in BAL (right). n=6 mice/group. ANOVA, *p<0.05.

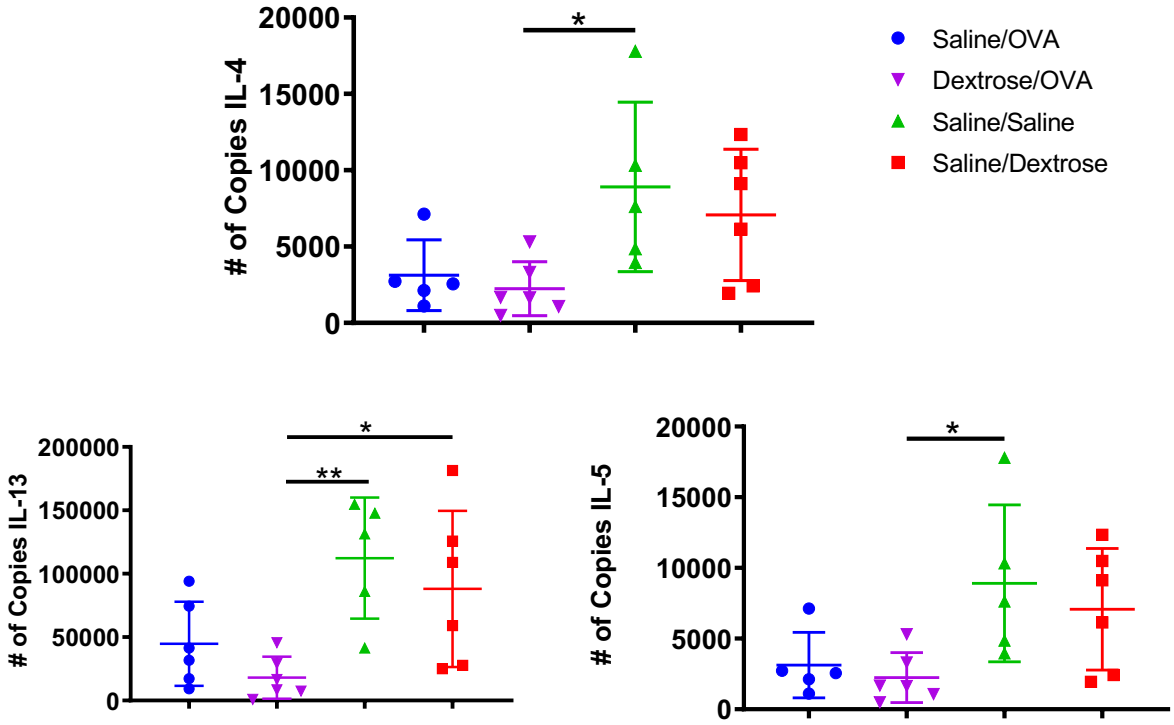



**Figure 29: Lung Th2 response in dextrose-exposed mice measured by qPCR.**

qPCR measurement of IL-4, IL-5, and IL-13 in lung tissue following tolerance model. n=6 mice per group. ANOVA, *p<0.05, **p<0.01.

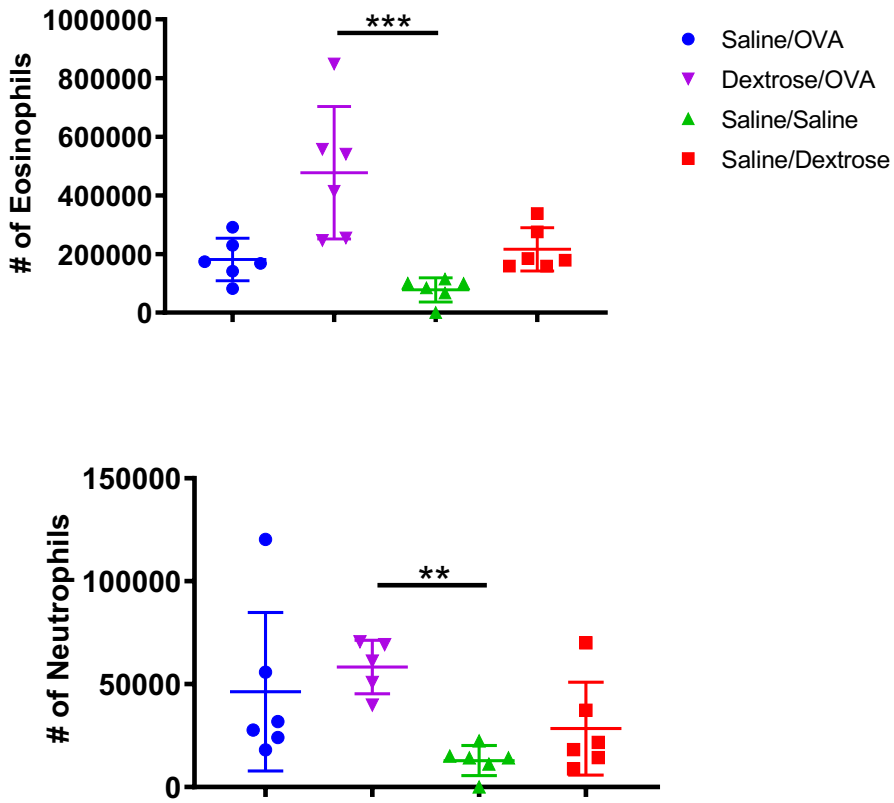



**Figure 30: Infiltration of eosinophils and neutrophils in BAL following mouse tolerance model.**

Flow cytometry quantification of CD45+/Siglec-F+ eosinophils and CD45+/Ly-6G+ neutrophils in BAL. n=6 mice/group. ANOVA, *p<0.05, **p<0.01.

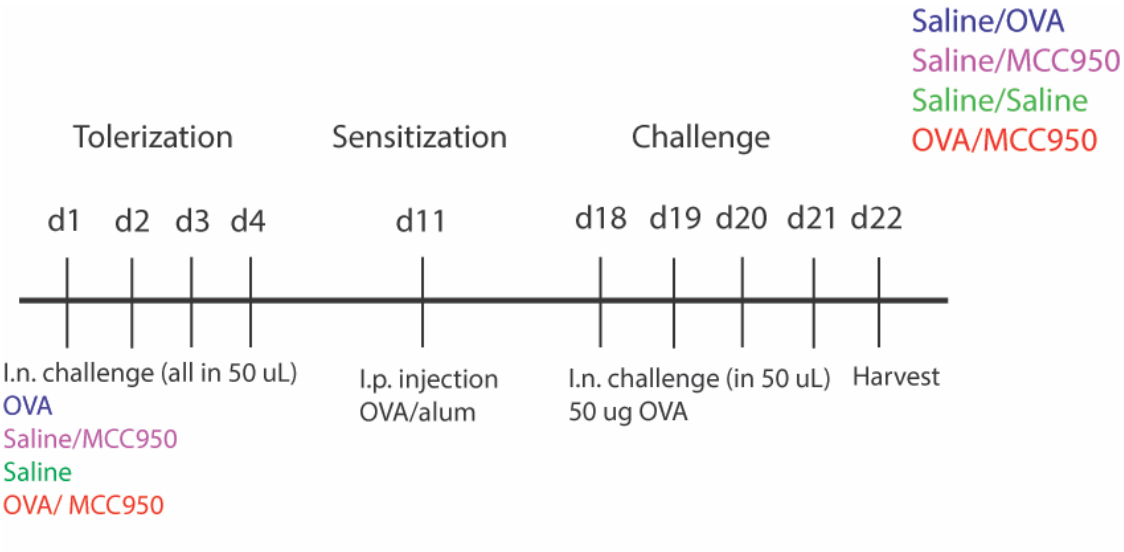



**Figure 31: Mouse model of induced antigen tolerance with and without MCC950.**

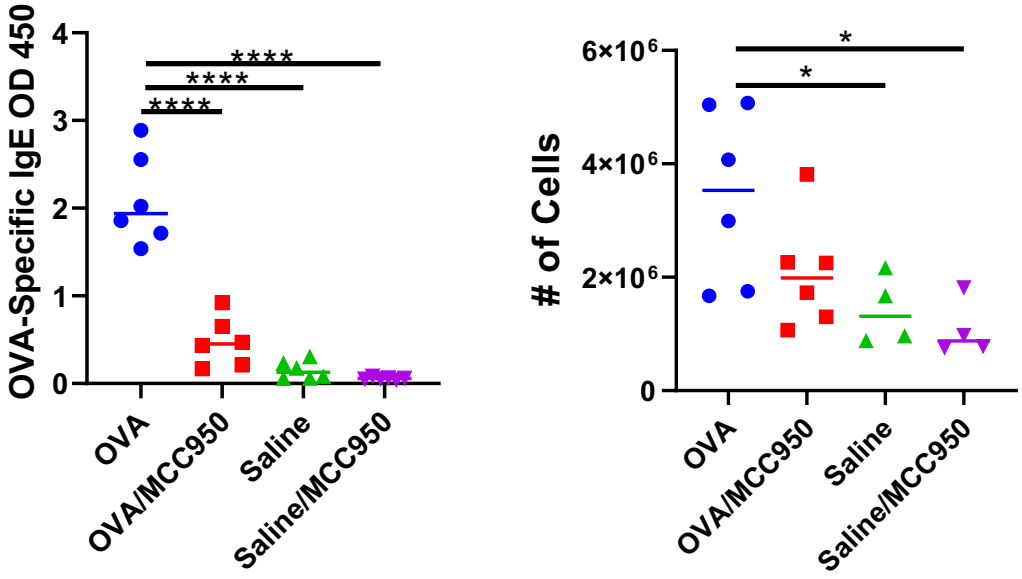



**Figure 32: Th2 response in MCC950 tolerized mice measured by antigen specific IgE and total cells in BAL.**

ELISA of OVA-specific IgE in serum (left) and total number of cells in BAL (right). N=6 mice/group, ANOVA followed by multiple comparisons. *p < 0.05, ****p < 0.0001.

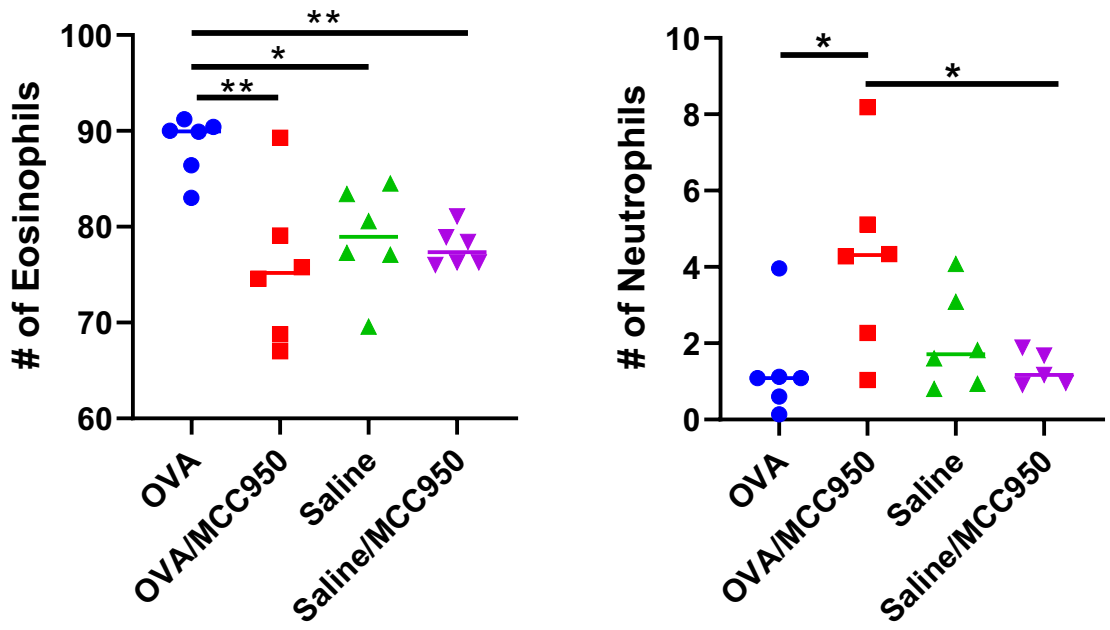



**Figure 33: Infiltration of eosinophils and neutrophils in BAL following mouse MCC950 tolerance model.**

Flow cytometry quantification of CD45+/Siglec-F+ eosinophils and CD45+/Ly6G+ neutrophils in BAL. n=6 mice/group ANOVA, *p < 0.05, **p < 0.01.

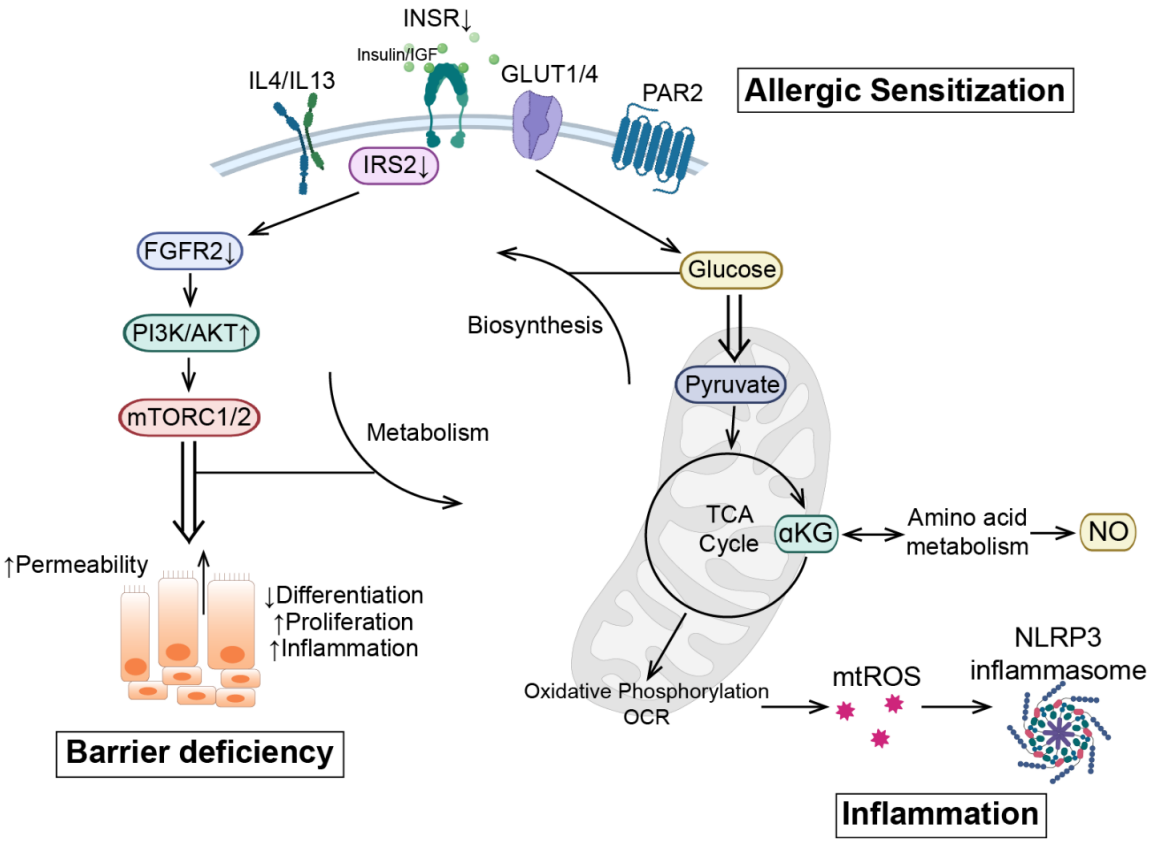



**Figure 34: Summary of epithelial cell bioenergetics pathways and their role in signaling for proliferation, differentiation and upstream regulation of glucose transport.**

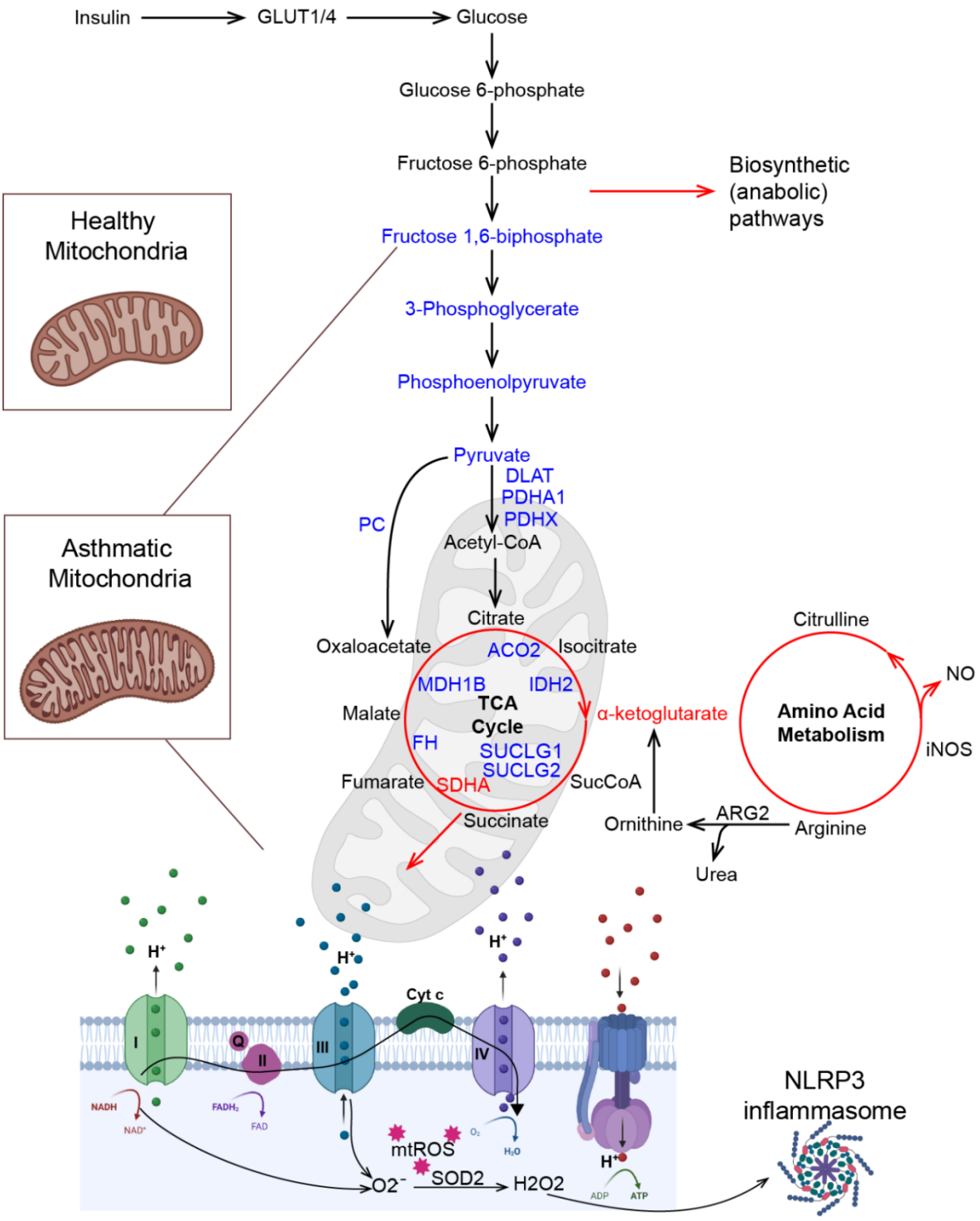



**Figure 35: Changes in mitochondrial function in asthma vs healthy epithelial cells.**

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