The Role of Metabolism in Th2 and Th17 Cells During Airway Inflammation

By

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To my mom and dad (Mark), without your love and encouragement nothing would have been possible. To my husband, Brian, thank you for your constant support and pushing me to constantly be a better person. To my pups, Shelby & Tyson, your companionship and love makes each day much brighter.

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LIST OF ABBREVIATIONS

AHR	Airway Hyperresponsiveness
APC	Antigen Presenting Cells
BAL	Bronchoalveolar Lavage
CPT1a	Carnitine Palmitoyltransferase 1A
CyTOF	Mass Cytometry by Time of Flight
DAMP	Damage-Associated Molecular Patterns
Dex	Dexamethasone
ECAR	Extracellular Acidification Rate
EMD	Earth Mover's Distance
FAO	Fatty Acid Oxidation
FAS	Fatty Acid Synthase
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GC	Glucocorticoid
GLS	Glutaminase
GLUD1	Glutamate Dehydrogenase 1
Glut1	Glucose Transporter 1
GOT1	Glutamate Oxaloacetate Transaminase 1
GRβ	Glucocorticoid Receptor β
HDM	House Dust Mite
HIF1α	Hypoxia Inducible Factor 1α
HK2	Hexokinase 2

ICS	Inhaled Corticosteroids
ILC2	Group 2 Innate Lymphoid cells
IFNγ	Interferon γ
IL	Interleukin
LABA	Long-Acting β-Agonists
LPS	Liposaccharide
MEM	Marker Enrichment Modeling
mTOR	Mechanistic (mammalian) Target Of Rapamycin
NK	Natural Killer
OCR	Oxygen Consumption Rate
OxPhos	Oxidative Phosphorylation
PAMP	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear cells
PDH	Pyruvate Dehydrogenase
PDHK1	PDH Kinase 1
PRR	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
TLR4	Toll-like Receptor 4
Teff	T Effector cells
TGFβ	Transforming Growth Factor β
Treg	Regulatory T cells
UMAP	Uniformed Manifold Approximation and Projection

CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

Asthma

Asthma is a worldwide public health concern with diverse pathological consequences. In 2017, 272.7 million people worldwide suffered from asthma and 495,100 individuals died that year as a result (1,2). Characterized by airway hyperresponsiveness (AHR), inflammation, remodeling, and increased mucus production, asthma is localized in the lower airway and results in chronic inflammation (3–5). Asthma is a heterogeneous disease that varies not only in severity but also in the main prevalent cell types and can develop in one of two manners (4,6). Environmental allergens can cause IgE-mediated inflammation termed either atopic or allergic asthma (5,7). The second way in which asthma can develop does not involve allergens, has a late in adulthood onset, and is not mediated by IgE (5,7). Although, how asthma was developed is important for clinical history, patients are divided by pathology and endotype (4,8).

Asthma can be defined by the phenotypic characteristics that make up the disease, the endotype which is the underlying inflammatory factors driving disease, or a combination of both (4). Endotypes can be broadly divided into inflammation with either high or low levels of Th2 associated cytokines and patients split evenly into these endotypes (9–12). Of particular interest to our study is the low Th2 endotype, neutrophilic asthma, which is associated with severe disease and resistance to glucocorticoids (GC) (13–15). Neutrophilic asthma is characterized by high levels of IL-

17 which help to recruit the neutrophils into the airway while still maintaining some eosinophilic inflammation (14,16–18). Th17 cells have been implicated to not only be the producers of IL-17 but also to be a GC-resistant cell type (19–24). Interestingly, dual Th2/Th17 cells are found in severe asthma and also contribute to GC-resistance (23,24).

Asthmatic patients will develop periodic or persistent coughing, wheezing, shortness of breath, and other symptoms of airway obstruction which cause them to seek medical attention (5,25). Patients are clinically assessed to determine disease persistence, severity, and treatment options (6). A stepwise approach is taken to treatment that is based on symptoms and physiological readings of lung function (6). For example, patients with persistent asthma are given low dose daily inhaled corticosteroids (ICS) to control disease, a class of GC (6). As patients develop worse symptoms as defined by the clinical criteria, the dosage of ICS is increased, and long-acting β-agonists (LABA) are added to the treatment plan (6). Severe asthmatics are prescribed the highest doses of ICS and given an individualized treatment plan, due to ICS not working well enough to control symptoms (25).

Induction of airway inflammation involves a wide array of cytokines, innate, and adaptive immune cells (**Figure1-1**) (26). Allergens, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) will act on pattern recognition receptors (PRRs), Toll-like receptor 2 (TLR2), or TLR4 to activate airway epithelial cells to begin cytokine secretion (26–29). IgE is released and activates the initial secretion of CCL5, CCL11, CXCL8, CXCL10, IL-4, IL-5, IL-13, and TLSP from the airway smooth muscle cells to recruit immune cells and promote airway

remodeling (26,30,31). Mast cells bind IgE and release mediators such as histamine (32). IL-5 and IL-13 recruit and promote the survival of eosinophils while CCL5 and CCL11 function as chemotaxis which enhances their trafficking in the lung (33). Eosinophils granules contain cytokines, major basic protein (MBP), and lipid mediators that are released upon activation (26,34,35). MBP is cytotoxic as it interferes with cell homeostasis and eosinophil peroxidase (EPO), also found within granules, generates ROS both creating damage in the airway (36). Secretion of CXCL8 by epithelial cells into the airway recruits neutrophils (26,37). Neutrophils will degranulate in the airway releasing neutrophil serine proteases (NSPs) that cause damage to the epithelium by destroying the lung matrix and breaking tissue down (37). The effects of the degranulation of both eosinophils and neutrophils result in the physiological changes that cause airway remodeling and inflammation during disease.(37)

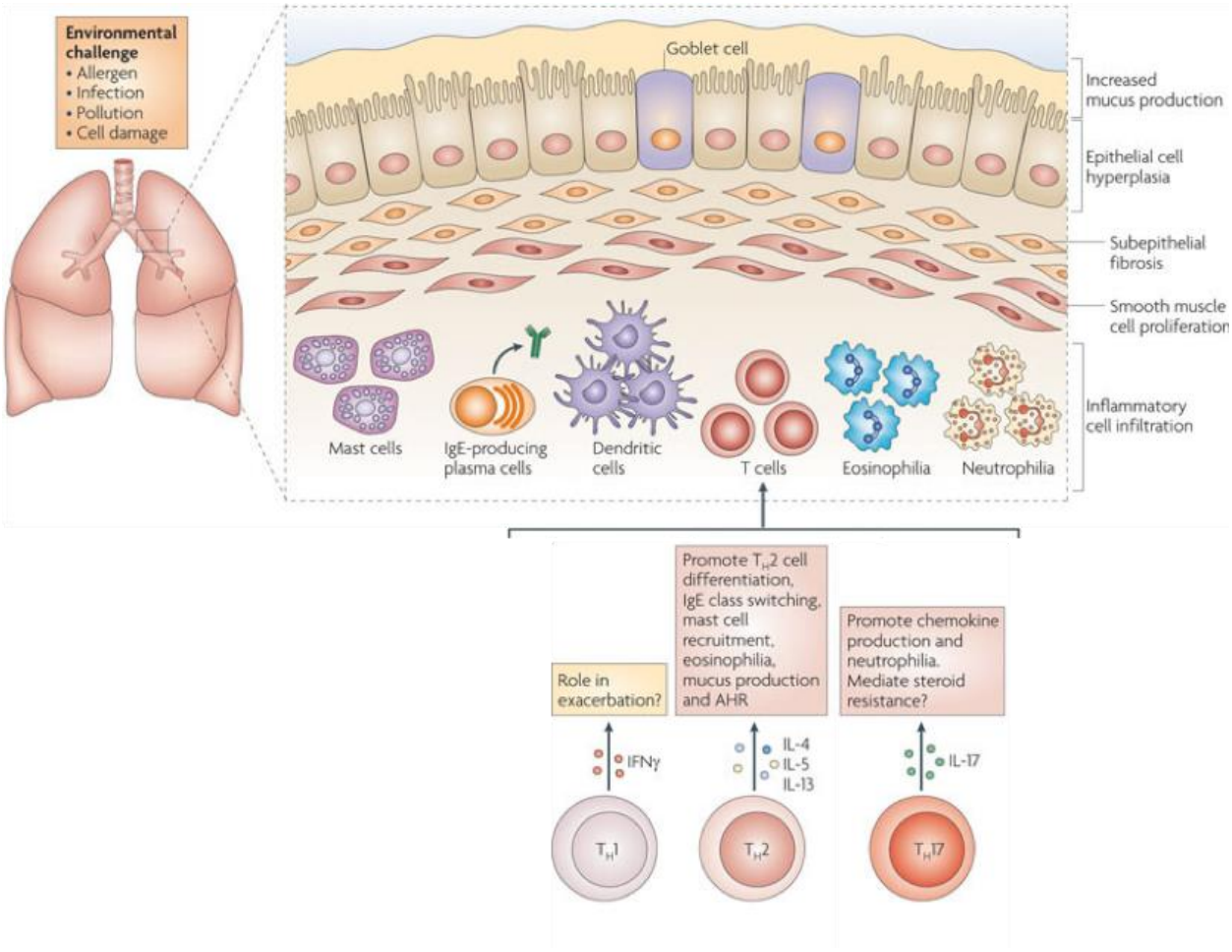

Figure 1-1. Immune cells involved in airway inflammation. Innate cells and the broad spectrum of T cells that are present during inflammation. Key missing cells are ILC2s. Adapted by permission from [**Springer Nature**]: [**Springer**] [**Nature Reviews Immunology**] [**Functions of T cells in Asthma: More than just Th2 cells, Clare M. Lloyd and Edith M. Hessel**], [**COPYRIGHT**] (2010) **(30)**

T Cells in Asthma Development

Due to the broad spectrum of cytokine secretion that can occur during airway inflammation and the variations in disease between patients, most CD4 T helper cell subtypes can be found in asthma (4,30,38,39). Although not as prevalent as other cell types, the role of Th1 cells in asthma has been associated as both protective and inflammatory (40–43). Th1 cells have also been implicated in severe disease and have been documented to exhibit GC resistance (42–44). Th1 cell differentiation occurs as a response to IL-12 released by innate cells at the site of inflammation and upregulation of IL-12 receptors on T cell surface as a response to TCR engagement (45,46). Innate immune cells secrete IFNγ allowing for further Th1 differentiation by activating the transcription of *Tbx21* which encodes Tbet that then activates IFNγ expression in Th1 cells so they can begin to produce it (45,47). IFNγ production in Th1 cells is maintained by transcription of STAT4 that is activated by IL-12 (45).

Th2 high or type-2 mediated asthma is one of the better studied endotypes of asthma. This endotype characterized by an influx of eosinophils and involves cytokines associated with Th2 cells such as IL-4, IL-5, and IL-13 (8,10,11,30,48,49). Other cells that can produce these cytokines are $\gamma\delta$ T cells and ILC2s, the latter of which has been shown to have an important role in driving disease severity (30,50–53). During allergic airway disease, naïve $CD4^+$ T cells are recruited to the airway by epithelium derived IL-25, IL-33, and TSLP (10,40,52,54–56). Recruited T cells are activated by IL-4, the origins of which are unknown, which then activates the transcription of STAT6 and

GATA3 to promote Th2 cells (10,57). Activated Th2 cells that localize to the airway by the chemokines CCL17 and CCL22 will then produce IL-4, IL-5, and IL-13 to mediate inflammation (11,48,58). These cytokines are crucial for eosinophil recruitment and maintenance, IgE production, tissue remodeling, and development of AHR (10,11,30).

Apart from Th2 mediated asthma, neutrophilic asthma falls within the Th2 low endotype in which disease is mediated by IL-17 production (16,17,20,59,60). Th17 cells have been associated with the production of IL-17 and contributing to GC-resistant in asthma (19,23,50,61,62). During inflammation, innate cells release IL-1β, IL-6, IL-23, and TFGβ which recruit naïve CD4 T cells to the lung tissue and start the differentiation process (46,63–67). Although IL-1β and IL-23 are not required to differentiate Th17 cells in culture and are associated with a pathogenic phenotype, *in vivo* studies have supported their requirement for functional Th17 cells (63,65,68–72). The synergistic effects of these cytokines on activated naïve CD4 T cells will upregulate the transcription of STAT3 and RORγt to differentiate into Th17 cells (64,66,73). Th17 cells will then release IL-17, IL-21, and IL-22 to mediate inflammation (64,66,73). There are several different IL-17 isoforms, however, in asthma it has been documented that the primary isoforms are IL-17A and IL-17F (20,74). Neutrophil recruitment is mediated by IL-6 and CXCL8 both of which are promoted by IL-17 (14,16).

T Cell Metabolism

It has been thought historically that cells primarily utilized oxidative phosphorylation (OxPhos) for optimal energy unless external pressures forced other pathways to become more active. Work by Otto Warburg in the 1920s on cancer

metabolism demonstrated that proliferating cells utilize aerobic glycolysis more so than OxPhos (75). The primary benefit of aerobic glycolysis to proliferating cells is that it provides a robust source of biosynthetic precursors and anabolic metabolism in a variety of pathways to support cell growth. Warburg's work came to be known as the Warburg effect and has shifted the paradigm not only in cancer but also in T cell metabolism. This hypothesis was validated in human CD4 T cells that required glycolysis for proliferation and cytokine production but not oxidative phosphorylation. The metabolic shift between OxPhos in naïve T cells to aerobic glycolysis is a result of both transcriptional and post-transcriptional regulators to support rapid T effector (Teff) cell proliferation (76,77). This shift in metabolism is not limited to glycolysis but also includes an increase in glutaminolysis (glutamine metabolism) (78–82). These changes support proliferation by giving the cells more access to amino acids, nucleotides, and lipids which are necessary for rapid cell expansion (76,77,83). Conversely, longer lived T cells such as T regulatory (Tregs) and memory T cells primarily rely on a catabolic metabolism primarily of fatty acid oxidation (FAO) (84,85). Despite this, Tregs still require low levels of glycolysis and can shift their metabolism depending on environmental cues (86–88). This emphasizes the importance of glycolysis for proliferative cells.

The glycolytic shift in Teff cells occurs upon antigen presentation to the TCR and engagement of the co-stimulatory signals such as CD28 (89–91). TCR engagement results in activation of the PI3K/Akt/mechanistic (mammalian) target of rapamycin (mTOR) signaling pathway (92–96). mTOR can form two complexes, mTORC1 and mTORC2, studies have shown that mTORC1 signaling is required for T cell metabolism

(92,93,97). Concurrently, there is upregulation of c-Myc and hypoxia inducible factor 1α (HIF1α) which are also necessary for glycolysis (79,83,98). In order to support the increased demand for glycolysis, glucose transporter 1 (Glut1) is also upregulated in T cells in a PI3K/Akt/mTOR dependent manner (99–102). These pathways work in concert to increase the glycolytic activity of activated T cells. However, as previously mentioned, glutamine is also required for T cell proliferation (78–82). Rapid glutamine uptake is required for TCR activation of mTORC1 (103–105). Not only does mTORC1 mediate T cell differentiation through metabolism, but without it STAT3 and STAT4 are downregulated thereby decreasing cytokine production in Th1 and Th17 cells (106). Metabolism not only assists in T cell proliferation but also in transcriptional regulation for differentiation.

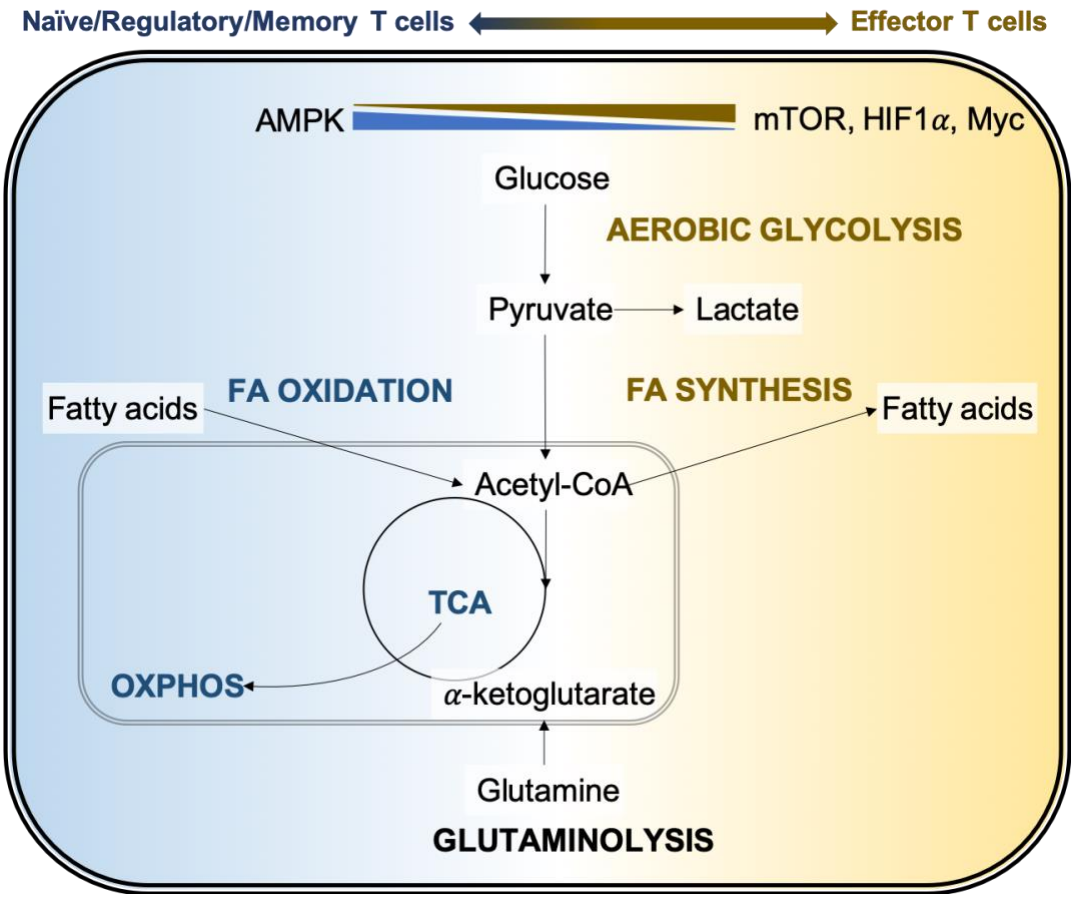

Figure 1-2. Metabolic pathways and regulators in T cells. Glycolysis, oxidative phosphorylation, FA oxidation, FA synthesis, and glutaminolysis all play important roles in T cell metabolism to drive necessary proliferation, differentiation, and function. Adapted from Metabolic Barriers to T Cell Function in Tumors, Ayaka Sugiura and Jeffrey C. Rathmell (2018) **(107)**.

The Rathmell lab has shown that T cells not only reprogram their metabolism upon activation but that T cell subsets differ in their metabolic requirements and each established specific programs that support their function and fate (77,84). Of the T cell subsets, metabolism in Th2 cells has been the least studied. Th2 cells differ from the other subtypes in their requirement of mTORC1, as studies have found that mTORC2 is the required pathway for differentiation of Th2 cells (92,93,106,108). Interestingly, Th2 cells have also been documented to have the highest glycolytic rates (84,109). They have also have demonstrated to not require high levels of glutamine to function (110). In the context of airway inflammation, Th2 cells have been documented to require the peroxisome proliferator-activated receptor γ (PPARγ) to produce IL-5 and IL-13 (111,112). PPARγ is a metabolic regulator which induces fatty acid uptake and has been shown to be needed in early T cell activation (113). This limited information on Th2 metabolism leaves an opening for more investigation and discovery of metabolic programming.

There are several metabolic points of inflection where T cells can differentiate into Th17 or into Tregs which contributes to the paradigm of plasticity in Th17/Treg differentiation. The involvement of Th17 cells in a wide array of autoimmune diseases has led to many studies on Th17 metabolism and the metabolic shifts that can result in Treg differentiation (74,114–116). Aerobic glycolysis is maintained in Th17 cells by preventing pyruvate from being converted into acetyl-CoA through the inhibition of pyruvate dehydrogenase (PDH) by PDH kinase (PDHK1) (117). In contrast, inhibition of PDHK1 during experimental autoimmune encephalomyelitis resulted in a decrease of Th17 cells and an increase in Tregs (117). Another regulator of glycolysis, HIF1α, has

been implicated in controlling the fate of Th17 cells (118–120). HIF1α binds to FoxP3 targeting it for degradation, preventing Treg differentiation, while also transcriptionally activating RORγt (121). Studies have also demonstrated that inhibition of the glycolytic regulator, mTOR, by the activation of AMP kinase (AMPK) results in impairment of Th17 cell generation and promotes Treg differentiation (122–124). Regulation of Th17 cells can also be mediated by inhibition of fatty acid synthesis (FAS), as it impairs the function of Th17 cells and promotes FAO to support Tregs (125).

Th17 cells have been described with several characteristic metabolic features. They can have the highest upregulation of Glut1 after activation relative to other T cell subsets (84,101). Moreover, high levels of glucose are able to induce IL-6 and IL-17 in Jurkat T lymphocytes suggesting that increased glucose availability can influence Th17 differentiation (126). In Th17 cells, the mTOR pathway has been documented to not only induce glycolysis but also promote STAT3 activity (106). Additionally, mTORC1 regulates Th17 differentiation by phosphorylating and activating S6K2 to promote RORγt translocation to the nucleus (127). Glutamine metabolism has been implicated by several studies to be required for functional Th17 cells both *in vitro* and *in vivo* (80,104,115). The Rathmell lab demonstrated that glutaminase was required for Th17 cell differentiation and function (80). Another study established that Th17 regulation can occur by glutamate oxaloacetate transaminase 1 (GOT1), as inhibition of GOT1 stops RORγt transcription and thereby decreases Th17 cells (115). Furthermore, there are data to suggest that Th17 cells are metabolically flexible between glycolysis and OxPhos (128). This is supported by data that similarities in the longevity of Th17 cells and transitional CD8 memory are a result of metabolic flexibility that allows the cells to

have better function and persistence (129). OxPhos has also been documented to be required for the differentiation of pathogenic Th17 cells (68). The biggest limitation of these studies examining metabolic flexibility is that they have been performed in culture. Franchi et al. outline how despite the flexibility Th17 cells showed *in vitro*, cells from an *in vivo* setting may not properly shift pathways when one was inhibited (128). Overall, the regulation of Th17 cells by various metabolic pathways and dependence on these pathways suggests that metabolic inhibitors may be ideal for the regulation of T cell differentiation and effector function during inflammation.

Glucocorticoids

Glucocorticoids (GCs) are used to treat a variety of immunological diseases from asthma to rheumatoid arthritis to cancer (130–132). They have a wide array of mechanisms through which they mediate cells, such as inducing cell death, repressing expression of inflammatory cytokines, and activating expression of anti-inflammatory cytokines (133–135). However, resistance to GCs has been documented as a problem in some of these diseases (132,136–138). GC resistance was first documented in asthmatic patients in 1968 who displayed eosinophilic GC resistance (139). Although any asthmatic endotype can develop GC-resistance, neutrophilic Th17 induced disease has a high prevalence of resistance (140,141).

There are some mechanisms by which cells have been found to become GC resistant. GC resistance can begin by altered signaling and increased expression of the glucocorticoid receptor isoform β (GRβ) has been associated with resistance (142). GCs suppress GATA3 preventing Th2 differentiation while increasing RORγt expression

allowing for Th17 cell development (**Figure 1-3**) (143–145). They can increase expression of IL-1 and TGFβ receptors, co-active STAT3, and increase IL-17 production (21,62,146–148). Th17 cells have been documented to bypass GC mediated apoptosis by expressing high levels of the anti-apoptotic, BCL-2 (62). Sensitivity to GCs was restored in Th17 cells when BCL-2 was knocked down (62). GCs have also been shown to be promoters of Th17 differentiation by decreasing IL-2 expression resulting in increased Th17 differentiation (145).

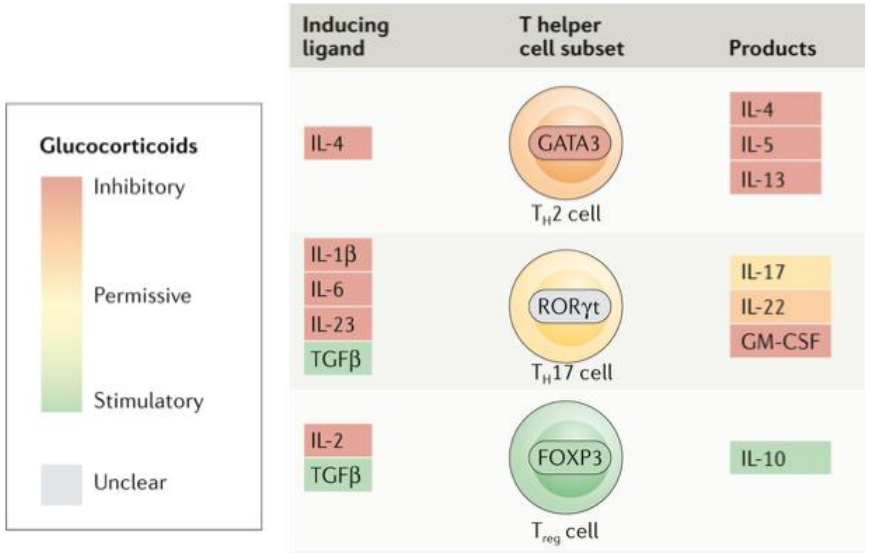

Figure 1-3. Glucocorticoid effects on T cells and associated cytokines. The response of Th2, Th17, and Tregs and their cytokines when treated with GCs. Reprinted by permission from **[Springer Nature]**: **[Springer] [Nature Reviews Immunology] [Glucocorticoids in T cell development, differentiation and function, Matthew D. Taves and Jonathan D. Ashwell], [COPYRIGHT]** (2020) **(148)**.

GC-resistance in Th17 cells has been well documented, but the mechanisms of this resistance have not been fully elucidated (19,23,149–152). Based on the similarities between cancer cells and Th17 cells in their metabolic flexibility, a possible metabolic mechanism of action by bypassing metabolic restraints may be hypothesized for Th17 cells (153,154). GCs inhibit glucose uptake resulting in cell death in some leukemic cells, however GC-resistant leukemic cells have been identified to bypass these effects by having an increased glycolytic metabolic profile compared to GC-sensitive cells (155–158). One study found that not only do GC-resistant leukemic cells have increased glycolysis and OxPhos, these cells also have an increase in glutamine and cholesterol metabolism and increased in the PI3K/Akt/mTOR and Myc signaling pathways (156). Both GC-resistant cell lines and primary leukemic cells from patients have had sensitivity restored once treated with glycolytic inhibitors (157). GC-resistant leukemic cells have a highly proliferative metabolic profile and were shown to shift between glucose bioenergetic pathways to fulfill their energy requirements (158). Targeting two pathways in these cells resulted in restored GC-sensitivity. In Burkitt lymphoma cells, a combination of GC and either a glycolysis inhibitor or a mitochondrial inhibitor also resulted in restored GC- sensitivity (159). These studies suggest that metabolic inhibition may be a promising target to augment the effects of GC-resistance.

Metabolic Inhibitors

After the discovery of the Warburg effect in cancer cells, antagonists of metabolism were recognized as a hopeful option to inhibit or kill cancer cells (160,161). The first antagonists of metabolism were glutamine analogs that had limited effects on

tumors, but unfortunately had high levels of toxicity (160). From these early studies, 6-diazo-5-oxo-l-norleucine (DON) was manufactured (160). DON has been a valuable glutaminase (GLS) inhibitor in glutaminolysis studies and is still frequently used *in vitro* (162,163). As more metabolic inhibitors were developed and discovered the main hypothesis was to use these inhibitors to disrupt cancer metabolism. Rapamycin is a bacterial antifungal metabolite that was identified as having inhibitory effects on mammalian cells (164). Rapamycin mediates metabolism by allosteric inhibition of mTORC1 (165,166). Cancer treatment was done with rapamycin analogs, as the pharmacokinetics of rapamycin prevented its use in patients (164). However, the analogs did not lead to a substantial reduction in solid tumors and were taken out of clinical trials (164). Another inhibitor sourced from nature was metformin (167). Metformin inhibits OxPhos, previously thought to inhibit at complex I of the mitochondria more recent studies suggest it may inhibit glycerophosphate dehydrogenase (168–170). Metformin is a successful treatment for type 2 diabetes and has a promising role in cancer (170–172). Breast cancer odds decrease in patients on long term metformin and animal studies have demonstrated a decrease in dosage need for chemotherapy when animals were dosed with metformin (172,173). These are just a selection of some of the well-established metabolic inhibitors, however, use in the mediation of immunity has been limited.

Although the main research focus for the development of new metabolic inhibitors remains on cancer, more studies are being done to determine if metabolic mediation in immune cells is a good target to modulate inflammatory diseases. Small molecules targeting glutamine metabolism are the updated variation on analogs (174).

Inhibitor of glutaminase, CB-839, has shown promising results in lung tumors, pulmonary fibrosis, graft-vs-host, colitis, and asthma in laboratory studies (80,175,176). Currently, three active clinical trials are investigating the effects of CB839 in cancer (177–179). Monoacylglycerol lipase (MAGL) inhibitors are being studied as potential targets for cancer and metabolic disorders as they can inhibit lipid metabolism (180). Glycolytic inhibition is frequently of interest but can lead to complications as glycolysis is still required for other functions in the body besides immunity and aberrant cancer cells (181). Glut1 is an easy metabolic target for small molecules and there have been several developed over the last couple of years that seem promising (181–183). Although there is much optimism for the promise of current developments of metabolic inhibitors, there remains much research to be done before these approaches will be used in patients.

Models of Asthma in Research

The complexity of asthma in humans cannot be replicated in an animal model, therefore researchers have been primarily using acute models of airway inflammation in an attempt to simulate different inflammation phenotypes (184,185). Mouse models that have been developed attempt to induce airway inflammation by mechanisms that are similar to how human allergic asthma develops. Most models involve sensitizing mice with an antigen several times for a period of weeks with a final challenge before experimental analysis (185,186). The route of sensitization traditionally has been via intraperitoneal injections, however intranasal and intratracheal are more commonly used as they can induce inflammation in the upper and lower airways,

respectively (186). There is also variation in the antigens used, as the antigen drives what type of immune response develops (185,186). Ovalbumin has a long history of use in mice due to accessibility and ability to induce a Th2 mediated response when used alone (185,186). However, ovalbumin does not induce an allergic response in human airways so it may not be ideal to mimic human response (186). House dust mite, *Alternaria alternata* extract, ragweed, and cockroach extract are antigens that can be used in mice and have been shown to be human allergens as well (185,186). Both house dust mite and *Alternaria* can establish Th2 mediated responses that include the recruitment of a broad range of inflammatory cells associated with asthma in mice (186). Models of airway inflammation recruit Th2 high responses but either under more chronic conditions or with complete Freund's adjuvant (CFA) can also recruit more Th17 cells (152). However, this an artificial model when compared to human allergens. McAlees et al. identified that LPS used in combination with ovalbumin induced a more neutrophilic response in mice due to TLR4 signaling stimulated by the LPS (27,187). The variety of options in models of airway inflammation allow researchers to use antigens and routes of sensitization that are optimized for their asthma endotype of interest.

Research Objectives

Severe, glucocorticoid resistant neutrophilic asthma is a major problem that is of great interest for many in the asthma field. It has been established that IL-17 is a major contributor to inflammation and that Th17 cells play an important role in the secretion of this cytokine. While Th17 cells are the main contributor of inflammation in neutrophilic

asthma, Th2 cells are still present. The presence of both of these $CD4^+$ T cells leads us to question whether there is a metabolic component driving the differences in levels of these two cell types and whether targeting metabolism could be an alternative approach to reducing T cell-mediated airway inflammation. The first aim of this dissertation was to determine if $CD4^+$ T cell specific metabolic differences can be identified in the PBMCs from healthy and asthmatic individuals. Secondly, I aimed to elucidate the metabolic differences between Th2 and Th17 cells both *in vitro* and *in vivo* during airway inflammation. The final aim was to establish metabolic inhibitors that could be used to supplement the use of glucocorticoids and assist in overcoming GC- resistance since it is a current barrier in the treatment of severe asthmatics.

CHAPTER II

ELEVATED METABOLIC PROFILE IN ASTHMATICS

A portion of this work adapted from manuscript originally published in *The Journal of Immunology*. Healey DCC, Cephus JY, Barone SM, Chowdhury NU, Dahunsi DO, Madden MZ, Ye X, Yu X, Olszewski K, Young K, Gerriets VA, Siska PJ, Dworski R, Hemler J, Locasale JW, Poyurovsky MV, Peebles Jr RS, Irish JM, Newcomb DC, and Rathmell JC. 2021. Targeting In Vivo Metabolic Vulnerabilities of Th2 and Th17 Cells Reduces Airway Inflammation. *J. Immunol*. 206:ji2001029.

Introduction

Asthma is a chronic inflammatory disease that affects approximately 25 million Americans and has increased incidence worldwide(188). Patients with asthma have increased airway inflammation, airway hyperresponsiveness (AHR), remodeling, and mucus production that is driven in part by increased T cell activation (20,152). Asthma is not a uniform disease and many different phenotypes and endotypes of asthma exist, ranging from mild-intermittent to severe asthma (6). T cell-mediated immune responses are important in airway inflammation, airway hyperresponsiveness (AHR), remodeling, and mucus production (30,40,189,190). These T cell-mediated responses can result in a variety of phenotypes, including eosinophilic asthma driven by Th2 cells or neutrophilic asthma which can be driven by Th17 or Th2/Th17 cells (20,23,152). Although both Th2 and Th17 cells can contribute to asthma, the metabolic programs of

these subsets and how they may impact disease or potential therapy have not previously been well described.

Metabolomic studies in asthma have been primarily focused on overall metabolism changes such as an increase in purine or arginine metabolism (191,192). Interestingly, there are metabolic biomarkers measurable in urine that shift in children who have instances of wheezing who later develop asthma (193). However, studies on the metabolomics of immune cells in asthma have been limited (67,194–197). Studies have demonstrated that there are measurable differences in the immune populations found in the BAL and PBMCs of asthmatics, specifically circulating T cells (61,198,199). Serum and sputum of asthmatics have identified increased lactate compared to healthy individuals indicating more aerobic glycolysis (67,197). Moreover, CD4 T cells isolated from stimulated asthmatic peripheral blood mononuclear cells (PBMCs) produced increased levels of lactate compared to cells isolated from healthy controls (197). Limitations on determining immune cell, specifically T cell, metabolism lie in the availability of sample types and research tools. Our objective was to determine both overall and CD4 specific metabolic differences between healthy and asthmatic individuals. We aimed to address this problem by using mass cytometry (CyTOF) which would allow for single-cell measurements by a high dimensional approach. Metabolic proteins have been integrated into CyTOF panels and allowed for a novel way to identify metabolic differences in T cells with single cell resolution (200,201).

Materials and Methods

Segmental Allergen Challenge and Metabolomics

Mild, atopic asthma patients were recruited to the Vanderbilt Asthma, Sinus, Allergy Program as previously described (202). Patients recruited were between 20-46 years old and had positive skin test to aeroallergens and/or inhaled allergen provocation to qualify for the study (**Table 2-1**). Volunteers consented to the IRB protocol (number: 051158) which was approved by the Vanderbilt University Committee for the Protection of Human Subjects Patients. BAL fluid was sampled from left upper lobe of the lung, then participants were challenged with an allergen known to exacerbate inflammation. Twenty-four hours following segmental allergen challenge, BAL fluid was sampled from the same section of lung after 24 hours as described previously (202). Lactate levels were measured using a colorimetric lactate kit (Sigma #MAK064). Metabolomics on the BAL was measured by high-resolution nontargeted Q Exactive–mass spectrometry (QE-MS) as previously described (117).

Recruitment of Asthma Patients for CyTOF

Patients for which PBMCs were collected were aged 25-47 years old and were a mixture of males and females (**Table 2-3**). Mild asthmatics were characterized by the NHLBI classification of asthma severity including symptoms, medications, quality of life, and lung function (20). Severe asthmatics were characterized by their ability to meet criteria set by the Severe Asthma Research Program. All asthmatic patients were taking asthma medications, were clinically stable, and were not undergoing an exacerbation. Human studies were approved by the Vanderbilt University Committee for the Protection of Human Subjects (IRB number: 111034).

PBMC Processing

Blood from mild and severe asthmatics was collected EDTA tubes, stored at 4 degrees, and processed within 24 hours. Blood from healthy patients was collected from filters obtained from the Nashville Red Cross. Conical tubes were filled with 25 mLs of Ficoll (GE Healthcare # 17-1440-02) and then 25 mLs of blood was carefully layered on top without disturbing the surface. Tubes were then centrifuged for 30 minutes at room temperature with the brake off so as not to disturb the buffy coat. Carefully, the buffy coat containing the PBMCs was removed and placed into a fresh tube. Buffy coat was washed with PBS, red cell removal was done with ACK lysis buffer (VWR # 10-548E) for 2 minutes and washed with RPMI + 10% FBS. Finally, cells are pelleted and resuspended in 10% DMSO in FBS for long term storage in liquid nitrogen.

CyTOF Analysis on PBMCs

Cells were rapidly thawed and recovered in RPMI + 10% FBS. After all samples were thawed, 5 million live cells from each sample were used, and stained on the same day. First, the cells were stained using cisplatin (Fluidigm #201198) to capture dead cells and then washed with PBS plus 1% BSA. Cells were stained for surface antibodies, permeabilized with cold methanol, stained for intracellular antibodies, and finally stained using intercalator (Fluidigm #201192) to identify live cells. The metabolic antibodies used were commercially available western-blot antibodies that were first validated for flow cytometry, and then custom conjugated to metals using Fluidigm conjugation kits. The antibodies in the staining panel are as follows: CD45-141, CD19-142, CD5-143, ATP5a-144, CD4-145, CD8a-146, pSTAT5-147, CPT1a-148, p4E-BP1-

149, CD134(OX40)-150, Grim19-151, pAkt-152, CD62L-153, CD3-154, CD27-155, p38-156, pSTAT3-158, GLUD1-159, Tbet-161, CD69-162, Glut1-163, CytoC-164, CD44-166, GATA3-167, RORγ-168, CD25-169, HLA-DR-170, pERK1/2-171, Ki-67-172, Granzyme B-173, CD279(PD-1)-174, pS6-175, and CD127-176. Cells were stored in intercalator at 4 degrees for less than one week. Before each run, samples were washed 2x in PBS and 2x in diH2O with a final resuspension in 1X EQ Four Element Calibration Beads (Fluidigm #201078). Approximately 10^6 cells were collected from each sample using a Helios Mass Cytometer (Fluidigm) through the Vanderbilt Cancer and Immunology Core. Data pre-processing was done in Cytobank for clean-up and appropriately setting scales. Data analysis was then performed in R using Uniformed Manifold Approximation and Projection (UMAP) for dimensionality reduction, FlowSOM for clustering, and Marker Enrichment Modeling (MEM) for quantification of phenotypic differences between populations (203,204). Analysis was done on the concatenated files of each cohort which included 8,000 cells from each patient.

Statistical Analyses

Statistical analyses were performed on Prism using Student's t-test or one-way ANOVA unless otherwise stated. Statistically significant results are indicated by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. CyTOF statistical analysis was performed on R.

Results

BAL fluid in asthmatics has elevated metabolic pathways

While lactate has been reported to be elevated in murine models of airway inflammation and both PBMCs and sputum of asthmatic patients, the metabolic

programs of asthma-associated T cells remain poorly described (67,197,205). To directly determine if lactate levels are increased in asthmatic patients after an allergen challenge, we sampled the bronchoalveolar lavage (BAL) fluid from the same airway in a cohort of mild asthmatics (**Table 2-1**) before and after segmental allergen challenge. Consistent with previous studies, the BAL fluid collected after allergen challenge contained significantly higher concentrations of lactate than that collected from matched samples prior to challenge (**Figure 2-1A**). Matched BAL fluid samples before and after allergen challenge were then analyzed by high resolution mass-spectrometry metabolomics to broadly identify changes in metabolites (**Figure 2-1B**). While the abundance of many metabolites was unchanged, a number were significantly increased following the allergen challenge. Consistent with elevated glutamine metabolism, glutamate levels were increased after allergen challenge. Further pathway analysis of these significantly enriched metabolites identified higher levels of several pathways, including alanine, aspartate, and glutamate metabolism and the citric acid cycle consistent with elevated mitochondrial metabolism (**Table 2-3**). Allergic responses in the airways of asthma patients, therefore, induce increases in both glucose and glutamine metabolism.

Characteristics of mild asthmatics for BAL analysis

	Female	Male
No.	20	10
Age (y)	31.6	27.7
Race (%)		
African American	10%	20%
Asian	0%	0%
Caucasian	90%	80%
Other	0%	0%
Allergen (%)		
Cat	40%	20%
Dust Mite	35%	20%
Grass Pollen	10%	50%
Ragweed	15%	10%

Table 2-1. Characteristics of mild asthmatics for BAL analysis. Demographics and allergen sensitivity for mild asthmatics that underwent the segmental allergen challenge.

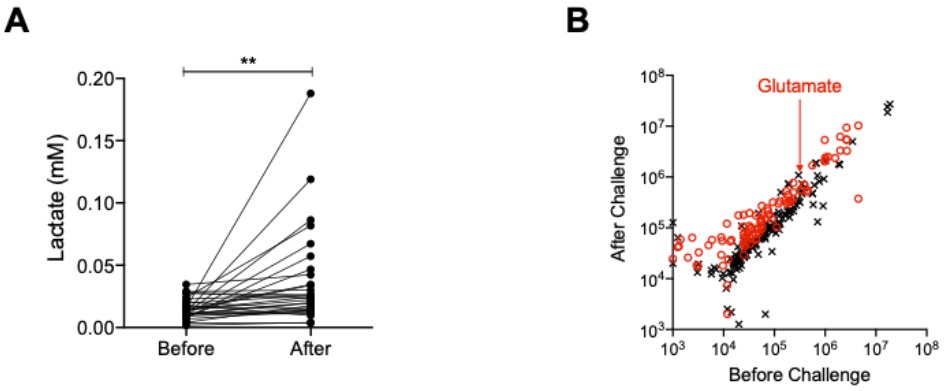

Figure 2-1. Asthmatics have elevated metabolic pathways in BAL fluid. A. Lactate was measured by absorbance in BAL fluid collected from mild asthmatic patients before and after segmental allergen challenge (n=30). **B.** BAL fluid in segmental allergen challenge was

analyzed using high-resolution mass spectrometry metabolomics and significantly altered metabolites ($p<0.05$) are indicated in red. ** $p<0.01$, unpaired t test. Samples gathered by Ryszard Dworski and data analyzed by Peter Siska.

Pathways Enriched in BAL of Mild Asthmatics	FDR
Aminoacyl tRNA biosynthesis	1.17×10^{-6}
Alanine, Aspartate, & Glutamate Metab.	7.07×10^{-4}
Citric Acid Cycle	0.0025
Nitrogen Metab.	0.0064
Arginine and Proline Metab.	0.0411
Valine, Leucine, & Isoleucine Biosynthesis	0.0411

Table 2-2. Enriched metabolic pathways from asthmatic BAL. Pathways that were enriched in mild asthmatics after segmental allergen challenge as measured by QE-MS and significance was calculated by False Discovery Rate (FDR). Samples gathered by Ryszard Dworski and data analyzed by Jason Locasale.

PBMCs from asthmatics from than healthy individuals

PBMCs from asthmatic patients may show systemic immunologic changes that reflect inflammation in the airway (197). To test if the immune and metabolic characteristics of T cell populations were altered in mild and severe asthma. PBMCs from healthy, mild, and severe asthma participants (**Table 2-3**) were analyzed by mass cytometry using antibody panels that included markers of cell identity, activation and immune state, as well as glucose, glutamine, and mitochondrial metabolism. Our panel was designed to examine primarily the T cell population. Initially, CD45+ cells were analyzed to identify if there were differences between our patient cohorts. The data were concatenated from each of the cohorts and viSNE analysis was done to visualize the multi-dimensional data into two dimensions (206). The cells were primarily stratified into 3 main population clusters; left, middle, and right (**Figure 2-2**). The right cluster was CD3+ cells which included our main cell type of interest, CD4. The $CD4^+$ cells were localized on the lower right cluster and high expression for CD5, CD27, and CD127. These markers indicate high activation of these cells and T memory cells are either being activated or just present in the PBMCs. Transcription factor expression in this cluster was much more modest when compared to all the available markers. Our panel included markers that were not only associated with T cells which defined the other clusters (**Figure 2-3**). The left cluster is the B cell population as it is CD19+ and HLA-DR+. The middle cluster expressed HLA-DR, granzyme B, pSTAT3, and pSTAT5 which suggests it contains a combination of antigen presenting cells (APCs) and natural killer (NK) cells. Although $CD4^+$ T cells can express HLA-DR as well, our data did not indicate any CD4+ HLA-DR^+ cells were present in the PMBCs. Interestingly, the middle cluster

had the highest expression of the metabolic markers that were included in our panel (**Figure 2-4**). Strikingly, there were visual differences between healthy individuals compared to either the mild or severe asthmatics in which clusters were populated. One of the biggest drivers of the differences was the cluster that expressed HLA-DR, which indicated that asthmatics have more APCs and NK cells (**Figure 2-3**).

Characteristics of mild and severe asthmatics for PBMC CyTOF analysis

	Mild Asthmatics	Severe Asthmatics
No.	5	6
Age (y)	32.6	37.9
Female (%)	2 (40%)	4 (66.7%)
Race (%)		
African American	20%	17%
Asian	0%	0%
Caucasian	60%	83%
Other	20%	0%
Ethnicity (%)		
Non-Hispanic	80%	100%
Hispanic	20%	0%
Allergies	100%	83%

Table 2-3. Characteristics of mild and severe asthmatics for PBMC CyTOF analysis.
Demographics of mild and severe asthmatics that donated PBMCs for CyTOF analysis.

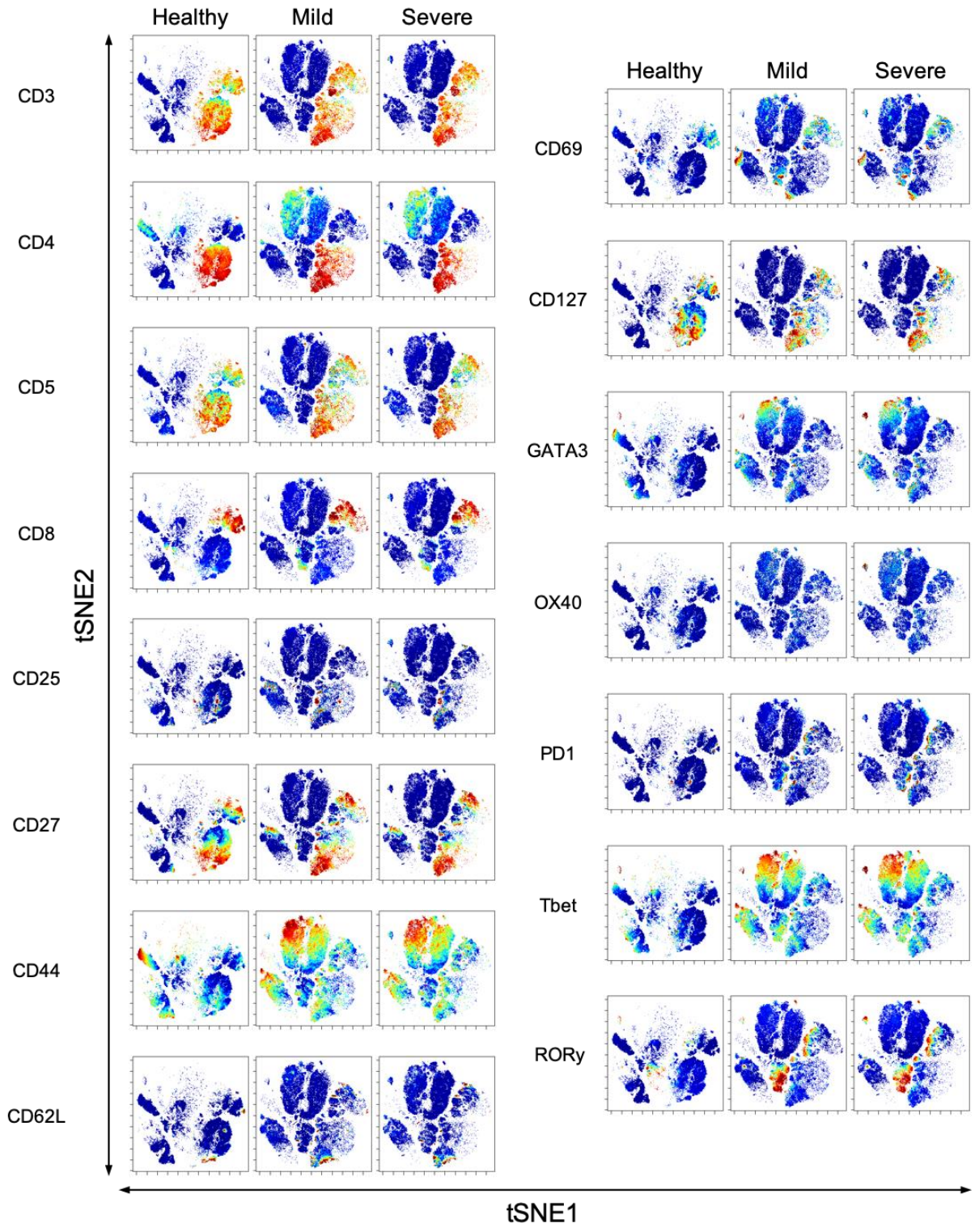

Figure 2-2. Differences in the patient cohorts among T cell associated markers. viSNE analysis done on equal sampling of 100,000 CD45+ cells from concatenated samples of patient cohorts.

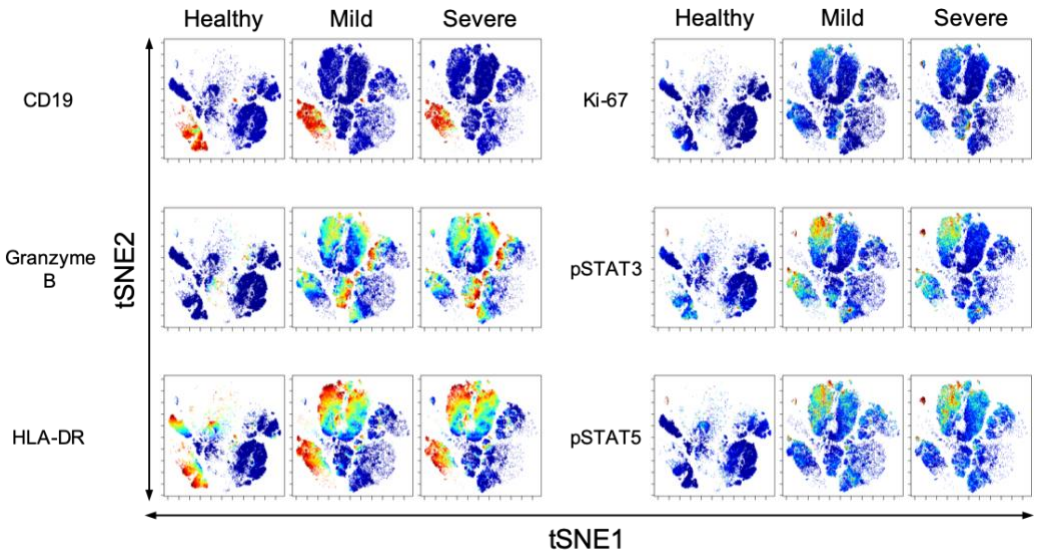

Figure 2-3. Differences in the patient cohorts among markers associated with B cells, APCs, and NK cells. viSNE analysis done on equal sampling of 100,000 CD45+ cells from concatenated samples of patient cohorts.

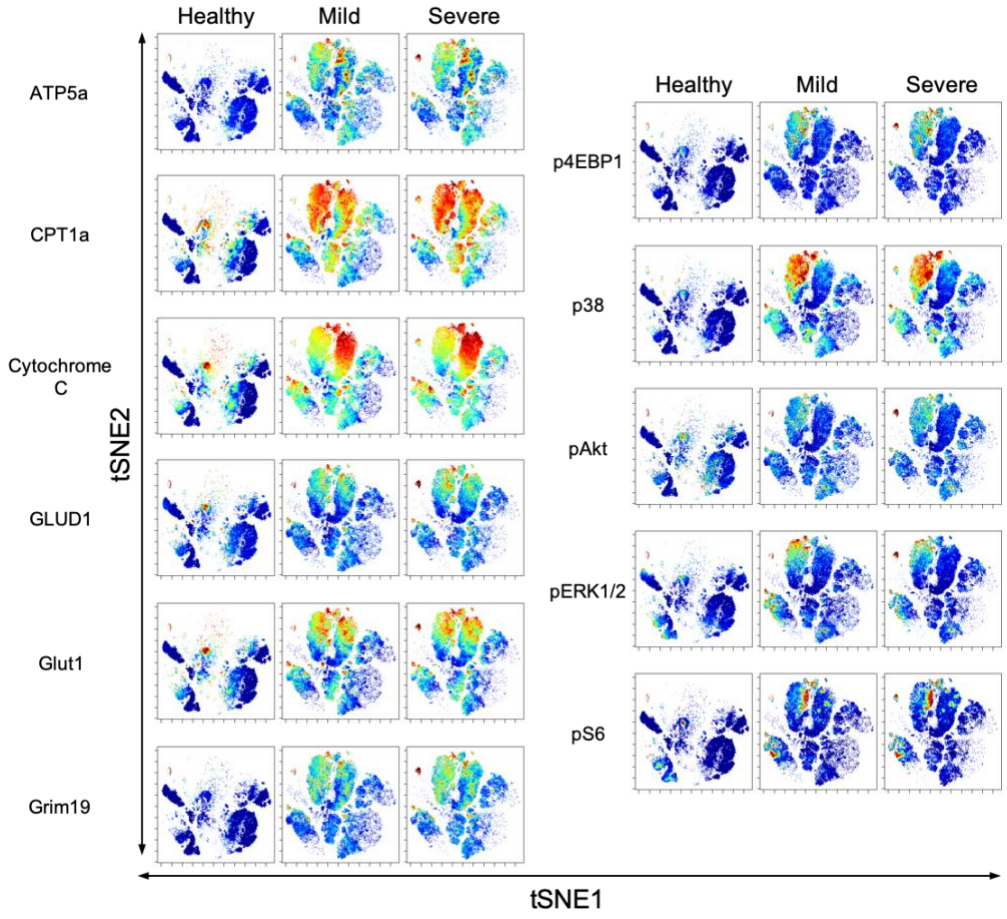

Figure 2-4. Differences in the patient cohorts among metabolism associated markers. viSNE analysis done on equal sampling of 100,000 CD45+ cells from concatenated samples of patient cohorts.

CD4 cells are more metabolically active in asthmatic donors

The clusters suggest that within the CD4 T cells there are differences between healthy and asthmatics (**Figure 2-2**). CD4 T cells from healthy, mild, or severe asthma donors were analyzed to establish the degree of similarity between these T cell populations and to identify population clusters. After concatenation of the mass cytometry data from healthy, mild, and severe asthmatics, UMAP, an analysis tool used for dimensionality reduction, revealed that T cells from each donor type had distinct phenotypes based on the density plots on the UMAP axes (**Figure 2-5A**). Earth Mover's Distance (EMD) quantifies the overall degree of difference between populations and healthy individuals who had large pairwise EMD scores from UMAP when compared to either mild or severe asthmatics (207). Both mild and severe forms of asthma can lead to significant shifts in the overall peripheral blood T cell populations and phenotypes. Surprisingly, CD4 T cells from these mild and severe asthmatic donors differed from each other only modestly and were each most different from healthy donor CD4 T cells.

T cell populations were next subdivided to examine phenotypic clusters. FlowSOM was applied to the mass cytometry data to separate CD4 T cells into distinct populations on the UMAP axes (203). FlowSOM clusters 2, 3, 4, and 7 were largely represented by T cells from healthy donors, while clusters 1, 5, 6, 8, 9, and 10 were predominantly from mild and severe asthma donors, with clusters 5, 6, and 10 more abundant in severe asthmatics (**Figure 2-5B**). Marker Enrichment Modeling (MEM) can identify and quantify cell markers that drive overall phenotypes and differences between these clusters (204). This approach was applied to transcriptional and metabolic markers in each cluster in the three cohorts. Healthy individuals had lower expression of

each of the examined markers in all clusters compared to T cell clusters from the mild and severe asthma cohorts (**Figure 2-5C**). The metabolic proteins included in the analyses were broadly elevated in the asthmatic cohorts and clusters, although Glutamate Dehydrogenase 1 (GLUD1, a glutaminolysis enzyme) was only modestly different between the T cell donors and across each T cell subset and Carnitine Palmitoyl-Transferase 1a (CPT1a, a lipid oxidation enzyme) was particularly elevated in peripheral blood T cells from severe asthmatics. The transcription factors Tbet, GATA3, and RORγ, which drive Th1, Th2, and Th17 cells, respectively, all showed enrichment in mild and severe asthmatics. The enrichment of both GATA3 and RORγ expression across these subsets suggests that both Th2 and Th17 cells may be present in the peripheral blood of mild and severe asthmatics. These data demonstrate that the basal state of CD4 T cells in asthmatics express markers of greater metabolic and inflammatory activity or capacity than those of healthy individuals.

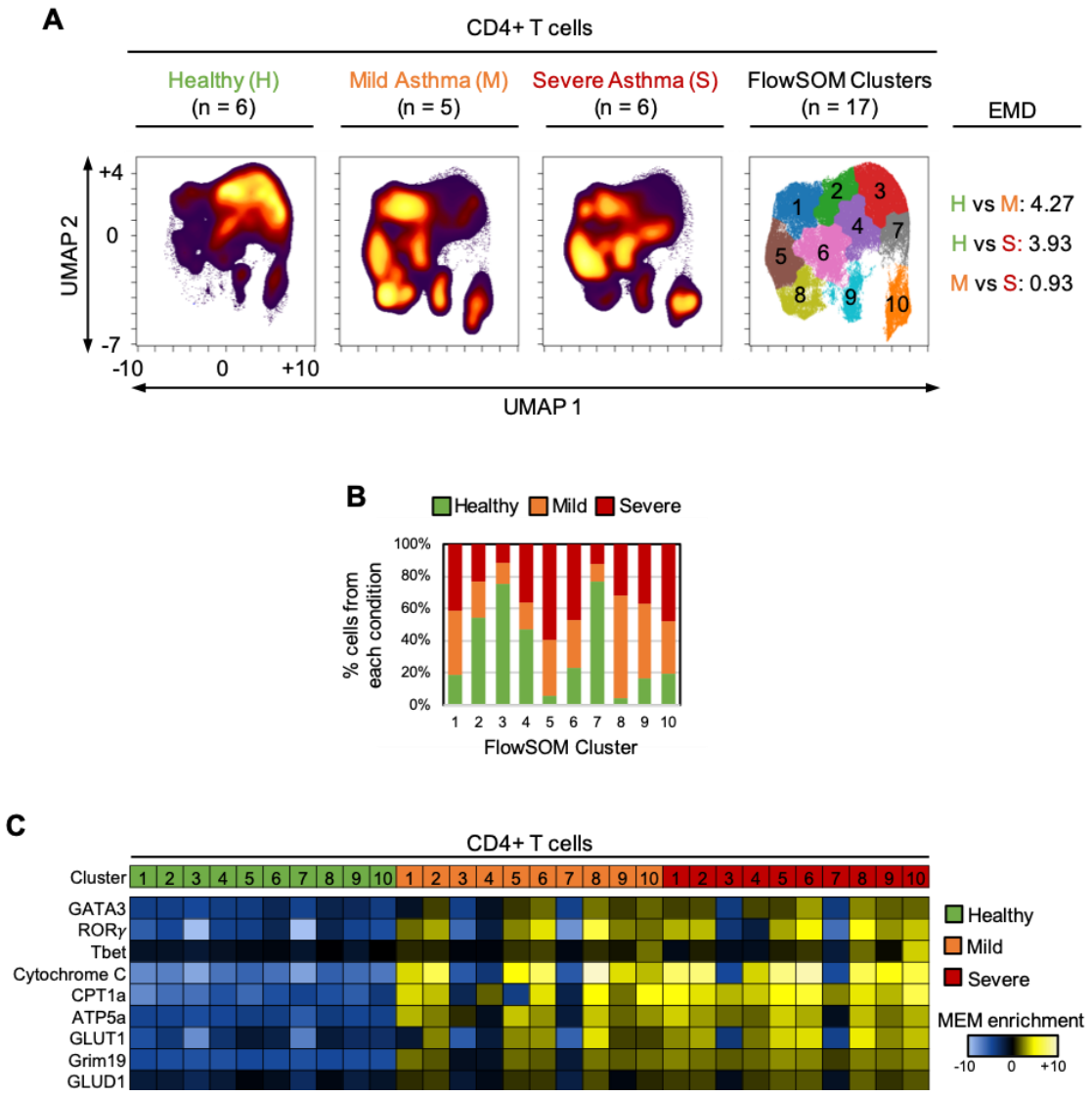

Figure 2-5. Asthmatics have more metabolically active CD4 cells circulating in peripheral blood. A. Concatenated data from CyTOF on cells from healthy, mild, and severe asthmatic participants were analyzed using UMAP for dimensionality reduction and FlowSOM for clustering. Cell density for each condition shown on UMAP axes and FlowSOM clusters indicated in different colors on the UMAP axes for concatenated cohort. Earth Movers Distance (EMD) was applied to quantify the overall degree of difference between each group. **B.** The abundance of cell from healthy, mild, or severe asthmatics data are shown for each FlowSOM cluster in (**A**). **C**. MEM enrichment scores are shown for metabolic markers and transcription factors from the FlowSOM clusters of each disease state. ** p<0.01, unpaired t test. Data analysis by Sierra Barone.

Discussion

Our study and others demonstrate that there is a measurable metabolic shift in the BAL that occurs in asthmatics (67,197). We identify using mass spectroscopy that there is not only a shift in glycolysis by an increase in lactate but also an increase in glutamate. Airway inflammation thus creates an environment that alters both overall metabolism and CD4 T cell metabolism in the BAL (197). These metabolomic data do not, however, differentiate the metabolic programs of specific cell types and rather reflect the overall change. To overcome this challenge to understand the metabolism of T cells in asthma, we developed a single cell-based assay for T cell metabolism using CyTOF. We found this metabolic shift can also be observed in PBMCs from asthmatics. Both mild and severe asthmatics have more B cells, T cells, and HLA-DR expressing cells. The B cell cluster had a minimal expression of metabolic markers, however some of these markers did increase from the healthy to the asthmatic patients. Interestingly, the HLA-DR expressing cells in mild and severe asthmatics had the highest expression of the metabolic markers. This cluster most likely consists of macrophages and dendritic cells which have been documented to require increased metabolic activity for turnover and function in lung diseases (208–213). These markers in the various pathways associated with activated macrophages and dendritic cells were all increased (214–216). The metabolic proteins were not as highly expressed in T cells but there was an increase in the asthmatics compared to the healthy patients. This correlated with T cells that had higher markers of activation.

$CD4^+$ T cells from both mild and severe asthmatics established significant phenotypic and metabolic changes in peripheral blood relative to peripheral blood $CD4^+$

T cells from healthy donors. Airway inflammation thus appears to have a systemic impact that is evident in the altered expression of peripheral blood $CD4^+$ T cell metabolic markers. Chronic inflammation, such as during autoimmunity, can result in systemic alterations. This could explain why increases in metabolic activity were not just limited to a small population of antigen specific CD4+ T cells. The metabolic proteins in the CD4+ T cells were generally coordinated and increased in both mild and severe asthma. There were some exceptions and the T cell population with the greatest CD4 contribution from severe asthma patients also had selectively high expression of Glut1. Likewise, F1/F0 ATPase component ATP5a and mitochondrial lipid transporter CPT1a were selectively increased in some T cells from severe asthma patients. In contrast, GLUD1 was specifically expressed in a population of CD4 T cells present in all asthmatics but enriched in severe cases. Levine et al. established that CD8 T cells had a high metabolic profile during a transition stage early in differentiation that decreased once cells were terminally differentiated (201). Our data suggest that chronically active CD4 T cells have that same highly metabolic phenotype.

Surprisingly, mild and severe asthmatics indicated modest metabolic differences between each other and each showed a greater difference from the healthy control. The similarity of T cells in mild and severe asthma may be a result of analyzing T cells from peripheral blood rather than from the site of inflammation, because individuals in neither cohort were experiencing inflammatory exacerbations when PBMCs were obtained, or as a consequence of the multiple treatments that severe asthmatics take to control inflammation. Metabolic markers may provide a valuable addition to T cell phenotyping

for asthma and further suggest that glucose and glutamine pathways are active in T cells from asthma patients and can discriminate between mild and severe disease.

CHAPTER III

INCREASED GENE EXPRESSION UPON T CELL ACTIVATION IN *ALTERNARIA ALTERNATA* INDUCED AIRWAY INFLAMMATION

A portion of this work adapted from manuscript originally published in *The Journal of Immunology*. Healey DCC et al. 2021. Targeting In Vivo Metabolic Vulnerabilities of Th2 and Th17 Cells Reduces Airway Inflammation. *J. Immunol*. 206:ji2001029.

Introduction

Gene expression analysis of asthmatic patients and mouse models has been an important tool for researchers. Studies have focused on the identification of asthma phenotypes, changes in the airway due to glucocorticoids, and more (109,217–220). However, there has only been one study that investigated gene expression changes of metabolism in T cells. Tibbitt et al. ran single-cell RNA sequencing (scRNAseq) to examine naïve, Th1, and Th2 cell metabolism in a house dust mite model of airway inflammation (109). They found that genes in Th2 cells from the BAL were enriched for glucose metabolism, fatty acid oxidation, and fatty acid synthesis. Interestingly, they observed that *Slc2a1* (Glut1) expression was lower in Th2 cells than in Tregs. We were interested in the metabolism of Th2 cells that localized to the lung and how that compared to Tibbitt's study which focused on Th2 cells that had infiltrated into the airway. We used the *Alternaria alternata* extract model which induced a potent Th2 and eosinophilic response (221,222).

Materials and Methods

Mice

C57BL/6 mice were purchased from Jackson Laboratory at 8-12 weeks of age. Experiments on airway inflammation utilized female mice while male mice were used for all differentiation experiments and *in vitro* studies. All procedures were performed under protocols approved by the Institutional Animal Care and Utilization Committee (IACUC) at Vanderbilt University Medical Center.

Alternaria alternate Airway Inflammation Induction

For the *Alternaria alternata* extract (Alt Ext) model, mice were intranasally administered with 75 μL of 7.5 μg Alt ext (Greer Laboratories #XPM1D3A2.5) or PBS on days 0, 3, 6, and 9. Lungs were harvested on day 7 for scRNA seq or day 10 for flow cytometry analysis.

Flow Cytometry Analysis

Intracellular cytokine staining was performed after a 4-hour restimulation using PMA/Ionomycin and GolgiStop. Cells were stained using a fixable viability dye and a CD4 antibody in PBS for 20 minutes at room temperature. Cells were then permeabilized prior to staining for either IL-4 or IL-17. After cells were divided equally into wells in a 96-well plate so that metabolic markers were individually stained. For metabolic staining, first a primary antibody was left to bind for 1 hour and after (depending on the primary antibody) either an anti-rabbit or an anti-mouse fluorescent antibody was used to stain the metabolic marker. Western antibodies for metabolic proteins were validated for flow cytometry.

Single-cell RNA Sequencing

Adult female mice were administered Alt Ext as previously described and lungs were harvested on day 7. Single cell lung suspensions were enriched for CD45+ cell using Miltenyi microbeads (Catalog #130-052-301) using manufacturer's protocol. CD45+ cells were further purified by cell sorting, and then CD45+ cells were loaded onto 10X Genomics Chromium Controller for single-cell RNA (scRNA) sequencing. CellRanger software (v3.0.2) was used with default parameters for library demultiplexing, fastq file generation, read alignment, and unique molecular identifiers (UMI) quantification to generate the gene expression matrix. Aggregated gene expression matrices containing numbers of UMIs per cell per gene were filtered to retain cells with at least 200 genes detected and less than 10% of total UMIs originating from mitochondrial RNA. Genes detected in more than 3 cells were retained for the following analysis. Dimension reduction (PCA, UMAP) and clustering were applied to the filtered matrix using Seurat (v3.2.0) with default parameters, except the top 20 dimensions of PCA were used for UMAP (223). R package clusterProfiler (v3.16.1) was used for the gene set enrichment analysis with the KEGG gene sets pulled from R database msigdbr_v7.1.1 (224). Data visualization was done using the respective analysis tools or custom scripts using ggplot2 (R package). Data are deposited for public access under accession GSE163572.

Results

Our metabolic analyses identified differences between Th2 and Th17 cells in vitro, but these metabolic profiles observed may not mimic those that occur in vivo

during active inflammation. To measure T cell metabolism in vivo, Alt Ext was used to sensitize mice and induce airway inflammation. This model resulted in increased numbers of $CD4^+$ T cells as well as IL-5, IL-1, and dual cytokine producing cells, although IL-17 producing cells were present in lower numbers (**Figure 3-1A**). Single-cell RNA (scRNA) sequencing was performed on CD45+ lung cells from control and mice subject to Alt Ext and a concatenation of PBS and Alt Ext groups was transformed into a UMAP with clustering (**Figure 3-1B**). Cells and cell clusters were assessed for expression of SELL (CD62L), IL-4, and IL-17 to identify resting, Th2 and Th17 cell populations respectively. (**Figure 3-1B**). While resting SELL+ and IL-4 producing cells were readily identified in specific cell clusters, the Alt Ext model led to few IL-17 producing cells detected by scRNA sequencing. We, therefore, compared clusters of $CD4^+$ cells with IL-4 producing cells (cluster 2) to a cluster enriched with resting SELL+ $CD4^+$ cells (cluster 1) to identify gene expression patterns associated with Th2 cytokine producing cells in airway inflammation (**Figure 3-1C**). In addition to increased expression of T cell receptor signaling genes, KEGG enrichment analysis indicated a significant increase in oxidative phosphorylation and a trend towards elevated glycolytic and glutamate metabolism gene expression in the IL-4 expressing cells (**Figure 3-1D**).

Because few IL-17 producing cells were detected by scRNA sequencing, T cells from lungs of mice sensitized to Alt Ext were next analyzed by flow cytometry. This approach allowed a greater number of T cells to be analyzed for expression of cytokines and metabolic markers of glycolysis (Glut1) or glutaminolysis (GLUD1) (**Figure 3-1E**). Both proteins had increased expression in cytokine producing cells compared to those that did not produce cytokines. Interestingly, GLUD1 expression was significantly

increased in IL-17 producing cells compared to IL-5 producing cells. These data suggest that despite activation increasing the metabolic output of Th2 cells, Th17 cells can demonstrate greater potential for glutamine metabolism *in vivo*.

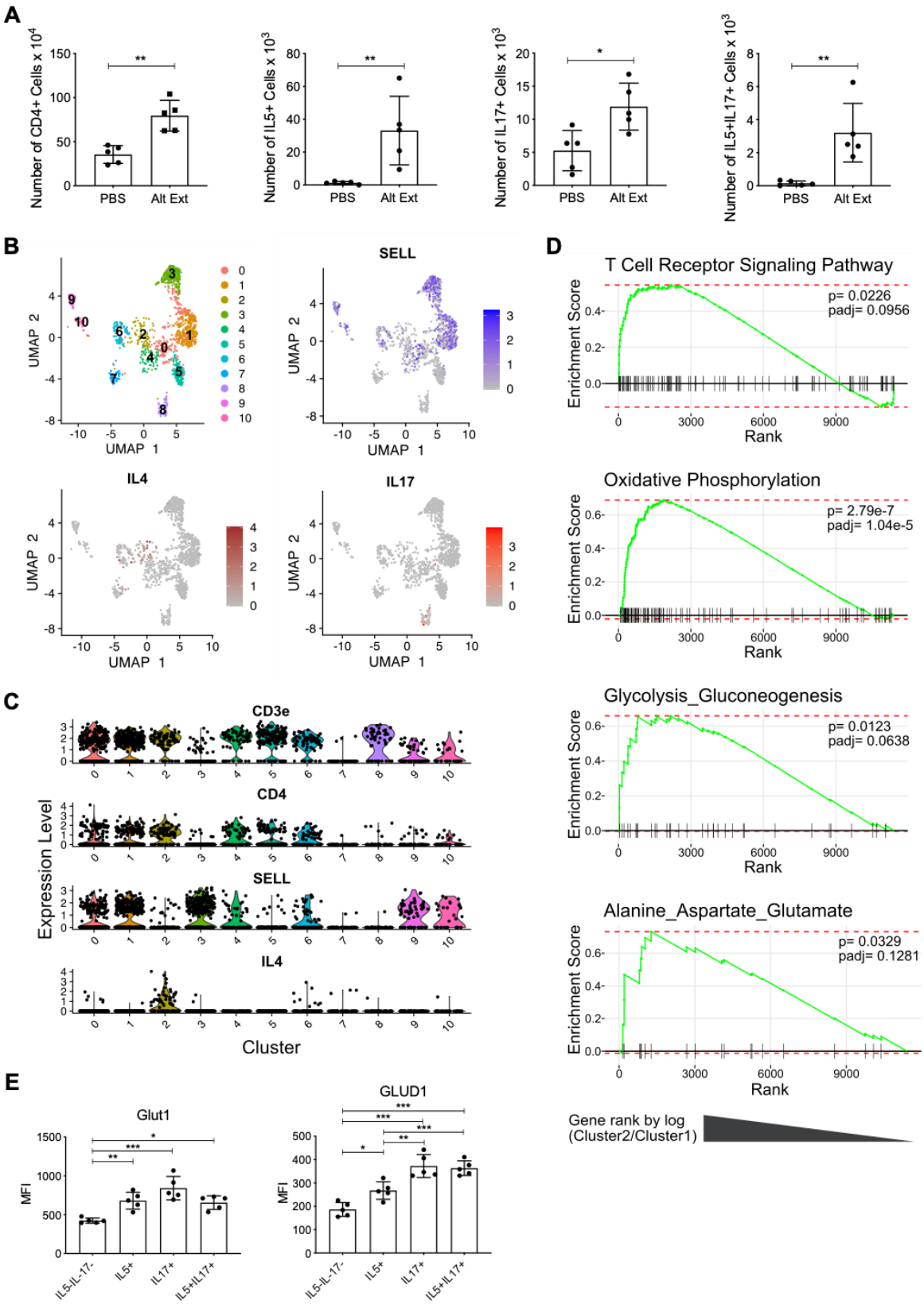
A
Number of CD4+ Cells x 10^4
Number of IL5+ Cells x 10^3
Number of IL17+ Cells x 10^3
Number of IL5+IL17+ Cells x 10^3
PBS
Alt Ext
B
UMAP 1
UMAP 2
SELL
IL4
IL17
C
CD3e
CD4
SELL
IL4
Expression Level
Cluster
D
T Cell Receptor Signaling Pathway
p= 0.0226
padj= 0.0956
Oxidative Phosphorylation
p= 2.79e-7
padj= 1.04e-5
Glycolysis_Gluconeogenesis
p= 0.0123
padj= 0.0638
Alanine_Aspartate_Glutamate
p= 0.0329
padj= 0.1281
Enrichment Score
Rank
Gene rank by log
(Cluster2/Cluster1)
E
Glut1
GLUD1
MFI
IL5-IL-17-
IL5+
IL17+
IL5+IL17+

Figure 3-1. CD4$^+$ T cells from *Alternaria* extract challenged mice increase metabolic gene expression. Mice were challenged with either PBS or Alt ext. **A.** Lymphocytes were isolated from dissociated lungs and analyzed by intracellular flow cytometry to quantitate total and cytokine-producing CD4 T cells (n=5 per group) at day 10. **B-D**. Lung infiltrating CD45+ cells were collected from control and Alt ext mouse lungs at day 7 and analyzed by scRNAseq (n=3). Concatenated data from scRNA seq on all lung cells from PBS or Alt ext mice were analyzed using UMAP for dimensionality reduction. **B**. UMAPs showing the gene expression of SELL (CD62L), IL-4, and IL-17. **C**. Violin plots showing the gene expression level per cluster of CD3e, CD4, SELL, and IL-4. **D**. GSEA plots of enriched gene sets that were enriched in IL-4 expressing cells compared to SELL cells in CD4 T cells (Cluster2/Cluster1). **E**. Expression of glucose transporter 1 (Glut1) and glutamate dehydrogenase 1 (GLUD1) proteins was measured by flow cytometry in different CD4 T cell cytokine producing populations. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, unpaired t test and one-way ANOVA. Data generated by Dawn Newcomb and Norwin Chowdhury. scRNAseq data analysis by Xiang Ye.

Discussion

Th2 airway inflammation induced by Alt Ext led to a metabolically active Th2 population by gene expression. The scRNA sequencing showed that IL-4 producing CD4 T cells expressed gene signatures of increased oxidative and a trend towards increased glycolysis and glutamate metabolism. We did not identify significant changes in fatty acid oxidation or synthesis (109). However, the enrichment in these pathways is consistent with the metabolic requirements that have been identified by other studies (84,101,225). Unfortunately, the model did not identify sufficient numbers of IL-17 producing CD4 T cells to determine differences between Th2 and Th17 cells. However, flow cytometry analysis was able to provide more information about the metabolic differences between Th2 and Th17 cells. These data suggest that cytokine producing T cells in the lung had increased glycolysis and glutaminolysis relative to those T cells not secreting cytokines. CD4 T cells producing IL-17 alone or in combination with IL-5 displayed the highest expression of Glut1 or GLUD1. This model also developed a smaller Th17 population which also indicated a high capacity for metabolic activity, particularly glutamine metabolism. These data suggest that both Th2 and Th17 cells are metabolically active in asthma, although IL-17 producing Th17 cells may have a greater level of metabolic activity than IL-5 producing Th2 cells based on the expression of the metabolic markers Glut1 and GLUD1.

CHAPTER IV

DISTINCT METABOLIC PROGRAMS OF TH2 AND TH17 CELLS

A portion of this work adapted from manuscript originally published in *The Journal of Immunology*. Healey DCC et al. 2021. Targeting In Vivo Metabolic Vulnerabilities of Th2 and Th17 Cells Reduces Airway Inflammation. *J. Immunol*. 206:ji2001029.

Introduction

Historically, asthma has been characterized as primarily a Th2 driven disease due to the prominent roles of its associated cytokines in airway inflammation (189,226). However, early studies of asthmatics reported elevated levels of cytokines not established at that time as Th17 associated, including increased IL-1, IL-6, and IL-17 (226). As Th17 cells were recognized and came into prominence, their role in severe asthma became better elucidated (17,20,60,227). Th17 cells primarily produce IL-17 which helps to promote CXCL8 which recruits neutrophils to the site of inflammation in the airway (16,228). Despite current insights into Th17 cells in asthma, there are still gaps in our knowledge as to how mild type-2 mediated asthmatics can develop neutrophilic severe asthma and the development of dual Th2/Th17 cells present in some of these patients (19,23,24). These alterations in the T cell profile of patients may be metabolism driven.

The metabolic requirements and fluctuations in Th17 cells have been well studied both *in vitro* and *in vivo*. This is not only due to their role in a variety of diseases but also to their plasticity towards Treg cells (114,154,229–231). Meanwhile, the

literature on Th2 cell metabolism is much more limited (225). The Rathmell lab has established that although both Th2 and Th17 cells require glycolysis for proliferation, Th2 cells have higher glycolytic rates (84). Enriched metabolic pathways are not limited to glycolysis but also include both fatty acid oxidation and synthesis (109). The most extensive research on Th2 metabolism has centered on the mTOR pathway, which is a regulator of cell metabolism (76,79,225,232). There are two distinct complexes of which mTOR is the catalytic subunit, either mTORC1 or mTORC2. Studies have demonstrated that mTORC1 is required for T effector cell differentiation (92,97,108). However, Th2 cells also require mTORC2 as it upregulates downstream an AGC kinase, SGK1, which regulates Th2 differentiation (106,233).

In contrast to the limited information on Th2 metabolism, the studies on Th17 cells suggest their metabolism contributes to their plasticity (116,128). Proliferation and differentiation of Th17 cells require glycolysis, activation of the transcription factor, HIF1α, and active PDHK (84,117–121). Th17 cells utilize mTORC1 and are impaired by the energy sensor AMPK (inhibitor of mTOR activity) (122,123). Functionality of Th17 cells depends on glutaminolysis (78,80,81,104,115). A majority of these studies focus on the requirements for T cells to differentiate into Th17 versus Tregs, this axis makes Th17 cells the most metabolically flexible of the T effector cells (115,123,125,230). Importantly, Th2 and Th17 cells are implicated in severe neutrophilic asthma therefore identifying metabolic differences or vulnerabilities may be beneficial for alternative therapeutics (19,23,24). We aimed to determine the metabolic differences between Th2 and Th17 cells. We directly compared differentiated CD4 T cells *in vitro* measuring metabolites, extracellular flux, and the metabolic proteins in our CyTOF panel. In order

to identify the metabolic differences in airway inflammation, we used a HDM+LPS model that induced both Th2 and Th17 cells in the lung and developed neutrophilic inflammation. Lung Th2 and Th17 cells were measured for changes in their metabolic proteins.

Materials and Methods

Mice

C57BL/6 female mice were obtained from Jackson Laboratory at 7 weeks of age. All procedures were performed under protocols approved by the Institutional Animal Care and Utilization Committee (IACUC) at Vanderbilt University Medical Center.

T Cell in vitro Activation and Differentiation

CD4 T cells were isolated from the spleen of wild type C57BL6/J mice by negative separation using magnetic beads (Miltenyi Biotec). Cells were activated and differentiated using anti-CD3 (2.5 μg/mL) and a feeder layer of irradiated splenocytes. Th2 cells were differentiated by adding recombinant mouse (rm) IL-4 (80 ng/mL) and anti-IFNγ (10 μg/mL). Th17 cells were differentiated by adding rmIL-6 (80 ng/mL), recombinant human TGFβ (1.5 ng/mL), and anti-IFNγ (10 μg/mL). Cells were cultured for 5 days, with a split on day 3 into new wells and fresh media supplemented with 10 ng/mL IL-2 to promote continued proliferation. Metabolomics on differentiated cells was measured by high-resolution nontargeted Q Exactive–mass spectrometry (QE-MS) as previously described (117).

Flow Cytometry Analysis

Intracellular cytokine staining was performed after a 4-hour restimulation using PMA/Ionomycin and GolgiStop. Cells were stained using a fixable viability dye and a CD4 antibody in PBS for 20 minutes at 4 degrees. Cells were then permeabilized with Cytofix/Cytoperm (BD Bioscience #554722) prior to staining for either IL-4 or IL-17. Next, cells were incubated with rat serum and Fc Block for 30 minutes and then divided equally into wells in a 96-well plate so that metabolic markers were individually stained. For metabolic staining, first a primary antibody was left to bind for 1 hour at room temperature and after (depending on the primary antibody) either an anti-rabbit or an anti-mouse fluorescent antibody was used to stain the metabolic marker. Western antibodies for metabolic proteins were validated for flow cytometry. Transcription factor staining was performed on non-stimulated cells after viability and surface staining. The Foxp3/ Transcription Factor Staining Buffer set (eBioscience #00-5523-00) was used to permeabilize the cells and then the cells were stained for either GATA3 or RORγt. Samples were run on the Miltenyi MACSQuant and analysis was done using FlowJo software.

Extracellular Flux Analyses

Differentiated T cells were isolated by negative separation using magnetic beads (Miltenyi Biotec) to remove feeder layer of irradiated splenocytes and then counted. Cells were resuspended in Seahorse XF RPMI (Agilent #103336-100) supplemented glutamine for ECAR measurements or glutamine, sodium pyruvate, and glucose for OCR measurements. Cells were then seeded at 100,000 live cells per well on a XF96

plate (Agilent) was coated with Cell-Tak (BD Bioscience #354240) and rested for 30 minutes in a non-CO_2 incubator. Mito Stress assay to measure OCR was performed using a kit (Agilent #103015-100) containing oligomycin, FCCP, and rotenone/Antimycin A. Glycolysis Stress assay to measure ECAR was performed using glucose (Agilent #103577-100), oligomycin (Agilent), and 2-deoxy-D-glucose (Sigma-Aldrich # D6134). XF96 plate was placed on Agilent Seahorse XF96 bioanalyzer to record measurements.

Airway Inflammation Induction

Female mice at 8 weeks of age were intranasally administered with 50 μL of either PBS or 100 μg of house dust mite *Dermatophagoides pteronyssinus* extract (Greer Laboratories #XPB70D3A25) plus 0.1 ug of LPS from *Escherichia coli* 0111:B4 (Sigma #L4391). Mice were challenged on Day 0, 7, and 14 and cells and tissues were harvested on day 15 for all endpoints except for airway hyper-responsiveness measurements, which were performed on day 16. Inhibitor treatments were initiated on Day 0 and given daily throughout the 14 days of airway inflammation induction. Glut1 inhibitor (KL-11743, Kadmon Corporation) was dosed daily by oral gavage at 75 mg/kg as prescribed by the company (182,183). GLS inhibitor (CB839, Calithera Biosciences) was dosed twice daily via oral gavage at 200 mg/kg as described previously (175,176).

Airway Hyperresponsiveness Measurement

Mice were anesthetized using pentobarbital sodium, a small incision (0.25 cm) was made in the trachea to insert a tracheostomy tube. Mice were then placed in a chamber and mechanically ventilated at 150 breaths/minute with 0.2ml tidal volume using the Flexivent (SciReq). AHR was measured after administration of aerosolized

saline (vehicle) followed by increasing concentrations of acetyl-ß-methacholine (12–50 mg/kg body weight) (234).

Histopathology

To measure airway inflammation, lungs were perfused, inflated and instilled with 800 μL of neutral-buffered formalin overnight at room temperature. Lungs were transferred to 70% ethanol and paraffin embedded. Tissue sections (5μm) were stained with hematoxylin and eosin (H&E) or Periodic Acid-Schiff (PAS) stain, and slides were quantified and scored according to following scale by a pathologist blinded to the experimental groups to score inflammation. The scoring system for H&E staining was a 0 to 3 scoring system: 0 indicated no inflammatory cells; 1, a few inflammatory cells; 2, increased accumulation of inflammatory cells; and 3, abundant accumulation of inflammatory cells. The scoring system for PAS- staining was: 0, no PAS-positive cells; 1, <5% PAS-positive cells; 2, 5% to 10% PAS-positive cells; 3, 10% to 25% PAS-positive cells; and 4, >25% PAS-positive cells.

Collection of Bronchoalveolar Lavage (BAL) Fluid and Determining Inflammatory Cell Infiltration

A tracheostomy tube was inserted and attached to a syringe. The lungs were flushed using 800 μl of saline and as much fluid as possible was withdrawn gently using the syringe. An aliquot of the BAL was taken for cell count using Trypan Blue to determine the number of live cells. Another aliquot was spun onto a slide where the cells were fixed and stained. A representative two hundred cells were counted from the

slide and classified as macrophages, eosinophils, neutrophils, or lymphocytes. The remaining BAL fluid was spun down and the supernatant was stored for later analysis by Luminex cytokine analysis.

Luminex

Cytokine levels in the BAL supernatants were measured using a MILLIPLEX Mouse High Sensitivity T cell Panel kit (Millipore #MHSTCMAG-70K) for IL-4, IL-5, IL-17A, and IL-1β by Luminex assay through the Vanderbilt Hormone Assay and Analytical Services Core.

Single Cell Suspension from Lungs

Lungs were harvested then placed into gentleMACS C Tubes (Miltenyi Biotec) containing RPMI+10% FBS, Collagenase IA (Sigma #C2674), and DNase I (DN25). C Tubes were place on the gentleMACS Dissociator (Miltenyi Biotec), program *m_lung_02.01* was run twice, and then they were places in a 37 degree incubator for 1 hour. EDTA was then added to all the tubes to stop the reaction and cells were pelleted. After cells were resuspended in media and strained through a 70 μm filter to get a single cell suspension. Cells were then used for flow cytometry analysis and cell counting.

Statistical Analyses

Statistical analyses were performed on Prism using either Student's t-test or one-way ANOVA unless otherwise stated. Statistically significant results are indicated by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Results

Differences in metabolites between Th2 and Th17 cells

Analyses of human asthmatic samples identified that CD4 T cells had increased expression of metabolic markers and it appeared that both Th2 and Th17 cells were present to contribute to increased metabolism in asthma. The Alt Ext experiment identified metabolic changes in Th2 cells by scRNAseq and helped to establish some metabolic differences between Th2 and Th17 cells. We were interested in first determining differences *in vitro* between the metabolic programs of Th2 and Th17 cells. Using a published metabolomics data set in which the metabolomic profiles from Th2 cells had not been previously analyzed (117), we compared metabolites from *in vitro* differentiated murine Th2 and Th17 CD4 T cells to naïve CD4 T cells (**Figure 4-1A**). As previously described, both Th2 and Th17 cells utilized distinct metabolic programs and metabolite profiles from naïve CD4 T cells and each other (84,117). To identify how these metabolic programs most differed, metabolic pathway analysis was performed on significantly different metabolites between Th2 and Th17 cells. Similar to increased glutamate metabolism observed in segmental allergen challenge in asthmatic patients, the most altered pathway in Th17 cells relative to Th2 cells was alanine, aspartate, and glutamate metabolism, including increased levels of glutamate and aspartate (**Figure 4-1B**). The shift in the ratio of glutamine and glutamate in Th2 and Th17 cells further supports a greater role for glutaminolysis in Th17 cells (**Figure 4-1C**) (80).

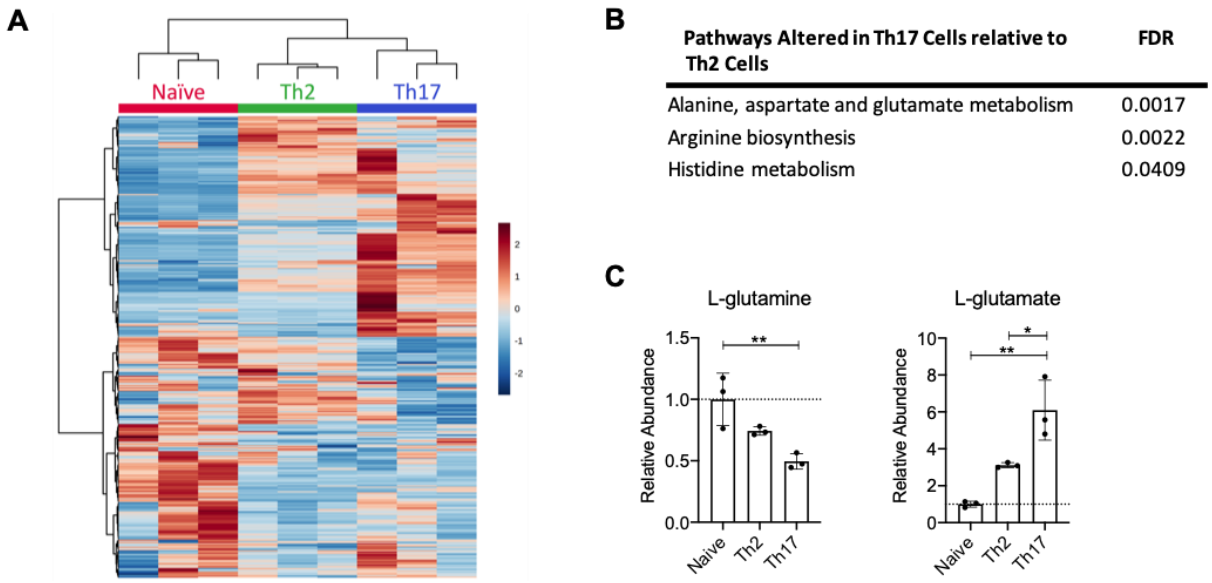

Figure 4-1. Th2 and Th17 cells have distinct metabolic programs. A-B. High resolution metabolomics data using Q-Exactive-MS spectrometry of CD4 T cells differentiated in either naïve, Th2, or Th17 media conditions for 5 days (n=3) was analyzed using Metaboanalyst (**A**) to generate a heat map of differentially abundant metabolites and (**B**) perform pathway analysis of statistically different metabolites ($p<0.05$) between Th2 and Th17 cells. Note that while all samples were collected for each T cell subset and analyzed by QE-MS together, naïve and Th17 data were previously published and are re-analyzed here together with Th2 data **(117)**. **C**. Glutaminolysis associated metabolites measured in naïve and differentiated Th2 and Th17 cells (n=3). * $p<0.05$, ** $p<0.01$, unpaired t test and one-way ANOVA. Data generated by Val Gerriets.

Modest differences in the metabolic rates of Th2 and Th17 cells

The rates of glucose and mitochondrial metabolism were next measured in murine Th2 and Th17 cells. Extracellular acidification rate (ECAR) reflects cellular rates of lactate secretion and the contribution of glycolysis can be tested by providing T cells glucose in extracellular flux assays. CD4 T cells were differentiated *in vitro* into Th2 and Th17 subsets and were found to have similar ECAR measurements, with Th2 cells demonstrating a trend towards increased glycolytic rate, maximal capacity, and additional reserve that can be induced when mitochondrial oxidative metabolism is suppressed by treatment with the mitochondrial inhibitor, oligomycin (**Figure 4-2A, Figure 4-2B**). As ECAR measures decreased extracellular pH and does not account for alternative fates for glucose, Th17 cells may continue to require glucose and instead more effectively shunt glucose metabolism intermediates into different metabolic pathways. In particular, Th17 cells had modestly increased levels of glucose-derived intermediates in the pentose phosphate pathway and one carbon metabolism when compared to Th2 cells (**Figure 4-2C**) (102). Moreover, inhibition of mTORC1 which results in decreased glycolysis, impaired both Th2 and Th17 cells (**Figure 4-2D**). In contrast to ECAR, measurement of oxygen consumption rates (OCR), which indicate mitochondrial oxidative phosphorylation, showed that Th17 cells had modestly higher basal and maximal respiratory rates and significantly more mitochondrial ATP production (**Figure 4-2E, Figure 4-2F**).

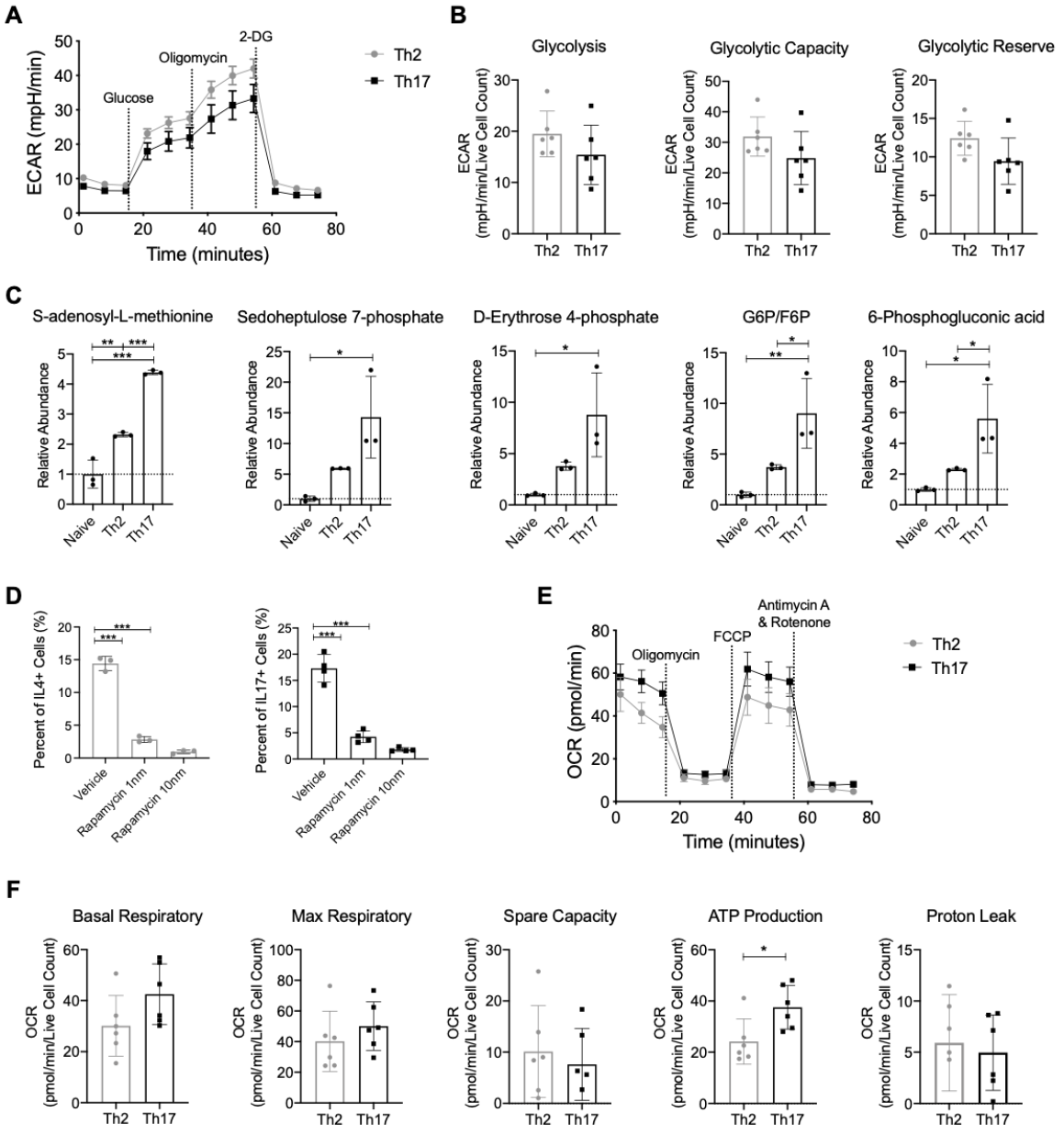

Figure 4-2. Differences in metabolic flux between Th2 and Th17 cells. A-B. Th2 and Th17 cells were differentiated *in vitro* (n=4) and flux measurements of extracellular acidification rates (ECAR) were measured. **C.** Pentose phosphate pathway and one-carbon metabolites measured in naïve and differentiated Th2 and Th17 cells (n=3). **D**. Cytokine production measurements by intracellular flow of differentiated Th2 (n=3) and Th17 (n=4) cells that were treated with Rapamycin. **E-F**. Th2 and Th17 cells were differentiated *in vitro* (n=4) and flux measurements of oxygen consumption rates (ECAR) were measured.* p<0.05, ** p<0.01, unpaired t test and two-way ANOVA.

Th2 cells have a higher metabolic activity by protein expression

We were interested in determining whether there were differences in the expression of metabolic proteins between Th2 and Th17 cells. Cytokines were produced in differentiated cells ranging from between 20-30% producing IL-4 and about 10% producing IL-17 of each population (**Figure 4-3A**). The metabolic protein expression in these cytokine producing cells was measured and Th2 cells had overall the highest expression of most proteins (**Figure 4-3B**). The only exception was hexokinase II (HKII) which was more highly expressed in Th17 cells. Together, these data suggest that both *in vitro* stimulated Th2 and Th17 cells are highly metabolically active and while Th2 cells appear to have greater glycolytic capacity, Th17 cells more predominantly utilize mitochondrial oxidative phosphorylation. These data also suggest that in contrast to *in vivo* Alt Ext studies *in vitro* Th2 cells have more metabolic activity as measured by protein expression.

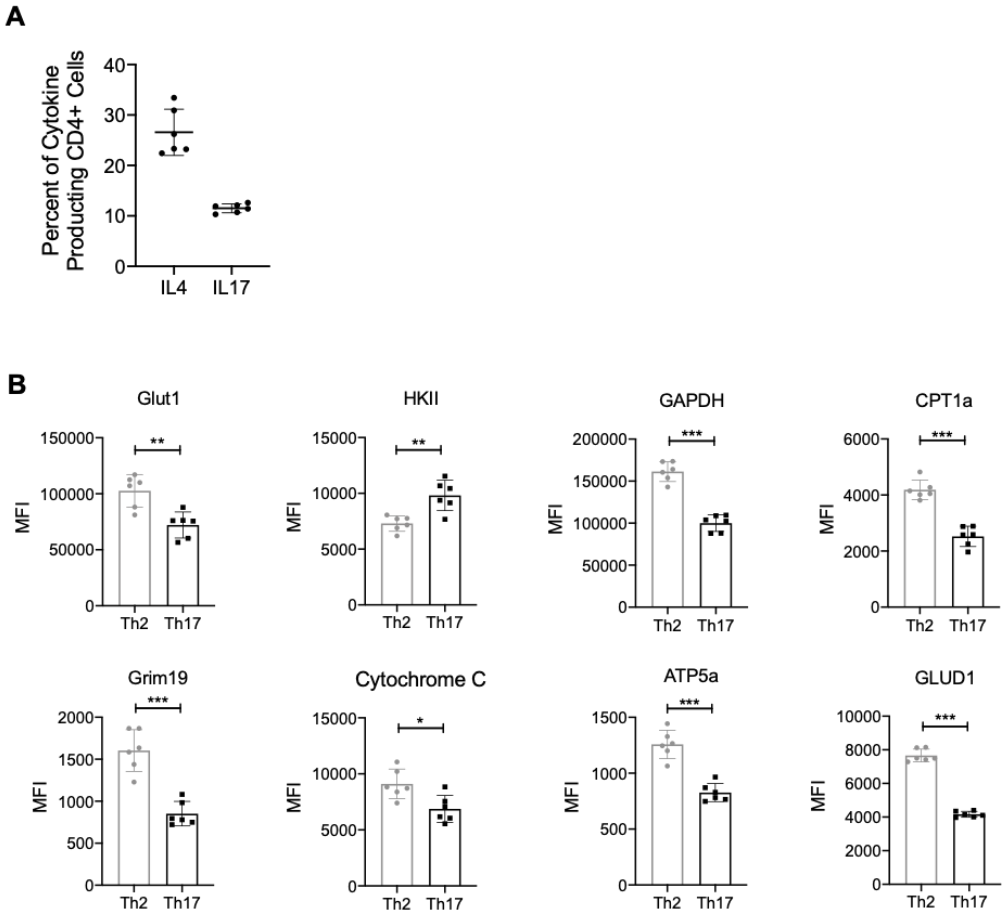

Figure 4-3. Differences in metabolic protein expression between Th2 and Th17 cells. Th2 and Th17 cells were differentiated *in vitro* (n=6). **A**. Percent of cytokine production of differentiated cells was measured. **B**. Expression of various metabolic proteins was measured by MFI in the differentiated cytokine producing CD4 T cells. Proteins include glucose transporter 1 (Glut1), hexokinase 2 (HK2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), carnitine palmitoyltransferase 1A (CPT1a), Grim19 (a mitochondrial complex I protein), Cytochrome C, ATP5a (a mitochondrial complex V protein), and glutamate dehydrogenase 1 (GLUD1).* $p<0.05$, ** $p<0.01$, unpaired t test.

A model of mixed Th2 and Th17 airway inflammation induced with house dust mite and LPS

Next, we were interested to further explore the metabolic differences between Th2 and Th17 cells *in vivo* during airway inflammation. The Alt Ext model (**Figure 3-1**) suggested *in vivo* metabolic phenotypes of Th2 and Th17 cells but did not generate a sufficiently robust Th17 response characteristic of neutrophilic severe asthma. We therefore established a modified version of a murine model previously described to promote Th17 CD4 T cells and neutrophilia following administration of house dust mite (HDM) in combination with LPS to activate TLR4 (**Figure 4-4A**) (187). Sensitized mice developed increased airway hyperresponsiveness (**Figure 4-5A**) and lungs showed thickening of the basement membrane, increased inflammation, immune cell infiltration, and increased numbers of goblet cells (**Figure 4-5B**). Cellular and immunological analyses indicated HDM+LPS challenged mice had significantly increased total cells in the BAL and increased immune infiltrating cells, including a sharp increase in both eosinophils and neutrophils (**Figure 4-5C**). Cytokine levels were measured from the BAL fluid and a variety of inflammatory cytokines characteristic of both Th2 and Th17 cells, including IL-4, IL-13, and IL-17, were found to be increased following HDM+LPS challenge (**Figure 4-5D**).

We assessed the functional phenotypes of CD4 T cells in the lungs of HDM+LPS sensitized mice as in the Alt Ext model. As expected, the total number of live CD4 T cells was significantly higher after HDM+LPS challenge (**Figure 4-4B, Figure 4-5E**). While there was a trend towards increased numbers of CD4 T cells producing only IL-4, the numbers of CD4 T cells producing IL-17 alone or both IL-4 and IL-17 were

significantly increased following sensitization (**Figure 4-5F**). These data demonstrate that HDM+LPS challenge promoted an increased innate inflammatory infiltrate response and increased IL-17 production by T cells in the lung environment to mimic some key features of severe Th17-mediated neutrophilic asthma.

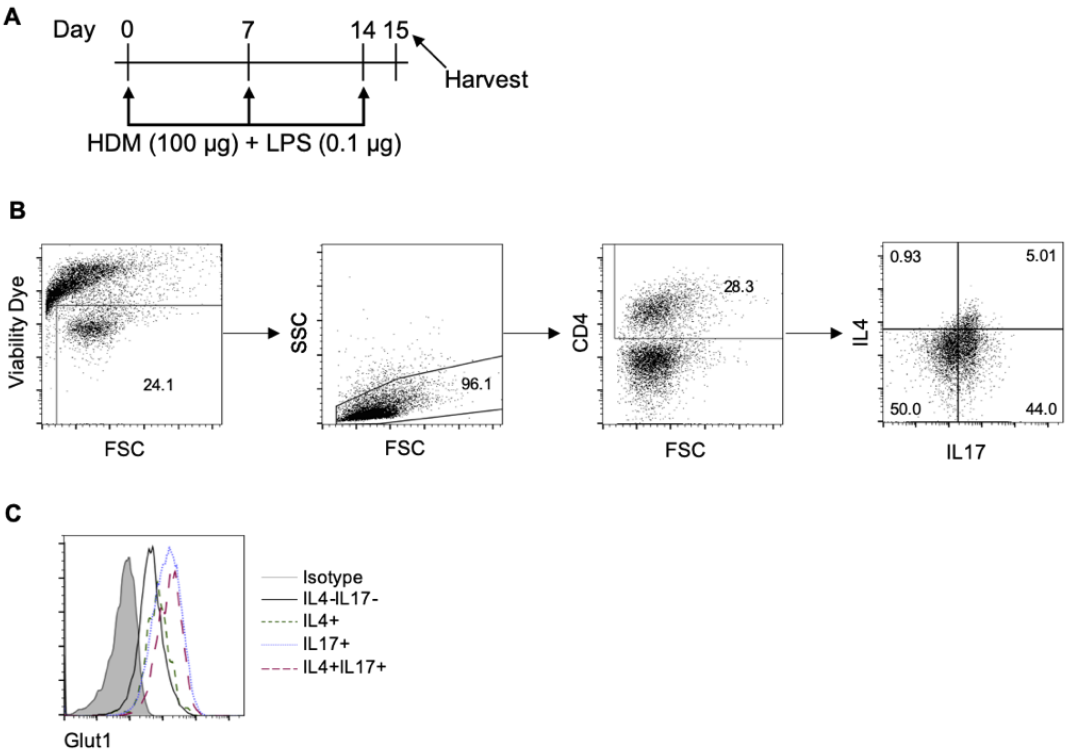

Figure 4-4. Airway inflammation model and gating strategy. A. Experimental design for induced airway inflammation. **B-C**. Representative gating strategy for homogenized lung cells of a mouse challenged with HDM+LPS. **B**. Gates for live cells, lymphocytes, CD4 T cells, and cytokine-producing cells. **C**. Histogram of Glut1 expression for the cells producing and not producing cytokines.

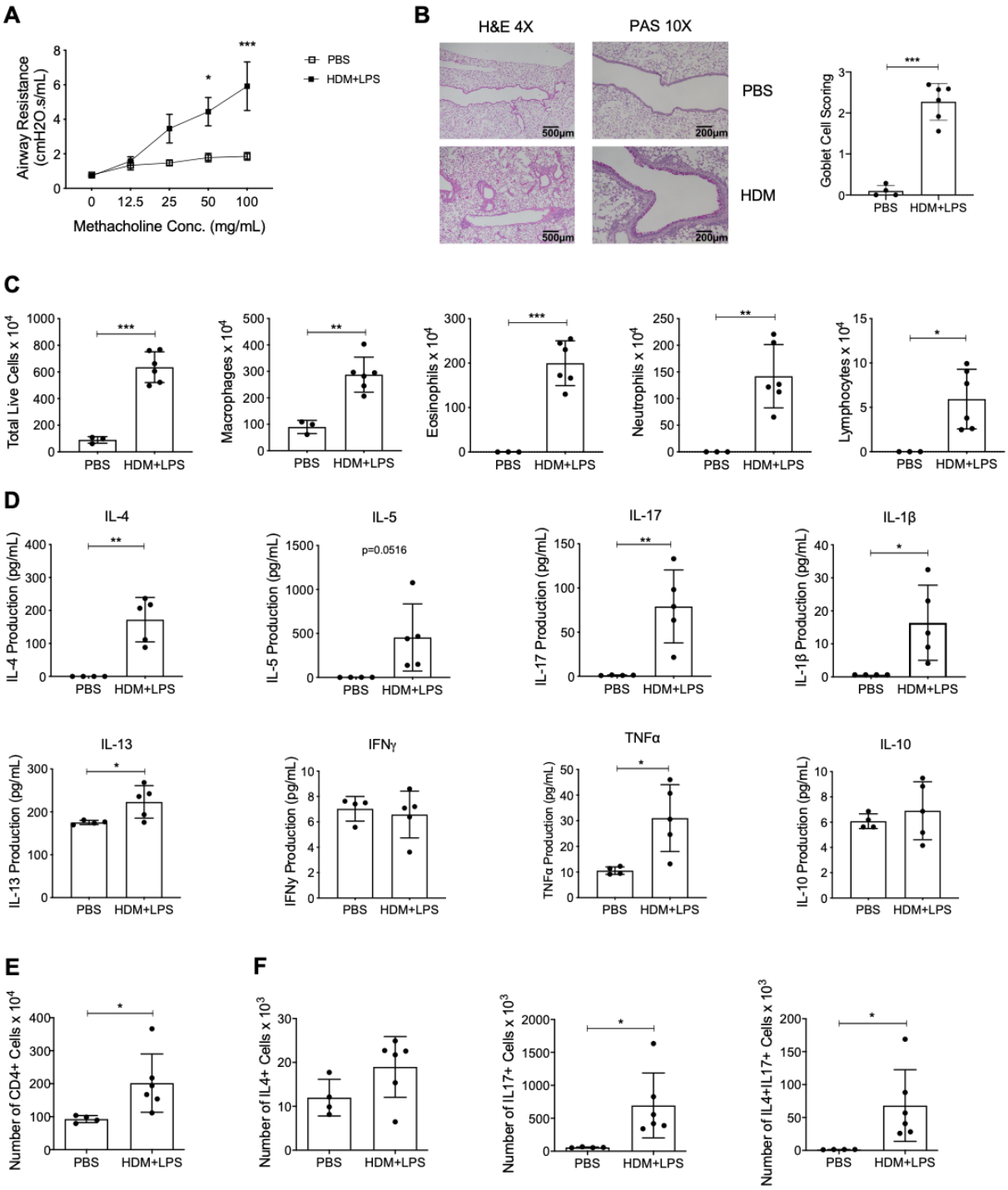

Figure 4-5. Neutrophilic airway inflammation is induced upon challenge of HDM+LPS.
Mice were challenged with either PBS (n=3) or HDM+LPS (n=6) to elicit airway inflammation. **A**. Airway hyperresponsiveness (AHR) was measured with increasing doses of methacholine

challenge. **B**. H&E and PAS staining of lungs from PBS or HDM+LPS sensitized mice with corresponding scoring of goblet cells. **C-D**. BAL fluid from the lung was analyzed. **C**. Infiltrating cells were quantified, 200 cells were counted, and total numbers were calculated from the total numbers of each. **D**. Production of Th2, Th17, and inflammation associated cytokines measured by multiplex. **E-F**. Lymphocytes were isolated from dissociated lung and analyzed by intracellular flow cytometry to quantitate (**E**) total and (**F**) cytokine-producing CD4 T cells in the lung. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, unpaired t test.

Th17 cells have higher markers of metabolism *in vivo*

The metabolic phenotype of activated CD4 T cells in lung inflammation has not been described at a single-cell resolution. Our *in vitro* data based on protein marker expression suggested Th2 and Th17 cells have a high metabolic capacity with a potential differential usage of glucose and glutamine metabolism. Therefore, we tested the expression of metabolic markers for these pathways on cytokine-producing cells from HDM+LPS airway inflammation. Animals were sensitized to HDM+LPS and analyzed by flow cytometry for co-expression of cytokines and metabolic markers. All cytokine producing T cells were found to have greater expression of metabolic protein markers for glucose and glutamine than CD4 T cells that did not express IL-4 or IL-17 (**Figure 4-4C, Figure 4-6**). These IL-4 and IL-17 negative CD4 T cells may reflect poorly activated or resting cells rather than T cells participating in the inflammatory response. Consistent with the generation of a strong Th17 inflammatory response in this model, CD4 T cells producing IL-17 alone or IL-4 and IL-17 had the greatest expression of all metabolic markers. By gating on cytokine producing cells, we determined that Glut1, Hexokinase 2 (HK2), and GAPDH in the glycolytic pathway, GLUD1 in the glutamine metabolism pathway, CPT1a in the mitochondrial lipid oxidation pathway, and Grim19, Cytochrome C, and ATP5a in mitochondrial electron transport were all elevated relative to non-cytokine producing cells. Interestingly, IL-17 alone and IL-4 and IL-17 dual producing CD4 T cells had the highest levels of expression across all markers. The degree of metabolic potential, therefore, appears to parallel the inflammatory potential of CD4 T cells in airway inflammation.

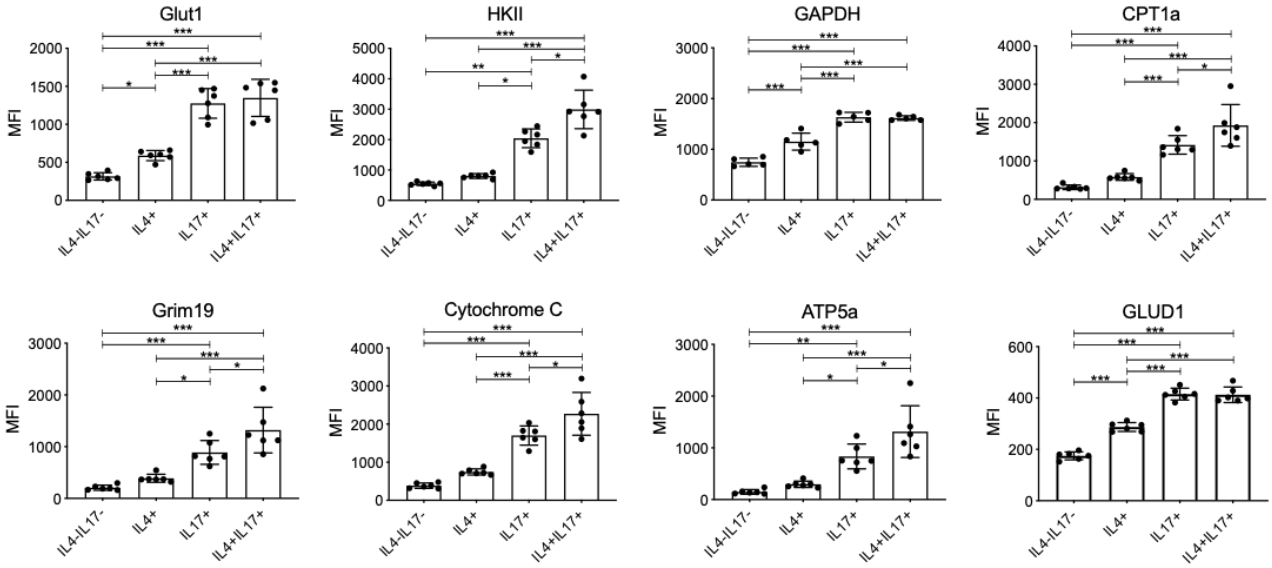

Figure 4-6. IL-17 producing cells have a higher metabolic activity *in vivo*. Expression of various metabolic proteins was measured by MFI in different CD4 T cell cytokine producing populations during murine airway inflammation. Proteins include glucose transporter 1 (Glut1), hexokinase 2 (HK2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), carnitine palmitoyltransferase 1A (CPT1a), Grim19 (a mitochondrial complex I protein), Cytochrome C, ATP5a (a mitochondrial complex V protein), and glutamate dehydrogenase 1 (GLUD1). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, one way ANOVA.

Discussion

Asthma is an inflammatory disease driven in part by different T cell subsets that vary with the clinical subtype (6,235). T cell subsets and functional populations are now known to have diverse metabolic requirements to activate, proliferate, and function (80,84,89,101,102,117). To date, however, the metabolic differences directly between Th2 and Th17 subsets have not been well established and metabolic changes in these cells have not been tested in airway inflammation (84,92,93,101,106,225). The kinase, mTOR, is an important metabolic regulator which uses environmental signals to activate one of the two mTOR signaling complexes (mTORC) which lead to T cell differentiation (92,93,97,106). While Th2 cells may rely on mTORC2 more for function than mTORC1, inhibition of mTORC1 by rapamycin suppressed cytokine production by both populations (106). Our *in vitro* metabolomics results support this finding and flux analyses suggest that Th2 cells perform aerobic glycolysis at a higher rate than Th17 cells. Th17 cells may, however, shunt glucose-derived metabolites into other pathways to a greater extent (101). In contrast to lower lactate secretion, Th17 cells appeared to have a higher overall rate of oxygen consumption and mitochondrial respiration. These differences are supported by the literature on Th2 and Th17 metabolism (84,93,109,225). However, contrary to our hypothesis differentiated Th2 cells had significantly increased expression of most of the metabolic proteins compare to Th17 cells. This finding was not recapitulated when the same proteins were measured from *in vivo* T cells. These data highlight the need for *in vivo* studies to validate metabolomics done *in vitro*, as the environment can be contributing to differences.

Our distinct model for neutrophilic asthma is characterized by both Th2 and Th17 populations and increased infiltrating cells in the airway. The lung cytokine producing T cell subsets expressed proteins characteristic of high capacity for metabolic activity and this was particularly evident in IL-17 producing cells. The data show that all cytokine producing T cells in the lung have greater metabolic potential and are likely more metabolically active than those T cells not secreting cytokines. Moreover, CD4 T cells producing IL-17 alone or in combination with IL-4 displayed the highest expression of all metabolic markers. The high metabolic rates of Th2 and Th17 cells and findings that genetic deficiency of *Slc2a1* (Glut1) or *Gls* can protect against inflammation suggest that pharmacologic inhibition of those proteins may protect from airway inflammation (80,101,236).

CHAPTER V

METABOLIC INHIBITION CAN REDUCE AIRWAY INFLAMMATION AND COOPERATE WITH GLUCORTICOIDS TO ENHANCE THEIR EFFECTS

A portion of this work adapted from manuscript originally published in *The Journal of Immunology*. Healey DCC el al. 2021. Targeting In Vivo Metabolic Vulnerabilities of Th2 and Th17 Cells Reduces Airway Inflammation. *J. Immunol*. 206:ji2001029.

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Introduction

Glucocorticoids (GCs) are one of the primary long-term treatments for asthma currently prescribed and although effective in mild forms of disease, severe Th17 neutrophilic forms of asthma respond poorly to this treatment (6,19,140,237,238). There are many possible mechanisms of GC action and through which GC-resistance may develop. These mechanisms include reduced translocation of the glucocorticoid receptor (GR), upregulation of cytokines, or phosphorylation of kinases (140,239,240). Th17 cells, in particular, can resist apoptosis, continue to produce cytokines, and induce neutrophilic damage even in the presence of GCs (19,20,23,241). Additionally, it is well known that long term use of glucocorticoids can result in many side effects including

those that cause systemic metabolic alterations that highlight a need to reduce GC dosage or use (239,242,243).

The Warburg effect, long described in cancer cells, is the use of aerobic glycolysis which is also found in proliferating T effector cells (89,96,244). The similarities between cancer and T cell metabolism allow for findings in one context to be studied in the other to determine if it would also be applicable. Although few studies have examined the role of GCs in T cell metabolic alteration, they have determined in CD8 cells that there is a suppression of fatty acid metabolism and the mTOR pathway (245,246). More studies have been done on cancer cells, specifically T lineage acute lymphoblastic leukemia (T-ALL), that are GC-resistant (155,156,158). These studies demonstrate that GC-resistant T-ALL cells have increased in glycolysis, glutamate metabolism, and oxidative phosphorylation. Importantly, inhibition of glucose uptake or glycolysis could restore sensitivity to previously GC-resistant leukemia cells (155,157).

Targeting metabolism can be one way in which T cells may be modulated to bypass GC-resistance and suppress airway inflammation. Glucose inhibitors have been documented to reduce disease in a T cell dependent manner in models of systemic lupus erythematosus (163). Combined glycolysis and glutaminolysis inhibition also impaired T cell responses and showed improved outcomes for allograft rejection (247). Glutaminolysis inhibition has been established to decrease CD4 activation and protect against graft-vs-host disease, inflammatory bowel disease (80,82). Pharmaceutical inhibitors of glycolysis and glutaminolysis are being developed as an alternative or adjuvants to cancer therapy (175,176,182,183). We aimed to determine the impact of metabolic inhibition on Th2 and Th17 cells. We tested whether metabolic inhibition can

be a mechanism by which to decrease T cell-dependent airway inflammation. Furthermore, we investigated if pharmacological inhibition of glucose or glutamine metabolism may potentially augment the effects of glucocorticoids during airway inflammation.

Materials and Methods

Mice

C57BL/6 female mice were obtained from Jackson Laboratory at 7 weeks of age. $GLS^{fl/fl}$ mice were reconstituted at Vanderbilt from mice generated at Duke and crossed in our facilities to CD4-CRE transgenic mice. All procedures were performed under protocols approved by the Institutional Animal Care and Utilization Committee (IACUC) at Vanderbilt University Medical Center.

Airway Inflammation Induction

Female mice at 8 weeks of age were intranasally administered with 50 μL of either PBS or 100 μg of house dust mite *Dermatophagoides pteronyssinus* extract (Greer Laboratories #XPB70D3A25) plus 0.1 ug of LPS from *Escherichia coli* 0111:B4 (Sigma #L4391). Mice were challenged on Day 0, 7, and 14 and cells and tissues were harvested on day 15 for all endpoints except for airway hyper-responsiveness measurements, which were performed on day 16. Inhibitor treatments were initiated on Day 0 and given daily throughout the 14 days of airway inflammation induction. Glut1 inhibitor (KL-11743, Kadmon Corporation) was dosed daily by oral gavage at 75 mg/kg

as prescribed by the company (182,183). GLS inhibitor (CB839, Calithera Biosciences) was dosed twice daily via oral gavage at 200 mg/kg as described previously (175,176).

Inhibitor Treatments

Inhibitor treatments *in vivo* were initiated on Day 0 and given daily throughout the 14 days of airway inflammation induction. Glut1 inhibitor (KL-11743, Kadmon Corporation) was dosed daily by oral gavage at 75 mg/kg as prescribed by the company dissolved in 0.5% methylcellulose and 0.25% Tween-80 (182,183). GLS inhibitor (CB839, Calithera Biosciences) was dosed twice daily via oral gavage at 200 mg/kg as described previously dissolved in 25% (w/v) hydroxypropyl- b-cyclodextrin in 10 mmol/L citrate, pH 2 (175,176). Dexamethasone was injected in the intraperitoneal daily for the last 5 days before harvest at 2.5 mg/kg. Inhibitor treatments *in vitro* were dosed at Day 0 of T cell differentiation and either carried out to Day 3 for experiments combining inhibitors and GCs or Day 5 for experiments examining inhibitors alone.

Airway Hyperresponsiveness Measurement

Mice were anesthetized using pentobarbital sodium, a small incision (0.25 cm) was made in the trachea to insert a tracheostomy tube. Mice were then placed in a chamber and mechanically ventilated at 150 breaths/minute with 0.2ml tidal volume using the Flexivent (SciReq). AHR was measured after administration of aerosolized saline (vehicle) followed by increasing concentrations of acetyl-ß-methacholine (12–50 mg/kg body weight) (234).

Collection of Bronchoalveolar Lavage (BAL) Fluid and Determining Inflammatory Cell Infiltration

A tracheostomy tube was inserted and attached to a syringe. The lungs were flushed using 800 μl of saline and as much fluid as possible was withdrawn gently using the syringe. An aliquot of the BAL was taken for cell count using Trypan Blue to determine the number of live cells. Another aliquot was spun onto a slide where the cells were fixed and stained. A representative two hundred cells were counted from the slide and classified as macrophages, eosinophils, neutrophils, or lymphocytes. The remaining BAL fluid was spun down and the supernatant was stored for later analysis by Luminex cytokine analysis.

Luminex

Cytokine levels in the BAL supernatants were measured using a MILLIPLEX Mouse High Sensitivity T cell Panel kit (Millipore #MHSTCMAG-70K) for IL-4, IL-5, IL-17A, and IL-1β by Luminex assay through the Vanderbilt Hormone Assay and Analytical Services Core.

Single Cell Suspension from Lungs

Lungs were harvested then placed into gentleMACS C Tubes (Miltenyi Biotec) containing RPMI+10% FBS, Collagenase IA (Sigma #C2674), and DNase I (DN25). C Tubes were place on the gentleMACS Dissociator (Miltenyi Biotec), program *m_lung_02.01* was run twice, and then they were places in a 37 degree incubator for 1 hour. EDTA was then added to all the tubes to stop the reaction and cells were pelleted.

After cells were resuspended in media and strained through a 70 μm filter to get a single cell suspension. Cells were then used for flow cytometry analysis and cell counting.

T Cell in vitro Activation and Differentiation

CD4 T cells were isolated from the spleen of wild type C57BL6/J mice by negative separation using magnetic beads (Miltenyi Biotec). Cells were activated and differentiated using anti-CD3 (2.5 μg/mL) and a feeder layer of irradiated splenocytes. Th2 cells were differentiated by adding recombinant mouse (rm) IL-4 (80 ng/mL) and anti-IFNγ (10 μg/mL). Th17 cells were differentiated by adding rmIL-6 (80 ng/mL), recombinant human TGFβ (1.5 ng/mL), and anti-IFNγ (10 μg/mL). Cells were cultured for 5 days, with a split on day 3 into new wells and fresh media supplemented with 10 ng/mL IL-2 to promote continued proliferation. Differentiated cells were cultured with inhibitors and GC starting on Day 0 and the compounds were supplemented if cells were split on Day 3. Compounds were dissolved in DMSO (KL-11743, CB839, and rotenone) or diH_2O (2DG and Dex).

Flow Cytometry Analysis

Intracellular cytokine staining was performed after a 4-hour restimulation using PMA/Ionomycin and GolgiStop. Cells were stained using a fixable viability dye and a CD4 antibody in PBS for 20 minutes at 4 degrees. Cells were then permeabilized with Cytofix/Cytoperm (BD Bioscience #554722) prior to staining for either IL-4 or IL-17. Transcription factor staining was performed on non-stimulated cells after viability and surface staining. The Foxp3/ Transcription Factor Staining Buffer set (eBioscience #00-

5523-00) was used to permeabilize the cells and then the cells were stained for either GATA3 or RORγt. Non-stimulated differentiated cells were also washed with PBS, stained for mitochondrial tracking and ROS, and then for CD4. Reagents used were DCFDA (ThermoFisher #C6827), MitoTracker Green (ThermoFisher #M7514), MitoSOX (ThermoFisher # M36008), and TMRE (ThermoFisher # T669). Samples were run on the Miltenyi MACSQuant and analysis was done using FlowJo software.

Statistical Analyses

Statistical analyses were performed on Prism using either Student's t-test or one-way ANOVA unless otherwise stated. Statistically significant results are indicated by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Results

Mitochondrial changes in Th2 and Th17 cells *in vitro* upon metabolic inhibition

We were interested in establishing how different metabolic inhibitors not only impacted the functionality of Th2 and Th17 cells, but also what was the mitochondrial effects. Two Glut1 inhibitors (2DG, KL-11743), a GLS inhibitor (CB839), a GC (Dex), and a mitochondrial inhibitor (rotenone) were used on differentiated CD4 T cells. All the compounds impaired Th2 viability, although only 2DG and Dex significantly decreased IL-4 production (**Figure 5-1A, Figure 5-1B**). Interestingly, GATA3 expression increased with most of the compounds except for 2DG (**Figure 5-1C**). Th17 viability was reduced by all the compounds, and all but KL-11743 decreased IL-17 production (**Figure 5-1D, Figure 5-1E**). All the compounds decreased RORγt expression (**Figure 5-1F**). These

compounds therefore impact CD4 T cell proliferation but have differing effects on cytokine production and the expression of transcription factors. Interestingly, the two Glut1 inhibitors did not have the same effects. This discrepancy could be due to differences in the mechanism of action since 2DG results in an accumulation of P-2DG whereas KL-11743 just blocks glucose uptake.

We next used mitochondrial stains to determine the effects of the compounds on mitochondrial biology. Cellular staining with the mitochondrial mass, MitoTracker, increased in Th2 cells with most of the compounds, whereas it decreased in Th17 cells (**Figure 5-2A**). Th2 and Th17 cells had an increase in mitochondrial ROS, MitoSox, when treated with most of the compounds (**Figure 5-2B**). However, cellular ROS, as measured by DCFDA, did not change in either cell type by any of the compounds (**Figure 5-2C**). Finally, TMRE was used to measure mitochondrial membrane potential. The compounds, except for 2DG, increased Th2 mitochondrial potential (**Figure 5-2D**). Conversely, only Dex impaired mitochondrial potential in Th17 cells. These data identified that metabolic inhibitors and GCs impact the mitochondria differently in Th2 and Th17 cells.

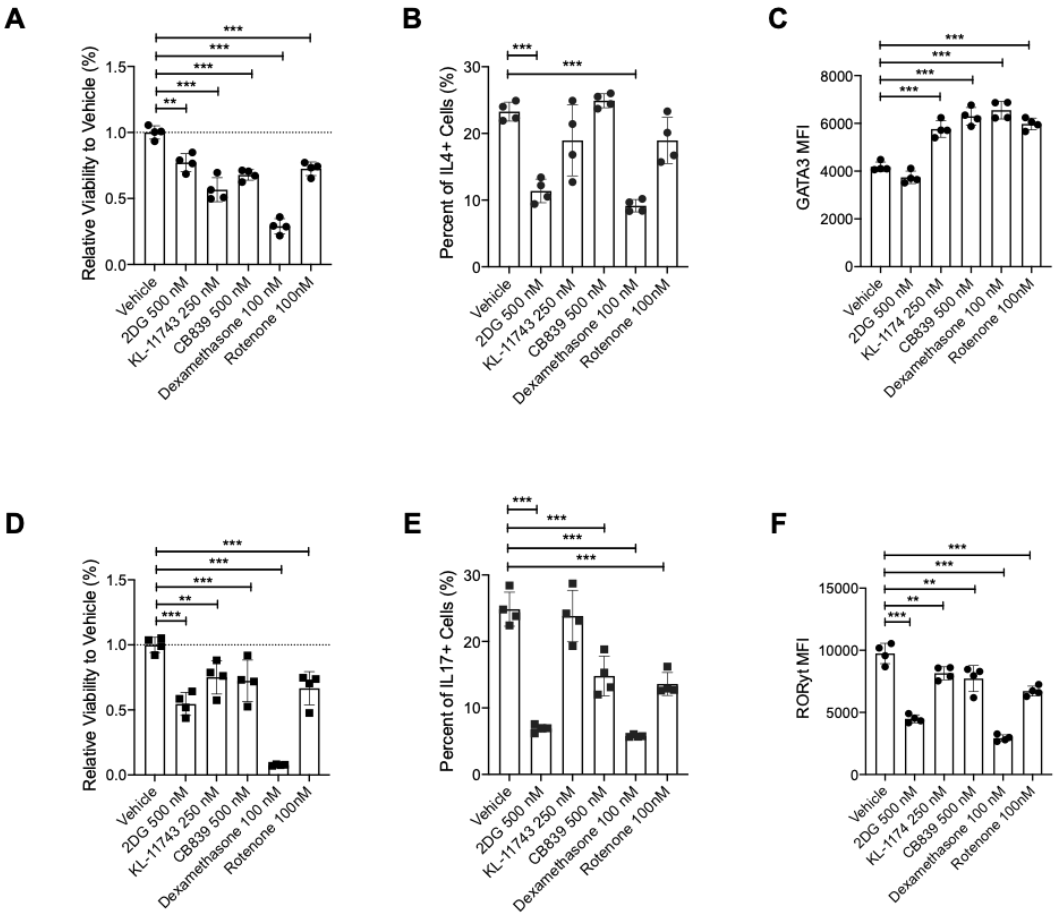

Figure 5-1. Metabolic inhibition impairs Th2 and Th17 cells. A-C. CD4 cells differentiated in Th2 polarizing media for 5 days (n=4). Cells were analyzed by flow to determine number of live cells (**A**), cytokine producing (**B**), and transcription factor expression (**C**). **D-F**. CD4 cells differentiated in Th2 polarizing media for 5 days (n=4). Cells were analyzed by flow to determine number of live cells (**D**), cytokine producing (**E**), and transcription factor expression (**F**). Live cell numbers are relative to the number of live cells in the vehicle treated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, one-way ANOVA

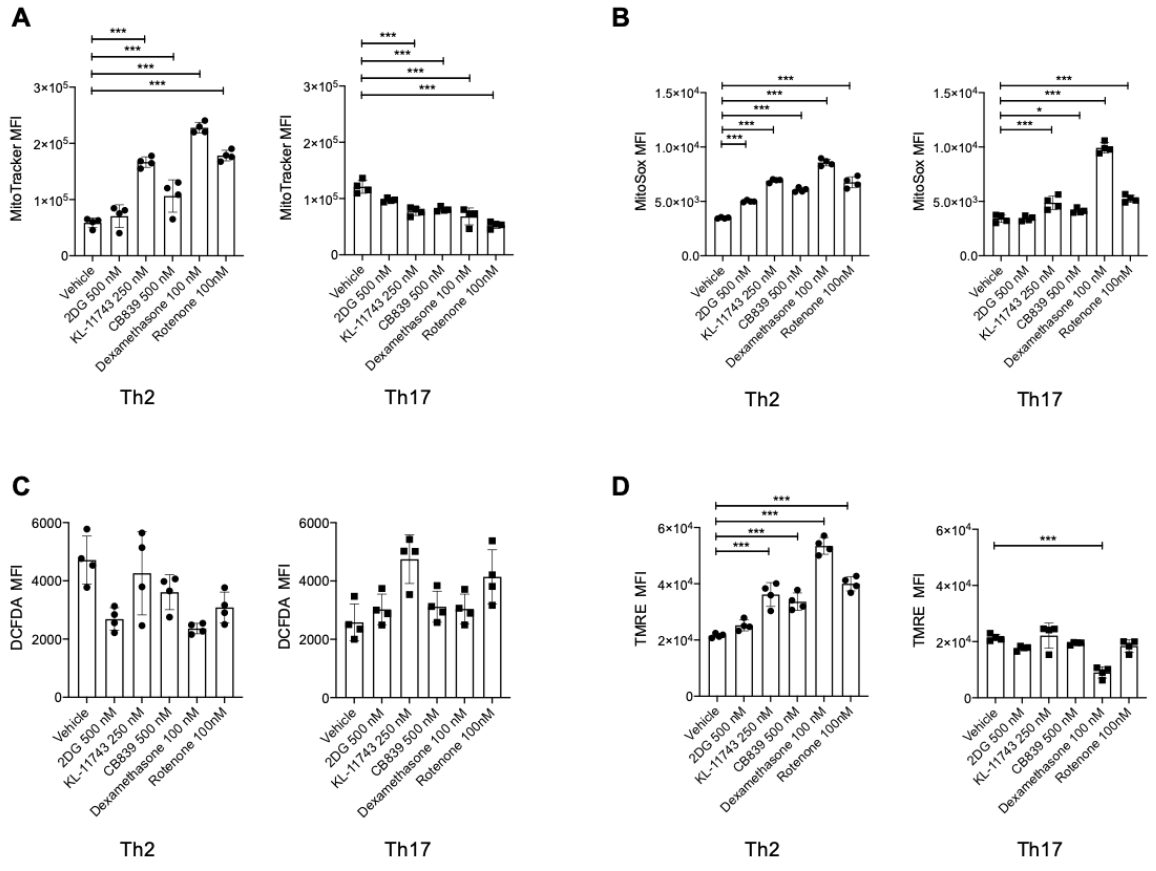

Figure 5-2. Metabolic inhibition results in alteration of mitochondrial output in Th2 and Th17 cells. A-D. CD4 cells differentiated in Th2 polarizing or Th17 polarizing media for 5 days (n=4). **A**. Cells were stained with MitoTracker Green to quantitate mitochondria. **B**. Cells were stained with MitoSox to measure mitochondrial superoxide. **C**. Cells were stained with DCFDA to measure cellular ROS. **D**. Cells are stained with TMRE to measure mitochondrial membrane potential. * p<0.05, ** p<0.01, *** p<0.001, one-way ANOVA.

GLS is required for cytokine producing T cells in the lung

Glutaminolysis is important for proliferating T cells, and our data suggest that Th17 cells in particular require this pathway to be active to function (**Figure 3-1E, Figure 4-1B, Figure 4-1C, Figure 4-6**) (78–80). We used $GLS^{fl/fl}$-CD4Cre (GLS KO) or WT mice to induce airway inflammation using our HDM+LPS model. There were trends towards more infiltrating cells in the BAL in WT mice that had been challenged, however GLS KO had an increased number of lymphocytes in the BAL (**Figure 5-3A**). Despite this increase in BAL cell numbers, T cells in GLS KO mice did not show increased cytokine secretion similar to control T cells (**Figure 5-3B**). These data suggest that glutaminase inhibition may selectively inhibit cytokine producing CD4 T cells at the site of inflammation.

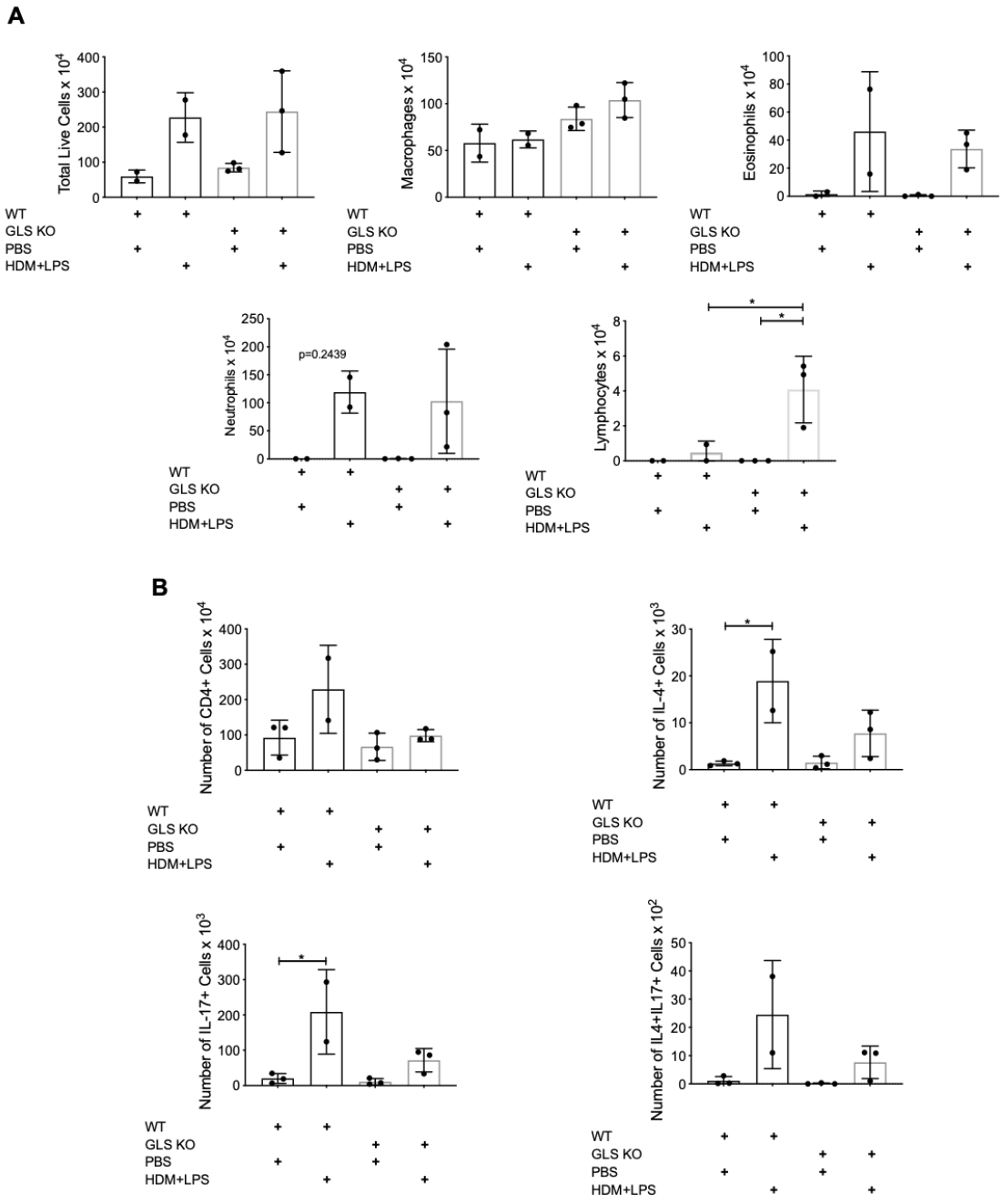

Figure 5-3. GLS is required for cytokine producing CD4 cells in the lung. A-B. $GLS^{fl/fl}$-CD4Cre (GLS KO) mice or WT mice were challenged with PBS or HDM+LPS (n=2-3). **A**. BAL fluid from the lung was analyzed. Infiltrating cells were quantified by counting cells and calculating numbers from the totals of each. **B**. Lymphocytes were isolated from dissociated lung and analyzed by intracellular flow cytometry to quantitate total and cytokine-producing CD4 T cells in the lung. * $p<0.05$, two-way ANOVA.

Glut1 inhibition has modest effects

Based on the high expression of all metabolic markers in inflammatory IL-4 and IL-17 producing CD4 T cells in the lung, we hypothesized that metabolic inhibitors of pathways may decrease markers of airway inflammation. Because glycolysis first requires glucose uptake and both Glut1 and HK2 were elevated in cytokine-producing cells, glucose uptake and Glut1 were inhibited using previously established doses of KL-11743 (182,183). Animals were sensitized using HDM+LPS to develop a primary Th17 airway response and were treated daily with KL-11743. This treatment resulted in a trend towards decreased infiltration of each cell type measured in the BAL, although only eosinophils were significantly reduced (**Figure 5-4A**). Cytokine levels in the BAL fluid were also measured and while Th2 cytokines IL-5, IL-13, and Th17 cytokine IL-17 were unchanged, IL-1β was reduced (**Figure 5-4B**). Consistent with moderate changes caused by Glut1 inhibition in this HDM+LPS Th17 model of airway inflammation, numbers of lung total and cytokine-producing CD4 T cells were unchanged by KL-11743 (**Figure 5-4C**).

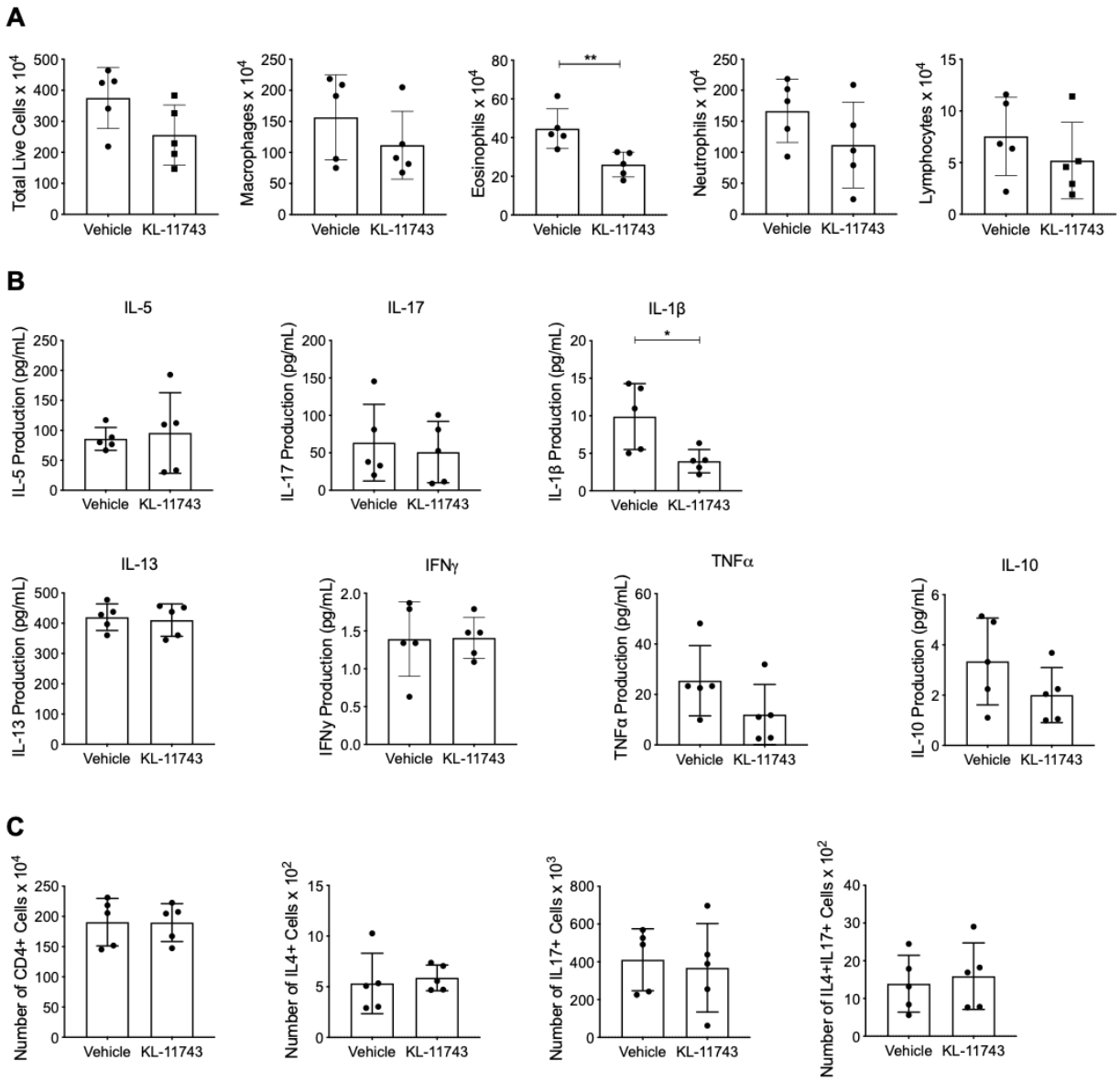

Figure 5-4. Glut1 targeting has modest impact on infiltrating cells into the BAL. A-D. Mice were challenged with HDM+LPS and dosed once daily with either vehicle (n=6) or KL-11743 (n=6). **A-B**. BAL fluid from the lung was analyzed. **A**. Infiltrating cells were quantified by counting cells and calculating numbers from the totals of each. **B**. Production of Th2, Th17, and inflammation associated cytokines were measured by multiplex. **C**. Lymphocytes were isolated from dissociated lung and analyzed by intracellular flow cytometry to quantitate total and cytokine-producing CD4 T cells in the lung. * $p<0.05$, ** $p<0.01$, unpaired t test.

GLS inhibition can reduce airway inflammation

The effect of inhibiting GLS to suppress glutamine metabolism was next tested in HDM+LPS induced airway inflammation. Mice were challenged and treated daily using either vehicle or the GLS inhibitor, CB839. Similar to Glut1 inhibition, CB839 caused a trend towards decreased BAL-infiltrating cells of all types, although only eosinophils were significantly decreased (**Figure 5-5A**). Cytokine levels in the BAL fluid were significantly decreased by CB839 treatment for the Th2 cytokine IL-5, the Th17 cytokine IL-17, as well as IL-1β (**Figure 5-5B**). However, other cytokines associated with inflammation and an anti-inflammatory cytokine (IL-10) were unchanged. GLS inhibition by CB839 also led to a modest trend toward decreased total CD4 T cells in the BAL and a significant decrease in IL-17 producing CD4 T cells (**Figure 5-5C**). Targeting T cell metabolism by blocking Glut1 or GLS may, therefore, provide protection against airway inflammation.

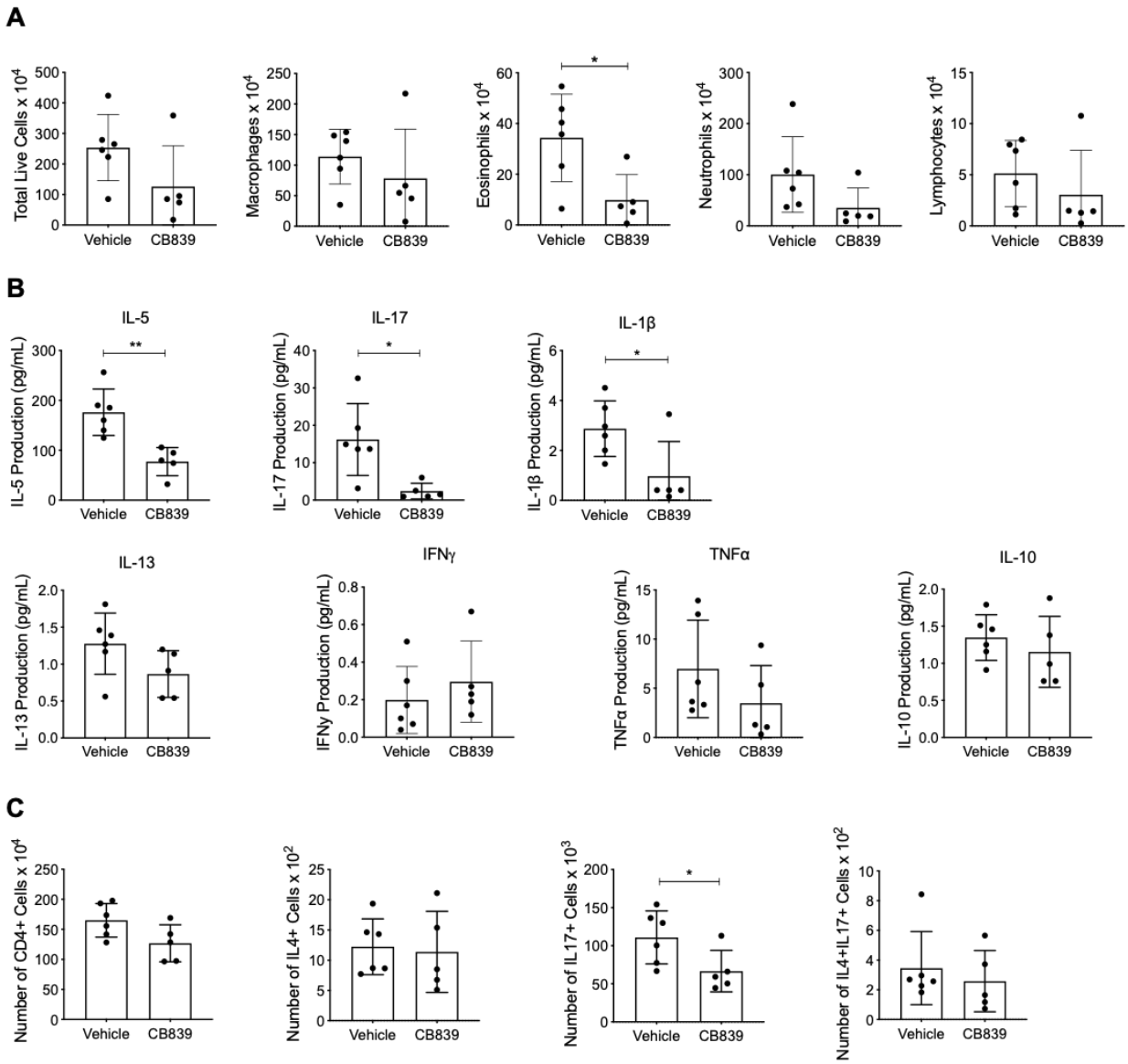

Figure 5-5. GLS targeting decreases BAL cell infiltration and IL-17 producing cells found in the lung. A-C. Mice were challenged and dosed once daily with either vehicle (n=6) or CB839 (n=5). **A-B**. BAL fluid from the lung was analyzed. **A**. Infiltrating cells were quantified by counting cells and calculating numbers from the totals of each. **B**. Production of Th2, Th17, and inflammation associated cytokines were measured by multiplex. **C**. Lymphocytes were isolated from dissociated lung and analyzed by intracellular flow cytometry to quantitate. Total and cytokine-producing CD4 T cells in the lung. * $p<0.05$, ** $p<0.01$, unpaired t test.

Glucocorticoids and metabolic inhibition can cooperate to decrease T cell viability and cytokine production *in vitro*

Given the high metabolic rate of IL-17 producing CD4 T cells and the ability of Glut1 and GLS inhibitors to reduce markers of inflammation in our Th17 neutrophilic airway inflammation model, we sought to determine if these inhibitors could enhance the effect of GCs to specifically reduce Th17 cell survival and inflammatory functions. Th2 and Th17 cells were differentiated *in vitro* for 3 days in the presence of a GC (dexamethasone, Dex), KL-11743, or CB839 alone, or with GC combined with each metabolic inhibitor. Viability and IL-4 production in Th2 cells were decreased by each compound, with Glut1 inhibition and a combination of GC and Glut1 inhibitor having the greater effects (**Figure 5-6A, Figure 5-6B**). Despite GATA3 expression minimally decreased by the GC and inhibitors, the combination of Glut1 inhibitor and GC had a very large impact on expression (**Figure 5-6C**). Th17 cell viability was decreased by each compound except for the GLS inhibitor, however the combination of GC and inhibitor further reduced viability (**Figure 5-6D**). Similar to reduced Th17 cell viability, GC or Glut1 inhibition reduced IL-17 production (**Figure 5-6E**). The expression of RORγt by the compounds mimicked the decrease in Th17 viability, as GC in combination with inhibitor had the greatest effects (**Figure 5-6F**). Th2 and Th17 cells each had similar effects to the GC and inhibitors. While GLS inhibition did not have a significant effect on its own in this setting, the combination of either Glut1 or GLS inhibitor enhanced the ability of the GC to reduce cytokine production.

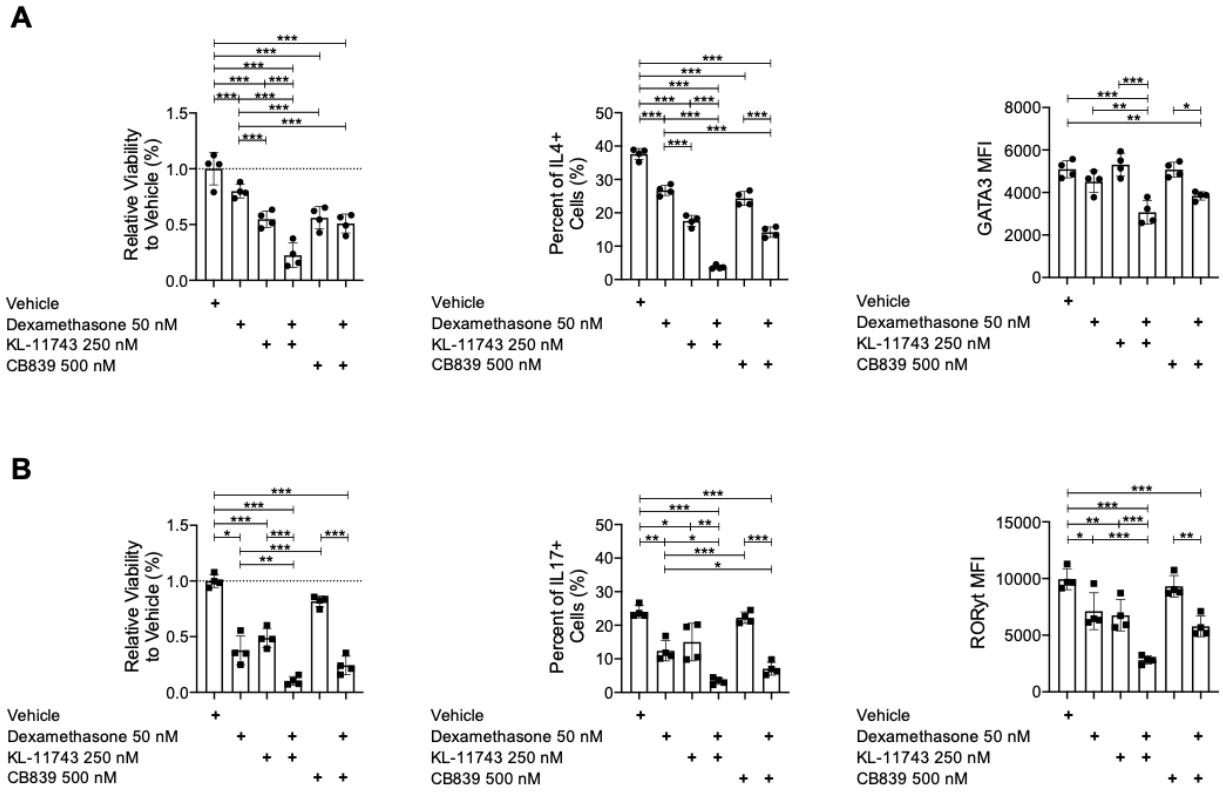

Figure 5-6. GC and metabolic inhibitor in combination impact Th2 and Th17 cells. A-C. CD4 cells differentiated in Th2 polarizing media for 3 days (n=4). Cells were analyzed by flow to determine number of live cells (**A**), cytokine producing (**B**), and transcription factor expression (**C**). **D-F**. CD4 cells differentiated in Th17 polarizing media for 3 days (n=4). Cells were analyzed by flow to determine number of live cells (**D**), cytokine producing (**E**), and transcription factor expression (**F**). Live cell numbers are relative to the number of live cells in the vehicle treated. * p<0.05, ** p<0.01, *** p<0.001, one-way ANOVA. Representative of 2 experiments.

Dexamethasone has no effects on HDM+LPS model of airway inflammation

We next tested whether a glucocorticoid, dexamethasone, would decrease cells present during airway inflammation. During the last 5 days of the model, either PBS or dexamethasone was injected into the peritoneum of the mice. At this dose and treatment regimen, no differences were observed between groups in the infiltrating cells of the BAL (**Figure 5-7A**). No differences were also observed in the total lung $CD4^+$ T cells nor in the cytokine producing cells (**Figure 5-7B**).

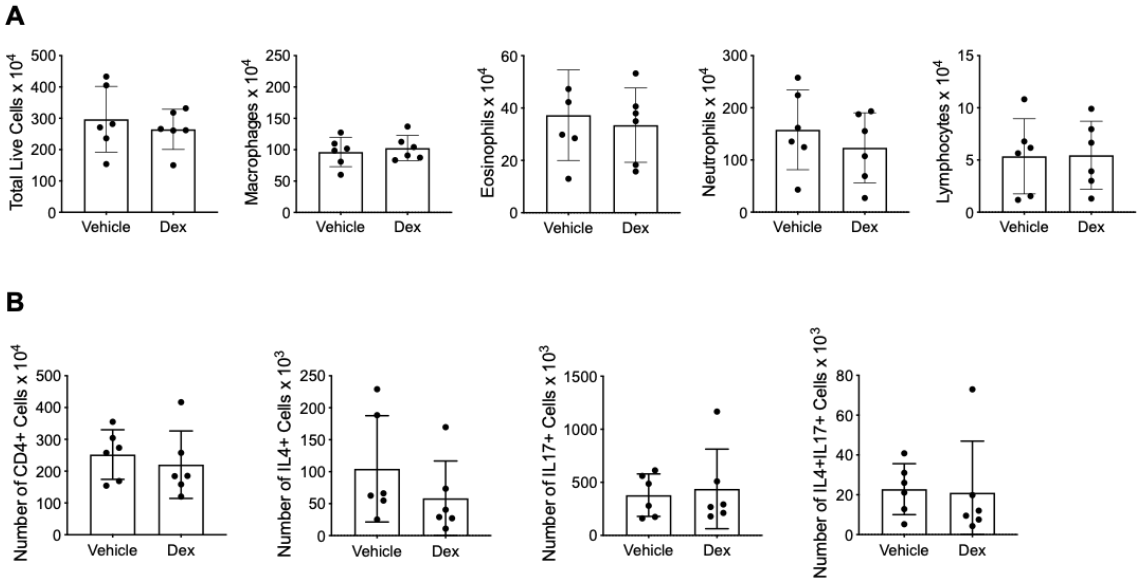

Figure 5-7. Dexamethasone has no effects on airway inflammation. A-B. Mice were challenged and ip injected for the last 5 days with either PBS (vehicle) or dexamethasone (Dex) (n=6). **A**. BAL fluid from the lung was analyzed, infiltrating cells were quantified by counting cells, and calculating numbers from the totals of each. **B**. Lymphocytes were isolated from dissociated lung and analyzed by intracellular flow cytometry to quantitate total and cytokine-producing CD4 T cells in the lung. No significance, unpaired t test. Representative of 2 experiments.

Combination of a glucocorticoid and a GLS inhibitor as a potential therapeutic

Finally, we assessed whether a combination treatment of metabolic inhibitor with Dex *in vivo* would affect airway inflammation, specifically on lung function by measuring AHR. The Glut1 inhibitor was tested alone and in combination with Dex *in vivo* (**Figure 5-8A**). There were no differences observed in any of the groups. The GLS inhibitor was also tested alone or in combination with Dex *in vivo* (**Figure 5-8B, Figure 5-8C**). Overall, no differences were observed between vehicle treated mice compared to treated mice at low concentrations of methacholine (**Figure 5-8B**). However, airway resistance was significantly decreased when CB839 is used in combination with a GC at the highest methacholine dose (**Figure 5-8C**). Together, these data show that the high potential metabolic activity of IL-17 producing cells can be pharmacologically targeted and this approach may cooperate to augment the immune suppressive properties of GC.

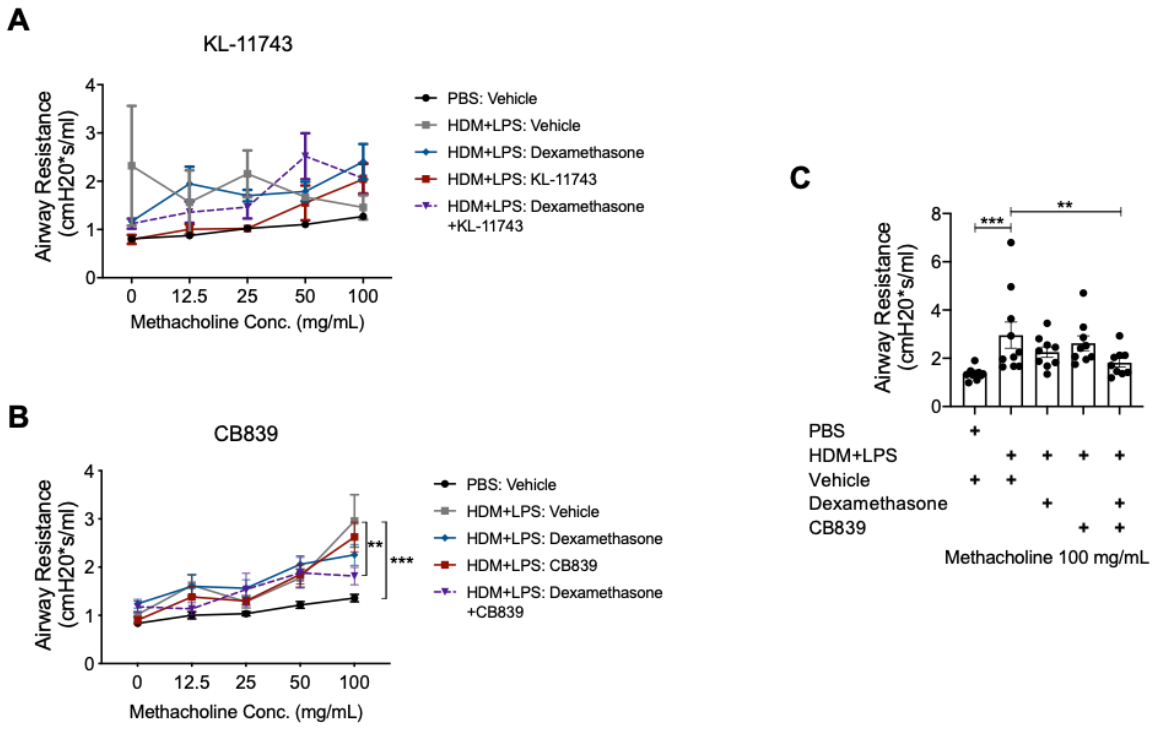

Figure 5-8. Combination of GC and GLS inhibition *in vivo* decreases AHR. A-C. AHR measurements 2 days following last challenge for mice sensitized either with either PBS or HDM+LPS. **A**. Mice were treated with either vehicle or KL-11743 daily and the last 5 days either received ip injections of dexamethasone or PBS (n=4-5). AHR measurements of response to increasing methacholine challenge. **B-C**. Mice were treated with either vehicle or CB839 daily and the last 5 days either received ip injections of dexamethasone or PBS (n=9-10). **B**. AHR measurements of response to increasing methacholine challenge. **C**. AHR measurement at a methacholine challenge of 100 mg/mL. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, one-way ANOVA.

Discussion

Studies from asthmatic patients and murine models have reported high levels of lactate and an IL-1 dependent increase in glycolysis (67,197,205). Metabolic shifts occur upon activation of naïve T cells that assist in differentiation and functionality (80,84,89,101,102,117). Modulation of metabolism can suppress T cell mediated responses and improve outcomes (82,122,163,247). GLS deficient T cells have decreased tissue localization and are highly present in the airway. Despite an increase of lymphocytes in the airway, there were no changes to the neutrophilic recruitment. GLS is required for cytokine production of both Th2 and Th17 cells (80). Pharmacological inhibitors can impair Th2 and Th17 cell viability and cytokine production *in vitro*. Due to the metabolic differences between Th2 and Th17 cells, some inhibitors impacted their function differently. Th2 cells had an increase in mitochondrial mass, ROS, and potential as well as increased GATA3 expression. These changes were probably due to compensatory mechanisms due to cellular stress in an attempt to maintain function (57,248,249). Alternatively, Th17 cells had a decrease in mitochondrial mass, potential, and RORγt expression while they increased their mitochondrial ROS. These data in Th17 cells suggest that not only is there an impairment in function, but that it may be specific to the pathogenic Th17 cells as they have been documented to decrease when there is an increase in mitochondrial ROS (68).

We tested if acute *in vivo* pharmacological inhibition could provide protection from T cell mediated airway inflammation. Using Glut1 and GLS inhibitors we found that each could minimize some aspects of airway inflammation when provided as single

agent therapeutics in this mixed model of Th2 and Th17 airway inflammation. Eosinophilia was reduced in both cases and some T cell cytokines were less abundant in BAL fluid. GLS inhibition may have resulted in a broader inhibition, as the type 2 immunity cytokine IL-5 as well as the number of IL-17 producing cells and the amount of IL-17 in the BAL fluid were decreased. This finding is consistent with prior work suggesting a particularly high dependence of Th17 cells on glutaminolysis and that targeting this pathway can impair IL-17 producing cells (80,250).

Standard treatment for asthmatic patients is inhaled glucocorticoid steroids, yet resistance in a Th17-mediated manner has been observed in populations of severe neutrophilic asthmatics (6,140,237,238). Our model of airway inflammation did not respond to GC treatment and maintained cytokine producing CD4 T cell populations. Although we expected IL-17 producing cells and neutrophil infiltration to not be impacted, we hypothesized that there would be decreased IL-4 producing cells as well as eosinophils. However, Th2 and Th17 cells decrease viability and cytokine production *in vitro* which suggests that the HDM+LPS model could be ideal for studying all GC-resistance. Our *in vitro* data also indicated that a combination of metabolic inhibitor and a GC resulted in overall decreased viability, cytokine production, and reduction of transcription factor expression. Although *in vivo* the Glut1 inhibitor did not have any significant effects, the combination of GC and the GLS inhibitor may yet be a promising avenue to bypass GC resistance. We demonstrated that metabolic inhibitors could mediate inflammation *in vivo* and this was done by disrupting mitochondrial functions based on the *in vitro* data. These data also suggest that using a two-pronged approach

of a glucocorticoid and a metabolic inhibitor may be a mechanism by which to bypass GC-resistance in asthma.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

Asthma is a complex disease that is characterized by a variety of phenotypes (6,39,251). Milder phenotypes of asthma typically have increased eosinophilic airway inflammation that is predominantly driven by $CD4^+$ Th2 cells producing IL-4, IL-5, and IL-13 (30). While patients with severe asthma can have increased eosinophilic airway inflammation that is driven by Th2 cells, these patients may also have increased neutrophils and IL-17 producing cells, including $CD4^+$ Th17 cells, in the airway (20,23,152). Glucocorticoid resistance by Th17 cells is an issue that impacts some severe asthmatics and has been implicated in other inflammatory diseases, such as Crohn's (19,20,23,141,241). Mechanisms of Th17 cell resistance to GCs remain uncertain. Consistent with a metabolic contribution to GC sensitivity, however, glucose uptake rates could determine GC response and cell death in acute lymphoblastic leukemia (157). T cell metabolism is important in determining $CD4^+$ T cell subset differentiation and targeting the essential metabolic programs of these cells may provide a new approach to modulate immune responses, suppress Th2 and Th17 cell-driven airway inflammation, and bypass GC resistance. (30,152)

My dissertation addresses several gaps in knowledge that are important for both the asthma and immunometabolism fields. First, I demonstrate that asthmatic patients have an altered metabolic profile that is measurable from their circulating PBMCs. This metabolic alteration is also extended to their $CD4^+$ T cell population. Secondly, Th2 and

Th17 cells have distinct metabolic profiles *in vitro*. These differences were also apparent in the Alt Ext and HDM+LPS models of airway inflammation; however, the *in vivo* data suggest that highly activated T cells require a variety of metabolic pathways. Next, my data establishes that metabolic inhibition could be a promising therapeutic target for mediating airway inflammation, as both the Glut1 and GLS inhibitors reduced some aspect of inflammation. Finally, I show that using a combination of a GC and a metabolic inhibitor could improve the therapeutic efficacy of GC-resistant asthmatics.

Conclusions and Significance

While phenotypic differences had been observed in the PBMCs from asthmatic patients compared to healthy individuals, there had not been a good way to determine the immunometabolic differences at a single-cell level. In Chapter 2, I used mass cytometry to specifically determine the metabolic differences in $CD4^+$ T cells between healthy, mild, and severe asthmatics. What was most striking about the data is how different healthy individuals looked compared to asthmatic patients. Some of those differences were driven by the different cell types, however the primary change was in the metabolism. This is the first time that metabolic differences at a single cell level have been demonstrated to drive changes in immune cells circulating during airway inflammation. Specifically, metabolic alterations drove the differences between cohorts in CD4 T cells. Mass cytometry has opened a new tool for metabolic analysis that has only recently begun to be harnessed as evidenced by two manuscripts published in the last 6 months, not including the manuscript this work is published in (200,201).

Dr. Dawn Newcomb's lab generated scRNAseq data sets from $CD4^+$ T cells isolated from *Alternaria alternata* extract challenged mice. Chapter 3, explores the metabolic differences between CD4 T cells from these experiments. Due to the limited number of Th17 cells in the scRNAseq, we were unable to compare differences between Th2 and Th17 cells. However, we were able to show that gene enrichment pathways for cell metabolism were increased in activated Th2 cells compare to naïve CD4 T cells. Additionally, Th17 cells were measurable by flow cytometry in the lung. This allowed us to measure Glut1 and GLUD1 expression and compare Th2 and Th17 cells directly. Th17 cells had higher expression markers for metabolic activity despite not being the predominant T cell type in this model of airway inflammation. This chapter would not have been possible without help from Dr. Xiang Ye, Norwin Chowdhury, and Dr. Dawn Newcomb.

The metabolic differences between Th2 and Th17 cells observed in the Alt Ext model of airway inflammation were a good starting point in determining metabolic shifts. In Chapter 4, I determined the differences between Th2 and Th17 cells both *in vitro* and *in vivo*. The metabolic profiles of Th2 and Th17 cells are different. Interestingly, *in vitro* Th2 cells had a high metabolic activity as measure by proteins, whereas *in vivo* Th17 cells have the higher activity as also measured by protein expression. Th17 literature has suggested that there are two types of Th17 cells, non-pathogenic and pathogenic (68–70). Pathogenic Th17 cells are IL-1β and IL-23 driven, members of the Rathmell lab have found that metabolic differences in Th17 cells depend on the cytokine milieu in which they are differentiated (unpublished data Gabriela Andrejeva and Ayaka Sugiura). The differences I observed between the T cells *in vivo* and *in vitro* could be due to

nonpathogenic Th17 cells in one setting versus pathogenic in another. Moreover, this reiterates the importance of not relying just on data observed *in vitro* since there could be unknown environmental factors *in vivo* that affect how cells behave. The key finding was that cytokine producing cells during airway inflammation have an increased metabolic profile which is particularly pronounced in IL-17 producing CD4 T cells.

Despite the metabolic differences we observed in Th2 and Th17 cells, these effector subsets also displayed many similarities. In Chapter 5, I determined if metabolism could be exploited to decrease CD4 T cells in order to reduce airway inflammation and to bypass GC-resistance. The data demonstrated that metabolic inhibitors altered mitochondrial mass, ROS production, and membrane potential, although not in precisely the same manner in Th2 and Th17 cells. These differences were likely due to their specific metabolic requirements and response to metabolic stress. Additionally, GLS deficiency in T cells was demonstrated to reduce CD4 T cells in the lung during airway inflammation (80). Importantly, *in vivo* metabolic inhibition of either Glut1 or GLS decreased the number of infiltrating cells in the bronchoalveolar lavage (BAL). A combination of a glucocorticoid and either Glut1 or GLS inhibition further decreased T cell viability and cytokine production and targeting GLS together with dexamethasone reduced AHR. Together these findings suggest a potential novel avenue for therapy for airway inflammation to overcome severe steroid-resistant asthma.

Future Directions

Generating more comprehensive immunometabolism data using CyTOF

CyTOF could be used to generate data with a more in depth look at innate immune cells, metabolism, and cellular activation with the development of a new panel. One of the biggest questions when examining the asthmatic patient data in Chapter 2 is what are the other cell types present in the PBMCs that could be contributing to the metabolic shift of asthmatics compared to healthy controls. The highly metabolically activate cells that were not T cells have a high expression of HLA-DR so they are most likely macrophages or dendritic cells. CyTOF should be run on more asthmatic PBMCs and a new panel created. The new panel would include more innate and innate-like cell markers, such as CD11b, CD11c, CD15, CD16, CD20, CD56, and TCR$\gamma\delta$ to better understand the immunometabolism in asthmatic patients. The new panel would also include more metabolic antibodies that could differentiate between activation points in different pathways, such as mTOR. A limitation I experienced with the panel used was the lack of detection of phosphorylated antibodies since the PMBCs were not activated prior to staining. I specifically wanted to measure the metabolism of $CD4^+$ T cells straight from the patient that had not been stimulated. However, not only to better measure the phosphorylated antibodies but also to determine whether cells that are already highly metabolic can further increase their metabolism, stimulated and unstimulated samples should be run. Based on a CyTOF study from the Rathmell lab performed on tumor infiltrating T cells in patients, the ideal stimulation of cells would be with anti-CD3 and anti-CD28 (91).

Identifying metabolic alterations in obese asthmatics

Obesity has been linked to modifications in immune cell responses, specifically causing an increase of $CD4^+$ T cells in the adipose tissue (252). CD4+ T cells from obese mice have been documented to have an altered metabolic profile towards increased mitochondrial oxidation of glucose (253). Obese mice infected with influenza and treated with metformin had improved survival compared to non-treated mice (253). Additionally, obesity in asthma has been associated with a more severe phenotype that is GC resistant (254,255). Obesity can contribute to the development of asthma or it can make pre-existing asthma severity increase (254). Due to the overlap between asthma and obesity, it is of interest to determine how metabolism is affected in Th2 and Th17 cells when these two conditions overlap. CyTOF could be run on PBMCs from normal BMI healthy individuals, clinically obese non-asthmatic individuals, normal BMI asthmatics, and clinically obese asthmatics to identify if there are metabolic differences present in the CD4+ T cells. Moreover, our HDM+LPS model could be induced in high fat diet and low fat diet mice to not only determine if there are metabolic alterations in the Th2 and Th17 cells but also to be able to treat the mice with metabolic inhibitors. There is a greater potential for the benefits of metabolic inhibitors in asthma during obesity due to the promising results of metformin during influenza infection.

Determining metabolic gene expression from Th17 cells in airway inflammation

I was interested in measuring metabolism in a manner that most likely indicated activation of the various metabolic pathways. I decided to measure metabolic proteins since gene expression does not always correlate with a functional output of protein.

However, I was able to use scRNAseq to show genetically metabolic activation in Th2 cells in Chapter 3. The biggest limitation of this experiment was that the Alt Ext model we used did not have enough Th17 cells to directly compare to Th2 cells. scRNAseq should be run on the HDM+LPS model and cells pooled from about 6 separate mice per group. This would ensure that there are not only enough Th2 and Th17 cells, but also that there is a good number of dual Th2/Th17 cells that were produced in this model. This would also contribute to the work done by Tibbitt et al. which documented the metabolic differences between Th1 and Th2 cells during airway inflammation (109).

Metabolomics on Th2 and pathogenic Th17 cells

One of the surprising findings from Chapter 4 is that the metabolism between Th2 and Th17 cells differ *in vitro* when compared to *in vivo*. I hypothesize one of the reasons for this difference is that the protocol in our lab generates non-pathogenic Th17 cells. It would be interesting to differentiate pathogenic Th17 cells using IL-1β and IL-23 and then compare the metabolism of those cells with Th2 cells. Extracellular flux analysis on CD4 T cells from *in vivo* would allow us to validate the differences seen *in vitro*. After the lungs had been removed from HDM+LPS challenged mice cell sorting would be done to separate the Th2 and Th17 cells and then run the assay. A study limitation was that despite observing dual IL-4 IL-17 producing cells *in vivo, in vitro* I was unable to differentiate dual producing cells. Successful differentiation of dual producing cells would allow for more specific metabolomic studies on cells that are a clear candidate of GC-resistance (23,24).

Combination therapy and assessing effects on inflammation

Combination therapy of GC and the GLS inhibitor decreased airway inflammation in mice challenged with a high concentration of methacholine in Chapter 5. These mice had low overall response to methacholine when compared to mice in Chapter 4. Mice that were given the GLS inhibitor had to be orally gavaged twice daily which could have led to increased blood levels of cortisol due to extra stress. The GLS inhibitor can be incorporated into food pellets and given to mice in a less stressful manner. This would help to determine if there are changes to inflammation at lower methacholine doses. These changes could give us a better idea of how effective the combination therapy could be. Additionally, histopathology of the lungs could be assessed to determine the effects of the combination therapy on lung tissue.

Future studies should focus on assessing the implication of immune metabolism not only in the CD4 T cells of asthmatics but also in the other immune cells prevalent during inflammation as my work demonstrated a global metabolic shift. Additionally, the promising data from the metabolic inhibitors and GC was based on my model of acute airway inflammation. In order to better assess the effectiveness of these inhibitors, they should be used in a chronic model (a month or longer) which would have more severe airway remodeling and AHR. Finally, Th17 associated GC-resistance is not only limited to asthma therefore studies should be done to test if metabolic inhibitors can also improve the efficacy of GC in other diseases, such as rheumatoid arthritis.

Concluding Remarks

My dissertation identified specific metabolic activity in Th2 and Th17 cells that have not been previously documented during airway inflammation. I also establish a new avenue for metabolic inhibitors as potential mediators of airway inflammation and augmenting the effects of GCs. Inhibitors of cell metabolism alone or in combination with other therapies may provide a new approach to treat therapy-resistant asthma and other inflammatory diseases.

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