Targeting In Vivo Metabolic Vulnerabilities of Th2 and Th17 Cells Reduces Airway Inflammation


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Targeting In Vivo Metabolic Vulnerabilities of Th2 and Th17 Cells Reduces Airway Inflammation


T effector cells promote inflammation in asthmatic patients, and both Th2 and Th17 CD4 T cells have been implicated in severe forms of the disease. The metabolic phenotypes and dependencies of these cells, however, remain poorly understood in the regulation of airway inflammation. In this study, we show the bronchoalveolar lavage fluid of asthmatic patients had markers of elevated glucose and glutamine metabolism. Further, peripheral blood T cells of asthmatics had broadly elevated expression of metabolic proteins when analyzed by mass cytometry compared with healthy controls. Therefore, we hypothesized that glucose and glutamine metabolism promote allergic airway inflammation. We tested this hypothesis in two murine models of airway inflammation. T cells from lungs of mice sensitized with Alternaria alternata extract displayed genetic signatures for elevated oxidative and glucose metabolism by single-cell RNA sequencing. This result was most pronounced when protein levels were measured in IL-17-producing cells and was recapitated when airway inflammation was induced with house dust mite plus LPS, a model that led to abundant IL-4 and IL-17–producing T cells. Importantly, inhibitors of the glucose transporter 1 or glutaminase in vivo attenuated house dust mite + LPS eosinophilia, T cell cytokine production, and airway hyperresponsiveness as well as augmented the immunosuppressive properties of dexamethasone. These data show that T cells induce markers to support metabolism in vivo in airway inflammation and that this correlates with inflammatory cytokine production. Targeting metabolic pathways may provide a new direction to protect from disease and enhance the effectiveness of steroid therapy. The Journal of Immunology, 2021, 206: 1127–1139.

Asthma is a chronic inflammatory disease that affects ∼25 million Americans and has increased incidence worldwide (1). Patients with asthma have increased airway inflammation, airway hyperresponsiveness (AHR), remodeling, and mucus production that is driven in part by increased T cell activation (2). Asthma is not a uniform disease and many different phenotypes and endotypes of asthma exist, ranging from mild-intertmittent to severe asthma (3). Use of asthma therapeutics, including glucocorticoids (GCs) and β-agonists, are major defining criteria for asthma phenotypes (3). Patients with severe asthma are on high dose–inhaled GCs and bursts of oral GCs, yet many times do not respond well to therapy. Therefore, alternative approaches are needed to treat patients with uncontrolled asthma.

T cell–mediated immune responses are important in airway inflammation, AHR, remodeling, and mucus production. 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 (4). Although patients with severe...
asthma may 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 (2, 5, 6). T cell metabolism is important in determining CD4 T cell subset differentiation and targeting the essential metabolic programs of these cells may now allow a new approach to modulate immunity and suppress Th2 and Th17 cell–driven lung inflammation and asthma (4, 5).

The metabolic requirements of naive T cells change upon activation to develop into functional T cell subsets that each establish unique metabolic programs and requirements (7–12). Although resting T cells use an oxidative and catabolic metabolism, T effector (Teff) cells become highly glycolytic during expansion. This glycolytic phenotype is dependent on induction of the glucose transporter 1 (Glut1), as deletion of Glut1 impairs inflammatory T cell function in vivo (7). Although Teff cells continue to employ mitochondrial oxidative phosphorylation, this occurs to a lesser extent than in T regulatory or T memory cells, and Th17 cells can be more oxidative than other Teff cells (13). In addition to glucose, activated T cells can also convert glutamine to glutamate using glutaminase (GLS) in the process of glutaminolysis to support proliferation (11, 14, 15). Th17 cells rely heavily on glutaminolysis for differentiation, IL-17 production, and Th17–mediated inflammatory responses; however, other Teff subsets, such as Th1 cells, are not dependent on glutamine metabolism (11). Despite a growing literature on the metabolism of T cells, the metabolic profile of Th2 cells and the in vivo metabolic programs and requirements of Th2 and Th17 subsets remain poorly understood in airway inflammation.

Targeting metabolism can be one way in which T cells may be modulated to suppress airway inflammation. Glucose inhibitors have been shown to reduce disease in a T cell–dependent manner in models of systemic lupus erythematosus (16). Combined glycolysis and glutaminolysis inhibition also impaired T cell responses and showed improved outcomes for allograft rejection (17). In asthma, metabolic studies have largely focused on glycolysis. The serum and sputum of asthmatics have been shown to have increased lactate compared with healthy individuals indicating more aerobic glycolysis (18, 19). Moreover, CD4 T cells isolated from stimulated asthmatic PBMCs produced increased levels of lactate compared with cells isolated from healthy controls (19). Murine models also reported increased amounts of lactate in the bronchoalveolar lavage (BAL) and an increase in glycolysis in an IL–1–dependent manner (18, 20). These studies suggest that targeting metabolism may suppress lung inflammation, yet metabolic changes were measured in bulk tissues or in settings unable to discern the state of each functional cell type.

In this study, we establish the in vivo metabolic requirements of cytokine-producing Th2 and Th17 cells and test potential approaches to inhibit metabolism as a mechanism to decrease T cell–dependent airway inflammation. We hypothesized that Th2 and Th17 cells would have distinct metabolic programs and that pharmaceutical inhibition of glucose or glutamine metabolism may decrease allergen-induced airway inflammation and potentially augment the effects of GCS. Using patient samples and two models of murine airway inflammation this study shows that T cells in asthma have elevated markers of glucose and glutamine metabolism, with this effect particularly pronounced in IL-17–producing CD4 T cells. Importantly, in vivo metabolic inhibition of either Glut1 or GLS decreased the number of infiltrating cells and inflammation in the BAL. A combination of a GC 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.

**Materials and Methods**

**Segmental allergen challenge and metabolomics**

Mild, atopic asthma patients were recruited to the Vanderbilt Asthma, Sinus, and Allergy Program as previously described (21). Patients recruited were between 20 and 46 y old and had positive skin test to aeroallergens and/or inhaled allergen provocation to qualify for the study (Supplemental Fig. 1A). Exclusion criteria for the study were smoking and pregnancy for women. Volunteered consented to the institutional review board 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 hour following segmental allergen challenge, BAL fluid was sampled from the same section as described previously (21). Lactate levels were measured using a colorimetric lactate kit (no. MA064; Sigma-Aldrich). Metabolomics on the BAL and the differentiated T cells was measured by high-resolution nontargeted Q Exactive–mass spectrometry as previously described (9).

**Recruitment of asthma patients for mass cytometry**

Patients for which PBMCs were collected were aged 25–47 y old and were a mixture of males and females (Supplemental Fig. 1C). Mild asthmatics were characterized by the National Heart, Lung, and Blood Institute classification of asthma severity including symptoms, medications, quality of life, and lung function (3). 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 acute exacerbation. Human studies were approved by the Vanderbilt University Committee for the Protection of Human Subjects (institutional review board number: 111034).

**Mass cytometry analysis on PBMCs**

Cells were rapidly thawed and recovered in RPMI 1640 + 10% FBS. After all samples were thawed, 5 million live cells from each sample were used, and all samples were stained on the same day. First, the cells were stained using cisplatin (no. 201198; Fluidigm) to capture dead cells and then washed with PBS plus 1% BSA. Cells were stained for surface Abs, permeabilized with cold methanol, stained for intracellular Abs, and finally stained using intercalator (no. 201192; Fluidigm) to identify live cells. The metabolic Abs used were commercially available Western blot Abs that were first validated for flow cytometry and then custom conjugated to metals using Fluidigm conjugation kits. Cells were stored in intercalator at 4˚ for <1 wk. Before each run, samples were washed twice in PBS and twice in deionized H2O with a final resuspension in 1X EQ Four Element Calibration Beads (no. 201078; Fluidigm). Approximately 1 × 10^6 cells were collected from each sample using a Helios Mass Cytometer (Fluidigm) through the Vanderbilt Cancer and Immunology Core. Data preprocessing was done in Cytobank for cleanup and appropriately setting scales. Data analysis was then performed in R using uniform manifold approximation and projection (UMAP) for dimensionality reduction, self-organizing maps (SOMs) using the FlowSOM algorithm for clustering, and marker enrichment modeling for quantification of phenotypic differences between populations (22, 23). Analysis was done on the concatenated files of each cohort, which included 8000 cells from each patient.

**Mice**

C57BL/6 mice were purchased from The Jackson Laboratory at 8–12 wk of age. Experiments on airway inflammation used female mice, whereas 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 Use Committee at Vanderbilt University Medical Center.

**T cell in vitro activation, differentiation, and flow cytometry**

CD4 T cells were isolated from the spleen of wild-type C57BL/6J 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 mouse rIL-4 (80 ng/ml) and anti–IFN-γ (10 μg/ml). Th17 cells were differentiated by adding mouse rIL-6 (80 ng/ml), human rTGF-β (1.5 ng/ml), and anti–IFN-γ (10 μg/ml). Cells were cultured for 5 d, with a split on day 3 into new wells and fresh media supplemented with 10 ng/ml IL-2 to
promote continued proliferation. Intracellular cytokine staining was performed after a 4-h restimulation using PMA/ionomycin and GolgiStop. Cells were stained for viability and surface markers and permeabilized with Cytofix/Cytoperm (no. 554722; BD Biosciences) prior to staining for cytokines and metabolic markers. Transcription factor staining was performed on nonstimulated cells after surface staining and using the Foxp3 Transcription Factor Staining Buffer set (no. 00-5523-00; eBioscience).

Airway inflammation induction and inhibitor treatments

For the Alternaria alternata extract (Alt Ext) model, mice were intranasally administered with 75 µl of 7.5 µg Alt Ext (no. XP11D3A.25; Greer Laboratories) or PBS on days 0, 3, 6, and 9. Lungs were harvested on day 7 or 10 for end point analysis. For the house dust mite (HDM) model, mice were intranasally administered with 50 µl of either PBS or 100 µg of HDM Dermatophagoides pteronyssinus extract (no. XPB70D3A25; Greer Laboratories) plus 0.1 µg of LPS from Escherichia coli 011:1B4 (no. L4391; Sigma-Aldrich). Female mice were challenged on days 0, 7, and 14, and cells and tissues were harvested on day 15 for all end points except for AHR measurements, which were performed on day 16. Inhibitor treatments were initiated on day 0 and given daily throughout the 14 d of airway inflammation induction. Glut1 inhibitor (KL-11743; Kadmon) 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 (24, 25). GLS inhibitor (C8391; 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) (26, 27). Dexamethasone was injected in the i.p. daily for the last 5 d before harvest at 2.5 mg/kg.

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<sup>+</sup> cells using Miltenyi Biotec MicroBeads (catalog no. 130-052-301) using manufacturer’s protocol. CD45<sup>+</sup> cells were further purified by cell sorting, and then, CD45<sup>+</sup> cells were loaded onto 10<sup>6</sup> Chromium Controller for single-cell RNA (scRNA) sequencing. Cell Ranger software (v3.0.2) was used with default parameters for library demultiplexing, fastq file generation, read alignment, and unique molecular identifiers (UMIs) 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 <10% of total UMIs originating from mitochondrial RNA. Genes detected in more than three 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 (28). R package clusterProfiler (v3.16.1) was used for the gene set enrichment analysis with the Kyoto Encyclopedia of Genes and Genomes gene sets pulled from R database msigdb_v7.1.1 (29). Data visualization was done using the respective analysis tools or custom scripts using ggplot2 (R package). All the scripts used are available upon request. Data are deposited for public access under https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE163572.

Collection of 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 200 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.

Histopathology

To measure airway inflammation, lungs were perfused, inflated, and infiltrated with 800 µl of neutral-buffered formalin overnight at room temperature. Lungs were transferred to 70% ethanol and paraffin embedded. Tissue sections (5 µm) were stained with 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 0–3 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, 5–10% PAS-positive cells; 3, 10–25% PAS-positive cells; and 4, >25% PAS-positive cells.

Luminex

Cytokine levels in the BAL supernatants were measured using a MILLIPLEX Mouse High Sensitivity T Cell Panel kit (no. MHSTCMAG-70K; Millipore) for IL-4, IL-5, IL-17A, and IL-1β by Luminex assay through the Vanderbilt Hormone Assay and Analytical Services Core.

AHR

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/min with 0.2 ml tidal volume using the Flexivent (SciReg). AHR was measured after administration of aerosolized saline (vehicle), followed by increasing concentrations of acetyl-β-methacholine (12–50 mg/kg body weight) (30).

Statistical analyses

Statistical analyses were performed on Prism using either Student 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. Mass cytometry and scRNA sequencing statistical analysis were performed on R.

Results

CD4 cells are more metabolically active in asthmatic donors

Although 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 (18–20). To directly determine if lactate levels are increased in asthmatic patients after an allergen challenge, we sampled the BAL fluid from the same airway in a cohort of mild asthmatics (Supplemental Fig. 1A) 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 (Fig. 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 (Fig. 1B). Although 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 (Supplemental Fig. 1B). Allergic responses in airways of asthma patients, therefore, induce increases in both glucose and glutamine metabolism.

PBMCs from asthmatic patients may show systemic immunologic changes that reflect inflammation in the airway (19). 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 (Supplemental Fig. 1C) were analyzed by mass cytometry using Ab panels that included markers of cell identity, activation, and immune state, as well as glucose, glutamine, and mitochondrial metabolism. 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 T cell population clusters. After concatenation of the mass cytometry data from healthy, mild, and severe asthmatics, UMAP 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 (Fig. 1C). Earth mover’s distance (EMD) quantifies the overall degree of difference
between populations and healthy individuals had large pairwise EMD scores from UMAP when compared with either mild or severe asthmatics (31). 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. SOMs using the FlowSOM algorithm were applied to the mass cytometry data to separate CD4 T cells into distinct populations on the UMAP axes (22). SOM clusters 2, 3, 4, and 7 were largely represented by T cells from healthy donors, whereas 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 (Fig. 1C). Marker enrichment modeling can identify and quantify cell markers that drive overall phenotypes and differences between these clusters (23). 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 with T cell clusters from the mild and severe asthma cohorts (Fig. 1E). The metabolic proteins included in the analyses were broadly elevated in the asthmatic cohorts and clusters, although glutamate dehydrogenase 1 (GLUD1; glutaminolysis enzyme) was only modestly different between the T cell donors and across each T cell subset, and CPT1a (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 suggest 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 capacity than those of healthy individuals.

### Distinct metabolic programs of Th2 and Th17 cells

Analyses of human asthmatic samples showed that CD4 T cells had increased expression of metabolic markers and it appeared that both

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**FIGURE 1.** Asthmatics have more metabolically active CD4 cells circulating in peripheral blood. (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 mass-spectrometry metabolomics, and significantly altered metabolites (p < 0.05) are indicated in red. (C) Concatenated data from mass cytometry on cells from healthy, mild, and severe asthmatic participants were analyzed using UMAP for dimensionality reduction and SOMs using the FlowSOM algorithm for clustering. Cell density for each condition shown on UMAP axes and SOM clusters shown in different colors on the UMAP axes for concatenated cohort. EMD was applied to quantify the overall degree of difference between each group. (D) The abundance of cell from healthy, mild, or severe asthmatics are shown for each SOM cluster shown in (C). (E) Marker enrichment modeling enrichment scores are shown for metabolic markers and transcription factors from the SOM clusters of each disease state. **p < 0.01, unpaired t test.
Th2 and Th17 cells were present to contribute to increased metabolism in asthma. The relative differences between the metabolic programs of Th2 and Th17 cells, however, have not been previously described. Using a published metabolomics dataset in which the metabolomic profiles from Th2 cells had not been previously analyzed, we compared metabolites from in vitro differentiated murine Th2 and Th17 CD4 T cells to naive CD4 T cells (Fig. 2A) (9). As previously described, both Th2 and Th17 cells used distinct metabolic programs and metabolite profiles from naive CD4 T cells and each other (8, 9). 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 (Fig. 2B). The shift in the ratio of glutamine and glutamate in Th2 and Th17 cells further supports a greater role for glutaminolysis in Th17 cells (Supplemental Fig. 2A) (11).

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 showing a trend toward 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 (Fig. 2C, Supplemental Fig. 2B). As ECAR measures decreased extracellular pH and does not account for alternative fates for glucose, Th17 cells may require the same or additional glucose than Th2 cells 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 with Th2 cells (Supplemental Fig. 2C) (10). Moreover, inhibition of mTOR signaling complex (mTORC1), which results in decreased glycolysis, impaired both Th2 and Th17 cells (Supplemental Fig. 2D). In contrast to ECAR, measurement of oxygen consumption rates, which indicate mitochondrial oxidative phosphorylation, showed that Th17 cells had a modestly higher basal and maximal respiratory rates and significantly more mitochondrial ATP production (Fig. 2D, Supplemental Fig. 2E). Together, these data suggest that both in vitro stimulated Th2 and Th17 cells are highly metabolically active, and although Th2 cells appear to have greater glycolytic capacity, Th17 cells more predominantly use mitochondrial oxidative phosphorylation.

Increased gene expression upon T cell activation in Alternaria extract–induced airway inflammation

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-17, and dual cytokine-producing cells, although IL-17–producing cells were present in lower numbers (Fig. 3A). scRNA sequencing was performed on CD45R+ 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 (Fig. 3B). Cells and cell clusters were assessed for expression of SELL (CD62L), IL-4, and IL-17 to identify resting and Th2 and Th17 cell populations, respectively (Fig. 3B). Although 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 (Fig. 3C). In addition to increased expression of TCR signaling genes, Kyoto Encyclopedia of Genes and Genomes enrichment analysis showed a significant increase in oxidative phosphorylation and a trend toward elevated glycolytic and glutamate metabolism gene expression in the IL-4–expressing cells (Fig. 3D).

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 of T cells to be analyzed for expression of cytokines and metabolic markers of glycolysis (Glut1) or glutaminolysis (GLUD1) (Fig. 3E). Both proteins had increased expression in cytokine-producing cells compared with those that did not produce cytokines. Interestingly, GLUD1 expression was significantly increased in IL-17–producing cells compared with IL-5–producing cells. These data suggest that despite activation increasing the metabolic output of Th2 cells, Th17 cells can demonstrate a greater potential for glutamine metabolism in vivo.

A model of mixed Th2 and Th17 airway inflammation induced with HDM and LPS

The Alt Ext model suggested in vivo metabolic phenotypes but did not generate a robust Th17 response characteristic of 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 HDM in combination with LPS to activate TLR4 (Supplemental Fig. 3A) (32). Sensitized mice developed increased AHR (Fig. 4A), and lungs showed thickening of the basement membrane, increased inflammation, immune cell infiltration, and increased numbers of goblet cells (Fig. 4B). Cellular and immunological analyses showed 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 (Fig. 4C). Cytokine levels were measured from the BAL fluid and a variety of inflammatory cytokines characteristic of both Th2 and Th17 cells, including IL-4 and IL-17, were increased following HDM + LPS challenge (Fig. 4D).

We next 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 (Fig. 4E, Supplemental Fig. 3B). Although there was a trend toward 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 (Fig. 4F). 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.

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 high metabolic capacity with a potential differential usage of glucose and glutamine metabolism. Therefore, we tested 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 coexpression of cytokines and metabolic markers. All cytokine-producing T cells were found to have greater expression of metabolic protein markers for glucose and glutamine metabolism than CD4 T cells that did not express IL-4 or IL-17 (Fig. 5, Supplemental Fig. 3C). 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. These included 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. Interestingly, 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.

Metabolic inhibition can reduce airway inflammation

Given the high expression of all metabolic markers in inflammatory IL-4– and IL-17–producing CD4 T cells in the lung, metabolic inhibitors were evaluated to determine if targeting these 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 (24, 25). 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 toward decreased infiltration of each cell type measured in the BAL, although only eosinophils were significantly reduced (Fig. 6A). Cytokine levels in the BAL fluid were also measured, and although Th2 cytokine IL-5 and Th17 cytokine IL-17 were unchanged, IL-1β was reduced (Fig. 6B). 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 (Fig. 6C).

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 toward decreased BAL-infiltrating cells of all types, although only eosinophils were significantly decreased (Fig. 7A). Cytokine levels in the BAL fluid were significantly decreased by CB839 treatment for the Th2 cytokine IL-5, the Th17 cytokine IL-17, and IL-1β (Fig. 7B). 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 (Fig. 7C). Targeting T cell metabolism by blocking Glut1 or GLS may, therefore, provide protection against airway inflammation.

GCs and metabolic inhibition can cooperate to decrease T cell viability and cytokine production

GCs are one of the primary long-term treatments for asthma currently prescribed (3, 33), and although effective in mild forms of disease, severe Th17 neutrophilic forms of disease respond poorly to this treatment (3, 34–36). Given the high metabolic rate of IL-17–producing CD4 T cells and ability of Glut1 and GLS
FIGURE 3. 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 scRNA sequencing (scRNAseq) (n = 3). Concatenated data from scRNAseq 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 CD3ε, CD4, SELL, and IL-4. (D) Gene set enrichment analysis plots of enriched gene sets that were enriched in IL-4–expressing cells compared with SELL cells in CD4 T cells (cluster 2/cluster 1). (E) Expression of Glut1 and 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.
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 reduce Th17 cell survival and inflammatory functions. Th17 cells were differentiated in vitro for 3 d in the presence of a GC (dexamethasone), KL-11743 alone, or CB839 alone, or with GC combined with each metabolic inhibitor. Th17 cell viability was decreased by each individual compound, with GC treatment or Glut1 inhibition having greater effects than GLS inhibition alone (Fig. 8A). However, the combination of GC and inhibitor further reduced Th17 cell viability. Similar to reduced Th17 cell viability, GC or Glut1 inhibition reduced IL-17 production (Fig. 8A). Although 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 IL-17 production. We next assessed whether combination treatment in vivo would have an effect on airway inflammation, specifically on lung function by measuring AHR. Based on the glutamine metabolic program of Th17 cells the inhibitor CB839 was tested alone and in combination with dexamethasone in vivo (Fig. 8B). Overall, no difference was observed between vehicle-treated mice compared with treated mice at low concentrations of methacholine (Supplemental Fig. 3D). However, airway resistance was significantly decreased when CB839 is used in combination with a GC at the highest methacholine dose. 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.

Discussion
Asthma is an inflammatory disease driven in part by different T cell subsets that vary with the clinical subtype (3, 37). T cell subsets and functional populations are now known to have diverse metabolic requirements to activate, proliferate, and function (7–12). To date, however, these changes have not been well established or tested in airway inflammation. In this study, we show that the
metabolic phenotype of peripheral blood T cells from mild and severe asthmatics differ from healthy controls and that modulating metabolic pathways may provide a new approach to shift T cell fate. Th2 airway inflammation induced by Alt Ext led to a metabolically active Th2 population by gene expression. This model also developed a smaller Th17 population that also showed a high capacity for metabolic activity, particularly glutamine metabolism. In a distinct model for neutrophilic asthma characterized by both Th2 and Th17 populations, cytokine-producing T cell subsets also expressed proteins characteristic of high capacity for metabolic activity, and this was also particularly evident in IL-17–producing cells. Importantly, these metabolic profiles can be pharmacologically targeted in vivo to protect from features of airway inflammation and disease. Because GCs are standard therapy for many asthma patients, we also tested if metabolic inhibition could augment the effects of GCs and found that inhibition of either Glut1 or GLS increased T cell death and reduced cytokine production and AHR. These data suggest that targeting metabolism may provide a new direction in asthma and may enhance GC therapy effectiveness.

A metabolic shift occurs in the lungs of asthmatic patients and in T cells from animal models of asthma. Surprisingly, CD4 T cells from both mild and severe asthmatics also showed 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 altered expression of peripheral blood T cell metabolic markers. These proteins 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. Surprisingly, mild and severe asthmatics showed modest metabolic differences between each other, and each showed a greater difference from the healthy control. It is possible this similarity of T cells in 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.

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 has not previously been well described. The kinase, mTOR, is an important metabolic regulator that uses environmental signals to activate one of the two mTORC that lead to T cell differentiation (38). Although Th2 cells may rely on mTORC2 more than mTORC1 (37), inhibition of mTORC1 by rapamycin suppressed cytokine production by both populations. Our in vitro metabolomics results support this finding, and flux analyses suggest that Th2 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 (7). In contrast to lower lactate secretion, Th17 cells appeared to have a higher overall rate of oxygen consumption and mitochondrial respiration. In the Alt Ext model, the scRNA sequencing showed that IL-5–producing CD4 T cells expressed gene signatures of increased oxidative and a trend toward increased glycolysis and glutamate metabolism. Both in vivo models of airway inflammation suggested that although all cytokine-producing T cells in the lung were more metabolically active than those T cells not secreting cytokines. CD4 T cells producing IL-17 alone or in combination with IL-4 or IL-5 displayed the highest expression of all metabolic markers. It remains an important question if this difference reflects general feature of IL-17–producing T cells in vivo or if the particular airway inflammation model employed in this study more strongly drove the metabolism of Th17 than Th2 cells.

The high metabolic rates of Th2 and Th17 cells and findings that genetic deficiency of SLC2A1 (Glut1) or GLS can protect against inflammation suggested that pharmacologic inhibition of those proteins may provide protection from airway inflammation (7, 11, 39). Previous work showed that T cell–specific genetic loss of GLS dampened airway inflammation (11). Our current study tested if acute in vivo pharmacological inhibition could also provide protection and expands on the analysis of the metabolic dependencies of T cells in 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,

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Glut1 targeting has modest impact on infiltrating cells into the BAL. (A–C) Mice were challenged with HDM + LPS and dosed once daily with either vehicle (n = 6) or KL-11743 (n = 6). (A and 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.
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 (11, 40).

FIGURE 7. 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 and 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.

FIGURE 8. GC and metabolic inhibitor in combination decrease Th17 cell viability, cytokine production, and in vivo decrease AHR. (A) CD4 cells were differentiated in Th17 polarizing media for 3 d (n = 4). Cells were analyzed by intracellular flow to determine number of live cells and cytokine-producing CD4 Th17 cells. Live cell numbers are relative to the number of live cells in the vehicle treated. (B) AHR measurement 2 d following last HDM challenge for mice sensitized with either PBS or HDM + LPS and then treated with vehicle, dexamethasone, and CB839 at a methacholine challenge of 100 mg/ml (n = 9–10). *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.
A standard treatment for asthmatic patients is inhaled GC steroids, yet resistance in a Th17-mediated manner has been observed in populations of severe neutrophilic asthmatics (3, 33–35). Our data suggest that metabolic inhibitors used in combination with GCs may be a promising approach by which to bypass GC resistance. GC resistance by Th17 cells is an issue not only limited to asthma, other inflammatory diseases, such as Crohn disease, implicate Th17 cells as the main resistance cell type (41). 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 (42). Together with our findings, these data suggest that metabolic inhibitors may enhance sensitivity to GC and restore Th17 cell responsiveness to this therapy.

Inhibitors of cell metabolism alone or in combination with other therapies may provide a new approach to treat therapy-resistant disease asthma and other inflammatory diseases. Previous studies that have shown that blocking glycolysis either through glucose transporter inhibitors or glucose analogs results in improved disease in lupus and prevention of allograft rejection in a T cell–mediated manner, suggesting that glucose transport inhibitors are effective mediators of inflammation not only in a Th17 cell–dependent manner (16, 17). Furthermore, inhibition of glutaminolysis may prevent allograft rejection and protect against graft-versus-host disease and inflammatory bowel disease (11, 17). These data suggest that using a two-pronged approach of a GC and a metabolic inhibitor may be mechanism by which to bypass GC resistance in asthma.

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