Published in final edited form as:

Clin Exp Allergy. 2017 September ; 47(9): 1170–1184. doi:10.1111/cea.12947.

Fueling the Mechanisms of Asthma: Increased Fatty Acid Oxidation in Inflammatory Immune Cells May Represent a Novel Therapeutic Target

Amir A. Al-Khami^{1,2,3}, Mohamed A. Ghonim^{1,4}, Luis Del Valle^{1,5}, Salome V. Ibba¹, Liqin Zheng¹, Kusma Pyakurel¹, Samuel C. Okpechi¹, Jone Garay¹, Dorota Wyczechowska¹, Maria D. Sanchez-Pino¹, Paulo C. Rodriguez⁶, A. Hamid Boulares¹, and Augusto C. Ochoa^{1,7,*}

¹Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, LA

²Deptment of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA

³Faculty of Science, Tanta University, Tanta, Egypt

⁴Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

⁵Deptment of Pathology, Louisiana State University Health Sciences Center, New Orleans, LA

⁶University of Augusta Cancer Center, Augusta, GA

⁷Department of Pediatrics, Louisiana State University Health Sciences Center, New Orleans, LA

Abstract

Background—Increasing evidence has shown the close link between energy metabolism and the differentiation, function, and longevity of immune cells. Chronic inflammatory conditions such as parasitic infections and cancer trigger a metabolic reprogramming from the preferential use of glucose to the up-regulation of fatty acid oxidation (FAO) in myeloid cells, including macrophages and granulocytic and monocytic myeloid-derived suppressor cells. Asthma is another chronic inflammatory condition where macrophages, eosinophils, and polymorphonuclear cells play an important role in its pathophysiology.

Objective—We tested whether FAO might play a role in the development of asthma-like traits and whether the inhibition of this metabolic pathway could represent a novel therapeutic approach.

Methods—OVA and house dust mite (HDM)-induced murine asthma models were used in this study.

Results—Key FAO enzymes were significantly increased in the bronchial epithelium and inflammatory immune cells infiltrating the respiratory epithelium of mice exposed to OVA or HDM. Pharmacologic inhibition of FAO significantly decreased allergen-induced airway

*Corresponding Author: Augusto C. Ochoa, Louisiana State University Health Sciences Center, 1700 Tulane Avenue, Room 910, New Orleans, LA 70112. Phone: 504-210-2828; Fax: 504-210-2970; aochoa@lsuhsc.edu.

Conflict of Interest

The authors declare no conflict of interest.

hyperresponsiveness, decreased the number of inflammatory cells, and reduced the production of cytokines and chemokines associated with asthma.

Conclusions and clinical relevance—These novel observations suggest that allergic airway inflammation increases FAO in inflammatory cells to support the production of cytokines, chemokines, and other factors important in the development of asthma. Inhibition of FAO may therefore provide a novel therapeutic approach for the treatment of asthma by re-purposing existing drugs that block FAO and are approved for the treatment of heart disease.

Keywords

Immunometabolism; fatty acid oxidation; airway hyperresponsiveness; inflammation; asthma

Introduction

Increasing evidence demonstrates the close association between energy metabolism and the differentiation and function of various immune cell subsets (1–3). Resting T and B cells primarily rely on oxidative phosphorylation (OXPHOS) of glucose to maintain baseline functions. Activation signals delivered through antigen or cytokine receptors shift their metabolism to glycolysis, which is a faster (although less efficient) way of producing ATP and synthesizing nucleotides and proteins necessary for cell proliferation and function (4,5). However, this normal sequence of events changes significantly with disease. For example, chronic inflammatory conditions such as parasitic infections and cancer cause myeloid cells to undergo metabolic reprogramming, preferentially using fatty acids as the source of ATP to support their functions (6–8). In murine cancer models, myeloid-derived suppressor cells (MDSC) show an increased fatty acid oxidation (FAO), and the use of pharmacologic FAO inhibitors blocks their immunosuppressive functions and enhances anti-tumor T cell mediated immunotherapy (6,7).

Asthma is a chronic obstructive airway disease that affects over 300 million people worldwide and is increasing in incidence. Patients with allergic asthma display a chronic airway inflammation mediated primarily by type 2 T helper (Th2) cells and characterized by the infiltration of the bronchoalveolar mucosa with inflammatory immune cells such as dendritic cells, macrophages, eosinophil, and neutrophils, which ultimately lead to the characteristic airway hyperresponsiveness (AHR) (9–11). Current treatments primarily rely on mixtures of inhaled corticosteroids and bronchodilators, with some patients benefiting from antibodies against IgE or IL5 (12,13). Therefore, novel insights into the underlying mechanisms of asthma may suggest new prevention and/or treatment approaches. As such, our goal was to determine whether inflammatory cells in asthma would undergo metabolic reprogramming as shown in other chronic inflammatory conditions and to determine the potential therapeutic effect of FAO inhibition.

The results showed a significant increase in the expression of key FAO enzymes in murine models of the disease. These enzymes included carnitine palmitoyltransferase 1 (CPT1) and 3-hydroxyacyl-CoA dehydrogenase (HADHA). CPT1, the first and rate-limiting enzyme of the FAO pathway, regulates the entry of long-chain fatty acids into the mitochondria through converting acyl-CoAs to acylcarnitine derivatives. HADHA, on the other hand, is a

trifunctional enzyme that catalyzes the final three steps in the FAO cycle. Importantly, FAO inhibition blocked AHR, recruitment of inflammatory immune cells, and production of allergen-specific IgE and asthma-associated cytokines and chemokines. Thus, the data suggest that FAO support the function of inflammatory cells in asthma, and as such its inhibition may represent a novel therapeutic strategy.

Methods

Murine Asthma Models

Asthma models were established using OVA or house dust mite (HDM) exposure of male C57BL/6J or Balb/c mice (6–8 week old) from the Jackson Laboratories. Experiments were approved by the LSUHSC Institutional Animal Care and Usage Committee (Number is 3277). For the OVA-induced asthma model, mice were sensitized by intraperitoneal injection of 50 µg Grade V chicken OVA (Sigma-Aldrich) mixed with 2 mg aluminum hydroxide in saline once a week for 2 weeks. After two weeks, mice were challenged with aerosolized 3% OVA for 30 minutes (14,15). Mice were left untreated or treated with intraperitoneal injection of the CPT1 inhibitor etomoxir (Sigma-Aldrich) or the HADHA inhibitor ranolazine (Sigma-Aldrich) at 50 mg/Kg (both diluted in saline) 30 minutes after challenge. Mice received another intraperitoneal injection of 50 mg/Kg etomoxir or ranolazine 24 hours later (30 minutes after AHR assay). Sera and organs were then collected 24 hours later (Figure 1A). When indicated, mice were intravenously injected with 0.2 ml liposomal clodronate (ClodronateLiposomes.com) 8 hours prior to the airway challenge to deplete blood monocytes (16). Control mice were injected with 0.2 ml control (PBS) liposomes (ClodronateLiposomes.com). For the HDM-induced asthma model, mice were anaesthetized with isoflurane and sensitized intranasally with 25 µl saline (control) or 1 mg/ml whole HDM (*Dermatophagoides pteronyssinus*) extract (Greer Labs) three times a week for 5 weeks (14,17). Mice were euthanized two days after the last HDM exposure to determine the expression of FAO mediators (Figure 1C). To test the effect of FAO inhibition against chronic, HDM-induced asthma-like traits, mice were HDM-challenged daily for 3 consecutive days at the end of the fifth week, followed by intraperitoneal injection of 50 mg/Kg etomoxir 30 minutes after each challenge. One day later, mice received another etomoxir injection 24 hours later (30 minutes after AHR assay), and sera and organs were harvested after another 24 hours (Figure 8A).

AHR

AHR to inhaled methacholine was measured 24 hours after OVA challenge by recording enhanced pause (Penh) by whole body plethysmography (EMKA Systems). In brief, baseline readings were taken and averaged for three minutes after animals were placed in a barometric plethysmographic chamber. Saline or increasing concentrations (12.5 – 100 mg/ml) of aerosolized methacholine were nebulized. Readings were taken and averaged for three minutes after each nebulization, and Penh representing AHR was calculated (18).

Immunohistology

Lungs were fixed in formalin for histologic analysis or used for bronchial alveolar lavage (BAL) collection (18). Sections were processed for H&E staining or immunohistochemistry

staining with antibodies to CPT1 (8F6AE9 Abcam; 1:100) or HADHA (ab54477 Abcam; 1:500). Some sections were also stained with antibodies to CPT1 or HADHA together with F4/80 (SP115 Abcam; 1:100), Ly-6G (RB6-8C5 ebioscience; 1:500), Siglec-F (E50-2440 BD Biosciences; 1:500), or CD45R (RA3-6B2; BD Biosciences; 1:500). Protocols were described previously (7). BAL fluids were subjected to cytopsin and stained with Diff-Quik (IMEB).

Flow cytometry

Lungs were digested with DNase and Liberase (Roche USA) at 37°C for 45 minutes. Single cell suspensions were stained with mouse antibodies against Siglec-F (E50-2440), F4/80 (BM8), CD11b (M1/70), CD11c (HL3), MHC-II (AF6-120.1), CD4 (H129.19), and CD45R (RA3-6B2). Antibodies were obtained from BD Biosciences; F4/80 was from ebioscience. Samples were analyzed using a Gallios flow cytometer (Beckman Coulter), and the results were analyzed with FlowJo software (TreeStar).

Real-time PCR

Total RNA from purified cells was isolated using the RNeasy Mini Kit (Qiagen). cDNA was generated using iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed for CPT1, HADHA, Acyl CoA dehydrogenase (ACADM), peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC1 β), arginase I, and Nitric Oxide Synthase 2 (NOS2) using Taqman primers (Applied Biosystems). Gene expression was calculated relative to 18s rRNA using 2^{-CT} method.

Assessment of IgE, cytokines, and chemokines

A sandwich ELISA was used to quantify OVA-specific IgE (Serotec) and total IgE (ebioscience), as described by the manufacturer. Cytokines and chemokines were assessed with Bio-Rad Bio-Plex (Bio-Rad Laboratories) or the Millipore Milliplex (Millipore), per the manufacturer's instructions.

Data analysis

Experiments were repeated at least twice. Data were analyzed by either Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism software. Results were expressed as mean $\pm$ SEM of values from at least five mice per group unless stated otherwise. *P* values are presented in figures as *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; or ****, *P* < 0.0001.

Results

Inflammatory immune cells from OVA sensitized and challenged mice have an increased expression of FAO enzymes

Recent work has shown that myeloid cells isolated from chronic inflammatory disease models such as parasitic infections or cancer had an increased expression of FAO enzymes that paralleled changes in their functions (6–8). We aimed to determine whether asthma models could cause a similar metabolic reprogramming of myeloid cells. We used the OVA-

induced asthma model (Figure 1A), which involves the requirement for systemic priming with an adjuvant and is commonly used in mechanistic studies. In addition, we used a model based on the inhaled delivery of a true allergen, HDM (Figure 1C). This model involves mucosal sensitization within the lungs with allergen, thus better representing human asthma. The expression of FAO enzymes, CPT1 and HADHA, was initially tested by immunohistochemistry. As expected, there was a significant inflammatory cell infiltration of the peribronchial and alveolar tissues in mice exposed to OVA or HDM. More importantly, the expression of CPT1 and HADHA was markedly increased in allergic lungs compared to controls (Figure 1B and 1D). However, the increase in OVA-induced HADHA did not reach statistical significance (Figure 1B). Interestingly, the apical ciliary border of the bronchial epithelium was also positive for these enzymes in control and allergic lungs (Figure 1B and 1D). Isotype control staining for CPT1 and HADHA is shown in Supplemental Figure 1. Given the similar results in both asthma models, we used the OVA model to conduct additional experiments.

To determine which of the inflammatory cells had an increased expression of FAO enzymes, we performed double immunofluorescence labeling with CPT1 or HADHA and various immune cell markers in lung sections of OVA-exposed mice. The results showed the most abundant expression of both CPT1 and HADHA in F4/80⁺ macrophages, while Ly-6G⁺ neutrophils, Siglec-F⁺ eosinophils, and CD45R⁺ B cells expressed significantly lower levels of both enzymes (Figure 2A and 2B).

FAO inhibition blocks AHR and OVA-specific IgE production

Next we tested the effect of etomoxir, a specific CPT1 inhibitor, in mice sensitized and challenged with OVA. As shown in Figure 3A, mice were treated with etomoxir 30 minutes post challenge. AHR was measured 24 hours after treatment. A second dose of etomoxir was given 30 minutes after AHR assay, and lungs, spleens, and peripheral blood were harvested 24 hours later (day 16). Although measuring lung resistance is considered a more reliable way of evaluating the lung function, Penh has been shown to correlate well with invasive measures of AHR (19). Moreover, our studies consistently showed an increased AHR in allergen-exposed C57BL/6 mice compared to control mice (14,15,17). In line with these reports, AHR to methacholine was significantly increased in OVA-sensitized and challenged mice versus controls, while etomoxir-treated mice had a significant reduction in AHR (Figure 3B). We also tested the effect of the HADHA inhibitor ranolazine. Similarly, there was a significant, although moderate, decrease in AHR (Figure 3C). We then asked whether these effects were specific to the C57BL/6J mice. Therefore, we repeated the OVA sensitization and challenge followed by treatment in Balb/c mice. The results confirmed the protective effect of etomoxir or ranolazine in Balb/c mice exposed to OVA (Figure 3D).

We next tested whether FAO inhibition changed IgE levels in allergen-exposed mice. Noticeable levels of OVA-specific IgE were induced in BAL fluids and sera of OVA-exposed animals over controls. However, etomoxir treatment significantly reduced the production of OVA-specific IgE (Figure 3E). In addition and consistent with previous reports (20), total serum IgE was markedly increased upon OVA exposure but in much higher amounts than OVA-specific IgE. Therefore, although etomoxir treatment slightly decreased the levels of

total serum IgE, this effect did not achieve statistical significance (Figure 3F). Moreover, the reduction in IgE levels was not caused by a systemic decrease in immunoglobulins since the concentrations of total serum IgG or IgM remained unchanged (Supplemental Figure 2).

FAO inhibition suppresses OVA-induced inflammation

As shown by H&E staining, a single etomoxir treatment resulted in a major decrease in the inflammatory cells infiltrating the bronchoalveolar tissue of OVA-sensitized and challenged mice (Figure 4A). As expected, this was also accompanied by a reduction in the number of cells expressing CPT1 (Figure 4B). To start understanding the mechanism, we stained for apoptosis using TUNEL assay; however, this did not show an increase in the number of apoptotic cells in the lungs of mice treated with etomoxir (Figure 4C). To further study other possible mechanisms by which FAO inhibition caused such a significant response, inflammatory immune cells were isolated from the BAL fluids or single cell suspensions of whole lung tissue. As previously demonstrated by immunohistology, the number of total leukocytes, eosinophils, macrophages, and lymphocytes were significantly decreased in the BAL fluids of OVA-exposed mice treated with etomoxir (Figure 5A). These changes were confirmed by flow cytometry analysis of lung single cell suspensions, which showed significant decreases in the percentages of Siglec-F⁺ eosinophils, F4/80⁺CD11b⁺CD11c⁻ interstitial macrophages (but not F4/80⁺CD11b⁻CD11c⁺ alveolar macrophages), CD11c⁺MHC-II⁺ dendritic cells, CD4⁺ T cells, and CD45R⁺ B lymphocytes compared to untreated mice (Figure 5B). However, this was not caused by a systemic immunosuppressive effect since the numbers of different immune subsets essentially remained unchanged in the spleens of etomoxir-treated mice compared to control mice (Figure 5C). It is also of note that the percentages of these immune cells in lungs and spleens correlated well with their absolute numbers (Supplemental Figure 3).

FAO inhibition reduces cytokines and chemokines associated with OVA-induced allergic airway inflammation

We also tested the effect of FAO inhibition on the production of cytokines and chemokines known to mediate inflammatory responses in asthma. As predicted, OVA sensitization and challenge significantly increased the levels of inflammatory cytokines and chemokines in BAL fluid. Treatment with etomoxir significantly decreased the levels of the cytokines IL-4, IL-5, and IL-6 and the chemokines CCL2 (MCP-1), CCL4 (MIP-1 β), CCL11 (eotaxin), CXCL1 (KC), CXCL5 (LIX), CXCL9 (MIG), and CXCL10 (IP-10) (Figure 6A and Supplemental Figure 4A). This effect was also reflected in the serum levels of several cytokines such as IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, and TNF α and chemokines such as CCL5 (RANTES) and CXCL1 (KC) (Figure 6B and Supplemental Figure 4B). These changes, however, were not the result of a systemic immunosuppressive effect of etomoxir since the levels of other cytokines such as IFN γ , G-CSF and GM-CSF did not change significantly in BAL fluid or serum (Supplemental Figure 5A and 5B, respectively).

Effect of FAO inhibition on lung macrophages

Given the importance of macrophages in allergic airway inflammation (16,21,22) and the significance of FAO in alternatively activated (M2) macrophages (8,23), we tested the expression of FAO enzymes in F4/80⁺ lung macrophages. Double immunofluorescence

labeling with F4/80 and CPT1 showed a significant increase in the number of F4/80⁺ CPT1⁺ macrophages in the lungs of OVA-sensitized and challenged mice. Treatment with etomoxir markedly reduced the number of F4/80⁺ CPT1⁺ macrophages (Figure 7A). Furthermore, while the levels of mRNA coding for the FAO enzymes CPT1, HADHA, and PGC1 β were increased in macrophages sorted from allergen-exposed lungs, etomoxir treatment reduced these enzymes to normal levels (Figure 7B). There were no significant changes in the expression of ACADM (Figure 7B). In addition, we tested the mRNA levels for arginase I and NOS2, factors important in the pathophysiology of asthma. As shown in Figure 6C, lung macrophages from allergic mice had significantly higher levels of arginase I and lower levels of NOS2 compared to controls, confirming an M2 phenotype. However, FAO inhibition with etomoxir significantly reduced arginase I and conversely elevated NOS2 expression.

We next sought to test the role of macrophages in the protective effects induced by FAO inhibitors. For this, we used intravenous injection of liposomal clodronate to deplete circulating inflammatory monocytes, presumed progenitors of lung-recruited macrophages that have been shown to play a role in allergic asthma and considered pathogenic, whereas resident alveolar macrophages have been found to restrain allergic lung inflammation (16,21). Consistent with these reports, our data showed that liposomal clodronate alone decreased the number of total leukocytes, eosinophils, and macrophages in the BAL fluids of OVA-exposed mice (Figure 7D). Moreover, liposomal clodronate reduced the production of BAL fluid and serum IL-5 (Figure 7E). Control (PBS) liposomes did not have any effects on OVA-exposed mice (data not shown). Of interest, combining liposomal clodronate with etomoxir treatment further enhanced these protective responses (Figure 7D and 7E), suggesting that while FAO inhibition in macrophages may contribute to the therapeutic effects of etomoxir, other cells may also be involved.

FAO inhibition attenuates the inflammatory immune response in a chronic HDM asthma model

We further confirmed our observations by testing the effect of FAO inhibition against already established, chronic asthma-like traits induced by HDM, a true human allergen (Figure 8A). Interestingly and consistent with the OVA model of asthma, etomoxir treatment resulted in a significant decrease in AHR in HDM-exposed mice (Figure 8B). Furthermore, etomoxir markedly blocked the recruitment of eosinophils, macrophages, and lymphocytes into lungs, with a comparable decrease in the total number of leukocytes (Figure 8C). FAO inhibition in the HDM model also blocked the production of IL-5 in the BAL fluid and serum compared to untreated mice (Figure 8D).

Discussion

Increasing evidence has demonstrated that the differentiation, function, and longevity of immune cells are closely associated with the use of distinct metabolic pathways to produce energy (1–3). These observations have been studied in several murine chronic inflammatory conditions such as parasitic infections and cancer (6–8). In these diseases, myeloid cells undergo a metabolic reprogramming, shifting from OXPHOS of glucose to the oxidation of fatty acids to sustain their functions. Allergic asthma is a disease primarily mediated by Th2

cells, which produce IL4 and IL5 that promote the production of IgE and chemokines, recruiting macrophages, dendritic cells, eosinophils, and neutrophils to the bronchoalveolar tissues where they play important roles in the pathophysiology of the disease (9–11). Despite the extensive information on the role of immune mechanisms in asthma, little is known about the metabolic requirements of the inflammatory cells.

The data demonstrated that exposure to OVA or HDM in murine asthma models led to an increased expression of FAO enzymes in inflammatory cells, particularly macrophages, infiltrating the bronchoalveolar tissues. These macrophages also expressed higher levels of arginase I but lower level of NOS2 than controls, thus indicating a phenotype of M2 macrophages. This observation is similar to that reported in *Heligmosomoides polygyrus* helminth infection where M2 macrophages also underwent a metabolic reprogramming and exhibited an increased expression of FAO enzymes (8). Inhibition of FAO in this disease resulted in an increased parasite load as a result of M2 macrophages losing their ability to control parasite proliferation. In contrast, in cancer we and others have reported that tumor-infiltrating dendritic cells and MDSC also up-regulate FAO and concomitantly develop the ability to suppress T cell proliferation and IFN γ production (6,7,24,25). However, inhibition of FAO increased the anti-tumor T cell responses and significantly improved the therapeutic efficacy of chemotherapy and adoptive cellular therapy (6,7,24,25).

The results shown here demonstrated the increased expression of FAO mediators particularly in F4/80⁺ macrophages infiltrating the lungs of mice sensitized and challenged with OVA, while granulocytes (neutrophils and eosinophils) expressed significantly lower levels. In keeping with this, activated eosinophils have previously been found to display a reduced number of mitochondria and oxidative metabolism (26). In addition to inflammatory cells, we noticed an increased expression of FAO enzymes in the brush border of bronchial cells in both control and allergic mice. Recent publications have shown an increased number of mitochondria, the organelle where FAO takes place, in the apical region of bronchial epithelial cells kept in culture (27). It is possible that this localization of mitochondria is a result of the need for ATP to power the function of bronchial cilia.

More important was the significant therapeutic effect caused by the inhibition of FAO using two different pharmacologic agents that target CPT1 and HADHA. The molecular mechanisms by which FAO inhibitors induced major therapeutic effects remain unclear. Although in vivo data showed a significant decrease in the number of inflammatory cells in the lungs, there was no increase in the apoptosis in the lungs of these mice. We found that these FAO inhibitors did not cause the apoptosis of LPS-stimulated macrophages (data not shown) or MDSC (7) in vitro. Moreover, the decrease in the inflammatory cell subpopulations appeared to be limited to the lungs since they did not change significantly in the spleens. This effect also appeared to be selective in that interstitial macrophages (F4/80⁺CD11b⁺CD11c⁻), but not alveolar macrophages (F4/80⁺CD11b⁻CD11c⁺), were decreased after FAO inhibition. Interestingly, our data showed that the depletion of circulating inflammatory monocytes, assumed progenitors of lung-recruited macrophages, attenuated the inflammatory immune response. This is consistent with a recent study showing that recruited monocytes, but not resident alveolar macrophages, could elicit allergic lung inflammation (16,21). A prior investigation also demonstrated that M2

macrophage transfer into fungus-induced allergic mice increased the inflammatory response and collagen deposition, thus promoting asthma-like traits (22). Of note, our results showed that combining the depletion of circulating inflammatory monocytes with FAO inhibition led to better protective responses compared with either treatment alone. These results suggest that FAO inhibition in macrophages as well as other inflammatory cells may contribute to the therapeutic effects of etomoxir treatment.

The rapid and significant reduction in the numbers of inflammatory cells upon FAO inhibition may in part explain the dramatic decrease in the production of cytokines and chemokines associated with the asthma-like response. However, this process again appeared to be selective since not all cytokines and chemokines tested were reduced. It is therefore likely that allergen-sensitized inflammatory myeloid cells in the inflamed tissues are highly dependent on FAO to produce ATP required to support their functions, making them highly sensitive to a sudden inhibition of FAO. In contrast, cells that are distant from this site or those that are not allergen-sensitized may not be dependent on FAO and therefore are not sensitive to FAO inhibition. The apoptosis of these cells could also be a rapid event, in which case we might not observe apoptotic cells 48 hours after treatment. Furthermore, the potential effect of FAO inhibition on other lymphoid immune cells in the allergic airway microenvironment remains to be carefully determined, especially since cells like regulatory T (Treg) cells and memory T cells use FAO as a source of energy (3,28). In fact, reports have shown that the inhibition of FAO restrains Treg cell differentiation and function (7,29,30). This is intriguing because, although Treg cells are known to control asthma (31,32), the net result of FAO inhibition appears to be a reduced allergic inflammatory response. Therefore, further investigation of the immunometabolism of these cells in the setting of allergic asthma is warranted.

The decreased OVA-specific IgE and the results on B cells are also very interesting, but the mechanisms remain unclear. The results showed that FAO inhibition reduced the numbers of B cells in the lungs and to a lesser extent in the spleens. However, recent reports showed that B cells use glycolysis to support their functions (33). In fact, inhibition of glycolysis using the pyruvate dehydrogenase kinase inhibitor dichloroacetate down-regulated B cell proliferation and antibody (IgG and IgM) secretion both in vitro and in vivo (33). However, the rapid decrease of FAO-dependent myeloid cells caused by FAO inhibitors might affect the number and function of IgE-producing B cells. Since murine IgE has an in vivo half-life of 12 hours (34) and levels of IgE in serum were measured 48 hours after treatment, this might in part explain the significant and rapid decrease in OVA-specific IgE.

With the continued rise in the incidence of asthma and the significant number of asthmatics who do not respond to existing medications (13), there is a need for new therapies. This study provides evidence that FAO inhibition may provide a novel approach to the treatment of asthma and possibly other hypersensitivity diseases. Currently, three FAO inhibitors (two inhibitors of HADHA and one inhibitor of CPT1) are approved for clinical use in the treatment of cardiovascular disease. Etomoxir, a potent inhibitor of CPT-1, was not developed for clinical use because of hepatotoxicity with chronic oral use (35); however, it may be possible that other administration routes or short-term usage may circumvent the toxicities associated with chronic use. Ranolazine, a piperazine derivative that inhibits

HADHA, is approved for the treatment of unstable angina. In conclusion, it is possible that new therapeutic approaches can be developed from understanding the distinct energy metabolism of chronic inflammatory diseases such as asthma or other immunological diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded in part by R01AI112402 to ACO; P30GM114732 to AAA and AHB (Program Director, ACO); HL072889 to AHB; and from LA CaTS Center (U54GM104940) to AAA and ACO

References

1. Ghesquiere B, Wong BW, Kuchnio A, Carmeliet P. Metabolism of stromal and immune cells in health and disease. *Nature*. 2014; 511:167–176. [PubMed: 25008522]
2. Murray PJ, Rathmell J, Pearce E. SnapShot: Immunometabolism. *Cell Metab*. 2015; 22:190. [PubMed: 26154058]
3. Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. *Immunity*. 2013; 38:633–643. [PubMed: 23601682]
4. Buck MD, O'Sullivan D, Pearce EL. T cell metabolism drives immunity. *J. Exp. Med*. 2015; 212:1345–1360. [PubMed: 26261266]
5. Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, Qiu J, Kretz O, Braas D, van der Windt GJ, Chen Q, Huang SC, O'Neill CM, Edelson BT, Pearce EJ, Sesaki H, Huber TB, Rambold AS, Pearce EL. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. *Cell*. 2016; 166:63–76. [PubMed: 27293185]
6. Al-Khami AA, Rodriguez PC, Ochoa AC. Metabolic reprogramming of myeloid-derived suppressor cells (MDSC) in cancer. *OncImmunology*. 2016:e1200771. [PubMed: 27622069]
7. Hossain F, Al-Khami AA, Wyczzechowska D, Hernandez C, Zheng L, Reiss K, Valle LD, Trillo-Tinoco J, Maj T, Zou W, Rodriguez PC, Ochoa AC. Inhibition of Fatty Acid Oxidation Modulates Immunosuppressive Functions of Myeloid-Derived Suppressor Cells and Enhances Cancer Therapies. *Cancer Immunol. Res*. 2015; 3:1236–1247. [PubMed: 26025381]
8. Huang SC, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, Beatty W, Love-Gregory L, Lam WY, O'Neill CM, Yan C, Du H, Abumrad NA, Urban JF Jr, Artyomov MN, Pearce EL, Pearce EJ. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat. Immunol*. 2014; 15:846–855. [PubMed: 25086775]
9. Lambrecht BN, Hammad H. The immunology of asthma. *Nat. Immunol*. 2015; 16:45–56. [PubMed: 25521684]
10. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. *Nat. Rev. Immunol*. 2013; 13:9–22. [PubMed: 23154224]
11. Zaynagetdinov R, Sherrill TP, Kendall PL, Segal BH, Weller KP, Tighe RM, Blackwell TS. Identification of myeloid cell subsets in murine lungs using flow cytometry. *Am. J. Respir. Cell Mol. Biol*. 2013; 49:180–189. [PubMed: 23492192]
12. Bosnjak B, Stelzmueller B, Erb KJ, Epstein MM. Treatment of allergic asthma: modulation of Th2 cells and their responses. *Respir. Res*. 2011; 12:114. [PubMed: 21867534]
13. Holgate ST, Polosa R. Treatment strategies for allergy and asthma. *Nat. Rev. Immunol*. 2008; 8:218–230. [PubMed: 18274559]
14. Ghonim MA, Pyakurel K, Ju J, Rodriguez PC, Lammi MR, Davis C, Abughazleh MQ, Mansy MS, Naura AS, Boulares AH. DNA-dependent protein kinase inhibition blocks asthma in mice and modulates human endothelial and CD4(+) T-cell function without causing severe combined immunodeficiency. *J. Allergy Clin. Immunol*. 2015; 135:425–440. [PubMed: 25441643]

15. Ghonim MA, Pyakurel K, Ibba SV, Al-Khami AA, Wang J, Rodriguez P, Rady HF, El-Bahrawy AH, Lammi MR, Mansy MS, Al-Ghareeb K, Ramsay A, Ochoa A, Naura AS, Boulares AH. PARP inhibition by olaparib or gene knockout blocks asthma-like manifestation in mice by modulating CD4(+) T cell function. *J. Transl. Med.* 2015; 13:225. [PubMed: 26169874]
16. Zaslona Z, Przybranowski S, Wilke C, van RN, Teitz-Tennenbaum S, Osterholzer JJ, Wilkinson JE, Moore BB, Peters-Golden M. Resident alveolar macrophages suppress, whereas recruited monocytes promote, allergic lung inflammation in murine models of asthma. *J. Immunol.* 2014; 193:4245–4253. [PubMed: 25225663]
17. Ghonim MA, Pyakurel K, Ibba SV, Wang J, Rodriguez P, Al-Khami AA, Lammi MR, Kim H, Zea AH, Davis C, Okpechi S, Wyczzechowska D, Al-Ghareeb K, Mansy MS, Ochoa A, Naura AS, Boulares AH. PARP is activated in human asthma and its inhibition by olaparib blocks house dust mite-induced disease in mice. *Clin. Sci. (Lond).* 2015; 129:951–962. [PubMed: 26205779]
18. Naura AS, Kim H, Ju J, Rodriguez PC, Jordan J, Catling AD, Rezk BM, bd Elmageed ZY, Pyakurel K, Tarhuni AF, Abughazleh MQ, Errami Y, Zerfaoui M, Ochoa AC, Boulares AH. Minocycline blocks asthma-associated inflammation in part by interfering with the T cell receptor-nuclear factor kappaB-GATA-3-IL-4 axis without a prominent effect on poly(ADP-ribose) polymerase. *J. Biol. Chem.* 2013; 288:1458–1468. [PubMed: 23184953]
19. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit Care Med.* 1997; 156:766–775. [PubMed: 9309991]
20. Mehrotra P, Hollenbeck A, Riley JP, Li F, Patel RJ, Akhtar N, Goenka S. Poly (ADP-ribose) polymerase 14 and its enzyme activity regulates T(H)2 differentiation and allergic airway disease. *J. Allergy Clin. Immunol.* 2013; 131:521–531. [PubMed: 22841009]
21. Jiang Z, Zhu L. Update on the role of alternatively activated macrophages in asthma. *J. Asthma Allergy.* 2016; 9:101–107. [PubMed: 27350756]
22. Moreira AP, Cavassani KA, Hullinger R, Rosada RS, Fong DJ, Murray L, Hesson DP, Hogaboam CM. Serum amyloid P attenuates M2 macrophage activation and protects against fungal spore-induced allergic airway disease. *J. Allergy Clin. Immunol.* 2010; 126:712–721. [PubMed: 20673988]
23. Vats D, Mukundan L, Odegaard JI, Zhang L, Smith KL, Morel CR, Wagner RA, Greaves DR, Murray PJ, Chawla A. Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. *Cell Metab.* 2006; 4:13–24. [PubMed: 16814729]
24. Herber DL, Cao W, Nefedova Y, Novitskiy SV, Nagaraj S, Tyurin VA, Corzo A, Cho HI, Celis E, Lennox B, Knight SC, Padhya T, McCaffrey TV, McCaffrey JC, Antonia S, Fishman M, Ferris RL, Kagan VE, Gabrilovich DI. Lipid accumulation and dendritic cell dysfunction in cancer. *Nat. Med.* 2010; 16:880–886. [PubMed: 20622859]
25. Cubillos-Ruiz JR, Silberman PC, Rutkowski MR, Chopra S, Perales-Puchalt A, Song M, Zhang S, Bettigole SE, Gupta D, Holcomb K, Ellenson LH, Caputo T, Lee AH, Conejo-Garcia JR, Glimcher LH. ER Stress Sensor XBP1 Controls Anti-tumor Immunity by Disrupting Dendritic Cell Homeostasis. *Cell.* 2015; 161:1527–1538. [PubMed: 26073941]
26. Peachman KK, Lyles DS, Bass DA. Mitochondria in eosinophils: functional role in apoptosis but not respiration. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98:1717–1722. [PubMed: 11172017]
27. Xu W, Janocha AJ, Leahy RA, Klatte R, Dudzinski D, Mavrikis LA, Comhair SA, Lauer ME, Cotton CU, Erzurum SC. A novel method for pulmonary research: assessment of bioenergetic function at the air-liquid interface. *Redox. Biol.* 2014; 2:513–519. [PubMed: 24624341]
28. Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. *Science.* 2013; 342:1242454. [PubMed: 24115444]
29. Gerriets VA, Kishton RJ, Nichols AG, Macintyre AN, Inoue M, Ilkayeva O, Winter PS, Liu X, Priyadharshini B, Slawinska ME, Haeblerli L, Huck C, Turka LA, Wood KC, Hale LP, Smith PA, Schneider MA, MacIver NJ, Locasale JW, Newgard CB, Shinohara ML, Rathmell JC. Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *J. Clin. Invest.* 2015; 125:194–207. [PubMed: 25437876]
30. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, Sullivan SA, Nichols AG, Rathmell JC. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs

- are essential for effector and regulatory CD4⁺ T cell subsets. *J. Immunol.* 2011; 186:3299–3303. [PubMed: 21317389]
31. Jin HS, Park Y, Elly C, Liu YC. Itch expression by Treg cells controls Th2 inflammatory responses. *J. Clin. Invest.* 2013; 123:4923–4934. [PubMed: 24135136]
32. Ulges A, Klein M, Reuter S, Gerlitzki B, Hoffmann M, Grebe N, Staudt V, Stergiou N, Bohn T, Bruhl TJ, Muth S, Yurugi H, Rajalingam K, Bellinghausen I, Tuettenberg A, Hahn S, Reissig S, Haben I, Zipp F, Waisman A, Probst HC, Beilhack A, Buchou T, Filhol-Cochet O, Boldyreff B, Breloer M, Jonuleit H, Schild H, Schmitt E, Bopp T. Protein kinase CK2 enables regulatory T cells to suppress excessive TH2 responses in vivo. *Nat. Immunol.* 2015; 16:267–275. [PubMed: 25599562]
33. Caro-Maldonado A, Wang R, Nichols AG, Kuraoka M, Milasta S, Sun LD, Gavin AL, Abel ED, Kelsoe G, Green DR, Rathmell JC. Metabolic reprogramming is required for antibody production that is suppressed in anergic but exaggerated in chronically BAFF-exposed B cells. *J. Immunol.* 2014; 192:3626–3636. [PubMed: 24616478]
34. Vieira P, Rajewsky K. The half-lives of serum immunoglobulins in adult mice. *Eur. J. Immunol.* 1988; 18:313–316. [PubMed: 3350037]
35. Vickers AE. Characterization of hepatic mitochondrial injury induced by fatty acid oxidation inhibitors. *Toxicol. Pathol.* 2009; 37:78–88. [PubMed: 19234235]

Figure 1. Expression of FAO enzymes in the bronchopulmonary tissue of allergen-exposed mice (A) A model for OVA-induced asthma-like traits in mice. (B) Upper panel Shows H&E staining in lung sections from normal or OVA-exposed mice. Middle panel shows immunohistochemistry for CPT1. Lower panel shows immunohistochemistry for HADHA. The bar graphs (left panels) indicate the quantification of the number of positive cells in five 40X fields. (C) A model for HDM-induced asthma-like traits in mice. (D) Upper panel Shows H&E staining in lung sections from normal or HDM-exposed mice. Middle panel shows immunohistochemistry for CPT1. Lower panel shows immunohistochemistry for

HADHA. The bar graphs (left panels) also show the number of positive cells counted in five 40X fields. Data = mean $\pm$ SEM; n = 3 mice/group; **, $P < 0.01$; ****, $P < 0.0001$.

Figure 2. Expression of FAO enzymes in immune cells infiltrating the bronchopulmonary tissue of OVA-exposed mice

(A) Double Immunofluorescence labeling in lung sections from OVA-exposed mice using CPT1 and F4/80 (macrophages), Ly-6G (neutrophils), Siglec-F (eosinophils), or CD45R (B cells). The bar graph (lower panel) shows the quantification of the percentages of CPT1 positive myeloid cells (merge) among CPT1-expressing inflammatory cells (far left), counted in three 200X fields. (B) Double Immunofluorescence labeling in lung sections from OVA-exposed mice using HADHA and F4/80 (macrophages), Ly-6G (neutrophils), Siglec-F (eosinophils), or CD45R (B cells). The bar graph (lower panel) shows the

percentages of HADHA positive myeloid cells (merge) among HADHA-expressing inflammatory cells (far left), counted in three 200X fields. Data = mean $\pm$ SEM; n = 3 mice/group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Effect of FAO inhibition on AHR and IgE production in OVA-exposed mice

(A) A model for the treatment of OVA-induced asthma-like traits in mice. (B) One day after OVA challenge and treatment with etomoxir, AHR to inhaled methacholine was measured in unrestrained conscious C57BL/6J mice. (C) The experiment was repeated as in (B) except that mice were treated with ranolazine. AHR was measured one day after challenge and treatment. (D) The OVA sensitization and challenge followed by treatment with etomoxir or ranolazine were conducted on Balb/c mice. AHR was measured one day after allergen exposure and treatment. (E) Two days post OVA challenge and treatment with etomoxir in C57BL/6J mice, BAL fluids and sera were collected to measure the levels of OVA-specific IgE. (F) The levels of total IgE were measured in sera of the indicated conditions. Data = mean $\pm$ SEM; n = 5 mice/group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Figure 4. FAO inhibition reduces inflammation and CPT1 expression without induction of apoptosis

Lungs were harvested two days after OVA challenge and treatment with or without etomoxir. Normal lung tissues were included as a control. (A) H&E staining in lung sections. (B) Immunohistochemistry for CPT1. (C) Lungs sections were examined for apoptosis by TUNEL assay. A brain tumor tissue was stained as a positive control. Data represents 3 mice/group.

Figure 5. Effect of FAO inhibition on the frequency of inflammatory immune cells in lungs and spleens

Two days after OVA challenge and treatment with or without etomoxir, the indicated organs were harvested. (A) BAL cells were collected and stained to quantify the total number of cells and different subpopulations, including eosinophils, macrophages, and lymphocytes. (B) Whole lungs were removed and digested into single cell suspensions to determine by flow cytometry the percentages of Siglec-F⁺ eosinophils, F4/80⁺CD11b⁺CD11c⁻ interstitial macrophages, F4/80⁺CD11b⁻CD11c⁺ alveolar macrophages, CD11c⁺MHC-II⁺ dendritic cells, CD4⁺ T cells, and CD45R⁺ B cells. (C) Spleens were also harvested and the

subpopulations were determined by flow cytometry. Data = mean $\pm$ SEM; n = 5 mice/group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

(a) BAL fluid **(b) Serum**

Figure 6. Effect of FAO inhibition on cytokines and chemokines linked to OVA-induced asthma-like traits

The levels of the indicated cytokines and chemokines were determined in BAL fluids (A) and sera (B) collected from mice sacrificed on day two of OVA challenge and etomoxir treatment. Data = mean $\pm$ SEM; n = 5 mice/group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Figure 7. FAO inhibition decreases the frequency of macrophages expressing FAO enzymes (A) Double immunofluorescence labeling with CPT1 and F4/80 (macrophages) in lung sections from OVA-exposed mice treated with or without etomoxir. Normal lungs were included. Data represents 3 mice/group. (B and C) F4/80⁺ macrophages were sorted from the lungs of OVA-exposed mice treated with or without etomoxir. Macrophages sorted from normal lungs were used as a control. Quantitative RT-PCR analysis of (B) the FAO-associated enzymes CPT1, HADHA, ACADM, and PGC1 β and (C) the macrophage-linked markers arginase I and NOS2 was conducted on the purified macrophages. (D–E) Mice were

OVA-sensitized and challenged and then treated with etomoxir as outlined in Figure 3A except that the indicated groups were intravenously injected with 0.2 ml liposomal clodronate (CL) 8 hours before challenge. Two days after OVA challenge and treatments, organs were harvested. (D) BAL cells were collected and stained to enumerate the total number of cells, eosinophils, macrophages, and lymphocytes. (E) The concentrations of serum IL-5 were measured. Data in (B–E) = mean $\pm$ SEM; n = 5 mice/group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Figure 8. FAO inhibition restrains HDM-induced allergic airway inflammation

(A) A model for the treatment of HDM-induced asthma-like traits in mice. (B) One day after the last HDM challenge and etomoxir treatment, AHR to inhaled methacholine was measured in unrestrained conscious C57BL/6J mice. (C) Two days after the last HDM challenge and etomoxir treatment, BAL cells were collected and stained to quantify the total number of cells and immune cells, including eosinophils, macrophages, and lymphocytes.

(D) The levels of IL-5 were determined in sera. Data = mean $\pm$ SEM; n = 5 mice/group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript