PULMONARY INFLAMMATION & INJURY IN A MOUSE MODEL OF NON-ALCOHOLIC STEATOHEPATITIS

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✷ ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver condition that affects millions of individuals in the United States, of which approximately twenty percent of cases progress to non-alcoholic steatohepatitis (NASH). NASH is characterized by macrovascular steatosis and persistent inflammation in the liver, which can lead to fibrosis. Evidence suggests potential effects of NAFLD and NASH on the development of pulmonary pathologies, but the interaction between the liver and the lung is not well understood. In this study, we assessed the impact of NASH development on lung inflammation and fibrosis over time. Male C57BL/6J mice were fed control (10% kCal) or high-fat (HFD) (60% kCal) diets. Liver tissue, lung tissue, and bronchoalveolar lavage (BAL) fluid were collected after 1, 3, and 6 months of feeding. Histopathologic evaluation of livers from HFD-fed mice at 6 months confirmed the development of NASH. In the lung, we observed histopathologic alterations, including inflammatory cell infiltration, lipid-laden macrophages, septal damage, and epithelial thickening at 6 months. Gene expression analysis of whole lung tissue revealed changes in genes related to inflammation (IL-1B), fibrosis (CTGF), and lipid metabolism (ApoA1). These results characterize an association of pulmonary complications during simple steatosis to NASH transition, suggesting lung-liver crosstalk.

1 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver condition that is estimated to affect upwards of thirty percent of individuals in the United States, with even more at risk due to the rising obesity epidemic.[3,5,19] NAFLD is characterized by the accumulation of fat in the liver, known as steatosis. It is estimated that twenty percent of patients diagnosed with NAFLD progress to non-alcoholic steatohepatitis (NASH).[21] NASH is a severe, chronic liver disease characterized by persistent inflammation and immune cell infiltration. NASH may also progress to fibrosis and cirrhosis of the liver–this irreversible scarring of tissue can further lead to uncontrolled cell growth, cancer, and death. NASH is becoming the leading indication for liver transplantation in both the U.S. and worldwide.[2,14,16]

Both NAFLD and NASH are associated with systemic effects that manifest in pathologies across the body, including cardiovascular disease, metabolic syndrome, and chronic kidney conditions.[1] Emerging evidence also suggests that NAFLD and NASH may impact the development of pathologies in the lung. Several longitudinal observational studies have detailed an association between NAFLD and decreased measurements of lung function (e.g. forced expiratory volume and vital capacity).[7,11,12,15] In addition, patients with chronic obstructive pulmonary disease (COPD) showed increased incidence of both NAFLD and NASH.[20] Although there is rising clinical evidence of a relationship between NASH and reduced pulmonary function, the interplay between the liver and the lung remains largely unexplored.

A central aspect of NAFLD and NASH that may contribute to lung injury is inflammation. Key players in this response are macrophages, phagocytic cells of the innate immune system. In the liver, macrophages are known to take up surrounding fat through endocytosis. This causes the macrophages to become activated and release pro-inflammatory mediators such as cytokines, small proteins important in inflammatory cell signaling.[14] These mediators enter the bloodstream and can exert effects in other tissues of the body through systemic circu-

lation, leading to observed comorbidities.[1,9,14] We hypothesize that these inflammatory mediators accumulate in the lung, causing pulmonary injury, inflammation, and the disruption of key signaling pathways by dysregulating genes related to lipid metabolism and inflammation, including *Il-1B, Ctgf, Lxr, ApoA1, and Abca1*. The present study was designed to test this hypothesis and characterize the development of lung injury in a high fat diet mouse model of NASH. The results of our study provide data on lung-liver crosstalk, which may be useful for the development of new approaches for clinical management of pulmonary pathology related to NASH.

2 METHODOLOGY

ANIMALS & TREATMENTS

Wild type male C57BL/6J mice (6-8 weeks, $n = 5-9$/group) were fed control (10% kCal) or a high-fat diet (HFD) (60% kCal) for 1, 3, and 6 months. Food consumption was monitored weekly. Animal protocols were approved by Rutgers University IACUC.

HISTOLOGICAL ANALYSIS

Liver and lung tissue were collected, fixed in 10% formalin or inflated and fixed in 4% paraformaldehyde, respectively, and cut into 5-µm sections and stained with hematoxylin & eosin (H&E). Lung sections were evaluated for characteristics of lung injury and inflammation, including inflammatory cell infiltration, septal damage, and epithelial thickening. Liver sections were examined for the histopathological characteristics of NAFLD (e.g. fat accumulation) and NASH (e.g. fat accumulation and inflammatory cell infiltration) based on established criteria.[2,16]

BRONCHOALVEOLAR LAVAGE (BAL) CELL AND PROTEIN MEASUREMENT

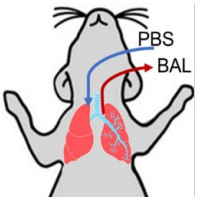

FIGURE 1

BAL fluid was collected by slowly instilling and withdrawing 1 mL of ice-cold (4°C) PBS into the lungs of mice through a cannula in the trachea (FIGURE 1). This fluid was centrifuged at 300xg for 8 minutes. Cell pellets were resuspended in 1 mL of PBS. Viable cells (10 µl) were counted on a hemocytometer using trypan blue dye exclusion. Cytospins were prepared by centrifugation of 10^4 cells BAL fluid onto microscope slides using a Shandon cytospin (Thermo Scientific). Cells were fixed in methanol and stained with Giemsa to visualize BAL cell populations. Total protein content in cell free BAL was quantified using a BCA protein kit (Pierce Biotechnologies Inc.) with bovine serum albumin as the standard. All samples were assayed in triplicate at 562 nm using a spectrophotometer.

MRNA ISOLATION AND RT-QPCR ANALYSIS

Total RNA was extracted from lung tissue using TRIzol™ Reagent and bead TissueLyser LT (Qiagen). cDNA was generated using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time quantitative PCR (RT-qPCR) was performed on a QuantStudio 6 system using commercially available Power SYBR® Green gene expression assays (Applied Biosystems). Data were normalized to β-actin and presented as fold change relative to 1 month CTRL mice. Fold changes in gene expression were calculated using $\Delta\Delta Ct$ method where $Ct(target\ gene) - Ct(\beta - act) = \Delta Ct$; $\Delta Ct - average\ \Delta Ct(1\ Month\ CTRL) = \Delta\Delta Ct$; and $2 - \Delta\Delta Ct = fold\ change$.

STATISTICAL ANALYSIS

Data are presented as mean + SE and were analyzed using 2-way ANOVA and Sidak's multiple comparisons test. A p-value ≤0.05 was considered statistically significant.

3 RESULTS

HIGH-FAT DIET INDUCES NASH AND CAUSES HISTOPATHOLOGICAL CHANGES IN THE LUNG

To confirm the development of NASH, we assessed histopathological changes in the liver. Livers from mice fed a HFD for 1 and 3 months exhibited steatosis, or the accumulation of lipid droplets in the liver, but no inflammatory cell infiltration, indicating NAFLD (data not shown). The most prominent changes in the liver were observed 6 months following consumption of a HFD; these included more severe steatosis and infiltration of inflammatory cells

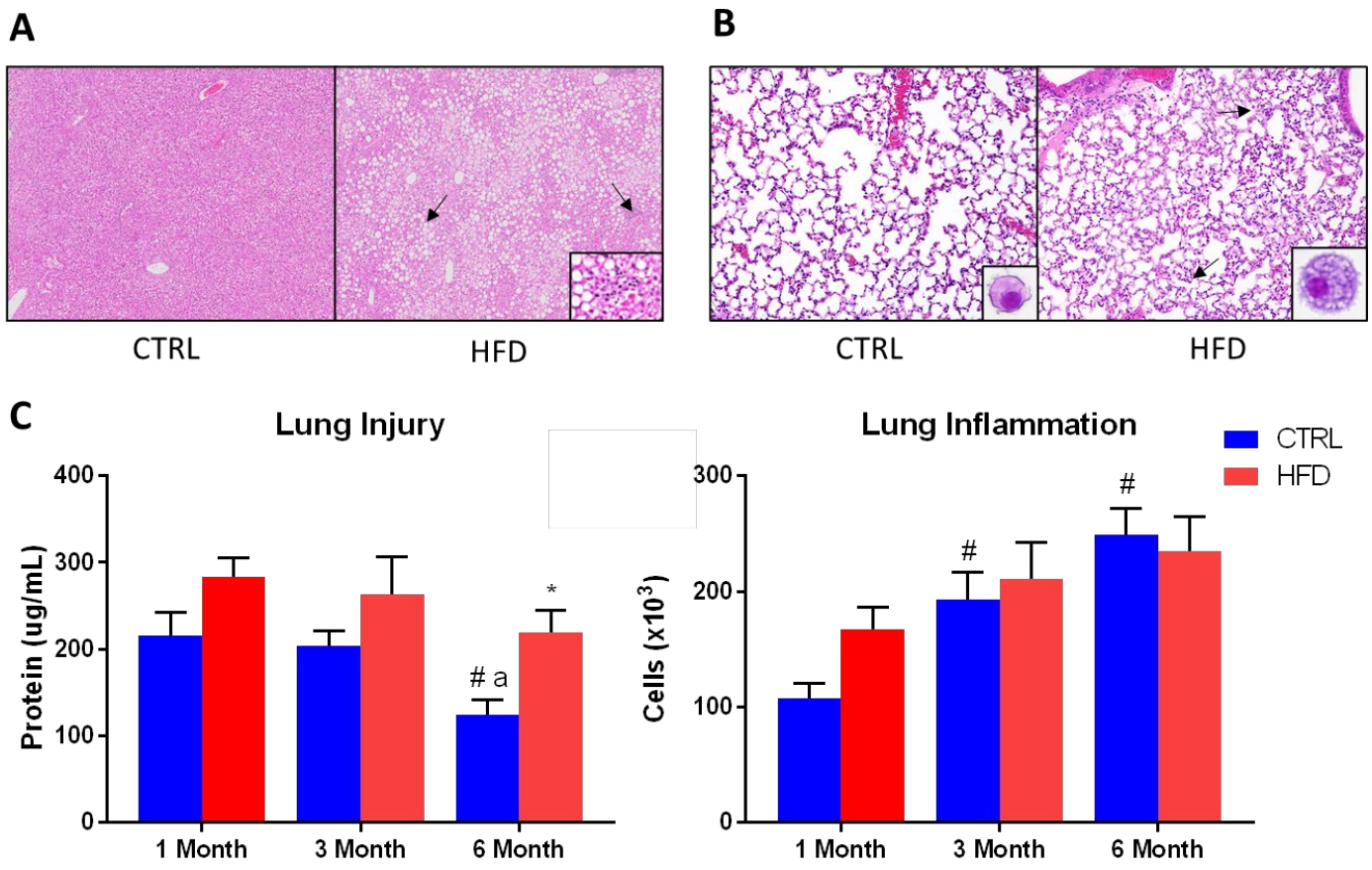

FIGURE 2: *Representative images of H&E stained sections of liver (PANEL A) and lung (PANEL B) from mice fed control (CTRL) or high fat diets (HFD) for 6 months. PANEL A, left arrow indicates steatosis, right arrow and inset indicates inflammation. PANEL B, top arrow indicates inflammatory cell infiltration and bottom arrow indicates epithelial thickening. PANEL B insets highlight representative macrophages from Giemsa-stained cytopsins, including macrophages with a large, lipid-laden appearance in HFD-fed mice. Original magnification (A) 4x or (B) 20x, inserts 40x. (C) BAL collected from mice 1, 3, and 6 months after a control (CTRL) or high fat diet (HFD) was assessed for protein and cell content. Bars, mean + SE (n=5-10).*

**Significantly different from CTRL fed mice.*

#Significantly different from 1 month.

aSignificantly different from 3 month.

(FIGURE 2A). Significant increases in body weights, increases in total serum cholesterol, and decreased glucose tolerance in HFD-fed mice confirmed this was metabolic syndrome-related NASH (data not shown). We next assessed alterations in lung histology. After 6 months, inflammatory cell infiltration, septal damage, and epithelial thickening were observed in HFD-fed mice relative to mice fed the control diet (FIGURE 2B). The accumulation of large macrophages in the lung that appeared lipid-laden was also noted in the histology. Further examination of cell cytospins also revealed the presence of large, vacuolated, and potentially lipid-laden macrophages (FIGURE 2B inserts). We further investigated lung injury and inflammation by quantifying levels of BAL protein and cells, respectively (FIGURE 2C). Alt-

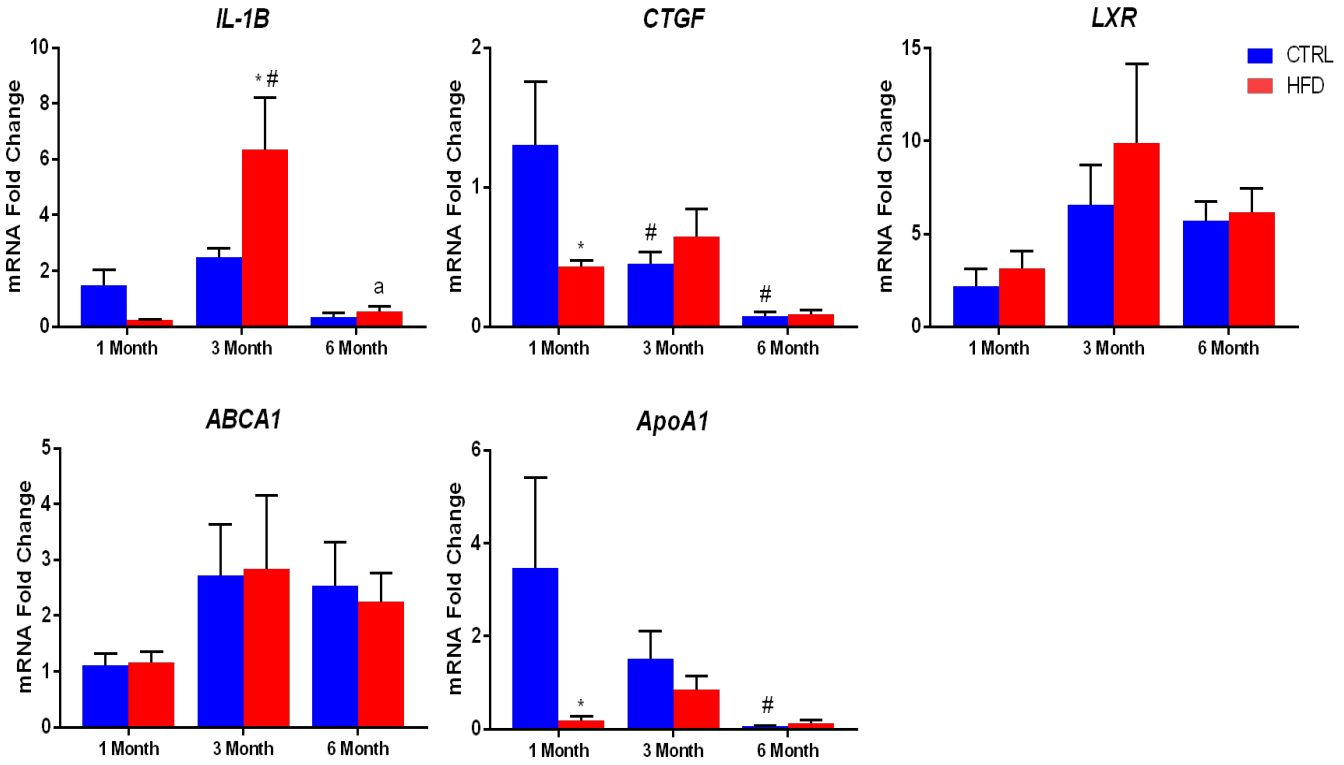

FIGURE 3: *Lung tissue collected 1, 3, and 6 months after control (CTRL) or high fat diet (HFD) from mice were analyzed for gene expression by RT-qPCR. Data were normalized relative to ß-actin and presented as fold change relative to 1 Month CTRL fed mice. Bars, mean + SE (n=3-7).*

**Significantly different from CTRL fed mice.*

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hough increased lung injury was noted in HFD-fed mice at 6 months as measured by BAL protein, this may be due to a reduction in BAL protein in control mice at this time. Surprisingly, a time related increase in lung inflammation was observed in control mice at 3 and 6 months. Cell counts were unaffected by administration of the HFD.

HIGH FAT DIET DISRUPTS EXPRESSION OF INFLAMMATORY AND LIPID METABOLISM RELATED GENES

To better understand degrees of inflammatory changes in the lung following a HFD, we analyzed expression of inflammatory and lipid-related genes. Expression of interleukin 1 beta (*Il-1b*), a key early response proinflammatory gene, was significantly increased in mice fed a HFD when compared to con-

trol mice at 3 months. *Il-1b* expression returned to control levels by 6 months. The expression of connective tissue growth factor (*Ctgf*), a gene indicative of fibrosis and tissue remodeling, was significantly decreased 1 month following HFD-feeding. Interestingly, control mice displayed a significant decrease in *Ctgf* at 3 and 6 months when compared to 1 month. Similarly, expression of apolipoprotein A1 (*Apoa1*), a lipid chaperone, was decreased 1 month following HFD when compared to control-fed mice. ApoA1 expression was similarly reduced in the 6-month control-fed mice when compared to 1-month control mice. There were no changes in expression of liver X receptor (*Lxr)*, a nuclear receptor involved in lipid homeostasis, and its target ATP-binding cassette transporter 1 (*Abca1)*, a lipid transporter.

4 DISCUSSION

The inflammatory and fibrogenic effects of NASH in the liver have been well characterized.[2,14] Emerging clinical evidence suggests that NAFLD and NASH are associated with pulmonary injury, but crosstalk between the lung and the liver in NASH has not been investigated.[7,11,12,15] In these studies, we used a mouse model of HFD to induce NASH and investigate associated injury and inflammation in the lung. We found that NASH was associated with histopathological alterations in lung tissue 6 months post HFD feeding; moreover, expression of genes related to inflammation and lipid metabolism was dysregulated throughout NAFLD development, including the progression to NASH. These results provide insights into the interplay between liver and lung inflammation and highlight potential inflammatory pathways for crosstalk between the tissues.

Based on established criteria, we confirmed the development of NASH in mice fed a HFD as we observed increased steatosis and inflammatory cell infiltration at 6 months.[2,16] This was correlated with pulmonary histopathological changes in the HFD-fed mice at this time. Further assessment by a pathologist will be completed to confirm these histopathological changes. Although there was no evidence of pulmonary fibrosis in these animals, we noted the appearance of lipid-laden macrophages in the lung. These cells have been shown to be associated with fibrosis in other disease states, and may contribute to the development of lung fibrosis at later time points in NASH.[18] Interestingly, histopathological changes in the lung were not reflected by increases in BAL protein or cell counts, which are markers of pulmonary alveolar epithelial damage and leaky vasculature, or infiltration of immune cells into the lung in response to injury.[17] It may be that NASH is not associated with epithelial barrier dysfunction and that pathologic alterations involve other mechanisms of injury. For example, it is possible that resident macrophages present in the lung are activated following HFD and that they drive lung inflammation. Further studies are needed to explore this possibility. We also noted a decrease in BAL protein content at 6 months and increases in BAL cells in control mice; this may be indicative of age-related changes in tissue structure or vasculature, or in basal inflammatory activity.[4] HFD-fed mice seem to also mimic this age-related trend of increasing inflammation, although not significantly.

We speculated that histopathological changes in the lung of animals fed a HFD might be driven by differential expression of genes related to inflammatory proteins and cytokines. In this context, our gene expression analyses revealed increases in *Il-1b* at 3 months in HFD-fed mice, which suggests that during the development of NASH, the lung responds to hepatic inflammation by upregulating inflammatory gene expression. IL-1β is an early response cytokine known to promote inflammation; thus, increases in inflammatory cells in the lung of mice fed a HFD may be mediated in part by this cytokine. We also observed early downregulation of *Apoa1* in HFD-fed mice at 1 month. ApoA1 is a lipid chaperone that promotes the efflux of cholesterol; it has been shown to have anti-inflammatory and anti-fibrotic effects in the lung.[6,8] The observed decrease in ApoA1 may further exacerbate inflammation in the lung in response to a HFD. Although we did not observe lung fibrosis during the histopathological analysis, we assessed changes in CTGF gene expression as a measure of fibrotic extracellular matrix tissue remodeling.[10,13] *Ctgf* expression was decreased in HFD-fed mice at 1 month, suggesting that a HFD may suppress fibrotic mechanisms in the lung early

in the process of NASH development. This might be a compensatory response to prevent fibrosis induced by other growth factors generated in the lung in response to the HFD. A similar decrease in *Ctgf* expression at 3 and 6 months in the control mice indicates that the HFD may be mimicking age-related effects in mice as early as 1 month.

While these data provide preliminary characterization of pulmonary changes in a mouse model of NASH, experiments to confirm the presence of lipid-laden macrophages by staining for lipids as well as an assessment of histopathological changes by a pathologist will be conducted. Future studies will also be performed to further elucidate mechanisms of high fat diet-associated effects on the lung, including assessment of systemic markers of lipid dysregulation, liver inflammation and dysfunction, and other inflammatory signaling pathways in the lung. Developing our understanding of the interplay between the lung and the liver can help identify the mechanisms by which disease can influence distant pathologies, and how inflammation in particular can be controlled to limit pathological comorbidities in patients.

5 Conclusion

Overall, these data demonstrate that HFD-induced NASH leads to pulmonary histopathological changes. Moreover, these changes may be driven by the dysregulation of key mediators involved in inflammation and lipid metabolism. This analysis of lung-liver crosstalk in NASH highlights potential for the clinical management of pulmonary complications associated with NASH■

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