Qualitative Histopathological Analysis of Bleomycin-Induced Lung Injury

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Recent evidence has suggested that the accumulation of cholesterol-laden "fatty macrophages" in the lung may play a key role in the presentation of ALI 1, a condition that affects nearly 200,000 people per year in the United States. Studying the pathways of these cells suggests that preventing the formation and build-up of these macrophages could prevent many of the characteristic symptoms of acute lung injury. Specifically, this experiment aims to explore the effect that ACAT1 inhibition by K604 has on fatty macrophage formation and outcomes of ALI. To test this, different groups of mice were administered either PBS (as control), ITB (intratracheal bleomycin), PBS + K604, or ITB + K604. After the mice were sacrificed, the left lung lobes were extracted, inflated, embedded, and sectioned for microscopic scanning and analysis with the ImageJ software. The lung tissue was characterized using 3 unbiased parameters: alveolar wall thickness (μm), cell infiltration (number of nuclei), and percent white space. Simultaneously, a cholesterol assay was performed using cells extracted from mouse bronchoalveolar lavage (BAL). The results show that K604 was successful at preventing ITB-induced increases in cell infiltration, consolidation, and alveolar thickening as well as lipid accumulation in macrophages by limiting the formation of cholesterol esters through ACAT1 inhibition. In this respect, this experiment shows that K604 might be a viable pharmaceutical target in the treatment of ALI.

Introduction

Acute lung injury (ALI) is a condition primarily characterized by inflammation, thickening of alveolar walls, and immune cell invasion. Currently, options for pharmaceutical treatments for acute lung injury are scarce 2, and most could be categorized as "physical" (such as mechanical ventilation or fluid management). Unfortunately, these treatments often have their own drawbacks that could lead to further deterioration in patients, so more focus is currently being placed on discovering more viable and long-term pharmaceutical solutions.

Many of the symptoms found during ALI are primarily driven by macrophages, as errors in macrophage activation or function can exacerbate the damages brought upon by ALI. One of the most pressing issues revolves around the dysregulation of lipid homeostasis [1] in macrophages. Under normal circumstances, cholesterol is excreted from macrophages and out of the cells through active transport, but with ALI, the macrophages are overwhelmed, leading to an accumulation of what is often termed "fatty macrophages" 1. The build-up of these macrophages results in more lipid and cholesterol accumulation in the cells, causing further damage to the lung.

More specifically, when cholesterol levels in macrophages are high, increased esterification occurs in the cell with the help of the mitochondrial enzyme ACAT1 (acetyl-CoA acetyltransferase 1), leading to a conversion from cholesterol to cholesterol esters 1. This process is regarded as integral to lipid droplet formation in the cells and contributes to the formation of fatty macrophages. However, it was discovered that K604 (a specific acyl-CoA:cholesterol acyltransferase 1 inhibitor) could prevent ACAT1 from functioning by acting as a competitive inhibitor of the mitochondrial enzyme 3, and in turn, halt the formation of cholesterol esters 4. Since it is strongly believed that the presence of fatty macrophages is a major contributing factor behind injury in ALI and that the accumulation of these macrophages is a direct result of the creation of cholesterol esters 5, this experiment attempted to explore the extent of the effectiveness of inhibiting ACAT1 through an unbiased method in assessing the degree of lung injury driven by cholesterol-laden macrophages in ALI.

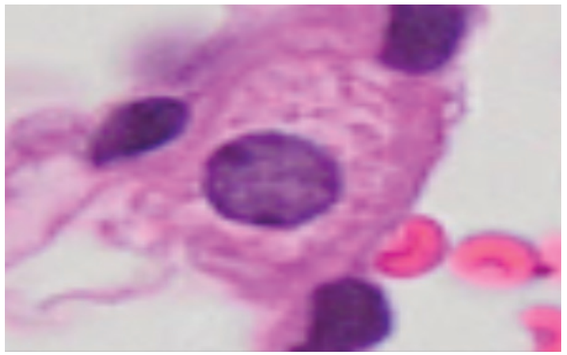

Fig. 1: "Fatty" macrophages with great lipid and cholesterol accumulation. (represented by light purple buildup)

Healthy and fully functioning macrophages are essential to effective injury response and repair during ALI. Macrophages are considered the "first line of defense" against pathogens and release substances into the lungs that will help give rise to a stronger inflammatory response. In this experiment, intratracheal-bleomycin was administered directly to the mice to simulate acute lung injury; specifically, ITB causes the mice to exhibit some main features of ALI that are studied in this experiment, including an increase in neutrophils in BALF (50-60%), pulmonary edema, and lung pathology6. K604 was also similarly administered directly through the lungs.

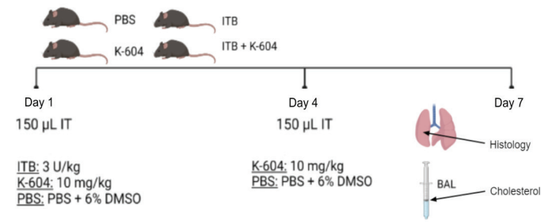

Fig. 2: Timeline of experimental design. Histology and cholesterol occur on Day 7.

Methods

Animal Use

Both male and female wild-type C57BL6/J mice (6-8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed in groups of 4 per cage (separated by sex) under standard conditions; they were provided food and water ad libitum and received humane care in compliance with Rutgers University IACUC-approved protocols adhering to the NIH guidelines for the care and use of laboratory animals 7. Equal numbers of male and female mice were used, even though males are traditionally more susceptible to ALI and its symptoms 8. The mice were treated with either PBS, intratracheal bleomycin, K604, or both ITB and K604 throughout the experiment; all were sacrificed seven days after initial intratracheal administration of K604 or bleomycin (Santa Cruz, Cat# sc-200134B).

K604 and Bleomycin Administration

The mice were first anesthetized with isoflurane and received a single intratracheal administration of either 50 uL PBS, 10 mg/kg K604, 3 U/kg bleomycin, or 10 mg/kg K604 + 3U/kg bleomycin. All volumes were brought up to 150 uL with 6% DMSO (dimethyl sulfoxide). The mice were observed to fully ensure that the complete dose was correctly administered and that the mice recovered from the anesthesia. 72 hours later, the mice were re-anesthetized and administered 50 uL PBS or 10 mg/kg K604 (intratracheally).

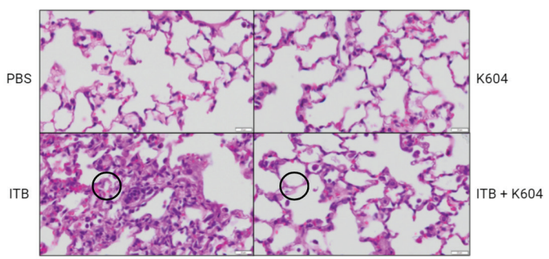

Fig. 3: Representative histopathological images from each condition, based on the average of 10 areas. Images were captured from both males and females from all four groups. The healthiness of the lung was determined by 3 parameters: alveolar wall thickness, cell infiltration (number of nuclei), and percent white space. The bottom right analysis shows a significantly healthier condition as the ITB-treated mice were administered the K604 treatment: on the other hand, as seen in the bottom left, all 3 parameters point towards unhealthy ALI symptoms.

The mice were sacrificed seven days after initial intratracheal administration.

BAL Cell Cholesterol Quantitation

After sacrificing the mice, 10,000 to 20,000 cells were extracted from the BAL fluid for cholesterol analysis. The concentration of total cholesterol, free cholesterol, and cholesterol esters in the BAL cell samples were all measured. A bioluminescent Cholesterol/Cholesterol Ester-Glo™ Assay (Promega, Madison, WI) was utilized and luminescence was recorded on a SpectraMax®M2 multi-mode microplate reader (Molecular Devices, San Jose, CA) utilizing SoftMax Pro software v5.3.

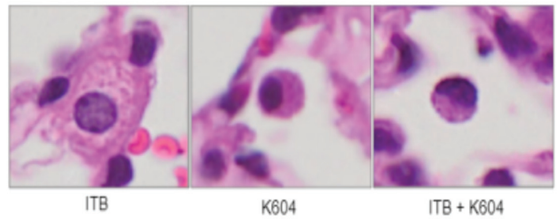

Fig. 4: Very large macrophages such as the one in the far left are often observed in the fluid of ITB-treated mice, leading to lipid accumulation in the lungs. On the other hand, administration of K-604 to ITB-treated mice appears to reduce the number of large macrophages in the cells when compared to ITB alone. Even in mice not treated with ITB, K604 has no negative effects on macrophage growth, as seen in the middle image.

Histology

After the BAL fluid collection, the large left lung lobe was extracted, inflated, fixed in paraformaldehyde (3%), and embedded in paraffin. Sections of 4 uM were stained with hematoxylin and eosin for histopathological analysis. Each slide was scanned at 40x

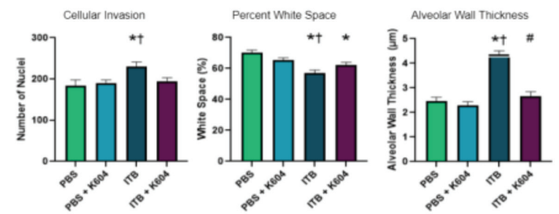

Fig. 5: Administering ITB increases the degree of cellular invasion (more nuclei), decreases the percent white space, and increases average alveolar wall thickness. On the other hand, treating with K604 seems to mitigate all three of these symptoms, directing them back toward the direction of the control and K604 specimens.

with the VS120 Virtual Slide Microscope (Olympus, Waltham, MA) and viewed under the OlyVIA viewing software for virtual slide images (Olympus). From each histological slide, 10 randomly chosen areas were captured and analyzed to determine the healthiness of the lung through 3 parameters: average alveolar wall thickness, cell infiltration (number of nuclei), and tissue consolidation (percent of open white tissue space) using ImageJ. These parameters were picked specifically to observe the impact and prevalence of described "fatty macrophages." After all data

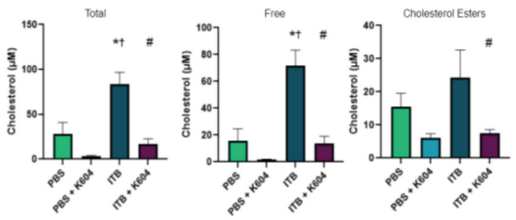

Figure 6: ITB increases the amount of total cholesterol, free cholesterol, and cholesterol esters (per 1 x 104 cells). Total cholesterol (83.59±13.26 vs 27.82±13.17) and free cholesterol (72.63±11.68 vs 15.51±9.11) are increased in BAL macrophages compared to control. Compared to ITB alone, co-administration of K604 mitigates the increase in total cholesterol (16.94±5.71 vs 83.59±13.26), free cholesterol (13.79±5.4 vs 71.63 ±11.68), and cholesterol esters (7.45±1.19 vs 24.23±8.39). Data are representative of 3 separate experiments, where n=5-16 per group. $p < 0.05$ when compared with PBS (*), PBS + K604 (†), and ITB (#).

was collected and recorded, the Prism software was used to consolidate all numbers into graphs.

Results/Discussion

Data gathered from the experiment successfully demonstrated that ACAT1 inhibition may be a viable target to improve the condition of the lung in ALI by maintaining a healthy macrophage status. With reduced levels of cholesterol, both free and esters, the macrophages had a much smaller chance of turning "fatty" and consequently the lungs showed signs of improvement across all three measured parameters (alveolar wall thickness, cell infiltration, and percent white space).

Preliminary observations of the histological cells in ImageJ displayed general trends across the four groups of mice: PBS (control), ITB (intratracheal bleomycin), K604 (only the ACAT1 inhibitor), and ITB + K604 (intratracheal bleomycin and the inhibitor). Administration of ITB led to thickening of the alveolar walls across the entire area, simulating ALI (Figure 3). The average alveolar cell wall length is notably thicker in the ITB group than all other three groups, including the treated mice. Concurrently, there seemed to be a much higher degree of immune cell invasion in the ITB group compared to the others.

The three parameters that were used to measure the healthiness of an area of the lung were: average alveolar wall thickness, cell infiltration, and percent white space. Optimally, a healthy lung would look very similar to the control lung shown in Figure 3: thin but reasonably sized walls, an average number of nuclei proportional to the space covered, and a generous percent of white space. In this respect, it can also be seen that the K604 treatment alone has no negative effects on the condition of the lung; the second group administered just K604 appeared as healthy as the control. Even more interestingly, the mice that were administered ITB but also treated with K604 showed promising signs of improvement in regard to all three parameters.

Zooming into the histological images, the difference in the state of the macrophages can be observed between the three experimental groups. Figure 4 shows how varying "types" of macrophages appear based on the level of cholesterol accumulation. "Fatty macrophages" (or large macrophages, categorized as diameter > 8 μm) were observed much more often in the ITB-administered mice compared to the control or K604-treated mice, further indicating that K604 seems to have very visible benefits for the state of the lung.

More concretely, the graphs in Figure 5 numerically illustrate the benefits of K604 administration to the mice in all three parameters. The ITB + K604 mice generally had reduced cellular infiltration (1.94.94 +/- 2.20 vs 229.72 +/- 3.318), a higher percent of white space (62.34 +/- 0.42% vs 57.04 +/- 0.54 %), and less severe alveolar wall thickening (2.66 +/- 0.05 vs 4.35 +/- 0.04) compared to the ITB alone animals. Therefore, K604 was helpful in preserving the condition of the macrophages by preventing cholesterol buildup, which in turn assists in maintaining the health of the entire lung. Additionally, the conditions of the ITB + K604 mice seemed to be almost similar to the control and K604-only mice with regards to all three characteristics.

In addition to maintaining the average alveolar wall thickness, cell infiltration, and percent white space to reasonable values similar to healthy control mice, K604 is also observed to reduce the formation and build-up of cholesterol esters directly. As shown in Figure 6, the ITB+K604 animals have lower levels of total cholesterol, free cholesterol, and cholesterol esters compared to the ITB animals. Through utilizing the Cholesterol/Cholesterol Ester-GloTM Assay kit (Promega©) and extracting cells from the BAL fluid, it can be seen that administering ITB significantly increases levels of all cholesterol in the cells and lungs, henceforth explaining the accumulation of "fatty macrophages" observed in the histological images. On the other hand, with K604 administration, cholesterol levels were very noticeably lower (total: 16.94±5.71 vs 83.59±13.26, free: 13.79±5.4 vs 71.63 ±11.68, cholesterol esters: 7.45±1.19 vs 24.23±8.39). This reduction suggests that K604 prevents cholesterol accumulation.

Thus, based on collected data, ACAT1 inhibition, by K604 or a similar compound, could be considered as a viable treatment option for ALI.

Conclusion

In closing, this experiment aimed to determine the effectiveness of inhibiting ACAT1, through K604, on the formation of fatty macrophages in ALI. The results were promising, as the data demonstrates that the K604 was effective in preventing both cholesterol ester formation and accumulation of fatty macrophages. In turn, this development led to generally healthier lungs, as observed under ImageJ, with values for alveolar cell wall thicknesses, number of nuclei, and percent white space returning closer to the values observed in the control animals. The data shows that K604 could be a realistic pharmaceutical treatment for acute lung injury, especially since there is a current lack of this in regards to ALI; however, given that this is a newer idea, further experimentation must be performed to investigate any underlying mechanisms and potential side effects of this ACAT1 inhibition. Ultimately, with more robust (and less fatty) macrophages, there could be a much stronger line of defense in the lung in response to pathogens involved in ALI, and thus protect patients against this condition to a much better degree.

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