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Salton Sea aerosol exposure in mice induces a pulmonary response distinct from allergic inflammation

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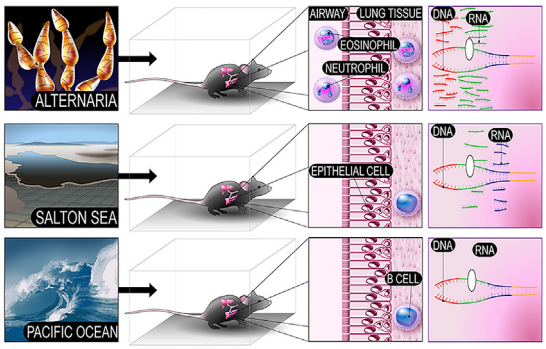
HIGHLIGHTS

- Mice exposed to allergen induced an allergic inflammation.
- Mice exposed to Salton Sea aerosol induced non-allergic inflammation genes.
- Mice exposed to Pacific Ocean aerosol showed no lung response.
- Salton Sea aerosols may promote lung inflammation, synergize with allergen response.

GRAPHICAL ABSTRACT



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ABSTRACT

In communities surrounding the Salton Sea, high rates of asthma are associated with high aerosol dust levels. However, the Salton Sea itself may play an additional role in pulmonary health. Therefore, to investigate a potential role of the Salton Sea on pulmonary health, we exposed mice to aerosolized Salton Sea water for 7 days and assessed tissue responses, including cellular infiltration and gene expression changes. For reference, mice were also exposed to aerosolized fungal allergen (*Alternaria* sp.) and Pacific Ocean aerosols. Exposure to aerosolized *Alternaria* sp. induced dramatic allergic inflammation, including neutrophil and eosinophil recruitment to the bronchoalveolar lavage fluid (BALF) and lung tissue. By contrast, Salton Sea "spray" induced only B cell recruitment to the lung tissue without increased inflammatory cell numbers in BALF. However, there were consistent gene expression changes suggestive of an inflammatory response. The response to the Salton Sea spray was notably distinct from the response to Pacific Ocean water, which induced some B cell recruitment but without an inflammatory gene expression profile. Our studies suggest that soluble components in Salton Sea water promote induction of a unique inflammation-associated response, though any relationship to asthma remains to be explored.

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Abbreviations: BALF, bronchoalveolar lavage fluid; IgE, immunoglobulin E; PM, particular matter; SMPS, scanning mobility particle sizer; PCA, Principal Component Analysis; AMS, aerosol mass spectrometer; ROS, reactive oxygen species.

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1. Introduction

The Salton Sea is a 345 mi^2 inland body of water located in California's Riverside and Imperial counties. The Sea is primarily fed by agricultural runoff as well as inflow from the Alamo, New, and Whitewater rivers. In recent decades, the Sea has been undergoing a rapid retreat. This retreat is causing increased exposure of dry lakebed (playa), resulting in increased dust production which is spreading throughout the region and impacting the surrounding population. Moreover, the drying Sea has become hypersaline, at approximately 74 parts per thousand, over twice that of Pacific Ocean water (Bureau of Reclamation, 2020). The consequent rapid change in the Sea's ecology has resulted in periodic algal blooms, and fish and migratory bird die-offs (Carmichael and Li, 2006; Xu et al., 2016). Pesticide and herbicide use from agricultural areas located to the southeast and northwest of the Sea (Xu et al., 2016), as well as heavy metal contamination from elements such as selenium (Zhou et al., 2017), paint an overall picture of ill-health in the Sea itself.

This ecosystem's ill health is also reflected in the surrounding communities. The human population surrounding the Salton Sea includes a high proportion of migrant workers, with high rates of poverty and poor access to health care. Area residents suffer from one of the highest rates of childhood asthma in California at 20%–22.4%, compared to an average of 14.5% for the rest of the State (Farzan et al., 2019). Predictably, the surrounding area also has one of the highest rates of hospitalization for asthma (California Environmental Protection Agency, 2018), making it a serious health crisis in an already underserved community.

Asthma is a disease of airway restriction, defined as an increase in airway hyperreactivity, and usually associated with allergies (referred to as "atopic" asthma), characterized by increased immunoglobulin E (IgE) production, Th2 cytokine secretion and the recruitment of eosinophils to the lungs (Bousquet et al., 2000). Atopic asthma exacerbations can occur in response to exposure to environmental or household allergens (Wark and Gibson, 2006). High levels of particulate matter (PM) have also been known to exacerbate asthma (Guarnieri and Balmes, 2014). Unfortunately for the communities surrounding the Salton Sea, there are many potential allergens and asthma exacerbating particles. The region has consistently high levels of particulate matter between 10 μm and 2.5 μm in diameter (PM_{10}) and under 2.5 μm in diameter ($PM_{2.5}$; Environmental Protection Agency, 2012; Evan, 2019; Frie et al., 2019). Indoor household allergens, such as *Alternaria alternata*, and other fungi, are also prevalent (Sinclair et al., 2018). Up to 70% of patients with fungal allergies show a positive skin test response to *Alternaria* (Bush and Prochnau, 2004). Additionally, household *Alternaria* exposure is linked to an increased odds ratio for developing asthma symptoms (Salo et al., 2006).

However, the region's rampant asthma may have more complex origins than simple dust levels, largely pointing to the Salton Sea itself. Studies have identified a variety of pesticides, including DDT, organophosphates and pyrethroid, in both the water and the sediment of sites around and within the Sea (LeBlanc and Kuivila, 2008). Organophosphates have been linked to increased risk of childhood asthma (Shaffo et al., 2018). Additionally, the Sea experiences periodic algal blooms and has been shown to contain low levels of microcystin-LR and YR, cyanotoxins known to cause ill-health (Carmichael and Li, 2006). These microcystins have been shown to cause damage to the lungs after chronic exposure (Li et al., 2016; Wang et al., 2016). Some algal blooms, such as red tides off the coast of Florida, have also been directly linked to the development of asthma and asthma exacerbations (Fleming et al., 2007; Zaias et al., 2011). Additionally, cyanobacteria, which make up a large part of algal blooms, may serve directly as sensitizing allergens, exacerbating the harmful effects of the algae (Bernstein et al., 2011).

Despite suggestive associations between the Salton Sea and asthma, more direct mechanistic information on Salton Sea aerosols and their potential impact on pulmonary health are still needed. To address this issue, we began studies to simulate chronic aerosol exposures in a mouse model of pulmonary inflammation. In the present study, we focused on the direct effect of Salton Sea "spray" aerosols on lung responses.

2. Materials and methods

2.1. Water sample collection

Two batches of Salton Sea water were collected at the edge of Salton City. The first was collected on March 2nd, 2019 (33°19′25.9″N 115°56′18.3″W) and the second was collected on May 13th, 2020 (33°19′53.2″N 115°56′30.0″W). Water samples were collected with a homemade raft; because aerosols are most likely generated at the surface layer of the sea, the design of the raft aims to collect water from the top few centimeters of the water column. Four sampling ports were square distributed, sticking out of the bottom of the raft at a length of 2 in., to ensure sampling surface water only and avoiding floating debris. Two 4.96 m poles were installed and used to move the raft to places with large depth. Water samples were taken by a hand pump on the shore. The whole system was sterilized thoroughly by bleach solution and flushed by MilliQ water before being used on site. More than 2 L of water samples were taken before sample collection to rinse the system. The collected water was stored on ice while transported to the University of California, Riverside campus. Once there, water samples were stored at 4 °C until processed.

Pacific Ocean water was also collected in two batches. The first was collected at Torrey Pines on March 9th, 2019. The second was also collected at Torrey Pines on October 2nd, 2020 (32°55′51.4″N 117°15′37.7″W). Water was collected directly by containers without using the raft since ocean water is relatively well mixed due to tides. Samples were stored at 4 °C until processed.

2.2. Water processing

Before using for aerosolization studies, the water was filtered through an acid-washed, sterilized glass funnel using a sterile 0.2 μm filter (47-mm diameter; Pall Supor 200 Sterile Grid filters, Pall Corporation, Por Washington) into an acid-washed sterilized collecting flask below via vacuum filtration. After filtration, filtrate was either stored at 4 °C or as aliquots archived at −80 °C for long-term storage. The pH of all filtrates was measured; all filtrates were approximately pH 7.0 (±0.8%). The filtered water was stored for various periods from weeks to several months between collection and use in the chamber exposure studies; we were unable to detect any difference in the magnitude of the reported effects on mouse lung responses associated with storage time.

2.3. Animals

Animal studies were performed in accordance with UCR institutional IACUC and NIH guidelines and approved protocols. Adult male and female (8–9 weeks old) C57BL/6 J mice were purchased from Jackson Labs, Sacramento. Mice were acclimated for one week in the University of California, Riverside SPF vivarium before being placed into the exposure chamber when they were 9–10 weeks old. Mice were kept 3–4 to a cage and given food and water ad libitum, with bedding being changed at least once weekly. A 12-hour day/night cycle was provided.

Exposure studies were performed in dual animal chambers (an exposure chamber and a control chamber) developed from the chamber described in Peng (2019).[23] When in the exposure chamber, mice were given a mixture of dry filtered air (0.5–1 lpm) and aerosolized spray (dried by silica gel, 3.5–4.5 lpm) with a total particle concentration of approximately 1500 μg m^{-3}. The three types of PM were generated from solutions of *Alternaria alternata* and *Alternaria tenuis* filtrate (Greer Laboratories, Lenoir, NC, USA; 0.4 g/L), Salton Sea water (133-

200× dilution), or Pacific Ocean water (40× dilution) with proper concentrations or dilution ratios, respectively. Example of typical exposure PM levels for different PM types are shown in Fig. 1a with weekly averaged PM level being 1425 μg m^{-3} for *Alternaria*, 1377 μg m^{-3} for Pacific Ocean spray, and 1523 μg m^{-3} for Salton Sea spray. Sample aerosolization was accomplished by using a homemade nebulizer with silica-gel dryers (Peng et al., 2019). Mice in the control chamber were given filtered dry air (5.0 lpm) only, with other conditions the same as the exposure chamber, including bedding replacement, food and water supplies, and corresponding day/night cycle. Particulate matter was only monitored within the exposure chamber by a scanning mobility particle sizer (SMPS, including Series 3080 Electrostatic Classifier and Ultrafine Condensation Particle Counter 3776, TSI) to assist in maintaining stable PM concentration of 1500 μg m^{-3}. Concentration was similar to our previous study in Peng et al. (2018),[24] where 1500 μg m^{-3} of *Alternaria* was sufficient to generate neutrophil and eosinophil recruitment to the lungs. Relative humidity (40–60%) and ammonia (weekly averaged $[NH_4] < 25$ ppm) were selectively measured in some of the exposures to ensure consistent quality control. For each exposure, we used an equal number of male and female mice. Each exposure had a control air cohort that matched the number and sex of the exposure group. The number of mice for each exposure is as follows: 8 mice for the 3/2/2019 Salton Sea collection, 10 mice for the 5/13/2020 Salton Sea collection, 4 mice for the 3/9/2019 Pacific Ocean collection, 6 mice for the 10/2/2020 Pacific Ocean collection, 10 mice for the *Alternaria* exposure.

After 7 days, the mice were removed from the chamber, anesthetized via isoflurane and sacrificed by cervical dislocation. The mice were then processed for either RNA extraction and flow cytometry or histological analysis. For the RNA extraction/flow cytometry mice, BALF was collected via 3 injections of 0.8 mL PBS, after which the right lung lobe was extracted and flash frozen in liquid nitrogen and kept at −80 °C until RNA extraction, while the left lobe was digested using 0.5 mg/mL collagenase D (Roche Diagnostics, Mannheim, Germany), 50 U/mL DNAse I (Sigma Aldrich, St. Louis, USA) in RPMI 1640 (Gibco, Grand Island, USA) supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, USA) preheated to 37 °C. The lung was left to digest for 30 min at 37 °C before being diced into small (~1–2 mm) sections and pushed through a cell strainer (Corning, Corning, USA). The cell strainer was rinsed with RPMI 1640 with 10% heat-inactivated FBS before being centrifuged and resuspended for use in Flow Cytometry. For the histological mice, the lung was inflated with 0.7 mL of a 1:1 OCT:PBS mixture before being flash frozen via liquid nitrogen in an OCT block.

2.4. Flow cytometry

BALF and post-digested lungs were centrifuged at 1500 rpm before being resuspended in 100 μL of a 1:50 dilution of Mouse BD FC block (BD Pharmingen, San Jose, USA; Clone 2.4G2) in FACS Buffer. Cells were stained using fluorescent antibodies: anti-CD45 FITC (BioLegend, San Diego, USA; Clone 30-F11), anti-CD19 PE-Cy5 (eBioscience, San Diego, USA; Clone MB19-1), anti-CD3 Alexa Fluor 700 (BioLegend, San Diego, USA; Clone 17A2), anti-Ly6G BV510 (BioLegend, San Diego, USA; Clone 1A8), anti-CD11b BV421 (BioLegend, San Diego, USA; Clone M1/70), anti-CD11c PE-Cy7 (BioLegend, San Diego, USA; Clone N418) and anti-SiglecF APC (BioLegend, San Diego, USA; Clone S17007L). Samples were run on a MoFlo Astrios (Beckman Coulter, Carlsbad, USA). Gating and analysis were performed using FlowJo (Version 10.71, Ashland, USA). Note that the figures show different Y-axis ranges in order to best illustrate the magnitude of the differences in cells recovered from lavage versus tissue; however, the absolute values are also presented in the text.

2.5. RNA extraction

RNA was extracted using TRIzol© (Ambion, Carlsbad, USA). Briefly, ~100 mg of frozen lung tissue was placed in a mortar, covered with liquid nitrogen, then ground into dust using a pestle before adding to TRIzol©. Chloroform was added, mixed and centrifuged. The aqueous phase was removed and mixed with isopropanol and centrifuged, leaving a pellet which was then washed 3× with 75% ethanol before drying at room temperature. The pellet was resuspended in DEPC-Treated water (Ambion, Austin, USA). Concentration and purity of RNA was checked via NanoDrop 2000 (Thermo Scientific, Carlsbad, USA).

2.6. NanoString analysis

Purified RNA was analyzed using an nCounter® Sprint Profiler (NanoString Technologies, Seattle, USA) with the nCounter® Mouse Immunology Panel according to manufacturer protocols. Gene expression was analyzed using the nSolver® 4.0 software (NanoString Technologies, Seattle, USA). Statistical analysis was done using nSolver® Advanced Analysis 2.0 (NanoString Technologies, Seattle, USA); false discovery rates (FDR) were calculated, using the Benjamini-Hochberg method.

Differences in lung immune gene expression profiles (from nCounter® Mouse Immunology Panel) for each mouse sampled were

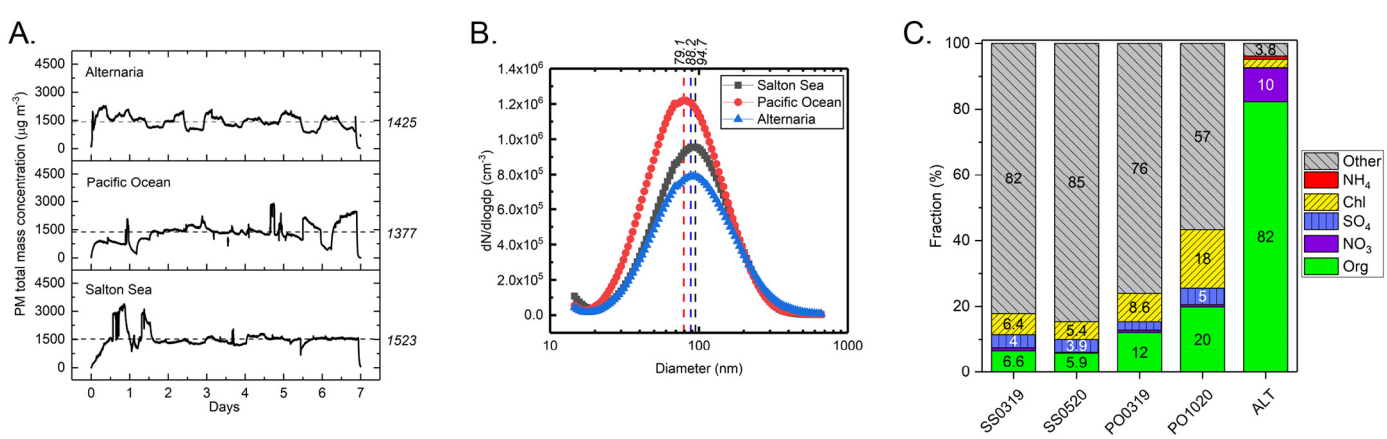


Fig. 1. Quantification of *Alternaria* (ALT), Pacific Ocean (PO) and Salton Sea (SS) aerosols. PM mass concentration was measured by a scanning mobility particle sizer. Chemical composition was determined by AMS. (A) PM mass concentration during 7-day exposure of SS, PO and ALT. Dash line shows the 7-day averaged mass concentration of PM. All units are in μg m^{-3}. (B) Averaged mobility diameter distribution of different PM used in exposure experiments. (C) Chemical composition of dry particulate matters generated from different materials collected in different season (mm/yr). Other includes metals (sodium, calcium, magnesium), trace metals and other inorganics. (Key: SS0319, Salton Sea/March 2019; SS0520, Salton Sea/May 2020; PO0319, Pacific Ocean/March 2019; PO1020, Pacific Ocean/October 2020; ALT, Alternaria filtrate).

analyzed using Principal Component Analyses (PCA; Pielou, 1984) using the "prcomp" function in R version 4.0.3 (R version 4.0.3; R Core Team, 2020). Normalized and log transformed gene expression data matrices were constructed as data points were projected onto the 2-D plane, such that the variance is maximized. As dimensions were reduced, they spread out in two directions to explain most of the differences in the data. X-axes (labeled as PC1) in the ordination space represent the first principal component, which separates data points to represent the most variation in the dataset; y-axes (labeled as PC2) are orthogonal to PC1 and separate data points to represent the next greatest amount of variation within these gene expression datasets, across exposure types. We used the *ggplot2* package (Wickham, 2009) and the "stat ellipse" function, with 95% confidence intervals, to visualize these PCA plots in R (R version 3.2.1; R Core Team, 2017).

2.7. Histology

OCT embedded lungs were sectioned at 20 μm in a Cryostat. Sections were stored at −80 °C until staining. Before staining with H&E, slides were fixed with 4% PFA for 10 min. Histological images were taken using a Keyence BZ-X710 (Keyence Corporation of America, Itasca, USA).

2.8. Aerosol mass spectrometry

Chemical composition of aerosolized particles was measured by an HR-ToF-AMS (DeCarlo et al., 2006) Particles were generated using the same atomizer system as chamber exposures, as described by Peng et al. (2018). The outlet of our atomizer system was split into two ports, with one connected to the sampling inlet of the aerosol mass spectrometer (AMS) and the other venting through a HEPA filter. The Salton Sea and Pacific Ocean stock samples were diluted 10× with MilliQ water to generate particles at suitable concentrations. *Alternaria* solutions were the same concentrations as those used in chamber exposures. ToF-AMS Analysis Toolkit 1.57 and PIKA 1.16 on Igor Pro 6.36 were used in data processing.

2.9. Statistical analysis

All statistical analysis was done using GraphPad Prism 6 (GraphPad, San Diego, USA). p-Value was calculated using the Mann-Whitney *U* test for nonparametric data. We analyzed multivariate homogeneity of group dispersions (variances) using PERMDISP2 procedures in the R package *vegan*, with the function "betadisper" (Oksanen et al., 2016) in R. Euclidean distances between objects and group centroids were handled by reducing the original distances to principal coordinates. We used Tukey's Honest Significant Difference methods as "TukeyHSD.betadisper" to create 95% confidence intervals on differences between mean distance-to-centroids across exposures, as compared with mice in chambers containing control air.

3. Results

3.1. Control of exposure to aerosol particulate levels

To ensure consistent levels of simulated chronic environmental aerosol exposures, our environmental chamber system was set up to continuously monitor suspended aerosols by particulate size as well as steady-state mass concentrations. In this study, we performed exposure studies using aerosolized suspensions generated from aqueous solutions of *Alternaria* filtrate, Salton Sea water, and Pacific Ocean water. Mass concentrations of PM generated from different sources were stable over 7 days, with averaged mass concentrations around 1500 μg m^{-3} (±10%; Fig. 1a). Average size distributions of the different types of PM were generated using the same atomizer system, under identical conditions (Fig. 1b). Only minor differences in average particle size were observed between samples; peak particle mobility diameter for Pacific Ocean PM was 79.1 nm; *Alternaria* PM was 88.2 nm, and Salton Sea PM was 94.7 nm. Because the injection method was consistent between each exposure, minor differences in PM size distribution were primarily due to composition differences of each aerosolized solution. Moreover, differences in particle densities were seen with higher "salty" PM densities, as compared to the "organic" PM (*Alternaria* PM 1.36 g cm^{-3} < Pacific Ocean PM 1.96 g cm^{-3} < Salton Sea PM 2.07 g cm^{-3}). Importantly, a majority of PM was either fine (PM with a diameter between 0.1 μm–2.5 μm) or ultrafine (PM with a diameter of less than 0.1 μm), with the vast majority of PM under 1 μm in mobility diameter. This is critical to consistent exposure effects, as ultrafine PM is expected to be able to travel deep into lung tissue down to the alveoli.

PM surface area has been proposed to be an important factor in studies on the health effects of PM on lungs. However, as all three types of PM used in this experiment were generated from aqueous solutions, with no inert components; the particles were – by their nature – highly water soluble. Accordingly, particle size could change within the lung due to the high relative humidity of the lung microenvironment (Löndahl et al., 2007); therefore, for comparability across different material exposures, we chose to control the total mass concentration of the different types of PM instead of total surface area.

Due to the complexity of multiple factors, including particle size and depth of penetration into lung tissue, as well as the extent of animal activity, age, or relative humidity fluctuation, it is difficult to estimate the actual dose of material deposited in the lung of a mouse over the course of a 7-day exposure. Since we maintained a target mass concentration in the chamber, with similar particle size distributions across each of three aerosol suspensions, we expect that particle suspensions of all three materials were delivered in similar fashion. Moreover, since the particles were all generated using aqueous solutions, it is likely that all particles coming into direct contact with lung tissue (i.e., alveolar or airway epithelium) will similarly fully dissolve and release their components to diffuse into the tissue.

3.2. PM chemical composition analysis by aerosol mass spectrometer

Since the aerosol suspensions of particles were all using aqueous solutions with no inert particulate matter, the biological impacts of the exposures are expected to be based on the release of soluble components of particulates into the tissue, rather than on the particulate physical properties. Thus, we determined the soluble composition of the aqueous solutions using AMS. There was a large difference in the organic fraction between PM from the Salton Sea (5.9–6.6%), Pacific Ocean (12–20%) and the *Alternaria* (82%). Additionally, *Alternaria* PM had a notable fraction of NO_3 (10%) compared to the others (<0.1%). Detectable levels of NH_4 (1.01%) were only measured for the *Alternaria* PM. There were also key differences between the "salty" PMs (Salton Sea and Pacific Ocean). The Salton Sea PM was lower in organic content than the Pacific Ocean PM. In contrast, the fraction of metal ions and other inorganics were higher in the Salton Sea PM than in the Pacific Ocean PM (Fig. 1c).

3.3. Alternaria elicited allergic immune cell recruitment to lungs

To assess whether Salton Sea exposure can trigger allergic asthma, it was important to establish reference characteristics of a canonical allergic lung inflammation. Thus, we exposed C57BL/6 J mice to *Alternaria alternata* and *Alternaria tenuis* filtrate mixture (hereafter referred to as "*Alternaria*") at a chamber mass concentration of approximately 1500 μg m^{-3} for 7 days. A group of mice were held in the exposure chamber, while a control group was simultaneously held in a parallel chamber that had only filtered air pumped into it. Following the exposure, BALF and lung tissues were assessed for inflammatory cell infiltration. H&E staining of the lung showed marked cellular infiltration around the airways compared to the controls (Fig. 2a), indicating

an inflammatory response to the *Alternaria*. Additionally, there was a significant increase in the number of cells in the BALF ($1.7 \times 10^6 \pm 2.7 \times 10^5$ vs. $2.5 \times 10^5 \pm 4.9 \times 10^4$ control, $p < 0.0001$; Fig. 2b) compared to the controls.

BALF cells were stained for analysis by flow cytometry to identify infiltrating inflammatory cells. The differential proportions of neutrophils ($CD11b^+$, $Ly6G^+$), eosinophils ($CD11c^-$, Siglec F^+), T cells (SSC^{low}, $CD3^+$), and B cells (SSC^{low}, $CD19^+$) were quantified as a proportion of $CD45^+$ cells, with the remaining cells mainly being alveolar macrophages. Similar to our previous studies (Peng et al., 2018), the BALF of *Alternaria* exposed mice showed an expected increase in neutrophils ($72.1 \pm 11.1\%$ vs. $0.2 \pm 0.1\%$ control, $p < 0.0001$; Fig. 2d) and eosinophils ($8.3 \pm 1.7\%$ vs. ~0% control, $p < 0.0001$; Fig. 2c). T cells made up a higher, though small, percent of the BALF after *Alternaria* exposure ($2.5 \pm 0.4\%$ vs $0.2 \pm 0.1\%$ control, $p < 0.0001$; Fig. 2f). B cells were essentially undetectable in the BALF ($0.2 \pm 0.1\%$ vs. $0.2 \pm 0.1\%$ control; Fig. 2e).

Infiltrating inflammatory cells may be limited to the interstitial compartment of the tissue, and so might not be detected among lung lavage cells. Differences in BALF versus tissue infiltrating cells may also reveal differences in the way inflammatory cells are recruited as well as differences in their impact on tissue remodeling, which has a critical impact on airway resistance. Thus, tissue infiltrating cells were also isolated by enzymatic digestion of lung tissues and stained for analysis by flow cytometry. Interestingly, we found that while there were some similarities in the types of cells detected, the proportions of different infiltrating cell types were notably different. For example, the proportion of neutrophils in *Alternaria* exposed lungs, while higher than in the control lungs ($21.8 \pm 1.3\%$ vs. $11.1 \pm 1.8\%$ control, $p < 0.01$; Fig. 2d), was nonetheless smaller than the 70% + proportion of neutrophils in the BALF. In the case of eosinophils, there also were low numbers of cells detected in controls; however, *Alternaria*-exposed lung actually showed a higher proportion in the digested tissue ($12.3 \pm 1.0\%$ vs. $3.6 \pm 0.3\%$ control $p < 0.01$; Fig. 2c) compared to ~8.3% in the BALF. These contrasting ratios of neutrophils and eosinophils in BALF versus digested tissue are consistent with the possibility that neutrophils may play a more important role in clearing microbes from alveolar and airway spaces, while eosinophils are more important in the interstitial spaces, where they may contribute to tissue remodeling.

Lymphocytes were also more easily detected in lung digests compared to BALF. T cells were higher in digested tissue ($13.6 \pm 0.9\%$ vs. $13.9 \pm 1.4\%$ control) compared to BALF, but the *Alternaria* exposed lungs showed no significant difference compared to controls (Fig. 2f); an expected increase in recruited CD4 T cells[21] was likely diluted by the infiltrating granulocytes. The percentage of B cells was also higher in digests ($8.8 \pm 0.6\%$ vs. $17.4 \pm 1.0\%$ control, $p < 0.01$) than in BALF, but the proportion of B cells detected in exposed lungs was decreased relative to controls, also possibly due to the increased proportion of granulocytes (Fig. 2e).

3.4. Response to Salton Sea and Pacific Ocean water

With the inflammatory response to *Alternaria* exposure as a reference, we exposed mice to filtered and aerosolized Salton Sea water. Exposures were performed using water samples collected at different times and sites at the Sea, but all exposures were performed at a similar mass concentration. Following the exposures, mice were analyzed in the same manner as the *Alternaria* exposure. In contrast to the picture in *Alternaria* exposed mice, the lungs from mice exposed to aerosolized Salton Sea water did not contain granulocyte recruitment in either the BALF or digested lung tissue. Moreover, H&E stained lung sections (Fig. 3a) showed no evidence for cellular recruitment after exposure to aerosolized Salton Sea water. Total BALF cell counts also showed no differences between exposure and control ($5.2 \times 10^5 \pm 1.6 \times 10^5$ vs. $3.8 \times 10^5 \pm 7.9 \times 10^4$ control; Fig. 3b). Flow cytometry analysis of digested lung tissue revealed minimal inflammatory cell recruitment: eosinophils ($3.7 \pm 0.6\%$ vs. $2.4 \pm 0.5\%$ control; Fig. 3c), neutrophils (12.2 ± 1.2 vs. $15.3 \pm 2.7\%$ control; Fig. 3d), and T cells ($10.7 \pm 0.7\%$ vs. $10.2 \pm 0.8\%$ control; Fig. 3f) showed no significant differences. Interestingly, B cells in digested lung tissue were increased after exposure to the Salton Sea water ($18.5 \pm 1.5\%$ vs. $12.3 \pm 0.7\%$ control, $p < 0.05$; Fig. 3e). All four cell types were essentially not present in the BALF (data not shown).

To determine whether this response was due to unique characteristics of the Salton Sea spray particles or a general response to Sea spray, we exposed mice to aerosolized Pacific Ocean water, also collected at multiple dates. Communities living near the Pacific Ocean do not show the high asthma rates found near the Salton Sea, so any differences observed may provide clues to potential links between Salton Sea aerosols and asthma. Inflammatory cell recruitment from Salton Sea and Pacific Ocean exposures turned out to be very similar, as there was no difference in the BALF cell counts ($3.8 \times 10^5 \pm 7.6 \times 10^4$ vs $2.6 \times 10^5 + 3.3 \times 10^4$ control; Fig. 3b) nor increase in the percentage of tissue digest eosinophils ($2.6 \pm 0.3\%$ vs. $2.8 \pm 0.3\%$ control; Fig. 3c), neutrophils ($11.9 \pm 2.7\%$ vs. $16.8 \pm 6.8\%$ control; Fig. 3d), or T cells ($15.5 \pm 1.3\%$ vs. $10.7 \pm 1.4\%$ control; Fig. 3f). Moreover, there was a similar significant increase in B cell percentage ($21.1 \pm 1.2\%$ vs. $13.9 \pm 1.9\%$ control, $p < 0.05$; Fig. 3e). Once again, all four cell types were essentially absent in the BALF (data not shown).

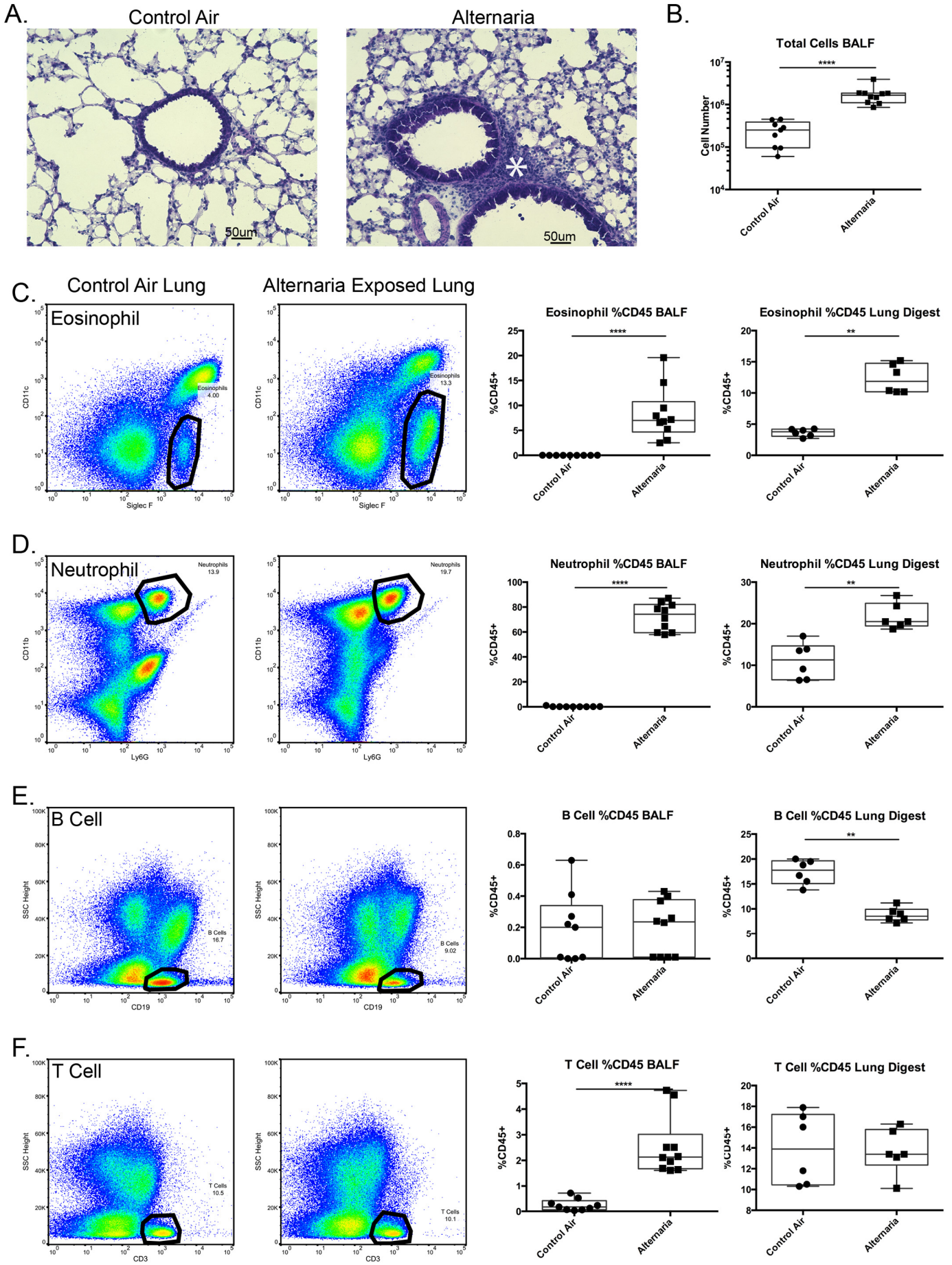
3.5. Gene expression changes in the response to aerosols

While assays for cellular infiltrates and histological changes can reveal significant inflammatory responses to exposures, more subtle tissue responses were revealed from analyses of gene expression profiles. In these studies, we focused on a subset of immune-associated genes assayed using a panel of short sequence tag probes (NanoString). This method quantifies expressed genes by direct counting of hybridized tagged gene probes and includes a set of general "housekeeping gene" probes. As a set, this approach allowed broad internal normalization of the assayed gene expression profiles, which in turn enabled direct comparisons of different RNA profiles from different samples and studies. Principal Component Analysis (PCA) of gene expression profiles collapses the complex gene expression data sets, and helps provide overall comparisons among individual mice in different treatment groups.

Our reference allergic inflammatory response to *Alternaria* exposure illustrates a reproducible and characteristic gene expression pattern, as seen by the distance between centroids from the control air group to the *Alternaria* group ($d = 19.84$) in this PCA ordination; we observed tight clustering within the control air group and *Alternaria* groups and clear differentiation between the groups in the PCA, despite multiple replicates (Fig. 4a). We identified 213 differentially regulated genes (FDR < 0.10) of which 166 were significantly upregulated vs 47 which were significantly downregulated (Fig. 4b). These genes are diverse in function, but the strongest change in regulation falls into immune defense responses and chemokine production, consistent with the observed recruitment of inflammatory cells into the lung. Among the top 20 regulated genes are Ig receptors (*Fcgr2b, Pigr, Fcgr3*), chemokines (*Cxcl3, Ccl9, Ccl8, Ccl3 Ccl22*), immune regulatory genes (*Tgfbi, Lilrb4, IL33, Ctss, Ptafr, Ctsc*) and innate immune genes (*Cfb, Muc1*).

By contrast, analysis of gene expression profiles from exposures to aerosolized Salton Sea water revealed a distinctively different pattern (Fig. 5a). The PCA shows an overall distinction between control air and Salton Sea exposed groups; however, the extent of these orthogonal axes did not explain as much of the variance, nor did they illustrate as great of a separation as was detected for the *Alternaria* exposures. Euclidean distance between centroids of control air and Salton Sea exposed groups ($d = 3.08$) within the PCA ordination was shorter than was found in the PCA for the *Alternaria* exposures.

This exposure triggered significant gene expression changes, with 151 differentially expressed genes (Fig. 5b). Of these, 146 genes were


A.
Control Air
Alternaria
50um
50um
B.
Total Cells BALF
Cell Number
****
Control Air
Alternaria
C.
Control Air Lung
Alternaria Exposed Lung
Eosinophil
CD11c
Siglec F
Eosinophils 4.00
Eosinophils 13.3
Eosinophil %CD45 BALF
Eosinophil %CD45 Lung Digest
%CD45+
D.
Neutrophil
CD11b
Ly6G
Neutrophils 13.9
Neutrophils 19.7
Neutrophil %CD45 BALF
Neutrophil %CD45 Lung Digest
E.
B Cell
SSC Height
CD19
B Cells 16.7
B Cells 9.02
B Cell %CD45 BALF
B Cell %CD45 Lung Digest
F.
T Cell
CD3
T Cells 10.5
T Cells 10.1
T Cell %CD45 BALF
T Cell %CD45 Lung Digest

significantly upregulated while only 5 were significantly downregulated (FDR < 0.10). The regulated genes were primarily associated with phosphorylation pathways (Jak1, Jak2, Jak3, Stat5b, Tnfrsf14), T cell activation (Ifnar1, Ifngr1), and NF-KB signaling (Ikbkb). Additionally, a preference toward MHC I/Th1 response predominates (Ifnar1, Ifngr1, Tap1), although there were some Th2-related receptors upregulated (Il6ra). It should be noted that the magnitude of gene expression changes, while statistically significant, were relatively small, with the vast majority (134 of the 146 upregulated genes) showing less than 0.5 Log_2 fold change. Despite the lower magnitude changes, the regulated genes were consistent across replicate exposures and multiple Salton Sea samples, illustrated by the clustering of the Salton Sea data points relative to control data in the PCA.

As noted above, both Salton Sea and Pacific Ocean exposures induced some recruitment of B cells into lung tissue. Interestingly, this similarity was not seen in the gene expression profiles; in replicate studies with different Pacific Ocean samples, aerosolized Pacific Ocean exposed mice showed no significant changes in gene expression compared to controls (Fig. S2). It is likely that if there were any induced genes related to B cell recruitment, they were not represented in the probe set used. More importantly however, these comparisons suggest that Salton Sea water exposures had a characteristic biological effect, unrelated to any general effect of exposure to sea water.

Our analysis of gene regulation in response to *Alternaria* versus Salton Sea exposures showed that each induced a reproducible and characteristic set of gene expression changes, with tight clustering within the groups and little intergroup overlap. Additionally, both Salton Sea and *Alternaria* produced responses distinct from the Pacific Ocean exposed mice (Fig. 6a). It is especially notable, however, that the *Alternaria* and Salton Sea exposures each induced strikingly different sets of genes (Fig. 6b; Pacific Ocean excluded due to a lack of differentially expressed genes). Of the 166 upregulated genes in *Alternaria* exposed mice and the 146 in the Salton Sea exposed mice, only 55 are upregulated in both. Even more notable is that of the 47 downregulated genes in the *Alternaria* exposed mice and 5 downregulated genes in the Salton Sea exposed, only 1 was downregulated in both. Additionally, 13 genes which were upregulated in Salton Sea exposed mice were downregulated in *Alternaria* while 2 genes which were upregulated in *Alternaria* exposed mice were downregulated in mice exposed to aerosolized Salton Sea. Thus, while it appeared that both types of exposures regulated genes associated with at least some aspect of innate and adaptive immunity, their overall impacts were on rather different components of the immune or inflammatory response.

4. Discussion

The studies reported here were principally aimed at determining whether the aqueous components (which may include microbial components and toxins) in Salton Sea water might have effects on pulmonary tissues in response to chronic delivery into the lung as aerosolized particles. We found that aerosolized Salton Sea water was able to induce a distinct inflammatory gene expression profile despite a lack of cell recruitment to the BALF as well as neutrophil and eosinophil recruitment to the lung tissue. It is important to note that these studies do not test the biological effects of actual dust generated at the Salton Sea exposed playa; these effects are the subject of ongoing studies and will be reported separately. Since the impact of dust exposure among residents in the region is dependent on a variety of factors, including prevailing wind patterns, dust events, and proximity to the Salton Sea, such studies will need to take these other additional factors into account.

To investigate the effect that Salton Sea spray may have on the communities surrounding the Sea, we exposed C57BL/6 mice to approximately 1500 μg m^{-3} of aerosolized Salton Sea for 7 days. While this is meant to simulate a chronic exposure condition, there are still some limitations associated with our model. Residents surrounding the Salton Sea are exposed to variable levels of aerosols over a period of years, unlike the consistent 7-day exposure in our study. As perfectly matching both the exposure time and aerosol concentration the residents are exposed to is impractical, we focused on a reasonable timeframe and aerosol concentration that showed demonstrable results. As this timeframe and concentration was sufficient to reliably induce large changes in *Alternaria* exposed mice and gene expression changes in Salton Sea exposed mice while the Pacific Ocean exposed mice showed no change, we believe our methodology is capable of providing real insights into the health effects of these aerosols. While direct measurement of airway hyperreactivity was beyond the scope of this study, our initial results call for future studies into this topic.

For this study, we used aerosols generated from filtered aqueous solutions. While this excluded the potential effects of larger inert dust particles, it allowed us to specifically focus on the effects of the dissolved components in the water. Separating the effects of the dissolved aqueous components and inert dust particles is critical as inert dust particles can have their own biological effects, including triggering of airway irritant receptors (Sellick and Widdicombe, 1971). Additionally, larger dust particles (e.g., 1 μm or larger) may affect the delivery of soluble components carried on their surface, since they would not be as capable of penetrating deep into alveolar spaces. Exclusion of these larger particles was also important for generating a consistent PM (all PM in the study had a mobility diameter well under 1 μm); thus, particle size was unlikely to be a limiting factor for distribution. Effects due directly to minor differences in particle size are most likely negligible, as exposure to Pacific Ocean water failed to induce gene expression changes, indicating that the specific composition of the aerosol is the primary driving agent for gene expression changes and cell recruitment. Our AMS breakdown was unable to pinpoint a broad category for reactive agents, as there was no consistent ratio between the composition and the effects. Thus, future studies should focus on specific components that may be present in the Salton Sea water. As we found some aspects of the NF-KB pathway upregulated (Ikbkb, RelA), care should be taken to investigate sources of reactive oxygen species (ROS). In particular, pesticides (LeBlanc and Kuivila, 2008) and heavy metal ions (Frie et al., 2019; D'Evelyn et al., 2021), both of which have been detected in the Salton Sea, should be investigated, as they are known to induce ROS (Abdollahi et al., 2004; Leikauf et al., 2020).

The studies reported here are only among the first steps in studies identifying the potential aerosols contributing to lung disease in residents near the Salton Sea. The sea spray aerosols produced at the Salton Sea surface are certainly not the only contributor to inhaled aerosols and the proportions of other components in the inhaled aerosols may vary

Fig. 2. Inflammatory cell recruitment due to *Alternaria* aerosols. Mice were exposed to either filtered control air or aerosolized *Alternaria* filtrate for 7 days in dual environmental chambers. Following exposure, bronchoalveolar lavage fluid (BALF) was collected and the left lobe was digested and analyzed via flow cytometry. (A) Lungs inflated with a 1:1 OCT:PBS solution and frozen in OCT blocks were sectioned and stained with H&E. (*, Interstitial infiltrate). (B) Total cells in the BALF were counted via hemocytometer (Left, Control air, n = 9; Right, *Alternaria*, n = 10). (C–F) Cells were represented as a percentage of $CD45^+$ cells in digested lungs or BALF. Representative dot plots for control air and *Alternaria* exposed are shown. Eosinophils are $CD45^+CD11c^-SiglecF^+$ (Left diagram: Left, Control air BALF, n = 9; Right, *Alternaria* BALF, n = 10; Right diagram: Left, Control Air Lung, n = 6, Right, *Alternaria* Lung, n = 6). Neutrophils are $CD45^+CD11b^+Ly6G^+$ (Left diagram: Left, Control air BALF, n = 9; Right, *Alternaria* BALF, n = 10; Right diagram: Left, Control air, n = 6, Right, *Alternaria,* n = 6). B cells are $CD45^+SSC^{low}CD19^+$ (Left diagram: Left, Control air BALF, n = 9; Right, *Alternaria* BALF, n = 10; Right diagram: Left, Control air, n = 6, Right, *Alternaria,* n = 6). T cells are $CD45^+SSC^{low}CD3^+$ (Left diagram: Left, Control air BALF, n = 9; Right diagram: Right, *Alternaria* BALF, n = 10; Left, Control air, n = 6, Right, *Alternaria,* n = 6). ** = $p < 0.01$; **** = $p < 0.0001$.

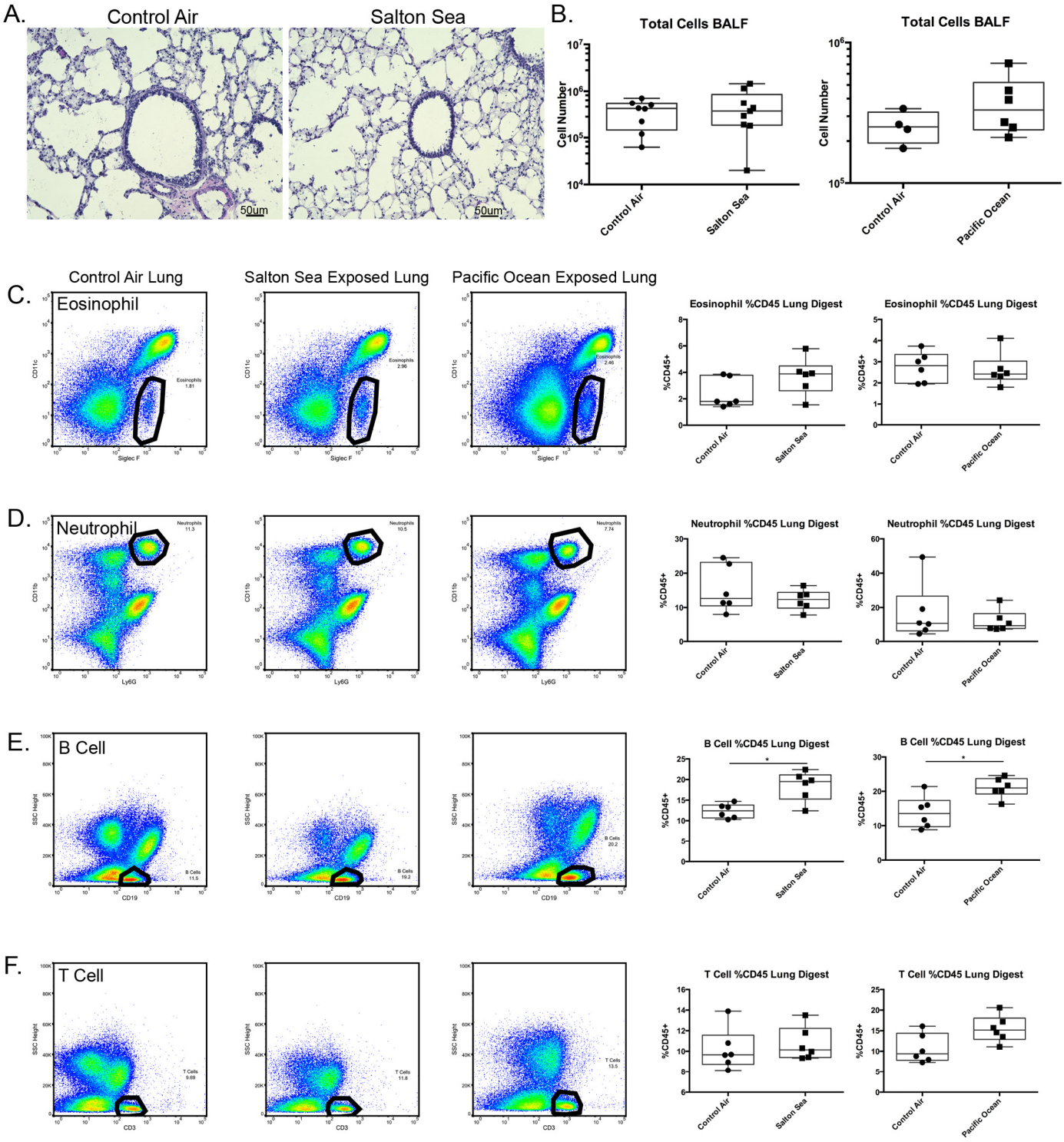


Fig. 3. Inflammatory cell recruitment due to Salton Sea and Pacific Ocean aerosols. Mice were exposed to filtered control air, filtered and aerosolized Salton Sea water, or filtered and aerosolized Pacific Ocean water for 7 days. BALF was collected and tissue was digested for flow cytometry. (A) Lungs were inflated with a 1:1 OCT:PBS mixture and frozen, sectioned and stained with H&E. (B) Total cells in the BALF were counted via hemocytometer (Left diagram: Left, Control air, n = 8; Right, Salton Sea, n = 9; Right diagram: Left, Control Air, n = 4; Right, Pacific Ocean, n = 6). (C–F) Digested lung was stained and analyzed via flow cytometry. Cells populations are represented as the percentage of $CD45^+$ cells. Representative dot plots for the control air, Salton Sea exposed, and Pacific Ocean exposed mice are shown. Aerosolized Salton Sea and Pacific Ocean exposed mice are compared to their contemporaneous controls. Eosinophils are $CD45^+CD11c^-SiglecF^+$ (Left diagram: Left, Control air, n = 6; Right, Salton Sea, n = 6; Right Diagram: Left, Control air, n = 6; Right, Pacific Ocean, n = 6). Neutrophils are $CD45^+CD11b^+Ly6G^+$ (Left diagram: Left, Control air, n = 6; Right, Salton Sea, n = 6; Right Diagram: Left, Control air, n = 6; Right, Pacific Ocean, n = 6). B cells are $CD45^+SSC^{low}CD19^+$ (Left diagram: Left, Control air, n = 6; Right, Salton Sea, n = 6; Right Diagram: Left, Control air, n = 6; Right, Pacific Ocean, n = 6). T cells are $CD45^+SSC^{low}CD3^+$ (Left diagram: Left, Control air, n = 6; Right, Salton Sea, n = 6; Right Diagram: Left, Control air, n = 6; Right, Pacific Ocean, n = 6). * = $p < 0.05$.

widely depending on the aggregation of sea spray among other ambient dust particles generated at the playa or more distant sources. Indeed, these studies should not be interpreted to suggest that Salton Sea water aerosols are the only source of potential aerosol toxins in the region. Also, actual exposures well depend on an individual's geographic position relative to the Salton Sea, seasonal winds, and other factors.

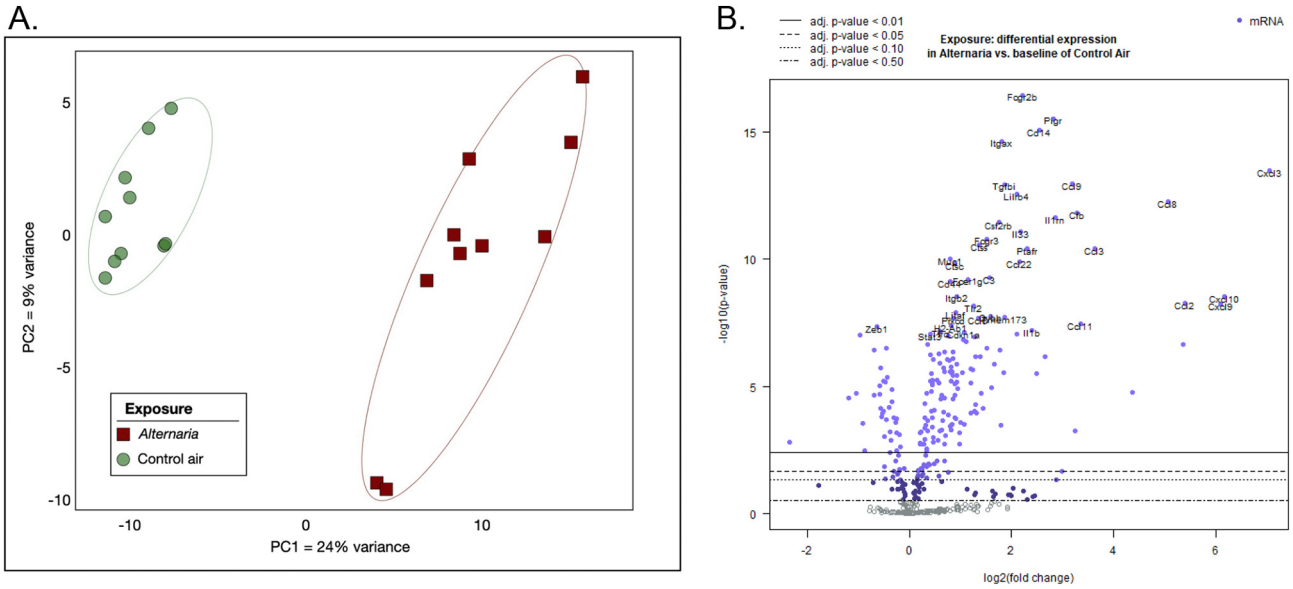


Fig. 4. Gene expression changes due to *Alternaria* aerosol exposure. After a 7-day exposure to either filtered control air (n = 10) or *Alternaria* filtrate (n = 10), lung RNAs were analyzed for gene expression using a defined immunology gene panel (NanoString); (A) PCA of the gene expression data with red squares representing mouse lung immune gene expression profiles from individual animals exposed to aerosolized *Alternaria* sp., as compared to green circles, which are from mouse samples exposed to control air. (B) Volcano plot depicting the differential expression profile of the *Alternaria* exposed mice compared to a baseline of control air. The X-axis depicts the log2 fold change while the Y-axis depicted the -log10 (Benjamini-Hochberg adjusted p-value). The 40 most significant gene by Benjamini-Hochberg adjusted p-value are labeled.

The main observation reported here is that soluble components of Salton Sea water are able to induce a unique pattern of gene expression changes in chronically exposed lungs, and that this pattern is strikingly distinct from the characteristic allergic inflammation induced by the common household fungal allergens in *Alternaria* filtrate. In the context of the observed high incidence of asthma in the Salton Sea region, our findings suggest that the Salton Sea water soluble components by themselves appear to induce significant lung responses, but they are clearly distinct from the characteristic allergic inflammatory responses typified by *Alternaria* exposures.

However, the distinctive effect of Salton Sea exposures does not entirely rule out potential impacts on asthma. A number of receptors were significantly upregulated, including Il6r, CD97, Ifngr1 and Ifnar1. IL-6 is associated with IL-4 production, which is critical for Th2 differentiation (Rincon and Irvin, 2012). The soluble form of Il6r has also been associated with asthma severity (Peters et al., 2017). CD97 is a known

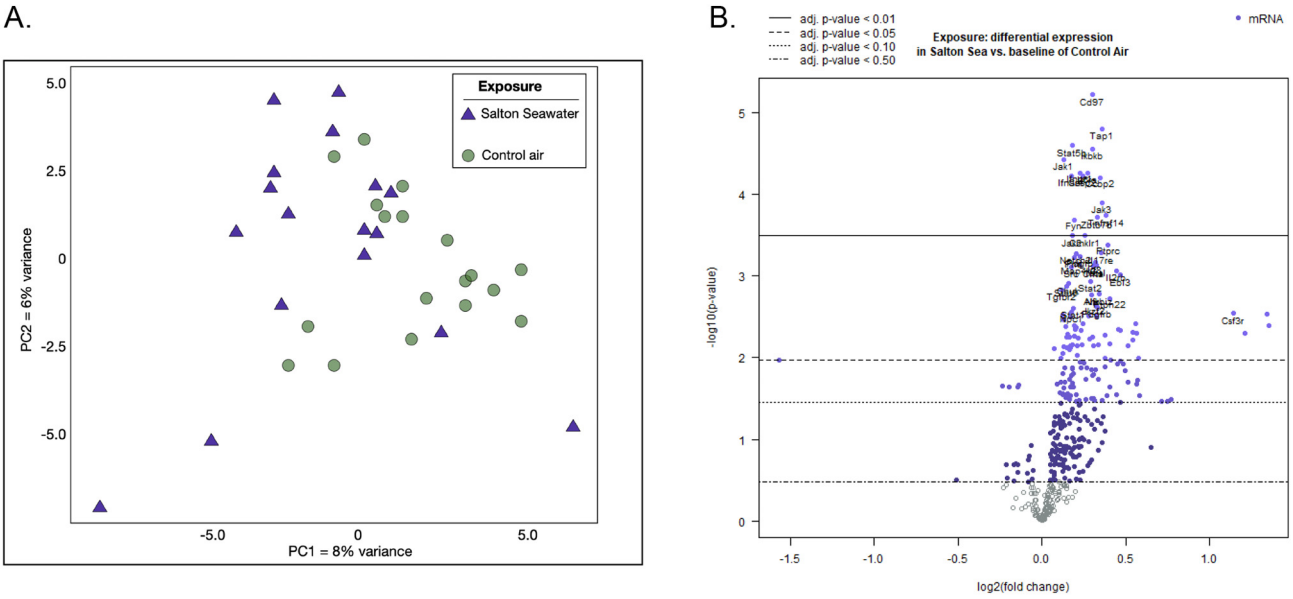


Fig. 5. Gene expression changes due to Salton Sea aerosol exposure. After a 7-day exposure to either filtered control air (n = 18) or aerosolized Salton Sea water (n = 17), lung RNAs were analyzed for gene expression using a defined immunology gene panel (NanoString). (A) PCA of the gene expression data with purple triangles representing mouse lung immune gene expression profiles from individual animals exposed to aerosolized Salton Sea water, as compared to green circles, which are from mouse samples exposed to control air. (B) Volcano plot depicting the differential expression profile of the aerosolized Salton Sea exposed mice compared to a baseline of control air. The X-axis depicts the log2 fold change while the Y-axis depicted the -log10 (Benjamini-Hochberg adjusted p-value). The 40 most significant gene by Benjamini-Hochberg (BH) adjusted p-value are labeled.

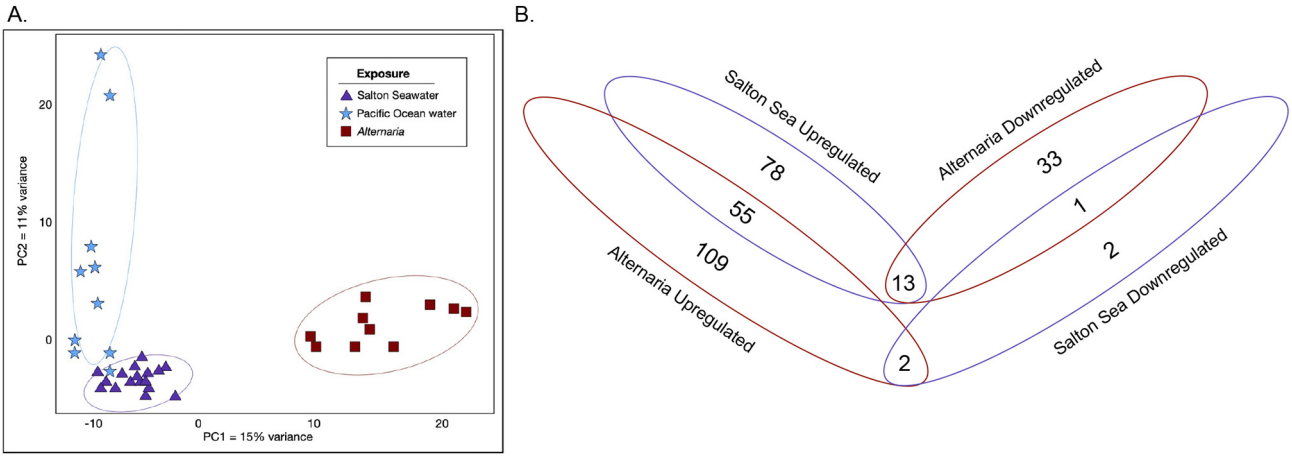


Fig. 6. Comparisons between *Alternaria*, Salton Sea and Pacific Ocean exposures. Mice were exposed to either control air, *Alternaria*, or aerosolized Salton Sea for 7-days before lung tissue was collected. Extracted RNA was analyzed using a Mouse Immunology Panel (NanoString). (A) PCAs were generated using the "prcomp" function in R (version 4.0.3), to compare Pacific Ocean (blue stars), Salton Sea (purple triangles) and *Alternaria* (red squares) exposures, and visualized PCA as in Methods. Comparisons are made between *Alternaria* exposed mice (n = 10) and their contemporaneous controls (n = 10) or aerosolized Salton Sea exposed mice (n = 17) and their contemporaneous controls (n = 18). 213 genes were differentially regulated in the *Alternaria* comparison (FDR < 0.10), of which 166 were upregulated and 47 were downregulated. 151 genes were differentially regulated in the Salton Sea comparison (FDR < 0.10), of which 146 were upregulated and 5 were downregulated. 55 genes were upregulated in both comparisons, 1 gene was downregulated in both comparisons, 13 were upregulated in the Salton Sea comparison but downregulated in the *Alternaria* comparison while 2 were upregulated in the *Alternaria* comparison but downregulated in the Salton Sea comparison. 78 genes were uniquely upregulated, and 2 genes were uniquely downregulated in the Salton Sea comparison while 109 genes were uniquely upregulated and 33 were uniquely downregulated in the *Alternaria* comparison.

costimulatory factor on CD4+ T cells (Capasso et al., 2006). In contrast to the previous receptors, Ifngr1 and Ifnar1 are associated with Th1 response. However, Th1-polarization has been linked to nonallergic asthma (Zoratti et al., 2014). Additionally, our previous studies showed that Th1 inflammatory responses could not only co-exist with allergic Th2 inflammatory responses, they could show additive effects (Li et al., 1998). Salton Sea exposures also induced a number of genes associated with signaling pathways, such Jak1, Jak2 and Jak3. As these signaling pathways are known to have critical roles in pulmonary eosinophilia, airway hyperreactivity and mucus hypersecretion (Hoshino et al., 2004), upregulation of these components could potentially provide additive or synergistic effects in the presence of other triggers, including environmental or household allergens.

5. Conclusions

Our results suggest two main points. First, Salton Sea exposure is unable to generate an inflammatory response similar to a potent allergen, as characterized in our study by *Alternaria*. However, aerosolized Salton Sea was able to trigger an inflammatory response distinct from a potent allergen, unlike aerosolized Pacific Ocean water, which did not trigger an inflammatory response. Thus, while Salton Sea spray may not be sufficient to generate asthma alone, it could play a key role in the progression to asthma or other inflammatory diseases. Future studies should explore the role of this inflammatory response in the context of the full range of aerosols to which the communities surrounding the Salton Sea are exposed.

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CRediT authorship contribution statement

Trevor A. Biddle: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Qi Li:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Mia R. Maltz:** Conceptualization, Formal analysis, Methodology, Software, Visualization, Writing – original draft. **Purvi N. Tandel:** Data curation, Visualization. **Rajrupa Chakraborty:** Data curation, Formal analysis, Methodology, Visualization. **Keziyah Yisrael:** Data curation. **Ryan Drover:** Data curation, Formal analysis. **David R. Cocker:** Supervision. **David D. Lo:** Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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