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UNIVERSITY OF CALIFORNIA SAN DIEGO

Acute Stress Decreases Innate Lymphoid Cell Subpopulations During Murine Lung Inflammation

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Payton Kyle Jensen

Committee in charge:

Professor Taylor A. Doherty, Chair
Professor Steven P. Briggs, Co-Chair
Professor Carolyn M. Kurle

2024

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The Thesis of Payton Kyle Jensen is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

DEDICATION

I would like to thank my mom, dad, and little sister for their encouragement, love, and support throughout my academic career. They have taught me more about life than any biology class ever could.

I would like to express my utmost gratitude and appreciation to Dr. Doherty for providing me with this incredible opportunity to conduct my master's research project in his lab. His guidance, leadership, and expertise has allowed me to prosper as a researcher and I am very grateful for his support over the years.

I would also like to acknowledge Steven Briggs and Carolyn Kurle for their participation as my committee members. Their lessons, feedback, and critiques will forever contribute to the quality and rigor of my work moving forward.

Next, I would like to thank Allyssa Strohm, Yung-An Huang, and Xinyu Wang for their incredible mentorship during my time in the Doherty lab. I truly appreciate the supportive role they've played in my personal and professional growth as a scientist. Their expansive knowledge of the subject matter along with their dedication to my personal development has helped make this research possible.

Finally, I want to thank the rest of the Doherty lab team for their assistance during experiments and for the close friendships we've made during my time at UCSD. Their dedication and passion for biology have helped me become a better scientist and I cannot express how proud I am to call these amazing people my colleagues and friends.

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LIST OF ABBREVIATIONS

Alt	*Alternaria alternata*
BPC	Burn Pit Constituents
BAL	Bronchoalveolar Lavage
COPD	Chronic Obstructive Pulmonary Disease
DMSO	Dimethyl sulfoxide
iILC	Inflammatory Innate Lymphoid Cells
ILC	Innate Lymphoid Cells
PAH	Polyaromatic Hydrocarbon
scRNA	Single-Cell Ribonucleic Acid
TCDD	2,3,7,8 tetrachlorodibenzo-p-dioxin

ACKNOWLEDGEMENTS

Results discussed here are currently under review for publication.

This thesis contains material as it appears in the paper that has been published titled, "*Short-term Exposure to Burn Pit Combustion Products Induces Neutrophilia and Expansion of ILC1-like Cells during Lung Inflammation in a STING-Dependent Manner*" by Jong Chan Kim

This thesis also contains material as it appears in the submitted manuscript, currently under review, titled, "*PIP-Seq Identifies Novel Heterogeneous Lung Innate Lymphocyte Population Activation after Combustion Product Exposure*" by Yung-An Huang.

VITA

2023 Bachelor of Science in Microbiology, University of California San Diego

2024 Master of Science in Biology, University of California San Diego

ABSTRACT OF THE THESIS

Acute Stress Decreases Innate Lymphoid Cell Subpopulations During Murine Lung Inflammation

by

Payton Kyle Jensen

Master of Science in Biology

University of California San Diego, 2024

Professor Taylor A. Doherty, Chair
Professor Steven P. Briggs, Co-Chair

Veterans returning from deployment in the Middle East have displayed chronic asthmatic-like symptoms after exposure to burn pits and sandstorms. These soldiers also encounter stressful environments during deployment, introducing another factor that could alter their immune response. In this thesis, we have investigated the impact of acute stress on lung inflammation in murine models, focusing on the role of innate lymphoid cells (ILCs) during

exposure to the allergen *Alternaria alternata* (*Alt*) and burn pit constituents (BPCs) which include dioxins, hydrocarbons, and particulate matter.

Our previous studies have shown that combinatory exposure to Alt and BPCs induces a mixed type 2 and non-type 2 inflammatory response, characterized by increased neutrophilia and a shift from ILC2 to ILC1-like populations. Single-cell RNA sequencing (scRNA-seq) of lung samples showed upregulation of neutrophilia-associated genes and stress-related genes in ILCs from Alt+BPC-challenged mice, indicating that stress exacerbates inflammatory responses.

Here, we present a novel model to reflect an acute stress event followed by inhalation of BPCs or Alt focusing on analysis of lung granulocytes and innate lymphoid cells. Results indicated a significant decrease in different ILC subpopulations of stressed mice after either allergen or BPC exposure. This decline reflects a significant reduction in neutrophilia during non-type 2 inflammation and trending decreases in eosinophilia during type 2 inflammation.

This study concludes that acute stress alters the immune response to lung inflammation. These findings suggest that stress, combined with BPC exposure, might contribute to the respiratory issues observed in exposed military personnel.

INTRODUCTION

In US military bases throughout the Middle East, burn pits are utilized to dispose of munition and domestic waste products, exposing many soldiers to hazardous combustion air pollutants (Olsen et al., 2022). Soldiers returning from deployment have reported various respiratory illnesses linked to burn pit exposure, including an elevated risk of asthma (Sharkey et al., 2016). Notably, previously healthy soldiers returned from deployment with dyspnea after exposure to burn pits and sandstorms. The majority of these soldiers had characteristics indicative of asthma including airway hyperresponsiveness and distal airway narrowing (Olsen et al., 2022). In severe cases, some had constrictive bronchiolitis and severe pulmonary fibrosis (Olsen et al., 2022). Lung biopsies of those severe cases revealed the presence of polycyclic aromatic hydrocarbons (PAHs), a known burn pit combustion product. A recent study shows that burn pit combustion products typically encompass a mixture of PAHs, dioxins from incomplete combustion, and particulate matter (Olsen et al., 2022). Further, sandstorms can disperse various types of allergens in the air, adding another possible trigger for this phenotype we observe in these soldiers (Yarahmadi et al., 2020).

In addition to chronic exposure to burn pits and allergens, soldiers also endure stressful environments related to their work. Immunologically, stress can result in a spectrum of effects depending on its intensity and duration (Dhabhar, 2014). Acute stress (stress that lasts a matter of minutes to hours) impacts the immune system differently compared to chronic stress (stress that can last for days, months, and even years). Acute stress often occurs in everyday life, which can be both beneficial and harmful. While acute stress may increase immunoprotection by amplifying white blood cell recruitment to infection sites, it can also heighten inflammation and immunopathology following antigen exposure (Viswanathan & Dhabhar, 2005; Tang et al.,

2022). On the other hand, chronic stress is known to suppress leukocyte proliferation, antibody production, and NK cell activity, hindering the immune system's ability to fight off infection (Dhabhar, 2014). Acute stress has been documented to increase neutrophil counts while decreasing both T and B-cell infiltration in the peripheral blood of mice treated with LPS (Tang et al., 2022). While combat scenarios have been documented as chronic, the collective effect of acute high-stress environments and air pollutant exposure on asthma remains largely unexplored.

Asthma is a heterogeneous disease categorized by either a type 2 immune response or a non-type 2 immune response. Type 2 immune responses are classically regulated by CD4+ T-helper 2 (Th2) cells which secrete protein messengers, called cytokines, including IL-4, IL-5, and IL-13 that drive tissue eosinophilia and mucus production (Voehringer, 2006). Conversely, non-type 2 asthma is typically characterized by high neutrophilia and mediated by Th1 and Th17 cells which secrete cytokines such as IL-17 and IFN-**γ** (Boonpiyathad et al., 2019)

Recent studies have highlighted the roles of innate lymphoid cells (ILCs) during both type 2 and non-type 2 asthma responses (Fort, 2006; Fallon, 2001). Functioning as innate counterparts to adaptive Th2 cells, ILC2s mount immediate responses compared to the adaptive T-cell response. ILCs are stationed within tissues and react to alarmin cytokines released upon tissue damage. ILCs are identified as CD45 positive, Lineage negative, and Thy-1.2 positive lymphocytes. Distinguished by the cytokines they produce, ILC subsets, including ILC1s, ILC2s, and ILC3s, mirror the responses of Th1, Th2, and Th17 cells, respectively. Importantly, ILCs play pivotal roles in asthma pathogenesis, as their activation by alarmins can trigger both non-type 2 and type 2 cytokine production (Paul and Zhu, 2010; Lambrecht et al., 2019).

Upon exposure to various allergens such as the fungal allergen *Alternaria alternata*, respiratory epithelial tissues initiate a cascade of responses, releasing alarmin proteins including

IL-33, thymic stromal lymphopoietin, and/or IL-25 (Divekar & Kita, 2015; Vivier et al. 2018). These alarmins activate ILC2s and trigger the production of type 2 cytokines (Divekar & Kita, 2015; Vivier et al. 2018). As a result, ILC2s secrete IL-5 and IL-13, contributing to downstream effects such as influx of eosinophils, mucus production, and airway hyperresponsiveness, which are hallmarks of the typical type 2 asthmatic phenotype (Paul and Zhu 2010; Lambrecht et al. 2019). Furthermore, these cytokines exert chemoattractant effects, drawing in other immune cell types, including eosinophils, Th2 cells, dendritic cells, and others to promote type 2 inflammation. We identify conventional ILC2s as being CD127+ST2+. However, several other ILC2 surface marker phenotypes exist including, inflammatory ILC2s (iILC2s) that are activated by IL-25 and lack the IL-33 receptor (ST2). The extent of CD127 expression on iILC2s is currently unknown. Interestingly, iILC2s can produce IL-17 making them ILC3-like cells and highlighting the plasticity that exists in ILC populations (Huang et al., 2014).

ILC3s are typically activated during lung infection by bacteria, viruses, and helminths, however, COPD (chronic obstructive pulmonary disease) murine models also indicate a prominent role of ILC3s (Hsu et al., 2021). In response to these pathogens, dendritic cells, inflammatory monocytes (IMs), and macrophages produce alarmins IL-1β or IL-23 to activate ILC3s (Yang et al., 2021). ILC3s have a homeostatic role through their production of IL-22 and IL-17A. IL-22 assists in preventing lung fibrosis and reducing lung airway inflammation while IL-17A is a pro-inflammatory cytokine that induces lung neutrophilia commonly found in COPD (Felton et al., 2018; Anthony et al., 2013). ILC3s are contained in the CD127+ST2- population.

Eosinophils and neutrophils are CD45+ leukocytes that release granule mediators that are initially beneficial for anti-microbial responses, aiding in the elimination of pathogens and inducing airway inflammation. However, over time, the persistence of inflammatory mediators

released by these granulocytes can become toxic to epithelial cells, exacerbating inflammation (Mincham et al., 2021; Minai-Fleminger & Levi-Schaffer, 2009). Neutrophils are isolated as CD45+SiglecF-Ly6G+, while eosinophils are CD45+SiglecF+Ly6C-CD11b+ and CD11c-.

Our laboratory has primarily focused on elucidating the role of ILCs during airway type 2 inflammatory responses. To better understand the effects of ILCs after burn pit exposure, we examined the effects of key burn pit constituents in an asthmatic mouse model. Our novel burn pit constituent (BPC) model contains a mixture of particulate matter (PM_4), dioxins (2,3,7,8-TCDD), and polyaromatic hydrocarbons (BaP in DMSO) which are reflective of combustion products found in biopsies of soldiers exposed to burn pits (Olsen et al., 2022). To investigate the combinatory role BPCs and type-2 allergens have on lung inflammation, *Alternaria alternata*, an allergen common to dust storms in the Middle East, was added (Yarahmadi et al., 2020).

In mice intranasally challenged with an *Alternaria* and BPC mixture (Alt+BPC), we noted increased airway neutrophilia in addition to preserved eosinophilia, suggesting the presence of a non-type-2 and type-2 innate immune response. To characterize ILC responses during the non-type-2 and type-2 responses, we analyzed ILC subpopulations and discovered a modest ILC2 to ILC1 shift (Kim, 2023). Single-cell RNA (scRNA) sequencing was performed with mouse lung samples enriched for ILCs after exposure to Alt+BPC or Alt to broadly investigate the expression markers of inflammatory cells responsible for this phenotype. We found that several ILC and natural killer cell populations from Alt+BPC-challenged mice upregulate genes associated with neutrophilia and airway remodeling compared to the *Alternaria* control (Huang et al., Unpublished). Additional analysis of Alt+BPC scRNA revealed that stress-related genes were upregulated in ILC1 and ILC2 subpopulations, indicating that cellular stress could play an important role in the phenotype observed in soldiers (Huang et al., Unpublished).

These findings provided valuable insights that laid the groundwork for our investigation into burn pit exposure and its potential contribution to the asthmatic phenotype. However, the influence of stress on allergic asthma or BPC exposure remains largely unexplored.

In this study, we investigated the effect of acute stress by implementing a six-hour restraint followed by a three-day intranasal challenge with the designated Alt or BPC. In mice challenged with Alt, restraint had minimal effects on overall granulocytes, only depicting trending decreases in eosinophilia. However, we noted decreased overall ILC counts specifically in ILC3-like population (which could include iILC2s), and ILC2 populations. To further investigate the role of stress on BPC exposure, mice were subjected to the same restraint model and were intranasally challenged with BPCs. We found that the restraint groups contained significantly lower neutrophils in the airway compared to the high neutrophilia found in previous studies. To identify the cause of this decreased neutrophilia in the airway, we isolated ILC subpopulations and discovered that both ILC1 and ILC3-like cell populations decreased after restraint. These findings suggests that in type 2 and non-type 2 immune responses, acute stress can reduce ILC subpopulations corresponding to a decline in the dominant granulocyte populations.

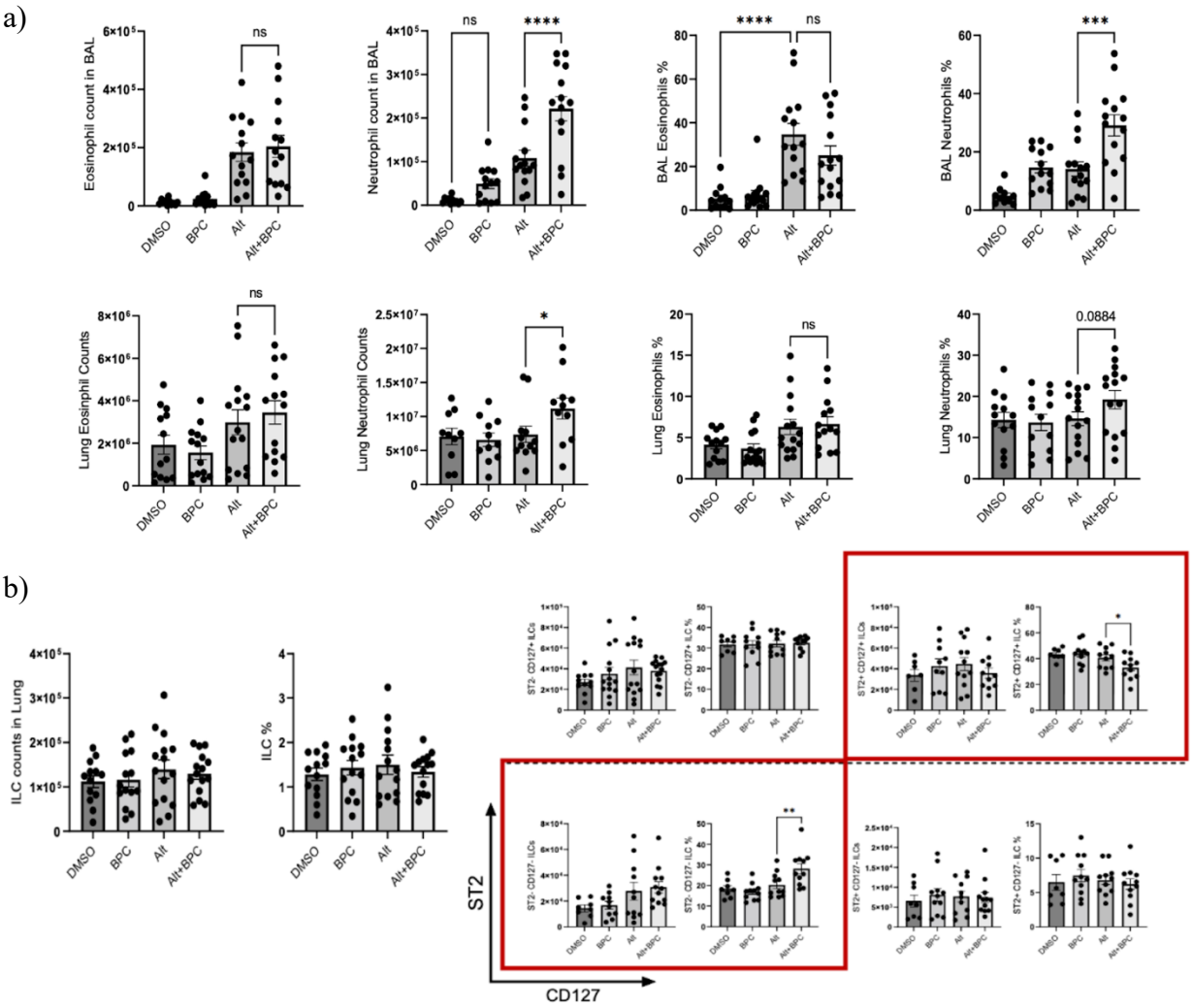


Figure 1: Burn Pit exposure induces neutrophilic inflammation and ILC2 to ILC1-like shift in the lungs.

BAL (Bronchoalveolar lavage) fluid and whole lung samples of wild-type mice intranasally challenged for three days with an Alt+BPC mixture and euthanized on the third day. Samples were collected and tagged with fluorescently labeled antibodies for quantification of cell populations. DMSO serves as a vehicle control while Alt serves as a positive control. (A) Eosinophil and neutrophil total cell count and percent in the vehicle control DMSO (Dimethyl sulfoxide), BPC (burn pit constituent), Alt (*Alternaria alternata*), and Alt+BPC challenged mice. (B) Cell counts and percent of total ILCs in the Lungs. ILC subsets depicting total cell counts and percent of total ILCs are shown. ILC subsets are categorized based on CD127 and ST2 expression. Percents of eosinophils, neutrophils, and total ILCs are represented as a ratio of gated cells in the CD45+ population. BPC contains a pollutant mixture described in the methods. Bars depict the mean while the error bars represent SEM. Significance was determined using one-way ANOVA followed by Fisher's LSD test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Published data courtesy of Jong Chan Kim, 2023.

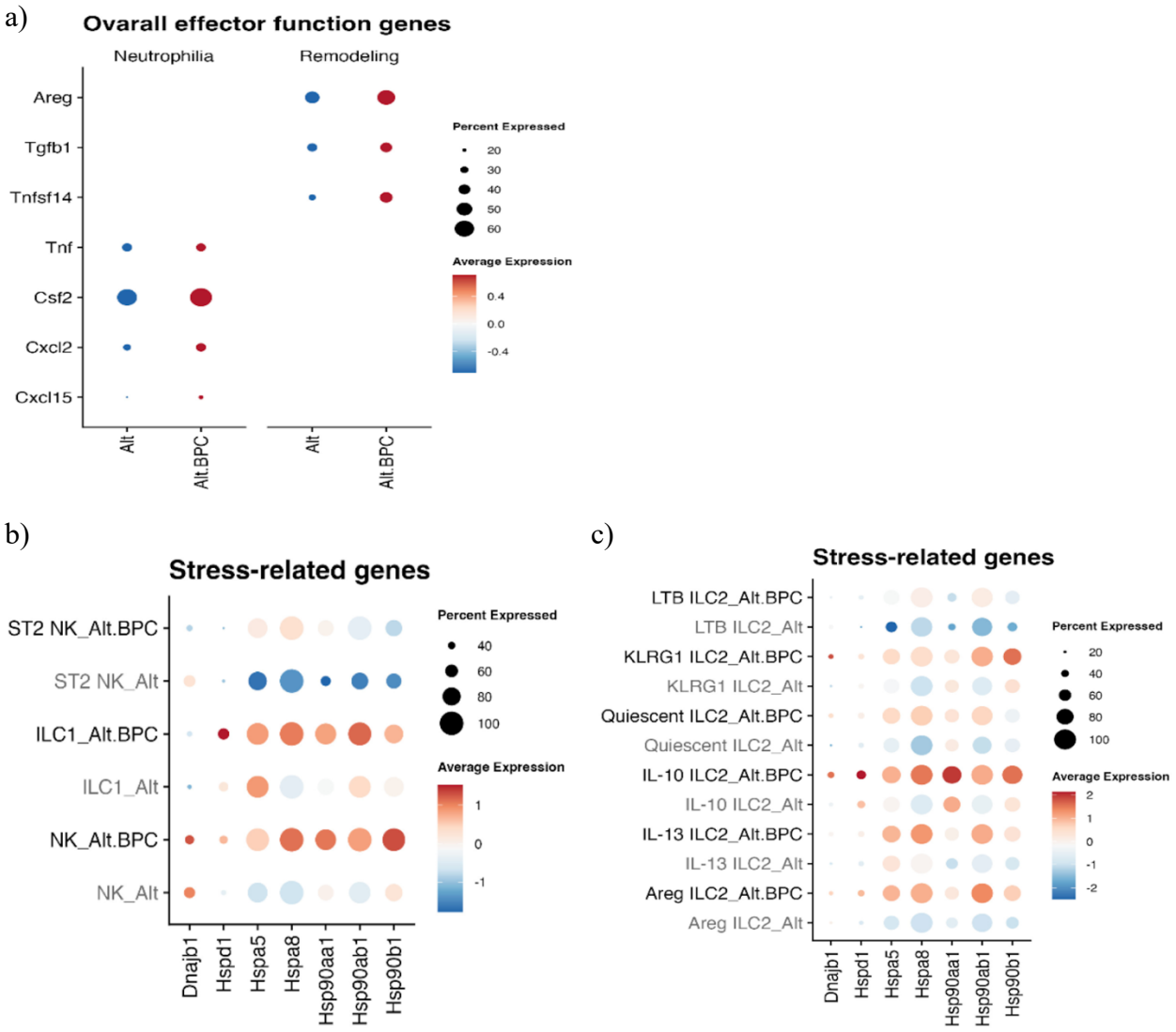


Figure 2: Single-cell RNA sequencing of Alt+BPC and Alt control-challenged mice

Wild-type mice intranasally challenged with Alt or Alt+BPC for three consecutive days had lung samples collected and placed in a single-cell suspension. ILC-enriched samples were preserved for single-cell RNA (scRNA) sequencing. (A) Average and percent scRNA expression of genes associated with neutrophilia and airway remodeling in Alt and Alt+BPC challenged mice specifically highlighting gene markers associated with neutrophilia (tnf, csf2, cxcl2, cxcl15) and airway remodeling (Areg, Tgfb1, Tnfsf14). (B) Average and percent scRNA expression of stress-related genes among NK and ILC1 subsets in Alt and Alt+BPC challenged mice. (C) Average and percent scRNA expression of stress-related genes among ILC2 subsets in Alt and Alt+BPC challenged mice. Unpublished data courtesy of Yung-An Huang.

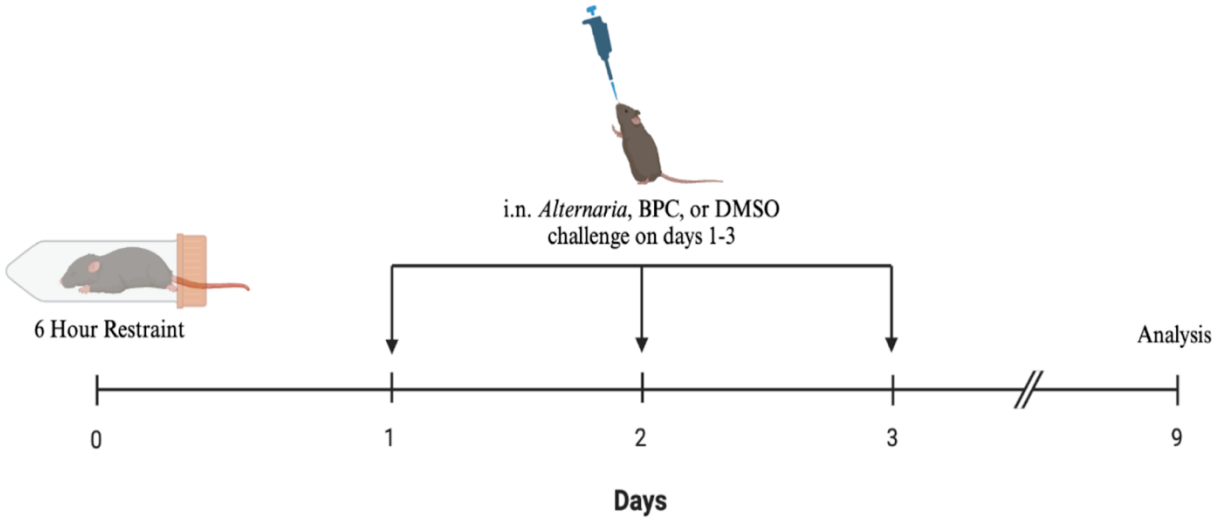


Figure 3: Restraint stress and lung inflammation model.

A murine timeline depicting a 6-hour restraint in a 50 mL falcon tube followed by single dose intranasal (i.n.) challenges of *Alternaria alternata*, BPC, or DMSO for three consecutive days. Mice are euthanized and analyzed 6 days after the final intranasal challenge.

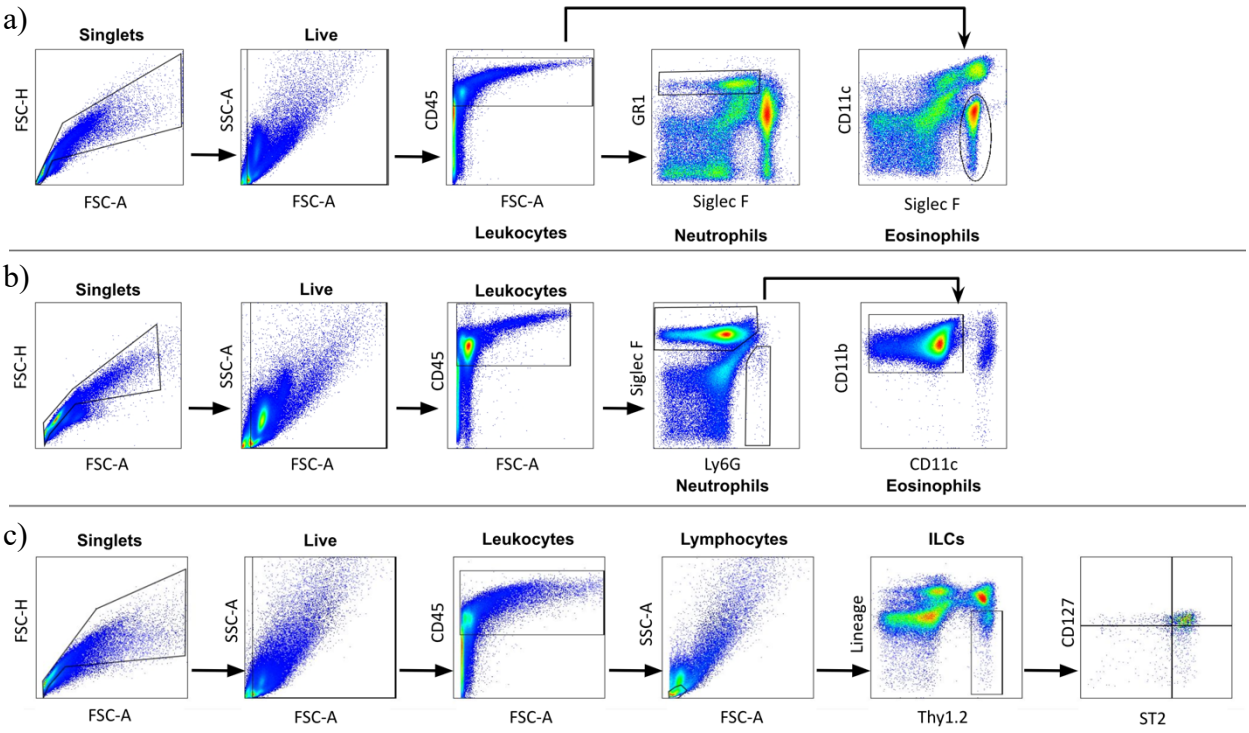


Figure 4: Gating schemes utilized to isolate granulocytes and ILC subsets in the lung and BAL.

Analysis of fluorescently labeled BAL and lung samples using flow cytometry to isolate specific cell populations. (A) Our traditional granulocyte gating scheme is used to isolate neutrophils and eosinophils in the BAL and lung. The first and second gates delineate singlet cells and live cells respectively. CD45+ cells are categorized as leukocytes with hematopoietic origin. Of that CD45+ population, neutrophils were gated as GR1+SiglecF- while eosinophils were gated as CD11c-SiglecF+. (B) The revised granulocyte gating scheme was used to isolate neutrophils and eosinophils in the BAL and lung. The first, second, and third gates serve the same function as in A, however, neutrophils were gated as SiglecF-Ly6G+ while eosinophils were gated as SiglecF+Ly6G-CD11b+CD11c- leukocytes. (C) Our gating scheme was utilized to isolate lung ILCs and ILC subsets with the first, second, and third gates serving the same function as in A and B. Lymphocytes were then sorted based on size (FSC) and granularity (SSC), then ILCs were gated as Lineage-Thy1.2+. ILC subsets were further categorized based on CD127 and ST2 expression.

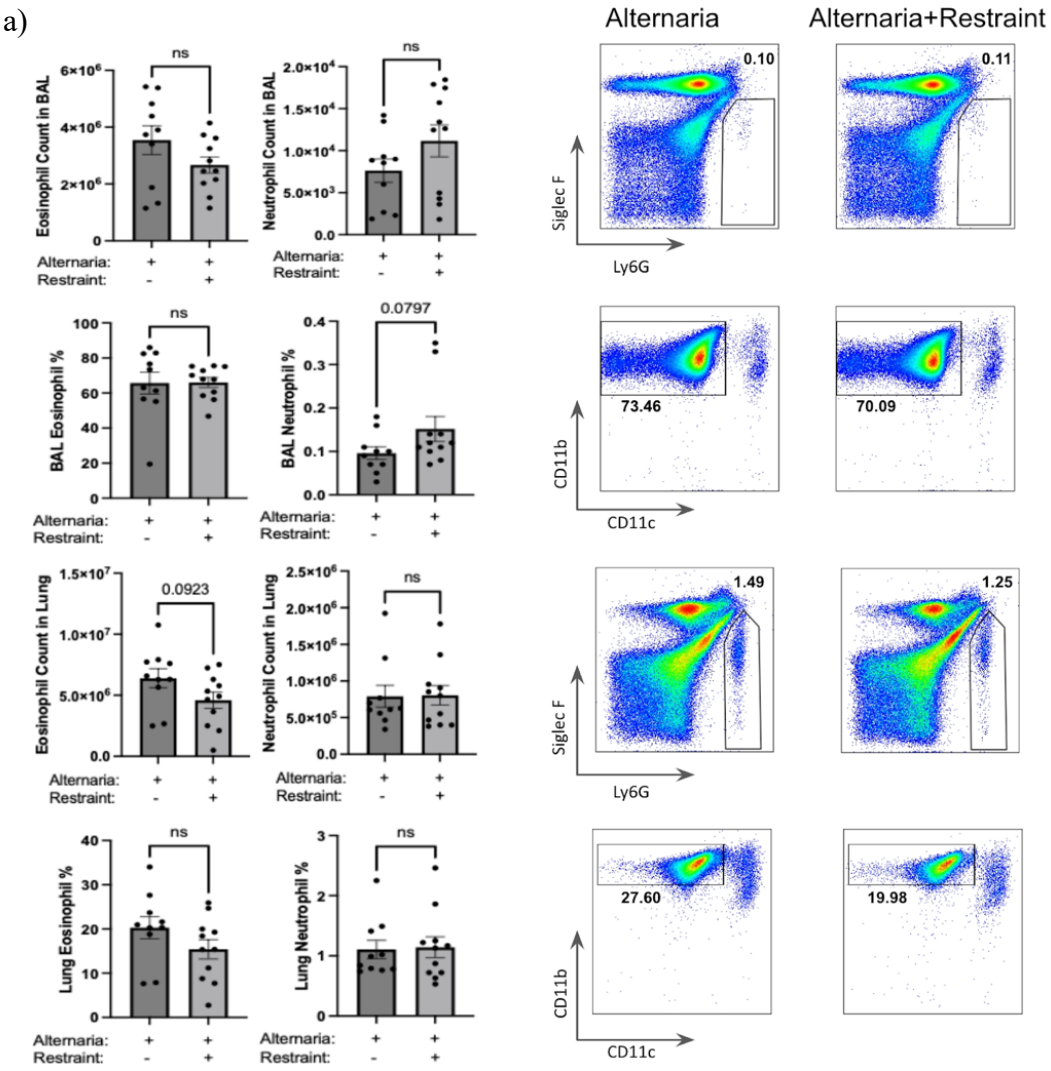


Figure 5: Stress and Alternaria exposure induces decreases in ILC2 and CD127+ST2- ILC populations

Wild-type mice were restrained for 6 hours to mimic acute stress. They were then intranasally challenged for 3 consecutive days with Alternaria alternata (Alt) and euthanized six days later. Their BAL and lung samples were fluorescently labeled to isolate cell populations using flow cytometry. An Alt control without restraint was used to isolate the effects of restraint. (A) Total count and percent of neutrophils and Eosinophils in the BAL fluid and lung of restraint-induced stressed mice challenged with Alt. Respective flow cytometry plots are depicted alongside the bar graphs with a number next to the gate representing its percentage of CD45+ cells. (B) Total count and percent of ILCs and ILC subpopulations in the lung of restraint-induced stressed mice challenged with Alt. Representative flow cytometry plots are depicted with numbers in each quadrant representing the percent of the total ILC population. Bars depict the mean while the error bars represent SEM. Significance was determined using one-way ANOVA followed by Fisher's LSD test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

b)

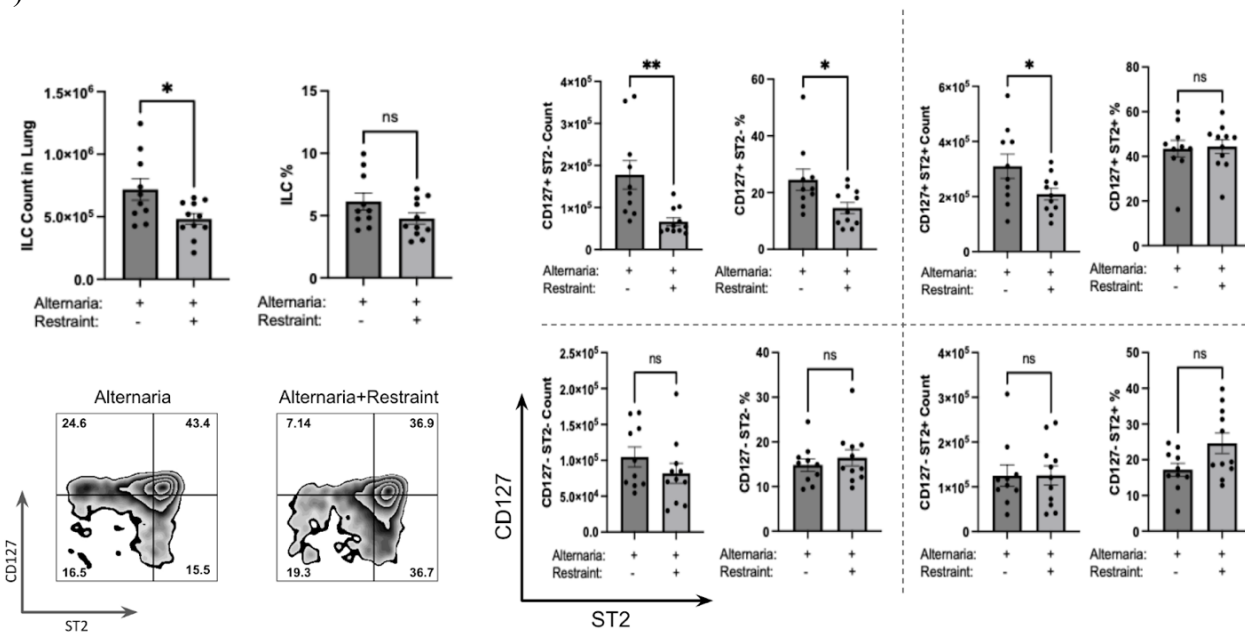


Figure 5 continued: Stress and Alternaria exposure induces decreases in ILC2 and CD127+ST2- ILC populations

Wild-type mice were restrained for 6 hours to mimic acute stress. They were then intranasally challenged for 3 consecutive days with Alternaria alternata (Alt) and euthanized six days later. Their BAL and lung samples were fluorescently labeled to isolate cell populations using flow cytometry. An Alt control without restraint was used to isolate the effects of restraint. (B) Total count and percent of ILCs and ILC subpopulations in the lung of restraint-induced stressed mice challenged with Alt. Representative flow cytometry plots are depicted with numbers in each quadrant representing the percent of the total ILC population. Bars depict the mean while the error bars represent SEM. Significance was determined using one-way ANOVA followed by Fisher's LSD test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

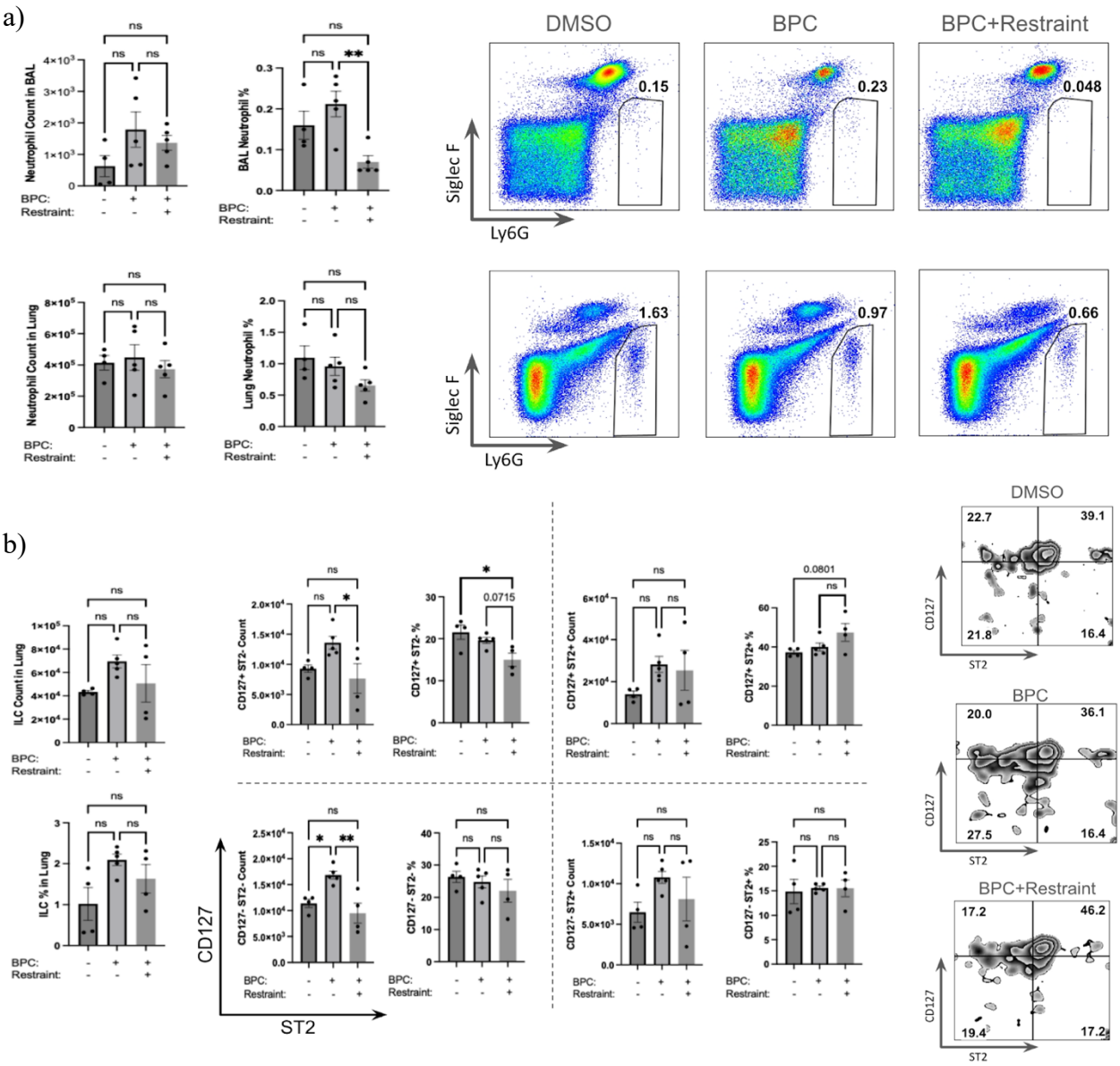


Figure 6: Stress and Burn Pit exposure induce decreases in neutrophilia, ILC1, and CD127+ST2- ILC subpopulations.

Wild-type mice were restrained for 6 hours to mimic acute stress. They were then intranasally challenged for 3 consecutive days with either DMSO (vehicle) or BPCs and euthanized six days later. Their BAL and lung samples were fluorescently labeled to isolate cell populations using flow cytometry. (A) Total count and percent of neutrophils in the BAL fluid and lung of restraint-induced stressed mice challenged with BPCs. Respective flow cytometry plots are depicted alongside the bar graphs with a number next to the gate representing its percentage of CD45+ cells. (B) Bar graphs quantifying the total count and percent of ILCs and ILC subpopulations in the lung of restraint-induced stressed mice challenged with BPCs. Representative flow cytometry plots are depicted with numbers in each quadrant representing the percent of the total ILC population. Bars depict the mean while the error bars represent SEM. Significance was determined using one-way ANOVA followed by Fisher's LSD test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

To assess the combinatory role of BPCs and *Alternaria* on type 2 lung inflammation, mice were intranasally challenged over three days with an Alt+BPC mixture. DMSO in PBS was administered as vehicle control, Alternaria dose of 20 µg was dissolved in DMSO or in PBS, and BPC contained 20µg $PM_{4.0}$, 24 ng of 2,3,7,8-TCDD, and 5 ng BaP in DMSO. Bronchoalveolar lavage (BAL) fluid was collected to assess cell populations in the airway while whole lung samples were processed to gain a broad perspective of the entire lung. Both samples were surface stained with fluorescent antibodies and analyzed using flow cytometry in order to specifically analyze granulocytes and ILCs (Figure 4a; Figure 4c).

In the airway, leukocyte-derived (CD45+) SiglecF+CD11c- eosinophils were observed in the Alt+BPC challenged group, which is indicative of a type 2 response, but significant change compared to the *Alternaria* (Alt) control group was not observed (Figure 1a). However, substantial CD45+GR1+SiglecF- neutrophil infiltration in the BAL can be seen in the Alt+BPC group, indicating a neutrophilic non-type 2 response (Figure 1a). In the lung, significant increases in neutrophil numbers can be observed in Alt+BPC mice while eosinophil numbers remain similar to the Alt-only group (Figure 1a). Lineage-Thy1.2+ ILCs remain constant overall, but interestingly CD127+ST2+ ILC2s decrease while CD127-ST2- ILC1-like populations increase among the Alt+BPC group compared to the Alt-only group (Figure 1b). When we take into consideration the consistent overall ILC cell counts and percent, these findings support the concept of an ILC2 to ILC1-like shift occurring within the Alt+BPC group.

To gain a broader understanding of genomic expression associated with Alt+BPC exposure, single-cell RNA (scRNA) sequencing of lung cells from mice challenged with Alt+BPC and *Alternaria* only was performed (Figure 2). Transcription factors, cytokine, and chemokines associated with neutrophilia (*tnf, csf2, cxcl2, cxcl15*) and airway remodeling (*Areg,*

Tgfb1, Tnfsf14) were upregulated in the combinatory Alt+BPC group (Figure 2a). ILC1 and ILC2 populations were further categorized and analyzed for changes in stress-related gene expression. All subsets of the ILC1 and ILC2 populations demonstrated upregulation of stress-related genes in Alt+BPC mice (Figure 2b and 2c). Cellular stress of innate cells could result in immune system exhaustion. Considering the physical stress soldiers encounter on the battlefield, this could result in a synergistic effect between cellular stress and allergen exposure on the innate immune system.

To assess the synergistic effects of Alt and BPC-related cell stress while also accurately reflecting the stressful environment soldiers endure, we introduced an acute six-hour restraint to the mice model (Figure 3). In this model, mice were restrained once in a 50 mL conical tube for 6 hours and then intranasally challenged the next day with Alt or BPC over three consecutive days. Various iterations of the stress model with different endpoints were tested, however, to minimize individual differences between mice, the one-week endpoint depicted in Figure 3 was adopted. To better separate neutrophils from myeloid cells such as monocytes, we used individual Ly6C and Ly6G antibodies instead of GR1 which binds to both Ly6C and Ly6G. Ly6G has been reported to be a more specific marker for murine neutrophils (Misharin et al., 2013; Viswanathan & Dhabhar, 2005). Figure 4b depicts this revision to our gating scheme. BAL and lung samples were taken from intranasally challenged mice and surface stained for flow cytometric analysis.

We first assessed the effects of *Alternaria* exposure and restraint stress to evaluate how stress alone can influence a type-2 immune response. Mice were challenged via intranasal administration of 30 μg of *Alternaria* for 3 days. We found no statistical difference in the numbers of SiglecF+Ly6C-CD11b+CD11c- eosinophils and SiglecF-Ly6G+ neutrophils in both

BAL and lung samples in restrained mice compared to mice that did not undergo restraint. However, we do notice a trending decline in eosinophils in both BAL and lung samples (Figure 5a). In our lymphocyte populations, we found a significant reduction in Lineage-Thy1.2+ ILC populations with restraint while the proportions of ILCs in total leukocyte populations remained insignificant with restraint. Specific subpopulations of ILCs including CD127+ST2+ ILC2s, and CD127+ST2- ILC3-like cells saw a significant decrease with restraint (Figure 5b). The CD127+ST2- ILC3-like cells decreased in both number and percentage while CD127+ST2+ ILC2s only displayed a significant decrease in total cell counts with restraint (Figure 5b).

We then focused on the effects of burn pit constituents and stress on lung inflammation. We utilized DMSO as a vehicle for burn pit constituent intranasal administration. BPC alone induced a neutrophilic dominant response in the airway, but with restraint, SiglecF-Ly6G+ neutrophils were significantly reduced (Figure 6a). Although total CD45+Lineage-Thy1.2+ ILC populations remained similar among the two groups, ILC subpopulations depicted some significant differences. Specifically, within the CD127-ST2- ILC1 and CD127+ST2- ILC3-like populations, overall cell counts were reduced significantly with restraint (Figure 6b). The proportion of leukocyte-derived cells did not decrease with restraint in ILC subpopulations or granulocyte populations.

The consistent decline in CD127+ST2- ILC3-like populations in two immunologically distinct models of airway inflammation (Both Alt and BPC restraint models) indicates that acute stress could reduce CD127+ST2- ILC3-like populations in the lung. These results also indicate that stress can reduce neutrophilia in non-type 2 lung inflammation through the depletion of CD127+ST2- ILC3-like cells and CD127-ST2- ILC1s.

DISCUSSION

Our study aimed to model some of the key conditions soldiers might experience leading to respiratory issues attributed to burn pit constituents (BPCs) and other environmental factors encountered in the Middle East (Olsen et al., 2022). By developing an innate murine model to reflect combined BPC and allergen exposure, we observed a mixed type 2 and non-type 2 inflammatory response in the lungs. Intranasal challenges with *Alternaria alternata* (*Alt*) and BPCs over three days followed by BAL fluid and whole lung sample analysis revealed significant insights into the cellular mechanisms involved.

Preliminary work from our lab showed that exposure to Alt+BPCs led to a heightened neutrophilic response (CD45+GR1+SiglecF- neutrophilia) compared to controls (Figure 1a) using the gating scheme depicted in Figure 4a (Kim, 2023). This was accompanied by stable eosinophilic levels (CD45+SiglecF+CD11C- eosinophilia) typically associated with type 2 inflammation. Isolation of ILCs and their subpopulations revealed that the increased neutrophilia was correlated to ILC dynamics in the lung, specifically a shift from CD127+ST2+ ILC2s to CD127-ST2- ILC1-like cells (Kim, 2023). Eosinophil granules, which can alter smooth muscle contraction and promote oxidative stress, might contribute to the respiratory phenotype observed in soldiers exposed to burn pits (Minai-Fleminger & Levi-Schaffer, 2009). Additionally, the neutrophilic influx and airway fibrosis impact found in COPD models could be relevant given the similarities between BPCs and cigarette smoke constituents (Mincham et al., 2021; Fowles & Dybing, 2003).

Single-cell RNA sequencing (scRNA-seq) data from Huang et al provided an unbiased genomic foundation for our lab's observations, revealing the upregulation of cytokines, chemokines, and transcription factors linked to neutrophilia and airway remodeling in Alt+BPC-

challenged mice. Notably, stress-related genes were upregulated in ILC1 and ILC2 cells, indicating that stress plays a crucial role in modulating immune responses (Huang et al., Unpublished). The upregulation of heat shock proteins (hsps) suggests a protective mechanism against protein misfolding due to stress (Muralidharan & Mandrekar, 2013).

In mice challenged with Alternaria alternata, we saw no significant differences in leukocyte-derived SiglecF+Ly6C-CD11b+CD11c- eosinophilia or SiglecF-Ly6G+ neutrophilia with restraint when compared to the no restraint control. However, we do notice a trending decrease in the total number of eosinophils in the BAL and lung samples. In our lymphocyte population, we observed a significant decline in total CD45+Lineage-Thy1.2+ ILCs. Among these ILC subpopulations, there was also a significant decrease in the CD127+ST2- mixed ILC3-like population and CD127+ST2+ ILC2 population. This decrease in CD127+ST2+ ILC2s with restraint reflects the trending decline in SiglecF+Ly6C-CD11b+CD11c- eosinophils in the lung and BAL samples. A decline in CD127+ST2- iILC2s, progenitor cells for mature ILC2s, could explain this decrease in ILC2 effector cells and overall ILC populations since ILC2s make up the biggest proportion of ILCs (Yang et al., 2021). These findings suggest that acute stress could impair the immune system's ability to mount an effective ILC2-derived type 2 response.

In the context of non-type 2 lung inflammation induced by BPCs, acute stress led to a significant reduction in CD45+SiglecF-Ly6G+ neutrophils. The decline in ILC1s and CD127+ST2- ILC3-like mixed populations likely contributes to this reduced neutrophilia. IL-17 production by ILC3-like cells and IFN-γ from ILC1s are known to promote neutrophilic infiltration, suggesting that their decrease due to stress results in less neutrophilic inflammation (Lindén, 2001; Hamid & Tulic, 2009). Using this non-type 2 driven acute stress model as a reference, long-term consequences of reduced neutrophilia after stress could result in a skew

towards type 2 inflammation with chronic stress. However, this hypothesis needs to be tested in chronic models to accurately understand the effects of chronic stress and BPC exposure on lung airway inflammation.

This study demonstrates that acute stress significantly downregulates specific ILC subpopulations thus impacting both type 2 and non-type 2 lung inflammation. The depletion of CD127+ST2- mixed ILC populations, which could include progenitor, multipotent iILC2s, and other ILC3-like cells, offers an explanation for the observed alterations in immune responses under stress. While our murine model is used to mimic stress and BPC exposure in soldiers, it may not accurately encompass the full experience of these soldiers during their deployment. Additionally, this model lacks a chronic stress component which could have different impacts on the innate immune response and may reflect the soldier's experience during deployment more accurately (Viswanathan & Dhabhar, 2005). This study also lacks detailed insights into the ILC and granulocyte kinetics. Cytokine staining of IL-5, IL-17, IFN-γ, and others could explain causal relationships between ILCs and granulocytes. Future research should focus on the specific cell types within the CD127+ST2- mixed population to pinpoint the mechanisms driving these changes. Exploring the effects of Alt+BPC and stress using this model could hopefully provide further insights into the neutrophilic phenotype we explored in earlier experiments. Utilizing scRNA sequencing in stressed Alt+BPC-challenged mice could further elucidate the genomic pathways involved. Additionally, a chronic stress model may provide more comprehensive insights into the lung inflammation phenotype experienced by soldiers returning from deployment, better reflecting their prolonged exposure to stressful environments and potential respiratory hazards.

This thesis contains material as it appears in the paper that has been published titled, "*Short-term Exposure to Burn Pit Combustion Products Induces Neutrophilia and Expansion of ILC1-like Cells during Lung Inflammation in a STING-Dependent Manner*" by Jong Chan Kim

This thesis also contains material as it appears in the submitted manuscript, currently under review, titled, "*PIP-Seq Identifies Novel Heterogeneous Lung Innate Lymphocyte Population Activation after Combustion Product Exposure*" by Yung-An Huang

MATERIALS AND METHODS

Mice

Female C57BL/6J mice aged 6-12 weeks old were obtained from Jackson Laboratories (Bar Harbor, ME). All studies are approved through the University of California, San Diego Institutional Animal Care and Use Committee.

Lung Inflammation and Restraint Models

For mice used in the stress model, stress was induced using 50 mL flacon tubes with angular holes cut into the caps. Wild-type mice were placed into the falcon tube with their tails protruding out of the angular holes in the caps. Their tails were taped down to restrict movement for six consecutive hours. Mice were not physically squeezed and felt no pain. Non-restrained mice were left in their cages for the duration of the experiment.

Mice were then intranasally challenged with Alternaria alternata extract (Greer, Lenoir, NC) diluted in PBS or Burn Pit Combustion products (Alt+BPC challenged mice). Mice were challenged with 30 µg of Alternaria alternata intranasally three times over three days and euthanized at respective time points described in the results. Burn Pit Combustion product was prepared with 0.6ng 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) in 1% DMSO (Accustandard, New Haven, CT) and 20 µg PM4.0 (National Institute of Standards and Technology, Gaithersburg, MD) with 5 ng Benzo[a]pyrene (BaP) (Thermo Fisher Scientific Chemicals, Waltham, MA) in 100% DMSO (Thermo Fisher Scientific Chemicals, Waltham, MA). Mice were challenged with this BPC mixture three times over three days and euthanized at respective time points described in the results. In Alt+BPC, a mixture of 30µg of Alternaria alternata and the BPC mixture described above was intranasally administered to wild-type mice over three days and subsequently euthanized 24 hours later (Kim, 2023)

Single-cell RNA sequencing

Wild-type mice intranasally challenged with Alt or Alt+BPC for three consecutive days had lung samples collected and placed in a single-cell suspension. ILCs from Alt and Alt+ BPC treated mice were enriched from two single-cell suspended lungs by Mouse Pan-ILC Enrichment Kit (STEMCELL Technologies, Vancouver, British Columbia, Canada). ILC-enriched samples were collected in PIPseqTM T100 3′ Single Cell Capture and Lysis Kit and processed via sequencing service (Fluent BioSciences, Watertown, MA) (Huang et al., Unpublished).

Barcode-gene matrices acquired from the PIPseeker pipeline were further analyzed with the R package Seurat (v4.3.0). To exclude doublets and low-quality cell populations, doublets were identified by R package scDblFinder (v1.15.1) and expressed less than 200 or greater than 4000 genes or had mitochondrial genes greater than 5%. These cells were filtered from our dataset (Huang et al., Unpublished).

Alt and Alt+BPC datasets had Seurat's workflow of SCtransform normalization and integration performed to generate the primary clustering at the resolution of 0.3. Uniform manifold approximation and projection (UMAP) was utilized for dimensionality reduction and easier visualization of the data. For deeper ILC analysis, clusters with positive expression of *Ptprc*, a CD45+ marker, and *Thy1*, a CD90 marker underwent a secondary clustering at a higher resolution of 1.0 (Huang et al., Unpublished).

The datasets used are available in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) repository (GSE261844). R scripts are available through GitHub (https://github.com/tdohertylab/Alt.BCP_ILC-enriched-lung_scRNAseq).

BAL and Lung Processing

Bronchoalveolar lavage (BAL) fluid was collected using 2% bovine serum albumin (BSA) (Sigma, St Louis, MO). Supernatant from the first BAL was collected and stored at -20℃ for future ELISA analysis. The whole lung was collected into RPMI and promptly dissociated into a single-cell suspension using the Miltenyi Lung Digest Kit and Dissociator per the company's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were counted using the Novocyte flow cytometer based on size and granularity (ACEA, San Diego, CA).

Flow Cytometry

One million lung and BAL fluid cells were stained for surface staining, with the utilization of CD16/32 (Biolegend, San Diego, CA) to block Fc receptors for 5 minutes. In the Alt+BPC experiments, Eosinophils were gated as CD45+CD11c-SiglecF+ cells, while neutrophils were gated as CD45.2+Siglec F-GR1+ cells using CD45.2 (PerCP), Siglec-F (PE), GR-1 (APC), and CD11c (FITC) markers and fluorophores. In the stress experiments, eosinophils were gated as CD45+SiglecF+Ly6C-CD11b+CD11c- cells while neutrophils were gated as CD45+SiglecF-Ly6G+ using CD45.2 (PerCP), SiglecF (PE), CD11b (APC), CD11c (FITC), Ly6G (Pacific Blue), and Ly6C (PE-Cy7). ILCs were identified as Lineage-Thy1.2+ lymphocytes and stained using CD45.2 (PerCP), Thy1.2 (Pacific Blue), and a lineage cocktail (FITC). The lineage cocktail comprised markers from Biolegend (CD3e, Ly-6G/Ly-6C, CD11b, CD45R/B220, and TER-119), along with CD11c, NK1.1, CD5, FceR1, TCR-β, and TCR-γδ. ILC subsets were surface stained for ST2 (APC) and CD127 (PE-Cy7) expression.

Statistical Analysis

Statistical analysis was performed with PRISM Software (GraphPad Software, La Jolla, CA). P-values were obtained using the Mann-Whitney test or the unpaired t-test and a P-value of less than 0.05 was considered statistically significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

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