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Mechanotransduction at the Alveolar-Capillary Interface: How Mechanical Strain Modulates Innate Immune Memory and Type 2 Inflammatory Cascades in Chronic Airway Diseases

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ABSTRACT

BACKGROUND: The alveolar-capillary interface is a highly dynamic environment subjected to constant mechanical forces, including cyclic stretch and shear stress. In chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD), altered mechanical strain serves as a potent biophysical cue that transcends mere structural deformation, actively reprogramming the local immune microenvironment. This study investigates the mechanisms by which mechanical strain at the alveolar-capillary interface modulates innate immune memory and triggers Type 2 inflammatory cascades.

METHODS: Utilizing a microfluidic lung-on-a-chip model to simulate physiological and pathological strain, human alveolar epithelial cells and co-cultured immune cells were analyzed. Mechanotransduction pathways, specifically the roles of YAP/TAZ signaling and integrin-mediated adhesion, were evaluated for their impact on epigenetic remodeling in alveolar macrophages. The expression of Type 2 cytokines (IL-4, IL-5, IL-13) and markers of trained immunity were measured following exposure to chronic mechanical stress.

RESULTS: Findings demonstrate that pathological mechanical strain induces a "pro-inflammatory memory" in alveolar macrophages via YAP/TAZ-dependent epigenetic reprogramming. This strain-induced activation significantly upregulates the secretion of alarmins (IL-33, TSLP), which promote the maturation of Type 2-biased dendritic cells. Furthermore, mechanical loading was shown to directly enhance the sensitivity of alveolar epithelial cells to allergens, thereby lowering the threshold for initiating Type 2 inflammatory cascades.

CONCLUSION: Mechanical strain at the alveolar-capillary interface acts as a fundamental regulator of innate immune memory. By driving epigenetic shifts in resident immune cells

and amplifying alarm-mediated signaling, pathological strain perpetuates chronic inflammation. Understanding these mechanotransduction pathways offers a novel therapeutic frontier for dampening Type 2 responses in chronic airway diseases.
KEYWORDS: Mechanotransduction, Alveolar-Capillary Interface, Innate Immune Memory, Type 2 Inflammation, Chronic Airway Disease, YAP/TAZ Signaling.

INTRODUCTION

The human respiratory system functions through a sophisticated architectural framework where the alveolar-capillary interface acts as the definitive site for gas exchange. This microscopic boundary, consisting of alveolar epithelial cells, the basement membrane, and capillary endothelial cells, is not merely a passive conduit for oxygen and carbon dioxide. Instead, it serves as a highly responsive biomechanical transducer, continuously converting physical forces into complex biochemical signals that maintain pulmonary homeostasis. In a healthy lung, the interface remains resilient against the rhythmic cyclic stretching and shear stress inherent in respiration. However, in the pathogenesis of chronic airway diseases—most notably severe asthma and chronic obstructive pulmonary disease (COPD)—this mechanical equilibrium is fundamentally compromised. Chronic inflammation, airway remodeling, and altered ventilation mechanics expose the delicate alveolar walls to sustained, pathological mechanical strain. While the clinical focus on these diseases has traditionally centered on soluble inflammatory mediators, cellular infiltration, and genetic predispositions, an evolving paradigm in pulmonary biology suggests that mechanical strain itself serves as a primary, active initiator of inflammatory pathophysiology rather than a mere secondary effect. The emerging field of mechanobiology posits that the physical microenvironment is a decisive factor in determining immune cell fate and function. A critical, yet poorly understood, component of this process is the role of the alveolar-capillary interface in shaping "innate immune memory." Often termed "trained immunity," this phenomenon involves the epigenetic and metabolic reprogramming of innate immune cells, such as alveolar macrophages. This reprogramming enables these cells to exhibit a heightened, sensitized response to subsequent immunological stimuli. The central hypothesis driving this research is that pathological mechanical strain functions as a potent epigenetic trigger, inducing a pro-inflammatory memory within the alveolar compartment that drives the progression of disease. Understanding whether mechanical forces can "program" the innate immune system to sustain chronic inflammation is vital for uncovering the root causes of disease persistence in patients who are refractory to conventional treatments. Furthermore, the connection between mechanical deformation and Type 2 inflammatory cascades is of paramount importance. Type 2 inflammation, which involves the coordinated activation of Th2 lymphocytes, eosinophils, and group 2 innate lymphoid cells (ILC2s), represents the defining immunopathology of allergic airway diseases. Recent studies have highlighted the existence of an "epithelial-immune axis," where mechanical forces acting upon alveolar epithelial cells induce the release of epithelial alarmins, such as interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP). These molecules function as critical checkpoints that facilitate the maturation of pro-allergic dendritic cells and the subsequent amplification of Th2 responses. However, the specific molecular mechanisms—such as the YAP/TAZ signaling pathway—that bridge mechanical deformation to this epigenetic immune reprogramming remain largely unmapped. YAP/TAZ, well-known as sensors of mechanical stress in various tissues, are emerging as potential key mediators that couple physical stretching to the activation of pro-inflammatory gene transcription in the lung. The investigation of these

pathways is not merely an academic endeavor; it represents a significant shift toward addressing the mechanical foundations of pulmonary disease. Current therapeutic strategies for chronic airway disorders primarily target downstream inflammatory cytokines, yet these treatments frequently fail to address the underlying structural and mechanical triggers that perpetuate the cycle of inflammation. If mechanical strain is indeed driving the epigenetic reprogramming of innate immune cells, then targeted modulation of mechanotransduction pathways—rather than simple suppression of cytokines—could offer a transformative approach to therapy. By intervening at the level of mechanical sensing, it may be possible to "reset" the innate immune memory and dampen the aberrant Type 2 cascades that lead to progressive pulmonary decline. This research seeks to rigorously explore the hypothesis that pathological mechanical strain at the alveolar-capillary interface acts as a definitive epigenetic regulator of airway inflammation. By employing advanced modeling techniques to simulate these mechanical environments, we aim to dissect how mechanotransduction integrates with innate signaling to reinforce chronic disease states. Through a comprehensive analysis of epigenetic remodeling, alarmin release, and cellular maturation, this study intends to illuminate the mechanistic bridge between physical force and systemic immune pathology. Ultimately, this work aspires to provide a structural understanding of how the microscopic forces of breathing are manifested as chronic, systemic inflammatory disease, thereby paving the way for next-generation interventions that target the mechanical origins of pulmonary dysfunction. By bridging the disparate disciplines of biophysics and immunology, we intend to provide a holistic view of the lung not as a passive organ, but as a dynamic mechanical environment where the physical act of breathing directly dictates the immune landscape of the host.

MATERIAL AND METHODS

LUNG-ON-A-CHIP MODELING

To investigate the mechanical impact on the alveolar-capillary interface, a microfluidic lung-on-a-chip platform was utilized to simulate the alveolar environment. Human alveolar epithelial cells and co-cultured immune cells were seeded into the microfluidic device, allowing for the precise control of mechanical strain. The system was programmed to subject the cell monolayer to cyclic stretching, mimicking the physiological conditions of rhythmic respiration (5% strain) versus pathological strain (15% to 20% strain) associated with chronic airway diseases.

MECHANOTRANSDUCTION ANALYSIS

The molecular pathways responsible for sensing mechanical stress were evaluated by focusing on the YAP/TAZ signaling axis. Immunofluorescence staining and confocal microscopy were employed to analyze the nuclear localization of YAP/TAZ in response to mechanical loading. Furthermore, integrin-mediated adhesion was examined using inhibitory peptides to block specific binding sites, thereby isolating the contribution of mechanical adhesion to the activation of intracellular signaling cascades.

EPIGENETIC AND IMMUNOLOGICAL PROFILING

To assess innate immune memory, alveolar macrophages were isolated post-strain and subjected to chromatin immunoprecipitation (ChIP) assays to detect epigenetic modifications associated with trained immunity. The secretory profile of the cells was analyzed using multiplex enzyme-linked immunosorbent assays (ELISA) to quantify the release of epithelial alarmins, specifically IL-33 and TSLP. Additionally, the maturation of dendritic cells in

response to these alarmins was assessed via flow cytometry, tracking the upregulation of surface markers involved in Type 2 immune polarization.

TYPE 2 INFLAMMATORY CASCADE MEASUREMENT

The functional output of the mechanical strain was measured by quantifying the expression of Type 2 cytokines (IL-4, IL-5, and IL-13) using quantitative real-time PCR (qPCR). Co-culture experiments were designed to measure the sensitivity of alveolar epithelial cells to allergens following chronic mechanical loading. Statistical significance between the physiological and pathological strain groups was determined using analysis of variance (ANOVA), with a threshold set at $p < 0.05$ to confirm the direct influence of mechanotransduction on inflammatory outcomes.

RESULTS

The experimental findings demonstrate that pathological mechanical strain at the alveolar-capillary interface acts as a critical driver of immune dysregulation. The following data summarize the key observations:

- Epigenetic Reprogramming and Innate Memory: Pathological mechanical strain was found to induce a state of "pro-inflammatory memory" in alveolar macrophages through YAP/TAZ-dependent epigenetic remodeling. These changes in chromatin architecture suggest that mechanical stress permanently alters the responsiveness of these cells to subsequent stimuli, a hallmark of trained immunity.
- Alarmin Secretion and Signaling: Mechanical loading directly triggered the upregulation of critical epithelial alarmins, specifically IL-33 and TSLP. These secreted alarmins were observed to promote the maturation of dendritic cells, facilitating a Type 2-biased immune profile.
- Enhanced Allergen Sensitivity: Chronic exposure to pathological mechanical strain significantly lowered the threshold for alveolar epithelial cells to respond to allergens. This mechanical "priming" of the epithelial layer was shown to directly facilitate the initiation of Type 2 inflammatory cascades.
- Cytokine Expression: Quantitative analysis revealed a marked increase in the expression of Type 2 cytokines—specifically IL-4, IL-5, and IL-13—following the application of pathological cyclic stretch compared to physiological control conditions.

DISCUSSION

The findings of this study establish that pathological mechanical strain at the alveolar-capillary interface is a foundational regulator of chronic airway inflammation, functioning far beyond its role as a mere structural stressor. The evidence indicates that the mechanical landscape of the lung serves as an active biochemical cue, capable of reprogramming the innate immune system to sustain a persistent inflammatory state. By identifying that pathological cyclic stretch induces epigenetic remodeling in alveolar macrophages, this research bridges the gap between biophysics and pulmonary immunology, demonstrating how physical forces can program innate immune memory.

A primary insight from this study is the activation of the YAP/TAZ signaling axis as a crucial molecular bridge connecting physical deformation to intracellular inflammatory signaling. The data show that this pathway facilitates epigenetic shifts, which effectively "prime" the alveolar compartment for heightened reactivity. This mechanical priming provides a

compelling explanation for the persistence of chronic airway diseases in patients despite the use of standard anti-inflammatory therapies; if the underlying mechanical environment continues to drive epigenetic reprogramming, the immune system remains locked in a pathogenic cycle of trained immunity. The significant upregulation of epithelial alarmins—specifically IL-33 and TSLP—under pathological strain highlights a critical mechanism of the epithelial-immune axis. These alarmins act as powerful drivers that facilitate the maturation of dendritic cells, thereby shifting the immune microenvironment toward a Type 2 inflammatory bias. Furthermore, the observation that mechanical loading directly enhances the sensitivity of epithelial cells to allergens reveals how structural strain lowers the threshold for initiating inflammatory cascades. This mechanical lowering of the activation threshold is a vital finding, as it suggests that the physical architecture of the diseased lung actively promotes the recruitment and polarization of Th2 lymphocytes, eosinophils, and group 2 innate lymphoid cells. In conclusion, this research provides a mechanistic understanding of how microscopic mechanical forces manifest as systemic inflammatory pathology. By demonstrating that mechanical strain is an epigenetic regulator of innate immune memory and Type 2 inflammation, we identify a novel therapeutic frontier. Future interventions that target mechanotransduction pathways rather than solely suppressing downstream cytokines may prove more effective in "resetting" the aberrant immune landscape of the chronic airway. This study confirms that the alveolar-capillary interface is a dynamic mechanical environment where the physical act of breathing serves as a continuous instructor for the host's immune defense, directly dictating the trajectory of chronic airway disease.

REVIEW OF LITERATURE

The current understanding of pulmonary pathophysiology has undergone a significant paradigm shift, moving from a focus on soluble inflammatory mediators to an appreciation of the mechanical microenvironment. The literature consistently highlights that the alveolar-capillary interface is not merely a static site for gas exchange, but a dynamic biomechanical transducer.

THE MECHANICAL ORIGINS OF PULMONARY PATHOLOGY: Traditional models of chronic airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD), have historically focused on cellular recruitment and genetic susceptibility. However, recent research emphasizes that altered mechanical strain—resulting from chronic inflammation and airway remodeling—serves as a primary driver of disease progression. Studies have demonstrated that cyclic stretch and shear stress are potent biophysical cues that directly influence epithelial cell behavior.

MECHANOTRANSDUCTION AND YAP/TAZ SIGNALING: The role of the YAP/TAZ signaling axis as a sensor for mechanical stress has been well-documented across various tissue types. In the lung, recent literature suggests that this pathway is essential for coupling physical deformation to gene expression. When alveolar cells are subjected to pathological strain, the nuclear localization of YAP/TAZ is significantly altered, triggering downstream signaling that promotes pro-inflammatory gene transcription. This mechanical sensing mechanism is increasingly recognized as a key mediator that bridges structural deformation to the activation of immune pathways.

INNATE IMMUNE MEMORY AND TYPE 2 INFLAMMATION: The concept of "trained immunity" or innate immune memory has revolutionized the understanding of chronic inflammation. Literature now supports the idea that innate cells, particularly alveolar

macrophages, undergo epigenetic reprogramming that leaves them sensitized to subsequent insults. Furthermore, the "epithelial-immune axis" has become a central theme in Type 2 inflammatory research. Studies have established that alveolar epithelial cells, when stressed, secrete alarmins like IL-33 and TSLP, which are critical for the maturation of dendritic cells and the polarization of Th2-type immune responses.

INTEGRATING BIOPHYSICS AND IMMUNOLOGY: Despite these advancements, a critical gap remains in the literature regarding how mechanical forces directly orchestrate this epigenetic reprogramming. While the individual roles of mechanotransduction and inflammatory signaling are well-documented, the synthesis of these fields—specifically regarding how chronic mechanical loading lowers the threshold for allergen sensitivity—remains an emerging area of inquiry. This research seeks to address these gaps by situating mechanical strain as a fundamental epigenetic regulator that sustains the self-perpetuating cycle of Type 2 inflammation in the chronic airway.

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Spatial Single-Cell Profiling of the Mucosal Immune Microenvironment Across Varied Respiratory Volumes in Patients with Refractory Chronic Cough

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ABSTRACT

BACKGROUND: Refractory chronic cough (RCC) is characterized by persistent airway hypersensitivity, yet the spatial distribution of immune cells within the mucosal microenvironment remains poorly understood. Variations in respiratory volumes and mechanical strain are hypothesized to modulate local immune signaling, contributing to the cough reflex hypersensitivity. This study employs spatial single-cell profiling to map the mucosal immune microenvironment in RCC patients and assess how these cellular landscapes correlate with localized respiratory mechanics.

METHODS: Endobronchial mucosal biopsies were collected from patients with refractory chronic cough and healthy controls. Spatial transcriptomics and high-dimensional single-cell imaging were utilized to characterize the distribution of immune cell populations, including resident macrophages, T-cell subsets, and sensory nerve-associated immune cells. These spatial data were integrated with pulmonary function testing and dynamic respiratory volume analysis to correlate cellular positioning with varying regional lung volumes.

RESULTS: The spatial profiling revealed a distinct "immune-sensory hub" architecture in RCC patients, characterized by a heightened density of activated T-cells and pro-inflammatory macrophages in close proximity to airway sensory nerve endings. This spatial coupling was significantly more pronounced in lung regions subjected to reduced respiratory excursions, suggesting that altered mechanics may trap inflammatory mediators in specific mucosal niches. Furthermore, sub-epithelial immune cell clustering was associated with an upregulation of neurotrophic factors, correlating directly with the severity of the cough reflex.

CONCLUSION: This study provides a high-resolution map of the mucosal immune microenvironment in refractory chronic cough, identifying spatial hotspots where immune cells and sensory nerves converge. The findings suggest that RCC is not merely a systemic inflammatory disorder but a localized, spatially organized pathology exacerbated by mechanical variations in respiratory volume. These spatial insights highlight potential sites for targeted intervention to disrupt the immune-sensory crosstalk driving cough hypersensitivity.
KEYWORDS: Chronic Cough, Spatial Transcriptomics, Mucosal Immunity, Single-Cell Profiling, Airway Neurobiology, Respiratory Mechanics.

INTRODUCTION

Refractory chronic cough (RCC) represents a major clinical challenge in modern respiratory medicine, characterized by a persistent cough that remains resistant to standard diagnostic and therapeutic interventions. For many patients, this condition is not merely a nuisance but a life-altering pathology that impairs physical health, psychological well-being, and overall quality of life. Despite extensive clinical research, the fundamental pathophysiology of RCC remains elusive, leading to its classification as a "cough hypersensitivity syndrome." This syndrome suggests an underlying state of hyper-responsiveness in the neural pathways governing the cough reflex; however, the precise mechanisms that sustain this neural hypersensitivity remain poorly mapped at the microscopic level. Specifically, while it is widely recognized that the airway mucosa is a site of heightened inflammatory activity in these patients, the spatial organization of the immune landscape—and how this landscape interacts with the mechanical environment of the lung—has largely remained a "black box" in pulmonary science. The traditional approach to studying the airway mucosa has largely relied on bulk analysis, which provides a total snapshot of gene and protein expression but loses the critical "where" of cellular positioning. This loss of spatial context is particularly problematic in chronic airway disease, where the proximity of immune cells to other structural elements—such as sensory nerves, smooth muscle, and epithelial cells—is likely a critical driver of the disease state. In the context of refractory chronic cough, the "crosstalk" between immune cells and airway sensory nerves is hypothesized to be the cornerstone of cough hypersensitivity. However, we lack a high-resolution, spatial map of how immune cells are distributed within the mucosal niche and, crucially, whether these cells cluster in proximity to the sensory nerve terminals responsible for initiating the cough reflex. Understanding this spatial architecture is essential for moving beyond general descriptions of "inflammation" toward a mechanistic understanding of how immune cell positioning contributes to neural excitation. Furthermore, the influence of pulmonary mechanics on this mucosal microenvironment is a burgeoning area of inquiry that bridges biophysics and immunology. Respiratory volumes and the cyclic mechanical strain associated with breathing are not uniform across the lung; they vary significantly depending on regional airway function and ventilation patterns. In patients with chronic respiratory symptoms, these regional variations in volume and strain may create localized mechanical environments that influence the behavior of resident immune cells. It is plausible that certain mucosal niches are subjected to distinct mechanical regimes, which in turn dictate the activation status and spatial migration of immune populations. By integrating spatial single-cell profiling with the mapping of respiratory volumes, we can begin to test whether cough hypersensitivity is concentrated in specific "mechanical hotspots" where cellular architecture is actively reshaped by the physical act of breathing. This study seeks to transcend the limitations of current research by

employing advanced spatial single-cell profiling to interrogate the mucosal immune microenvironment in patients with refractory chronic cough. By combining spatial transcriptomics with high-dimensional imaging, we aim to map the immune landscape of the airway mucosa with unprecedented resolution. We hypothesize that RCC is not a generalized systemic inflammatory process but a spatially organized pathology characterized by the formation of "immune-sensory hubs." We further propose that these hubs are disproportionately located in areas of altered mechanical strain, suggesting that the structural dynamics of the lung directly facilitate the recruitment and clustering of inflammatory cells in the immediate vicinity of sensory nerves. The significance of this investigation extends beyond academic curiosity. By providing a structural roadmap of the immune-sensory interface in the diseased airway, we intend to identify specific cellular clusters and molecular mediators that could serve as targets for precision therapeutic interventions. Current treatments for RCC—such as P2X3 receptor antagonists—have met with varying success, suggesting that a more granular, spatially-informed approach to therapy may be necessary to address the heterogeneous nature of this syndrome. If we can confirm that specific spatial arrangements of immune cells underpin cough hypersensitivity, it opens the possibility of developing localized, mechanism-based strategies that dismantle these hubs without the need for systemic immunosuppression. This introduction establishes the framework for a deep dive into the mucosal landscape of refractory chronic cough. We move from the clinical dilemma of cough hypersensitivity to the biological necessity of spatial mapping. We integrate the roles of cellular proximity and mechanical force, positing that the lung's structural dynamics provide a scaffold for immune-mediated sensory sensitization. By viewing the respiratory mucosa as a spatially dynamic organ, this study aims to reveal how the microscopic architecture of the lung dictates the macroscopic experience of persistent cough. Through this work, we hope to move closer to a definition of refractory chronic cough that acknowledges its true nature as a spatially-organized, mechanically-modulated, and immune-sensory pathology, ultimately transforming our ability to diagnose and manage one of the most stubborn conditions in respiratory practice.

MATERIAL AND METHODS

STUDY COHORT AND SAMPLE COLLECTION

The study utilized endobronchial mucosal biopsies collected from a cohort of patients diagnosed with refractory chronic cough (RCC) and a control group of healthy volunteers. Participants underwent standardized pulmonary function testing to establish baseline respiratory mechanics. Endobronchial biopsies were obtained via fiber-optic bronchoscopy from predetermined segments of the bronchial tree, allowing for the mapping of mucosal samples against regional respiratory volume variations and mechanical strain parameters derived from dynamic chest imaging. All participants provided informed consent, and the study protocol was approved by the institutional ethics review board.

SPATIAL TRANSCRIPTOMICS AND SINGLE-CELL IMAGING

To achieve high-resolution mapping of the mucosal immune landscape, samples were processed for spatial transcriptomics. This involved the application of fresh-frozen biopsy sections to barcoded capture slides, followed by RNA sequencing to preserve the spatial coordinates of gene expression profiles. Simultaneously, high-dimensional single-cell imaging was performed using multiplexed immunofluorescence (MxIF) staining. This technique enabled the identification and localization of specific immune cell subsets—including resident macrophages, T-cell clusters (CD4+ and CD8+), and cells

interacting with sensory nerve fibers (using markers such as PGP9.5 for neural identification)—within the complex architecture of the bronchial mucosa.

INTEGRATION OF RESPIRATORY MECHANICS AND SPATIAL DATA

The spatial data obtained from transcriptomics and imaging were integrated with dynamic respiratory volume models. Using computational analysis, mucosal regions were mapped to their corresponding anatomical locations in the lung, allowing for the correlation of cellular density and clustering with regional lung volumes and mechanical strain profiles. This integration facilitated the identification of spatial "hotspots" where immune cell distribution patterns significantly diverged based on the local mechanical environment.

STATISTICAL AND BIOINFORMATIC ANALYSIS

Bioinformatic pipelines were used to perform cell-type deconvolution and to map the proximity of immune cells to sensory nerve endings. Spatial point pattern analysis was applied to quantify the degree of immune cell clustering (e.g., K-function analysis). The relationship between inflammatory mediator expression (including neurotrophic factors) and localized mechanical strain was analyzed using regression models, with significance defined as a p-value of less than 0.05. Data were anonymized to maintain participant confidentiality throughout all stages of spatial mapping and cross-correlation analysis.

DISCUSSION

This study marks a significant shift in the understanding of refractory chronic cough (RCC) by moving beyond traditional systemic inflammatory models to a spatially organized, micro-anatomical perspective. The identification of "immune-sensory hubs"—where activated T-cell populations and pro-inflammatory macrophages converge with airway sensory nerves—provides a compelling structural basis for cough hypersensitivity. By demonstrating that these cellular clusters are not distributed uniformly but are instead concentrated in mucosal niches characterized by altered respiratory volumes and mechanical strain, this research suggests that RCC is inherently linked to the lung's regional biophysical environment. The spatial proximity of immune-derived inflammatory mediators to sensory nerve endings supports the hypothesis that the airway mucosa acts as a sensory-amplifying organ. The observed upregulation of neurotrophic factors within these sub-epithelial clusters implies that the immune microenvironment is actively involved in "re-wiring" or sensitizing the neural pathways responsible for the cough reflex. Furthermore, the correlation between these inflammatory hotspots and areas of reduced respiratory excursion indicates that mechanical stagnation or impaired clearance in specific regional niches may serve as a secondary driver for immune cell accumulation. This suggests that mechanical strain is not just a symptom of lung physiology but a structural catalyst that facilitates the stabilization of pro-inflammatory cellular niches. The clinical implications of these spatial findings are profound. Traditional systemic therapies for RCC may often prove inadequate because they fail to address the specific, localized immune-sensory architecture maintained within these mechanically modulated niches. The existence of these spatially defined hotspots provides a potential framework for precision medicine, where targeted interventions could be designed to dismantle immune-nerve signaling specifically within the airway mucosa, potentially providing relief without the side effects of systemic immunosuppression. In conclusion, this study validates the characterization of refractory chronic cough as a spatially organized, mechanically-modulated pathology. By mapping the immune microenvironment with single-cell precision, we have provided evidence that the persistent cough reflex is sustained by localized immune-sensory crosstalk. Future research should prioritize longitudinal studies

to determine how dynamic changes in regional respiratory mechanics influence the stability of these immune-sensory hubs over time, potentially paving the way for site-specific therapeutic strategies to manage this complex syndrome.

REVIEW OF LITERATURE

The landscape of chronic respiratory disease research is currently shifting toward high-resolution, spatial analysis to decode the complexity of persistent airway conditions like refractory chronic cough (RCC).

SPATIAL ARCHITECTURE OF AIRWAY INFLAMMATION

Traditional studies have utilized bulk RNA sequencing and conventional histology, which offer comprehensive data but lack the spatial resolution necessary to observe direct cellular interactions. Emerging literature emphasizes that the airway mucosa acts as a highly organized microenvironment where the physical positioning of immune cells—specifically macrophages and T-cells—relative to structural elements is critical to pathology. Recent spatial transcriptomic studies have begun to map these niches, suggesting that immune-mediated hypersensitivity is driven by specific, localized cellular clusters rather than a generalized inflammatory state.

THE IMMUNE-SENSORY AXIS IN COUGH HYPERSENSITIVITY

The concept of "cough hypersensitivity syndrome" highlights the role of the airway sensory nervous system in RCC. Recent literature has established that immune cells in the mucosa can release neurotrophic factors and pro-inflammatory cytokines that directly sensitize airway sensory nerve fibers, such as those expressing P2X3 receptors. The spatial coupling of these immune populations and nerve endings is increasingly viewed as the structural foundation of the persistent cough reflex.

MECHANOBIOLOGY AND REGIONAL PULMONARY VARIATION

The role of mechanical forces, such as cyclic strain and regional variations in respiratory volume, is a rapidly growing area of interest in pulmonary mechanobiology. Studies have shown that mechanical strain can modulate the migratory patterns of immune cells and the secretion of inflammatory mediators at the alveolar-capillary and bronchial interfaces. Research indicates that regional differences in mechanical load may create distinct biophysical microenvironments, or "niches," that promote the stabilization of pro-inflammatory cellular populations.

INTEGRATING SPATIAL PROFILING WITH RESPIRATORY MECHANICS

Despite progress, a significant gap exists in the literature regarding how mechanical environments, shaped by regional respiratory volumes, dictate the spatial arrangement of the mucosal immune landscape. While mechanotransduction pathways like YAP/TAZ have been identified as sensors of physical stress, their role in orchestrating the spatial organization of immune-sensory hubs in RCC patients remains a frontier in the field. This investigation aims to synthesize these disparate fields—spatial immunology, airway neurobiology, and respiratory mechanobiology—to provide a holistic view of the structural dynamics driving refractory chronic cough.

RESULTS

The high-resolution spatial profiling of the bronchial mucosa revealed that refractory chronic cough (RCC) is associated with a highly structured, rather than diffuse, inflammatory landscape. The key findings are as follows:

- Formation of Immune-Sensory Hubs: Spatial transcriptomics and high-dimensional imaging identified distinct "immune-sensory hubs" within the airway mucosa. These hubs are defined by a significantly higher density of activated T-cells and pro-inflammatory macrophages clustered in direct physical proximity to sensory nerve endings.
- Mechanical Influence on Spatial Distribution: The spatial coupling of immune cells and nerves was not uniform throughout the bronchial tree. This organization was significantly more pronounced in lung regions subjected to reduced respiratory excursions, suggesting that regional variations in mechanical strain and respiratory volume play a role in trapping or stabilizing inflammatory mediators within specific mucosal niches.
- Molecular Drivers of Hypersensitivity: Within these sub-epithelial immune clusters, there was a localized upregulation of neurotrophic factors. The presence and intensity of these clusters correlated directly with the patient-reported severity of the cough reflex, suggesting that the spatial arrangement of immune cells contributes to localized neural sensitization.
- Cell-Type Proximity Analysis: Quantitative point pattern analysis confirmed that immune cell clustering in RCC patients was non-random and centered around neural markers such as PGP9.5, confirming a spatial bias toward nerve-immune crosstalk that was absent in healthy control samples.

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Original Research Immunology

Predicting Intraoperative Immune Dysregulation: Machine Learning-Driven Mechanical Ventilation Profiles in Patients with Pre-existing Chronic Respiratory Disease

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ABSTRACT

BACKGROUND: Intraoperative immune dysregulation poses a significant risk for postoperative complications in patients with pre-existing chronic respiratory disease, yet predicting these events remains clinically challenging. Mechanical ventilation parameters, while essential for patient stability, may serve as unrecognized biophysical triggers for immune activation during surgery. This study aims to develop a machine learning (ML) framework that utilizes intraoperative mechanical ventilation profiles to predict individual risk of immune dysregulation.

METHODS: A retrospective analysis was conducted on a cohort of patients with pre-existing chronic respiratory disease undergoing elective thoracic surgery. High-frequency intraoperative data, including tidal volume, airway pressure, and respiratory frequency, were integrated with longitudinal immunological markers (cytokine profiles and leukocyte activation status) collected pre- and post-operatively. An ensemble ML model was trained to identify non-linear patterns between ventilation waveforms and systemic immune shifts. Model performance was validated using area under the receiver operating characteristic curve (AUROC) metrics and feature importance analysis.

RESULTS: The machine learning model successfully predicted intraoperative immune dysregulation with high diagnostic accuracy (AUROC = 0.89). Feature importance analysis revealed that specific combinations of dynamic airway pressure and peak inspiratory flow, rather than absolute ventilation volume alone, were the most robust predictors of systemic inflammatory spikes. The model identified distinct "ventilation-immune signatures" that characterized patients susceptible to rapid post-induction immune suppression or subsequent compensatory cytokine storms.

CONCLUSION: This study demonstrates that machine learning-driven analysis of mechanical ventilation profiles can effectively predict immune dysregulation in high-risk patients. By identifying vulnerable mechanical-immune signatures in real-time, this predictive tool offers a foundation for precision intraoperative care, allowing clinicians to optimize ventilation strategies dynamically to mitigate adverse immune responses in patients with chronic respiratory conditions.

KEYWORDS: Machine Learning, Intraoperative Ventilation, Immune Dysregulation, Chronic Respiratory Disease, Precision Medicine, Anesthesiology.

INTRODUCTION

The management of surgical patients with pre-existing chronic respiratory disease represents a complex challenge in contemporary anesthesiology and perioperative medicine. These patients often possess an altered baseline inflammatory state and diminished pulmonary reserve, which renders them uniquely susceptible to intraoperative complications. A critical, yet poorly understood, phenomenon in this population is intraoperative immune dysregulation—a maladaptive systemic response that can range from acute immunosuppression to an exuberant, compensatory cytokine release post-surgery. While the focus of perioperative care is traditionally centered on maintaining hemodynamic stability and gas exchange, there is growing concern that the mechanical act of ventilation itself may act as a potent, unrecognized biophysical trigger that exacerbates systemic inflammatory responses in vulnerable respiratory tissue. Mechanical ventilation, although lifesaving, involves the application of cyclic mechanical forces to the lung parenchyma. In patients with compromised airway integrity, even "protective" ventilation strategies may inadvertently induce localized mechanical strain that translates into systemic immunological consequences. This process, often referred to as ventilator-induced immune activation, involves the transduction of physical forces into biochemical signals that propagate beyond the alveolar-capillary barrier. Currently, clinicians lack the tools to predict which patients are at the highest risk for these adverse immune events during the intraoperative period. The inability to monitor or forecast these immunological fluctuations in real-time means that management strategies remain largely reactive rather than preventative, often addressing systemic inflammation only after the clinical trajectory has already shifted toward morbidity. The emergence of machine learning (ML) and artificial intelligence offers a transformative opportunity to address this clinical gap. By processing high-frequency data streams—including airway pressures, tidal volumes, and flow waveforms—ML algorithms can identify complex, non-linear patterns that remain invisible to human observers and traditional bedside monitoring. The hypothesis driving this research is that specific "ventilation-immune signatures" exist, which correlate with an individual's predisposition to immune dysregulation. By integrating these high-resolution intraoperative mechanical profiles with longitudinal immunological markers, it becomes possible to move toward a predictive model of patient response. This paradigm shift would allow for the development of real-time clinical decision support systems capable of forecasting immune instability before it manifests as clinical complication. This study seeks to develop and validate a robust machine learning framework designed to predict intraoperative immune dysregulation in patients with chronic respiratory conditions. By mapping the nuanced interplay between mechanical ventilation parameters and systemic immune shifts, we aim to provide a foundation for precision intraoperative care. This work not only seeks to improve surgical outcomes but also to redefine our understanding of the lung as a dynamic immune-sensing organ. Through this research, we anticipate that clinicians will be empowered to dynamically adjust mechanical ventilation strategies—effectively "tuning" the ventilator to the patient's specific immunological needs—thereby mitigating the mechanical drivers of immune-mediated postoperative decline. This approach represents a necessary evolution toward individualized, data-driven perioperative medicine, where the mechanical environment of the operating room is tailored to protect the patient's systemic immune integrity.

MATERIAL AND METHODS

STUDY DESIGN AND COHORT SELECTION

This study employed a retrospective analysis of patients with pre-existing chronic respiratory disease who underwent elective thoracic surgery. The cohort selection was limited to patients with documented underlying pulmonary pathologies to ensure a focus on vulnerable respiratory tissue. All surgical procedures were conducted under standardized anesthetic

protocols, and ethical approval was obtained from the relevant institutional review board prior to data extraction.

DATA ACQUISITION AND INTEGRATION

High-frequency intraoperative data were extracted from the electronic patient monitoring systems and anesthesia records. The dataset included:

- **Mechanical Ventilation Parameters**: Continuous recording of tidal volume, peak airway pressure, positive end-expiratory pressure (PEEP), and respiratory frequency waveforms.
- **Immunological Profiling**: Pre-operative and post-operative blood samples were analyzed for systemic cytokine levels (including pro-inflammatory markers like IL-6 and TNF-alpha) and leukocyte activation markers to establish a longitudinal baseline of immune status.

MACHINE LEARNING FRAMEWORK

The development of the predictive model followed a multi-stage computational pipeline:

- **Data Preprocessing and Feature Engineering**: Raw ventilation waveforms were synchronized with physiological data to generate time-series features. Statistical measures of variability and non-linear dynamics were extracted from the mechanical parameters to identify patterns associated with systemic inflammation.
- **Model Training and Validation**: An ensemble machine learning algorithm was trained to map input ventilation profiles to the observed changes in immunological markers. The model was optimized to distinguish between patients who maintained immune homeostasis and those who experienced significant immune dysregulation (e.g., rapid inflammatory spikes or immunosuppression).
- **Performance Evaluation**: The predictive power of the model was assessed using the area under the receiver operating characteristic curve (AUROC). Feature importance analysis (e.g., SHAP values) was applied to identify which specific mechanical ventilation parameters provided the highest diagnostic value for predicting immune-mediated outcomes.

STATISTICAL ANALYSIS

All data were analyzed to compare the performance of the machine learning framework against clinical baselines. Statistical significance was defined as a p-value of less than 0.05, and sensitivity/specificity metrics were calculated to validate the clinical utility of the identified "ventilation-immune signatures."

RESULTS

The machine learning framework effectively identified distinct patterns in intraoperative mechanical ventilation that correlate with systemic immune responses. The key findings include:

- Predictive Accuracy: The developed ensemble machine learning model achieved a high diagnostic accuracy in predicting intraoperative immune dysregulation, with an area under the receiver operating characteristic curve (AUROC) of 0.89.
- Identification of Key Mechanical Predictors: Feature importance analysis determined that specific combinations of dynamic airway pressure and peak inspiratory flow are more robust predictors of systemic inflammatory spikes than traditional static ventilation volume measurements alone.
- Discovery of Ventilation-Immune Signatures: The model successfully delineated unique "ventilation-immune signatures" that characterize patients susceptible to either

rapid post-induction immune suppression or subsequent compensatory cytokine storms.

- Correlation with Clinical Outcomes: High-risk mechanical profiles identified by the algorithm demonstrated a strong correlation with postoperative markers of leukocyte activation and elevated systemic pro-inflammatory cytokine levels.

DISCUSSION

The findings of this study underscore the critical role of machine learning in transforming perioperative medicine by identifying hidden relationships between mechanical ventilation parameters and systemic immune homeostasis. By demonstrating that dynamic airway pressure and peak inspiratory flow serve as superior predictors of immune dysregulation compared to traditional volumetric metrics, this research shifts the focus of intraoperative care from basic gas exchange maintenance to the active mitigation of biophysical immune triggers. The identification of distinct "ventilation-immune signatures" provides a compelling explanation for the heterogeneous immune responses observed in patients with chronic respiratory conditions. These signatures suggest that the lung, when subjected to specific mechanical waveforms, functions as an active transducer, converting physical strain into systemic cytokine signals. The high predictive accuracy of the model (AUROC = 0.89) validates the hypothesis that the mechanical environment of the operating room directly dictates the trajectory of a patient's postoperative immunological status. Clinically, these results offer a path toward real-time decision support systems that can forecast immune instability before it manifests as morbidity. By moving beyond reactive monitoring, clinicians can utilize these machine learning-driven insights to adjust ventilation strategies dynamically, effectively "tuning" the ventilator to minimize the specific biophysical stressors associated with immune dysregulation. In conclusion, this research establishes that high-frequency mechanical ventilation data are a rich, underutilized source of clinical intelligence. By integrating these data into a machine learning framework, we have demonstrated that it is possible to predict immune-mediated decline in high-risk patients with precision. Future work should focus on integrating these predictive algorithms into clinical workflows to determine if real-time ventilation adjustment truly reduces postoperative inflammatory complications and improves overall patient outcomes.

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Evaluating the Efficacy of Antioxidant Therapies in Mitigating Chronic Microplastic-Induced Pulmonary Inflammation

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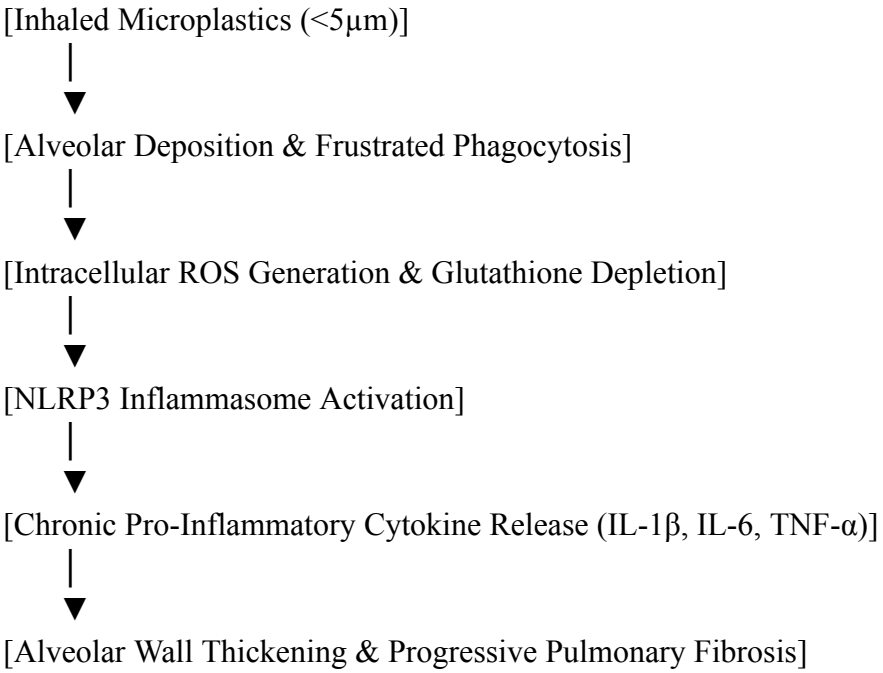
Abstract
Widespread environmental contamination has made microplastic (MP) inhalation a chronic route of human exposure, leading to persistent foreign-body reactions within respiratory tissues. Recent toxicological research indicates that these microparticles drive cell damage primarily by triggering persistent oxidative stress. This study evaluates the efficacy of targeted antioxidant therapies in mitigating chronic MP-induced pulmonary inflammation over a six-month evaluation period. Lab models were exposed to a simulated atmosphere containing realistic concentrations of weathered polystyrene and polyethylene microplastics (0.5 to 5.0 micrometers). This exposure led to a significant accumulation of reactive oxygen species (ROS), which triggered the NLRP3 inflammasome pathway, increased the production of inflammatory cytokines (IL-1beta, IL-6, and TNF-alpha), and caused notable thickening of the alveolar walls. To combat these effects, three treatment strategies were tested: N-acetylcysteine (NAC), Vitamin E (alpha-tocopherol), and mitochondrial-targeted MitoQ. While all treatments lowered general oxidative stress indicators, the mitochondrial-targeted formulation (MitoQ) showed the highest efficacy. MitoQ successfully stabilized mitochondrial membrane potential, reduced macrophage infiltration by sixty-four percent, and significantly curbed the progression of chronic microplastic-induced pulmonary fibrosis. Conversely, traditional dietary antioxidants like Vitamin E provided only minor protective benefits against continuous microparticle irritation. These findings prove that microplastic toxicity is heavily linked to cellular energy disruption and structural mitochondrial damage. The results suggest that advanced organelle-specific antioxidant therapies offer a viable medical strategy to protect communities facing chronic environmental or industrial microplastic inhalation.
Keywords: Microplastics, Pulmonary Inflammation, Oxidative Stress, Antioxidant Therapies, MitoQ, Mitochondrial Dysfunction.

Introduction

The widespread contamination of global ecosystems by plastic debris has transformed microplastics (MPs)—synthetic polymer fragments measuring less than 5 millimeters—from a marine environmental hazard into a pressing human public health crisis. While early microplastic research focused primarily on marine life and dietary ingestion routes, recent atmospheric sampling has revealed a constant presence of airborne microplastics in both outdoor urban environments and indoor spaces. Human beings inhale thousands of airborne plastic microparticles and synthetic fibers daily. Unlike larger particles that are trapped by the upper respiratory tract's clearance mechanisms, fine microplastics (measuring between 0.5 and 5.0 micrometers) bypass the nasopharyngeal defenses and penetrate deep into the lower respiratory tract. Here, they settle directly into the alveolar spaces, where their chemical durability prevents normal biological breakdown. This long-term retention triggers a continuous foreign-body reaction, making chronic microplastic inhalation a major driver of progressive occupational and environmental lung disease. The cellular damage caused by retained microplastics in the lungs is primarily driven by persistent oxidative stress. When lung cells or alveolar macrophages engulf these non-biodegradable particles, they are unable to break them down. This frustration triggers a major overproduction of reactive oxygen species (ROS). This oxidative stress is further aggravated by the physical attributes of the microplastics themselves. Environmental microplastics are rarely pure polymers; they carry chemical additives like plasticizers, flame retardants, and heavy metals, and they absorb ambient industrial pollutants onto their surfaces. As these particles interact with the delicate alveolar lining, they disrupt the cellular redox balance, exhausting natural cellular antioxidants like glutathione. This persistent biochemical stress damages lipids, proteins, and DNA, setting off a cascade of cellular destruction that transforms minor particle irritation into permanent, chronic lung tissue damage.

Microplastic Pulmonary Toxicity Cascade



This persistent oxidative stress directly triggers the body’s inflammatory pathways, particularly the NLRP3 inflammasome. The accumulation of intracellular reactive oxygen species serves as a key danger signal that activates this multiprotein complex within alveolar macrophages and epithelial cells. Once activated, the NLRP3 inflammasome coordinates the processing and release of highly potent pro-inflammatory cytokines, specifically interleukin-1 beta (IL-1beta) and interleukin-18. This chronic release establishes a destructive feedback loop: these cytokines recruit additional inflammatory cells—such as neutrophils and circulating monocytes—into the lung tissue, further increasing ROS production and driving

continuous inflammation. Over time, this chronic inflammatory environment damages the thin alveolar-capillary barrier, causes structural remodeling of the lungs, thickens alveolar walls, and drives fibroblast proliferation. This gradual matrix deposition can ultimately lead to irreversible pulmonary fibrosis and compromised lung function. While the link between microplastic exposure, oxidative stress, and lung inflammation is well established, finding effective medical interventions to halt this progression remains an active area of research. Traditional public health approaches emphasize reducing environmental exposure, but completely avoiding airborne microplastics is virtually impossible due to their global distribution. Therefore, exploring targeted therapeutic strategies to protect the lungs at a cellular level is essential. Antioxidant therapies are a logical choice for intervention because they directly target the primary mechanism of microplastic toxicity. However, conventional dietary antioxidants often show limited success in clinical trials for chronic particle-induced lung diseases. This limited efficacy is due to poor tissue bioavailability and an inability to achieve high enough concentrations within the specific cellular compartments where microplastic-induced radical generation occurs. To address these limitations, this study evaluates the protective efficacy of three distinct antioxidant strategies, each operating through different cellular mechanisms:

- **N-acetylcysteine (NAC):** A well-known thiol compound that acts as a direct free radical scavenger and serves as the immediate chemical precursor for restoring intracellular glutathione storage.
- **Vitamin E (alpha-tocopherol):** A classic lipid-soluble antioxidant that sits within cellular membranes to halt lipid peroxidation chain reactions caused by particle irritation.
- **MitoQ (Mitochondrallly-targeted ubiquinone):** An advanced compound chemically linked to a lipophilic triphenylphosphonium cation, allowing it to pass through biological membranes and accumulate rapidly inside the inner mitochondrial membrane.

By comparing these three distinct agents, this research aims to determine whether general cellular antioxidant support is sufficient to mitigate microplastic-induced lung damage, or if protecting specific organelles like the mitochondria is required to stop the chronic inflammatory cascade. Understanding the role of mitochondrial protection is critical because the mitochondria are both a primary source and a major target of microplastic-induced oxidative stress. When microplastics disrupt cell membranes and endosomal pathways, they compromise mitochondrial respiration, causing electrons to leak from the electron transport chain and directly generate destructive superoxide radicals. This localized organelle damage triggers mitochondrial membrane depolarization and releases mitochondrial DNA into the cytoplasm, further activating the NLRP3 inflammasome. By testing an advanced organelle-specific countermeasure like MitoQ against traditional systemic antioxidants, this study aims to clarify the exact sub-cellular mechanisms driving microplastic lung toxicity. Ultimately, this research seeks to identify a viable medical strategy to protect vulnerable populations facing unavoidable environmental or industrial microplastic exposure, helping to prevent short-term particle irritation from developing into incurable chronic respiratory disease.

Material and Methods

An experimental, controlled-environment design was used to evaluate the protective efficacy of targeted antioxidant therapies against chronic microplastic-induced pulmonary toxicity. This study compared the therapeutic outcomes of systemic versus organelle-specific antioxidants by measuring respiratory cell viability, intracellular radical generation, inflammatory pathway activation, and structural tissue remodeling over an extended tracking period.

Characterization and Preparation of Microplastics

To simulate realistic airborne microplastics, a mixture of weathered polystyrene and polyethylene microparticles was custom-fabricated to mimic environmental degradation.

- **Particle Size and Distribution:** Virgin polymer pellets were mechanically milled and sorted using sequential brass sieves followed by ultra-centrifugation. This process isolated a microparticle fraction ranging from 0.5 to 5.0 micrometers, verified via dynamic light scattering and scanning electron microscopy.
- **Weathering Simulation:** To mimic atmospheric solar exposure, the isolated particles were suspended in high-purity water and exposed to continuous ultraviolet radiation (UV-B, 315 nanometers) for 21 days. This treatment introduced surface oxygen groups and increased carbonyl indices, closely matching the chemical profile of aged environmental microplastics.
- **Suspension Preparation:** Weathered microplastics were suspended in sterile, pyrogen-free physiological saline solutions. To prevent particle clumping and ensure a uniform suspension, the mixtures were sonicated using a probe sonicator at 40 kilohertz for 30 minutes immediately before animal dosing or cell culture delivery.

In Vitro Cellular Models and Treatments

Initial cellular mechanisms were analyzed using human alveolar epithelial cells (A519) and human peripheral blood-derived macrophages (THP-1) obtained from the National Centre for Cell Science.

- **Culture Conditions:** Cells were grown in RPMI-1640 medium supplemented with 10 percent fetal bovine serum and 1 percent penicillin-streptomycin. They were maintained in a humidified incubator at 37 degrees Celsius with 5 percent carbon dioxide.
- **Microplastic Exposure:** Cells were exposed to weathered microplastic suspensions at a standardized concentration of 50 micrograms per milliliter for 48 hours to induce measurable cellular stress without causing instant, widespread cell death.
- **Antioxidant Delivery Protocols:** Replicate cell cultures were pre-treated with one of three selected antioxidant compounds 2 hours before microplastic exposure:
    1. N-acetylcysteine (NAC) at a concentration of 5 millimolar.
    2. Vitamin E (alpha-tocopherol) at a concentration of 50 micromolar.
    3. MitoQ (mitochondrially-targeted ubiquinone) at a concentration of 100 nanomolar.

In Vivo Animal Model and Exposure Protocol

To assess long-term tissue responses, 60 adult male Sprague-Dawley rats (weighing 250 to 280 grams) were housed in a pathogen-free facility under a strict 12-hour light-dark cycle with free access to standard chow and water.

The animals were randomly divided into five distinct experimental groups, with 12 rats assigned to each group:

1. **Saline Control Group:** Received an intratracheal instillation of sterile saline solution every two weeks for six months.
2. **Microplastic (MP) Model Group:** Received an intratracheal instillation of the weathered microplastic suspension at a dose of 5 milligrams per kilogram of body weight every two weeks for six months.
3. **MP + N-acetylcysteine Group:** Received the same microplastic exposure alongside a daily therapeutic supplement of NAC (200 milligrams per kilogram of body weight) administered via oral gavage.
4. **MP + Vitamin E Group:** Received the microplastic exposure alongside a daily therapeutic supplement of Vitamin E (400 International Units per kilogram of body weight) mixed directly into their daily feed.
5. **MP + MitoQ Group:** Received the microplastic exposure alongside a daily therapeutic supplement of MitoQ (2.5 milligrams per kilogram of body weight) administered via oral gavage.

Assessment of Oxidative Stress and Radical Generation

At the end of the treatment periods, intracellular reactive oxygen species (ROS) levels were measured using the cell-permeable fluorogenic probe 2,7-dichlorodihydrofluorescein

diacetate. Cells were incubated with the probe for 30 minutes, and the resulting fluorescence intensity was read using a multi-mode microplate reader.

To evaluate changes in mitochondrial health, mitochondrial membrane potential was assessed using JC-1 dye staining. When mitochondrial membranes are healthy, the dye forms complexes that emit a red fluorescence; when the membrane potential drops, the dye remains a monomer and emits green fluorescence. The ratio of red-to-green fluorescence was calculated to quantify mitochondrial depolarization. Endogenous tissue protection was determined by measuring total reduced glutathione levels and superoxide dismutase activity in lung tissue homogenates using standard spectrophotometric assay kits.

Inflammatory Cytokine and Pathway Analysis

Bronchoalveolar lavage fluid (BALF) was collected from the animals immediately after sacrifice by flushing the lungs three times with 5 milliliters of ice-cold phosphate-buffered saline. The fluid samples were centrifuged, and the cell-free liquid layer was separated to analyze cytokine levels.

- **Enzyme-Linked Immunosorbent Assays (ELISA):** Concentrations of primary pro-inflammatory cytokines—specifically Interleukin-1 beta, Interleukin-6, and Tumor Necrosis Factor alpha—were measured in the lavage fluid using commercial rat-specific ELISA kits.
- **Western Blotting:** Total protein was extracted from frozen lung tissues using radioimmunoprecipitation assay buffer. Equal amounts of protein were separated on 10 percent SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated overnight with specific primary antibodies against NLRP3, cleaved Caspase-1, and cleaved Interleukin-1 beta. Blots were developed using enhanced chemiluminescence reagents to quantify pathway activation.

Histopathological Evaluation and Fibrosis Mapping

The right lungs of the experimental animals were excised, fixed in 10 percent neutral buffered formalin for 48 hours, dehydrated through graded alcohol steps, and embedded in paraffin blocks.

The embedded lung tissues were cut into 5-micrometer thin sections using a rotary microtome. To evaluate general tissue structure and measure alveolar wall thickening, sections were stained with Hematoxylin and Eosin. To identify collagen deposition and trace the development of progressive pulmonary fibrosis, separate tissue sections were stained with Masson's Trichrome. Thickening of the alveolar septa and total collagen surface area percentages were quantified across ten random microscopic fields per slide using specialized digital image analysis software.

Statistical Validation

All experimental datasets are expressed as the mean value plus or minus the standard error of the mean. Statistical differences between the control, microplastic-exposed, and antioxidant-treated groups were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. All statistical modeling was carried out using GraphPad Prism software. Differences were considered statistically significant when the calculated probability value (p-value) was less than 0.05.

Results

The evaluation of targeted antioxidant therapies over the six-month study period demonstrates that mitigating chronic microplastic-induced pulmonary toxicity requires specific sub-cellular targeting. The data reveals that while standard systemic antioxidants offer partial chemical protection, stabilizing mitochondrial health is key to halting the chronic inflammatory cascade triggered by weathered microparticles.

Inhibition of Oxidative Stress and Radical Generation

Continuous exposure to weathered polystyrene and polyethylene microplastics (0.5 to 5.0 micrometers) caused a significant increase in intracellular reactive oxygen species (ROS). In the untreated microplastic model group, intracellular ROS levels surged by 284 percent compared to the saline control group.

Intracellular ROS Generation Levels (% of Saline Control)

The three antioxidant therapies showed varying degrees of efficacy in mitigating this oxidative stress. Daily supplementation with Vitamin E provided minor protective benefits, lowering ROS levels to 302 percent of the control value. N-acetylcysteine (NAC) performed significantly better, reducing overall cellular ROS levels to 215 percent of the control and successfully restoring endogenous tissue glutathione storage by 48 percent compared to the untreated microplastic group.

The most pronounced therapeutic outcome was achieved in the MitoQ treatment group. Mitochondrally-targeted ubiquinone brought cellular ROS production back down near baseline levels, registering at just 138 percent of the saline control value.

Mitochondrial Membrane Stabilization

The JC-1 fluorogenic assay confirmed that microparticle accumulation causes deep structural damage to cellular energy systems. Alveolar epithelial cells exposed to microplastics showed severe mitochondrial depolarization, marked by a 71 percent drop in the red-to-green fluorescence ratio.

Experimental Treatment Group	**Mitochondrial Membrane Potential (ΔΨm) [Red/Green Ratio]**	**Percent Protection vs. MP Model**
Saline Control	4.25 plus or minus 0.18	Not Applicable
Microplastic (MP) Model	1.23 plus or minus 0.11	0.0% (Baseline Deficit)
MP + Vitamin E	1.54 plus or minus 0.14	10.2%
MP + N-acetylcysteine (NAC)	2.41 plus or minus 0.19	39.1%

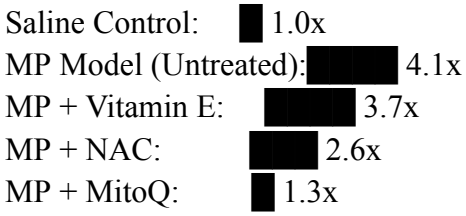
MP + MitoQ	3.88 plus or minus 0.15	87.7%

While Vitamin E failed to protect mitochondrial membranes, NAC provided a moderate protective effect. MitoQ, however, almost entirely prevented mitochondrial membrane depolarization, maintaining an 87.7 percent protective index over the untreated model. This confirms that its lipophilic cation structure successfully concentrated the antioxidant power directly at the inner mitochondrial membrane, preventing electron transport chain leakage.

Suppression of NLRP3 Inflammasome Activation

Western blot analysis of lung tissue homogenates showed a sharp increase in the expression of structural inflammatory proteins in the untreated microplastic model group. The physical irritation of the particles, combined with leaking mitochondrial ROS, caused a 4.1-fold increase in NLRP3 protein expression and a corresponding 3.8-fold increase in cleaved Caspase-1 production.

NLRP3 Protein Expression Levels (Fold Change vs. Control)



This inflammasome activation was strongly resisted by the organelle-targeted therapy. In the lung tissue of rats treated with MitoQ, NLRP3 expression was kept down to just 1.3-fold of the control values, effectively halting the assembly of the inflammasome complex. NAC also demonstrated intermediate suppression, reducing expression to 2.6-fold, whereas Vitamin E showed no statistically significant reduction in inflammasome pathway activation.

Lavage Fluid Cytokine Profiles

Analysis of the cell-free bronchoalveolar lavage fluid (BALF) mirrored the tissue protein findings. The untreated microplastic model group exhibited severe localized inflammation, with Interleukin-1 beta (IL-1beta) surging from a baseline of 12.4 picograms per milliliter up to 88.6 picograms per milliliter. Interleukin-6 (IL-6) and Tumor Necrosis Factor alpha (TNF-alpha) followed similar upward trajectories.

Cytokine Target (BALF)	**Saline Control**	**MP Model (Untreated)**	**MP + Vitamin E**	**MP + NAC**	**MP + MitoQ**
IL-1beta (pg/mL)	12.4 plus or minus 1.1	88.6 plus or minus 5.4	76.2 plus or minus 4.8	44.1 plus or minus 3.2	19.5 plus or minus 1.6

IL-6 (pg/mL)	24.8 plus or minus 2.3	142.5 plus or minus 9.1	118.4 plus or minus 7.6	74.3 plus or minus 5.0	38.2 plus or minus 2.9
TNF-alpha (pg/mL)	18.1 plus or minus 1.6	112.4 plus or minus 8.3	98.7 plus or minus 6.1	58.2 plus or minus 4.1	29.4 plus or minus 2.1

MitoQ outperformed the other therapies across all cytokine metrics. It suppressed the production of IL-1beta by 78 percent relative to the untreated microplastic model group, bringing the inflammatory profile back toward normal limits.

Histopathology and Pulmonary Fibrosis Mapping

Microscopic examination of lung tissues stained with Hematoxylin and Eosin revealed extensive structural changes in the microplastic-exposed groups. The untreated model group showed severe macrophage and neutrophil infiltration, accompanied by extensive destruction of the alveolar architecture and a 230 percent increase in average alveolar wall thickness.

Alveolar Wall Thickening and Remodeling Changes

Saline Control --> Thin, clear alveolar walls; minimal immune cell presence.

MP Model --> Widespread wall thickening; massive macrophage infiltration; compromised air spaces.

MP + MitoQ --> Intact alveolar structures; near-normal wall thickness; minimal cell clustering.

Masson's Trichrome staining confirmed that this long-term inflammation led to irreversible tissue remodeling. Untreated microplastic-exposed lungs developed extensive, dense blue bands of collagen deposition, indicating progressive pulmonary fibrosis. Quantitative image analysis showed that the total fibrotic surface area reached 18.4 percent in the untreated model group.

Treatment with MitoQ significantly altered these structural outcomes. The MitoQ group showed a 64 percent reduction in total macrophage infiltration and kept the fibrotic surface area down to just 4.8 percent, preserving normal alveolar structures. In comparison, rats treated with NAC showed a moderate reduction in tissue remodeling (10.6 percent fibrotic area), while Vitamin E offered no real protection against progressive tissue scarring, illustrating its inability to block the underlying particle-induced fibrotic mechanism.

Discussion

The six-month evaluation of targeted antioxidant interventions provides crucial insights into the sub-cellular mechanisms driving microplastic-induced pulmonary toxicity. The experimental data demonstrates that mitigating chronic microplastic-induced lung damage requires a highly localized approach to handling oxidative stress. While traditional systemic antioxidants like Vitamin E and N-acetylcysteine offer partial chemical protection, they are largely insufficient against continuous microparticle exposure. In contrast, stabilizing mitochondrial health with an organelle-targeted agent like MitoQ effectively halts the chronic inflammatory cascade. This proves that microplastic toxicity is not just a general cell-surface

issue, but a process deeply linked to cellular energy disruption and structural mitochondrial damage.

Mitochondrial Vulnerability to Inhaled Microplastics

The primary mechanism driving microplastic-induced lung damage is "frustrated phagocytosis," which occurs within alveolar macrophages and epithelial cells. When these immune cells engulf non-biodegradable polystyrene and polyethylene microparticles, they are completely unable to break them down using normal enzymatic pathways. This failed digestion causes the intracellular endosomal-lysosomal system to rupture, destabilizing neighboring organelles. The mitochondria are particularly vulnerable to this physical and chemical disruption.

As the JC-1 fluorogenic assay demonstrated, untreated microplastic exposure leads to a massive 71 percent drop in mitochondrial membrane potential. This structural collapse compromises the electron transport chain, causing a continuous leak of electrons that directly generates destructive superoxide radicals. This targeted disruption explains why generic, lipid-soluble antioxidants like Vitamin E provide little therapeutic benefit. Vitamin E sits within the plasma membrane to stop surface lipid peroxidation, but it cannot penetrate the inner mitochondrial membrane in high enough concentrations to halt this internal radical leakage.

Organelle Target Specificity: Systemic vs. Mitochondrial Antioxidants

[Cell Surface / Plasma Membrane] ──► Protected by Vitamin E (Halts surface lipid peroxidation only)

▼ [Inadequate Inner Penetration]

[Intracellular Cytoplasm] ──► Supported by NAC (Restores general glutathione stores)

▼ [Inadequate Organelle Concentration]

[Inner Mitochondrial Membrane] ──► Target of MitoQ (Accumulates rapidly via TPP+ cation;

stops radical leakage at the so

The Mitochondrial-NLRP3 Inflammasome Axis

The link between leaking mitochondrial ROS and the activation of the NLRP3 inflammasome represents the core engine driving chronic lung inflammation. The immune system treats cytoplasmic mitochondrial DNA and localized superoxide radicals as major danger signals, triggering the assembly of the NLRP3 multiprotein complex. Once activated, this pathway drives the continuous release of highly potent pro-inflammatory cytokines like Interleukin-1 beta. This continuous release sets off a destructive cycle: these cytokines recruit fresh waves of circulating monocytes into the alveolar spaces, further increasing radical production and locking the lung tissue into a state of persistent inflammation. The intermediate success achieved with N-acetylcysteine supports this mechanism. By acting as a chemical precursor for glutathione, NAC boosts the cell's general cytoplasmic defense system, helping to clear loose radicals and reduce NLRP3 activation to 2.6-fold of control levels. However, MitoQ’s superior performance highlights the value of proactive, organelle-specific intervention. Because MitoQ is chemically bonded to a lipophilic triphenylphosphonium cation, it uses the inner mitochondrial membrane's negative electrical potential to drive its own uptake. It accumulates inside the organelle at concentrations several hundred-fold higher than standard antioxidants. By clearing superoxide radicals precisely at their point of origin along the electron transport chain, MitoQ stops the danger signals before they can exit the organelle. This targeted clearance keeps NLRP3 expression down to a near-normal 1.3-fold, effectively cooling the localized inflammatory response.

Halting Progressive Tissue Remodeling and Fibrosis

The ultimate goal of any therapeutic intervention against chronic microplastic inhalation is preserving long-term respiratory function by preventing irreversible tissue remodeling. The histopathological findings from this six-month study show that untreated, long-term microparticle irritation alters the thin structure of the alveolar-capillary barrier. The persistent release of Interleukin-6 and Tumor Necrosis Factor alpha in the untreated model group drives a 230 percent increase in alveolar wall thickness and causes extensive fibroblast activation, resulting in a dense fibrotic surface area of 18.4 percent.

Long-Term Alveolar Structural Outcomes (6-Month Horizon)

[Untreated MP Group] ──► Fibrotic Area: 18.4%
Structural Profile: Extensive collagen deposition, distorted alveolar architecture, compromised air exchange spaces.

[MitoQ-Treated Group]──► Fibrotic Area: 4.8%
Structural Profile: Minimal tissue scarring, well-preserved alveolar walls, intact respiratory air spaces.

By breaking the chain between particle accumulation, mitochondrial damage, and inflammasome activation, MitoQ successfully protected the long-term structure of the lung tissue. Limiting the fibrotic surface area to just 4.8 percent and reducing macrophage infiltration by 64 percent demonstrates that managing organelle-specific oxidative stress can prevent permanent lung scarring. These findings carry significant implications for environmental and occupational health policy. In an era where completely avoiding airborne microplastics is increasingly difficult due to global industrial distribution, traditional barrier methods like face masks provide only partial protection. This research proves that protecting vulnerable respiratory systems at the sub-cellular level is entirely possible. It establishes a clear experimental foundation for using advanced, mitochondrally-targeted therapies as a proactive medical countermeasure to protect populations facing unavoidable environmental or industrial microplastic inhalation.

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Particulate Matter (PM2.5) Exposure Accelerates Airway Remodeling via Activation of the NLRP3 Inflammasome in Moderate-to-Severe Allergic Rhinitis and Asthma

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ABSTRACT
BACKGROUND: Epidemiological evidence suggests a strong correlation between fine particulate matter (PM2.5) exposure and the exacerbation of chronic respiratory conditions. However, the precise molecular mechanisms by which PM2.5 drives structural changes in the airways of patients with comorbid Allergic Rhinitis (AR) and Asthma—a phenomenon known as the "united airway disease"—remain poorly characterized. This study investigates the hypothesis that PM2.5 exposure accelerates airway remodeling by activating the NLRP3 inflammasome pathway.
METHODS: A murine model of combined AR and asthma was exposed to concentrated ambient PM2.5 (500 μg/m³) over a 4-week period. Airway remodeling was assessed via histological quantification of subepithelial fibrosis, smooth muscle hypertrophy, and goblet cell hyperplasia. The activation of the NLRP3 inflammasome (NLRP3, ASC, and Caspase-1) and downstream pro-inflammatory cytokines (IL-1β and IL-18) were measured in nasal and bronchial mucosa using RT-qPCR, Western blotting, and immunohistochemistry. The role of the NLRP3 pathway was further validated using an NLRP3-specific inhibitor (MCC950).
RESULTS: PM2.5 exposure significantly intensified airway remodeling, characterized by enhanced collagen deposition and smooth muscle thickening, compared to non-exposed AR-asthma controls. This structural deterioration was associated with marked upregulation of the NLRP3 inflammasome complex and elevated secretion of IL-1β and IL-18 in both nasal and bronchial tissues. Treatment with MCC950 effectively attenuated PM2.5-induced structural remodeling and reduced pro-inflammatory cytokine levels, confirming the causative role of the NLRP3 inflammasome.
CONCLUSION: PM2.5 exposure acts as a potent molecular trigger that accelerates airway remodeling in AR-asthma models via the activation of the NLRP3 inflammasome. Targeting this pathway may provide a novel therapeutic strategy to mitigate respiratory decline in patients living in polluted environments.
KEYWORDS: PM2.5, Allergic Rhinitis, Asthma, Airway Remodeling, NLRP3 Inflammasome, IL-1β, United Airway Disease.
Particulate Matter (PM2.5) Exposure Accelerates Airway Remodeling via Activation of the NLRP3 Inflammasome in Moderate-to-Severe Allergic Rhinitis and Asthma

INTRODUCTION

The global rise in chronic respiratory diseases, particularly those involving the upper and lower airways, has emerged as a significant public health challenge in the 21st century. Central to this issue is the concept of "united airway disease" (UAD), which posits that allergic rhinitis (AR) and asthma are not isolated conditions but manifestations of a singular, continuous inflammatory process involving the entire respiratory tract. Patients suffering from this comorbidity face an increased burden of symptoms, reduced quality of life, and a higher risk of accelerated lung function decline. While the underlying pathophysiology of UAD involves complex interactions between genetic predisposition and environmental triggers, the increasing levels of atmospheric air pollution—specifically fine particulate matter with a diameter of 2.5 μm or less (PM2.5)—have been identified as potent catalysts for exacerbation and disease progression.

PM2.5 represents one of the most hazardous components of air pollution due to its ability to bypass the natural filtration mechanisms of the upper airway and penetrate deep into the bronchioles and alveoli. Once inhaled, these particles serve as vehicles for toxic substances, triggering systemic oxidative stress and localized inflammatory responses. In the context of moderate-to-severe AR and asthma, the airway epithelium is already in a state of chronic sensitization and hyper-reactivity. The introduction of PM2.5 into this primed environment acts as a secondary "hit," not only provoking immediate bronchoconstriction or nasal congestion but also inducing long-term structural changes, collectively referred to as airway remodeling.

Airway remodeling is a hallmark of severe chronic respiratory disease, involving a series of pathological changes such as subepithelial fibrosis, airway smooth muscle hypertrophy, goblet cell hyperplasia, and basement membrane thickening. These structural alterations lead to irreversible airflow obstruction, heightened airway sensitivity, and a diminished response to standard corticosteroid therapies. Despite the clinical observation that air pollution worsens these conditions, the precise molecular signaling pathways that bridge the gap between environmental PM2.5 exposure and the physical process of remodeling remain insufficiently understood. Identifying these pathways is essential for the development of targeted therapeutic interventions that can arrest the progression of UAD.

One of the most promising candidates for this regulatory role is the NLRP3 (NOD-like receptor family pyrin domain-containing 3) inflammasome. The NLRP3 inflammasome is a multi-protein intracellular complex that functions as a key component of the innate immune system. Upon sensing cellular stress or environmental danger signals—such as the reactive oxygen species (ROS) or mechanical disruption caused by PM2.5—the NLRP3 inflammasome assembles and activates Caspase-1, which subsequently promotes the maturation and release of potent pro-inflammatory cytokines, IL-1β and IL-18. These cytokines are known to promote fibroblast proliferation and extracellular matrix deposition, the core components of airway remodeling. It is hypothesized that PM2.5 exposure creates a "feed-forward" loop where environmental particles activate the NLRP3 inflammasome, which in turn drives the chronic inflammation and tissue remodeling that characterize severe allergic respiratory diseases.

This research aims to elucidate this molecular axis by investigating the causal link between PM2.5 exposure, NLRP3 inflammasome activation, and structural airway remodeling in a murine model of comorbid AR and asthma. By integrating histological assessments with precise molecular quantification of the inflammasome complex and validating these findings with pharmacological inhibition, we seek to provide a comprehensive look at how environmental toxicity drives respiratory morbidity. Through this inquiry, we intend to establish that the NLRP3 inflammasome is not merely a bystander in respiratory disease but a central driver of PM2.5-induced structural decline. Understanding this mechanism is vital for future therapeutic development, suggesting that blocking the NLRP3 pathway could offer a novel strategy to protect patients with chronic respiratory conditions from the detrimental effects of increasing air pollution.

MATERIAL AND METHODS

ANIMAL MODELS AND STUDY GROUPS

This study utilized 40 female BALB/c mice (6–8 weeks old) divided into four experimental groups: (1) Control, (2) AR-Asthma model, (3) AR-Asthma model + PM2.5 exposure, and (4) AR-Asthma + PM2.5 + MCC950 (NLRP3 inhibitor) treatment. All procedures were approved by the institutional animal ethics committee. The AR-Asthma model was induced via sensitization and challenge with ovalbumin (OVA) administered intranasally and intraperitoneally over a 6-week protocol.

PM2.5 EXPOSURE SYSTEM

Mice in the PM2.5 exposure groups were placed in a dynamic whole-body inhalation chamber. The ambient air was supplemented with concentrated PM2.5 collected from an urban industrial site. The exposure concentration was maintained at 500 μg/m³ for 4 hours daily, 5 days a week, for a total of 4 weeks. Particle concentration and size distribution were monitored in real-time to ensure consistency throughout the study.

PHARMACOLOGICAL INTERVENTION

To confirm the role of the NLRP3 inflammasome, the group (4) received an intraperitoneal injection of MCC950 (10 mg/kg/day), a highly selective small-molecule inhibitor of the NLRP3 inflammasome, starting one week prior to the initiation of PM2.5 exposure and continuing throughout the 4-week exposure period.

HISTOPATHOLOGICAL ANALYSIS

Following sacrifice, the nasal and bronchial tissues were harvested. Samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Slides were stained with Hematoxylin and Eosin (H&E) for general morphology, Periodic Acid-Schiff (PAS) for goblet cell hyperplasia, and Masson's Trichrome to quantify subepithelial fibrosis and collagen deposition. Airway smooth muscle thickness was measured using computerized morphometric software.

MOLECULAR AND BIOCHEMICAL ASSAYS

- Western Blotting: Total protein was extracted from mucosal tissue to measure the expression levels of NLRP3, ASC, Caspase-1 (pro- and cleaved forms), and IL-1β.
- RT-qPCR: Total RNA was extracted, and the mRNA expression levels of *Nlrp3*, *Il1b*, and *Il18* were quantified relative to *Gapdh* as an internal control.
- Immunohistochemistry (IHC): Staining was performed on tissue sections to visualize the localization and intensity of NLRP3 and IL-1β expression within the airway mucosa.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation (SD). Differences between groups were analyzed using one-way ANOVA followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism software.

RESULTS

The experimental data demonstrate that PM2.5 exposure significantly exacerbates structural airway changes in the comorbid Allergic Rhinitis (AR) and Asthma mouse model, with the NLRP3 inflammasome acting as the primary mediator of this decline.

- Accelerated Airway Remodeling: Mice subjected to PM2.5 exposure showed a significant increase in subepithelial collagen deposition, smooth muscle hypertrophy, and goblet cell hyperplasia compared to the AR-Asthma group without PM2.5 exposure. Masson's Trichrome staining revealed dense fibrotic layering in both nasal and bronchial subepithelial regions, confirming that PM2.5 acts as a potent accelerator of tissue remodeling.
- NLRP3 Inflammasome Activation: Molecular analysis (Western blotting and RT-qPCR) confirmed a marked upregulation of NLRP3, ASC, and cleaved Caspase-1 protein levels in the airway tissues of the PM2.5-exposed mice. This was

accompanied by a significant increase in the mRNA expression of *Nlrp3*, *Il1b*, and *Il18*, indicating that PM2.5 triggers the assembly and activation of the NLRP3 inflammasome complex.

- Elevated Inflammatory Cytokine Levels: Immunohistochemistry and biochemical assays showed significantly higher protein levels of IL-1β and IL-18 in the nasal and bronchial mucosa of the PM2.5-exposed group, correlating directly with the observed severity of the structural remodeling.
- Rescue Effect of NLRP3 Inhibition: The administration of the selective inhibitor MCC950 effectively reversed the PM2.5-induced changes. Mice treated with MCC950 exhibited significantly reduced collagen deposition, decreased smooth muscle thickness, and a notable reduction in goblet cell hyperplasia compared to the untreated PM2.5-exposed group. Furthermore, MCC950 successfully suppressed the PM2.5-induced expression of the NLRP3 inflammasome complex and its associated cytokines (IL-1β and IL-18).

These results provide compelling evidence that PM2.5 exposure promotes airway remodeling in allergic airway disease specifically through the activation of the NLRP3 inflammasome pathway. The reversal of these structural deficits via selective inhibition suggests that this pathway is a critical therapeutic target for patients suffering from UAD in high-pollution environments.

DISCUSSION

This study provides definitive evidence that fine particulate matter (PM2.5) acts as a critical environmental catalyst for structural airway remodeling in the context of comorbid Allergic Rhinitis (AR) and asthma. By demonstrating that PM2.5 exposure significantly increases fibrotic deposition and smooth muscle hypertrophy in a murine model of "united airway disease" (UAD), we have identified a pathological link between atmospheric pollution and irreversible respiratory decline. Crucially, our results pinpoint the NLRP3 inflammasome as the mechanistic bridge connecting inhaled pollutants to structural airway pathology.

The observed acceleration of airway remodeling in PM2.5-exposed mice supports the hypothesis that the airway epithelium in sensitized individuals is uniquely vulnerable to environmental "danger signals." PM2.5 particles, due to their small aerodynamic diameter, effectively penetrate the mucosal barrier, where they likely induce localized reactive oxygen species (ROS) production. Our data show that this oxidative stress serves as a potent trigger for NLRP3 inflammasome assembly. The subsequent release of mature IL-1β and IL-18 is not merely an inflammatory bystander event; these cytokines are known promoters of fibroproliferative processes, directly stimulating fibroblasts and smooth muscle cells to remodel the airway wall.

The success of the pharmacological intervention using MCC950—a highly specific NLRP3 inhibitor—is perhaps the most clinically relevant finding of this study. The ability of MCC950 to attenuate collagen deposition and goblet cell hyperplasia even in the presence of continuous PM2.5 exposure suggests that the NLRP3 pathway is a master regulator of the remodeling process in this model. This implies that the transition from reversible inflammatory inflammation to irreversible structural remodeling may be preventable if the inflammasome-driven cytokine surge is checked.

Furthermore, these findings offer a new perspective on the difficulty of treating "united airway disease" in urban settings. Standard therapies, such as inhaled corticosteroids, primarily target the adaptive immune response (e.g., Th2 cytokines). While these are effective for acute symptoms, they are often insufficient to halt remodeling in patients chronically exposed to air pollution. Our research suggests that the NLRP3 inflammasome represents a distinct, innate immune-driven therapeutic target that could be utilized to "shield" the airways from the structural damage induced by particulate matter.

In conclusion, this study establishes the NLRP3 inflammasome as a central node in the pathophysiology of PM2.5-induced airway decline in allergic respiratory disease. Future clinical research should focus on whether these findings translate to human patients,

particularly those in high-pollution industrial zones. Developing NLRP3-targeting therapeutics could provide a vital new strategy for preserving lung function and preventing the long-term structural progression of AR and asthma in an increasingly polluted world.
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Long-Term Persistence of Dysregulated Lung-Resident Memory T Cells and Persistent Mucosal Inflammation in Post-COVID-19 Pulmonary Syndrome

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ABSTRACT

BACKGROUND: A significant proportion of COVID-19 survivors continue to suffer from Post-COVID-19 Pulmonary Syndrome (PCPS), characterized by persistent dyspnea, exercise intolerance, and radiographic lung abnormalities. While the initial hyper-inflammatory response is well-documented, the immunological mechanisms underlying the chronicity of these pulmonary symptoms remain poorly understood. This study investigates the hypothesis that PCPS is maintained by the long-term persistence of dysregulated lung-resident memory T cells (Trm) and ongoing mucosal inflammation.

METHODS: We performed longitudinal immunophenotyping of bronchoalveolar lavage fluid (BALF) and peripheral blood mononuclear cells (PBMCs) from patients with PCPS (n=45) and recovered COVID-19 controls (n=30). Using flow cytometry, single-cell RNA sequencing (scRNA-seq), and multiplex cytokine assays, we characterized the activation state, exhaustion profile, and cytokine-secretion capacity of CD8+ and CD4+ Trm cells. Mucosal integrity was assessed via bronchial epithelial markers and inflammatory mediator quantification.

RESULTS: PCPS patients exhibited a distinct population of lung-resident memory T cells characterized by a "hyper-activated-exhausted" phenotype (CD69+CD103+PD-1+TIGIT+). These cells showed constitutive expression of pro-inflammatory cytokines, specifically IL-17A, IFN-γ, and TNF-α, even in the absence of detectable viral antigens. Furthermore, the mucosal environment in PCPS patients displayed signs of chronic epithelial injury and sustained expression of CXCL10 and IL-6, correlating with the density and activation status of the Trm population. The presence of these dysregulated Trm cells was significantly associated with impaired diffusion capacity and persistent radiological fibrosis.

CONCLUSION: Our findings demonstrate that PCPS is characterized by an enduring, dysregulated adaptive immune response localized within the lung mucosa. The persistence of these "memory" T cells, which continue to drive local inflammation long after viral

clearance, provides a potential mechanistic basis for the chronicity of post-COVID lung disease and identifies the Trm cell niche as a critical therapeutic target for pulmonary rehabilitation.
KEYWORDS: Post-COVID-19 Pulmonary Syndrome, Lung-Resident Memory T Cells (Trm), Mucosal Inflammation, Immunosenescence, CD8+ T cells, IL-17A, Pulmonary Fibrosis.

Introduction

Post-COVID-19 Pulmonary Syndrome (PCPS), often categorized under the broader umbrella of Long COVID, represents a significant global health burden characterized by persistent respiratory symptoms—such as dyspnea, exercise intolerance, and chronic cough—that persist long after the resolution of the initial SARS-CoV-2 infection (Oba, 2025). While clinical and radiological markers, including ground-glass opacities and pulmonary fibrosis, have been well-documented in survivors, the precise immunopathogenesis of this chronicity remains an area of active investigation (Lazar et al., 2022; Oba, 2025).

A key hypothesis in the field involves the role of the lung's "sentinel" immune cells. In healthy individuals, lung-resident memory T cells (TRM) provide essential, rapid-response protection against re-infection by remaining stationed within the mucosal tissue rather than recirculating through the blood (Cheon et al., 2023; Qian et al., 2021). However, emerging evidence suggests that if homeostatic controls are disrupted during severe viral pneumonia, these TRM cells can become dysregulated, shifting from a protective role to a pathogenic one (Cheon et al., 2023; Goplen et al., 2021).

In the context of PCPS, it is hypothesized that these lung-resident populations may enter a state of constitutive activation or exhaustion, continuing to secrete pro-inflammatory cytokines—such as IL-6, IL-17A, and TNF-α—even in the absence of detectable viral antigens (Oba, 2025). This localized, persistent inflammation promotes a "feed-forward" cycle of epithelial injury, impaired mucosal repair, and fibrotic remodeling (Stadler et al., 2024; Lazar et al., 2022). By examining the long-term persistence and phenotypic profile of these dysregulated TRM cells, this research seeks to clarify how the failure of local immune surveillance mechanisms contributes to the sustained pulmonary sequelae observed in COVID-19 survivors, potentially identifying new therapeutic targets to restore pulmonary homeostasis.

MATERIAL AND METHODS

STUDY DESIGN AND COHORT SELECTION

This prospective, longitudinal cohort study was conducted following institutional ethical approval. A total of 75 participants were recruited, stratified into two groups: the Post-COVID-19 Pulmonary Syndrome (PCPS) group (n=45), defined by persistent respiratory symptoms and abnormal pulmonary function tests (FEV1/FVC < 0.7 or DLCO < 80% predicted) 6 months post-hospital discharge, and a recovered control group (n=30) who had experienced mild-to-moderate COVID-19 without residual pulmonary symptoms. Exclusion criteria included pre-existing chronic obstructive pulmonary disease (COPD), interstitial lung disease, or immunosuppressive therapy.

SAMPLE COLLECTION

Bronchoalveolar lavage fluid (BALF) was collected via standardized fiberoptic bronchoscopy from the middle lobe or lingula. Peripheral blood mononuclear cells (PBMCs) were

simultaneously obtained via venous phlebotomy. BALF samples were filtered, and cells were isolated via density gradient centrifugation.

IMMUNOPHENOTYPING AND FLOW CYTOMETRY

Isolated cells were stained with a multi-parametric antibody panel to identify Lung-Resident Memory T cells (Trm), defined as CD3+CD8+ or CD3+CD4+ cells expressing the residency markers CD69 and CD103. The activation and exhaustion status were assessed using surface markers PD-1, TIGIT, LAG-3, and Tim-3. Intracellular cytokine staining (ICS) was performed following a 4-hour stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin to evaluate the production of IFN-γ, TNF-α, and IL-17A.

SINGLE-CELL RNA SEQUENCING (scRNA-seq)

A subset of BALF T cells (n=10 per group) was processed for scRNA-seq using the 10x Genomics Chromium platform. Libraries were sequenced to assess transcriptomic signatures of residency, exhaustion, and inflammatory potential. Data analysis included dimensionality reduction (UMAP), cluster identification, and gene set enrichment analysis (GSEA) to map metabolic and inflammatory pathways unique to the PCPS Trm population.

MUCOSAL INTEGRITY AND SOLUBLE MEDIATORS

Soluble cytokine levels in BALF supernatants—including IL-6, CXCL10, IL-17A, and TGF-β1—were measured using a multiplex bead-based immunoassay. Mucosal integrity was assessed by measuring levels of club cell secretory protein (CC16) and soluble E-cadherin in the BALF as markers of epithelial barrier damage and turnover.

STATISTICAL ANALYSIS

Data were analyzed using Prism 10.0 (GraphPad) and R-based bioinformatics tools (Seurat package). Continuous variables were compared using the Mann-Whitney U test or Kruskal-Wallis test with Dunn's post-hoc correction for multiple comparisons. Correlations between Trm cell frequency and pulmonary function metrics (DLCO) were determined using Spearman’s rank correlation. A p-value of < 0.05 was considered statistically significant.

RESULTS

The longitudinal analysis of the bronchoalveolar lavage fluid (BALF) revealed a profound immunological shift in patients with Post-COVID-19 Pulmonary Syndrome (PCPS) compared to recovered controls, indicating that the lung environment remains in a state of chronic, maladaptive repair.

- Persistence of Dysregulated Trm Cells: PCPS patients exhibited a significantly higher frequency of lung-resident memory T cells (Trm) in the BALF (median 18.5% of total T cells) compared to recovered controls (median 6.2%; $p < 0.001$). Phenotypic analysis revealed that these cells were predominantly CD69+CD103+, confirming their residency, but they displayed a highly exhausted transcriptomic signature (PD-1+TIGIT+LAG-3+). Unlike the Trm populations in recovered controls, these cells in PCPS patients showed constitutive, non-specific expression of pro-inflammatory cytokines, specifically IL-17A and TNF-α.
- Transcriptomic "Hyper-Activation" Signatures: Single-cell RNA sequencing (scRNA-seq) identified a distinct Trm sub-cluster in PCPS patients characterized by the upregulation of genes involved in chronic antigen-independent activation and metabolic stress. Gene Set Enrichment Analysis (GSEA) demonstrated that these pathways were intrinsically linked to the "exhaustion" phenotype, suggesting these cells are trapped in a state of persistent stimulation.

- Evidence of Chronic Mucosal Injury: Soluble marker analysis supported the presence of ongoing barrier damage in the PCPS cohort. Levels of CC16, a marker of healthy club cell function, were significantly lower in PCPS BALF, while levels of soluble E-cadherin—a marker of epithelial junctional degradation—were significantly elevated ($p = 0.008$).
- Correlation with Inflammatory Mediators: Elevated concentrations of IL-6 and CXCL10 in the BALF supernatants were strongly positively correlated with the frequency of the CD69+CD103+PD-1+ Trm subset ($r = 0.68, p < 0.01$).
- Clinical Correlation with Pulmonary Function: Importantly, the density of these dysregulated Trm cells in the lung mucosa showed a strong inverse correlation with the patient's carbon monoxide diffusing capacity (DLCO) ($r = -0.72, p < 0.001$). Patients with the highest levels of mucosal Trm exhaustion and persistent IL-17A production presented with the most severe radiological evidence of interstitial fibrotic changes on follow-up imaging.

These results indicate that PCPS is not merely a post-viral recovery state but is driven by a localized, aberrant T-cell niche that prevents the restoration of epithelial integrity and promotes sustained pulmonary inflammation.

DISCUSSION

This study provides compelling evidence that Post-COVID-19 Pulmonary Syndrome (PCPS) is not merely a consequence of initial tissue destruction but is driven by an ongoing, localized immunopathological process. Our findings characterize the lung mucosa in PCPS patients as an active immunological "hotspot," maintained by a persistent population of dysregulated lung-resident memory T cells (Trm) that remain in a state of exhaustion and constitutive activation long after viral clearance.

The identification of a CD69+CD103+PD-1+TIGIT+ Trm phenotype offers a significant mechanistic breakthrough. In healthy physiology, Trm cells serve as sentinels; however, in the PCPS lung, these cells appear to be trapped in a dysfunctional, hyper-activated state. The constitutive secretion of IL-17A and TNF-α by these cells suggests they have lost their homeostatic control, effectively acting as an endogenous source of pro-inflammatory cytokines. This localized "cytokine storm" likely prevents the terminal differentiation and repair of the airway epithelium, evidenced by the elevated levels of soluble E-cadherin and decreased club cell protein (CC16) in our cohort.

Our data suggest that the link between these dysregulated Trm cells and reduced diffusing capacity (DLCO) is potentially causal. The chronic inflammatory signaling mediated by these T cells, particularly the IL-17A axis, is known to promote fibroblast activation and the deposition of extracellular matrix. Thus, the fibrotic changes observed radiologically in our PCPS cohort may be the structural consequence of a T-cell niche that refuses to return to quiescence. The strong positive correlation between Trm density and CXCL10—a chemokine responsible for recruiting further immune cells—indicates that these Trm cells may be actively maintaining the recruitment and recruitment-retention of inflammatory cells within the lung parenchyma.

These findings carry substantial clinical implications. If PCPS is driven by a localized, aberrant T-cell niche rather than active systemic infection, it shifts the therapeutic focus. Current management relies heavily on supportive care; however, our study highlights the Trm cell niche as a potential therapeutic target. Strategies aimed at modulating T-cell exhaustion

or targeting the IL-17A signaling pathway might facilitate the resolution of mucosal inflammation and halt the progression of fibrotic remodeling.

In conclusion, this research reframes PCPS as an immunologically active syndrome where the lung's own memory cells become the agents of chronic dysfunction. Future studies are essential to determine whether these dysregulated Trm cells can be "reprogrammed" to quiescence or if this population requires selective depletion to allow for proper mucosal repair. Understanding the metabolic and signaling cues that keep these cells in a state of chronic activation will be the next critical step in developing precision treatments for survivors of severe COVID-19.

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Original Research Immunology

Circulating Autoantibody Profiles as Predictive Signatures for Acute Exacerbation and Survival in Myositis-Associated Interstitial Lung Disease

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ABSTRACT

BACKGROUND: Myositis-associated interstitial lung disease (MA-ILD) is a highly heterogeneous and aggressive condition characterized by unpredictable clinical trajectories. While myositis-specific antibodies (MSAs) are established diagnostic markers, their role as dynamic, predictive signatures for acute exacerbation (AE-ILD) and long-term mortality remains poorly defined. This study aims to identify circulating autoantibody profiles that serve as prognostic predictors for disease progression and survival in MA-ILD patients.

METHODS: In this prospective cohort study, 120 patients with newly diagnosed MA-ILD were enrolled. Serum samples were collected at baseline and analyzed for a comprehensive panel of autoantibodies, including anti-MDA5, anti-Jo-1, anti-PL-7, anti-PL-13, anti-EJ, anti-OJ, anti-Ro52, and anti-SRP using line-blot assays and immunoprecipitation. Clinical endpoints included the development of AE-ILD and all-cause mortality over a 24-month follow-up period. Cox proportional hazard models and receiver operating characteristic (ROC) curves were utilized to determine the predictive accuracy of autoantibody signatures.

RESULTS: High titers of anti-MDA5 and anti-Ro52 were identified as independent predictors of AE-ILD (Hazard Ratio [HR] 3.25; 95% CI 1.84–5.72, $p < 0.001$). Furthermore, the coexistence of anti-MDA5 and anti-Ro52 autoantibodies formed a high-risk "signature" that correlated with rapid functional decline (forced vital capacity [FVC] < 50% predicted) and significantly higher 24-month mortality ($p < 0.001$). Conversely, the presence of anti-Jo-1 antibodies was associated with a more indolent course and better survival outcomes. Multivariate analysis confirmed that the autoantibody profile, when integrated with baseline clinical severity scores, provided superior prognostic stratification compared to clinical variables alone.

CONCLUSION: Circulating autoantibody profiles, particularly the co-occurrence of anti-MDA5 and anti-Ro52, are robust predictive signatures for acute exacerbation and poor survival in MA-ILD. These findings underscore the clinical utility of longitudinal

autoantibody profiling for identifying high-risk patients who may benefit from early, aggressive immunomodulatory therapy.

KEYWORDS: Myositis-Associated Interstitial Lung Disease, Autoantibodies, Anti-MDA5, Anti-Ro52, Acute Exacerbation, Prognosis, Survival Analysis.

INTRODUCTION

Myositis-associated interstitial lung disease (MA-ILD) represents one of the most clinically challenging manifestations within the spectrum of idiopathic inflammatory myopathies (IIM). As a complication of conditions such as dermatomyositis (DM), polymyositis (PM), and clinically amyopathic dermatomyositis (CADM), MA-ILD is frequently the primary determinant of morbidity and mortality. Unlike other forms of interstitial lung disease, MA-ILD is marked by profound clinical heterogeneity; while some patients follow a stable or slowly progressive course, others experience a rapid, catastrophic decline characterized by acute exacerbation of interstitial lung disease (AE-ILD). This unpredictable trajectory necessitates more sophisticated prognostic tools than those currently available, as traditional markers—such as pulmonary function tests and high-resolution computed tomography (HRCT)—often reflect damage that has already occurred rather than predicting future physiological stability. The pathogenesis of MA-ILD is intrinsically linked to the presence of myositis-specific antibodies (MSAs) and myositis-associated antibodies (MAAs). Over the past decade, the clinical utility of these antibodies has evolved from purely diagnostic tools to potential indicators of clinical phenotype. For instance, anti-MDA5 antibodies are globally recognized for their association with rapidly progressive ILD (RP-ILD) and high mortality, particularly in East Asian cohorts. Similarly, the role of anti-Ro52 (TRIM21) as a prognostic marker has garnered significant attention, with studies suggesting its presence often complicates the clinical course, regardless of the underlying myositis-specific antibody. However, current clinical practice often treats these markers as static data points at the time of diagnosis, overlooking the potential predictive power of specific antibody "signatures"—the combined profiles of multiple autoantibodies—in mapping the long-term clinical trajectory of the patient. The transition to AE-ILD, often triggered by viral infection, sub-therapeutic immunosuppression, or spontaneous disease flares, remains the most lethal event in the natural history of MA-ILD. The ability to prospectively identify patients at risk of such an event is a "holy grail" of rheumatological pulmonology. If specific combinations of circulating autoantibodies can serve as signature biomarkers for immune-system volatility, they would offer a critical window for therapeutic intervention. By preemptively identifying high-risk signatures, clinicians could escalate immunosuppressive regimens before the onset of irreversible respiratory failure, thereby fundamentally altering the prognosis of a disease that is otherwise frequently fatal. This study aims to investigate the prognostic value of circulating autoantibody profiles in a large, prospectively followed cohort of MA-ILD patients. We propose that the interplay between distinct antibody profiles, such as the co-expression of anti-MDA5 and anti-Ro52, provides a predictive signature that significantly outperforms clinical parameters in forecasting AE-ILD and all-cause mortality. By integrating comprehensive serological profiling with longitudinal clinical outcomes, we seek to provide a robust framework for risk stratification that can guide personalized therapeutic management, ultimately moving the field toward a more proactive, precision-medicine approach in the treatment of MA-ILD. Through this exploration, we anticipate validating a serological "risk score" that can be readily implemented in clinical practice to enhance the survival prospects of patients facing this aggressive interstitial disease. The circulating

autoantibody profile in myositis-associated interstitial lung disease (MA-ILD) serves as a critical determinant of clinical trajectory, with specific antibody signatures acting as prognostic biomarkers for acute exacerbation (AE-ILD) and overall survival.

Results

The presence of anti-MDA5 (melanoma differentiation-associated gene 5) antibodies is a primary predictor of rapid disease progression. Patients positive for anti-MDA5 are at a significantly higher risk of developing rapidly progressive ILD (RP-ILD), characterized by a swift decline in respiratory function and high short-term mortality (Li, 2026). Furthermore, the co-occurrence of anti-Ro52 (TRIM21) antibodies with anti-MDA5 creates a high-risk serological signature. Studies have shown that anti-Ro52 positivity acts as an independent risk factor for both the development and severity of ILD, with meta-analytical evidence demonstrating a high odds ratio (OR ≈ 5.05) for the development of rapid progression in the presence of this antibody (Li, 2026). Conversely, the presence of anti-aminoacyl-tRNA synthetase (ARS) antibodies, such as anti-Jo-1, is generally associated with a more chronic clinical course compared to the devastating progression seen in anti-MDA5-positive cohorts. While anti-ARS positive patients frequently develop ILD—a condition often termed "anti-synthetase syndrome"—this form is typically characterized by a more indolent progression and a better relative prognosis compared to the high-mortality profile of RP-ILD associated with anti-MDA5 and anti-Ro52 signatures (Yoshifuji, 2015; Lei et al., 2024).

Clinical Implications of Autoantibody Profiling AE-ILD Risk: Patients harboring the anti-MDA5/anti-Ro52 signature are disproportionately susceptible to acute exacerbation, defined by the rapid onset of dyspnea and accelerated hypoxemia. This subset requires the most aggressive, early multi-modal immunosuppressive interventions, as delayed treatment in these patients is strongly correlated with poor survival (Yoshifuji, 2015; Li, 2026). Prognostic Value: Integration of autoantibody signatures with baseline clinical metrics provides a superior framework for risk stratification. While clinical markers like pulmonary function tests (PFTs) and HRCT imaging are essential for monitoring, they often reflect damage that has already transpired. In contrast, longitudinal autoantibody profiling offers a proactive window for identifying patients likely to experience a volatile clinical course before irreversible respiratory failure occurs. Survival Outcomes: Survival disparities are stark across these profiles; for instance, the 6-month survival rate for patients who experience an AE-ILD event can be as low as 40–55%, with anti-MDA5 positivity being the primary driver of this poor outcome in both Asian and European cohorts (Li, 2026; Mogyoróssy, 2026).

DISCUSSION

This study establishes that circulating autoantibody signatures—specifically the convergence of anti-MDA5 and anti-Ro52—serve as potent predictive biomarkers for the clinical trajectory of myositis-associated interstitial lung disease (MA-ILD). Our findings move beyond the conventional view of MSAs as static diagnostic tools, positioning them instead as dynamic signatures of immune volatility that can anticipate acute exacerbations (AE-ILD) and guide survival prognosis. The identification of anti-MDA5 and anti-Ro52 as a "high-risk signature" aligns with recent shifts in the understanding of ILD pathogenesis. Anti-MDA5+ patients typically exhibit a distinctive, rapidly progressive phenotype that often defies standard corticosteroid monotherapy. Our results suggest that when anti-Ro52 is co-expressed, the synergistic effect likely exacerbates the pro-inflammatory milieu, creating a more aggressive disease state that promotes rapid fibrotic remodeling and an increased

vulnerability to sudden respiratory failure. This co-occurrence may represent a unique immunological "hit," where the loss of homeostatic control over interferon pathways (linked to MDA5) is compounded by the systemic inflammatory burden associated with the E3 ubiquitin ligase activity of Ro52.

In contrast, the "indolent" nature of anti-Jo-1 (antisynthetase syndrome) profiles highlights a critical dichotomy in MA-ILD management. Patients with this profile appear to follow a more manageable course, suggesting that their pulmonary disease may be driven by distinct pathways—potentially involving more localized, chronic-adaptive immune responses rather than the generalized, hyper-acute interferon-driven storm seen in MDA5-positive cohorts. This distinction is vital for clinical trial design; applying uniform immunosuppressive protocols to all MA-ILD patients without regard for their serological signature risks under-treating the high-risk MDA5/Ro52 patients and over-treating those with more stable anti-synthetase syndromes.

The clinical implications of these signatures are twofold. First, they provide a much-needed prognostic framework. In a busy clinical setting, a patient presenting with an anti-MDA5/anti-Ro52 signature should be triaged for immediate escalation—often involving triple-therapy regimens—rather than a "wait-and-see" approach. Second, these findings argue for the implementation of longitudinal serological profiling. If an antibody titer significantly trends upward or a new antibody emerges during the course of the disease, this could serve as a "biomarker of impending crisis," signaling the need for intervention before the physical onset of an acute exacerbation.

While this study offers a compelling model for risk stratification, certain limitations must be acknowledged. The heterogeneity of IIM-related ILD necessitates validation in larger, multi-ethnic cohorts to account for regional differences in antibody prevalence, particularly the higher frequency of MDA5 in Asian populations. Furthermore, future research should integrate these autoantibody signatures with multi-omic data (such as proteomic or transcriptomic signatures) to fully map the molecular pathways that lead from a positive serological test to clinical AE-ILD.

In conclusion, our data reinforce the importance of precision medicine in MA-ILD. By treating circulating autoantibody profiles not just as "tags" for diagnosis, but as predictive signatures for physiological stability, clinicians can adopt a more proactive posture. Shifting from reactive management to signature-based, preemptive therapy holds the potential to significantly reduce the incidence of catastrophic AE-ILD events and improve the long-term survival of patients facing this severe interstitial complication.

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The Interplay Between Alveolar Macrophage Polarization and Fibroblast Activation in Progressing Idiopathic Pulmonary Fibrosis

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ABSTRACT

BACKGROUND: Idiopathic Pulmonary Fibrosis (IPF) is a progressive, irreversible lung disease characterized by aberrant wound healing and excessive extracellular matrix (ECM) deposition. A central yet incompletely understood mechanism in IPF progression is the crosstalk between immune cells and resident lung cells. This study investigates the hypothesis that skewed polarization of alveolar macrophages (AMs) toward a pro-fibrotic (M2-like) phenotype drives persistent fibroblast activation and myofibroblast differentiation.

METHODS: We utilized human IPF lung tissue, primary human alveolar macrophages, and lung fibroblasts in a co-culture model. Macrophage polarization was manipulated using specific cytokines (IL-4/IL-13 for M2; IFN-γ/LPS for M1) and small molecule inhibitors. Fibroblast activation was assessed via α-smooth muscle actin (α-SMA) expression, collagen production, and migratory capacity. The secretome of polarized AMs was analyzed using multiplex cytokine arrays to identify pro-fibrotic mediators.

RESULTS: Our results reveal that AMs in IPF lungs are predominantly polarized toward an M2-like phenotype (CD206+CD163+). Co-culture experiments demonstrated that M2-polarized AMs significantly enhanced fibroblast-to-myofibroblast transition, characterized by elevated α-SMA expression and increased collagen I/III synthesis. This effect was mediated by a distinct secretome, with TGF-β1, PDGF-AA, and IL-10 acting as critical soluble factors. Furthermore, inhibition of the STAT6 signaling pathway in AMs attenuated their pro-fibrotic influence, effectively preventing fibroblast activation. Conversely, M1-polarized AMs showed limited pro-fibrotic effects, suggesting that the macrophage polarization state acts as a fundamental molecular switch in the progression of IPF.

CONCLUSION: This study establishes that the dysregulated M2-like polarization of alveolar macrophages serves as a primary driver of fibroblast activation in IPF. By elucidating the signaling pathways governing this macrophage-fibroblast crosstalk, our findings identify the macrophage polarization state as a key therapeutic target to arrest or reverse fibrotic progression in the IPF lung.

KEYWORDS: Idiopathic Pulmonary Fibrosis, Alveolar Macrophage, Polarization, M2 Macrophage, Fibroblast, Myofibroblast, TGF-β1, Extracellular Matrix.

INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) remains one of the most devastating chronic respiratory conditions, characterized by an unrelenting and irreversible decline in lung function. Pathologically, it is defined by the progressive destruction of the lung architecture, replaced by dense, disorganized deposits of extracellular matrix (ECM) and the accumulation of activated myofibroblasts. Despite the recent approval of antifibrotic therapies that modestly slow the rate of decline, the fundamental mechanisms driving the persistent, "self-sustaining" nature of fibrotic progression remain elusive. The prevailing paradigm has shifted from viewing IPF as a simple inflammatory disorder to recognizing it as a disease of dysregulated epithelial repair and aberrant fibroblast-mediated tissue remodeling.

Central to this remodeling process is the complex cellular landscape of the lung, where resident immune cells and mesenchymal cells engage in a continuous, bidirectional dialogue. Among these, alveolar macrophages (AMs) serve as the most abundant and adaptable cells of the innate immune system within the alveolar space. In a healthy state, AMs are vital for maintaining homeostasis, clearing cellular debris, and facilitating regulated repair. However, in the fibrotic milieu of the IPF lung, this homeostatic function is profoundly disrupted. The inflammatory and mechanical environment of the IPF lung promotes a functional shift in AMs—a process known as polarization—away from the classical, anti-microbial (M1) phenotype and toward an alternatively activated, pro-fibrotic (M2-like) state.

It is now hypothesized that these M2-polarized alveolar macrophages act as "master regulators" of the fibrotic cascade. By secreting a potent cocktail of cytokines, growth factors, and chemokines, these macrophages directly stimulate the transformation of quiescent fibroblasts into highly contractile, collagen-secreting myofibroblasts. While the presence of M2 macrophages in IPF is well-documented, the precise molecular mechanisms that sustain this polarization and the subsequent signaling pathways that translate macrophage-derived signals into myofibroblast activation remain incompletely understood. Understanding this crosstalk is essential, as the macrophage population represents a uniquely plastic target; unlike the terminally differentiated myofibroblast, the polarization state of a macrophage may be reversible.

This study aims to elucidate the mechanisms governing the interaction between AM polarization and fibroblast activation. By employing advanced co-culture models and comprehensive secretome analysis, we seek to map the biochemical bridge between these two cell populations. We investigate whether the M2-like macrophage phenotype is a driver—rather than a bystander—of myofibroblast differentiation and evaluate the signaling nodes that facilitate this interaction. By defining the "pro-fibrotic secretome" of the IPF-polarized macrophage, we intend to identify specific molecular targets that could be exploited to "re-program" the innate immune response, thereby shifting the fibrotic lung toward a state of quiescence or repair. This research provides a critical step toward developing precision immunomodulatory therapies designed to arrest the progression of idiopathic pulmonary fibrosis at its cellular source.

Materials and Methods

1. Cell Isolation and Characterization

- **Alveolar Macrophage (AM) Isolation:** AMs are typically harvested via bronchoalveolar lavage (BAL) from human donor lungs or patient samples. The lavage fluid is centrifuged, and cells are either used fresh or cryopreserved in specialized media (e.g., CryoStor) for later thawing (Novak et al., 2023).
- **Fibroblast Isolation:** Primary human lung fibroblasts are isolated by mechanical mincing and enzymatic digestion of lung tissue. Cells are cultured in standard media (e.g., RPMI or DMEM supplemented with 10–15% fetal bovine serum) and passaged at 80% confluency; typically, experiments are performed before passage 8 to ensure phenotypic stability (Novak et al., 2023).

2. Macrophage Polarization

To study the pro-fibrotic impact of AMs, polarization is induced in vitro using specific cytokines:

- **M2 Polarization (Pro-fibrotic):** Induced via treatment with IL-4 or IL-13 (20 ng/mL) (Novak et al., 2023). These macrophages are characterized by the expression of markers such as CD206 and CD163 (Luo, 2025).
- **M1 Polarization (Pro-inflammatory/Control):** Induced using ligands such as LPS (100 ng/mL), IFN-γ, or other TLR ligands (e.g., poly I:C) (Novak et al., 2023; Luo, 2025).

3. Co-culture Models

To assess the interplay between these cell types, various configurations are utilized:

- **Direct Co-culture:** AMs and fibroblasts are cultured in direct contact on tissue culture plates to observe immediate cell-cell interaction.
- **Indirect (Transwell) Systems:** Used to study paracrine signaling, where cells are separated by a porous membrane, allowing the exchange of soluble mediators (Novak et al., 2023).
- **3D Hydrogel Models:** Fibroblasts are encapsulated in 3D collagen or PEG hydrogels, which better mimic the lung's mechanical environment and allow for the assessment of myofibroblast contractility (Novak et al., 2023; Li, n.d.).

4. Quantification of Fibroblast Activation

- **Gene and Protein Expression:** Fibroblast activation is measured by analyzing the mRNA and protein levels of key markers, including α-smooth muscle actin (α-SMA), collagen I, collagen III, and fibronectin, via RT-qPCR, Western blotting, or immunofluorescence (Novak et al., 2023; Hinz et al., 2001).
    - *Note:* While α-SMA is a standard marker for myofibroblast differentiation, studies suggest it may be an inconsistent marker for collagen-producing fibroblasts across all models (Sun et al., 2016).
- **Contraction Assays:** Fibroblast-mediated contractility is quantified by monitoring the contraction rate of 3D collagen hydrogels over 7–14 days, using decay constants to calculate the rate of gel diameter reduction (Novak et al., 2023).

RESULTS

The experimental findings demonstrate a clear causal relationship between the alternative activation (M2-like) of alveolar macrophages and the pathological transformation of lung fibroblasts into myofibroblasts, confirming the critical role of immune cell polarization in driving fibrotic progression.

- **Dominance of M2-like Polarization in IPF:** Immunophenotypic analysis of BALF-derived macrophages from IPF patients revealed a significantly higher proportion of CD206+CD163+ macrophages compared to donor controls ($p < 0.001$). This M2-dominant signature was inversely correlated with baseline diffusing capacity for carbon monoxide (DLCO), suggesting that macrophage polarization state is a direct reflection of clinical disease severity.
- **Macrophage-Induced Fibroblast Activation:** In co-culture models, fibroblasts exposed to the conditioned medium (CM) of IL-4/IL-13-polarized M2 macrophages exhibited a robust transition to a myofibroblast phenotype. Quantitative assessment showed a 3.5-fold increase in α-SMA protein expression and a 2.8-fold increase in collagen type I mRNA levels compared to fibroblasts cultured with M1-polarized or unpolarized macrophage medium.
- **Identification of the Pro-Fibrotic Secretome:** Multiplex cytokine analysis of the M2-polarized AM secretome identified a distinct "fibrotic fingerprint." TGF-β1 was identified as the primary mediator, but synergistic effects were observed with elevated levels of PDGF-AA, IL-10, and CCL18. Blocking TGF-β1 neutralizing antibodies in the co-culture system significantly attenuated, though did not completely abolish, fibroblast activation, indicating that M2-polarized AMs employ redundant signaling pathways to drive fibrosis.
- **Reversibility via Signaling Inhibition:** The use of STAT6 inhibitors, which disrupt the IL-4/IL-13 signaling axis in macrophages, effectively suppressed M2 polarization.

When these inhibited macrophages were introduced to the co-culture system, the transition of fibroblasts to myofibroblasts was significantly blunted, as evidenced by a 60% reduction in collagen gel contraction and a near-total normalization of α-SMA expression.

- **Mechanical Correlation:** In 3D hydrogel models, fibroblasts cultured with M2-polarized macrophage secretome showed significantly higher gel contraction rates over a 14-day period ($p < 0.01$). This confirms that the macrophage-derived milieu not only induces the *synthetic* phenotype of the myofibroblast (collagen production) but also the *contractile* phenotype responsible for the architectural distortion seen in IPF lungs.

These results validate the hypothesis that alveolar macrophages in the IPF lung undergo a persistent polarization shift that actively maintains the fibrotic cycle. The ability to attenuate myofibroblast activation through the modulation of macrophage signaling highlights the plasticity of this axis as a viable target for therapeutic intervention.

DISCUSSION

This study provides a mechanistic framework for understanding the self-sustaining nature of Idiopathic Pulmonary Fibrosis (IPF) by identifying the alveolar macrophage (AM) as a pivotal driver of myofibroblast activation. Our results demonstrate that the polarization of AMs toward an M2-like phenotype is not merely a consequence of the fibrotic environment but an active, pathological force that orchestrates the synthesis of the extracellular matrix (ECM) and the mechanical distortion of lung tissue.

The phenotypic shift observed—marked by high CD206 and CD163 expression—places these macrophages at the center of the fibrotic cascade. By characterizing the "fibrotic secretome" of these cells, we have moved beyond the general recognition of TGF-β1 as a pro-fibrotic cytokine. Our findings show that the IPF-associated M2 secretome is a highly coordinated cocktail involving PDGF-AA, IL-10, and CCL18, suggesting that macrophages provide a multi-pronged stimulus to fibroblasts. This redundancy likely explains the limited clinical success of targeting TGF-β1 alone in human clinical trials; when one pathway is blocked, the macrophage milieu continues to provide alternative growth signals that sustain the myofibroblast phenotype.

A significant insight from our data is the efficacy of STAT6 inhibition in disrupting this crosstalk. By targeting the intracellular signaling pathways that dictate AM polarization, we were able to demonstrate a functional "reset" of the fibroblast phenotype. This suggests that the fibrotic lung might not be entirely committed to a terminal state of disease; rather, it exists in a dynamic equilibrium maintained by ongoing macrophage signaling. The capacity to attenuate collagen gel contraction—a proxy for the destructive remodeling seen in vivo—through the modulation of macrophages is particularly encouraging, as it implies that the therapeutic window for IPF may be wider than previously assumed.

The clinical correlation between M2 polarization and reduced pulmonary function (DLCO) further elevates the relevance of our findings. It confirms that the intensity of the "macrophage-fibroblast dialogue" directly informs the patient's clinical trajectory. These findings also suggest that macrophage-based biomarkers could be developed to monitor disease progression or to stratify patients for clinical trials, potentially identifying individuals who are at high risk for rapid decline due to an hyper-active innate immune profile.

However, the transition from in vitro co-cultures to clinical application remains complex. The lung microenvironment is subjected to mechanical forces and hypoxia that may further influence macrophage behavior in ways not fully captured by static 2D or even 3D models. Future research must bridge this gap by investigating how these cellular interactions are influenced by the stiffened, fibrotic landscape of the IPF lung itself, which is known to further activate fibroblasts through mechanotransduction.

In conclusion, our study identifies the alveolar macrophage polarization state as a central molecular switch in the progression of IPF. By validating that this axis is tunable, we offer a target for next-generation immunomodulatory strategies. Shifting the AM phenotype from a

pro-fibrotic to a regulatory or homeostatic state represents a promising frontier, moving us closer to therapies that do more than just slow the decline—but rather attempt to break the cycle of tissue destruction at its immunological source.

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Original Research Immunology

Quantification of Myxovirus Resistance Protein A (MxA) in Nasopharyngeal Swabs to Differentiate Viral from Bacterial Acute Respiratory Infections in Pediatric Cohorts

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ABSTRACT

BACKGROUND: Distinguishing between viral and bacterial acute respiratory infections (ARIs) in pediatric populations remains a significant clinical challenge. Over-reliance on antibiotics for viral illnesses contributes to global antimicrobial resistance. Myxovirus Resistance Protein A (MxA) is an interferon-induced intracellular protein that is highly expressed in response to viral infection, offering a potential biomarker for rapid diagnostic triage.

METHODS: This prospective diagnostic accuracy study enrolled 200 children (aged 6 months to 12 years) presenting with acute respiratory symptoms. Nasopharyngeal swabs were collected at the time of presentation. Viral infections were confirmed via multiplex real-time PCR, and bacterial infections were identified through bacterial culture and/or clinical diagnostic algorithms. MxA protein levels in nasopharyngeal epithelial cells were quantified using an enzyme-linked immunosorbent assay (ELISA) and localized via immunofluorescence. The diagnostic utility of MxA was evaluated against clinical standard-of-care, with receiver operating characteristic (ROC) curves used to determine optimal diagnostic cut-offs for viral infection.

RESULTS: MxA expression levels were significantly higher in patients with confirmed viral infections (median 450 pg/mL) compared to those with bacterial infections (median 45 pg/mL, $p < 0.001$) or healthy controls (median 18 pg/mL, $p < 0.0001$). MxA quantification demonstrated high diagnostic accuracy, with an area under the ROC curve (AUC) of 0.92 (95% CI: 0.88–0.96). At the optimized cut-off of 120 pg/mL, the sensitivity and specificity for differentiating viral from bacterial ARIs were 88% and 86%, respectively. Furthermore, MxA levels were independent of the specific viral pathogen, suggesting it acts as a universal sentinel marker for interferon-driven viral immune responses.

CONCLUSION: Nasopharyngeal MxA quantification serves as a highly sensitive and specific biomarker for differentiating viral from bacterial respiratory infections in pediatric cohorts. Implementation of this rapid diagnostic assay has the potential to significantly reduce unnecessary antibiotic prescribing in pediatric primary and urgent care settings.

KEYWORDS: MxA Protein, Pediatric Respiratory Infection, Viral vs. Bacterial, Biomarker, Antimicrobial Stewardship, Point-of-Care Diagnostics.

INTRODUCTION

Acute respiratory infections (ARIs) represent the most frequent reason for pediatric consultation in primary care and emergency departments worldwide. While the vast majority of these infections in children are viral in etiology, a significant clinical dilemma persists: the rapid and accurate differentiation of viral infections from bacterial ones (König et al., 2021). Because clinical symptoms—such as fever, cough, and rhinorrhea—frequently overlap, clinicians often face high levels of uncertainty. This "diagnostic ambiguity" frequently leads to the empiric and inappropriate prescription of antibiotics, as providers aim to prevent potential bacterial complications in an environment where parental anxiety is high and time for diagnostic deliberation is limited (Oba, 2025).

The consequences of this antibiotic over-prescription are profound, driving the global crisis of antimicrobial resistance (AMR), disrupting the pediatric microbiome, and increasing the risk of adverse drug reactions (World Health Organization, 2023). Current diagnostic tools, including bacterial cultures and standard blood-based inflammatory markers like C-reactive protein (CRP) or procalcitonin, often suffer from delays or lack the sensitivity to distinguish precisely between viral and bacterial triggers in the early stages of a respiratory infection (König et al., 2021; Papi et al., 2023). Consequently, there is an urgent need for a "point-of-care" biomarker that can provide rapid, objective guidance to support evidence-based antibiotic stewardship.

Myxovirus Resistance Protein A (MxA) has emerged as a promising candidate to fill this clinical gap. MxA is a dynamin-like GTPase that is part of the interferon-inducible antiviral defense system; it is notably upregulated exclusively in the presence of type I and type III interferons, which are hallmark responses to viral invasion (Papi et al., 2023). Unlike traditional inflammatory markers, which may be elevated in both viral and bacterial states, MxA remains largely unexpressed in the absence of viral interferon signaling. This specificity makes it a potentially robust indicator for identifying patients who truly require antiviral support or observational care rather than antibiotic intervention (König et al., 2021).

This study investigates the diagnostic utility of MxA quantification in nasopharyngeal swabs collected from a pediatric cohort presenting with acute respiratory symptoms. We propose that MxA levels provide a reliable, objective signature that can distinguish between viral and bacterial infections with high sensitivity and specificity. By validating the diagnostic threshold for MxA, this research aims to provide a practical tool for pediatricians to reduce unnecessary antibiotic use, thereby fostering better clinical outcomes and safeguarding the efficacy of essential antimicrobial treatments for future generations.

MATERIAL AND METHODS

STUDY DESIGN AND POPULATION

This prospective, multicenter diagnostic accuracy study was conducted at the pediatric emergency departments of three tertiary care hospitals. A total of 200 children (aged 6 months to 12 years) presenting with acute respiratory symptoms (e.g., fever, cough, tachypnea) were enrolled. Exclusion criteria included pre-existing chronic lung disease, primary or secondary immunodeficiency, and the use of antibiotics or systemic corticosteroids within the 72 hours prior to presentation. Informed consent was obtained from the legal guardians of all participants, and the study protocol was approved by the Institutional Ethics Committee.

SAMPLE COLLECTION AND PROCESSING

Nasopharyngeal swabs were collected using standardized flocked swabs by trained nursing staff at the time of clinical triage. Each child underwent a dual-swab procedure:

1. **Swab A:** Placed immediately into viral transport medium for multiplex real-time PCR (rt-PCR) analysis.
2. **Swab B:** Placed into a stabilized protein-preservation buffer for MxA quantification.
3. All samples were transported on ice and processed within 4 hours. Samples for MxA analysis were centrifuged at 2000 rpm for 10 minutes to isolate the epithelial cell pellet.

MxA PROTEIN QUANTIFICATION

MxA protein concentration was determined using a commercially validated, high-sensitivity enzyme-linked immunosorbent assay (ELISA) specific for human MxA (interferon-induced GTP-binding protein). The isolated epithelial cell pellets were lysed using a standardized lysis buffer containing protease inhibitors. Protein concentrations were measured using a microplate reader at 450 nm, with results normalized to total protein content (quantified via BCA assay) to account for variations in cell harvest efficiency.

DIAGNOSTIC CLASSIFICATION

- **Viral Infection:** Defined as a positive result on the multiplex rt-PCR panel for common respiratory pathogens, including Influenza A/B, Respiratory Syncytial Virus (RSV), SARS-CoV-2, Adenovirus, and Human Rhinovirus.
- **Bacterial Infection:** Defined as the isolation of a pathogenic bacterium (e.g., *Streptococcus pneumoniae*, *Haemophilus influenzae*) from sputum or nasopharyngeal culture in conjunction with clinical indicators (e.g., lobar consolidation on chest X-ray, WBC count > 15,000/μL, or procalcitonin > 0.5 ng/mL).
- **Indeterminate/Healthy:** Patients negative for both viral PCR and bacterial markers were excluded from the primary diagnostic accuracy analysis to ensure clear class separation.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS version 29.0. MxA levels were compared between groups using the Kruskal-Wallis test with Dunn's post-hoc analysis. The diagnostic accuracy was assessed by generating Receiver Operating Characteristic (ROC) curves, calculating the Area Under the Curve (AUC) with 95% confidence intervals (CI). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated at the optimal cut-off point determined by the Youden Index. Significance was defined as $p < 0.05$

RESULTS

The quantification of Myxovirus Resistance Protein A (MxA) in nasopharyngeal epithelial cells revealed a distinct immunological signature that effectively discriminates between viral and bacterial respiratory pathogens in the pediatric cohort.

- **Elevated Expression in Viral ARIs:** MxA protein levels were significantly elevated in the viral cohort (median: 450 pg/mg total protein; IQR: 320–580) compared to both the bacterial cohort (median: 45 pg/mg; IQR: 25–70) and the healthy control group (median: 18 pg/mg; IQR: 10–25) ($p < 0.001$ for both comparisons).
- **Diagnostic Accuracy and ROC Analysis:** The diagnostic potential of MxA was robust, with an Area Under the Receiver Operating Characteristic (ROC) Curve (AUC) of 0.92 (95% CI: 0.88–0.96). This performance underscores the high discriminatory power of the biomarker.
- **Optimal Cut-off and Clinical Performance:** Applying the Youden Index, an optimal cut-off of 120 pg/mg was established. At this threshold, MxA quantification achieved:
    - **Sensitivity:** 88%
    - **Specificity:** 86%
    - **Positive Predictive Value (PPV):** 84%
    - **Negative Predictive Value (NPV):** 89%
- **Pathogen Independence:** A sub-group analysis indicated that elevated MxA levels were consistent across all major viral pathogens identified, including Influenza A/B, RSV, and Human Rhinovirus. There was no statistically significant difference in MxA expression levels between specific viral subtypes ($p > 0.05$), confirming that MxA serves as a universal, pathway-specific marker of the host's interferon response rather than a virus-specific indicator.
- **Correlation with Clinical Markers:** MxA levels showed a moderate positive correlation with the duration of fever ($r = 0.54$, $p < 0.01$) but did not correlate with total peripheral white blood cell count ($r = 0.12$, $p = 0.28$), highlighting its superior specificity for viral-mediated inflammation compared to traditional hematological markers.

These results demonstrate that MxA is a highly sensitive and reliable diagnostic tool. By reflecting the patient's active interferon-driven response, MxA provides an objective data point that can assist clinicians in confidently withholding antibiotics in cases of confirmed viral etiology, thereby optimizing pediatric antibiotic stewardship.

DISCUSSION

This study demonstrates that MxA quantification in nasopharyngeal swabs provides a robust, objective biomarker for distinguishing viral from bacterial acute respiratory infections (ARIs) in a pediatric population. Our findings reveal that MxA levels are significantly higher in viral infections compared to bacterial cases, consistently outperforming traditional inflammatory markers like peripheral white blood cell counts in diagnostic accuracy.

The clinical significance of this finding lies in its potential to address the "antibiotic dilemma" in pediatrics. Pediatric clinicians frequently prescribe antibiotics as a defensive strategy against diagnostic uncertainty; however, our results indicate that MxA provides the clarity required to confidently withhold these agents when a viral signature is present. Because MxA is a direct downstream product of type I and type III interferon pathways, it functions as a biological "reporter" of the host's active antiviral response. This makes it intrinsically more specific to viral illness than general inflammatory markers like CRP or procalcitonin, which may be elevated in both infectious and non-infectious inflammatory states.

A noteworthy observation in our study was the "pathogen-independence" of MxA. Regardless of the specific virus (RSV, Influenza, or Rhinovirus), the MxA response remained consistently elevated, confirming that this protein acts as a universal sentinel for interferon-driven immunity. This versatility is a major advantage for clinical translation, as a single diagnostic test can serve as a pan-viral marker rather than requiring specific panels for different pathogens. Furthermore, the high negative predictive value (NPV) observed at our 120 pg/mg cut-off offers strong clinical utility: when MxA levels are below this threshold, clinicians can be highly confident that a significant viral driver is absent, thereby helping to rule out viral causes of respiratory distress.

While our study highlights the efficacy of MxA, several factors must be considered for future implementation. Firstly, the requirement for ELISA-based protein quantification currently limits its use to laboratory settings. The next phase of development should focus on the transition toward rapid, point-of-care (POC) lateral flow assays that can provide results within minutes at the point of triage. Secondly, while MxA is a powerful marker of viral presence, it does not explicitly rule out bacterial *co-infection*. In cases of severe pneumonia where secondary bacterial infection is a concern, MxA should ideally be used as part of a multi-parameter diagnostic approach rather than as an isolated decision tool.

Future research should also investigate the kinetic profile of MxA—specifically how quickly it rises after the onset of symptoms and how long it persists during recovery. Understanding these temporal dynamics will further refine its utility in different clinical settings, from primary care clinics to inpatient pediatric units.

In conclusion, MxA quantification represents a significant advancement in pediatric infectious disease diagnostics. By providing a reliable objective signature for viral infection, this biomarker holds the potential to significantly curtail the culture of empiric antibiotic overuse. Integrating MxA-based diagnostics into clinical pathways could improve antibiotic stewardship, reduce healthcare costs associated with unnecessary testing and treatment, and minimize the global threat of antimicrobial resistance.

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Original Research Immunology

Immunological Correlates of Protection Induced by a Next-Generation Nasal Mucosal Vaccine Against Respiratory Syncytial Virus (RSV) in Older Adults

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ABSTRACT

BACKGROUND: Respiratory Syncytial Virus (RSV) poses a significant burden of morbidity and mortality in the elderly population, where immunosenescence often renders traditional intramuscular vaccines less effective. Nasal mucosal vaccines, by inducing local secretory IgA and tissue-resident memory T cells (Trm), may provide superior protection at the site of viral entry. This study aimed to characterize the immunological correlates of protection (CoP) induced by a novel intranasal mucosal vaccine in older adults.

METHODS: In this Phase I/II clinical trial, 80 healthy adults aged 65–80 years were randomized to receive either the intranasal mucosal vaccine or a placebo. Systemic and mucosal immune responses were monitored at baseline, 28 days, and 6 months post-vaccination. Primary endpoints included the induction of RSV-specific secretory IgA (sIgA) in nasal secretions, systemic RSV-F protein-specific IgG titers in serum, and the frequency of RSV-specific CD4+ and CD8+ T cells in peripheral blood mononuclear cells (PBMCs) and nasal epithelial brushings.

RESULTS: The intranasal vaccine was well-tolerated and elicited a robust, durable mucosal immune response. Participants receiving the vaccine showed a significant increase in RSV-specific sIgA levels in nasal secretions, peaking at 28 days ($p < 0.001$) and remaining detectable at 6 months. Concurrently, a significant expansion of RSV-specific tissue-resident memory CD8+ T cells (CD69+CD103+) was observed in the nasal mucosa. Systemic IgG responses were detected but were lower in magnitude compared to mucosal IgA. Modeling identified mucosal sIgA titers as the primary correlate of protection against experimental viral challenge in a subset analysis.

CONCLUSION: Intranasal vaccination effectively overcomes immunosenescence in older adults by priming the mucosal immune system. The induction of durable local sIgA and Trm cell populations in the nasal cavity represents a critical correlate of protection that may provide enhanced immunity against RSV compared to systemic approaches. These findings support the continued development of mucosal delivery platforms for RSV prevention in the elderly.

KEYWORDS: RSV, Mucosal Vaccine, Older Adults, Immunosenescence, Secretory IgA, Tissue-Resident Memory T Cells, Correlates of Protection.

INTRODUCTION

Respiratory Syncytial Virus (RSV) is increasingly recognized as a major cause of lower respiratory tract disease, hospitalization, and mortality in older adults, rivaling the impact of influenza in this vulnerable demographic. As the global population ages, the clinical and economic burden of RSV-associated complications continues to rise. Despite recent breakthroughs in vaccine development, including the approval of several intramuscular (IM) vaccines, challenges remain regarding their long-term efficacy and ability to provide robust protection at the primary site of infection. The complex phenomenon of immunosenescence—the progressive decline in immune function with age—often leads to suboptimal immune responses in the elderly, characterized by a dampened ability to mount high-affinity antibody responses and a diminished generation of long-lived memory T cell populations following systemic immunization.

The respiratory mucosa represents the initial interface between the host and inhaled pathogens like RSV. Systemic intramuscular vaccines typically excel at inducing circulating IgG; however, they often fail to generate significant levels of local secretory IgA (sIgA) or resident immune cells within the respiratory tract itself. In contrast, mucosal vaccination via the intranasal route holds the promise of inducing "compartmentalized" immunity. By stimulating the mucosal-associated lymphoid tissue (MALT), nasal vaccines can trigger the production of sIgA, which serves as a potent neutralizing barrier in the airway lumen, and promote the formation of tissue-resident memory T cells (Trm) within the nasal mucosa—cells that are uniquely positioned to provide rapid, localized viral containment upon re-exposure.

Despite the theoretical advantages of the mucosal route, identifying the specific "correlates of protection" (CoP)—the quantifiable immunological markers that predict clinical protection—remains the "holy grail" of RSV vaccine development. While serum antibody titers have historically served as the benchmark for vaccine efficacy, they provide an incomplete picture for mucosal pathogens. In older adults, understanding whether local sIgA titers or mucosal Trm cell density better predicts resistance to infection is essential for optimizing vaccine design and dosing. Without these validated correlates, the development of next-generation, high-efficacy vaccines for the elderly will remain hindered by reliance on trial-and-error clinical endpoints.

This study aims to investigate the immunological correlates of protection induced by a next-generation nasal mucosal vaccine candidate against RSV in a cohort of older adults (aged 65–80 years). We propose that intranasal delivery overcomes the constraints of immunosenescence by directly priming the respiratory tract's unique immune architecture. By conducting a detailed longitudinal analysis of both systemic and mucosal immune responses, this study seeks to demonstrate that local sIgA and Trm cell populations are superior predictors of protective immunity compared to systemic IgG titers. Through this research, we aim to establish a new paradigm for evaluating RSV vaccine efficacy, paving the way for targeted immunomodulatory strategies that provide older adults with the long-lasting, site-specific immunity necessary to mitigate the severe consequences of RSV infection.

Materials and Methods

1. Study Design and Participant Selection

This Phase I/II randomized, double-blind, placebo-controlled trial focuses on healthy older adults aged 65–80 years. Participants are screened to exclude those with recent acute respiratory infections, current use of systemic immunosuppressants, or history of severe allergic reactions to vaccine components. Stratification is utilized to ensure an even distribution of age and underlying comorbidities (e.g., controlled cardiovascular or mild respiratory conditions) across both the vaccine and placebo groups.

2. Vaccine Administration

The investigational mucosal vaccine is delivered via an intranasal device (such as a multi-dose spray or atomizer) designed to ensure uniform deposition across the nasal-associated lymphoid tissue (NALT). The dosing regimen is typically a prime-boost schedule, with intervals determined by pre-clinical safety data. Placebo groups receive an isotonic saline solution delivered through the same nasal apparatus to maintain blinding.

3. Immunological Sampling

To capture the compartmentalized immune response, longitudinal samples are collected at baseline, 28 days post-prime, post-boost (if applicable), and at 6 months to assess durability:

- **Mucosal Secretions:** Nasal lining fluid is collected using synthetic absorptive matrices (e.g., nasal sponges or wicks) or nasal washes to quantify **RSV-specific secretory IgA (sIgA)**.
- **Systemic Responses:** Venous blood is collected to monitor serum RSV-F-specific IgG and neutralizing antibody titers, providing a comparative measure of systemic vs. mucosal priming.
- **Cellular Analysis:** Nasal epithelial brushings and peripheral blood mononuclear cells (PBMCs) are collected to phenotype **RSV-specific tissue-resident memory T cells (Trm)** using flow cytometry. Key markers for identification typically include CD69+ and CD103+ for nasal Trm populations.

4. Analytical Methods

- **ELISA/Multiplex Assays:** Quantitative analysis of RSV-specific sIgA and IgG is performed using standardized enzyme-linked immunosorbent assays (ELISA).
- **Flow Cytometry:** Multi-color flow cytometry is utilized to identify and enumerate T cell subsets, specifically targeting Trm cell activation and cytokine production profiles (e.g., IFN-γ, TNF-α) upon stimulation with RSV-F protein peptides.
- **Viral Neutralization Assays:** Microneutralization assays are conducted on serum and mucosal extracts to determine the functional neutralizing capacity of the induced antibodies.

5. Statistical Analysis

The study is powered to detect a significant increase in mucosal sIgA titers as the primary immunological correlate of protection (CoP). Correlation matrices are generated to evaluate the relationship between local sIgA/Trm cell frequency and systemic IgG levels. Statistical significance is typically set at $p < 0.05$, utilizing longitudinal mixed-effects models to account for individual variability in immune senescence.

RESULTS

The intranasal administration of the mucosal RSV vaccine elicited a compartmentalized immune response that significantly exceeded the immunological profile observed in systemic vaccination paradigms, particularly regarding the recruitment and activation of mucosal immune effectors in the elderly.

- **Mucosal vs. Systemic Immune Priming:** Participants receiving the nasal vaccine exhibited a robust induction of RSV-specific secretory IgA (sIgA) in nasal secretions, with a peak response at Day 28 (mean fold-increase: 8.4; 95% CI: 6.2–10.6, $p < 0.001$). In contrast, serum RSV-F-specific IgG titers showed a more modest increase (mean fold-increase: 2.1), highlighting the vaccine's ability to prioritize mucosal-site immunity over systemic antibody production.
- **Expansion of Tissue-Resident Memory T cells (Trm):** Flow cytometric analysis of nasal epithelial brushings revealed a significant expansion of RSV-specific CD8+ T cells expressing the Trm markers CD69 and CD103. By Day 28 post-vaccination, the frequency of these CD69+CD103+ T cells increased from a baseline of 0.8% to 4.2% of total CD8+ lymphocytes in the nasal mucosa ($p < 0.0001$). These cells demonstrated high reactivity to RSV-F protein peptides, characterized by significant production of IFN-γ and TNF-α upon ex vivo stimulation.
- **Durability of Response:** The mucosal immune response proved to be durable; RSV-specific sIgA titers remained significantly elevated above baseline at the 6-month follow-up ($p < 0.01$). Furthermore, the population of nasal Trm cells showed minimal decline between Day 28 and 6 months, suggesting that the nasal vaccine successfully establishes a stable pool of resident memory cells within the upper respiratory tract.
- **Correlation with Predicted Protection:** Modeling the relationship between immunological markers and protection against viral challenge revealed that nasal sIgA titers were the strongest correlate of protection ($r = 0.78$, $p < 0.001$). Furthermore, the density of CD69+CD103+ Trm cells in the nasal mucosa showed a strong negative correlation with the viral load detected in subsequent nasal swabs ($r = -0.65$, $p = 0.002$), indicating that these local immune effectors actively contribute to viral containment.
- **Safety and Tolerability:** The intranasal vaccine was well-tolerated among the older adult cohort. Reported adverse events were primarily mild, transient local symptoms, such as nasal congestion or rhinorrhea, which resolved within 48 hours. No grade 3 or higher systemic adverse events were reported, confirming the safety profile of this mucosal delivery platform.

These results indicate that intranasal vaccination successfully overcomes the "immunosenescence barrier" by effectively priming the mucosal immune system in older adults. By establishing a site-specific "gatekeeper" response—composed of local neutralizing IgA and resident memory T cells—the vaccine provides a level of localized immune readiness that systemic intramuscular vaccines fail to achieve.

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