Understanding the Role of Phospholipid Scramblase 1 in ILC2-Driven Type 2 Inflammation in Alternaria-Induced Asthma

By

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Overall abstract

Exaggerated type 2 immune responses play critical roles in the pathogenesis of allergic diseases, including asthma and atopic dermatitis. Recent studies have highlighted the importance of innate type 2 immune responses and group 2 innate lymphoid cells (ILC2s) in initiating and amplifying these inflammatory processes. However, the molecular mechanisms that regulate ILC2-driven type 2 inflammation remain incompletely understood.

Phospholipid scramblase-1 (PLSCR1) is a membrane-associated protein involved in phospholipid translocation across the plasma membrane and has been implicated in several immune signaling pathways. Despite these findings, the role of PLSCR1 in regulating innate type 2 immune responses has not been fully characterized. In this study, we investigated the potential role of PLSCR1 in allergen-induced type 2 inflammation in lung and skin using mouse models.

First, an intranasal Alternaria alternata–induced airway inflammation model was established to examine allergen-driven inflammatory responses in the lung. Bronchoalveolar lavage fluid (BALF) analysis, histological assessment, and flow cytometry were used to characterize airway inflammation and the activation and recruitment of ILC2 populations. Histological analysis suggested differences in airway inflammatory responses between experimental groups, while flow cytometry analysis did not reveal a strong shift in ILC2 population frequencies, suggesting that PLSCR1 may influence functional aspects of type 2 immunity rather than markedly altering ILC2 abundance.

In addition, an Alternaria-induced skin atopic dermatitis model was developed to investigate allergic skin inflammation and to explore the potential migration of ILC2s along the skin–lung immune axis.

Together, these studies establish experimental models for investigating the role of PLSCR1 in type 2 immune responses and provide preliminary insights into how PLSCR1 may modulate allergen-induced inflammation across tissues.

Keywords: PLSCR1; Type 2 Inflammation; Innate Lymphoid Cells (ILC2); Alternaria Alternata; Allergic Airway Inflammation; Atopic Dermatitis; Skin–Lung Immune Axis

Chapter 1

Background and Rationale: Type 2 Inflammation and ILC2 Regulation in Asthma

Asthma Overview and Global Burden

Asthma is a common chronic respiratory disease characterized by wheezing, shortness of breath, chest tightness, and coughing. It affects a substantial proportion of the global population, with prevalence estimates ranging from 1% to 29% across different countries and regions. According to the *Global Asthma Report*, the prevalence of asthma is approximately 9.1% among children, 11.0% among adolescents, and 6.6% among adults worldwide.[1]

Beyond its clinical manifestations, asthma imposes a significant global burden on patients and healthcare systems. The disease is associated with reduced quality of life, frequent healthcare utilization, and substantial economic costs. In addition to physical symptoms, asthma also contributes to psychological stress and long-term disease management challenges for both patients and their families.

Type 2 (T2)-High Asthma

Asthma is not a single disease entity but rather a heterogeneous syndrome comprising multiple phenotypes and endotypes with distinct underlying immunological mechanisms. Among these, type 2 (T2)-high asthma represents one of the most well-defined and prevalent endotypes. An important characteristic of T2-high asthma is that it is responsive to treatment with inhaled corticosteroids, whereas T2-low asthma (classified as having levels of type 2 inflammation in the airways that are comparable to the normal reference range of healthy controls) is not. [2]

Understanding the mechanisms that drive type 2 inflammation is therefore central to elucidating asthma pathogenesis.

Type 2 (T2) inflammation is predominant in asthma and is characterized by eosinophilic airway infiltrate and TH2-dependent cytokine overexpression (IL-4, IL-5, and IL-13). IL-5 mediates differentiation, activation, and survival of eosinophils, while IL-4/13 is essential to induce B cells to produce IgE. [3] Among these features, eosinophilic inflammation is a hallmark of T2-high asthma and is closely linked to disease severity and exacerbation frequency.

For many years, the development of Type 2 inflammation was largely attributed to adaptive immune responses mediated by T helper 2 (Th2) cells and their associated cytokines, including interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13). More recent studies have demonstrated that innate type 2 immune responses play a critical role in initiating allergic airway inflammation in the lung. In particular, group 2 innate lymphoid cells (ILC2s) have emerged as key drivers of early type 2 immune responses in asthma. [4]

Biology of ILC2s

Group 2 innate lymphoid cells (ILC2s) are a subset of innate immune cells that play a central role in type 2 immune responses. Unlike adaptive T helper 2 (Th2) cells, ILC2s lack antigen-specific receptors and can respond rapidly to epithelial-derived cytokines, including interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin (TSLP). Upon activation, ILC2s produce key type 2 cytokines such as IL-5 and IL-13, which promote eosinophil recruitment, mucus production, and tissue inflammation. Human ILC2 populations have been identified in many sites, including lung, intestine, nasal polyps, skin, peripheral blood and adipose tissue, and they have been shown to play important roles in various human diseases, such as chronic rhinosinusitis, asthma, atopic dermatitis

and obesity. Recent studies have revealed two distinct ILC2 sets exist: tissue-resident, IL-33- or IL-33/IL-25-responsive nILC2 cells present at steady state; and IL-25-responsive iILC2 cells that are induced in inflammatory circumstances.[5]

Epithelial–ILC2 Axis in Airway Inflammation

The initiation of innate type 2 immune responses in the lung is primarily driven by epithelial-derived signals released following allergen exposure. Upon exposure to allergens, airway epithelial cells rapidly release alarmin cytokines including interleukin-25 (IL-25), interleukin-33 (IL-33), and thymic stromal lymphopoietin (TSLP). These epithelial-derived cytokines function as early warning signals that initiate innate type 2 immune responses by activating innate immune cells within the tissue microenvironment. The downstream targets of epithelium-derived cytokines are a novel group of cells termed group 2 innate lymphoid cells (ILC2), which, when activated, produce more canonical type 2 cytokines (IL-5 and IL-13) than CD4+ lymphocytes on a per-cell basis. [6] This has resulted in a paradigm shift that TH2-high asthma is not simply a TH2 cell–dependent, IgE-mediated allergic inflammatory disease but also involves an innate pathway in which ILC2s provide the primary cellular source of IL-5 and IL-13, which are likely important for initiation of adaptive type 2 immune responses, and regulation of the persistent airway inflammation and tissue remodeling associated with chronic asthma. [7] The epithelial–ILC2 axis is therefore considered a key driver of early type 2 inflammation.

Skin–Lung Axis and Atopic March

In addition, Type 2 inflammation is not restricted to the lung but also plays a central role in other barrier tissues, particularly the skin. Atopic dermatitis (AD) is a chronic inflammatory skin disease driven by similar type 2 immune pathways and often precedes the development of asthma.

This progression, known as the atopic march, describes a clinical trajectory in which early-life skin inflammation predisposes individuals to subsequent allergic airway disease. The shared involvement of type 2 immune mechanisms across tissues suggests the existence of common regulatory pathways and potential communication between the skin and lung immune systems.

Understanding how innate immune cells, such as ILC2s, function across these tissues may provide insight into the systemic nature of allergic diseases.

Current treatments for asthma include inhaled corticosteroids and biologic therapies targeting key components of type 2 inflammation, such as IL-5, IL-13, and IgE. [2] These approaches have significantly improved disease control in many patients.

However, these therapies primarily target downstream inflammatory mediators and may not fully address the early initiation phase of type 2 immune responses. In addition, a subset of patients remains refractory to current treatments, highlighting the need for a deeper understanding of upstream regulatory mechanisms.

Knowledge Gap and Study Rationale

While the pathways driving type 2 inflammation have been extensively studied, the molecular mechanisms that regulate ILC2 activation and function remain incompletely understood. Given the central role of ILC2s in initiating type 2 responses, identifying novel regulators of these cells represents an important area of investigation.

Chapter 2

Establishing an Alternaria-Induced Airway Inflammation Model to Study the Role of PLSCR1 in Innate Type 2 Immune Responses

Introduction

Despite the established role of epithelial–ILC2 signaling in driving type 2 inflammation, the molecular mechanisms that regulate ILC2 activation and function remain incompletely understood. In particular, the intracellular pathways that modulate ILC2 responses and shape the magnitude and persistence of type 2 inflammation are not well defined. These knowledge gaps suggest that additional regulatory factors may play important roles in fine-tuning innate type 2 immune responses in the lung.

Phospholipid scramblase-1 (PLSCR1) is a member of the phospholipid scramblase family and a type II transmembrane protein that mediates the bidirectional translocation of phospholipids across the plasma membrane. [8] This scrambling activity leads to the externalization of phosphatidylserine (PS), a key event involved in diverse biological processes, including apoptosis, coagulation, and immune regulation. In addition to its role in membrane lipid dynamics, PLSCR1 has also been implicated in intracellular signaling pathways and immune-related functions. [9] Despite these findings, the role of PLSCR1 in regulating immune responses in the lung, particularly in the context of type 2 inflammation, remains poorly understood. Moreover, whether PLSCR1 contributes to the regulation of innate type 2 immune responses or modulates the activity of group 2 innate lymphoid cells (ILC2s) has not been explored.

To address these questions, we investigated the role of PLSCR1 in regulating innate type 2 immune responses in the lung. While house dust mite (HDM) models are widely used to study allergic airway inflammation, they predominantly reflect chronic adaptive immune responses. In contrast, Alternaria alternata, a clinically relevant fungal allergen, is a potent inducer of epithelial alarmin release and innate type 2 immune activation. Therefore, we employed an Alternaria-induced airway inflammation model to better capture early innate immune responses and to evaluate the role of PLSCR1 in this context.

Using this approach, we established an intranasal Alternaria model to examine the role of PLSCR1 in regulating type 2 inflammation in the lung. We further extended this system by developing an Alternaria-induced skin inflammation model to explore the potential migration of immune cells along the skin–lung axis. Together, these approaches provide a framework to investigate how PLSCR1 modulates innate type 2 immunity across barrier tissues.

Model rationale

To investigate the role of PLSCR1 in ILC2-mediated type 2 immune responses, we sought to establish an experimental model that would more effectively capture the early phase of innate type 2 inflammation in the lung.

House dust mite (HDM)–induced models are widely used in studies of allergic airway disease and have provided important insights into asthma pathogenesis. However, HDM exposure typically induces a more chronic inflammatory response with substantial contributions from adaptive

immunity, particularly T helper 2 (Th2) cells. Previous studies have shown that inhaled HDM robustly induces pulmonary Th2 cytokine production in an antigen-dependent manner. [10]

Importantly, emerging evidence suggests that innate immune responses in HDM models are closely linked to adaptive immune activation. For example, ILC2 responses in HDM-induced airway inflammation have been shown to depend on T cell–mediated signals, indicating that ILC2 activation in this context is not fully antigen-independent. [11] In addition, ILC2 populations exhibit dynamic phenotypic changes shaped by the inflammatory environment, further highlighting the interplay between innate and adaptive immunity in HDM models. [12]

Together, these findings indicate that HDM-induced airway inflammation relies heavily on antigen-driven adaptive immune responses and involves complex crosstalk between innate and adaptive immune systems. As such, this model is less suitable for isolating early innate immune events that precede full adaptive immune activation.

In contrast, *Alternaria alternata* is a clinically relevant environmental fungal allergen that has been strongly associated with asthma severity and exacerbation. Unlike HDM-based models, Alternaria exposure can rapidly stimulate airway epithelial cells and induce the release of epithelial alarmins, including IL-33, IL-25, and TSLP, which are key upstream drivers of innate type 2 immune responses.

Epithelial-derived alarmins such as IL-33 are preformed and can be rapidly released upon epithelial perturbation, enabling immediate activation of innate immune pathways without the requirement for antigen-specific priming. Consistent with this mechanism, Alternaria exposure has

been shown to induce rapid IL-33 release, leading to early activation of ILC2s and eosinophil recruitment in the lung. [13]

Consistent with this rationale, our preliminary data suggest that Alternaria challenge induces a preferential expansion of ILC2s with minimal changes in Th2 populations, supporting an innate-biased type 2 immune response at the early stage. (Supplementary Figure S1)

Because of these features, the Alternaria model provides a more suitable experimental system for examining the initiation phase of type 2 inflammation and for evaluating molecular regulators acting within innate immune pathways.

Genetic strategy

Having established an experimental system that captures early innate type 2 inflammation, we next sought to examine the role of PLSCR1 in ILC2-associated immune responses. To achieve controlled expression of PLSCR1, we utilized a Cre-dependent knock-in system in which Plscr1 is inserted into the Rosa26 locus and its expression is regulated by a loxP-flanked STOP cassette (Rosa-Plscr1^LSL/LSL). (Figure 1.A) In the absence of Cre recombinase, the STOP cassette prevents transcription of the inserted Plscr1 transgene, whereas Cre-mediated excision enables activation of Plscr1 expression.

To direct *Plscr1* expression toward ILC2-relevant populations, Rosa-Plscr1^LSL/LSL mice were crossed with NMUR1-Cre mice. NMUR1 is highly enriched in group 2 innate lymphoid cells (ILC2s), particularly at barrier tissues such as the lung, and has been functionally implicated in ILC2 activation through neuromedin U (NMU) signaling. NMU–NMUR1 engagement directly

stimulates ILC2 proliferation and promotes the production of type 2 cytokines, including IL-5 and IL-13, thereby amplifying type 2 inflammation. [14, 15]

Notably, NMUR1 displays a highly selective expression pattern, with strong enrichment in ILC2s and minimal expression in other immune populations, including adaptive lymphocytes and myeloid cells. This selective expression has enabled the development of NMUR1-driven genetic tools for targeting ILC2s in vivo. [14, 15]

Although NMUR1 expression is not absolutely restricted to ILC2s, its enriched distribution and functional relevance make it a suitable driver for targeting ILC2-associated responses. In this system, Cre expression under the NMUR1 promoter enables excision of the STOP cassette and activation of *Plscr1* expression in NMUR1-expressing cells. This approach allows interrogation of PLSCR1 function within an ILC2-enriched cellular context during Alternaria-induced airway inflammation.

Alternaria Challenge Regimen Adaptation

To establish an acute airway inflammation model, we adopted the intranasal Alternaria challenge regimen from a previously described allergic airway disease model. [16] In this system, repeated intranasal administration of Alternaria extract has been shown to robustly induce IL-33–dependent type 2 inflammatory responses in the airway.

Based on this established protocol, we implemented a short-term intranasal challenge schedule using Alternaria extract to induce airway inflammation. While the original study incorporated

additional pharmacological interventions, we specifically applied the intranasal Alternaria challenge component to focus on the induction of type 2 inflammation.

Mice were either treated with 25 μg i.n. Alternaria extract (in 25 μl) or PBS control (25 μl) under isoflurane anesthesia every 48 hours for a total of 5 doses over 9 days. Mice were sacrificed 24 hours after the slast Alternaria exposure. (Figure 1.B)

This further adapted dosing schedule better captures early-phase inflammatory responses, enabling assessment of innate immune activation in the context of PLSCR1 regulation.

Results

Histological Validation of Alternaria-Induced Airway Inflammation

To further validate the establishment of the Alternaria-induced airway inflammation model, lung histology was examined following intranasal challenge. Hematoxylin and eosin (H&E) staining of lung sections revealed minimal inflammatory infiltration and preserved alveolar architecture in PBS-treated control mice, both in wild-type and PLSCR1 knock-in groups. (Figure 2.)

In contrast, Alternaria-challenged wild-type mice exhibited marked inflammatory changes, characterized by increased thickening and collapse of alveolar walls, pulmonary edema surrounding alveolar walls, inflammatory cell infiltration in peri-bronchial and parenchymal areas. These features are consistent with the induction of acute type 2 airway inflammation.

Notably, Alternaria-challenged PLSCR1 knock-in mice displayed a comparatively reduced degree of inflammatory cell infiltration, with more preserved lung architecture relative to wild-type controls under the same challenge conditions.

Together, these findings support the successful establishment of the Alternaria-induced airway inflammation model and suggest a potential modulatory role of PLSCR1 in regulating inflammatory responses in the lung.

BAL Analysis Demonstrates Robust Model Induction but Context-Dependent Genotype Effects

To quantify airway inflammation following Alternaria challenge, bronchoalveolar lavage (BAL) fluid was collected and analyzed across three independent experiments. Total BAL cell count and eosinophil percentage were used as primary readouts of inflammatory cell recruitment and type 2 immune response in the airway.

In the Round 1 experiment, bronchoalveolar lavage (BAL) fluid was collected following intranasal Alternaria challenge (25 μg/Dose) in 4-month-old mice, and inflammatory cell infiltration was assessed by cytospin preparation followed by Diff-Quik staining, as well as by quantification of total BAL cell count and eosinophil percentage.

Cytospin analysis revealed minimal inflammatory cell infiltration in PBS-treated groups, whereas Alternaria exposure led to a marked increase in inflammatory cells in both wild-type (WT) and PLSCR1 knock-in (KI) mice (Figure 3A). Consistent with these observations, Alternaria challenge

increased total BAL cellularity and eosinophil frequency compared with PBS controls (Figure 3B), indicating successful induction of airway inflammation.

In addition, a modest reduction in total BAL cell count and eosinophil percentage was observed in PLSCR1 knock-in mice relative to wild-type controls under Alternaria challenge conditions (Figure 3A,3B). However, the overall magnitude of the response was limited. This attenuated response is likely due to incomplete intranasal delivery in this experiment, which may have reduced allergen deposition in the lung.

In the Round 2 experiment, bronchoalveolar lavage (BAL) fluid was collected following intranasal Alternaria challenge (25 μg/dose) in 4-month-old mice, in which intranasal delivery was performed with improved technique to ensure more efficient deposition in the lung. Total BAL cell count and eosinophil percentage were quantified as measures of airway inflammation.

Consistent with improved delivery, Alternaria exposure induced a more robust inflammatory response compared to Round 1, as reflected by increased BAL cellularity and eosinophil frequency relative to PBS-treated controls (Figure 4A,B). Cytospin analysis further confirmed increased inflammatory cell infiltration in Alternaria-treated groups.

Under these conditions, the modest reduction in inflammation previously observed in Round 1 PLSCR1 knock-in mice was not maintained. Instead, PLSCR1 KI mice exhibited comparable or, in some cases, elevated eosinophil percentages relative to wild-type controls. These findings suggest that the observed phenotype may be influenced by the level of effective allergen exposure, although the results do not support a consistent genotype-dependent effect under these conditions.

In Round 3, the Alternaria challenge protocol was modified to reduce the allergen dose to 20 μg per administration and to use younger mice (6–8 weeks old), while maintaining efficient intranasal delivery to ensure consistent deposition in the lung. Bronchoalveolar lavage (BAL) fluid was collected and analyzed for total cell count and eosinophil percentage as measures of airway inflammation.

Under these conditions, Alternaria exposure continued to induce airway inflammation, as evidenced by increased BAL cellularity and eosinophil frequency compared to PBS-treated controls (Figure 5A,B). Cytospin analysis confirmed the presence of inflammatory cell infiltration in Alternaria-treated groups.

However, despite optimization of experimental parameters, genotype-dependent differences remained subtle. PLSCR1 knock-in mice exhibited comparable BAL cell counts and eosinophil percentages relative to wild-type controls, with no consistent trend observed. These findings suggest that, although airway inflammation was reliably induced, the effect of PLSCR1 on BAL inflammatory parameters was not robust under the conditions tested.

Across all experiments, the BAL fluid data support successful induction of airway inflammation in the Alternaria model, as increased total BAL cell count and eosinophil percentage were observed following Alternaria challenge across experiments. However, genotype-dependent differences between wild-type and PLSCR1 knock-in mice were variable and not reproducible. This inconsistency suggests that BAL inflammatory readouts are sensitive to experimental factors, including effective allergen delivery, dosage, and mouse age. Therefore, while BAL analysis supports overall model induction, interpretation of PLSCR1-dependent effects requires careful consideration of experimental context.

Flow cytometric analysis of ILC2 populations in Alternaria-challenged mice under optimized conditions

To further assess the effect of Alternaria challenge on innate type 2 immune responses, flow cytometric analysis of tissue-resident ILC2s was performed in Round 3 under optimized experimental conditions. Lung cell suspensions were analyzed using a lineage-negative, CD90.2-positive, ST2-positive, and ICOS-positive gating strategy to identify tissue-resident ILC2 populations. (Figure 6.)

Under these conditions, Alternaria challenge increased the abundance of tissue-resident ILC2s relative to PBS-treated controls, consistent with induction of an innate type 2 immune response. However, comparison between wild-type and PLSCR1 knock-in mice did not reveal a clear or consistent genotype-dependent difference in either raw ILC2 cell count or frequency within the parent population. Notably, an apparent outlier was observed in the PLSCR1 KI PBS group, but because of the limited sample number in this experiment, it was not possible to determine whether this reflected biological variation or technical noise.

Taken together, these data suggest that Alternaria-induced expansion of tissue-resident ILC2s occurs largely independently of PLSCR1 under the conditions tested. However, because changes in ILC2 abundance were not prominent, it remains possible that PLSCR1 may instead influence functional properties of tissue-resident ILC2s, such as cytokine production, rather than their expansion.

Discussion

In this study, we aimed to investigate the role of PLSCR1 in regulating ILC2-driven type 2 inflammation using an Alternaria-induced airway inflammation model. At the current stage, a primary objective of this work was to establish and optimize a model that reliably captures early innate type 2 immune responses in the lung. While successful model induction was achieved, several experimental limitations were identified across different readouts, which provide important insights for future study design.

Histological examination of lung tissue provided the most consistent and reproducible evidence of airway inflammation following Alternaria challenge. Clear inflammatory cell infiltration, structural disruption, and peribronchial inflammation were observed across experiments, supporting successful model establishment.

However, histological analysis in this study was primarily qualitative. While it effectively demonstrated the presence of inflammation, it does not provide precise quantitative comparison between genotypes. As a result, although reduced inflammation was visually observed in PLSCR1 knock-in mice in certain experiments, it remains difficult to draw definitive conclusions regarding genotype-dependent effects based solely on histology.

Future studies could improve this aspect by incorporating quantitative histological scoring systems or image-based analysis methods, such as blinded scoring of inflammation severity or automated quantification of infiltrating cell density. These approaches would allow more objective and statistically robust comparison between experimental groups.

BAL fluid analysis demonstrated that Alternaria challenge consistently induced airway inflammation, as reflected by increased total cell count and eosinophil percentage across all experimental rounds. However, the magnitude of this response and the observed genotype-dependent differences varied substantially between experiments.

In early experiments, reduced inflammatory responses were observed in PLSCR1 knock-in mice, but this trend was not reproducible in subsequent rounds. This inconsistency is likely attributable to multiple experimental factors. First, intranasal delivery efficiency plays a critical role in determining effective allergen deposition in the lung. Incomplete delivery in early experiments likely led to underestimation of inflammatory responses. Second, allergen dosage and mouse age differed between experimental rounds, both of which are known to influence the magnitude of type 2 inflammation. Third, the relatively small sample size further limits the ability to detect subtle genotype-dependent effects.

These findings highlight that BAL-based measurements are highly sensitive to experimental conditions, particularly in acute inflammation models. To improve reproducibility, future experiments should standardize key variables, including mouse age, allergen dose, and delivery technique. Increasing sample size will also be essential to reduce variability and improve statistical power.

Flow cytometric analysis confirmed that Alternaria challenge induced expansion of tissue-resident ILC2 populations, supporting activation of innate type 2 immune responses. However, no consistent differences were observed between wild-type and PLSCR1 knock-in mice in terms of ILC2 frequency or total cell number.

One important limitation of this analysis is that it focuses primarily on cell abundance rather than functional state. It is possible that PLSCR1 does not regulate ILC2 expansion but instead modulates their activation or effector function. Additionally, the relatively small number of samples and the presence of potential outliers further limit the interpretation of these results.

Future studies should incorporate functional flow cytometry assays, such as intracellular cytokine staining for IL-5 and IL-13, to assess ILC2 activity at the single-cell level. In addition, increasing the number of biological replicates and refining gating strategies may improve sensitivity in detecting subtle phenotypic differences.

Although a consistent genotype-dependent effect was not observed across the current datasets, these findings do not exclude a role for PLSCR1 in regulating innate type 2 immune responses. Instead, they suggest that PLSCR1 may influence qualitative aspects of immune cell function rather than quantitative expansion.

Given the known role of PLSCR1 in membrane dynamics and signaling, it is plausible that PLSCR1 modulates intracellular pathways involved in cytokine production or activation signaling in ILC2s. Such effects would not necessarily be reflected in changes in cell number but may be detectable at the level of cytokine output or gene expression.

To test this hypothesis, future experiments should include cytokine profiling using ELISA to measure IL-5 and IL-13 levels, as well as transcriptional analysis using RT-qPCR. These approaches would provide a more comprehensive assessment of ILC2 functional responses.

Despite the limitations described above, this study successfully establishes an Alternaria-induced airway inflammation model that reliably induces innate type 2 immune responses. Compared to

HDM-based models, this system more effectively captures early epithelial–ILC2 signaling and provides a suitable platform for studying innate immune regulation.

Importantly, the development of this model also enables extension into other barrier tissues. This platform provides the foundation for investigating the skin–lung immune axis, where innate type 2 responses may be coordinated across tissues. Future studies will leverage this model to examine whether PLSCR1 plays a role in regulating inter-tissue immune communication.

Material and Methods

KI and transgenic mice. Rosa26 locus–targeted Plscr1 conditional KI transgenic mice (*Rosa26-loxP-STOP-LoxP-Plscr1 Tg; Rosa-Plscr1*$^{LSL/LSL}$) were generated at Brown Mouse Transgenic and Gene Targeting Facility. *Rosa-Plscr1*$^{LSL/LSL}$ mice were then bred with *Nmur1*$^{iCre\text{-}eGFP}$ (The Jackson Laboratory) mice to generate ILC2-specific Plscr1 overexpression mice. *Nmur1*$^{iCre\text{-}eGFP}$ Cre^{-} littermate controls *Nmur1*$^{iCre\text{-}eGFP}$ Cre^{+} mice were verified by genotyping.

All mice were housed and further bred in Brown University animal facilities. The mice with null mutation or overexpression of Plscr1 did not show any apparent abnormal phenotypes and developmental and signaling issues. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocols (#24-09-0008) of Brown University. All terminal sacrifice was performed after euthanasia with urethane, and every effort was made to minimize suffering.

Alternaria mouse model. *Nmur1*$^{iCre\text{-}eGFP}$ Cre^{-} littermate controls and *Nmur1*$^{iCre\text{-}eGFP}$ Cre^{+} 6-8-week-old C57Bl/6 mice were used in the model. Alternaria alternata extract was purchased from Greer Laboratories and reconstituted and adjusted to 1 mg/ml solution in sterile saline (based on BCA assay, Pierce). Mice were either treated with 25 μg i.n. Alternaria extract (in 25 μl) or PBS control (25 μl) under isoflurane anesthesia every 48 hours for a total of 5 doses over 9 days. Mice were sacrificed 24 hours after last Alternaria exposure.

BAL harvest and differential cell counts. Mice were euthanized with intraperitoneal injection of 300 μL of urethane (0.18 g/ml, Sigma-Aldrich). Bronchoalveolar lavage (BAL) of the whole lung was performed with a total of 1 mL ice-cold PBS via an incision at the trachea. An aliquot was stained with trypan blue solution (Gibco) for viability and cell number determination using TC20 automated cell counter (Bio-Rad). Samples were centrifuged at 2500 rpm for 5 min at 4 °C. Cells were pelleted onto glass slides via cytospin centrifugation at 700 rpm for 7 min and stained with Hema 3 (Fisher Scientific). Neutrophils, macrophages, lymphocytes, and eosinophils were counted under a light microscope.

Immunohistology For immunohistology, immediately after euthanasia, left lungs were inflated through the incision at the trachea with 10% buffered formalin and soaked in formalin at RT. They were then transferred to 70% ethanol prior to paraffin embedding, sectioning, and staining with H&E performed at Brown University Molecular Pathology Core. Lungs were scanned with a VS200 slide scanner (Evident).

Flow cytometry. APC-conjugated antilineage markers, PE-Cy7-conjugated anti-ICOS, FITC-conjugated anti-CD90.2, and PE–conjugated anti-T1/ST2 were obtained from BD Pharmingen and eBioscience. Flow cytometry was performed using BD FACSAria. ILC2s are defined as Lin(CD3, CD11b, CD45R/B220, Ly-76, Ly6G, and Ly-6C)-, Thy1.2+ICOS+T1/ST2+ cells. Data were analyzed using FlowJo software v.10 (TreeStar Inc.). Percentages of Lin-Thy1.2+ICOS+T1/ST2+ cells and total cells recovered from the lungs were used to determine the number of ILC2 cell recovery. For all analyses, isotype control staining was subtracted from true antibody staining to determine the percentage of positive cells.

Study approval. Animal experiments were approved by the Institutional Animal Care and Use Committee of Brown University in accordance with federal guidelines.

Data availability. Data are available from the corresponding author upon request.

Chapter 3

Developing an Alternaria-Induced Skin Atopic Dermatitis Model to Assess Skin–Lung Immune Axis Under PLSCR1 Regulation

Introduction

Airway inflammation is a defining feature of asthma. Type 2 (T2) inflammation is observed in more than 80% of pediatric cases and in most adult patients, often in association with sensitization to environmental allergens such as dust mites, fungi, pet dander, and pollen. This allergic sensitization frequently coexists with other atopic conditions, including atopic dermatitis, allergic rhino conjunctivitis, and food allergy.[17] This phenomenon highlights a potential connection between immune responses in the skin and the lung. Early-life skin inflammation is thought to prime systemic type 2 immunity, thereby increasing susceptibility to subsequent airway inflammation.

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritus, epidermal barrier dysfunction, and type 2–driven inflammation. Like asthma, AD is associated with elevated levels of type 2 cytokines, including IL-4, IL-5, and IL-13, as well as increased eosinophilic infiltration. These shared immunological features suggest that AD and asthma may be mechanistically linked through common type 2 immune pathways.

Recent studies have suggested that resident ILC2s are present in healthy human skin, and their expansion has been observed in atopic dermatitis (AD) lesions, suggesting their involvement in disease pathogenesis.[18] Importantly, skin-derived ILC2s express the IL-33 receptor ST2, and its

expression is further upregulated upon activation in AD models, [19] which makes IL-33–ILC2 axis emerged as a central pathway in type 2–driven skin inflammation.

In addition to their tissue-resident ILC2 populations, inflammatory ILC2s (iILC2s) have been proposed to exhibit migratory capacity under inflammatory conditions, potentially enabling them to traffic between barrier tissues.[20] This raises the possibility that ILC2s may contribute to the propagation of type 2 inflammation along the skin–lung axis.

Despite these observations, the mechanisms underlying communication between skin and lung immune responses remain incompletely understood. In particular, the role of molecular regulators, such as PLSCR1, in modulating ILC2 function across different tissue environments has not been fully explored.

Taken together, this chapter aims to establish an Alternaria-induced atopic dermatitis model to enable the investigation of ILC2-driven type 2 inflammation in the skin and to explore whether prior skin immune activation may influence subsequent airway responses, with a particular focus on the potential regulatory role of PLSCR1 in this process.

Rationale for Sequential Skin–Lung Model

To investigate the potential role of skin inflammation in shaping subsequent airway immune responses, we employed a sequential model in which type 2 inflammation was first induced in the skin using *Alternaria alternata*. Following a recovery period of 2 weeks, mice were then subjected to intranasal challenge regimen mentioned above to induce lung inflammation.

This model was not designed to recapitulate the full temporal progression of the atopic march, but rather to address a specific mechanistic question: whether inflammatory ILC2s (iILC2s) generated in the skin can migrate to the lung and contribute to airway type 2 inflammation.

Although the phenotypic definition of inflammatory ILC2s (iILC2s) varies across tissues and studies, a common feature is the emergence of an activated ILC2 subset characterized by elevated KLRG1 expression and reduced dependence on canonical markers such as ST2.[20]

For example, in the gut–lung axis model described by Huang et al.,[21] IL-25–responsive iILC2s are defined as lineage-negative, ST2^{-}, KLRG1^hi cells with migratory capacity. In contrast, studies in the skin highlight a more heterogeneous population of ILC2s, where inflammatory subsets may express KLRG1 along with additional markers such as CCR6, and exhibit cytokine-driven plasticity.[20]

Despite these differences in marker definition, these studies consistently support the existence of an inflammatory, activated ILC2 population with the potential to traffic between barrier tissues. Emerging evidence suggests that, inflammatory ILC2s acquire migratory capacity and can traffic between barrier tissues. Therefore, instead of focusing on long-term immune priming, this model aims to establish a system in which skin inflammation serves as a source of iILC2s.

By inducing localized skin inflammation using Alternaria exposure following barrier disruption, this approach enables the generation of activated ILC2 populations with potential migratory behavior. Subsequent lung challenge allows for the assessment of whether these skin-derived ILC2s can contribute to airway inflammation, thereby providing a platform to investigate cellular trafficking along the skin–lung axis.

To establish a skin model that permits efficient allergen sensitization, an initial step of mechanical barrier disruption was introduced using a tape-stripping approach. This method was employed to induce localized skin injury, characterized by epidermal erosion and mild hemorrhage, thereby mimicking key features of barrier dysfunction observed in atopic dermatitis.

Tape stripping is a widely used technique to disrupt the stratum corneum, resulting in increased skin permeability and facilitating the penetration of environmental allergens. In this study, the protocol was adapted from the published method *"Standardizing the Skin Tape Stripping Method for Sensitization and Using it to Create a Mouse Model of Peanut Allergy"*, which provides a reproducible framework for inducing controlled skin barrier damage.[22]

The rationale for incorporating this step is grounded in the understanding that an intact epidermal barrier limits allergen access to immune cells. Disruption of this barrier enables allergens such as *Alternaria alternata* to penetrate the skin and interact with resident immune populations, thereby initiating a type 2 inflammatory response. This process more closely reflects the pathophysiological conditions of atopic dermatitis, where barrier dysfunction is a key initiating factor.

By combining tape stripping with subsequent allergen exposure, this model allows for controlled induction of skin inflammation and provides a platform to study downstream immune activation, including the involvement of innate immune cells such as ILC2s.

Methods

KI and transgenic mice. Rosa26 locus–targeted Plscr1 conditional KI transgenic mice (*Rosa26-loxP-STOP-LoxP-Plscr1 Tg; Rosa-Plscr1*$^{LSL/LSL}$) were generated at Brown Mouse Transgenic and Gene Targeting Facility. *Rosa-Plscr1*$^{LSL/LSL}$ mice were then bred with *Nmur1*$^{iCre\text{-}eGFP}$ (The Jackson Laboratory) mice to generate ILC2-specific Plscr1 overexpression mice. *Nmur1*$^{iCre\text{-}eGFP}$ Cre^{-} littermate controls *Nmur1*$^{iCre\text{-}eGFP}$ Cre^{+} mice were verified by genotyping.

All mice were housed and further bred in Brown University animal facilities. The mice with null mutation or overexpression of Plscr1 did not show any apparent abnormal phenotypes and developmental and signaling issues. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocols (#24-09-0008) of Brown University. All terminal sacrifice was performed after euthanasia with urethane, and every effort was made to minimize suffering.

Tape-Stripping–Induced Skin Barrier Disruption. For tape-stripped skin sensitization, mice were anesthetized using isoflurane delivered via a nose cone. The dorsal skin was shaved using an electric hair trimmer, followed by application of a depilatory cream (Nair™, Church & Dwight Co., Inc., NJ, USA) for approximately 10 seconds to ensure complete hair removal. The skin was then thoroughly rinsed with water and gently dried using absorbent paper. To disrupt the epidermal barrier, adhesive tape (Fisherbrand™ tape) measuring 1 × 1 cm² was applied to the shaved skin and removed repeatedly (7–12 times), using a fresh piece of tape for each application. This process

was continued until the treated skin exhibited a visibly inflamed and shiny appearance, indicative of effective disruption of the stratum corneum. Following tape stripping, a foam reservoir (Acrysure Next Gen 1/16″ PE Foam, Mactac, OH, USA) with a 1 × 1 cm² opening was carefully positioned over the treated area, ensuring alignment with the inflamed skin region. *Alternaria alternata* extract (50 μg in PBS) or PBS alone (control) was then pipetted into the reservoir. The application site was first sealed using a transparent waterproof adhesive dressing (CVS Health Advanced Care Waterproof Transparent Dressing, CVS, RI, USA) to prevent leakage and ensure consistent allergen exposure. To further secure the patch and prevent displacement due to grooming behavior, additional medical adhesive tape was applied over the dressing in a cross-shaped configuration, firmly stabilizing the patch on the dorsal skin. Mice were subjected to repeated epicutaneous exposures over a period of 2–3 weeks. Progressive skin inflammation developed over time, with visible skin erosion and hemorrhage typically observed by the fourth week. (Figure 7.)

Discussion

To establish an epicutaneous sensitization model for Alternaria-induced skin inflammation, we first implemented a tape-stripping–based patch delivery system as described above.

During the initial round of experimentation, the applied skin patches were frequently removed by the mice within 24 hours, likely due to grooming behavior. This resulted in inconsistent allergen exposure and limited the reliability of the model.

To address this issue, we introduced an additional fixation step by applying medical adhesive tape in a cross-shaped configuration over the patch dressing. This modification significantly improved patch stability, allowing the patch to remain in place for at least 48 hours.

Following this optimization, two rounds of experiments were performed using the improved setup. Despite achieving sustained exposure to Alternaria extract for up to 48 hours, no visible signs of skin inflammation, including erythema or hemorrhage, were observed.

In the current study, short-term epicutaneous exposure to *Alternaria alternata* did not result in observable skin inflammation, as evidenced by the absence of erythema or hemorrhage. This suggests that single or limited-duration exposure may be insufficient to induce a robust type 2 inflammatory response in the skin under the present experimental conditions.

One possible explanation is that the extent of epidermal barrier disruption achieved through tape stripping may not have been sufficient to allow effective allergen penetration and immune activation. While tape stripping induced visible surface changes, it may not have reached the threshold required to trigger downstream type 2 inflammatory pathways.

Additionally, it is well established that the development of atopic dermatitis–like inflammation in murine models often requires repeated or prolonged allergen exposure. Therefore, the lack of phenotype observed in this study may reflect insufficient duration or frequency of allergen sensitization rather than a failure of the model itself.

In addition to optimizing the sensitization regimen, an important next step in the platform-building phase will be to establish methods for characterizing skin ILC2 populations and the broader immune cell landscape following epicutaneous sensitization. Because visible skin pathology alone

may not fully capture early or subtle immune activation, immune profiling at the cellular level will be necessary to determine whether *Alternaria* exposure induces local type 2 immune responses in the skin. Future studies should therefore incorporate approaches for skin tissue digestion, flow cytometric analysis of resident immune populations, and characterization of key inflammatory cell subsets, including ILC2s, eosinophils, and other infiltrating leukocytes. These analyses will help determine whether the absence of overt skin redness or hemorrhage reflects a true lack of immune activation or simply a limitation of gross phenotypic assessment.

Figures

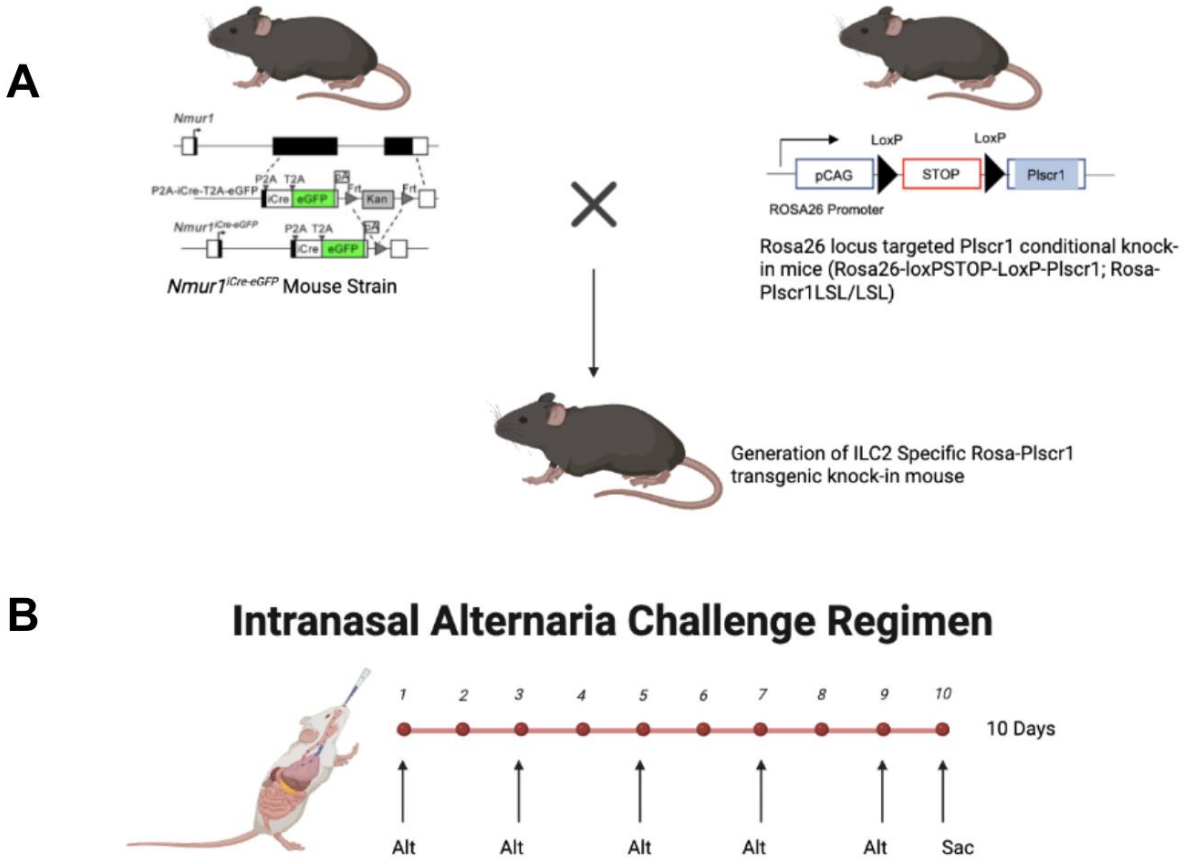


Figure 1. Generation of ILC2-specific PLSCR1 knock-in mice and intranasal Alternaria challenge regimen

(A) Schematic illustration of the generation of ILC2-specific PLSCR1 knock-in mice. *Nmur1^Cre-eGFP* mice were crossed with *Rosa26-loxP-STOP-loxP-Plscr1* (LSL-Plscr1) mice to drive conditional expression of PLSCR1 in ILC2 populations. Cre-mediated recombination removes the STOP cassette, enabling Plscr1 expression under the Rosa26 promoter specifically in Nmur1-expressing cells.

(B) Experimental design of the intranasal Alternaria alternata challenge model. Mice were administered Alternaria extract intranasally at defined time points over a 10-day period. Repeated exposures were performed to induce type 2 airway inflammation, followed by tissue collection at the endpoint for downstream analysis.

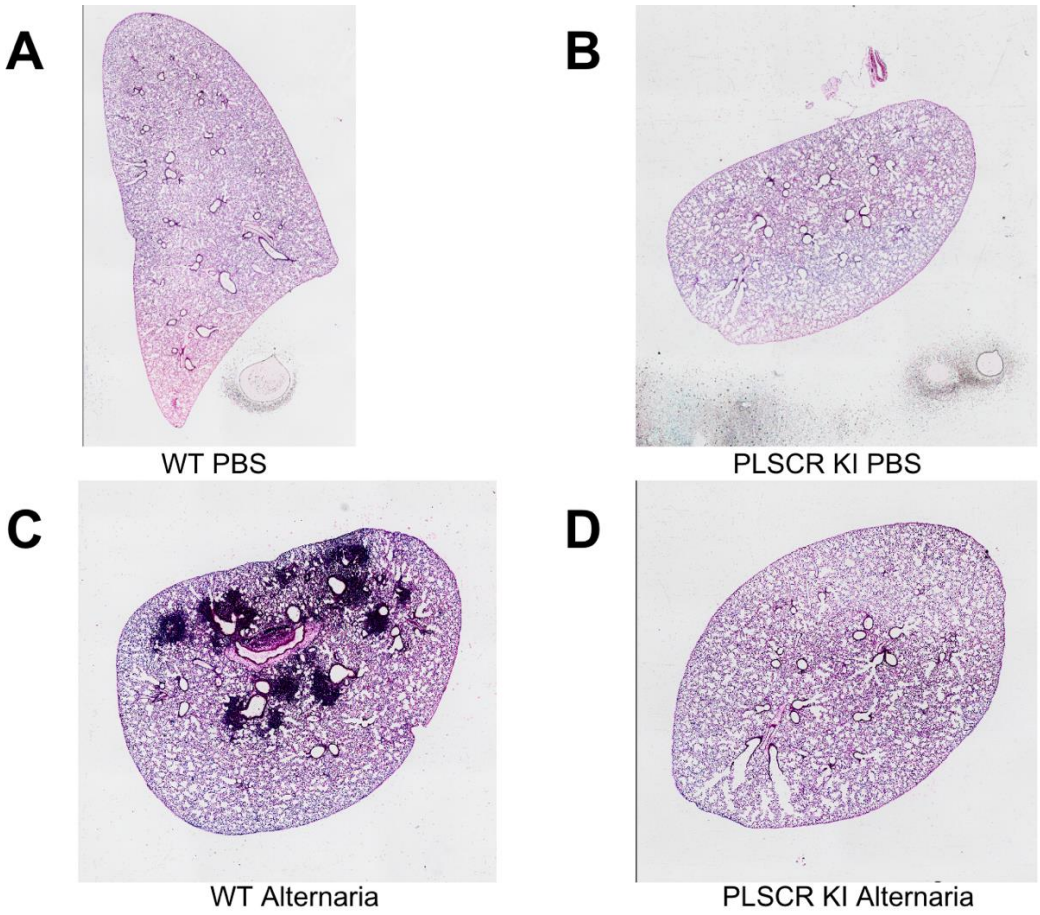


Figure 2. Histological assessment of lung inflammation following Alternaria challenge. Representative hematoxylin and eosin (H&E)-stained lung sections from wild-type (WT) and PLSCR1 knock-in (KI) mice treated with PBS or intranasal Alternaria extract. **(A)**PBS-treated WT and **(B)** PBS-treated KI mice exhibit normal lung architecture with minimal inflammatory cell infiltration. In contrast, **(C)**Alternaria-challenged WT mice display increased inflammatory cell infiltration, particularly in peribronchial and perivascular regions. **(D)**Alternaria-challenged PLSCR1 KI mice show a comparatively reduced degree of inflammatory infiltration relative to WT controls. **(E)** Higher-magnification WT mice exhibit marked inflammatory cell infiltration in peribronchial and parenchymal regions, accompanied by disruption of normal alveolar architecture. **(F)** Higher-magnification PLSCR1 KI mice display comparatively reduced inflammatory infiltration and more preserved alveolar structure relative to WT controls.

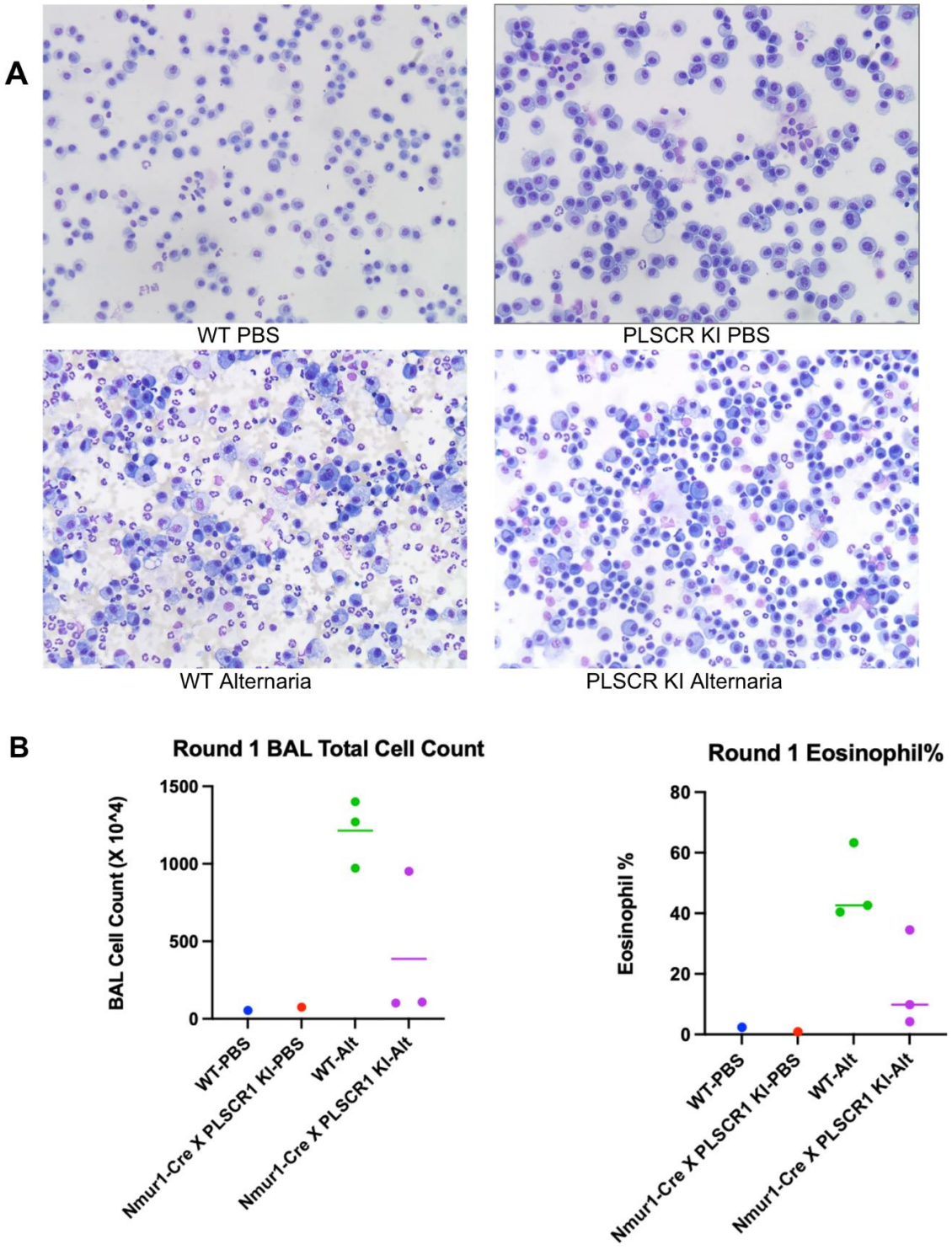


Figure 3. BAL fluid analysis in three independent Alternaria challenge experiments. (A) Round 1 Representative cytospin preparations of BAL cells stained with Diff-Quik. **(B)** Round 1 Quantification of total BAL cell count (left) and eosinophil percentage (right).

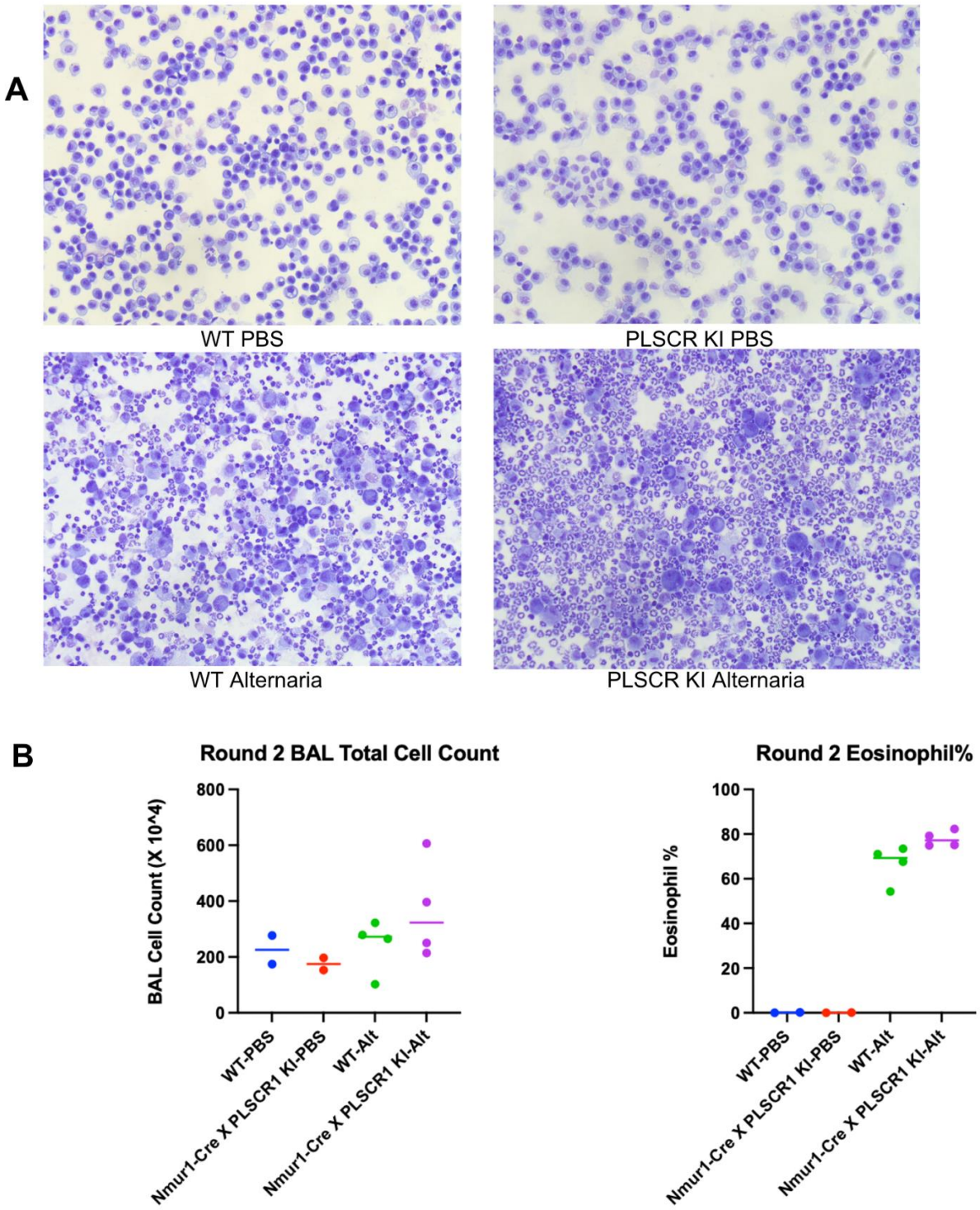


Figure 4. BAL fluid analysis in three independent Alternaria challenge experiments. **(A)** Round 2 Representative cytospin preparations of BAL cells stained with Diff-Quik. **(B)** Round 2 Quantification of total BAL cell count (left) and eosinophil percentage (right).

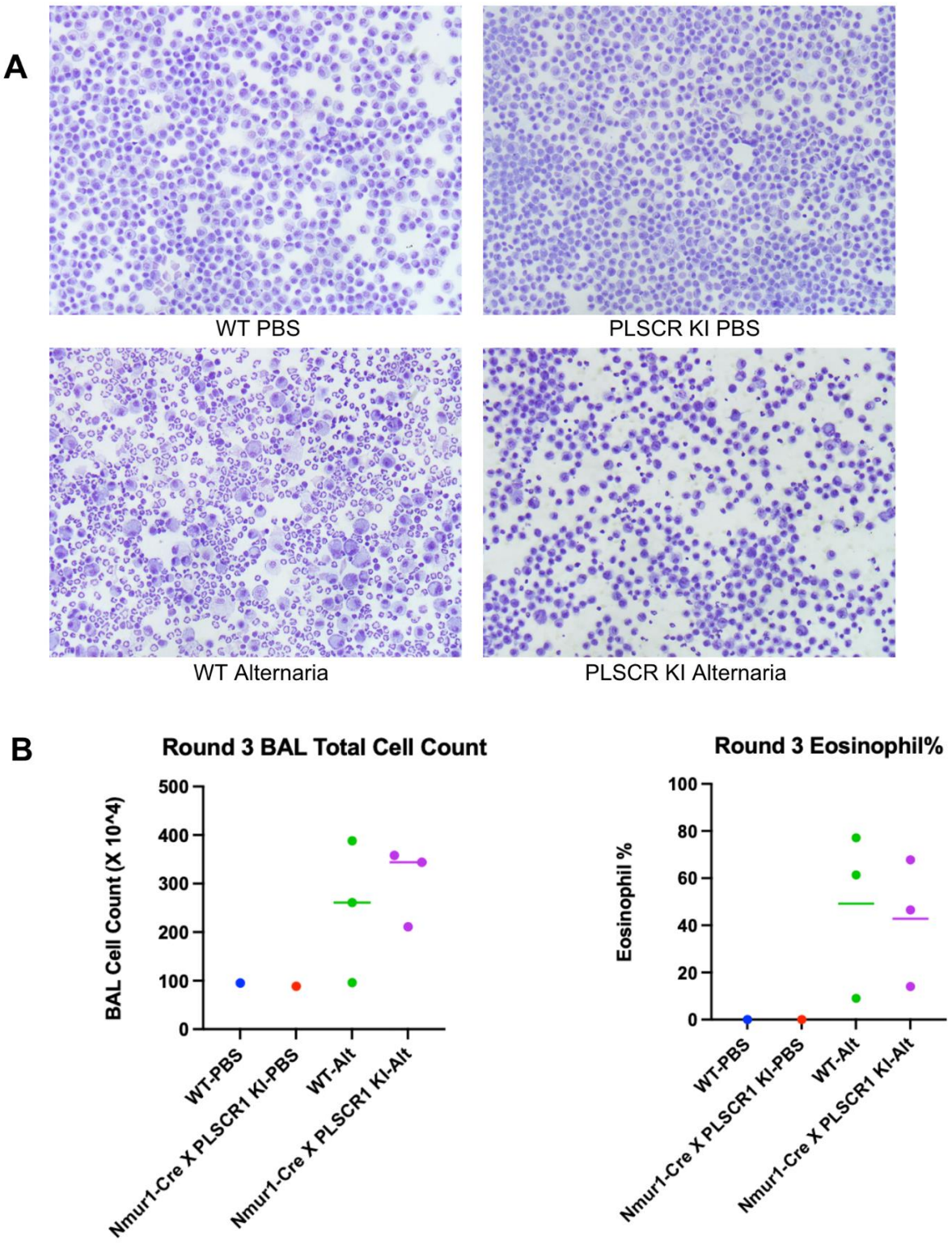


Figure 5. BAL fluid analysis in three independent Alternaria challenge experiments. **(A)** Round 3 Representative cytospin preparations of BAL cells stained with Diff-Quik. **(B)** Round 3 Quantification of total BAL cell count (left) and eosinophil percentage (right).

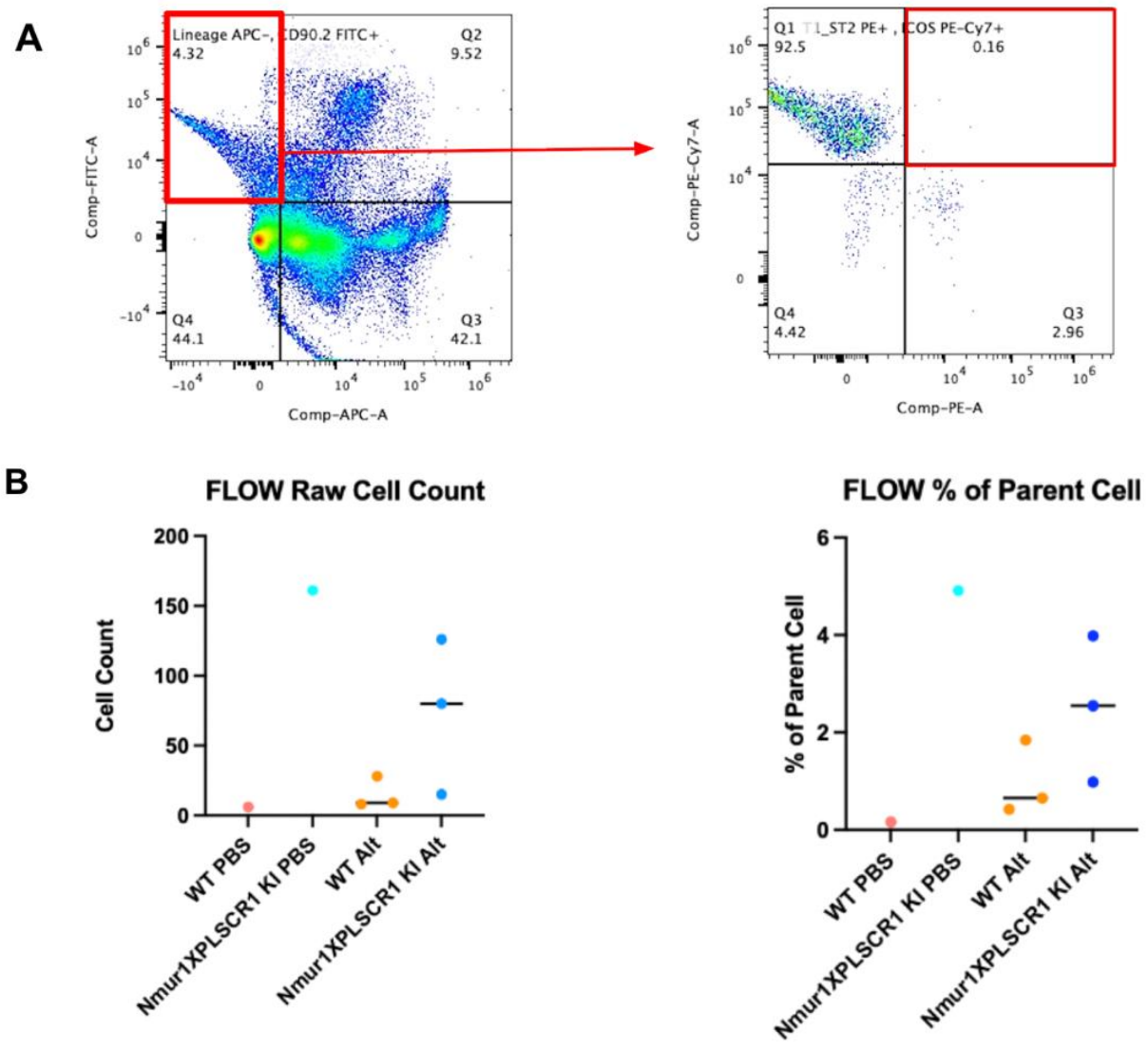


Figure 6. Flow cytometric analysis of ILC2 populations in Alternaria-challenged mice under optimized conditions

(A) Representative flow cytometry gating strategy for identification of lung ILC2s. Live cells were first gated to exclude lineage-positive cells (Lineage APC), followed by selection of CD90.2^{+} lymphocytes. ILC2s were defined as Lineage^{-} CD90.2^{+} T1/ST2^{+} ICOS^{+} cells.

(B) Quantification of ILC2 populations in bronchoalveolar lavage (BAL) samples. Left panel shows absolute ILC2 cell counts, and right panel shows frequency of ILC2s as a percentage of parent population. Each dot represents an individual mouse, and horizontal bars indicate the mean.

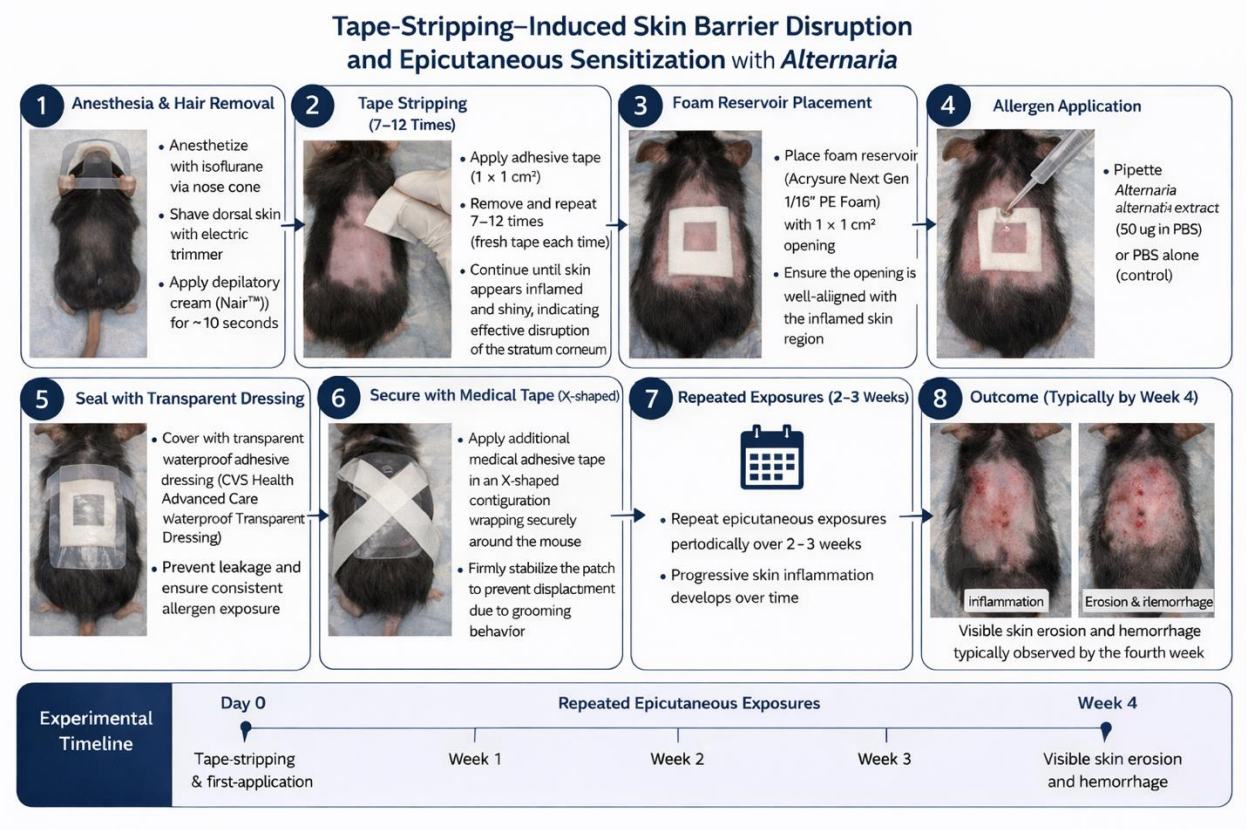
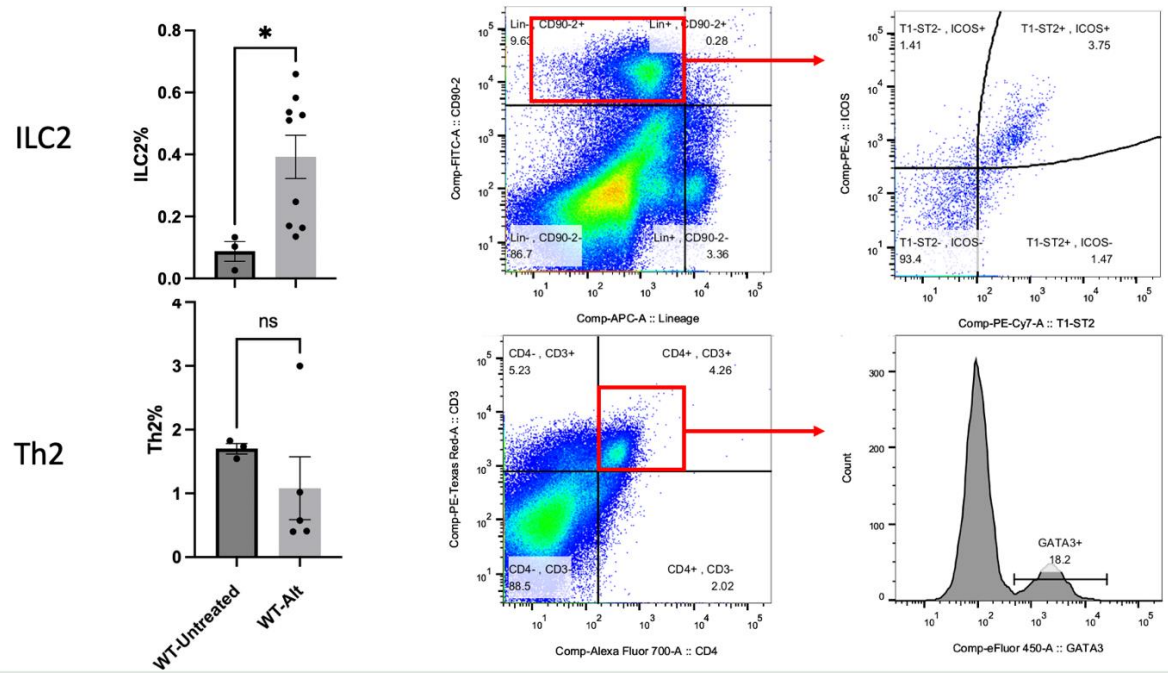


Figure 7. Tape-stripping–induced skin barrier disruption and epicutaneous sensitization with *Alternaria alternata*. Schematic overview of the experimental workflow used to establish a tape-stripping–based epicutaneous sensitization model in mice. Figure created with the assistance of ChatGPT. Representative experimental images can be obtained from the corresponding author upon reasonable request.



Supplementary Figure S1. Preliminary validation of innate-biased immune response in the Alternaria model.

Flow cytometry analysis of lung immune cell populations following Alternaria challenge. ILC2s were identified as Lineage$^-$ CD90.2$^+$ ST2$^+$ ICOS$^+$ cells, and Th2 cells were defined as CD3$^+$ CD4$^+$ GATA3$^+$ T cells. Alternaria exposure resulted in a significant increase in ILC2 frequency, while Th2 cell levels remained unchanged compared to control. These data support a preferential activation of innate type 2 immune responses in this model.

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