Indoor Rewilding of Laboratory Mice Recalibrates Pulmonary Mucosal Immunity and Mechanics

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ABSTRACT

Laboratory mice raised under specific-pathogen-free (SPF) conditions experience restricted microbial and antigenic exposure, which favours an immature immune system and limits their translational value for respiratory research. While microbial enrichment in "dirty" mouse models restores immune maturation, its impact on integrated respiratory function and model transferability to human disease remains understudied. Here, we tested whether ecological exposure through indoor rewilding of SPF-reared mice could reshape immune complexity and recalibrate pulmonary physiology. Two-month-old female C57BL/6J mice were housed for three months under SPF or indoor-rewilding conditions and assessed for immune, mechanical, and systemic parameters. Rewilded mice exhibited expanded pulmonary immune subsets, increased dendritic-cell immune checkpoint, with TNF/IFN-γ activation coupled to regulatory IL-10 signaling. Despite sustained exposure, the alveolar-capillary barrier integrity was preserved. Functionally, respiratory oscillometry revealed improved pulmonary mechanics, including lower airway resistance, higher compliance, and reduced airway responsiveness to methacholine. Systemic cytokine analyses indicated compartmentalized pulmonary immune activation, maintaining an overall anti-inflammatory balance. Importantly, no external pathogens were introduced. Together, these findings demonstrate that indoor rewilding reestablishes coordinated lung immune and mechanical homeostasis in SPF-reared mice, providing a safe and scalable model for studying human-like mucosal immunity and respiratory physiology with broad implications for preclinical respiratory research and therapeutic testing.

Keywords: Rewilding, lung mechanics, airway hyperresponsiveness, pulmonary mucosal immunity, translational models, ecological validity.

INTRODUCTION

While SPF mice provide the controlled, infection-free models essential for reproducible research, this very cleanliness imposes a significant biological constraint: a poorly trained immune system. In contrast to humans, who encounter a diverse array of microbes and antigens, SPF mice develop limited lymphocytic and inflammatory profiles, leading to distorted infection responses and compromised translational fidelity of findings from murine disease models [1,2]. To bridge this gap, so-called "dirty" mouse models have emerged, which deliberately reintroduce ecological complexity. Approaches such as embryo transfer into wild dams ("wildlings"), co-housing with feral mice, and rewilding in secure outdoor enclosures have been shown to restore many hallmarks of immune maturity, including expanded granulopoiesis, more robust antibody and cytokine responses, and human-like patterns of pathogen susceptibility [3–7]. However, these "naturalization" paradigms remain logistically complex, seasonally constrained, and carry risks of zoonotic transmission, alongside varying effects depending on the exposure setup, thus restricting their scalability and reproducibility [8,9].

The lungs, being in constant contact with the external environment, are particularly affected by the SPF conditions. Respiratory resilience, i.e. the ability of the lung tissue to resist, adapt, and recover from insults while maintaining efficient gas exchange, requires coordinated epithelial-endothelial integrity, immune surveillance, and mechanical performance, all of which are shaped by environmental exposure. SPF housing, by shielding animals from microbial, antigenic, and particulate challenges (e.g., allergens), profoundly limits the maturation of pulmonary immunity. The scarce presence of lung parenchymal mast cells in SPF mice [10], in clear contrast to their abundant presence in human lungs [11,12], exemplifies the simplified leukocyte repertoires in laboratory mice that would translate into unrealistic mimicking of human lung physiology and pathology [13]. Interestingly, recent studies in "dirty" mouse models have showed that ecological exposure induces pulmonary immune remodeling, including recruitment of parenchymal mast cells akin to feral mice [14], and modulation of allergen-induced hyperresponsiveness, which can be either heightened [15] or attenuated [16] depending on the exposure setup. However, whether such ecological enrichment also remodels pulmonary properties, particularly its mechanical performance, has never been tested.

In this study, we developed a novel indoor rewilding paradigm within a non-SPF animal facility that combines high ecological complexity, controllable experimental parameters, and year-round operational convenience. This semi-natural vivarium replicates key elements of feral mouse habitat (e.g., farmland

soil, vegetation, invertebrates, manure, and organic substrates), whose composition and renewal are precisely controlled, while maintaining standardized conditions for temperature, humidity, light cycles, and predator exclusion. This paradigm allows to study how ecological exposure recalibrates host physiology in a reproducible, biosecure, and scalable setting, while promoting animal welfare through natural behaviors like foraging and burrowing.

Leveraging this platform, we conducted the first comprehensive assessment of pulmonary and systemic immunity, barrier integrity, and partitioned respiratory mechanics in SPF-reared mice after three months of indoor rewilding. It is well established that lung function is closely coupled to immune activity, and that persistent immune activation and parenchymal alterations drive the progressive decline in pulmonary performance characteristic of chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [17]. Hence, we sought to determine whether ecological immune enrichment could translate into measurable changes in lung mechanical function. Our results demonstrate that indoor rewilding diversifies the pulmonary immune landscape while enhancing mechanical efficiency and maintaining barrier integrity, a combination not previously characterized in “dirty” mouse models. These findings position indoor rewilding as a complementary and translational tool for modelling human-like mucosal immunity and pulmonary physiology, with broad implications for respiratory disease research and preclinical therapy development.

MATERIALS AND METHODS

Ethics statement

All procedures involving mice were carried out in accordance with the Canadian Council on Animal Care (CCAC) guidelines and were approved by the Animal Care Committee (CPAUL-2, approval number: 23-1342). Female mice were chosen to reduce confounding factors related to breeding and limit aggression within the vivarium.

Animals

Two-month-old female C57BL/6J mice were purchased from Jackson Laboratories (strain #000664). Upon arrival, mice were randomly assigned to standard SPF housing or to an indoor semi-natural vivarium. Both groups were maintained at ~22°C, ~45%RH, on a 12:12-hour light/dark cycle, and had *ad libitum* access to food and water.

Semi-natural vivarium

Our indoor rewilding platform consisted of a 12m^2 enclosure (**Figure 1A, Supplementary Video 1**) established within a non-SPF room ordinarily dedicated to farm animals at the IUCPQ Research Center animal facility (Québec-City, Canada). Before initiating the experimental protocol, 28 two-month-old female mice were randomly allocated to SPF (14) or Rewild (14). Afterwards, all mice were ear-tagged with ear punches, and baseline health status and body weight were recorded. Both groups were comparable at baseline, with no differences in health or weight. SPF group remained in conventional SPF housing in individually ventilated cages (IVCs). Rewild group was released into the semi-natural vivarium for a three-month rewilding period, with continuous infrared video monitoring (**Supplementary Video 2**). After rewilding period, mice were captured using Longworth traps then transferred to standard cages that contained soil, plants, and other natural elements from the vivarium, to maintain ecological exposure until testing. Rewild group was weighed and examined prior to experimentation (no abnormal phenotypes were detected) and had at least 24-hours of acclimation prior to testing to minimize stress.

Muscular force and motor coordination testing

At endpoint (5-month-old mice), seven mice per group (randomly selected) (cohort 1, also used for FlexiVent, see **Fig. 1A**) underwent grip strength and rotarod testing during the light phase in a dedicated room after a 30-min acclimation. Forelimb grip strength was measured in a single session following the

modified SHIRPA and using a digital force meter (Ametek® Chatillon Columbus Instruments), as indicated before [18]. Each mouse performed three trials (1-min intervals); peak force (PK) and last available force (LAVF) were recorded and normalized to body weight. Neuromotor coordination and endurance were evaluated using a Rotarod apparatus (Panlab, Harvard Bioscience). Mice were trained on two consecutive days (4 RPM for 5-min). On the third day, mice underwent two testing trials (30-min inter-trial interval) with linear acceleration from 4 to 40 RPM over 300-sec; the latency-to-fall was averaged.

In vivo respiratory mechanics assessment

Partitioned oscillometry was used to assess *in vivo* respiratory mechanics in mice *via* the FlexiVent® system (FX module 2, FlexiWare version 8.3, SCIREQ, Montréal, Canada), following well-established protocols [19–23].

Mechanical ventilation

Mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). At surgical depth of anesthesia, a tracheostomy was performed, and an 18-gauge metal cannula secured with suture. Mice were ventilated supine at 10mL/kg tidal volume, 150 breaths/min, PEEP 3cmH_2O, with room air as carrier gas. Pancuronium bromide (100µL at 20µg/ml, intramuscular) suppressed spontaneous respiratory efforts. Anesthetic depth was monitored throughout the experiment using electrocardiography.

Baseline lung mechanics

Two deep inflations to 30 cmH_2O were performed before data collection [24]. Baseline respiratory mechanics were then assessed through three cycles (1-min intervals) of perturbations sequence: a Deep-inflation (yielding inspiratory capacity, IC), followed by Snapshot-150, Quick Prime-3, and pressure-controlled partial pressure-volume (PVs-P) maneuvers. The Snapshot-150 maneuver (1.25-sec sinusoidal oscillation at 2.5Hz, single-frequency forced oscillation technique, FOT) provided single-compartment estimates of respiratory system resistance (Rrs), elastance (Ers), and the normalized work of breathing (WOBn). The Quick Prime-3 multifrequency perturbation (1-20Hz, broadband FOT) was used to fit the constant-phase model and derive Newtonian resistance (Rn), tissue damping (G), tissue elastance (H), and hysteresivity (η = G/H) [25]. PVs-P loops were recorded using a stepwise inflation to 30cmH_2O,

yielding the Salazar-Knowles shape parameter K (volume-independent compliance). Measurements with coefficient of determination (COD) <0.9 were excluded; remaining values were averaged per mouse.

Methacholine challenge

After baseline recordings, increasing concentrations of aerosolized methacholine (0, 3, 10, 100, 300 mg/ml in PBS; Sigma-Aldrich) were delivered for 10-sec at 50% duty cycle via an integrated Aeroneb Lab nebulizer (rinsed with PBS between doses) [19,23]. To minimize derecruitment, two deep-inflations to 30 cmH_2O preceded each dose. After each nebulization, alternating Snapshot-150 and Quick Prime-3 perturbations were repeated 8-10 times, with ~8 sec of tidal ventilation between maneuvers. Measurements with COD <0.9 were excluded. For each dose, the maximal post-dose values (Δ from dose 0 mg/ml baseline) for Rrs, Ers, Rn, G, H, and WOBn were used to draw the concentration-response curves, and compute AUCs.

Bronchoalveolar lavage (BAL)

In cohort 2, mice under deep ketamine/xylazine anesthesia were tracheotomized, and a bronchoalveolar lavage was performed by doing three sequential 1-mL PBS injections/recoveries [26,27]. Recovered volumes were pooled and recorded. Samples were centrifuged (500 g, 5 min, 4°C). Cell pellets were resuspended in 200 µL PBS for total/differential counts and flow cytometry, while BAL fluid (BALF) were aliquoted and stored at -80°C for albumin ELISA and MSD multiplex cytokines/chemokines. Total BAL cell counts used an automated cell counter (Corning CytoSmart Cell Counter, Axion AxISVue, Cell-Count v3). Cytospins (Cytospin III, Shandon Scientific) were stained (Hema 3, Thermo Fisher, #122-911) for differential counts under light microscopy.

Blood collection and plasma preparation

Blood was obtained by cardiac puncture into EDTA tubes and centrifuged (2000 g, 10 min, 4°C). Plasma was aliquoted and stored at -80°C until use for ELISAs (erythropoietin, corticosterone) and MSD multiplex cytokines/chemokines.

Lung tissue processing and leukocyte isolation

Lungs were excised, and left, right superior, and post-caval lobes were processed fresh and finely minced in ice-cold GNK buffer (PBS supplemented with glucose, NaCl, and KCl), then digested for 45 min at 37°C with gentle inversion every 10 min in equal volume of GNK buffer containing 1 mg/ml collagenase

IV (Worthington, #LS004188), 0.2mg/ml DNase I (Sigma, #10104159001), and 10% FBS (Gibco). Samples were then mechanically dissociated through a 70-µm cell strainer. Following PBS wash and centrifugation (500 g, 5 min, 4°C), RBC lysis (ammonium chloride solution) was performed for 4 min at room temperature, quenched with PBS, and suspension filtered (40 µm). Final pellets were resuspended in PBS containing 1% FBS and counted using an automated cell counter, excluding dead cells stained with trypan blue. For fluorescence minus one (FMO) controls, pooled samples were used. This well-established protocol yields robust recovery of viable $CD45^+$ cells for downstream multiparametric flow cytometry [26–29].

Flow cytometry

BAL cells and lung single-cell suspensions were stained with fluorochrome-conjugated antibodies (**Supplementary Table 1**). Data were acquired on a BD LSR Fortessa (BD Biosciences) and analyzed using FlowJo v10. Gating strategies were based on FMO controls and are provided in **Supplementary Fig. 1-4**.

RNA extraction and quantitative RT-PCR

Total RNA from frozen right-middle lobes was extracted (TRIzol reagent), quantified (Infinite-2000, TECAN), reverse-transcribed (High-Capacity cDNA kit, Thermo-Fisher, #4368814), and amplified using SYBR chemistry (Thermo-Fisher, #A46109). Primers (**Supplementary Table 2**) were designed with melting temperatures around 60°C, and their specificity verified *in silico* (Primer-BLAST, NCBI). Amplifications were performed on a LightCycler 480 II system (Roche) using the following cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec, and a final melting curve analysis (95°C for 1 min, 55°C for 30 sec, 95°C for 30 sec). Expression was normalized to RPLP0 and calculated by 2^(−ΔΔCt).

Protein extraction and immunoblotting

Frozen right-inferior lobes were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors (Roche). Equal amounts of protein (30 µg) were resolved by SDS-PAGE and transferred onto PVDF or nitrocellulose. Membranes were blocked with 5% non-fat dry milk in TBS-Tween and probed overnight at 4°C with primary antibodies, followed by HRP-conjugated secondary antibodies, and detected by ECL (Immobilon® ECL UltraPlus Western HRP Substrate). Details regarding all antibodies are provided in **Supplementary Table 3**. Total proteins were used for normalization (No-Stain reagent

on PVDF, Ponceau-S on nitrocellulose). Densitometry used ImageJ, and representative lanes from the immunoblots are displayed for each protein of interest.

Enzyme-linked immunosorbent assays (ELISA)

Commercial ELISA kits were used according to manufacturer instructions for quantification of plasma erythropoietin (EPO, Abcam, #Ab270893), plasma corticosterone (Biotechne, #NBP3-23556), and BALF albumin (Abcam, #207620). Results were normalized to appropriate standard curves.

Multiplex MSD cytokines/chemokines profiling

BALF and plasma cytokines/chemokines were quantified using a V-PLEX Mouse Cytokine 19-Plex Kit (MSD) following the manufacturer's protocol. Plates were read on a MESO® QuickPlex SQ-120 Multiplex Imager. Data were analyzed using MSD-Workbench software. Results were $\log_{10}$-transformed prior to statistical analysis to account for skewed distributions.

Cecal pathogen monitoring

Under a Class II biosafety cabinet, cecal feces were collected aseptically from SPF and rewilded mice using sterile instruments and DNase/RNase-free tubes. Samples were snap-frozen and stored at -80°C until shipment on dry ice to Charles River Research Animal Diagnostic Services (RADS). Pathogen screening was performed using the PCR Rodent Infectious Agent (PRIA®) panels. Results are summarized in **Supplementary Table 4**.

Statistical analyses

All statistical analyses were performed using GraphPad Prism v10. Data distribution was assessed with the Shapiro-Wilk normality test. For comparisons between two independent groups, unpaired two-tailed Student's t-tests were used for normally distributed data, while Mann-Whitney tests were applied for non-parametric data. For body weight trajectories, methacholine challenge curves and cytokine log-transformed values, repeated-measures two-way ANOVA was performed with Šidák's *post hoc* correction for multiple comparisons. AUCs across doses were compared by unpaired two-tailed t-tests. Significance threshold was set to $p < 0.05$.

Patient and public involvement

Patients and/or the public were not involved in the design, conduct, reporting, or dissemination plans of this research.

CRediT authorship contribution statement

Mohamed Lala Bouali, Amir Mohamed Kezai, Papa Yaya Badiane, and Sébastien S. Hébert established the indoor vivarium. **Mohamed Lala Bouali**: Conceptualization, Methodology, Validation, Formal Analysis, Software, Investigation, Supervision, Data Curation, Writing - Original Draft, Visualization, Writing - Review & Editing. **Amir Mohamed Kezai**: Investigation. **Marie-Josée Beaulieu**: Methodology, Investigation, Formal Analysis, Data Curation. **Joanny Roy**: Methodology, Investigation, Formal Analysis, Data Curation. **Papa Yaya Badiane**: Investigation. **Véronique Lévesque**: Investigation. **Luc Fillion**: Resources. **Luc Vallières**: Conceptualization, Validation, Resources, Writing - Review & Editing. **Marie-Renée Blanchet:** Conceptualization, Methodology, Validation, Resources, Supervision, Writing - Review & Editing. **Sébastien S. Hébert**: Conceptualization, Methodology, Validation, Formal Analysis, Resources, Funding Acquisition, Project Administration, Writing - Review & Editing.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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RESULTS

Rewilding preserves a lean body weight trajectory and improves neuromuscular performance

We first compared the physiological status of two-month-old female C57BL/6 mice obtained from The Jackson Laboratory, housed for 90 days either in an SPF facility or in the semi-natural vivarium (**Fig. 1A**). During this period, no mice contracted pathogens (**Supplementary Table 4**), as revealed by PCR testing of feces designed to screen for a range of viruses, bacteria, and parasites, confirming that our enclosures and procedures were biosecure.

Body weight gain was significantly different between the two groups (**Fig. 1B**). At baseline (day 0), body weights were similar (SPF: 18.05 ± 0.70 g; Rewild: 17.93 ± 0.65 g; $p = 0.98$). After three months, SPF mice had gained 3 times more weight (27.11 ± 0.80 g; Δ +9.1, $p < 0.0001$) than rewilded mice (21.13 ± 0.63 g; Δ +3.2, $p < 0.0001$). A mixed-effects analysis confirmed a significant time-group interaction ($F(1,30) = 62.8$, $p < 0.0001$), indicating that body weight gain was strongly influenced by housing conditions. At necropsy, abdominal fat was frequently observed in SPF mice, but not in rewilded mice (not shown).

Neuromuscular performance consistently improved under rewilding conditions. Rewilded mice appeared more vigorous and active in the vivarium (**Supplementary Videos 1-2**). Forelimb grip strength was significantly higher for both peak force (**Fig. 1C**) and last available force (**Fig. 1D**). Motor coordination and endurance, assessed by rotarod latency-to-fall, were also enhanced in rewilded mice (**Fig. 1E**), indicating better coordination and vigilance. Overall, rewilded mice resisted excess weight gain and performed better functionally, contrasting with the overweight and reduced activity of SPF mice.

Plasma endocrine profiling offered complementary systemic readouts. Stress levels were evaluated by measuring corticosterone at the morning circadian trough, which did not differ between groups (**Fig. 1F**), indicating no chronic activation of the stress axis and no persistent stress response detectable after handling at the end of the rewilding period. Conversely, circulating erythropoietin (EPO) was significantly lower in rewilded mice (**Fig. 1G**). Since EPO secretion reflects renal hypoxia sensing, this decrease is consistent with lower basal hypoxic signaling. However, given that EPO can be modulated by factors such as physical activity, hematocrit, or hormonal influences, these data should be interpreted as descriptive rather than mechanistic.

Taken together, these results demonstrate that indoor rewilding is safe, non-stressful, and beneficial for the physical and metabolic fitness.

Rewilding remodels baseline lung mechanics and blunts airways hyperresponsiveness.

We next examined the effects of rewilding on basal lung mechanics and airway responsiveness to methacholine challenge using partitioned oscillometry and quasi-static pressure-volume (PVs-P) analyses with the FlexiVent system (**Fig. 2A**).

At rest, partitioned oscillometry revealed a distinct mechanical profile in rewilded mice compared to SPF controls (**Fig. 2B**). Newtonian resistance (Rn), which indexes the resistance of the central and large conducting airways, was significantly lower in rewilded mice, consistent with reduced airway tone or larger conducting calibers. In contrast, tissue damping (G), a measure of energy dissipation within the lung parenchyma, was higher, while tissue elastance (H), a proxy for parenchymal stiffness, remained unchanged ($p = 0.1881$, not shown). Consequently, hysteresivity ($\eta = G/H$), which quantifies the fraction of elastic energy lost during each breathing cycle and serves as indicator of ventilation heterogeneity, was increased. The total respiratory resistance (Rrs), which conflates both airway and tissue components, was modestly elevated, because the rise in G outweighed the fall in Rn. Importantly, the normalized work of breathing (WOBn), which indexes the energy required to inflate the lungs, was reduced in rewilded mice, indicating a lower energetic cost of ventilation despite increased parenchymal dissipation.

Quasi-static PVs-P analysis further supported enhanced distensibility. Static elastance (Est), representing the overall stiffness of the respiratory system, was significantly lower (**Fig. 2B**), with a concomitant rise in the parameter K, a volume-independent proxy of compliance. Together, these changes indicate more compliant lungs with improved alveolar recruitment at low pressures in rewilded mice, independent of body size or weight. Inspiratory capacity (IC) showed a trend toward elevation ($p = 0.0865$). Overall, this combination of freer central airways, higher parenchymal compliance, and slightly increased damping supports more efficient ventilation and indicates that rewilded lungs operate in a state of improved mechanical efficiency and airway recruitment. This mechanical heterogeneity may reflect localized immune-tissue interactions, as supported by the immunological findings described later.

Given that airway reactivity is altered in lung disease such as asthma and is often tested in SPF-based mouse models, we tested whether the baseline phenotype observed in **Fig. 2B** altered airway

responsiveness to constrictive stress. Following increased, non-cumulative, doses of aerosolized methacholine (0 to 300 mg/ml), SPF mice exhibited a steep, dose-dependent increase in airway and tissue resistive parameters, whereas rewilded mice showed significantly blunted responses across intermediate and high doses, revealing a protective phenotype with blunted airway responsiveness across both central and peripheral compartments (**Fig. 2C**). The rise in ΔRrs and ΔErs was markedly attenuated in rewilded mice across multiple doses (adjusted tests, $p < 0.05$ at 30 to 300 mg/ml). Partitioning this response revealed reduced large-airway reactivity (ΔRn, $p < 0.05$ at 30 to 300 mg/ml) and diminished tissue damping (ΔG, $p < 0.05$ at 30 to 300 mg/ml). Consistently, the rise in ΔWOBn was also dampened in rewilded mice ($p < 0.05$ at 30 to 300 mg/ml). By contrast, ΔH increased with dose in both groups without any difference (not shown), indicating that rewilding selectively protected against resistive rather than elastic loading. Consistent with decreased sensitivity to methacholine, rewilded mice exhibited significantly lower AUCs for ΔRrs, ΔRn, ΔG and ΔWOBn, but not for ΔH (**Fig. 2D**).

Together, these data demonstrate that rewilded mice exhibit reduced central airway resistance, higher compliance, and attenuated airway responsiveness, indicating a mechanically more efficient and less reactive lung compared to SPF controls.

Rewilding densifies and reshapes lung immune compartment

We next examined whether functional remodeling of the lung was accompanied by changes in immune composition (**Fig. 3A**). Cytospin differentials showed selective increases in lymphocytes, macrophages, and neutrophils, while eosinophils remained unchanged (**Fig. 3B-C**). This profile indicates an enriched but non-eosinophilic milieu, consistent with a diversified leukocyte landscape without allergic-type profile. Flow cytometry of BAL cells confirmed these findings (**Fig. 3D**). Counts of $CD45^+$ cells per BAL volume were higher in rewilded mice, with expansions of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and natural killer T cells (NKT).

$CD45^+$ leukocytes were significantly higher in rewilded lungs (**Fig. 3E**). Within this expanded leukocyte pool, $CD4^+$ and $CD8^+$ T cells were significantly increased, with a modestly higher CD4/CD8 ratio ($p = 0.0311$) suggesting a shift toward a regulatory-dominant immune state compatible with enhanced immune surveillance and mucosal tolerance in rewilded lungs. Further, natural killer cells (NK) and NKT cells were also significantly increased, while B cells remained unchanged (**Fig. 3E**). Notably, type-2 conventional dendritic cells (cDC2s; identified as $CD11c^+$ $MHCII^+$ $CD11b^+$ SIRPα+; see

Supplementary Fig. 4 and table 1) were similar between groups, but their phenotype shifted toward higher PD-L1 (MFI, $p = 0.0001$) and PD-L2 (MFI, $p = 0.0267$) expression in rewilded mice, whereas CD200 expression was unchanged (**Fig. 3E**). These results demonstrate selective enrichment of lymphoid and innate-like populations in the airspaces and lung parenchyma, accompanied by increased checkpoint tone on dendritic cells, suggestive of heightened immune surveillance without eosinophilia.

Rewilding calibrates lung inflammatory-regulatory programs and enhance systemic anti-inflammatory balance

At the transcriptional level, rewilded lungs showed higher expression of TNF, CCL4, CXCL10, and IL-10 compared to SPF controls (all $p \leq 0.03$), while IL-4, IL-5, IL-6, INF-γ, CXCL15, and TGF-β1 remained unchanged (**Fig. 4A**). This pattern suggests a mixed but balanced state coupling effector readiness (TNF, CCL4, CXCL10) with local regulation (IL-10). At the protein level, rewilded lungs showed increased platelet endothelial cell adhesion molecule-1 (PECAM-1) and intercellular adhesion molecule-1 (ICAM-1), while vascular cells adhesion molecule-1 (VCAM-1) remained unchanged, compatible with enhanced but controlled leukocyte trafficking in the lung microvasculature (**Fig. 4B**).

Further analysis of circulating cytokines/chemokines in BALF revealed a selective pro-inflammatory enrichment under rewilding (**Fig. 4C**). The radar plot highlighted elevated IFN-γ, TNF, CXCL1, CXCL2, and CCL3, IL-2 and IL-6 (all $p \leq 0.0454$, individual t-tests), consistent with an activated but non-allergic pulmonary milieu. Furthermore, plasma levels of the same mediators suggest that systemic inflammation is not present in rewilded mice. Plasma cytokine radar plots showed reductions in pro-inflammatory drivers, notably IL-6 and IL-17 (all $p \leq 0.0384$, individual t-tests) while other cytokines remained stable (**Fig. 4D**). In contrast, plasma IL-10 was slightly increased in rewilded mice. Accordingly, regulatory ratios IL-10/IL-6 and IL-10/IL-17 were significantly increased, supporting a systemic anti-inflammatory balance (**Fig. 4E**).

Together, these results indicate that indoor rewilding shifts the pulmonary environment toward enhanced effector readiness while simultaneously promoting systemic immune restraint, offering a dual profile of local vigilance and systemic regulation.

Alveolar-capillary barrier integrity is preserved under rewilding

We finally assessed whether the abovementioned changes translated into measurable disruption of the alveolar-capillary barrier, a critical determinant of gas exchange and respiratory health. Immunoblot analyses of whole lung protein extracts showed comparable abundance of the tight-junction proteins zona occludens 1 (ZO-1), occludin, and claudin-5, as well as of the adherens junction protein vascular-endothelial (VE)-cadherin (**Fig. 5A**). Consistently, albumin concentrations in BALF did not differ significantly across the groups, indicating no detectable protein extravasation into the airspaces (**Fig. 5B**). Thus, despite denser leukocyte traffic and elevated cytokines, the alveolar-capillary barrier remained intact.

DISCUSSION

"Dirty" mouse models have advanced our understanding of how ecological exposures shape the immune system, although their effects beyond immune remodeling, particularly on vital barrier organs physiology remain largely underexplored. The lung, as a continuously exposed barrier organ requiring the integration of mechanical performance, immune vigilance, and tissue integrity, represents a particularly sensitive system to test such effects. In this study, we demonstrate that indoor rewilding restores immune complexity within a relatively short period (90 days) while recalibrating pulmonary biomechanics and systemic homeostasis in SPF-reared adult mice. Accordingly, ecological enrichment extends beyond immunological recalibration to support organ-level functional and tissular resilience.

Our work extends previous advances from "dirty" models such as fecal microbiota transfer (FMT) *via* oral gavage or coprophagy, naturalization protocols, and (outdoor) rewilding approaches that have linked microbial complexity to immune maturation [1,2,15,16,30–33]. In addition to organic materials, our approach incorporates living vegetation and invertebrates within a controlled indoor environment, thereby providing ecological enrichment while maintaining experimental containment. Unlike previous rewilding paradigms, which can expose animals to climate variations, predators, and unpredictable pathogens such as helminths or ectoparasites, the indoor rewilding framework preserves biosecurity and reproducibility and can be readily adapted to existing non-SPF infrastructures. Routine health monitoring confirmed that no external pathogens were introduced during the rewilding period (**Supplementary Table 4**), validating the biosafety of the approach. Embedding this paradigm within an accredited animal facility further streamlines essential operations such as colony transfer, health surveillance, and longitudinal experimental design, offering a scalable and standardized platform for modeling immune-functional integration studies under semi-natural conditions.

The pulmonary immune remodeling observed in rewilded mice reiterates but also extends prior wildling findings. Airspaces and parenchyma displayed denser leukocyte subsets, with expanded $CD4^+$ and $CD8^+$ T cells, NKT and NK populations, and elevated immune checkpoint expression (PD-L1/PD-L2) on cDC2s. This configuration supports surveillance and tolerance rather than allergy, as eosinophils and IL-4/IL-5 responses were absent. The absence of eosinophilic infiltration, despite adult-phase exposure, likely reflects the establishment of local regulatory control during the three-month rewilding period, which conditioned tolerance rather than hypersensitivity to environmental cues. The cytokine profile within the lung, marked by TNF, IFN-γ, CXCL1/CXCL2, CCL3/CCL4, and IL-10 upregulation, reflects

a vigilant yet balanced immunophenotype. Despite this local activation, systemic cytokine patterns leaned toward anti-inflammatory balance (elevated IL-10/IL-6 and IL-10/IL-17 ratios), consistent with compartmentalized mucosal homeostasis uncommon in SPF mice. These cytokine contrasts were paralleled by increased PECAM-1 and ICAM-1 expression, promoting selective leukocyte trafficking without excessive inflammation [34,35]. This arrangement resembles human mucosal immunophysiology, where immune readiness coexists with structural containment [36–38].

Despite intensified immune activity, alveolar-capillary barrier integrity remained intact. Tight- and adherens-junction proteins (ZO-1, occludin, claudin-5, VE-cadherin) and BALF albumin were unchanged, indicating preserved barrier function and demonstrating that immune trafficking can occur without structural compromise. Notably, in inflammatory models, TNF/ICAM-1 upregulation usually accompanies junctional breakdown [39]. Thus, indoor rewilding promotes a phenotype of regulated tissular resilience which separates immune activation from barrier alteration, likely mediated through coordinated adhesion and checkpoint mechanisms (PECAM-1, PD-L pathways). Clinically, this distinction is critical since barrier dysfunction drives asthma, COPD, and fibrosis.

Functionally, the mechanical consequences of this immune recalibration were mirrored in FlexiVent analysis. Rewilded mice exhibited lower central airway resistance, improved compliance, and blunted methacholine-induced reactivity, hallmarks of more efficient lung mechanics. These enhancements suggest balanced immune-epithelial interactions and tissue maintenance, limiting edema formation and blunting pro-contractile cascades classically linked to AHR. This immune-mechanical homeostasis may explain the paradoxical association of elevated TNF with reduced AHR in rewilded mice. In SPF asthma models, TNF promotes airway hyperresponsiveness, and anti-TNF agents alleviate symptoms [40–43]. Here, concurrent IL-10 elevation and PD-L1/PD-L2 upregulation likely buffer TNF's contractile signaling, redirecting it toward coordination rather than pathology. This context-specific modulation underscores how immunological immaturity in SPF mice can distort cytokine function and thus therapeutic predictability.

Comparing our findings to previous studies provides further insight. Islam et al. [16] reported that SPF mice colonized with wild-derived gut microbiota showed reduced house-dust-mite (HDM)-induced allergic inflammation and AHR *via* TNF-dampening and metabolic modulation, consistent with our finding of reduced airway reactivity under methacholine. Conversely, Ma et al. [15] found that wild-

inherited microbiota amplified Th2 inflammation in HDM-challenged offspring. Unlike that model, our study examined baseline physiology and direct smooth-muscle reactivity, revealing a freer, non-hyperreactive airway state. Together, these contrasts emphasize the role of exposure context (direct *vs* transgenerational, baseline *vs* allergen-challenged) in determining whether rewilding fosters tolerance or pathology.

Rewilded mice also exhibited systemic adaptations consistent with their remodeled lung physiology, including improved neuromuscular performance and reduced plasma EPO levels despite higher physical output, suggesting more efficient pulmonary ventilation and tissular oxygenation, reducing hypoxic drive. Stable corticosterone levels indicate that indoor rewilding and immune enrichment did not trigger chronic stress. These findings highlight systemic adjustments favoring energy efficiency and metabolic stability, positioning the lung as both a target and contributor to overall organismal resilience in ecologically enriched environments.

Taken together, indoor rewilding establishes a balance between immune vigilance and biomechanical efficiency rarely seen in SPF animals. By restoring immune-functional integration, this model enhances predictive power for conditions like asthma, COPD, and fibrosis, where immune and mechanical factors intersect, and strengthens preclinical testing for translatable therapies. Future longitudinal, sex-balanced studies could examine the longevity of these effects, possible sex differences, and interactions with disease-susceptible genotypes. Whether similar functional findings can be reproduced in other “dirty” models remains to be tested.

In summary, ecological realism bridges the gap between reductionist SPF systems and more physiologically relevant models. Indoor rewilding promotes human-like mucosal immunity, preserves epithelial and endothelial barriers, and enhances respiratory mechanics, offering an optimized framework for advancing mucosal immunology and respiratory medicine.

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FIGURES

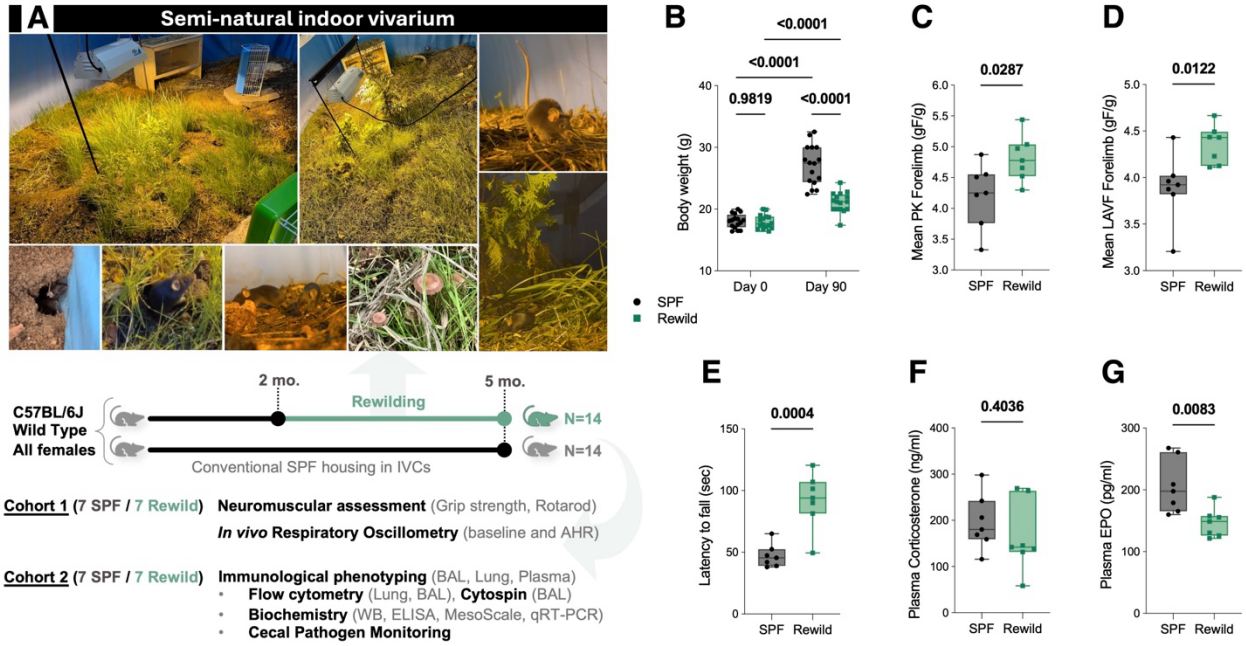

Figure 1. Rewilding promotes the maintenance of a lean body weight and enhances neuromuscular and systemic performance. (A) Representative images of the semi-natural indoor vivarium and schematic of the experimental design. Female C57BL/6J mice were housed under SPF or rewilding (semi-natural vivarium) conditions for 90 days. Cohort 1 (N = 7/group) was used for neuromuscular testing (grip strength, rotarod) and pulmonary mechanics using forced oscillation technique (FOT); while cohort 2 (N = 7/group) for immunophenotyping, biochemical assays, and fecal pathogen monitoring. **(B)** Body weight at baseline (day 0) and endpoint (day 90). **(C-D)** Forelimb grip strength assessed as peak force (PK) and last available force (LAVF), normalized to body weight. **(E)** Neuromotor coordination and endurance assessed by latency to fall on the rotarod. **(F)** Plasma corticosterone and **(G)** erythropoietin (EPO) levels measured by ELISA. Data are shown as individual values overlaid on box-and-whisker plots, where the box spans the interquartile range (25th-75th percentile), the line indicates the median, and whiskers extend from the minimum to the maximum. Statistical comparisons were performed using two-way repeated measures ANOVA with Šidák's multiple comparison test for body weight, and unpaired two-tailed Student's t-tests for all other comparisons.

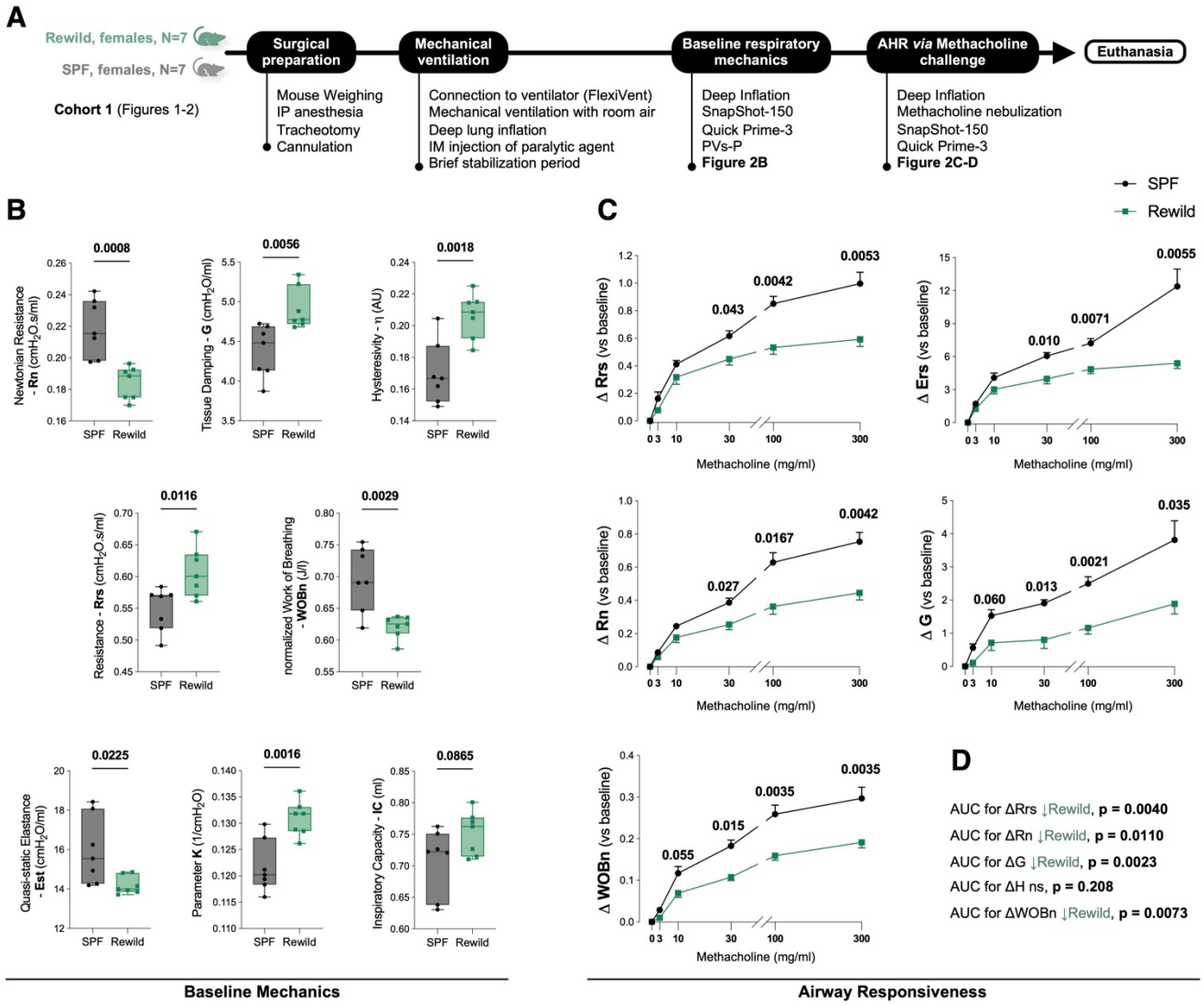

Figure 2. Rewilding remodels baseline lung mechanics and blunts airway responsiveness. (A) Experimental workflow for *in vivo* pulmonary function testing by oscillometry (FOT) using the FlexiVent system, including baseline assessment of lung mechanics and methacholine challenge to evaluate airway hyperresponsiveness (AHR). **(B)** Baseline FlexiVent readouts obtained from Deep-inflation, Snapshot-150, Quick Prime-3 and pressure-controlled partial pressure-volume (PVs-P) maneuvers. Each value represents the mean of three valid cycles per animal; comparisons between SPF and rewilded groups were made using unpaired two-tailed Student's t-tests. **(C)** FlexiVent dose-response curves to nebulized methacholine (0-300 mg/ml) showing changes (Δ vs. baseline) in Rrs, Ers, Rn, G, and WOBn. Curves show group means of peak responses; statistical comparisons were performed by two-way repeated measures ANOVA with Šidák's correction for multiple comparisons. **(D)** Area under the curve (AUC)

analyses confirm blunted AHR in rewilded mice for ΔRrs, ΔRn, ΔG, and ΔWOBn, with no significant difference for ΔH. Means were compared using unpaired two-tailed Student's t-test.

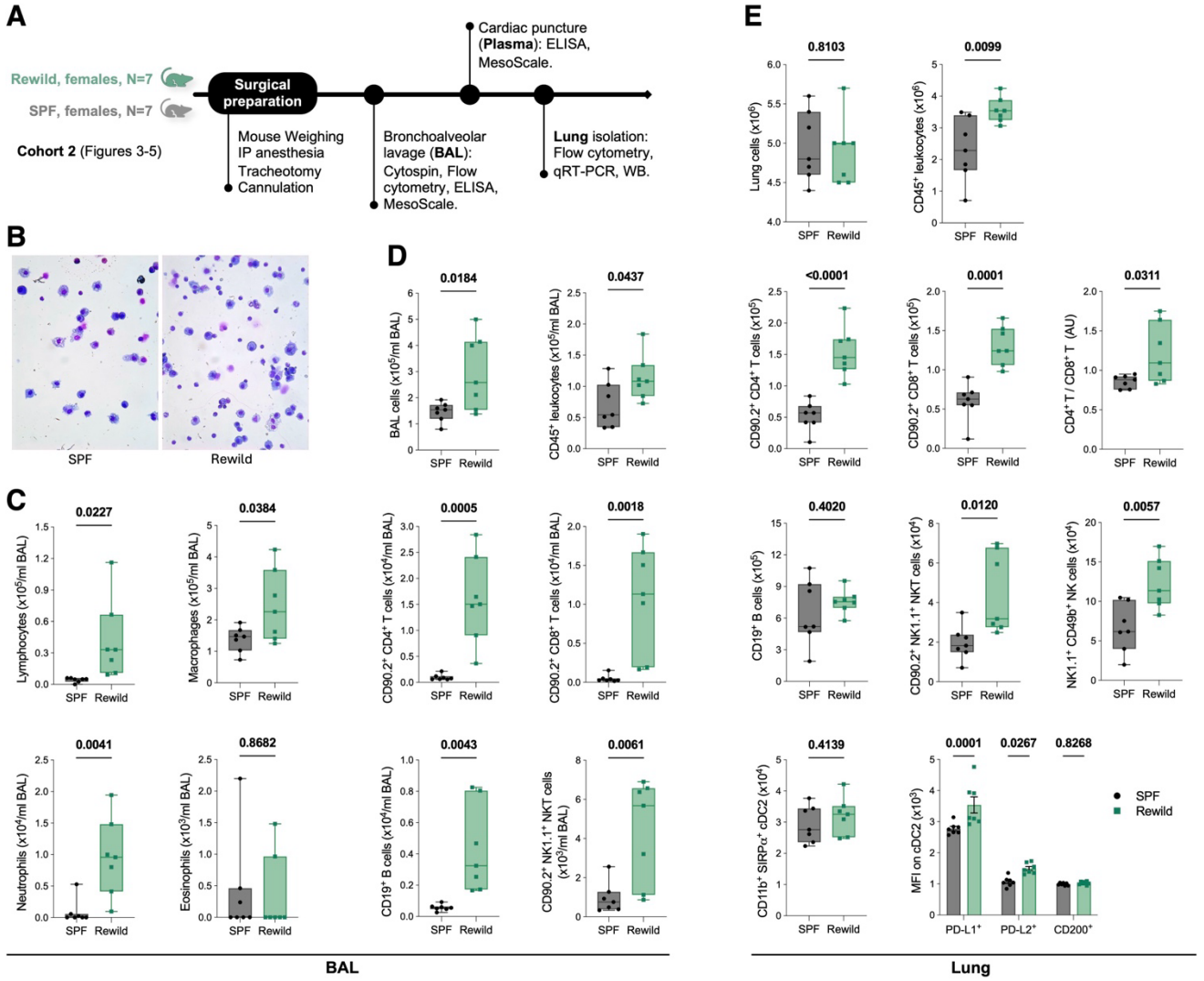

Figure 3. Rewilding densifies airway leukocytes and reshapes lung immune compartments. (A) Experimental workflow for immunophenotyping of bronchoalveolar lavage (BAL), lung and plasma compartments. **(B)** Representative cytospin images of BAL cells stained with modified May-Grünwald–Giemsa. **(C)** Differential BAL cytology showing increased lymphocytes, macrophages, and neutrophils but unchanged eosinophils in rewilded mice. **(D)** BAL flow cytometry showing higher leukocyte counts and expansions of CD4$^+$ and CD8$^+$ T cells, B cells, and NKT cells. **(E)** Lung single-cell suspensions showing higher leukocyte numbers and selective rewilding-increases in CD4$^+$, CD8$^+$, NK, and NKT cells, with stable total cell counts and B cells. Median fluorescence intensity (MFI) analysis of lung conventional cDC2 subset revealed upregulation of PD-L1 and PD-L2, but not CD200. Data are shown as individual values or as mean ± SEM for MFI histograms. Means were compared using unpaired two-tailed Student's t-test.

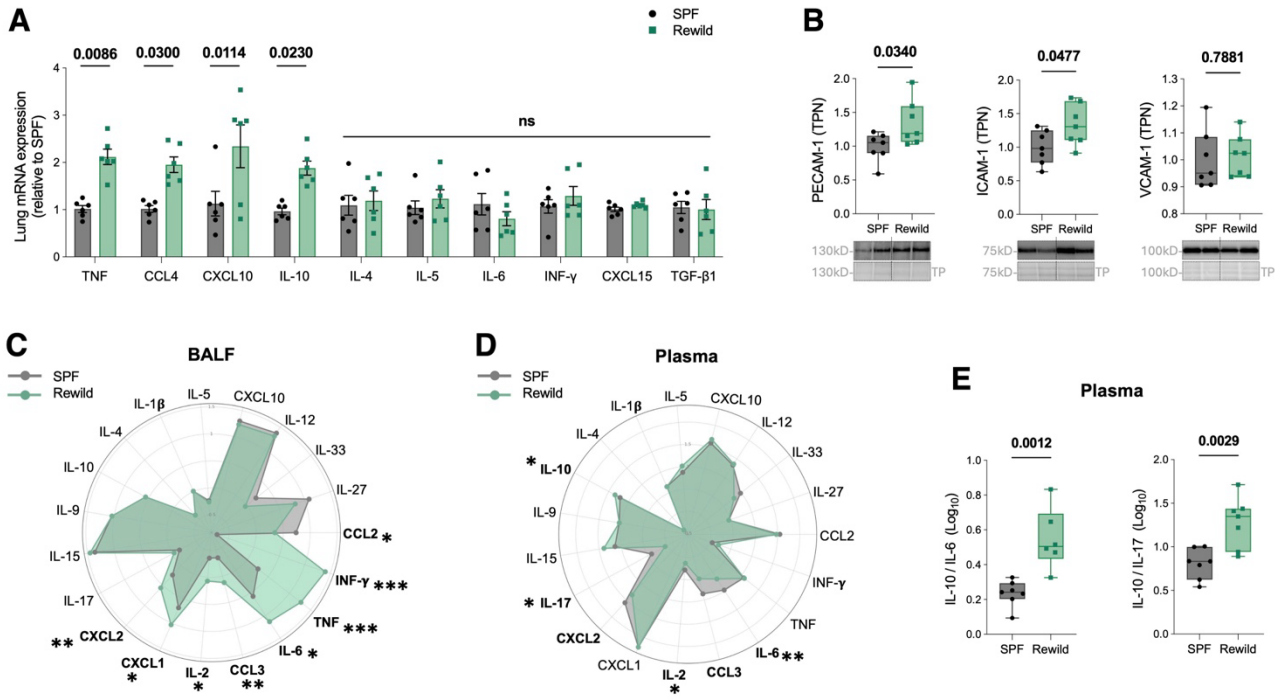

Figure 4. Rewilding calibrates lung inflammatory-regulatory programs with localized airway activation and systemic restraint. (A) Relative mRNA expression of inflammatory and regulatory genes in lung tissue measured by qRT-PCR, normalized to the housekeeping gene RPLP0. Statistical analysis was performed on Log_2-transformed values using two-way ANOVA with Šidák's correction for multiple comparisons. Adjusted p-values are shown; ns: non-significant. **(B)** Immunoblot analysis of vascular adhesion proteins PECAM-1, ICAM-1 and VCAM-1 in whole-lung homogenates. Representative blots and total protein normalization controls are shown; Comparisons were made using unpaired two-tailed Student's t-tests. **(C)** Radar plot of bronchoalveolar lavage fluid (BALF) cytokines and chemokines measured by multiplex MesoScale assay. Values represent group means of log_{10}-transformed concentrations. The rewild group displayed broader cytokine activation in the airways, consistent with localized immune enrichment. Asterisks (*) indicate statistically significant differences between groups (unpaired two-tailed Student's t-tests. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$). **(D)** Radar plot of plasma cytokines and chemokines measured by multiplex MesoScale assay (similar analysis as in BALF) illustrating a restrained profile in rewilded mice. Panels **(C)** and **(D)** are shown side by side to enable direct comparison of local (airway) versus systemic cytokine landscapes. **(E)** Ratios of systemic cytokines (log_{10}-transformed), IL-10/IL-6 and IL-10/IL-17, highlighting increased systemic anti-inflammatory balance. Means were compared using unpaired two-tailed Student's t-test.

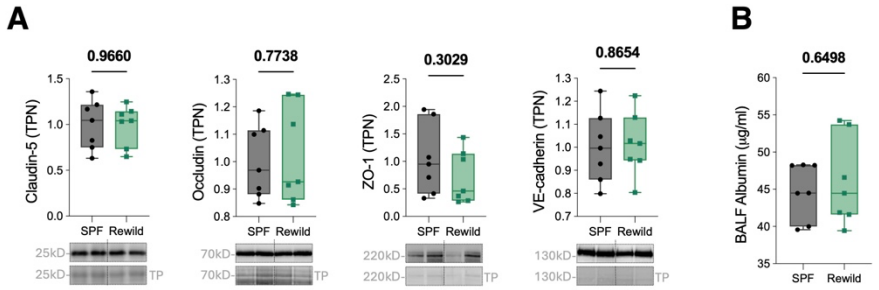

Figure 5. Alveolar-capillary barrier integrity remains preserved under rewilding. (A) Immunoblot quantification of junctional proteins claudin-5, occludin, ZO-1, and VE-cadherin from whole-lung homogenates. Representative blots with total protein normalization controls are shown. **(B)** Albumin extravasation into airways assessed by ELISA in BALF, as a proxy for barrier permeability. Means were compared using unpaired two-tailed Student's t-test.

SUPPLEMENTARY MATERIALS

Supplementary Figure 1. Representative gating strategy for BAL lymphocyte subsets. Sequential gating from Time, FSC/SSC, singlets, live cells, and CD45+ leukocytes, followed by lineage markers (CD90.2, CD4, CD8, CD19, NK1.1, CD49b, CD11b).

Supplementary Figure 2. Representative gating strategy for lung lymphocyte subsets. Sequential gating from Time, FSC/SSC, singlets, live cells, and CD45+ leukocytes, followed by lineage markers (CD90.2, CD4, CD8, CD19, NK1.1, CD49b, CD11b).

Supplementary Figure 3. Representative gating strategy for lung granulocyte subsets. Sequential gating from Time, FSC/SSC, singlets, live cells, and CD45+ leukocytes, followed by lineage markers (CD11b, CD11c, CCR3, Siglec-F, Ly6G).

Supplementary Figure 4. Representative gating strategy for lung dendritic cell subsets. Sequential gating from Time, FSC/SSC, singlets, live cells, and CD45+ leukocytes, followed by conventional DCs markers (CD11b, MHCII, CD11c, CD103, SIRPα, XCR1), with further assessment of PD-L1, PD-L2, and CD200 median fluorescence intensities (MFIs).

Supplementary Table 1. List of fluorochrome-conjugated antibodies used in flow cytometry panels for lung and BAL leukocyte profiling.

Supplementary Table 2. List of forward and reverse primers designed for amplification of murine cytokine, chemokine and housekeeping genes. Primer sequences were used to quantify mRNA expression in Lung tissue by SYBR Green-based qRT-PCR.

Supplementary Table 3. List of primary and secondary antibodies used for protein detection by immunoblot. Dilutions used, host species, suppliers, and catalog numbers are indicated.

Supplementary Table 4. Pathogen monitoring of SPF and rewilded mice performed by Charles River's PRIA® panel. The table lists viral, bacterial, and parasitic agents tested in cecal feces; all results were negative, confirming the absence of pathogen introduction during the rewilding period.

Supplementary Video 1. Indoor rewilding vivarium setup. The vivarium was enriched with farmland soil (~30-40 cm in depth), dried hay, living grass and plants, a small wooden shed, wood chips and bark shedding, rocks and pebbles, mixed chicken and cow manure, fungi, and a variety of invertebrates (worms, ants, grasshoppers, flies), with full exclusion of predators. Food and water were provided *ad libitum* at dedicated stations inside the vivarium and monitored daily. In addition to standard lab chow, wild bird seeds were scattered within the enclosure. Refreshments of organic material (e.g., hay, manure, bark, moss) occurred every 3-4 weeks to maintain antigenic stimulation.

Supplementary Video 2. Behavioral activity of rewilded mice. Infrared recordings showing spontaneous locomotor and exploratory behaviors of C57BL/6J mice during the three-month indoor rewilding period, illustrating enhanced physical activity and natural behaviors such as food caching and tunnel exploration.