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Physiological microbial exposure has a temporally limited inhibitory effect on lung ILC2 responses to allergens in mice

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Abstract

The impact of microbes on restraining the immune response to allergens has been extensively studied and is a key element of the hygiene hypothesis. Lung type 2 innate lymphoid cell responses to airway allergens can be inhibited by administration of a number of microbial products; however, it is unclear whether such an effect would be observed with natural infections and how sustained any observed inhibitory effects would be. To answer these questions, we used a murine model of physiological microbial exposures through cohousing SPF laboratory mice with pet store mice and examined the acute type 2 response to intranasally delivered fungal allergen extract *Alternaria alternata*. We found laboratory mice cohoused with pet store mice for two weeks display a suppressed ILC2 response to *A. alternata* which resulted in reduced eosinophilia. By comparison, mice cohoused for at least two months had ILC2 and eosinophil responses similar to SPF mice despite dramatic changes to the composition of the immune cell populations in the lungs. Lung ILC2 in two-month cohoused mice were still sensitive to subsequent inflammatory cues, as administration of poly(I:C) was able to suppress ILC2 activation and eosinophilia equally well in SPF and two-month cohoused animals. These findings suggest ILC2 dynamically respond to their environment and are not easily desensitized long-term.

Introduction

Allergic asthma affects 300 million people globally, with the incidence increasing over the last century[1]. The most common type of allergic asthma is mediated by type 2 immunity, characterized by cytokines interleukin-4 (IL-4), IL-5, and IL-13, and recruitment of eosinophils, mast cells, and other immune cells into the lungs[2] (Holgate 2012). Historically, $CD4^+$ type 2 T helper cells (Th2) have been thought to be the main producers of IL-5 and IL-13 and thus studied as the cellular mediators of allergic asthma. However, since the discovery and characterization of the tissue resident innate lymphoid cells (ILCs) in the past decade, the large contribution of ILC2 in allergic responses has been appreciated[3]. Specifically, lung-resident ILC2 rapidly respond to inflammatory signals induced in the lung epithelium, particularly IL-33, by protease-containing allergens[4, 5] and are necessary for a maximal adaptive response from Th2 against allergens[6, 7].

The hygiene hypothesis and the related "old friends" hypothesis posit that improved sanitation and hygiene reduce our exposure to pathogens and commensals and this reduced microbial exposure leads to inappropriate responses to allergens[8, 9]. Retrospective and prospective studies of children have found compelling links between exposures to microbes and allergic sensitization. For example, having older siblings, starting day-care at an early age, and growing up on a farm are thought to be proxies for increased microbial exposures and all are associated with lower risk to allergic sensitization[10, 11, 12, 13, 14, 15]. Additional studies have more directly made the connection between microbes and allergies; high endotoxin levels in house dust, increased intestinal microbial diversity, and lack of antibiotic use in infancy are all associated

with lower risk of atopy[16, 17, 18, 19]. While not all studies investigating these links between microbes and allergic responses report significant results[20], the human studies have motivated basic research into the effects of microbial exposure on the immune response to allergens in animal models, in order to tease apart potential mechanisms for these phenomena. Microbial products or synthetic analogs such as unmethylated CpG DNA, household dust containing LPS, bacterial lysate OM-85, and the double stranded RNA analog poly(I:C) all decrease allergic sensitization in various allergic asthma models[21, 22, 23, 24, 25, 26] albeit some studies reported low levels of LPS actually worked as an adjuvant and increased allergic responses in certain allergic models[27, 28]. Intranasal administration of the *Heligmosomoides polygyrus*-derived protein HpARI interferes with ILC2-activating cytokine IL-33 and prevents sensitization to airway allergens[29, 30]. Deliberate infection with a gammaherpesvirus that infects the lungs was also found to be protective in an allergic asthma model[31]. Various microbes and microbial products can interfere with the response of lung ILC2 to allergens suggesting an early block in the nascent type-2 immune response. In particular, type I and II interferons induced by acute viral infection or microbial products reduce IL-5 and IL-13 production by ILC2[25, 32, 33, 34]. Most of these studies focus on the acute effects of infection by individual pathogens or transient treatment with microbial products: the short- and long-term effects of diverse, physiologically acquired infections on the immune response to inhaled allergens has not been assessed.

Improved animal models of human diseases such as allergic asthma would be highly useful for clinicians and basic researchers, especially for answering questions related to how immune experience impacts allergic responses. Our group has the unique opportunity to study the

effects of physiological microbial exposure on immune responses in mouse models. As we have previously published, inbred laboratory mice cohoused with a pet store mouse continuously for two months acquire natural infections, resulting in an immune-experienced phenotype[35]. Through this prior work we have demonstrated that the gene signature of peripheral blood mononuclear cells (PBMC) of cohoused mice more closely resembles the gene signature of human PBMC, while SPF mice PBMC more closely resemble neonatal cord blood[35]. We sought to utilize this "dirty" mouse model to test whether microbial exposures from pet store mice could alter the lung ILC2 response to airway allergens at timepoints both early and late in cohousing. Timing of transmission of microbes through cohousing is not instantaneous but happens within the first two weeks of introduction of the pet store mouse. After two months of cohousing infections are generally resolved or in a chronic or latent phase, but signs of continued inflammation are present, such as elevated serum cytokine levels[36]. We therefore tested the innate immune response to *A. alternata* (*Alt*) after short-term (two-week) cohousing, modeling a response to an allergen in the midst of an immune response to infection, as well as after long-term (at least two-month) cohousing, to model response to allergen in a healthy individual that has prior microbial experience. Short-term cohousing led to a substantial reduction in the ILC2 and eosinophil responses to intranasal *Alt* administration, in keeping with prior studies investigating the acute effects of deliberate infections and treatment with microbial products. However, diverse microbial experience did not lead to permanent changes in reactivity to inhaled allergens; despite changes in lung immune cell composition and a delay in the response to *Alt* exposure, ILC2 activation and lung eosinophilia was restored in long-term cohoused mice. Furthermore, the type-2 response of long-term cohoused animals could be

105 inhibited by acute treatment with poly(I:C), similar to the inhibition observed in SPF animals.
106 Together, these data indicate lung ILC2 are highly sensitive to recent inflammatory and
107 inhibitory signals in the tissue microenvironment, but prior microbial experience does not lead
108 to sustained reprogramming of the ILC2 response to inhaled allergens. These findings suggest
109 the immune system is “reset” for type-2 responses to allergens following acute control of
110 microbial infections, with implications for developing treatments for allergic diseases.

Results

Diverse microbial exposure leads to transient impairment of lung ILC2 and eosinophil responses to intranasal *A. alternata*. We utilized an established mouse model, involving intranasal exposure with *A. alternata* extract (*Alt*), evaluating the innate immune response in the lungs at 24 hours[37]. Both C57BL/6 (B6) and (C57BL/6 x BALB/c)$_{F1}$ IL-5$^{wt/venus}$ (IL-5v F1) animals were used, the latter to serve as a reporter for IL-5 production. Shortly before sacrifice, a fluorescent anti-CD45 antibody was injected intravenously so that cells in the vasculature (CD45 iv^{+}) could be distinguished from cells in the lung parenchyma and airspace (CD45 iv^{-}, Fig S1a), providing a more flexible way to analyze lung-resident immune cell populations than, for example, bronchoalveolar lavage. As expected, there were few eosinophils in the lungs of SPF mice given intranasal PBS 24 hours prior, but after *Alt* treatment this population increased significantly (Fig S1a, b). Previous studies indicate that the type-2 response to allergens can be inhibited by exposure to microbial products[25]. One example is the *H. polygyrus* protein HpARI, known to bind and interfere with ILC2 activating cytokine IL-33[30]. Indeed, intranasal administration of HpARI concurrent with *Alt* exposure led to significantly reduced lung eosinophilia (Fig S1b). Likewise, HpARI inhibited production of IL-5 by lung ILC2 in response to *Alt* treatment, as revealed by reduced frequency and expression intensity of the IL-5 reporter and reduced surface expression of the IL-33 receptor ST2 and high affinity IL-2 receptor CD25 (Fig S1c-f). These results demonstrate ILC2 responses to *Alt* can be inhibited by microbial products in B6 mice and IL-5v F1 reporter mice.

While those studies confirm acute intranasal exposure to microbial products restrains the ILC2 response to *Alt*, it was unclear whether physiological microbial exposure would have similar effects. To answer this, we cohoused SPF B6, BALB/c, and IL-5v F1 animals with mice purchased from local pet stores. As expected[35], we observed seroconversion against common murine pathogens and the diversity increased over the cohousing period (two weeks vs two months, Fig S1g-h). We also assayed serum cytokine and chemokine levels over time after cohousing, observing levels of most factors (including TNF-α, CXCL10, and IL-6) peaked at approximately 10-14 days of cohousing and then plateaued, at elevated levels relative to SPF mice, up to at least two months (Fig 1a, Fig S1i). These findings align with published results showing the frequency of $CD44^{high}$ blood $CD8^{+}$ T cells (indicative of antigen experience) also peaked at approximately two weeks post cohousing[35].

These data suggest the ~2-week time point is suitable to assess the short-term effects of recent microbial exposure physiologically transmitted through mouse-to-mouse contact. Similar to the effects of acute treatment with defined microbial products, short-term cohousing led to a significant reduction in the appearance of lung parenchymal eosinophils following *Alt* exposure; while eosinophil numbers increased >40-fold in SPF mice, this was limited to only a ~7-fold increase in two-week cohoused mice (Fig 1b). The frequency and expression levels of IL-5 $venus^{+}$ lung ILC2 was also reduced in two-week cohoused mice (Fig 1c-d), while the total numbers of ILC2 were not impacted by cohousing and decreased slightly in number 24 hours after *Alt* treatment in both groups (Fig S2a), perhaps due to the reported phenomenon of it being more difficult to extract activated lymphocytes from tissues[38]. We considered that

cohousing may have affected IL-5 producing $CD4^+$ T cells in the lung, but the population of parenchymal $CD4^+$ T cells was similarly small in two-week cohoused and SPF animals, and no significant change in the number of IL-5 venus expressing $CD4^+$ T cells was induced by cohousing or *Alt* treatment (Fig S2b-d). These results demonstrate physiological transmission of natural murine pathogens limits ILC2 responses against an airway allergen and reduces lung eosinophilia.

To determine the extent to which physiological microbial exposure led to a sustained change in the lung response to allergens, we next assessed mice that had been cohoused long-term, for at least two months. In sharp contrast to our findings using short-term cohoused mice, the response to *Alt* in two-month cohoused animals was indistinguishable from that in SPF animals, inducing similar numbers of parenchymal eosinophils (Fig 1e) and similar production of IL-5 venus by lung ILC2 at 24 hours after *Alt* treatment (Fig1f-g). Together, these data suggest that acute physiological microbial exposure does indeed provoke an impaired type-2 response to an inhaled allergen, but this blunted response is not sustained long-term despite ongoing systemic inflammation above levels observed in SPF mice.

Delayed initial response to *A. alternata* in long-term cohoused mice. It was possible the lungs of long-term cohoused mice revert to the characteristics of SPF mice, despite the effects of the cumulative infectious history (Fig S1g) and sustained changes in serum cytokines and chemokines (Fig 1a)[35], leading to identical responses to intranasal *Alt*. To assess this possibility, we first examined the cellular composition of the lungs in SPF and long-term co-housed mice.

Multiple immune cell populations were increased in number in cohoused animals (Fig 2a, see Fig S3a-c for gating strategy), and previously published whole lung RNA sequencing revealed that the overall gene expression in the lungs is significantly altered by cohousing[39]. However, the size of the ILC2 population was unchanged and eosinophils only modestly higher in mice cohoused for at least two months (Fig 2a). Lung ILC2 were characterized in more detail and in addition to being equal in number in SPF and two-month cohoused mice, the cells had a similar surface phenotype, including CD44, ST2, CD25, KLRG1, and CD127 (Fig 2b). The transcription factor Gata3 was also similarly expressed in these two groups (Fig 2c).

We next assessed the response to *Alt* in SPF and ≥2-month cohoused mice kinetically. One of the first steps in the type 2 response to acute airway allergen is the release of IL-33 from lung cells[37, 40]. This alarmin is stored in the nucleus of cells but is rapidly secreted upon allergen sensing [41]. We collected bronchoalveolar lavage fluid (BALF) from the lungs of SPF and two-month cohoused mice one hour after *Alt* treatment and measured IL-33 by ELISA. IL-33 was lower in *Alt* treated long-term cohoused mice than SPF mice, while IL-33 levels in BALF were low in all PBS treated control mice (Fig 3a). To determine whether this decreased IL-33 would have an impact in type 2 cytokine levels immediately after allergen treatment, BALF and lung samples were collected 4.5 hours after *Alt* exposure to measure IL-5 and IL-13 levels by ELISA. Reflecting the lower IL-33, cohoused mice also had reduced IL-5 and IL-13 in the lungs and BALF (although IL-13 in the BALF did not reach statistical significance) (Fig 3b-c and Fig S4a-b). We also measured IL-5 and IL-13 in the lungs of SPF and two-month cohoused BALB/c mice and observed a similarly reduced cytokine response at this acute timepoint in this mouse strain (Fig

198 S4c-d). This, along with consistent responses in previous experiments between B6 mice and IL-
199 5v F1 reporter mice, demonstrates the observed phenomena in cohoused mice are not specific
200 to one particular inbred strain of laboratory mice. To test to what extent the lower initial
201 amounts of IL-5 and IL-13 were due entirely to reduced initial IL-33, or if two-month cohoused
202 mouse ILC2 had reduced sensitivity to IL-33, mice were treated intranasally with recombinant
203 IL-33 and IL-5 and IL-13 were measured 4.5 hours later. We found IL-5 was slightly reduced in
204 cohoused mouse lungs, but lung IL-13 and BALF IL-5 and IL-13 were unchanged between SPF
205 and cohoused (Fig 3d-e, Fig S4e-f). These results suggest that lower IL-5 production 4.5 hours
206 after *Alt* treatment in two-month cohoused mouse lungs is in large part due to reduced IL-33,
207 but may also reflect reduced sensitivity to IL-33 by ILC2 in those animals.
208
209 Together, these findings suggest sustained changes in the immune cell composition of the lungs
210 and altered initial responses toward *Alt* in long-term cohoused mice. Nevertheless, these
211 alterations evidently do not prevent lung ILC2 activation and efficient eosinophil infiltration 24
212 hours after *Alt* exposure in two-month cohoused mice (Fig 1). This suggests the type-2 response
213 to allergens is delayed but not inhibited by long-term cohousing with pet store mice.
214
215 **Response to repeated *A. alternata* exposure is similar in SPF and two-month cohoused mice**.
216 It was possible the altered initial response toward *Alt* in long-term cohoused mice would impact
217 the effects of repeated allergen exposure. To test this, we adapted a model of repeated airway
218 *Alt* treatment in SPF and two-month cohoused animals. We treated mice intranasally with the
219 allergen every other day three times and analyzed 24 hours after the last dose in order to focus

on the innate immune response. After repeated exposure to *Alt*, there was an expansion of ILC2 in the lungs of both SPF and two-month cohoused mice (Fig 4a), and a subsequent recruitment and/or expansion of eosinophils, neutrophils, and conventional ($Foxp3^-$) and regulatory ($Foxp3^+$) $CD4^+$ T cells in both groups of mice (Fig 4b-e). While most of these cell populations were slightly elevated in PBS-treated two-month cohoused mice compared to PBS-treated SPF mice, reflecting the steady-state changes (Fig 2a), *Alt* treatment led to cell populations of approximately equal size in SPF and ≥two-month cohoused lungs. The magnitude of the recruited or expanded populations may therefore be slightly decreased in two-month cohoused mice (since the number of cells was higher in these mice before treatment); however, a type 2 allergic response clearly was not substantially inhibited in ≥two-month cohoused mice after repeated allergen exposure.

The similar responses by SPF and two-month cohoused mice to repeated *Alt* treatment, as well as at 24-hours following a single treatment, demonstrate a threshold of activation of ILC2 is reached in two-month cohoused mice, prompting a type 2 immune response despite sustained alterations to the immune cell composition of the lungs and serum cytokines and chemokines which accompany long-term cohousing.

Acute microbial stimulus is capable of re-suppressing type 2 responses to *A. alternata* in two-month cohoused mice. We established ILC2 responses to *Alt* could be inhibited by microbes introduced through cohousing two weeks after initiating cohousing with a pet store mouse, but responses at two months of cohousing were similar to SPF mouse responses (Fig 1). Serum

cytokine levels in cohoused mice over time demonstrated that inflammation peaks around 2 weeks and is lower by two months, although still elevated over SPF mice (Fig 1a). Certain inflammatory cytokines, such as interferons, inhibit ILC2 responses[25, 32, 33, 34], and the increased level of inflammation at two weeks may account for the suppression of these cells early but not late after cohousing. On the other hand, it was possible long-term cohoused mice were now resistant to the ability of acute microbial product exposure to impair type-2 responses against allergens. For example, the elevated levels of interferon-induced factors found at steady-state in serum (Fig 1a)[36] might lead to sustained desensitization of some responses to interferons. Alternatively, sustained levels of interferons in two-month cohoused mice may be below the threshold to modulate ILC2s. To test these possibilities, we pre-treated SPF and two-month cohoused mice intranasally with poly(I:C) one day before treating with *Alt* and examined the response 24 hours after allergen exposure. As previously described[25], poly(I:C) pretreatment suppressed IL-5 production from ILC2 and eosinophil recruitment into the lungs, and also caused reduced expression of ST2 and CD25 on ILC2, reflecting reduced levels of activation of these cells (Fig 5a-e). Two-month cohoused mice pre-treated with poly(I:C) before *Alt* treatment similarly had reduced lung eosinophilia (Fig 5a), reduced IL-5 venus expression in ILC2 (Fig 5b-c), and reduced surface expression of ST2 and CD25 on ILC2 (Fig 5d-e), demonstrating the type-2 immune response to allergens in these animals is still sensitive to blockade by acute production of inflammatory factors, such as interferons.

Discussion

In addition to genetic factors, an individual's history of microbial exposures is an important component that can influence the susceptibility to atopic diseases such as allergic asthma. We used the "dirty mouse" model, physiologically acquired natural murine pathogens and commensals through cohousing laboratory mice with pet store mice, to test the effects of diverse microbial experience on responses to a fungal allergen delivered to the airways. We found short-term cohousing (two weeks) did indeed lead to a suppressed type 2 responses to *Alt*. However, the ability to mount a type-2 response against single or repeated exposures to the allergen was restored in long-term (at least two months) cohoused mice, despite reduced initial induction of ILC2 activation.

A central finding from our studies is that prior microbial experience does not permanently reprogram flexibility in immune reactivity: while short-term cohousing resulted in inhibition of this response, this was restored in long-term cohoused mice. These findings imply – despite sustained changes in inflammatory factors in the serum and immune cell composition of the lungs – the immune system can "reset," in this case restoring the ability to mount a type-2 immune response to an allergen. Furthermore, despite diverse microbial experience in long-term co-housed mice, they were still responsive to blockade of this type-2 response by acute exposure to inflammatory cues (modeled by poly(I:C)).

These findings support underlying concepts of the hygiene/"old friends" hypotheses, but argue it is recent microbial exposures, rather than the cumulative infectious history, which dictates

type-2 responsiveness to allergens. This conclusion has logical appeal, since it would behoove the immune system to remain flexible for appropriate responses independent of previous microbial exposures – for instance, being able to mount a type-2 immune response against helminthic infections despite a history of numerous type-1 responses against intracellular pathogens. Our studies showed physiological, unsynchronized transmission of microbes (including several viruses) causes transient blockade of type-2 responses to allergens but that this effect is largely lost once acute infections are under control. This immunological "resetting" is in contrast to responses to other immune challenges, such as infection by *Listeria monocytogenes*, *Plasmodium berghei*, induction of sepsis and influenza vaccination, all of which are sustainably altered in ≥two-month cohoused mice[35, 36, 39]. Hence, several aspects of the immune response changed long-term following diverse microbial experience, but the ability to mount a type-2 response to inhaled allergens is evidently not one of these.

In epidemiological studies, human subjects may have had months, years, or decades of microbial exposures (depending on the age of participants). The identities and timing of the full range of bacteria, viruses, and fungi an individual has been exposed to in their lifetime cannot be determined, but these exposures are believed to be an important component in determining one's susceptibility to developing allergic conditions. The cohoused mouse model we have employed in this study provides the benefits of animal models – genetic homogeneity and genetic tools such as fluorescent gene reporters, short experimental timeframes, and ability to characterize cellular and molecular responses within the tissues – but also allows us to study

304 the impacts of the physiological microbial exposures on immune responses that cannot be
305 addressed with SPF animals[42, 43].
306
307 Whether ILC2 would be more, less, or equally responsive to airway allergen in cohoused versus
308 SPF mice was not obvious prior to our studies, as previous publications could support opposing
309 hypotheses. For example, two-month cohoused mice showed evidence of strong type-1
310 immune responses, yet also displayed evidence of type 2 responses, including elevated IL-5, IL-
311 13 and IgE levels in the serum, consistent with a history of, or ongoing, parasitic infections[35, 36].
312 Some parasitic infections impede subsequent allergic responses[44, 45, 46]. Conversely, this type 2
313 signature may have been an indicator of immune cells poised to robustly respond to additional
314 type 2 stimuli such as an airway allergen. In humans, certain severe respiratory infections in
315 infants and children, such as respiratory syncytial virus and rhinovirus, are associated with
316 increased likelihood of wheeze and allergic asthma, potentially through vulnerability of
317 unresolved lung tissue damage[47, 48]. We did indeed observe evidence of prior lung infections,
318 with increases of many immune cell populations in the lungs such as neutrophils; however, this
319 did not result in increased sensitivity to allergens. In fact, two-month cohoused mice had
320 reduced IL-33 levels in the BAL after *Alt* treatment. Other studies have demonstrated type I and
321 II interferons can suppress IL-5 and IL-13 production from lung ILC2[25, 33, 34]. IFN-γ was elevated
322 over SPF mice in the serum of two-month cohoused mice, and an interferon signaling signature
323 was characterized from PBMC sequencing[35, 36], but the peak in inflammatory cytokines,
324 including IFN-γ, was around two weeks after cohousing (Fig 1a), the time at which *Alt* responses
325 were suppressed. We found acute administration of poly(I:C) before *Alt* treatment could

suppress ILC2 responses in both SPF and two-month cohoused mice, suggesting a new spike in interferons could once again suppress ILC2 responses to allergens in microbially-experienced mice.

One potential concern about using the cohoused mouse model is the heterogeneity in the timing and range of microbial transfer might lead to high variability in immune phenotype or immune responses. In recently published work, the contributions of individual pathogens on the immune phenotype of cohoused mice were tested, and we found that no single microbe or combination of infections could explain the observed variability in immune activation[39]. Moreover, we found the variability in responses in cohoused mice was not substantially greater than that of SPF mice, which is similar to what has been reported in other publications using cohoused mice[35, 36, 39]. To press this point, we chose to present data from all experiments conducted rather than show representative experimental repeats, in order to illustrate the full range of responses observed after cohousing.

In conclusion, testing airway allergic responses in a mouse model of microbial exposure due to physiological transmission of murine pathogens and commensals has revealed that infectious history does not have a robust and lasting effect on reducing allergic airway responses, unlike acute exposure to microbial products. Despite the long-lasting impacts that microbial exposure through cohousing has on the immune response systemically and in the lungs, it may be that acute triggers of inflammation are the relevant inhibitors of allergic immune responses. These

findings have direct implications for how susceptibility to type-2 immune responses against allergens can be effectively managed.

Methods

Mice

Six- to 8-week old female C57BL/6 (B6) and BALB/cJ mice were purchased from Charles River (via the National Cancer Institute) *il5*$^{venus/venus}$ BALB/c mice were kindly provided by Dr. Kiyoshi Takatsu, Toyama University, Toyama, Japan[49]. These mice were bred in-house with B6 mice for one generation to produce (B6 x BALB/c)$_{F1}$ mice heterozygous for the cytokine reporter (called IL-5v F1). Pet store mice were purchased from Twin Cities area pet stores and cohoused with SPF mice as described[35]. Only female mice were used because of the ethics of introducing a new mouse to a cage of adult male mice; males cannot be cohoused as this creates animal welfare concerns due to fighting, aggression, and social defeat. Screening for infectious agents was done as described[35]. Mice were used for experiments between 14 and 17 days or 60 and 120 days post-cohousing (2wk CoH and >2mo CoH, respectively in figures). Upon euthanasia, a small number of cohoused mice were found to have overt lung pathology, which did not correlate with the appearance or behavior of the live mice. Pathology consisted of pale white or gray tissue, mottled in appearance and of a tougher consistency than healthy lungs, making up at least an estimated 25% of the outer surface of the lungs, up to 100%. We did not find this pathology significantly impacted the results of the studies and were left in the analysis. Animals were maintained in A-BSL3 under specific pathogen–free, or germ free conditions at the University of Minnesota. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Serum cytokine measurements

SPF mice were bled prior to cohousing with pet store mice, as well as on various days during the 60-day cohousing conditioning period. Serum cytokines and chemokines were quantitated according to manufacturer instructions using a ProcartaPlex custom 7-plex panel (CXCL10, IL-1β, IL-4, IL-6, IL-10, IFN-γ, and TNF-α; Invitrogen) using a Luminex 200 with Bio-plex Manager Software 5.0. Samples with a reading below the limit of detection were assigned a concentration of 0 pg/ml.

Airway administration of *A. alternata* extract, HpARI, rIL-33, and poly(I:C)

Alternaria alternata extract (*Alt*, Greer Laboratories, Lenoir, NC, 100 μg in 40 μl PBS for B6 and F1 mice, 50 μg in 50 μl PBS for BALB/c mice), recombinant IL-33 (R&D Systems, Minneapolis, MN, 200 ng/dose in 40 μl), or PBS were administered intranasally (i.n.) once or three times (days 0, 2, and 4) to mice anesthetized with isoflurane. In some experiments, recombinant HpARI[30] (10μg) was mixed with the *Alt*. In some experiments mice were pre-treated with i.n. poly(I:C) (Invivogen, San Diego, CA, 50μg in 40 μl PBS) 24 hours before *Alt* treatment.

Lung and BAL cytokine measurements

At the indicated time points, mice were killed by an overdose of isoflurane. The trachea was cannulated, and the lungs were lavaged two times with HBSS (2 x 0.5 ml). Lungs were then collected. The BAL fluid and lungs were stored at -80°C for cytokine assays. Lungs were homogenized with a glass dounce in 1.0 ml PBS with Halt protease and phosphatase inhibitor (Thermo Scientific). The homogenates were centrifuged at 10,000 rpm at 4°C for 15 min, and the protein concentrations in the supernatant were quantified with the BCA Protein Assay kit

(Thermo Scientific). The levels of IL-5, IL-13, and IL-33 in lung homogenate and BAL supernatants were measured by Quantikine ELISA kits (R&D systems) per manufacturer instructions.

Flow cytometry

At the indicated time points, mice were killed by an overdose of isoflurane. Lymphocytes were isolated from lungs as previously described[50], except no Percoll step was performed. Instead, after the GentleMax tissue dissociation step, red blood cells were lysed by incubating for 10 minutes in 10 ml ACK buffer and washed in 20 ml RPMI with 3% FBS. In experiments where Tregs were identified, 50 µg of Treg-Protector (anti-ARTC2.2) nanobodies (BioLegend) were injected i.v. 15–30 minutes prior to mouse sacrifice as described (Borges da Silva 2018). Direct *ex vivo* staining was performed as described previously[51] with fluorochrome-conjugated antibodies (purchased from BD Biosciences, BioLegend, eBioscience, Cell Signaling Technology, Tonbo or Thermo Fisher Scientific). For discrimination of vascular-associated lymphocytes in non-lymphoid organs, *in vivo* i.v. injection of FITC, vF450, or BUV605 conjugated CD45 antibody was performed as described[52]. For survival assessment, cells were stained with Live/Dead (Tonbo Biosciences) For detection of intracellular factors surface stained cells were permeabilized, fixed and stained by using the eBioscience Foxp3 staining kit, according to manufacturer instructions. Flow cytometric analysis was performed on LSR Fortessa (BD Biosciences) and data was analyzed using FlowJo software (Treestar).

Statistics

Unless noted, measurements were taken from distinct samples. GraphPad Prism was used to determine statistical significance. Student unpaired two-tailed t-test, one-way ANOVA, or two-way ANOVA with Tukey's multiple comparisons test was used when appropriate. For two-way ANOVA tests, only relevant comparisons were shown (e.g. we did not report SPF PBS vs. >2mo CoH ALT). In Figure 5 when six groups were compared only comparisons that reached statistical significance were reported in order to improve clarity. A p value < 0.05 was considered statistically significant.

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Author contributions

K.E.B. designed and performed the experiments and analyzed and interpreted the data. K.I., M.J.P, D.A.W., R.T., T.A.K., J.X., and T.S.G. performed experiments. H.J.M. provided the HpARI reagent. S.C.J. and H.K. supervised the project. K.E.B. and S.C.J. wrote the manuscript. H.K., T.S.G. and H.J.M. edited the manuscript.

Competing interests

The authors declare no competing interests.

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606 **Figures and Figure Legends**

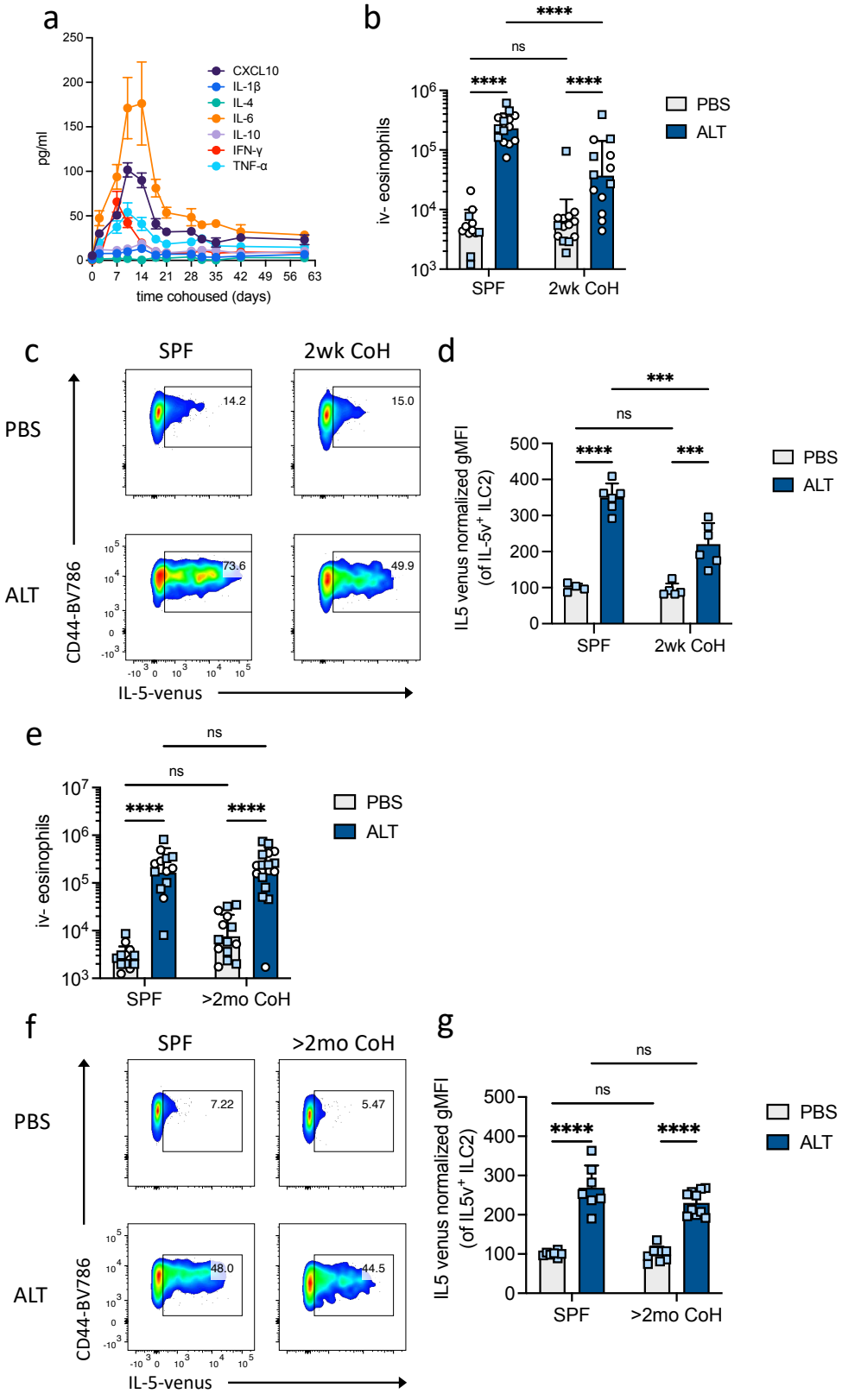

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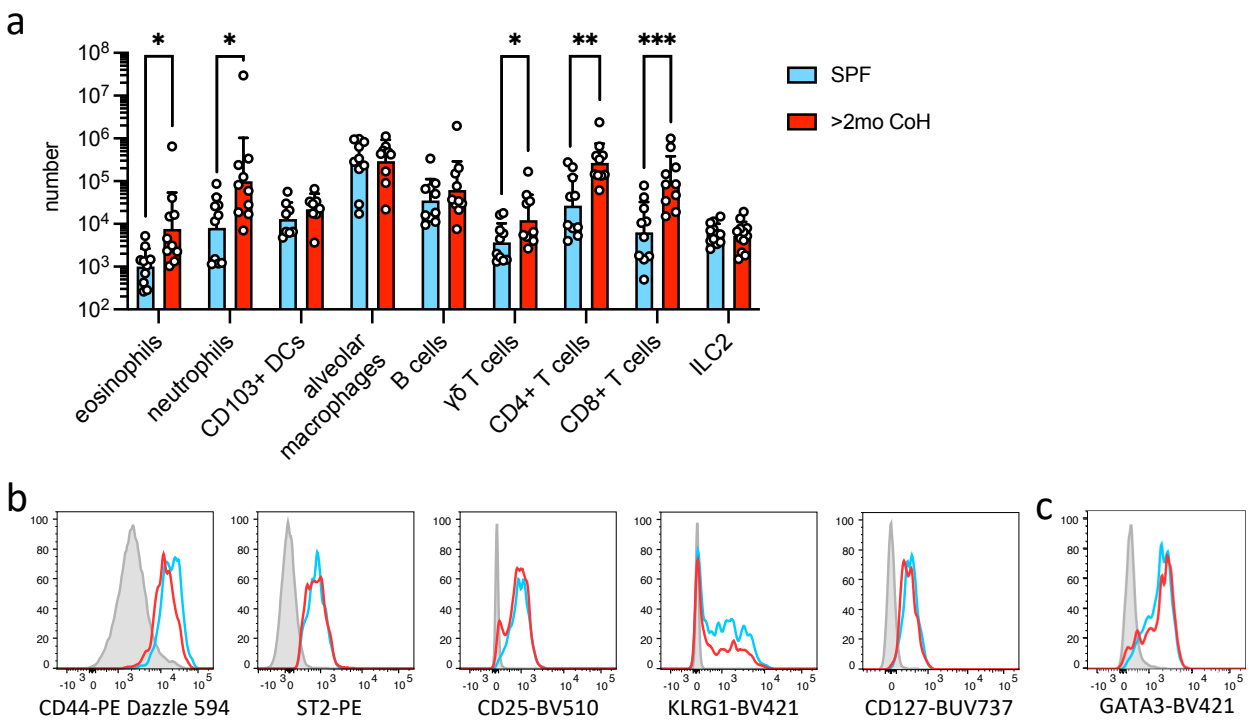
Fig 1. Mice cohoused for two weeks with pet store mice have inhibited eosinophil and ILC2 responses to intranasal *A. alternata* treatment. a, Kinetic changes in serum levels of cytokines and chemokines in C57BL/6 (B6) mice after cohousing with a pet store mouse (*n* = 8). The same cohort of animals were repeatedly measured over time. **b**-**g**, Mice were treated with intranasal phosphate buffered saline (PBS) or *A. alternata* extract in PBS (ALT) and analyzed 24 hours later. Mice were B6 (white circles) or B6xBALB/c IL-5$^{WT/venus}$ (IL-5v F1, light blue squares). Mice were cohoused with pet store mice for approximately two weeks (2wk CoH, b-d) or at least two months (>2mo CoH, e-g), or were age-matched specific pathogen free (SPF) mice. **b**, **e**, Number of eosinophils in the lungs and airways (negative for an intravascular CD45 antibody) 24 hours after intranasal treatment. **c**-**d** and **f**-**g**, IL-5 venus expression within lung ILC2 of IL-5v F1 SPF and 2wk CoH mice 24 hours after PBS or ALT treatment. **c**, **f**, Representative flow plots of IL-5 expression in lung ILC2. **d, g**, Normalized IL-5 venus gMFI of IL-5 venus^{+} lung ILC2. **b**, Pooled from four B6 experiments and two IL-5v F1 experiments (*n* = 12-14/group). **d**, Pooled from two IL-5v F1 experiments (*n* = 4-6/group). **e**, Pooled from 3 B6 experiments and four IL-5v F1 experiments (*n* = 13-16/group). **g**, Pooled from four IL-5v F1 experiments (*n* = 7-10/group). **a**, Symbols show mean +/- SD. **b**, **d**, **e**, **f**, Bar graphs show mean + SD of log-transformed values. *P* values were determined with a 2-way ANOVA with Tukey's multiple comparisons test; ns $p > 0.05$, *** $p < 0.001$, **** $p < 0.0001$.



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628 **Fig 2. Mouse lung populations are altered by cohousing but ILC2 phenotypes are unchanged.**

629 **a**, Lung immune cell populations of SPF and 2mo CoH B6 mice were identified and quantified by

630 flow cytometry. All enumerated cells were i.v. $CD45^-$ except alveolar macrophages, which had

631 an i.v.-intermediate phenotype and were not gated on i.v. status. Pooled from three

632 experiments (*n* = 8-12/group). Bar graphs show mean + SD of log transformed values. *P* values

633 were determined with a Student's t-test (two-tailed). Eosinophils had unequal variance and t-

634 test was conducted with Welch's correction; no symbol $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p <$

635 0.001. **b**, Representative surface protein expression of lung ILC2 in SPF (blue) and >2mo CoH

636 (red) mice. $I.v.^+$ $CD19^+$ cells in gray are included as a negative or low expression control. **c**,

637 Representative intracellular GATA3 expression of lung ILC2 cells. $I.v.^+$ $Lineage^+$ cells in gray are

638 included as a negative or low expression control. Representative of three experiments.

639

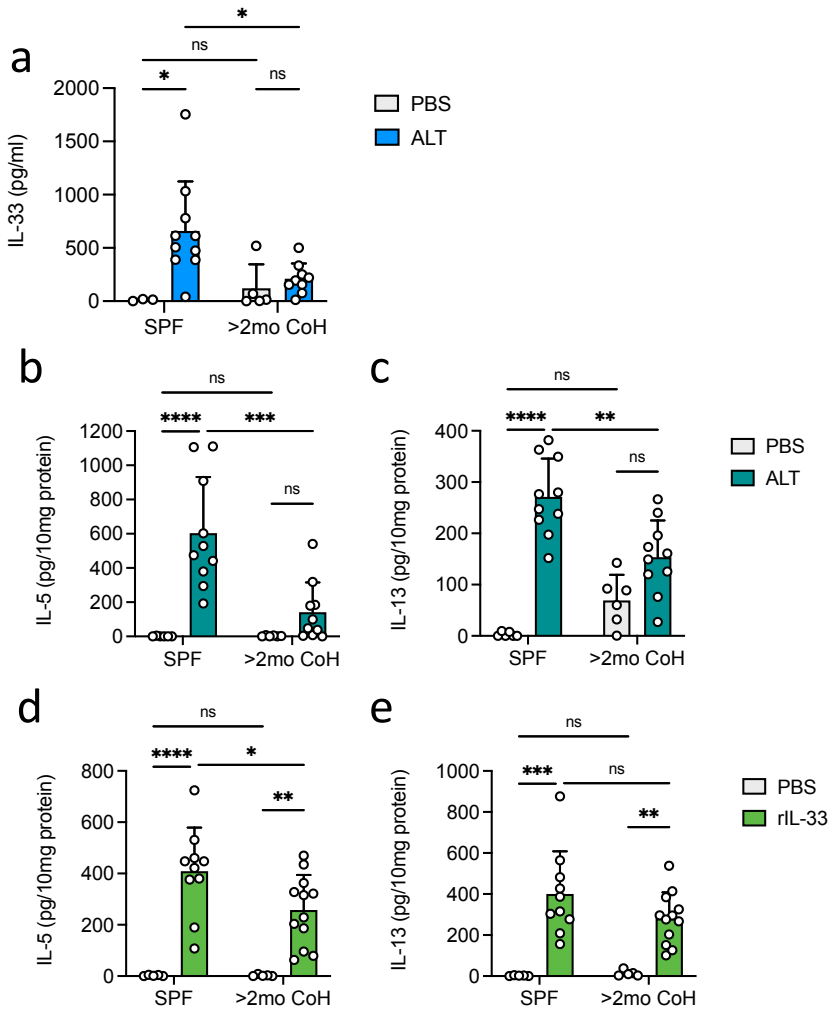


Fig 3. Acute IL-33, IL-5 and IL-13 responses after *A. alternata* exposure are reduced in two-month cohoused mice. a, IL-33 in the bronchoalveolar lavage fluid (BALF) of SPF and >2mo CoH B6 mice one hour after ALT exposure, detected by ELISA. **b**-**e**, ALT (b-c), recombinant IL-33 (rIL-33, d-e) or control phosphate buffered saline (PBS) were given intranasally to B6 mice and lungs and BAL were collected 4.5 hours later. IL-5 and IL-13 in the lung homogenates were detected by ELISA and normalized to the amount of total protein in the samples. Bar graphs show mean + SD. *P* values were determined with 2-way ANOVA with Tukey's multiple comparisons test; ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

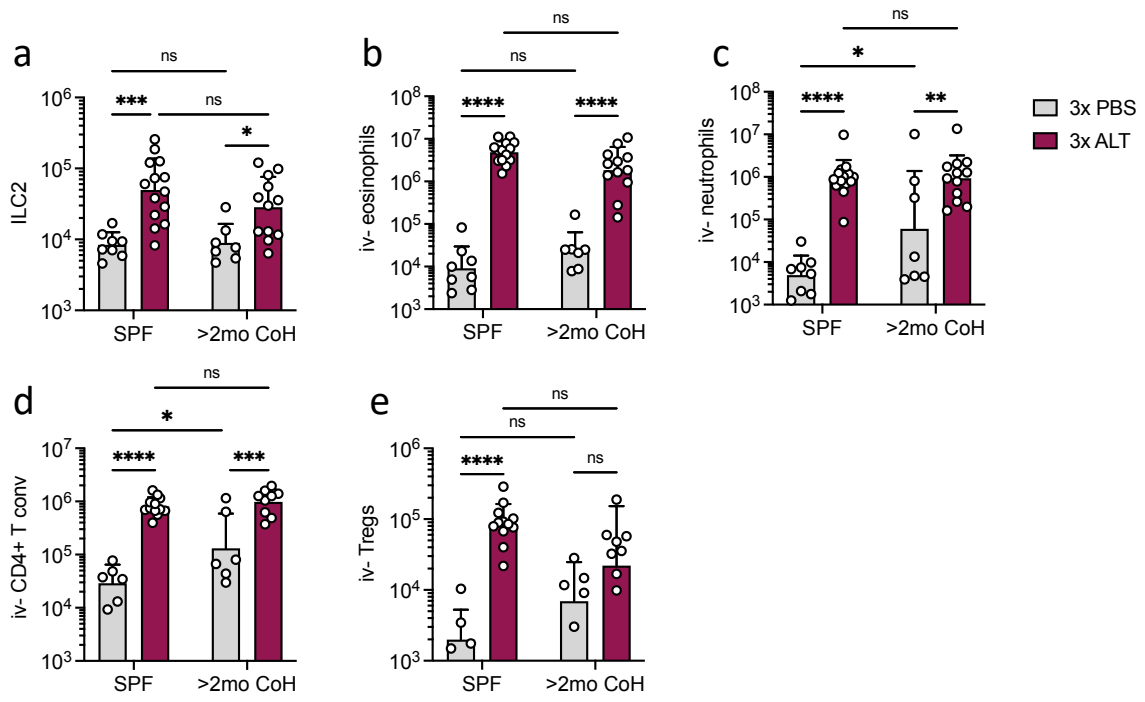


Fig 4. Comparable immune infiltration in SPF and two-month cohoused mice after repeated *A. alternata* exposure. SPF and >2mo CoH B6 mice were treated on days 0, 2, and 4 with intranasal PBS or ALT and on day 5 lungs were harvested for flow cytometric analysis. All enumerated cells were i.v. $CD45^-$. **a**, ILC2, **b**, eosinophils, **c**, neutrophils, **d**, $CD4^+$ conventional T cells ($Foxp3^{neg}$), and **e**, Tregs ($Foxp3^+$ $CD4^+$ T cells). Pooled from 3-4 experiments (n = 4-14/group). Bar graphs show mean + SD of log transformed values. P values were determined with two-way ANOVA with Tukey's multiple comparisons test; ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

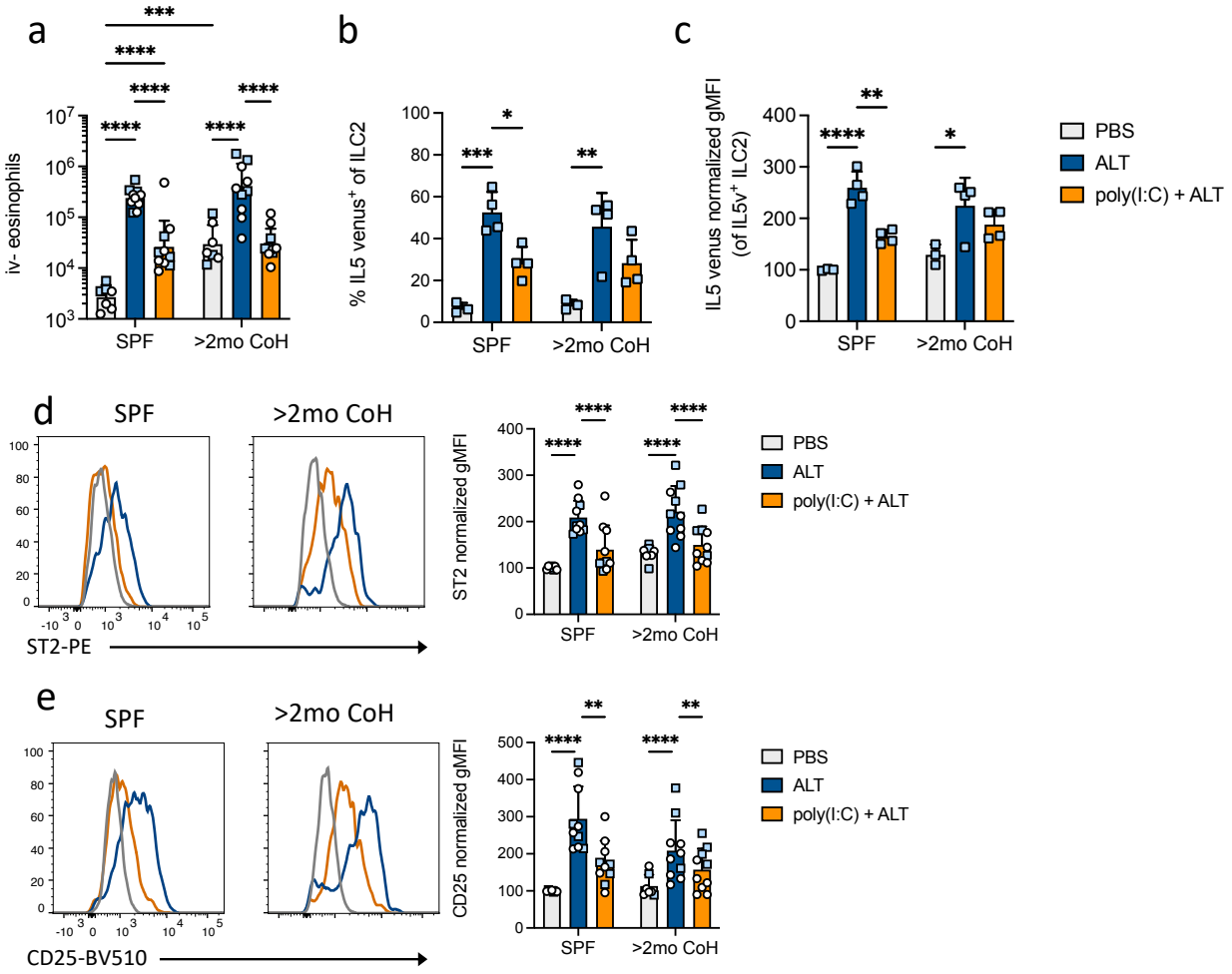
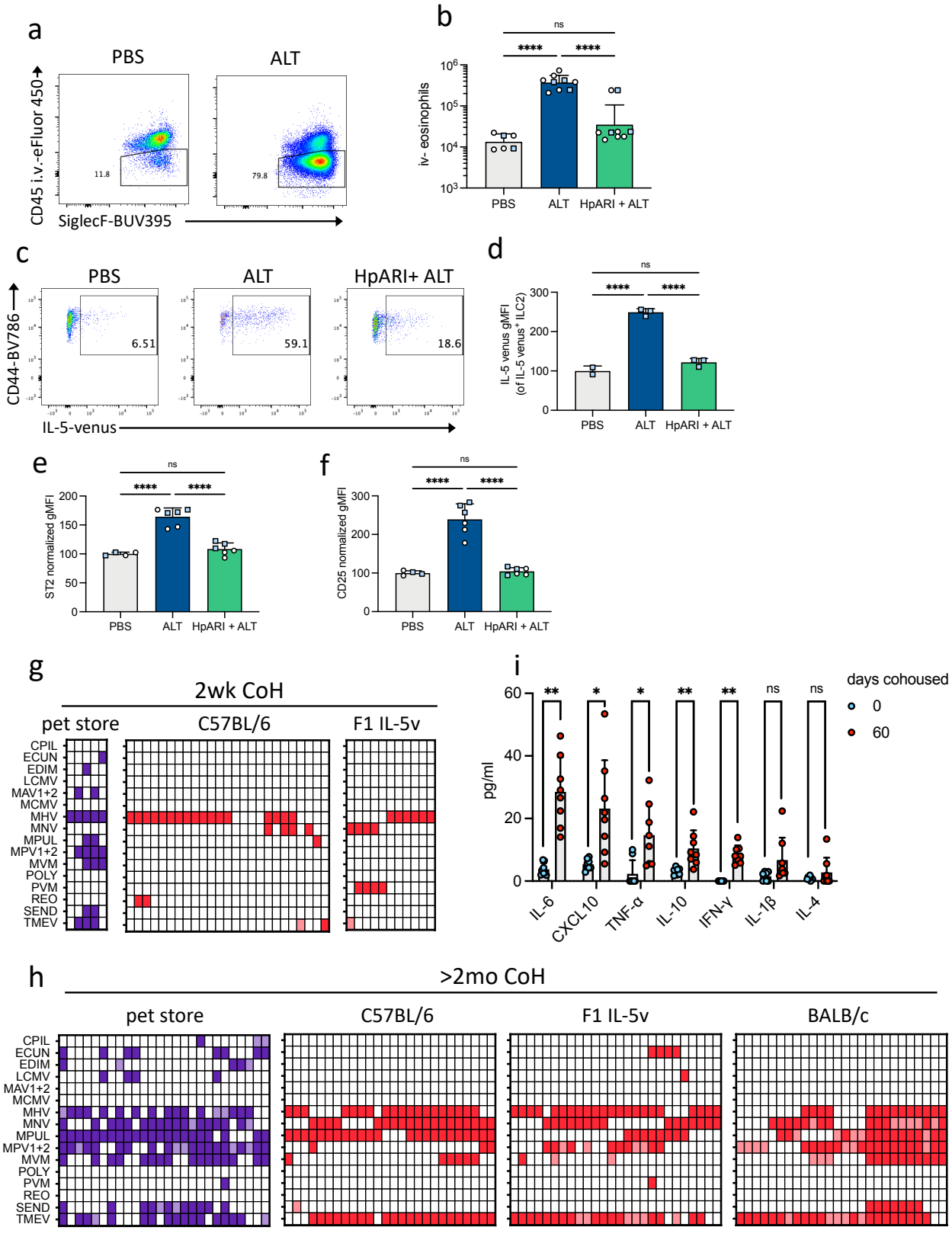


Fig 5. Poly(I:C) suppresses ILC2 and eosinophil responses to ALT in SPF and two-month cohoused mice. SPF or >2mo CoH B6 (white circles) or IL-5v F1 (light blue squares) mice were treated with intranasal poly(I:C) or left untreated 24 hours before intranasal PBS or ALT and analyzed by flow cytometry 24 hours later. **a**, Number of lung i.v. CD45^{-} eosinophils. Bar graph shows mean + SD of log transformed values. **b**, Percent of lung ILC2 expressing IL-5 venus. **c**, Normalized gMFI of IL-5 venus, gated on IL-5 venus^{+} lung ILC2. **d**, Representative histograms and summary data of ST2 expression on lung ILC2. **e**, Representative histograms and summary data of CD25 expression on lung ILC2. **a**, **d**-**e**, Pooled from two B6 and two IL-5v F1 experiments (*n* = 7-10 group). **b**-**c**, Pooled from two IL-5v F1 experiments (*n* = 3-4/group). **b**, **c**, **d**, **e**, Bar

669 graphs show mean + SD. *P* values were determined with two-way ANOVA with Tukey's multiple

670 comparisons test; no symbol $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.


a
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ALT
CD45 i.v.-eFluor 450
SiglecF-BUV395
11.8
79.8
b
ns
****
****
iv- eosinophils
PBS
ALT
HpARI + ALT
c
PBS
ALT
HpARI+ ALT
CD44-BV786
IL-5-venus
6.51
59.1
18.6
d
IL-5 venus gMFI (of IL-5 venus+ ILC2)
e
ST2 normalized gMFI
f
CD25 normalized gMFI
g
2wk CoH
pet store
C57BL/6
F1 IL-5v
CPIL
ECUN
EDIM
LCMV
MAV1+2
MCMV
MHV
MNV
MPUL
MPV1+2
MVM
POLY
PVM
REO
SEND
TMEV
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days cohoused
0
60
pg/ml
IL-6
CXCL10
TNF-α
IL-10
IFN-γ
IL-1β
IL-4
h
>2mo CoH
pet store
C57BL/6
F1 IL-5v
BALB/c

Fig S1. *A. alternata* response is susceptible to inhibition by microbial factors. a, Eosinophils ($CD45^+$ $SiglecF^+$ $CD11b^+$ $CD11c^-$ cells) were identified by being in the lung parenchyma or airways by being unlabeled by a fluorescently tagged anti-CD45 antibody that was injected intravenously three minutes before euthanasia. Shown are SPF C57BL/6 (B6) mice treated 24 hours prior with intranasal PBS or *A. alternata* (ALT). **b**, Number of eosinophils in the lungs and airways (negative for an intravascular CD45 antibody) 24 hours after intranasal treatment with PBS, ALT, or ALT co-administered with HpARI. Mice were B6 (white circles) or B6xBALB/c IL-$5^{WT/venus}$ (IL-5v F1, light blue squares). Bar graph shows mean + SD of log transformed values. **c**, Representative flow plots of IL-5 venus expression in IL-5v F1 lung ILC2. **d**, Normalized IL-5 venus gMFI of IL-5 $venus^+$ lung ILC2. **e**-**f**, Normalized ST2 (e) and CD25 (f) of B6 and IL-5v F1 mice. **b** and **e**-**f**, Pooled from one B6 and one IL-5v F1 experiment (*n* = 5-9/group). **d**, Data from one experiment in IL-5v F1 mice (*n* = 2-3/group). Bar graphs show mean + SD. *P* values were determined with one-way ANOVA with Tukey's multiple comparisons test; ns $p > 0.05$, **** $p < 0.0001$. **g**, **h**, Blood samples were collected and tested for antibodies against common murine pathogens. Each column represents an animal. Each row indicates a pathogen. Filled boxes indicate positive results, lighter shaded boxes indicate equivocal (weak positive) results. Empty boxes indicate negative results. **g**, Serology results from representative pet store, cohoused B6 and F1 IL-5v mice cohoused for approximately two weeks at the time of blood collection. **h**, Serology results from representative pet store and cohoused B6, F1 IL-5v, and BALB/c mice cohoused for at least two months at the time of blood collection (*n* = 26/group). CPIL = *Clostridium piliforme*, ECUN = *Encephalitozoon cuniculi*, EDIM = rotavirus, LCMV = lymphocytic choriomeningitis virus, MAV1+2 = mouse adenovirus 1 and 2, MCMV = murine cytomegalovirus,

694 MHV = mouse hepatitis virus, MNV = murine norovirus, MPUL = *Mycoplasma pulmonis*,
695 MPV1+2 = mouse parvovirus type 1 and type 2, MVM = minute virus of mice, POLY = polyoma
696 virus, PVM = pneumonia virus of mice, REO = reovirus, SEND = murine respirovirus (Sendai
697 virus), TCMV = GDVII Theiler's murine encephalomyelitis virus. **i**, Serum cytokine/chemokine
698 levels of B6 mice days 0 and 60 after cohousing with a pet store mouse, (n = 8). These are from
699 the same data in figure 1a. Bar graphs show mean + SD. *P* values were determined with a
700 Wilcoxon matched-pairs signed rank test; ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

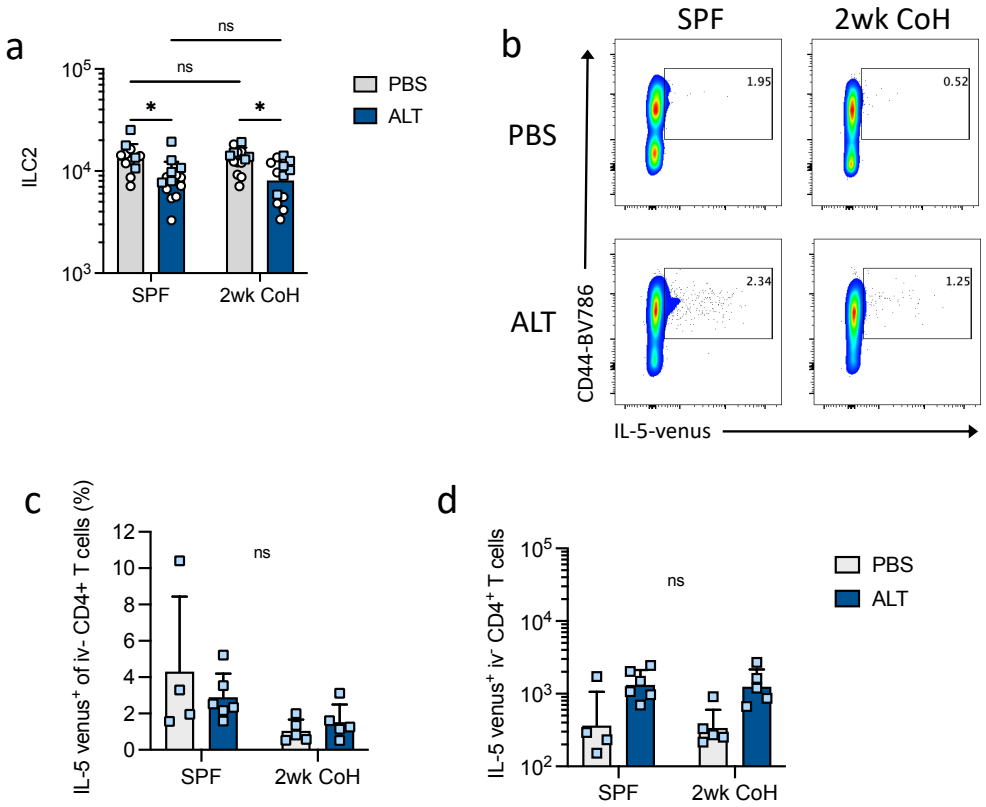
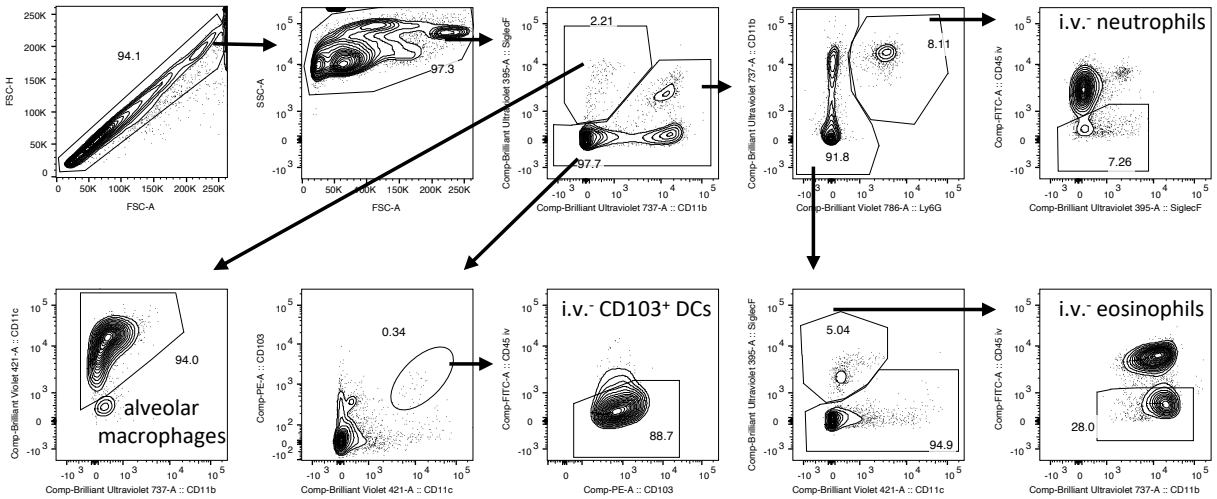


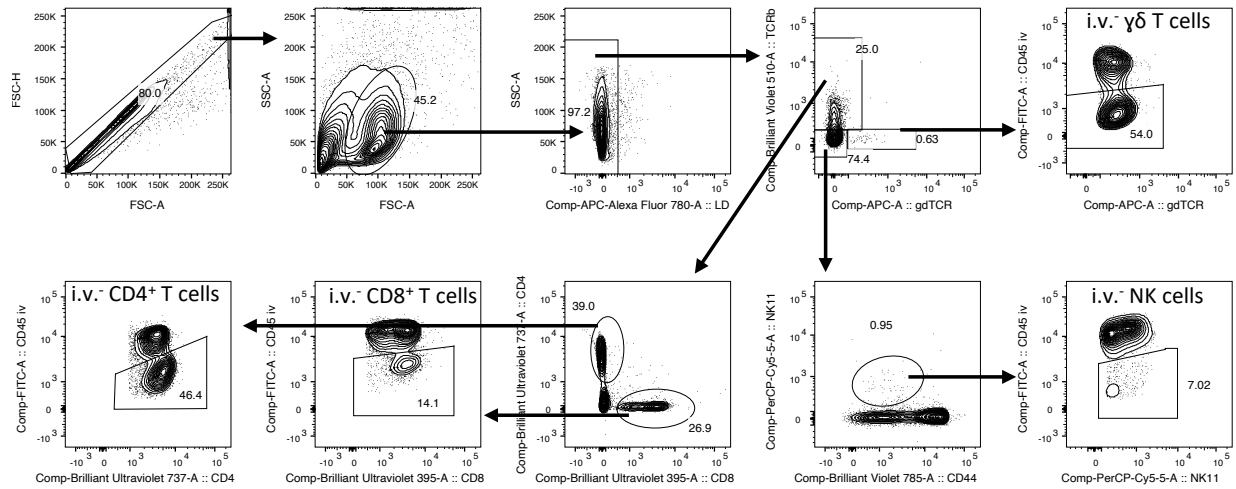
Fig S2. ILC2 numbers and CD4+ T cell data from two-week cohoused mice.

Flow analysis 24 hours after intranasal PBS or ALT treatment. Mice were B6 (white circles) or IL-5v F1 (light blue squares) and were cohoused with pet store mice for approximately two weeks or were age-matched SPF controls. **a**, Number of lung ILC2. **b**-**c**, IL-5 venus expression within lung (i.v. CD45$^-$) CD4$^+$ T cells of IL-5v F1 SPF and 2wk CoH mice 24 hours after PBS or ALT treatment. **b**, Representative flow plots of IL-5 venus expression in lung CD4$^+$ T cells. **c**, Percent IL-5 venus$^+$ and **g**, normalized IL-5 venus gMFI of IL-5 venus$^+$ lung CD4$^+$ T cells. **a**, Pooled from four B6 experiments and two IL-5v F1 experiments, *n* = 12-14 per group. **b**-**d**, Pooled from two IL-5v F1 experiments, *n* = 4-6 per group. Bar graphs show mean + SD. *P* values were determined with a two-way ANOVA with Tukey's multiple comparisons test; ns $p > 0.05$, * $p < 0.05$.

a Gating neutrophils, CD103+ DCs, eosinophils, alveolar macrophages (untreated cohoused mouse)



b Gating γδ T cells, CD4⁺ T cells, CD8⁺ T cells, NK cells (untreated cohoused mouse)



c Gating ILC2, B cells (untreated cohoused mouse), first gated on singlets, lymphocytes, live cells

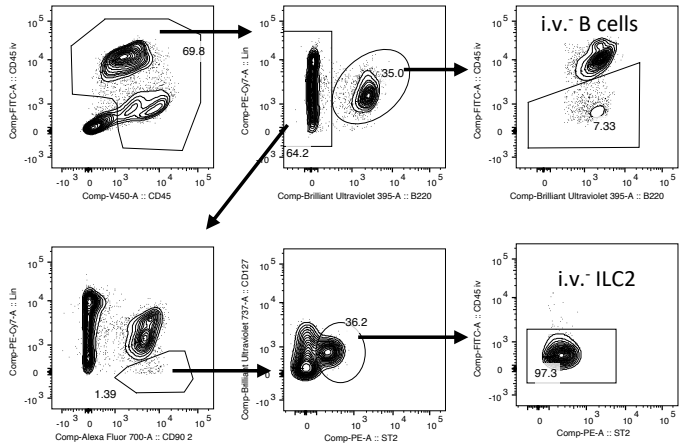


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Fig S3. Gating strategy for flow cytometry. Example flow cytometry gating used to identify (**a**) alveolar macrophages and intravenous negative neutrophils, CD103+ dendritic cells, eosinophils, (**b**) γδ T cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, (**c**) ILC2 and B cells. The example plots are from >2mo CoH B6 untreated mice as in Figure 2.

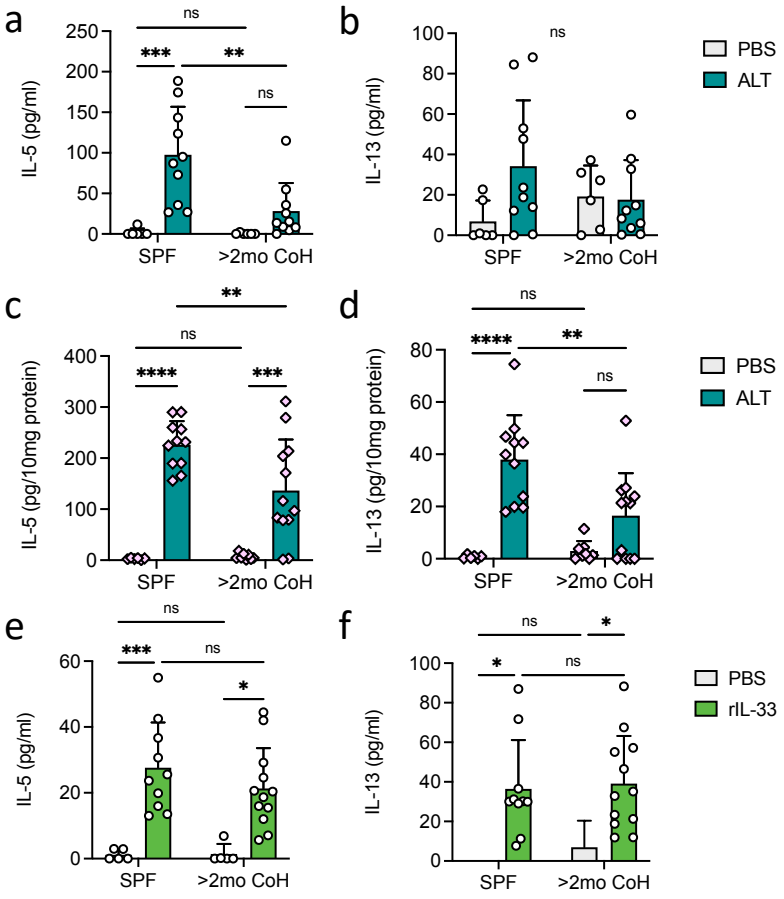

Fig S4. BALF and lung cytokine measurements.

a-**b**, **e**-**f**, B6 mice (white circles) or **c**-**d**, BALB/c mice (pink diamonds) were cohoused with pet store mice for at least two months (>2mo CoH) or were age-matched SPF controls. ALT (**a**-**d**), recombinant IL-33 (rIL-33, **e**-**f**) or control PBS were given intranasally to mice and lungs and bronchoalveolar lavage fluid (BALF) were collected 4.5 hours later. IL-5 and IL-13 in the lung homogenates (**c**-**d**) and BALF (**a**-**b**, **e**-**f**) were detected by ELISA and lung concentrations were normalized to the amount of total protein in the samples. Bar graphs show mean + SD. *P* values were determined with a 2-way ANOVA with Tukey's multiple comparisons test; ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.