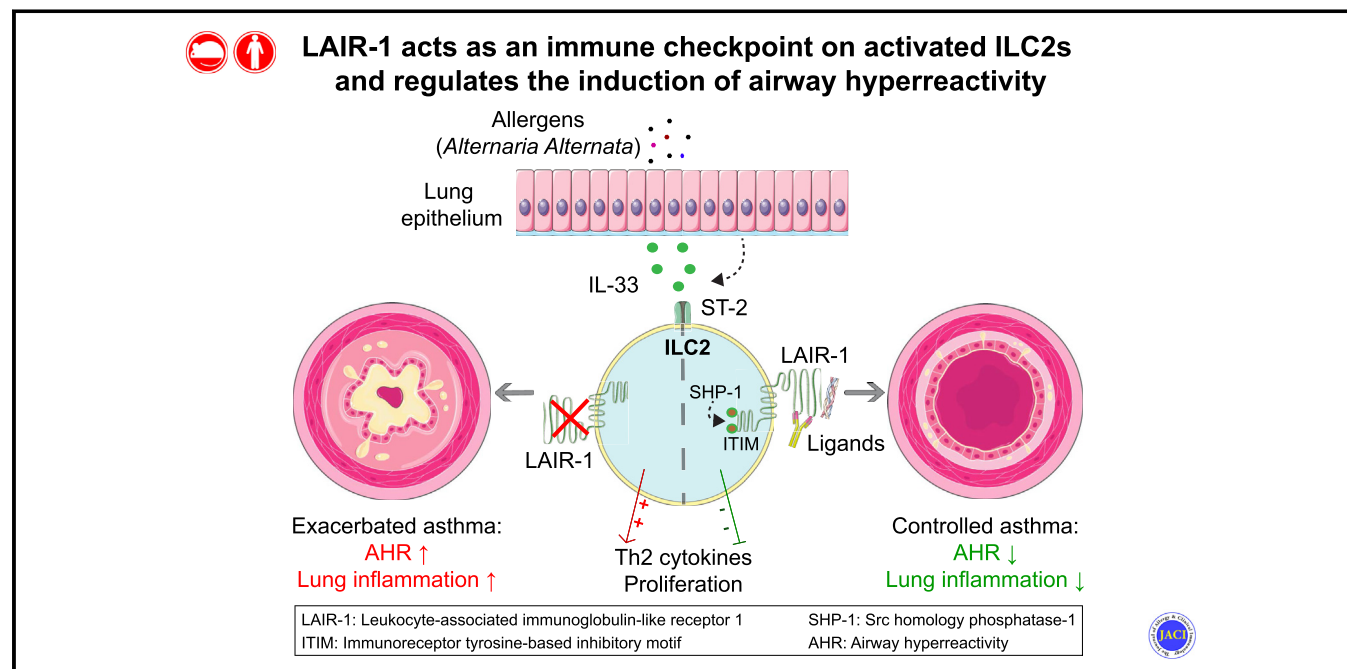


LAIR-1 acts as an immune checkpoint on activated ILC2s and regulates the induction of airway hyperreactivity

Doumet Georges Helou, PhD, Pedram Shafiei-Jahani, MS, Benjamin P. Hurrell, PhD, Jacob D. Painter, MS, Christine Quach, MS, Emily Howard, BS, and Omid Akbari, PhD *Los Angeles, Calif*

GRAPHICAL ABSTRACT



Background: Type 2 innate lymphoid cells (ILC2s) are relevant players in type 2 asthma. They initiate eosinophil infiltration and airway hyperreactivity (AHR) through cytokine secretion. Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) is an inhibitory receptor considered to be an immune checkpoint in different inflammatory diseases.

Objective: Our aim here was to investigate the expression of LAIR-1 and assess its role in human and murine ILC2s.

Methods: Wild-type and LAIR-1 knockout mice were intranasally challenged with IL-33, and pulmonary ILC2s were sorted to perform an *ex vivo* comparative study based on RNA sequencing and flow cytometry. We next studied the impact of LAIR-1 deficiency on AHR and lung inflammation by using knockout mice and adoptive transfer experiments in *Rag2*^{-/-} *IL2rg*^{-/-} mice. Knockdown antisense strategies and humanized mice were used to assess the role of LAIR-1 in human ILC2s.

Results: We have demonstrated that LAIR-1 is inducible on activated ILC2s and downregulates cytokine secretion and effector function. LAIR-1 signaling in ILC2s was mediated via inhibitory pathways, including SHP1/PI3K/AKT, and LAIR-1 deficiency led to exacerbated ILC2-dependent AHR in IL-33 and *Alternaria alternata* models. In adoptive transfer experiments, we confirmed the LAIR-1-mediated regulation of ILC2s *in vivo*. Interestingly, LAIR-1 was expressed and inducible in human ILC2s, and knockdown approaches of *Lair1* resulted in higher cytokine production. Finally, engagement of LAIR-1 by physiologic ligand C1q significantly reduced ILC2-dependent AHR in a humanized ILC2 murine model.

Conclusion: Our results unravel a novel regulatory axis in ILC2s with the capacity to reduce allergic AHR and lung inflammation. (J Allergy Clin Immunol 2021;■■■■:■■■■-■■■■.)

From the Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles.

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Corresponding author: Omid Akbari, PhD, University of Southern California, Keck School of Medicine, Department of Molecular Microbiology and Immunology, NRT 5505, 1450 Biggy St, Los Angeles, CA 90033-9605. E-mail: akbari@usc.edu. 0091-6749/\$36.00

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Type 2 innate lymphoid cells (ILC2s) are newly discovered tissue-resident immune cells belonging to the family of non-cytotoxic ILCs. Unlike B and T cells, ILC2s lack the expression of lineage (Lin) markers and specific antigen receptors.^{1,2} Mainly residing in adventitial cuffs within collagen-rich regions, ILC2s are in tight interaction with resident structural cells that release nonspecific alarmins, including IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), to trigger immune activation under stress conditions.³⁻⁶ In particular, necrosis and active necroptosis are the principal mechanisms for the IL-33 secretion that is associated with increased pulmonary epithelial permeability.^{7,8} Pulmonary ILC2s are associated with adventitial stromal cells, which are known as a fibroblast-like subset implicated in alarmin secretion and matrix remodeling.⁴ Because of their location, ILC2s are among the first immune responders to these endogenous danger signals, promoting their activation and proliferation. Activated ILC2s mimic T_H2 cells in secreting copious amounts of classical T_H2 effector cytokines, including IL-5, IL-9, and IL-13.^{2,9} During the past decade, numerous studies have documented the role of ILC2s in different diseases, such as type 2 diabetes mellitus,^{10,11} chronic rhinosinusitis,¹² and liver fibrosis,¹³ but mainly in allergic asthma type 2 inflammation.¹⁴⁻¹⁶

Allergic asthma is the most frequent type of asthma characterized by airway hyperreactivity (AHR) and T_H2 cell-mediated immune responses. Exposure to exogenous stimuli, including inhaled allergens, triggers specific symptoms ranging from chest pain, cough, and wheezing to acute bronchoconstriction in life-threatening attacks.^{17,18} The inflammatory cascade is initiated with alarmin secretion in the lungs, which instructs a type 2 response associated with massive recruitment of eosinophils in response to IL-5.^{19,20} Representing key upstream players, ILC2s have become a potential therapeutic target in asthma.²¹ Novel strategies are considering the modulation of inhibitory and costimulatory receptors defining activated ILC2s, such as CD200R,²² TIGIT,²³ TNFR2,²⁴ ICOS, and ICOS-L.²⁵ In this context, we have recently demonstrated the therapeutic potential of a novel programmed cell death protein 1 (PD-1) agonist in allergic asthma through the downregulation of ILC2s.²⁶ Other inhibitory receptors, such as the leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1), are yet to be explored in ILC2s.

LAIR-1, also known as CD305, was first discovered in 1997 and described as a novel inhibitory receptor having a single immunoglobulin-like domain and a cytoplasmic tail equipped with 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs).²⁷ LAIR-1 is broadly expressed and inhibits the activation of different immune populations, including B and T lymphocytes, natural killer cells, dendritic cells (DCs), and monocytes.²⁷⁻³¹ It has been reported that LAIR-1 binds to collagens with high affinity through the conserved sequence of Gly-Pro-Hyp collagen repeats³² and a single residue, arginine 65.³³ Moreover, collagen has been reported to induce and enhance the expression of LAIR-1.³⁴ The first component of complement C1q also contains a collagen-like region and is therefore considered as a functional ligand for LAIR-1 among other immune receptors.³⁵⁻³⁷ Because LAIR-1 is an ITIM-containing receptor,

Abbreviations used

AHR:	Airway hyperreactivity
AKT:	Protein kinase B
APC:	Allophycocyanin
BAL:	Bronchoalveolar lavage
Cy7:	Cyanine 7
DC:	Dendritic cell
FITC:	Fluorescein isothiocyanate
ILC2:	Type 2 innate lymphoid cell
ITIM:	Immunoreceptor tyrosine-based inhibitory motif
KO:	Knockout
LAIR-1:	Leukocyte-associated Ig-like receptor-1
Lin:	Lineage
PD-1:	Programmed cell death protein 1
PE:	Phycoerythrin
rh:	Recombinant human
rm:	Recombinant mouse
RNAseq:	RNA sequencing
SHP-1:	Src homology phosphatase-1
TCR:	T-cell receptor
USC:	University of Southern California
WT:	Wild-type

its engagement mainly leads to Src homology phosphatase-1 (SHP-1) recruitment, promoting the transduction of negative signaling.^{27,30,38} To date, studies have demonstrated a critical role for LAIR-1 in the immune imbalance defining autoimmune diseases and cancers. LAIR-1 cross-linking limits rheumatoid arthritis³⁴ and SLE progression,³¹ whereas it decreases immune cell cytotoxicity and improves oncogenic resistance in cancer.^{39,40}

In the present study, we have highlighted for the first time 2 major findings. First, we demonstrated that LAIR-1 is inducible on pulmonary ILC2s and controls their activation mainly through signal transduction downstream of Src homology phosphatase-1 (SHP-1). Second, we used the knockout (KO) mouse to assess the protective anti-inflammatory role of LAIR-1 in ILC2-dependent AHR. In a translational approach, we also demonstrated a major role for LAIR-1 in the regulation of human ILC2s both *in vitro* and *in vivo* by utilizing a humanized mouse model. Therefore, our data clearly introduced LAIR-1 as an inhibitory receptor in ILC2s that could serve as a potential therapeutic target to treat ILC2-related disorders, including asthma and allergic diseases.

METHODS

Mouse experiments

All mice were bred in the animal facility of the Keck School of Medicine, University of Southern California (USC). The experimentation protocols were approved by the USC Institutional Animal Care and Use Committee and implemented in accordance with the principles of the Declaration of Helsinki. Wild-type (WT) C57BL/6, *Lair1* KO (LAIR-1 KO), and *Rag2/Il2rg* double-KO (*Rag2*^{-/-}*Il2rg*^{-/-}) mice were purchased from Jackson Laboratory (Bar Harbor, Me). The experiments were performed with 6- to 8-week-old age- and sex-matched mice.

Tissue digestion and pulmonary ILC2 sorting

Mice were intranasally challenged on 3 consecutive days with 0.5 or 1 µg of carrier-free recombinant mouse (rm) IL-33 (rmIL-33) (ThermoFisher,

Waltham, Mass) or PBS. On day 4, lungs were collected and processed as described previously.^{26,41} Briefly, lung tissue was cut into small pieces and incubated in type IV collagenase (400 U/mL [Worthington Biochemicals, Lakewood, NJ]) at 37°C for 60 minutes. To obtain a single-cell suspension, the lung tissue digest was passed through a 70- μ m cell strainer (Falcon, Glendale, Ariz). In some experiments, blood, bone marrow, ear skin, and visceral adipose tissue samples were collected and prepared for flow cytometry, as previously described.^{11,42} The following antibodies were used to identify and sort pulmonary ILC2s: allophycocyanin (APC)-cyanine 7 (Cy7) anti-mouse CD45 (clone 30-F11) and phycoerythrin (PE)-Cy7 anti-mouse CD127 (clone A7R34) (both from BioLegend, San Diego, Calif); PerCP-eFluor710 anti-mouse ST2 (clone RMST2-2 [eBioscience, San Diego, Calif]), PE anti-mouse LAIR-1 (clone 113 [ThermoFisher Scientific]), and fluorescein isothiocyanate (FITC) anti-mouse Lin cocktail (including T-cell receptor [TCR]- $\gamma\delta$ [clone eBioGL3 [eBioscience]], TCR- β [clone H57-597], CD3e [clone 145-2C11], CD45R [clone RA3-6B2], Gr-1 [clone RB6-8C5], CD11c [clone N418], CD11b [clone M1/70], Ter119 [clone TER-119], Fc ϵ R1 [clone MAR-1], CD5 [clone 53-7.3], and NKp46 [clone 29A1.4] [all from BioLegend]). APC anti-mouse KLRG1 (clone 2F1/KLRG1 [BioLegend]), PE anti-mouse MAIR-V (clone TX70 [BioLegend]), APC anti-mouse CD200R (clone OX110 [Invitrogen, Waltham, Mass]), and APC anti-mouse PD-L1 (clone 10F.9G2 [BioLegend]) were added for immunophenotyping experiments. When indicated, BV421 anti-mouse CD135 (Flt3; clone A2F10), APC anti-mouse LPAM-1 (integrin $\alpha 4\beta 7$; clone DATK32) and BV510 anti-mouse CD25 (clone PC61) (all from BioLegend) were used to identify ILC2 precursors in the bone marrow. To exclude dead cells, fluorescent live/dead fixable stains (ThermoFisher) were used in all experiments according to manufacturer's instructions. Pulmonary ILC2s were sorted on a FACSARIA III system (Becton Dickinson, Franklin Lakes, NJ). The sort purity was always 95% or higher, and the percentage of LAIR-1⁺ ILC2s from WT mice was approximately 25% to 35%. Cells were cultured in RPMI medium (Gibco, Waltham, Mass) supplemented with 10% heat-inactivated FBS and 100 U/mL of penicillin-streptomycin (GenClone, San Diego, Calif).

RNAseq

The sorted ILC2s (5×10^4 /mL) were incubated with rmIL-2 (10 ng/mL) and rmIL-7 (10 ng/mL) for 24 hours. Total RNA was isolated by using a Micro-RNAeasy kit (Qiagen, Hilden, Germany). In total, 10 ng of input RNA was used to produce cDNA for downstream library preparation. Samples were sequenced on a NextSeq 500 system (Illumina, San Diego, Calif). Raw reads were aligned, normalized, and further analyzed by using Partek Genomics Suite software (version 7.0, Partek Inc). Normalized read counts were tested for differential expression by using Partek's gene-specific analysis algorithm. RNA sequencing (RNAseq) data supporting the findings of this study have been deposited in Genbank with the primary accession code GSE171819.

Intracellular staining and cytokine quantification

ILC2s were cultured in the presence of rmIL-2 (10 ng/mL) and rmIL-7 (10 ng/mL). When indicated, ILC2s were incubated with purified mouse C1q (10 μ g/mL [Complement Technology, Tyler, Tex]) or the SHP-1 inhibitor tyrosine phosphatase inhibitor-1 (15 μ M [MedChemExpress, Monmouth Junction, NJ]).^{39,43} A Cytofix/Cytoperm kit (BD Biosciences) was used and followed by intracellular staining with PE anti-p65 (clone 532301 [R&D Systems]), BV421 anti-protein kinase B AKT (anti-AKT) (pS473) (clone M89-61 [BD Biosciences]), PE-Cy7 anti-BCL-2 (clone 10C4 [eBioscience]), and unconjugated anti-SHP-1 (clone SR41-02 [ThermoFisher]) followed by Alexa Fluor 647 goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove, Pa). Intracellular staining was performed using the Foxp3 Transcription Factor Staining Kit (ThermoFisher) according to the manufacturer's instructions, and APC anti-mouse Ki67 (SolA15 [ThermoFisher]) was used to assess proliferation. In some experiments, the CellTrace Violet Cell Proliferation Kit (ThermoFisher) was also used according to the manufacturer's instructions. In parallel, the levels of IL-5, IL-6, and IL-13 were measured in supernatants by using Legendplex multiplex kits (BioLegend), and the data were analyzed via LEGENDplex data analysis software (version 8.0). GM-CSF was quantified by using an ELISA kit from BioLegend.

Induction and assessment of AHR

Mice were intranasally challenged for 3 days with mouse rmIL-33 (0.5 μ g per mouse in 50 μ L [BioLegend]) to induce AHR or for 5 consecutive days with 100 μ g of *Alternaria alternata* extracts (Greer Laboratories). Control mice were challenged with PBS, and lung function was evaluated on the day following the last challenge. In some experiments, AHR was induced in *Rag2*^{-/-}*Il2rg*^{-/-} mice after an intravenous adoptive transfer of total pulmonary ILC2s (50×10^3) sorted from IL-33-challenged WT and LAIR-1 KO mice. Lung function was assessed by direct measurement of lung resistance and dynamic compliance in restrained, tracheostomized, and mechanically ventilated mice by using the FinePointe RC system (Buxco Research Systems, Harvard, Mass) under general anesthesia. Mice were sequentially challenged with aerosolized PBS (baseline), followed by increasing doses of methacholine ranging from 5 to 40 mg/mL. Maximum lung resistance and minimum compliance values were recorded during a 3-minute period after each methacholine challenge. AHR data were analyzed by repeated measurements of a general linear model.

Assessment of lung inflammation

To collect the bronchoalveolar lavage (BAL) fluid, lungs were washed with 3 mL of ice-cold PBS. BAL supernatant was collected to quantify cytokines by using Legendplex multiplex kits (BioLegend). Principal immune populations were identified in the BAL fluid by using the following antibodies: PE-Cy7 anti-mouse CD45 (clone 30-F11 [BioLegend]), APC-Cy7 anti-mouse CD11c (clone N418 [BioLegend]), PE anti-mouse Siglec-F (clone E50-2440 [BD Biosciences]), BV421 anti-mouse CD11b (clone M1/70 [BD Biosciences]), APC anti-mouse Gr-1 (clone RB6-8C5 [BioLegend]), FITC anti-mouse CD19 (clone 6D5 [BioLegend]), and PerCP-Cy5.5 anti-mouse CD3 (clone 17A2 [BioLegend]). Lung tissue was processed and ILC2s were identified as already described, whereas the expression of C1q in CD11c⁺ cells was assessed by using APC-Cy7 anti-mouse CD11c (clone N418 [BioLegend]) and FITC anti-C1q (clone JL-1 [Invitrogen]). When indicated, intracellular staining was performed to evaluate cytokine production by using APC anti-mouse/anti-human IL-5 (clone TRFK5 [BioLegend]) and PE anti-mouse IL-13 (clone eBio13A [eBioscience]) after 4 hours of *in vitro* incubation with 50 μ g/mL of PMA (Sigma-Aldrich, St Louis, Mo), 500 μ g/mL of ionomycin (Sigma-Aldrich), and 1 μ g/mL of Golgi plug. To exclude dead cells, fluorescent live/dead fixable stains (ThermoFisher Scientific) were used according to the manufacturer's instructions. CountBright Absolute Count Beads were used to count lung and BAL fluid immune cells (Invitrogen). Acquisition was performed on a BD FACSCanto II cell analyzer (BD Biosciences) by using BD FACSDiva software (version 8.0.1). Data were analyzed with FlowJo software (version 10 [TreeStar]). In some experiments, 1 lobe per lung was collected for histology and stored in paraformaldehyde 4% buffered in PBS. The lungs were embedded in paraffin and cut into 4- μ m sections for hematoxylin and eosin staining or trichrome staining. Histology images were acquired on a Leica DME microscope and Leica ICC50HD camera (Leica, Wetzlar, Germany), and ImageJ was used for analysis.

Culture of human ILC2s and adoptive transfer

All human studies were approved by the USC institutional review board and conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all human participants. Human peripheral blood ILC2s were isolated from total PBMCs as previously described.²⁶ Briefly, human fresh blood was first diluted 1:1 in PBS and transferred to SepMateTM-50 separation tubes (Stemcell Technologies, Vancouver, British Columbia, Canada) prefilled with 15 mL of Lymphoprep (Axis-Shield). Samples were centrifuged at 1200 g for 10 minutes to collect PBMCs. A CRTH2 MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was then used according to the manufacturer's conditions to isolate CRTH2⁺ cells. The following antibodies were used to identify and sort human ILC2s: FITC anti-Lin, FITC anti-CD235a (clone HI264), FITC anti-Fc ϵ R1a (clone AER-37), FITC anti-CD1a (clone HI149), FITC anti-CD123 (clone 6H6), FITC anti-CD5 (clone L17F12), APC-Cy7 anti-CD45 (clone HI30), PE anti-CD294 (CRTH2) (clone BM16), and PE-Cy7 anti-CD127 (clone A019D5).

(all from BioLegend). The purified ILC2s were cultured in complete RPMI medium with recombinant human (rh) IL-2 (rhIL-2) (20 ng/mL), rhIL-7 (20 ng/mL), and rhIL-33 (20 ng/mL) for 3 days. For knockdown experiments, 5 μ M *Lair-1* antisense morpholino oligonucleotides AGACATGGCCC AGGTCCCAGCAGT or random control oligonucleotides (Gene Tools, LLC, Philomath, Ore) were added to the culture as free uptake oligonucleotides at hour 0 and hour 36. When indicated, LAIR-1 expression was assessed on freshly isolated or cultured ILC2s by using Alexa Fluor 647 anti-human LAIR-1 (clone NKTA255 [BioLegend]). Intracellular staining with eFluor450 anti-human Ki67 (clone 20Raj1 [eBioscience]) was performed to assess proliferation. Human IL-5, IL-6, and IL-13 were quantified in supernatants by using Legendplex multiplex kits from BioLegend.

For the adoptive transfer experiments, *in vitro*-cultured ILC2s (50×10^3) were intravenously injected into *Rag2*^{-/-}*Il2rg*^{-/-} mice. To induce AHR, mice were intranasally challenged with 1 μ g of rhIL-33 for 3 consecutive days. On days 4 and 5, the mice received 50 μ g of purified human C1q (Complement Technology) or PBS via the intraperitoneal route as described before.⁴⁴ The intraperitoneal route was chosen because C1q is a high-molecular-weight protein (410–462 kDa) and it therefore has a limited diffusion via the intranasal route.^{45,46} On day 6, lung function was measured before BAL and lung collection for flow cytometry analysis.

Statistical analysis

Data are representative of at least 2 independent experiments and are presented as means $\pm$ SEMs (except for RNAseq data). The 2-tailed Student *t* test was applied for comparisons between 2 groups. For multi-group comparisons, we used 1-way ANOVA with the Tukey *post hoc* test. Data were analyzed with Prism Software (GraphPad Software Inc). Error bars represent SEMs. *P* values less than .05 were considered to denote statistical significance (**P* < .05; ***P* < .01; ****P* < .001).

RESULTS

LAIR-1 is inducible on pulmonary ILC2s

LAIR-1 is broadly and constitutively expressed on different immune populations, including monocytes, natural killer cells, and B and/or T cells.⁴⁷ To elucidate the expression of LAIR-1 on ILC2s, C57BL/6 WT mice were challenged for 3 consecutive days with rmIL-33 or with PBS and lungs were collected at day 4 (Fig 1, A). Pulmonary ILC2 expression was assessed by utilizing flow cytometry by gating on CD45⁺Lin⁻ST2⁺CD127⁺ cells as described before²⁶ (Fig 1, B). LAIR-1 was not expressed on naive pulmonary ILC2s, whereas intranasal stimulation with IL-33 induced a LAIR-1⁺ ILC2 population (Fig 1, C and D and see Fig E1, A in the Online Repository at www.jacionline.org).⁴⁸ Similarly, LAIR-1 was not expressed on skin and adipose tissue-resident ILC2s at the basal state; however, it was weakly expressed on bone marrow and blood ILC2s (see Fig E1, B–H). To explore the particularities of this induced subset, we sorted both LAIR-1⁻ and LAIR-1⁺ ILC2s and established a differential expression profiling by using RNAseq analysis. LAIR-1⁺ ILC2s presented a different transcriptional profile, as revealed by principal component analysis (Fig 1, E). This analysis provides unperturbed information on the clustering of LAIR-1⁻ and LAIR-1⁺ ILC2 samples based on the highest variability in the overall data set. LAIR-1 expression was associated with upregulation of 950 genes and downregulation of 875 genes (Fig 1, F). In particular, genes related to inflammation and ILC2 activation, such as *Il13*, *Il9*, and *Nfkb*, were downregulated in LAIR-1⁺ ILC2s. Along with *Lair1* gene induction, a battery of genes related to tissue remodeling, such as *Loxl2*, *Coll8a1*, *Cd9*, and *Ecm1*, were among the most upregulated (Fig 1, G and see Fig E1, I). In parallel, the

trichrome histologic staining suggested lung tissue remodeling and collagen deposition following repeated IL-33 challenge (see Fig E2, A in the Online Repository at www.jacionline.org). C1q was reported as a potential natural ligand that engages LAIR-1 through a noncomplement mechanism.³⁶ Furthermore, both flow cytometry and transcriptional data suggested that C1q is mainly and constitutively expressed by antigen-presenting cells (DCs and macrophages) in mouse and human lungs (Fig E2, B–E⁴⁹). To confirm the regulatory role of the LAIR-1 axis, we treated both ILC2 subsets with C1q or PBS and assessed cytokine production (Fig 1, H). In line with the transcriptional data, LAIR-1⁺ ILC2s secreted less IL-5 and IL-13 *in vitro*. In addition, C1q significantly decreased IL-5 and IL-13 production in LAIR-1⁺ ILC2s but not in LAIR-1⁻ ILC2s (Fig 1, I and J). Altogether, our data indicate that LAIR-1 is inducible on ILC2s, is functional, and participates in ILC2 downregulation.

Lack of LAIR-1 on pulmonary ILC2 increases effector function and T_H2 cytokine secretion

To better assess the implication of LAIR-1 on ILC2s, we introduced the previously characterized LAIR-1 KO mouse,⁵⁰ which has a targeted deletion of the *Lair1* gene. WT and LAIR-1 KO mice were intranasally challenged with rmIL-33 for 3 consecutive days to compare the transcriptional profile of fluorescence-activated cell sorting–sorted ILC2s (Fig 2, A). The absence of LAIR-1 resulted in 1181 significantly modulated genes (counts of <20 [*P* $\leq$.05]) displayed as a heatmap for differential gene expression (Fig 2, B). Interestingly, 326 genes were consistently and commonly modulated in our 2 transcriptional analyses: (1) LAIR-1⁻ vs LAIR-1⁺ ILC2s from WT mice and (2) LAIR-1 KO vs WT ILC2s. Relevant inflammatory genes, including *Il13*, *Il9*, *Tnfrsf8*, and *Stat5b* were upregulated in LAIR-1⁻ and LAIR-1 KO ILC2s, whereas mainly antioxidant and anti-inflammatory genes, such as *Gclm*, *Prdx6*, *Nqo1*, *Dok2*, and *CD200r1* were consistently downregulated in comparison with LAIR-1⁺ and WT ILC2s (Fig 2, C). This further highlights a central anti-inflammatory role for LAIR-1 in activated ILC2s. To better explore the LAIR-1 mechanism of action, we performed pathway analysis using gene-level statistics to compute pathway-level scores. Interestingly, the PI3K/AKT signaling, T_H2 cell, and NF- κ B activation pathways were among the highly affected pathways (Fig 2, D). As ILC2s functionally mimic T_H2 cells, we first compared the expression of cytokine-related genes in activated WT and LAIR-1 KO ILC2s. A series of genes encoding T_H2 cytokines, including *Il5*, *Il13*, *Il9*, and *Csf2*, were significantly upregulated in the absence of LAIR-1 (Fig 2, E). At the protein level, ILC2s from LAIR-1 KO mice exhibited higher production of IL-5, IL-13, IL-6, and GM-CSF (Fig 2, F–I), confirming the transcriptional data. Taken together, these results indicate that the LAIR-1 axis can negatively regulate relevant inflammatory pathways and dampen cytokine production in activated ILC2s.

LAIR-1 mediates regulation of ILC2s utilizing SHP-1/PI3K/AKT signaling circuits

The PI3K/AKT pathway is a principal regulator of cell activation, differentiation, proliferation, and survival.⁵¹ According to our pathway analysis, PI3K/AKT was one of the mainly affected pathways in activated LAIR-1–deficient ILC2s.

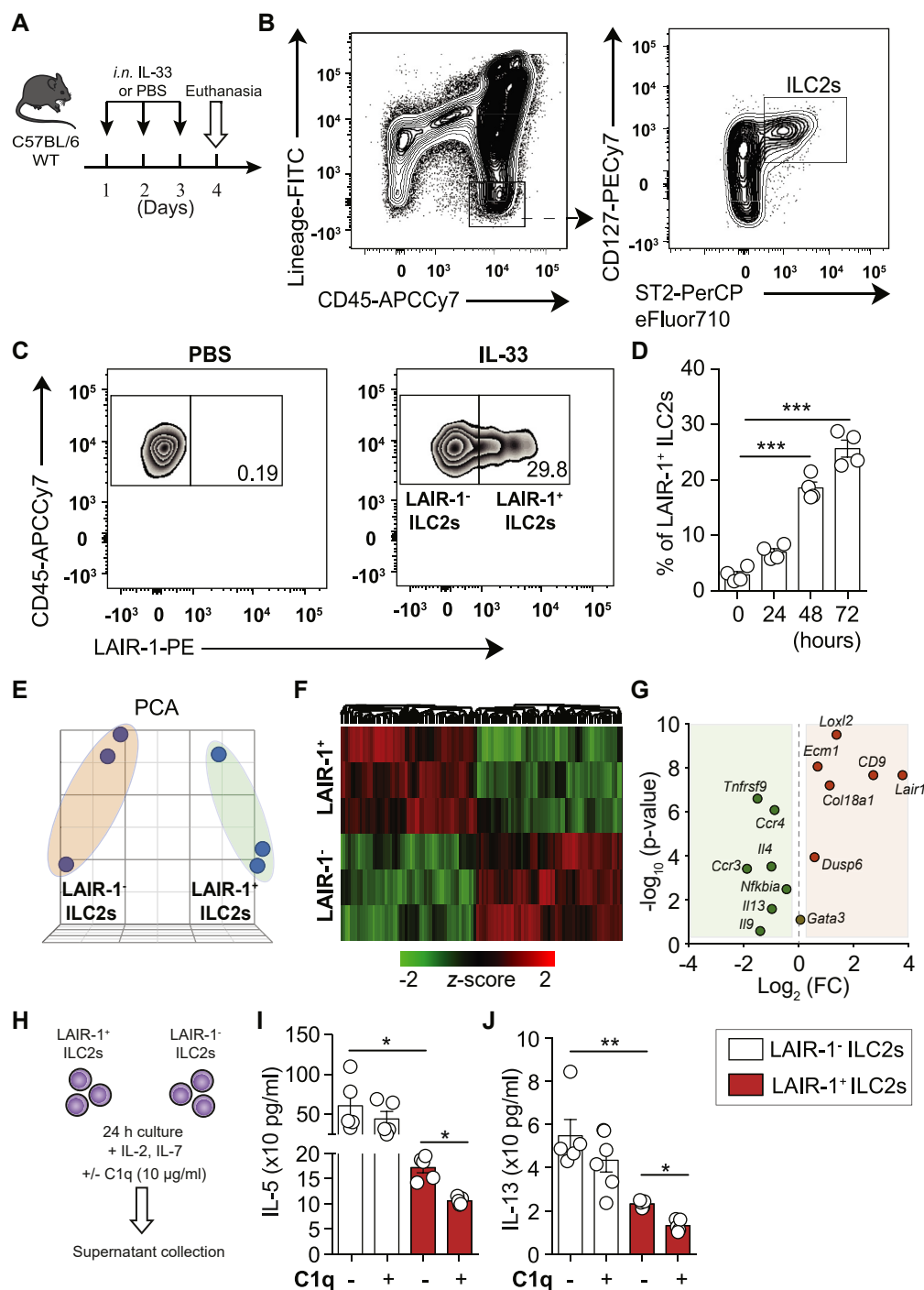


FIG 1. IL-33 induces an LAIR-1⁺ population in pulmonary ILC2s. **A-J**, C57BL/6 WT mice were intranasally (i.n.) challenged for 3 consecutive days with 0.5 µg of rIL-33 or with PBS, and lungs were collected on day 4. **B**, Pulmonary ILC2s identified as CD45⁺Lin⁻CD127⁺ST2⁺ cells. **C**, Representative flow cytometry plots showing LAIR-1 expression on ILC2s from PBS- and IL-33-challenged mice. **D**, LAIR-1 expression kinetics on ILC2s in response to *in vivo* IL-33 challenges, represented as percentages of LAIR-1⁺ ILC2s. **E-J**, LAIR-1⁻ and LAIR-1⁺ ILC2s were sorted from IL-33-challenged mice and incubated with rIL-2 (10 ng/mL) and rIL-7 (10 ng/mL) for 24 hours. **E**, Principal component analysis (PCA) plot showing the clustering of LAIR-1⁻ and LAIR-1⁺ ILC2 samples based on uncorrelated variables. **F**, Heatmap representation of total differentially regulated genes, generated with a gene-specific analysis algorithm (counts of <20 [*P* ≤ .05]; *n* = 3). **G**, Focused volcano plot representing a list of relevant and significantly regulated genes in LAIR-1⁺ ILC2s as compared with in LAIR-1⁻ ILC2s. **H-J**, LAIR-1⁻ and LAIR-1⁺ ILC2s were cultured with or without C1q at a concentration of 10 µg/mL, and supernatants were collected after 24 hours. **I** and **J**, Levels of IL-5 (**I**) and IL-13 (**J**) quantified by using the LEGENDplex immunoassay. Data are presented as means ± SEMs (1-way ANOVA); **P* < .05; ***P* < .01; and ****P* < .001.

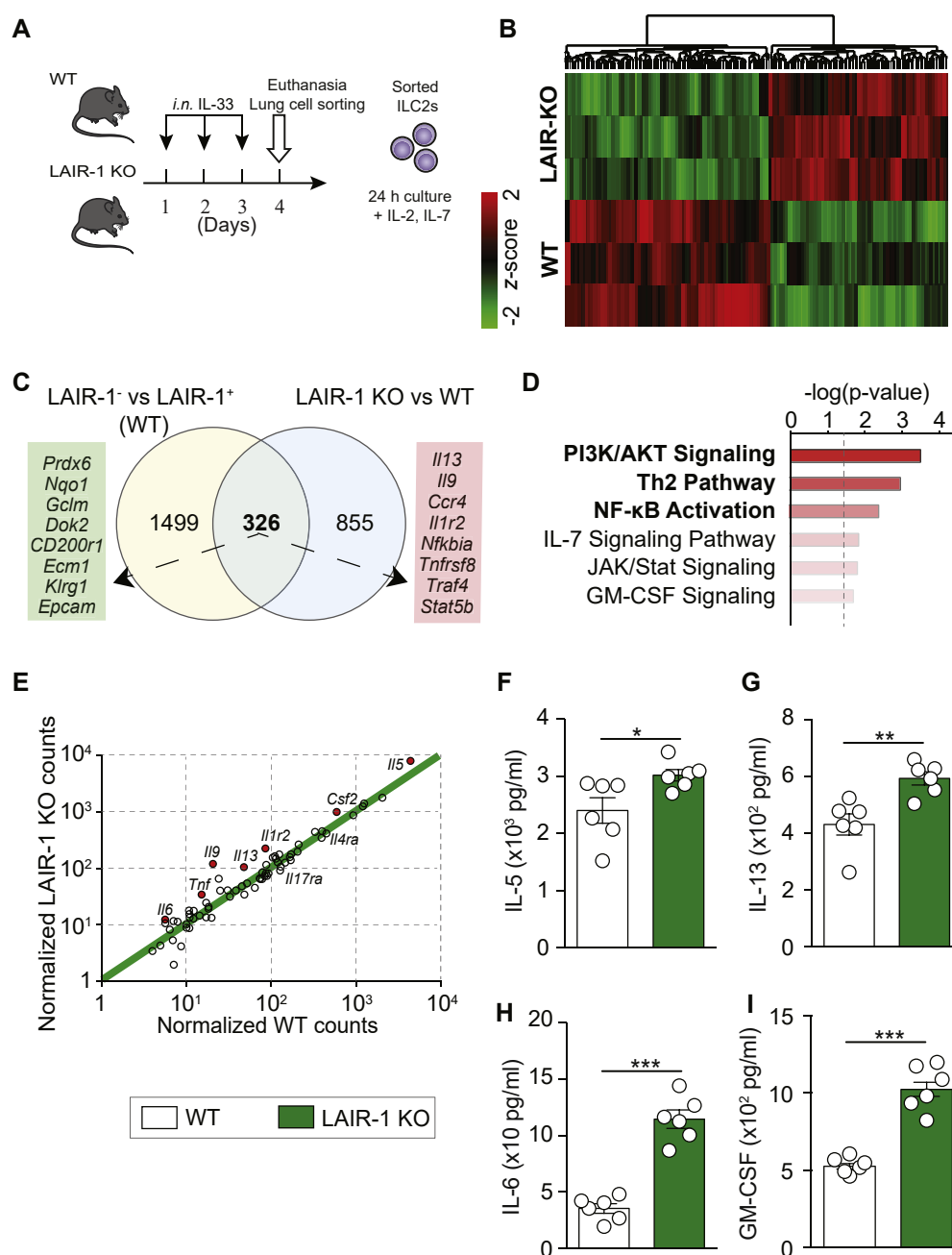


FIG 2. LAIR-1 deficiency increases ILC2 activation and cytokine production. **A-I**, WT and LAIR-1 KO mice were challenged intranasally for 3 consecutive days with 0.5 μ g of rmlIL-33. On day 4, pulmonary the ILC2s were sorted and cultured with rmlIL-2 (10 ng/mL) and rmlIL-7 (10 ng/mL) for 24 hours. **B**, Heatmap representation of total differentially regulated genes, generated with a gene-specific analysis algorithm (counts of <20 [$P \leq .05$]; $n = 3$). **C**, Venn diagram highlighting a list of commonly regulated genes in 2 bulk RNAseq procedures: LAIR-1⁻ versus LAIR-1⁺ from WT mice and LAIR-1 KO versus WT ILC2s. Green list represents downregulated genes, and red list represents upregulated genes in LAIR-1⁻ and LAIR KO ILC2s. **D**, Pathway enrichment analysis enriched for relevant upregulated genes in LAIR-1 KO ILC2s. **E**, Differentially expressed cytokine and cytokine receptor genes plotted as the normalized counts in WT versus LAIR-1 KO ILC2s. Red dots represent significantly upregulated relevant genes ($P \leq .05$; $n = 3$). **F-H**, Levels of IL-5 (**F**), IL-13 (**G**), and IL-6 (**H**) quantified by using LEGENDplex bead-based immunoassay. **I**, Level of GM-CSF quantified by ELISA. Data are presented as means $\pm$ SEMs (unpaired 2-tailed Student t test); * $P < .05$; ** $P < .01$; *** $P < .001$. Cell, mouse, and human images are provided with permission from Servier Medical Art. JAK/Stat, JAK-STAT Janus kinase-signal transducer and activator of transcription.

Consistently, several studies have highlighted a constitutive interaction between LAIR-1 and SHP-1,^{38,39} a phosphatase that directly inhibits PI3K/AKT downstream signaling as well as NF- κ B⁵² (Fig 3, A). To investigate these interactions in ILC2s, we assessed the effect of LAIR-1 on the expression of relevant downstream players at the transcriptional and protein levels. Interestingly, a series of genes related to the PI3K pathway, NF κ B activation, apoptosis, and proliferation were significantly upregulated in LAIR-1 KO activated ILC2s compared with in WT ILC2s, whereas *Ptpn6*, which encodes SHP-1, was significantly downregulated (Fig 3, B). Using flow cytometry, we also observed a significantly lower expression of SHP-1 in LAIR-1 KO ILC2s than in WT ILC2s (Fig 3, C). The lack of LAIR-1 was also associated with higher expression of AKT, p65 (which is a principal component of the canonic NF- κ B pathway), and the antiapoptotic factor BCL-2 (Fig 3, D-F). Moreover, proliferation was improved, as revealed by ki67 intranuclear staining (Fig 3, G) and CellTrace Violet staining (see Fig E3, A in the Online Repository at www.jacionline.org). To further confirm the implication of SHP-1 in ILC2 activation, we used the specific inhibitor tyrosine phosphatase inhibitor-1 at a concentration of 15 μ M to modulate SHP-1 activity *in vitro*. Interestingly, SHP-1 inhibition in ILC2s led to increased *in vitro* proliferation and enhanced IL-5 production (see Fig E3, B and C). Moreover, the LAIR-1 ligand C1q increases the expression of SHP-1 in ILC2s (see Fig E3, D). Altogether, these results support the involvement of LAIR-1–SHP-1 interactions in the control of ILC2 activation.

LAIR-1 regulates lung function and inflammation in ILC2-dependent AHR

Given the regulatory role of LAIR-1 in pulmonary ILC2s, we next hypothesized that this receptor may reduce AHR and lung inflammation. WT and LAIR-1 KO mice received either IL-33 or PBS via the intranasal route for 3 consecutive days. One day after the last challenge, lung function was assessed by direct measurement of lung resistance and dynamic compliance in anesthetized tracheostomized mice by using the FinePointe RC system (Buxco Research Systems), followed by BAL fluid and lung tissue sample analysis. Although IL-33 significantly increased lung resistance in both genotypes, lung resistance in LAIR-1 KO mice was significantly higher than in WT mice (Fig 4, A). Consistently, the results of dynamic compliance showed lower response in LAIR-1 KO mice than in WT mice (Fig 4, B). This suggested that LAIR-1 reduces IL-33–induced AHR. The principal immune populations in the BAL fluid were identified and quantified by flow cytometry (see Fig E4, A-E in the Online Repository at www.jacionline.org). Consistent with AHR, the total number of immune cells (CD45⁺) in the BAL fluid of IL-33–challenged mice was significantly higher in LAIR-1 KO mice than in WT mice (Fig 4, C). Eosinophilia, which is known to be a main feature in type 2 asthma, was significantly higher in LAIR-1 KO mice than in WT mice, as was the number of inflammatory CD11b⁺ DCs (Fig 4, D and E and see Fig E4, A). In the lungs, ILC2 count was higher in LAIR-1 KO mice following IL-33 challenge, as was the percentage of IL-5⁺IL-13⁺ ILC2s (Fig 4, F and G and see Fig E5, A in the Online Repository at www.jacionline.org). The exacerbated lung inflammation in LAIR-1–deficient mice was also confirmed by histology (Fig 4, H). Expectedly, the lack of LAIR-1 improved ILC2 viability, confirming our previous *in vitro* data (see Fig E5, B); however, it had no significant effect on the expression of other

receptors, including MAIR-V, KLRG1, PD-L1, and CD200R (see Fig E5, C-H). Altogether, these results reveal that LAIR-1 dampens IL-33–induced lung inflammation and reduces AHR.

To address LAIR-1–specific implication in ILC2-dependent asthma, we adoptively transferred activated WT or LAIR-1 KO ILC2s into *Rag2*^{-/-}*Il2rg*^{-/-} mice that lacked all lymphoid cells. The recipient mice were then challenged with IL-33 for 3 consecutive days. On day 4, AHR and BAL fluid eosinophil and pulmonary ILC2 numbers were assessed (Fig 4, I). As expected, in the absence of any adoptive transfer, *Rag2*^{-/-}*Il2rg*^{-/-} mice did not develop AHR in response to IL-33. Consistent with the previous observations in LAIR-1 KO mice, here we observed that mice receiving LAIR-1 KO ILC2s displayed significantly higher lung resistance and increased eosinophil recruitment as compared with in those receiving mice WT ILC2s (Fig 4, J and K). In addition, the percentage of CD45⁺Lin⁻ cells and, consequently, the number of LAIR-1 KO ILC2s in the lungs were significantly higher (Fig 4, L and M). Therefore, these models overall confirm our previous results and underline the protective role of LAIR-1 in IL-33–induced AHR and pulmonary inflammation through the regulation of ILC2s.

LAIR-1 downregulates *A alternata*–induced AHR and limits lung inflammation

We next investigated whether LAIR-1 conserves its immunoregulatory role in a clinically relevant AHR model induced by the fungal allergen *A alternata*, which is known to be associated with type 2 inflammation.^{53,54} WT and LAIR-1 KO mice were intranasally challenged with *A alternata* extracts (100 μ g per mouse) for 5 consecutive days, followed by AHR and lung inflammation assessment (Fig 5, A). Consistent with the IL-33 model, lung resistance was higher in LAIR-1 KO mice than in WT mice (Fig 5, B). This was associated with an increased number of CD45⁺ cells in the BAL fluid (Fig 5, C). In particular, LAIR-1 KO mice displayed significantly higher numbers of eosinophils, T cells, and B cells but similar recruitment of neutrophils and CD11c⁺ cells (Fig 5, D-H). We further addressed whether LAIR-1 is inducible in ILC2s and regulates their activation in response to a widespread fungal allergen. Interestingly, our results revealed that exposure of mice to *A alternata* induces LAIR-1 expression on about 30% to 40% of pulmonary ILC2s (Fig 5, I). In parallel, the lack of LAIR-1 was associated with an increased number of pulmonary ILC2s as well as with an increased percentage of IL-5⁺IL-13⁺ ILC2s (Fig 5, J and K). Taken together, these results confirm the inhibitory role of LAIR-1 in a clinically relevant asthma model and demonstrate the capacity of LAIR-1 to control the type 2 immune response.

LAIR-1 is inducible and controls the effector function of human ILC2s

ILC2s are now considered to be relevant immune players in human type 2 asthma⁵⁵⁻⁵⁷ despite their low percentage among PBMCs (see Fig E6 in the Online Repository at www.jacionline.org). To assess the translational potential of our data, we explored whether LAIR-1 was expressed and whether it modulated human ILC2 activation, as demonstrated in the mouse. To this end, we assessed the expression of LAIR-1 on freshly sorted ILC2s and on cultured ILC2s. Human ILC2s were identified and sorted as CD45⁺Lin⁻CD127⁺CRTH2⁺ cells (Fig 6, A

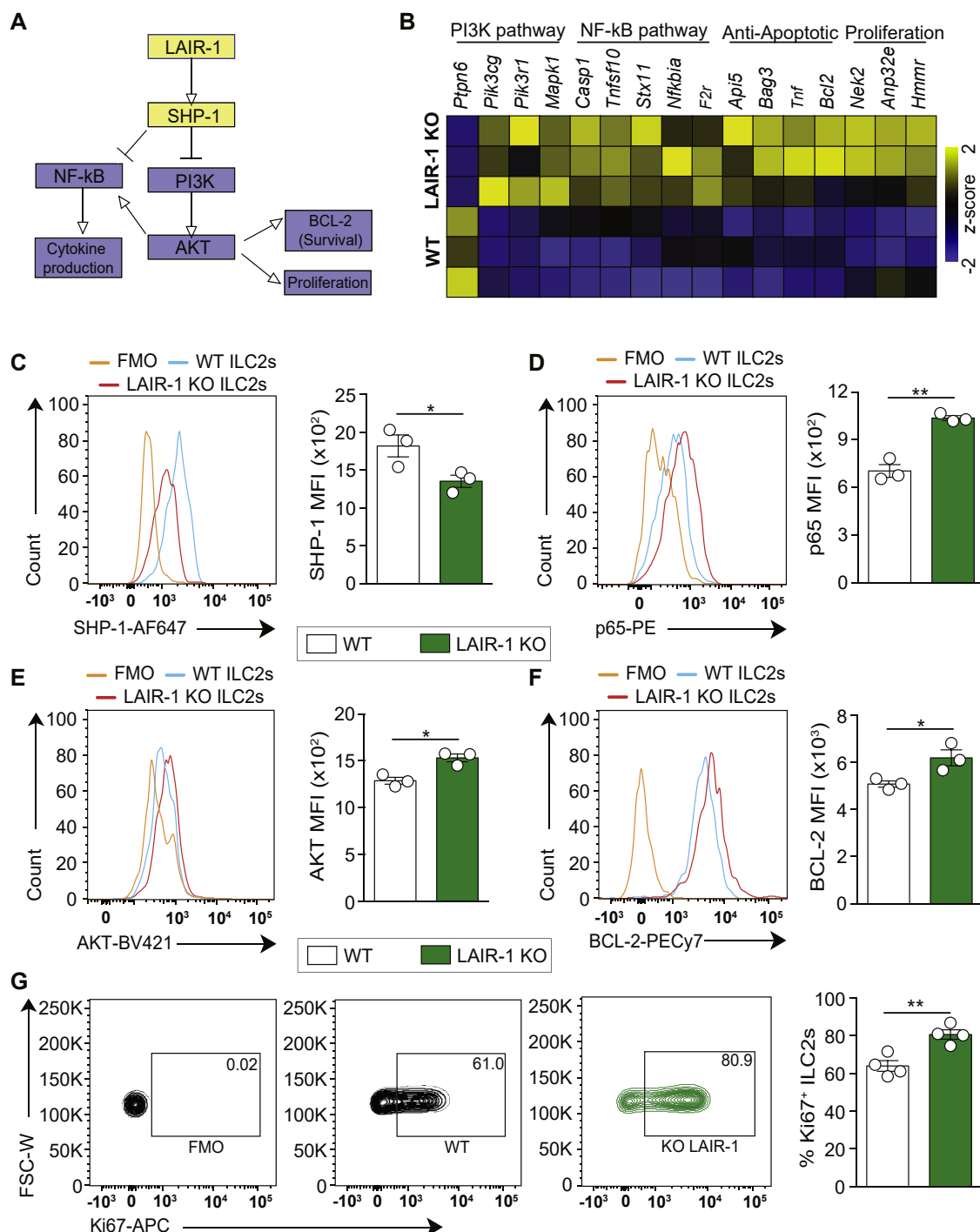


FIG 3. LAIR-1 signaling involves the PI3K/AKT and NF-κB pathways. **A-G**, WT and LAIR-1 KO mice were challenged intranasally for 3 consecutive days with 0.5 μg of rmlL-33. On day 4, pulmonary ILC2s were sorted and cultured with rmlL-2 (10 ng/mL) and rmlL-7 (10 ng/mL) for 24 hours. **A**, Predicted LAIR-1 downstream signaling cascade. **B**, Heatmap representation of differentially regulated genes involved in LAIR-1 signaling pathways. A gene-specific analysis algorithm was used to test for differential expression of genes in WT vs LAIR-1 KO ILC2s (counts of <20 [$P < .05$; $n = 3$]). **C-F**, Representative histogram (left) with the corresponding mean fluorescence intensity (MFI) quantification (right) of SHP-1 (**C**); p65 (**D**); AKT (**E**), and BCL-2 (**F**) expression in WT and LAIR-1 KO ILC2s. **G**, Representative flow cytometry plots of Ki67 intranuclear staining and corresponding quantification (right) presented as the percentage of proliferative ILC2s. Data are presented as means ± SEMs (unpaired 2-tailed Student t test); * $P < .05$; ** $P < .01$. FSC, Forward scatter.

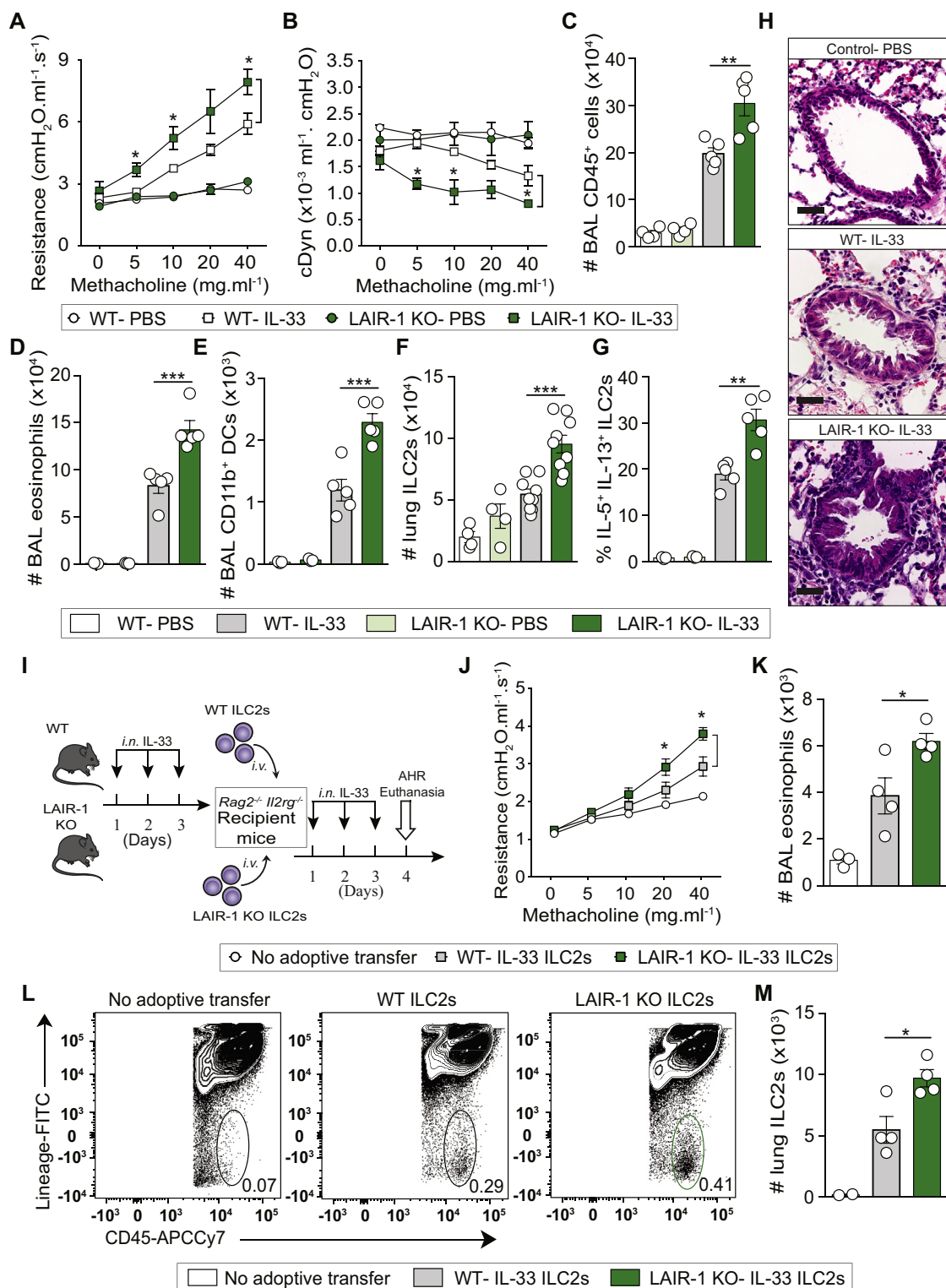


FIG 4. LAIR-1 deficiency exacerbates ILC2-dependent AHR and lung inflammation. WT and LAIR-1 KO mice were challenged for 3 consecutive days with $0.5 \mu\text{g}$ of rmlL-33. On day 4, AHR and lung inflammation were assessed. **A** and **B**, Lung resistance (**A**) and dynamic compliance (**B**) ($n = 5$). **C**, Total number of CD45⁺ cells in the BAL fluid. **D** and **E**, Total number of eosinophils (**D**) and CD11b⁺ DCs (**E**) in the BAL fluid. **F**, Total number of pulmonary ILC2s gated as CD45⁺ Lin⁻ ST2⁺ CD127⁺ cells. **G**, Percentage of IL-5⁺ and IL-13⁺ ILC2s identified by intracellular staining. **H**, Hematoxylin and eosin (H&E) staining of lung sections (scale bar = $50 \mu\text{m}$). **I-M**, WT and LAIR-1 KO mice were challenged for 3 consecutive days with $0.5 \mu\text{g}$ of rmlL-33. On day 4, pulmonary ILC2s were sorted and adoptively transferred into Rag2^{-/-} Il2rg^{-/-} mice via the intravenous route. Recipient mice were then challenged intranasally for 3 consecutive days with $0.5 \mu\text{g}$ of rmlL-33 before the assessment of AHR and lung inflammation. **J**, Lung resistance ($n = 4$). **K**, Total number of eosinophils in the BAL fluid. **L**, Gating of Lin⁻ cells from live CD45⁺ cells. **M**, Total number of pulmonary ILC2s gated as CD45⁺ Lin⁻ ST2⁺ CD127⁺ cells. Data are presented as means $\pm$ SEMs (1-way ANOVA); * $P < .05$; ** $P < 0.01$; *** $P < .001$.

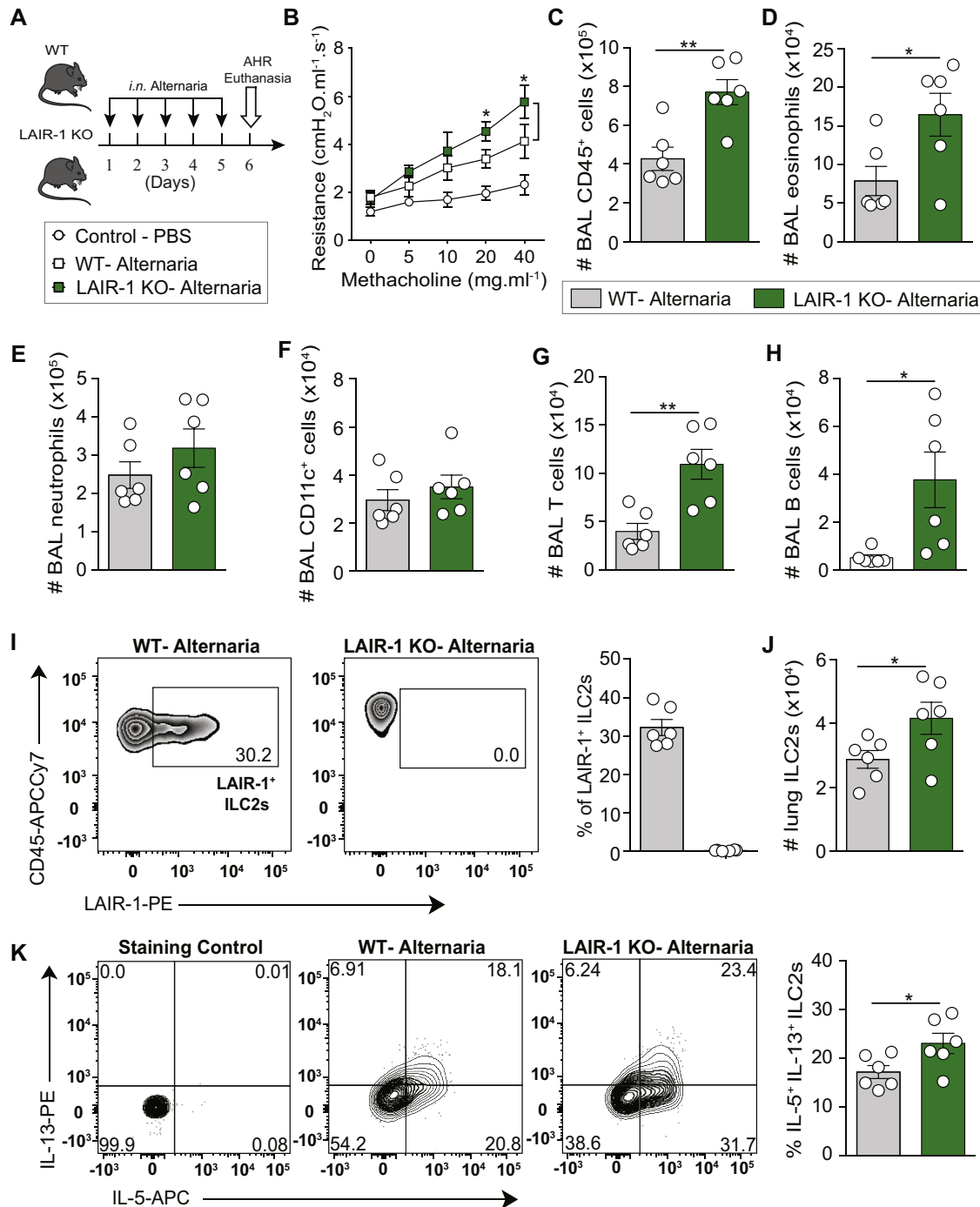


FIG 5. LAIR-1 deficiency significantly increases *A. alternata*-induced pulmonary inflammation. **A-K**, WT and LAIR-1 KO mice were challenged intranasally for 5 consecutive days with 100 μ g of *A. alternata* extracts. On day 6, AHR and lung inflammation were assessed. **B**, Lung resistance measured in response to increasing concentrations of methacholine ($n = 4$). **C**, Total number of CD45⁺ cells in the BAL fluid. **D-H**, Total number of eosinophils (**D**), neutrophils (**E**), CD11c⁺ cells (**F**), T cells (**G**), and B cells (**H**) in the BAL fluid. **I**, Percentage of LAIR-1⁺ ILC2s in the lungs. **J**, Total number of pulmonary ILC2s. **K**, Percentage of IL-5⁺ and IL-13⁺ ILC2s. Data are presented as means $\pm$ SEMs (unpaired 2-tailed Student *t* test); **P* < 0.05; ***P* < 0.01.

and *B*). Interestingly, we first observed that LAIR-1 was expressed on circulating ILC2s in the basal state (at hour 0) and was then significantly induced in response to rhIL-33 (at hour 72) (Fig 6, C). Because LAIR-1 was remarkably expressed on human ILC2s, we performed functional tests using specific LAIR-1

antisense morpholino oligonucleotides to address the effect of a transient knockdown of the *Lair1* gene on human ILC2 activation in response to IL-33. As a validation of our approach, we observed a significant decrease in LAIR-1 expression on morpholino-treated ILC2s (Fig 6, D). Then, we demonstrated that morpholino

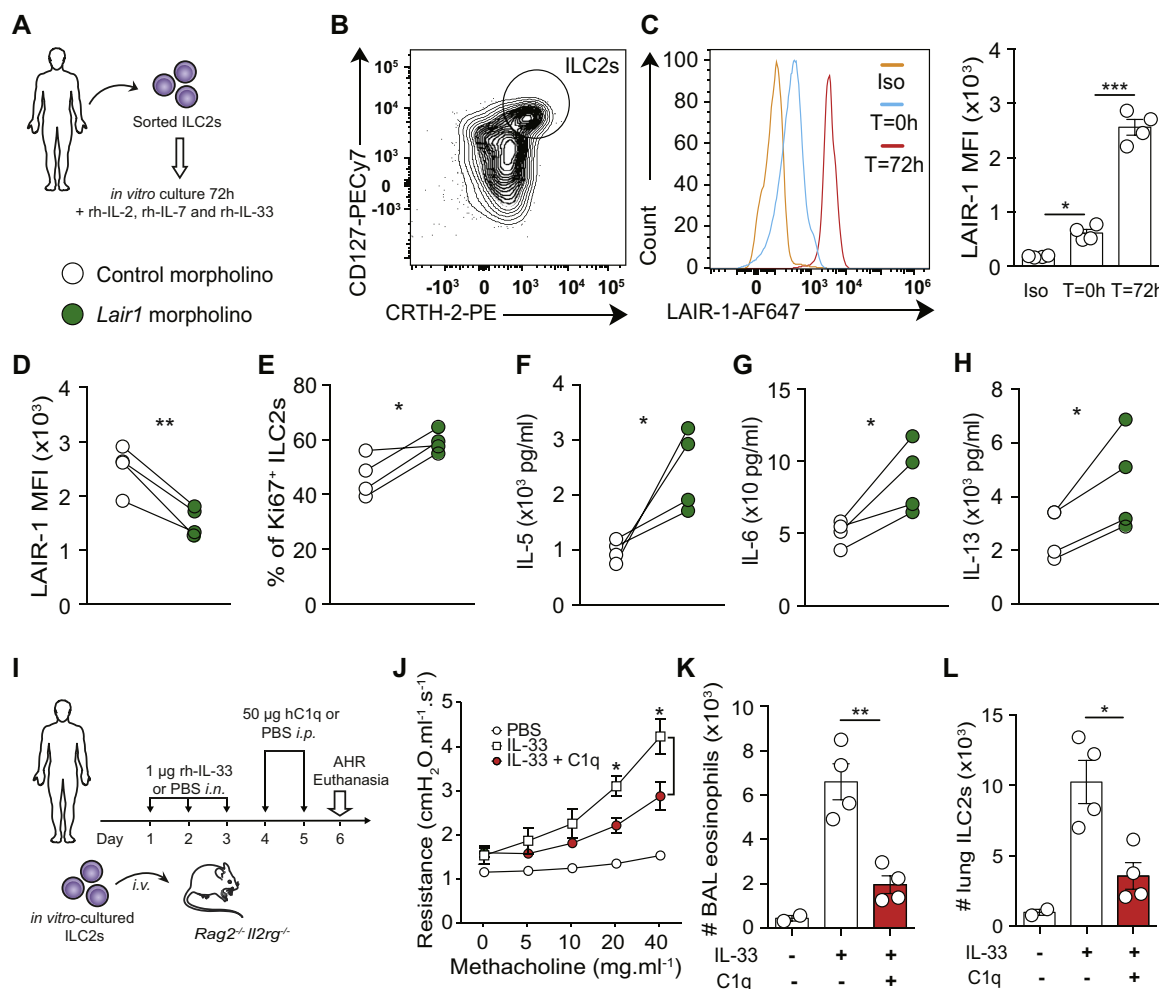


FIG 6. LAIR-1 is expressed and functional on human ILC2s. **A-H**, Human ILC2s were sorted from PBMCs and cultured in the presence of rhIL-2, rhIL-7, and rhIL-33 with LAIR-1 morpholino antisense oligonucleotides or control oligonucleotides. **B**, Gating strategy of human ILC2s identified as CD45⁺ Lin[−] CD127⁺ CRTH2⁺ cells. **C**, Representative histogram of LAIR-1 expression on human ILC2s and corresponding quantification (right). **D** and **E**, Effect of morpholino treatment on LAIR-1 expression presented as mean fluorescence intensity (MFI) (**D**) and Ki67⁺ ILC2s percentage (**E**). **F-H**, Levels of IL-5 (**F**), IL-6 (**G**), and IL-13 (**H**). **I-L**, *In vitro*-cultured human ILC2s were adoptively transferred into *Rag2*^{−/−} *Il2rg*^{−/−} mice. Recipient mice were intranasally challenged with 1 μ g of rhIL-33 or PBS for 3 consecutive days and intraperitoneally (i.p.) injected with human C1q or PBS on days 4 and 5. Measurement of lung function and inflammation followed on day 6. **J**, Lung resistance ($n = 4$). **K**, Total number of eosinophils in the BAL fluid gated as CD45⁺ SiglecF⁺ CD11c[−] cells. **L**, Total number of human ILC2s in lungs gated as CRTH2⁺ CD127⁺ cells. **D-H**, Data presented as means $\pm$ SEMs (paired 2-tailed Student *t* test). The 1-way ANOVA test was used for multiple comparisons. **P* < .05; ***P* < .01; ****P* < .001.

treatment along with IL-33 stimulation increases ILC2 proliferation, as revealed after 72 hours of culture by using the ki67 intranuclear staining (Fig 6, E). In parallel, quantification of cytokine levels in the supernatant showed that specific *Lair1* antisense morpholino is able to induce a significant upregulation in IL-5, IL-6, and IL-13 production as compared with in control sequences (Fig 6, F-H). Lastly, we tested the effect of LAIR-1 engagement on human ILC2s *in vivo* by using a humanized mouse model. Briefly, *Rag2*^{−/−} *Il2rg*^{−/−} mice lacking lymphoid cells were reconstituted with human peripheral blood ILC2s and intranasally challenged for 3 consecutive days with PBS or rhIL-33 to induce AHR. On days 4 and 5, the mice received PBS or human C1q, a potential physiologic ligand for LAIR-1, via the intraperitoneal route. On day 6, we assessed AHR and lung inflammation

(Fig 6, J). Strikingly, lung resistance in the C1q-treated mice was significantly reduced as compared with in the control mice (Fig 6, J). In line with lung resistance, the C1q-treated mice showed abrogated lung inflammation associated with decreased eosinophilia and a lesser number of human ILC2s (Fig 6, K and L). Taken together, these results indicate that LAIR-1 is highly implicated in the control of human ILC2 activation, suggesting a promising therapeutic approach against type 2 asthma.

DISCUSSION

The present study is, to our knowledge, the first to elucidate the expression, the signaling pathways, and the inhibitory potential of the LAIR-1 axis in ILC2s in the context of allergic asthma. We

demonstrated that the transmembrane glycoprotein inhibitory receptor LAIR-1 is inducible on mouse ILC2s following activation. LAIR-1 deficiency increases type 2 cytokine production in association with an upregulation of PI3K/AKT signaling. We further demonstrated the protective role of LAIR-1 in ILC2-dependent asthma. Interestingly, LAIR-1 was expressed and inducible on human ILC2s, and it downregulated their activation both *in vitro* and *in vivo*, suggesting a new potential target in allergic asthma.

LAIR-1 is a broadly expressed immune receptor, playing an inhibitory role on the majority of immune cells.^{27,47,58} In neutrophils, LAIR-1 is lost during differentiation and is expressed only following stimulation on mature cells.³⁰ No study has yet investigated the conditions under which ILC2s could express LAIR-1. Here, we have demonstrated that ILC2 precursors in the bone marrow and circulating ILC2s display a weak expression of LAIR-1, whereas tissue-resident ILC2s do not express it. In agreement with recent studies highlighting the flexible nature of activated ILC2s,^{1,59,60} pulmonary ILC2s could express LAIR-1 only following stimulation. This suggests that LAIR-1 is induced on highly activated ILC2s to dampen their functions and that its expression could necessitate a special inflammatory microenvironment. Besides the downregulation of genes related to inflammation, LAIR-1⁺ ILC2s highly express genes related to extracellular matrix generation and tissue remodeling. For instance, the gene encoding LOXL2, an enzyme that promotes collagen cross-linking,^{39,61} was highly upregulated in LAIR-1⁺ ILC2s. In parallel, several studies have reported airway remodeling and collagen deposition in different asthma models.⁶²⁻⁶⁴ Therefore, ILC2s may help in providing an optimal microenvironment for LAIR-1 expression while sustaining bidirectional modulatory interactions with collagen-producing lung cells.⁶

Lacking antigen-specific receptors, secretion of type 2 cytokine represents the main contribution of ILC2s in orchestration of the immune response. Our data demonstrated at the transcriptional and protein levels that LAIR-1 controls T_H2 cytokine production in ILC2s along with SHP-1 activation and downregulation of the PI3K/AKT and NF- κ B signaling pathways. Therefore, we suggest here that the recruitment of SHP-1 phosphatase by the LAIR-1 intracellular domain mediates an inhibitory cascade in ILC2s, leading to decreased cytokine production and proliferation. Previous studies have shown that following LAIR-1 engagement, ITIM phosphorylation is a key event driving suppression of the main immune pathways, including IFN- α production in plasmacytoid DCs^{31,35} and TCR signaling in T cells.⁶⁵ Interestingly, a recent study has also demonstrated that LAIR-1–SHP-1 interaction triggers CD8⁺ T cell exhaustion in lung tumors.³⁹ Therefore, our findings support previous studies and decrypt the relevant mechanisms associated with LAIR-1 activity in pulmonary ILC2s.

A balance between activating and inhibitory signals is required to avoid inappropriate immune responses. Because of its broad inhibitory role, LAIR-1 acts as an immune checkpoint and is involved in cancer, as well as in autoimmune and infectious diseases. In particular, the development of rheumatoid arthritis is associated with an imbalanced expression of LAIR-1 on T cells and osteoclasts.^{66,67} Furthermore, decreased LAIR-1 expression on B cells and plasmacytoid DCs worsens SLE progression.^{31,47} LAIR-1 also suppresses neutrophil inflammatory functions in viral bronchiolitis.^{68,69} Although LAIR-1 cross-linking has become a promising therapeutic strategy against several diseases,

only 1 study has investigated LAIR-1 involvement in allergic contexts and reported that LAIR-1 reduces cytokine production by DCs and limits T-cell proliferation in the allergic contact dermatitis model.⁷⁰ Here, we have for the first time investigated the implication of LAIR-1 in allergic asthma by using IL-33 and *A. alternata* models. Our data support a protective role for LAIR-1 in allergic airway inflammation, as LAIR-1 deficiency is associated with exacerbated AHR and eosinophilia. Using adoptive transfer experiments, we demonstrated that LAIR-1 can suppress ILC2-dependent asthma primarily through ILC2 inhibition.

Human cohorts have confirmed the involvement of ILC2s in asthma progression and severity.^{71,72} Although mouse asthma models are very useful to elucidate immunologic mechanisms, we further validated our data in human ILC2s to be more clinically relevant. Interestingly, circulating ILC2s from healthy donors express LAIR-1. Similarly to what occurs in mice, this expression is significantly inducible in response to IL-33, suggesting a critical role for LAIR-1 in ILC2 homeostasis and regulation. Because of the absence of specific LAIR-1 inhibitors and agonists to date, we used the morpholino gene-silencing technique and C1q to modulate LAIR-1 activity *in vitro* and *in vivo*, respectively. A transient knockdown of *Lair1* in ILC2s led to increased proliferation and secretion of type 2 cytokines in response to IL-33, revealing a remarkable inhibitory role in human ILC2s. C1q has been used in different human studies for its capacity to mediate immunosuppressive effect through LAIR-1 engagement.^{35,65,73} It has also been reported as a potent inhibitor in an ovalbumin-induced allergic asthma model.⁴⁴ Because C1q is among the main components of the total protein serum content,^{37,74} it is very plausible that circulating ILC2s are highly exposed and may be regulated by C1q. To further develop our translational approach, here we have developed a humanized mouse model reconstituted with human ILC2s and treated with human C1q after IL-33 challenge. Interestingly, C1q significantly reduces AHR and eosinophilia mainly through the regulation of ILC2s. Existing single-cell RNAseq⁴⁹ shows that luminal macrophages and activated DCs are the major sources of C1q in the lungs of healthy individuals and patients with asthma (see Fig E2, E). As ILC2s localize with DC subsets in lungs,^{4,75} this might suggest the existence of an unrecognized regulatory axis mediated by C1q–LAIR-1 interactions in allergic asthma.

On the basis of diverse experimental strategies, this study has provided evidence for the implication of LAIR-1 in mouse and human ILC2s, and consequently in ILC2-dependent asthma. Therefore, our findings could be associated with clinical implications via the therapeutic modulation of LAIR-1 in allergic asthma.

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Clinical implication: LAIR-1 engagement may serve as potential therapeutic target for the treatment of ILC2-related diseases such as allergic asthma.

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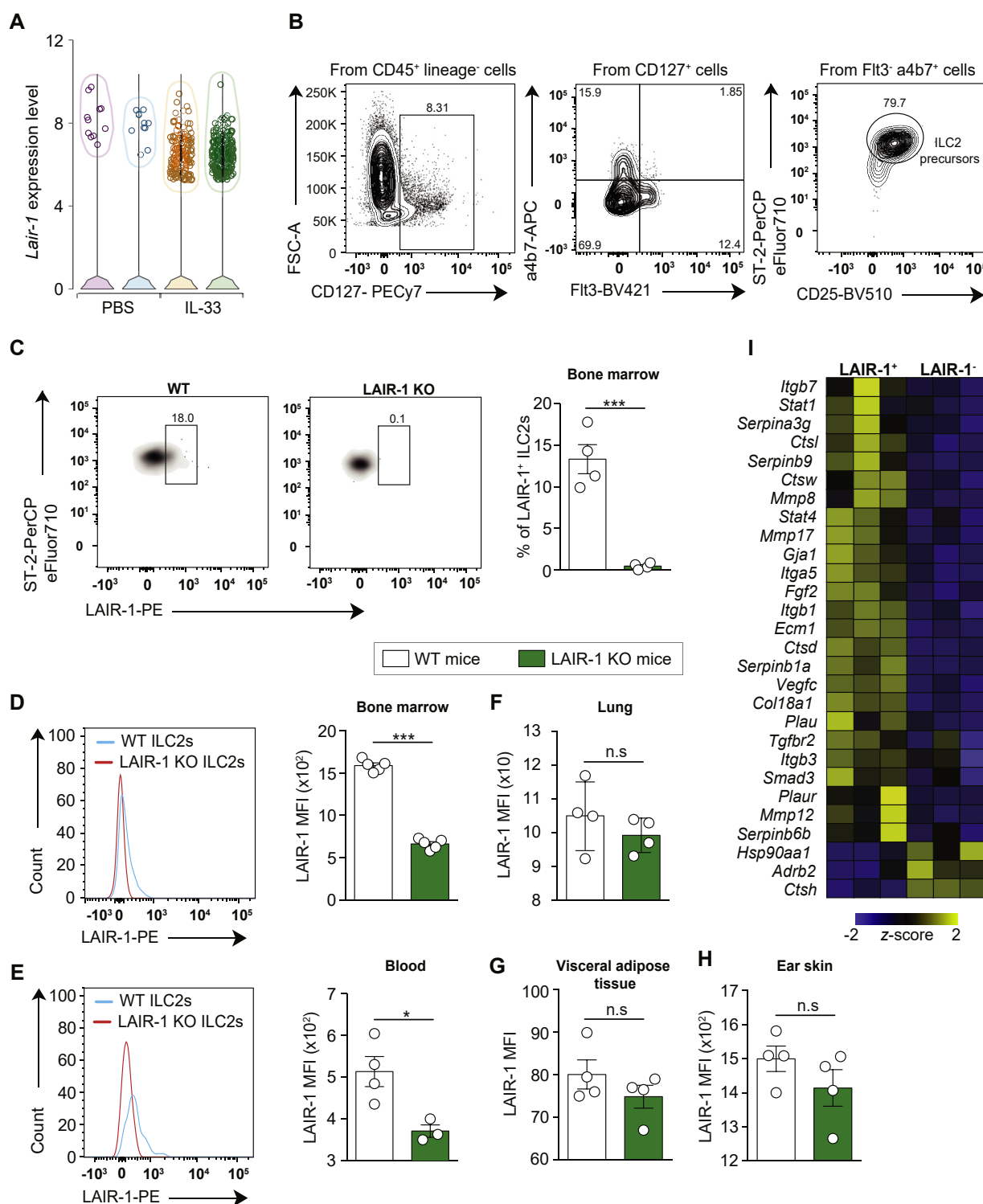


FIG E1. Characterization of LAIR-1 expression on ILC2s in different tissues. **A**, Violin plot of distribution of *Lair-1* expression on PBS- and IL-33-treated ILC2 clusters. **B-H**, LAIR-1 expression was assessed on naive WT ILC2s from different tissues, with KO ILC2s serving as the staining control. **B**, Gating strategy of ILC2 precursors in the bone marrow, identified as CD45⁺Lin⁻CD127⁺integrin α 4b7⁺Fit3⁺CD25⁺ and ST2⁺ cells. **C**, Flow cytometry plots showing the percentage of LAIR-1⁺ ILC2 precursors in the bone marrow and corresponding quantification (right). **D**, Representative histogram of the expression of LAIR-1 on ILC2 precursors in the bone marrow and corresponding quantification (right) presented as mean fluorescence intensity (MFI). **E**, Representative histogram of the expression of LAIR-1 on blood ILC2s and corresponding quantification (right) presented as MFI. **F-H**, Graphs representing the MFI of LAIR-1 expression on ILC2s from lung (F), visceral adipose tissue (G), and ear skin (H). **I**, Heatmap representation of differentially regulated genes involved in tissue remodeling. A gene-specific analysis algorithm was used to test for differential expression of genes in LAIR-1⁺ versus LAIR-1⁻ ILC2s (counts of <20 [$P < .05$]; $n = 3$). Data are presented as means $\pm$ SEMs (unpaired 2-tailed Student t test); * $P < .05$; *** $P < .001$. n.s., Not significant; n.s., not significant.

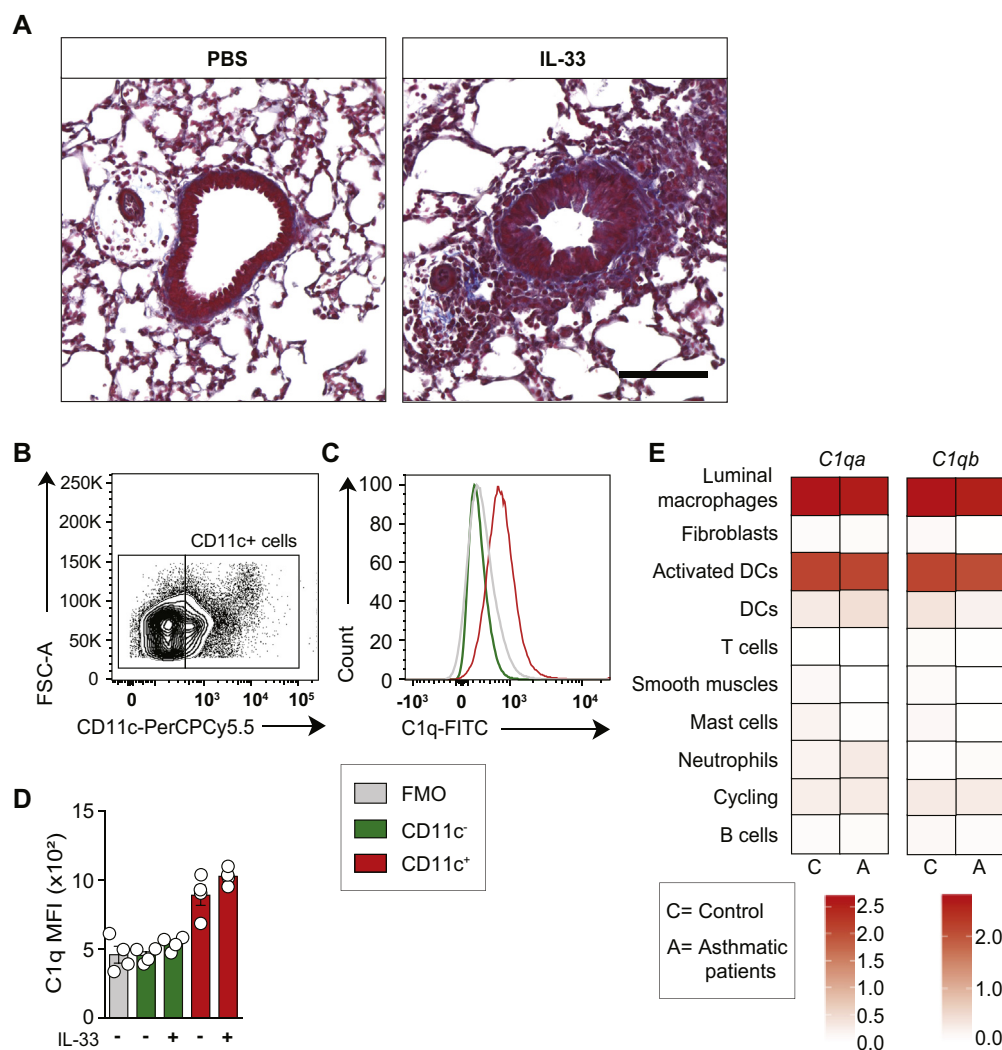


FIG E2. Pulmonary tissue remodeling and C1q expression in lung inflammation. C57BL/6 mice were challenged intranasally for 3 consecutive days with 1 μ g of rIL-33 or PBS. **A**, Trichrome histologic staining of lung sections (original magnification, $\times 20$; scale bars = 100 μ m). **B**, Gating of CD11c⁺ cells in the lungs. **C**, Representative histogram of the expression of C1q on CD11c⁺ and CD11c⁻ pulmonary cells. **D**, Graph representing the mean fluorescence intensity (MFI) of C1q expression on CD11c⁺ and CD11c⁻ pulmonary cells. **E**, Heatmaps generated from publicly available human single-cell RNAseq (<https://asthma.cellgeni.sanger.ac.uk/>) to show the expression levels of *C1qa* and *C1qb* in the principal immune cells from control and asthmatic patient lungs. FSC, Forward scatter.

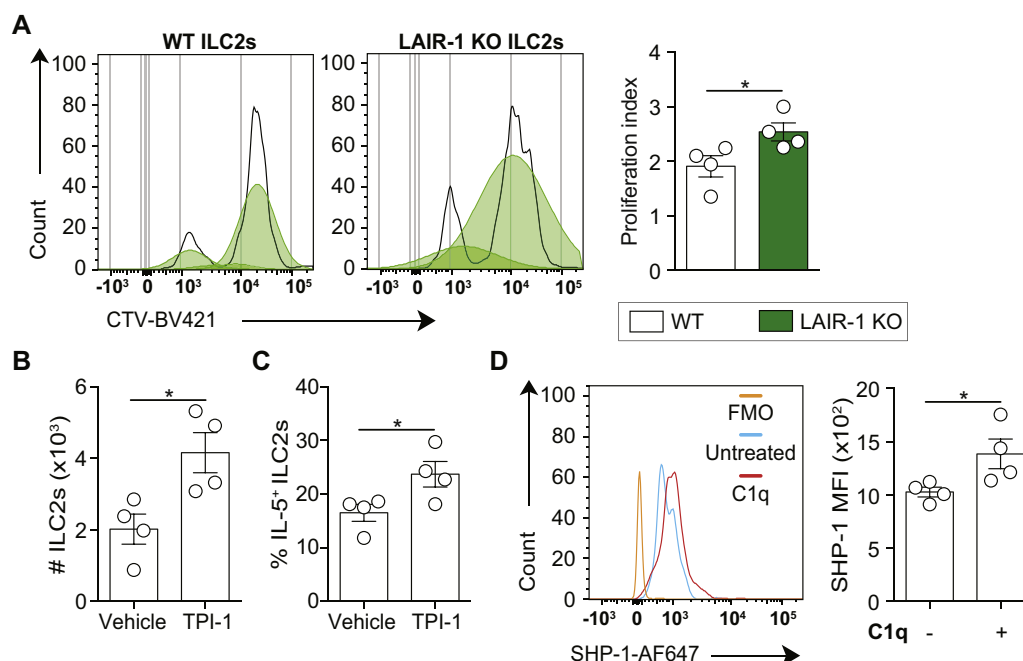


FIG E3. Effect of LAIR-1 deficiency and SHP-1 modulation on ILC2 activation. **A-E**, WT and LAIR-1 KO mice were challenged intranasally for 3 consecutive days with 0.5 μ g of rmIL-33. On day 4, pulmonary ILC2s were sorted and cultured overnight with rmIL-2 (10 ng/mL) and rmIL-7 (10 ng/mL). When indicated, WT ILC2s were treated with 15 μ M TPI-1 or 10 μ g/mL of C1q. **A**, Representative histograms of CellTrace Violet staining in ILC2s and corresponding proliferation index. **B**, Effect of TPI-1 treatment on total number of ILC2s in culture. **C**, Effect of TPI-1 treatment on the percentage of IL-5⁺ ILC2s assessed by intracellular staining. **D**, Representative histogram of SHP-1 expression in ILC2s and corresponding mean fluorescence intensity (MFI) quantification. Data are presented as means $\pm$ SEMs (unpaired 2-tailed Student *t* test); **P* < .05. FSC, Forward scatter.

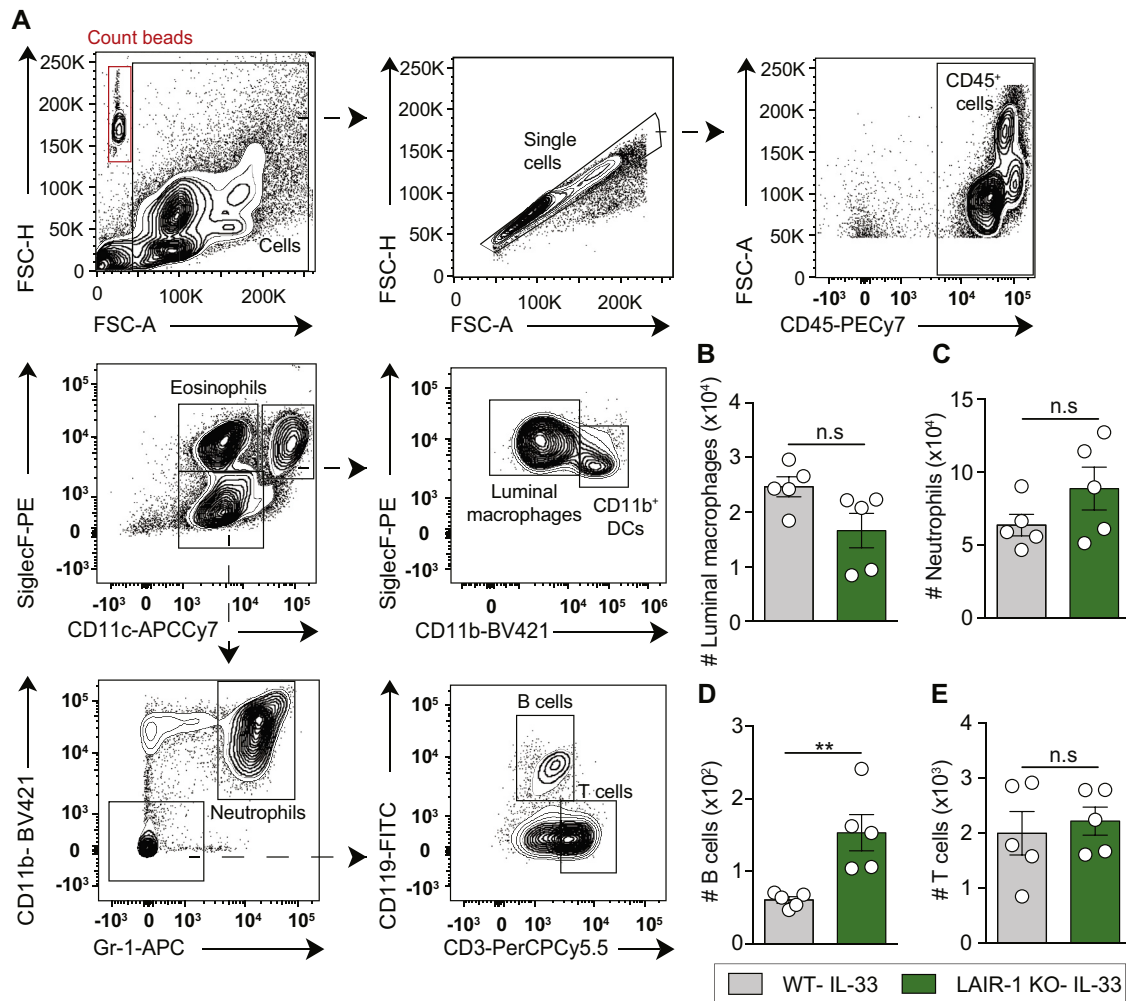


FIG E4. Quantification of BAL fluid immune populations in WT and LAIR-1 KO mice. **A-E**, WT and LAIR-1 KO mice were challenged intranasally for 3 consecutive days with 0.5 μ g of rmlIL-33. On day 4, BAL fluid and lungs were collected for flow cytometry analysis. **A**, Gating strategy of main immune populations in the BAL fluid. **B-E**, Absolute count of luminal macrophages (**B**), neutrophils (**C**), B cells (**D**), and T cells (**E**) in the BAL fluid. Data are presented as means $\pm$ SEMs (unpaired 2-tailed Student *t* test); ***P* < .01. FSC, Forward scatter; n.s., not significant.

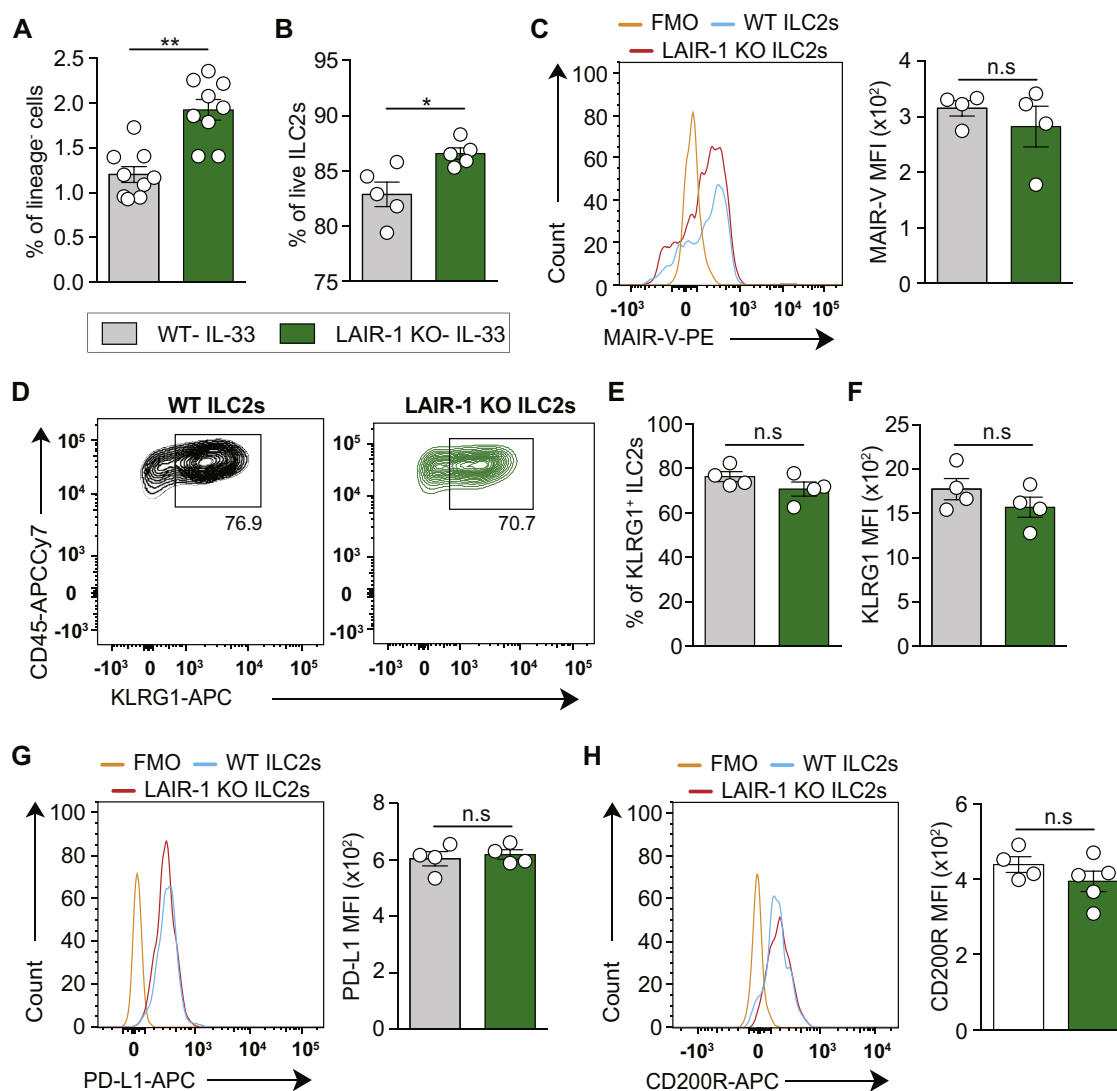


FIG E5. Impact of LAIR-1 deficiency on the expression of other immune receptors in ILC2s. WT and LAIR-1 KO mice were challenged intranasally for 3 consecutive days with 0.5 μ g of rml-33. On day 4, lungs were collected for flow cytometry analysis. **A**, Percentage of CD45⁺Lin⁻ cells in the lungs. **B**, Percentage of live ILC2s in the lungs assessed by using live/dead fixable stains. **C**, Representative histogram of MAIR-V expression in ILC2s and corresponding mean fluorescence intensity (MFI) quantification. **D-F**, Representative histograms of KLRG1 expression in ILC2s (**D**) and corresponding percentages of KLRG1⁺ ILC2s (**E**), as well as MFI quantification (**F**). **G** and **H**, Representative histogram of PD-L1 (**G**) and CD200R (**H**) expression in ILC2s and corresponding MFI quantification. Data are presented as means $\pm$ SEMs (unpaired 2-tailed Student *t* test); * $P < .05$; ** $P < .01$. n.s., Not significant.

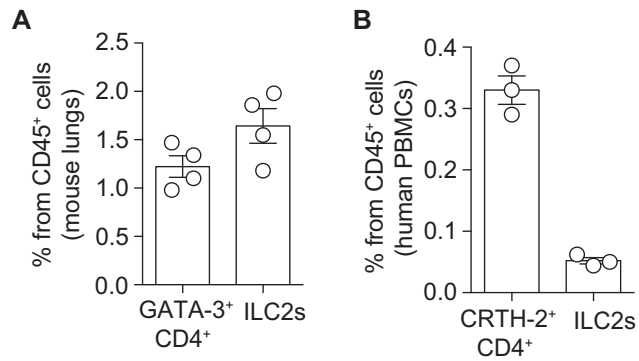


FIG E6. Estimation of the percentage of ILC2s and T_H2 cells. **A**, Percentage of GATA-3⁺ CD4⁺ T cells and ILC2s from lung CD45⁺ cells in the mouse. **B**, Percentage of CRTH2⁺ CD4⁺ T cells and ILC2s from CD45⁺ cells in human PBMCs. Data are presented as means ± SEMs.