PNAS

Supporting Information for

CD45 alleviates airway inflammation and lung fibrosis by limiting expansion and activation of ILC2s

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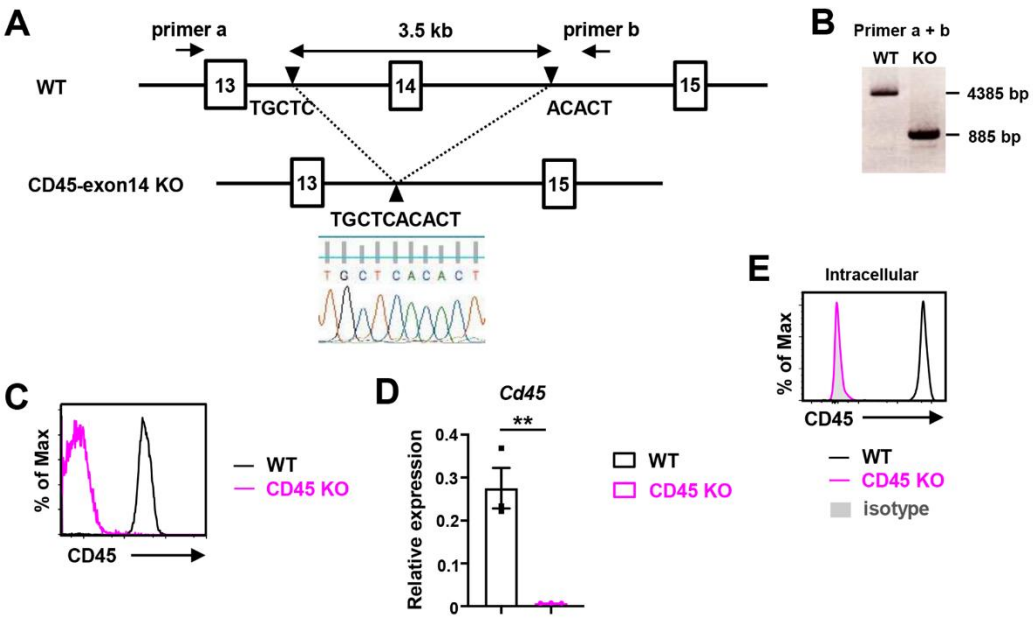

Fig. S1. Generation of CD45 knockout (KO) mice.

(A) Schematic illustration of the CD45 KO allele. Horizontal arrows indicate the primers for genotyping. DNA sequencing was performed to confirm the deletion of exon 14 in the CD45 locus. (B) Genotyping PCR to detect the CD45 KO allele by primers a and b, as shown in A. (C) Flow cytometric analysis of CD45 expression on ILC2s of CD45 KO mice (CD45 KO) and wild-type mice (WT). (D) Real-time PCR analysis showing the mRNA expression on the 5' side of exon 14 of CD45 in ILC2s from indicated mice (n = 3). (E) Intracellular staining of CD45 in ILC2s from indicated mice after culture with brefeldin A for 6 hours. Data represent at least two independent experiments with similar results (B, C, and E). Data are mean ± SD with Student's *t*-test (D) and pooled from two independent experiments. **, $p < 0.01$.

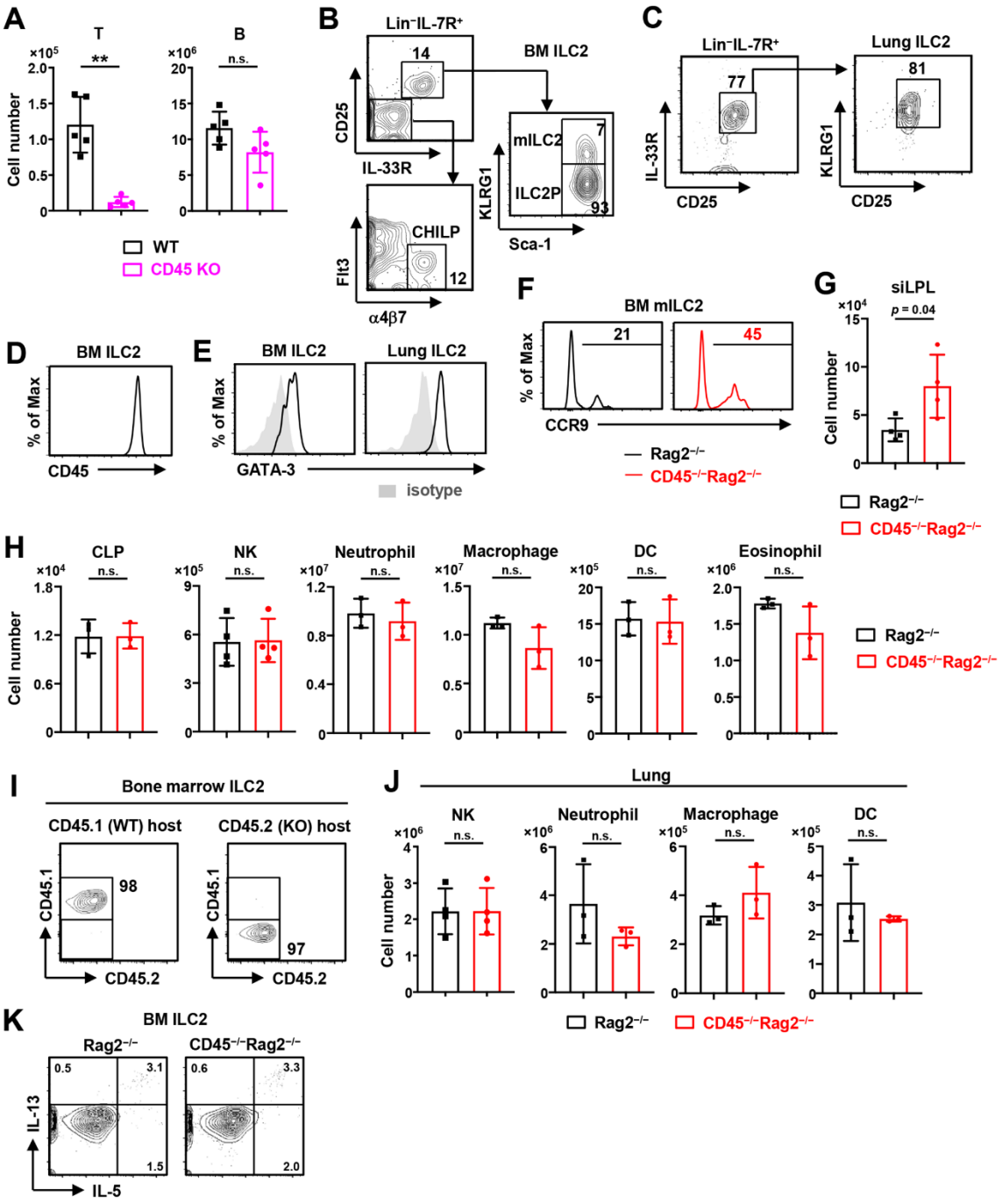

Fig. S2. Phenotypes of CD45$^{-/-}$ and CD45$^{-/-}$Rag2$^{-/-}$ mice.

(A) Numbers of T cells and B cells in the bone marrow of CD45 knockout mice (CD45 KO) and wild-type mice (WT) (n = 5). (B) Gating strategy for CHILPs, ILC2Ps, and mILC2s in the bone marrow. (C) Gating strategy for lung ILC2. (D) Expression of CD45 in the gate of bone marrow ILC2 (Lin$^-$IL-7R$^+$Sca-1$^+$CD25$^+$ST2$^+$). (E) Expression of GATA-3 in the gate of bone marrow ILC2 and lung ILC2 (Lin$^-$IL-7R$^+$CD25$^+$ST2$^+$KLRG1$^+$). (F and G) Flow cytometric analysis of CCR9$^+$ mILC2s in the bone marrow (F) and numbers of CCR9$^+$ ILC2s in the lamina propria of the small intestine (G) of CD45$^{-/-}$Rag2$^{-/-}$ and control Rag2$^{-/-}$ mice (n = 4). (H) Numbers of CLPs (Lin$^-$IL-7R$^+$c-kit^{int}Sca-

$1^{int}Flt3^{+}$), NK cells ($CD3^{-}NK1.1^{+}$), neutrophils ($Gr\text{-}1^{+}CD11b^{+}$), macrophages ($CD11b^{+}F4/80^{+}Gr\text{-}1^{-}CD11c^{-}$ Siglec-F^{-}), dendritic cells (DC) ($CD11c^{+}Gr\text{-}1^{-}CD11b^{-}$), and eosinophils ($CD11b^{+}$Siglec-$F^{+}$ $Gr\text{-}1^{-}CD11c^{-}$) in the bone marrow of $CD45^{-/-}Rag2^{-/-}$ and $Rag2^{-/-}$ mice (n = 3−4). (I) Genetically marked CD45.1 WT and CD45 KO mice of the same age underwent parabiosis surgery and were analyzed after eight weeks. Flow cytometric analysis for partner-derived and host-derived cells in ILC2s of the bone marrow. (J) Numbers of NK cells, neutrophils, macrophages, and dendritic cells (DC) in the lung of $CD45^{-/-}Rag2^{-/-}$ and $Rag2^{-/-}$ mice (n = 3−4). (K) Flow cytometric analysis of IL-5^{+} and IL-13^{+} bone marrow ILC2s after stimulation with PMA and ionomycin for 4 hours. Data are mean ± SD with Student's *t*-test and pooled from 2−3 independent experiments (A, G, H, and J). Data represent at least two independent experiments with similar results (B, C, D, E, F, I, and K). **, $p < 0.01$; n.s., not significant.

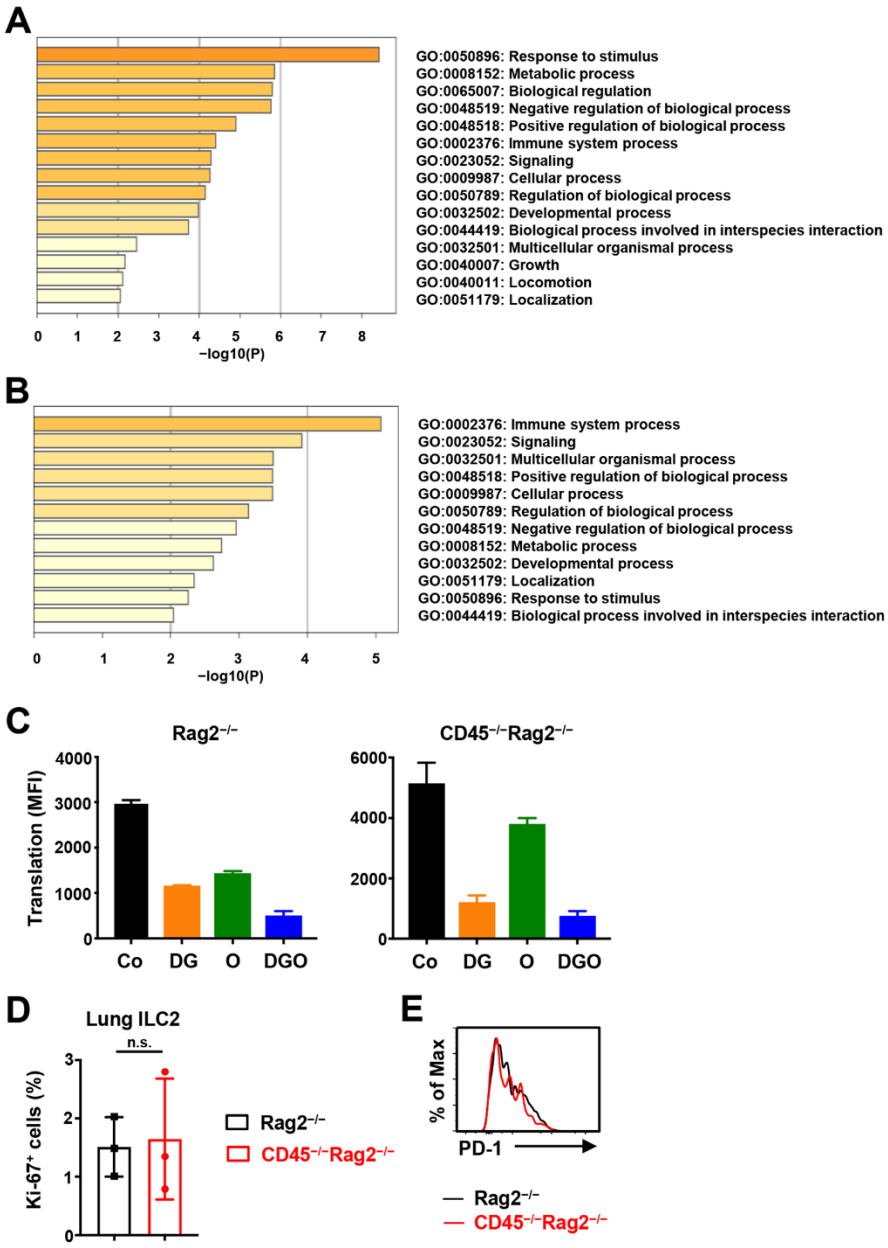

Fig. S3. Gene expression and metabolism in CD45$^{-/-}$ ILC2s.

(A and B) Bar chart depicting the enrichment analysis of differentially expressed genes in bone marrow ILC2Ps (A) and mILC2s (B) between CD45$^{-/-}$Rag2$^{-/-}$ and control Rag2$^{-/-}$ mice. Normalized enrichment scores were calculated using Metascape. (C) Translation levels assessed by OP-puromycin mean fluorescence intensity (MFI) in ILC2s from the lung of CD45$^{-/-}$Rag2$^{-/-}$ mice and Rag2$^{-/-}$ mice, analyzed using modified SCENITH method in the control condition (Co) or after the addition of 2-DG (DG), oligomycin (O) or both inhibitors (DGO) (n = 3). (D) Frequency of proliferating Ki-67^{+} ILC2s in the lung (n = 3). (E) Expression of PD-1 on lung ILC2s from CD45$^{-/-}$Rag2$^{-/-}$ and Rag2$^{-/-}$ mice. Data are mean ± SD with Student's *t*-test and pooled from two independent experiments (D). Data represent at least two independent experiments with similar results (C and E). n.s., not significant.

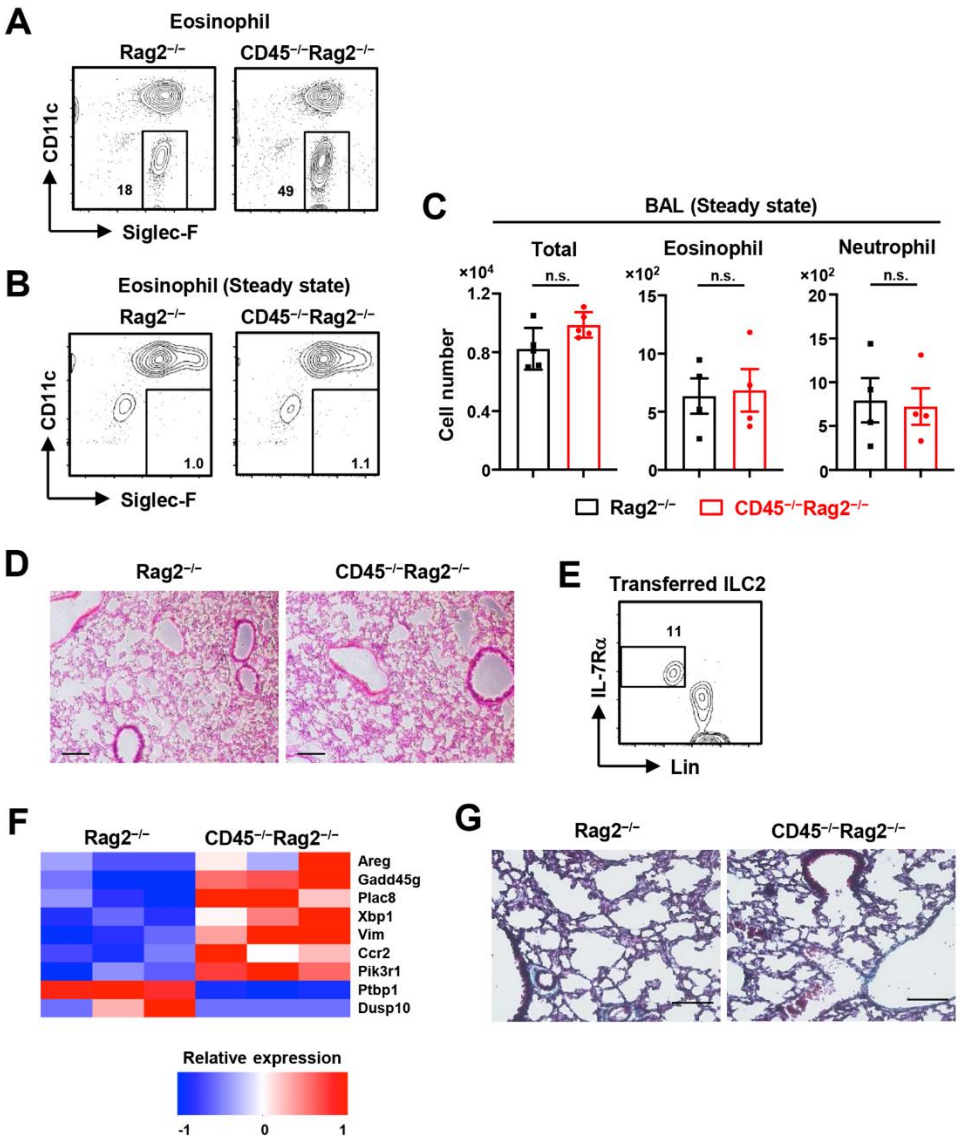

Fig. S4. Lung inflammation and activation of ILC2s in CD45$^{-/-}$Rag2$^{-/-}$ mice.
(A and B) Flow cytometric analysis of eosinophils in the lung of CD45$^{-/-}$Rag2$^{-/-}$ and control Rag2$^{-/-}$ mice after intranasal administration with papain for three consecutive days (A) or in the steady state (B). (C) Numbers of total cells, eosinophils, and neutrophils in the BAL fluid of the lung from CD45$^{-/-}$Rag2$^{-/-}$ and Rag2$^{-/-}$ mice in the steady state (n = 4). (D) H&E staining of the lung from CD45$^{-/-}$Rag2$^{-/-}$ and Rag2$^{-/-}$ mice in the steady state. Scale bar, 100 μm. (E) IL-7Rα KO mice were adoptively transferred with lung ILC2s. Flow cytometric analysis of transferred IL-7Rα^{+} ILC2s in the IL-7Rα KO recipient mice lung. (F) Heat map of differentially regulated genes activating mILC2s from the bone marrow of CD45$^{-/-}$Rag2$^{-/-}$ and Rag2$^{-/-}$ control mice in the steady state with three biological replicates. (G) Masson's trichrome staining of the lung from CD45$^{-/-}$Rag2$^{-/-}$ and Rag2$^{-/-}$ mice in the steady state. Scale bar, 100 μm. Data represent at least two independent experiments with similar results (A, B, D, E, and G). Data are mean ± SD with Student's *t*-test and pooled from two independent experiments (C). n.s., not significant.

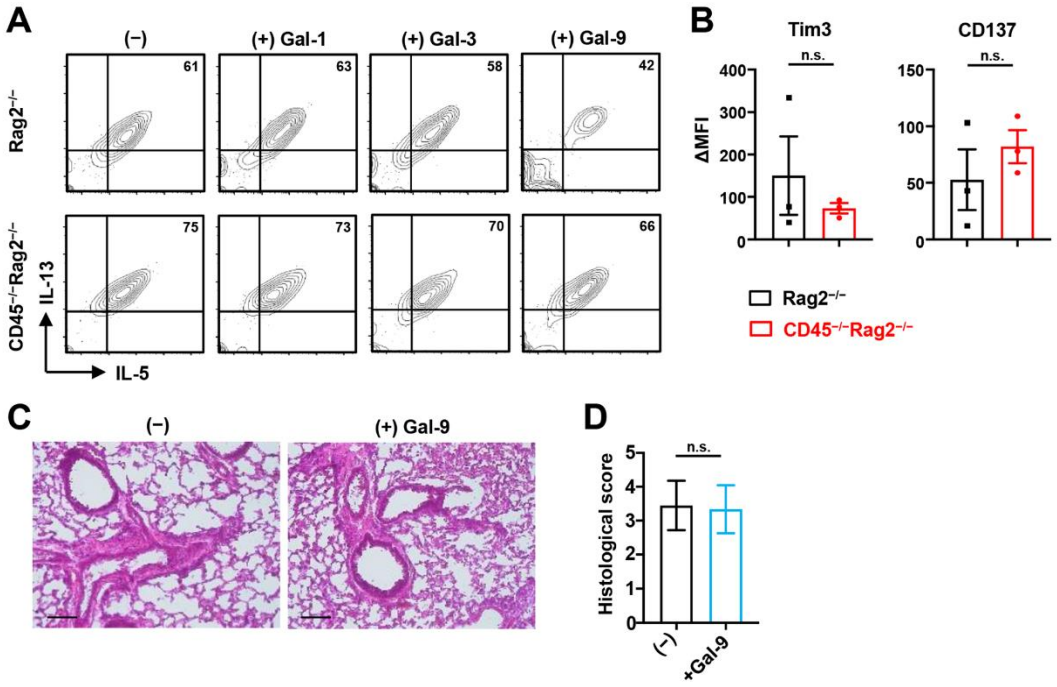

Fig. S5. Phenotypes of CD45$^{-/-}$Rag2$^{-/-}$ mice administrated with Gal-9.
(A) Flow cytometric analysis of IL-5^{+}IL-13^{+} ILC2s from the lung of CD45$^{-/-}$Rag2$^{-/-}$ and control Rag2$^{-/-}$ mice after ex vivo culture with IL-2, IL-7, IL-33, and with (+) or without (−) 5 μg/ml galectin (Gal)-1, Gal-3, or Gal-9. (B) Difference in mean fluorescence intensity (ΔMFI) between Tim3 or CD137 and isotype control in ILC2s from the lung of indicated mice at steady state (n = 3). (C and D) Mice were administered 100 μg papain for three consecutive days to induce airway inflammation and with (+Gal-9) or without (−) 30 μg mouse recombinant Gal-9 on the second and third days. H&E staining (C) and histological scores (D) of the lung from CD45$^{-/-}$Rag2$^{-/-}$ mice (n = 3). Scale bar, 100 μm. Data are mean ± SD with Student's *t*-test and pooled from two independent experiments (B and D). Data represent at least two independent experiments with similar results (A and C). n.s., not significant.

SI Material and Methods

Mice

All mice were maintained under specific pathogen-free (SPF) conditions at the Experimental Research Center for Infectious Diseases, Institute for Life and Medical Sciences, Kyoto University. All procedures were carried out under Sevoflurane anesthesia to minimize animal suffering. The Animal Experimentation Committee of the Institute for Life and Medical Sciences, Kyoto University, approved all mouse protocols.

Cell isolation

Cells were isolated from the bone marrow, lung, BAL fluid, spleen, and small intestines. Bone marrow cells were collected from the femur. After perfusion with PBS, the lungs were minced with scissors and incubated at 37°C for 1 hour in RPMI 1640 medium containing 10% FBS, 1 mg/ml collagenase D and 50 μg/ml DNase (Roche). Digested lung suspension was gently passed twice through a 24G needle, and leukocytes were separated by centrifugation through 30% Percoll.

Cell culture

ILC2s were sorted by a FACSAria cell sorter (BD Biosciences) into a tube with RPMI 1640 medium containing 10% FBS, 50 μM 2-mercaptoethanol and 10 mM HEPES (pH 7.4). To assess ex vivo production of IL-5 and IL-13, ILC2s were cultured with 10 ng/ml IL-2 and 1 ng/ml IL-33, with or without 10 ng/ml IL-7, and with or without galectin-1 or galectin-3 or galectin-9 (5 μg/ml) in 96-well round-bottom microplate at 37°C in 5% CO_2 for three days, and all cells were stained with the relevant antibodies for flow cytometry. In addition, for a 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) uptake assay, ILC2s were cultured with 20 ng/ml IL-2 and 20 ng/ml IL-33 in RPMI 1640 medium containing 10% FBS without glucose and then incubated with 10 μM 2-NBDG (Thermo Fisher Scientific) at 37°C for 20 minutes.

Flow cytometry and antibodies

Cells were surface-stained for 30 minutes at 4°C in PBS containing 0.2% BSA and 0.05% NaN_3 with fluorescent dye- or biotin-conjugated antibodies. The following fluorescent dye- or biotin-conjugated antibodies were used: anti-CD3ε (145-2C11), TCRβ (H57-597), CD4 (RM4.5), CD8α (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (MB19-1), CD25 (7D4), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CCR9 (CW-1.2), NK1.1 (PK136), γδTCR (GL-3), IL-33R (DIH9), c-kit (2B8), Sca-1 (E13-161.7), Flt3 (A2F10), CD45R/B220 (RA3-6B2), CD127 (A7R34), KLRG1 (2F1), α4β7 (DATK32), FcεRI (MAR-1), Ter119 (Ter119), ICOS (C398.4A), IL-5 (TRFK5), IL-13 (eBio13A), Gr-1 (RB6-8C5), Siglec-F (E50-2440), F4/80 (BM8), p-STAT5 (47), p-Lck (Y394) (A18002D), p-Lck (Y505) (A17013A), p-Fyn (Y530) (polyclonal), p-Lyn (Y507) (5B6), Ki-67 (SolA15), GATA-3 (16E10A23), Bcl-2 (A19-3), and rabbit IgG (Poly4064) (Thermo Fisher Scientific, BD Bioscience, and BioLegend). Biotinylated monoclonal antibodies were detected with PE- or Brilliant Violet 421-conjugated streptavidin (Thermo Fisher Scientific). The lineage marker cocktail contained antibodies against CD3ε, CD4, CD8α, CD19, CD11b, CD11c, NK1.1, Gr-1, FcεRI, and Ter-119.

For intracellular staining, cells were stained for surface antigens, fixed, permeabilized, and stained using the Foxp3 Staining Buffer Set and IC Fixation Buffer (Thermo Fisher Scientific) or Cytofix/Cytoperm Fixation/Permeabilization solution kit (BD bioscience). Cells were analyzed on a FACSVerse or LSRFortessa flow cytometers (BD Biosciences) using FlowJo software. In the figures, values in quadrants, gated areas, and interval gates indicate the percentages in each population.

Digital RNA-seq (dRNA-seq) and data analysis

ILC2Ps ($Lin^-IL\text{-}7R^+CD25^+ST2^+KLRG1^-$) and mILC2s ($Lin^-IL\text{-}7R^+CD25^+ST2^+KLRG1^+$) in the bone marrow and ILC2s ($Lin^-IL\text{-}7R^+CD25^+ST2^+KLRG1^+$) in the lung were freshly isolated from mice. Two hundred cells were sorted into a tube using a FACS Aria II for digital RNA sequencing (dRNA-seq). In the plot of PCA, 95% confidence ellipses for each group are shown. Heat maps were drawn on the gene counts, normalized to the total counts of all genes using heatmap.2 from the R package gplots. Log2 fold changes were calculated between $CD45^{-/-}$ and control ILC2s to identify differentially expressed genes. Genes were considered differentially expressed when they had $\log_2$ fold changes of ≥ 1 or ≤ −1 and *p* values ≤ 0.01.

Adoptive transfer of ILC2s

Lung ILC2s were isolated from control $Rag2^{-/-}$ or $CD45^{-/-}Rag2^{-/-}$ mice. Approximately 1×10^5 ILC2s were transferred into IL-7Rα-deficient recipients by intravenous injection (i.v.). Then, mice were intranasally administrated with papain every day for three days.

Papain-induced pulmonary inflammation

Papain (50 μg or 100 μg) in 50 μl of sterile PBS was intranasally administrated into the recipient mice on day 1, day 2, and day 3. On day 4, an animal feeding catheter (No. 5202S, Fuchigami Kikai Co., Ltd., Muko, Japan) was inserted into the trachea, and 1ml of PBS was infused into the lung through the catheter and aspirated three times to obtain BAL fluid. Then, the lung samples were collected after perfusion. For in vivo ILC2 depletion, mice were administered i.p. with 250 μg anti-mouse Thy1.2 (30H12) antibody (BioXCell) and simultaneously received i.n. with 60 ng anti-mouse Thy1.2 antibody at two and four days before administration of papain. To assess the influence of galectin-9 on lung inflammation, mouse recombinant galectin-9 (30 μg) was administered intravenously into the mice on day 2 and day 3.

Bleomycin-induced pulmonary fibrosis

Mice were intraperitoneally (i.p.) injected with 4 mg/kg bleomycin in 50 μl of sterile PBS. After seven days, the BAL fluid and lung were collected for analysis. Masson's trichrome staining evaluated collagen content and distribution. Additionally, mice were monitored daily for survival until death, or euthanasia was performed for 45 days. Mice that lost 20% of their body weight were considered to have reached humane endpoints and were euthanized according to the animal experiment protocol.

Mixed bone marrow chimera

CD45.1 × CD45.2 F1 WT hosts mice were irradiated with a single dose of 9 Gy and then i.v. injected with a 1:1 mixture of 6×10^6 congenic WT (CD45.1) and $CD45^{-/-}$ bone marrow cells. Eight weeks later, ILC2s from bone marrow and lung were isolated and analyzed by flow cytometry.

Real-time RT-PCR

Total RNA was isolated and reverse-transcribed using random primers. cDNA was analyzed by real-time RT-PCR using SYBR Green PCR Master Mix (Qiagen) in an ABI7500 Real-Time PCR System (Applied Biosystems). PCR results were normalized to corresponding levels of Hprt mRNA in cDNA from thymocytes from WT mice. The following primers were used for real-time RT-PCR: Hprt, 5'-GTTGGATACAGGCCAGACTTTGTTG-3' and 5'-GATTCAACTTGCGCTCATCTTAGGC-3'; CD45, 5'-CCTGTATCTAAACCTGAGTC-3' and 5'-GACATAGGCAAGTAGGGACA-3'.

Parabiosis

Female 8-week-old congenic WT (CD45.1) and $CD45^{-/-}$ (CD45.2) C57BL/6J mice were surgically connected in parabiosis. The lateral skin from the elbow to the knee in each mouse was sutured, the forelimbs and hindlimbs were tied together, and the skin incisions were closed using surgical adhesive. After surgery, the mice were maintained on water containing antibiotics for about two weeks to prevent infection and analyzed five weeks later.

Galectin staining by flow cytometry

$CD45^{-/-}$ or control ILC2s were resuspended in 1 μg/ml recombinant mouse galectin-9 for 30 minutes. Then, cells were washed and resuspended in anti-mouse galectin-9 antibody for 30 minutes. After staining, cells were washed and analyzed immediately on a flow cytometer.

Metabolic profiling by modified SCENITH method with OP-puromycin

Cells were plated at 2×10^6 cells/ml in 96-well plates. Cells were cultured and treated for 45 minutes at 37°C, 5% CO_2 with control (Co), 2-deoxy-D-glucose (DG; 100 mM; Sigma-Aldrich), oligomycin (O; 1 μM; Sigma-Aldrich) or a combination of both drugs (DGO). O-propargyl (OP)-puromycin (20 μM) from Click-iT Plus OPP Alexa Fluor 488 Protein Synthesis Assay Kit (Thermo Fisher Scientific) was added into the culture medium for the last 30 minutes. Cells were washed in cold PBS and stained with primary conjugated antibodies against different surface markers for 20 minutes at 4°C in a staining buffer (PBS containing 0.2% BSA). After washing with the staining buffer, cells were fixed and permeabilized using Cytofix/Cytoperm Fixation and Permeabilization Solution Kit (BD Biosciences). Then, OP-puromycin incorporation was detected following the manufacturer's instructions of Click-iT Plus OPP Alexa Fluor 488 Protein Synthesis Assay Kit and using flow cytometry.

Statistical analysis

All data are presented as means ± SD. Comparisons between two samples were performed using an unpaired two-tailed Student's *t*-test. One-way ANOVA analysis followed by Turkey's multiple-comparison test was used for multiple group comparisons. **, $p < 0.01$; n.s., not significant.