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Supplemental Information

Group 2 Innate Lymphoid Cells Exacerbate Amebic Liver Abscess in Mice

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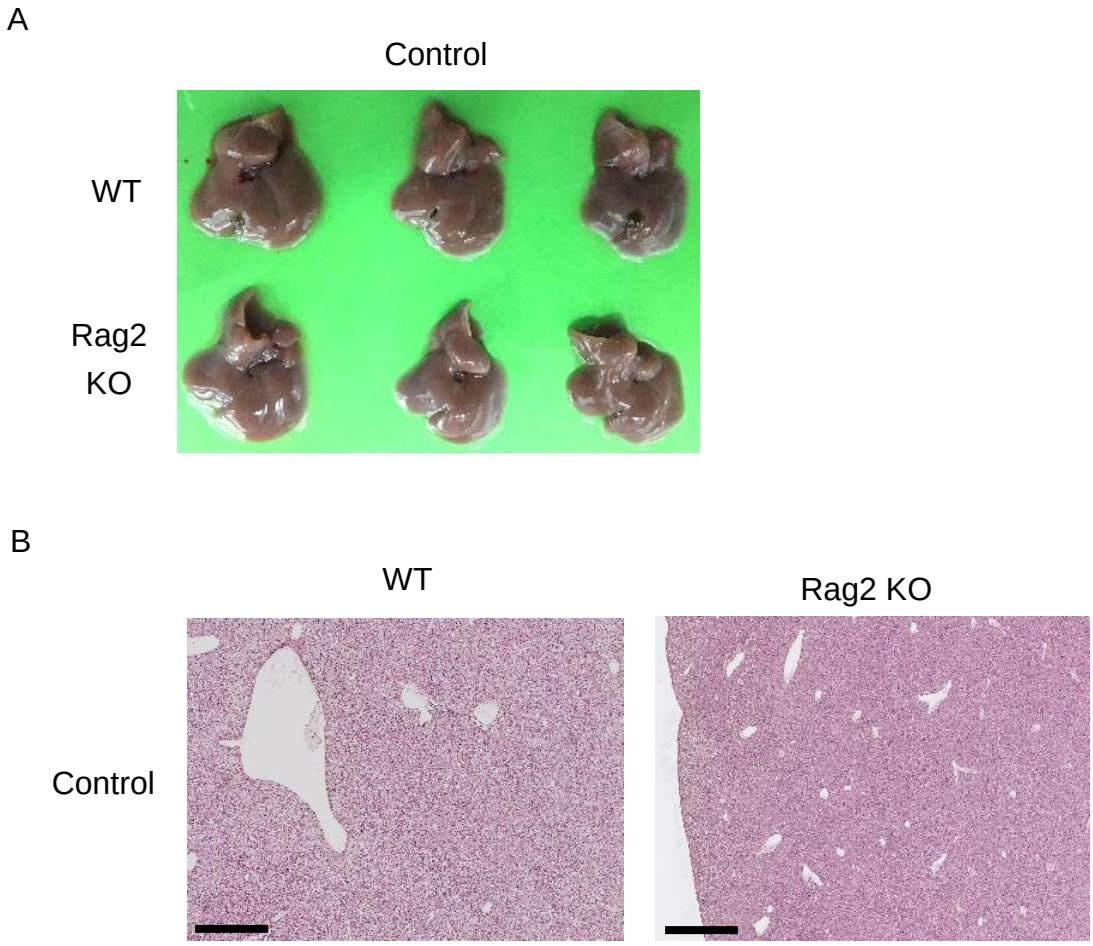
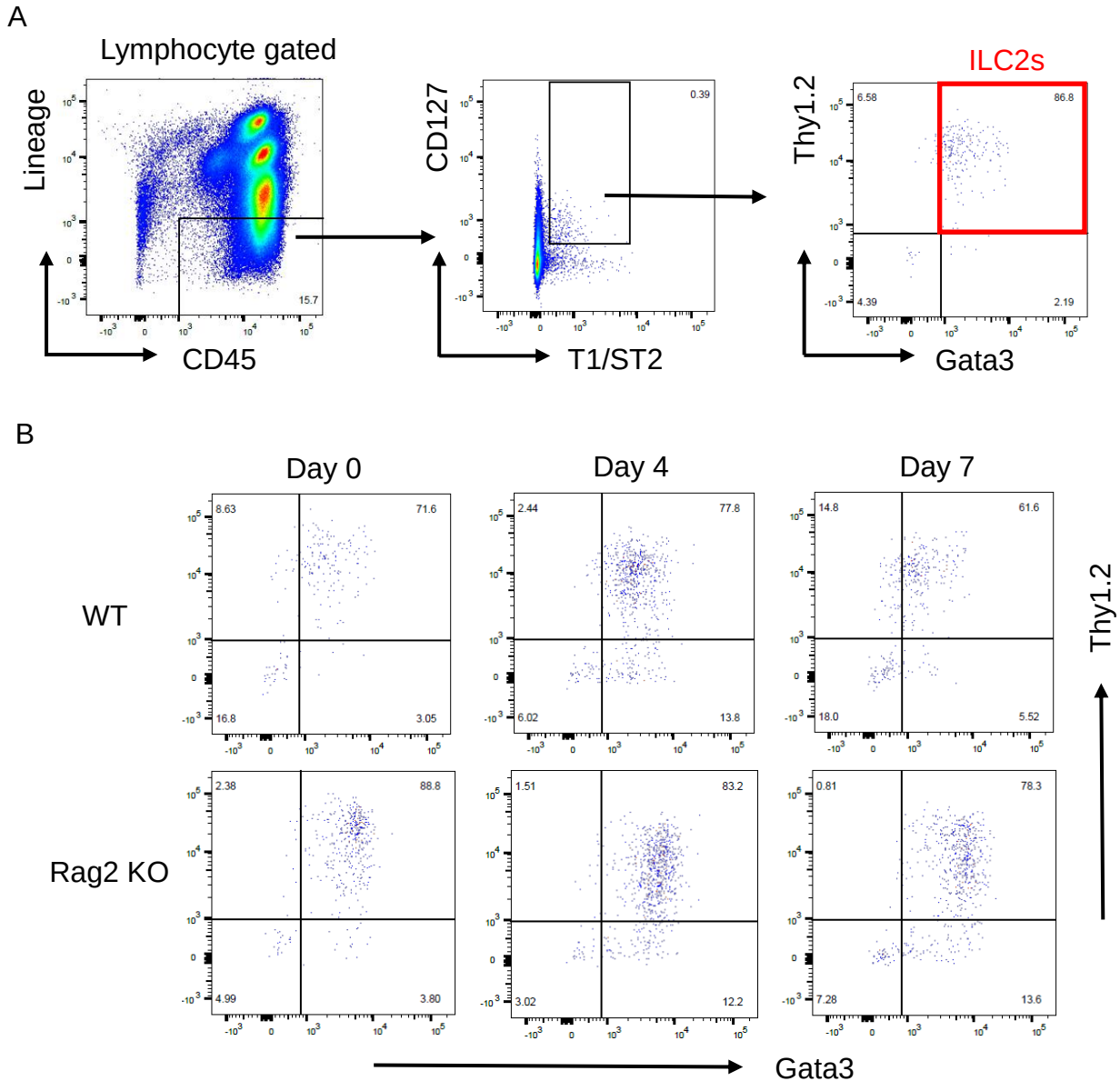
Supplemental figure 1



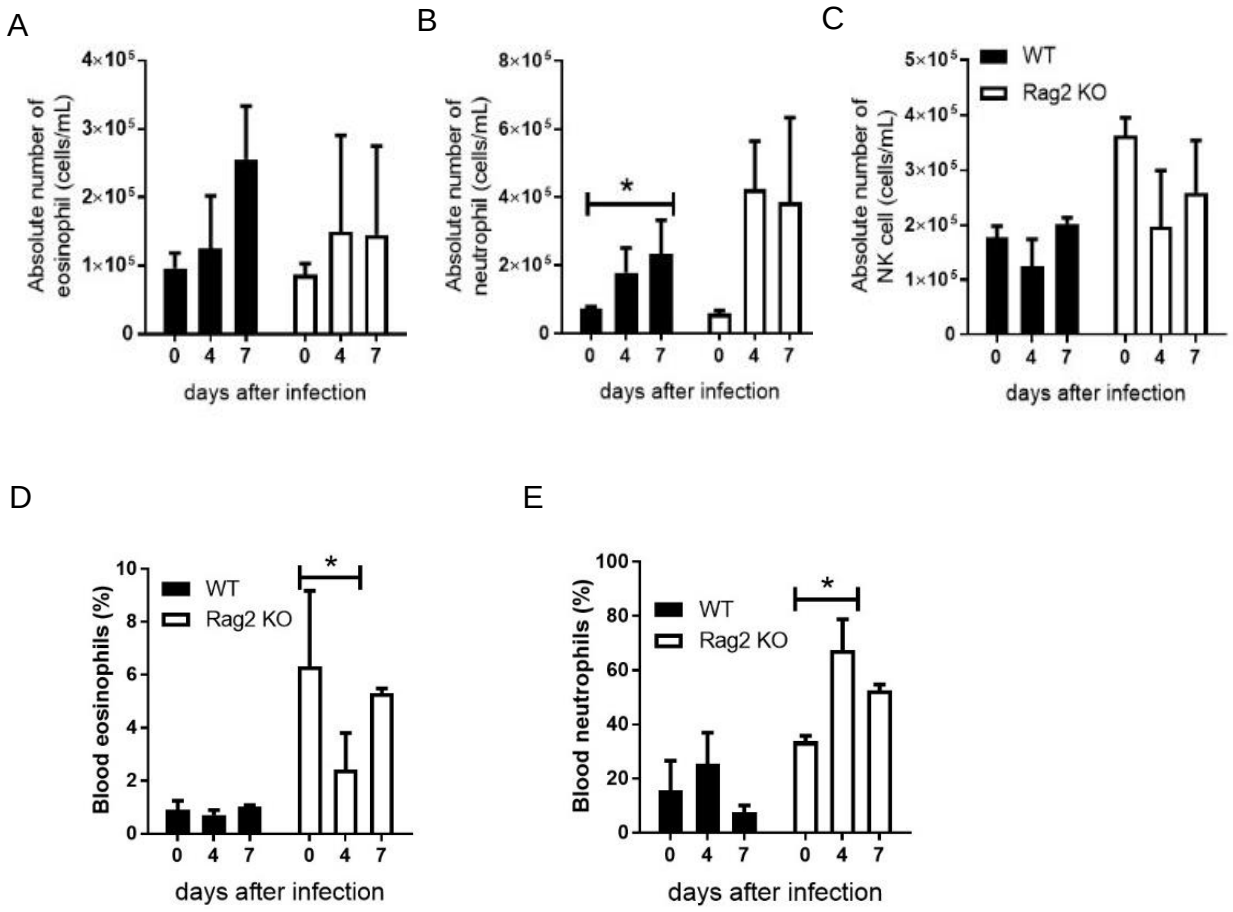
Supplemental figure 1. The observation of the livers from control WT and Rag2 KO mice. Related to Figure 1. (A) Macro observation of the livers from uninfected WT and Rag2 KO mice as a negative control (n = 3). (B) Histology of liver tissues from uninfected WT and Rag2 KO mice stained with H&E. Representative figures are shown. Original magnification: × 2.5 and Scale bar = 1 mm.

Supplemental figure 2



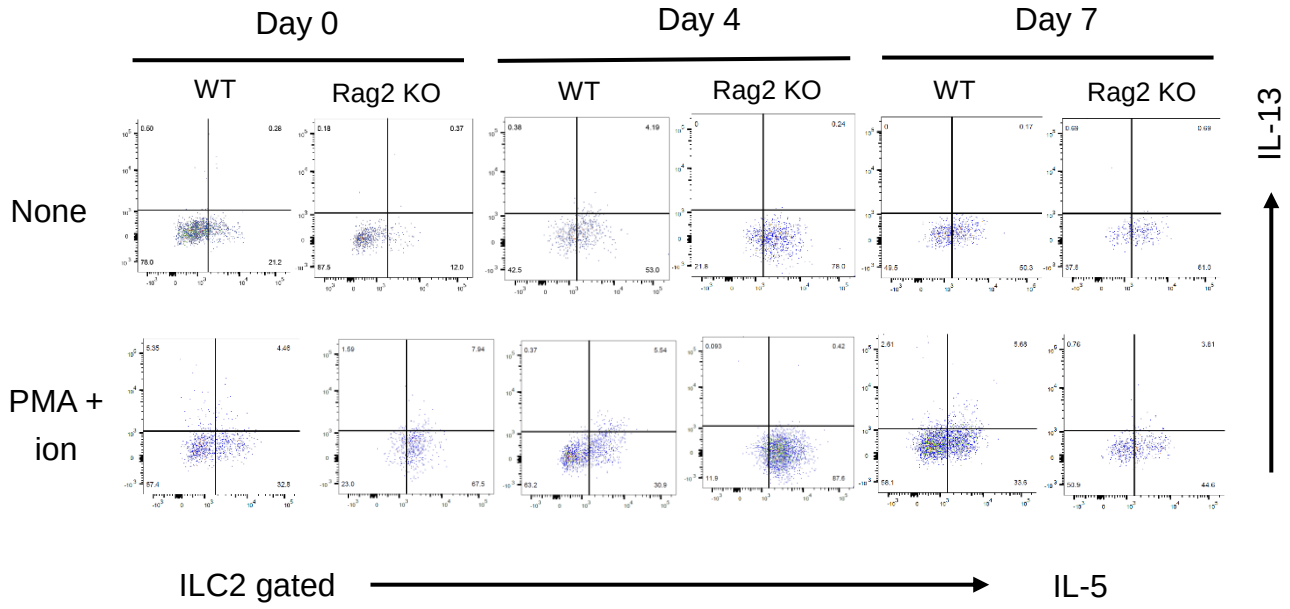
Supplemental figure 2. The kinetics of ILC2s in the livers from WT and Rag2 KO mice after *E. histolytica* injection. Related to Figure 1. Hepatic lymphocytes were collected from WT and Rag2 KO mice on day 0 (naïve), 4 and 7 after *E. histolytica* injection. (A) Gating strategy of hepatic ILC2s in naïve WT mice by flow cytometry. Hepatic ILC2s were detected as a lineage$^-$ CD45$^+$ Gata3$^+$ Thy1.2$^+$ T1/ST2$^+$ gated population. A red square on the dot plot showed hepatic ILC2s. (B) Flow cytometry of hepatic ILC2s in WT and Rag2 KO mice after *E. histolytica* injection. Representative dot plots are shown after gating as in (A). Data are representative of three independent experiments with similar results.

Supplemental figure 3



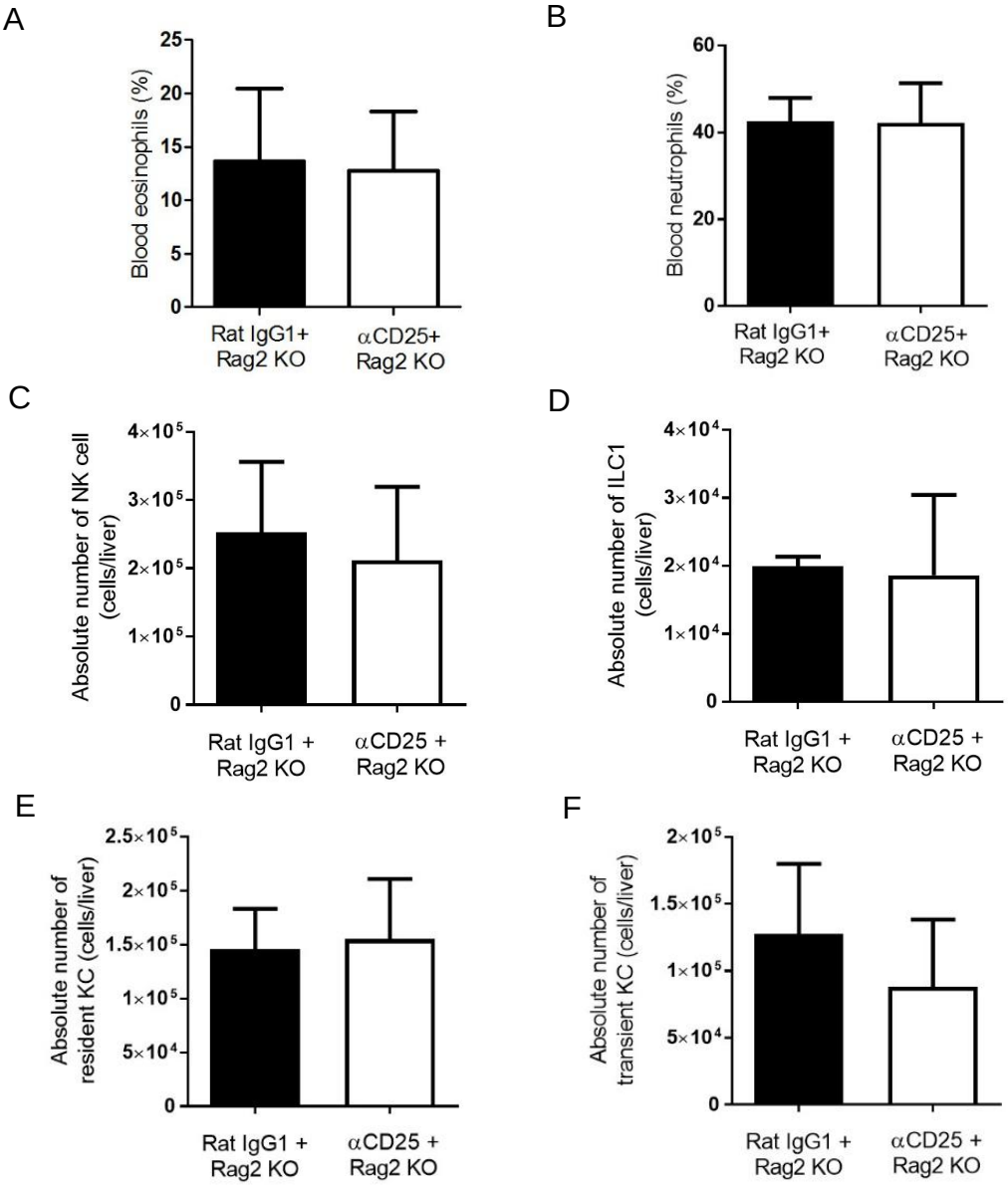
Supplemental figure 3. The kinetics of immune cells in the livers from WT and Rag2 KO mice during ALA formation. Related to Figure 1. The absolute numbers of eosinophil (A; MHC class II$^-$ SiglecF$^+$ CD11b$^+$ cells), neutrophil (B; CD11b$^+$ Gr-1$^+$ F4/80$^-$ MHC class II$^-$ SiglecF$^-$ cells), NK cell (C; CD49b$^+$ NK1.1$^+$ NKp46$^+$ CD3$^-$ MHC class II$^-$ cells), and the proportion of blood eosinophil (D) and neutrophil (E) were analyzed by flow cytometry. Hepatic immune cells and blood cells were isolated from WT and Rag2 KO mice on days 0 (naïve), 4 and 7 after *E. histolytica* injection (n = 3 per time point in each group). Data are representative of three independent experiments with similar results. Statistically significant differences between day 0 and indicated time points in each group are indicated with *P* value (*$P < 0.05$, ANOVA). Each point shows the mean ± standard deviation (SD).

Supplemental figure 4



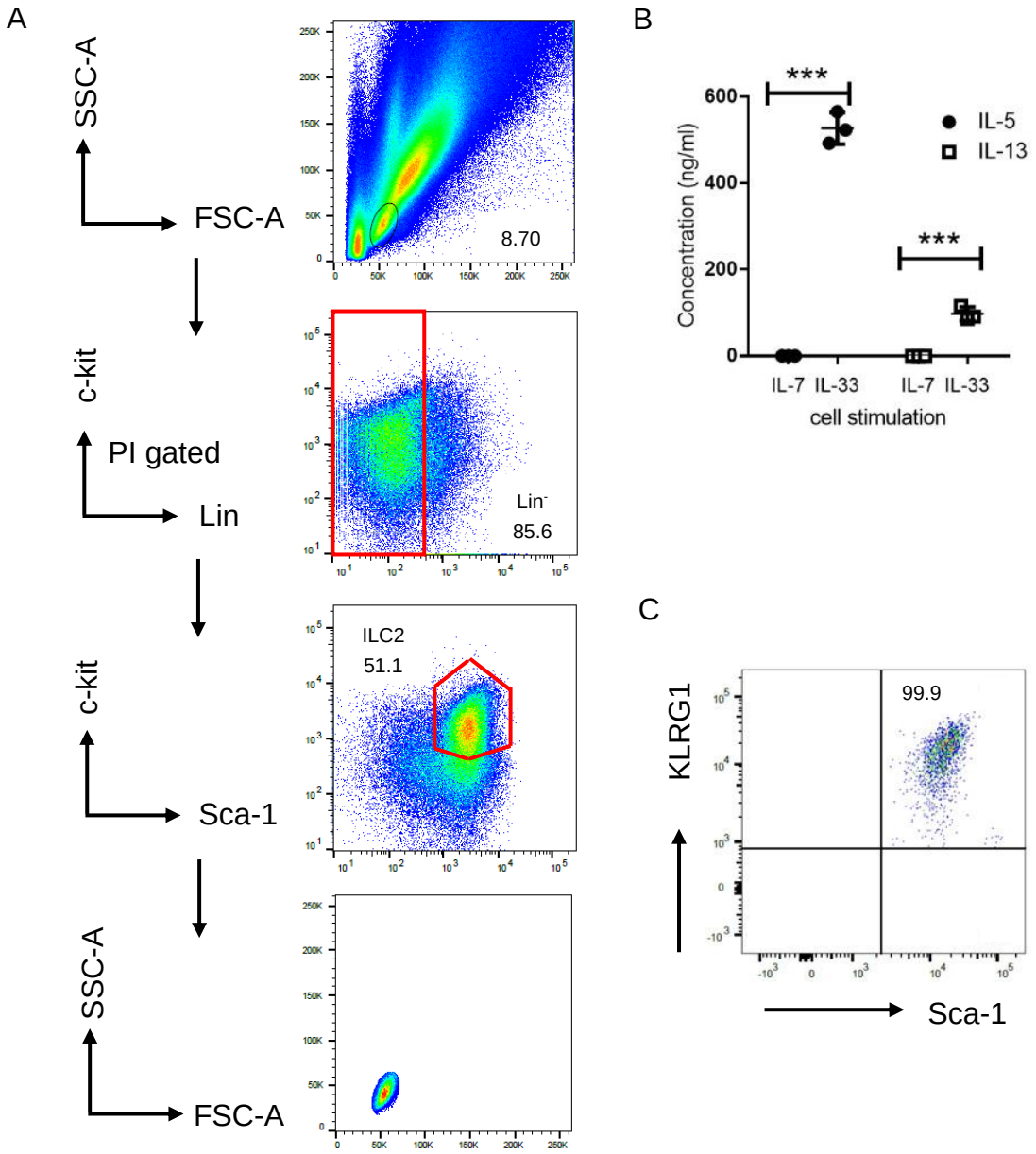
Supplemental figure 4. Dot plots of IL-5$^+$ and IL-13$^+$ ILC2s in the livers from WT and Rag2 KO mice during ALA formation. Related to Figure 2. Flow cytometry of IL-5 and IL-13 producing ILC2s in the livers from WT and Rag2 KO mice on days 0, 4, and 7 (n =3 per each group). The hepatic lymphocytes were stimulated with PMA and ionomycin for 12 hrs. Brefeldin A was added for the last 9 hrs. IL-5 and IL-13-producing ILC2s were detected by intracellular cytokine staining. Representative dot plots were shown after gating ILC2s. Data are representative of three independent experiments with similar results.

Supplemental figure 5



Supplemental figure 5. The effect of αCD25 mAb treatment on other immune cells. Related to Figure 3. (A-F) The proportion of eosinophil (A) and neutrophil (B) in the blood and the absolute numbers of hepatic NK cell (C; KLRG1$^+$ CD49b$^+$ NK1.1$^+$ NKp46$^+$ cells), ILC1 (D; KLRG1$^-$ CD49b$^-$ NK1.1$^+$ NKp46$^+$ cells), resident Kupffer cell (KC) (E; CD11b^{lo} F4/80^{hi} Gr1$^-$ cell) and transient inflammatory monocyte-derived KC (F; CD11b^{hi} F4/80^{lo} Gr1$^-$ cells) were analyzed by flow cytometry. Immune cells in the liver and blood were isolated from Rag2 KO mice treated 2 doses administration of αCD25 mAb (n = 4 in each group). Each data shows the mean ± standard deviation (SD).

Supplemental figure 6



Supplemental figure 6. Sorting strategy and phenotype of transferred ILC2s. Related to Figure 4. The sorting strategy of ILC2s from the mesentery of naïve WT mice by flow cytometry for *in vivo* transfer. Transferred ILC2s were sorted as a c-kit^{+} Sca-1^{+} lineage^{-} PI^{-} cell population (A). IL-5 and IL-13 production by sorted ILC2s co-cultured with IL-33 or IL-7 were measured by Bioplex (B). Statistically significant differences between IL-7 and IL-33 stimulation in each cytokine production are indicated with *P* value (***$P < 0.01$, unpaired *t*-test). Each point shows the mean ± standard deviation (SD). Confirmation of transferred ILC2s presenting in the liver on day 4 after ameba injection by flow cytometry (C; Gata3^{+} CD45^{+} lineage^{-} gated).

Supplemental figure 7

Biotin-conjugated antibodies	Clone
CD11b	M1/70
CD11c	N418
CD3ε	145-2C11
CD19	MB19-1
Ly6G/Ly6C (Gr1)	RB6-8C5
F4/80	BM8
TER-119/Erythroid cells	TER-119
FcεR1α	MAR-1
NK1.1	PK136

Supplemental figure 7. The list of lineage markers for hepatic ILC2 staining. Related to Figure 1 and Transparent Methods. Hepatic lymphocytes were stained for detecting ILC2s with biotin-conjugated antibodies of these lineage markers.

Transparent Methods

Mice. Age- and sex-matched C57BL/6 wild-type (WT) male mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). $Rag2^{-/-}$ (Rag2 KO) mice (Stock #RAGN12), $Rag2^{-/-}\gamma_c{}^{-/-}$ (DKO) mice (Stock #4111) mice were purchased from Taconic. $IL\text{-}33^{GFP/GFP}$ (IL-33-deficient) mice (Oboki et al., 2010) were kindly provided by Dr. Nakae, Hiroshima University. All mice were maintained under specific pathogen-free conditions and offered food and water ad libitum. All mice used were 8-10 weeks old.

Parasite culture and Infection. *E. histolytica* strain JPN51 was kindly gifted from Dr. Eric Houpt, University of Virginia. Trophozoites were serially passaged *in vivo* by intra-cecally inoculating *E. histolytica* to mice, collected from cecal contents and then cultured *in vitro* at 37 °C in Biostate-Iron-Serum-33 (BIS-33) media supplemented with heat-inactivated 10% adult bovine serum, 25 U/ml penicillin and 25 mg/ml streptomycin. An intra-portal vein inoculation procedure was precisely described in a previous publication (Deloer et al., 2016; Goddard et al., 2016). In brief, trophozoites were collected from flasks by cooling them on ice for 3-5 minutes, were then washed by PBS twice. Mice were anesthetized with 1-2% isoflurane (Wako, Tokyo, Japan) using inhalation anesthesia apparatus and were maintained body temperature using the heating pad. Each portal vein

was exteriorized from the peritoneum, and 50 µl of 2 × 10^5 trophozoites were injected into the portal vein ~ 10 mm below the liver at an angle < 5° to the vein, with the bevel facing up. After removed the needle, the injection site was held under pressure with a sterile cotton swab for 3 min. Once mice were stopped bleeding at the injection site of the portal vein, the peritoneal linings and then the skin was sutured with sterile 4-0 vicryl suture and taper needle using a simple continuous or interrupted suture pattern. Uninfected mice were injected 50 µl of PBS into the portal vein in the same way. After the surgical procedure was complete, mice were maintained on a heating pad for recovery in bedding-free, clean cages for a minimum of 20 min.

RNA isolation and quantitative real-time PCR. After sacrifice, 100 µl of liver homogenates were collected and stored at -80°C. Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The first-stranded cDNA synthesis was done using the PrimeScript RT reagent kit (Takara, Tokyo, Japan) according to the manufacturer's instruction. Real Time-qPCR was performed with QuantiTect® Primer Assay (QIAGEN) for detecting IL-5 (QT00099715), IL-13 (QT00099554), IL-25 (QT00134645) and IL-33 (QT00135170) using QuantiTect® SYBR Green PCR kit (QIAGEN) on the Quant Studio™ 7 Flex Real-Time PCR System

(Applied Biosystems, Life Technologies™, CA, USA). The manufacturer does not open the primer sequences for these cytokines. The cycle time value of each cytokine gene was normalized with GAPDH of the same sample; fold induction for each gene expression was calculated by the ΔCt method. Results obtained each PCR were pooled and statistically analyzed.

Histology and assessment of ALA formation. Liver tissues were removed and fixed in 10% neutral buffered formalin and then embedded in paraffin. After cutting into round slices, the tissue sections were stained with hematoxylin and eosin (H&E) and examined microscopically using NanoZoomer (Hamamatsu Photonics, Shizuoka, Japan). For the assessment of ALA formation, the area and the number of ALA in the liver section of each group of a mouse were calculated by NDP.view 2 software. The area of ALA showed an area of all abscesses in a liver section of each mouse of the group. The number of ALA showed the average of the number of ALA in non-sequential three liver sections from each mouse of the group.

Isolation of hepatic lymphocytes. On indicated days after infection, mice were dissected and hepatic lymphocytes were prepared as described previously (Yajima et al., 2004).

Briefly, liver tissue was removed and separately placed in gentle MACS Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) containing 3 ml of HBSS. The resulting homogenate suspension was filtered with 100 μm EASY strainers (Greiner Bio-one, Tokyo, Japan) and pelleted by centrifugation. The pellet was resuspended in RPMI 1640 with 10% FBS containing 33% Percoll (GE Healthcare Bio-Sciences, Uppsala, Sweden) and 10% heparin sodium, then centrifuged at 940 x g for 20 min. Cells at the bottom of the tube were harvested and washed extensively before use.

Antibodies for flow cytometry and immunofluorescence. All cells were stained with various combinations of mAbs. The following mAbs were purchased: biotin-conjugated anti-CD3ε (clone 145-2C11; BioLegend, Tokyo Japan), anti-CD11b (clone M1/70; BioLegend), anti-CD45R/B220 (clone RA3-6B2; BioLegend), anti-I-A/I-E (clone M5/114.15.2; BioLegend), anti-Ly-6G/Ly-6C (Gr-1, clone RB6-8C5; BioLegend), anti-TER-119/ Erythroid Cells (clone TER-119; BioLegend), anti-FcεRIα (clone MAR-1; BioLegend), anti-CD11c (clone N418; Affymetrix, Tokyo, Japan), anti-F4/80 (clone BM8; Affymetrix), anti-CD19 (clone MB19-1; Affymetrix), anti-NK1.1 (CD161, clone PK136; TONBO biosciences, San Diego, CA, USA) and PerCP-Cy5.5 Streptavidin (BioLegend) as the lineage markers (Supplemental figure 7). FITC-conjugated anti-

T1/ST2 (clone DJ8) was purchased from MD Biosciences (Oakdale, MN, USA). PE-conjugated anti-GATA3 (clone L50-823), anti-SiglecF (clone E13-161.7), anti-KLRG1 (clone 2F1), PE-Cy7-conjugated anti-CD127 (clone SB/199), anti-CD117 (c-kit, clone 2B8) and FITC-conjugated anti-NKp46 (CD335, clone 29A1.4), anti-Ly-6A/E (Sca-1, clone E13-161.7) were purchased from BD Biosciences (San Jose, CA, USA). Allophycocyanin (APC)-conjugated anti-CD127 (clone SB/199), anti-IL-5 (clone-TRFK5), anti-CD49b (clone Dx5), anti-IL-17RB (clone 9B10), APC-Cy7-conjugated anti-CD25 (clone PC61), anti-CD45 (clone 30-F11), FITC-conjugated anti-CD11b (clone M1/70), anti-NK1.1 (clone PK136), PE-conjugated anti-Ly-6G/Ly-6C (Gr-1, clone RB6-8C5) and PE-Cy7-conjugated anti-Thy1.2 (CD90.2 clone 30-H12), anti-CD45 (clone 30F11), anti-F4/80 (clone BM8) were purchased from BioLegend. PE-Cy7-conjugated anti-IL-13 (clone eBio13A) and PE-conjugated anti-IL-5 (clone TRFK5) were purchased from Affymetrix. The stained cells were acquired and analyzed in a FACSVerse flow cytometer (BD Biosciences). The data were analyzed using FlowJo v10 software (FlowJo, LLC, Ashland, OR, USA). The following anti-mouse antibodies used for immunofluorescence: anti-mouse IL-33 antibody (Catalog # AF3626; R&D Systems), Alexa Fluor® 647-conjugated anti-GATA3 (Clone L50-823, Catalog # 560078; BD Pharmingen^{TM}), PE-conjugated anti-IL-33 receptor (T1/ST2) (Clone U29-93 Catalog #

566311; BD Pharmingen™), Alexa Fluor® 488-conjugated donkey anti-goat IgG (H+L) (Catalog # A11055; Thermo Fisher Scientific), Alexa Fluor® 555-conjugated donkey anti-rabbit IgG (H+L) (Catalog # A31572; Thermo Fisher Scientific), anti-phycoerythrin antibody (Catalog # PA5-35006; Thermo Fisher Scientific) and anti-mouse CD16/32 (Fc Block, clone 2.4G2; purified in our lab).

Intracellular cytokine staining. Hepatic lymphocytes were incubated without any stimulation or with 20 ng/ml PMA (Sigma) and 1 μg/ml ionomycin (Invitrogen) for 12 h at 37°C in 5% CO_2, and 10 μg/ml brefeldin A (Sigma-Aldrich) added 3 h later, at a concentration of 1×10^6 in RPMI containing 10% FCS. After culture, cells were stained with various combinations of mAbs. After surface staining, cells were subjected to staining of intracellular-cytokine and transcription factor using the manufacturer's instructions. In brief, for transcription factor staining, 1 ml of fix and permeabilization solution (Foxp3 transcription staining kit, eBiosciences) was added to the cell suspension with mild mixing and placed for 20 min at RT. Fixed cells were washed with 2 ml of Perm/Wash solution (eBiosciences) twice and were stained intracellularly with various combinations of antibodies for 30 min at RT. For intracellular cytokine staining, cells were fix and permeabilization with IntraPrep Permeabilization Reagent (Beckman

Coulter, Brea, CA, US). Samples were acquired in a FACSVerse flow cytometer (Becton, Dickinson, Franklin Lakes, NJ, US) and analyzed by FlowJo V10 software.

ELISA and Bioplex. Supernatants of the liver homogenates from mice at the indicated times after *E. histolytica* injection were obtained by centrifugation at 440 × g for 3 min at 4 °C. For the detection of IFN-γ in the culture supernatants of the hepatic lymphocytes, 5 × 10^5 cells/ well of whole hepatic lymphocytes were stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) for 3 days, then IFN-γ secretion in the supernatants were measured with a DuoSet ELISA development system (DY485, R&D Systems) according to the manufacturer's instructions. Briefly, 96-well immune plates were coated with a goat-anti-mouse IFN-γ affinity-purified antibody (Ab) as the capture Ab. Following primary incubation, samples were treated with biotinylated goat anti-mouse IFN-γ mAb to detect these cytokines. Plates were subsequently incubated with streptavidin conjugated to HRP and visualized using a substrate solution. For the measurement of IL-5 and IL-13 by Bioplex, 1 × 10^4 of sorted mesentery ILC2s were co-cultured with IL-7 or IL-33 (10 ng/ml) for 5 days. Then, IL-5 and IL-13 secretion in the supernatants were measured with Bio-Plex Pro mouse cytokine GI 23-Plex (Panel #M60009RDPD, Bio-Rad) according to the manufacturer's instructions and detected by Bioplex 200 (Bio-Rad). The data were

analyzed by Bio-Plex Manager Software.

***In vivo* depletion of ILC2s and neutralization of IL-5.** To deplete ILC2s, anti-mouse (α) CD25 (IL-2Rα) mAb (clone PC-61.5.3; BioXCell, West Lebanon, NH, USA) was intraperitoneally injected into Rag2 KO mice at a dose of 200 μg/ mouse every 2 days from 3 days before *E. histolytica* infection. The isotype-matched control IgG (Rat IgG1, clone HRPN) was obtained by BioXCell. For the confirmation of ILC2-depletion, hepatic lymphocytes from the mice administrated αCD25 mAb was purified and analyzed a proportion of lineage$^-$ CD45$^+$ Gata3$^+$ Thy1.2$^+$ T1/ST2$^+$ ILC2s using flow cytometer. To neutralize IL-5, αIL-5 mAb (clone TRFK5; BioXCell) was intraperitoneally injected into Rag2 KO mice at a dose of 250 μg/ mouse every 2 days from 3 days before *E. histolytica* infection. Rat IgG1 for αCD25 mAb (clone HRPN) was used as the isotype-matched control IgG for αIL-5 mAb.

Adoptive transfer of ILC2s. Naïve ILC2s were isolated from the mesentery of WT mice as described (Moro et al., 2015) and cultured in 96-well round bottom plate with RPMI-1640 medium (Sigma) containing 10% FCS, 50 μM 2-mercaptoethanol (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), 1× non-essential amino acids

(Sigma), 10 mM HEPES (Sigma), and 1 mM sodium pyruvate (Gibco) and IL-2 (10 ng/ml) (Moro et al., 2015). A total of 2×10^6 cultured naïve ILC2s were intravenously transferred into the naïve Rag2$^{-/-}\gamma_c^{-/-}$ double KO mice one day before *E. histolytica* injection.

Immunofluorescence.

Infected or uninfected liver tissues were harvested, submerged into 20% sucrose overnight and embedded into O.C.T compound (Sakura Finetek U.S.A., Inc., Torrance, CA) to be snap frozen using liquid nitrogen. Sectioning was performed on a cryostat (Leica CM 1950) at 3.5μm thickness. Thoroughly air-dried sections were fixed with ice-cold acetone/methanol (1:1 mixture) for 5 min, air-dried again, and stored, if needed, in -80 °C until use. After being rehydrated with PBS for 5 min sections were blocked with 5% FBS in PBS-T (0.05% Tween) and Fc Block (5 μg/mL) in a moisture box for 2h at room temperature. Sections were stained with anti-mouse IL-33 antibody (1:50 dilution, 1% FBS in PBS-T), PE-conjugated anti-IL-33 receptor (T1/ST2) (1:200) and Alexa Fluor® 647-conjugated anti-GATA3 (1:50) overnight at 4 °C. Slides were rinsed with PBS-T on a shaker for 30 min at 4 °C. To amplify PE-conjugated antibody for primary staining, sections were labeled with rabbit anti-phycoerythrin antibody (1:400) for 1h at

4 °C, washed for 30 min again, further incubated with Alexa Fluor® 555-conjugated donkey anti-rabbit IgG (H+L) antibody (1:1600) for 1h at 4 °C as previously described (Yoshizawa et al., 2018). To visualize non-labeled purified antibody for primary staining, like anti-IL-33, Alexa Fluor® 488-conjugated donkey anti-goat IgG (H+L) (1:800) was used for secondary antibody simultaneously. After final wash for 30 min, sections were mounted with ProLong™ Diamond Antifade with DAPI (Thermo Fisher Scientific). Leica TCS SP8 laser scanning confocal microscope equipped with an acousto-optical beam splitter (AOBS) system (Leica Camera AG, Wetzlar, Germany) with a 40x oil objective (PL APO, NA1.25) was also used for image acquisition with LAS AF software. Image processing and analyses were carried out using for Fiji/ImageJ (http://rsbweb.nih.gov/ij/) as well.

Statistics. Figures and Statistical significance were evaluated GraphPad Prism software (GraphPad, San Diego, CA, USA). Mice were allocated to experimental groups based on their genotypes and treatments. There is no mouse to exclude from the analyses. For assessments of ALA and analyses of hepatic lymphocytes infiltration, unpaired 2-tailed Student's t-test was performed when 2 experimental groups were compared. For the kinetics of lymphocyte number and mRNA expression compared with day 0 in each group

and *in vivo* transfer experiment, 1-way ANOVA was performed. *P* values with <0.05 were considered to represent a significant difference.

Study approval. This study was approved by the Committee of Ethics on Animal Experiments of the Nagasaki University (the approval number of animal experiments; 1502181226, the approval number of recombinant DNA experiments; 1902201550) and RIKEN. Experiments were carried out under the control of the Guidelines for Animal Experiments of both Nagasaki University and RIKEN.

Supplemental References

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