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Unconventional ST2- and CD127-negative lung ILC2 populations are induced by the fungal allergen *Alternaria alternata*



To the Editor:

In the last decade, group 2 innate lymphoid cells (ILC2s) have been recognized for their ability to drive type 2 responses in experimental animal models in the absence of T cells.^{1,2} When mice are intranasally challenged with *Alternaria alternata*, a fungal allergen associated with severe asthma and fatal exacerbations in humans,³ ILC2s are potentially activated via the IL-33/ST2 (IL-33R) axis to robustly secrete type 2 cytokines.⁴⁻⁶ Animal models, including *A alternata*-induced lung inflammation in mice, have provided critical insight into pathways of ILC2 regulation that appear to apply to ILC2 responses in human disease.⁷ Thus, accurate identification of ILC2s in the lungs of mice is critical to future understanding of their biology and contribution to allergic disease. We assessed whether the conventional ILC2 identification markers ST2 (IL-33R) and CD127 (IL-7R) sufficiently include all type 2 cytokine-producing ILCs in the mouse lung.

Wild-type (WT) mice were challenged intranasally with *A alternata* for 3 consecutive days, and 24 hours after the last challenge, bronchoalveolar lavage (BAL) and lung tissue were harvested. Mice challenged with *A alternata* demonstrated increased peribronchial inflammation (Fig 1, A), robust BAL and lung eosinophil recruitment (Fig 1, B), as well as elevated BAL IL-5 and IL-13 levels (Fig 1, C). Conventional ILC2s were identified as CD45⁺Lineage⁻Thy1.2⁺ST2⁺CD127⁺ lymphocyte-sized cells (Fig 1, D; Fig E1, A; see this article's [Methods section](#) in the Online Repository at www.jacionline.org). As expected, total conventional ILC2s expanded after *A alternata* challenge (Fig 1, E). Unexpectedly, ILC populations lacking either ST2 or CD127 or both proliferated significantly, with reduced apoptosis, and represented approximately 40% of the total ILC population (Fig 1, E; see Fig E1, B, in this article's Online Repository at www.jacionline.org). All ILC subsets varied within 10% before, during, and after *A alternata* (Fig E1, C) challenges and also displayed robust increases in IL-5 (Fig 1, F and G; see Fig E1, D), IL-13 (see Fig E2, A, in this article's Online Repository at www.jacionline.org), and GATA-3 (Fig 1, G; see Fig E2, B) expression. Furthermore, all 4 subpopulations also highly expressed CD25 (IL-2R α), killer lectin like receptor G1 (KLRG1), inducible T-cell costimulator (ICOS), stem cell antigen-1 (Sca-1), c-Kit, CD44, and CD69, and expression of these markers (except c-Kit) was upregulated by *A alternata* (see Fig E3 in this article's Online Repository at www.jacionline.org). RAG2 knockout mice that lack B and T cells but have ILCs showed similar ILC subset activation after *A alternata* challenges (see Fig E4 in this article's Online Repository at www.jacionline.org). Overall, though conventional ILC2s

represented the highest total Th2 cytokine-producing ILCs, the sum of the 3 unconventional populations in WT mice approximated 40% of the total ILC2 burden.

To confirm that processing for ILC cytokines using calcium ionophore (see this article's [Methods section](#)) did not influence the presence of IL-5 production by the different ILC subsets, we performed *A alternata* challenges in heterozygous IL-5 reporter (Red5) mice. CD45⁺Lineage⁻Thy1.2⁺ lymphocytes were gated, and all subsets (based on ST2 and CD127) showed significant increases in IL-5 expression after *A alternata* challenges (Fig 1, H), though the 2 ST2⁻ populations showed the lowest overall % IL-5 production, suggesting a heterogeneous mix of ILCs in these subsets. Using the same mice, we found that unconventional ILC2s represented approximately 25% of all IL-5⁺ CD45⁺Lineage⁻Thy1.2⁺ cells (Fig 1, I). Thus, in WT mice and reporter mice, 25% to 40% of ILC2s are negative for either ST2 or CD127 or both.

In light of recent reports of ILC plasticity,^{8,9} we investigated whether *A alternata* also induced ILC1 and ILC3 responses. Surprisingly, IFN- γ was detected in the BAL and increased significantly after *A alternata* challenge (see Fig E5, A, in this article's Online Repository at www.jacionline.org) though IL-17A was not detected (not shown). IFN- γ ⁺ ILCs were increased in percent and total numbers (Fig E5, B), whereas IL-17A⁺ ILCs were largely increased in total numbers only in challenged mice (Fig E5, C). We found that conventional and unconventional ILC2s expressed similar levels of the ILC1 surface marker IL-18R,⁹ and expression was nearly doubled after *A alternata* challenge (Fig E5, D). All 4 populations also expressed low levels of ROR γ t (Fig E5, E) and T-bet (Fig E5, F) relative to GATA-3, and expression was not significantly different after *A alternata* challenge. To elucidate whether the mixed ILC phenotypes were due to hybrid cells or heterogeneous populations, we investigated the simultaneous expression of ILC1, ILC2, and ILC3 cytokine production (see Fig E6, A and B, in this article's Online Repository at www.jacionline.org). We detected low levels of IL-5⁺IFN- γ ⁺ ILC2s and IL-5⁺IL-17A⁺ ILC2s in each population, and the frequency of double-positive cells within each ILC2 subpopulation increased with *A alternata* challenge. IL-17⁺ cells largely appeared to be distinct from IL-5⁺ cells though overall IL-5⁺IFN- γ ⁺ and IL-5⁺IL-17A⁺ ILC2s were increased after *A alternata* challenge (Fig E6, A and B). In aggregate, these results suggest that the ILC populations contain a heterogeneous mixture of hybrid ILCs and distinct ILC1s and ILC3s.

We next compared these ILC populations using RNA sequencing and quantitative PCR with fluorescence-activated cell-sorted lung ILC subsets (see Fig E7, A, in this article's Online Repository at www.jacionline.org; see this article's [Methods section](#)) from mice challenged with *A alternata*. In agreement with our flow cytometry protein-level data, we found high levels of ILC2-defining surface markers, transcription factors, and cytokine transcripts expressed by all 4 subpopulations, relative to ILC1 and ILC3 signatures (Fig 2, A and B). Importantly, RNA-Seq and quantitative PCR confirmatory studies demonstrated that the ST2⁻ populations express reduced levels of Il1rl1 transcript (encodes ST2), and the CD127⁻ populations express less Il7ra transcript (Fig E7, B and C). Consistent with the protein data, the ST2⁺ populations expressed more ILC2-related transcripts (Gata3, Il5, Il9, Il13, and Rora) compared with the ST2⁻ counterparts. The ST2⁺CD127⁻ population overall expressed the greatest number of ILC2-related transcripts, whereas the

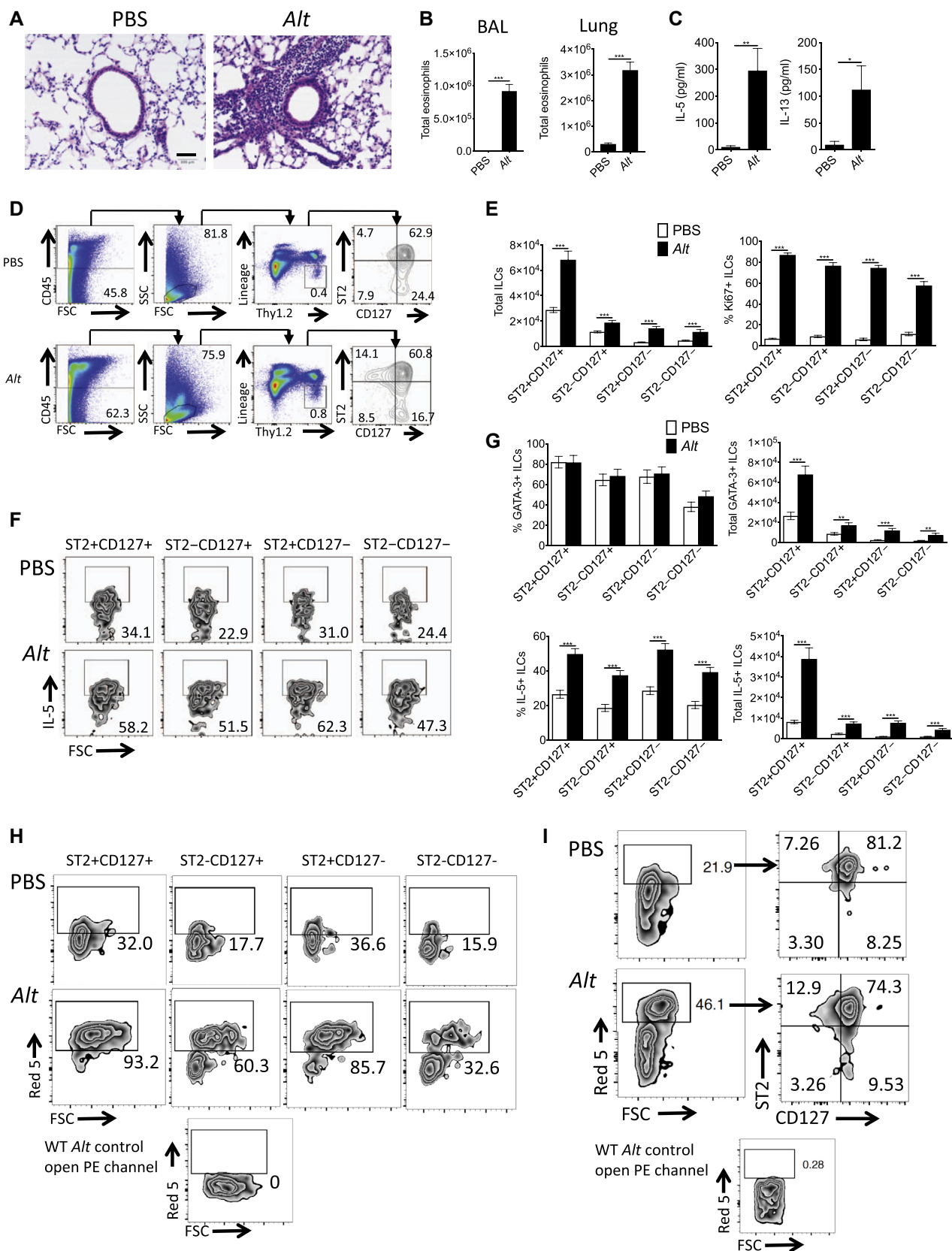


FIG 1. Unconventional ILC2 populations are induced by *A. alternata* (*Alt*). Hematoxylin and eosin–stained lung sections (scale bar = 100 μ m) (**A**), eosinophils (**B**), and IL-5 and IL-13 from PBS- or *Alt*-challenged mice (**C**). Identification, frequency (**D**), and total numbers (**E**) of ILCs. Ki67⁺ frequency within ILC subpopulations (Fig 1, **E**). Frequency and total number of IL-5⁺ and GATA-3⁺ ILCs (**F** and **G**). % IL-5⁺ ILC2s gated from all Thy1.2⁺ ILCs (**H**) and ILC ST2/CD127 gating from all IL-5⁺ ILCs (**I**) from Red5 mice. FSC, Forward scatter; PE, phycoerythrin. Data shown are representative of 2 to 9 independent experiments with 3 mice per group. **P* < .05, ***P* < .01, ****P* < .001, unpaired *t* test.

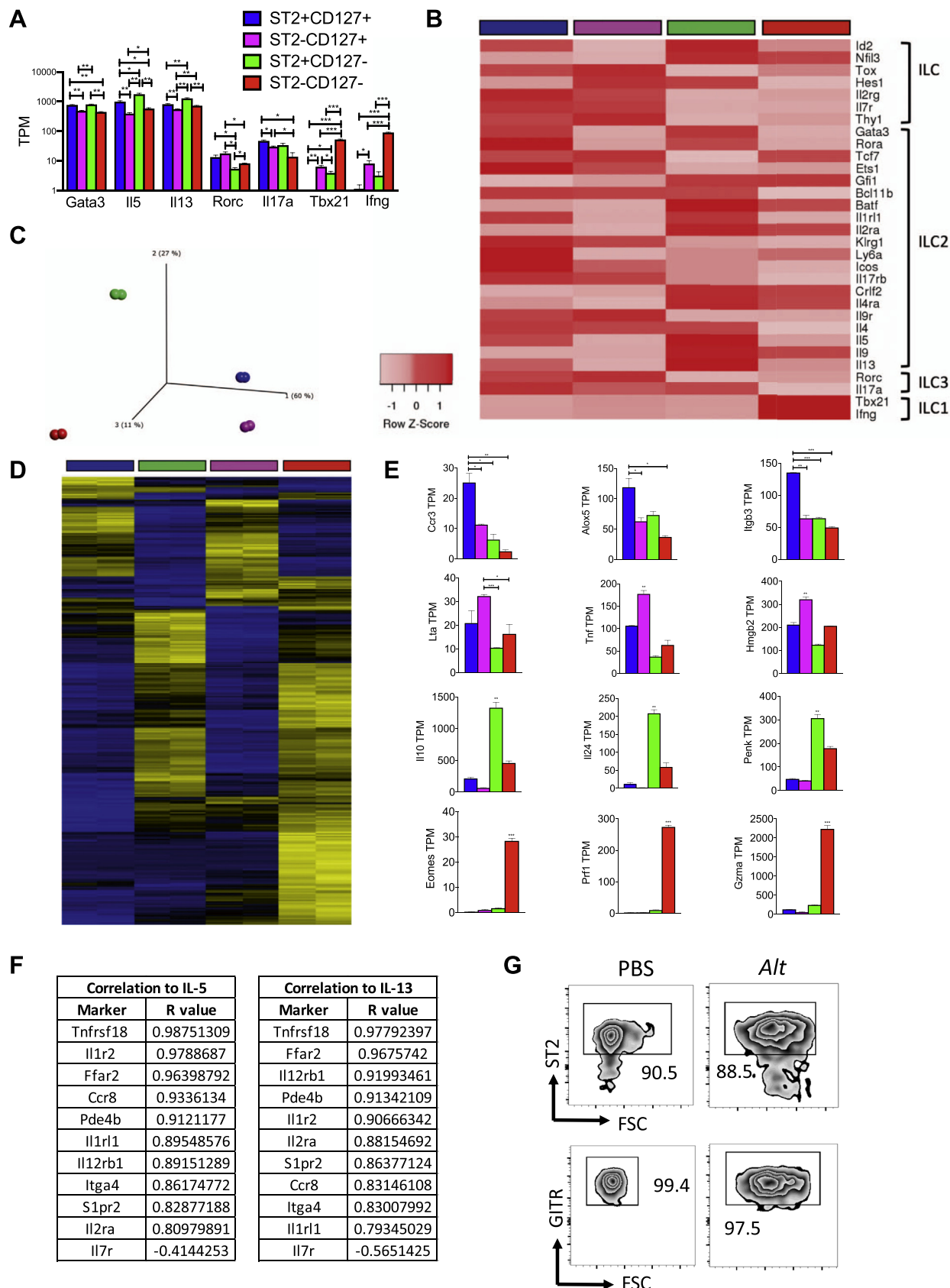


FIG 2. ILC subpopulations contain ILC2s as well as heterogeneous ILC transcriptomes. RNA sequencing-derived transcript levels (**A**) and heat map (**B**) of ILC1, ILC2, and ILC3 transcripts after *A. alternata* (*Alt*) challenge (Fig 2, *B*). PCA plot (**C**), heat map (**D**), and differentially expressed candidate genes (**E**) after *Alt* challenge. Transcript correlations with IL-5 and IL-13 (**F**). **G**, % ST2 and GITR of IL-5⁺ Thy1.2⁺ ILCs from Fig 1, *I*. *A-F*, Data shown are representative of a single experiment with 2 replicates that include pooled lungs from 9 mice. *G*, Representative of 3 mice per group. *FSC*, Forward scatter; *GITR*, glucocorticoid-induced TNFR family related gene; *TPM*, transcripts per million. **P* < .05, ***P* < .01, ****P* < .001, unpaired *t* test. Unless indicated by bars, stats in Fig 2, *E*, compared with all other groups.

ST2⁺CD127⁺ population generally expressed the lowest number of ILC2-related transcripts (Fig 2, A and B). The CD127⁺ populations had the greatest ILC3 signature compared with the CD127⁺ counterparts, whereas the ST2⁺CD127⁺ population had the greatest ILC1 signature. Furthermore, principal-component analysis showed that ST2⁺CD127⁺ and ST2⁺CD127⁺ populations are more similar, compared with the ST2⁺CD127⁺ and ST2⁺CD127⁺ populations, which are individually more unique (Fig 2, C and D). Ccr3, Alox5, and Itgb3 transcripts were increased in the double-positive population and ST2⁺CD127⁺ ILCs showed increased expression of Lta, Tnf, and Hmgb2 (Fig 2, E). ST2⁺CD127⁺ ILCs showed elevated levels of anti-inflammatory cytokines Il10 and Il24, and the ST2⁺CD127⁺ ILCs had ILC1/NK transcript increases in Eomes, Gzma, and Prf1. These results suggest that all populations contain ILC2s as well as unique transcripts supporting overall heterogeneity in the 4 subsets.

Finally, we correlated various transcript markers to the type 2 cytokine transcripts Il5 and Il13 across all populations. Tnfrsf18, which encodes glucocorticoid-induced TNFR family related gene (GITR), showed the strongest correlation with IL-5 and IL-13 transcripts, well above that of Il1rl1 (ST2) and Il7r (CD127) transcripts (Fig 2, F). Mirroring the transcript results, we found that GITR expression more accurately identified IL-5⁺ ILCs from Red5 mice by FACS compared with ST2 in both PBS- and *A alternata*-challenged mice (Fig 2, G). Thus, our work strongly suggests that current methods of ILC2 identification do not sufficiently include the entire type 2 cytokine-producing ILC2 population. Furthermore, our studies highlight the heterogeneity and complexity of ILC responses to *A alternata*, a natural allergen associated with severe asthma.

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Prevalence and characteristics of adult shellfish allergy in the United States



To the Editor:

Shellfish allergy (SA) is one of the most common food allergies worldwide^{1,2} and the third most common in US children.³ Although the isolation of specific shellfish allergens and immunologic mechanisms has improved the understanding of SA pathophysiology,⁴ knowledge gaps remain regarding the prevalence, distribution, and determinants of SAs among US adults.⁵ Consequently, this study estimates national SA prevalence, characteristics, and severity within a large, nationally representative sample of 40,443 US adults surveyed between October 2015 and September 2016. Details regarding survey development, complex survey sampling, weighting, statistical methodologies, and outcomes measures for food allergy assessment—including their unique strengths and limitations—are detailed in Gupta et al.⁶

In short, reported allergies were considered convincingly IgE-mediated if reported symptoms included at least 1 stringent symptom (see Fig E1 in this article's Online Repository at www.jacionline.org) upon ingestion of the responsible allergen. Classification of physician-confirmed and severe allergies is summarized in Fig E2 in this article's Online Repository at www.jacionline.org. For analyses comparing characteristics of crustacean-allergic versus mollusk-allergic adults, individuals reporting both allergies were excluded. Coastal residence was determined at the county level via residential ZIP using National Oceanic and Atmospheric Administration definitions of coastal state, coastal county, and shoreline counties⁷ not bordering the Great Lakes. Please note that because ZIP codes were queried only for participants' current residence, this may lead to inaccurate estimates for respondents who had moved since developing SA.

Population-weighted demographic and atopic disease characteristics are presented in Table I. Mean age of SA diagnosis was 17.7 years (95% CI, 16.6%-18.7%), and mean age of adult-onset SA was 28.3 years (95% CI, 26.9%-29.8%). SA prevalence by allergen types and age categories is presented in Table E1 in this article's Online Repository at www.jacionline.org. Overall,

METHODS

Mice

Six- to 8-week-old female C57BL/6 WT mice were obtained from The Jackson Laboratory. RAG2^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, Me) and bred in-house. Red5 mice were obtained from Dr Nunzio Bottini (UCSD) and originated from Dr Richard Locksley (UCSF) and bred in-house. All animal experiments were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

In vivo A alternata models

WT mice were challenged intranasally with PBS or 50 µg of *A alternata* extract (Greer, NC, lot number 299382) in 40 µL daily for 3 days and euthanized 24 hours after the last challenge for BAL collection and lung tissue harvest. *A alternata* was diluted in PBS. BAL was collected using 2% BSA; the first draw was 500 µL, and the second through fifth draws were 600 µL each. For experiments in which ILCs were isolated, mice were challenged with *A alternata* 4 times over 10 days to further expand the ILC population. For time course experiments, mice were challenged with *A alternata* on D0, D1, and D2. Mice were euthanized on D0 (baseline), D1, D2, D3, and D7. BAL was centrifuged at 1500 rpm for 5 minutes at 4°C; the supernatant was stored at -20°C for ELISA, and the cells were analyzed by flow cytometry. Lungs were digested using Mouse Lung Dissociation Kit (Miltenyi Biotec, San Diego, Calif) according to the manufacturer's protocol and subsequently filtered with a 40-µm cell strainer.

Flow cytometry

BAL and lung single-cell suspensions were counted on a Novocyte (Acce Biosciences, San Diego, Calif) or an Accuri (BD) flow cytometer and prepared for staining by washing with a solution of 2% FBS and 0.01% sodium azide in PBS. Cells were incubated with an mAb to CD16/CD32 for 10 minutes at 4°C to block Fc receptors and then stained for 30 minutes at 4°C with various combinations of antibodies to surface markers. All antibodies were purchased from BioLegend unless otherwise noted. To identify eosinophils (defined as CD45⁺Siglec-F⁺CD11c⁻), BAL and lung cells were stained with PerCP-conjugated anti-CD45.2, phycoerythrin (PE)-conjugated anti-Siglec-F (BD), and fluorescein isothiocyanate-conjugated anti-CD11c.

To identify ILCs (defined as CD45.2⁺lineage⁻Thy1.2⁺ lymphocyte-sized cells) and conventional ILC2s (defined as CD45.2⁺lineage⁻Thy1.2⁺ST2⁺CD127⁺ lymphocyte-sized cells), lung cells were stained with fluorescein isothiocyanate-conjugated lineage cocktail (anti-CD3e, anti-GR-1, anti-CD11b, anti-B220, anti-Ter119), anti-CD11c, anti-NK1.1, anti-CD5, anti-FcεR1, anti-TCR-β, and anti-TCRγδ; PerCP-conjugated anti-CD45.2; eFluor 450-conjugated anti-Thy1.2 (ThermoFisher); APC-conjugated anti-ST2; and PE-Cy7-conjugated anti-CD127. To determine ILC surface marker expression, cells were stained with PE-conjugated anti-CD25, anti-C-Kit, anti-inducible T-cell costimulator, anti-killer lectin like receptor G1, anti-CD44, anti-stem cell antigen-1 (ThermoFisher, Waltham, Mass), anti-IL18R (ThermoFisher), anti-CD69, or PE/Cy7 anti-mouse CD357 (GITR) (BioLegend, San Diego, Calif). In addition, in select experiments ILCs were stained with PE Annexin V (BioLegend) according to the manufacturer's protocol.

To determine nuclear transcription factor expression, surface-stained cells were fixed and permeabilized with a transcription factor staining kit (ThermoFisher) according to the manufacturer's protocol and stained for 30 minutes at 4°C with PE-conjugated anti-GATA-3 (ThermoFisher), anti-Ki67 (ThermoFisher), anti-T-Bet, or anti-RORγt (ThermoFisher). In select experiments, some of the aforementioned antibodies were conjugated to different fluorochromes: BV605-conjugated anti-T-Bet, and PE-eFluor 610-conjugated RORγt (ThermoFisher).

To determine ILC cytokine-producing potential, lung cells were incubated for 3 hours at 37°C with a cell stimulation cocktail consisting of phorbol 12-myristate 13-acetate, ionomycin, brefeldin A, and monensin (ThermoFisher) in RPMI 1640 media supplemented with penicillin/streptomycin, 10% FBS, glutamine, and 2-mercaptoethanol (ThermoFisher). Stimulated cells were surfaced stained for ILCs as described earlier and fixed and permeabilized

with an intracellular cytokine staining kit (BD) according to the manufacturer's protocol and stained for 30 minutes at 4°C with PE-conjugated anti-IL-5 or anti-IL-13, eFluor 506-conjugated anti-IL-17A, and APC-Cy7-conjugated anti-IFN-γ. In select experiments, IL-13 was conjugated to APC-eFluor 780 (ThermoFisher). Finally, stained samples were analyzed with a Novocyte (Acce Biosciences) or Accuri (BD) flow cytometer, and ILCs stained with Zombie Aqua (BioLegend) or 4'-6-diamidino-2-phenylindole, dihydrochloride (ThermoFisher) to discriminate live and dead cells were sorted for subsequent *in vitro* stimulation experiments and transcript quantification with a FACS Aria II (BD) or a FACS Aria Fusion (BD) at the UCSD Human Embryonic Stem Cell Core Facility.

ELISA

ELISAs for IL-5 (R&D, Minneapolis, Minn), IL-13 (R&D), and IFN-γ (ThermoFisher) were performed on BAL supernatants according to the manufacturers' protocol. ELISA plates were read on a model 680 microplate reader (Bio-Rad, Hercules, Calif) at 450 nm.

RNA extraction

ILC RNA was extracted using RNA Easy Plus Micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Before RNA-seq library preparation, samples were quantified as described previously^{E1,E2} and quality of RNA assessed by Fragment Analyzer (Advance Analytical, Santa Clara, Calif). All samples had an RNA integrity number greater than 7.5.

Quantitative PCR

RNA was reverse-transcribed using SuperScript IV VILO Master Mix (ThermoFisher) according to the manufacturer's protocol. Transcript levels were measured with an MX3000P quantitative PCR system (Stratagene, La Jolla, Calif) using TaqMan Universal PCR Master Mix (ThermoFisher) with the following TaqMan assays (ThermoFisher): Hprt (Mm00446968_m1), Il1rl1 (Mm00516117_m1), Il7r (Mm00434295_m1), Gata3 (Mm0048683_m1), Il5 (Mm00439646_m1), Il13 (Mm00434204_m1), Rorc (Mm01261022_m1), Il17a (Mm00439618_m1), Tbx21 (Mm00450960_m1), and Ifng (Mm01168134_m1). Transcripts were measured relative to Hprt.

RNA sequencing

Purified total RNA (≈5 ng) was amplified following the Smart-seq2 protocol.^{E3,E4} Briefly, mRNA was captured using poly-dT oligos and directly reverse-transcribed into full-length cDNA using the described template-switching oligo.^{E3,E4} cDNA was amplified by PCR for 15 cycles and purified using AMPure XP magnetic bead (0.9:1 (vol:vol) ratio, Beckman Coulter, Brea, Calif). From this step, for each sample, 1 ng of cDNA was used to prepare a standard NextEra XT sequencing library (NextEra XT DNA library prep kit and index kits; Illumina, San Diego, Calif). Barcoded Illumina sequencing libraries (NextEra; Illumina) were generated using an automated platform (Biomek FXP, Beckman Coulter). Both whole-transcriptome amplification and sequencing library preparations were performed in a 96-well format to reduce assay-to-assay variability. Quality control steps were included to determine total RNA quality and quantity, the optimal number of PCR preamplification cycles, and fragment library size. The reference genome was mm10 (mouse genome). None of the samples failed quality controls and were pooled at equimolar concentration, loaded, and sequenced on the Illumina Sequencing platforms (HiSeq2500, NovaSeq6000, Illumina). Libraries were sequenced to obtain more than 10 million 50-bp reads mapping uniquely to mRNA mm10 reference.

Histology

For select experiments, the left side of the lung was fixed by perfusion with 4% paraformaldehyde, sectioned, and stained. Hematoxylin and eosin and

periodic acid-Schiff stainings were performed at the Histology Core at the University of California, San Diego Moores Cancer Center.

Data analysis and statistics

Flow cytometry data were analyzed using FlowJo version 10.4.1 (Flowjo, Ashland, Ore). ELISA data were analyzed using GraphPad Prism or elisaanalysis.com. Quantitative PCR data were analyzed on Microsoft Excel by the delta-delta Ct method relative to Hprt. For all experiments, statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, Calif). Unpaired *t* tests (2-tailed) were performed for all experiments. *P* values of less than .05 were considered statistically significant.

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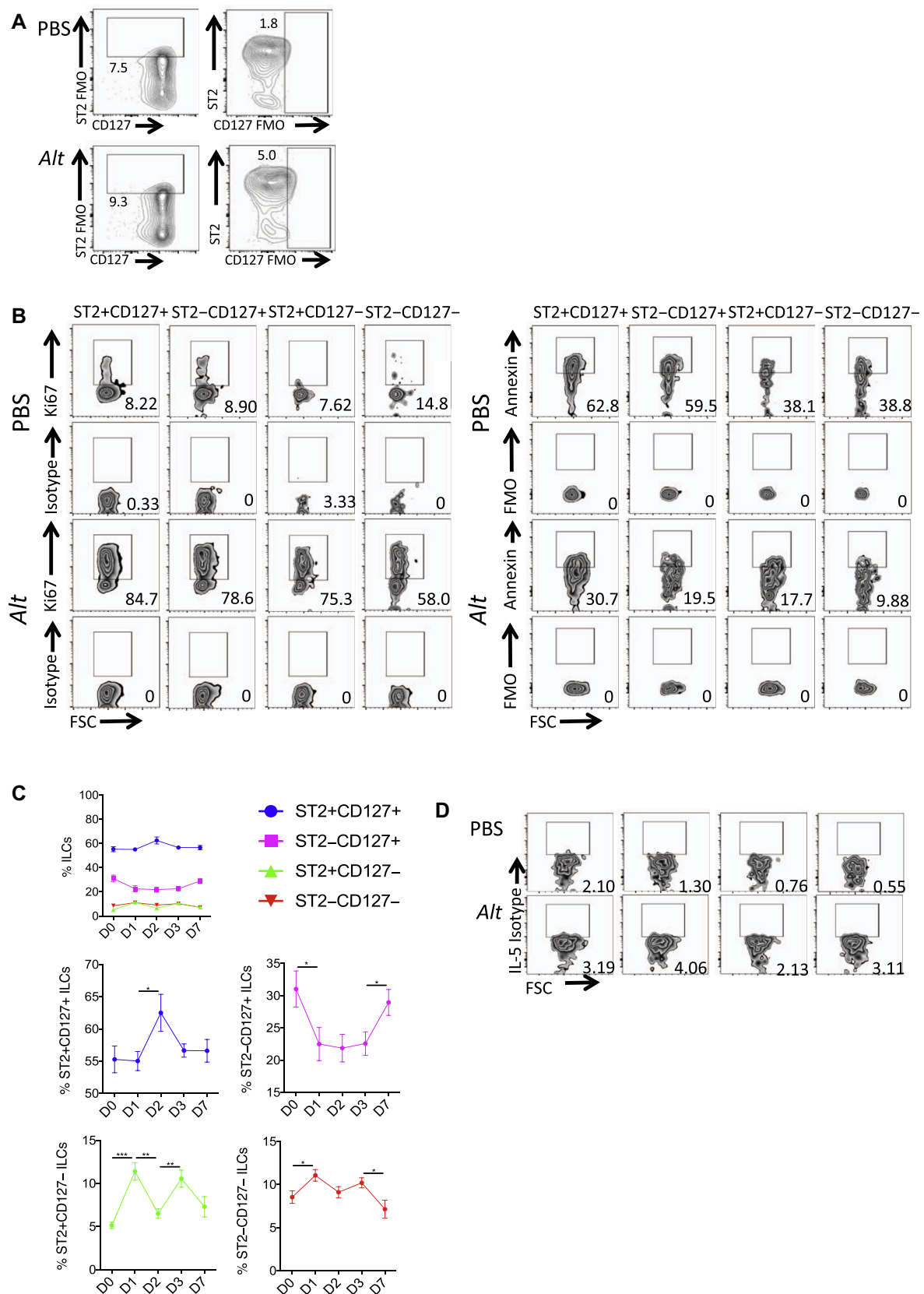


FIG E1. ILC subpopulation gating, proliferation, apoptosis, and kinetics after *A. alternata* (Alt) challenge. **A**, Representative FMO controls for ILC subset gating. **B**, FACS plots of Ki67⁺ (left) frequency and Annexin V⁺ (right) frequency within ILC subpopulations. **C**, Frequency of ST2⁺CD127⁺, ST2⁻CD127⁺, ST2⁺CD127⁻, and ST2⁻CD127⁻ cells within the total ILC population collectively (top) and individually (bottom). **D**, Isotype controls for intracellular IL-5 staining. FMO, Fluorescence minus one; FSC, forward scatter. Data shown are representative of 3 independent experiments with 2 to 3 mice per group. **P* < .05, ***P* < .01, ****P* < .001, unpaired *t* test.

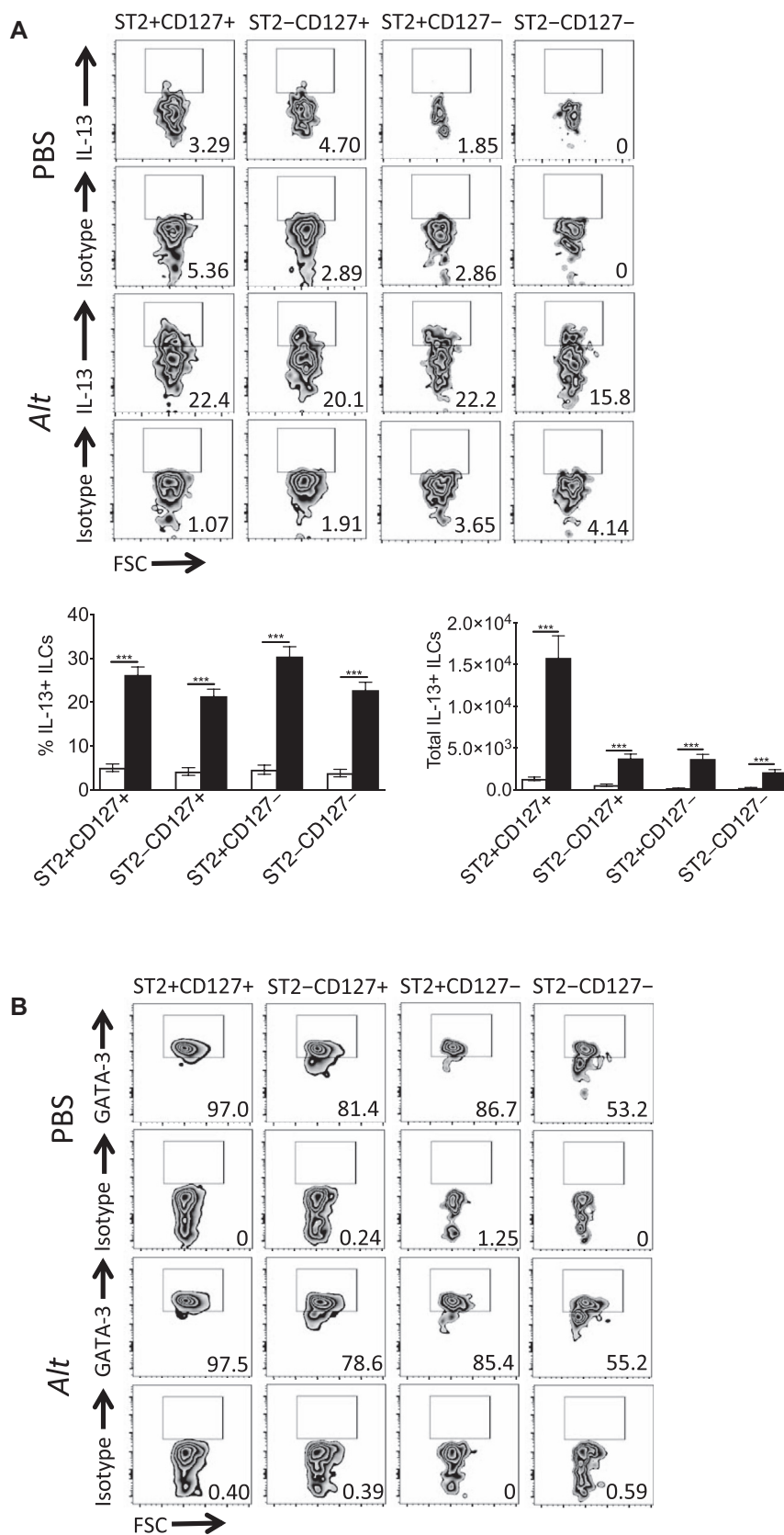


FIG E2. **A**, Frequency and total number of IL-13⁺ ILCs. **B**, frequency of GATA3. *Alt*, *A. alternata*; *FSC*, forward scatter. Data shown are representative of 2 to 9 independent experiments with 3 mice per group. **P* < .05, ***P* < .01, ****P* < .001, unpaired *t* test.

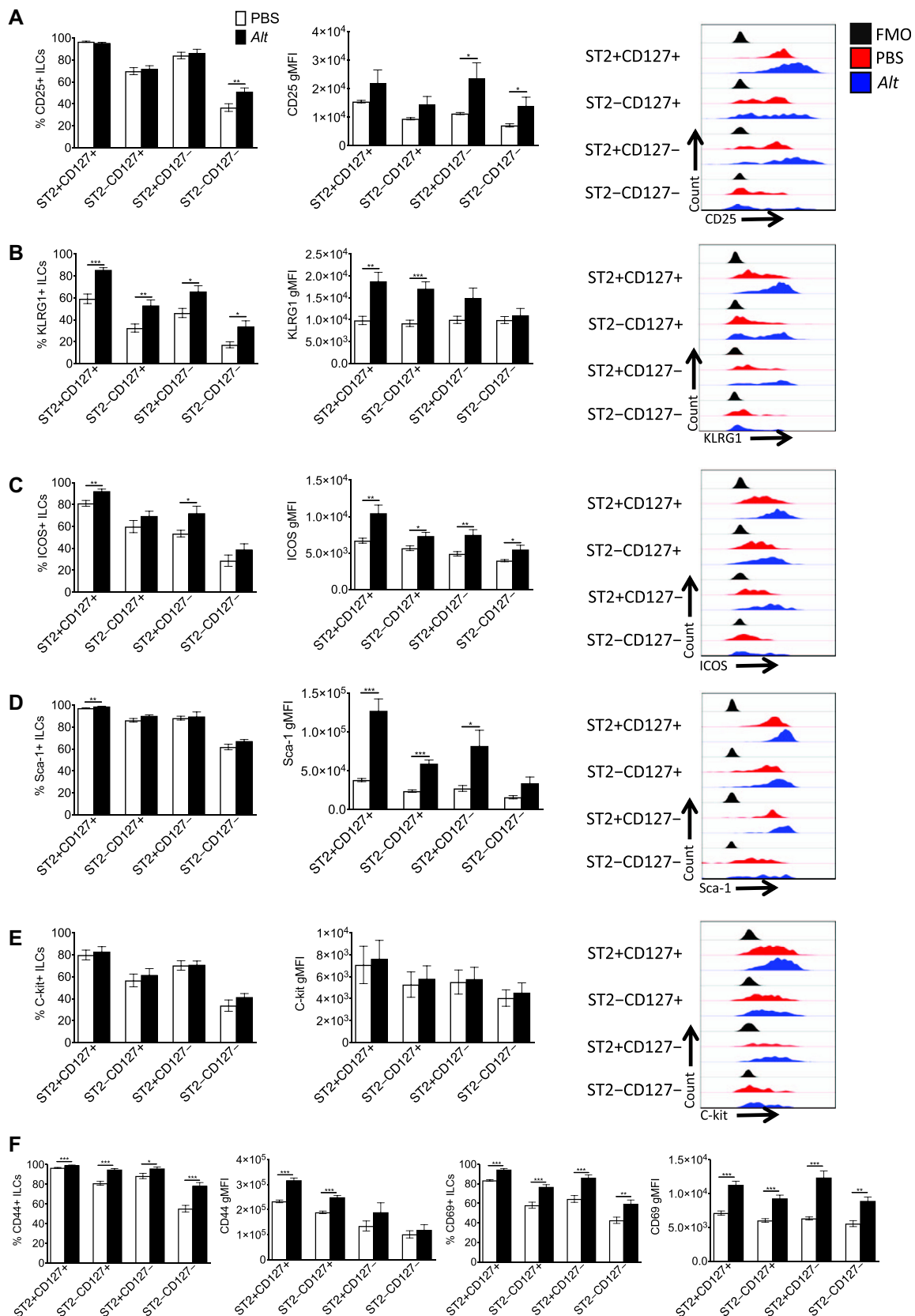


FIG E3. Levels of ILC2 surface marker expression after *A. alternata* (Alt) challenge. **A**, CD25⁺ frequency within ILC subpopulations and CD25 gMFI. **B**, KLRG1⁺ frequency within ILC subpopulations and KLRG1 gMFI. **C**, ICOS⁺ frequency within ILC subpopulations and ICOS gMFI. **D**, Sca-1⁺ frequency within ILC subpopulations and Sca-1 gMFI. **E**, C-kit⁺ frequency within ILC subpopulations and C-kit gMFI. **F**, CD44⁺ frequency within ILC subpopulations and CD44 gMFI and CD69⁺ frequency within ILC subpopulations and CD69 gMFI. FMO, Fluorescence minus one; ICOS, inducible T-cell costimulator; KLRG1, Killer lectin like receptor G1; MFI, mean fluorescence intensity; Sca-1, stem cell antigen-1. Data shown are representative of 2 to 3 independent experiments with 3 mice per group. **P* < .05, ***P* < .01, ****P* < .001, unpaired *t* test.

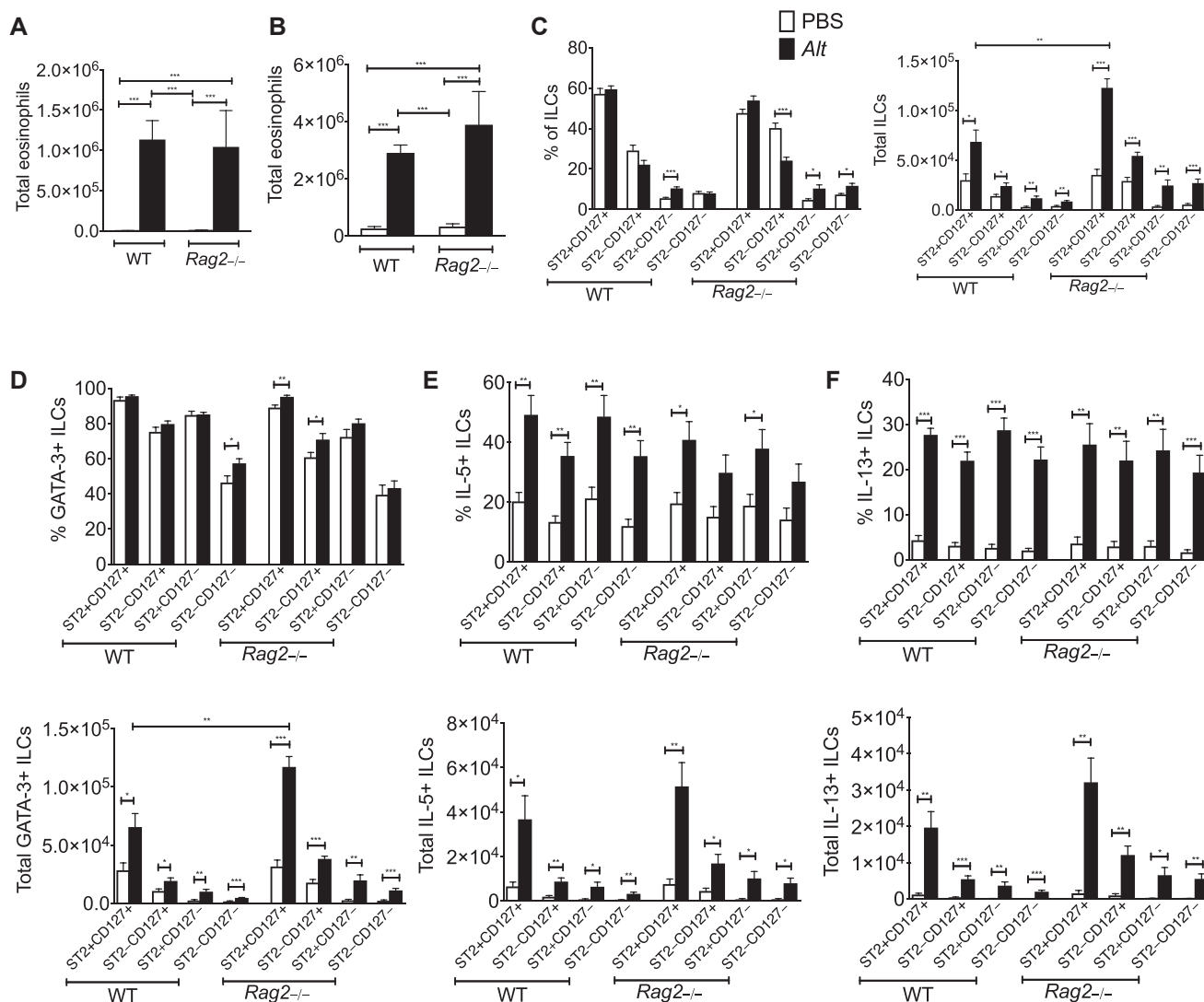


FIG E4. Unconventional ILC2 changes after *A. alternata* (Alt) challenge in RAG2 knockout mice. Total number of eosinophils in BAL (**A**) and lung (**B**). **C**, Frequency and total number of ILCs in PBS- and Alt-challenged mice. **D**, GATA-3 frequency within ILC subpopulations and the total number of GATA-3⁺ ILCs. **E**, IL-5⁺ frequency within ILC subpopulations and the total number of IL-5⁺ ILCs. **F**, IL-13⁺ frequency within ILC subpopulations and the total number of IL-13⁺ ILCs. Data shown are representative of 2 independent experiments with 3 mice per group. **P* < .05, ***P* < .01, ****P* < .001, unpaired *t* test. Comparisons between Alt and PBS groups unless indicated.

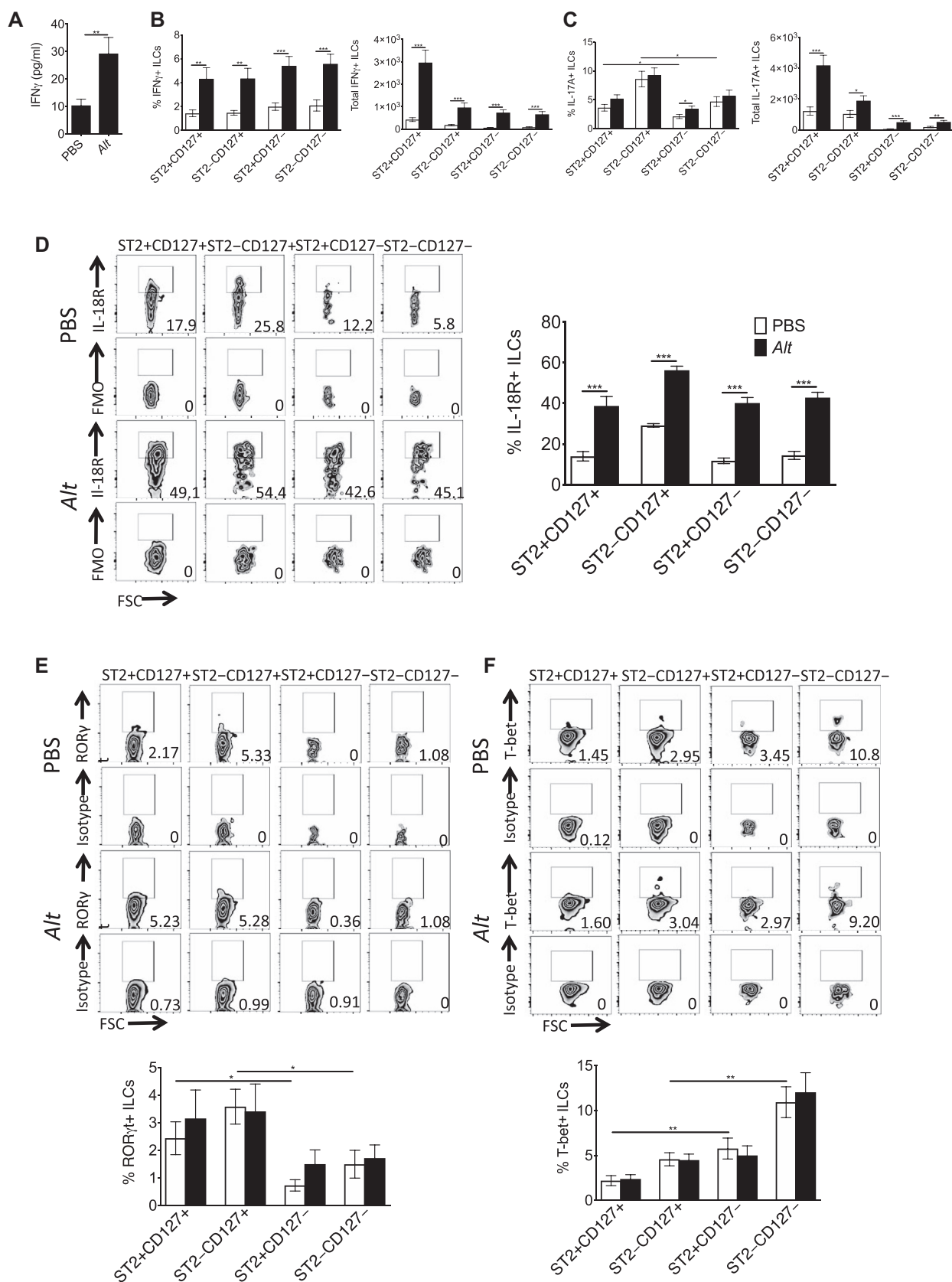


FIG E5. Levels of ILC1 and ILC3 markers after *A. alternata* (Alt) challenge. **A**, BAL IFN- γ . **B**, IFN- γ + frequency and total number of IFN- γ + ILCs. **C**, IL-17A+ frequency and total number of IL-17A+ ILCs. **D**, Frequency and total IL-18R+ ILC subpopulations. **E**, Frequency and total ROR γ t+ subpopulation. **F**, Frequency and total T-bet+ ILC subpopulation. FSC, Forward scatter. Data shown are representative of 2 to 5 independent experiments with 3 mice per group. * $P < .05$, ** $P < .01$, *** $P < .001$, unpaired t test.

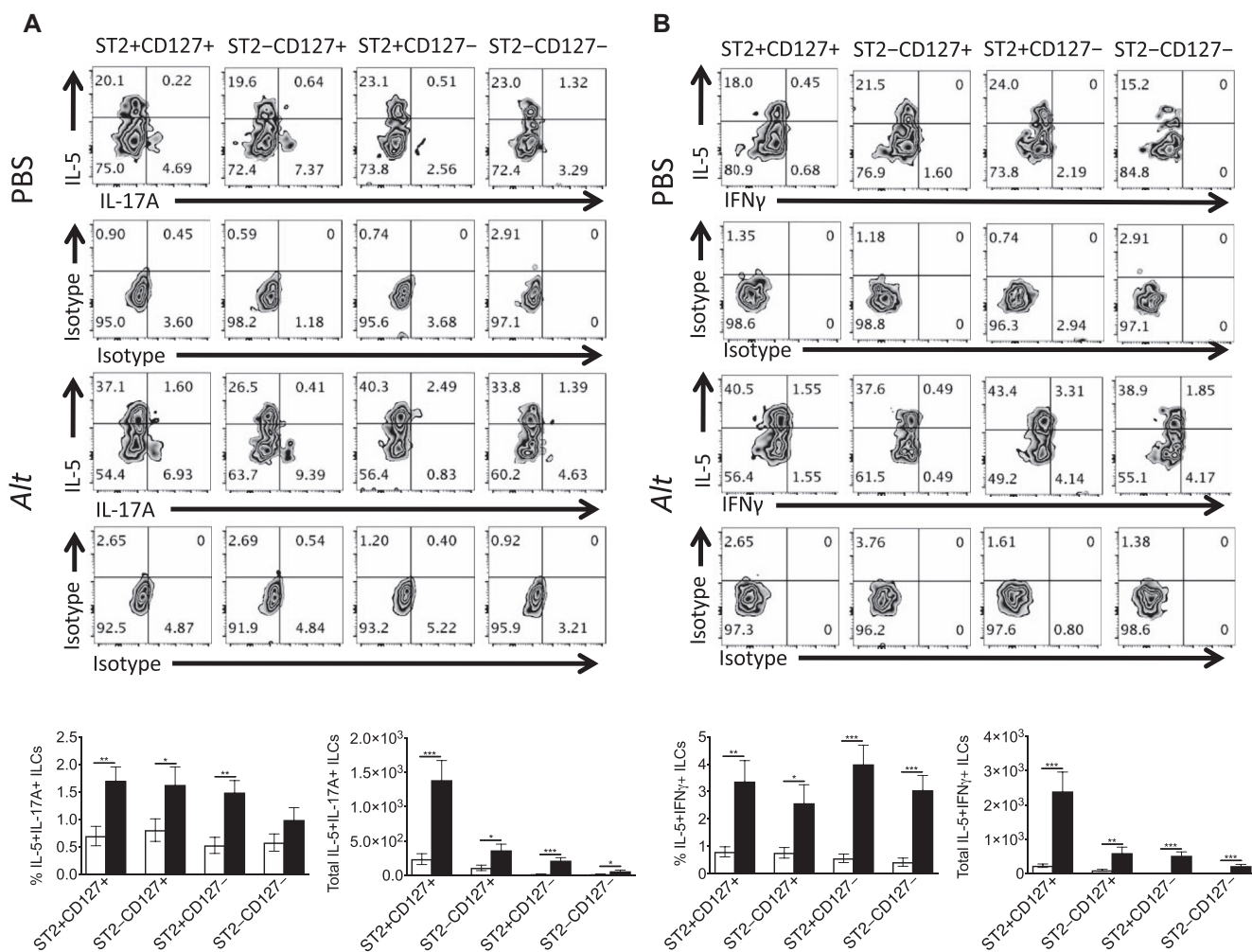


FIG E6. ILC1 and ILC3 cytokine levels in ILC2 subpopulations after *A. alternata* (*Alt*) challenge. **A**, IL-5⁺IL-17A⁺ frequency within ILC subpopulations and total number of IL-5⁺IL-17A⁺ ILCs. **B**, IL-5⁺IFN- γ ⁺ frequency within ILC subpopulations and total number of IL-5⁺IFN- γ ⁺ ILCs. Data shown are representative of 2 to 5 independent experiments with 3 mice per group. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001, unpaired *t* test.

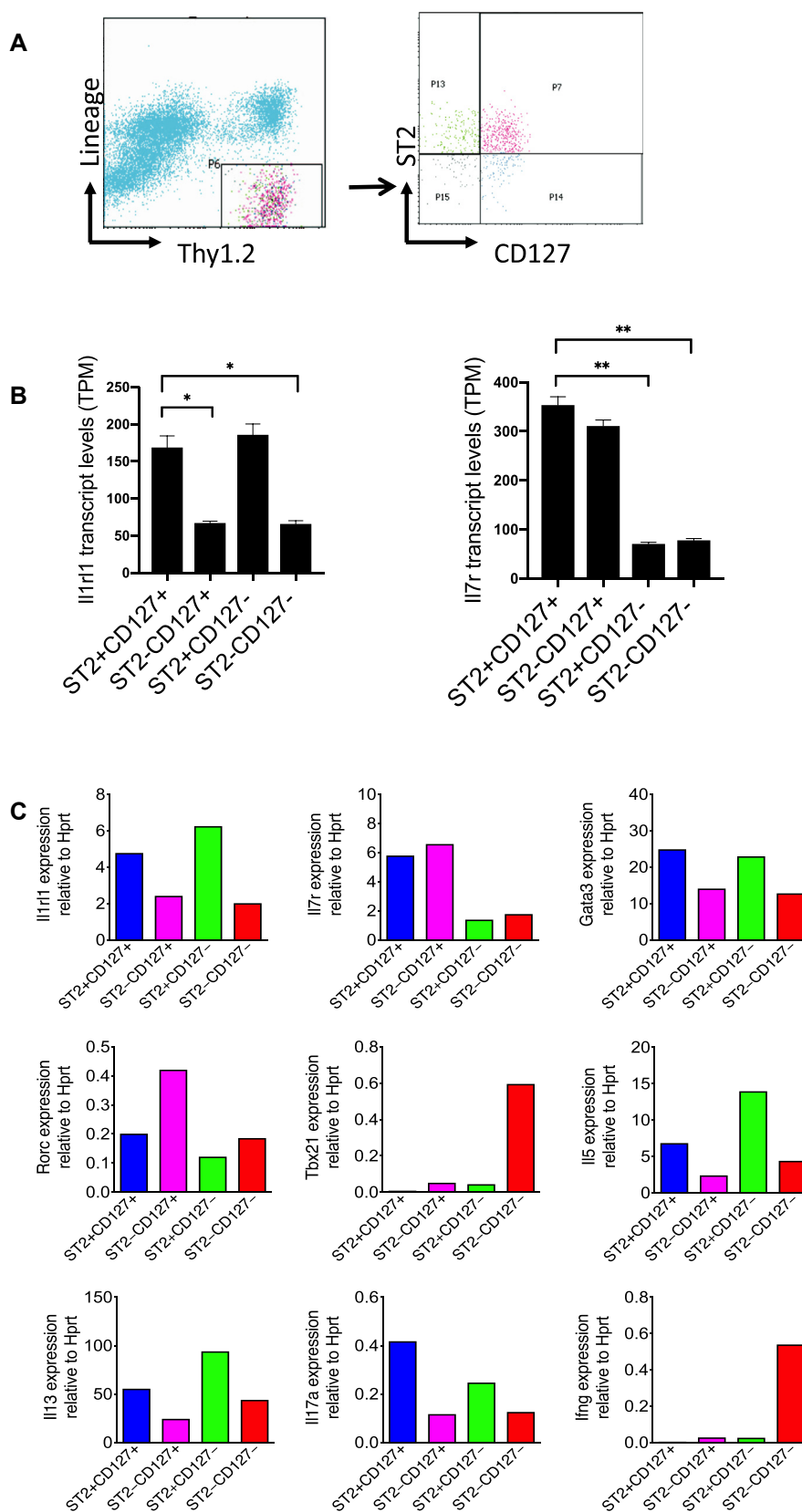


FIG E7. Transcript changes in sort-purified stimulated ILCs. **A**, Sort plot for purification of ILC subsets based on ST2 and CD127 staining. **B**, RNA-seq transcripts per million (TPM) levels for Il1r1 and Il7r in ILC subsets. **C**, Quantitative PCR of ILC subsets for Il1r1, Il7r, Il5, Il13, Il17a, and Ifng transcripts after *A. alternata* challenges. Data shown are representative of 2 to 4 replicates. * $P < .05$, ** $P < .01$, unpaired t test.