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Lung-resident CD69^{+}ST2^{+} Th2 cells mediate long-term type 2 memory to inhaled antigen in mice

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ABSTRACT

Background: Chronic airway diseases, such as asthma, are characterized by persistent type 2 immunity in the airways. Our knowledge is limited regarding the mechanisms that explain why type 2 inflammation continues in these diseases.

Objective: To investigate the mechanisms involved in long-lasting immune memory in the lungs leveraging mouse models.

Methods: Naïve mice were exposed intranasally (i.n.) to ovalbumin (OVA) antigen with *Alternaria* extract as an adjuvant. Type 2 memory was analyzed by parabiosis model, flow cytometry with *in-vivo* antibody labeling, and i.n. OVA recall challenge. Gene-deficient mice were used to dissect the mechanisms.

Results: In the parabiosis model, mice previously exposed i.n. to OVA with *Alternaria* showed more robust antigen-specific immune responses and airway inflammation than mice with circulating OVA-specific T cells. After a single airway exposure to OVA with *Alternaria*, CD69^{+}ST2^{+} Th2-type T cells, which highly express type 2 cytokine mRNA and lack CD62L expression, appeared in the lung tissues within 5 days and persisted for at least 84 days. Upon re-exposure to OVA *in vivo*, these cells produced type 2 cytokines quickly without involving circulating T cells. Development of tissue-resident CD69^{+}ST2^{+} Th2 cells and long-term memory to an inhaled antigen were abrogated in mice deficient in ST2 or IL-33, but not in those deficient in TSLP receptor.

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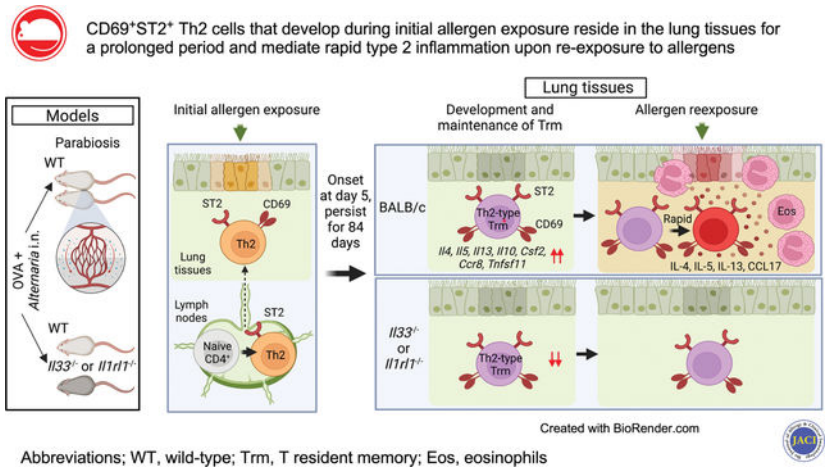
Conclusion: $CD69^+ST2^+$ Th2 memory cells develop quickly in the lung tissues after initial allergen exposure and persist for a prolonged period. The ST2/IL-33 pathway may play a role in the development of immune memory in the lungs to certain allergens.

Capsule Summary

Kobayashi *et al.* demonstrated that initial airborne exposure to allergens induces allergen-specific Th2 cells that reside within the lung tissues for a prolonged period and respond quickly to secondary exposure to the allergens.

Graphical Abstract



Abbreviations; WT, wild-type; Trm, T resident memory; Eos, eosinophils

Keywords

IL-33; tissue-resident memory T cells; Th2 cells; allergy; allergens; IL-5

INTRODUCTION

Chronic airway diseases, such as asthma and chronic rhinosinusitis (CRS), are characterized by recurrent and persistent airway inflammation and tissue remodeling. Th2-type $CD4^+$ T cells that recognize allergens and other environmental factors have been considered a key player in these diseases.[1] However, a major question remains regarding why these T cells persist in the respiratory mucosa and cause recurrent airway inflammation and disease exacerbations. In a clinical study, transplantation of lungs from donors with mild asthma produced asthma in non-asthmatic recipients, whereas transplantation of non-asthmatic lungs to an asthmatic patient ameliorated the disease,[2] suggesting that the immune cells transferred together with lung tissues initiate the immunopathology of asthma. Fixed drug eruption, in which an allergic response recurs at the same site each time a drug is taken systemically, also suggests the potential roles of antigen-specific T cells that reside in the skin tissues.[3] Therefore, an investigation into the biology and function of tissue-resident memory Th2 cells would help us to understand the immunologic mechanisms of chronic and recurrent allergic diseases and develop novel therapeutic strategies to treat them.

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Memory T cells can be divided into subpopulations based on their trafficking patterns.[4] Central memory T (Tcm) cells recirculate through blood and secondary lymphoid organs, and effector memory T (Tem) cells recirculate from blood to non-lymphoid tissues.[4] Tissue-resident memory T (Trm) cells have recently been identified in several barrier organs, including the skin, intestines, and lungs,[3–5] and are limited in their ability to recirculate, but they can remain in non-lymphoid tissues for a prolonged period.[6] A growing body of literature points to a role for Trm cells in immune-mediated diseases, such as alopecia areata, inflammatory bowel disease, psoriasis, and multiple sclerosis.[7–10] In contrast to Tcm and Tem cells, Trm cells likely have strong effector functions and provide frontline protection against pathogens in mucosal organs.[3, 11, 12] In this regard, $CD8^+$ Trm cells have been studied extensively in the context of infection.[5] However, our knowledge of the $CD4^+$ cell arm of Trm cells and their roles in allergic diseases is limited.

Recently, repeated airborne exposures to the house dust mite (HDM) allergen were found to induce long-lived, HDM-specific Trm cells within the lung tissues.[13, 14] Moreover, in HDM-sensitized and -challenged mice, Th2-type Trm cells induced airway hyperreactivity and excessive mucus production in the airway mucosa while circulating Th2 cells promoted perivascular eosinophilic inflammation.[15] These previous observations demonstrate the existence of Th2-type Trm cells within the lung tissues of sensitized animals and implicate their functional significance in airway pathology; however, little is known of the mechanisms involved in their development and persistence in the lungs. Our knowledge is also limited regarding the functional and molecular characterization of Th2-type Trm cells compared with conventional Th2 cells.

To fill these gaps, we used mouse models to examine the development and persistence of antigen-specific memory in the lungs. Generally, inhalation of innocuous ovalbumin (OVA) protein results in immunologic tolerance.[16] Among various environmental factors, the association between asthma and exposure to fungi, such as *Alternaria*, has been recognized clinically and epidemiologically.[17–23] Therefore, to investigate antigen-specific memory, we used OVA as a model antigen and *Alternaria* extract as an inhaled adjuvant. We found that when naïve mice were exposed intranasally (i.n.) to OVA together with *Alternaria* extract, OVA-specific Th2 cells developed in the lung tissues within several days. These antigen-specific Th2 cells persisted for several months without any additional stimulation and, upon re-exposure to OVA antigen *in vivo*, produced airway inflammation and pathology more robustly than circulating Th2 cells. Furthermore, these tissue-resident Th2 cells - in particular, the $CD69^+ST2^+$ double-positive population - highly transcribed type 2 cytokine messenger RNA (mRNA) and produced cytokines quickly upon antigen encounter without involving the circulating Th2 cells. Thus, Th2-type Trm cells in the lung tissues may serve as tissue sentinels to airborne allergens and play a pivotal role in initiating antigen-specific type 2 memory responses and chronic airway inflammation in the respiratory mucosa.

METHODS

Mice

BALB/cJ, C.129-*Il4*^{tm1Lky}/J (4Get, *Il4*^{eGFP}), BALB/cByJ (*Ptprc*$^{b/b}$ encoding CD45.2), and CByJ.SJL(B6)-*Ptprc*^{a}/J (*Ptprc*$^{a/a}$ encoding CD45.1) mice were purchased from the Jackson

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Laboratory (Bar Harbor, ME). *Il1rl1*$^{-/-}$ mice [24] and *Il33*$^{Cit/Cit}$ mice (interleukin [IL]-33-deficient mice due to insertion of the citrine cassette into the *Il33* gene)[25] were gifts from Dr. Andrew N. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK). IL-5 reporter *Il5*^{venus} mice[26] were a gift from Dr. Kiyoshi Takatsu (University of Toyama, Toyama, Japan). 4get mice and *Il1rl1*$^{-/-}$ mice were crossed to produce *Il4*^{eGFP}*Il1rl1*$^{-/-}$ mice. The mice were maintained under specific pathogen-free conditions at the Mayo Clinic (Rochester, MN, and Scottsdale, AZ). Female mice were used at 7–12 weeks of age. All protocols and procedures for handling the mice were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC).

Reagents

Endotoxin-free OVA was prepared from specific pathogen-free chicken eggs (Charles River Laboratories, Wilmington, MS) under sterile conditions, as previously described.[27] The absence of endotoxin (<0.5 EU/mg protein) was verified using the Limulus Amebocyte Lysate (LAL) Assay (Wako Chemicals, Richmond, VA). *Alternaria alternata* extract (XPM1C3A25, lot#282854) and crude HDM extract (RMB82M, lot#387032) were purchased from Greer Laboratories (Lenoir, NC). *Alternaria* extract contained undetectable levels of endotoxin (<0.0500 EU/mg, Kinetic LAL Analysis, Charles River, Charleston, SC). The amount of *Alternaria* extract (e.g.,100 μg) in this study refers to the total extract; the extract contains 14.5 % protein.

Airway sensitization and challenge of mice

To investigate the local immune responses in the lungs, the administration of antigens and adjuvants and *in-vivo* challenge were performed intranasally (i.n.). Naïve wild-type (WT) or gene-deficient mice were lightly anesthetized with isoflurane and administered 100 μg OVA i.n. with or without 100 μg *Alternaria* extract in 50 μl PBS once on day 0, or 10 μg OVA i.n. with 200 μg HDM extract in 50 μl PBS on days 0 and 7. On days 42, 43, and 44, the mice were challenged i.n. with 10 μg OVA. To verify the role of IL-33, *Il33*$^{Cit/Cit}$ mice were administered 10 ng IL-33 i.n. on days 7, 8, and 9. Alternatively, in some experiments, mice were challenged by i.n. OVA on days 21, 22, and 24. Twenty-four hours after the last OVA challenge, mice were euthanized, and bronchoalveolar lavage (BAL) fluids were collected by cannulating the trachea and performing lavage in triplicate using Hank's balanced salt solution (0.5, 0.25, and 0.25 ml). BAL cell numbers were counted, and differentials were determined in Wright-Giemsa-stained cytospin preparations. More than 200 cells were counted using conventional morphologic criteria. The lungs were also collected, homogenized in 0.5 ml PBS, and centrifuged at 10,000×g at 4°C for 15 min. The protein concentrations in the supernatant were quantified with a Pierce™ BCA Protein Assay kit (Thermo Fisher, Rockford, IL). Cytokine levels in the supernatants were measured using ELISA, as described below. Alternatively, mice were exposed i.n. to OVA plus *Alternaria* on day 0 and challenged by i.n. OVA on day 5. Six hours later, the lungs were collected for cytokine analyses. In some experiments, mice were exposed i.n. to OVA plus *Alternaria* twice, on days 0 and 7, and the immune cells in the mediastinal lymph nodes (mLNs) and lung tissues were analyzed on day 11 by flow cytometry or by culturing them *in vitro* with OVA, as described below.

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Flow cytometry analyses of immune cells in the lung tissues

Naïve mice were exposed i.n. once on day 0 to OVA plus *Alternaria* or OVA plus HDM as described above. Mice were euthanized and lungs were collected on day 3, 5, 28, 56, or 84, as described in the figure legends. To differentiate the cells in circulation from those in the lung tissues, cells were labeled *in vivo* by intravenous (i.v.) injection[28] of 2 μg PerCP-Cy5.5-conjugated anti-mouse CD45 antibody (Ab) (clone 30-F11, BioLegend, San Diego, CA) in 200 μl PBS 5 min before euthanasia. Single-cell suspensions of lungs were prepared by digesting harvested lungs with a cocktail of collagenases in the presence of DNase, as previously described.[29] The cells were preincubated with Fc receptor blocker and stained with combinations of the following antibodies: anti-CD4 (clone RM4–5, BD), anti-ST2 (clone DJ8, MD Bioproducts), anti-CD69 (clone H1.2F3, BD), anti-CCR8 (clone SA214G2, BioLegend), and anti-CD62L (clone MEL-14, BD). Samples were acquired with FACSCalibur, FACSCanto, and LSRFortessa flow cytometers (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ), and the data were analyzed using FlowJo V10 (FlowJo).

Long-term memory responses in the lungs

Naïve mice were exposed i.n. to OVA plus *Alternaria* once on day 0. On day 45, mice were challenged i.n. once with 10 μg OVA, and lungs were collected 6 hours later. Mice were labeled by injection of anti-mouse CD45 Ab *in vivo* 5 min before euthanasia, as described above. Lung homogenates were analyzed for the levels of cytokines and chemokines. Lung single-cell suspensions were analyzed by flow cytometry, as described above. In some experiments, to prevent an influx of lymphocytes into the lung tissues from circulation, mice were treated systemically by intraperitoneal (i.p.) injection of FTY720 (Cayman Chemical, Ann Arbor, MI)[30] (20 μg in 100 μl PBS) for 3 days before OVA challenge; FTY720 causes internalization of the G protein-coupled sphingosine-1-phosphate receptor 1 (S1PR1) and prevents lymphocyte egress from lymph nodes.[30]

Parabiosis model

We used a parabiosis model to evaluate the roles of tissue-resident lymphocytes—compared with circulating lymphocytes—in producing airway inflammation. We followed the published protocol to create the model with slight modifications.[31] Briefly, on day 0, BALB/cByJ (CD45.2) mice were exposed i.n. to OVA plus *Alternaria* and then co-housed with naïve CD45.1 mice (BALB/cByJ background) for 14 days. To perform parabiosis surgery, the mice were anesthetized and their lateral skin was shaved and disinfected. Mirrored incisions were made on the lateral aspects of both mice from forelimb to hindlimb. Sutures were placed around the olecranon and knee joints to secure the upper and lower extremities. The dorsal and ventral skin were approximated with sutures. Antibiotics and analgesics were administered as recommended by the Mayo Clinic IACUC. The co-joined pairs recovered and rested for 28 days to establish neovascularization and circulation of immune cells between the parabionts. The shared circulation was verified by collecting peripheral blood and analyzing the composition of $CD4^+$ T cells (i.e., CD45.1 vs. CD45.2). On days 43, 44, and 45, each parabiont was challenged individually by i.n. administration

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of 10 μg OVA and euthanized 24 hours later. BAL fluids and lung tissues were analyzed for inflammatory cells, tissue pathology, and levels of cytokines and chemokines.

In-vitro culture of $CD4^+$ T cells from mLN and lung cells

Naïve mice were exposed i.n. to OVA plus *Alternaria* twice, on days 0 and 7. On day 11, mLN and lung cells were collected, and single-cell suspensions were prepared, as described above. $CD4^+$ T cells were isolated using the EasySep™ Mouse $CD4^+$ Cell Isolation Kit (StemCell Technologies, Vancouver, Canada). Splenocytes were collected from naïve BALB/c mice. $CD4^+$ T cells (1×10^5/well) and splenocytes as antigen-presenting cells (3×10^5/well) were cultured with or without 100 μg/ml OVA for 4 days, as previously described.[32] Cell supernatants were analyzed for levels of IL-4, IL-5, and IL-13 by ELISA.

Sorting Th2 cell subpopulations and NanoString gene expression analysis

Naïve mice were exposed i.n. to OVA plus *Alternaria* once on day 0 and were *in-vivo* labeled with anti-CD45 Ab 5 min before euthanasia. On day 7 or day 28, mLNs and lungs were collected, and single-cell suspensions were enriched for $CD4^+$ T cells using the EasySep Mouse $CD4^+$ Cell Isolation Kit. Cells were then stained with anti-CD4, anti-ST2, and anti-CD69 antibodies, as described above. The following *in-vivo* cell populations were sorted with a FACSAria flow cytometer (BD Biosciences): lung $CD45^+CD4^+$ (i.e., $CD4^+$ T cells within the lung blood vessels), $CD45^-CD4^+ST2^-$ (i.e., $CD4^+ST2^-$ T cells within the lung tissues), $CD45^-CD4^+CD69^-ST2^+$, and $CD45^-CD4^+CD69^+ST2^+$ cells, and mLN $CD45^-CD4^+ST2^-$ and $CD45^-CD4^+ST2^+$ cells. The cells were lysed, and the total RNA was extracted and purified, as described previously.[32] mRNA was probed with the nCounter analysis platform (NanoString, Seattle, WA) using the 561-gene mouse immunology profiling panel, following the protocol recommended by the manufacturer. Data were analyzed using the nSolver Analysis software package (NanoString).

ELISAs

The lung levels of IL-4, IL-5, IL-13, CCL17, and CCL22 were measured by the Quantikine ELISA kit (R&D Systems), and CCL24 was measured by the DuoSet ELISA kit (R&D), according to the manufacturers' instructions. Plasma levels of OVA-specific IgE were measured by ELISA as previously described.[16]

Statistical analysis

Data are presented as means ± standard error of means (SEMs) for the numbers of mice or experiments indicated. The statistical significance of differences between the various treatment groups was assessed by using Student's *t*-tests, Mann-Whitney U test, and Wilcoxon signed-rank test, depending on data distribution and study design, and *P* values less than 0.05 were considered statistically significant.

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RESULTS

The parabiosis model suggests pivotal roles for tissue-resident memory cells

A case study in lung transplant patients who developed asthma after transplant surgery[2] suggested the potential roles of non-circulating lung-resident immune cells in initiating the immunopathology of asthma. Because it is technically challenging to perform lung transplantation in mice, we investigated the roles of lung-resident immune cells using a parabiosis model. Although OVA - a model antigen - is an excellent tool to monitor antigen-specific memory in mice, airway exposure to endotoxin-free OVA protein induces immunologic tolerance.[33, 34] Furthermore, exposure to the fungus *Alternaria* is a risk factor for developing asthma in humans.[17, 20–23] Therefore, to induce type 2 immune memory to OVA antigen in mice, we administered endotoxin-free OVA protein i.n. to naïve mice together with *Alternaria alternata* extract as an adjuvant.[16, 35] In our previous studies, exposure of naïve mice to OVA alone without *Alternaria* did not sensitize them to OVA.[16, 35] In contrast, when mice previously exposed i.n. to OVA antigen with *Alternaria* extract were challenged i.n. with OVA alone, they developed airway eosinophilia and increased lung levels of type 2 cytokines (Supplemental Fig. E1). Furthermore, when mice were exposed i.n. to *Alternaria* extract alone (without OVA), they did not develop OVA-specific IgE antibodies. These mice did not develop airway eosinophilia when challenged i.n. with OVA, suggesting the specificity of OVA antigen responses (Supplemental Fig. E1). These immunologic responses were abolished in *Rag1*$^{-/-}$ mice, suggesting that *Alternaria* extract promotes the development of Th2-type memory to OVA protein.

To compare the functions of circulating versus tissue-resident memory cells, we used a parabiosis model in conjunction with this OVA plus *Alternaria* model. To do this, naïve BALB/cByJ mice (CD45.2) were exposed i.n. to OVA with *Alternaria* extract only once (Fig. 1A). Fourteen days later, when the CD45.2 mice established immune memory to OVA, they were co-joined with naïve congenic mice (CD45.1) by parabiosis surgery. After an additional 28 days (to establish blood circulation between parabionts), the memory responses of each mouse were examined by i.n. challenge with OVA antigen. After the parabiosis surgery, 41% of circulating CD4$^+$ T cells in the mice previously exposed to OVA plus *Alternaria* (i.e., CD45.2 mice) were derived from the host, and 58% were from the co-joined naïve parabiont (i.e., CD45.1 mice) (Fig. 1B). In addition, 61% of circulating CD4$^+$ T cells in the naïve parabiont were from the host, suggesting that the parabionts exchanged circulating CD4$^+$ T cells, whereas those from the naïve parabiont were slightly more prevalent in circulation than those from the mice exposed previously to OVA plus *Alternaria* (i.e., memory parabionts).

When the mice were challenged by i.n. administration of OVA, the memory parabionts developed robust peribronchial and perivascular inflammation, whereas the naïve parabionts showed minimal airway inflammation (Fig. 1C). The memory parabionts showed a significantly higher number of airway lymphocytes and eosinophils compared with the OVA-challenged naïve parabionts (P<0.05, Fig. 1D). The memory parabionts had significantly higher lung levels of IL-5, IL-13, CCL17, and CCL22 compared with the OVA-challenged naïve parabionts (P<0.05), whereas no differences in IL-4 and CCL24 were

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observed (Fig. 1E). These findings suggest that a one-time airway exposure to OVA in the presence of a type 2 adjuvant (*Alternaria*) is sufficient to generate immunologic memory to the OVA antigen and that non-circulating Th2 cells within the lung tissues play a major role in mediating antigen-specific recall responses and airway pathology.

Airborne allergen exposure induces Th2 cell subpopulations in the lung tissues

To investigate the early stage of development of $CD4^+$ Th2-type memory cells in the lung tissues, we performed a short kinetic study by exposing naïve BALB/c mice to OVA plus *Alternaria* (Fig. 2A). Because the lungs are highly vascularized, we injected mice with PerCP-Cy5.5-conjugated anti-CD45 Ab 5 min prior to euthanasia to differentiate between circulating and tissue-resident lymphocytes.[28] In naïve mice, few $CD4^+$ T cells were found in the lung tissues (i.e., *in-vivo* $CD45^-$) but many were identified in circulation (i.e., *in-vivo* $CD45^+$, Fig. 2B). As soon as 3 days after i.n. exposure to OVA plus *Alternaria*, $CD4^+$ T cells started to appear in the lung tissues and increased rapidly until day 5 (Fig. 2B, 2C, $P<0.05$).

We characterized these tissue T cells using antibodies to Th2 cell marker ST2 (i.e., IL-1RL1)[36] and C-type lectin CD69. CD69 identifies tissue-resident $CD4^+$ and $CD8^+$ T cells[5] as it antagonizes the cell surface expression of S1PR1 and suppresses the trafficking of lymphocytes from tissues to circulation and lymphatics.[37, 38] On day 3, approximately 20% of $CD4^+$ T cells within the lung tissues expressed ST2 (Fig. 2D, 2E). On day 5, the proportion of $ST2^+$ cells increased further, and approximately 30% became double-positive for both ST2 and CD69. In contrast, in the intravascular space (i.e., *in-vivo* $CD45^+$), few $CD4^+$ cells were positive for ST2, and the $CD69^+ST2^+$ double-positive cell population was undetectable. To verify the antigen-specificity of $CD4^+$ T cells in the lungs, mice were challenged i.n. with OVA alone (Fig. 2F). Mice previously exposed to OVA plus *Alternaria* responded to OVA challenge within 6 hours and produced significantly higher amounts of IL-4, IL-5 and IL-13 as compared to those challenged with PBS (Fig. 2G, $p<0.05$ and $p<0.01$). In contrast, mice previously exposed to OVA alone or *Alternaria* alone produced minimal type 2 cytokines when challenged with OVA, suggesting that an airway adjuvant (*Alternaria*) is necessary to establish antigen-specific immune memory within the lung tissues.

$CD69^+ST2^+$ Th2 cells mediate long-term memory and a quick, antigen-specific recall response

To examine whether these antigen-specific Th2 cells persist in the lung tissues for a long period, we extended the time after allergen exposure. Naïve BALB/c mice were exposed once i.n. to OVA plus *Alternaria*, and no exogenous antigen was provided for the next 45 days (Fig. 3A). The memory cell function within the lung tissues was examined by i.n. OVA challenge after treating the mice with the S1PR1 agonist FTY720[30] to minimize the influence of circulating lymphocytes; FTY720 causes internalization of S1PR1 and prevents lymphocyte egress from lymph nodes.[30] Forty-five days after the single exposure to OVA with *Alternaria*, $CD4^+$ T cells were detectable within the lung tissues (i.e., *in-vivo* $CD45^-$), and >25% presented the $CD69^+ST2^+$ phenotype (Fig. 3B). Administration of FTY720 reduced circulating $CD4^+$ T cells by 94% but did not affect the number of lung tissue $CD4^+$ T cells, including $CD69^-ST2^+$ and $CD69^+ST2^+$ Th2 cells (Fig. 3B, 3C). An i.n. challenge

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with OVA quickly increased the lung levels of type 2 cytokines in 6 hours, including IL-4, IL-5, and IL-13 as well as T cell chemokine CCL17 (Fig. 3D). The levels of these cytokines/chemokines were not affected by FTY720, suggesting that they were derived from Th2 cells within the lung tissues with no or minimal contribution from the circulating Th2 cells.

To investigate the real-time transcription of type 2 cytokines, we used an IL-5 reporter *Il5*^{venus} mouse model.[26, 39] Mice were exposed to OVA plus *Alternaria* and challenged i.n. with OVA 45 days later (Fig. 4A). Similar to WT mice (Fig. 3), systemic treatment with FTY720 significantly reduced circulating CD4$^+$ T cells by 93% (P<0.05) but did not affect those in the lung tissues of *Il5*^{venus} mice (Fig. 4B and 4C). After i.n. challenge with OVA, increased expression of IL-5-venus was observed in the *in-vivo* CD45$^-$ cell population (Fig. 4D), suggesting that they were derived from the cells within the tissues. By the scatter plot, IL-5-venus-positve cells were identified mainly within the lymphocytic population (Supplemental Fig E2). Furthermore, within the CD4$^+$ lymphocyte populations, IL-5-venus-positive cells were highly enriched in the CD69$^+$ST2$^+$ cell population, whereas IL-5-venus-negative cells were distributed among several populations (Fig. 4D); approximately 75% of the IL-5-venus signal was derived from the CD69$^+$ST2$^+$ double-positive cells (Fig. 4E). The IL-5-venus-positive ST2$^+$ cells were not affected by FTY720. Altogether, after a single antigen exposure, the memory CD69$^+$ST2$^+$ Th2 cells were resident in the lung tissues for at least 45 days and provided a quick source of IL-5 when the animals were re-exposed to the antigen.

IL-33 is necessary for Th2-type memory in the lungs

To examine the mechanisms involved in the development of long-lasting type 2 memory in the lungs, we speculated on the potential roles of factors produced in airway mucosa. A recent study suggested that three epithelium-derived cytokines - IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) - play pivotal roles in type 2 immunity in the lungs of mice infected with the nematode *Nippostrongylus brasiliensis*.[40] We also previously found that chronic airway inflammation induced by repeated exposure to natural allergens, such as *Alternaria* and HDM, is dependent on IL-33 and TSLP, but not on IL-25.[29] Therefore, to examine the roles of IL-33 and TSLP, WT mice or mice deficient in receptors or ligands for these molecules were exposed once i.n. to OVA with or without *Alternaria* extract, and the immunologic memory was examined by i.n. OVA challenge 42 days later (Fig. 5A). Naïve mice previously exposed to OVA alone did not generate airway inflammation when challenged with OVA alone (Fig. 5B). In contrast, when the mice previously exposed to OVA plus *Alternaria* were challenged with OVA, they developed airway eosinophilia and increased lung levels of IL-5 and IL-13. These memory responses were significantly suppressed in mice deficient in IL-33 receptor ST2 (i.e., *Il1rl1*$^{-/-}$) or IL-33 protein (i.e., *Il33*$^{Cit/Cit}$) (P<0.05 and P<0.01). In contrast, mice deficient in TSLP receptor (i.e., *Crlf2*$^{-/-}$) developed airway eosinophilia and increased type 2 cytokines roughly comparable to WT mice, suggesting that the IL-33/ST2 pathway is involved in the development of long-term type 2 memory in the lungs and that the TSLP pathway is unlikely to be involved.

The roles of IL-33 in the differentiation and development of antigen-specific Th2 cells have been controversial.[24, 41, 42] Therefore, we examined whether the early development of Th2

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cells is affected in *Il1rl1*$^{-/-}$ mice. To this end, IL-4eGFP reporter mice[43] that mark CD4^{+} T cells transcribing a canonical Th2 cytokine *Il4* were crossed with WT or *Il1rl1*$^{-/-}$ mice. The development of Th2 cells was analyzed by gating out the CXCR5^{+} T follicular helper cells. After i.n. exposure to OVA with *Alternaria*, the proportion and number of IL-4eGFP^{+} Th2 cells increased in both the lung-draining mediastinal lymph nodes (mLNs) and lungs of *Il1rl1*$^{+/+}$ control mice compared with naïve mice (Fig. 5D, 5E). The number of Th2 cells in the mLNs was not affected in IL-33 receptor-deficient *Il1rl1*$^{-/-}$ mice. However, the number of Th2 cells in the lungs decreased by 70% (P<0.05). We further analyzed the functions of these Th2 cells by *in-vitro* culture. WT or *Il1rl1*$^{-/-}$ mice were exposed i.n. to OVA with *Alternaria*, and we stimulated CD4^{+} T cells isolated from mLNs and lungs with OVA antigen *in vitro* for 4 days (Fig. 5F). CD4^{+} T cells from both the mLNs and lungs of WT mice produced a large quantity of type 2 cytokines, including IL-4, IL-5, and IL-13 (Fig. 5G). CD4^{+} T cells from the mLNs of *Il1rl1*$^{-/-}$ mice produced amounts of type 2 cytokines comparable to those of WT mice; in contrast, CD4^{+} T cells from the lungs of *Il1rl1*$^{-/-}$ mice produced significantly less type 2 cytokines (P<0.01).

To verify the role of the IL-33/ST2 pathway in *Alternaria*-induced type 2 immunity in the lungs, we supplemented IL-33 to the IL-33-deficient *Il33*$^{Cit/Cit}$ mice several days after the initial allergen exposure (Supplemental Fig. E3). *Il33*$^{Cit/Cit}$ mice that had been exposed to OVA+*Alternaria* showed minimal airway eosinophil and increase in type 2 cytokines when they were challenged i.n. with OVA alone 42 days later. This long-term memory response was significantly enhanced when mice were administered IL-33 i.n. after the initial allergen exposure (P<0.05). Altogether, these findings suggest that the IL-33/ST2 pathway is dispensable for differentiation of antigen-specific Th2 cells in mLNs but plays a major role in type 2 immunity in the lungs, perhaps regulating Th2 cell recruitment to the lungs and/or their maturation and maintenance in the tissues.

IL-33 promotes development and/or maintenance of CD69^{-}ST2^{+} and CD69^{+}ST2^{+} CD4^{+} T cells in the lung tissues after exposure to Alternaria

To examine the roles of IL-33 in the dynamics of lung Th2 cells, we performed a long kinetic study using IL-33-deficient mice. WT mice or IL-33-deficient *Il33*$^{Cit/Cit}$ mice were exposed once i.n. to OVA plus *Alternaria*, and CD4^{+} cells within the lung tissues were monitored for up to 84 days (Fig. 6A). In WT mice, 28 days after a single exposure to OVA plus *Alternaria*, many CD4^{+} T cells were identified in the lung tissues (i.e., *in-vivo* CD45^{-}CD4^{+} cells) (Fig. 6B, 6C); the number of these CD4^{+} T cells declined over the next 56 days but did not reach the levels of naïve mice. The numbers of tissue CD4^{+} cells were significantly lower in *Il33*$^{Cit/Cit}$ mice, specifically on day 56 (P<0.05).

Differentials of these CD4^{+} cells based on fluorescence-activated cell sorting (FACS) showed that approximately 40% of lung tissue CD4^{+} T cells were ST2^{+} with roughly equal proportions of CD69^{-} and CD69^{+} cells on day 28 in WT mice (Fig. 6D). On day 56, the frequency of total ST2^{+} cells increased to 60% of tissue CD4^{+} T cells, mainly reflecting the increase in CD69^{+} cells (Fig. 6E). In IL-33-deficient *Il33*$^{Cit/Cit}$ mice, the prevalence of lung CD69^{-}ST2^{+} cells and CD69^{+}ST2^{+} cells was significantly lower than in WT mice throughout the experimental period (P<0.05, P<0.01).

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To examine the reproducibility of the observations in other allergens, we performed a parallel experiment by using HDM. Like the *Alternaria* model, many $CD4^+$ T cells were identified in the lung tissues of WT mice 28 days after a single exposure to OVA plus HDM extract (Supplemental Fig. E4). However, in contrast to the *Alternaria* model, the prevalence of lung $CD69^-ST2^+$ cells and $CD69^+ST2^+$ cells in *Il33*$^{Cit/Cit}$ mice was roughly comparable to that in WT mice. Altogether, these findings suggest that $CD69^+ST2^+$ Th2-type memory cells develop in the lungs after a single airway exposure to allergens while the mechanisms of their development may vary depending on the nature of allergens.

Lung $ST2^+CD69^+$ cells express a distinct set of transcripts

To characterize lung $CD69^+ST2^+$ cells, we performed a NanoString gene expression profile assay that enables quantification of mRNA transcripts with small copy numbers and without amplification.[44] Naïve BALB/c mice were exposed once i.n. to OVA plus *Alternaria*, and the $CD69^-ST2^+$ and $CD69^+ST2^+$ cell populations were sorted from the day 7 lung tissues (Fig. 7A, 7B). Controls included $ST2^+$ Th2 cells in the mLNs, $CD69^-ST2^-$ double-negative cells in the lungs and mLNs, and total intravascular $CD4^+$ T cells in the lungs (Fig. 7B). Heat map analysis showed that several canonical transcripts associated with Th2 cells (e.g., *Gata3*, *Il1rl1*, *Il4, Il5*, *Il10,* and *Il13*) were commonly represented in all $ST2^+$ cell populations regardless of their origins (Fig. 7C). However, when they were compared to each other, distinct sets of genes were upregulated or downregulated in each population. For example, compared with $ST2^+$ cells in mLNs, lung $CD69^-ST2^+$ cells more highly expressed *Gata3*, *Il1rl1, CD69, Icos*, and *Cxcr4* and downregulated an adhesion molecule, *Sell* (Fig. 7D, Supplemental Fig. E5); the *Sell* gene (which encodes CD62L) was further downregulated in lung $CD69^+ST2^+$ cells. The transcripts for *Il4*, *Il5*, *Il13*, *Il10*, *Csf2*, *Tnfsf11*, and *Ccr8* were higher in lung $CD69^+ST2^+$ cells compared with lung $CD69^-ST2^+$ cells. In contrast, expression of *Tcf7* was lowest in lung $CD69^+ST2^+$ cells among all cell populations. Flow cytometry analyses confirmed that ST2 (encoded by *Il1rl1*) and CD62L protein expression were highest and lowest, respectively, in lung $CD69^+ST2^+$ cells compared with other $ST2^+$ cell populations (Fig. 7E, 7F). Higher expression of CCR8 was observed in lung $CD69^+ST2^+$ cells than in lung $CD69^-ST2^+$ cells.

To examine the kinetic changes in these observations, the $CD69^-ST2^+$ and $CD69^+ST2^+$ cell populations were sorted from the day 28 lung tissues and analyzed by a NanoString assay (Supplemental Fig. E6). The transcripts for *Il5*, *Il10*, *Csf2*, and *Ccr8* were higher in lung $CD69^+ST2^+$ cells compared with lung $CD69^-ST2^+$ cells on day 28. In contrast, expression of *Sell* and *Tcf7* was lower in $CD69^+ST2^+$ cells compared with $CD69^-ST2^+$ cells. In the $CD69^+ST2^+$ cell population, expression of *Il2*, *Bcl2* and *Tnfsf14* was higher on day 28 as compared with day 7 while *Il4*, *Ccr4*, and *Sell* were lower on day 28 (Supplemental Fig. E6). Altogether, these findings suggest that $CD69^+ST2^+$ cells within the lung tissues likely represent an active population of Th2 cells by highly expressing type 2 cytokine transcripts and are poised to stay within the lung tissues (i.e., lowest expression of CD62L).

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DISCUSSION

Persistent and recurrent airway inflammation is a major feature of chronic airway disorders, such as asthma and CRS. Th2 cells are considered a key cell population in the pathogenesis of allergic airway disorders.[45] However, our knowledge is limited to explain why and how type 2 immunity persists in the airways of these disorders. To address this, we examined the development and persistence of antigen-specific Th2-type memory in the lung tissues using mouse models. The major findings are that antigen-specific Th2 cells develop in the lung tissues within several days after respiratory exposure to allergens and persist for several months and maintain their robust responsiveness to the antigen. Furthermore, among Th2 cell populations, lung $CD69^+ST2^+$ cells are likely to be the most reactive population as they highly express type 2 cytokine transcripts (Fig. 7) and produce cytokines quickly when they encounter a cognate antigen *in vivo* (Fig. 4). The $CD69^+ST2^+$ cells remained in the lungs for at least 84 days after the initial allergen exposure (Fig. 6) and responded to the antigen without involving the circulating T cells (Fig. 3), suggesting that lung $CD69^+ST2^+$ cells are the *bona fide* Th2-type Trm cells.

$CD8^+$ Trm cells are broadly distributed in mucosal barrier tissues[46–48] and serve as the first responders during pathogen reinfection.[49, 50] Although much less is known regarding $CD4^+$ Trm cells, Th2-type Trm cells have been reported previously in mice repeatedly exposed to HDM.[13–15] In this study, by using a “expose and leave” strategy in which mice received a single dose of airway allergen exposure and were left untouched, we found that few $CD4^+$ T cells were present in the lung tissue compartment of naïve BALB/c mice. However, a single exposure to OVA protein with *Alternaria* adjuvant rapidly drove OVA-specific Th2 cells in the lung tissues in 3 days (Fig. 2), which became $CD69^+ST2^+$ Trm cells and persisted for at least 12 weeks without exogenous OVA antigen (Fig. 6). Previously, $CD4^+$ Trm cells were found in the lung tissues 6 days after the primary i.n. exposure to HDM extract.[13] We add to this knowledge by demonstrating that these Th2-type Trm cells start to develop as early as 3 days after *Alternaria* exposure (Fig. 2) and that they serve as a quick and primary source of type 2 cytokine upon re-exposure the allergen (Fig. 4). A previous study using i.p. sensitization followed by repeated airway OVA challenge also showed that Th2-type Trm cells persisted in the lungs for more than 2 years.[51] Thus, antigen-specific Th2-type Trm cells likely develop quickly in the lungs upon airborne exposure to various allergens and remain in the lungs for a prolonged period, which may explain the persistence of type 2 immunity in allergic airway disorders.

The contribution of Th2-type Trm cells to allergic airway disorders may not be limited to their ability to persist but also likely involves their robust ability to initiate immunopathology. The S1PR1 agonist FTY720 blocks lymphocyte egress from lymphoid organs, thereby reducing circulating lymphocytes.[52, 53] We found that FTY720 treatment reduced circulating $CD4^+$ T cells by 93%, but production of type 2 cytokines and CCL17 in OVA-challenged mice was not affected (Fig. 3). In the parabiosis model, upon re-exposure to OVA, the mice that were previously exposed i.n. to OVA plus *Alternaria* (thus having OVA-specific Trm cells in the lungs) showed significantly higher lung levels of type 2 cytokines, eosinophilic inflammation, and tissue pathology than mice with circulating OVA-specific T cells, underscoring the importance of Trm cells in mediating antigen-induced

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immunopathology. Interestingly, no differences in the lung levels of IL-4 were observed between OVA-exposed memory and naïve parabionts, leading us to speculate that the IL-5 and IL-13 responses are mediated by Th2-type Trm cells, and that the IL-4 response is mediated by circulating memory Th2 cells, such as Tem and Tcm cells. Another parabiosis study using HDM extract found that Th2-type Trm cells were found mainly near the airway and induced mucus production and airway hyperresponsiveness whereas circulating memory Th2 cells were preferentially localized in the parenchyma and were responsible for eosinophil and T cell recruitment to the lungs.[15] These findings suggest pivotal roles for Th2-type Trm cells in defining the magnitudes and anatomical locations of allergen-induced memory responses in the lungs.

The mechanisms involved in the development and maintenance of Trm cells are an active area of research in the fields of both $CD4^+$ and $CD8^+$ Trm cells. $CD8^+$ Trm cells reportedly utilize CD69, CD103, and IL-15 for their maintenance and survival, although these requirements appear to vary among the models.[5] CD69 is considered a universal phenotypic marker for both $CD4^+$ and $CD8^+$ Trm cells.[4, 5, 53, 54] The receptor for S1P (S1PR1) allows lymphocytes to drain from the tissue sites by recognizing high concentrations of S1P in the blood and lymph.[55] CD69 binds to the S1PR1 protein and prevents its cell surface expression.[37, 38] CD69-deficient $CD8^+$ T cells therefore show defective Trm cell development in both skin and lungs.[56, 57] The importance of CD69 in promoting tissue residency likely applies to allergen-specific $CD4^+$ Trm cells as well. For example, repeated exposure to HDM or *Aspergillus* extract induced development of Th2 cells and IL-9-producing T cells, respectively, which highly express CD69 and reside in the lungs.[13, 54, 58] We also found in this study that $CD4^+ST2^+$ T cells expressed CD69 during the early development of Trm in mice exposed to *Alternaria* (Fig. 3), and long-term kinetics showed that $CD69^+ST2^+$ T cells remained in the lung tissues longer than their $CD69^-$ counterparts (Fig. 6). The $CD69^+ST2^+$ T cells were also identified in the lungs of mice exposed to HDM (Supplemental Fig. E4), suggesting that the cell population is a common feature of type 2 immunity to various allergens. Further studies will help us to understand the molecular mechanisms to explain how CD69 is involved in the development and maintenance of $CD69^+ST2^+$ Th2 cells in the lung tissues.

$CD8^+$ Trm cells are thought to undergo a differentiation program in response to tissue-derived cues, such as exogenous ATP, IL-15, and TGF-β.[59–62] Lung epithelial cells produce a variety of cytokines, including IL-33, TSLP, and IL-25,[63] which likely play an important role in the development and/or maintenance of $CD4^+$ Trm cells in the lung tissues. In a study using triple-knockout mice (i.e., deficient in receptors for IL-33, IL-25, and TSLP) infected with the parasitic nematode *Nippostrongylus Brasilliensi*, Th2 cells that were primed in draining lymph nodes (dLNs) were shown to undergo terminal differentiation in the lungs, influenced by these epithelium-derived cytokines.[40] In this study, the development of $CD69^+ST2^+$ Trm cells in the lungs in response to *Alternaria* exposure was compromised in mice deficient in IL-33 (Fig. 6), and IL-33 was dispensable for Th2 cell differentiation in dLNs (Fig. 5). In contrast, $CD69^+ST2^+$ Trm cells were preserved in IL-33-deficient mice when they are exposed to HDM (Supplemental Fig. E4), suggesting heterogeneity depending on the nature of allergens. In $CD8^+$ T cells, TNF-α, IL-33, and TGF-β acted together to suppress expression of *Klf2*, *Sell*, and *S1pr1*[64] - all of which are essential for

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lymphocytes to exit from the tissue compartment.[52] In ILC2s, IL-33 was shown to promote uptake of environmental nutrients, such as glucose and fatty acid,[65] and increase the fitness of ILC2s to reside in the lung tissues. Thus, IL-33 may regulate tissue recruitment and/or retention of Trm cells. Further studies will be necessary to examine the roles of IL-33 and potentially other cytokines and molecules in the lungs in the development of Trms in response to *Alternaria*, HDM, and other airborne allergens. Studies are also necessary to elucidate the molecular and cellular mechanisms to explain how these factors promote the development and/or persistence of Th2-type Trms. For example, do they act directly on Th2 cells or indirectly through activation of other cells in the lungs, such as ILC2s, mast cells and eosinophils?

In summary, we found in this project that Th2-type Trm cells developed quickly within the lung tissues after initial allergen exposure and persisted in the tissues for months, playing a pivotal role in inducing lung pathology upon re-exposure to the allergens. The IL-33/ST2 pathway likely plays a major role in the establishment of Th2-type Trm cells in response to *Alternaria*, reminding us of the large-scale genome-wide association studies implicating *IL33* and its receptor *IL1RL1* in asthma.[66] $CD8^+$ and $CD4^+$ Trm cells accelerate host protection from reinfection with microbes at barrier sites.[4] However, a growing body of evidence also points to the roles of Trm cells in the pathogenesis of immune-mediated diseases.[7–10] Our parabiosis model (Fig. 1) indicates the pivotal roles for antigen-specific immune cells that are resident in the lungs, including $CD8^+$ and $CD4^+$ Trm and potentially B cells, in mediating lung pathology upon re-exposure to allergens. Further studies will be necessary to investigate the roles of Th2-type Trm cells in the pathogenesis of chronic inflammation of mucosal tissues, including but not limited to asthma, CRS, atopic dermatitis, and eosinophilic esophagitis. Given the persistent nature of Trm, it will be also important to understand whether Trm cells that develop in a pediatric period persist into adulthood.[67] The molecular mechanisms underlying the development and maintenance of Th2-type Trm should also be elucidated further. To this end, it would be highly valuable to identify the specific genes and endogenous molecules necessary for $CD4^+$ Trm cell development and create Trm cell-deficient animals to pinpoint their exact contribution to the pathogenesis of chronic airway and allergic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ab	antibody

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BAL	bronchoalveolar lavage
CRS	chronic rhinosinusitis
dLNs	draining lymph nodes
eGFP	enhanced green fluorescent protein
FACS	fluorescence-activated cell sorting
HDM	house dust mite
IL	interleukin
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
LAL	limulus amebocyte lysate
LN	lymph node
mLN	mediastinal lymph node
mRNA	messenger RNA
OVA	ovalbumin
S1PR1	sphingosine-1-phosphate receptor 1
Tcm	central memory T
Tem	effector memory T
Trm	tissue-resident memory T
TSLP	thymic stromal lymphopoietin
WT	wild-type

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Key Messages

- A single exposure to inhaled allergens for naïve mice induces the generation of Th2 cells that persist in the lung tissues for more than 8 weeks.
- Lung-resident $CD69^{+}ST2^{+}$ Th2 cells respond quickly to allergen re-exposure and promote lung pathology.
- The IL-33/ST2 pathway plays a role in the generation of tissue-resident Th2 cells to certain allergens.

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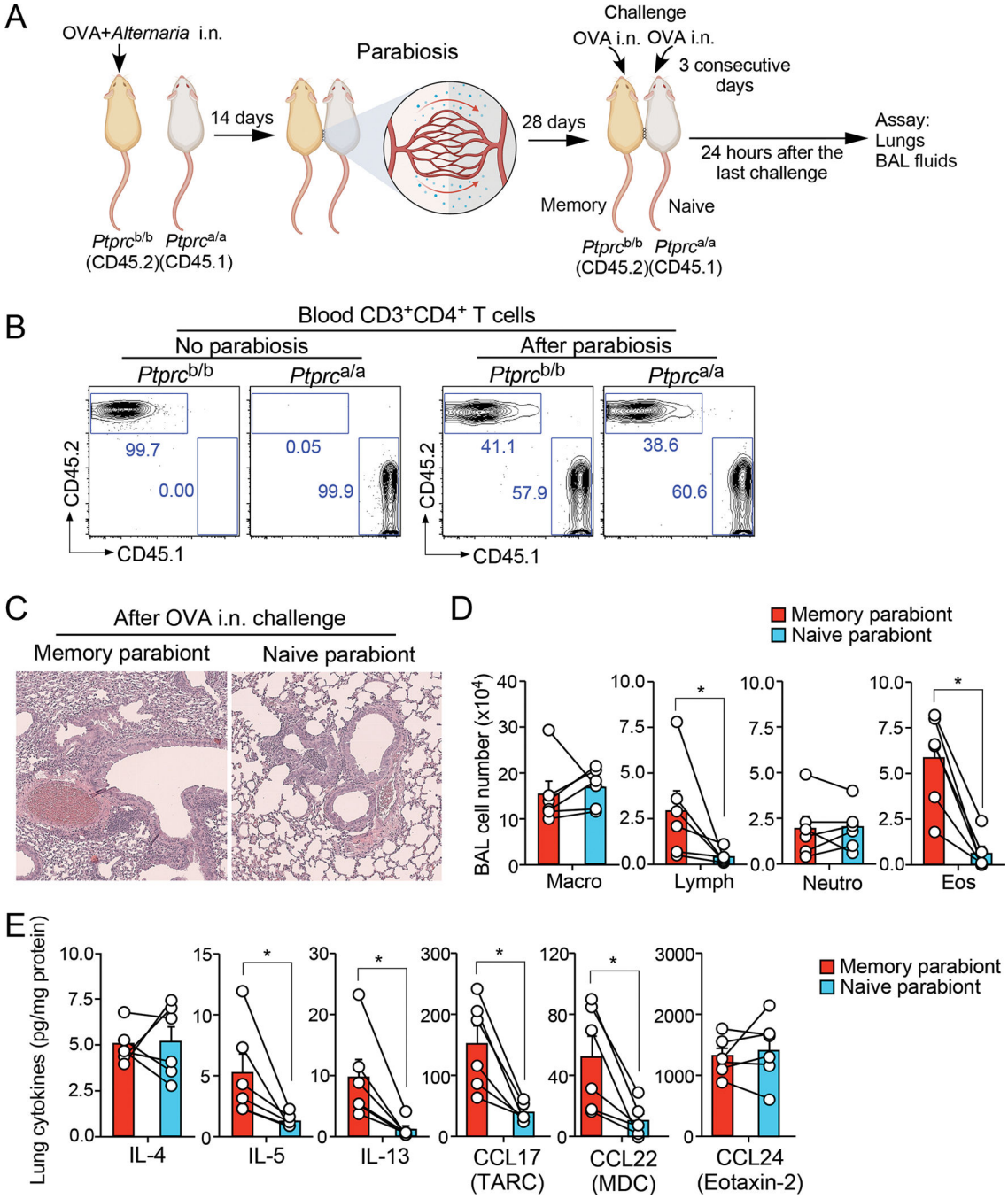

Figure 1.

Tissue lymphocytes are necessary to produce strong memory responses to inhaled antigens. (A) Schematic overview of the experimental protocol. (B) Representative scattergrams of CD45.1 and CD45.2 expression on the $CD3^+CD4^+$ lymphocyte population from peripheral blood analysis by FACS, 21 days after parabiosis surgery. (C) Representative photomicrographs of lungs collected and examined by H&E stain 24 hours after the last i.n. OVA challenge. (D) Total number of cells in BAL fluids and (E) lung levels of cytokines and chemokines. Data are pooled from three experiments and presented as mean ± SEM (n=6

pairs). Each dot represents one mouse, and the paired mice are connected by lines. *P<0.05 between the groups indicated.

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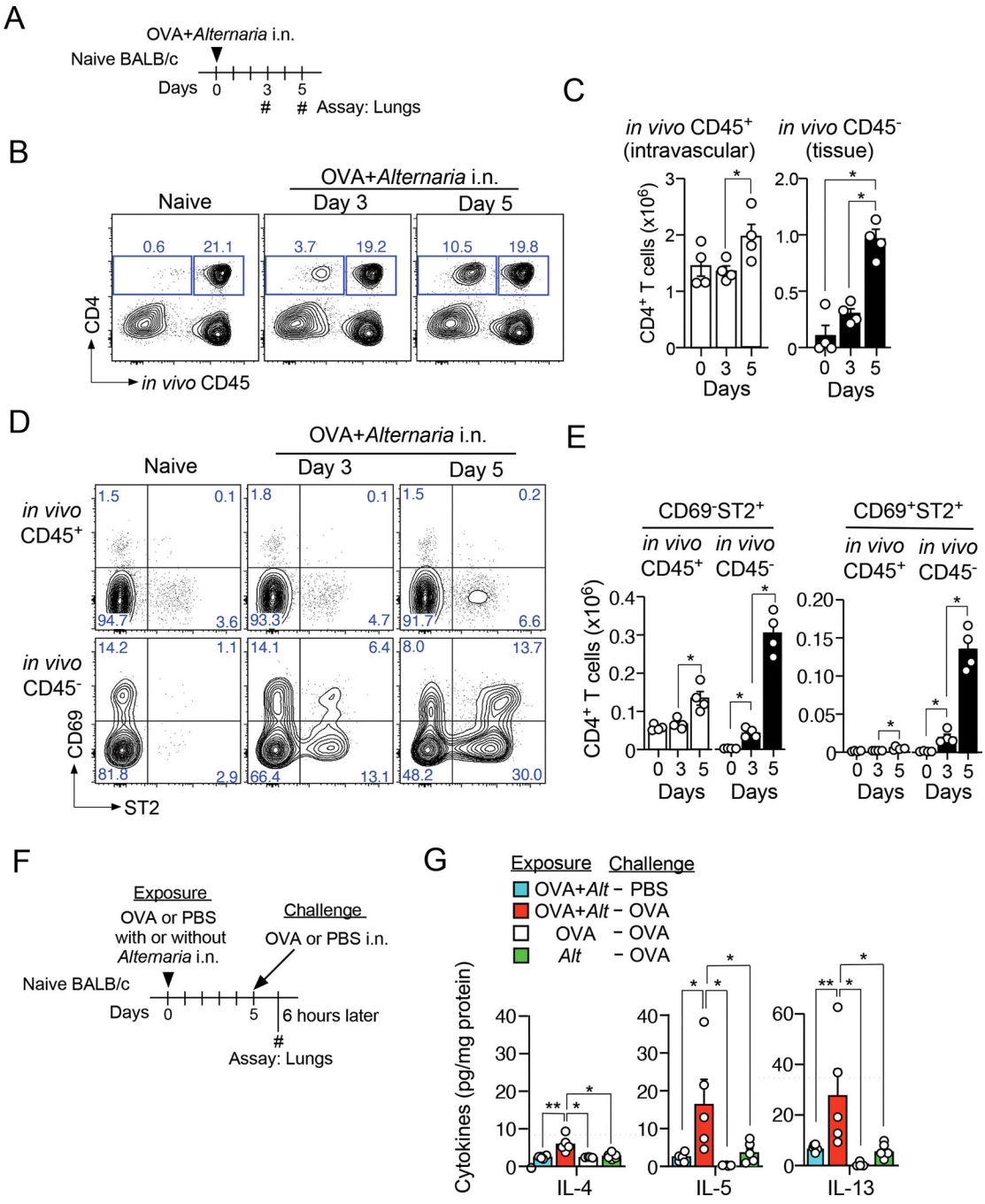

Figure 2.

Antigen-specific Th2 cells accumulate in the lung tissues within 5 days of allergen exposure in naïve mice. (A) Schematic overview of the experimental protocol. (B-E) Lungs were collected on days 0, 3, or 5 immediately after i.v. injection with anti-CD45 Ab, and single-cell suspensions were analyzed by FACS. (B) Representative scattergrams showing CD4 expression and *in-vivo* CD45 labeling of lymphocytes. (C) Numbers of each cell population. Data are presented as mean ± SEM (n=4) and are representative of two experiments. (D) Representative scattergrams showing CD69 and ST2 expression on the *in-vivo* CD45-

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positive (intravenous) and -negative (tissue) $CD4^+$ lymphocyte populations. (E) Numbers of each cell population. Data are presented as mean ± SEM (n=4) and are representative of two experiments. (F) Schematic overview of the experimental protocol for Panel G. (G) Lung levels of cytokines. *P<0.05 and **P<0.01 between the groups indicated. Data are presented as mean ± SEM (n=4–5) and are representative of two experiments. *Alt*, *Alternaria*.

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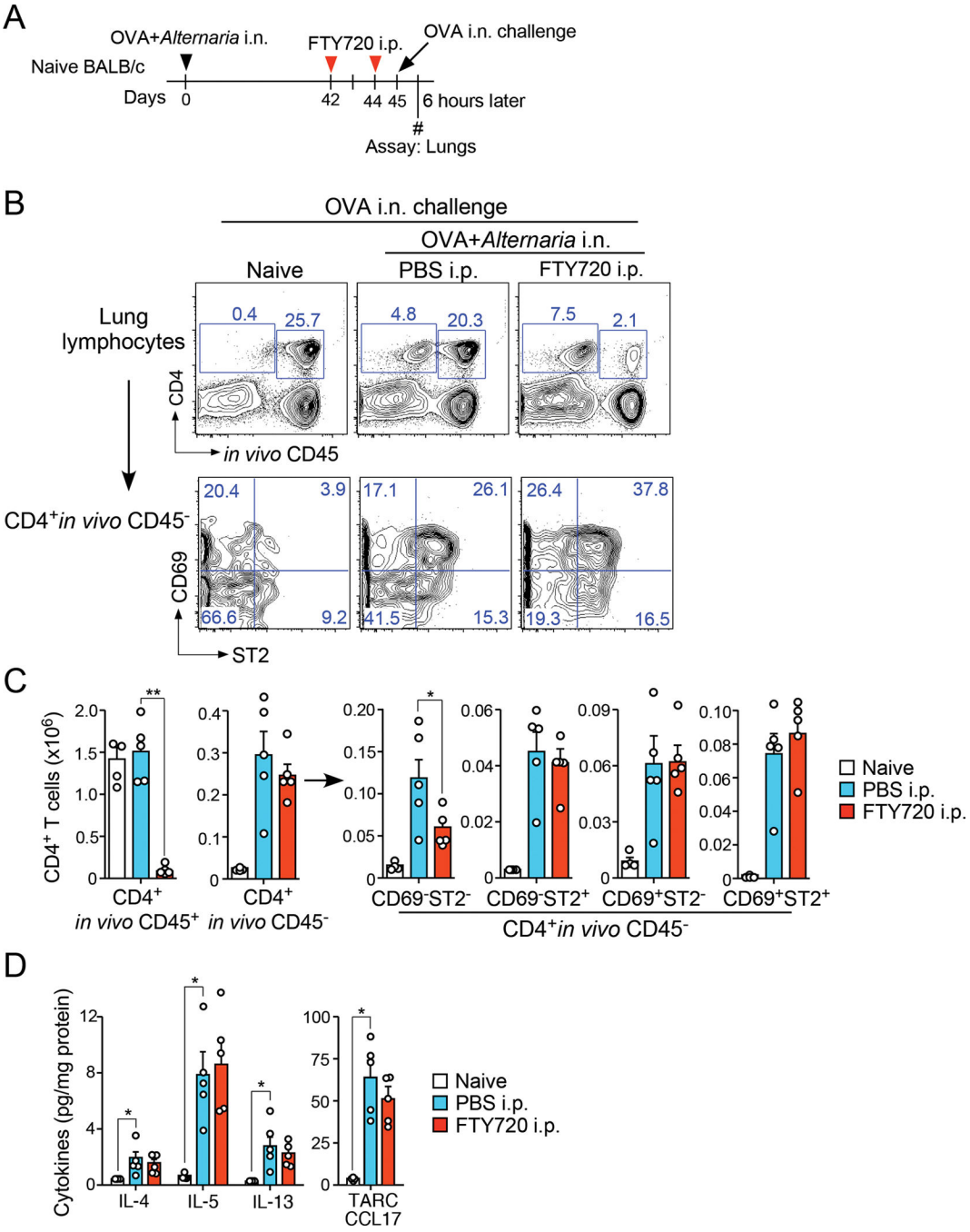

Figure 3.

Th2-type memory cells persist in the lung tissues for more than 6 weeks after the initial inhaled allergen exposure. (A) Schematic overview of the experimental protocol. (B–D) Six hours after i.n. OVA challenge, lungs were collected and analyzed by FACS and ELISA. (B) Representative scattergrams showing expression of CD4 and *in-vivo* CD45 labeling (upper panels), and expression of CD69 and ST2 on the *in-vivo* CD45-negative (tissue) $CD4^+$ cell population (lower panels). (C) Numbers of each cell population. (D) Lung levels of

cytokines and CCL17. Data are presented as mean ± SEM (n=4–5 in each group). Each dot represents one mouse. *P<0.05 and **P<0.01 between the groups indicated.

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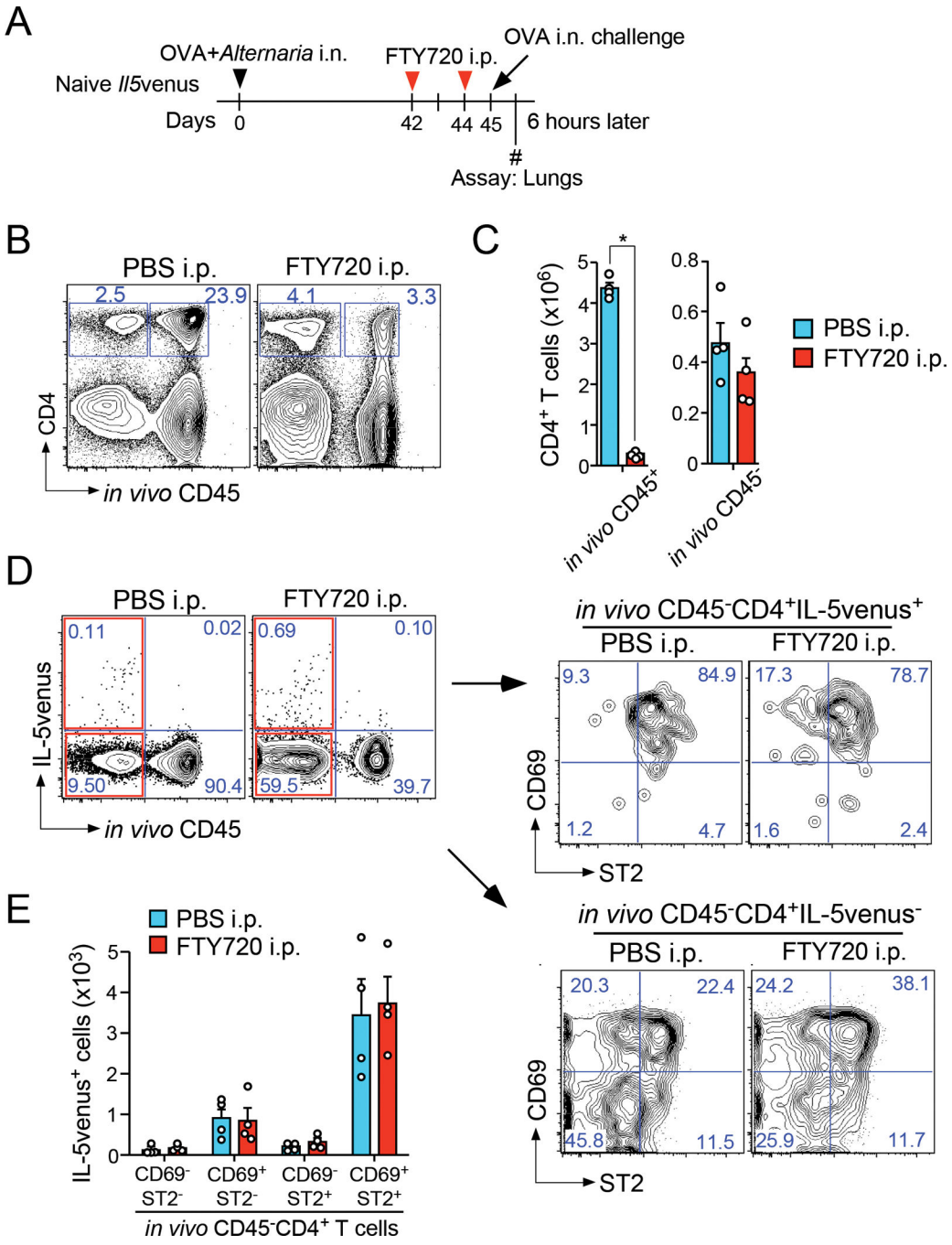

Figure 4.

ST2$^+$CD69$^+$CD4$^+$ memory T cells in the lung tissues quickly produced IL-5 in response to OVA challenge *in vivo*. (A) Schematic overview of the experimental protocol. Six hours after i.n. OVA challenge, lungs were collected and analyzed by FACS. (B) Representative scattergrams showing expression of CD4 and *in-vivo* CD45 labeling. (C) Numbers of *in-vivo* CD45-positive (intravenous) and -negative (tissue) CD4$^+$ T cells. (D) Representative scattergrams showing expression of IL-5-venus and *in-vivo* CD45 labeling. The IL-5-venus-positive and -negative cell populations were further analyzed for their expression of ST2 and

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CD69. (E) Numbers of each cell population. Data are presented as mean ± SEM (n=4 in each group). Each dot represents one mouse. *P<0.05 between the groups indicated.

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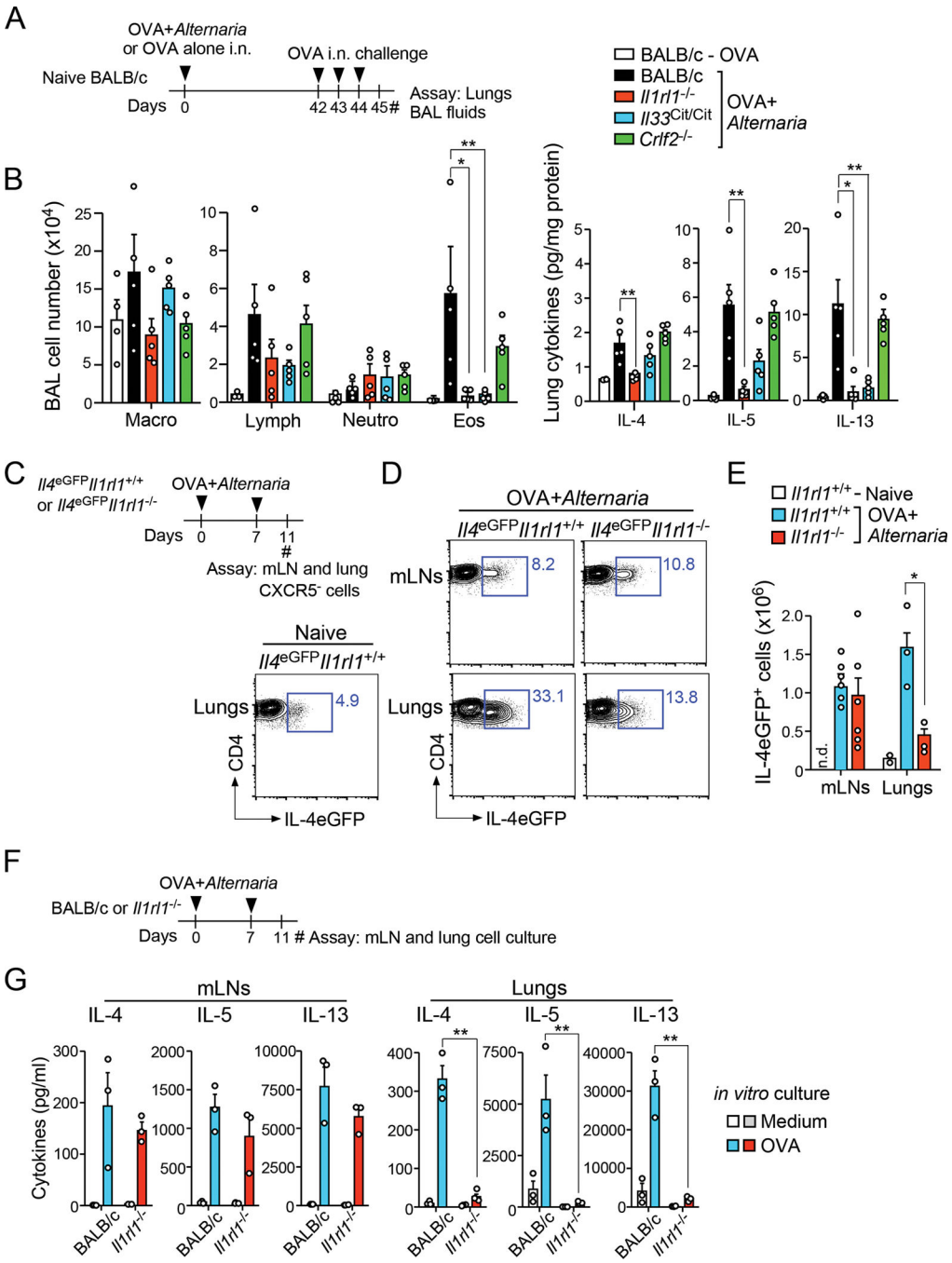

Figure 5.

The IL-33/ST2 pathway is indispensable for memory type 2 responses in the lungs. (A) Schematic overview of the experimental protocol for Panel B. (B) Total number of cells in BAL fluids, and lung levels of cytokines. Data are presented as mean ± SEM (n=4–5 in each group) and are representative of two experiments. (C) Schematic overview of the experimental protocols for Panels C and D. (D) Representative scattergrams showing expression of IL-4eGFP in the $CXCR5^{-}CD4^{+}$ cell population in mLN and lung lymphocytes. (E) Numbers of $IL\text{-}4eGFP^{+}$ $CXCR5^{-}CD4^{+}$ T cells. Data are presented as

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mean ± SEM (n=3–6 in each group) and are representative of two experiments. N.D.; not determined. (F) Schematic overview of the experimental protocol for Panel G. (G) $CD4^+$ T cells were isolated from mLNs and lungs and cultured with splenocytes from naïve mice with or without OVA antigen. The levels of cytokines in the supernatants were measured by ELISA. Data are presented as means ± SEM (n=3). *P<0.05 and **P<0.01 between the groups indicated.

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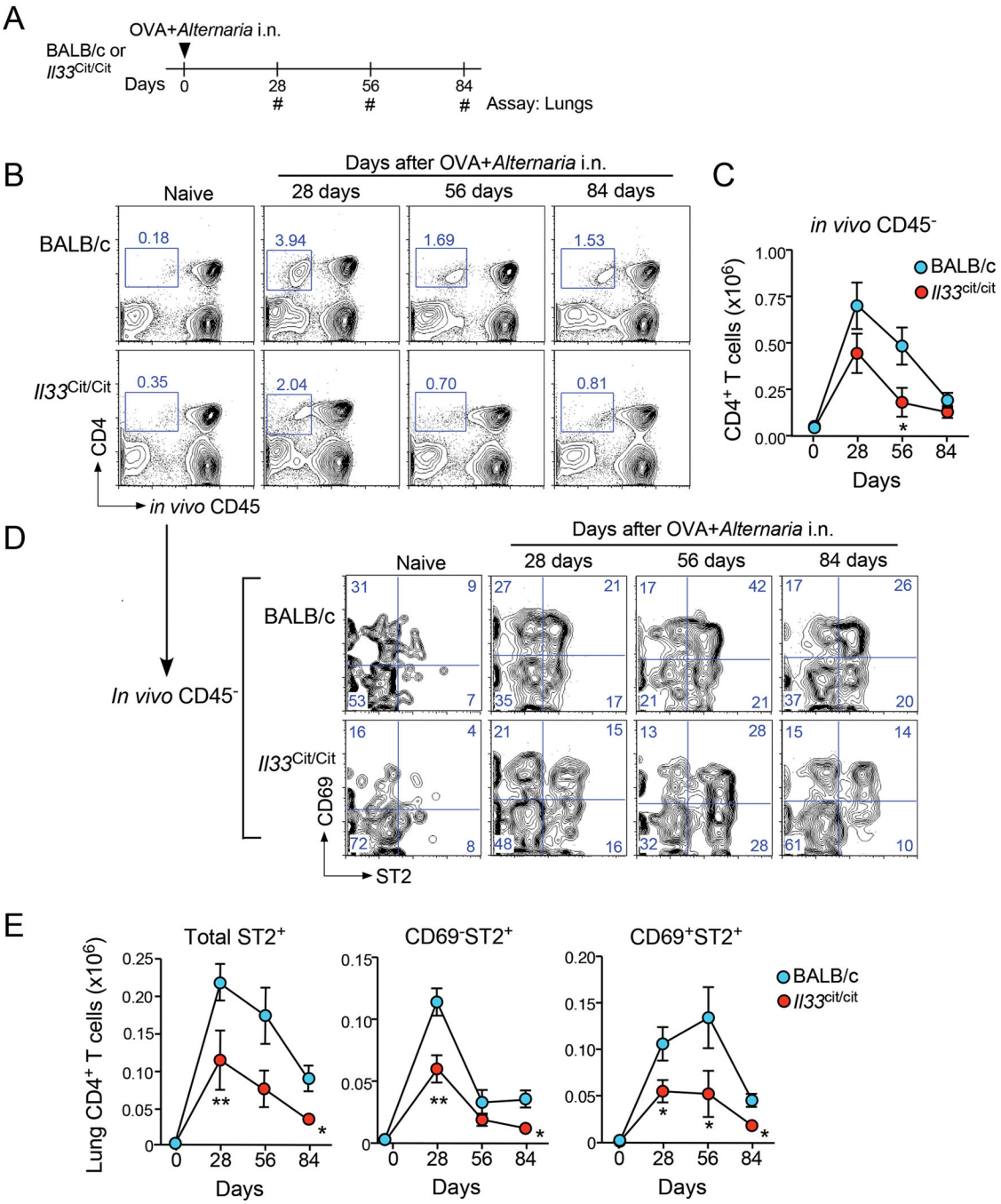

Figure 6.

Development and long-term presence of CD69$^+$ST2$^+$CD4$^+$ memory T cells in the lung tissues in response to *Alternaria* exposure are dependent on IL-33. (A) Schematic overview of the experimental protocol. (B–E) Lungs were collected immediately after i.v. injection with anti-CD45 Ab, and single-cell suspensions were analyzed by FACS. (B) Representative scattergrams showing CD4 expression and *in-vivo* CD45 labeling of lymphocytes. (C) Kinetic change in the numbers of *in-vivo* CD45-negative (tissue) CD4$^+$ T cells. Data are presented as mean ± SEM (n=3–5). (D) Representative scattergrams showing CD69 and

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ST2 expression on the *in-vivo* CD45-negative (tissue) $CD4^+$ lymphocyte population. (E) Kinetic changes in the numbers of each cell population. Data are presented as mean ± SEM (n=3–5) and are representative of two experiments. $^{**}P<0.05$ and $^{**}P<0.01$ compared with WT BALB/c mice at the same time point.

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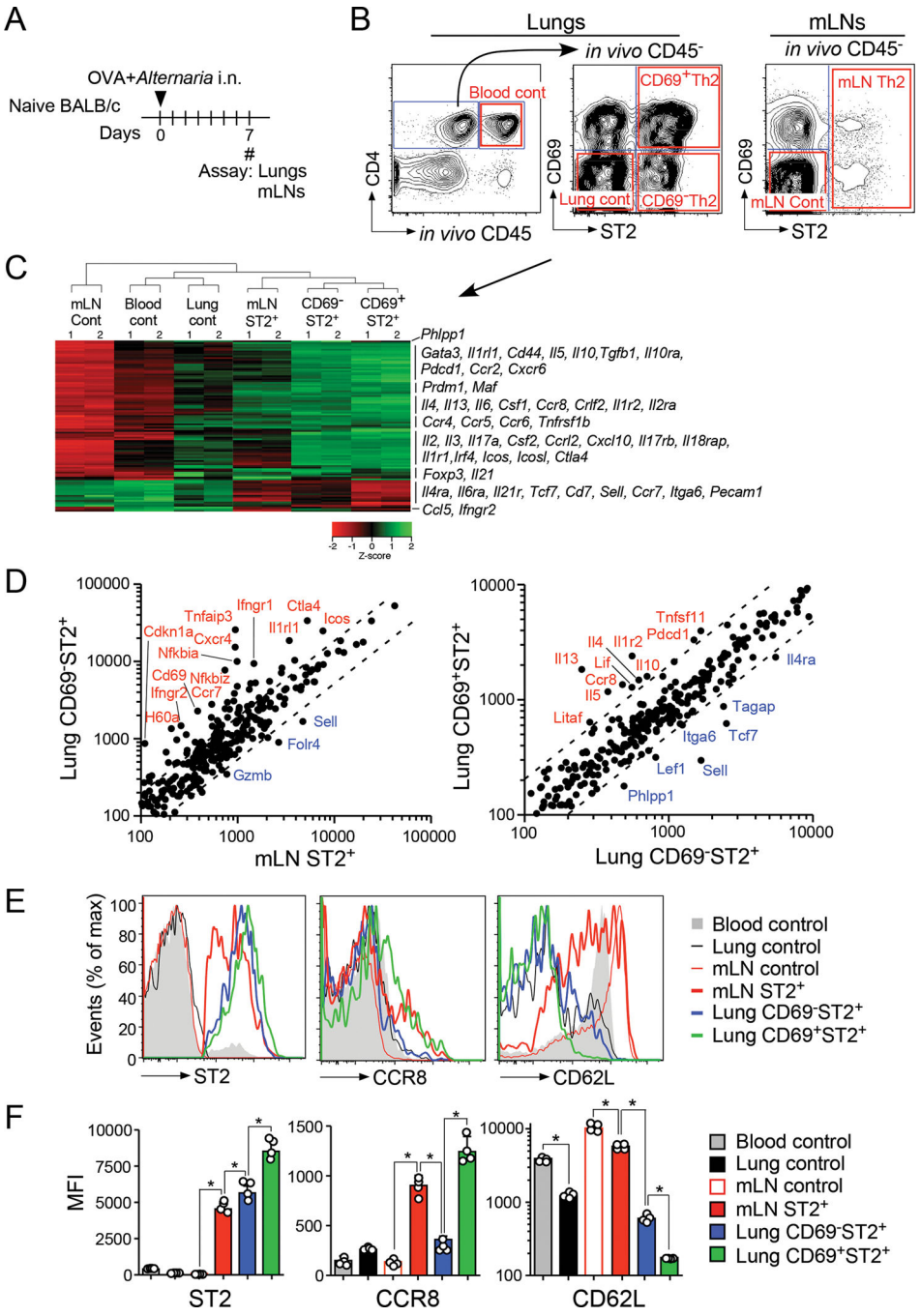

Figure 7.

Analyses of gene expression in several ST2$^+$CD4$^+$ T cell populations. (A) Schematic overview of the experimental protocol. (B) Representative scattergrams showing CD4 expression and *in-vivo* CD45 labeling of lymphocytes. Expression of ST2 and CD69 was examined by gating the *in-vivo* CD45-negative CD4$^+$ cell populations in the lungs and mLNs. The cell populations marked by the red squares are those sorted for gene expression analyses. (C and D) mRNA expression in sorted lymphocyte populations was analyzed by nCounter Gene Expression Assay (NanoString). (C) Heat map showing gene

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expression from the cells sorted and pooled from two separate experiments. Representative genes in each cluster are indicated on the right. (D) Scattergrams showing the differences in gene expression between $ST2^{+}CD4^{+}$ cells in mLN versus $CD69^{-}ST2^{+}$ $CD4^{+}$ cells in the lungs (left panel) and between $CD69^{-}ST2^{+}$ $CD4^{+}$ cells and $CD69^{+}ST2^{+}$ $CD4^{+}$ cells in the lungs (right panel). Dotted lines indicate 2-fold differences between the populations. (E and F) Lungs and mLNs were collected as described in Panel A, and single-cell suspensions from the specimens were analyzed by FACS by gating indicated cell populations. (E) Representative histograms. (F) Mean fluorescent intensity of each protein. Data are presented as mean ± SEM (n=4 in each group). Each dot represents one mouse. $^{**}P<0.05$ and $^{**}P<0.01$ between the groups indicated.