

Antiapoptotic serine protease inhibitors contribute to survival of allergenic T_H2 cells

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Background: The mechanisms that regulate maintenance of persistent T_H2 cells and potentiate allergic inflammation are not well understood.

Objective: The function of serine protease inhibitor 2A (Spi2A) was studied in mouse T_H2 cells, and the serine protease inhibitor B3 (*SERPINB3*) and *SERPINB4* genes were studied in T_H2 cells from patients with grass pollen allergy.

Methods: Spi2A-deficient T_H2 cells were studied in *in vitro* culture or *in vivo* after challenge of Spi2A knockout mice with ovalbumin in alum. Expression of *SERPINB3* and *SERPINB4* mRNA was measured in *in vitro*-cultured T_H2 cells and in *ex vivo* CD27⁺CD4⁺ cells and innate lymphoid cell (ILC) 2 from patients with grass pollen allergy by using quantitative PCR. *SERPINB3* and *SERPINB4* mRNA levels were knocked down in cultured CD27⁺CD4⁺ cells with small hairpin RNA.

Results: There were lower levels of *in vitro*-polarized T_H2 cells from Spi2A knockout mice ($P < .005$) and *in vivo* after ovalbumin challenge ($P < .05$), higher levels of apoptosis (Annexin V positivity, $P < .005$), and less lung allergic inflammation (number of lung eosinophils, $P < .005$). *In vitro*-

polarized T_H2 cells from patients with grass pollen allergy expressed higher levels of both *SERPINB3* and *SERPINB4* mRNA (both $P < .05$) compared with unpolarized CD4 T cells. CD27⁺CD4⁺ from patients with grass pollen allergy expressed higher levels of both *SERPINB3* and *SERPINB4* mRNA (both $P < .0005$) compared with CD27⁺CD4⁺ cells. ILC2 expressed higher levels of both *SERPINB3* and *SERPINB4* mRNA (both $P < .0005$) compared with ILC1. Knockdown of either *SERPINB3* or *SERPINB4* mRNA (both $P < .005$) levels resulted in decreased viability of CD27⁺CD4⁺ compared with control transduced cells.

Conclusion: The Serpins Spi2A in mice and *SERPINB3* and *SERPINB4* in allergic patients control the viability of T_H2 cells. This provides proof of principle for a therapeutic approach for allergic disease through ablation of allergic memory T_H2 cells through *SERPINB3* and *SERPINB4* mRNA downregulation. (J Allergy Clin Immunol 2017;■■■■:■■■■-■■■■.)

Key words: T_H2 cells, apoptosis, memory, grass pollen allergy

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Persistent T_H2 cells are the principal cell population responsible for the maintenance of chronic allergic inflammation and the rapid relapse of acute allergic inflammation on allergen re-exposure.¹⁻³ The mechanisms that regulate the maintenance of persistent T_H2 cells and potentiate allergic inflammation remain elusive. Strong candidates for these persistent T_H2 cells are circulating human CD4⁺ T cells, which express the prostaglandin D₂ receptor (CRTH2) and have a phenotype of terminally differentiated central effector/memory T cells based on T_H2 cytokine production, gene expression profile, and the ability to respond to allergens.^{4,5} In mice the differentiation of effector T_H2 cells into persistent T_H2 cells (referred to as memory T_H2 cells) requires escape from apoptosis.^{6,7} Such persistent T_H2 cells retain the cytokine expression profile of the effector cell.⁸⁻¹⁰

Compared with T_H1 and CD8⁺ effector cells, T_H2 cells are relatively resistant to apoptosis and refractory to the classical TNF receptor family-induced, activation-induced cell death (AICD) pathway of apoptosis.¹¹ Release of executioner cathepsins, such as cathepsins B and L, from the lysosome triggers cell death in response to several stimuli in a variety of physiologic settings.¹²⁻¹⁴ We identified serine protease inhibitor 2A (Spi2A) as a physiologic inhibitor of the lysosomal death pathway in mice.¹⁵ Spi2A, which is encoded by the *Serpina3g* gene on mouse chromosome 12,¹⁶ is unusual for a serine protease inhibitor (Serp) in that it inhibits not only serine proteases but also cysteine cathepsins and resides in the cytosol and nucleus.¹⁵ In human subjects squamous cell carcinoma antigen (SCCA) 1 (*SERPINB3*), which belongs to the ovalbumin (OVA)-Serp family,¹⁷ inhibits both serine proteinases, such as chymotrypsin, and cysteine proteinases, such as cathepsin L, K, S, and papain,¹⁸ whereas the

Abbreviations used

AICD:	Activation-induced cell death
APC:	Allophycocyanin
BAL:	Bronchoalveolar lavage
CRTH2:	Prostaglandin D ₂ receptor
FACS:	Fluorescence-activated cell sorting
ICS:	Intracellular staining
ILC1:	Innate lymphoid cell 1
ILC2:	Innate lymphoid cell 2
KO:	Knockout
OVA:	Ovalbumin
OVA-tet:	I-A ^b tetramer (APC) containing the OVA peptide 323-339
PE:	Phycoerythrin
PerCP:	Peridinin-chlorophyll-protein complex
PMA:	Phorbol 12-myristate 13-acetate
S-C:	Sensitization-challenge
SCCA:	Squamous cell carcinoma antigen
Serpin:	Serine protease inhibitor
shRNA:	Small hairpin RNA
Spi2A:	Serine protease inhibitor 2A
STAT:	Signal transducer and activator of transcription
TCR:	T-cell receptor

closely related Serpin SCCA-2 (SERPINB4) is able to inhibit serine proteinases, such as cathepsin G and mast cell chymase.¹⁹ Both Spi2A in mice^{15,20} and SCCA-1²¹⁻²³ and SCCA-2^{24,25} in human subjects are potent inhibitors of apoptosis.

We now show in mice and human subjects that intracellular Serpins are required for the survival of disease-causing memory T_H2 cells. Spi2A was upregulated in T_H2 but not T_H1 effectors in response to T-cell receptor (TCR) stimulation and cytokines. Development of T_H2 cells after immunization with OVA was impaired in Spi2A knockout (KO) mice, as was the development of memory allergic inflammatory responses in the lung. Both *SERPINB3* and *SERPINB4* were expressed in human T_H2 cells after *in vitro* polarization and in memory T_H2 cells from patients with pollen allergy. Importantly, knockdown of either *SERPINB3* or *SERPINB4* resulted in a drastic impairment of memory T_H2 cell survival. We conclude that antiapoptotic Serpins are potential targets for ablation of disease-causing memory T_H2 cells.

METHODS**Mice**

Spi2A KO mice were on the C57BL/6 background.²⁶ Wild-type C57BL/6 mice were obtained from Charles River Laboratories (Bar Harbor, Me). All mice were maintained in accordance with UK Home Office regulations.

Human subjects

Participants with grass pollen-induced seasonal allergic rhinoconjunctivitis (n = 8) provided blood samples and answered symptom questionnaires (Table I). Blood samples were collected during the grass pollen season (May-July). The study was approved by the South West London REC3 Research Ethics Committee and the Research Office of the Royal Brompton and Harefield NHS Foundation Trust.

T_H1 and T_H2 polarization *in vitro*

Mice. By using the protocol from Boonstra et al,²⁷ spleen and lymph node cells were harvested from Spi2A KO and wild-type mice, positively sorted with magnetic beads (Miltenyi Biotec, Bergisch Gladbach,

TABLE I. Subjects' characteristics

	SAR (n = 8)
Sex (male/female)	5/3
Age (y), mean (range)	31 (20-48)
Allergen grass-specific IgE, kU/L, mean (SD)	62.5 ± 13.3
Total IgE, kU/L, mean (SD)	28.9 ± 328.30
Allergen skin prick test (mm ²), mean (SD)	10.3 (2.4)

Distribution of age, sex, specific IgE levels, and skin prick test responses. SAR, Seasonal allergic rhinitis.

Germany) for CD4⁺CD62L⁺CD44⁻ cells, and then seeded at 10⁶/mL in RPMI containing anti-CD3 (2 μg/mL) and anti-CD28 (5 μg/mL) mAbs. For T_H1 polarization, cells were cultured with neutralizing anti-IL-4 mAb (10 μg/mL) and rIL-12 (10 ng/mL) for 4 days. For T_H2 polarization, cells were cultured with neutralizing anti-IFN-γ mAb (10 μg/mL) and rIL-4 (10 ng/mL) for 4 days. Fresh T_H2 polarization medium plus IL-2 (10 ng/mL) was added to these cultures every 48 hours. All reagents were from eBioscience (San Diego, Calif).

Human subjects. Heparinized venous blood was collected from patients with grass pollen-induced allergy and seasonal allergic rhinitis. Whole blood was diluted 1:1 with RPMI 1640 media (Invitrogen), layered on Histopaque-1077 (Sigma-Aldrich, St Louis, Mo) density gradient (density = 1.077 g/L), and centrifuged for 25 minutes at 1136g at room temperature. The PBMC layer was collected, washed, and resuspended in RPMI 1640. Cell viability was greater than 95%, as determined by using trypan blue exclusion. Naive CD4⁺CD45RA⁺ T cells were negatively selected and enriched by using the EasySep Human Naive CD4⁺ T Cell Isolation Kit (STEMCELL Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's instructions. The purity of fractionated cell populations determined by using fluorescence-activated cell sorting (FACS) analysis with phycoerythrin (PE)-conjugated anti-CD45RA (5H9; BD Biosciences, San Jose, Calif) was 99%. Purified CD4⁺CD45RA⁺ cells (10⁶/mL) were cultured in RPMI 1640 (Life Technologies, Gaithersburg, Md) supplemented with 10% FCS, 2 mmol/L L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), and 100 μg/mL streptomycin (Life Technologies). Cells were stimulated with plate-bound anti-CD3 (1 μg/mL; clone OKT3) and anti-CD28 (2 μg/mL; 37407; R&D Systems, Abingdon, United Kingdom) and rIL-2 (50 U/mL; R&D Systems). rIL-12 (2.5 ng/mL; R&D Systems) and anti-IL-4 (5 μg/mL; clone MP4-25D2; BD Pharmingen) were added to direct T_H1 differentiation. rIL-4 (12.5 ng/mL; NBS Biologicals, Cambridge, United Kingdom), anti-IFN-γ (5 μg/mL; clone B-B1; Invitrogen, Paisley, United Kingdom), and anti-IL-10 (5 μg/mL; clone JES3-9D7; Invitrogen) were added for T_H2 differentiation.

After 4 days, the cells were expanded under the same conditions in the absence of anti-CD3 or anti-CD28. Cells were then restimulated every 7 days. When required, cells were activated with phorbol 12-myristate 13-acetate (PMA; 5 ng/mL; Sigma-Aldrich) and ionomycin (500 ng/mL; Merck Millipore, Hertfordshire, United Kingdom) for 4 hours. In addition, cell lysates were collected to investigate gene expression by using RT-PCR, and cell supernatants were analyzed for cytokines and chemokines by using a human cytokine/chemokine magnetic bead panel 96-well plate assay (Milliplex Map Kit; Millipore, Danvers, Mass) and a Luminex xMAP Magpix platform (Merck Millipore), according to the manufacturer's instructions.

Flow cytometry

For extracellular staining, the following mAbs were used: anti-CD4-allophycocyanin (APC)-Cy7 (BioLegend, San Diego, Calif), anti-T1/ST2-fluorescein isothiocyanate (FITC; clone DJ8; MD Biosciences, Oakdale, Minn), and anti-CD27-APC (eBioscience). For intracellular staining (ICS) with anti-IL-4-PE-Cy7, anti-IFN-γ-Pacific Blue, and IL-5-PE

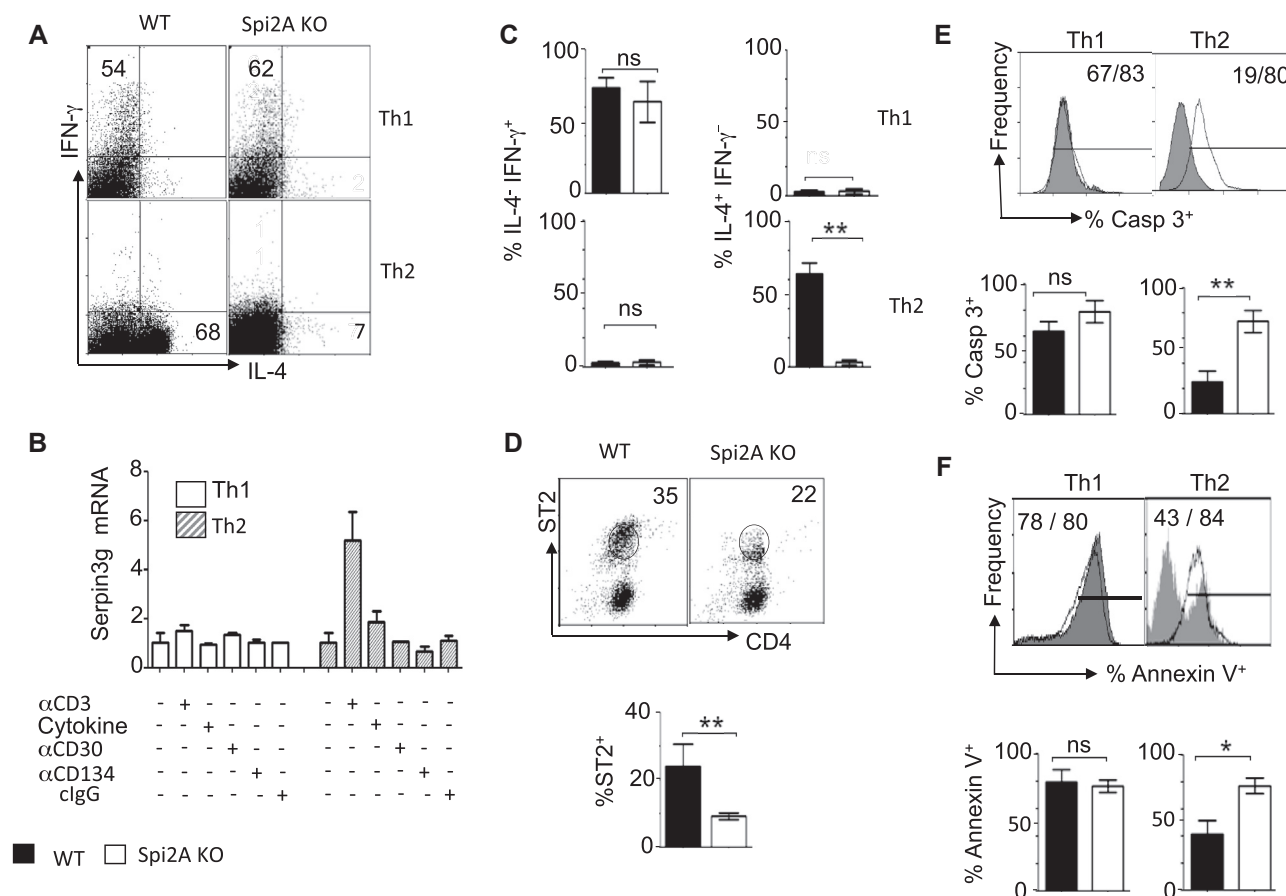


FIG 1. Differentiation and survival of T_H2 cells from Spi2A KO mice. **A**, Flow cytometric plots for T_H1 - or T_H2 -polarized CD4 T cells from wild-type (WT) or Spi2A KO mice after ICS for IL-4 or IFN- γ . Percentage IL-4 $^{+}$ IFN- γ $^{-}$ and IL-4 $^{-}$ IFN- γ $^{+}$ cells in quadrants are indicated. **B**, Level of *Serpin3g* mRNA (fold change over that of naive CD4 T cells [CD4 $^{+}$ CD62L $^{+}$]) in T_H1 or T_H2 cells after no stimulation (–) or stimulation with mAb or cytokine (IFN- γ for T_H1 and IL-4 for T_H2). **C**, Histograms of mean percentage IL-4 $^{+}$ IFN- γ $^{-}$ and IL-4 $^{-}$ IFN- γ $^{+}$ polarized CD4 $^{+}$ cells ($\pm$ SEM, $n = 6$). **D**, Flow cytometric plots after staining for ST2 and CD4 on WT and Spi2A KO T_H2 -polarized cells. Percentages of ST2 $^{+}$ CD4 $^{+}$ cells in plots are indicated. **E**, Flow cytometric histogram for WT (filled histograms) or Spi2A KO (line) T_H1 and T_H2 cells after ICS for activated caspase-3. Percentage of activated caspase-3-positive cells (line gate) is indicated as WT/Spi2A KO. Histograms show mean $\pm$ SEM percentages of activated caspase-3-positive cells ($n = 6$). **F**, Flow cytometric histogram for WT (solid histogram) or Spi2A KO (line) T_H1 and T_H2 cells after staining with Annexin V. Percentage of Annexin V $^{+}$ cells (line gate) is indicated for WT/Spi2A KO mice. Histograms show mean $\pm$ SEM percentage of Annexin V-positive cells ($n = 6$). * $P < .05$ and ** $P < .005$. ns, $P > .05$ (not significant).

(eBioscience), fixation and permeabilization were carried out according to the manufacturer's protocol (Cytofix/Cytoperm; BD Biosciences). Spleen cell suspensions were generated by means of mechanical disaggregation of whole spleens in RPMI with 10% FBS and then filtered through gauze to remove particular matter. Splenocytes (10^6 /mL) were stimulated with PMA (50 ng/mL), ionomycin (1 μ g/mL; Sigma-Aldrich), and brefeldin A (10 μ g/mL; eBioscience) for 5 hours and then stained with I-A ^{b} tetramer (APC) containing the OVA peptide 323-339 (OVA-tet), anti-CD4-APC-Cy7, and anti-T1/ST2-FITC in RPMI with 10% FBS for 1 hour at 37°C with 7-actinomycin D to identify dead cells.²⁸ Cells were stained with OVA-tet (AAHAEINEA; National Institutes of Health Tetramer Facility, Atlanta, Ga) or negative control CLIP peptide/I-A ^{b} tetramer. Cells were then subjected to ICS. Apoptosis was measured by using Annexin V staining or intracellular anti-active caspase-3, according to the manufacturer's protocol (BD Biosciences). CD4 $^{+}$ T cells were immunostained with anti-human CD45RO-PE (clone UCHL1) and CD45RA-FITC (clone 5H9; BD Biosciences) to check enrichment of human CD4 $^{+}$ CD45RA $^{+}$ naive T cells from isolated PBMCs on day zero.

Each week, resting cells were stimulated with PMA and ionomycin for 1 hour and brefeldin A for 4 hours before surface immunostaining with

antihuman CD4-peridinin-chlorophyll-protein complex (PerCP; clone L200) and intracellular immunostaining with anti-human IL-5-APC (clone TRFK), anti-human IL-13-PerCP-cy5.5 (clone JES-5AZ), anti-human GATA-3-PE-Cy7 (clone L50823), anti-human IFN- γ -PE Cy5.5 (clone B27), and anti-human T-bet V450 (clone 04-46) antibodies (BD Biosciences), according to the manufacturer's protocol (Cytofix-Cytoperm, BD Biosciences). Type 2 innate lymphoid cells (ILC2s; Lin $^{-}$ CD44 $^{+}$ CD127 $^{+}$ CD161 $^{+}$ CRTH2 $^{+}$) and type 1 innate lymphoid cells (ILC1s; Lin $^{-}$ CD44 $^{+}$ CD127 $^{+}$ CD161 $^{+}$ CRTH2 $^{-}$ CD117 $^{-}$ NKPA4 $^{-}$)²⁹ were immunophenotyped and FACS purified (>90% purity) from PBMCs from 6 patients with grass pollen allergy by using the following cell-surface antibodies: CD127-PE-Cy5, CD45-AF700, CD117 $^{+/-}$ -BV785 (BioLegend), CD294 (CRTH2)-BV711, CD336-PerCP-Cy5.5, CD161-APC, Fc ϵ RI α -FITC, and lineage cocktail (CD3, CD14, CD19, CD20, and CD56) (FITC; BioLegend). Cells were sorted directly into RLT buffer before gene expression analysis by using RT-PCR.

Analysis of Serpin gene expression

In vitro T_H1 - or T_H2 -polarized cultures were reseeded in fresh nonpolarizing medium for 1 day with culture media containing IL-2 alone (10 ng/mL) or

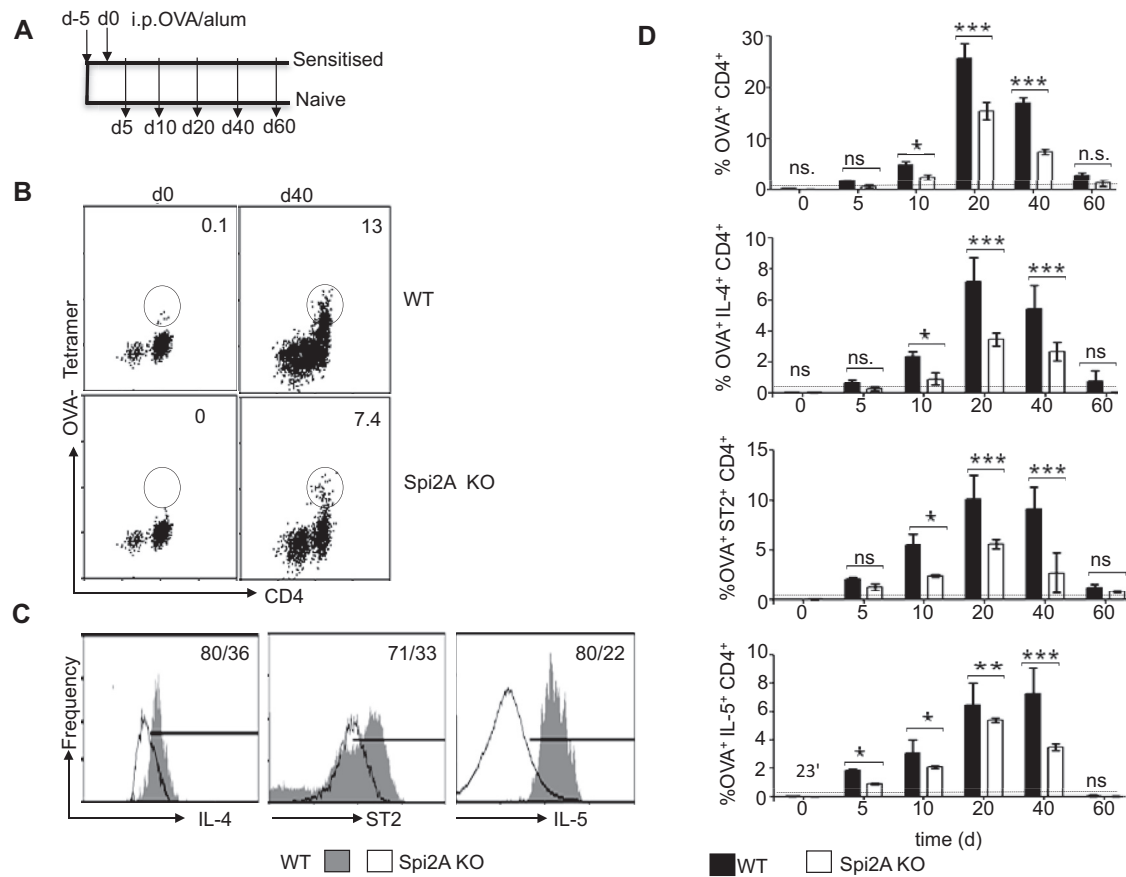


FIG 2. T_H2 responses in immunized Spi2A KO mice. **A**, Experimental protocol. Arrows indicate intraperitoneal (*i.p.*) injections of OVA plus alum on days -5 and 0 and the times at which mice were analyzed. **B**, Flow cytometric plots after staining with OVA₃₂₉₋₃₃₇/I-A^b tetramer (OVA-tet) and anti-CD4 before (day 0) and 40 days after the final OVA plus alum immunization. Percentage of OVA-tet⁺CD4⁺ cells is indicated. **C**, Histograms (solid, WT; line, Spi2A KO) after staining for ST2 on gated OVA-tet⁺CD4⁺ cells after ICS. Histograms show IL-4 or IL-5 on gated OVA-tet⁺CD4⁺ cells after ICS. Percentage positive population (line gate) is indicated for WT/Spi2A KO, respectively. **D**, Histograms for mean percentage of OVA-tet⁺CD4⁺, mean percentage of OVA-tet⁺CD4⁺IL-4⁺, mean percentage of OVA-tet⁺CD4⁺ST2⁺, and mean percentage of OVA-tet⁺CD4⁺IL-5⁺ cells of total splenocytes ($\pm$ SEM, $n = 6-8$). * $P < .05$, ** $P < .005$, and *** $P < .0005$. ns, $P > .05$ (not significant). Broken horizontal lines show the limit of detection.

with either IL-4 (10 ng/mL), IFN- γ (10 ng/mL), anti-CD3 (5 μ g/mL), anti-CD30 (50 μ g/mL), anti-OX40 (50 μ g/mL), or anti-CD30 (50 μ g/mL) plus OX40 (50 μ g/mL; eBioscience). Each condition was performed in duplicate.

Total RNA from each sample was obtained by using the RNeasy Plus Micro Kit (Qiagen, Manchester, United Kingdom) and QiaShredder spin columns, according to the manufacturer's instructions. Real-time PCR reactions were run with the SYBR Green PCR Master mix (Applied Biosystems, Foster City, Calif) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Primer sequences for *Serpina3g* (Spi2A gene) and β_2 -microglobulin were from Winkler et al.³⁰ Levels of *Serpina3g* mRNA are expressed as fold over β_2 -microglobulin control mRNA.³¹ Primer sequences of human *IL4*, *IL13*, *GATA3*, and *TBET* were from Shamji et al.³² Primer sequences for *SERPINB3* (forward 5'-AGC CGC GGT CTC GTG C-3' and reverse 5'-GGC AGC TGC AGC TTC TG-3') and *SERPINB4* (forward 5'-AGC CAC GGT CTC TCA G-3' and reverse 5'-GCA GCT GCA GCT TCC A-3') were from Mitsuishi et al.³³ The level of human mRNA was expressed as fold over *EFA2* control mRNA.³²

Gene knockdown of *SERPINB3* and *SERPINB4*

The following GIPZ lentiviruses were used (Thermo Fisher Scientific Biosciences GmbH, Paisley, United Kingdom): small hairpin RNA (shRNA)

mir *SERPINB3* or *SERPINB4*, nonsilencing GIPZ lentiviral shRNA mir, and GAPDH-GIPZ lentiviral shRNA mir positive control. In addition, nontransduced cells were used as controls. CD4⁺CD27⁻ cells were transduced with lentivirus (multiplicity of infection = 3) by means of centrifugation at 37°C for 90 minutes in Polybrene (4 μ g/mL), stimulated with anti-CD3 (0.5 μ g/mL) and anti-CD28 (0.5 μ g/mL) for 4 days, and then analyzed by using flow cytometry for CD4 and green fluorescent protein expression. The viability of CD27⁻CD4⁺ T cells was assessed by using trypan blue exclusion.

OVA-alum T_H2 model

Naive Spi2A KO and wild-type mice were immunized (sensitization) by means of intraperitoneal injection of OVA (100 μ g; Grade V; Sigma-Aldrich) and alum (4 mg; Pierce, Rockford, Ill) on days 0 and 5. Mice were subjected to 3 daily intranasal challenges with OVA in PBS (0.5% wt/vol) at various time points after sensitization to induce airway inflammation (sensitization-challenge [S-C]). In addition, some mice underwent intranasal challenge alone. For both S-C and challenge mice, analysis was made 1 day after the last intranasal challenge. For bronchoalveolar lavage (BAL) fluid extraction, the lungs of mice were perfused 5 times with lidocaine chloride in PBS (0.4% wt/vol; Sigma-

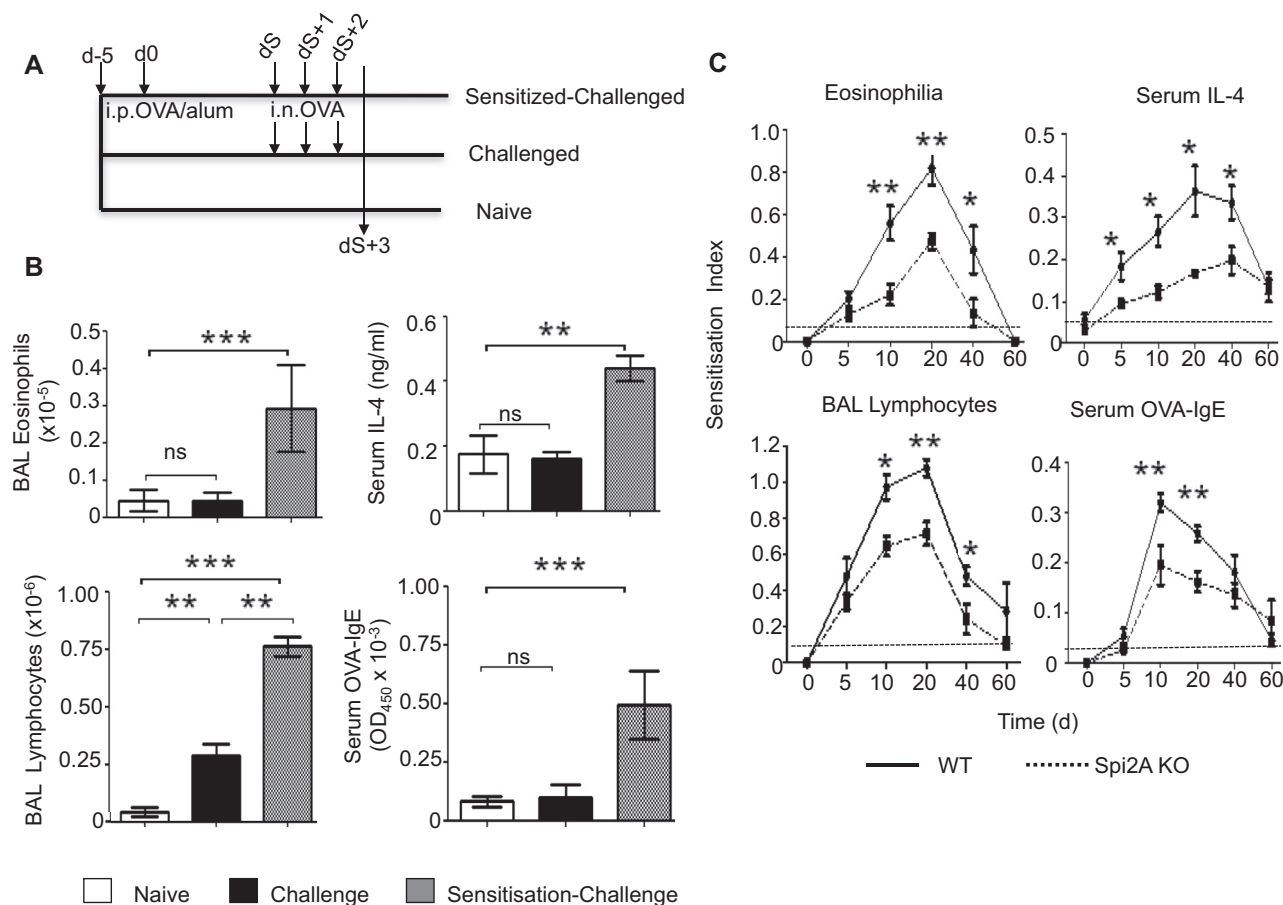


FIG 3. Diminished inflammatory allergic responses in Spi2A KO mice. **A**, Experimental protocol. Arrows indicate intraperitoneal (*i.p.*) injections of OVA plus alum on days -5 and 0 in sensitized mice, which were then challenged (S-C mice) after a certain period of time (*dS*) with intranasal (*i.n.*) injection of OVA once per day on 3 consecutive days (*dS*, *dS*+1, and *dS*+2). Analysis was performed on naive mice (*N*) or challenge-only mice (*C*) 1 day after the last intranasal challenge (*dS*+3). **B**, Histograms of mean $\pm$ SEM ($n = 6-8$) eosinophil and lymphocyte numbers in BAL fluid and concentration of IL-4 and OVA-IgE in the sera in experimental groups of mice. S-C mice were challenged 10 days after sensitization. **C**, Line graphs of allergic memory index for BAL fluid eosinophils or lymphocytes and serum IL-4 or OVA-IgE levels versus time before challenge after sensitization. Memory Index = (Response in S-C mice) - (Response in challenge mice). * $P < .05$, ** $P < .005$, and *** $P < .0005$, ns, $P > .05$ (not significant).

Aldrich) and centrifuged at 400g to obtain BAL fluid cells and supernatants. Cells (about 300 cells per mouse) underwent the cytospin process and were stained with modified Giemsa staining (Sigma-Aldrich) to enumerate numbers of infiltrating eosinophils and lymphocytes. To measure the sensitization index (SI) for the lung, T_H2 response was calculated as follows:

$$SI = (\text{Response in S-C mice}) - (\text{Response in challenge mice}).$$

ELISA

Serum IgE concentrations were measured, as previously described.³⁴ Concentrations of IL-4 and IFN- γ were measured with Luminex technology (Invitrogen/Millipore/Luminex, Waltham, Mass). Detection levels for IL-4 and IFN- γ were 3.1 and 2.3 ng/mL, respectively.

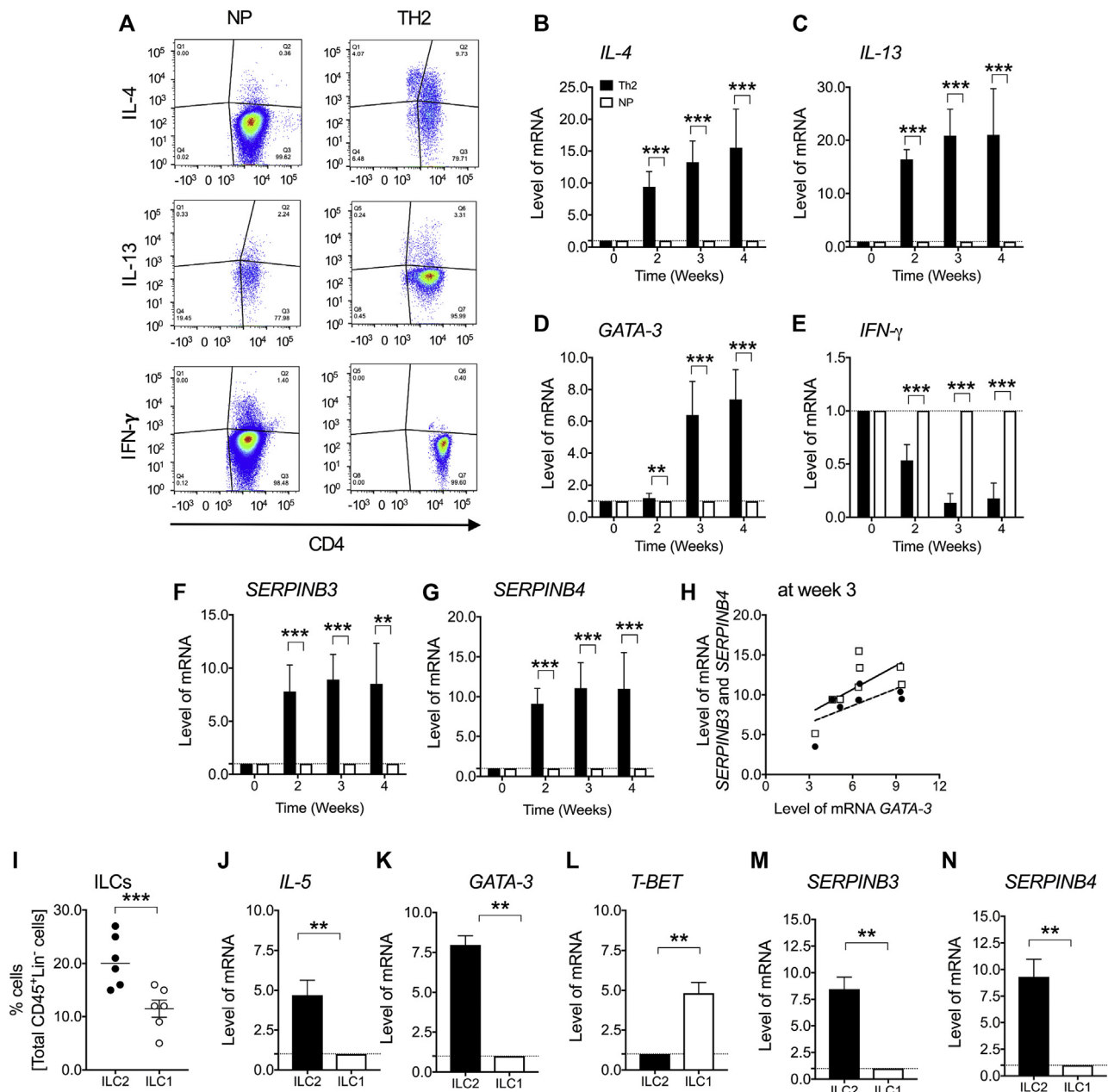
Statistical analysis

Significance values were calculated by using 2-way ANOVA with the Bonferroni posttest or 2-tailed *t* test (Prism software; GraphPad Software, La Jolla, Calif).

RESULTS

Spi2A is required for development of mouse T_H2 cells

We examined the role of the cathepsin-specific Serpin Spi2A in cell death of polarized T_H2 cells *in vitro*. We used a non-antigen-based system in which naive CD4 T cells (CD4⁺CD62L⁺) were magnetic bead sorted from wild-type C57BL/6 mice and then stimulated through the TCR and CD28 after culture under appropriate polarizing conditions to generate T_H1 or T_H2 cells.²⁷ This was verified by using ICS and flow cytometry to detect signature cytokines (Fig 1, A). We determined whether Spi2A mRNA was upregulated in T_H1 or T_H2 cells. Stimulation through the TCR with anti-CD3 antibody induced expression of Spi2A mRNA in T_H2 cells (5-fold greater than the naive level) but not T_H1 cells (Fig 1, B). Furthermore, stimulation of the IFN- γ receptor did not induce Spi2A expression in T_H1 cells, whereas stimulation through the IL-4 receptor resulted in Spi2A mRNA upregulation in T_H2 cells (2-fold greater than the naive level; Fig 1, B). Costimulation through both CD30 and OX40 (CD134) TNF receptor



family members also failed to induce *Spi2A* mRNA in either T_H1 or T_H2 cells (Fig 1, B). We conclude that TCR and cytokine stimulation results in *Spi2A* upregulation in T_H2 but not T_H1 cells.

To examine the requirements for *Spi2A* in CD4 T-cell differentiation, we measured numbers of T_H1 ($IFN-\gamma^+IL-4^-CD4^+$) and T_H2 ($IFN-\gamma^-IL-4^+CD4^+$) cells in cultures of *Spi2A* KO CD4 T cells. On day 4 of culture, we did not observe any

significant difference in the percentage of T_H1 cells from *Spi2A* KO compared with wild-type mice (Fig 1, A). However, when the cultures were polarized for T_H2 development and then rested until day 8, we observed a 9-fold decrease ($P = .0023$) in the percentage of T_H2 cells ($IFN-\gamma^-IL-4^+CD4^+$) from *Spi2A* KO mice compared with those from wild-type mice (Fig 1, C) and a corresponding decrease in IL-4 secretion (see Fig E1 in this article's

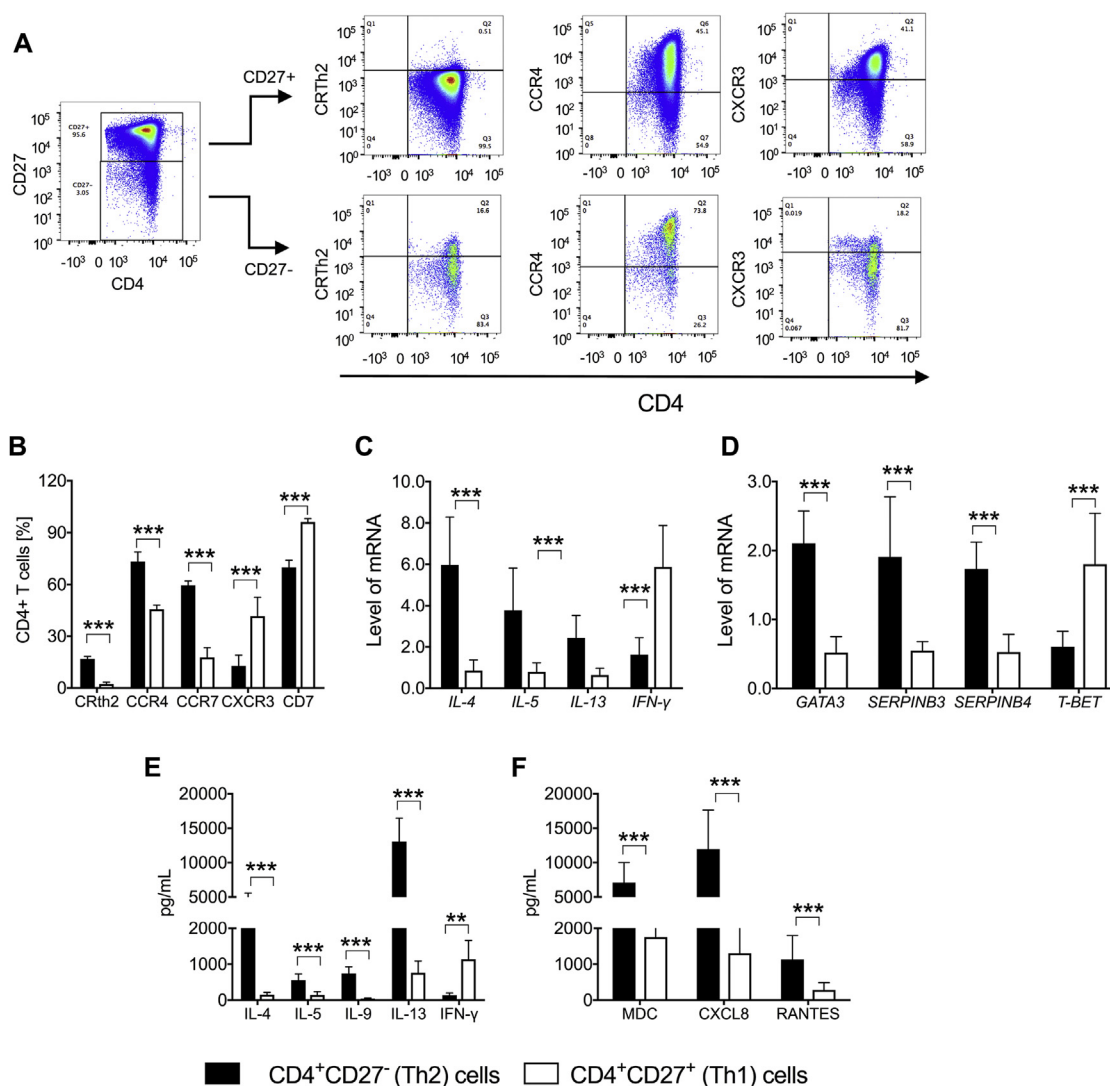


FIG 5. Expression of *SERPINB3* and *SERPINB4* in memory T_H2 cells from allergic patients. **A**, Representative flow cytometric data for $CD4^+$ T cells gated for CD27 expression and analyzed for cell-surface markers. **B**, Histogram for mean $\pm$ SEM ($n = 8$ patient samples) percentage positive for surface markers of total CD4 in either the CD27⁺ or CD27⁻ populations. **C** and **D**, Mean $\pm$ SEM ($n = 8$ patient samples) levels of target mRNA (fold change compared with the *EFA2* housekeeping gene) in $CD4^+$ CD27⁺ and $CD4^+$ CD27⁻ cells 18 hours after stimulation with anti-CD3/CD28. **E** and **F**, Mean $\pm$ SEM ($n = 8$ patient samples) concentration of cytokines and chemokines 18 hours after stimulation with anti-CD3/CD28. ** $P < .005$ and *** $P < .0005$.

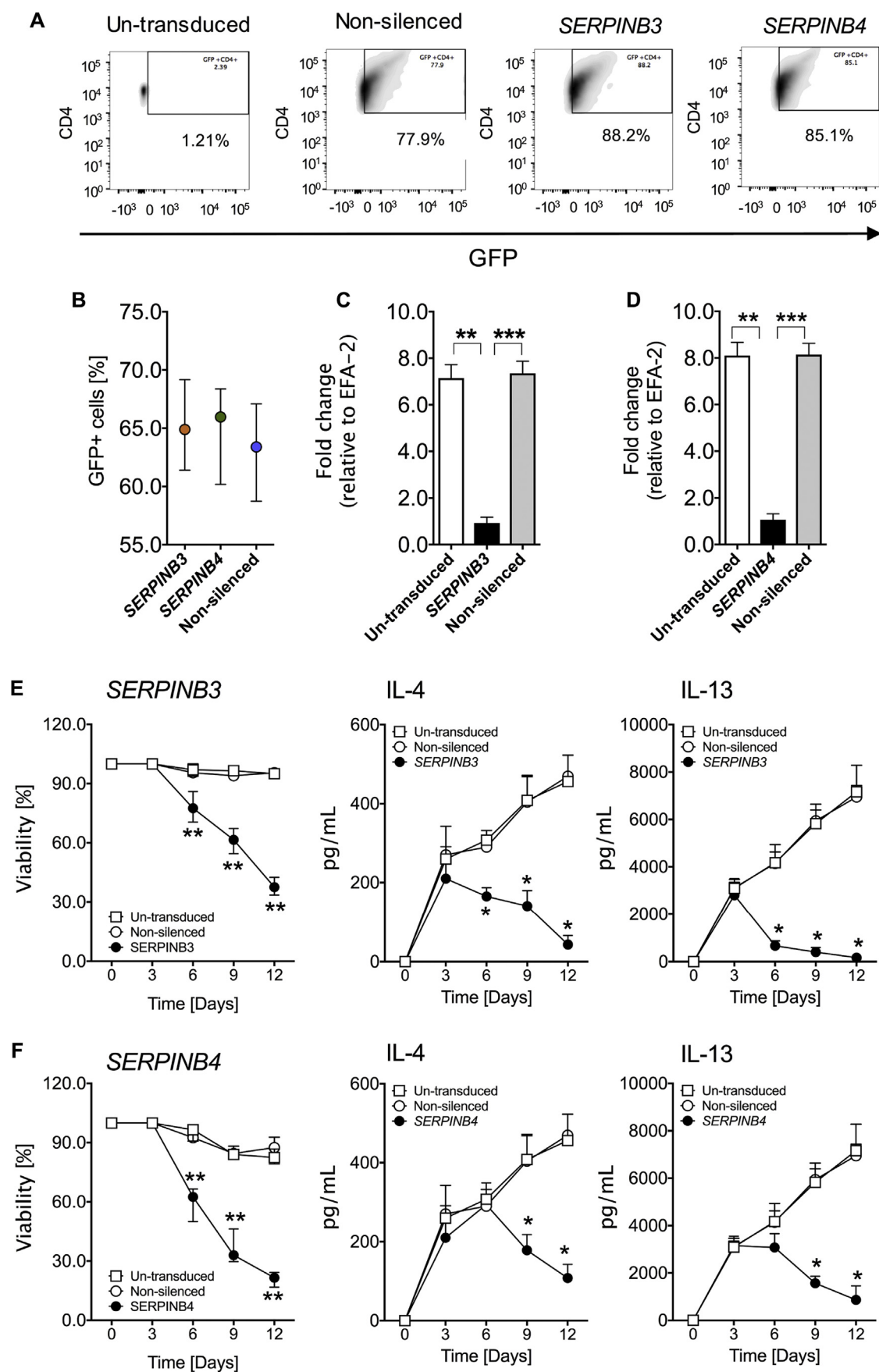
Online Repository at www.jacionline.org). As an additional differentiation marker, we examined expression of the IL-1 receptor family member ST2, which is expressed on persistently activated T_H2 cells.³⁵ We observed a significant decrease in the percentage of ST2⁺CD4⁺ cells ($P = .0027$) in cultures from Spi2A KO compared with wild-type mice (Fig 1, D). The specific requirement of T_H2 cells for Spi2A was presumably due to upregulation through TCR and IL-4 stimulation, whereas T_H1 cells did not up-regulate Spi2A and therefore were not affected in Spi2A KO mice.

Spi2A KO T_H2 cells are susceptible to apoptosis

We examined the mechanism through which Spi2A facilitated differentiation of T_H2 cells *in vitro*. We determined whether

Spi2A was required for either activation or proliferation of T_H2 cells. Labeling studies with carboxyfluorescein succinimidyl ester showed that the decrease in Spi2A KO T_H2 cells was not due to decreased proliferation (see Fig E2 in this article's Online Repository at www.jacionline.org). Furthermore, Spi2A KO T_H2 cells did not display any decrease in activation status, as indicated by CD69 expression (see Fig E3 in this article's Online Repository at www.jacionline.org).

We then examined the susceptibility of T_H2 cells to apoptosis. ICS for activated caspase-3 in the cytoplasm (Fig 1, E) or phosphatidylserine expression on the plasma membrane with Annexin V (Fig 1, F) revealed that T_H1 cells underwent higher levels of apoptosis compared with T_H2 cells. This is consistent with earlier reports of the refractory nature of T_H2 cells to classic AICD.¹¹ However, we observed that Spi2A KO T_H1 cells were no more



susceptible to apoptosis than wild-type T_H1 cells. In contrast, we observed a significant increase in the percentage of activated caspase-3-positive ($P = .0032$; Fig 1, E) and Annexin V-positive ($P = .0071$; Fig 1, F) Spi2A KO T_H2 cells compared with wild-type cells. We conclude that Spi2A is required for *in vitro* differentiation of T_H2 cells by ensuring survival through suppression of apoptosis.

Diminished numbers of T_H2 cells after challenge of Spi2A KO mice

We determined whether Spi2A controlled the level of T_H2 cell development *in vivo*. Mice were immunized on days 0 and 5 (Fig 2, A), and numbers of OVA-specific CD4 T cells were measured in the spleen by staining with I-A^b tetramers refolded with OVA peptide (amino acid sequence 323-339 [OVA-tet]), followed by flow cytometry (Fig 2, B, and see Fig E4 in this article's Online Repository at www.jacionline.org).^{56,37} Within the population of OVA-tet⁺CD4⁺ cells, we measured numbers of phenotypically T_H2 cells based on the effector phenotype of either intracellular IL-4 or IL-5 production (Fig 2, C). In addition, we also used expression of the ST2 differentiation marker³⁸ for T_H2 cells, which is important because CD4 T cells that are negative for T_H1 or T_H2 cytokine production can also form long-lived T_H2 effector cells.⁷ The representative flow cytometric experiment in Fig 2, C, shows that on day 40 after the last OVA immunization, we observed a robust population of OVA-tet⁺CD4⁺ cells in the spleens of wild-type mice, most of which exhibited an ST2⁺IL-4⁺IL-5⁺ phenotype indicative of T_H2 cells. In contrast, the percentage of OVA-tet⁺CD4⁺ cells was about 2-fold lower in Spi2A KO mice, and of these, about 3-fold fewer expressed phenotypic markers of T_H2 differentiation (overall 6 times lower level of T_H2 cells in Spi2A KO compared with wild-type cells).

Longitudinal analysis of T_H2 cells in wild-type mice revealed that the number of T_H2 phenotype OVA-tet⁺ST2⁺CD4⁺ or OVA-tet⁺IL-4⁺CD4⁺ cells increased to a maximal level on day 20 and then decreased steadily to a residual memory level at day 60 (Fig 2, D). For OVA-tet⁺IL-5⁺CD4⁺ cells, the peak of the response was on day 40, with a sharp decrease to a memory level at day 60. T_H2 cells were detectable in both wild-type and Spi2A KO mice by day 5 after immunization, but after day 10, we observed significantly fewer T_H2 cells in Spi2A KO compared with wild-type mice (OVA-tet⁺CD4⁺ST2⁺: $P = .027$; OVA-tet⁺CD4⁺IL-4⁺: $P = .03$; Fig 2, D). The deficit in the level of T_H2 cells in Spi2A KO mice persisted at the peak of the response on day 20 for ST2⁺CD4⁺ and IL-4⁺CD4⁺ cells and until the memory phase at day 60 (OVA-tet⁺ST2⁺CD4⁺: $P = .0004$; OVA-tet⁺IL-4⁺CD4⁺: $P = .0003$). We conclude that Spi2A determines the numbers of T_H2 effector and memory cells throughout the T_H2 response to OVA *in vivo*.

Diminished inflammatory allergic responses in Spi2A KO mice

To determine the physiologic relevance of Spi2A in T_H2 cell survival, we examined allergic inflammation in the lungs of Spi2A KO mice after OVA challenge. Wild-type mice were sensitized by means of intraperitoneal injection with OVA in alum to generate T_H2 cells as before (Fig 2), challenged after various lengths of time with repeated intranasal injection of OVA in PBS (S-C mice),³⁹ and then analyzed 1 day later (Fig 3, A and B). We observed a robust T_H2 -mediated inflammatory response in the lungs of S-C mice, as evidenced by the recovery of eosinophils and lymphocytes from BAL fluid (Fig 3, B). The lung inflammatory response was dependent on sensitization because the level of eosinophil and lymphocyte influx in challenge-only mice was significantly less than in S-C mice (Fig 3, B). OVA-specific IgE levels were significantly greater in the S-C group compared with the challenge group because they were sensitized twice by means of intraperitoneal injection and had more time to develop a B-cell response. This response is a T_H2 response because of the presence of the adjuvant alum, which promotes T_H2 skewing of the immune response (Fig 3, B). The difference in allergic inflammation response (as indicated by either numbers of BAL eosinophils or lymphocytes or concentration of serum IL-4 or OVA-IgE) in S-C mice compared with challenge-only mice was calculated and used as an indication of the relative magnitude of the allergic response to OVA (allergic index). In contrast, we did not observe any decrease in T_H1 responses in Spi2A KO mice, as evidenced by serum levels of IFN- γ or anti-OVA IgG_{2a} (see Fig E5 in this article's Online Repository at www.jacionline.org).

We examined T_H2 -driven inflammatory lung responses in Spi2A KO mice. There was a significant decrease in allergic memory indices in BAL fluid (number of eosinophils, $P = .003$; number of lymphocytes, $P = 0.03$) and serum (IL-4 concentration, $P = .002$; OVA-IgE concentration, $P = .004$) in Spi2A KO mice compared with those in wild-type mice on day 20 after immunization (Fig 3, C). The deficit in the allergic memory response also extended to 40 days after immunization. Taken together, we conclude that the defect in T_H2 memory development in Spi2A KO mice results in decreased allergic inflammatory responses in the lung.

SERPINB3 and SERPINB4 are upregulated in human T_H2 cells

Human subjects lack a homologue of mouse *Serpina3g*, which encodes Spi2A, but express the OVA Serpins SCCA-1 and SCCA-2, which, like Spi2A, are inhibitors of apoptotic cysteine cathepsins.^{14,16} Therefore we determined whether SCCA-1 and SCCA-2 have an equivalent role in human T_H2 cells to Spi2A in mouse T_H2 cells. We examined the development of T_H2 cells from

FIG 6. Expression of *SERPINB3* and *SERPINB4* is required for memory T_H2 cell survival. **A**, Representative flow cytometric data for green fluorescent protein (GFP)⁺ percentage of lentivirus-transduced CD27⁺ CD4⁺ T cells. **B**, Mean $\pm$ SEM ($n = 8$ patient samples) GFP⁺ percentage of lentivirus-transduced CD27⁺ CD4⁺ T cells. **C**, Mean $\pm$ SEM ($n = 8$ patient samples) level of *SERPINB3* mRNA (fold change compared with the *EFA2* housekeeping gene). **D**, Mean $\pm$ SEM ($n = 8$ patient samples) level of *SERPINB4* mRNA (fold change compared with the *EFA2* housekeeping gene). **E** and **F**, Mean $\pm$ SEM percentage viability ($n = 8$ patient samples) of CD27⁺ CD4⁺ T_H2 cells and concentrations of IL-5 and IL-13 in culture supernatants after transduction with lentiviruses. * $P < .05$, ** $P < .005$, and *** $P < .0005$.

allergic patients (Table 1) after culture under T_H2 cell polarization conditions.⁴⁰ After 3 weeks of culture, we observed the development of T_H2 cells, as evidenced by increased intracellular expression of IL-4, compared with nonpolarized CD4 T cells (Fig 4, A). Over the course of 4 weeks, we observed sustained and significant increases in expression of the genes encoding the T_H2 cytokines IL-4 ($P = .0002$; Fig 4, B), IL-5 ($P = .0002$; Fig 4, C), and the GATA-3 transcription factor ($P = .0002$; Fig 4, C) compared with CD4 T cells cultured under nonpolarizing conditions. Culture under T_H2-polarizing conditions resulted in the corresponding downregulation of the T_H1-specific T-bet gene expression compared with CD4 T cells cultured under nonpolarizing conditions ($P = .002$; Fig 4, E). We observed the sustained and significant upregulation of *SERPINB3* ($P = .006$; Fig 4, F) and *SERPINB4* ($P = .006$; Fig 4, G) compared with expression in unpolarized cells from as early as week 1 of polarization. Comparison of the expression of *SERPINB3* ($r = 0.54$, $P = .0014$) and *SERPINB4* ($r = 0.71$, $P < .0001$) with that of the T_H2 master transcription factor GATA-3 revealed the significant correlation of both Serpin genes with *GATA3* expression (Fig 4, H). We conclude that the genes encoding the antiapoptotic OVA Serpins SCCA-1 and SCCA-2 are upregulated in human T_H2 cells.

Innate lymphoid cell 2 (ILC2) has been implicated in lung inflammation through production of T_H2 cytokines, whereas innate lymphoid cell 1 (ILC1) has been implicated in the production of T_H1 cytokines.⁴¹ ILC1 and ILC2 were FACS purified from the blood of patients with pollen allergy (Fig 4, I). Numbers of ILC2 from these patients were greater than those of ILC1s ($P < .01$). ILC2 counts were confirmed by upregulation of *IL5* (Fig 4, J) and *GATA3* (Fig 4, K) mRNA compared with that in ILC1s (both $P = .002$), and ILC1 counts were confirmed by upregulation of T-bet mRNA compared with that in ILC2 ($P = .002$; Fig 4, L). *Ex vivo* ILC2s expressed about 9 times more *SERPINB3* (Fig 4, M) and *SERPINB4* (Fig 4, M) mRNA than ILC1 (both $P = .002$). This implies a role for SCAA-1 and SCCA-2 in ILC2, as well as T_H2 cells, in patients with pollen-induced allergic responses.

The surface molecule CRTH2 is a marker of human memory T_H2 cells,⁵ and expression of CRTH2 (prostaglandin D₂ receptor 2, CCR4, and CCR7) and T_H2 cytokines is associated specifically with pathogenic terminally differentiated CD27[−]CD4⁺ memory cells in allergic patients.⁴ Flow cytometry on *ex vivo* CD4 T cells from patients with grass pollen allergy (Fig 5, A) revealed that the CD27[−]CD4⁺ population contained significantly greater levels ($P = .002$) of CRTH2⁺, CCR4⁺, and CCR7⁺ memory T_H2 cells compared with CD27⁺CD4⁺ cells (Fig 5, B). The T_H2 phenotype of CD27[−]CD4⁺ cells from allergic patients was confirmed by significantly increased expression of the genes encoding IL-4, IL-5, and IL-13 (all $P = .0002$) compared with CD27⁺CD4⁺ cells after 18 hours of *in vitro* stimulation with anti-CD3 antibody (Fig 5, C). We observed significant upregulation of both *SERPINB3* ($P = .0002$) and *SERPINB4* ($P = .0002$) expression in this memory T_H2 cell population (CD27[−]CD4⁺) compared with that in a non-T_H2 cell population (CD27⁺CD4⁺) from allergic patients (Fig 5, D). The T_H2 phenotype of CD4⁺CD27[−] cells was also confirmed by significantly increased secretion of the T_H2 signature cytokines IL-4, IL-5, IL-9, and IL-13 (all $P = .0002$; Fig 5, E) and the T_H2 signature chemokines human macrophage-derived chemokine ($P = .007$), CXCL8 ($P = .0002$), and RANTES (CCL5; $P = .01$; Fig 5, F) in CD27[−] compared with CD27⁺CD4⁺ cells. Conversely, the T_H1

phenotype CD27⁺CD4⁺ population was confirmed by the increased expression of *IFNG* (Fig 5, C) and T-bet (Fig 5, D) mRNA and secretion of IFN- γ (Fig 5, E) compared with that in CD27[−]CD4⁺ cells (all $P < .0005$). Therefore in our patient group CD27[−]CD4⁺ cells are significantly enriched for memory T_H2 cells. These data indicate that both genes encoding the SCCA-1 and SCCA-2 OVA Serpins are specifically upregulated in allergy-associated memory T_H2 cells from human patients.

***SERPINB3* and *SERPINB4* promote the viability of human allergic T_H2 cells**

We next determined the functional significance of *SERPINB3* and *SERPINB4* upregulation in T_H2 cells from patients with grass pollen allergy. We purified CD4 T cells from allergic patients and then transduced them with lentivirus encoding control or shRNA specific for either *SERPINB3* or *SERPINB4*. Transduced cells were identified based on expression of green fluorescent protein (Fig 6, A), which was encoded on a bicistronic message with shRNA. At least 85% of total cells were transduced with either control or *SERPINB3* and *SERPINB4* shRNAs (Fig 6, B). We observed an about 80% knockdown in both *SERPINB3* and *SERPINB4* messages after transduction with shRNA (Fig 6, C).

We next cultured transduced CD4 T cells with anti-CD3/IL-2, and after about 6 days, we observed a significant and sustained decrease in the number of viable CD27[−]CD4⁺ cells transduced with either *SERPINB3* and *SERPINB4* shRNA compared with cells transduced with control shRNA (Fig 6, E). The impaired viability persisted for up to 12 days, at which point control-transduced CD27[−]CD4⁺ cells retained greater than 80% viability, whereas cells lacking *SERPINB3* or *SERPINB4* expression were less than 30% viable (Fig 6, E and F). The reduced viability also resulted in a decrease in IL-4 and IL-13 production (Fig 6, E and F). We conclude that ablation of either *SERPINB3* or *SERPINB4* gene expression decreases the viability of clinically relevant T_H2 memory cells.

DISCUSSION

We show that the chymotrypsin-like Serpin Spi2A facilitated the survival of T_H2 effectors *in vivo* and that ablation of Spi2A resulted in alleviation of allergic inflammatory responses in the lung. We also demonstrate that the OVA Serpins SCAA-1 and SCAA-2 protect allergic memory T_H2 cells from death in patients with grass pollen-induced allergic rhinitis. Therefore, despite being members of different Serpin families, Spi2A and SCAA-1/SCAA-2 are functionally equivalent in their ability to ensure the survival of memory T_H2 cells in both mice and human subjects.

The antiapoptotic activities of SCAA-1,^{21–23} SCAA-2,^{24,25} and Spi2A^{15,20} are well known and mediated by inhibition of cathepsins B and L in cytoplasm. In the present study we show that the cytoprotection afforded by SCAA-1/SCAA-2 and Spi2A extends to human and mouse T_H2 cells. There are important differences between the roles Spi2A plays in T_H2 cells compared with other T cells. We found no evidence for a role for Spi2A in T_H1 cell survival. However, use of a more physiologically relevant model for *in vivo* T_H1 development than OVA plus alum immunization would be useful to explore this possibility more fully. Spi2A is a physiologic target of the nuclear factor κ B transcription factor^{15,42} and a target of signal transducer and activator of

transcription (STAT) transcription⁴³ induced by cytokines (eg, IL-15³¹ and IL-7⁴⁴) and growth factors (eg, erythropoietin).²⁶ Therefore the differential effect of TCR signals that upregulate Spi2A in T_H2 cells but not T_H1 cells might be due to the additive effects of IL-4 through STAT6 and the lack of an effect by IFN- γ through STAT3. However, Spi2A can be induced by IFN- γ in activated macrophages,⁴³ and therefore it is possible that in different physiologic settings IFN- γ can affect T_H1 cell survival through Spi2A upregulation. SCCA-1/SCCA-2 expression has been noted previously in human mononuclear cells,⁴⁵ and intriguingly, it has been reported that deficiency in mouse *Serpina3a*, which is thought to be the homologue of human SCAA-1/SCAA-2, reduces airway hyperresponsiveness by limiting goblet cell hyperplasia.⁴⁶ In contrast to our findings with OVA-specific IgE levels in our *Serpina3g* (Spi2A) KO mice, levels of HDM-specific IgG₁ and IgE were no different between *Serpina3a* KO and wild-type mice.⁴⁶ The mouse strain (C57Bl/6 vs BALB/c), allergen (HDM vs OVA), and especially route of sensitization (lung vs intraperitoneal) might explain the discrepancy. Therefore it will be important when evaluating the physiologic relevance of *SERPINB3* and *SERPINB4* in humanized mice to use a variety of allergenic models.

A major question for the development of T_H2 memory is the following: How do T_H2 memory cells and their precursors escape apoptosis? *In vitro* polarization studies have concluded that compared with T_H1 effectors, T_H2 cells are relatively resistant to classical AICD.¹¹ Our *in vitro* polarization study also shows higher levels of apoptosis in T_H1 cells than in T_H2 cells. However, the differences in apoptosis observed for T_H1 and T_H2 cells might also be affected by differences in the culture conditions used to generate each population. Despite this caveat, our results show clearly that Spi2A protects T_H2 but not T_H1 cells from apoptosis and therefore point to the lysosomal pathway of cell death controlling T_H2 cell survival. Spi2A deficiency reduced T_H2 cell numbers *in vivo* but did not affect IL-13 production in our lung inflammation model. Although *SERPINB3* and *SERPINB4* mRNA is expressed in human ILC2, we do not know whether Spi2A is expressed in mouse ILC2. Further studies will clarify whether Spi2A, SCAA-1, and SCAA-2 control the survival of other producers of allergic cytokines (eg, ILC2) in patients with lung inflammatory disease.

Factors affecting the initiation and course of allergic inflammation act in a narrow window in early life or even perinatally.⁴⁷ The refractory nature of allergic disease to cytokine therapy, which seeks to shift the T_H2 balance to T_H1, can be explained in terms of the stability of T_H2 memory cells that develop very early in life.⁴⁸ Therefore it would be advantageous to be able to eliminate the persistent T_H2 memory cells that give rise to atopic⁴⁹⁻⁵¹ and allergic diseases, such as asthma,⁵² through induction of apoptosis.^{49,50,52-54} We show that the targeting of *SERPINB3* and *SERPINB4* messages by shRNA drastically impaired the viability of memory T_H2 cells in patients with grass pollen allergy. Furthermore, expression of *SERPINB3* and *SERPINB4* in ILC2s implies that mRNA knockdown can also ablate allergenic innate immune system cells. Alleviation of allergic asthma by means of *in vivo* *GATA3* mRNA knockdown in T_H2 cells provides support for the concept of targeting memory and effector T_H2 cells by using *SERPINB3* and *SERPINB4* mRNA knockdown.⁵⁵ It remains to be determined whether *SERPINB3* and *SERPINB4* also control memory CD8 T-cell responses in human subjects, as is the case for Spi2A in mice.^{31,44} A localized

therapeutic approach targeting memory T_H2 cells would potentially deplete the reservoir of allergic disease and therefore complement antiallergic drugs and cytokine therapy.

Key messages

- Upregulation of *Spi2A* protects mouse T_H2 cells from apoptosis and determines the number of effector and memory T_H2 cells *in vivo*.
- *Spi2A* inactivation reduces the development of allergic inflammation in mice.
- The genes *SERPINB3* and *SERPINB4* are upregulated in memory T_H2 cells and ILC2s from allergic patients.
- The viability of allergenic memory T_H2 cells is decreased by *SERPINB3* and *SERPINB4* knockdown.
- There is proof of principle for a therapeutic approach to allergic disease through ablation of allergic memory T_H2 cells by means of mRNA *SERPINB3* and *SERPINB4* downregulation.

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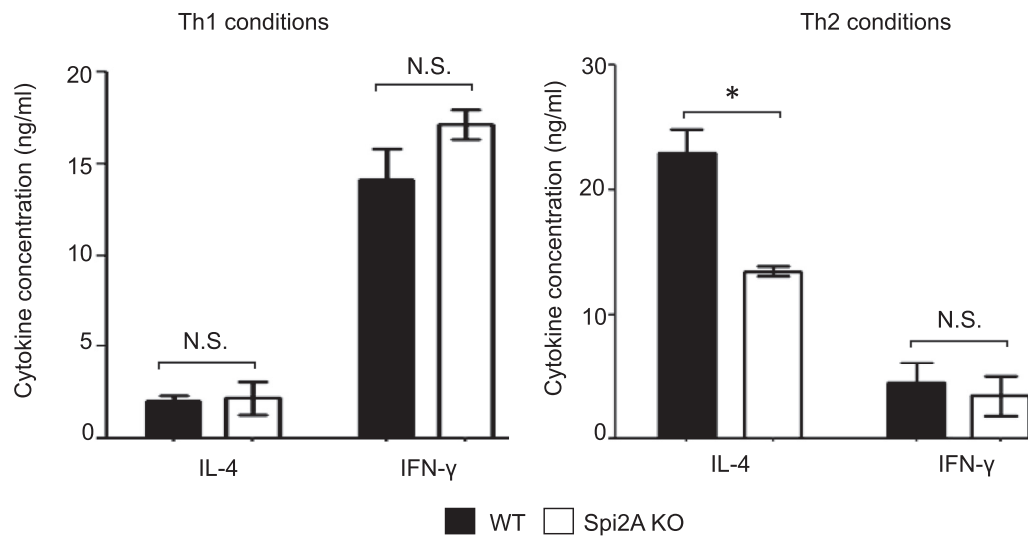


FIG E1. Extracellular cytokine levels. Multiplex ELISA was used to detect IL-4 and IFN- γ levels in culture supernatants. Freshly isolated splenocytes were magnetically sorted from CD4⁺CD62L⁺ WT and Spi2A KO cells and then cultured under T_H1 or T_H2 conditions. Culture supernatants were obtained on days 4 (T_H1) and 8 (T_H2) and analyzed by using multiplex ELISA (Invitrogen/Luminex). *N.S.*, Not significant. **P* < .05.

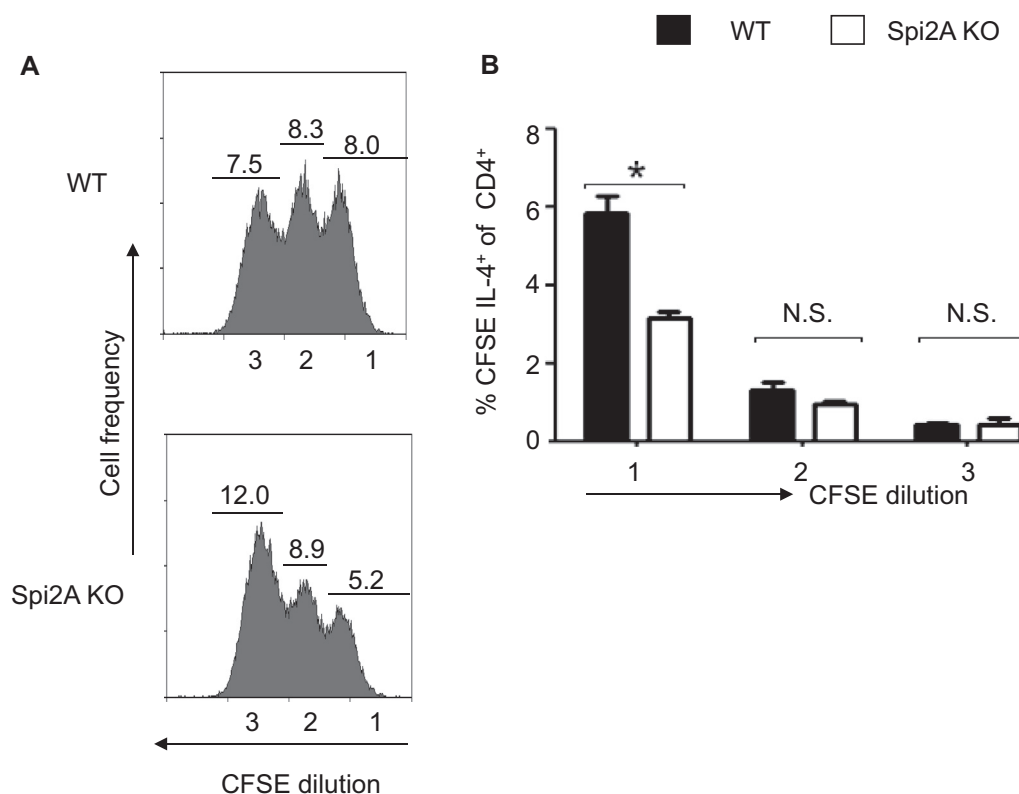


FIG E2. Cell division of T_H1 and T_H2 cells. **A**, $CD4^+CD62L^+$ splenocytes from WT and Spi2A KO mice were purified by means of magnetic sorting and then cultured under T_H2 culture conditions after labeling with carboxyfluorescein succinimidyl ester (CFSE). Proliferation was measured by using 2-fold CFSE dilution over 4 days. Generations 1, 2, and 3 are shown, and each is the percentage of total cells. **B**, Mean $\pm$ SEM IL-4⁺ percentage at a given CFSE dilution ($n = 4$ cultures) for 3 dilutions of CFSE. * $P < .05$. N.S., Not significant.

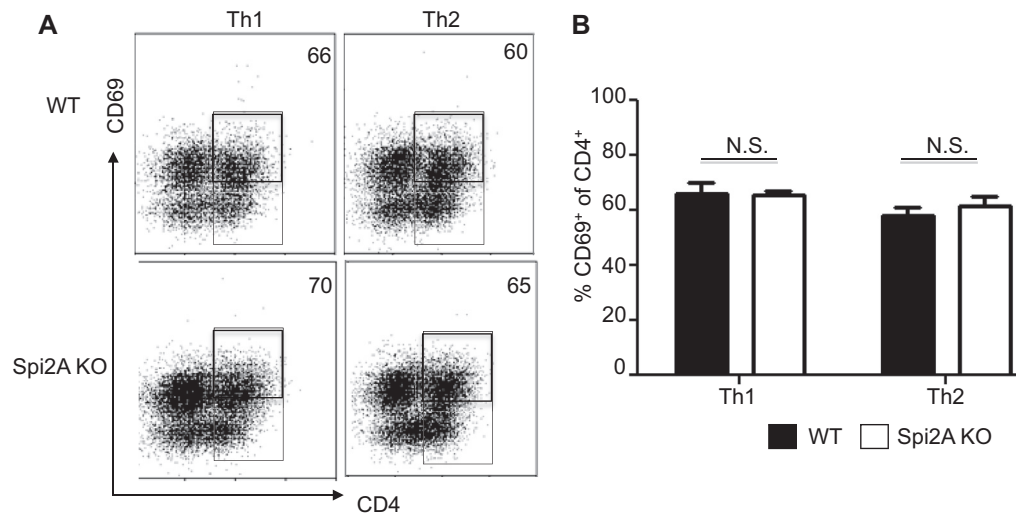


FIG E3. Activation of polarized CD4 T cells. **A**, Expression of the T-cell activation marker CD69 was measured after 24 hours in CD62L⁺CD4⁺ cells polarized to either T_H1 or T_H2 conditions. Gates for total CD4⁺ and CD69^{hi}CD4⁺ cells are shown. Percentage of CD69⁺ of CD4⁺ cells is indicated. **B**, Mean $\pm$ SEM CD69⁺ percentage of CD4⁺ cells (n = 4 cultures). *N.S.*, Not significant.

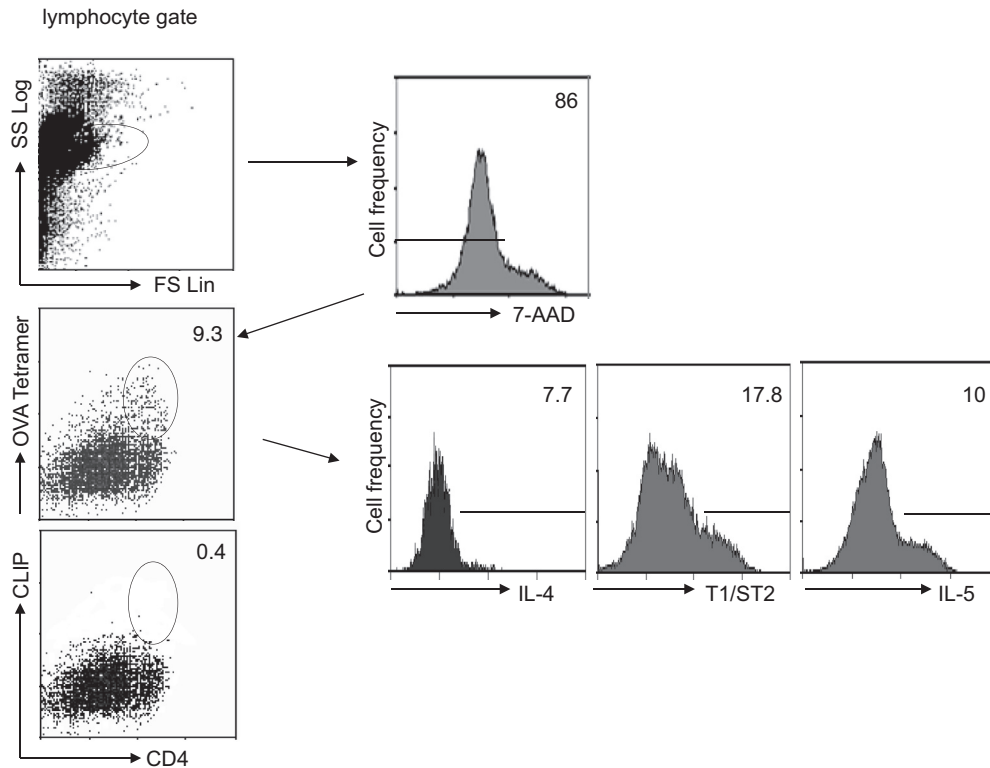


FIG E4. Analysis of cells after MHC class II tetramer staining. Flow cytometric analysis using the OVA/MHC II tetramer. Wild-type C57BL/6 mice were immunized with 100 μ g of OVA plus 4 mg of alum on days 0 and 5 and killed on day 15. Isolated splenocytes were then restimulated *in vitro* with PMA plus ionomycin and IL-2. Lymphocytes were first gated by using light side scatter (SS) and forward scatter (FS) and then gated for 7-actinomycin D-negative viable cells. Viable lymphocytes were analyzed for staining with anti-CD4 antibody and I-A^b tetramer with OVA peptide (region 329-337 AAHAEINEA) or CLIP peptide. CD4⁺I-A^b tetramer-positive cells were analyzed for staining with anti-T1/ST2 antibody or ICS with IL-4 and IL-5 antibodies. Percentage positive value of each gated population is indicated in the *top right corner*. 7-AAD, 7-Actinomycin D.

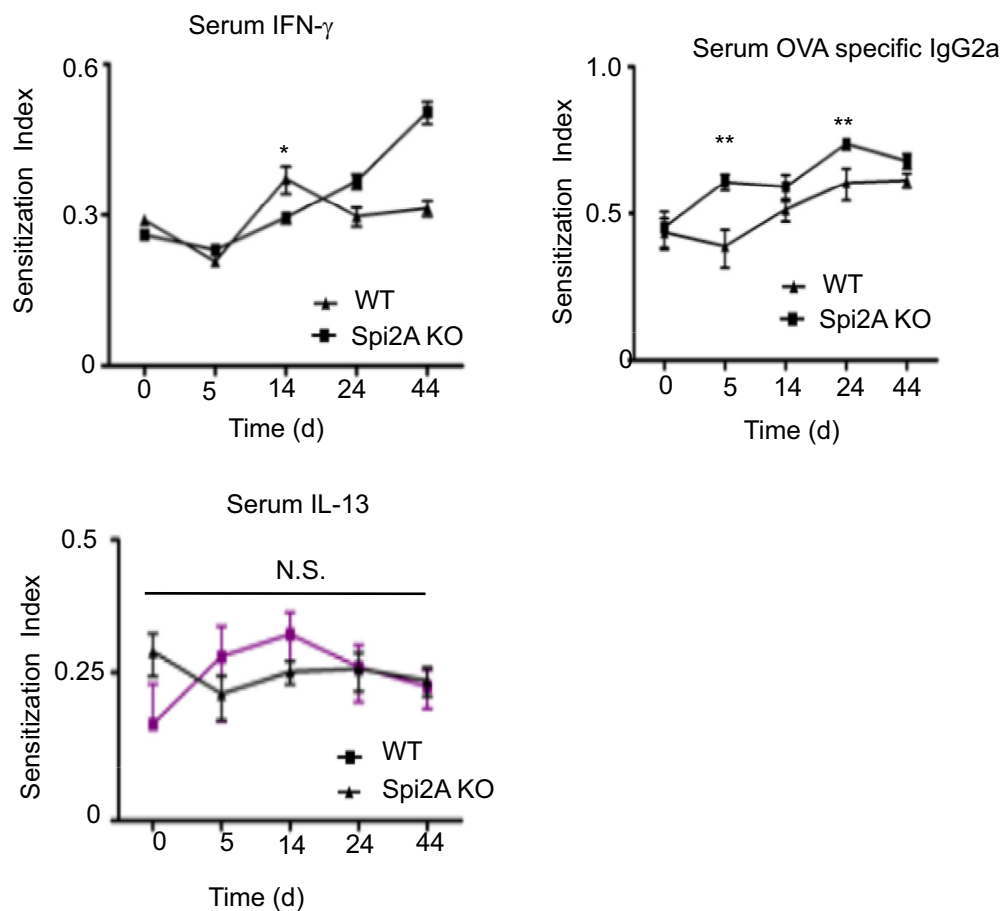


FIG E5. Effect of Spi2A deficiency on serum markers after OVA plus alum immunization. Wild-type C57BL/6 mice and Spi2A KO mice were subjected to sensitization and challenge with OVA, as described in Fig 3, A. At various time points, serum levels of immunologic indicators were determined, and sensitization indices were calculated. Mean $\pm$ SEM sensitization indices are shown (n = 6 cultures). * P < .05 and ** P < .05. N.S., Not significant.