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Anti-apoptotic Serine Protease Inhibitors contribute towards the survival of allergenic Th2 cells

Mohamed H. Shamji, PhD. FAAAAI[a,b], **Jeff N. Temblay, PhD**[c], **Wei Cheng, PhD**[c], **Susan M. Byrne, PhD**[c], **Ellen Macfarlane, MSc**[a,b], **Amy R. Switzer, MSc**[a,b], **Natalia DC Francisco, MSc**[a,b], **Fedina Olexandra**[a], **Fabian Jacubczik, MSc**[c], **Stephen R. Durham, MD**[a,b], and **Philip G. Ashton-Rickardt, PhD**[c]

[a]Immunomodulation and Tolerance Group, National Heart and Lung Institute, Imperial College London, London, SW3 6LY, United Kingdom and MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, London, United Kingdom

[b]Allergy and Clinical Immunology, Inflammation, Repair and Development, National Heart and Lung Institute, Imperial College London, London, SW3 6LY, United Kingdom and MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, London, United Kingdom

[c]Section of Immunobiology, Division of Inflammation and Immunology, Department of Medicine, Faculty of Medicine, Imperial College London. London W2 1PG. UK

Abstract

Background—The mechanisms regulating the maintenance of persistent Th2 cells that potentiate allergic inflammation are not well understood.

Objective—The function of Serine Protease Inhibitor 2A (Spi2A) was studied in mouse Th2 cells and Serine Protease Inhibitor (*SERPIN*) B3 and B4 genes were studied in Th2 cells from grass pollen allergic individuals.

Methods—Spi2A deficient Th2 cells were studied *in vitro* culture or *in vivo* after challenge of Spi2A Knock-Out (KO) mice with ovalbumin in alum. The expression of *SERPIN B3* and *B4* mRNA was measured *in vitro* cultured Th2 cell and in *ex vivo* $CD27^{-}$ $CD4^{+}$ and ICL2 cells from grass pollen allergic individuals using quantitative PCR. *SERPIN B3* and *B4* mRNA levels were knocked down in cultured $CD27^{-}$ $CD4^{+}$ cells with shRNA.

Results—There were lower levels of *in vitro* polarized Th2 cells from Spi2A KO mice ($P<0.005$) and *in vivo* after OVA challenge ($P<0.05$), higher levels of apoptosis (annexin V positivity $P<0.005$) and less lung allergic inflammation (number of lung eosinophils $P<0.005$). *In vitro* polarized Th2 cells from grass pollen allergic individuals expressed higher levels of both *SERPIN B3* and *B4* (both $P<0.05$) mRNA compared to un-polarized CD4 T cells. $CD27^{-}CD4^{+}$ from grass

Corresponding author: Professor Philip G. Ashton-Rickardt, Section of Immunobiology, Division of Immunology and Inflammation, Department of Medicine, Faculty of Medicine, Imperial College London, Norfolk Place, London W21PG, UK. Phone: (+44) 20-7594-2762, p.ashton-rickardt@imperial.ac.uk.

pollen allergic individuals expressed higher levels of both *SERPIN B3* and *B4* (both P<0.0005) mRNA compared to $CD27^{+}CD4^{+}$ cells. ICL2 cells expressed higher levels of both *SERPIN B3* and *B4* (both P<0.0005) mRNA compared to ICL1 cells. Knock-down of either *SERPIN B3* or *B4* (both P <0.005) Mrna levels resulted in decreased viability of $CD27^{-}$ $CD4^{+}$ compared to control transduced cells.

Conclusion—The serpins Spi2A in mice and Serpin B3 and B4 in allergic individuals, control viability of Th2 cells. This provides proof-of-principle for a therapeutic approach for allergic disease through the ablation of allergic memory Th2 cells through mRNA *SERPIN B3* and *B4* down-regulation.

Keywords

Th2 cells; apoptosis; memory; grass pollen allergy

INTRODUCTION

Persistent Th2 cells are the principal cell population responsible for the maintenance of chronic allergic inflammation and the rapid relapse of acute allergic inflammation upon re-exposure to allergens [1–3]. The mechanisms regulating the maintenance of persistent Th2 cells that potentiate allergic inflammation remains elusive. Strong candidates for these persistent Th2 cells are circulating human CD4+ T cells, which express the prostaglandin D2 receptor (CRTH2) and have a phenotype of terminally differentiated T central effector/memory T cells, based on Th2 cytokine production, gene-expression profile, and the ability to respond to allergens [4, 5]. In mice, the differentiation of effector Th2 cells into persistent Th2 cells (referred to as memory Th2 cells) requires the escape from apoptosis [6, 7]. Such persistent Th2 cells retain the cytokine expression profile of the effector cell [8–10].

Compared to Th1 and $CD8^{+}$ effectors, Th2 cells are relatively resistant to apoptosis and are refractory to classical Tumor Necrosis Factor Receptor (TNF-R) family induced, activation induced cell death (AICD) pathway of apoptosis [11]. The 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 physiological settings [12, 1314]. We have identified serine protease inhibitor (Spi) 2A as a physiological inhibitor of the lysosomal pathway of death in mice [15]. Spi2A, encoded by the *Serpina3g* gene on mouse chromosome 12[16], is unusual for a serine protease inhibitor (or serpin) in that it inhibits not only serine proteases but also cysteine cathepsins and resides in the cytosol and nucleus [15]. In humans, SCCA-1 (SERPINB3), which belongs to the ovalbumin-serine proteinase inhibitor (ova-serpin) family [17], inhibits both serine proteinase such as chymotrypsin and cysteine proteinases such as cathepsin L, K, S and papain [18], while the 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-1 [21–23] and SCCA-2 in humans [24, 25] are potent inhibitors of apoptosis.

We now show in mice and man that intracellular serpins are required for the survival of disease-causing memory Th2 cells. Spi2A was up-regulated in Th2 but not Th1 effectors in response to T cell receptor (TCR) stimulation and cytokines. The development of Th2 cells after immunization with ovalbumin (OVA) was impaired in Spi2A KO mice as was the

development of memory allergic inflammatory responses in the lung. Both *SERPINB3* and *SERPINB4* were expressed in human Th2 cells after *in vitro* polarization and in memory Th2 from pollen allergy patients. Importantly, knock-down of either *SERPINB3* or *SERPINB4* resulted in a drastic impairment of memory Th2 cell survival. We conclude that anti-apoptotic serpins are potential targets for the ablation of disease causing memory Th2 cells.

METHODS

Mice

Spi2A KO mice were on the C57BL/6 background [26]. Wild type C57BL/6 mice were obtained from Charles River Laboratories. 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.

Th1 and Th2 polarization *in vitro*

Mice—Using the protocol from [27] spleen and lymph node cells were harvested from Spi2A KO and wild-type mice, and positively sorted by magnetic beads (Myltenyi Biotech) for $CD4^+$ $CD62L^+$ $CD44^-$ cells then seeded at (10^6/ml) in RPMI media containing anti-CD3 (2μg/ml) and anti-CD28 (5μg/ml) monoclonal antibodies (mAb). For Th1 polarization, cells were cultured with neutralising anti-IL-4 mAb (10μg/ml) and recombinant IL-12 (10ng/ml) for 4d. For Th2 polarization, cells were cultured with neutralizing anti-IFNγ mAb (10μg/ml) and recombinant IL-4 (10ng/ml) for 4d. These cultures were given fresh Th2 polarization medium plus IL-2 (10ng/ml) every 48 hrs. All reagents were from E-bioscience.

Human—Heparinized venous blood was collected from grass pollen-induced allergic subjects with seasonal allergic rhinitis. Whole blood was diluted 1:1 with RPMI-1640 media (Invitrogen, UK) and layered on Histopaque-1077 (Sigma-Aldrich, UK) density gradient (density 1,077) and centrifuged for 25 minutes at 1136 *x g* at room temperature. The PBMC layer was collected, washed and resuspended in RPMI-1640. The cell viability was greater than 95%, as determined by trypan blue exclusion. Naïve $CD4^+CD45RA^+$ T cell were negatively selected and enriched using EasySep™ Human Naïve CD4+ T Cell Isolation Kit (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. The purity of fractionated cell populations determined by FACS analysis using PE-conjugated anti-CD45RA (BD bioscience, 5H9) was 99%. Purified $CD4^+CD45RA^+$ cells (10^6/ml) were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 2 mM 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; R&D systems,

37407, Abingdon, UK) and rIL-2 (50 U/ml; R&D systems, Abingdon, UK). To direct Th1 differentiation, rIL-12 (2.5 ng/ml; R&D Systems, Abingdon, UK) and anti-IL-4 (5 μg/ml; clone MP4-25D2; BD Pharmingen) were added. For Th2 differentiation, rIL-4 (12.5 ng/ml; NBS Biologicals, Huntingdon, Cambridge, U.K.), anti-IFN-γ (5 μg/ml; clone B-B1; Invitrogen, Paisley, UK), and anti-IL-10 (5 μg/ml; clone JES3-9D7; Invitrogen, Paisley, UK) were added. After 4 days the cells were expanded under the same conditions in the absence of anti-CD3 or anti-CD28. Cells were then re-stimulated every 7 days. When required, cells were activated with PMA (5 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (500 ng/ml; Merck Millipore, Hertfordshire, UK) for 4 h. In addition, cell lysates were collected to look at gene expression by RT-PCR and cell supernatants were analyzed for cytokines and chemokines using a human cytokine/chemokine magnetic bead panel 96-well plate assay (Milliplex Map Kit, Millipore, MA, USA) and a Luminex xMAP Magpix platform (Merck Millipore, Hertfordshire, UK), according to the manufacturer's instructions.

Flow Cytometry

For extracellular staining the following mAb were used: anti-CD4-APC-Cy7 (Biolegend), anti-T1/ST2-FITC (clone: DJ8, MD Biosciences), anti-CD27-APC (Ebioscience). For ICS with anti-IL-4–PE-Cy7, anti-IFNγ-Pacific Blue and IL-5-PE (Ebioscience) the fixation and permeabilization was carried out according the manufacturer's protocol (Cytofix-Cytoperm, BD Biosciences). Spleen cell suspensions generated by mechanical disaggregation of whole spleens in in RPMI with 10% fetal bovine serum (FBS) then filtered through gauze to remove particular matter. Splenocytes (10^6/ml), were stimulated with Phorbol Myristate Acetate (PMA) (50ng/ml) and Ionomycin (1μg/ml) (Sigma-Aldrich) and Brefeldin A (10μg/ml) (ebioscience) for 5hr prior then stained with OVA-tet, anti-CD4-APC-Cy7 and anti-T1/ST2-FITC in RPMI with 10% fetal bovine serum (FBS) for 1h at 37°C with 7-actinomycin D (7-AAD) to identify dead cells [28]. Cells were stained with I-A^b tetramer (APC) containing the OVA peptide 323–339 [AAHAEINEA] (NIH Tetramer Facility, USA) (OVA-tet) or negative control CLIP peptide/I-A^b tetramer. Cells were then subjected to ICS. The staining and gating procedure is shown in Supplementary Figure S4. Apoptosis was measured using Annexin V staining or intracellular anti-Active Caspase 3 according to the manufacturer's protocol (BD Biosciences, San Jose, CA). To check the enrichment of human $CD4^+CD45RA^+$ naïve T cells from isolated PBMCs on day zero, $CD4^+$ T cells were immunostained with antihuman CD45RO-PE (clone: UCHL1) and CD45RA-FITC (clone: 5H9) (BD Biosciences, San Jose, CA). Each week, resting cells were stimulation of PMA, Ionomycin for 1 hr and Brifeldin A for 4h prior surface immunostaining with antihuman CD4-Percp (clone: L200) and intracellular immunostaining with anti human IL-5-APC (clone: TRFK), anti human IL-13-Percp cy5.5 (clone: JES-5AZ), antihuman GATA-3-PE-Cy7 (clone: L50823), anti human IFN-γ-PE Cy5.5, clone: B27) and antihuman T-bet V450 (clone: 04–46) antibodies (BD Biosciences, San Jose, CA) according the manufacturer's protocol (Cytofix-Cytoperm, BD Biosciences). ILC2 ($Lin^-CD44^+CD127^+CD161^+CRTH2^+$) and ILC1 ($Lin^-CD44^+CD127^+CD161^+CRTH2^-CD117^-NKP44^-$) [29] were immunophenotyped and FACS-purified (>90% purity) from PBMCs from six grass pollen allergic subjects using the cell surface antibodies: CD127-PE-Cy5, CD45-AF700, $CD117^{+/-}$-BV785 (Biolegend, London, UK), CD294 (CRTH2)-BV711, CD336-PerCP-Cy5.5, CD161–APC, FcεRIα-FITC, and lineage Cocktail (CD3, CD14, CD19, CD20, CD56) (FITC)

(Biolegend, London, UK). Cells were directly sorted into RLT-buffer prior to gene expression analysis by RT-PCR.

Analysis of *Serpin* gene expression

In vitro Th1 or Th2 polarized cultures were re-seeded in fresh non-polarizing medium for 1d prior with culture media containing IL-2 alone (10ng/ml) or with either: IL-4 (10ng/ml), IFN-γ (10ng/ml), anti-CD3 (5μg/ml), anti-CD30 (50μg/ml), anti-OX40 (50μg/ml), anti-CD30 (50μg/ml) plus OX40 (50μg/ml) (eBioscience). Each condition was performed in duplicate. Total RNA from each sample was obtained using the RNeasy® Plus Micro Kit (Qiagen, UK) and QiaShredder spin columns (Qiagen, UK) according to the manufacturer's instructions. Real-time PCR reactions were run using the SYBR Green PCR Master mix (Applied Biosystems) on a 7900HT Fast Real–Time PCR System (Applied Biosystems). Primer sequences for *Serpina3g* (Spi2A gene) and β-*2microglobulin* came from [30]. Level of *Serpina3g* mRNA is expressed as fold-over β-*2microglobulin* control mRNA [31]. Primer sequences of human *IL-4, IL-13, GATA-3, T-BET* came from [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′] came from [33]. The level of human mRNA was expressed as fold-over *Elongation Factor 2 (EFA-2)* control mRNA [32].

Gene knockdown of *SERPINB3* and *SERPINB4*

The following GIPZ lentiviruses were used (Thermo Fisher Scientific Biosciences GmbH, Paisley, UK): shRNAmir *SERPINB3* or S*ERPINB4*, non-silencing – GIPZ lentiviral shRNAmir and GAPDH-GIPZ lentiviral shRNAmir positive control. In addition, non-transduced cells were used as controls. $CD4^+CD27^-$ cells were transduced with lentivirus (MOI=3) by centrifugation at 37°C for 90 minutes in polybrene (4μg/ml) then stimulated with anti-CD3 (0.5μg/mL) and anti-CD28 (0.5μg/ml) for 4d and then analysed by flow cytometry for CD4 and GFP expression. The viability of $CD27^-CD4^+$ T cells was assessed by trypan blue exclusion.

OVA-Alum Th2 model

Naïve Spi2A KO and wild-type mice were immunized (sensitization, S) by i.p. injection of OVA (100μg) (Grade V, Sigma-Aldrich, Poole UK) and Alum (4mg) (Pierce) on d0 and d5. To induce airway inflammation (sensitization-challenge, S-C), mice were subjected to 3 daily i.n. challenges with OVA in PBS (0.5% w/v) at various time points after sensitization . In addition some mice underwent i.n. challenge alone (challenge, C). For both S-C and C mice analysis was made 1d after the last i.n. challenge. For BAL extraction, the lungs of mice were perfused 5 times with Lidocaine chloride in PBS (0.4% w/v, Sigma-Aldrich, Poole UK) and centrifuged at 400 × *g* to obtain BAL cells and supernatant. Cells (about 300 cells per mouse) underwent cytospin and were stained using a modified Giemsa stain (Sigma-Aldrich) to enumerate numbers of infiltrating eosinophils and lymphocytes. To measure the sensitization index (SI) for the lung Th2 response was calculated: SI= (Response in S-C mice) – (Response C mice).

ELISA

The concentration of serum IgE was measured as before [34]. The concentrations of IL-4 and IFN-γ were measured by Luminex™ (Invitrogen/Millipore/Luminex Corp). The detection levels for IL-4 and IFNγ were 3.1ng/ml and 2.3ng/ml respectively.

Statistical analysis

Significance values were calculated using two-way ANOVA with Bonferroni post-test or two-tailed t test (GraphPad software; Prism). CFM imaging of fixed cells was calculated (GraphPad software; Prism) using Mann-Whitney U tests (GraphPad software; Prism).

RESULTS

Spi2A is required for the development of mouse Th2 cells

We examined the role of the cathepsin-specific, serpin Spi2A in the cell death of polarized Th2 cells *in vitro*. We used a non-antigen based system in which naïve CD4 T cells ($CD4^+$ $CD62L^+$) were magnetic bead sorted from wild-type C57BL6 mice then stimulated through the TCR and CD28 after culture under the appropriate polarizing conditions to generate Th1 or Th2 cells[27]. This was verified by intracellular staining (ICS) and flow cytometry to detect signature cytokines (Figure 1A). We determined whether *Spi2A* mRNA was up-regulated in Th1 or Th2 cells. Stimulation through the TCR with anti-CD3 antibody induced the expression of *Spi2A* mRNA only in Th2 cells (5-fold above naïve level) but not Th1 cells (Figure 1B). Furthermore, stimulation of the IFN-γ receptor also failed to induce *Spi2A* expression in Th1 cells whereas stimulation through the IL-4 receptor resulted in *Spi2A* mRNA up-regulation in Th2 cells (2-fold above naïve level) (Figure 1B). Co-stimulation through both CD30 and OX-40 (CD134) TNFR-family members also failed to induce *Spi2A* mRNA in either Th1 and Th2 cells (Figure 1B). We conclude that TCR and cytokine stimulation results in *Spi2A* up-regulation in Th2 but not Th1 cells.

To examine the requirements for Spi2A in CD4 T cell differentiation, we measured the level of Th1 (IFN-γ^+ IL-4^- $CD4^+$) and Th2 (IFN-γ^- IL-4^+ $CD4^+$) cells in cultures of Spi2A KO CD4 T cells. On day (d) 4 of culture we did not observe any significant difference in the percentage of Th1 cells from Spi2A KO compared to wild-type (Figure 1A). However, when the cultures were polarized for Th2 development then rested until d8 we observed a 9-fold decrease (P=0.0023) in the percentage of Th2 cells Th2 (IFN-γ^- IL-4^+ $CD4^+$) from Spi2A KO mice compared to wild-type (Figure 1C) and a corresponding decrease in IL-4 secretion (Supplementary Figure S1). As an additional differentiation marker we examined the expression of the IL-1 receptor family member ST2, which is expressed on persistently activated Th2 cells [35]. We observed a significant decrease in the percentage of $ST2^+$ $CD4^+$ cells (P=0.0027) in cultures from Spi2A KO mice compared to wild-type (Figure 1D). The specific requirement of Th2 cells for Spi2A was presumably due to up-regulation through TCR and IL-4 stimulation, whereas Th1 cells did not up-regulate Spi2A and were so were not affected in Spi2A KO mice.

Spi2A KO Th2 cells are susceptible to apoptosis

We examined the mechanism by which Spi2A facilitated the differentiation of Th2 cells *in vitro*. We determined whether Spi2A was required for either the activation, or proliferation of Th2 cells. Labeling studies with CFSE showed that the decrease in the level of Spi2A KO Th2 cells was not due to decreased proliferation (Supplementary Figure S2). Furthermore, Spi2A KO Th2 cells did not display any decrease in activation status as indicated by CD69 expression (Supplementary Figure S3). We then examined the susceptibility of Th2 cells to apoptosis. ICS for activated caspase 3 in the cytoplasm (Figure 1E) or phosphotidyl serine expression on the plasma membrane with annexin V (Figure 1F) revealed that Th1 cells underwent higher levels of apoptosis compared to Th2 cells. This is consistent with earlier reports of the refractory nature of Th2 cells to classic AICD [11]. However, we observed that Spi2A KO Th1 cells were no more susceptible to apoptosis than where wild-type Th1 cells. In contrast, we observed a significant increase in the percentage of activated caspase 3-positive (P=0.0032) (Figure 1E) and annexin V-positive (P=0.0071) (Figure 1F) Spi2A KO Th2 cells compared to wild-type. We conclude that Spi2A is required for the *in vitro* differentiation of Th2 by ensuring survival through the suppression of apoptosis.

Diminished levels of Th2 cells after challenge of *Spi2A* KO mice

We determined whether Spi2A controlled the levels Th2 cells development *in vivo*. Mice were immunized on day (d) 0 and d5 (Figure 2A) and the level of OVA-specific CD4 T cells 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 (Figure 2B, Supplementary Figure S4) [3637]. Within the population of OVA-tet$^+$ CD4$^+$ cells we measured the levels of phenotypically Th2 cell based on the effector-phenotype of either intracellular IL-4 or IL-5 production (Figure 2C). In addition, we also used the expression of the ST2 differentiation marker [38] for Th2 cells, which is important as CD4 T cells that are negative for Th1 or Th2 cytokine production can also form long-lived Th2 effectors cells [7]. The representative flow-cytometry experiment in Figure 2C, shows that on d40 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 Th2 cells. In contrast, the percentage of OVA-tet$^+$ CD4$^+$ was about 2-fold lower in Spi2A KO mice and of these about 3-fold fewer expressed phenotypic markers of Th2 differentiation (overall 6-times lower level of Th2 cells in Spi2A KO compared to wild-type).

Longitudinal analysis of Th2 cells in wild-type mice revealed that the level of Th2 phenotype OVA-tet$^+$ ST2$^+$ CD4$^+$ or OVA-tet$^+$ IL-4$^+$ CD4$^+$ cells increased to a maximal level on d20 then decreased steadily to a residual memory level at d60 (Figure 2D). For OVA-tet$^+$ IL-5$^+$ CD4$^+$ cells the peak of the response was on d40 with a sharp decline to a memory level at d60. Th2 cells were detectable in both wild-type and Spi2A KO mice by d5 after immunization but after d10 that we observed significantly less Th2 cells in Spi2A KO compared to wild-type (OVA-tet$^+$ CD4$^+$ ST2$^+$ P=0.027; OVA-tet$^+$ CD4$^+$ IL-4$^+$ P =0.03) (Figure 2D). The deficit in the level of Th2 cells in Spi2A KO mice persisted at the peak of the response on d20 for (ST2$^+$ CD4$^+$ and IL-4$^+$ CD4$^+$) and until the memory phase at d60 (OVA-tet$^+$ ST2$^+$ CD4$^+$ P=0.0004; OVA-tet$^+$ IL-4$^+$ CD4$^+$ P =0.0003). We conclude that

Spi2A determines the level of Th2 effectors and memory cells throughout the Th2 response to OVA *in vivo*.

Diminished inflammatory allergic responses in *Spi2A* KO mice

To determine the physiological relevance of Spi2A in Th2 cell survival we examined allergic inflammation in the lungs of Spi2A KO mice after OVA challenge. Wild-type mice were sensitized by i.p. injection with OVA in alum to generate Th2 cells as before (Figure 2) then after various lengths of time challenged with repeated intra-nasal injection of OVA in PBS (Sensitization/Challenge – SC mice) [39] and then analyzed one day later (Figure 3A and B). We observed a robust Th2 –mediated inflammatory response in the lungs of SC mice as evidenced by the recovery of eosinophils and lymphocytes from broncheoalveolar lavages fluid (BAL) (Figure 3B). The lung inflammatory response was dependent on sensitization because the level of eosinophil and lymphocyte influx in challenge only mice (C) was significantly less than in S-C mice (Figure 3B). OVA-specific IgE levels were significantly higher in the S-C group compare to the C group because they were sensitized twice by i. p. injection and had more time to develop a B-cell response. The nature of the response is a Th2 response because of the presence of the adjuvant Alum, which promotes a Th2 skewing of the immune response (Figure 3B). The difference in allergic inflammation response (as indicated by either number of BAL eosinophils or lymphocytes, concentration of serum Il-4 or OVA-IgE) in SC compared to C 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 Th1 responses in Spi2A KO mice as evidenced by serum levels of IFN-γ or anti-OVA IgG2a (Supplementary Figure S5).

We examined Th2 driven inflammatory lung responses in Spi2A KO mice. There was a significant decrease in the allergic memory indices in the BAL (number of eosinophils P=0.003, number of lymphocytes P=0.03) and serum (concentration IL-4 P=0.002, concentration of OVA-IgE P=0.004) in Spi2A KO mice compared to wild-type on d20 after immunization (Figure 3C). The deficit in the allergic memory response also extended to 40d after immunization. Taken together we conclude that the defect in Th2 memory development in Spi2A KO results in decreased allergic inflammatory responses in the lung.

SERPINB3 and *SERPINB4* are up-regulated in human Th2 cells

Humans 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 if SCCA-1/2 have an equivalent role in human Th2 cells to Spi2A in mouse Th2 cells. We examined the development of Th2 cells from allergic patients (Table 1) after culture under Th2 cell polarization conditions [40]. After 3 weeks of culture we observed the development of Th2 cells as evidenced by increased intracellular expression of IL-4 compared to non-polarized CD4 T cells (Figure 4A). Over the course of 4 weeks we observed sustained and significant increased in the expression of the genes encoding the Th2 cytokines *IL-4* (P=0.0002, Figure 4B), *IL-5* (P=0.0002, Figure 4C) and the *GATA-3* transcription factor (P=0.0002, Figure 4C) compared to CD4 T cells cultured alongside under non-polarizing conditions. Culture under Th2 polarizing conditions resulted in the corresponding down-regulation of the Th1-specific *T-bet* gene expression

compared to CD4 T cells cultured alongside under non-polarizing conditions (P=0.002, Figure 4E). We observed the sustained and significant up-regulation of *SERPINB3* (P=0.006, Figure 4F) and *SERPINB4* (P=0.006), Figure 4G), compared to un-polarized cells from as early as week 1 of polarization. Comparison of the expression of *SERPINB3* (r=0.54, p=0.0014) and *SERPINB4* (r=0.71, p<0.0001) with that of the Th2-master transcription factor GATA-3, revealed the significant correlation of both serpin genes with *GATA-3* expression (Figure 4H). We conclude that the genes encoding the anti-apoptotic ova-serpins SCCA-1 and SCCA-2 are up-regulated in human Th2 cells.

Innate helper 2 cells (ICL2) cells have been implicated in lung inflammation through the production of Th2 cytokines, whereas Innate helper 1 cells (ICL1) have been implicated in the production of Th1 cytokines [41]. ICL2 and ICL1 cells were FACS-purified from the blood of pollen allergy patients (Figure 4I). The levels of ILC2 cells from these patients were higher than those of ILC1 cells (P<0.01). ICL2 cells were confirmed by the up-regulation of *IL-5* (Figure 4J) and *GATA-3* (Figure 4K) mRNA compared to ICL1 cells (both P=0.002) and ICL1 cells were confirmed by the up-regulation of *T-bet* mRNA compared to ICL2 cells (P=0.002) (Figure 4L). *Ex vivo* ICL2 cells expressed about 9-times more *SERPINB*3 (Figure 4M) and S*ERPINB4* (Figure 4M) mRNA than ICL1 cells (both P=0.002). This implies a role for SCAA-1 and SCCA-2 in ICL2 cells as well as Th2 cells in pollen allergic responses.

The surface molecule-CRTH2 is a marker of human memory Th2 cells [5] and the expression of CRTH2 (Prostaglandin D_2 receptor 2 (DP_2), CCR4 (C-C chemokine receptor type 4) and CCR7 (C-C chemokine receptor type 7) as well as Th2 cytokine expression are specifically associated with pathogenic terminally differentiated $CD27^-$ $CD4^+$ memory cells in allergic patients [4]. Flow-cytometry on *ex vivo* CD4 T cells from grass pollen allergic patients (Figure 5A) revealed that the $CD27^-$ $CD4^+$ population contained significantly higher levels (P=0.002) of $CRTH2^+$, $CCR4^+$ and $CCR7^+$ memory Th2 cells compared to $CD27^+CD4^+$ cells (Figure 5B). The Th2-phenotype of $CD27^-$ $CD4^+$ cells from allergy patients was confirmed by the significantly increased expression of the genes encoding *IL-4*, *IL-5*, and *IL-13* (all P=0.0002) compared to $CD27^+CD4^+$ cells after 18 hours of *in vitro* stimulation with anti-CD3 antibody (Figure 5C). We observed the significant up-regulation of both *SERPINB3* (P=0.0002) and *SERPINB4* (P=0.0002) expression in this memory Th2 cell population ($CD27^-$ $CD4^+$) compared a non-Th2 cell population ($CD27^+CD4^+$) from allergic patients (Figure 5D). The Th2-phenotype of $CD4^+CD27^-$ cells was also confirmed by the significantly increased secretion of the Th2-signature cytokines [4]-IL-4, IL-5, IL-9 and IL-13 (all P=0.0002) (Figure 5E) and Th2-signature chemokines[4] – MDC (Human macrophage-derived chemokine) (P=0.007), CXCL8 (C-X-C motif) ligand 8 (P=0.0002) and RANTES (Chemokine (C-C motif) ligand 5 (also CCL5) (P=0.01)) (Figure 5F) in $CD27^-$ compared to $CD27^+$ $CD4^+$ cells. Conversely, the Th1 phenotype $CD27^+$ $CD4^+$ population was confirmed by the increased expression of *IFN-γ* (Figure 5C) and *T-bet* (Figure 5D) mRNA and secretion of IFN-γ (Figure 5E) compared to $CD27^-$ $CD4^+$ cells (all P<0.0005). Therefore in our patient group $CD27^-$ $CD4^+$ cells are significantly enriched for memory Th2 cells. These data indicate that both the genes encoding the SCCA-1 and SCCA-2 ova-serpins are specifically up-regulated in allergy associated memory Th2 cells from human patients.

SERPINB3 and *SERPINB4* promote the viability of human allergic Th2 cells

We next determined the functional significance of *SERPINB3* and *SERPINB4* up-regulation in Th2 cells from grass-pollen allergic patients. We purified CD4 T cells from allergic patients then transduced with lentivirus encoding control or shRNA specific for either *SERPINB3* or *SERPINB4*. Transduced cells were identified by the expression of GFP (Figure 6A), which was encoded on a bi-cistronic message with shRNA. At least 85% of total were transduced with either control or *SERPINB3* and *SERPINB4* shRNAs (Figure 6B). We observed about an 80% knockdown in both *SERPINB3* and *SERPINB4* messages after transduction with shRNA (Figure 6C). We next cultured transduced CD4 T cells with anti-CD3/IL-2 and after about 6d observed a significant and sustained decreased in the number of viable $CD27^{-}$ $CD4^{+}$ cells transduced with either *SERPINB3* and *SERPINB4* shRNA compared with cells transduced with control shRNA (Figure 6E). The impaired viability persisted for up to 12 days at which point control transduced $CD27^{-}$ $CD4^{+}$ cells retained >80% viability whereas cells lacking *SERPINB3* or *SERPINB4* expression were <30% viable (Figure 6E and 6F). The reduced viability also resulted in the decrease of IL-4 and IL-13 production (Figure 6E and 6F). We conclude that ablation of either *SERPINB3* or *SERPINB4* gene expression decreases the viability of clinically relevant Th2 memory cells.

DISCUSSION

We show that the chymotrypsin-like serpin, Spi2A facilitated the survival of Th2 effectors *in vivo* and that ablation of Spi2A resulted in the alleviation of allergic inflammatory responses in the lung. We also demonstrate that the ova-serpins SCAA-1 and SCAA-2 protect allergic memory Th2 cells from death in 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 Th2 cells in both mice and humans.

The anti-apoptotic activity of both SCAA-1 [21–23] and SCAA-2 [24, 25] and Spi2A [15, 20] are well known and are mediated by the inhibition of cathepsins B and L in the cytoplasm. In the present study, we show that the cyto-protection afforded by SCAA-1/SCAA-2 and Spi2A extends to human and mouse Th2 cells. There are important differences between the roles Spi2A plays in Th2 compared to other T cells. We found no evidence for a role for Spi2A in the survival of Th1 cells. However the use of a more physiologically relevant model for *in vivo* Th1 development than OVA+ alum immunization would be useful to more fully explore this possibility. Spi2A is a physiological target of the NF-κB transcription factor [15, 42] and a target of STAT transcription [43] induced by cytokines (e.g. IL-15 [31], IL-7 [44]) and growth factors (e.g. erythropoietin [26]. Therefore, the differential effect of TCR signals that up-regulate Spi2A in Th2 cells but not Th1 cells`, may 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 so it is possible that in different physiological settings IFN-γ may affect Th1 cell survival through Spi2A up-regulation. SCCA-1/SCCA-2 expression has been noted previously in human mononuclear cells [45] and intriguingly it has been reported that deficiency in mouse *Serpinb3a*, which is thought to be the homologue of human SCAA-1/SCAA-2, reduces airway hyper-responsiveness by

limiting goblet cell hyperplasia [46]. In contrast to our findings with OVA-specific IgE levels in our *Serpina3g* (Spi2A) KO mouse, levels of HDM-specific IgG1 and IgE l were no different between *Serpinb3a* KO and wild-type mice[46]. The strain of the mice (C57Bl/6 versus BALB/c), allergen (HDM vs OVA) and especially the route of sensitization (lung vs ip) might explain the discrepancy. It will be important therefore when evaluating the physiological relevance of SERPINB3 and SERPINB4 in humanized mice to use a variety of allergenic models.

A major question for the development of Th2 memory is how do Th2 memory cells and their precursors escape apoptosis? *In vitro* polarization studies have concluded that compared to Th1 effectors, Th2 cells are relatively resistant to classical AICD [11]. Our *in vitro* polarization study also show higher levels of apoptosis in Th1 cells compared to Th2 cells. But the differences in apoptosis observed for Th1 and Th2 cells may also be affected by differences in the culture conditions used to generate each population. Despite this caveat our results clearly show that Spi2A protects Th2 cells but not Th1 cells from apoptosis and so point to the lysosomal pathway of cell death controlling Th2 cell survival. Spi2A deficiency reduced the level of Th2 cells *in vivo* but did not affect the production of IL-13 in our lung inflammation model. Although *SERPINB3* and *SERPINB4* mRNA is expressed in human ICL2 cells we do not know if Spi2A is expressed in mouse ICL2 cells. Further studies will clarify if Spi2A, SCAA-1 and SCAA-2 control the survival of other producers of allergic cytokines (such as ICL2 cells) in lung inflammatory disease.

The 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 that seeks to shift the Th2 balance to Th1 may be explained in terms of the stability of Th2 memory cells that develop very early in life [48]. Therefore it would be advantageous to be able to eliminate the persistent Th2 memory cells that give rise to atopic diseases [49–51] and allergic diseases such as asthma [52] through the 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 Th2 cells in grass-pollen allergic patients. Furthermore, the expression of *SERPINB3* and *SERPINB4* in ICL2 cells implies that mRNA knock-down may also ablate allergenic innate immune system cells. The alleviation of allergic asthma by *in vivo GATA-3* mRNA knock-down in Th2 cells provides support for the concept of targeting memory and effector Th2 cells by *SERPINB3* and *SERPINB4* mRNA knock-down [55]. It remains to be determined whether *SERPINB3* and *SERPINB4* also control memory CD8 T cell responses in humans as is the case for Spi2A in mice [31,44]. A localized therapeutic approach targeting memory Th2 cells would potentially deplete the reservoir of allergic disease and so complement anti-allergic drugs and cytokine therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Th2 cells
T-helper cell 2 cells

Th1 cells
T-helper cell 1 cells

Serine Protease Inhibitor
Serpin

SCCA-1
Squamous Cell Carcinoma Antigen-1

SCCA-1
Squamous Cell Carcinoma Antigen-2

Spi2A
Serine Protease Inhibitor 2A

OVA
ovalbumin

KO
knock-out

CD
cluster of differentiation (maker)

TCR
T cell receptor

mAb
monoclonal antibody

PBMC
peripheral blood mononuclear cell

IL
interleukin

IFN
interferon

AICD
activation induced cell death

BAL
broncheoalveolar lavages fluid

Ig
immunoglobulin

ICL2 cell
Innate helper 2 cell

ICL1
Innate helper 1 cell

shRNA
small hairpin ribonucleic acid

NF-κB
nuclear factor kappa B

STAT
signal transducer and activator of transcription

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Clinical Implications

- The up-regulation of *Serpin 2A (Spi2A)* protects mouse Th2 cells from apoptosis and determines the number of effector and memory Th2 cells *in vivo*
- *Spi2A* in-activation reduces the development of allergic inflammation in mice
- The genes *SERPINB3* and *SERPINB4* are up-regulated in memory Th2 and innate helper 2 (ICL2) cells from allergy patients
- The viability of allergenic memory Th2 cells is decreased by *SERPINB3* and *SERPINB4* knock-down
- Proof-of-principle for a therapeutic approach for allergic disease through the ablation of allergic memory Th2 cells through mRNA *SERPIN B3* and *B4* down-regulation

CAPSULE SUMMARY

The inactivation of genes encoding anti-apoptotic, serine protease inhibitors, results in the ablation of allergenic Th2 cells in both mouse models and *in ex vivo* memory Th2 cells from pollen allergy patients.

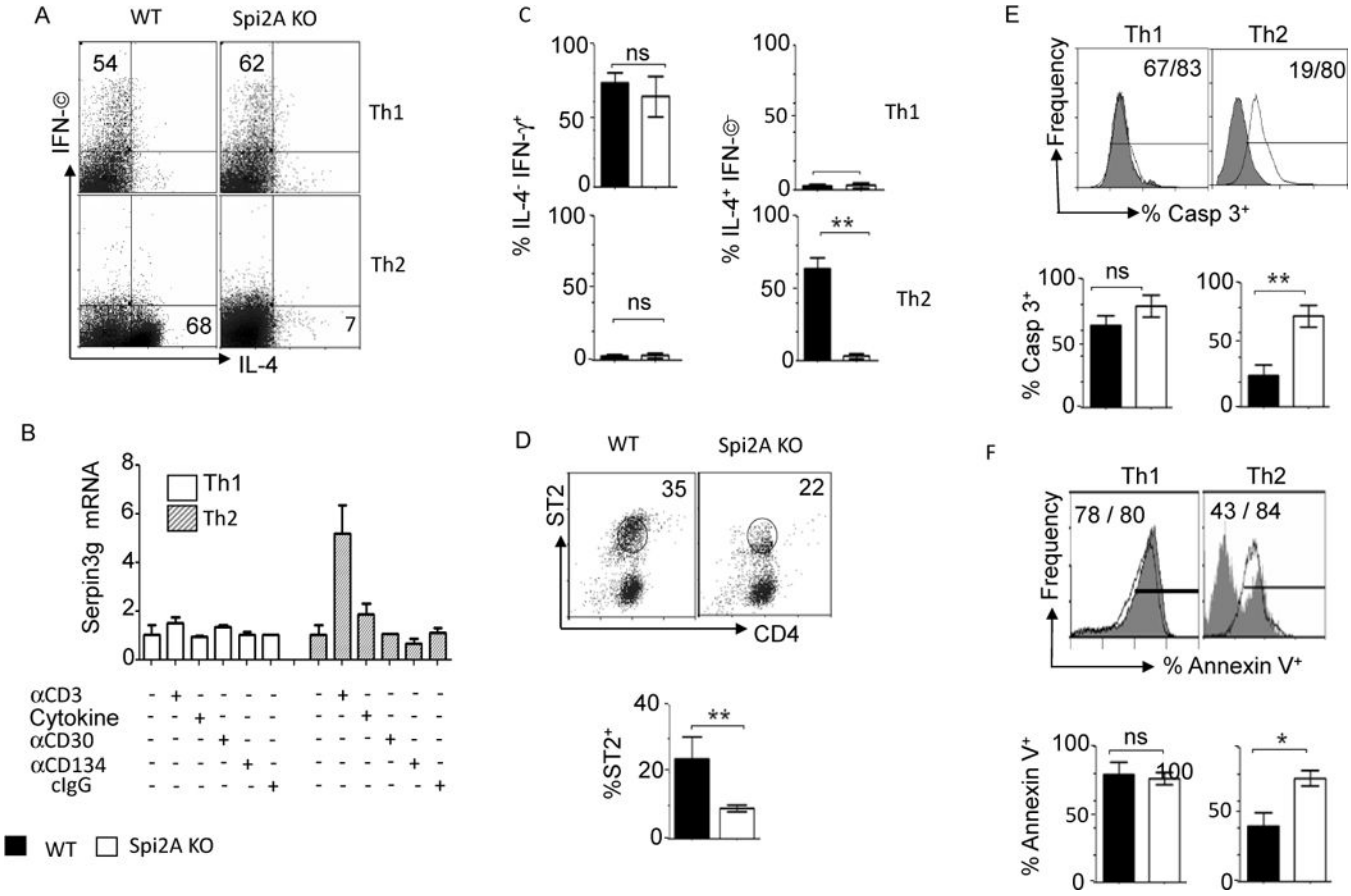

Figure 1. Differentiation and survival of Th2 cells from Spi2A KO mice

(A) Flow cytometry plots for Th1 or Th2 polarized CD4 T cells from wild-type (WT) or Spi2A KO 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 *Serpina3g* mRNA (fold-change over that of naïve CD4 T cells (CD4$^+$CD62L$^+$)) in Th1 or Th2 cells after no-stimulation (−) or stimulation with mAb or cytokine (IFN-γ for Th1 and IL-4 for Th2). (C) Histograms of mean percentage IL-4$^+$ IFN-γ$^-$ and IL-4$^-$ IFN-γ$^+$ of polarized CD4$^+$ cells (± SEM, n=6). (D) Flow cytometry plots after staining for ST2 and CD4 on WT and Spi2A KO Th2 polarized cells. Percentages of ST2$^+$ CD4$^+$ cells in plots are indicated. (E) Flow cytometry histogram for WT (filled) or Spi2A KO (line) Th1 and Th2 cells after ICS for activated caspase 3. Percentage activated caspase 3-positive (line gate) is indicated WT/Spi2A KO. Histograms of mean percentage activated caspase 3-positive (± SEM, n=6). (F) Flow cytometry histogram for WT (filled) or Spi2A KO (line) Th1 and Th2 cells after staining with Annexin V. Percentage Annexin V-positive (line gate) is indicated for WT/Spi2A KO. Histograms of mean percentage Annexin V-positive (± SEM, n=6). P <0.05 *; P <0.005 **; P>0.05 Not Significant (N.S.).

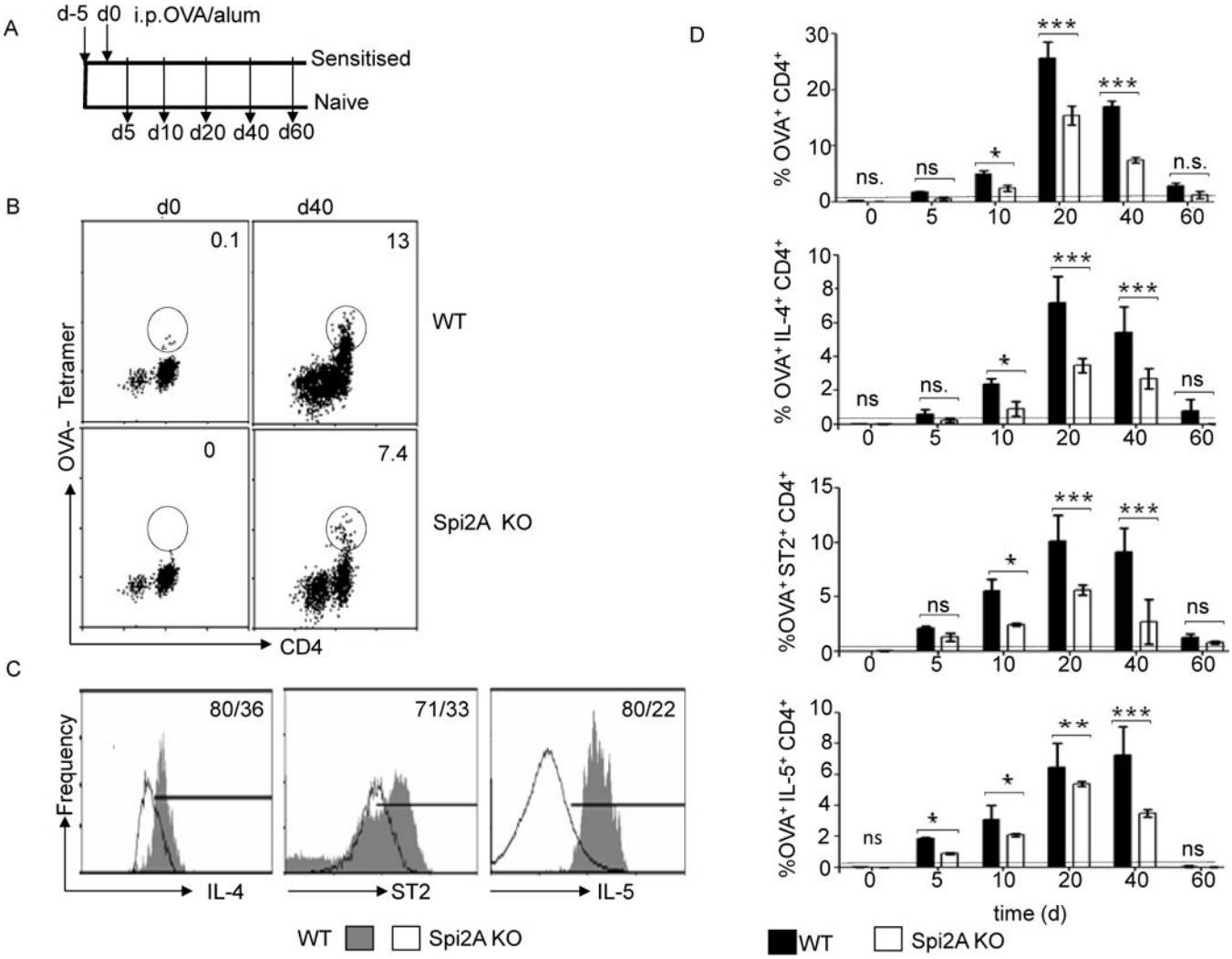

Figure 2. Th2 responses in immunized Spi2A KO mice

(A) Experimental protocol. Arrows indicate i.p. injections of OVA + alum on d-5 and d0 and the times at which mice were analyzed (B) Flow cytometry plots after staining with $OVA_{329–337}$/I-A^b tetramer (OVA tet) and anti-CD4 before (d0) after 40d after the final OVA + alum immunization. Percentage OVA tet^+ $CD4^+$ cells is indicated. (C) Histograms (filled WT, line Spi2A KO) after staining for ST2 on gated OVA tet^+ $CD4^+$ cells. Histograms after ICS for IL-4 or IL-5 on gated OVA tet^+ $CD4^+$ cells. Percentage positive population (line gate) is indicated for WT/Spi2A KO, respectively. (D) Histograms for mean percentage OVA tet^+ $CD4^+$, mean percentage OVA tet^+ $CD4^+$ IL-4^+, mean percentage OVA tet^+ $CD4^+$ $ST2^+$ and mean percentage OVA tet^+ $CD4^+$ IL-5^+ of total splenocytes (± SEM, n=6–8). P <0.005 **; P <0.0005 ***; P>0.05 Not Significant (n.s.). Broken horizontal lines show the limit of detection.

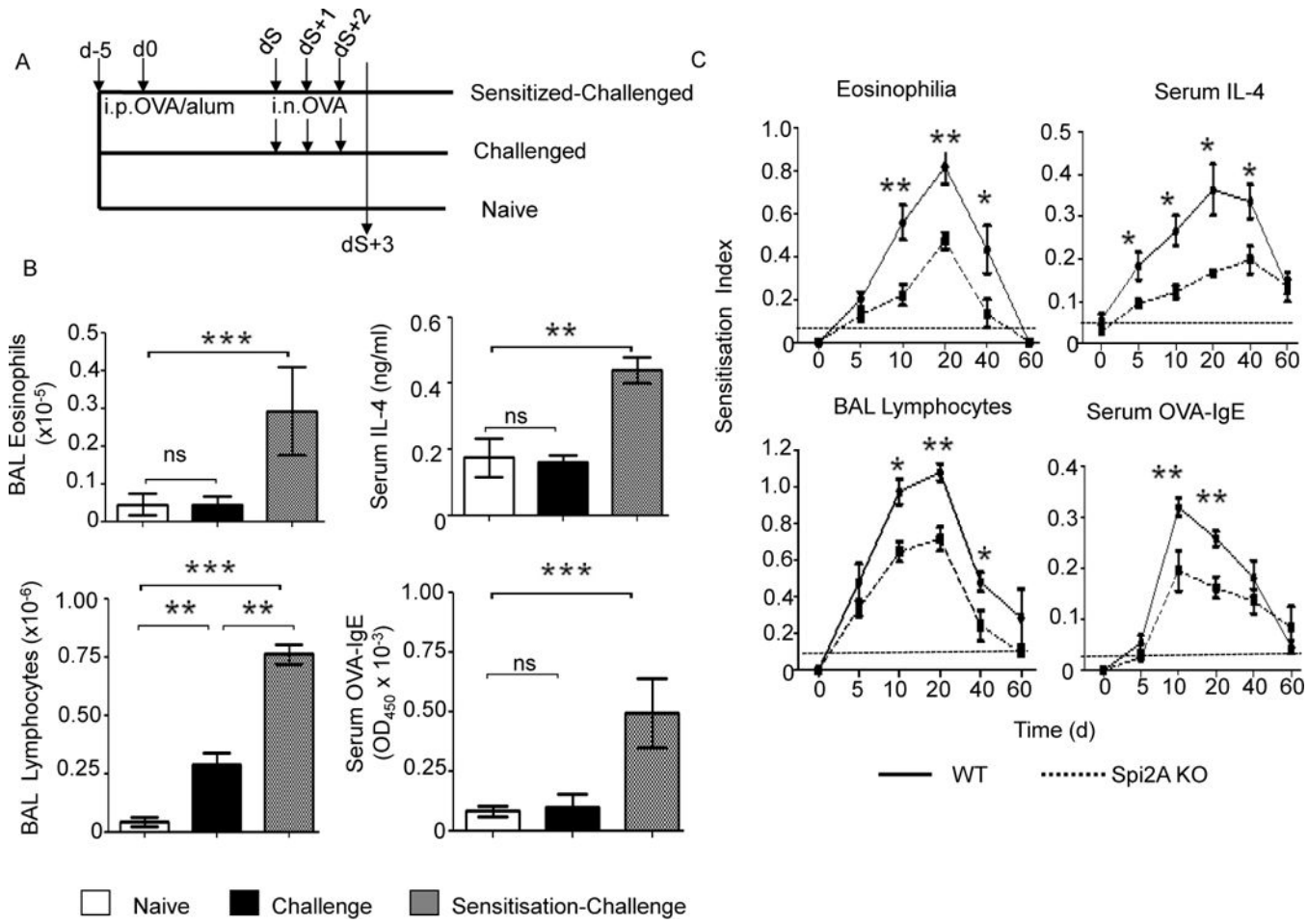

Figure 3. Diminished inflammatory allergic responses in Spi2A KO mice

(A) Experimental protocol. Arrows indicate i.p. injections of OVA + alum on d-5 and d0 in Sensitized mice which were then challenged (S-C mice) after a certain period of time (dS) by i.n. injection of OVA, one per day on 3 consecutive days (dS, dS+1,dS+2). Analysis was performed on naïve mice (N) or challenge only mice (C) one day after the last i.n. challenge (dS+3). (B) Histograms of mean (± SEM, n=6–8) eosinophils and lymphocytes numbers in BAL and concentration of IL-4 and OVA-IgE in the serum in experimental groups of mice. S-C mice were challenged 10d after sensitisation. (C) Line graphs of allergic memory index for BAL eosinophils or lymphocytes and serum IL-4 or OVA-IgE versus time prior to challenge after sensitization. Memory Index= (Response in S-C mice) – (Response C mice). P< 0.05*; P <0.005**; P<0.0005***; P>0.05, Not Significant (N.S.).

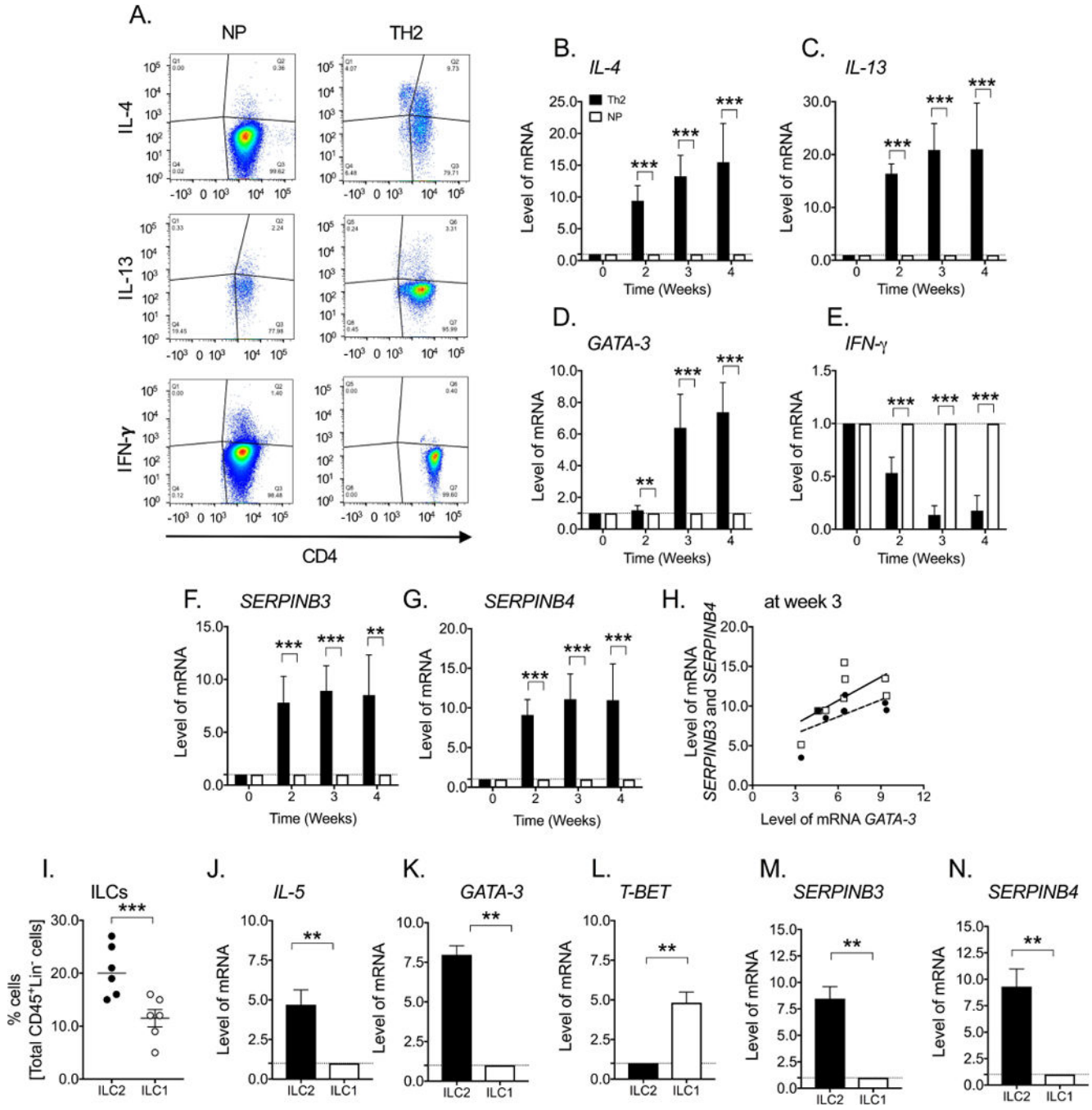

Figure 4. Expression of *SERPINB3* and *SERPINB4* in human Th2 cells

(A). Representative flow-cytometry data illustrating expression of Th2/Th1 cytokines in non-polarized (NP) and polarized Th2 cells at week 3. (B–G). Mean (± SEM n= 8 patient samples) level of target mRNA (fold-change compared to EFA-2 housekeeping gene) in Th2 polarized and non-polarized (NP) cells. (H) Relationship between Th2 master regulator *GATA-3* and S*ERPINB3* (filled symbols) and *SERPINB4* (open symbols) mRNA levels in polarized Th2 cells from individuals after 3 weeks of culture. (I) Percentage of ILC2 and ILC1 cells of $CD45^{+}Lin^{-}$ PBMC from pollen allergy patients (n=6) (horizontal line

indicates mean percentage). Mean levels of (J) *IL-5*, (K) *GATA-3*, (L) *T-BET*, (M) *SERPINB3* and (N) *SERPINB4* mRNA in ICL2 cells relative to the ICL1 cells. P< 0.05*; P <0.005**; P<0.0005***.

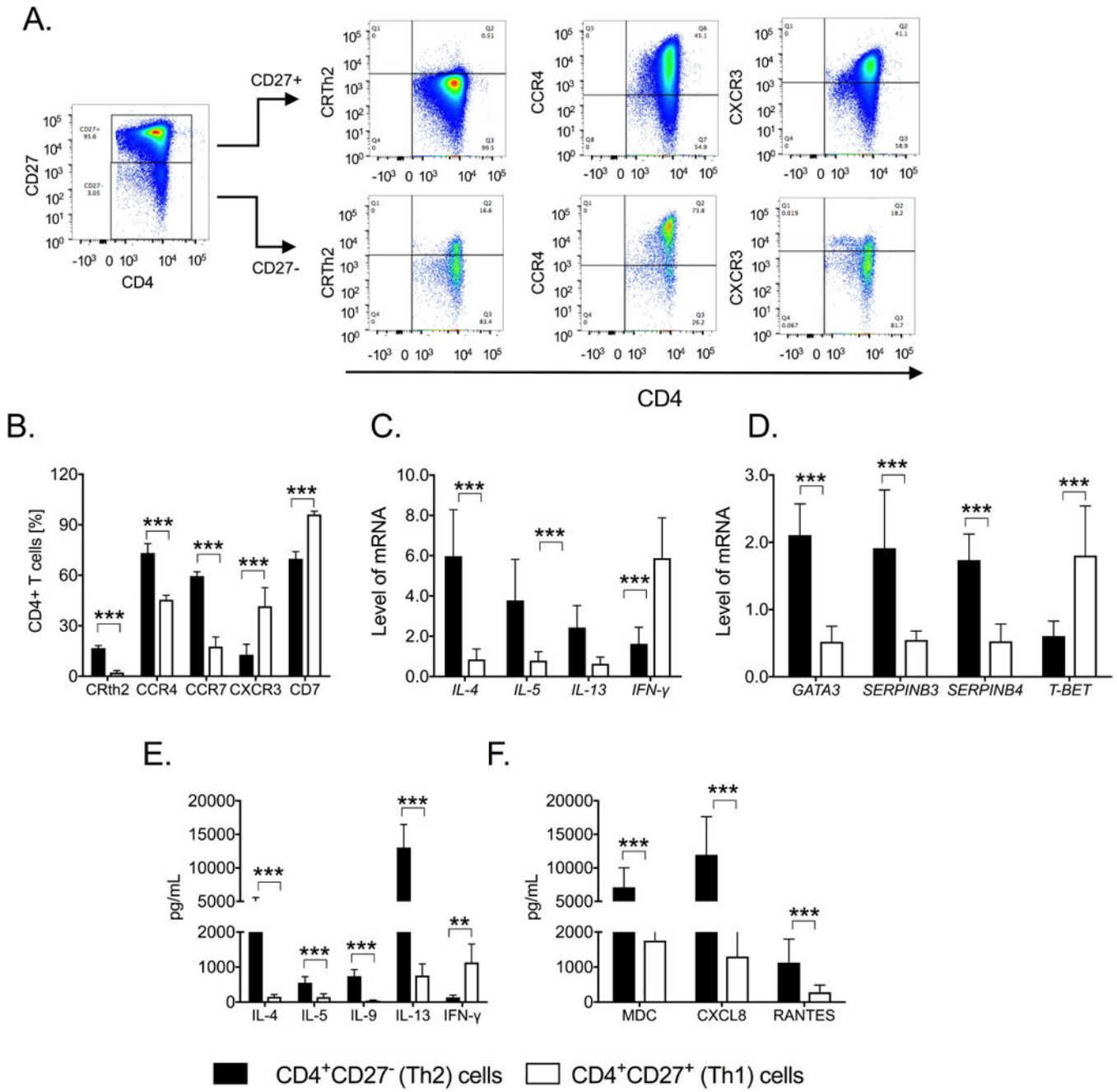

Figure 5. Expression of *SERPINB3* and *SERPINB4* in memory Th2 cells from allergic patients

(A) Representative flow-cytometry data for $CD4^+$ T cells gated for CD27 expression and analyzed for cell surface markers. (B) Histogram for mean percentage (± SEM n= 8 patient samples) positive for surface markers of total CD4 in either $CD27^+$ or $CD27^-$ populations. (C and D) Mean (± SEM n= 8 patient samples) level of target mRNA (fold-change compared to EFA-2 housekeeping gene) in $CD4^+$ $CD27^+$ and $CD4^+$ $CD27^-$ cells 18h after stimulation with anti-CD3/CD28. (E and F) Mean (± SEM n= 8 patient samples) concentration of cytokine and chemokine 18h after stimulation with anti-CD3/CD28. P< 0.05*;P <0.005** ; P<0.0005***.

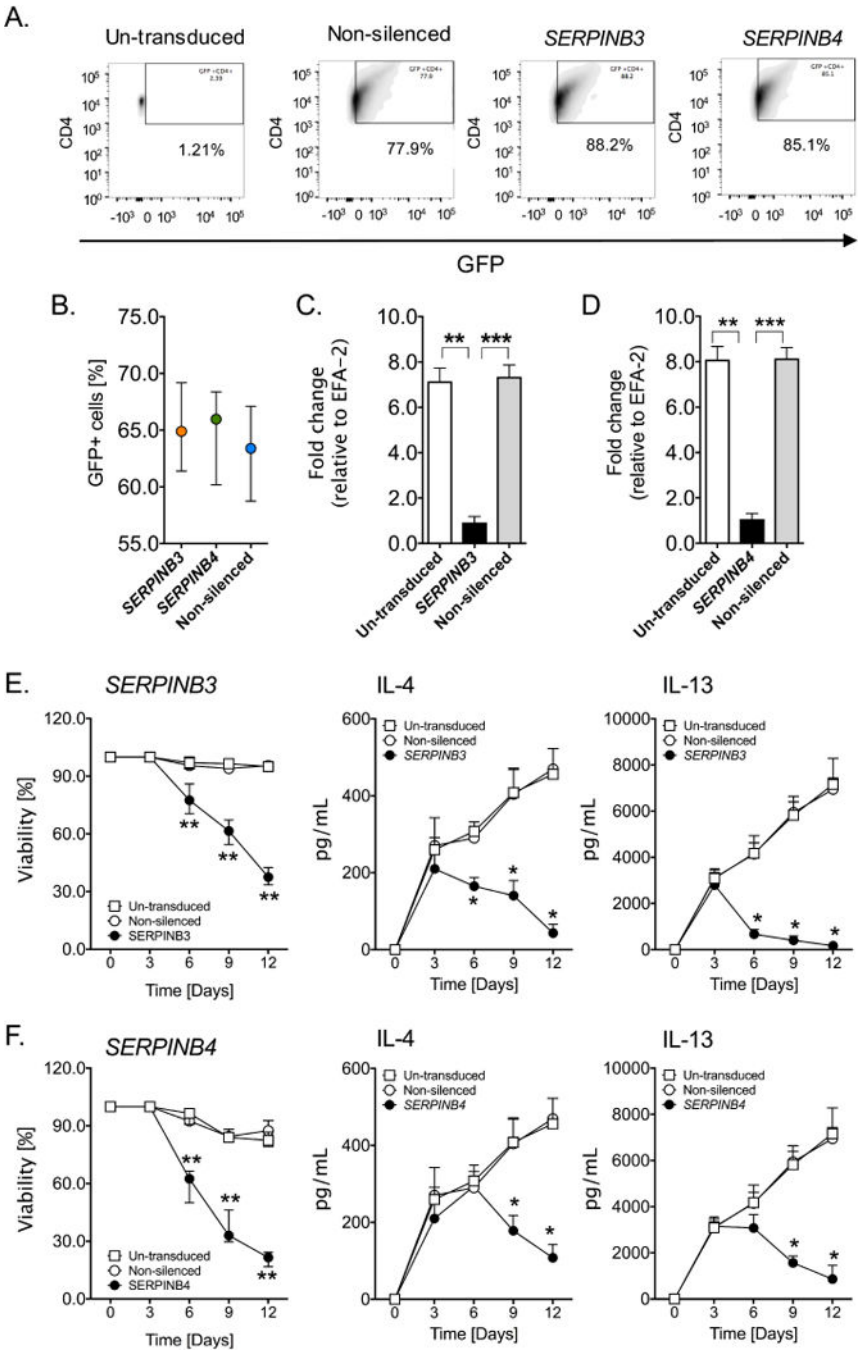

Figure 6. Expression of *SERPINB3* and *SERPINB4* are required for the survival of memory Th2 cells

(A) Representative flow-cytometry data for percentage GFP$^+$ of lentivirus transduced CD27$^-$ CD4 T cells. (B). Mean (± SEM n= 8 patient samples) percentage GFP$^+$ of lentivirus transduced CD27$^-$ CD4 T cells. (C) Mean (± SEM n= 8 patient samples) level of *SERPINB3* mRNA (fold-change in compared to EFA-2 housekeeping gene). (D) Mean (± SEM n= 8 patient samples) level of *SERPINB4* mRNA (fold-change in compared to EFA-2 housekeeping gene). (E and F) Mean percentage viability (± SEM n= 8 patient samples) of

$CD27^{-}$ $CD4^{+}$ Th2 cells and concentrations of IL-5 and IL-13 in culture supernatant after transduction with lentiviruses. P< 0.05*; P <0.005**; P<0.0005***.

Table I

Subject characteristics

	SAR (n=8)
Gender (M:F)	5:3
Age (mean, range)	31 (20:48)
Allergen Grass Specific IgE (mean, SD)	62.5 ± 13.3
Total IgE (mean, SD)	28.9 ± 328.30
Allergen skin prick test (mm^2) (mean, SD)	10.3 (2.4)

Distribution of age, gender, specific IgE, skin prick test.