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*

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 D_2 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 (Serpin) 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)–Serpin family,[17] inhibits both serine proteinases, such as chymotrypsin, and cysteine proteinases, such as cathepsin L, K, S, and papain,[18] whereas the

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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-1[21-23] 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 Biotech, Bergisch Gladbach, Germany) for $CD4^+CD62L^+CD44^-$ cells, and then seeded at 10^6/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).

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.

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 1136*g* 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 Naïve $CD4^+$ 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 (10^6/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

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