Supplemental Information

Development of aging-related emphysematous and lymphoma-like lesions is enhanced by the lack of secretoglobin 3A2 in mouse lungs

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Supplemental Materials and Methods

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Supplemental Materials and Methods

Evaluation of elastic fiber content

To determine the amount of elastic fibers in mouse lung tissue, quantification of elastic fibers was performed by referring to the quantitative evaluation method of angiogenesis and tumors[1]. In the images of Victoria blue (VB)-stained alveoli, areas of 60 × 60 μm^2 (360 pixels × 360 pixels) without adjacent bronchi and blood vessels were cropped, and the quantification of elastic fiber content was performed using ImageJ software (NIH, Bethesda, MD, USA). The numbers of cropped alveoli images analyzed ranged 99–181 using 3–5 lung tissue

sections from three mice for each age. The target 24-bit RGB image was separated into 8-bit grayscale images for each of the red, green, and blue channels using the split channels command, and the red channel image was subtracted from the blue channel image using image calculator. The threshold was set at ≥ 31, and the elastic fiber signal for each alveolus was quantified by obtaining the gray intensity within the threshold range.

Cell culture

Raw264 [a mouse monocyte/macrophage (Mφ) cell line] cells and MH-S (a mouse alveolar Mφ cell line) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute medium (RPMI)-1640 medium supplemented with 10% FBS and 1 % penicillin-streptomycin mixed solution (Nacalai Tesque, Kyoto, Japan) at 37°C in 5% CO_2 and 95% air. RAW264 and MH-S cells were plated at 5.0×10^5 cells/mL in a 35-mm-diameter dish and cultured for 16 hours in normal maintenance medium with and without SCGB3A2 (1 μg/mL).

Fluorescent immunostaining

Fluorescent immunostaining was performed to evaluate surfactant protein (SP)-A, SP-B, SP-C, and SP-D expression in the lungs of aging mice. In brief, after deparaffinization, sections were washed with pure water, and the sections were reacted with 5% milk (Morinaga Milk, Tokyo, Japan) in PBS (pH 7.4) for 1 h at room temperature to block nonspecific proteins. Primary antibodies against SP-A (H-148, sc-13977, Santa Cruz Biotechnology, Dallas, TX, USA), SP-B (WRAB-SPB, Seven Hills Bioreagents, Cincinnati, OH, USA), SP-C (FL-197, sc-13979, Santa Cruz Biotechnology), and SP-D (H-120, sc-13980, Santa Cruz Biotechnology) diluted 3200-fold with 5% milk in PBS were reacted with slides at 4°C for 16–18 h. After the primary antibody reaction, biotin-labeled secondary antibody, supplemented with normal goat serum, was added to the tissues and reacted for 30 min at room temperature followed by reaction with FITC-labeled avidin and biotin complex for 30 min at room temperature in the dark. (Vectastain Elite ABC Kit, Vector Laboratories). After washing the tissues, cell nuclei were stained with 0.1 μg/mL of 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (Thermo Fisher Scientific) in the dark at room temperature. Tissues were washed and sealed with CC/Mount (Diagnostic

Biosystems, Pleasanton, CA, USA). The localization of SPs was observed under an FV1200 confocal laser microscope (Olympus).

Supplemental Figure S1. Evaluation of alveolar elastic fiber content

The amount of elastic fibers in the pulmonary alveoli of wild-type (WT) and *Secretoglobin* (*Scgb) 3a2*-knockout (KO) mice from day 0 to 2 years (y) is presented as a bar graph and a scatter plot. Black bars and circles: WT; white bars and circles: *Scgb3a2*-KO. The number of alveoli images analyzed (shown in parenthesis) was as follows: day 0, WT (108) and KO (99); day 1, WT (129) and KO (142); day 5, WT (111) and KO (99); day 7, WT (116) and KO (122); day 15, WT (111) and KO (135); 8 weeks of age (8 w), WT (113) and KO (181); 1 year of age (1 y), WT (116) and KO (137); 1.5 y, WT (115) and KO (103); 2 y, WT (122) and KO (141). Data are presented as the mean ± standard deviation (SD). Significant differences between WT and *Scgb3a2*-KO mice at each age were analyzed using Student's *t*-test (*$p < 0.05$, **$p < 0.01$). Differences among ages within the WT or KO group were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer post hoc test. Different letters indicate significant differences between WT mice of different ages (a–e) and KO mice of different ages (A–F). $p < 0.05$ for day 0 vs. day 5 in WT, day 1 vs. day 5 in WT, and 8 w vs. 1 y in KO, and $p < 0.01$ for the others.

Supplemental Figure S2. Expression of SCGB3A2 during lung development/maturation

A. Immunohistochemistry for Secretoglobin (SCGB) 3A2 (i-viii). Representative immunohistochemistry results. i: day 0; ii: day 1; iii: day 5; iv: day 7; v: day 15; vi: 8 weeks (8 w), vii: 1-year-old wild-type (WT) mouse lung as a reference; viii: 8 w *Secretoglobin* (*Scgb) 3a2*-knockout (KO) mouse lung as a negative control with no SCGB3A2 staining, indicating the specificity of the SCGB3A2 antibody used. Arrows: secretory-like images, Brown: immunopositive signal for SCGB3A2, blue: nuclei. Scale bars: 50 μm.
B. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for SCGB3A2 during development. Four mice (two males and two females) from day 0 to day 15 and eight mice (four males and four females)

from 8 w were analyzed. qRT-PCR results (each sample analyzed in triplicate) for SCGB3A2 mRNA expression in WT mouse lungs from day 0 to day 15 and 8 w presented as a bar graph and scatter plot. Data are presented as the mean ± standard deviation (SD). Differences between ages were examined by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer post hoc test (*$p < 0.05$).

Supplemental Figure S3. A rare pathology and the effect of SCGB3A2 on macrophage polarity

A. Representative hematoxylin and eosin (HE)-stained images revealing a high level of macrophage accumulation observed in the lungs of aged wild-type (WT) and *Secretoglobin* (*Scgb) 3a2*-knockout (KO) mice and their Ym1/2 immunohistochemical staining images (i-iv). i: HE-stained image of the lung of 1.5-year-old (1.5 y) WT mouse, ii: HE-stained image of the lung of 2-year-old (2 y) *Scgb3a2*-knockout (KO) mouse, iii: immunohistochemistry for Ym1/2 (marker for macrophage pneumonia) in 1.5 y WT mouse, iv: HE-stained image of the lung of 2 y *Scgb3a2*-KO mouse. White arrowheads: macrophages. Brown indicated by black arrowheads: Ym1/2 immunopositivity. Blue in iii and iv: nuclei. Scale bars: 20 μm.
B. Expression of macrophage polarity marker genes in macrophage cell lines in the presence and absence of SCGB3A2 by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (i-iv). i: expression of type I macrophage marker *Tumor necrosis factor-alpha* (*Tnf-α)* mRNA in Raw 264, ii: expression of type II macrophage marker *Interleukin-10* (*Il-10*) mRNA in Raw 264, iii: expression of *Tnf-α* mRNA in MH-S cells, and iv: expression of *Il-10* mRNA in MH-S in the presence (SCGB) or absence (Cont.) of SCGB3A2. N = 3–5, each performed in triplicate. Data are presented as the mean ± standard deviation (SD). Differences between Cont. and SCGB were analyzed by Student's *t*-test (*$p < 0.05$).

Supplemental Figure S4. Expression of SPs in the mouse lungs during aging process

Representative fluorescent immunostaining for Surfactant proteins (SPs) of lung tissues from wild-type (WT) and *Secretoglobin* (*Scgb) 3a2*-knockout (KO) mice from 8 weeks (8 w) to 2 years (2 y) old (Ai–x – Di-x). A: SP-A; B: SP-B; C: SP-C; D: SP-D; i: 8 w, WT, ii: 8 w, *Scgb3a2*- KO, iii: 0.5 y, WT, iv: 0.5 y, *Scgb3a2*- KO, v: 1 y, WT, vi: 1 y, *Scgb3a2*- KO, vii: 1.5 y, WT, viii: 1.5 y, *Scgb3a2*- KO, ix: 2 y, WT, 2 y, *Scgb3a2*- KO, green: each SP; blue: nuclei stained with 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI). Scale bar: 10 μm.

Supplemental Figure S5. *Serpina1a* expression in lung tissues of WT and *Sgcb3a2* KO mice.

Serpina1a expression in wild-type (WT) and *Secretoglobin* (*Scgb) 3a2*-knockout (KO) mouse lungs at 8 weeks of age is presented as a bar graph and scatter plot. *Serpina1a* mRNA was detected in WT mouse lungs at 8 weeks, but it was barely detectable in KO mouse. Five male WT mice and four male KO mice were used. The experiments were performed in triplicate per sample. mRNA expression in WT and KO mouse lungs was too low to be accurately analyzed at 0.5 to 2 years of age, and the data are not presented. For WT and KO mouse lungs from 0.5 to 2 years, the same samples used in Fig.5A were used.

Supplemental Figure S6. Gene expression in the lungs of 3-month-old WT and *Scgb3a2*-KO mice.

Significantly different genes identified by RNA sequencing (2-fold changes, $p < 0.01$) are presented in a heatmap. KO lungs: KOP3, KOP4, and KOP5; WT lungs: WTP2, WTP3, WTP4, and WTP.

Supplemental Figure S7. Changes of BALF and serum SCGB3A2 concentrations during aging

Enzyme-linked immunosorbent assay (ELISA) was performed to measure Secretoglobin (SCGB) 3A2 concentrations in bronchoalveolar lavage fluid (BALF) (A) and serum (B) from wild-type (WT) mice from 8 weeks (8 w) to 2 years (2 y) of age using a modification of a previously described method[2], and the results are presented

in a bar graph and scatter plot. In this experiment, mouse uteroglobin-related protein 1/ SCGB3A2 (mUGRP1/SCGB3A2) antibody was used. SCGB3A2 levels in BALF significantly increased at 0.5 years of age and remained constant until 2 years of age. SCGB3A2 levels in serum appeared to increase at 1 and 1.5 years of age, but no statistical significance was observed. A: N = 5–8. The age, sex, and number of mice analyzed were as follows: 8 w: 4 males (m), 4 females (f); 0.5 y: 3 m, 3 f; 1 y: 5 f; 1.5 y: 5 f; 2 y: 6 m, 1 f. B: N = 4–8. The age, sex, and number of mice analyzed were as follows: 8 w: 4 m, 4 f; 0.5 y: 3 m, 4 f; 1 y: 4 f; 1.5 y: 4 f; 2 y: 5 m, 1 f. Data are presented as the mean ± standard deviation (SD). Significant differences between 8 weeks old and other ages were analyzed using Student's *t*-test (*$p < 0.05$, 8 w vs. 0.5, 1, 1.5, and 2 y).

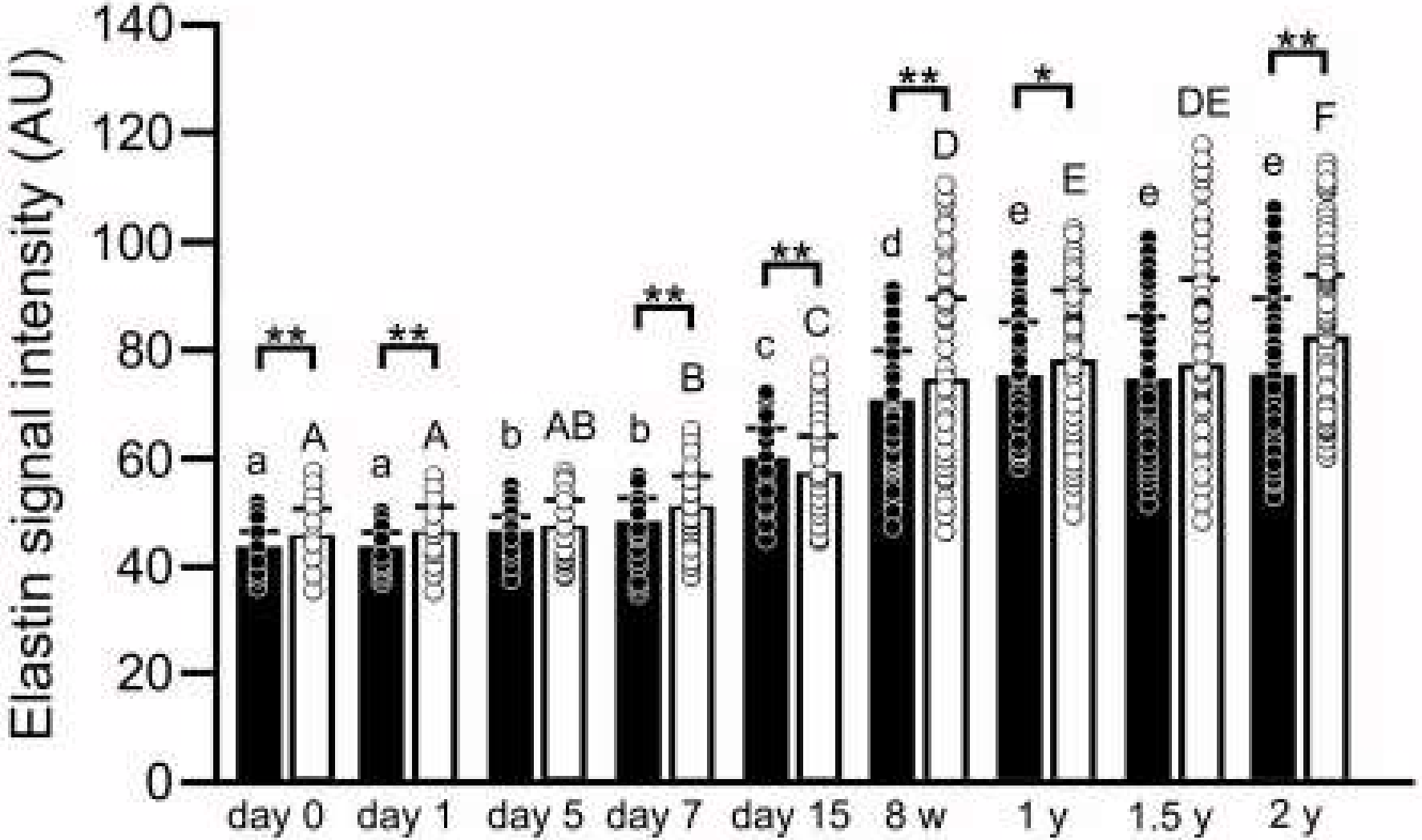
Supplemental Figure S8. Schematic relationship among aging, major lung SPs and physiological changes in the lungs of SCGB3A2-deficient mice

In wild-type (WT) mouse lungs, the expression of Secretoglobin (SCGB) 3A2, SCGB1A1, Surfactant protein (SP)-A, B, C, and D remained constant during aging. In *Secretoglobin* (*Scgb) 3a2*-knockout (KO) mouse lungs, the expression of SPs in young adults (8 weeks old) was comparable to that of WT mice at both mRNA and protein levels (high). With aging, SP mRNA expression decreased significantly in KO as compared with WT lungs. The results of RNA sequencing, which indicated that KO mice have an inherently hyperactive immune system at 3 months old, suggest that the aging-dependent activation of the immune system may occur in KO mouse lungs. Furthermore, SCGB3A2 changes the polarity of macrophages from inflammatory type I to anti-inflammatory type II, suggesting that in SCGB3A2-deficient lungs, macrophages are likely to enhance inflammatory action in SCGB3A2-deficient lungs. SCGB3A2 deficiency is also associated with a possible reduction of Alpha-1-Antitrypsin (A1AT) expression, all together resulting in the increased risks of lung inflammation and emphysema.

Supplemental References

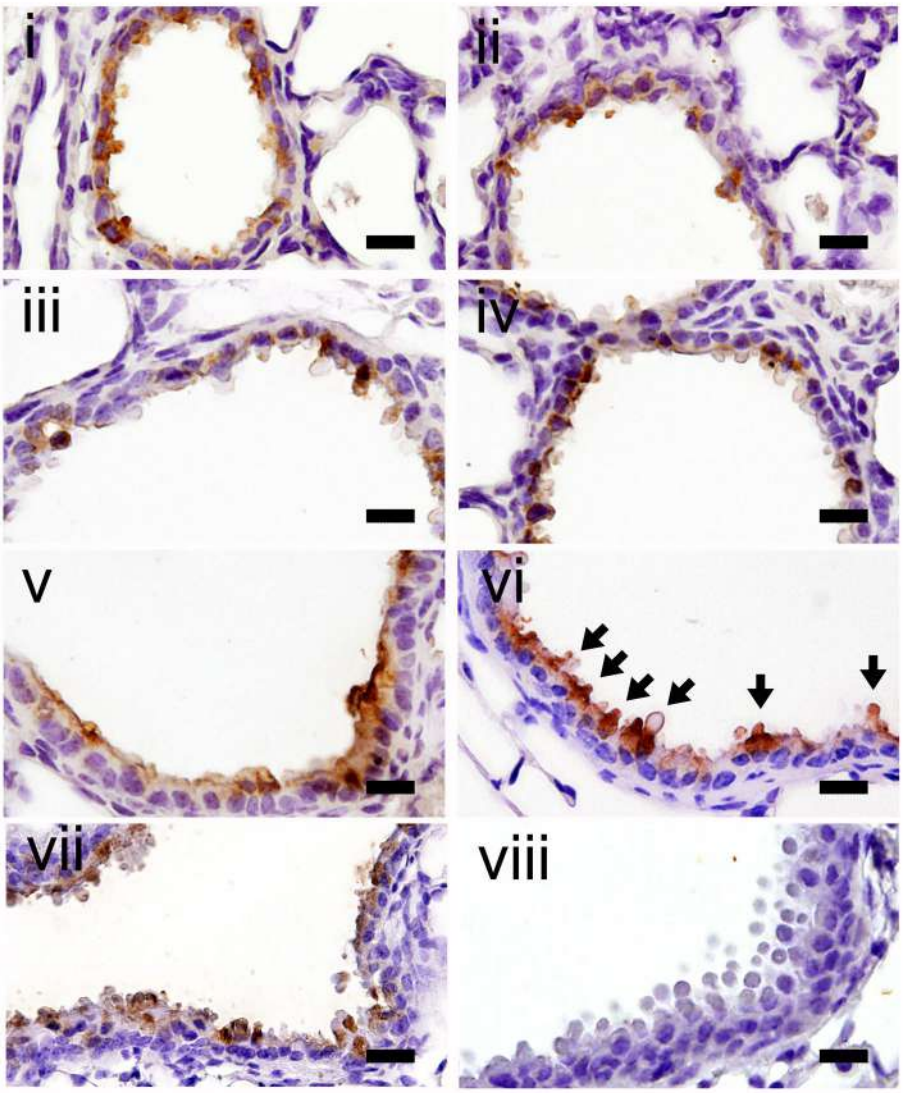
1 Wild R, Ramakrishnan S, Sedgewick J *et al.* Quantitative assessment of angiogenesis and tumor vessel architecture by computer-assisted digital image analysis: effects of VEGF-toxin conjugate on tumor microvessel density. *Microvasc Res*. 2000;59:368-376.

2 Chiba Y, Kurotani R, Kusakabe T *et al.* Uteroglobin-related protein 1 expression suppresses allergic airway inflammation in mice. *Am J Respir Crit Care Med*. 2006;173:958-964.



Supplemental Figure S1

A



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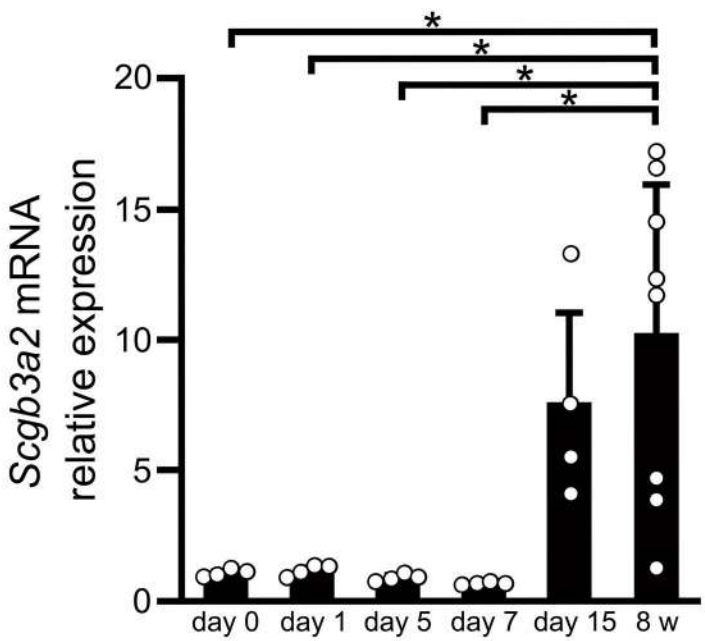

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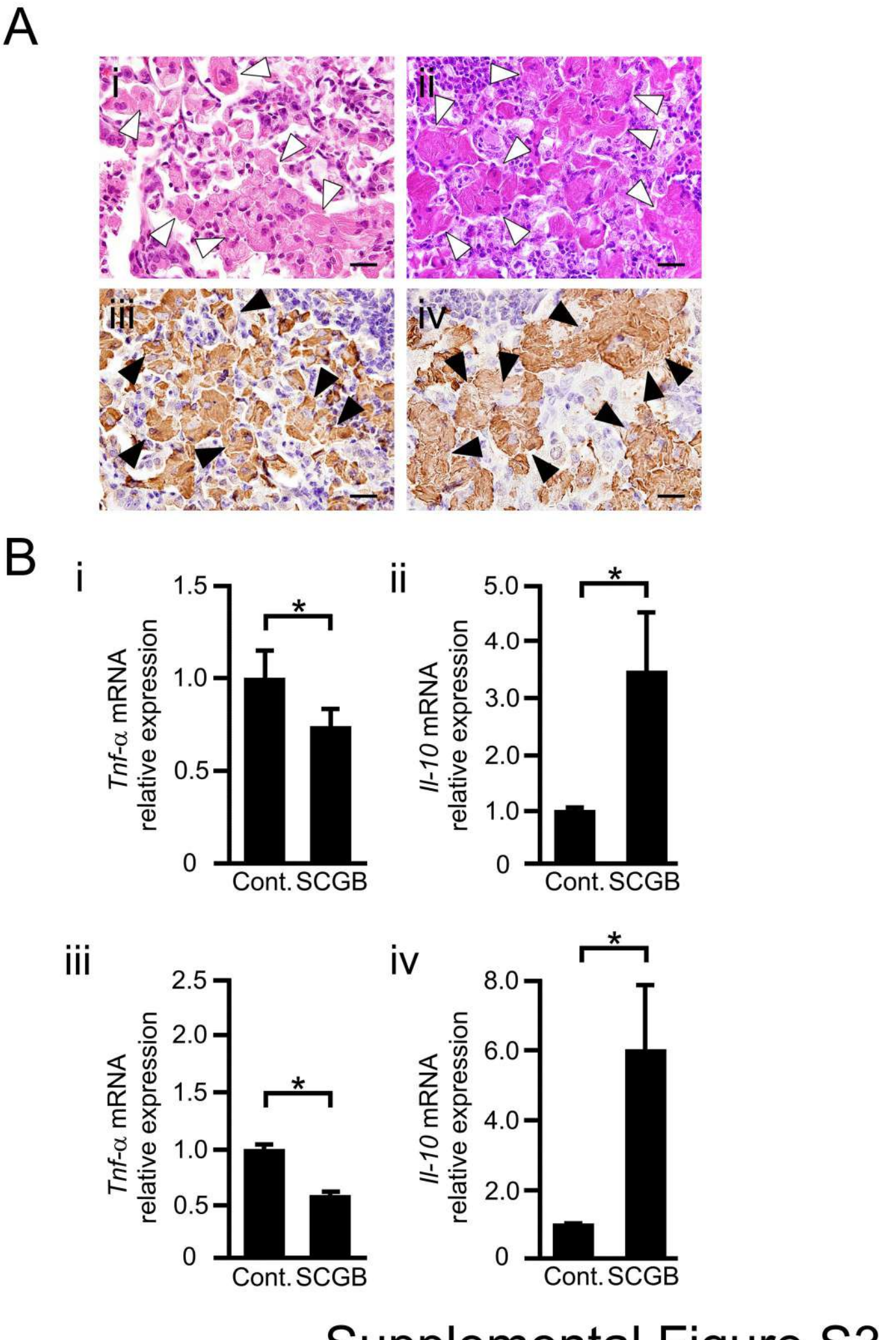

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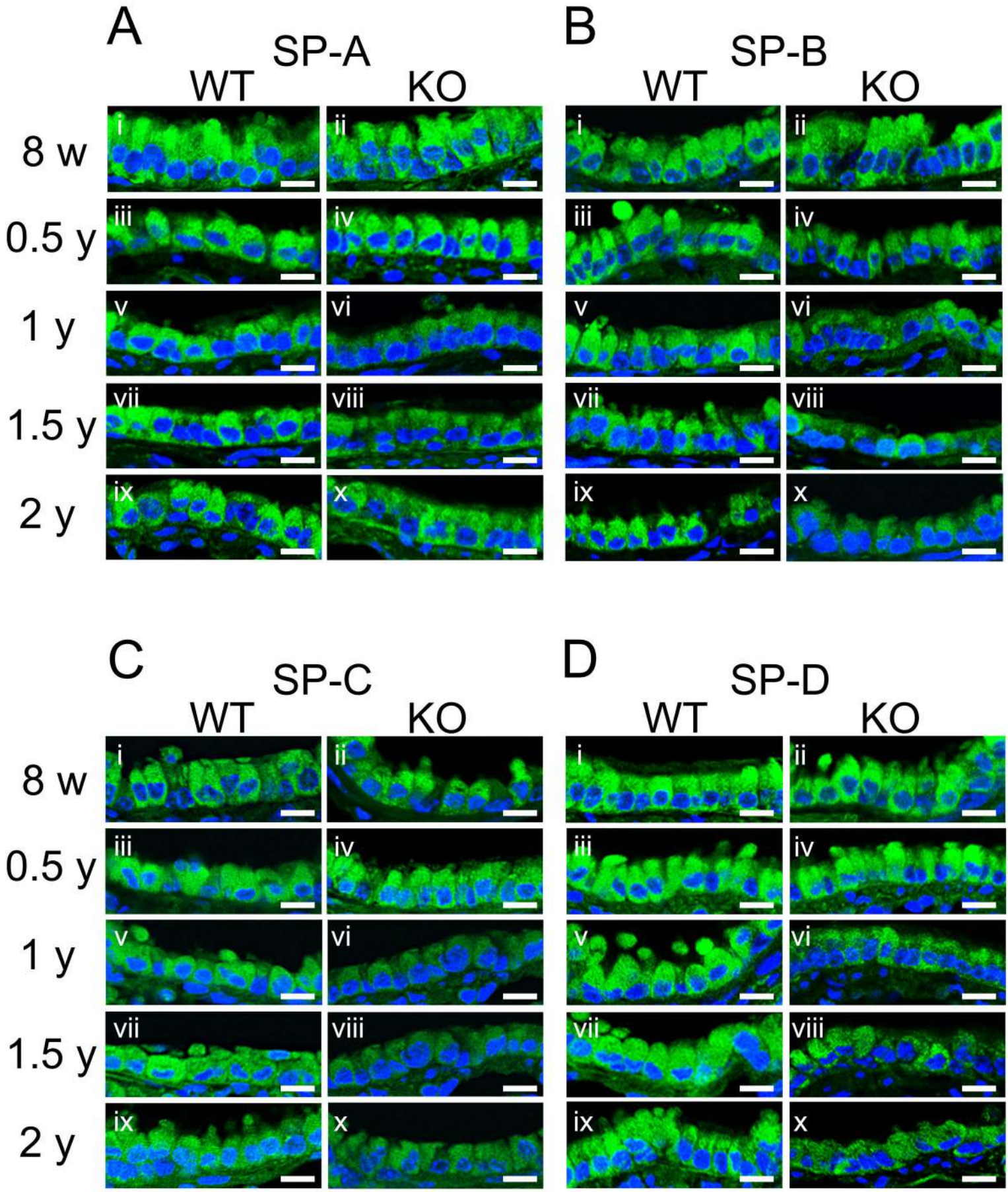

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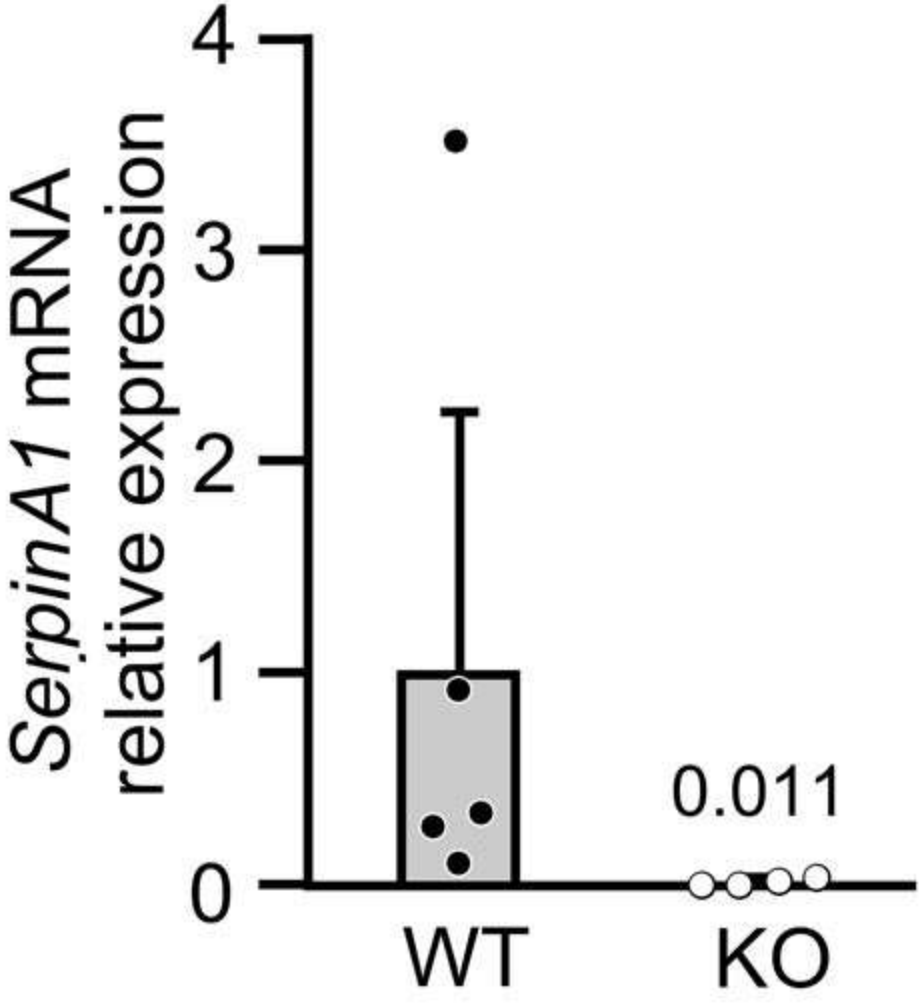

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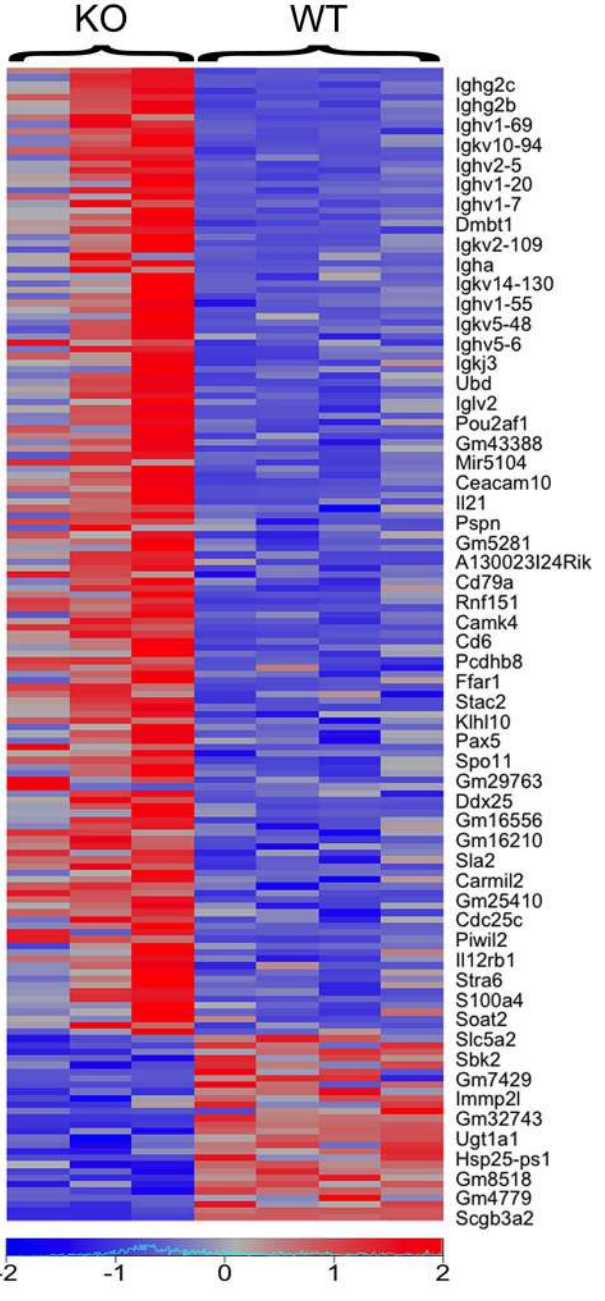

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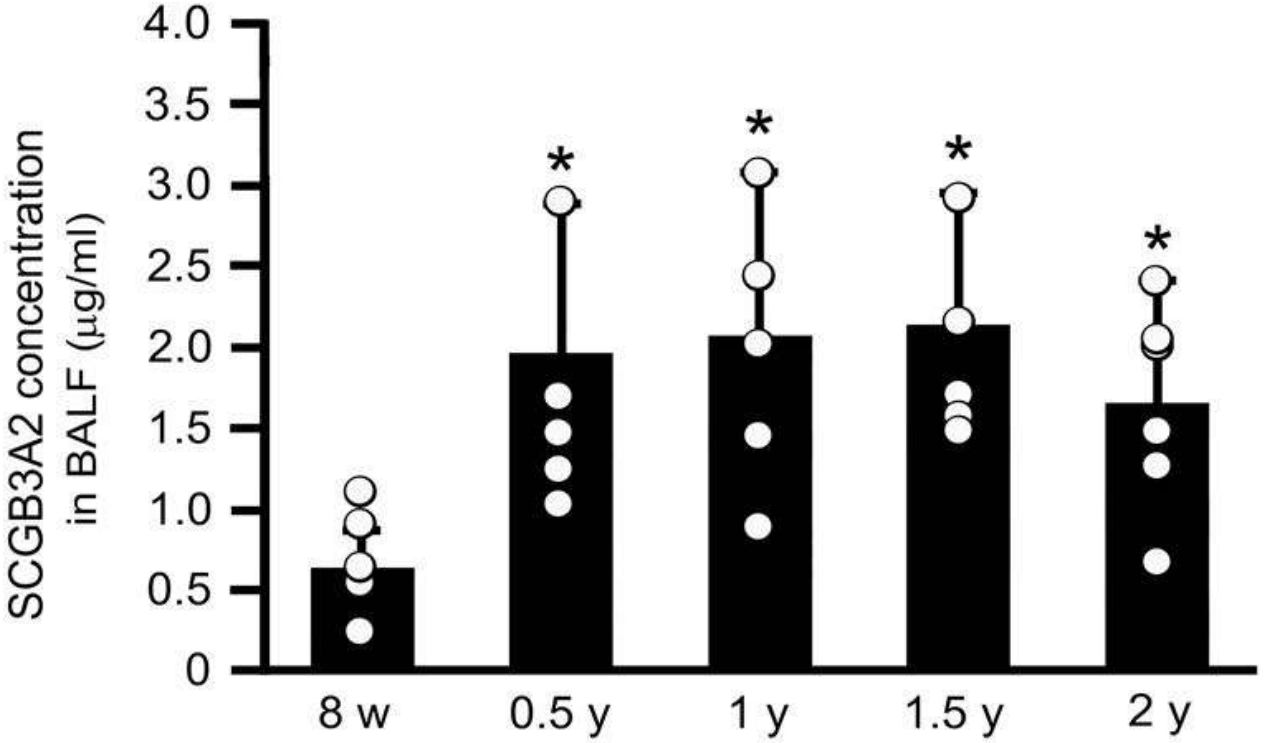
A



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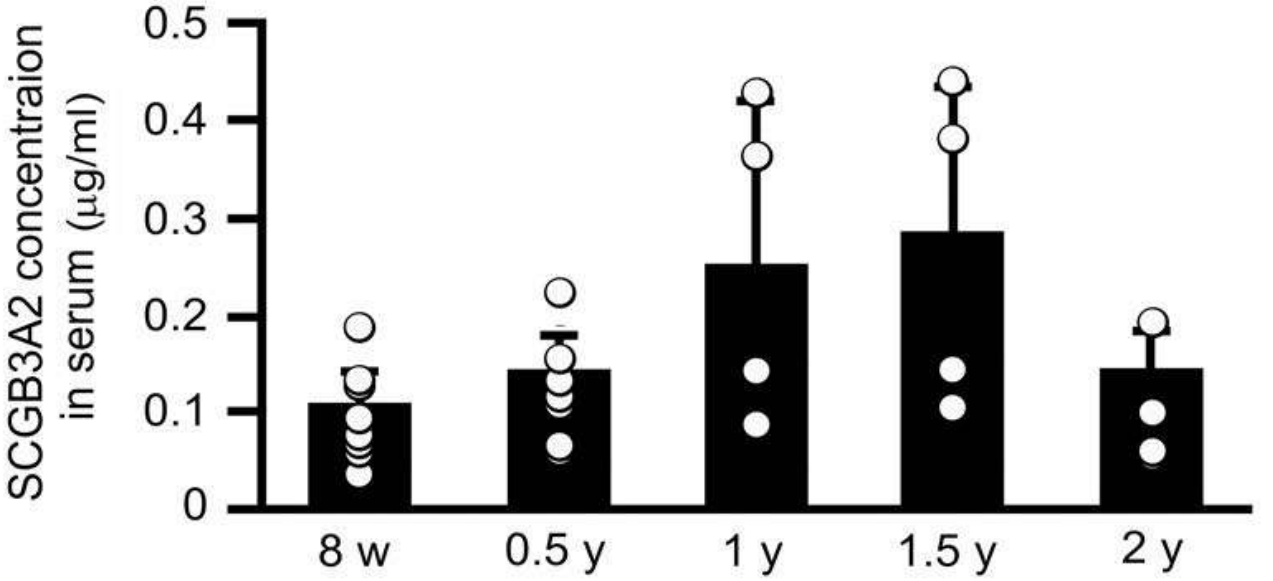
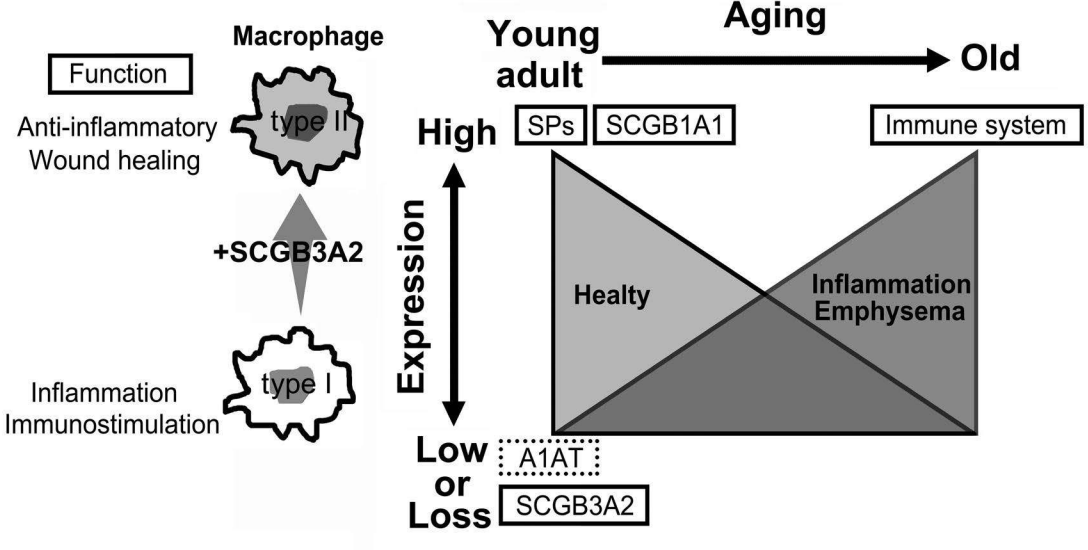


Supplemental Figure S7



Supplemental Figure S8

Supplemental Table S1. Primers used for qRT-PCR

target gene	sequence（5' → 3'）
Scgb1a1	FWD: GCTCAGCTTCTTCGGACATC
	REV: CTCTTGTGGGAGGGTATCCA
Scgb3a2	FWD: GACTGCATTCCAAAGTCCCG
	REV: GAGAAGGGCAGTGGCAGAATAACC
Sftpa	FWD: TAAGAAGCCAGAGAACCAGGTAGG
	REV: CTCAGTGATGTAAAGTGGACGAAGG
Sftpb	FWD: CTGCTTCCTACCCTCTGCTG
	REV: TCCTCACACTCTTGGCACAG
Sftpc	FWD: TAGCCCCGAGTGAGCGAGCA
	REV: GTGGGTGTGGAGGGCTTGGC
Sftpd	FWD: TTTGAGGATGCCCAGGAGATGTGC
	REV: AGGAAAGCAGCCTTGTTGTGG
Tnf-alpha	FWD: ATGAGCACAGAAAGCATGATC
	REV: CAGAGCAATGACTCCAAAGTA
Il-10	FWD: ACAGCCGGGAAGACAATAAC
	REV: TCATTTCCGATAAGGCTTGG
Serpina1a	FWD: TCCTTCCAACACCTCCTCCA
	REV: CCTTGGGTTCCCTTCTCCAC
β-actin	FWD: TGGCACCACACCTTCTACAATGAG
	REV: GGGTCATCTTTTCACGGTTGG

qRT-PCR: quantitative reverse transcription-polymerase chain reaction, *Scgb1a1:* Secretoglobin family 1A member 1*, Scgb3a2:* Secretoglobin family 3A member 2*, Sftpa:* Surfactant protein A*, Sftpb:* Surfactant protein B*, Sftpc:* Surfactant rotein *C, Sftpd:* Surfactant protein D*, Tnf-alfa*: Tumor necrosis factor-alpha, *Il-10:* Interleukin-10, *Serpina1a*: serine (or cysteine) peptidase inhibitor, clade A (Mus musculus), as known as Alpha-1- Antitrypsin, FWD: Forward primer, REV: Reverse primer

Supplemental Table S2. Pathology of lungs of aging mice. Number of positives/number of animals (%)

age	8 w		0.5 y		1 y		1.5 y		2 y	
pathology/genotype	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
normal	3/3 (100)	6/6 (100)	8/8 (100)	4/5 (80)	4/9 (44)	0 (0)	1/5 (20)	0(0)	5/10 (50)	0 (0)
lymphocyte infiltration	0 (0)	0 (0)	0 (0)	1/5 (20)	0 (0)	5/5 (100)	0 (0)	1/10 (10)	0 (0)	0 (0)
lymphocyte aggregates	0 (0)	0 (0)	0 (0)	0 (0)	5/9 (56)[a]	0 (0)	4/5 (80)	9/10 (90)	5/10 (50)	8/9 (89)
Lymphoma	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1/9 (11)[b]
Alveolar hyperplasia, focal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1/5 (20)[c]	0 (0)	1/10 (10)[d]	0 (0)	0 (0)
Macrophage pneumonia	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1/5 (20)[e]	0 (0)	0 (0)	1/9 (11)[f]
Other lesions	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2/10 (20)[g]	0 (0)	2/9 (22)[g]

WT: wild-type, KO: *Secretoglobin* (*Scgb*) *3a2*-knockout, 8 w: 8 weeks old, 0.5 y to 2 y: 0.5 years old to 2 years old

a: Focus of alveolar inflammation

b: Lymphoma found in 1 of 9 mice with lymphocyte aggregation (lymphoma suspected in 2 mice)

c: Tumor or hyperplasia observed in one of the mononuclear infiltrated mice

d: Lymph node plasma cell hyperplasia with lymphoid aggregates

e: Detected in 1 of 5 mice with lymphocyte aggregation

f: Detected in 1 of 9 mice with lymphocyte aggregation

g: Small focus or filtration of alveolar macrophages with lymphoid infiltration or aggregation

Supplemental Table S3. Pathology of spleen of aging mice. Number of positives/number of animals (%)

age	8 w		0.5 y		1 y		1.5 y		2 y	
pathology/genotype	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
normal	3/3 (100)	3/3 (100)	6/8 (75)	4/5(80)	0/9 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/10 (0)	0/8 (0)
white pulp fusion	0 (0)	0 (0)	2/8 (25)	2/5 (40)	9/9 (100)	5/5 (100)	5/5 (100)	5/5 (100)	10/10 (100)	8/8 (100)
Extramedullary hematopoiesis	0 (0)	0 (0)	2/8 (25)	4/5 (80)	1/5 (20)	5/5 (100)	2/5 (40)	5/5(100)	10/10 (100)	8/8 (100)
fibrosis	0 (0)	0 (0)	0 (0)	3/5 (60)	9/9 (100)	5/5 (100)	5/5 (100)	5/5 (100)	10/10 (100)	7/8 (88)
metaplasia in white pulp	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5/5 (100)	0(0)	5/5 (100)	9/10 (90)	8/8 (100)
Other	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)	1/5（20）[a]	0(0)	2/8（25）[b]
erythropoiesis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)	1/5 (20)	10/10 (100)	8/8 (100)

The white pulp fusions began to be observed in both WT and KO spleens at 0.5 years of age. The fusion of WT spleens were found in a small portion of the tissue while that of KO spleens was larger and occupied wider areas of the spleen than WT. Extramedullary hematopoiesis (erythroid element, megakaryocytes and myeloid cells) in the splenic red pulp was observed from 0.5 years of age in both WT and KO, however the prevalence was much higher in KO than WT mice. They became more pronounced at 2 years of age. Fibrosis was observed from 1 year of age in WT and from 0.5 years of age in KO. a: Increase of plasma cells, b: Increase in plasma cells and Russell bodies. WT: wild-type, KO: *Secretoglobin* (*Scgb*) *3a2*-knockout, 8 w: 8 weeks old, 0.5 y to 2y: 0.5 years old to 2 years old