ONLINE SUPPORTING INFORMATION

CD69 Signaling in Eosinophils Induces IL-10 Production and Apoptosis via the Erk1/2 and JNK Pathways, Respectively

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SUPPLEMENTARY METHODS

Lung cell preparation

Mouse lungs were harvested and digested in a collagenase–DNase mixture from the Lung Dissociation Kit (Miltenyi, Bergisch Gladbach, Germany), according to the manufacturer's instructions. A single cell suspension was obtained by passing the lung cells through a 70-μm cell strainer (Thermo Fisher Scientific, MA, USA).

Eosinophil purification

Splenocytes were incubated with biotin-conjugated anti-CD19, anti-CD90.2, and anti-CD8α antibodies (BD Pharmingen, CA, USA), followed by incubation with anti-biotin–conjugated magnetic beads (Miltenyi, Bergisch Gladbach, Germany). Purity and survival rates were greater than 90%. The FACSAria III (BD Biosciences, NJ, USA) cell sorter was used to isolate eosinophils for quantitative polymerase chain reaction examination. Eosinophils were gated as CD19-CD90.2-Gr-1intSiglec-F+, and the purity of eosinophils was >99%.

RNA isolation

RNA from purified eosinophils was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany). PrimeScript RT Master Mix (Takara, Shiga, Japan) was used to reverse transcribe the extracted RNA to the first-strand cDNA.

Flow cytometric analysis and $CD69^+$-Eosinophils sorting

To analyze CD69 expression in ovalbumin (OVA)-induced asthma mice, single-cell suspension from lung tissues was stained with the monoclonal antibodies CD45, CD11b, Gr-1, Siglec-F (BD Biosciences, NJ, USA), and CD69 (Miltenyi, Bergisch Gladbach, Germany) and/or corresponding isotype IgG for 30 min at 4°C. FACS Canto II flow cytometer and Aria III (BD Biosciences, NJ, USA) were used to measure the samples. $CD69^+$-Eosinophils from healthy and asthmatic mouse lung and spleen were sorted by identification as $CD45^+CD11b^{Hi}Gr\text{-}1^{Int}Siglec\text{-}F^{Hi}CD69^+$and $CD90.2^-CD19^-Gr\text{-}1^{Int}Siglec\text{-}F^{Hi}CD69^+$, respectively. FlowJo software (BD Biosciences, NJ, USA) was used to analyze the data obtained.

Immunofluorescence staining

Section samples from purified eosinophils or frozen lung tissues were incubated with anti-mouse SiglecF (1 μg/mL; eBioscience, CA, USA), followed by anti-mouse CD69 (2.5 μg/mL; Santa Cruz Biotechnology, TX, USA) overnight; thereafter, the samples were incubated with 1/200 secondary antibodies for 1 h at room temperature (RT). Hoechst (2.0 μg/mL; Dojindo, MD, USA) was used to perform counterstaining for 10 min at RT. The FV3000 confocal microscopes (Olympus, Tokyo, Japan) were used to measure these section samples, and FV10-ASW FluoView software (Olympus, Tokyo, Japan) was used to analyze them.

Western blot

M-PER lysing buffer (Thermo Fisher Scientific, MA, USA) containing phosphatase/protease inhibitors (Cell Signaling Technology, MA, USA) was used to prepare the whole-cell protein extracts. A total of 25-μg protein was loaded onto 7.5% polyacrylamide gels (Bio-Rad, CA, USA) and then transferred to polyvinylidene fluoride membrane (Bio-Rad, CA, USA). Membranes were blocked using the Intercept Blocking Buffer (LI-COR, NE, USA) for 1 h at RT and stained with antibodies for total or phosphorylated Erk1/2, STAT5, Jak3, JNK (Cell Signaling Technology, MA, USA), and β-actin (Santa Cruz Biotechnology, TX, USA) overnight at 4°C. Odyssey software 3.0 (LI-COR, NE, USA) was used to quantify the proteins, and Image Studio Lite software 5.2.5 (LI-COR, NE, USA) was used to analyze the images.

SUPPLEMENTARY TABLE

Table S1. Primer List

	Forward	Reverse
GAPDH	5′-TGCCCAGAACATCATCCCTG-3′	5′-TCAGATCCACGACGGACACA-3′
GATA3	5′-GCCTGCGGACTCTACCATAA-3′	5′-CATTAGCGTTCCTCCTCCAG-3′
IFNγ	5′-ACTCAAGTGGCATAGATGTGG-3′	5′-AAGACTTCAAAGAGTCTGAGGTAG -3′
IL-4	5′-ACACCACAGAGAGTGAGCTCG-3′	5′-TGCAGCTCCATGAGAACACTAG-3′
IL-10	5′-ATTTGAATTCCCTGGGTGAGAAG-3′	5′-CACAGGGGAGAAATCGATGACA-3′
IL-13	5′-AAAACTGCAGCAAGACCGTG-3′	5′-CCAGAGCCCACTGCTTCAAT-3′
IL-17A	5′-TACAGTGAAGGCAGCAGCGATC-3′	5′-ACATTCTGGAGGAAGTCCTTGG-3′
RORγ	5′-ACTGAGGCCATTCAGTATGTGG-3′	5′-TGCACATTCTGACTAGGACGAC-3′
T-bet	5′-CCCACAAGCCATTACAGGATGT-3′	5′-TGCCTTCTGCCTTTCCACAC-3′
TGF-β	5′-ACCATCCATGACATGAACCGG-3′	5′-AGCAGTTCTTCTCTGTGGAGC-3′

SUPPLEMENTARY FIGURES

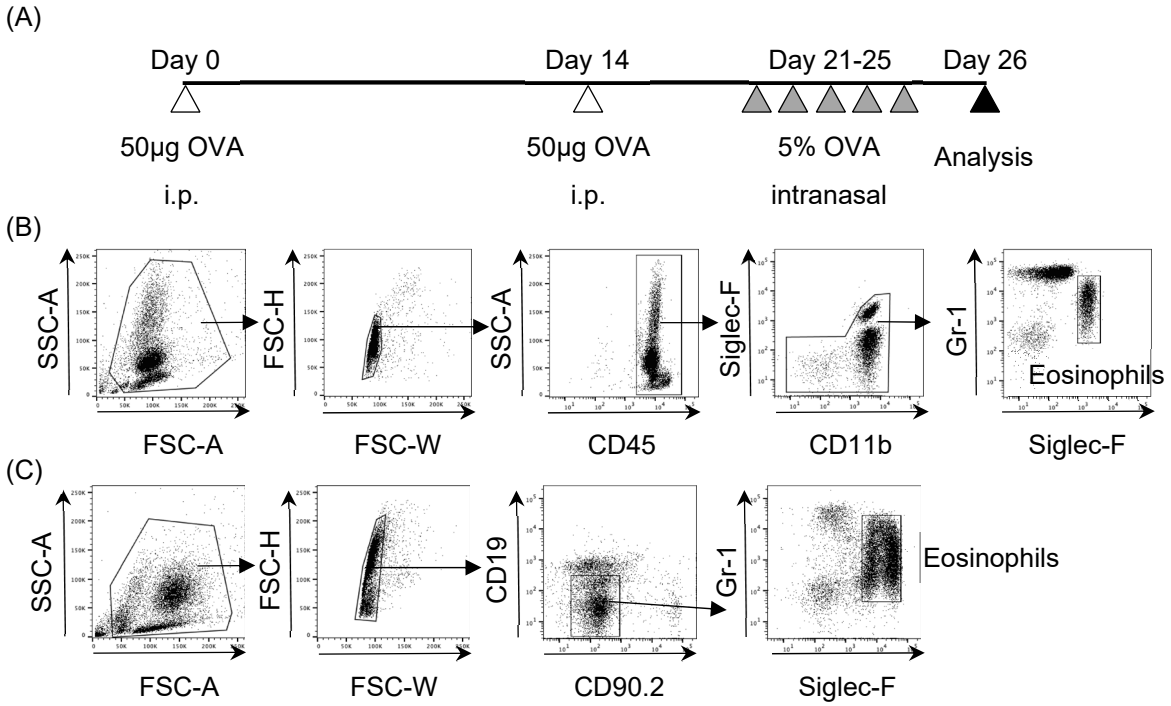

Figure S1. Schema of experimental protocol and eosinophil isolation

(A) Experimental design of OVA-induced asthma mice model. (B) and (C) Eosinophil gate strategy using flow cytometry analysis. Eosinophils from healthy and asthmatic mouse lung and spleen were identified as $CD45^{+}CD11b^{Hi}Gr\text{-}1^{Int}Siglec\text{-}F^{Hi}$ and $CD90.2^{-}CD19^{-}Gr\text{-}1^{Int}Siglec\text{-}F^{Hi}$, respectively. i.p.; indicates intraperitoneal.

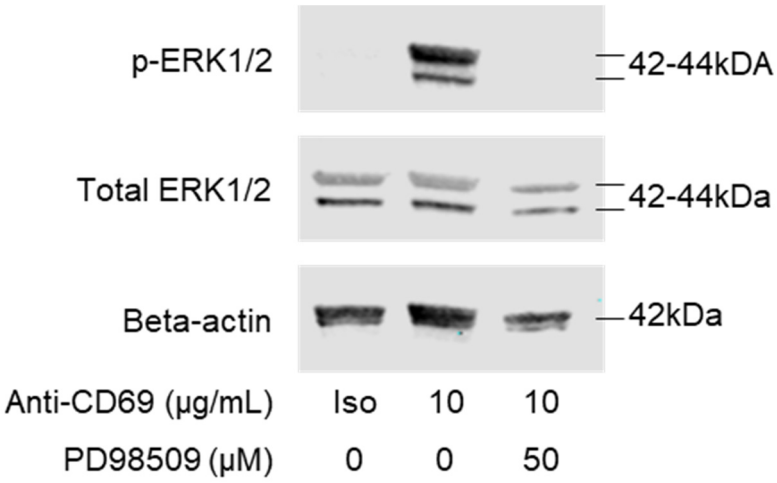

Figure S2. Phosphorylation of Erk1/2 following CD69 crosslinking on eosinophils in the presence of PD98509

Western blot analysis for phosphorylated and total Erk1/2 was shown. PBS or 50 μM PD98509 was incubated for 1 h prior to CD69 crosslinking on eosinophils purified from IL-5Tg mice for 30 min.

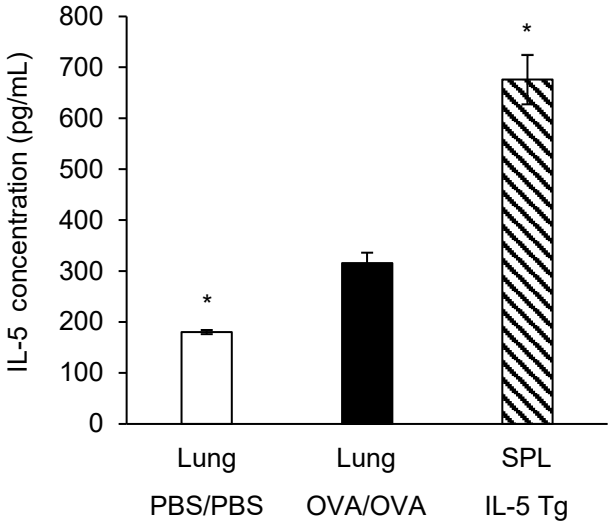

Figure S3. IL-5 protein in the supernatant

The IL-5 concentration in supernatants obtained from lung cells of healthy mice (PBS/PBS), lung cells of OVA-induced asthma mice (OVA/OVA), and splenocytes (2 million cells/250 μL/well) from IL-5Tg mice for 24 h incubation was measured using an ELISA kit. Data are expressed as mean ± SEM (n = 6 for each group). * indicates a significant difference compared with healthy mice ($p < 0.05$). SPL; spleen.