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This information is current as of July 30, 2015.

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J Immunol 2015; 195:1273-1281; Prepublished online 26 June 2015;
doi: 10.4049/jimmunol.1500531
<http://www.jimmunol.org/content/195/3/1273>

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Spred1, a Suppressor of the Ras–ERK Pathway, Negatively Regulates Expansion and Function of Group 2 Innate Lymphoid Cells

Mayu Suzuki,^{*,†} Rimpei Morita,^{*,†} Yasuko Hirata,^{*,†} Takashi Shichita,^{*,‡} and Akihiko Yoshimura^{*,†}

Cytokines from group 2 innate lymphoid cells (ILC2s) have been implicated in acute allergic responses, such as papain-induced lung inflammation. However, the means of homeostatic regulation of ILC2s have not been established. In this study, we demonstrated that *Spred1*, a negative regulator of the Ras–ERK pathway, plays an important role in the proliferation and apoptosis of ILC2s and in cytokine secretion from ILC2s. Intranasal administration of papain stimulated IL-5 and IL-13 production in the lung, which was enhanced when *Spred1* was deleted. In vitro, *Spred1*^{−/−} ILC2s proliferated faster than wild type ILC2s did and produced higher levels of cytokines in response to IL-33. On the contrary, a MEK inhibitor suppressed ILC2 proliferation and cytokine production. *Spred1* deficiency resulted in stabilization of GATA3, which has been shown to play essential roles in the maintenance and cytokine production of ILC2. These data suggest that *Spred1* negatively regulates ILC2 development and functions through the suppression of the Ras–ERK pathway. *The Journal of Immunology*, 2015, 195: 1273–1281.

Allergic asthma, the most common form of asthma, is a chronic inflammatory disease characterized by increased levels of Th2 cell–type (type 2) cytokines, including IL-4, IL-5, IL-9, and IL-13, in the lungs (1). IL-4 is necessary for class switching and the production of IgE from B cells, IL-5 induces eosinophilia, and IL-13 plays an important role in the effector phase of asthma by inducing airway remodeling, hyperresponsiveness, and mucus production. Th2 cells have been implicated as a possible source of these type 2 cytokines, but the recent discovery of innate lymphoid cells (ILCs) raises the possibility that group 2 ILCs (ILC2s) may in fact be innate sources of type 2 cytokines, especially IL-5 and IL-13 (2–4). For example, papain, a plant-derived cysteine protease that is known to cause occupational asthma (5), was shown to induce asthmatic airway inflammation in *Rag*-deficient mice (6), and ILC2s were subsequently demonstrated to play a critical role in this acute inflammation model (7). ILC2-derived IL-13 has been

shown to be critical in that it promotes the migration of activated lung dendritic cells into the draining lymph nodes to induce differentiation of Th2 cells, suggesting that ILC2s are also involved in type 2 adaptive immune responses (8).

ILC2s produce IL-5 and IL-13 in a process dependent on GATA3, but not RORα and RORγt (9). ILC2s are negative for lineage markers, although they express CD25, c-Kit (CD117), Sca-1, ST2 (IL-33 receptor), Thy-1 (CD90), and IL-7Rα (CD127) (7). Consistent with their expression of IL-17RB (IL-17B and IL-25 receptor) and ST2 (IL-33 receptor component), ILC2s produce cytokines in response to IL-25 and IL-33 (2). Lung-resident ILC2s have also been shown to contribute to airway hyperreactivity induced by virus or allergen challenge (10–14). Unlike gut ILC2s (2), lung ILC2s critically require IL-33 to produce type 2 cytokines in vitro (7). Because intranasal administration of papain fails to induce lung inflammation in *Il33*^{−/−} mice (6), IL-33 is also important in ILC2 activation in vivo.

IL-33 is a member of the IL-1 cytokine family, along with IL-1α, IL-1β, and IL-18 (15). It consists of an IL-1 receptor accessory protein and ST2 (16). IL-1 receptor family signaling activates the transcription factor NF-κB and the mitogen-activated protein MAPKs p38, JNK, and ERK1/2 (17). In the case of ILC2 activation by IL-33, p38 inhibitor has been reported to suppress IL-33–induced production of IL-5 and IL-13, although JNK and NF-κB inhibitors do not; this is because p38-mediated phosphorylation of the transcription factor GATA3 is critical for cytokine production from ILC2s (9). However, the roles of ERK1/2 in ILC2s have not been examined.

We have discovered a family of negative regulators of growth factor– and cytokine-induced ERK activation, which we have called Sprouty-related Ena/VASP homology 1 domain-containing proteins (*Spred*) (18). *Spred1* downregulates the ERK signaling pathway through recruiting the Ras–GAP neurofibromin–neurofibromatosis type 1 to the membrane (19), and mutations in human *SPRED1* have been discovered in Legius syndrome, which resembles a mild neurofibromatosis type 1 syndrome (20, 21). We previously reported that *Spred1*^{−/−} mice are more sensitive to a chronic OVA-induced asthma model (22). The levels of IL-5 and

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Received for publication March 6, 2015. Accepted for publication June 2, 2015.

This work was supported by a Grant-in-Aid for Scientific Research (S) (2522105) from Japan Society for the Promotion of Science and an Intramural Research Grant (22-4) for Neurological and Psychiatric Disorders of National Center Hospital of Neurology and Psychiatry from the Uehara Memorial Foundation (to A.Y.), and by Precursory Research for Embryonic Science and Technology, the SENSHIN Research Foundation, the Kanae Foundation for the Promotion of Medical Science, and the Takeda Science Foundation (to T.S.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AD, atopic dermatitis; BALF, bronchoalveolar lavage fluid; BM, bone marrow; CLP, common lymphoid progenitor; ILC, innate lymphoid cell; ILC2, group 2 innate lymphoid cell; MEF, mouse embryonic fibroblast; PAS, periodic acid–Schiff; PI, propidium iodide; *Spred*, Sprouty-related Ena/VASP homology 1 domain-containing protein; ST2-MEF, MEF stably expressing ST2; WT, wild type.

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IL-13 were increased in the lungs of OVA-immunized *Spred1*-deficient mice, but the source of these cytokines has not yet been identified, because the differentiation of *Spred1*^{-/-} T cells was normal.

In this study, we demonstrate that *Spred1* negatively regulates papain-induced lung inflammation via inhibition of the IL-33-induced activation of ERK signaling pathway. *Spred1* suppresses both secretions of type 2 cytokines from ILC2s and proliferation apoptosis of ILC2s via the stabilization of GATA3. *Spred1* was highly expressed in lung ILC2s, and *Spred1* levels were increased in response to IL-33, suggesting that *Spred1* is involved in a negative feedback loop affecting the IL-33-activated ERK signaling pathway. The *Spred1*-ERK pathway is shown to be a novel therapeutic target for airway inflammation associated with asthma.

Materials and Methods

Mice

Spred1^{-/-} mice have been described previously (22). C57BL/6J mice were purchased from Tokyo Laboratory Animals Science (Tokyo, Japan). Mice were kept in specific pathogen-free facilities at Keio University. All experiments using mice were approved by the Animal Ethics Committee of Keio University and performed according to the Animal Ethics Committee's guidelines.

Cell lines

HEK293T cells and mouse embryonic fibroblasts (MEFs) were cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine.

Airway inflammation

For papain-induced acute lung inflammation, mice were treated intranasally with 30 µg papain in 40 µl PBS or PBS alone on days 0, 3, and 6. For histopathologic examination, lungs were fixed in 4% paraformaldehyde. Fixed lungs were embedded in paraffin, cut into 5-µm sections, and stained with periodic acid-Schiff (PAS) for goblet cell hyperplasia analysis as described previously (23). For bronchoalveolar lavage fluid (BALF) or whole-lung analysis, mice were treated intranasally with 40 µg papain in 20 µl PBS or PBS alone for three consecutive days. At 24 h after the last administration, mice were sacrificed and BALF (in 1 ml PBS) or lungs were collected. Cytokines in BALF were measured with ELISA according to the manufacturer's protocol (eBioscience, San Diego, CA).

Abs and reagents

FITC-conjugated anti-CD11b (M1/70), CD3e (145-2C11), Gr-1 (RB6-8C5), B220 (RA3-6B2), NK1.1 (PK136), c-Kit (2B8), PE-conjugated anti-Sca-1 (D7), PerCP-Cyanine5.5-conjugated anti-CD25 (PC61.5), PE-Cyanine7-conjugated streptavidin, APC-conjugated anti-CD11b (M1/70), CD127 (A7R34), biotin-conjugated anti-CD127 (A7R34), Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (N418), TER-119 (TER-119), and NK1.1 (PK136) were purchased from eBioscience. FITC-conjugated anti-TER-119 (TER-119), PE-conjugated anti-CD4 (RM4-5), PerCP-Cyanine5.5-conjugated streptavidin, APC-conjugated streptavidin, anti-c-Kit (2B8), human CD2 (LFA-2), biotin-conjugated anti-CD3e (145-2C11), and B220 (RA3-6B2) were purchased from BioLegend (San Diego, CA). PerCP-conjugated anti-Ki-67 (M-19) mouse mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PE-conjugated anti-Siglec-F (E50-2440) was purchased from BD Pharmingen (Franklin Lakes, NJ). FITC-conjugated anti-T1/ST2 (DJ8) was purchased from MD Bioproducts (Zurich, Switzerland). Anti-phospho-p44/42 MAPK (E10) mouse mAb, and anti-p44/42 MAPK (137F5) rabbit mAb were purchased from Cell Signaling Technology (Danvers, MA). Anti-α-Tubulin (DM1A) mouse mAb was purchased from Sigma-Aldrich (St. Louis, MO). Anti-GATA3 rabbit polyclonal Ab (ab106625) and Histone H3 rabbit polyclonal Ab (ab1791) were purchased from Abcam (Cambridge, U.K.). Recombinant mIL-2, mIL-4, mIL-7, and mIL-33 were purchased from PeproTech (Rocky Hill, NJ). Recombinant mTSLP was purchased from eBioscience. Recombinant mIL-25 was purchased from BioLegend. IFN-γ neutralizing Ab (R4-6A2), anti-CD3e (2C11), and anti-CD28 (57.31) Abs were produced in house. Papain was purchased from Wako Pure Chemical Industries (Osaka, Japan). U0126 was purchased from Promega (Madison, WI). SP600125, SB203580, and MG-132 were purchased from Calbiochem (San Diego, CA).

Cell preparation

Lung cells were prepared according to the modified method of Veinotte et al. (24). In brief, lungs from mice were incubated in HBSS(+) containing 1% BSA, 1 mg/ml Collagenase D, and 1 mg/ml DNaseI (Roche, Basel, Switzerland) for 40 min at 37°C. Digested lungs were passed through a 100-µm strainer and washed. Lymphocytes were isolated using Percoll density (44 and 67%; GE Healthcare, Chalfont St. Giles, U.K.) centrifugation at 2000 rpm for 30 min, after which cells in the interface were collected and washed. Bone marrow (BM) cells were collected from femora and tibiae. After lysis of RBCs, each cell was used for sorting or analysis by flow cytometry.

Th2 preparation and in vitro differentiation

Naive CD4⁺ T cells were prepared from the spleens of IL-4 hCD2 BAC Tg mice and cultured in Th2 cell culture conditions as described previously (23).

Flow cytometry sorting and analysis

All cells were incubated with 2.4G2 to block Fc receptors before staining. Lung ILC2s and BM-derived immature ILC2s were stained with Abs of lineage markers (CD3, CD11b, Gr-1, B220, NK1.1, and Ter-119), Sca-1, CD25, CD127, and c-Kit. For isolation of common lymphoid progenitors (CLPs), prepared BM cells were stained with Abs of lineage markers (CD3, CD11b, CD11c, Gr-1, B220, NK1.1, and Ter-119), Sca-1, CD127, and c-Kit. For isolation of Th2 cells, cultured CD4⁺ T cells were stained with Abs of CD4 and hCD2. For detection of eosinophils, BALF cells were stained with Abs of CD11b and Siglec-F. For analysis of apoptotic cells, isolated and cultured BMILC2s were stained using Annexin V-Biotin Apoptosis Detection Kit (BioVision, Milpitas, CA) according to the manufacturer's protocol. For analysis of proliferating cells, isolated and cultured BMILC2s were stained using a BrdU Flow Kit (BD Pharmingen) according to the manufacturer's protocol. BD FACS Aria II was used for cell sorting and phenotypic analysis, BD Canto II was used for phenotypic analysis, and FlowJo × 10.0.7r2 was used for data analysis.

Culture of lung ILC2s and BMILC2s

Sorted lung ILC2s were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM glutamine, 0.05 mM 2-ME, 100 ng/ml mIL-33, 50 ng/ml mIL-2, and 10 ng/ml mIL-7. To generate BMILC2, sorted immature NH cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM glutamine, 0.05 mM 2-ME, 10 ng/ml mIL-33, and 10 ng/ml mTSLP, and half of the medium was replaced every 2–3 d for 1 wk.

Immunoblotting

Nuclear extracts were prepared according to the method described by Chan et al. (25), with a slight modification. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were blocked and probed with the primary Abs. The membranes were then probed with HRP-conjugated anti-mouse IgG or anti-rabbit IgG as secondary Abs and visualized using Chemi-Lumi One L or Chemi-Lumi One Super Western-blotting detection reagents (Nacalai Tesque, Kyoto, Japan) and LAS-3000mini (Fujifilm, Tokyo, Japan).

Lung explant culture

Lungs were perfused with HBSS(+) and cut into small pieces. The lung explants from each animal were divided into six parts that were cultured separately in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM glutamine, and 0.05 mM 2-ME and pretreated with the indicated dose of U0126 for 1 h prior to stimulation with 10 µg/ml papain for 18 h.

Retrovirus construction and transduction

Full-length ST2L containing the C-terminal FLAG expression vector of MSCV-IRES-Puro (MSCV-Puro-ST2L-c-Flag) was provided by Dr. M. Tago (26). MSCV-Puro-ST2L-c-Flag vectors and retrovirus packaging vectors were cotransfected into HEK293T cells according to the polyethylenimine method to obtain the virus. MEFs were infected with the virus containing 8 µg/ml polybrene.

RNA isolation and real-time PCR

Total RNA was extracted using RNAiso Plus (Takara Bio, Otsu, Japan) or RNeasy Plus Micro (Qiagen, Hilden, Germany) and subjected to reverse

transcription using a High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA). PCR analysis was performed using an iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA) and SsoFast EvaGreen Supermix (Bio-Rad). All primer sets yielded a single product of the correct size. The following primer sets were used for real-time PCR: *spred1* (5'-GGCCTAGACATTCAGAGCCG-3' and 5'-TAGTCTCATCCCCACAGTGG-3'); *gapdh* (5'-GCCTCGTCCCGTAGAC-3' and 5'-GGCAACAATCTCCACT-3'); *actb* (5'-TCACCCACACTGTGCCCATCTACG-3' and 5'-GAGCCTCAGGGCA-TCGGAACCGCTC); *gata3* (5'-CTGGAGGAGGAACGCTAATG-3' and 5'-AGTAGAAGGGTTCGAGGAA-3'); *cdkn2b* (5'-CGCGGTGTAAAATAGCAAGG-3' and 5'-TTTCAGTTCACAGGGGAAGG-3'); and *tnfrsf21* (5'-TG-TGTGTCAGTATCCGAAAG-3' and 5'-AGATCCATTCTCCCGTTC-3') (Eurofins Genomics, Luxembourg).

Statistics

For comparison between two groups, significance levels were determined with paired, two-tailed Student *t* test; *p* < 0.05 was considered statistically significant.

Results

Spred1-deficiency exacerbates allergen-induced lung inflammation

We previously reported that *Spred1*-deficient mice were more sensitive than wild type (WT) mice to OVA-induced airway inflammation (22). Although *Spred1* had little effect on Th2 cell differentiation, IL-5 and IL-13 levels were higher in *Spred1*^{-/-} mice than in WT mice. Accordingly, we suspected that *Spred1*

regulates ILC2s. To examine this possibility, we used the papain-induced lung inflammation model, consisting of an acute inflammation depending on ILC2s (7). Intranasal administration of papain resulted in the accumulation of the type 2 cytokines IL-5 and IL-13 in BALF (Fig. 1A). These cytokine levels were significantly elevated in papain-treated *Spred1*^{-/-} mice compared with WT mice (Fig. 1A). In addition, Siglec-F⁺CD11b⁺ eosinophils were significantly increased in the BALF of papain-treated *Spred1*^{-/-} mice compared with WT mice (Fig. 1B). PAS staining also revealed increased mucus production by the airway goblet cells of papain-treated *Spred1*^{-/-} mice compared with those of WT mice (Fig. 1C). These results indicate that *Spred1*^{-/-} mice produced higher levels of type 2 cytokines and were more sensitive than WT mice to papain-induced lung inflammation.

Next, to investigate whether ILC2 levels are regulated by *Spred1*, we performed flow cytometric analysis of ILC2 fractions using whole-lung mononuclear cells. As shown in Fig. 1D, the Lin⁻Sca1⁺c-Kit⁺CD127⁺CD25⁺ ILC2 fraction was markedly elevated in lung tissue from papain-stimulated *Spred1*^{-/-} mice compared with WT mice, although no significant differences between the genotypes were observed when tissues were treated with PBS as a control. We also examined a more robust Ag-driven model of asthma using a house dust mite Ag as described previously (27). Similar results were observed in mice stimulated with house dust mite extract (Supplemental Fig. 1A, 1B). These data

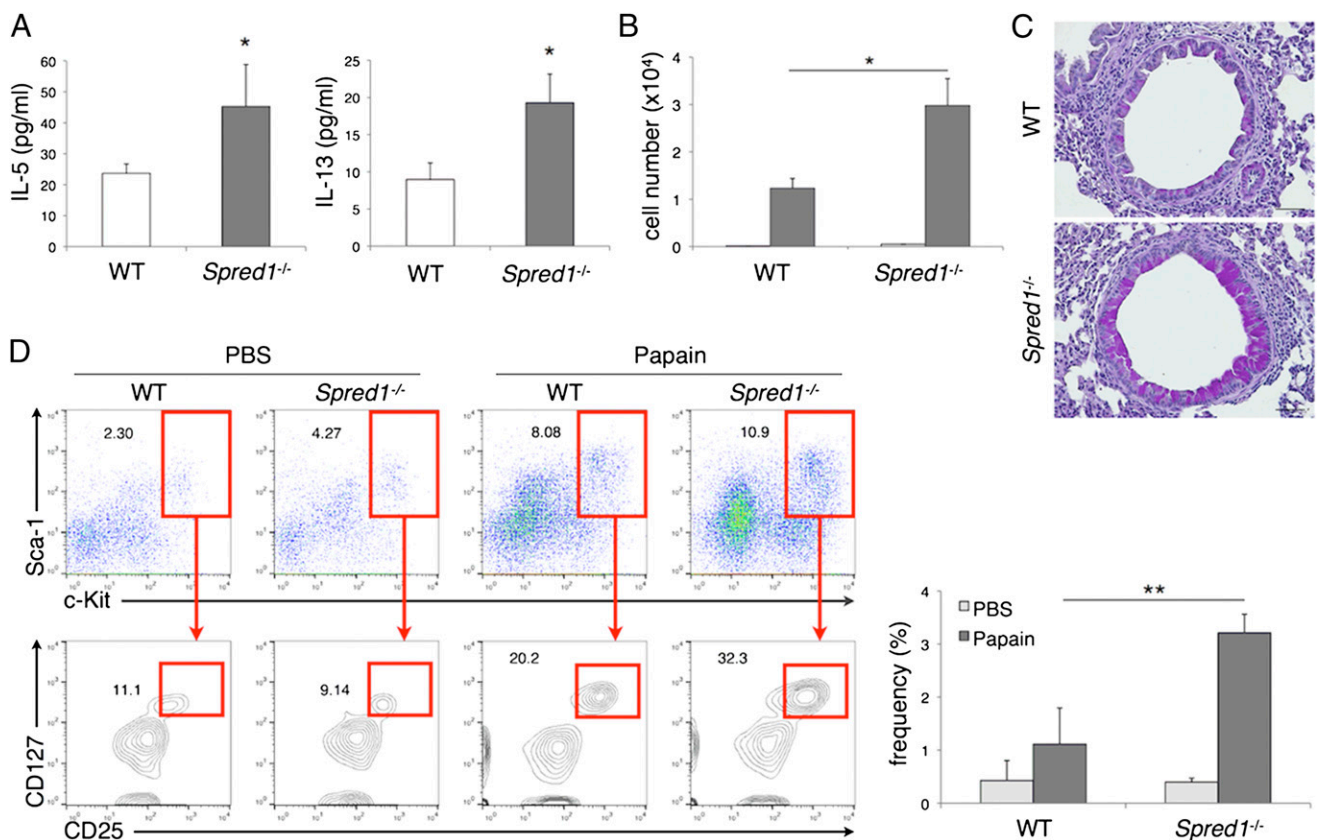


FIGURE 1. Papain-induced lung inflammation is exacerbated in *Spred1*-deficient mice. (A) WT or *Spred1*^{-/-} mice were treated with papain for three consecutive days; 24 h after the last administration, IL-5 and IL-13 in BALF were analyzed with ELISA. (B) WT or *Spred1*^{-/-} mice were treated with PBS (white bar) or papain (gray bar) for three consecutive days; 24 h after the last administration, eosinophils in BALF were counted with flow cytometry. (C) Mucus secretion in the lungs was analyzed using PAS staining of papain-treated WT or *Spred1*^{-/-} mice. Scale bars, 50 μ m. (D) WT or *Spred1*^{-/-} mice were treated with PBS or papain for three consecutive days; 24 h after the last administration, whole lung cells from these mice were counted with flow cytometry. Lin⁻ cells were gated for the analysis of c-Kit and Sca-1 expression. Lin⁻Sca1⁺c-Kit⁺ cells were gated as indicated by red boxes (top) and further analyzed for CD25 and CD127 expression (bottom). The numbers in the plots indicate the percentages of cells in the gates. The graph shows the cell frequencies of ILC2s in Lin⁻ cells. Data are representative of at least three independent experiments. Error bars show SD. **p* < 0.05, ***p* < 0.01.

suggest that allergen treatment enhances ILC2 accumulation in the lungs of *Spred1*^{-/-} mice, resulting in increased IL-5 and IL-13 levels in the BALF of *Spred1*^{-/-} mice.

Spred1 expression is induced by IL-33 in ILC2s

To define the role of Spred1 in ILC2 activation, we first compared the expression pattern of *Spred1* in T cells with that in ILC2s. *Spred1* mRNA levels were markedly higher in lung ILC2s than in in vitro differentiated CD4⁺IL-4⁺ Th2 cells (see *Materials and Methods*) or Lin⁻CD127⁺ Sca-1^{int}c-Kit^{int} CLPs in BM, which are common progenitors of Th2 cells and ILC2s (Fig. 2A). This suggests that Spred1 plays a more important role in ILC2s than in T cells. Consistent with the low levels of Spred1 expression in CLPs, there were no significant differences in the frequencies of Lin⁻Sca-1⁺c-Kit⁻CD25⁺CD127⁺ immature ILC2s in BM (28) between WT and *Spred1*^{-/-} mice (Fig. 2B). It has been reported that IL-33 is required for papain-induced airway inflammation and ILC2 activation (7). Consistently, ILC2s isolated from lung tissues secreted IL-5 and IL-13 in response to IL-33, but not in response to IL-25 or TSLP (Fig. 2C). We also compared expression levels of the IL-33 receptor ST2 in Th2 cells and lung ILC2s (Fig. 2D). Although the expression of ST2 in Th2 cells was modest, that in ILC2s was much higher. IL-33 strongly enhanced *Spred1* mRNA levels in lung ILC2s in vitro (Fig. 2E). A similar IL-33-mediated induction of *Spred1* expression was observed in MEFs stably expressing ST2 (ST2-MEFs; Fig. 2F). These data support our

hypothesis that Spred1 is a negative feedback regulator of IL-33 signaling.

IL-33-mediated ERK activation is inhibited by Spred1 and is important in ILC2 function

Halim et al. (7) have demonstrated that papain-induced IL-5 and IL-13 secretion is significantly suppressed by anti-IL-33 Ab, indicating that the IL-33 released by papain stimulation is involved in IL-5 and IL-13 secretion. Furthermore, in *Rag2*^{-/-} *Il2rg*^{-/-} mice, which have no ILC2s, IL-5 and IL-13 production are also decreased, supporting the hypothesis that ILC2s are a source of these cytokines in this model (7).

Because IL-33 has been shown to activate MAP kinases via a TRAF6-independent mechanism (15, 29), and because Spred1 is a specific negative regulator of the Ras-ERK pathway, we next investigated the effect of Spred1 and ERK on IL-33-mediated ILC2 activation. To do so, we used an ex vivo lung explant culture, which mimics the physiological state of the lung. Consistent with our in vivo model (Fig. 1A), IL-5 and IL-13 were also significantly elevated in the culture supernatants of papain-treated *Spred1*^{-/-} mouse lung explants compared with their WT counterparts (Fig. 3A). In addition, MEK1/2 inhibitor U0126 strongly suppressed IL-5 and IL-13 secretion from lung explant cultures stimulated with papain or IL-33 in a dose-dependent manner (Fig. 3B, 3C).

To demonstrate that Spred1 negatively regulates IL-33-mediated ERK activation through biochemical methods, we used BM-

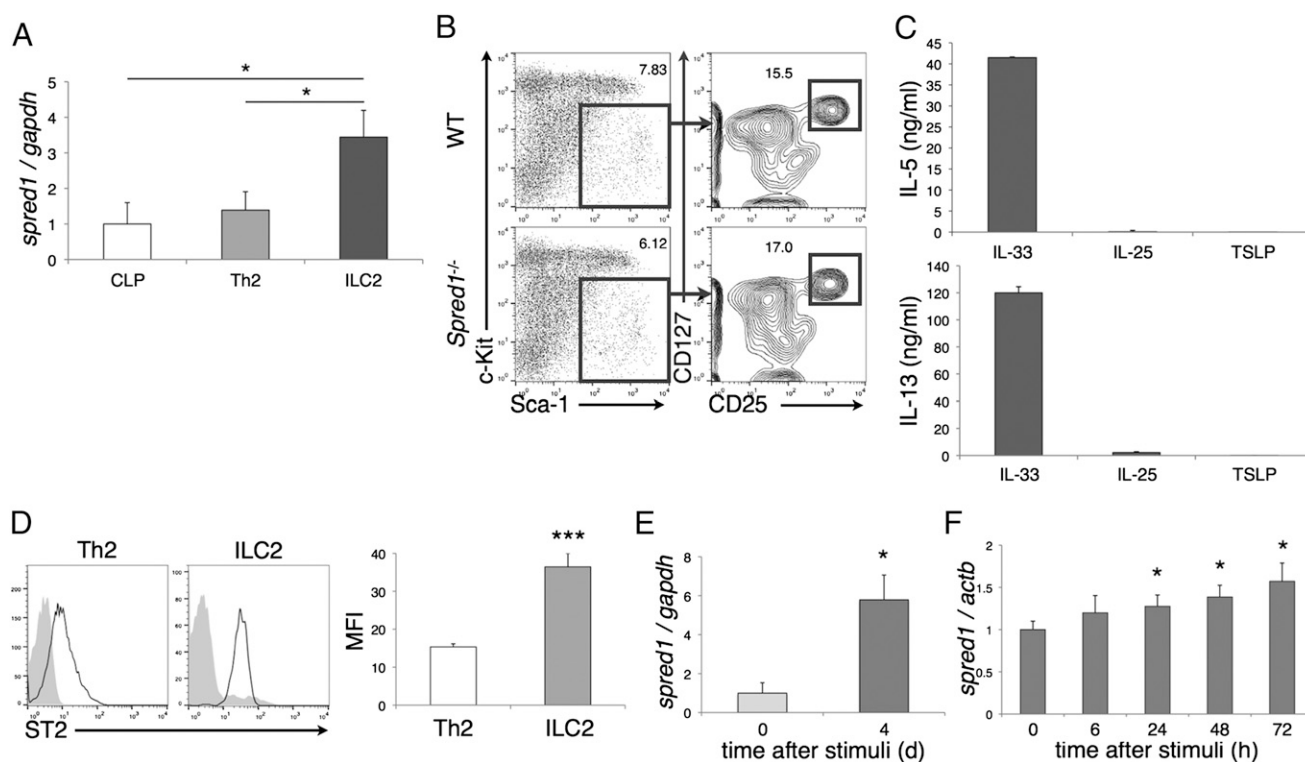


FIGURE 2. Spred1 expression is increased via the IL-33 signaling pathway. (A) Quantitative PCR analysis of *Spred1* transcripts in indicated cells. Lin⁻CD127⁺Sca-1^{int}c-Kit^{int} CLPs were sorted from BM cells, Th2 cells were differentiated from splenic naive T cells, CD4⁺IL-4⁺ cells were sorted on day 6, and ILC2s were sorted from lungs. (B) BM immature ILC2s were identified using flow cytometry. Lin⁻ cells were gated for the analysis of c-Kit and Sca-1 expression (left) and further analyzed for CD25 and CD127 expression (right). The numbers in the plots indicated the percentages of cells in the indicated gates. (C) Purified lung ILC2s from WT mice were incubated with IL-2 and IL-7 supplemented with indicated cytokines for 4 d, after which IL-5 and IL-13 in the culture supernatants were analyzed using ELISA. (D) ST2 expression in indicated cells (black line histograms). Shaded gray histograms show IgG control (with Th2 cells as control) or Lin⁻Sca-1⁻c-Kit⁻ cells (with ILC2s as control). The graph shows MFI of ST2 in indicated cells. (E) Quantitative PCR analysis of *Spred1* transcripts in purified lung ILC2s stimulated with IL-33 for the indicated times. (F) Quantitative PCR analysis of *Spred1* transcripts in ST2-MEFs stimulated with IL-33 for the indicated times. Data are representative of at least three independent experiments. Error bars show SD. **p* < 0.05, ****p* < 0.001.

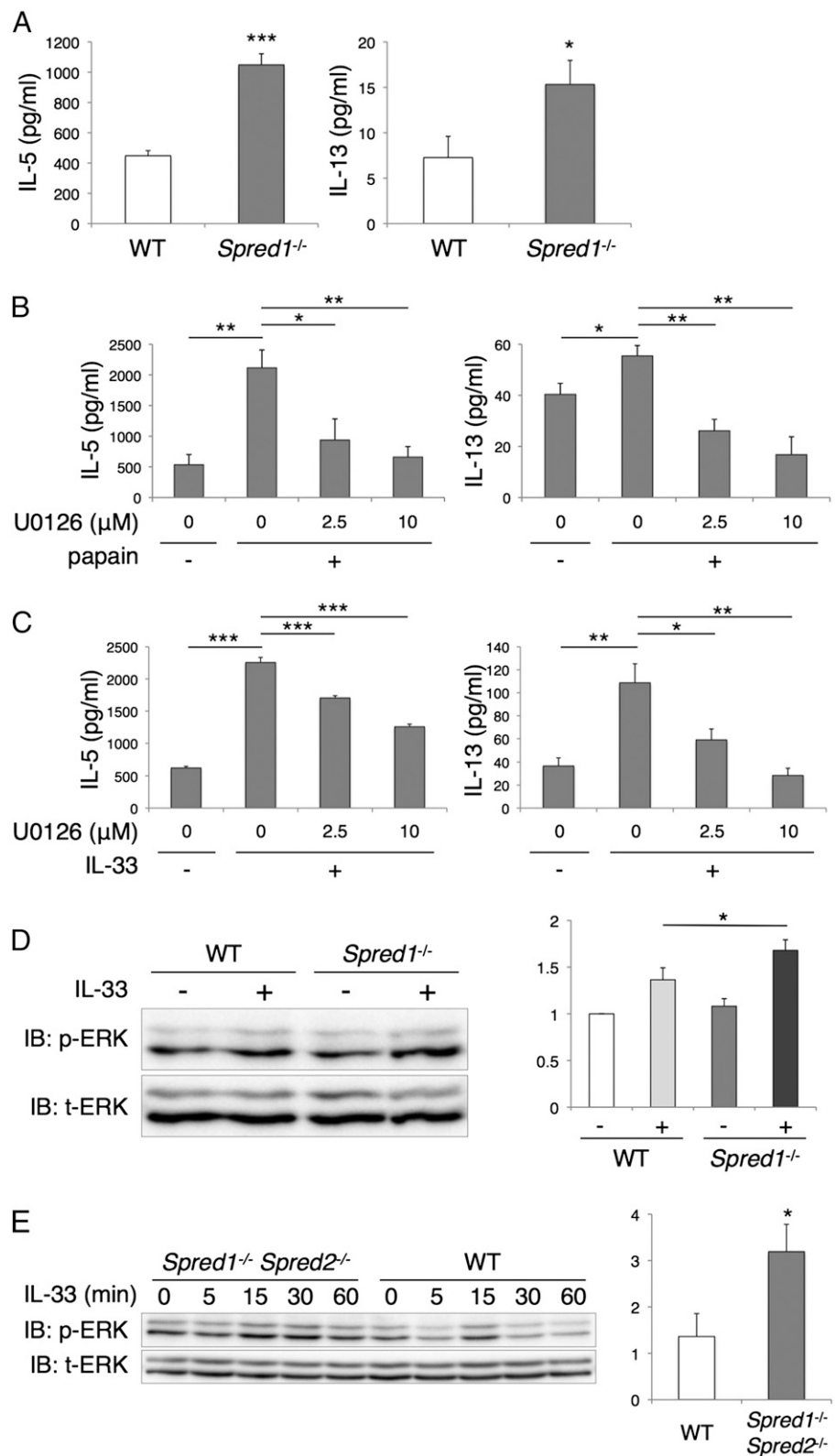


FIGURE 3. ERK signaling pathway is important in ILC2 function. **(A)** WT or *Spred1*^{-/-} mouse lung explants were stimulated with papain for 18 h, after which IL-5 and IL-13 in the culture supernatants were analyzed with ELISA. **(B)** WT mouse lung explants were pretreated with the indicated dose of U0126 for 1 h prior to papain stimulation for 18 h, after which IL-5 and IL-13 in the culture supernatants were analyzed with ELISA. **(C)** WT mouse lung explants were pretreated with the indicated dose of U0126 for 1 h prior to IL-33 stimulation for 18 h, after which IL-5 and IL-13 in the culture supernatants were analyzed with ELISA. **(D)** BMILC2s were stimulated with or without 100 ng/ml IL-33 for 30 min, after which phosphorylation and expression levels of ERK1/2 were detected by immunoblotting. Arbitrary densitometric units normalized with *t*-ERK bands are shown in the graph. **(E)** ST2-MEFs were stimulated with IL-33 for the indicated times, after which phosphorylation and expression levels of ERK1/2 were detected by immunoblotting. Arbitrary densitometric units of band of each 15 min after stimulation normalized with *t*-ERK bands are shown in the graph. Data are representative of at least three independent experiments. Error bars show SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

derived ILC2s (BMILC2s) (28). Flow cytometric analysis confirmed that BMILC2s were Lin⁻Sca-1⁺CD25⁺CD127⁺, although the cell surface expression of c-Kit in BMILC2s was broader than lung ILC2s (Supplemental Fig. 2A). BMILC2s secreted IL-5 and IL-13 in response to epithelial cell-derived cytokines, IL-33, IL-25, and TSLP (Supplemental Fig. 2B). Among these cytokines, IL-33 was the most effective to stimulate BMILC2s, like lung

primary ILC2s (Fig. 2C). Because we could obtain relatively large number of BMILC2s compared with lung ILC2, we used BMILC2 to define the biochemical role of *Spred1*. As shown in Fig. 3D, IL-33-mediated ERK phosphorylation was enhanced in BMILC2s from *Spred1*^{-/-} mice compared with WT mice. Likewise, stronger ERK activation induced by IL-33 was also observed in *Spred1/2*-deficient ST2-MEFs compared with WT ST2-MEFs

(Fig. 3E). These data lend further support to the notion that *Spred1* is a negative feedback regulator of IL-33-mediated ERK activation.

Spred1 regulates ILC2 proliferation and survival

Because the ILC2 fraction was increased in papain-treated, *Spred1*-deficient mice in vivo (Fig. 1D) and because ILC2s isolated from the lung treated with U0126 were restricted to proliferate in vitro (Fig. 4A), we examined the effect of *Spred1* deficiency and the

MEK inhibitor on ILC2 proliferation and survival. To evaluate the effect of *Spred1* deficiency on apoptosis, we completed an apoptotic assay using Annexin V and propidium iodide (PI). The population of Annexin V⁺PI⁺ live cells was greater in BMILC2s from *Spred1*^{-/-} mice than in those from WT mice (Fig. 4B). In keeping with this finding, U0126 significantly decreased the survival of BMILC2s (Fig. 4C). In addition, cell growth rate as assessed by BrdU incorporation was greater in BMILC2s from *Spred1*^{-/-} mice than in those from WT mice (Fig. 4D) and was

FIGURE 4. *Spred1* suppresses ILC2 survival and proliferation. **(A)** Quantification of live cells among purified lung ILC2s from WT mice and incubated with IL-33, IL-2, and IL-7 in the presence or absence of U0126, assessed on days 2, 4, and 6. **(B)** Apoptosis in BMILC2s from WT or *Spred1*^{-/-} mice was assessed using Annexin V and PI staining on day 8. The numbers in the plots indicate the percentages of cells in the quadrant. The graph shows the percentages of live cells (AnnexinV⁻PI⁻). **(C)** Apoptosis in BMILC2s cultured with or without U0126 for 24 h was assessed with Annexin V and PI staining. The numbers in the plots indicate the percentages of cells in the quadrant. The graph shows the percentages of live cells (AnnexinV⁻PI⁻). **(D)** Incorporation of BrdU by BMILC2s from WT or *Spred1*^{-/-} mice. The graph shows the percentages of BrdU⁺ cells. **(E)** Incorporation of BrdU by BMILC2s cultured with or without U0126 for 24 h. The graph shows the percentages of BrdU⁺ cells. Data are representative of at least three independent experiments. Error bars show SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

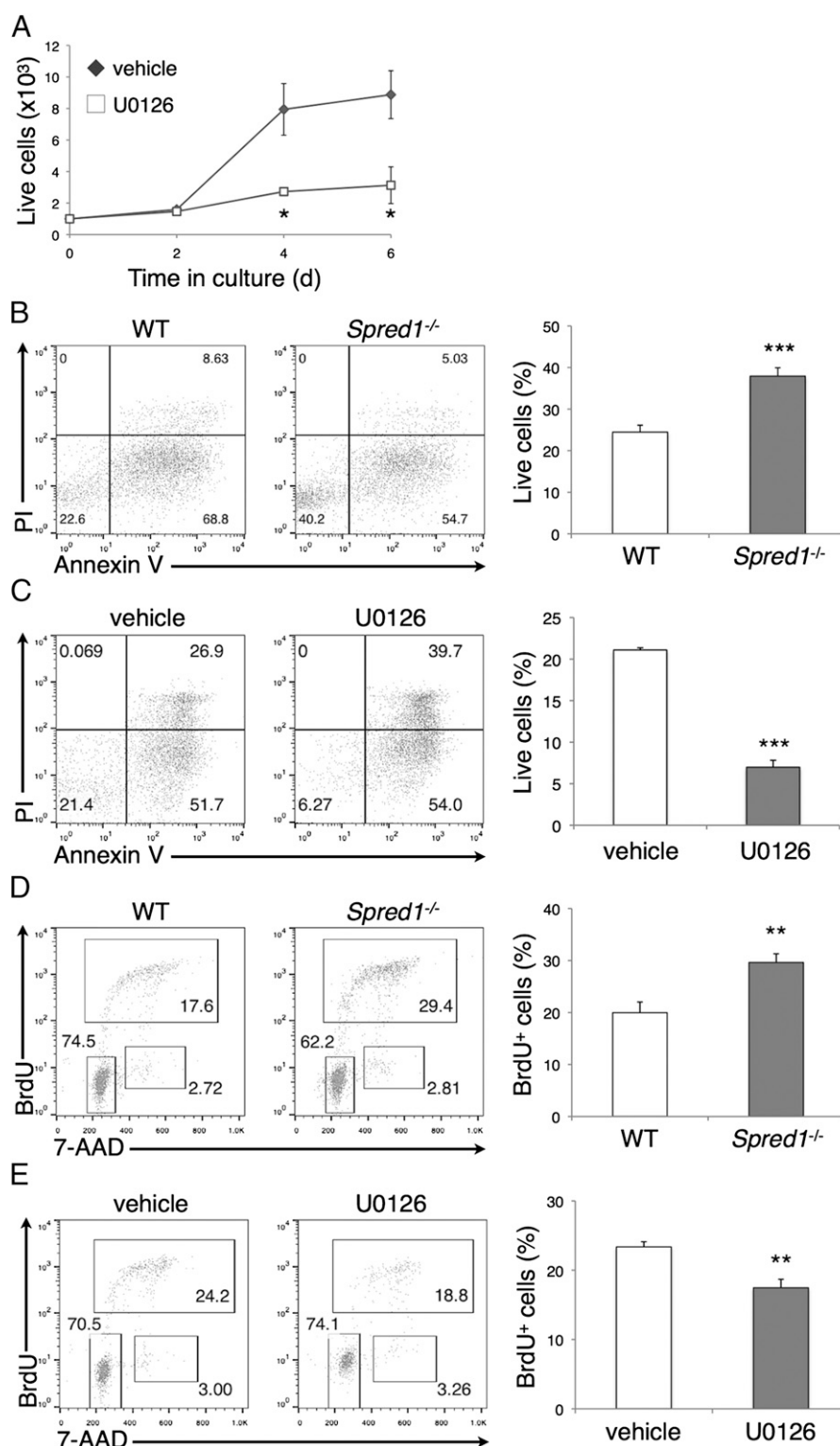
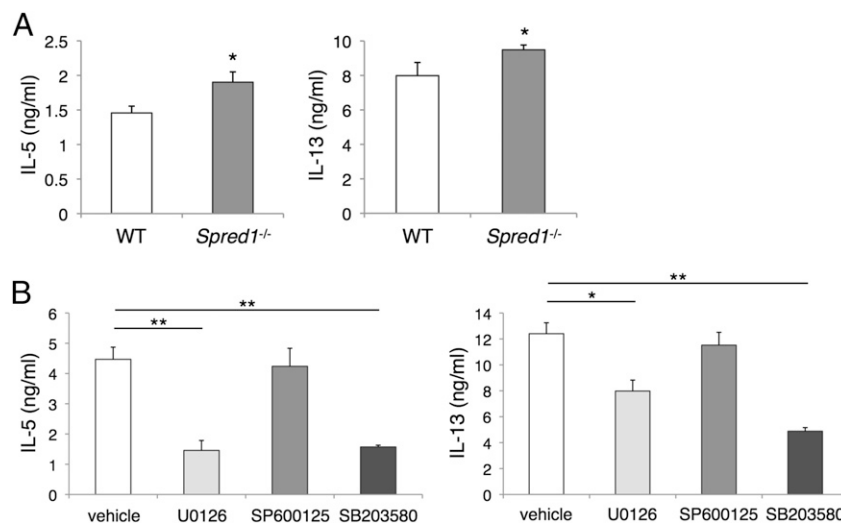


FIGURE 5. *Spred1* regulates IL-5 and IL-13 production from ILC2s. **(A)** Purified lung ILC2s from WT or *Spred1*^{-/-} mice were incubated with IL-33, IL-2, and IL-7 for 2 d, after which IL-5 and IL-13 in the culture supernatants were analyzed with ELISA. **(B)** Purified lung ILC2s from WT mice were incubated with IL-33, IL-2, and IL-7 in the presence or absence of 5 μ M of each of the indicated compounds for 2 d, after which IL-5 and IL-13 in the culture supernatants were analyzed with ELISA. **p* < 0.05, ***p* < 0.01.



also decreased by U0126 treatment (Fig. 4E). These results suggest that *Spred1* reduces the survival and cell cycle progression of ILC2s through suppressing the ERK signaling pathway.

Spred1 regulates IL-5 and IL-13 production from ILC2s

The enhanced expression of cytokines in BALF from papain-treated *Spred1*^{-/-} mice (Fig. 1A) could be due not only to the greater number of ILC2s in these mice, but also to enhanced production of cytokines from *Spred1*^{-/-} ILC2s. To examine this possibility, we measured cytokine levels in the culture media of purified lung ILC2s on day 2, because there were no differences in the cell numbers between U0126-treated and untreated lung ILC2s (Fig. 4A). Both IL-13 and IL-5 levels were slightly but significantly higher in *Spred1*^{-/-} ILC2s than in WT ILC2s (Fig. 5A). It has been reported that p38 is deeply involved in cytokine production from ILC2s (9). Consistently, a p38 inhibitor SB203580 strongly inhibited IL-5 and IL-13 production from lung ILC2s, whereas a JNK inhibitor SP600125 did not (Fig. 5B). We found that U0126 also significantly inhibited IL-5 and IL-13 production from lung ILC2s (Fig. 5B). Collectively, these results suggest that *Spred1* and the ERK signaling pathway regulate cytokine production from ILC2s.

Spred1 regulates GATA3 stabilization in ILC2s

It has been reported that GATA3 plays a crucial role in the type 2 cytokine expression in ILC2s and in expansion and survival of

ILC2s (9, 30). Because the GATA3 protein is stabilized via the activated ERK signaling pathway in Th2 cells (31), we examined the effect of *Spred1* deficiency on the expression levels of GATA3 in ILC2s. As shown in Fig. 6A, protein expression levels of GATA3 were higher in *Spred1*^{-/-} BMILC2s than in WT BMILC2s, whereas, as expected, there was no significant difference in the transcripts of GATA3 between *Spred1*^{-/-} and WT BMILC2s (Fig. 6B). Treatment of ILC2s with a MEK inhibitor resulted in the reduction of GATA3 content, whereas a proteasome inhibitor MG132 reverted the effect of the MEK inhibitor (Fig. 6C). It has been shown that GATA3 suppresses the expression of a death receptor *tnfrsf21* and a cell cycle inhibitor *cdkn2b* in ILC2s (30). As expected, expression of these genes was reduced in *Spred1*^{-/-} ILC2s (Fig. 6D). These data are consistent with our observations that the MEK inhibitor enhanced apoptosis and G1 arrest in BMILC2s (Fig. 4C, 4E), whereas *Spred1*^{-/-} BMILC2s were more resistant to apoptosis and proliferative than WT BMILC2s were (Fig. 4B, 4D). These data suggest that GATA3 is a target of the *Spred1*-ERK system in ILC2s.

Discussion

A recent study demonstrated that Lin⁻Sca-1⁺c-Kit⁺CD25⁺CD127⁺ ILC2s are critical for protease allergen-induced airway inflammation (7); however, the regulatory mechanisms of ILC2s remain to be elucidated. IL-33 is required for IL-5 and IL-13 production by lung ILC2s (7, 15). GATA3 plays essential roles in type 2

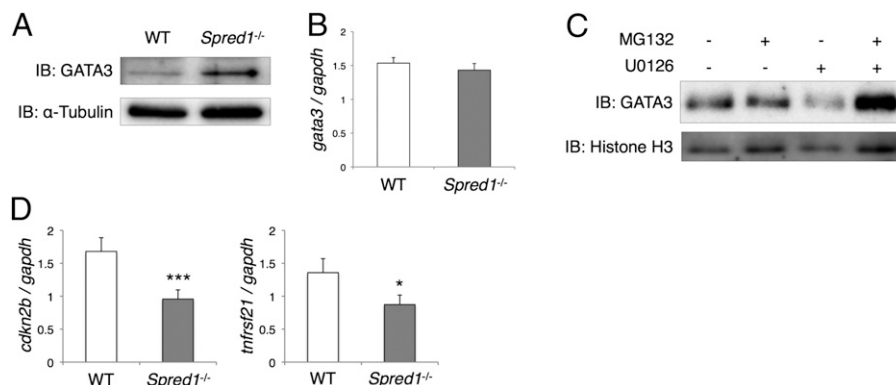


FIGURE 6. *Spred1* regulates GATA3 stabilization in ILC2s. **(A)** Expression levels of GATA3 and α -Tubulin in BMILC2s were detected by immunoblotting. **(B)** Quantitative PCR analysis of *gata3* transcripts in BMILC2s. **(C)** BMILC2 treated with indicated inhibitors for 6 h and expression levels of GATA3 and Histone H3 in nuclear extract were detected with immunoblotting. **(D)** Quantitative PCR analysis of *cdkn2b* and *tnfrsf21* transcripts in BMILC2s. Data are representative of at least two independent experiments. Error bars show SD. **p* < 0.05, ****p* < 0.001.

cytokine production and cell survival and proliferation in both Th2 and ILC2s (30, 32). IL-33-activated p38 phosphorylates GATA3, resulting in nuclear translocation of GATA3, which activates type 2 cytokine production, whereas JNK is not deeply involved in ILC2 function (9). Our study suggests that the ERK signaling pathway also participates in regulating the cellular expression levels of GATA3 protein, as has been proposed in the case of Th2 cells (31). Interestingly ERK does not directly phosphorylate GATA3, but induces ubiquitination and degradation in Th2 cells (31). We could not directly demonstrate that ERK induces stabilization of GATA3 in ILC2s because of the low number of ILC2s available from mice. However, because GATA3 protein levels were enhanced in *Spred1*^{-/-} ILC2 cells but reduced by the MEK inhibitor, and the proteasome inhibitor enhanced GATA3 contents, a similar mechanism may be present in ILC2s. In addition, the ERK signaling pathway is critical for the proliferation of lung ILC2s and for the development of ILC2s from BM immature ILC2s in response to IL-33. These results suggest that ERK plays an important role in both cytokine production and the proliferation and development of ILC2s.

Spred1 has been shown to regulate mast cell and eosinophil proliferation negatively in response to IL-3 and IL-5, respectively (22, 33). *Spred1* also inhibits cytokine production from mast cells in response to IgE cross-linking (34). Because *Spred1* is a highly specific inhibitor for the Ras-ERK pathway (19), it is likely that hyperactivation of ERK is a mechanism driving the hyperproliferation and higher cytokine production that is observed in *Spred1*^{-/-} ILC2s. Indeed, we observed stronger ERK phosphorylation in *Spred1*^{-/-} BMILC2s in response to IL-33. IL-33-induced ERK phosphorylation was not very strong; however, for this reason, we could not rule out the possibility of involvement of other ILC2-activating cytokines, such as IL-25 and TSLP. Yet we did not observe any strong activation of lung ILC2s in response to IL-25 or TSLP, suggesting that IL-33 is the most critical activator of ILC2s.

We observed that the IL-5 and IL-13 levels in the BALF after stimulation with papain were 2–3-fold higher in *Spred1*^{-/-} mice than in WT mice. *Spred1*-deficient ILC2s contain higher levels of GATA3 protein, which may account for the more abundant production of cytokines from *Spred1*^{-/-} ILC2s. However, *Spred1*^{-/-} ILC2s only produced ~20% more cytokines than WT ILC2s did when we used similar numbers of ILC2s. At the same time, the ILC2 fraction was twice as large in *Spred1*^{-/-} mouse lungs as in WT mouse lungs. Furthermore, BMILC2 expansion from immature ILC2s was 2-fold higher in *Spred1*^{-/-} BM cells than in WT BM cells. These data suggest that *Spred1* has a more profound effect on proliferation than on cytokine production. The reason for this difference is not clear at present.

As we used straight *Spred1*^{-/-} mice for our papain-induced asthma model, we could not rule out the possibility of the involvement of nonhematopoietic cells, such as epithelial cells and endothelial cells. In fact, protease allergens have been shown to induce expression of IL-25 and TSLP via MAP kinases (35). IL-33 levels were slightly higher in MEFs from *Spred1*^{-/-} mice than in those from WT mice (data not shown). Therefore, further effort to explore the effect of *Spred1* in epithelial cells in papain-induced airway inflammation is needed to gain insight into the mechanisms of *Spred1* in asthma.

ILC2s are involved in several disorders such as asthma (7), atopic dermatitis (AD) (36), and obesity (37, 38). In AD, ILC2s are enriched in inflamed skin lesions and are necessary for the induction of mouse AD-like disease (36). In obesity, ILC2s act as a protective factor against obesity through their promotion of the beiging of white adipose tissue (37) and their regulation of adipocyte precursor

numbers and fates (38). We have shown that *Spred1*-deficient mice develop allergic phenotypes with age (A. Yoshimura, unpublished observations) and that their body weights are lower than those of WT mice (22). These phenotypes can be explained, at least in part, by enhanced ILC2 activity in *Spred1*^{-/-} mice.

In conclusion, our results reveal a previously unrecognized role of the ERK signaling pathway and *Spred1* in ILC2s. Our study suggests that the ERK signaling pathway, including *Spred1*, could be a new target for the treatment of asthma.

Acknowledgments

We thank R. Komine, S. Tsuruta, and M. Asakawa for technical assistance, Y. Ushijima for preparing the manuscript, and R. Nakagawa and A. Kimura for discussion.

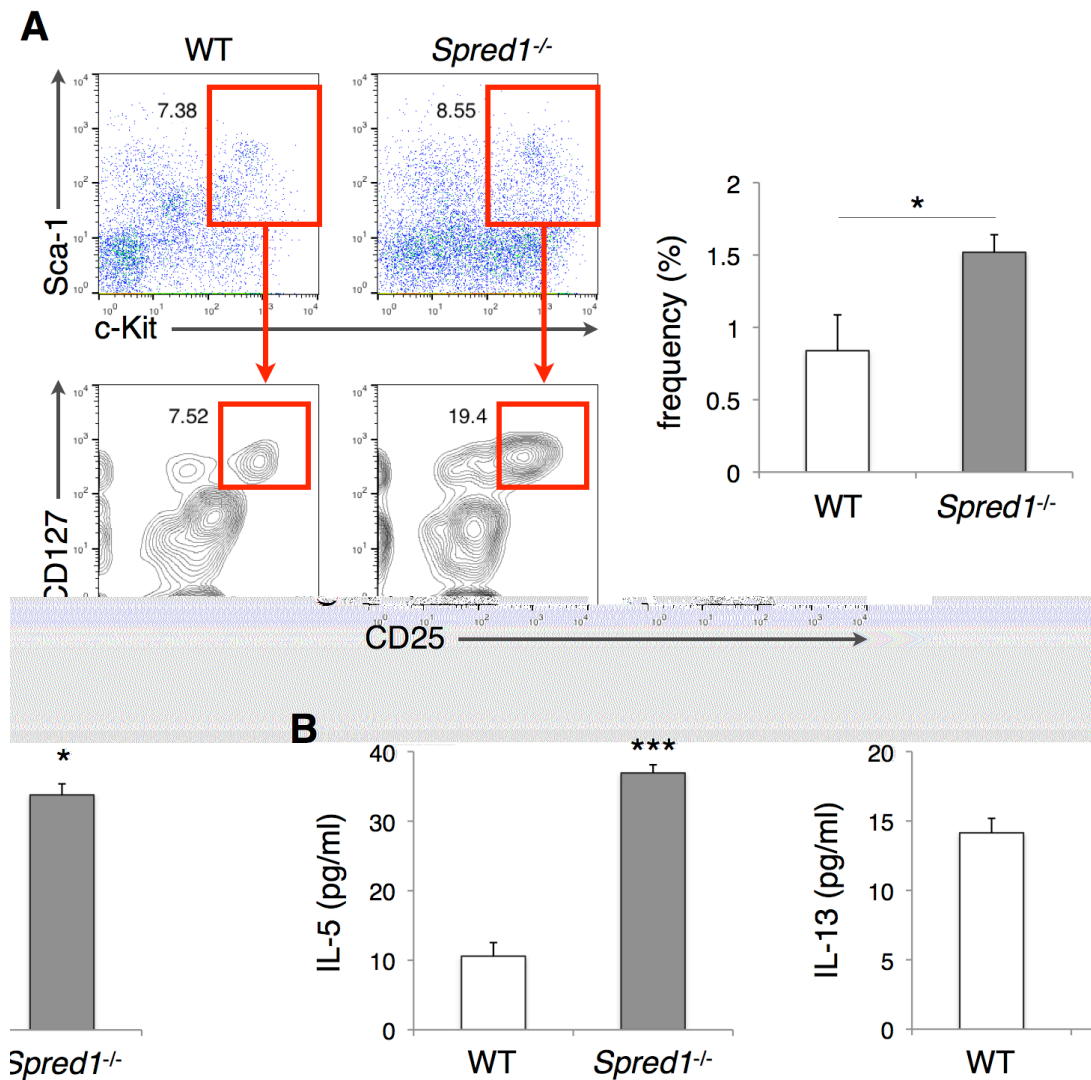
Disclosures

The authors have no financial conflicts of interest.

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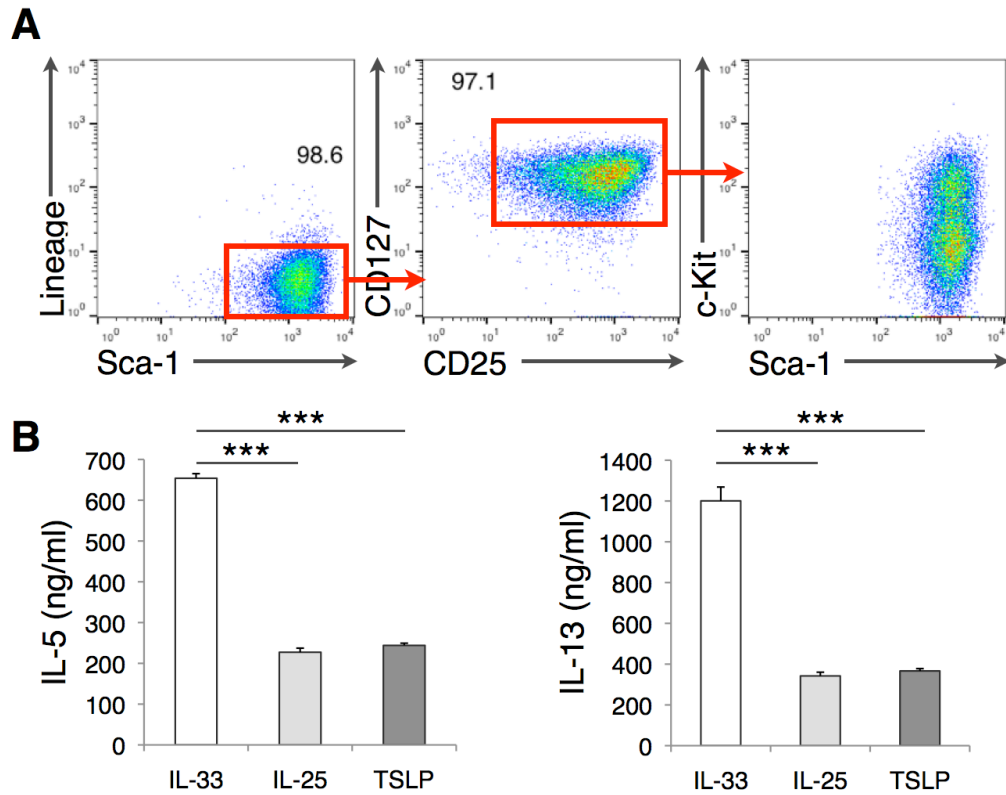
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Supplemental Figure 1. Increase of ILC2s in *Spred1*-deficient mice in the HDM-induced lung inflammation model.

(A) WT or *Spred1*^{-/-} mice were treated intranasally with 50 µg/20 µl house dust mite *Dermatophagoides pteronyssinus* (HDM) extract for seven consecutive days; 24 hours after the last administration, whole lung cells from these mice were analyzed by flow cytometry. Lin⁻ cells were gated for the analysis of c-Kit and Sca-1 expression. Lin⁻Sca-1⁺c-Kit⁺ cells were gated as indicated by red boxes (upper panels) and analyzed for CD25 and CD127 expression (lower panels). The graph shows the cell frequencies of ILC2s in Lin⁻ cells (N=3). (B) IL-5 and IL-13 in BALF were analyzed by ELISA. Data are representative of three independent experiments. Error bars show SD. *p < 0.05. (N=3).



Supplemental Figure 2. BMILC2s show similar phenotypes to mature ILC2s.

(A) Surface markers of BMILC2s from WT mice were analyzed by flow cytometry.

Representative data of three independent experiments.

(B) BMILC2s from WT mice were incubated with IL-2 and IL-7 supplemented with indicated cytokines for four days, then IL-5 and IL-13 in the culture supernatants were analyzed by ELISA. Error bars show SD. *** $p < 0.001$. (N=3).