Spred-1 negatively regulates allergen-induced airway eosinophilia and hyperresponsiveness

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T helper 2 cytokines, including interleukin (IL)-4, IL-5, and IL-13, play a critical role in allergic asthma. These cytokines transmit signals through the Janus kinase/signal transducer and activator of transcription (STAT) and the Ras–extracellular signal-regulated kinase (ERK) signaling pathways. Although the suppressor of cytokine signaling (SOCS) family proteins have been shown to regulate the STAT pathway, the mechanism regulating the ERK pathway has not been clarified. The Sprouty-related Ena/VASP homology 1–domain-containing protein (Spred)-1 has recently been identified as a negative regulator of growth factor–mediated, Ras-dependent ERK activation. Here, using Spred-1–deficient mice, we demonstrated that Spred-1 negatively regulates allergen-induced airway eosinophilia and hyperresponsiveness, without affecting helper T cell differentiation. Biochemical assays indicate that Spred-1 suppresses IL-5–dependent cell proliferation and ERK activation. These data indicate that Spred-1 negatively controls eosinophil numbers and functions by modulating IL-5 signaling in allergic asthma.

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Abbreviations used: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BMEo, BM–derived eosinophil; EGFP, enhanced GFP; ERK, extracellular signal-regulated kinase; PAS, periodic acid-Schiff; Spred, Sprouty-related EVH1-domain-containing protein.

Asthma is characterized by a variable degree of airflow obstruction, airway hyperresponsiveness (AHR; defined by enhanced airflow obstruction in response to nonspecific stimuli), mucus overproduction, and chronic airway inflammation. Numerous eosinophils and lymphocytes infiltrate peribronchial tissues in asthmatics. Th2 cells are the predominant lymphocyte population that infiltrates the airways of asthmatics, and the cytokine products of Th2 cells play essential roles in airway eosinophilia, AHR, and serum IgE in animal models (1). Eosinophils are produced in bone marrow, and recent observations in both mice and humans suggest that pulmonary allergen exposure results in both increased output of eosinophils from hemopoietic tissues and increased migration of these cells to the lung (2–4). It is the accumulation of activated eosinophils during the late-phase response to allergen exposure that ultimately results in progressive inflammatory tissue damage. In addition, pulmonary eosinophilia in response to allergen challenge is associated with elevated levels of eosinophil-derived proteins in both the lungs and peripheral blood (5, 6). However, the specific mechanisms that alter eosinophilopoiesis in asthma are poorly understood.

Eosinophil production is predominantly regulated by the Th2 cytokine, IL-5 (7, 8). IL-5 receptor consists of IL-5–specific α-chain and the common β-chain that is shared by the IL-3 and GM-CSF receptor (9). In addition to the JAK–STAT pathway, the Ras–extracellular signal-regulated kinase (ERK) pathway has also been implicated in signaling of IL-5 and other cytokines (10–12), and this pathway is shown to be important for IL-5–dependent cell survival (12). Therefore, the Ras-ERK signals seem to be important for eosinophilia in asthma; however, the regulation of this path-

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way and its contribution to the disease have not been clarified.

ERK activation is initiated by binding of Grb2 to the phosphorylated tyrosine residues of the receptor or phosphorylated adaptor molecules such as Shc, FRS-2, IRS-1/2, SHP-2, and Gab-1. The complex of Grb2 and SOS activates Ras by GTP loading. Ras-GTP recruits Raf-1 to the plasma membrane (13, 14). Raf-1 is phosphorylated and activated by not well-defined kinases with complex regulatory mechanisms (15). Activated Raf phosphorylates and activates the dual-specific kinase MEK, which phosphorylates and activates ERK. The regulation of this pathway has been suggested to be quite important for cell proliferation and differentiation. Recently, Sprouty family proteins were identified as negative regulators for several growth factor-induced ERK activation including FGF and EGF (9, 16). Four Sprouty homologues are found in mammals. We cloned an additional Sprouty-related family of novel membrane bound molecules, Sprouty-related Ena/VASP homology 1–domain-containing proteins (Spreds; reference 17). Three members of Spreds were identified in mammals (18), which have a Sprouty-related COOH-terminal cysteine-rich domain in addition to the NH_2-terminal Ena/VASP homology 1 domain. Like Sproutys, Spred-1, Spred-2, and Spred-3 also down-regulate Ras/ERK signaling. As Spred inhibits active Ras-induced ERK activation, Spred might modulate the unidentified activation steps of Raf by a novel mechanism. Spred/Sprouty family proteins have emerged as a negative regulator of the ERK pathway; however, details of their physiological function and molecular mechanism remain to be investigated.

In the present paper, we generated Spred-1 knockout mice and examined the function of Spred-1 on the development of asthma and associated eosinophilia. We show that Spred-$1^{-/-}$ mice exhibited exaggerated allergen-induced AHR, eosinophilia, and mucus production in a murine allergic asthma model. Spred-$1^{-/-}$ mice also showed increased responsiveness, especially ERK signals, to IL-5 and subsequent overexpression of IL-13 in eosinophils. Thus, it is conceivable that the down-regulation of Spred-1 in the airways has a significant role in prolonged airway eosinophilia and asthma phenotypes. We propose the possibility that Spred-1 may present a novel therapeutic target for the treatment of asthma.

RESULTS

Generation of Spred-$1^{-/-}$ mouse and assessment of T cell development

We found that Spred-1 is highly expressed in hematopoietic cells (unpublished data). To examine the function of Spred-1 in Th1/Th2 cell differentiation and diseases, we generated mice lacking the Spred-1 gene by homologous recombination techniques (Fig. 1). To obtain the loss-of-function mutant, exons containing the kit-binding domain and Sprouty-related COOH-terminal cysteine-rich domains were deleted. Loss of Spred-1 protein expression was confirmed by Western blotting of the tissue extracts. Spred-1 protein was detected in the brain of the WT mice but not in that of the mutant mice. Offspring were born within the Mendelian expectation ratio from intercrosses of heterozygotes as well as incrosses of homozygotes. This indicates that Spred-1 is not necessary for fertility and development. Adult Spred-$1^{-/-}$ mice appeared to be healthy, except that a slight lower body weight, a shortened face, and a kinky tail, but they showed no apparent abnormalities in most organs (unpublished data). No abnormalities of peripheral hematopoietic cell population and number, CD4/CD8 profiles of spleen and thymus, or B cell development were observed in Spred-$1^{-/-}$ mice (unpublished data).

Enhanced OVA-induced asthma phenotypes in Spred-$1^{-/-}$ mice

Because the ERK pathway is shown to be involved in the Th1/Th2 balance (19–21), we investigated the role of Spred-1 in a Th2-type disease, bronchial asthma, using an OVA-

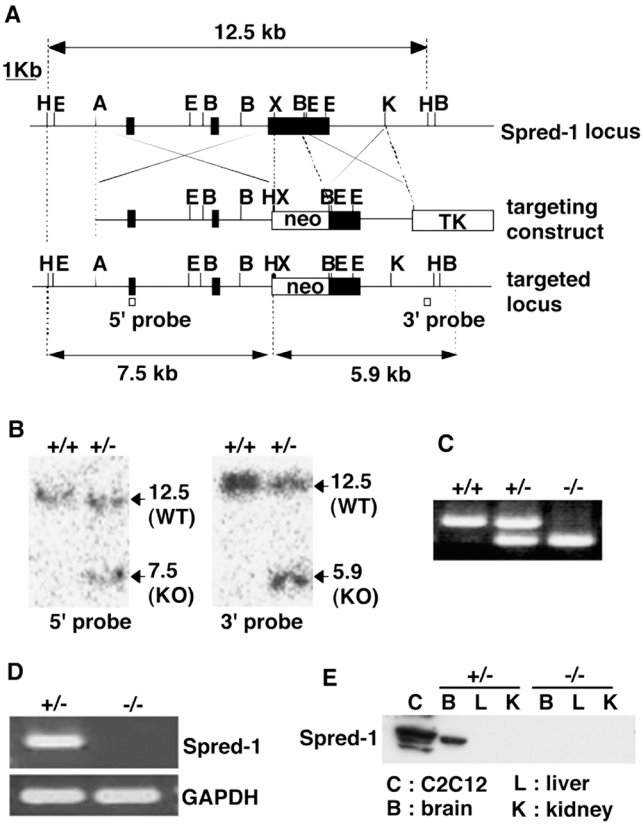

Figure 1. Generation of Spred-1–deficient mice. (A) Schematic representations of wild-type and mutant loci of the Spred-1 gene together with the targeting vector. Exons (from the second to fourth) for genes encoding Spred-1 are represented by black boxes. The neomycin-resistance gene driven by a PGK promoter (pgk-neo) and the gene coding the thymidine kinase driven by an HSV promoter (hsv-tk) are indicated by white boxes. EcoRV-digested genomic DNA fragments were detected by probe. Restriction sites: E, EcoRI; Ev, EcoRV; H, HindIII; A, ApaI; K, KpnI; X, XhoI. (B) Representative Southern blot analysis with HindIII-digested DNA. The positions of probes are shown in A. (C) Genomic PCR analysis of tails of indicated mice. (D) RT-PCR analysis of Spred-1 and control GAPDH expression in total RNA of brains of wild-type and Spred-$1^{-/-}$ mice. (E) Western blotting of brain, liver, and kidney with anti–Spred-1 antibody. C2C12 cells that express Spred-1 endogenously at high levels are shown as a positive control.

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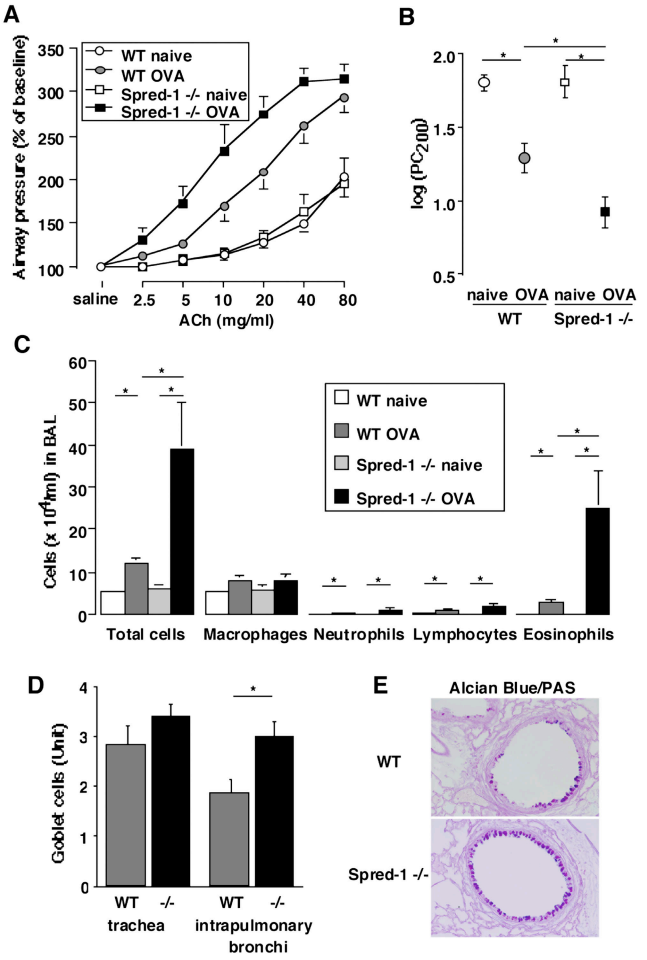

Figure 2. Effects of Spred-1 inactivation on asthmatic responses in an OVA-induced asthma model. Mice were sensitized and aerosol challenged with OVA. Airway responsiveness was determined by the acetylcholine-dependent change in airway pressure in saline-treated control and OVA-sensitized/-challenged LM and Spred-1–deficient mice (A) and the calculation of PC_{200} (B). (C) Effects of Spred-1 depletion on cell counts in BAL fluid. *, $P < 0.05$ by analysis of variance with Bonferroni correction. (D) Semiquantitative analysis of the abundance of PAS-positive mucus-containing cells. Lung tissue sections were obtained from formalin-fixed, paraffin-embedded lung tissue prepared and stained with Alcian blue/PAS. PAS-positive cells were defined as the average of the sore as described in Materials and Methods. *, $P < 0.05$ by Kruskal-Wallis test with Mann-Whitney U test. (E) Representative lung tissue sections. Original magnification, 200.

induced asthma model. After systemic sensitization to OVA and aerosolized OVA challenges, airway responsiveness to acetylcholine aerosol was measured using an invasive technique (22). WT mice demonstrated AHR to acetylcholine, and Spred-$1^{-/-}$ mice exhibited significantly enhanced AHR compared with WT mice (Fig. 2, A and B). OVA sensitization and challenge resulted in an increased number of eosinophils in bronchoalveolar lavage (BAL) fluid in WT mice. Spred-$1^{-/-}$ mice after an OVA challenge displayed a further increase in eosinophils in BAL fluids compared with WT mice, although there was no significant difference in the number of lymphocytes and neutrophils (Fig. 2 C). There was no difference in OVA-induced asthma between C57BL/6 mice and WT (Spred-$1^{+/+}$) littermates (unpublished data). Alcian blue/periodic acid-Schiff (PAS) staining was performed to examine the levels of mucus hyperproduction. After OVA challenge, mild staining in the airways of WT mice cells was noted, and staining levels were increased significantly in the intrapulmonary bronchi of Spred-$1^{-/-}$ mice (Fig. 2, D and E), indicating enhanced goblet cell metaplasia in Spred-$1^{-/-}$ lung. In the absence of sensitization and challenges, no substantial differences were apparent in ARH to acetylcholine, inflammatory cells in BAL fluids, and Alcian blue/PAS staining cells in the airways between Spred-$1^{-/-}$ and WT mice.

Next, we directly measured cytokines and eotaxin levels in mouse BAL fluids. The challenge with OVA increased the concentrations of IL-13 and eotaxin in Spred-$1^{-/-}$ mice significantly more than in WT mice (Fig. 3 A), reflecting marked eosinophilia in the airways of Spred-$1^{-/-}$ mice. However, there was no difference in the concentration of IL-4, IL-5, or IFN-γ in BAL fluids after OVA challenges between Spred-$1^{-/-}$ and WT mice.

Th1/Th2 responses in Spred-$1^{-/-}$ T cells

Enhanced AHR in Spred-$1^{-/-}$ mice may be explained by a simple increased Th2 response, although IL-4 levels were not altered. Therefore, we measured serum IgE levels that are determined by Th1/Th2 balance. The concentrations of total IgE and OVA-specific IgE in Spred-$1^{-/-}$ mice were similar to those in WT controls (Fig. 4 A). There were no significant differences in serum IgA levels among the groups (Fig. 4 A). $CD4^+$ T cells were isolated from draining lymph node cells from paratracheal and mediastinal lymph nodes after OVA

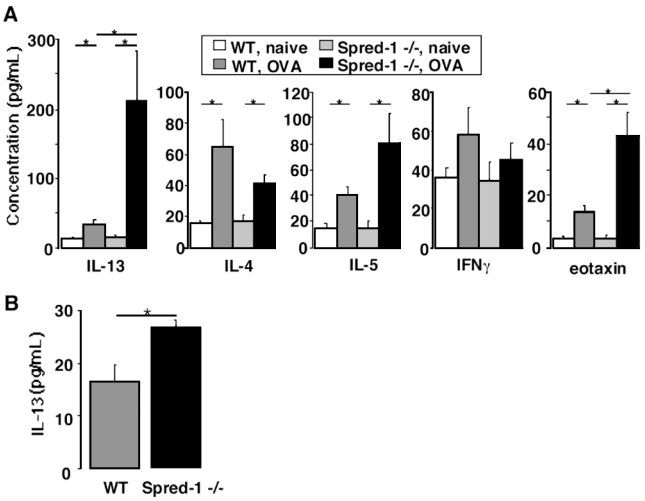

Figure 3. Elevated IL-13 levels in BAL fluid of Spred-1–deficient mice. (A) Effects of Spred-1 inactivation on cytokine levels in BAL fluid in an OVA-induced asthma model. Data are means ± SEM of at least six mice per group. *, $P < 0.05$ by analysis of variance with Bonferroni correction. (B) Eosinophils isolated from BAL of OVA-sensitized/-challenged WT and Spred-1–deficient mice were incubated at 10^6 cells/ml for 24 h. The secretion of IL-13 from Spred-1–deficient mouse eosinophils was significantly higher than that from WT mouse eosinophils ($n = 6$). Data are expressed as the mean ± SEM of six mice. *, $P < 0.05$ by Student's *t* test.

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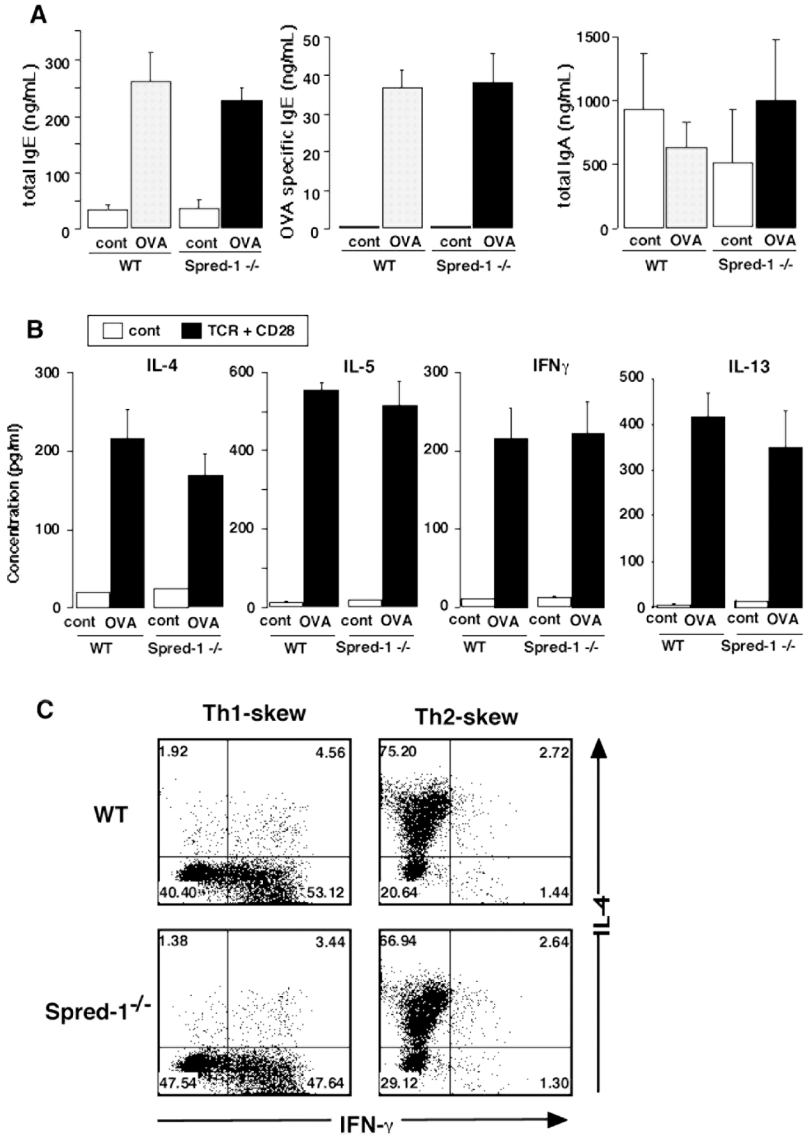

Figure 4. Intact Th1/Th2 response in Spred-1–deficient mice. (A) Serum concentration of total IgE, OVA-specific IgE, and total IgA in an OVA-induced asthma model. Data are means ± SEM of at least six mice per group. (B) Cytokine production from CD4$^+$ T cells after stimulation with anti-TCR plus anti-CD28 mAbs. CD4$^+$ T cells were isolated from paratracheal and mediastinal lymph node cells from WT and Spred-1–deficient mice after OVA challenges and cultured with anti-TCR plus anti-CD28 mAbs in vitro for 48 h. Cytokine production in the supernatants was assessed by ELISA. All data are means ± SEM. (C) CD4$^+$ T cells from WT and Spred-1–deficient mice were stimulated for 5 d with anti-TCR plus anti-CD28 mAbs in the presence of IL-12 and anti–IL-4 mAb (Th1-skewed condition) or IL-4 and anti–IL-12 mAb (Th2-skewed condition). The generation of Th1 and Th2 cells was assessed by cytoplasmic staining with IL-4 and IFN-γ. The absolute numbers of cells harvested were similar in these cultures. The percentages of cells in each quadrant are shown. Three independent experiments were performed with similar results.

challenges and stimulated in vitro with anti-TCR plus anti-CD28 mAbs. IL-4, IL-5, IL-13, and IFN-γ production from CD4$^+$ T cells was similar between Spred-1$^{-/-}$ and WT mice (Fig. 4 B). These data suggest that Spred-1 does not affect Th1/Th2 differentiation and cytokine production from T cells.

To confirm these conclusions, we assessed the development of Th1 and Th2 cells from naive CD4$^+$ T cells in WT and Spred-1$^{-/-}$ mice. T cells were stimulated with anti-TCR plus anti-CD28 mAbs, and the population of IL-4– or IFN-γ–producing cells was analyzed by intracellular FACS staining. The ratio of IL-4– and IFN-γ–producing T cells was comparable between Spred-1$^{-/-}$ and WT mice. IL-4 and IFN-γ levels in the culture supernatant were also comparable (unpublished data). We also tested the generation of Th1 and Th2 cells under a Th1- or Th2-skewed condition. Naive CD4$^+$ T cells were stimulated with anti-TCR mAb in the presence of IL-4 plus anti–IL-12 mAb or of IL-12 plus anti–IL-4 mAb. There were no significant differences in the generation of IL-4–dependent Th2 cells or IL-12–dependent Th1 cells between Spred-1$^{-/-}$ and WT T cells (Fig. 4 C). These data suggested that the development of asthma phenotypes is enhanced in Spred-1$^{-/-}$ mice through the up-regu-

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lation of a limited repertoire of Th2 cytokines, such as IL-13, and that this is not due to the enhancement of antigen-specific Th2 immune responses.

In addition to T cells, eosinophils produce Th1 and Th2 cytokines, including IL-5 and IL-13 (23, 24). Next, we investigated IL-13 expression in accumulated eosinophils in the airways of OVA-challenged mice (Fig. 3 B). Eosinophils were isolated from BAL fluids and incubated in vitro for the evaluation of IL-13 production. Airway eosinophils isolated from Spred-1$^{-/-}$ mice secreted significantly higher levels of IL-13 than those from WT mice (Fig. 3 B). These findings indicate that the IL-13 production from eosinophils is augmented in Spred-1$^{-/-}$ mice.

Increased eosinophilia in response to IL-5 but not to IL-13 in Spred-1$^{-/-}$ mice

It has been shown that IL-5 is critical for the induction of eosinophilia (25–27), as is IL-13 for the development of eosinophilic inflammation and AHR (28, 29). Thus, to elucidate the mechanism of enhanced OVA-induced asthma phenotypes and preferential IL-13 production in Spred-1$^{-/-}$ mice, we analyzed the response of eosinophils to these cytokines. Intratracheal administration of recombinant mouse IL-13 increased eosinophil counts in BAL fluids in WT mice; however, the level of eosinophilia after IL-13 instillation in Spred-1$^{-/-}$ mice was comparable to that in WT mice (unpublished data). Intraperitoneal injection of recombinant mouse IL-5 into WT mice induced an increase in the eosinophil number in peripheral blood as reported previously (30). In Spred-1$^{-/-}$ mice, IL-5 injection induced a prominent and prolonged increase in peripheral eosinophil counts (Fig. 5 A).

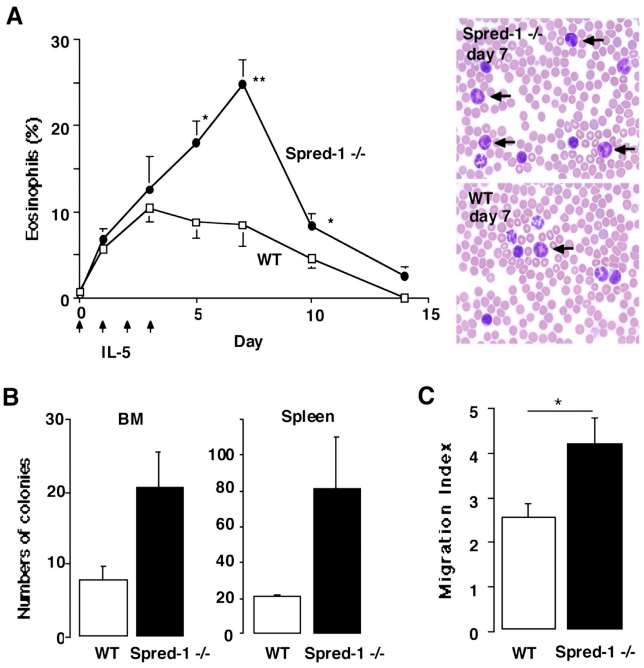

Figure 5. Eosinophil response to IL-5 or to eotaxin and IL-13 production from isolated eosinophils. (A) Recombinant mouse IL-5 (10,000 U/d) was injected intraperitoneally into WT or Spred-1–deficient mice for 4 d. Differential counts were performed by examination of blood smear stained with a modified Wright-Giemsa stain at various time points (left). Typical appearances of blood smear from WT and Spred-1–deficient mice at day 7 are shown (right). Original magnification, 400. *, $P < 0.05$ and **, $P < 0.01$ by Student's *t* test. (B) Colony formation of eosinophil progenitors in bone marrow and splenic cells. Bone marrow or splenic cells were plated in methylcellulose containing 10 ng/ml IL-5. On day 14, the number of colonies was counted microscopically. (C) Eotaxin-induced eosinophil chemotaxis. Eosinophils from bone marrow of WT or Spred-1–deficinet mice were allowed to migrate to 100 ng/ml eotaxin through polycarbonate filter for 60 min. The migrated cells were counted. Data are reported as migration index and they are calculated as follows: (number of cells migrating to chemoattractant)/(number of cells migrating to vehicle). Data are means ± SEM of six experiments. * $P < 0.05$ by Mann-Whitney U test.

To confirm the hyperresponsiveness of Spred-1$^{-/-}$ eosinophil progenitors to IL-5 in vitro, IL-5–dependent colonies were counted from the bone marrow and spleen of Spred-1$^{-/-}$ and WT mice. Bone marrow and splenic cells were cultured with IL-5 as the only supportive cytokine to enumerate eosinophil precursors. In this medium, the number of colonies from Spred-1–deficient bone marrow and splenic cells was more than those from WT cells (Fig. 5 B). These data suggest that Spred-1$^{-/-}$ mice contain more eosinophil progenitors than WT mice or that the hematopoietic progenitors of Spred-1$^{-/-}$ mice are more sensitive to IL-5.

Because the Ras–ERK pathway has been shown to regulate eosinophil chemotaxis (31), we performed the chemotaxis experiment with eosinophils derived from bone marrow of Spred-1$^{-/-}$ or WT mice. Chemotaxis of eosinophils from Spred-1$^{-/-}$ mice was accelerated in response to eotaxin compared with WT mice (Fig. 5 C). These findings indicate that Spred-1$^{-/-}$ mice exhibit the hyperresponsiveness of eosinophil to IL-5 and to eotaxin and enhanced/sustained eosinophilia.

Spred-1 inhibits IL-5–mediated ERK activation and cell proliferation

IL-5 activates the propagation of signals principally via the JAK–STAT pathways, especially STAT5, and the Ras-MAPK pathways (32). To elucidate the molecular mechanism by which the inactivation of Spred-1 enhances IL-5 responses, we analyzed the JAK-STAT and Ras–MAPK pathways using an IL-5–dependent cell line, Y16 (33). Although forced expression of WT or ΔC-Spred-1 did not affect IL-5–induced JAK2 and STAT5 activation, WT Spred-1 reduced Raf-1 and ERK activation, and the dominant negative ΔC mutant of Spred-1 augmented Raf-1 and ERK activation (Fig. 6 A).

Other tyrosine kinases such as Lyn and JAK2 have been shown to enhance eosinophil differentiation (34). Therefore, we analyzed the interaction of Spred-1 with Raf-1, Lyn, and JAK2 kinases in Y16 cells transfected with WT Flag-Spred-1. The expression levels of Lyn in Y6 cells was very low, so we could not conclude the interaction between Lyn and Spred-1. Raf-1 was coimmunoprecipitated with Spred-1 as reported previously (17). However, JAK2 was not coimmunoprecipitated with Spred-1 (Fig. 6 B). These data further support our

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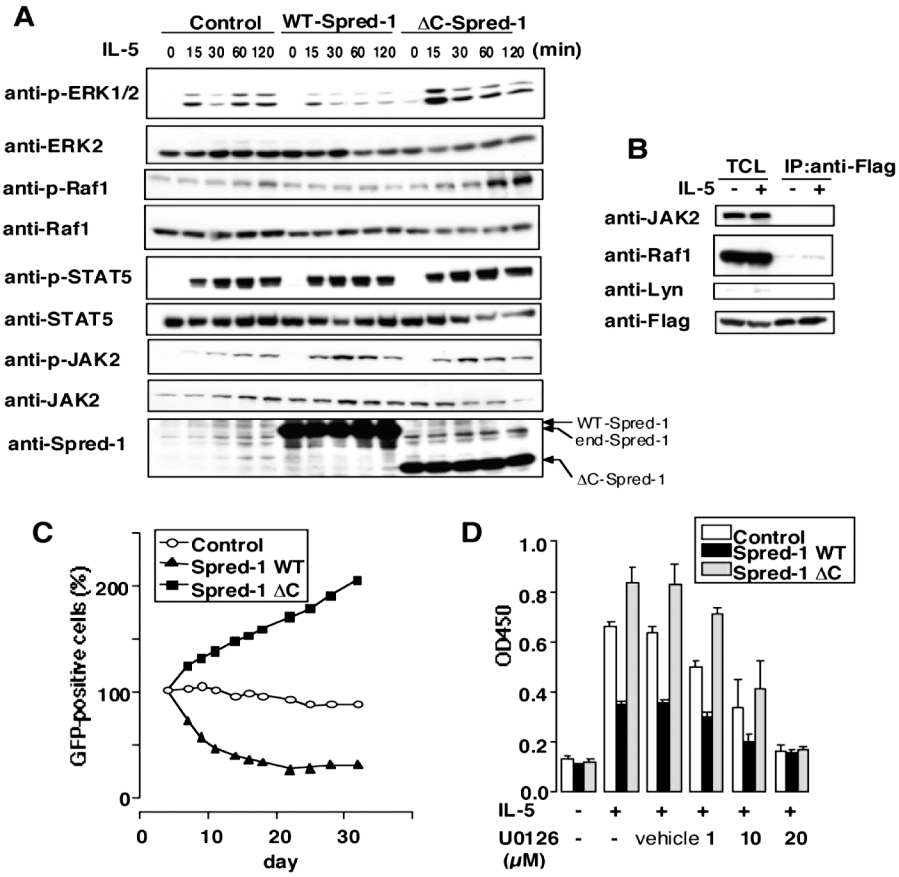

Figure 6. Effect of WT and ΔC Spred-1 on IL-5–induced ERK activation and proliferation of Y16 cells. (A) Y16 cells transfected with Flag-tagged WT or ΔC Spred-1 were stimulated with 50 U/ml IL-5 for indicated periods. Cell extracts were immunoblotted with the indicated antibodies. (B) Interaction of Spred-1 with Raf1, Lyn, and JAK2. Y16 cells were transfected with WT Flag-Spred-1. Anti-Flag immunoprecipitates (IP) from Y16 cells with or without incubation of 50 U/ml IL-5 were blotted (IB) with the indicated antibodies. TCL, total cell lysates. (C) Retroviruses carrying Flag-tagged WT and COOH-terminal–truncated Spred-1 (ΔC-Spred-1). cDNAs in pMY-IRES-GFP vector were produced by transfection of the PLAT-E packaging cell line. Y16 cells were infected with viruses for 24 h in the presence of 5 U/ml IL-5. The changes of the proportion of GFP-positive cells with 5 U/ml IL-5 for indicated periods were assayed by flow cytometer. (D) Effect of MEK inhibitor, U0126, on IL-5–induced proliferation. Y16 cells transfected with WT or ΔC Spred-1 were preincubated with buffer, vehicle DMSO, or U0126 for 30 min and cultured with 50 U/ml IL-5 for 48 h. Cell proliferation was measured by colorimetric assay using WST-8 and 1-methoxy PMS.

proposal that the effect of Spred-1 is specific to the downstream of JAK2 (i.e., the Ras–Raf–ERK pathway).

Next, we examined the effect of WT and ΔC-Spred-1 on IL-5–dependent proliferation of Y16 cells. WT and ΔC-Spred-1 cDNAs were introduced into Y16 cells with enhanced GFP (EGFP) using a bicistronic retrovirus vector pMY-IRES-EGFP (35). Because the infected cells expressed both EGFP and Myc-tagged Spred-1, the percentage of infected cells was determined as the EGFP-positive rate by flow cytometry. When Y16 cells were cultured in a medium containing 5 U/ml IL-5 for 4 wk, the proportion of control IRES-EGFP–infected cells was unchanged; however, the population of WT Spred-1–infected cells decreased, whereas that of ΔC-Spred-1–infected cells increased (Fig. 6 C). The MEK inhibitor, U0126, blocked IL-5–induced proliferation of ΔC-Spred-1–transfected Y16 cells as efficiently as parental Y16 cells (Fig. 6 D). These results suggest that the inactivation of Spred-1 enhances IL-5 responses through augmenting Ras–MAPK activity.

To further confirm the effect of Spred-1 on IL-5–dependent signals in more physiological conditions, BM–derived eosinophil (BMEo) cells were analyzed (Fig. 7). Activations of Raf-1 and ERK2 were augmented in Spred-1$^{-/-}$ BMEos compared with WT BMEos, but JAK2 activation was not affected (Fig. 7). These results further supported that Spred-1 inhibits the Ras–ERK pathway downstream of IL-5/JAK2 and that the hyperresponsiveness of Spred-1$^{-/-}$ eosinophils to IL-5 may be explained by enhanced ERK activation.

DISCUSSION

We have recently identified Spreds and determined that Spred-1 interacts with Ras and inhibits growth factor–induced Raf kinase activation (17). The present paper demonstrated that Spred-1 inactivation by a dominant negative mutant enhances IL-5 signals via the Ras–MAPK pathway and IL-5–induced cell proliferation, whereas Spred-1 overexpression suppresses IL-5–induced Ras–MAPK activity. Spred-1–deficient mice exhibit augmented eosinophilia

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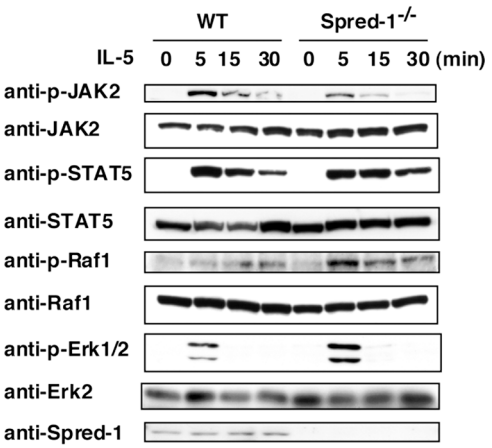

Figure 7. The activation of Raf-1, ERK2, and JAK2 in IL-5–stimulated eosinophils from Spred-1$^{-/-}$ and WT mice. Eosinophils derived from bone marrow were stimulated with 1,500 U/ml IL-5 for indicated periods. Cell extracts were immunoblotted with the indicated antibodies.

in response to IL-5. It has been suggested that the Ras–MAPK pathway might regulate Th cell development (19–21, 36). By using mouse models, we demonstrated that Spred-1 inactivation increases allergen-induced expression of IL-13 and magnifies asthma phenotypes without affecting Th2 differentiation including IL-4 or IgE levels. We further demonstrated that IL-13 production by airway eosinophils is up-regulated in Spred-1–deficient mice after allergen challenges. IL-5 (25–27) and IL-13 (28, 29) are critical for the development of asthma. Therefore, these data suggest that Spred-1 plays an important regulatory role in asthma by modulating the signaling of a limited repertoire of Th2 cytokines.

IL-4 has been suggested to be important for the generation of allergen-specific Th2 cells during sensitization (37), and the roles of Ras–MAPK activation in IL-4 production and Th2 differentiation have been investigated. Although some reported no effect on IL-4 production in T cells when inhibiting ERK activity (37), others report a decrease (19, 38) or increase in IL-4 expression and subsequent Th2 differentiation (20, 21). In the present paper, we showed that IL-4 and IFN-γ production in vitro from Th cells in response to anti-TCR from Spred-1–deficient mice is normal. Furthermore, the levels of IL-4 in the airways and serum IgE after allergen challenge in vivo in Spred-1–deficient mice were comparable to those in WT mice. Thus, we consider that Spred-1 may not influence Th2 differentiation.

The higher numbers of IL-5–dependent colonies from the bone marrow and spleen from Spred-1$^{-/-}$ mice than those from WT mice suggest that Spred-1–deficient mice contain an increased number of eosinophil progenitors. The mechanism for this is not clear at present. Our paper shows that Spred-1 inactivation by a dominant negative mutant enhances IL-5 signals via the Ras–MAPK pathway and IL-5–induced cell proliferation using the IL-5–dependent murine B cell line, Y16. Therefore, Spred-deficient eosinophil progenitor cells may respond more efficiently to IL-5. However, we could not rule out the possibility that Spred-1–deficient mice contain a higher number of stem cells, which respond better to the stem cell factor. Allergen-induced eosinophilia is probably due to a higher response of progenitors to IL-5. IL-5 activates several kinases, including Btk, JAK2, Lyn, and Raf-1, as well as the phosphatase SHP2 (32, 39, 40). In eosinophils, JAK2 and Lyn appear to be important for cell proliferation and survival, whereas Raf-1 seems to play a central role in regulating cell function, such as degranulation, adhesion, and survival (41). Because IL-5–induced eosinophilia is markedly enhanced and prolonged in Spred-1–deficient mice, Spred-1 might affect eosinophil proliferation and/or survival in response to IL-5.

Our present data suggest that Spred-1 regulates IL-13 expression through the inactivation of Ras–MAPK signals. This hypothesis is supported by the previous findings that eosinophils have the potential to produce IL-13 (24, 42) and that the Ras–ERK pathway mediates induction of IL-13 expression in T cells (43) and mast cells (44). The downstream functional target molecules of Ras–MAPK in IL-13 induction in response to IL-5 have not been clarified. It has been reported that the IL-5 receptor expression on airway eosinophils is down-regulated after an inhaled allergen challenge and that this is associated with a loss of IL-5 responsiveness (45, 46). However, it is unlikely that Spred-1 modulates IL-5 receptor expression because a specific inhibitor of MAPK kinase (MEK) 1 had no effect on the down-regulation of IL-5 receptor α-chain in eosinophils (47).

We demonstrated that the IL-5–mediated proliferation of Y16 cells is inhibited by WT Spred-1 transfection, whereas it is increased by ΔC-Spred-1 in the present paper. Furthermore, a MEK inhibitor, U0126, blocked IL-5–induced proliferation in Y16 cells, and the inhibitory effect of U0126 was similar between parental Y16 cells and ΔC-Spred-1 transfectants. These data suggest that Spred-1 regulates IL-5–dependent proliferation by modulating the ERK pathway. It has already been demonstrated that pharmacological inhibitor of ERK phosphorylation attenuates allergen-induced airway reactions (48). Our findings and the previous paper suggest that the Ras–ERK pathway is critical in the development of eosinophilic inflammation and AHR.

Given the variety of cellular distribution of Spred-1 expression including airway epithelial cells, endothelial cells (49), hematopoietic progenitor cells (unpublished data), and eosinophils in the present paper, it is unlikely that all of the asthma phenotypes in Spred-1$^{-/-}$ mice are due to Spred-1 deficiency on eosinophils. In addition, other Spred family members might also regulate eosinophilia and allergen-induced AHR. However, our data clearly demonstrate that Spred-1$^{-/-}$ mice showed prominent airway eosinophilia and intact Th1/Th2 differentiation, and Spred-1$^{-/-}$ eosinophils are more sensitive to IL-5–induced proliferation and Raf-1/ERK activation, as well as eotaxin-induced migration in vitro. Therefore, it is possible that hyperresponsive eosinophils to IL-5 and eotaxin in Spred-1$^{-/-}$ mice may predominantly contribute to the airway pathology in these mice.

Our studies provide evidence that Spred-1 is critical for the IL-5 response and IL-13 production and for allergen-

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induced asthma without influencing Th2 development. Although the relationship between total IgE and asthma prevalence is well known (50), an atopic status is not an associated finding in severe asthma (51). There is considerable evidence to support a critical role for the involvement of Th2 cytokines and eosinophils both in atopic and in nonatopic asthma. In particular, IL-5 is critical for eosinophilia, and IL-13 has the potential to modulate airway inflammation and AHR independently of IgE in animal models. Therefore, we propose that Spred-1 may be a useful therapeutic target to compensate for atopic and nonatopic asthma.

MATERIALS AND METHODS

Mice. A genomic library from the 129/SV mouse strain (Stratagene) was screened with a cDNA probe of the mouse Spred-1, and several overlapping positive clones, including from second to fourth exons, were identified. The targeting vector was constructed by replacing the fourth exon with a pgk-neo cassette while preserving 5-Kb (left arm) and 3-Kb (right arm) flanks of homologous sequences (Fig. 1 A). The hsv-TK gene was inserted for negative selection. Homologous recombination in murine embryonic stem cells was performed as described previously (52) and was confirmed by Southern blot analysis. The chimeric mice were backcrossed to C57BL/6 three times. The resultant F3 mice were intercrossed to obtain the offspring for analysis. Genomic PCR was performed as described previously (53). The following primer sets were used: Spred-1 WT, 5′-GCAGACTACAGACATCCGGACATGTGG-3′ and 5′-CGCATGGCCCCAATGATACCGGCAAG-3′; Spred-$1^{-/-}$, 5′-CGAGATCAGCAGCCTCTGTTCCACATAC-3′ and 5′-CCAAGAGAGCTGAGGATGAACTCACCG-3′.

RT-PCR. To detect Spred-1, total RNA was extracted from the bone marrow, spleen, and brain using TRIzol reagent (GIBCO BRL). RT-PCR was performed using a GeneAmp RNA PCR kit (PE Biosystems) according to the manufacturer's instructions. The specific primers for Spred-1 were 5′-GATGAGCGAGGAGACGGCGAC-3′ and 5′-GTCTCTGAGTCTCTCTCCACGGA-3′.

Sensitization and challenge. 10–12-wk-old C57BL/6 mice, Spred-$1^{-/-}$ mice, or their WT littermates were sensitized with intraperitoneal injections of 20 μg OVA (Grade V; Sigma-Aldrich) plus 2.25 mg aluminum hydroxide (Pierce Chemical Co.) on days 1 and 14. On days 26–28, mice received aerosol challenge containing either saline or 1% OVA for 20 min/d.

Measurement of airway responsiveness. On day 30, 36 h after the last aerosol challenge, mice were ventilated to measure AHR to acetylcholine aerosol as described previously (22, 54). Airway opening pressure was measured with a differential pressure transducer and continuously recorded. Stepwise increases in the acetylcholine dose were given with an ultrasonic nebulizer. All animal experiments were approved by the Committee on Animal Research, Faculty of Medicine, Kyushu University. The data were expressed as the provocative concentration 200 (PC_{200}), the concentration at which airway pressure was 200% of its baseline value, and PC_{200} was calculated by log-linear interpolation for individual mice. Lower log PC_{200} values represent greater AHR. The serum levels of total and OVA-specific IgE were analyzed by ELISA with rat anti–mouse IgE (Serotec Ltd.).

BAL and cytokine measurements. Mice were exsanguinated with a lethal dose of pentobarbital, and their lungs were gently lavaged with 1 ml of 0.9% saline via a tracheal cannula. Total and differential BAL cell counts were performed as described previously (22). Samples were centrifuged at 2,000 revolutions/min for 10 min, and the supernatants were stored at −80°C. Mouse IL-4, IL-5, IL-13, and IFN-γ were quantified using ELISA kits (Biosource International) according to the manufacturer's protocols. Measurement of cytokine production in BAL fluid was assessed by ELISA assays.

Histological assessment. Lungs were fixed with 10% formalin, and tissue sections were stained with Alcian blue/PAS to determine the presence of mycin glycoconjugates (22). The numerical scores for the abundance of PAS-positive mucus-containing cells in each airway were determined as follows: 0, <5% PAS-positive cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75% (36).

Collection of lung cells and lymph node cells. Enzymatic digestion of the lungs was performed with collagenase type 1A, hyaluronidase, and DNase, and the samples were filtered through a 52-μm nylon mesh. The erythrocytes were removed by lysis. Cells were stained with FITC-labeled anti-NK1.1 mAb, PE-labeled anti-CD19 mAb, PerCP-labeled anti-CD3 mAb, and allophycocyanin-labeled anti-CD4 mAb. After flow cytometric analysis using a FACSCalibur with CellQuest software (BD Biosciences), the absolute numbers of lung immune cells were calculated. Draining lymph node cells were collected from paratracheal and mediastinal lymph nodes, and $CD4^+$ T cells isolated with a magnetic cell sorter (Miltenyi Biotec) were stimulated with a combination of 30 μg/ml anti-TCR mAb (H57-597) and 1 μg/ml anti-CD28 mAb (PV-1) for 48 h. ELISA assays for cytokine production were performed as described previously (54).

Cell purification and induction of helper T cells. Naive $CD4^+$ T cells isolated from spleens were stimulated with a combination of anti-TCR mAb and anti-CD28 mAb, and 10 U/ml rIL-12 and anti–IL-4 mAb (11B11) were added for Th1 development, while 100 U/ml rIL-4 and anti–IL-12 mAb (C15.6 and C17.8) were added for Th2 development. ELISA assays for cytokine production and intracellular cytokine staining were performed as described previously (54).

In vitro colony assay. Single cell suspensions were isolated from the bone marrow or spleen of WT and Spred-$1^{-/-}$ mice. The erythrocytes were removed by lysis using NH_4Cl and 3×10^3 cells were plated in methylcellulose (Methocult3434; StemCell Technologies Inc.) containing 10 ng/ml of IL-5. On day 14, the numbers of colonies were counted microscopically.

Isolation of lung eosinophils. BAL was performed in OVA-sensitized and -challenged mice by three repeated lavages with RPMI 1640 containing penicillin-streptomycin at 25 cm H_2O. The lavage fluid was collected and incubated for 30 min to remove macrophages from the cell suspension by adherence to plastic. Eosinophils were isolated by a negative selection strategy, removing B cells and T cells with Ab-conjugated magnetic beads (MACS; Miltenyi Biotec) specific for CD45-R (B220) and CD90 (Thy1.2), respectively. The purity of the recovered eosinophils was confirmed to be >98% by staining cytospin preparations with Diff-Quik (neutrophils comprise the contaminating cell populations). Eosinophils were cultured at 10^6 cells/ml for the indicated times.

IL-5 and IL-13 treatment in vivo. Recombinant murine IL-5 (10,000 U/d) was injected intraperitoneally into WT- or Spred-1–deficient mice for 4 d (30). Differential counts were performed by examination of blood smears stained with a modified Wright-Giemsa stain at various time points.

IL-13 administration was performed as described previously (22). A recombinant murine IL-13 solution (0.5 g) or a vehicle solution was instilled intratracheally on days 1, 3, and 5. BAL was performed on day 6, 24 h after the last instillation, and BAL cell differentials were determined.

Cell lines and cultures. IL-5–dependent murine cell line, Y16 was cultured in an RPMI 1640 medium containing 5% FBS, 50 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 5 U/ml IL-5 (55). The retrovirus-packaging cell line PLAT-E was maintained in DMEM containing 10% FBS.

Bone marrow cells from the femoral bone of Spred-$1^{-/-}$ mice or their WT littermates were cultured in RPMI 1640 supplemented with 100 U/ml murine IL-5, 30% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol for 2 wk to generate populations of BMEos that were >95% pure.

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Chemotaxis assay. The chemotaxis assay was performed in a 96-well disposal chemotaxis plate (5-μm pore size; Neuro Probe). In brief, eotaxin was diluted in RPMI 1640 with 0.1% BSA and placed in the bottom wells (27 μl), 25 μl of cell suspension at 4×10^6 cells/ml was placed on the top well of the chamber, which was separated from the bottom well by a polycarbonate filter. The plate was incubated for 60 min at 37°C in a humidified incubator with 5% CO_2. The cells remaining on top of the filters were absorbed off, the filter tops were carefully washed, and the plates were centrifuged to pellet all cells on the undersides of the filters. The filters were removed, and cells in the bottom wells were counted by light microscopy. Data are reported as a migration index, calculated as follows: (number of cells migrating to chemoattractant)/(number of cells migrating to vehicle).

Cell proliferation assay. Cell proliferation was assayed using a Cell Counting Kit (Dojindo). In brief, 10^3 Y16 cells were plated in 96-well plates in RPMI 1640 medium with IL-5 and cultured for 48 h. After adding WST-8 and 1-methoxy PMS, optical density for 450 nm was measured.

Plasmids, transfection, and infection. WT Spred-1 and COOH-terminal–truncated Spred-1 (ΔC-Spred-1) were subcloned into pMY-IRES-GFP vectors for retrovirus infection as described previously (56). In brief, pMY-IRES-GFP vectors were transfected into a PLAT-E packaging cell line using the transfection reagent FuGENE 6 (Boehringer) to obtain the viruses (35). Y16 cells (2×10^5 cells) were infected with viruses on a RetroNectin (TaKaRa)-coated plate for 24 h in the presence of 5 U/ml IL-5. Cells were washed three times with PBS, resuspended in RPMI 1640, 5% FBS containing 5 U/ml IL-5, and incubated for the indicated times. 10^4 cells were analyzed for GFP fluorescence on a flow cytometer.

Immunochemical analysis. Immunoprecipitation and immunoblotting were performed using anti-STAT5, anti-ERK2 (Santa Cruz Biotechnology, Inc.), anti-Flag (M2; Sigma-Aldrich), anti–phospho-TAT5, anti–phospho-ERK2 (Cell Signaling), and anti–phospho-Ser338-Raf-1 (Upstate Biotechnology) antibodies as described previously (56, 57). Anti–Spred-1 antibody was prepared by immunizing rabbits (17).

Statistical analysis. Values were expressed as the mean ± SEM. Differences among groups were analyzed using unpaired Student's *t* tests or an analysis of variance together with a post-hoc Bonferroni analysis. Nonparametric data were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test. p-values <0.05 were considered to be significant.

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