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Growth Factors, Cytokines, Cell Cycle Molecules

Thymic Stromal Lymphopoietin Contributes to Myeloid Hyperplasia and Increased Immunoglobulins, But Not Epidermal Hyperplasia, in RabGEF1-Deficient Mice

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Mice overexpressing the proallergic cytokine thymic stromal lymphopoietin (TSLP) in the skin develop a pathology resembling atopic dermatitis. RabGEF1, a guanine nucleotide exchange factor for Rab5 GTPase, is a negative regulator of IgE-dependent mast cell activation, and *Rabgef1*$^{-/-}$ and TSLP transgenic mice share many similar phenotypic characteristics, including elevated serum IgE levels and severe skin inflammation, with infiltrates of both lymphocytes and eosinophils. We report here that *Rabgef1*$^{-/-}$ mice also develop splenomegaly, lymphadenopathy, myeloid hyperplasia, and high levels of TSLP. *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice, which lack TSLP/TSLP receptor (TSLPR) signaling, had levels of blood neutrophils, spleen myeloid cells, and serum IL-4, IgG1, and IgE levels that were significantly reduced compared with those in *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice. However, *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice, like Rag1- or eosinophil-deficient *Rabgef1*$^{-/-}$ mice, developed cutaneous inflammation and epidermal hyperplasia. Therefore, in *Rabgef1*$^{-/-}$ mice, TSLP/TSLPR interactions are not required for the development of epidermal hyperplasia but contribute to the striking myeloid hyperplasia and overproduction of immunoglobulins observed in these animals. Our study shows that RabGEF1 can negatively regulate TSLP production *in vivo* and that excessive production of TSLP contributes to many of the phenotypic abnormalities in *Rabgef1*$^{-/-}$ mice. However, the marked epidermal hyperplasia, cutaneous inflammation, and increased numbers of dermal mast cells associated with RabGEF1 deficiency can develop via a TSLPR-independent pathway, as well as in the absence of Rag1 or eosinophils. ***(Am J Pathol 2010, 177:2411–2420; DOI: 10.2353/ajpath.2010.100181)***

Thymic stromal lymphopoietin (TSLP) is an interleukin (IL)-7–like cytokine initially identified as a growth and differentiation factor for lymphocytes, and later implicated as an important regulator of allergic inflammation.[1–5] TSLP binds to the TSLP receptor complex, which consists of an IL-7 receptor α chain (IL-7Rα) and a TSLP receptor (TSLPR) chain.[1–3] TSLPR is an atypical type I cytokine receptor that binds TSLP with low affinity, but the binding affinity with TSLP is markedly enhanced when TSLPR is incorporated into the heterodimeric IL-7Rα/TSLPR receptor complex.[5] TSLP treatment results in the expansion and maturation of human myeloid dendritic cells and the induction of OX40L expression in these cells.[3,6] TSLP-treated myeloid dendritic cells can promote inflammatory responses by inducing OX40-OX40L–dependent Th2 cytokine production in naïve CD4 T cells and by secreting chemokines that recruit Th2 cells and granulocytes.[3,7,8] Moreover, TSLP can induce mast cells to produce Th2 cytokines independently of IgE stimulation.[9] Recently, TSLP has been shown to promote survival, cytokine and chemokine release, and up-regula-

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tion of adhesion molecules in human eosinophils.[10] These observations suggest that TSLP can orchestrate allergic inflammation by activating cells of both the adaptive and the innate immune systems. In accord with this notion, transgenic mice expressing TSLP under the regulation of the keratinocyte-specific promoters for keratin-14[11] or keratin-5[12] develop atopic dermatitis-like skin lesions, with inflammatory infiltrates including eosinophils and lymphocytes, and also exhibit pronounced splenomegaly, lymphadenopathy, and elevated serum levels of IgE and IgG1 antibodies.[11,12] In addition to the development of a disorder resembling atopic dermatitis, mice that overproduce TSLP in the skin are also more susceptible to antigen induced airway inflammation and hyperresponsiveness, suggesting that TSLP contributes to the so-called "atopic march" which is hypothesized to link atopic dermatitis with the later onset of asthma.[13,14]

The mouse ortholog of RabGEF1 (also known as Rabex-5, Rabaptin-5 associated exchange factor for Rab5) was first identified in a survey of differentially expressed mRNA transcripts in mouse mast cells activated by aggregation of high affinity IgE receptors (FcεRI).[15] RabGEF1-deficient (*Rabgef1*$^{-/-}$) mast cells exhibit delayed receptor internalization, elevated and prolonged intracellular signaling events, and enhanced cytokine and mediator release in response to FcεRI- or Kit-dependent activation.[15–17] In addition to its role as a negative regulator of mast cell activation, RabGEF1 can contribute to intracellular vesicular trafficking by catalyzing the conversion of GDP to GTP on members of the Rab5 family of small GTPases, molecules known to be important for early endosome fusion during receptor internalization and for endocytic vesicular trafficking.[18–20]

We previously reported that deletion of RabGEF1 protein in the mouse results in substantial morbidity, including skin lesions characterized by marked epidermal hyperplasia and hyperkeratosis, infiltration of lymphocytes, eosinophils, and other inflammatory cells, and increased numbers of dermal mast cells.[15] Also, the serum of RabGEF1-deficeint mice contains high concentrations of IgE and histamine.[15] Notably, phenotypic abnormalities very similar to those of *Rabgef1*$^{-/-}$ mice also have been observed in transgenic mice which overexpress TSLP in the skin.[11,12] In this study, we show that *Rabgef1*$^{-/-}$ mice exhibit markedly elevated levels of TSLP and IL-4, develop splenomegaly, lymphadenopathy, and expansion of myeloid cells in the blood, bone marrow, and spleen, and produce large numbers of B cells in the spleen. To investigate the contribution of TSLP to the dermatitis and other aspects of the pathology of *Rabgef1*$^{-/-}$ mice, we disrupted TSLP signaling in these mice by breeding *Rabgef1*$^{+/-}$ mice with TSLP receptor-deficient (*TSLPR*$^{-/-}$) mice, which lack expression of TSLPR and are unresponsive to TSLP.[21] Our data indicate that TSLPR-mediated signaling contributes substantially to the development of myeloid hyperplasia, splenomegaly, and high levels of serum IL-4, IgE, and IgG1 in *Rabgef1*$^{-/-}$ mice. By contrast, we show that the spontaneous cutaneous inflammation, increased numbers of dermal mast cells, and marked epidermal hyperplasia and hyperkeratosis observed in *Rabgef1*$^{-/-}$ mice can occur in the absence of TSLPR-mediated signaling, as well as in *Rabgef1*$^{-/-}$ mice which lack mature lymphocytes or eosinophils.

Materials and Methods

Mice

Rabgef1$^{-/-}$ mice on a mixed C57BL/6 and 129/SvEv genetic background[15] were backcrossed to C57BL/6J or BALB/cJ mice for at least 10 generations. No live *Rabgef1*$^{-/-}$ pups have ever been obtained on the C57BL/6 strain, whereas ~19% of the two-week-old pups of BALB/c *Rabgef1*$^{+/-}$ parents were identified as RabGEF1 knock-outs. We created (C57BL/6 × BALB/c)F_1 RabGEF1-deficient mutants by mating C57BL/6-*Rabgef1*$^{+/-}$ and BALB/c-*Rabgef1*$^{+/-}$ mice; ~12% of the two-week-old (C57BL/6 × BALB/c)F_1 pups were genotyped as RabGEF1 knock-outs. Like 129/B6-*Rabgef1*$^{-/-}$ mice, all BALB/c or (C57BL/6 × BALB/c)F_1 *Rabgef1*$^{-/-}$ mice spontaneously developed severe dermatitis. Skin lesions were clearly visible at ~3 weeks after birth when the mice were weaned. However, (C57BL/6 × BALB/c)F_1-*Rabgef1*$^{-/-}$ mice developed skin lesions and signs of morbidity more slowly than did BALB/c-*Rabgef1*$^{-/-}$ mice. *Rag1*$^{-/-}$ mice (C.129S7(B6)-Rag1^{tm1Mom}/J) that had been backcrossed to Balb/cAnNTac for seven generations and eosinophil-deficient *GATA1*$^{\Delta dbl}$ mice (C.Cg-Gata1^{tm6Sho}/J) that had been backcrossed to BALB/c for eight generations were obtained from the Jackson Laboratory (Bar Harbor, ME). TSLP receptor–deficient (*TSLPR*$^{-/-}$) mice on BALB/c background[21] were bred in our facility. *Fcer1a*$^{-/-}$ mice[22] on the BALB/c background were kindly provided by J.-P. Kinet at Harvard School of Medicine. *Rag1*$^{-/-}$, *Fcer1a*$^{-/-}$, *TSLPR*$^{-/-}$, and *GATA1*$^{\Delta dbl}$ mice were mated to BALB/c-*Rabgef1*$^{+/-}$ mice to generate single or double mutant mice.

All animal care and experimentation were conducted in compliance with current National Institutes of Health guidelines and with the approval of the Stanford University Institutional Animal Care and Use Committee.

Flow Cytometry

Cells were blocked with unconjugated anti-CD16/CD32 on ice for 10 minutes and then stained with PE-labeled anti–Gr-1 (RB6-8C5, 0.3 μg/ml), FITC-labeled anti–Mac-1 (M1/70, 2.5 μg/ml), and PE-labeled anti-B220 (RA3-6B2, 1 μg/ml) antibodies (BD Biosciences, San Jose, CA). The expression of cell surface markers was analyzed on a FACSCaliber (BD Biosciences) using FlowJo Software (version 8.7.1). Dead cells (stained with 7-aminoactinomycin D (Sigma, St. Louis, MO) or propidium iodide (PI; Invitrogen, Carlsbad, CA) were not included in the analyses.

Peripheral Blood and Bone Marrow Leukocyte Counts

Numbers of total leukocytes, neutrophils, monocytes, and lymphocytes in heparinized blood were quantified using the Abbott Cell-Dyn 3500 automated hematology analyzer. We quantified nucleated bone marrow cells recovered from two femora plus two tibiae per mouse.

Histology and Immunohistochemistry

Formalin-fixed tissues were embedded in paraffin, and 4-μm tissue sections were stained with hematoxylin and eosin or with 0.1% (vol/vol) toluidine blue (LabChem, Inc., Pittsburgh, PA) , pH 1.0 (for detection of mast cells). To quantify eosinophils, paraffin-embedded skin sections were stained with Congo red (Sigma-Aldrich, St. Louis, MO) as previously described.[23] Numbers of dermal mast cells or eosinophils were counted in 8–12 consecutive microscopic fields. Epidermal thickness and numbers of mast cells or eosinophils per horizontal ear cartilage field length (mm) or per mm^2 abdominal skin area were quantified using Image-Pro Plus Version 6.0 software (Media Cybernetics, Bethesda, MD).

For localization of TSLP protein, skin sections were incubated with goat anti-mouse TSLP antibody (2 μg/ml, R&D Systems, Minneapolis, MN) or with normal goat IgG (R&D Systems), at 4°C for 42 hours, washed, and with biotinylated rabbit-anti-goat IgG for 30 minutes at room temperature. After incubating with ABC solution (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, positive staining was detected by DAB (3,3′-diaminobenzidine; Sigma-Aldrich) and sections were counterstained with Meyer's hematoxylin. For detection of a keratinocyte marker, a mouse anti-human keratin monoclonal antibody (AE1/AE3) (LifeSpan BioSciences, Seattle, WA), that has reactivity to the 19 known human epidermal keratins and can also react with mouse tissue, was used to stain cytokeratin using a Vector^R M.O.M. detection kit (Vector Laboratories, Inc. Burlingame, CA).

ELISA

A single-cell suspension of splenocytes was prepared by mechanical disaggregation of the whole spleen, and red blood cells were lysed in RBC lysing buffer (Sigma-Aldrich, St. Louis, MO). Ear skin, spleen, and splenocyte lysates were prepared by sonicating the tissuses in Tper EDTA-free lysis buffer (Pierce, Rockford, IL) containing protease inhibitors (Roche, Indianapolis, IN), and total protein concentrations in the supernatants were measured by a Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA) as in ref.[24] Concentrations of TSLP protein in serum, cell, or tissue lysates were measured by ELISA (R&D Systems). Serum IL-4 was measured by ELISA (BD Biosciences). Total serum IgE and IgG1 titers were measured by ELISA as described.[24]

MPO Assay

100 μl MPO substrate buffer (50 mmol/L phosphate buffer pH 6.0, 0.05% [vol/vol] H_2O_2 [Sigma-Aldrich, St. Louis, MO], 0.4 mg/ml o-phenylenediamine dihydrochloride [OPD tablets; Sigma-Aldrich]) was added to 50 μl of the diluted supernatants of ear skin or spleen lysates (prepared as described above) or to recombinant MPO (CalBiochem, Gibbstown, NJ) used as the standard protein. After 5 to 10 minutes, 50 μl 1 N HCl was added to stop the reaction, and OD was measured by a plate reader.

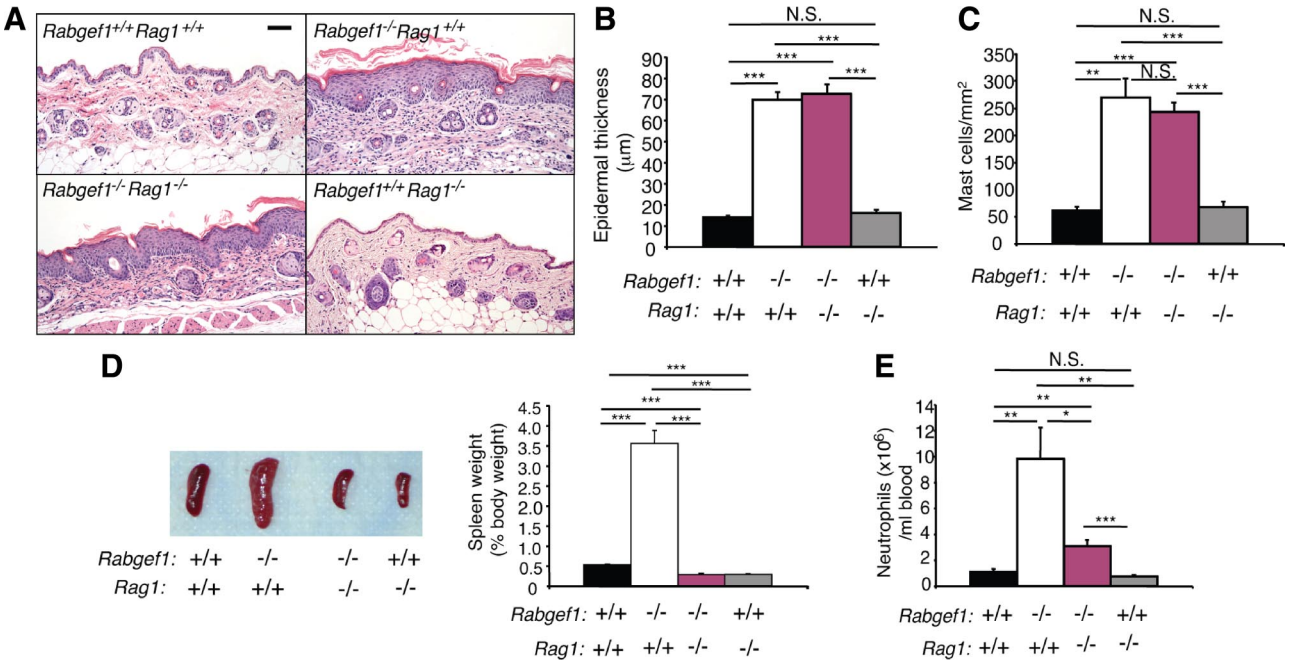

Figure 1. Variable Rag1-dependence of different phenotypic abnormalities in *Rabgef1*$^{-/-}$ mice. *Rabgef1*$^{+/-}$ mice were bred with *Rag1*$^{-/-}$ mice to generate Rag1-deficient *Rabgef1*$^{+/-}$ mice, and mice with each of the four possible genotypes were analyzed. **A:** Representative microscopic images of histological sections of abdominal skin stained with hematoxylin and eosin; Scale bar = 50 μm. Epidermal thickness (**B**) and mast cell numbers (**C**) in the dermis of abdominal skin. **D:** Gross images and weight (as the percentage of body weight) of the spleens (**E**). Numbers of neutrophils in the peripheral blood. All mice were of the BALB/c strain and 4–5 weeks old. Data shown are the mean + SEM from three to eight mice for histological analysis of the skin, from seven to 15 mice for assessment of spleen weights, and from seven to 10 mice for neutrophil counts in the blood. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; N.S., not significant ($P > 0.05$).

Statistics

Results represent means ± or + SEM. Differences between results were assessed for statistical significance by the unpaired Student's *t*-test, 2-tailed.

Results

Rabgef1$^{-/-}$ *Mice Develop Myeloid Hyperplasia and Expansion of B Lymphocytes in the Spleen*

We found that, in addition to developing severe dermatitis and elevated serum IgE levels,[15] *Rabgef1*$^{-/-}$ mice developed splenomegaly and markedly enlarged lymph nodes (see Supplemental Figure S1A at *http://ajp.amjpathol.org*). *Rabgef1*$^{-/-}$ mice also developed significant increases in numbers of neutrophils and/or monocytes in the spleen (see Supplemental Figure S1B at *http://ajp.amjpathol.org*), bone marrow (see Supplemental Figure S1C at *http://ajp.amjpathol.org*), and peripheral blood (see Supplemental Figure S1D at *http://ajp.amjpathol.org*) of these mice. In addition to the changes observed in the myeloid compartment, the numbers of B lymphocytes were substantially increased in the spleen but were significantly lower in the bone marrow of *Rabgef1*$^{-/-}$ mice compared to those in the wild-type mice (see Supplemental Figure S1E at *http://ajp.amjpathol.org*). Thus, deletion of RabGEF1 expression resulted in an expansion of myeloid cells in the blood, spleen, and bone marrow, and abnormal B lymphopoiesis, with significant increases in B cell numbers in the spleen.

Role of Adaptive Immunity in the Development of the Phenotypic Abnormalities of Rabgef1$^{-/-}$ *Mice*

To assess whether adaptive immunity contributed to the development of spontaneous dermatitis, epidermal hypersplasia, increased numbers of dermal mast cells, and other pathology in RabGEF1-deficient mice, we generated *Rabgef1*$^{-/-}$*Rag1*$^{-/-}$ mice by breeding RabGEF1 mutant mice with *Rag1*$^{-/-}$ mice, which can't produce mature lymphocytes because of their inability to undergo V(D)J recombination of immunoglobulin and T cell receptor genes.[25] A lack of Rag1 had no significant effect on epidermal thickness, epidermal hyperkeratosis, or dermal mast cell num-

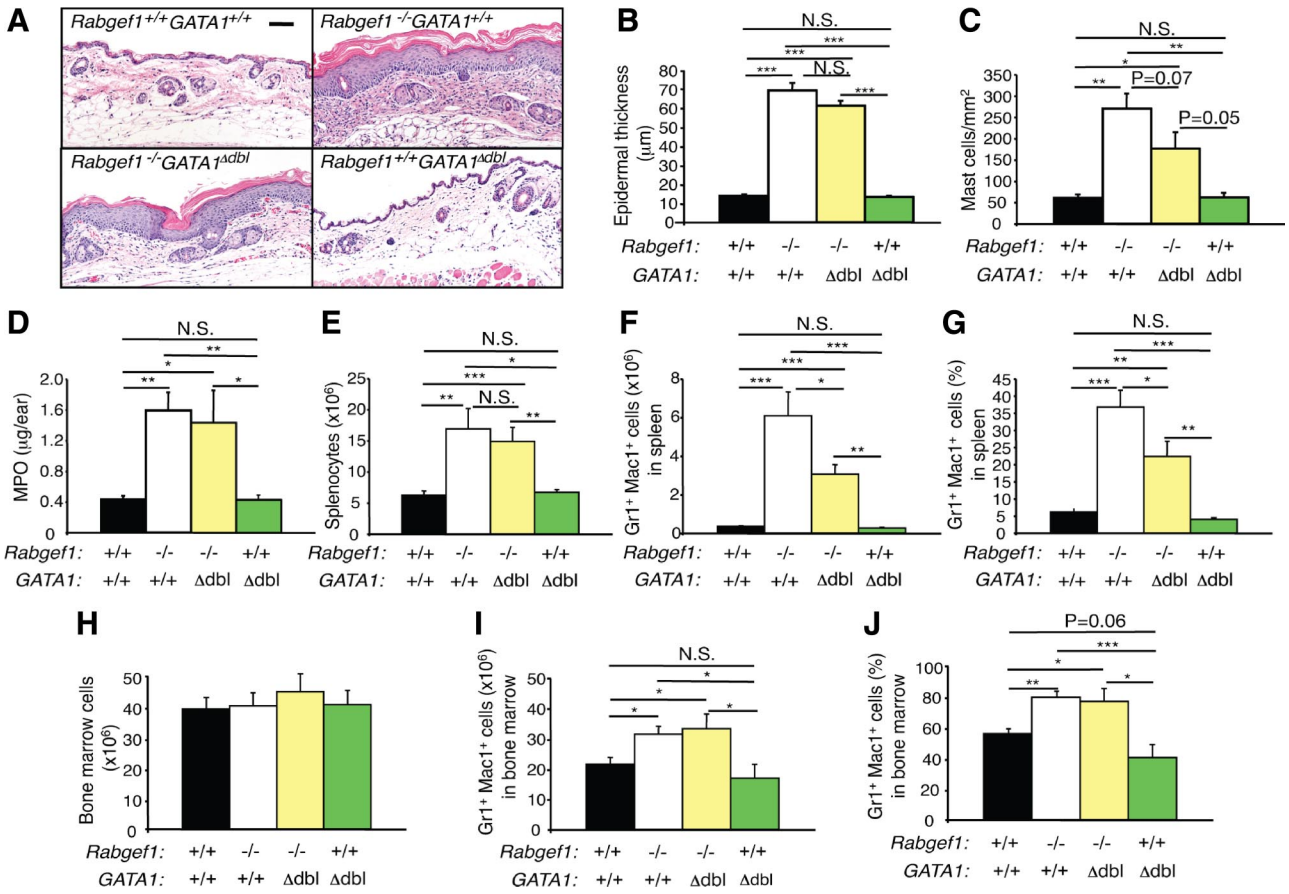

Figure 2. Development of skin pathology and expansion of myeloid cells in eosinophil-deficient *Rabgef1*$^{-/-}$ mice. *Rabgef1*$^{+/-}$ mice were bred with *GATA1*$^{\Delta dbl}$ mice to generate eosinophil-deficient *Rabgef1*$^{+/-}$ mice, and mice with each of the four possible genotypes were analyzed. **A:** Representative microscopic images of histological sections of abdominal skin stained with hematoxylin and eosin; Scale bar = 50 μm. Epidermal thickness (**B**) and mast cell numbers (**C**) in the dermis of abdominal skin. **D:** Myeloperoxidase (MPO) levels in the ear skin. **E:** Total leukocyte numbers in the spleen. **F** and **G:** Numbers (**F**) and percentages (**G**) of Gr1$^+$Mac1$^+$cells in the spleen, as assessed by flow cytometry. **H:** Total leukocyte numbers in the bone marrow. **I** and **J:** Numbers (**I**) and % (**J**) of Gr1$^+$Mac1$^+$ cells in the bone marrow, as assessed by flow cytometry. All mice were of the BALB/c strain and 4–6 weeks old. Data shown are the mean + SEM from three to eight mice per group for analyses of the skin, from six to nine mice per group for analyses of the spleen, and from five to nine mice per group for analyses of the bone marrow. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; N.S., not significant ($P > 0.05$).

bers in *Rabgef1*$^{-/-}$ mice (Figure 1, A–C). However, *Rabgef1*$^{-/-}$*Rag1*$^{-/-}$ spleens were notably smaller than *Rabgef1*$^{-/-}$*Rag1*$^{+/+}$ spleens and also slightly but significantly smaller than the spleens of the corresponding wild-type mice (Figure 1D). Both *Rabgef1*$^{-/-}$*Rag1*$^{-/-}$ and *Rabgef1*$^{-/-}$*Rag1*$^{+/+}$ mice exhibited increased neutrophil numbers in the blood compared to those observed in the wild-type mice, but numbers of circulating neutrophils in *Rabgef1*$^{-/-}$*Rag1*$^{-/-}$ mice were significantly reduced compared to those of *Rabgef1*$^{-/-}$*Rag1*$^{+/+}$ mice (Figure 1E). These data indicate that while adaptive immunity is not essential for the development of aspects of skin pathology in *Rabgef1*$^{-/-}$ mice, it does contribute to the development of lymphohematopoietic abnormalities in these animals. Our results also show that the high levels of IgE and IgG1 immunoglobulin production in *Rabgef1*$^{-/-}$ mice (which do not occur in *Rabgef1*$^{-/-}$*Rag1*$^{-/-}$ mice) do not contribute significantly to the development of the assessed aspects of animals' skin pathology. In accord with our findings with *Rabgef1*$^{-/-}$*Rag1*$^{-/-}$ mice, we also found that *Rabgef1*$^{-/-}$ mice which lack the α chain of the FcεRI[22] (the IgE binding component of the high affinity IgE receptor complex) and thus are unable to respond to FcεRI-dependent cell activation, exhibit skin pathology which is similar in severity to that of *Rabgef1*$^{-/-}$ mice expressing the wild-type FcεRI α chain (see Supplemental Figure S2, A–D at *http://ajp.amjpathol.org*).

Eosinophils Are Not Required for Epidermal Hyperplasia or Myeloid Hyperplasia in Rabgef1$^{-/-}$ *Mice*

We found extensive eosinophil infiltration in the skin, bone marrow, spleen, lymph nodes, stomach, and liver of *Rabgef1*$^{-/-}$ mice (see Supplemental Figure S3A at *http://ajp.amjpathol.org* and unpublished data). To assess the role of eosinophils in the phenotypic abnormalities of RabGEF1-deficient mice, we generated eosinophil-deficient *Rabgef1*$^{-/-}$ mice by breeding *Rabgef1*$^{+/-}$ mice with eosinophil-deficient *GATA1*$^{\Delta dbl}$ mice.[26] *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ and *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ mice exhibited very similar increases in epidermal thickness and hyperkeratosis (Figure 2, A and B). Although there was a slight (~34%) reduction in mast cell numbers in the inflamed abdominal skin of *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ mice compared to values for *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ mice ($P = 0.07$ by unpaired Student's *t*-test) (Figure 2C), mast cell numbers in the inflamed ear skin of *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ and *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ mice were very similar (see Supplemental Figure S3B at *http://ajp.amjpathol.org*). MPO levels in the ear skin were also very similar between *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ and *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ mice and were highly elevated in *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ and *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ mice compared to those in the wild-type or *Rabgef1*$^{+/+}$*GATA1*$^{\Delta dbl}$ mice (Figure

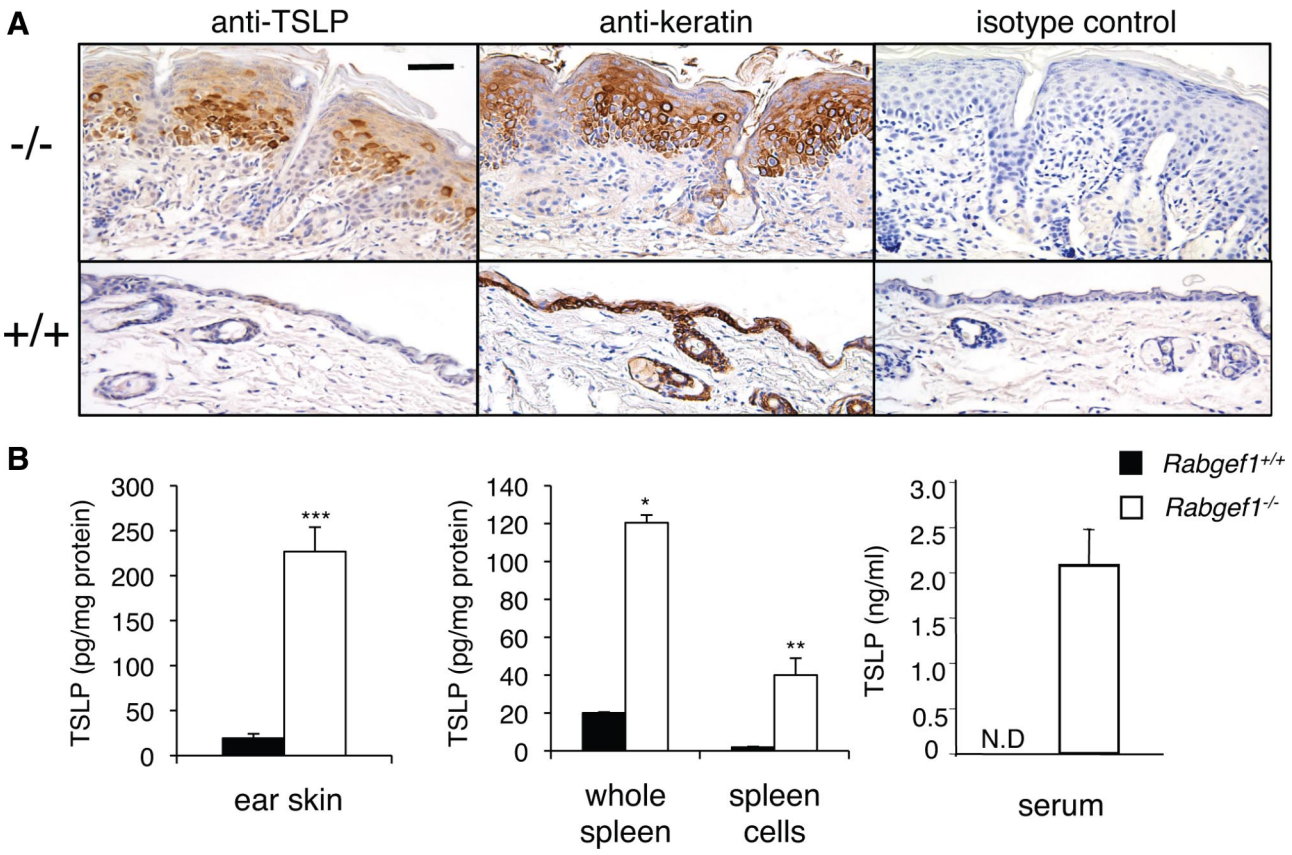

Figure 3. *Rabgef1*$^{-/-}$ mice have high levels of TSLP. **A:** Localization of TSLP and keratin in the skin of *Rabgef1*$^{+/+}$ (+/+) and *Rabgef1*$^{-/-}$ (−/−) mice by immunohistochemistry; Scale bar = 50 μm. **B:** Concentrations of TSLP protein in ear skin, whole spleens, splenocytes, and serum of *Rabgef1*$^{+/+}$ and *Rabgef1*$^{-/-}$ mice by ELISA. Data shown in **B** are the mean + SEM from five to seven mice per group. All mice were of the BALB/c strain and 4–6 weeks old. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus *Rabgef1*$^{+/+}$ controls. N.D., not detectable.

2D). Like *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ mice, *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ mice exhibited enlarged spleens (Figure 2E), as well as expansion of Gr1^{+}Mac1^{+} cells in the spleen (Figure 2, F and G) and bone marrow (Figure 2, H–J). The numbers and % of Gr1^{+}Mac1^{+} cells in the spleen of *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ mice were reduced compared to those in *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ mice, probably largely reflecting the absence of eosinophils in such animals (Figure 2, F and G). However, there were no detectable differences in the numbers and % of Gr1^{+}Mac1^{+} cells in the bone marrow between *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ and *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ mice (Figure 2, I and J). Moreover, the peripheral blood of *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ and *Rabgef1*$^{-/-}$*GATA1*$^{\Delta dbl}$ mice exhibited similar levels of WBC, neutrophilia and lymphopenia (see Supplemental Figure S4, A–E at *http://ajp.amjpathol.org*). Taken together, these data indicate that eosinophils are not required for the development of epidermal hyperplasia, myeloid hyperplasia, blood neutrophilia, or blood lymphopenia in *Rabgef1*$^{-/-}$ mice, and that, depending on the anatomical site, eosinophils make little or no contribution to the increased numbers of dermal mast cells in the skin lesions of these animals.

TSLPR-Dependent Signaling Is Not Required for Epidermal Hyperplasia in Rabgef1$^{-/-}$ *Mice*

The targeted overexpression of TSLP in keratinocytes in mice results in an atopic dermatitis-like skin disease accompanied by massive lymphadenopathy and splenomegaly,[11,12] increased numbers of eosinophils,[11,12] elevated serum IgE levels,[11,12] myeloid hyperplasia,[11,12] and abnormal B lymphogenesis.[27] Similar to *Rabgef1*$^{-/-}$ mice, the keratinocyte-specific TSLP transgenic mice develop skin disease that occurs with minimal contribution of T or B lymphocytes.[12] We detected strikingly elevated levels of TSLP in *Rabgef1*$^{-/-}$ mice in keratinocytes (by immunohistochemistry), and in the ear skin, whole spleen, splenocytes, and serum (by ELISA) (Figure 3, A and B), raising the possibility that TSLP contributes to the development of pathology in these animals. To test this hypothesis, we bred *Rabgef1*$^{+/-}$ mice with TSLP receptor-deficient (TSLPR$^{-/-}$) mice[21] to disrupt the signaling pathway mediated by TSLPR in these animals.

Rabgef1$^{-/-}$*TSLPR*$^{-/-}$ mice were grossly indistinguishable from *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice (see Supplemental Figure S5A at *http://ajp.amjpathol.org*). Both *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice were smaller than the wild-type or *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice and developed prominent skin lesions (see Supplemental Figure S5, A and B at *http://ajp.amjpathol.org*). Like *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice, *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice also exhibited high concentrations of serum TSLP (see Supplemental Figure S5C at *http://ajp.amjpathol.org*). Histological analysis of the skin lesions in *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice showed comparable levels of epidermal hyperplasia and hyperkeratosis in their skin lesions (Figure 4A). These observations were confirmed by morphometric measurements showing that *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice developed skin lesions with similar increases in epidermal thickness (Figure 4B and see Supplemental Figure S5D at *http://ajp.amjpathol.org*) and dermal mast cell numbers (Figure 4C and see Supplemental Figure S5E at *http://ajp.amjpathol.org*), whether cutaneous lesions were sampled from ear pinnae (Supplemental Figure S5, D and E at *http://ajp.amjpathol.org*) or abdominal skin (Figure 4, B and C). On the other hand, numbers of eosinophils in the dermis of *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ skin were reduced to approximately the wild-type levels (see Supplemental Figure S3A at *http://ajp.amjpathol.org*), suggesting that TSLPR may regu-

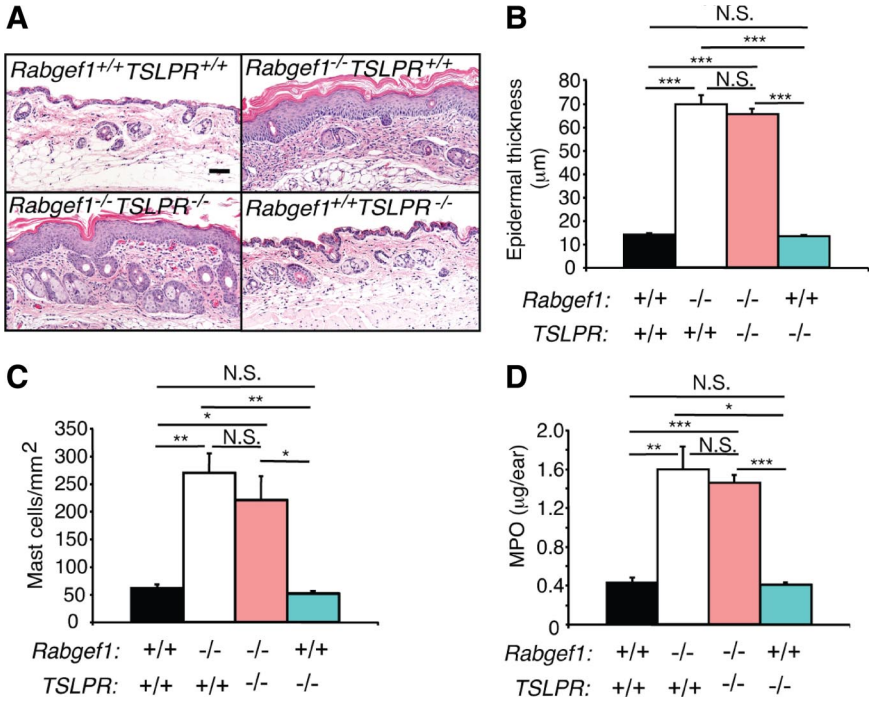

Figure 4. TSLPR-independent development of skin pathology in *Rabgef1*$^{-/-}$ mice. **A:** Representative microscopic images of abdominal skin sections of *Rabgef1*$^{+/+}$*TSLPR*$^{+/+}$ (wild type), *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$, *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$, and *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice. Sections were stained with hematoxylin and eosin; Scale bar = 50 μm. Images of skin of *Rabgef1*$^{+/+}$*TSLPR*$^{+/+}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice are identical to those labeled *Rabgef1*$^{+/+}$*GATA1*$^{+/+}$ and *Rabgef1*$^{-/-}$*GATA1*$^{+/+}$ in Figure 2A, because these images represent the same genotypes in these figures (ie, wild-type mice and mice deficient only in RabGEF1). Epidermal thickness (**B**) and mast cell numbers (**C**) in the dermis of abdominal skin. **D:** Myeloperoxidase (MPO) levels in the ear skin. All mice were of the BALB/c strain and 4–6 weeks old. Data shown are the mean + SEM from three to eight mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; N.S., not significant ($P > 0.05$).

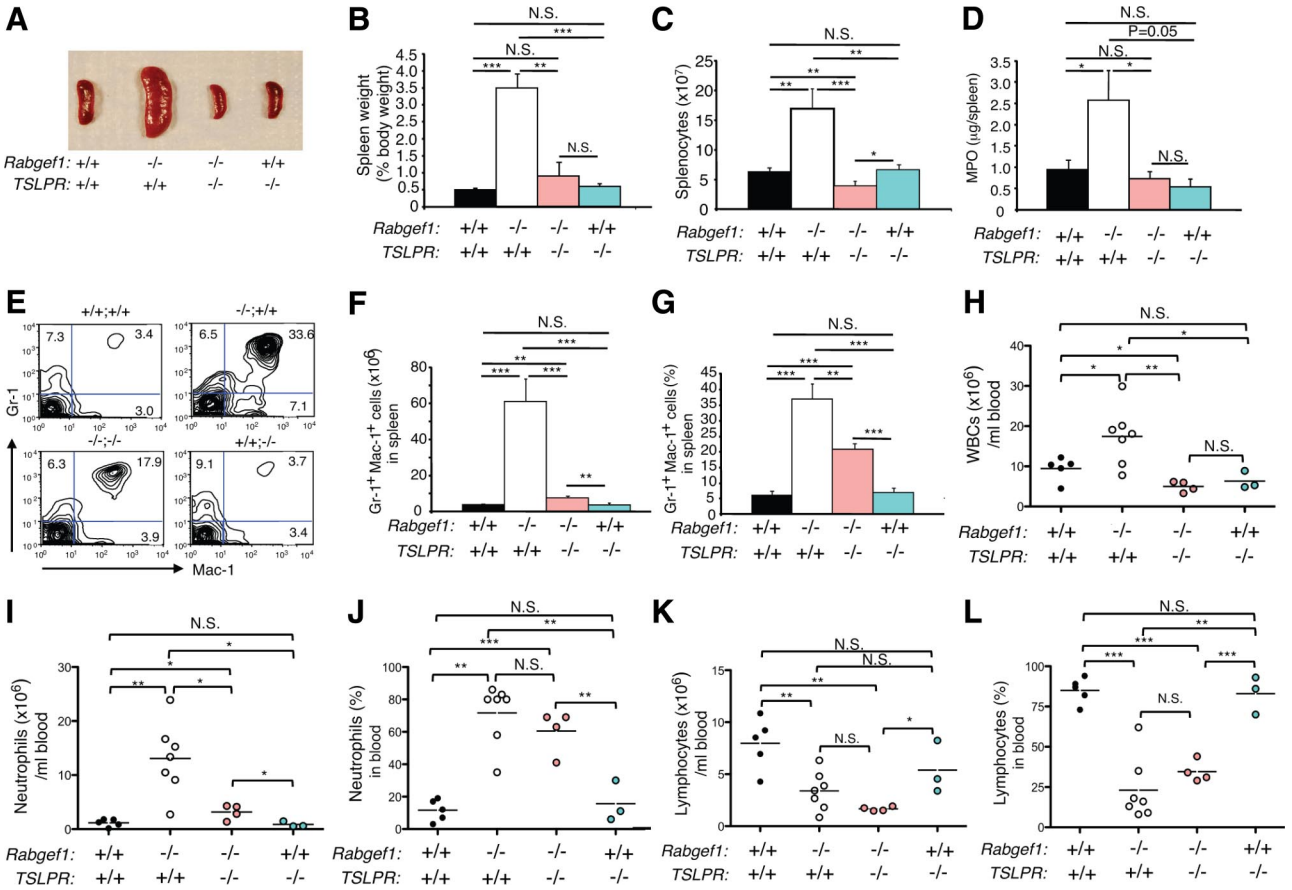

Figure 5. TSLPR contributes to myeloid hyperplasia and elevated blood neutrophils in *Rabgef1*$^{-/-}$ mice. **A:** Gross images of spleens of *Rabgef1*$^{+/+}$*TSLPR*$^{+/+}$ (wild type), *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$, *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$, and *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice. **B–D:** Spleen weight (as the % of body weight) (**B**), total leukocyte numbers (**C**), and myeloperoxidase (MPO) levels (**D**). **E:** Representative FACS profiles of spleen cells stained with anti-Gr1 and anti-Mac1 antibodies in *Rabgef1*$^{+/+}$*TSLPR*$^{+/+}$ (+/+;+/+), *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ ($^{-/-}$;+/+), *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ ($^{-/-}$;$^{-/-}$), and *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ (+/+;$^{-/-}$) mice. Numbers shown in each quadrant are percentages of total viable spleen cells. **F** and **G:** Numbers (**F**) and % (**G**) of Gr1^{+}Mac1^{+} cells in the spleens analyzed by flow cytometry. **H–L:** Total white blood cells (WBCs) (**H**), and numbers (**I** and **K**) and % (**J** and **L**) of neutrophils (**I** and **J**) and lymphocytes (**K** and **L**) in the peripheral blood. All mice were of the BALB/c strain and 4–6 weeks old. Data shown are from four to 12 mice per group for analyses of spleens and three to seven mice per group for analyses of blood. Horizontal bars indicate mean values. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; N.S., not significant ($P > 0.05$).

late the recruitment and/or survival of this granulocyte in the skin of *Rabgef1*$^{-/-}$ mice. Myeloperoxidase (MPO) activity, an index for myeloid cell infiltration,[28] measured in the ear skin was substantially higher in both *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice than in the wild-type or *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice, but was similar between *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice (Figure 4D). Taken together, our results show that TSLPR-dependent signaling is dispensable for the development of some aspects of the skin pathology in *Rabgef1*$^{-/-}$ mice, such as the epidermal hyperplasia and hyperkeratosis, and the increases in numbers of dermal mast cells and dermal content of MPO.

TSLPR-Dependent Signaling Promotes Myeloid Hyperplasia and Increased Numbers of B Cells in the Spleen, and Elevated Levels of Serum IL-4, IgE, in Rabgef1$^{-/-}$ *Mice*

In contrast to the development of skin lesions in Rab-GEF1-deficient mice, which was largely TSLPR-independent, spleens of *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice were significantly smaller than those of *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice, even when corrected for body weight (Figure 5, A and B), and were also slightly smaller (Figure 5A) and contained significantly fewer cells (Figure 5C) than those of the corresponding wild-type or *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice. The MPO levels in *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ spleens were likewise reduced to levels observed in the wild-type and *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ spleens (Figure 5D). Furthermore, the numbers and % of Gr1^{+}Mac1^{+} cells in the spleens of *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice (Figure 5, E–G) were markedly reduced compared to those in *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ spleens but still slightly but significantly higher than those in wild-type or *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice (Figure 5, E–G). By contrast, the numbers and % of Gr1^{+}Mac1^{+} cells in the bone marrow were similar between *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice and were significantly higher than those in wild-type or *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$mice (see Supplemental Figure S6 at *http://ajp.amjpathol.org*).

Analyses of peripheral blood leukocytes revealed findings similar to those obtained in the spleen. Numbers of white blood cells (WBCs) and neutrophils in the peripheral blood of *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice were highly elevated compared to those in the wild-type, *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$, or

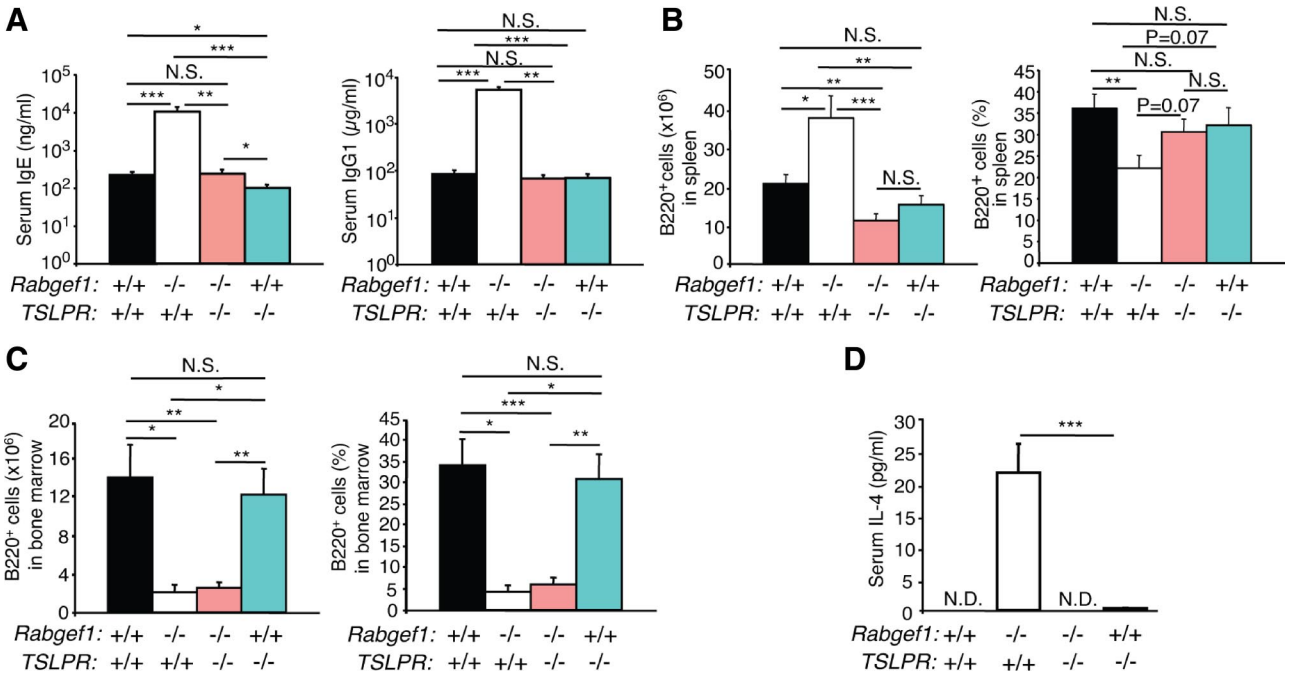

Figure 6. TSLPR contributes to increases in serum IgE and IgG1, splenic B cells, and serum IL-4 levels in *Rabgef1*$^{-/-}$ mice. **A:** Concentrations of serum IgE and IgG1. **B:** Numbers and percentages of B220^{+} cells in the spleen, as assessed by flow cytometry. **C:** Numbers and percentages of B220^{+} cells in the bone marrow, as assessed by flow cytometry. **D:** Concentrations of serum IL-4. Data shown are the mean + SEM from four to 15 mice per group for analyses of serum immunoglobulins, from three to seven mice per group for quantification of B220^{+} cells, and from nine to 14 mice per group for measurement of serum IL-4. N.D., not detectable. All mice were of the BALB/c strain and 4–6 weeks old. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; N.S., not significant ($P > 0.05$).

Rabgef1$^{+/+}$*TSLPR*$^{-/-}$ mice (Figure 5, H and I). *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice exhibited much reduced numbers of blood WBCs and neutrophils compared to *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$mice (Figure 5, H and I), but the numbers and % of neutrophils in *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ blood were still elevated significantly compared to values for the peripheral blood of the wild-type or *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice (Figure 5, 1 and J). The slight increase in numbers of blood neutrophils in *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice may have reflected the myeloid hyperplasia in the bone marrow of these animals (see Supplemental Figure S6, A–C at *http://ajp.amjpathol.org*). Notably, *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice exhibited similar numbers and % of peripheral blood lymphocytes, values which were lower than those in the wild-type or *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice (Figure 5, K and L). These results indicate that, in *Rabgef1*$^{-/-}$ mice, TSLP/TSLPR interactions can enhance myeloid cell production in the spleen and result in higher levels of neutrophils in the peripheral blood but are not required for the marked lymphopenia in the peripheral blood.

We next evaluated whether TSLP/TSLPR interactions influenced immunoglobulin (Ig) production or aspects of B cell development in RabGEF1-deficient mice. In contrast to the markedly increased concentrations of serum IgG1 and IgE detected in *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice, levels of IgG1 and IgE in *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice were very similar to those in wild-type mice (Figure 6A). However, we detected a significant reduction in total serum IgE concentrations in *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice (100.7 ± 21.9 ng/ml) compared to those in the wild-type (224.8 ± 41.4 ng/ml) or *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ (243.6 ± 64.0 ng/ml) mice. The numbers of B220^{+} cells in the spleen of *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice were substantially reduced compared to those in *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ spleen and slightly but significantly reduced compared to those in the spleen of the wild-type mice (Figure 6B). However, disruption of TSLPR-mediated signaling in *Rabgef1*$^{-/-}$ mice did not appear to alter the % or numbers of B220^{+} cells in the bone marrow, as *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ and *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice exhibited similar levels of B220^{+} cell deficiency in the bone marrow (Figure 6C).

TSLP has been shown to induce IL-4 production in CD4^{+} T cells and to promote Th2 lymphocyte differentiation in the absence of exogenous IL-4 and antigen-presenting cells.[29,30] We detected strikingly increased concentrations of IL-4 in the serum of *Rabgef1*$^{-/-}$*TSLPR*$^{+/+}$ mice (Figure 6D). However, IL-4 was not or only barely detectable in the serum of wild-type, *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$, and *Rabgef1*$^{+/+}$*TSLPR*$^{-/-}$ mice (Figure 6D). These results indicate that the striking increase in serum IL-4 levels observed in RabGEF1-deficient mice is largely dependent on TSLPR-mediated signaling and suggest that TSLPR-dependent enhancement of IL-4 production may contribute to the markedly increased numbers of splenic B cells and serum IgE and IgG1 concentrations in *Rabgef1*$^{-/-}$ mice.

Discussion

We found that *Rabgef1*$^{-/-}$ mice exhibited markedly elevated serum and tissue concentrations of TSLP, indicating that RabGEF1 is a negative regulator of TSLP levels *in vivo*. We do not know whether RabGEF1 has this effect through direct or indirect mechanisms, but it is known

that epithelia of the skin, intestine, and respiratory tract are the major sources of TSLP[3] and that production of TSLP in these epithelial cells can be induced by active vitamin D_3 (1a,25-$(OH)_2D_3$) or its low calcemic analog,[31] proinflammatory cytokines,[32–35] and various TLR ligands.[33,35] In addition, bone marrow–derived cells, including human mast cells[36] and mouse basophils,[37] also can produce TSLP. While current understanding of the mechanisms regulating TSLP production is rather limited, NF-κB–binding sites are present in the promoters of both human and mouse TSLP genes,[35] and there is evidence supporting an involvement of NF-κB pathways in the regulation of TSLP production.[33–35,38] RabGEF1 has been shown to be a potent inhibitor of NF-κB–dependent signaling.[39] Therefore, in *Rabgef1*$^{-/-}$ mice, it is possible that enhancement of NF-κB–dependent signaling may contribute to the overproduction of TSLP in keratinocytes and perhaps other TSLP-producing cells.

TSLP has been strongly implicated in the pathogenesis of human atopic dermatitis and in some mouse models of atopic dermatitis.[4,40,41] Many of the phenotypic abnormalities observed in *Rabgef1*$^{-/-}$ and keratinocyte-specific TSLP transgenic mice can also be detected in RXRα$\beta^{ep-/-}$ mice, that have keratinocyte-specific ablation of the retinoid X receptors,[11] and each of these three types of mutant mice expresses high levels of TSLP in the skin. In addition, in a mouse model of atopic dermatitis elicited by repeated epicutaneous sensitization with ovalbumin, *TSLPR*$^{-/-}$ mice express markedly decreased eosinophil infiltration, and levels of Th2 cytokines, in the skin.[42] Finally, patients with atopic dermatitis express copious amount of TSLP in their skin lesions.[7] Such observations in human subjects, together with those obtained with TSLP transgenic or *TSLPR*$^{-/-}$ mice, have strongly implicated TSLP in the development of allergic skin inflammation.

However, our finding that *Rabgef1*$^{-/-}$*TSLPR*$^{-/-}$ mice which are incapable of responding to TSLP stimulation continued to exhibit skin lesions with some features of atopic dermatitis (including epidermal hypersplasia, dermal inflammation, and increased numbers of dermal mast cells) points to the existence of a TSLP/TSLPR-independent mechanism for the development of cutaneous epidermal hyperplasia and chronic inflammation, at least in mice. Notably, we showed that chronic skin lesions with substantial epidermal hyperplasia and increased numbers of dermal mast cells can also develop in *Rabgef1*$^{-/-}$ mice which lack Rag1 or eosinophils. By contrast, the skin pathology elicited by exogenous administration of TSLP required the presence of T cells and eosinophils.[43] Taken together, our findings indicate that *Rabgef1*$^{-/-}$ mice develop chronic inflammation of the skin with increased levels of MPO in the dermis, increased numbers of dermal mast cells, and epidermal hyperplasia by a robust mechanism that does not require the participation of TSLP/TSLPR-depending signaling, adaptive immunity or eosinophils. By contrast, we found that TSLP/TSLPR-dependent signaling was required for the increased numbers of eosniophils observed in the skin lesions of *Rabgef1*$^{-/-}$ mice, and it is possible that TSLP/TSLPR-dependent signaling also may influence the recruitment, survival, and/or functional activation of other subsets of leukocytes in the skin lesions of *Rabgef1*$^{-/-}$ mice.

Although TSLPR was not essential for the development of cutaneous inflammation, epidermal hypersplasia, or increased numbers of mast cells in the skin lesions of *Rabgef1*$^{-/-}$ mice, other striking phenotypic abnormalities of *Rabgef1*$^{-/-}$ mice, including splenic myeloid cell expansion, abnormal B cell development, and enhanced serum levels of IL-4 and IgE and IgG1 immunoglobulins, were significantly and in some cases entirely dependent on TSLPR-dependent pathways. By contrast, the myeloid hyperplasia and reduced B cell levels in the bone marrow of *Rabgef1*$^{-/-}$ mice developed independently of the expression of TSLPR. While the mechanisms by which TSLPR signaling contributes to the noncutaneous phenotypic abnormalities in *Rabgef1*$^{-/-}$ mice remain to be fully elucidated, both myeloid and B cells express TSLPR and can respond to TSLP.[27]

In conclusion, our findings show that RabGEF1 can critically regulate the homeostasis of the cutaneous and lymphohematopoietic systems and that one effect of RabGEF1 deficiency is to develop strikingly elevated levels of TSLP, but that only some of the phenotypic abnormalities observed in *Rabgef1*$^{-/-}$ mice depend on the TSLP/TSLPR signaling pathway. Indeed, the finding that *Rabgef1*$^{-/-}$ mice can develop striking epidermal hyperplasia and skin inflammation with increased numbers of mast cells in the absence of TSLPR-dependent signaling identifies a TSLP/TSLPR-independent mechanism for developing skin pathology with some of the features of "allergic type" cutaneous inflammation. Given some of the histological similarities between the chronic skin lesions which develop in *Rabgef1*$^{-/-}$ mice and those observed in atopic dermatitis and other chronic skin diseases in humans, we propose that *Rabgef1*$^{-/-}$ mice may be useful in revealing new insights into interactions between the cutaneous and immune systems which contribute to the development and progression of such skin disorders.

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