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Regulator of Calcineurin 1 (Rcan1) Is Required for the Development of Pulmonary Eosinophilia in Allergic Inflammation in Mice

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The presence of eosinophils in the lung is often regarded as a defining feature of asthma. On allergen stimulation, numbers of eosinophils and their progenitors are increased in both the bone marrow and lungs. Eosinophil progenitors provide an ongoing supply of mature eosinophils. Here, we report that deficiency in the regulator of calcineurin 1 gene (*Rcan1*) leads to a near-complete absence of eosinophilia in ovalbumin-induced allergic asthma in mice. In the absence of *Rcan1*, bone marrow cells produce significantly fewer eosinophils *in vivo* and *in vitro* on interleukin-5 stimulation. Importantly, eosinophil progenitor populations are significantly reduced in both naïve and ovalbumin-challenged *Rcan1*^{-/-} mice. Bone marrow cells from *Rcan1*^{-/-} mice are capable of developing into fully mature eosinophils, suggesting that *Rcan1* is required for eosinophil progenitor production but may not be necessary for eosinophil maturation. Thus, *Rcan1* represents a novel contributor in the development of eosinophilia in allergic asthma through regulation of eosinophil progenitor production. (Am J Pathol 2011, 179:1199–1210; DOI: 10.1016/j.ajpath.2011.05.022)

A nationwide survey found that more than half (54.6%) of the U.S. population test positive to one or more allergens.¹ Allergic asthma is a chronic inflammatory disease that is characterized by eosinophil infiltration. Eosinophils

are prominent effector cells in allergic asthma.^{2–4} Several studies have established a causative link between eosinophils and allergic lung diseases.^{5–8} Targeting eosinophils using anti-IL-5 antibodies has been considered as a therapeutic approach for the treatment of asthma. In steady state, eosinophil progenitors constantly egress from the bone marrow into the blood and circulate to peripheral tissues. In allergic diseases, the bone marrow releases increased numbers of eosinophil progenitor cells that migrate to the site of allergic inflammation, where they provide a constant supply of mature eosinophils.^{9–13} Molecular mechanisms governing eosinophil progenitor production remain incompletely defined.

Down syndrome patients who overexpress regulator of calcineurin 1 (*Rcan1*) have an association with immune disorders including allergy and asthma.^{14–16} Hypereosinophilic syndrome was reported in a fetus harboring trisomy 21.¹⁷ Eosinophilic pericardial effusion was also documented in a neonate with Down syndrome.¹⁸ The proform of eosinophil major basic protein has been identified as a maternal serum marker for Down syndrome.¹⁹ Increased incidence of allergic asthma in patients with Down syndrome has been reported in a recent large survey study.¹⁵ These findings suggest an association of *Rcan1* with eosinophils and asthma.

The human *RCAN1* gene was previously known as *DSCR1* (Down syndrome critical region 1).²⁰ Additional names for *Rcan1* include *Adapt78* (the gene is transiently induced during cell adaptation),²¹ myocyte-enriched calcineurin interacting protein 1 (MCIP1),²² calcipressin 1,²³ and calcineurin binding protein 1 (CBP1).²⁴ The human *RCAN1* gene is located on chromosome 21. *Rcan1* is widely expressed in various tissues, including heart, lung, kidney, brain, muscle, liver, and testis.^{22,25,26} The *RCAN1* gene

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consists of seven exons, of which exons 1 to 4 can be alternatively transcribed.²⁷ Deletion of exons 5 and 6 from the mouse *Rcan1* gene leads to deficiency of the Rcan1 protein.²⁸ These *Rcan1*^{-/-} mice are viable and fertile and provide a useful tool for the study of Rcan1 function.²⁸

Published reports on Rcan1 function are concerned largely with calcineurin activity. Experiments in different organisms and cell types have showed a dual function for Rcan1, which can act as either an inhibitor^{29,30} or a facilitator^{24,28,31-33} of calcineurin activity, depending on the cellular context. In yeast and in *Cryptococcus neoformans*, Rcan1 has the characteristics of a calcineurin activator,^{24,32,33} and in a cardiac hypertrophic mouse model Rcan1 functions as a calcineurin facilitator.^{28,31} In contrast, in other experimental models, Rcan1 inhibits calcineurin activity *in vitro* and *in vivo*.^{23,29,30} Recently, we found that Rcan1 is an inhibitor of calcineurin in mast cells.³⁴

Our finding that Rcan1 is a negative regulator of IgE-mediated mast cell activation³⁴ prompted us to determine what role Rcan1 has in allergic asthma. Surprisingly, we found that *Rcan1* deficiency leads to near-complete absence of eosinophilia in ovalbumin-induced asthma in mice. The number of eosinophil progenitors was significantly reduced in *Rcan1*^{-/-} mice, and calcineurin activity was reduced in *Rcan1*^{-/-} eosinophil progenitors. Thus, Rcan1 represents a novel mechanism in the development of eosinophilia in allergic asthma, likely by regulating eosinophil progenitor cell numbers.

Materials and Methods

Animals

The *Rcan1* gene was targeted for deletion by standard homologous recombination in embryonic stem cells (Sv129 strain), followed by generation of chimeric mice, which were subsequently bred to pass the targeted allele into the germline in the C57BL/6 genetic background, as described elsewhere.²⁸ These mice were originally provided by Dr. Jeffery Molkentin (Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH). The protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with guidelines of the Canadian Council on Animal Care.

Antibodies

Antibodies to phospho-JNK (Thr 183/Tyr 185), JNK, phospho-p38 MAPK (Thr 180/Tyr 182), phospho-Stat5, Stat5, phospho-p44/42, p44/42, phospho-Gsk3 β , and Gsk3 β were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to p38 MAPK and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to GATA-1 was purchased from Novus Biologicals (Littleton, CO). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD117 (c-kit) monoclonal antibody and FITC-rat IgG2a were purchased from Cedarlane Laboratories (Hornby, ON, Canada). FITC-conjugated rat anti-mouse IgE (IgG1) and FITC-rat IgG1 were purchased from BD Biosciences (San Jose, CA).

Allergen Sensitization and Challenge

Mice were immunized intraperitoneally with 10 μ g ovalbumin (OVA) (grade V; Sigma-Aldrich, St. Louis, MO) in 0.1 mL saline on days 0, 2, 4, 6, 8, 10, and 12. On days 40, 43, and 46, mice were challenged intranasally with 200 μ g OVA (20 μ L of 10 mg/mL) in saline or with saline alone.

Bronchoalveolar Lavage, Lung Tissue Processing, and Histology

Bronchoalveolar lavage fluid (BALF) was obtained by lavaging the lung with 1 mL of phosphate buffer solution containing soybean trypsin inhibitor (100 μ g/mL). The BALF was centrifuged and the supernatants were stored at -80°C until use. Cell pellets were resuspended in 1 mL of PBS. The BALF cells were counted, cytopun, and stained using a Diff-Quik assay (Fisher Scientific, Pittsburgh, PA). At least 200 cells per slide were examined and were characterized as mononuclear cells, neutrophils, or eosinophils. The remaining cells were homogenized in 250 μ L of 0.5% cetyltrimethylammonium chloride and centrifuged at 4°C for 30 minutes at 16,000 $\times$ *g*. The supernatants were used for eosinophil peroxidase (EPO) and myeloperoxidase (MPO) assay.

The right lung was homogenized (at maximum speed for 20 seconds; PowerGen 125; Fisher Scientific) in 50 mmol/L HEPES buffer (4 μ L/mg lung) containing soybean trypsin inhibitor (100 μ g/mL). The homogenate was centrifuged at 4°C for 30 minutes at 16,000 $\times$ *g*. The supernatant was stored at -80°C for later cytokine analysis. The pellet was resuspended and homogenized in 0.5% cetyltrimethylammonium chloride (4 μ L/mg lung) and centrifuged as described above. The clear extract was used for EPO and MPO assays. One third of the left lung was excised and fixed in 10% formalin overnight and then in 100% ethanol for paraffin embedding and sectioning. Slides were subjected to staining with H&E or PAS.

ELISA and Cytokine Multiplex Assay

Cytokines in the cell-free supernatants were determined using DuoSet ELISA kits obtained from R&D Systems (Minneapolis, MN). Cell-free culture supernatants were also analyzed for cytokines using a Bio-Plex Pro mouse 23-plex group 1 cytokine assay (Bio-Rad, Hercules, CA), which detects 23 cytokines and chemokines. The assay was performed according to the manufacturer's protocol and was read on a Bio-Plex 200 HTF multiplex array system. Data were analyzed using Bio-Plex Manager version 6.0 software (Bio-Rad).

MPO and EPO Assay

Myeloperoxidase activity in the lung was determined as a marker for neutrophils. Briefly, samples in duplicate (75 μ L) were mixed with equal volumes of the substrates (3 mmol/L 3,3',5,5'-tetramethylbenzidine dihydrochloride, 120 μ mol/L resorcinol, and 2.2 mmol/L H₂O₂) for 2 minutes. The reaction was stopped by adding 150 μ L of 2 mol/L H₂SO₄. The optical density was measured at 450

nm. For EPO assay, 75 μ L of substrate solution (3 mmol/L o-phenylenediamine dihydrochloride, 50 mmol/L chloride-free HEPES pH 8.0, 6 mmol/L KBr, and 8.8 mmol/L H_2O_2) was added to 75 μ L of each sample. After 30 minutes, the reaction was stopped with 150 μ L of 2 mol/L H_2SO_4 and the absorbance was read at 490 nm.

Fluorescence-Activated Cell Sorting Analysis and Sorting of Eosinophil Lineage-Committed Progenitors

Eosinophil progenitor cells were analyzed or sorted according to published protocols.³⁵ Briefly, bone marrow cells were stained with antibodies to the IL-5R α chain, c-Kit, CD34, and Sca-1, as well as a lineage cocktail including antibodies to CD3, CD4, CD8, B220, Gr-1, and CD19. Eosinophil progenitors were identified as Lin[−]Sca-1[−]CD34⁺IL-5R α ⁺c-Kit^{low} cells. Mature eosinophils were defined as Siglec-F⁺ or Siglec-F⁺IL-5R α ⁺ cells. In some experiments, to acquire bone marrow cells containing eosinophil progenitors (for Western blot analysis, real-time PCR, or calcineurin activity assay), bone marrow cells were stained with antibodies to CD34, Sca-1, and lineage markers (CD3, CD4, CD8, B220, Gr-1, and CD19). CD34⁺Lin[−]Sca-1[−] and CD34[−]Lin[−]Sca-1[−] bone marrow cells were obtained by cell sorting.

The following antibodies were purchased from BD Biosciences: PE-CD3, PE-CD4, PE-CD8, PE-B220, PE-Gr-1, PE-CD19, PE-Sca-1, Alexa Fluor 488-IL-5R α chain, PerCP-Cy5.5-c-Kit, Alexa Fluor 647-CD34, and PE-Siglec-F.

Colony-Forming Assay

Bone marrow cells were collected by flushing the tibia of wild-type and *Rcan1*^{−/−} mice. The erythrocytes were removed by lysis using NH_4Cl . Bone marrow cells (9×10^3 cells) were cultured in Methocult GF3434 methylcellulose medium (StemCell Technologies, Vancouver, BC, Canada) containing 50 ng/mL of IL-5. On day 12, the numbers of colonies were counted microscopically.

IL-5-Induced Eosinophil Production in Vivo

Recombinant murine IL-5 (rmIL-5, 10 μ g/day; PeproTech, Rocky Hill, NJ) was injected intraperitoneally into wild-type and *Rcan1*^{−/−} mice for 6 days (days 0 to 5). Differential counts were performed by examination of blood smears stained with Diff-Quik at various time points.

Bone Marrow-Derived Eosinophil Isolation and Culture

Mouse primary bone marrow-derived eosinophils were cultured as described elsewhere.³⁶ Bone marrow cells were collected from the femurs and tibiae of wild-type and *Rcan1*^{−/−} mice. Red blood cells were lysed in H_2O for 15 seconds followed by the addition of 2 $\times$ phosphate buffer. The bone marrow cells were cultured at 1×10^6 cells/mL in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL pen-

icillin, 10 μ g/mL streptomycin, 25 mmol/L HEPES (Invitrogen), 50 μ mol/L 2-mercaptoethanol (Sigma-Aldrich), 100 ng/mL recombinant murine stem cell factor (rmSCF; PeproTech), and 100 ng/mL recombinant murine FLT3 ligand (rmFLT3-L; PeproTech) from days 0 to 4. On day 4, the medium containing SCF and FLT3-L was replaced with medium containing 50 ng/mL rmIL-5 only. On day 8, the cells were moved to new flasks and maintained in fresh medium supplemented with rmIL-5.

Chemotaxis Assay

The assay was performed in a 24-well Transwell plate (Corning Life Sciences, Corning, NY) with a 5.0- μ m pore size polycarbonate membrane. The medium (600 μ L) containing 50 ng/mL rmIL-5 was placed in the lower chamber. Recombinant mouse eotaxin (rmEotaxin; PeproTech) was added to the lower chamber as a chemoattractant. Bone marrow-derived eosinophils in 100 μ L (2×10^6 cells/mL) were placed in the upper chamber. Cells were incubated for 60 minutes at 37°C to permit migration across the membrane. The Transwell inserts were gently removed, and the cells in the bottom wells were counted.

Real-Time Quantitative PCR

The mRNA levels of *Rcan1* and *GATA-1* were quantified using Assays-on-Demand reagents containing TaqMan MGB probes and TaqMan master mix (Applied Biosystems, Foster City, CA) on a sequence detection system (ABI Prism 7000, Applied Biosystems). *GAPDH* was used as an endogenous reference. The Assays-on-Demand reagents for *Rcan1* target the larger *Rcan1* isoform (*Rcan1*-1). Data were analyzed using the relative standard curve method according to the manufacturer's protocol. A mean value of target gene after *GAPDH* normalization at the time point showing highest expression was used as a calibrator to determine the relative levels of *Rcan1* at different conditions. PCR products were resolved on a 2% agarose gel and were stained with ethidium bromide (Invitrogen, Carlsbad, CA).

Calcineurin Activity Assay

Phosphatase activity was measured using a calcineurin assay kit (Biomol International-Enzo Life Sciences, Plymouth Meeting, PA). To obtain the eosinophil progenitor cell population, bone marrow cells from wild-type and *Rcan1*^{−/−} mice were incubated with antibodies to CD34 and Sca-1 and with a lineage cocktail including antibodies to CD3, CD4, CD8, B220, Gr-1, and CD19. CD34⁺Lin[−]Sca-1[−] cells were obtained by cell sorting. Lysates were prepared from CD34⁺Lin[−]Sca-1[−] bone marrow cells or mature eosinophils cultured from bone marrow cells. Phosphatase activity was measured according to the manufacturer's instructions.

Western Blot Analysis

Cell lysates (20 to 30 μ g) were subjected to electrophoresis in 12% SDS-polyacrylamide gels. Gels were trans-

ferred to polyvinylidene difluoride membrane, blotted with primary and secondary antibodies, and detected by an enhanced chemiluminescence detection system (Western Lightning Plus-ECL; PerkinElmer, Norwalk, CT).

Thymidine Incorporation Assay

Bone marrow cells were collected from the tibiae and femurs of wild-type and *Rcan1*^{-/-} mice. Bone marrow cells were passed through a nylon filter in a single-cell suspension and were resuspended in 100 μ L sort buffer. Cells were stained with anti-CD34 antibody (Alexa Fluor 647) and a cocktail of phycoerythrin (PE)-conjugated antibodies to lineage markers (CD3, CD4, CD8, B220, Gr-1, and CD19) and Sca-1. Lineage or Sca-1⁺ cells were discarded by cell sorting. Remaining cells were further separated into CD34⁺ and CD34⁻ cell populations using a FACSria fluorescence-activated cell sorting system (BD Biosciences). Cells were resuspended in medium containing 100 ng/mL mSCF and 100 ng/mL FLT3-ligand and seeded in 96-well plates (1.85×10^5 cells/well). Cells were allowed to recover overnight and were then pulsed with 1 μ Ci of [³H]thymidine for 8 hours. Cells were harvested and incorporation was measured on a Wallac 1409 scintillation β counter (Wallac Oy, Turku, Finland). To determine eosinophil proliferation, bone marrow-derived eosinophils (2×10^5 cells/well) from wild-type and *Rcan1*^{-/-} mice were pulsed with 1 μ Ci of [³H]thymidine for 8 hours in the presence of IL-5 (50 ng/mL). Thymidine incorporation was then measured.

Statistical Analysis

Analysis of variance and the paired Student's *t*-test were used for statistical evaluation of data. *P* values of <0.05 were considered significant. Except as specified, data are expressed as means $\pm$ SEM.

Results

Near-Complete Blockade of OVA-Induced Airway Eosinophilia in *Rcan1*-Deficient Mice

We have found that *Rcan1* negatively regulates IgE-mediated mast cell activation.³⁴ This finding prompted us to examine the role of *Rcan1* in allergic asthma by using an OVA-induced asthma model in mice. To determine whether *Rcan1* regulates lung inflammation in allergic asthma, *Rcan1*^{-/-} mice and *Rcan1*^{+/+} mice were sensitized and challenged with OVA. After systemic sensitization to OVA and aerosolized OVA challenges, airway inflammation was examined. Histological analysis revealed that *Rcan1*^{-/-} mice showed reduced inflammatory cells in the lung, compared with that in wild-type mice (Figure 1). Cells in BALF were obtained for differential cell counts. An OVA-induced increase of inflammatory cells in the BALF was seen in *Rcan1*^{+/+} mice. In contrast, total cell number in BALF was reduced by 53% in *Rcan1*^{-/-} mice, relative to OVA-treated *Rcan1*^{+/+} mice (Figure 2A). Analysis of differential cell counts revealed

Figure 1. *Rcan1* deficiency leads to reduced allergic inflammation in the lung. *Rcan1*^{+/+} and *Rcan1*^{-/-} mice were systemically sensitized with OVA and challenged with aerosolized OVA. Lung and BALF were collected 24 hours after the last OVA challenge. Lung sections from saline or OVA-challenged mice were stained with H&E or PAS for microscopic examination. Tissue morphology and airway mucus production were similar in both *Rcan1*^{+/+} and *Rcan1*^{-/-} mice under basal conditions. In OVA-challenged mice, H&E staining revealed a much milder inflammatory cell infiltration in *Rcan1*^{-/-} mice and PAS staining revealed significantly reduced goblet cell hyperplasia and mucous secretion. Representative images were chosen from 14 mice (in three independent experiments) for OVA-challenged mice and from six mice (in three independent experiments) from saline-treated control mice. Scale bar = 100 μ m.

that the number of neutrophils and mononuclear cells was similar in both *Rcan1*^{+/+} and *Rcan1*^{-/-} mice. Importantly, very little increase of eosinophil numbers could be seen in OVA-challenged *Rcan1*^{-/-} mice (Figure 2A). EPO activity is an eosinophil marker, and the OVA-induced increase of EPO activities in BALF and lung homogenates was dramatically reduced in *Rcan1*^{-/-} mice (Figure 2, B and C). MPO is a neutrophil marker, and the OVA-induced levels of MPO activity were similar in both wild-type and *Rcan1*^{-/-} mice (Figure 2E). Thus, *Rcan1* deficiency selectively blocked airway eosinophilia.

We also examined *Rcan1* expression in the lung of OVA-challenged or saline-treated mice. *Rcan1* was constitutively expressed in the lung, as determined by real-time PCR analysis. Of note, the level of *Rcan1* was decreased in the lung of OVA-challenged mice (see Supplemental Figure S1, A and B, at <http://ajp.amjpathol.org>). To examine whether eosinophils express *Rcan1*, mature eosinophils cultured from wild-type mouse bone marrow cells were used to isolate RNA. To examine

Figure 2. Rcan1 deficiency leads to near-complete absence of eosinophilia in the lung. $Rcan1^{+/+}$ and $Rcan1^{-/-}$ mice were systemically sensitized with OVA and challenged with aerosolized OVA. Lung and BALF were collected 24 hours after the last OVA challenge. **A:** Cells in the BALF were obtained for differential cell counts (Diff-Quik staining). Total cell number in the BALF was reduced by 53% in $Rcan1^{-/-}$ mice, compared with OVA-treated $Rcan1^{+/+}$ mice. Importantly, very little increase of eosinophil numbers could be seen in OVA-challenged $Rcan1^{-/-}$ mice. **B** and **C:** Lung and BALF were collected for measuring activity of EPO, an eosinophil marker. Little EPO activity could be observed in $Rcan1^{-/-}$ mice after OVA challenge. **D:** Cell pellets in the BALF were also used to determine activity of MPO, a neutrophil marker. No major difference in MPO activity was found between $Rcan1^{+/+}$ and $Rcan1^{-/-}$ mice ($n = 14$ mice in the OVA group and $n = 6$ mice in the saline group, in three independent experiments). * $P < 0.05$ versus the OVA-treated $Rcan1^{+/+}$ group.

whether eosinophil progenitors express *Rcan1*, bone marrow cells from wild-type mice were stained with antibodies to CD34, Sca-1, and the lineage markers CD3, CD4, CD8, B220, Gr-1, and CD19. CD34⁺Lin[−]Sca-1[−] and CD34[−]Lin[−]Sca-1[−] bone marrow cells were obtained by cell sorting and were used for RNA isolation. Mouse bone marrow-derived mast cells after IgE activation were used as a positive control for *Rcan1* expression. *Rcan1* was found in both eosinophils and eosinophil progenitors (see Supplemental Figure S1C at <http://ajp.amjpathol.org>).

Rcan1 Deficiency Leads to Reduced Eosinophil Production in Vitro and in Vivo

Next, we characterized the role of *Rcan1* in eosinophil production *in vitro* and *in vivo*. Eosinophils originate from the bone marrow and circulate in the blood. To determine

whether *Rcan1* regulates the number of eosinophils in the blood, peripheral blood cells from $Rcan1^{-/-}$ and $Rcan1^{+/+}$ mice with or without exposure to OVA challenge were stained with antibodies to IL-5R α and Siglec-F and were analyzed by flow cytometry. In naïve animals, peripheral blood eosinophil counts were similar in both $Rcan1^{-/-}$ and $Rcan1^{+/+}$ mice (Figure 3, A and B). Systemic sensitization to OVA and aerosolized OVA challenge induced an increase of eosinophils in the blood of $Rcan1^{+/+}$ mice. Notably, $Rcan1^{-/-}$ mice showed a dramatically impaired response to OVA. Minimal eosinophilia was observed in the blood of $Rcan1^{-/-}$ mice (Figure 3, A and B). Thus, *Rcan1* deficiency prevented an OVA-induced increase of eosinophils in the blood, suggesting that eosinophil production was reduced because of *Rcan1* deficiency.

Eosinophil production *in vivo* is regulated by a specific set of growth factors, including IL-5.³⁷ To determine whether growth factor-induced eosinophil production *in vivo* is regulated by *Rcan1*, $Rcan1^{-/-}$ and $Rcan1^{+/+}$ mice were injected with IL-5 intraperitoneally for 6 days. The number of eosinophils in the peripheral blood was examined by differential cell counts for 12 days. Injection of IL-5 induced increased numbers of eosinophils in $Rcan1^{+/+}$ mice. By contrast, $Rcan1^{-/-}$ mice showed an approximately 50% reduction in eosinophils, relative to $Rcan1^{+/+}$ mice, throughout the time period when IL-5-induced eosinophilia was most prominent (day 4 to day 10) (Figure 3, C and D). On day 12, animals were sacrificed, and the blood and bone marrow cells were stained with antibodies to Siglec-F and IL-5R α for flow cytometry analysis. Similarly, $Rcan1^{-/-}$ mice showed fewer eosinophils in the bone marrow, compared with $Rcan1^{+/+}$ mice (Figure 3, E and F). Thus, *Rcan1* contributed to IL-5-induced production of eosinophils *in vivo*.

In vitro experiments were performed to further evaluate the role of *Rcan1* in eosinophil production. Bone marrow cells were cultured under eosinophil-promoting conditions *in vitro* by using growth medium containing SCF and FLT3-L, followed by IL-5, as described elsewhere,³⁸ or by using a conventional colony-forming assay. The same number of bone marrow cells from $Rcan1^{-/-}$ and $Rcan1^{+/+}$ mice was used for cell culture on day 0. After culture in growth medium, the yield of mature eosinophils was enumerated on day 14. $Rcan1^{-/-}$ bone marrow cells produced significantly fewer eosinophils, compared with $Rcan1^{+/+}$ bone marrow cells (Figure 3G). Similarly, in a colony-forming assay, $Rcan1^{-/-}$ bone marrow cells produced fewer eosinophil colony-forming units, compared with $Rcan1^{+/+}$ bone marrow cells (Figure 3H). Eosinophil morphology was examined by light microscopy. No difference could be observed between $Rcan1^{-/-}$ and $Rcan1^{+/+}$ eosinophils (data not shown). Thus, $Rcan1^{-/-}$ bone marrow cells produced fewer eosinophils when cultured *in vitro*.

Reduced Eosinophil Progenitors in Rcan1-Deficient Mice

Eosinophils originate from progenitor cells in the bone marrow. We reasoned that the potential mechanisms that

Figure 3. *Rcan1* deficiency leads to reduced eosinophil production *in vitro* and *in vivo*. **A** and **B**: *Rcan1*^{+/+} and *Rcan1*^{-/-} mice were systemically sensitized with OVA and challenged with aerosolized OVA. Peripheral blood was collected 24 hours after the last OVA challenge. Cells were stained with antibodies to Siglec-F and IL-5R α . **A**: Representative dot plots are shown for Siglec-F⁺ and IL-5R α ⁺ cells (eosinophils) from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice. **B**: Summaries of the percentage of Siglec-F⁺ and IL-5R α ⁺ cells (eosinophils) are shown. OVA challenge induced increase of eosinophils in *Rcan1*^{+/+} mice but not in *Rcan1*^{-/-} mice. Data are reported as means $\pm$ SEM ($n = 8$ mice in each group from two independent experiments). * $P < 0.05$ versus the OVA-treated *Rcan1*^{+/+} group. **C–F**: Recombinant murine IL-5 (10 μ g/day, once daily) was injected intraperitoneally into *Rcan1*^{+/+} and *Rcan1*^{-/-} mice for 6 days (days 0 to 5). Peripheral blood was collected at various time points and examined under Diff-Quik staining. Differential cell counts were performed by examination of blood smears. The percentage of eosinophils was enumerated by counting 200 cells/slide. Data are reported as means $\pm$ SEM ($n = 6$ mice in each group from two independent experiments). **C**: * $P < 0.05$ versus the *Rcan1*^{-/-} group. Typical appearances of eosinophils (arrows) in blood smears are shown in **D** for samples from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice at day 8 after IL-5 injection. Scale bar = 20 μ m. On day 12, mice were sacrificed, and bone marrow cells were stained with antibodies to Siglec-F and IL-5R α and analyzed by flow cytometry. **E**: Siglec-F⁺ and IL-5R α ⁺ cells were identified as eosinophils. **F**: *Rcan1*^{-/-} mice showed reduced number of eosinophils. Data are reported as means $\pm$ SEM ($n = 6$ mice in each group from two independent experiments). * $P < 0.05$ versus the *Rcan1*^{+/+} group. **G**: Bone marrow cells from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice were cultured in medium supplemented with rmSCF, rmFLT3-L and rmIL-5 *in vitro* for 14 days. The same number of bone marrow cells (BMCs) was used at the beginning of the culture on day 0 (D0). Mature bone marrow-derived eosinophils (BMEOs) were examined on day 14 (D14) under Diff-Quik staining and by flow cytometry. Data obtained by microscopic examination are reported as means $\pm$ SEM from 12 mice in four independent experiments. * $P < 0.05$ versus the *Rcan1*^{+/+} D14 group. **H**: Eosinophil colony-forming assay. Bone marrow cells (9×10^3 cells) were cultured in methylcellulose medium containing 50 ng/mL of rmIL-5 for 12 days, and the numbers of colonies were counted. Bone marrow cells from *Rcan1*^{-/-} mice produced fewer eosinophil colonies. Data are reported as means $\pm$ SEM of eight independent experiments in triplicate. * $P < 0.05$ versus the *Rcan1*^{+/+} group.

contribute to the reduced eosinophil production *in vivo* and *in vitro* may include a reduction in the number of eosinophil progenitors in the bone marrow or impaired developmental capacity of eosinophil progenitors.

Eosinophil progenitors in the bone marrow have recently been identified as Lin⁻Sca-1⁻CD34⁺IL-5R α ⁺c-Kit^{low} cells.¹⁰ To determine whether *Rcan1* regulates eosinophil progenitors, bone marrow cells from *Rcan1*^{-/-} and *Rcan1*^{+/+} mice were stained with various antibodies and analyzed by flow cytometry for enumeration of Lin⁻Sca-1⁻CD34⁺IL-5R α ⁺c-Kit^{low} cells, as described elsewhere.¹⁰ Naïve *Rcan1*^{-/-} mice showed significantly reduced numbers of eosinophil progenitors, compared with *Rcan1*^{+/+} mice (Figure 4, A–C). Systemic sensitization to OVA and aerosolized OVA challenges induced a strong increase (116%) in the number of eosinophil pro-

genitors in the bone marrow of wild-type mice. Importantly, OVA-challenged *Rcan1*^{-/-} mice showed severely reduced numbers of eosinophil progenitors, compared with *Rcan1*^{+/+} mice (Figure 4, A, D, and E). Thus, both naïve and OVA-challenged *Rcan1*^{-/-} mice demonstrated severely reduced numbers of eosinophil progenitors in the bone marrow.

To determine whether *Rcan1* influences the developmental capacity of the eosinophil progenitors, Lin⁻Sca-1⁻CD34⁺IL-5R α ⁺c-Kit^{low} cells were obtained from bone marrow by cell sorting. The same numbers of sorted eosinophil progenitors (400 cells) from *Rcan1*^{-/-} and *Rcan1*^{+/+} mice were used in the cell culture. Notably, similar numbers of eosinophil CFU were observed in both *Rcan1*^{-/-} and *Rcan1*^{+/+} mice after culture for 20 days (Figure 5A). Likewise, eosinophils obtained by culturing

Figure 4. Rcan1 deficiency leads to reduced eosinophil progenitor cell production *in vivo*. **A:** Bone marrow cells from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice were stained with antibodies against lineage markers including Sca-1, CD34, IL-5R α chain, and c-Kit. Eosinophil progenitor cells (EoPs) are identified as Lin⁻Sca-1⁻CD34⁺IL-5R α ⁺c-Kit^{low} cells. Representative flow cytometry profiles are shown. **B** and **C:** Summaries of the flow cytometry analysis show that bone marrow cells from naïve *Rcan1*^{-/-} mice contain significantly reduced number of EoPs. The percentage of EoPs in total bone marrow cells (**B**) or in CD34⁺ cells (**C**) is reduced. Data are reported as means $\pm$ SEM ($n = 8$ mice from four independent experiments). **D** and **E:** *Rcan1*^{+/+} and *Rcan1*^{-/-} mice were systemically sensitized with OVA and challenged with aerosolized OVA (as described in *Materials and Methods*). Bone marrow cells were analyzed by flow cytometry. OVA challenge increased EoPs in *Rcan1*^{+/+} mice. Bone marrow cells from OVA-challenged *Rcan1*^{-/-} mice contain reduced number of EoPs. The percentage of EoPs in total bone marrow cells (**D**) or in CD34⁺ cells (**E**) is reduced. Data are reported as means $\pm$ SEM ($n = 8$ mice from four independent experiments). * $P < 0.05$ versus the *Rcan1*^{+/+} group.

bone marrow cells from both *Rcan1*^{-/-} and *Rcan1*^{+/+} mice for 14 days in growth medium showed similar fluorescence intensities for Siglec-F (Figure 5B). No morphological difference could be seen between *Rcan1*^{-/-} and *Rcan1*^{+/+} eosinophils (Figure 5C). Thus, Rcan1 does not appear to influence the developmental capacity of eosinophils *in vitro*.

We further examined whether Rcan1 affects the proliferation capacity of eosinophils or eosinophil progenitors. CD34⁺Lin⁻Sca-1⁻ and CD34⁻Lin⁻Sca-1⁻ bone marrow cells were sorted by flow cytometry. These freshly isolated bone marrow cells and bone marrow-derived eosinophils were pulsed with [³H]thymidine, and the incorporation of [³H]thymidine was determined. No difference in [³H]thymidine incorporation was seen between *Rcan1*^{-/-} and *Rcan1*^{+/+} CD34⁺ cells or between *Rcan1*^{-/-} and *Rcan1*^{+/+} CD34⁻ cells (Figure 5D). Similarly, no difference in [³H]thymidine incorporation was seen between *Rcan1*^{-/-} and *Rcan1*^{+/+} eosinophils (Figure 5E). Thus, Rcan1 does not affect the proliferation of eosinophils or eosinophil progenitors.

Decreased Calcineurin Activity in *Rcan1*^{-/-} CD34⁺ Bone Marrow Cells

The function of Rcan1 is largely associated with calcineurin activity; however, little is known about calcineurin in eosinophils. To determine calcineurin expression in eosinophils, bone marrow-derived eosinophils from *Rcan1*^{-/-} and *Rcan1*^{+/+} mice were stimulated with IL-5 or left unstimulated. Cell lysates were analyzed for calcineurin A by Western blot analysis. We found that calcineurin is constitutively expressed in eosinophils. IL-5 stimulation did not change the expression level of calcineurin. No difference in calcineurin expres-

sion level was observed between *Rcan1*^{-/-} and *Rcan1*^{+/+} eosinophils (Figure 6A).

To examine calcineurin expression in eosinophil progenitors, bone marrow cells from *Rcan1*^{-/-} and *Rcan1*^{+/+} mice were depleted with lineage markers and Sca-1. CD34⁺Lin⁻Sca-1⁻ and CD34⁻Lin⁻Sca-1⁻ bone marrow cells were obtained by cell sorting. The CD34⁺ cell population contains eosinophil progenitors. CD34⁻ cells were also included as controls. These cells were subjected to Western blot analysis for calcineurin A and a calcineurin activity assay. No difference in calcineurin level was observed between CD34⁺ cells from *Rcan1*^{-/-} versus *Rcan1*^{+/+} mice or between CD34⁻ cells from *Rcan1*^{-/-} versus *Rcan1*^{+/+} mice (Figure 6B). Importantly, the calcineurin activity assay revealed that CD34⁺Lin⁻Sca-1⁻ bone marrow cells from *Rcan1*^{-/-} mice showed a decreased level of calcineurin activity, compared with *Rcan1*^{+/+} mice (Figure 6C).

Eosinophil lineage development is controlled by several classes of transcription factors. Of these transcription factors, GATA-1 is the most important for eosinophil lineage specification.³⁹ To examine whether Rcan1 regulates eosinophil production via GATA-1, we determined GATA-1 expression in eosinophil progenitors. Bone marrow cells from *Rcan1*^{-/-} and *Rcan1*^{+/+} mice were depleted with lineage markers and Sca-1. CD34⁺Lin⁻Sca-1⁻ and CD34⁻Lin⁻Sca-1⁻ bone marrow cells were obtained by cell sorting and analyzed by RT-PCR for GATA-1 expression. CD34⁺ cells from both *Rcan1*^{-/-} and *Rcan1*^{+/+} mice expressed similar levels of GATA-1. A similar level of GATA-1 was also observed in CD34⁻ cells (see Supplemental Figure S2A at <http://ajp.amjpathol.org>). To examine GATA-1 expression at the protein level, CD34⁺Lin⁻Sca-1⁻ bone marrow cells from *Rcan1*^{-/-} and *Rcan1*^{+/+} mice were obtained by cell sorting and then were fixed, permeabilized, and stained for

Figure 5. Rcan1 deficiency does not affect eosinophil maturation *in vitro*. **A:** Bone marrow cells were stained with various antibodies to identify Lin⁻Sca-1⁻CD34⁺IL-5R α ⁺c-Kit^{low} cells as eosinophil progenitor cells (EoPs). Fluorescence-activated cell-sorted EoPs (400 cells) were plated in methylcellulose medium containing 50 ng/mL of IL-5. After *in vitro* culture for 20 days, EoPs from both Rcan1^{+/+} and Rcan1^{-/-} mice produced a similar number of colonies. Data are reported as means $\pm$ SEM of four independent experiments. **B:** Representative histograms show that *in vitro* cultured bone marrow-derived eosinophils from both Rcan1^{+/+} and Rcan1^{-/-} mice express similar levels of Siglec-F. **C:** Bone marrow-derived eosinophils cultured *in vitro* from both Rcan1^{+/+} and Rcan1^{-/-} mice show similar morphology under Diff-Quik staining. Scale bar = 20 μ m. **D:** Bone marrow cells from wild-type and Rcan1^{-/-} mice were stained with anti-CD34 (Alexa Fluor 647) and a cocktail of antibodies to lineage markers (CD3, CD4, CD8, B220, Gr-1, and CD19) and Sca-1 (PE). Lineage⁺ or Sca-1⁺ cells were discarded by cell sorting. Remaining cells were further separated into CD34⁺ and CD34⁻ cell populations and pulsed with [³H]thymidine for 8 hours. Incorporation was measured on a Wallac β counter. Data are reported as means $\pm$ SEM from three independent experiments in triplicate. **E:** To determine eosinophil proliferation, bone marrow-derived eosinophils from wild-type and Rcan1^{-/-} mice were pulsed with [³H]thymidine for 8 hours in the presence of IL-5, and thymidine incorporation was then measured. Data are reported as means $\pm$ SEM from four independent experiments in triplicate.

GATA-1, c-Kit, and IL-5R α by multicolor staining. A distinct population of IL-5R α ⁺c-Kit^{low} cells was identified as eosinophil progenitors. We found that eosinophil progenitors from both Rcan1^{-/-} and Rcan1^{+/+} mice expressed similar levels of GATA-1 (see Supplemental Figure S2, B and C, at <http://ajp.amjpathol.org>). Thus, GATA-1 expression in eosinophil progenitors is not affected by Rcan1 deficiency.

Effects of Rcan1 on Eosinophil Migration and IL-4 Production, but Not Apoptosis

After entering the circulation, eosinophils are recruited into inflammatory sites. Eotaxins play an important role in eosinophil recruitment into the lung.⁴⁰ To determine

whether Rcan1 regulates eosinophil trafficking, bone marrow-derived Rcan1^{-/-} and Rcan1^{+/+} eosinophils were subjected to a chemotaxis assay in a Transwell plate using eotaxin (CCL11) as a chemoattractant. Rcan1^{-/-} eosinophils showed modestly reduced migration toward eotaxin (Figure 7A). This reduced migration capacity of Rcan1^{-/-} eosinophils may contribute to reduced eosinophilia in the lung *in vivo*, as seen in OVA-treated Rcan1^{-/-} mice.

Eosinophils express various adhesion molecules on their surface, including α 4 integrin.⁴¹ To determine whether Rcan1 deficiency leads to abnormal α 4 integrin expression, the α 4 integrin level on eosinophils was examined by flow cytometry. Bone marrow-derived eosinophils from both Rcan1^{-/-} and Rcan1^{+/+} mice expressed similar levels of α 4 integrin (Figure 7B). Stimulation of eosinophils with eotaxin for 1 hour induced an increase of α 4 integrin expression. Eotaxin-induced increase of α 4 integrin expression was similar in both Rcan1^{-/-} and Rcan1^{+/+} eosinophils (Figure 7B), suggesting that Rcan1 deficiency does not affect the expression of the adhesion molecule α 4 integrin.

To examine whether Rcan1 regulates eosinophil signal transduction, bone marrow-derived eosinophils were

Figure 6. Effects of Rcan1 on calcineurin expression or activity in eosinophils and their progenitors. **A:** Eosinophils cultured from the bone marrow of Rcan1^{+/+} and Rcan1^{-/-} mice were treated with IL-5 (500 ng/mL) for 5, 20, or 60 minutes or left untreated (NT). Cell lysates were analyzed by Western blotting for calcineurin. **B:** CD34⁺Lin⁻Sca-1⁻ and CD34⁻Lin⁻Sca-1⁻ bone marrow cells were obtained by cell sorting (see Materials and Methods). Eosinophils cultured from the bone marrow of wild-type mice (BMEo) were included as a control. Cell lysates were probed for calcineurin and actin. **C:** CD34⁺Lin⁻Sca-1⁻ and CD34⁻Lin⁻Sca-1⁻ bone marrow cells were obtained by cell sorting and subjected to a calcineurin activity assay. Data are reported as means $\pm$ SEM (n = 8 mice in three independent experiments). * P < 0.05 versus the Rcan1^{+/+} group.

Figure 7. Effects of *Rcan1* deficiency on eosinophil migration and function. **A:** The migration capacity of bone marrow-derived eosinophils from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice was examined using a Transwell assay. Eotaxin (CCL11, 1 to 1000 ng/mL) was used as a chemoattractant. Eosinophils from *Rcan1*^{-/-} mice show decreased migration capacity. Data are reported as means $\pm$ SEM of three independent experiments. **P* < 0.05 versus the *Rcan1*^{+/+} group. **B:** Eosinophils from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice were treated with eotaxin (100 ng/mL) for 1 hour. Expression of α 4 integrin was analyzed by flow cytometry. Gray open profile, isotype antibody; black open profile, no-treatment group; black solid profile, eotaxin-treated group. Similar levels of eotaxin-induced α 4 integrin expression were seen in both *Rcan1*^{+/+} and *Rcan1*^{-/-} eosinophils. **C:** Bone marrow-derived eosinophils from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice were stimulated with IL-5 (500 ng/mL) for various lengths of time. Total cell lysates were prepared for Western blotting. A similar pattern of IL-5 induced phosphorylation is seen in both *Rcan1*^{+/+} and *Rcan1*^{-/-} eosinophils. **D:** *Rcan1* deficiency leads to impaired IL-4 production. Eosinophils from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice were stimulated with IL-5 (500 ng/mL) or with IL-5 and eotaxin (100 ng/mL) for various lengths of time. Untreated cells (NT) served as control. Cell-free supernatants were collected for the detection of IL-4 by enzyme-linked immunosorbent assay. Error bars indicate standard error from six independent experiments. **P* < 0.05 versus the *Rcan1*^{-/-} group.

treated with IL-5. Phosphorylation of various signaling molecules was examined. No major difference in phosphorylation patterns of Stat5, MAP kinase p44/42, p38, and Gsk3 β was observed between *Rcan1*^{-/-} and *Rcan1*^{+/+} eosinophils (Figure 7C).

We reasoned that eosinophil survival might also affect eosinophilia in the lung. Others have shown that Rcan1 regulates CD4 T-cell apoptosis.³⁰ We attempted to determine whether Rcan1 regulates eosinophil apoptosis. Mature eosinophils were obtained by culturing bone marrow cells from *Rcan1*^{+/+} and *Rcan1*^{-/-} mice. After eosinophil maturation, IL-5 was withdrawn from the culture medium to induce apoptosis. Apoptotic cells were monitored for 1, 2, and 3 days by using annexin V and propidium iodide staining. Withdrawal of IL-5 induced a similar number of apoptotic cells in both *Rcan1*^{+/+} and *Rcan1*^{-/-} eosinophils (data not shown). Thus, Rcan1 appears to have no effect on eosinophil apoptosis. In addition, the baseline level of eosinophils in the intestine was examined. Both *Rcan1*^{+/+} and *Rcan1*^{-/-} mice appear to have similar levels of eosinophils in the intestine (see Supplemental Figure S3 at <http://ajp.amjpathol.org>).

In local tissues, eosinophils produce inflammatory mediators, such as IL-4, that contribute to airway inflammation. To examine whether Rcan1 regulates production of cytokines and chemokines, eosinophils from *Rcan1*^{-/-} and *Rcan1*^{+/+} mice were stimulated with IL-5 or with IL-5 and eotaxin. Cell-free supernatants were harvested to examine cytokines and chemokines using a cytokine multiplex assay that detects 23 cytokines and chemokines. The majority of the tested cytokines and chemokines were undetectable. Of note, IL-4 was the only cytokine found to be reduced in *Rcan1*^{-/-} eosinophils after stimulation with IL-5 or with IL-5 and eotaxin (Figure 7D). No major differences of IL-9, IL-13, interferon γ (IFN γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) production were observed between *Rcan1*^{-/-} and *Rcan1*^{+/+} eosinophils (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). These findings suggest that Rcan1 deficiency does not lead to overall eosinophil functional deficiency but rather that Rcan1 selectively affects eosinophil migration and IL-4 production.

Discussion

Asthma is associated with marked infiltration of eosinophils in the lung. Knowledge of the essential role of eosinophils in allergic asthma seen in eosinophil-deficient mice^{7,8} and in humans²⁻⁴ has led to the concept of targeting eosinophils as a therapeutic approach for the treatment of asthma. However, clinical trials using anti-IL-5 antibodies to target eosinophils failed to eliminate tissue eosinophils and did not improve clinical symptoms in patients with asthma.^{42,43} These findings highlight the importance of eosinophils in asthma and an urgent need for identifying mechanisms that control lung eosinophilia in allergic asthma.

In the present study, we identified Rcan1 as a novel signaling molecule essential for allergen-induced eosinophilia. Notably, *Rcan1*^{-/-} mice showed a near-complete

absence of eosinophilia in the BALF, lung, and blood in OVA-challenged mice. Further analysis revealed that *Rcan1*^{-/-} mice possess reduced numbers of eosinophil progenitor cells in the bone marrow. Eosinophil progenitor cells provide a constant supply of mature eosinophils in the blood and tissues. The number of eosinophil progenitors is known to be increased in allergic asthma.⁹⁻¹³ We found that allergen challenge induces a significant increase of eosinophil progenitors in wild-type mice, but not in *Rcan1*^{-/-} mice. Thus, the reduced number of eosinophil progenitor cells likely contributes to the lack of eosinophilia in OVA-challenged *Rcan1*^{-/-} mice. Accordingly, *Rcan1* represents a new target in the control of the eosinophil population in allergic asthma.

The near-complete absence of eosinophilia in *Rcan1*^{-/-} mice prompted us to determine various functional and developmental features of eosinophils, including maturation, migration, signal transduction, and cytokine production. *In vivo*, IL-5 induced fewer eosinophils in the blood in *Rcan1*^{-/-} mice, compared with *Rcan1*^{+/+} mice; *in vitro*, bone marrow cells from *Rcan1*^{-/-} mice produced fewer eosinophils by cell culture. These reduced eosinophil numbers *in vivo* and *in vitro* are likely due to reduced numbers of eosinophil progenitor cells in the bone marrow. This contention is based on our observation that the same number of eosinophil progenitor cells obtained by cell sorting from both *Rcan1*^{-/-} and *Rcan1*^{+/+} mice resulted in a similar number of eosinophil colonies in cell culture. Eosinophil progenitor cells isolated from *Rcan1*^{-/-} mice appear to develop normally into mature eosinophils with normal morphology and Siglec-F expression. This finding suggests that mechanisms governing eosinophil maturation are distinct from eosinophil progenitor regulation. This notion is consistent with other reports that the *dblGATA* enhancer is not essential for eosinophil differentiation *ex vivo*,³⁷ but is critical for the generation of eosinophil progenitors.³⁸

We observed that the decrease in the number of eosinophils in the BALF of OVA-treated *Rcan1*^{-/-} mice is more dramatic than the decrease in the number of eosinophil progenitors in *Rcan1*^{-/-} mice. Of note, in *Rcan1*^{-/-} mice eosinophils showed reduced migration capacity in response to the chemoattractant eotaxin. Thus, it is likely that the decreased numbers of eosinophil progenitors, together with the impaired eosinophil migration capacity, collectively contribute to the severe deficiency of eosinophilia in *Rcan1*^{-/-} mice.

Eosinophils contribute to lung inflammation by producing inflammatory mediators such as IL-4. We found that *Rcan1*^{-/-} eosinophils produced less IL-4 in response to stimulation with IL-5 or with IL-5 and eotaxin. Accordingly, *Rcan1* likely regulates allergic inflammation by reducing the number of eosinophils and their mediator production capability.

It is noteworthy that eosinophils developed from both *Rcan1*^{-/-} and *Rcan1*^{+/+} mice showed a similar pattern of phosphorylation of various signaling molecules, including Stat5, p38, p44/42 MAP kinase, and Gsk3 β , in response to IL-5 stimulation. Likewise, eosinophils from both *Rcan1*^{-/-} and *Rcan1*^{+/+} mice showed similar levels of α 4 integrin expression. These findings suggest that *Rcan1* deficiency

does not generate an overall functional deficiency on eosinophils. Rather, *Rcan1* modulates specific aspects of eosinophil function, including migration and IL-4 production.

The positive regulatory role of *Rcan1* in eosinophil progenitors and eosinophilia development in allergic lung inflammation seen in the present study is in contrast to the negative effects of *Rcan1* in mast cells.³⁴ *Rcan1* deficiency leads to enhanced calcineurin activity, increased NF κ B and NFAT activation, and increased cytokine production by mast cells *in vitro*.³⁴ In the present study, *Rcan1* deficiency led to reduced calcineurin activity in eosinophil progenitors. The contrasting effects of *Rcan1* on mast cells and eosinophil progenitors suggest that *Rcan1* has distinct effects on specific cell types or tissues. The opposite effects of *Rcan1* on calcineurin activity have been reported previously. *Rcan1* can function as an inhibitor^{29,30} or a facilitator^{24,28,31-33} of calcineurin. The exact mechanisms of such opposite effects are unclear. One possibility is that *Rcan1* is associated and regulated by different molecules in different cell types. *Rcan1* has been found to be physically associated with various molecules, including TAK1,⁴⁴ TAB2,⁴⁴ GSK-3 β ,⁴⁵ Raf1,⁴⁶ and calcineurin. TAK1 is able to convert *Rcan1* from an inhibitor to a facilitator of calcineurin signaling by direct phosphorylation of *Rcan1*.⁴⁴ Similarly, GSK-3 β is able to regulate *Rcan1* function.^{45,47} Mehta et al⁴⁷ showed that the GSK-3 β phosphorylation site on *Rcan1* is required for its activating effects on calcineurin but not for its inhibitory effects on calcineurin, and suggested that the inhibitory role of *Rcan1* is likely through competing with other substrates for docking onto calcineurin. To date, there is no report on the role of TAK1 in eosinophils and their progenitors or in mast cells, and only limited information on the role of GSK-3 β in eosinophils and mast cells. There is a possibility that the molecules associated with *Rcan1* such as TAK1 and GSK-3 β may contribute to the distinct role of *Rcan1* in functional regulation of eosinophil progenitors and mast cells.

Our findings, together with those of others, suggest that information generated from one cell type regarding *Rcan1* function cannot be readily generalized to other cell types. This notion may be particularly important, considering that *Rcan1* has been found in various tissues and cells^{22,25,26} and that studies on *Rcan1* have been performed in various different experimental models, including yeast.^{48,49}

In conclusion, we have identified novel roles for *Rcan1* in an important regulatory mechanism of eosinophilia in allergic asthma. *Rcan1* deficiency leads to reduced production of eosinophil progenitor cells, impaired eosinophil migration capacity, and reduced eosinophilia in the lung. Thus, *Rcan1* may serve as a potential target for the treatment of allergic asthma.

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