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***Polycomb* Group Gene Product Ring1B Regulates Th2-Driven Airway Inflammation through the Inhibition of Bim-Mediated Apoptosis of Effector Th2 Cells in the Lung**

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Polycomb group (*PcG*) gene products regulate the maintenance of *homeobox* gene expression in *Drosophila* and vertebrates. In the immune system, *PcG* molecules control cell cycle progression of thymocytes, Th2 cell differentiation, and the generation of memory CD4 T cells. In this paper, we extended the study of *PcG* molecules to the regulation of in vivo Th2 responses, especially allergic airway inflammation, by using conditional Ring1B-deficient mice with a CD4 T cell-specific deletion of the *Ring1B* gene (*Ring1B*^{-/-} mice). In *Ring1B*^{-/-} mice, CD4 T cell development appeared to be normal, whereas the differentiation of Th2 cells but not Th1 cells was moderately impaired. In an Ag-induced Th2-driven allergic airway inflammation model, eosinophilic inflammation was attenuated in *Ring1B*^{-/-} mice. Interestingly, *Ring1B*^{-/-} effector Th2 cells were highly susceptible to apoptosis in comparison with wild-type effector Th2 cells in vivo and in vitro. The in vitro experiments revealed that the expression of Bim was increased at both the transcriptional and protein levels in *Ring1B*^{-/-} effector Th2 cells, and the enhanced apoptosis in *Ring1B*^{-/-} Th2 cells was rescued by the knockdown of *Bim* but not the other proapoptotic genes, such as *Perp*, *Noxa*, or *Bax*. The enhanced apoptosis detected in the transferred *Ring1B*^{-/-} Th2 cells in the lung of the recipient mice was also rescued by knockdown of *Bim*. Therefore, these results indicate that Ring1B plays an important role in Th2-driven allergic airway inflammation through the control of Bim-dependent apoptosis of effector Th2 cells in vivo. *The Journal of Immunology*, 2010, 184: 4510–4520.

Helper CD4 T cell-dependent immune responses are controlled by various functional T cell subsets including Th1, Th2, and Th17 cells (1–5). Th1 cells produce IFN- γ , whereas Th2 cells produce IL-4, IL-5, and IL-13 and are involved in type I hypersensitivity. Several transcription factors that control Th1/Th2/Th17 cell differentiation have been revealed. Among them, GATA3 appears to be a key factor for Th2 cell differentiation (6–8), T-bet for Th1 cells (9), and retinoic acid-related orphan receptor γ and α for Th17 cells (10, 11). Chromatin modification of the IL-4/IL-5/IL-13 locus occurs during Th2 cell differentiation (12–14) and is primarily mediated by GATA3 (15).

Th2 cells play an important role in the pathogenesis of allergic asthma via the induction of allergen-specific IgE production, airway inflammation, airway hyperresponsiveness, and mucus hyperproduction (16–20). The administration of allergens adsorbed with alum induces reproducible allergen-specific acquired immune responses that are dependent on Th2 cells producing IL-4, IL-5, and IL-13. A subsequent allergen challenge via the airway causes the rapid activation and recruitment of Th2 cells, resulting in enhanced vascular permeability, mucus hypersecretion, and cellular infiltration into the lung tissue, particularly eosinophilic infiltration in the perivascular and peribronchiolar regions.

There are many important differences between humans and mice in terms of the anatomical, physiological, and immunological contributors to disease (21, 22). Caution in interpretation of the results of the studies in mice is necessary, and knowledge and limitations of the various approaches used are essential (23). The majority of mouse models are acute and so do not address the permanent airway remodeling seen in chronic asthmatics. However, these models do provide the opportunity to dissect the complex mechanisms controlling the accumulation of inflammatory leukocytes in the lung. Although there are limitations when mouse models are translated to the human diseases, much has been learned from these investigations.

After Ag recognition by the TCR, naive CD4 T cells undergo clonal expansion followed by differentiation into functional effector Th cells (24, 25). After Ag elimination, the life span of Ag-activated Th cells is effectively controlled to maintain homeostasis. Apoptosis allows for the decline of Th cell numbers during the contraction phase (24, 26). Two concepts for the induction of T cell death during the shutdown of an immune response have been described: activation-induced cell death and activated T cell autonomous death (ACAD) (25). Although activation-induced cell death seems to be most important for the elimination of chronically activated and potentially autoreactive T cells

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Abbreviations used in this paper: ACAD, activated T cell autonomous death; BAL, bronchoalveolar lavage; Eos, eosinophil; HPRT, hypoxanthine phosphoribosyltransferase; Lym, lymphocyte; M ϕ , macrophage; N.D., not detected; Neu, neutrophil; PAS, periodic acid-Schiff; *PcG*, *Polycomb* group; PI, propidium iodide; PRC, *Polycomb* repressive complex; siRNA, small interfering RNA; Tg, transgenic.

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(27). ACAD also contributes to the downregulation of T cell numbers during the contraction phase (28). ACAD is regulated by intrinsic cell death pathways and involves members of the Bcl2 family. The Bcl2 family members consist of three subfamilies (29, 30). The first group, typified by Bcl2 and Bcl-x_L, is antiapoptotic and includes all four of the short peptide regions, BH1–4, that characterize the family. The second group, composed in T cells of Bax and Bak, are the actual killers of the cell and include only three of the four homology domains, BH1–3. The BH3-only proteins, including Bim and Noxa, are the third group of the Bcl2 family proteins and share the BH3 domain, a 9-aa sequence. In contrast to the other groups, BH3-only proteins are regulators of cell death rather than effectors and are localized in various organelles and in the cytoplasm (31).

The *Polycomb* group (*PcG*) gene products form multimeric complexes and mediate the silencing activity of specific genes including the *homeobox* genes both in invertebrates and vertebrates (32, 33). Ring1B, Ring1A, Bmi-1, Mel-18, M33, Pc2, Rae-28/Mph1, and Mph2 are members of a multimeric protein complex similar to the *Polycomb* repressive complex (PRC)1 identified in *Drosophila*. Another *PcG* complex, PRC2, contains Eed, Suz12, EzH1, and EzH2 and possesses an intrinsic histone H3-K27 methyltransferase activity (34–36). Lack of individual components of PRC1 results in apoptosis and the loss of proliferative responses of immature lymphoid cells (37, 38). The requirement of Ring1B during early mammalian development (39) and inactivation of the X chromosome by catalyzing monoubiquitination of histone H2A (40–42) have been demonstrated. In mature lymphocytes, *PcG* gene products play several roles in the differentiation process and cell fate. Mel-18 controls Th2 cell differentiation through the regulation of GATA3 expression (43), and Bmi-1 controls the stability of GTAT3 protein in developing Th2 cells

(44). Moreover, Bmi-1 controls memory CD4 T cell survival through the direct repression of the *Noxa* gene (45). However, the roles of *PcG* gene products in *in vivo* immune responses mediated by effector Th2 cells still remain unknown.

In this study, we investigated the roles of Ring1B in Ag-induced allergic airway inflammation, an *in vivo* immune response mediated by effector Th2 cells, using conditional Ring1B-deficient mice with a CD4 T cell-specific deletion of the *Ring1B* gene. Our results indicate that Ring1B plays a crucial role in allergic airway inflammation through the control of Bim-dependent apoptosis of effector Th2 cells in the lung.

Materials and Methods

Mice

Ring1B^{fl/fl} mice that carry the *Ring1B* exon 2 flanked with loxP sites were described previously (41). CD4-Cre transgenic (Tg) mice were purchased from Taconic Farms (Germantown, NY). The mice used in this study were backcrossed to C57BL/6 more than eight times. C57BL/6 mice were purchased from Clea (Tokyo, Japan). TCRβδ-knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice in this study were 6–8 wk of age and were maintained under specific-pathogen-free conditions. Animal care was carried out in accordance with the guidelines of Chiba University (Chiba, Japan).

Immunofluorescent staining and flow cytometry analysis

In general, 10⁶ cells were incubated on ice for 30 min with the appropriate staining reagents according to a standard method (46). The reagents used in this study were as follows: anti-CD4-FITC (RM4-5), anti-CD4-PE (RM4-5), anti-CD4-APC (RM4-5), anti-TCRβ-PE (H57-597), anti-CD3ε-PE (145-2C11), anti-γC-PE, anti-CD25-PE (3C7), anti-CD69-PE (H1.2F3), anti-IL-2Rβ-PE (TM-β1), anti-CD62L-PE (MEL-14), anti-CD44-PE (IM7), and anti-IL-4Rα-PE were purchased from BD Pharmingen (San Diego, CA). Anti-CD8-

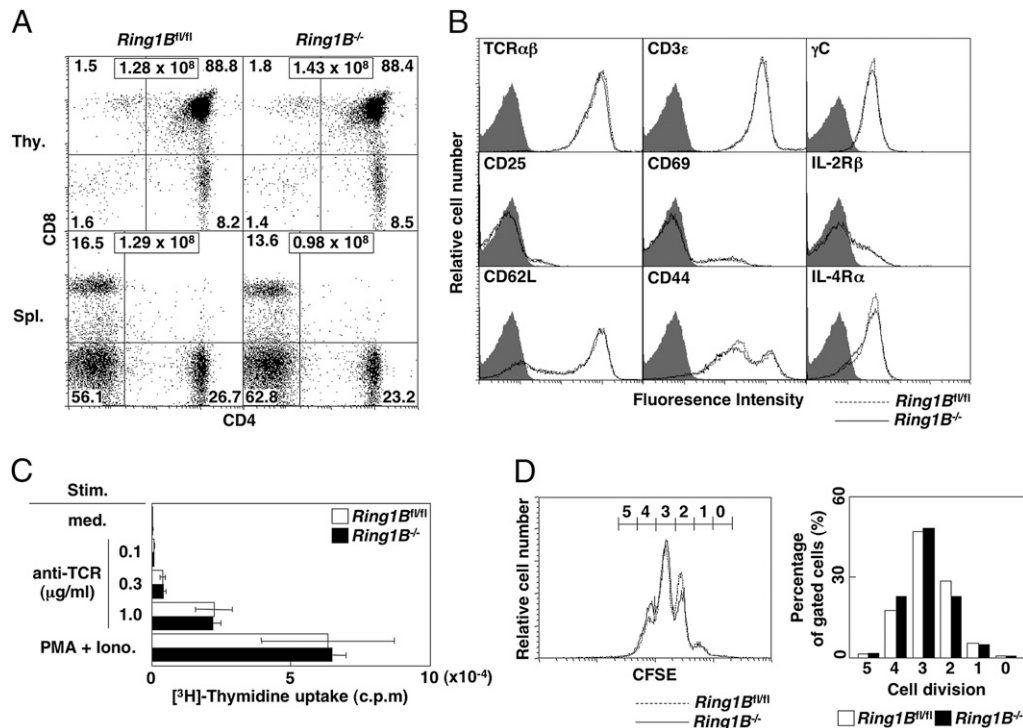


FIGURE 1. Phenotype and proliferative responses of splenic CD4 T cells from *Ring1B^{fl/fl}* and *Ring1B^{-/-}* mice. **A**, Representative CD4/CD8 profiles of thymocytes and splenocytes from *Ring1B^{fl/fl}* and *Ring1B^{-/-}* mice are shown. The yields of thymocytes and splenocytes are shown in boxed numbers. **B**, Each histogram depicts the expression of the indicated cell surface marker Ags on electronically gated splenic CD4 T cells from *Ring1B^{fl/fl}* (dotted line) and *Ring1B^{-/-}* mice (solid line). Background stainings are shown as shaded areas. Six individual mice in each group showed similar results. **C**, The proliferative response of splenic CD4 T cells from *Ring1B^{fl/fl}* mice (□) and *Ring1B^{-/-}* mice (■) are shown by [³H]thymidine incorporation. Splenic CD4 T cells were stimulated with immobilized anti-TCRβ mAb (0.1–1 μg/ml) or PMA (50 ng/ml) plus ionomycin (500 nM). Three independent experiments were performed with similar results. **D**, Splenic CD4 T cells were labeled with CFSE and stimulated with immobilized anti-TCRβ mAb in the presence of IL-4 for 48 h. Percentages of the cells in the gates representing numbers of cell division (0–5) are shown in the right panel.

Cy5 was prepared in our laboratory. Anti-Ly5.1-APC was purchased from eBioscience (San Diego, CA). Anti-FcγII and III mAb (2.4G2) were used as culture supernatants. Flow cytometry analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA), and results were analyzed with CellQuest software (BD Biosciences). Intracellular staining of IL-4 and IFN-γ was performed as described previously (47). APC-conjugated anti-IFN-γ mAb (XMGI.2) and PE-conjugated anti-IL-4 mAb (11B11) were used for detection and were purchased from BD Pharmingen.

Proliferation assay

Splenic CD4 T cells (2×10^5) were stimulated in 200-μl cultures for 40 h with immobilized anti-TCRβ mAb (H57-597) or PMA (50 ng/ml) and ionomycin (500 nM). [^{3} H]Thymidine (37 kBq/well) was added to the stimulation culture for the last 16 h, and the incorporated radioactivity was measured by using a beta plate (43). Where indicated, splenic CD4 T cells were labeled with CFSE and then stimulated with anti-TCRβ mAb in the presence of IL-4 for 2 d (48).

In vitro Th1/Th2 cell differentiation cultures and adoptive cell transfer

CD4 T cells were first isolated using an autoMACS Sorter (Miltenyi Biotec, Auburn, CA), then those with naive phenotype (CD44^{low}) were further purified using a FACS Vantage cell sorter (BD Biosciences) yielding a purity of >98% as described previously (49). Naive splenic CD4 T cells were stimulated with 3 μg/ml immobilized anti-TCRβ mAb (H57-597) in the presence of 30 U/ml IL-2, and 1–100 U/ml IL-12 (BD Pharmingen) and anti-IL-4 mAb (11B11) for Th1 cell differentiation, or 1–100 U/ml IL-4 (Toyobo, Osaka, Japan) and anti-IFN-γ mAb (R4-6A2) for Th2 cell differentiation as described previously (49). In some experiments, splenic CD4 T cells were stimulated with immobilized anti-TCRβ mAb (3 μg/ml)

plus anti-CD28 mAb (1 μg/ml). For adoptive cell transfer, splenic CD4 T cells purified from *Ring1B*^{fl/fl} and *Ring1B*^{-/-} Ly5.1 OT-II OVA-specific TCR Tg mice were stimulated with OVA peptide (0.3 μM, residues 323–339; ISQAVHAAHAEINEAGR synthesized by BEX, Tokyo, Japan) plus APC (irradiated splenocytes) under Th2 culture conditions for 6 d in vitro as described previously (50). The effector Th2 cells (1×10^7) were transferred i.v. into Ly5.1 C57BL/6-recipient mice.

Measurement of cytokines

The in vitro-differentiated Th2 cells were restimulated with 3 μg/ml immobilized anti-TCRβ mAb for 24 h. Splenocytes from sensitized and challenged mice were collected 24 h after the last challenge and cultured with 100 μg/ml OVA (Sigma-Aldrich, St. Louis, MO) and 2.5 μg/ml anti-IL-4Rα mAb (BD Pharmingen) for 48 h. The concentration of IL-4, IL-5, IL-13, and IFN-γ in the culture supernatant was measured by ELISA as described previously (43).

Sensitization and airway challenge with OVA

Mice were sensitized by an i.p. injection of 100 μg OVA adsorbed to 4 mg alum [Al(OH)₃] on day 0. OVA dissolved in PBS (100 μg per 30 μl) was administered intranasally to each mouse on days 7, 8, and 9.

Collection of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed 24 h after the last OVA challenge as described previously (51). A total of 100,000 viable BAL cells were cytocentrifuged onto slides by a Cytospin 4 (Thermo Electron, Waltham, MA) and stained with May-Grünwald-Giemsa solution (Merck, Darmstadt, Germany). Two hundred leukocytes were counted on each slide. Cell types were identified using morphological criteria. The mRNA

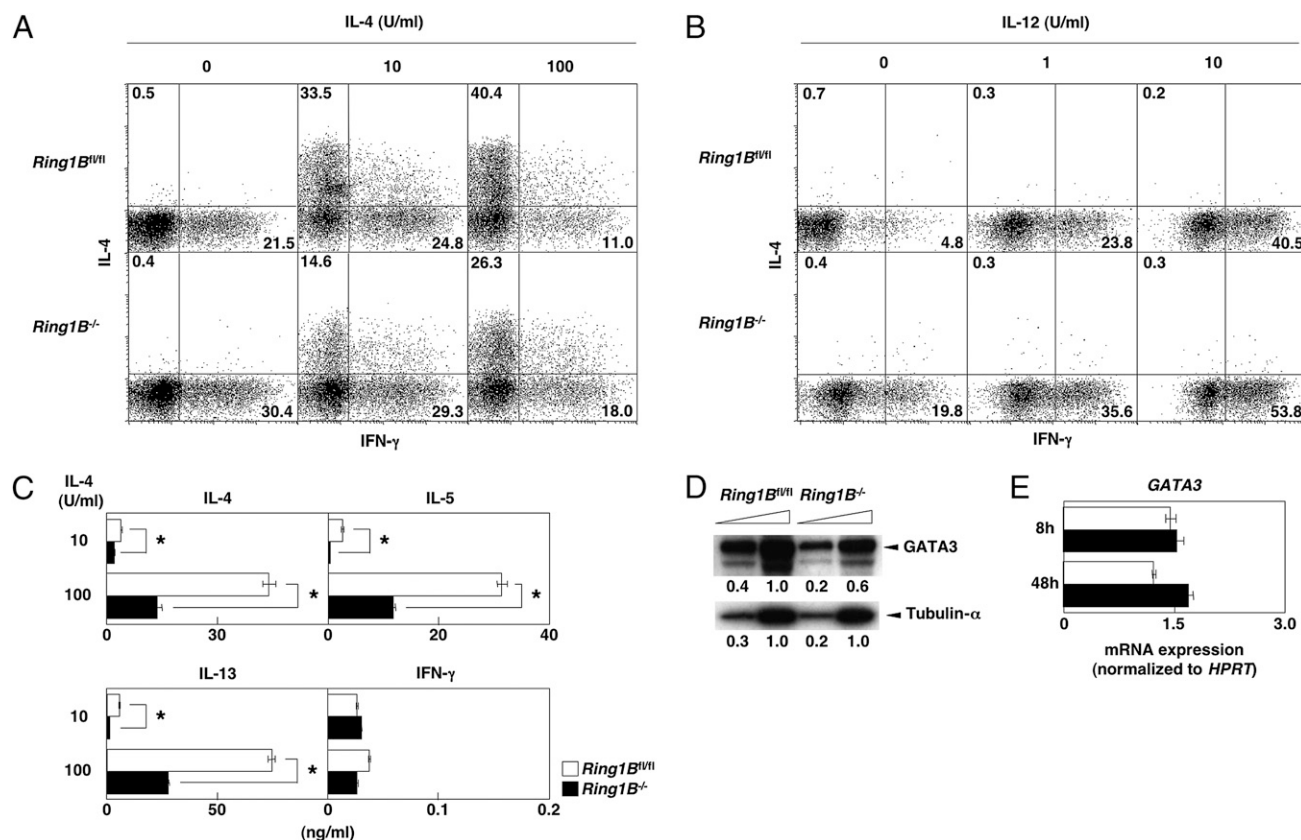


FIGURE 2. In vitro Th1/Th2 cell differentiation in splenic CD4 T cells from *Ring1B*^{-/-} mice. *A* and *B*, Intracellular IFN-γ/IL-4 profiles are shown with percentages of cells in each area. Naive splenic CD4 T cells from *Ring1B*^{-/-} mice were stimulated with immobilized anti-TCRβ mAb in the presence of IL-2 and indicated doses of IL-4 (*A*, Th2 conditions) or and indicated doses of IL-12 (*B*, Th1 conditions) for 5 d. *C*, On day 5, the in vitro-generated Th2 cells were restimulated with immobilized anti-TCRβ mAb for 24 h, and the production of IL-4, IL-5, IL-13 and IFN-γ in the culture supernatant was determined by ELISA (**p* < 0.01). *D*, On day 5, expression of GATA3 and tubulin-α in Th2 cells cultured with 100 U/ml IL-4 was analyzed by immunoblotting with 3-fold serial dilutions of cell lysates. Arbitrary densitometric units are shown under each band. *E*, Expression of GATA3 mRNA in cells cultured under Th2 conditions (100 U/ml IL-4) for indicated hours was assessed by quantitative PCR analysis. The mRNA expression levels were normalized to HPRT expression. Three independent experiments were performed with similar results.

expression levels of *IL-4*, *IL-5*, *IL-13*, *IFN- γ* , *Eotaxin-2*, *GATA3*, and *T-bet* by cells recovered by BAL were measured by quantitative PCR analysis.

Lung histology

Lungs were recovered 24 h after the last OVA challenge and infused with 10% (v/v) formalin in PBS for fixation. The lung samples were sectioned, stained with H&E or periodic acid-Schiff (PAS) reagent, and examined for pathological changes under a light microscope at $\times 200$ magnification. The number of infiltrated mononuclear cells or eosinophils in the peribronchiolar regions was calculated by direct counting in four different fields per slide as described previously (51).

Lung mononuclear cell preparation

The lungs were sliced into small cubes and then incubated for 30 min in 5 ml RPMI 1640 solution containing collagenase type III (2000 U/ml) (Worthington, Lakewood, NJ) and trypsin inhibitor (0.3 mg/ml) (Sigma-Aldrich). Lung mononuclear cells were separated by centrifugation on Percoll (GE Healthcare, Buckinghamshire, U.K.).

Immunoblotting

Immunoblotting for the detection of GATA3, Bim^{EL}, Bim^L, Arf, and Puma was performed as described previously (52). The mAbs specific for mouse Bim and Puma were purchased from Cell Signaling Technology (Beverly, MA), and the mAb specific for mouse Arf was purchased from Upstate Biotechnology (Lake Placid, NY).

Quantitative PCR analysis

Total RNA was isolated using TRIzol reagent. cDNA was synthesized using oligo(dT) primer and Superscript II RT (Invitrogen Life Technologies, Carlsbad, CA). Quantitative RT-PCR was performed as previously described using an ABI PRISM 7500 Sequence Detection System (49). The primers and TaqMan probes for the detection of mouse *Muc5ac*, *Gob5*, *IL-4*, *IL-5*, *IL-13*, *Eotaxin-2*, *T-bet*, and hypoxanthine phosphoribosyltransferase (HPRT) were purchased from Applied Biosystems (Foster City, CA). The probes for the detection of the other genes in this study were purchased from Roche Diagnostics (Basel, Switzerland). Primers for the Roche Diagnostics probes were as follows: HPRT forward, 5'-CCTGGTTCATCATCGCTAATC-3'; HPRT reverse, 5'-TCCTCCTCAGACCGCTTTT-3'; *GATA3* forward, 5'-TTATCAAGCCCAAGC-GAAG-3'; *GATA3* reverse, 5'-TGGTGGTGGTCTGACAGTTC-3'; *IFN- γ* forward, 5'-ATCTGGAGGAAGTGGCAAAA-3'; *IFN- γ* reverse, 5'-TTCAAGACTTCAAAGATCTGAGG-3'; *Bim* forward, 5'-GGAGACGAGTTC-AACGAACTT-3'; *Bim* reverse, 5'-AACAGTTGTAAGATAACCATTTG-AGG-3'; *Noxa* forward, 5'-CAGATGCCTGGGAAGTCG-3'; *Noxa* reverse, 5'-TGAGCACACTCGTCTTCAA-3'; *Bax* forward, 5'-GTGAGCGGCT-GCTTGTCT-3'; *Bax* reverse, 5'-GGTCCCGAAGTAGGAGAGGA-3'; *Perp* forward, 5'-GACCCAGATGCTTGTTTTC-3'; *Perp* reverse, 5'-ACCAG-GGAGATGATCTGGAA-3'; *Mcl1* forward, 5'-GGCTTGTCAAACAAAG-AGG-3'; *Mcl1* reverse, 5'-TCTTATTAGATATGCCAGACCAG-3'; *Bcl2* forward, 5'-GCCTCTTCACCTTTCAGCAT-3'; *Bcl2* reverse, 5'-CTGCTTT-TTATTTCATGAGGTACATT-3'; *Bcl-x_L* forward, 5'-TGACCACCTAGAGC-CTTGGA-3'; *Bcl-x_L* reverse, 5'-TGTTCCTGAGATCCACAA-3'; *Ring1B* (exon2) forward, 5'-ACAACGAAACCTCAGGTAA-3'; and *Ring1B* (exon2) reverse, 5'-CCAGTTTATGAGGCTATCTCC-3'. Gene expression was normalized using the HPRT signal.

Knockdown assay

For the knockdown of proapoptotic genes, we used the Mouse T cell Nucleofector Kit (amaxa) according to the manufacturer's protocol. The small interfering RNAs (siRNAs) for knockdown of Bim (s62987), Noxa (s81670), Bax (s62875), Perp (s82105), and the negative control (1, AM4635) were purchased from Applied Biosystems.

Detection of apoptotic cells

For the detection of apoptotic cells, we used the Annexin V^{FTC} or Annexin V^{APC} apoptosis detection kit II (BD Biosciences) according to the manufacturer's protocol. For cell transfer experiments, effector Th2 cells cultured from OT-II Tg splenic CD4 T cells were transferred i.v. into Ly5.2⁺ C57BL/6 host animals, and the mice were challenged by intranasal administration of OVA on 3 consecutive days after cell transfer. Lymphoid cells in the lung were purified 12 h after the last challenge. For knockdown assays in vitro, effector Th2 cells were transfected with siRNA and cultured for 3 h with IL-2 (10 U/ml). Then, the cells were cultured with medium alone for 16 h, and the levels of apoptosis were determined. For measurement of apoptosis of siRNA knockdown cells in the lung, 1×10^7 cells prepared as stated above were

transferred i.v. into TCR $\beta\delta$ -knockout host mice. The mice were intranasally injected with PBS two times from a day after transfer. Twelve hours after the last injection, the lymphoid cells were analyzed. Percent rescue was calculated using a formula as follows: (number of Annexin V⁺ cells in control siRNA transfected *Ring1B*^{-/-} cells) - (number of Annexin V⁺ cells in Bim siRNA transfected *Ring1B*^{-/-} cells) / (number of Annexin V⁺ cells in control siRNA transfected *Ring1B*^{-/-} cells) - (number of Annexin V⁺ cells in control siRNA transfected *Ring1B*^{fl/fl} cells) $\times 100$ (%).

Data analysis

Statistical analysis was performed using the two-tailed Student *t* test. Values are presented as the mean $\pm$ SD.

Results

Phenotypic and functional characterization of peripheral CD4 T cells in CD4⁺ T cell-specific *Ring1B*-deficient mice

The aim of this study was to clarify the role of the *PcG* molecule Ring1B in Th2-dependent in vivo immune responses, such as allergic airway inflammation, using conditional Ring1B-deficient (*Ring1B*^{-/-}) mice with a CD4⁺ T cell-specific deletion of the *Ring1B* gene. We crossed mice containing a loxP-flanked Ring1B gene (41) with mice Tg for the Cre recombinase gene controlled by the CD4 promoter. The resulting mice were designated *Ring1B*^{-/-} mice throughout this paper. Non-Tg *Ring1B*^{fl/fl} littermates served as controls in all experiments.

First, we examined T cell development in the thymus in *Ring1B*^{fl/fl} and *Ring1B*^{-/-} mice (Fig. 1A). CD4/CD8 profiles of thymocytes and splenocytes of *Ring1B*^{-/-} mice showed normal development of CD4 and CD8 T cells. The cell surface expression of TCR β , CD3 ϵ , common γ -chain, CD25, CD69, IL-2R β , CD62L, CD44, and IL-4R α on splenic CD4 T cells of *Ring1B*^{-/-} mice was comparable to those of *Ring1B*^{fl/fl} mice (Fig. 1B). CD4⁺CD25⁺ regulatory T cells, TCR β ⁺NK1.1⁺ NKT cells in the spleen of *Ring1B*^{-/-} mice and in vitro-differentiated induced regulatory T cells were normally developed in comparison with those of *Ring1B*^{fl/fl} mice (data not shown). In addition, anti-TCR β mAb-induced proliferative responses of splenic CD4 T cells were indistinguishable between *Ring1B*^{fl/fl} and *Ring1B*^{-/-} mice (Fig. 1C). The anti-TCR β mAb-induced cell division of *Ring1B*^{-/-} splenic CD4 T cells assessed by CFSE-labeling experiments was similar to that of *Ring1B*^{fl/fl} splenic CD4 T cells (Fig. 1D). We obtained similar results for the development of CD4 T cells and the anti-TCR β mAb-induced proliferation and cell division of splenic CD4 T cells in pLck-Cre *Ring1B*^{-/-} mice, which are another line of mice with a T cell-specific deletion of *Ring1B* gene (data not shown). Moreover, cell cycle progression assessed by BrdU and 7-aminoadenine D staining were indistinguishable between *Ring1B*^{fl/fl} and *Ring1B*^{-/-} splenic CD4 T cells (data not shown). Thus, no obvious defect in the phenotype and proliferative responses of *Ring1B*^{-/-} splenic CD4 T cells was noted.

Impaired Th2 cell differentiation of *Ring1B*^{-/-} CD4 T cells in vitro

Next, we assessed the capability of *Ring1B*^{-/-} CD4 T cells to differentiate into Th1/Th2 cells in vitro. Naive splenic CD4 T cells (CD4⁺CD44^{low}) were cultured under Th1 or Th2 conditions, and Th1/Th2 cell differentiation was assessed by intracellular cytokine staining and ELISA (Fig. 2). Under Th2 conditions, Th2 cells were generated in an exogenous IL-4 dose-dependent manner in the control *Ring1B*^{fl/fl} T cell cultures, and the generation of Th2 cells in *Ring1B*^{-/-} mice was moderately impaired at all concentrations of IL-4 tested (Fig. 2A). In contrast, Th1 cell differentiation in *Ring1B*^{-/-} mice was not inhibited but rather enhanced under Th1 conditions (Fig. 2B). The production of IL-4, IL-5, and IL-13 in the in vitro-generated *Ring1B*^{-/-} Th2 cells as in Fig. 2A was significantly decreased (Fig. 2C). The expression of GATA3 protein was

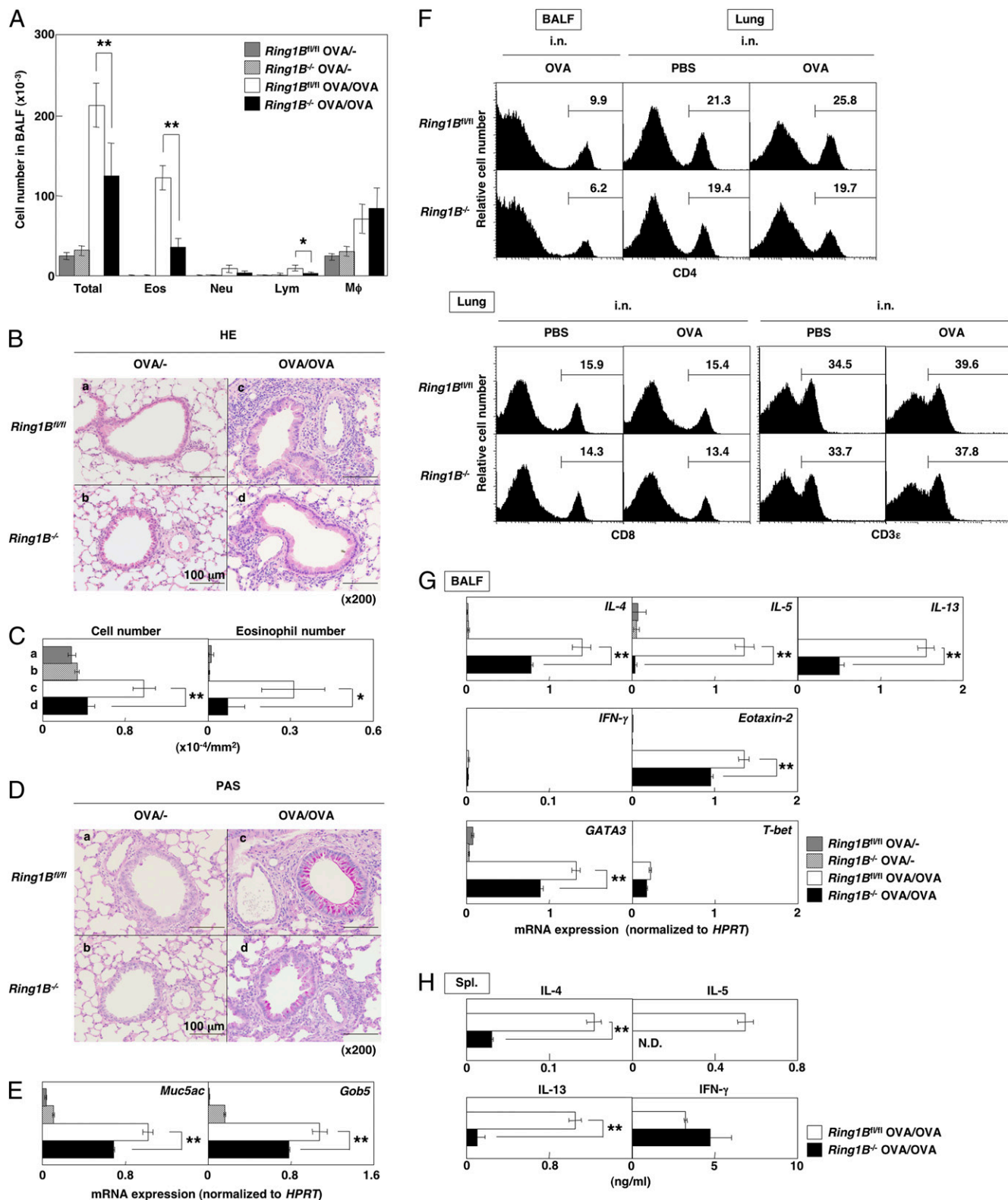


FIGURE 3. Reduced airway inflammation in *Ring1B*^{-/-} mice. Airway inflammation was induced with OVA sensitization and challenges. **A**, Total numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), and macrophages (Mφ) in the BAL fluid of *Ring1B*^{fl/fl} and *Ring1B*^{-/-} mice 24 h after the last challenge are shown. The results were obtained using the percentages of each cell type, total cell number per milliliter, and the volume of BAL fluid recovered. Samples were collected 24 h after the last OVA challenge. Mean values with SDs ($n = 5$) are shown. Three independent experiments were done with similar results ($*p < 0.05$; $**p < 0.01$). **B**, The level of OVA-induced airway inflammation in *Ring1B*^{-/-} mice was examined by histological analysis. Ag-induced leukocyte infiltration into the lung was evaluated using H&E staining. **a** and **b**, *Ring1B*^{fl/fl} and *Ring1B*^{-/-}, respectively, with OVA priming but not OVA challenge; **c** and **d**, *Ring1B*^{fl/fl} and *Ring1B*^{-/-}, respectively, with OVA priming and OVA challenge. Magnification $\times 200$. **C**, The numbers of infiltrated mononuclear cells and eosinophils in the perivascular and peribronchiolar regions were calculated by direct counting from four different fields per slide. The mean values with SDs ($n = 5$) are shown ($*p < 0.05$; $**p < 0.01$). **D**, Ag-induced goblet cell hyperplasia was evaluated by PAS staining. Representative photographic views of *Ring1B*^{fl/fl} and *Ring1B*^{-/-} mice are shown. OVA/-, with OVA priming but not OVA challenge; OVA/OVA, with OVA priming and OVA

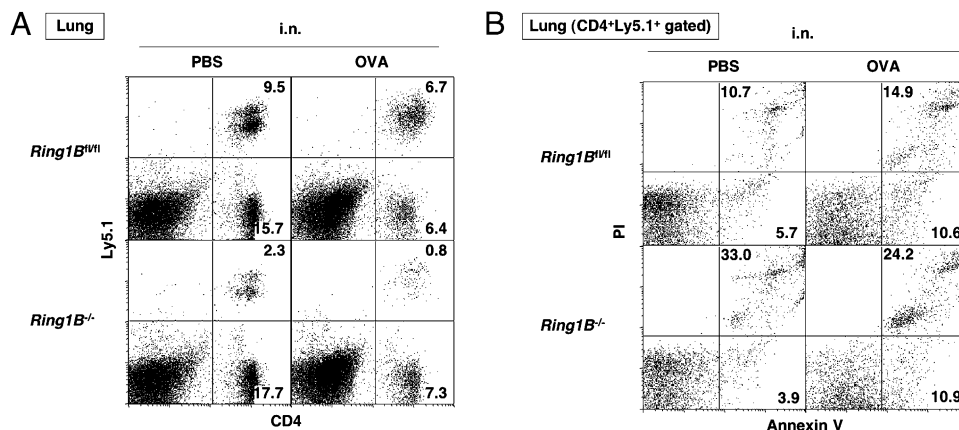


FIGURE 4. Increased cell death in *Ring1B^{-/-}* Th2 cells in the lung. In vitro-differentiated effector Th2 cells from C57BL/6 Ly5.1⁺ OT-II Tg *Ring1B^{-/-}* mice were transferred into C57BL/6 Ly5.2⁺ mice. The mice were sensitized three times by intranasal injection of OVA in PBS from a day after transfer. Lymphocytes were purified 12 h after the last injection. **A**, A representative Ly5.1/CD4 profile of the cells in the lung. **B**, The Annexin V/PI staining profiles were determined in electronically gated CD4⁺Ly5.1⁺ donor cells in the lung. Three experiments were performed with similar results. OVA, with OVA challenge; PBS, with PBS challenge.

slightly but reproducibly decreased in *Ring1B^{-/-}* Th2 cells (Fig. 2D), whereas the mRNA expression of *GATA3* appeared not to be altered in *Ring1B^{-/-}* Th2 cells (Fig. 2E), suggesting a post-transcriptional regulation of the *GATA3* expression by Ring1B. We confirmed moderately but reproducibly impaired Th2 cell differentiation in pLck-Cre *Ring1B^{-/-}* CD4 T cells (data not shown). These results indicate that the differentiation of CD4 T cells into Th2 cells is moderately impaired in the absence of Ring1B.

Impaired Th2-driven allergic airway inflammation in *Ring1B^{-/-}* mice

We used an OVA-induced airway inflammation model to evaluate the levels of Th2 cell-dependent in vivo immune responses in *Ring1B^{-/-}* mice. In BAL fluid from the OVA-immunized and OVA-intranasally challenged *Ring1B^{-/-}* mice, total infiltrating leukocytes, eosinophils, and lymphocytes were significantly decreased (Fig. 3A). In addition, we examined the histological changes in the lungs of allergy-induced *Ring1B^{-/-}* mice by H&E staining (Fig. 3B). Few inflammatory cells were observed in the lungs of *Ring1B^{fl/fl}* and *Ring1B^{-/-}* mice that did not receive the OVA challenge (Fig. 3Ba, 3Bb). Substantial numbers of mononuclear cells infiltrated the peribronchiolar regions in *Ring1B^{fl/fl}* mice after the OVA challenge (Fig. 3Bc); however, the levels of mononuclear cell infiltration were reduced in *Ring1B^{-/-}* mice (Fig. 3Bd). The numbers of infiltrated cells and eosinophils were determined by direct counting, and significantly decreased numbers of infiltrated mononuclear cells and eosinophils were detected in the peribronchiolar regions in allergy-induced *Ring1B^{-/-}* mice (Fig. 3C). We then examined the levels of mucus hyperproduction by PAS staining. Representative staining profiles of the bronchiolar regions in allergy-induced *Ring1B^{-/-}* mice are shown (Fig. 3D). No specific staining was detected in *Ring1B^{fl/fl}* and *Ring1B^{-/-}* mice without the OVA challenge (Fig. 3Da, 3Db). Moderate staining was noted in

Ring1B^{fl/fl} bronchioles, whereas the staining levels were slightly but reproducibly decreased in the *Ring1B^{-/-}* bronchioles (Fig. 3Dc, 3Dd). Consequently, we examined the mRNA expression of *Muc5ac* and *Gob5* in the lungs of *Ring1B^{-/-}* mice (Fig. 3E). Expression of both was mildly reduced in *Ring1B^{-/-}* mice compared with *Ring1B^{fl/fl}* mice after OVA challenge.

As shown in Fig. 3A, we observed a reduction in infiltrating lymphocytes in the BAL fluid. Thus, we examined what population of lymphocytes was decreased in allergy-induced *Ring1B^{-/-}* mice. Interestingly, the percentage of CD4⁺ cells in BAL fluid and in the lung of *Ring1B^{-/-}* mice was moderately decreased (BALF OVA: *Ring1B^{fl/fl}*, 9.9% versus *Ring1B^{-/-}*, 6.2%; lung PBS: *Ring1B^{fl/fl}*, 21.3% versus *Ring1B^{-/-}*, 19.4%; and lung OVA: *Ring1B^{fl/fl}*, 25.8% versus *Ring1B^{-/-}*, 19.7%), whereas no obvious difference was observed in the percentages of CD8⁺ and CD3⁺ cells in the lung (Fig. 3F). Following OVA challenge, the population of CD4⁺CD44^{high}CD62L^{low} and CD4⁺CD44^{high}CD62L^{high} cells in the BAL fluid and in lung tissue was indistinguishable between *Ring1B^{fl/fl}* and *Ring1B^{-/-}* mice (data not shown).

The mRNA expression of Th2 cytokines *IL-4*, *IL-5*, and *IL-13*, the eosinophil-specific chemokine *Eotaxin-2* and *GATA3* in the BAL fluid cells was significantly decreased, whereas the expression of *IFN-γ* and *T-bet* was very low level, and no apparent difference was observed in the allergy-induced *Ring1B^{-/-}* mice in comparison with that of *Ring1B^{fl/fl}* mice (Fig. 3G). The impaired *IL-5* production in *Ring1B^{-/-}* mice was most prominently and reproducibly detected in this assay system. In addition, we assessed the ratio of *GATA-3/T-bet* mRNA expression, and the ratio was slightly decreased in BAL fluid cells in allergy-induced *Ring1B^{-/-}* mice compared with *Ring1B^{fl/fl}* mice (*Ring1B^{fl/fl}*, 6.0 versus *Ring1B^{-/-}*, 5.1). We also examined *GATA3* protein expression in lung tissue in allergy-induced *Ring1B^{-/-}* mice, and no apparent difference was observed between *Ring1B^{fl/fl}* and *Ring1B^{-/-}* mice (data not shown).

challenge. Magnification ×200. **E**, The expression levels of *Muc5ac* and *Gob5* mRNA by cells in the lung were assessed by quantitative PCR analysis. The mRNA expression levels were normalized to HPRT expression. **a** and **b**, *Ring1B^{fl/fl}* and *Ring1B^{-/-}*, respectively, with OVA priming but not OVA challenge; **c** and **d**, *Ring1B^{fl/fl}* and *Ring1B^{-/-}*, respectively, with OVA priming and OVA challenge. **F**, Representative CD4 profiles in BAL fluid and CD4, CD8, and CD3 profiles in lung tissue recovered from *Ring1B^{fl/fl}* and *Ring1B^{-/-}* mice on which BAL had also been performed. **G**, The expression levels of *IL-4*, *IL-5*, *IL-13*, *IFN-γ*, *Eotaxin-2*, *GATA3*, and *T-bet* mRNA by BAL cells were examined by quantitative PCR analysis. The mRNA expression levels were normalized to HPRT expression. **H**, Splenocytes from allergy-induced *Ring1B^{-/-}* mice were stimulated with 100 μg/ml OVA in vitro for 48 h, and the production of *IL-4*, *IL-5*, *IL-13*, and *IFN-γ* in culture supernatants was determined by ELISA. Three independent experiments were performed with similar results. Eos, eosinophil; Neu, neutrophil; Lym, lymphocyte; Mφ, macrophage; N.D., not detected.

Next, splenocytes from the OVA-immunized and OVA-challenged *Ring1B^{fl/fl}* and *Ring1B^{-/-}* mice were stimulated with Ag in vitro, and the production of Th1/Th2 cytokines was assessed. As shown in Fig. 3H, the production of IL-4, IL-5, and IL-13 was substantially reduced in the *Ring1B^{-/-}* groups. These results indicate that Th2-driven OVA-induced allergic airway inflammation is attenuated in *Ring1B^{-/-}* mice.

Apoptotic cells were increased in the transferred *Ring1B^{-/-}* Th2 cells in vivo

Next, we assessed the levels of apoptosis in *Ring1B^{-/-}* effector Th2 cells after adoptive transfer using Annexin V and propidium iodide (PI) staining. OVA-specific $\alpha\beta$ TCR Tg (OT-II Tg) splenic CD4 T cells from *Ring1B^{fl/fl}* or *Ring1B^{-/-}* C57BL/6 Ly5.1 mice were stimulated with OVA peptide and normal C57BL/6 Ly5.1 mouse APCs under Th2 culture conditions in vitro for 6 d. Effector Th2 cells were adoptively transferred into Ly5.2 C57BL/6 host animals, and the mice were challenged by an intranasal injection of OVA in PBS or PBS alone each day for 3 d after cell transfer. Twelve hours after the last challenge, the number of transferred CD4⁺Ly5.1⁺*Ring1B^{-/-}* donor Th2 cells in the lung was reduced in comparison with that of *Ring1B^{fl/fl}* Th2 cells (PBS: *Ring1B^{fl/fl}*, 9.5% versus *Ring1B^{-/-}*, 2.3%; and OVA: *Ring1B^{fl/fl}*, 6.7% versus *Ring1B^{-/-}*, 0.8%) (Fig. 4A). Furthermore, higher numbers of Annexin V⁺ cells were detected in transferred Ly5.1 *Ring1B^{-/-}* Th2 cells (the sum of upper and lower right quadrants; PBS: *Ring1B^{fl/fl}*, 16.4% versus *Ring1B^{-/-}*, 36.9%; and OVA: *Ring1B^{fl/fl}*, 25.5% versus *Ring1B^{-/-}*, 35.1%) (Fig. 4B). These results indicate that *Ring1B^{-/-}* Th2 cells are more susceptible to apoptosis regardless of the intranasal challenge of OVA.

Ring1B^{-/-} effector Th2 cells were more susceptible to cell death in vitro

Next, we performed in vitro experiments to obtain additional insights into the molecular mechanisms underlying the Ring1B-mediated control of apoptosis in effector Th2 cells. Effector Th2 cells were

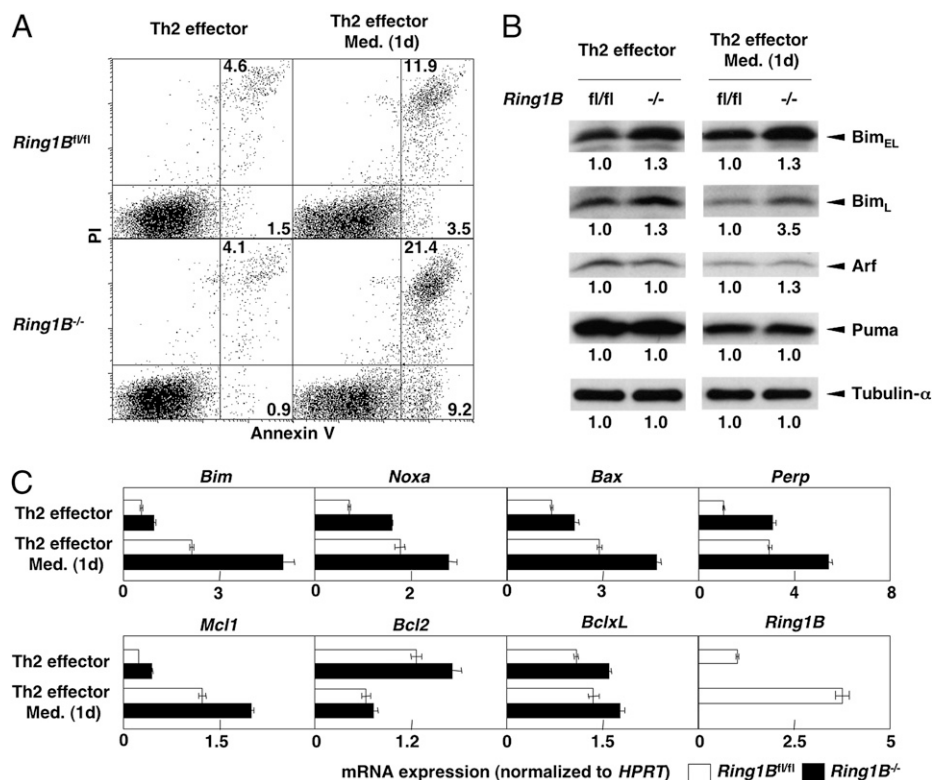
cultured with medium alone for 24 h (cytokine withdrawal), and Annexin V/PI staining profiles of these cells were analyzed (Fig. 5A). The levels of Annexin V⁺PI⁻ and Annexin V⁺PI⁺ cells were indistinguishable between *Ring1B^{fl/fl}* and *Ring1B^{-/-}* effector Th2 cells. In contrast, the levels of both Annexin V⁺PI⁻ and Annexin V⁺PI⁺ cells were significantly increased in *Ring1B^{-/-}* Th2 cells after cytokine withdrawal (Annexin V⁺PI⁻: *Ring1B^{fl/fl}*, 3.5% versus *Ring1B^{-/-}*, 9.2%; and Annexin V⁺PI⁺: *Ring1B^{fl/fl}*, 11.9% versus *Ring1B^{-/-}*, 21.4%). Similar results were obtained in *Ring1B^{-/-}* Th1 cells (data not shown).

We then assessed the expression levels of various proapoptotic and antiapoptotic molecules in the effector Th2 cells after cultivation in medium alone for 24 h. Interestingly, the expression of Bim protein, particularly the Bim_L isoform, was increased in *Ring1B^{-/-}* Th2 cells after cytokine withdrawal, whereas no obvious difference was observed in the expression of the other proapoptotic proteins Arf and Puma (Fig. 5B). In addition, mRNA expression of several proapoptotic genes *Bim*, *Noxa*, *Bax*, and *Perp* were significantly increased in *Ring1B^{-/-}* Th2 cells, whereas no decrease in the expression of the antiapoptotic genes *Mcl1*, *Bcl2*, and *Bcl-x_L* was detected (Fig. 5C). Ring1B knockout was clearly evident in the mRNA expression of *Ring1B* in *Ring1B^{-/-}* Th2 cells (Fig. 5C). The expression of Fas, Fas ligand, and TNFR1 α on the cell surface and the expression of Bcl2 protein were indistinguishable between *Ring1B^{fl/fl}* and *Ring1B^{-/-}* Th2 cells even after cytokine withdrawal (data not shown).

Apoptosis following cytokine withdrawal in *Ring1B^{-/-}* effector Th2 cells was rescued by knockdown of Bim

To assess the contribution of Bim in the apoptosis of *Ring1B^{-/-}* effector Th2 cells, we knocked down *Bim* in *Ring1B^{-/-}* effector Th2 cells and assessed the levels of apoptotic cell death after cytokine withdrawal (Fig. 6A). As expected, after transfection with the control siRNA, the numbers of Annexin V⁺PI⁻ and Annexin V⁺PI⁺ cells were increased in *Ring1B^{-/-}* Th2 cells in comparison with those

FIGURE 5. *Ring1B^{-/-}* Th2 cells are highly susceptible to apoptotic cell death in vitro. In vitro differentiated effector *Ring1B^{-/-}* Th2 cells were cultured with medium alone for 24 h. A, Each sample was subjected to Annexin V/PI staining. B, Expression of Bim (isoforms, EL and L), Arf, Puma, and tubulin- α in cells cultured under indicated conditions was analyzed by immunoblotting. Arbitrary densitometric units are shown under each band. C, Expression of *Bim*, *Noxa*, *Bax*, *Perp*, *Mcl1*, *Bcl2*, and *Bcl-x_L* mRNA in cells cultured under indicated conditions was assessed by quantitative PCR analysis. The mRNA expression levels were normalized to HPRT expression. Three independent experiments were performed with similar results.



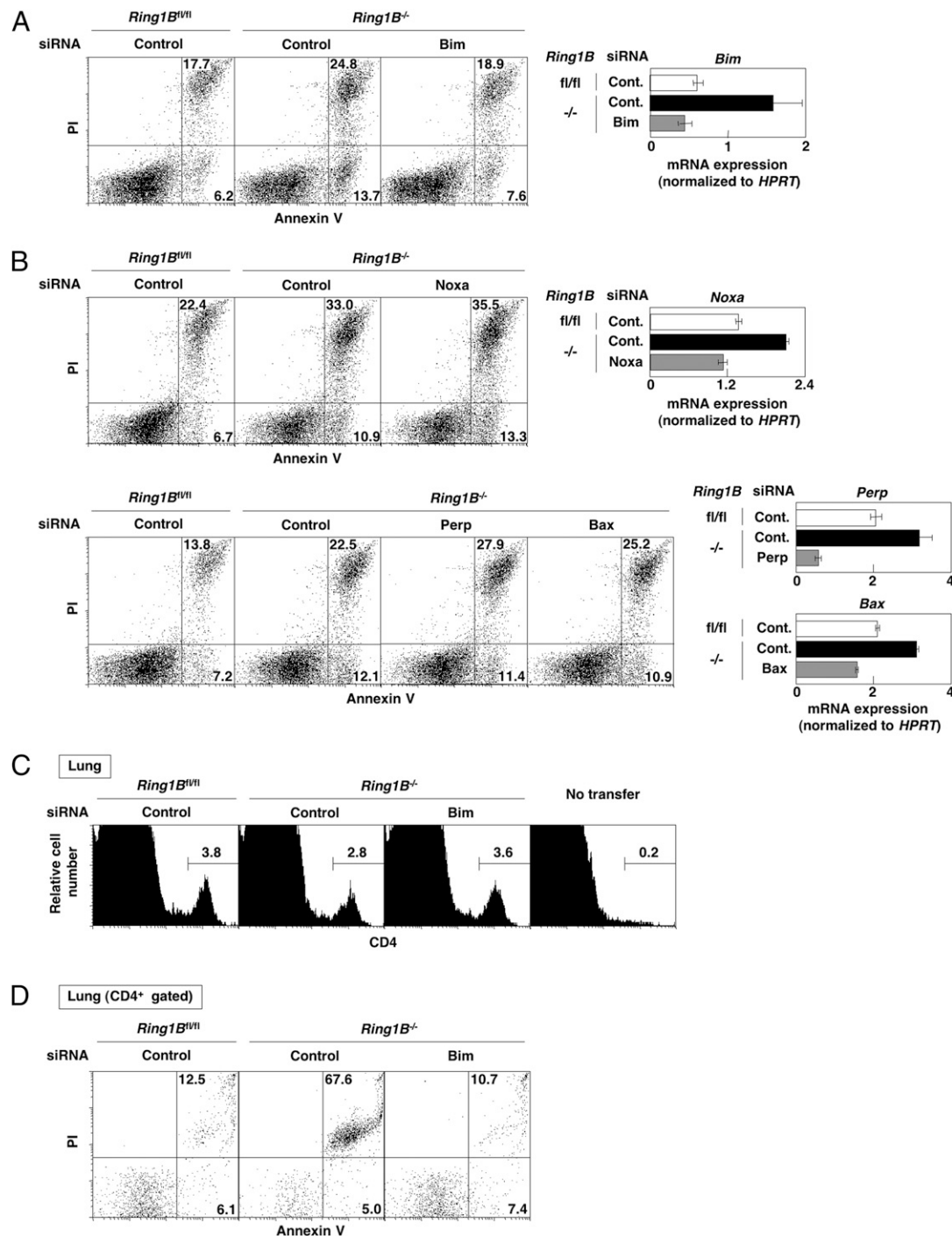


FIGURE 6. Susceptibility to apoptosis of *Ring1B*^{-/-} Th2 cells is rescued by knockdown of *Bim*. *A* and *B*, Control, *Bim*, *Noxa*, *Perp*, or *Bax* siRNA was transfected into in vitro-differentiated effector *Ring1B*^{-/-} Th2 cells, and the cells were then cultured with medium alone for 16 h. Each sample was subjected to Annexin V/PI staining (left panels). Expression of *Bim*, *Noxa*, *Perp*, and *Bax* mRNA in the cells transfected with siRNA specific for each gene was assessed by quantitative PCR analysis (right panels). The mRNA expression levels were normalized to HPRT expression. Three independent experiments were performed with similar results. *C*, The indicated cells were transferred into TCRβδ-knockout mice, and mononuclear cells in the lung were purified 36 h later. Representative CD4 profiles of the cells in the lung are shown. *D*, Annexin V/PI staining profiles of electronically gated CD4⁺ donor cells in the lung shown in *C*.

in *Ring1B*^{fl/fl} Th2 cells (Annexin V⁺PI⁻: *Ring1B*^{fl/fl} control siRNA, 6.2% versus *Ring1B*^{-/-} control siRNA, 13.7%; and Annexin V⁺PI⁺: *Ring1B*^{fl/fl} control siRNA, 17.7% versus *Ring1B*^{-/-} control siRNA, 24.8%). The levels of Annexin V⁺PI⁻ and Annexin V⁺PI⁺ cells were significantly reduced when *Bim* siRNA was transfected (Annexin V⁺PI⁻: *Ring1B*^{-/-} control siRNA, 13.7% versus *Ring1B*^{-/-} *Bim* siRNA, 7.6%; and Annexin V⁺PI⁺: *Ring1B*^{-/-} control siRNA, 24.8% versus *Ring1B*^{-/-} *Bim* siRNA, 18.9%). Fig. 6A, right panel,

shows the efficient knockdown of mRNA expression of *Bim* in *Bim* siRNA-transfected *Ring1B*^{-/-} Th2 cells.

We next tested the effect of the knockdown of the other proapoptotic genes, *Noxa*, *Perp*, and *Bax*, and found that no obvious rescue effect was observed by the knockdown of these three genes (Fig. 6B). The efficiency of the knockdown of mRNA expression of these three genes is shown in the right panels. In addition, the increased susceptibility to apoptosis in *Ring1B*^{-/-} effector Th2 cells after cytokine withdrawal was

not altered in Ring1B^{-/-}Noxa^{+/-} effector Th2 cells (data not shown). Taken together, these results indicate that Bim plays an important role in the induction of apoptosis in Ring1B^{-/-} effector Th2 cells after cytokine withdrawal in vitro.

Finally, to examine whether Bim-dependent apoptotic cell death is involved in the induction of cell death in Ring1B^{-/-} Th2 cells in the lung, we assessed the levels of apoptosis in Ring1B^{-/-} Th2 cells that were transferred into recipient syngeneic mice after the knockdown of Bim. Splenic CD4 T cells from Ring1B^{fl/fl} or Ring1B^{-/-} mice were cultured under Th2 conditions in vitro for 5 d and were then transfected with control or Bim siRNA. Then, the cells were adoptively transferred into TCRβδ-knockout host mice in which no endogenous T cells develop. The numbers of transferred CD4⁺Ring1B^{-/-} donor Th2 cells in the lung were reduced in comparison with Ring1B^{fl/fl} Th2 cells after transfection with the control siRNA, and the numbers of CD4⁺Ring1B^{-/-} donor Th2 cells were rescued by transfection with Bim siRNA (Ring1B^{fl/fl} control siRNA, 3.8% versus Ring1B^{-/-} control siRNA, 2.8% versus Ring1B^{-/-} Bim siRNA, 3.6%). Then, Annexin V/PI staining of the electronically gated CD4 T cells was assessed. A dramatic increase in the numbers of Annexin V⁺ cells was detected in the Ring1B^{-/-} Th2 cell transfer group, whereas this was not observed in Ring1B^{-/-} Th2 cells transfected with Bim siRNA (the sum of upper and lower right quadrants; Ring1B^{fl/fl} control siRNA, 18.6% versus Ring1B^{-/-} control siRNA, 72.6% versus Ring1B^{-/-} Bim siRNA, 18.1%) (Fig. 6D). The rescue from apoptosis by Bim siRNA was 76.9 ± 33.5% (n = 5). These results indicate that Bim plays an important role also in the induction of apoptosis in Ring1B^{-/-} effector Th2 cells in the lung.

Discussion

In this paper, we demonstrate that Ring1B plays an important role in the induction of Th2-driven allergic airway inflammation through the control of Bim-dependent apoptosis of effector Th2 cells in vivo. We used mice with a CD4⁺ T cell-specific deletion of the Ring1B gene, and therefore, their developmental processes and early T cell development in the thymus were apparently normal (Fig. 1). Because PcG products control the expression of numerous genes in various different tissue types during development (32, 33), this study is the first definitive analysis on the role of a PcG molecule in the in vivo animal models of asthma. Although there are various reports indicating that Bim plays an important role in the prevention of autoimmunity (53–56), little is known about the role of BH3-only proteins in allergic responses. The current study provides clear evidence indicating that allergic airway inflammation is controlled by a BH3-only protein Bim.

We previously reported that Mcl-1 and Bmi-1 controls Th2 cell differentiation through distinct mechanisms; namely, Mcl-1 regulates GATA3 transcription, and Bmi-1 regulates the stability of GATA3 protein (43, 44). In this paper, we show that another PcG molecule, Ring1B, plays a role in Th2 cell differentiation. We have observed that the expression level of GATA3 protein was moderately but reproducibly reduced in Ring1B^{-/-} Th2 cells, whereas the mRNA expression of GATA3 appeared not to be altered in Ring1B^{-/-} Th2 cells (Fig. 2D, 2E). This result is similar to that of Bmi-1-deficient Th2 cells (44). Therefore, Ring1B may cooperate with Bmi-1 to control the stability of GATA3 protein and facilitate Th2 cell differentiation. In the current study, however, we observed a more dramatic phenotype in Ring1B^{-/-} Th2 cells (i.e., accelerated apoptotic cell death in the lung [Figs. 4, 6]). Given that both Th2 cell differentiation and the number of functional effector Th2 cells present in the lung contribute to the extent of Th2-driven allergic airway inflammation, the attenuation of allergic airway inflammation in Ring1B^{-/-} mice appears to be more dependent on the latter mechanism.

BH3-only proapoptotic proteins are essential for the initiation of developmentally programmed cell death and stress-induced apoptosis

(57). Bim and Puma especially appear to play a crucial role in activated T cell death induced by the loss of essential cytokines during the shut-down of T cell responses (28, 58, 59). In this study, we show that Ring1B^{-/-} Th2 cells are highly susceptible to apoptotic cell death in vivo and in vitro (Figs. 4, 5) and that the expression of Bim protein, but not Puma protein, was increased in Ring1B^{-/-} Th2 cells following cytokine withdrawal (Fig. 5B). In addition, the enhanced susceptibility to apoptosis of Ring1B^{-/-} Th2 cells was rescued by the knockdown of Bim (Fig. 6A). Enhanced apoptosis detected in the transferred Ring1B^{-/-} Th2 cells in the lung tissue was also rescued by the knockdown of Bim in Ring1B^{-/-} effector Th2 cells in vivo (Fig. 6D). These results indicate that Ring1B represses the expression of Bim in effector Th2 cells and supports the survival of effector Th2 cells after cytokine withdrawal or after migration into the lung, which is a low cytokine environment. Because the expression of Puma protein in Th2 cells was not affected in the absence of Ring1B even after cytokine withdrawal (Fig. 5B), Puma may not be involved in the Ring1B-mediated control of cell death in effector Th2 cells in the lung.

By a chromatin immunoprecipitation assay, we detected low but significant levels of binding of Ring1B and Bmi-1 at the Bim locus in effector Th2 cells, and the binding of Bmi-1 was decreased in the absence of Ring1B (A. Suzuki and T. Nakayama, unpublished observation). Thus, the Ring1B/Bmi-1 complex may bind directly to the Bim locus and repress its transcription. In addition, other possible mechanisms may be involved in the Ring1B-mediated repression of the expression of Bim. The expression of Bim mRNA is controlled by the activation of the forkhead-like transcription factor FOXO3a (60). However, this regulation may not be active in Th2 cells because the expression of FOXO3a protein in the nucleus was similar between Ring1B^{fl/fl} and Ring1B^{-/-} Th2 cells (A. Suzuki and T. Nakayama, unpublished observation). It has also been reported that the proapoptotic activity of Bim is enhanced by the activation of the JNK pathway through direct phosphorylation (61, 62). However, the involvement of the JNK pathway is also unlikely because the expression levels of JNK and phosphorylated JNK were indistinguishable between Ring1B^{fl/fl} and Ring1B^{-/-} Th2 cells (A. Suzuki and T. Nakayama, unpublished observation).

We previously reported that Bmi-1 controls the generation of memory Th1/Th2 cells through repression of the Noxa gene (45). Although the expression of Noxa was significantly increased in memory Bmi-1^{-/-} Th2 cells, no obvious difference in the expression of Bim mRNA was observed in memory Bmi-1^{-/-} Th2 cells (45). In the current study, knockdown of Noxa did not rescue the cell death in Ring1B^{-/-} effector Th2 cells (Fig. 6B). It is known that Bim binds all Bcl-2 prosurvival proteins, such as Mcl-1, Bcl2, and Bcl-x_L, whereas Noxa binds Mcl-1 but not Bcl-2 or Bcl-x_L (63). Such a selective binding feature may explain the preferential roles of Bim and Noxa. Noxa expression is induced by IL-7 (64), and therefore, Noxa may play a more important role in the apoptosis of memory T cells, which are known to be highly dependent on IL-7 (45, 65, 66). Thus, these reports and the results in the current study indicate that Bim may play an important role in effector Th2 cells early at the contraction phase in the environment with low cytokine concentration, whereas Noxa appears to be more important during the memory phase.

In summary, the results of this study indicate that Ring1B plays a key role in Th2-driven allergic airway responses through the repression of Bim-dependent apoptosis of effector Th2 cells.

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Disclosures

The authors have no financial conflicts of interest.

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