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Regnase-1 degradation is crucial for interleukin-33- and interleukin-25-mediated ILC2 activation

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Abstract

Group-2 innate lymphoid cells (ILC2s) are a critical innate source of type-2 cytokines in allergic inflammation. Although ILC2s are recognized as a critical cell population in the allergic inflammation, the regulatory mechanism(s) of ILC2s are less well understood. Here, we show that Regnase-1, an immune-regulatory RNase that degrades inflammatory mRNAs, negatively regulates ILC2 function, and that IκB kinase (IKK) complex-mediated Regnase-1 degradation is essential for IL-33– and IL-25–induced ILC2 activation. ILC2s from Regnase-1AA/AA mice expressing a Regnase-1 S435A/S439A mutant resistant to IKK complex-mediated degradation, accumulated Regnase-1 protein in response to IL-33 and IL-25. IL-33– and IL-25–stimulated Regnase-1AA/AA ILC2s showed reduced cell proliferation and type-2 cytokine (IL-5, IL-9, and IL-13) production and increased cell death. In addition, Il2ra and Il1rl1, but not Il5, Il9, or Il13, mRNAs were destabilized in IL-33–stimulated Regnase-1AA/AA ILC2s. In vivo, Regnase-1AA/AA mice showed attenuated acute type-2 pulmonary inflammation induced by the instillation of IL-33, IL-25, or papain. Furthermore, the expulsion of Nippostrongylus brasiliensis was significantly delayed in Regnase-1AA/AA mice. These results demonstrate that IKK complex-mediated Regnase-1 degradation is essential for ILC2s-mediated type-2 responses both in vitro and in vivo. Therefore, controlling Regnase-1 degradation is a potential therapeutic target for ILC2-contributed allergic disorders.
Introduction

Group-2 innate lymphoid cells (ILC2s) produce a large quantity of type-2 cytokines including IL-5, IL-9, and IL-13 and thus are considered an innate counterpart of Th2 cells (1-4). ILC2s do not express a specific antigen receptor but quickly respond to epithelial-derived cytokines such as IL-33 and IL-25 (also known as IL-17E) (4). This epithelial cytokine-mediated ILC2 activation is sufficient to induce allergic pulmonary inflammation evoked by the instillation of papain (5, 6), house dust mite (7, 8), or *Alternaria alternata* (9, 10) in mice. ILC2s are also essential for early host protection against helminth infections including *Nippostrongylus brasiliensis* (*N. brasiliensis*) (3, 11). In addition to acute type-2 inflammation, ILC2s contribute to chronic phase of type-2 inflammation by activating Th2 cells (12, 13) and by acquiring a memory-like phenotype (14). Indeed, ILC2s are implicated in human chronic type-2 disorders including chronic rhinosinusitis (15, 16) and allergic asthma (17-19). Thus, a deeper understanding of the regulatory mechanisms of ILC2 activation is essential to develop therapeutic measures for type-2 immunity associated disorders.

Regnase-1 (also known as Zc3h12a or Mcpip1) is a PIN-like RNase that destabilizes mRNAs of a set of inflammatory genes (20). *Regnase-1*+/− mice spontaneously develop autoinflammatory disease caused by aberrant activation of macrophages and CD4+ T cells (20, 21). Regnase-1 is constitutively expressed in macrophages and fibroblasts to prevent “leaky” cytokine production in the resting state (22). Upon stimulation via TLRs or IL-1R, IκB kinase (IKK) complex, composed of IKKα, IKKβ, and IKKγ (NEMO), phosphorylates Regnase-1 resulting in
ubiquitin-proteasome–dependent degradation that allows cells to rapidly upregulate mRNAs of inflammatory genes (22). Regnase-1 harbors a canonical IKK-target motif, DSGxxS, and a targeted mutation in this motif (S435A/S439A) confers Regnase-1 resistance to IKK complex-mediated degradation and stronger decay activity against Il6 (22). In addition, S435/S439 motif is required for Regnase-1 degradation downstream of IL-17R signaling (23).

Because IL-33R (composed of ST2 and IL-1RAcP) shares its signaling molecules with TLR/IL-1R, stimulation of cells with IL-33 activates myeloid differentiation factor 88 (MyD88)–dependent signaling (24, 25). IL-1R-associated kinase 4 (IRAK4), IRAK1, and TNF receptor–associated factor 6 (TRAF6) are activated downstream of MyD88, and, in turn, IKK complex phosphorylates IκBα to activate the transcription factor NF-κB (25). Another epithelial cytokine, IL-25, signals through IL-17RA and IL-17RB (26), which recruit Act1 as an adaptor molecule (27, 28), indicating IL-25 shares its signaling molecules with IL-17 (29). Act1 associates with TRAF6 to induce NF-κB activation through the IKK complex-mediated degradation of IκBα (30, 31). Therefore, Regnase-1 degradation might be controlled by the IKK complex downstream of IL-33 and IL-25. Alternatively, Regnase-1 may contribute to the regulation of IL-33– and IL-25–induced type-2 responses. Although Regnase-1 is initially considered a critical negative regulator of Th1/Th17 responses (20, 21, 32, 33), Regnase-1 also controls Th2 development (34), and the expression of Th2-related genes, including Il5, Il9, Il13, and Gata3, was increased in Regnase-1−/− CD4+ T cells (21, 34, 34).
These observations prompted us to investigate the role of Regnase-1 in the activation of ILC2s by IL-33 and IL-25.

Because Regnase-1−/− mice spontaneously develop Th1/Th17 inflammation and die within a few months after birth (20, 21, 35), we took advantage of Regnase-1AA/AA mutant mice where Regnase-1 alleles are mutated to encode Regnase-1 S435A/S439A protein that is resistant to IKK complex-mediated degradation (23). In this study, we show that Regnase-1 undergoes S435/S439 motif–dependent degradation downstream of IL-33 and IL-25, and that Regnase-1 degradation is crucial for IL-33– and IL-25–induced ILC2 activation both in vitro and in vivo.
Results

IL-33 and IL-25 induce Regnase-1 accumulation in Regnase-1<sup>AA/AA</sup> ILC2s.

To examine whether Regnase-1 protein is expressed in ILC2s and is controlled downstream of IL-33 and IL-25 signaling, we used in vitro expanded BM ILC2s. ILC2s (CD45<sup>+</sup>Lin<sup>-</sup>CD90.2<sup>+</sup>CD25<sup>+</sup>Sca<sup>-</sup>1) sorted from BM of WT mice were c-Kit-negative as previously described for BM ILC2s (Supplemental Figure 1A and B) (36, 37) and in vitro expanded by IL-2, IL-33, and IL-25. After expansion and rest, ILC2s were stimulated with IL-2 plus IL-33 (IL-2/33) or IL-2 plus IL-25 (IL-2/25) for varying periods and Regnase-1 expression was examined by immunoblotting. IL-2/33 stimulation induced a slowly migrating band within 15 min (Figure 1A) indicating Regnase-1 phosphorylation at S494/S513 by IRAK1 (22, 23). Then, Regnase-1 expression slightly decreased from 30 min, and recovered by 120 min after stimulation (Figure 1A and C). This is similar to that of LPS–stimulated macrophages, although Regnase-1 levels showed greater dynamic change in macrophages (22). Regnase-1 phosphorylation was maintained for 3 days in IL-2/33–stimulated ILC2s, and Regnase-1 level gradually decreased over time (Figure 1B and C). When ILC2s were stimulated with IL-2/25, Regnase-1 decreased at 30 min, and maintained low expression level over time (Figure 1C and D). Although Regnase-1 is phosphorylated at S494/S513 by TANK-binding kinase 1 (TBK1) and inducible IκB kinase (IKK-i) downstream of IL-17 (23), slowly migrating Regnase-1 was not detected in IL-2/25–stimulated ILC2s. Regnase-1 gradually decreased upon long-term stimulation with IL-2/25 and had almost disappeared by day 3 (Figure 1C and E). To examine whether
the IKK-target motif controls Regnase-1 levels downstream of IL-33 and IL-25, we
used BM ILC2s from Regnase-1WT/WT and Regnase-1AA/AA mice. Because
Regnase-1AA/AA ILC2s did not expand well in response to IL-33 or IL-25 (discuss
below), we only used freshly isolated BM ILC2s for comparing ILC2s from
Regnase-1WT/WT and Regnase-1AA/AA mice. Freshly isolated Regnase-1WT/WT and
Regnase-1AA/AA ILC2s expressed comparable levels of canonical ILC2s markers
(Supplementary Figure 1C). As shown in Figure 1F and G, intracellular Regnase-1
staining showed that both IL-2/33 and IL-2/25 stimulation induced a slight decrease in
Regnase-1 at 1 h, which subsequently recovered in WT ILC2s. In Regnase-1AA/AA
ILC2s, Regnase-1 was not decreased in response to IL-2/33 or IL-2/25 and accumulated
over time. After 3 days of culture, high levels of Regnase-1 accumulated in IL-2/33–
and IL-2/25–stimulated Regnase-1AA/AA ILC2s (Figure 1H and I). Compared with
IL-2/25, IL-2/33 induced markedly higher Regnase-1 expression in Regnase-1AA/AA
ILC2s. Although phosphorylated Regnase-1 was not observed in IL-2/25-stimulated
WT ILC2s, slowly migrating Regnase-1 accumulated in IL-2/25 stimulated
Regnase-1AA/AA ILC2s, suggesting IL-25, similar to IL-17, induces TBK/IKK–
dependent Regnase-1 phosphorylation. Therefore, IKK complex controlled Regnase-1
degradation downstream of IL-33 and IL-25 in ILC2s similar to that downstream of
TLR/IL-1 and IL-17, respectively.

Regnase-1 degradation is essential for IL-33– and IL-25–induced ILC2 activation.
Next, we explored the role of IKK target motif–dependent Regnase-1 degradation in ILC2. Compared with IL-2 alone, IL-2/33 stimulation induced robust cell proliferation (Figure 2A) and production of type-2 cytokines, IL-5, IL-9, and IL-13 (Figure 2B) from BM ILC2s. IL-2/25 stimulation also induced cell proliferation (Figure 2A) and IL-5 and IL-13, but not IL-9, production (Figure 2B). Regnase-1AA/AA ILC2s showed severely attenuated cell proliferation (Figure 2A) and cytokine production (Figure 2B) in response to IL-2/33. Cell proliferation (Figure 2A) and IL-5 and IL-13 production (Figure 2B) were mildly but significantly decreased in IL-2/25–stimulated Regnase-1AA/AA ILC2s. We also observed defective IL-5 and IL-13 production from Regnase-1AA/AA ILC2s when we used TSLP, another STAT5 activating cytokine, instead of IL-2, suggesting that the importance of Regnase-1 downstream of IL-33 and IL-25 rather than downstream of IL-2 signaling (Supplementary Figure 2A). When ILC2s were stimulated with PMA/Ionomycin, which bypass the activation of IRAKs or Act1, Regnase-1WT/WT and Regnase-1AA/AA ILC2s produced comparable amount of the cytokines (Supplementary Figure 2A). Furthermore, we showed that TPCA-1, an IKKβ inhibitor which suppresses Regnase-1 degradation in IL-1β–stimulated fibroblasts (22), suppressed IL-33– and IL-25–induced IL-5 and IL-13 production from WT ILC2s (Supplementary Figure 2B). Therefore, the defective cytokine production in IL-33– or IL-25–stimulated Regnase-1AA/AA ILC2s should be due to the accumulation of Regnase-1 rather than non-specific abnormality caused by the mutation.

IL-2Rα (also known as CD25) (38) and ST2 (also known as IL-1RL1 or IL-33Rα) (39) are critical components of the IL-2 and IL-33 receptors, respectively.
The co-stimulatory molecule ICOS is essential for ILC2 activation (40-42). Compared with IL-2 alone, IL-2/33 stimulation upregulated all three molecules in WT ILC2; however, this upregulation was significantly attenuated in Regnase-1<sup>AA/AA</sup> ILC2 (Figure 2, C and D). ICOS and ST2 levels were slightly but significantly down-regulated in IL-2/25–stimulated Regnase-1<sup>AA/AA</sup> ILC2. IL-2Rα was not upregulated by IL-2/25 stimulation even in WT cells. Because forced expression of Regnase-1 induces cellular apoptosis (43, 44), we examined ILC2 cell death after cytokine stimulation. Both IL-2/33 and IL-2/25 induced higher cell death (7-aminoactinomycin-D (7-AAD)<sup>+</sup> and/or annexin-V<sup>+</sup> cells) in Regnase-1<sup>AA/AA</sup> ILC2s compared with WT cells (Figure 2, E and F). Intracellular IL-5 and IL-13 staining showed decreased cytokine staining in IL-2/33–, but not IL-2/25–, stimulated Regnase-1<sup>AA/AA</sup> ILC2s (Figure 2, G and H). IL-9 production promotes ILC2 survival and cytokine production in an autocrine manner (45, 46). However, exogenous IL-9 did not rescue the reduced IL-5/IL-13 production from Regnase-1<sup>AA/AA</sup> ILC2s (Supplemental Figure 2C and D).

To assess whether decreased cytokine production and surface molecule expression in Regnase-1<sup>AA/AA</sup> ILC2 occurred at the mRNA level, Regnase-1<sup>WT/WT</sup> and Regnase-1<sup>AA/AA</sup> BM ILC2s were stimulated with IL-2/33 or IL-2/25 for 1 or 2 days. The expression of Il5, Il9, Il13, Icos, and Il2ra were upregulated by IL-2/33 stimulation, and Regnase-1<sup>AA/AA</sup> ILC2s showed significantly decreased expression of these mRNAs other than Icos mRNA (Figure 3A). Although Il1rl1 (which encodes ST2) was not upregulated by IL-2/33 stimulation in WT cells, IL-2/33 stimulation over time
decreased Il1rl1 levels in Regnase-1AA/AA ILC2s, and the difference between
Regnase-1WT/WT and Regnase-1AA/AA ILC2s was statistically significant on day 2.
IL-2/25 stimulation also induced the upregulation of Il5, Il9, Il13, Icos, and Il2ra in
ILC2s (Figure 3B). However, the levels of these mRNAs and Il1rl1 were comparable
between Regnase-1WT/WT and Regnase-1AA/AA ILC2s except for Il9 on day 1. Although
Gata3, the critical transcription factor for ILC2 functions (47), was a Regnase-1-target
gene in CD4+ T cells (21, 34), Gata3 levels were not decreased in Regnase-1AA/AA
ILC2s (Supplemental Figure 3). In addition, levels of Il17ra and Il17rb, components
of IL-25R, were comparable between Regnase-1WT/WT and Regnase-1AA/AA ILC2s.
These data demonstrate that the IKK-mediated control of Regnase-1
degradation is essential for IL-33– and IL-25–induced ILC2 activation. Defective
IKK-mediated Regnase-1 degradation in ILC2s induces enhanced cell death by IL-33
and IL-25 stimulation, and reduces the mRNA levels of IL-33–inducible, but not IL-25–
inducible, genes.

Regnase-1 accumulation in IL-33–stimulated ILC2s destabilizes Il2ra and Il1rl1.
Because several mRNA levels were downregulated in IL-33–stimulated Regnase-1AA/AA
ILC2s, we next sought to identify direct target mRNA(s) for Regnase-1 downstream of
IL-33 in ILC2s. Regnase-1WT/WT and Regnase-1AA/AA BM ILC2s were stimulated for 24
h (Figure 4A) or 48 h (Figure 4B) with IL-2/33 followed by actinomycin D treatment
to stop mRNA transcription. Then, the mRNA half-lives within the cells were examined.
Il2ra and Il1rl1 were more rapidly degraded in Regnase-1AA/AA ILC2s compared with
WT cells after 24 h (Figure 4A) and 48 h (Figure 4B) stimulations, while Il5, Il9, Il13, and Icos showed comparable half-lives between Regnase-1WT/WT and Regnase-1AA/AA ILC2s. Regnase-1 started to accumulate after 4 h stimulation with IL-2/33 in Regnase-1AA/AA ILC2s (Figure 1E). However, the mRNA half-lives for Il2ra and Il1rl1 were comparable between Regnase-1WT/WT and Regnase-1AA/AA ILC2s at this time point (Supplemental Figure 4), suggesting relatively high-level Regnase-1 accumulation is required to destabilize Il2ra and Il1rl1.

These results suggest that Regnase-1 accumulation in IL-33–stimulated Regnase-1AA/AA ILC2s destabilizes Il2ra and Il1rl1 mRNAs. Destabilization of the mRNAs may contribute to reduced IL-2R and IL-33R expression and reduced response to the cytokines in IL-33–stimulated Regnase-1AA/AA ILC2s.

Regnase-1AA/AA mice show attenuated IL-33–, IL-25–, or papain-induced acute allergic pulmonary inflammation.

Next, we investigated the in vivo role of Regnase-1 degradation. Regnase-1WT/WT and Regnase-1AA/AA mice were i.n. administered IL-33 for 4 consecutive days, and lung inflammation was analyzed 24 h after the final challenge (Figure 5A). IL-33–induced lung inflammation was attenuated in Regnase-1AA/AA mice (Figure 5B-D). Although IL-33 increased the numbers of CD45+ hematopoietic cells and eosinophils in the lungs (Figure 5E and Supplemental Figure 5A-D) and bronchoalveolar lavages (BALs) (Supplemental Figure. 6A-D) of WT mice, these cells did not accumulate in Regnase-1AA/AA mice. Lung CD11c+ eosinophils represent activated eosinophils (48).
and were accumulated in the lungs of IL-33 administered Regnase-1WT/WT, but not Regnase-1AA/AA, mice (Figure 5E and Supplemental Figure 5D). Numbers of ST2+ILC2s (CD45+Lin−ST2+CD90.2+Sca-1+ cells) (Figure 5E and Supplemental Figure 5F) and percentages of IL-5/IL-13 producing ILC2s (within CD45+Lin−ST2+ cells) (Figure 5F and Supplemental Figure 5G) in the lungs were markedly lower in IL-33 administered Regnase-1AA/AA mice compared with WT mice. Concomitantly, IL-5 and IL-13 concentrations in BAL fluids (BALFs) were significantly lower in IL-33 administered Regnase-1AA/AA mice (Supplementary Figure 6E). IL-13 induces lung mucin production (49). We found that Periodic acid-Schiff (PAS)-positive cells (Figure 5C) and the expression of Muc5ac, which encodes a mucin protein, (Figure 5G) were decreased in the lungs of Regnase-1AA/AA mice administered IL-33. Similar to IL-33, Regnase-1AA/AA mice showed decreased IL-25–induced (Figure 6A) pulmonary inflammation (Figure 6B-D). Eosinophil accumulation in lungs (Figure 6E and Supplemental Figure 7A-C) was abrogated in Regnase-1AA/AA mice. Eosinophil counts in BALs (Supplemental Figure 8A-C) also showed decreased trend in Regnase-1AA/AA mice. As previously reported (50, 51), lung instillation of IL-25 induced ST2 KLRG-1+, rather than ST2+, ILC2s (Figure 6E and Supplemental Figure 7D-F). Numbers of ST2 KLRG-1+ ILC2s (CD45+Lin−CD90.2+ST2−KLRG-1− cells) (Figure 6E and Supplemental Figure 7D) and percentages of IL-5/IL-13 producing ILC2s (within CD45+Lin−CD90.2+ cells) (Figure 6F and Supplemental Figure 7E) in the lungs were significantly lower in Regnase-1AA/AA mice compared with WT mice. BALF IL-5 levels showed decreased
trend in Regnase-1AA/AA mice (Supplementary Figure 8D). PAS-positive cells (Figure 6C) and Muc5ac expression (Figure 6G) were decreased in the lungs of IL-25 administered Regnase-1AA/AA mice. Although lung administration of IL-25 upregulated the expression of Il33 in WT mice probably through the induction of inflammation, it was abrogated in Regnase-1AA/AA mice (Supplementary Figure 7G).

To examine a more physiologically relevant model, we used papain, an allergen that induces ILC2-mediated type-2 pulmonary inflammation (5, 6). Regnase-1WT/WT and Regnase-1AA/AA mice were i.n. administered papain for 4 consecutive days, and analyzed 24 h after the final challenge (Figure 7A). Papain induced lung inflammatory cell infiltration even in Regnase-1AA/AA mice (Figure 7B-D). Substantial numbers of CD45+ hematopoietic cells accumulated in the lungs (Figure 7E and Supplemental Figure 9A) and BALs (Supplemental Figure 10A and B) in Regnase-1AA/AA mice. However, the accumulation of eosinophils in lungs (Figure 7E and Supplemental Figure 9A-C) and BALs (Supplemental Figure 10A-C) was severely abrogated in Regnase-1AA/AA mice. Non-eosinophil inflammatory cells including monocytes, neutrophils, macrophages, and lymphocytes were accumulated in the lungs and BALs of Regnase-1WT/WT and Regnase-1AA/AA mice (Supplemental Figure 9G and 10E). This phenotype is similar to that observed for ST2-deficient mice (52). Numbers of ST2+ILC2s (Figure 7E and Supplemental Figure 9D and E) and percentages of IL-5/IL-13 producing ILC2s (Figure 7F and Supplemental Figure 9F) in the lungs, and BALF IL-5 and IL-13 concentrations (Supplementary Figure 10D) were significantly lower in papain administered Regnase-1AA/AA mice. PAS-positive
cells (Figure 7C) and Muc5ac expression (Figure 7G) were decreased in the lungs of papain administered Regnase-1AA/AA mice. Overall, Regnase-1AA/AA mice showed milder papain-induced lung pathology scores compared with WT mice (Figure 7D).

Il33 was upregulated by a single papain administration in WT mice as previously reported (52), and it was not abrogated in Regnase-1AA/AA mice (Supplementary Figure 9H). Therefore, the defective response to IL-33 may explain the phenotype observed in papain administered Regnase-1AA/AA mice.

Regnase-1AA/AA mice are susceptible to helminth infection.

Finally, we assessed the role of Regnase-1 degradation in N. brasiliensis infection. Regnase-1WT/WT and Regnase-1AA/AA mice were infected with L3 larvae of N. brasiliensis (Figure 8A). Intestinal worm burden was higher in Regnase-1AA/AA mice than WT mice on day 7, but not on day 5, post infection (Figure 8B) indicating delayed worm expulsion in Regnase-1AA/AA mice. Concomitantly, lung infiltration of CD45+ hematopoietic cells and eosinophils was significantly lower in Regnase-1AA/AA mice (Figure 8C). Although the difference of ST2+ILC2s numbers between Regnase-1WT/WT and Regnase-1AA/AA mice was relatively small and only statistically significant on day 7 (Figure 8C), the percentage of IL-5/IL-13 producing ILC2s was lower in Regnase-1AA/AA mice on days 5 and 7 (Figure 8D).

Taken together, these observations demonstrate that Regnase-1 degradation plays a critical role in activating ILC2 in vivo and in acute type-2 pulmonary inflammation.
Regnase-1 has been described as a negative regulator of Th1/Th17 inflammatory responses by controlling mRNA stability (20, 21, 32, 33). In this study, we showed that Regnase-1 also contributes to type-2 immune responses by regulating IL-33- and IL-25-mediated ILC2 activation. Although Regnase-1 was identified as an LPS-inducible gene (20), macrophages and fibroblasts constitutively expressed Regnase-1 protein (22). Once cells are activated through TLR/IL-1R, Regnase-1 undergoes degradation leading to a temporal decrease of Regnase-1 followed by its de novo synthesis. The canonical IKK-target motif (DSGxxS) in Regnase-1 (S435/439) is essential for TLR/IL-1R induced Regnase-1 degradation. This study demonstrated that the IKK-target motif in Regnase-1 was also essential for Regnase-1 degradation in IL-33–stimulated ILC2s, as Regnase-1 accumulated in IL-2/33–stimulated Regnase-1AA/AA ILC2s. In addition to IKKs, IRAK1 phosphorylates Regnase-1 at S494/S513, to form a slowly migrating Regnase-1 by immunoblotting, promoting the subcellular translocation from the endoplasmic reticulum to the cytoplasm, in which the IKK complex engages to phosphorylated Regnase-1 to facilitate its degradation (22, 23). Upon stimulation of ILC2s with IL-2/33, a slowly migrating Regnase-1 signal was observed within 15 min by immunoblotting. Therefore, because IL-33R shares signaling molecules with TLR/IL-1R (25), in IL-2/33–stimulated ILC2s, Regnase-1 can undergo IRAK1-mediated quick phosphorylation followed by IKK-mediated degradation as in TLR/IL-1R–stimulated cells.
Regnase-1 is also involved in IL-17–induced immune responses (32, 33, 53).

Regnase-1 S435/S439 controls Regnase-1 degradation in IL-17–stimulated fibroblasts, and TBK1/IKKi are substitutes for the role of IRAK1 (23). Here, we showed that Regnase-1 accumulated in IL-2/25–stimulated Regnase-1AA/AA ILC2s. Although a slowly migrating Regnase-1 was barely detectable in IL-2/25–stimulated WT ILC2s, it accumulated in Regnase-1AA/AA ILC2s. Thus, TBK1/IKKi-mediated Regnase-1 phosphorylation exists downstream of IL-25, but phosphorylated Regnase-1 might be quickly degraded in WT cells. Therefore, because IL-17 and IL-25 belong to the same cytokine family and share Act1 as an adaptor molecule which interacts with Regnase-1 to induce TBK1/IKKi-mediated phosphorylation (23, 29), Regnase-1 may be controlled downstream of IL-25 in ILC2s by a similar mechanism in IL-17–stimulated cells.

IL-33 induced greater Regnase-1 accumulation in Regnase-1AA/AA ILC2s compared with IL-25. IL-33 was reported to have a greater ILC2-activating capacity compared with IL-25 both in vitro and in vivo (7, 54). This stronger ILC2 activation by IL-33 may explain the higher accumulation of Regnase-1 in IL-2/33–stimulated Regnase-1AA/AA ILC2s. Because of the higher Regnase-1 accumulation, IL-2/33–stimulated Regnase-1AA/AA ILC2s might show a greater decrease in cell proliferation and cytokine production, and increase in cell death compared with IL-2/25–stimulated cells. Forced Regnase-1 expression induced cellular apoptosis (44), indicating Regnase-1 accumulation in IL-2/33– or IL-2/25–stimulated Regnase-1AA/AA ILC2s might increase cell death. Intracellular IL-5/IL-13 staining was comparable between IL-2/25–stimulated Regnase-1WT/WT and Regnase-1AA/AA ILC2s and IL-25–inducible
mRNA levels were not decreased in Regnase-1AA/AA ILC2s. Thus, increased ILC2 cell death might decrease cytokine production from IL-2/25–stimulated Regnase-1AA/AA ILC2s. In addition to increased cell death, IL-2/33 stimulation decreased cytokine production on a per-cell-basis and destabilized II2ra and II1rl1 mRNAs in Regnase-1AA/AA ILC2s. Although the expression of II5, II9, and II13 mRNAs was decreased in IL-2/33–stimulated Regnase-1AA/AA ILC2s, their stabilities were comparable between Regnase-1WT/WT and Regnase-1AA/AA ILC2s. Therefore, II5, II9, and II13 mRNA might not be direct targets for Regnase-1 in ILC2s, and their reduced expression in IL-2/33–stimulated Regnase-1AA/AA ILC2s might be a secondary effect of decreased signaling from IL-2R and IL-33R. Taken together, the decreased expression of IL-2R and IL-33R, and increased cell death might explain decreased cytokine production from IL-2/33–stimulated Regnase-1AA/AA ILC2s.

Because ILC2s are largely tissue-resident (55), the BM ILC2s used for in vitro studies might not share the same characteristics as ILC2s residing in the lungs. However, the results from in vivo IL-33 and IL-25 administration models were similar to those of in vitro experiments with BM ILC2s. IL-33– and IL-25–induced pulmonary inflammation was almost completely abrogated in Regnase-1AA/AA mice. Thus, the role of Regnase-1 degradation might be universal to several ILC2 populations present in different organs. Because IL-33- and IL-25-mediated ILC2 activation plays a central role in acute type-2 pulmonary inflammation induced by allergens (5, 6) or helminth infection (3, 11, 56), defective ILC2 responses should explain attenuated papain-induced lung inflammation and reduced early resistance to N. brasiliensis.
infection in Regnase-1AA/AA mice. In addition to ILC2s, defects in other innate and adaptive cell populations might be involved in the overall phenotype of Regnase-1AA/AA mice observed in the current study, as Regnase-1 also controls Th2 activation downstream of T cell receptor signaling (34).

Il2ra and Il1rl1 mRNAs were destabilized in IL-33–stimulated, but not in IL-25–stimulated, Regnase-1AA/AA ILC2s. This might be explained by the amount of Regnase-1 accumulated in ILC2s. Il2ra and Il1rl1 half-lives were comparable between Regnase-1WT/WT and Regnase-1AA/AA ILC2s at 4 h after IL-2/33 stimulation, but were significantly decreased in Regnase-1AA/AA ILC2s at 24 and 48 h after IL-2/33 stimulation. Therefore, relatively high-level Regnase-1 accumulation might be required to destabilize mRNAs, and Regnase-1 accumulated in IL-2/25–stimulated Regnase-1AA/AA ILC2s might not be sufficient to destabilize the mRNAs. Although the defect in cytokine production in IL-25–stimulated Regnase-1AA/AA ILC2 was milder than that observed in IL-33–stimulated cells, the in vivo response to IL-25 administration was almost completely abrogated in Regnase-1AA/AA mice. Because IL-25 administration induced the upregulation of Il33 in Regnase-1WT/WT, but not in Regnase-1AA/AA, mice, the phenotype observed in IL-25 administered Regnase-1AA/AA mice should be the sum of the reduced response of the mice to both IL-25 and IL-33. Further study is required to clarify the exact differential role of Regnase-1 and its degradation downstream of IL-33 and IL-25.

The stability of Regnase-1 targets found in CD4+ T cells, such as Icos and Gata3 (21, 34), were not different between Regnase-1WT/WT and Regnase-1AA/AA ILC2s.
Regnase-1 might control mRNAs in a cell type-specific manner by interacting with specific partner(s) in the cell population. Alternatively, although Icos and Gata3 mRNAs were stabilized in Regnase-1–deficient CD4+ T cells, excessive Regnase-1 expression in Regnase-1AA/AA cells might not affect the stability.

Because Regnase-1AA/AA ILC2s expressed unusually high-levels of Regnase-1 after IL-33– or IL-25–stimulation, the role of Regnase-1 discovered in this study might be specific because of its “overexpression”. Even so, our study demonstrated the potential therapeutic use of Regnase-1 degradation inhibitor(s) for treating allergic disorders. For instance, IKKβ inhibitor(s) are candidates for anti-asthmatic agents by inhibiting NF-κB (57, 58). In addition to suppressing NF-κB, our results suggest that IKKβ inhibitor(s) decrease ILC2 activity by increasing cellular Regnase-1 levels. Because ILC2s and their activation are implicated in the pathogenesis of severe and treatment-resistant asthma (18, 19, 59), Regnase-1 degradation inhibitor(s) might be beneficial for treating severe/refractory asthma.

In summary, we showed that Regnase-1 degradation was essential for ILC2-mediated type-2 responses both in vitro and in vivo. Although we only studied acute type-2 inflammation models, ILC2s might be also involved in chronic and treatment-resistant allergic disorders (15-19). Therefore, controlling Regnase-1 activity is a potential therapeutic target for severe and/or uncontrolled allergic disorders.
Methods

Mice

C57BL/6 WT mice were purchased from Oriental Yeast (Tokyo, Japan).

C57BL/6-background Regnase-1 AA/AA mice were generated as described (23). Mice were maintained under specific pathogen-free conditions at the animal facilities of Hyogo College of Medicine.

Reagents

Rabbit anti-mouse-Regnase-1 monoclonal antibody (mAb) (23) and recombinant human IL-33 (60) were prepared as described. Recombinant human IL-2 and mouse IL-9 were purchased from PeproTech (Hamburg, Germany). Recombinant and mouse IL-25 and mouse TSLP was purchased from R&D systems (Minneapolis, MN). Actinomycin-D, anti-DNP mouse IgE (SPE-7), ionomycin (A23187), papain from papaya latex, and phorbol 12-myristate 13-acetate (PMA) were from Sigma-Aldrich Japan (Tokyo, Japan).

TPCA-1 was from abcam (Cambridge, UK). Anti-mouse-IgE mAb (23G3) was purchased from Southern Biotechnology Associates, Inc (Birmingham, AL, USA).

Anti-mouse-ST2 mAb (DJ8) was purchased from MD Bioproducts (St Paul, MN, USA).

Anti-mouse-ERK2 mAb (C-14) was purchased from Santa Cruz (Dallas, TX, USA).

ECL anti-rabbit IgG HRP-Linked and ECL Prime Western Blottin Detection Reagent were purchased from GE healthcare (Little Chalfont, UK). Biotin-anti-rabbit-IgG was purchased from Vector Laboratories (Burlingame, CA, USA). Annexin-V and 7-AAD were purchased from BD Bioscience (San Jose, CA, USA).
Alexa-Fluor-488-streptavidin was purchased from Invitrogen (Carlsbad, CA, USA).

Collagenase and recombinant bovine DNase-I were purchased from Wako Pure Chemicals (Osaka, Japan). mAbs specific for mouse B220 (RA3-6B2), CD3ε (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD16/32 (93), CD25 (PC61), CD45 (30F11), CD49b (DX5), CD11b (M1/70), CD11c (N418), CD16/32 (93), CD25 (PC61), CD45 (30F11), CD49b (DX5), CD90.2 (53-2.1), c-Kit (2B8), ICOS (CD15-F9), IL-5 (TRFK5), IL-13 (eBio13A), KLRG-1 (2F1/KLRG1), Gr-1 (RB6-8C5), Sca-1 (D7), and Siglec-F (E50-2440) were purchased from eBioscience (San Diego, CA, USA), BioLegend (San Diego, CA, USA), or BD Bioscience.

Mouse models

Mice were anesthetized by i.p. injection of Somnopentyl (51.8 µg/g as sodium pentobarbital; Kyoritsu Seiyaku, Tokyo, Japan) and IL-33 (100 ng/50 µL/dose), IL-25 (100 ng/50 µL/dose), papain (10 µg/50 µL/dose), or PBS (50 µL/dose as a control) was i.n. administered for 4 consecutive days. Mice were euthanized 24 h after the final i.n. challenge and analyzed. For *N. brasiliensis* infection, mice were subcutaneously inoculated with 500 L3 larvae of *N. brasiliensis*. Worm burden in the small intestine and lung inflammation were analyzed on days 5 and 7 post infection. *N. brasiliensis* was maintained in male SD rats as previously described (61).

Preparation of ILC2s and in vitro culture

Lineage-negative cells were enriched from BM cells using a Lineage Cell Depletion Kit and AutoMACS Separator (Miltenyi Biotech, Gladbach, Germany). Then, cells were
Fc-blocked with anti-CD16/32, incubated with biotin-anti-CD25-antibody and anti-DNP-IgE, then stained using FITC-anti-B220, -CD3, -CD4, -CD8, -CD11b, -CD11c, -CD49b, and -IgE antibodies (lineage markers) as well as PE-anti-CD90.2, PerCP-Cy5.5-streptavidin, APC-anti-CD45, and Pacific Blue-anti-Sca-1.

CD45<sup>+</sup>Lin<sup>+</sup>CD90.2<sup>+</sup>CD25<sup>+</sup>Sca-1<sup>+</sup> cells were sorted as BM ILC2s using FACS Aria III (BD Biosciences) (Supplementary Figure 1A). In some experiments, ILC2s were expanded for 10 days in complete medium containing IL-2 (3 ng/mL), IL-33 (50 ng/mL), and IL-25 (50 ng/mL). Then, cells were rested in medium only containing IL-2 (3 ng/mL) for 2–3 days before used for experiments. ILC2s were cultured in 96-well palates at 1 × 10<sup>3</sup> cells/200 µL/well in complete medium containing the combinations of IL-2 (3 ng/mL), TSLP (10 ng/mL), IL-9 (50 ng/mL), IL-33 (100 ng/mL) or IL-25 (100 ng/mL) for varying periods. In some experiments, ILC2s were stimulated with PMA (30 ng/mL) and ionomycin (500 µg/mL). For cytokine production, cell proliferation, or surface molecule expression, cells were cultured for 3 days. Then, the supernatant was harvested and analyzed by ELISA, and cells were subjected to FACS analysis. For intracellular cytokine staining, Golgi-Stop was added for the last 4 h of culture and cells were subjected to FACS analysis. To analyze mRNA half-life, ILC2s were stimulated with IL-2 (3 ng/mL) and IL-33 (100 ng/mL) for 4, 24, or 48 h. Then, actinomycin D (2 µg/mL) was added to culture medium to stop transcription. Cells were further cultured for 0, 1, 2, and 4 h and were collected for quantitative PCR analysis.

**Histological examination**
Mice were perfused via the right ventricle with 10 ml of PBS. Lung left lobes were dissected from mice and were immediately fixed in 4% paraformaldehyde for 24 h at 4°C. Samples were embedded in paraffin, and then 4-μm coronal sections were cut with a microtome TU-213 (Yamato Kohki Industrial, Asagiri, Japan) and stained with H&E or PAS. Samples were evaluated under an AX80/DP72 microscope (Olympus Life Science, Tokyo, Japan). The lung pathology scores were determined by a blinded investigator in terms of bronchial and interstitial cellular infiltration, and mucus production (all 0 to 5 points, thus the maximum potential score is 15).

Preparation of lung and BAL cells

To prepare lung cells, lung left lobes were dissected from mice and infused with complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum, 2-ME (50 μM), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL)) containing collagenase (150 U/ml) and DNase I (10 μg/mL). Then, the samples were minced and digested for 50 min at 37°C. Cell suspensions were filtered using a cell strainer and red blood cells were lysed. For FACS analysis, cells were suspended in ice-cold staining buffer (1% BSA in PBS). For intracellular cytokine staining, lung cells were cultured in complete medium containing PMA (30 ng/mL) and ionomycin (500 μg/mL) in the presence of Golgi-Stop (BD Biosciences) for 4 h. BAL was performed with three aliquots of 1 mL of PBS per mouse. After centrifugation, BALFs were collected and kept at −80°C for ELISA, and cells were suspended in ice-cold staining buffer for FACS analysis.
**FACS analysis**

Cells prepared from in vitro culture, lungs, or BALs were washed in ice-cold staining buffer, Fc-blocked with anti-CD16/32 antibody, then incubated with each antibody (1/100) for 15 min and washed twice with staining buffer. Intracellular cytokine and Regnase-1 staining were performed using BD Cytofix/Cytoperm™ Plus Fixation/permeabilization Kit (BD Biosciences). For intracellular Regnase-1 staining, anti-mouse Regnase-1 mAb and IgG from normal rabbit sera (as a control) (19 µg/ml) were used as primary antibodies. Biotin-conjugated anti-rabbit-IgG mAb (1/500) was used as a secondary antibody, and Alexa-Fluor-488-streptavidin (1/500) was used as a tertiary reagent. Data were acquired using a FACS Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 7.6.1, Tree Star Inc., Ashland, OR, USA). To investigate lung inflammatory cells, Lung total hematopoietic cells were defined as CD45\(^+\) singlet cells (**Supplementary Figure 5A**). From the CD45\(^+\) population, eosinophils (Autofluorescence\(\text{CD11b}^+\text{Siglec-F}^+\)), monocytes/neutrophils (Autofluorescence\(\text{CD11b}^+\text{CD11c}^{\text{int}}\text{Siglec-F}^+\)), macrophages (Autofluorescence\(\text{CD11c}^{\text{high}}\text{Siglec-F}^{\text{high}}\)), lymphocytes (Autofluorescence\(\text{CD11b}^+\text{FSC}^{\text{low}}\text{SSC}^{\text{low}}\)), and ILC2s (Lin\(\text{ST2}^+\text{CD90.2}^+\text{Sca-1}^+\)) were defined (**Supplementary Figure 9A-E**). In IL-25 administered mice, ILC2s were divided into ST2\(^+\) (Lin\(\text{ST2}^+\text{CD90.2}^+\text{ST2}^+\)) and ST2\(^+\)KLRG-1\(^+\) (Lin\(\text{CD90.2}^+\text{ST2}^+\text{KLRG-1}^+\)) ILC2s (**Supplementary Figure 7D**). BAL total hematopoietic cells were defined as CD45\(^+\) singlet cells. From the CD45\(^+\) population, eosinophils
(CD11b+CD11c-Siglec-F+), neutrophils (CD11b+CD11c-Gr-1-Siglec-F), macrophages (CD11c\textsuperscript{high}Siglec-F\textsuperscript{high}), and lymphocytes (CD11b+CD11c-FSC\textsuperscript{low}SSC\textsuperscript{low}) were defined (Supplementary Figure 10B and C).

**ELISA**

Cytokine concentrations in culture supernatants and BALFs were analyzed by mouse IL-5 and IL-13 ELISA DuoSet™ ELISA Kits (R&D Systems) and the mouse IL-9 ELISA MAX™ Deluxe Kit (eBioLegend).

**Quantitative PCR**

Total RNAs from in vitro cultured cells and lungs were isolated using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and Sepasol RNA-I Super G (Nakarai, Kyoto, Japan), respectively. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Gene expression levels were quantified using TaqMan Gene Expression Assays (Applied Biosystems), Premix Ex Taq™ (Perfect Real Time), and the Thermal cycler dice RT-PCR system (Takara Bio Inc., Otsu, Japan), with an initial denature at 95°C for 30 s, followed by 40–45 cycles of denaturing at 95°C for 5 s and annealing and extension at 60°C for 30 s. The TaqMan probes used in this study were Il5 (Mm00439646_m1), Il9 (Mm00434305_m1), Il13 (Mm00434204_m1), Il33 (Mm00505403_m1), Icos (Mm00497600_m1), Il2ra (Mm01340213_m1), Il17r1 (Mm00516117_m1), Il17ra (Mm00434214_m1), Il17rb (Mm00444709_m1), Gata3
(Mm00484683_m1), and 18S rRNA (Applied Biosystems). Results are shown relative to eukaryotic 18S rRNA levels as determined by the 2-ΔΔC(t) method.

**Immunoblotting**

Cells were lysed in lysis buffer (1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing a Complete Mini protease inhibitor cocktail (Roche; Penzberg, Germany). After removing debris by centrifugation, the cell lysates were separated by SDS-PAGE and were transferred onto an Immobilon Membrane (Millipore; Billerica, MA). Proteins on membranes were visualized with ECL Prime Western Blotting Detection Reagent and ImageQuant LAS4000mini (GE Healthcare). Rabbit anti-mouse Regnase-1 mAb (1.9 µg/ml) and rabbit anti-mouse ERK2 mAb (1/1000) were used as primary antibodies, and ECL anti-rabbit IgG HRP-linked (1/4000) was used as the detection antibody. The quantification of the bands were performed using ImageQuant TL(ver8.1; GE Healthcare). The Regnase-1 levels were shown as relative to ERK2 levels.

**Statistical analysis**

Two-tailed Student’s t-test (to compare two groups), one-way ANOVA followed by Tukey’s test (to compare more than two groups), and two-way ANOVA followed by Bonferroni’s test (to analyze time-course experiments) were performed using Prism 6 (version 6.0h, GraphPad Software, La Jolla, CA, USA). P values less than 0.05 were considered statistically significant.
Study approval

All animal experiments were performed with the approval of, and in accordance with, the guidelines of the Institutional Animal Care Committee of Hyogo College of Medicine (No.14-007, No.16-006, No.16-066, and No.19-015).
Author Contributions

K.M. designed the study, performed experiments, analyzed data, and wrote the manuscript; H.T. generated Regnase-1<sup>AA/AA</sup> mice and provided critical experimental materials, performed experiments and analyzed data; K.Y. performed *N. brasiliensis*-infection experiments and analyzed data; T.A., A.F., S. Akasaki, A.K., E.K. performed experiments and analyzed data; and S. Akira generated Regnase-1<sup>AA/AA</sup> mice and provided critical experimental materials, and supervised the study. T.Y. analyzed data and supervised the study. All authors discussed the results and revised the manuscript.
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Figure 1. IkB kinase complex controls Regnase-1 degradation in ILC2s downstream of IL-33 and IL-25.

(A–E) In vitro expanded bone marrow (BM) ILC2s were stimulated with IL-2 plus IL-33 (IL-2/33; A–C) or IL-2 plus IL-25 (IL-2/25; C–E) for the indicated periods. The expression levels of Regnase-1 and ERK2 were determined by immunoblotting. Representative immunoblotting images (A, B, D, and E) and densitometry quantification of Regnase-1 levels (mean (n=3) ± SD) (C) are shown. (F–I) Freshly isolated BM ILC2s from Regnase-1^WT/WT (blue) and Regnase-1^AA/AA (red) mice were stimulated with IL-2/33 or IL-2/25 for the indicated periods. (F and G) Intracellular Regnase-1 levels were analyzed by FACS. Representative flow cytometry plots (F) and the quantified graph (mean (n=3) ± SD) (G) are shown. (H and I) The expression levels of Regnase-1 and ERK2 were determined by immunoblotting. Representative immunoblotting images (H) and densitometry quantification of Regnase-1 levels (mean (n=3) ± SD) (I) are shown. Data are representative of two or three independent experiments. Significance was determined by one-way ANOVA followed by Tukey’s test; **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant. Arrows indicate Regnase-1 (Reg1), Arrowheads indicate phosphorylated Regnase-1 (p-Reg1). MFI; mean fluorescence intensity.
Figure 2. IκB kinase complex-mediated Regnase-1 degradation is essential for IL-33- and IL-25-induced ILC2 activation.
Bone marrow ILC2s from Regnase-1WTWT (blue) and Regnase-1AA/AA (red) mice were stimulated with IL-2 alone, IL-2 plus IL-33 (IL-2/33), or IL-2 plus IL-25 (IL-2/25) for 3 days. (A) Live cell number was quantified by FACS. (B) The concentrations of IL-5, IL-9, and IL-13 in the culture supernatants were determined by ELISA. (C and D) Cell surface expressions of ICOS, IL-2Rα, and ST2 were analyzed by FACS. Filled gray histogram indicates unstained control. (E and F) Cells were stained with annexin-V and 7-AAD, and the percentage of dead cells was quantified by FACS. (G and H) Golgi-Stop was added at the final 4 h of a 3-day culture, and intracellular cytokine levels were analyzed by FACS. Filled gray histogram indicates unstained control. Data are representative of more than three independent experiments. Representative flow cytometry plots (C, E, and G) and mean (n=3) ± SD (A, B, D, F, H) are shown. MFI; mean fluorescence intensity. Significance was determined by one-way ANOVA followed by Tukey’s test (A, B, and D) or two-tailed Student’s t-test (F and H); *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant.
Figure 3. Reduced IL-33-inducible mRNA expression in Regnase-1AA/AA ILC2s.
Bone marrow ILC2s from Regnase-1WT/WT (blue) and Regnase-1AA/AA (red) mice were stimulated with IL-2 plus IL-33 (IL-2/33; A) or IL-2 plus IL-25 (IL-2/25; B) for 1 or 2 days. mRNA levels for Il5, Il9, Il13, Icos, Il2ra, and Il1rl1 were determined by quantitative PCR. Data are representative of two independent experiments. Mean (n=3) ± SD are shown. Significance was determined by two-way ANOVA followed by Bonferroni’s test; **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant.
Figure 4. *Il2ra* and *Il1rl1* are destabilized in IL-33-stimulated Regnase-*I^AA/AA* ILC2s.

Bone marrow ILC2s from Regnase-*I^WT/WT* (blue) and Regnase-*I^AA/AA* (red) mice were stimulated with IL-2 plus IL-33 for 24 (A) or 48 h (B), and then treated with actinomycin D for the indicated periods. mRNA levels for *Il5*, *Il9*, *Il13*, *Icos*, *Il2ra*, and *Il1rl1* were determined by quantitative PCR and remaining mRNAs after actinomycin D treatment (relative to 0 h) were calculated. Data are representative of two independent experiments. Mean (n=3) ± SD are shown. Significance was determined by two-way ANOVA followed by Bonferroni’s test; *P<0.05, **P<0.01.
Figure 5. Attenuated IL-33–induced pulmonary inflammation in Regnase-1AA/AA mice.

(A) Experimental schema. Regnase-1WT/WT (blue) and Regnase-1AA/AA (red) mice were intranasally administered with IL-33 (100 ng/dose) or PBS (control; Cont) for 4 consecutive days. (B–D) Histological examination. Representative H&E staining (B) and Periodic acid–Schiff staining (PAS) (C) of lung left lobes (Scale bar = 50 μm) and the quantified pathology scores of IL-33–treated mice (D) are shown. (E) Total numbers of CD45+ cells (CD45+ singlet cells), eosinophils (CD45+Autofluorescence−CD11b+Siglec–F+), CD11c+eosinophils (CD45+Autofluorescence−CD11b+Siglec–F+CD11c+), and ST2+ILC2s (CD45−Lin−ST2+CD90.2+Sc1−1+) in the lung left lobes were quantified by FACS. (F) Lung cells were stimulated with PMA and Ionomycin for 4 h. The percentage of IL-5 and IL-13 producing cells within CD45+Lin−ST2+ILC2s were quantified by intracellular cytokine staining and FACS. (G) mRNA levels for Muc5ac in the lungs were determined by quantitative PCR. Data are representative of three independent experiments. Representative images (B and C) and mean (n=3 for control and n=6 for IL-33) ± SEM (D–G) are shown. Significance was determined by two-tailed Student’s t-test (D) or one-way ANOVA followed by Tukey’s test (E–G); *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant.
Figure 6. Attenuated IL-25–induced pulmonary inflammation in Regnase-1AA/AA mice.

(A) Experimental schema. Regnase-1WT/WT (blue) and Regnase-1AA/AA (red) mice were intranasally administered with IL-25 (100 ng/dose) or PBS (control; Cont) for 4 consecutive days. (B–D) Histological examination. Representative H&E staining (B) and Periodic acid-Schiff staining (PAS) (C) of lung left lobes (Scale bar = 50 µm) and the quantified pathology scores of IL-25–treated mice (D) are shown. (E) Total numbers of CD45+ cells (CD45+ singlet cells), eosinophils (CD45+ Autofluorescence+CD11b+Siglec-F-), CD11c+ eosinophils (CD45+ Autofluorescence+CD11b+Siglec-F+CD11c+), and ST2-KLRG1+ILC2s (CD45+Lin-CD90.2+ST2-KLRG1+) in the lung left lobes were quantified by FACS. (F) Lung cells were stimulated with PMA and Ionomycin for 4 h. The percentage of IL-5 and IL-13 producing cells within CD45+Lin-CD90.2+ILC2s were quantified by intracellular cytokine staining and FACS. (G) mRNA levels for Muc5ac in the lungs were determined by quantitative PCR. Data are representative of two independent experiments. Representative images (B and C) and mean (n=3 for control and n=6) ± SEM (D–G) are shown. Significance was determined by two-tailed Student’s t-test (D) or one-way ANOVA followed by Tukey’s test (E–G); *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant.
Figure 7. Attenuated papain-induced pulmonary inflammation in Regnase-1AA/AA mice.

(A) Experimental schema. Regnase-1WT/WT (blue) and Regnase-1AA/AA (red) mice were intranasally administered with papain (Papa; 10 µg/dose) or PBS (control; Cont) for 4 consecutive days. (B–D) Histological examination. Representative H&E staining (B) and Periodic acid-Schiff staining (PAS) (C) of lung left lobes (Scale bar = 50 µm) and the quantified pathology scores of IL-17– treated mice (D) are shown. (*) Total numbers of CD45$^+$ cells (CD45$^+$ singlet cells), eosinophils (CD45$^+$ Autofluorescence$^+$ CD11b$^+$ Siglec-F$^+$), CD11c$^+$ eosinophils (CD45$^+$ Autofluorescence$^+$ CD11b$^+$ Siglec-F$^+$ CD11c$^+$), and ST2$^+$ ILC2s (CD45$^+$ Lin$^-$ ST2$^+$ CD90.2$^+$ Sca-1$^+$) in the lung left lobes were quantified by FACS. (F) Lung cells were stimulated with PMA and Ionomycin for 4 h. The percentage of IL5$^+$ and IL13$^+$ producing cells within CD45$^+$ Lin$^-$ ST2$^+$ ILC2s were quantified by intracellular cytokine staining and FACS. (G) mRNA levels of Muc5ac in the lungs were determined by quantitative PCR. Data are representative of two independent experiments. Representative images (B and C) and mean (n=3 for control and n=7 for papain) ± SEM (D–G) are shown. Significance was determined by two-tailed Student's t-test (D) or one-way ANOVA followed by Tukey's test (E–G); *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant.
Figure 8. *Regnase-1*^AA/AA^ mice are susceptible to *Nippostrongylus brasiliensis* infection.

(A) Experimental schema. *Regnase-1*^WT/WT^ (blue) and *Regnase-1*^AA/AA^ (red) mice were subcutaneously infected with *Nippostrongylus brasiliensis* (*N.b.*). The mice were analyzed at day 5 and 7 post-infection. (B) The numbers of worms recovered from small intestines were counted. (C) Total numbers of CD45^+^ cells (CD45^+^ singlet cells), eosinophils (CD45^+^ Autofluorescence^CD11b^Siglec-F^+^), and ST2^+^ILC2s (CD45^+^Lin^ST2^CD90.2^Sca-1^*) in the lung left lobes were quantified by FACS. (D) Lung cells were stimulated with PMA and Ionomycin for 4 h. The percentage of IL-5 and IL-13 producing cells within CD45^+^Lin^ST2^ILC2s were quantified by intracellular cytokine staining and FACS. Pooled data from two independent experiments are shown (mean ± SEM). n=7 (day 0 WT and AA), n=9 (day 5 WT), n=8 (day 5 AA), n=10 (day 7 WT), and n=9 (day 7 AA). Significance was determined by two-way ANOVA followed by Bonferroni’s test; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant.