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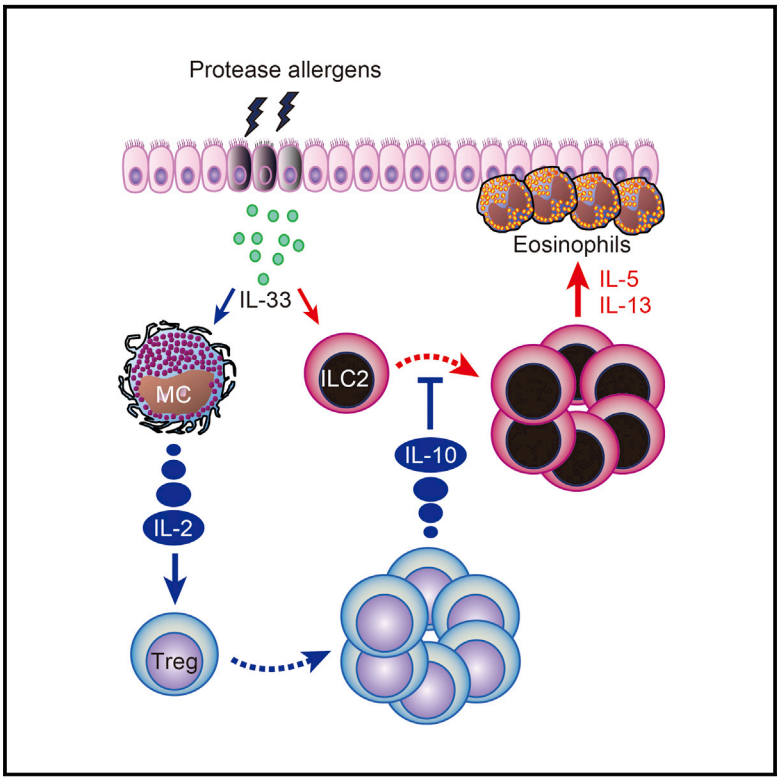
An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers

Graphical Abstract



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In Brief

The role of mast cells (MCs) in non-Th2 cell- and non-IgE-mediated allergic disorders is unknown. Nakae and colleagues show that IL-33-stimulated MC-derived IL-2 enhances expansion of numbers of regulatory T cells, thereby suppressing of allergic inflammation.

Highlights

- MC-deficient mice exhibit exacerbated papain-induced lung inflammation
- Such lung inflammation is associated with reduced numbers of Treg cells
- IL-2 produced by IL-33-stimulated mast cells promotes Treg cell expansion
- MCs suppress papain-induced airway inflammation by numbers of Treg cells

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An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers

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SUMMARY

House dust mite-derived proteases contribute to allergic disorders in part by disrupting epithelial barrier function. Interleukin-33 (IL-33), produced by lung cells after exposure to protease allergens, can induce innate-type airway eosinophilia by activating natural helper (NH) cells, a member of group 2 innate lymphoid cells (ILC2), to secrete Th2 type-cytokines. Because IL-33 also can induce mast cells (MCs) to secrete Th2 type-cytokines, MCs are thought to cooperate with NH cells in enhancing protease or IL-33-mediated innate-type airway eosinophilia. However, we found that MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice exhibited exacerbated protease-induced lung inflammation associated with reduced numbers of regulatory T (Treg) cells. Moreover, IL-2 produced by IL-33-stimulated MCs promoted expansion of numbers of Treg cells, thereby suppressing development of papain- or IL-33-induced airway eosinophilia. We have thus identified a unique anti-inflammatory pathway that can limit induction of innate-type allergic airway inflammation mediated by NH cells.

INTRODUCTION

House dust mites (HDMs) are a major source of allergens in patients with allergic disorders such as atopic dermatitis, asthma, and rhinitis (Gregory and Lloyd, 2011), and administration of HDM extracts to mice causes allergic airway inflammation with similarities to that seen in asthma (Johnson et al., 2004). HDM-derived antigens can induce barrier disruption of lung epithelial cells, thereby facilitating allergen sensitization that is dependent on Toll-like receptor 4 (TLR4) (Hammad et al., 2009; Trompette et al., 2009). In particular, HDM-derived cysteine proteases such as Der p1 can disrupt the tight junctions between epithelial cells, which are thought to contribute to development of allergic disorders (Herbert et al., 1995; Nakamura et al., 2006; Wan et al., 1999).

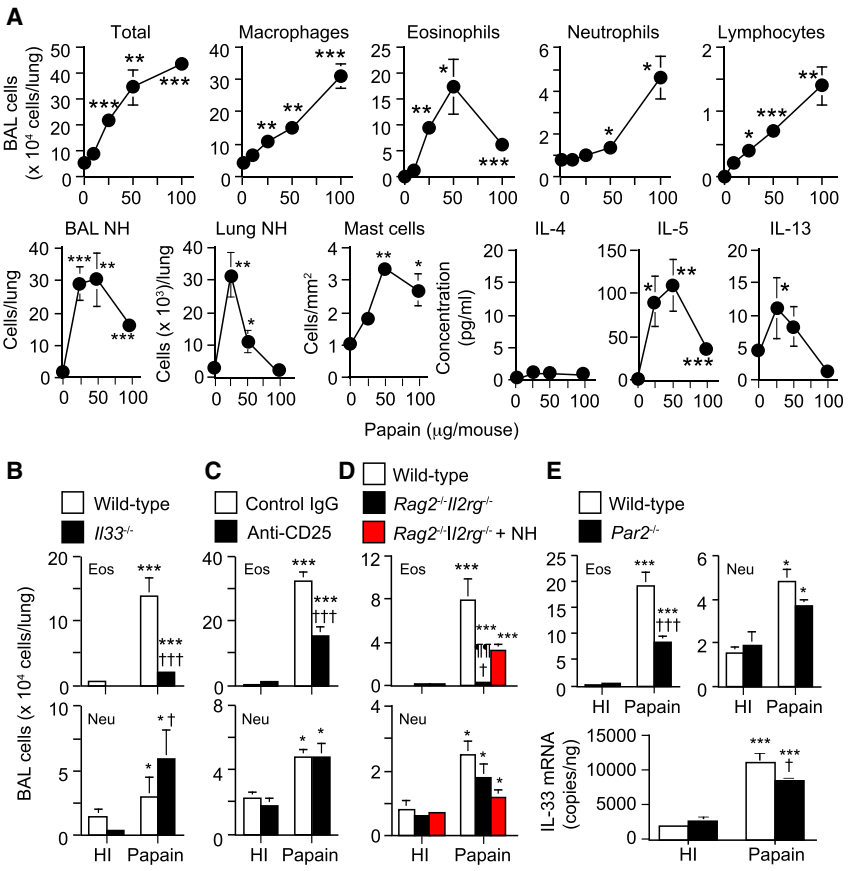

Figure 1. Lung Natural Helper Cells Contribute to Papain-Induced Innate-Type Airway Eosinophilia

(A) Cell numbers and concentrations of cytokines in BALF and lungs from C57BL/6N wild-type mice 24 hr after the final inhalation of various doses of papain (n = 5–6).

(B–D) Numbers of eosinophils and neutrophils in BALF from mice 24 hr after papain inhalation (25 μg) (heat-inactivated papain [HI], n = 3–6; and papain, n = 10–21). Wild-type and *Il33*$^{-/-}$ mice (B), *Rag2*$^{-/-}$ mice injected with anti-mouse CD25 mAb and control IgG (C), and wild-type, *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice, and *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice which had been engrafted with NH cells from wild-type mice (D).

(E) Numbers of eosinophils and neutrophils in BALF and expression of IL-33 mRNA in the lungs from wild-type and *Par2*$^{-/-}$ mice 24 hr after papain inhalation (25 μg). Heat-inactivated papain [HI], n = 7–8 for BALF, and n = 5 for IL-33 mRNA; and papain, n = 20–21 for BALF, and n = 8 for IL-33 mRNA.

(F) IL-33 expression in lungs from C57BL/6N wild-type mice after papain inhalation (25 μg). Red, IL-33 staining; blue, DAPI staining. Scale bars represent 100 μm. The data show the mean ± SEM (A) or mean + SEM (B–E). *p < 0.05, **p < 0.01, and ***p < 0.005 versus 0 μg papain (A) and HI (B–E); †p < 0.05 and †††p < 0.005 versus papain-treated wild-type mice (B, D, E) and papain-treated control IgG-injected *Rag2*$^{-/-}$ mice (C); and ††p < 0.01 versus papain-treated *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice engrafted with NH cells (*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ + NH).

Similarly, the plant-derived cysteine protease, papain, which is homologous to HDM-derived Der p1 and human cathepsin B (Chua et al., 1988), is known to induce airway inflammation in humans and is a cause of occupational asthma (Milne and Brand, 1975). In mice, we and others showed that papain can induce production of an alarmin, interleukin-33 (IL-33), a member of the IL-1 family of cytokines, by lung epithelial cells, followed by induction of IL-5 and IL-13 production by lung natural helper (NH) cells, resulting in development of IL-4 and IL-13-STAT6-dependent eosinophilic airway inflammation even in the absence of acquired immune cells such as T and B cells (Halim et al., 2012; Oboki et al., 2010). Thus, it is thought that pathogen- or plant-derived proteases, which are distinct from pathogen-associated molecular patterns, can initiate antigen-non-specific allergic inflammation via activation of the innate immune system by causing release of alarmins such as IL-33 from damaged epithelial cells (Kiss et al., 2007), which is later followed by establishment of antigen-specific allergic responses via activation of the adaptive immunity (Sokol et al., 2008).

Like NH cells, mast cells (MCs) are considered to be major effector cells in certain allergic disorders and can secrete a variety of inflammatory mediators in response to IL-33 even in the absence of immunoglobulin E (IgE) (Ho et al., 2007; Iikura et al., 2007). Therefore, we hypothesized that MCs can cooperate with NH cells in enhancing protease and IL-33-induced innate-type airway inflammation. However, in the present study, we found that IL-33-stimulated MCs can play a suppressive role in this setting by promoting an expansion of regulatory T (Treg) cell numbers that is dependent on MC-derived IL-2. Thus, MCs can negatively regulate the initiation phase of papain and IL-33-induced innate-type airway inflammation.

RESULTS

NH Cells Contribute to Protease-Mediated Eosinophilia

Inhalation of papain by mice resulted in the dose-dependent development of airway inflammation accompanied by accumulation of macrophages, eosinophils, neutrophils, lymphocytes, and natural helper (NH) cells in the bronchoalveolar lavage fluid (BALF), increased numbers of NH cells and MCs in the lungs, and increased concentration of IL-5 and IL-13, but not IL-4, in the BALF (Figure 1A). Inhalation of a high dose of papain

(100 μg), but not of a lower dose of papain (25 μg), resulted in hemorrhage associated with destruction of blood vessel walls, as well as lung epithelial cells, presumably reflecting the strong proteinase activities of papain (Kamijo et al., 2013). Indeed, high-dose papain-mediated airway inflammation is considered to be a model for COPD (Chapman, 2008). The histology of lungs from mice treated with 25 μg papain resembled that seen in asthma (in that eosinophils are prominent), whereas the lung pathology in mice treated with 100 μg papain had features of COPD, including enlarged airspaces (Figure S1A). The pathology induced by 50 μg papain showed an intermediate phenotype (Figure S1A). In association with increases in the numbers of eosinophils in BALFs (Figure S1C), the expression of IL-33 mRNA in the lungs (Figure S1B), the numbers of MCs in the lungs (Figure S1C), and numbers of NH cells in the BALFs and the lungs (Figure S1D) were significantly increased 24 hr after the 1st or 2nd papain inhalation. Consistent with previous studies (Halim et al., 2012; Oboki et al., 2010), airway eosinophilia, but not neutrophilia, was significantly attenuated 24 hr after the 3rd papain inhalation in *Il33*$^{-/-}$ mice, in *Rag2*$^{-/-}$ mice, which had been depleted of NH cells by injection of anti-CD25 mAb, and in *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice, which lack NH cells as well as T, B, NKT, and NK cells (Moro et al., 2010) (Figures 1B–1D). As reported previously (Halim et al., 2012), the impaired airway eosinophilia 24 hr after the 3rd papain inhalation that was seen in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice was partially restored to that seen in wild-type mice by injection of NH cells into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (Figure 1D). In addition, papain-IL-33-NH-cell-mediated airway eosinophilia and lung expression of IL-33 mRNA 24 hr after the 3rd papain inhalation were also significantly, albeit partially, suppressed in mice deficient in protease-activated receptor 2 (Par2) (Figure 1E). Immunoreactive IL-33 protein was detected in the nuclei of alveolar epithelial cells from wild-type mice, but not *Il33*$^{-/-}$ mice, 24 hr after the 3rd inhalation of papain, but not after inhalation of heat-inactivated papain (Figure 1F). This suggests that IL-33 released from alveolar epithelial cells by papain, at least in part through Par2, activates lung NH cells, followed by induction of airway eosinophilia (hereinafter called papain-IL-33-NH-cell-mediated airway eosinophilia).

MCs Suppress NH Cell-Mediated Eosinophilia

Because IL-33 induces mast cells (MCs) to secrete Th2 type-cytokines (Ho et al., 2007; Iikura et al., 2007), MCs are thought to cooperate with NH cells in enhancing protease or IL-33-mediated innate-type airway eosinophilia. Unexpectedly, MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice exhibited exacerbated papain-IL-33-NH-cell-mediated airway eosinophilia in comparison with wild-type mice after inhalation of low-dose papain (Figure 2A). In contrast, the responses in the two groups were not significantly different when the mice were treated with high-dose papain, which induced strong responses in all groups (Figure S2A). Likewise, papain-IL-33-NH-cell-mediated airway eosinophilia was exacerbated in *Rag2*$^{-/-}$ mice compared with wild-type mice after inhalation of low-dose papain (Figure 2B), but not in the strong reactions that occurred after inhalation of high-dose papain (Figure S2A). In accord with these findings, the amounts of IL-5 and IL-13, but not IL-4, in the BALF and airway inflammation were increased in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Rag2*$^{-/-}$ mice compared with wild-type mice after inhalation of low-dose papain (Figures 2A and 2B; Figures S2A–S2C). In addition, NH cells in the BALF and the lungs of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Rag2*$^{-/-}$ mice and MCs in the lungs of *Rag2*$^{-/-}$ mice were significantly increased in numbers compared with wild-type mice after inhalation of low-dose papain (Figure 2C), although the proportion of IL-5^{+} IL-13^{+} NH cells in the BALFs and that of IL-2^{+} c-Kit^{+} FcεRIα^{+} MCs in the lungs was comparable in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, *Rag2*$^{-/-}$ mice and wild-type mice (Figure S2D), and in *Rag2*$^{-/-}$ mice and wild-type mice (Figure S2E), respectively. Similarly, in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Rag2*$^{-/-}$ mice, airway inflammation (as assessed by eosinophil numbers, amounts of IL-5 and IL-13 in BALF, or lung histology) was exacerbated versus amounts in wild-type mice after inhalation of low-dose IL-33 (Figure 2D; Figures S2F and S2G), but such differences were not observed in the strong responses that occurred after inhalation of high-dose IL-33 (Figure S2H). In certain mouse disease models, different results have been reported regarding the role of MCs based on studies in *Kit* mutant MC-deficient mice (such as *Kit*$^{W/W\text{-}v}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) versus MC-depleted mice which lack abnormalities in c-Kit (Reber et al., 2012; Rodewald and Feyerabend, 2012). In the present model system, we confirmed that the exacerbated airway eosinophilia induced by low-dose IL-33 was also observed in MC-depleted Mas-TRECK mice (Figure S2I).

We also found that the amounts of IL-5 and IL-13 in the BALF from papain-treated mice were substantially lower than those from IL-33-treated mice (Figures 2A, 2B, and 2D), perhaps because these cytokines, which contain cysteine residues, can be directly degraded by papain (Figure S2J).

Taken together, these observations suggest that MCs and *rag*-dependent immune cells suppress airway eosinophilia induced by optimal doses of papain or IL-33.

Treg Cells Can Suppress NH Cell-Mediated Eosinophilia

The above observations suggest that MCs and Rag-dependent immune cells such as T, B, and/or NKT cells can play regulatory roles in airway eosinophilia induced by certain doses of papain and IL-33. Lymphocytes were very sparse in BALF from saline-treated wild-type mice, but significantly increased in number in BALF from papain-challenged wild-type mice (Figure 1A). After inhalation of papain or IL-33, the proportion and number of CD25^{+}Foxp3^{+} Treg cells in CD4^{+} T cells in BALF from wild-type mice increased (Figure 3A). In accord with this, the proportion and/or number of Treg cells was significantly increased in thoracic LNs, but not the spleen, from papain- or IL-33-treated wild-type mice compared with naive wild-type mice (Figure 3A). Like IL-5 and IL-13 (Figure S2J), IL-33 also can be degraded by papain (data not shown). Indeed, we could not detect IL-33 in the BALF of wild-type mice after papain inhalation (data not shown), despite the ability of papain to induce increased expression of IL-33 in the nuclei of airway epithelial cells (Figure 1F), perhaps because papain-induced IL-33 was degraded by papain. Therefore, the amounts of IL-33 in the BALF of papain-treated mice were substantially lower than those in the BALF of IL-33-treated mice, and this was associated with different numbers of Treg cells in papain- and IL-33-treated mice. In addition, when lymphocytes were cultured with papain, heat-inactivated papain, or IL-33, papain, but not heat-inactivated papain or IL-33, induced cell death (data not shown). Such effects of papain might account for why numbers of Treg cells in the BALF, but not in LNs, might be smaller in papain-treated mice than in IL33-treated mice.

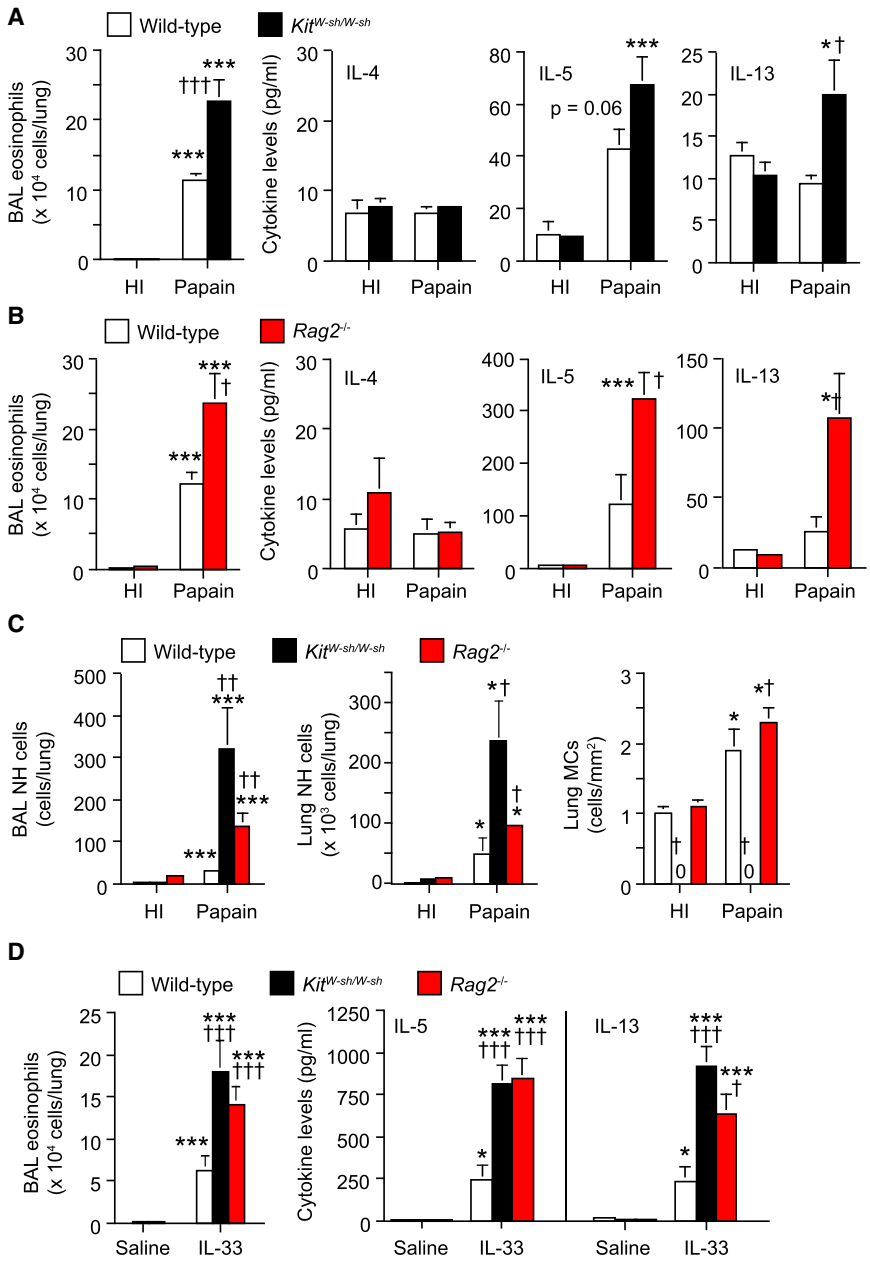

Figure 2. Exacerbated Papain-IL-33-NH-Cell-Mediated Airway Eosinophilia in *Rag2*-Deficient and Mast-Cell-Deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ Mice

(A and B) Numbers of eosinophils and levels of IL-4, IL-5, and IL-13 in BALF from wild-type mice and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (heat-inactivated papain [HI], n = 5; and papain; n = 24–25) (A) and wild-type mice and *Rag2*$^{-/-}$ mice (heat-inactivated papain [HI], n = 5; and papain; n = 10–25). (B) 24 hr after the final inhalation of low-dose papain (25 μg).

(C) Numbers of NH cells and MCs in BALF and/or lungs from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, and *Rag2*$^{-/-}$ mice 24 hr after the final inhalation of low-dose papain (25 μg) (heat-inactivated papain [HI], n = 5–10; and papain; n = 5–10).

(D) Numbers of eosinophils and levels of IL-4, IL-5, and IL-13 in BALF from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, and *Rag2*$^{-/-}$ mice 24 hr after the final inhalation of low-dose IL-33 (0.1 μg) (saline, n = 4–6; and IL-33, n = 11–15). The data show the mean + SEM. *p < 0.05 and ***p < 0.005 versus HI (A–C) and saline (D), and †p < 0.05 and †††p < 0.005 versus papain-treated wild-type mice (A–C) and IL-33-treated wild-type mice (D).

The exacerbated airway eosinophilia seen in *Rag2*$^{-/-}$ mice after papain or IL-33 inhalation was attenuated in *Rag2*$^{-/-}$ mice after transfer of Treg cells to produce numbers similar to those seen in wild-type mice (Figure 3B). These observations suggest that Treg cells are crucial for regulation of papain-IL-33-NH-cell-mediated innate-type airway eosinophilia, while effector T cells are not. In support of this conclusion, the reduced proportion of Treg cells in thoracic LNs after papain or IL-33 inhalation also correlated inversely with the exacerbated airway eosinophilia in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice or Mas-TRECK mice compared with wild-type mice (Figures 3C–3E; Figure S3). Nevertheless, the proportion of Treg cells in thoracic LNs was comparable between saline-treated wild-type and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice or Mas-TRECK mice (Figures 3D and 3E; Figure S3). Injection of Treg cells into *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice resulted in the same attenuating effect as noted above for *Rag2*$^{-/-}$ mice (Figure 3F). Thus, MCs can influence expansion of numbers of Treg cells during papain-IL-33-NH-cell-mediated airway eosinophilia.

Mast Cells Induce Treg Cell Number Expansion

We observed close associations between mast cells and Treg cells in the lungs of mice after papain inhalation (Figure S4A). To investigate whether MCs might directly influence Treg cell differentiation in mice, we co-cultured mouse CD4$^+$ CD25$^-$CD62L$^+$ naive splenic T cells with wild-type mouse bone-marrow-cell-derived cultured MCs (BMCMCs) in the presence or absence of IL-33. The proportion of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells was increased in the co-cultures in the presence of IL-33 (Figure 4A). Compared to results obtained with naive T cells, a more substantial expansion of CD4$^+$CD25$^+$Foxp3$^+$ Treg cell numbers was observed in co-cultures of whole splenic CD4$^+$ T cells (that included resident Treg cells) and wild-type BMCMCs compared with culture of whole splenic CD4$^+$ T cells alone, even without any exogenous immunostimulant (i.e., in medium alone: 1.9%; Figure 4B). Moreover, addition of IL-33 to such MC-whole CD4$^+$ T cell co-cultures, but not to whole CD4$^+$ T cell cultures that lacked MCs, markedly expanded the population of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells (Figures 4B and 4D). We also found that the FoxP3$^+$ Treg cells, which expanded in the co-cultures, expressed the transcription factor Helios (Figure 4B). When CD4$^+$ CD25$^+$ T cells enriched from the spleen were co-cultured with wild-type BMCMCs in the presence of

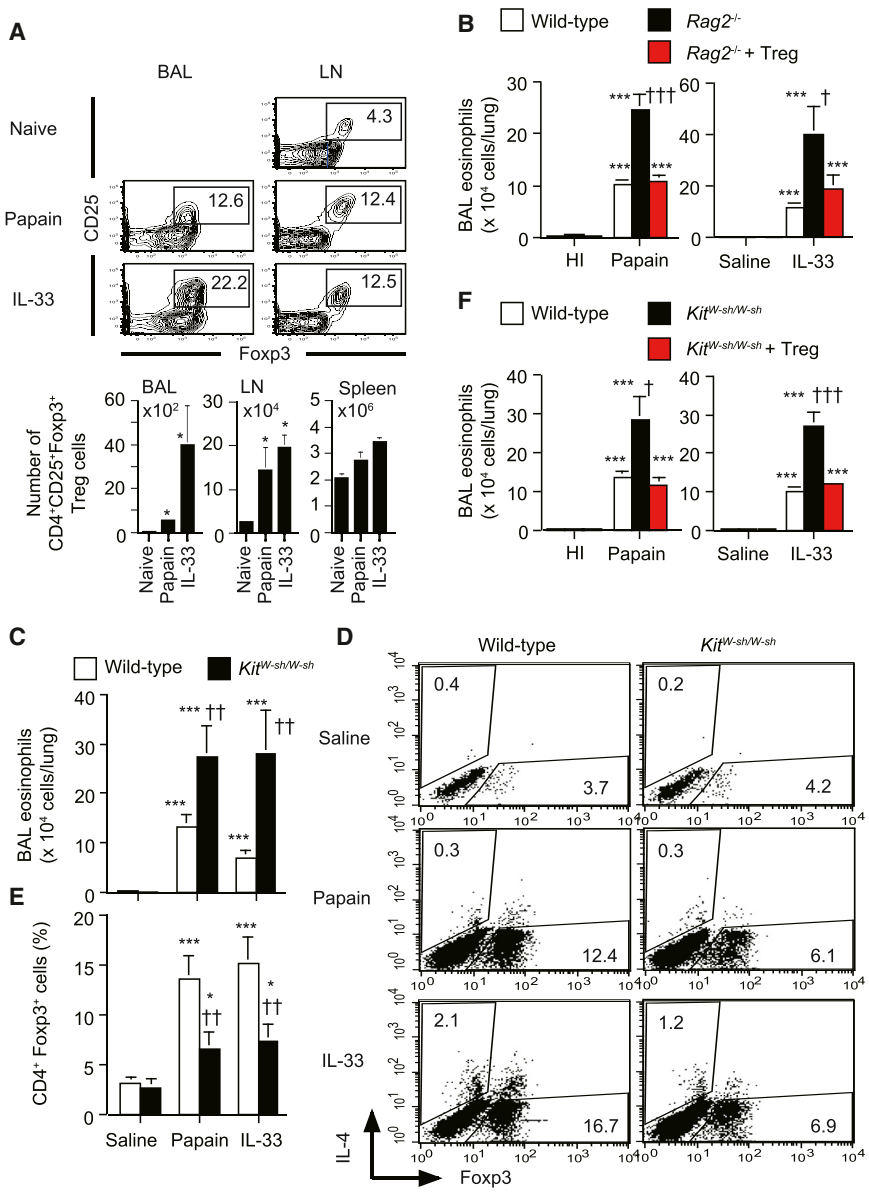

Figure 3. Evidence that Treg Cells and Mast Cells Are Necessary for Suppression of Papain-IL-33-NH-Cell-Mediated Airway Eosinophilia

(A) Proportions of $CD4^+CD25^+Foxp3^+$ Treg cells in BALF, thoracic LNs and spleen from naive, papain-inhaled and/or IL-33-inhaled C57BL/6J wild-type mice. Since the number of lymphocytes in BALF from naive mice was so small, the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells in these mice was hardly detectable. Data show representative results from three independent experiments.

(B) Numbers of eosinophils in BALF from wild-type mice, *Rag2*$^{-/-}$ mice, and *Rag2*$^{-/-}$ mice which had been engrafted with $CD4^+CD25^+$ Treg cells from spleens of wild-type mice (*Rag2*$^{-/-}$ + Treg) 24 hr after the final inhalation of 25 μg of papain or 0.1 μg of IL-33 (heat-inactivated papain [HI], n = 4–5; versus papain, n = 16; and saline, n = 5, versus IL-33, n = 13).

(C–E) Numbers of eosinophils in BALF (C), and (D and E) proportions of $CD4^+CD25^+Foxp3^+$ Treg cells in thoracic LNs from wild-type mice and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice 24 hr after the final inhalation of 25 μg of papain or 0.1 μg of IL-33 (saline, n = 9; papain, n = 10; and IL-33, n = 10). FACS data show representative results in (D).

(F) Numbers of eosinophils in BALF from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been engrafted with $CD4^+CD25^+$ Treg cells from spleens of C57BL/6J wild-type mice (*Kit*$^{W\text{-}sh/W\text{-}sh}$ + Treg) 24 hr after the final inhalation of 25 μg of papain or 0.1 μg of IL-33. Data show the mean + SEM (HI, n = 4–5, versus papain, n = 10–15; saline, n = 5, versus IL-33, n = 13). *$p < 0.05$ and ***$p < 0.005$ versus HI or saline (B, C, and F), and †$p < 0.05$ and ††$p < 0.001$ versus papain-treated or IL-33-treated wild-type mice (B, C, and F) and versus papain-treated or IL-33-treated *Rag2*$^{-/-}$ + Treg groups or *Kit*$^{W\text{-}sh/W\text{-}sh}$ + Treg groups (B and F).

IL-33, $CD4^+CD25^+Foxp3^+$ Treg cells were significantly expanded (Figure 4C). These in vitro results are consistent with the conclusion that IL-33-stimulated BMCMCs increase numbers of Treg cells primarily via expansion of numbers of committed Tregs, but might also induce some de novo Treg cell differentiation.

BMCMCs derived from mice deficient in IL-1RL1 (also called ST2) and MyD88, which are components of the IL-33 receptor and IL-33 signaling, respectively, failed to enhance Treg cell expansion upon stimulation with IL-33 (Figure 4D). IL-33 can stimulate MCs to secrete IL-6 (Figure S4C), which is a key cytokine for Th17-cell differentiation. In contrast, we detected no significant effect of MC-derived IL-6 on IL-33-mediated expansion of Treg cell number; nor did MCs expand Th17 or Th2 cell numbers (Figure 4D; Figure S4D), probably because we did not use any TCR-mediated stimuli such as anti-CD3 mAb in the MC-whole $CD4^+$ T cell co-cultures. In support of this conclusion, MHC class II (*H2-Ab1*)-deficient BMCMCs efficiently induced IL-33-mediated Treg cell expansion in MC-whole $CD4^+$ T cell co-cultures (Figure 4E). In addition, Treg cell expansion in number in those co-cultures was not induced by LPS or IL-1β (Figure S4E), or by IgE (Figures S4E and S4G), IL-13, IL-25, or TSLP (Figure S4F), even though the signal transduction pathway involving LPS and IL-1β via TLR4 and IL-1R, respectively, is shared by IL-33 via IL-33R.

We also found that IL-33 enhanced $CD4^+CD25^+Foxp3^+$ Treg cell number expansion from human peripheral blood-derived $CD4^+$ T cells in the presence, but not absence, of peripheral blood stem-cell-derived cultured human MCs in vitro (Figure 4F). We observed that the effect of IL-33 in human cells was less than that in mouse cells, perhaps because of the smaller number of Treg cells in $CD4^+$ T cells isolated from human peripheral blood compared to those from mouse spleens.

Next, to assess whether in vitro-expanded Treg cells can suppress airway inflammation in vivo, we administered IL-33 intranasally (i.n.) immediately after injecting *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice with $CD4^+CD25^+$ Treg cells derived from co-culture of $CD4^+$ T cells

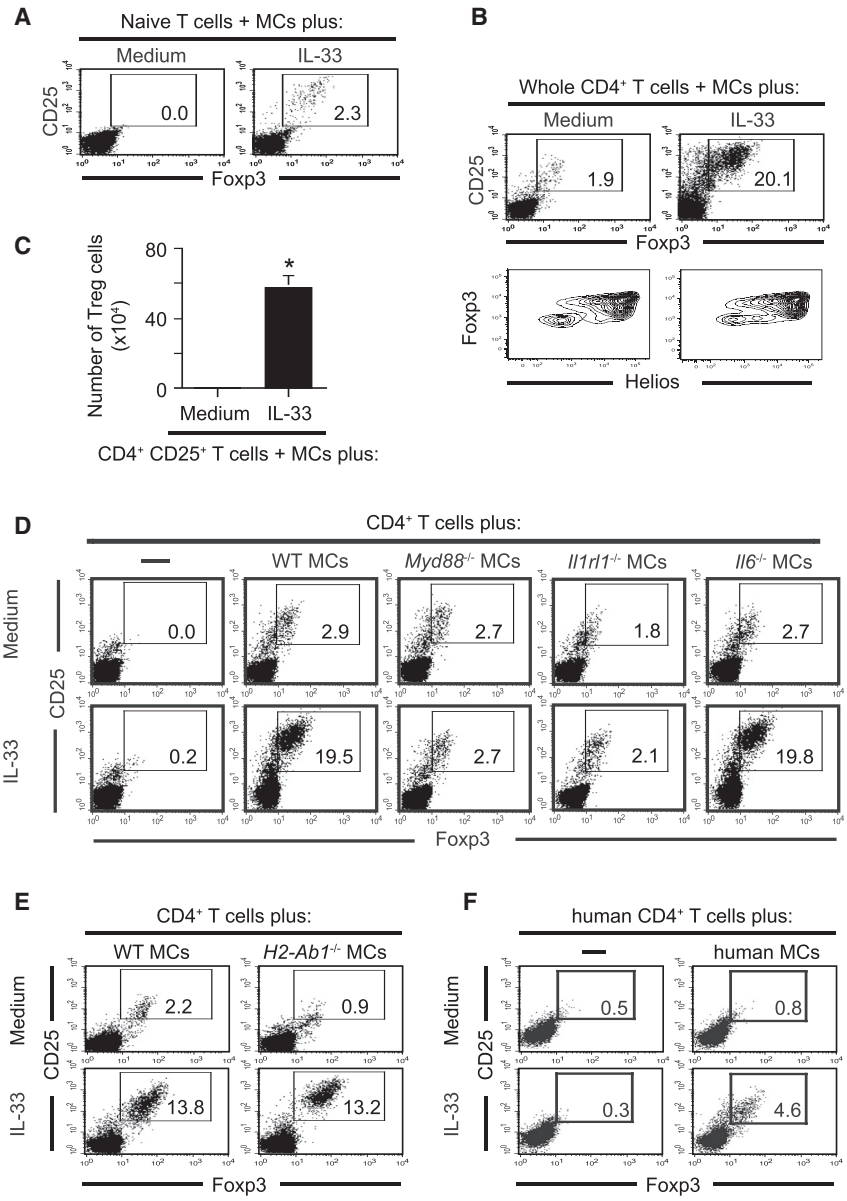

Figure 4. IL-33-Stimulated Mast Cells Can Enhance Treg Cell Expansion In Vitro

(A–F) Mouse (A–E) or human (F) CD4$^+$ T cells were cultured with and/or without mouse bone-marrow-derived cultured mast cells (MCs) (A–E) or human peripheral blood stem-cell-derived cultured MCs (F) in the presence and absence of IL-33 for 3 days. The proportions and numbers of CD25$^+$Foxp3$^+$ and/or Helios$^+$ Treg cells among c-Kit-negative CD4$^+$ T cells were assessed by flow cytometry. Data show representative results from 3–5 batches of MCs per experiment and from 2–3 independent experiments, each of which gave similar results (A, B, D–F). (A) Co-culture of mouse CD4$^+$CD25$^-$CD62L$^+$ naive T cells and wild-type MCs. (B) Proportion of CD25$^+$Foxp3$^+$ Treg cells, and expression of Helios in CD4$^+$ CD25$^+$ T cells in co-culture of mouse CD4$^+$ T cells (not naive T cells) and wild-type MCs in the presence of IL-33. (C) Co-culture of mouse CD4$^+$CD25$^+$ Treg cells and wild-type MCs. After flow cytometric analysis, the number of Treg cells was calculated. Data show the mean + SEM (n = 3). (D) Culture of mouse splenic CD4$^+$ T cells with and without wild-type, *Myd88*$^{-/-}$, *Il1rl1*$^{-/-}$, or *Il6*$^{-/-}$ MCs. (E) Co-culture of mouse CD4$^+$ T cells with wild-type or *H2-Ab1*$^{-/-}$ MCs. (F) Co-culture of human peripheral blood-derived CD4$^+$ T cells with and without human peripheral blood stem-cell-derived cultured MCs.

and wild-type BMCMCs in the presence of IL-33. Eosinophil counts in the BALF were significantly decreased in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been engrafted with wild-type, but not *Il10*$^{-/-}$, CD4$^+$CD25$^+$ Treg cells (Figure 5A). These results suggest that the ability to secrete IL-10 is essential for the immunoregulatory function of the CD4$^+$CD25$^+$ Treg cells that expanded in response to IL-33-mediated MC activation in vitro.

Microarray analysis indicates that mouse NH cells express IL-10R1 mRNA (Moro et al., 2010). NH cells in BALF from papain- or IL-33-treated wild-type mice expressed IL-10R1 (Figure 5B). Moreover, proliferation by NH cells in response to IL-33, but not to IL-2, IL-25, or IL-2 + IL-25, was significantly inhibited by the addition of 0.5-50 ng/ml IL-10 (Figure 5C) or 10 ng/ml IL-10 (Figure 5D). In association with the inhibition of NH cell proliferation by IL-10, the amounts of IL-5 and IL-13 in the culture supernatants were also reduced in this setting (Figures 5C and 5D). In addition, we found that IL-10R1 mRNA also was expressed in human ILC2 (Figure 5E). Proliferation and IL-13 production by human ILC2s in response to IL-33 plus IL-2 was significantly suppressed when human ILC2s were co-cultured with human Treg cells (Figure 5F). These observations suggest that Treg cell-derived IL-10 may regulate NH cell function directly.

MC-Derived IL-2 in Treg Cell Number Expansion

MC-derived soluble factors and co-stimulatory molecules each can be important for MC-dependent T cell activation (Kashiwakura et al., 2004; Nakae et al., 2005). We found that IL-33- and BMCMC-mediated Treg cell number expansion was largely, but not completely, inhibited by using a transmembrane filter to separate whole CD4$^+$ T cells and BMCMCs (Figure 6A), suggesting that both soluble molecules and cell-cell proximity (perhaps because this allows engagement of membrane-associated molecules) are required for full induction of Treg cells. In addition to IL-6, IL-13, and TNF (Figure S4C), we found that IL-33 stimulation induced BMCMCs to produce TGF-β1 and IL-2 (Figure S5A), which are critical for Treg cell development and expansion in number, respectively (Josefowicz et al., 2012), but not IL-10 (Figure S5A). Similarly, MCs purified from lungs produced IL-2 in response to 10 ng/ml IL-33 in vitro (20 ng/ml versus 273 ng/ml after medium versus IL-33 stimulation). Notably, *Il2*$^{-/-}$ BMCMCs, in contrast to *Tgfb1*$^{-/-}$ or *Il10*$^{-/-}$ BMCMCs, failed to expand Treg cells in MC-whole CD4$^+$

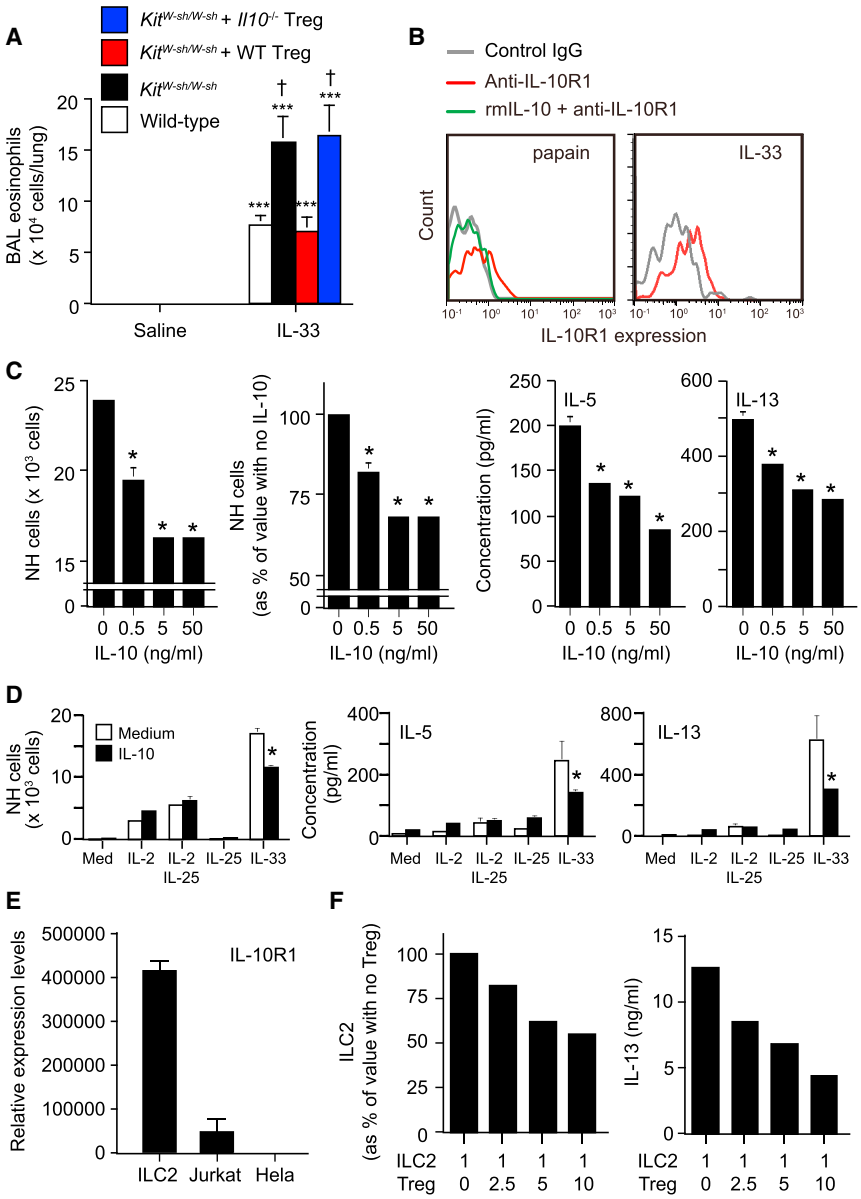

Figure 5. Suppression of Airway Eosinophilia by Treg Cells Derived from IL-33-Stimulated MCs

(A) Numbers of eosinophils in BALF from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with IL-33- and MC-derived wild-type CD4$^+$CD25$^+$ Treg cells (*Kit*$^{W\text{-}sh/W\text{-}sh}$ + WT Treg) or *Il10*$^{-/-}$ CD4$^+$CD25$^+$ Treg cells (*Kit*$^{W\text{-}sh/W\text{-}sh}$ + *Il10*$^{-/-}$ Treg) at 24 hr after the final IL-33 or saline inhalation. Data show the mean + SEM (saline, n = 5; and IL-33, n = 10). ***p < 0.005 versus saline, and †p < 0.001 versus values from wild-type or *Kit*$^{W\text{-}sh/W\text{-}sh}$ + WT Treg groups.

(B) Expression of IL-10R1 on NH cells from BALF of C57BL/6J-wild-type mice after the final inhalation of 25 μg of papain or 0.1 μg of IL-33. BAL cells were incubated with anti-IL-10R1 mAb or isotype matched control IgG in the presence and/or absence of rmIL-10. The expression of IL-10R1 on NH cells (gated by NH cell markers) in BAL cells was determined by flow cytometry. Gray lines = isotype-matched control IgG staining, red lines = anti-mouse IL-10R1 mAb staining or a green line = anti-mouse IL-10R1 mAb staining in the presence of rmIL-10. Since the proportion of lymphocytes in BALF from naive mice was very small, the proportion of NH cells in these mice was hardly detectable. Data show representative results from one of three mice.

(C) Numbers of NH cells and percent inhibition of numbers of NH cells, and IL-5 and IL-13 production by NH cells, in response to 10 ng/ml IL-33 in the presence and absence of 0.5 2 and 50 ng/ml IL-10. Data show the mean + SD (n = 3). *p < 0.05 versus 0 (no added IL-10).

(D) Numbers of NH cells, and IL-5 and IL-13 production by NH cells, in response to 10 ng/ml IL-2, 10 ng/ml IL-25, 10 ng/ml IL-33, and IL-2 + IL-25 in the presence and absence of 10 ng/ml IL-10. Data show the mean + SD (n = 3) *p < 0.05 versus Medium.

(E) Expression levels of IL-10R1 mRNA in human ILC2s, Jurkat cells and HeLa cells were determined by quantitative PCR. Data show mean + SEM (n = 3).

(F) Human ILC2s were co-cultured with and without human Treg cells in the presence of 50 ng/ml IL-33 and 10 U/ml IL-2. Data show representative results from the two independent experiments performed, each of which gave similar results.

T cell co-cultures in the presence of IL-33 (Figures 6B–6D). Indeed, IL-2 production was also detected by immunohistochemistry in lung mast cells from papain- or IL-33-treated wild-type, but not *Il2*$^{-/-}$ or *Il1rl1*$^{-/-}$, mice (Figures S5B–S5E). Moreover, addition of neutralizing mAbs for IL-2, ICAM-1, CD86, or 4-1BBL, but not PD-L1, ICOSL, OX40L, CD80, or FasL, significantly decreased Treg cell expansion (Figure 6E). Thus, the co-stimulatory and adhesion molecules ICAM-1, CD86, and 4-1BBL and especially MC-derived IL-2 contribute to the Treg cell expansion in numbers observed in this system.

Next, to investigate the role of IL-2 produced by MCs in vivo, we administered IL-33 intranasally to *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with wild-type or *Il2*$^{-/-}$ BMCMCs. In wild-type BMCMC-engrafted mice, the numbers of eosinophils were reduced to the levels observed in wild-type mice after IL-33 inhalation (Figure 7A). In contrast, engraftment with *Il2*$^{-/-}$ BMCMCs did not result in normalization of the exacerbated response to intranasal IL-33, and neither did engraftment with *Il1rl1*$^{-/-}$ BMCMCs (Figure 7A). In agreement with this, the decrease in the proportion of Treg cells in thoracic LNs of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice after IL-33 inhalation was reversed by engraftment of the mice with wild-type, but not *Il2*$^{-/-}$ or *Il1rl1*$^{-/-}$, BMCMCs (Figures 7B and 7C). Similar results were observed in these mice after papain inhalation (Figure 7D). When wild-type BMCMCs were co-cultured with lung epithelial cells (ECs) from wild-type or *Il33*$^{-/-}$ mice in the presence of papain or heat-inactivated papain, wild-type BMCMCs co-cultured with wild-type ECs, but not with *Il33*$^{-/-}$ ECs, produced IL-2 after papain stimulation (Figure 7E). These observations suggest that IL-33, induced by papain, stimulates MCs via IL-1RL1 to produce IL-2, which can in turn suppress papain/IL-33/NH-cell-mediated airway eosinophilia by promoting Treg-cell expansion in numbers.

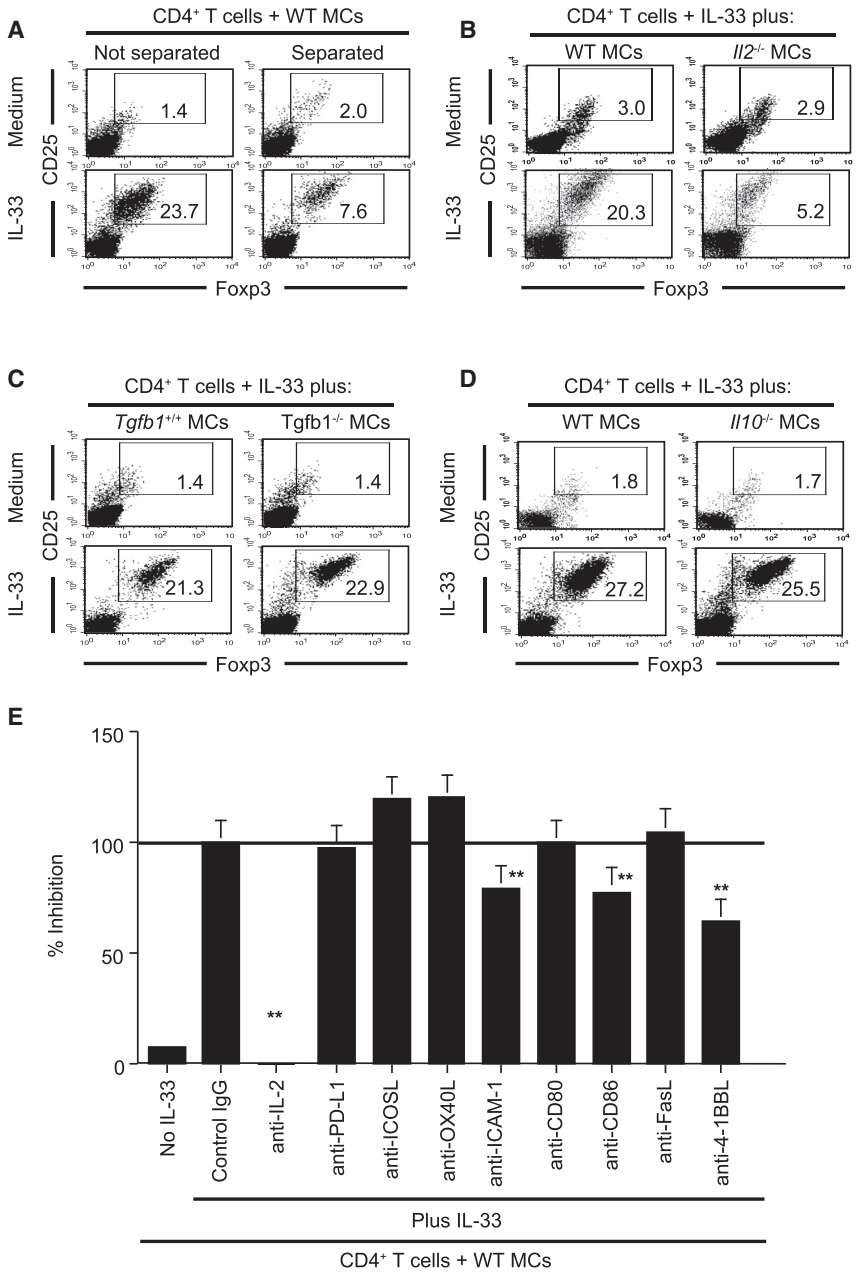

Figure 6. Importance of MC-Derived IL-2 and Costimulatory Molecules for IL-33- and MC-Mediated Treg Cell Expansion In Vitro

(A–E) Mouse CD4$^+$ T cells (not naive T cells) were cultured with and/or without mouse bone-marrow-derived cultured MCs (MCs) in the presence and absence of IL-33 for 3 days. The proportion of CD25$^+$Foxp3$^+$ Treg cells among c-Kit-negative CD4$^+$ T cells was assessed by flow cytometry. (A) Co-culture of CD4$^+$ T cells with wild-type MCs separated by transmembrane filters (Separated) or cultured together (Not separated). (B) Co-culture of CD4$^+$ T cells with wild-type or *Il2*$^{-/-}$ MCs in the presence and absence of IL-33. (C) Co-culture of CD4$^+$ T cells with *Tgfb1*$^{+/+}$ or *Tgfb1*$^{-/-}$ MCs in the presence and absence of IL-33. (D) Co-culture of CD4$^+$ T cells with wild-type or *Il10*$^{-/-}$ MCs in the presence and absence of IL-33. (E) Co-culture of CD4$^+$ T cells with wild-type MCs in the presence of neutralizing mAb for adhesion/co-signaling molecules. (A–D) Data show representative results from 3–5 batches of MCs per experiment and from the 2–3 independent experiments performed, each of which gave similar results. (E) Data show the mean + SEM (n = 5). **p < 0.01 versus control IgG groups.

DISCUSSION

It is now well established that IL-33 can induce Th2 cell-type inflammation accompanied by eosinophils, i.e., IL-33 can have pro-inflammatory effects (Chackerian et al., 2007; Kondo et al., 2008; Kurowska-Stolarska et al., 2008; Schmitz et al., 2005; Xu et al., 2008). Although IL-33 also can attenuate cardiomyocyte hypertrophy and cardiac fibrosis after pressure overload (Sanada et al., 2007), the molecular mechanisms accounting for these effects of IL-33 are not fully understood. In the present study, we provide additional evidence that the plant-derived protease papain, which activates innate immune responses in a manner distinct from that of pathogen-associated molecular patterns such as LPS, can induce secretion of an alarmin, IL-33, by lung epithelial cells to induce NH-cell-mediated acute allergic inflammation in the lungs of naive mice, and showed that this effect of papain on lung epithelial cells is in part dependent on Par2.

We found that IL-33 protein was barely detectable in lung epithelial cells from heat-inactivated papain-treated wild-type mice. By contrast, high amounts of IL-33 protein expression were observed in lung epithelial cells of naive and PBS-treated IL-33-reporter mice (Pichery et al., 2012). In contrast, constitutive IL-33 protein expression was detected in airway epithelial cells from PBS-treated IL-33-reporter mice, although the levels of IL-33 protein expression were lower in these cells and the frequency of IL-33-positive cells was much lower in lungs from PBS-treated IL-33-reporter mice than antigen-challenged IL-33-reporter mice (Hardman et al., 2013). We do not know the reason for the differences among our results and those of Pichery et al. and Hardman et al. and can only speculate that they might reflect differences in the animals' housing conditions (i.e., under SPF conditions or conventional conditions) and/or other factors that remain to be defined.

Although IgE-stimulated mast cells are well known to have potent effector cell functions in the pathology of allergic disorders, we have provided multiple lines of evidence that IL-33-stimulated mast cells can play a regulatory role in the development of NH-cell-mediated non-antigen-specific protease-induced acute inflammation. Our evidence indicates that MCs can do this by producing IL-2 that in turn enhances Treg cell expansion in naive mice that lack antigen-specific IgE. IgE

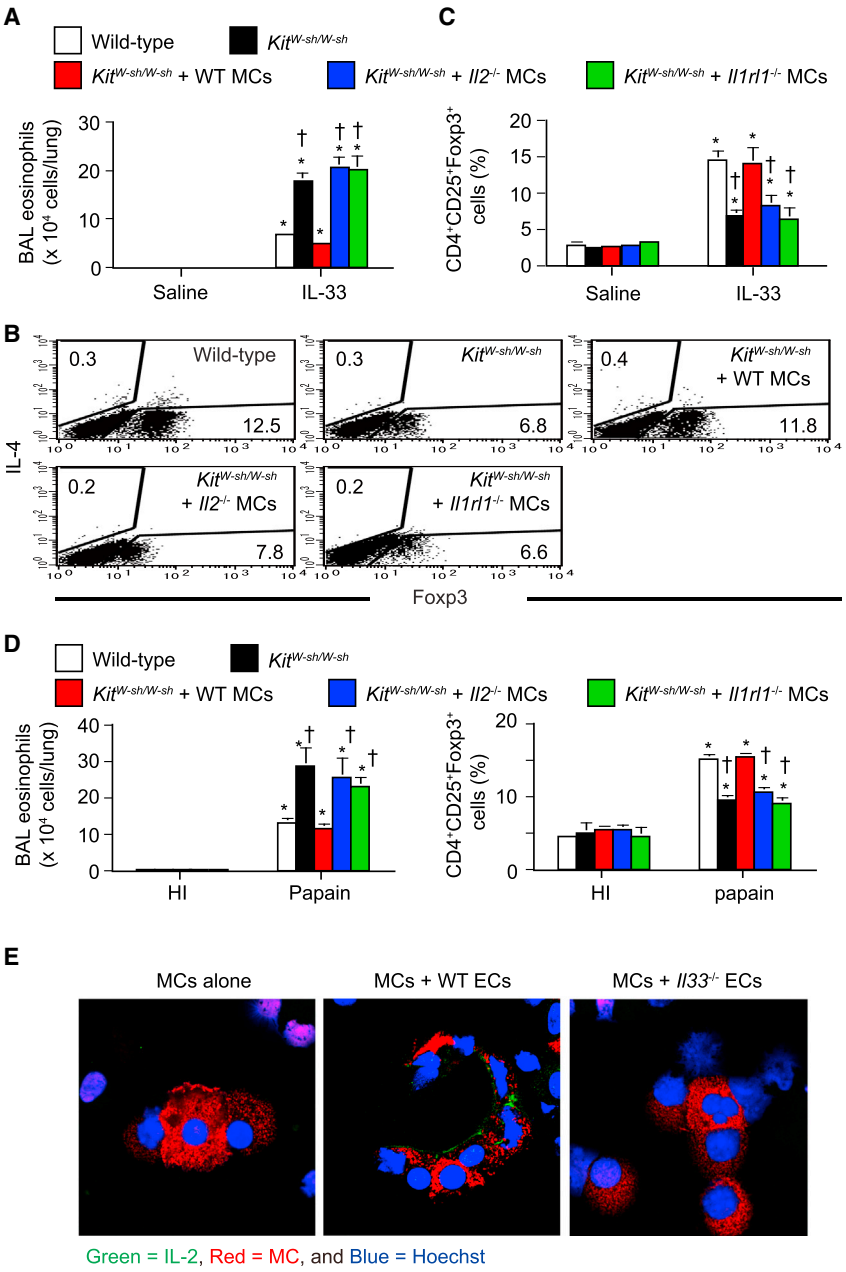

Figure 7. Importance of IL-2 Produced by IL-33-Stimulated Mast Cells for Suppression of Papain-IL-33-NH-Cell-Mediated Airway Eosinophilia

(A–D) Mice were intranasally administered 0.1 μg IL-33 or saline, or 25 μg papain or heat-inactivated papain (HI) for 3 days (once per day). Numbers of eosinophils in BALF and proportions of Foxp3$^+$ Treg cells among CD4$^+$ cells in thoracic LNs from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been engrafted with wild-type (WT), *Il2*$^{-/-}$, or *Il1rl1*$^{-/-}$ MCs before the start of IL-33 or saline challenge) at 24 hr after the final IL-33 or saline inhalation (saline, n = 5; IL-33, n = 10) (A–C) or at 24 hr after the final papain or heat-inactivated papain (HI) inhalation (HI, n = 4–6; papain, n = 10–11) (D). Data show the mean + SEM (A, C, and D). *p < 0.005 versus saline or HI and †p < 0.05 versus wild-type mice or *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been engrafted with wild-type MCs (*Kit*$^{W\text{-}sh/W\text{-}sh}$ + WT MCs). FACS data show a representative result in each IL-33-treated group, as shown in (C).

(E) IL-2 production by wild-type MCs co-cultured with or without lung epithelial cells (ECs) from wild-type (WT) or *Il33*$^{-/-}$ mice in the presence of papain and monensin for 6 hr. Green, IL-2; Red, MC; and Blue, Hoechst. Scale bars represent 10 μm. Representative results in each group are shown. No staining was obtained with the isotype control for the anti-IL-2 Ab (data not shown).

and specific Ag, but not IL-33, can induce degranulation of MCs (Ho et al., 2007; Iikura et al., 2007). By contrast, we found that IL-33-stimulated MCs, but not IgE and Ag-stimulated MCs, can induce Treg cell expansion. Moreover, IgE and Ag-stimulated mast cells and Treg cells can suppress each other's function in the setting of anaphylaxis (Gri et al., 2008; Piconese et al., 2009). Thus, MCs appear to play distinctly different, indeed opposite, roles in the induction of the acute phase of papain-induced innate allergic inflammation in naive mice (in which MCs downregulate the response) and in antigen-sensitized mice bearing antigen-specific-IgE (in which MCs have a major role in initiating and amplifying the response).

MCs can negatively regulate innate or adaptive immune responses in mice. For example, MCs have been reported to promote peripheral tolerance to skin allografts (Lu et al., 2006). In contrast to wild-type mice, MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that received donor-specific transfusion and anti-CD154 Ab treatment to induce tolerance exhibited significantly impaired survival of allogenic skin grafts (Lu et al., 2006). It was proposed that Treg-cell-derived IL-9 induced MC activation in the allografts, and that MC activation, and perhaps IL-10, contributed to the effectiveness of Treg-cell-mediated allograft tolerance (Lu et al., 2006). These findings are consistent with the interpretation that MCs function downstream of Treg cells in enhancing tolerance to skin allografts, but the exact relationship between mast cells and Treg cells in this model remains to be determined. In other settings, MCs have been reported to exert anti-inflammatory or immunosuppressive effects via the production of histamine (Hart et al., 1998) or by unknown mechanisms (Depinay et al., 2006). It has also been reported that IgG-stimulated MC-derived IL-10 or IgE-stimulated MC-derived IL-2 can have effects that suppress the chronic phase of local inflammation in certain models of contact hypersensitivity (Grimbaldeston et al., 2007; Hershko et al., 2011). Finally, after hematopoietic cell transplantation, graft versus host disease (GVHD) was severely exacerbated in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (median survival time, MST = 13 days versus 60 days in WT mice; p < 0.0001) (Leveson-Gower et al., 2013). However, the survival of C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice during

GVHD was significantly improved if the mice were engrafted intraperitoneally (i.p.) with BMCMCs from WT C57BL/6 mice but not from IL-10-deficient C57BL/6 mice. These data and other findings in that study (e.g., showing that Treg cell numbers and function were similar in the C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ and wild-type mice) indicate that the presence of mast cells can significantly reduce GVHD pathology in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice independently of Treg, by decreasing proliferation of conventional T cells by a mechanism involving IL-10. The present study identifies another mechanism of MC-dependent negative regulation of inflammation, in which IL-33-activated MCs function upstream of Treg cells, and independently of antigen-specific IgE, in enhancing Treg-dependent downregulation of the acute phase of papain-IL-33-NH-cell-mediated airway inflammation.

Can mast cells exhibit such a function in humans, as well as in mice? Although evaluating this question in humans in vivo is difficult, we have addressed it by analyzing co-cultures of peripheral blood progenitor-cell-derived cultured human MCs and human peripheral blood-derived CD4$^+$ T cells. We found that IL-33 can enhance polyclonal expansion of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in co-cultures of human MCs and human T cells in vitro, but that IL-33 had little or no effect in the absence of MCs. While the clinical relevance of these findings is not yet clear, our data indicate that co-culturing MCs and T cells in the presence of IL-33 represents an approach for efficiently generating large numbers of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in vitro.

Taken together, our findings suggest that IL-33 and MCs can play an anti-inflammatory or immunosuppressive negative-feedback role based on a pathway comprising IL-33-stimulated induction of MC-derived IL-2 leading to expansion of IL-10-producing CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (by a mechanism that is enhanced by mast cell-T cell proximity). The Treg cells can then downregulate tissue inflammation.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J and C57BL/6N wild-type (Japan SLC), C57BL/6N-*Il33*$^{-/-}$ (Oboki et al., 2010), C57BL/6J-Mas-TRECK (Otsuka et al., 2011), C57BL/6-*Rag2*$^{-/-}$, C57BL/6-*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$, C57BL/6-*Il6*$^{-/-}$, C57BL/6-*Il10*$^{-/-}$, C57BL/6-*Par2*$^{-/-}$ and 129 × B6-*Tgfb1*$^{+/-}$, C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$, C57BL/6-*Myd88*$^{-/-}$, C57BL/6-MHC class II (*H2-Ab1*)$^{-/-}$, C57BL/6-*Il2*$^{-/-}$, C57BL/6-Foxp3-GFP, and C57BL/6-*Il1rl1*$^{-/-}$ mice were used. All experiments were conducted in accordance with the institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation.

Papain- and IL-33-Mediated Airway Inflammation

Mice were treated with papain (Calbiochem; 25 μg as a low-dose protocol and 50–100 μg as a high-dose protocol) or recombinant human IL-33 (rhIL-33: PeproTech; 0.1 μg as a low-dose protocol and 5 μg as a high-dose protocol) in 20 μl of sterile, pyrogen-free 0.9% NaCl ("saline") i.n. for 3 days (one inhalation per day) unless otherwise specified. As controls, mice were treated with an equal volume of heat-inactivated papain or saline alone.

Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) was collected from naive mice or mice 24 hr after the last challenge with papain or IL-33, as described elsewhere (Oboki et al., 2010).

Detection, Isolation, Culture, and Transfer of Mouse NH Cells

BALF was collected at 24 hr after the last inhalation of papain, heat-inactivated papain or saline. IL-1RL1$^+$ CD25$^+$ NH cells in 7-aminoactinomycin D$^-$ Lin$^-$ Sca1$^+$ CD127$^+$ cells and IL-10R1 expression in Lin$^-$ CD90.1$^+$ Sca-1$^-$ IL-1RL1$^+$ CD25$^+$ NH cells were analyzed on a FACS Canto II (BD Bioscience).

NH cells were isolated as described previously (Moro et al., 2010) with minor modifications. For transfer of NH cells into *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice, freshly isolated NH cells were cultured in the presence of 10 ng/ml rmIL-2 at 37°C for 1–2 months (Furusawa et al., 2013). The expanded NH cells (4 × 10^6 cells/mouse) were then intravenously injected into *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice. One day after injection, the mice were intranasally treated with papain as described above.

NH cells (5,000 cells/well in a 96-well round-bottom plate) were cultured in the presence and absence of 10 ng/ml rmIL-33, rmIL-25, and rmIL-2, with and without 10 ng/ml IL-10, at 37°C for 5 days. The concentrations of IL-5 and IL-13 in the culture supernatants were measured by ELISA.

Mouse Mast Cell–T Cell Co-culture

T cells (1 × 10^6 cells/well [including approx. 5%–10% CD4$^+$ CD25$^+$ Treg cells] in a 24-well plate) purified as described above were co-cultured for 3 days with BMCMCs (5 × 10^5 cells/well) in the presence and absence of 100 ng/ml rhIL-33 (PeproTech and R&D Systems). To separate MCs and T cells, we used a Cell Culture Insert (0.4 μm pore size: BD Falcon) with CD4$^+$ T cells placed in the lower wells and BMCMCs placed in the upper wells in the presence and absence of rmIL-33 for 3 days. Foxp3 and Helios expression in c-Kit-negative CD4$^+$CD25$^+$ T cells, and IL-4, IL-13, and IL-17 expression in c-Kit-negative CD4$^+$ T cells, were analyzed on a FACSCalibur (Becton Dickinson), FACS Canto II (Becton Dickinson), or MACSQuant (Miltenyi Biotech).

Human Mast Cell–T Cell Co-culture

Human cells were collected with the approval of the Ethical Review Board of the National Research Institute for Child Health & Development in Japan. The CD4$^+$ T cells (2 × 10^6 cells; containing approx. 1%–2% of CD4$^+$ CD25$^+$ Treg cells) were co-cultured with and without peripheral blood-derived cultured mast cells (5 × 10^5 cells) in the presence and absence of 100 ng/ml rhIL-33 for 4 days. The co-cultures of T cells and mast cells used cells obtained from the same donor. Foxp3 expression in CD4$^+$CD25$^+$ T cells was analyzed by FACSCalibur.

Human ILC2-Treg Cell Co-culture

Human ILC2 were prepared as described previously (Mjösberg et al., 2012), with minor modifications. CD4$^+$ CD25^{high} CD127$^{dim/-}$ Treg cells were enriched from PBMCs of healthy donors using a human CD4$^+$ CD25$^+$ CD127$^{dim/-}$ regulatory T cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions and then sorted on a FACS Aria.

ILC2 (1 × 10^4 cells/well) in 96-well round-bottom plates were co-cultured with different numbers of autologous Treg cells in the presence of 10 U/ml rhIL-2 and 50 ng/ml rhIL-33 at 37°C for 3 days. After 3 days, the cells were harvested and sorted again with a FACS Aria. The expression levels of IL-5 and IL-13 were determined by quantitative PCR.

Statistical Analyses

Unless otherwise specified, data show the mean ± /+ SEM and were evaluated for statistical significance using the two-tailed Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.06.021.

AUTHORS CONTRIBUTIONS

H.M., K.A., H.U., A.N., K. Oboki, T.O., A.M., K. Motomura, S.T., K. Miyauchi, S.Y., S. Narushima, N.K., K. Moro, K.S., S.J.G., and S. Nakae performed the research and/or analyzed the data. M.I., H. Suto, T.T., H.K., M.A., S.J.G., C.A.A., S.K., M.K., K. Sudo, H. Saito, and K. Matsumoto contributed reagents/materials/analytical tools. H.M., K.A., H.U., K. Oboki, T.O., and S. Nakae designed the study. H.M., S.J.G., H. Saito, and S. Nakae wrote the paper.

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