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Interleukin-33 requires CMRF35-like molecule-1 expression for induction of myeloid cell activation

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Abstract

Background: IL-33 is a potent activator of various cells involved in allergic inflammation, including eosinophils and mast cells. Despite its critical role in Th2 disease settings, endogenous molecular mechanisms that may regulate IL-33-induced responses remain to be defined. We have recently shown that eosinophils express CMRF35-like molecule (CLM)-1. Yet, the role of CLM-1 in regulating eosinophil functions is still elusive.

Methods: CLM-1 and CLM-8 expression and cellular localization were assessed in murine bone marrow-derived and/or peritoneal cells at baseline and following IL-33 stimulation (flow cytometry, western blot). IL-33-induced mediator release and signaling were assessed in wild-type (wt) and *Clm1*$^{-/-}$ cells and mice.

Results: BM-derived eosinophils express high levels of glycosylated CLM-1. IL-33 induced a rapid, specific, concentration- and time-dependent upregulation of CLM-1 in eosinophils (*in vitro* and *in vivo*). *Clm1*$^{-/-}$ eosinophils secreted less IL-33-induced mediators than wt eosinophils. CLM-1 co-localized to ST2 following IL-33 stimulation and was required for IL-33-induced NFκB and p38 phosphorylation. Th2 cytokine (e.g., IL-5, IL-13) and chemokine (e.g., eotaxins, CCL2) secretion was markedly attenuated in IL-33-treated *Clm1*$^{-/-}$ mice. Subsequently, IL-33-challenged mice displayed reduced infiltration of mast cells, macrophages, neutrophils, and B cells. Despite the markedly impaired IL-33-induced eotaxin expression in *Clm1*$^{-/-}$ mice, eosinophil accumulation was similar in wt and *Clm1*$^{-/-}$ mice, due to hyperchemotactic responses of *Clm1*$^{-/-}$ eosinophils.

Conclusions: CLM-1 is a novel regulator of IL-33-induced eosinophil activation. These data contribute to the understanding of endogenous molecular mechanisms regulating IL-33-induced responses and may ultimately lead to receptor-based tools for future therapeutic intervention in IL-33-associated diseases.

Interleukin-33 (IL-33) is a member of the IL-1 cytokine family, which includes IL-1 and IL-18 (1). Over the past years, it has become increasingly apparent that IL-33 has a key role in initiating Th2 immune responses, such as host defense against nematodes, and allergic responses, such as asthma (2). Under baseline conditions, IL-33 localizes to the nucleus of structural cells (e.g., epithelial and endothelial cells) (3). However, following tissue damage, IL-33 is released from dying cells in the inflammatory site (4). Thus, IL-33 is considered an innate defense cytokine acting as an alarmin (similar to IL-1α and high-mobility group box chromosomal protein-1 (HMGB-1)) (3–5).

Interleukin-33 is a potent activator of various cells including eosinophils. In fact, activation of eosinophils with IL-33 leads to the secretion of various cytokines and increased expression of adhesion molecules (6, 7). The roles of IL-33 in Th2-associated disease settings are nicely demonstrated by *in vivo* experiments showing that IL-33 is sufficient and required for the induction of Th2 cytokines, IgE production, eosinophilia, and various mucosal pathologies (even in the absence of an adaptive immune system) (8, 9). Importantly, a recent large-scale genome-wide association study that was conducted in more than 10 000 asthmatic patients has identified the IL-33/IL-33R axis as a risk factor in asthma (10). Thus,

IL-33 is a critical mediator of Th2-associated diseases. Interestingly, and despite the central roles that IL-33 exerts in Th2 settings, the presence of endogenous molecular mechanisms that regulate IL-33-induced cellular effects is currently unknown.

The CMRF35-like molecule (CLM) family members consist of nine transmembrane glycoprotein receptors mapping to mouse chromosome 11D. Of these, only CLM-1 and CLM-8 possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their intracellular domains and are thus able to suppress cellular activation by recruitment of phosphatases (11–13). Interestingly, however, CLM-1 can display opposing inhibitory and co-activating functions as it can associate with the p85α subunit of the PI3K signaling cascade, thus co-activating LPS-induced mast cell responses (14, 15). We have recently established that CLM-1 is highly expressed by tissue eosinophils and that it inhibits eosinophil chemotaxis by specifically suppressing their response to eotaxins (16). Nonetheless, the full spectrum of CLM-1-regulated responses especially in eosinophils and in the context of Th2 immunity remains unclear. This is especially important because CLM-1 family members have undergone strong positive evolutionary selection and are genetically associated with various immune diseases including psoriasis, atopic dermatitis, and colitis (17).

In this study, we demonstrate that IL-33 upregulates the expression of CLM-1 in eosinophils (*in vitro*) and that *in vivo* administration of IL-33 induces rapid upregulation of CLM-1 in additional cells including neutrophils and macrophages. Furthermore, we show that CLM-1 is required for IL-33-induced Th2 cytokine and chemokine production *in vitro* and *in vivo*. To the best of our knowledge, this is the first demonstration of a cell surface receptor (other than receptors belonging to the IL-1R family) that is involved in IL-33-dependent responses.

Materials and methods

Mice

The generation of *Clm1*$^{-/-}$ mice and the commercial source of *Clm8*$^{-/-}$ mice were previously described (16, 18). Wild-type (wt) C57BL/6 mice were obtained from Harlan Laboratories (Rehovot, Israel) and grown in-house. In all experiments, age-, weight-, and sex-matched mice were used and housed under specific-pathogen-free conditions according to the protocols approved by the Tel-Aviv University Institutional Animal Care Unit.

Bone marrow (BM)-derived eosinophils and mast cells

Mouse low-density (LD) BM-derived eosinophils, BM-derived mast cells, and BM-derived macrophages were generated as previously described (19–22). Eosinophil LDBM-derived cultures consisted approximately 90% Siglec-F$^+$/SSC^{high} cells with eosinophil morphology. These cultures also contained 3.56 ± 0.32% mast cells (defined as FcεRI$^+$/C-kit$^+$ cells) and 0.02 ± 0.003% innate lymphoid type 2 (ILC2) cells (defined as CD45$^+$/Lin$^-$/ICOS$^+$/Sca1$^+$ cells).

Eosinophil purification

Highly purified spleen eosinophils were obtained by positive selection of Siglec-F$^+$ cells using biotinylated anti-Siglec-F (clone ES22-10D8), followed by avidin-conjugated magnetic beads (MACS, Miltenyi Biotech, Teterow, Germany) from six mice resulting in >97% pure eosinophils. Notably, this enrichment protocol did not induce eosinophil death for at least 24 h. Similarly, this method was used to further purify LDBM-derived eosinophils to a purity of 99%.

Western blot

Cell lysates were prepared from freshly isolated BM-derived eosinophils or macrophages (5–10 × 10^6) in RIPA lysis buffer. Samples were either left untreated or pretreated with PNGase F (Sigma, Rehovot, IL, USA) and thereafter analyzed on a 12% SDS-PAGE. The gels were electrotransferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). The membranes were incubated with Armenian hamster anti-mouse CLM-1 (18) or with rat anti-mouse CLM-8 (R&D Systems, Minneapolis, MN, USA) (4°C, overnight) followed by goat anti-Armenian hamster or goat anti-rat antibodies conjugated with HRP (Jackson ImmunoResearch, West Grove, PA, USA) and detected using ECL (Pierce, Rockford, IL, USA).

Cell culture and cytokine stimulation (*in vitro*)

Mouse BM-derived eosinophils and MCs (5 × 10^5 cells/200 μl) were stimulated with IL-33 (0–100 ng/ml). For screening experiments assessing CLM-1 and CLM-8 expression following cytokine treatment, IL-33, IL-4, IL-13, IL-1β, IL-25, and TNF-α (Peprotech, Rehovot, Israel) were used (50 ng/ml, 24 h, 37°C). Thereafter, the cells were stained with anti-CLM-1 or anti-CLM-8 followed by an appropriate secondary antibody (described below). In some experiments, BM-derived eosinophils were treated with CLM-1-Fc fusion protein (5 μg/ml) or control antibody (anti-gp120) (kindly provided by Menno Van Lookeren Campagne, Genentech) and stimulated with IL-33 (100 ng/ml, 24 h, 37°C). Thereafter, the culture supernatant was obtained and IL-6 assessed.

Real-time PCR (qPCR)

RNA samples from highly purified primary eosinophils were subjected to reverse transcription analysis using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis was performed using the CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) in conjunction with the ready-to-use fast-start SYBR Green I Master reaction kit (Roche Diagnostic Systems, Rotkreuz, Switzerland). Results were normalized to *Hprt* cDNA as previously described (20). The primers that were used in this study were as follows: *Ccl2,* Fwd-cctgtcatgcttctgggcctgc, Rev-ggggcgttaactgcatctggctg; *Hprt*, Fwd-gtaatgatcagtcaacgggggac, Rev-ccagcaagcttgcaaccttaacca.

IL-33 administration (*in vivo*)

Wt and *Clm1*$^{-/-}$ mice were challenged with recombinant IL-33 (1 μg/200 μl saline/mouse) for 3 consecutive days. Twenty-four hours after the last administration, the mice were killed and the peritoneal cavity was washed (PBS supplemented with 2.5% FCS) and centrifuged. Total and differential cell counts were determined using a hemocytometer and modified Giemsa staining; given that the peritoneal cavity was lavaged with 10 ml of PBS, the cytokine/chemokine content was diluted and nondetectable. Thus, peritoneal fluids were concentrated using centrifugal membrane filtration (cutoff: 3000 MW) to a volume of 50–60 μl and brought to a total volume of 200 μl to avoid differences that are due to the filtration processes. Samples were kept at −20°C until assessed.

Phosphoflow

IL-33-induced p38 and NFκB phosphorylation following IL-33 activation was assessed and analyzed using a protocol previously described by Krutzik et al (23). Briefly, cells were fixed in 4% paraformaldehyde/PBS and permeabilized using ice-cold methanol. Thereafter, the cells were stained with the following anti-phospho-specific antibodies (Cell Signaling, Danvers, MA, USA): phospho-p38 (pT180, pY182), phospho-NFκB p65 (pS536). Antibodies were either conjugated to Alexa Fluor 488 or 647 dyes. For phosphoflow analysis, the mean fluorescent intensity (MFI) for each intracellular signaling molecule in wt and *Clm1*$^{-/-}$ eosinophil at time point 0 minutes was measured to identify basal phosphorylation levels. Following confirmation of no significant differences in basal MFI levels between groups for each molecule, MFI value for each time point was normalized to baseline and expressed as fold-change over baseline.

Enzyme-linked immunosorbent assay (ELISA)

IL-6, IL-4, IL-13, and IL-5 were measured by ELISA MAX (Biolegend, San Diego, CA, USA). The lower detection limits for IL-6, IL-4, IL-13, and IL-5 were 15.6, 3.9, 3.9, and 7.8, respectively. CCL11, CCL17, CCL24, and CCL2 were determined using Duo-Set (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions. The lower detection limits for CCL11, CCL17, CCL24, and CCL2 were 15.6, 31.2, 15.6, and 15.6, respectively.

Flow cytometry

For surface flow cytometric staining, BM-derived and peritoneal lavage cells (3×10^5 cells in 200 μl HBSS supplemented with 1% BSA, 0.1% sodium azide) were blocked with 2.4G2 antibody (BD Pharmingen, San Jose, CA, USA). Thereafter, the cells were stained (30 min, 4°C) with anti-CCR3-FITC (R&D Systems, 10 μl/1 $\times 10^6$ cells), anti-CD11b-PerCP-Cy5.5, anti-FcεRI-PE, and anti-c-kit-APC-eFlour 780 (all purchased from eBioscience (San Diego, CA, USA) and stained according to the manufacturers' instructions), anti-CLM-1-purified (Genentech, 1 μg/ml), anti-CLM-8-purified (R&D Systems, 1 μg/ml), anti-rat Dylight647, and anti-Armenian hamster Dylight488 (Jackson, 1 : 400). Events were acquired by a Gallios flow cytometer system (Beckman Coulter, Brea, CA, USA), and data were analyzed using the Kaluza (Beckman Coulter) or FlowJo (TreeStar, Ashland, OR, USA) software on at least 10 000–50 000 events. Surface molecule expression was calculated by defining the delta MFI between the specific antibody stain and the isotype-matched control antibody and normalized to the average baseline expression.

Immunofluorescence

For immunofluorescent studies, BM-derived eosinophils were stimulated with IL-33 (Peprotech, Rehovot, IL, 0–100 ng/ml, 24 h). Thereafter, the cells were stained with rat anti-mouse ST2 (eBioscience), Armenian hamster anti-mouse CLM-1 (18) followed by Alexa Fluor 594-conjugated rabbit anti-rat antibody and Dylight488-conjugated goat anti-Armenian hamster (Jackson ImmunoResearch, West Grove, PA, USA) and mounted with DAPI Fluoromount G (Southern Biotech, Birmingham, AL, USA). Images were captured using an Olympus AX70 fluorescent microscope (Center Valley, PA, USA) equipped with a DP72 camera. Image analysis was performed using Photoshop CS2 software (Adobe, San Jose, CA, USA). Quantitation of ST2 and CLM-1 co-localization was conducted as described by Bolte et al (24) using Mander's coefficient, which was defined as the ratio of positive co-localization pixels to the total intensity in the channel, and varies from 0 (no overlap) to 1 (100% co-localization between images). Mander's coefficient was calculated using JACoP plugin for ImageJ (ImageJ 64 1.43, NIH, Bethesda, MD, USA).

Statistical analysis

Data were analyzed by ANOVA followed by Tukey *post hoc* test or Student's *t*-test using GraphPad Prism 5 (San Diego, CA, USA). Data are presented as mean ± SD, and values of $P < 0.05$ were considered statistically significant.

Results

Expression of CLM-1 and CLM-8 in eosinophils, macrophages, and mast cells

We have recently demonstrated that tissue eosinophils express high levels of CLM-1 in comparison with other myeloid cells such as macrophages and dendritic cells (16). Consistent with our previous findings, wt LDBM-derived eosinophils expressed markedly increased levels of CLM-1 on their surface in comparison with BM-derived macrophages (Fig. 1A), whereas CLM-8 expression was similar in eosinophils and macrophages (Fig. 1A). Because mast cells were also shown to express various CLM family members (13, 25, 26), we compared the expression of CLM-1 and CLM-8 in LDBM eosinophils to that of BM-derived mast cells (MCs). BMMCs displayed comparable levels of CLM-1 and CLM-8 to eosinophils (Fig. 1A).

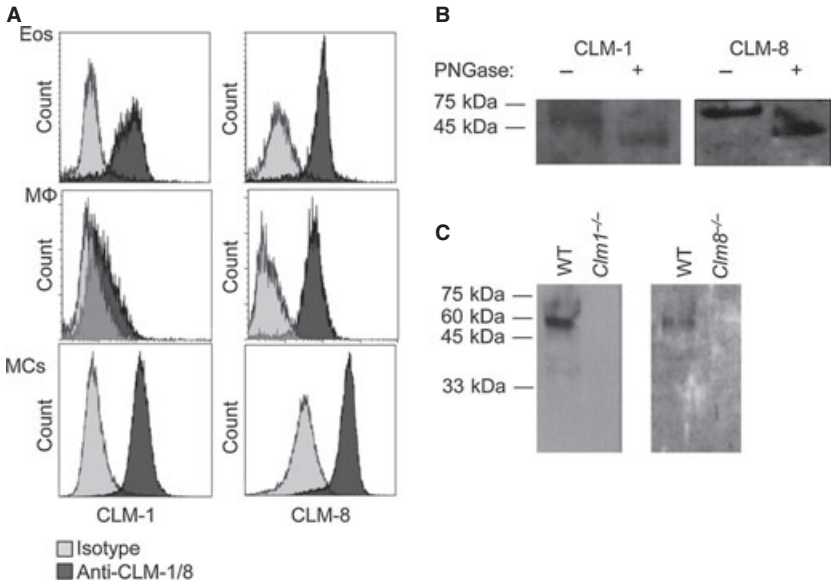

Figure 1 Expression of CLM-1 and CLM-8 eosinophils. Low-density bone marrow (BM)-derived eosinophils (Eos), BM-derived macrophages (Mϕ), and BM-derived mast cells (MCs) were stained with anti-CLM-1/CLM-8 antibodies or control antibodies. A representative histogram (A) is shown. In (B), the expression of CLM-1 and CLM-8 was assessed in wild-type eosinophils by western blot analysis before (-) and after (+) glycosidase treatment (PNGase F). The specificity of anti-CLM-1 and anti-CLM-8 antibody was assessed by western blot in lysates from wild-type (WT), *Clm1*$^{-/-}$, and *Clm8*$^{-/-}$ eosinophils. Data are representative of one experiment from an $n = 3$.

Biochemical characterization of CLM-1 in eosinophils

Western blot analysis revealed that CLM-1 and CLM-8 in eosinophils are expressed in their n-glycosylated form because PNGase F reduced the size of CLM-1 and CLM-8 from approximately 60 kDa to approximately 45 kDa (Fig. 1B) (27). In order to confirm that the observed bands correspond with CLM-1 and CLM-8, the specificity of our anti-CLM-1 and anti-CLM-8 antibodies was assessed using the cells obtained from *Clm1*$^{-/-}$ and *Clm8*$^{-/-}$ cells (Fig. 1C).

IL-33 upregulates the expression of CLM-1 *in vitro*

We have previously reported that the expression of CLM-1 is increased in settings of airway allergic inflammation and in peripheral blood eosinophils of patients with allergic rhinitis (16). This suggested that factors present in the allergic inflammatory milieu may upregulate CLM-1 (and perhaps CLM-8) expression. To assess this hypothesis, a screening approach was undertaken and LDBM eosinophils were incubated with various cytokines that are associated with Th2 diseases, including IL-33, IL-4, IL-13, IL-25, IL-1β, and TNF-α. Only IL-33 was capable of increasing the expression of CLM-1 and CLM-8 in LDBM eosinophils (Fig. 2A–D). Subsequent experiments verified that IL-33 increased the expression of CLM-1 and CLM-8 in LDBM eosinophils in a concentration- (Fig. 2E,F) and time-dependent fashion with both CLMs rapidly upregulated (reaching peak expression after 5 h) (Fig. 2G,H). IL-33-induced CLM-1 overexpression was specific to eosinophils because IL-33 failed to increase the expression of CLM-1 in BM-derived macrophages and BMMCs (Fig. S1). Yet, IL-33 induced the expression of CLM-8 in BMMCs, but not macrophages (Fig. S1).

In vivo administration of IL-33 upregulates the expression of CLM-1

We next determined whether IL-33 is also capable of increasing the expression level of CLM-1 and CLM-8 *in vivo*. To this end, IL-33 was injected to the peritoneal cavity of wt mice. Twenty-four hours after IL-33 administration, the expression of CLM-1 and CLM-8 was assessed on various cell types. Although *in vitro*, CLM-1 induction by IL-33 was specific to eosinophils (Fig. 2, Fig. S1), *in vivo*, IL-33 induced the expression of CLM-1 (but not CLM-8) in various cells including eosinophils (Fig. 2I), macrophages (Fig. 2J), and neutrophils (Fig. 2K), but not in mast cells (Fig. 2L). Notably, innate lymphoid type 2 cells (ILC2) did not express CLM-1 or CLM-8 (Fig. S2 and not shown). Collectively, these data demonstrate that CLM-1 is an IL-33-induced gene in various IL-33-responsive cells and especially eosinophils.

CLM-1 is required for IL-33-induced eosinophil and mast cell activation

To further define the role of CLM-1 in IL-33-induced effects, an *in vitro* approach was undertaken using eosinophils as a model for IL-33-responsive, CLM-1-expressing

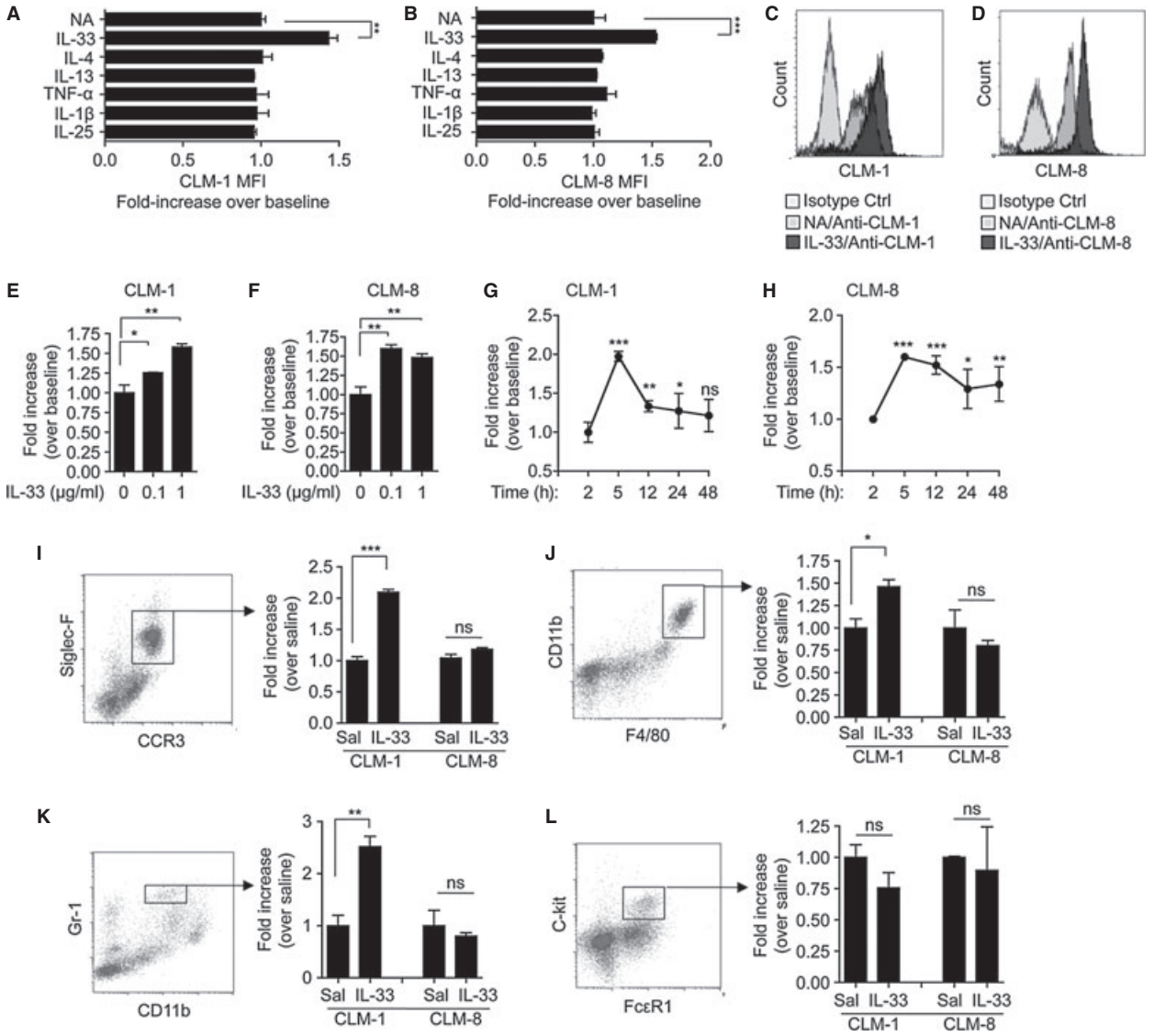

Figure 2 Regulation of CLM-1 and CLM-8 by IL-33. Low-density bone marrow (BM)-derived eosinophils were stimulated with the indicated cytokines (100 ng/ml) and the expression of CLMs was assessed after 24 h (A–B). Representative histogram plot for IL-33-induced CLM-1/-8 expression is shown (C–D) [NA – nonactivated, IL-33 – IL-33 activated]. IL-33-induced concentration-dependent (E-F) and time-dependent (G-H) upregulation of CLM-1/-8 expression in eosinophils is shown. IL-33-induced CLM-1/-8 expression following intraperitoneal injection of IL-33 (2 μg/mouse) is shown in eosinophils (I), macrophages (J), neutrophils (K), and mast cells (L), $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, nonsignificant.

cells. To this end, *Clm1*$^{-/-}$ LDBM eosinophils were generated and stimulated with increasing concentrations of IL-33. Despite the predicted inhibitory role of CLM-1 (13, 16, 18, 26), CLM-1 was required for IL-33-induced mediator release from eosinophils (Fig. 3). IL-33-stimulated wt LDBM eosinophils secreted readily detectable levels of pro-inflammatory cytokines (e.g., IL-6, Fig. 3A) and Th2-associated cytokines (i.e., IL-4 and IL-13, Fig. 3B,C). *Clm1*$^{-/-}$ LDBM eosinophils displayed markedly decreased levels of the aforementioned IL-33-induced mediators (Fig. 3A–C). Although our LDBM-derived eosinophil cultures contained approximately 90% Siglec-F^{+} cells that correspond with eosinophil morphology, there may still be additional (noneosinophil) cellular populations that could respond to IL-33 stimulation. Thus, the LDBM cell cultures were further enriched for eosinophils using positive selection of Siglec-F^{+} cells, yielding a purity of 99%. Similar to our findings with the regular LDBM-derived eosinophil culture, highly enriched IL-33-activated *Clm1*$^{-/-}$ eosinophils displayed a marked reduction in IL-33-induced IL-6 secretion (Fig. 3D). Furthermore, highly purified primary *Clm1*$^{-/-}$ spleen eosinophils showed decreased mRNA expression of IL-33-induced

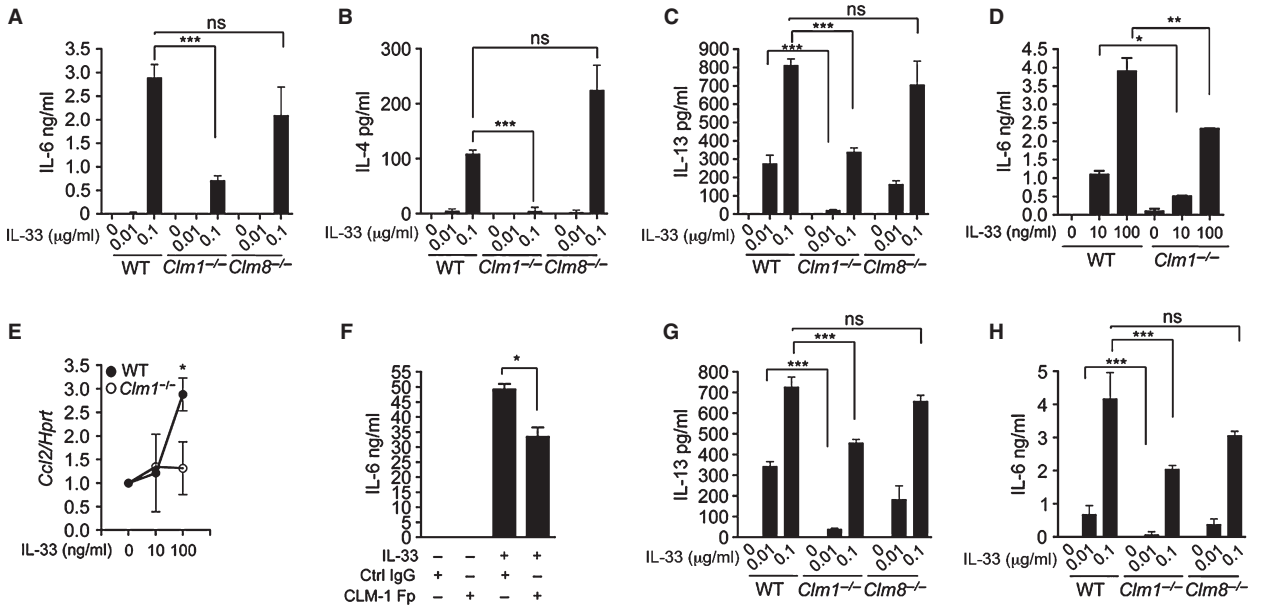

Figure 3 CMRF35-like molecule-1, but not CLM-8, is required for IL-33-induced cellular activation. Low-density bone marrow (BM)-derived eosinophils (A-C), highly enriched low-density BM-derived eosinophils (D), highly enriched spleen eosinophils (E), and BM-derived mast cells (G-H) were obtained from wild-type (WT), *Clm1*$^{-/-}$, and/or *Clm8*$^{-/-}$ mice. The cells were activated with the indicated concentrations of IL-33 and cytokine/chemokine expression was assessed in the culture supernatant for IL-6 (A, D, F, H), IL-4 (B), and IL-13 (C, G) secretion. In (F), LDBM-derived wild-type eosinophils were stimulated with IL-33 in the presence of CLM-1 IgG1 Fc fusion protein (Fp) or control IgG1 (Ctrl) and IL-6 assessed. *Ccl2* mRNA levels were determined by qPCR analysis and normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns, nonsignificant. Data are representative from at least $n = 3$ independent experiments.

CCL2 expression in comparison with wt spleen eosinophils (Fig. 3E).

Because CLM-1 may have uncontrolled effects on eosinophil differentiation that may result in altered mediator release, an additional approach was taken. LDBM-derived wt eosinophils were obtained and incubated with an IgG1 CLM-1 Fc fusion protein that competes with ligand binding of membrane-expressed CLM-1, thus mimicking the lack of CLM-1 expression in *Clm1*$^{-/-}$ cells. Consistently, eosinophils treated with an IgG1 CLM-1 Fc fusion protein displayed significantly decreased levels of IL-33-induced IL-6 secretion (Fig. 3F).

Our previous findings suggested that IL-33 might be also co-regulated by CLM-8 (Fig. 2B). Thus, we were interested to define whether the requirement for IL-33-induced effects was specific to CLM-1 or a shared phenomenon with CLM-8. Hence, *Clm8*$^{-/-}$ LDBM eosinophils were activated with IL-33 as well. IL-33-activated *Clm8*$^{-/-}$ LDBM eosinophils displayed similar cytokine secretion as wt cells (Fig. 3A–C).

Importantly, the inability of LDBM *Clm1*$^{-/-}$ eosinophils to respond to IL-33 was not due to altered IL-33 receptor expression because wt and *Clm1*$^{-/-}$ LDBM eosinophils displayed comparable levels of ST2 and IL-1RAcP (Fig. S3).

Next, we were interested to determine whether the requirement for CLM-1 in IL-33-induced mediator release was eosinophil specific or a generalized phenomenon in additional CLM-1-bearing, IL-33-responsive cells. For this, wt and *Clm1*$^{-/-}$ BMMCs were stimulated with IL-33 and mediator release was assessed. Similar to our findings with eosinophils, *Clm1*$^{-/-}$ but not *Clm8*$^{-/-}$ BMMCs displayed significantly decreased responsiveness to IL-33-induced IL-6 and IL-13 release (Fig. 3G,H). Consistent with previous reports, IL-33 alone did not cause any significant cytokine/chemokine secretion from BM-derived macrophages, a phenomenon that was indifferent between wt and *Clm1*$^{-/-}$ cells (28).

CLM-1 is co-localized to ST2 and is required for IL-33-induced NFκB and p38 activation

The requirement for CLM-1 in IL-33-induced mediator release suggested a possible interaction between CLM-1 and IL-33 receptors. Despite our efforts to demonstrate the binding of CLM-1 and ST2 by co-immunoprecipitation experiments, we were unable to precipitate CLM-1 or ST2 from eosinophils using the currently available antibodies. Thus, as an alternative approach, immunofluorescent analysis was performed to define the localization of CLM-1 and ST2 before and after IL-33 stimulation. CLM-1 was expressed on the surface of eosinophils in a punctate pattern (Fig. 4A), which was further enhanced following IL-33 stimulation, thus confirming our flow cytometry data (Figs 2 and 4A). Interestingly, CLM-1 was partially co-localized with ST2 at baseline conditions (i.e., nonstimulated cells) and IL-33 stimulation increased the co-localization of CLM-1 with ST2 (Fig. 4A, B). Quantitation of CLM-1 and ST2 co-localization following

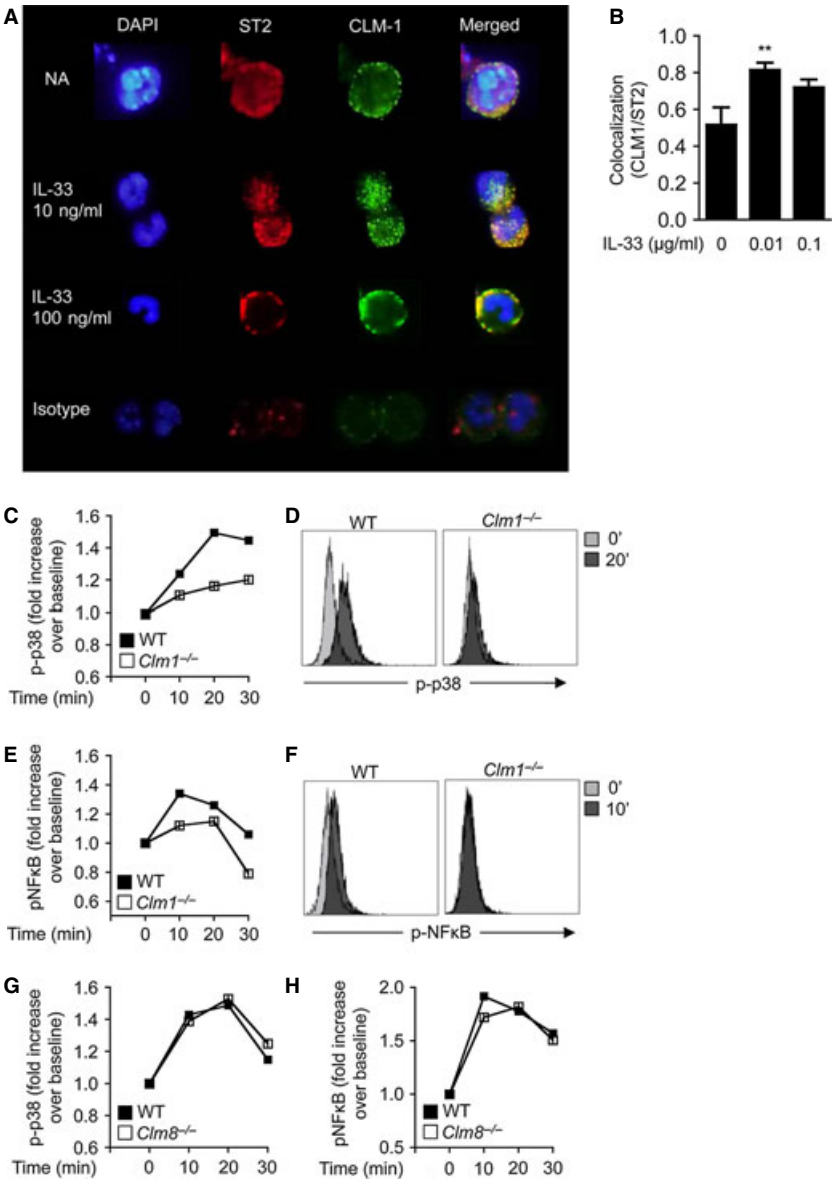

Figure 4 CMRF35-like molecule-1 (CLM-1) co-localizes to ST2 in eosinophils and regulates IL-33-induced p38 and NFκB activation. LDBM eosinophils were stimulated with IL-33 (0–100 ng/ml, 24 h) and stained with DAPI (blue), anti-ST2 (red), and anti-CLM-1 (green) or isotype-matched control antibodies. Receptor localization was assessed by fluorescent microscopy (A), and quantitative analysis is shown (B). Wild-type (WT), *Clm1*$^{-/-}$, and *Clm8*$^{-/-}$ LDBM eosinophils were stimulated with IL-33 (100 ng/ml) for the indicated time points (C, E, G, H). Phosphorylation of p38 and NFκB was assessed by intracellular flow cytometry. Kinetic analyses (C, E, G, H) and representative histogram plots (D, F) are shown. Data are representative from at least $n = 3$ independent experiments. $^{**}P < 0.01$.

IL-33 treatment revealed that 0.1 μg/ml of IL-33 induced a significant increase in CLM-1:ST2 co-localization (Fig. 4B).

In support of a functional and mechanistic requirement for CLM-1 in IL-33-induced eosinophil activation, *Clm1*$^{-/-}$ LDBM eosinophils displayed markedly decreased IL-33-induced NFκB and p38 phosphorylation (Fig. 4C–F). Consistent with the inability of CLM-8 to regulate IL-33-induced mediator release from LDBM eosinophils and BMMCs, IL-33-activated *Clm8*$^{-/-}$ LDBM eosinophils displayed similar p38 and NFκB phosphorylation (Fig. 4G,H). Notably, IL-33 did not activate JNK and ERK phosphorylation in LDBM eosinophils (data not shown).

CLM-1 is required for IL-33-induced mediator release and consequent cellular infiltration *in vivo*

To further establish the requirement for CLM-1 in IL-33-induced cellular responses, a direct *in vivo* approach was used

in which IL-33 was injected into the peritoneal cavity of wt and *Clm1*$^{-/-}$ mice and mediator release as well as cellular recruitment was assessed (29).

Administration of IL-33 to wt mice resulted in a marked increase in various chemokines including CCL11, CCL24, and CCL2, but not CCL17 (Fig. 5A–C and data not shown), as well as the Th2 cytokines IL-5 and IL-13 (Fig. 5D,E). Strikingly, the levels of CCL11, IL-5, and IL-13 were nearly abolished in IL-33-treated *Clm1*$^{-/-}$ mice (Figs 5A, 5D,E) and the levels of CCL24 and CCL2 markedly reduced (Fig. 5B,C). Consistently, IL-33-induced total cell counts were attenuated in *Clm1*$^{-/-}$ mice (Fig. 5F). Flow cytometric analysis of peritoneal cells revealed that the infiltration of mast cells, macrophages, neutrophils, and B cells was significantly reduced in the peritoneal cavity of IL-33-treated *Clm1*$^{-/-}$ mice (Fig. 5G–J). However, despite the low level of IL-33-induced CCL11 and CCL24 in the peritoneum of IL-33-treated *Clm1*$^{-/-}$ mice, eosinophilic infiltration was similar between wt and *Clm1*$^{-/-}$ mice (Fig. 5K). This effect was likely due to the fact that CLM-1 is a negative regulator of eotaxin-induced eosinophil chemotaxis ((16) and Fig. S4). Indeed, both IL-33-treated wt and *Clm1*$^{-/-}$ mice showed a comparable and marked reduction in peripheral blood eosinophilia, a sign of active recruitment into the tissue ((30) and Fig. 5L).

Discussion

Interleukin-33 is a potent cytokine belonging to the IL-1 cytokine family (1). Recent studies highlight IL-33 as a key factor in allergic inflammatory reactions and nematode infections mainly by its ability to activate immune cells and elicit type-2 immune responses (1, 8, 31–33). While such studies have been largely focused on the expression, processing, and/or effector functions of IL-33, endogenous molecular pathways that regulate IL-33-induced effects are largely unknown. We now report a novel requirement for CLM-1 in IL-33-induced effects especially in eosinophils. We demonstrate that: (1) CLM-1 is differentially expressed by various myeloid cells including eosinophils, mast cells, and macrophages, but not ILC2 cells; (2) IL-33 induces a rapid and significant increase in CLM-1 expression; (3) CLM-1 (but not CLM-8) is required for IL-33-induced mediator release and subsequent inflammatory responses; and finally, (4) CLM-1 co-localizes with ST2 and regulates IL-33-induced NFκB and p38 activation. Collectively, our results demonstrate a novel physiological role for CLM-1 in IL-33-induced inflammatory effects. To the best of our knowledge, this is the first report of an endogenous non-IL-1-related family receptor that is required for IL-33-induced responses.

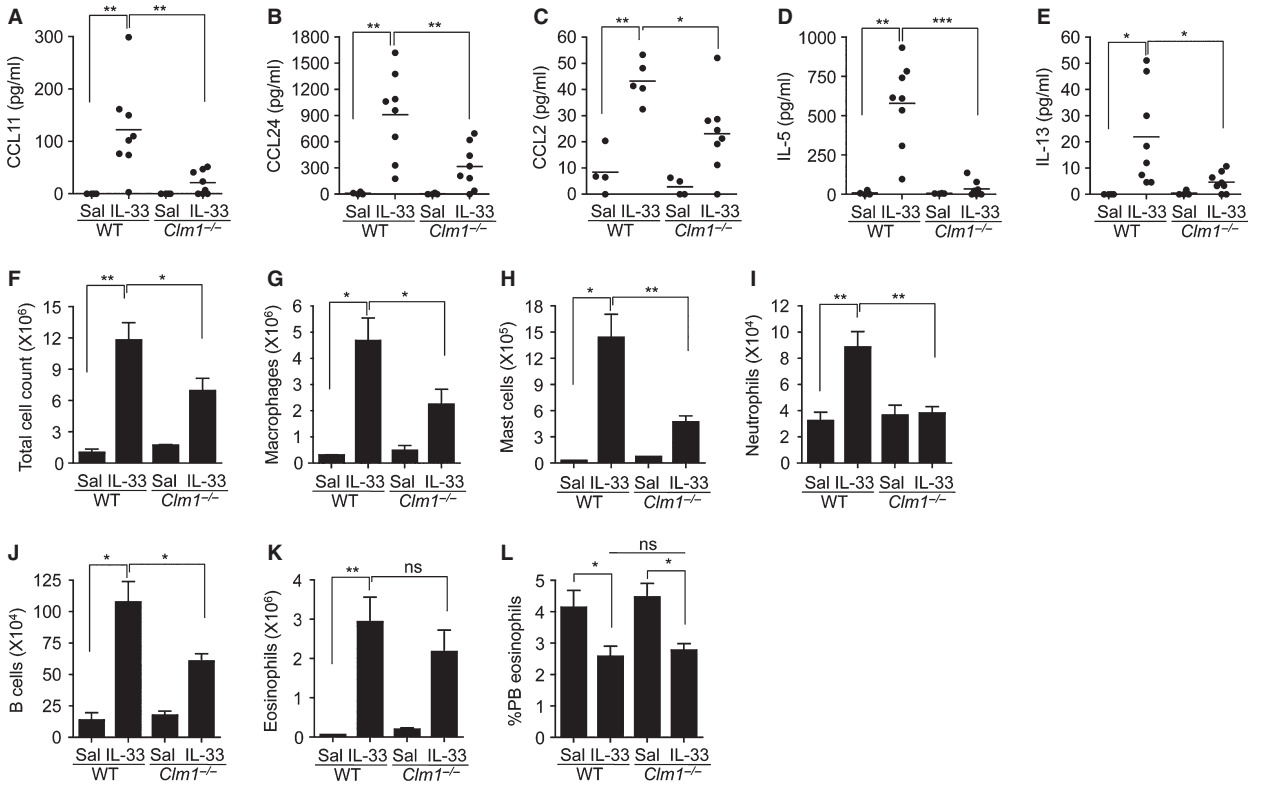

Figure 5 CLM-1 is required for IL-33-induced peritoneal inflammation. Wild-type (WT) and *Clm1*$^{-/-}$ mice were intraperitoneally challenged with IL-33. Thereafter, peritoneal lavage was performed, and the expression of CCL11 (A), CCL24 (B), CCL2 (C), IL-5 (D), and IL-13 (E) was assessed. IL-33-induced peritoneal cellular infiltration was assessed (F-K). Eosinophil levels in the peripheral blood (PB) were determined by flow cytometry (L). Each dot represents one mouse; data are from at least $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, nonsignificant.

CMRF35-like molecule-1 is an ITIM-bearing cell surface receptor that belongs to a larger family of type I transmembrane glycoproteins (i.e., CLM-1-9), which are predominantly expressed by myeloid cells (13). Of the nine CLM family members, only CLM-1 and -8 possess ITIMs in their intracellular domain and are thus expected to deliver inhibitory signals by the recruitment of phosphatases that suppress the activities of other CLM receptors (13–15, 18, 34–37). Despite its predicted inhibitory activity, our results demonstrate a functional requirement for CLM-1 in IL-33-induced cellular activation. This may be explained by the unique intracellular signaling motif that CLM-1 contains. The intracellular domain of CLM-1 contains two ITIM motifs, one immunoreceptor tyrosine-based switch motif (ITSM) (38) and a p85α phosphoinositide 3-kinase-binding motif, indicating the ability of CLM-1 to interact with multiple signaling intermediates (13). Supporting this, CLM-1 has been shown to associate with the FcRγ chain (which contains ITAMs) and recent studies demonstrate that CLM-1 (and its human orthologue, CD300f) can partner with SHP-1 and -2 as well as with PI3K (14, 15, 34). Furthermore, CLM-1 has been shown to display opposing activation and inhibitory functions in mast cell activation (14). Thus, our data further support the notion that under certain circumstances, CLM-1 may serve as a co-activator rather than a classical 'inhibitory' receptor.

While our data demonstrate that CLM-1 is required for IL-33-induced cellular activation, it is important to note that additional IL-33-responsive cells do not express CLM-1. For example, $CD4^+$ T cells and ILC2 cells can both respond to IL-33, but they do not express CLM-1. Despite this, we observed a significant attenuation of IL-33-induced responses *in vivo*. Given the lack of CLM-1 expression by the aforementioned cells, these effects are likely not driven by altered activity of $CD4^+$ T cells and/or ILC2 cells in *Clm1*$^{-/-}$ mice. Rather (and consistent with previous publications), our data suggest that intraperitoneal injection of IL-33 induces the secretion of Th2-associated cytokines from other cells such as mast cells, macrophages, and/or eosinophils (29). An alternative interpretation may be that CLM-1-expressing cells in the peritoneal cavity either support IL-33-induced activities of other IL-33-responsive cells that do not express CLM-1.

A recent study assessing the function of CLM-1 in a mast cell-dependent model of allergic airway inflammation and a dermatitis model suggests an inhibitory function for CLM-1, as *Clm1*$^{-/-}$ mice displayed an exaggerated disease phenotype (39). The differences between our findings and the latter study may be due to numerous factors including the relative expression of additional inflammatory cytokines besides IL-33, which may be regulated by CLM-1. Alternatively, the abundance of IL-33-responsive non-CLM-1^+ cells (e.g., ILC2s and $CD4^+$ T cells) and the nature of CLM-1 ligands can be different as well. Moreover, CLM family members are capable of forming both homo- and heterodimers. Thus, the signaling capacity of CLM-1 may be dependent on the expression of additional CLM family members (40). Defining the full expression and functional spectrum of CLM family members in additional cells and in response to additional triggers will likely shed more light into these interactions and their relevance in IL-33-associated pathologies as well.

Our data demonstrate a significant requirement for CLM-1 in IL-33-induced cellular responses. This conclusion bears the limitation that the majority of our studies compared functional responses between IL-33-activated wt and *Clm1*$^{-/-}$ LDBM-derived eosinophils. In order to draw a stronger conclusion regarding the requirement of CLM-1 in IL-33-induced responses, additional strategies to activate CLM-1 (such as CLM-1 receptor cross-linking) in the presence of IL-33 are required. Unfortunately, the current available antibodies could not activate CLM-1 in BM-derived eosinophils and purified eosinophils from IL-5 transgenic mice did not secrete any mediator in response to IL-33 (data not shown). Despite these limitations, our *in vivo* studies (although not directly assessing the role of CLM-1 in eosinophils) and ligand blocking studies using a CLM-1 Fc fusion protein corroborate the functional requirement of CLM-1 in IL-33-induced cellular responses.

Current models describing the *in vivo* functions of IL-33 secretion and consequent effector functions emphasize the importance of IL-33 in the context of tissue injury (mainly epithelial cells) and cell death (mainly necrosis) (3, 5, 41). Interestingly, the identified ligands for CLM-1 are phosphatidylserine and ceramide, both of which are molecules that are associated with cell death and tissue injury (39, 42). Thus, it is likely that CLM-1 ligands will be readily available in settings where IL-33 is secreted. Directly related, eosinophils, mast cells, and macrophages have been all implicated in tissue repair processes and can be activated by various danger signals (43, 44). Taken together, this raises the notion that CLM-1 will regulate cellular functions in settings of increased tissue damage and subsequent repair processes.

Despite the finding of decreased eosinophil-attracting chemokines in the peritoneal lavage of IL-33-treated *Clm1*$^{-/-}$ mice, the overall eosinophilic infiltrate was similar between IL-33-treated wt and *Clm1*$^{-/-}$ mice. This result is not surprising especially because we have recently shown that CLM-1 is a specific negative regulator of eosinophil chemotaxis in response to eotaxins (16). Thus, *Clm1*$^{-/-}$ eosinophils are hypersensitive to eotaxin stimulation and even low amounts of eotaxins could induce a strong chemotactic response. Taken together, our data suggest that similar to other ITIM-bearing receptors such as PIR-B, which can regulate multiple cellular responses (e.g., chemotaxis, differentiation, innate immune responses, and adhesion) (45–49), CLM-1 may regulate a broader range of eosinophil responses and effector functions.

In summary, we demonstrate that CLM-1 is a novel endogenous regulator of IL-33-induced responses in various cells and especially in eosinophils. Collectively, these data provide insight into the molecular mechanisms that are required for IL-33-induced responses. Such understanding may ultimately lead to the development for future therapeutic strategies in inflammatory diseases involving IL-33.

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Author contributions

D.S., I.M., D.K.A., H.R, and A.M. performed the experiments; D.S. and A.M. designed the experiments and analyzed the data; D.S. and A.M. wrote the manuscript.

Conflicts of interest

All authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression of CLM-1 and CLM-8 in macrophages and mast cells following IL-33 stimulation.

Figure S2. Expression of CLM-1 in innate lymphoid type 2 cells (ILC2).

Figure S3. Expression of ST2 and IL-1RAcP in murine eosinophils.

Figure S4. CLM-1 is a negative regulator of eotaxin-induced eosinophil responses.

Data S1. Interleukin-33 requires CMRF35-like molecule-1 expression for induction of myeloid cell activation.

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