An interleukin-33 gene polymorphism is a modifier for eosinophilia in rats

Running head: *Il33* polymorphism for eosinophilia

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ABSTRACT

In previous studies, we identified a loss-of-function mutation in the *Cyba* gene as the primary cause of hereditary eosinophilia in the MES rat strain. We also identified a modifier locus for eosinophilia named *eos3* in rats. In this study, we examined the interleukin-33 (*Il33*) gene as a candidate for the *eos3* and found a missense nucleotide substitution in the gene, which resulted in a G171S amino acid substitution in the IL-33 protein. Recombinant IL-33 isoform with the G171S substitution had approximately 50% of activity of normal isoform in NF-κB-dependent reporter assay, and reduced bioactivity (~65% of normal) to provoke eosinophilia when injected into mice. In a genetic association study using (ACI x MES) x MES backcross rats, we found that the effects of polymorphic *Il33* alleles on blood eosinophil level were manifested only in rats with loss of *Cyba* function. In these rats, the blood eosinophil level was significantly lower (~50%) in heterozygotes for the ACI allele of *Il33* compared with homozygotes for the MES allele. Oddly however, eosinophilic MES rats had blood IL-33 content below the detectable limits. These results suggest that the *Il33* gene polymorphism could be a modifier of eosinophilia in rats.

Keywords: eosinophil, gene polymorphism, interleukin-33, modifier, rat

INTRODUCTION

The Matsumoto Eosinophilia Shinshu (MES) is a rat strain that develops blood eosinophilia (>500/μl) and eosinophil-related inflammatory lesions in many organs after 8 weeks of age as hereditary traits.[1-5] In a previous study, we performed chromosomal mapping and positional cloning of the genes for eosinophilia in MES rats using (ACI x MES) x MES backcross progeny, and demonstrated that the primary cause of eosinophilia in MES rats is a loss-of-function mutation in the gene for cytochrome b(-245), alpha polypeptide (*Cyba*) on chromosome 19.[6,7] Also, another quantitative trait locus for blood eosinophil level named *eos3* was found between the *D1Rat123* and *D1Rat30* marker loci on chromosome 1.[8] It appeared that the *eos3* allele of MES increased, while that of ACI decreased, the blood eosinophil levels of backcross rats. The gene for the *eos3* locus has not yet been determined. Of note, this chromosomal region contains the interleukin-33 (*Il33*) gene.

IL-33 is a cytokine that is expressed mainly by stromal cells, such as endothelial and epithelial cells.[9,10] IL-33 is suggested to function as an alarmin that is released upon cell damage and alerts the immune cells to endogenous trauma such as physical stress or infection.[11] IL-33 binds the transmembrane receptor interleukin-1 receptor-like 1 (IL1RL1), which subsequently associates with interleukin-1 receptor accessory protein (IL1RAP) to enable IL-33-dependent activation of NF-κB.[12] T-helper type-2 (Th2) cells abundantly express the receptor molecules.[13] In Th2 cells, IL-33 upregulates production of Th2-associated cytokines including IL-4, IL-5, and IL-13, which activate eosinophil development and function. Eosinophils also express IL1RL1/IL1RAP receptors on the cell surface and are directly activated by IL-33.[14-18] Several studies using mouse models have highlighted a role of IL-33 in eosinophilia. Administration of IL-33 to mice stimulates eosinophil differentiation in the bone marrow, provokes profound eosinophilia, and exacerbates eosinophil-mediated airway inflammation[15,19] or allergic conjunctivitis.[20] Also, transgenic over-expression of IL-33 leads to spontaneous pulmonary eosinophilic inflammation in mice.[21] Conversely, suppression of IL-33 by anti-IL-33 antibody treatment inhibits inflammation in murine models of allergic asthma[22], and allergic rhinitis.[23] Also, *Il33* gene-knock out mice showed attenuated eosinophil influx into the bronchoalveolar

lavage fluid, airway hyperresponsiveness, and pulmonary inflammation in ovalbumin (OVA)-induced allergic response as well as a substantially diminished lipopolysaccharide-induced systemic inflammatory response.[24] Few clinical studies also suggest the functional importance of IL-33 in the eosinophil-related diseases.[25] Intriguingly, a single nucleotide polymorphism (SNP), which is located proximal to the start codon of *IL33* gene was shown to be associated with blood eosinophil counts in humans.[26] *IL33* gene polymorphisms were demonstrated to be a risk factor also for nasal polyposis[27] and Japanese cedar pollinosis[28], in which eosinophils play a key role. The functional relevance of the SNPs is unknown; however, these observations suggested that a latent *Il33* polymorphism influenced blood eosinophil levels in our backcross rats.

In this study, we explore this possibility by seeking nucleotide alterations in the *Il33* gene of rats and examining its influence on the function of the gene product. We also evaluate the modifying effects of polymorphic *Il33* alleles on eosinophilia.

RESULTS

ACI rats have a missense nucleotide polymorphism in the *Il33* gene

Sequence analysis of *Il33* cDNA revealed two nucleotide substitutions between ACI and MES rats. While one of these substitutions was silent (618T > C), the other substitution (511G > A) was a missense mutation, such that codon 171 was changed from GGT in MES to AGT in ACI, which resulted in a glycine to serine substitution (G171S) in the IL-33 protein (Figure 1a). The corresponding amino acid in the mouse (173rd residue) and human IL-33 (176th residue) is glycine, indicating that the MES allele is an ancestral type to the ACI allele.

The 511G >A nucleotide substitution in *Il33* cDNA is located in exon 7 of the gene. To examine if the nucleotide substitution is a mutation specific to ACI rats, 9 inbred rat strains (BDIX, BN, DA, DONRYU, IS, LEW, SHR, TM, and WBN/Kob) were screened for the substitution by PCR amplification of the exon 7 sequence of the *Il33* gene from genomic DNA of rats followed by nucleotide sequencing. The missense nucleotide substitution was found in BDIX, suggesting that the substitution is not specific to ACI but rather is a polymorphism in laboratory rat strains.

A G171S amino acid substitution in IL-33 does not influence proteolysis by caspase-3

The human and mouse IL-33 is a physiological substrate for caspase-3.[29,30] Thus, IL-33 is inactivated in cells by caspase-mediated proteolysis during apoptosis. The caspase-3 cleavage site is located at Asp178 within human IL-33 ([175]DGVD[178]) and Asp175 within mouse IL-33 ([172]DGVD[175]). This caspase-3 cleavage motif is conserved in rat IL-33 ([170]DGVD[173]). Notably, the G171S amino acid substitution in IL-33 of ACI rats is located within this motif (Figure 1a). The influence of the G171S substitution on proteolysis by caspase-3 was then investigated. Both IL-33 isoforms showed a similar profile in a proteolysis assay with serially diluted caspase-3 (Figure 1b), indicating that the G171S substitution did not influence susceptibility of IL-33 to proteolysis by caspase-3.

IL-33 isoform with a G171S amino acid substitution has reduced signaling activity at the cellular level

The influence of the G171S amino acid substitution on IL-33 activity was then investigated. Recombinant IL-33 isoforms were made, in which the 171st amino acid residue was either glycine (MES type) or serine (ACI type), and given to cultured cells. The activity of IL-33 was then assessed by the NF-κB reporter assay. First, it was confirmed that the HEK293 cells did not respond to recombinant rat IL-33 if it was not transfected with expression plasmid constructs for the rat IL1RL1 and IL1RAP co-receptors (data not shown). Both IL-33 isoforms induced NF-κB-dependent luciferase activity in a concentration-dependent manner (Figure 2). The IL-33 isoform with G171S of the ACI type showed approximately 50% activity of the MES type at all doses examined.

IL-33 isoform with a G171S amino acid substitution has reduced activity to provoke eosinophilia in vivo

To determine whether the IL-33 isoform with G171S of ACI type was also less potent in vivo, we then compared the activity of IL-33 isoforms in a mouse model. Recombinant mouse IL-33 as a positive control provoked blood eosinophilia when administered to mice (Figure 3). Recombinant IL-33 isoforms of both MES and ACI types also prvoked eosinophilia in mice; however, an increase in the blood eosinophil levels was attenuated in mice administered with the ACI isoform (490 ± 139/μl; $P<0.05$) compared with the mice treated with the MES isoform (756 ± 123/μl). Thus, both in vitro and in vivo studies indicated that the ACI allele of *Il33* was hypomorphic compared with the ancestral MES allele.

The effects of *Il33* polymorphism were manifested only in backcross rats with loss of *Cyba* function

(ACI x MES) x MES backcross rats[6] were then genotyped for the *Il33* polymorphism, and the effects of the polymorphic alleles on blood eosinophil levels in the rats were evaluated. All rats heterozygous for the mutant *Cyba^{mes}* gene (*Cyba^{mes/ACI}*) had blood eosinophil levels within the normal range (<250/μl). In these rats, the blood eosinophil level was not influenced by the *Il33* genotype (59 ± 36 versus 56 ± 35; *P*=0.66), indicating that the polymorphic alleles did not influence the steady state blood eosinophil level (Table 1). In contrast, influence of the *Il33* polymorphism on blood eosinophil level was manifested in backcross rats homozygous for the mutant *Cyba^{mes}* gene (*Cyba^{mes/mes}*) and hence with loss of *Cyba* function. In these rats, the blood eosinophil level was significantly lower in heterozygotes for the ACI allele of *Il33* (110 ± 127/μl) compared with homozygotes for the MES allele (202 ± 175/μl; *P*<0.0001). Thus, the ACI allele for the *Il33* gene attenuated, while the MES allele augmented blood eosinophilia when paired with loss of *Cyba* function (and probably tissue damage).

MES rats have normal plasma IL-33 level

To evaluate an etiological relevance of IL-33 in the eosinophilia of MES rats, plasma and organ IL-33 levels were determined. A preliminary ELISA assay of a MES rat detected IL-33 in all organs examined (spleen, mesenteric lymph node, bone marrow, stomach, large intestine, and liver), but not in plasma (data not shown). IL-33 contents in plasma, spleen (as a lymphoid tissue), and stomach (as a gastrointestinal tissue) were then measured in additional MES and normal SD rats. MES rats over 8 weeks of age had blood eosinophilia (Figure 4). All rats, however, had plasma IL-33 level below the detectable limits (<6.85 pg/ml) in the ELISA assay at all ages (data not shown). The IL-33 contents in the spleen were significantly lower in MES rats compared with SD rats at 6 weeks of age. The IL-33 contents were lower also in the stomach of MES rats than that of SD rats at 8 weeks of age. MES and SD rats had similar IL-33 contents in both organs at other ages.

DISCUSSION

The data presented herein suggested that an *Il33* gene polymorphism is a modifier for eosinophilia in rats. This study was initiated following our previous identification of the *eos3*

as a quantitative trait locus for blood eosinophil level of (ACI x MES) x MES backcross rats.[8] Based on the data obtained in the backcross rats, it was expected that a gene product of the ACI allele on *eos3* had lower activity to increase blood eosinophil count than that of the MES allele. Consistent with this expectation, *Il33* allele of ACI was hypomorphic in increasing blood eosinophil count compared with that of MES in the in vivo activity assay. The hypomorphic nature was verified in the in vitro reporter assay. Also, the magnitude of decrease in IL-33 bioactivity of ACI rats (50~65%) agreed well with the magnitude of decrease of blood eosinophil count (54%) in the *Cyba*$^{mes/mes}$ homozygous backcross rats with the ACI allele of *Il33*. Furthermore, the *Il33* genotype did not influence blood eosinophil count in the *Cyba*$^{mes/ACI}$ heterozygous (and hence normal) backcross rats. This was consistent with the observation in humans that the SNP close to the *IL33* locus showed stronger association with atopic asthma than nonatopic asthma.[26] Also, this mode of *Il33* action in the backcross rats agreed with the proposed role of IL-33 as an alarmin. IL-33 is constitutively expressed in the nucleus of endothelial and epithelial cells. Release from these cells upon damage is necessary for IL-33 to act as an alarmin and transmit signals to the immune system including eosinophils.[11] Admittedly though, the results presented in this report are not sufficient to substantiate the hypothesis that polymorphic *Il33* is indeed *eos3*. There remained the possibility that other gene(s), which are linked to the *Il33* on chromosome 1 is actually *eos3*. To definitively prove the identity of *Il33* and *eos3*, it would be necessary to generate knock-in MES rats in which *Il33* gene is replaced by the ACI allele, and verify reduction of the blood eosinophil level in the rats. This is not a realistic strategy.

The most puzzling observation was the normal blood IL-33 levels of MES rats. Patients with hypereosinophilia or pulmonary eosinophilia had marginally elevated circulating IL-33 levels.[31] Also, the IL-33 level in nasal secretions was significantly elevated in patients with allergic rhinitis.[32] Furthermore in mouse ovalbumin or helminthic parasites-induced airway inflammation models, pulmonary eosinophilia was associated with increased levels of IL-33 in the bronchoalveolar lavage fluid.[33-35] The primary cause of eosinophilia in MES rats is a loss-of-function mutation in the *Cyba* gene.[7] Thus, homozygosity for the mutant *Cyba*^{mes} allele (*Cyba*$^{mes/mes}$) was prerequisite for development of eosinophilia in (ACI x MES) x MES backcross rats, while none of the rats heterozygous for the mutant allele (*Cyba*$^{mes/ACI}$)

developed eosinophilia.[6] It was expected that the homozygous rats had organ damages because of infiltration of overproduced eosinophils that might have caused IL-33 release and exacerbation of eosinophilia. We could not explore this possibility because blood and organ samples of the backcross rats were not kept. Instead, we examined plasma and organ IL-33 content in MES rats. If the hypothesis described above is correct, we would expect that MES rats with the inherent $Cyba^{mes/mes}$ genotype and eosinophil-related inflammatory lesions in organs had elevated plasma IL-33 levels. Contrary to our expectation, the plasma IL-33 content remained at undetectable levels in eosinophilic MES rats. Rather, the IL-33 content was significantly lower in the spleen and stomach of MES rats than normal SD rats when they were in the early stages of eosinophilia. Relevance of the reduction of organ IL-33 content to eosinophilia in MES rats is unclear. In addition to its function as an alarmin, IL-33 is considered to act in the cell nucleus as a modulator of gene expression.[36] Nuclear IL-33 in particular sequesters NF-κB and reduces NF-κB-triggered gene expression to dampen proinflammatory signaling.[37,38] Our preliminary NF-κB-dependent reporter assay, however, did not reveal the sequestering activity for rat IL-33 over-expressed in the cells (data not shown). Even though the discrepancy of normal plasma IL-33 level and mechanisms for the reduction of organ IL-33 content remained elusive, it was possible that reduced IL-33 content altered gene expression profile in organs and contributed to mediate eosinophilia in MES rats. The *Il33* polymorphic alleles might have exerted their differential effects in the backcross rats through this activity.

In summary, we identified a polymorphism in the *Il33* gene as a potential factor to modify eosinophilia in rats. Our findings reinforce the proposed role of IL-33 as an alarmin and implication of *IL33* gene polymorphisms in eosinophil-related diseases.

MATERIALS AND METHODS

Polymorphism survey of the rat *Il33* gene

mRNA was extracted from the spleen of MES and ACI/NSlc rats with the QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, England). First-strand cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Amersham Biosciences) and was then subjected to PCR amplification. cDNA fragments covering the entire coding region of the rat *Il33* gene were amplified by the primer pairs of Il33-1 (5'-CACTGCAGGAAAGTACATCC-3') and Il33-2

(5'-TAACCCAGAAGGCACAGACC-3'). The PCR products were directly sequenced using a BigDye Cycle Sequencing FS Ready Reaction Kit and an ABI310 PRISM DNA Analyzer (Applied Biosystems).

Genomic DNA of inbred rat strains, BDIX, BN, DA, DONRYU, IS, LEW, SHR, TM, and WBN/Kob was obtained from the National BioResource Project for the Rat in Japan. Chromosomal DNA sequences containing exon 7 of the *Il33* gene were obtained from the genomic DNA by PCR with the primer pairs of Il33-exon7F (5'-CCACAACAGGAGTTTTGAAGG-3') and Il33-exon7R (5'-CAAATGTGGCTGTCACGTAGC-3'), and sequenced.

Production of recombinant rat IL-33

Bacterial expression and purification of recombinant rat IL-33 were performed as described for human IL-33[19] with modifications. The expression construct was made with the pET-32a(+) plasmid (Novagen/Merck Millipore, Darmstadt, Germany), such that a truncated IL-33 (starting with the 109th amino acid residue) was fused to the C-terminal of thioredoxin and histidine tags. PCR was used to amplify *Il33* cDNA fragments from MES and ACI rats and C57BL/6J mouse, which were subsequently inserted into the *Kpn*I/*Eco*RI site of the pET-32a(+) plasmid. *E. coli* BL21(DE3)pLysS (Novagen/Merck Millipore) was transformed with the expression plasmid constructs. Expression of IL-33 proteins in *E. coli* was induced by addition of a final concentration of 1 mM of isopropyl β-D-1 thiogalactopyranoside to an exponentially growing bacterial culture of 25 ml in LB medium at 28 . After 4 hours of induction, bacteria were harvested by centrifugation and disrupted by resuspension in 1 ml of B-PER Bacterial Protein Extraction Reagent (Thermo Scientific Pierce, Rockford, IL, USA), followed by mild sonication. The recombinant IL-33 was purified from the crude bacterial lysate using a Ni-NTA affinity column (GE Healthcare, Uppsala Sweden) according to the manufacturer's instructions. Endotoxin was removed by using Detoxy-Gel Endotoxin Removing Columns (Thermo Scientific Pierce). Purity of recombinant proteins was confirmed by SDS-PAGE and Coomassie blue staining. The IL-33 concentration was determined by Quantikine Mouse IL-33 Immunoassay kit (R&D Systems, Minneapolis, MN, USA). This assay also recognizes rat IL-33. The amount of rat IL-33 measured was expressed as mouse IL-33 equivalent.

In vitro caspase-3 cleavage assay

Recombinant human caspase-3 was obtained as described[39] with modifications. The bacterial expression construct was made with the pET-21a(+) plasmid (Novagen), such that a truncated caspase-3 (starting with the 29th amino acid residue) was fused to the histidine tag. PCR was used to amplify caspase-3 cDNA fragments from the HepG2 cell line, which were subsequently inserted into the *Nde*I/*Xho*I site of the pET-21a(+) plasmid. Bacterial expression and purification of recombinant caspase-3 was performed as described above. Five μg of recombinant IL-33 was incubated with recombinant caspase-3 for 2 hrs at 37 , followed by SDS-PAGE and Coomassie blue staining.

In vitro assay of recombinant IL-33 activity

An in vitro assay for recombinant rat IL-33 activity was performed as described for human IL-33[13] with modifications. Expression constructs for the rat IL1RL1 and IL1RAP co-receptors for IL-33 were made by inserting cDNA fragments of the gene into the pCI-neo plasmid (Promega, Madison, WI, USA) and pEF6/V5-His plasmid (Invitrogen, Carlsbad, CA, USA), respectively. The human embryonic kidney cell line HEK293 was obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan, and cultured in DMEM supplemented with 10% fetal calf serum. The cells were grown in 24-well plates and co-transfected with 400 ng of the rat IL1RL1 expression plasmid, 200 ng of IL1RAP expression plasmid, 400 ng of the NF-κB reporter plasmid pGL4.32[*luc2P*/NF-κB-RE/Hygro] (Promega), and 4 ng of the control reporter plasmid phRL-TK (Promega) using HilyMax Transfection Reagent (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Twenty-four hours after transfection, recombinant IL-33 was added to the cells. After 5 hours, the cells were harvested, and the luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega). The reporter firefly luciferase activity was normalized with the *Renilla* luciferase activity. The assays were conducted in triplicate, and the experiments were repeated 3 times.

In vivo assay of recombinant IL-33 activity

Female BALB/c mice of 9 weeks of age were purchased from Japan SLC, Inc (Hamamatsu, Japan). After 1 week of habituation under clean conventional conditions at the Institute of Experimental Animals, Shinshu University, mice were injected intraperitoneally with PBS or 100 ng of recombinant IL-33 daily for 7 days. On the 8th day, blood samples were collected

into a heparinised syringe from the jugular vein of mice under ether anesthesia. This protocol was consistent with similar studies on recombinant human IL-33[13,29] and was chosen following extensive dose and time-course studies, using an IL-33 dose range from 0.05 to 1.0 µg per mouse per injection, given on 1-7 consecutive days. The numbers of eosinophils in the blood were counted with an automated cell counter (XT-2000iV, Sysmex Co., Ltd., Kobe, Japan). All animal experimentation was carried out in accordance with the Regulations for Animal Experimentation of Shinshu University.

Quantification of the plasma and organ IL-33 contents

MES rats of 4, 6, 8, 10, and 12 weeks of age, which were maintained at the Institute of Experimental Animals, Shinshu University, were used. Age-matched normal SD rats were purchased from Japan SLC. This rat strain was used as a control because it had the identical Il33 allele to MES rats, but had a normal *Cyba* gene and hence normal blood eosinophil levels. Blood samples were collected into a heparinised syringe from the jugular vein of rats under ether anesthesia. An aliquot of the blood was used for eosinophil quantification, and then plasma sample was obtained after centrifugation. After euthanasia by cervical dislocation, the organs were collected. Parts of organs were conventionally processed, formalin-fixed, paraffin-embedded, sliced to 3 µm and stained with hematoxylin-eosin (HE). Parts (~100 mg) of the spleen and stomach were homogenized in 500 µl of passive lysis buffer (Promega) supplemented with 5 µl of protease inhibitor cocktail (nacalai tesque, Kyoto, Japan). Cleared cell lysate was obtained after centrifugation at 13,000 rpm for 5 min. IL-33 content in the plasma and cell lysates was determined by Quantikine Mouse IL-33 Immunoassay kit according to the manufacture's instructions. The amounts of IL-33 in the cell lysates were normalized by the total protein concentration.

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DISCLOSURE/CONFLICT OF INTEREST

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The authors declare no conflict of interest.

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Titles and legends to figures

Figure 1 MES and ACI rat strains show a polymorphism for the *Il33* gene. **(a)** Alignment of partial nucleotide and deduced amino acid sequences (numbered) for the *Il33* cDNA derived from MES and ACI rat strains. Nucleotide and amino acid substitutions are indicated in bold. The putative caspase-3 cleavage motif is underlined. **(b)** Caspase-3 proteolysis assay for rat IL-33. Recombinant rat IL-33 was incubated with the indicated concentration of recombinant caspase-3, followed by analysis by SDS-PAGE and Coomassie blue staining. M: molecular weight marker.
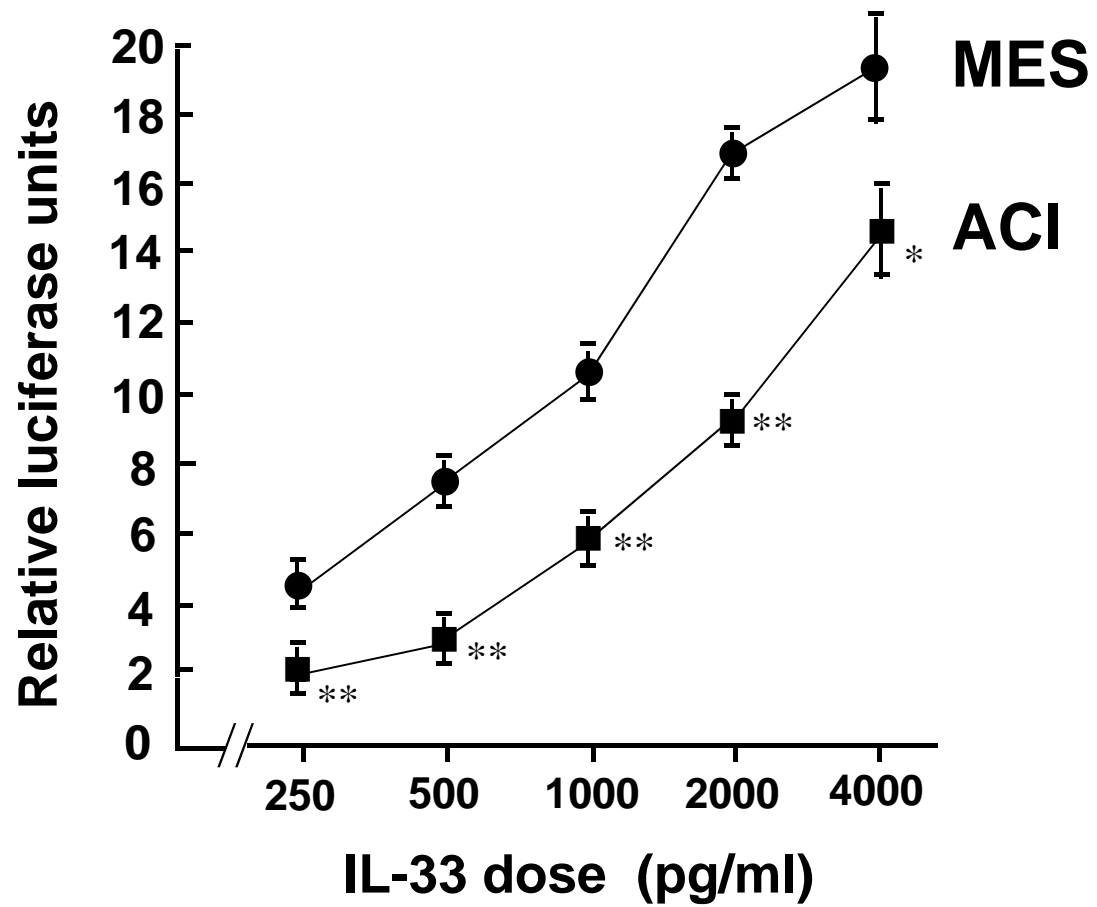
Figure 2 IL-33 isoform with a G171S amino acid substitution has reduced signaling activity at the cellular level. Comparison of activity of rat IL-33 isoforms as determined by NF-κB reporter assay (mean $\pm$ SD; n=3). Data presented here represent 3 independent experiments. Statistical differences between the MES and ACI isoforms at each dose were evaluated by Student's *t* test. $*P < 0.05$ and $**P < 0.01$ versus MES isoform.

Figure 3 IL-33 isoform with a G171S amino acid substitution has reduced activity to provoke eosinophilia in vivo. Comparison of blood eosinophil levels of mice administered recombinant IL-33 isoforms of the mouse, MES, and ACI type (mean $\pm$ S.D; n=5). Statistical differences between the groups were evaluated by one-way analysis of variance (one-way ANOVA) followed by the Tukey-Kramer post hoc test. $*P < 0.05$; $**P < 0.01$.
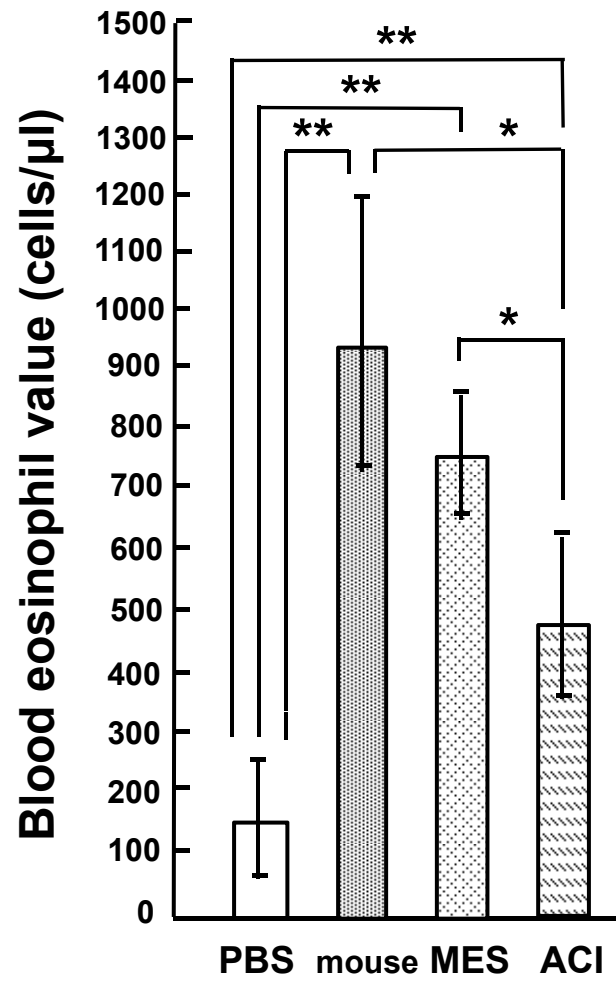
Figure 4 MES rats have low organ IL-33 contents when they are in the early stages of eosinophilia. Comparison of the blood eosinophil level **(a)**, and spleen **(b)** and **(c)** stomach IL-33 content of SD and MES rats (mean $\pm$ S.D; n=3). Statistical differences between the strains were evaluated by Student's *t* test. $*P < 0.05$; $***P < 0.001$.
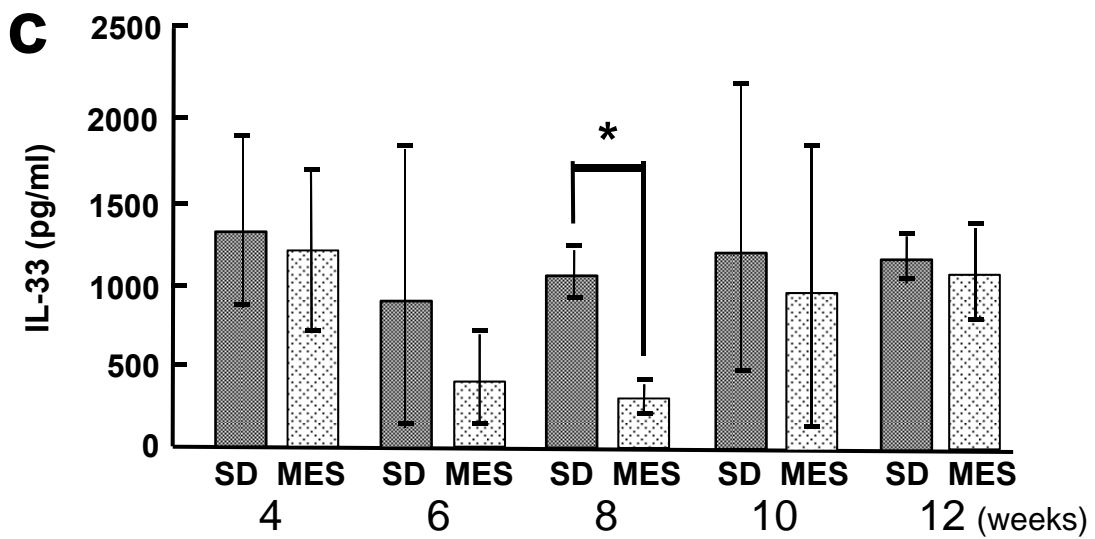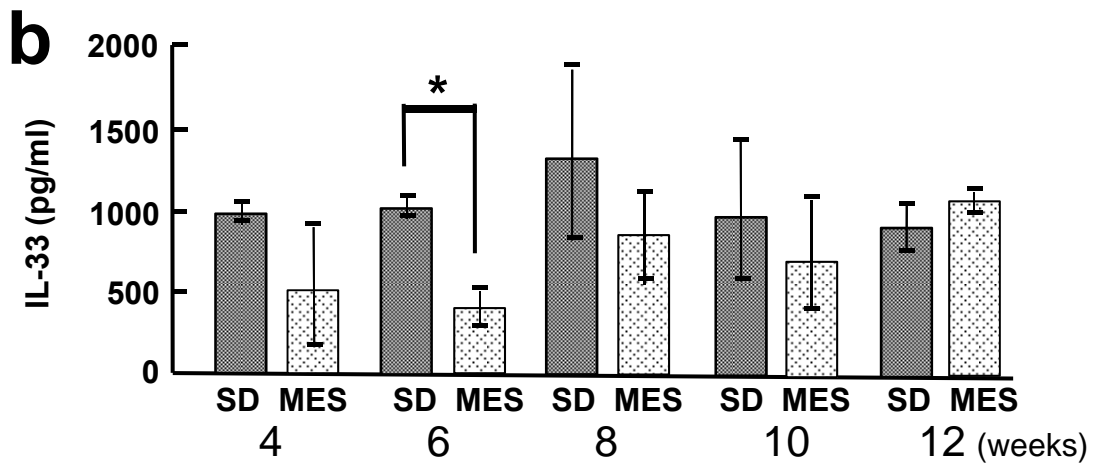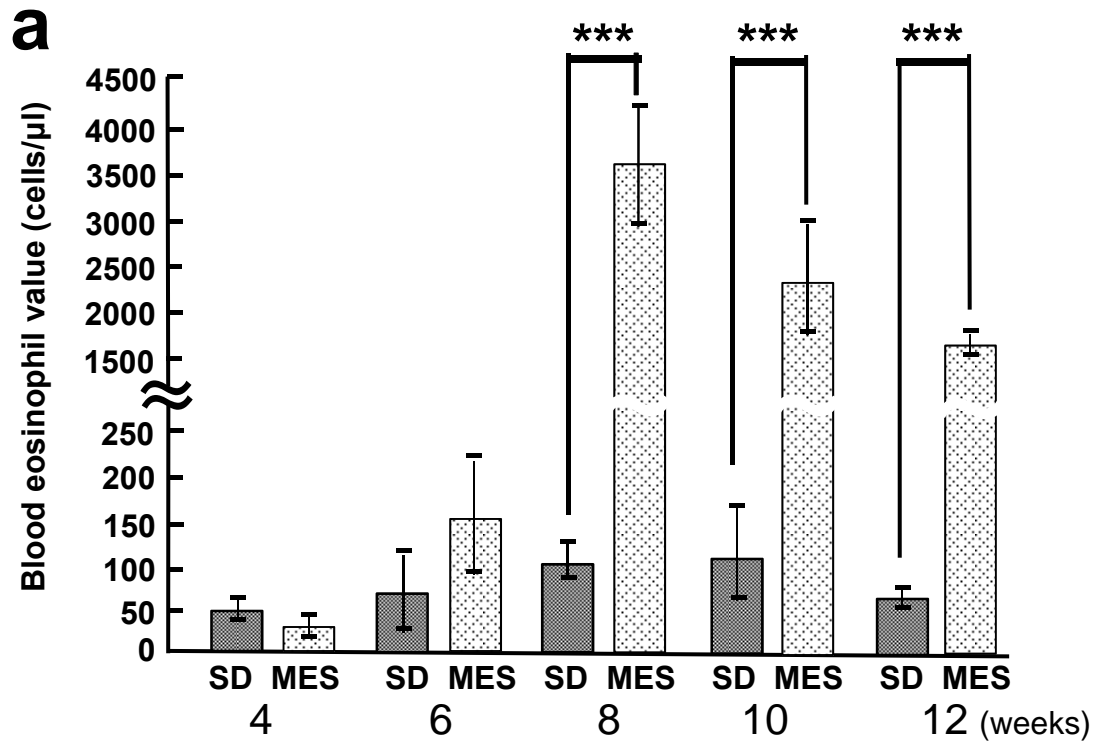
a

ACI

TCA GGA GAC **A**GT GTG GAT GGG
^{168}S   G   D   **S**   V   D   G^{174}

MES

TCA GGA GAC **G**GT GTG GAT GGG
^{168}S   G   <u>D   **G**   V   D</u>   G^{174}

Caspase-3 cleavage motif

b
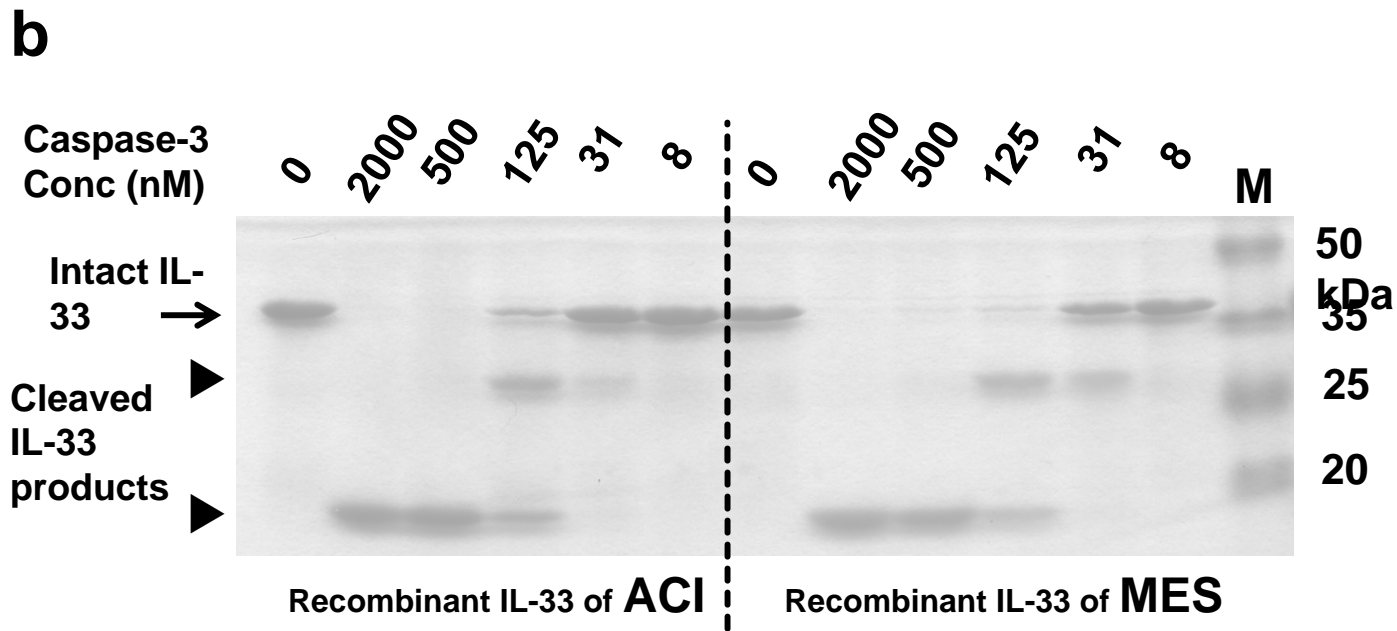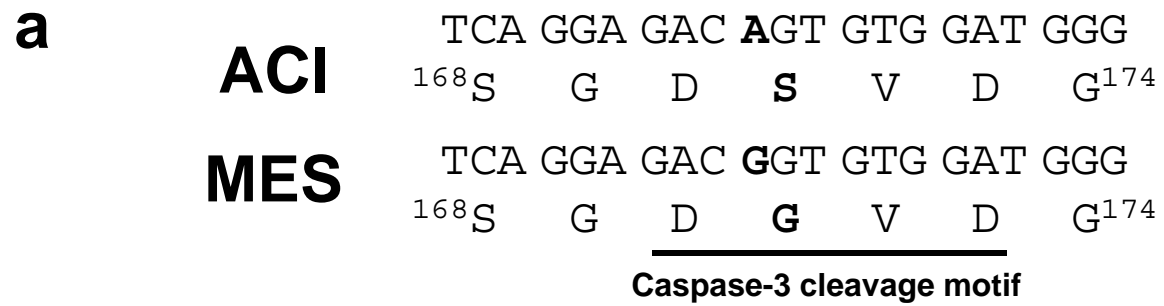
Figure 1.

Figure 2.

Figure 3

Figure 4.

Table 1. Comparison of blood eosinophil level (cells/µl) of (ACI x MES) x MES backcross rats classified by the genotype at *Cyba* and *Il33* loci.

	Il33 Genotype		
	MES/MES	MES/ACI	*P* value**
Cyba^{mes/mes}	202 ± 175* (n=81)	110 ± 127 (n=87)	< 0.0001
Cyba^{mes/ACI}	59 ± 36 (n=79)	56 ± 35 (n=81)	0.66

*Values are expressed as mean ± S.D.

**P values were calculated by the Student's *t* test.