Attenuated Airway Eosinophilic Inflammations in IL-38 Knockout Mouse Model

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Summary: Background: The role of IL-38, a new member of the IL-1 family, in airway eosinophilic inflammatory conditions such as asthma is unclear. To investigate the role of IL-38 in airway eosinophilic inflammation, an IL-38-gene deficient (KO) murine asthma model was analyzed.

Methods: The numbers of eosinophils and neutrophils, and levels of IL-5, IL-13 and IL-17A protein and mRNA in bronchoalveolar lavage fluid (BALF) and lung tissue were compared between wild-type (WT) and IL-38-KO mice after OVA sensitization and challenge. The effects of additional purified recombinant mouse (rm) IL-38 protein were investigated in the IL-38-KO murine asthma model.

Results: The IL-38 and IL-5 mRNA in WT mice was significantly higher after OVA challenge than after saline challenge (p<0.05). The number of airway eosinophils in IL-38-KO mice was significantly lower than in WT mice after OVA challenge (p<0.01). BALF analysis confirmed the lower number of airway eosinophils in IL-38-KO mice and showed that this was significantly associated with lower IL-5 protein levels (r=0.92, p<0.0001). However, the additional rm IL-38 protein did not neutralize airway eosinophilia in IL-38-KO mice.

Conclusion: IL-38 may enhance airway eosinophilic inflammation in asthma through IL-5 induction.

Key words Airway inflammation, Cytokine, Asthm, IL-38, Eosinophils

INTRODUCTION

The interleukin-1 family includes seven ligands with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ), three receptor antagonists (IL-1Rα, IL-36Ra, IL-38), and an anti-inflammatory cytokine (IL-37) [1]. The IL-1 family plays a major role in not only acute and chronic inflammation, but also allergic inflammation. IL-18 plays a well-known role in Th1 polarization and can act as a co-factor for Th2 cell development and IgE production [2-5]. IL-33 affects various types of cells including eosinophils, Th2 cells, basophils, mast cells and type 2 innate lymphoid cells (ILC2), and can also induce Th2 cytokines [6-7]. IL-
IL-38 (IL-1F10) was originally identified in silico and was previously named IL-1HY2 in 2001 [9-10]. The IL-38 gene is located in the IL-1F cluster on chromosome 2 next to the genes encoding IL-1Ra and IL-36Ra. IL-38 shares 41% and 43% homology with IL-1Ra and IL-36Ra, respectively, and has a three-dimensional structure similar to IL-1Ra [11]. IL-38 can act as an IL-36 antagonist [12]. Like IL-1β and IL-18, IL-38 lacks a signal peptide and has no consensus cleavage site [9]. van de Veerdonk and coworkers [12] have reported that IL-38 inhibits Candida albicans-induced neutrophilic inflammation via suppression of IL-17A and IL-22 production. We, along with Boutet et al., have reported the anti-inflammatory effects of IL-38 in a mouse model of rheumatoid arthritis (RA) [13,14]. Previous reports have indicated that IL-38 is involved in the development of some inflammatory diseases such as ankylosing spondylitis, primary Sjögren’s syndrome, and systemic lupus erythematosus [15-17]. Moreover, we recently reported that IL-38 was expressed in tumor cells of various cancers, and that IL-38 expression was associated with poor survival of lung adenocarcinoma patients [18].

A previous study has found that serum IL-38 levels in children with asthma were significantly higher than those in healthy subjects [19]. We have also found that IL-38 mRNA was expressed in normal human lung [20]. However, the roles of IL-38 in allergic inflammatory conditions such as asthma and eosinophilic inflammation are still unknown. We established a purified recombinant mouse (rm) IL-38 protein and also developed an enzyme-linked immunosorbent assay (ELISA) system for detection of mouse IL-38 protein. The present study was designed to investigate the roles of IL-38 in asthma and eosinophilic inflammation using a murine asthma model induced by ovalbumin (OVA) sensitization and challenge.

MATERIALS AND METHODS

Study design

C57BL/6NCrl (B6) wild-type (WT) mice were purchased from Charles River Japan (Yokohama, Japan). IL-38-knockout (KO) mice (Il1f10tm1Lex/Mmucd), backcrossed 9 times with C57BL/6NCrl, were purchased from the Mutant Mouse Regional Resource Center (University of California Davis, West Sacramento, CA) [21]. All mice used in this study were females aged 6-8 weeks and were separated by sex in the study. Mice were bred under specific pathogen free (SPF) conditions and standardized diets were provided at the animal facility (Kurume University Animal Center). Less than 6 mice were bred in a cage (218 [W] × 320 [D] × 133 [H] mm, Floor Area: 481 cm²) to minimize animal suffering and distress. The method of euthanasia was cervical dislocation or exsanguination under unconscious anesthesia. All procedures were approved by the Committee for Ethics of Animal Experiments, Kurume University (approval No. 050-058, March 30th, 2016), and animal care was provided in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication No. 86-23).

The experimental procedure, shown in Figure 1, has been described previously [3, 22]. B6 IL-38-KO mice and control B6 WT mice (4 to 18 mice per group, see the figure legends) were treated twice with an intraperitoneal injection of 10 μg sterile chicken ovalbumin (OVA, grade V, Sigma–Aldrich Chemical, St. Louis, MO) emulsified with 4 mg of sterile aluminum hydroxide (Alu-Gel-S Suspension, Serva Electrophoresis GmbH, Heidelberg, Germany) in a total volume of 200 μL. The injections were given at day 0 and 5. These mice were challenged for 20 min with 0.9% saline (vehicle) or 5% OVA in 0.9% saline, given via the airways by an ultrasonic nebulizer (Omron NE-U07, Tokyo, Japan) in a closed box. Lung tissues and lung tissue extracts were obtained 4, 8 and 24 h after OVA challenge (inhalation) or control saline for examination of mRNA expression. Mouse lung tissues at 24 h after OVA challenge were obtained. Sampling of bronchoalveolar lavage fluid (BALF) and determination of airway hyperresponsiveness (AHR) were undertaken at 24 h after OVA challenge (Figure 1A). To investigate the neutralizing effects of IL-38 in vivo, the rm IL-38 protein (5 μg/body) was additionally injected once intraperitoneally before OVA challenge on day 18 (female, 6-8 wk, n= 9 to 10) (Figure 1B). Three mice died prior to euthanasia due to fighting.

Histological analysis

For the histological analysis, mice were euthanized by intraperitoneal injection of pentobarbital sodium (2.5–5 mg per mouse). After the thorax had been opened, the trachea was dissected free from the underlying soft tissues, and a 0.8-mm tube was inserted through a small incision in the trachea. The lung tissues were immediately fixed by intratracheal instillation of 10% buffered formalin (pH 7.40) for 15–20 min at a constant pressure of 25 cm H₂O. After gross examination, the extracted tissues were placed into 10% buffered formalin and further fixed for at least 24 h. Fixed lung tissues were prepared with hematoxylin...
and eosin (HE) and direct fast scarlet 4BS (DFS) for eosinophil staining [23,24]. The number of airway inflammatory cells was counted by two independent investigators in a blinded manner using a digitalized microscope system (BZ-9000, BioRevo, Keyence Japan, Tokyo) and the average counts per person were used as data.

**Messenger ribonucleic acid (mRNA) expression determined by quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR)**

Mice were euthanatized by intraperitoneal injection of pentobarbital sodium (2.5–5 mg per mouse). Lung tissues were obtained 4, 8, or 24 h after challenge, homogenized immediately (Polytron PT2100, Kinematica AG, Littau, Switzerland), and then total RNA was purified from the homogenate with Trizol reagent (Invitrogen Life Technologies Japan, Tokyo, Japan). Total RNA was converted to cDNA using a commercial kit (Quantitect reverse transcription kit, Qiagen, Hilden, Germany) as described previously [25]. Real-time quantitative PCR (qPCR) with intercalation (SYBR green master mix, Qiagen) of the mouse IL-5, IL-13, IL-17, IL-38, and β-actin genes was performed using a thermal cycler (Mx3000p PCR machine, Stratagene, La Jolla, CA). Each specific primer set was purchased from Qiagen Co. (Hilden, Germany). Expression of the mouse IL-5, IL-13, IL-17A and IL-38 genes relative to that of the β-actin gene was calculated using the comparative threshold cycle method, as described previously [13,25].

**Analysis of BALF obtained from mice**

Total cell counts (TCCs) were calculated using a hemocytometer after trypan blue staining. The differential cell counts were obtained from cytospin prepa-

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**Fig. 1.** Design of the mouse allergen airway inflammation model and the characteristics of the purified recombinant mouse IL-38 protein.

A) WT (n=18) and IL-38-KO (n=16 to 18) mice were sensitized by intraperitoneal injection of a mixture with 10 μg sterile chicken ovalbumin (Sigma-Aldrich Chemical, St. Louis, MO) and 4 mg of emulsified sterile aluminum hydroxide (Heidelberg, Germany) (OVA) in a total volume of 200 μL, on days 0 and 5, and continuously challenged by inhalation of 0.9% saline (OVA/saline) or 5% OVA (OVA/OVA) via an ultrasonic nebulizer for 20 minutes on day 18. The sampling was performed on day 19.

B) Comparison of airway inflammation among WT, untreated IL-38-KO and IL-38-KO mice treated with a single intraperitoneal injection of rmIL-38 protein (5 μg/body) (n=9 to 10 per each group).

AHR=airway hyperresponsiveness, IL-38-KO=IL-38 knockout, OVA=ovalbumin, WT=wild type.
rations, as reported previously [3, 22]. The supernatants of BALF and sera were kept at −30°C for measurements.

Airway responsiveness after OVA or saline challenge in WT- and IL-38 KO mice

AHR to aerosolized acetylcholine (ACH, Sigma-Aldrich Japan, Tokyo, Japan) was tested 24 h after OVA or saline challenge, as described previously [3]. Briefly, under mechanical ventilation (150 breaths/min, tidal volume 10 mL/kg, and positive end-expiratory pressure 2 cmH2O) (Buxco FinePointe RC; Data Science International, MN) after anesthetization and intratracheal intubation, mean airway resistance of mice was measured automatically after inhalation of 0.9% saline at the baseline followed by increasing doses of aerosolized ACH (doubling doses from 0 to 160 mg/mL in 20 μL) via a nebulizer (inhalation for 30 s and response for 3 min). The provocative concentration 200 (PC200) for ACH was calculated when a 200% increase in airway resistance from the baseline was observed [3].

Establishment and characteristics of recombinant mouse IL-38 protein and monoclonal antibody to IL-38 protein

A purified rm IL-38 protein was established in our laboratory. Briefly, full-length mouse IL-38 cDNA with a 6 × His tag at the C-terminal, converted to a Spodoptera frugiperda (SF)-type codon, was synthesized using the GenBank sequence (AY071844.1) (Figure 2A). XbaI and Bgl II were used as restriction enzymes (Figure 2B). The synthesized mouse IL-38 cDNA was subcloned into a plasmid vector (pPSC8, Protein Sciences Co., CT, USA) from the BMH expression vector (Biomatik, Wako Pure Chemical Industries, Osaka, Japan) and designed as pPSC8/mouse IL-38 (Figure 2B). To investigate the characteristics of mouse IL-38 protein, its synthesized cDNA was subcloned into the pEF1α-IRES-AcGFP1 vector (Clontech Laboratories, Inc., Mountain View, CA, USA) (Figure 2C). The pEF1α-IRES/mouse IL-38 plasmid was transfected into P815 cells (a murine myeloma cell line) using a Gene Pulser II (Bio-Rad, Hercules, CA). Mouse IL-38 protein was detected from the supernatant of lysed mouse IL-38 cDNA-transfected P815 cells using Western blotting. We established rat anti-mouse IL-38 monoclonal antibodies (mAb) (clone 4A, 36A, and 41A) using the rm IL-38 protein, as reported previously [26]. These anti-mouse IL-38 mAbs were applicable for detection of mouse IL-38 protein by Western blotting and enzyme-linked immunosorbent assay (ELISA) (Figure 2D), but not for detection of specific mouse IL-38 protein in paraffin-embedded mouse tissues (data not shown). To investigate the biological kinetics of the purified rm IL-38 protein in vivo, the serum levels of mouse IL-38 protein were measured in WT mice (female, 6-8 wk, n= 6) by ELISA before, and at 10 and 30 min, and 1, 3, 6 and 24 h, after intraperitoneal injection of 5 μg of the rm IL-38 protein (Figure 2E).

Measurement of cytokine levels by ELISA system

A mouse IL-38 protein was detected by the ELISA system we developed with rat mAbs (clone 36A as the first antibody and clone 4A as the second antibody) (Figure 2D). The levels of mouse IL-5, IL-13, and IL-17A protein were measured using commercial ELISA kits (eBioscience, San Diego, CA, USA). The lower limit of detection for IL-38, IL-5, IL-13 and IL-17A by measurement of absorbance at 450 nm was 3.125 ng/mL, 4 pg/mL, 4 pg/mL, and 4 pg/mL respectively. Half the value of the lower limit of detection for each biomarker was used for statistical analysis when the value was not detectable.

Statistical analysis

All data were expressed as means ± standard error of the mean (SEM). Differences between two, and three or more groups, were compared using unpaired Student’s t test with the exact Wilcoxon test, Fisher’s exact test, and analysis of variance (ANOVA) with the Tukey Honestly Significant Difference Test or Wilcoxon rank test, respectively. Spearman correlation coefficient (r) was used to evaluate correlations between quantitative variables. Differences at P<0.05 were considered to be statistically significant. Histological analyses conducted by two investigators were evaluated statistically using the Inter-Rater Reliability Coefficient. JMP 12.2.0 (SAS Institute Japan, Tokyo, Japan) was used for statistical analysis.

RESULTS

Characteristics and biological kinetics of recombinant mouse IL-38 protein in vitro and in vivo

Western blotting systems (Figure 3A) detected IL-38 protein from supernatant of cell lysate of mouse IL-38 cDNA-transfected P815 cells (Lane 3 and 4). IL-38 protein was not detected from cell lysate supernatant of P815 cells (Lane 5) or supernatants of mouse IL-38 cDNA transfected P815 cells (Lane 6 and 7). However, the IL-38 protein levels from the same samples of Lanes 6 and 7 were measured by ELISA sys-
Fig. 2. Establishment and characteristics of recombinant mouse IL-38 protein.
A) A full-length mouse IL-38 cDNA.
B) A synthesized mouse IL-38 cDNA in vector. The rm IL-38 protein was purified from SF9 and expressed in SF+ cells (Wako Pure Chemical Industries, Osaka, Japan) co-transfected with baculovirus AcNPV (Protein Sciences Corporation, Meriden, CT, USA) and pPSC8/mouse IL-38 using affinity chromatography.
C) The pEF1α-IRES/mouse IL-38.
D) Establishment of mouse IL-38 sandwich ELISA system. Color development was performed with streptavidin-bound horseradish peroxidase (Millipore, Tokyo, Japan) and a color development substrate solution (ELISA POD Substrate TMB kit, Nakarai Tesque, Kyoto, Japan) by measurement of absorbance at 450 nm. Doubling concentrations (3.125 to 200 ng/mL) of the purified recombinant mouse IL-38 protein were used as standard controls.
E) Biological kinetics of the additional purified rm IL-38 protein in vivo (WT mice, female, 6-8 wk, n= 6).
tem (29.5 ng/mL and 62.4 ng/mL, respectively). Taken together, the data indicated that mouse IL-38 protein is soluble, despite the fact that the IL-38 gene does not have a signal peptide in vitro.

The serum levels (mean ± SEM) of mouse IL-38 protein were below the lower limit of detection before injection, and the peak serum level of mouse IL-38 protein (311 ± 42 ng/mL) was observed at 10 min after injection. No IL-38 protein was detectable in mouse serum at 6 h after injection. The mean (± SEM) half-life (tR1/2R) of mouse IL-38 protein in serum after injection was 64.7 (± 11.9) min (Figure 3B).

**Mouse model study**

Levels of mRNA for IL-5, IL-13 and IL-17A in the lungs of WT mice were maximal at 8 h after OVA challenge, and higher than baseline at 4 h and 24 h (data not shown). Therefore, we examined mRNA expression in the lungs of mice at 8 h after OVA challenge. The mean level of mRNA for IL-38 (normalized against β-actin ± SEM) in the lungs of WT mice was significantly (p = 0.0348) higher at 8 h after OVA challenge than after saline challenge (Figure 4A). Levels of mRNA for IL-5, IL-13 and IL-17A in WT mice after OVA challenge were significantly higher (p
than those after saline challenge (Figure 4B). In contrast, the expression of mRNA for IL-5 and IL-13, but not IL-17A, in IL-38-KO mice was significantly (p = 0.0340, p = 0.0049, and p = 0.0166, respectively) lower than that in WT mice after OVA challenge.

Pathological examination revealed that the number of eosinophils after OVA challenge (mean ± SEM, cells/bronchus) in IL-38-KO mice (0.8 ± 0.4, p = 0.0313) was significantly lower than in WT mice (3.1 ± 1.3) at 24 hours after OVA challenge, and higher than that after saline challenge in both IL-38-KO (0.0 ± 0.0) and WT (0.0 ± 0.0) mice (Figure 5A).

BALF analysis showed that the number (mean ± SEM, ×10⁴ cells/mL) of eosinophils (4.7 ± 1.4) in IL-38-KO mice was significantly (p = 0.0090) lower than that in WT mice (15.2 ± 3.6) 24 h after OVA challenge (Figure 5B). However, there was no significant difference in TCCs, lymphocytes, neutrophils, and macrophages between WT and IL-38-KO mice after

![Image of pathological examination and BALF analysis](image-url)

**Fig. 5.** Attenuation of airway inflammation in IL-38 KO mice at 24 h after OVA challenge and the effects of additional recombinant mouse IL-38 protein on IL-38-KO mouse models. 
A) Pathological examination of HE- and DFS-stained lung tissues from WT (d, e, f) and IL-38-KO mice (j, k, l) after OVA challenge revealed focal eosinophilic inflammation predominantly in peribronchial areas when compared with WT (a, b, c) and IL-38-KO (g, h, i) mice after saline challenge. Quantitative analysis after DFS staining was performed to determine the number of airway eosinophils (inter-rater reliability coefficient = 0.97) (n=5 per each group). Bars=100 μm (×200) and 50 μm (×400).
B) Total and differential cell counts in BALF (n=16 to 18 per each group).
C) Protein levels in BALF supernatants determined by ELISA (n=16 to 18 per each group).
D) Correlation between the number of eosinophils and levels of IL-5 and IL-13 protein in BALF.
E) AHR on ACh (n=4 to 6 per each group).
F) Total and differential cell counts in BALF among WT, untreated IL-38-KO and treated IL-38-KO mice (n=16 to 18 per each group).
G) Protein levels in BALF supernatants determined by ELISA among WT, untreated IL-38-KO and treated IL-38-KO mice (n=16 to 18 per each group).

* p<0.05, ** p<0.01, and *** p<0.0001 versus saline challenge

p<0.05 and ** p<0.01 versus WT mice

AHR=airway hyperresponsiveness, DFS=direct fast scarlet 4BS, IL-38 KO=IL-38-knockout, ND=not detected, NS=not significant, OVA=ovalbumin, WT=wild type
Levels of IL-5 (p = 0.0278) and IL-13 (p = 0.0396) protein in the BALF of IL-38-KO mice were significantly lower than those in WT mice after OVA challenge (Figure 5C). Levels of IL-5 protein in WT (p < 0.0001) and IL-38-KO mice (p < 0.0001) and IL-13 protein in WT (p = 0.0396), but not those in IL-38-KO mice (p = 0.1388), were significantly higher after OVA challenge than after saline challenge. However, no IL-38 or IL-17A protein was detected in the BALF of WT and IL-38-KO mice. There was a significant positive correlation between the number of eosinophils and the level of IL-5 protein (r = 0.92, p < 0.0001), but not IL-13 protein (r = 0.30, p = 0.0515) in BALF from WT and IL-38-KO mice after OVA challenge (Figure 5D). Next, we examined the AHR. However, a 200% increase in airway resistance from the baseline was not significant between IL-38-KO and WT mice. Moreover, a previous study supporting our results found that the level of IL-38 protein was increased in children with asthma, and that this was negatively associated with the percentage of peripheral regulatory T cells [19]. In a fungal infection model, IL-38 attenuated neutrophilic inflammation via IL-17 suppression [12,13].

To our knowledge, our study is the first to report a correlation between airway eosinophilic inflammation and IL-38 production in asthma. We found that airway eosinophilic inflammation in IL-38-KO mice was significantly attenuated as compared with WT mice after OVA challenge, based on both pathological and BALF analyses. This mouse model study showed that expression of both IL-5 and IL-13 mRNA (lung tissues extracts) and protein (BALF supernatant) in IL-38-KO mice was significantly lower than that in WT mice after OVA challenge. There was a significantly positive correlation between the absolute number of eosinophils and the levels of IL-5 protein, but not IL-13 protein, in BALF of both WT and IL-38-KO mice. However, we could not confirm any correlation between airway eosinophilic inflammation and IL-38-positive cells because the immunostaining of fixed mouse lung tissue with our established anti-mouse IL-38 mAb was unsuccessful. We investigated whether anti-mouse IL-38 polyclonal antibody could alter airway eosinophilic inflammation in vivo in this murine model. The number of eosinophils did not change in the presence or absence of antibody administration in WT mice after OVA challenge (data not shown). We were unable to examine whether our established anti-mouse IL-38 polyclonal and monoclonal antibodies had neutralizing activity because our purified rm IL-38 protein had no anti- or pro-inflammatory effect in vitro. One reason may have been that the mouse IL-38 protein was in soluble form, as we have reported previously [13]. We also investigated whether treatment with rm IL-38 protein could alter airway eosinophilic inflammation in vivo in this IL-38-KO murine model. Enhancement of airway eosinophilic inflammation was evident in IL-38-KO mice treated with rm IL-38 protein, but not to a significant degree in comparison with untreated IL-38-KO mice. The protocol involved a single intraperitoneal injection of rm IL-38 protein before OVA challenge. We found that the half-life of soluble form in purified rm IL-38 protein was short, at around 65 min after injection in vivo (Figure 3B).

A previous study supporting our results found that the level of IL-38 protein was increased in children with asthma, and that this was negatively associated with the percentage of peripheral regulatory T cells [19]. In a fungal infection model, IL-38 attenuated neutrophilic inflammation via IL-17 suppression [12,13].

Our study had some limitations. First, an IL-38 transgenic murine model and the neutralizing mAb to IL-38 protein have not been prepared yet. Secondary, IL-38 protein could not be detected in some specimens by our ELISA system. Our ELISA system had a 3.1 ng/mL lower limit of detection for mouse IL-38 protein. Therefore, development of a new ELISA system with higher sensitivity will be necessary. Third, we could not obtain the predetermined threshold (PC200) in B6 background IL-38-KO and WT mice. There was no significant increase in airway hyperresponsiveness of B6 IL-38-KO and WT mice, as reported previously [28,29].

**CONCLUSION**

Our present findings suggest that IL-38 may contribute to airway eosinophilic inflammation in allergic diseases. We hope that our data will lead to a better un-
derstanding of the biological roles of IL-38.

CONFLICT OF INTEREST: The authors have no conflicts of interest to declare.

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