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## Original Article

## Decreased expression of airway epithelial Axl is associated with eosinophilic inflammation in severe asthma

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## Abbreviations:

BAL, bronchoalveolar lavage; GM-CSF, granulocyte macrophage colony-stimulating factor; HBEC, human bronchial epithelial cells; HDM, house dust mite extracts; hr, human recombinant; IL, interleukin; LPS, lipopolysaccharide; SARP, Severe asthma research program; Th, T helper; TSLP, thymic stromal lymphopoietin; U-BIOPRED, Unbiased BIOMarkers in Prediction of REspiratory Disease outcomes

## ABSTRACT

**Background:** Airway epithelium-derived cytokines are critical to provoke and perpetuate type 2 inflammation in asthma. Yet it is poorly understood how this epithelial cell-driven inflammatory response is negatively regulated. We previously reported that Axl receptor tyrosine kinase was expressed by basal cells in the airway epithelium and had a role in defining their stem cell identity. However, whether and how Axl regulates airway type 2 inflammation remains unknown.

**Methods:** We performed immunofluorescence staining to compare Axl expression in airway epithelium between non-asthmatic subjects, mild-moderate asthma and severe asthma. We confirmed this result by interrogating public databases of global gene expression in endobronchial biopsies. We then quantified eosinophil numbers infiltrating into the trachea of wild-type or Axl-knockout mice that were intranasally treated with house dust mite extracts (HDM). Cell-based assays using siRNA targeting Axl were further performed to identify molecules involved in Axl-mediated regulation of inflammation.

**Results:** Histological assessments and transcriptome analyses revealed decreases in protein and mRNA of Axl in airway basal cells of severe asthmatics. This reduction of Axl expression was correlated with infiltration of eosinophils and mast cells in severe asthmatics. Eosinophil infiltration was more evident in the trachea of Axl-knockout mice in response to repetitive HDM administration. siRNA-mediated knockdown of Axl increased mRNA and protein expression of granulocyte macrophage-colony stimulating factor (GM-CSF) in human bronchial epithelial cells.

**Conclusions:** Axl kinase expressed by basal cells may suppress excessive eosinophilic inflammation via inhibition of GM-CSF in the airway. Axl reduction has clinical implications for the pathogenesis of severe asthma.

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## Introduction

Epithelial cells cover the surface of the airways, sense inhaled allergens and pathogens via pattern recognition receptors and orchestrate prompt and adequate activation of the immune system for pulmonary host defence.<sup>1</sup> Growing evidence indicates that asthmatic patients are defective in airway epithelial barrier function.<sup>2</sup> The dysfunctional epithelial cells produce excessive amounts of interleukin (IL)-33,<sup>3–5</sup> thymic stromal lymphopoietin (TSLP)<sup>6,7</sup>

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and granulocyte macrophage colony-stimulating factor (GM-CSF)<sup>8</sup> that activate and perpetuate type 2 inflammation leading to airway wall remodelling in asthma.<sup>9</sup> Although a number of type 2 immunity-associated signaling molecules that are triggered by airway epithelial cells have been discovered, little is known about how the epithelium-driven type 2 inflammatory responses are negatively regulated.

Tyros3, Axl and Mertk are members of the TAM receptor tyrosine kinase family and have an essential role in the resolution of inflammation. TAM receptors promote efferocytosis in macrophages and inhibit Toll-like receptor signaling in dendritic cells<sup>10–13</sup> by indirectly recognising phosphatidylserine on apoptotic cells via their ligands Gas6 and Protein S.<sup>14</sup> TAM receptors have distinctive functions in tissue homeostasis and negative regulation of inflammation: Mertk acts as a tolerogenic receptor at homeostasis and during immunosuppression; Axl is induced by type 1 inflammatory stimuli and is important for resolution of acute inflammation triggered by influenza A virus infection; Tyro 3 in dendritic cells is critical for negative regulation of type 2 immunity for allergy or parasite infection.<sup>15–18</sup>

Airway basal cells act as stem cells in airway epithelium of human and mouse.<sup>19–23</sup> In addition to the stem cell activity, a pro-inflammatory role of basal cells in triggering type 2 inflammation by producing IL-33 and TSLP is beginning to be unravelled.<sup>24,25</sup> We have recently reported that Axl, but not Mertk or Tyro3, was selectively expressed by basal cells in human and mouse airways and that Axl defined the stem cell identity of basal cells by driving cell cycle re-entry and inducing symmetric cell division required for the proper expansion of epithelial cells.<sup>26</sup> We also previously demonstrated that Axl expression was diminished in airway macrophages of asthmatics, which was associated with reduced efferocytosis of macrophages in asthma.<sup>27</sup> However, it is not known whether and how Axl in basal cells contributes to type 2 inflammatory responses in airways. Here we found the reduction of Axl protein in bronchial basal cells in severe asthma, which was negatively correlated with the number of eosinophils and mast cells infiltrating into the mucosa. These results are underpinned by a murine model indicating that Axl, which was expressed by basal cells, inhibits house dust mite extracts (HDM)-induced eosinophilic inflammation in the murine trachea. Mechanistically, *in vitro* assays using primary human bronchial epithelial cells and its cell line revealed that Axl kinase suppressed GM-CSF production upon HDM stimulation.

## Methods

### Patient population

The use of human bronchial biopsy specimens was approved by the Ethics Committee at the Tohoku University School of Medicine (approval number: 2021-1-556) and the Sagami National Hospital (approval number: 2017-031). Endobronchial biopsy samples were examined from seven non-asthmatic subjects, six patients with mild-moderate asthma and ten patients with severe asthma. Subjects were recruited from Sagami National Hospital between June 2005 and April 2015. Non-asthmatic subjects had no history of respiratory symptoms and undertook endobronchial biopsies due to investigation of abnormal shadows on a chest X-ray, although the final pathological examination did not reveal any tumors. The diagnosis of asthma and classification of its severity met the Global Initiative for Asthma guidelines criteria. Experiments using primary human bronchial epithelial cells were approved by the Ethics Committee at the Tohoku University School of Medicine (approval number: 2019-1-353). Isolation and culture of human bronchial epithelial cells was followed by a previous

publication.<sup>28</sup> Lung tissues were provided by patients who underwent lung resection due to primary lung cancer. We verified that the tissues had no pathological findings of tumors or overt fibrosis. All patients provided informed consent. This study was performed in accordance with the Code of Ethics of the World Medical Association.

### Global gene expression analyses

Transcriptome of specimens from endobronchial epithelial brushing (Severe asthma research program, SARP; accession no. GSE63142) or from bronchial biopsies (Unbiased BIOMarkers in Prediction of REspiratory Disease outcomes (U-BIOPRED) Project; accession no. GSE76227) were analysed using GEO2R in the Gene Expression Omnibus at the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>).

### Immunofluorescence staining for human endobronchial biopsies and murine tracheal tissues

Immunofluorescence staining for human biopsies or for murine tracheal epithelium was performed as previously described.<sup>26</sup> Briefly endobronchial biopsy specimens were fixed with 10% formalin, embedded in paraffin and cut at a 3- $\mu$ m thickness. After deparaffinization, heat-induced antigen retrieval was performed with the antigen unmasking solution at pH9 (Nichirei Biosciences, Japan) at 121 °C for 5 min by autoclave. The sections were then blocked and incubated overnight at 4 °C with the following primary antibodies: goat anti-human Axl polyclonal antibody (#AF154, R&D; 2  $\mu$ g/mL) and rabbit anti-human/mouse Keratin5 polyclonal antibody (#PRB-160P, BioLegend; 1  $\mu$ g/mL). Secondary antibodies were used as follows: Alexa Fluor 647-donkey anti-goat antibody and TRITC-donkey anti-rabbit antibody (Jackson ImmunoResearch). Slides were mounted and nuclei were stained with Pro-Long Diamond antifade reagent with DAPI (ThermoFisher Scientific). Images were collected on a Nikon C2si confocal microscope system using a 20  $\times$  /0.75 Plan Apo or 60  $\times$  /1.40 Plan Apo  $\lambda$  oil objective through NIS-Elements C (Nikon) with Z spacing of 0.150  $\mu$ m. Images were processed and analysed using NIS-Elements AR (Nikon).

### Staining for eosinophils and mast cells in human bronchial tissues

Eosinophils and mast cells were visualised using Eosinophil-Mast Cell Stain Kit according to the manufacturer's instruction (ScyTek Laboratories). Nuclei were counterstained with haematoxylin. Images were obtained on an OLYMPUS BX53 microscope using a 20 $\times$ /0.50 or 40 $\times$ /0.75 UPlanFL N objective and captured using a DP71/u-TV1X-2 camera through a DP controller (Olympus).

### Mice

The generation of Axl-knockout mice has been described previously.<sup>29</sup> All Axl-knockout mice used in this study were backcrossed to C57BL/6J background for more than 10 generations. C57BL/6J wild-type mice were purchased from CLEA Japan (Tokyo, Japan). All experiments were approved by the Tohoku University Animal Experiment Ethics Committee. 8–10-week-old female mice were used for all *in vivo* experiments.

### House dust mite extracts-induced airway type 2 inflammation model

Mice were anesthetized with isoflurane and intranasally administered with house dust mite extracts (*Dermatophagoides*

*farinae*; ITEA, Tokyo, Japan) as previously described.<sup>30,31</sup> Collection of bronchoalveolar lavage (BAL) and dissection of trachea and lung tissues were performed, as described previously.<sup>16,26</sup>

#### Mouse cell isolation from tracheal and lung tissues and flow cytometry

Single cell suspensions were prepared from tracheas or lungs of naïve mice or HDM-treated mice by incubation with Collagenase A (Sigma–Aldrich) or Liberase TM (Roche) and DNase I (Sigma–Aldrich). After staining with Zombie UV Fixable Viability Kit (BioLegend) followed by Fc receptor blocking, cells were stained with the following cocktail of antibodies: PerCP/Cy5.5-anti Ly-6G (#127615 [1A8], BioLegend; 1 µg/mL), APC/Fire750-anti CD3 (#100247 [17A2], BioLegend; 2 µg/mL), APC/Fire750-anti NK1.1 (#108751 [PK136], BioLegend; 1 µg/mL), APC/Fire750-anti CD220 (#103259 [RA3-6B2], BioLegend; 1 µg/mL), Brilliant Violet 421-anti CD11c (#117329 [N418], BioLegend; 0.5 µg/mL), Brilliant Violet 510-anti CD45 (#103138 [30-F11], BioLegend; 0.5 µg/mL), Brilliant Violet 650-anti CD11b (#101239 [M1/70], BioLegend; 0.5 µg/mL), PE-anti Axl (#FAB8541P [175128], R&D; 10-fold dilution), PE/CF594-anti Siglec-F (#562757 [E50-2440], BD Biosciences; 1 µg/mL), PE/Cy5-anti MHC class II (#107611 [M5/114.15.2], BioLegend; 1 µg/mL). Cells were run on BD FACS Fortessa (BD Biosciences) with CountBright Absolute Counting Beads (ThermoFisher Scientific) and analysed using FlowJo (BD Life Sciences). Gating strategy was previously described.<sup>26</sup>

#### Cell culture

An immortalized human bronchial epithelial cell line, BEAS-2B cell, was maintained in BEGM Bronchial Epithelial Cell Growth Medium routinely containing hydrocortisone (Lonza). Isolation and culture of human bronchial epithelial cells were previously described.<sup>28</sup>

#### Immunofluorescence staining for human bronchial epithelial cells

For immunofluorescence staining for Keratin5 and P63, human bronchial epithelial cells were cultured on an eight-well chamber slide (ThermoFisher Scientific) coated with rat tail collagen I (Corning). Cells were fixed with 4% PFA for 30 min and then blocked and permeabilized with 10% rabbit serum (Dako) and 0.3% Triton X in PBS for 30 min. Cells were then stained for 24 h at 4 °C with rabbit anti-Keratin 5 (Biolegend, #905501, 1 µg/mL) or rabbit anti-P63 (Abcam, ab124762 [EPR5701], 3.3 µg/mL) followed by a secondary antibody conjugated with Alexa Fluor 647 (Life Technologies, 1:100) for 1 h. F-actin was visualized by Alexa Fluor 555-conjugated phalloidin. Cell nuclei were identified using ProLong Diamond antifade reagent with DAPI (Life Technologies).

#### Flow cytometry and ELISA for evaluation of Axl and Gas6

To evaluate whether Axl kinase was fully phosphorylated or if Axl on cells was totally occupied by endogenous Gas6, human recombinant (hr) Gas6 (R&D Systems) at 100 ng/mL or 500 ng/mL of final concentration was added to culture 15 min before cell collection. Prior to the exogenous supplementation of hrGas6, we did not replace cell culture medium because small amounts of apoptotic cells in culture was essential and enough for activation of Axl kinase *in vitro*.<sup>32</sup> To determine the presence of Axl and Gas6 by flow cytometry, cells were harvested using 1 mM or 5 mM EDTA/PBS for 10 min at 37 °C. Here we did not use trypsin to collect cells as protease could cleave epitopes recognised by the following antibodies. After staining with LIVE/DEAD Fixable Near-IR Dead Cell

Stain Kit (ThermoFisher Scientific) followed by Fc receptor blocking, cells were stained with PE-anti human Axl (#FAB154P, R&D Systems; 10-fold dilution), APC-anti human Axl (#FAB154A, R&D Systems; 10-fold dilution) or biotinylated goat anti human Gas6 (#BAF885, R&D Systems; 2 µg/mL). Streptavidin-PE (ThermoFisher Scientific) was used for detection of biotinylated anti Gas6. Cells were run on BD FACS Fortessa (BD Biosciences) and analysed using FlowJo (BD Life Sciences). For inhibition of Axl kinase activity, BGB 324 (Selleckchem), a selective Axl kinase inhibitor,<sup>33</sup> was used. The amounts of phosphorylated Axl and total Axl in cell lysates were determined as previously described.<sup>34</sup>

#### siRNA transfection

siRNA-mediated knockdown of Axl was performed as previously described.<sup>34</sup> Briefly Silencer Select siRNAs and lipofectamine RNAiMAX were used to introduce specific single siRNAs (ThermoFisher Scientific; Axl-siRNA1 (#s1845), Axl-siRNA2 (#s1846) and non-target-siRNA (Silencer Select Negative control #1)). Knockdown efficacy with different concentrations of siRNA and lipofectamine was evaluated by qRT-PCR and ELISA and we employed 2.5 nM siRNA and 0.1% lipofectamine solution in this study.

#### Stimulation of BEAS-2B cells and primary human bronchial epithelial cells with pro-inflammatory insults

Three days after the siRNA transfection, cells were stimulated with HDM (ITEA; 0.4, 4, 40 µg/mL as indicated in Fig. 6), human recombinant IL-4 + human recombinant IL-13 (R&D; 30 ng/mL in each) or human recombinant tumour necrosis factor (TNF)-α (R&D; 10 ng/mL) for 6 h (qRT-PCR) or 24 h (ELISA).

#### Quantitative real-time PCR

Total RNA extraction, cDNA synthesis and quantitative real-time PCR were performed as previously described.<sup>26</sup> Primers were purchased from TAKARA BIO (ACTB, HA067803; AXL, HA268303; GAS6, HA032830; CSF2, HA126039; IL33, HA299316) or were synthesized according to sequences registered in public resources<sup>35–38</sup> (PrimerBank ID: CCL5, 22538813c1; CCL24, 22165426c1; CXCL8, 10834978a1. RTPrimerDB ID: RPLP0, 2507; CCL11, 4000; CXCL1, 280). Real-time PCR was conducted using the StepOnePlus Real-Time PCR system (ThermoFisher Scientific). The relative expression levels of the specific mRNAs were calculated by the  $2^{-\Delta\Delta Ct}$  method. RPLP0 was used as an endogenous control.

#### ELISA for GM-CSF

The concentration of GM-CSF in culture supernatants was determined by ELISA according to manufacturer's instruction (Human GM-CSF Quantikine ELISA Kit, #DGM00, R&D systems).

#### Statistics

All data are expressed as medians (with interquartile ranges) unless otherwise indicated. Statistical tests were performed using GraphPad Prism 8 (GraphPad Software, CA, USA). Difference in the distribution of the genders were analysed using the Chi square test. For comparison between three or two groups, the Kruskal–Wallis test followed by the Dunn's multiple comparison test or the Mann–Whitney test was performed respectively unless otherwise indicated. Statistical correlation analyses were performed using Pearson's test. P-values less than 0.05 were considered significant.

## Results

### *A decrease in the expression of Axl kinase in the bronchial epithelium from patients with severe asthma*

As we sought to determine whether and how Axl kinase in airway basal cells regulated type 2 inflammatory responses during the pathogenesis of asthma, we first assessed Axl protein expression in airway epithelium of endobronchial biopsies from non-asthmatic subjects and patients with mild-moderate asthma or severe asthma. The patients' characteristics were shown in Table 1. Patients with severe asthma were treated with high-dose inhaled corticosteroids but not regularly with oral corticosteroids (Table 1). Immunofluorescence staining revealed that Axl protein was present predominantly in basal cells marked as Keratin5-expressing cells in bronchial epithelium of non-asthmatic subjects and patients with mild-moderate asthma (Fig. 1a). Whereas the Axl protein expression in Keratin5-positive basal cells was reduced in patients with severe asthma (Fig. 1a). The percentage of Axl<sup>+</sup>Keratin5<sup>+</sup> cells to total Keratin5<sup>+</sup> cells was significantly decreased in severe asthma (81.3% [67.6–90.2], the median with 5–95 percentile) compared to that in non-asthma (93.6% [88.9–97.5];  $P = 0.0023$ ) and in mild-moderate asthma (92.2% [91.7–94.8];  $P = 0.0048$ ; Fig. 1b).

To validate our histological assessment of the Axl reduction in airway epithelium of severe asthma, we interrogated global gene expression datasets of bronchial epithelial specimens in two independent severe asthma cohorts (SARP (NCBI gene expression omnibus accession GSE63142<sup>39</sup>) and U-BIOPRED (accession GSE76227<sup>40</sup>)). We found that Axl mRNA expression in the bronchial epithelium was significantly decreased in severe asthma compared to non-asthmatic subjects ( $P = 0.035$ ) and mild-moderate asthma ( $P = 0.011$ ) in the SARP cohort (Fig. 1c). The decrease in Axl expression in severe asthma was reproducible in the U-BIOPRED cohort, although the transcriptome of non-asthmatic subjects was not available in the cohort ( $P = 0.0239$ ; Fig. 1c). No alteration of mRNA expression was observed for other TAM receptors (Mertk,

Tyro3) and their ligand Gas6 in SARP and U-BIOPRED cohorts (Supplementary Fig. 1). Protein S, a ligand for Tyro 3, was significantly decreased in severe asthma compared to non-asthma and mild-moderate asthma, however, this was not reproducible in U-BIOPRED cohort (Supplementary Fig. 1).

### *Negative correlation between Axl-positive basal cells and eosinophils or mast cells infiltrating into bronchial mucosa in asthmatic patients*

To determine whether a decrease in the expression of Axl protein expression in bronchial basal cells was associated with persistent type 2 inflammation which was a main component of inflammatory phenotypes of severe asthma,<sup>41</sup> we evaluated the number of eosinophils and mast cells as effector cells during type 2 inflammation in the endobronchial biopsy specimens. To determine the location of these cells under a light microscope, we used Vital New Red solution to specifically detect eosinophils with granular red cytoplasmic staining and Astra Blue solution to visualise mast cells stained metachromatically with toluidine blue.<sup>42,43</sup> Infiltration of eosinophils and mast cells into bronchial submucosal tissues was observed and more evident in severe asthma than in mild-moderate asthma (Fig. 2a–c). There was a negative correlation between Axl-expressing basal cells and eosinophils or mast cells in bronchial mucosa from asthmatic patients (Fig. 2d, e). These data suggested that the decreased expression of Axl kinase in basal cells of bronchial epithelium might be associated with augmented type 2 inflammation in severe asthma.

### *Increased eosinophils in the trachea of Axl-knockout mice during an HDM-induced type 2 inflammation in vivo*

To confirm that Axl had a role in eosinophilic inflammation in a murine model, we repetitively instilled HDM into C57BL/6 wild-type mice or Axl-knockout (Axl<sup>−/−</sup>) mice. We first confirmed that HDM administration induced robust type 2 inflammatory

**Table 1**  
Patient characteristics in histological analyses.

|                                         | Non-asthma (n = 7) | Mild-moderate asthma (n = 6) | Severe asthma (n = 10) | P value |
|-----------------------------------------|--------------------|------------------------------|------------------------|---------|
| Men/women                               | 4/3                | 5/1                          | 6/4                    | 0.5518  |
| Age                                     | 64 (60–76)         | 41 (35–63)                   | 61 (31–71)             | 0.4195  |
| Body mass index (kg/m <sup>2</sup> )    | 22.7 (18.3–29.1)   | 24.6 (22.3–26.2)             | 24.4 (22.3–26.7)       | 0.6996  |
| No. of smokers (never/ex-smoke/current) | 3/4/0              | 2/4/0                        | 5/5/0                  | 0.6350  |
| Smoking history (pack-years)            | 25 (0–61)          | 5 (0–14)                     | 5 (0–23)               | 0.5183  |
| Symptom duration (year)                 | NA                 | 7 (1–18)                     | 11 (4–33)              | 0.2181  |
| Atopy, no. (%)                          | ND                 | 3 (50)                       | 7 (70)                 | 0.4237  |
| Atopic dermatitis, no. (%)              | ND                 | 4 (67)                       | 4 (40)                 | 0.3017  |
| Sinusitis, no. (%)                      | ND                 | 4 (67)                       | 3 (30)                 | 0.1523  |
| Blood examination                       |                    |                              |                        |         |
| Eosinophil count (/μL)                  | 70 (50–110)        | 255 (88–375)                 | 195 (105–410)          | 0.0874  |
| Total IgE (U/mL)                        | ND                 | 263 (45–2385)                | 134 (60–1581)          | 0.9311  |
| Pulmonary function test                 |                    |                              |                        |         |
| FEV <sub>1</sub> (% predicted)          | ND                 | 103.4 (84.3–113.6)           | 78.8 (57.2–100.9)      | 0.1106  |
| FVC (% predicted)                       | ND                 | 115.3 (99.6–128.7)           | 89.0 (74.2–116.0)      | 0.0992  |
| FEV <sub>1</sub> /FVC (%)               | ND                 | 80.0 (77.8–83.5)             | 72.5 (61.0–81.7)       | 0.2198  |
| PC20-Ach (μg/mL)                        | ND                 | 5430 (1162–20,000)           | 20,000 (1684–20,000)   | 0.6177  |
| Controller medications, no. (%)         |                    |                              |                        |         |
| ICS alone                               | NA                 | 2 (33)                       | 0 (0)                  | 0.0510  |
| ICS + LABA                              | NA                 | 4 (67)                       | 10 (100)               | 0.0510  |
| ICS (μg daily dose)                     | NA                 | 800 (700–800)                | 1600 (1520–1600)       | 0.0001  |
| LAMA                                    | NA                 | 3 (50)                       | 5 (50)                 | >0.9999 |
| LTRA                                    | NA                 | 4 (67)                       | 7 (70)                 | 0.8892  |
| OCS                                     | NA                 | 0 (0)                        | 0 (0)                  | >0.9999 |

Data are presented as the median (with interquartile ranges). Data were analysed with Chi-square test, Kruskal–Wallis test followed by Dunn's multiple comparison test or Mann–Whitney test.

FEV<sub>1</sub>, forced expiratory volume in 1 s; FVC, forced vital capacity; PC20-Ach, the provocation concentration of inhaled acetylcholine that causes a decrease in FEV<sub>1</sub> of 20%; ICS, inhaled corticosteroids (equivalent dose to budesonide); LABA, long-acting  $\beta_2$  agonists; LAMA, long-acting muscarinic antagonists; LTRA, leukotriene receptor antagonists; OCS, oral corticosteroid; NA, not applicable; ND, no data.

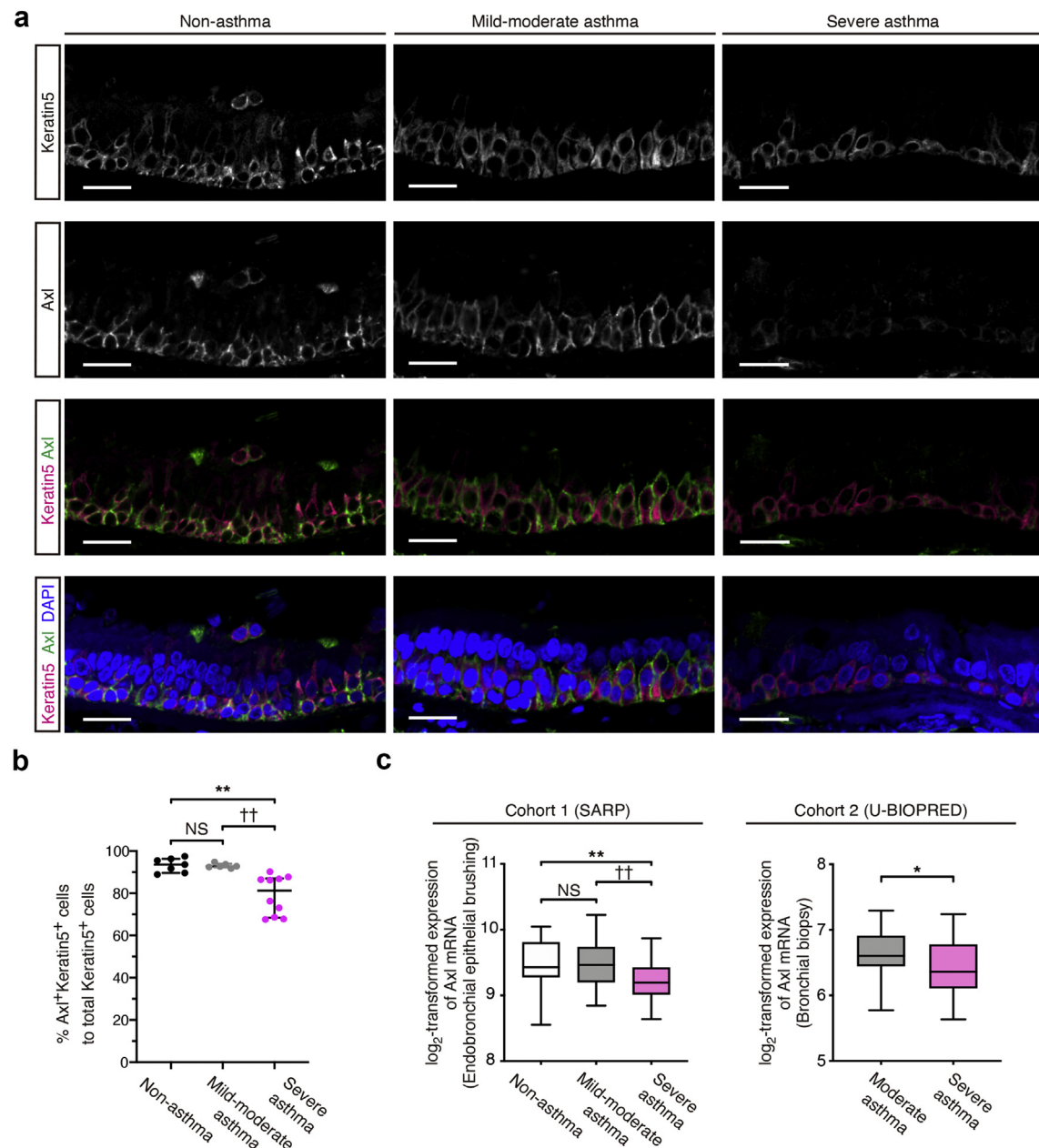


**Fig. 1.** Axl kinase is down-regulated in the airway epithelium of patients with severe asthma. **(a)** Representative immunostaining of human bronchial epithelium for Keratin5 (a basal cell marker) and Axl in non-asthmatic subjects ( $n = 7$ ), patients with mild-moderate asthma ( $n = 6$ ) and patients with severe asthma ( $n = 10$ ). Scale bars, 25  $\mu\text{m}$   $n$  indicates the number of patients. **(b)** Percent of Axl-expressing Keratin5<sup>+</sup> basal cells to total Keratin5<sup>+</sup> basal cells in non-asthmatic subjects ( $n = 7$ ), patients with mild-moderate asthma ( $n = 6$ ) and patients with severe asthma ( $n = 10$ ). Data represents the median with 5–95% percentile and statistical significance determined by the Kruskal–Wallis test followed by Dunn’s multiple comparison test. \*\* $P < 0.01$  compared with non-asthma; † $P < 0.01$  compared with mild-moderate asthma. N.S. indicates “not significant”. **(c)** Airway epithelial transcriptome of two severe asthma cohorts were interrogated for Axl expression. Data from Cohort 1 represent endobronchial epithelial brushings of non-asthmatic subjects ( $n = 27$ ), mild-moderate asthma patients ( $n = 72$ ) and severe asthma patients ( $n = 56$ ) from the Severe Asthma Research Program (SARP; accession: GSE63142). Data shown as a logarithmic scale, median with 5–95% percentile and statistical significance analyzed by the Kruskal–Wallis test followed by Dunn’s multiple comparison test. \*\* $P < 0.01$  compared with non-asthmatic; † $P < 0.01$  compared with mild-moderate asthma. N.S. indicates “not significant”. Data from Cohort 2 represent bronchial biopsies of moderate asthma patients ( $n = 35$ ) and severe asthma patients ( $n = 56$ ) in the Unbiased BIOMarkers in Prediction of REspiratory Disease outcomes (U-BIOPRED) Project (accession: GSE76227). Data shown as a logarithmic scale, median with 5–95% percentile and statistical significance analysed by the Mann–Whitney test. \* $P < 0.05$  compared with moderate asthma.

responses in C57BL/6 mice by showing an increase in the number of eosinophils in BAL fluid and an increase in the number of mucous-producing cells in bronchiolar epithelium (Supplementary Fig. 2).

Localization of basal cells in murine airways is anatomically distinct from that in human airways. In mice, basal cells are confined to the trachea and main bronchi (*i.e.* only extrapulmonary airways),<sup>22</sup> whereas these cells exist throughout airways from the trachea to bronchioles in human (*i.e.* both extrapulmonary and

intrapulmonary airways).<sup>44,45</sup> Thus, using the mouse HDM model, we here investigated eosinophilic inflammation in the trachea and in the lungs without main bronchi.

Because pro-inflammatory stimuli, such as lipopolysaccharide (LPS), are known to up-regulate Axl protein expression,<sup>15</sup> we asked whether cell-types that had not expressed Axl protein at homeostasis acquired its expression after HDM administration. Immunofluorescence staining indicated that Axl protein was still

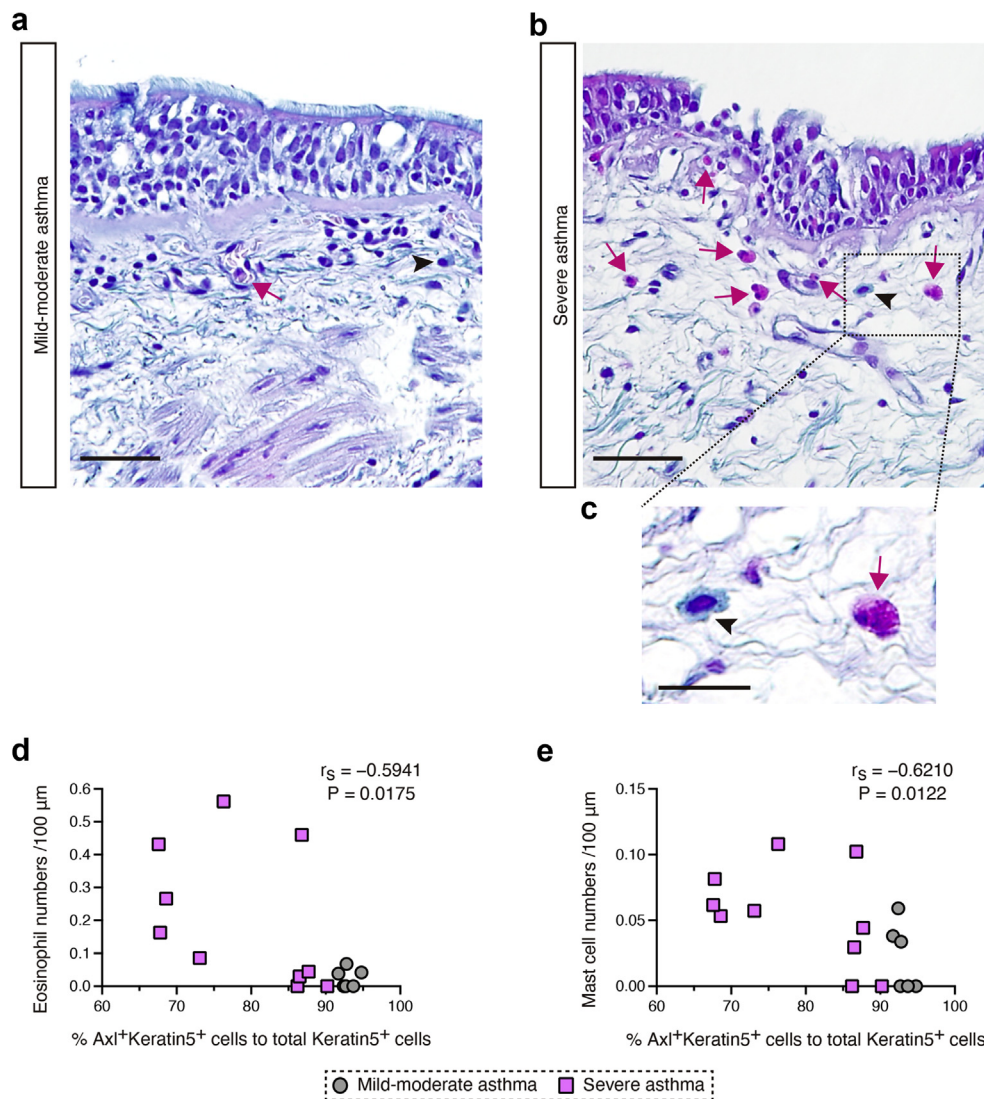


**Fig. 2.** Eosinophils and mast cells are more evident in severe asthma with lower expression of Axl kinase in airway basal cells. (a–c) Visualization of eosinophils (red arrows) and mast cells (black arrowheads) in bronchial biopsy specimens of patients with mild-moderate asthma (a) or severe asthma (b). (c) is a higher magnified image from (b). Scale bars, 25 μm (a and b) and 20 μm (c). Vital New Red solution enabled specific detection of eosinophils with granular red cytoplasmic staining and Astra Blue solution visualized mast cells that were stained metachromatically with blue. Nuclei were counterstained with haematoxylin. Representatives of six patients with mild-moderate asthma and of ten patients with severe asthma. (d–e) Correlation between the percentage of Axl<sup>+</sup> basal cells to total basal cells and the number of eosinophils (d) or mast cells (e) in the airway of patients with asthma. Grey circles or pink squares indicate mild-moderate asthma or severe asthma, respectively. The number of eosinophils and mast cells per 100 μm of basement membrane are shown.  $r_s$ , Spearman's correlation coefficient.

predominantly present in Keratin5-positive basal cells in HDM-treated mice (Fig. 3a). In addition, immune cells resident in mucosal tissues of the trachea including CD11c<sup>hi</sup>/MHC class II<sup>hi</sup> dendritic cells also did not express Axl protein at homeostasis or following HDM-induced inflammation (Fig. 3b). Taken together, repetitive HDM instillation did not alter cell-types expressing Axl protein in the murine trachea.

We then evaluated inflammatory responses to HDM in the trachea of wild-type or Axl<sup>-/-</sup> mice. At a steady state, there was no significant differences of submucosal inflammation or the numbers of eosinophils and neutrophils in the trachea between C57BL/6 wild-type mice and Axl<sup>-/-</sup> mice (Fig. 4a–d). After intranasal HDM administration, the number of eosinophils and neutrophils dramatically increased in lung tissues but not in the trachea of wild type mice (Fig. 4b–d and Supplementary Fig. 3), suggesting that the trachea exhibited less inflammatory responses to HDM than lung tissues. However, inflammatory cell infiltration in epithelial and

submucosal layers, oedema and angiogenesis in submucosal tissues of tracheas were augmented in Axl<sup>-/-</sup> mice treated with HDM (Fig. 4a). Furthermore, the number of eosinophils was significantly increased in the trachea of Axl<sup>-/-</sup> mice after HDM administration compared to wild-type mice (Fig. 4b, c). By contrast, there was no significant difference of neutrophil counts in the trachea after HDM instillation between wild-type mice and Axl<sup>-/-</sup> mice (Fig. 4b, d). Collectively these data suggest that Axl in epithelial basal cells inhibits the recruitment of eosinophils into tracheal tissues in response to HDM stimulation.

#### siRNA-mediated knockdown of Axl increases the expression of GM-CSF in human bronchial epithelial cells in vitro

To identify how Axl kinase regulates inflammation, we first used a human bronchial epithelial cell line (BEAS-2B cell) which was immortalized by SV40 from normal human bronchial epithelial

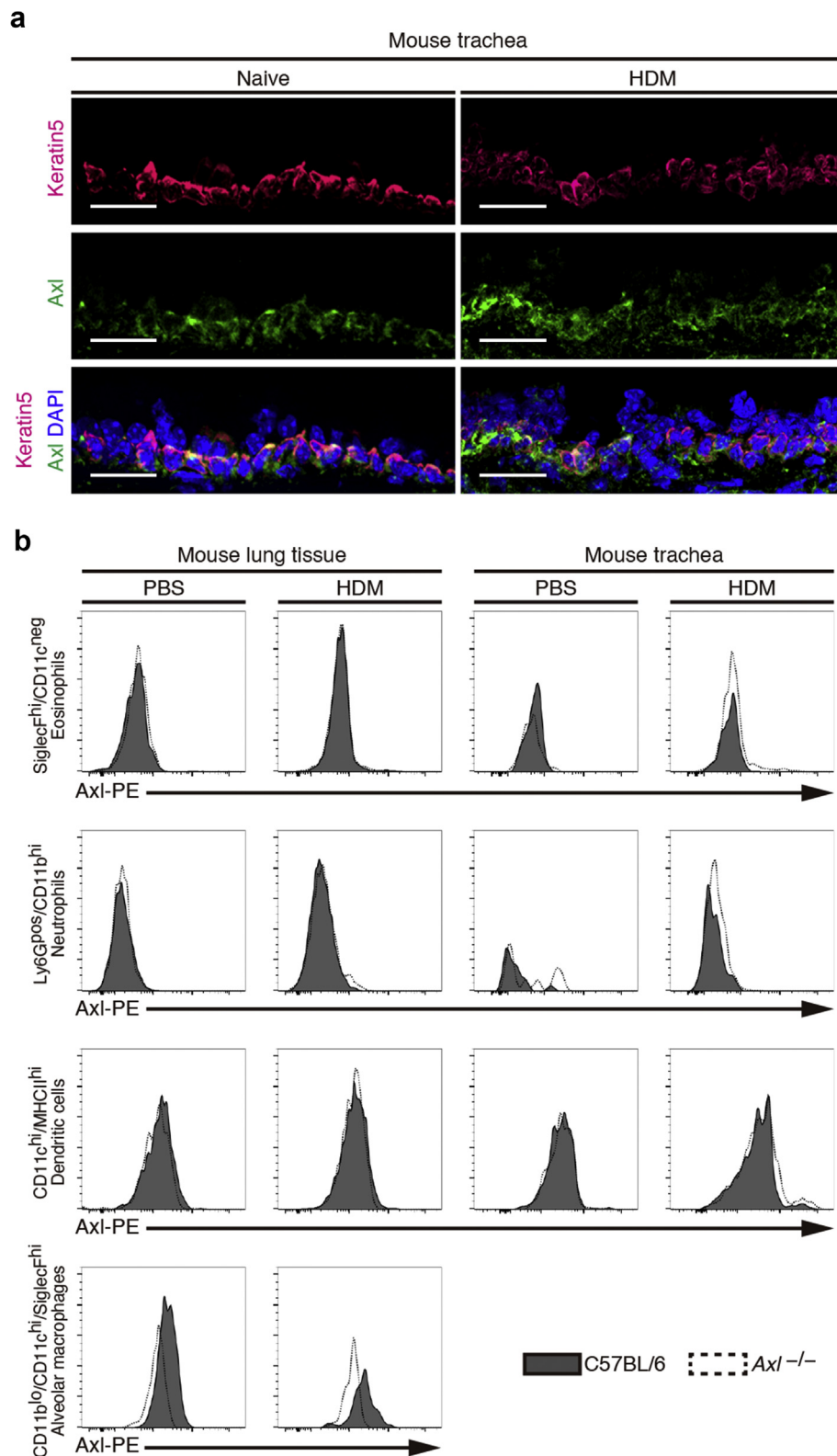


**Fig. 3.** Axl is predominantly expressed by airway basal cells in mice at steady state and after house dust mite extract administration. **(a)** Localization of Axl in murine tracheal epithelium in naive C57BL/6 mice and house-dust mite extracts (HDM)-treated C57BL/6 mice. Keratin5, magenta; Axl, green; DAPI, blue. Scale bars, 25  $\mu$ m. **(b)** Axl expression on eosinophils, neutrophils, dendritic cells and alveolar macrophages within lung tissues (left panels) or tracheas (right panels) from C57BL/6 or Axl-knockout mice that were treated with PBS or HDM. No alveolar macrophages were detected in the tracheas. C57BL/6, solid line/shaded; Axl-knockout, dotted line/open.

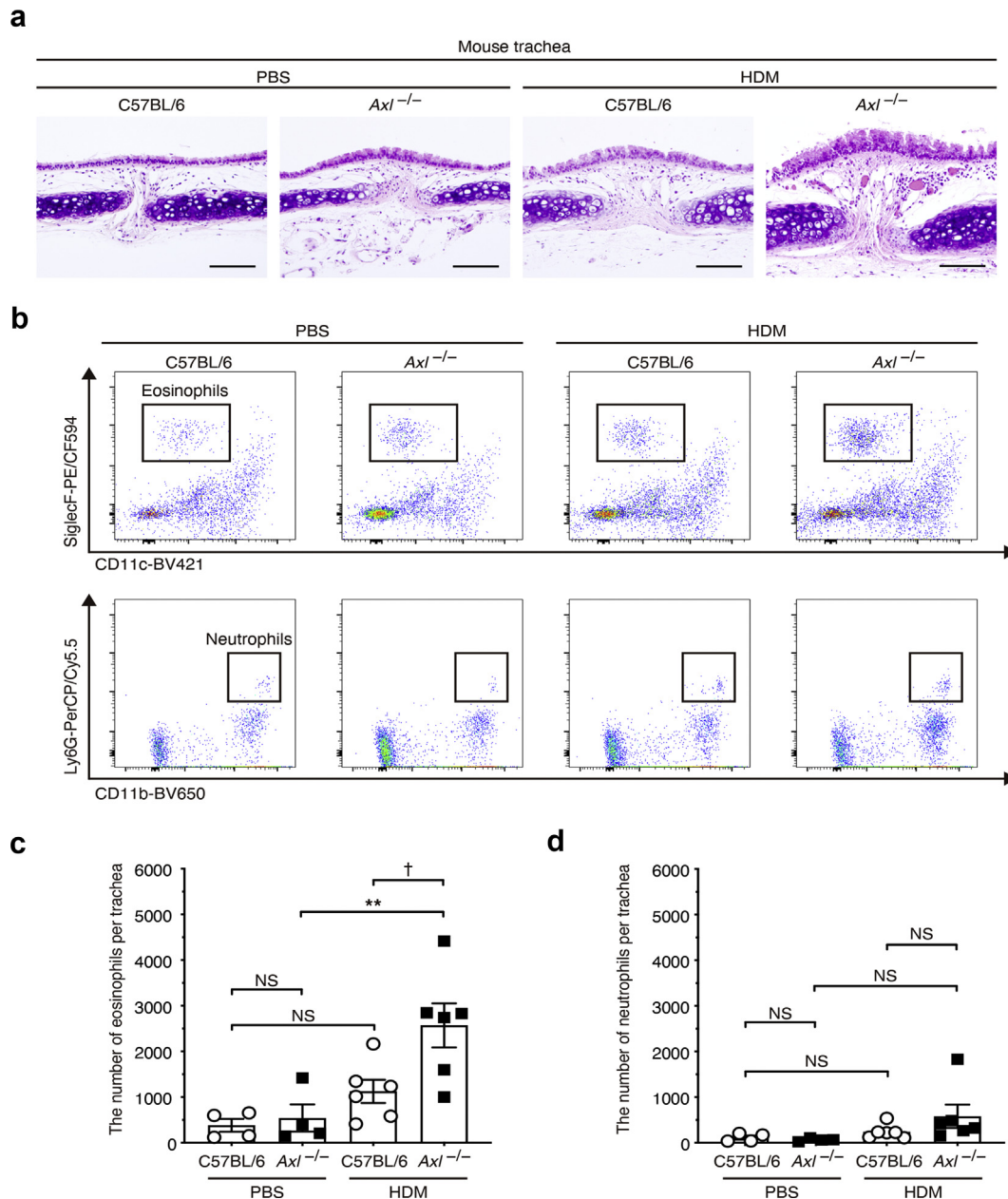


**Fig. 4.** House dust mite extract (HDM)-induced eosinophil infiltration is increased in the trachea of *Axl*-knockout mice *in vivo*. **(a)** Representative hematoxylin-eosin staining of tracheas from C57BL/6 wild-type mice or *Axl*-knockout mice (*Axl*<sup>-/-</sup>) after repetitive intranasal administration of PBS or house dust mite extracts (HDM). Scale bars 100  $\mu$ m. Representative of  $n = 5$  in each group. **(b)** Representative (of  $n = 4$ –6 mice in each group) flow-cytometric analyses of eosinophils (upper panels) and neutrophils (lower panels) in the trachea. **(c)** and **(d)** Absolute numbers of eosinophils **(c)** and neutrophils **(d)** infiltrating the tracheas. Data are combined from two independent experiments (containing 4–6 mice per group) and shown as mean  $\pm$  s.e.m. White circles and black squares represent individual C57BL/6 or *Axl*<sup>-/-</sup> mice, respectively. \*\* $P < 0.01$  vs. *Axl*<sup>-/-</sup> treated with PBS, † $P < 0.01$  vs. C57BL/6 mice treated with HDM. NS, “not significant” by one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test.

cells and maintained in culture media containing hydrocortisone.<sup>46</sup> This cell line maintains phenotypes of airway basal cells and expresses basal cell markers (Keratin5<sup>47</sup> and P63<sup>48</sup>) and was confirmed to constitutively express *Axl* at both mRNA and protein levels (Supplementary Fig. 4A, B). Its ligand Gas6 was also endogenously expressed by BEAS-2B cells (Supplementary Fig. 4A) and exogenous supplementation of hrGas6 did not increase phosphorylation of *Axl* in BEAS-2B cells (Supplementary Fig. 4C). A selective inhibitor for *Axl* kinase, BGB324, decreased phosphorylation of *Axl* in BEAS-2B cells (Supplementary Fig. 4D). These data suggested that *Axl* kinase in BEAS-2B cells was functional and fully activated in an autocrine or paracrine manner *in vitro*.

To test whether *Axl* kinase regulated the expression of cytokines and chemokines in airway epithelial cells under inflammatory conditions, we treated BEAS-2B cells with either (1) PBS as vehicle, (2) HDM, (3) interleukin (IL)-4 and IL-13 or (4) tumour necrosis factor (TNF)- $\alpha$  after siRNA-mediated knockdown of *Axl* protein and then evaluated expression levels of genes involved in pro-inflammatory responses by qRT-PCR (Fig. 5a). The knockdown efficacies of siRNA targeting *Axl* (*Axl*-siRNA1 and *Axl*-siRNA2) were 95.5% and 92.7% at a mRNA level (Supplementary Fig. 4E) and 99.0% and 98.8% at a protein level (Supplementary Fig. 4F). Through this screening, knockdown of *Axl* up-regulated mRNAs of *CSF2*, *CCL5*, *CCL11* and *CXCL8* when cells were stimulated with HDM (Fig. 5B).

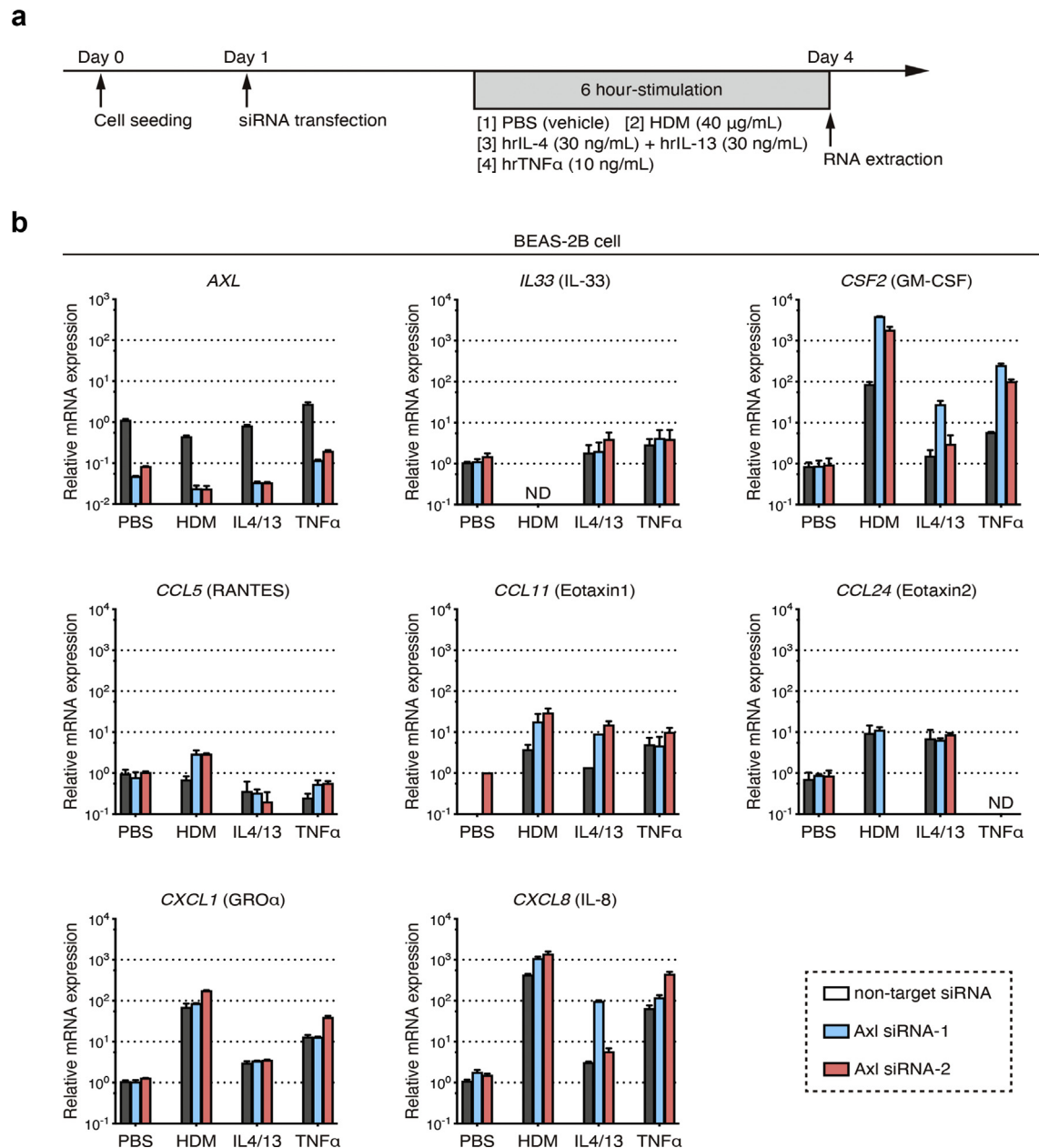


**Fig. 5.** mRNA expressions of cytokines and chemokines in BEAS-2B cells during pro-inflammatory stimuli *in vitro*. **(a)** Experimental design. The human bronchial epithelial cell line (BEAS-2B cell) was maintained in culture media containing hydrocortisone and one day later non-targeting siRNA, or two siRNAs for Axl (named as Axl siRNA-1 and Axl siRNA-2) were added. Six hours prior to cell collection for total RNA extraction, cells were treated with [1] PBS (as vehicle), [2] house dust mite extracts (HDM, 40  $\mu$ g/mL), [3] human recombinant (hr) interleukin (IL)-4 (30 ng/mL) and hrIL-13 (30 ng/mL) or [4] hr tumour necrosis factor (TNF)- $\alpha$  (10 ng/mL). **(b)** qRT-PCR analyses showing expression of genes encoding cytokines and chemokines. A normalized expression value is calculated by  $2^{-\Delta\Delta C_t}$  method.

This pattern of gene expressions was similar in cells treated with TNF- $\alpha$  in the presence of Axl-targeted siRNAs (Fig. 5b). Because the increase in mRNA expression of CSF2 encoding GM-CSF was dramatically higher compared to the other genes such as CXCL8 (Fig. 5b), we further confirmed that CSF2 mRNA expression was up-regulated by HDM in a dose-dependent manner (Fig. 6a). This HDM-induced CSF2 expression was further increased when the Axl gene was silenced (Fig. 6a). In addition, GM-CSF protein secretion in culture supernatants was significantly increased upon HDM stimulation after knockdown of Axl (Fig. 6b).

We finally sought to validate the Axl-mediated GM-CSF suppression in primary human bronchial epithelial cells (HBEC)

*in vitro*. Characteristics of patients were shown in Supplemental Table 1. Immunofluorescence staining revealed that HBEC expressed Keratin5 and P63 as selective markers for airway basal cells (Fig. 6c). A flow cytometric analysis indicated positive cell surface expression of Axl on HBEC (Fig. 6d). Gas6 was also present on the surface of HBEC as shown in Figure 6e. Exogenous addition of hrGas6 at final concentration of 500 ng/mL did not increase the presence of Gas6 on HBEC (Fig. 6e, f), suggesting that HBEC also produced endogenous Gas6 which could saturate Axl receptor on these cells under a regular culture condition. Knockdown of Axl increased CSF2 mRNA expression in PBS- or HDM-treated HBEC (Fig. 6g).

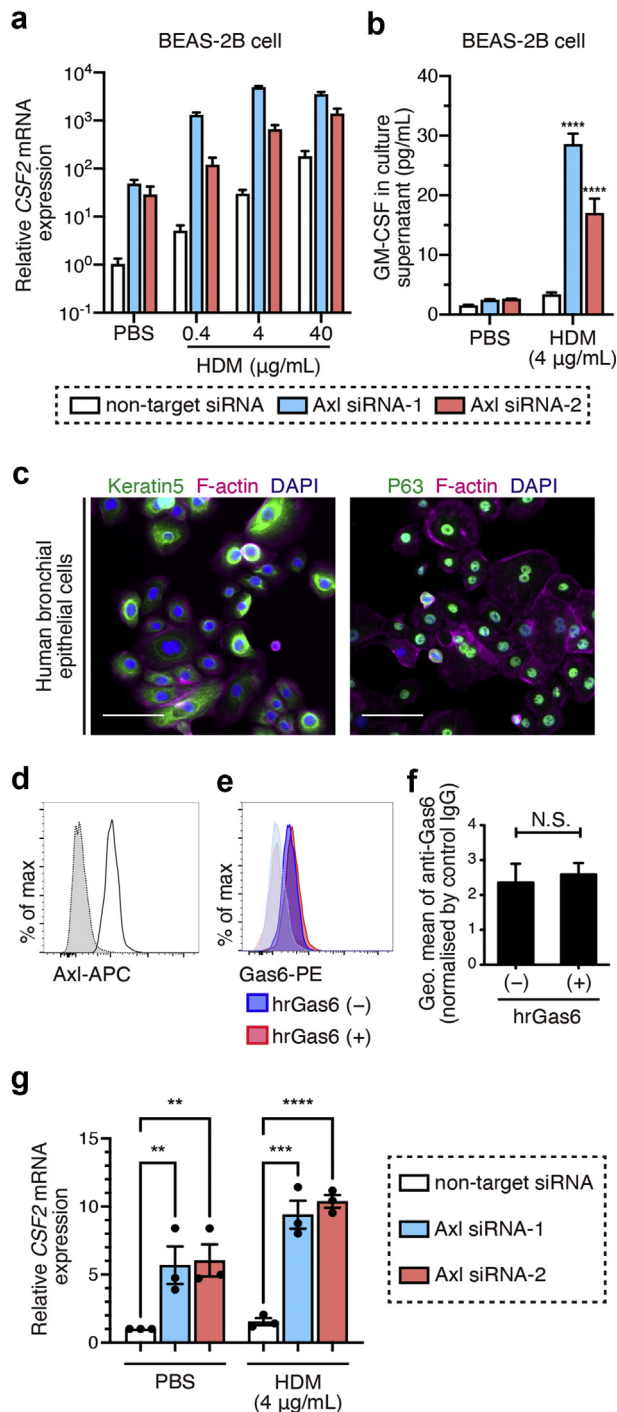


**Fig. 6.** Axl knockdown increases the expression of GM-CSF in response to house dust mite extracts (HDM) in human bronchial epithelial cells *in vitro*. (a) Relative expression of CSF2 encoding GM-CSF in BEAS-2B cells during HDM treatment. Data represents mean and SD of three culture replicates. The y axis represents a log scale. (b) Quantification of GM-CSF protein in culture supernatants. Data are representative of three independent experiments and indicate mean and SD of three culture replicates. Two-way analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison test. \*\*\*\*P < 0.0001 vs. non-target siRNA-transfected cells treated with 4 µg/mL of HDM. ND indicates "not detected". (c) Immunostaining of Keratin 5 (a left panel) and P63 (a right panel) in human bronchial epithelial cells (HBEC). F-actin is visualized by phalloidin (magenta). Nuclei is stained with DAPI (blue). Scale bars, 20 µm. (d) A flow cytometry analysis showing the expression of Axl on the cell surface of HBEC. Specific staining, solid line/open; isotype control, dotted line/shaded. (e) A flow cytometric analysis of the presence of Gas6 on the cell surface of HBEC. "hr" indicates human recombinant. Blue or red represents cells without hrGas6 or supplemented with hrGas6 (500 ng/mL). Light shadow shows control IgG. (f) Quantification of the

## Discussion

In this study, we demonstrated that Axl-expressing basal cells were decreased in severe asthma compared to mild-moderate asthma or non-asthmatics. This was validated by other two cohorts (SARP and U-BIOPRED) at mRNA levels. The reduction of Axl-expressing basal cells was significantly associated with higher infiltration of eosinophils and mast cells in mucosa of severe asthma. In the HDM-induced type 2 inflammation model, Axl diminished infiltration of eosinophils in the tracheal tissue. GM-CSF production driven by HDM is negatively regulated by Axl in human bronchial epithelial cells *in vitro*, which may explain the *in vivo* findings. The role of Axl as a suppressor of eosinophilic inflammation may be coincident with our human pathological findings. Taken together our results give new insights into how the TAM receptor tyrosine kinase Axl regulates type 2 immunity and contributes to prevention of eosinophile infiltration of patients with severe asthma.

Severe asthma is predominantly characterized by persistent type 2 inflammation, with activation and recruitment of eosinophils and mast cells despite high-dose therapy with inhaled glucocorticosteroids.<sup>41</sup> Once airway epithelial cells recognize type 2 immunity-mediated insults, such as proteases and pathogen-associated molecular patterns from inhaled allergens, these barrier cells begin to release a wide variety of humoral factors including cytokines (e.g. IL-1, IL-25, IL-33, TSLP and GM-CSF), chemokines (e.g. RANTES and eotaxins) and alarmins (e.g. adenosine triphosphate), which can subsequently organize dendritic cells to mount Th2 cell-mediated immunity with augmentation of ILC2, eosinophil and mast cell function.<sup>49,50</sup> Cytokines including GM-CSF are associated with the severity of asthma.<sup>8,51</sup> Mechanistically, epithelial GM-CSF attracts dendritic cells in response to allergic sensitization in the lung,<sup>52,53</sup> in addition to its well-recognized role in survival of eosinophils.<sup>54</sup> Moreover, a phase 2 randomized placebo-controlled trial showed that an anti-GM-CSF monoclonal antibody improved airway obstruction in uncontrolled eosinophilic asthma.<sup>55</sup> Given these previous reports, our data highlights a novel role for Axl in the negative regulation of type 2 inflammatory responses, possibly by down-regulation of GM-CSF. This might explain the strong negative correlation between the reduction of Axl-expressing basal cells and the increased eosinophils or mast cells in asthmatic patients in our pathological study.

Axl is expressed by a variety of cell-types including myeloid cells and epithelial cells.<sup>10–12</sup> Although a growing number of studies implicate Axl in cellular proliferation, tissue repair and anti-inflammation, fine-tuning of its function appears to be dependent on the cell-type and inflammatory environment. In myeloid cells, during viral infection-induced type 1 inflammation, Axl promotes efferocytosis of airway macrophages with subsequent resolution of inflammation<sup>16</sup> and matures dendritic cells leading to the induction of antiviral adaptive immunity in mice.<sup>17</sup> Furthermore, during helminth infection-induced type 2 inflammation, Axl and Mertk in conjunction with IL-4 and IL-13 induces the proliferation and tissue repair function of lung and peritoneal macrophages.<sup>56</sup> However, in an HDM-induced asthma mouse model, Axl on airway macrophages does not regulate eosinophilic inflammation,<sup>18</sup> but

presence of Gas6 on the surface of HBEC as shown by geometric (geo.) mean of intensity of PE-conjugated anti-Gas6 normalized by corresponding PE-conjugated control IgG. Data show mean and SD of three independent experiments. Unpaired t-test was performed. N.S. indicates not significant. (g) Relative expression of CSF2 encoding GM-CSF in HBEC during PBS or HDM treatment. Data represents mean and SD of three individuals. Two-way analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison test. \*\*P < 0.01 \*\*\*P < 0.001, \*\*\*\*P < 0.0001 vs. non-target siRNA-transfected cells.

functions in apoptotic cell clearance.<sup>27</sup> In the context of epithelial cells, we have recently reported that Axl is critical for basal cell proliferation and consequent epithelial repair during influenza A virus-triggered airway injury.<sup>26</sup> Moreover, the current study indicates that Axl negatively regulates HDM-induced eosinophil infiltration into the trachea of mice. Importantly, Axl-mediated suppression of GM-CSF production depends on stimulation by HDM, but not by type 2 cytokines IL-4 and IL-13. These data suggest that Axl negatively regulates intracellular pro-inflammatory pathways in a cell-type-specific or an inflammatory niche-dependent manner.

It remains unclear how Axl mRNA expression is down-regulated in airway epithelium of patients with severe asthma in this study, whereas there were no significant differences of Axl mRNA expression between non-asthmatics and patients with mild-moderate asthma. Previous publications provided the evidence that airway epithelial cells of severe asthma differed in their phenotypes and functions from those of mild-moderate asthma.<sup>57,58</sup> Although several up-stream signaling pathways need to be considered, a possible answer may be that transcription of the Axl gene is epigenetically modified with an increase in severity of asthma. This idea is supported by a recent study demonstrating that hypermethylation of CpG loci of the Axl gene is significantly correlated with higher risk of asthma-related symptoms in childhood.<sup>59</sup> Another explanation may be that microRNAs differentially expressed in airway basal cells in severe asthma compared to those in mild-moderate asthma<sup>60</sup> regulate Axl gene expression. Additional studies are needed to evaluate the epigenetic status of the Axl gene or microRNA profiles in airway basal cells of asthmatics. In addition, we did not evaluate how Axl expression was altered in human peripheral lungs of asthmatics because of limited availability of resected lung tissues of patients with asthma.

We observed that mRNA expression of protein S in endobronchial epithelial brushing samples from the SARP cohort was decreased in severe asthma compared to non-asthma or mild-moderate asthma. A recent publication has indicated protective roles of protein S in allergic asthma by suppressing Th2-mediated inflammation.<sup>61</sup> In addition, Tyro3, a receptor for protein S, is known to negatively regulate type 2 inflammatory responses in mice.<sup>18</sup> Taken together, the decreased expression of protein S in bronchial epithelial cells might be associated with type 2 inflammation in severe asthma.

In the current study, we analysed the Axl expression in bronchial specimens from patients with severe asthma including both eosinophilic and non-eosinophilic phenotypes. Therefore, it remains to be elucidated how the Axl kinase-associated pathway is influential in these phenotypes of severe asthma. Further studies will be required to determine the role of Axl kinase on the pathology of severe asthma.

In summary, our results reveal a trachea-specific requirement of Axl for the suppression of excessive eosinophilic inflammation induced by HDM exposure. The failure of this Axl-mediated inhibition of type 2 inflammatory responses might be associated with eosinophile infiltration observed in the airways of severe asthma.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2022.02.010>.

### Conflict of interest

NF was supported by the Japan Society for the Promotion of Science KAKENHI (Grant Number 18K15942) and MSD Life Science Foundation Public Interest Incorporated Foundation. YT was supported by GSK Japan Research Grant 2019. The rest of the authors have no conflict of interest.

### Authors' contributions

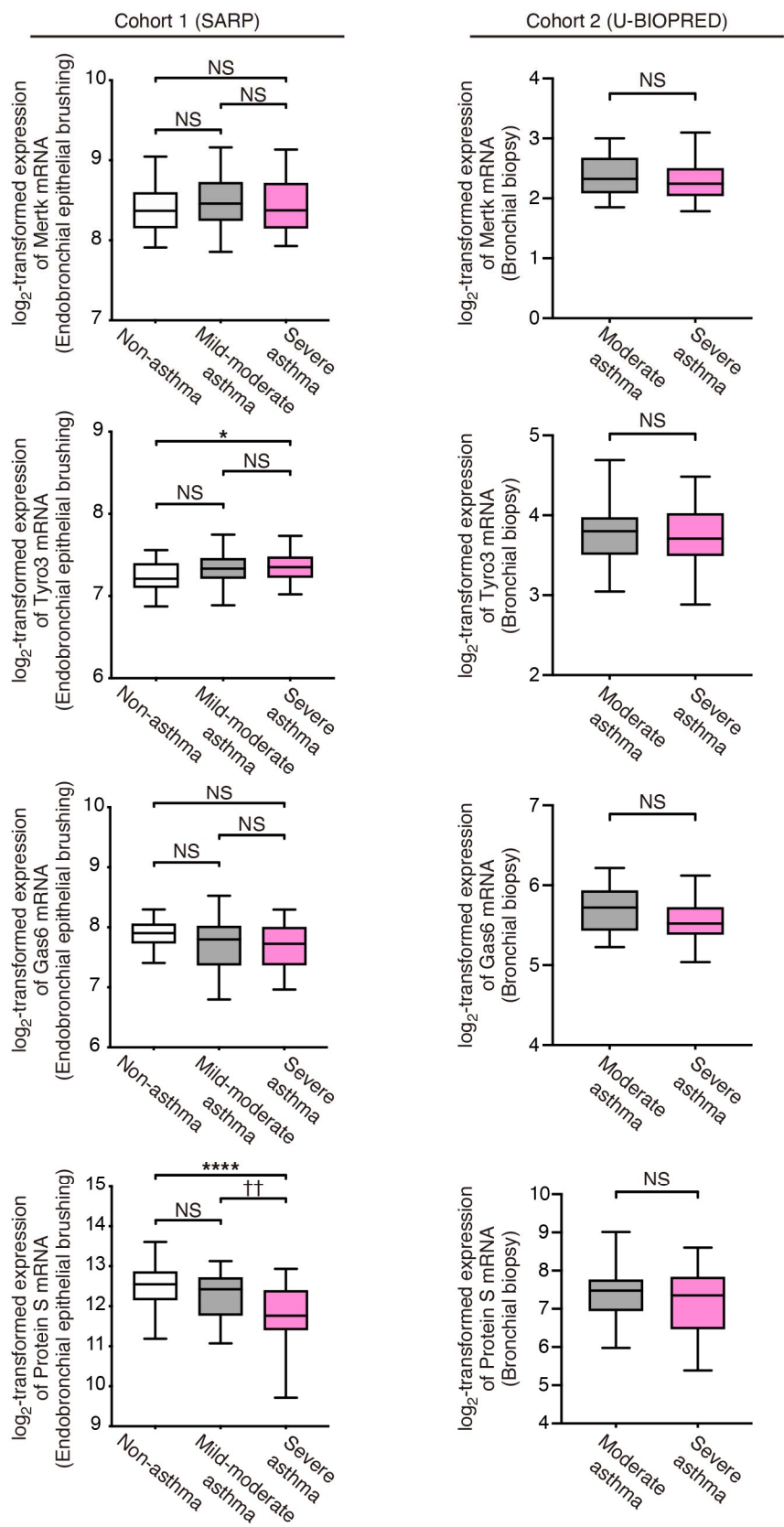
KI, NF, TH, MI and HS designed the study and wrote the manuscript. KI, NF, YKA, IS, MY, KO, YT, TS, TI, TN, YKY, HA, YO, SM and MT contributed to data collection. KI, NF, TH, MI and HS interpreted the results. All authors read and approved the final manuscript.

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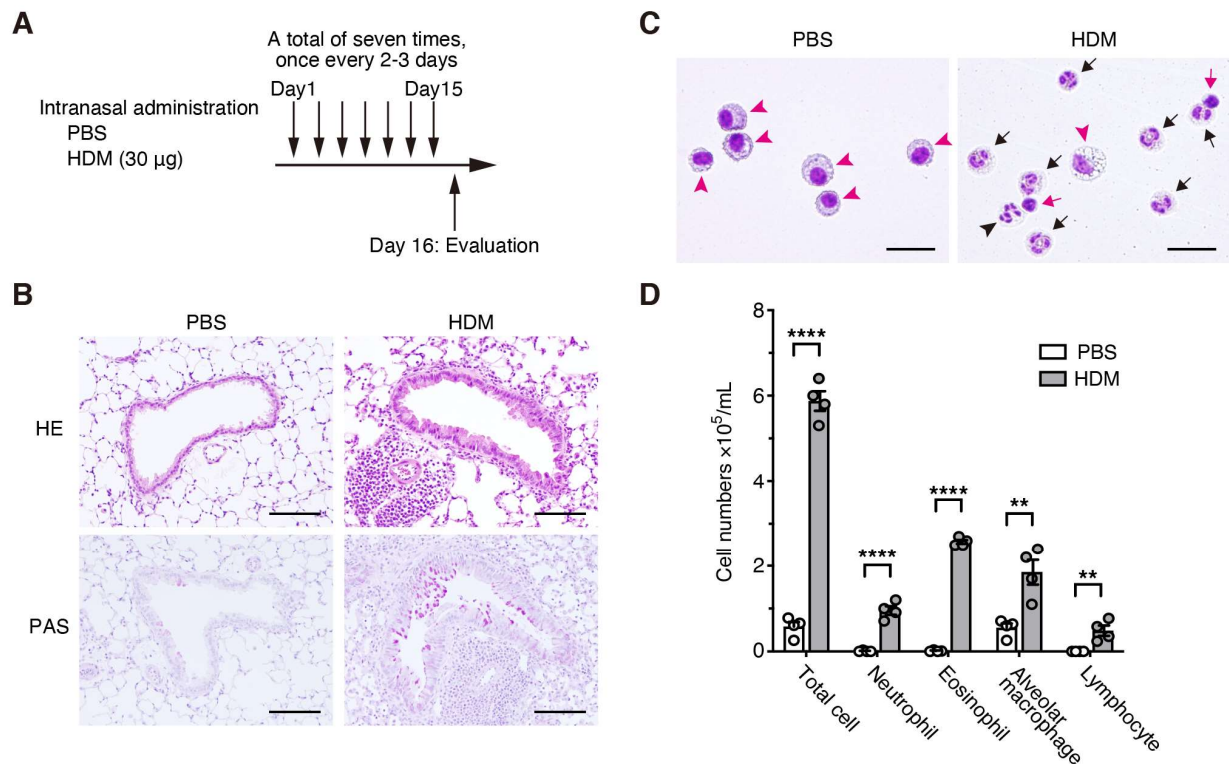
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Supplementary figures



Supplementary Figure 1 Itakura K, et al.

**Supplementary Figure 1** Microarray expression profiling of TAM receptors other than Axl and their ligands in two independent cohorts of airway epithelium from severe asthma patients. mRNA expression of genes encoding Mertk, Tyro3, Gas6 and Protein S was shown. Data of Cohort 1 were from endobronchial epithelial brushing specimens of non-asthmatic subjects (n=27), mild-moderate asthma patients (n=72) and severe asthma patients (n=56) in Severe Asthma Research Program (SARP; accession: GSE63142). Data represented median with 5-95% percentile and were analysed with Kruskal-Wallis test followed by Dunn's multiple comparison test. \*P<0.05, \*\*\*\*P<0.0001 compared with healthy; ††P<0.01 compared with mild-moderate asthma. NS indicates "not significant". Data of Cohort 2 were from bronchial biopsies of moderate asthma patients (n=35) and severe asthma patients (n=56) in Unbiased BIOMarkers in Prediction of REspiratory Disease outcomes (U-BIOPRED) Project (accession: GSE76227). Data represented median with 5-95% percentile and were analysed with Mann-Whitney test.



**Supplementary Figure 2 Itakura K, et al.**

**Supplementary Figure 2** Validation of type 2 inflammatory responses in lung tissues from C57BL/6 mice treated with house dust mite extracts (HDM).

(A) An experimental protocol. HDM (30 µg/mouse) or PBS was intranasally given to C57BL/6 female mice once every 2-3 days for a total of seven times.

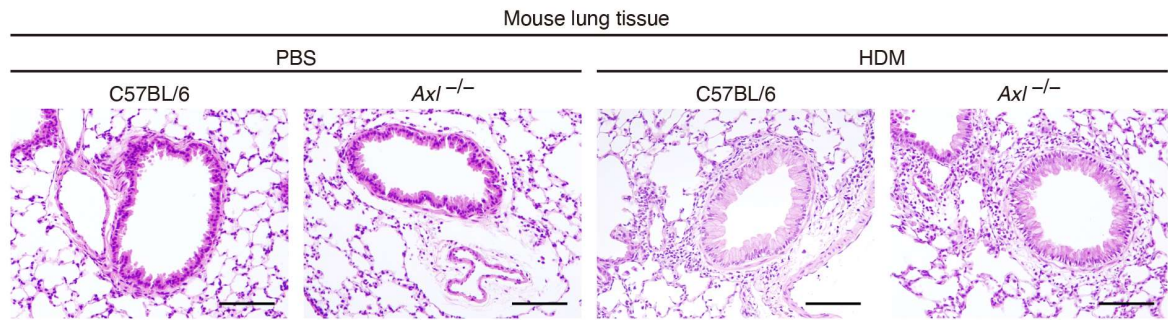
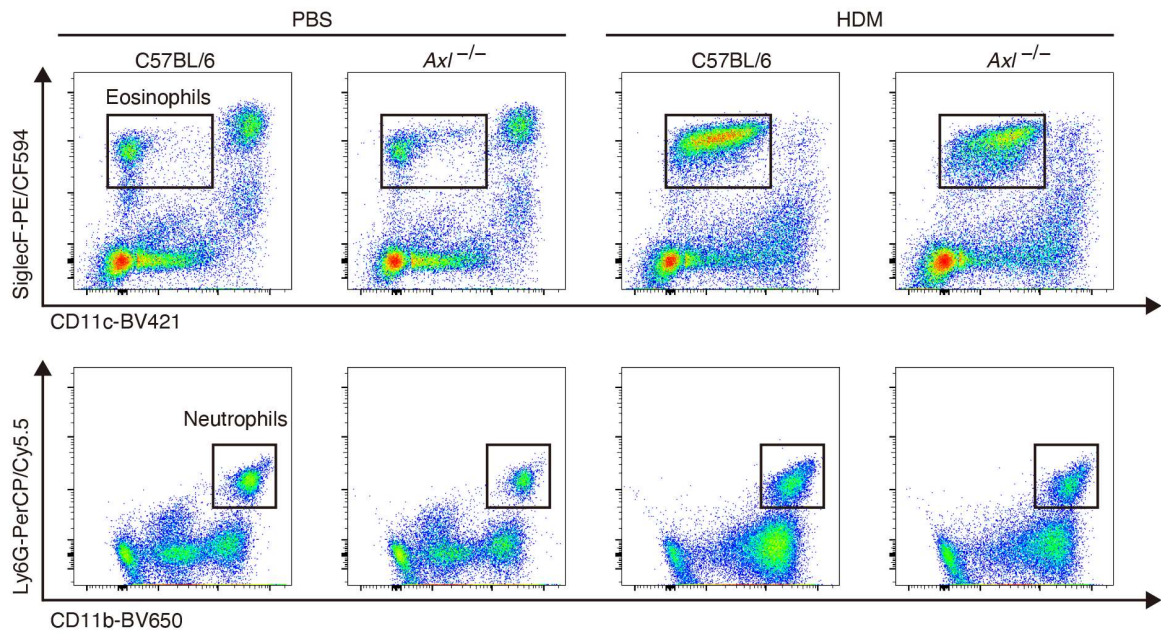
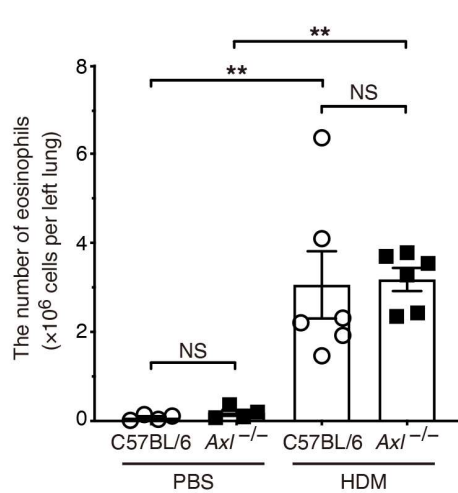
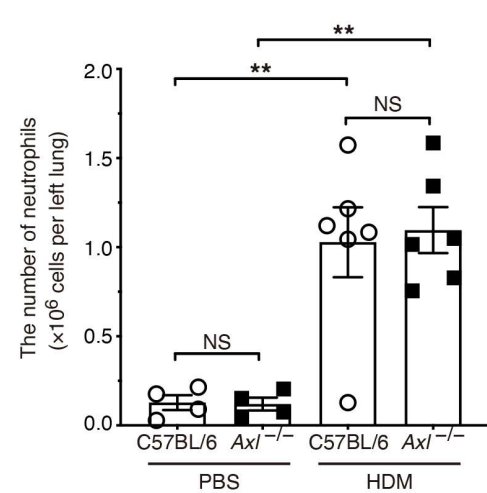
(B) Hematoxylin-eosin (HE, upper panels) and Periodic acid-Schiff (PAS, lower panels) staining of lung tissues from C57BL/6 female mice given PBS or HDM. Scale bars, 100 µm.

(C) Representative Diff-Quik staining of cells from bronchoalveolar lavage fluid (BALF) of C57BL/6 mice treated with PBS or HDM. Red arrowheads, alveolar macrophages; black arrowheads, neutrophils; black arrows, eosinophils; red arrows, lymphocytes. Scale bars, 20 µm.

(D) Quantification of BALF cell numbers. Data are shown as mean ± s.e.m. Open or shaded circles represent individual mice of C57BL/6 treated with PBS or HDM, respectively.

\*\*P<0.01, \*\*\*\*P<0.0001 vs. PBS, by Mann-Whitney test.



**A****B****C****D**

**Supplementary Figure 3 Itakura K, et al.**

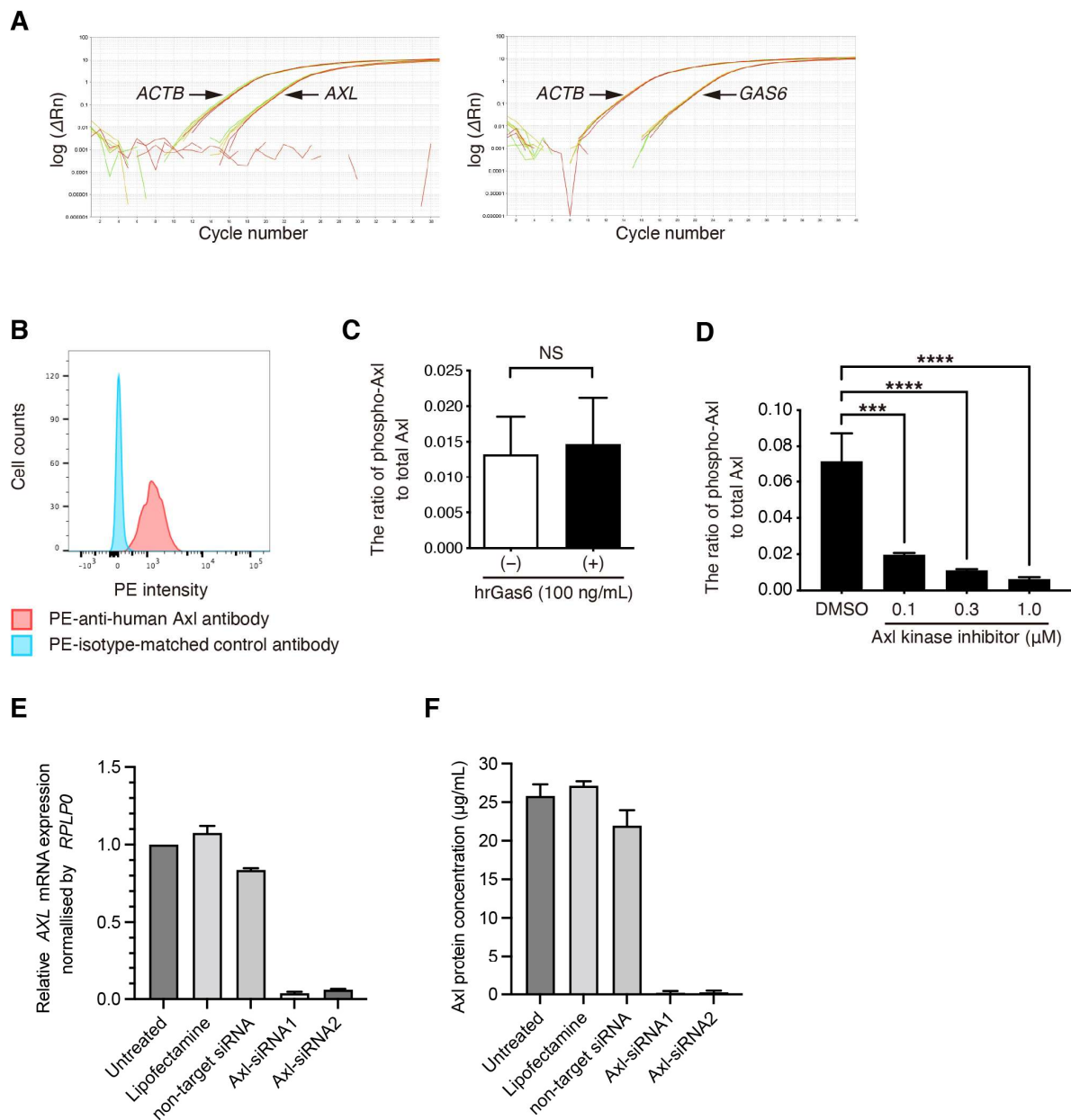
**Supplementary Figure 3** House dust mite-induced eosinophil infiltration is not altered in lung tissues of *Axl*-knockout mice.

(A) Representative hematoxylin-eosin staining of lung tissues from C57BL/6 wild-type mice or *Axl*-knockout mice (*Axl*<sup>-/-</sup>) after intranasal repetitive administration of PBS or house dust mite extracts (HDM). Scale bars, 100  $\mu$ m.

(B) Representative flow-cytometric analyses of eosinophils (upper panels) and neutrophils (lower panels) in lung tissues.

(C), (D) Absolute numbers of eosinophils (C) and neutrophils (D) infiltrating in left lung tissues. Data are combined from two independent experiments and shown as mean  $\pm$  s.e.m. White circles or black squares represent individual mice of C57BL/6 or *Axl*<sup>-/-</sup>, respectively.

\*\*P<0.01 vs. PBS. NS, “not significant” by one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test.



**Supplementary Figure 4 Itakura K, et al.**

**Supplementary Figure 4** Autocrine/paracrine activation of Axl kinase in an immortalised human bronchial epithelial cell line (BEAS-2B cell) *in vitro*.

(A) Amplification plots of real time RT-PCR indicate mRNA expression of *AXL* (left) and *GAS6* (right) in BEAS-2B cells. Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye.  $\beta$ -actin encoding *ACTB* is used as a housekeeping gene.

- (B) A flow-cytometric analysis indicates cell surface expression of Axl on BEAS-2B cells. Histogram in red or blue shows cells stained with phycoerythrin (PE)-conjugated anti-human Axl monoclonal antibody or PE-conjugated isotype-matched control antibody, respectively.
- (C) Exogenous supplementation of human recombinant Gas6 (hrGas6) at 100 ng/mL did not increase phosphorylation of Axl in BEAS-2B cells. Phosphorylated Axl and total Axl were quantified by ELISA. Data show mean and SD of three independent experiments. Unpaired t-test was performed. NS indicates “not significant”.
- (D) An inhibitor for Axl kinase named BGB324 decreased phosphorylation of Axl in BEAS-2B cells in a dose-dependent manner, indicating that Axl kinase was functional in culture. Data represented mean with SD of triplicate cultures and were analysed with one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test. \*\*\*P = 0.0001, \*\*\*\*P < 0.0001 vs. cells treated with 0.1% DMSO as a vehicle control.
- (E, F) Knockdown efficacies of siRNAs targeting Axl (Axl-siRNA1 and Axl-siRNA2) at a mRNA level (E) and a protein level (F) in BEAS-2B cells. Data show mean and SD of three independent experiments.

**Supplementary Table1** Characteristics of patients for a primary human bronchial epithelial cell assay

|           | Sex  | Age | Smoking (pack-years) | COPD | Asthma |
|-----------|------|-----|----------------------|------|--------|
| Patient 1 | Male | 62  | 61                   | no   | no     |
| Patient 2 | Male | 68  | 125                  | no   | no     |
| Patient 3 | Male | 49  | 0                    | no   | no     |