

**Pathological study of eosinophil-driven mechanisms
in novel murine models of allergic and fibrotic pulmonary disease**

アレルギー性および線維性肺疾患の新規マウスモデルにおける好酸球誘
導性機序の病理学的研究

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ABSTRACT

Pathological study of eosinophil-driven mechanisms in novel murine models of allergic and fibrotic pulmonary disease

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This doctoral thesis presents two novel eosinophil-driven murine models for allergic bronchopulmonary aspergillosis (ABPA) and idiopathic pulmonary fibrosis (IPF) employing the spontaneously eosinophilic Yama mouse strain. ABPA and IPF, despite being triggered by distinct causes—fungal hypersensitivity and repetitive epithelial injury, respectively—exhibit shared clinical characteristics, such as chronic inflammation, abnormal tissue remodeling, and advancing fibrosis. Eosinophils have become essential mediator cells in both allergic and non-allergic pulmonary disorders. Although, their specific role in chronic lung fibrosis remains insufficiently understood.

The Yama strain, distinguished by persistent, IL-5-independent peripheral eosinophilia, offers a distinctive biological background to examine eosinophil function without dependence on genetic modification or external cytokine activation. These investigations were conducted to elucidate the role of intrinsic eosinophilia in modulating inflammatory pathways, Th2-mediated immune responses, epithelial damage, and fibrotic remodeling in two separates yet physiologically related disease models. This dissertation utilizes the Yama mouse in an allergen-induced ABPA model and a repeated bleomycin-induced IPF model to clarify prominent eosinophil-dependent pathways contributing to chronic

pulmonary diseases and to identify potential TGF- β -independent mechanisms of fibrosis relevant to human disease.

The ABPA study demonstrated that sensitization and challenge with the *Aspergillus fumigatus* allergen Asp f1 resulted in sustained peripheral eosinophilia, increased serum IgE levels, and significant airway remodeling in Yama mice. In comparison to BALB/c mice, Yama mice demonstrated significantly heightened goblet cell hyperplasia, mucus obstruction, peribronchial inflammation, and collagen accumulation. Differential gene expression analysis demonstrated substantial elevation of Th2-associated cytokines (IL-4, IL-5, IL-6, IL-13) and *Colla1*, but TGF- β expression exhibited minimal change. The data suggest that airway fibrosis in Yama mice primarily occurs via TGF- β -independent mechanisms, possibly facilitated by IL-13 and eosinophil-derived mediators, including eosinophil cationic protein. The findings confirm the Yama strain as a reliable and physiologically optimal model that mirrors the immunopathological characteristics of severe human ABPA.

In the IPF model, repeated intranasal administration of bleomycin resulted in enduring and progressive lung fibrosis in Yama mice, exceeding the temporary and self-resolving fibrotic response seen in C57BL/6 controls. Yama mice exhibited significant interstitial thickening, many fibroblastic foci, alveolar collapse, and considerably elevated collagen deposition. In accordance with the ABPA findings, Yama mice exhibited significant overexpression of Th2 cytokines (IL-4, IL-5, IL-13), sustained elevation of IL-6, and increased expression of *Colla1*. Notably, although fibrosis was severe, TGF- β expression rose only marginally, indicating

that fibrogenesis in this model is primarily influenced by alternate profibrotic pathways involving eosinophil-derived proteins and Th2/IL-6 signaling, rather than traditional TGF- β –dependent processes. This observation illustrates the variability of pathogenic mechanisms in human IPF, where TGF- β activity does not consistently correspond with disease severity.

These investigations collectively illustrate that spontaneous eosinophilia markedly enhances both allergic and non-allergic fibrotic processes, emphasizing eosinophils as pivotal effector cells that connect inflammation with the structural remodeling of pulmonary tissue. The Yama mouse serves as a robust and adaptable preclinical model for investigating eosinophil-mediated pathways in chronic lung illness, potentially aiding in the formulation of targeted therapies approaches for eosinophil-related fibrosis.

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GENERAL INTRODUCTION

INTRODUCTION

1.1 Background

Respiratory diseases remain a significant global health burden, with chronic and progressive conditions such as allergic bronchopulmonary aspergillosis (ABPA) and idiopathic pulmonary fibrosis (IPF) contributing substantially to morbidity and mortality worldwide (Asano and Oguma, 2025; Dietschmann *et al.*, 2020; Kondoh *et al.*, 2022; Raghu *et al.*, 2016). ABPA is a hypersensitivity lung disorder caused by immune responses to *Aspergillus fumigatus* (*Af*), a ubiquitous fungal pathogen (Kurup and Grunig, 2001; Trivedi and Lloyd, 2007). Globally, ABPA affects an estimated 4.8 million people, imposing a substantial healthcare and socioeconomic burden (Roboubi *et al.*, 2023). Immunopathologically, ABPA is characterized by Th2-dominated inflammation, eosinophilia, elevated IgE levels, mucus hypersecretion, and central bronchiectasis (Moss, 2005; Nikaein *et al.*, 2015). Chronic inflammation and persistent immune activation in the bronchial tree can result in irreversible airway remodeling and fibrosis (Knutsen and Slavin, 2011; Kumar and Foster, 2002; Nikaein *et al.*, 2015).

ABPA typically occurs in individuals with pre-existing respiratory diseases, such as asthma or cystic fibrosis, in whom the fungus colonizes the tracheobronchial tree (Oshero, 2012; Patel and Greenberger, 2019). Clinically, ABPA manifests as recurrent productive cough, episodic wheezing, dyspnea, and fever, often accompanied by malaise, weight loss, and chest pain (McCrary, 2019). Despite extensive immunological and molecular study, the precise mechanisms

underlying disease progression and reversibility remain poorly understood (Luo *et al.*, 2024).

IPF, on the other hand, is a chronic and progressive interstitial lung disease of unknown etiology, with a median survival rate of only 3–5 years after diagnosis (Degryse *et al.*, 2010; Degryse and Lawson, 2011; Kondoh *et al.*, 2022; Raghu *et al.*, 2016). Patients experience progressive dyspnea, dry cough, and deteriorating pulmonary function, with an increasing global prevalence, including in Japan (Kondoh *et al.*, 2022; Lederer and Martinez, 2018; Ley Kondoh *et al.*, 2011; Wang *et al.*, 2025). The pathogenesis of IPF involves repetitive alveolar epithelial injury, abnormal wound repair, and excessive extracellular matrix (ECM) deposition, ultimately leading to irreversible distortion of the lung parenchyma (Lederer and Martinez, 2018; She *et al.*, 2021; Wijsenbeek and Cottin, 2020). Despite advances in antifibrotic therapies, such as nintedanib and pirfenidone, the mechanisms responsible for disease initiation and progression remain incompletely defined (Amati *et al.*, 2023; Guo *et al.*, 2023; Miao *et al.*, 2023).

1.2 Limitations of conventional animal models

Although animal models have greatly advanced our understanding the immunopathogenesis of ABPA and IPF, conventional murine systems exhibit major limitation in reproducing the chronic and progressive features of these diseases (Bushkirk *et al.*, 2014; Dietschmann *et al.*, 2020; Kurup and Grunig, 2001; Lee *et al.*, 2025; Moore and Hogaboam, 2008; Song *et al.*, 2022). In ABPA research, existing models typically employ acute sensitization and short-term allergen

challenges using *A. fumigatus* extracts, resulting in transient inflammation rather than the persistent hypersensitivity observed in humans (Buskirk *et al.*, 2014; Kurup and Grunig, 2001; McCrary, 2019). BALB/c mice, commonly used for allergic airway studies due to its Th-2-prone immune profile, develop only mild peribronchial inflammation with limited eosinophil recruitment and minimal mucus accumulation following *A. fumigatus* challenge (Buskirk *et al.*, 2014; Garcia *et al.*, 2010; Hogaboam *et al.*, 2000; Dietschmann *et al.*, 2020). These models therefore insufficiently represent the severe and persistent immunopathology observed in human ABPA (Hogaboam *et al.*, 2000; Kurup and Grunig, 2001; Luo *et al.*, 2024).

Similarly, the conventional single-dose bleomycin (BLM) model of pulmonary fibrosis in C57BL/6 mice induces only transient fibrotic changes, which resolve spontaneously within several weeks (Degryse and Lawson, 2011; Lee *et al.*, 2025; Moore and Hogaboam, 2008; Song *et al.*, 2022). This self-limiting nature of single-dose BLM-induced fibrosis contrast with the irreversible and progressive fibrosis observed in human IPF, where fibrotic lesion expands and coalesces over time, leading to architectural distortion and respiratory failure (Degryse *et al.*, 2010; Degryse and Lawson, 2011; Gul *et al.*, 2023; Lederer and Martinez, 2018; Redente *et al.*, 2021; Song *et al.*, 2022). Recent studies have attempted to enhance the chronicity of IPF models through repeated dosing, yet they still inadequately reproduce the persistent and severe pathology of IPF (Gul *et al.*, 2023; Lederer and Martinez, 2018; Moore and Hogaboam, 2008; Redente *et al.*, 2021). Thus, there remains an urgent need for animal models that faithfully reflect the

immunopathological complexity of chronic eosinophilic inflammation and fibrogenesis.

1.3 Yama mouse: a unique model

The Yama mouse is a unique inbred strain that exhibits spontaneous and stable peripheral blood eosinophilia independent of IL-5 overexpression (Yamada *et al.*, 2024; Yamada *et al.*, 2025). This distinct phenotype provides a powerful platform to investigating the eosinophil-mediated immunopathology under physiological regulation, without artificial cytokine stimulation (Yamada *et al.*, 2024; Yamada *et al.*, 2025). In previous studies, Yama mice have been applied in the investigation of allergic and eosinophil-associated disorders, providing insights into eosinophil activation and tissue infiltration under physiological conditions (Yamada *et al.*, 2024; Yamada *et al.*, 2025). The absence of external cytokine manipulation ensures that observed pathophysiological changes directly reflect intrinsic eosinophil activity rather than artificially induced immune dysregulation. Considering that eosinophils play important roles in allergic inflammation, tissue remodeling, and fibrosis, Yama mouse represents as a valuable and currently underutilized model for studying eosinophil-associated diseases such as ABPA and IPF (Agarwal *et al.*, 2014; Hogaboam *et al.*, 2000; She *et al.*, 2021; Wijsenbeek and Cottin., 2020).

1.4 The role of eosinophils in lung diseases

Eosinophils are multifunctional leukocytes that have been traditionally associated with allergic and parasitic diseases. However, accumulating evidence indicates their broader involvement in chronic inflammatory and fibrotic lung disorders, including ABPA and IPF (Agarwal *et al.*, 2013; Agarwal *et al.*, 2014; Hogaboam *et al.*, 2000; She *et al.*, 2021; Wijsenbeek and Cottin, 2020). In ABPA, eosinophils are recruited to the airway following fungal antigen exposure and contribute to tissue injury through the release of, e.g., cytotoxic granule proteins, cytokines, and reactive oxygen species (Kurup and Grunig, 2001; Moss, 2005; Nikaein *et al.*, 2015; Trivedi and Lloyd, 2007). Agarwal *et al.*, (2014) reported abundant pulmonary eosinophil infiltration in untreated ABPA patients, suggesting their pathogenic role in driving tissue damage and remodeling.

Beyond their inflammatory functions, eosinophils also play a crucial role in fibrogenesis. They secrete profibrotic mediators such as transforming growth factor- β (TGF- β), interleukin-4 (IL-4), and interleukin-13 (IL-13), which promote fibroblast proliferation, collagen synthesis and ECM deposition (Akuhota and Weller, 2012; Al-muhsen *et al.*, 2013; Fulkerson and Rothenberg, 2023; She *et al.*, 2021; Wijsenbeek and Cottin, 2020). In both models of allergic airway inflammation and pulmonary fibrosis, eosinophil-derived cytokines have been shown to promote collagen synthesis and tissue remodeling (She *et al.*, 2021; Wijsenbeek and Cottin, 2020). Despite these insights, the precise mechanisms by which eosinophils drive the transition from chronic inflammation to irreversible

fibrosis remain poorly defined, largely due to the lack of suitable in vivo systems that sustain eosinophil activity over time.

1.5 Objectives

Despite significant advances, the mechanisms by which eosinophils mediate chronic inflammation and fibrosis in the lung remain incompletely elucidated. Current mouse models of ABPA and IPF are insufficient for reproducing the persistent eosinophilic inflammation, airway remodeling, and progressive fibrogenesis observed in patients (Buskirk *et al.*, 2014; Dietschmann *et al.*, 2020; Kurup and Grunig, 2001; Lee *et al.*, 2025; Moore and Hogaboam, 2008; Song *et al.*, 2022). The spontaneous eosinophilia of the Yama mouse provides a unique experimental platform to dissect the eosinophil-driven mechanisms of diseases without the confounding effects of exogenous cytokine overexpression (Yamada *et al.*, 2024; Yamada *et al.*, 2025).

The present study was conducted with the following objectives:

1. To establish a novel murine model of ABPA using Yama mice. This objective aimed to characterize *A. fumigatus* antigen-induced airway inflammation, with particular attention to eosinophil infiltration, mucus hypersecretion, mast cell involvement, and tissue remodeling, and to compare these responses with those of BALB/c mice (Garcia *et al.*, 2010; Hogaboam *et al.*, 2000; Kurup and Grunig, 2001; McCrary, 2019; Moss, 2005).

2. To develop a progressive and severe model of idiopathic pulmonary fibrosis (IPF) using Yama mice. Employing a repeated-dose bleomycin (BLM) protocol, this objective sought to reproduce the chronic and irreversible fibrotic features of human IPF, and to compare disease progression between Yama and C57BL/6 mice were to elucidate the contribution of eosinophilia to fibrosis progression (Degryse *et al.*, 2010; Degryse and Lawson, 2011; Gul *et al.*, 2023; Lederer and Martinez, 2018; Yamada *et al.*, 2024).
3. To elucidate eosinophil-driven mechanisms of inflammation and fibrosis. Through histopathological, hematological, and gene expression analyses, the study aimed to clarify the roles of eosinophil-derived mediators in fibroblast activation and extracellular matrix deposition (Agarwal *et al.*, 2014; Buskirk *et al.*, 202; Fulkerson and Rothenberg, 2023; She *et al.*, 2021; Wijssenbeek and Cottin, 2020).

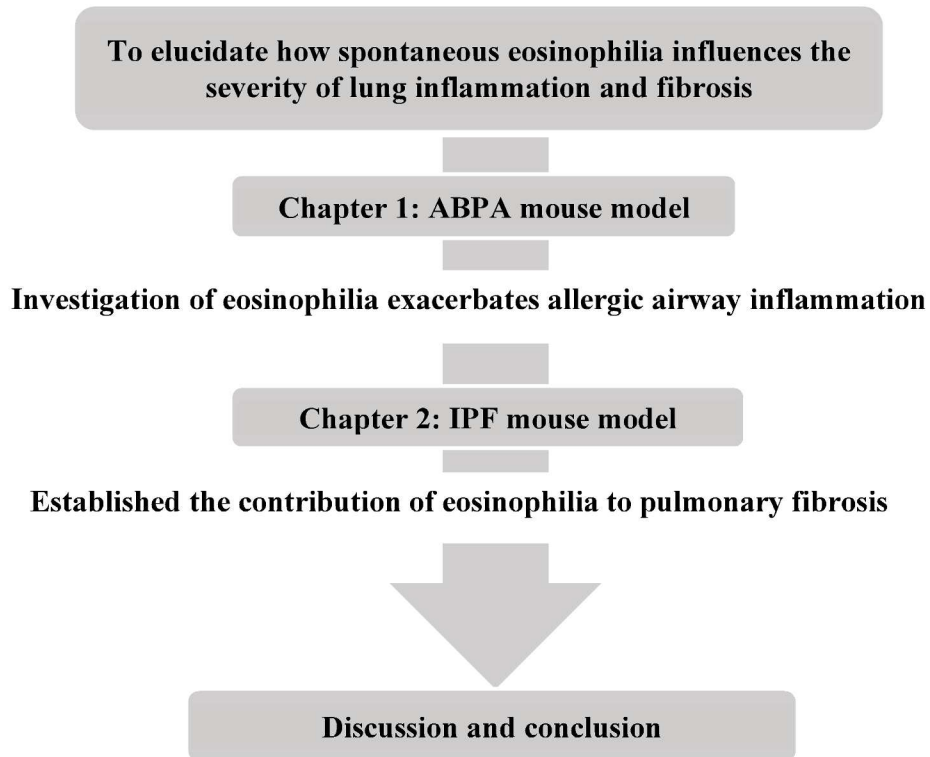
OBJECTIVE AND STRUCTURE OF THE THESIS

This thesis aims to elucidate how spontaneous eosinophilia influences the severity of lung inflammation and fibrosis. We established eosinophilic mouse models to investigate the role of eosinophils in airway inflammation, mucus production, and fibrotic remodeling.

In chapter 1: To investigate whether eosinophilia exacerbates allergic airway inflammation, an ABPA-like model was developed in Yama mice by using the *Aspergillus fumigatus* allergen (Asp f1). Hematological, serological, histopathological, immunohistochemical, and molecular analyses were performed to assess inflammation, allergic response, cytokine expression, and mucus hypersecretion.

In chapter 2: To determine the contribution of eosinophilia to pulmonary fibrosis, a bleomycin-induced model was established in Yama mice. Lung fibrosis was evaluated through hematological, biochemical, histopathological, immunohistochemical, and gene expression analyses of collagen and fibrotic markers.

OBJECTIVE



LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications:

Ramandani, D., Hasegawa, M., Yamada, Y., Yoshizaki, K., Sakurai, M., and Morimoto, M. (2025). Establishment of a novel murine model of allergic bronchopulmonary aspergillosis using an eosinophilic mutant mouse. *J Vet Med Sci*. doi: [10.1292/jvms.25-0425](https://doi.org/10.1292/jvms.25-0425)

Ramandani, D., Hasegawa, M., Yamada, Y., Yoshizaki, K., Sakurai, M., and Morimoto, M. (2025). A novel murine model of idiopathic pulmonary fibrosis with persistent and marked features using Yama mice. *Jpn J Vet Sci*. (in press)

CHAPTER 1

**Establishment of a novel murine model of allergic bronchopulmonary
aspergillosis using an eosinophilic mutant mouse**

ABSTRACT

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity lung disorder caused by chronic exposure to *Aspergillus fumigatus* (*Af*). Existing mouse models do not fully capture the severity of ABPA. This study developed a novel murine model of ABPA using the spontaneously eosinophilic Yama mouse. Female Yama and BALB/c mice were sensitized and challenged with Asp f1, a major allergen derived from *Af*. Peripheral eosinophil counts, serum IgE levels, and histopathological changes in the lungs, including goblet cell hyperplasia, mucus plugging, collagen deposition, eosinophil cationic protein (ECP), and CD117/c-kit expression, were assessed. Cytokine and fibrosis-related gene mRNA expression of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-13 (IL-13), transforming growth factor- β (TGF- β), and Colla1 was also measured. Compared to BALB/c mice, Asp f1-challenged Yama mice exhibited sustained eosinophilia, increased IgE, pronounced goblet cell hyperplasia with mucus plugging, collagen deposition, and ECP expression. Yama mice also showed more severe peribronchial inflammation and pulmonary fibrosis. RT-PCR analysis demonstrated substantial increases in IL-4, IL-5, IL-6, IL-13, and Colla1 transcripts, indicating a Th2-dominant immune profile and enhanced fibrotic activity. The minimal differences in TGF- β expression between Asp f1- and saline-treated mice indicate that fibrosis in Yama mice may proceed via a TGF- β -independent pathway. These findings establish the Yama mouse as a novel model for ABPA research, closely replicating the immunopathological features of severe human ABPA. This model provides a reliable platform to investigate

eosinophil-driven lung fibrosis mechanisms and evaluate hypersensitivity responses contributing to human ABPA onset and progression.

INTRODUCTION

Allergic bronchopulmonary aspergillosis (ABPA) is a lung disorder characterized by an immune response against *Aspergillus fumigatus* (*Af*) (Kurup and Grunig, 2001; Trivedi and Lloyd, 2007). Asp f1 is highly toxic, antigenic, and contains biologically active substances for mice (Garcia *et al.*, 2010; Kurup and Grunig, 2001). The toxin and proteolytic enzymes may disrupt epithelial integrity, facilitating antigen translocation across the mucosal barrier (Garcia *et al.*, 2010; Kurup and Grunig, 2001). ABPA predominantly affects individuals with underlying respiratory conditions, such as asthma and cystic fibrosis, wherein it colonizes the tracheobronchial tree (Oshero, 2012; Patel and Greenberger, 2019). The disease commonly presents with a worsening productive cough or episodic wheezing, often accompanied by dyspnea, fever, malaise, weight loss, hemoptysis, and chest pain (Mc Crary, 2019).

ABPA is a substantial global health burden, estimated to affect more than 4.8 million people (Roboubi *et al.*, 2023). The characteristic immune response involved in this disease comprises recurrent pulmonary inflammation, mucus hypersecretion, eosinophilia, elevated IgE levels, and central bronchiectasis (Moss, 2005). Moreover, its pathogenesis is driven by Th2-dominated immunity and fungal-induced allergy, which ultimately results in progressive airway injury (Nikaein *et al.*, 2015). However, the mechanisms involved in the progression and reversibility of ABPA are not yet fully understood (Luo *et al.*, 2024).

Existing animal models cannot accurately reproduce the complex immunopathology of ABPA, particularly in terms of severe immunopathogenesis

and the mechanism of *Af*-induced allergies (Kurup and Grunig, 2001; Luo *et al.*, 2024). In humans, ABPA-associated tissue damage may lead to severe and prolonged asthma, as well as progressive pulmonary fibrosis (Knutsen, 2006; Kumar and Foster, 2002). Conventional mouse models typically rely on acute sensitization and short-term allergen challenge, which cause transient inflammation rather than the chronic hypersensitivity that characterizes human ABPA (Buskirk *et al.*, 2014; Kurup and Grunig, 2001; Mc Crary, 2019). Moreover, most mouse strains exhibit only a moderate Th2-dominant response, limited eosinophil recruitment, and rapid clearance of *A. fumigatus* antigens. Consequently, major clinical characteristics were poorly reproduced in such models (Hogaboam *et al.*, 2000).

Agarwal *et al.* (2014) reported an abundance of pulmonary eosinophils in untreated patients with ABPA, thereby suggesting a primary proinflammatory role of these cells in the disease. Furthermore, eosinophils contribute to tissue fibrosis in asthma and pulmonary fibrosis (Trivedi and Lloyd, 2007; Yamada *et al.*, 2024). However, the mechanisms underlying chronic *Af* exposure and hypersensitivity to fibrosis remain unclear. Prior studies on BALB/c mice have focused primarily on allergic inflammation, with limited examination of fibrotic progression (Garcia *et al.*, 2010; Osheroov, 2012; Trivedi and Lloyd, 2007). Histopathological changes revealed that *Af*-challenged BALB/c mice developed mild peribronchial inflammatory infiltrates composed primarily of lymphocytes and scattered eosinophils, with limited interstitial involvement (Asano *et al.*, 2018; Buskirk *et al.*, 2014; Hogaboam *et al.*, 2000). Additionally, occasional goblet cell hyperplasia

has also been observed, accompanied by minimal accumulation of luminal mucus (Buskirk *et al.*, 2014; Hogaboam *et al.*, 2000; Knutsen and Slavin, 2011). These results demonstrate the importance of developing more representative models to better understand the interactions between fungal antigens, immune responses, and fibrogenesis in ABPA pathogenesis.

The present study established an inbred strain with spontaneous eosinophilia—designated the Yama mouse—as a new animal model of ABPA. The Yama mice exhibited persistent peripheral blood eosinophilia without artificial induction, offering a unique opportunity to investigate eosinophil-activating mechanisms. Furthermore, these mice manifested pronounced peribronchial and interstitial inflammation characterized by dense eosinophilic infiltrates along with extensive goblet cell hyperplasia, severe mucus plugging and mast cell infiltration. To establish the disease pathology, comparative analyses of hematology, histopathology, and gene expression were performed in Yama and BALB/c mice following *Aspergillus* antigen sensitization and challenge to induce experimental ABPA.

MATERIALS AND METHODS

Animal models

This study utilized 6-week-old female Yama mice (ICR), which were kept in our laboratory, along with BALB/c mice purchased from CLEA Japan, Inc. (Tokyo, Japan). BALB mice, the most commonly used ABPA model, served as the control group. The study included a total of 24 mice, which were randomly assigned to four groups ($n = 6$ per group). All the animals were housed at the Advanced Research Center for Laboratory Animal Science (ARCLAS), Yamaguchi University. The animals were maintained under the conventional environment in polycarbonate cages (CL-0138) with EcoChip bedding (CLEA) and mouse igloos. They were kept in a controlled environment ($24 \pm 2^{\circ}\text{C}$, 50–70% humidity) with a 12-hr light/dark cycle. They were provided with standard rodent chow (CE-2, CLEA) and tap water *ad libitum*. All experimental protocols complied with the institutional animal care guidelines and were approved by the Animal Research Committee of Yamaguchi University (Approval Number 589).

Sensitization and challenge

The sensitization protocol involved intraperitoneal injections of 1 μg of recombinant Asp f1 antigen (Indoor Biotechnologies®, Charlottesville, MA, USA) combined with 2 mg of aluminum hydroxide (Sigma-Aldrich, Saint Louis, MO, USA) in 150 μL of PBS on days 0 and 7. Control mice received PBS only. For the challenge, mice were administered 5 μg of Asp f1 in 50 μL of saline intranasally on days 14, 15, and 16. Control mice received saline alone. All

intranasal administrations were performed under anesthesia using a mixture of 10 mg/kg xylazine (Bayer, Tokyo, Japan) and 80 mg/kg ketamine (Daiichi Sankyo Co., Ltd., Tokyo, Japan). Sensitization and challenge dose selection were based on prior reports (Garcia *et al.*, 2010). All mice were euthanized at the end of the experiment (day 23). Euthanasia was performed under anesthesia using a mixture of 10 mg/kg xylazine (Bayer) and 80 mg/kg ketamine (Daiichi Sankyo Co., Ltd.) with cervical dislocation. The study protocol and schedule are shown in Fig. 1A.

Peripheral eosinophil count

Blood samples were collected on days 0, 14, and 23, and subsequently processed using Hinkelman's staining solution, in accordance with previously described methods (Morimoto *et al.*, 1998). The stained samples were examined under a light microscope, and eosinophils were manually counted using a hemocytometric counting chamber (Erma Sales Co., Ltd., Saitama, Japan).

Histopathological analysis

The mice were euthanized seven days after the final antigen challenge. Lung samples were frozen for subsequent RNA extraction, whereas the remaining tissues were fixed in 10% neutral-buffered formalin and processed routinely for paraffin embedding. Four-micrometer-thick sections were stained with hematoxylin-eosin (HE), alcian blue-periodic acid-Schiff (AB-PAS), and picrosirius red (PSR) (ScyTek Laboratories, Inc., Logan, UT, USA). Goblet cell quantification was performed by counting positively stained cells in five randomly

selected high-power fields (40× magnification) on AB-PAS–stained lung sections, with goblet cells identified based on combined AB-PAS positivity, indicating acidic and neutral mucins. Collagen accumulation was assessed by quantifying the percentage of Picrosirius red–positive areas in lung sections, representing collagen deposition, using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry was performed using anti-mouse eosinophil cationic protein (ECP) IgG (diluted 1:400; Avicera Bioscience Inc., Santa Clara, CA, USA) and anti-human CD117/c-kit (diluted 1:800; Agilent Technologies Inc., Santa Clara, CA, USA). Antigen retrieval was performed using 0.1% trypsin for ECP, and heat-induced epitope retrieval pH 6 for CD117/c-kit. ECP-positive eosinophils and c-kit/CD117-positive mast cells were quantified by counting positively stained cells in five randomly selected high-power fields (40× magnification) in lung sections.

Measurement of serum IgE

Serum was isolated from blood samples collected on day 23 from the Yama and BALB/c mice by centrifugation at 2,000 x g for 10 min at 4°C (Yamada *et al.*, 2024; Yamada *et al.*, 2025). Thereafter, the serum IgE levels were quantified using an LBIS™ Mouse IgE ELISA kit (Fujifilm Wako Pure Chemical, Osaka, Japan), as per the manufacturer's guidelines.

Gene expression analysis

Total RNA was extracted from frozen lung tissues using RNeasy® Plus Mini Kit (QIAGEN, Tokyo, Japan), according to the manufacturer's protocol. The samples were incubated at 37°C for 60 min, followed by 95°C for 5 min, and subsequently on ice for 5 min to create cDNA using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Target genes included: interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-13 (IL-13), transforming growth factor- β (TGF- β), and Colla1 mRNA. The target genes were quantified using 1 μ L cDNA samples amplified by TaqMan® Gene Expression Assays (IL-4: Mm00445259-m1, IL-5: Mm00439646-m1, IL-6: Mm0046190-m1, IL-13: Mm00434204-m1, TGF- β : Mm01178820-m1, Colla1: Mm00801632-gH, Thermo Fisher Scientific Inc., Waltham, MA, USA) and a CFX96 Touch Real-Time PCR Detection system (Biorad Laboratories, CA, USA). For amplification, the following cycle was repeated 60 times: 95°C, 1 min; 95°C, 15 sec; and 60°C, 1 min. Housekeeping genes 18S rRNA (Mm03928990-g1, Thermo Fisher Scientific Inc., Waltham, MA, USA) were used and subjected to standardization with IL-4, IL-5, IL-6, IL-13, TGF- β , and Colla1 (Yamada *et al.*, 2024; Yamada *et al.*, 2025).

Statistical analysis

Statistical analyses were performed using JMP Pro software version 15 (SAS Institute, Tokyo, Japan). Data are presented as mean $\pm$ standard deviation (SD). Comparisons between two groups were conducted using the Student's *t*-test, whereas comparisons among multiple groups or time points were performed using

one-way ANOVA followed by Tukey's honestly significant difference (HSD) test for multiple comparisons, as appropriate. A p -value < 0.05 was considered statistically significant.

RESULTS

Peripheral blood eosinophils and serum IgE

At baseline (day 0), the Yama mice exhibited significantly higher eosinophil counts than the BALB/c control mice ($p < 0.05$). Following Asp f1 sensitization and challenge, the eosinophil counts increased markedly in the Yama mice on day 14 and subsequently returned to baseline values (Fig. 1B). Serum total IgE levels were elevated following Asp f1 exposure in both BALB/c and Yama mice, and were almost 2-fold higher than those in the control mice (Fig. 1C).

Histopathological analysis

Histopathological analysis with HE staining revealed peribronchial and interstitial inflammatory infiltrates in the Asp f1-challenged Yama mice, comprising eosinophils and mononuclear cells (Fig. 2). Conversely, the BALB/c mice exhibited modest peribronchial inflammation of mononuclear cells and eosinophils with interstitial involvement. The saline-treated controls of both the strains demonstrated no significant histopathological changes, thus confirming the specificity of the Asp f1-induced response. Furthermore, AB-PAS staining revealed prominent goblet cell hyperplasia, along with luminal mucus accumulation in the bronchi of Asp f1-challenged Yama mice. In contrast, the Asp f1-challenged BALB/c mice exhibited only mild variations in their goblet cells. However, the saline-treated control groups of both the strains exhibited minimal to no goblet cell hyperplasia (Fig. 3).

Moreover, PSR staining revealed moderate collagen deposition in the lungs of the Asp f1-challenged Yama mice, which was consistent with advanced fibrotic remodeling. In contrast, the BALB/c mice exhibited mild fibrosis, whereas the saline-treated control groups of both the strains demonstrated minimal collagen accumulation (Fig. 4). Additionally, ECP staining revealed marked eosinophil infiltration in the lungs of the Asp f1-challenged Yama mice. However, compared to them, the BALB/c group exhibited minimal eosinophil infiltration (Fig. 5).

CD117/c-kit immunohistochemistry further revealed distinct strain-dependent responses. In BALB/c mice, the number and distribution of mast cells showed fair differences between Asp f1-challenged and control groups. In contrast, Asp f1-challenged Yama mice exhibited a significant increase in mast cell numbers around the bronchi and within the peribronchial interstitial. This accumulation indicates mast cell activation in association with eosinophilic and Th2-dominant inflammation. (Fig. 6).

Expression of cytokines

The relative IL-4, IL-5, IL-6, IL-13, TGF- β , and Colla1 mRNA expression in the BALB/c-saline group was set as the baseline. In comparison to this value, substantial upregulation of IL-4 was observed in the BALB/c-Asp f1 group. Interestingly, the Yama-saline group demonstrated increased relative IL-4 expression even without antigen challenge, which further increased upon Asp f1 administration in the Yama-Asp f1 group ($p < 0.05$) (Fig. 7A). Conversely IL-5 expression exhibited a slight decrease in the BALB/c-Asp f1 group. The Yama-

saline group displayed an expression level comparable to that of the BALB/c-saline group. In contrast, the Yama-Asp fl group demonstrated notable upregulation of IL-5 expression ($p<0.05$) (Fig. 7B). The relative IL-6 expression in the BALB/c-Asp fl group demonstrated moderate elevation. Additionally, a mild increase was observed in the Yama-saline group, with the highest expression in the Yama-Asp fl group ($p<0.05$) (Fig. 7C). IL-13 expression showed slightly increase in the Yama-saline group. IL-13 expression was also significantly elevated in both strains after Asp fl sensitization and challenge. However, the increase was markedly higher in Yama mice than in BALB/c mice ($p<0.05$) (Fig. 7D).

The TGF- β relative expression was significantly upregulated in the BALB/c-Asp fl group ($p<0.05$). In contrast, expression in the Yama-saline group was moderately reduced and only modestly elevated following antigen exposure in the Yama-Asp fl group (Fig. 7E). Moreover, the relative Col1a1 expression was moderately increased in the BALB/c-Asp fl group. Furthermore, the relative expression levels remained near baseline in the Yama-saline group, whereas a pronounced upregulation was observed in the Yama-Asp fl group ($p<0.05$) (Fig. 7F).

DISCUSSION

The present study established a novel murine model of ABPA using eosinophilic Yama mice. This model successfully reproduced several key features of human ABPA—including eosinophilia, elevated IgE levels, mucus hypersecretion, and pulmonary fibrosis (Agarwal *et al.*, 2013; Luo *et al.*, 2024; McCrary, 2019; Yamada *et al.*, 2024). The Yama-Asp f1 group that was subjected to the Asp f1 allergen exhibited significantly higher peripheral eosinophil counts than other groups. This finding suggests that Yama mice may offer an advantage as a novel model for ABPA, given that eosinophilia is a hallmark hematological feature of the disease (Asano and Oguma, 2025). The highest eosinophil counts in the Asp f1-challenged groups of both the Yama and BALB/c mice were observed on day 14 as a characteristic response to ABPA. By day 23, the eosinophil levels in Yama mice had returned to baseline. At this stage, the most notable features that reflected both human and experimental ABPA were the movement of eosinophils from the blood into the airways and their infiltration into the lung interstitial. Wild-type mice exposed intranasally to *Af* antigens also develop eosinophilia in the lungs, blood, and bone marrow (Dietschmann *et al.*, 2020; Kay *et al.*, 2004; Kurup and Grunig, 2001). Notably, the eosinophil counts in the Yama mice were significantly higher than those in the BALB/c mice, thereby reflecting a more robust Th2-driven inflammatory response.

Furthermore, the total serum IgE levels were elevated following Asp f1 exposure in both the BALB/c and Yama mice. However, the control groups of both the strains maintained relatively low levels of IgE. In ABPA, *Af* exposure drives B

cells to undergo switch recombination for IgE production, which is mediated by cytokines such as IL-4 and IL-13 (Agarwal *et al.*, 2014; Asano and Oguma, 2025; Kurup and Grunig, 2001). The elevated IgE binds to high-affinity FcεRI receptors on mast cells and basophils, consequently initiating degranulation and the release of inflammatory mediators that lead to bronchoconstriction, mucus hypersecretion, and tissue remodeling (Possa *et al.*, 2013; Trivedi and Lloyd, 2007). Both the mouse strains exhibited a significant elevation in IgE levels at equivalent levels, following the Asp f1 challenge.

This discrepancy between IgE levels and disease severity parallels observations in human ABPA, wherein the total IgE concentration serves as a diagnostic marker of acute disease activity but does not reliably indicate progression to fibrotic stages (Agarwal *et al.*, 2013; Agarwal *et al.*, 2014). In patients, the IgE levels typically plateau or decline once irreversible tissue remodeling occurs. At this stage, fibrosis is primarily driven by persistent eosinophilic inflammation and profibrotic signaling, rather than by active IgE-mediated hypersensitivity (Agarwal *et al.*, 2013; Agarwal *et al.*, 2014; Knutsen and Slavin, 2011). The findings indicate that elevated total IgE levels mainly reflect acute hypersensitivity responses. The progression to severe fibrotic pathology in Yama mice appears to be driven by persistent eosinophilic inflammation and subsequent profibrotic mechanisms rather than by IgE-mediated effects alone (Agarwal *et al.*, 2013; Garcia *et al.*, 2010; Knutsen and Slavin, 2011).

In this study, the Asp f1-challenge induced stronger Th2 responses airway remodeling, including peribronchial-interstitial inflammation, goblet cell

hyperplasia, lung fibrosis, and mast cell infiltration (Takazono and Sheppard, 2017; Tracy *et al.*, 2016). The Asp f1-challenged Yama mice exhibited more prominent airway changes, dense eosinophil and mononuclear cell infiltration—primary lymphocytes—and mast cell infiltration. In contrast, the BALB/c mice exhibited milder changes with moderate interstitial involvement with the same inflammatory cells as Yama mice. The control groups displayed no significant inflammation, thus confirming that these airway alterations were induced explicitly by Asp f1 exposure (Possa *et al.*, 2013; Trivedi and Lloyd, 2007). AB-PAS staining further demonstrated extensive goblet cell hyperplasia and luminal mucus accumulation in the Asp f1-challenged Yama mice, which was markedly greater than that observed in the BALB/c mice. This pathological change may be related to *Af*-derived proteases, which can damage airway epithelial cells and trigger the release of proinflammatory cytokines, consequently increasing mucus production (Davis and Wypych, 2021; De-Luca *et al.*, 2017; Hogaboam *et al.*, 2000).

Importantly, the severity of the pathological changes in the airways observed in the Yama mice following Asp f1 challenge was significantly greater than that in the BALB/c mice, thereby demonstrating enhanced immunopathological reactivity in this eosinophilic background of the former group. This elevated eosinophil response is consistent with the role of eosinophils in ABPA pathogenesis, particularly in the release of profibrotic mediators, such as ECP (Asano and Oguma, 2025; Kay *et al.*, 2004; Possa *et al.*, 2013; Ueki *et al.*, 2018). Our findings further support this theory, as ECP immunostaining demonstrated significantly greater eosinophilic infiltration of the lungs in the Yama-Asp f1 mice than in the Yama-

saline controls, and even lower counts in both the BALB/c groups. The release of proinflammatory mediators and epithelial damage caused by *Af*-derived proteases exacerbates inflammation and increases the exposure of the immune system to *Af*-associated allergens, thereby triggering a strong Th2-type immune response (Agarwal *et al.*, 2014; Dietschmann *et al.*, 2020; Luo *et al.*, 2024). Together, the *Af* antigens and hypersensitivity likely contribute to the development of progressive fibrosis (Trivedi and Lloyd, 2007; Ueki *et al.*, 2018; Yamada *et al.*, 2024).

In the Yama mice, Asp f1 challenge markedly upregulated IL-4, IL-5, IL-6, and IL-13 mRNA expression relative to the baseline, which was consistent with an immunological response to *Af* (Agarwal *et al.*, 2014; Luo *et al.*, 2024; Moss, 2005; Nikaein *et al.*, 2015). The Asp f1-challenged BALB/c mice also exhibited elevated levels of these cytokines compared with those in the saline group. However, this increase was less pronounced than in the Yama mice, which further supports the use of Yama mice as a more reliable ABPA model. These results also confirm that ABPA pathogenesis is mediated by the Th2 response (Agarwal *et al.*, 2014; Knutsen, 2006; Kurup and Grunig, 2001; Oshero, 2012). Moreover, IL-4 promotes IgE production and allergic inflammation, and myofibroblast activation via the IL-4R α /STAT6 pathway independently of TGF- β (De-luca *et al.*, 2017; Kurup and Grunig, 2001; Luo *et al.*, 2024). IL-5 facilitates eosinophil activation and recruitment, whereas IL-6 sustains inflammation and contributes to airway remodeling (Luo *et al.*, 2024). IL-13 expression was also significantly elevated in both strains after Asp f1 sensitization and challenge, with a greater increase in Yama mice. IL-13 acts synergistically with IL-4 to promote mucus hypersecretion,

airway remodeling, and fibrotic changes by stimulating fibroblast activation and extracellular matrix production (De-luca *et al.*, 2017; Kurup and Grunig, 2001; Moss, 2005). The more pronounced IL-13 response in Yama mice may therefore explain the enhanced airway remodeling and collagen deposition observed in this strain. Collectively, these cytokines orchestrate the allergic and inflammatory processes central to ABPA pathogenesis.

TGF- β mRNA expression demonstrated distinct patterns between the two mouse strains in this study. In BALB/c mice, Asp f1 challenge resulted in a marked upregulation of TGF- β , which aligns with its established function as a central profibrotic mediator that promotes fibroblast activation and extracellular matrix deposition (Yamada *et al.*, 2024; Yang *et al.*, 2022). In contrast, Yama mice exhibited a relative downregulation of TGF- β expression, despite displaying more severe fibrotic remodeling characterized by extensive collagen deposition and Col1a1 overexpression. This unexpected discrepancy indicates that alternative or complementary pathways may mediate fibrosis in Yama mice. Specifically, eosinophil-mediated mechanisms involving cytokines such as IL-4, IL-5, and IL-13, as well as effector molecules including eosinophil cationic protein and osteopontin, may sustain fibrogenesis independently of TGF- β (Yamada *et al.*, 2024; Yang *et al.*, 2022). Furthermore, histological assessment revealed that mast cell numbers were significantly increased in Asp f1-challenged Yama mice but only a few in BALB/c mice. Activated mast cells can release histamine, proteases, and profibrotic cytokines such as IL-13 and TGF- β , thereby amplifying Th2-type inflammation and promoting airway remodeling (Kurup and Grunig, 2001; Trivedi

and Lloyd, 2007). This selective mast cell activation in Yama mice may therefore further contribute to the IL-13–dominant fibrotic response observed in this model. The contribution of osteopontin requires further exploration in the future.

Previous ABPA models relied on repetitive exposure to fungal conidia or sensitization to crude extracts (Hogaboam *et al.*, 2024; Roboubi *et al.*, 2023; Takazono and Sheppard, 2017). Recombinant Asp f1 is a defined allergen with improved reproducibility and specificity, compared to the crude extracts used formerly. The spontaneous eosinophilic phenotype of the Yama mice eliminates the requirement for IL-5 induction or exogenous cytokine administration, thereby enabling the study of eosinophil-driven pathologies under physiologically relevant conditions. Moreover, the rapid induction of both eosinophilic inflammation and fibrotic remodeling following sensitization and challenge underscores the reliability and efficiency of this model in studying ABPA pathogenesis (Asano *et al.*, 2018; Yamada *et al.*, 2025; Yamada *et al.*, 2024).

However, this model does not completely reproduce the chronic fungal colonization characteristic of human ABPA, as it employs a recombinant antigen instead of live *Af* (Garcia *et al.*, 2010; Knutsen and Slavin, 2011; Kumar and Foster, 2002; Namvar *et al.*, 2022). Future studies incorporating chronic fungal exposure are required to validate the applicability of this model to long-term disease progression. Although Yama mice provide a unique opportunity to explore IL-5-independent eosinophilia (Yamada *et al.*, 2024), the precise mechanisms underlying their spontaneous phenotypes remain incompletely characterized (Yamada *et al.*, 2025).

In summary, this study established the Yama mouse as a promising preclinical model for ABPA research. The inherent eosinophilia of this strain, along with its exaggerated fibrotic and Th2 responses to Asp f1 challenge, closely mirror the immunopathological features of severe human ABPA. Consequently, this model provides a robust platform for elucidating the mechanisms of eosinophil-driven lung fibrosis and evaluating the hypersensitivity responses that initiate and exacerbate ABPA pathogenesis.

FIGURES

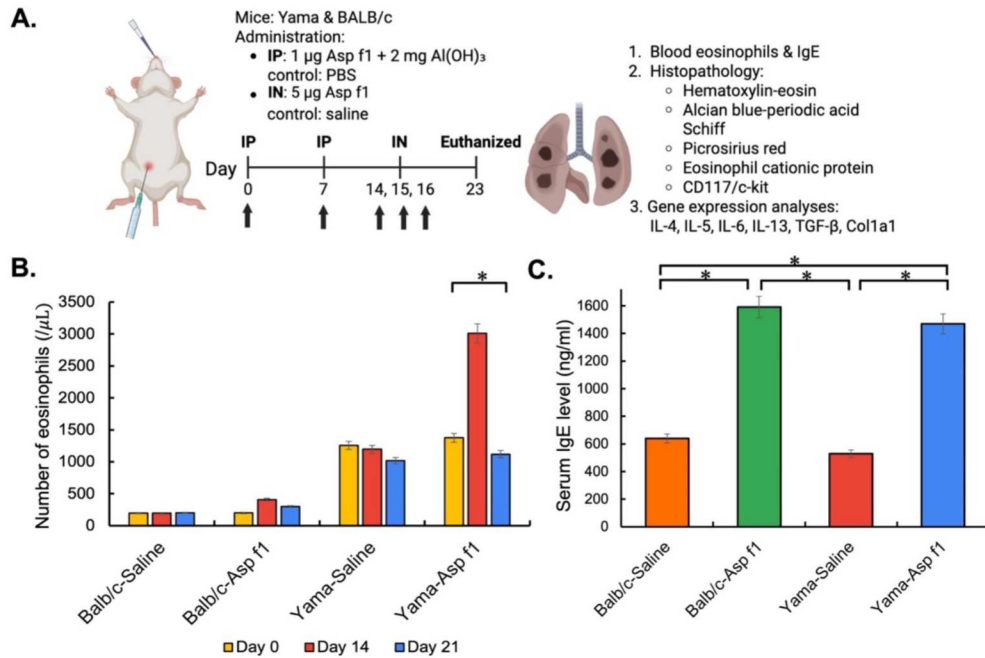


Figure 1. (A) Schematic illustration of the experimental protocol showing intraperitoneal (IP) sensitization and intranasal (IN) challenge. (B) The number of eosinophils in the peripheral blood prior to and following the experiment (n=6). Data are presented as mean $\pm$ SD (*p<0.05). (C) Serum IgE levels determined by ELISA (n=5). Data are presented as mean $\pm$ SD (*p<0.05).

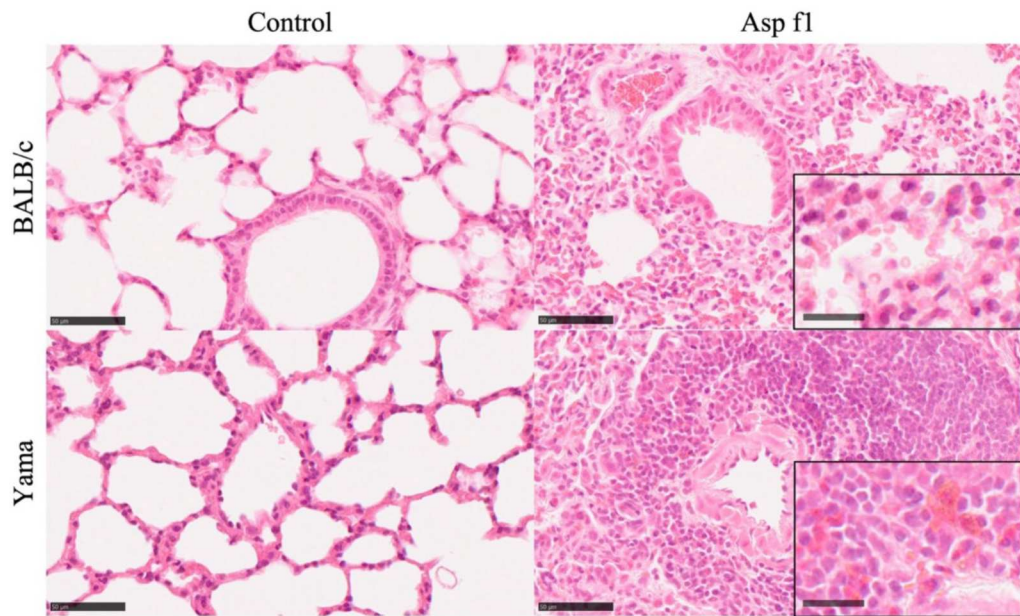


Figure 2. Histological analysis of lung sections from the Asp fl-challenged and saline-treated BALB/c and Yama mice stained with hematoxylin and eosin. The bars are 50 µm, and the inset bars are 25 µm.

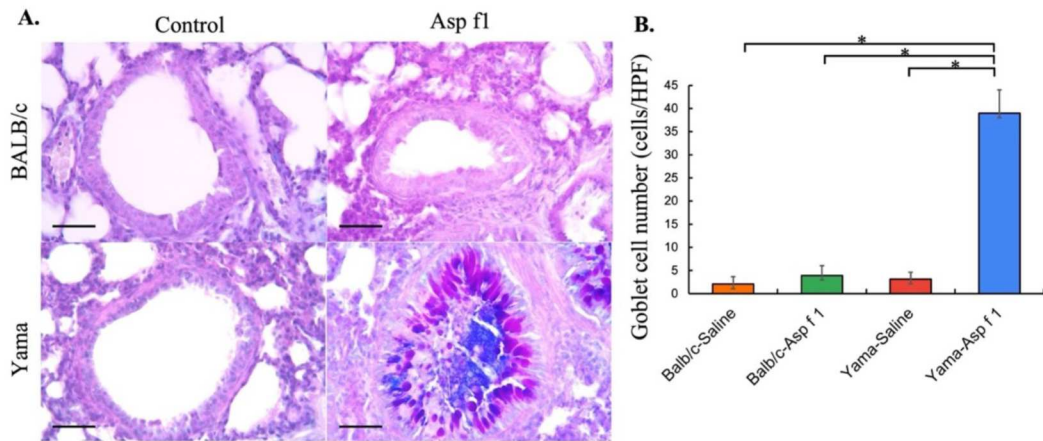


Figure 3. (A) Representation of goblet cell hyperplasia via alcian blue-periodic acid-Schiff's mucus staining and goblet cell counting. Bars=50 μ m. (B) Goblet cell numbers in the Yama-Asp-f1 mice were significantly higher than in the other groups (*p<0.05).

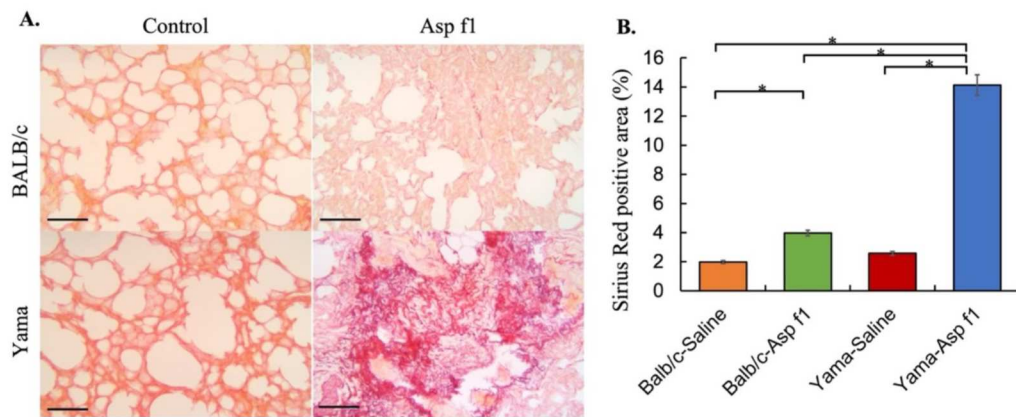


Figure 4. (A) Representation of picrosirius red (PSR) staining of the lungs from both Yama and BALB/c mice (Asp f1-treated as well as controls). Bars=50 μm. (B) Ratio of PSR-positive area of the lung (n=6). Data are presented as mean ±SD (*p<0.05).

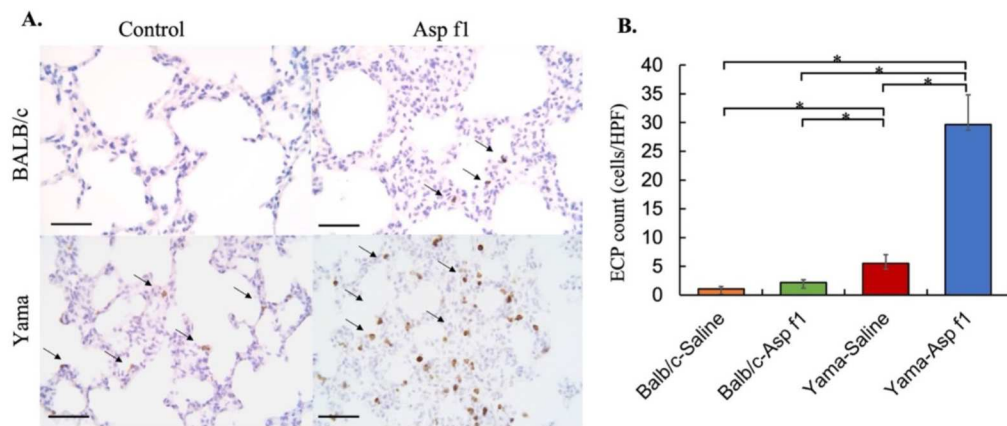


Figure 5. (A) Representation of eosinophilic cationic protein (ECP) immunohistochemistry of the lungs from both Yama and BALB/c mice (Asp f1-treated as well as controls). Arrows indicate ECP-positive eosinophils. Bars=50 μ m. (B) The number of eosinophils infiltration in the lung (n=6). Data are presented as mean $\pm$ SD (*p<0.05).

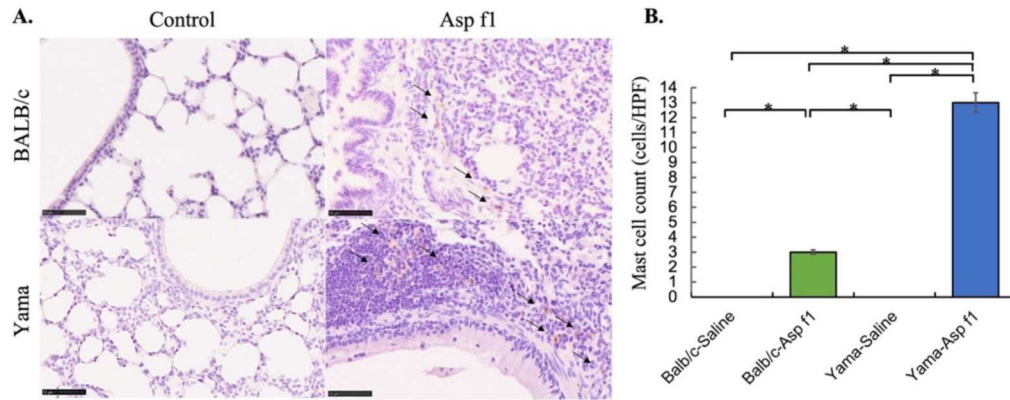


Figure 6. (A) Representation of CD117/c-kit immunohistochemistry of the lungs from both Yama and BALB/c mice (Asp fl-treated as well as controls). Arrows indicate CD117/c-kit-positive mast cells. Bars=50 μ m. (B) The number of mast cells infiltration in the lung (n=6). Data are presented as mean $\pm$ SD (*p<0.05).

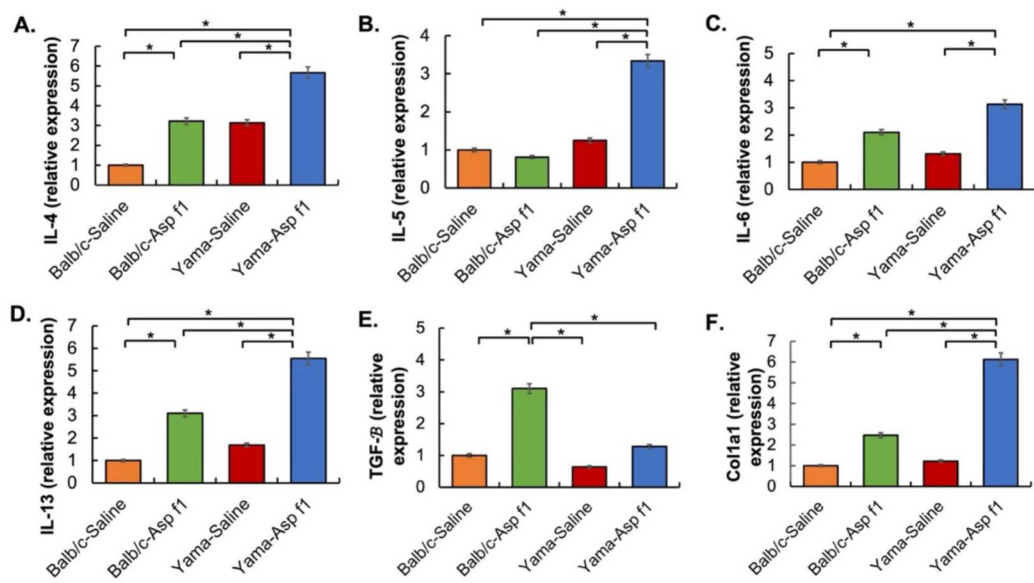


Figure 7. Relative expression of interleukin-4 (IL-4) (A), IL-5 (B), IL-6 (C), IL-13 (D), transforming growth factor- β (TGF- β) (E), and Col1a1 (F) in the lungs of both Yama and BALB/c mice (n=6). Data are presented as mean $\pm$ SD (*p<0.05).

CHAPTER 2

A novel murine model of idiopathic pulmonary fibrosis with persistent and marked features using Yama mice

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease characterized by excessive collagen deposition and irreversible scarring. Reliable animal models are essential for elucidating IPF pathogenesis and evaluating antifibrotic therapies. However, conventional bleomycin (BLM) models generally induce only transient and moderate fibrosis. Considering the profibrotic role of eosinophils, we developed a novel IPF model using the spontaneous eosinophilic mutant Yama mice strain to achieve persistent and severe fibrosis. Male Yama and C57BL/6 mice (6 weeks old, n=24) received eight intranasal administrations of BLM (2 mg/kg) at two-week intervals, while controls received saline. Peripheral eosinophil counts were monitored, and tissues were analyzed histologically and biochemically. Yama mice exhibited a sharp eosinophil peak after the first BLM exposure, followed by a gradual decline. Histological analysis revealed markedly enhanced fibrosis, greater collagen deposition, and higher Ashcroft scores in Yama mice compared with C57BL/6 mice. Alcian blue–PAS-positive fibroblast foci, a characteristic feature of human IPF, were abundant in Yama mice. Immunohistochemistry demonstrated increased expression of ECP. Moreover, mRNA expression of Th2 cytokines (IL-4, IL-5, IL-13) and profibrotic mediators (IL-6, Colla1) was significantly higher in Yama mice. This repeated BLM administration model successfully reproduces the key pathological features of severe IPF and emphasizes the pivotal role of eosinophils in fibrogenesis.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive interstitial lung disease with poor prognosis, showing a median survival of 3–5 years (Degryse *et al.*, 2010; Degryse and Lawson, 2011; Kondoh *et al.*, 2022; Raghu *et al.*, 2016). Patients typically present with dyspnea, cough, and progressive decline in pulmonary function, and its prevalence has been increasing globally, including in Japan (Kondoh *et al.*, 2022; Lederer and Martinez, 2018; Ley *et al.*, 2011; Wang *et al.*, 2025). Experimental models are crucial for understanding fibrotic mechanisms and evaluating potential therapies. Among these, administration of bleomycin (BLM) to the airway is the most commonly used model (Lederer and Martinez, 2018; Moore and Hogaboam, 2008; Redente *et al.*, 2021). However, the conventional single-dose BLM-induced murine model generally produces only moderate and transient fibrosis, failing to replicate the chronic and progressive remodeling characteristic of human IPF (Degryse and Lawson, 2011; Lee *et al.*, 2025; Moore and Hogaboam, 2008; Song *et al.*, 2022).

The pathogenesis of IPF involves repetitive alveolar epithelial injury, aberrant repair processes, and excessive extracellular matrix (ECM) accumulation, ultimately resulting in structural distortion and irreversible lung dysfunction (Lederer and Martinez, 2018; She *et al.*, 2021; Wijsenbeek and Cottin, 2020). While fibrotic lesions with collagen overexpression are defined by histological features, they are not faithfully reproduced in traditional BLM models (Gul *et al.*, 2023; Redente *et al.*, 2021; Song *et al.*, 2022). Despite their widespread use in preclinical studies, including evaluations of antifibrotic agents such as nintedanib

(Amati *et al.*, 2023; Guo *et al.*, 2023; Miao *et al.*, 2023), existing mouse models remain insufficient.

A novel animal model is needed to more accurately reflect the complex and progressive pathogenesis of IPF (Degryse *et al.*, 2010; Degryse and Lawson, 2011; Gul *et al.*, 2023; Yamaguchi *et al.*, 2017). In conventional repetitive BLM administration models, fibrosis can be sustained for a longer period than single-dose protocols; however, several studies have noted that these models still exhibit partial spontaneous resolution and do not fully recapitulate the progressive and irreversible features of human IPF (Degryse *et al.*, 2010; Redente *et al.*, 2021). Emerging evidence suggests that eosinophils are involved in fibrosis, and several studies have reported a correlation between eosinophil activity and fibrosis progression (Aceves, 2014; Adamko *et al.*, 2013; Fulkerson and Rothenberg, 2023). Eosinophils release profibrotic cytokines such as TGF- β and IL-4, which stimulate fibroblast activation and ECM deposition (Akuhota and Weller, 2012; Al-muhsen *et al.*, 2013; Fulkerson and Rothenberg, 2023).

Yama mice possess a unique genetic background characterized by persistent eosinophilia without IL-5 overexpression (Yamada *et al.*, 2025), which has been implicated in tissue remodeling and the amplification of fibrotic responses (Yamada *et al.*, 2024). This eosinophil-driven environment may enable Yama mice to develop fibrosis that is more progressive and self-perpetuating than that observed in existing BLM-based models. Importantly, as demonstrated in this study, fibrosis in Yama mice continues to progress even after the final BLM administration, rather than ending ongoing spontaneous resolution. Therefore,

Yama mice may provide a more pathophysiology-relevant model for studying the persistent and progressive characteristics of human IPF.

MATERIALS AND METHODS

Animal models

Male Yama mice maintained in our laboratory and C57BL/6 mice obtained from CLEA Japan, Inc. (Tokyo, Japan) were used (6–8 weeks old). A total of 24 mice were included and randomly divided into four groups (n = 6 per group): C57BL/6-Saline, C57BL/6-BLM, Yama-Saline, and Yama-BLM. The control group was C57BL/6 mice, the most commonly used strain in BLM-induced IPF models. All animals were housed at the Advanced Research Center for Laboratory Animal Science (ARCLAS), Yamaguchi University, under conventional conditions. Environmental parameters were maintained at $24 \pm 2^{\circ}\text{C}$ and 50–70% humidity, with a 12-hour light/dark cycle. Standard rodent chow (CE-2, CLEA Japan Inc.) and tap water were provided *ad libitum*. All experimental procedures were conducted in accordance with institutional guidelines for animal care and were approved by the Animal Research Committee of Yamaguchi University (Approval Number 589). As part of animal welfare measures, mice exhibiting coughing due to lung injury were euthanized.

Bleomycin administration

Experimental groups received eight intranasal administrations of BLM (2 mg/kg body weight) (Cayman Chemical Co., Ann Arbor, MI, USA) every two weeks, while the control group received saline. The dose of BLM was based on a previous report (Redente *et al.*, 2021). The intranasal administration is less invasive and easier to perform repeatedly (Santa *et al.*, 2023). All intranasal administrations were performed under anesthesia using a mixture of xylazine 10

mg/kg body weight (Bayer, Tokyo, Japan) and ketamine 80 mg/kg body weight (Daiichi Sankyo Co., Ltd., Tokyo, Japan). All mice were euthanized at the end of the experiment (14 days after the final administration) under anesthesia with a combination of xylazine 10 mg/kg body weight and ketamine 80 mg/kg body weight, with cervical dislocation. Mice were euthanized 14 days after the last BLM administration to analyze the established fibrotic phase, which normally develops after day 14 of the last dose in BLM-induced lung injury, as well as to allow the acute inflammatory reaction to subside (Moore and Hogaboam, 2008). The overall study design is depicted in Fig. 1A.

Peripheral eosinophil count

Following the collection of blood samples one week after each treatment, the samples were then processed using Hinkelman's staining solution in accordance with previously described procedures (Morimoto *et al.*, 1998). Eosinophils were enumerated manually under a light microscope using a hemocytometer chamber (Erma Sales Co., Ltd., Saitama, Japan).

Histopathological analysis

The left lung lobe was fixed in 10% neutral-buffered formalin and routinely processed for paraffin embedding. Sections (4 μ m thick) were stained with hematoxylin and eosin (HE), alcian blue (pH 2.5)–periodic acid–Schiff (AB-PAS), and picrosirius red (PSR) (ScyTek Laboratories, Inc., Logan, UT, USA). PSR staining was used to evaluate collagen deposition, and the percentage of PSR-

positive area relative to the total lung area was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The Ashcroft scoring system was used to determine the severity of pulmonary fibrosis, in which histological changes are graded on a semiquantitative scale from 0 (normal lung architecture) to 8 (severe fibrotic distortion) based on the extent of alveolar wall thickening and fibrotic remodeling (Cui et al., 2003). Immunohistochemistry was performed using anti-mouse eosinophil cationic protein (ECP) IgG (diluted 1:400; Avicera Bioscience Inc., Santa Clara, CA, USA). A secondary antibody for ECP was used with a goat anti-rabbit polyclonal (Histofine® Simple Stain™ Max PO (R), Nichirei Biosciences Inc, Tokyo, Japan). Antigen retrieval was conducted by incubating tissue sections in 0.1% trypsin for ECP. The number of ECP-positive cells was counted in five randomly selected high-power fields (HPF; 400x magnification; 2.04 mm²). The histological and quantitative analyses were performed under blinded conditions.

Wet/dry weight ratio assay

The superior lobe lung was excised, rinsed of blood, and weighed immediately. Samples were dried in a drying oven at 60°C for 72hr, and the wet/dry weight ratio was calculated. The ratio was calculated as: (W/D ratio) = W (g)/D (g) × 100% (Miao *et al.*, 2023).

Measurement of hydroxyproline

Hydroxyproline content was quantified using the QuantiChrom™

Hydroxyproline Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Absorbance was measured at 560 nm using a 2030 Multilabel Reader ARVO X4 (PerkinElmer, Waltham, MA, USA). Hydroxyproline concentrations were determined from a standard curve and expressed as hydroxyproline $\mu\text{g/mL}$. Collagen conversions were determined 1 $\mu\text{g/mL}$ hydroxyproline is equivalent to 7.69 $\mu\text{g/mL}$ collagen.

Gene mRNA expression analysis

Total RNA was isolated from the remaining frozen lung tissues using RNeasy® Plus Mini Kit (QIAGEN, Tokyo, Japan), in accordance with the manufacturer's protocol. The samples were incubated at 37°C for 60 min, then subjected to 95°C for 5 min, and subsequently placed on ice for 5 min to synthesize cDNA using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The target genes comprised interleukin-4 mRNA (IL-4), interleukin-5 mRNA (IL-5), interleukin-6 mRNA (IL-6), interleukin-13 mRNA (IL-13), transforming growth factor- β mRNA (TGF- β), and Colla1 mRNA. The target genes were quantified with 1 μL of cDNA samples were amplified using TaqMan® Gene Expression Assays (IL-4: Mm00445259-m1, IL-5: Mm00439646-m1, IL-6: Mm0046190-m1, IL-13: Mm00434204-m1, TGF- β : Mm01178820-m1, Colla1: Mm00801632-gH, Thermo Fisher Scientific Inc., Waltham, MA, USA) and a CFX96 Touch Real-Time PCR Detection system (Biorad Laboratories, CA, USA). The subsequent cycle was performed 60 times for amplification. 95°C for 1 min; 95°C for 15 sec; and 60°C for 1 min. Housekeeping genes (18S rRNA)

(Mm03928990-g1, Thermo Fisher Scientific Inc., Waltham, MA, USA) were utilized and standardized with IL-5, IL-4, IL-6, IL-13, TGF- β , and Colla1. Relative mRNA expression level in the C57BL/6-saline group was used as the baseline.

Statistical analysis

Statistical analyses were performed using JMP Pro software version 15 (SAS Institute, Tokyo, Japan). Data are expressed as mean $\pm$ standard deviation (SD). Quantitative data were analyzed using statistical tests following confirmation of normality and homogeneity. Comparisons of peripheral blood eosinophil levels across multiple groups or time points were performed using ANOVA followed by Tukey's HSD post hoc test for multiple comparisons. Additionally, comparisons between two groups were conducted using the Student's *t*-test. A *p*-value <0.05 was considered statistically significant.

RESULTS

Peripheral blood eosinophils

At baseline (day 0), Yama mice exhibited significantly higher peripheral blood eosinophil counts compared with C57BL/6 control mice ($p<0.05$). Following BLM administration, blood eosinophils were measured biweekly. Yama-BLM mice demonstrated markedly elevated eosinophil counts compared to C57BL/6-BLM mice. In Yama-BLM mice, eosinophil counts peaked at week 2 and gradually decreased toward the end of the examination. In contrast, C57BL/6-BLM mice showed a modest increase at week 2, returning to near baseline levels by the end of the observation period (Fig. 1B).

Histopathological analysis and hydroxyproline content

Histopathological analysis of lung sections from the Yama and C57BL/6 mice stained with HE staining. Both control C57BL/6 and Yama mice show normal alveolar architecture with thin septa and minimal inflammation. C57BL/6-BLM mice showed moderate interstitial thickening and inflammatory infiltration involving both peribronchiolar and interstitial areas. Yama-BLM mice demonstrate extensive and diffuse pulmonary fibrosis involving both peribronchiolar and interstitial areas, with marked interstitial fibrosis, collapse of alveolar structures, and dense inflammatory cell infiltration (Fig. 2). BLM-treated Yama mice, numerous AB–PAS-positive fibroblast foci were observed along the alveolar walls. In contrast, the BLM-treated C57BL/6 mice exhibited only a few in fibroblast foci. However, the saline-treated control groups of both strains

exhibited minimal to no fibroblast foci (Fig. 3A). Ashcroft's scoring confirmed significantly greater fibrosis severity in Yama mice than C57BL/6 mice (Fig. 3B).

PSR staining revealed significantly higher collagen deposition and collagen-positive area as a percentage of total lung area (%) of the BLM-treated Yama mice, which was consistent with severe fibrotic remodeling. In contrast, the C57BL/6 mice exhibited mild collagen deposition, whereas the saline-treated control groups of both strains demonstrated minimal collagen accumulation (Fig. 4A-B). Yama mice exhibited a greater increase in the wet/dry ratio following BLM administration compared to C57BL/6 mice (Fig. 4C). Hydroxyproline assays further demonstrated that Yama-BLM mice correspond with greater collagen deposition compared with C57BL/6-BLM mice (Fig. 4D-E). Immunohistochemical analysis of ECP revealed marked eosinophil infiltration in the lungs of the BLM-treated Yama mice (Fig. 5A). However, the C57BL/6 group exhibited minimal eosinophil infiltration (Fig. 5B).

Expression of cytokines

Relative mRNA expression level of IL-4, IL-5, IL-6, IL-13, TGF- β , and Colla1 in the C57BL/6-saline group was used as the baseline. BLM treatment increased IL-4 mRNA expression by 1.31-fold was identified in the C57BL/6-BLM group. Furthermore, the Yama-saline group displayed enhanced relative IL-4 mRNA expression even without treatment (1.45-fold), which increased 2.84-fold upon BLM injection in the Yama-BLM group ($p<0.05$) (Fig. 6A). Relative IL-5 mRNA expression demonstrated a modest increase in the C57BL/6-BLM group

(1.69-fold). The Yama-saline group presented an mRNA expression level comparable to that of the C57BL/6-BLM group (1.77-fold). In contrast, the Yama-BLM group observed a remarkable 4.36-fold increase in IL-5 mRNA expression ($p<0.05$) (Fig. 6B). IL-6 mRNA expression in the C57BL/6-BLM group exhibited an elevation (2.42-fold). The Yama-saline group already showed an enhanced baseline (3.45-fold), with the largest relative mRNA expression detected in the Yama-BLM group (7.06-fold, $p<0.05$) (Fig. 6C).

BLM treatment boosted IL-13 mRNA expression in both Yama and C57BL/6 mice, respectively (3.65-fold and 5.13-fold). The Yama-saline group demonstrated a slightly increased mRNA expression 1.42-fold (Fig. 6D). The TGF- β mRNA expression in the C57BL/6-BLM mice was significantly upregulated (3.48-fold, $p<0.05$). While the Yama-saline group showed slightly lower baseline levels (0.96-fold) and moderate elevation in the Yama-BLM group (2.40-fold) (Fig. 6E). Moreover, the relative Col1a1 mRNA expression was considerably enhanced in the C57BL/6-BLM group (2.54-fold). The relative mRNA expression levels modestly rose in the Yama-saline group (1.78-fold), however a considerable upregulation was found in the Yama-BLM group (9.20-fold, $p<0.05$) (Fig. 6F).

DISCUSSION

IPF is still one of the most severe interstitial lung diseases, and the therapeutic options remain very limited (Amati *et al.*, 2023; Miao *et al.*, 2023). The prognosis of patients is generally very poor, and therefore, the establishment of animal models that can faithfully reproduce the pathophysiology of IPF is strongly required (Guo *et al.*, 2023; Hutchinson *et al.*, 2015; Ley *et al.*, 2011). To date, the BLM-induced fibrosis model has been widely used. However, this model is well known to be transient and self-limiting (Herrera *et al.*, 2023; Lee *et al.*, 2025). For this reason, it does not reflect the progressive and irreversible fibrosis consistent with the irreversible fibrosis characteristic of human IPF (Lee *et al.*, 2025; Redente *et al.*, 2021; Song *et al.*, 2022). In other words, although the BLM model has contributed to fibrosis research, it still has a fundamental limitation (Redente *et al.*, 2021; Song *et al.*, 2022). Therefore, the development of new mouse models is necessary.

In the present study, we established a novel murine model of IPF using Yama mice, which exhibit spontaneous hypereosinophilia (Yamada *et al.*, 2024; Yamada *et al.*, 2025). Compared with the commonly used C57BL/6 mice, Yama mice showed much higher peripheral eosinophil counts at baseline and sustained eosinophilia after BLM administration (Yamada *et al.*, 2024; Yamada *et al.*, 2025). The eosinophil levels were likely to have returned to baseline by the end of the study because of eosinophil migration into the airways and infiltration of the lung interstitium. This feature is very important because eosinophils have repeatedly

been reported to be related to fibrotic diseases (Aceves, 2014; Adamko *et al.*, 2013; Akuhota and Weller, 2012). In fact, eosinophil infiltration into damaged lung tissue has been reported in BLM-induced pulmonary fibrosis (Adamko *et al.*, 2013; Akuhota and Weller, 2012; Cui *et al.*, 2003). It is known that eosinophils release several fibrogenic cytokines, such as TGF- β 1 and IL-4, and also cytotoxic proteins. These mediators aggravate alveolitis and epithelial injury, ultimately promoting tissue remodeling (Al-muhsen *et al.*, 2013; Cui *et al.*, 2003; Fulkerson and Rothenberg, 2023).

Therefore, the presence of eosinophilia in Yama mice can explain why they showed more severe fibrosis than C57BL/6 mice. In our experiment, eosinophil counts in Yama mice reached a peak at 2 weeks after BLM treatment and then decreased gradually. This finding is consistent with previous reports that eosinophils act as early responders to lung injury and release pro-fibrotic mediators, which form the basis of subsequent fibrosis (Al-muhsen *et al.*, 2013; Cui *et al.*, 2003; Fulkerson and Rothenberg, 2023). On the other hand, in C57BL/6 mice, eosinophil mobilization was very limited. This difference between the two strains may be one reason why Yama mice developed more severe fibrotic changes. In summary, it can be said that the inherent eosinophilia of Yama mice is one of the key elements of this model.

Histopathological evaluation also demonstrated that the Yama model reproduces the essential features of human IPF. In Yama mice, the lung exhibited diffuse interstitial thickening, collapse of alveolar structure, and marked infiltration of mononuclear cells and eosinophils (Redente *et al.*, 2021; Santa *et al.*,

2023; Song *et al.*, 2022; Yamaguchi *et al.*, 2017). Especially, Alcian blue–PAS–positive fibroblastic foci, which are known as the hallmark lesions of human IPF (Yamaguchi *et al.*, 2017), were abundant in Yama mice but only sporadic in C57BL/6 mice. Furthermore, marked eosinophil infiltration was confirmed by ECP staining, suggesting that immune effector cells and fibroblasts may interact to accelerate fibrosis (Aceves, 2014; Adamko *et al.*, 2013; Cui *et al.*, 2003). Taken together, these histological findings provide solid evidence that Yama mice reproduce the pathological hallmarks of human IPF.

Biochemical and histological analyses supported these observations. PSR staining and hydroxyproline assay, Yama-BLM mice showed significantly more collagen deposition than C57BL/6-BLM mice. This clearly indicates that the fibrotic process in Yama mice is more aggressive and sustained. Because excessive collagen accumulation is the most important characteristic of human IPF, the present finding strongly suggests that Yama mice more closely reproduce the continuous extracellular matrix remodeling observed in IPF patients (Degryse and Lawson, 2011; Guo *et al.*, 2023; Hutchinson *et al.*, 2015).

In addition, the lung wet/dry ratio was also increased in Yama mice. In acute models, such an increase is sometimes interpreted as pulmonary edema (Amati *et al.*, 2023; Miao *et al.*, 2023). However, in the present chronic setting, it is more reasonable to interpret the increase as reflecting a reduction of aerated lung volume and increased parenchymal density caused by collagen accumulation and tissue remodeling (Moore and Hogaboam, 2008; Raghu *et al.*, 2018; Redente *et al.*, 2021). Thus, both biochemical and physiological findings consistently suggest

that Yama mice develop more severe fibrosis than conventional strains.

Cytokine analysis further revealed important mechanistic differences. In Yama mice, IL-4 and IL-5 were elevated at baseline and further increased after BLM treatment. IL-13 was also strongly induced. These cytokines are known as typical Th2 cytokines, and together they promote eosinophilic inflammation, fibroblast activation, and collagen synthesis (Adamko *et al.*, 2013; Franzen *et al.*, 2024; Santa *et al.*, 2023; Wolters *et al.*, 2013). In addition, Yama mice showed persistently high IL-6 levels. IL-6 has been reported to stimulate fibroblast proliferation, promotes Th17 responses, and induces epithelial-to-mesenchymal transition (Al-muhsen *et al.*, 2013; Franzen *et al.*, 2024; Santa *et al.*, 2013; Yamada *et al.*, 2024). IL-6 elevation is also reported to be associated with poor prognosis in human IPF (Lederer and Martinez, 2018; Raghu *et al.*, 2016). Therefore, the cytokine environment in Yama mice may mimic the severe phenotype of IPF patients. In summary, Yama mice presented a cytokine profile characterized by a combination of Th2 dominance and IL-6 elevation, which may explain their severe phenotype.

One particularly interesting finding was the role of TGF- β . In C57BL/6 mice, BLM administration induced strong TGF- β expression, consistent with its well-established function as a key profibrotic cytokine (Adamko *et al.*, 2013; Franzen *et al.*, 2024; Kliment *et al.*, 2011; Santa *et al.*, 2023). However, in Yama mice, TGF- β expression increased only moderately despite the presence of more severe fibrosis and marked overexpression of Col1a1. This observation suggests that Yama mice may develop fibrosis through mechanisms that are not entirely

dependent on TGF- β signaling. This study did not assess downstream mediators like Smad2/3; however, a previous study indicates that TGF- β signaling may be subject to intrinsic negative-feedback control. The TGF- β promotes rapid activation of Smad3, followed by a gradual downregulation of Smad3 expression in lung fibroblasts, thus limiting further TGF- β signaling (Zhao and Gerverd, 2002). This process may partially figure out how TGF- β mRNA levels in our model exhibit a modest rise despite significant fibrosis. Similar findings, specifically the dissociation between TGF- β signaling and collagen accumulation, have been reported in other Yama-based disease models (Yamada *et al.*, 2024), reinforcing the notion that this strain exhibits a distinct regulatory profile in fibrogenesis.

Therefore, Th2-associated cytokines (IL-4 and IL-13) and IL-6-driven inflammatory signaling are possibly to serve as major drivers of enhanced fibrotic response in Yama mice. Alternative fibrosis-related pathways—such as Wnt/ β -catenin, Notch signaling, and osteopontin (Miao *et al.*, 2023; She *et al.*, 2021; Yamada *et al.*, 2024) may profoundly contribute to the extensive fibrosis evident in this model and require investigation in upcoming studies. Moreover, the characteristic hypereosinophilic response in Yama mice may create a highly profibrotic tissue microenvironment, facilitated by the release of eosinophil granule proteins, fibroblast activation, and modulation of tissue remodeling, ultimately resulting in more pronounced fibrosis despite the absence of strong TGF- β upregulation. Given that the pathogenic mechanisms of human IPF are heterogeneous, this result increases the translational value of Yama mice. Thus,

this model may reflect the diversity of pathogenic mechanisms seen in clinical cases.

In conclusion, Yama mice showed pulmonary fibrosis with severe extracellular matrix deposition, characteristic histological lesions, and a cytokine profile dominated by Th2 and IL-6. In C57BL/6 mice. While TGF- β is the main driver of fibrosis, immune-related pathways are important in Yama mice. This difference is highly relevant to clinical IPF, because patients' immune signatures vary, and responses to antifibrotic drugs are heterogeneous (Amati *et al.*, 2023; Miao *et al.*, 2023). Therefore, the Yama-BLM model is not only a more severe fibrosis model but also a model reflecting disease heterogeneity. It is a useful platform to investigate noncanonical mechanisms and to test new therapies targeting eosinophil and Th2 pathways. Finally, this model may facilitate identification of therapeutic targets for immune-modulating strategies in preclinical research and thus may contribute to expanding therapeutic options in IPF.

FIGURES

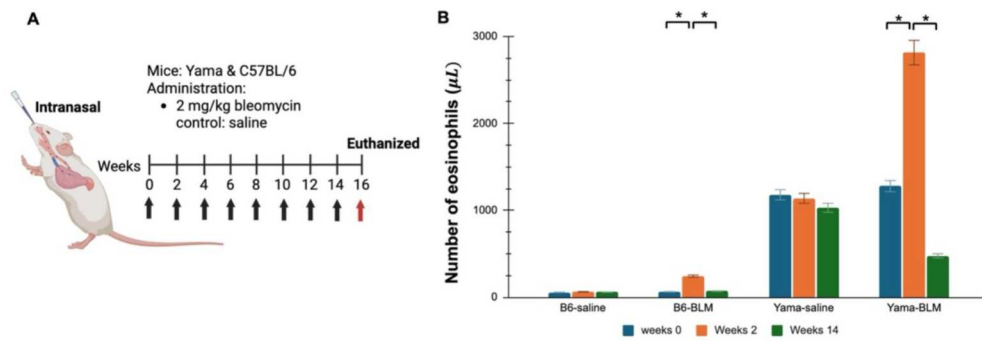


Figure 1. (A) Schematic illustration of the protocol and research schedule. (B) The number of eosinophils in the peripheral blood before and following the experiment (n=6). Data are presented as mean $\pm$ SD (* p <0.05).

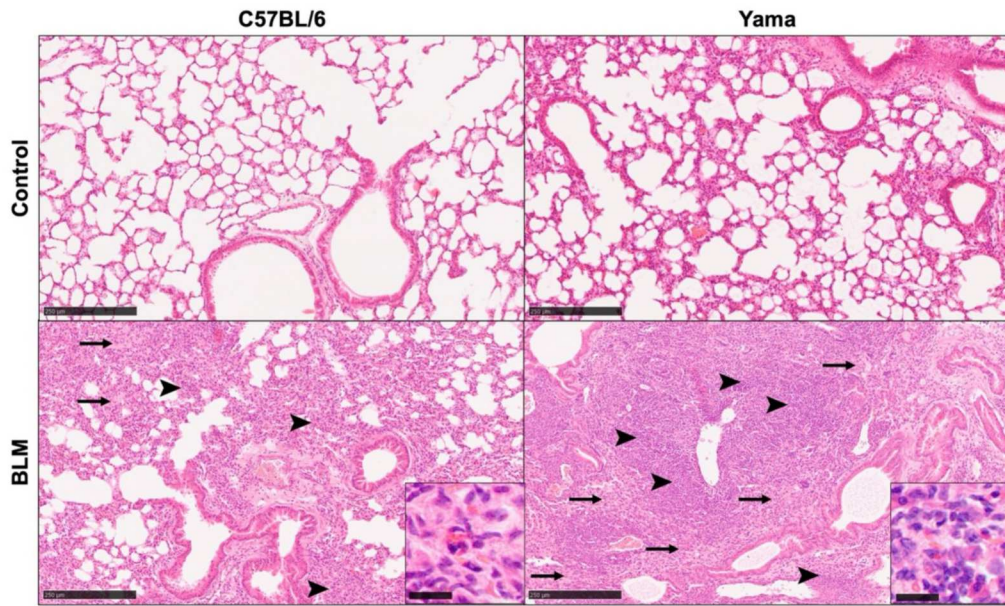


Figure 2. Histological analysis of lung sections from the BLM-challenged and saline-treated C57BL/6 and Yama mice stained with hematoxylin and eosin (HE). Saline-treated C57BL/6 and Yama mice exhibit normal alveolar architecture with thin septa and minimal inflammation. C57BL/6-BLM mice show moderate interstitial thickening (black arrows) with inflammatory infiltration (black arrowheads) in peribronchiolar and interstitial regions. Yama-BLM mice display extensive, diffuse pulmonary fibrosis with marked interstitial thickening (black arrows), alveolar collapse, and dense inflammatory cell infiltration (black arrowheads) throughout peribronchiolar and interstitial areas. The inset indicates eosinophil and neutrophil. The bars=250 μ m and the inset bars=25 μ m.

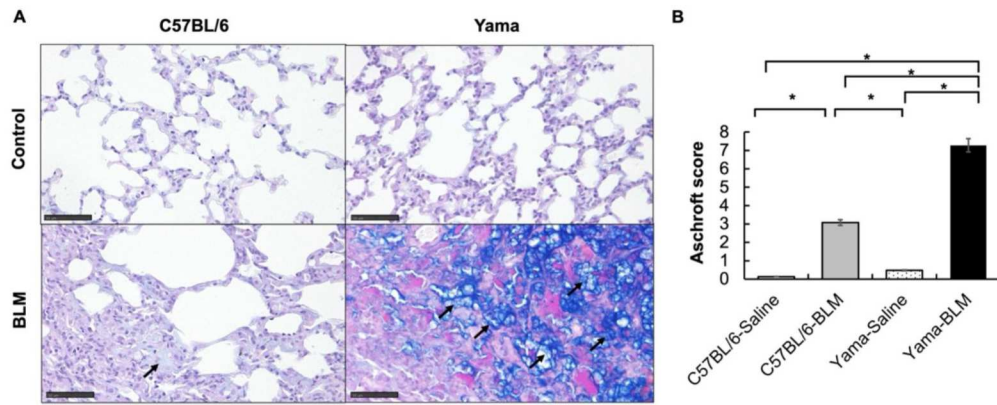


Figure 3. A. Representation of fibrotic foci via Alcian Blue-Periodic acid-Schiff's (AB-PAS) staining. B. Aschroft score represents C57BL/6 mice and Yama mice (n=6). Black arrows indicate the fibrotic foci. Data are presented as mean $\pm$ SD (* p <0.05). Bars=50 μ m.

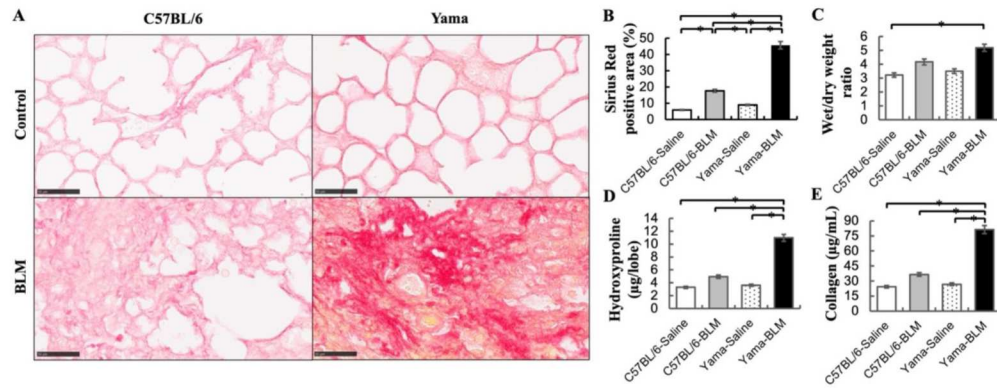


Figure 4. (A) Representation of Picrosirius red (PSR) staining of the lungs from both Yama and C57BL/6 (BLM-treated as well as controls). (B) Percentage of PSR-positive area. (C) Wet/dry weight lung ratio. (D) Hydroxyproline amount in μg/lobe. (E). Collagen amount in μg/mL. Bars=50 μm. (n=6). Data are presented as mean ±SD (* p <0.05).

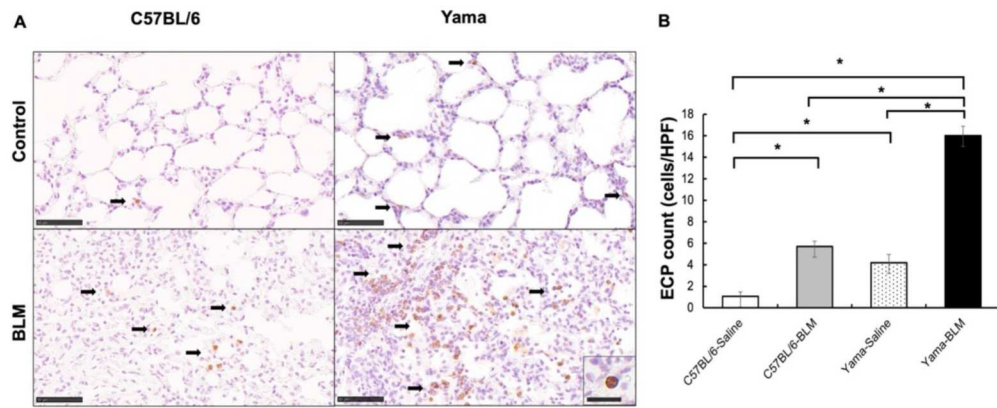


Figure 5. (A) Representation of eosinophilic cationic protein (ECP) immunohistochemistry of the lungs from both Yama and C57BL/6 mice (BLM-treated as well as controls). Black arrows indicate ECP-positive eosinophils (cytoplasm). Bars=50 μ m. (B) The number of eosinophils in the lung (n=6; five randomly selected HPF 400x). Data are presented as mean $\pm$ SD (* p <0.05).

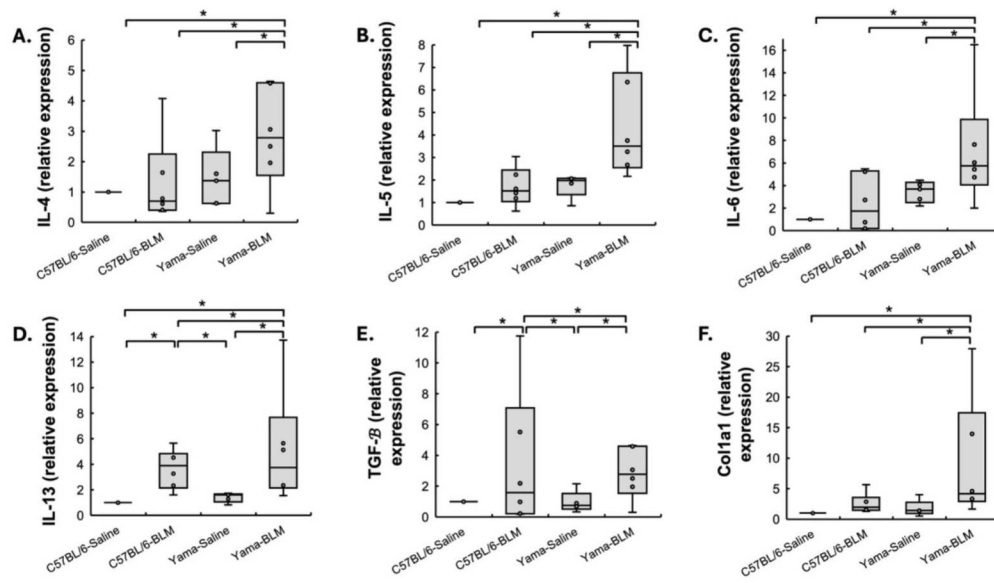


Fig. 6. Relative mRNA expression of interleukin-4 (IL-4) (A), IL-5 (B), IL-6 (C), IL-13 (D), transforming growth factor- β (TGF- β) (E), and Col1a1 (F) in the lungs of both Yama and C57BL/6 mice ($n=6$). Data are presented as mean $\pm$ SD (* $p<0.05$).

GENERAL DISCUSSION

GENERAL DISCUSSION AND CONCLUSION

Both ABPA and IPF are chronic, progressive pulmonary disorders characterized by persistent inflammation, tissue remodeling, and fibrosis. Although they share common pathogenic features, they differ in their predominant mediators and immunological profiles (Agarwal *et al.*, 2014; Adamko *et al.*, 2013). The present study established two novel murine models using the eosinophilic Yama mice to elucidate the eosinophil-driven mechanisms underlying these distinct yet interconnected pulmonary disorders. The findings collectively indicate that the inherent eosinophilia in Yama mice exacerbates both inflammatory and fibrotic processes, providing new insights into the important role of eosinophils in chronic lung pathology (Akuhota and Weller, 2012; Aceves, 2014 Kurup and Grunig, 2001).

In the first model, intranasal administration of the *Aspergillus fumigatus* antigen Asp f1 induced a substantial Th2-type immune response, as demonstrated by significant peripheral and tissue eosinophilia, elevated total IgE, mucus hypersecretion, and airway remodeling (Davis and Wypych, 2021, De-Luca *et al.*, 2017). These features closely resemble human ABPA, in which eosinophilic inflammation and mucus plugging are key factors in disease progression (Dietschmann *et al.*, 2020; Garcia *et al.*, 2010). Compared with BALB/c mice, Yama mice showed more extensive peribronchial and interstitial eosinophilic infiltration, enhanced mucus accumulation, and increased collagen deposition, suggesting that pre-existing eosinophilia amplifies Th2 activation and enhances fibrogenic potential (Hogaboam *et al.*, 2000; Kay *et al.*, 2004).

Eosinophils in Yama mice possibly enhance disease severity by releasing profibrotic mediators, such as ECP, IL-4, IL-5, and IL-13, which collectively promote fibroblast activation and ECM production (Knutsen, 2006; Knutsen and Slavin, 2011; Kumar and Foster, 2002; Kurup and Grunig, 2001). Interestingly, substantial fibrosis developed despite only moderate TGF- β expression, suggesting that eosinophil-mediated fibrogenesis in ABPA may occur through a TGF- β -independent pathway, predominantly facilitated by IL-13 and ECP (Kurup and Grunig, 2001; Luo *et al.*, 2024; McCrary, 2019). This finding corresponds with recent reports indicating that IL-13 can directly stimulate collagen gene expression and airway remodeling independently of TGF- β , supporting the existence of a unique eosinophil–IL-13 axis in allergic fibrosis.

In the second model, repetitive intranasal administration of BLM in Yama mice produced persistent and progressive fibrosis, in contrast to the transient and self-limiting fibrosis typically observed in conventional C57BL/6 models (Lederer and Martinez, 2018; Lee *et al.*, 2025). Histopathological analyses revealed severe alveolar collapse, interstitial thickening, fibroblastic foci, and extensive collagen deposition—changes closely resembling those seen in human IPF lesions (Ley *et al.*, 2011; Miao *et al.*, 2023; Moore and Hogaboam, 2008). Eosinophil infiltration was significantly increased in Yama mice, consistent with previous studies showing that eosinophils were related to the production of fibrogenic cytokines, epithelial injury, and impaired tissue repair (Yamada *et al.*, 2024; Yamada *et al.*, 2025).

Cytokine profiling indicated a Th2-dominant immunological background characterized by increased mRNA levels of IL-4, IL-5, and IL-13, accompanied by

a consistent increased IL-6 expression (Franzen *et al.*, 2024; Lee *et al.*, 2025; Raghu *et al.*, 2016). These cytokines affect synergistically to sustain fibroblast proliferation, enhance ECM synthesis, and induce mucus production, thereby promoting chronic remodeling and fibrosis (Fulkerson and Rothenberg, 2023; Gul *et al.*, 2023; Raghu *et al.*, 2018). In Yama mice, TGF- β expression exhibited only a modest rise, despite the occurrence of more pronounced fibrosis and significant overexpression of Col1a1. This observation indicates that Yama mice may acquire fibrosis through pathways that are not entirely dependent on TGF- β signaling.

Although this study did not evaluate downstream mediators such as Smad2/3, previous study suggests that TGF- β signaling may be regulated by inherent negative-feedback mechanisms. In lung fibroblasts, TGF- β stimulates a rapid activation of Smad3, which is followed by a gradual downregulation of Smad3 expression, thereby restricting additional TGF- β signaling (Zhao and Gerverd, 2002). This mechanism may explain why TGF- β mRNA levels in our model demonstrate modest elevation despite extensive fibrosis. Similar dissociation results between TGF- β expression and collagen deposition, have been reported in other Yama-based disease models (Yamada *et al.*, 2024), supporting the idea that this strain possesses a unique regulatory profile in cellular fibrogenesis (Kliment *et al.*, 2011; Kondoh *et al.*, 2022; Redente *et al.*, 2021). Such TGF- β -independent fibrotic pathways have also been reported in certain subsets of human IPF patients, reflecting disease heterogeneity and the existence of numerous concurrent profibrotic cascades (Santa *et al.*, 2023; She *et al.*, 2021).

Together, ABPA and IPF models highlight an essential pathogenic concept relevant to human diseases: eosinophils play a key role as mediators of chronic pulmonary inflammation and fibrogenesis. In ABPA, eosinophil activation is primarily triggered by IgE-mediated hypersensitivity and Th2 cytokine signaling, whereas in IPF, eosinophils contribute to disease progression through innate and regulatory mechanisms involving cytokine secretion and tissue-remodeling factors independent of antigen-specific induction (Kurup and Grunig, 2001; Garcia *et al.*, 2010; Gharaee and Phan, 1998).

Thus, Th2-associated cytokines (e.g., IL-4 and IL-13) and IL-6-dependent inflammatory signaling may act as primary catalysts for the exacerbated fibrotic response in Yama mice. Additional fibrosis-related pathways, including Wnt/ β -catenin, Notch signaling, and osteopontin, may also contribute to the pronounced fibrosis observed in this model and could be investigated in future studies (Miao *et al.*, 2023; She *et al.*, 2021; Yamada *et al.*, 2024). The distinctive hypereosinophilic state in Yama mice may create a highly profibrotic tissue microenvironment driven by the secretion of eosinophil granule proteins, activation of fibroblasts, and alteration of tissue remodeling, ultimately leading to enhanced fibrosis despite modest TGF- β upregulation. The combination of these mechanisms suggests that eosinophilic inflammation represents a unique pathogenic route connecting allergic airway illness and idiopathic fibrosis.

The Yama mouse, characterized by persistent peripheral eosinophilia without artificial cytokine induction, provides a robust and physiologically relevant model for investigating eosinophil-dependent processes under natural

immunological conditions (Yamada *et al.*, 2024; Yamada *et al.*, 2025). Its ability to replicate both ABPA-like and non-allergic IPF-like fibrotic pathologies within the same genetic background underscores the central role of eosinophils as a unifying factor in chronic pulmonary disorders. This dual-model approach integrates immunologically distinct yet pathophysiologically related diseases, demonstrating that dysregulated eosinophil activity can induce both Th2-mediated and non-Th2 fibrotic remodeling (Aceves, 2014; Asano and Oguma, 2025).

In conclusion, the present study demonstrates that the Yama mouse serves as a powerful and versatile preclinical model for investigating eosinophil-mediated lung disease. The capacity of this model to reproduce both allergic (ABPA) and non-allergic (IPF) fibrotic processes highlights the broad pathogenic potential of eosinophil activation and Th2 cytokine signaling in chronic respiratory conditions. Future research should focus on identifying specific eosinophil-derived mediators, such as osteopontin and other fibrosis-related pathways that contribute to TGF- β -independent fibrosis, and on evaluating therapeutic strategies targeting eosinophil activation or IL-13 signaling. These findings connect allergic and idiopathic forms of lung fibrosis and emphasize eosinophils as a central pathologic factor in the etiology of chronic interstitial lung disorders (Buskirk *et al.*, 2014; Luo *et al.*, 2024; Redente *et al.*, 2021; Wang *et al.*, 2025).

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