의학박사 학위논문

노인 여성 천식에서 프로게스테론 유도 억제인자 (progesterone-induced blocking factor, PIBF) 의 작용 기전 및 치료 효과

Progesterone-induced blocking factor (PIBF) as a Potential Therapeutic Target for Elderly Female Asthma

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Progesterone-induced blocking factor (PIBF) as a Potential Therapeutic Target for Elderly Female Asthma

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2021 년 2 월

감사의 글

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Abstract

Background: Elderly females with asthma tend to experience higher rate of morbidity and mortality compared to other patient subgroups of asthma. However, underlying mechanism of their susceptibility is largely unknown. PIBF (Progesterone-induced blocking factor) is induced by stimulation of progesterone and known to have immunomodulatory roles.

Objective: To determine the role of PIBF in asthma of postmenopausal women

Methods: The levels of PIBF were measured in serum of healthy controls and patients with asthma. The effect of menopause and lack of PIBF on asthma was investigated using ovariectomized mice and PIBF hetero mice, respectively. To evaluate therapeutic effect of PIBF, 35 kDa recombinant PIBF was administered during OVA challenge in murine asthma model and in vitro experiments using NK cells.

Results: PIBF levels were decreased in serum samples of postmenopausal patients with asthma. By establishing OVA-induced murine asthma models, we have observed that ovariectomy and lack of PIBF respectively exacerbated airway inflammation. Treatment with 35kDa recombinant PIBF reduced OVA-induced airway inflammation in ovariectomized mice. In vitro experiments also reported inhibitory role of recombinant PIBF on NK cell-mediated lysis of K562 cells.

Conclusions: PIBF could be a potential therapeutic agent for elderly females with asthma whose airway inflammation is largely dependent on progesterone-PIBF related pathway. Further studies are warranted to better understand the mechanism underlying anti-inflammatory effect of PIBF in the context of asthma.

Key words: Progesterone-induced blocking factor, menopause, asthma, airway inflammation

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1. INTRODUCTION

1.1. Asthma in the elderly patients

Asthma is a chronic airway inflammatory disease causing a substantial healthcare burden worldwide. Even though asthma can develop at any time in life, clinical presentations vary according to the age of patients. Asthma in the elderly patients, often classified as aged 65 years and older, is featured with higher morbidity and mortality compared with patients in younger ages. (1, 2) The causes of high disease burden in this population are complex and multifactorial. Structurally, aging process results in reduction of elastic recoil, decreased respiratory muscle strength, and increased chest wall rigidity. (3) Altered immune system, such as 'immunosenescence' and 'inflamm-aging' may also have an impact on severity of asthma, by increasing susceptibility to airway infection and resistance to corticosteroid treatment. (1) In addition to these age-related changes, multiple comorbid conditions affecting control status of asthma, impaired cognitive and physical function, adverse effects from polypharmacy, and psychosocial factors contribute to poor outcome in the elderly asthma. (4) Among the elderly, female subgroup needs additional attention as they experience more exacerbations and side effects of corticosteroids, stronger association with depression and obesity, as well as higher mortality than male asthmatics in the same age group. (5, 6) This might be a result of interplay between biological susceptibility, hormonal change after menopause, and socioeconomic status. (7)

Given the rapid speed of population aging, asthma in the elderly population is an important health issue. Deeper understanding of distinct pathophysiology and specialized management strategies are required.

1.2. Female hormones and asthma – epidemiological data

Accumulated epidemiological data support the impact of female sex hormones on development or severity of asthma. Across the women's life span, alterations of sex hormones differently affect presentations of asthma. (8) Prior to puberty, asthma is more prevalent and severe in males compared to females. However, it becomes more common in females after puberty. Even though the reason for this gender switch is not well known, drastic alteration of sex hormones may play a central role. In some female patients with asthma, respiratory symptoms worsen during preovulatory or perimenstrual period. (9, 10) Pregnancy also affects severity of asthma, and about a third of patients experience deteriorated symptoms during their pregnancies. (11) Menopause is another contributor to respiratory symptoms in females. In 2016, Triebner and colleagues reported increased new-onset asthma and respiratory symptoms in postmenopausal women compared with nonmenopausal women. (12) When analyzing follow-up data of 67,872 adult women without asthma in France, surgical menopause was related to increased risk of asthma onset. Association between natural menopause and increased incidence of asthma was observed only in overweight/obese women, which was not observed in subjects with normal weight. (13) A meta-analysis conducted by McCleary et al. presented onset of menopause was related to higher risk of current asthma and wheezing. (14)

Similar to endogenous change in sex hormones, exogenous female sex hormones such as contraceptives or hormone replacement therapy also modulates risk of asthma. (14, 15) In 2020, Nwaru and colleagues reported reduced risk of newly developed asthma in women who had ever used hormonal contraceptives. (15) They also found that use of hormonal contraceptives was associated with reduced risk of severe asthma exacerbation in women of reproductive age. (16) Even though involvement of female sex hormones in respiratory health and disease seems evident, precise role of estrogen and/or progesterone is difficult to delineate given numerous confounding factors including age, body mass index, parity or gravidity,

smoking, and other comorbidities.

1.3. Female hormones and asthma – experimental data

Persistent studies have been conducted to elucidate underlying mechanism of interaction between female sex hormones and asthma. The impact of estrogens in various medical conditions has been reported including respiratory diseases. (17) However, less attention has been paid to progesterone, which is the second major endogenous female sex hormone mostly produced in the ovaries. In 1995, Piccinni and colleagues noted increased production of Th2 cytokines of Th1 cell lines in the presence of progesterone. (18) Similarly, progesterone stimulation led to increased concentrations of IL-4 and IL-13 from human peripheral blood mononuclear cells (PBMC). (19) When reviewing the animal experiments, in ovalbumin (OVA)-challenged male mice, administration of medroxyprogesterone via esophagus exacerbated bronchial eosinophilic inflammation and enhanced airway hyperresponsiveness. (20) When establishing OVA-challenged asthma model in ovariectomized (OVX) rat, single subcutaneous injection of progesterone preceding OVA-challenge reduced lung inflammation. Meanwhile, progesterone treatment enhanced IL-10, IL-1β, and TNF-α production in incubated bronchoalveolar lavage (BAL) cells and increased IL-4 in bone marrow cells compared to the untreated rats. (21) In ovariectomized murine model of allergic asthma induced by house dust mite allergen, supplement of progesterone accelerated eosinophilic inflammation and mucous cell metaplasia when exposed to tobacco smoking concomitantly. (22, 23) In contrast, Hall and colleagues suggested a protective role of progesterone in influenza A infection. In their mice inoculated by influenza A, exogenous progesterone promoted recovery of airway inflammation by increasing TGF-β, IL-6, IL-22, cellular proliferation and upregulating the epidermal growth factor amphiregulin (AREG). (24) In murine model of ozone exposure, inhalation of

progesterone reduced the expression of markers of airway remodeling, such as matrix metalloproteinase-8 (MMP8), MMP9, hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), α-smooth muscle actin (α-SMA), and glycogen synthase kinase-3β (GSK-3β) in BAL fluid or lung tissues, as well as peri-bronchial collagen deposition. (25) Meanwhile, subcutaneous injection of progesterone failed to affect cell apoptosis of lung in bleomycin-injured murine model. (26) These data collectively implicate the presence of progesterone dependent pathway in various pathologic conditions and immunomodulatory role of progesterone. However, conflicting results may come from different target tissues and physiologic and/or pathologic backgrounds of hosts. Moreover, as most studies observed on the final alteration brought by administration of progesterone, underlying processes induced by progesterone remain largely unknown.

1.4. Progesterone-induced blocking factor (PIBF)

During pregnancy, maintaining high concentration of progesterone is essential. Progesterone inhibits myometrial contraction and regulates maternal immune response to prevent rejection of fetal-semi-allograft. The immunomodulatory effect of progesterone is mediated by PIBF whose serum level positively correlates with serum progesterone level. (27) In normal pregnancy, progesterone activates lymphocytes and decidual CD56+ cells to generate PIBF which providing immunotolerance state for fetus by modulating adaptive immune system and suppressing natural killer (NK) cell activity. (28) In line with this, expression of PIBF and proportion of PIBF+ lymphocytes are reduced in patients with recurrent spontaneous abortions and threatened preterm pregnancy termination. (29, 30)

The full-length form of PIBF is composed of 756 amino acids with molecular weight of 90 kDa. (31) The full length PIBF was reported to be located within the nucleus and

participate in regulation of cell cycles. (32) Alternative splicing of PIBF results in various lengths of isoforms which might work as cytokines. Immunofluorescence microscopic examination revealed diffuse distribution of 35 kDa PIBF in cytoplasm. Other researchers reported that activated cells secreted 34 kDa PIBF. (33) The biologically active part of PIBF is the 48 kDa N-terminus.

As normal pregnancy is featured by decreased NK activity and Th2 shift, PIBF is thought to play a key role in maintaining this circumstance. (34) In mitogen activated lymphocytes from non-pregnant participants, PIBF inhibited NK activity by increasing IL-10 and decreasing IL-12. (35) The lymphocytes from pregnant women with history of multiple unexplained abortions showed lower IL-10 and higher IL-12 expressed cells compared with those from healthy pregnant women. (36) When using spleen cells from Balb/c mice incubated with Con A with or without PIBF, significantly higher level of IL-3, IL-4, and IL-10 was noted in cells cultured with PIBF. There was no difference in the level of IFN-γ. (37) Based on these results, Kozma and colleagues investigated whether PIBF is involved in the STAT6/STAT4 pathway, whose initiation is via IL-4 receptor. They suggested that PIBF induced STAT6 activation by binding glycosylphosphatidylinositol (GPI) anchored protein which forms a heterodimer with IL-4Rα. (38) In addition, PIBF was suggested to downregulate release of arachidonic acid. (39) Thus, subsequent decreased prostaglandin synthesis resulted in reduction in IL-12 production, which finally inhibited cytotoxic activity of NK cells. (40)

Since PIBF had immunomodulatory effects, there have been attempts to find its role as an immunotherapy for cancer treatment. (41) As expected, PIBF were found in several malignancies and considered to accelerate proliferation and/or invasion of cancer cells. (42) The overexpression of PIBF was observed in breast cancer, cervical cancer, epithelial ovarian cancer, lymphoma, leukemia, and glioblastoma (32, 43-45) In mouse model of spontaneous

cancers such as prostate, testicular, and lung cancer, administration of antiprogesterone (mifepristone) significantly prolonged survivals. Moreover, oral mifepristone showed palliative effect in various advanced cancers refractory to treatment. However, larger clinical trials and deeper understanding of PIBF is required. Despite the largely unknown mechanism, modulation of PIBF is still regarded as a novel treatment strategy for both inflammatory disease and cancer waiting for further research. (45)

1.5. Possible role of PIBF in asthma of postmenopausal females

As aforementioned, elderly women make up a vulnerable population in asthma. They experience more severe form of diseases and adverse effects of corticosteroids (the mainstay of asthma treatment), namely diabetes and osteoporosis. Therefore, novel treatment strategies are urgently required to manage these patients. Since adverse disease outcomes in elderly females are at least partly due to depletion of female hormones, we could assume that modulation of hormonal effects may reverse the change from menopause. Given the immunomodulatory function of PIBF, we sought to investigate the role of PIBF in pathophysiology of asthma and evaluate its potential as therapeutic agent, particularly among elderly females.

2. MATERIALS AND METHODS

2.1. Study subjects

To understand the distribution of serum PIBF in female asthmatics, we obtained serum of 94 asthmatics and 154 healthy controls. The diagnosis of asthma was based on Global Initiative for Asthma (GINA) guidelines. The healthy subjects were composed of visitors in our medical center for regular health screening. They had no history of respiratory diseases, malignancies, and endocrinologic disorders. Particularly in female subjects, those under hormonal replacement therapy or using oral contraceptives were excluded. In addition, subjects having history of hysterectomy and oophorectomy were also excluded. All subjects gave informed consent for participating the research. This study was approved by the Institutional Review Board of Asan Medical Center (IRB No. 2018-1169)

2.2. Mice

Female C57BL/6 mice aged 6 to 8 weeks were purchased from the Jackson laboratory (Bar Harbor, ME, USA). PIBF hetero (+/-) mice were kindly donated from professor In-Jeoung Baek in Asan medical center. Animals were housed in groups of five per cage in a light- and temperature- controlled room with free access to food and water. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Asan Medical Center.

2.3. Ovariectomy

Mice were anesthetized by intraperitoneal (i.p.) injection of 100 to 150 μl ketamine and xylazine mixture (0.5 μg/ml, Yuhan, Seoul, Korea and 7 mg/ml, Bayer, Germany, respectively)

and undergone surgical ovariectomy via medial back incision on day -7. Prior to surgery, the fur on the back was shaved and the skin was sterilized with a 10% iodine solution. A midline incision was made followed by second small incision on lateral part of peritoneum to enter the abdominal cavity. Each ovary was grasped and drawn from the abdominal cavity through the incisions. Heated scissors were used to remove the ovaries. The remained uterine horn and remnant fallopian tubes were returned into the abdomen cavity and the abdominal wall incision was closed using 4-0 silk stitches. Lastly, skin incision was closed using silk and swabbed with 10% iodine solution. Mice were then returned to clean cages on heating pads.

2.4. Ovalbumin (OVA) driven allergic asthma model and treatment of PIBF

OVA/alum was used to induced allergic airway inflammation. One week after ovariectomy, We intraperitoneally injected 75 μg of OVA (Sigma-Aldrich) and 2 mg of aluminum hydroxide (Imject Alum, Thermo Scientific) on day 1 and 15, followed by intranasal administration of 50 μ OVA on day from 22 to 24. Twenty-four hour after the last OVA intranasal challenge, lungs and bronchoalveolar lavage fluid were collected for histological analysis and ELISA.

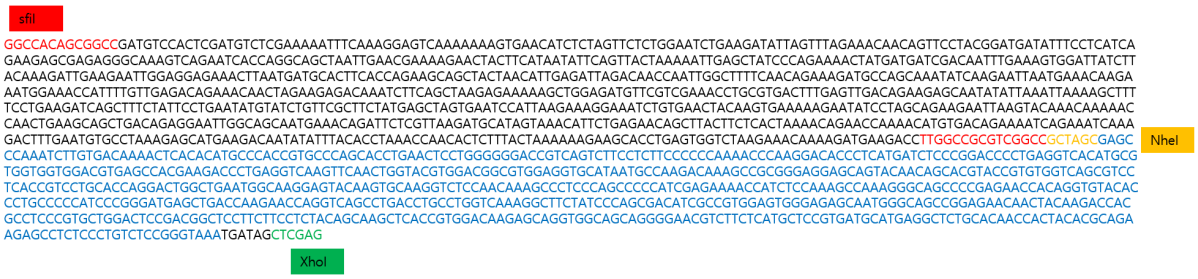
In the experiments using PIBF +/- mice and WT mice for control group, progesterone was injected in all mice on day 1, 7, and 14 to control the level of systemic concentration of progesterone. In the experiments evaluating the therapeutic effect of PIBF in allergic airway inflammation, we intraperitoneally injected PIBF, dissolved in PBS, on day 22, 23, 24 consecutively, with 0.5, 5, 50mg.

2.5. 35kDa recombinant PIBF

To make human recombinant 35 kDa secretory PIBF, we derived the template of 35 kDa PIBF using PIBF exon 1 and 18 specific primers from human PBMC and cancer cell lines including

A549, HeLa, K562 and THP-1 (Figure 1). Next, template was inserted into TA cloning vector for amplification and sequencing. After conformation via sequencing, the template was moved to expression vector, pET21a, as Histidine-tagged form for small scale production of recombinant 35 kDa PIBF in E.coli system. Final confirmation was done by coomassie staining and silver staining after purification using metal-ion affinity chromatography (Figure 2 and 3). For mass production of 35 kDa PIBF to use in animal study, recombinant protein was produced by Ybiologics (Daejeon, Korea) using above template and HEK 293F cell expression system. In addition, to prolong the half-life of recombinant, we added Fc-domain to C-terminal of recombinant (Figure 4).

A



B

Figure 1. 35 kDa PIBF DNA (A) and amino acid sequence (B)

The black letters indicate the sequence of 35 kDa PIBF and the blue letters indicate C-terminal linked Fc protein sequence.

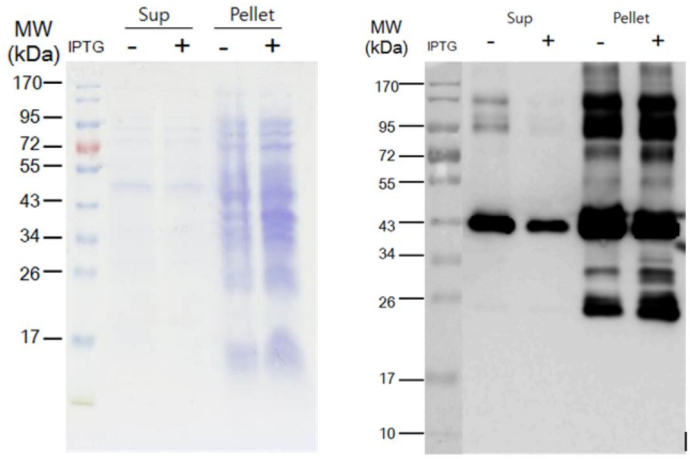


Figure 2. The results of small scale human recombinant 35 kDa PIBF expression in E.coli expression system (Left: Coomassie staining, right: western blot)

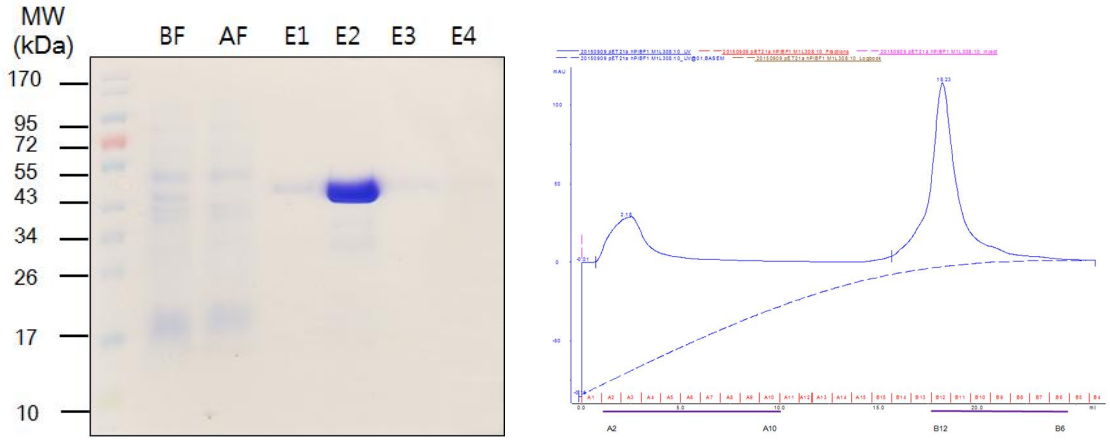
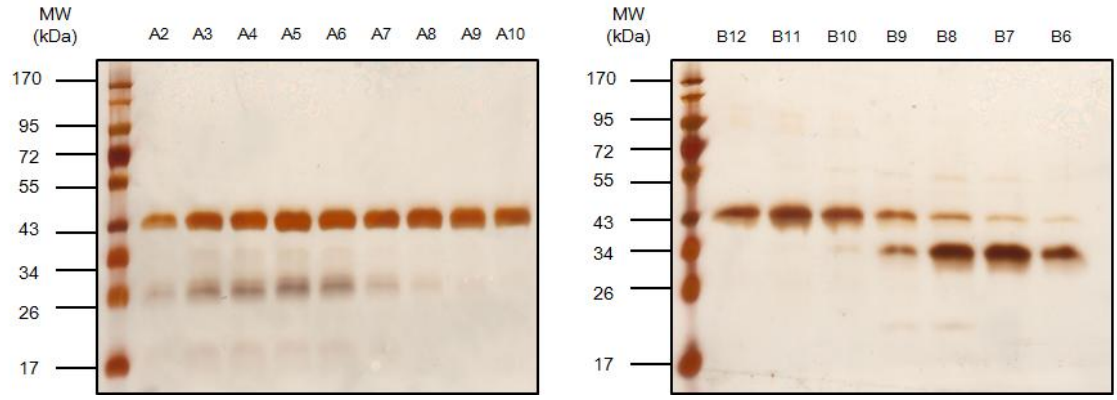


Figure 3. Purification of manual manufactured recombinant 35 kDa PIBF (Left: 1st purification with Talon, right: 2nd purification with FPLC)

A



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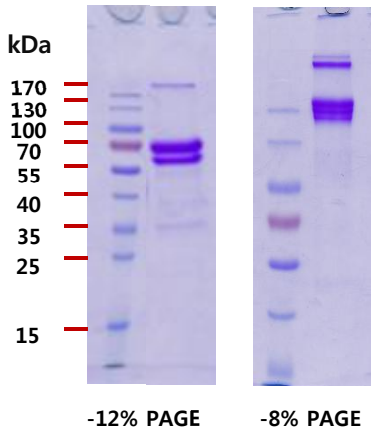


Figure 4. A. Confirmation of purified recombinant 35 kDa PIBF with silver staining. B. Coomassie staining of mass produced recombinant 35 kDa PIBF after purification (Fc added form, left: non-reduced, right: reduced)

2.6. Evaluation of histological change in lung tissue

Lungs were removed and fixed in 5% formalin, paraffin-embedded and cut into 4-μm sections, and stained with hematoxylin and eosin (H&E). Degrees of lung tissue inflammation and mucus secretion were graded on a scale of 0 to 4 (0, none; 1, minimal; 2, mild; 3, moderate; 4, severe).

2.7. Bronchoalveolar lavage (BAL) fluid analysis

Briefly, mice were anesthetized with 100 to 150 μl ketamine and xylazine mixture, the trachea was exposed, and a cannula was inserted. The lungs were washed 2 times with 1ml of sterile PBS injected through the cannula. Cells were separated by centrifugation at 1,000 x g for 10 minutes at 4°C, and the pellets were resuspended with RBC lysis buffer (StemCell Technologies, Vancouver, BC, Canada) to remove RBCs. The number of total immune cells was counted. For differential cell counts, BAL cells were cytospun onto slides (StatSpin CytoFuge 12; Iris, Norwood, Mass) at 400 x g for 5 minutes at room temperature, followed by a Diff-Quik stain (Sysmex, Kobe, Japan) and common fixation for light microscopy with synthetic mounting medium (Histomount; Ted Pella, Redding, CA, USA). A total of 300 cells were used in each preparation to examine proportion of eosinophils, macrophages, neutrophils, and lymphocytes using standard morphological criteria.

2.8. ELISA

To produce antibodies detecting all alternative, secreted forms of PIBF, we have searched common amino-acid sequences between each variant. There was no single common sequence of 25, 35, 48 and 89 kDa PIBF forms, instead, we identified 3 common sequences between each forms; peptide 1: SSEEREGKVRITRQLIERKEL (22 mer, common sequence of 35, 48

& 89), peptide 2: ETNLQLREKAGDVRRNLRDFELT (24 mer, common sequences of 35, 48 & 89), peptide 3: EPKHVTENQKSKTLNVPKEHEDN (24 mer, common sequences of 25, 35 & 89). Peptide synthesis, immunization to rabbit and polyclonal antibodies purification were performed by AbFrontier (Seoul. Korea).

For evaluation of PIBF levels in human serum, we used indirect ELISA system using secreted PIBF polyclonal antibodies. Briefly, serum was diluted a thousand-fold in PBS and coated 96-well ELISA plates (Corning, NY, USA). Next, 1 ug/ml of rabbit-anti-PIBF-polyclonal antibody was added into serum coated plates after blocking procedure. Two hours after incubation, the-thousand folds diluted HRP-conjugated goat anti-rabbit-IgG polyclonal antibody (Enzo life sciences, NY, USA) was added, and colorimetric development was done with BioFX TMB solution (Surmodics IVD, Inc, MN, USA).

In the case of animal experiments, IL-4, IL-5, IL-13, Eotaxin, IL-17 and IP-10 were assessed in BAL fluids using ELISA Duoset (R&D systems, Minneapolis, MN, USA) following the manufacturer's guidelines. To evaluate the levels of OVA specific immunoglobulins in serum, the mouse ELISA Quantitation Sets (Bethyl laboratories, Montgomery, TX, USA) were used.

2.9. NK cytotoxicity test

The target cell line K562 (2 x 10^7 cells) were labeled with the green fluorescent dye PKH-67 Fluorescent Cell Liker Kits (Sigma-Aldrich) following the manufacturer's instruction. NK92MI cells (8 x 10^6 cells) were treated with 100 or 500 nM recombinant PIBF for 1hr at 37^{O}C. Labeled target cells were transferred in 96-well U-bottom culture plate at 50,000 cells per well density and then mixed with NK92MI cells (at a ratio 1 to 25). The plate was incubated for 2hr at 37^{O}C in a 5% CO_2 incubator. Target cells were stained with propidium iodide (Sigma-

Aldrich), and the percentages of dead target cells showing GFP-positive and PI-positive were determined by flow cytometry.

2.10. Data analysis

Histologic analyses were performed by 2 independent investigators who were blinded to the sample identification number throughout the analysis. The data shown represents mean ± SEM. Comparisons between 2 groups were performed by using the Student t-test or Mann-Whitney U-test, depending on the normality of distribution. P values were considered statistically significant when less than 0.05. All statistical analyses were conducted and plots were created with GraphPad Prism, versions 7.0 and 8.0 (GraphPad Software, Inc, La Jolla, CA, USA).

3. RESULTS

3.1. PIBF is decreased in serum of postmenopausal asthmatic females

We measured PIBF in in the serum of patients with asthma and healthy controls. The clinical characteristics of the study subjects are summarized in Table 1. There was no sex difference in of PIBF concentration in normal subjects. Among females, PIBF levels in serum were significantly reduced in patients with asthma compared with normal subjects (Figure 5A). In female asthmatics, PIBF levels were further reduced in the patients aged 60 and older (Figure 5B).

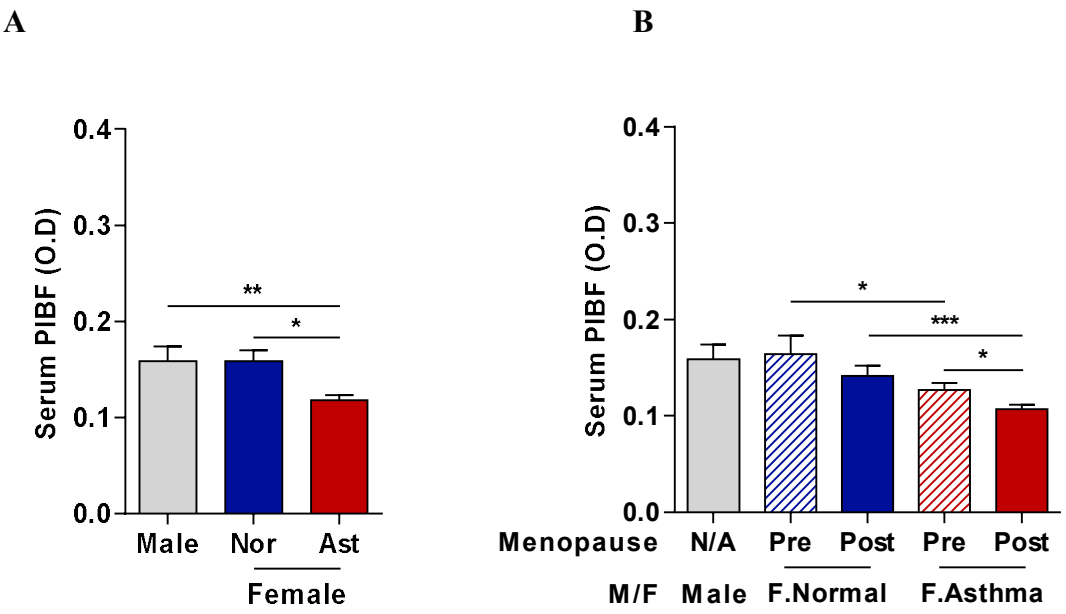


Figure 5. PIBF is decreased in serum of postmenopausal asthmatic females

PIBF expression was decreased in serum of female patients with asthma (n = 94) compared to female healthy controls (n = 102). (A) Among the females with asthma, postmenopausal patients (n = 45) had significantly lower level of PIBF compared to the premenopausal patients (n = 49). (B)

*P<0.05, **P <0.01 and ***P<0.001

Table 1. Comparison of characteristics of patients with asthma according to menopause

Characteristics	Premenopausal asthmatics	Postmenopausal asthmatics	P value
n	49	45	
Age, yr	38.45±6.88	71.87±5.03	0.000
Eosinophils	430.86±292.50	166.96±132.33	0.000
IgE	332.14±646.35	104.43±161.41	0.024
FEV1 % predicted	79.88±13.56	73.38±16.83	0.041
FEV1/FVC	0.76±0.10	0.67±0.11	0.000
ICS dose (Low to medium/High)	43/6	30/15	0.014

FEV1, Forced expiratory volume in 1 second; FVC, Forced vital capacity; ICS, Inhaled corticosteroids

Table 2. Subgroup analysis of PIBF expression according to clinical characteristics

	FEV1		Eosinophils		IgE*		ICS dose	
Subgroup	≥80% (n=42)	<80% (n=52)	≥300 (n=40)	<300 (n=53)	≥100 (n=37)	<100 (n=38)	Low to medium (n=73)	High (n=21)
PIBF	0.125 ±0.087	0.122 ±0.060	0.132 ±0.096	0.115 ±0.049	0.140 ±0.100	0.104 ±0.042	0.125 ±0.078	0.118 ±0.056

FEV1, Forced expiratory volume in 1 second; FVC, Forced vital capacity; ICS, Inhaled corticosteroids

One asterisk (*) indicates p value smaller than 0.05.

3.2. Ovariectomy (OVx) induced exacerbation of airway inflammation in murine asthma model

We aimed to assess the influence of menopause on asthma using animal model. To represent the human scenario of asthma in postmenopausal females, allergic asthma model was established in ovariectomized mice (Figure 6). As expected, there was no induced airway inflammation in control mice regardless of ovariectomy. However, in the mice with allergic asthma, ovariectomized induced enhancement of airway inflammation. First, as shown in Figure 7., OVA challenge caused significant increases in the number of total cells and eosinophils in BALF, which was more prominent in the ovariectomized mice. The higher levels of IL-4, IL-5, IL-13, and eotaxin were detected in asthma group after OVA-challenge as compared with control mice. Ovariectomy further increased their levels in BALF (Figure 8). Meanwhile, although OVA-challenge resulted in elevation of IL-17 and IP-10 in BALF, their levels were comparable irrespective of ovariectomy (Figure 9). In serum of mice with allergic asthma, levels of OVA-specific IgE, IgG1, and IgG2c was elevated. They also did not report difference according to ovariectomy, except for IgG2, which was decreased in ovariectomized mice (Figure 10 and 11). Enhanced airway inflammation was evidenced by histopathological characteristics including thickened bronchial wall, peribronchial edema, greater infiltration of inflammatory cells (Figure 12). Histological lung evaluations also revealed a marked inflammation in ovariectomized mice. These data collectively demonstrate that ovariectomy exacerbated airway inflammation in allergic asthma of mice.

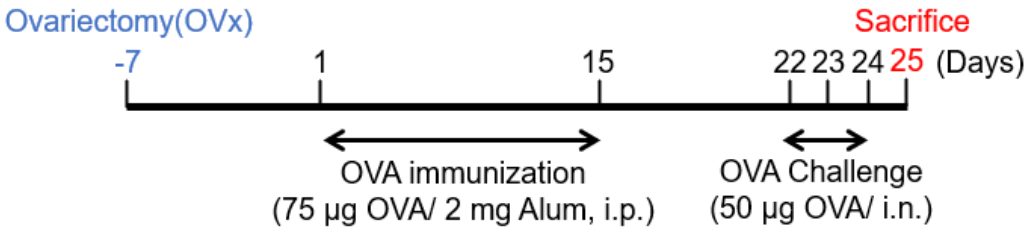


Figure 6. Protocol for the establishment of OVA-induced allergic asthma model in ovariectomized mice

Animal model protocol for ovariectomy and OVA-induced allergic asthma.

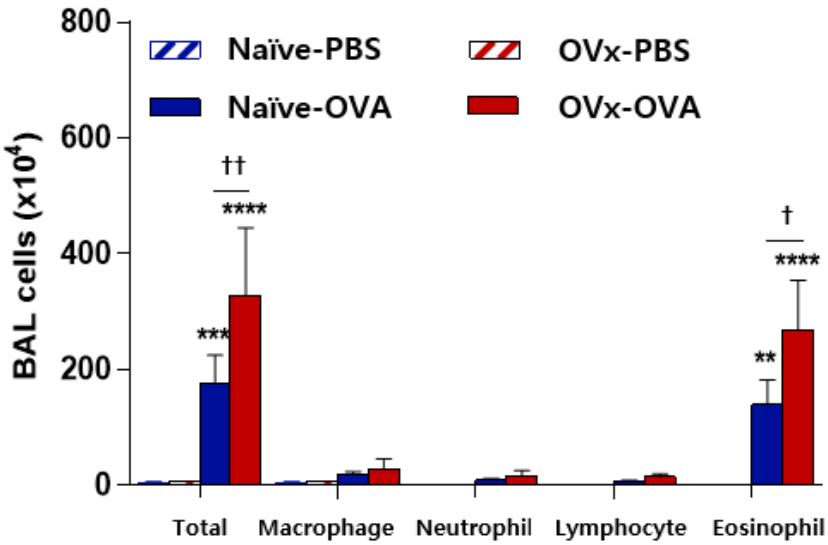


Figure 7. Ovariectomy exacerbated airway inflammation in murine asthma model

The number of inflammatory cells and their composition in BAL fluid.

** P<0.01: vs naïve-PBS, *** P<0.001: vs naïve-PBS, **** P<0.0001: vs naïve-PBS

† P<0.05: vs naïve-OVA, †† P<0.01: vs naïve-OVA

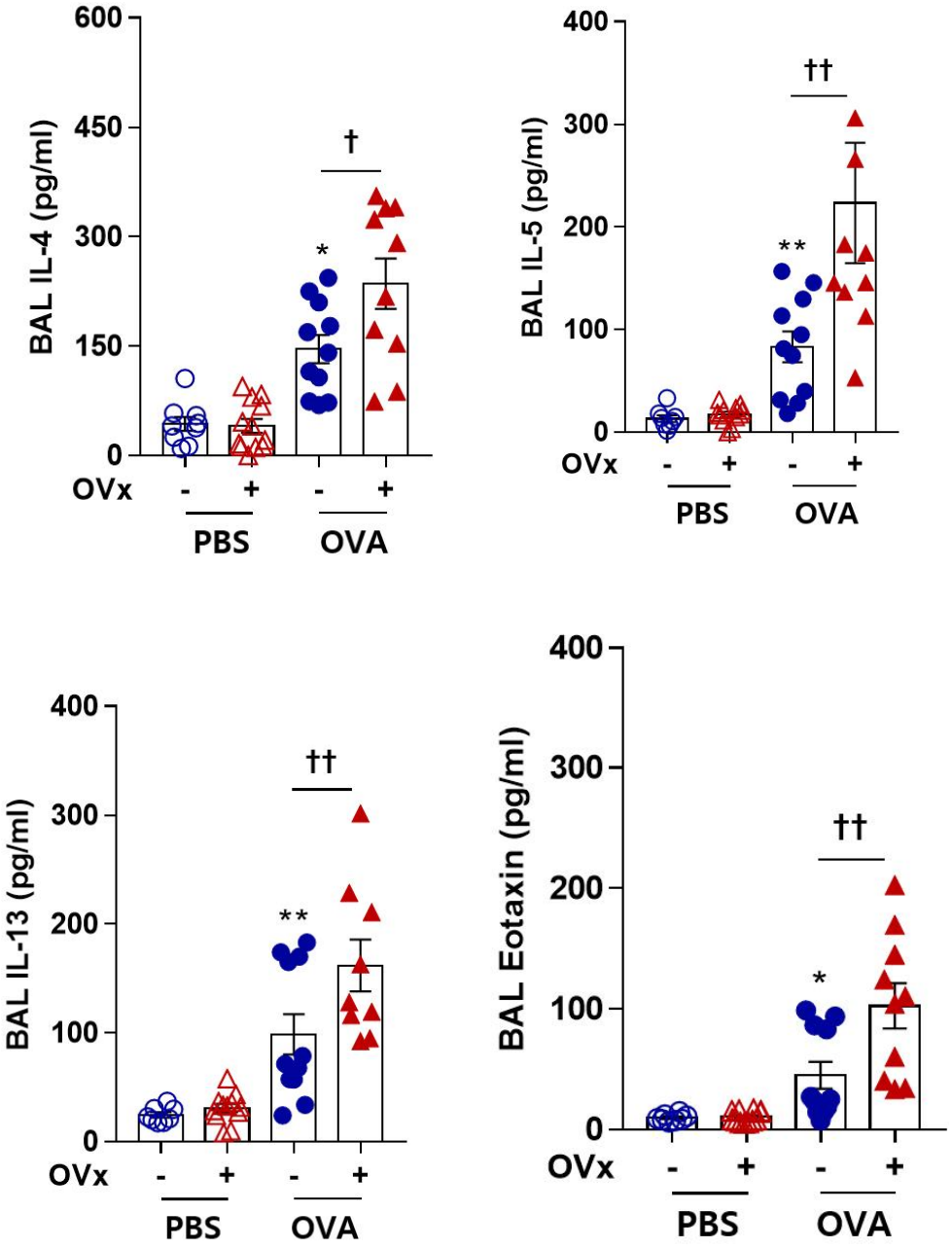


Figure 8. Ovariectomy enhanced type 2 cytokine production in murine asthma model

The expression of IL-4, IL-5, IL-13, and eotaxin was measured in BAL fluid.

* P<0.05: vs naïve-PBS, ** P<0.01: vs naïve-PBS,

† P<0.05: vs naïve-OVA, †† P<0.01: vs naïve-OVA

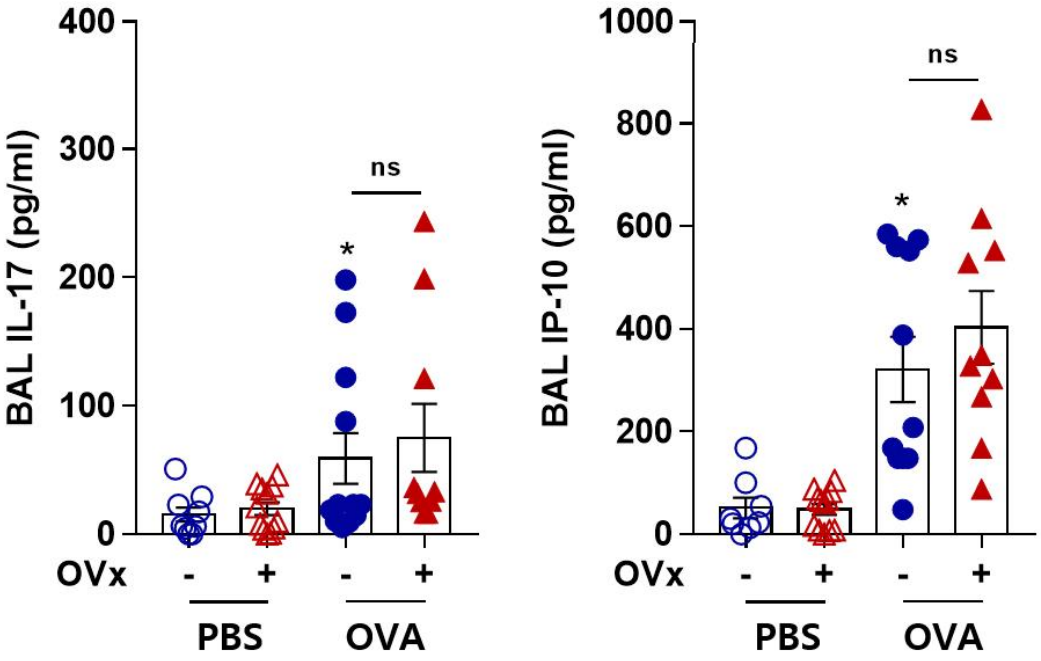


Figure 9. Effect of ovariectomy on production of IL-17 and IP-10 in BAL fluid of murine asthma model

The expression of IL-17 and IP-10 was measured in BAL fluid.

* P<0.05: vs naïve-PBS, ** P<0.01: vs naïve-PBS,

† P<0.05: vs naïve-OVA, †† P<0.01: vs naïve-OVA

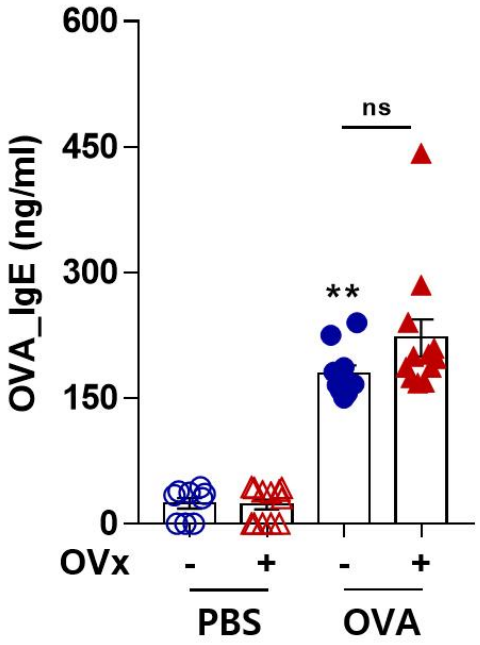


Figure 10. Effect of ovariectomy on the levels of OVA-specific IgE in serum of murine asthma model

The expression of OVA specific IgE was measured in serum of mice.

* P<0.05: vs naïve-PBS, ** P<0.01: vs naïve-PBS,

† P<0.05: vs naïve-OVA, †† P<0.01: vs naïve-OVA

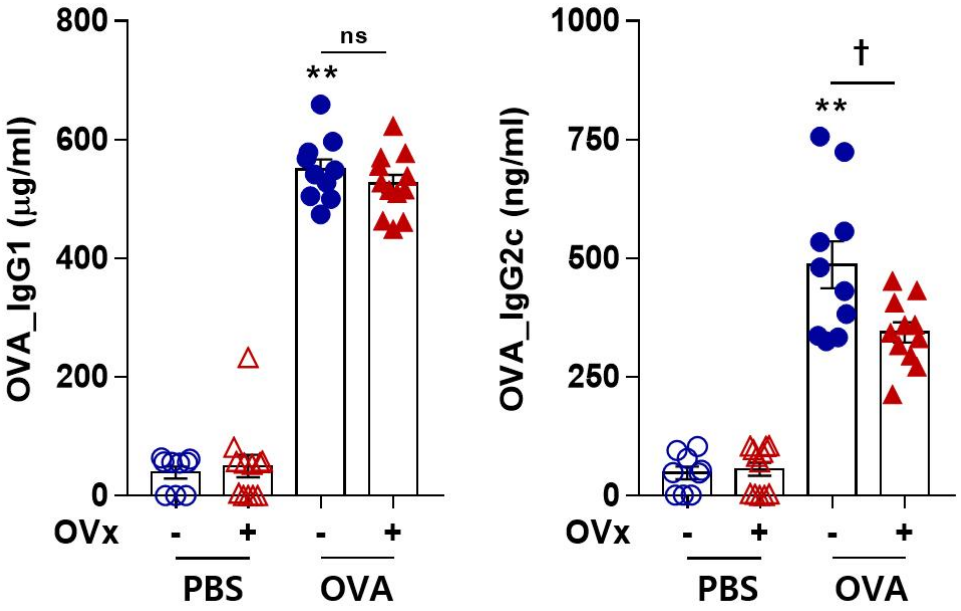


Figure 11. Effect of ovariectomy on the levels of OVA-specific IgG in serum of murine asthma model

The expression of OVA-specific IgG1 and IgG2c was measured in serum of mice.

* P<0.05: vs naïve-PBS, ** P<0.01: vs naïve-PBS,

† P<0.05: vs naïve-OVA, †† P<0.01: vs naïve-OVA

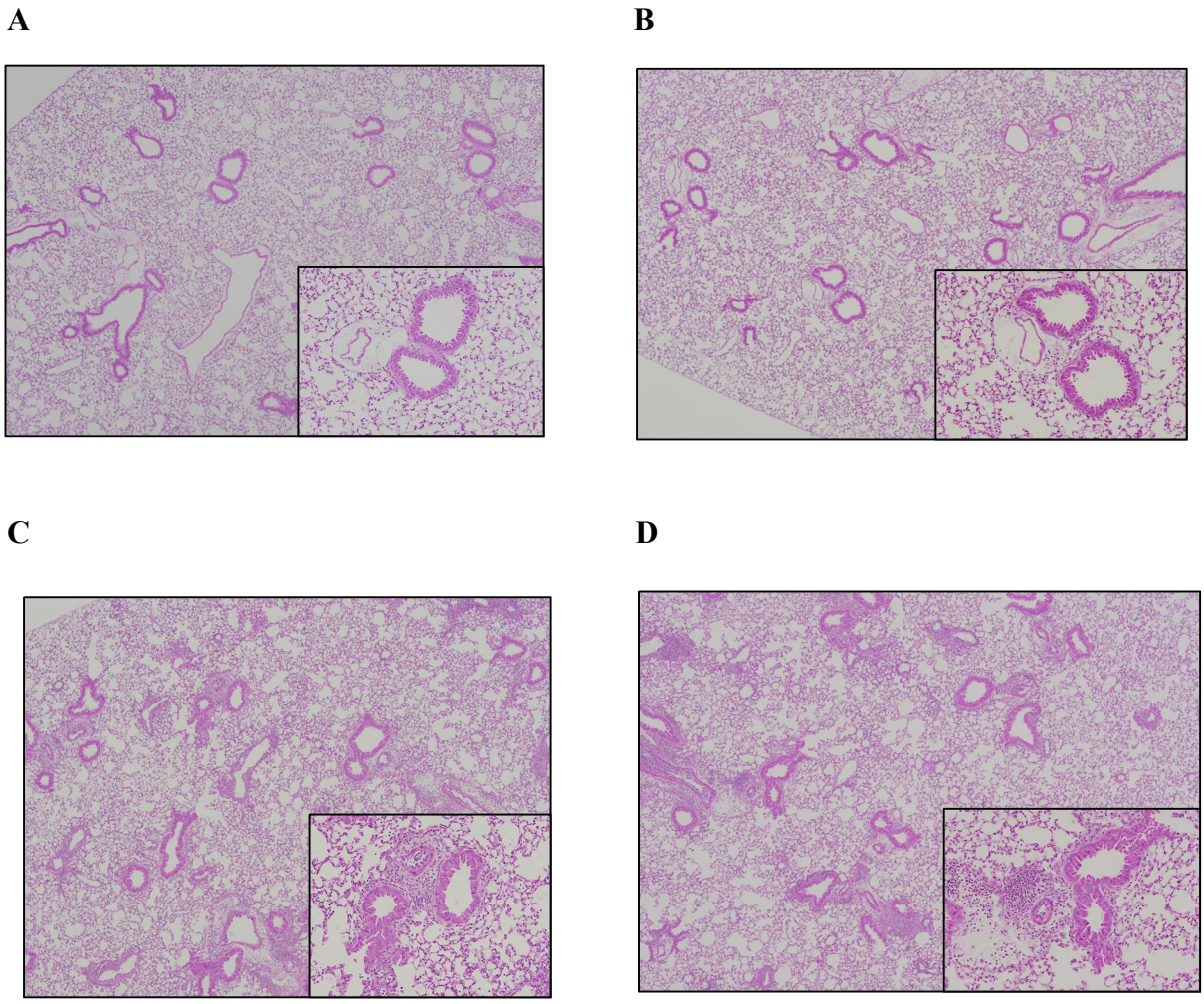


Figure 12. Tissue inflammation of the lungs was aggravated in ovariectomized mice

Hematoxylin and eosin (H&E) staining, 100x magnification

A. Naïve-PBS

B. OVx-PBS

C. Naïve-OVA

D. OVx-OVA

3.3. The lack of PIBF enhanced airway inflammation in ovariectomized mice

In order to assess the role of PIBF in airway inflammation, we used mice deficient for PIBF. OVA induced asthma model was established in both PIBF +/- and WT mice. They underwent ovariectomy a week prior to the first sensitization and progesterone was regularly administered to control level of progesterone among the mice (Figure 13). As shown in Figure 14, OVA challenge led to eosinophilic airway inflammation, which was more prominent in PIBF +/- when compared with WT mice. Increased BALF levels of IL-4, IL-5, IL-13, IL-17, IP-10, and eotaxin were observed in asthmatic mice, as expected. Among them, IL-4, IL-13, eotaxin, and IP-10 were higher in PIBF +/- mice compared to WT mice (Figure 15 and 16). Elevation of serum OVA specific IgE and IgG2c was also more prominent in PIBF deficient mice (Figure 17 and 18). In accordance, OVA challenge caused more severe lung inflammation in PIBF deficient mice (Figure 19). Taken together, these results suggest that lack of PIBF exacerabted OVA-induced airway inflammation.

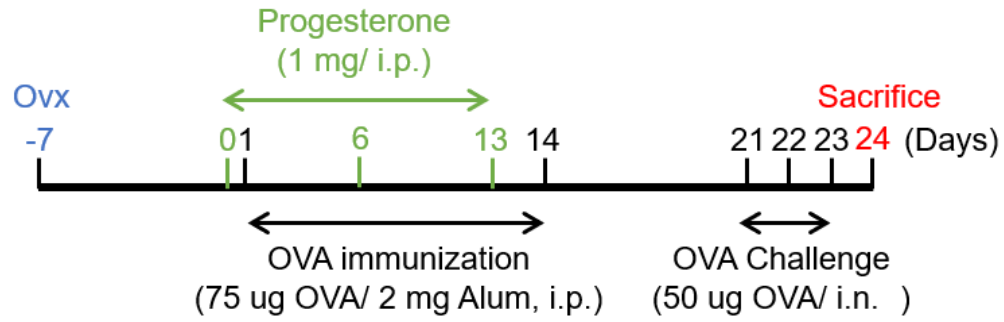


Figure 13. Animal model protocol for ovariectomy, OVA-induced allergic asthma combined with supplement of progesterone

Animal model protocol for OVA-induced allergic asthma in mice with controlled systemic progesterone level.

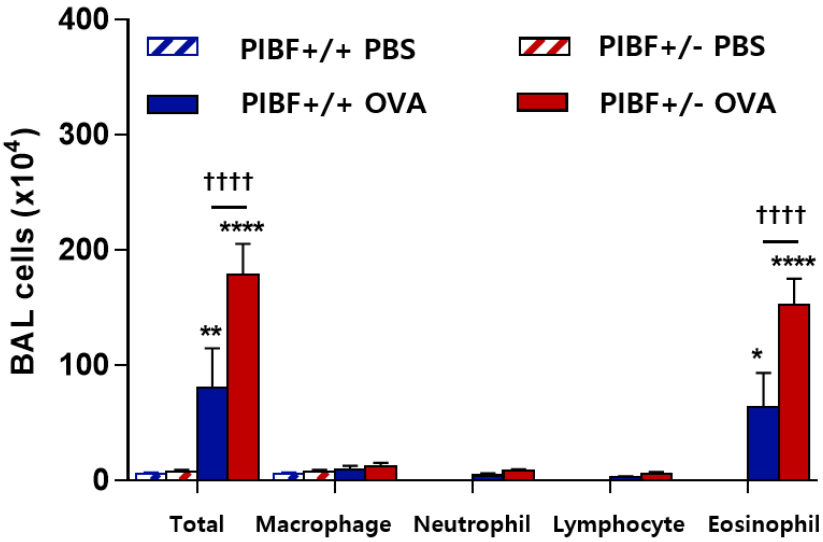


Figure 14. Lack of PIBF enhanced airway inflammation in ovariectomized mice

The number of inflammatory cells and their composition in BAL fluid.

* P<0.05: vs PIBF+/+ PBS, ** P<0.01: vs PIBF+/+ PBS, **** P<0.0001: vs PIBF+/+ PBS

†††† P<0.0001: vs PIBF+/+ OVA

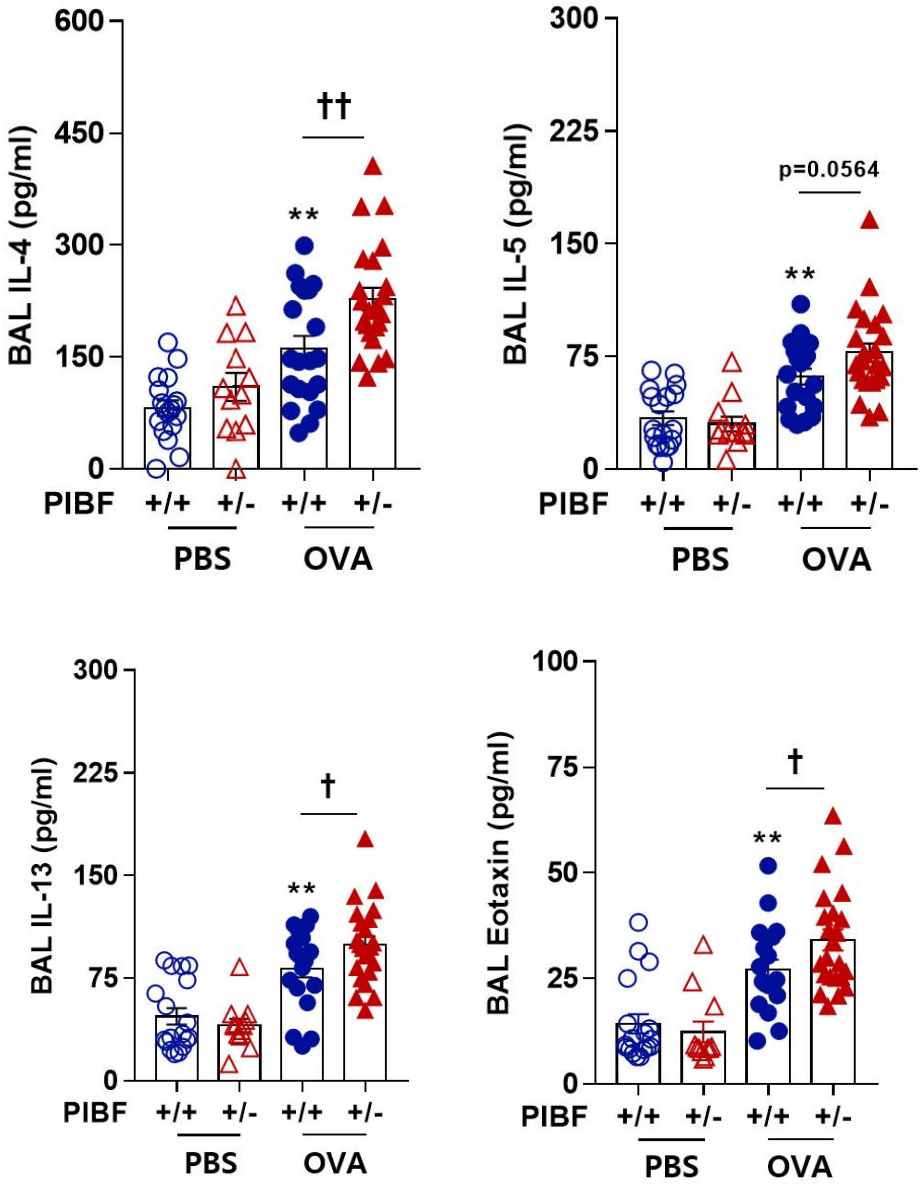


Figure 15. Lack of PIBF affected cytokine production in BAL fluid in murine asthma model

The expression of type 2 cytokines was measured in BAL fluid.

* $P<0.05$: vs PIBF+/+ PBS, ** $P<0.01$: vs PIBF+/+ PBS, *** $P<0.001$: vs PIBF+/+ PBS

**** $P<0.0001$: vs PIBF+/+ PBS, † $P<0.05$: vs PIBF+/+ OVA, †† $P<0.01$: vs PIBF+/+ OVA

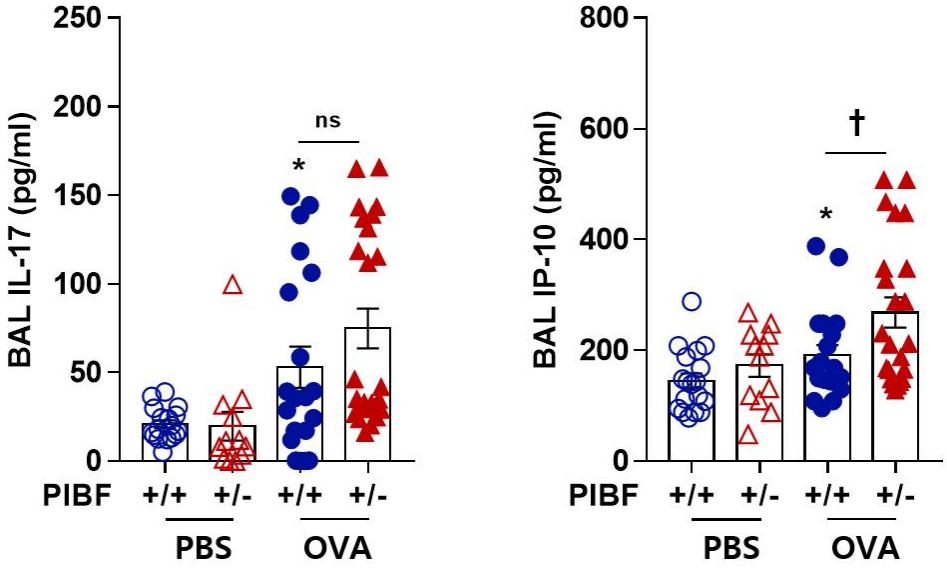


Figure 16. Lack of PIBF affected cytokine production in BAL fluid in murine asthma model

The expression of IL-17 and IP-10 was measured in BAL fluid.

* P<0.05: vs PIBF+/+ PBS, ** P<0.01: vs PIBF+/+ PBS, *** P<0.001: vs PIBF+/+ PBS

**** P<0.0001: vs PIBF+/+ PBS, † P<0.05: vs PIBF+/+ OVA, †† P<0.01: vs PIBF+/+ OVA

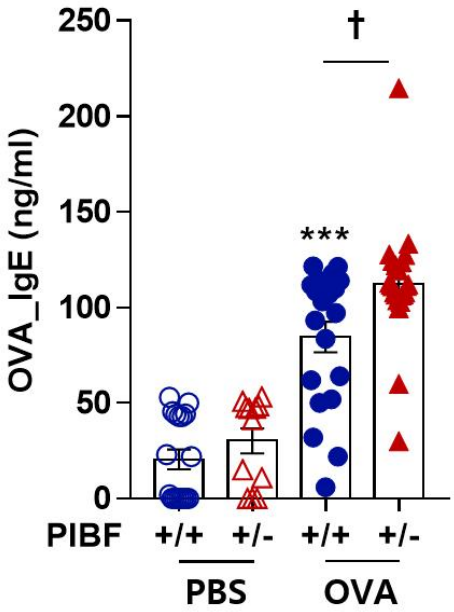


Figure 17. Lack of PIBF affected the levels of immunoglobulin in serum of murine asthma model

The expression of OVA specific IgE was measured in serum of mice.

* P<0.05: vs PIBF+/+ PBS, ** P<0.01: vs PIBF+/+ PBS, *** P<0.001: vs PIBF+/+ PBS

**** P<0.0001: vs PIBF+/+ PBS, † P<0.05: vs PIBF+/+ OVA, †† P<0.01: vs PIBF+/+ OVA

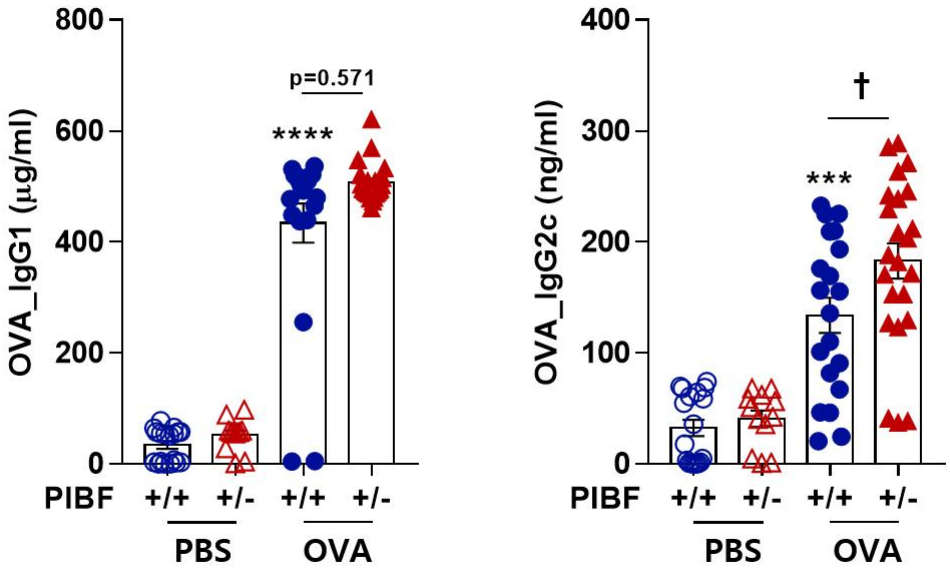


Figure 18. Lack of PIBF affected the levels of immunoglobulin in serum of murine asthma model

The expression of OVA specific IgG1 and IgG2c was measured in serum of mice.

* P<0.05: vs PIBF+/+ PBS, ** P<0.01: vs PIBF+/+ PBS, *** P<0.001: vs PIBF+/+ PBS

**** P<0.0001: vs PIBF+/+ PBS, † P<0.05: vs PIBF+/+ OVA, †† P<0.01: vs PIBF+/+ OVA

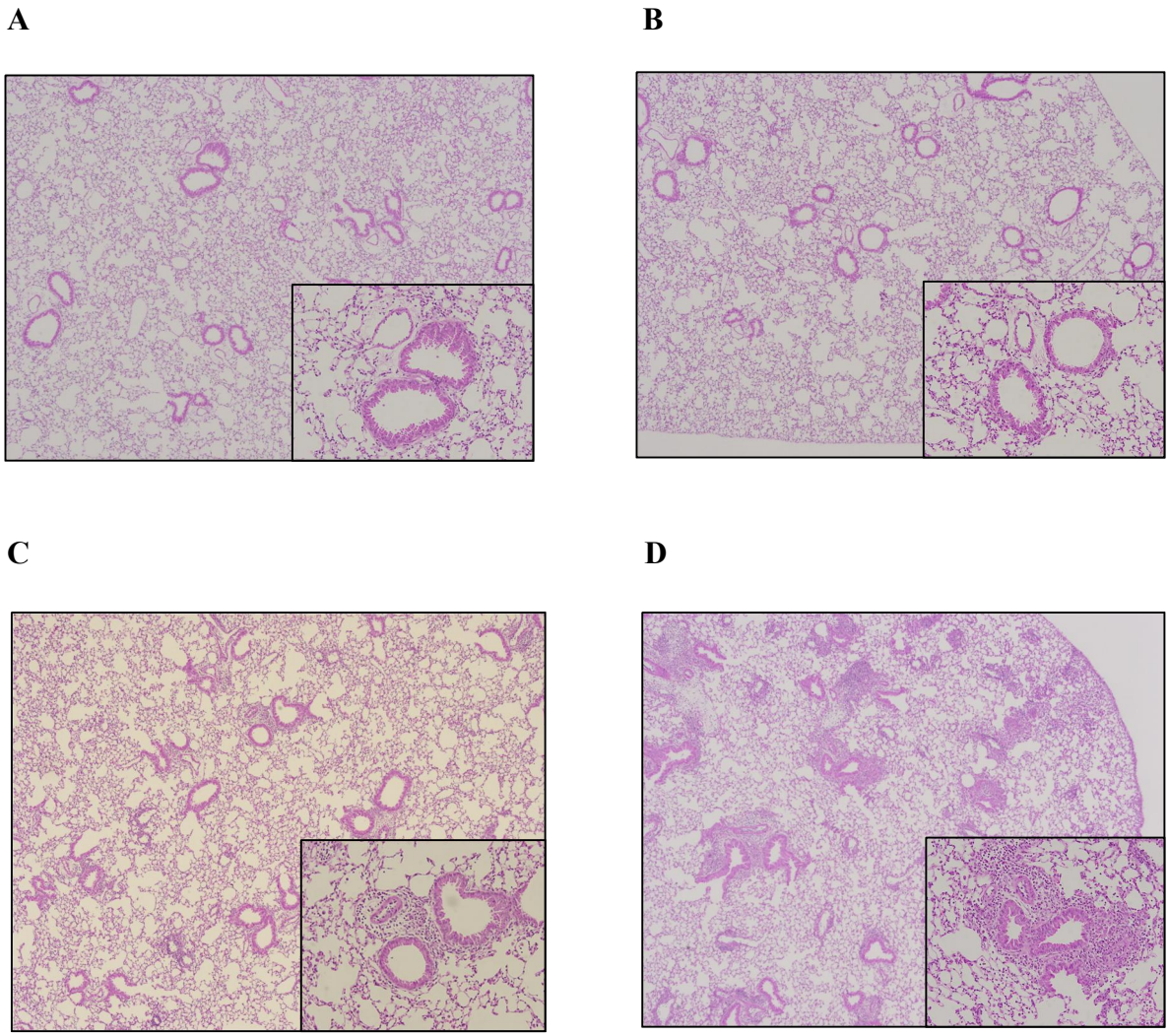


Figure 19. Tissue inflammation of the lungs was aggravated in PIBF deficent mice

Hematoxylin and eosin (H&E) staining, 100x magnification

A. PIBF+/+ PBS

B. PIBF+/− PBS

C. PIBF+/+ OVA

D. PIBF+/− OVA

3.4. Treatment with 35kDa PIBF suppressed the lysis of NK cells

When NK92MI cells treated with 100 or 500 nM recombinant PIBF were mixed with K562 cells, 500nM 35kDa PIBF suppressed the lysis of NK cells (Figure 20). Meanwhile, when using human leukemic monocyte lymphoma U937 and Jurkat T cells to assess the effect of PIBF on production of cytokines, we could not observe consistent results regarding production of TNF-α and IL-8 regardless of concentration of PIBF (Figure 21 and 22).

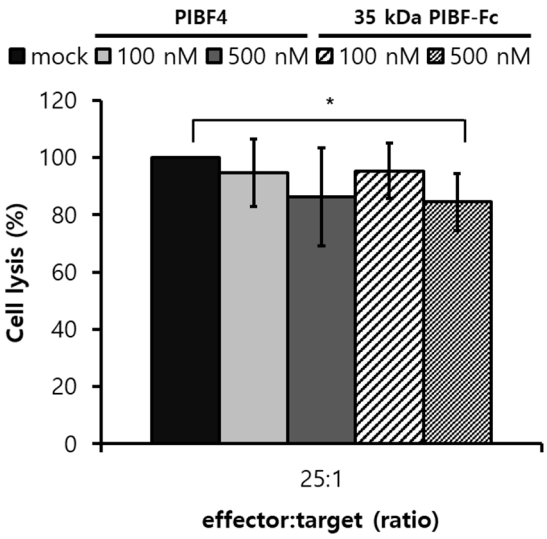


Figure 20. Treatment with 35 kDa PIBF suppressed the lysis of NK cells

NK cell lysis according to PIBF treatment with different isotypes and concentrations.

*P<0.05

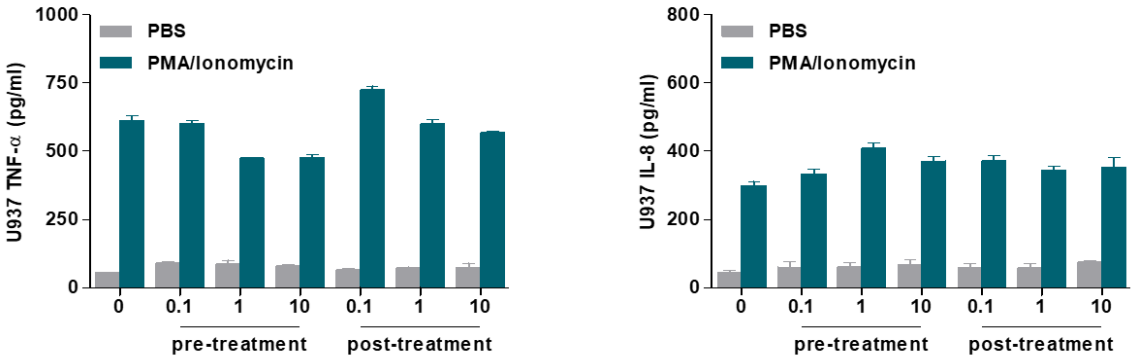


Figure 21. Effect of PIBF on cytokine production of U937 cells

The expression of TNF-α and IL-8 was measured in U937 cells when PIBF was treated before and after activation using PMA/Ionomycin.

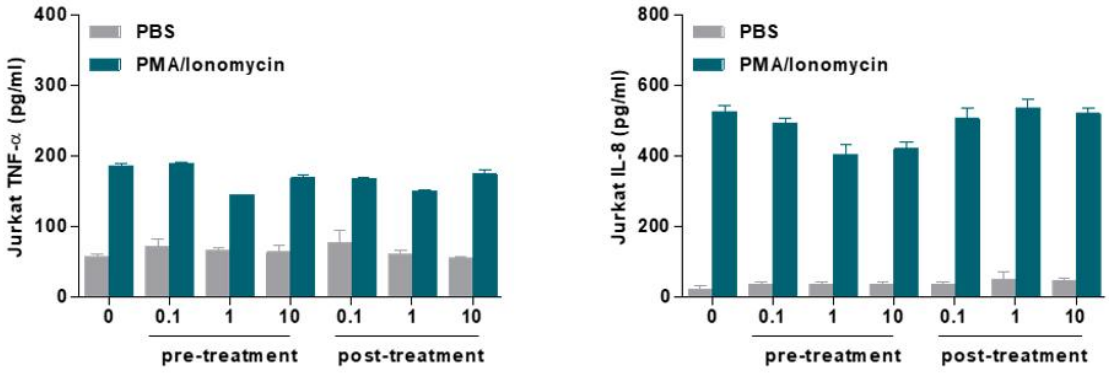


Figure 22. Effect of PIBF on cytokine production of Jurkat cells

The expression of TNF-α and IL-8 was measured in Jurkat cells when PIBF was treated before and after activation using PMA/Ionomycin.

3.5. 35kDa recombinant PIBF attenuated airway inflammation in ovariectomized mice

We then investigated the treatment effect of 35kDa recombinant PIBF in postmenopausal asthma in vivo. In active treatment group, PIBF was administered intraperitoneally 30 minutes before OVA challenge, at four different doses (0, 0.5, 5, and 50 mg/mouse) (Figure 23). These effects were compared according to the preceding ovariectomy. As expected, more prominent airway inflammation was observed in mice who underwent ovariectomy as compared with those who did not. The anti-inflammatory effect of PIBF was also noted only in ovariectomized mice. The higher dose of PIBF exerted the greater effect (Figure 24). In BAL fluid, the total cells and eosinophils significantly decreased when given 50mg of PIBF (Figure 25). Regarding cytokines in BAL fluid, the levels of IL-4, IL-5, IL-13, eotaxin, and IL-17 decreased, while IP-10 increased by treatment with PIBF (Figure 26 and 27). In serum, OVA-specific IgE gradually decreased in PIBF dose dependent manner. Meanwhile, OVA-specific IgG2 gradually increased and OVA-specific IgG1 was not affected by PIBF (Figure 28 and 29). H&E staining of lung tissue sections revealed reduction of histologic inflammation in mice treated with PIBF relative to controls (Figure 30). These results demonstrated therapeutic effect of PIBF in OVA-induced allergic asthma model.

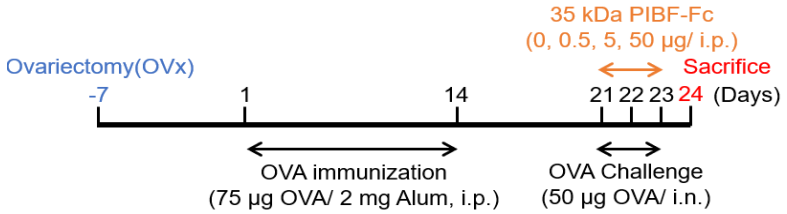


Figure 23. Murine model of allergic asthma model treated with recombinant PIBF

The recombinant 35kDa PIBF was administered interperitoneally on the days when mice were challenged with OVA. All mice underwent ovariectomy preceding establishment of OVA-induced asthma model.

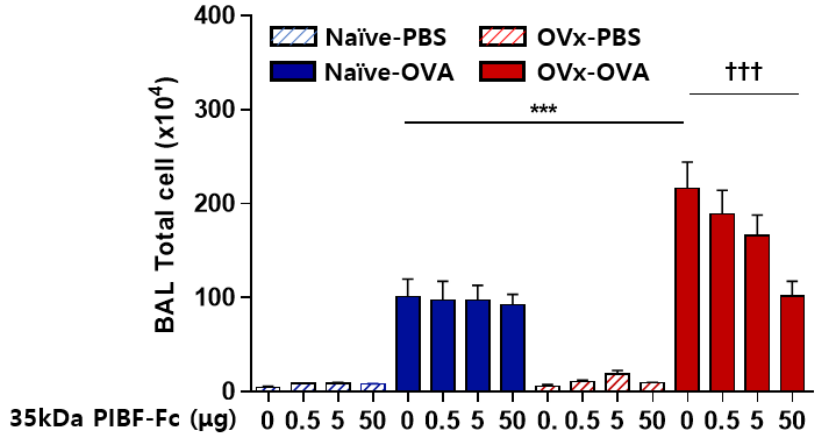
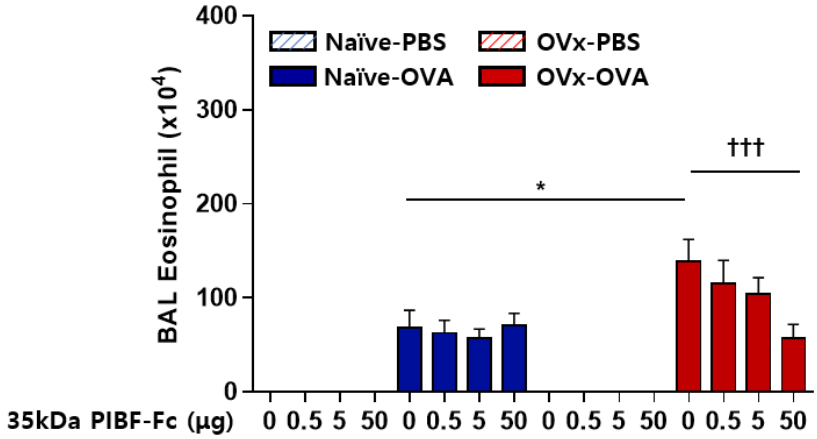
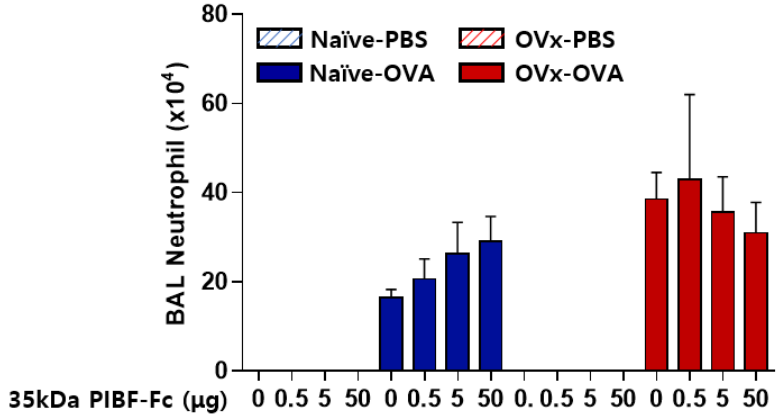


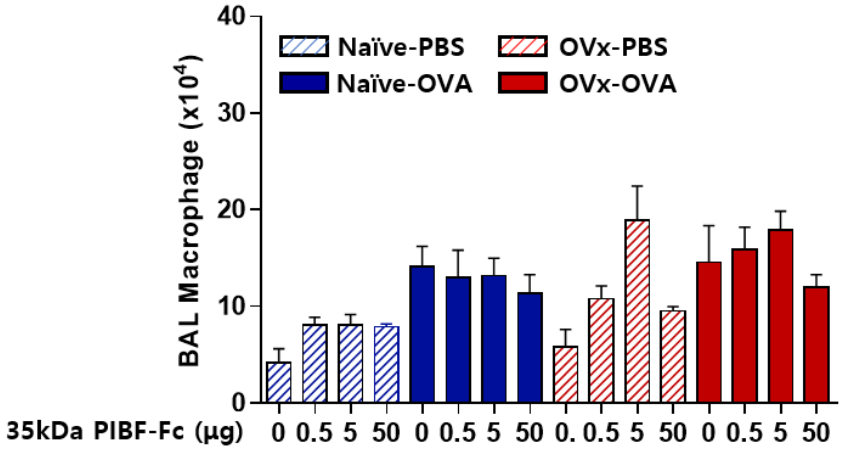
Figure 24. 35kDa recombinant PIBF attenuated airway inflammation in ovariectomized mice

The number of total cells in BAL fluid according to treatment with PIBF.

* P<0.05: vs naïve-OVA, *** P<0.001: vs naïve-OVA, ††† P<0.001: vs OVx-OVA


Naïve-PBS
OVx-PBS
Naïve-OVA
OVx-OVA
BAL Eosinophil (x10^4)
400
300
200
100
0
*
†††
35kDa PIBF-Fc (μg)
0 0.5 5 50 0 0.5 5 50 0 0.5 5 50 0 0.5 5 50


Naïve-PBS
OVx-PBS
Naïve-OVA
OVx-OVA
BAL Neutrophil (x10^4)
80
60
40
20
0
35kDa PIBF-Fc (μg)
0 0.5 5 50 0 0.5 5 50 0. 0.5 5 50 0 0.5 5 50


Naïve-PBS
OVx-PBS
Naïve-OVA
OVx-OVA
BAL Macrophage (x10^4)
40
30
20
10
0
35kDa PIBF-Fc (μg)
0 0.5 5 50 0 0.5 5 50 0. 0.5 5 50 0 0.5 5 50

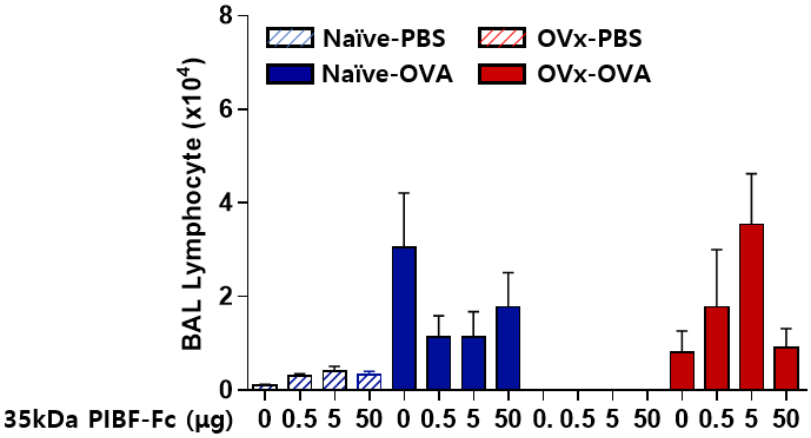
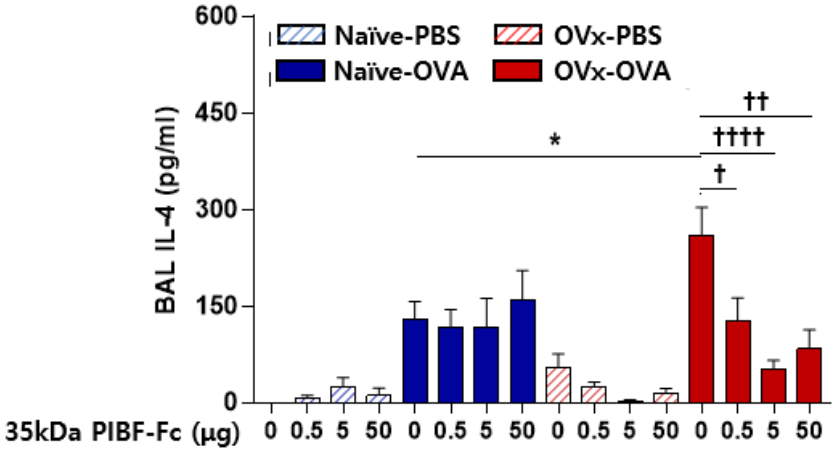
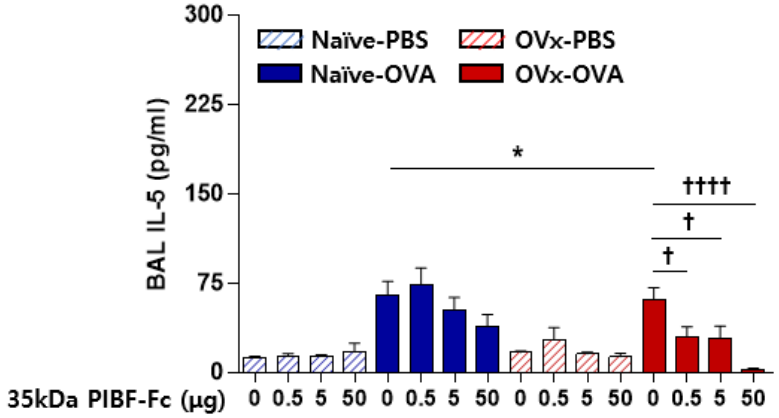


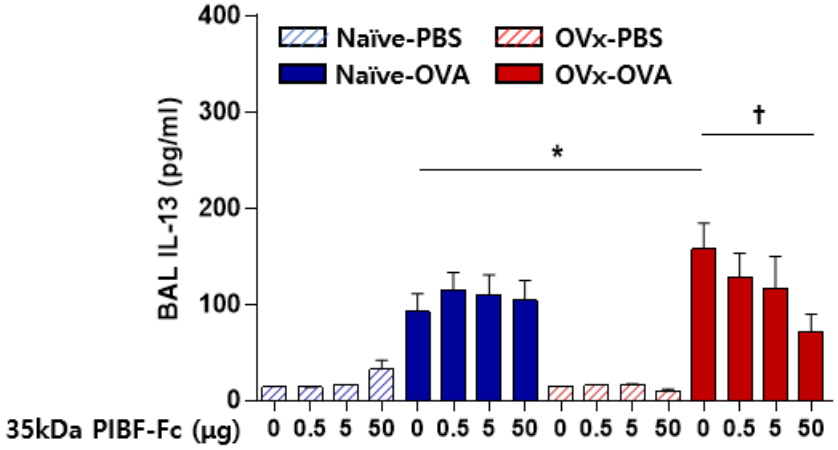
Figure 25. 35kDa recombinant PIBF attenuated airway inflammation in ovariectomized mice

The number of inflammatory cells and their composition in BAL fluid.

* P<0.05: vs naïve-OVA, *** P<0.001: vs naïve-OVA, ††† P<0.001: vs OVx-OVA


Naïve-PBS
OVx-PBS
Naïve-OVA
OVx-OVA
BAL IL-4 (pg/ml)
600
450
300
150
0
*
††
††††
†
35kDa PIBF-Fc (μg)
0 0.5 5 50 0 0.5 5 50 0 0.5 5 50 0 0.5 5 50


Naïve-PBS
OVx-PBS
Naïve-OVA
OVx-OVA
BAL IL-5 (pg/ml)
300
225
150
75
0
*
††††
†
†
35kDa PIBF-Fc (μg)
0 0.5 5 50 0 0.5 5 50 0 0.5 5 50 0 0.5 5 50


Naïve-PBS
OVx-PBS
Naïve-OVA
OVx-OVA
BAL IL-13 (pg/ml)
400
300
200
100
0
*
†
35kDa PIBF-Fc (μg)
0 0.5 5 50 0 0.5 5 50 0 0.5 5 50 0 0.5 5 50

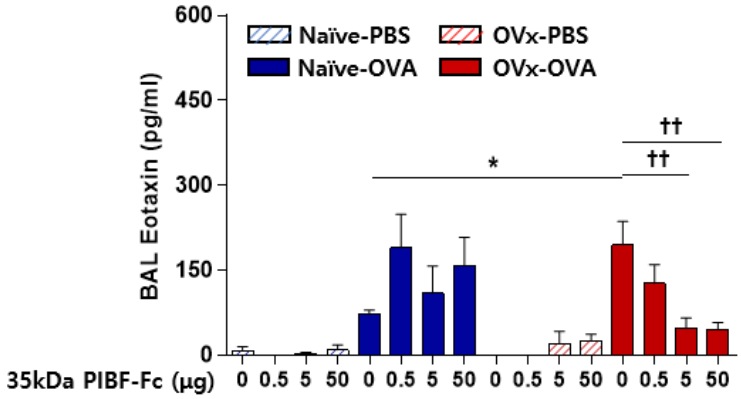


Figure 26. 35kDa recombinant PIBF reduced Th2 cytokines in BAL fluid of ovariectomized mice

The expression of type 2 cytokines was measured in BAL fluid.

* P<0.05: vs naïve-OVA, ** P<0.01: vs naïve-OVA, † P<0.05: vs OVx-OVA

†† P<0.01: vs OVx-OVA, ††† P<0.001: vs OVx-OVA, †††† P<0.0001: vs OVx-OVA

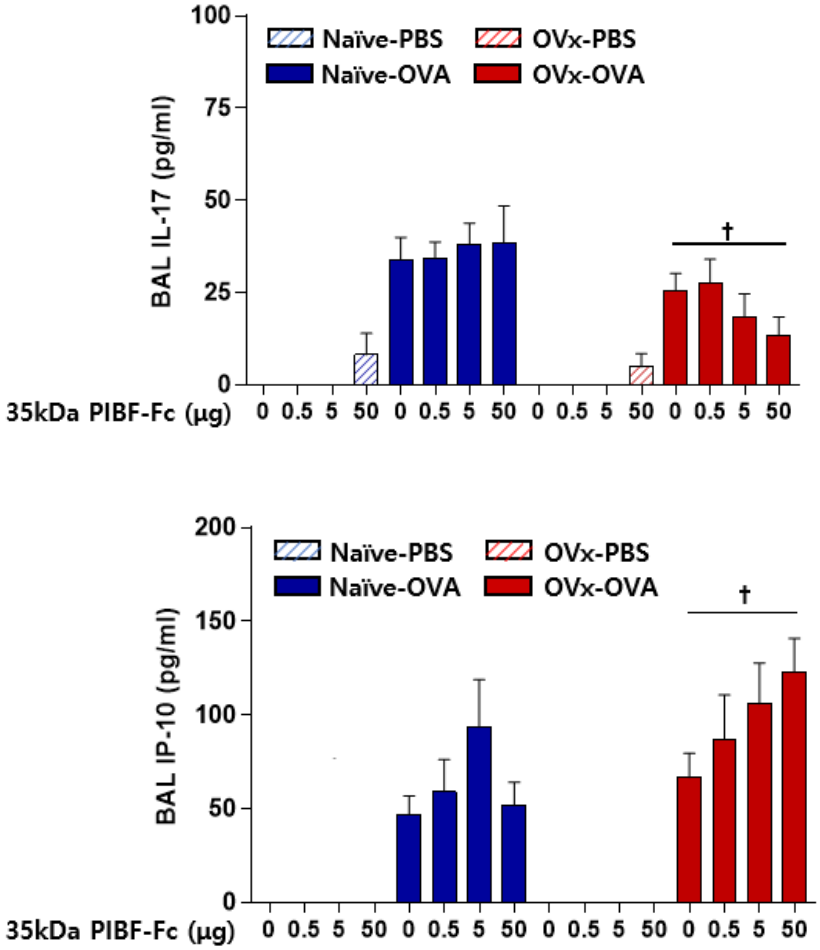


Figure 27. Change of IL-17 and IP-10 in BAL fluid of ovariectomized mice according to the treatment with 35kDa recombinant PIBF

The expression IL-17and IP-10 was measured in BAL fluid.

* P<0.05: vs naïve-OVA, ** P<0.01: vs naïve-OVA, † P<0.05: vs OVx-OVA

†† P<0.01: vs OVx-OVA, ††† P<0.001: vs OVx-OVA, †††† P<0.0001: vs OVx-OVA

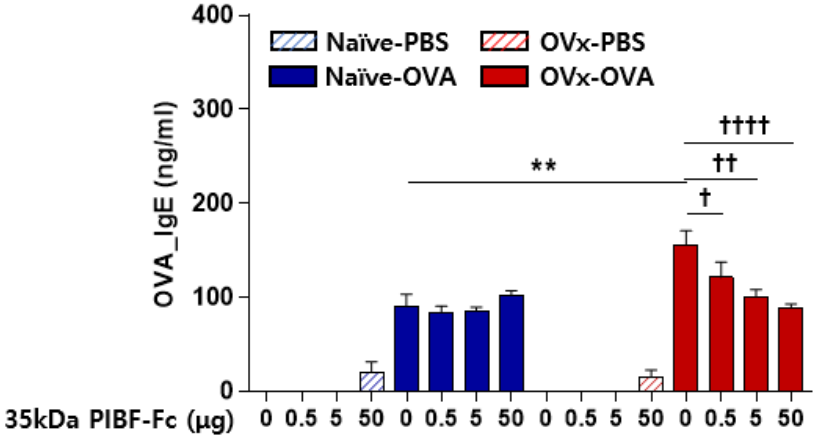


Figure 28. Supplement of PIBF affected the levels of immunoglobulin in serum of murine asthma model

The expression of OVA specific IgE was measured in serum of mice.

* P<0.05: vs naïve-OVA, ** P<0.01: vs naïve-OVA, † P<0.05: vs OVx-OVA

†† P<0.01: vs OVx-OVA, ††† P<0.001: vs OVx-OVA, †††† P<0.0001: vs OVx-OVA

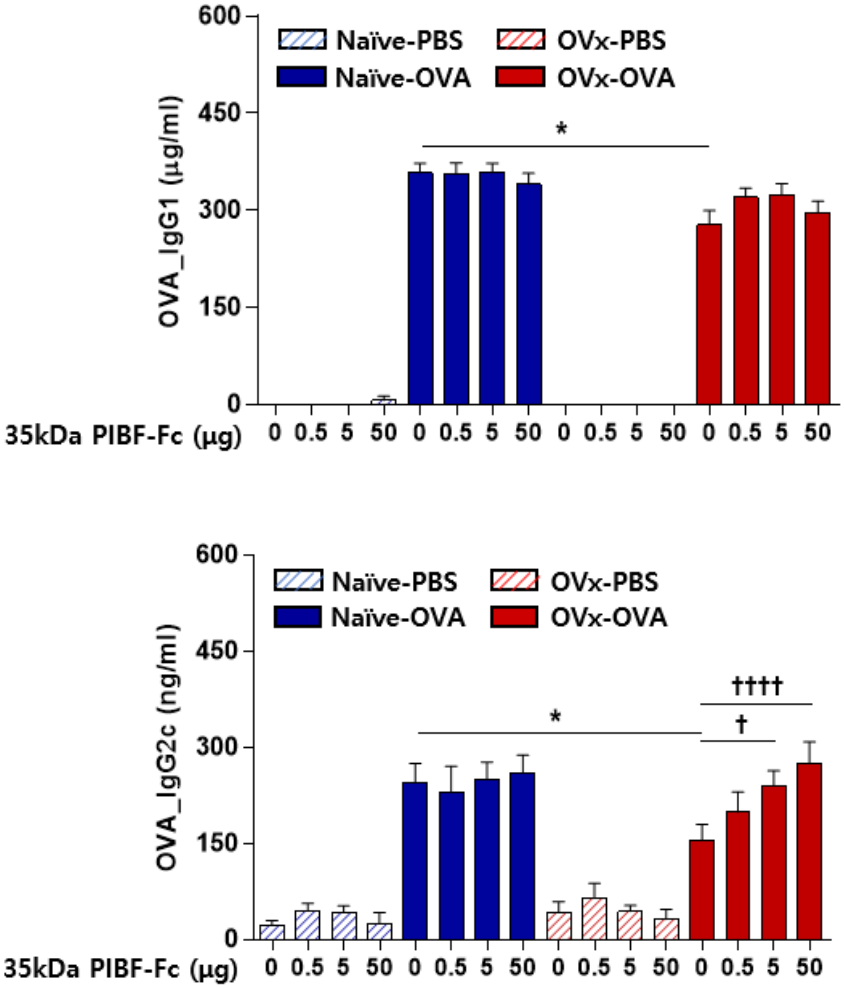


Figure 29. Supplement of PIBF affected the levels of immunoglobulin in serum of murine asthma model

The expression of OVA specific IgG1 and IgG2c was measured in serum.

* P<0.05: vs naïve-OVA, ** P<0.01: vs naïve-OVA, † P<0.05: vs OVx-OVA

†† P<0.01: vs OVx-OVA, ††† P<0.001: vs OVx-OVA, †††† P<0.0001: vs OVx-OVA

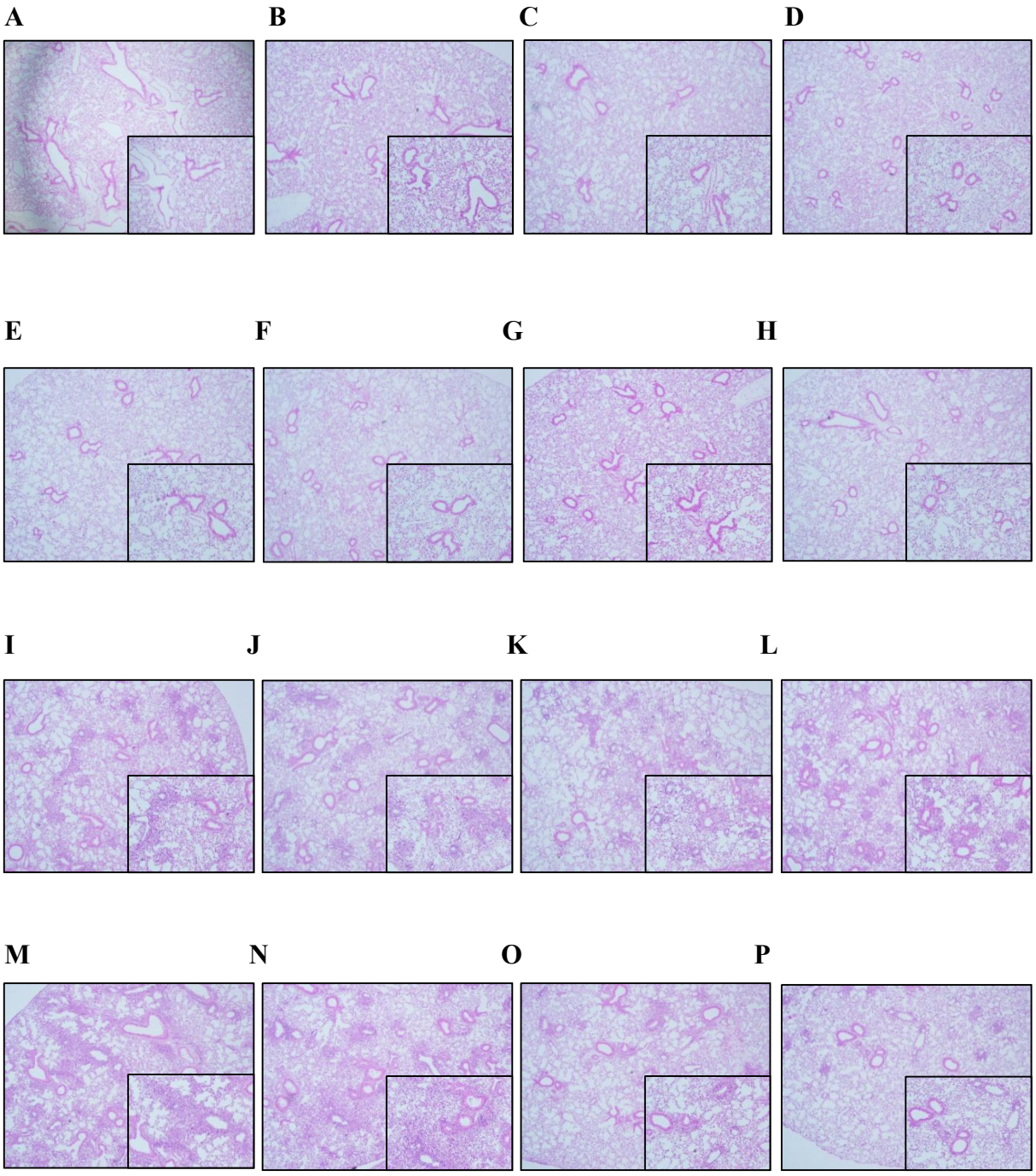


Figure 30. Airway inflammation was reduced in 35kDa PIBF treated mice

Hematoxylin and eosin (H&E) staining, 100x magnification

A. Naïve-PBS + PIBF 0μg, B. Naïve-PBS + PIBF 0.5μg, C. Naïve-PBS + PIBF 5μg

D. Naïve-PBS + PIBF 50μg, E. OVx-PBS + PIBF 0μg, F. OVx-PBS + PIBF 0.5μg

G. OVx-PBS + PIBF 5μg, H. OVx-PBS + PIBF 50μg, I. Naïve-OVA + PIBF 0μg

J. Naïve-OVA + PIBF 0.5μg, K. Naïve-OVA + PIBF 5μg, L. Naïve-OVA + PIBF 50μg

M. OVx-OVA + PIBF 0μg, N. OVx-OVA + PIBF 0.5μg, O. OVx-OVA + PIBF 5μg

P. OVx-OVA + PIBF 50μg

4. DISCUSSION

We demonstrated prominent downregulation of PIBF in serum of female asthmatics in their postmenopausal period. In murine allergic asthma model induced by OVA/alum, preceding ovariectomy resulted in aggravation of airway inflammation. Compared to the wile type mice, PIBF deficient mice showed enhanced airway inflammation when challenged with OVA. When 35kDa PIBF was administered intranasally, attenuated inflammation was noted in allergic asthma model of ovariectomized mice. In vitro experiment reported the inhibitory role of PIBF on NK cell lysis. These data implicate the role of PIBF in female asthma and its potential as a therapeutic agent.

There is a growing body of evidence that progesterone suppresses inflammation and oxidative stress in pathologic conditions such as brain injury, rheumatoid arthritis, and multiple sclerosis. (46-48) In asthma, despite the conflicting results, several studies have reported protective effects of female sex hormones including progesterone. (15) However, identifying the role of specific sex hormone is challenging due to various confounding factors such as BMI, smoking and history of menstruation. The effect of hormone may also differ whether it is exogenous or endogenous. Moreover, since estrogen and estrogen fluctuate according to menstrual cycle interacting mutually, delineating the role of each hormone is particularly difficult in premenstrual females. Accordingly, postmenopausal status is simpler situation compared to premenopausal status due to consistent deficiency of female sex hormones.

When evaluating serum levels of PIBF in asthmatics and healthy controls, we have observed significantly lower level in elderly female asthmatics. Both asthma and menopause seemed to be associated with downregulation of PIBF, since having asthma was related to decreased PIBF expressions among patients in premenopausal period. Serum PIBF was measured in the previous studies related to pregnancy. Women having unexplained infertility

had the lower serum PIBF levels compared to fertile women. (49) Serum PIBF was also reported as a predicting factor for successful pregnancy in patients who had undergone invitro fertilization (IVF) procedure. (50) In pregnant women, lower maternal serum concentration of PIBF was related to preterm delivery. (51) However, majority of studies have investigated PIBF in women in reproductive age. Thus, there is scarce of data regarding serum PIBF level in post-menopausal women. We have found that serum PIBF was still detectable in post-menopausal women. Some researchers suggested that progesterone was not the only factor affecting the level of PIBF and proportion of cells expressing progesterone receptors could also attribute to the production of PIBF. (49) However, in this study, we have not measured female sex hormones including estrogen and progesterone. Therefore, we could not analyze the correlation between progesterone and PIBF among our participants.

In murine model of allergic asthma, ovariectomy exacerbated airway inflammation. This model reflected the development of asthma in postmenopausal females. The similar effect of ovariectomy in female mice was previous reported in 2017. Compared to sham operated Balb/c mice, ovariectomized mice had more leukocytes in BAL fluid when allergic asthma model was induced using OVA. Histologic findings of lung showed significantly increased eosinophils and mast cells in ovariectomized mice. They also reported higher level of IL-4 and lower level of IL-17 in BAL fluid compared with sham operated mice. (52) By contrast, Riffo-Vasques reported the opposite results in mice that ovariectomy preceding establishment of allergic asthma reduced lung inflammation and airway hyperresponsiveness to methacholine. Interestingly, when ovariectomy was conducted between sensitization and challenge of OVA, it tended to augment eosinophilic inflammation and recruit more IL-5. (49) In allergic asthma model of rat, ovariectomized rats reported reduced number of total mononuclear cells, neutrophils, and eosinophil counts in BAL fluids compared with sham rats. Regarding

cytokines of BAL fluid, ovariectomy did not affect basal level of cytokines. However, in supernatants of 4-hour incubated BAL cells, more IL-10, less IL-1β, and less TNF-α was observed in ovariectomized rat than those of sham rats. (21) In our experiments, development of OVA-induced allergic asthma was featured with prominent type 2 inflammation in ovariectomized mice supported by inflammatory cells, cytokines in BAL fluid, histologic findings, as well as serum immunoglobulins.

To further investigate the role of PIBF, we compared the airway inflammation in PIBF wild type and hetero mice. The estrous cycle of mice repeats every four to five days, composed of 4 stages including proestrus, estrus, metestrus, and diestrus phases. (53) As a result, mice used in the experiments might have had different levels of progesterone according to the menstrual cycle. Since progesterone works as a stimulant for production of PIBF, the effect of serum progesterone needed to be controlled not to confound the effect of PIBF. Therefore, we conducted ovariectomy and administered same dose of progesterone to equalize the effect of progesterone. We could not use PIBF knock out mice due to the fatality. Compared to PIBF+/+ mice, PIBF +/- mice had more eosinophilic airway inflammation with increase of Th2 cytokines in BAL fluid. Histology of lungs also revealed more severe form of inflammation in PIBF deficient mice. Collectively, lack of PIBF led to exacerbated allergic airway inflammation suggesting its substantial role in female asthma.

As we have observed the impact of PIBF in development of allergic asthma in female mice, we sought to assess the therapeutic effect of PIBF. We used recombinant 35kDa PIBF for the experiments. We have observed that concomitant supplement of PIBF during challenge with OVA attenuated eosinophilic inflammation in a dose dependent manner. It showed no effect on neutrophils, macrophages, and lymphocytes in BAL fluid. Along with inflammatory cells, IL-4, IL-5, IL-13, and eotaxin also reduced. Serum level of OVA-specific IgE decreased

with treatment with 35kDa PIBF accompanied with elevation of OVA-specific IgG2c. Histologic findings of lungs also showed attenuated inflammation in mice treated with PIBF. These treatment effects were only observed in allergic asthma model in ovariectomized mice, while no significant change was noted in mice with intact ovaries.

Thus far, researches on PIBF have been mostly focused on pregnancies and malignancies featured with notably increased PIBF. The full-length PIBF was reported to reside in the nucleus affecting the cell cycle and cleaved PIBF isoforms were suggested to act as cytokines. In line with the precious studies, we observed inhibitory role of PIBF in NK cell lysis. (54) However, we could not observe the effect on altered cytokine release in cell experiments. Bartho et al. suggested that PIBF inhibited both IL-12 production and TNFα. (35) Kozma and colleagues reported that PIBF induced Jak1 phosphorylation and activation of STAT6 by binding to PIBF-receptor-IL-4α complex. (38) Further investigations are needed to understand the mechanism of inhibitory effect of PIBF on eosinophilic inflammation.

Elderly females are the most challenging subgroup in patients with asthma. The substantial disease burden in this population is attributed to not only severity of airway inflammation but also adverse effects of corticosteroids. Frequent asthma exacerbations require more corticosteroids, which results in additional comorbidities making a vicious cycle. Even though biologic agents are currently available as an effective way of saving corticosteroids, most of them target only type 2 inflammation. Another limitation of newly developed medications is exclusion of elderly population. Since most clinical trials exclude elderly patients aged over 80, the effective treatment strategies are hart to establish. In this regard, our study suggested a novel treatment option for the elderly females once their asthma is affected by menopause.

5. CONCLUSIONS

In conclusion, PIBF was reduced in postmenopausal female asthmatics and supplement of recombinant PIBF showed therapeutic effect in mice model of postmenopausal asthma. Thus, PIBF could be a potential therapeutic agent for elderly females with asthma whose airway inflammation is largely dependent on progesterone-PIBF related pathway. Further studies are warranted to better understand the mechanism underlying anti-inflammatory effect of PIBF in the context of asthma.

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국문요약

배경: 노인 여성 천식 환자들은 다른 천식 환자들에 비해서 높은 이환율과 사망률을 보이지만, 그 기전에 대해서는 아직 밝혀진 바가 거의 없다. 프로게스테론 유도 억제인자, 이하 PIBF는 프로게스테론에 의해 생산이 촉진되며 면역체계를 조절하는 것으로 알려져 있다.

목표: 폐경 후 여성 천식에서 PIBF 역할을 탐색해보고자 한다.

방법: 우선 천식 환자와 건강 대조군의 혈청에서 PIBF를 측정하여 비교한다. 폐경과 PIBF의 부족이 천식에 미치는 영향을 확인하기 위해서 난소절제를 시행한 쥐와 PIBF 이종 쥐를 각각 사용하여 OVA 로 유도한 천식 모델을 구축하고 기도염증의 정도를 비교한다. PIBF의 치료효과를 평가하기 위해 35kDa 재조합 PIBF를 천식을 유도한 쥐와 자연살해세포에 처리하여 본다.

결과: PIBF는 폐경 후 천식환자의 혈청에서 감소되어 있었다. 쥐의 OVA 유도 천식 모델에서 난소절제술을 시행한 경우와 PIBF 이종 쥐를 사용하였을 경우 각각에서 기도 염증이 심화되는 것을 확인할 수 있었다. 35kDa 재조합 PIBF를 처리하였을 경우 난소절제술을 시행한 천식 유도 쥐의 기도염증과, K562 세포의 자연살해세포에 의한 용해가 억제되었다.

결론: PIBF는 기도염증이 프로게스테론-PIBF 경로에 의존적인 노인 여성 천식 환자에서 잠재적인 치료제가 될 수 있을 것으로 기대된다. 앞으로 천식에서 PIBF의 항염증 효과를 밝히는 추가적인 연구가 필요하겠다.