岩手医科大学
審査学位論文
（博　士）

A Phosphatidylinositol 3-kinase Inhibitor strongly suppressed Pulmonary Vascular Remodeling of Allergic Vasculitis in Murine Model

Journal:	*Experimental Lung Research*
Manuscript ID	UELR-2015-0204.R1
Manuscript Type:	Original Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Oikawa, Yuka; Iwate Medical University School of Medicine, Division of Pulmonary Medicine, Allergy and Rheumatology, Department of Internal Medicine Sasaki, Nobuhito; wate Medical University School of Medicine, Division of Pulmonary Medicine, Allergy and Rheumatology, Department of Internal Medicine Niisato, Miyuki; Iwate Medical University School of Medicine, Division of Pulmonary Medicine, Allergy and Rheumatology, Department of Internal Medicine Nakamura, Yutaka; Iwate Medical University School of Medicine, Division of Pulmonary Medicine, Allergy and Rheumatology, Department of Internal Medicine Yamauchi, Kohei; Iwate Medical University School of Medicine, Department of Internal Medicine, Division of Pulmonary Medicine, Allergy and
Keywords:	PI3K inhibitor, allergic vasculitis, murine model, IL-4, IL-5, IL-13, TGF-β, C57BL/6, remodeling, eosinophil

A Phosphatidylinositol 3-kinase Inhibitor strongly suppressed Pulmonary Vascular Remodeling of Allergic Vasculitis in a Murine Model

Yuka Oikawa, Nobuhito Sasaki, Miyuki Niisato, Yutaka Nakamura, Kohei Yamauchi.
Division of Pulmonary Medicine, Allergy and Rheumatology, Department of Internal Medicine, Iwate Medical University School of Medicine, Morioka, Japan

Supported by the Ministry of Education, Science and Culture, Japan (#26461194)

Running title: Effects of PI3K inhibitor on allergic vasculitis

Correspondence: Kohei Yamauchi, MD, PhD
Professor
Division of Pulmonary Medicine, Allergy and Rheumatology
Department of Internal Medicine, Iwate Medical University School of Medicine
19-1 Uchimaru, Morioka 020-8505, Japan
Tel : +81-19-651-5111, ex 2332
Fax : +81-19-626-8040
e-mail: kyamauch@iwate-med.ac.jp

Abstract

Objectives: We investigated the effects of pan-class I PI3K inhibitor, ZSTK474 on vascular remodeling using a murine model of allergic vasculitis with eosinophil infiltration. **Methods:** C57BL/6 mice were sensitized with OVA. The positive controls were exposed to aerosolized OVA daily for 7 days. The other group of mice were administered ZSTK474 (30 mg/kg, p.o. daily) in parallel with daily exposure to aerosolized OVA for 7 days. On the 3rd and 7th day, bronchoalveolar lavage (BAL) was performed and the lungs were excised for pathological analysis. Cell differentials were determined and the concentrations of IL-4, IL-5, IL-13 and TGF-β in BAL fluid were measured. **Results:** The total cell numbers and eosinophil numbers in BALF were greatly reduced in the ZSTK474 treated group on the 3rd and 7th day after exposure to OVA. The numbers of total white blood cells and eosinophils in the peripheral blood were significantly reduced in the ZSTK474 treated group on the 3rd and 7th day after exposure to OVA. The concentrations of IL-4, IL-5 and IL-13 in

BAL fluids were also reduced significantly on the 3rd day in the ZSTK474 treated group. The concentrations of TGF-β in BAL fluids were also reduced significantly on the 3rd and 7th day in the ZSTK474 treated group. The pathological scores reduced significantly in the ZSTK474 treated group compared to the control group. **Conclusion:** The PI3K inhibitor, ZSTK474 suppressed pulmonary vascular remodeling in the murine model of allergic vasculitis with eosinophil infiltration. PI3K signal transduction may have a critical role in the immunological process that induces allergic vasculitis.

Key words: PI3K inhibitor, allergic vasculitis, murine model, IL-4, IL-5, IL-13, TGF-β, C57BL/6, remodeling

Abbreviation used in this article

BAL :bronchoalveolar lavage

BALF :bronchoalveolar lavage fluid

p.o. : *per os*

OVA: ovalbumin

IL-4: interleukin-4

IL-5: interleukin-5

IL-13: interleukin-13

TGF-β: transforming factor β

Introduction

Eosinophilic granulomatous polyangitis (EGPA) is characterized by bronchial asthma, eosinophilia, and systemic necrotizing vasculitis with or without granulomas (1-3). The main lesions of EGPA are located in medium and small-sized vessels. The vasculitis of EGPA causes serious damage to the skins, nerves, digestive canals, lung and other organs. Although many clinical trials have been performed, an effective therapy for EGPA has not been established yet.

The mechanism of EGPA remains unclear. Eosinophils are the most dominant cells in the blood and extravascular tissues in EGPA, and are known to release cytotoxic products such as major basic protein, eosinophil-derived neurotoxin, and cytokines including TGF-β (4, 5).

We previously reported a murine model of pulmonary allergic vasculitis, which was induced by repeated inhalative exposure of OVA to C57BL/6 mice sensitized with OVA (6). We demonstrated that the small pulmonary arteries of the OVA-exposed mice were occluded with accumulated

myofibroblasts and collagen deposition on the 7th day.

Phosphatidylinositol-3,4,5-trisphosphate (PIP3), which is generated from phosphatidylinositol-4,5-bisphosphate by phosphatidylinositol 3-kinase (PI3K), is known to play important roles in various physiological functions *via* PI3K/Akt signaling pathway (7, 8). Since it has been reported that PI3K is involved in cell growth, cell survival and cell cycle progression, the inhibitors of PI3K have drawn much attention due to their anti-cancer actions (9). However, several reports elucidated the anti-inflammatory actions of the inhibitors of PI3K (10). Palframan et al. reported that eosinophil recruitment from bone marrow in response to IL-5 was suppressed by the inhibitors of PI3K through their inhibitory actions on the expression of chemokines induced by IL-5(11). In addition, Duan et al. reported that IL-5, IL-13 and eotaxin levels in bronchoalveolar lavage fluid were suppressed by the inhibitor of PI3K in murine asthma model (12).

In this regard, we examined the effects of pan-class I PI3K inhibitor, ZSTK474 on the histological changes in our model of allergic pulmonary

vasculitis. This study may contribute to finding a therapy for allergic vasculitis.

Methods and Materials

Animals

Female C57/BL6 mice (6-8 wk old) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed under specific pathogen-free conditions following a 12-hour light-dark cycle and fed a standard laboratory diet and given water *ad libitum*. The estrous cycle of the female mice was not synchronized in the present experiment. The obtained results were thought to be the average from the female mice in various stages of the estrous cycle. All experiments described in this study were performed according to the guidelines for the care and use of experimental animals as determined by the Japanese Association for Laboratory Animals Science in 1987.

Administration of ZSTK474

ZSTK474 was synthesized by the Research Laboratory of Zenyaku Kogyo Co., Ltd. (Tokyo, Japan). One hundred μl of ZSTK474 (9mg/ml) suspended in 0.75% hypromellose (Wako, Japan) in water as a solid dispersion (13) were administered through a flexible feeding tube (for mice, size:ϕ □□9x70mm, CL-4563)(CLEA Japan, Inc). Therefore, ZSTK474 (30mg/kg) was delivered reliably to the stomach of each mouse. ZSTK474 was administered to mice once daily during 1 week of OVA inhalation in the ZSTK474-treated mice. Prior to the experiment, we investigated the optimal dose of ZSTK474 in terms of the relationship between the doses of ZSTK474 (1~ 100 mg/kg) and their effects on the severity of vasculitis. In addition, taking into consideration the lethal dose and the effective dose in the *in vivo* study of previous paper (13), we decided to perform the current experiment using 30 mg/kg of ZSTK474. The hypromellose suspension without ZSTK474 was administered with a flexible feeding tube to the control mice.

Immunization and Aerosolization protocol

The mice were sensitized according to the methods described in a previous paper (14). Each mouse of the positive control group was placed in a plastic chamber (10 cm × 15 cm × 25 cm) and exposed to aerosolized OVA (5 mg/ml in 0.9% saline) for 1 h daily until the 7th day as shown in Figure1. The aerosolized OVA was produced by a Pulmo-Aide Compressor/Nebulizer (Devilbiss) (Sunrise Medical HHG, Inc. Somerset, PA, USA) at a flow rate of 5-7 liter/min. The ZSTK474-treated group of mice was exposed to aerosolized OVA for 1 h daily until the 7th day and in parallel, administered ZSTK474 as described above (Fig. 1).

Collection and measurement of specimens

After being exposed to aerosolized OVA every day over a period of one week, the mice with or without ZSTK474 were killed by cutting the femoral artery on the 3rd and 7th day, 24 hours after the final inhalation, and blood, BALF and lung tissue were collected as shown in Figure 1. As a

control, after being exposed to aerosolized saline instead of OVA every day over one week, samples were collected.

BALF was collected analyzed according to the methods described in a previous paper (6). The numbers of cells recovered per mouse were then expressed as the mean and standard deviation (SD) for each treatment group.

After harvesting the BALF, the lungs were fixed with 10% neutral buffered formalin and embedded in paraffin. These 3-μm-thick sections were stained with hematoxylin eosin (HE) and Elastica- Masson (EM).

Semi-quantitative analysis of pathological changes in the pulmonary arteries

The extent of histological changes in pulmonary arteries was assessed as described previously (6, 15). In brief, the tissue was cut into sections 3 μm thick, stained with HE and EM, and evaluated by light microscopy. Histological scores were determined according to the criteria (6, 15). We scored 5 vessels whose diameters ranged from 20 to 50 μm in each of the

lung tissue sections and the average was considered as the histological index of one mouse.

Immunohistochemical staining for α smooth muscle actin

We adopted the biotin-streptavidin system using a Histofine Kit (Nichirei, Tokyo, Japan) for the immunohistochemical staining. We used anti- α smooth muscle actin (α-SMA) (Spring Bioscience, Fremont, CA, USA). The detail was previously reported (6).

Cytokine measurement

BALF was used for the measurement of IL-4, IL-5, IL-13 and TGF-β concentration using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Mann-Whitney U Test was used in the analysis of results. All values are

expressed as means ± SD. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of ZSKT474 on eosinophil numbers in peripheral blood and in BALF

The total cell number and the number of eosinophils in BALF on the 3rd day and 7th day were dramatically increased compared with those before OVA inhalation (Fig.2). The total cell number and the number of lymphocytes, eosinophils and neutrophils in BALF on the 3rd day were decreased significantly in the ZSTK474-treated mice compared with those of the control positive mice (Fig.2).

The number of eosinophils in BALF on the 7th day was decreased significantly in the ZSTK474-treated mice compared with those of the positive control mice (Fig.2).

After repetitive exposure to OVA, the total white blood cell numbers in peripheral blood slightly increased on the 3rd day in the positive control

mice. In contrast, the total white blood cell numbers in the ZSTK474-treated mice were significantly lower on the 3rd and 7th day than those of the positive control (Fig.3A).

The blood eosinophil counts in the positive control increased after OVA inhalation. The blood eosinophil counts in the ZSTK474-treated mice were significantly lower on the 3rd and 7th day than those in the positive control ($P<0.05$) (Fig.3B).

Cytokine concentration in BALF

We measured the concentrations of IL-4, IL-5, IL-13, and TGF-β in BALF on the 3rd and 7th day in the positive control group mice and in the ZSTK474-treated group mice as described in Methods. As shown in Figure 4A, 4B and 4C respectively, the concentrations of IL-4, IL-5 and IL-13 in the BALF of the positive control mice peaked on the 3rd day. The concentrations of IL-4, IL-5 and IL-13 in BALF of the ZSTK474-treated mice were significantly lower on the 3rd day than those of the positive control.

In contrast, the concentration of IL-4, IL-5 and IL-13 in BALF on the 7th day was rather higher in the ZSTK474-treated group compared with those of the positive control.

The concentrations of TGF-β in the BALF of the positive control group increased until the 7th day after OVA inhalation and the concentrations of TGF-β in the BALF of the ZSTK474-treated mice decreased significantly on the 3rd and 7th day compared with those of the positive control (Fig.4D).

Effects of ZSTK474 on the histological changes in pulmonary arteries.

In the positive control group mice, almost all small pulmonary arteries were highly obstructed due to the accumulation of cellular components including myofibroblasts proliferation (Fig.5a)(6, 15, 16). In addition of myofibroblasts, collagenous materials were observed (Fig. 7a).

In contrast, the histological changes of the small pulmonary arteries in the ZSTK474-treated mice were markedly reduced compared to those of the positive controls (Fig.5b, Fig.7b).

Semiquantitative analysis of the histological vascular changes on the 7th

day in the positive controls and the ZSTK474-treated mice was performed as described in "Methods" to determine the severity index.

The severity index in the ZSTK474-treated group mice was significantly reduced compared to that in the positive controls (Fig.6).

α-SMA staining demonstrated myofibroblasts occupied intravascular space of pulmonary arteries on the 7th day in the positive control (Fig. 7c). In contrast, myofibroblasts were not seen on the 7th day in the ZSTK-treated mice (FIG.7d).

Discussion

We established a murine model with allergic vasculitis with eosinophil infiltration (6). In this model, we observed prominent eosinophil infiltration perivascular space of pulmonary arteries and intravascular proliferation of myofibroblasts (6, 15,16). With this model, we have evaluated inhibitory effects of imatinib mesylate and rapamaycin on allergic vasculitis by decrease of TGF−β (15, 16). The present study demonstrated that the PI3K

inhibitor ZSTK474 inhibited the histological changes of vasculitis in the pulmonary arteries of the OVA-exposed mouse model of AGA and reduced the number of eosinophils in BALF on the 3rd day after OVA inhalation. ZSTK474 lowered the TGF-β concentration in the BALF of OVA-sensitized mice after OVA inhalation.

IL-4, IL-5 and IL-13 are thought to play critical roles in the eosinophilopoiesis, and chemotaxis, survival and activation of eosinophils. Especially, the proliferation and differentiation of eosinophils are known to be regulated by IL-5 (17- 21). Palframan et al. reported that mobilization of bone marrow eosinophils is a critical early step in their trafficking to the lung during an allergic inflammatory reaction. PI3K-I blocked eosinophil release induced by IL-5 in their perfused bone marrow system (11). In addition, concerning eosinophil recruitment, Lim et al demonstrated that PI3Kγ-deficient mice challenged with OVA had significantly reduced numbers of bronchoalveolar lavage and peribronchial eosinophils compared with wild mice. They also reported that there was no significant

difference in the number of bone marrow or circulating peripheral blood eosinophils when comparing wild mice and PI3Kγ-deficient mice, suggesting that the trafficking of eosinophils into the lung was reduced in PI3Kγ-deficient mice (22). In contrast to their results, the numbers of eosinophils in both BALF and peripheral blood were markedly reduced in the ZSTK474-treated mice compared with those of positive control on the 3rd and 7th day (Fig.3B). Lim et al used PI3Kγ-deficient mice and we used pan-class I PI3K inhibitor, ZSTK474. A PI3K pathway other than PI3Kγ may be involved in the trafficking of eosinophils from the bone marrow to blood or in the proliferation of eosinophils in bone marrow.

In our experiments, the concentrations of IL-4, IL-5 and IL-13 in the BALF of OVA-exposed mice (positive control) increased on the 3rd day and decreased on 7th day. On the other hand, TGF-β concentration increased until 7th day. TGF-β has the capacity to suppress the inflammatory actions of lymphocytes and cytokine production from immune cells. In this regard, the reduction of Th2 cytokines on the 7th day

may be due to anti-inflammatory cytokines such as TGF-β or tolerization in response to OVA stimulation.

On the other hand, Th2 cytokine concentrations in the BALF of the ZSTK474-treated mice on the 3rd day were markedly reduced compared with those of the positive control mice. PI3K plays a pivotal role in the recruitment and activation of certain inflammatory cells. Lee et al suggested that the PI3K/Akt pathway was involved in the production of Th2 cytokines such as IL-4, IL-5, IL-13 in an OVA sensitized murine asthma model (23). On the other hand, the concentrations of Th2 cytokines in the BALF of the ZSTK474-treated mice on the 7th day were increased compared with those of the positive control mice. We also observed similar results in the case of rapamycin-treated mice in a murine model of pulmonary allergic vasculitis (15). When the PI3k/Akt/mTOR pathway is blocked, another pathway might be employed later to induce the Th2 cytokine production. In addition, it is possible that inhibition of TGF-β production by PI3k inhibitor might induce an increase of Th2

cytokines on the 7th day. However, further investigation is needed to elucidate these mechanisms.

TGF-β is known to be a key molecule involved in tissue fibrosis including subepithelial fibrosis in asthma and pulmonary fibrosis (24, 25). In our experimental model, we previously reported a marked increase in the TGF-β concentration in the BALF of the OVA sensitized mice after OVA exposure (15). The present study demonstrated that ZSTK474 decreased the TGF-β1 concentration in the BALF of the OVA sensitized mice after OVA exposure. Consistent with the present results, Koizumi et al demonstrated that the mTOR inhibitor, rapamycin reduced the TGF-β1 concentration in the BALF of the OVA sensitized mice after OVA exposure (15). In this regard, the PI3K/Akt/mTOR pathway is thought to be involved in the regulation of the TGF-β1 concentration in the airway of the murine model with pulmonary allergic vasculitis.

We demonstrated that ZSTK474 strikingly suppressed the vascular remodeling of pulmonary arteries in the OVA sensitized mice after OVA

exposure. Critical events of the vascular remodeling in allergic vasculitis with eosinophilia include destruction of the basement membrane and intraluminal proliferation of myofibroblasts (6). It has been well known that TGF-β plays a pivotal role in pulmonary fibrosis and fibroblast proliferation (26). In our results, ZSTK474 suppressed the vascular remodeling through both inhibiting fibroblast proliferation by PI3K/Akt/mTOR and reducing the collagen production by decreasing TGF-β expression (27, 28)(Fig.5a,b, Fig.7a,b,c,d) However, it remains still unclear whether allergic pulmonary vasculitis was actually suppressed or simply delayed ZSTK474. This needs to be clarified by extension of the experimental protocol in the next step.

In summary, we speculated on the mechanism of inhibitory effects of ZSTK474 on allergic pulmonary vasculitis as follows: 1) ZSTK474 suppressed the production of Th2 cytokines such as IL-4, IL-5 and IL-13 from $CD4^+$ T lymphocytes sensitized with OVA in our experimental murine model. 2) The reduced production of Th2 cytokines especially IL-5

caused a decrease of eosinophils recruited into the lung tissue. 3) ZSTK474 might inhibit eosinophil mobilization from the bone marrow and blood, and recruitment from blood to lung. 4) The vascular remodeling including myofibroblast proliferation and ECM deposition did not progress because of the reduced level of TGF-β concentration in the lung tissue. ZSTK474 inhibited the TGF-β induced fibroblast proliferation.

In conclusion, ZSTK474 attenuated the vascular remodeling of the murine model with allergic pulmonary vasculitis by reducing the eosinophil recruitment and TGF-β production in the lung.

Acknowledgement

The authors would like to thank Miss M Niisato for technical assistance, and are grateful to Mr Brent Bell for his critical reading of the manuscript.

Declaration of Interest

The authors report no conflicts of interest.

References

1. Savige J, Gillis D, Benson E, Davies D, Esnault V, Falk RJ, Hagen EC, Jayne D, Jennette JC, Paspaliaris B, Pollock W, Pusey C, Savage CO, Silvestrini R, van der Woude F, Wieslander J, Wiik A: International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA). Am J Clin Pathol. 1999;111:507- 513.
2. Jennette JC, Falk RJ: Clinical and pathological classification of ANCA-associated vasculitis: what are the controversies? Clin Exp Immunol. 1995;101 (Suppl 1):18-22.
3. Hellmich B, Gross WL: Recent progress in the pharmacotherapy of Churg-Strauss syndrome. Expert Opin Pharmacother. 2004;5:25-35.
4. Klion AD, Bochner BS, Gleich GJ, Nutman TB, Rothenberg ME, Simon HU, Wechsler ME, Weller PF, The Hypereosinophilic Syndromes Working Group: Approaches to the treatment of hypereosinophilic syndromes: a workshop summary report. J Allergy Clin Immunol. 2006;117:1292-1302.
5. Keogh KA, Specks U: Churg-Strauss syndrome: update on clinical, laboratory and therapeutic aspects. Sarcoidosis Vasc Diffuse Lung Dis. 2006;23:3-12.
6. Yamauchi K, Sasaki N, Niisato M, Kamataki A, Shikanai T, Nakamura Y, Kobayashi H, Suwabe A, Kanno H, Sawai T, Inoue H: Analysis of pulmonary allergic vasculitis with eosinophil infiltration in asthma model of mice. Exp Lung Res. 2010;36:227-236.
7. Leevers SJ, Vanhaesebroeck B, Waterfield MD: Signalling through phosphoinositide 3-kinases: the lipids take centre stage. Curr Opin Cell Biol. 1999;11:219-225.
8. Anderson KE, Jackson SP: Class I phosphoinositide 3-kinases. Int J Biochem Cell Biol. 2003;35:1028-1033.
9. Kumar CC, Madison V: AKT crystal structure and AKT-specific inhibitors. Oncogene. 2005;24:7493-7501.

10. Fukao T, Yamada T, Tanabe M, Terauchi Y, Ota T, Takayama T, Asano T, Takeuchi T, Kadowaki T, Hata J, Koyasu S: Selective loss of gastrointestinal mast cells and impaired immunity in PI3K-deficient mice. Nat Immunol. 2002;3:295-304.
11. Palframan RT, Collins PD, Severs NJ, Rothery S, Williams TJ, Rankin SM: Mechanisms of acute eosinophil mobilization from the bone marrow stimulated by interleukin 5: the role of specific adhesion molecules and phosphatidylinositol 3-kinase. J Exp Med. 1988;188: 1621-1632.
12. Duan W, Aguinaldo Datiles AM, Leung BP, Vlahos CJ, Wong WS: An anti-inflammatory role for a phosphoinositide 3-kinase inhibitor LY294002 in a mouse asthma model. Int Immunopharmacol. 2005;5: 495-502.
13. Haruta K, Mori S, Tamura N, Sasaki A, Nagamine M, Yaguchi S, Kamachi F, Enami J, Kobayashi S, Yamori T, Takasaki Y; Inhibitory effects of ZSTK474, a phosphatidylinositol 3-kinase inhibitor, on adjuvant-induced arthritis in rats. Inflamm Res. 2012;61:551-562.
14. Ohkawara Y, Lei XF, Stampfli MR, Marshall JS, Xing Z, Jordana M: Cytokine and eosinophil responses in the lung, peripheral blood and bone marrow compartments in a murine model of allergen-induced airways inflammation. Am J Respir Cell Mol Biol. 1997;16: 510-520.
15. Suzuki N, Sasaki N, Utsumi Y, Nagashima H, Nakamura Y, Yamashita M, Yamauchi K, Sawai T: Effects of imatinib mesylate on pulmonary allergic vasculitis in a murine model. Int J Rheum Dis. 2013;16:455-462.
16. Koizumi R, Sasaki N, Nakamura Y, Suzuki N, Sawai T, Yamauchi K: Rapamycin attenuates pulmonary allergic vasculitis in murine model by reducing TGF-β production in the lung. Allergol Int. 2014;63:457-66.
17. Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K: Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for

interleukin 4 in cell recruitment but not mucus production. J Exp Med. 1997;186:1737-1747.

18. Lee JJ, McGarry MP, Farmer SC, Denzler KL, Larson KA, Carrigan PE, Brenneise IE, Horton MA, Haczku A, Gelfand EW, Leikauf GD, Lee NA: Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. J Exp Med. 1997;185:2143-2156.
19. Pope SM, Brandt EB, Mishra A, Hogan SP, Zimmermann N, Matthaei KI, Foster PS, Rothenberg ME: IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. J Allergy Clin Immunol. 2001;108:594-601.
20. Foster PS, Mould AW, Yang M, Mackenzie J, Mattes J, Hogan SP, Mahalingam S, Mckenzie AN, Rothenberg ME, Young IG, Matthaei KI, Webb DC: Elemental signals regulating eosinophil accumulation in the lung. Immunol Rev. 2001;179:173-181.
21. Herrick CA, Bottomly K: To respond or not to respond: T cells in allergic asthma. Nat Rev Immunol. 2003;3:405-412.
22. Lim DH, Cho JY, Song DJ, Lee SY, Miller M, Broide DH: PI3K gamma-deficient mice have reduced levels of allergen-induced eosinophilic inflammation and airway remodeling. Am J Physiol Lung Cell Mol Physiol. 2009;296:L210-219.
23. Lee KS, Lee HK, Hayflick JS, Lee YC, Puri KD: Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. FASEB J. 2006;20:455-465.
24. Raghow B, Irish P, Kang AH: Coordinate regulation of transforming growth factor beta gene expression and cell proliferation in hamster lungs undergoing bleomycin-induced pulmonary fibrosis. J Clin Invest. 1989 ;84:1836-1842.
25. Broekelmann TJ, Limper AH, Colby TV, McDonald JA: Transforming growth factor beta 1 is present at sites of extracellular

matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci U S A. 1991;88:6642-6646.

26. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. 2008;214:199-210.

27. Kulkarni AA, Thatcher TH, Olsen KC, Maggirwar SB, Phipps RP, Sime PJ: PPAR-γ ligands repress TGFβ-induced myofibroblast differentiation by targeting the PI3K/Akt pathway: implications for therapy of fibrosis. PLoS One. 2011;6:e15909.

28. Le Cras TD, Korfhagen TR, Davidson C, Schmidt S, Fenchel M, Ikegami M, Whitsett JA, Hardie WD: Inhibition of PI3K by PX-866 prevents transforming growth factor-alpha-induced pulmonary fibrosis. Am J Pathol. 2010;176:679-686.

Figure Legends

Figure 1: Experimental protocol

Mice were sensitized on days 0 and 5 of the protocol by an intraperitoneal injection of 0.5 ml aluminum hydroxide-precipitated antigen containing 8 µg OVA. Mice were exposed daily to the aerosolized OVA produced by a Pulmo-Aide Compressor /Nebulizer at a flow rate of 5-7 liter/min in the plastic chamber as described in "Methods". ZSTK474 was administered as 30mg/kg orally once daily during 1 week as described in "Methods".

Figure 2: The effect of ZSTK474 on cell differentials in BALF

Hatched columns: OVA-sensitized mice (n=12) with exposed to saline (control). Open columns: OVA-sensitized mice (n=12) with exposed to OVA (positive control). Closed columns: OVA-sensitized mice (n=12) with exposed to OVA and treated with ZSTK474. **T**: total cells; **A**: alveolar macrophages; **L**: lymphocytes; **E**: eosinophils; **N**: neutrophils. Data are means ± SD * $p<0.01$, * * $p<0.05$

Figure 3A: The effect of ZSTK474 on total white blood cell counts in peripheral blood

Open circles: OVA-sensitized mice with exposed to OVA (positive control), Closed circles: OVA-sensitized mice with exposed to OVA and treated with ZSTK474 as described in "Methods". Samples on the day 0 were collected before OVA exposure. Samples on the 3rd and 7th day were collected after OVA exposure. Data are means ± SD * $p<0.01$

Figure 3B: The effect of ZSTK474 on eosinophil counts in peripheral blood

Open circles: OVA-sensitized mice with exposed to OVA (positive control), Closed circles: OVA-sensitized mice with exposure to OVA and treated with ZSTK474. * $p<0.01$

Figure 4: Cytokine concentration in BALF

Concentrations of IL-4, IL-5, IL-13, and TGF-β in BALF are shown.

Figure 4A, IL-4; Figure 4B, IL-5; Figure 4C, IL-13; Figure 4D, TGF-β .

Open circles: OVA-sensitized mice with exposed to OVA (positive control).

Closed circles: OVA-sensitized mice with exposure to OVA and treated with ZSTK474. Data are means ± SD *p<0.01, **p<0.05.

Figure 5. Effects of ZSTK474 on allergic pulmonary vascular remodeling

a: Pulmonary artery totally occluded by intraluminal myofibroblasts (a yellow arrow head) on the 7th day in the positive control (HE staining). Perivascular infiltration including eosinophil was prominent (a yellow arrow). **b**. Intraluminal myofibroblast accumulation was not observed (a yellow arrow head) on the 7th day in the ZSTK474-treated mice (HE staining).

Figure 6. Effects of ZSTK474 on severity of vascular changes

Open circles: histological scores of the positive control on the 7th day (n = 6). Closed circles: the ZSTK474-treated mice on the 7th day (n = 6). Data

are given as mean ± SD and *p<0.01.

Figure 7. Effects of ZSTK474 on allergic pulmonary vascular pathological changes evaluated with EM staining and Immunostaining for α-SMA

a: Intraluminal space of the pulmonary artery was occupied by myofibroblasts and greenish colagenous material on the 7th day in the positive control (a yellow arrow). The vascular wall was partially disrupted. (EM staining).

b: Intravascular myofibroblasts were not seen in the pulmonary artery and only blood cells were observed in the lumen on the 7th day in the ZSTK474-treated mice (a yellow arrow). The vascular wall was intact. (EM staining)

c: The intraluminal myofibroblasts on the 7th day in the positive control were positively stained with anti-α-SMA (a yellow arrow). The lumen of the pulmonary artery was totally occluded by α-SMA-positive myofibroblasts.

d: No α-SMA positive cells were seen in the lumen of the pulmonary artery on the 7th day in the ZSTK474-treated mice. Smooth muscle cells in vascular wall were positively stained. (a yellow arrow)

Figure 1

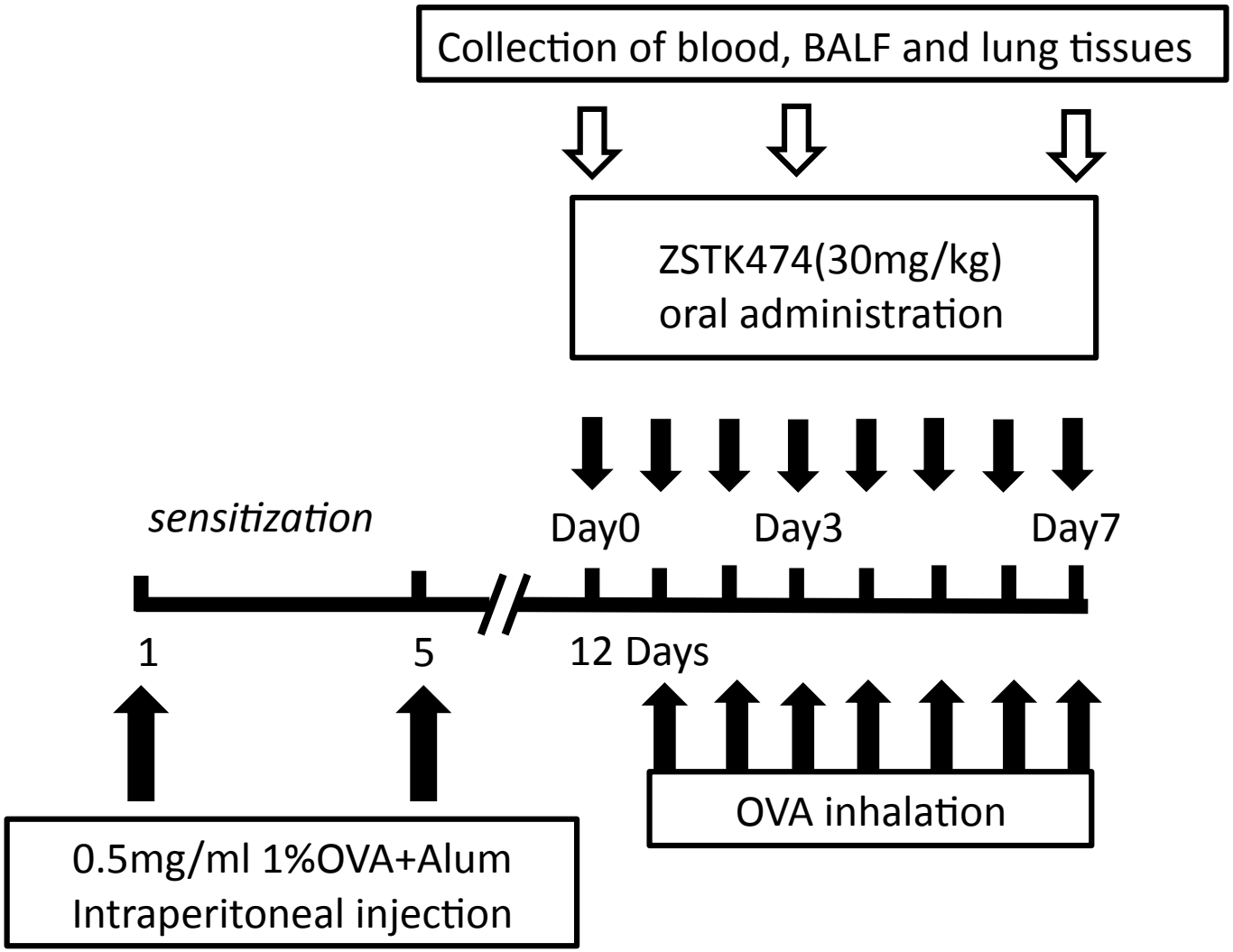

Collection of blood, BALF and lung tissues
ZSTK474(30mg/kg)
oral administration
sensitization
Day0
Day3
Day7
1
5
12 Days
OVA inhalation
0.5mg/ml 1%OVA+Alum
Intraperitoneal injection

Figure 2

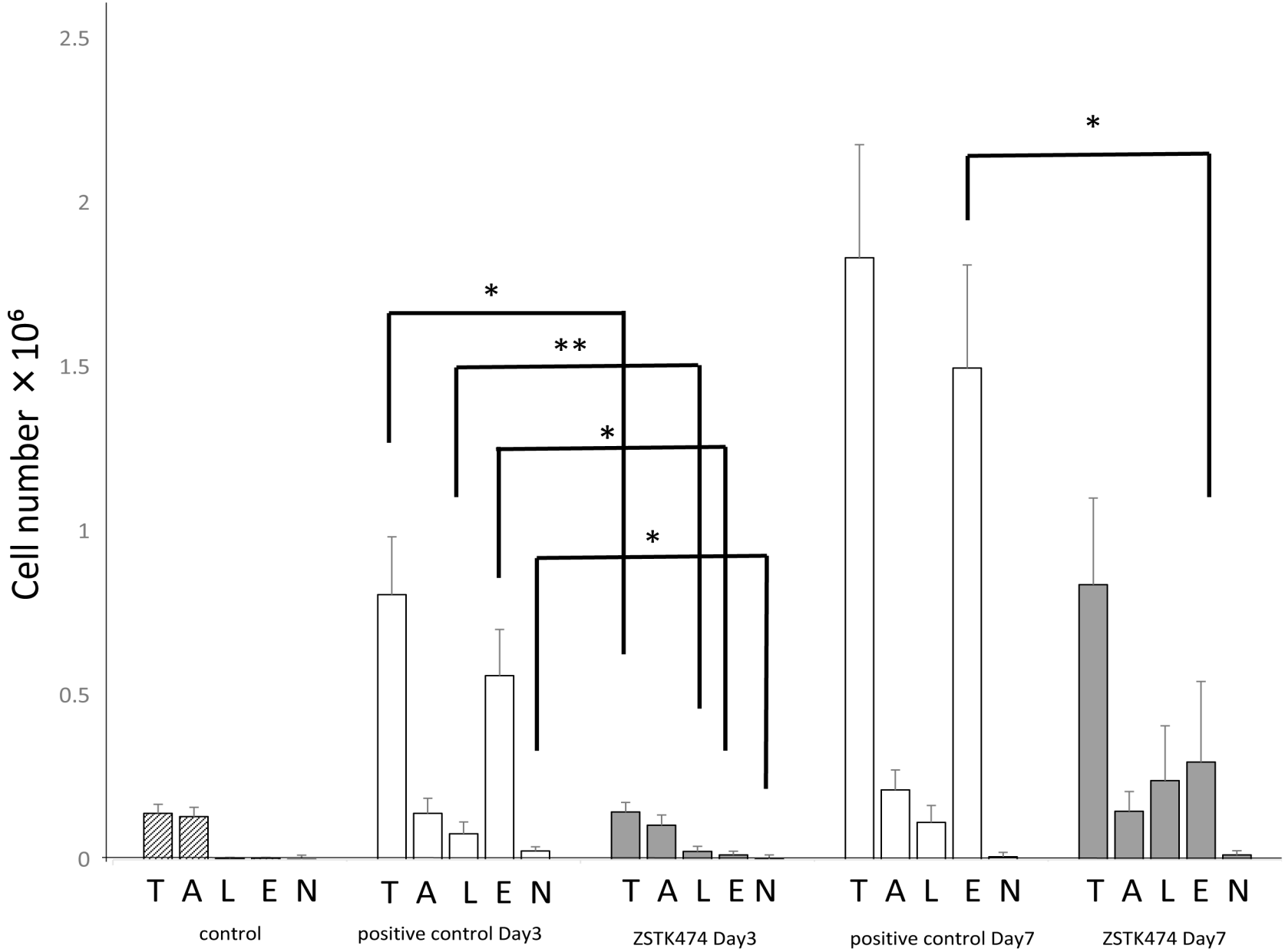

Cell number × 10⁶
2.5
2
1.5
1
0.5
0
*
**
*
*
*
T A L E N
T A L E N
T A L E N
T A L E N
T A L E N
control
positive control Day3
ZSTK474 Day3
positive control Day7
ZSTK474 Day7

Figure 3A

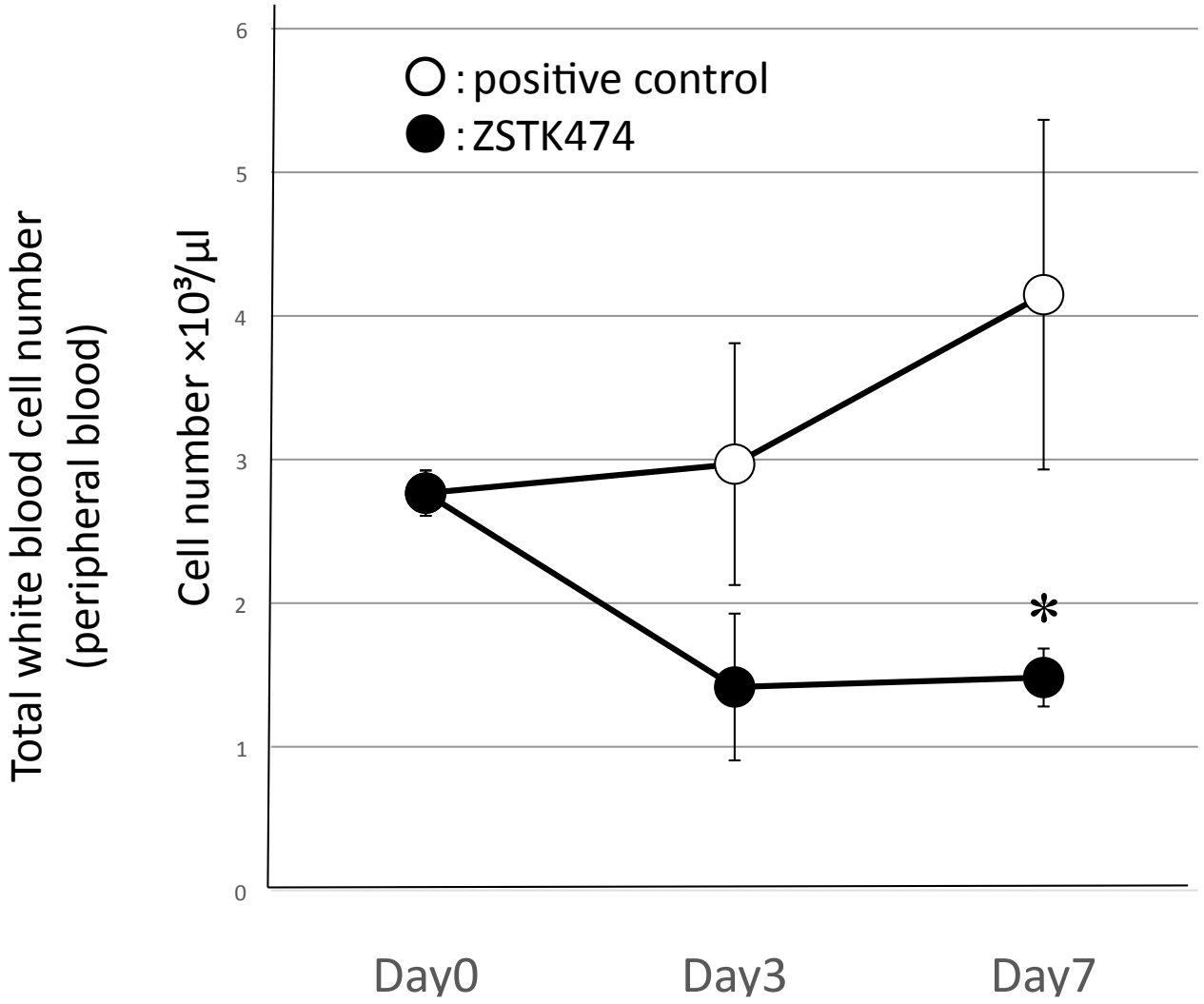

Total white blood cell number
(peripheral blood)
Cell number ×10³/μl
○ : positive control
● : ZSTK474
*
0
1
2
3
4
5
6
Day0
Day3
Day7

Figure 3B

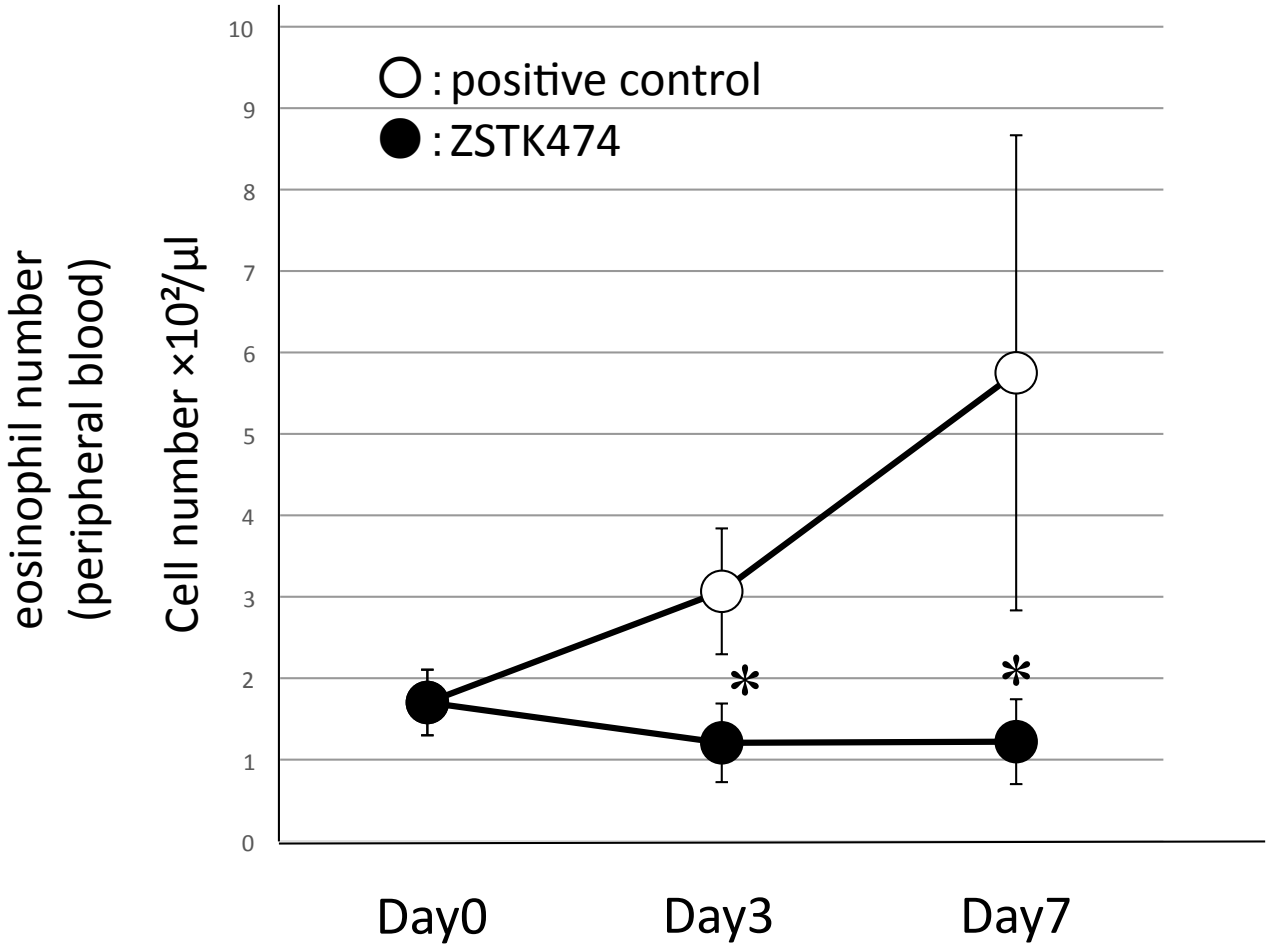

eosinophil number
(peripheral blood)
Cell number ×10²/μl
○ : positive control
● : ZSTK474
10
9
8
7
6
5
4
3
2
1
0
*
*
Day0
Day3
Day7

Figure 4A

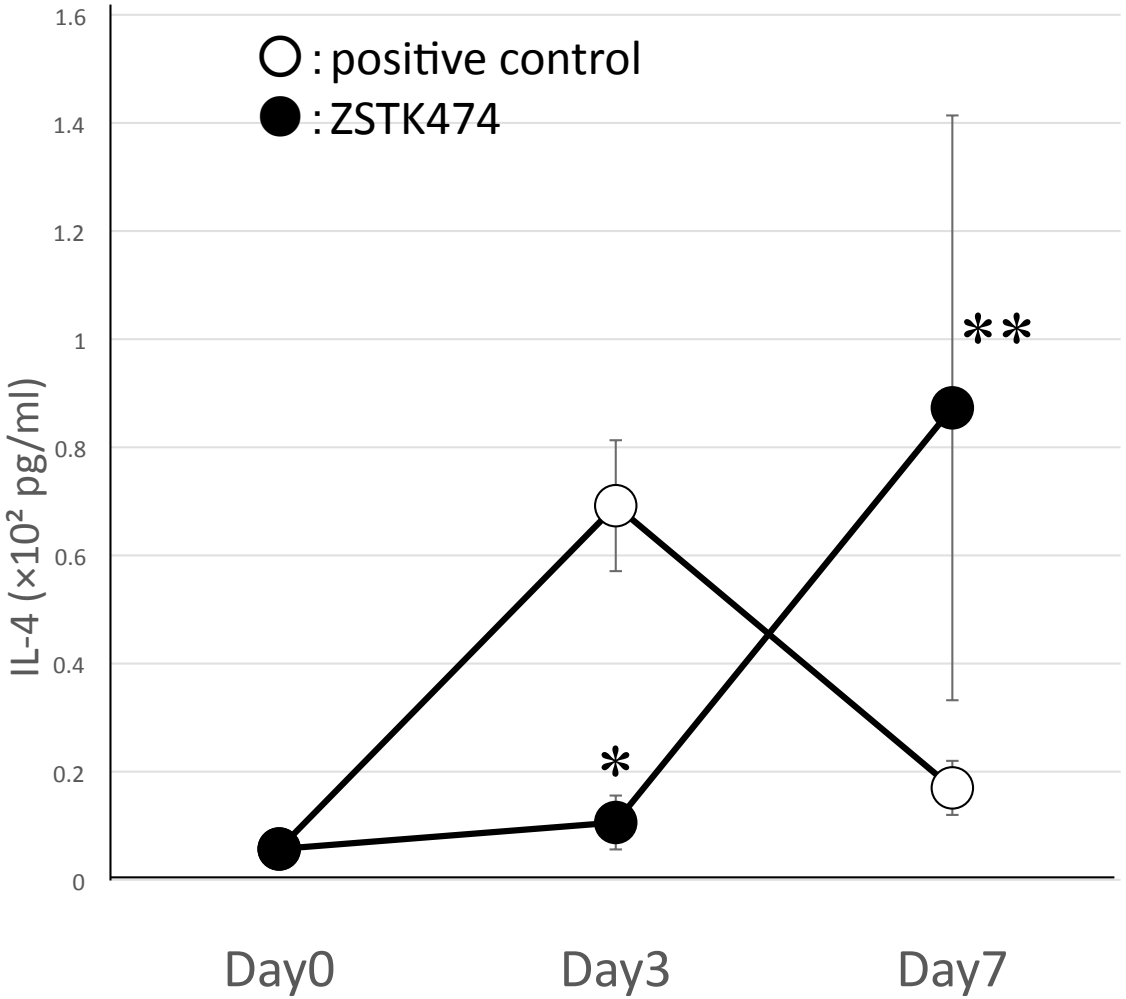

○ : positive control
● : ZSTK474
IL-4 (×10² pg/ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
Day0
Day3
Day7

Figure 4B

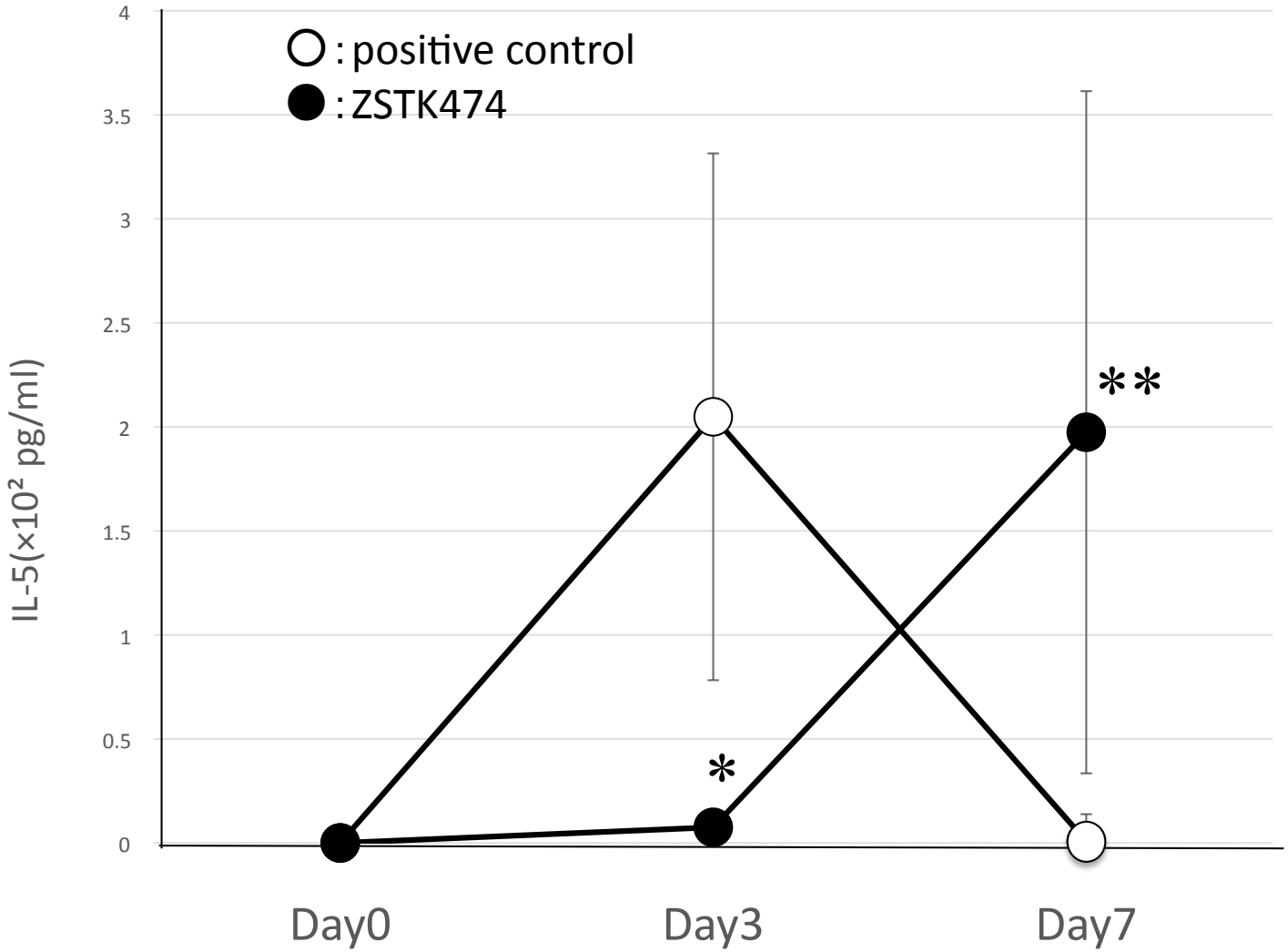

○ : positive control
● : ZSTK474
IL-5(×10² pg/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
**
Day0
Day3
Day7

Figure 4C

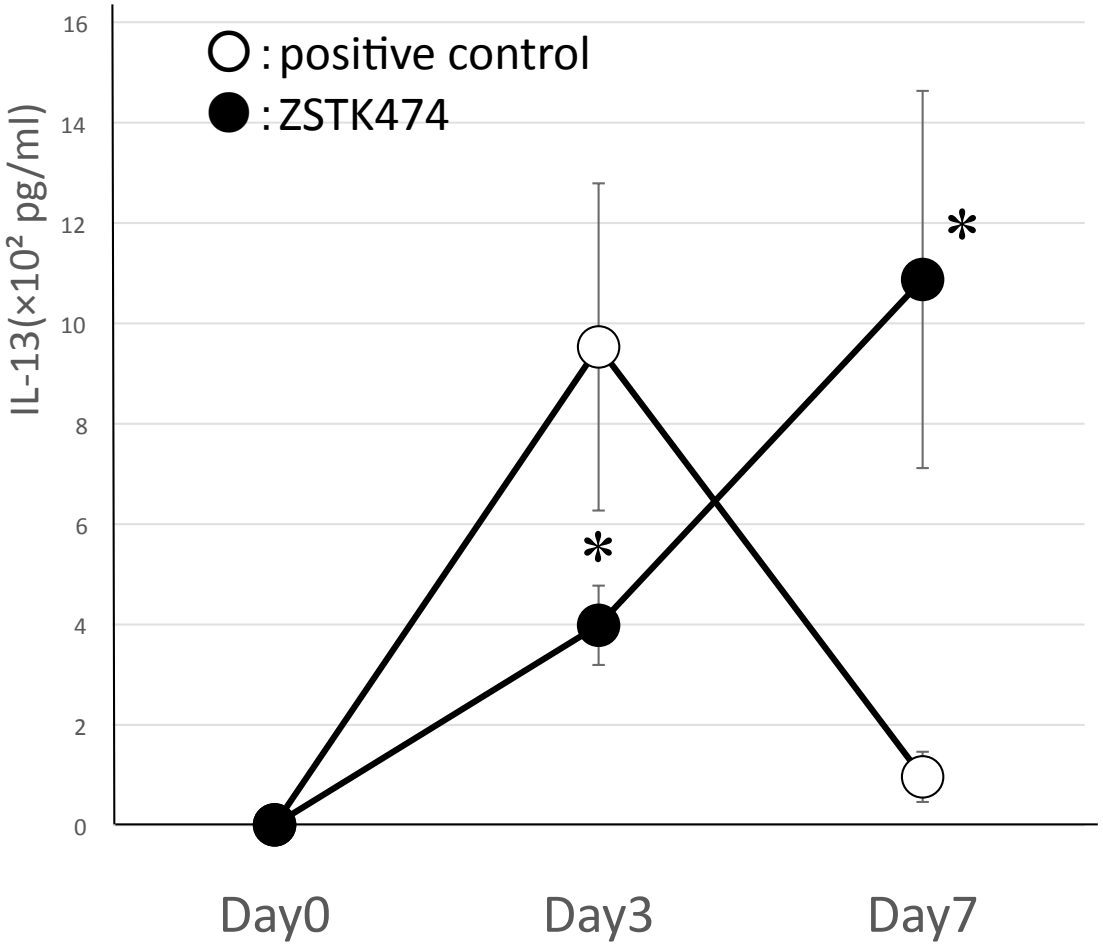

○ : positive control
● : ZSTK474
IL-13(×10² pg/ml)
16
14
12
10
8
6
4
2
0
*
*
Day0
Day3
Day7

Figure 4D

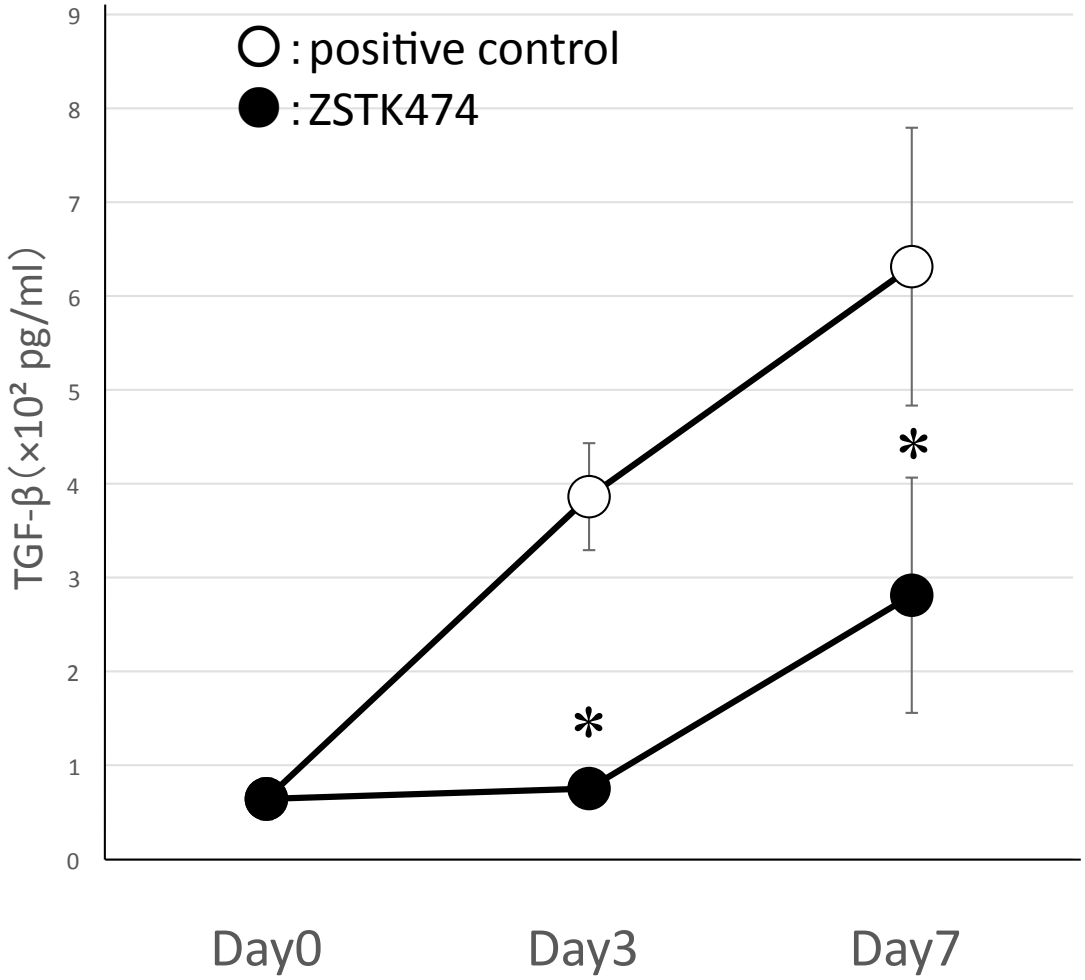
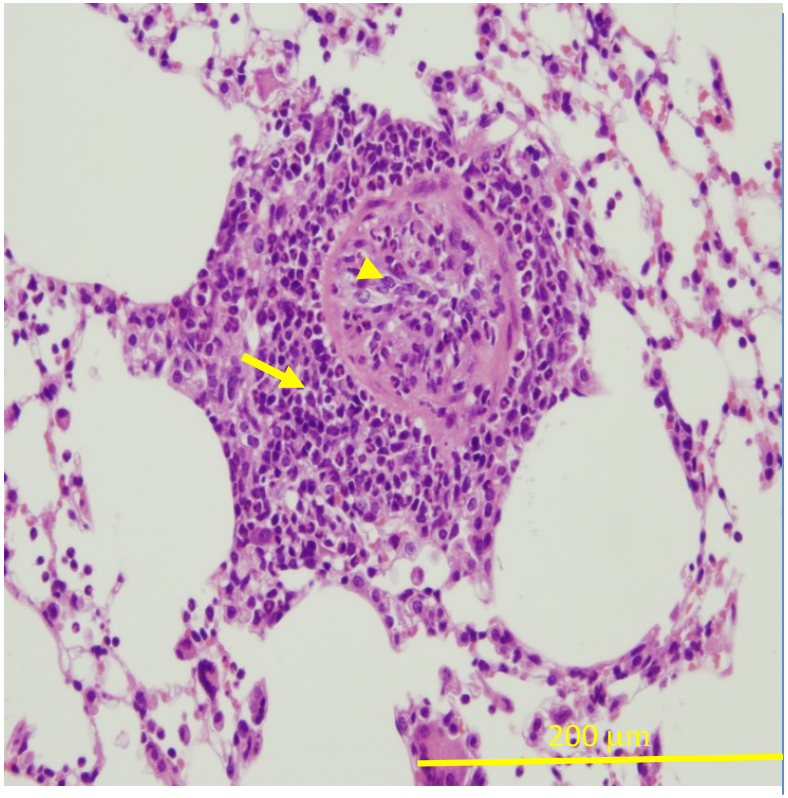

○ : positive control
● : ZSTK474
TGF-β（×10² pg/ml）
0
1
2
3
4
5
6
7
8
9
*
*
Day0
Day3
Day7

Figure 5

a



b

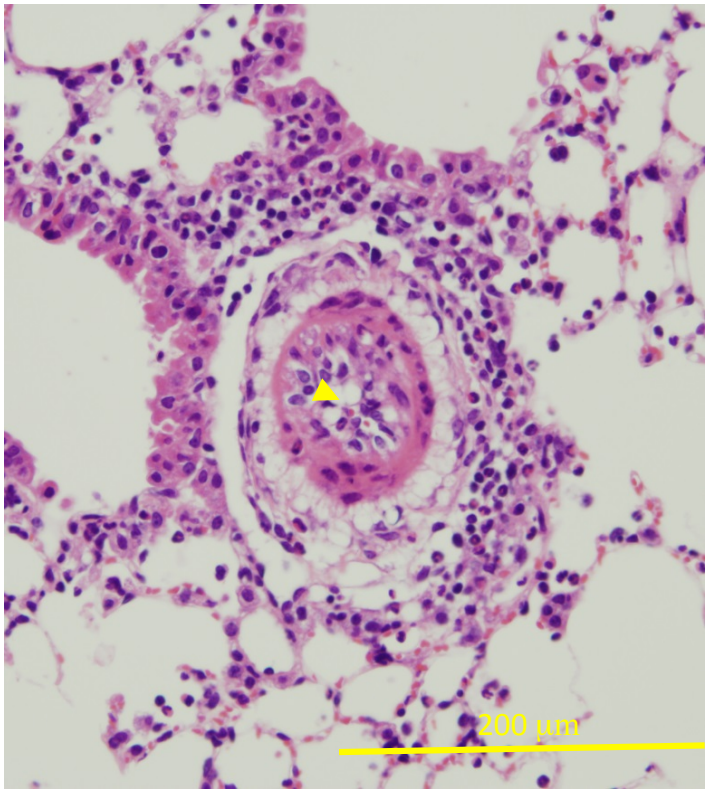


Figure 6

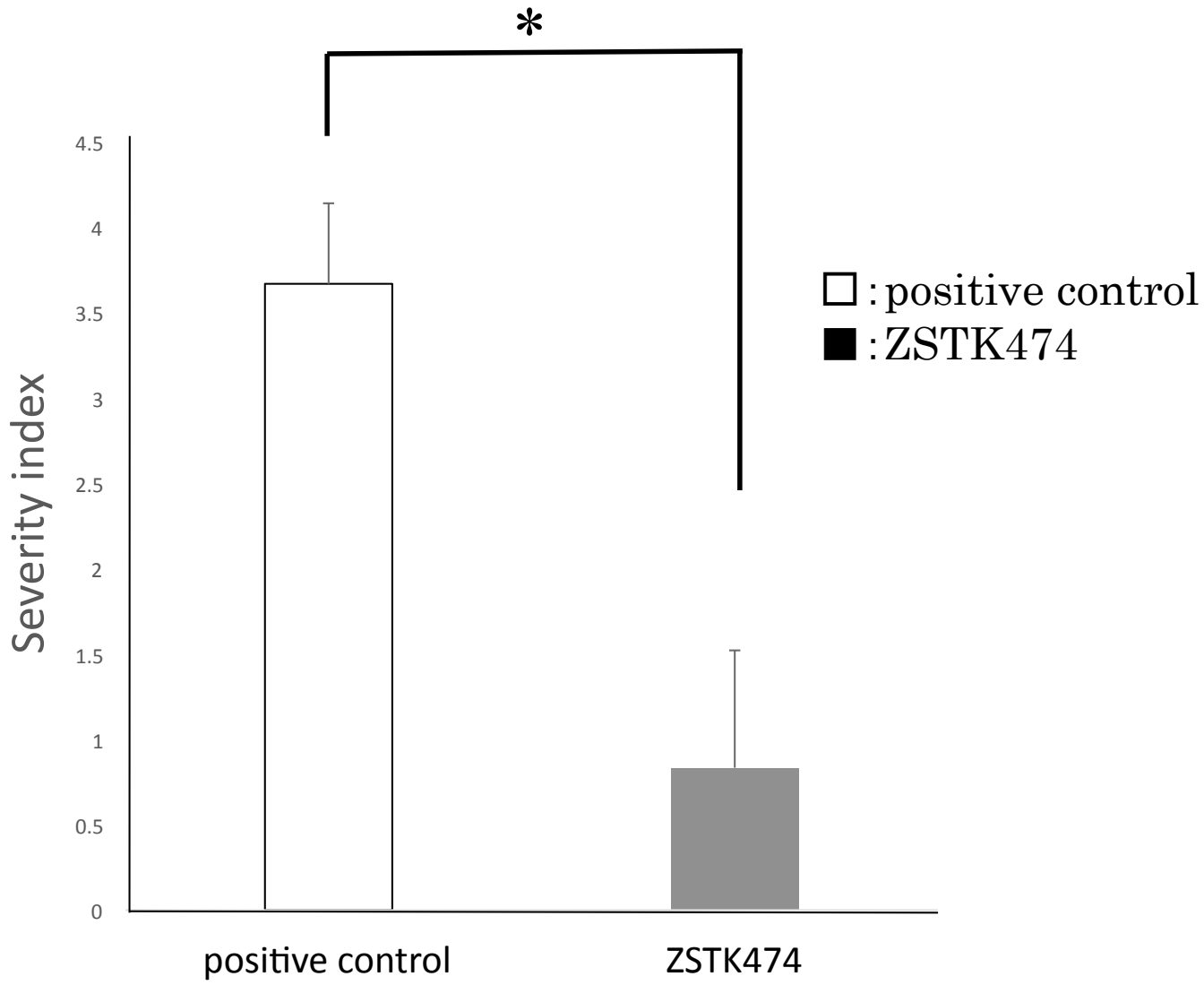

*
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Severity index
□ : positive control
■ : ZSTK474
positive control
ZSTK474

Figure 7