

ORIGINAL ARTICLE

Comprehensive attenuation of IL-25-induced airway hyperresponsiveness, inflammation and remodelling by the PI3K inhibitor LY294002

PING HUANG,¹ YAN LI,^{1,2} ZE LV,¹ JINGJING WANG,³ QIAN ZHANG,⁴ XIUJUAN YAO,⁴ CHRIS J. CORRIGAN,⁵ KEWU HUANG,² WEI WANG¹ AND SUN YING^{1,5}

¹Department of Immunology, School of Basic Medical Sciences, Capital Medical University, ²Department of Respiratory and Critical Care Medicine, Beijing Chao-Yang Hospital, Capital Medical University & Beijing Institute of Respiratory Medicine, ³Department of Laboratory Animal Sciences, Capital Medical University, ⁴Department of Respiratory Medicine, Beijing Tongren Hospital, Capital Medical University, Beijing, China, and ⁵Division of Asthma, Allergy & Lung Biology, King's College London, MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, London, UK

ABSTRACT

Background and objective: Existing *in vitro* and *in vivo* studies suggest that both IL-25 and phosphoinositide 3-kinases (PI3Ks) exhibit broad effects on the functions of immune cells implicated in the pathogenesis of asthma. Whether the blockade of PI3K signalling directly inhibits the asthma relevant pathogenetic changes induced by IL-25 in an *in vivo* condition is still unclear. Using an established IL-25-induced murine model of asthma, we undertook a comprehensive evaluation of the effects of co-administered LY294002, a pharmacological pan-inhibitor of PI3K on IL-25-induced changes on this model, with particular regard to airway remodelling.

Methods: BALB/c mice were serially intranasally challenged with IL-25 according to an established protocol to induce airway inflammation, hyperresponsiveness (AHR) and remodelling. In an additional subgroup LY294002 was administered intranasally. Lung function and airway cytokine and chemokine concentrations and cellular infiltration and remodelling changes assessed by histology and immunohistochemistry were measured at specific time points.

Results: Intranasal administration of LY294002 significantly inhibited IL-25-induced AHR and recruitment of inflammatory cells into bronchoalveolar lavage fluid. LY294002 also attenuated IL-25-induced increased concentrations of cytokines and chemokines in lung tissue. Histological and immunohistochemical analysis showed that LY294002 also significantly inhibited IL-25-induced lung tissue eosinophilia, mucus production, collagen deposition, smooth muscle hypertrophy and angiogenesis.

Conclusion: The PI3K pan-inhibitor LY294002 attenuated not only IL-25-induced asthma-like AHR and airway inflammation but also remodelling in this model, suggesting that PI3K is a major downstream messenger for IL-25 and that targeting this pathway might reduce

SUMMARY AT A GLANCE

The phosphoinositide 3-kinase (PI3K) pan-inhibitor LY294002 effectively attenuated IL-25-induced airway inflammation, hyperresponsiveness and remodelling in a murine asthma model in an *in vivo* condition.

asthma symptoms in the short term and airway remodelling in the longer term.

Key words: airway inflammation, asthma, interleukin-25, phosphoinositide 3-kinase inhibitors, remodelling.

Abbreviations: α -SMA, alpha-smooth muscle actin; AHR, airway hyperresponsiveness; ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; CBA, cytometric bead array; CK2, casein kinase 2; CXCL-1, CXC chemokine ligand-1; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GSK3 β , glycogen synthase kinase-3 β ; H&E, haematoxylin and eosin; IL, interleukin; KC, keratinocyte chemoattractant; LT, leukotriene; MT, Masson's trichrome; mTOR, mammalian target of rapamycin; OVA, ovalbumin; PAS, periodic acid-Schiff; PI3K, phosphoinositide 3-kinase; TGF- β , transforming growth factor- β ; Th2, T-helper 2; TNF- α , tumour necrosis factor alpha; VEGF, vascular endothelial growth factor.

INTRODUCTION

Asthma is one of the most prevalent threats to public health owing to its chronicity and potentially life-threatening exacerbations. Asthma is estimated to affect 300 million people worldwide, a prevalence which increases annually.¹ Chronic airway inflammation, remodelling and hyperresponsiveness (AHR) with airflow obstruction are the major characteristics of asthma, while airway remodelling might contribute to treatment refractoriness. The pathogenesis of asthma is not yet entirely

Correspondence: Sun Ying, Department of Immunology, School of Basic Medical Sciences, Capital Medical University, #10 Xitoutiao, Youanmenwai, Fengtai District, Beijing 100069, China. Email: ying.sun@ccmu.edu.cn

Received 11 November 2015; invited to revise 11 January and 25 March 2016; revised 3 March and 5 April 2016; accepted 20 May 2016 (Associate Editor: Darryl Knight).

clear. Current therapy for asthma is primarily focused on control of symptoms, including inhaled and systemic corticosteroids, inhaled beta-agonists and oral leukotriene (LT) receptor antagonists.² Unfortunately, in approximately 10% of patients, symptoms cannot be controlled by maximal, optimally delivered therapy, resulting in increased morbidity and costs,³ thereby driving researchers to identify potential new therapeutic targets.

IL-25 has been implicated in asthma pathogenesis at least partly because it promotes T-helper 2 (Th2)-type immune responses. In laboratory mice, intranasal administration of IL-25 alone has been shown to induce human asthma-like features in the airways, including Th2 cytokine production, epithelial cell hyperplasia, eosinophil infiltration, increased mucus secretion and AHR.⁴ We have further previously demonstrated that chronic intranasal challenge with IL-25 alone in this murine model is also sufficient to cause airway remodelling.⁵

Phosphoinositide 3-kinases (PI3Ks) are a family of intracellular signalling proteins involved in a number of cellular processes, such as survival, proliferation, differentiation and migration, and also involved in the pathogenesis of various diseases, including cancer, inflammation and allergy.⁶ It has been shown that administration of LY294002, a pharmacological pan-inhibitor of PI3K, considerably reduced bronchial inflammation and AHR in a murine asthma model induced by sensitization and challenge with ovalbumin (OVA).^{7,8} We further demonstrated that LY294002 significantly inhibited IL-25-induced expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) by endothelial cells, mediators which may be involved in airway remodelling in asthma, in an *in vitro* condition.^{9,10}

The purpose of this study was to extend our *in vitro* findings to examine comprehensively the effects of LY294002 in our IL-25-induced murine model of asthma in an *in vivo* condition, with particular regard to its possible effects on airway remodelling. We hypothesized that the PI3K pan-inhibitor LY294002 not only attenuates IL-25-induced airway inflammation, but also inhibits IL-25-induced airway remodelling in an *in vivo* condition.

METHODS

Experimental animals

Female BALB/c mice (8–10 weeks of age) were obtained from Vital River Laboratories (Beijing, China), housed under constant temperature and light cycles and maintained in a pathogen-free mouse facility located in the Department of Laboratory Animal Sciences, Capital Medical University, Beijing, China. All animal experiments were strictly conducted under protocols approved by the Institutional Animal Care and Use Committee at Capital Medical University.

Administration of IL-25, LY294002 and vehicle control

The IL-25-induced murine intranasal challenge model of asthma was established as described previously.⁵ To

investigate the inhibitory effect of the PI3K pan-inhibitor LY294002 on IL-25-induced changes in the model, mice were randomly assigned to three groups, including an IL-25 (mIL25, 2 µg/50 µL in saline; R&D Systems, Minneapolis, MN, USA) challenged group (positive control), an IL-25 + LY294002 (80 µg/50 µL in 0.1% dimethyl sulfoxide (DMSO) in saline; Merck Chemicals Ltd, Nottingham, UK) challenged group and a 0.1% DMSO in saline-challenged group (vehicle control). Mice were first treated with LY294002 or saline containing 0.1% DMSO (50 µL in each case) and then challenged 2 h later with IL-25 or an equivalent volume of saline. All mice received intranasal administration of the appropriate agents once daily from day 1 to day 6, and then once every other day until day 36, followed by observation for a further 17 days after the cessation of administration. The schedule of administration is summarized in Figure S1 (Supplementary Information).

Measurement of AHR

AHR was measured on days 3, 7, 19, 31, 37 and 53, 24 h after the most recent challenge in subsets of mice in each group by detecting lung resistance to methacholine (Sigma-Aldrich, Beijing, China) using the FlexiVent system (Scireq Inc., Montreal, QC, Canada) as described previously.^{11,12} Day 0 served as the baseline measurement.

BALF collection and analysis

Following the measurement of AHR, bronchoalveolar lavage fluid (BALF) was collected as described previously and the total cells were counted.⁵ Cytospins were prepared from the cell pellets and differential cell counts performed following Congo red and/or haematoxylin staining.

Histological staining

Sections (5-µm thickness) were cut from fixed embedded lung tissues and stained with haematoxylin and eosin (H&E) (Sigma-Aldrich), periodic acid-Schiff (PAS) (Beijing Yi Li Fine Chemistry Co Ltd, Beijing, China) and Masson's trichrome (MT) blue (Nanjing Mindit Biochemistry Co Ltd, Nanjing, China) to examine inflammatory cellular infiltration, mucus productions and collagen deposition, respectively.^{5,13} Congo red (Beijing Solarbio Technology Co Ltd, Beijing, China) staining was used to detect eosinophil infiltration into the lung tissue.⁵ PAS-stained sections were photographed at ×20 magnification and then graded against standards as described in a previous report.¹³ Congo red-stained sections were photographed at ×40 magnification and Congo red-stained eosinophils quantified objectively using IMAGE-PRO PLUS (Media Cybernetics, Inc., Rockville, MD, USA) software as described, with results expressed as percentages of the total nucleated cells staining positively with Congo red.⁵

Immunohistochemistry

Immunohistochemistry of alpha-smooth muscle actin (α-SMA, 1:200) and CD31 (endothelial cells) (1:50) was employed as markers of smooth muscle hypertrophy

and angiogenesis, respectively. The primary monoclonal antibodies against mouse α -SMA and CD31 were purchased from Abcam (Hong Kong, China). The thickness of peribronchial α -SMA staining¹⁴ and the total numbers of CD31⁺ vascular endothelial cells per unit area of lung sections were measured objectively on a Leica DM6000B microscope (Leica, Wetzlar, Germany) connected with a Leica Application Suite, version 3.6 programmed with IMAGE-PRO PLUS software.

Measurements of concentrations of lung total collagen, cytokines, chemokines and growth factors

Right lung tissue (100 mg) was homogenized as previously described.⁵ Supernatants of homogenized lung tissue were collected and stored at -80°C until used. The amount of total lung collagen was measured using the Sircol collagen assay kit (Biocolor, Belfast, UK).⁵ Concentrations of IL-5, IL-13, eotaxin, VEGF and transforming growth factor- β (TGF- β) in lung tissue were quantified using commercial ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The concentrations of IL-1 β , IL-6, TNF- α and the murine IL-8 homologue (CXC chemokine ligand-1 (CXCL-1)KC) in lung tissue homogenates were measured using a commercial BD cytometric bead array (CBA) platform (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions.⁵

Statistical analysis

The results were analysed using the statistical software package GRAPHPAD PRISM 5.01 (GraphPad, San Diego, CA, USA). Data in the different groups were analysed and compared by two-way analysis of variance (ANOVA) to determine whether there were significant differences between the treatment groups, followed by Bonferroni post-tests for multiple comparison of the means. All data were expressed as the mean $\pm$ SEM. *P*-values <0.05 were considered statistically significant.

RESULTS

LY294002 effects on IL-25-induced AHR and airway inflammation

Intranasal challenge with IL-25, as compared with DMSO diluent control, significantly increased AHR to methacholine, while concurrent treatment with LY294002 abrogated this increase from day 3 (although the differences were not statistically significant until day 7) up to day 37 (Fig. 1). Concurrently, LY294002 significantly attenuated the mean total numbers of cells, eosinophils, lymphocytes and neutrophils in the BALF of the IL-25-challenged animals, particularly at days 19 and 31 when the peak of cellular infiltration was observed (Fig. 2), as well as infiltration of inflammatory cells into lung tissue (Fig. S2, Supplementary Information).

LY294002 effects on IL-25-induced lung tissue eosinophilia

Analysis of Congo red-stained lung sections showed that concurrent treatment with LY294002 also significantly attenuated IL-25-induced eosinophil infiltration into the lung peribronchial and perivascular regions, evident from day 7 when significant infiltration was first observed, until the end of the study (day 53) (Fig. 3, illustration of results is limited to day 7, 19 and 37 outcomes).

LY294002 effects on airway goblet cell hyperplasia and mucus production

Analysis of PAS-stained lung sections showed that concurrent treatment with LY294002 markedly inhibited IL-25-induced airway goblet cell and mucous hyperplasia, evident from day 7, when significant hyperplasia was first observed until the end of the study (day 53) (Figs S3A (Supplementary Information), 4A).

LY294002 effects on IL-25-induced extracellular lung matrix protein deposition

Analysis of MT blue-stained lung sections showed abundant collagen deposition around the airways and lung vasculature in the IL-25-challenged mice from day 31 as compared with the DMSO-challenged mice, while concurrent treatment with LY294002 subjectively reduced this (Fig. S3B, Supplementary Information). This was accompanied by significant, objective reduction of the mean total lung collagen concentration in the IL-25-challenged, LY294002-treated animals from day 31, when elevated collagen deposition was first apparent, until the end of the study (day 53) (Fig. 4B).

LY294002 effects on IL-25-induced airway smooth muscle hypertrophy

Immunostaining of lung sections for α -SMA (Fig. S3C, Supplementary Information) showed that intranasal inhalational challenge of mice with IL-25 induced airway smooth muscle hypertrophy evident from day 19 until cessation of challenge, but resolving later, as compared with control DMSO challenge. Concurrent treatment with LY294002 completely inhibited IL-25-induced smooth muscle hypertrophy over this entire time period (Fig. 4C).

LY294002 effects on IL-25-induced submucosal vascularity

Immunostaining of lung sections for CD31 (Fig. S3D, Supplementary Information) showed that challenge with IL-25 induced a significant elevation of the mean number of CD31-positive blood vessels in the lung tissues which was apparent from day 19 and persisted until the end of the study (day 53), as compared with DMSO challenge. Again, concurrent treatment with LY294002 completely abrogated this increase in vascularity over the entire duration of the study (Fig. 4D).

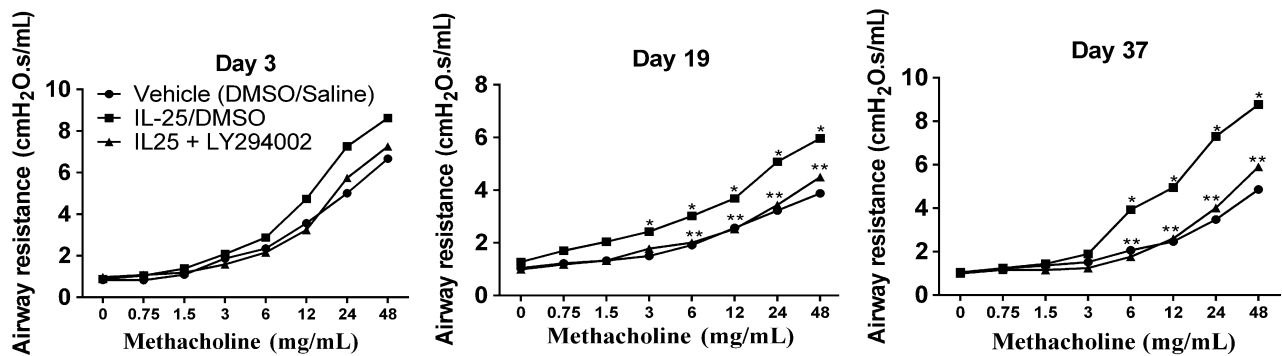


Figure 1 Airway resistance of vehicle-treated (DMSO/saline, ○), IL-25/DMSO-treated (■) and IL-25/LY294002-treated (▲) mice at individual time points ($n = 5$ in each group at each time point). * $P < 0.05$ between vehicle-challenged and IL-25/DMSO-challenged mice, ** $P < 0.05$ between IL-25/DMSO-challenged and IL-25 + LY294002-challenged groups.

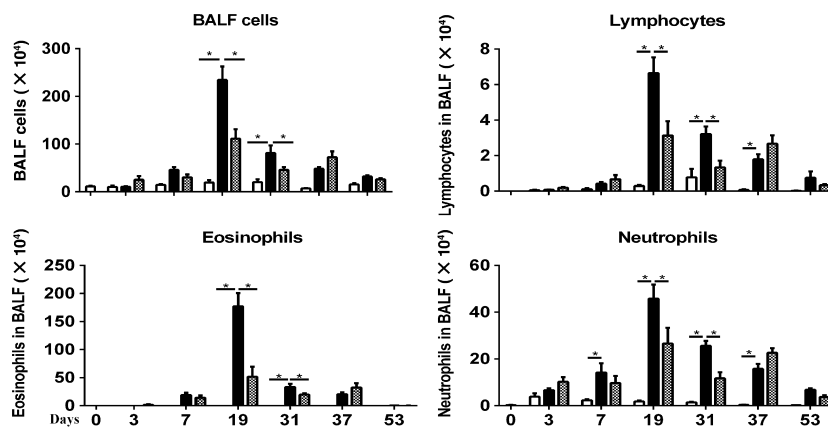


Figure 2 Total cells, eosinophils, neutrophils and lymphocytes in bronchoalveolar lavage fluid (BALF). Data are expressed as mean $\pm$ SEM ($n = 5$ in each group at each time point). * $P < 0.05$ versus vehicle-challenged or IL-25/DMSO-challenged groups. □, vehicle (DMSO/saline); ■, IL-25/DMSO; ▨, IL-25/LY294002.

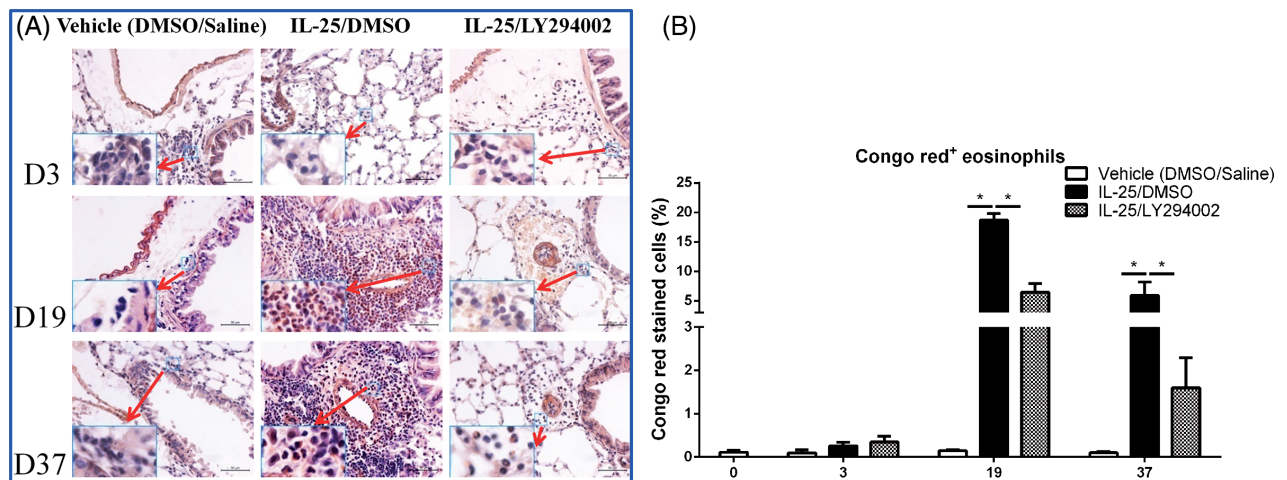


Figure 3 (A) Representative photomicrographs of Congo red⁺ eosinophils in lung sections. (B) Quantitative analysis using IMAGE-PRO PLUS software. Data were collected from three independent experiments (stained three times, i.e. three sections from each murine lung at each time point) and are expressed as mean $\pm$ SEM ($n = 5$ mice in each group at each time point). * $P < 0.05$. □, vehicle (DMSO/saline); ■, IL-25/DMSO; ▨, IL-25/LY294002.

LY294002 effects on expression of cytokines, chemokines and growth factors

ELISA and CBA were employed to measure concentrations of the Th2 cytokines IL-5 and IL-13, the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α , the

chemokines eotaxin and KC (CXCL-1, a homologue of human IL-8) and the growth factors VEGF and TGF- β in supernatants of lung tissue homogenates from mice of the respective groups. The results showed that challenge with IL-25 significantly increased the mean

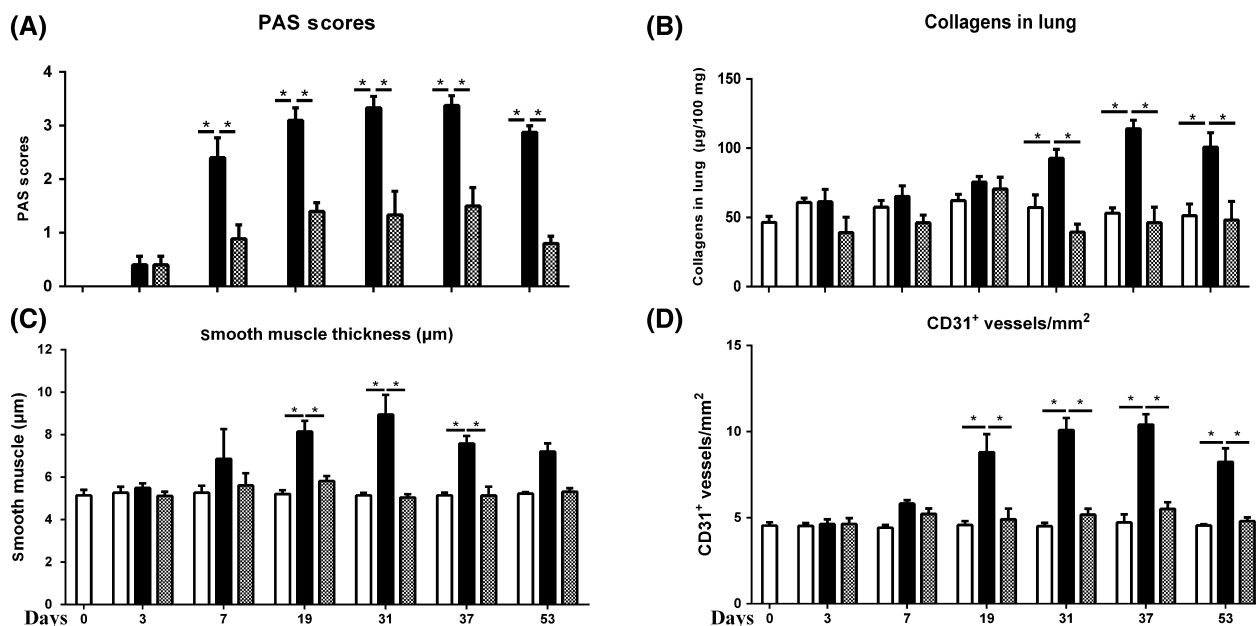


Figure 4 (A) Mucus score based on periodic acid-Schiff (PAS) staining. (B) Total lung collagen (µg/100 mg of total protein). (C) Quantitative analysis of thickness of alpha-smooth muscle actin-positive muscle was performed as described previously.^{5,14} (D) CD31⁺ blood vessels per unit area. Data were collected from three independent experiments (stained three times, i.e. three sections from each murine lung at each time point) and are expressed as mean $\pm$ SEM ($n = 5$ mice in each group at each time point). * $P < 0.05$. □, vehicle (DMSO/saline); ■, IL-25/DMSO; ▒, IL-25/LY294002.

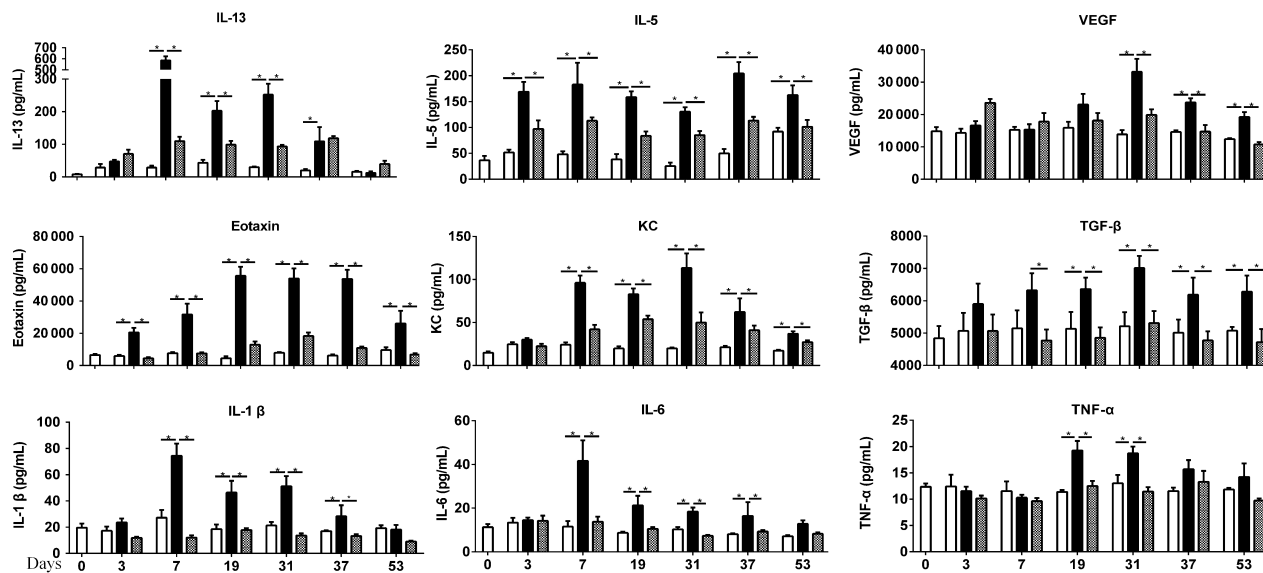


Figure 5 Concentrations of cytokines, chemokines and growth factors in lung homogenates from vehicle-challenged, IL-25/DMSO-challenged and IL-25/LY294002-challenged mice at the time points shown. Data were collected from three independent experiments (ELISA repeated three times using samples from each murine lung at each time point) and are expressed as mean $\pm$ SEM ($n = 5$ mice in each group at each time point). * $P < 0.05$. □, vehicle (DMSO/saline); ■, IL-25/DMSO; ▒, IL-25/LY294002.

concentrations of all of these mediators in the homogenates, albeit with distinct time courses, as compared with diluent control DMSO challenge (Fig. 5). Again, concurrent treatment with LY294002 attenuated these increases to a degree such that their mean concentrations were no longer significantly different to those measured in DMSO diluent control challenge animals.

DISCUSSION

In a recent previous study,⁵ we showed that chronic exposure of the airways of mice to IL-25 by nasal challenge recapitulates many of the pathophysiological features of human asthma, including AHR, inflammation and various indices of airway remodelling such as

smooth muscle hypertrophy and neovascularization. Here, we extend these studies to show that concurrent treatment with the PI3K pan-inhibitor LY294002 is able to abolish or attenuate all of these IL-25-induced phenomena. Other previous studies have demonstrated that blockade of IL-25 can inhibit airway inflammation and Th2 cytokine production in animal models of asthma,^{14–16} and that such changes were also attenuated in p110 δ - and PI3K- γ -deficient animals^{17–20} in the presence of PI3K inhibitors.^{7,8,21,22} This likely reflects the wide involvement of PI3Ks in the intracellular signalling essential for numerous aspects of cellular function, including immune cells implicated in asthma.^{23–29} Our study further demonstrates that PI3K is a central second messenger for all of the wide-ranging actions of IL-25 in the asthmatic airways and, critically, that PI3K inhibition may inhibit not only airway inflammation and AHR but also remodelling.²⁶

Eosinophil-active Th2 cytokines and chemokines, in particular IL-5, IL-13 and eotaxin, are implicated in the pathogenesis of the eosinophil-rich airway inflammation which typically characterizes asthma.³⁰ Treatment of IL-25-challenged mice with LY294002 clearly attenuated production of these mediators in the present study, in line with a previous study⁷ showing that the same inhibitor was able to reduce the concentrations of these mediators in the BALF of OVA-sensitized and challenged mice. In both studies, this was associated with reduced lung tissue and airway eosinophilia, which might reflect reduced production of eosinophil-active mediators and/or the fact that eosinophil migration is also partly PI3K-dependent.²³

Reduction of IL-25-induced expression of Th2 cytokines and infiltration of eosinophils by LY294002 may also at least partly account for the reduced goblet cell hyperplasia and hypertrophy observed in the treated animals in this study, as Th2 cytokines and eosinophils have been implicated as contributing factors to these phenomena.^{31–34} LY294002 treatment also reduced IL-25-induced deposition of collagen around the airways and the concentration of lung total collagens. These data are in line with a previous study showing reduced peribronchial fibrosis in PI3K γ -deficient mice, possibly as a result of reduced numbers of peribronchial TGF- β 1-expressing cells and attenuated Smad 2/3 signalling.²⁶

LY294002 is a relatively non-specific inhibitor of PI3K, but given the plethora of possible alternative signalling pathways it remains a relatively specific indicator of the involvement of PI3K as distinct from other signalling pathways. Its use is a logical extension of our previous published studies^{9,10} suggesting that PI3K signalling predominantly drives proliferation of and autocrine VEGF production by vascular endothelial cells, a process contributing to a key element of airway remodelling (neovascularization) in asthma. Clearly, however, some role for other signalling pathways cannot be ruled out and is indeed likely. Other protein kinases such as CK2, mTOR and GSK3 β are also 'off-target' protein kinases potentially targeted by LY294002,^{35,36} but all three of these kinases are actually located downstream of PI3K or PI3K/Akt signalling pathways, and our previous studies on IL-25-induced angiogenesis suggest relatively little Akt involvement.¹⁰ Another

limitation of this study is that we confined ourselves to addressing the effects of LY294002 prior to administering IL-25; it might also be informative to further explore whether LY294002 may limit or even reverse the established, asthma-related features in this IL-25-induced model of asthma.

TGF- β has been shown to be involved in the migration and proliferation of airway smooth muscle cells, which is in turn thought to contribute to airway smooth muscle hyperplasia.^{37,38} In this study, the mean concentration of TGF- β in lung tissues was significantly reduced by LY294002 treatment of the IL-25-challenged mice, consistent with the hypothesis that TGF- β is partly responsible for this hyperplasia. TGF- β is produced principally by eosinophils and lymphocytes,³³ and so, the reduced production of this mediator observed in this study might, at least partly, reflect the reduction of eosinophil and lymphocyte infiltration into the airways by LY294002.

Our previously reported *in vitro* experiments have shown that IL-25 enhances proliferation of blood vascular endothelial cells and angiogenesis at least partly by upregulating the expression of bFGF, VEGF and their receptors by endothelial cells, while LY294002 significantly inhibits IL-25-induced expression of VEGF and angiogenesis.^{9,10} Our present data showing that LY294002 significantly inhibited IL-25-induced angiogenesis in an *in vivo* condition are consistent with this and suggest that the effect may at least partly reflect the reduction of local lung VEGF production.

In summary, the PI3K inhibitor LY294002 effectively attenuates IL25-induced AHR, inflammation and remodelling in this murine model of chronic asthma. These data clearly implicate PI3Ks in the pathogenesis of asthma and delineate PI3K signalling as a possible therapeutic target at least for the downstream effects of IL-25 in asthma, where suitable interruption of this signalling might not only ameliorate ongoing airway inflammation and AHR but also alter the natural history of chronic airway remodelling.

Acknowledgements

We acknowledge financial support from the National Natural Science Foundation of China (81102250, 81373177 and 81471594), the Scientific Research Common Program of the Beijing Municipal Commission of Education (KM201110025005 and KM201410025006) and the Special Project Fund for Clinical Research of the Chinese Medical Association (08020410119).

REFERENCES

- Masoli M, Fabian D, Holt S, Beasley R; Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 2004; **59**: 469–78.
- National Asthma Education and Prevention Program. Expert Panel Report 3 (EPR-3): guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. *J. Allergy Clin. Immunol.* 2007; **120** (Suppl. 5): S94–138.
- Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am. J. Respir. Crit. Care Med.* 2000; **162**: 2341–51.

- 4 Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, Menon S, Seymour B, Jackson C, Kung TT *et al.* New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. *J. Immunol.* 2002; **169**: 443–53.
- 5 Yao XJ, Huang KW, Li Y, Zhang Q, Wang JJ, Wang W, Liu J, Lv Z, An YQ, Ding YZ *et al.* Direct comparison of the dynamics of IL-25- and 'allergen'-induced airways inflammation, remodelling and hypersensitivity in a murine asthma model. *Clin. Exp. Allergy* 2014; **44**: 765–77.
- 6 Takeda M, Ito W, Tanabe M, Ueki S, Kihara J, Kato H, Tanigai T, Kayaba H, Sasaki T, Chihara J. The pathophysiological roles of PI3Ks and therapeutic potential of selective inhibitors in allergic inflammation. *Int. Arch. Allergy Immunol.* 2010; **152**(Suppl. 1): 90–5.
- 7 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.
- 8 Kwak YG, Song CH, Yi HK, Hwang PH, Kim JS, Lee KS, Lee YC. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J. Clin. Invest.* 2003; **111**: 1083–92.
- 9 Corrigan CJ, Wang W, Meng Q, Fang C, Wu H, Reay V, Lv Z, Fan Y, An Y, Wang YH *et al.* T-helper cell type 2 (Th2) memory T cell-potentiating cytokine IL-25 has the potential to promote angiogenesis in asthma. *Proc. Natl. Acad. Sci. U. S. A.* 2011; **108**: 1579–84.
- 10 Wang W, Fan YQ, Lv Z, Yao XJ, Wang W, Huang KW, Meng Q, Fang CL, Lee TH, Corrigan CJ *et al.* Interleukin-25 promotes basic fibroblast growth factor expression by human endothelial cells through interaction with IL-17RB, but not IL-17RA. *Clin. Exp. Allergy* 2012; **42**: 1604–14.
- 11 Jonasson S, Hedenstierna G, Hedenstrom H, Hjoberg J. Comparisons of effects of intravenous and inhaled methacholine on airway physiology in a murine asthma model. *Respir. Physiol. Neurobiol.* 2009; **165**: 229–36.
- 12 Riesenfeld E, Allen GB, Bates JH, Poynter ME, Wu M, Aimiand S, Lundblad LK. The temporal evolution of airways hyperresponsiveness and inflammation. *J. Allergy Ther.* 2012; **1**: 1–7.
- 13 Rickel EA, Siegel LA, Yoon BR, Rottman JB, Kugler DG, Swart DA, Anders PM, Tocker JE, Comeau MR, Budelsky AL. Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. *J. Immunol.* 2008; **181**: 4299–310.
- 14 Gregory LG, Jones CP, Walker SA, Sawant D, Gowers KH, Campbell GA, McKenzie AN, Lloyd CM. IL-25 drives remodelling in allergic airways disease induced by house dust mite. *Thorax* 2013; **68**: 82–90.
- 15 Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, Liu YJ, Zhu Z, Dong C. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J. Exp. Med.* 2007; **204**: 1509–17.
- 16 Ballantyne SJ, Barlow JL, Jolin HE, Nath P, Williams AS, Chung KF, Sturton G, Wong SH, McKenzie AN. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. *J. Allergy Clin. Immunol.* 2007; **120**: 1324–31.
- 17 Pinho V, Souza DG, Barsante MM, Hamer FP, DeFreitas MS, Rossi AG, Teixeira MM. Phosphoinositide-3 kinases critically regulate the recruitment and survival of eosinophils in vivo: importance for the resolution of allergic inflammation. *J. Leukoc. Biol.* 2005; **77**: 800–10.
- 18 Salogni L, Musso T, Bosio D, Mirolo M, Jala VR, Haribabu B, Locati M, Sozzani S. Activin A induces dendritic cell migration through the polarized release of CXC chemokine ligands 12 and 14. *Blood* 2009; **113**: 5848–56.
- 19 Del PA, Vermi W, Dander E, Otero K, Barberis L, Luini W, Bernasconi S, Sironi M, Santoro A, Garlanda C *et al.* Defective dendritic cell migration and activation of adaptive immunity in PI3Kgamma-deficient mice. *EMBO J.* 2004; **23**: 3505–15.
- 20 Ali K, Bilancio A, Thomas M, Pearce W, Gilfillan AM, Tkaczuk C, Kuehn N, Gray A, Giddings J, Peskett E *et al.* Essential role for the p110delta phosphoinositide 3-kinase in the allergic response. *Nature* 2004; **431**: 1007–11.
- 21 Ali K, Camps M, Pearce WP, Ji H, Rucke T, Kuehn N, Pasquali C, Chabert C, Rommel C, Vanhaesebroeck B. Isoform-specific functions of phosphoinositide 3-kinases: p110 delta but not p110 gamma promotes optimal allergic responses in vivo. *J. Immunol.* 2008; **180**: 2538–44.
- 22 Endo D, Gon Y, Nunomura S, Yamashita K, Hashimoto S, Ra C. PI3Kγ differentially regulates Fc ε RI-mediated degranulation and migration of mast cells by and toward antigen. *Int. Arch. Allergy Immunol.* 2009; **149**: 66–72.
- 23 Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I *et al.* Function of PI3K γ in thymocyte development, T cell activation, and neutrophil migration. *Science* 2000; **287**: 1040–6.
- 24 Nashed BF, Zhang T, Al-Alwan M, Srinivasan G, Halayko AJ, Okkenhaug K, Vanhaesebroeck B, Hayglass KT, Marshall AJ. Role of the phosphoinositide 3-kinase p110delta in generation of type 2 cytokine responses and allergic airway inflammation. *Eur. J. Immunol.* 2007; **37**: 416–24.
- 25 Takeda M, Ito W, Tanabe M, Ueki S, Kato H, Kihara J, Tanigai T, Chiba T, Yamaguchi K, Kayaba H *et al.* Allergic airway hyperresponsiveness, inflammation, and remodeling do not develop in phosphoinositide 3-kinase gamma-deficient mice. *J. Allergy Clin. Immunol.* 2009; **123**: 805–12.
- 26 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**: L210–9.
- 27 Thomas M, Edwards MJ, Sawicka E, Duggan N, Hirsch E, Wymann MP, Owen C, Trifilieff A, Walker C, Westwick J *et al.* Essential role of phosphoinositide 3-kinase gamma in eosinophil chemotaxis within acute pulmonary inflammation. *Immunology* 2009; **126**: 413–22.
- 28 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**: 455–65.
- 29 Doukas J, Eide L, Stebbins K, Racanelli-Layton A, Dellamary L, Martin M, Dneprovskaia E, Noronha G, Soll R, Wrasidlo W *et al.* Aerosolized phosphoinositide 3-kinase gamma/delta inhibitor TG100-115 [3-[2,4-diamino-6-(3-hydroxyphenyl) pteridin-7-yl]phenol] as a therapeutic candidate for asthma and chronic obstructive pulmonary disease. *J. Pharmacol. Exp. Ther.* 2009; **328**: 758–65.
- 30 Chung KF. Targeting the interleukin pathway in the treatment of asthma. *Lancet* 2015; **386**: 1086–96.
- 31 Xie S, Sukkar MB, Issa R, Khorasani NM, Chung KF. Mechanisms of induction of airway smooth muscle hyperplasia transforming growth factor-β. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2007; **293**: 245–53.
- 32 Gang C, Nasreen K. TGF-β1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir. Res.* 2006; **7**: 2.
- 33 Elovic A, Wong DT, Weller PF, Matossian K, Galli SJ. Expression of transforming growth factors-alpha and beta1 messenger RNA and product by eosinophils in nasal polyps. *J. Allergy Clin. Immunol.* 1994; **93**: 864–9.
- 34 Fernandes DJ, Mitchell RW, Lakser O, Dowell M, Stewart AG, Solway J. Do inflammatory mediators influence the contribution of airway smooth muscle contraction to airway hyperresponsiveness in asthma? *J. Appl. Physiol.* 2003; **95**: 844–53.
- 35 Gharbi SI, Zvelebil MJ, Shuttleworth SJ, Hancox T, Saghir N, Timms JF, Waterfield MD. Exploring the specificity of the PI3K family inhibitor LY294002. *Biochem. J.* 2007; **404**: 15–21.
- 36 Arrowsmith CH, Audia JE, Austin C, Baell J, Bennett J, Blagg J, Bountra C, Brennan PE, Brown PJ, Bunnage ME *et al.* Corrigen-dum: the promise and peril of chemical probes. *Nat. Chem. Biol.* 2015; **11**: 887.

- 37 Nakagome K, Dohi M, Okunishi K, Tanaka R, Kouro T, Kano MR, Miyazono K, Miyazaki J, Takatsu K, Yamamoto K. IL-5-induced hypereosinophilia suppresses the antigen-induced immune response via a TGF- β -dependent mechanism. *J. Immunol.* 2007; **179**: 284–94.
- 38 Vargaftig BB, Singer M. Leukotrienes mediate murine bronchopulmonary, hyperreactivity, inflammation, and part of mucosal metaplasia and tissue injury induced by recombinant murine interleukin-13. *Am. J. Respir. Cell Mol. Biol.* 2003; **28**: 410–9.

Supplementary Information

Additional supplementary information can be accessed via the *html* version of this article at the publisher's website.

Figure S1 Schedule of murine challenge.

Figure S2 Haematoxylin and eosin (H&E)-stained lung sections showing that LY294002 significantly reduced IL-25-induced infiltration of inflammatory cells into the peribronchial and perivascular regions of the lung tissues as compared with DMSO-treated vehicle control groups.

Figure S3 Representative photomicrographs of (A) periodic acid-Schiff (PAS)-stained lung sections from vehicle control-challenged, IL-25/DMSO-challenged and IL-25/LY294002-challenged mice, (B) Masson's trichrome blue-stained lung sections from vehicle control-challenged, IL-25/DMSO-challenged and IL-25/LY294002-challenged mice, (C) alpha-smooth muscle actin (α -SMA) immunoreactivity in lung sections from vehicle control-challenged, IL-25/DMSO-challenged and IL-25/LY294002-challenged mice, and (D) CD31 immunoreactive blood vessels in lung sections from vehicle control-challenged, IL-25/DMSO-challenged and IL-25/LY294002-challenged mice at various time points.

SUPPLEMENTARY INFORMATION

Comprehensive attenuation of IL-25-induced airways hyperresponsiveness, inflammation and remodelling by the PI3K inhibitor LY294002

Ping Huang¹, Yan Li^{1,2}, Ze Lv¹, Jingjing Wang³, Qian Zhang⁴, Xiujuan Yao⁴, Chris J Corrigan⁵,
Kewu Huang², Wei Wang¹ and Sun Ying^{1,5}

¹the Department of Immunology, School of Basic Medical Sciences, Capital Medical University, Beijing, P.R. China; ²the Department of Respiratory and Critical Care Medicine, Beijing Chao-Yang Hospital, Capital Medical University & Beijing Institute of Respiratory Medicine, Beijing, P.R. China; ³the Department of Laboratory Animal Sciences, Capital Medical University; ⁴the Department of Respiratory Medicine, Beijing Tongren Hospital, Capital Medical University, and ⁵King's College London, MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, Division of Asthma, Allergy & Lung Biology, London UK.

Figure S1-Schedule of murine challenge.

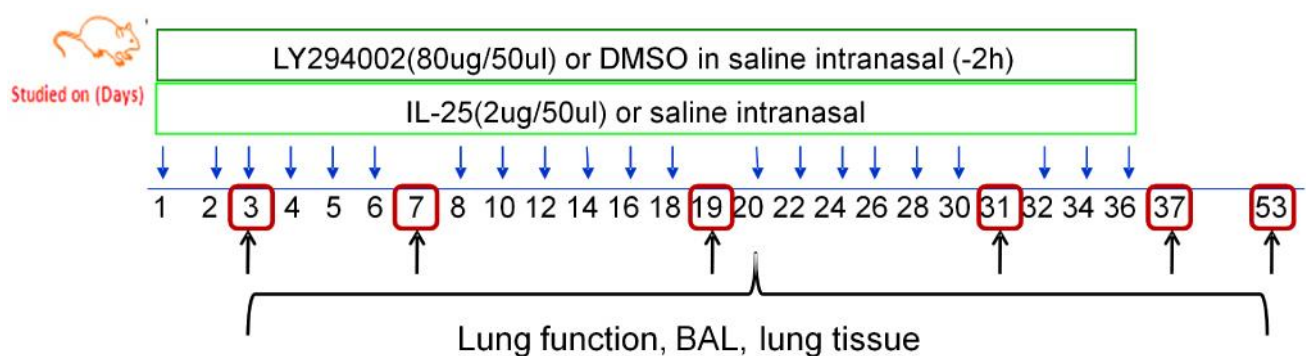


Figure S2-H&E-stained lung sections showing that LY294002 significantly reduced IL-25-induced infiltration of inflammatory cells into the peribronchial and perivascular regions of the lung tissues as compared with DMSO-treated vehicle control groups.

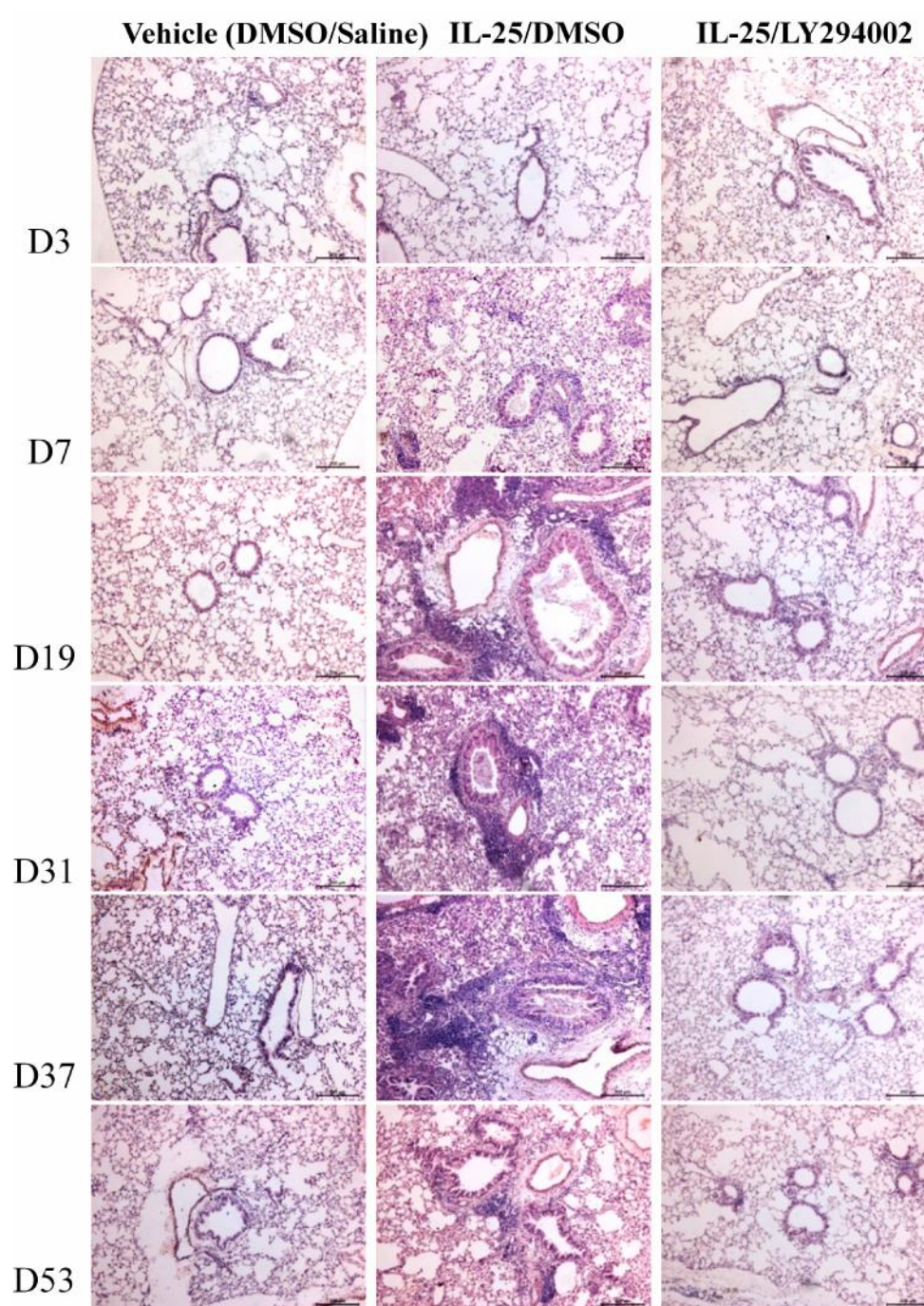
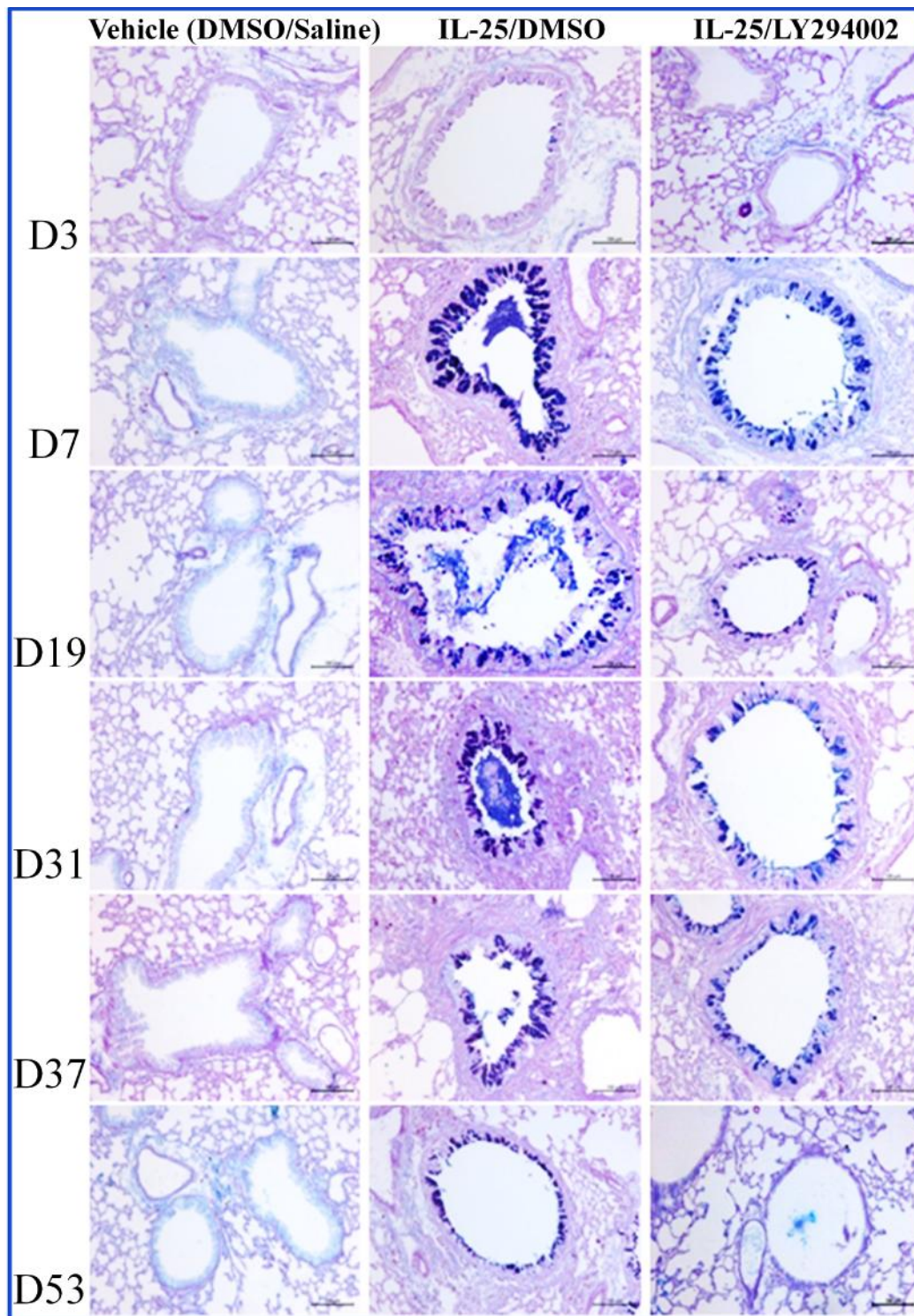
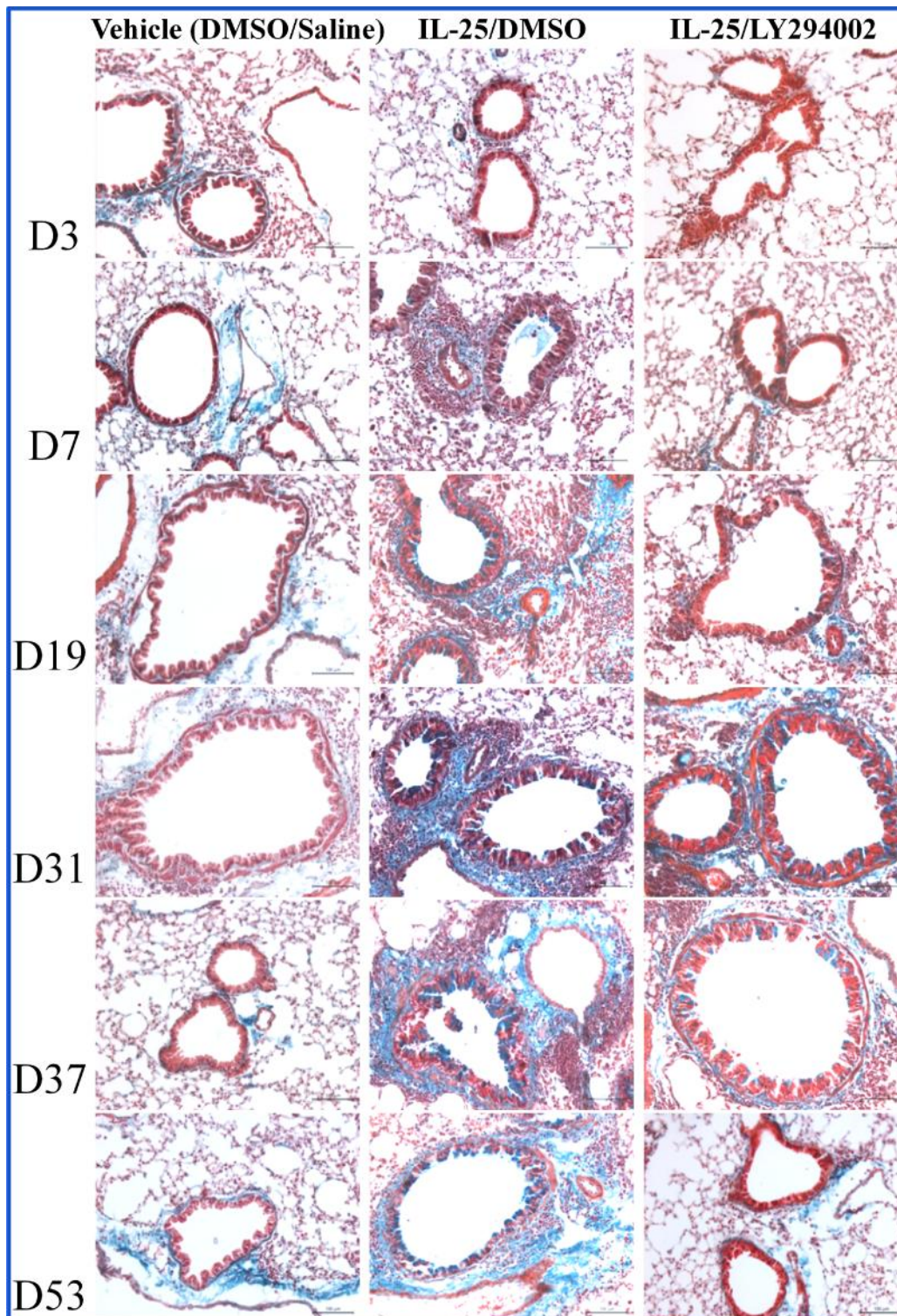


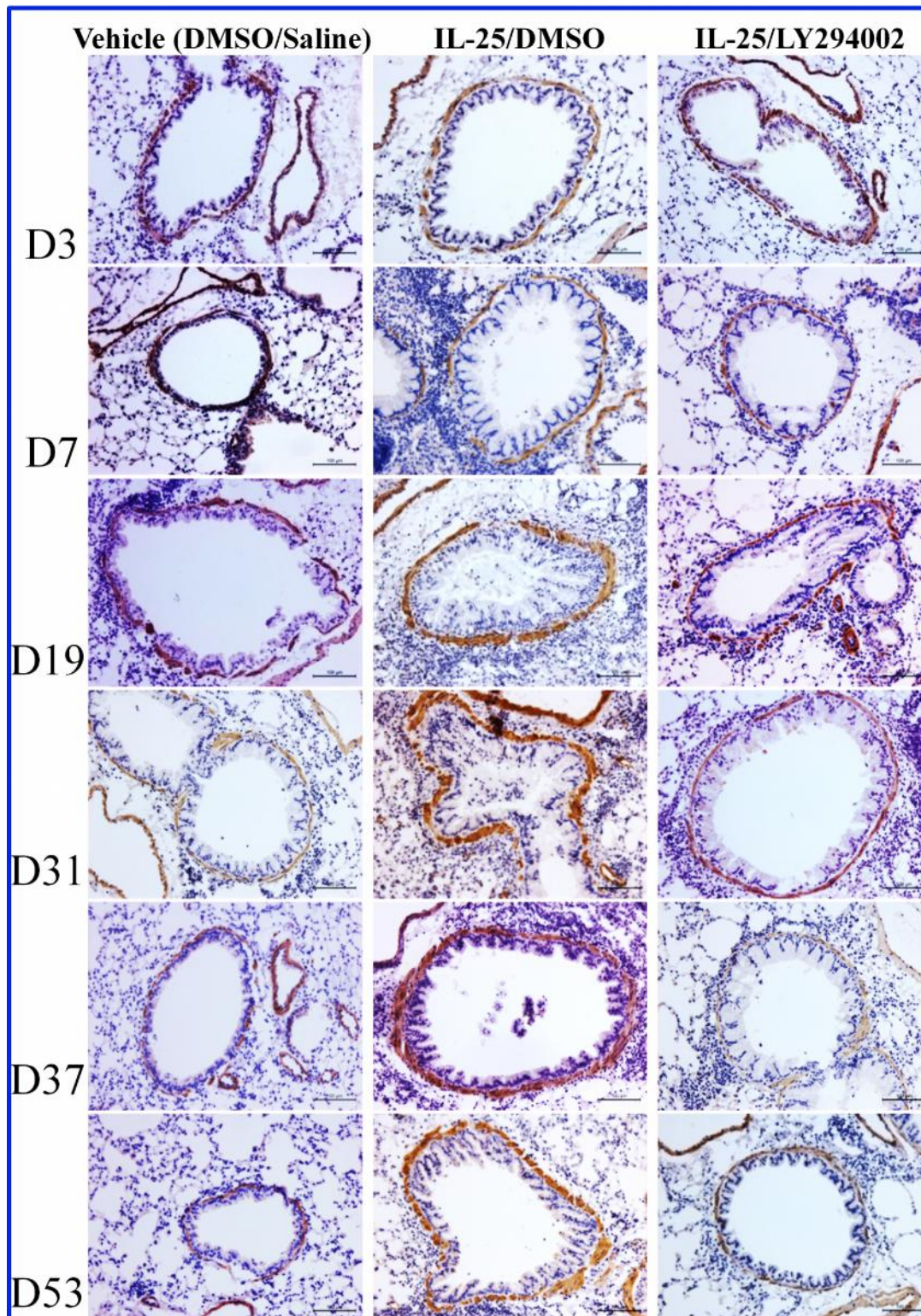
Figure S3 –



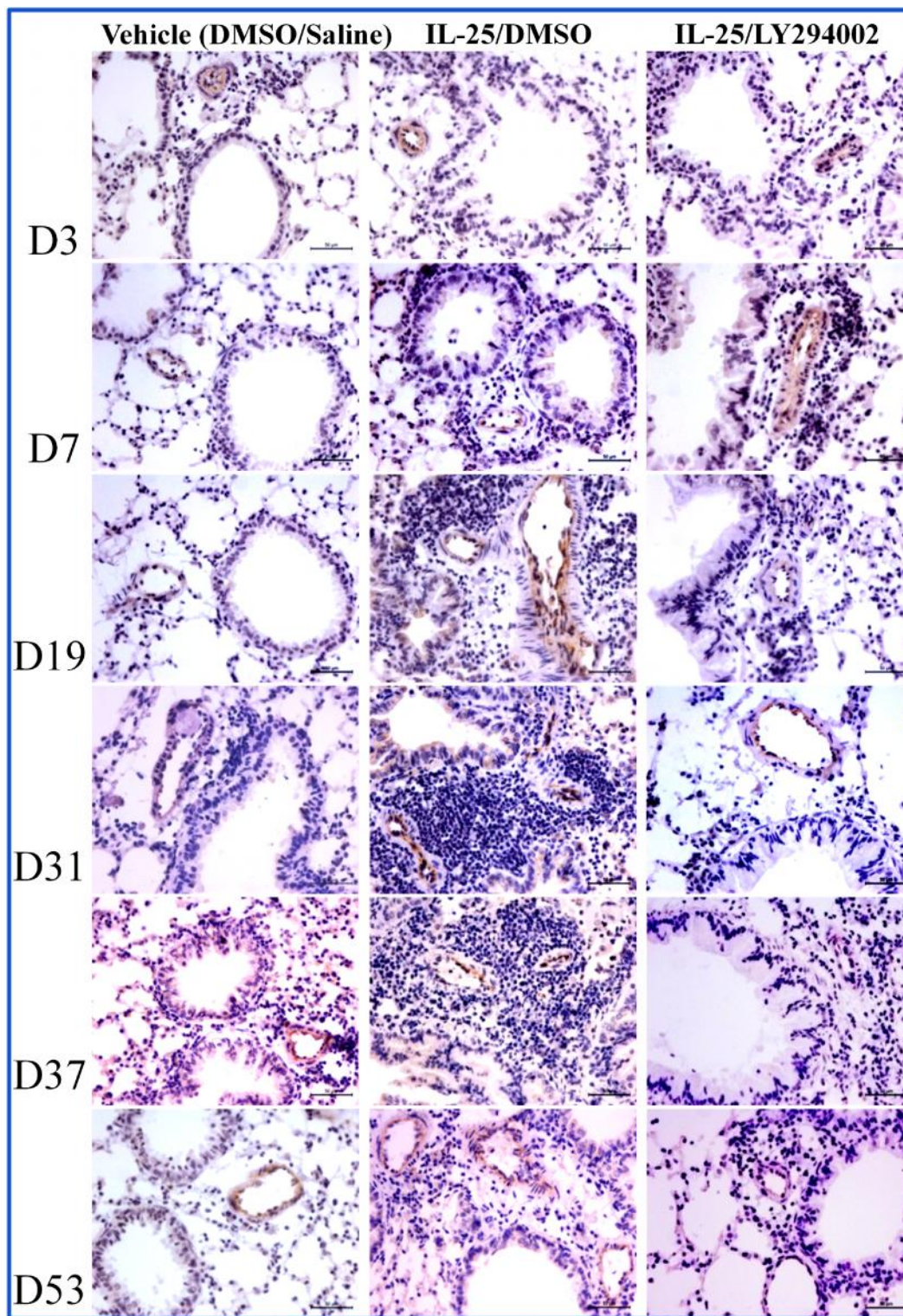
(A) Representative photomicrographs of periodic acid-Schiff (PAS) stained lung sections from vehicle control-, IL-25/DMSO- and IL-25/ LY294002 -challenged mice at various time-points as indicated (original magnification x 20).



(B) Representative photographs of Masson's trichrome-blue-stained lung sections from vehicle control-, IL-25/DMSO- and IL-25/LY294002-challenged mice at various time-points as indicated (original magnification x 20).



(C) Representative photographs of α -SMA immunoreactivity in lung sections from vehicle-, IL-25/DMSO- and IL-25/LY294002-challenged mice at various time-points as indicated (original magnification x 20).



(D) Representative photographs of CD31 immunoreactive blood vessels in lung sections from vehicle-, IL-25/DMSO- and IL-25/LY294002-challenged mice at various time-points as indicated (original magnification x 20).