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Title: The effects of IL-33 on airways collagen deposition and matrix metalloproteinase expression in a murine surrogate of asthma

Running Title: IL-33 and airways fibrosis in asthma

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Summary

It has been suggested that interleukin 33 (IL-33) plays an important role in the pathogenesis of asthma through a variety of pathways, but its role in airways fibrosis in asthma has not been fully elucidated. In the present study we evaluated changes in the expression of extracellular matrix proteins (ECMs) as well as matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in an IL-33-induced, antigen-independent murine surrogate of asthma as well as a conventional surrogate employing per-nasal challenge of mice previously sensitised to produce an IgE response to ovalbumin (OVA). In addition, in *in vitro* experiments we explored the direct effects of IL-33 on the proliferation and function of murine fibroblasts. Per-nasal administration of IL-33 alone was sufficient to induce airways deposition of ECMs, including collagens I, III, V and fibronectin, to a degree comparable with that observed in the OVA-sensitised and challenged mice. These changes were associated with a local imbalance between the expression of extracellular MMPs and TIMPs. Per-nasal challenge of mice with IL-33 also induced elevated airways expression of connective tissue growth factor (CTGF) and fibroblast growth factor receptor 4 (FGFR4), two key

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facilitators of local fibrosis, again to a degree compatible with that observed in OVA-sensitised and challenged mice. Deletion of the ST2 gene, which encodes the IL-33 receptor, abrogated these fibrotic changes in the airways in the OVA surrogate. *In vitro*, IL-33 significantly increased the proliferation and expression of collagen III by murine lung fibroblasts. These data suggest that direct exposure of murine airways to IL-33 is able to induce local fibrotic changes, at least partially through effects of signalling through the IL-33/ST2 axis on fibroblast function and local expression of MMPs and their inhibitors, and other fibrosis-related proteins.

Keywords: asthma, fibrosis, remodelling, IL-33, pathogenesis.

Abbreviations: AHR, airway hyperresponsiveness; CTGF, connective tissue growth factor; ECM, extracellular matrix; IHC, immunohistochemistry; IL-33, interleukin-33; MMP, matrix metalloproteinase; OVA, ovalbumin; TIMP, tissue inhibitor of metalloproteinase.

Introduction

Asthma causes suffering in more than 300 million people worldwide, a number expected to reach 400 million by the year 2020.^{1, 2} Asthma is a chronic respiratory disease characterised by airways smooth muscle hyperresponsiveness combined with a varying degree of chronic inflammation of the airways submucosa, resulting in recurrent wheezing, chest tightness and shortness of breath.³ These inflammatory

changes, which exacerbate occlusion of the lumen of the airways, are potentially reversible in those patients showing satisfactory therapeutic benefit from conventional anti-asthma therapy such as inhaled corticosteroids and β 2-adrenergic agonists, as well as oral leukotriene inhibitors.⁴ Airways occlusion may, however, become irreversible at least partly because of structural changes (“remodelling”) in the airways associated with the inflammation, including mucous hyperplasia, subepithelial extracellular matrix deposition, smooth muscle hypertrophy and bronchial neoangiogenesis.⁵ Clearly it remains a priority fully to define the mechanisms of these irreversible changes, so that in the future their development may hopefully be pre-empted.

Interleukin-33 (IL-33) is an IL-1-like pro-inflammatory epithelial alarmin which was first discovered and named in 2005. IL-33 expression is increased in the airways mucosa of patients with severe asthma. It activates nuclear factor kappa-B (NF- κ B),⁶ mitogen-activated protein kinase (MAPK) and possibly other signalling pathways through its receptor ST2, resulting in increased expression of IL-4, IL-5 and IL-13 and other Th2 cytokines, thereby further aggravating airways inflammation.^{7, 8} Specific inhibition of IL-33 or its receptor ST2 has been reported to abrogate airways inflammation, mucus hypersecretion and airways hyperresponsiveness in murine surrogates of asthma^{9, 10} Our own previous findings have shown that direct, per-nasal application of IL-33 alone to the respiratory tract can induce typical asthma-like features in mice, including airways eosinophilic mucosal infiltration, hyperresponsiveness and neoangiogenesis.^{11, 12} Recent studies indicate that IL-33 is

able to act exert some of these effects indirectly by activating airways mucosal ILC2 cells, inducing the latter to produce Th2-type cytokines.^{13, 14} These findings strongly imply a pivotal role for IL-33 in the pathogenesis of the chronic airways inflammation observed in many patients with asthma.

The laydown of interstitial matrix proteins causing fibrosis and potentially irreversible airways obstruction is pathognomonic of asthma in humans. In the present study we address the hypothesis that IL-33, acting through its receptor ST2, promotes this fibrosis by direct activation of airways fibroblasts, by increasing the local production of other fibroblast growth factors and finally by upsetting the equilibrium between the local fibrinolytic activities of matrix metalloproteinases and their inhibitors. To accomplish this we measured these changes following direct application of IL-33 to the murine respiratory tract and compared them with those observed in a conventional murine surrogate of asthma induced by ovalbumin sensitisation and challenge, the latter both in wild type mice and those with deletion of the gene encoding the IL-33 receptor ST2.

Materials and methods

Animals

Female BALB/c mice (6–8 weeks old, Vital River Laboratory, Beijing, China) were kept in a pathogen-free environment in the Department of Laboratory Animal

Sciences, Capital Medical University, Beijing, China. *St2*^{-/-} mice (BALB/c background) were kindly provided by Dr. Andrew McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.). All experiments were approved by the Institutional Animal Care and Use Committee of Capital Medical University, Beijing, China.

IL-33 challenge and OVA challenge

Murine surrogates of asthma-induced by IL-33 or OVA challenge were prepared and studied as previously described.¹² Briefly, recombinant murine IL-33 (rmIL-33, 100 ng in 50 μ L saline, R&D Systems, Minneapolis, MN, USA) or saline control was administered per-nasally directly to the airways of two groups of mice daily from days 18-23 from the commencement of the experiment, then every 2 days until day 53. Further groups of mice were sensitised by intraperitoneal injection with OVA on days 0 and 12 of the experiment, then challenged per-nasally with a solution of OVA or saline control on the same days as those challenged with IL-33. Subgroups of mice in each group were euthanised at various, fixed time points until day 70 (17 days after the final challenge). To further explore possible effects of the IL-33/ST2 axis on airways fibrosis, a further group of *St2*^{-/-} gene deleted mice was also sensitised to, and challenged with OVA or saline control and subgroups of these animals euthanised and examined on days 24 and 48 (Figure 1a).

Lung immunohistochemistry

Immunohistochemistry was employed to quantify immunoreactivity for the most common biological markers of airways fibrosis in asthma, including collagens I, III and V, the matrix metalloproteinases MMP1, MMP2, MMP9, MMP12 and MMP13, fibroblast growth factor receptor 4 (FGFR4), connective tissue growth factor (CTGF), fibronectin and tissue inhibitor of metalloproteinase-1 (TIMP1). The primary antibodies employed were purchased from Abcam (Cambridge, UK), Novus Biologicals (Littleton, CO) or Biorbyt (Carrickfergus, UK). The sources and optimised concentrations of the individual primary antibodies are summarised in Table

1. Substitution of the primary antibody with PBS was employed as a negative control.

Paraffin sections were de-waxed in xylene, rehydrated in serially reduced concentrations of ethanol, then subjected to antigen retrieval by incubating in citrate or EDTA buffer. Endogenous peroxidase was quenched by incubating with 3% H₂O₂.

The sections were then blocked with 1% BSA (Solarbio, Beijing, China) in PBS and incubated with primary antibody overnight at 4°C. After washing, the sections were incubated with appropriate secondary antibodies then washed again and incubated with an HRP-labelled third layer antibody. Finally, the areas of the sections binding to the specific, first layer antibodies were identified by colour development with 3,

3'-diaminobenzidine (Solarbio, Beijing, China), then the entire sections

counterstained with heamatoxylin and analysed by microscopy. Immunoreactivity

was measured using a Nikon microscope (Nikon, Tokyo, Japan) connected with a

computer imaging system and analysed using proprietary software (Image-Pro Plus

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6.0, Media Cybernetics, Silver Spring, USA). The data were expressed as percentages of the positively stained areas in the entire lung sections as previously described.^{11, 12}

Effects of IL-33 on the proliferation and expression of fibronectin and collagen III by murine fibroblasts

Fibroblast proliferation was measured using a commercial kit (Cell Counting Kit-8, Dojindo, Japan). Murine lung fibroblasts MLg [Mlg2908](ATCC®CCL-206™) were propagated in monolayer culture according to the manufacturer's instructions. When the cells were approximately 80% confluent they were detached (0.25% trypsin, Gibco Laboratories, Paisley, Scotland) then washed in PBS buffer. After washing, cells (2000 cells/100 µL/well) were seeded into 96-well plates then incubated at 37 °C for 12 hours before adding rmIL-33 (R&D Systems, Minneapolis, MN, USA) at final concentrations of 10, 50 or 100ng/mL or normal saline control in a total volume of 150µL in culture medium without foetal bovine serum (FBS). Cells were also cultured in medium containing FBS as a positive control. Cellular proliferation in the presence of saline control and in the absence of FBS was set as baseline (100% proliferation). These concentrations of IL-33 were as used in our previous study.¹⁵ After incubation for a further 48 hours, 10µL of CCK-8 solution were added to each well and incubation allowed to proceed for a further 4 hours. The absorbance at 450 nm was measured with Perkin Elmer EnSpire® Multimode Plate Reader (Waltham, MA, USA).

A Western blotting platform was used to measure the expression of collagen III and fibronectin by murine lung fibroblasts. Cells were processed as above; briefly, MLg cells were seeded into 6-well plates (10^5 cells/2 mL/well), incubated until 50% confluent then serum starved for 12 hours. IL-33 was added to the plates at a final concentration of 100ng/mL and the cells incubated for a further 48 hours. Following incubation, 50 μ L of precooled RIPA were added to each well with thorough shaking on ice until the cells were fully lysed. The lysates were then centrifuged at 12000 rpm for 20 min at 4 °C then the supernatants gently aspirated and transferred to a fresh precooled microcentrifuge tube on ice. 8 μ L aliquots of the lysates were mixed with 2 μ L of protein loading buffer (10% SDS, 5% 2-mercaptoethanol, 50% glycerol and 0.5% bromophenol blue in 250 mM Tris-HCl, pH 6.8). After heating to 95 °C for 10 min, the proteins in the lysate were separated using 10% SDS-PAGE. Separated proteins in the gels were electrophoretically transferred to a nitrocellulose membrane (250 mA, 2 hr). The membrane was then blocked with 5% skimmed milk in PBS containing 0.05% Tween 20 (PBS-T buffer) for 1 hr at room temperature. After washing the membrane with PBS-T, it was incubated with the same primary antibodies as used in the immunohistochemical analysis above against collagen III (1:1000, Abcam, Cambridge, UK) and fibronectin (1:1000, Abcam, Cambridge, UK) and an anti- β -actin antibody (1:2000, Abcam, Cambridge, UK) to serve as a loading control diluted in PBS-T containing 0.25% BSA and the membrane incubated overnight at 4 °C. After washing the membrane with PBS-T again, the bound antibodies were detected by staining with a 1:10000 dilution of goat anti-mouse or

anti-rabbit IRDye (according to the species of origin of the primary antibody) (LI-COR Biosciences, Lincoln, NE, United States) in 10 mL of PBS-T buffer for 1 hr at room temperature. After final washing, the membranes were imaged and analysed using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, United States).

Statistical analysis

All data were processed using GraphPad Prism7.0 software (GraphPad, San Diego, USA). Differences between experimental groups were analysed using one-way ANOVA or two-way ANOVA followed by a Bonferroni post test or the Student's unpaired t-test. For all tests, *P*-values less than 0.05 were considered significant.

Results

IL-33 increases lung ECM protein deposition

Compared with saline, repeated, per-nasal challenge of the murine airways with IL-33 alone resulted in deposition of extracellular matrix proteins in and around the airways, with distinct time courses. Significantly elevated expression of immunoreactivity for collagen I was apparent from day 48 and persisted until the end of the study (day 70, Figure 1b and c). Significantly elevated collagen III/V immunoreactivity was apparent from day 36 and again persisted until the end of the study (Figure S1 and Figure S2). OVA sensitised and challenged mice showed similar changes, except that the onset of elevated expression of immunoreactivity for collagens I and III was later than with

IL-33 challenge (Figure 1 and Figure S1). IL-33 challenge also promoted the deposition of fibronectin in and around the murine airways, although this effect was more delayed and less extensive than that associated with OVA challenge (Figure 2).

IL-33 induces elevated CTGF and FGFR4 expression

Global immunoreactivity for CTGF was significantly elevated in and around the airways of the IL-33-challenged mice, with a transient time course similar to that observed in association with OVA challenge except that, in the latter case CTGF immunoreactivity was significantly elevated somewhat earlier (from day 20) (Figure 3). Compared with saline, IL-33 challenge also induced significantly elevated expression of FGFR4 immunoreactivity commencing from day 24 and persisting until the end of the experiment. A similar time course of elevated expression on FGFR4 was observed following OVA challenge, although the extent of the changes was much more obvious with this stimulus (Figure 4).

IL-33 regulates expression of MMPs and TIMPs

Per-nasal challenge of the airways of the mice with IL-33 alone was also sufficient transiently and significantly to elevate expression of immunoreactivity for MMP1 and MMP-13 in and around the airways between days 36 and 54, a time course of elevation similar to that observed following OVA challenge (Figure 5, Figure S4). Both IL-33 and OVA challenge induced significantly elevated expression of immunoreactivity for MMP9 and MMP12, apparent from day 24 and transient in the case of MMP9, while persisting until the end of the experiment in the case of MMP12

(Figure 7, Figure S3). The only notable difference between the effects of IL-33 and OVA challenge on airways MMP expression was observed in the case of MMP2, where IL-33 challenge did not significantly alter MMP2 expression whereas OVA challenge caused a significant increase in the expression of MMP2 immunoreactivity apparent from day 24 which persisted until the end of the experiment (Figure 6). Finally, both IL-33 and OVA challenge resulted in a transient, significant elevation of the expression of immunoreactivity for TIMP1 apparent from day 36 (Figure 8).

IL-33 promotes fibroblast proliferation and collagen production

In vitro, rmlL-33 significantly promoted the proliferation of murine lung fibroblasts (Figure 9). Furthermore, IL-33 stimulation resulted in elevated expression of collagen III by these cells, although we observed no effect of IL-33 on the production of fibronectin at the concentration employed.

Effects of the IL-33/ST2 axis on pulmonary fibrosis

To further clarify the possible contribution of IL-33/ST2 axis signalling to airways fibrosis in this animal asthma surrogate, we repeated the challenge experiments with OVA in *St2^{-/-}* mice. Knockdown of the ST2 gene significantly attenuated the effects of OVA challenge in increasing the expression of immunoreactivity for fibronectin, CTGF, FGFR4, MMP2, MMP9 and MMP12 in the airways compared with OVA-challenged WT mice (Figure 10, Figure S5).

Discussion

Previous studies from ourselves and others have strongly implicated IL-33 in playing a key role in the pathogenesis of the airways mucosal inflammation which typically accompanies human asthma.⁷⁻¹² In our own previous studies we showed that direct, per-nasal application of IL-33 alone to the murine airway is sufficient cause asthma-like inflammation, including subepithelial deposition of collagens.^{11, 12} We have here greatly extended these observations to focus on the effects of IL-33 on the pro-fibrotic inflammatory milieu in the airways. We have shown that direct, per-nasal administration of IL-33 to the airways alters the balance of expression of matrix metalloproteinases and their inhibitors and also promotes the expression of key, pro-fibrotic proteins acting on fibroblasts, including CTGF and FGFR4. In order further to underline the relevance of these effects of IL-33 in asthma, we have shown that these IL-33-induced changes were broadly, although not completely comparable with those observed in a more “classical” murine surrogate of asthma employing OVA sensitisation and challenge. Furthermore, these effects of IL-33 were significantly, but not completely abrogated in IL-33 receptor deficient (*St2^{-/-}*) mice sensitised and challenged with OVA. IL-33 directly induced proliferation and collagen production by lung fibroblasts *in vitro*. Taken together, our data provide further, strong support of the hypothesis that activation of the IL-33/ST2 signalling pathway plays a key role in the development of airways fibrosis.

Recent studies have similarly implicated IL-33 or its receptor ST2 in the pathogenesis of fibrosis in various organs such as the lung parenchyma, the liver, the heart and the skin.¹⁶⁻¹⁹ IL-33 transgenic mice develop pulmonary fibrosis.²⁰ In a bleomycin-induced surrogate of pulmonary fibrosis,²⁰ administration of exogenous IL-33 amplified fibrosis and collagen synthesis, possibly through acting on macrophages and ILC2 cells, the latter cells being sources of production of other pro-fibrotic cytokines such as TGF- β and IL-13. Such mechanisms may partly explain why, in the present study, IL-33 did not directly induce the synthesis of fibronectin by murine fibroblasts *in vitro*, whereas per-nasal administration of IL-33 *in vivo* did enhance expression of fibronectin in the murine airways in a manner compatible with that observed in the OVA-challenged mice. This and other indirect effects of IL-33 *in vivo* might result from its actions on cells other than fibroblasts causing the release of additional mediators, including ILC2s, macrophages and mast cells.^{21, 22}

In the present study, IL-33-treated mice developed elevated airways expression of FGFR4 and CTGF, two important factors involved in fibrosis. CTGF, a member of the CNN protein family, plays an important role in wound healing and tissue repair, and has been reported to act synergistically with TGF- β in sustaining fibrosis.²³ Moreover, previous studies have shown that over expression of CTGF promotes fibrosis in a variety of tissues or organs, or conversely that blockade or knock down of CTGF can significantly reduce this.²⁴⁻²⁷ FGFR4 signalling has been reported to synergise with that of TGF- β together to activate injury-primed renal fibroblasts.²⁸ Our previous data demonstrate that IL-33 promotes the expression of TGF- β and bFGF.¹¹ It is

reasonable to postulate, therefore, that IL-33 acts on the one hand to promote the synthesis of FGF and TGF- β , and on the other hand to increase the expression of FGFR4, both of which promote fibroblast collagen synthesis.

Both MMPs and TIMPs play an important role in airways remodelling.²⁹ MMPs are capable of degrading extracellular matrix proteins during inflammation to promote remodelling, while TIMPs are natural inhibitors of MMPs.³⁰⁻³² Thus, the equilibrium of expression of MMPs and TIMPs likely determines the degree of deposition of extracellular matrix. Our present data clearly show that exogenous administration of IL-33 can alter the expression of MMPs and TIMPs simultaneously in the airways.

Knock down of the *St2* gene significantly reduced collagen deposition around the airways in the OVA-challenged mice concomitantly with significantly reduced expression of MMPs. This further underlines the specificity of the effects of IL-33 in airways remodelling in influencing matrix metalloproteinase expression in the airways inflammatory process associated with asthma. It is worth noting again, however, that IL-33 challenge alone did not precisely recapitulate the effects of OVA sensitisation and challenge because, for example, under the particular conditions of our experiments it did not result in elevation of MMP2 expression. While we cannot discern the precise explanation for this, we speculate that one possible explanation is that, being a murine surrogate of “allergic asthma”, the airways inflammation induced in the OVA sensitisation/challenge model is IgE-dependent, whereas we previously detected no role for IgE in the airways inflammation induced by direct IL-33 challenge.¹¹ In this regard, it is interesting that deletion of the *St2* gene partially but

significantly reduced OVA-induced MMP2 expression, suggesting that the IL-33/ST2 axis does contribute, at least partially and indirectly, to the production of MMP2 in asthma.

The broadly contemporaneous profile of development of expression of MMP1, MMP9 and MMP13 is potentially of mechanistic significance because these MMPs can activate each other, ³³⁻³⁵ although one study ³⁶ has suggested an inhibitory role for MMP9 in bleomycin-induced pulmonary fibrosis, so that the possibility of a protective role for some of these proteinases in the induction of fibrosis must also be considered. On the other hand, we demonstrate spatial and temporal differences in the expression of some of these proteins, for example the relatively delayed, elevated expression of MMP12 and TIMP1. Finally, when attempting to interpret the role of these proteinases and their inhibitors in regulating airways fibrosis, it should be borne in mind that MMPs can not only cleave extracellular matrix but also directly cleave a variety of cytokines, thereby activating them. For example, MMP2 and MMP9 can directly activate TGF- β in this manner.³⁷

In summary, IL-33 alone can cause airways submucosal fibrotic changes very similar to those observed in conventional surrogates of asthmatic mucosal inflammation triggered by IgE-dependent mechanisms, suggesting that IL-33 plays a similarly important role in the pathogenesis of the airways submucosal fibrotic changes which characterise human asthma. Our data also suggest that these effects of IL-33 reflect not only a direct action on airways fibroblasts but also indirect effects mediated via

other cells and mediators within the airways mucosa. Our ongoing studies are directed at further clarification of these mechanisms.

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Disclosures

The authors declare no conflicts of interest.

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Figure 1. (a) Schedule of murine challenge. (b) Representative photomicrographs of collagen I immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (c) Quantitative analysis of collagen I immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure 2. IL-33 induced fibronectin deposition in the lungs of challenged animals. (a) Representative photomicrographs of fibronectin immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of fibronectin immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure 3. IL-33 promotes CTGF expression in the lungs of challenged animals. (a) Representative photomicrographs of CTGF immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of CTGF immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure 4. IL-33 promotes FGFR4 expression in the lungs of challenged animals. (a) Representative photomicrographs of FGFR4 immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of FGFR4 immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure 5. IL-33 promotes MMP1 expression in the lungs of challenged animals. (a) Representative photomicrographs of MMP1 immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of MMP1 immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure 6. IL-33 fails to promote MMP2 expression in the lungs of challenged animals. (a) Representative photomicrographs of MMP2 immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of MMP2 immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure 7. IL-33 promotes MMP9 expression in the lungs of challenged animals.(a) Representative photomicrographs of MMP9 immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of MMP9 immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure 8. IL-33 promotes TIMP1 expression in the lungs of challenged animals.(a) Representative photomicrographs of TIMP1 immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of TIMP1 immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure 9. IL-33 promotes fibroblast proliferation and collagen production.(a) Representative photomicrographs of western blot assay. (b) IL-33 promotes fibroblast proliferation. (c, d) Quantitative analysis of fibronectin and collagen III expression. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group in proliferation experiment and $n = 3$ in each group in western blot). $*P < 0.05$.

Figure 10. Effect of ST2 receptor knock down on airways fibrosis in an OVA challenged asthma model on 24 days. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Supplementary Information

Title: The effects of IL-33 on airways collagen deposition and matrix metalloproteinase expression in a murine surrogate of asthma

Running Title: IL-33 and airways fibrosis in asthma

Gao An,¹ Xin Zhang,¹ Wenjun Wang², Qiong Huang,¹ Yan Li,¹ Chris J Corrigan,³ Wei Wang¹ and Sun Ying¹

¹Department of Immunology, School of Basic Medical Sciences, Capital Medical University, Beijing, China, ²Department of Respiratory and Critical Care Medicine, Beijing Chao-Yang Hospital, Capital Medical University & Beijing Institute of Respiratory Medicine, Beijing, China, and ³Faculty of Life Sciences & Medicine, School of Immunology & Microbial Sciences, Asthma UK Centre in Allergic Mechanisms of Asthma King's College London.

Word Count (Introduction through Discussion): 5927

Figures: 15

Tables: 1

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Senior author: Sun Ying

Figure S1. IL-33 induced collagen III deposition in the lungs of challenged animals. (a) Representative photomicrographs of collagen III immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of collagen III immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure S2. IL-33 induced collagen V deposition in the lungs of challenged animals. (a) Representative photomicrographs of collagen V immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of collagen V immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

Figure S3. IL-33 promotes MMP12 expression in the lungs of challenged animals. (a) Representative photomicrographs of MMP12 immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of MMP12 immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

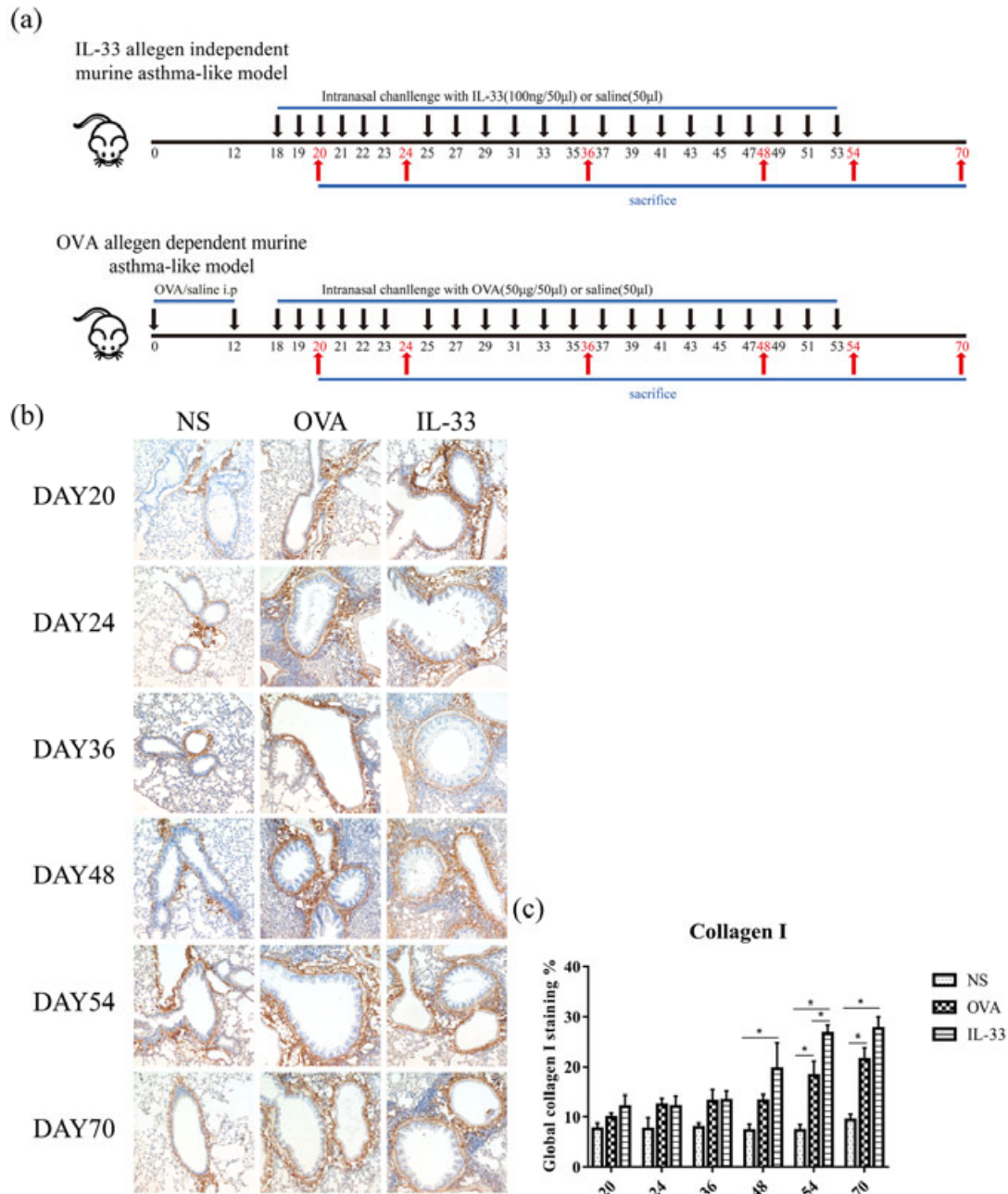
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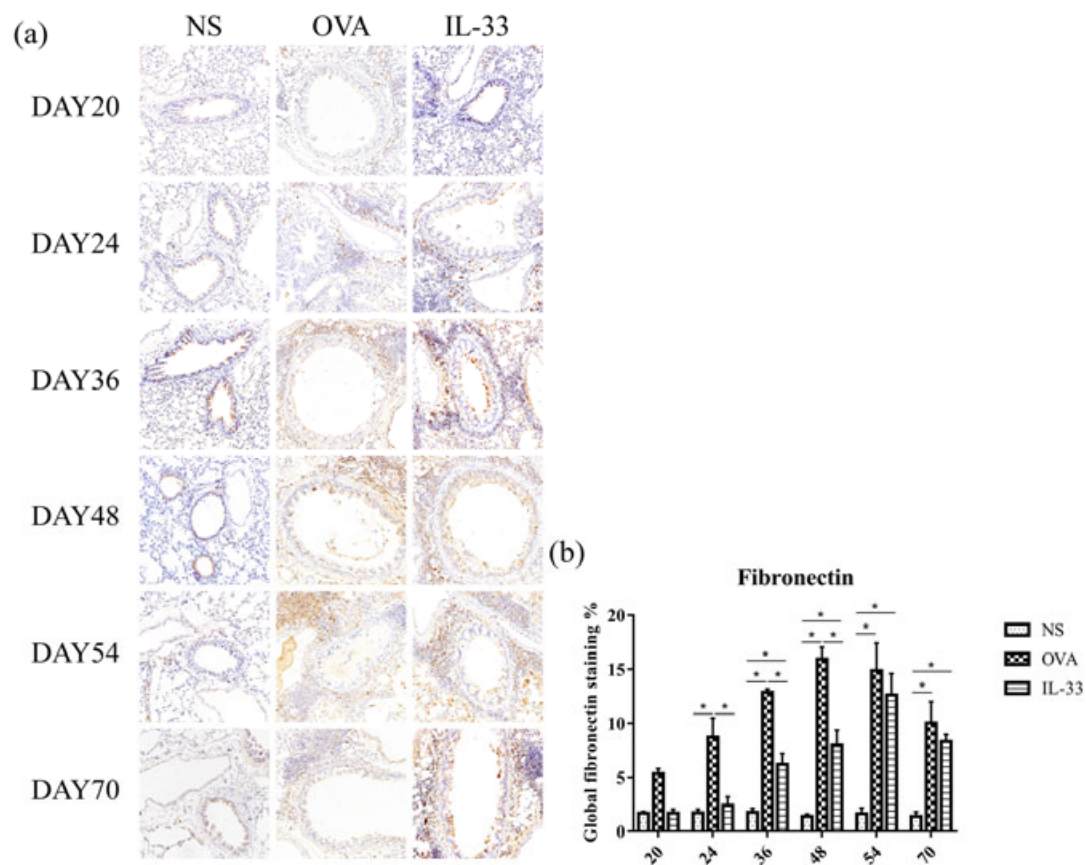
Figure S4. IL-33 promotes MMP13 expression in the lungs of challenged animals. (a) Representative photomicrographs of MMP13 immunoreactivity in lung sections from saline (NS)-, ovalbumin (OVA)- and IL-33-challenged mice at various time points as indicated (original magnification $\times 20$). (b) Quantitative analysis of MMP13 immunoreactivity. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

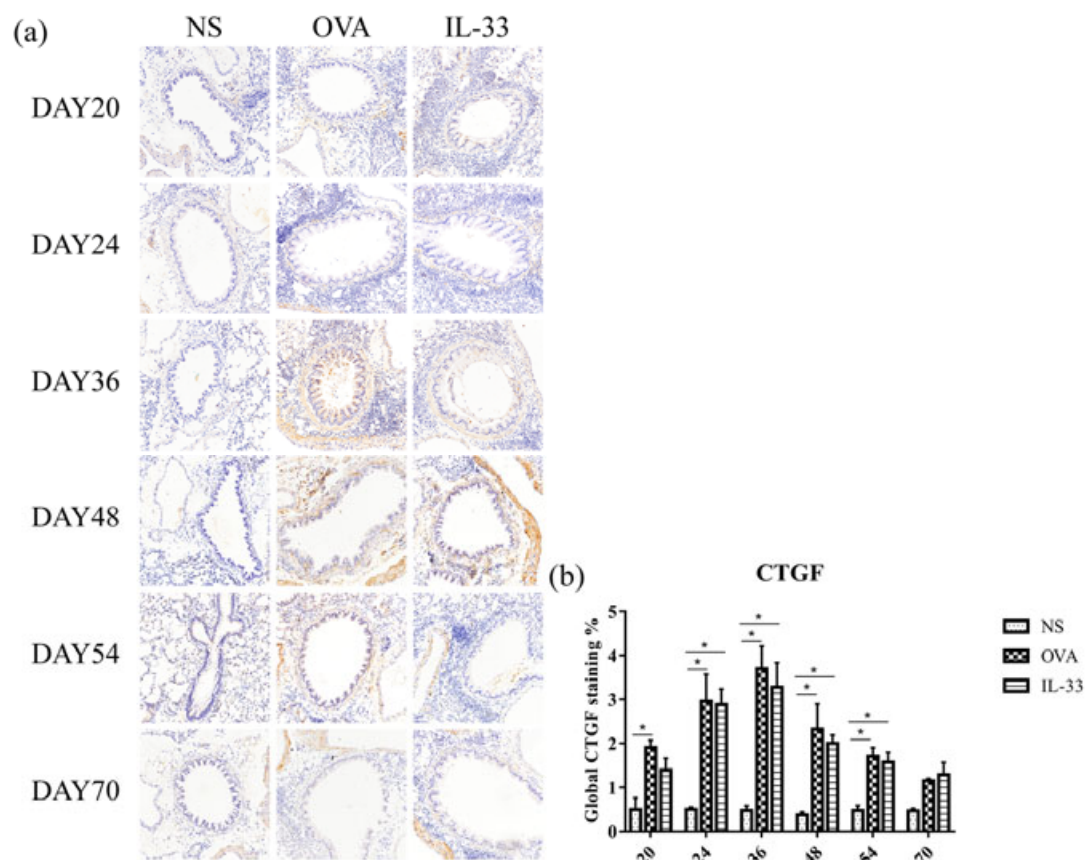
Figure S5. Effect of ST2 receptor knock down on airways fibrosis in an OVA challenged asthma model on 48 days. The data are expressed as the mean $\pm$ SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

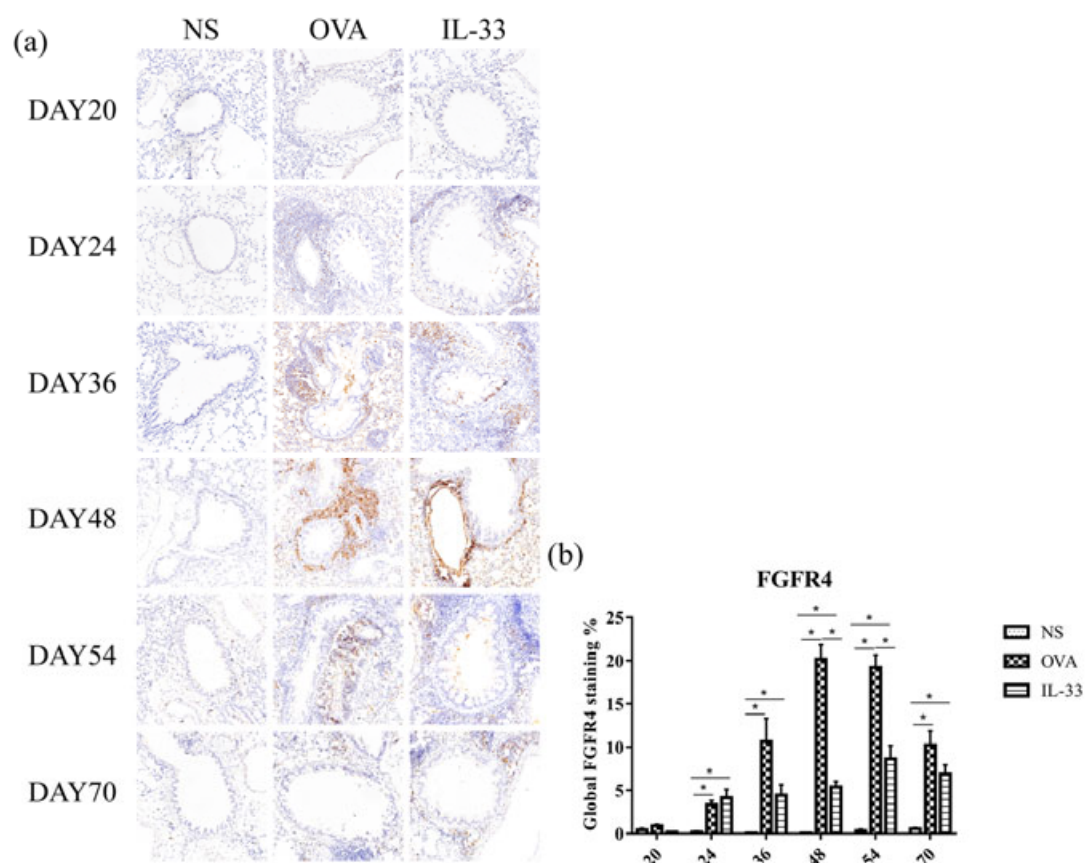
Table 1 Summary of primary antibodies used for immunohistochemistry (Rb rabbit, M mouse).

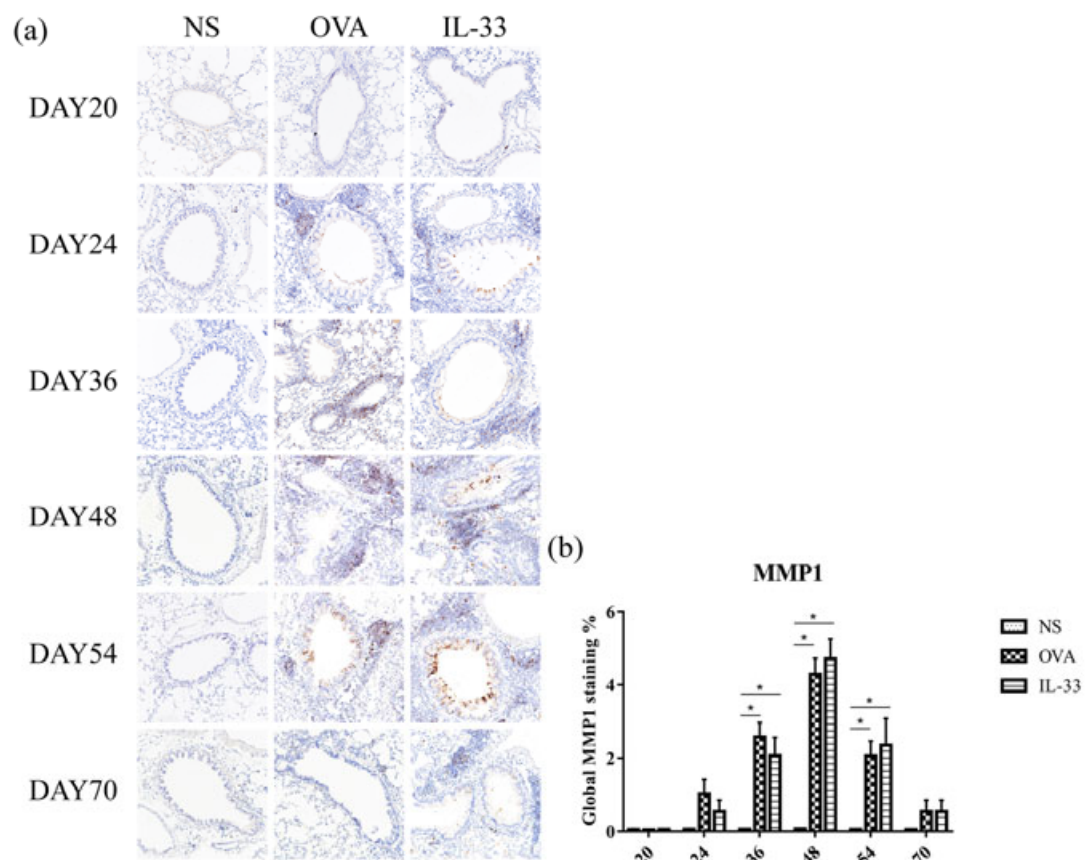
Antibody	Dilution ratio	Host	Company	Cat. #
Anti-Collagen I antibody	1:300	Rb	Abcam	ab34710
Anti-Collagen III antibody	1:300	Rb	Abcam	ab7778
Anti-Collagen V antibody	1:300	Rb	Abcam	ab7046
Anti-MMP1 antibody	1:100	Rb	Novus Biologicals	Nbp1-72209
Anti-MMP2 antibody	1:300	Rb	Abcam	Ab37150
Anti-MMP9 antibody	1:300	Rb	Abcam	Ab38898
Anti-MMP12 antibody	1:100	Rb	Abcam	Ab66157
Anti-MMP13 antibody	1:200	Rb	Abcam	Ab39012
Anti-CTGF antibody	1:300	Rb	Abcam	Ab6992
Anti-TIMP1 antibody	1:100	Rb	Biorbyt	Orb11483
Anti-Fibronectin antibody	1:300	M	Abcam	Ab6328
Anti-FGFR4 antibody	1:300	M	Abcam	Ab44971

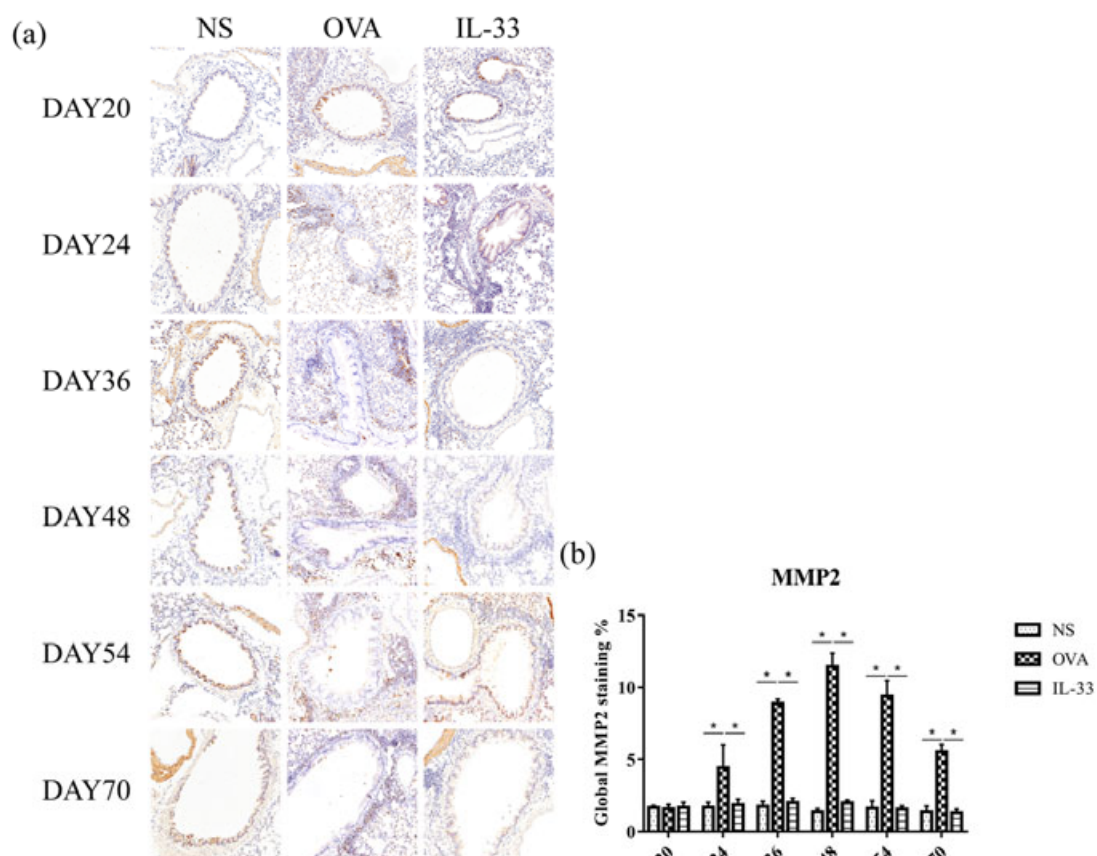


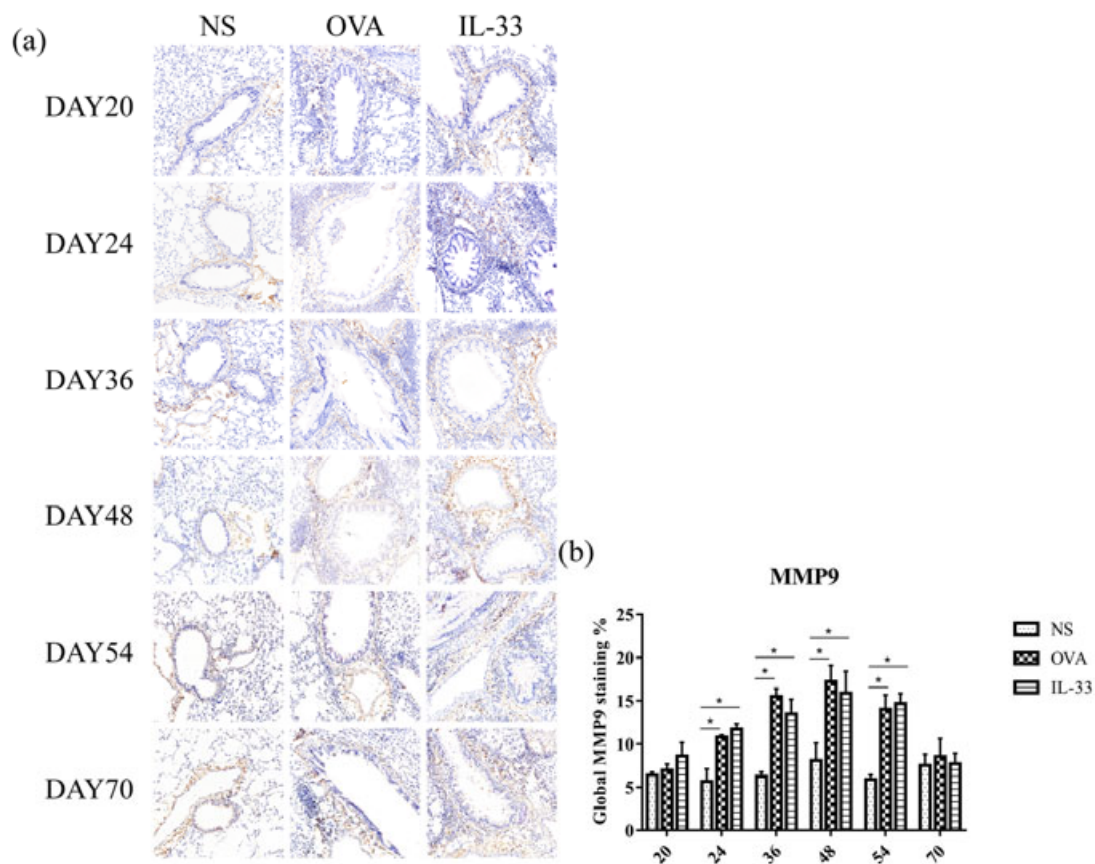


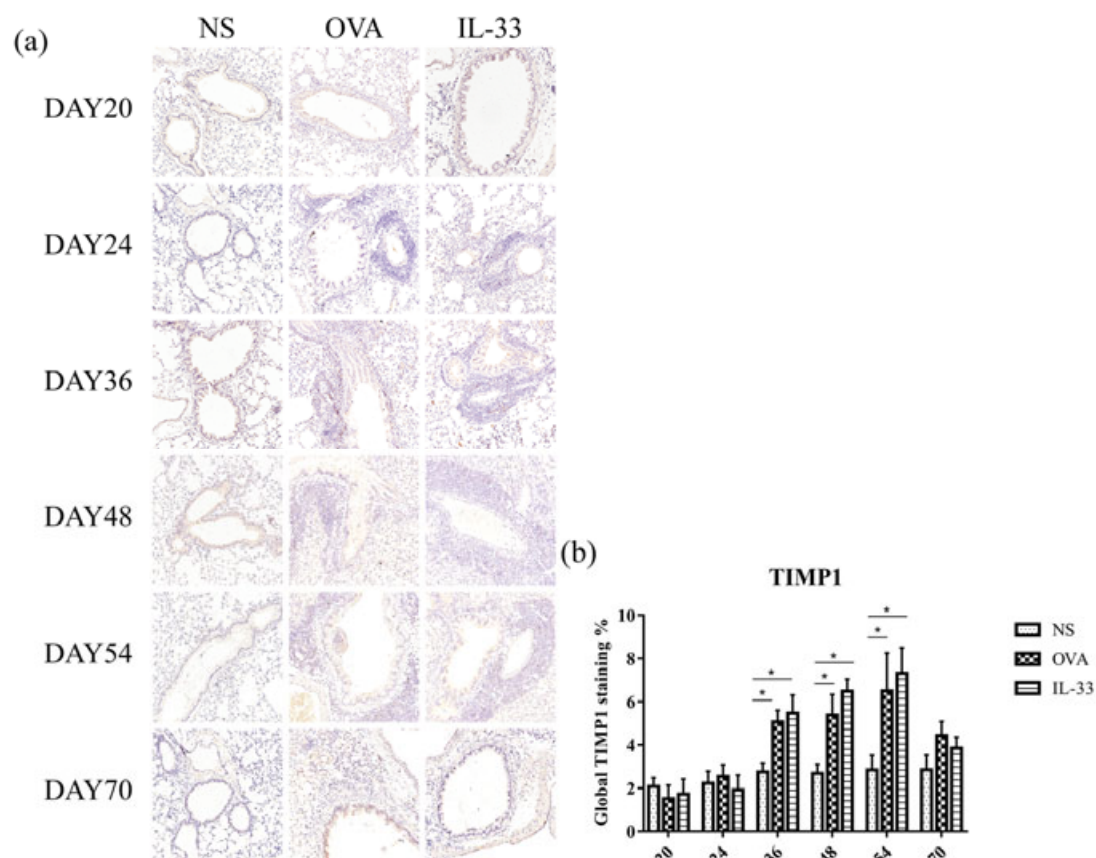


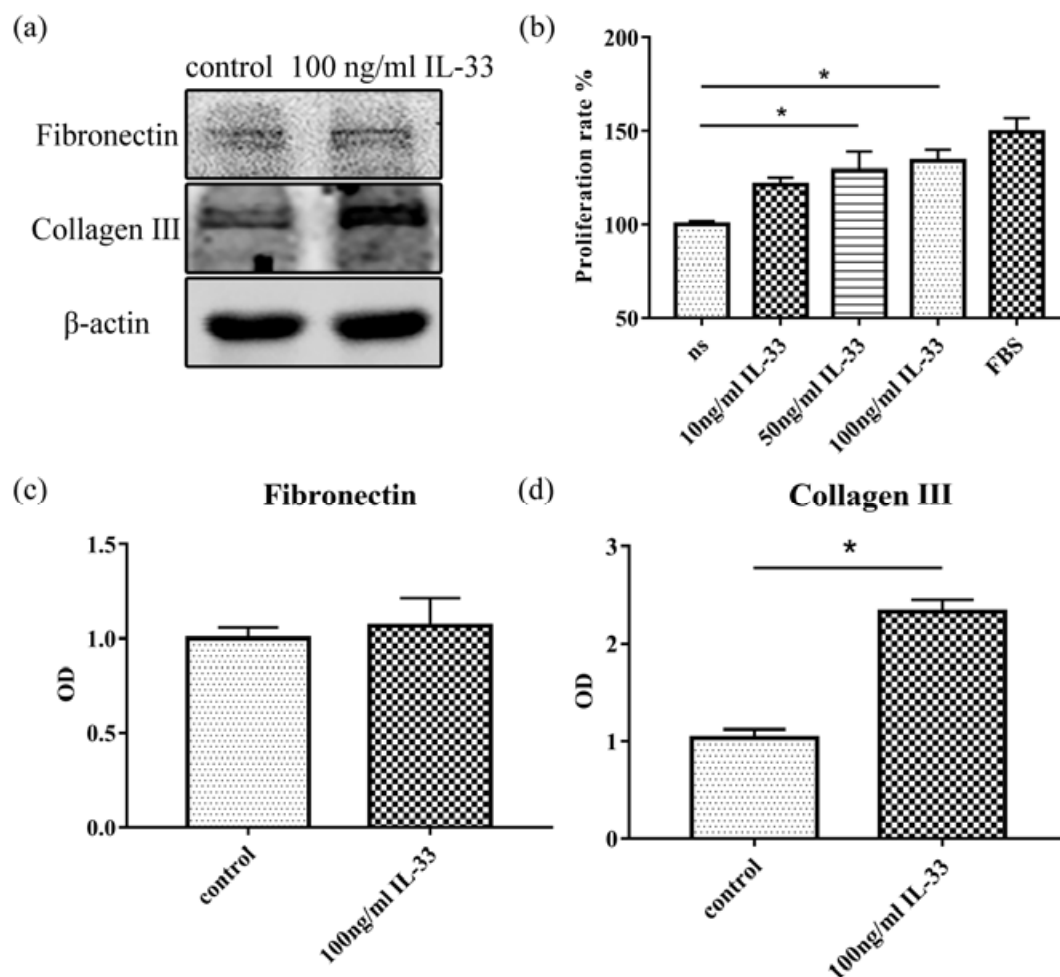


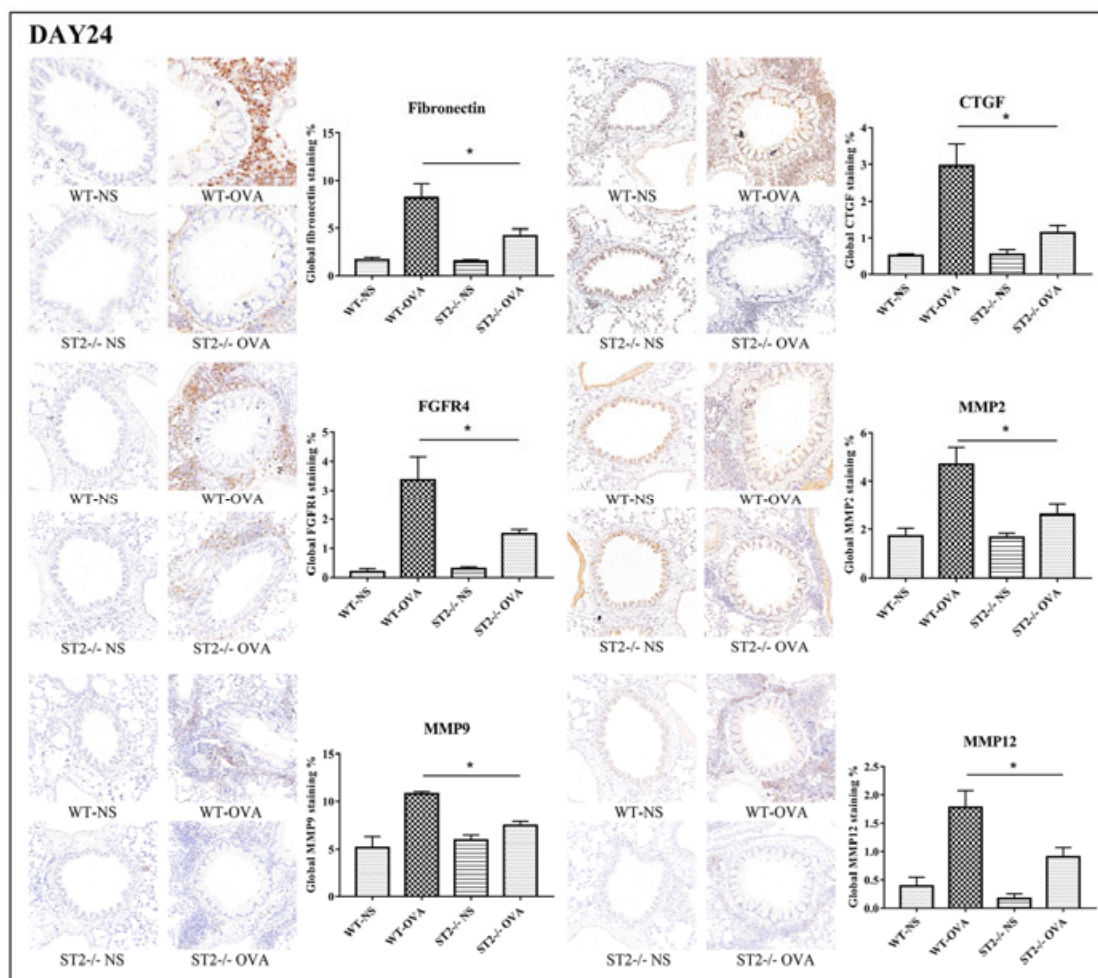












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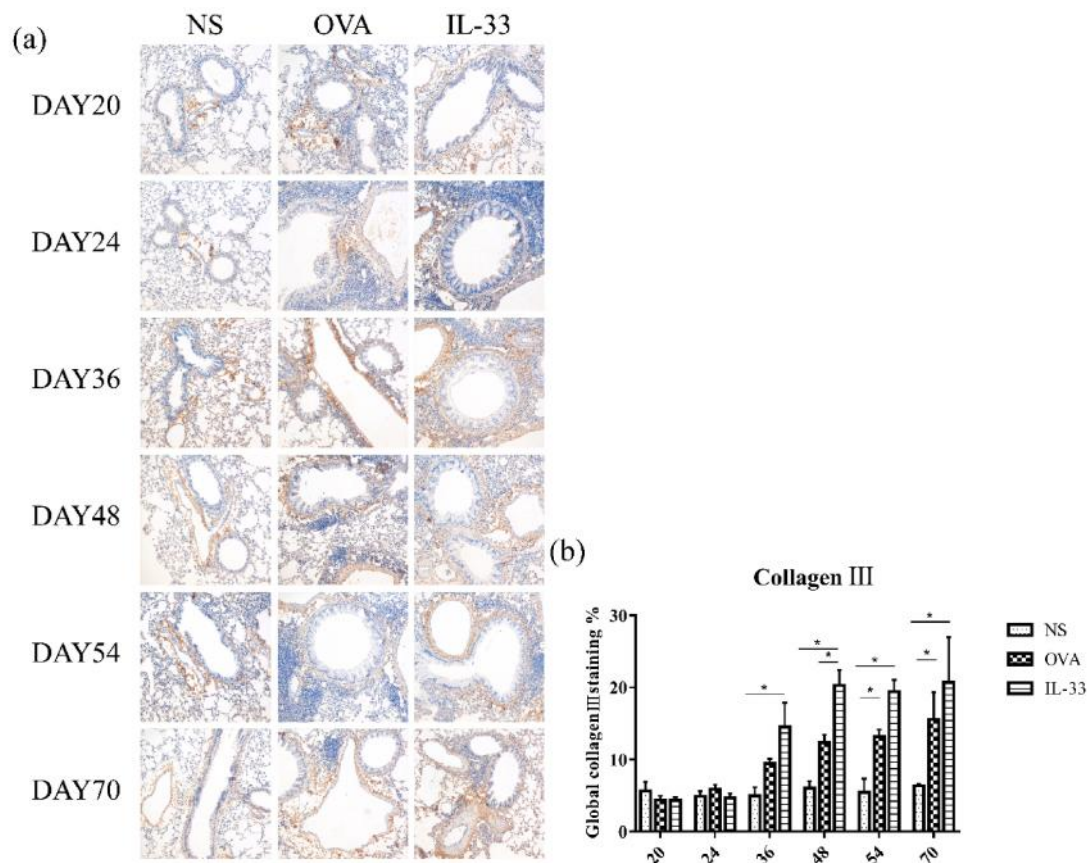


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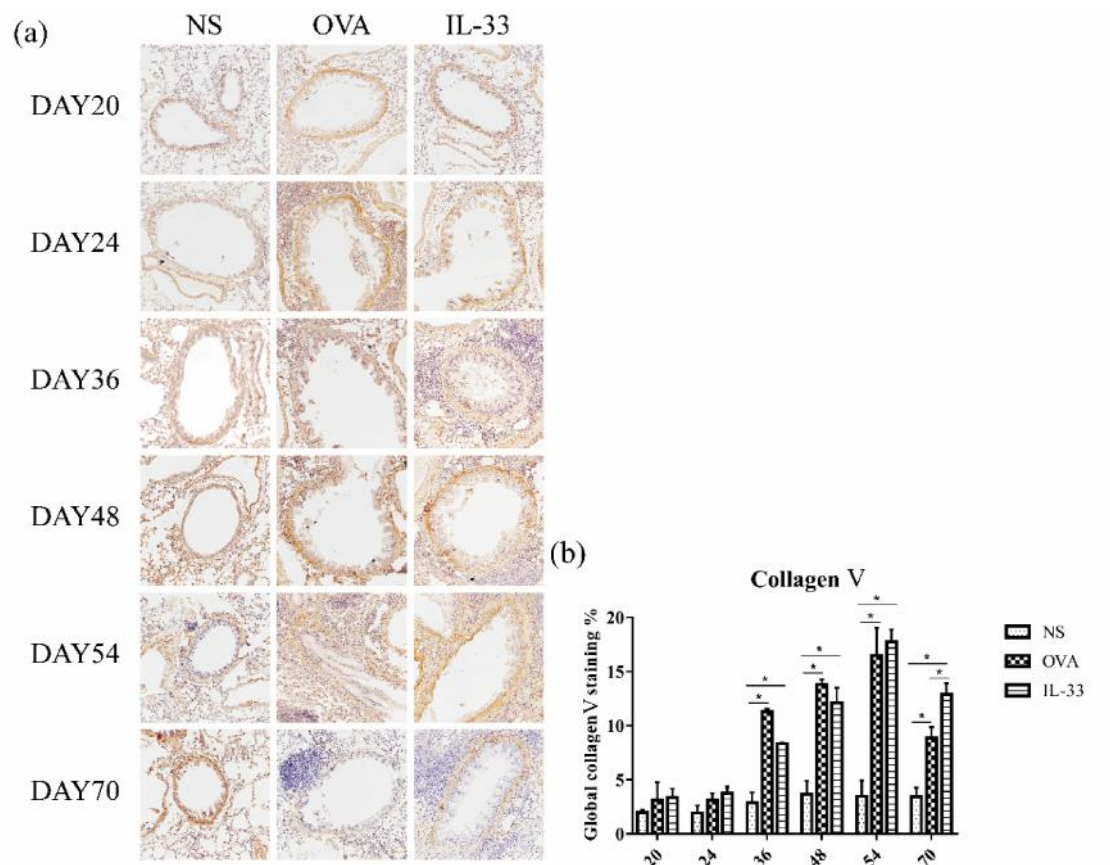


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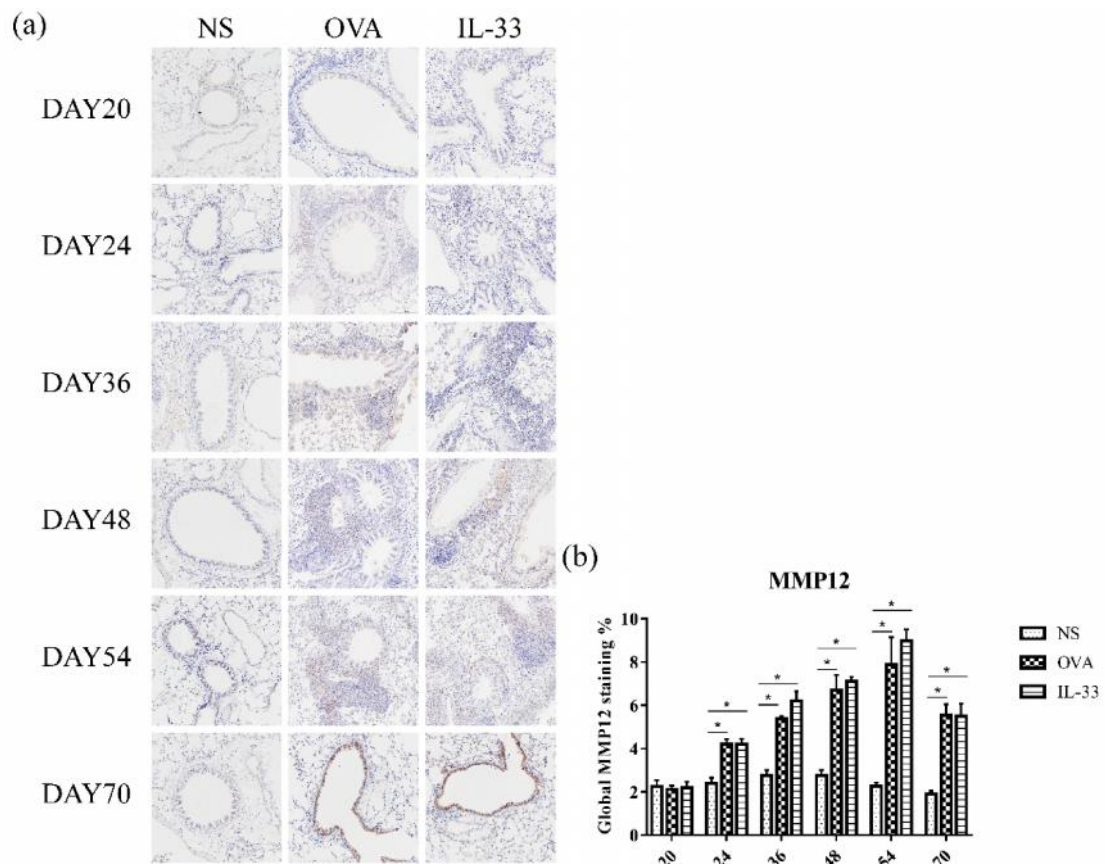


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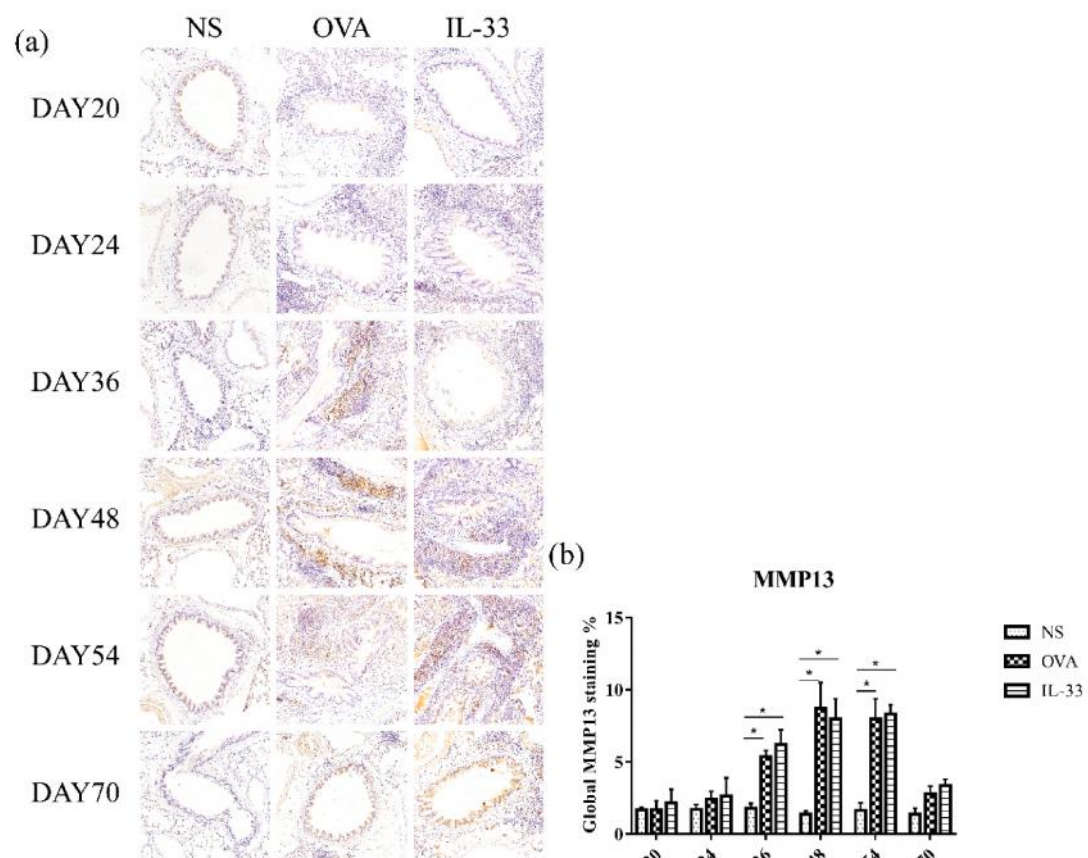


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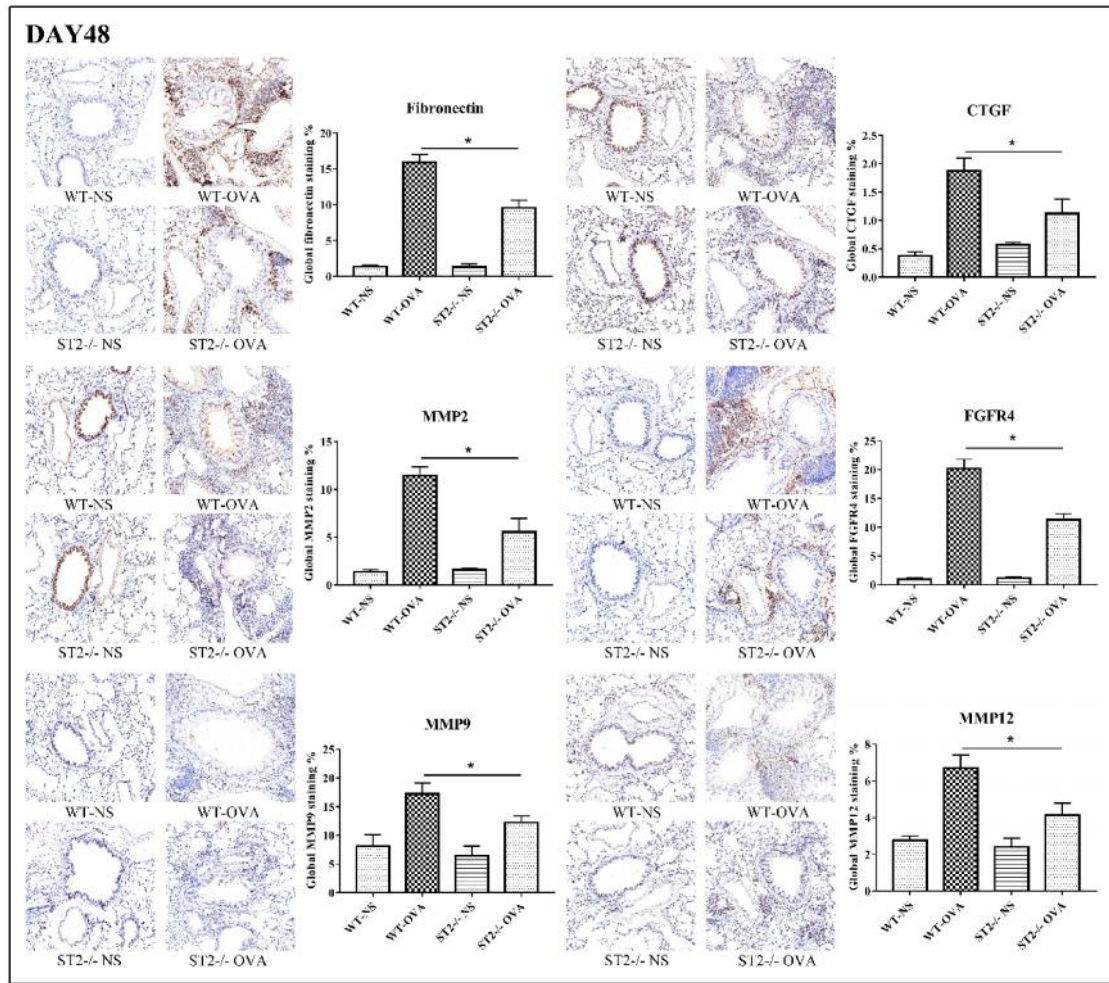


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