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ORIGINAL ARTICLE

Combined blockade of IL-25, IL-33 and TSLP mediates amplified inhibition of airway inflammation and remodelling in a murine model of asthma

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ABSTRACT

Background and objective: **Isolated blockade of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) has been shown to reduce airways inflammation and hyperresponsiveness in murine asthma model. The hypothesis that combined blockade of all three cytokines can accomplish this more effectively has never been addressed.**

Methods: **We studied a murine asthma model employing sensitization and challenge with ovalbumin (OVA) or saline control. To discern the effects of IL-33 blockade, we compared outcomes in strain identical, wild-type and IL-33 receptor (*St2*$^{-/-}$) gene-deleted mice. We then examined, in the *St2*$^{-/-}$ animals, the effects of additional, single or combined blockade of IL-25 and TSLP with blocking antibodies. Outcomes included airways reactivity, inflammatory cellular infiltration, epithelial cell metaplasia, deposition of fibrosis-related proteins, local Th2-type cytokine expression and total and specific serum IgE concentrations measured by ELISA and quantitative immunohistochemistry.**

Results: ***St2*$^{-/-}$ gene deletion significantly reduced airways reactivity, inflammatory cellular infiltration, lung tissue expression of Th2 cytokines and fibrosis related proteins and serum total IgE in response to OVA sensitization and challenge. Additional administration of anti-IL-25 and anti-TSLP blocking antibodies to the *St2*$^{-/-}$ mice further significantly reduced inflammation, Th2 cytokine expression, airways fibrosis and IgE production, while anti-TSLP alone reduced eosinophil infiltration and local IL-4 expression. The airways inflammatory cellular infiltrate and lung tissue expression of Th2 cytokine, but not fibrosis-related proteins were also reduced in the presence of isotype identical, control antibodies.**

Conclusion: **Combined blockade of these three cytokines may better ameliorate airways pathological changes in this murine asthma model, with implications for human asthma.**

SUMMARY AT A GLANCE

Combined blockade of IL-25, IL-33 and TSLP may augment disease control and further reduce airways inflammation and remodelling in chronic asthma.

Key words: asthma, interleukin-25, interleukin-33, remodelling, thymic stromal lymphopoietin.

INTRODUCTION

Although current standard therapies alleviate symptoms in most asthma patients,[1] no known therapeutic strategy effectively retards associated airways remodelling changes. Consequently, delineation of such strategies remains an urgent priority.

The alarmin cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) play possible roles in the pathogenesis of asthma. We and others have previously shown that IL-25 and IL-33 applied directly to the airways are sufficient to induce asthma-like features, including eosinophil infiltration, Th2-type responses, epithelial cell metaplasia, remodelling and neovascularization in mice,[2–10] while blockade of IL-25 and IL-33 or their receptors significantly abrogates these changes.[7,11]

Further, asthmatic patients show elevated expression of TSLP in bronchoalveolar lavage fluid (BALF).[12] TSLP transgenic mice develop spontaneous airways inflammation, whereas knockdown of the TSLP receptor greatly attenuates airways inflammation in a murine asthma model.[13] Therapy with Tezepelumab, a TSLP blocking antibody can significantly reduce symptomatology in patients with uncontrolled asthma.[14]

Together, these studies are consistent with the hypothesis that the alarmin cytokines play very significant complementary, partly overlapping and partly

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distinct roles in the pathological changes associated with human asthma. Although previous studies have addressed the effects of blockade of single alarmins in patients with asthma and animal models,[7,11,13] we are not aware of any studies addressing the effects of combined blockade. In the present study we address the hypothesis that, in a murine asthma model involving ovalbumin (OVA) sensitization and challenge, deletion of the *St2* gene encoding the IL-33 receptor, which completely abrogates IL-33/ST2 signalling, reduces airways inflammation, hyperresponsiveness and remodelling, while additional blockade of IL-25 and/or TSLP signalling with blocking antibodies exerts additional, partly overlapping and partly unique effects on these outcomes.

METHODS

Experimental animals

Female wild-type BALB/c (Vital River Laboratory, Beijing, China) and $St2^{-/-}$ (BALB/c background) mice (kindly provided by Dr. Andrew McKenzie, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) were kept in a pathogen-free environment in the Department of Laboratory Animal Sciences, Capital Medical University, Beijing, China. All experiments were approved by the Institutional Animal Care and Use Committee of the Capital Medical University.

OVA challenge and antibody blockade

A murine model of asthma based on OVA sensitization and challenge was employed as previously described.[5,6] $St2^{-/-}$ animals treated additionally with commercial, specific blocking antibodies or isotype-matched IgG control antibodies were injected 2 h prior to the initial OVA sensitization or challenge on day 0 and day 18 (Appendix S1, Fig. S1A and Table S1 in Supplementary Information).

Measurement of airways resistance

The FlexiVent, Scireq, Inc., Montreal, Canada apparatus was used to measure pulmonary mechanical parameters following inhalation of physiological saline and serially increasing concentrations of methacholine solution as described previously[8,15] (Appendix S1 in Supplementary Information).

Total cellular and differential counts in BALF

BALF was collected for total cellular and differential counts as described previously[8,15] (Appendix S1 in Supplementary Information).

Lung histology

Paraffin-embedded lung sections (4 μm thickness) were cut and stained with HE, periodic acid–Schiff (PAS), Masson's trichrome and Congo red respectively for evaluation of airways inflammation, mucus production, collagen deposition and eosinophil infiltration as in our previously published protocols [5,6,8–10,15] (Appendix S1 in Supplementary Information).

Lung immunohistochemistry

Immunohistochemistry (IHC) was employed to quantify immunoreactivity for the most common biological markers of airways fibrosis in asthma, including matrix metalloproteinases (MMP) 2, 9 and 12, fibroblast growth factor receptor 4 (FGFR4), connective tissue growth factor (CTGF) and fibronectin[6,10] (see Appendix S1 in Supplementary Information). The sources and optimized concentrations of the individual primary antibodies used are summarized in Table S1 in Supplementary Information.

Determination of cytokine concentrations in lung homogenates

The right lungs from the experimental animals were weighed then suspended in 500 μL/100 mg PBS containing protease inhibitor and 1% Triton X 100, then homogenized. Homogenate concentrations of IL-4, IL-5, IL-13 and eotaxin were measured using commercial ELISA kits (eBioscience, San Diego, CA) as previously described.[5,6,8–10,15]

Measurement of total serum IgE

Total serum IgE was measured using a commercial ELISA kit (eBioscience) according to the manufacturer's instructions.[5,6,8–10]

Statistical analysis

Data were processed using GraphPad Prism7.0 software (GraphPad, San Diego, CA). Lung function data were compared by two-way ANOVA (analysis of variance) to determine whether there were significant differences between the treatment groups, followed by Tukey's post hoc test to allow for multiple comparisons of the means. Differences between experimental groups in other experiments were analysed using one-way ANOVA followed by Tukey's post hoc test. For all tests, P values less than 0.05 were considered significant.

RESULTS

Effects of blockade of IL-25 and TSLP in OVA-challenged $St2^{-/-}$ mice

Airways hypersensitivity and infiltration of inflammatory cells

OVA sensitization and challenge of the wild-type animals clearly increased their mean total airways resistance in response to serially increasing concentrations of methacholine. This response was partially but significantly attenuated in the OVA-challenged $St2^{-/-}$ mice compared with the wild-type animals, but remained significantly elevated compared with that of the saline sensitized and challenged control group (Fig. 1A). Concomitant administration of the anti-IL-25 and anti-TSLP blocking antibodies, alone or in combination, to the $St2^{-/-}$ mice did not further significantly modify this effect.

The mean numbers of total cells, eosinophils, macrophages, lymphocytes and neutrophils were significantly attenuated in the BAL fluid of the $St2^{-/-}$ mice compared with the wild-type controls following OVA challenge, although again remained significantly elevated compared with the saline-challenged controls (Fig. 1B,C and Fig. S1B in Supplementary Information).

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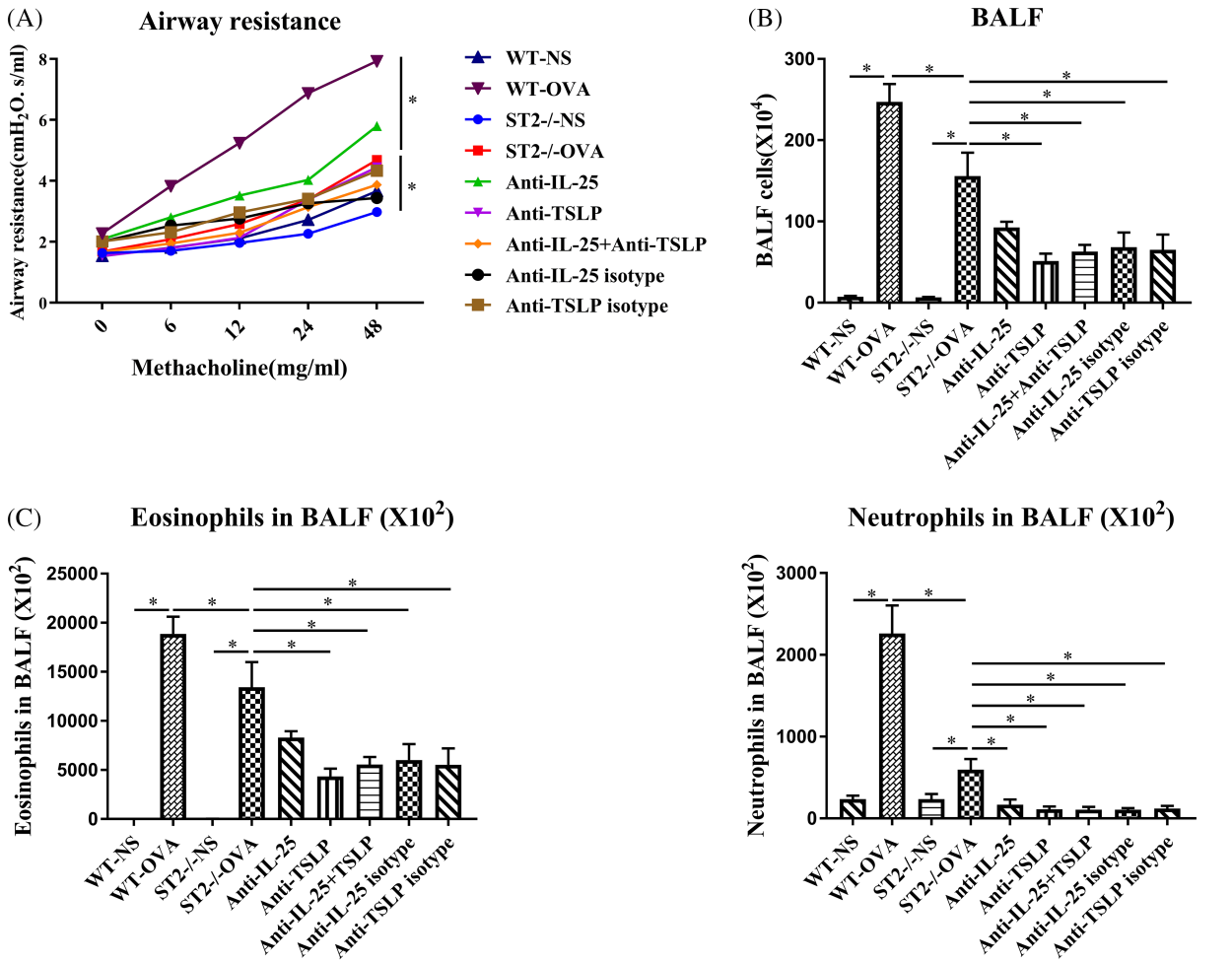


Figure 1 Inhibitory effects of *St2* gene ablation (comparing WT and $St2^{-/-}$ animals) in ovalbumin (OVA) and normal saline (NS) sensitized and challenged mice and additional inhibitory effects of anti-IL-25, anti-TSLP or both in the $St2^{-/-}$ animals on airway resistance and BALF inflammatory cellular infiltration. (A) Airway resistance to methacholine on day 24. (B) Total cells and (C) eosinophils and neutrophils in BALF. The data are expressed as the mean ± SEM ($n = 5$ in each group). $^*P < 0.05$.

Concomitant blockade of IL-25 in the $St2^{-/-}$ mice with a neutralizing antibody further significantly attenuated the mean numbers of lymphocytes and neutrophils, but not total cells, eosinophils and macrophages. In contrast, concomitant blockade of TSLP in the $St2^{-/-}$ mice further significantly attenuated the mean numbers of total cells, eosinophils, lymphocytes and neutrophils, but not macrophages (Fig. 1B,C and Fig. S1B in Supplementary Information). Combined blockade of IL-25 and TSLP in the $St2^{-/-}$ mice showed a net effect similar to that of either antibody used alone (Fig. 1B,C and Fig. S1B in Supplementary Information). Interestingly, the isotype-matched control antibodies were as effective as the specific anti-IL-25 and anti-TSLP antibodies in reducing the mean numbers of total inflammatory cells and their subsets in the BALF of the OVA-challenged $St2^{-/-}$ mice.

Airways inflammation and mucus production

HE staining revealed significantly elevated peribronchial inflammatory cellular infiltration in the lung parenchyma of the wild-type mice challenged with OVA compared with saline control (Fig. 2). Again, the extent of this phenomenon was significantly reduced in the $St2^{-/-}$ mice. While additional, individual blockade of IL-25 or TSLP with neutralizing antibodies in the $St2^{-/-}$ mice somewhat further attenuated this infiltration, it was significantly reduced only with combined blockade. PAS staining showed significantly elevated mucus production in the airways of the OVA-challenged wild-type mice compared with saline control, which was not further attenuated in the OVA-challenged $St2^{-/-}$ mice or by additional, single or combined blockade of IL-25 and TSLP (Fig. S2A in Supplementary Information).

Airways collagen laydown and eosinophilic infiltration

Masson's trichrome blue staining revealed no significant difference in the extent of collagen deposition in and around the airways of the OVA-challenged wild-type and $St2^{-/-}$ mice, as compared with saline controls (Fig. S2B in Supplementary Information), neither was there any additional effect of the IL-25 and TSLP blocking antibodies, either alone or in combination.

Congo red staining revealed that peribronchial infiltration of eosinophils into the lung parenchyma of the

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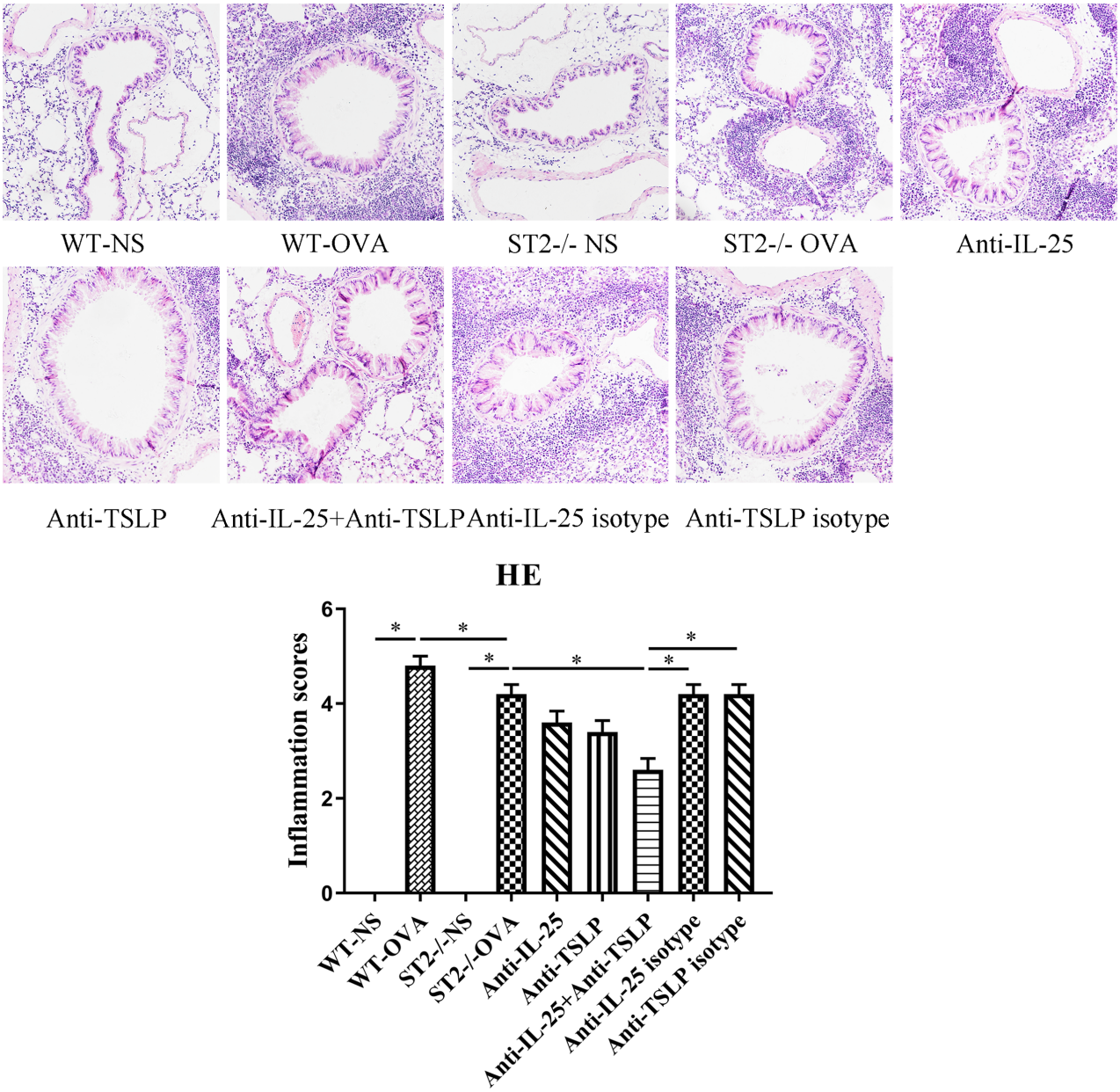


Figure 2 Inhibitory effects of *St2* gene ablation (comparing WT and $St2^{-/-}$ animals) in ovalbumin (OVA) and normal saline (NS) sensitized and challenged mice and additional inhibitory effects of anti-IL-25, anti-TSLP or both in the $St2^{-/-}$ animals on peribronchial recruitment of inflammatory cells. Representative photomicrographs and quantitative analysis of the inflammation scores from H&E stained lung sections. Original magnification 20×. The data are expressed as the mean ± SEM ($n = 5$ in each group at each time point).*$P < 0.05$.

$St2^{-/-}$ mice compared with wild-type mice challenged with OVA was significantly attenuated (Fig. 3). Additional blockade of TSLP, TSLP and IL-25 together but not IL-25 alone in the $St2^{-/-}$ mice further attenuated the mean percentage of Congo red positive eosinophils comprising the inflammatory cellular infiltrate, although this change was not statistically significant (Fig. 3).

Concentrations of lung tissue mediators and serum total and OVA-specific IgE

ELISA revealed that the mean lung homogenate concentrations of the Th2 cytokines IL-4, IL-5 and IL-13 and the chemokine eotaxin were significantly elevated in the wild-type mice challenged with OVA compared with saline controls, while the mean concentrations of IL-4, IL-5 and IL-13, but not eotaxin were significantly reduced in the OVA-challenged $St2^{-/-}$ mice. Blockade of TSLP, alone or in combination with IL-25 further reduced the mean concentration of IL-4 in the homogenates of the OVA-challenged $St2^{-/-}$ mice (Fig. 4), whereas only combined blockade of IL-25 and TSLP significantly further reduced the mean concentrations of IL-5 and IL-13 (Fig. 4). Neither of the blocking antibodies, alone or in combination significantly further altered the mean concentration of eotaxin (Fig. S1C in Supplementary Information).

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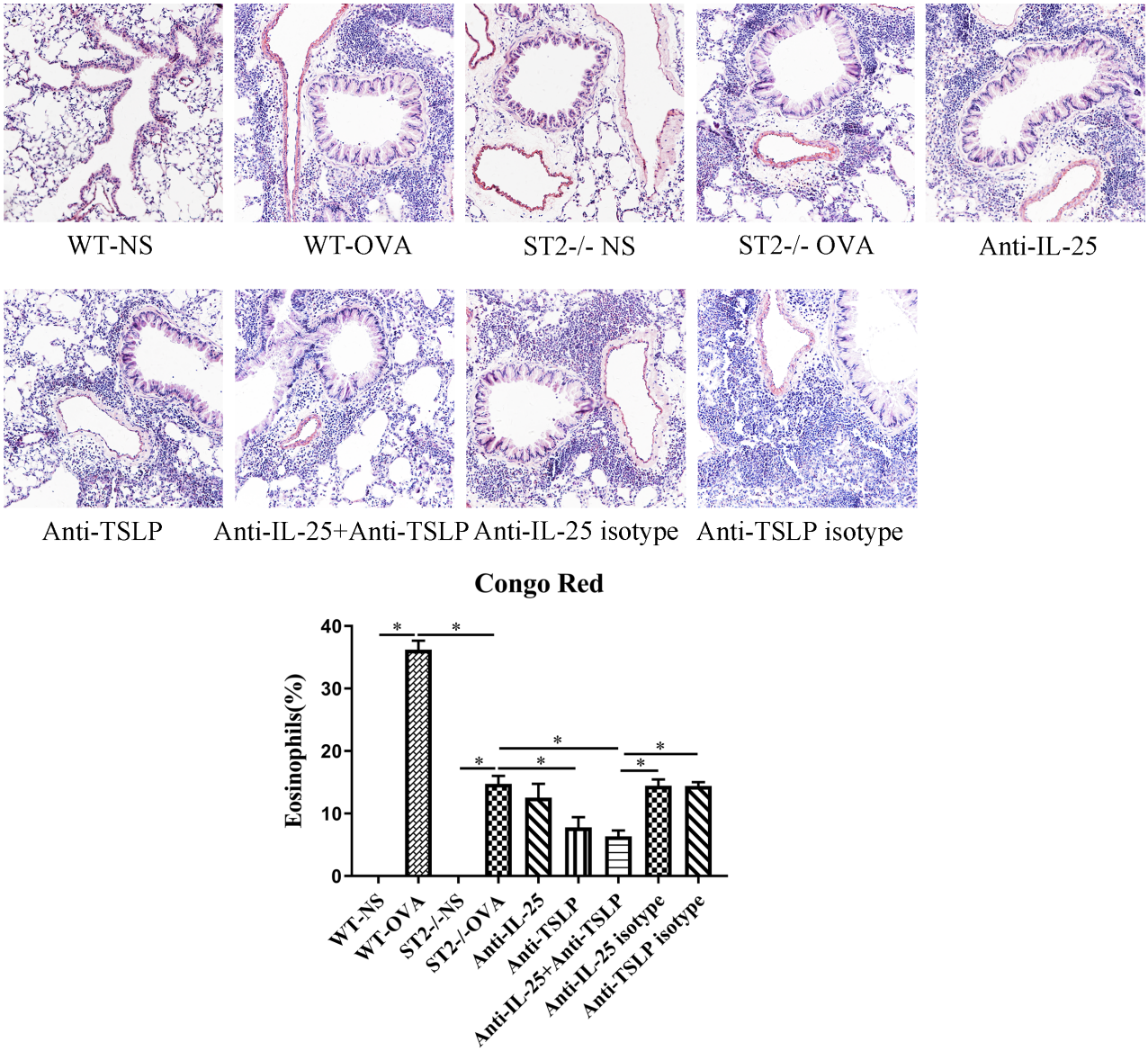


Figure 3 Inhibitory effects of *St2* gene ablation (comparing WT and $St2^{-/-}$ animals) in ovalbumin (OVA) and normal saline (NS) sensitized and challenged mice and additional inhibitory effects of anti-IL-25, anti-TSLP or both in the $St2^{-/-}$ animals on peribronchial accumulation of eosinophils. Representative photomicrographs and quantitative analysis of Congo red-stained lung sections from each group. Original magnification 20×. The data are expressed as the mean ± SEM ($n = 5$ in each group at each timepoint). $^*P < 0.05$.

Concomitantly, the mean total and OVA-specific serum concentrations of IgE were significantly elevated in the wild-type mice challenged with OVA compared with saline control. These changes were significantly, but not completely attenuated in the OVA-challenged $St2^{-/-}$ mice, in which blockade of both TSLP and IL-25, but neither in isolation significantly further attenuated total, but not OVA-specific IgE (Fig. 4 and Fig. S1C in Supplementary Information).

Airways expression of fibrosis-associated features

IHC revealed that the mean global expression of immunoreactivity for the airways remodelling associated proteins FGFR4, CTGF, fibronectin and MMP 2, 9 and 12 was significantly elevated in the wild-type mice sensitized and challenged with OVA as compared with saline control. Mean global expression of all of these proteins was partially but significantly attenuated in the OVA challenged $St2^{-/-}$ mice and was in turn, with the exception of MMP9, further significantly reduced by combined blockade of TSLP and IL-25, but neither mediator in isolation in the $St2^{-/-}$ mice (Figs 5,6 and Figs S3,S4 in Supplementary Information).

DISCUSSION

This is the first ever approach, so far as we are aware, to delineate the individual contributions of the alarmin cytokines IL-33, IL-25 and TSLP to pathological

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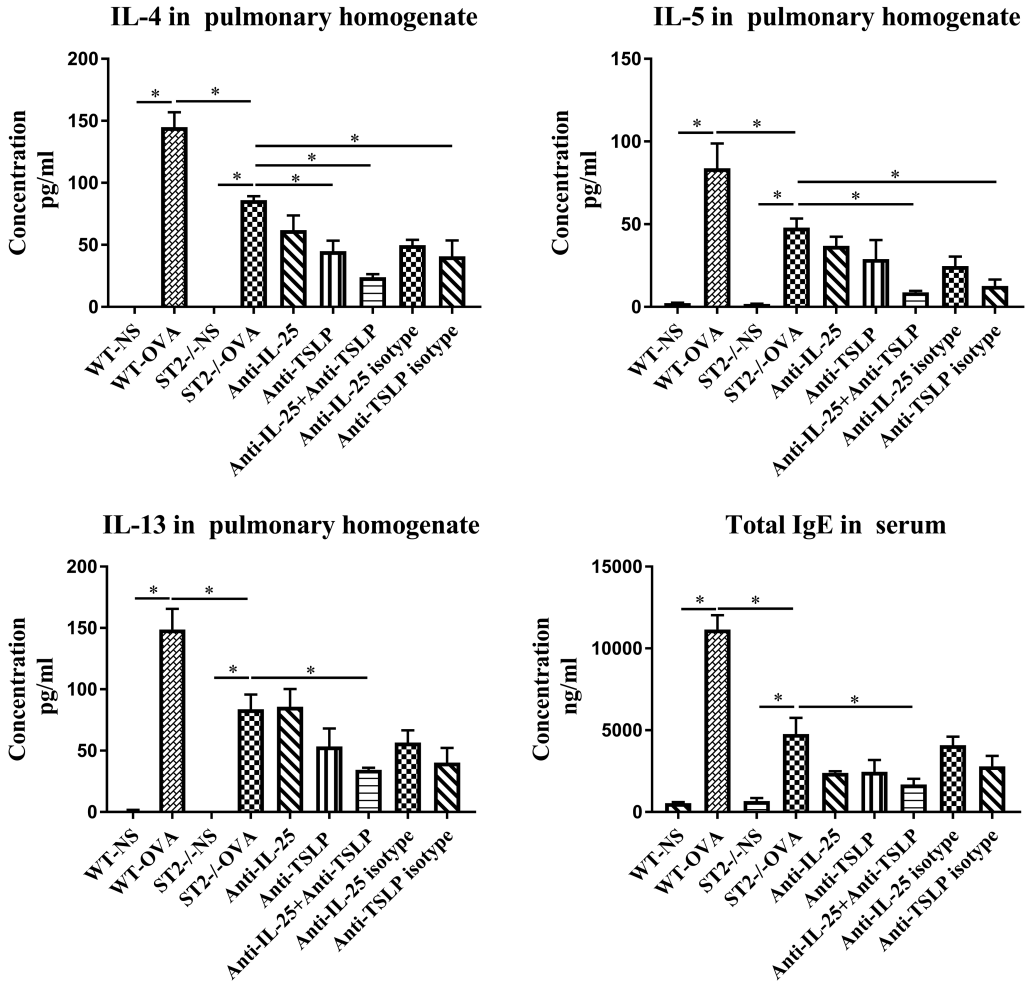


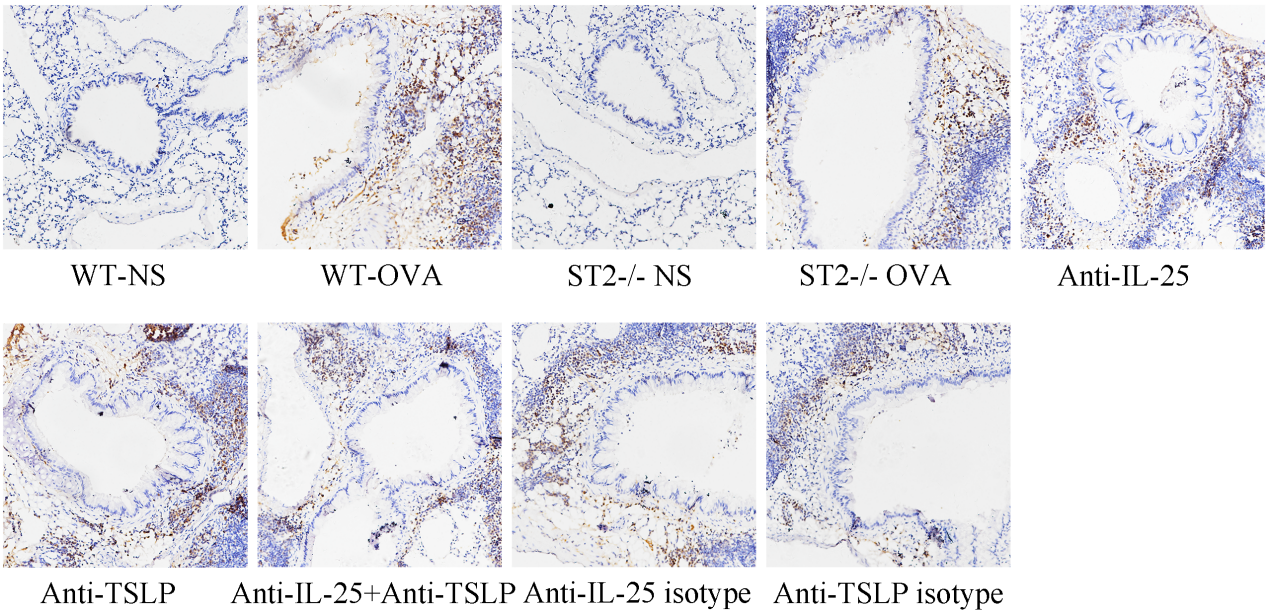
Figure 4 Inhibitory effects of *St2* gene ablation (comparing WT and $St2^{-/-}$ animals) in ovalbumin (OVA) and normal saline (NS) sensitized and challenged mice and additional inhibitory effects of anti-IL-25, anti-TSLP or both in the $St2^{-/-}$ animals on Th2-type cytokine concentrations in lung homogenates and total serum IgE. The data are expressed as the mean ± SEM ($n = 5$ in each group at each time point). $*P < 0.05$.

changes in a well-established animal model of human asthma. We first examined the effects of $St2^{-/-}$ gene deletion, which completely abrogates IL-33/ST2 signalling, comparing with wild-type controls, then further investigated the effects of anti-IL-25 and/or anti-TSLP in the $St2^{-/-}$ animals. Our present study affirms and extends findings of several previous studies addressing isolated deletion of IL-33/ST2 signalling[7,16–18] showing that this significantly abrogates, but does not abolish airways hyperreactivity, inflammatory cellular infiltration, local Th2 cytokine expression, global IgE production and airways expression of factors other than collagen implicated in fibrotic airways remodelling changes implicated in the pathogenesis of irreversible airways obstruction in human asthma.

Our data are further consistent with possibility that, on a background of deletion of IL-33/ST2 signalling, blockade of the remaining epithelial alarmins IL-25 and TSLP, particularly concomitant blockade, has the potential further to inhibit the infiltration of inflammatory cells into the airways and lung parenchyma, as well as local expression of the Th2 cytokines IL-4, IL-5 and IL-13. We furthermore observed variable, additional inhibition of a range of factors implicated in airways remodelling. These data are consistent with the hypothesis that, for the treatment of chronic, severe human asthma, and particularly as prophylaxis against long term airways remodelling, combined blockade of these alarmins is more effective than isolated blockade of any.

Blockade of IL-33 signalling in the $St2^{-/-}$ mice was associated with a clear attenuation of the marked infiltration of eosinophils, lymphocytes and neutrophils into the airways lumen in association with OVA sensitization and challenge. The effect on macrophage numbers was less profound, likely reflecting the fact that these are majority 'resident' airways luminal cells. The anti-TSLP administrated to the OVA-challenged $St2^{-/-}$ mice significantly further attenuated the influx of eosinophils, lymphocytes and neutrophils, whereas the anti-IL-25 antibody further attenuated lymphocytes and neutrophils, suggesting that in the absence of IL-33 signalling, TSLP and IL-25 have additional roles to play in the recruitment of inflammatory cells to the airways. The relative contributions of individual alarmins may,

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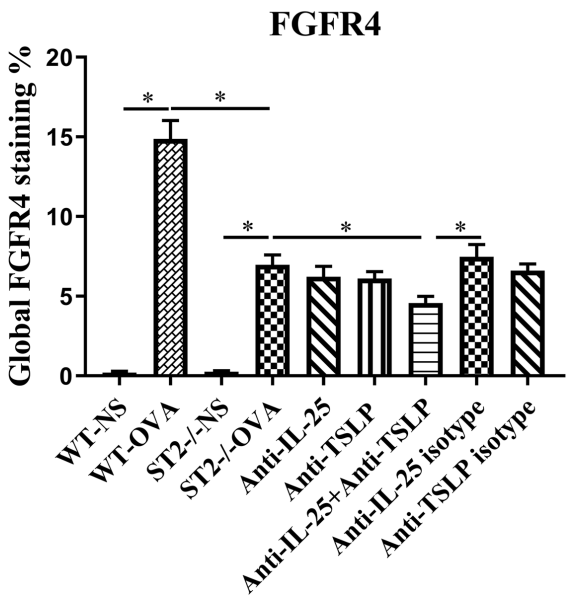


Figure 5 Inhibitory effects of *St2* gene ablation (comparing WT and $St2^{-/-}$ animals) in ovalbumin (OVA) and normal saline (NS) sensitized and challenged mice and additional inhibitory effects of anti-IL-25, anti-TSLP or both in the $St2^{-/-}$ animals on FGFR4 expression in lung tissue sections. Representative photomicrographs and quantitative analysis of FGFR4 immunoreactivity. Original magnification 20×. The data are expressed as the mean ± SEM ($n = 5$ in each group at each time point). $^{*}P < 0.05$.

in addition, vary according to the site of the epithelial surface involved and the nature of the disease process.

In our hands, combined administration of IL-25 and TSLP blocking antibodies to the $St2^{-/-}$ mice further reduced the expression of Th2 cytokines within the lung parenchyma, as well as total IgE production. This further suggests a possible role for all three alarmins in regulating the local production of these Th2 cytokines in the airways and, by extension, IgE synthesis since only IL-4 and IL-13 can switch B cells to IgE production.

In contrast, in our hands abrogation of the effects of all three alarmins did not influence local mucus production, epithelial metaplasia, collagen laydown and eotaxin expression in the airways, whereas only abrogation of IL-33 signalling affected airways hypersensitivity to methacholine, suggesting that this is the total extent of the contribution of the alarmins to this phenomenon, as least insofar as it reflects short-term changes in the airways in our model. Similarly, a recent study using a similar murine model suggested that deletion of the TSLP receptor does not abrogate airways epithelial metaplasia and mucus secretion.[19] Another recent study[20] using a similar model suggests that IL-25 blockade abrogates airways hyperresponsiveness employed a higher concentration of anti-IL-25 antibody than in the present study, and the possible contribution of altered mucus production to this effect was not specifically addressed. Overall these data suggest that the

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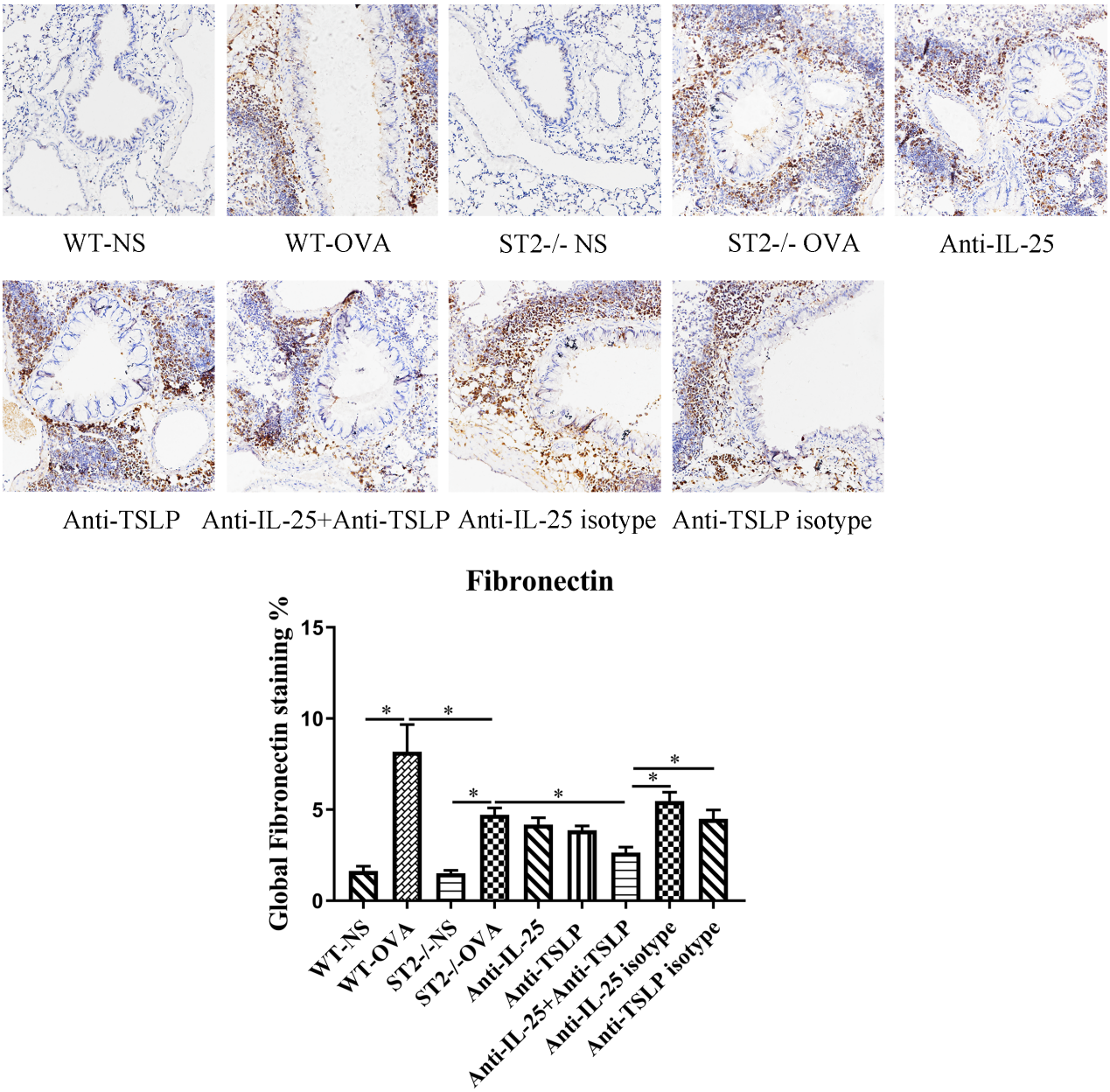


Figure 6 Inhibitory effects of *St2* gene ablation (comparing WT and $St2^{-/-}$ animals) in ovalbumin (OVA) and normal saline (NS) sensitized and challenged mice and additional inhibitory effects of anti-IL-25, anti-TSLP or both in the $St2^{-/-}$ animals on fibronectin expression in lung tissue sections. Representative photomicrographs and quantitative analysis of fibronectin immunoreactivity. Original magnification 20×. The data are expressed as the mean ± SEM ($n = 5$ in each group at each time point). $^{*}P < 0.05$.

alarmin cytokines do not contribute to mucus secretion, at least independently, although in another study it was suggested that combined blockade of IL-13 and IL-33 might be a more effective strategy to reduce production of mucus in the airways.[21]

Previous studies suggest significant roles for CTGF, FGFR4, fibronectin and MMP in the pathogenesis of asthma, most likely by participating in the mechanisms of deposition of extracellular matrix and local fibrosis.[8–11,18–22] Using a similar murine model we previously reported that deletion of the *St2* gene significantly inhibits the expression of these molecules.[10] Our present data suggest that additional combined blockade of IL-25 and TSLP has the potential further to reduce the expression of these airways remodelling-associated proteins.

Interpretation of our data is subject to some limitations engendered by the complexity of the protocol. One could argue that our study might ideally have been performed using a protocol ensuring complete blockade of the alarmins, individually and collectively at all times during the sensitization and challenge procedures. Such a protocol would however be extremely complex and difficult to achieve realize and validate in practice. It is, therefore, conceivable that we have not uncovered the full potential for individual and combined blockade of

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the alarmins to abrogate asthma pathology using the present experimental system. A second interpretational problem relates to our observed effects of the anti-TSLP and anti-IL-25 isotype control antibodies, the inhibitory effects of which were with some outcome measures (the observed effects on airways luminal inflammatory cellular infiltration and bronchial hyperresponsiveness, but not the observed effects on tissue and peribronchial inflammation, tissue eosinophil infiltration and expression of the markers of remodelling) statistically equivalent to those of the specific antibodies when applied to the OVA challenged *St2*$^{-/-}$ mice. These effects were not additive when the isotype controls were employed together (data not shown) suggesting a non-specific effect. We speculate that they at least partly involve interactions of these antibodies with the IgG Fc receptors FcγRIIB1 and FcγRIIB2, which are expressed by most leukocytes. Because they have ITIM sequences and are inhibitory Fc receptors, they do not induce phagocytosis.[23–25] If so, any study employing isotype-matched, irrelevant antibodies as controls in studies in which the active antibodies are employed to block the actions of protein targets in vivo is subject to these potential confounding effects.

It is now clear that complex interactions between cytokines, immune cells and structural cells play a role in the development and progression of asthma. It is equally clear that blockade of single, downstream cytokine targets, while reducing the severity of disease in subsets of patients is unlikely to be suitable for all. Our present data, as well as other studies suggesting that IL-25, IL-33, and TSLP have been reported jointly to promote Th2 polarization, neoangiogenesis and tissue remodelling in asthma[21,22] clearly reaffirm the conclusion that combined blockade may be indicated for the treatment of some patients with severe asthma. Of course, such ambitions must be tempered by the spectres of immune suppression, practicality and cost effectiveness.

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Author contributions: Conceptualization: G.A., W.W., C.J.C., W.W., Y.C., Y.S. Data curation: G.A., W.W. Formal analysis: G.A., W.W., X.Z., Q.H., Q.L., S.C., X.D., C.J.C., K.H., W.W., Y.C., Y.S. Methodology: G.A., W.W., X.Z., Q.H., Q.L., S.C., X.D., C.J.C., K.H., W.W. Writing—original draft: G.A., W.W., X.Z., Q.H., Q.L., S.C., X.D. Writing—review and editing: G.A., W.W., C.J.C., K.H., W.W., Y.C., Y.S. Resources: K.H., W.W., Y.S. Project administration: Y.C., Y.S. Supervision: Y.S.

Abbreviations: ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; CTGF, connective tissue growth factor; FGFR4, fibroblast growth factor receptor 4; IHC, immunohistochemistry; IL-25, interleukin-25; IL-33, interleukin-33; MMP, matrix metalloproteinase; OVA, ovalbumin; PAS, periodic acid–Schiff; TSLP, thymic stromal lymphopoietin.

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Supplementary Information

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

Appendix S1 Additional methods.
Figure S1 Protocol of murine experiments, numbers of macrophages and lymphocytes in BALF, concentrations of eotaxin in pulmonary homogenates and OVA-specific IgE in the serum of the OVA challenged *St2*$^{-/-}$ mice.
Figure S2 Additional blockade of IL-25, TSLP or both did not significantly alter mucus secretion and collagen deposition in the airways of the OVA challenged St2$^{-/-}$ mice.
Figure S3 Additional blockade of both IL-25 and TSLP further reduced expression of CTGF and MMP2 in OVA challenged St2$^{-/-}$ mice.
Figure S4 Additional blockade of both IL-25 and TSLP further reduced expression of MMP12 but not MMP9 in OVA challenged St2$^{-/-}$ mice.
Table S1 Summary of primary antibodies used for immunohistochemistry and antibody blockade (Rb rabbit, M mouse).

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SUPPLEMENTARY INFORMATION

Combined blockade of IL-25, IL-33 and TSLP mediates amplified inhibition of airway inflammation and remodelling in a murine model of asthma

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Appendix S1-Additional Methods

OVA challenge and antibody blockade

Briefly, groups of wild-type and *St2*$^{-/-}$ mice were sensitised by intraperitoneal injection with OVA on days 0 and 12, then challenged per-nasally daily with a solution of OVA or saline control on days 18 to 23 (6 consecutive days). *St2*$^{-/-}$ animals additionally treated with commercial, specific blocking antibodies or isotype-matched IgG control antibodies were injected (20 μg/200 μL/mouse) with LEAF^{TM} purified rat anti-mouse IL-25 (clone 35B), LEAF^{TM} purified rat IgG1κ or rat IgG2aκ isotype identical antibodies (Biolegend, CA, USA) or rat anti-mouse TSLP (clone 152614, R&D Systems, Minneapolis, MN) (see Table S1) 2 hours prior to the initial OVA sensitisation on day 0. In addition, on day 18 only, solutions of these antibodies (20 μg/50 μL/mouse) were administered per-nasally 2 hours prior to the per-

nasal OVA/diluent challenge. These animals were finally euthanised and examined on day 24 (Fig. S1(a)).

Measurement of airways resistance

Mice were anesthetised by intraperitoneal injection of 2% sodium pentobarbital at a dosage of 160 mg/kg on day 24 of the experiment, then placed on an anatomical table and the trachea exposed through a skin incision with blunt dissection of the underlying connective tissue. After exposing the trachea it was opened with ophthalmic scissors then intubated with an intubation needle connected to a small animal lung function meter (FlexiVent, Canada) with the animal in a supine position and ventilated at a rate of 150 breaths/min, a tidal volume of 10 ml/kg and 2 cmH_2O of positive end-expiratory pressure (PEEP). The FlexiVent apparatus was used to measure pulmonary mechanical parameters following inhalation of physiological saline and serially increasing concentrations of methacholine solution (6, 12, 24 and 48 mg/mL). Concentration-response curves of methacholine-induced changes in airways resistance were constructed.

Total cellular and differential counts in bronchoalveolar lavage fluid

Following measurement of lung function, the mice were retained on an anatomical table and 0.8 mL sterile PBS was injected into the trachea. Following repeated flushing and aspiration, the bronchoalveolar lavage fluid (BALF) was collected. BALF samples were centrifuged at 1500 rpm for 10 min at 4°C, then red blood cells lysed by adding 200 μL red blood cell lysate solution to the cell pellet and incubating for 4 min at room temperature. PBS (1 mL) was then added to the cell suspension to stop the reaction, then the cells re-centrifuged at 1500 rpm for 10 min at 4°C. The cell pellets were resuspended in 1 mL PBS and the total cells counted using an automatic counter. The cells were then pelleted again by centrifugation at 2000 rpm

for 10 min. Smear slides of the cell suspension were then prepared and differential cell counts performed after staining with Congo red. The data were expressed as percentages and absolute numbers of macrophages, eosinophils, neutrophils and lymphocytes.

Lung histology

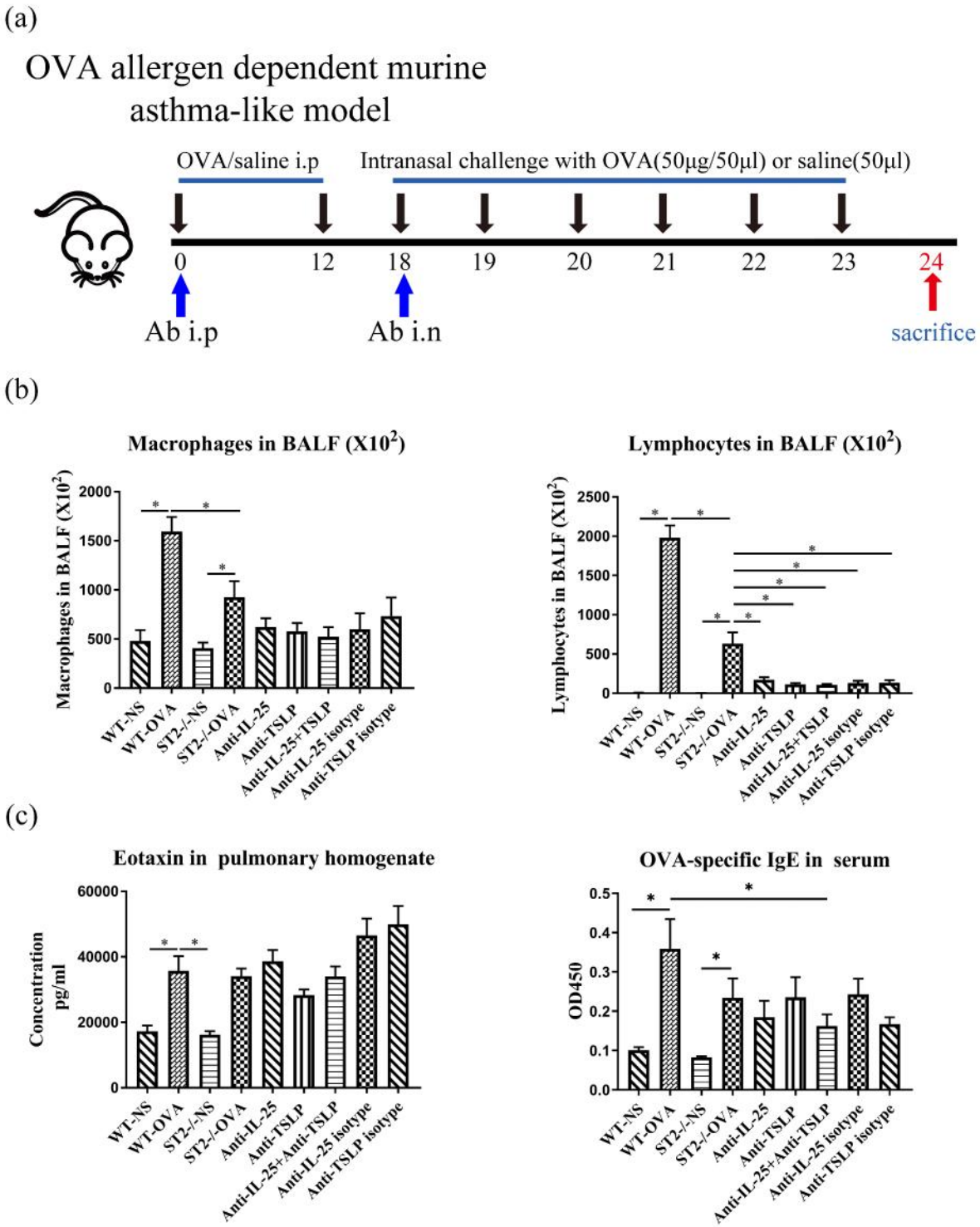
Following collection of BALF, the mice were euthanized prior to removal of the lungs. After washing the lungs with pre-cooled PBS to remove blood and debris, the left lungs were isolated and fixed with 10% formalin for 24 hours. After dehydration, the lungs were embedded in paraffin. Lung sections (4 μm thickness) were then prepared using an automatic slicer, dried at 60°C then stored at room temperature for later use. Objective indices of airways inflammation, mucus production (goblet cell hyperplasia, collagen deposition and eosinophil infiltration were evaluated following staining of the sections with H&E, Periodic Acid–Schiff (PAS), Masson's Trichrome and Congo Red, respectively. In H&E stained sections, the area around the trachea was scored on a scale from 0 to 5: 0, no inflammation; 1, a few inflammatory cells; 2, a layer of inflammatory cells around the structure evaluated; 3, ring of inflammatory cells containing 2 to 4 layers; 4, focal inflammatory cells clustered around the structure; 5, intense inflammatory infiltrate. The following scoring system was used to assess goblet cell hyperplasia in PAS stained sections: 0 normal; 1 minimal, goblet cell hyperplasia in large bronchioles; 2 mild, goblet cell hyperplasia in large and medium bronchioles; 3 moderate, goblet cell hyperplasia in large, medium, and some small bronchioles; and 4 marked, goblet cell hyperplasia in all airways. For each tissue section, photomicrographs of 4 discrete microscopic fields around the major airways were selected for objective analysis of collagen deposition and eosinophil infiltration. Collagen deposition was expressed as the mean fraction of the total areas of the fields staining positively with the

collagen (Masson's trichrome) stain. Eosinophil numbers were expressed as the mean fraction of the total cells in the fields staining with the Congo Red stain.

Lung immunohistochemistry

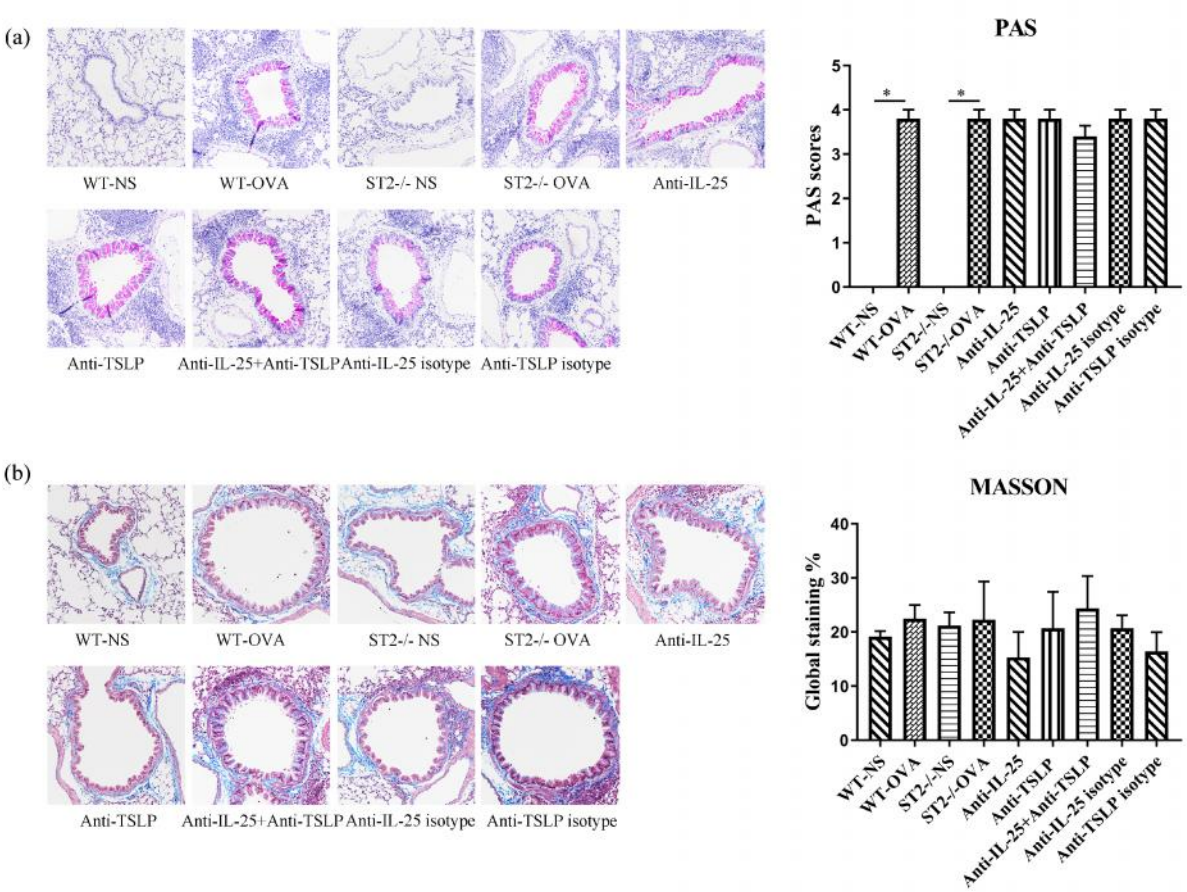
Immunohistochemistry was employed to quantify immunoreactivity for the most common biological markers of airways fibrosis in asthma, including matrix metalloproteinases (MMP) 2, 9 and 12, fibroblast growth factor receptor 4 (FGFR4), connective tissue growth factor (CTGF) and fibronectin. The primary antibodies employed were purchased from Abcam (Cambridge, UK). The sources and optimised concentrations of the individual primary antibodies are summarised in Table S1. Substitution of the primary antibody with PBS was employed as a negative control. Paraffin embedded sections were de-waxed in xylene, rehydrated in serially reducing concentrations of ethanol, then subjected to antigen retrieval by incubating in citrate or EDTA buffer. Endogenous peroxidase was quenched by incubating with 3% H_2O_2. 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 a horseradish peroxidise (HRP)-labelled, third layer antibody. Signals were visualised by colour development with the HRP substrate 3,3'-diaminobenzidine (DAB, Solarbio, Beijing, China). Immunoreactivity was quantified objectively using a Nikon microscope (Nikon, Tokyo, Japan) connected with a computer imaging system with proprietary software (Image-Pro Plus 6.0, Media Cybernetics, Silver Spring, USA). The data were expressed as percentages of the positively stained areas in the entire lung biopsy sections.

Figure S1. Protocol of murine experiments, numbers of macrophages and lymphocytes in BALF, concentrations of eotaxin in pulmonary homogenates and OVA-specific IgE in the serum of the OVA challenged *St2*$^{-/-}$ mice.



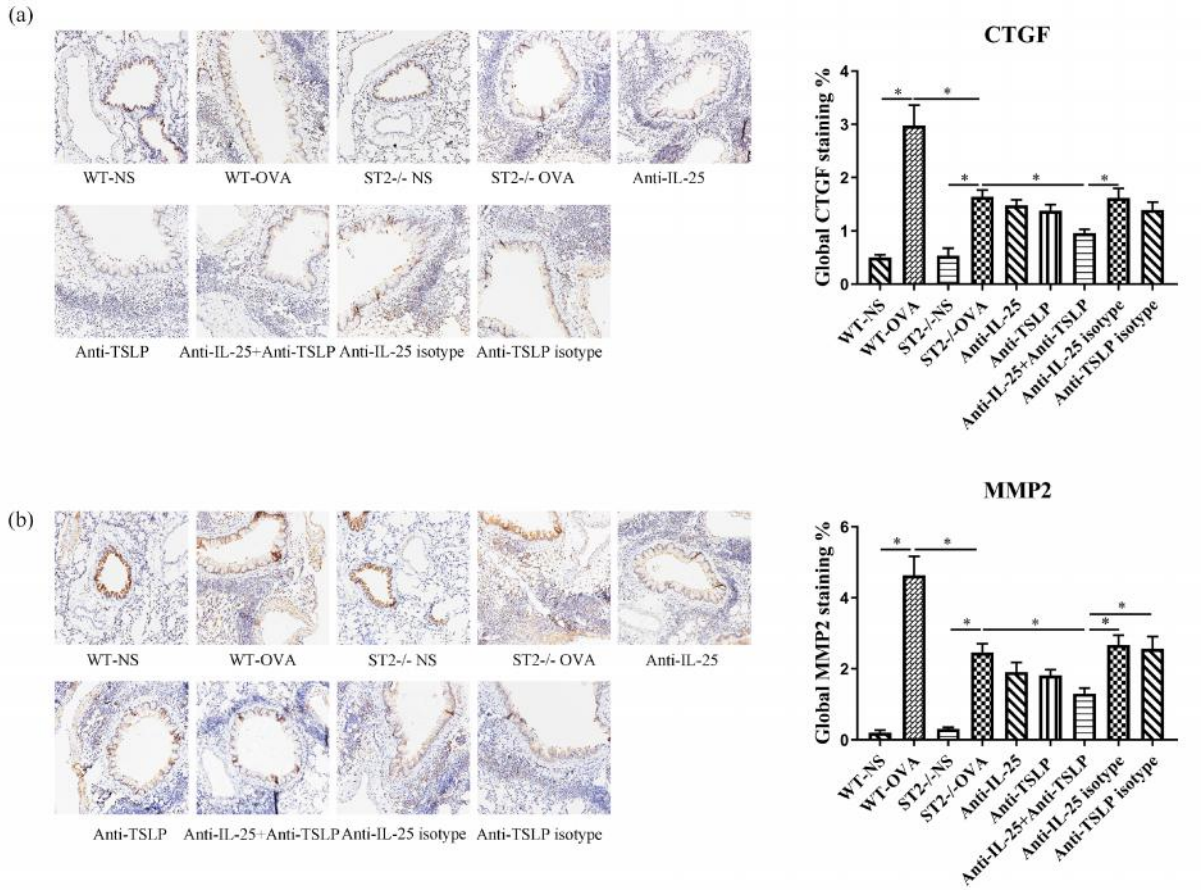
(a) Schedule of murine sensitisation and challenge. i.p: intraperitoneal; i.n: intranasal.; Ab: blocking antibody or isotype identical control. (b) Macrophages and lymphocytes in BALF. (c) Effect of single and combined blockade of IL-25 and TSLP on the production of eotaxin in the lung homogenates and OVA-specific IgE in serum of OVA/saline (NS) sensitised and challenged *St2*$^{-/-}$ mice. The data are expressed as the mean ± SEM (n = 5 in each group at each time point). *P < 0.05.

Figure S2. Additional blockade of IL-25, TSLP or both did not significantly alter mucus secretion and collagen deposition in the airways of the OVA challenged St2$^{-/-}$ mice.



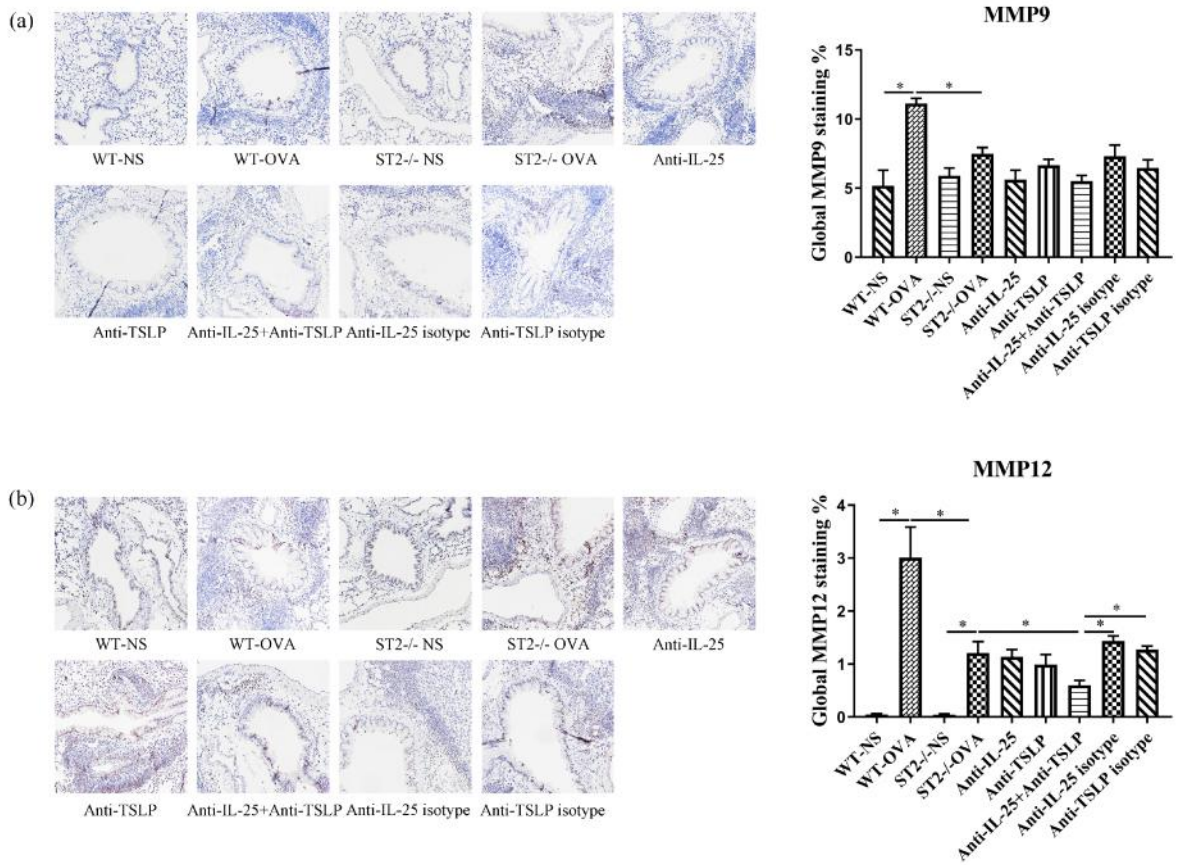
(a) Representative photomicrographs and quantitative analysis of PAS-stained lung sections from each group. Original magnification ×20. (b) Representative photographs and quantitative analysis of Masson-stained lung sections from each group. Original magnification ×20. The data are expressed as the mean ± SEM (n = 5 in each group at each time point). *$P < 0.05$.

Figure S3. Additional blockade of both IL-25 and TSLP further reduced expression of CTGF and MMP2 in OVA challenged St2$^{-/-}$ mice.



(a) Representative photomicrographs and quantitative analysis of CTGF immunoreactivity in lung sections. Original magnification ×20. (b) Representative photomicrographs and quantitative analysis of MMP2 immunoreactivity in lung sections. Original magnification ×20. The data are expressed as the mean ± SEM (n = 5 in each group at each time point). *P < 0.05.

Figure S4. Additional blockade of both IL-25 and TSLP further reduced expression of MMP12 but not MMP9 in OVA challenged $St2^{-/-}$ mice.



(a) Representative photomicrographs and quantitative analysis of MMP9 immunoreactivity in lung sections. Original magnification ×20. (b) Representative photomicrographs and quantitative analysis of MMP12 immunoreactivity in lung sections. Original magnification ×20. The data are expressed as the mean ± SEM (n = 5 in each group at each time point). *P < 0.05.

Table S1. Summary of primary antibodies used for immunohistochemistry and antibody blockade (Rb rabbit, M mouse).

Antibody	Dilution ratio	Host	Company	Cat. #
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-Fibronectin antibody	1:300	M	Abcam	Ab6328
Anti-CTGF antibody	1:300	Rb	Abcam	Ab6992
Anti-FGFR4 antibody	1:300	M	Abcam	Ab44971
Anti-IL-25 antibody	/	Rat	Biolegend	514404
Anti-TSLP antibody	/	Rat	R&D	MAB555
Rat IgG1, κ Isotype	/	Rat	Biolegend	400432
Rat IgG2a, κ Isotype	/	Rat	Biolegend	400544