

Chitinase-1 inhibition attenuates airway hyperresponsiveness and type 2 airway inflammation in allergen-driven and TGF- β -driven murine models of airway remodeling

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Abstract

Background

Airway remodeling limits the reversibility of airflow obstruction in asthma, and no approved therapy prevents it. Chitinase-1 (CHIT1) is a macrophage- and neutrophil-derived enzyme that promotes transforming growth factor (TGF)- β signaling, and kasugamycin (KSM) was recently identified as a CHIT1 inhibitor with antifibrotic activity in pulmonary fibrosis models. Whether CHIT1 inhibition modifies airway physiology or type 2 inflammation in allergen-driven disease is unknown, and the direction of any effect is not predictable: CHIT1 is reported as pathogenic in fibrotic lung disease but protective in allergic airway inflammation.

Methods

KSM was evaluated in three murine protocols with five animals per group. In a chronic house dust mite (HDM) model, male C57BL/6 mice received intranasal HDM for three weeks with intraperitoneal KSM at 12.5–100 mg/kg from day 8. In two inducible lung-specific TGF- β transgenic (TG) protocols, KSM was given either concurrently with doxycycline-induced transgene expression (female, 50 mg/kg) or after induction was established (male, 100 mg/kg). Airway hyperresponsiveness (AHR) to methacholine, bronchoalveolar lavage fluid (BALF) cellularity, lung histology, type 2 cytokines and fibrosis-related gene expression were assessed.

Results

In all three protocols, KSM reduced AHR and total BALF cellularity, with concordant reductions in macrophages, lymphocytes, eosinophils and neutrophils. The effect was reproduced in male and female animals, across doses, and whether treatment began before or after the fibrogenic stimulus was established. In the HDM model, interleukin (IL)-4, IL-5 and IL-13 fell at the highest dose to concentrations comparable with saline controls, with large effect sizes (Cohen's d 1.8–2.1), although these reductions did not survive correction for the fifteen pairwise comparisons implied by the six-group design. Fibrosis-related transcripts were directionally lower after KSM, but median power was 0.27 and lung collagen was not measured.

Conclusions

CHIT1 inhibition reduced airway hyperresponsiveness and airway inflammation, including type 2 cytokines, in three independent models differing in driving stimulus, sex, dose and treatment timing. This convergence identifies CHIT1 as a candidate target in airway remodeling and provides the first physiologic evidence that its inhibition modifies airway function. Confirming an effect on structural

fibrosis requires a prospectively powered study with quantitative endpoints and verified target engagement.

Background

Airway remodeling is a central obstacle in asthma management.[1] Chronic uncontrolled inflammation and recurrent exacerbations drive structural alterations that can result in fixed airflow obstruction, poor disease control, reduced responsiveness to inhaled corticosteroids, and permanent respiratory impairment.[2] Remodeling is driven in large part by TGF- β -mediated fibrosis involving collagen deposition, fibroblast activation, and basement-membrane thickening, and is often considered irreversible.[3, 4] Although achieving adequate asthma control remains the cornerstone of prevention,[5] this approach does not halt structural progression in all patients, and some individuals continue to develop these changes despite optimal management.[6] Biologics have transformed the management of severe type 2-high asthma, yet none has been shown to directly prevent or reverse remodeling,[7] underscoring an unmet need for therapies that act on structural rather than purely inflammatory endpoints.

Chitin-chitinase interactions have emerged as candidate drivers of chronic airway disease. Chitinases degrade chitin, a polysaccharide of fungi, nematodes, and arthropods,[8] and contribute to pulmonary homeostasis and immune balance.[9] In the absence of chitinase activity, chitin polymers accumulate in the airway, producing epithelial stress, chronic innate immune activation, and age-related fibrotic lung disease.[10] Conversely, cigarette smoke, environmental toxins, and other stressors upregulate chitinase expression, and both chitin fragments and the enzymes themselves can activate macrophages, epithelial cells, and neutrophils, promoting inflammation, alveolar destruction, and remodeling.[11] Members of the chitinase family have been implicated in asthma in a manner that is not uniform: chitinase-3-like-1 (CHI3L1) and acid mammalian chitinase promote type 2 responses,[12] whereas CHIT1 has been reported to engage protease-activated receptor-2 on airway epithelium[13] and, in *Chit1*-deficient mice, to restrain allergic inflammation through TGF- β -dependent induction of Foxp3⁺ regulatory T cells.[14]

CHIT1 is expressed predominantly by macrophages and neutrophils,[15, 16] and circulating and airway chitotriosidase levels are elevated in asthma and chronic obstructive pulmonary disease in proportion to disease severity.[17] Kasugamycin (KSM), an aminoglycoside-like antibiotic in agricultural use, was recently identified as a CHIT1 inhibitor that blocks the interaction between CHIT1 and TGF- β receptor-associated protein 1 and attenuates bleomycin- and TGF- β -induced pulmonary fibrosis in mice.[18] That work established antifibrotic activity in fibrosis models but did not address allergen-driven disease, did not evaluate airway physiology, and did not examine type 2 cytokines, leaving open the question most relevant to asthma: whether pharmacologic CHIT1 inhibition alters airway responsiveness, and whether any benefit depends on suppression of type 2 immunity.

We therefore evaluated KSM in three murine protocols spanning an allergen-driven model of chronic airway inflammation and two independent TGF- β -driven protocols, with AHR as a physiologic endpoint

alongside airway inflammation, histology, type 2 cytokines, and fibrosis-related gene expression.

Methods

Animals

Male and female C57BL/6 mice (5–6 weeks old) were housed under specific pathogen-free conditions in a conventional animal facility. Lung-specific, doxycycline-inducible TGF- β transgenic (TG) mice on a C57BL/6 background and wild-type (WT) littermates were used; transgene expression was induced by doxycycline (0.5 g/L) in drinking water as previously described.[19] All procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University (IACUC No. 2017 – 0349) and conducted in accordance with institutional and national ethical guidelines. Reporting follows the ARRIVE 2.0 guidelines; the completed checklist is provided as Additional file 1.

Animals were maintained under the standard conditions of the institutional barrier facility: a 12-hour light/dark cycle, ambient temperature $22 \pm 2^\circ\text{C}$, relative humidity $50 \pm 10\%$, and irradiated chow and autoclaved water available ad libitum. Transgenic and wild-type genotypes were confirmed by PCR of tail-derived genomic DNA.

Sample size, randomization, blinding, and exclusions

No formal sample-size calculation was performed; group sizes were based on our previous experience with these models. Five animals per group were analyzed in all three protocols (70 animals in total; Table 1). A retrospective power analysis for the observed effect sizes is reported in the statistical analysis section. Animals of the same sex and age were allocated to treatment groups without formal randomization. Investigators performing airway physiology, bronchoalveolar lavage cell counting, and histologic assessment were not blinded to treatment allocation. Group allocation is reported in Table 1. The number of animals initially allocated to each group is not stated.

Experimental design

Three protocols were used (Fig. 1).

Protocol A (allergen-driven model; Fig. 1C). Male mice were challenged intranasally with HDM extract (30 μg in sterile saline) three times per week for three weeks. From day 8, KSM was administered intraperitoneally at 12.5, 25, 50, or 100 mg/kg three times per week for two weeks. Control animals received intranasal saline and intraperitoneal vehicle. Mice were sacrificed one day after the final injection.

Protocol B (concurrent treatment in TGF- β TG mice; Fig. 1A). Female TGF- β TG or WT mice received doxycycline water for 13 days. During the same period, KSM (50 mg/kg) or vehicle was administered

intraperitoneally three times per week (six injections total). Mice were sacrificed one day after the final injection.

Protocol C (delayed treatment in TGF- β TG mice; Fig. 1B). Male TGF- β TG or WT mice received doxycycline water for 7 days to establish transgene expression. From day 8, KSM (100 mg/kg) or vehicle was administered intraperitoneally three times per week (three injections total), and mice were sacrificed on day 13.

Protocols B and C differ concurrently in sex, dose, number of injections, and induction period. They were therefore analyzed as two independent tests of the same hypothesis rather than as a comparison of treatment timing, and no statistical comparison was made between them.

Drug preparation and dosing rationale

KSM was dissolved for parenteral administration and given by intraperitoneal injection; vehicle-treated animals received an equal volume of vehicle alone. Doses of 50 and 100 mg/kg were selected on the basis of the exposures shown to inhibit CHIT1 activity and attenuate pulmonary fibrosis in mice,[18] and the range was extended downward to 12.5 mg/kg in Protocol A to explore dose dependence. Animals were monitored daily for signs of ill health. No formal toxicity assessment was undertaken.

Measurement of airway hyperresponsiveness

AHR to inhaled methacholine (MCh; Sigma-Aldrich, St. Louis, MO, USA) was assessed on the day of sacrifice using the forced oscillation technique (FlexiVent; SCIREQ, Montreal, QC, Canada). Mice were anesthetized, tracheostomized, and mechanically ventilated. Aerosolised phosphate-buffered saline or MCh at increasing concentrations (6.25, 12.5, 25.0, 50.0, and 100.0 mg/mL) was delivered for 10 seconds via a nebulizer connected to the ventilator. AHR was quantified as total respiratory system resistance (Rrs) derived from the single-compartment snapshot perturbation, expressed in cmH₂O·s/mL, and the maximal value of the three highest consecutive measurements at each concentration was used.

Bronchoalveolar lavage

BALF was obtained by lavage with 1 mL of Hanks' balanced salt solution (HBSS) via a tracheal cannula. Recovered fluid was centrifuged and the cell pellet resuspended in 300 μ L HBSS. Total cell counts were determined with a hemocytometer using trypan blue exclusion. For differential counts, cytopsin preparations (Cytospin 3; ThermoFisher Scientific, Waltham, MA, USA) were stained with hematoxylin and eosin (Hemacolor, Merck, Darmstadt, Germany), and 200 cells per slide were classified by an observer who was not blinded to treatment allocation.

Histological analysis

Lung tissue not used for BALF collection was fixed in 4% formalin and paraffin-embedded. Sections (3–4 μm) were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome. Slides were examined by light microscopy at the magnifications indicated in the figure legends. Histologic findings are presented as representative images and were assessed qualitatively; no morphometric quantification of collagen area, airway smooth muscle thickness, or goblet cell number was performed.

Lung homogenate and cytokine measurement

Following BALF collection, remaining lung tissue was homogenized in lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich) using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA). Homogenates were incubated, centrifuged, and the supernatant filtered through a 0.45- μm membrane (Gelman Science, Ann Arbor, MI, USA) and stored at -20°C until assay. Concentrations of interleukin (IL)-4, IL-5, and IL-13 were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All samples were assayed in duplicate. Cytokine concentrations are reported per milliliter of homogenate; no correction for total protein content was applied, so between-group differences could in principle reflect differences in tissue input as well as in cytokine production.

Analysis of mRNA expression

Total RNA was extracted from lung tissue using TRIzol reagent (Ambion, Life Technologies, Carlsbad, CA, USA). Complementary DNA was synthesized using reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT) primers. Quantitative real-time PCR was performed with SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) and gene-specific primers (Bioneer, Daejeon, Korea) on a StepOnePlus system (Applied Biosystems, Foster City, CA, USA). Expression was normalized to an endogenous housekeeping transcript and calculated by the $\Delta\Delta\text{Ct}$ method relative to the WT vehicle group. Values are reported as relative expression within each panel and are not directly comparable between panels. All primers were designed against murine sequences; primer sequences are given in the primer table available from the corresponding authors.

The distinction between endogenous and transgene-derived TGF- β 1 requires comment, because the transgene in this model is not of murine origin. The inducible construct is tet-O-CMV-hTGF- β 1-SV40, comprising a human TGF- β 1 cDNA carrying cysteine-to-serine substitutions at codons 223 and 225 that prevent latency-associated peptide binding, followed by SV40 rather than human 3' untranslated sequence.[19] The murine and human coding sequences are 85% identical at the nucleotide level, so species discrimination cannot be assumed from the choice of target gene alone. Two lines of evidence indicate that the assay reported here measures the endogenous murine transcript. First, the primers were designed against murine *Tgfb1* and the construct lacks the human 3' untranslated region. Second, and decisively, wild-type littermates carry no copy of the construct and therefore contain no human template; had the assay detected the transgene, wild-type cycle thresholds would have been

undetermined and a finite fold change relative to a wild-type baseline could not have been computed. The defined wild-type baseline and bounded induction in Figs. 6F and S1F are only obtainable if the amplicon is present in wild-type lung. Accordingly, TGF- β 1 expression in these panels denotes host-derived endogenous *Tgfb1*, not the inducing transgene, and is interpreted as secondary autoinduction of the endogenous gene by transgene-derived TGF- β 1 protein.

Statistical analysis

Data are presented as mean $\pm$ SEM. Individual animal-level values are not available and are therefore not plotted. AHR dose-response curves were analyzed by two-way repeated-measures analysis of variance (ANOVA) with group and MCh concentration as factors, followed by pairwise comparison at the highest MCh concentration; the resulting significance levels are indicated in the figures. BALF counts, cytokine concentrations, and gene expression were compared by one-way ANOVA followed by Bonferroni correction for multiple comparisons. With six groups in Protocol A and four groups in Protocols B and C, the corrected two-sided thresholds for pairwise comparisons were 0.0033 and 0.0083 respectively; we report both uncorrected p values and the corrected threshold so that readers can judge each comparison directly. With five observations per group, normality could not be verified, and the parametric results should therefore be read as approximate. Standardized effect sizes (Cohen's d) computed from the group means and dispersion shown in the figures are available from the corresponding authors.

A retrospective power analysis was performed to interpret non-significant comparisons; it is reported for transparency and not as a substitute for prospective sample-size calculation. Power was computed from the group means and dispersion presented in the figures, and the resulting estimates are therefore approximate. With n equal to 5 per group, a two-sided α of 0.05, and the effect sizes observed, statistical power was 0.68–0.84 for the type 2 cytokine comparisons but ranged from 0.08 to 0.55 across the fibrosis-related transcripts, with a median of 0.27. Detecting the observed differences in fibronectin and α -smooth muscle actin at 80% power would have required approximately 114 and 44 animals per group respectively. Non-significant transcript findings are therefore interpreted as inconclusive rather than as evidence of absence.

Analyses were performed with SPSS v18.0 (IBM, Chicago, IL, USA). A two-sided p value below 0.05 was considered nominally significant. No correction was applied across the three protocols, which were analyzed separately. Given the group sizes and the number of endpoints, all analyses should be regarded as exploratory and hypothesis-generating.

Table 1
Group sizes and animal disposition.

Protocol	Model	Sex	Group	Analyzed (n)
A	HDM (allergen)	M	Saline control	5
A	HDM (allergen)	M	HDM + vehicle	5
A	HDM (allergen)	M	HDM + KSM 12.5 mg/kg	5
A	HDM (allergen)	M	HDM + KSM 25 mg/kg	5
A	HDM (allergen)	M	HDM + KSM 50 mg/kg	5
A	HDM (allergen)	M	HDM + KSM 100 mg/kg	5
B	TGF- β TG, concurrent	F	WT + vehicle	5
B	TGF- β TG, concurrent	F	WT + KSM 50 mg/kg	5
B	TGF- β TG, concurrent	F	TG + vehicle	5
B	TGF- β TG, concurrent	F	TG + KSM 50 mg/kg	5
C	TGF- β TG, delayed	M	WT + vehicle	5
C	TGF- β TG, delayed	M	WT + KSM 100 mg/kg	5
C	TGF- β TG, delayed	M	TG + vehicle	5
C	TGF- β TG, delayed	M	TG + KSM 100 mg/kg	5

F, female; HDM, house dust mite; KSM, kasugamycin; M, male; TG, transgenic; WT, wild type.

Results

KSM attenuates HDM-induced airway hyperresponsiveness and airway inflammation

To test whether CHIT1 inhibition modifies allergen-driven airway disease, male mice were challenged intranasally with HDM for three weeks and treated with KSM from day 8 at doses from 12.5 to 100 mg/kg (Fig. 1C). Five animals were studied per group (Table 1).

Chronic HDM exposure produced a marked increase in airway resistance across escalating MCh concentrations relative to saline-challenged controls. KSM treatment shifted the dose-response curve downward at all four doses (Fig. 2A). The reduction was not monotonically dose-dependent: the lowest resistance at 100 mg/mL MCh was observed at 25 mg/kg, whereas 100 mg/kg produced an intermediate effect. AHR in KSM-treated animals nevertheless remained above that of saline controls at every dose, indicating partial rather than complete normalization of airway responsiveness.

HDM exposure increased total BALF cellularity approximately 30-fold over control, with increases in macrophages, lymphocytes, eosinophils, and neutrophils (Fig. 2B). Macrophages accounted for the largest absolute increment, comprising roughly 40% of the total inflammatory infiltrate. KSM reduced total BALF cellularity at all doses, and reductions were concordant across all four differential cell populations, with the largest effects at 25 and 100 mg/kg. Eosinophils and neutrophils were reduced in parallel with macrophages rather than selectively, indicating a broad rather than lineage-restricted anti-inflammatory effect.

Lung-homogenate concentrations of the type 2 cytokines IL-4, IL-5, and IL-13 were elevated after HDM exposure (Fig. 2C-E). KSM produced graded, dose-ordered reductions in all three cytokines. At 100 mg/kg, IL-4 fell by 27%, IL-5 by 53%, and IL-13 by 30% relative to HDM-exposed vehicle controls, with large standardized effect sizes (Cohen's d 1.8 to 2.1) and uncorrected p values of 0.014, 0.010, and 0.032 respectively. None of these comparisons met the Bonferroni-corrected threshold of 0.0033 required by the six-group design, and we therefore do not describe them as statistically significant. Their interpretation nevertheless differs from that of a null result: the point estimates were large, the ordering across doses was monotonic for IL-5 and IL-13, and at the two highest doses all three cytokines returned to concentrations comparable with those of saline-challenged control animals. Retrospective power for these three comparisons was 0.68–0.84 (statistical summary available on request), so the analysis was reasonably sensitive to effects of this size.

Histologically, HDM-exposed lungs showed peribronchial and perivascular mononuclear infiltration on H&E staining, goblet cell hyperplasia on PAS staining, and increased peribronchial trichrome-positive material (Fig. 3). KSM-treated animals showed visibly less peribronchial cellular infiltration, most evident at the intermediate and higher doses. Changes in PAS-positive goblet cells and in trichrome-stained peribronchial material were less consistent across doses. These assessments were qualitative and are not supported by morphometric quantification.

KSM reduces AHR and airway inflammation in two independent TGF- β transgenic protocols

We next asked whether the same endpoints responded to KSM when remodeling was driven by TGF- β rather than by allergen, using two protocols that differed in sex, dose, and the timing of treatment relative to transgene induction (Fig. 1A, 1B). Because these variables differed concurrently, the protocols are reported as independent replications and were not compared statistically.

In the concurrent-treatment protocol (female mice, 50 mg/kg, $n = 5$ per group; Fig. 4), doxycycline-induced TG mice developed markedly increased airway resistance relative to WT controls. KSM given throughout the induction period reduced AHR across the MCh dose-response curve to values approaching those of WT animals (Fig. 4A). Total BALF cellularity was increased approximately 13-fold in vehicle-treated TG mice, with macrophages comprising the majority of the infiltrate, and was reduced by KSM (Fig. 4B). Reductions were also seen in lymphocytes, eosinophils, and neutrophils. Histologic

examination showed prominent peribronchial and perivascular inflammation and increased peribronchial trichrome staining in TG mice, both visibly reduced after KSM (Fig. 4C).

In the delayed-treatment protocol (male mice, 100 mg/kg, n = 5 per group; Fig. 5), TG mice again showed increased AHR relative to WT controls, and KSM reduced airway resistance across the curve (Fig. 5A). Total BALF cellularity and all differential populations were reduced by KSM (Fig. 5B). Peribronchial inflammation and trichrome staining were attenuated, although both remained visibly greater than in WT controls (Fig. 5C).

The concordance of these two protocols is informative precisely because they differ. Reductions in AHR and in macrophage-predominant BALF inflammation were observed in female and male animals, at 50 and 100 mg/kg, and whether treatment began before or after transgene expression was established. The direction of effect was therefore robust to sex, dose, and treatment timing. The present design does not permit the individual contributions of these variables to be resolved, and no inference about the relative efficacy of earlier versus later treatment can be drawn from these data.

Fibrosis-related gene expression shows consistent but statistically unconfirmed reductions

To examine whether the reduction in inflammation was accompanied by changes in remodeling-associated transcripts, we measured lung mRNA for collagen Ia1, collagen III, fibronectin, tissue inhibitor of metalloproteinase (TIMP)-1, α -smooth muscle actin (α -SMA), and endogenous murine TGF- β 1 in the concurrent-treatment protocol (Fig. 6). As set out in the Methods, the TGF- β 1 panel reports the host's own transcript and not the human transgene that drives the model.

Vehicle-treated TG mice showed higher expression of collagen Ia1 (approximately 7.5-fold), collagen III (5.2-fold), fibronectin (6.9-fold), and endogenous TGF- β 1 (4.0-fold) than WT controls. After KSM, mean expression was lower for all four transcripts: collagen Ia1 by 58%, collagen III by 93%, fibronectin by 38%, and endogenous TGF- β 1 by 86%. The direction of change was thus concordant across all four extracellular matrix and growth factor transcripts measured, and for collagen III and endogenous TGF- β 1 the treated means approached WT levels. TIMP-1 and α -SMA, which are not matrix constituents, did not follow this pattern (see below).

These comparisons must nevertheless be interpreted with caution, for a quantitative reason. With five animals per group, retrospective power ranged from 0.08 for fibronectin to 0.55 for collagen Ia1, with a median of 0.27 across the six transcripts (statistical summary available on request). Only collagen Ia1 reached a nominal p value below 0.05, and no transcript met the Bonferroni-corrected threshold of 0.0083. A study with this power would be expected to miss most real effects of the size observed; detecting the fibronectin difference at 80% power would have required approximately 114 animals per group. The absence of statistical significance is therefore uninformative about whether an effect exists, and should be read as neither confirmation nor refutation.

TIMP-1 and α -SMA were not altered in a consistent direction (Fig. 6D, 6E). TIMP-1 was numerically higher after KSM, which we regard as uninterpretable at a power of 0.09. Because the TGF- β 1 panel reflects endogenous rather than transgene-derived transcript, its reduction is best read as attenuation of the secondary autoinduction loop through which exogenous TGF- β 1 protein amplifies host TGF- β 1 transcription, rather than as an effect of KSM on the inducing stimulus itself, which is under doxycycline control and not a pharmacologic target here.

In the delayed-treatment protocol, transcript levels showed the same directional pattern but with smaller differences and no statistically significant treatment effects (Additional file 2, Fig. S1). In that protocol, collagen Ia1 and fibronectin differed significantly between WT and TG animals, confirming transgene-driven induction, but the KSM-versus-vehicle comparisons within TG animals were not significant.

Taken together, the transcript data are directionally concordant with the reduction in inflammation but do not constitute statistical evidence of an antifibrotic effect. We did not measure lung collagen content biochemically or quantify collagen area morphometrically. The fibrotic endpoint is therefore unresolved rather than negative, and resolving it would require both quantitative protein-level measurement and a substantially larger sample.

Discussion

Across an allergen-driven model and two independent TGF- β -driven protocols, pharmacologic inhibition of CHIT1 with KSM reduced airway hyperresponsiveness and airway inflammation. The effect on AHR is, to our knowledge, the first physiologic evidence that CHIT1 inhibition modifies airway function, and it was reproduced in male and female animals, at two doses, and whether treatment was initiated before or after the fibrogenic stimulus was established. Type 2 cytokines were reduced with large effect sizes in the allergen model, returning to control concentrations at the higher doses, although these reductions did not survive correction for the fifteen pairwise comparisons implied by the six-group design. Fibrosis-related transcripts moved consistently in the same direction, but the study was too small to test them: median power for those comparisons was 0.27. The pattern across endpoints is therefore coherent, and the principal claim we make is directional and physiologic rather than mechanistic.

KSM was identified as a CHIT1 inhibitor that disrupts the interaction between CHIT1 and TGF- β receptor-associated protein 1, thereby attenuating TGF- β signaling and fibrosis in bleomycin- and TGF- β -driven models of pulmonary fibrosis.[18] Our findings extend that pharmacology in three directions relevant to obstructive airway disease: to an allergen exposure that itself contains chitin, to airway physiology as an endpoint, and to type 2 immunity. The chitin content of HDM makes this model a particularly direct test of the chitin-chitinase axis, since the substrate and the enzyme are simultaneously present in the airway. [10, 11] The physiologic endpoint matters independently of the fibrotic one: AHR is the functional abnormality that defines asthma, and no previous study of a chitinase inhibitor has measured it.

Our results bear on an unresolved question about the direction of CHIT1's contribution to allergic airway disease. Other chitinase-family members, including acid mammalian chitinase and CHI3L1, promote

type 2 inflammation, and their neutralization attenuates allergic responses.[12] By contrast, *Chit1*-deficient mice display enhanced type 2-mediated airway inflammation and reduced Foxp3⁺ regulatory T cells, which has been interpreted as evidence that CHIT1 restrains allergic inflammation.[14] Pharmacologic inhibition in our hands reduced rather than aggravated type 2 cytokines and airway responsiveness, aligning CHIT1 with the other family members rather than with a protective role. Two considerations may account for the difference from the genetic model.

First, lifelong genetic deletion and short-term pharmacologic inhibition are not equivalent perturbations. Constitutive absence of chitinase activity permits progressive accumulation of chitin polymers in the airway, which is by itself sufficient to cause epithelial stress, innate immune activation, and fibrotic lung disease.[10] A two-week partial blockade initiated in adult animals is unlikely to reach that threshold. A *Chit1*-null phenotype may therefore reflect the downstream consequences of substrate accumulation over the lifespan rather than the loss of an acute signaling function, and the two experiments may not be measuring the same thing.

Second, CHIT1 has enzymatic and non-enzymatic activities that need not be inhibited in parallel. Because KSM acts at least in part by blocking a protein-protein interaction with TGF- β receptor-associated protein 1,[18] partial pharmacologic blockade may affect the scaffolding function through which CHIT1 amplifies TGF- β signaling differently from the catalytic activity that generates immunomodulatory chitin fragments. Our data do not test this possibility. We did not measure chitinase enzymatic activity, CHIT1 protein, regulatory T cells, or TGF- β pathway activation, and we therefore cannot distinguish this explanation from several alternatives, including an effect of KSM unrelated to CHIT1. Discriminating among them requires the experiments listed below and is the principal question raised by this work.

One further observation is reported without interpretation. In all three protocols macrophages constituted the dominant BALF population, and CHIT1 is expressed predominantly by macrophages and neutrophils.[15, 16] In the allergen model, eosinophils and neutrophils were reduced in parallel with macrophages rather than selectively. Whether this reflects action on a myeloid compartment that produces the enzyme, or a downstream consequence of reduced type 2 cytokine production, cannot be determined from these data, since both changed together.

Existing therapeutic options act only partially on airway structure. Inhaled corticosteroids and monoclonal antibodies attenuate some remodeling-associated measures,[20–22] but none has been shown to reverse established structural fibrosis,[23] and bronchial thermoplasty remains limited by procedural complexity and variable efficacy.[24, 25] Earlier chitinase inhibitors, including demethylallosamidin and the clinical-stage compound OATD-01, have been evaluated largely for inflammatory endpoints or in idiopathic pulmonary fibrosis rather than in allergen-driven airway disease. [26, 27] Positioning CHIT1 inhibition as a candidate approach to airway remodeling in asthma therefore addresses a genuine gap, provided the structural claim is held to quantitative standards that the present study does not meet.

Several limitations require emphasis, two of which are fundamental rather than incremental.

The first is the absence of target engagement data. Neither chitinase enzymatic activity nor CHIT1 protein or transcript levels were measured in lung tissue or BALF. We therefore cannot establish that the observed effects were mediated by CHIT1 inhibition at all. KSM is structurally related to the aminoglycosides, which have independently reported anti-inflammatory properties, and an off-target mechanism cannot be excluded. That inhibition of lung chitinase activity has been demonstrated for KSM at comparable exposures[18] provides indirect support but is not evidence about these animals. This is the single most important omission in the study, and it constrains every mechanistic statement above.

The second is statistical power. With five animals per group and no prospective sample-size calculation, median power across the fibrosis-related transcripts was 0.27, and no transcript comparison met the Bonferroni-corrected threshold. Power for the type 2 cytokine comparisons was higher, at 0.68–0.84, but those comparisons also failed correction for multiple testing. Consequently, positive findings in this study carry an elevated risk of being false positives, and negative findings carry a high risk of being false negatives; the fibrosis endpoints in particular are inconclusive rather than negative. We have restricted the title and conclusions to airway responsiveness and inflammation and make no claim of a demonstrated antifibrotic effect.

Further limitations follow. We did not quantify fibrosis at the protein level: histology was assessed qualitatively without morphometry, and no hydroxyproline or biochemical collagen assay was performed. Neither randomization nor blinding was performed, and both are recognized sources of bias in the estimates reported here. The two TGF- β protocols differed simultaneously in sex, dose, injection number, and induction period, so their concordance supports robustness of direction but cannot isolate the contribution of treatment timing, and no inference about early versus delayed treatment is available from this design. No positive comparator such as dexamethasone was included, so the magnitude of the KSM effect cannot be placed on a therapeutic scale. Dose-response in the HDM model was non-monotonic for AHR, which may reflect small group sizes, saturation of target inhibition, or genuine biological complexity; these cannot be distinguished here. Systemic toxicity and pharmacokinetics were not characterized. Finally, all three protocols used short exposures in young adult mice, which model neither the duration nor the age distribution of established human asthma.

The confirmatory study these results call for is specific. It should measure chitinase activity and CHIT1 expression in lung and BALF alongside quantitative fibrosis endpoints, in a single design with unified sex, dose, and treatment timing, powered prospectively for the transcript effect sizes reported here, which implies group sizes an order of magnitude larger than ours for the weaker endpoints. It should include a corticosteroid comparator, and it should assess regulatory T cells and TGF- β pathway activation directly in order to test the reconciliation proposed above against the *Chit1*-null phenotype. Until target engagement is demonstrated, CHIT1 inhibition in asthma should be regarded as a hypothesis supported by directional evidence rather than an established pharmacologic strategy.

Conclusions

In murine models of allergen-driven and TGF- β -driven airway disease, CHIT1 inhibition with kasugamycin reduced airway hyperresponsiveness reproducibly across differing sex, dose, and treatment timing, and reduced airway inflammation including type 2 cytokines, which returned to control concentrations at the higher doses. These reductions were large in magnitude but did not survive correction for multiple comparisons, and fibrosis-related transcripts, although directionally concordant, were assessed at a median power of 0.27 and remain inconclusive. Because target engagement was not verified, the mechanism is undetermined and an effect unrelated to CHIT1 cannot be excluded. We therefore present these findings as directional and hypothesis-generating: they identify airway hyperresponsiveness as a tractable endpoint for chitinase inhibition and specify the confirmatory experiments, principally demonstration of target engagement together with quantitative fibrosis measurement in an adequately powered design, that would be required before CHIT1 inhibition could be advanced as an antiremodeling strategy in asthma.

Abbreviations

α -SMA

alpha-smooth muscle actin

AHR

airway hyperresponsiveness

ARRIVE

Animal Research:Reporting of In Vivo Experiments

BALF

bronchoalveolar lavage fluid

CHIT1

chitinase-1

Dox

doxycycline

H&E

hematoxylin and eosin

HDM

house dust mite

IACUC

Institutional Animal Care and Use Committee

IL

interleukin

KSM

kasugamycin

PAS

periodic acid-Schiff

SEM

standard error of the mean

TG

transgenic

TGF- β

transforming growth factor beta

TIMP-1

tissue inhibitor of metalloproteinase-1

WT

wild type

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (IACUC No. 2017-0349) and were conducted in accordance with institutional and national guidelines for the care and use of laboratory animals. Consent to participate is not applicable to this study.

Consent for publication

Not applicable.

Availability of data and materials

Group-level summary data supporting the findings of this study are presented in the figures. The underlying individual animal-level data are not available and therefore could not be deposited in a public repository. Additional information regarding the analyses is available from the corresponding authors upon reasonable request.

Competing interests

S-RK, HH, J-WP, HJP, and J-HL declare that they have no competing interests.

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Authors' contributions

HJP, J-HL, and S-RK conceived and designed the study. S-RK and HH performed the animal experiments and acquired the data. S-RK and HH analyzed the data. J-WP provided resources and supervised the

work. S-RK drafted the manuscript. HJP and J-HL critically revised the manuscript. All authors read and approved the final version prior to submission.

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Authors' information

Not applicable.

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Figures

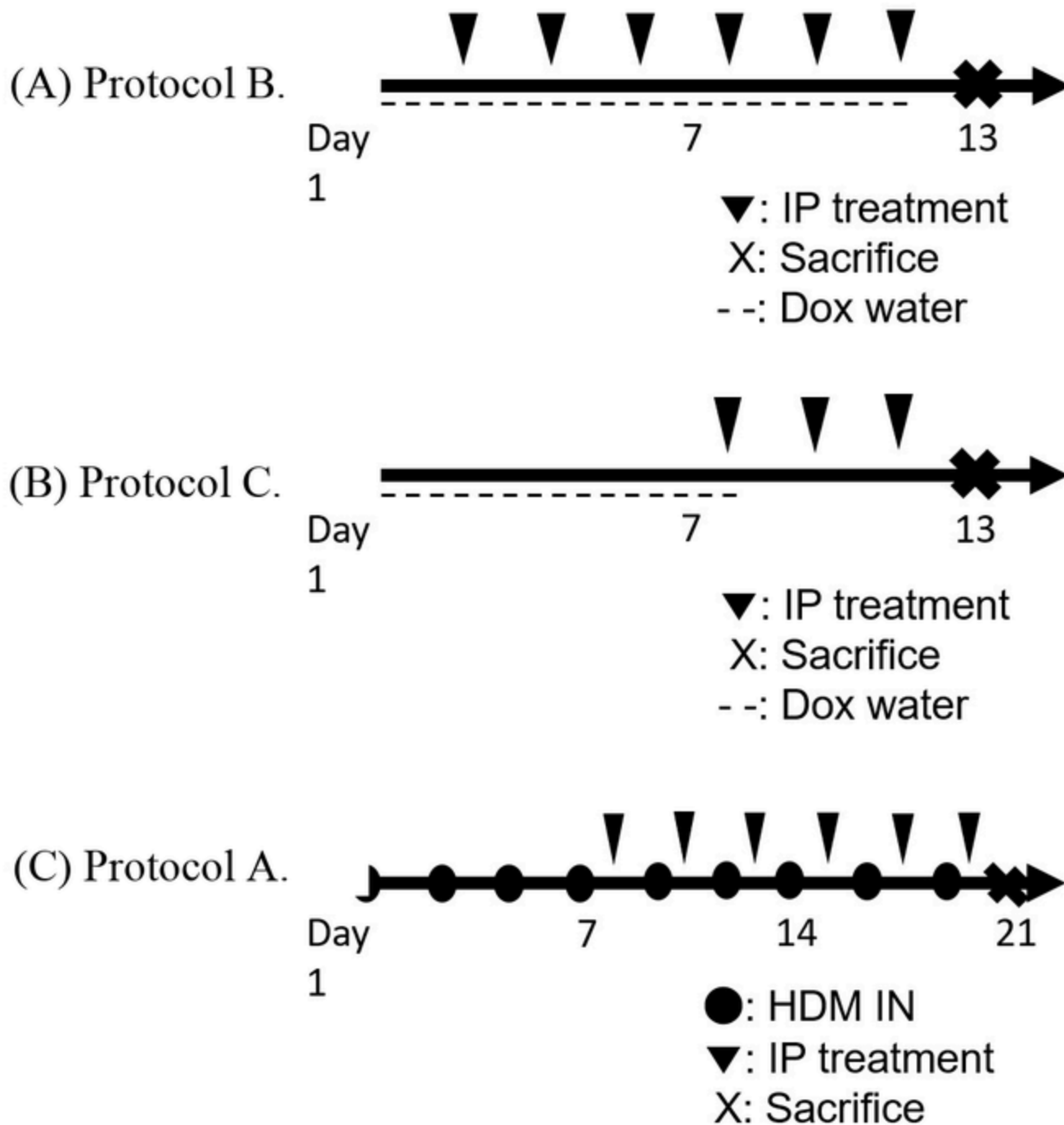
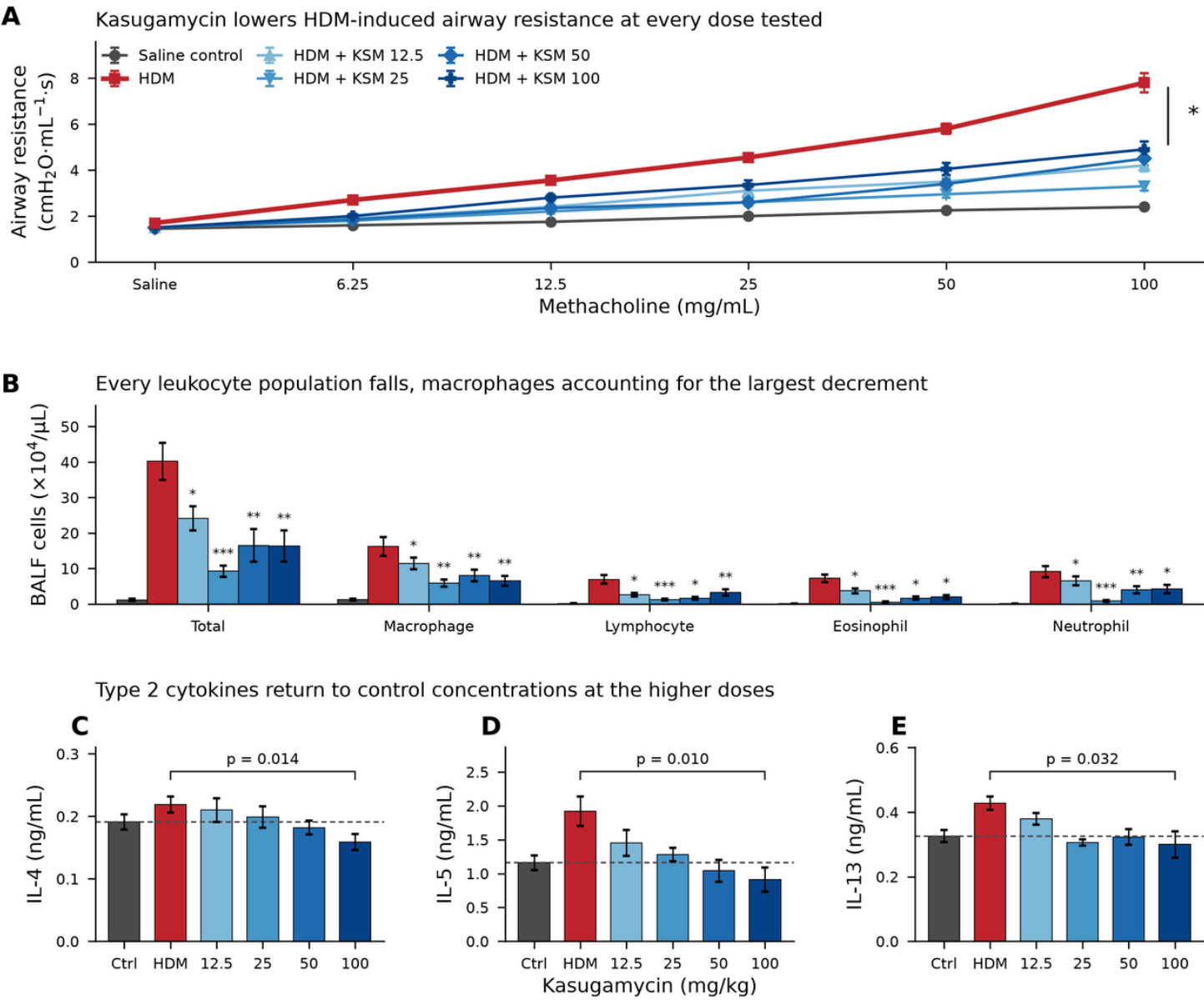


Figure 1

Experimental protocols. (A) Protocol B, concurrent treatment in female TGF- β transgenic mice: doxycycline drinking water for 13 days with kasugamycin (KSM) 50 mg/kg intraperitoneally three times weekly (six injections), sacrifice on day 13. (B) Protocol C, delayed treatment in male TGF- β transgenic mice: doxycycline for 7 days, then KSM 100 mg/kg intraperitoneally three times weekly from day 8 (three injections), sacrifice on day 13. (C) Protocol A, allergen-driven model: intranasal house dust mite (HDM) three times weekly for three weeks with KSM 12.5–100 mg/kg intraperitoneally three times weekly from day 8, sacrifice one day after the final injection. Filled triangles, intraperitoneal injection; filled circles,

intranasal HDM; cross, sacrifice; dashed line, doxycycline drinking water. Dox, doxycycline; IP, intraperitoneal.



n = 5 per group; error bars, SEM. Asterisks in (B) denote comparison with HDM + vehicle. Brackets in (C-E) give uncorrected p values; the Bonferroni-corrected threshold for this six-group design is 0.0033. Dashed line, saline-control mean. Ctrl, saline control.

Figure 2

Kasugamycin attenuates HDM-induced airway hyperresponsiveness and airway inflammation. (A) Airway resistance in response to escalating aerosolised methacholine. (B) Total and differential bronchoalveolar lavage fluid (BALF) cell counts; asterisks denote comparison with the HDM + vehicle group. (C-E) Lung-homogenate concentrations of IL-4 (C), IL-5 (D) and IL-13 (E); the dashed line marks the saline-control mean and brackets give uncorrected p values. Data are mean $\pm$ SEM with n = 5 per group. (A) was analyzed by two-way repeated-measures ANOVA and (B-E) by one-way ANOVA with Bonferroni correction; with six groups the corrected threshold for pairwise comparisons is $p < 0.0033$.

Effect sizes and retrospective power for the cytokine panels are given in the Methods. BALF, bronchoalveolar lavage fluid; HDM, house dust mite; IL, interleukin; KSM, kasugamycin.

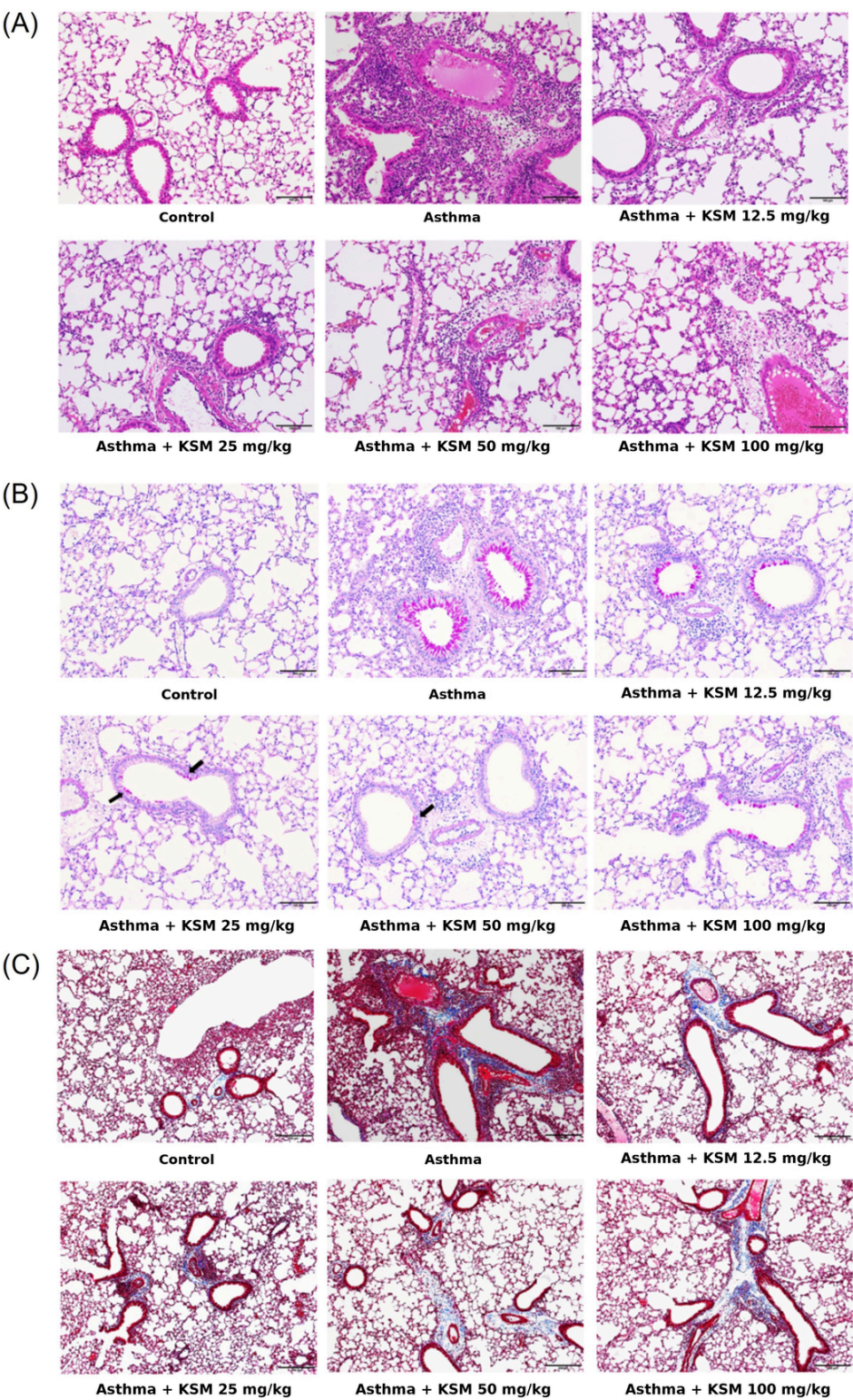


Figure 3

Histologic findings in the HDM model. Representative lung sections stained with (A) hematoxylin and eosin (x200), (B) periodic acid-Schiff (x200), and (C) Masson's trichrome (x100) from control, HDM-

exposed, and HDM-exposed KSM-treated mice at 12.5–100 mg/kg. Panel labels denote the treatment group of each tile. Arrows in (B) indicate periodic acid-Schiff-positive goblet cells. Findings were assessed qualitatively at the magnifications indicated; no morphometric quantification was performed. HDM, house dust mite; KSM, kasugamycin.

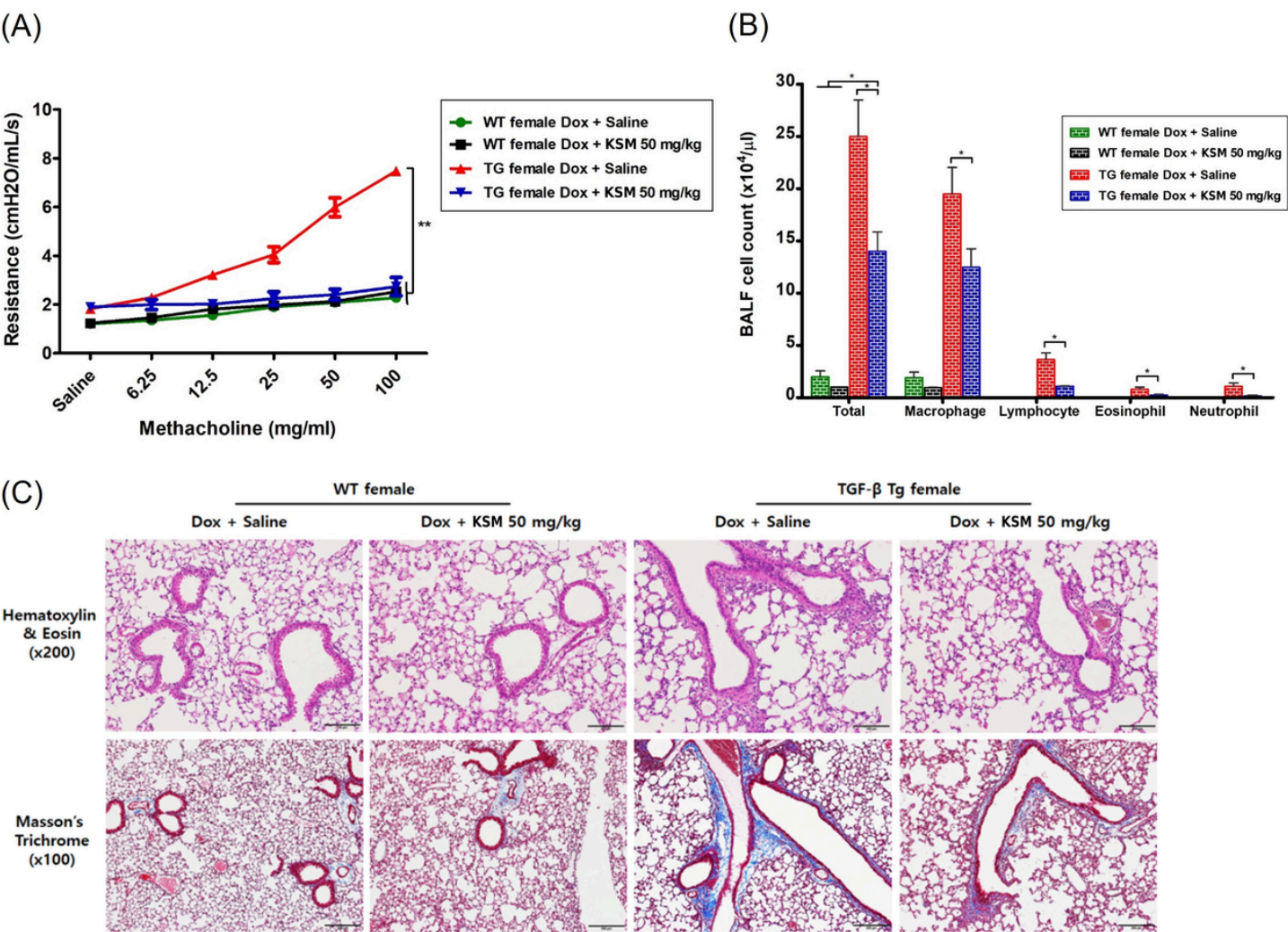


Figure 4

Kasugamycin reduces AHR and airway inflammation when given concurrently with TGF- β induction (female mice, 50 mg/kg). (A) Airway resistance in response to escalating methacholine. (B) Total and differential BALF cell counts. (C) Representative lung histology (hematoxylin and eosin, x200; Masson's trichrome, x100). Data are mean $\pm$ SEM (n = 5 per group); individual animal values are not plotted. *p < 0.05, **p < 0.01. AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; Dox, doxycycline; KSM, kasugamycin; TG, transgenic; WT, wild type.

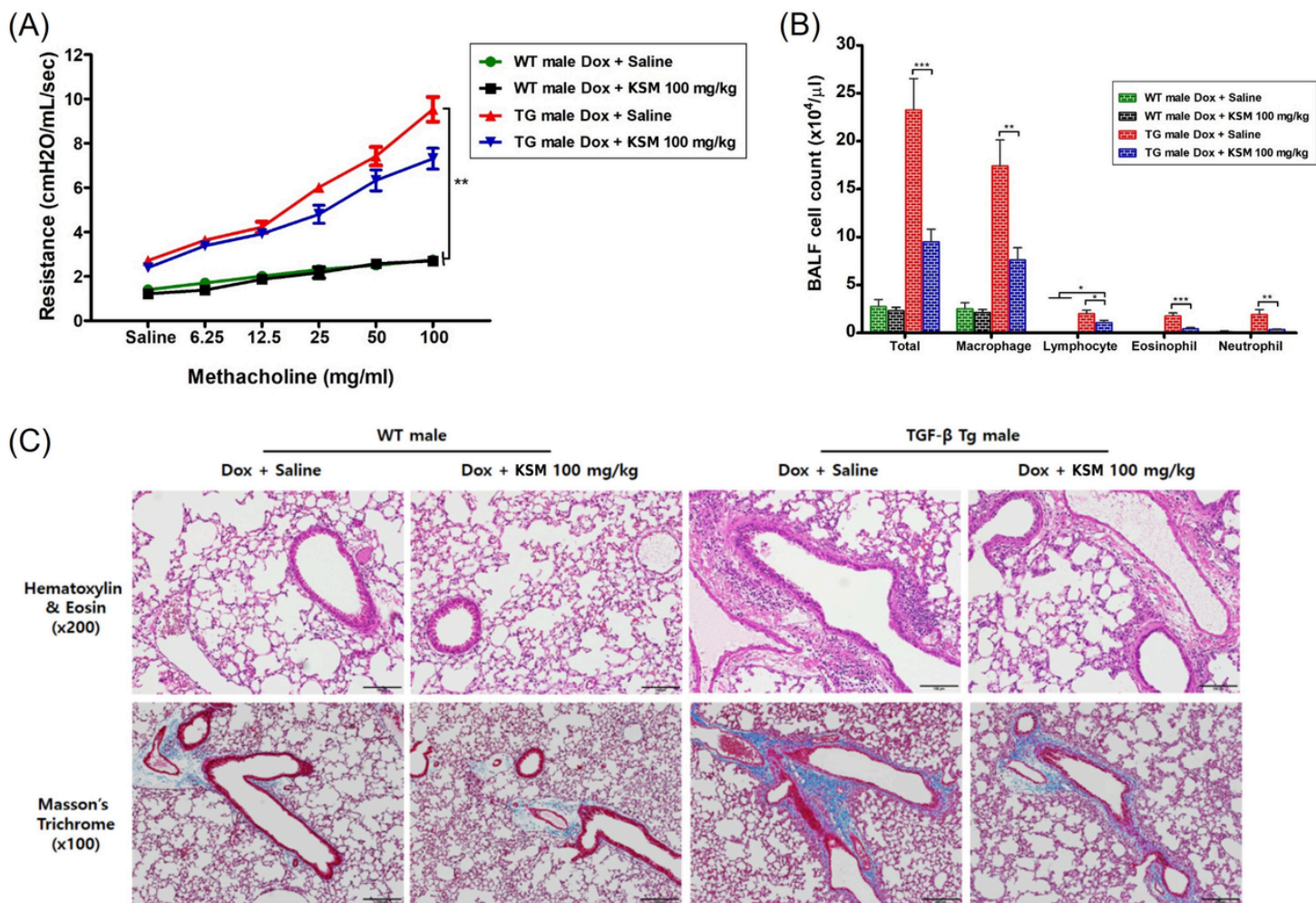


Figure 5

Kasugamycin reduces AHR and airway inflammation when given after TGF- β induction is established (male mice, 100 mg/kg). Panels and statistics as in Figure 4. Protocols shown in Figures 4 and 5 differ concurrently in sex, dose, injection number, and induction period and were not compared statistically; they are presented as independent replications. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The four matrix and growth-factor transcripts fall after kasugamycin; TIMP-1 and α -SMA do not follow
Concurrent treatment, female TGF- β transgenic mice, 50 mg/kg. Retrospective power for each comparison is printed in the panel.

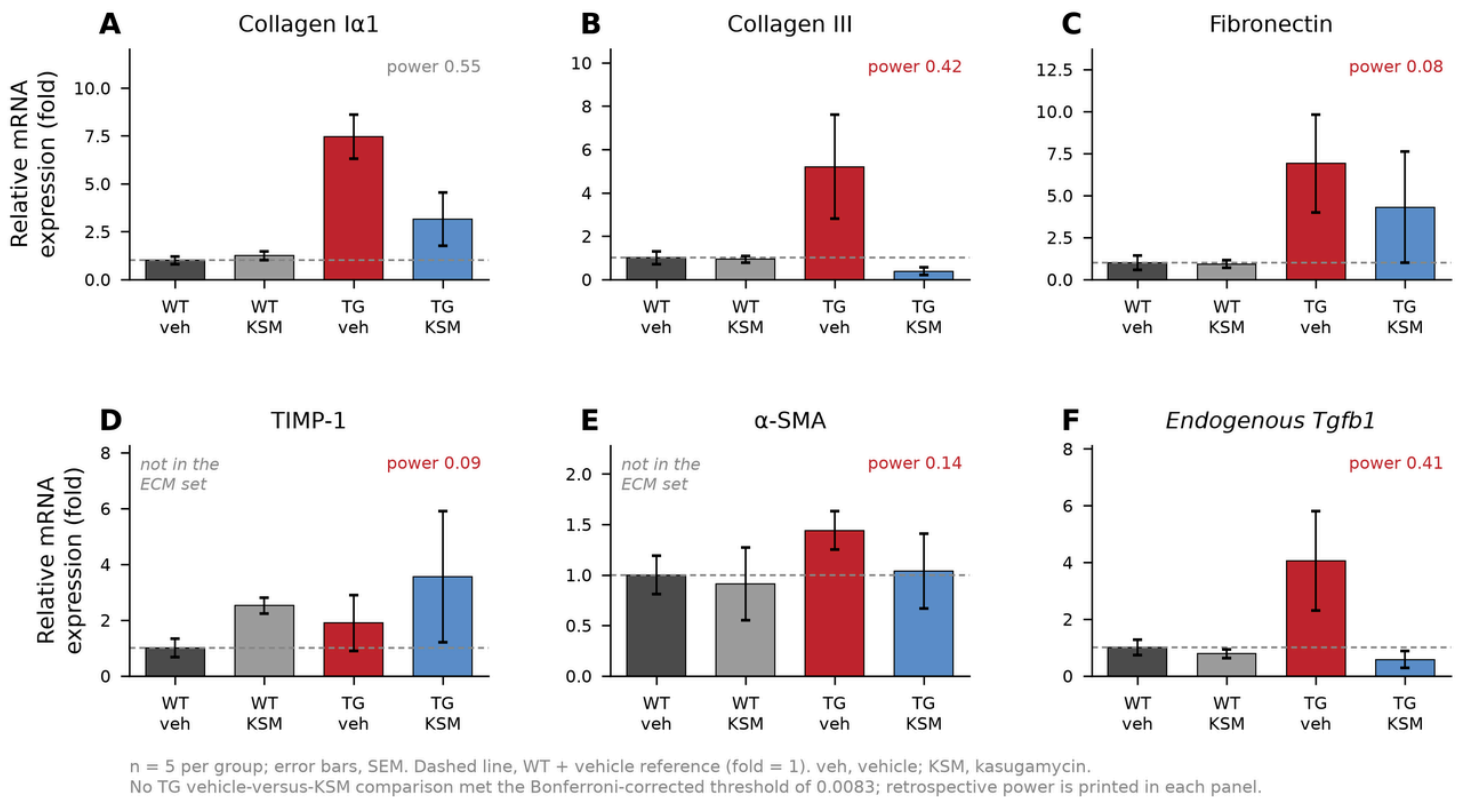


Figure 6

Fibrosis-related gene expression in the concurrent-treatment protocol. Relative lung mRNA expression of (A) collagen I α 1, (B) collagen III, (C) fibronectin, (D) TIMP-1, (E) α -SMA and (F) endogenous murine *Tgfb1*, normalized to an endogenous housekeeping transcript and expressed relative to the female WT vehicle group; the dashed line marks that reference (fold = 1). The four extracellular matrix and growth factor transcripts (A-C, F) were lower after KSM, whereas TIMP-1 and α -SMA, which are not matrix constituents, did not follow this pattern. Data are mean $\pm$ SEM with n = 5 per group. No TG vehicle-versus-KSM comparison met the Bonferroni-corrected threshold of p < 0.0083; the retrospective power of each comparison is printed in the corresponding panel and ranged from 0.08 to 0.55 (median 0.27), so no panel is conclusive. α -SMA, α -smooth muscle actin; KSM, kasugamycin; TG, transgenic; TIMP, tissue inhibitor of metalloproteinase; veh, vehicle; WT, wild type.

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