Materials and Methods

Animals

C57BL/6 inbred mice were purchased and maintained in a specific pathogen-free (SPF) environment at the Model Animal Research Center of Nanjing University. Wistar rats were purchased from Charles River Laboratories (Beijing, China) and maintained in the SPF animal room of Wowu Pharmaceutical Company in Huzhou, China. All animal manipulations in this study were conducted in accordance with the criteria of the Institutional Animal Care and Use Committee (IACUC) of the Model Animal Research Center of Nanjing University (Nanjing, China).

Establishment of gene KO mouse lines

We established *Gnat1*$^{-/-}$, *Gnat2*$^{-/-}$ and *Gnat3*$^{-/-}$ mouse lines using CRISPR-Cas9 technology (1, 2). A pair of sgRNAs targeting the first exon of *Gnat1* (left, 5′-GCTGAAAGAGGATGCTGAGAAGG-3′; right, 5′- GGAGTGCTTCTCCTCAGCGCTGG-3′) was first cloned into the pGL3-U6-sgRNA vector with the BbsI and BsaI double restriction sites, and the resultant constructs were used as PCR templates to obtain T7-sgRNA amplicons. The T7 promoter was added via sense PCR primers. After transcription and purification in vitro, the sgRNA was coinjected with Cas9 mRNA (purchased from the Nanjing Biomedical Research Institute of Nanjing University) into C57BL/6 zygotes. The targeted mice were screened by the genotyping of tail genomic DNA, and two lines with 11- and 4-bp deletions were chosen as founders. Using different pairs of sgRNAs, *Gnat2* and *Gnat3* KO mice were generated by the same strategy. The following sgRNAs were used to generate Gnat2 KO mice: left, 5′-GAACGTGCTGCAGTCCATCCTGG -3′ and right, 5′- TGCATAGTCAATGCCTAGTGTGG -3′. The following sgRNA pair was designed to generate *Gnat3* KO mice: left, 5′-GGATTGATTATGTCAATCCTAGG -3′ and right, 5′- GGCTTTCACAATAGCTAGGATGG -3′. The lines with 11- and 32-bp deletions of *Gnat2* and 19- and 4-bp deletions of *Gnat3* were used as the respective founders. The *Gnat* single KO mice were crossed for at least 5 generations to create double KO mice and for at least 10 generations to obtain triple KO mice for subsequent research. All sgRNAs mentioned above were designed using a CRISPR tool (http://www.e-crisp.org/E-CRISP/designcrispr.html). The primer sequences are list in the Table 1.

Reporter cells of TAS2R10/14

The expressive vectors harboring human Gα16/gust44, TAS2R10 and TAS2R14 cDNAs were used for establishing the reporter cells of TAS2R10/14 as previously reported(3). We co-transfected Gα16/gust44 with TAS2R10 or TAS2R14 vectors into HEK293T cells by using LipoMax transfection reagent (Sudgen, Nanjing). Twenty-four hours after transfection, the transfected HEK293T cells were digested with trypsin and then inoculated the isolated cells into black 96 wells plate (Costar, 3603) containing 200ul DMEM medium with 5% fetal bovine serum. Forty-six hours after transfection, the culture medium was replaced with serum-free DMEM medium and starved the cells for 2 hours. Then, fluo-4 dye was added to each well and incubated for 30-60 minutes. Immediately after treatment with bitter substances, the fluorescent signals were measured by microplate reader (BioTek, Synergic H1). The fluorescence intensity reflects the level of cytosolic calcium or TAS2Rs activation.

Isometric contraction measurement

Measurement of ASM isometric contraction was performed as previously described. Briefly, left extrapulmonary bronchi of WT and *Gnat* KO mice (age 8–10 weeks, both sexes) were isolated and immersed in cold Krebs solution (118.07 mmol/L NaCl, 4.69 mmol/L KCl, 2.52 mmol/L $CaCl_2$, 1.16 mmol/L $MgSO_4$, 1.01 mmol/L NaH_2PO_4, 25 mmol/L $NaHCO_3$, 11.1 mmol/L glucose). An approximately 1.5 mm segment was threaded onto two steel wires (40 mm in diameter) and then mounted in a small-vessel wire myograph chamber (610-M; Danish Myo Technology, Aarhus, Denmark) containing Krebs solution with a bubbling gas mixture (5% CO_2 and 95% O_2) at 37°C. After equilibration for 30 minutes, the variation in tension was recorded by a PowerLab recording device (AD Instruments, Australia). K^+ (60 mmol/L) was administered to obtain a resting tension of approximately 5-mN for proper preloading and was equilibrated for 30 minutes. After the administration of an additional 60 mmol/L K^+ the contractile responses to other stimuli were measured.

Primary mouse ASMC culture

The tracheae and extrapulmonary bronchi of mice (aged 6–8 weeks, including both males and females) were dissected from the surrounding tissues in ice-cold Krebs buffer, and ASM was disassociated from cartilage. The ASM was washed with 1 ml of 5X pen/strep (Life Technologies) in PBS five times then transferred into 1 ml of digestion buffer (0.2% collagenase, 0.15% trypsin) and cut into small pieces. After shaking for 30 minutes in a 37°C water bath, the mixture was triturated with a pipette tip several times, and the digestion process was halted by the addition of DMEM (12100046, Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco). The mixture was centrifuged at 1000 g for 3 minutes, and the resultant cell pellet was resuspended for culture. The cells were cultured and passed in DMEM supplemented with 10% FBS and 1x pen/strep. ASMCs cultured for 10 days were subjected to subsequent experiments.

Measurement of Ca^{2+} signals

Primary ASM of mice and HEK293T cells (100–500) were plated in each well of a 96-well plate (Costar, 3603) and cultured overnight. When the cells reached 80–95% density, fluo-4 dye was added, and the calcium signal was measured using a Fluo-4 Direct™ Calcium assay kit (Invitrogen, F10472) according to the manufacturer's instructions. Fluo-4 fluorescence was measured at an excitation wavelength of 494 nm and an emission wavelength of 516 nm with a microplate reader (BioTek, Synergic H1). After recording the basal fluorescence value of the resting cells for 20 seconds, reagents were added, and the resultant fluorescence was recorded immediately for 5 minutes at 2 second intervals. F_0 was defined as the initial fluorescence under resting conditions, while the dynamics of the Ca^{2+} signals were determined by $\%\Delta F/F_0$.

Immunofluorescence analysis

Cover slips were coated with 1% gelatine and then placed into the wells of a 24-well plate. Primary ASMCs (500–2000) were thereafter inoculated into the wells and cultured in DMEM supplemented with FBS for 60 minutes. After treatments with different reagents, the cells were immobilized by 4% paraformaldehyde (pH 7.4 in PBS) (Sigma) for 10 minutes and then washed three times with PBS. The coverslips were then blocked with 1% BSA and incubated with

individual primary antibodies overnight at 4°C. The primary antibodies used in the present study were rabbit anti-MAChR M3 (1:100, ab126168, Abcam) and mouse anti-Gnat 1 (1:100, sc-136143, Santa Cruz Biotechnology) or mouse anti-Gαi 2 (1:250, 67007-1-Ig, Proteintech). After a 1-hour incubation with the secondary antibodies and DAPI (BioSharp) at room temperature, the coverslips were washed and mounted with 50% glycerol in PBST. The secondary antibodies used in the present study were Alexa Fluor 546-conjugated donkey anti-rabbit IgG (A10040, Life Technologies), Alexa Fluor 546-conjugated donkey anti-mouse IgG (A10036, Life Technologies), Alexa Fluor 488-conjugated goat anti-rabbit IgG (A11008, Life Technologies), and Alexa Fluor 488-conjugated donkey anti-mouse IgG (A21202, Life Technologies). To detect colocalization, the coverslips were observed by a GE DeltaVision OMX multifunctional ultra-high resolution microscopic imaging system.

Western blot analysis

Proteins were analyzed by western blot as previously reported (4, 5). Briefly, ASMs isolated from the tracheae and extrapulmonary bronchi of control and KO mice were frozen in liquid nitrogen, rapidly transferred to a precooled acetone solution containing 10% TCA and 10 mM DTT, and stored at -80°C overnight. Then, the ASM was homogenized in a water solution containing 10% TCA and 10 mM DTT and pelleted by centrifugation. After washing with acetone and diethyl ether, the pellets were thoroughly dissolved and measured with a bicinchoninic acid kit (Pierce BCA protein assay kit, Pierce Biotechnology, Rockford, Ill). The proteins were sampled with 5x sample buffer (10% SDS, 20% glycerol, 0.05% bromophenol blue, 10 mmol/L β-mercaptoethanol, 200 mmol/L Tris-HCl, 8 mol/L urea) and denatured at 95°C for 5–10 minutes. After resolution by SDS-PAGE, the proteins were transferred to an activated polyvinylidene fluoride membrane and blocked with 5% nonfat milk in RIS-buffered saline with Tween 20, followed by sequential incubation with primary and horseradish peroxidase-conjugated secondary antibodies. The following primary antibodies were employed: MAChR M3 (1:1000, ab126168, Abcam), Gnat 1 (1:1000, ab74059, Abcam), Gnat2 (1:1000, ab97501, Abcam), Gnat3 (1:1000, E-AB-16465, Elabscience), β-Actin (AC15, 1:5000; Sigma-Aldrich), p-RLC (#3675, 1:1000, CST), total RLC (1:1000, rabbit, donated by James T Stull), FLAG (1:1000, clone M2, F1804, Sigma–Aldrich), HA (1:1000, rabbit mAb, C29F4), and GFP (1:1000, GF28R, MA5–15256, Thermo Fisher Scientific).

Plasmid construction

Full-length *MAChR M3* and *Gnat2* were amplified from a mouse liver cDNA library, whereas *Gnat1* and *Gnat3* (full-length; 1–1029 nt; 1029–1139 nt) were synthesized by Genscript (Nanjing, China), and fragments of *Gnat1* with differing lengths (1–1029 nt; 1029–1139 nt) were amplified from the full-length plasmid. The PCR primer pairs are shown in Supplement Table 1. The fragments were inserted into the appropriate tagged expression vectors with a ClonExpress MultiS one-step cloning kit (C113, Vazyme, Nanjing, China). *MAChR M3* was cloned into pCMV-FLAG, and *Gnat1* fragments were cloned into pCMV-HA (both via XhoI and NotI multiple cloning sites); *Gnat1* (1029–1139) containing the H5 region was cloned into pEGFP-C3 via Not I and Xho I sites. The Gnat2 and Gnat3 plasmids were constructed as described above. The plasmid graph is shown in Fig S4.

The following plasmids for the human bitter receptor and its Gα subunit were purchased from Hongxun Biotechnology Company (Nanjing, China): pDsRed2-ER-SST3-T2R10/14,

pcDNA3.1(+) - Gα16/gust44, and pDsRed2-ER.

Coimmunoprecipitation of *Gnats* and M3-type acetylcholine receptor (MAChR M3)

Immunoprecipitation was performed to assess interactions between *Gnats* and MAChR M3. HEK 293T cells (2×10^5) were plated in 60-mm dishes and cultured for 24 hours before being transfected with the expression plasmids using LipoMax transfection reagent (Sudgen Biotech, Nanjing, China, 32012). Forty-eight hours after transfection, the cells were homogenized with 600 µl of lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), and 1% Triton X-100) supplemented with 1 mM PMSF and 1X proteinase inhibitor cocktail (Roche, Switzerland). The homogenate was incubated on ice for 30 minutes and centrifuged at 12,000 rpm for 15 minutes. Then, 500 µl of the resultant supernatant was added to cleaned FLAG beads (Smart-Lifesciences, SA042005) and incubated overnight at 4°C, while 80 µl of the remaining supernatant was preserved as the total protein sample. After centrifugation at 5,000 g, the pellets were washed twice with low-salt buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) and once with high-salt buffer (500 mM NaCl, 50 mM Tris-HCl, pH 7.4). The washed pellets were then sampled for Western blot analysis.

Asthma mouse model and measurement of respiratory resistance

Asthma model generation and respiratory resistance detection were performed as previously reported (4, 6). Mice of both sexes were used at 8–10 weeks of age. The mice were sensitized with 100 µg of ovalbumin (OVA, Sigma-Aldrich, St Louis, MO in 200 µl of aluminium hydroxide (Inject Alum, Pierce) by intraperitoneal injection on days 0 and 14. On days 24, 25 and 26, the mice were challenged with aerosolized 1.5 ~2% OVA in PBS for 60 minutes. Twenty-four hours after the last challenge, the mice were sedated with 250 to 300 mg/kg avertin, intubated with a blunt mouth 18-gauge metal needle, and ventilated, after which airway resistance measurements were taken as previously described. Respiratory resistance was measured by the flexiVent system (SCIREQ). The ventilator was set to generate a 10 mL/kg tidal volume at a frequency of 150 breaths per minute. The Rrs was measured by a perturbation of SnapShot. The Rn was measured by a perturbation of Quick prime-3 (SCIREQ, Montreal, Quebec, Canada). Sequential doses of 2.0, 4.0, 8.0, 16, 32 and 64 mg/mL MCh were nebulized to challenge the airway until a dose resulted in a sustained airway resistance that was approximately four- to five-fold higher than that at the baseline. At three minutes after the last MCh inhalation, the bitter tastant kudinoside A (3 µg) or chloroquine (150 µg) was nebulized over a 10 second period. Resistance measurements (Raw, cm H_2O ml^{-1} s^{-1}) were taken every 30 seconds throughout the experiment.

The bronchoalveolar lavage fluid (BALF) was collected by instillation of the lung with 500µl of PBS, and then centrifuged 100µl of BALF (1500 rpm x5min at 4°C) onto a slide so as to adhere the cells firmly on the slide in a circle of 1 cm diameter. The cells were stained with Diff-Quik Stain (Leagene, Beijing, China) and examined under a microscopy. The eosinophil percentages over the total inflammatory cells were calculated within at least three fields per mouse. The total cells were counted with a haemocytometer.

Measurement of respiratory resistance in rats

Wistar rats aged 46–62 days were subjected to COPD therapy. To establish the COPD disease model, the animals were administered SO_2 via inhalation twice a week for 7 weeks. The COPD

phenotypes were evaluated by whole-body plethysmography (WBP) with an EMMS animal respiratory function detection system (EMM Company, AAC060). After successful establishment of the COPD model, the rats were treated with different reagents by nebulized inhalation for 30 minutes once a day for six days a week. During this period, SO_2 was inhaled, but the frequency was reduced to once a day until the 14th week of the experiment. Respiratory function was measured with a flexivent forced concussion pulmonary function detector with a negative pressure-driven forced expiration (NPFE) module (SCIREQ, FX-4). Airway resistance was evaluated by calculating the forced expiratory volume in 0.2 seconds (FEV0.2) and the forced vital capacity (FVC) ratio (FEV0.2/FVC). After measurement, the left lung was dissected, fixed and stained with haematoxylin and eosin (HE) for the analysis of infiltrated inflammatory cells in the lung parenchyma and interstitial structure.

Acute lung injury mouse model

Aerosolized LPS was utilized to induce acute lung injury and inflammation(7, 8). Female mice at age 8-12 weeks were exposed simultaneously to aerosolized 8ml LPS (2mg/ml) in a custom-built cylindrical chamber connected to an air nebulize, and allowed the mice to inhale LPS (sigma, 0111:B4, L4130) for 60 minutes. The mice of KE-A treatment group were then exposed to aerosolized 8ml KE-A (150μg/ml) for three times (respectively at 1h, 6h, 24h after LPS treatment), and inhaled for 60 minutes. All the mice were euthanized at 48 hours after LPS exposure. The left lungs were fixed with 4% paraformaldehyde (PFA) for histological examination. The right lungs were sampled immediately for real-time PCR and Luminex assays.

Luminex assays

Lung tissues were frozen in liquid nitrogen and homogenized in 500μl lysis buffer (50mM Tris-HCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 10mM sodium β-glycerophosphate, 50mM NaF, 5mM NaPPi, 1X Protease inhibitors cocktail). After incubated in ice for 30 minutes, the homogenizations were centrifuged twice at 12000rpm for 10min at 4°C. The protein concentration of the resultant supernatant was measured with a bicinchoninic acid kit (Pierce BCA protein assay kit), and all samples were adjusted to the same protein concentration for subsequent experiments. The protein level of multiple inflammatory cytokines was detected by Luminex assays kit (LX-MultiDTM-31, R&D systems), and this assay was served by LabEx (Shanghai, China).

Real-time PCR

Quantitative RT-PCR was performed as described previously(9). Briefly, total RNA was extracted from the mice lungs by using RNAiso Plus (Takara Bio). Then, 1 μg total RNA was reversely transcribed with the HiScript® Q RT SuperMix (Vazyme, R123) according to the manufacturer's instructions. Real-time quantitative qPCR was performed using the ABI QuantStudio 5 system with Taq pro universal SYBR qPCR Master Mix (Vazyme, Q712). The primers for target genes are listed in Table 2. The *36b4* was used as reference gene.

Histology

The tissues were immersed in 4% PFA at 4°C overnight and dehydrated in a graded series of ethanol. The tissues were embedded in paraffin and cut into 10-μm-thick sections for standard HE staining. The thickness of the smooth muscle layer was measured from the innermost edge to the

outermost edge using ImageJ software (National Institutes of Health, Bethesda, Md). The standard of immune cell infiltration scores: 0=none, 1=sporadic scattered, 2=diffused distribution, 3=focal diffused/occasionally clustered, 4=multifocal diffused/clumped, 5=large area visible/random visual field are all visible distribution.

Modified Movat's pentachrome stain was used to distinguish the smooth muscle, collagen, ground substances and fibrinoids in the lung tissues of rats with COPD and control rats. After the routine dewaxing of paraffin sections and dehydration with a series of increasing concentration of ethanol solution, the sections were stained sequentially according to the manufacturer's instructions (Shanghaiyuanye Bio-Technology, R20396). Mucin and ground substances were stained blue with Alcian blue, nuclei and elastic fibres were stained black with Weigert haematoxylin solution, fibrinoids and muscles were stained red with Crocein Scarlet/Acid Fuchsin, and collagen and reticular fibres were stained yellow with saffron.

The AB-PAS staining was used for staining acidic mucin protein in the lung tissue of asthmatic animals. The sections were stained sequentially according to the manufacturer's instructions (Shanghaiyuanye Bio-Technology, R23046). Glycogen and neutral mucin were stained purplish red, Acidic mucins (sulfomucins and salivary mucins) and hyaluronic acid were stained blue. After PAS staining, at least three random airways were scored as follows: 1 = nil (<5% PAS^+ goblet cell staining), 2 = mild (5-30% PAS^+ goblet cells), 3 = moderate (30–60% PAS^+ goblet cells), and 4 = severe (>60% PAS^+ goblet cells). The total score was then divided by the number of airways examined to give an average severity score per airway, the mucus-goblet index.

To assess the LPS-induced pulmonary injury of mice, we used a small animal acute lung injury (ALI) model scoring scheme generated by the American Thoracic Society (ATS) as previously described(10, 11). Briefly, three random fields of lung tissue were scored in a blinded manner. The following parameters were analyzed for the ATS/ALI scoring system: (A) neutrophils in the alveolar space (none = 0, 1-5 cells = 1, > 5 cells = 2); (B) neutrophils in the interstitial space/septae (none = 0, 1-5 cells = 1, > 5 cells = 2); (C) hyaline membranes (none = 0, one membrane = 1; > 1 membrane = 2); (D) proteinaceous debris in air spaces (none = 0, one instance = 1, > 1 instance = 2); (E) alveolar septal thickening (< 2x mock thickness = 0, 2-4x mock thickness = 1, > 4x mock thickness = 2). Scores were calculated as followed: [(20 x A) + (14 x B) + (7 x C) + (7 x D) + (2 x E)] / 100. Final scores were obtained by averaging three fields per mouse. The defuse alveolar damage (DAD) scores were determined as followed: 1 = absence of cellular sloughing and necrosis; 2 = uncommon solitary cell sloughing and necrosis; 3 = multifocal (>3) cellular sloughing and necrosis with uncommon septal wall hyalinization; 4 = multifocal (> 75% of field) cellular sloughing and necrosis with common and/or prominent hyaline membranes. The final DAD score determined by the average of at least three fields per mouse.

Immunohistochemistry analysis of macrophages

Briefly, lung sections were dewaxed in xylene, rehydrated, and incubated with 0.5% hydrogen peroxide in methanol for 10 minutes to inhibit endogenous peroxidases. The sections were incubated with an anti-EMR1 antibody (1:200, 27044-1-AP, Proteintech) overnight at 4°C, and immunodetection was performed using a biotinylated secondary Ig reagent (UltraSensitiveTMS-P (Mouse/Rabbit), MAB biotechnologies) and DAB solution (MAB biotechnologies). The nuclei were counterstained with haematoxylin solution.

Statistical analysis

The data are presented as the means ±SDs. Statistical analyses were performed using Prism 6 software. Differences among the groups were determined by two-way ANOVA or the unpaired two-tailed Student's t-test. A paired two-tailed t-test was also used in some experiments to evaluate differences. The significance levels were set as follows: not significant (NS), $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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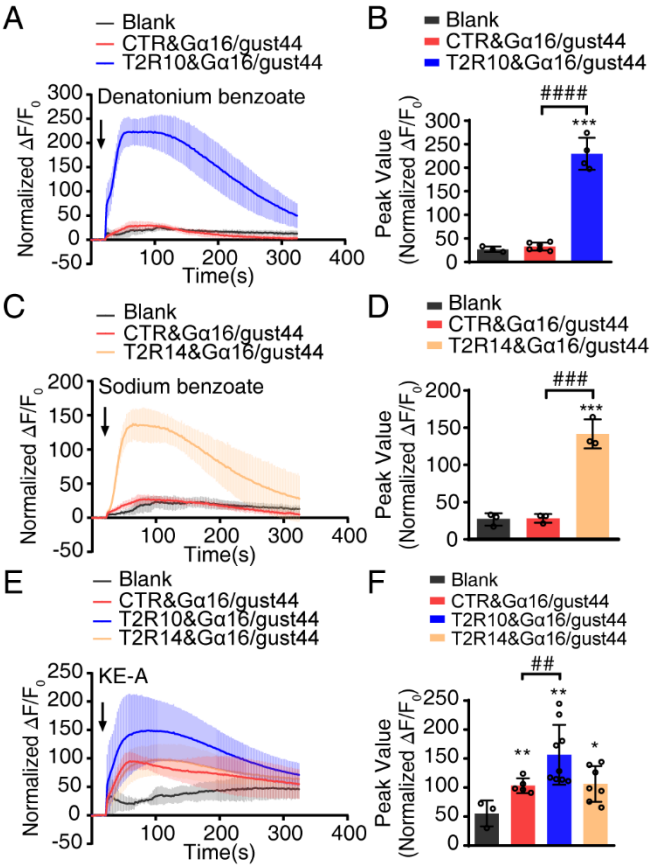

Fig. S1. KE-A is a TAS2R agonist. HEK293T cells were co-transfected with expression vectors encoding TAS2R10 or TAS2R14 plus G_{16}/$gust_{44}$, and the transfectants were used as TAS2R10 (blue) and TAS2R14 reporters (orange), respectively. The reporters were then incubated with fluo-4 for cytosolic visualization. Upon treating the cell reporters with KE-A (50 μM), sodium benzoate (1 mM) and denatonium benzoate (1 mM), the cytosolic calcium levels were measured. A and B: The cell reporters were treated with the TAS2R10 agonist denatonium benzoate; C and D: The cell reporters were treated with sodium benzoate, the agonist of TAS2R14. E and F: The reporter cells were treated with KE-A. # compared with the blank control, * compared with the control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n>3.

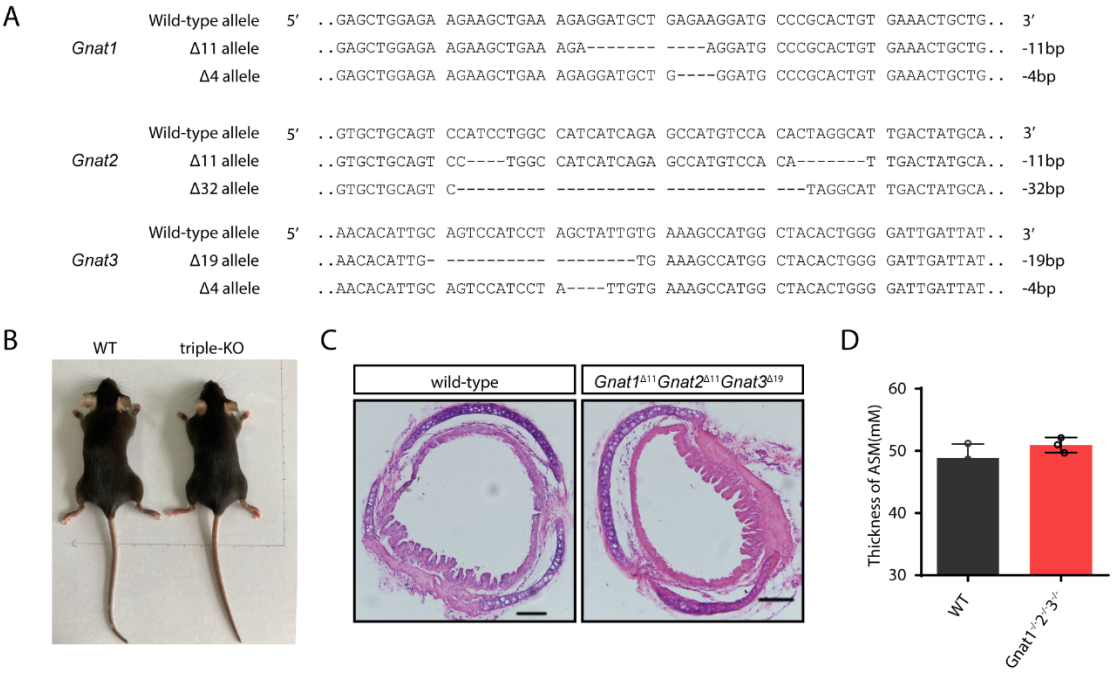

Figure S2. Generation of *Gnats* knockout mice. A: Sequences of six mutant alleles in ES cell clones. B: Images of wild-type and *Gnat1*$^{-/-}$ *Gnat* 2$^{-/-}$ *Gnat* 3$^{-/-}$ mice at 8 weeks old. C: Representative haematoxylin and eosin staining of left extrapulmonary bronchi from wild-type and triple knockout mice (Scale bar= 100μm). D: *Gnat*s deletion did not alter the thickness of the bronchus (n=3). The data are presented as the mean ± SD; unpaired Student's *t*-test.

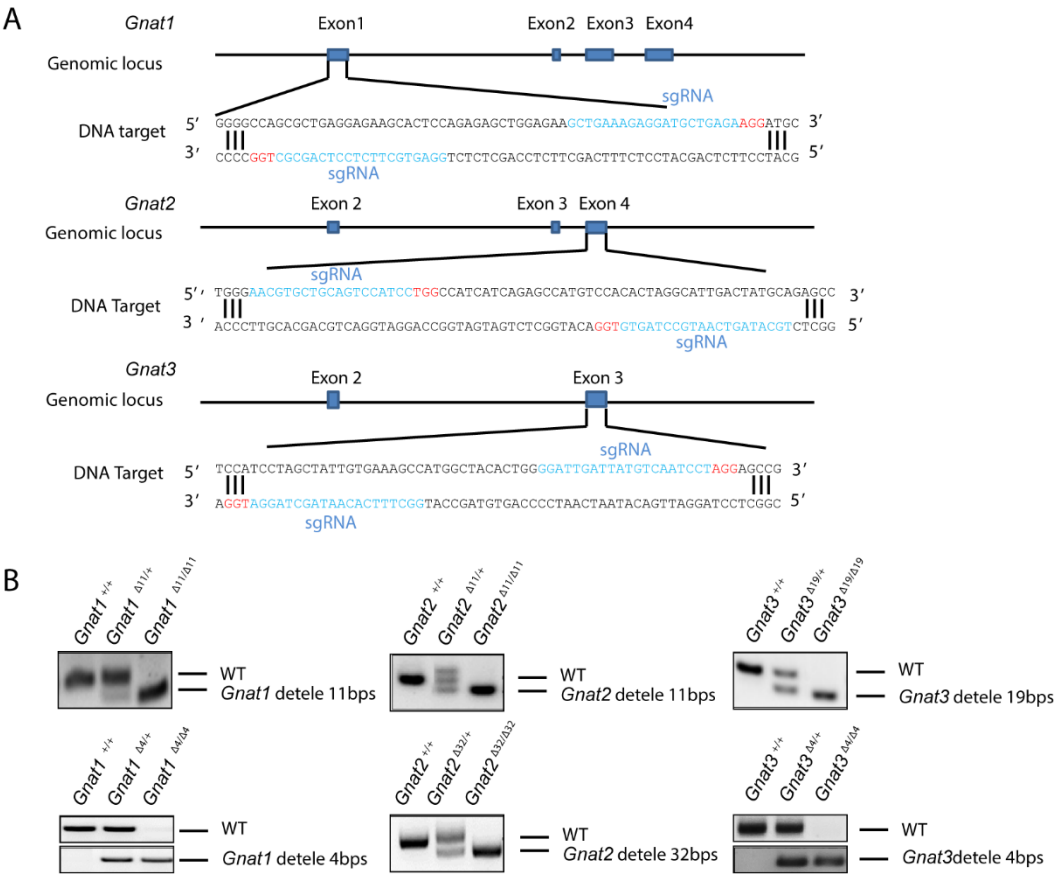

Figure S3. Generation of *Gnats* knockout mice. A: Schematic of the Cas9/sgRNA-targeting sites in *Gnat1, 2*, and *3*. The sgRNA target sequence is underlined. The protospacer-adjacent motif (PAM) sequence is labelled in red. B: The genotyping patterns of *Gnats* knockout mice.

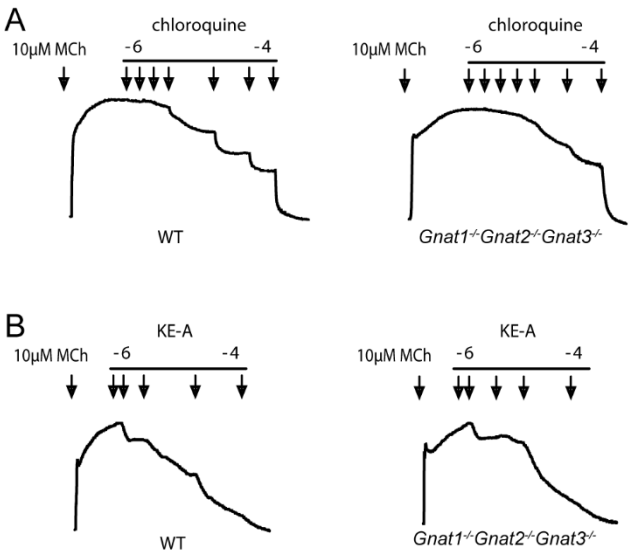

Figure S4. Typical recording traces of the dose-responsive relaxation of chloroquine (A) and KE-A (B) in the control and *Gnats* triple knockout (TKO) mice.

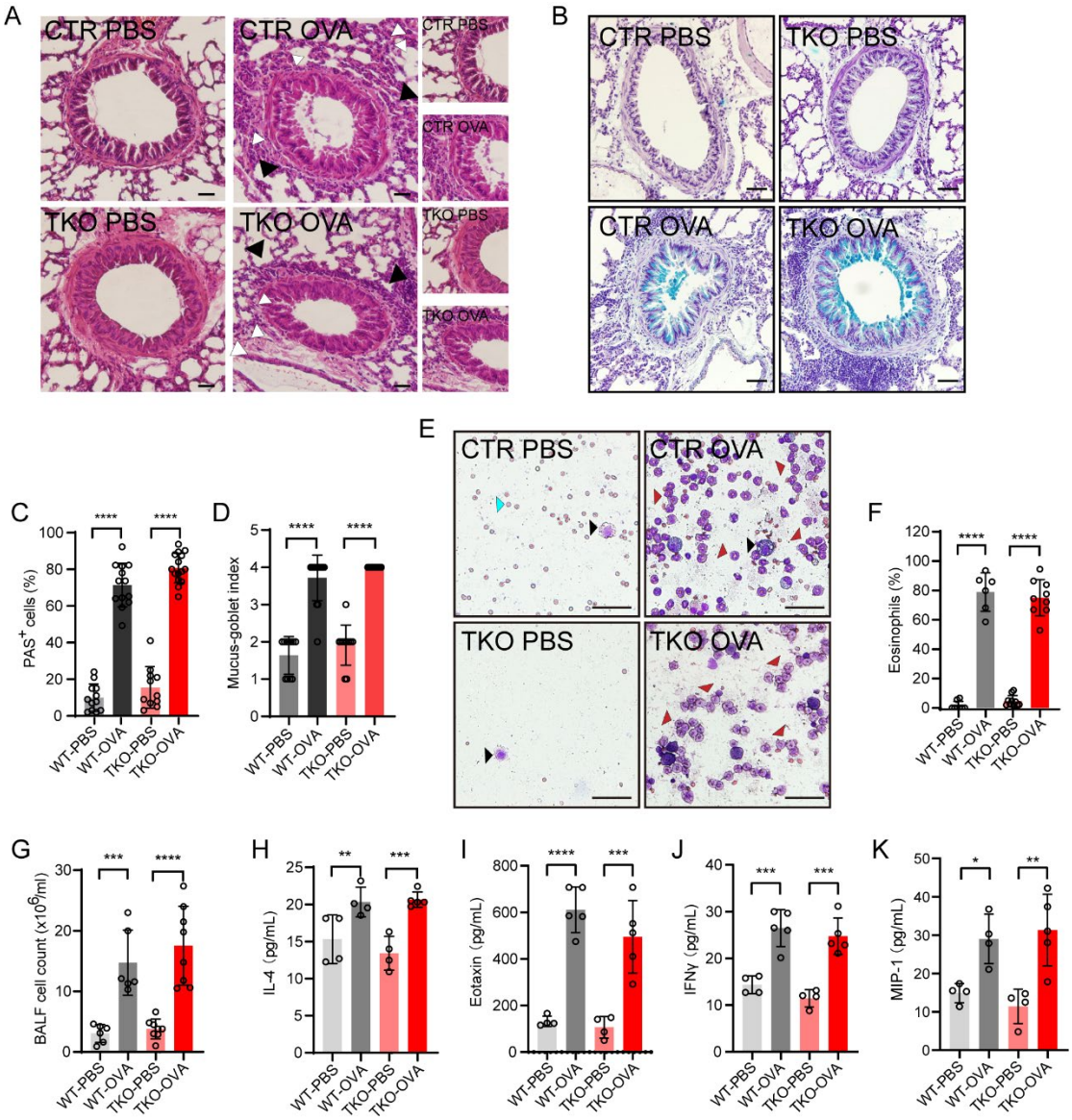

Figure S5. Asthmatic characteristics of control and *Gnats* triple knockout (TKO) mice. A: Control and mutant mice displayed comparable asthmatic pathologies, eg. increased inflammatory cell infiltration and swelled epithelial goblet cells. The black arrows indicate the positions with inflammatory cell infiltration. The white arrows indicate the eosinophil infiltration (Scale bar= 50μm). B: PAS staining showed the increase of acidic mucin protein (stained in blue) in the airway after OVA treatment. C: Statistic graphs represent the percentage of mucus-positive cells in the airways. D: Quantification of mucus production by mucus-goblet index. E: Eosinophil cells in the bronchoalveolar lavage fluid (BALF) were stained by Diff-Quik Stain and visualized under a microscope. The red arrows indicate the eosinophils, black arrows indicate the monocytes, blue arrows indicate the erythrocytes. Scale bars=50 μm. F: Ratio of eosinophils in the inflammatory cells of BLAF. G: Total cells in the BALF. H-K: The protein levels of cytokines, eotaxin (CCL11) and macrophage inflammatory protein-1 (MIP-1) in the lung homogenization were measured by the Luminex assay.

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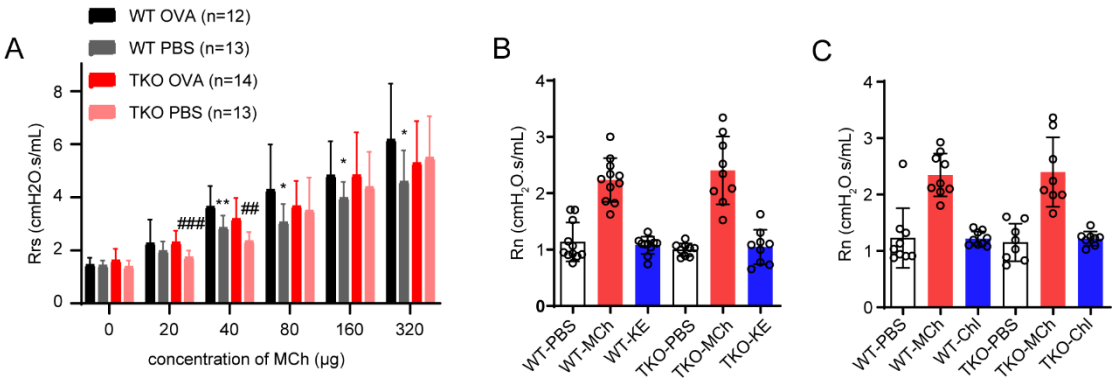

Fig S6. Pulmonary reactivity of control mice and *Gnats* triple knockout mice (TKO). A: Asthmatic animals displayed higher airway response to MCh compared with control groups. * compared with the CTR PBS group as determined by an unpaired Student's t-test; #compared with the TKO PBS group as determined by an unpaired Student's *t*-test. B-C: The values of central airway resistance (Rn) were comparable among WT and TKO groups treated with KE or Chl. *P < 0.05, **P < 0.01, **** P < 0.0001, unpaired Student's *t*-test and Two-way ANOVA.

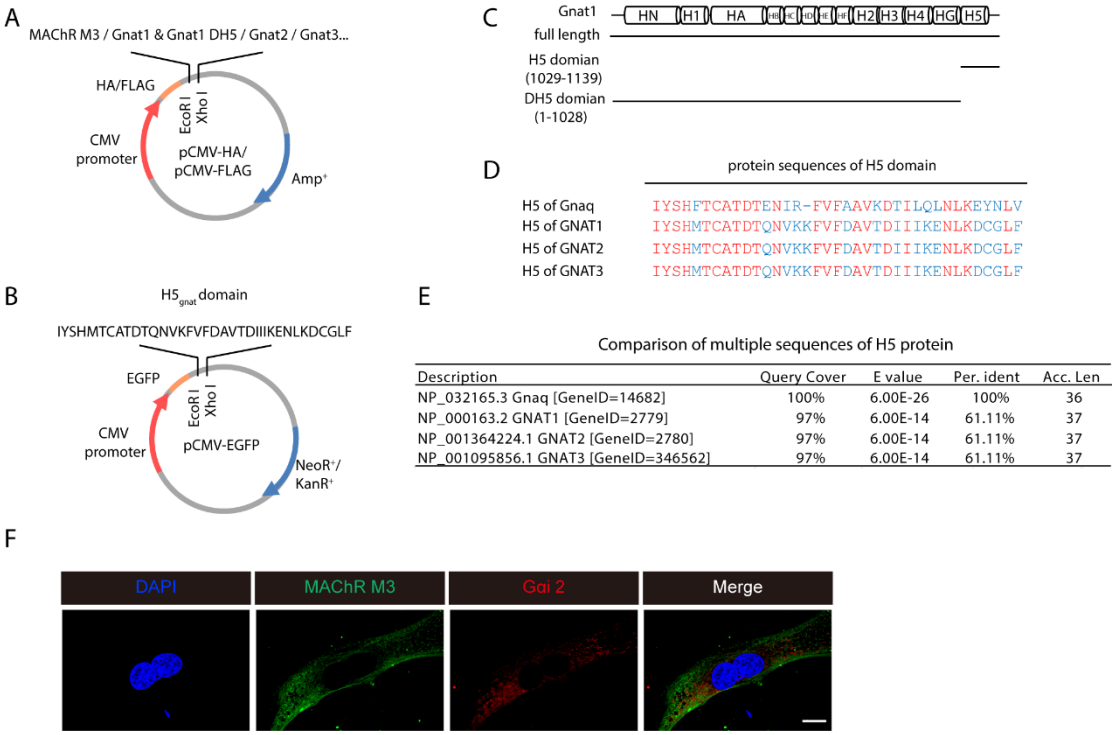

Figure S7. Construction of expression plasmids encoding the recombinant Gnat variants. A: Gene fragments encoding the intact M3 receptor and Gnat1/2/3 as well as Gnat1 with a truncated H5 sequence (Gnat1 DH5) were subcloned into the pCMV-HA/FLAG vector via EcoR I/Xho I restriction sites. B: The DNA fragment encoding the Gnat1 H5 protein was subcloned into the pCMV-EGFP vector through EcoRI/XhoI. C: Gnat1 variants, full-length Gnat1 H5 domain and Gnat1 without the H5 domain (DH5). D: Alignment of the H5 sequences of Gnaq and Gnats. Note that the H5 sequences of Gnat1, Gnat2 and Gnat3 are identical. E: The similarities of the H5 domains in Gnaq and Gnats. F: There is no co-localization between Gαi 2 and MAChR M3 In the airway smooth muscle cells. MAChR M3 stained in Green, red indicted Gαi 2, yellow indicates co-localization. (Scale bar =10μm)

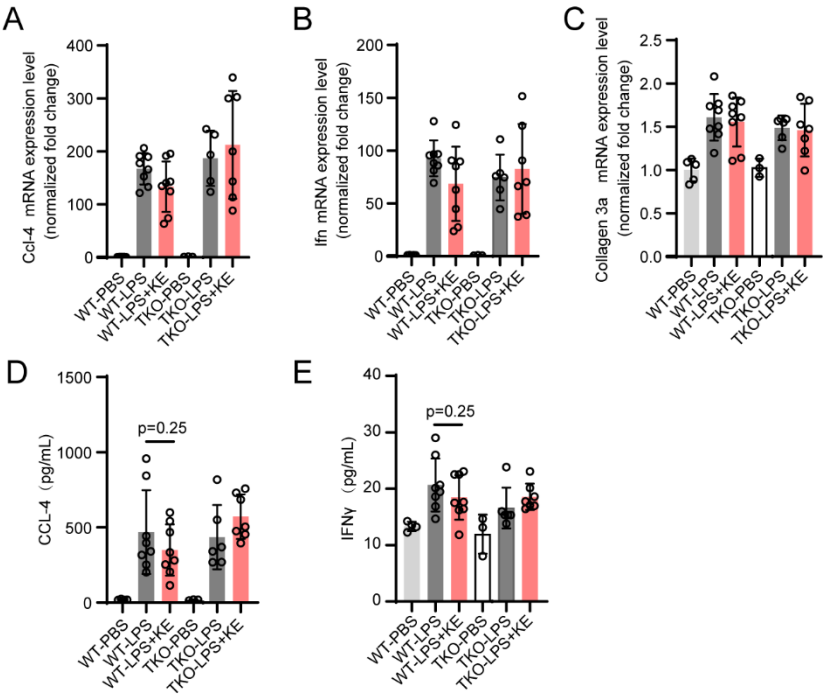

Figure S8. The expression of inflammatory cytokines and collagen3a in the mice with acute lung injury. A-C: The mRNA expression of inflammation-associated genes in the lung tissue (n= 3-8). D-E: The protein concentrations of CCL-4 and IFNγ in the lung lysates (n= 3-8).

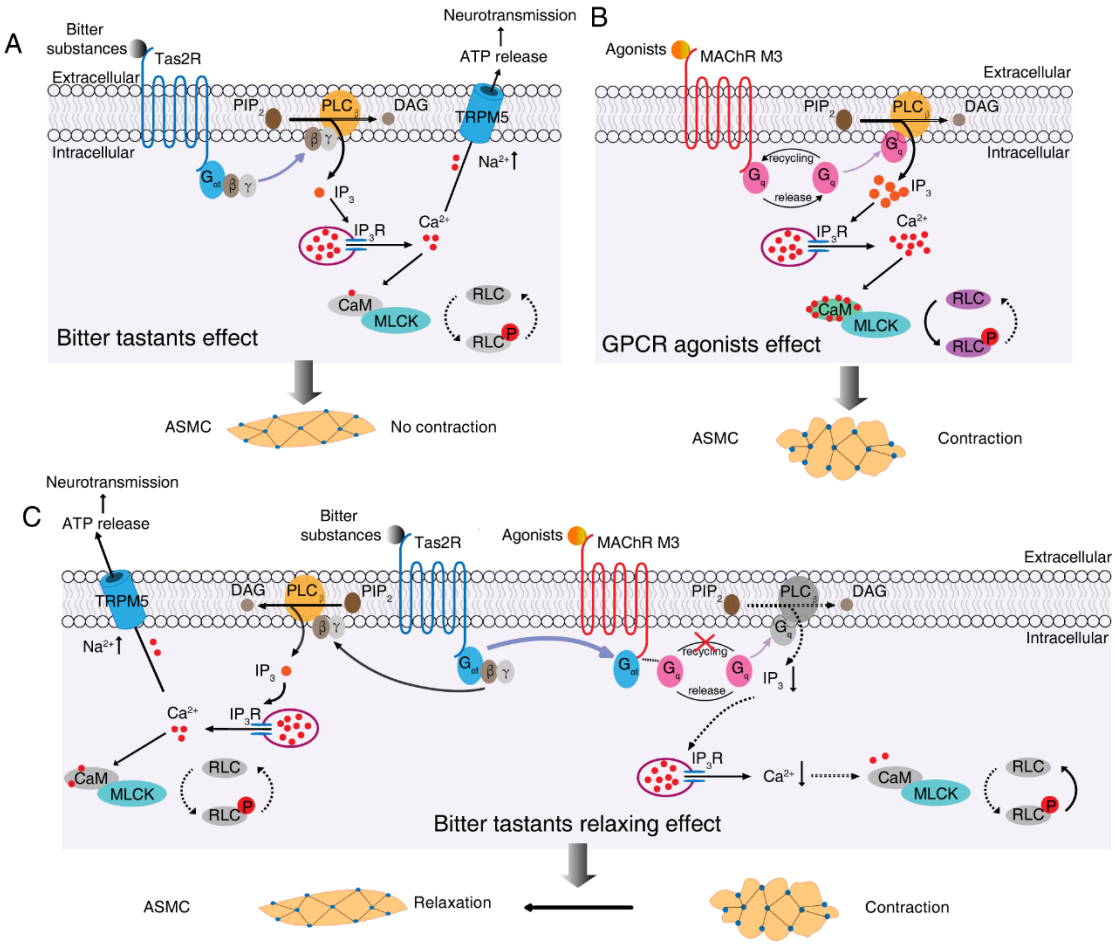

Figure S9. Working model of the relaxation initiated by bitter substances. A: The sensation signaling pathway of the bitter taste receptor. B: The Gq-associated signaling pathway of AChR-mediated ASM contraction. C: Gnat released after Tas2R agonistic activation binds with MAChR M3 and inhibits Gq recycling, calcium release and RLC phosphorylation, thereby relaxing the contraction of ASM.

Primer name	Sequence
Gnat1Δ11 Forward primer	AGAAGCACTCCAGAGAGCTG
Gnat1Δ11Reverse primer	ACACCCCTACCCAGAAGCAG
Gnat2Δ11 Forward primer	TGTCATCTATGGGAACGTGC
Gnat2Δ11 Reverse primer	ATCACGTACCGCACAGCTTG
Gnat3Δ19 Forward primer	AACAAGAATGCATGGAGTTT
Gnat3Δ19 Reverse primer	ACATAATCAATCCCCAGTGT
Gnat1Δ4 common Forward primer	CAGGTCTGTGGAGAGCCAGT
Gnat1Δ4 Reverse primer	ACAGTGCGGGCATCCCAGCA
Gnat1Δ4-WT Reverse primer	CAGTTTCACAGTGCGGGCATCCTTCTCAG
Gnat2Δ32 Forward primer	AGGGTCCAGTTAAAAGCTTA
Gnat2Δ32 Reverse primer	ACTTCTTCTCCTGCTTCCAC
Gnat3Δ4 common Forward primer	ATTGCAGGATCATCCATAAG
Gnat3Δ4 Reverse primer	CCATGGCTTTCACAATAGGA
Gnat3Δ4-WT Reverse primer	CCCAGTGTAGCCATGGCTTTCACAATAGCT

Table S1. The primer sequence for genotyping of *Gnat*s knockout mice.

Mouse gene	Forward 5’ to 3’	Reverse 5’ to 3’
IL-1β	AAAGATGAAGGGCTGCTTCC	TTCTCCACAGCCACAATGAG
TNFα	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
CCL-4	TTCCTGCTGTTTCTCTTACACCT	CTGTCTGCCTCTTTTGGTCAG
CSF 3	ATGGCTCAACTTTCTGCCCAG	CTGACAGTGACCAGGGGAAC
TGFβ	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
Collagen 3A	CTGTAACATGGAAACTGGGGAAA	CCATAGCTGAACTGAAAACCACC
36b4	ATCCCTGACGCACCGCCGTGA	TGCATCTGCTTGGAGCCCACGT

Table S2. The sequences of Real-time RCR primers for testing the inflammation of acute lung injury model.