Mas Receptor Activation Attenuates Allergic Airway Inflammation via Inhibiting JNK/CCL2-induced Macrophage Recruitment

Luna Hong
Department of Pulmonary and Critical Care Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.

Ming Chen
Department of Pulmonary and Critical Care Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.

Jianting Shi
Department of Pulmonary and Critical Care Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.

Qiujie Wang
Department of Pulmonary and Critical Care Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.

Yimin Guo
Department of Pulmonary and Critical Care Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.

Shanying Liu
Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Medical Research Center, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.

Ruijian Pan
Departments of Electric Power Engineering, South China University of Technology, Guangzhou, China

Xiaoqing Yuan
Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Medical Research Center, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.

Shanping Jiang (✉ jiangshp@mail.sysu.edu.cn)
Department of Pulmonary and Critical Care Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China. https://orcid.org/0000-0002-6222-0282

Research

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Abstract

Background: Defective absorption of acute allergic airway inflammation is involved in the initiation and development of chronic asthma. After allergen exposure, there is a rapid recruitment of macrophages around the airways, which promote acute inflammatory responses. The Ang-(1-7)/Mas receptor axis reportedly plays protective roles in various tissue inflammation and remodeling processes *in vivo*. However, the exact role of Mas receptor and their underlying mechanisms during the pathology of acute allergic airway inflammation remains unclear.

Methods: Mas receptor expression was assessed in ovalbumin-induced acute asthmatic murine model. Then we estimated the anti-inflammatory role of Mas receptor *in vivo* and explored expressions of several known inflammatory cytokines as well as phosphorylation levels of MAPK pathways. Mas receptor functions and underlying mechanisms were studied further in the human bronchial epithelial cell line (16HBE).

Results: Mas receptor expression decreased in acute allergic airway inflammation. Multiplex immunofluorescence co-localized Mas receptor and EpCAM, indicated that Mas receptor may function in the bronchial epithelium. Activating Mas receptor through AVE0991 significantly alleviated macrophage infiltration in airway inflammation, accompanied with down-regulation of CCL2 and phosphorylation levels of MAPK pathways. Further studies in 16HBE showed that AVE0991 pre-treatment inhibited LPS-induced or anisomycin-induced CCL2 increase and THP-1 macrophages migration via JNK pathways.

Conclusion: Our findings suggested that Mas receptor activation significantly attenuated CCL2 dependent macrophage recruitments in acute allergic airway inflammation through JNK pathways, which indicated that Mas receptor, CCL2 and phospho-JNK could be potential targets against allergic airway inflammation.

Background

Asthma, characterized by allergic airway inflammation, affects about 10% of adults and an even greater portion of children [1]. The most effective treatment for asthma is inhaled corticosteroids, with or without long-acting β_2-agonist and muscarinic antagonists. However, even with appropriate inhaled therapy, a significant number of patients' asthma is still poorly controlled. The Measuring Asthma GINA Control Study, conducted in primary care, pulmonology, and allergic clinics, found that only 13.6% of participants had well-controlled asthma according to GINA criteria [2]. It is widely accepted that recruited eosinophils, neutrophils, macrophages, and $CD4^+$ T cells are powerful mediators and lead to persistent inflammation [3]. The inability to resolve ongoing inflammation may be crucial for the initiation and development of chronic asthma.

Macrophages, one of abundant immune cells in the lung, play an important role in allergen-induced airway inflammation in asthma [4]. After allergen exposure, there is a rapid recruitment of macrophages, which promotes acute inflammatory responses [5]. Hadjigol et al. reported that macrophage depletion

reduced inflammatory infiltrates and airway hyper-responsiveness (AHR) induced by ovalbumin (OVA) treatment [6]. The chemokine (C-C motif) ligand 2 (CCL2) is a known cytokine which functions in recruitment of macrophages to inflammation sites [7]. Blockade of CCL2/CCR2 signaling pathway inhibits recruitment of inflammatory macrophages and exerts protective function in OVA-induced allergic asthma [8]. Hence, pharmacological suppression of CCL2 signaling and its upstream pathways might exhibit crucial therapeutic potential in resolving inflammatory macrophages and attenuating airway inflammation.

In recent years, the ACE2/Ang-(1–7)/Mas receptor axis has attracted more and more attention for its anti-inflammatory and anti-remodeling effects in different pathological conditions. Ang(1–7) is a 7-amino acid peptide hormone of the renin-angiotensin system, acting on the G protein-coupled receptor Mas [9]. Barroso LC et al. reported that Ang(1–7) promotes neutrophilic inflammation resolution through NF-κB inhibition in arthritic mice [10]. In orthotopic breast tumors, Ang(1–7) serves as an anti-fibrotic agent to reduce tumor growth and fibrosis by reducing MAPK activity [11]. An *in vitro* study by Mei Jiang et al. revealed that the Ang-(1–7)/Mas receptor axis may drive anti-inflammatory effects through JNK/FoxO1 signaling pathway in LPS-induced macrophages [12]. Besides, Ang-(1–7) acts on Mas receptor to suppress p38 MAPK and NF-κB pathway and alleviate Ang□-induced ICAM-1, VCAM-1, and CCL2 expression in HUVECs [13]. In lung diseases, especially asthma, Ang(1–7) reportedly reduces lung inflammation and attenuates airway remodeling, while Mas receptor has been detected in the epithelium and bronchial smooth muscles [14–18]. However, the exact role of Mas receptor in bronchial epithelium during the pathology of acute allergic airway inflammation remains unclear.

Here, we showed that Mas receptor expression decreased in acute allergic airway inflammation and co-localized Mas receptor with EpCAM (bronchial epithelium marker). *In vivo* studies showed that AVE0991 significantly alleviated airway inflammation, especially macrophage infiltration, accompanied with down regulation of CCL2 and phosphorylation levels of MAPK pathways. *In vitro* studies in 16HBE showed that AVE0991 pre-treatment inhibited LPS- or anisomycin-induced CCL2 increase and THP-1 macrophages migration via JNK pathway. Our data suggests that besides Mas receptor, CCL2 and phospho-JNK serve as potential targets for allergic airway inflammation.

Methods

Animals

All animals' care and experimental procedures were approved by the Animal Research Committee of Sun Yat-sen University (application number 2017189). Twelve animals total were used in our experiments. Male BALB/c mice aged six to eight weeks, purchased from Beijing Vital River Laboratory Animal Technology Company, were housed in pathogen-free conditions. All mice were maintained in a 12/12h light-dark cycle with well temperature-control and were given free access to standard food and tap water.

OVA Immunization, Challenge, and AVE0991 Treatment

As shown in Figure S1, the mice were sensitized with intraperitoneal injections (*i.p.*) of 10μg OVA (A5503; Sigma) emulsified in 1mg aluminium hydroxide (122261; SERVA) for a total volume of 200ul on days 1, 7, and 14. Beginning on day 21, sensitized mice were challenged by 2.5% OVA (atomized for 30min on 7 consecutive days) [19-21]. Control mice were given saline *i.p.* (200μl per mouse) and challenged with saline at the same time points. During the challenge period, half of OVA groups were treated with 1mg/(kg·d) AVE0991 (B1007; APExBio;) by visible intratracheal administration an hour before atomization (Figure S2).

Blood and Lung Samples

Twenty-four hours after the last atomization, mice were anesthetized with sodium pentobarbital. The blood was collected and centrifuged (3000rpm for 10min at 4℃) to isolate plasma for an ELISA. The left lung lobe was harvested for histological analysis, while the right lung was snapped frozen in liquid nitrogen and kept at -80℃ for further analysis.

Histopathology, Immunohistochemistry, and Immunofluorescence

The left lungs were perfused with PBS, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4μm-thick slices. Sections were stained with H&E, PAS, and Masson's trichrome for the analysis of morphology, inflammation, and airway remodeling. Relative semi-quantitative analyses were performed according to a previously described protocol [22]. Sections were deparaffinized and rehydrated followed by microwaved antigen retrieval in citric acid buffer (pH 6.0) and endogenous peroxidase blocking with 3% H_2O_2. Thereafter, sections were blocked with 5% BSA for 30 min at room temperature and incubated with an anti-Mas receptor antibody (D222656; Sangon Biotech) overnight at 4°C in a humidified chamber. The staining was revealed followed by incubation with an HRP-conjugated secondary antibody for 1 h at room temperature and the diaminobenzidine substrate kit for peroxidase. Counterstaining was carefully performed using hematoxylin. For immunofluorescence, after 5% BSA blocking, sections were incubated with anti-F4/80 antibody (GB11027; Servicebio) or anti-ECP antibody (ab181697; Abcam) overnight at 4°C, followed by incubation with an FITC-conjugated secondary antibody (BA1105; BOSTER). To co-localize Mas receptor with bronchial epithelium or airway smooth cells, sections were incubated with an anti-Mas receptor antibody on the first day. After incubation with the FITC-conjugated secondary antibody, sections were incubated with an anti-EpCAM antibody (GB11274; Servicebio) or anti-α-smooth muscle actin antibody (ab5694; abcam) for another night, followed by incubation with a Cy3-conjugated secondary antibody (BA1032; BOSTER). Nuclei were visualized with DAPI and sections were photographed with an inverted fluorescence microscope (Olympus IX71). Fluorescence integrated density measurements [23] were made using ImageJ software (version 5.0, NIH)

Enzyme-Linked Immunosorbent Assay (ELISA)

Concentration of cytokines in plasma and lung section lysates were measured by an ELISA kit (Mouse IL-4 ELISA Kit, 555232, BD Bioscience; Mouse IL-10 ELISA Kit, 555252, BD Bioscience) following the manufacturer's instructions. First, we coated microwells with capture antibody overnight at 4℃ and

washed them three times with wash buffer. Then, we blocked the plates with assay diluents followed by another three washes. Standards and samples were added into microplates, incubated two hours at room temperature and washed five times. After further incubation with a working detector and seven washes, substrate reagents were added. The plates were incubated for 30 min, and then the reactions were stopped by adding stop solution. The color intensity was measured at 450nm with λ correction 570nm (Tecan, Zurich, Switzerland). IL-4 and IL-10 proteins' content was calculated via standard curves.

Cell Culture and Treatment

The human bronchial epithelial cells (16HBE) were cultured in RPMI1640 media (C11875500BT; Gibco) containing 10% FBS (1027016; Gibco) supplemented with 1% penicillin/streptomycin (SV30010; Hyclone) and maintained at 37℃ in a humidified incubator with 5% CO_2. 16HBE cells were seeded into six-well plates at a concentration of 1×10^5cells/ml, and incubated with serum-free culture medium for 12 h when the 16HBE cells reached 80% confluence. After starvation, we pre-treated the16HBE cells with AVE0991(10uM) for two hours and then stimulated cells with LPS (1ug/ml; L2880; Sigma) for four hours or anisomycin (5uM; B6674; APExBio) for one hour. Cells were harvested for further analysis. To produce conditioned media to be used subsequently in vitro assays, cells were thoroughly washed in PBS after stimulation and incubating for another 24h in non-supplemented RPMI 1640.

Immunocytofluorescent

16HBE cells were seeded into 96-well plates at a concentration of 1000 cells/well and incubated about 24 h. Then, cells were fixed with 4% paraformaldehyde with or without permeation by 0.1% Triton X-100. After blocking with 5% BSA for 15 min, cells were incubated with primary antibody overnight at 4℃ followed by incubations with goat anti-rabbit IgG secondary antibody, Cy3-conjugate, or FITC-conjugate. Nuclei were visualized with DAPI and images were photographed with an inverted fluorescence microscope (Olympus IX71).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from lung section and cultured cells using RNAiso Plus (9109; Takara) according to the manufacturer's instructions. About 500ng of RNA was reversed-transcribed to cDNA using PrimerScript™ RT Master Mix (RR036A; Takara). qRT-PCR analysis was performed in a LightCycler 480 instrument (Roche, Basel, Switzerland) using TB Green™Premix ExTaq™ (RR820A; Takara). The relative comparative CT method was applied to compared gene expression levels between two groups with the equation $2^{-\triangle\triangle Ct}$. The gene-specific primer sequences used for mRNA are listed in Table S1.

Western Blot

Lung tissues and harvested 16HBE cells were lysed by protein extraction regent RIPA buffer (V900854; Sigma-Aldrich) supplemented with protease inhibitor and phosphatase inhibitor (P1045; Beyotime). Lysates were diluted in protein loading buffer (LT102S; EpiZyme) and heated at 99.5 ℃ for ten min. The

proteins were separated by 12.5% SDS-PAGE gels (PG112; EpiZyme) and transferred to polyvinylidene difluoride membranes (PVDF; UWF011C; Merk-Millipore). The membranes were blocked with 5% bovine serum albumin for one hour and incubated at 4℃ overnight with primary antibodies specific to Mas receptor (D222656; Sangon Biotech), CCL2 (DF7577; Affinity), phospho-JNK (4668; CST), phospho-ERK1/2 (4370; CST), phospho-p38 (4511; CST), and GAPDH (5174; CST). Then, the membranes were incubated with HRP conjugate secondary antibodies for one hour and visualized using ECL chemiluminescence assay via the Mini Chemi Imaging System (SAGECREATION). The target protein's relative expression level was normalized to internal control (GAPDH), while the densitometric quantification of band intensity was calculated with ImageJ 5.0 software.

Transwell Assays

Cell migration assays were performed using a 24-well transwell apparatus with a 0.4μm pore size (353095; Corning). Approximately 2×10^5 PMA (70-CS0001; MultiSciences) induced THP-1 macrophages with a volume of 200μL were loaded into the upper chambers of the transwell plates. 600μL conditioned media was collected and added to the lower chambers. In inhibition experiments, conditioned media was pre-incubated with anti-CCL2 neutralizing antibody (16-7096; eBioscience) for one hour [24]. After24-h incubation, the cells were fixed with 4% paraformaldehyde and stained with crystal violet. Non-migrating cells on the upper surface of the membrane were gently removed. The numbers of migrated cells were counted in five randomly chosen fields per insert using ImageJ software.

Statistical analysis

All quantitative data are presented as mean±standard deviation from at least three independent experiments. Statistical analysis was conducted using GraphPad Prism software version 5.0 and SPSS version 20. The chi-square test, or Kruskal-Wallis test for non-parametric variables, and Student's *t*-test, or ANOVA analysis for parametric variables, were used to identify statistically significant data. A *P*-value 0.05 was considered statistically significant.

Results

Mas receptor expression is decreased in acute allergic airway inflammation

Based on the evidence that chronic OVA challenges reduced the area stained for Mas receptor by 40% [17], we wondered whether decreased expression of Mas receptor existed in acute allergic airway inflammation as well. Mice were sensitized and challenged by OVA as illustrated in Figure S1A. Consecutive OVA atomization for seven days resulted in obvious inflammatory cells' infiltration, mucus hyper secretion, collagen formation, and higher scores in semi-quantitative analysis (Figure S1B). As expected, the decrease of Mas receptor could be detected in both mRNA and proteins (Fig. 1A-B). In immunohistochemistry (IHC) analyses, the positive staining of Mas receptor is mainly present in the bronchial epithelium, while the mean density is significantly lower in OVA treated mice (Fig. 1C). Mas receptor expression is decreased in acute allergic airway inflammation, which is possibly involved in the

early stage pathogenesis of asthma. In order to determine where Mas receptor may function in the lungs, we looked for co-localization between Mas receptor and EpCAM (bronchial epithelium markers) or α-SMA (airway smooth markers). Mas receptor reportedly localize primarily to bronchial epithelium and smooth muscle [17]. Here, our lab works demonstrated that Mas receptor only distributed throughout bronchial epithelium where it co-localized with EpCAM rather than smooth muscle. Intensity traces from original images showed almost overlap between Mas receptor and EpCAM, while α-SMA did not co-localize with Mas receptor (Fig. 1D-E). Thus, the expression of Mas receptor decrease in bronchial epithelium may play important roles in acute allergic airway inflammation.

Activating Mas receptor attenuates macrophage infiltration in acute allergic airway inflammation

To identify the function of Mas receptor in acute allergic airway inflammation, we established a mice model of acute asthma and pre-treated asthma mice with AVE0991 (agonist of Mas receptor) one hour before the OVA challenge (Figure S2). Co-localization of Mas receptor and EpCAM makes intratracheal infusion AVE0991 activating Mas receptor on airway epithelial cells credible. Compared with the group treated with OVA only, after intratracheal administration of AVE0991, the inflammatory cells' recruitment was reduced and the goblet cells' hyperplasia was alleviated, and collagen synthesis decreased. In a semi-quantitative analysis, AVE0991 treatment could decrease relative scores (Fig. 2A). Recruitment of macrophages in asthma was significantly prevented by AVE0991 treatment, with a four-fold reduction in F4/80 + areas (Fig. 2B). In addition, activating Mas receptor obviously reduced infiltrating eosinophils in asthma (Fig. 2C). IL-4, a pro-inflammatory factor in allergic inflammtion, was significantly increased in plasma and lung lysates of asthma mice in comparison with normal mice, while IL-10, a anti-inflammation factor, exhibited an opposite result. As expected, activating Mas receptor in asthma mice reversed the imbalance between IL-4 and IL-10 (Fig. 2D).

Activating Mas receptor inhibits production of CCL2 and decreases acute allergic airway inflammation through MAPK pathways

Activating Mas receptor *in vivo* attenuates allergic airway inflammation, especially macrophage and eosinophil infiltration. It was reported that activating Mas receptor through Ang-(1–7) promoted resolution of inflammation by inducing apoptosis of eosinohil via ERK1/2 pathway [15]. But the underlying mechanism of decreased macrophages remains unclear. In fact, after allergen exposure, there is a rapid recruitment of macrophages to promote inflammatory responses, which exhibits crucial therapeutic potential [5]. We hypothesized that AVE0991 may affect the expression of important cytokines or chemokines, thus decreasing macrophage infiltration. We detected mRNA levels of several known inflammatory cytokines and chemokines. As qRT-PCR analysis showed, increased expression of CCL2 in asthma mice could be reversed by activating Mas receptor, and CCL4 and TNF-α displayed similar tendency. IL-25 and IL-33 revealed similar expressions between normal and asthma mice, although pre-treatment with AVE0991 resulted in decreased expression. There are no differences between asthma and AVE0991 treated asthma mice in CCL3 and TSLP (Fig. 3A). We next evaluated the protein expression of CCL2 in lung tissue by western blot and the results were in accord with qRT-PCR (Fig. 3B).

Lee YG et al. reported that bronchial epithelium secreted CCL2 to recruited alveolar macrophages and regulate airway inflammation in allergic asthma [25].We found that the recruitment of macrophages was parallel with the expression of CCL2, which can be restrained by activating Mas receptor. Owing to the important role of MAPK signaling pathways in the inflammation regulation, we further investigated the effect of activating Mas receptor on phosphorylation levels of MAPK pathways. Western blot analysis of lung tissues lysates revealed that OVA-induced increase of phospho-JNK, phospho-ERK1/2, and phospho-p38 could be inhibited by AVE0991 treatment (Fig. 3C). These data suggests that activating Mas receptor inhibited production of CCL2 and thus decreased acute allergic airway inflammation as well as macrophage recruitment, probably through MAPK pathways.

Activating Mas receptor attenuates CCL2 dependent THP-1 macrophages migration via JNK Pathways

Based on the results of *in vivo* studies, we hypothesized that AVE0991, functioning on Mas receptor of bronchial epithelium, inhibits CCL2 dependent macrophage recruitment via MAPK pathway. Because the human bronchial epithelial cell line (16HBE) has relatively high expression of both Mas receptor and CCL2 (Figure S3A-B), we selected 16HBE for the following *in vitro* study and explored the underlying mechanisms of the Mas receptor' anti-inflammatory role. 16HBE cells were pre-treated with 10 μM AVE0991 for two hours and then stimulated with 1 μg/ml LPS for four hours [26, 27]. The cells were harvested at the indicated time to detect the mRNA level of CCL2. Compared with LPS treatment alone, activating Mas receptor before LPS stimulation inhibited LPS-induced CCL2 mRNA expression (Fig. 4A). To examine the chemotactic activity of monocytes towards bronchial epithelium, we added PMA-induced THP-1 macrophages to the inserts of a transwell system containing conditioned media from 16HBE cells under different stimulations. Conditioned media from LPS-induced 16HBE cells significantly increased migration of THP-1 macrophages. In turn, conditioned media from 16HBE cells which were pre-treated with AVE0991 and stimulated by LPS further exerted an anti-chemotactic role. To determine whether CCL2 plays an important role in chemotactic activity of THP-1 macrophages, we used an anti-CCL2 neutralizing antibody to block CCL2. The migration rate of THP-1 macrophages decreased under this condition, which was consistent with AVE0991 pre-treatment (Fig. 4B). Based on previous evidence that AVE0991 treatment *in vivo* suppressed MAPK pathway activation, we further investigated the exact pathway associated with CCL2 expression. The phosphorylation of JNK increased significantly due to LPS stimulation, which was faded by activating Mas receptor, while AVE0991 treatment had no effect on increasing phospho-ERK1/2. There was no statistically significant difference in the phosphorylation level of p38 among all groups (Fig. 4C). We then employed anisomycin to activate JNK specifically [28, 29]. As expected, specific up-regulation of phospho-JNK, increasing expression of CCL2 and enhancing THP-1 macrophages migration induced by anisomycin were diminished upon pretreatment with AVE0991 (Fig. 4D-F). Anisomycin-induced THP-1 macrophages migration was blocked by an anti-CCL2 neutralizing antibody (Fig. 4F). These findings indicated that activating Mas receptor attenuated CCL2 dependent THP-1 macrophages via inhibiting phosphorylation of JNK specifically.

Discussion

We focused on elucidating (1) the expression of Mas receptor in OVA-induced acute allergic asthma and (2) whether activating Mas receptor could attenuate inflammatory cells, especially macrophage infiltration *in vivo* and *in vitro*. Our results demonstrated that Mas receptor mainly functioned in bronchial epithelium and activating Mas receptor attenuates allergic airway inflammation, especially CCL2 dependent macrophages recruitment via JNK pathways.

In acute allergic airway inflammation, we found that after consecutive OVA challenge for seven days, the expression of Mas receptor decreased significantly in lung tissue. The decrease probably presented in the whole pathological process of asthma, because in chronic asthma mice model, after OVA challenge from days 21 to 46, decreased expression of Mas receptor in lung tissue was also detected through western blot and immunohistochemical [17, 18]. Generally, decreased expression of Mas receptor was involved in the occurrence and development of many diseases. In the hippocampus of diabetic rats, the decreased expression of Mas receptor was involved in Alzheimer's disease like functional and pathological changes in streptozotocin-induced diabetes [30]. In balloon-injured rat aorta, the decrease of Mas receptor expression could be observed in the injured aorta, while valsartan could improve the expression of Mas receptor and reduce the neointimal hyperplasia of aorta [31]. Besides, chronic hypoxia-induced pulmonary hypertension also presented decreased expression of Mas receptor in the right ventricle of rats, which contributed to right ventricular remodeling and fibrosis [32]. Most of the studies mentioned above were mainly chronic diseases, and were related to tissue regeneration and remodeling. For acute inflammatory diseases, there were few studies discussing about changes of Mas receptor expression. In fact, different expression patterns of Mas receptor might exist in different stages of the disease. For example, the expression level of Mas receptor was significantly increased in acute phase and decreased in post-acute phase during the process of peripheral nerve injury, reflecting the transformation from anti-inflammatory to pro-inflammatory [33]. Compared with chronic OVA stimulation, our study found that even a short-term OVA stimulation could significantly reduce the expression of Mas receptor, which suggested that the decrease of Mas receptor might involve in the initiation and development of asthma in the early stage. Hence, it was of great significance to study the role of Mas receptor in acute allergic airway inflammation. Considering that Ang-(1–7) exerted a short duration of biological effect *in vivo* for rapid degradation by ACE which was rich in lung [34], we employed AVE0991, a synthetic non-peptide agonist of Mas receptor, in subsequent experiments to further evaluate the role of Mas receptor in acute allergic airway inflammation. Different from studies in MG Rodrigue-Machado [17, 18], we further performed the immunofluorescence double staining to co-localize Mas receptor with EpCAM, which confirmed that Mas receptor mainly acted on airway epithelial cells. It suggested that the method of intratracheal infusion AVE0991 to activate Mas receptor was credible.

Indeed, MG Rodrigue-Machado's research of AVE0991 focused more on airway and vascular remodeling in chronic asthma [18]. Nevertheless, we found that pretreatment of AVE0991 activating Mas receptor before OVA challenge significantly attenuated acute airway inflammation and decreased macrophage infiltration. In our study, increase in IL-4 induced by OVA was blocked by AVE0991. IL-4 was important cytokine for alternative activation of infiltrated macrophages and enhancement of allergic inflammation [35]. IL-10, a potent anti-inflammatory cytokine [36], exhibited an opposite result to IL-4. Our data argued

that AVE0991 could serve as an important tool for treatment of acute allergic airway inflammation. As for signaling pathways, the Ang-(1–7)/Mas receptor axis reportedly inhibited phosphorylation of JNK, ERK1/2, and p38 in different physiological conditions [37, 38, 39]. To elucidate the mechanisms by which Mas receptor mediated their effects on acute allergic airway inflammation, we investigated MAPK signaling pathways that were involved in asthma, including JNK, ERK1/2, and p38. *In vivo* studies revealed that activating Mas receptor inhibited the phosphorylation of JNK, ERK1/2, and p38 induced by OVA. In chronic asthma, MG Rodrigue-Machado discussed more about the protective effect of AVE0991 on pathology and changes in expression of renin-angiotensin axis related receptors and angiotensin peptides, rather than signaling pathways [18]. Researches based on Ang-(1–7) focused more on the role of ERK pathway in eosinophil infiltration and airway remodeling [14, 15, 16, 17]. In fact, all three subfamilies of MAPKs were supposedly involved in the pathogenesis of asthma. The phosphorylation states of all three MAPK members were up-regulated in asthma animal models, which identified them as potential novel targets for asthma treatment [40]. Various small-molecular inhibitors of MAPK tested in animal models of asthma hold potential for treatment of severe corticosteroid resistant asthma and warrant further clinical investigation. The ERK1/2 signaling pathway reportedly played a more important role in the process of airway remodeling rather than acute allergic airway inflammation, and p-ERK1/2 inhibitor effectively inhibited airway remodeling in chronic asthma [41]. Kim et al. reported that inhibition of p38 MAPK might attenuate allergen-induced airway inflammation and vascular leakage through modulation of VEGF expression in mice [42]. JNK pathway regulated various physiological conditions including inflammatory responses, expression of proteins and so on [43]. An only JNK inhibitor, SP600125, showed significant reduction of eosinophil, lymphocyte, neutrophil, and macrophage counts in BALF as well as decreased expression of pro-inflammatory cytokines in an OVA-induced murine acute asthma model [44].

Although multiple pathways and cytokines were involved in pathogenesis of acute allergic airway inflammation, one of critical pathways and its crucial downstream might be inhibited by Mas receptor activation and fail to induce inflammation. We detected mRNA levels of several known cytokines in lung samples, including IL-25, IL-33, TSLP [45], TNF-α, and chemokines (CCL2, CCL3, CCL4) [46, 47]. Our results demonstrated that an apparent increased expression of CCL2 in asthma mice could be sharply inhibited by AVE0991. CCL2 is closely associated with airway inflammation [48, 49], especially in recruiting macrophages [7]. Agache et al. observed a significant correlation between increased levels of CCL2 and asthma severity and fast LF decline [50]. Viral-induced exacerbation was accompanied by increased levels of CCL2. Although RSV-induced exacerbation was resistant to steroid treatment, inhibition of CCL2 function suppressed features of disease, including AHR and macrophage infiltration [51]. Previous studies demonstrated that either blocking the CCR2 or neutralizing CCL2 could effectively block the recruitment of inflammatory macrophages [52]. Though MG Rodrigue-Machado had mentioned that Ang-(1–7) prevented CCL2 increase induced by OVA, a further analysis of underlying mechanism was not performed [17]. In fact, there were evidences which suggested JNK pathway regulated the expression CCL2 under different processes. In the tumor microenvironment, increase of fatty acid oxidation promoted JNK activation, which enhanced the expression of CCL2 [53]. In non-alcoholic

steatohepatitis, JNK activation promoted the release of CCL2 and thus attracted macrophages to injured liver [54]. *In vitro* model of neuroinflammation presented upregulation of CCL2, while JNK inhibitor dose-dependently inhibited LPS-induced CCL2 upregulation [55]. In acute asthma, it was reported that OVA activated TLR-2/JNK pathway signaling and increase CCL2 secretion *in vivo*, while no further validation of TLR2/JNK/CCL2 was performed [56]. Thus, the axis linking JNK and CCL2 in asthma remains unclear.

Since researches on Mas receptor activated by AVE0991 or Ang-(1–7) were mainly *in vivo* studies and mostly discussed about the effect of ERK pathway on eosinophil infiltration and airway remodeling [14, 15, 16, 17, 18], the crucial pathway and its downstream molecules remained unclear in acute allergic airway inflammation especially in macrophage recruitment and further validation of underlying mechanism *in vitro* were needed. According to our studies *in vivo*, activation of Mas receptor attenuated acute allergic airway inflammation probably through the mechanism that activating Mas receptor inhibited JNK/CCL2 pathway and reduced macrophage recruitment. Hence, we investigated it next at cellular level to explore the key molecule and pathway. The bronchial epithelium was the major source for CCL2 in allergic lung inflammation [57]. Based on the findings that the recruitment of macrophages was parallel to the up-regulation of CCL2 and that Mas receptor was co-localized with EpCAM, we explored the underlying mechanisms in 16HBE, a human bronchial epithelial cell line. Our work revealed that activating Mas receptor directly inhibited CCL2 secretion and THP-1 macrophages migration stimulated by LPS via decreasing phosphorylation of JNK. LPS-induced conditioned media promoted THP-1 macrophages migrations, while anti-CCL2 neutralizing antibodies blocked the migrations. The specific effect of JNK pathways on CCL2 was further proved by anisomycin, a JNK activator. Different from results of *in vivo* studies, AVE0991 treatment did not inhibit the phosphorylation of ERK induced by LPS *in vitro*. Previous studies had shown that ERK signaling pathway was mainly related to airway remodeling, especially the proliferation and migration of airway smooth muscle cells [58, 59]. Similarly, AVE0991 could not inhibit anisomycin-induced phosphorylation of p38, which was discussed more on differentiation of T cells as well as the proliferation and migration of airway smooth cells [60, 61]. However, the phosphorylation of JNK was coincident *in vivo* and *in vitro*. Indeed, airway epithelial JNK activation was found critical in initiation of allergic inflammation, especially house dust mite, fungi or OVA induced TLR2/JNK activation [56]. Rhinoviruses infection of airway epithelial cell frequently produced higher levels of inflammatory cytokines via TLR4/JNK pathway and exacerbated inflammation in asthma, causing worse airway obstruction and symptoms [62]. In clinical, systemic glucocorticoid (GC) inhibited phosphorylation of JNK in bronchial mucosal cells in GC responsiveness asthma, while the phenomenon was not observed in GC-resistant asthmatic subjects [63]. Thus, the JNK pathway could serve as a potential target for future therapy in asthma.

Conclusion

In conclusion, this study provides evidence that activating Mas receptor exerts anti-inflammatory roles through JNK pathway, especially in CCL2 dependent macrophage recruitments *in vivo* and *in vitro*. (Fig. 5). Mas receptor, CCL2, and phospho-JNK may serve as potential therapeutic targets for acute allergic airway inflammation.

Abbreviations

OVA: ovalbumin

16HBE: the human bronchial epithelial cell line

THP-1: human myeloid leukemia mononuclear cells

MAPK: mitogen-activated protein kinase

JNK: c-Jun N-terminal kinase

ERK: extracellular regulated protein kinases

CCL2: C-C Motif Chemokine Ligand 2

Declarations

Ethics approval and consent to participate

All animals' care and experimental procedures were approved by the Animal Research Committee of Sun Yat-sen University (application number 2017189). The study does not involve any human data.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

All authors declare no conflicts of interest.

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Authors' contributions

SYL, XQY and SPJ conceived the study design and supervised the scientific work. LNH, QJW, YMG and RJP performed the experiments. MC and JTS analyzed the data. LNH wrote the paper.

All authors contributed to and approved the final manuscript.

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Figures

Figure 1

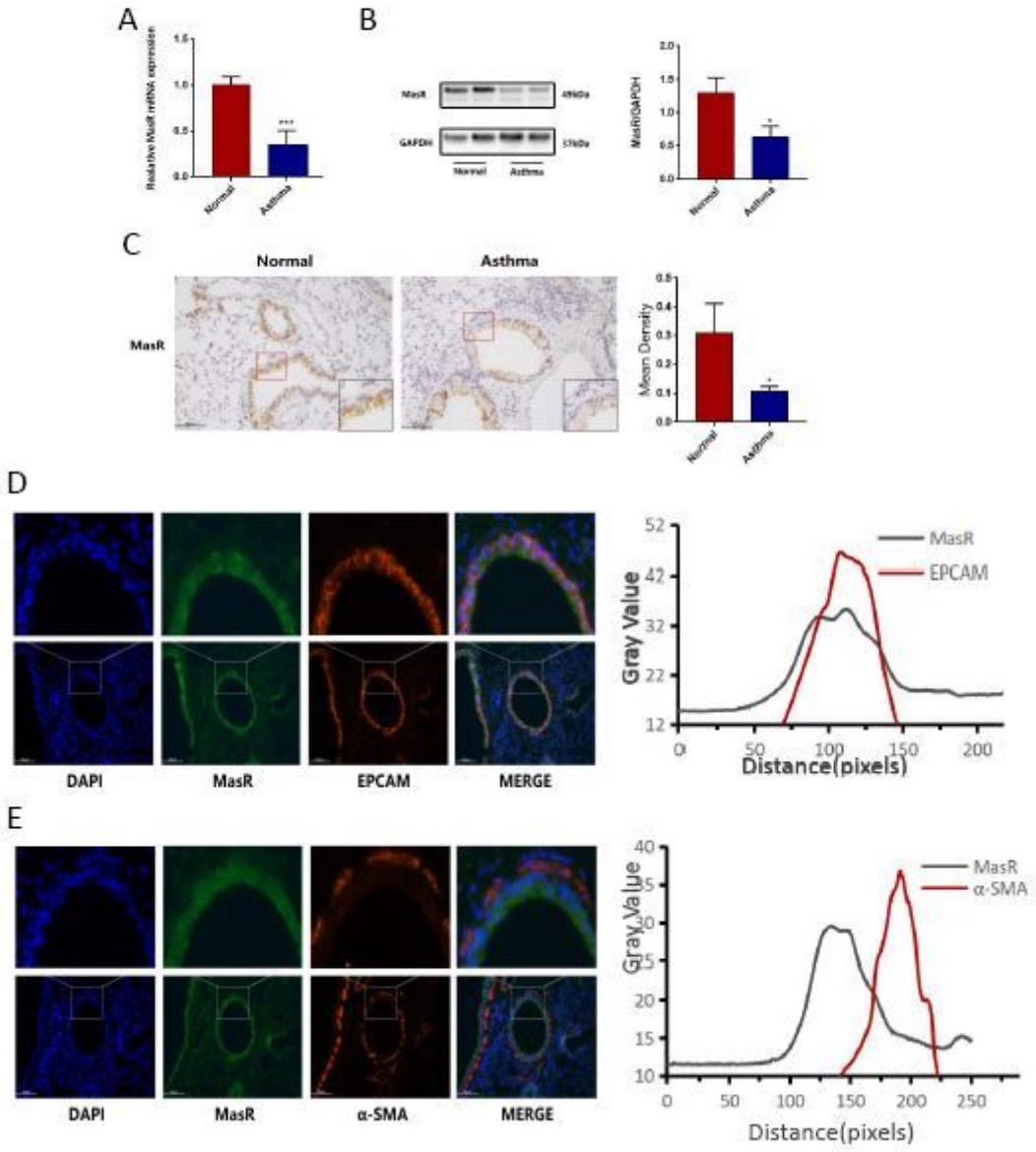

Figure 1

Mas receptor expression is decreased in acute allergic airway inflammation. A. The mRNA expression of Mas receptor in lung assessed by qRT-PCR. B. The protein expression of Mas receptor in lung assessed by Western Blot. C. Representative images of Mas receptor immunohistochemistry staining of mouse lung sections as shown at a magnification of 200X. The mean density of Mas receptor in bronchial epithelium was calculated. D. Representative images of multiplex immunofluorescence stained with Mas receptor and EpCAM. Intensity traces from original image are plotted at right. E. Representative images of

multiplex immunofluorescence stained with Mas receptor and α-SMA. Intensity traces from original image are plotted at right. (n=4, *P□0.05, **P□0.01, ***P□0.001 vs. normal mice)

Figure 2

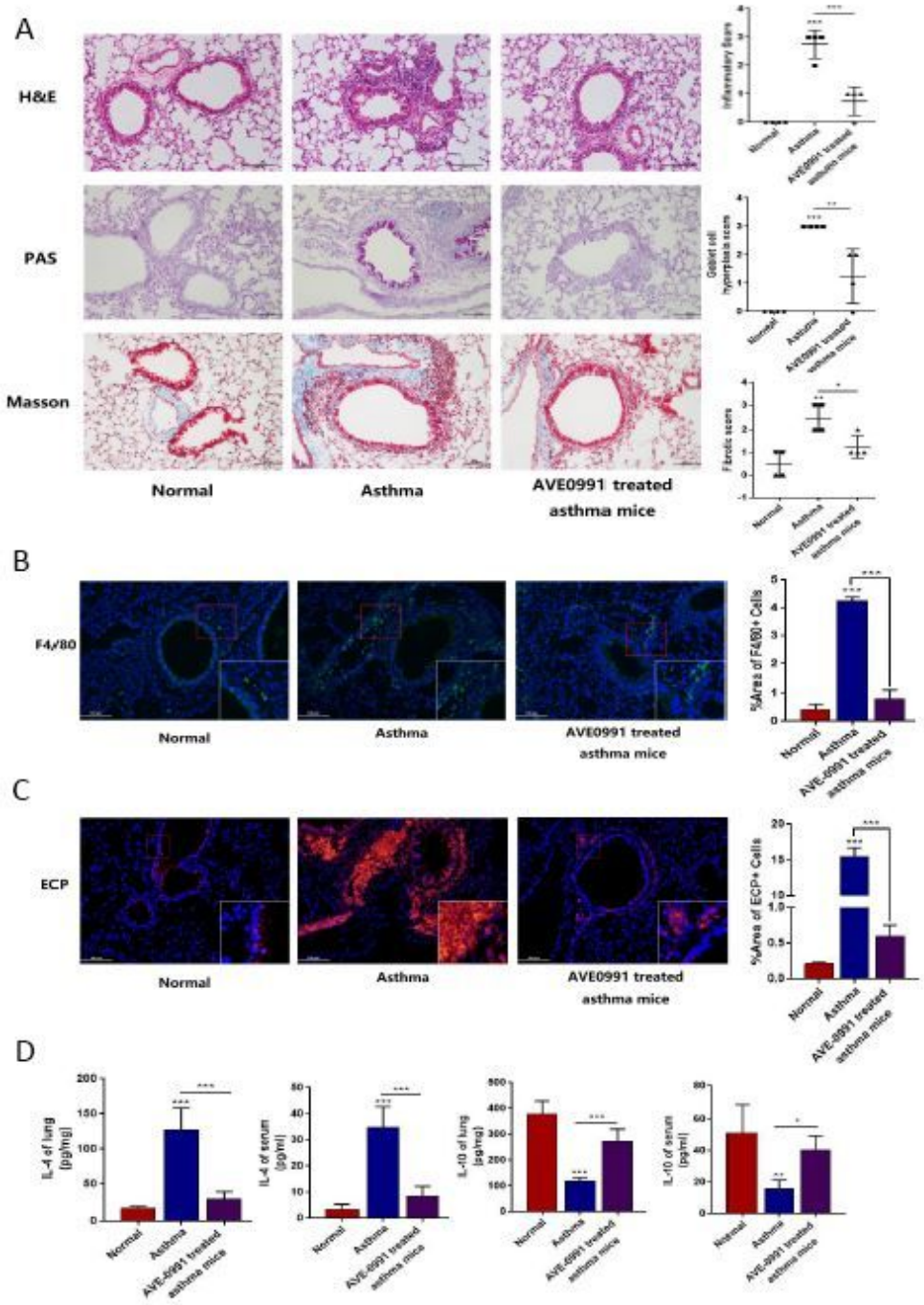

Figure 2

Activating Mas receptor attenuates macrophage infiltration in acute allergic airway inflammation. A. Representative images of H&E, PAS and Masson staining of mouse lung sections as shown at a magnification of 200X. Scatter plots represent semi-quantitative analysis of histopathological data from

H&E, PAS and Masson staining. B. Representative images of F4/80 immunofluorescent staining of mouse lung sections as shown at a magnification of 200X. The areas of F4/80+ cells were calculated. C. Representative images of ECP immunofluorescent staining of mouse lung sections as shown at a magnification of 200X. The areas of ECP+ cells were calculated. D. IL-4 and IL-10 levels in lung lysates and plasma assessed by ELISA. (n=4, *P<0.05, **P<0.01, ***P<0.001 vs. normal mice)

Figure 3

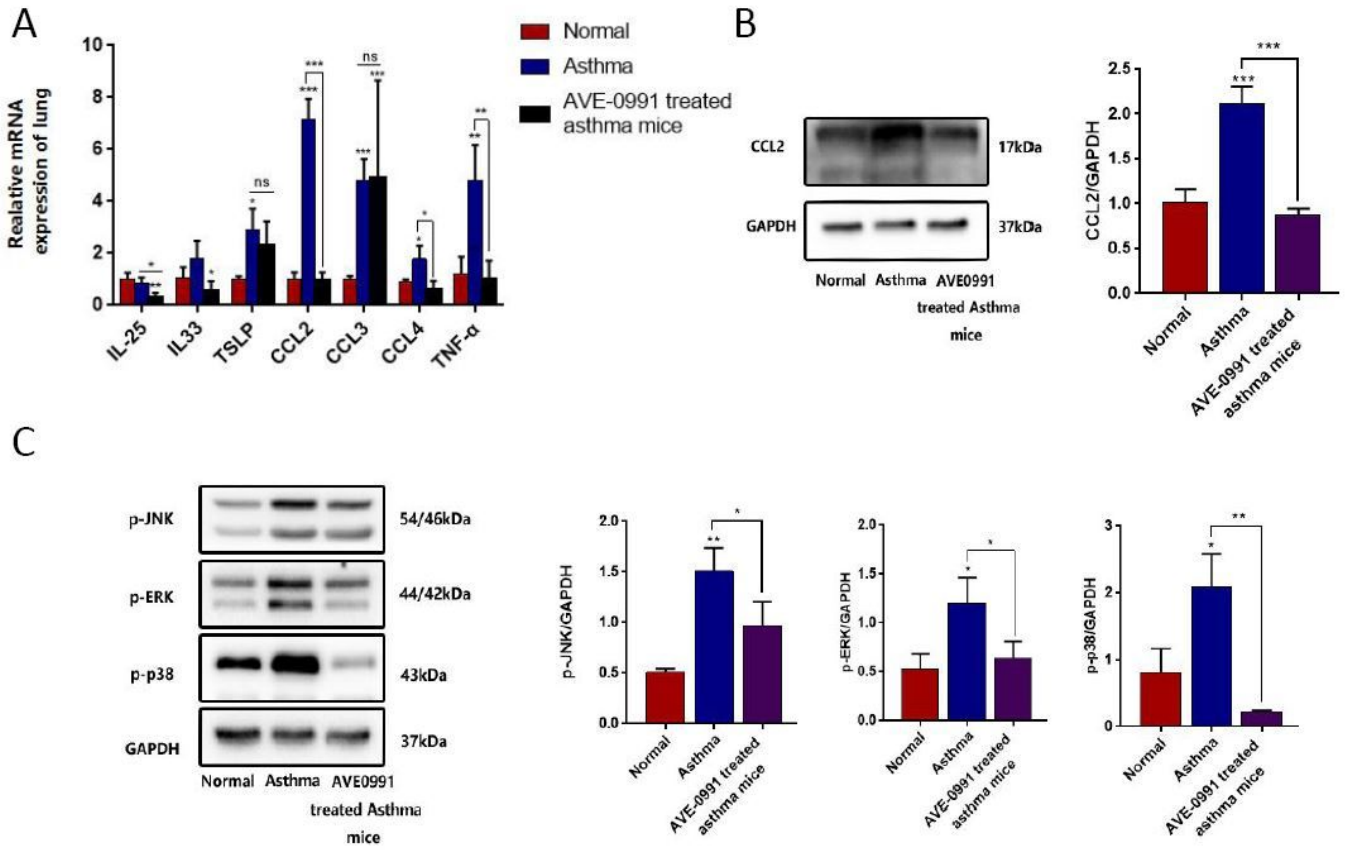

Figure 3

Activating Mas receptor inhibits production of CCL2 and decreases allergic airway inflammation through MAPK pathways. A. The mRNA levels of IL-25, IL-33, TSLP, CCL2,CCL3, CCL4 and TNF-α in lung tissues estimated by qRT-PCR. B. The protein expression of CCL2 in lung tissues estimated by Western Blot. C. Representative blots showing relative levels of p-JNK, p-ERK1/2 and p-p38 (normalized to GAPDH) in lung tissues. (n=4, *P<0.05, **P<0.01, ***P<0.001 vs. normal mice)

Figure 4

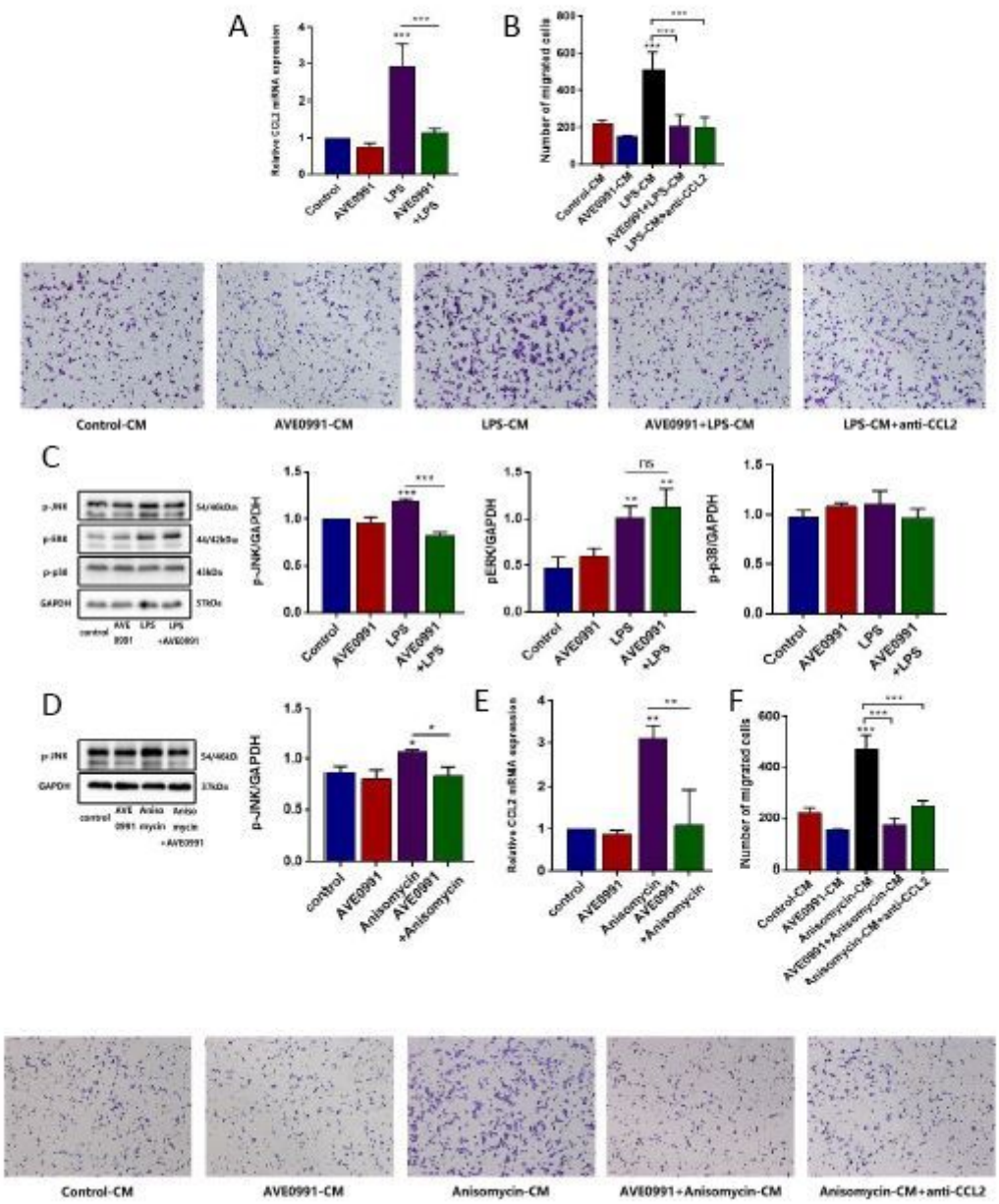

Figure 4

Activating Mas receptor attenuates CCL2 dependent THP-1 macrophages migration via JNK pathways. A, E. The mRNA levels of CCL2 in 16HBE under different stimulations estimated by qRT-PCR. B, F. Representative images of THP-1 macrophages migration stained with crystal violet as shown at a magnification of 100X. A histogram analysis of migrated cell counts was shown. C. Representative blots showing relative levels of p-JNK, p-ERK1/2 and p-p38 (normalized to GAPDH) in 16HBE under different stimulations. D. Representative blots showing relative levels of p-JNK (normalized to GAPDH) in 16HBE under different stimulations. (n=3, *P<0.05, **P<0.01, ***P<0.001vs. Control).

Figure 5

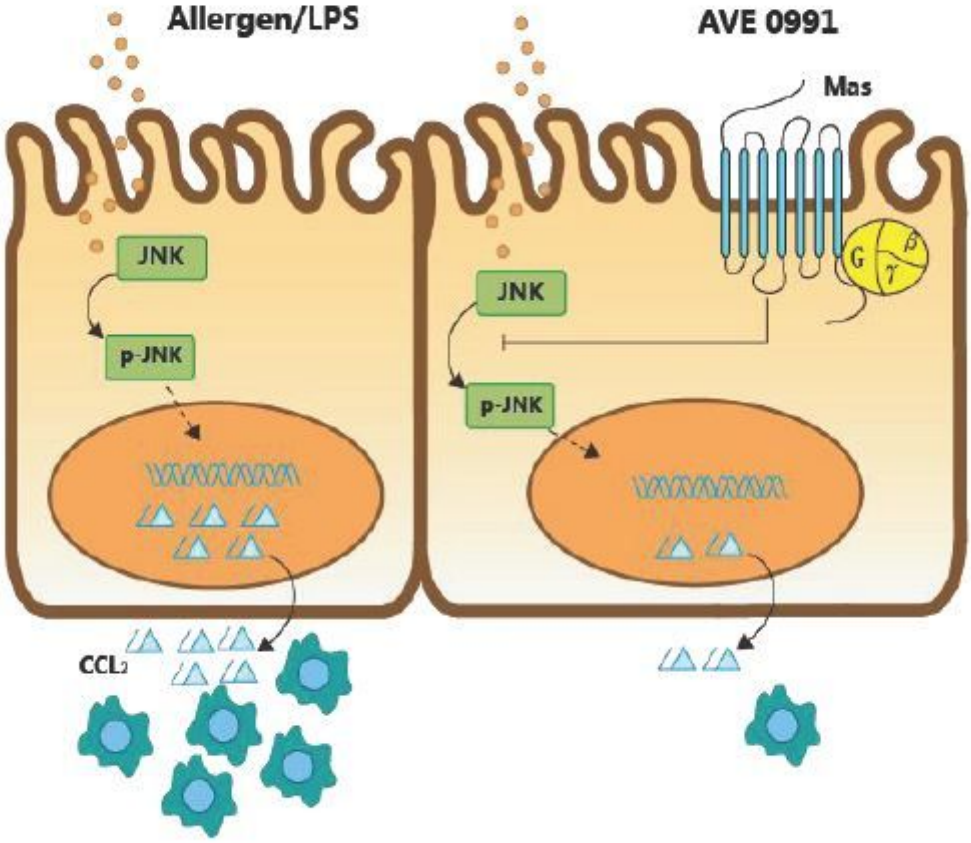

Figure 5

Illustrative model shows that activating Mas receptor attenuates CCL2 dependent macrophage recruitment via JNK pathway.

Supplementary Files

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- SupplementaryMaterialsMasR.pdf