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REVIEW ARTICLE

Sonic hedgehog (Shh) and CC chemokine ligand 2 signaling pathways in asthma

Xiang-Zhi Wang[a], Hang-Hu Zhang[a,b], Yu-Ling Qian[c], Lan-Fang Tang[a,*]

[a]Department of Pulmonology, Children's Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang, China; [b]Department of Pediatrics, Shaoxing People's Hospital, Shaoxing, Zhejiang, China; [c]Zhejiang University School of Medicine, Hangzhou, Zhejiang, China

Abstract: Asthma is a chronic inflammatory disease of the airways in which many cells are involved, including mast cells, eosinophils, T lymphocytes, and so on. During the process, many chemokines and mediators are released to engage in recruiting and activating eosinophils and other inflammatory cells. Also, some signaling pathways are involved in the pathobiology of asthma. Sonic hedgehog (*Shh*) is one of the members of hedgehog gene families. Shh signaling plays a critical role in the embryonic development, including the lung. Previous findings from our team reveal that *Shh* is involved in the asthma pathogenesis. Recombinant *Shh* could induce the CC chemokine ligand 2 (CCL2) overexpressing and Smo inhibitor GDC-O449 could inhibit CCL2 expression in airway epithelial cells, monocytes, or macrophages. Hence, we reviewed the effects of Shh and CCL2 signaling pathways, and the interaction between signaling pathways in asthma.

Keywords: Asthma; CC chemokine ligand 2 (CCL2); Signaling pathway; Sonic hedgehog (Shh)

1. INTRODUCTION

Asthma is a chronic inflammatory disease of the airways in which many cells are involved, including mast cells, eosinophils, T lymphocytes, and so on. The typical symptoms and signs of asthma include wheezing, shortness of breath, chest tightness, and cough especially at night and/or early morning. During the process, many chemokines and mediators are released to engage in recruiting and activating eosinophils and other inflammatory cells. Specific chemokines delegate the different stages of asthmatic disease.[1] Also, some signaling pathways were involved in the mechanism of asthma.

2. SONIC HEDGEHOG SIGNALING PATHWAY

Sonic hedgehog (*Shh*) is one of the members of hedgehog (*Hh*) gene families, which contains two ligands of Shh, 12-transmembrane receptors patched (Ptch) and smoothened (Smo), and the downstream Cubitus interruptus (Ci) transcription factor (Gli family) (Fig. 1). Hh pathway is activated when Hh (Shh, Ihh, or Dhh) binds to their repressive receptor Ptch, and then alleviates its repression on the signaling transducer (Smo). The unleashed Smo activates the Gli transcription factors, which results in the expression of downstream target genes (e.g., *Gli1* and *PTCH1)*. In vertebrates, there are three Gli proteins, Gli1, Gli2, and Gli3. Gli1 acts as a transcriptional activator that boosts the Shh signal in a positive feedback loop, whereas Gli2 and Gli3 mostly function as a Shh-regulated transcriptional activator or repressor. The 20-kDa NH2-terminal domain of Shh (Shh-N) has all the signaling functions while the 25-kDa COOH-terminal domain (Shh-C) is in charge of the autoprocessing of this protein. Shh-N purified from cell extracts has gone through palmitoylation and cholesterol modification.[2] Set7, a lysine methyltransferase, mediated methylation is an innovative post-translational modification of Gli3, which positively regulates the transactivity of Gli3 and the activation of Shh signaling. Gli3 methylation conduced to the tumor growth and metastasis in nonsmall cell lung cancer in vitro and in vivo.[3] The Gli transcription factors transduce Hh signaling in the well-characterized Drosophila melanogaster. Mammalian glycerol uptake/transporter 1 (Gup1), a homolog of *Saccharomyces cerevisiae* Gup1, is a negative regulator of N-terminal palmitoylation of Shh and may contribute to biological actions of Shh.[4] A novel miR-326–Gli2/Smo feedback loop is reported to act as an important crosstalk between miRNAs and the Shh signaling pathway during the embryonic development.[5] Shh multimerization is driven by self-assembly underpinned by the law of mass action.[6]

Shh signaling plays critical roles in embryonic development. During embryogenesis, Shh acts a role in the development of all three primary germ layers. In the early ectoderm, Shh is participated in the development of both neuroectodermic structures (e.g., neural tube, brain, cerebellum, and neural crest cells) and epithelial ectodermic structures (e.g., epidermis and hair follicles). In the early mesoderm, Shh involves in the development of extraembryonic mesodermic structures such as hematopoietic cells and angiogenic factors. Finally, in the early endoderm, Shh is linked to the development of the lung, pancreas, and gastrointestinal tract. In adults, Shh is active both in respiratory stem cells that participate in repairing damage to airways and in gastrointestinal stem cells. Meanwhile, it helps maintain epithelial asymmetry and differentiation.[7] Downregulation of Shh signaling results in neonatal abnormalities and Shh up regulation may induce cancer. Previous studies have found that a 1.7 Kb fragment, which is located in the 100 Kb upstream of the Shh coding sequence, includes a functional element for Shh expression in the endodermal organs.[8] Shh pathway plays a major role in the evolution of epithelial appendages, including feather, hair, tooth, tongue papilla, lung, and foregut.[9] Recent evidences have shown that a Shh/miR-206/brain-derived neurotrophic factor

*Address correspondence: Prof. Lan-Fang Tang, Department of Pulmonology, Children's Hospital of Zhejiang University School of Medicine, 3333, Binsheng Road, Hangzhou 310052, China. E-mail address: 6195007@zju.edu.cn (L.-F. Tang).

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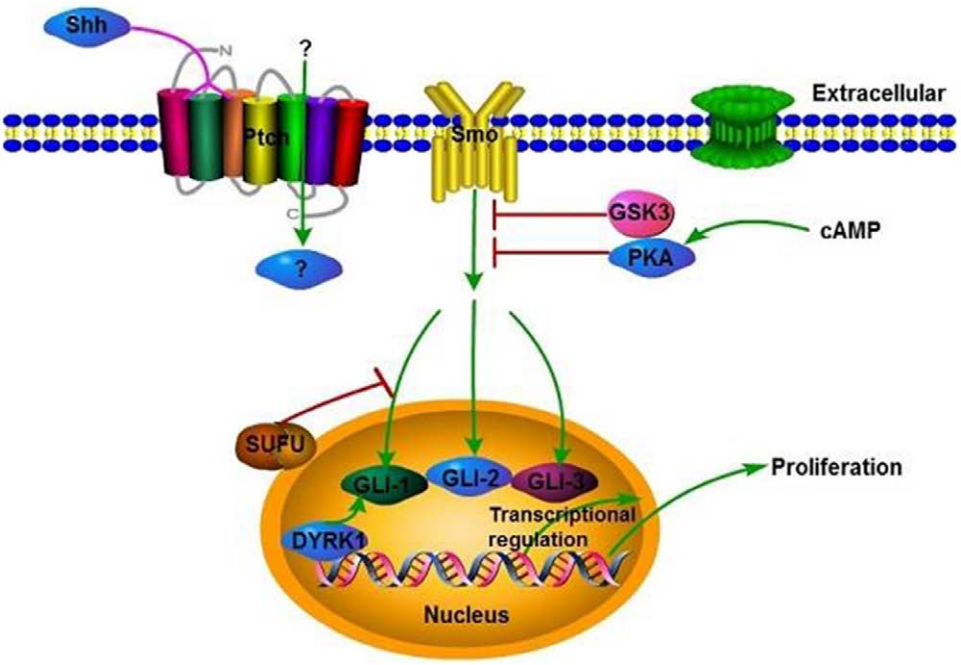

Fig. 1 Sonic hedgehog (Shh) signaling pathway. The Shh pathway includes mainly of Shh protein, transmembrane receptor Patched (Ptch), Smoothened (Smo), and the downstream GLI transcription factor. In the absence of Shh protein, Ptch1 interacts with Smo and inhibits it from being active, therefore blocking the signaling. Upon Shh binding to Ptch, Smo is released from the inhibition and it activates transcription through the Gli zinc finger transcription factors.

cascade can coordinate innervation and formation of airway smooth muscle.[10] The ventral and segmented expression of *Sox9* (a chondrogenic gene) in tracheal primordial, which is under Shh modulated by bone morphogenetic protein 4 (Bmp4) and Noggin (an anti-chondrogenic gene), is vital for patterning and formation of tracheal cartilage.[11] Shh mediates platelet-derived growth factor-induced vascular smooth muscle cells phenotypic modulation via regulation of Krüppel-like factor 4 (KLF4).[12] Shh acts on the progenitor pool for choroid plexus epithelial cells and choroid plexus pericytes, which indicated the important role of coordinating the development of two disparate yet functionally dependent structures—the choroid plexus vasculature and its ensheathing epithelium.[13] The bone morphogenetic proteins and Shh seem to be the key regulators, which involve in the formation of new bone and in the repairing of fractures.[14] *Pitrm1* (a metalloendopeptidase gene) is regulated downstream of Gli3 and Shh in the mouse limb, and expressed in muscle progenitors.[15] The data show a novel Shh forkhead-box (Fox) family transcription factors (Foxf)-fibroblast growth factor 18 (Fgf18)-Shh circuit in the palate development molecular network, in which Foxf1 and Foxf2 regulate palatal shelf growth downstream of Shh signaling by repressing Fgf18 expression in the palatal mesenchyma, partly to guarantee maintenance of Shh expression in the palatal epithelium.[16] James et al found that Shh is the target of G protein-coupled receptors (GPCRs) coupled to cAMP and protein kinase A, and may play additional considerable roles in the development, plasticity, tissue repair, cancer, and other processes.[17] Hh-interacting protein (HHIP) is highly expressed in the endothelial cells, while down-regulated during angiogenesis in some human tumors, which implies that modulation of HHIP could play a role in tumor angiogenesis.[18] Activation of Hh signaling pathway is protected from lipopolysaccharide-induced pulmonary microvascular endothelial cell damage.[19]

3. SONIC HEDGEHOG SIGNALING PATHWAY AND ASTHMA

Recent evidences have indicated an innovative role for Shh signaling in the crosstalk between pulmonary cells and infiltrating leukocytes during the induction of an allergic asthma. A recent study using murine models of asthma pathology showed that repression of Gli activity in T cells can decrease the recruitment and differentiation of Th2 cells to the lung during asthma, and lung epithelium, endothelium and innate immune cells, particularly eosinophils, also underwent Hh/Gli signaling.[20] Meta-analysis indicated that a subset of normal lung function genes, including *HHIP*, family with sequence similarity 13 member A and *PTCH1* regulating lung function in general populations, were related with abnormal lung function in asthma in

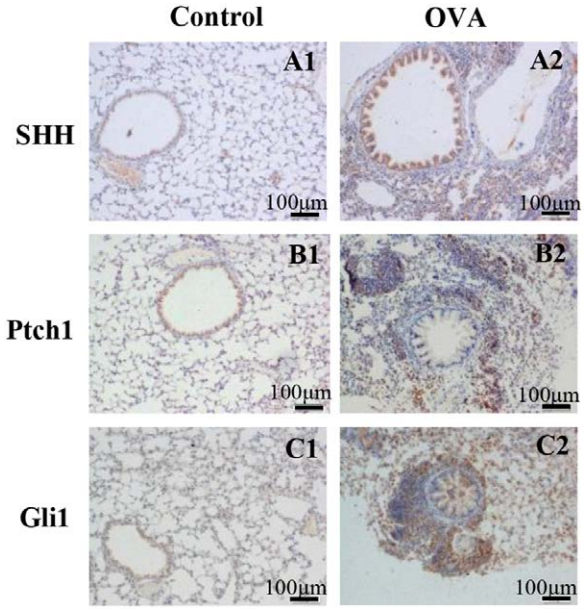

Fig. 2 Activation of Sonic hedgehog (Shh) signaling pathway in OVA (mouse model)-induced asthma model. A, A1 and A2 were Shh immunohistochemistry in control and asthma groups, respectively. B, B1 and B2 were control group and asthma group (Patched) Ptch1 immunohistochemistry. C, C1 and C2 are Gli1 immunohistochemistry in the control group and the asthma group.

non-Hispanic whites and African Americans,[21] which indicates that Shh signaling pathway was very important for the development of asthma. Previous study using a naphthalene-induced lung injury and compensatory lung growth murine model suggested that the overexpression of Shh and local pulmonary Sca-1$^+$CD34$^+$ CD45$^-$Pecam$^-$ cells were stimulated and had some potential to apply in the airways.[22] In house dust mite mouse model, the expression of CD4$^+$ T cells from lung improved levels of Smo transcript, which indicated active Hh signaling. Therefore, inflamed tissue released Shh to local T cells. This signaling lead to transcriptional changes, increased interleukin-4 (IL-4) production, and improved Th2 responses, signaling to other immune effector cells, maintaining allergic inflammation, and further aggravating disease.[23] In vivo, we found that Shh signaling pathway was activated in asthmatic model (Fig. 2), while cyclopamine, a Smo protein inhibitor, may relief the alveolar inflammation and abnormal remodeling in the airway (Fig. 3). This confirmed that Shh is involved in the mechanism of asthma as well. However, there were several negative results. In Ptch1 conditional knock-out mice in which the Hh receptor Ptch1 was inactivated in the T cell lineage, absence of Ptch1 did not result in an activation of canonic Hh signaling in peripheral T cells. They also subjected the mutant mice to three different disease models, which named allogeneic bone marrow transplantation mimicking graft-versus-host disease, allergic airway inflammation as a model of asthma and growth of adoptively transferred melanoma cells as a means to test tumor surveillance by the immune system. Nonetheless, they were neither able to demonstrate any differences in the disease courses nor in any pathogenic parameter in these three models of adaptive immunity. Therefore, they concluded that the Hh receptor Ptch is dispensable for T cell function in vitro as well as in vivo.[24]

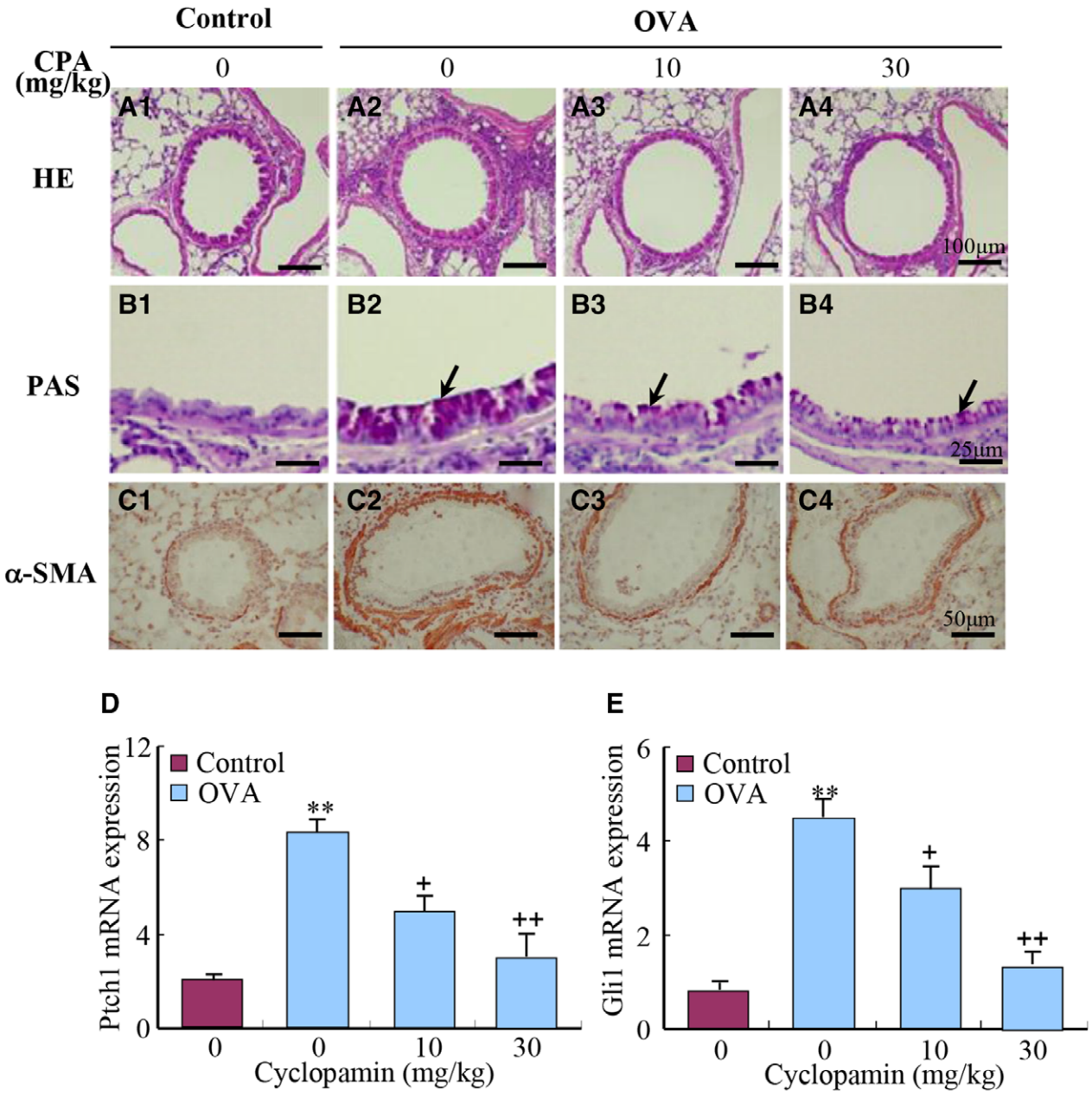

Fig. 3 Effect of Sonic hedgehog (Shh) inhibitor on airway remodeling in asthmatic model. A, Hematoxylin-eosin (HE) staining; B, periodic acid-Schiff (PAS) staining; C, α-smooth muscle actin (α-SMA) immunohistochemistry; D, reverse transcription-polymerase chain reaction (RT-PCR) detection of Ptch1 mRNA; E, RT- PCR assay for Gli1 mRNA levels (mean ± SD, *$p < 0.05$, **$p < 0.01$ compared with control; + $p < 0.05$, ++$p < 0.01$ compared with 0 mg/kg cyclopamin).

4. CC CHEMOKINE LIGAND 2

CC chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), is a member of the CC subfamily and attracts blood monocytes migrating into the lung, and then turns into bronchoalveolar macrophages (BAMs). CCL2 is produced by a great deal of cells, including monocytes, macrophages, lymphocytes, fibroblasts, endothelial, epithelial cells, and mast cells in response to much stimuli, such as lipopolysaccharide, IL-1, tumor necrosis factor-α, platelet-derived growth factor, interferon-γ (IFN-γ), or 12-o-tetradecanoylphorbol 13-acetate.[1] CCL2 relates with the CC chemokine receptor type-2 (CCR2) receptor and causes intracellular signal transduction (Fig. 4). CCR2 is a G-protein-coupled receptor that is expressed in monocytes, natural killer cells, T cells, and B cells. The coding gene for CCL2 is situated on chromosome 17q11.2 and gene for its receptor CCR2 is located on chromosome 3p21.3.

Many studies have indicated that the nuclear factor (NF)-κB-like binding site and the AP-1/GC box binding site has 90 and 68 base pairs, respectively, which are located on the upstream of the transcriptional start site, are indispensable for cytokine induction of the human CCL2 expression in reaction to an inflammatory stimulus.[25,26] NF-κB has five members: p65 (RelA), RelB, c-Rel, the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52, respectively.[27] It was originally recognized as a nuclear factor and was involved in the expression of a great deal of genes in different types of cells.[28] There are two closely located NF-κB binding sites, A1 and A2 sites. A2 site is similar to the κB sites, while the A1 site has a one base mismatch with the consensus sequence.[26] In the promoter region of the E-selectin gene, two NF-κB sites are closely situated, which are indispensable for the improved transcription of this gene. This collaboration has been reported to be mediated by high mobility group protein I(Y), HMG-I(Y), a minor groove DNA-binding protein that bent DNA upon binding. HMG-I(Y) can steady the binding and similarly of multicomponent protein–DNA complexes, including NF-κB and ATF-2. The κB-2 site includes an A-T-rich region in its center, which is analogous to the κB/HMG-I(Y) motif found in the IFN-β enhancer.[26] Because two NF-κB complexes collaborate for the human *CCL2* gene transcription, HMG-I(Y) may possibly play a role in increasing the assembly of the two NF-κB complexes that bound to the enhancer region of the *CCL2* gene. Further investigation is indispensable to decide whether HMG-I(Y) is involved in the human *CCL2* gene regulation.[28]

In the distal regulatory region of the *CCL2* gene, two novel polymorphisms have been discovered. The polymorphisms situated at positions –2518 (G or A) and –2076 (A or T) inter-relates to the considerable transcriptional start site of the gene. This genetic diversity in the immune response thus may be responsible for clinical differences in organ inflammation and disease severity.[29] Recent evidences have found that in a cohort of children, the frequency of the –2518G polymorphism in the *CCL2* gene regulatory region is obviously increased in asthmatic children than those in the controls and nonasthmatic atopic children. The appearance of the CCL2 G allele also is related with asthma severity and blood eosinophil level in asthmatic children.[30] The evidence suggests that there are important relationships between carrying G at 2518 of the gene regulatory region of CCL2 and the emergence of asthma, and between asthma severity and homozygosity for the G allele. CCL2 could prompt undifferentiated T-lymphocyte populations toward an IL-4-generating Th2-type cell.[31] IL-4 could upregulates eotaxin, which is the most potent chemoattractant for eosinophils.[32] The effect of G allele seems to be dose-dependent.[30]

5. CCL2 AND ASTHMA

CCL2 have an important role in the pathogenesis of asthma because of its ability to attract eosinophils and monocytes, activate basophils and mast cells, and induce the release of leukotriene C4 into the airway, all of which promoting airway hyperresponsiveness.[33] CCL2 was obviously upregulated after challenge in asthmatic patients.[34] Furthermore, the neutralization of CCL2 during the allergic airway response reduced histamine in the bronchoalveolar lavage, and CCL2 was involved in the induction of changes in airway resistance in a cockroach Ag-induced murine model and normal mice.[33] These results supported an important role for CCL2 in the asthmatic response.

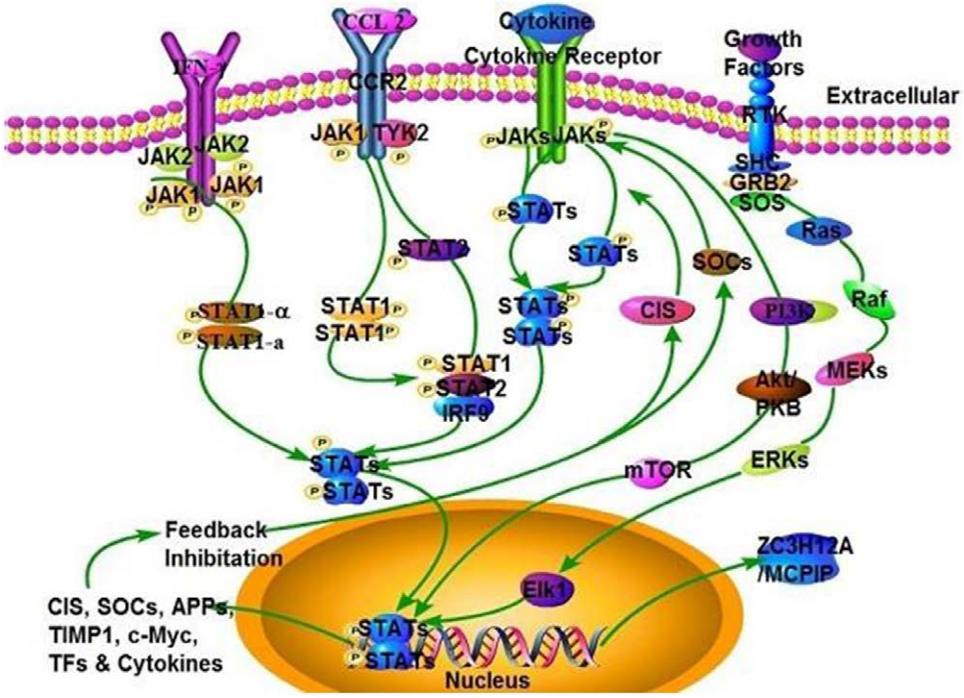

Fig. 4 Possible signaling pathway of CC chemokine ligand 2 (CCL2). CCL2 binds with CC chemokine receptor type-2 (CCR2) to activate (Janus kinases) JAK/STAT pathway and regulate the expression of target genes.

Jhony and his colleagues found that Aspirin-Triggered-Resolvin D1 (AT-RvD1) apparently decreased CCL2 and interleukin-8 (IL-8) production when comparing to cells treated with IL-4. More importantly, these effects were lipoxin A_4/formy1 peptide receptor 2 (ALX/FPR2) receptor dependent and partly related with the downregulation of STAT6 and NF-κB pathways by AT-RvD1.[35] A recent study used an ovalbumin-induced allergic asthma model that received anti-CCL2 antibody or CCR2 antagonist prior to the challenge, and found that the frequencies of both IL-17-secreting T helper (Th)17 and CD8 (Tc)17 cells were increased significantly. Meanwhile, when they blocked CCL2/CCR2 axis, they noted that the frequency of Th17 was greatly reduced but not the Tc17 cell, which indicates a selective effect of CCL2 on the recruitment of Th17 cells.[36] This model also examined the effect of chemerin on CCL2 expression in activated lung epithelial cells in vitro.[37] A study in a human rhinovirus (HRV)-induced airway hyper-responsiveness and inflammation in mouse model showed that epithelial cell and macrophage CCL2 may play a role in HRV-induced asthma exacerbations.[38] More importantly, Naibing et al found that mRNA expression of IL-2 as well as CCL2 and MCP-3 in the mouse lung was increased very early (within 2 h) after allergen challenge while IL-13-induced IL-1 and -2 expression, and IL-13-induced extracellular signal-regulated kinase 1/2 phosphorylation and CCL2 and MCP-3 production was restrained by RNA interference.[39] In a mouse allergic asthma model, depletion of alveolar macrophages restrained Th2-type allergic lung inflammation and its consequent airway remodeling. Furthermore, in allergic subjects with mild asthma, airway allergen challenge crooked the pattern of alveolar macrophages gene expression toward high levels of the receptor for MCP1 (CCR2/MCP1R) and expression of M2 phenotypic proteins; although many proinflammatory genes were apparently suppressed, CCL2/MCP-1 gene expression was obvious in the bronchial epithelial cells in a mouse allergic asthma model.[40]

Th2 cytokines (e.g., IL-4 and IL-13) and CCL2 participated in bronchial hyperreactivity and remodeling in allergic asthma. IL-4 and IL-13 up-regulated gene expression and apparently increased the release of CCL2 from bronchial epithelial cells. Both cytokines could activate p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and Janus kinase-2 (JAK-2) activity. To investigate the inhibitory activities of p38 MAPK, ERK, and JAK-2, Ip et al pretreated the cells with their inhibitors SB203580, PD98059, and AG490, and they found the production of IL-4-induced and IL-13-induced CCL2 was significantly suppressed. In summary, the activation of p38 MAPK, ERK, and JAK-2 was vital for IL-4-induced and IL-13-induced CCL2 release in human bronchial epithelial cells.[41] CCL2 was known to be regulated by oxidative stress, cytokines, and growth factors. In addition, CCL2 was also correlated with hypoxic regulation. CCL2 could be induced by both hypoxia and CoCl2 in human astrocytes, and the promoter of CCL2 had hypoxia response elements, which could bind hypoxia inducible factor-1 (HIF-1).[42]

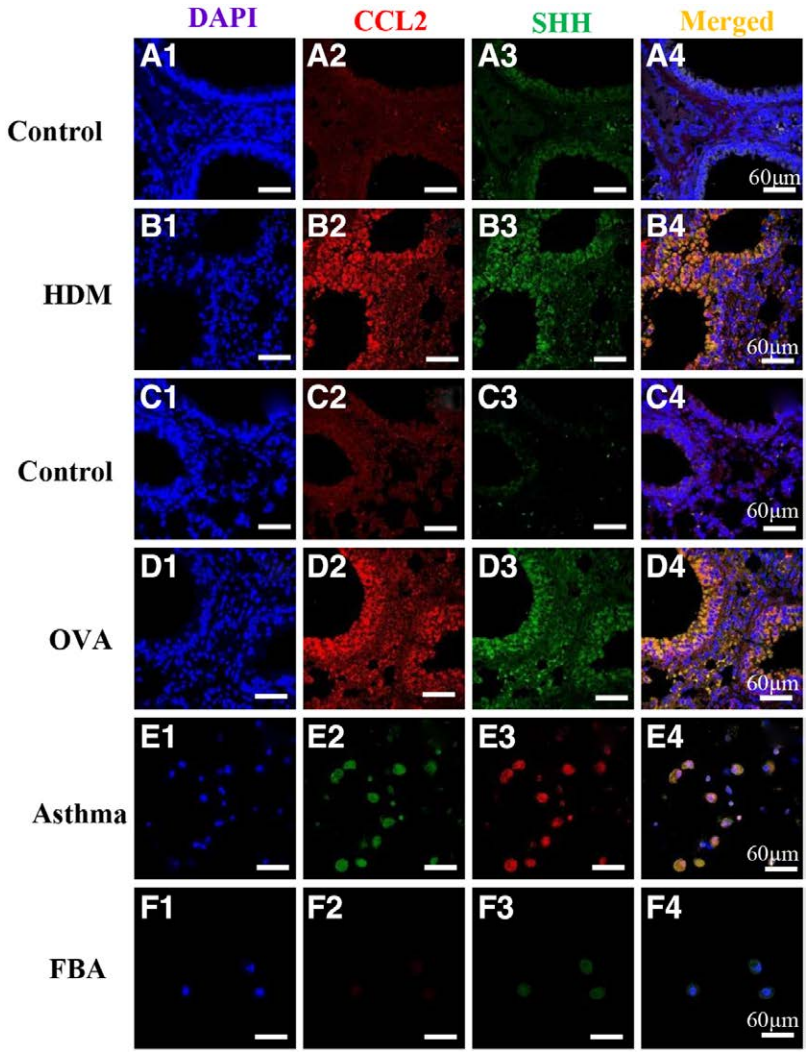

Fig. 5 Immuofluorescence staining of Sonic hedgehog (Shh) and CC chemokine ligand 2 (CCL2). A–D, In the OVA and HDM-induced murine model; E, Bronchoalveolar lavage fluid cells in asthmatic patients; F, Bronchoalveolar lavage fluid cells in patients with airway foreign body as controls.

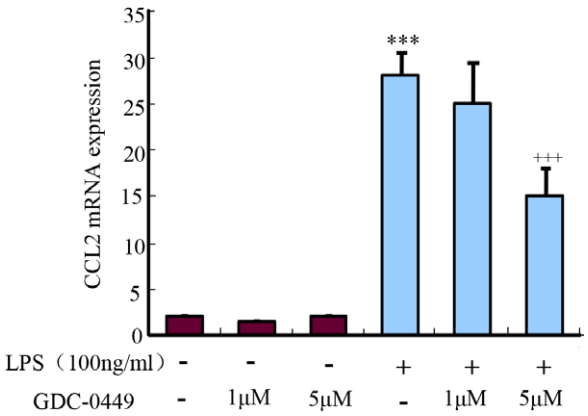

Fig. 6 Sonic hedgehog (Shh) regulates CC chemokine ligand 2 (CCL2) expression in macrophages. Effect of Shh stimulation and Smo inhibitor GDC-0449 on the expression of CCL2 mRNA in macrophage cell line. Data were expressed as mean ± SEM (n = 5, per group). $^{*}p < 0.05$ compared with control. $^{+}p < 0.05$ compared with 5 μM GDC-0449 alone.

6. INTERACTION BETWEEN SHH AND CCL2 SIGNALING PATHWAY IN ASTHMA

CCL2 induced hepatocellular carcinoma (HCC) cell invasion and epithelial–mesenchymal transition, which was accompanied by the activation of Hh signaling and increase the expression of Smo and Gli1.[43] In HCC cells, cancer-associated fibroblasts also secreted CCL2, CCL5, CCL7, and chemokine ligand 16 promotes HCC metastasis through the coordinate activation of Hh and transforming growth factor beta pathways.[44] In a mouse model of Hh signaling-dependent tumors, activation of Hh signaling in keratinocytes via expression of a constitutively activated, mutant Smo results in enhanced TGF-β signaling. Because of TGF-β signaling activation, the expression of CCL2 was improved in the tumor microenvironment (TME), whereas CCR2 expression was improved in myeloid-derived suppressor cells (MDSC). Circulating MDSCs migrated toward the CCL2-enriched TME and remain to foster an immunosuppressive TME.[45] All these articles clarified the interaction between Shh and CCL2; therefore, we supposed that Shh and CCL2 signaling pathway also were interacted. Additionally, as demonstrated by immunofluorescence, Shh and CCL2 interacted in airway epithelium in HDM and OVA mouse model, whereas they were observed mainly in exfoliated airway epithelium cells and inflammatory cells (Fig. 5). Our previous study revealed that the downstream molecules of Shh signaling pathway were activated in monocytes or macrophages in inflammation, although Shh was not obvious. Moreover, recombinant Shh could induce the CCL2 overexpression, and Smo inhibitor GDC-O449 could inhibit CCL2 expression in the airway epithelial cells and monocytes or macrophages (Fig. 6). Our data revealed a possible novel mechanism that Shh regulated the proliferation of airway epithelial cells through its downstream transcription factor Gli2, which may dominate the expression of cyclin D1 and cyclin E1,

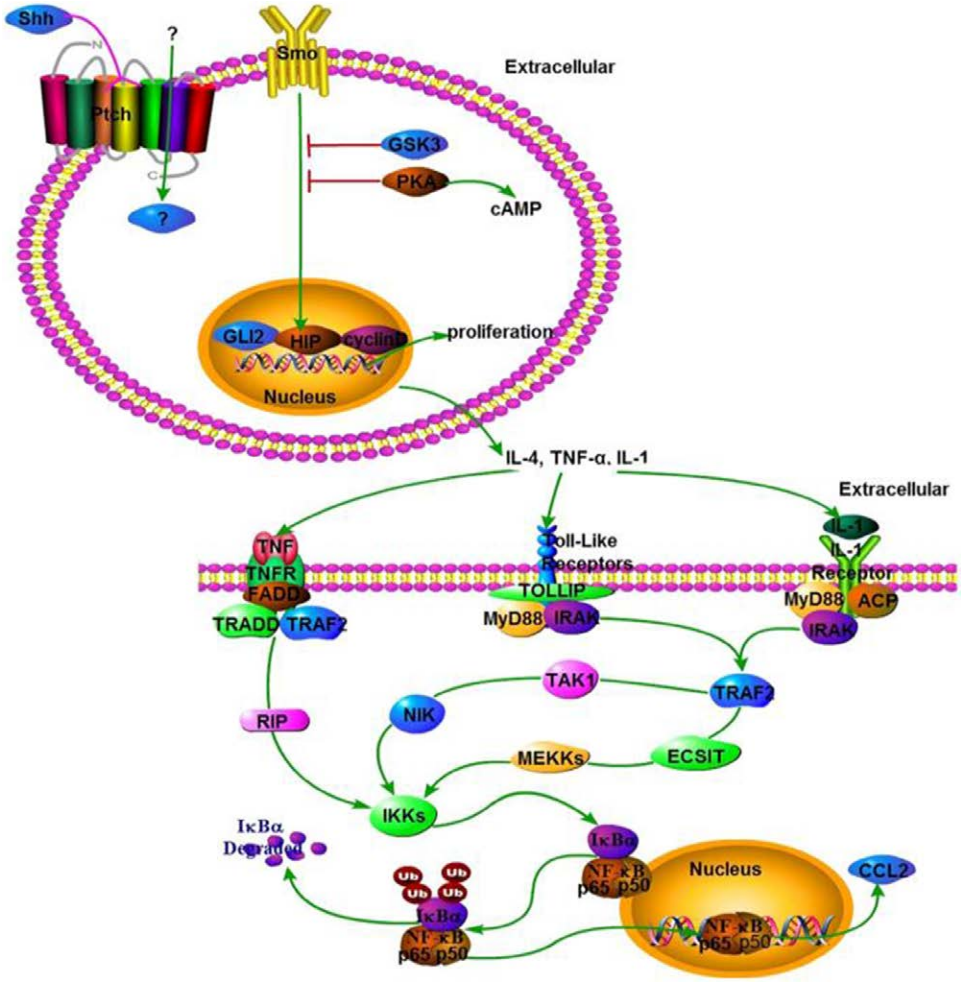

Fig. 7 Possible interaction between Sonic hedgehog (Shh) and CC chemokine ligand 2 (CCL2) signaling pathways in asthma. Shh signaling pathway is activated and releases some inflammatory factors, such as IL-1, TNF-α, and IL-4. These inflammatory factors may bind with their receptors to activate NF-κB, which is located on the upstream of the transcriptional start site of *CCL2* gene; therefore, it may upregulate the expression of CCL2 in the airway epithelium and BAMs in asthma and participate in the occurrence and development of asthma.

Table

Principal clinical studies with Shh and CCL2 signaling pathways in asthma

Signaling pathway	First author [ref.] year	The type of asthma	Summary of outcomes
Shh	Standing ASI [20] 2017	Allergic asthma	Shh signaling was activated, enhancing the Th2 response
Shh	Li X [21] 2011	Allergic asthma	A subset of the genes, including *HHIP* and *Ptch1,* which regulated lung function
Shh	Furmanski AL [23] 2013	Allergic asthma	$CD4^+$ T cells from lung improved levels of Smo transcript, which increased IL-4 production and Th2 responses aggravating allergic asthma
CCL2	Campbell EM [33] 1999	Allergic asthma	CCL2-mediated airway hyperreactivity
CCL2	Holgate ST [34] 1997	Allergic asthma	CCL2 was obviously upregulated after allergen challenge
CCL2	de Oliveira JR [35] 2015	Allergic asthma	AT-RvD1 decreased CCL2 production
CCL2	Wang A [36] 2015	Allergic asthma	CCL2 recruited Th17 cells to the lung
CCL2	Zhao L [37] 2014	Allergic asthma	Chemerin suppressed allergic asthma by inhibiting CCL2 production
CCL2	Schneider D [38] 2013	Allergic asthma	CCL2 contributed to HRV-induced airway hyperresponsiveness and inflammation
CCL2	Gu N [39] 2009	Allergic asthma	Intelectin was required for IL-13 induced CCL2 expression and promoted allergic airway inflammation
CCL2	Lee YG [40] 2015	Allergic asthma	*CCL2* gene expression was obvious in the bronchial epithelial cells in allergic asthma
CCL2	Ip WK [41] 2006	Allergic asthma	IL-4 and IL-13 up-regulated CCL2 expression
CCL2	Baay-Guzman GJ [42] 2012	Allergic asthma	HIF-1 expression was associated with CCL2 expression in airway inflammatory cells

CCL2 = CC chemokine ligand 2; Shh = Sonic hedgehog.

and upregulated the expression of CCL2 in the airway epithelium and BAMs. CCL2 bound with CCR2 activated JAK/signal transducer and activator of transcription (STAT) signaling pathway and increased the expression of regulatory RNase 1 (also known as ZC3H12A/MCPIP), and further promoted monocytes change into BAMs, which is involved in the mechanism of asthma (Fig. 7; Table).

In conclusion, asthma is a common chronic respiratory disease with a rising incidence. To date, there has been substantial research progress regarding the mechanism of asthma. Some evidences have showed that Shh and CCL2 are highly correlated with the pathology of asthma; however, how Shh and CCL2 interact is important in asthma and remains to be elucidated. Further investigation should focus on delineating their interacted pathways and mechanism using animal models as well as women and men with various phenotypes of asthma. Understanding the pathways may be helpful for us to better understand the mechanism of asthma and explore new therapeutics of asthma.

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