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Long-chain non-coding RNA n337374 relieves symptoms of respiratory syncytial virus-induced asthma by inhibiting dendritic cell maturation via the CD86 and the ERK pathway

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Abstract
Background: In this study, we investigated the relationship between long-chain non-coding RNAs (lncRNAs) and respiratory syncytial virus (RSV)-exacerbated asthma.
Methods: Transcriptome microarray was used to detect differentially expressed lncRNAs in dendritic cells (DCs) co-cultured with RSV-infected human airway epithelial cells and DCs infected with RSV. The identified downregulation of lncRNA n337374 was validated using fluorescence RT-qPCR. LncRNA n337374-overexpressing DCs and RSV-exacerbated asthmatic mouse models were established. Airway hyper-reactivity and bronchoalveolar lavage fluid (BALF) were examined, and pathological changes in lung tissues were observed in mice. Surface molecules in DCs were detected by flow cytometry and RT-qPCR and the expression of CD86 and mitogen-activated protein kinases was determined by western blot.
Results: In an RSV-exacerbated asthmatic mouse model, the airway wall was thickened, luminal stenosis was observed, a large number of inflammatory cells were infiltrated in the lung tissue, lung function was impaired, and counts of inflammatory cells in the BALF were increased. The overexpression of lncRNA n337374 ameliorated these pathological changes and improved impaired lung function and inflammation in an asthmatic mouse model. In DCs co-cultured with RSV-infected human airway epithelial cells, CD86 expression was promoted and ERK was markedly phosphorylated. When lncRNA n337374-overexpressing DCs were used in the co-cultures, the expression of CD86 and phosphorylated ERK was decreased.
Conclusion: The results suggest that lncRNA n337374 overexpression may suppress DC maturation by downregulating the CD86 and ERK pathway, subsequently relieving the symptoms of RSV-induced asthma. LncRNA n337374 may be a promising target in the treatment of RSV infection-induced asthma.
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Introduction

Asthma is a heterogeneous disease defined by a history of respiratory symptoms, such as wheezing, shortness of breath, and chest tightness.[1] Viral respiratory infections are important and common triggers of asthma exacerbations in children.[2,3] Respiratory syncytial virus (RSV), an enveloped, non-segmented negative-strand RNA virus, is the most common cause of severe acute lower respiratory illness in children, which, in some cases, leads to infant hospitalization and even death.[4,5] Increasing studies have provided convincing evidence that RSV-induced asthma exacerbation in early childhood is an important causative factor for subsequent onset of asthma later in life.[6] Though the pathology of RSV-exacerbated model was reported to be involved in complement activation,[7] the underlying mechanism remains incompletely characterized.

Long-chain, non-coding RNAs (lncRNAs) extensively exert regulatory effects on apoptosis, proliferation, and differentiation by modulating gene expression at different levels rather than encoding proteins.[8] The function of lncRNAs in asthma has received much attention, and some lncRNAs, such as lncRNA MEG3 and GAS5, have been found to play a role in the development of asthma.[9,10] Nonetheless, little is known about the involvement of lncRNAs in RSV-induced asthma exacerbation model and the underlying mechanisms.

Dendritic cells (DCs) are crucial for the induction of adaptive immunity, tolerance, or allergic responses in the lung.[11] DCs have been recognized as the most powerful antigen-presenting cells, functioning in the initiation of asthma.[12] It has long been demonstrated that RSV infection leads to DC maturation.[13] In our previous study, we found that primary rat airway epithelial cells exposed to RSV-induced functional maturation of rat myeloid dendritic cells (mDCs).[14] Based on these findings, we used human immature monocyte-derived dendritic cells (iMDDCs) in co-culture with RSV-infected human airway epithelial cells and an RSV-induced asthma exacerbation mouse model to investigate promising, differentially expressed lncRNAs (DELs) that may be implicated in RSV-exacerbated asthma and elucidate the underlying molecular mechanisms. Moreover, human whole-genome microarray technology, cluster analyses, function and pathway enrichment analysis, fluorescence real-time quantitative PCR (RT-qPCR), flow cytometry, and western blot analysis were applied in this study.

Materials and methods

Cell preparation and culture

Human BEAS-2B bronchial epithelial cells were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China, and cultured in Dulbecco's modified Eagle's medium (DMEM). Human iMDDCs were generated from peripheral blood monocytes and cultured as described previously.[15]

RSV culture and titer determination

RSV Strain Long, an A subtype RSV (Guangzhou Biotest Bioengineering, Guangzhou, China) was cultured on monolayers of Hep-2 cells in RPMI-1640 medium. RSV was harvested as demonstrated in our previous study,[14] and a multiplicity of infection of 1 (MOI = 1) was used in subsequent analyses.

Cell grouping and co-culture of BEAS-2B bronchial epithelial cells and iMDDCs

Three experimental groups were used, including the human dendritic cell (HDC)-BEAS+RSV group, HDC-RSV group, and HDC-CONTROL group. In the HDC-BEAS+RSV group, a suspension of human BEAS-2B bronchial epithelial cells was inoculated into the top chamber of a Transwell co-culture system (37°C, 5% CO_2). RSV solution was added to the top chamber and cultured for 2 h. Consequently, iMDDCs were added to the bottom chamber of the Transwell system at a 1:1 ratio of iMDDCs to BEAS-2B for a 48-h co-culture. In the HDC-RSV group, RSV viral solution was inoculated into the top chamber and the same volume of iMDDC suspension was added to the bottom chamber. In the HDC-CONTROL group, the same procedure was used but the RSV viral solution was replaced with vehicle.

HTA 2.0 transcriptome microarray assay and bioinformatic analyses

The cells in the bottom chamber were collected to extract total RNA. The prepared GeneChips were scanned using Affymetrix® GeneChip Command Console (AGCC). The data were normalized using the Robust Multichip Analysis (RMA) algorithm.

Differentially expressed genes (DEGs) and lncRNAs between different groups were filtered using the random variance model (RVM) *t*-test.[16] Gene ontology (GO)[17] function and pathway enrichment analyses were performed for the DEGs. Pathway analysis was carried out using Kyoto Encyclopedia of Genes and Genomes (KEGG),[18] Biocarta, and Reactome.[19]

Fluorescence RT-qPCR

The expression of DELs was detected using fluorescence RT-qPCR. Briefly, 2 mg of purified RNA was used to establish a 20-μl reverse transcription system. The resulting cDNA was amplified by PCR (1 μL cDNA, 1 μL each of upstream and downstream primers, 10 μL 2 × MIX, and 7 μL sterile water) involving initial denaturation (95°C for 10 min) and 40 cycles of amplification (95°C denaturation for 30 s, 57°C annealing for 30 s, and 75°C extension for 45 s).

Infection of iMDDCs with n337374-overexpressing lentiviruses

Lentivirus overexpressing lncRNA n337374 (MOI = 25) was transfected with 293T cell (GENECHEM Co.) in DMEM containing 10% fetal bovine serum (FBS). Green fluorescent protein (GFP) expression was observed under a fluorescence microscope to evaluate transfection efficiency after 48 h. The supernatant containing lentivirus particles was collected

and titrated. Consequently, the viral solution at MOI = 20 was used to transfect iMDDCs. After 72 h, the n337374 mRNA expression from the gene corresponding to lncRNA n337374 was detected using fluorescence RT-qPCR. As a control, the iMDDCs were transfected with empty vector GV367 (MOI = 20) or exposed to culture medium using the same procedure. After transfection, the iMDDCs were co-cultured with human BEAS-2B bronchial epithelial cells infected with RSV using the Transwell system for 48 h as mentioned above.

Establishment of an lncRNA n337374-overexpressing RSV-exacerbated asthmatic mouse model

For the study, 7-week-old, female specific pathogen-free BALB/c mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. Twelve mice were randomly divided into the normal group, ovalbumin (OVA)+RSV group, and OVA+RSV+ LV-n337374-EGFP group. The experimental protocol was approved by the Ethical Committee of Shanghai Jiaotong University School of Medicine for Laboratory Animals.

In the normal group, each mouse was intraperitoneally injected with 0.2 mL sterile saline solution on days 0, 7, and 14. In the form of nasal drops, each mouse was administered 30 μL of sterile saline solution on days 21, 23, and 25 and 25 μL of sterile saline solution was administered on days 28 and 29.

In the OVA+RSV group, each mouse was intraperitoneally injected with 0.2 mL of freshly prepared OVA aluminum hydroxide solution containing 20 μg OVA and 2.25 mg aluminum hydroxide on days 0, 7, and 14. On days 21, 23, and 25, each mouse was administered 30 μL of sterile saline solution in the form of nasal drops. On days 28 and 29, each mouse was inoculated 25 μL of 10^7 pfu RSV in the form of nasal drops. In the OVA+RSV+LV-n337374-EGFP group, each mouse received the same treatment as the mice in the OVA+RSV group. Moreover, on days 21, 23, and 25, each mouse received 30 μL of 10^8 pfu LV-n337374-EGFP in the form of nasal drops. On day 34, the right posterior lung lobe tissues of each mouse were collected and prepared. Pathological changes in lung tissue sections (5-μm thick) were observed under a light microscope.

Detection of mouse airway hyper-reactivity

Pulmonary functions of the mice were detected using an animal pulmonary function-non-invasive airway mechanism detection system. Methacholine (Mch) at the same volume of saline (10 μL) was nebulized at increasing concentrations in the order of 0, 3.125, 6.25, 12.5, 25, and 50 mg/mL. Mean special airway resistance (sRAW) values were recorded under different Mch concentrations and used as indicators to assess the airway reactivity of the mice.

Classification of bronchoalveolar lavage fluid (BALF) cells

BALF of mice in each group was collected as previously described.[20] A total of 300 blood cells in BALF were counted to calculate the percentage of inflammatory cells including leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

Detection of surface molecules and western blot analysis

Surface molecules consisting of HLA-DR, CD40, CD80, CD83, and CD86 of DCs were detected using fluorescence RT-qPCR and flow cytometry. Protein levels of p-ERK1/2, p-JNK, and p-p38 in DCs were analyzed using western blot analysis.[14]

Statistical analyses

Statistical analyses were performed using SPSS software version 22.0 (IBM Software and Systems, NY, USA). All data were expressed as the mean ± standard deviation. Mean values between two groups were compared using t-test. Mean values of more than two groups were compared using one-way analysis of variance (ANOVA). $P < 0.05$ indicated a significant difference.

Results

LncRNA n337374 was significantly downregulated in DCs upon RSV stimulation

To unravel the possible lncRNAs related to RSV-exacerbated asthma, DEGs and DELs were screened among the HDC-CONTROL group, HDC-RSV group, and HDC-BEAS+RSV group. As a result, 25 DEGs and 29 DELs with $P < 0.05$ were identified between the HDC-BEAS +RSV and HDC-RSV group. Most of them were upregulated genes and lncRNAs, and only five genes and two lncRNAs were downregulated. The results of cluster analysis showed that the expression levels of these genes and lncRNAs were markedly different among the three groups (Figure 1A).

According to GO function enrichment analysis, the upregulated genes were significantly enriched in a number of pro-inflammatory immune reaction-related biological processes (Figure 1B), whereas the downregulated genes were significantly related to thyroid hormone catabolic process and protein O-linked glycosylation-related biological processes (Figure 1C). Moreover, the upregulated genes were consistently involved in the chemokine signaling pathway, cytokine–cytokine receptor interaction, and NF-κB signaling pathway (Figure 1D).

Of the identified DELs, the top 6 DELs with the largest fold changes were selected to be validated using fluorescence RT-qPCR. As shown in Figure 1E, the expression of the gene corresponding to lncRNA n337374 was significantly downregulated in the HDC-BEAS+RSV group compared with the HDC-CONTROL group and the HDC-RSV group ($P < 0.01$), in concordance with the results of the microarray assay. This suggested that lncRNA n337374 might play an important role in RSV-exacerbated asthma and was therefore selected for the subsequent analyses.

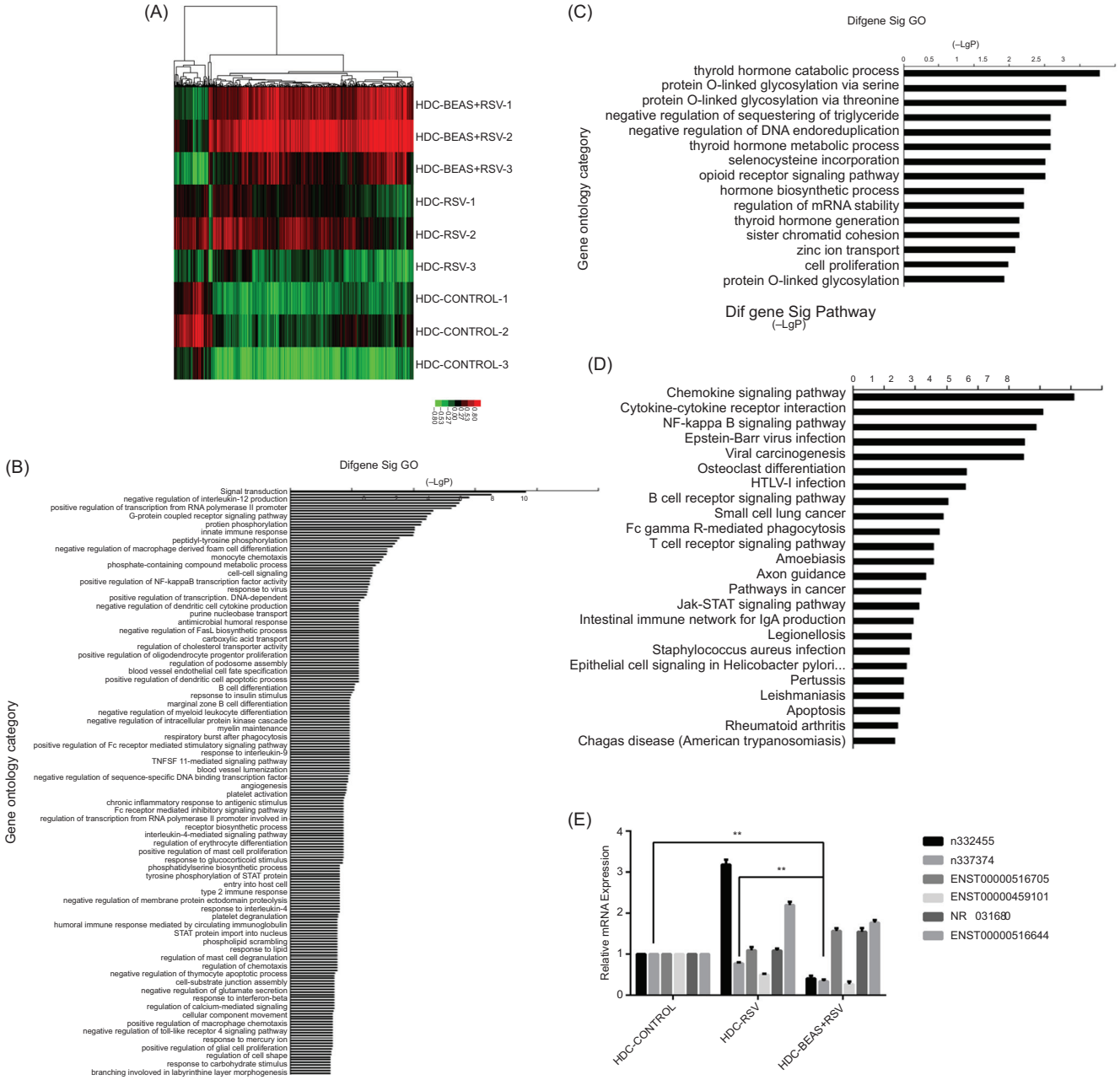

Figure 1 Identification, functional analysis, and validation of differentially expressed genes and lncRNAs. Cluster analysis of differentially expressed genes and lncRNAs (A). Red and green colors indicate upregulated and downregulated genes and lncRNAs, respectively. Biological processes that the upregulated genes are involved in (B). Biological processes that the downregulated genes are involved in (C). Signaling pathways significantly related to the upregulated genes (D). Detection of six differentially expressed lncRNAs in DCs by fluorescence real-time quantitative PCR (E).

Successful overexpression of lncRNA n337374 in DCs by infection with lentiviruses

We infected 293T cells with lncRNA n337374-overexpressing lentiviruses. After 48 h of infection, GFP expression was observed in almost all 293T cells under a fluorescence microscope (Figure 2A), indicating high transfection efficiency of the n337374-overexpressing lentiviruses in 293T cells. Therefore, n337374 lentiviruses were used to infect the target cells (iMDDCs) to achieve lncRNA n337374 overexpression. It was observed that n337374 expression was dramatically higher in DCs transfected with lncRNA n337374-overexpressing lentivirus (n337374-DC group) compared with DCs transfected with empty GV367 vector (GV367-DC group) and exposed to medium (CONTROL group, Figure 2B). These results indicate that lncRNA n337374 was successfully overexpressed in DCs by infection with lncRNA n337374-overexpressing lentiviruses.

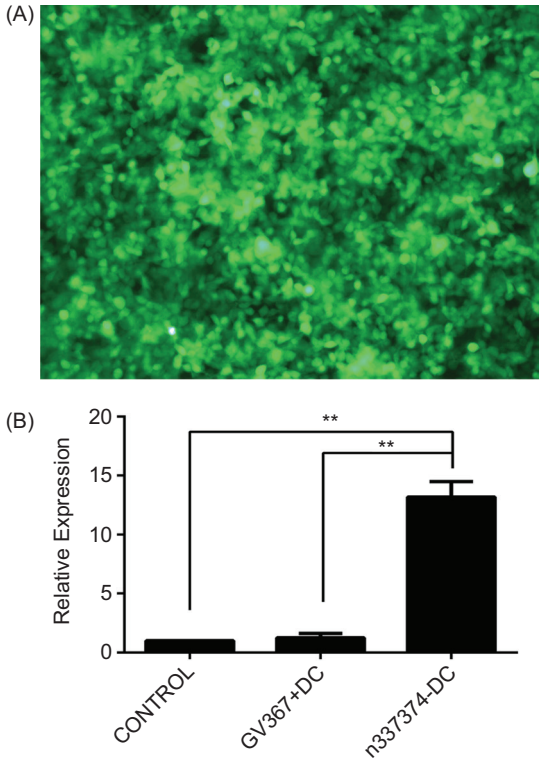

Figure 2 Transfection efficiency of lentiviruses overexpressing lncRNA n337374. Observation of green fluorescence in 293T cells transfected with lncRNA n337374- overexpressing lentiviruses (100×) (A). Green fluorescence suggests lncRNA n337374 overexpression. Relative expression of lncRNA n337374 in DCs transfected with lncRNA n337374-overexpressing lentiviruses or empty vector GV367 (B). **p < 0.01.

lncRNA n337374 overexpression relieved the symptoms of asthma in the RSV-exacerbated asthmatic mouse model

To study the effect of lncRNA n337374 overexpression on RSV-exacerbated asthma *in vivo*, the lncRNA n337374-overexpressing RSV-exacerbated asthmatic mouse model was established by intra-nasal administration of lncRNA n337374-overexpressing lentiviruses. Compared with the lung tissues of mice in the normal group (Figure 3A), the lung tissues in the OVA+RSV group showed noticeable airway wall thickening, luminal stenosis, and infiltration of a large amount of inflammatory cells (Figure 3B). This proved that the RSV-exacerbated asthma model was developed successfully in mice. Moreover, the OVA+RSV+LV-n337374-EGFP group had reduced airway wall thickening and luminal stenosis and decreased inflammatory cell infiltration in lung tissues (Figure 3C). Based on these morphological results, we concluded that lncRNA n337374 overexpression alleviated RSV-exacerbated asthmatic syndromes.

The sRAW values of mice in the OVA+RSV group and the OVA+ RSV+LV-n337374-EGFP group gradually increased with the increase in Mch concentration. At 0, 6.25, and 12.50 mg/mL Mch concentrations, sRAW values of the mice were significantly decreased in the OVA+RSV+LV-n337374-EGFP group compared with the OVA+RSV group ($P < 0.01$, Figure 3D). Cell classification and cell counting in the BALF showed significant increments in the total number of leukocytes, neutrophils, lymphocytes, monocytes and eosinophils in the OVA+RSV group relative to the normal group ($P < 0.05$). The OVA+RSV+LV-n337374-EGFP group had obviously decreased amounts of leukocytes, neutrophils, lymphocytes, monocytes, and eosinophils than the OVA+RSV group ($P < 0.05$, Figure 3E). These observations implied

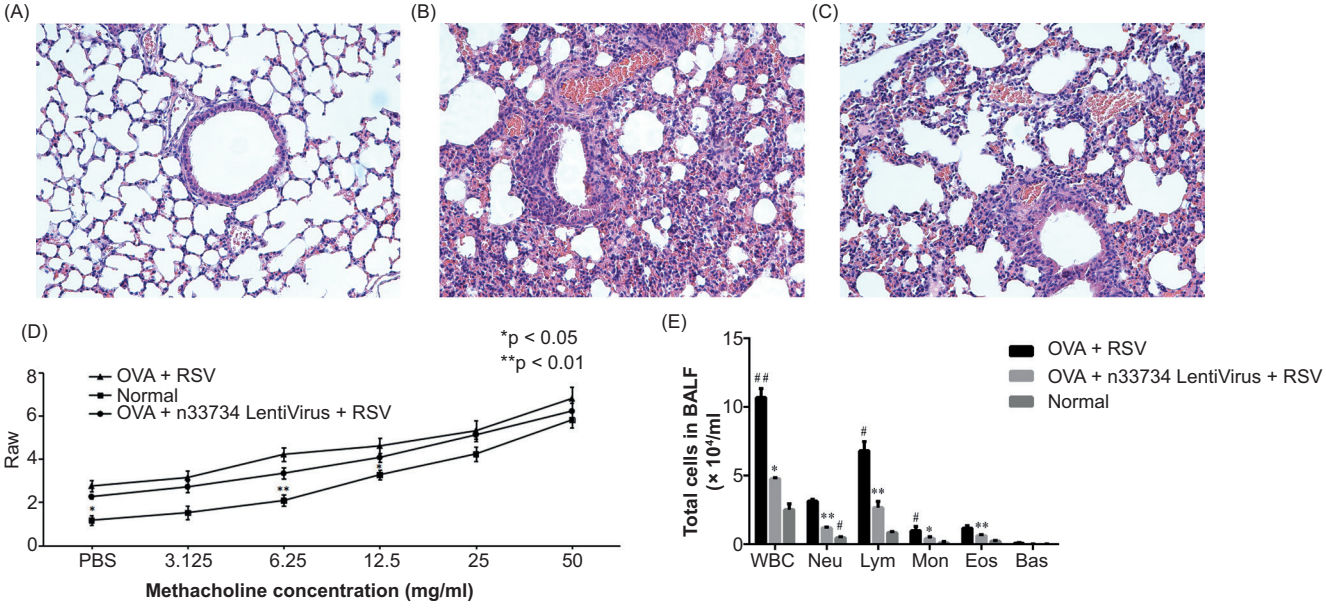

Figure 3 Overexpression of lncRNA n337374 attenuates asthmatic syndrome in RSV-exacerbated asthma mice. Representative images of lung tissue sections of mice from the normal group (A), the OVA+RSC group (B), and the OVA + n337374 lentivirus + RSV group (C), respectively (A–C); sRAW value of different treatment groups (D). Cell classification and cell counting results of leukocytes in the BALF of different treatment groups (E). * p < 0.05, ** p < 0.01 *vs.* the OVA+RSV group; # p < 0.05, ## p < 0.01 *vs.* the normal group.

that lncRNA n337374 overexpression improved the impairment of pulmonary function and inflammation caused by RSV- exacerbated asthma.

Overexpression of lncRNA n337374 compromised the upregulation of surface molecule CD86 and p-ERK1/2 protein in DCs exposed to RSV stimulation

By using fluorescence RT-qPCR, it was observed that CD86 mRNA expression was obviously elevated in the HDC-BEAS+RSV group compared with the HDC-RSV group ($P < 0.01$). However, the elevation of CD86 was significantly decreased in the n337374-HDC-BEAS+RSV group but not in GV367-HDC-BEAS+RSV group (Figure 4A). Similarly, flow cytometry results showed that the HDC-BEAS+RSV group had a remarkable increase in CD86 expression levels in comparison with the HDC-RSV group ($P < 0.01$), whereas the n337374-HDC-BEAS+RSV group had significantly decreased CD86 expression levels compared with the GV367-HDC-BEAS+RSV group ($P < 0.01$, Figure 4B–C).

As mitogen-activated protein kinases (MAPKs) are involved in all aspects of asthma, they have emerged as promising targets for the development of therapies against asthma.[21] Phosphorylated extracellular signal-regulated kinase (p-ERK), Jun kinase (p-JNK), and p38 were assessed using western blot analysis. Compared with the HDC-RSV group, the HDC-BEAS+RSV group showed a significantly higher expressions of p-ERK1/2 and p-JNK ($P < 0.01$, Figure 4D–E). Moreover, there was a significant decrease in p-ERK1/2 in the n337374-HDC-BEAS+RSV group compared with that of the GV367-HDC-BEAS+RSV group ($P < 0.01$, Figure 4D–E). However, the expression levels of p-JNK and p-38 were significantly increased in n337374-HDC-BEAS+RSV group compared with that of GV-367-HDC-BEAS+RSV group ($P < 0.05$, Figure 4D–E). These results revealed that RSV infection resulted in upregulation of CD86 and enhanced ERK phosphorylation in DCs, which was partly suppressed by lncRNA n337374 overexpression.

Discussion

Asthma has become a severe global health problem affecting all age groups, especially children.[22] RSV infection is a leading causative factor for acute asthma in children.[23] To date, the association between lncRNAs and RSV-exacerbated asthma has not been studied in detail. For the first time, to our knowledge, this study highlighted the potential lncRNAs participating in RSV-induced asthma using both *in vivo* and *in vitro* analyses. Using human transcriptome array analysis, we determined that a total of 25 DEGs and 29 DELs were possibly related to RSV-stimulated exacerbation asthma *in vitro*. Furthermore, GO function and pathway enrichment analysis uncovered that these dysregulated genes were significantly correlated with several pro-inflammatory immune reaction-related biological processes, the chemokine signaling pathway, the cytokine–cytokine receptor interaction pathway, and the NF-κB signaling pathway. Chemokines participate in the development and progression of asthma via the regulation of inflammatory cells, angiogenesis, and airway hyper-reactivity.[24] NF-κB acts as a switch for the inflammatory stimulus signals during the differentiation and maturation process of DCs.[25] NF-κB induces the expression of

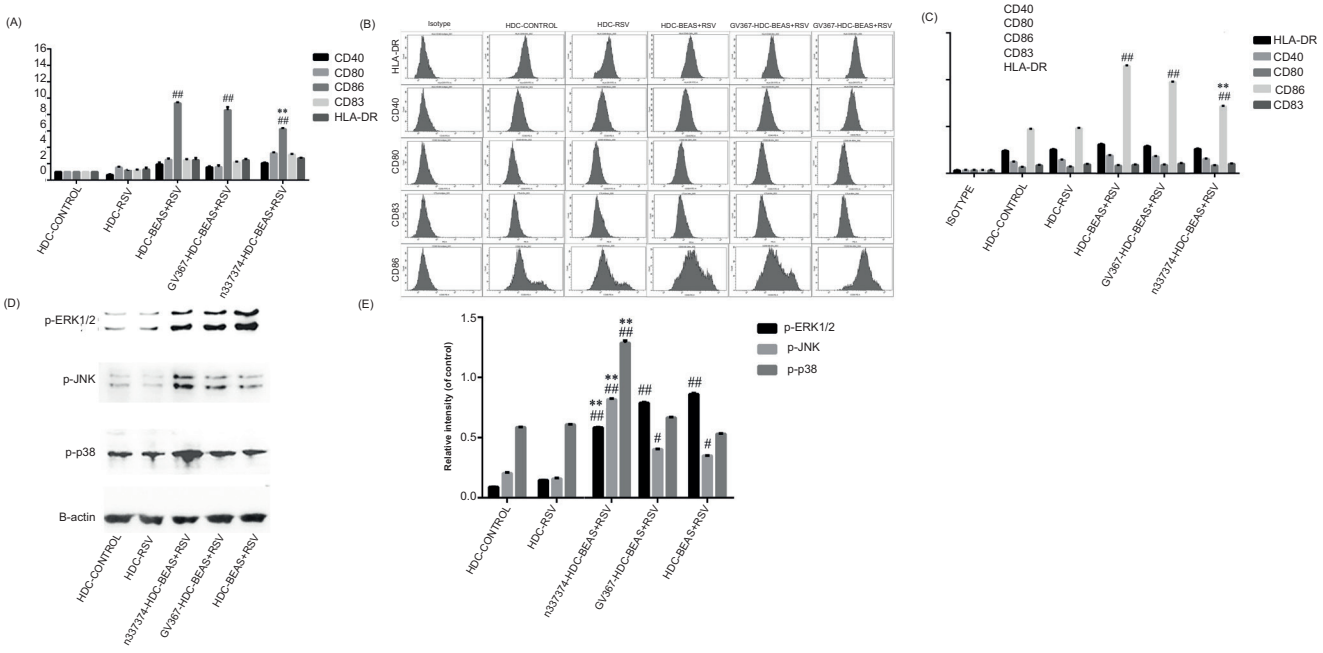

Figure 4 Effect of lncRNA n337374 overexpression on the surface molecules and MAPK pathways in DCs. Detection of surface molecules in DCs of different groups using fluorescence real-time quantitative PCR (A). Evaluation of surface molecules in DCs of different groups using flow cytometry (B–C). Western blot analysis of p-ERK, p-JNK, and p-p38 proteins in different groups (D–E). **p < 0.01 *vs.* the GV367-HDC-BEAS+RSV group; #, ##p < 0.05, p < 0.01 *vs.* the HDC-RSV group.

co-stimulatory molecules CD80 and CD86.[26] Therefore, it can be speculated that these dysregulated genes may participate in the development and progression of asthma by regulating the maturation and differentiation of DCs.

In this study, downregulation of lncRNA n337374 was successfully confirmed in DCs using fluorescence RT-qPCR, implying that lncRNA n337374 was associated with RSV-exacerbated asthma. Therefore, we developed an lncRNA n337374-overexpressing RSV- exacerbated asthmatic mouse model by nasal administration of the virus.

In asthmatic mouse models, lncRNA n337374 overexpression relieved asthmatic symptoms, as evidenced by evaluation of pulmonary function, observation of pathological sections of lung tissues, and classification and counting of BALF leukocytes. To our knowledge, this is the first report to demonstrate a relation between lncRNAs and RSV-exacerbated asthma, which provides a novel direction for asthma treatment.

CD40 and CD40 ligands, two co-stimulatory surface molecules on T cells, are implicated in DC differentiation and maturation.[27] HLA-DR, another co-stimulatory molecule, positively correlates with the proliferation and activation abilities of T cells.[28] CD83 is a marker molecule for DC maturation.[29] A recent study shows that knockdown of CD40 and CD86, two co-stimulatory molecules highly expressed in DCs, has synergistic effects on the suppression of allergic responses.[30] In our previous study, we found that in co-culture with RSV-infected primary rat airway epithelial cells, DCs presented functional maturation with increased expression of surface co-stimulatory molecules MHC II and CD86.[12] We thus speculate that DC maturation may relate to lncRNA n337374 overexpression in RSV-exacerbated asthma. In the present study, we used lncRNA n337374-overexpressing lentiviruses to transfect iMDDCs and achieve lncRNA n337374 overexpression, which were further co-cultured with RSV-infected bronchial epithelial cells. Expression of HLA-DR, CD40, CD80, CD83, and CD86 in DCs was detected using RT-qPCR and flow cytometry. Among these five surface molecules, the expression level of CD86 in DCs was enhanced in response to RSV infection, which was consistent with our previous study.[12] Furthermore, upregulation of CD86 stimulated by RSV infection was partly inhibited by lncRNA n337374 overexpression, suggesting that lncRNA n337374 overexpression may inhibit DC maturation through downregulating CD86, which could be a possible mechanism by which lncRNA n337374 helps defend against RSV-induced asthma.

The MAPK pathway is commonly activated in important physiological processes such as cell proliferation, differentiation, and immune regulation.[31] Activation of p38 MAPK signaling is observed in bronchial tissues from patients with asthma.[32] The ERK pathway is associated with airway remodeling in asthma.[33] JNK signaling is reported to mediate allergic airway inflammation.[34] Extensive evidence shows that the p38, JNK, and ERK pathways play a role in modulating the maturation, differentiation, and function of DCs and the expression of cell surface molecules such as CD86.[35,36] In the present study, strong phosphorylation of ERK1/2, p38 and JNK, was observed in DCs co-cultured with RSV-infected bronchial epithelial cells. The phosphorylation of ERK1/2 was alleviated in response to lncRNA n337374 overexpression, while phosphorylation of p38 and JNK was enhanced more. This indicates that the inhibitory effect of lncRNA n337374 overexpression on DC maturation may be partly mediated by the ERK pathway, thereby ameliorating RSV-induced asthmatic syndromes. Although phosphorylation of p38 and JNK was enhanced by lncRNA n337374, the asthmatic symptoms were relieved in RSV-exacerbated asthma mice. Therefore, we hypothesized that the decreased pERK1/2 by lncRNA n337374 might function more than the increased phosphorylation of p-JNK and p-p38 in RSV-exacerbated asthma. However, this hypothesis warrants further validation.

In summary, this is the first study on lncRNAs associated with RSV-induced asthma. We discovered a novel lncRNA, lncRNA n337374, which is involved in this process. By *in vivo* and *in vitro* studies, we found that lncRNA n337374 overexpression might attenuate RSV-induced asthmatic symptoms by suppressing DC maturation via decreasing CD86 expression and prohibiting the ERK pathway. Our study suggests that lncRNA n337374 might be a novel candidate target for therapy against asthma.

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Conflict of interest

The authors declare that they have no conflict of interest.

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