

Dexamethasone can attenuate the pulmonary inflammatory response via regulation of the lncH19/miR-324-3p cascade

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Research

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Abstract

Objective: To investigate lncRNAs and their roles in regulating the pulmonary inflammatory response under treatment of Dexamethasone (Dex).

Methods: IL-1 β (10 ng/mL) and LPS (1 μ g/mL) was used to induce an inflammatory cell model with A549 cells, and the results showed that IL-1 β performed better against LPS. Dex with different concentration was used to attenuate inflammation by IL-1 β , and its effect was assessed by RT-PCR to detect the inflammatory related mRNA, including IK β - α , IKK β , IL-6, IL-8, and TNF- α . And ELISA to detect the inflammatory cytokines TNF- α , IL-6 and IL-8. RT-PCR was used to quantify levels of lncRNAs, including lncMALAT1, lncHotair, lncH19, and lncNeat1. lncH19 was most closely correlated with the inflammatory response, which was induced by IL-1 β and attenuated by Dex. Among the lncRNAs, the level of lncH19 exhibited the highest increase following treatment with 1 μ M and 10 μ M Dex. Therefore, lncH19 was selected for further function study. lncH19 expression was inhibited by shRNA transduced by lentivirus. Cell assays for cell proliferation and apoptosis as well as RT-PCR, western blot, and ELISA for inflammatory related genes were conducted to confirm the functions of lncH19. Predicted target miRNAs of lncH19 included the following: hsa-miR-346, hsa-miR-324-3p, hsa-miR-18a-3p, hsa-miR-18b-5p, hsa-miR-146b-3p, hsa-miR-19b-3p and hsa-miR-19a-3p. Following estimation by RT-PCR, hsa-miR-346, hsa-miR-18a-3p and hsa-miR-324-3p showed consistent patterns in A549 NC and A549 shlncH19. miRNA inhibitor was transfected into A549 NC and A549 shlncH19 cells, and expression levels were determined by RT-PCR. Hsa-miR-324-3p was inhibited the most relative to hsa-miR-346 and hsa-miR-18a-3p and was subjected to further function study. RT-PCR, ELISA and western blotting for inflammatory related genes detection were conducted to validate the functions of the target hsa-miR-324-3p.

Results: Dex with 1 μ M and 10 μ M were shown to be effective in attenuating the inflammatory response. During this process, lncH19 significantly increased in expression ($P < 0.05$). Dex with 1 μ M was for further study. Under IL-1 β treatment with or without Dex, the inhibition of lncH19 lead to an increase cell proliferation, a decrease in cell apoptosis, an increase in the protein level of inflammatory-related genes, the phosphorylation of P65, ICAM-1 and VCAM-1, and inflammatory cytokines. Following prediction of the targets of lncH19 and validation by RT-PCR, miR-346, miR-18a-3p and miR-324-3p were found to be negatively correlated to lncH19. Additionally, Dex increased the expression of lncH19, but the expression of the miRNAs was reduced. Among miRNAs, miR-324-3p was the most markedly down-regulated following treatment of miRNA inhibitors. The MTS assay and cell apoptosis assay showed that the miR-324-3p inhibitor inhibited cell proliferation and induced cell apoptosis, thereby significantly attenuating the inflammatory response, which reversed the effect of lncH19 in regulating cell proliferation and the secretion of inflammatory cytokines ($P < 0.05$). Therefore, lncH19 might regulate miR-324-3p during Dex treatment in pulmonary inflammatory response.

Conclusion: Dex can attenuate the pulmonary inflammatory response via regulation of the lncH19/miR-324-3p cascade.

Introduction

Pulmonary inflammatory defense was initiating by mobilizing inflammatory cells and phlogistic factors into the lung [1]. Airway epithelial cells activation is the first line of defense for airway and lung from inflammation [2]. Epithelial cells recognized microorganisms through toll-like receptors (TLRs), activated NF- κ B signaling pathways and increased airway epithelial inflammatory cytokines such as IL-8, IL-1 α and tumor necrosis factor- α (TNF- α) [3].

However, immune imbalance caused by excessive or chronic inflammation can lead to a variety of lung diseases, including asthma, acute lung injury and diffuse interstitial disease, etc [4]. The inflammatory cytokines are involved in inducing, prolonging and amplifying the inflammatory response inducing airway hyperresponsiveness (AHR) by promoting the growth, proliferation and differentiation of eosinophils, promoting the generation of allergy-specific immunoglobulin E (IgE). Wilson, S. J. found that some of the therapeutic efficacy of inhaled corticosteroids was mediated through inhibition of NF- κ B regulated gene expression [5]. NF- κ B is a transcription factor regulating many of the cytokine and adhesion molecule genes expressed in allergic inflammation, cytokine including colony-stimulating factor and TNF- α , adhesion molecules including intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule-1 (VCAM-1) [5]. Dex is a synthetic glucocorticoid that exerts anti-inflammatory effects by inhibiting NF- κ B signaling pathway [6]. However, the molecular mechanism of Dex in pulmonary inflammatory diseases remains unclear.

Long non-coding RNAs (lncRNAs) are involved in many biological processes, such as cell growth, differentiation, the cell cycle, and the progress and metastasis of cancer [7][8]. Researchers have suggested that inflammatory cytokines, especially NF- κ B play a critical role in regulating lncRNAs in human diseases [9]. miR-489 targeting CHRF in repressing MyD88 and Smad in pulmonary fibrosis [10], differential expressed NEAT1 correlated with increased inflammation was observed in COPD [11], protein phosphatase ϵ (PTPRE) was involved in accelerating pulmonary allergic inflammation [12]. Therefore, we hypothesized that specific lncRNAs might regulate the pathologic processes underlying pulmonary inflammation.

In this study, we targeted specific lncRNAs that have been reported to be correlated with the inflammatory response to characterize their mechanistic roles in lung inflammatory diseases. The inflammatory response was induced by IL-1 β and LPS using previously described methods in A549 [13][14][15] and was then attenuated using Dex. We quantified changes in lncRNA expression during treatment of IL-1 β with Dex in A549 cells. We also determined the functional roles of candidate lncRNAs in regulating the inflammatory response under treatment of Dex in the pulmonary inflammation cell model. Because the functions of lncRNAs are usually performed by targeting miRNAs, we identified the miRNA targets of lncRNAs as well as quantified their expression levels to characterize their functions.

Materials And Methods

Cell culture and treatment

A549 were obtained from the Shanghai Cell Bank (Shanghai, China) and were cultured at 37°C with 5% CO₂. Cells were maintained on DMEM high-glucose culture medium with 10% fetal bovine serum (Gibco, USA). Cells were cultured, amplified and passaged. After 3 days, cells were digested and pelleted by centrifuge. Cell morphology was observed using a light microscope and suspended at a concentration of 1 × 10⁶/mL. For inflammatory cell model, IL-1 β (peprotech, USA) with 10 ng/mL or LPS (sigma, USA) with 1 μ g/mL was added and reached for 8 hours, 16 hours and 24 hours. For the effect of Dex (sigma, USA), Dex with different concentration (10nM, 100nM, 1000nM, 10000nM) and IL-1 β with 10 ng/mL were added at the same time for 24 hours. For the functions of lncH19 and miR-324-3p, IL-1 β with 10 ng/mL and Dex with 1 μ M were added at the same time, the cells and supernatant were harvested for further studies.

MTS assay

Cells were at a density of 1×10^6 /mL, and 100 μ L/well were seeded into 96-well plates. IL-1 β with 10 ng/mL was applied to cells with or without Dex (1 μ M) for 24 hours after transfection with or without miRNA inhibitor for 24 hours. Cells were examined at 1, 2 and 3 day. MTS was added. After 3 hours incubation, the optical density of the cells was detected at 490 nm.

Cell apoptosis assay

5×10^5 cells/well were seeded in 6-well plates overnight at 37°C. After treatment, cells were then pelleted and washed with PBS. 1 \times binding buffer was used to resuspend the cells, 5 μ L 7-AAD staining solution and 5 μ L of APC-conjugated Annexin V were then added. Samples were tested using a FACS Calibur flow cytometer, and the percentage of apoptotic cells was measured.

Lentivirus preparation and infection

LncH19 sequence comes from NCBI with the number NR_002196.2. The antisense sequence of LncH19 (5'-CGGCAAGAAGCGGGTCTGTTTCTTT-3') was synthesized and was cloned to the LV3 vector together with the inverted repeat sequence. The empty vector LV3 was used as a control. The lentivirus solution was prepared by Shanghai Majorbio, China. A549 cells were seeded in 96-well plates at a density of 3×10^4 cells/well. Virus solution was diluted in a 10 \times gradient at five different concentrations with DMEM medium. The supernatant with culture medium was discarded from each well and supplemented with 100 μ L of the virus solution at a different concentration in each well. Saline solution, instead of a virus solution, was used as a control. Cells were incubated at 37°C with 5% CO₂ for 24 hours. The cell culture supernatant was replaced with fresh prepared 100 μ L DMEM medium. Cells were then incubated for 72 hours. RT-PCR was performed to detect the expression of LncH19. miRNA inhibitor and the negative control were transfected using lipofectamine 3000 per the manufacturer's protocol (Thermo Fisher, USA).

ELISA assay to detect inflammatory cytokines

Cytokine concentrations in the cell culture supernate were determined using an ELISA kit for TNF- α , IL-6, and IL-8 (Cusabio, Wuhan, China). Briefly, 96-well plates were precoated with TNF- α , IL-6- and IL-8-specific human antibodies, and human TNF- α , IL-6, and IL-8 were used for the standard titration curve. Samples and standards were added and incubated for 120 minutes at 37°C. Biotin-labeled antibody was added and incubated for 60 minutes at 37°C. After washing three times, HRP-avidin was added, and samples were then incubated for 60 minutes at 37°C. Following three washes, TMB substrate was added, and samples were incubated for 25 minutes at 37°C and protected from light. Stop solution was added, and photo spectrometry was performed at 450 nm.

RT-PCR

mRNAs of the inflammatory related gene, including IK β - α , IKK β , IL-6, IL-8 and TNF- α , lncRNAs and miRNAs were verified by RT-PCR. M-MLV Reverse Transcriptase (Promega, USA) was used to synthesize cDNA. PCR reactions were prepared with GoTaq qPCR Master Mix (Promega, USA) and performed on an ABI 7500 system (Applied Biosystem, USA). The PCR program was as follows, 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, and then 60°C for 30 seconds. The housekeeping genes GAPDH and U6 were used to normalize expression levels. Primers are shown in Table 1.

Western blotting

Cells were lysed in 1% SDS lysis buffer. The BCA assay was used to determine protein concentrations. 10% SDS-PAGE was used to separate the protein. Protein was then transferred onto polyvinylidene fluoride membranes. Nonfat milk in PBS was used to block the membrane at room temperature for 1 hours. The membrane was incubated overnight at 4°C with primary antibody (p-P65 abcam (ab76302), P65 CST (8242), ICAM-1 Santa Cruz (sc-107), VCAM-1 Santa Cruz (sc-13160), GAPDH Transgen (HC301). After several washes with PBS, membranes were incubated in blocking buffer with a secondary antibody coupled to horseradish peroxidase for 2 hours at room temperature. The complexes were formed on the membrane, and the membrane was then detected by ECLplus (Amersham Biosciences/GE Healthcare, Velizy, France).

Statistical analysis

The data are presented as the mean±standard deviation (SD), N=3. Non-parametric t test and Kruskal-Wallis test were used to analyze differences between two groups and differences among multiple groups respectively. $P < 0.05$ was considered to indicate a statistically significant difference. IBM SPSS statistics software 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Dex attenuates the inflammatory response

To investigate the effect of Dex, we induced the pulmonary inflammatory cell model with IL-1 β and LPS. A549 cells were applied for the assay and treated with IL-1 β at 10 ng/mL or LPS at 1 μ g/mL in DMEM culture medium. Cells incubated with the reagents after 8, 16 and 24 hours were collected, and total RNA was isolated. RT-PCR was conducted to confirm the expression of the inflammatory related mRNA, including IK β - α , IKK β , IL-6, IL-8, and TNF- α . Generally, the results showed that IL-1 β performed better against LPS, as the mRNA levels of most of the inflammatory cytokines, aside from IL-6 and TNF- α , were significantly up-regulated at 8hours; in addition, mRNA levels of all other cytokines were 2- to 4-fold greater at 24 hours (Figure 1A). The secretory cytokines in the cell culture medium, including IL-6, IL-8 and TNF- α were determined by ELISA. The results were consistent in showing that IL-1 β was generally more effective, and the effect can be observed at 8 hours (Figure 1B). Therefore, we decided to use IL-1 β for 24 hours incubation to induce the pulmonary inflammation cell model.

Following the same principle, we used the cell model to characterize the effect of Dex. We incubated A549 cells with IL-1 β and Dex at different concentrations, ranging from 10 nM to 10000 nM at 24 hours. As before, RT-PCR and ELISA were used to evaluate the effect of Dex. mRNA levels of inflammatory related genes, including IK β - α , IKK β , IL-6, IL-8, and TNF- α , significantly increased following treatment with IL-1 β ($P < 0.05$). After adding Dex, the mRNA level was reduced to baseline levels, indicating that supplementation with Dex can attenuate the inflammatory response (Figure 2A). With respect to the secretory inflammatory cytokines, the concentration of IL-6, IL-8, and TNF- α increased significantly following treatment with IL-1 β relative to the control group. However, when cells were supplemented with Dex, the concentration of cytokines decreased, and Dex at both 1 μ M and 10 μ M significantly decreased in concentration (Figure 2B). Therefore, these observations suggest that Dex plays a role in attenuating the inflammatory response.

Verification of the lncRNAs involved in inflammation attenuation

Based on our preliminary findings, we targeted four lncRNAs, lncMALAT1, lncHotair, lncH19, and lncNeat1 to determine which of these lncRNAs are involved in processes underlying inflammation attenuation [17-20]. RT-PCR

was conducted to determine lncRNA levels following treatment with IL-1 β and Dex. The results show that the four candidate lncRNAs were down-regulated following treatment with IL-1 β ; however, lncRNA levels increased following supplementation with Dex (Figure 3). In general, lncRNA levels can be most clearly observed by the treatment of Dex at 1 μ M and 10 μ M ($P < 0.05$). Among the lncRNAs, the level of lncH19 exhibited the highest increase, approximately 2-fold, following treatment with 1 μ M and 10 μ M Dex. Therefore, lncH19 and Dex with 1 μ M was selected for further function validation.

To verify the functions of lncH19, the corresponding shRNA was designed and infected to A549 cells by lentivirus. The performance of the specific shRNA was assessed by RT-PCR. The expression of lncH19 showed a 50% decrease after the specific shRNA transfection (Figure 4A). The cells were then treated with IL-1 β and Dex (1 μ M) to investigate the effect of decreasing the expression of lncH19. Cell proliferation was assessed via the MTS assay. Treatment of 1 μ M Dex clearly inhibited cell proliferation, which might only be a side effect. When the expression of lncH19 was induced, cell proliferation increased relative to IL-1 β -treated cells lacking lncH19 inhibition with or without Dex (Figure 4B). We then further validated the functions of lncH19 via the flow cytometry cell apoptosis assay. Compared to the percentage of apoptotic cells with and without Dex treatment, apoptotic cells increased approximately 2-fold when Dex was applied. Cell apoptosis was inhibited and reduced by 30–50% relative to IL-1 β -treated samples lacking lncH19 inhibition with or without Dex (Figure 4C & 4D). Western blotting was conducted to determine the protein level of the genes that might be involved in the inflammatory response, including P65, p-P65, ICAM-1 and VCAM-1. The protein level of P65 did not change. For the rest of the genes, supplementation of Dex clearly decreased protein levels, but inhibition of lncH19 can also increase protein levels (Figure 4E). Therefore, given the consistency of these results with those obtained via flow cytometry and the MTS assay, the proteins p-P65, ICAM-1, and VCAM-1 are likely involved in the regulation of Dex. The cell culture supernatant was then collected, and the concentration of inflammatory cytokines, including IL-6, IL-8, and TNF- α was determined. Cytokines decreased in concentration by approximately 50% following the addition of Dex. Moreover, the inhibition of lncH19 increased the concentration of inflammatory cytokines (Figure 4F). Therefore, Dex can attenuate the inflammatory response, and lncH19 plays an important role in inflammatory processes.

Validation of candidate miRNAs regulated by lncH19

Miranda v3.3a has been used to predict the target miRNAs of lncH19. miRNAs are presented in Table 2. Because expression of lncH19 was up-regulated following addition of Dex and because up-regulation of lncRNA tends to result in the downregulation of miRNAs, we searched for down-regulated miRNAs following addition of Dex. miR-346, miR-18a-3p, and miR-324-3p were down-regulated and were therefore chosen for further validation (Figure 5). The corresponding miRNA inhibitors were designed and transfected to A549 cells. The performance of the inhibitors was assessed via RT-PCR. For both miR-18a-3p and miR-324-3p, the corresponding inhibitors clearly decreased the expression of miRNAs by ~50%. The inhibitor of miR-324-3p was significantly more effective relative to that of miR-18a-3p (Figure 6A). Therefore, we further investigated the functions of miR-324-3p and divided into four groups, the first group, shNC+IL-1 β +Dex+miRNA NC, abbreviated as NC+miRNA NC, the second group, shNC+IL-1 β +Dex+miR-324-3p inhibitor, abbreviated as NC+miR-324-3p inhibitor, the third group, shlncH19+IL-1 β +Dex+miRNA NC, abbreviated as shlncH19+miRNA NC, the fourth group, shlncH19+IL-1 β +Dex+miR-324-3p inhibitor, abbreviated as shlncH19+miR-324-3p inhibitor. An MTS assay was conducted to assess cell proliferation. By comparing the NC+miRNA NC group to NC+miR-324-3p inhibitor group, cell proliferation increased significantly at 72 hours ($P < 0.05$). When cells were infected with shRNA targeting lncH19, cell proliferation increased from an OD value of 1.5 to 2.0 relative to NC+miRNA NC group. Cell proliferation was lower in shlncH19+miR-324-3p inhibitor, group compare to shlncH19+miRNA NC (Figure 6B). Therefore, the evidence suggests that the expression

of lncH19 inhibited cell proliferation, but the expression of miR-324-3p promoted cell proliferation. We also conducted cell apoptosis assays via flow cytometry to determine the functions of miR-324-3p. The inhibition of lncH19 reduced apoptotic cells by ~30%, but inhibition of miR-324-3p clearly enhanced cell apoptosis relative to the NC+miRNA NC group (Figure 6C). mRNA levels of IK β - α , IKK β , IL-6, IL-8 and TNF- α were all up-regulated when lncH19 was inhibited but down-regulated when miR-324-3p was simultaneously inhibited relative to the NC+miRNA NC group (Figure 6D). The cell culture supernatant was collected to determine the concentration of the cytokines IL-6, IL-8 and TNF- α . The concentration of cytokines increased significantly when lncH19 was inhibited by shRNA relative to the NC+miRNA NC group. However, the concentration of cytokines decreased significantly when the expression of miR-324-3p was simultaneously inhibited, indicating that the inflammatory response was suppressed (Figure 6E). Western blotting was performed to determine the protein levels of inflammation-related genes, including p-P65, P65, ICAM-1 and VCAM-1. There was no significant change in the protein level of P65. Inhibition of lncH19 increased the protein levels of p-P65, ICAM-1 and VCAM-1 relative to the NC+miRNA NC group. However, protein levels were decreased when miR-324-3p was down-regulated by an inhibitor simultaneously. These findings suggest that miR-324-3p is involved in lncH19 regulation. lncH19 might target and inhibit the expression of miR-324-3p and thereby regulate inflammation.

Discussion

In this study, IL-1 β -induced inflammatory A549 cells were attenuated by Dex to aid the study of lncRNAs. Initially, we targeted lncMALAT1, lncHotair, lncH19, and lncNeat1 and evaluated their expression levels. The results showed that lncH19 was most closely correlated with symptoms of inflammation, as its expression decreased following addition of IL-1 β to cells but increased following supplementation of Dex. The expression of lncH19 was negatively correlated with miR-324-3p. We showed that Dex attenuates the inflammatory effect by targeting a cascade involving lncH19 and miR-324-3p.

Pulmonary inflammatory disease such as asthma is a disease associated with increase inflammation level and loss of bronchial epithelial cells. Increased level of inflammatory cytokines and cell apoptosis are one of the characters of bronchial epithelial cells of patients of pulmonary inflammatory diseases [16][17][18]. Therefore, drugs with function of inflammation suppression and benefit survival of epithelial cells would improve the outcome of pulmonary inflammatory disease. In the current study, we used IL-1 β induced A549 cells to mimic inflammatory epithelial cells. Based on this inflammatory epithelial cell model, we revealed that lncH19 is suppressed upon IL-1 β inducing and it is partially restored by Dex, suggesting its role in inflammatory epithelial cell.

lncH19 was discovered in the 11p15.5, H19/IGF2 locus [19]. It is highly expressed in the fetus but begins to be silenced after birth with its expression restricted to a few tissues, such as the mammary gland, uterus and adrenal gland [20]. Existing research suggests that mutation of H19 in mouse zygotes causes prenatal lethality, indicating that H19 plays an important role in cell growth and development [21]. Therefore, our findings are consistent with previous studies in that lncH19 can regulate cell proliferation. In addition, we suggested that lncH19 is involved in modulating apoptosis in inflammatory epithelial cell. Notably, lncH19 has been shown to be correlated with the NF- κ B pathway and is thus involved in the regulation of cell growth in multiple myeloma [9]. NF- κ B is an important family of transcription factors involved in cell differentiation, apoptosis and immunity. Activation of the NF- κ B pathway is involved in most cell behavior in cancer transformation, such as the inhibition of cell differentiation and cell apoptosis, promotion of cell proliferation, angiogenesis and potential metastasis [22]. Currently evidence shows that NF- κ B plays a role in pro-inflammatory mediator including cytokine, chemokines, and cell adhesion molecules production in inflammatory cells and structural cells such as lung epithelial cells [23]. However, its relationship with

lncH19 remains unclear in the context of lung inflammatory epithelial cells. In the present study, we show that induction of the inflammatory response by IL-1 β reduced the expression of lncH19, while supplementation of Dex, which can attenuate the inflammatory response, significantly increased the expression of lncH19. The up regulation of lncH19 expression is correlated with the attenuation of inflammatory response in lung epithelial cells; these finds indicate that lncH19 is likely involved in anti-inflammatory processes associated with pulmonary inflammatory disease. However, since lncRNAs usually inhibit targets, lncH19 might not directly target NF- κ B. Therefore, we suspected that lncH19 targets and inhibits NF- κ B and possibly other anti-inflammatory factors.

Further study of lncH19 revealed that the expression of miR-346, miR-17a-3p and miR-324-3p were inhibited by lncH19 over-expression. Following detection of cell proliferation and cell apoptosis, inhibition of miR-324-3p revealed a highly significant correlation between cell proliferation and apoptosis. Therefore, we suspected that lncH19 regulates the inflammatory response by regulating miR-324-3p. MiR-324-3p is a commonly expressed miRNA that regulates processes underlying cancer. The main target of miR-324-3p is SMAD7. According to Xu et al., the miR-324-3p/SMAD7 axis played a significant role in regulating cell growth and apoptosis in nasopharyngeal cancer [24]. Overexpression of SMAD7 is associated with the development of skin, pancreatic, lung, and colon cancer in the processes of cell growth and apoptosis [25]. Previous studies have shown that SMAD7 is correlated with the NF- κ B pathway and therefore regulates the cell cycle in cancers [26]. Inflammatory often changed the expression of p65, ICAM-1, and VCAM-1, their expression levels can be used as indicators of the inflammatory response. Previous studies have also shown that p65 is associated with the activation of NF- κ B [5]. By characterizing the expression of the cytokines, we have confirmed that both lncH19 and miR-324-3p could be important factors in regulating the inflammatory response. Therefore, regulation of the inflammatory response might be achieved through the regulation of SMAD7/NF- κ B pathways via a regulatory network between lncH19 and miR-324-3p in which lncH19 targets miR-324-3p. However, this proposed regulatory cascade requires further validation for future.

Our results have shown that Dex can attenuate the inflammatory response in IL-1 β induce inflammatory cells. The lncH19/miR-324-3p axis might play an important role in regulating the inflammatory response and thus cell proliferation and cell apoptosis. Future studies should examine additional genes targets of the lncH19/miR-324-3p axis, including SMAD7 and NF- κ B, to further elucidate the regulatory processes associated with the lncH19/miR-324-3p axis. Furthermore, the expression of lncH19/miR-324-3p should be quantified in animal model and pulmonary inflammatory disease patients before and after the use of corticosteroids, the anti-inflammatory effect of Dex in asthma are regulated by the lncH19/miR324-3p pathways in animal model, the correlation between lncH19 and miR-324-3p expression changes in specific pulmonary inflammatory disease such as asthma is also what we would need to study in the future, as our hypothesized mechanism is based on a IL-1 β induce epithelial cell model. We can also search for possible ncRNAs by RNA-seq technology, because lncRNA may also interact with other ncRNAs.

Conclusion

In summary, we demonstrated here for the first time that Dex can attenuate the inflammatory response in inflammatory epithelial cell, an important pathological change closely related to pulmonary inflammatory disease such as asthma, via regulation of the lncH19/miR-324-3p cascade.

Declarations

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Authors' contributions

Conceived and designed the experiments: FX, YC, CZ, performed the experiments: CGX, XDL, analyzed the data: FX, CGX, XDL, contributed reagents/materials/analysis tools: FX, ZLH, wrote the paper: YC, CZ. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All procedures were conducted according to the protocols and guidelines approved by Ethics Committee of Children's Hospital of Chongqing Medical University and were performed following the declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' information

Not available

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Tables

Name	sequence(5'-3')
H-IL-6-F	ACTCACCTCTTCAGAACGAATTG
H-IL-6-R	CCATCTTTGGAAGGTTTCAGGTTG
H-IL-8-F	GACCACACTGCGCCAACAC
H-IL-8-R	CTTCTCCACAACCCTCTGCAC
TNF- α -F	CTGCACTTTGGAGTGATCGG
TNF- α -R	GCTTGAGGGTTTGCTACAACAT
H-I κ κ β -F	CGATGGCACAATCAGGAAACAGGT
H-I κ κ β -R	ATTGGGGTGGGTCAGCCTTCTC
H-IKB- α -F	ACACCTTGCCTGTGAGCAGG
H-IKB- α -R	AGCACCCAAGGACACCAAAA
β -Actin-F	CATGTACGTTGCTATCCAGGC
β -Actin-R	CTCCTTAATGTCACGCACGAT
H-lncMALAT1-F	AAAGTCCGCCATTTTGCCAC
H-lncMALAT1-R	ACAACCTCGCATCACCGGAAT
H-lncHotair-F	GCAGTGGGGAACCTCTGACTC
H-lncHotair-R	TTGAGAGCACCTCCGGGATA
H-lncH19-F	GACATCTGGAGTCTGGCAGG
H-lncH19-R	CTGCCACGTCCTGTAACCAA
H-lncNeat1-F	GGCAGGTCTAGTTTGGGCAT
H-lncNeat1-R	CCTCATCCCTCCCAGTACCA
hsa-mir-346-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCGCTC
hsa-mir-346-F	GGTCTCTGTGTTGGGCGTCT
hsa-miR-18a-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCAGAA
hsa-miR-18a-3p-F	ACTGCCCTAAGTGCTCCTT
hsa-mir-324-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCAGCA
hsa-mir-324-3p -F	CCCCTGCCCCAGGTGCT
hsa-miR-146b-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCAGA
hsa-miR-146b-5p-F	GCCCTGTGGACTCAGTT
hsa-miR-18b-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAACT
hsa-miR-18b-5p-F	GCGTAAGGTGCATCTAGTGCAG

Name	sequence(5'-3')
hsa-mir-19a-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGTT
hsa-mir-19a-F	TGTGCAAATCTATGCAAA
hsa-miR-19b-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGTT
hsa-miR-19b-3p-F	GTGTGCAAATCCATGCAA
Universe-R	GTGCAGGGTCCGAGGT
U6-F	CGCTTCGGCAGCACATATAC
U6-R	CGAATTTGCGTGTTCATCCTTG

Table 1 Primers of mRNA, lncRNA, and miRNA.

lncRNA	miRNA	numBindingSitesPredicted	numTargetsPer100bp	specificBindingSitesPredicted
NR_002196.2	hsa-miR-346	5	0.22	252;435;579;887;1114
NR_002196.2	hsa-miR-324-3p	5	0.22	569;580;723;831;1103
NR_002196.2	hsa-miR-18a-3p	3	0.13	577;674;828
NR_002196.2	hsa-miR-18b-5p	2	0.09	137;1667
NR_002196.2	hsa-miR-146b-3p	2	0.09	257;746
NR_002196.2	hsa-miR-19b-3p	1	0.04	2023
NR_002196.2	hsa-miR-19a-3p	1	0.04	2034

Table 2 Prediction of the target miRNAs of lncH19.

Figures

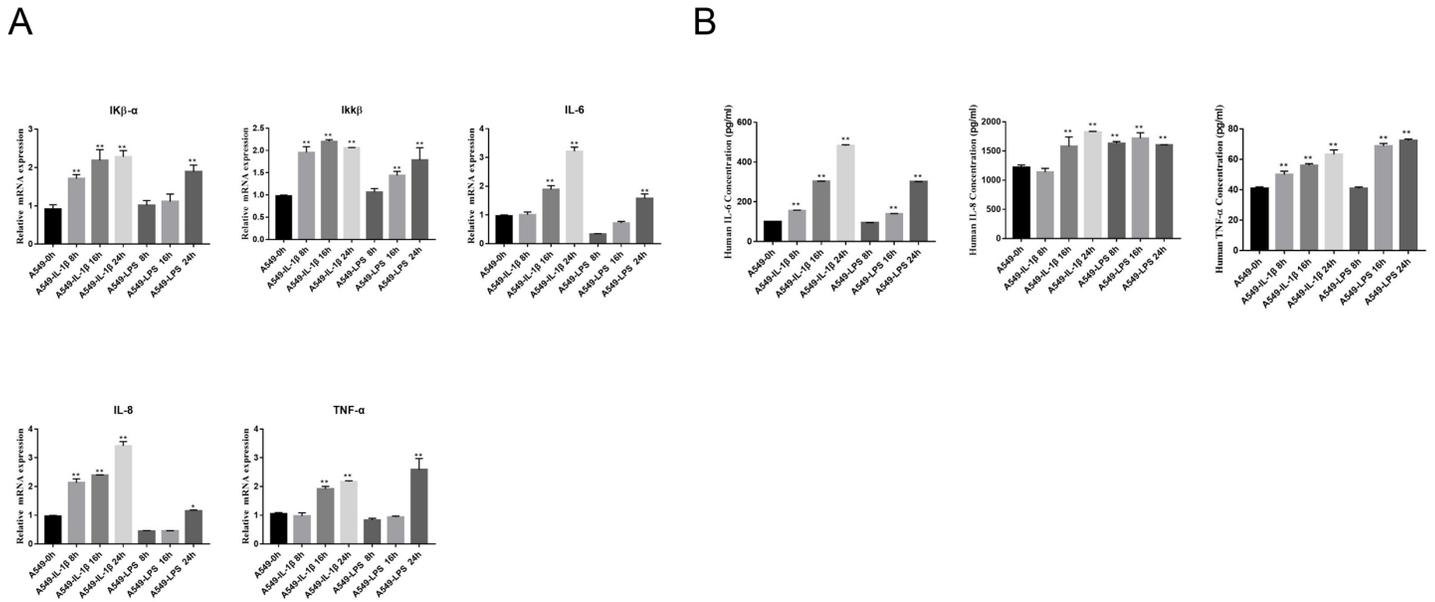


Figure 1

An asthma inflammatory cell model via A549 induced by different time of IL-1beta (10 ng/ml) or LPS (1 μg/ml) was established, and IL-1 beta proved to be a more robust inflammatory cell model. A. RT-PCR determination of the corresponding mRNA levels of the inflammatory genes IKbeta-alpha, IKKbeta, IL-6, IL-8, and TNF-alpha, GAPDH as housekeeping. B. Levels of the inflammatory cytokines IL-6, IL-8, and TNF-alpha determined by ELISA. Data are presented as mean±SD. N=3. **P < 0.01 vs. A549 0h. IL-1 beta: Interleukin-1 beta, LPS: lipopolysaccharide.

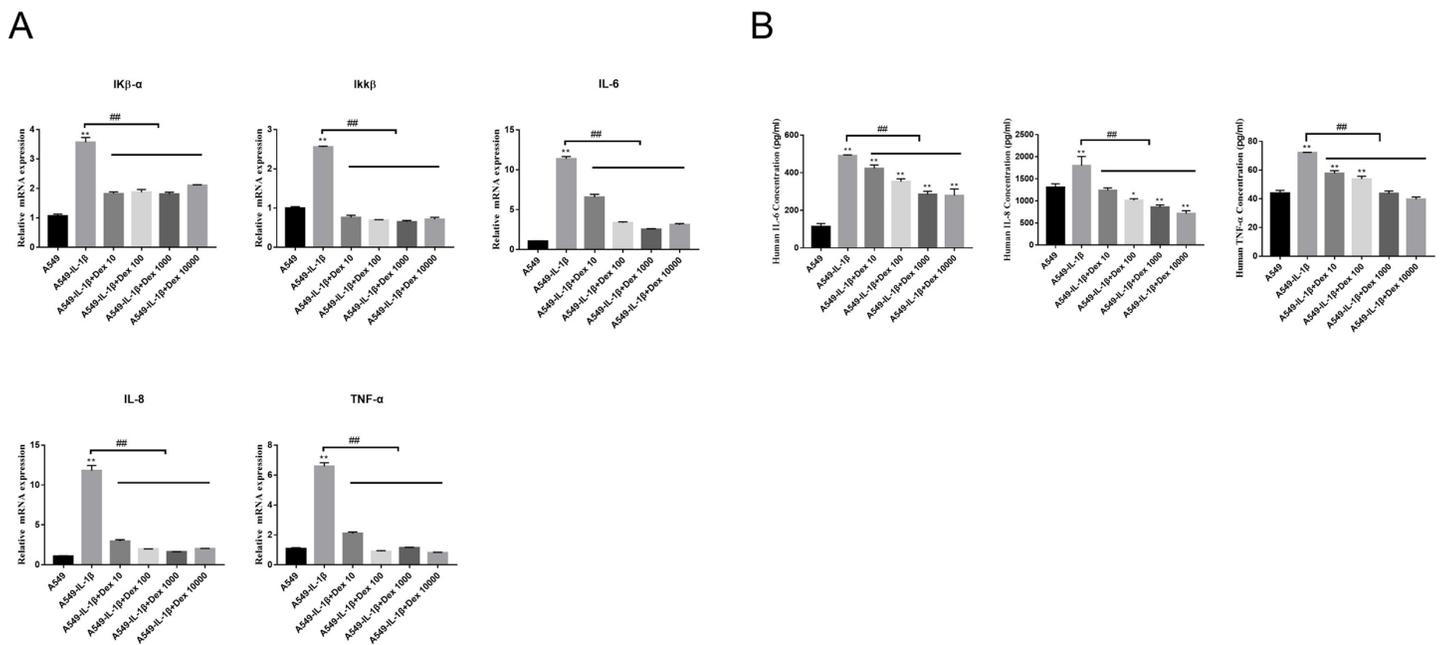


Figure 2

Different concentrations of Dex (10nM, 100nM, 1000nM, 10000nM) can attenuate the inflammatory response induced by IL-1 beta (10 ng/ml), IL-1 beta and Dex added at the same time as the stimuli. A. mRNA levels of the

inflammatory genes were determined by RT-PCR. B. Levels of the inflammatory cytokines determined by ELISA. Data are presented as mean±SD. N=3. *P < 0.05 vs. A549 0h, **P < 0.01 vs. A549 0h, ##P < 0.01. Dex: dexamethasone, IL-1 beta: Interleukin-1 beta.

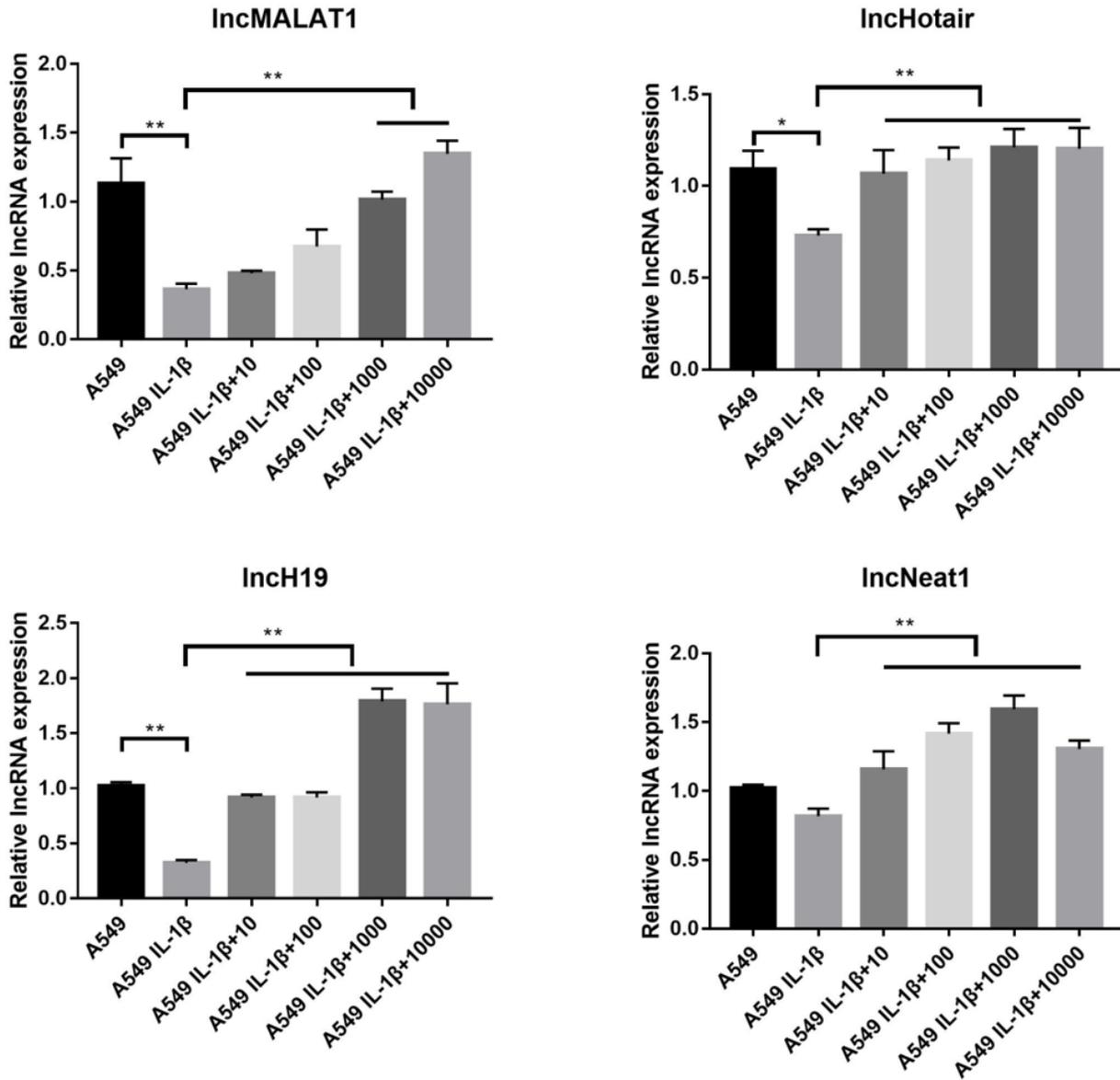


Figure 3

IncMALAT1, IncHotair, IncH19, and IncNeat1 were expressed less when A549 was treated by IL-1 beta (10 ng/ml) but were expressed more as the inflammatory response attenuated following application of Dex (1 μM), IL-1 beta and Dex added at the same time as the stimuli. Changes in IncH19 were the most pronounced. Data are presented as mean±SD. N=3. *P < 0.05, **P < 0.01. Dex: dexamethasone, IL-1 beta: Interleukin-1 beta.

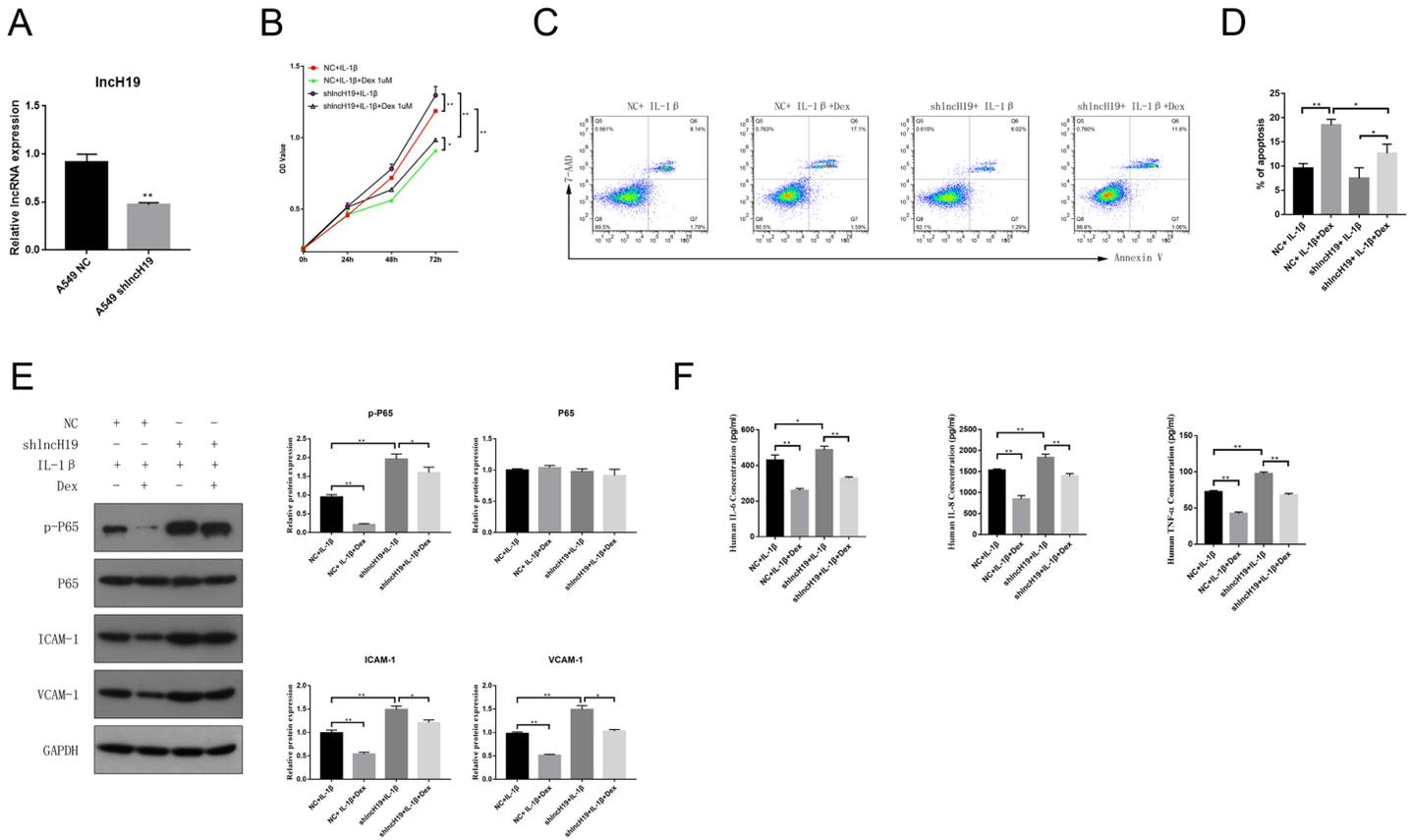


Figure 4

When IncH19 was inhibited while cells were treated with IL-1 beta (10 ng/ml) with or without Dex (1 μM) at the same time, cell proliferation increased, cell apoptosis decreased, and the protein levels of inflammatory-related genes increased, promoting the phosphorylation of P65, ICAM-1, VCAM-1, and inflammatory cytokines. A. IncH19 expression was reduced, indicating that a IncH19-inhibited cell line was generated. B. Cell proliferation of A549 NC and A549 shIncH19 cells treated by IL-1 beta with or without Dex. C. Representative cell apoptosis diagram measured by flow cytometry, upper left is fragment and damaged cell, upper right is late apoptosis and dead cell, lower left is normal cell of negative control, lower right is early apoptotic cell, the total of percent of apoptosis cells were calculated by the sum of the upper right and lower right. D. Percentage of apoptotic cells. E. Western blot for determining protein levels of inflammatory-related genes. F. ELISA for assessing levels of inflammatory cytokines. Data are presented as mean±SD. N=3. *P < 0.05, **P < 0.01. Dex: dexamethasone, IL-1 beta: Interleukin-1 beta.

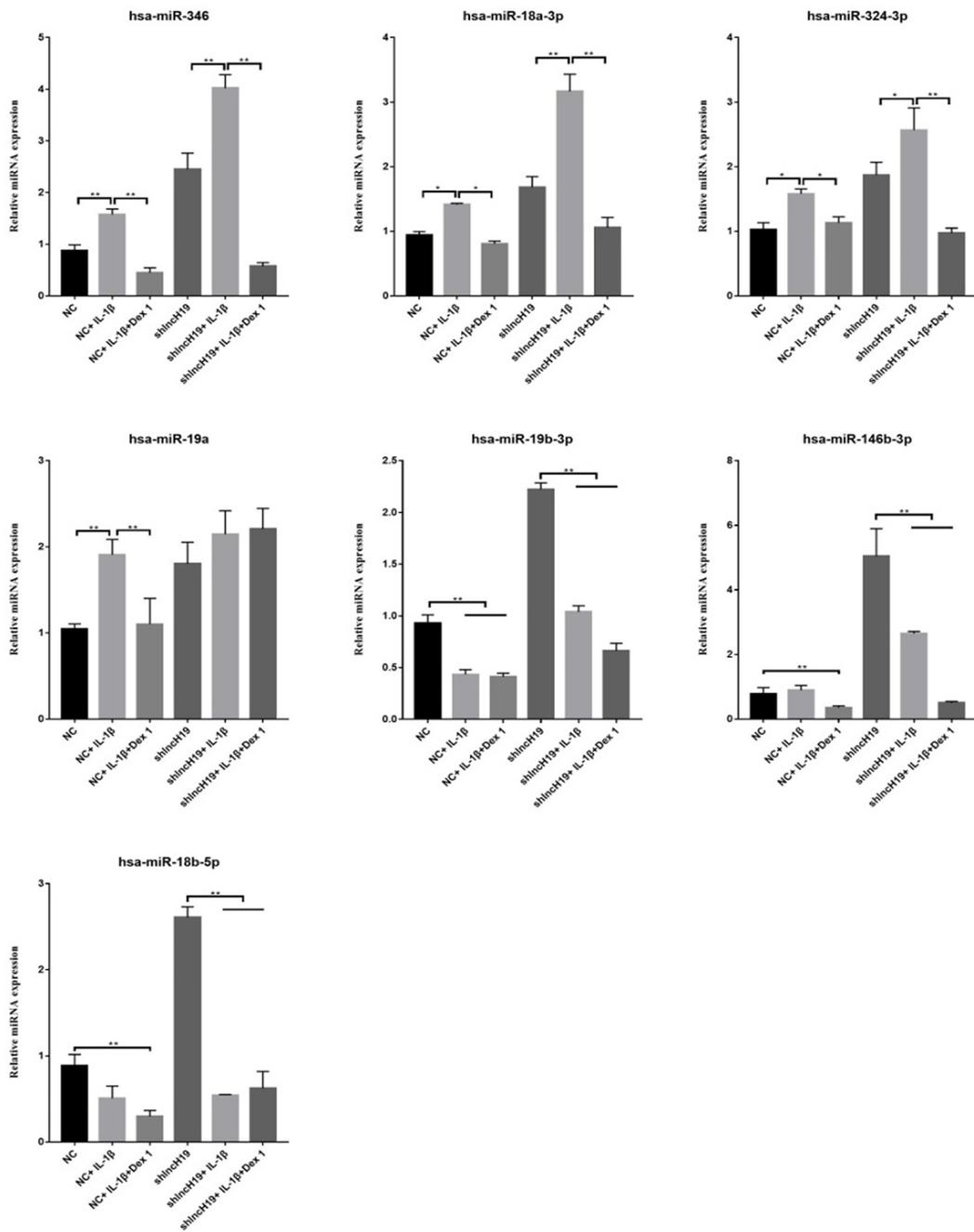


Figure 5

RT-PCR was conducted to determine the level of miRNAs predicted to be targets of lncH19 with or without treatment of IL-1 beta (10 ng/ml) or IL-1 beta (10 ng/ml) and Dex (1 μM) were added at the same time; trends in the expression of miR-346, miR-18a-3p, and miR-324-3p were consistent in A549 NC and A549 shlncH19. Data are presented as mean±SD. N=3. **P < 0.01. Dex: dexamethasone, IL-1 beta: Interleukin-1 beta.

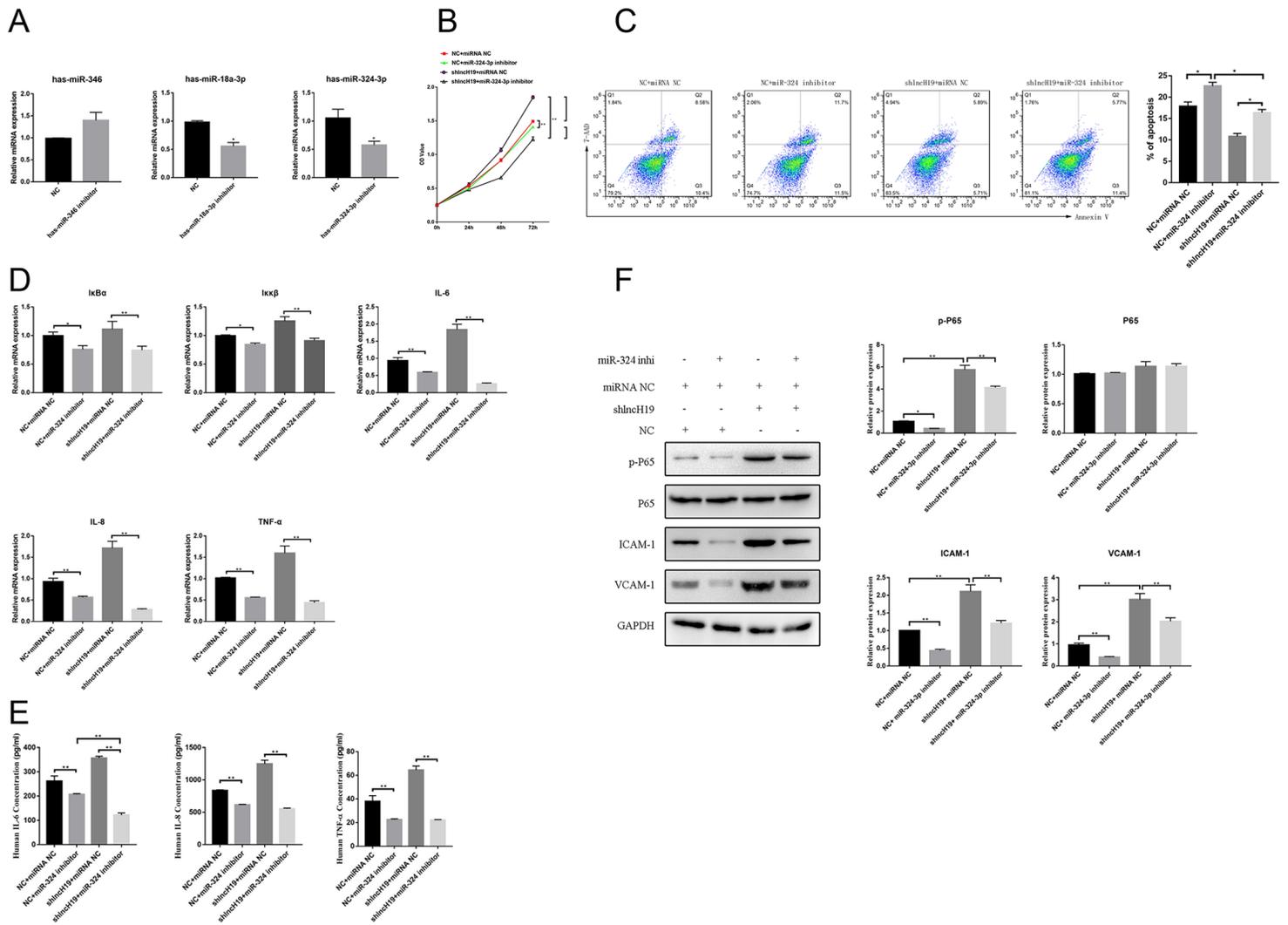


Figure 6

The attenuation of *Inch19* by miR-324-3p was inhibited as the inflammatory response attenuated under treatment with IL-1 beta (10 ng/ml) and with Dex (1 μ M) at the same time. A. Levels of candidate miRNAs determined by RT-PCR for examining the performance of the miRNA inhibitor. B. Cell proliferation determined by the MTS assay. C. Cell apoptosis determined by flowcytometry. D. mRNA levels of the inflammatory cytokines. E. ELISA for detecting levels of inflammatory cytokines. E. Western blot for detecting levels of inflammatory-related genes. Data are presented as mean \pm SD. N=3. *P < 0.05, **P < 0.01. Dex: dexamethasone. IL-1 beta: Interleukin-1 beta. shNC+IL-1 beta+Dex+miRNA NC, abbreviated as NC+miRNA NC, shNC+IL-1 beta+Dex+miR-324-3p inhibitor, abbreviated as NC+miR-324-3p inhibitor, shInch19+IL-1 beta+Dex+miRNA NC, abbreviated as shInch19+miRNA NC, shInch19+IL-1 beta+Dex+miR-324-3p inhibitor, abbreviated as shInch19+miR-324-3p inhibitor.