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

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Abstract

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

Methods: IL-1beta (10 ng/ml) was used to induce an inflammatory cell model with A549 cells. Dex was used to attenuate inflammation by IL-1beta, and its effect was assessed by RT-PCR to detect the inflammatory cytokines IKbeta-alpha, IKKbeta,, IL-6, IL-8, and TNF-alpha and ELISA to detect the inflammatory cytokines TNF-alpha, 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-1beta and attenuated by Dex, Thus, lncH19 was selected for further study. lncH19 expression was inhibited by shRNA transduced by lentivirus. Cell assays for cell viability and apoptosis as well as RT-PCR, western blot, and ELISA for inflammatory cytokines 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, hsa-miR-324-3p showed consistent patterns in A549 NC and A549 shlncH19. miRNA inhibitor was transfected into A549 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 study. RT-PCR, ELISA and Western Blot for cytokine detection were conducted to validate the functions of the target hsa-miR-324-3p.

Results: Dex was shown to be effective in attenuating the inflammatory response. During this process, lncH19 significantly increased in expression ($P < 0.05$). Under IL-1beta treatment with or without Dex, the inhibition of lncH19 lead to an increase cell viability, a decrease in cell apoptosis, an increase in the protein level of inflammatory-related genes, and the phosphorylation of P65, ICAM-1, 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 aforementioned 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 viability and induced cell apoptosis, thereby significantly attenuating the inflammatory response, which reversed the effect of lncH19 in regulating cell viability and the secretion of inflammatory cytokines ($P < 0.05$). Therefore, lncH19 might regulate miR-324-3p during Dex treatment.

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

Keywords: Asthma; inflammatory response; Dexamethasone; lncRNA; miRNA

Introduction

Asthma is a type of chronic inflammation that affects a variety of inflammatory cells, such as eosinophils, mast cells, basophils, lymphocytes, and monocytes. It is also characterized by mucus hypersecretion, airway hyperresponsiveness (AHR), and airway remodeling [1, 2]. These pathognomonic features are primarily mediated by antigen-specific CD4+ T helper type 2 (Th2) cells and the corresponding cytokines IL-5, IL-13, and IL-4 [2, 3]. These inflammatory cytokines are involved in inducing, prolonging and amplifying the inflammatory response by promoting the growth, proliferation and differentiation of eosinophils, promoting the generation of allergy-specific immunoglobulin E (IgE), and inducing AHR. Therefore, the inflammatory response is an important symptom associated with asthma. Dexamethasone (Dex) is a commonly used medicine to treat asthma [4], but the mechanism by which Dex mitigates the inflammatory response has yet to be elucidated.

Long non-coding RNAs (lncRNAs) are newly discovered RNAs, which are defined as RNA molecules with lengths that exceed 200 nt. lncRNAs are involved in many biological processes, such as cell growth, differentiation, the cell cycle, and the progress and metastasis of cancer [5, 6]. Much research has suggested that the abnormal expression of lncRNAs in human diseases is often due to the regulation of inflammatory cytokines, such as NF-kB [7]. Depending on their targets, lncRNAs can act as regulators for different biological processes. Therefore, we hypothesized that specific lncRNAs might regulate the pathologic processes underlying asthma.

In this study, we targeted specific lncRNAs that have been reported to be correlated with the inflammatory response to characterize their mechanistic roles in the inflammatory response in asthma. A549 cells were used in this study. The inflammatory response was induced by IL-1beta and LPS using previously described methods [8] and was then attenuated using Dex. We quantified changes in lncRNA expression during treatment of IL-1beta with Dex in A549 cells. We also determined the functional roles of candidate lncRNAs in regulating the inflammatory response under treatment of Dex in asthma. 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

A549 cells were obtained from the Shanghai Cell Bank (Shanghai, China) and were cultured at 37□ with 5% CO_2. 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 1×10^6/ml.

MTS assay

Cells were at a density of 1×10^6/ml, and 100 μl/well were seeded into 96-well plates. IL-1beta was applied to cells with or without Dex for 24 h after transfection with or without miRNA inhibitor for 24 h. Cells were examined at 1, 2, and 3 d. MTS was added. After 3 h 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□. Cells were then pelleted and washed with PBS. 1× binding buffer was used to resuspend the cells; 5 μl 7-AAD staining solution and 5 μl of APC-conjugated Annexin V were then added. Samples were tested using a FACSCalibur flow cytometer, and the percentage of apoptotic cells was measured.

Lentivirus preparation and infection

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× gradient at five different concentrations with DMEM medium. The supernatant with culture medium was discarded from each well and supplemented with 100 ul 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□ with 5% CO_2 for 24 h. The cell culture supernatant was replaced with fresh prepared 100 ul DMEM medium. Cells were then incubated for 72 h. RT-PCR was performed to detect the expression of lncH19. miRNA inhibitor and the negative control were transfected using lipofetamine 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 TNFα, IL-6, and IL-8 (Cusabio, Wuhan, China). Briefly, 96-well plates were precoated with TNFα-, IL-6- and IL-8-specific human antibodies, and human TNFα, IL-6, and IL-8 were used for the standard titration curve. Samples and standards were added and incubated for 120 min at 37□. Biotin-labeled antibody was added and incubated for 60 min at 37□. After washing three times, HRP-avidin was added, and samples were then incubated for 60 min at 37□. Following three washes, TMB substrate was added, and samples were incubated for 25 min at 37□ and protected from light. Stop solution was added, and photospectrometry was performed at 450 nm.

RT-PCR

mRNAs of the inflammatory cytokines, including IkB-alpha, Ikkbeta, IL-6, IL-8, and TNF-alpha, 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 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 h. The membrane was incubated overnight at 4□ with primary antibody (p-P65 abcam, P65 CST, ICAM-1 Santa, VCAM-1 Santa, GAPDH Transgen). After several washes with PBS, membranes were incubated in blocking buffer with a secondary antibody coupled to horseradish peroxidase for 2 h 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

When data fitted a normal distribution, comparisons were performed using independent t-tests and one-way and two-way ANOVAs. Significance was established as $P < 0.05$. GraphPad Prism software was used for statistical analysis.

Results

Dex attenuates the inflammatory response

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

Following the same principle, we used the cell model to characterize the effect of Dex. We incubated A549 cells with IL-1beta and Dex at different concentrations, ranging from 10 nM to 10000 nM at 24 h. As before, RT-PCR and ELISA were used to evaluate the effect of Dex. mRNA levels of inflammatory cytokines, including IKbeta-alpha, IKKbeta, IL-6, IL-8, and TNF-alpha, significantly increased following treatment with IL-1beta ($P < 0.05$). After adding Dex, the mRNA level was maintained at a level similar to the normal group, indicating that supplementation with Dex can attenuate the inflammatory response (Fig 2A). With respect to the secretary inflammatory cytokines, the concentration of IL-6, IL-8, and TNF-alpha increased significantly following treatment with IL-1beta relative to the control group. However, when cells were supplemented with Dex, the concentration of cytokines decreased, and Dex at both 1 uM and 10 uM significantly decreased in concentration (Fig 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. RT-PCR was conducted to determine lncRNA levels following treatment with IL-1beta and Dex. The results show that the four candidate lncRNAs were down-regulated following treatment with IL-1beta; however, lncRNA levels increased following supplementation with Dex (Fig 3). In general, lncRNA levels can be most clearly observed by the treatment of Dex at 1 uM and 10 uM ($P < 0.05$). Among the lncRNAs, the level of lncH19 exhibited the highest increase, approximately 2-fold, following treatment with 1 uM and 10 uM Dex. Therefore, lncH19 was selected for further validation.

To verify the functions of lncH19, the corresponding shRNA was designed and transfected to A549 cells by lentivirus. The performance of the shRNA was assessed by RT-PCR. Depending on the effect of the shRNA, lncRNA expression decreased by 50% (Fig 4A). The cells were then treated with IL-1beta and Dex (1 uM) to investigate the effect of decreasing the expression of lncH19. Cell viability was assessed via the MTS assay. Treatment of 1uM Dex clearly reduced cell viability, which might only be a side effect. When the expression of lncH19 was induced, cell viability increased relative to IL-1beta-treated cells lacking lncH19 inhibition with or without Dex (Fig 4B). We then further validated the functions of lncH19 via the flowcytometry 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-1beta-treated samples lacking lncH19 inhibition with or without Dex (Fig 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 (Fig 4E). Therefore, given the consistency of these results with those obtained via flowcytometry 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-alpha 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 (Fig 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 down-regulation 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 (Fig 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 (Fig 6A). Therefore, we further investigated the functions of miR-324-3p. An MTS assay was conducted to assess cell viability. By comparing the control group to a miR-324-3p inhibitor-treated group, cell viability decreased significantly at 72 h ($P < 0.05$). When cells were transfected with shRNA targeting lncH19, cell viability increased from an OD value of 1.5 to 2.0 relative to non-miRNA inhibitor-treated cells with and without shlncH19 transfection. Among cells transfected with only shlncH19 and those with both shlncH19 and miR-324-3p inhibitors, cell viability was lower if the expression of miR-324-3p was inhibited (Fig 6B). Therefore, the evidence suggests that the expression of lncH19 was negatively correlated with cell viability, but the expression of miR-324-3p was positively correlated with cell viability. We also conducted cell apoptosis assays via flowcytometry 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 A549 NC + miRNA inhibitor NC group (Fig 6C). mRNA levels of IkBalpha, Ikkbeta, IL-6, IL-8, and TNF-alpha were all up-regulated when lncH19 was inhibited but down-regulated when miR-324-3p was simultaneously inhibited relative to the A549 NC + miRNA inhibitor NC group (Fig 6D). The cell culture supernatant was collected to determine the concentration of the cytokines IL-6, IL-8, and TNF-alpha. The concentration of cytokines increased significantly when lncH19 was inhibited by shRNA relative to the A549 NC+miRNA inhibitor 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 (Fig 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 A549 NC + miRNA inhibitor NC group. However, protein levels were decreased when miR-324-3p was down-regulated by an inhibitor. 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-1beta-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-1beta 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 in asthma by targeting a cascade involving lncH19 and miR-324-3p.

lncH19 was discovered in the 11p15.5, H19/IGF2 locus [9]. lncH19 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 [10]. Current research suggests that mutation of H19 in mouse zygotes causes prenatal lethality, indicating that H19 plays an important role in cell growth and development [11]. Therefore, our findings are consistent with previous studies in that lncH19 is able to regulate cell activities, such as cell viability and cell apoptosis. Previously, lncH19 has been shown to be correlated with the NF-kB pathway and is thus involved in the regulation of cell growth in multiple myeloma [7]. NF-kB is an important family of transcription factors involved in cell differentiation, apoptosis and immunity. Activation of the NF-kB pathway is involved in most cell activities in cancer transformation, such as the inhibition of cell differentiation and cell apoptosis, promotion of cell proliferation,

angiogenesis and potential metastasis [12]. Currently, NF-kB also plays a role in the anti-inflammatory effects in asthma [12-15], which suggests that it can provide an important pathway for lncH19 targets and regulate the processes underlying the inflammatory effects of asthma. Induction of the inflammatory response by IL-1beta reduced the expression of lncH19. However, 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 asthma; therefore, lncH19 is likely involved in anti-inflammatory processes associated with asthma. However, since lncRNAs usually inhibit targets, lncH19 might not directly target NF-kB. Therefore, we suspected that lncH19 targets and inhibits NF-kB 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 negatively correlated with lncH19 expression. Following detection of cell viability and cell apoptosis, inhibition of miR-324-3p revealed a highly significant correlation between cell viability and apoptosis. Therefore, we suspected that lncH19 is correlated with miR-324-3p and regulates the inflammatory response by regulating miRNA. 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 [16]. SMAD7 encodes an intracellular protein that is a TGF-beta I antagonist [17]. Overexpression of SMAD7 is associated with the development of skin, pancreatic, lung, and colon cancer in the processes of cell growth and apoptosis [18, 19]. Previous studies have shown that SMAD7 is correlated with the NF-kB pathway and therefore regulates the cell cycle in cancers [20, 21]. p65, ICAM-1, and VCAM-1 were the common inflammatory cytokines [22, 23]. 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-kB [23]. By characterizing the expression of the aforementioned 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-kB 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.

Our results have shown that Dex can attenuate the inflammatory response in asthma. The lncH19/miR-324-3p axis might play an important role in regulating the inflammatory response and thus cell viability and cell apoptosis. Future studies should examine additional genes targets of the lncH19/miR-324-3p axis, including SMAD7 and NF-kB, 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 actual asthma patients, as our hypothesized mechanism is based on a A549 cell model. Data from actual patients are important for confirming the functions of the lncH19/miR-324-3p cascade and could contribute to asthma diagnoses as well as the development of therapeutic approaches to treat asthma.

Conclusion

In summary, we demonstrated here for the fist time that Dex can attenuate the inflammatory response in asthma via regulation of the lncH19/miR-324-3p cascade. The present study indicated that lncH19/miR-324-3p cascade maybe contribute to asthma diagnoses as well as the development of therapeutic approaches to treat asthma in patient.

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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 guidlines approved by Ethics Committee of Children's Hospital of Chongqing Medical Universityand were performed following the declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Fig 1. An asthma inflammatory cell model via A549 induced by different concentrations of IL-1beta and LPS was established, and IL-1beta proved to be a more robust inflammatory cell model. A. RT-PCR determination of the corresponding mRNA levels of the inflammatory cytokines IKbeta-alpha, IKKbeta, IL-6, IL-8, and TNF-alpha. B. Levels of the inflammatory cytokines IL-6, IL-8, and TNF-alpha determined by ELISA.

Fig 2. Different concentrations of Dex can attenuate the inflammatory response induced by IL-1beta. A. mRNA levels of the inflammatory cytokines were determined by RT-PCR. B. Levels of the inflammatory cytokines determined by ELISA.

Fig 3. lncMALAT1, lncHotair, lncH19, and lncNeat1 were expressed less when A549 was treated by IL-1beta but were expressed more as the inflammatory response attenuated following application of Dex. Changes in lncH19 were the most pronounced.

Fig 4. When lncH19 was inhibited while cells were treated with IL-1beta with or without Dex, cell viability 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. lncH19 expression was reduced, indicating that a lncH19-inhibited cell line was generated. B. Cell viability of A549 NC and A549 shlncH19 cells treated by 1L-1beta with or without Dex. C. Cell apoptosis measured by flowcytometry. D. Percentage of apoptotic cells in C. E. Western blot for determining protein levels of inflammatory-related genes. F. ELISA for assessing levels of inflammatory cytokines.

Fig 5. RT-PCR was conducted to determine the level of miRNAs predicted to be targets of lncH19; trends in the expression of miR-346, miR-18a-3p, and miR-324-3p were consistent in A549 NC and A549 shlncH19.

Fig 6. The attenuation of lncH19 by miR-324-3p was inhibited as the inflammatory response attenuated under treatment with IL-1beta and with Dex. A. Levels of candidate miRNAs determined by RT-PCR for examining the performance of the miRNA inhibitor. B. Cell viability 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.