Serum-derived exosomes from house dust mite-sensitized guinea pigs contribute to inflammation in BEAS-2B cells via the TLR4-NF-κB pathway

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Received April 15, 2021; Accepted July 21, 2021

DOI: 10.3892/mmr.2021.12387

Abstract. Airway epithelial cells, which are the first physical defense barrier against allergens, play a pivotal role in immunity, airway inflammation and airway remodeling. The damage and dysfunction of these cells trigger the development of airway inflammatory diseases. Exosomes, which exist in various bodily fluids, mediate cell-cell communication and participate in the immune response process. The present study aimed to investigate whether serum exosomes play a pro-inflammatory role in bronchial epithelial cells (BEAS-2B cells) and, if so, explore the underlying molecular mechanisms. A guinea pig model of House dust mite (HDM)-induced asthma was established by sensitizing the rodents with HDM and PBS, and serum-derived exosomes were harvested. It was found that serum-derived exosomes from HDM-sensitized guinea pigs displayed higher levels of exosomal markers than those from controls. Additionally, western blot analysis and reverse transcription-quantitative PCR indicated that serum-derived exosomes from HDM-sensitized guinea pigs carried heat shock protein 70 and triggered an inflammatory response in BEAS-2B cells via the toll-like receptor 4 (TLR4)-NF-κB pathway. However, TAK-242, an inhibitor of the expression of TLR4, blocked the activation of the TLR4-NF-κB pathway. These findings provided a novel mechanism for exosome-mediated inflammatory responses and a new perspective for the intervention of inflammatory airway disorders.

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Key words: exosomes, airway inflammation, epithelial cells, house dust mite, Toll like receptor 4, NF-κB, heat shock protein 70

Introduction

Inflammatory airway diseases, including asthma, chronic obstructive pulmonary disease (COPD), and other pulmonary inflammatory diseases, are characterized by limited airflow and airway hyperresponsiveness, which can aggravate the process of lung disorders further (1,2). As these lung diseases are associated with abnormal immune responses of the airway to inhaled allergens or toxic substances, an improved understanding of their physiopathology is required, particularly that of the first-line epithelial tissues (3). As innate immune sensors and modulators, airway epithelial cells play a pivotal role in mediating local innate and adaptive immune responses in airway microenvironments (4). Damaged airway epithelium-instigated abnormal airway barrier function responses trigger inflammatory airway diseases (5,6). When injured, airway epithelial cells release cytokines, including IL-25, IL-33 and thymic stromal lymphopoietin, that mediate the inflammatory response and airway remodeling of asthma (7-9). Therefore, airway epithelial cell-produced functional molecules mediate intercellular interactions and communications (10).

Intercellular informational communication plays a critical role in modulating physiopathological functions in organisms (11). Exosomes have recently drawn widespread attention for facilitating cell-to-cell communication and participating in various pathophysiological processes, including immune responses (12), antigen presentation (13), inflammatory responses (14), cell migration and cell proliferation (15). Various cell types, such as alveolar macrophages, stem cells, airway epithelial cells and eosinophils, secrete 30 to 120 nm-sized exosomes (3,16). Exosomes are also widely present in body fluids, such as plasma, serum, urine, breast milk and bronchoalveolar lavage fluids (BALF) (17,18). These nano-vesicles are formed by the inward budding of late endosomes that fuse with the cytoplasmic membrane and release intracellular vesicles into the extracellular space (19). Receptor cells can uptake exosomes in various ways to alter their phenotypic appearances and functions (20). Therefore, exosomes, comprising proteins, lipids and nucleic acids can release their contents to participate

in the intercellular transfer of information (19). In particular, exosomes can alter the biological functions of recipient cells via the transfer of mitochondria (21).

Recent studies have shown close associations between exosomes and inflammatory airway diseases (22-24). Exosomes are crucial in informational communication between asthma microenvironments and various cells (25). Eosinophils secrete exosomes in healthy subjects and patients with asthma, the latter of which is particularly enhanced (16), but stable patients with asthma and healthy subjects express exosomal microRNAs (miRNAs/miRs) differently (26). Additionally, miR-34a, miR-92b and miR-210 are enriched in exosomes, altering airway microenvironments upon asthma development (27). One study found exosome secretion in mice BALF to be significantly enhanced upon contact with ovalbumin compared with controls (28). Paredes *et al* (29) noted that exosomes from asthmatics induced leukotriene C4 and interleukin-8 (IL-8) secretions in airway epithelial cells (29). These extracellular vesicles can also mediate the pathogenesis of COPD (30). Plasma exosome concentrations are higher in patients with acute exacerbations of COPD and stable COPD than in healthy individuals (31). Reportedly, exosomal miR-21 from bronchial epithelial cells in patients with COPD promotes myofibroblast differentiation through hypoxia-inducible factor 1α (32). Clearly, exosomes from various sources mediate the pathogenesis of inflammatory airway disorders.

While most studies have focused on the promotion of the development of various cells or BALF exosome-induced airway inflammatory disorders, few have paid attention to serum-derived exosomes facilitating the pathogenesis of inflammatory airway diseases. Thus, the present study investigated whether serum-derived exosomes from House dust mite (HDM)-sensitized guinea pigs could alter the phenotypic appearances of bronchial epithelial cells and, if so, explore the underlying molecular mechanisms.

Materials and methods

Animals. Female guinea pigs (6-8 weeks old, n=20) were obtained from Hunan Changsha Tianqin Biotechnology (http://cstqsw.com) and acclimated for a week before the experiments. The animals were kept in a pathogen-free environment and fed *ad libitum*. Our research protocol was approved by the Animal Care Committee of Jiangxi Provincial People's Hospital Affiliated with Nanchang University (approval no. 2021-052; Nanchang, China).

Cells, reagents and antibodies. The human bronchial epithelial cell line BEAS-2B was obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. HDM extract was purchased from ALK-Abelló A/S, Dulbecco's modified Eagle's medium (DMEM) and ExoQuick Exosome Isolation Reagent were acquired from Thermo Fisher Scientific, Inc. Serum replacement was from Stemboscience, Inc., the PKH67 Green Fluorescent Cell Linker kit and DAPI were from Sigma-Aldrich (Merck KGaA), while paraformaldehyde, Giemsa's stain and Phosphotungstic acid hydrate were purchased from Beijing Solarbio Science & Technology Co., Ltd. The BCA Protein Quantitative kit was from CoWin Biosciences, polyvinylidene difluoride (PVDF) membranes were from MilliporeSigma, interleukin (IL)-4 and IL-13 ELISA kits were from Nanjing Jiancheng Bioengineering Institute. The Immunoglobulin E (IgE; cat. no. SFE40020) ELISA kit was purchased from Shanghai Shifeng, Inc. (https://shfeng-edu.biomart.cn), and IL-6 (cat. no. EK 106/2-96) and nerve growth factor (NGF; cat. no. EK 1141-96) ELISA kits were from Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd. TAK-242 (cat. no. HY-11109) and BAY 11-7082 (cat. no. HY-13453) were purchased from MedChemExpress, recombinant human heat shock protein 70 (rHSP70; cat. no. 11660-H07H) was from Sino Biological Technology Co., Ltd, and heat shock protein 70 (HSP70)-IN-1 (cat. no. M9273) was from AbMole Bioscience, Inc. Primary antibodies against CD63 (cat. no. ab68418), IKKα/β (cat. no. ab178870), phosphorylated (p)-IKKα/β (cat. no. ab194528) and HSP70 (cat. no. ab31010) were procured from Abcam, anti-TLR4 (cat. no. BA1717) was from Boster Biological Technology, and anti-p65 (cat. no. GB11997), anti-p-p65 (cat. no. GB11142-1), anti-β-actin (cat. no. GB11001) and a HRP-conjugated goat anti-rabbit IgG secondary antibody (cat. no. G1213) were purchased from Wuhan Servicebio Technology Co., Ltd.

Cell culture. BEAS-2B cells were cultivated in DMEM supplemented with 20% serum replacement (a cell culture supplement that replaces fetal bovine serum to maintain cell growth and reproduction *in vitro*), streptomycin (100 mg/ml) and penicillin (100 U/ml) at 37˚C in a 5% CO_2 atmosphere. The cells were passaged every 2 days in a 1:3 ratio. In cellular co-culture with serum-derived exosomes, serum replacement was used to prevent interference from fetal bovine serum-derived exosomes. BEAS-2B cells were co-cultured with serum-derived exosomes isolated from the HDM group and PBS group at 37˚C for 24 or 48 h separately. BEAS-2B cells were also pretreated with or without 5 μg/ml proteinase K (Beijing Solarbio Science & Technology Co., Ltd.), 5 μM BAY 11-7082, 5 μM HSP70-IN-1 and different concentrations of TAK-242 (100 or 300 nM) at 37˚C for 1 h, and then co-cultured with serum-derived exosomes from the HDM group at 37˚C for 24 h. BEAS-2B cells were treated with or without rHSP70 (1 or 10 μg/ml) at 37˚C for 4 h.

Animal model. The guinea pigs (200-250 g, female) were housed in a room maintained at moderate temperature (22±2˚C) and humidity (40-70%) with a 12 h light/dark cycle, and free access to food and water for the duration of the present study. They were adaptively fed for 7 days and randomly divided into two groups: Sham group (PBS treatment group, n=10) and HDM group (n=10). HDM extracts (100,000 U/ml) were diluted in a 0.1 mol/l PBS solution at concentrations of 2,000 U/ml, 4,000 U/ml and 8,000 U/ml. A HDM-induced asthma model was created as shown in Fig. 1. The guinea pigs in each group were intraperitoneally injected with pentobarbital (35 mg/kg). Subsequently, blood samples (5 ml per rodent) were withdrawn from the heart via cardiac puncture. The guinea pigs were euthanized by the immediate removal of the heart after exsanguination; death was confirmed when the animals developed cardiac arrest, respiratory arrest, corneal reflex arrest and rigor mortis. All procedures were conducted strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (33).

Lung histology. Harvested lung tissues from the guinea pigs were fixed in 10% formalin solution for 24 h at 37˚C and embedded in paraffin. After deparaffinization, 5 μm sections of these tissues were stained with hematoxylin for 5 min at 37˚C and eosin for 5 min at 37˚C (H&E) to observe morphology, including pulmonary edema, airway inflammation and airway epithelial injury under a light microscope (ECLIPSE CI; Nikon Corporation).

Total cell counts. Precipitated cell suspension was conducted with 1 ml PBS. A few droplets from the suspension were taken to the cell-count boards to determine the total cell count in BALF per ml. The remaining precipitated cells, including eosinophils, neutrophils and lymphocytes, were fixed in 4% paraformaldehyde solution for 30 min at 37˚C and stained with Wright-Giemsa for 20 min at 37˚C (at least 200 cells per sample) to deduce the percentage of cells under a light microscope [ECLIPSE CI; Nikon Corporation (magnification, x40)].

ELISA. BALF IL-4 and IL-13 concentrations and serum IgE levels from treated-guinea pigs and IL-6 and NGF contents in cell supernatants were quantified using ELISA kits. In brief, the guinea pigs were subjected to tracheotomy and washed with 5 ml ice-cold PBS three times before BALF was collected. The obtained BALF supernatants was centrifuged at 4˚C (250 x g, 10 min). Different concentrations of serum-derived exosomes (0, 50, 100, 200 μg/ml) from the HDM group were added to BEAS-2B cells simultaneously and incubated at 37˚C for 24 and 48 h. After incubation, cell supernatants were collected by centrifugation at 4˚C (1,000 x g, 15 min).

Exosome isolation and quantitation. Serum exosomes were isolated using the ExoQuick Exosomes Isolation Reagent, according to the manufacturer's recommended protocol. Briefly, serum from HDM-sensitized and PBS-sensitized guinea pigs was differentially centrifuged at 4˚C (2,000 x g, 30 min) to remove cells and debris. The supernatants were filtered through 0.22-μm filters to eliminate particles >220 nm, and a reagent mixture was added to the well until the solution was homogenous. The mixed suspension was then incubated at 4˚C for 30 min and centrifuged at 10,000 x g for 10 min at room temperature. Exosomes contained in the pellet at the bottom of the tube were re-suspended in 200 μl PBS. Serum-produced exosomal proteins were quantitated using the BCA Protein Assay kit, and estimated by reference to a standard curve generated from proteins [bovine serum albumin (BSA)] of known concentration.

Experimental groups. The samples were divided into three groups: Control group (untreated cells), S-exo treatment group (exosomes from the sham group) and H-exo treatment group (exosomes from the house dust mite group).

Transmission electron microscopy (TEM). The ultrastructure of exosomes was observed using TEM, referring to the methods described in a previous study (34). A 20 μl drop of the exosomal suspension was placed on parafilm and loaded to a carbon-coated grid for 2 min. A 2% phosphotungstic acid solution prepared with triple distilled water was used to stain the carbon-coated grid-loaded suspension for 30 sec at room temperature. The sample was then dried for 2 min under incandescent light, and the results were observed and images captured using a transmission electron microscope (JEM-1200EX; JEOL, Ltd.) at an acceleration voltage of 80 kV. The TEM images were cropped and scaled by Photoshop CS6 (Adobe Systems Incorporated).

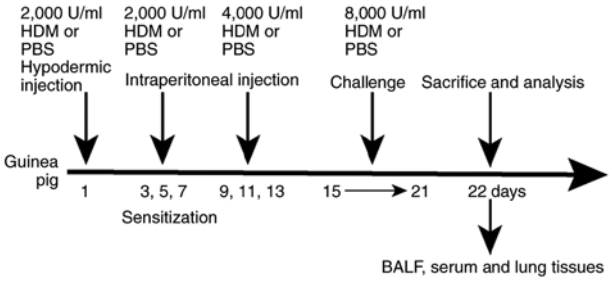

Figure 1. Summary of the study protocol. The HDM group guinea pigs were injected subcutaneously with 1,000 μl of 2,000 U/ml HDM on day 1, intraperitoneally injected with 1,000 μl of 2,000 U/ml HDM on days 3, 5 and 7, and intraperitoneally with 500 μl of 4,000 U/ml HDM on days 9, 11 and 13. Guinea pigs in the experimental group were then sensitized and challenged in atomized boxes crafted for the present study with 8,000 U/ml HDM extract from days 15 to 21, each time for 30 min. Sham group rodents were sensitized and challenged with PBS, instead of HDM. Subsequently, the animals were subjected to tracheotomy and washed with 5 ml ice-cold PBS three times before BALF was collected. Serum from the heart was collected using disposable needles, and lung tissues were harvested. HDM, house dust mite; BALF, bronchoalveolar lavage fluid.

Western blotting. After washing three times with precooled PBS and a protease inhibitor cocktail on ice for 30 min, cells were harvested in RIPA lysis buffer with 1 mM PMSF. The concentration of protein was measured using a BCA protein assay kit. Total proteins (30 μg per lane) were loaded, separated with 8-10% SDS-polyacrylamide gels, and transferred onto PVDF membranes. The PVDF membranes were blocked with 5% non-fat milk at room temperature for 2 h and then incubated with a 1:1,000 dilution of the specific primary antibodies at 4˚C overnight. Followed by washing with TBS with 1% Tween-20 three times (10 min each time), and incubation with horseradish peroxidase-conjugated secondary antibodies (1:3,000) at room temperature for 1 h. Then, the blots was visualized with enhanced chemiluminescent solution (Beijing Solarbio Science & Technology Co., Ltd.). Primary antibodies against the following were used: CD63, HSP70, TLR4, IKKα/β, p-IKKα/β, p65, p-p65 and β-actin. Band densities were analyzed using ImageJ software 6.0 (National Institutes of Health); the β-actin protein was used as an internal reference.

Exosome labeling. Exosomes were labeled with PKH67 (a novel fluorescent dye that labels living cells by binding to lipid molecules in membrane structures) for general cell membrane labelling according to the manufacturer's instructions, with minor modifications. In brief, 100 μl serum-derived exosomes from the HDM group were mixed with 1 ml Diluent C, and for control, 1 ml Diluent C was mixed with PBS. The Diluent C added to the experiment and control was prepared by mixing 1 μl PKH67 dye with 750 μl Diluent C. Next, 1 ml of 1% BSA (Beijing Solarbio

Table I. Sequences of primers used in reverse transcription-quantitative PCR.

Primer	Sequences (5'→3')
IL-6	F: GTAGTGAGGAACAAGCCAGAGC
	R: TACATTTGCCGAAGAGCCCT
NGF	F: AGACATCAAGGGCAAGGAGGTG
	R: GCTGTCAACGGGATTTGGGTC
IL-8	F: CAGTTTTGCCAAGGAGTGCTAA
	R: AAACTTCTCCACAACCCTCTGC
TNF-α	F: GCTGCACTTTGGAGTGATCG
	R: ATGAGGTACAGGCCCTCTGA
IL-1β	F: GCGGCATCCAGCTACGAAT
	R: AAGCCTCGTTATCCCATGTGTC
β-actin	F: CACCCAGCACAATGAAGATCAAGAT
	R: CCAGTTTTTAAATCCTGAGTCAAGC

IL, interleukin; NGF, nerve growth factor; TNF-α, tumor necrosis factor-α; F, forward; R, reverse.

Science & Technology Co., Ltd.) was added to the mixed experimental and control solutions to stop the dyeing, and an ExoQuick Exosome Isolation Reagent was used to precipitate the solutions and extract exosomes.

Immunofluorescence staining. To detect the NF-κB subunit, p65, in nuclei, BEAS-2B cells cultured in 6-well plates were treated with or without 100 μg/ml exosomes for 2 h at 37˚C. Briefly, BEAS-2B cells were fixed with 4.0% paraformaldehyde in PBS for 20 min at room temperature, washed with PBS three times, and permeabilized in 0.25% Triton X-100 at room temperature for 10 min. Non-specific binding was blocked with 3% BSA in PBS for 30 min at room temperature, and the cells were incubated with the p65 antibody at 4˚C overnight, followed by incubation with the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody the next day at room temperature for 1 h. Cells were then washed with PBS three times, nuclei were stained with DAPI for 5 min at room temperature, and detected with a fluorescence microscope (BX51; Olympus Corporation).

Reverse transcription-quantitative (RT-q)PCR. BEAS-2B cells were transferred into a tube containing TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and total RNA was extracted according to the manufacturer's instructions. cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and stored at -70˚C until further use. qPCR was performed using an SYBR Green Master Mix (Roche Diagnostics) to verify the differential expression of the genes. The thermocycling conditions for PCR were as follows: Initial denaturation for 10 min at 95˚C, followed by 40 cycles of 95˚C for 15 sec and extension at 60˚C for 1 min. RT-qPCR was performed in duplicate and normalized to β-actin. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method (35). The primer sequences are listed in Table I.

Statistical analysis. GraphPad Prism 6.0 (GraphPad Software, Inc.) was used for all statistical analyses. Data are expressed as the mean ± SD. An unpaired Student's t-test was used for comparisons between two groups, and one-way ANOVA followed by the Bonferroni post hoc test were employed for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference. Each experiment was repeated three times.

Results

Successful establishment of allergic asthma model in guinea pigs via HDM sensitization and challenge. Bronchial and alveolar septum structures in the sham group were normal, with fewer inflammatory cells infiltrating the lung tissues, but in the HDM group, bronchial mucosal edema, increased mucus secretion and observable inflammatory cell infiltration were observed (Fig. 2A). Serum IgE levels increased significantly in the HDM group compared with those in the sham group (Fig. 2B), as did IL-4 and IL-13 BALF levels (Fig. 2C) and total BALF cell numbers, with increases in inflammatory cells, such as lymphocytes, neutrophils and eosinophils (Fig. 2D). A model of allergic asthma was successfully established in guinea pigs via HDM sensitization and challenge.

BEAS-2B cells efficiently incorporated serum-derived exosomes. TEM revealed that exosome sizes in the two groups were in the range of 30-120 nm in diameter, and the vesicles appeared as double-layer circles (Fig. 3A and B). According to western blot analyses, the expression levels of exosome marker proteins, HSP70 and CD63, in the H-exo group were significantly higher than those in the S-exo group (Fig. 3C and D), as were serum exosomal protein amounts (Fig. 3E). The uptake of serum-derived exosomes by BEAS-2B cells was observed 24 h later; green fluorescence was perceived in BEAS-2B cell cytoplasm (Fig. 3F). The data demonstrated that BEAS-2B cells effectively absorbed serum-derived exosomes from the H-exo and S-exo groups.

Incubating BEAS-2B cells with serum-derived exosomes induces changes in gene expression profiles. The ability of serum-derived exosomes to stimulate a proinflammatory response in BEAS-2B cells was explored. The cells were cultured separately with or without 100 mg/ml serum-derived exosomes obtained from the H-exo and S-exo groups for 24 h, and cytokine expression was determined using RT-qPCR. The results showed that IL-6 and NGF secretions were higher in exosomes from the H-exo group than in those from the control group (Fig. 4A). To determine whether IL-6 and NGF levels in exosome-treated BEAS-2B cells were concentration-dependent; BEAS-2B cells were co-cultured with the concentration gradient of exosomes from the HDM group at different times (24 and 48 h). In the exosome-treated BEAS-2B cells, the increase in mRNA expression levels and secretion of IL-6 and NGF was only dose-dependent for 50 and 100 μg/ml. There was a decrease at 200 μg/ml (Fig. 4B-E). Therefore, serum-derived exosomes from the HDM group modulated BEAS-2B cell phenotypic appearances and enhanced the inflammatory responses.

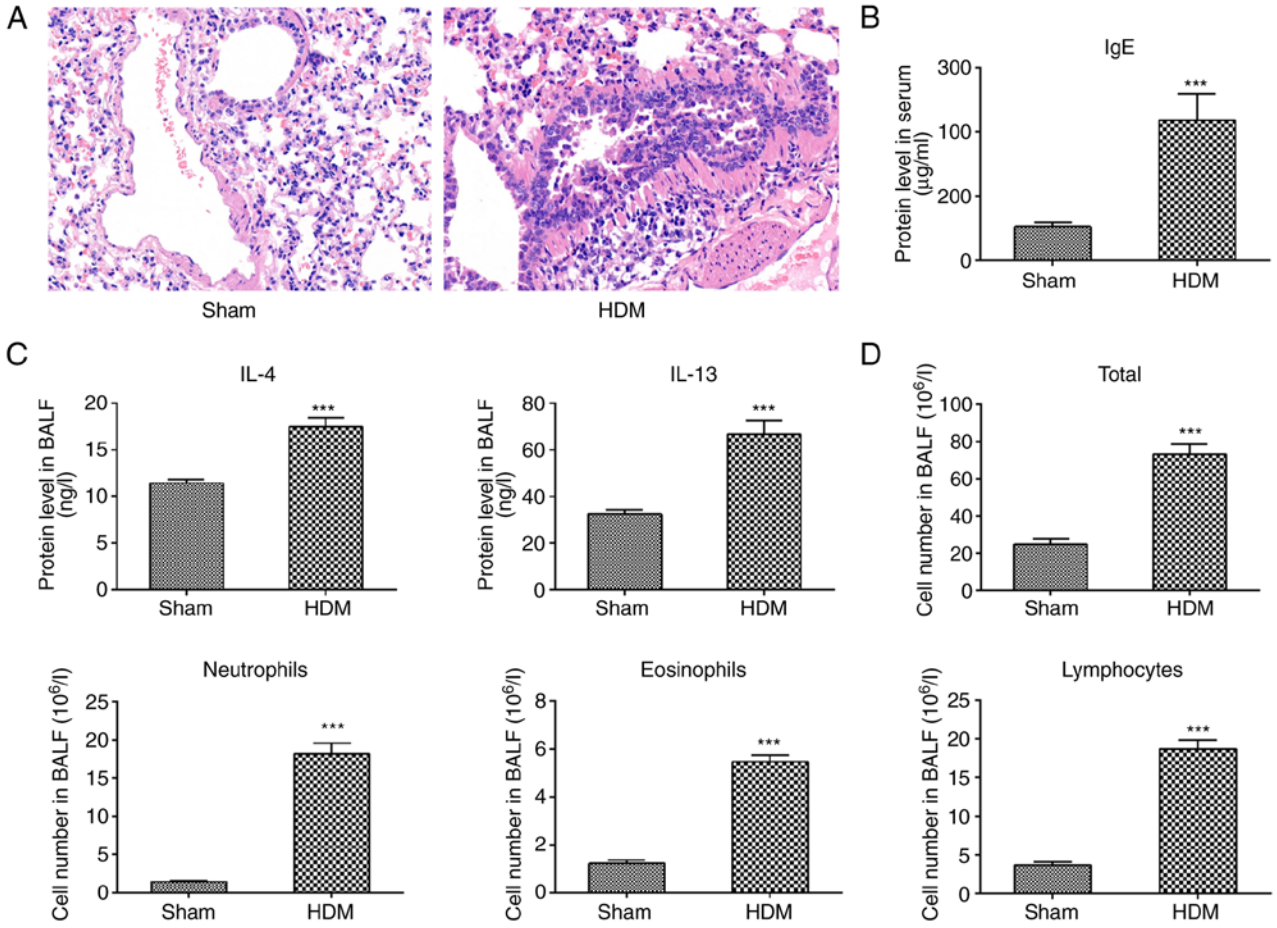

Figure 2. Allergy asthma model in guinea pigs was established successfully via HDM sensitization and challenge. (A) A lung tissue section depicting the level of airway inflammation (hematoxylin and eosin staining). Magnification, x200. (B) ELISA-determined Serum IgE levels. (C) ELISA-determined BALF IL-4 and IL-13 contents. (D) Total cell count and inflammatory cell count in BALF. ***P<0.001 vs. sham group. HDM, house dust mite; BALF, bronchoalveolar lavage fluid; IL, interleukin.

Serum-derived exosomes from the HDM group induces TLR4-NF-κB pathway activation in BEAS-2B cells. To determine the signaling pathway through which serum-derived exosomes from the HDM group promoted the inflammatory response in BEAS-2B cells, TLR4-NF-κB pathway protein levels were evaluated using western blot analysis, and the signaling events triggered by exosomes were observed. As presented in Fig. 5A, IKKα/β and p65 phosphorylation resulted in a rapid NF-κB activation 30 min after exosome addition. As expected, stimulation with exosomes significantly increased TLR4, p-IKKα/β and p-p65 expression levels, with maximum elevations noted at 2 or 4 h (Fig. 5A-D). Generally, NF-κB signaling activation is linked to p65 protein phosphorylation and translocation to the cell nucleus. Serum-derived exosomes from the HDM group stimulation resulted in the translocation of p65 from the cytoplasm to the nucleus (Fig. 5E and F).

TAK-242 reduces the expression of NF-κB phosphorylation in BEAS-2B cells. IL-6 and NGF mRNA levels in BEAS-2B cells were evaluated via RT-qPCR after blocking the TLR4-NF-κB pathway. It was demonstrated that IL-6 and NGF expression levels decreased when BEAS-2B cells were treated with serum-derived exosomes from the HDM group in the presence of the NF-κB inhibitor (BAY 11-7082) and TLR4 inhibitor (TAK-242) (Fig. 6A and B). TLR4, p-IKKα/β and p-p65 expression levels increased after BEAS-2B cell stimulation by serum-derived exosomes from the HDM group. To verify the TLR4 pathway protein levels obtained above, various concentrations of TAK-242 were added to BEAS-2B cells. TLR4, p-IKKα/β and p-p65 protein levels were partially inhibited in BEAS-2B cells by TAK-242 (Fig. 6C-F). These findings indicated that blocking TLR4 suppressed the exosome-mediated signaling pathway in BEAS-2B cells.

Serum-derived exosomes from the HDM group enable the transfer of HSP70 into BEAS-2B cells and contribute to cytokine secretion via the TLR4-NF-κB pathway. A variety of biological molecules, including nucleic acids (DNA, miRNA, RNA), proteins and lipids, have been classified as exosome contents (36). To investigate whether exosomal surface proteins have an effect on the production of cytokines, exosomes were treated with proteinase K before they were added to BEAS-2B cells. Cytokine upregulation was partially suppressed by proteinase K compared with the levels in the exosome group not treated with the enzyme (Fig. 7A), indicating that exosomal surface proteins prompted the increased expression of cytokines. Based on a previous finding that exosomal HSP70 from mycobacteria-infected macrophage cells induced an inflammatory response in uninfected macrophages (37), it was further examined whether HSP70 with

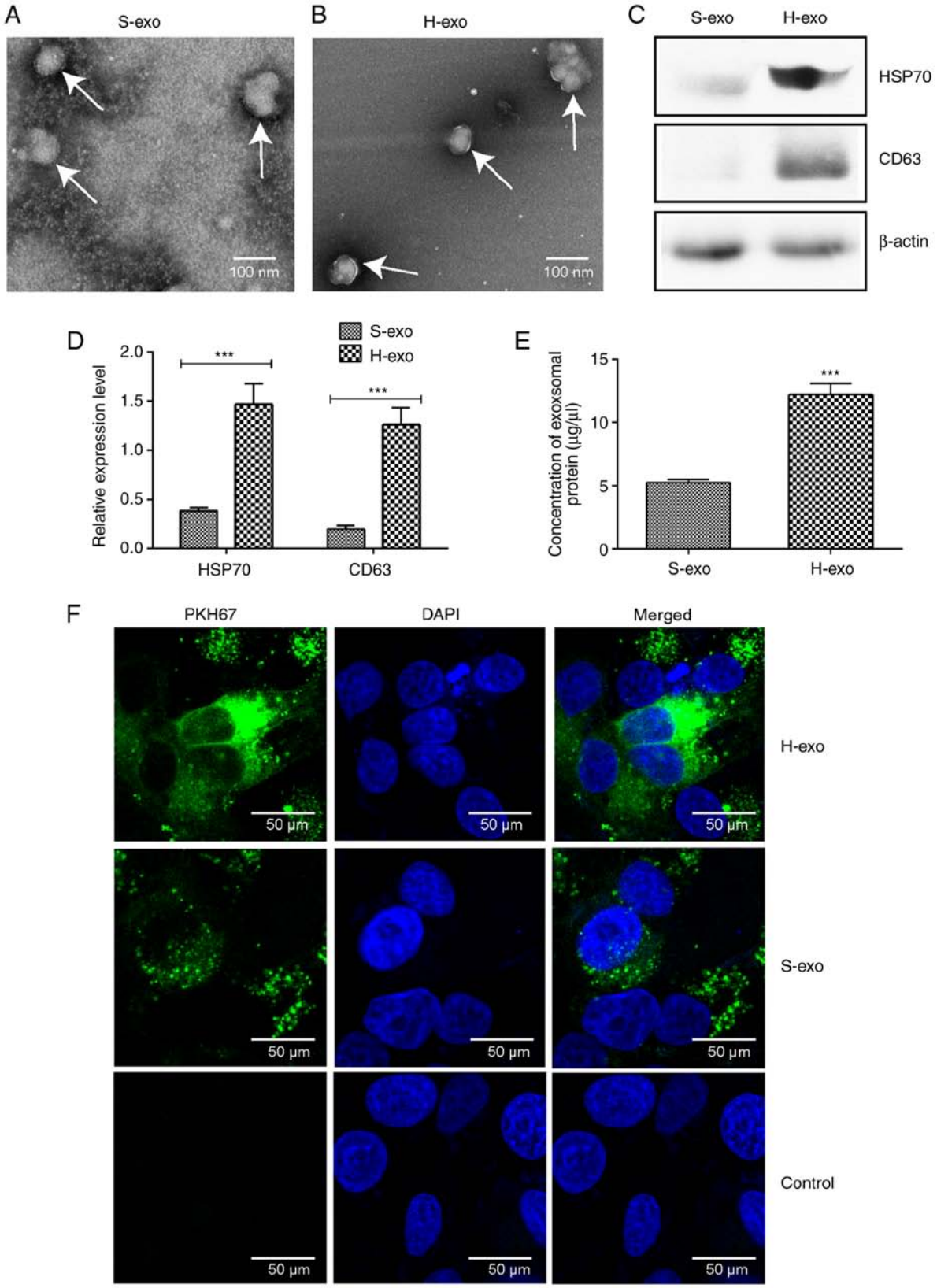

Figure 3. Characterization of serum-derived exosomes. (A and B) Morphology of S-exo and H-exo under transmission electron microscopy. Scale bar, 100 nm. (C and D) Immunoblot-determined CD63 and HSP70 expression levels. β-actin was used as a loading control. (E) BCA Protein Assay kit-determined S-exo and H-exo concentrations. (F) S-exo and H-exo uptake by BEAS-2B cells. Fluorescence microscopy images of BEAS-2B cells incubated with PKH67-labeled serum exosomes (green) or non-incubated exosomes. BEAS-2B cell nuclei were stained with DAPI stain (blue). Scale bar, 50 μm. ***P<0.001 vs. S-exo group. S-exo, exosomes from the sham group; H-exo, exosomes from the house dust mite group; HSP70, heat shock protein 70.

serum-derived exosomes from the HDM group could also exert a pro-inflammatory effect in recipient cells. HSP70-IN-1, an inhibitor of HSP70, suppressed the increased expression of IL-6 and NGF induced by serum-derived exosomes in

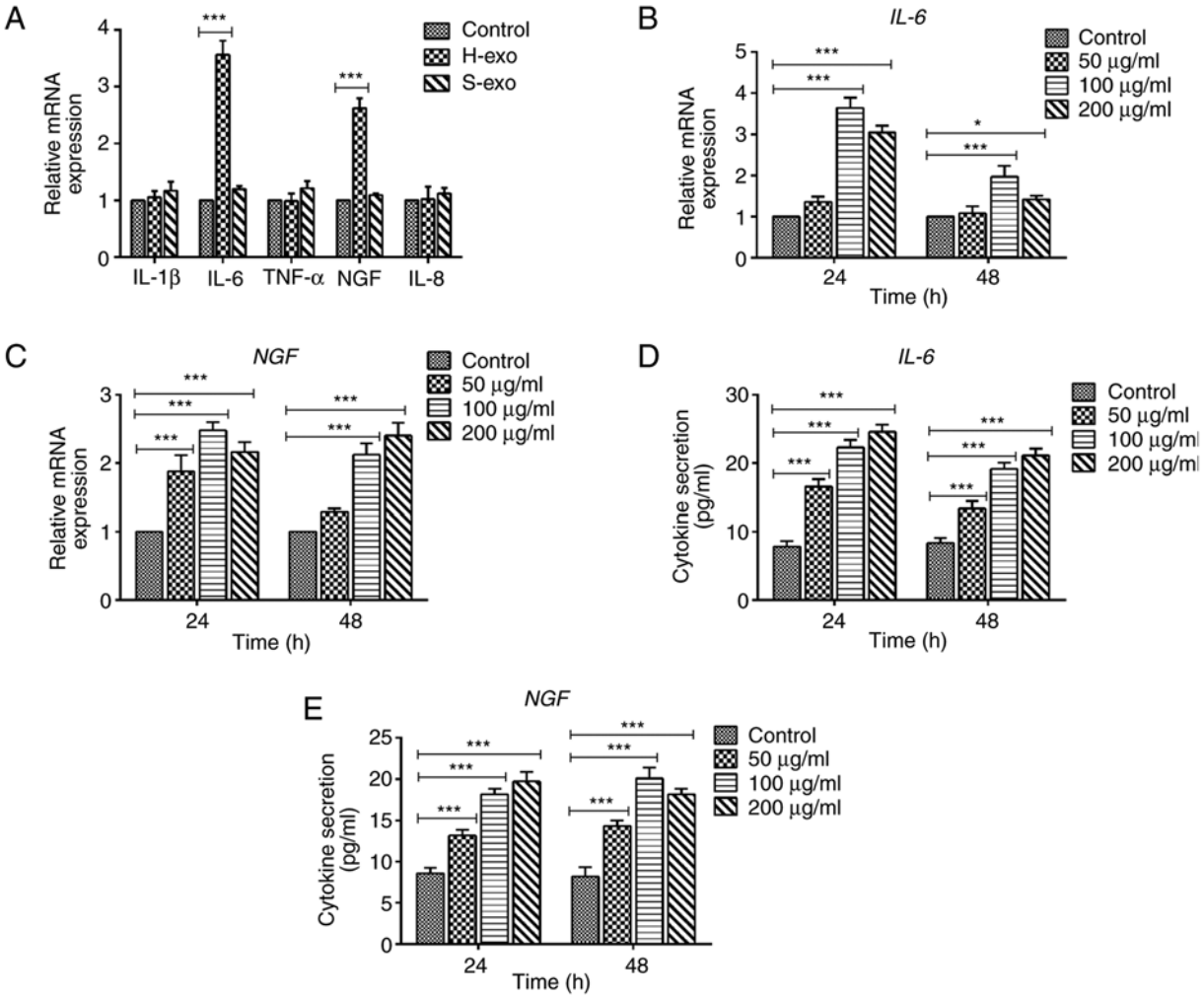

Figure 4. Effects of serum-derived exosomes on immune-related cytokine secretion in BEAS-2B cells. (A) RT-qPCR-determined mRNA levels of cytokines in BEAS-2B cells after addition of H-exo or S-exo. ***P<0.001 vs. control group (untreated cells). (B-E) RT-qPCR and ELISA-determined IL-6 and NGF expression levels in BEAS-2B cells at different time points and various concentrations of H-exo (0, 50, 100, 200 μg/ml). *P<0.05, ***P<0.001. S-exo, exosomes from the sham group; H-exo, exosomes from the house dust mite group; IL, interleukin; NGF, nerve growth factor; RT-qPCR, reverse transcription-quantitative PCR.

BEAS-2B cells (Fig. 7B). Various concentrations of rHSP70 were added to BEAS-2B cell cultures to mimic the role of exosomal proteins, and they promoted IL-6 and NGF mRNA levels in a dose-dependent manner (Fig. 7C); however, IL-6 and NGF expression levels were diminished after treatment with TAK-242 (Fig. 7D). Collectively, these data showed that exosomes-containing HSP70 from serum in the HDM group activated the TLR4-NF-κB signaling pathway in BEAS-2B cells.

Discussion

Previous investigations have shown that an alteration in the airway microenvironment can further aggravate inflammatory airway disorders, including chronic bronchitis, bronchiectasis and asthma (38,39). Airway epithelial cells, which are the first line of defense against different stimuli, play an essential role in maintaining the normal functioning of the airway microenvironment (3). Cell-to-cell communication is key to regulating the underlying mechanism of inflammatory lung diseases (40). Secreted soluble molecules, such as chemokines, cytokines and cell surface receptors, are also involved in this regulatory process (41). Exosomes, as extracellular functional units, have recently attracted research interest due to their crucial role in the pathogenesis of various diseases (18). The present study focused on the role of exosomes on the bronchial epithelium to mimic the effect of external stimuli on the airway microenvironment. In general, exosomes measure between 30 and 120 nm in size, but those from the H-exo group appeared smaller than the ones from the S-exo group (Fig. 3A and B), which was speculated to be possibly caused by partial exosomal fusion. However, the absence of a blank control group proved detrimental in the ability to make solid comparisons and inferences. Emerging evidence indicates that the secretion of exosomes from eosinophils in asthmatic subjects is higher than in healthy controls (16). Similarly, the protein levels of airway exosomal surface markers, including CD81, CD36 and HLA-DR, are significantly elevated in asthmatic subjects relative to healthy controls (42). As expected, the present study found that serum-derived exosomal surface molecules, such as CD63 and HSP70, increased in the H-exo group, but were notably attenuated in the S-exo group (Fig. 3C and D), possibly because the number of exosomes released from body fluids are not the same under different

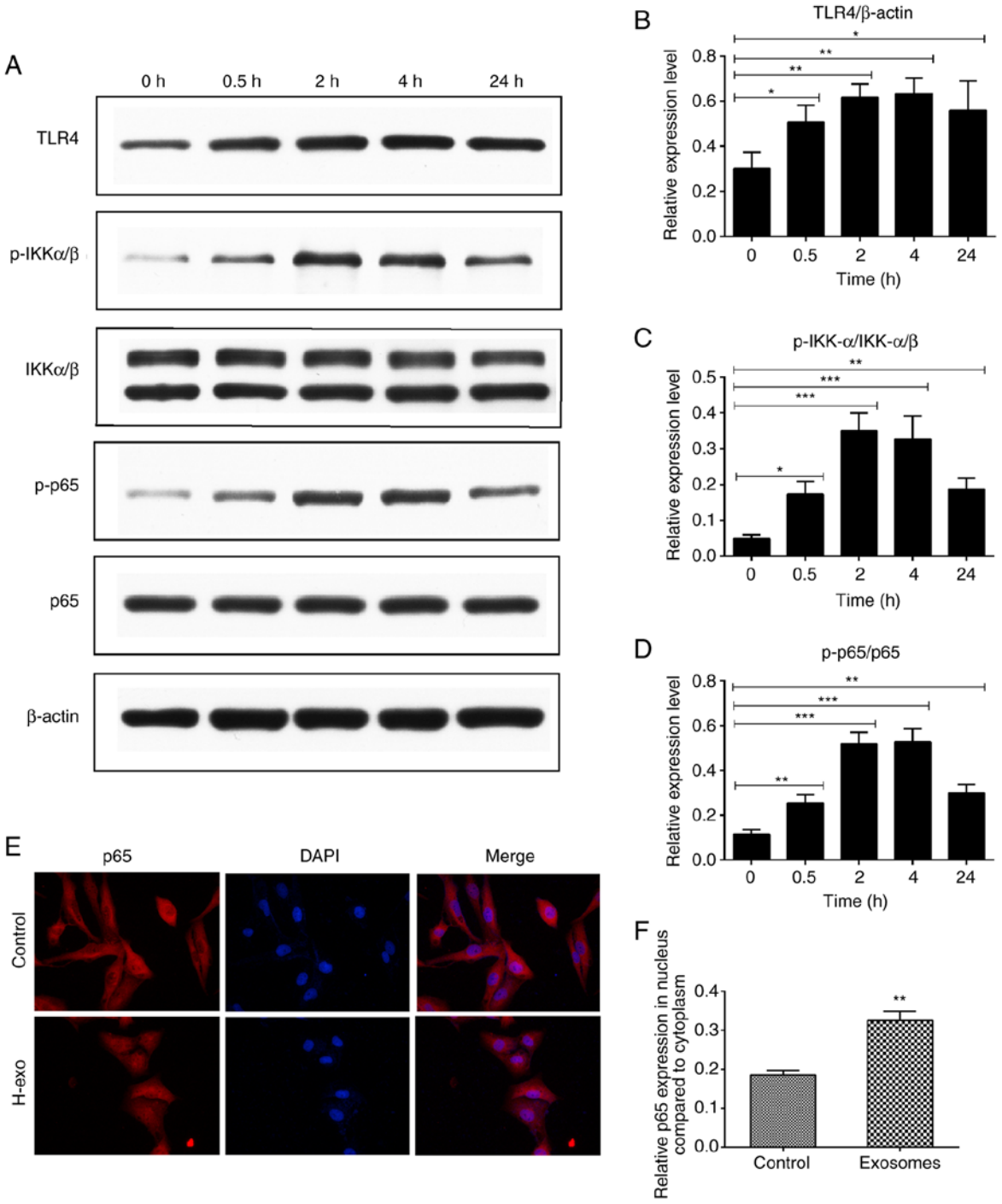

Figure 5. TLR4-NF-κB pathway activation in BEAS-2B cells. (A-D) TLR4-NF-κB pathway protein expression changes in BEAS-2B cells. *P<0.05, **P<0.01, ***P<0.001. (E and F) Representative images showing BEAS-2B cell incubation with or without H-exo from serum 2 h after the start of the process. Immunocytochemistry staining was performed using an anti-p65 antibody (red) and DAPI (blue) for nuclei staining. Magnification, x400. **P<0.01 vs. control group. H-exo, exosomes from the house dust mite group; TLR4, Toll-like receptor 4; p-, phosphorylated.

conditions, and the effects of these exosomes may also differ *in vivo* and *in vitro*.

In the past decade, exosomes, as mediators of intercellular crosstalk, have been shown to have the capacity to transfer their cargos to influence the physiological and pathological functions of receptor cells or parent cells. Valadi *et al* (43) demonstrated that exosomal RNA from mast cells in mice transferred to human mast cells and eventually translated into proteins in recipient cells. Exosomes from neutrophils are also reportedly rapidly up-taken by airway smooth muscle cells, and their potential proliferative ability increases after contact with lipopolysaccharides (44). Additionally, exosomes exist in biological fluids upon their release from cells and can be internalized by various cell types to alter their phenotypic appearances and functions (17,45). A previous study suggested that exosomes isolated from different body fluids enhance the production of inflammatory cytokines, such as IL-1β, TNF-α and IL-6, in monocytic cells (46). In the current study, BEAS-2B cells efficiently incorporated serum-derived exosomes from the

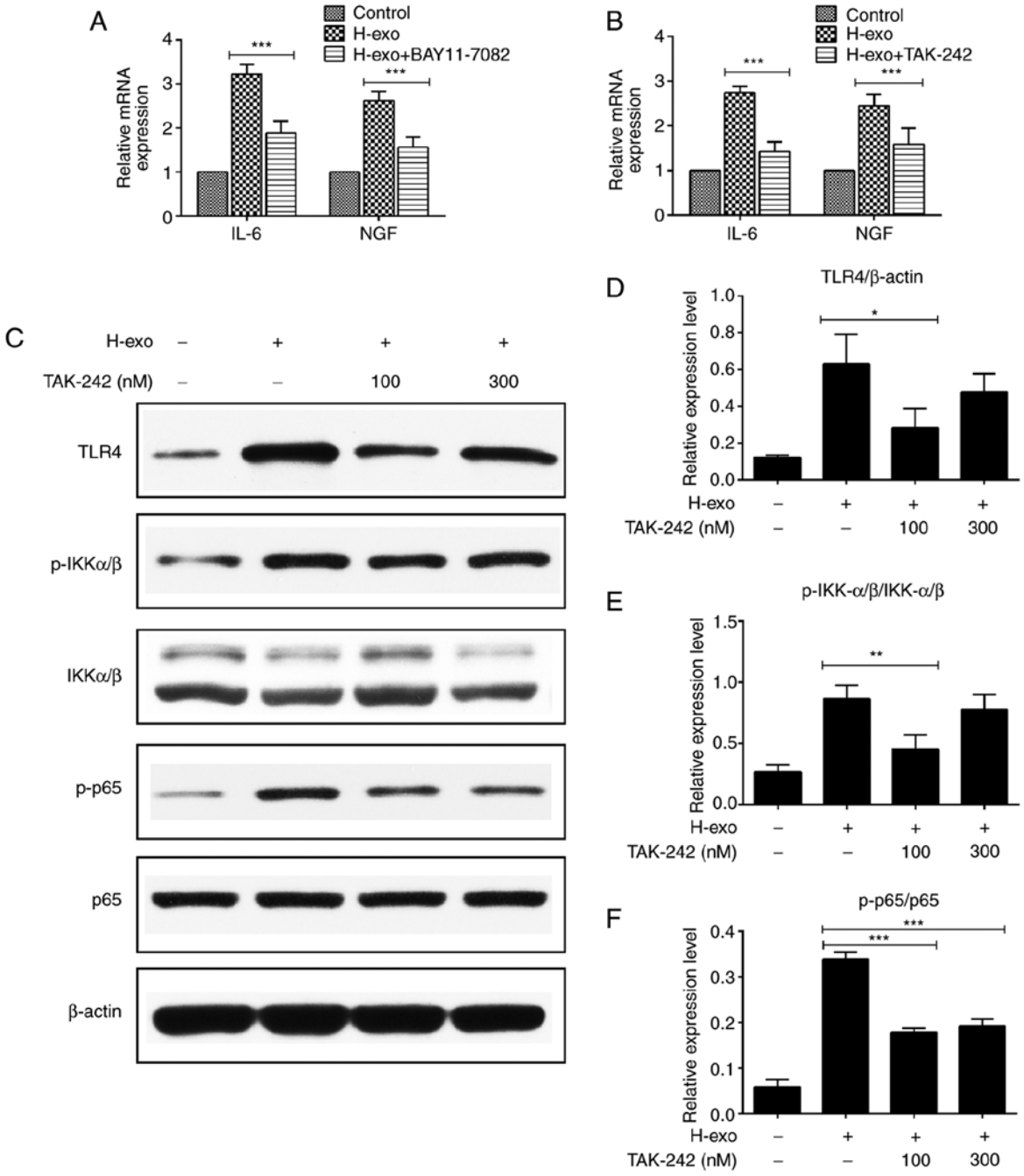

Figure 6. Inhibition of mRNA and protein expression levels in the TLR4-NF-κB pathway following TAK-242 addition to BEAS-2B cells. (A and B) IL-6 and NGF mRNA expression changes in BEAS-2B cells. (C-F) Western blotting-determined activation of TLR4-NF-κB in BEAS-2B cells. BEAS-2B cells were pretreated with various concentrations of TAK-242 and incubated with H-exo. *P<0.05, **P<0.01, ***P<0.001. H-exo, exosomes from the house dust mite group; IL-6, interleukin-6; NGF, nerve growth factor; TLR4, toll-like receptor 4; p-, phosphorylated.

HDM and sham groups (Fig. 3F). Because this part of the study focused on whether BEAS-2B cells could take up serum-derived exosomes, cell morphology images were not taken. Serum-derived exosomes from the HDM group interacted with BEAS-2B cells to induce increased IL-6 and NGF expression levels, but the increase was only concentration-dependent for 50 and 100 μg/ml exosome-treated cells (Fig. 4A-E). The exosomal treatments were normalized to the respective time controls, so the concentrations of cytokines for the different concentrations of the exosomes between the 24 and 48 h groups were not compared. IL-6 is a member of the interleukin family that plays a significant role in the occurrence and development of inflammatory airway disorders (47), while NGF, a member of growth factors, mediates the generation, proliferation, differentiation and maturation of inflammatory cells, and is involved in the mechanism of the airway neurogenic inflammatory response (48). No gene-expression changes were observed when serum-derived exosomes from the sham group were co-cultured with BEAS-2B cells (Fig. 4A), suggesting that these exosomes played a crucial role in maintaining the normal physiological function of the guinea pigs, possibly having no ability to alter the phenotypic appearances and functions of nearby or distant target cells.

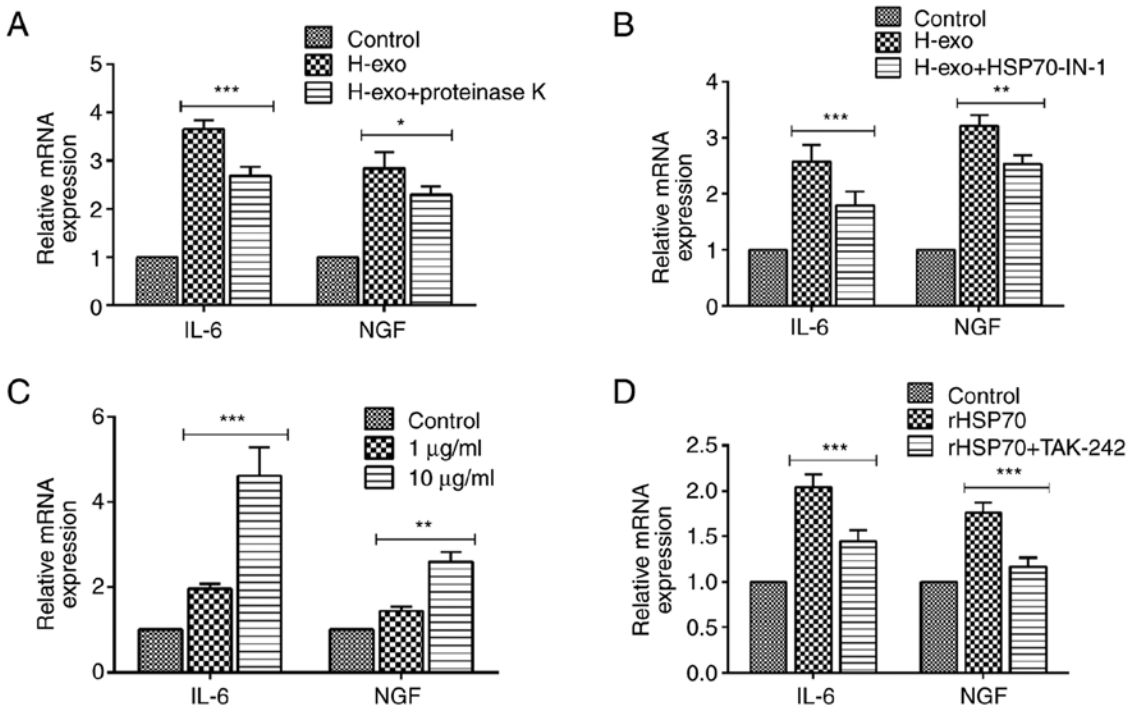

Figure 7. Exosomal surface HSP70 stimulation of IL-6 and NGF expression levels via the TLR4 pathway. (A and B) Relative mRNA expression levels of IL-6 and NGF following the addition of proteinase K. (C) Cytokine mRNA levels following the addition of various concentrations of rHSP70 to BEAS-2B cells. (D) IL-6 and NGF mRNA expression changes in the BEAS-2B cells incubated with 1 μg/ml rHSP70 after treatment with 100 nM TAK-242 for 24 h. *P<0.05, **P<0.01, ***P<0.001. H-exo, exosomes from the house dust mite group; IL-6, interleukin-6; NGF, nerve growth factor; TLR4, toll-like receptor 4; HSP70, heat shock protein 70; rHSP70, recombinant human HSP70.

Previous studies have also revealed that exosomes secreted by various cells or biological fluids promote local or systematic inflammatory processes to modulate the pathogeneses of various diseases (18,49). However, the underlying molecular mechanisms of the processes in the various diseases are different. According to a previous inquiry, airway epithelial cell apoptosis is prompted by exosomes from eosinophils in patients with asthma, and these exosomes can enhance the proliferation of bronchial smooth muscle cells via ERK1/2 activation (50). Mature dendritic cell-released exosomes can increase an inflammatory phenotype in the endothelium through membrane TNF-α, activating the NF-κB signaling pathway (51). Exosomes released from various body fluids allegedly also stimulate pro-inflammatory cytokine secretion via the NF-κB- and STAT3-mediated signaling pathway in a TLR-dependent manner (46), and plasma-derived exosomal mitochondrial DNA in patients with chronic heart failure induces IL-8 and IL-1β secretion via the TLR9-NF-κB pathway (52). These findings suggest that exosomes from different sources mediate cell-to-cell communication in a variety of ways.

We hypothesized that serum-derived exosomes in the HDM group could trigger BEAS-2B cell inflammation by activating the NF-κB pathway through TLR4. IKKα and IKKβ, subunits of the IIKK, are essential for IκB phosphorylation and NF-κB activation (53). The NF-κB family, including RelA/p65, RelB, Rel/c-Rel, p50 (p105/NF-κB1) and p52 (p100/NF-κB2), exert a crucial role in the pathology of the airway by modulating cytokine and chemokine secretion (54). In the present study, it was observed that these exosomes stimulated the increased expression of TLR4, p-IKKα/β and p-p65, and the NF-κB subunit, p65, translocated into the nuclei of BEAS-2B cells to induce cytokine secretion (Fig. 5A-F). However, after treatment with exosomes for 24 h, TLR-4, p-IKK-α/β and p-p65 expression levels were lower than those obtained from treatment with exosomes for 2 and 4 h, possibly because the proinflammatory effect of exosomes co-cultured with cells decreases over time, and the longer the reaction time, the more likely it is that exosomal content loses its activity. Blocking NF-κB and TLR4 downregulated cytokine expression levels (Fig. 6A and B). TAK-242 partially inhibited the TLR4-NF-κB signaling pathway and attenuated the protein expression levels in this pathway (Fig. 6C-F). However, 100 nM TAK-242 partially inhibited the activation of TLR4 and p-IKK-α/β more than 300 nM TAK-242 did, perhaps because volumes of TAK-242 below 100 nM produced a concentration-dependent effect, and volumes above 100 nM were in the plateau phase when interacting on BEAS-2B cells. On the other hand, the amount of TLR4 receptors in bronchial epithelial cells might have been insufficient, and its inhibitory effect may have been attenuated because of the high concentration of TAK-242. Therefore, exosomes from the HDM group could activate the TLR4-NF-κB pathway in BEAS-2B cells and contribute to the inflammatory response.

This research was significantly limited by the lack of exosome samples from the serum of patients with asthma, mainly because it is difficult to obtain serum samples from patients with moderate to severe asthma. Secondly, there is an ethical issue that the consent of patients with asthma is required before the obtained serum-derived exosomal samples are used for research. Thirdly, the extraction of serum-derived exosomes from animal models of asthma and co-culture with BEAS-2B cells are the preliminary experiments of our team. However,

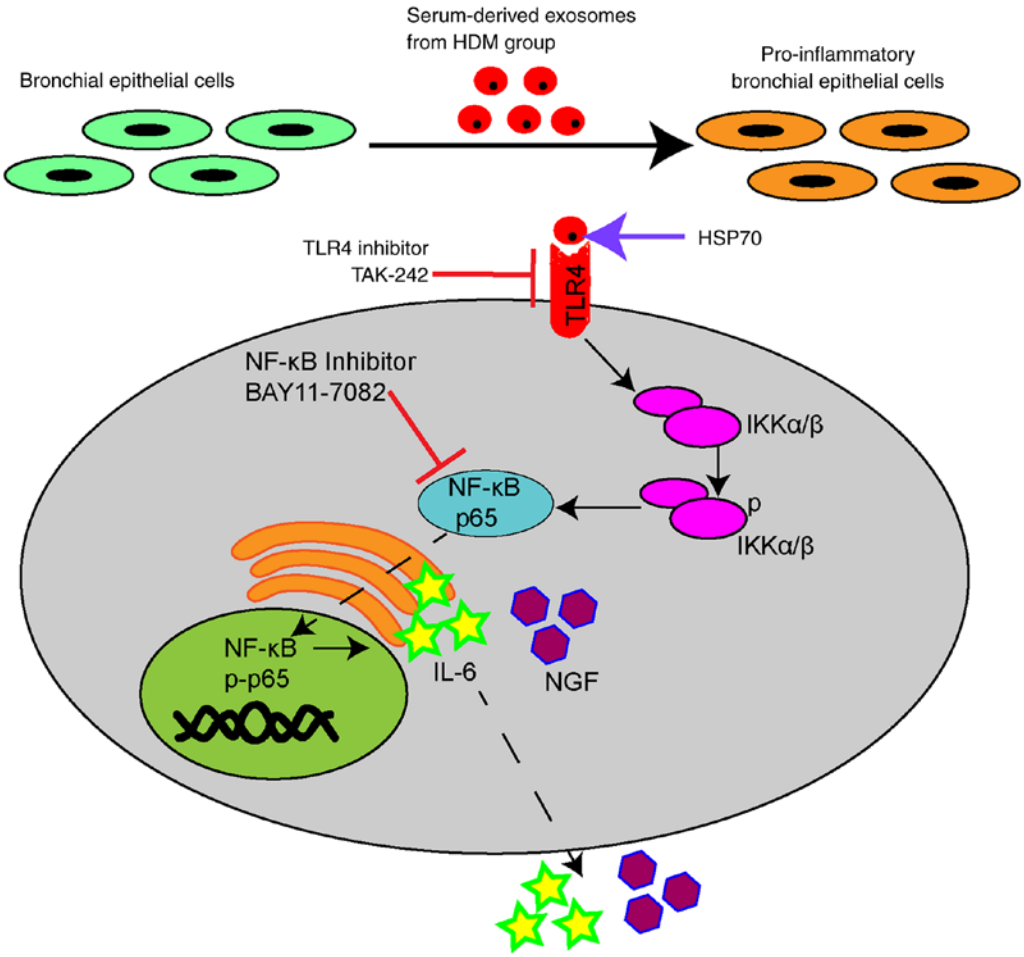

Figure 8. Schematic representation of exosome-mediated inflammatory response in BEAS-2B cells. Serum-derived exosomes from HDM group induces the transformation of bronchial epithelial cells into pro-inflammatory bronchial epithelia by activating the TLR4-NF-κB pathway via HSP70. HDM, house dust mite; IL-6, interleukin-6; NGF, nerve growth factor; TLR4, toll-like receptor 4; HSP70, heat shock protein 70; p-, phosphorylated.

research on the involvement of serum-derived exosomes in the pathogenesis of asthma is far from complete. Previous analysis of the expression of miR-125b in the serum exosomes of patients with different severities of asthma compared with healthy subjects showed an altered miR-125b content, and thus may have potential as a diagnostic marker for asthma (55). Therefore, further research must be conducted to determine whether serum-derived exosomes from asthmatic patients of different severity play a pro-inflammatory role in bronchial epithelial cells or other receptor cells, and this is our next research focus.

Exosomal surface molecules can mediate intracellular signaling pathways through direct contact with receptors on target cells. Anand *et al* (37) demonstrated that exosomal surface HSP70 levels from macrophages infected with mycobacteria are expressed higher than in controls, and HSP70 in exosome-treated macrophages activates NF-κB signaling to stimulate the release of TNF-α in uninfected macrophages. Circulating HSP70 levels from patients with asthma are relevant to the severity of disorders and the symptom of asthma and, therefore, may contribute to the pathogenesis of the disease (56). The present study showed that serum-derived exosomal HSP70 in the HDM group was higher than in the sham group, suggesting that exosomal surface HSP70 may be involved in the pathogenesis process. HSP70 on the surface of serum-derived exosomes from the HDM group could, therefore, alter BEAS-2B cell phenotypic appearances by regulating the TLR4-NF-κB signaling pathway (Fig. 7A-D). A previous study has shown that treatment with proteinase K-digested HSP70 in bone marrow-derived dendritic cells results in a reduction in HSP70-dependent cytokines (57). A previous study reported that the increased secretion of inflammatory factors could be markedly suppressed following the pretreatment of mesenchymal stem cells with proteinase K compared with a A549 exosome-treated group (58). As expected, the results of the current study indicated that the increases in cytokine concentrations were partially repressed by proteinase K and the HSP70 blocker compared with the exosome-treated group without the enzyme. Therefore, it should be considered that other exosomal components, including nucleic acids and other proteins, possibly participate in this inflammatory response and this should be further explored.

In conclusion, it was demonstrated that serum-derived exosomes interacted with BEAS-2B cells and could alter their phenotypic appearance. Additionally, the HSP70-modulated inflammatory effect on the surface of serum-derived exosomes from the HDM group upregulated IL-6 and NGF expression levels by activating the TLR4-NF-κB pathway (Fig. 8). Overall, exosomal presence in HDM-sensitized guinea pigs could be influential in the underlying mechanism of inflammatory airway diseases; however, blocking exosome-mediated

communication between cells would attenuate the inflammation, potentially partially relieving symptoms of inflammatory airway diseases in guinea pigs.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of China (grant nos. 81460004 and 82160006); the Jiangxi Provincial Cultivation Program for Academic and Technical Leaders of Major Subjects (grant no. 20172BCB22025); and the Jiangxi Provincial Natural Science Foundation General Project (grant no. 20202BAB206003).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JW and CL conceived and designed the present study. CL, XLH, XZ, ZFW and LXD performed the experiments. CL, JPL and XLH analyzed the experimental data. CL wrote the manuscript. CL and JW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care and Committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University (approval no. 2021-052; Nanchang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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