



Exosomal long non-coding RNA GAS5 suppresses Th1 differentiation and promotes Th2 differentiation via downregulating EZH2 and T-bet in allergic rhinitis

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ABSTRACT

The imbalance of helper T cell (Th) 1/Th2 differentiation is involved in the development of allergic rhinitis (AR). Recent studies reveal the regulatory function of exosomes on Th1/Th2 differentiation. However, the key mediator in exosomes that modulate such response remains unclear. In this study, the expression of long-noncoding RNA GAS5 (LncGAS5) was detected in exosomes which were isolated from AR patient nasal mucus (AR-EXO) and ovalbumin (OVA)-stimulated nasal epithelial cells (OVA-EXO). Th1/Th2 differentiation was induced in naïve CD4⁺ T cells, and the percentage of IFN- γ expressing cells (Th1 cells) and IL-4 expressing cells (Th2 cells) was detected using flow cytometry. The result showed that LncGAS5 was upregulated in AR epithelial samples, AR-EXO, and OVA-EXO. The coinubation of AR-EXO and CD4⁺ T cells suppressed Th1 differentiation and promoted Th2 differentiation, which is mediated by LncGAS5 in AR-EXO. The LncGAS5 in AR-EXO inhibited transcription and expression of EZH2, and it also inhibited T-bet expression at mRNA and protein levels. The gain-of-function and loss-of-function experiments suggested that LncGAS5 mediates Th1/Th2 differentiation partly through downregulating T-bet and EZH2. In summary, our findings demonstrated that LncGAS5 in AR epithelium-derived exosomes is the key mediator in Th1/Th2 differentiation, providing a possible therapeutic target of AR.

1. Introduction

Allergic rhinitis (AR), which is the most common allergic disease, has an ascending prevalence worldwide. It is reported that the prevalence of AR in US is 19.9 %, with the patient-reported adult AR over 30 % in all age groups, and the physician-diagnosed adult AR over 14 % in the whole American populations (Wallace and Dykewicz, 2017). Numerous studies have shown that the imbalance of immune responses between helper T cells (Th) 1 and Th2 cells is the immunological basis for AR (Haitchi and Holgate, 2004). Th1 cells are characterized by secreting cytokines such as interferon- (IFN-) γ , interleukin- (IL-) 2, IL-12, and tumor necrosis factor- (TNF-) α , which mediate cellular immune responses. Th2 cells are characterized by secreting cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13, which mediate fluid immune responses. Both Th1 and Th2 cells are differentiated by the same precursor cells Th0 cells, which are naïve CD4⁺ T cells (Di Lorenzo et al., 2017). Under physiological conditions, Th1 and Th2 cells regulate each other and maintain a balanced amount. Under pathological conditions, the balance is broken, and the percentage of Th2 cells is increased, while the

percentage of Th1 cells is reduced, causing an abnormal immune response (Kappen et al., 2017). Thus, promoting Th1 differentiation and suppressing Th2 differentiation may contribute to the therapy of AR.

Exosomes are nano-vesicles that are secreted by almost all kinds of cells (Boriachek et al., 2018). Commonly, exosomes are about 30–150 nm in diameter, and have a density of about 1.13–1.19 g/ml (Farooqi et al., 2018). The morphology of exosomes is like a “cup” or “disc” under a transmission electron microscope (Hessvik and Llorente, 2018). By detecting the specific protein markers, including CD9, CD81, CD63, TSG101, and heat shock protein 70 (Hsp70), exosomes can be isolated and identified (Cypriy et al., 2018). The main function of exosomes is “cell-cell communication”, transferring active molecules such as proteins, DNAs, mRNAs, and non-coding RNAs (ncRNAs), from one cell to another (Whiteside, 2018). Recent studies have revealed the effect of exosomes on Th cell differentiation (Zhang et al., 2014). For example, dendritic cell-derived exosomes and B cell-derived exosomes can increase the IL-4 level and promote Th2 differentiation (Huang et al., 2019; Admyre et al., 2007). Secreted by mucosal epithelium, nasal epithelium-derived exosomes are abundant in nasal mucus. Prior

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proteomic analyses of exosomes isolated from nasal mucus have identified multiple proteins that are specifically involved in the immune response in AR (Lasser et al., 2016). However, whether ncRNAs encapsulated by exosomes has an effect on Th cell differentiation remains unknown.

Long non-coding RNAs (lncRNAs) are a subset of ncRNAs longer than 200 nucleotides (Sun et al., 2018). They represent by far the largest fraction of noncoding genes, with estimated numbers in humans ranging from a relatively recent estimate of ~59000 (Iyer et al., 2015) to the NONCODE database listing of 102000 (Zhao et al., 2016). The regulatory functions of lncRNAs on the immune system are partly discovered (Mowel et al., 2018). For example, lncRNA Lnc-DC regulates the differentiation and activation profile of dendritic cells by binding STAT3 and preventing its dephosphorylation (Wang et al., 2014). LncRNA Growth Arrest Specific 5 (LncGAS5), whose gene is located at chromosome 1q25.1, was first discovered in growth-arrested fibroblasts in mice (Coccia et al., 1992). The study conducted by Mourtada-Maarabouni and her colleagues (Mourtada-Maarabouni et al., 2010) indicated that the inhibition of human T-cell proliferation by mTOR antagonists requires LncGAS5, suggesting that LncGAS5 may be involved in the T cell-related immune response. Interestingly, the previous study indicated that LncGAS5 can be encapsulated in exosomes, and exerts cell-cell interacting effect between macrophages and vascular endothelial cells (Chen et al., 2017).

Based on the above information, we hypothesize that under AR conditions, nasal epithelium-derived exosomes encapsulate LncGAS5, and transport them into CD4⁺ T cells, promoting allergic reactions via suppressing Th1 differentiation and increasing Th2 differentiation. To test the hypothesis, we isolated exosomes from AR nasal mucus and ovalbumin (OVA)-stimulated nasal epithelial cells. The result indicated that exosomal LncGAS5 broke the balance of Th1/Th2 partly through inhibiting the expression of T-bet and EZH2 (Enhancer of Zeste Homolog 2).

2. Materials and methods

2.1. Human nasal epithelial samples

AR patients and healthy individuals at a routine medical examination who were admitted in The First Affiliated Hospital of Zhengzhou University were included in this study. Nasal epithelial samples were scraped from the surface of the inferior nasal turbinate of patients with AR (n = 30) and healthy individuals (n = 30) using a plastic curette. The diagnosis of AR was confirmed by otolaryngologists based on the criterion of a typical history of AR over 2 years, with the clinical manifestations, including running nose, positive skin prick test against specific antigens, and serum specific IgE levels greater than 0.3 IU/ml. Individuals who have sinusitis were excluded. The clinical and demographic characteristics of the subjects were shown in Table 1.

Table 1

The clinical and demographic characteristics of the subjects.

Characteristics	Control (n = 30)	AR (n = 30)
Gender (male/female)	14/16	15/15
Age (mean)	26.1	25.3
Eosinophil %	2.18 ± 0.29	3.47 ± 0.38*
Th1/Th2 cell ratio	2.95 ± 0.31	2.03 ± 0.15*
Serum IgE (IU/ml)	39.12 ± 4.36	680.83 ± 82.24**
Serum IL-4 (pg/ml)	1.87 ± 0.16	3.11 ± 0.29**
Serum IL-2 (pg/ml)	2.29 ± 0.33	3.78 ± 0.45*
Serum IL-6 (pg/ml)	3.35 ± 0.21	7.07 ± 1.21*

* P < 0.05.

** P < 0.01.

2.2. Human nasal mucus samples

Nasal mucus samples were collected from the nasal cavity of patients with AR (n = 5) and healthy individuals (n = 5) using polyvinyl alcohol sponge. The sponge was placed in the nasal cavity against the head of the inferior nasal turbinate for 5 min. After the addition of 150 µl of phosphate buffer saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA) with protease inhibitor cocktail (Absin, Shanghai, China), the collected mucus was extracted from the sponge using centrifugation at 1500g at 4 °C for 30 min. After adding another 150 µl of PBS with protease inhibitor cocktail and the second time centrifugation, mucus was collected. Then the supernatant was collected after another centrifugation at 12,000g at 4 °C for 45 min followed by being diluted up to 4.5 ml of PBS in polypropylene tubes.

2.3. Isolation and purification of exosomes from nasal mucus

The isolation of exosomes from healthy mucus (control-EXO) and AR mucus (AR-EXO) were carried out as previously described (Thery et al., 2006). In brief, the supernatant of mucus samples was centrifuged at 12,000g at 4 °C for 45 min, and the supernatant was diluted in PBS in polypropylene tubes followed by the ultracentrifugation at 110,000g at 4 °C for 2 h. Then the pellet was resuspended in 4.5 mL of PBS and filtered through a 0.22 µm filter (Thermo Fisher Scientific) and collected in a fresh tube. The filtered suspension was ultracentrifuged at 110,000g at 4 °C for 70 min. Then the supernatant was collected, and the pellet containing exosomes was resuspended in the buffer.

2.4. Identification of exosomes

A total of 10 µl PBS was added into an equal volume of isolated exosomes to identify the exosomes. The sample was added dropwise to a 2 mm copper mesh. After keeping at room temperature for 1 min, the excess liquid was gently removed followed by the staining using 3 % (w/v) sodium phosphotungstate solution for 1 min at room temperature. Then the sample was gently washed and air-dried at room temperature. The shape of exosomes was observed under a transmission electron microscope (HT7830, HITACHI, Ltd., Tokyo, Japan). Western blot analysis was used to detect the expression of exosome markers CD63 (Rabbit Anti-CD63 antibody, ab217345, Abcam, Cambridge, UK) and CD81 (Mouse Anti-CD81 antibody, ab79559, Abcam).

2.5. Isolation of epithelium-derived exosomes and OVA exposure study

Human nasal epithelial cell line RPMI 2650 (CCL-30) was bought from the American Type Culture Collection (ATCC). Cells were cultured in the Eagle's Minimum Essential Medium (EMEM, ATCC, No. 30-2003) supplemented with 10 % fetal bovine serum (FBS) which was exosome depleted. To induce allergic reactions, RPMI 2650 cells were treated with ovalbumin (OVA, Grade V, Sigma-Aldrich, St Louis, MO, USA) at a dose of 2 mg/ml. Then the epithelium-derived exosomes were isolated from the OVA-treated RPMI 2650 cells (OVA-EXO) and untreated RPMI 2650 cells (EXO) as described above.

2.6. Quantitative real-time PCR (qRT-PCR)

Cells were lysed with TRIzol reagent (Invitrogen, Waltham, MA, USA), and the total RNA was extracted in accordance with the manufacturer's instructions. Reverse transcription was conducted using the PrimeScript RT reagent Kit (Takara, Dalian, China) using 0.1 µg of RNA, and the subsequent Real-time Quantitative PCR analysis was performed on an Applied Biosystems PRISM 7500 Sequence System (Thermo Fisher Scientific). GAPDH was used as the internal control. The relative expression of RNAs was calculated by $2^{-\Delta\Delta CT}$ or $2^{-\Delta CT}$ (Tong et al., 2014).

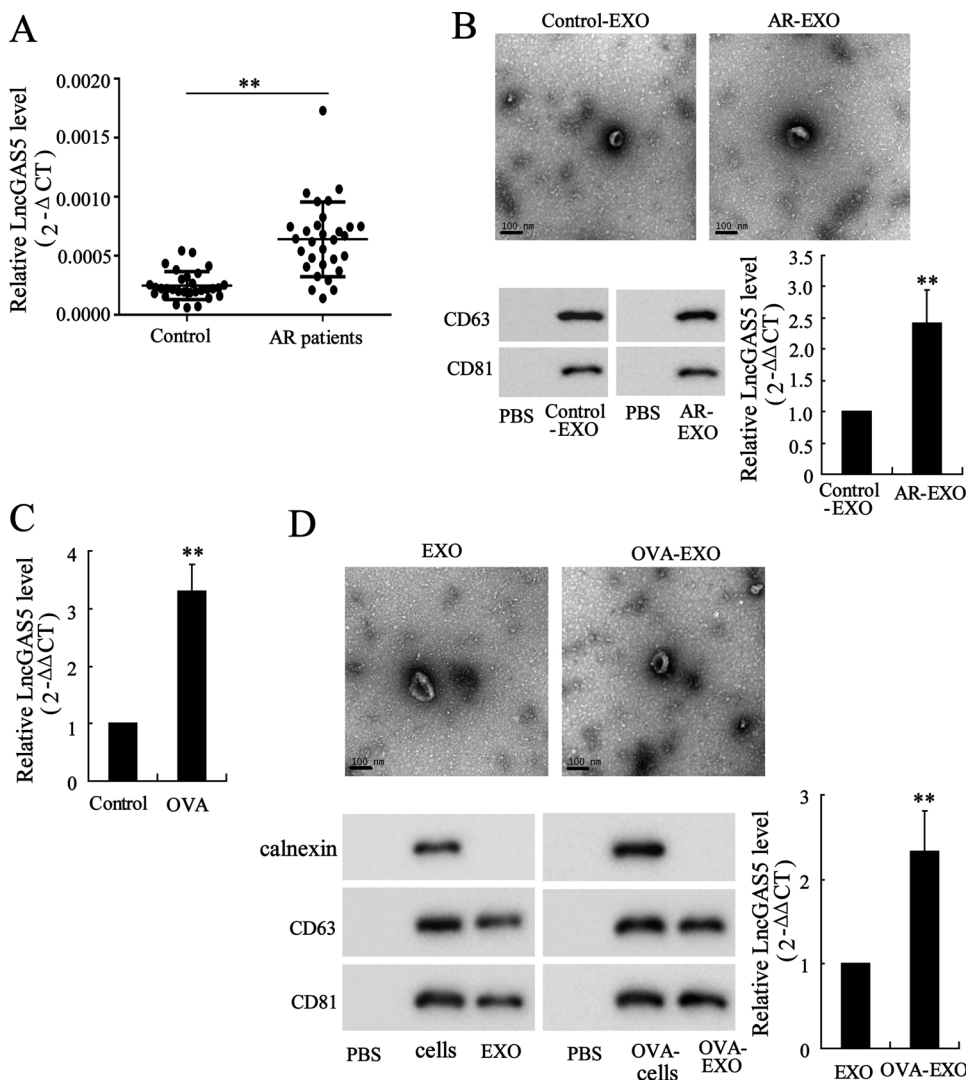


Fig. 1. LncGAS5 is upregulated in AR-EXO and OVA-EXO. (A) The different expressions of LncGAS5 in nasal epithelial samples were compared between AR patients ($n = 30$) and healthy controls ($n = 30$) using qRT-PCR. (B) The exosomes were respectively isolated from nasal mucus samples of AR patients ($n = 5$), named as the AR-EXO, and healthy controls ($n = 5$), named as the control-EXO, followed by the observation under the transmission electron microscope. The expression of exosome markers, CD63 and CD81, was determined using western blot analysis. The LncGAS5 expression in the AR-EXO and control-EXO was determined using qRT-PCR. (C) The differential expression of LncGAS5 was compared between human epithelial cells (RPMI 2650) with or without ovalbumin (OVA) simulation using qRT-PCR. (D) The exosomes were respectively isolated from OVA-induced RPMI 2650 cells, named as the OVA-EXO, and OVA-untreated cells, named as the EXO, followed by the observation under the transmission electron microscope. The expression of exosome markers, CD63 and CD81, and the endoplasmic reticulum marker, calnexin, were determined using western blot analysis. The LncGAS5 expression in OVA-EXO and EXO was determined using qRT-PCR. ** $p < 0.01$ vs Control or Control-EXO. The scale bar represents 100 nm. Values are presented as means $\pm$ SD and are representative of three individual experiments.

2.7. Western blot analysis

The cells were incubated in cell lysis buffer containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) to obtain protein extracts. The total protein content present was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). An equal concentration of the protein samples (50 μ g) was loaded on to 10%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and subjected to electrophoresis. Then the bands were transferred onto a polyvinylidene difluoride membrane (Millipore, Temecula, CA, USA) followed by being blocked with 5 % non-fat dry milk and incubated with primary antibodies [T-bet (ab91109), GATA-3 (ab199428), GAPDH (ab181602), and EZH2 (ab186006), all from Abcam] overnight at 4°C. The membranes were further incubated with HRP-bound peroxidase-conjugated secondary antibodies (ab205718, Abcam) at room temperature for 1 h. The bands were visualized and analyzed using a chemiluminescence system (Amersham Bioscience, Buckinghamshire, UK).

2.8. Isolation of CD4⁺ T cells and induction of differentiation

CD4⁺ T cells were isolated from peripheral blood mononuclear cells (PBMCs) which were derived from peripheral blood of AR patients by MACS following the manufacturer's instructions and labeled with carboxyfluorescein succinimidyl ester (CFSE). The induction of Th1

differentiation was performed by incubating CD4⁺ T cells with anti-CD3 (3 μ g/ml), anti-CD28 (5 μ g/ml), IL-12 (50 U/ml), and anti-IL-4 (10 mg/ml), as well as co-incubating with the control-EXO or AR-EXO for 7 d. The induction of Th2 differentiation was performed by incubating CD4⁺ T cells with anti-CD3 (3 μ g/ml), anti-CD28 (5 μ g/ml), IL-4 (20 ng/ml), anti-IFN- γ (20 μ g/ml), and IL-2 (2 ng/ml), as well as co-incubating with the control-EXO or AR-EXO for 7 d.

2.9. Identification of IFN- γ or IL-4 expressing cells

Flow cytometry was used to identify the percentage of cells which express IFN- γ or IL-4. In brief, cells were fixed with 2 % paraformaldehyde for 2 h at room temperature followed by the incubation with 0.5 % saponin for 30 min to increase the permeability of the cell membrane. After washing with PBS, the cells were incubated with FITC-labeled antibodies (anti-IFN- γ or anti-IL-4) for 1 h. The stained cells were analyzed with a flow cytometer (FACSCanto II; BD Biosciences, San Jose, CA, USA).

2.10. Enzyme-linked immunosorbent assay (ELISA)

Quantitative analysis of IFN- γ and IL-4 in cell culture supernatants was performed using ELISA procedures using Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

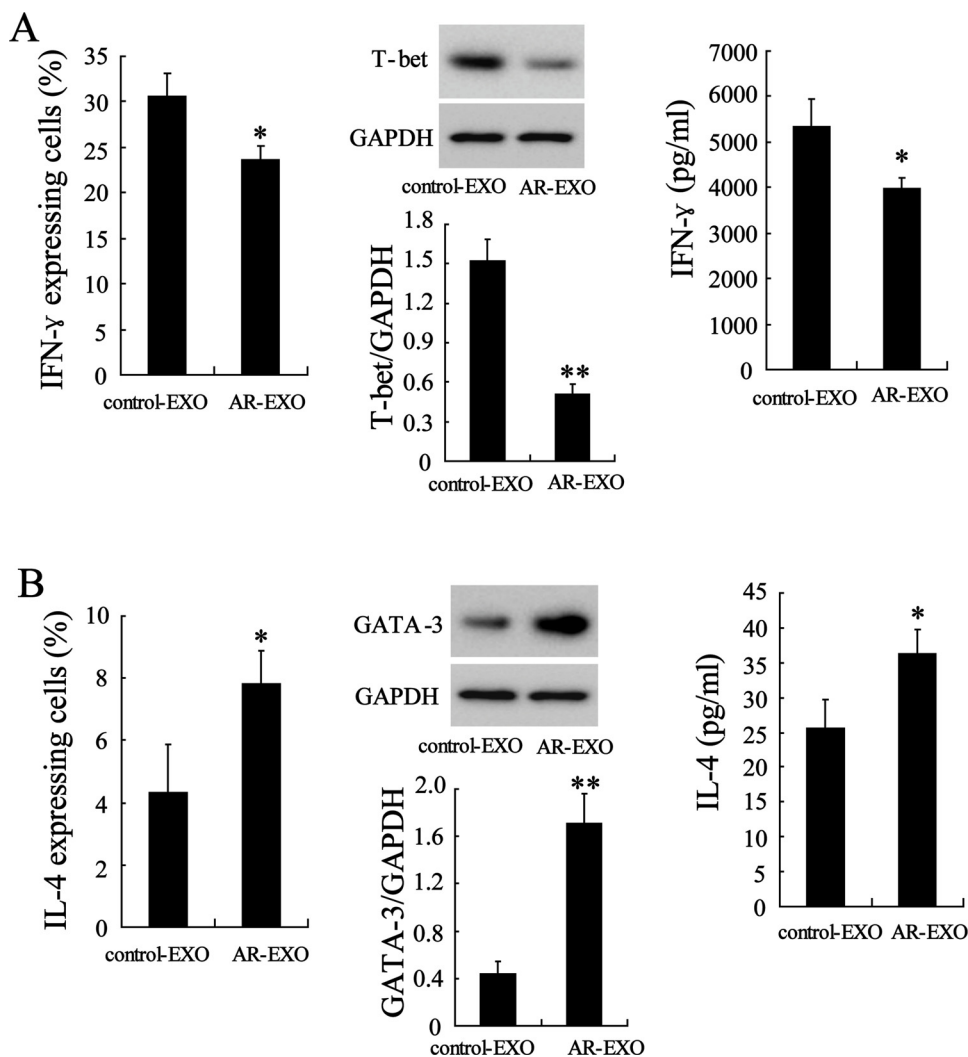


Fig. 2. Effect of AR-EXO coinubation on Th1/Th2 differentiation of CD4⁺ T cells. (A) The Th1 differentiation was induced in CD4⁺ T cells, and the AR-EXO or control-EXO was added for the 7-day coinubation. The percentage of IFN- γ expressing cells, T-bet protein level, and IFN- γ level in cell incubation supernatants were determined using flow cytometry, western blot analysis, and ELISA, respectively. (B) The Th2 differentiation was induced in CD4⁺ T cells, and the AR-EXO or control-EXO was added for the 7-day coinubation. The percentage of IL-4 expressing cells, GATA-3 protein level, and IL-4 level in cell incubation supernatants were determined using flow cytometry, western blot analysis, and ELISA, respectively. * $p < 0.05$, ** $p < 0.01$ vs control-EXO. Values are presented as means $\pm$ SD and are representative of three individual experiments.

2.11. Interfering LncGAS5 in OVA-EXO and CD4⁺ T cells

The short hairpin RNA (shRNA) against LncGAS5 was used to interfere the expression of LncGAS5. The shRNA against LncGAS5 (shRNA-LncGAS5) and its control (shRNA) were bought from Genechem (Shanghai, China). The RPMI 2650 cells were transiently transfected with shRNA-LncGAS5 or shRNA using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) for 48 h followed by the OVA stimulation. Then the exosomes (OVA-EXO^{shRNA-LncGAS5} and OVA-EXO^{shRNA}) were isolated as described above, respectively. The CD4⁺ T cells were transfected with lentivirus carrying the shRNA against LncGAS5 (shRNA-LncGAS5) and its control (shRNA) to downregulate LncGAS5 expression. The CD4⁺ T cells were also transfected with lentivirus carrying the shRNA against EZH2 or T-bet (shRNA-EZH2 or shRNA-T-bet) and its control (shRNA) to downregulate EZH2 or T-bet expressions before induction of differentiation. The 100 nmol/L shRNAs were used.

2.12. Luciferase reporter assay

293A cells were transfected with the luciferase reporter plasmids pGL3-EZH2 which contain the EZH2 promoter sequences that could bind with LncGAS5. Promoter constructs were generated by cloning the region of the human EZH2 promoter from -1702 to +52 and inserting it between the SacI and XhoI restriction sites of the pGL3-Basic vector. The luciferase reporter assay was conducted using the Luciferase Reporter Assay System (Promega, Madison, WI, USA) in accordance

with the manufacturer's instructions.

2.13. Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical differences between two groups were assessed using two-tailed Student's T-test. The comparisons among multiple groups were done using one-way analysis of variance (ANOVA), followed by Tukey post hoc testing. All the data were analyzed using SPSS 18 software (IBM). The difference at $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. LncGAS5 is upregulated in AR-EXO and OVA-EXO

The different expressions of LncGAS5 in nasal epithelial samples were compared between AR patients ($n = 30$) and healthy controls ($n = 30$). The result indicated that LncGAS5 was significantly upregulated in AR samples in comparison with healthy samples (Fig. 1A, $p < 0.01$). Then the exosomes were respectively isolated from nasal mucus samples of AR patients ($n = 5$), named as the AR-EXO, and healthy controls ($n = 5$), named as the control-EXO. The nano-vesicles with a diameter of 30–150 nm were seen in both groups under the transmission electron microscope, with the expression of exosome markers, CD63 and CD81 compared to PBS alone, and the quantitative analysis indicated that LncGAS5 expression in the AR-EXO was higher

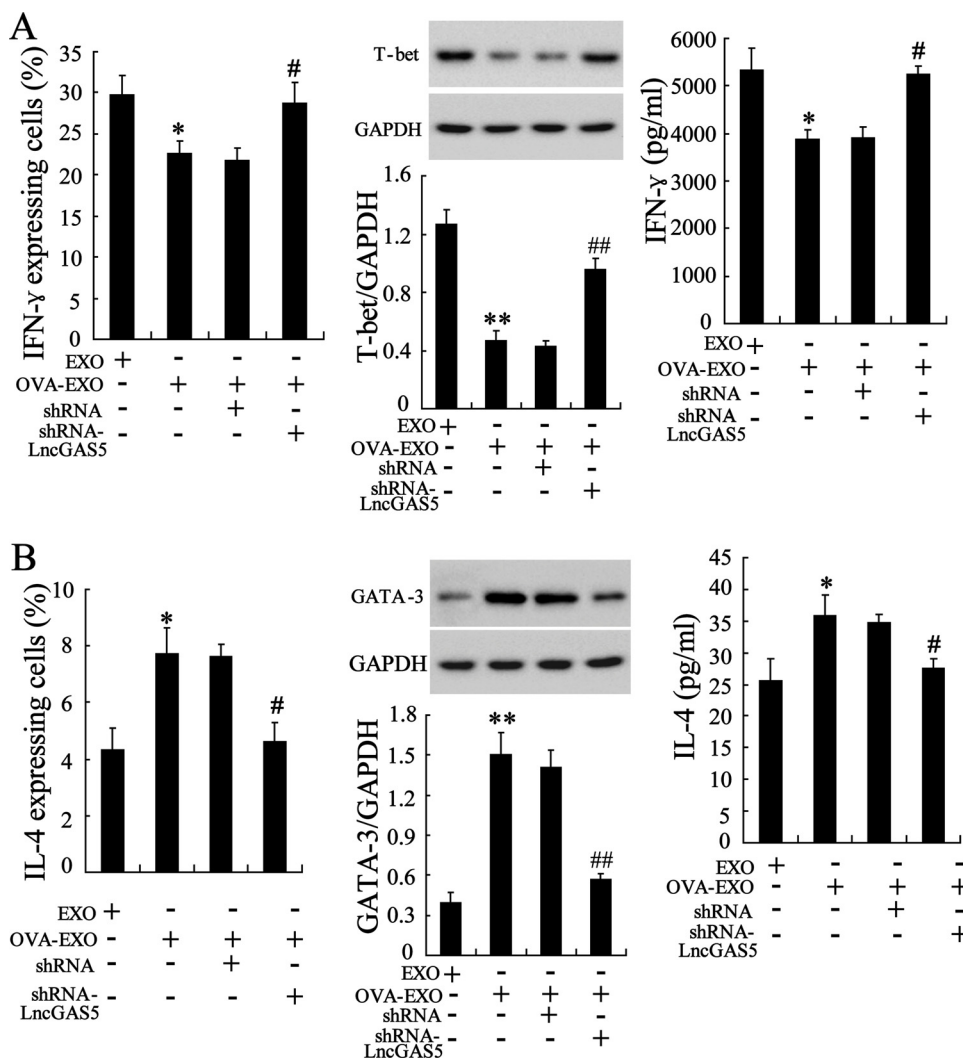


Fig. 3. LncGAS5 in OVA-EXO suppresses Th1 differentiation and promotes Th2 differentiation. (A) The Th1 differentiation was induced in CD4⁺ T cells, and the OVA-EXO^{shRNA-LncGAS5} or control (OVA-EXO^{shRNA}) was added for the 7-day co-cultivation. The percentage of IFN-γ expressing cells, T-bet protein level, and IFN-γ level in supernatants were determined respectively. (B) The Th2 differentiation was induced in CD4⁺ T cells, and the OVA-EXO^{shRNA-LncGAS5} or control (OVA-EXO^{shRNA}) was added for the 7-day co-cultivation. The percentage of IL-4 expressing cells, GATA-3 protein level, and IL-4 level in supernatants were determined respectively. *p < 0.05, **p < 0.01 vs EXO. #p < 0.05, ##p < 0.01 vs OVA-EXO^{shRNA}. OVA-EXO^{shRNA-LncGAS5} and OVA-EXO^{shRNA}, RPMI 2650 cells were transfected with shRNA-LncGAS5 or shRNA followed by the OVA stimulation, and the exosomes were isolated. Values are presented as means ± SD and are representative of three individual experiments.

than that in the control-EXO (Fig. 1B).

The differential expression of LncGAS5 was also compared between human epithelial cells (RPMI 2650) with or without OVA stimulation, which can induce allergic reactions. The result indicated that LncGAS5 was significantly upregulated in OVA-induced RPMI 2650 cells in comparison with OVA-untreated cells (Fig. 1C, $p < 0.01$). Then the exosomes were respectively isolated from OVA-induced RPMI 2650 cells, named as the OVA-EXO, and OVA-untreated cells, named as the EXO. The nano-vesicles with a diameter of 30–150 nm were seen in both groups under the transmission electron microscope, with the expression of CD63 and CD81 compared to PBS alone, and lack of endoplasmic reticulum marker (calnexin) compared to cell lysates, and the quantitative analysis indicated that LncGAS5 expression in the OVA-EXO was higher than that in the EXO (Fig. 1D). These data indicated that LncGAS5 was upregulated in the exosomes from mucus after the stimulation of allergic reactions.

3.2. AR-EXO co-cultivation suppresses Th1 differentiation and promotes Th2 differentiation

To explore the effect of AR-EXO on Th cell differentiation, the Th1 differentiation was induced in CD4⁺ T cells, and the AR-EXO or control-EXO was added for the 7-day co-cultivation. The result showed that AR-EXO reduced the percentage of IFN-γ expressing cells, reduced T-bet protein level, and reduced IFN-γ level in cell incubation supernatants (Fig. 2A). Meanwhile, the Th2 differentiation was induced in CD4⁺ T

cells, and the AR-EXO or control-EXO was added for the 7-day co-cultivation. The result showed that AR-EXO increased the percentage of IL-4 expressing cells, increased GATA-3 protein level, and increased IL-4 level in cell incubation supernatants (Fig. 2B). These data suggested that AR-EXO co-cultivation suppresses Th1 differentiation and promotes Th2 differentiation of CD4⁺ T cells.

3.3. LncGAS5 in OVA-EXO suppresses Th1 differentiation and promotes Th2 differentiation

Given that the promotion of Th2 differentiation was associated with AR-EXO co-cultivation, and that LncGAS5 was significantly upregulated in AR-EXO and OVA-EXO, we further investigated whether LncGAS5 in exosomes is involved in Th2 differentiation. The interference of LncGAS5 was conducted in OVA-EXO using shRNA against LncGAS5, and qRT-PCR confirmed the downregulation of LncGAS5 in OVA-EXO^{shRNA-LncGAS5} (Supplemental Fig. 1A). Then the Th1 differentiation was induced in CD4⁺ T cells, and the OVA-EXO^{shRNA-LncGAS5} or control (OVA-EXO^{shRNA}) were added for the 7-day co-cultivation. The result showed that OVA-EXO^{shRNA-LncGAS5} restored the percentage of IFN-γ expressing cells, T-bet protein level, and IFN-γ level in the supernatants, which were reduced by OVA-EXO co-cultivation (Fig. 3A). Meanwhile, the Th2 differentiation was induced in CD4⁺ T cells, and the OVA-EXO^{shRNA-LncGAS5} or control (OVA-EXO^{shRNA}) was added for the 7-day co-cultivation. The result showed that OVA-EXO^{shRNA-LncGAS5} reduced the enhancement of percentage of IL-4 expressing cells, GATA-3 protein

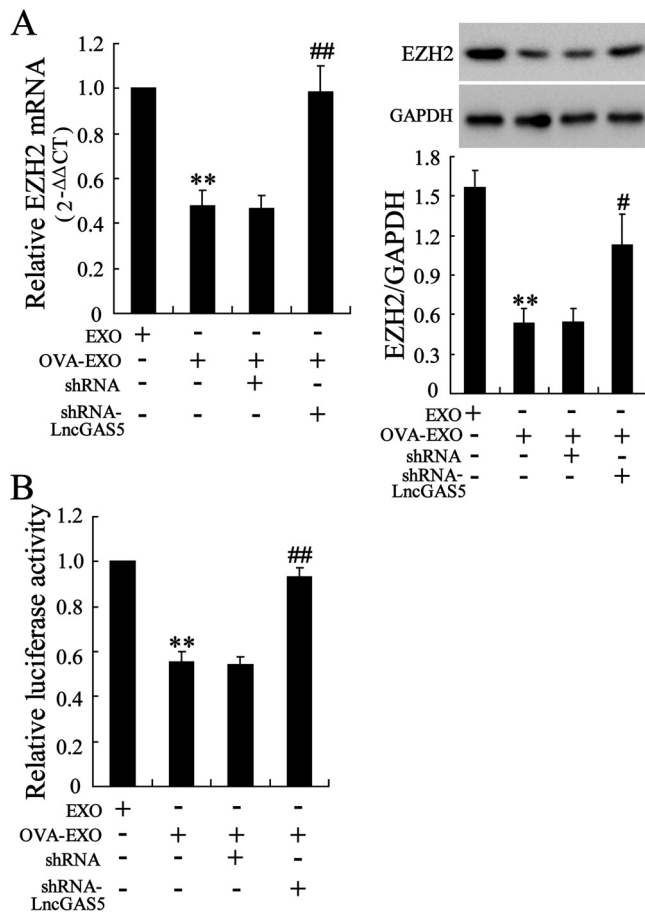


Fig. 4. LncGAS5 in OVA-EXO inhibits the transcription and expression of EZH2. The Th1 differentiation was induced in CD4⁺ T cells, and the OVA-EXO^{shRNA-LncGAS5} or control (OVA-EXO^{shRNA}) was added for the 7-day co-cubation. (A) The mRNA and the protein level of EZH2 were determined using qRT-PCR and western blot analysis. (B) The transcriptional activity of EZH2 was determined using luciferase reporter assay. ***p* < 0.01 vs EXO. #*p* < 0.05, ##*p* < 0.01 vs OVA-EXO^{shRNA}. OVA-EXO^{shRNA-LncGAS5} and OVA-EXO^{shRNA}, RPMI 2650 cells were transfected with shRNA-LncGAS5 or shRNA followed by the OVA stimulation, and the exosomes were isolated. Values are presented as means ± SD and are representative of three individual experiments.

level, and IL-4 level in the supernatants, which were raised by OVA-EXO co-cubation (Fig. 3B). These findings indicated that LncGAS5 in OVA-EXO suppresses Th1 differentiation and promotes Th2 differentiation.

3.4. LncGAS5 in OVA-EXO inhibits the transcription and expression of EZH2

Given that LncGAS5 in OVA-EXO reduced protein level of T-bet, we assumed that LncGAS5 might regulate T-bet via EZH2. To verify this hypothesis, the Th1 differentiation was induced in CD4⁺ T cells, and the OVA-EXO^{shRNA-LncGAS5} or control (OVA-EXO^{shRNA}) was added for the 7-day co-cubation. The result showed that OVA-EXO co-cubation reduced the mRNA and the protein level of EZH2, and OVA-EXO^{shRNA-LncGAS5} co-cubation negated such response (Fig. 4A). Meanwhile, OVA-EXO co-cubation reduced the transcriptional activity of EZH2, which was negated by OVA-EXO^{shRNA-LncGAS5} co-cubation, as shown by the modifications in the luciferase activity (Fig. 4B). Our findings indicated that LncGAS5 in OVA-EXO inhibited the transcription and expression of EZH2.

3.5. LncGAS5 in OVA-EXO downregulates T-bet at mRNA and protein levels

To elucidate the effect of LncGAS5 on T-bet expression, the Th1 differentiation was induced in LncGAS5-interfering CD4⁺ T cells, and the OVA-EXO^{shRNA-LncGAS5} or control (OVA-EXO^{shRNA}) was added for the 7-day co-cubation. The result showed that OVA-EXO co-cubation reduced the mRNA level of T-bet, and OVA-EXO^{shRNA-LncGAS5} co-cubation negated such response (Fig. 5A). Meanwhile, OVA-EXO co-cubation reduced the protein level of T-bet, and OVA-EXO^{shRNA-LncGAS5} co-cubation negated such response (Fig. 5B). In addition, MG132 (10 μmol/l), the proteasome inhibitor, was added to the co-cubation medium for 4 h to inhibit the degradation of T-bet via the proteasome degradation pathway. The addition of MG132 increased the protein expression of T-bet (Fig. 5B), suggesting the T-bet degradation was partly proteasome-dependent. However, such enhancement of T-bet protein expression was not affected by the addition of OVA-EXO^{shRNA-LncGAS5}, indicating that LncGAS5 did not affect the synthesis of T-bet protein.

3.6. LncGAS5 in OVA-EXO suppresses Th1 differentiation and promotes Th2 differentiation via regulating EZH2 and T-bet

The Th1 or Th2 differentiation was induced in CD4⁺ T cells, and the exosomes were added for the 7-day co-cubation. The co-cubation was conducted in 4 different groups: CD4⁺ T + OVA-EXO^{shRNA}, CD4⁺ T + OVA-EXO^{shRNA-LncGAS5}, CD4⁺ T^{shRNA} + OVA-EXO^{shRNA-LncGAS5}, and CD4⁺ T^{shRNA-EZH2} + OVA-EXO^{shRNA-LncGAS5}. The reduction of both mRNA and protein levels of EZH2 after knocking down EZH2 in CD4⁺ T cells was confirmed in Supplemental Fig. 1B. CD4⁺ T^{shRNA-EZH2} reduced the enhancement of percentage of IFN-γ expressing cells, T-bet protein level, and IFN-γ level in the supernatants, which were raised by OVA-EXO^{shRNA-LncGAS5} co-cubation (Fig. 6A). CD4⁺ T^{shRNA-EZH2} also restored the percentage of IL-4 expressing cells, GATA-3 protein level, and IL-4 level in the supernatants, which were reduced by OVA-EXO^{shRNA-LncGAS5} co-cubation (Fig. 6B). Meanwhile, the knockdown of EZH2 reduced both mRNA and protein levels of T-bet (Supplemental Fig. 1B).

Similar experiments were also conducted in T-bet-interfering CD4⁺ T cells. The co-cubation was conducted in 4 different groups: CD4⁺ T + OVA-EXO^{shRNA}, CD4⁺ T + OVA-EXO^{shRNA-LncGAS5}, CD4⁺ T^{shRNA} + OVA-EXO^{shRNA-LncGAS5}, and CD4⁺ T^{shRNA-T-bet} + OVA-EXO^{shRNA-LncGAS5}. The reduction of both mRNA and protein levels of T-bet after knocking down T-bet in CD4⁺ T cells was confirmed in Supplemental Fig. 1C. CD4⁺ T^{shRNA-T-bet} reduced the enhancement of percentage of IFN-γ expressing cells, T-bet protein level, and IFN-γ level in the supernatants, which were raised by OVA-EXO^{shRNA-LncGAS5} co-cubation (Fig. 6C). CD4⁺ T^{shRNA-T-bet} also restored the percentage of IL-4 expressing cells, GATA-3 protein level, and IL-4 level in the supernatants, which were reduced by OVA-EXO^{shRNA-LncGAS5} co-cubation (Fig. 6D). Taken together, our findings demonstrated that LncGAS5 in OVA-EXO suppresses Th1 differentiation and promotes Th2 differentiation via regulating EZH2 and T-bet.

4. Discussion

Extracellular vesicles, a heterogeneous group of membrane-enclosed carriers of biomolecules, refer to the pool of vesicles ranging in size from 30–1000 nm, including exosomes (derived from endosomal compartment), ectosomes (micro vesicles budding from plasma membrane) and apoptotic bodies (fragments of apoptotic cells) (Cypryk et al., 2018). Among them, the exosomes, which are less than 150 nm in diameter, were proposed to eliminate waste products initially (Johnstone et al., 1987). In recent years, exosomes are suggested to play a critical role in cell-cell communication, and are related to a large number of pathological and physiological functions, including adaptive

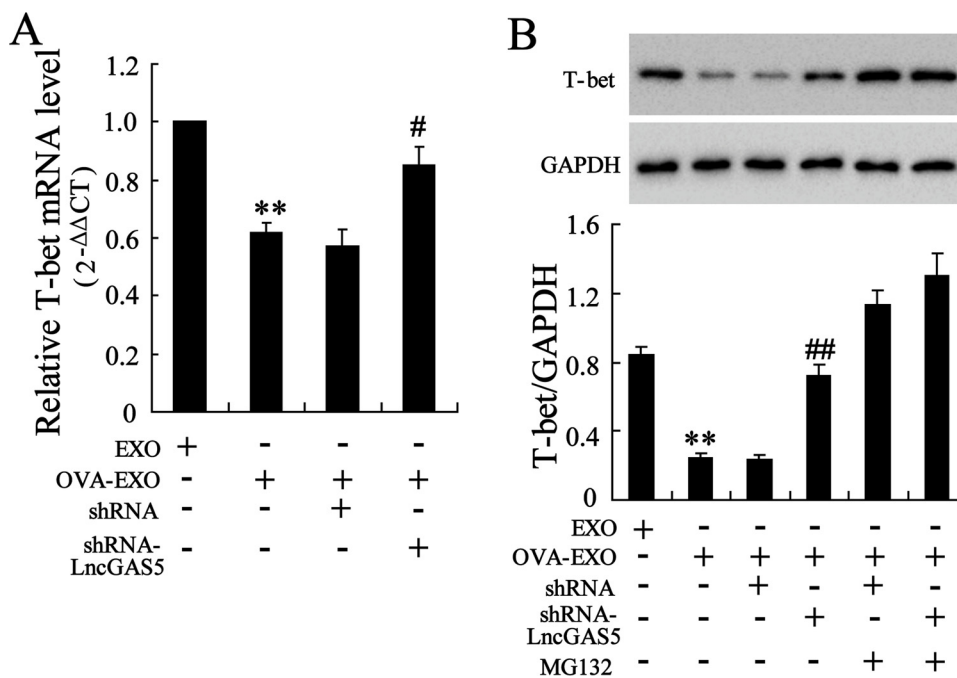


Fig. 5. LncGAS5 in OVA-EXO downregulates T-bet at mRNA and protein levels. RPMI 2650 cells were transfected with shRNA-LncGAS5 or shRNA followed by the OVA stimulation, and the exosomes (OVA-EXO^{shRNA-LncGAS5} and OVA-EXO^{shRNA}) were isolated. The Th1 differentiation was induced in LncGAS5-interfering CD4⁺ T cells, and the OVA-EXO^{shRNA-LncGAS5} or OVA-EXO^{shRNA} was added for the 7-day coincubation. (A) The mRNA level of T-bet was detected using qRT-PCR. (B) MG132 (10 μmol/L), the proteasome inhibitor, was added to the coincubation medium for 4 h to inhibit the degradation of T-bet via the proteasome degradation pathway. The protein level of T-bet was determined using western blot analysis. **p < 0.01 vs EXO. #p < 0.05, ##p < 0.01 vs OVA-EXO^{shRNA}. Values are presented as means ± SD and are representative of three individual experiments.

immune response (Record et al., 2014). As an allergic disease, AR is closely associated with dysregulated adaptive immune response, mainly referring to the imbalance of Th1 and Th2 cells. Th1 cells are characterized by secreting cytokines such as IFN-γ, and synthesizing specific protein like T-bet; while Th2 cells are characterized by secreting cytokines such as IL-4, and synthesizing specific protein like GATA-3 (Zhang et al., 2014). Notably, exosomes have been reported to participate in polarized differentiation of naïve CD4⁺ T cells into Th1/Th2 cells (Zhang et al., 2014). Huang et al. (2019) demonstrated that the exosomes derived from thymic stromal lymphopoietin-induced dendritic cells promote proliferation of CD4⁺ T cells, elevate the level of IL-4, and promote Th2 differentiation. Admyre et al. (2007) suggested that B cell-derived exosomes induce T-cell proliferation and Th2-like cytokine production. In the present study, the immunoregulatory effect of exosomes in AR was indicated. Specifically, the coincubation of exosomes, which were isolated from AR nasal mucus and from OVA-stimulated epithelial cells, and CD4⁺ T cells significantly reduced the percentage of Th1 cells and increased the percentage of Th2 cells, suggesting the vital role of exosome-mediated immune response in AR. In addition, our study revealed that LncGAS5 in epithelium-derived exosomes is the important mediator of cell-cell communication between epithelial cells and CD4⁺ T cells.

LncGAS5 is a growth suppressor that is up-regulated when cell growth inhibition is induced by starvation or rapamycin in T cells (Liu et al., 2015). Many studies have shown that LncGAS5 mainly acts as a tumor suppressor in regulating proliferation, apoptosis, migration, and invasion of tumor cells (Lu et al., 2019). The relationship between LncGAS5 and immune regulation has been discovered recently (Mayama et al., 2016). Sun et al. (2017) suggested that LncGAS5 inhibits the polarization of M2 macrophages and regulates autoimmune responses. Mourtada-Maarabouni et al. (2010) indicated that the inhibition of human T-cell proliferation by mTOR antagonists requires LncGAS5, suggesting that LncGAS5 regulates adaptive immune responses. Consistent with their study, our results showed that the up-regulation of LncGAS5 markedly suppresses Th1 differentiation and promotes Th2 differentiation, revealing the immunoregulatory function of LncGAS5 in AR. In addition, our findings proposed that such regulation is partly mediated by suppressing the expression of T-bet and EZH2.

EZH2 is a major component of polycomb repression complex 2,

which works with other components to carry out the histone methylation modification (Brand et al., 2019). As reported, EZH2 regulates the proliferation and differentiation of various kinds of cells such as hematopoietic stem cells, B lymphocytes and thymocytes. Also, it mediates the proliferation and apoptosis of allogeneic T cells (Guo et al., 2018; Chen et al., 2019). The function of inhibiting gene transcription of EZH2 is carried out by catalyzing the trimethylation of lysine at position 27 of histone 3 (H3K27me3) (Jacobsen et al., 2017). Except for the transcriptional inhibition function, recent studies also showed the transcriptional activation function of EZH2. Tong et al. (2014) found that EZH2 binds to and activates the promoter of T-bet gene, thereby up-regulating T-bet expression at the transcriptional level. They also found that EZH2 prevents the degradation of T-bet via the proteasome pathway, thereby upregulating T-bet at the post-translational level, and activating Th1 differentiation (Tong et al., 2014). In our work, we explored the effect of LncGAS5 on regulating EZH2 expression. The result showed that LncGAS5 in OVA-EXO inhibits the mRNA and protein levels of EZH2. In addition, the gain-of-function and loss-of-function experiments suggested that LncGAS5 mediates Th1/Th2 differentiation partly through EZH2. The specific regulatory mechanism between LncGAS5 and EZH2 needs further investigations.

T-bet is specifically expressed in Th1 cells, which promotes the differentiation of naïve CD4⁺ T cells into Th1 cells and positively regulates the expression of IFN-γ (Agnello et al., 2003). The high expression of T-bet is accompanied with the increase of Th1 specific cytokines, such as IFN-γ, IL-2, IL-12, and TNF-α. Except for inducing Th1 differentiation, T-bet also inhibits Th2 differentiation. Studies have shown that when the T-bet gene is weakened, the production of cytokines like IFN-γ is reduced in CD4⁺ T cells, and the Th2 differentiation is promoted, thus causing spontaneous asthma-like changes; while when the T-bet gene is completely deleted, the Th1 differentiation is totally inhibited, and only Th2 differentiation is conducted in CD4⁺ T cells (Oh and Hwang, 2014; Gagliani and Huber, 2017). Meanwhile, when the T-bet gene is introduced into Th2 cells, Th2 cells can still be reversed to Th1 cells even in a Th2 differentiation-induced medium (Hall, 2015). These data demonstrated that T-bet expression directly affects the differentiation of CD4⁺ T cells into Th1/Th2 cells. Therefore, T-bet has become a target for regulating Th1/Th2 differentiation and developing new medications. In the current study, we investigated the effect of LncGAS5 on regulating T-bet expression. The result showed

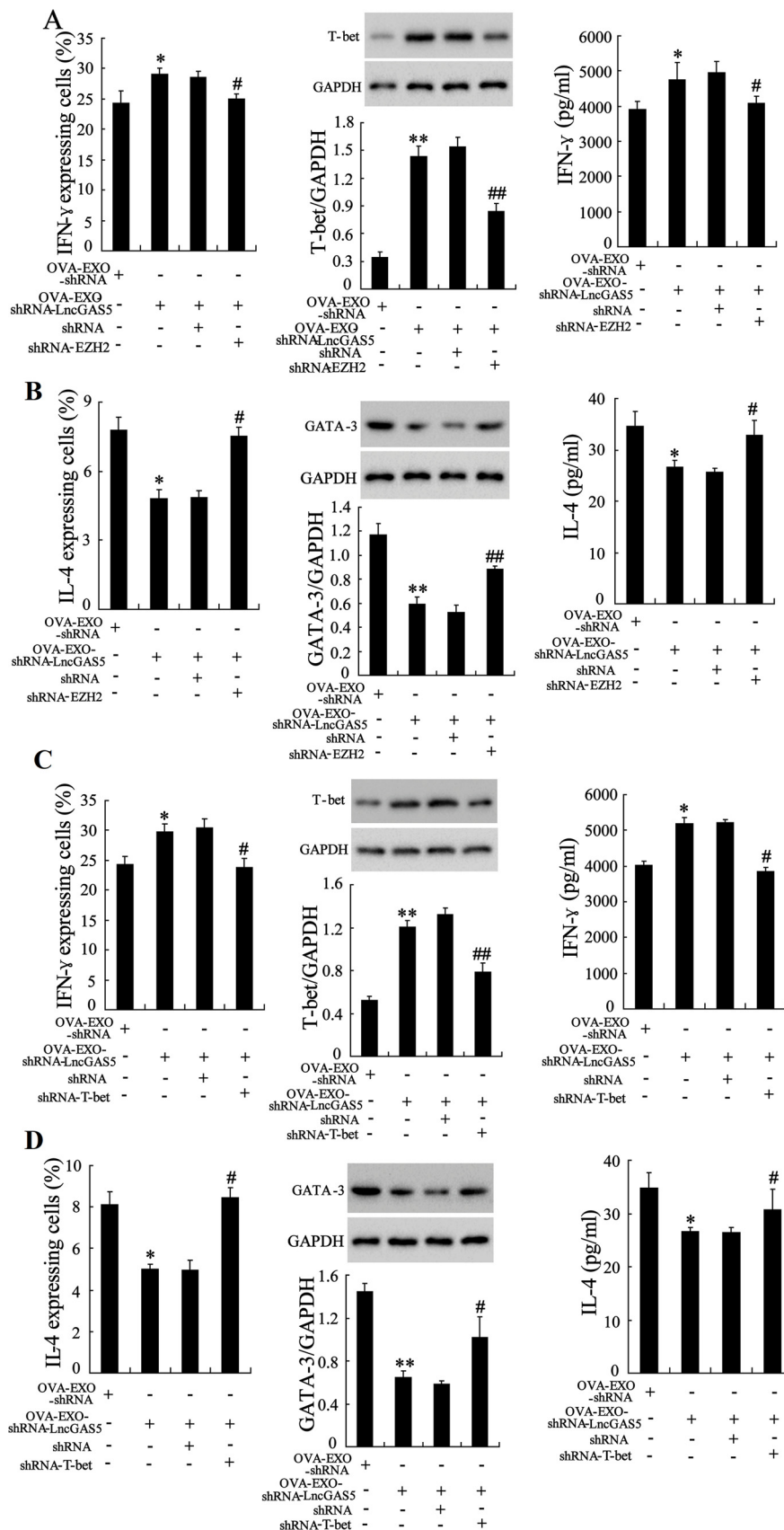


Fig. 6. LncGAS5 in OVA-EXO suppresses Th1 differentiation and promotes Th2 differentiation via regulating EZH2 and T-bet. The Th1 or Th2 differentiation was induced in CD4⁺ T cells, and the exosomes were added for the 7-day coincubation. The coincubation was conducted in 4 different groups: CD4⁺ T + OVA-EXO-shRNA, CD4⁺ T + OVA-EXO-shRNA-LncGAS5, CD4⁺ T-shRNA + OVA-EXO-shRNA-LncGAS5, and CD4⁺ T-shRNA-EZH2 + OVA-EXO-shRNA-LncGAS5. (A) The percentage of IFN- γ expressing cells, T-bet protein level, and IFN- γ level in supernatants were determined respectively. (B) The percentage of IL-4 expressing cells, GATA-3 protein level, and IL-4 level in supernatants were determined respectively. The Th1 or Th2 differentiation was induced in CD4⁺ T cells, and the exosomes were added for the 7-day coincubation. The coincubation was conducted in 4 different groups: CD4⁺ T + OVA-EXO-shRNA, CD4⁺ T + OVA-EXO-shRNA-LncGAS5, CD4⁺ T-shRNA + OVA-EXO-shRNA-LncGAS5, and CD4⁺ T-shRNA-T-bet + OVA-EXO-shRNA-LncGAS5. (C) The percentage of IFN- γ expressing cells, T-bet protein level, and IFN- γ level in supernatants were determined respectively. (D) The percentage of IL-4 expressing cells, GATA-3 protein level, and IL-4 level in supernatants were determined respectively. * $p < 0.05$, ** $p < 0.01$ vs CD4⁺ T + OVA-EXO-shRNA. # $p < 0.05$, ## $p < 0.01$ vs CD4⁺ T-shRNA + OVA-EXO-shRNA-LncGAS5. OVA-EXO-shRNA-LncGAS5 and OVA-EXO-shRNA, RPMI 2650 cells were transfected with shRNA-LncGAS5 or shRNA followed by the OVA stimulation, and the exosomes were isolated. CD4⁺ T-shRNA-EZH2 and CD4⁺ T-shRNA, CD4⁺ T cells were transfected with shRNA-EZH2 or shRNA before the coincubation. CD4⁺ T-shRNA-T-bet and CD4⁺ T-shRNA, CD4⁺ T cells were transfected with shRNA-T-bet or shRNA before the coincubation. Values are presented as means $\pm$ SD and are representative of three individual experiments.

that the interference of LncGAS5 significantly increased T-bet mRNA levels from 0.62 ± 0.035 to 0.85 ± 0.058 , which caused an elevation of 1.38 times, while the interference of LncGAS5 increased T-bet

protein levels from 0.23 ± 0.03 to 0.72 ± 0.07 , which caused an elevation of 3.12 times (Fig. 5). These data indicated that LncGAS5 regulated both the mRNA and the protein expressions of T-bet,

especially on the protein expression. However, LncGAS5 did not affect the synthesis of T-bet, as MG132 increased T-bet protein expression with or without interfering LncGAS5. Meanwhile, a study conducted by Tong et al. (2014) revealed that EZH2 regulated the posttranslational expression of T-bet, and our study indicated that EZH2 expression was regulated by LncGAS5. Consistent with their study, the knockdown of EZH2 reduced both mRNA and protein levels of T-bet (Supplemental Fig. 1B). We supposed that LncGAS5 may regulate T-bet expression indirectly via modulating EZH2 expression, although the specific interactions among LncGAS5, T-bet, and EZH2 deserves more investigations in the future.

In conclusion, our findings demonstrated that LncGAS5 in epithelium-derived exosomes is the key mediator in Th1/Th2 differentiation in AR, which provides a novel target of treating AR.

Ethics approval and consent to participate

This study was approved by the Institute Research Medical Ethics Committee of The First Affiliated Hospital of Zhengzhou University. Written informed consents were obtained from all the patients involved.

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None.

Author statement

Xiaoyuan Zhu: Conceptualization, Methodology, Writing- Original draft preparation, Supervision.

Xueping Wang: Software, Visualization, Writing-reviewing and Editing.

Ying Wang: Methodology, Writing-reviewing and Editing.

Yulin Zhao: Methodology, Writing-reviewing and Editing.

Availability of data and material

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

Declaration of Competing Interest

All authors declare that they have no competing interests.

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None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2019.11.009>.

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Supplemental Fig. 1 (A) RPMI 2650 cells were transfected with shRNA-LncGAS5 or shRNA followed by the OVA stimulation, and the exosomes (OVA-EXO^{shRNA-LncGAS5} and OVA-EXO^{shRNA}) were isolated. The qRT-PCR confirmed the downregulation of LncGAS5 in OVA-EXO^{shRNA-LncGAS5}. **(B)** CD4⁺ T cells were transfected with small hairpin RNA (shRNA) against EZH2. The mRNA and protein levels of EZH2 and T-bet were detected using qRT-PCR and western blot analysis. **(C)** CD4⁺ T cells were transfected with shRNA against T-bet. The mRNA and protein levels of T-bet were detected using qRT-PCR and western blot analysis.

**p<0.01 vs EXO or control (shRNA). ##p<0.01 vs OVA-EXO^{shRNA}.

