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Immunomodulatory features of MSC-derived exosomes decorated with DC-specific aptamer for improving sublingual immunotherapy in allergic mouse model

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Abstract

Introduction Sublingual immunotherapy (SLIT) is an effective and injection-free route for allergen-specific immunotherapy (AIT). Mesenchymal stromal/stem cell (MSC)-derived exosomes (Exo) has been identified as a novel delivery platform with immunomodulatory capacities. In addition, targeting agents such as aptamers (Apt) have been extensively used for specific delivery approaches such as direct delivery of allergen formulations to dendritic cells (DC) to improve the efficacy of specific immunotherapy. In this study, we assessed the effects of MSC-derived Exos containing ovalbumin (Ova) which decorated with DC-specific aptamer in allergic rhinitis mice model.

Materials and methods Exos were harvested from adipose tissue-derived MSCs, and Exo-Apt-Ova complex was formulated. Then, Ova-induced allergic asthma model was simulated and sensitized BALB/c mice were treated sublingually with Exo-Apt-Ova complex (5 µg Ova) twice weekly for 8 weeks. Ova-specific IgE levels in serum and concentrations of interferon-gamma (IFN-γ), interleukin (IL)-4, and transforming growth factor-beta (TGF-β) in the supernatant of cultured splenocytes were evaluated using enzyme-linked immunosorbent assay (ELISA). In addition, lung histologic analysis and nasopharyngeal lavage fluid (NALF) cell count were performed.

Results Administration of Ova-incorporated Apt-modified Exos dramatically increased IFN-γ and TGF-β levels, and decreased IL-4 and IgE levels. In addition, inflammatory responses in the lung tissue and the number of eosinophils in NALF decreased.

Conclusion SLIT using Exo-Ova (5 µg) decorated with DC-specific aptamer induced immunomodulatory responses and remarkably attenuated allergic airway inflammation in mice.

Keywords Mesenchymal stromal/stem cell (MSC), Sublingual immunotherapy (SLIT), Extracellular vesicle (EV), Exosome (Exo), Targeted drug delivery, Aptamer (Apt), Ovalbumin (Ova)

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Introduction

Allergic diseases are becoming a global health problem and allergic asthma, allergic rhinitis (AR), allergic conjunctivitis and skin-associated allergic diseases are among the most common [1, 2]. Airway allergic diseases are characterized by eosinophilic inflammation, mucosal hypersecretion, and airway hyperresponsiveness (AHR). Several lines of evidence have emphasized the critical role of the Th1/Th2 imbalance in triggering allergic reactions. In this case, hyperactivated Th2 cells release interleukin (IL)-4, IL-5, and IL-13 in high magnitude, which promote specific IgE secretion and recruit and activate eosinophils [3, 4].

Common strategies to control allergic reactions include allergen avoidance, pharmacotherapy, and specific immunotherapy. Although allergen avoidance is inevitable, and available medical treatments, including bronchodilators and inhaled corticosteroids only control symptoms and are not effective in suppressing disease progression, allergen-specific immunotherapy (AIT) is a well-designed therapeutic strategy to modify the immune responses to achieve sustained symptom relief and improved quality of life [5, 6]. Subcutaneous immunotherapy (SCIT), as the standard route of allergen-specific immunotherapy, consists of the administration of allergen extracts based on a protocol that includes build-up and maintenance phases that can last for 3–5 years. Recurrent invasive subcutaneous injections and the long duration of the treatment procedure may reduce patient compliance to start or even complete the treatment [7]. Therefore, the investigation of alternative routes such as sublingual immunotherapy (SLIT) has attracted much attention. SLIT is a non-invasive method in which allergens in the form of liquid drops, powder, tablet, or other compatible formulations are administered under the tongue by trained patients without clinic recruitment. These factors influence patient compliance with the treatment procedure [8, 9]. The exact mechanism of SLIT is not well defined, although it appears that allergens administered by the sublingual route are taken up by Langerhans dendritic cells (DC), and processed and presented in regional lymph nodes. A major concern regarding SLIT is the need for higher allergen doses compared to SCIT to achieve similar levels of efficacy [10]. In this case, innovative methods to reduce the allergen dosage could effectively improve the efficacy and safety of SLIT [11]. Exosomes (Exo) are biological nano-vesicles secreted by different types of cells both in physiological and pathological conditions and have been widely used as delivery systems in recent years [12]. Due to their low immunogenicity, high bioavailability and biodistribution, and similar content compared to their cellular source, mesenchymal stromal/stem cell (MSC)-isolated exosomes are considered as nano-sized delivery tools with immunomodulatory and immunoregulatory properties [13]. On the other hand, delivery of small-molecule therapeutic products to the desired cells or target site can boost the treatment outcomes by reducing the effective dose and ultimately reducing side effects. In addition, some approaches such as directed delivery, can enhance allergen presentation to antigen-presenting cells (APCs) [14–16]. Recently, aptamers (Apt) have been widely used as antibody replacements and as molecular directing agents to customize specific drug delivery. In this case, aptamers are characterized as artificial receptors, originally nucleotide of ribonucleotide sequences, and identified via an in vitro procedure called systematic evolution of the ligands by exponential enrichment (SELEX). In addition, aptamers have several benefits over monoclonal antibodies, such as lack of toxicity and immunogenicity, good thermal stability, ease of modification and production. These advantages of aptamers have led to their increased use to improve the efficacy of immunotherapy [17, 18].

In this study, we assessed the potential of sublingual immunotherapy in an allergic mouse model after administration of ovalbumin (Ova)-loaded exosome nanoparticles decorated with DC-specific Apt.

Materials and methods

Animals

Thirty BALB/c mice (female and 6–8 weeks old), were provided by the Animal Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Animals were housed in standard conditions (12 h/12 h dark/light cycle, 20–25 °C temperature, and 45–65% humidity) and free access to food and water.

Formulation of Apt-modified Ova-enriched MSC-exosomes

As explained earlier by Dehnavi et al., MSCs were obtained from the abdominal adipose tissue of BALB/c mice and exosomes were isolated from the fetal bovine serum (FBS)-free conditioned supernatant of MSCs culture [19]. Briefly, mouse adipose tissue was enzymatically digested with collagenase type I (Merck, Darmstadt, Germany), then centrifuged, and the cellular pellet was transferred to culture plates containing DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin. After 48 h, the media was refreshed to remove non-adherent cells and the culture continued until 80–80% confluent adherent cells were reached. These cells were validated as MSCs by microscopic morphology of adherent fibroblast-like cells, immunophenotyping using specific antibodies and flow cytometry technique for surface expression of CD34, CD44, CD45, and CD90 markers, and osteocyte differentiation capacity using specific differentiation media. The FBS-free conditioned supernatant of cultured MSCs was harvested

for exosome isolation using an exosome isolation kit (Exospin, MO, USA). Subsequently, the morphology of exosomes was characterized by Field Emission Scanning Electron Microscopy (FESEM) (KYKY-EM 3200) and the zeta potential, size, and polydispersity index were evaluated by dynamic light scattering (DLS) (Malvern Instruments Ltd, Malvern, UK). Finally, CD9 and CD63 surface expression of exosomes were analyzed by flow cytometry.

For the preparation of Apt-modified Exo, the 5′-COOH-modified aptamer with the sequence " 5′-TT TTTTTTTTGGCTGGCACTGGTCGAGGTATGTT GGGGCAGCT-3′ " [20] was conjugated to the surface amine group on the exosomes through EDC/NHS chemistry. For this purpose, 20 µL of 5′-COOH-modified Apt (25µM) was added to 4µL EDC/NHS solution (containing 92 µg EDC and 70 µg NHS), and then 450 µl phosphate buffer saline (PBS) was added and stirred at 4 °C for 4 h. The activated Apt was then added to 200 µg/ml exosomes solution at 5% w/w Apt/Exo ratio of Apt/Exo and incubated at 4 °C for 24 h. The successful conjugation of Apt to Exo was evaluated by 2.5% agarose gel electrophoresis.

In the next step, Ova was loaded into the Apt-modified exosomes based on our previous protocol [19, 21]. Briefly, 200 µl of prepared Ova solution (500 µg/ml) (Becton Dickinson Co, New Jersey, USA) was incubated with Apt-modified exosomes (200 µg/ml) for 6 h at 22 °C on a shaker. Finally, free Ova were eliminated from the Apt-Exo-Ova complex using an exosome isolation kit, and the amount of enriched Ova was determined by UV-HPLC Hewlett Packard 1100 series (Santa Clara, CA, USA).

Sensitization phase and SLIT

Animal sensitization was performed as previously described [22, 23]. Briefly, twenty five animals were received ovalbumin through two intraperitoneal (i.p) injections of 10 µg Ova in PBS adsorbed on aluminum hydroxide (alum) (Sigma, France) in 100 µL of PBS on days 0 and 14. Equivalent amounts of sterile PBS/alum were used for healthy control animals (n=5). The mice were then aerosolized daily for 4 consecutive days (on days 21 to 24) via a nebulizer (ZTH-2102 Zenith, Switzerland). On each day, animals were placed in a closed chamber and received 1% PBS-dissolved Ova solution as an aerosol for 20 min. On day 25, blood samples were collected for evaluation of total IgE levels. The sensitized mice (n=25) were then categorized into five groups (n=5).

SLIT treatment was initiated on day 28, for 8 weeks (twice weekly) by sublingual application of Ova (5 µg), Exo, and Exo-Ova (5 µg Ova) and Exo-Ova-Apt (5 µg Ova) in final volume of 25 µL in different groups (n=5 in each group) including sensitized animals. Notably, exosome-containing formulations were freshly prepared for each immunotherapy session. The positive control animals were sensitized mice that received 25 µL PBS. A negative control group consisting of not-sensitized mice receiving PBS (n=5) was also included. For sublingual procedure, animals were held on their back, therapeutic solution was administered under the tongue using a variable volume pipette (Eppendorf Research plus, Barkhausenweg, Hamburg, Germany), and then animals were held for one minute to avoid swallowing. At the end of treatment, on days 90 and 91 all animals underwent intranasal challenge with 1% Ova for 20 min. On day 92, the animals were sacrificed. In this case, animals were anesthetized through i.p injection of 100 µL ketamine/xylazine (containing 87.5 mg/kg and 12.5 mg/kg, respectively) and then euthanized via cervical dislocation method. Blood specimens were obtained via cardiac puncture, and sera were analyzed for specific IgE. Splenocytes were isolated, cultured, and analyzed for cytokine production. Nasopharyngeal lavage fluid (NALF) was also obtained from the nostrils for total cell and eosinophil counts. Finally, the lung tissues were separated for histopathologic assessment. Figure 1 provides a schematic representation of the study design and subsequent steps.

Assessing Ova-specific IgE concentrations

Blood specimens were obtained by cardiac puncture, and sera were collected after centrifugation (10 min at 1500 g). Ova-specific IgE levels were then evaluated via an enzyme-linked immunosorbent assay (ELISA) kit (East Biopharm, China) based on the manufacturer's protocols. Briefly, 100 µL of serum was added to the antigen pre-coated wells and incubated at 37∘C for 90 min. The wells were then washed and 100 µL of enzyme-conjugated secondary antibody was added and incubated for 30 min at 37∘C. Next, the wells were washed and 90µL of substrate was added for further incubation at 37∘C for 15 min. Finally, 50 µL stop solution was added and the color intensity was read at 450 nm to determine the exact concentrations based on the standard samples and the provided curve.

Measurement of cytokines secretion

Spleen tissues were collected, minced, washed with sterile PBS, and resuspended in 10% FBS and 1% penicillin/streptomycin-enriched RPMI 1640 media. Splenocytes were then cultured in duplicate and stimulated with Ova (5 µg/ml), 3% phytohaemagglutinin (PHA) and free media for 72 h at 37 °C. Next, the supernatants were obtained and interferon-gamma (IFN-γ), IL-4, and transforming growth factor-beta (TGF-β) cytokine levels were measured using ELISA kits (Karmania Pars Gene, Kerman, Iran).

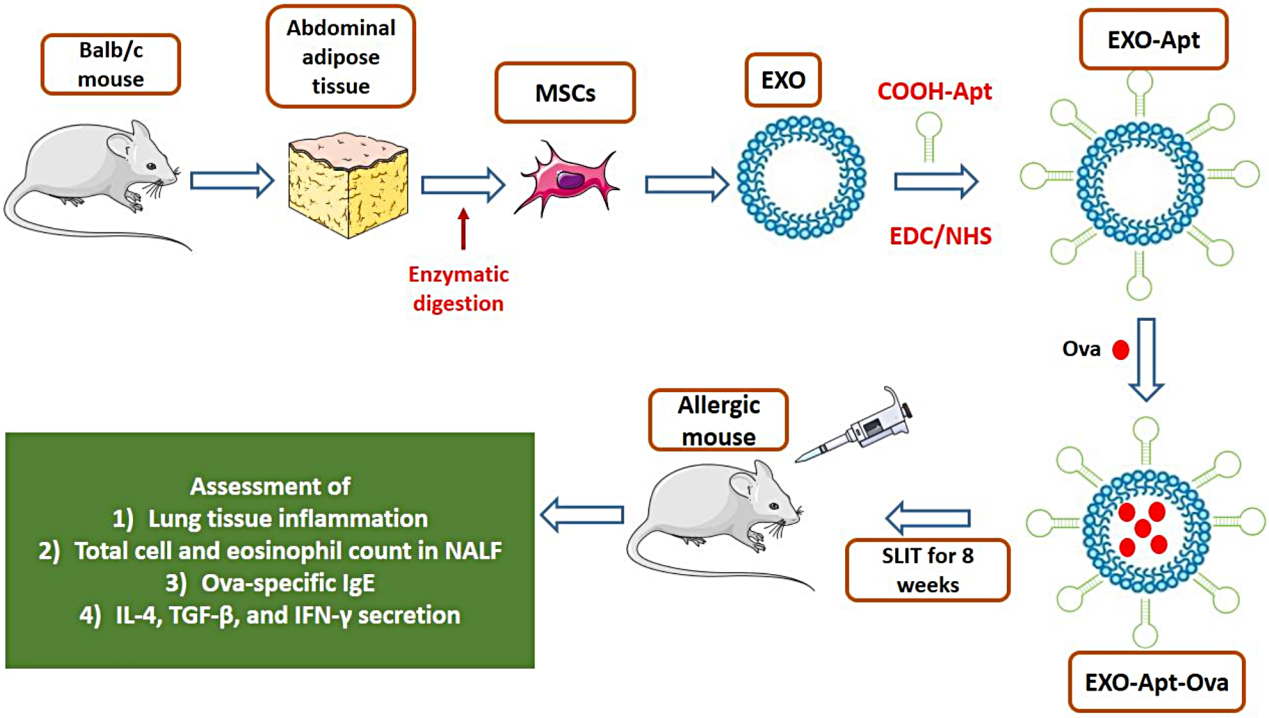

Fig. 1 Schematic representation of study design

Nasopharyngeal lavage fluid (NALF) analysis

To obtain NALF from mice, the trachea of sacrificed animals was cut out and 1 ml of sterile PBS was injected into the nasal cavity and gathered in a tube. The specimen was centrifuged (5 min, 2000 rpm, 4 ∘C), and the pellets were analyzed for total cell counting using Neubauer haemocytometer. In addition, the Wright-Giemsa stained smears were prepared to count and evaluate the percentage of eosinophils based on the morphology and stained granules.

Histological examination

The chests of scarified animals were opened and whole lung tissues were isolated. Then, tissues moved to 10% formaldehyde-contained tubes, embedded in paraffin, and sectioned (5 μm). Then, stained with hematoxylin and eosin (H&E) and evaluated by a blinded animal pathologist using a light microscope (Zeiss Jena, Germany).

Statistical analysis

GraphPad Prism (version 8.4.3) was applied for statistical analysis. All data are expressed as mean ± standard error of mean (SEM). Data were first analyzed using the Kolmogorov-Smirnov test to check the normal distribution of the data. Sensitized and control animals were compared by unpaired t-test for total IgE levels, while one-way ANOVA test was used to compare different groups of animals after the treatment procedure. In addition, $p < 0.05$ was considered as significant value.

Results

MSCs characterization

Characterization and validation of MSCs are provided in Fig. 2. As provided in Fig. 2a, microscopic evaluation of adherent cells showed fibroblast-like morphology. Also, in vitro osteogenic differentiation assay of isolated MSCs showed potential osteocyte differentiation validated by extracellular calcium deposits formation following alizarin red staining (Fig. 2b). in addition, as shown in Fig. 2c, high percentage of cells expressed CD44 (94.9%) and CD90 (95.2%) markers, while low levels of CD34 (1.31%) and CD45 (0.74%) surface expressions was observed.

Exosomes characterization

As shown in Fig. 3a, FESEM analysis was performed to determine the homogeneity and morphology of MSC-isolated exosomes. In addition, Fig. 3b shows the DLS analysis of the size of homogeneous spherical exosome nanoparticles which is consistent with the FESEM results. The average size of exosomes was 101.8 ± 15.9 nm. To further characterization, we evaluated the surface expression of CD9 and CD63 in MSC-derived exosomes by flow cytometry and the results showed 90.7% of exosomes expressed CD9, while 86.5% expressed CD63 (Fig. 3c).

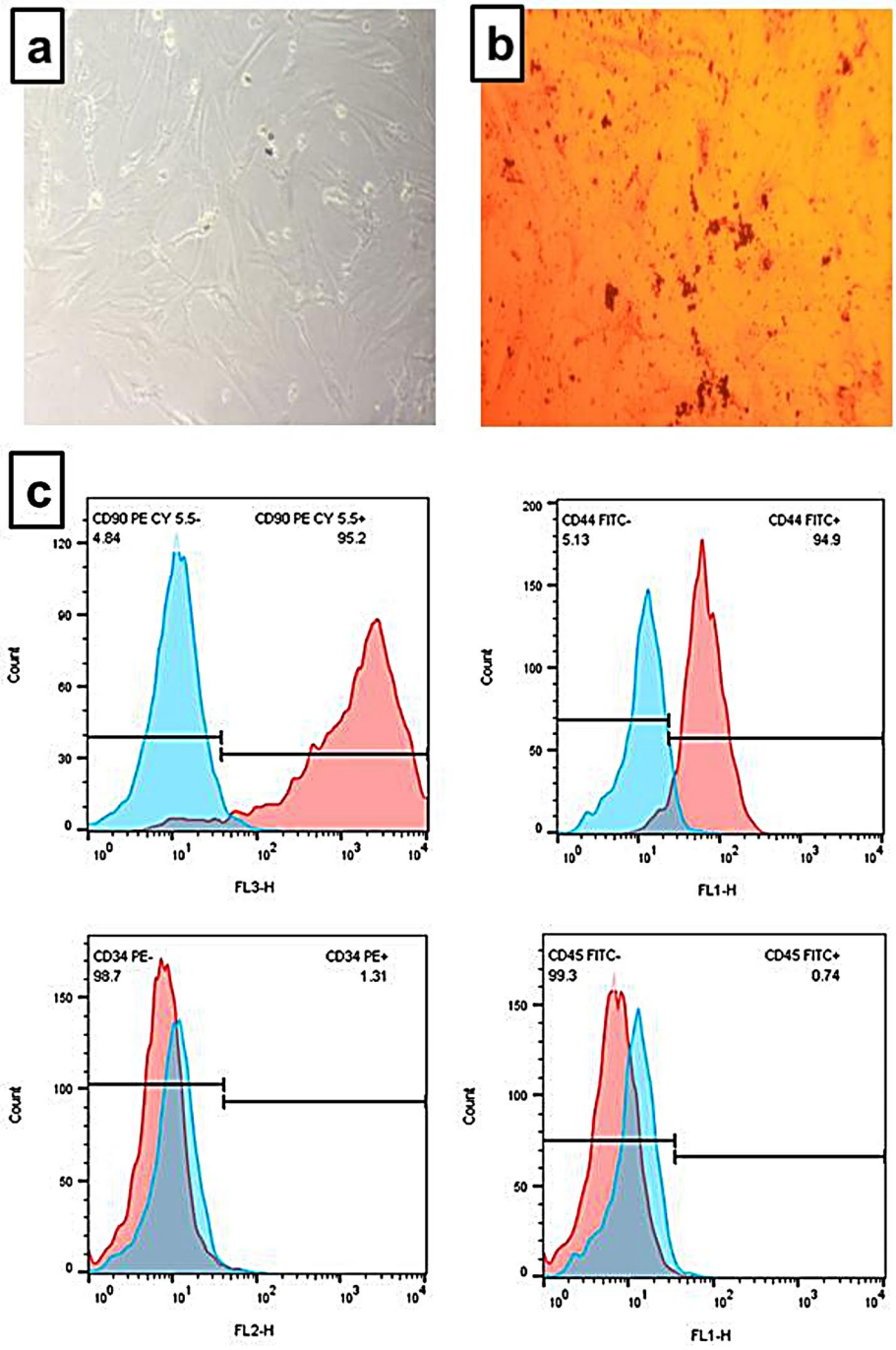

Fig. 2 Adipose tissues-derived MSCs characterization (**a**) Microscopic evaluation verified adherent fibroblast-like morphology of isolated MSCs; (**b**) Osteogenic differentiation assay validated osteogenesis potential following alizarin red staining of extracellular calcium deposits; and (**c**) Flow cytometry analysis showed highly expression of CD44 (94.9%) and CD90 (95.2%) and low levels of surface CD34 (1.31%) and CD45 (074%) markers

Monitoring of Exo-Apt synthesis

The successful attachment of the aptamer to the exosome surface was evaluated using agarose gel electrophoresis. As shown in Fig. 4, the Exo-Apt bio-conjugate did not transfer on the agarose gel due to its high molecular weight. In addition, the DLS analysis findings showed an increased in the size of exosomes following aptamer conjugation and the negative zeta potential of Exo-Apt complex intensified compared to exosomes due to the charge of DNA aptamer (Table 1).

Ova-enrichment of Apt-conjugated exosomes

The efficacy of Ova enrichment into the Apt-conjugated exosome was evaluated by UV-HPLC. Based on the

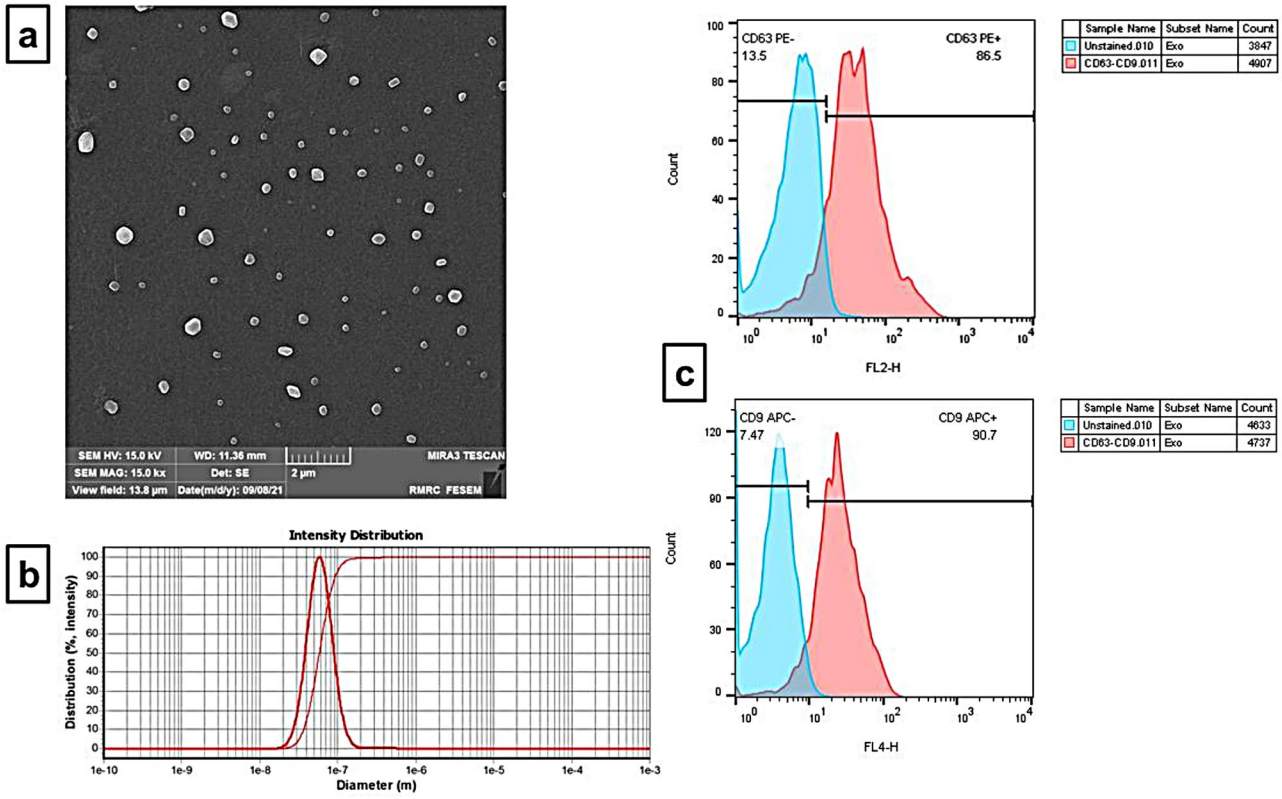

Fig. 3 Characterization of MSC-isolated exosomes (**a**) FESEM analysis verified the morphology of exosome nanoparticles; (**b**) DLS assessment showed that the average size of nanoparticles was 101.8 ± 15.9 nm; and (**c**) flow cytometry evaluation of CD9 and CD63 surface markers using specific antibodies showed that high percentage of exosome nanoparticles expressed CD9 (90.7%) and CD63 (86.5%)

obtained results, the quantity of the Ova enrichment in the Apt-conjugated exosome was 20 µg. In addition, DLS analysis is shown in Table 1 to compare the size, polydispersity index and zeta potential and FESEM analysis is represented in Fig. 5 to compare the morphology of free and formulated exosomes.

The Ova-specific IgE antibody alteration after SLIT

To evaluate the efficacy of SLIT on humoral immune responses, the ELISA assessment was conducted to measure the OVA-specific serum IgE. Although, the concentration of IgE was decreased in all experimental groups in comparison to the control Ova-sensitized PBS-treated group, a dramatic decrease was showed only in the Exo-Ova-Apt group after SLIT (Fig. 6).

Effect of SLIT on cytokine profile

The measurement of IFN-γ, IL-4, and TGF-β cytokine production in the splenocyte supernatant is shown in Fig. 7.

After SLIT, the secretion of IFN-γ, a major cytokine of Th1 cells, increased in all treatment groups in comparison to the sensitized PBS-treated group. In addition, treatment with Exo-Ova-Apt complex resulted in a dramatic reduction in IFN-γ secretion compared with Ova- and PBS-treated groups ($p<0.05$).

The results of IL-4 cytokine, a major cytokine of Th2 cells, concentration showed that all experimental groups showed a reduction in IL-4 levels compared to the PBS group. Also, there was a dramatic difference between Exo-Ova-Apt group and other experimental groups in the reduction of IL-4 ($p<0.0001$). Interestingly, after SLIT with Exo-Ova-Apt, we observed no statistical difference in IL-4 concentration compared to normal control mice.

In the case of TGF-β, all treatment groups (except the Ova-treated) secreted the high concentration of TGF-β cytokine in response to stimulation with Ova compared to the PBS-treated group. It was also observed that Exo-Ova-Apt treatment significantly elevated the TGF-β levels in comparison to the other experimental groups ($p<0.0001$).

Effect of SLIT on cell counting of NALF

As presented in Fig. 8, the numbers of eosinophils and total cell count in the NAL fluid were significantly reduced in all treatment groups (except Ova SLIT) in comparison to the PBS-treated group. Among the treatment groups which receive Exo, the mice receiving

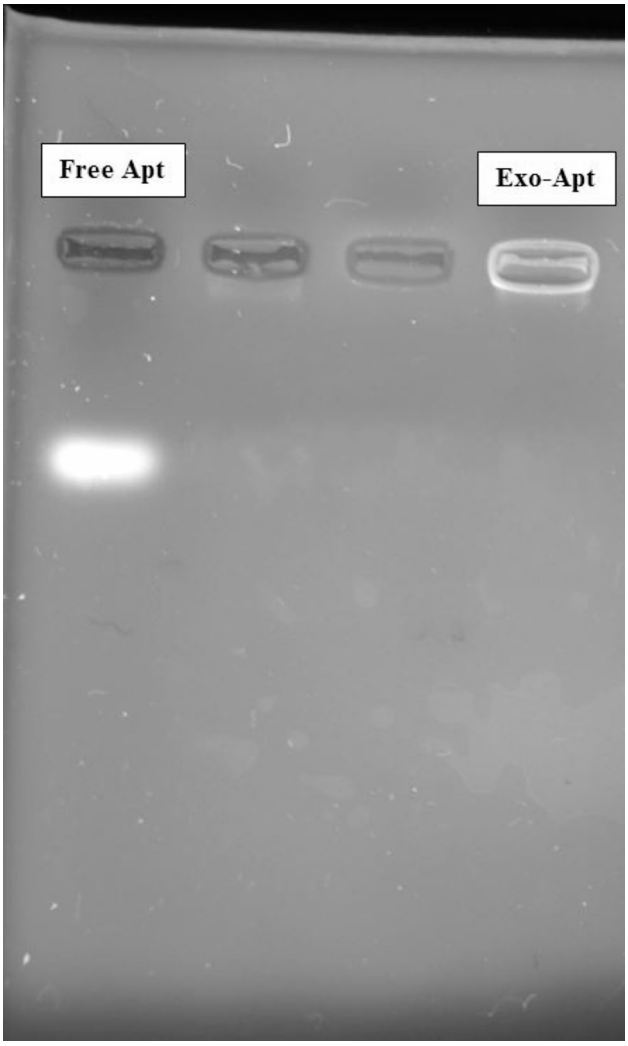

Fig. 4 Assessment of Exo-Apt bio-conjugate via agarose gel electrophoresis. As shown and labeled in the figure, free aptamer moved through agarose gel electrophoresis, while aptamer conjugated on the surface of exosomes remained immobile in the loading well (Full-length gel is presented in Supplementary Fig. 1)

Table 1 DLS characterization of free exosomes compared to Exo-Apt and Exo-Ova-Apt formulations

	Size (nm)	Polydispersity Index (PDI)	Zeta potential
Free exosome	101.8 ± 15.9	0.21 ± 0.06	-36 ± 3.1
Exo-Apt complex	109.3 ± 13.6	0.29 ± 0.01	-64 ± 6.9
Exo-Ova-Apt complex	114.1 ± 11.6	0.3 ± 0.02	-69 ± 7.3

Exo-Ova-Apt showed a significant reduction in total cell and eosinophil counts compared to the groups receiving Exo and Exo-Ova sublingually. Interestingly, the number of eosinophils in the NALF showed no statistical difference between Exo-Ova-Apt and normal control mice.

Histological analysis of lung tissue

Analysis of lung histopathological examinations in healthy control mice did not show any inflammation or infiltration of immune cells in the blood vessels and trachea (Fig. 9a). Additionally, examination of lung tissues demonstrated severe peribronchiolar and perivascular inflammation in PBS- and Ova-treated groups (Fig. 9b and c). Exo and Exo-Ova groups indicated mild to moderate infiltration of inflammatory cells and restricted epithelial degenerations (Fig. 9d and e). Furthermore, lung examination in the Exo-Ova-Apt-received mice indicated the local and slight infiltration of immune cells around a blood vessel (Fig. 9f).

Discussion

In this study, we evaluated the effects of Ova-enriched Apt-modified MSC-derived exosomes in an allergic asthma mouse model by sublingual immunotherapy. This nanoformulation showed excellent immunomodulatory capacities, decreased eosinophil infiltration to the NALF and alleviated local inflammation in lung tissue. In addition, administration of this complex could reduce the concentration of IL-4 (Th2 hallmark) in the cultured splenocyte supernatants along with the serum levels of Ova-specific IgE and increase the levels of IFN-γ (major Th1 cytokine) and TGF-β (regulatory T cell (Treg) hallmark) in the supernatant of cultured splenocytes. Our results indicated the successful shift from Th2 to Th1 and induction of immunomodulatory response of Tregs.

Sublingual immunotherapy is considered a non-invasive, safe and painless route of administration for allergic disorders with fewer side effects in comparison to subcutaneous immunotherapy [24, 25]. Accordingly, several lines of evidence have shown that the effects of SLIT is dependent on the allergen dosage, which subsequently increases the adverse effects [26, 27]. In addition, the use of nanoparticles such as exosomes as a targeting agent for specific allergens could facilitate the amount of allergen reaching the mucosal tissues in comparison to the soluble-free allergen administered sublingually [24]. On the other hand, it seems that current SLIT therapeutic approaches could not target DCs residing in the oral cavity. Therefore, targeted allergen delivery may improve the crosstalk between DCs and allergens [28, 29]. In this context, a DC-specific aptamer could enhance the immunomodulatory responses after SLIT.

In the current study, exosomes and aptamers were used as nanocarriers and delivery systems, respectively, to boost the therapeutic efficacy of SLIT in an allergic animal model. Similar to previous studies using nanoparticles (alone or targeted with aptamers) [23, 30–32], Our results showed that sublingual administration of exosome nanoparticles decorated with DC-specific aptamer

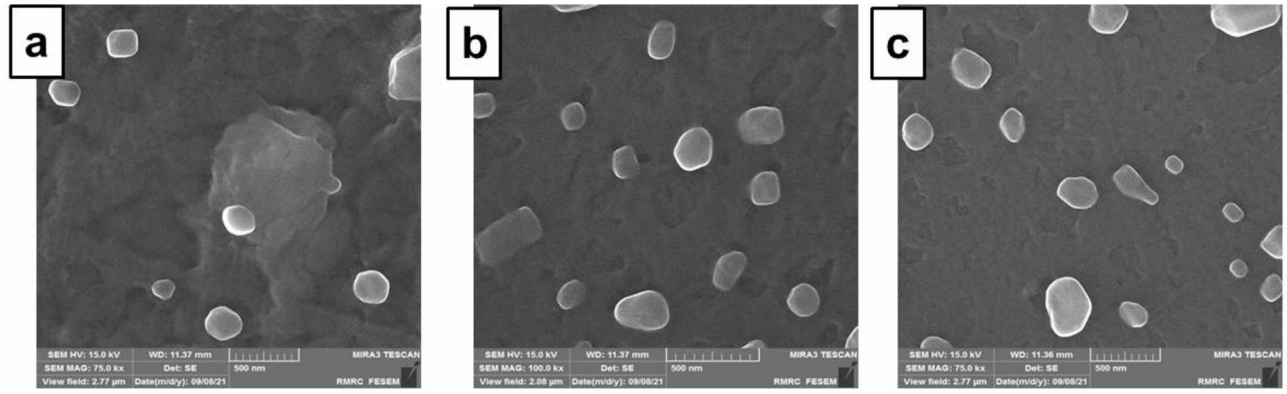

Fig. 5 FESEM analysis of Assessment of (**a**) free exosomes compared to (**b**) Exo-Apt and (**c**) Exo-Ova-Apt formulations

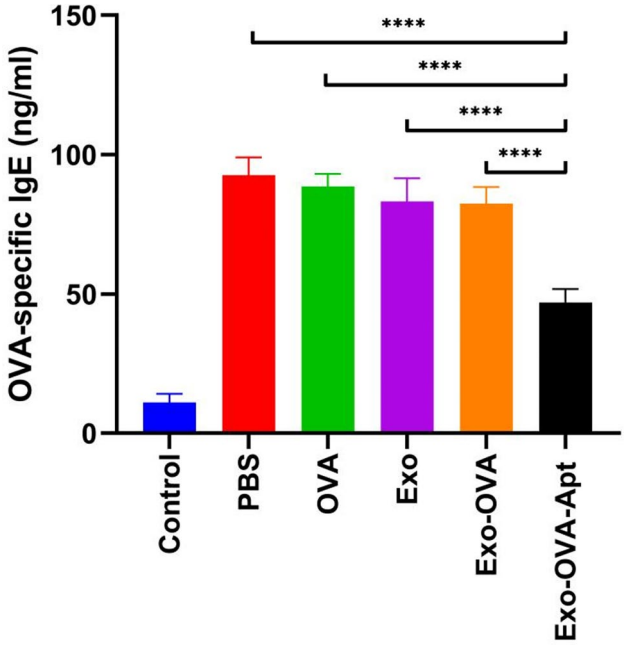

Fig. 6 Serum concentrations of Ova-specific IgE after SLIT in different control and treatment groups including Control (not-sensitized and not-treated healthy negative control animals), PBS (OVA-sensitized and PBS-treated positive control animals), OVA (OVA-sensitized and 5 µg OVA-treated animals), Exo (OVA-sensitized and Exo-treated animals), Exo-OVA (OVA-sensitized and Exo-OVA complex (containing 5 µg OVA)-treated animals), and Exo-OVA-Apt (OVA-sensitized and Exo-OVA-Apt complex (containing 5 µg OVA)-treated animals) ($n = 5$ in each group) (Data are presented as mean ± SEM) (One-Way ANOVA was applied to statistically compare different group and **** indicates $p < 0.0001$)

alleviated allergic inflammation and induced Th1 and Tregs immune responses.

Recently, exosomes have been widely used as promising agents for drug specific-targeting in various diseases. This biocompatible nanoscale delivery system has excellent stability and durability, and low toxicity and immunogenicity due to its endogenous entity [33–35]. Previously, Sun et al. demonstrated that curcumin-loaded exosomes had higher bioavailability, solubility, and immunomodulatory capacity than unformulated curcumin [36]. In addition, Haney et al. reported that exosome-formulated catalase could induce neuroprotective effects due to crossing the blood-brain barrier [37]. Similarly, our results showed that sublingual administration of exosome-formulated Ova significantly induced immunomodulatory responses and alleviated inflammation in the airways of mice compared to free Ova.

The MSC-isolated exosomes showed regulatory potential similar to that of MSCs [38, 39]. Several lines of evidence have identified the immunomodulatory capacities of MSCs or MSC-derived exosomes in inflammatory airway diseases. Adipose-isolated MSCs and collected exosomes were shown to alleviate AHR [40, 41]. In another study, MSC-derived secretome containing exosomes significantly suppressed anaphylaxis, decreased IL-4, IL-5, IL-13, and IgE concentrations, and increased regulatory cytokines including TGF-β and IL-10 [38]. These findings are consistent with our result which showed a remarkable enhancement in TGF-β and a significant reduction in the number of eosinophils and inflammatory cells in NALF in animals treated sublingually with MSC-derived exosome. In addition, the mentioned studies are consistent with the findings of Dong et al. who administered MSC-derived exosomes intratracheally [13], and Mun et al. [42], Dehnavi et al. [43], and Ren and colleagues [44], who administered MSC-derived exosomes intranasal, and Wang and colleagues who treated with MSC-derived exosome complex to evaluate regulatory responses in allergic rhinitis animal model [45]. Interestingly, it was shown that prophylactic treatment with MSC-derived exosomes could suppress allergic sensitization in animal model [46].

In our study, the surface of MSC-derived exosomes was functionalized with DC-specific aptamer and used to enhance the therapeutic efficacy of SLIT. To the extent that we are informed, this study is the first experiment in which exosomes were modified with DC-specific aptamer to enhance allergen-specific sublingual immunotherapy.

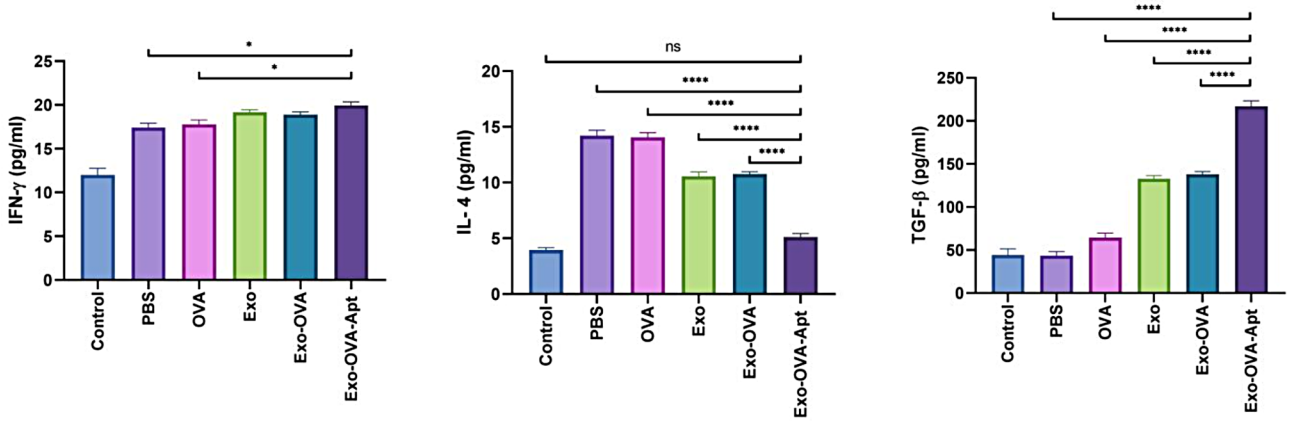

Fig. 7 Cytokine production in the supernatant of cultured splenocytes in different control and treatment groups including Control (not-sensitized and not-treated healthy negative control animals), PBS (OVA-sensitized and PBS-treated positive control animals), OVA (OVA-sensitized and 5 μg OVA-treated animals), Exo (OVA-sensitized and Exo-treated animals), Exo-OVA (OVA-sensitized and Exo-OVA complex (containing 5 μg OVA)-treated animals), and Exo-OVA-Apt (OVA-sensitized and Exo-OVA-Apt complex (containing 5 μg OVA)-treated animals) ($n = 5$ in each group) (Data are presented as mean ± SEM) (One-Way ANOVA was applied to statistically compare different group and ns, no significant.* $p < 0.05$ **** $p < 0.0001$)

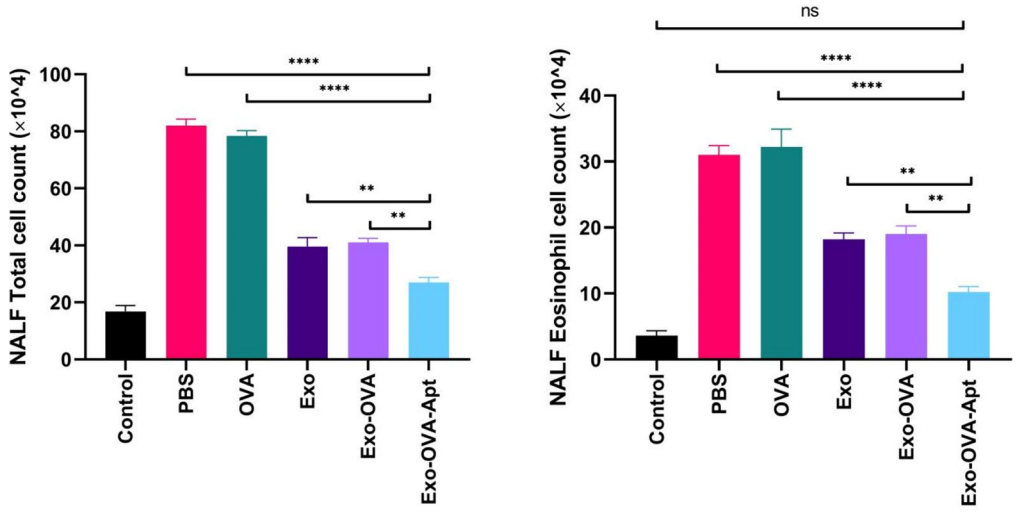

Fig. 8 Effects of SLIT on the number of total cell and eosinophil in NALF in different control and treatment groups including Control (not-sensitized and not-treated healthy negative control animals), PBS (OVA-sensitized and PBS-treated positive control animals), OVA (OVA-sensitized and 5 μg OVA-treated animals), Exo (OVA-sensitized and Exo-treated animals), Exo-OVA (OVA-sensitized and Exo-OVA complex (containing 5 μg OVA)-treated animals), and Exo-OVA-Apt (OVA-sensitized and Exo-OVA-Apt complex (containing 5 μg OVA)-treated animals) (n = 5 in each group) (Data are presented as mean ± SEM) (Data are presented as mean ± SEM) (One-Way ANOVA was applied to statistically compare different group and ns, no significant, ** $p < 0.01$, **** $p < 0.0001$)

Accordingly, we hypothesized that delivery of DC-specific aptamer with exosomes containing 5 μg Ova as a novel approach could improve the efficacy of SLIT and induce immunomodulatory responses in Ova-induced allergic rhinitis in BALB/c mice. The results showed that this formulation could efficiently enhance the efficacy of SLIT by increasing TGF-β and IFN-γ and decreasing IL-4 concentration, inflammatory cells and eosinophil infiltration. Previously, our group demonstrated that sublingual administration of exosomes containing 10 μg Ova significantly reduced inflammation and induced immunomodulatory responses in an animal model [19]. Thus, targeted therapy with a specific aptamer led to a reduction in allergen dose with the same efficacy compared to Ova-Exo complex. In agreement with our results, Shahbaz et al. reported that sublingual administration of 10 μg/5 μg Ova-loaded PLGA nanoparticles decorated with aptamer has a great potential effect on regulating Th1/Th2 balance, inducing Treg responses and alleviating airway inflammation [30]. Similarly, Sadeghi et al. showed that SLIT with aptamer-modified gold nanoparticles containing 5 μg Ova significantly reduced the levels of IgE and IL-4, increased the concentration of IFN-γ and TGF-β,

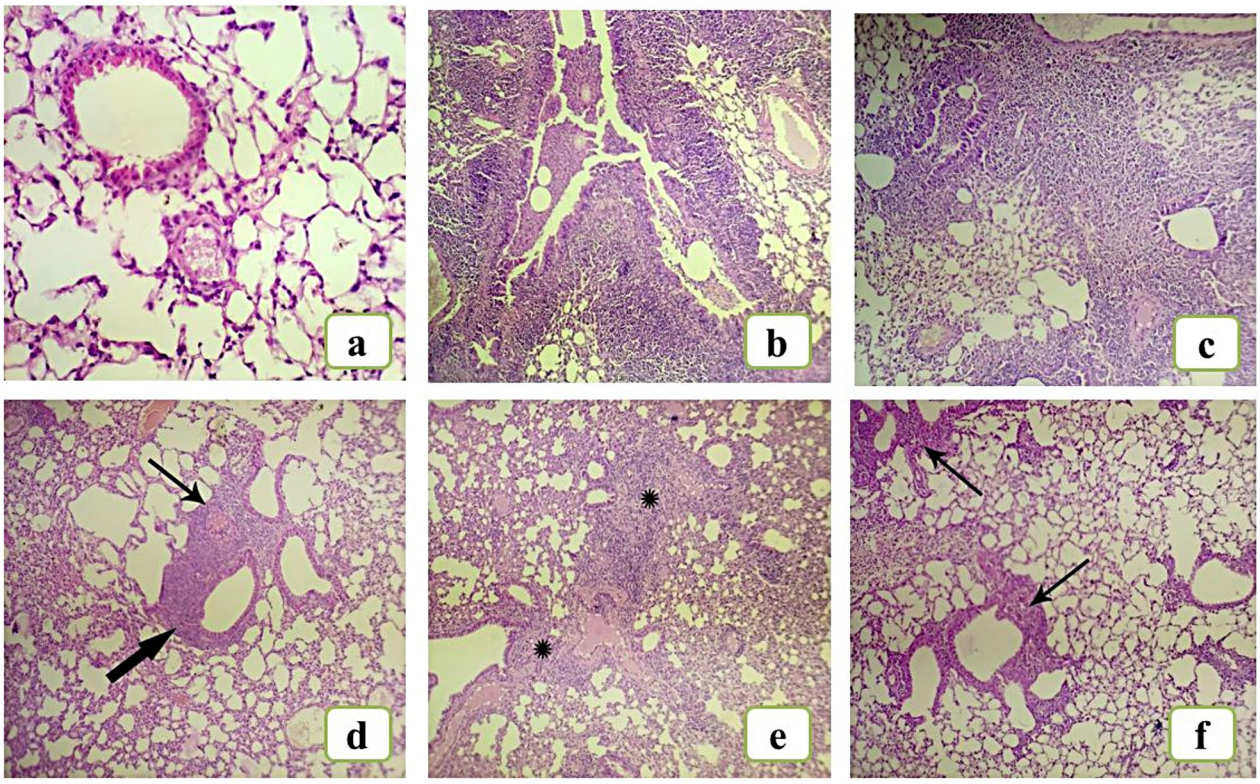

Fig. 9 Histopathological examination of lung tissue in BALB/c mice in experimental groups ((**a**) normal healthy mice; (**b**) Ova-sensitized PBS-received; (**c**) Ova-sensitized Ova-received; (**d**) Ova-sensitized Exo-received, arrows show limited cellular infiltrations and hyperemia ; (**e**) Ova-sensitized Exo-Ova-received, asterisks show cellular infiltrations; (**f**) Ova-sensitized Exo-Ova-Apt-received, arros show peribronchial cellular infiltrations) *(×400 and × 100 magnification microscopy of Hematoxylin and Eosin (H&E) stained sections)*

and also attenuated local inflammation in lung tissue [23].

Conclusion

Based on our findings, loading 5 µg Ova into the MSC-derived exosome could significantly improve the efficacy of sublingual immunotherapy in mice compared to free Ova. In addition, for the first time in the field of allergic immunotherapy of allergic asthma, we decorated exosome nanoparticles with DC-specific aptamer to solve the high dose of allergen required for SLIT. Our results showed that the Apt-Ova-Exo formulation containing 5 µg Ova shifted immune responses from Th2 to Th1, induced regulatory T cells, and reduced inflammation in lung tissue compared to other experimental groups. In addition, the results revealed a reduction in the required dose of Ova to achieve the same or even greater efficacy than free Ova or non-targeted Ova-loaded exosomes by facilitating allergen delivery to DCs in the sublingual mucosa. Thus, we believe that exosome-based and aptamer-directed immunotherapy may be beneficial for boost the effectiveness of SLIT for allergic rhinitis. Although, further detailed studies are demanded to reveal the efficacy of this complex in the future.

Supplementary Information

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Supplementary Material 1

Author contributions

MS contributed in Investigation, Methodology, Project Administration, and Writing Original Draft, SD contributed in Conceptualization, Methodology, Project Administration, and Writing, Review & Editing, AK contributed in Supervision and Validation, AAG contributed in Data Curation and Formal Analysis, AG contributed in Validation, MS contributed in Project Administration, AA contributed in Conceptualization, Methodology, Resources, and Wiritng, Review & Editing.

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Data availability

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

Declarations

Ethics approval and consent to participate

This study is based on the approved project entitled "Evaluation the efficacy of sublingually administrated OVA-encapsulated Adipose-derived mesenchymal

stem cell-derived exosome nanoparticles modified by DC-specific aptamer in allergic mouse model". All experiments were approved by the Institutional Animal Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Approval number: IR.AJUMS.ABHC.REC.1399.059) (Date of approval: 2021.1.12). In addition, this work has been reported in line with the ARRIVE guidelines 2.0.

Conflict of interest
All authors declare no potential conflict of interest.

AI declaration
The authors declare that they have not use AI-generated work in this manuscript.

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