



Specific Ag-guiding nano-vaccines attenuate neutrophil-dominant allergic asthma

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ABSTRACT

Polymorphonuclear neutrophils (PMN) are one fraction of the major inflammatory cells in allergic asthma (asthma, in short); the role of PMN in the asthma pathogenesis is not fully understood yet. This study aims to investigate the effects of specific Ag-guiding exosomes on suppressing the neutrophil-dominant airway inflammation. In this study, BALB/c mice were immunized with ovalbumin plus complete Freund adjuvant to induce an asthma model featured with neutrophil-dominant lung inflammation. The Ag specific PMN (sPMN)-targeting exosomes (tExo), that were exosomes carrying a complex of specific Ag/anti-CD64 Ab and Fas ligand, were constructed to be used to alleviate neutrophilic asthma in mice. We found that sPMNs were the major cellular component in bronchoalveolar lavage fluid (BALF) in asthma mice, while less than 3% PMNs in naive control mice. The sPMNs expressed higher levels of CD64, which formed complexes with Ag-specific IgG (sIgG). The sIgG/CD64 complex-carrying PMNs could be activated upon exposing to specific Ags. Exposure to tExos induced Ag-specific PMNs apoptosis. Administration of tExos efficiently suppressed experimental asthma. We conclude that a fraction of sPMN was identified in the airway of asthma mice. The sPMNs could be activated upon exposure to specific Ags. tExos could induce sPMNs apoptosis, that show the translational potential in the treatment of asthma.

1. Introduction

Allergic asthma (asthma, in short) is induced by aberrant immune responses to innocent Ags (Ag) by the immune system in the airway tissues (Borish, 2016). During asthma attacks, patients have breathing difficulties, cough and profound sputum (Xie et al., 2019). If medical intervention is not taken properly, asthma may develop complications, such as chronic airway inflammation and cardiovascular diseases (Khan, 2014). To date, the therapeutic effects of asthma are not satisfactory currently (Chung, 2017). Therefore, to further investigate the asthma pathogenesis and invent novel and effective remedies for the treatment

of asthma are necessary.

One of the major features of asthma is that the attacks are paroxysmal. The underlying mechanism of this phenomenon is the re-exposure to specific Ags induces mast cell activation. The activated mast cells release allergy-related mediators to evoke allergic attacks (Andersson et al., 2016). However, profound polymorphonuclear neutrophil (PMN) infiltration in the airway tissues was observed in asthma subjects (de Boer et al., 2015; Hosoki et al., 2016; Radermecker et al., 2018). The increase in IL-17 has been found in asthma that plays a role in neutrophil recruitment in the airways (Ramakrishnan et al., 2019). Cumulative research reports indicate that neutrophils are

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involved in the pathogenesis of both allergic asthma and non-allergic asthma (Radermecker et al., 2018). Yet, the underlying mechanism by which PMNs participate the paroxysmatic asthma attacks remains to be further elucidated.

PMNs are the major components of white blood cells in the body. Through the phagocytosing capacity, PMNs fight against invading microbes and clean senescent body cells (Manfredi et al., 2018). Besides, PMNs also have immune functions. By expressing immunoglobulin (Ig) G receptors CD64, CD32 and CD16, IgG can activate PMNs (Rosales, 2017). Activated PMNs release relevant mediators to participate immune interactions. Some of PMN-derived mediators, such as neutrophil elastase (NE) and tumor necrosis factor (TNF)- α , can induce inflammation in the local tissues (Marzano et al., 2018; Polverino et al., 2017). PMN-derived proteases can cause tissue destruction (Sandhaus and Turino, 2013). These phenomena are observed in asthma (Sandhaus and Turino, 2013). The airway tissues directly interact with the external environment. Exogenous Ags, such as house dust mites, can be absorbed into the airway tissues to induce specific antibody, such as IgE and IgG, production. It is well known that specific IgE can activate mast cells to induce asthma attacks (Galli and Tsai, 2012). Since PMNs express IgG receptors (Rosales, 2017), specific IgG (sIgG) may activate PMNs. The inflammatory mediators from activated PMNs may be involved in asthma attacks in an Ag-antibody reaction-dependent manner (Ray and Kolls, 2017); this needs to be further investigated.

It is recognized that exosomes are involved in immune responses and immune regulatory activities (Robbins et al., 2016). Exosomes are small vesicles (about 60–100 nm) exfoliated from cells (Robbins et al., 2016). Some cellular components may be encapsulated into exosomes. Thus, an exosome may carry several immune regulatory substances to fulfill designed immune regulatory functions (Chen et al., 2011).

In this study, a fraction of Ag-specific PMN (sPMN) in asthma mice was identified and characterized with pertinent immune analysis approaches. Exosomes with immune regulatory functions were constructed. The effects of the exosomes on suppressing experimental asthma were assessed.

2. Materials and methods

2.1. Reagents

Fas (sc420287) and CD64 (sc420305) CRISPR reagent kits, antibodies of NIMP-R14 (sc-59,338; Alexa Fluor® 488), MBP (F-6, Alexa Fluor® 546), CD154 (F-1, Alexa Fluor® 594), F4/80 (C-7, Alexa Fluor® 546), Fas (G-9, Alexa Fluor® 594), CD11c (N418, Alexa Fluor® 488), DEC205 (PN-15, Alexa Fluor® 594), CD64 (C-6) and FasL (NOK-1) were purchased from Santa Cruz Biotech (Santa Cruz, CA). The neutrophil isolation kit was purchased from Miltenyi Biotech (San Diego, CA). The anti-Ovalbumin antibody (6C8, ab17293) was purchased from Abcam (Cambridge, MA). FITC labeling kit, Freund adjuvant, FITC-dextran (MW =40 kDa) were purchased from Sigma Aldrich (St. Louis, MO). FasL protein, ELISA kits of IgE, IgG, IL-4, IL-5, IL-13, NE, TNF- α , MBP and ECP were purchased from Dekewu BioMart (Beijing, China). Reagents and materials for RT-qPCR, Western blotting and immunoprecipitation were purchased from Invitrogen (Carlsbad, CA).

2.2. Mice

BALB/c mice (6–8-week-old) were purchased from the Guangdong Experimental Animal Center (Guangzhou, China). DO11.10 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a specific pathogen free facility with accessing food and water freely. The experimental procedures were approved by the Animal Ethical Committee at Southern Medical University.

2.3. Immunization of mice

Mice were subcutaneously injected with ovalbumin (OVA, 0.1 mg/mouse) mixed in 0.1 mL complete Freund adjuvant (CFA or 0.1 mL Alum) on the back skin on day 0 and day 7, respectively. The mice were boosted through back skin injection with OVA (0.1 mg/mouse) mixed in 0.1 mL incomplete Freund adjuvant on day 14 and day 21, respectively. The mice were then challenged with nasal instillation (containing OVA 5 mg/mL) at 50 μ L per nostril daily from day 22 to day 27.

2.4. Assessment of asthma response

On day 28 of the sensitization time course, the asthma response was assessed in mice. The asthma response includes airway resistance, BALF levels specific IgE (sIgE), sIgG, IL-4, IL-5 and IL-13, bronchoalveolar lavage fluid (BALF) levels of NE and TNF- α , inflammatory cell counts in BALF, following published procedures (Girodet et al., 2011; Mo et al., 2017).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cytokine and Ig levels in BALF were determined by ELISA with commercial reagent kits following the manufacturer's instructions. To determine the serum levels of OVA-specific IgE (sIgE) and sIgG, microplates were coated with OVA (20 μ g/mL) and incubated overnight at 4 °C. After blocking with 1% bovine BALF albumin (BSA) for 30 min, BALF samples (diluted at 1:10) or BALF was added to the wells (0.1 mL/well) and incubated at 37 °C for 30 min. The plates were washed with phosphate-buffered saline containing 0.05 % Tween 20 (PBST) 3 times, biotinylated anti-mouse IgE or IgG (1:5,000) was added to the wells and incubated for 2 h at room temperature, washed with PBST 3 times. Peroxidase-labeled streptavidin was added to the Wells and incubated at room temperature for 2 h. The positive immune products in the wells were developed by TMB. The reactions were terminated by adding H₂SO₄ (2 M). The optical density of each well was recorded in a micro plate reader (BioTek Synergy LX) at 450 nm.

2.6. Assessment of mouse airway resistance

Following published procedures (Chen et al., 2019), tracheal intubation was performed under general anesthetization, mice were fixed in a closed body drawing box and mechanically ventilated. Mice were nebulized with methacholine at gradient dosages (0, 6.25, 12.5, 25, and 50 μ g/mL at 0.1 mL). Then, data from 5 s to 1 min after inhalation were collected, and the lung resistance was calculated and presented as fold changes.

2.7. Histology of the lung tissues

A piece of the lung tissues was excised and fixed with 4% formalin overnight. The tissues were processed for paraffin sections. The sections were stained with hematoxylin and eosin. The histology was observed under a light microscope.

2.8. Inflammatory scores

Inflammatory status in the lung tissues was evaluated using a semi-quantitative scale of 0 (normal)–5 (severe) based on inflammatory cell infiltration, epithelial damage, perivascular PMNs, peribroncholar PMNs, and edema as reported previously (Girodet et al., 2011).

2.9. Assessment of mouse airway epithelial barrier permeability

asthma mice were challenged with nasal drops containing OVA (5 mg/mL) mixed with FITC-dextran (MW =40 kDa; diluted to 5 mg/mL) at 50–100 μ L/nostril. The instillation was repeated 2 more times

within 30 min. Two hours later, the mice were sacrificed. Blood samples were collected. The BALF was isolated from the blood samples and analyzed by a fluorescence spectrometer (BioSpectrometer fluorescence, Eppendorf, Germany). The levels of FITC-dextran in the BALF were calculated against a standard curve.

2.10. Isolation of lung single cells (LSC)

The lungs were excised and cut into small pieces and incubated with collagenase IV (1 mg/mL) in the culture for 30 min at 37 °C with mild agitation. Single cells were filtered through a cell strainer (70 µm first, then 40 µm). The LSCs were collected by centrifugation at 500 g for 5 min.

2.11. Cell culture

Cells were cultured in RPMI1640 medium. The medium was supplemented with penicillin (100 U/mL), streptomycin (0.1 mg/mL), L-glutamine (2 mM) and 10 % fetal calf BALF. Before handling over further experiments, cell viability was assessed by the Trypan blue exclusion assay; it was greater than 99 %.

2.12. Flow cytometry (FCS, in short)

On the surface staining, cells were stained with fluorescence-labeled antibodies of interest (see Reagents for detail) or isotype IgG for 30 min on ice. After washing with phosphate-buffered saline (PBS) 3 times, the cells were analyzed with a flow cytometer (FACSCanto II). In the case of intracellular staining, cells were fixed with 1% paraformaldehyde containing 0.1 % Triton X-100 for 1 h, washed with PBS and subjected to the same procedures of the surface staining. The results were processed with FlowJo (TreeStar Inc., Ashland, OR) with the data of isotype IgG staining as gating references.

2.13. Purification of immune cells

Cells were labeled with pertinent antibodies (see figures in detail) conjugated with fluorescence. Immune cells were purified by FCS. The cell purity was checked by flow cytometry. If the purity did not reach or over 95 %, FCS was repeated with the cells.

2.14. Purification of Ag-specific PMNs

Single cells were isolated from the mouse lung tissues with allergic inflammation. The cells were cultured in the presence of OVA (the specific Ag; 5 µg/mL) overnight. Cells were harvested, stained with a neutrophil-isolation kit (containing antibodies of NIMP-R14 and CD11b) and anti-CD154 antibody. The NIMP-R14⁺ CD11b⁺ cells were isolated by the magnetic cell isolation procedures following the manufacturer's instruction, that were regarded as PMNs. CD154⁺ PMNs were purified by FCS, which were regarded as Ag-specific PMNs.

2.15. Assessment of cell apoptosis

Cells were stained with propidium iodide (PI) at 5 µg/mL for 15 min and stained with an annexin V reagent kit following the manufacturer's instructions. The cells were analyzed with a flow cytometer. The annexin V⁺ cells or PI⁺ annexin V⁺ cells were regarded as apoptotic cells.

2.16. Protein extract preparation

The following procedures were performed at 4 °C. Cells were incubated with a lysis buffer for 30 min and then centrifuged at 13,000 g for 10 min. The supernatant was collected and used as cytosolic proteins. The pellets were resuspended in a nuclear lysis buffer and incubated for

30 min. The lysates were centrifuged at 13,000 g for 10 min. The supernatant was collected and used as nuclear proteins.

2.17. Western blotting

Proteins (50 µg/well) were separated by SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% skim milk for 30 min, the membrane was incubated with antibodies (diluted to 1:500) of interest (see figures in detail) overnight at 4 °C, washed with TBST (Tris-buffered saline containing 0.1 % Tween 20) 3 times, incubated with the secondary antibodies (conjugated with horseradish peroxidase) for 2 h at room temperature, washed with TBST 3 times. The immunoblots on the membrane were developed by the enhanced chemiluminescence and photographed in an imaging station (UVP, Cambridge, UK).

2.18. Immunoprecipitation (IP)

The following procedures were performed at 4 °C. The precleaning step was omitted because the target products were IgG. Proteins prepared from relevant experiments were incubated with antibodies of interest at a ratio of 1:1 overnight to form immune complexes. The complexes were precipitated by incubating with protein G agarose beads for 2 h at room temperature. The beads were collected by centrifugation at 5000 g for 10 min. Proteins on the beads were eluted with an eluting buffer and analyzed by Western blotting.

2.19. Preparation of bone marrow-derived dendritic cells (BMDC)

The femurs were excised from BALB/c mice. The BMs were flushed out the femurs with a syringe and saline. Red blood cells in the BMs were lysed with a red blood cell lysis buffer (Sigma Aldrich). BM cells were cultured in RPMI1640 medium plus 40 ng/mL GM-CSF (Sigma Aldrich) and 20 ng/mL IL-4 (R&D Systems). On day 3, the RPMI-1640 complete medium was changed together with 20 ng/mL GM-CSF. On days 6 and 8, half of the supernatants were removed and replaced with 10 mL fresh complete medium with GM-CSF (40 ng/mL) and IL-4 (20 ng/mL). On day 9, non-adherent cells were collected and used as BMDCs. As assessed by flow cytometry, more than 95 % BMDCs were CD11c⁺ MHC II⁺.

2.20. Depletion of fas in BMDCs and CD64 in sPMNs

BMDCs or PMNs (5×10^6 cells per sample) were mixed with 15 µg Fas or CD64 CRISPR-carrying plasmids following the manufacturer's instruction. Electroporation was performed with the cells at 1650 V for 20 ms, one pulse was used for each sample. Cells were then cultured in 5 mL RPMI1640 medium. The efficiency of CRISPR was assessed by Western blotting 48 h after the treatment.

2.21. Preparation of sPMN-targeting exosomes (tExo)

Fas-deficient BMDCs (the depletion of Fas is to avoid BMDCs responding FasL) were cultured in the presence of FasL (0.1 mg/mL) and anti-CD64 antibody (0.1 mg/mL) overnight. The supernatant was collected and processed to purify the exosomes referring to published procedures (An et al., 2018). Briefly, supernatant was transferred into Ultra-Clear tubes (Beckman Coulter, Indianapolis, IN) at 4 mL/tube and centrifuged at 100,000 g in a Beckman Optima XL-70 ultracentrifuge for 12 min. Two milliliters supernatant was removed and 2 mL PBS was added to each tube and centrifuged at 100,000 g for 70 min. The last procedure was repeated 3 more times. The supernatant was removed. The pellets were resuspended in PBS; exosomes were further purified by the magnetic anti-CD9 antibody system following published procedures (Macias et al., 2019).

2.22. Treating asthma mice with tExos

After completing the immunization, asthma mice were treated with tExos (or control exosomes) via nasal instillation (50 μ L/nostril) daily from day 28 to day 34. Mice were sacrificed on day 35. The asthma parameters of each mouse were assessed.

2.23. Statistics

The data are presented as mean $\pm$ SEM. The difference between two groups was determined by the Student *t*-test. ANOVA followed by the Dunnett's test or Bonferroni test was performed for multiple comparisons. $P < 0.05$ was considered statistical significance. Pearson correlation coefficient test was performed to identify correlation between data from two groups.

3. Results

3.1. BALF PMN counts are positively correlated with asthma responses in mice

An asthma mouse model was developed with ovalbumin (OVA) as a specific Ag and complete Freud adjuvant (CFA) as an immunizing enhancer. The mice showed airway hyper-responsiveness, high inflammatory scores in the lung tissues, increase in Ag-specific IgG (sIgG), sIgE, mediators released from PMNs and Eos in BALF and enhanced airway

epithelial barrier permeability (Fig. 1A–H). It is noteworthy that although the sIgE levels were increased in the CFA group mice as compared with the saline control (SC) mice, the sIgG levels were even higher than that of sIgE ($p < 0.01$). Proinflammatory cytokines (IL-4, IL-5, IL-13 and IFN- γ) were increased in the serum of mice immunized with OVA/CFA (Fig. S1A–D in supplementary materials). BALF cellular components consisted of PMNs (26.9 % $\pm$ 5.0), Eos (12.7 % $\pm$ 1.3) and macrophages (10.9 % $\pm$ 2.8) in the OVA/CFA group (the CFA group), which were (2.5 % $\pm$ 0.8), (5.2 % $\pm$ 1.2) and (21.1 % $\pm$ 3.4), respectively, in the SC group (Fig. 1I–K, Fig. S2). A positive correlation was detected between the lung inflammatory scores and the sIgG levels in BALF, or BALF PMN frequency, or BALF NE levels, or BALF TNF- α levels (Fig. S3A–D). No correlation was found between the lung inflammatory scores and the BALF sIgE, Eos, M ϕ s, MBP, EPX (Fig. S3E–I).

For a comparison, in separate experiments, BALB/c mice were immunized with OVA/Alum (the Alum group), the conventional asthma protocol (Liu et al., 2019), to induce asthma. The Alum group mice showed asthma signs similar to the CFA group mice; the difference between the CFA group and the Alum group included higher levels of sIgE, MBP and EPX, higher frequency of Eo and lower PMN frequency in BALF (Fig. 1, Fig. S1–2). The results indicate that the OVA/CFA protocol induces the PMN-dominant lung inflammation in asthma mice while the OVA/Alum protocol induces the Eo-dominant lung inflammation in asthma mice.

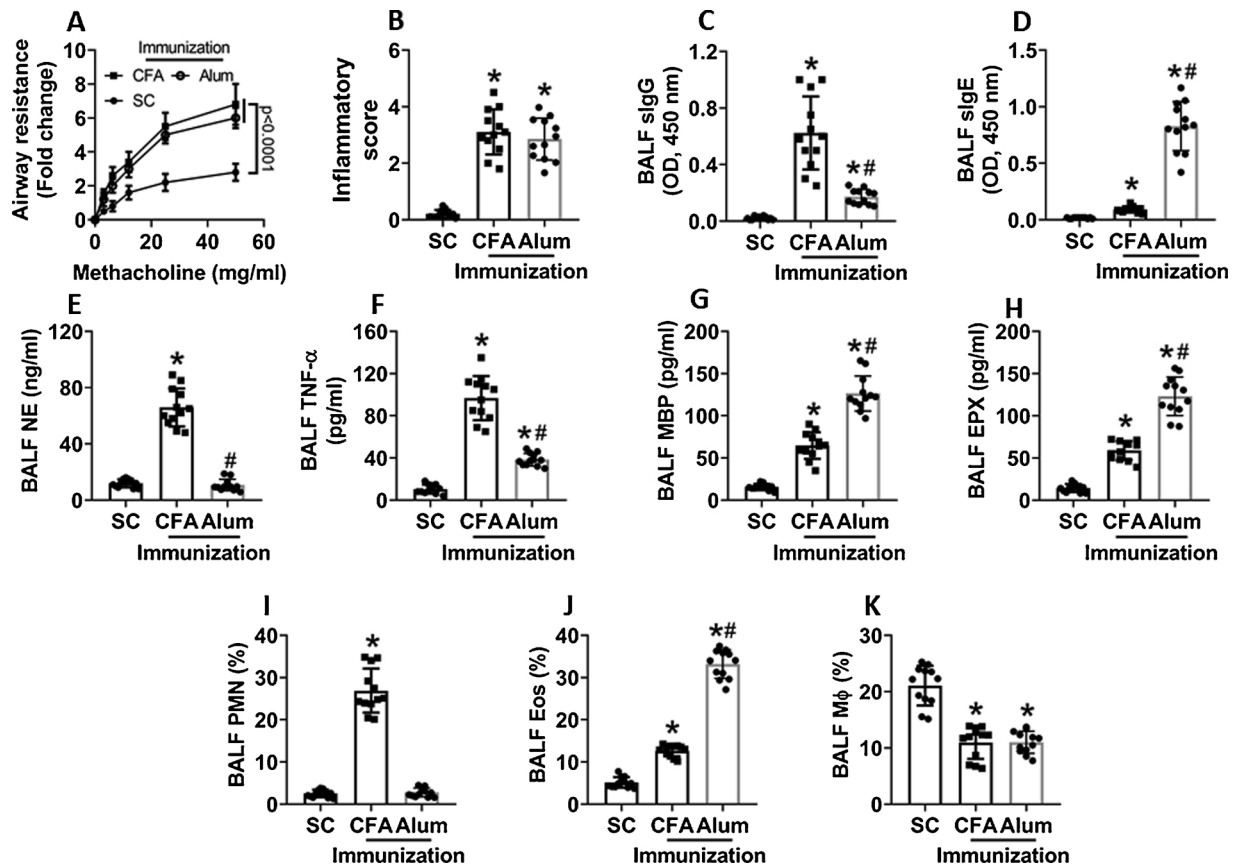


Fig. 1. Mouse airway allergic inflammation (asthma) is positively correlated with PMN number in BALF. BALB/c mice (12 mice per group) were immunized with ovalbumin (OVA)/complete Freud adjuvant (CFA) or OVA/Alum or saline (saline control; SC) to induce asthma. A, airway resistance in response to inhalation of methacholine. B, bars show inflammatory scores in mice. C–D, bars show antigen-specific IgG (sIgG) (C) or sIgE (D) levels in bronchoalveolar lavage fluids (BALF). E–H, bars show levels of NE (neutrophil elastase), TNF- α (tumor necrosis factor- α), MBP (major basic protein) and EPX (eosinophil peroxidase) in BALF. I–K, bars show frequencies of PMN, Eo and M ϕ in BALF (the FCS plots are presented in Fig. S2). The data of bars are presented as mean $\pm$ SEM of 12 mice per group. Each dot in bars presents data obtained from one mouse. * $p < 0.01$ (ANOVA followed by the Dunnett's test), compared with the SC group. #, $p < 0.01$ (the Student *t*-test), compared with the CFA group.

3.2. Specific Ag activates PMNs isolated from the airway tissues of asthma mice

As shown by Fig. S3, BALF sIgG is positively correlated with the frequency of BALF PMNs, suggesting that specific Ags may activate these PMNs. To test this, PMNs were isolated from the airway tissues (Fig. 2A) of OVA/CFA-immunized mice by FCS. The PMNs were exposed to OVA in the culture overnight. With the CD154-expression as an activation marker (Chiang et al., 2018; Zhou et al., 2020), we found that exposure to specific Ag (the OVA) markedly induced PMN activation (Fig. 2B–C), although this phenomenon was previously found in Ag-specific CD4⁺ T cell activation (Chiang et al., 2018). NE and TNF- α , the representative PMN mediators, were detected in culture supernatant (Fig. 2D–E). Exposure to bovine serum albumin (BSA, used as a non-specific Ag) did not activate PMNs, nor induce the mediator release from PMNs (Fig. 2B–E). The results demonstrate that there is a fraction of Ag-specific PMNs (sPMNs) in asthma mice. sPMNs can be activated upon exposure to specific Ags.

3.3. sIgG binds CD64 on the surface of PMNs to make the PMNs sensitized

We next isolated PMNs from naive mice by FCS. The PMNs were

exposed to sIgG (OVA-specific; labeled with an FITC-labeling kit following the manufacturer's instruction) at gradient concentrations in the culture for 2 h. The PMNs were analyzed by FCS. The results showed that sIgG bound to the surface of PMNs in a concentration-dependent manner (Fig. 3A–B). Depletion of CD64 (Fig. 3C) abolished the binding (Fig. 3A–B). A complex of sIgG and CD64 was identified in the PMNs by immunoprecipitation (IP) (Fig. 3D). The results indicate that sIgG can bind to CD64 on the surface of PMNs. Furthermore, we exposed the sIgG/CD64-bearing PMNs to OVA (the specific Ag) or BSA (an irrelevant Ag) in the culture overnight, that induced the PMNs to release NE and TNF- α into culture supernatant in the OVA-group, but not in the BSA group (Fig. 3E–F). The results indicate that, after the binding of sIgG to the CD64, PMNs are at the sensitized status. Re-exposure to specific Ags induces the sensitized PMNs to release preformed mediators to the microenvironment. Taking together, the data of Figs. 1–3 suggest that the sPMNs may be associated with the asthma pathogenesis.

3.4. Construction of tExos that contain a fusion protein of α CD64/OVA carried by exosomes

Aiming to alleviate or inhibit asthma by specifically removing sPMNs, we constructed tExos. The tExos are exosomes carrying complexes of anti-CD64 Ab (α CD64)/OVA/MHC II. Bone marrow-derived

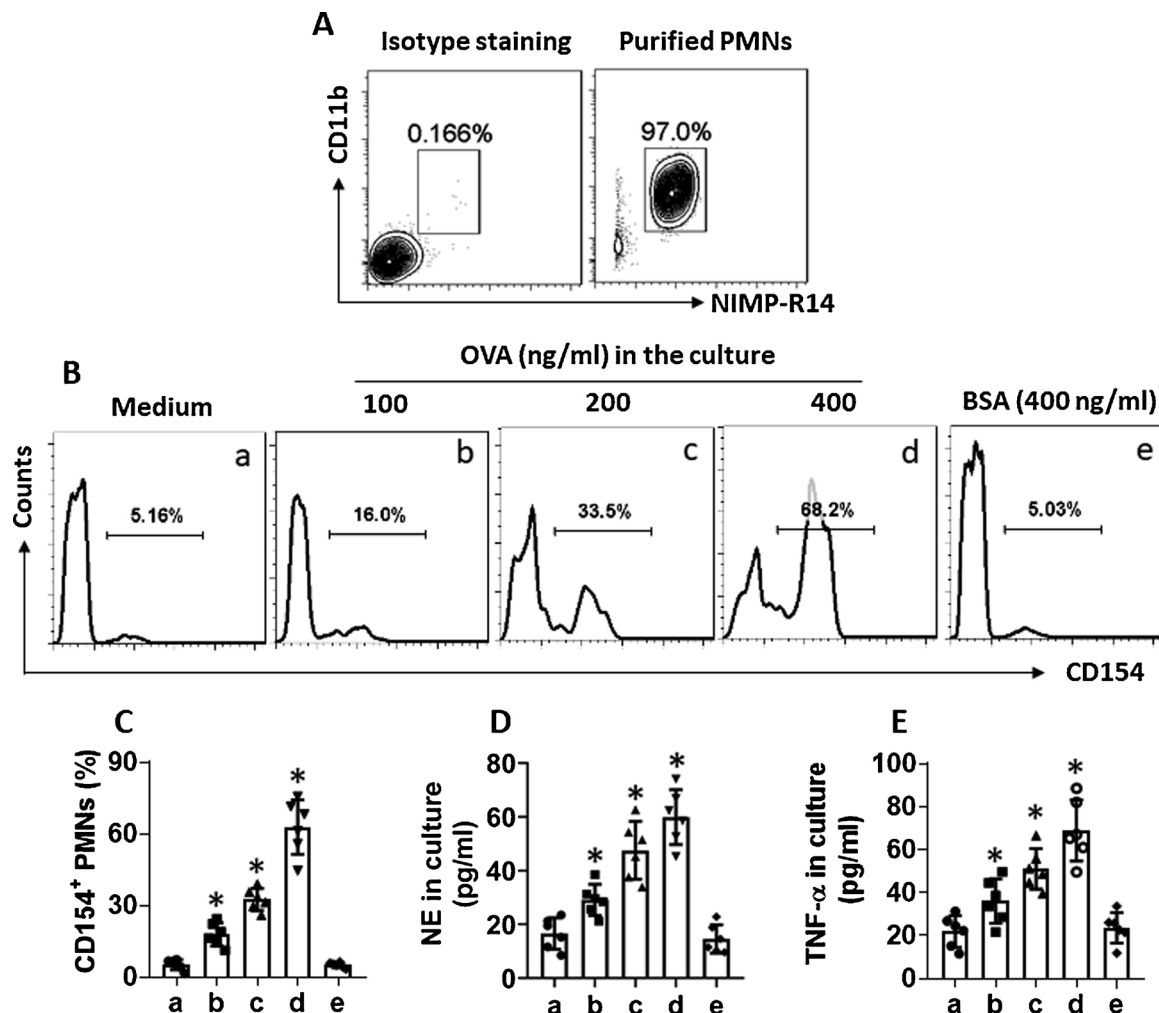


Fig. 2. specific Ags activate sPMNs isolated from the airway tissues of sensitized mice. PMNs were isolated from the airway tissues of asthma mice (6 mice per group) by FCS and exposed to OVA at indicated concentrations overnight. The cells were analyzed by flow cytometry. B, gated histograms show the frequency of CD154⁺ PMNs. C, bars show summarized CD154⁺ PMNs. D–E, culture supernatant was collected at the end of culture and analyzed by ELISA. Bars show levels of NE (D) and TNF- α (E) in culture supernatant. The data of bars are presented as mean $\pm$ SEM. Each dot in bars presents data obtained from one mouse. Statistics: ANOVA followed by the Dunnett's test. * $p < 0.01$, compared with group "a". A and B, the data are from one experiment that represent 6 independent experiments.

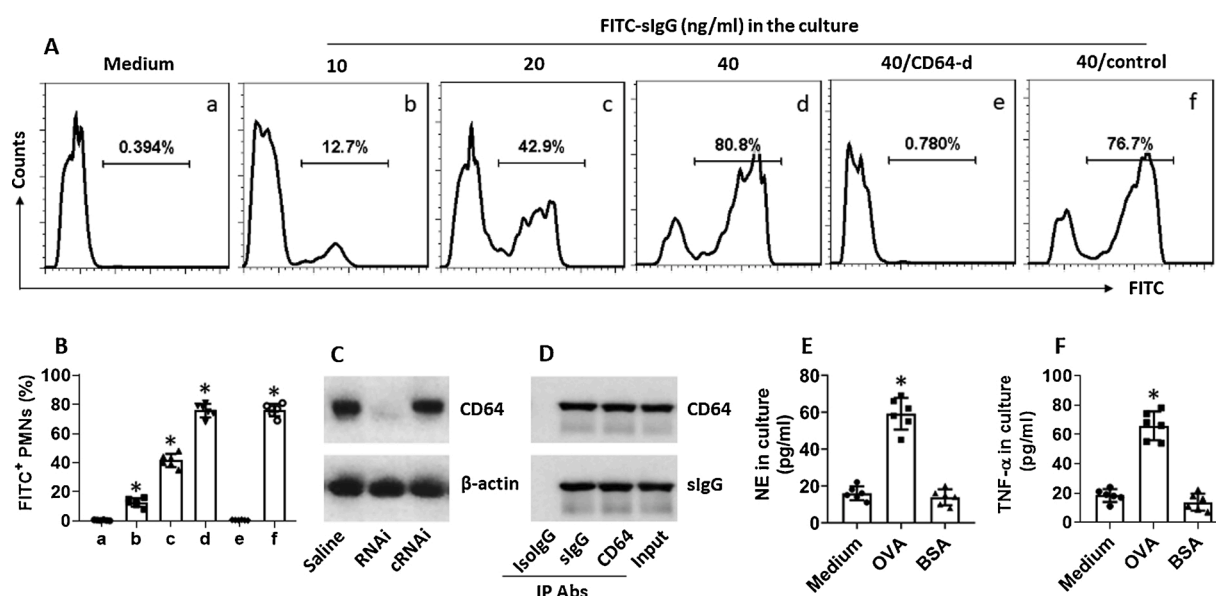


Fig. 3. sIgG sensitizes PMNs. PMNs were isolated from naïve mice by FCSS. A, PMNs were cultured in the presence of sIgG (OVA-specific; labeled with FITC) for 2 h and analyzed by flow cytometry. Gated histograms show the frequency of sIgG-bound PMNs. CD64-d: CD64-deficient PMNs (prepared by RNAi). Control: PMNs were treated with control RNAi reagents. B, summarized data of panel A. C, immunoblots show CD64 RNAi results. D, immunoblots show a complex of sIgG and CD64 (by IP). E–F, bars show levels of NE (E) and TNF-α (F) in culture supernatant (by ELISA). The data of bars are presented as mean ± SEM. Each dot in bars presents data obtained from one experiment. Statistics: ANOVA followed by the Dunnnett's test. * $p < 0.01$, compared with group "a" (panel B) or the medium group (E and F). Data of A, C and D are from one experiment that represent 6 independent experiments.

DCs (BMDCs) were generated and cultured in the presence of αCD64 Ab and OVA (the specific Ag used to develop the asthma animal model in this study) overnight. Exosomes were purified by CD9-bearing magnetic beads from the culture supernatant. Protein extracts of purified exosomes showed exosome markers (CD9, CD63 and CD81) (Fig. 4A). The proteins were analyzed by IP; complexes of αCD64/OVA/MHC II were

detected in protein extracts of the exosomes (Fig. 4B). We found that more than 80 % tExos could bind to sPMNs, but not naïve PMNs, nor those carrying OVA alone or αCD64 alone, or not carrying both OVA and αCD64, or sPMN with CD64 depletion (Fig. 4C–D). The data indicate tExos can bind sPMNs.

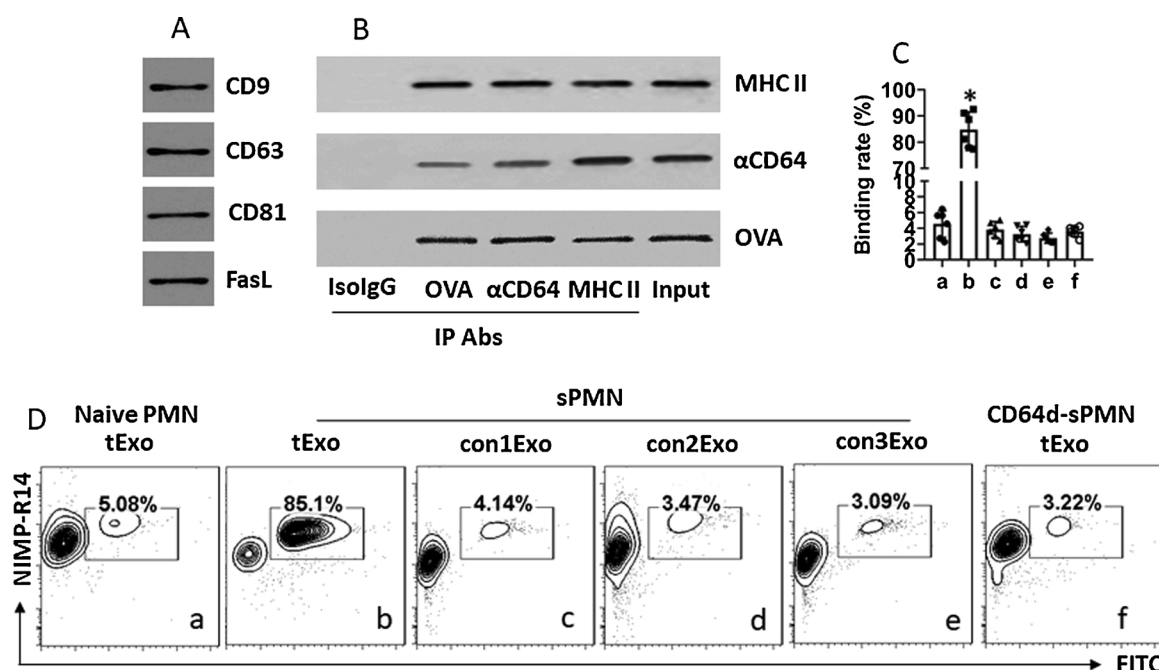


Fig. 4. tExos specifically bind sPMNs. A–B, proteins were extracted from tExos and analyzed by Western blotting and IP. Immunoblots show CD9, CD63, CD81 and FasL (A) and a complex of MHC II, αCD64 (anti-CD64 Ab) and OVA (B). C–D, naïve PMN, sPMN, CD64d-sPMN (CD64-deficient sPMN) were separately cultured in the presence of FITC-tExos (20 µg/mL) or control Exos for 30 min. The cells were then analyzed by flow cytometry to assess the frequency of tExo-bound cells. The bars show mean ± SEM of Exo-binding rate. The representative flow cytometry plots show the Exo-binding to PMNs. * $p < 0.0001$, compared with group a (ANOVA followed by the Dunnnett's test). con1Exo: Exosomes contain OVA but no αCD64 Ab; con2Exo: Exosomes contain αCD64 Ab but no OVA; con3Exo: exosomes contain neither OVA nor αCD64 Ab; CD64d-sPMN: CD64-deficient sPMNs.

3.5. tExos induce sPMN apoptosis

We then isolated PMNs from the asthma and naïve mouse airway tissues by FCS. PMNs were exposed to tExos or control exosomes in the culture overnight. We observed that exposure to tExos markedly induced sPMN apoptosis, that did not occur in naïve PMNs, or sPMNs treated with control exosomes (Fig. 5). The results demonstrate that tExos can induce sPMNs apoptosis.

3.6. tExos alleviate experimental asthma

To test if tExos have the translational potential in the treatment of asthma, an asthma mouse model was developed, of which mice were immunized with OVA/CFA. As shown by Fig. 6 and Fig. S5, the mice showed asthma, manifesting inflammation in the lung tissues, airway hyper responsiveness, high levels of NE, TNF- α , sIgE, sIgG, IL-4, IL-5, IL-13 and IFN- γ in BALF as well as high frequencies of PMNs, Eos and low frequency of M ϕ in BALF. Treating the mice with tExos through nasal instillation daily for 7 days efficiently suppressed asthma. It is noteworthy that administration of tExos not only reduced PMNs in BALF, but reduced Eos in BALF as well. The M ϕ number in BALF, however, was increased after the tExo treatment.

4. Discussion

Although the PMN infiltration in the airway tissues is common in the asthma pathology (Ray and Kolls, 2017), the underlying mechanism is not fully understood yet. The present study revealed that profound PMN infiltration was observed in the airway tissues as well as in BALF of asthma mice immunized with the OVA/CFA protocol. The PMN frequency in BALF was positively correlated with the inflammatory scores in the lung, while the frequency of Eos, the mostly discussed inflammatory cells in asthma (Johansson, 2014), was not correlated with the inflammatory scores in the lung. A fraction of PMNs in the asthma mouse airway tissues could be activated in an Ag-specific manner. Administration of tExos inhibited the Ag-specific PMNs and suppressed airway inflammation.

We found that the PMN frequency in BALF was positively correlated

with asthma response in mice immunized with OVA/CFA. It was recognized that PMNs play an important role in the pathogenesis of severe asthma (Ray and Kolls, 2017). Our data are consistent with the findings of clinical observation (Ray and Kolls, 2017) by showing that profound PMN infiltration in the lung tissues as well as in BALF. PMNs contain many chemical mediators, such as NE and TNF- α . By releasing these mediators, PMNs fulfill the defensive roles against invading microbes. However, these mediators can also cause host tissue injury. We also found high NE levels in the asthma mouse BALF. In addition, many murine asthma models show Eo dominant inflammation in the airways. For example, Chen et al. immunized mice with OVA/Alum and found an Eo dominant inflammation in the airways (Chen et al., 2017); Theiler et al. reported Eoia in the mouse airways after immunizing with OVA/Alum that could be alleviated by butyrate (Theiler et al., 2019). The present data show a neutrophil-dominant inflammation in the airways in a murine model. The difference between our results and previous reports (Chen et al., 2017; Theiler et al., 2019) may be because we used different adjuvants in the immunization. Similarly, by employing CFA plus Ags, neutrophilic inflammation was induced in the murine heart (Chen et al., 2020).

We also observed the frequency of Eos was increased in asthma mouse BALF. But the Eo frequency was not correlated with the asthma response, such as the inflammatory scores in the lung histology. Eosinophil infiltration is a common sign of allergic asthma (Yancey et al., 2017). By releasing toxic mediators, Eos contribute to the inflammation of allergic asthma (Yancey et al., 2017). Since it is still on debate whether Eos have the IgE receptors, the mechanism by which Eos activated by specific Ags is incompletely understood (Abdala-Valencia et al., 2018). The present data show that, by depleting PMNs, the asthma response was abrogated in mice, although no specific actions were performed on regulating Eos, the number of Eos was reduced in BALF as well. The results imply that PMNs may play a role in the activating Eos in the lung of asthma subjects; the underlying mechanism needs to be understood in further investigation.

Although it is well documented that PMNs are one of the inflammatory cells in asthma attacks (Ray and Kolls, 2017), the mechanism inducing PMNs activated has not been well determined. The present data show that a complex of CD64 and sIgG on the surface of PMNs, that

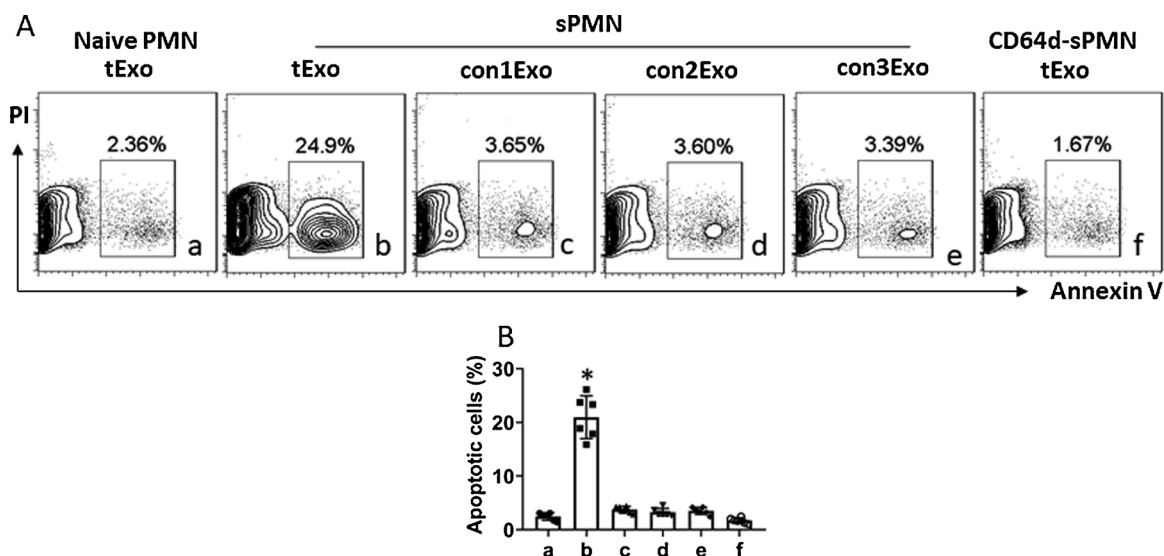


Fig. 5. tExos induce sPMNs apoptosis. PMNs were isolated from the lung tissues of asthma mice ($n = 10$); naïve PMNs, macrophages and DCs were isolated from the lung tissues of naïve mice ($n = 10$); CD4⁺ T cells were isolated from the lung tissues of DO11.10 mice ($n = 3$). The cells (10^6 cells/mL, in triplicate) were treated with tExos or control exosomes in the culture (20 μ g protein/mL) overnight as denoted above each subpanel of panel A. A, gated flow cytometry plots show apoptotic cell population, including Annexin V⁺ and Annexin V⁺ PI⁺ cells. B, the bars show mean $\pm$ SEM of apoptotic PMN counts in panel A. Statistics: ANOVA followed by the Dunnett's test. * $p < .0001$, compared with group "a". con1Exo: Exosomes contain OVA but no α CD64 Ab; con2Exo: Exosomes contain α CD64 Ab but no OVA; con3Exo: exosomes contain neither OVA nor α CD64 Ab; CD64d-sPMN: CD64-deficient sPMNs.

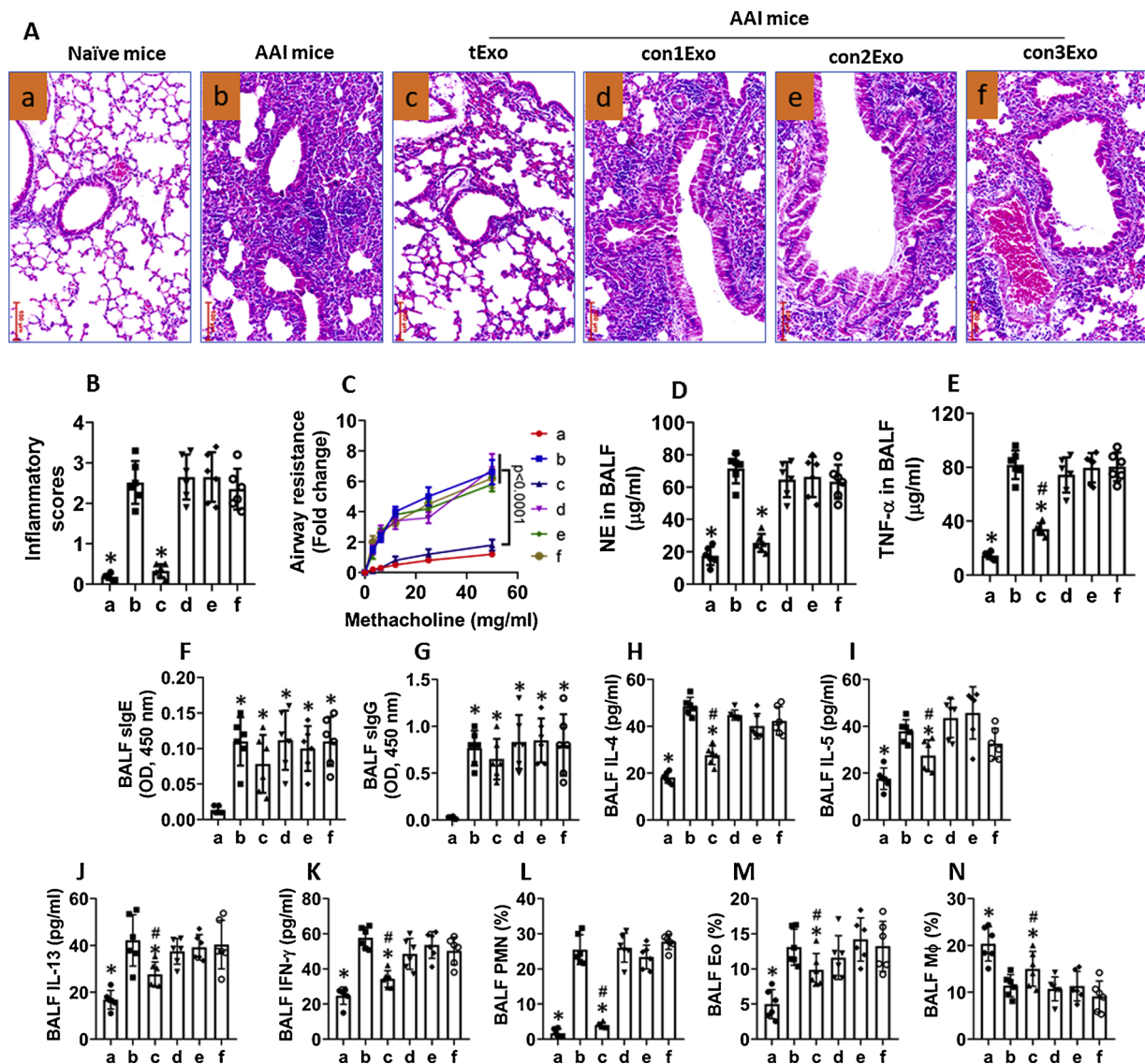


Fig. 6. tExos inhibit asthma in mice. asthma mice (6 mice per group) were treated with tExo-containing nasal drops (as denoted above each subpanel of A) daily for one week. A, representative mouse lung histology images ($\times 200$). B, inflammatory scores of the lung. C, airway resistance in response to methacholine challenge. D–E, NE and TNF- α levels in BALF. F–K, bars indicate the levels of sIgE (F), sIgG (G), IL-4 (H), IL-5 (I), IL-13 (J), IFN- γ (K), PMNs (L), Eos (M) and M ϕ s (N) in BALF (the FCS plots of L–N are presented in Fig. S5). The group labels of panel B–N are the same as panel A. The data of bars are presented as mean $\pm$ SEM of 6 mice per group. Statistics: ANOVA followed by the Dunnett's test. *, $p < 0.01$, compared with group b. #, $p < 0.05$, compared with group a. con1Exo: Exosomes contain OVA but no α CD64 Ab; con2Exo: Exosomes contain α CD64 Ab but no OVA; con3Exo: exosomes contain neither OVA nor α CD64 Ab.

confers the PMNs the ability to recognize specific Ags. This feature is similar to the IgE/Fc ϵ RI complex on the surface of mast cells, the major mechanism in evoking allergy attacks. It is a consensus that once sIgE binds the Fc ϵ RI on the surface of mast cells, the mast cells thus gain the ability to trigger allergic responses (Galli and Tsai, 2012). The mast cells are then at the “sensitized” status. Our data show that exposure to specific Ag can activate the sPMNs and induces sPMNs to release inflammatory mediators, such as NE and TNF- α , the representative inflammatory mediators in many inflammatory disorders, such as severe asthma (Leivo-Korpela et al., 2011) and inflammatory bowel disease (Silva et al., 2019). The data suggest that sPMNs are at the sensitized status. Once exposure to specific Ags, sPMNs can be activated and release inflammatory mediators to evoke inflammatory responses in the local tissues. The reasoning is well supported by the present data. By exposing to specific Ags, immunized mice showed inflammation in the airway tissues. Depletion of PMNs by tExos abolished the inflammation. Whether this phenomenon also plays a role in human diseases, such as severe asthma, is worth to be investigated.

Current therapies for asthma mainly include corticosteroids, anti-histamines and immunotherapy (Chung, 2017). If infections are complicated, antibiotics are required. In fact, administration of corticosteroids and anti-histamines can well control the asthma symptoms; but the therapeutic effects only last for a short time (Chung, 2017). Furthermore, using corticosteroids may result in many adverse effects and complications (Aldibbiat and Al-Sharefi, 2020). The present data shed light on the treatment of asthma. In the sPMN-dominant asthma, to prevent sPMN activation can suppress the asthma response. The tExos can specifically recognize the sPMNs and induce the sPMNs apoptosis. Thus, the tExo therapy does not affect other PMNs and do not have the possibility in affecting the defensive system of the body.

We found that, after immunizing mice with OVA/CFA, both Th1 and Th2 cytokines were up regulated in the airways. Other investigators also found this phenomenon. For example, Tokunaga et al. observed that the concentrations of IFN- γ , IL-5, IL-4, IL-13, and eotaxin in the BALFs were significantly increased in mice after immunization (Tokunaga et al., 2019). Raundhal et al. reported high IFN- γ levels were found in asthma

patients (Raundhal et al., 2015). The data showed fewer Mφs in BALF of the asthma mice. This may be because more PMNs in the CFA group and more Eos in the Alum group infiltrate in the lung, that results in the Mφ number is relevantly fewer.

In summary, the present study identified a fraction of PMNs is Ag-specific. Exposure to specific Ags can activate the sPMNs and induce sPMNs to release inflammatory mediators in the airway to induce the PMN-dominant lung inflammation. Administration of tExos can reduce sPMNs in the lungs and alleviate the PMN-dominant asthma in experimental mice. The data suggest that the tExo has the translational potential in the treatment of asthma.

Author contributions

MZZ performed all the experiments. MZZ, YL, HYH, LHM, GY, ZQL and CM took part some of the experiments. PCY and SWL organized the study and supervised experiments. PCY prepared the manuscript. All authors reviewed the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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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.2020.11.005>.

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