An epicutaneous therapeutic pollen-allergen extract delivery system in the allergic rhinitis mouse model: based on allergen loading on DC-specific aptamers conjugated nanogolds

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Abstract

Background: We investigated whether gold nanoparticles (GNPs) and dendritic cells (DC)-specific aptamer-modified GNPs (Apts-GNP) could be effective for epicutaneous immunotherapy (EPIT) in the case of pollen allergen extracts containing a variety of allergenic and nonallergenic components.

Methods: BALB/c mice were sensitized to total protein extract of *Platanus orientalis* pollen and then epicutaneously treated in different groups either with free *P. orientalis* total pollen extract, naked GNPs, the total extract loaded GNPs, and the total extract loaded Apts-GNPs with and without skin-penetrating peptides (SPPs). We then examined the specific IgE level (sIgE), total IgE concentration (tIgE) in the serum sample, IL-4, IL-17a, IFN-γ, and IL-10 cytokine concentrations in re-stimulated splenocytes with the total extract and the mixture of its recombinant allergens, nasopharyngeal lavage fluid (NALF) analysis, and histopathological analysis of lung tissue.

Results: The current study showed that the total extract-loaded GNPs, especially Pla. ext (50 µg)-GNPs, significantly decreased sIgE, tIgE, IL-17a, and IL-4 concentrations, immune cells and eosinophils infiltration in NALF, and increased IL-10 and IFN-γ concentrations compared with the PBS-treated group. In addition, histopathological analysis of lung tissue showed that it also led to a remarkable decrease in allergic rhinitis (AR)-associated inflammation and histopathological damage. The DC-targeted group showed greatest improvement in AR-related immune factors and had no histopathological damage compared with the same dose without aptamer.

Conclusion: Consequently, it seems that loading total protein extract on the GNPs and the Apt-modified GNPs could be an effective approach to improve EPIT efficacy in pollen-induced AR.

1. Introduction

Given the dramatic increase in the prevalence of IgE-mediated allergic diseases over the last century, there is an urgent need to develop effective treatments (1). More than 10–40% of the world's population suffers from allergic rhinitis (AR), which has also increased dramatically in recent years. In general, outdoor allergens such as pollen pretense a major risk for pollen allergy, which is the most common form of AR and accounts for more than 50% of all cases (2, 3). To date, allergen-specific immunotherapy (AIT) is the only cause-oriented treatment available for hypersensitivity reactions (1). In patients whose symptoms cannot be adequately controlled with medications, it reduces the immune system's sensitivity to allergens, including pollen. The most common forms of AIT today are subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) (4). SCIT is considered the gold standard, and SLIT has been shown to be equally effective and safe (5). However, both have some disadvantages, such as the time-consuming nature, the need for many medical appointments, and the possibility of local and systemic adverse reactions (6–9). Accordingly, novel approaches that alter the route of administration and formulation of allergens may be suitable to overcome these limitations (9).

It is intriguing that AIT can be administered by epicutaneous antigen application to the epidermis. Since the epidermis is not a vascular zone, this could decrease the risk of adverse reactions due to systemic side effects. Furthermore, the epidermis contains a large number of antigen-presenting cells (APCs), including langerhans cells, which may result in fewer allergen injection (10). Epicutaneous immunotherapy (EPIT) has attracted considerable attention in recent years. In addition, many studies have shown that EPIT can significantly increase IgG2a levels, decrease IgE levels, reduce Th2 responses with a large reduction in IL-13, IL-5, and IL-4, cytokine levels, increase regulatory T cells (Treg) responses with a substantial increase in transforming growth factor β (TGF-β), and cause a large reduction in eosinophils of bronchoalveolar lavage (1, 11, 12). It seems that, a major limitation of EPIT is the passage of allergens through the intact epidermis, which is largely confined by the stratum corneum (SC) (13). Therefore, the studies of EPIT field aim to increase efficacy by maximizing the passage of therapeutic agents through the skin and minimizing the allergen dose. Researchers propose a variety of innovative techniques to achieve this goal, which include nanoparticles (NPs) as drug delivery systems, microneedles coated with allergens, and fractional infrared laser ablation to create micropores in the skin (14–16). Gold nanoparticles (GNPs), offer a number of advantages as transdermal, biocompatible, and nontoxic delivery systems compared with conventional delivery methods, including improved immunogenicity, enhanced and accelerated uptake of the agents by macrophages and dendritic cells (DC), and may also serve as adjuvants and also exert an anti-inflammatory effect (17–19). Delivery of therapeutic agents to the target site or target cells can increase therapeutic efficacy by reducing the efficacious dose and ultimately reducing any adverse effects (20). In this context, we have already shown promising results for EPIT using functionalized GNPs in combination with DC-specific aptamers as nanoparticle-mediated targeted therapy, and of course in EPIT with a recombinant allergen. Our previous study also examined the effects of skin-penetrating peptides (SPPs) on transdermal delivery of therapeutic agents (12).

Since allergenic substances contain different molecules, it is reasonable to assure that EPIT with total pollen extract-loaded on DC-specific aptamers conjugated nanogolds would be as effective as EPIT with specific recombinant allergen. In this study, we therefore used GNPs and functionalized GNPs with DC-specific aptamers as transdermal targeted allergen delivery systems in allergic rhinitis mouse models for the first time in EPIT with a total pollen extract (*Platanus orientalis*) (Fig. 1). In addition, SPPs (mixture of TD1 and R7) were used to increase the permeability of intact skin and improve the efficacy of EPIT.

2. Materials and methods

2.1. Materials

Mouse IgE antibody, mouse interferon-gamma (IFN-γ), interleukin-10 (IL-10), interleukin-17 (IL-17), interleukin-4 (IL-4) ELISA kits and BCA (bicinchoninic acid) protein assay kit were purchased from Parstous Biotechnology Company (Iran). In addition, Parstous Biotechnology Company (Iran) provided the TMB and stop solution. Biotin anti-mouse IgE antibody and horseradish peroxidase-conjugated streptavidin were provided by Biolegend (USA). Trisodium citrate, Sodium tetrachloroaurate (III) ($HAuCl_4$),

and dithiothreitol (DTT) were purchased from Himedia (India), Sigma (USA), and SBS Genetech Company (China), respectively. Aluminum hydroxide (Al (OH) 3) was purchased from Sigma-Aldrich (USA). Phytohemagglutinin (PHA), RPMI 1640 culture medium, and their additives were provided by Gibco (UK). The DC -targeted aptamer (5'-thiol-T10-GGCTGGCACTGGTCGAGGTATGTTGGGCAGCT-3') previously developed by our group (21), produced by Bioneer (South Korea). Synthetic SPPs containing cyclic-TD1 (ACSSSPSKHCG) and R7 (an oligomer containing 7 L-arginine amino acids) were manufactured by Mebep Bioscience Company (China).

2.2. Animals

We acquired 50 female BALB/c mice aged 5–6 weeks (weight, 16–20 g) from the Pasteur Institute (Tehran, Iran). The mice were kept under specific pathogen-free conditions at the Animal Care Centre of the Nanotechnology Research Centre (Mashhad University of Medical Sciences, Mashhad, Iran) according to local animal care guidelines. It is important to mention that all experiments were done in accordance with the Animal Ethics Committee (IR.MUMS.MEDICAL.REC.1399.727) of Mashhad University of Medical Sciences.

2.3. Preparation of Pollen Allergenic Extracts

For the preparation of *P. orientalis* total pollen extract, as mentioned in the previous studies (22) with some minor modifications; Fresh *P. orientalis* pollen samples were collected during the main pollination months. To verify purity and eliminate contamination, microscopic examinations were performed. Subsequently, acetone defatting was performed prior to pollen extraction. 1 g *P. Orientalis* pollen was added to 100 ml of acetone with overnight shaking at 4°C. After incubation, the mixture was centrifuged at 15,000 g for 30 min at 4°C. The precipitates containing the pollen were dried at room temperature after the supernatant was removed. The dried pollen was extracted overnight at 4°C in 10 ml phosphate buffer (10 mM, pH 7). Finally, the supernatant was dialyzed in phosphate buffer (10 mM, pH 7) and then filtered (22). Protein concentration was determined using a BCA protein assay kit.

2.4. Expression and purification of recombinant Pla or 1, Pla or 2, Pla or 3 and cyclophilin allergens

To examine whether EPIT with Pla. ext-GNPs and Pla. ext-Apts-GNPs complex effectively modulated the immune response against the major allergens of the total extract, we examined the ex vivo immune responses after restimulation with *P. orientalis* total pollen extract and its recombinant major allergens. For the expression of the recombinant four major allergens of *P. orientalis* pollen (rPla or 1, rPla or 2, rPla or 3, and cyclophilin), we followed our previous laboratory study (22–25). After expression of the recombinant allergens in E. coli BL21CodonPlus (DE3) RIL (Stratagene, USA), a Ni- IDA metal affinity chromatography column (Parstous, Iran) was used to purify these recombinant proteins, which were then dialyzed and frozen for later applications. The IgE reactivity of these recombinant allergens with *P. orientalis* pollen–allergic patient sera was previously studied and confirmed by our laboratory. The concentration of these recombinant proteins was determined using a BCA protein assay kit (Parstous, Iran).

2.5. Preparation of Pla. ext-Apts-GNPs complex

2.5.1. Gold nanoparticles synthesis and characterization

GNPs were synthesized by a classical citrate reduction method (26). Briefly, 2.5 ml of trisodium citrate solution (38.8 mM) was rapidly added to 25 ml of boiling HAuCl4 solution (1 mM, 25 ml) and stirred vigorously in a 100 ml round-bottomed flask under reflux condenser. Then, this solution was boiled under reflux for 30 min, and the color changed from yellow to wine red. After cooling the GNP solution to room temperature, it was stored at 4°C. Beers law was used to calculate the GNP concentration. Dynamic light scattering (DLS) (Zetasizer (Nano- ZS, Malvern Instruments Ltd, Malvern, UK) and scanning electron microscopy (SEM) (MIRA3 TESCAN, Czech Republic) were used to evaluate the size, dispersion, zeta potential, and morphology of GNPs. The GNPs characterization was also performed using UV-vis spectroscopy (Mapada, China).

2.5.2. Apt-modified GNPs preparation

Binding of thiol-modified aptamers to GNPs was performed as described in our previous studies (12, 27). Thiolated aptamers were treated with 20 mM DTT (prepared in 10 mM Tris-HCl buffer, pH 7.4) for one hour at room temperature to cleave and activate the 5'-disulfide bonds of the aptamers. They were then washed three times with ethyl acetate to remove excess DTT. Subsequently, the prepared aptamers (140 µl, 5 µM) were added to the GNPs solution (1260 µl) and incubated in the dark at room temperature for 24 hours. To remove the residual and unconjugated aptamers, the solution was centrifuged at 13,000 rpm for 20 minutes, the supernatant was discarded, and the precipitate was washed three times with 4 mM trisodium citrate (500 µl). Finally, the pellet was resuspended in 4 mM trisodium citrate (500 µl) and stored at 4°C. Electrophoresis on a 2% agarose gel and DLS were used to evaluate and confirm the surface modification and formation of the Apt-modified GNPs.

2.5.3. The extract allergen loading on the Apt-modified GNPs

Protein binding to GNPs was performed as described in our previous study (12, 27). *P. orientalis* pollen protein extract (300 µl, 1 mg/ml in phosphate buffer 10 mM) was added to 1200 µl aptamer-modified GNPs and gently shaken overnight at 25°C. To remove the unbound extract allergens, the solution centrifuged at 13,000 rpm for 20 min and the supernatant discarded. Then, we washed it three times with phosphate buffer (10 mM, pH 7). Finally, Pla. ext-Apts-GNP complex was resuspended in phosphate buffer and stored at 4°C.

The loading efficiency of *P. orientalis* pollen extract on GNPs was evaluated using the BCA protein assay kit, and the concentration of bound protein allergens in the solution was calculated using the following formula: (amount of bound *P. orientalis* pollen extract = initial protein content in the mixture – protein content in the supernatant). Further, SDS-PAGE (Sodium dodecyl-sulfate polyacrylamide gel electrophoresis) of protein allergens isolated from GNPs was performed as described in our previous

study (12). Stringent conditions were used to detach and release the bound proteins from the GNP surface. In this regard, 30 µl GNPs-protein complexes were mixed with 10 µl 4X SDS-PAGE loading buffer (5% β-mercaptoethanol, 10% glycerol, 2% SDS, 0.005% bromophenol blue and 0.06 M Tris-HCL pH 6.8), heated at 100°C for 5 min, and then kept in a water bath at 42°C for 10 hours. Proteins were completely isolated after GNP removal (13,000 rpm for 5 min) and then loaded onto a SDS-PAGE gel (15%).

2.6. Mice sensitization

Mice were sensitized to the total protein extract of *P. orientalis* pollen by three subcutaneous injections in the back of the neck one week apart. These injections were made with 10 µg *P. orientalis* total pollen extract and 2 mg aluminum hydroxide according to a protocol from Mondoulet, L., *et al* (11). Negative control animals received sterile PBS and aluminum hydroxide by subcutaneous injection. Mice were then exposed to 1% (v/v) *P. orientalis* total pollen extract for 20 min on four consecutive days (days 21–24) via an aerosol delivery system (Fig. 2). Mice were randomly separated to 10 groups (n = 5). At the end of the sensitization period (day 25), blood samples were collected from the retro-orbital sinus. Blood-specific IgE levels (sIgE) were measured at the termination of the sensitization phase (day 25) to confirm sensitization.

2.7. Epicutaneous immunotherapy

An electric razor was used to shave the dorsal skin of mice to achieve epicutaneous desensitization. As described in the previous studies (12), after 24 hours, the total volumes of treatment solution for the different groups (Fig. 2) were applied to the backs of the mice by a patch (once a week for 8 consecutive weeks). Following the immunotherapy phase (days 85 and 86), all mice received intranasal administration of 1% (v/v) *P. orientalis* total pollen extract for 20 minutes, as described in the sensitization phase. At 72 hours after the last aerosolization challenge, the mice were euthanized, blood samples were collected by cardiac puncture, and serum was obtained for IgE level evaluating. Figure 2 illustrates the study design.

2.8. Serum specific IgE level (sIgE) and total IgE (tIgE) measurements

sIgE antibodies were determined using an enzyme-linked immunosorbent assay (ELISA) assay developed in-house. Briefly, microplates were coated with 50 µg/well the total pollen extract solution and incubated overnight at 4°C. After washing five times with wash solution (PBS with 0.05% Tween 20), the plates were blocked with blocking solution (Parstous, Iran) for 1 hour at 25°C. Following the second washing step, 100 µl serum samples were added to each well and incubated at room temperature for 3 hours. Immunoglobulin detection was then performed using 1:500 diluted biotin anti-mouse IgE antibody (BioLegend, USA), 1:3000 diluted horseradish peroxidase-conjugated streptavidin (BioLegend, USA) and TMB solution (Parstous, Iran) as enzyme substrate. The washing step was performed between each step. Finally, optical density (OD) was measured at 450 nm and 630 nm as a reference using an ELISA microplate reader (Statfax 2100 Microplate Reader, Awareness Technology, USA) 20 min after addition of

the chromogenic substrate. Total IgE antibody level (tIgE) in mouse serum was measured using a sandwich ELISA kit according to the manufacturer's instructions (Parstous, Iran).

2.9. Measurement of cytokine concentrations in supernatants of the cultured splenocytes

For splenocyte isolation, 72 hours after the last intranasal exposure to allergen extract, the spleen of each mouse was harvested, minced, and filtered with sterile filters. Cell suspensions were then washed twice with RBC lysis buffer and resuspended in RPMI 1640 medium containing 10% inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 U/ml penicillin. Afterwards, 1×10^6 cells/ml were cultured in 24-well flat-bottom culture plates and stimulated with *P. orientalis* total pollen extract (100 µg/ml), the mixture of recombinant allergens of *P. orientalis* pollen (rPla or 1, rPla or 2, rPla or 3, and cyclophilin) (each one 1µg/ml), PHA (2% v/v), and media (at 37°C for 72 h in a 5% CO2 incubator). The collected supernatants were analyzed for the concentration of IFN-γ, IL-4, IL-17, and IL-10 (Parstous, Iran).

2.10. Proliferative spleen cell responses

For the T-cell proliferation assay, as mentioned in the previous studies (12, 28), spleen cells (2×10^5 cells/well) were propagated in 96-well round-bottom culture plates in two sets of triplicate (at 37°C for 72 h in a 5% CO2 incubator) and was induced with *P. orientalis* total pollen extract (20 µg/well) and a mixture of its recombinant allergens (rPla or 1, rPla or 2, rPla or 3, and cyclophilin) (each one 1 µg/ml). PHA (2% v/v) and medium alone were used as positive control and blank, respectively. Cell proliferation was determined as previously described (28) and the stimulation index (SI) was calculated as follows: SI = (OD sample - OD blank)/ (OD positive control - OD blank).

2.11. Nasopharyngeal lavage and cell differential counting

The trachea of anesthetized mice was punctured to collect nasal lavage fluid (NALF) 72 hours after the last intranasal exposure to allergen extract. A 22-gage catheter was inserted into the trachea, 1 ml PBS was gently administered into the nasal cavity, and the NAL fluid was obtained from the nostrils. The separated sediment from the centrifuge of the NALF sample (2000 rpm for 7 min at 4°C) was resuspended in 500 µl PBS and then examined with a hemocytometer. Slides were prepared and stained with Giemsa. white cells NALF were differentiated from at least 500 cells per slide at 100× magnification.

2.12. Histology

For histological lung examination, the lungs of mice were separated 72 hours after the last intranasal exposure and fixed in formaldehyde (10%). After decalcification with hydrochloric acid, lung sections were serially cut and embedded in paraffin. The sections were stained with hematoxylin and eosin (H&E). After viewing under a light microscope, photographs were taken with a digital camera. Five randomly selected airway sections from each slide were examined for AR-associated histopathological damage and the degree of perivascular and peribronchiolar inflammation as markers of lung damage and inflammation. To blind the pathologists evaluating the slides, all markings were removed.

2.13. Statistical analysis

Final data were analyzed using GraphPad Prism version 8.2 software (version 8.4.2, California, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, and Mann-Whitney U test used for nonparametric data. Data are expressed as mean ± standard error of the mean (SEM). The P value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of the synthesized GNPs and Pla. ext-Apts-GNPs complex

DLS results showed that the synthesized GNPs were relatively monodisperse (polydispersity index (PDI) = 0.381). The size and zeta potential of the synthesized GNPs were 17.6 ± 4 nm and − 18.2 ± 2.1 mV, respectively. In contrast to bare GNPs, the Apts-GNPs moved during electrophoresis due to the negative charge caused by the aptamer on their surface (Supplementary 1). The successful binding of aptamer to the GNPs was also confirmed by increase in zeta potential (-46.6 mV). SEM results demonstrated GNPs monodispersity and a spherical morphology (Fig. 3C). *P. orientalis* total pollen extract binding to GNPs and Apt-GNPs complex was investigated via BCA assay and SDS-PAGE. As indicated by the BCA result, the concentration of the total pollen extract bound to the GNPs was 60 µg/ml. The SDS-PAGE results were also consistent with the BCA assay (Supplementary 2). The increase in GNPs size after binding of *P. orientalis* total pollen extract confirmed successful allergens binding to the GNPs (24.5 ± 4.4 nm). Figure 3B shows the UV-Vis spectra of bare GNPs and the two different complexes. The UV-Vis spectra of naked GNPs, Pla. ext-GNPs, and Pla. ext-Apts-GNPs showed absorbance peaks at approximately 520 nm, 523 nm, and 525 nm, respectively (Fig. 3), representing a range of 15 to 25 nm diameter (29, 30), indicating aptamer or allergens of *P. orientalis* total pollen extract binding to naked GNPs (31, 32).

3.2. Serum specific IgE level (sIgE) and total IgE (tIgE) concentration

As explained in the methods section, to confirm sensitization, mice blood samples were collected at the termination of the sensitization period (day 25) and IgE levels were determined. The increased levels of sIgE and tIgE in mice receiving the pollen extract together with alum compared with control mice receiving PBS, confirmed the establishment of a sensitized mouse model to allergens of *P. orientalis* total pollen extract (P < 0.0001 and P < 0.01, respectively, Fig. 4A and B).

As shown in Fig. 4C, serum sIgE levels after EPIT were also significantly higher in the PBS-treated group (sensitized) than in the normal group (nonsensitized) (P < 0.01). The Pla. ext (100 µg)-treated group revealed a partial reduction in sIgE levels (no significant difference), and the naked GNP-treated group displayed a statistically significant difference compared to the PBS-treated group (P < 0.05). Interestingly, sIgE levels were significantly lower in all GNP treatment groups than in the PBS treatment group (Fig. 4C).

Although EPIT with Pla. ext (10 µg)-GNPs showed a significant decrease in sIgE levels compared with the PBS-treated group ($P < 0.05$), the addition of aptamers to this complex (Pla. ext (10 µg)-Apts-GNPs) resulted in a further but non-significant decrease compared with the Pla. ext (10 µg)-GNPs-treated group. Furthermore, Pla. ext (10 µg)-Apts-GNPs in combination with mixed SPPs differed significantly from PBS in terms of sIgE levels ($P < 0.01$, Fig. 4C), but this group showed no significant difference compared with the same dose without SPPs. Compared with the naked GNP-treated group, only the DC-specific aptamer targeted groups displayed a significant decrease. Also, the lowest sIgE levels were observed in two DC-specific aptamer targeted groups ($P < 0.01$), these sIgE levels were not significantly different from the negative control group (Fig. 4C, shown with blue markers). It is worth noting that our results showed that all doses of the therapeutic complex Pla. ext (50 µg)-GNPs with a 2-fold reduction in allergen concentration and Pla. ext (10 µg)-GNPs, with a 10-fold reduction in allergen concentration, reduced sIgE levels to the level of treatment with the free extract (Pla. ext (100 µg)) or even more (not significant). Analysis of tIgE concentrations showed strong agreement with sIgE levels (Fig. 4D). Compared with the naked GNP-treated group, the Pla. ext (50 µg)-GNPs and the two aptamer-treated groups showed a significant decrease ($P < 0.05$). Interestingly, Pla. ext (10 µg)-Apts-GNP complexes together with SPPs had the lowest tIgE ($P < 0.01$). Consistent with the sIgE results, adding aptamers to this complex (Pla. ext (10 µg)-Apts-GNPs) led to a non-significant decrease in tIgE compared to non-targeted ones (Pla. ext (10 µg)-GNPs) (Fig. 4D).

3.3. Cell count analysis of NALF

There was a significant reduction in total cell number in the NALF of all GNPs-treated groups, except the naked GNPs-treated group, compared with the PBS-treated group (Fig. 5A). In addition, all groups treated with Pla. ext-GNPs showed a reduction in total immune cell number in NALF compared with the free extract-treated group, although these reductions were not significant. Moreover, all groups treated with GNPs revealed a significant decrease in the eosinophil count in NALF (Fig. 5B). The two groups treated with Pla. ext (50 µg)-GNPs (EPIT) and Pla. ext (50 µg)-GNPs (SCIT) showed the greatest decrease in these two parameters ($P < 0.05$) (Fig. 5). Consistent with IL-17a results, the decrease in the two items in the treatment groups with 10 µg extract dose (Pla. ext (10 µg)-GNPs, Pla. ext (10 µg)-Apts-GNPs, and Pla. ext (10 µg)-Apts- GNPs + SSPs) showed a non-significant difference compared with the group with the lowest counts (Pla. ext (50 µg)-GNPs (SCIT)).

3.4. Cytokines secreted by stimulated splenocytes after immunotherapy

An evaluation of the T-cell response was performed after inhalation exposure of mice to the total extract in mouse splenocytes by ex vivo allergen-specific cytokine analysis (IL-4, IFN-γ, IL-10, and IL-17a) (Fig. 6A and B). Reactivated splenocytes of the PBS-treated group (sensitized) with *P. orientalis* total pollen extract and the mixture of its recombinant allergens (rPla or 1, rPla or 2, rPla or 3, and cyclophilin) secreted a greater amount of IL-4 than the control group (non-sensitization) ($P < 0.01$ and $P < 0.001$, Fig. 6A.I and Fig. 6B.I). Analysis of IL-4 concentrations in reactivated splenocytes with *P. orientalis* total pollen extract showed that all treatment groups with Pla. ext-GNPs and Pla. ext-Apts-GNPs complexes significantly

down-regulated IL-4 production compared with the PBS-treated group (sensitized) (Fig. 6A.I). While the free extract treated group and naked GNP-treated group showed a limited reduction in concentration of IL-4, there were no statistically significant difference compared with the PBS-treated groups (Fig. 6A.I). Compared with the naked GNP-treated group, only the two aptamer-treated groups showed a significant decrease ($P < 0.05$, Fig. 6A.I). It is interesting to note that almost all therapeutic doses of Pla. ext-GNPs complex compared with free extract reduced the IL-4 concentration to the extent of free extract treatment or even more (non-significant). Analysis of IL-4 concentrations in reactivated splenocytes with the mixture of recombinant allergens of *P. orientalis* pollen (rPla or 1, rPla or 2, rPla or 3, and cyclophilin) gave consistent results with reactivation by *P. orientalis* total pollen extract. Moreover, in the two aptamers treated groups, there was a significant decrease compared to the non-targeted ones (Pla. ext (10 µg)-GNPs-treated group) ($P < 0.05$, Fig. 6B.I.).

Re-stimulation of lymphocytes with the pollen extract showed the following results for IFN-γ concentration: in all GNP-treated EPIT groups, IFN-γ concentration was more than twice the PBS-treated group (sensitized) (Fig. 6A.II.). This increase was significant only in the two Apts-GNP-treated groups (Pla. ext (10 µg)-Apts-GNPs and Pla. ext (10 µg)-Apts-GNPs + SSPs) ($P < 0.05$). In addition, all of our EPIT treatments with the Pla or ext-GNPs complex increased IFN-γ concentration compared with the free extract group, with the only significant increase in Pla. ext (10 µg)-Apts-GNPs, along with the SSP-treated group ($P < 0.05$), which also showed a significant increase compared to the naked GNP-treated group (Fig. 6A.II.). It is also worth noting that the "Pla. ext (50 µg)-GNPs" treatment groups significantly increased IFN-γ concentration compared with the subcutaneous treatment group at the same dose ($P < 0.05$, Fig. 6A.II.). Specific re-stimulation of splenocytes with the mixture of recombinant allergens of *P. orientalis* pollen revealed significant increase in IFN-γ concentration in most treated groups (Fig. 6B.II.). This increase was significant in the free extract, the Pla. ext-GNPs (100 and 50 µg) and both Apts-GNP treated groups. Moreover, this increase was significantly different in the free extract, Pla. ext-GNPs (100 and 50 µg) and Pla. ext (10 µg)-Apts-GNPs + SSPs) groups compared with the naked GNPs group (Fig. 6B. II.). Also, IFN-γ/IL-4 concentration ratio in the GNPs-treated groups after EPIT (Fig. 6C.I and Fig. 6C.II) indicated a polarization from a Th2 response to a Th1 response.

All treatment groups, including the GNPs, Pla. ext-GNPs, and Pla. ext-Apts-GNPs, exhibited a significant elevation in IL-10 concentration compared to the PBS-treated group in re-stimulated splenocytes with the extract ($P < 0.05$) (Fig. 6A.III.). While all groups treated with Pla. ext-GNPs increased IL-10 concentrations compared with the free extract-treated group, no significant difference was showed. Consistent with the other results of this study, the highest concentration of IL-10 was observed in the Pla. ext (10 µg)-Apts-GNPs-treated groups. The highest concentration of IL-10 was confirmed by the analysis of IL-10 concentration in re-stimulated splenocytes with the mixture of recombinant allergens, and this increase was significantly higher compared to the same dose without aptamer, naked GNP, and the free extract-treated groups. (Fig. 6B.III.).

In addition, a decrease in IL-17a concentration was observed in all GNP-treated EPIT groups compared with the PBS group (Fig. 6A.IV and Fig. 6B.IV). This decrease in re-stimulated splenocytes with the extract

was significant in the Pla. ext (50 µg)-GNPs and the two Apts-GNPs-treated groups (Pla. ext (10 µg)-Apts-GNPs and Pla. ext (10 µg)-Apts-GNPs + SSPs)(Fig. 6A.IV.). It should be noted that there were no statistically significant differences among the three groups. Moreover, compared to the naked GNP-treated group, these three treated groups showed a significant decrease (Fig. 6A.IV.). While all groups downregulated IL-17a levels more than the free allergen group, only the groups treated with Pla or ext (50 µg)-GNPs showed a significant difference ($P < 0.05$). Our results showed that all EPIT treatment groups with Pla. ext-Apts-GNPs and Pla. ext-GNPs complexes decreased IL-17a concentrations to the level of the negative control group. (Fig. 6A.IV and Fig. 6B.IV), shown with blue markers).

3.5. Proliferative response of splenocyte to *P. orientalis* total pollen extract

Figure 6 **Immune response analysis in splenocytes**. IL-4 **(I)**, IFN-γ **(II)**, IL-10 **(III)** and IL-17a **(III)** cytokine concentrations in the supernatant of splenocyte cultures following re-stimulation with the total extract **(A)** and the recombinant allergens **(B)** after EPIT. IFN-γ/ IL -4 concentration ratio in the different treated groups after re-stimulation with the total extract **(C.I)** and the recombinant allergens **(C.II)**. *P. Orientalis* extract specific proliferative response after re-stimulation with the total extract **(D.I)** and the recombinant allergens **(D.II).** Note: Normal: not sensitized, PBS treated: treated with PBS, GNPs: treated by naked GNPs (as a control group), Pla. ext (100 µg): treated by 100 µg total extract of *Platanus orientalis* pollen, Pla. ext (100 µg)-GNPs: treated with 100 µg total extract loaded on GNPs, Pla. ext (50 µg)-GNPs: treated by 50 µg total extract loaded on GNPs, Pla. ext (50 µg)-GNPs (SCIT): subcutaneous immunotherapy with “Pla. ext (50 µg)-GNPs”, Pla. ext (10µg)-GNPs: treated with 10 µg total extract loaded on GNPs, Pla. ext (10 µg)-Apts-GNPs: treated by aptamer conjugated and 10 µg total extract loaded on GNPs, Pla. ext (10 µg)-Apts-GNPs + SPP: treated with Pla. ext (10 µg)-Apts-GNPs accompanied by SPPs (mixed TD1 and R7). ns, no significance.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs. sensitized mice (shown with black marker), and Vs. not sensitized mice (shown with blue marker)

3.6. Histopathological analysis of lung

Histological examination of lung tissue at AR could be a valuable method to evaluate allergic airway inflammation. The lung section showed normal histologic structure of the alveolar wall and bronchial epithelial lining, and no inflammation, hemorrhage, or fibrosis were observed (Fig. 7A). As shown in Fig. 7B, lung section analysis of the PBS group revealed alveolar damages. Histologic results in the naked GNPs-treated group and the free extract treated groups showed perivascular mononuclear cell inflammation (Figs. 7C and 7D). The group treated with Pla. ext (100 µg)-GNPs showed peribronchiolar mononuclear cell inflammation (Fig. 7E). As shown in Figs. 7F, the histological examination of Pla. ext (50 µg)-GNPs group showed the alleviation of pulmonary inflammation and appeared like the normal group. The group treated with Pla. ext (10 µg)-GNPs showed peribronchiolar mononuclear cell inflammation (Fig. 7G). Subsequently, histological analysis of the DC-specific aptamer-targeted groups, including the Pla. ext (10 µg)-Apts-GNPs and Pla. ext (10 µg)-Apts-GNPs in combination with SSPs, showed no pathological changes (Figs. 7H and 7I). Finally, the subcutaneously treated group with Pla. ext

(50 µg)-GNPs exhibited moderate infiltration of mononuclear inflammatory cells in the alveolar spaces (Fig. 7J).

4. Discussion

According to the last study of our group by Koushki et al., EPIT using DC-specific aptamer-targeted GNPs as carriers of a recombinant allergen could effectively modulate allergic immune responses in a mouse model (12). However, the question remains whether this allergen-targeted delivery system can also be used for AIT in the case of pollen allergen extracts that contain a variety of allergenic and non-allergenic components. In this context, *P. orientalis* pollen total extract was used as a model of pollen total extract in EPIT to investigate the efficacy of DC-specific aptamers conjugated with GNPs in the pollen-induced allergic rhinitis mouse models. In addition, this study also examined whether the skin-penetrating peptides along with the complex could improve this allergen delivery system.

Our results showed total pollen protein extract has an efficient loading on the DC-specific aptamer coated GNP with a hydrodynamic size of 24.5 ± 4.4 nm. Several studies on the application of GNPs as transdermal carriers have shown that GNPs of this size can efficiently penetrate the epidermis (29, 33). In addition to this positive effect of GNPs, Our results demonstrated even naked GNPs significantly ameliorate some AR-related immune factors such as specific IgE, the number of eosinophil cells in NALF, and IL-10 production. Although the other immune factors were all improved, they were not statistically significant compared with the positive control group. Consistently, Barto *et al*. showed that intranasal treatment with GNPs in allergen-induced asthma mouse model prevents the accumulation of inflammatory cells and peribronchiolar fibrosis, as well as the production of pro-inflammatory cytokines, reactive oxygen species, and mucus (34).

In parallel with our results, previous studies have shown that citrate GNP has anti-inflammatory properties under various conditions. According to these studies, spherical citrate GNP with a diameter of 13 to 24 nm have been shown to possess anti-inflammatory properties by decreasing the inflammatory cytokines concentration and increasing the anti-inflammatory cytokines, as well as reducing the cyclooxygenase type 2 (35–41).

Moreover, our results showed that GNPs as allergen delivery system can stimulate more immunomodulatory effects and improve immune factors of AR compared with PBS and free extract, such as IgE antibody, cytokines concentration (IL-4, IFN-γ, IL-10 and IL-17a) and inflammatory cell recruitment in the nose. In this regard, the present study showed that treatment with allergen-GNPs complex led to a 2-fold reduction in allergen dose to achieve equal or greater efficacy in comparison with free total pollen extract. The Pla.ext (50 µg)-GNPs treated group significantly decreased total IgE antibody concentration, specific IgE level, IL-4, IL-17a concentration, infiltrated total immune cells, and eosinophils in NALF and increased IL -10 and IFN-γ cytokines compared with the PBS treated group. The pathological examination of lung tissue in this treatment group also revealed no signs of inflammation or pathological damage. According to various studies, AIT and EPIT with free allergens have a dose-dependent efficacy and

improve with an increasing dose of allergen (10, 42–44); our results seem to support the notion that GNPs could reduce the common allergen dose in EPIT (100 µg) (11, 45–50) by increasing immunogenicity and enhancing antigen uptake by APC (17, 19). Various studies have shown that GNPs improve antigen delivery to DCs (51, 52).

We also used specific aptamers to direct the allergen of interest to DCs as professional APCs. The addition of aptamers to this complex (Pla. ext-Apts-GNPs) resulted in a 10-fold reduction in allergen dose to achieve equal or greater efficacy compared to the free extract. Interestingly, this therapeutic complex increased EPIT efficiency, such that of all treatment groups, the greatest improvement in AR-related immune factors were obtained in the DC-specific aptamer targeted groups. Compared with the same dose without aptamer, all parameters improved, although this difference was statistically significant only at IL-4 and IL-10 concentrations. Furthermore, pathological examination of lung tissue in the aptamer-treated groups showed no evidence of inflammation or pathological damage compared with the same dose without aptamer, which showed peribronchiolar mononuclear cell inflammation, indicating the efficacy of targeting with DC specific aptamers. There is growing evidence that treatment with EPIT decreases clinical and allergen-specific Th2 responses in sensitized mice and enhances Treg responses in local and peripheral areas. When antigens were applied to the epidermis in EPIT, they could be taken up, processed, and presented to T lymphocytes by APCs such as langerhans cells, resulting in tolerance by inducing Treg cells (47, 53, 54). DCs act as a link between innate and adaptive immunity and therefore play a very important role in coordinating the immune response. Researchers have demonstrated that DC targeting increases antigen uptake and presentation by 100 to1000 fold. The researchers stated that targeting DCs by aptamers in the context of immunotherapy and vaccine development may be beneficial in directing appropriate immune responses (12, 21, 27, 55–58). These data suggest that efficient targeting with DC-specific aptamers in EPIT could lead to enhanced allergen delivery to DCs and, consequently, induction of tolerance and Treg cells. Therefore, according to our and previous studies, this approach represents a significant development in improving the efficacy of EPIT and SLIT (12) (27, 58).

Consistent with AIT studies, our data in this study demonstrated that this therapeutic complex can modulate the allergic immune response at the T-helper cell level, as evidenced by their cytokine levels (IL-4, IL-17a, IFN-γ and IL-10) (27, 58). This study also showed a significant increase in IFN-γ concentration and IFN-γ/IL-4 concentration ratio in the DC-specific aptamer targeted groups. Accompanying our study, several studies have shown that EPIT on intact skin leads to a deviation from a Th2 response to a Th1 response (12, 59).

In addition to regulating T helper responses, our treatment reduced the number of infiltrated immune cells, including eosinophils in NALF. The total extract in complex with GNPs, especially the Pla. ext (50 µg)-GNPs, and the DC-specific aptamer-targeted groups strongly reduced total cell and eosinophil counts in NALF. Inflammatory cells such as basophils, neutrophils, mononuclear cells, and especially eosinophils play a crucial role in the pathogenesis of allergic airway inflammation. They migrate into the nasal mucosa and cause inflammation, nasal epithelial damage, and subepithelial fibrosis by releasing various products. The number of nasal eosinophils is considered a valuable approach to evaluate eosinophilic

inflammation in individuals with allergic rhinitis (60–62). In parallel to these data, histopathological assessment of lung tissue demonstrated a reduction in inflammation, infiltration of immune cells, and pathological damage in all GNP-treated groups. Various studies have shown that victorious AIT ameliorates pathological lung damage (12, 27, 28, 58).

Consistent with previous research (12), our results did not provide outstanding documentation for the use of SPPs to overcome allergen delivery to the stratum corneum in EPIT, when comparing groups with and without SPP administration.

After immunotherapy accompanied by various immunotherapy studies (12, 28, 48, 58, 63), we observed a tendency to downregulate specific splenocyte proliferation after restimulation with *P. orientalis* total pollen extract and its recombinant allergens. These data indicate that regulation of allergen sensitivity is not limited to the lung and nose but also extends to the spleen; in addition to the reduction in cytokine production, splenocyte proliferation was also reduced compared with the positive control. We speculate that the reduced proliferative response may be due to the suppressive activity of the induced Treg cells by the release of suppressive cytokines such as TGF-β and IL-10 or by cell to cell contact in EPIT.

Based on a double-blind, placebo-controlled study by Senti *et al.* (10), local adverse effects in EPIT are likely to be dose-dependent, occurring more frequently with high-dose treatment. Therefore, based on our results, it could be concluded that GNPs and DC-specific aptamer-targeted GNPs could reduce local adverse effects by reducing the allergen dose in EPIT.

5. Conclusion

This study shows that GNPs and DC specific aptamer-modified GNPs can improve EPIT efficacy for total pollen extracts containing a variety of allergenic and non-allergenic components. GNPs, as carriers of pollen extracts, resulted in a 2-fold reduction in the allergen dose for EPIT. Moreover, addition of aptamers to this complex (Pla. ext-Apts-GNPs) led to a 10-fold reduction in the allergen dose for EPIT. The DC-specific aptamer targeted groups with and without SSPs showed the highest efficacy in decreasing sIgE levels, tIgE, Th2 cytokines concentration, and increasing Treg and Th1 cytokines concentrations. Therefore, we believe that loading total protein extract on the GNPs and the Apt-modified GNPs could be an effective approach to improve EPIT efficacy in pollen-induced allergic rhinitis. We suggest further investigation in other studies, for example, by examining IgG2a and IgG1a concentrations, and T-cell subsets in skin-draining lymph nodes using flowcytometry.

Declarations

Conflict of interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

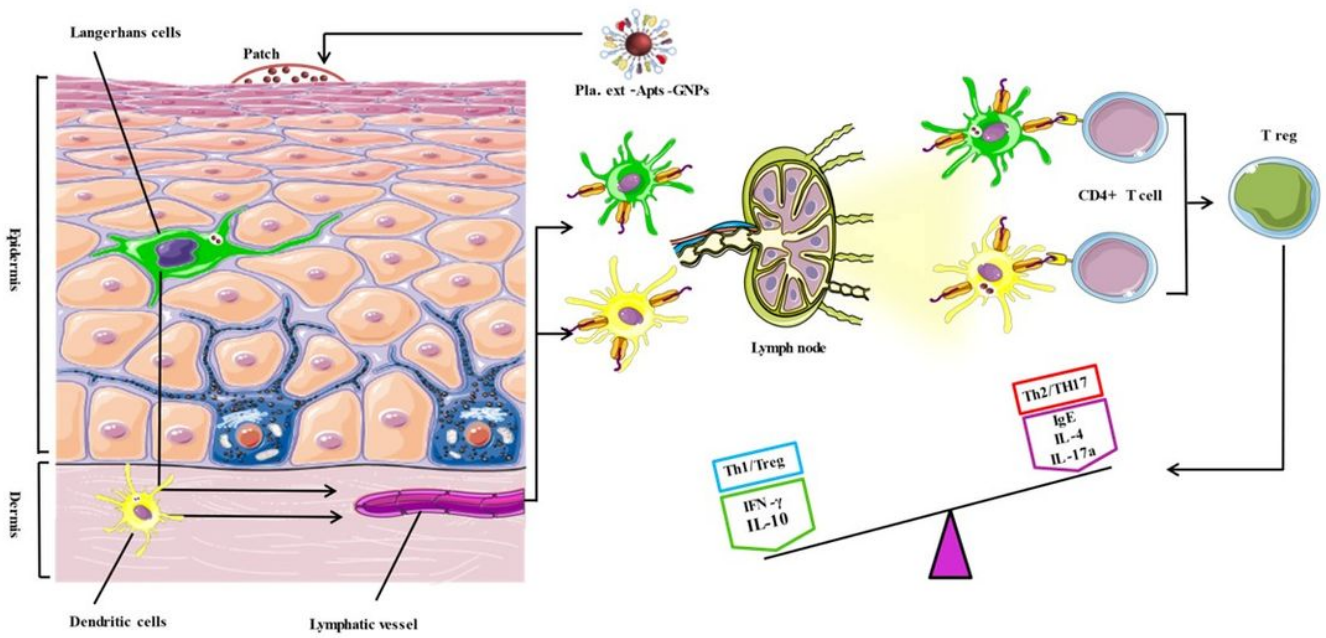

Figure 1

It is speculated that the Pla. ext-GNPs and the Pla. ext-Apts-GNPs complex are taken up by Langerhans cells (LCs) and dendritic cells (DCs) after application to intact skin and subsequently migrate to the draining lymph nodes of the skin. These APCs present antigens to T lymphocytes along with MHC-II under non-inflammatory conditions, leading to the induction of Tregs (Foxp3+CD62L+ Treg and Foxp3-LAP + Treg) during epicutaneous immunotherapy

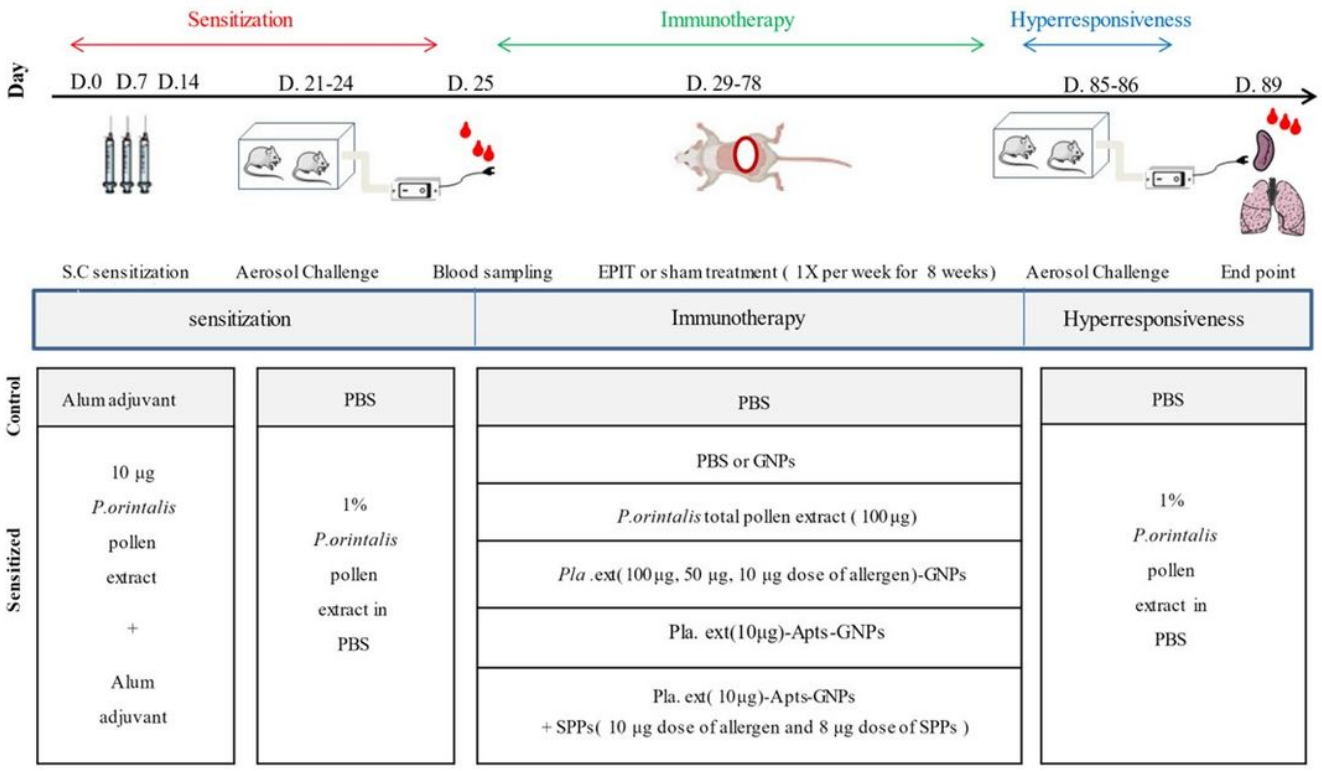

Figure 2

Study design. Mice were sensitized to *P.orintalis*pollen extract by 3 subcutaneous injections (with 1-week interval), together with aluminium hydroxide as adjuvant. Immunotherapy (48-hour application of a skin patch containing the therapeutic complex) was performed for 8 weeks with one treatment per week. The experiments were performed with 10 groups (n = 5 per group): 1) Normal: not sensitized, 2) PBS treated: sensitized and treated with PBS, 3) GNPs: treated by naked GNPs, 4) Pla. ext (100 µg): treated by 100 µg total extract of *Platanus orientalis* pollen, 5) Pla. ext (100 µg)-GNPs: treated with 100 µg the total extract loaded on GNPs, 6) Pla. ext (50 µg)-GNPs: treated by 50 µg the total extract loaded on GNPs, 7) Pla. ext (50 µg)-GNPs (SCIT): subcutaneous immunotherapy with “Pla. ext (50 µg)-GNPs”, 8) Pla. ext (10 µg)-GNPs: treated with 10 µg the total extract loaded on GNPs, 9) Pla. ext (10 µg)-Apts-GNPs: treated by aptamer conjugated and 10 µg the total extract loaded on GNPs, 10) Pla. ext (10 µg)-Apts-GNPs + SPP: treated with Pla. ext (10 µg)-Apts-GNPs accompanied by SPPs (mixed TD1 and R7). A blood sample was taken after sensitization (D 25) to confirm sensitization and after immunotherapy (D 89) to evaluate the effect of treatment

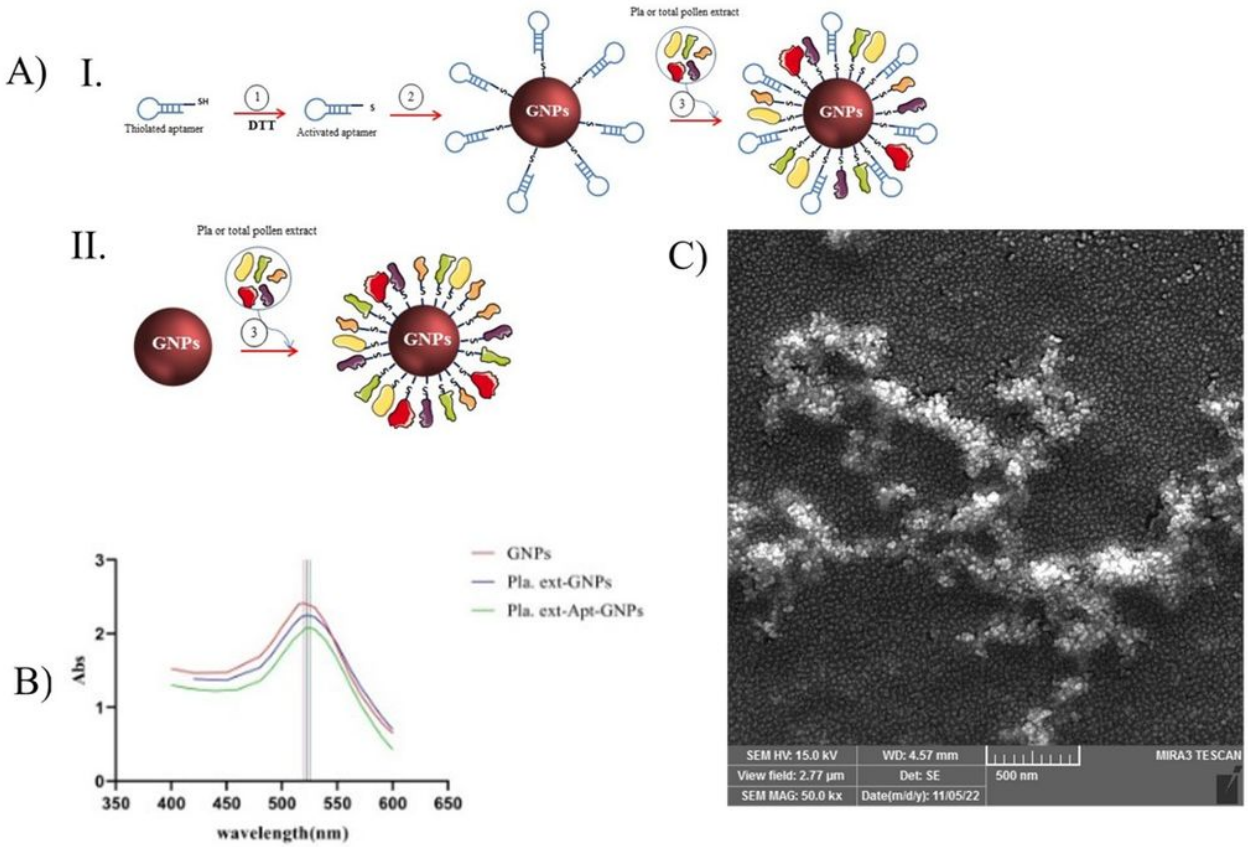

Figure 3

Synthesis and characterization of DC-Apt-functionalized GNPs loaded with pollen extract of *Platanus orientalis* (Pla. ext-Apts-GNPs) and GNPs loaded with pollen extract of *Platanus orientalis* (Pla. ext-GNPs). Schematic diagram of I: "Pla. ext-Apts-GNPs" and II: "Pla. ext-GNPs" preparation steps **(A)**. UV-Vis spectra of GNPs at each experimental step (n = 3) **(B)**. Scanning electron micrograph of Pla. ext-Apts GNPs (scale bar 500 nm) **(C)**

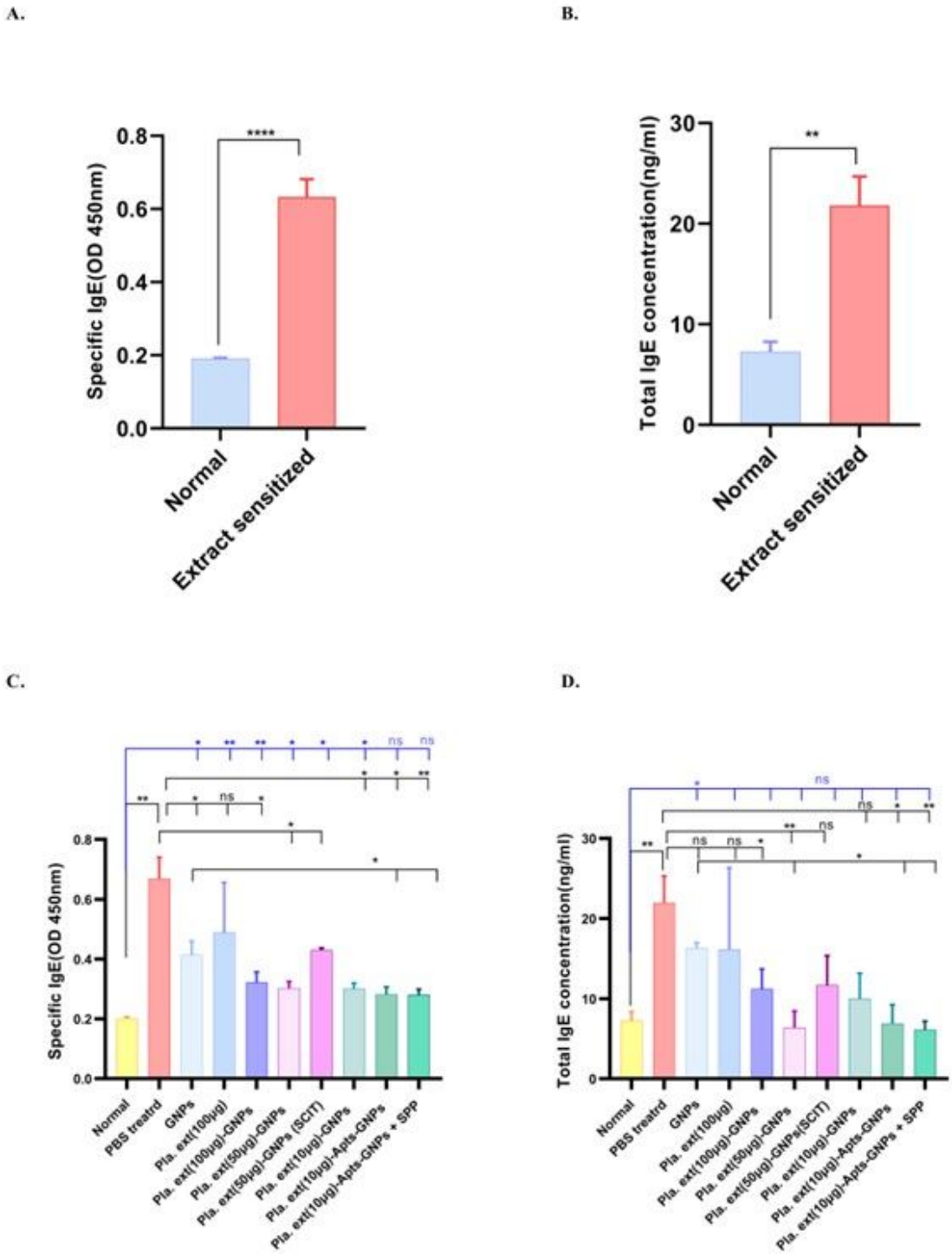

Figure 4

Specific IgE level and total IgE concentration after sensitization (A & B) and EPIT phase (C & D). Note: Normal: not sensitized, PBS treated: treated with PBS, GNPs: treated by naked GNPs (as a control group), Pla. ext (100 μg): treated by 100 μg total extract of *Platanus orientalis*pollen, Pla. ext (100 μg)-GNPs: treated with 100 μg total extract loaded on GNPs, Pla. ext (50 μg)-GNPs: treated by 50 μg total extract loaded on GNPs, Pla. ext (50 μg)-GNPs (SCIT): subcutaneous immunotherapy with “Pla. ext (50 μg)-

GNPs", Pla. ext (10 µg)-GNPs: treated with 10µg total extract loaded on GNPs, Pla. ext (10 µg)-Apts-GNPs: treated by aptamer conjugated and 10 µg total extract loaded on GNPs, Pla. ext (10 µg)-Apts-GNPs + SPP: treated with Pla. ext (10 µg)-Apts-GNPs accompanied by SPPs (mixed TD1 and R7). ns, not significance, ****p < 0.0001,*P < 0.05, **P < 0.01 vs. sensitized mice (shown with black marker), and vs. not sensitized mice (shown with blue marker)

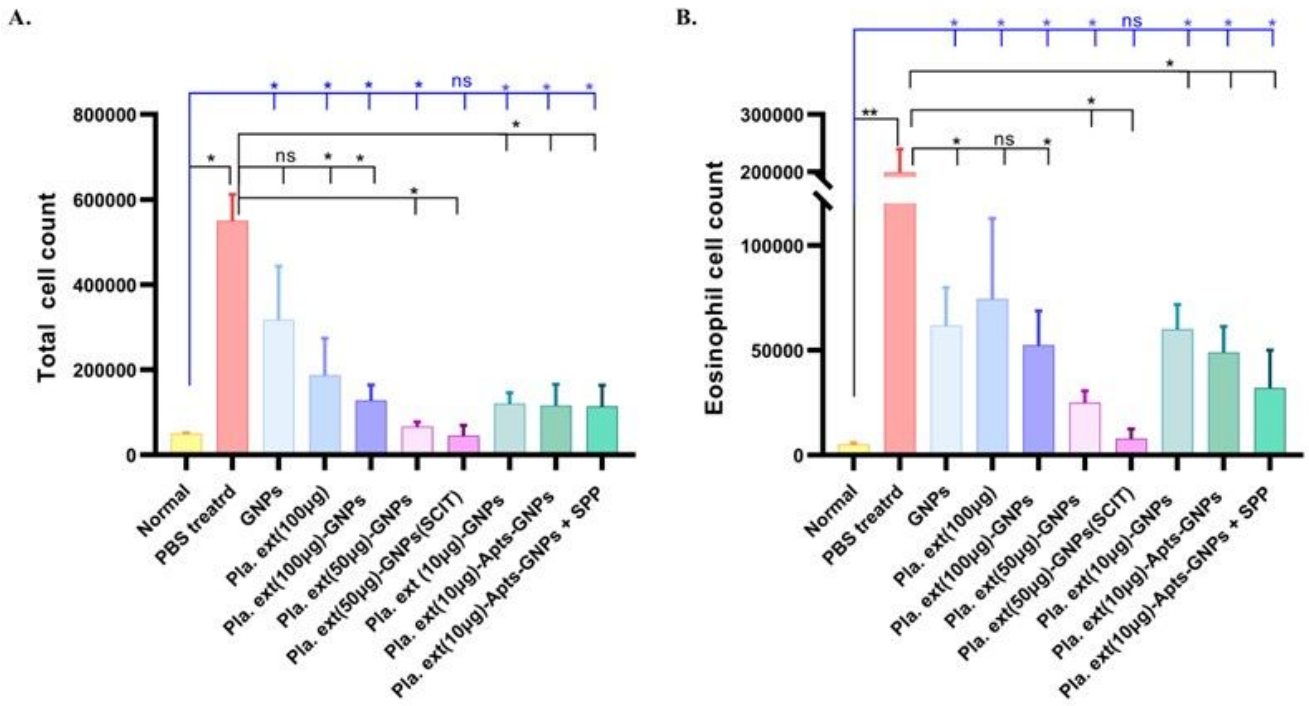

Figure 5

Evaluation of total cell count and eosinophil count in NALF after EPIT. Note: Normal: not sensitized, PBS treated: treated with PBS, GNPs: treated by naked GNPs (as a control group), Pla. ext (100 µg): treated by 100 µg total extract of *Platanus orientalis*pollen, Pla. ext (100 µg)-GNPs: treated with 100 µg total extract loaded on GNPs, Pla. ext (50 µg)-GNPs: treated by 50 µg total extract loaded on GNPs, Pla. ext (50 µg)-GNPs (SCIT): subcutaneous immunotherapy with "Pla. ext (50 µg)-GNPs", Pla. ext (10 µg)-GNPs: treated with 10 µg total extract loaded on GNPs, Pla. ext (10 µg)-Apts-GNPs: treated by aptamer conjugated and 10 µg total extract loaded on GNPs, Pla. ext (10 µg)-Apts-GNPs + SPP: treated with Pla. ext (10 µg)-Apts-GNPs accompanied by SPPs (mixed TD1 and R7). ns, not significant.*P < 0.05, **P < 0.01, vs. sensitized mice (shown with black marker), and vs. not sensitized mice (shown with blue marker).

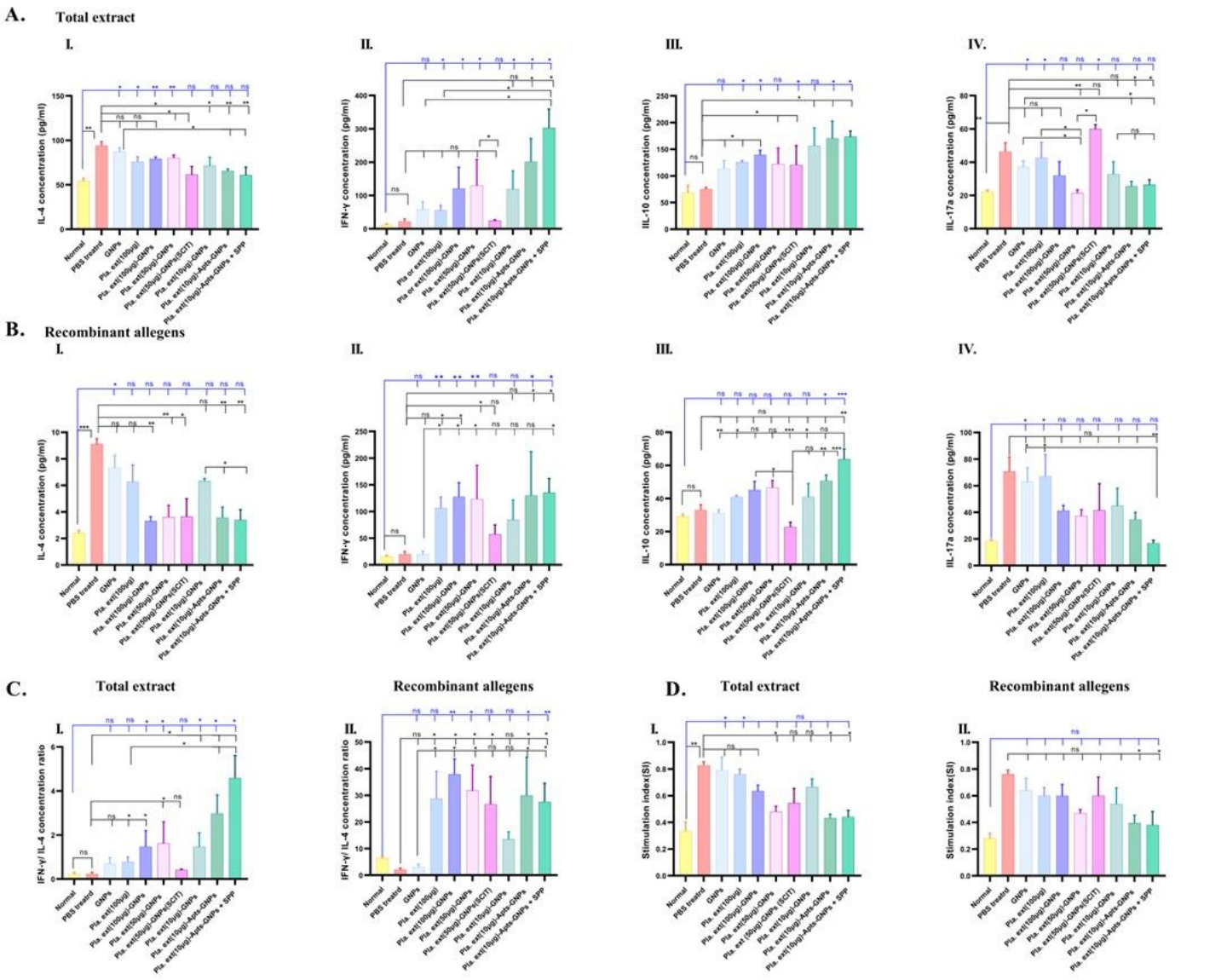

Figure 6

Immune response analysis in splenocytes. IL-4 **(I)**, IFN-γ **(II)**, IL-10 **(III)** and IL-17a **(III)**cytokine concentrations in the supernatant of splenocyte cultures following re-stimulation with the total extract **(A)**and the recombinant allergens **(B)** after EPIT. IFN-γ/ IL -4 concentration ratio in the different treated groups after re-stimulation with the total extract **(C.I)** and the recombinant allergens **(C.II)**. *P. Orientalis* extract specific proliferative response after re-stimulation with the total extract **(D.I)** and the recombinant allergens **(D.II).** Note: Normal: not sensitized, PBS treated: treated with PBS, GNPs: treated by naked GNPs (as a control group), Pla. ext (100 μg): treated by 100 μg total extract of *Platanus orientalis* pollen, Pla. ext (100 μg)-GNPs: treated with 100 μg total extract loaded on GNPs, Pla. ext (50 μg)-GNPs: treated by 50 μg total extract loaded on GNPs, Pla. ext (50 μg)-GNPs (SCIT): subcutaneous immunotherapy with “Pla. ext (50 μg)-GNPs”, Pla. ext (10μg)-GNPs: treated with 10 μg total extract loaded on GNPs, Pla. ext (10 μg)-Apts-GNPs: treated by aptamer conjugated and 10 μg total extract loaded on GNPs, Pla. ext (10 μg)-Apts-GNPs + SPP: treated with Pla. ext (10 μg)-Apts-GNPs accompanied by SPPs (mixed TD1 and R7). ns, no

significance.*P < 0.05, **P < 0.01, ***P < 0.001, vs. sensitized mice (shown with black marker), and Vs. not sensitized mice (shown with blue marker)

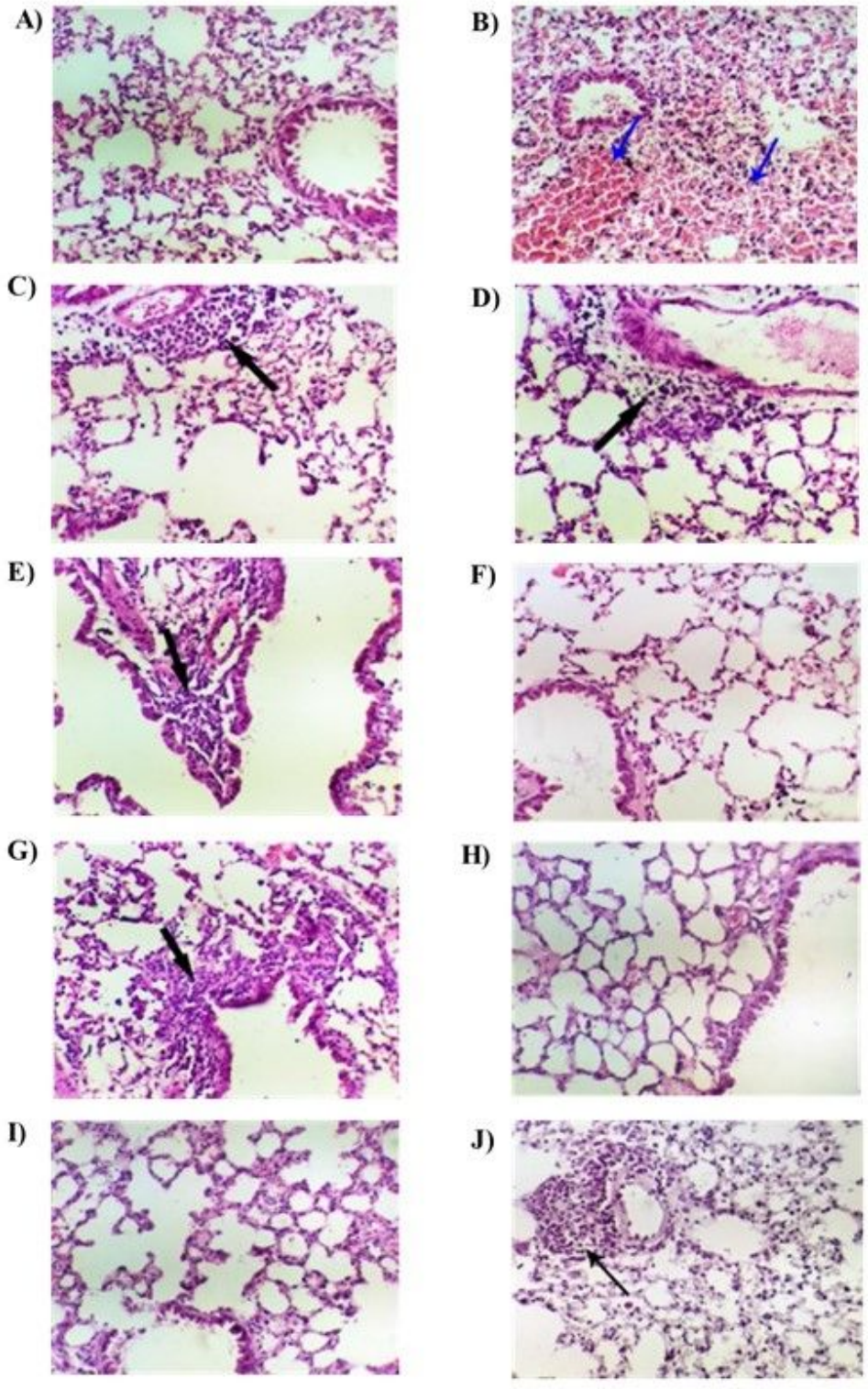

Figure 7

Lung histological analysis. Histopathological findings of lung in BALB/c mice in different groups: (A; not sensitized (Normal), B; PBS. (Alveolar damages with massive hemorrhage (arrows), C; GNPs (Perivascular

mononuclear cell inflammation (arrow), D; Pla. ext (100 µg). (Perivascular mononuclear cell inflammation (arrow) E; Pla. ext (100 µg)-GNPs. (Peribronchiolar mononuclear cell inflammation (arrow), F; Pla. ext (50 µg)-GNPs. (Histology of the lung was normal), G; Pla. ext (10 µg)-GNPs (peribronchiolar mononuclear cell inflammation (arrow), H; Pla. ext (10 µg)-Apts-GNPs. (Histology of the lung was normal), I; Pla. ext (10 µg)-Apts-GNPs +SPPs. (Histology of the lung was normal), J; Pla. ext (50 µg)-GNPs (SCIT)(moderate infiltration of mononuclear inflammatory cells in the alveolar spaces (arrow). (Staining with hematoxylin and eosin (H&E), 400× magnification)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx