



Poly-L-lysine-coated nanoparticles are ineffective in inducing mucosal immunity against group a streptococcus

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Abstract

Background: Group A Streptococcus (GAS) can cause a range of maladies, from simple throat infections to lethal complication, such as rheumatic heart disease. The M-protein, a bacterial cell surface protein, is the major virulence factor of GAS. Several attempts have been made over the past few decades to develop vaccines against GAS that employed peptides derived from the M-protein. One such approach used lipopeptides or lipid core peptide (LCP) systems that incorporated a B-cell epitope derived from the conserved region of the M-protein.

Methods: In the present study, we prepared different biodegradable polymer [dextran, poly-(lactic-coglycolic-acid) (PLGA), and poly-L-lysine] nanoparticles (NPs)-based delivery systems for a lipopeptide vaccine candidate (LCP-1). The NPs were characterised by their size, charge, morphology, antigen-presenting cells (APCs) uptake and subsequent APCs maturation efficacy, followed by in vivo nasal immunisation in mice.

Results: All produced NPs ranged in size from 100–205 nm, and their charge varied depending upon the nature of polymer. A high APCs uptake efficacy for dextran and poly-L-lysine NPs were observed, compared to PLGA NPs. Despite the high uptake by APCs, dextran and poly-L-lysine NPs failed to improve APCs maturation that resulted in low antibody titres. In contrast, while LCP-1 encapsulated into PLGA showed low APCs uptake, it induced significant maturation of DCs and higher antibody titres compared to other NPs.

Conclusions: Positively-charged poly-L-lysine NPs were non-immunogenic, while negatively charged PLGA NPs induced similar responses to antigens adjuvanted with cholera toxin B (CTB).

Keywords: Mucosal delivery, lipopeptides, nanoparticles, nasal, vaccine, PLGA, Poly-L-lysine

Introduction

Group A Streptococcus (GAS) infections represent a major health concern in developing countries due to poor access to health care facilities. Infections with GAS can lead to minor illnesses, such as pharyngitis, to complicated diseases, such as rheumatic heart disease (RHD) [1]. If untreated, GAS infections could be life-threatening; however, antibiotic therapy, particularly penicillin, can be effective in treating the bacterium. Long medication duration can lead to low patient compliance, and chances of re-infection after the completion of therapy are significant problems in disease management [2]. Therefore, the development of a vaccine could be a promising approach

to prevent diseases associated with GAS. GAS invades the body through nasal or respiratory mucosal tissues and then carries out systemic infection. An ideal GAS vaccine would provide both systemic and local protection at the invading sites. Traditional immunisation routes (parenteral routes) may induce systemic immunity, but fail to induce mucosal-specific local protection against administered antigens [3]. In contrast, mucosal immunisation routes, such as nasal, oral, pulmonary, vaginal, etc., are able to induce both mucosal and systemic responses against administered antigens [4]. Due to its anti-phagocytic function, the GAS cell-surface M-protein is a major virulence factor in GAS infections. However, the use of whole

proteins as a vaccine component may be associated with several disadvantages, such as the presence of impurities, unwanted side effects, allergies and manufacturing difficulties [5]. Importantly, some fragments of M-proteins could induce an autoimmune response. Hence, the whole protein is not favoured as an antigen for vaccine development [6]. Therefore, current GAS vaccine development is mainly focused on M-protein-derived peptide-based approaches. However, peptides, themselves, are non-immunogenic and require a delivery system or adjuvant to activate the immune system [6].

Nanoparticles (NPs) based on polymers are a promising platform to enhance the efficacy of mucosally-administered peptide antigens [3]. Cationic NPs have been shown to be effective in inducing mucosal immunity, while anionic NPs have only shown minimum improvement over soluble antigens [7]. We have previously shown that lipopeptides loaded into cationic liposomes or chitosan-based nanoparticles improved mucosal and systemic antibody titres in mice, compared to free lipopeptides, upon intranasal administration [8-10]. Poly-L-lysine is a well-known cationic polymer used for the delivery of DNA vaccines [11]. Other polymers, such as dextran and PLGA, were also reported for the intranasal delivery of lipopeptide vaccines [10,12]. However, intranasal immunisation with lipopeptide antigen loaded poly-L-lysine NPs is yet to be documented. Thus, we expected that NPs coated with poly-L-lysine would be preferentially taken up by antigen-presenting cells (APCs), and would generate a strong humoral response upon intranasal delivery. Different NPs incorporating PLGA, dextran and poly-L-lysine were prepared and characterised based on their size, charge and encapsulation efficiency of antigens. Additionally, we measured the capacity of these NPs to be taken up by APCs, and their subsequent effect in the maturation of APCs. Finally, mice were intranasally immunised with the NPs to compare the differences in their capacity to induce both mucosal and systemic immune responses.

Materials and methods

Poly-(lactic-co-glycolic-acid) (PLGA) (L:G, 50:50) (MW: 10,000-15,000) was purchased from PolySciTech® (United States). Dextran sodium (MW: 9,000-20,000), dichloromethane (DCM), poly(vinyl alcohol) (MW: 30,000-70,000), poly-L-lysine hydrobromide (MW: 15,000-30,000), phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate, and all other reagents were purchased at the available purity from Sigma-Aldrich (Castle Hill, NSW, Australia). Trimethyl chitosan (degree of quaternisations: 73%) was synthesised from chitosan, as described previously [13]. Antimouse IgG, IgG1, IgG2a and IgA antibodies conjugated to horseradish peroxidase were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All chemicals were used as received, without any purification. Millipore water was used in all formulations. Lipid core peptide vaccine candidate (LCP-1) (Figure 1) incorporating C-16 alkyl lipids (2-amino-D,L-hexadecanoic acid) chemically conjugated by a linker (Ser-Ser-Lys) to B-cell

epitope (J14; KQAEDKVKASREAKKQVEKALEQLEDKVK) and universal T-helper cell epitope (P25; KLIPNASLIENCTKAEL) were synthesised as previously described, using the microwave assisted solid phase peptide synthesis method and Boc chemistry [6,14]. Additionally, peptides (J14 and P25) were also synthesised by solid phase peptide synthesis using Fmoc chemistry [15].

Preparation of nanoparticles

The schematic representation for the preparation of NPs is shown in Figure 2. NP-1 was prepared by mixing cationic LCP-1 with anionic dextran in water. NP-2 was prepared by encapsulating NP-1 into PLGA using the double emulsion solvent evaporation method, as reported previously [16]. Briefly, aqueous NPs-1 solution was slowly added to PLGA solution in dichloromethane to prepare water-in-oil (w/o) emulsions. These emulsions were further sonicated and slowly added to the stabiliser solution (0.75% PVA in water) to prepare a water-in-oil-in-water (w/o/w) emulsion. Organic phase (dichloromethane) was evaporated by stirring the double emulsion for 6 h at room temperature. After evaporation, NPs-1-loaded PLGA particles (NPs-2) were subjected to centrifugation at 3,000 g for 3 min. The sediment and pellets were discarded,

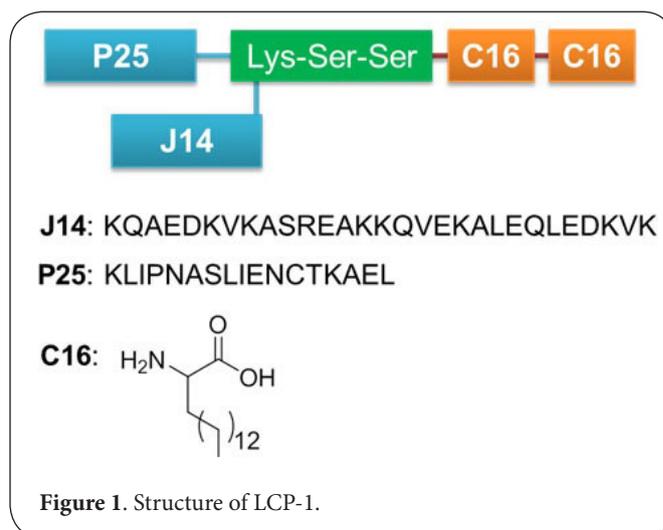


Figure 1. Structure of LCP-1.

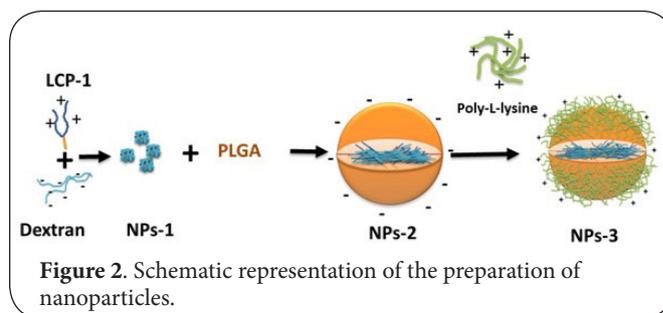


Figure 2. Schematic representation of the preparation of nanoparticles.

and the supernatant was further centrifuged at 15,000 g for 15 min to form pellets. The final supernatant was discarded and pellets were washed twice through dispersion in Millipore water, followed by centrifugation at 15,000 g for 15 min. The final pellets were redispersed in water. NPs-3 were prepared as above, except aqueous poly-L-lysine solution was added to NP-2 after evaporating dichloromethane, and stirred for a further 2 h at room temperature.

Particle size distribution and zeta potentials

The particle size, polydispersity index and zeta potential of the NPs were characterised by dynamic light scattering (DLS) using a Zetasizer (Zetasizer Nano Series ZS, Malvern Instruments, United Kingdom). Analyses of all the NPs were performed at least in triplicate.

Morphology

Transmission electron microscopy (TEM, JEOL Ltd, Japan) was used to visualise the surface morphology of the NPs. NPs-1 was visualised without any stain, while visualisation of NPs-2 and NPs-3 was done with 1% phosphotungstic acid stain. Briefly, a drop of NPs was settled into carbon-coated copper grid for 1 min and excess was wicked off using filter paper. A drop of staining solution was added and left for 30 s. Excess stain solution was removed using filter paper. The grid was air-dried for 5 min before observing them by microscope.

Antigen-presenting cells uptake and maturation studies

Spleens were harvested from naïve mice and processed as described previously without any modification [13]. The splenocytes cells were stimulated with NPs containing carboxyfluorescein conjugated to LCP-1 (2 µg) for APCs uptake studies [10,12]. In the maturation studies, a similar amount of LCP-1 (without carboxyfluorescein) containing NPs was used. The uptake of LCP-1 and the expression of costimulatory maturation markers by APCs were quantified by flow cytometry (LSR II Flowcytometer, BD Biosciences, CA, USA).

Immunisation studies

Swiss out-bred mice were used for the nasal immunisation study. The animal ethics and experimental protocols were carefully reviewed and approved by the Institute of Ethics Review Board (Griffith University, GU ref no.Gly-01-15 AEC) for animal-based work, in accordance with National Health and Medical Research Council (NHMRC) of Australia guidelines. Mice were divided into different groups (n=5 mice/group) and immunised with NPs bearing 10 µg of LCP-1. Mice were administered intranasally with a booster dose after 2 weeks post-primary immunisation. Positive control mice were administered with 10 µg cholera toxin B (CTB) and 20 µg of peptides (J14 and P25). The negative control group was administered with PBS. All of the formulations were administered at a volume of 10 µL (5 µL/nare). Samples (saliva) were collected after the 7th day of first boosts upon intraperitoneal injection with

pilocarpine solution (50 µL). Blood sample were collected on 14th day of post-primary immunization. Sera was separated after centrifuging blood at 10,000 g for 10 min. Both samples were stored at -80°C before final analysis. ELISA was performed on all samples to determine the production of J-14-specific antibodies by the previously reported method [8].

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey post-hoc tests were used for statistical analyses. *P*-values of <0.05 (*) were considered statistically significant.

Results and discussion

Vaccination is considered to be one of the greatest human weapons in the fight against a wide range of infectious diseases [17]. Gram-positive GAS bacteria invade humans through the respiratory mucosa, and could cause fatal complication, such as rheumatic heart disease. The M-protein is the major virulence factor for GAS infection. One of the directions for vaccine development is the use of conserved B-cell epitopes from the M-protein (e.g. J14) [5,18,19]. However, peptide-based vaccines are not immunogenic and require the addition of adjuvants or a delivery system for optimum efficacy. Recently, the J14 B-cell peptide epitope, along with the P25 universal T-helper epitope, were introduced into the lipid core peptide (LCP) system to form vaccine candidates against GAS (Figure 1) [20,21]. This lipopeptide, when delivered with the cationic liposome, chitosan, and PLGA-based nanocarriers significantly improved immune responses over free LCP-1 upon mucosal immunizations [8,9,13,15,16]. However, it is not clear if high immune responses are due to NPs material or its cationic charge. Therefore, we hypothesised that cationic poly-L-lysine bearing NPs would potentially improve the antigen-specific immune response of LCP-1 after intranasal immunisation.

Negatively charged NPs were prepared either by complexing LCP-1 with dextran (NPs-1) or encapsulating NPs-1 into PLGA (NPs-2). NPs-2 were further coated with poly-L-lysine to form positively charged NPs-3. Dextran, PLGA and poly-L-lysine were selected due to their biodegradability, low toxicity and adjuvanting properties [22]. Particle size plays a vital role in the ability to cross mucosal membranes and be taken up by APCs [19]. Therefore, all particles were produced in the size range of 100-205 nm. Specifically, DLS analysis showed that the diameter of NPs-1, NPs-2 and NPs-3 were 129±0.2 nm, 193±4 nm and 205±1 nm, respectively, and the polydispersity index were 0.21±0.01, 0.08±0.04, and 0.11±0.04, respectively, indicating a monodispersed size distribution of particles. TEM analysis of NPs confirmed these particle sizes (Figure 3). The surface-charge of NPs-1, NPs-2 and NPs-3 were -50±0.5 mV, -31±0.5 mV, and +15±1.0 mV, respectively.

APCs, such as dendritic cells (DCs) or macrophages, take up, process and present antigens to naïve T-cells through the major histocompatibility complex, which stimulates antibody response against specific antigens. To determine whether NPs

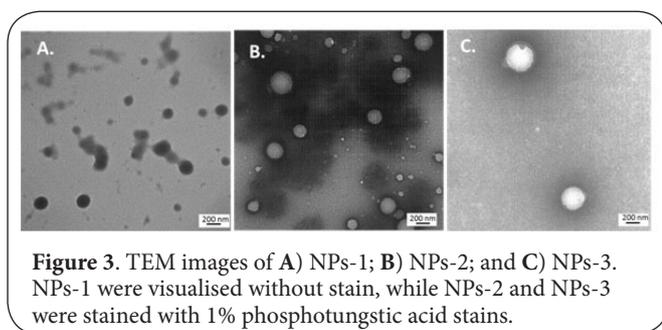


Figure 3. TEM images of A) NPs-1; B) NPs-2; and C) NPs-3. NPs-1 were visualised without stain, while NPs-2 and NPs-3 were stained with 1% phosphotungstic acid stains.

were able to be taken up by APCs, NPs were incubated with splenocyte-derived macrophages and dendritic cells (**Figure 4**). Dextran/LCP-1 bearing anionic NPs (NPs-1) and cationic poly-L-lysine coated PLGA NPs (NPs-3) were taken up by both DCs and macrophages significantly better than free LCP-1. NPs-1 were taken up by APCs preferentially, as expected, due to the well-documented recognition of dextran by mannose receptors present in the APCs [23]. Similarly, cationic NPs-3 were promptly taken up by DCs and macrophages. However, the uptake of LCP-1 was significantly reduced when lipopeptide was incorporated into PLGA-based polymers (NPs-2).

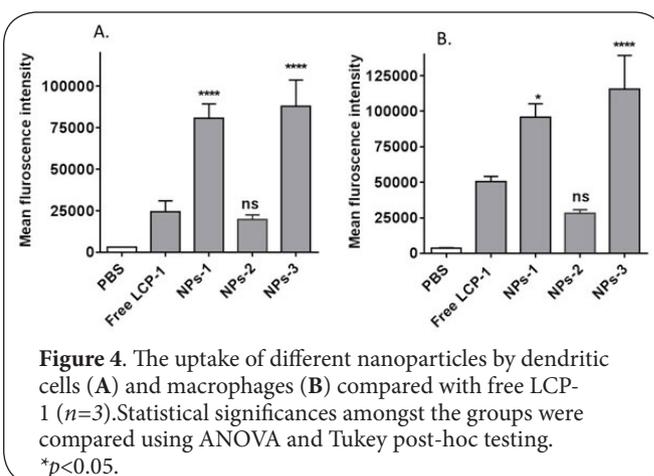


Figure 4. The uptake of different nanoparticles by dendritic cells (A) and macrophages (B) compared with free LCP-1 ($n=3$). Statistical significances amongst the groups were compared using ANOVA and Tukey post-hoc testing. * $p<0.05$.

The ability of the NPs to induce maturation of APCs were accessed. When APCs mature, they express different co-stimulatory molecules, such as CD40, CD80 and CD86. In general, LCP-1 and NPs-1 did not significantly increase the expression of markers at the tested lipopeptide concentration (**Figure 5**). Despite its low uptake, NPs-2 were able to induce a signifi-

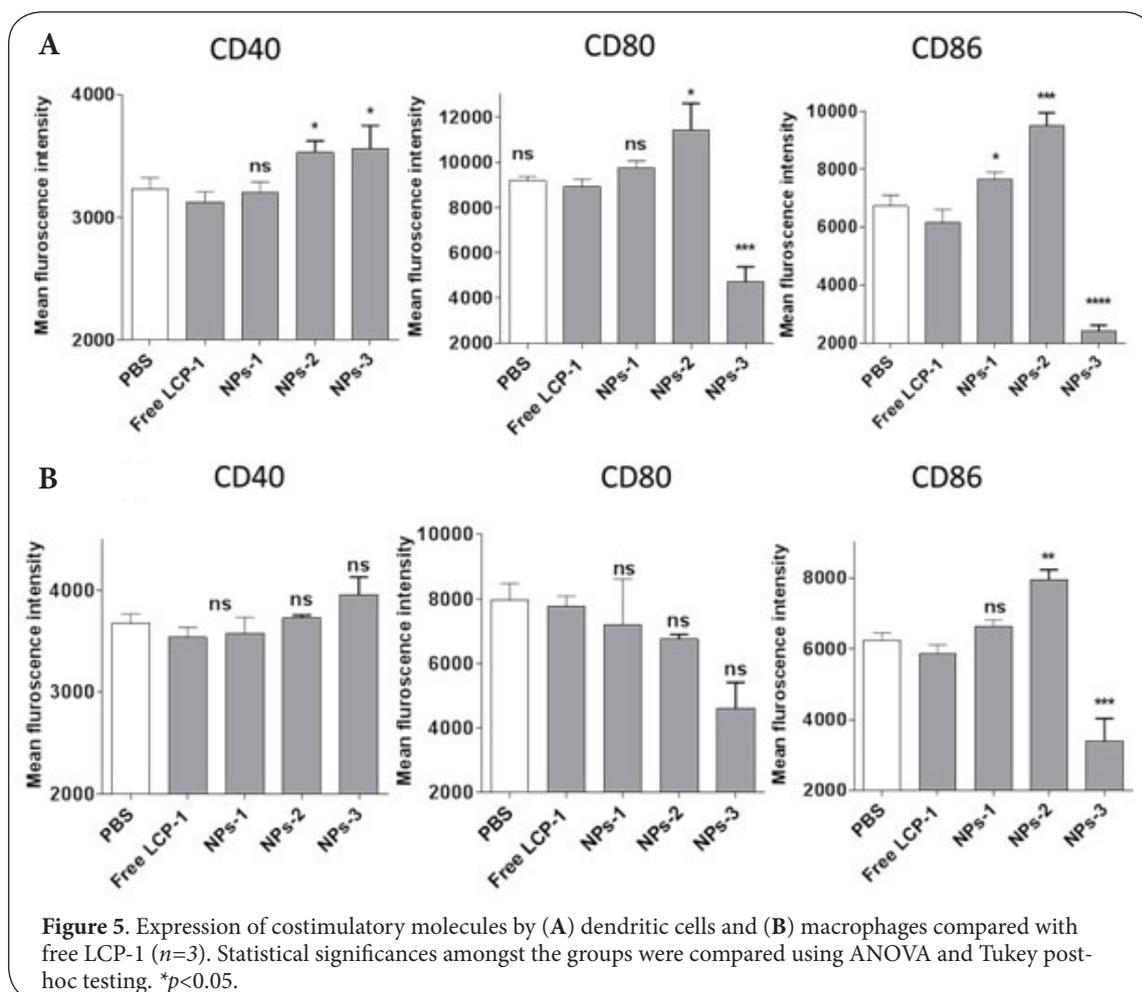


Figure 5. Expression of costimulatory molecules by (A) dendritic cells and (B) macrophages compared with free LCP-1 ($n=3$). Statistical significances amongst the groups were compared using ANOVA and Tukey post-hoc testing. * $p<0.05$.

cantly higher expression of costimulatory molecules in DCs. Interestingly, in spite of its high uptake, poly-L-lysine bearing NPs down-regulated the expression of CD80 and CD86 in DCs. The potential ability of poly-L-lysine to down-regulate an immune response had been suggested previously [24]. In contrast, the expression of CD40 was significantly enhanced by NPs-3. Similar trends in the expression of co-stimulatory molecules in macrophages was observed for all studied groups. Thus, NPs-3 might not be able to stimulate maturation of APCs and induce humoral immunity.

The production of antigen-specific mucosal and systemic antibody titres were examined following intranasal immunisation with NPs in Swiss outbred mice. A physical mixture of CTB with peptides (J14 and P25) was used as a positive control, while PBS was used as negative control. Following two immunisations, free LCP-1 and dextran complexed LCP-1 (NP-1) did not induce significant levels of systemic IgG titres (Figure 6A). In contrast, antibody titres were markedly improved when J14 peptide epitopes were administered with CTB. Interestingly, the incorporation of anionic dextran/LCP-1 into PLGA polymers (NPs-2) showed significant improvement in antibody titres, while additional cationic poly-L-lysine coating (NPs-3) did not induce production of the significant systemic antibody titres. These results were consistent with APCs maturation experiments where NPs-2 showed a high expression of co-stimulatory markers, while NPs-3 showed down-regulation of co-stimulatory markers. High levels of J14-specific systemic antibody titres are expected to reduce systemic dissemination of GAS, while the reduction of mucosal colonisation requires a high level of IgA antibodies in mucosal tissues. Except for the CTB-adjuvanted group, where two out of five mice produced J14-specific IgA titres, none other groups showed an induction of mucosal immunity (Figure 6B). Taken together, we observed that mice immunised with a low dose of LCP-1-bearing PLGA NPs generated a humoral immune response against the antigen, while the poly-L-lysine polymer did not provide an adjuvanting effect. This could be related

to the polymers capacity to downregulate APCs maturation.

Conclusions

In conclusion, we developed NPs-based delivery systems for lipopeptide-based vaccines. Although there was low uptake of PLGA-bearing NPs by APCs, they proved to be the most efficient in the maturation of APCs and the induction of systemic IgG antibodies compared to free lipopeptides or negative controls. In contrast to our expectations, although highly taken up by APCs, cationic poly-L-lysine bearing nanoparticles were unable to trigger *in vitro* maturation and antibody responses. Thus, nanoparticle charge is not the crucial factor for induction of humoral immune response in mucosal immunization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	NM	AKG	MRB	MFG	MS	IT
Research concept and design	✓	--	--	--	✓	✓
Collection and/or assembly of data	✓	✓	✓	✓	--	--
Data analysis and interpretation	✓	--	--	--	✓	--
Writing the article	✓	--	--	--	--	--
Critical revision of the article	✓	--	--	--	✓	✓
Final approval of article	✓	✓	✓	✓	✓	✓
Statistical analysis	✓	--	--	--	--	--

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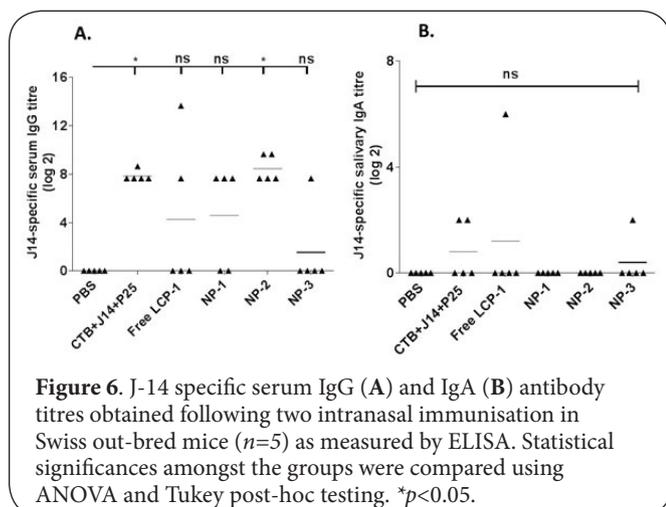
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