Intranasal SARS-CoV-2 spike-based immunisation adjuvanted with polyethyleneimine elicits mucosal and systemic humoral responses in mice

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A B S T R A C T
The SARS-CoV-2 pandemic continues despite the presence of effective vaccines, and novel vaccine approaches may help to reduce viral spread and associated COVID-19 disease. Current vaccine administration modalities are based on systemic needle-administered immunisation which may be suboptimal for mucosal pathogens. Here we demonstrate in a mouse model that small-volume intranasal administration of purified spike (S) protein in the adjuvant polyethyleneimine (PEI) elicits robust antibody responses with modest systemic neutralisation activity. Further, we test a heterologous intranasal immunisation regimen, priming with S and boosting with RBD-Fc. Our data identify small-volume PEI adjuvantation as a novel platform with potential for protective mucosal vaccine development.

1. Introduction
The primary antibody function correlating with protection from SARS-CoV-2 infection and COVID-19 disease is neutralisation. Neutralising antibodies engage the viral spike glycoprotein to interfere with virus-receptor binding and entry (Dejnirattisai et al., 2021). The dominant neutralising antibody target is the S receptor binding domain (RBD) (Dejnirattisai et al., 2021; Lan et al., 2020). Both S and the RBD have been prepared as soluble recombinant forms which can be readily expressed and purified (Kleanthous et al., 2021; Liu et al., 2020; Schaub et al., 2021), and have previously been shown to be safe and immunogenic in animal models and clinical trials (Li et al., 2021; Thanh Le et al., 2020; Wörzner et al., 2021). There are currently several SARS-CoV-2-S trimer-based vaccines in development for systemic administration that use adjuvants licensed for clinical use (Thanh Le et al., 2020).

Despite existing commercially available and experimental vaccines providing protection against both wild-type and some variants of concern (VoCs) of SARS-CoV-2, the emergence of more highly mutated VoCs, such as Omicron, have highlighted the need for supplementary or alternative approaches to improve protection against new strains (Karim and Karim, 2021; Otto et al., 2021), which are undermining global healthcare systems (Georgiadis and Georgiadis, 2021). Consequently, clinical trials are already underway to respond to the ongoing need for modified booster immunisations that aim to improve protection against key VoCs (Choi et al., 2021). There is therefore a continuing need for vaccination approaches that are easy to administer and offer favourable immune profiles against variants of SARS-CoV-2. One possible solution is the development of mucosal vaccination approaches, particularly a readily administrable liquid, or nebulised intra-nasal immunisation, such as the live-attenuated influenza vaccine, Flumist® (Carter and Curran, 2011; Stark et al., 2022).

Intranasal delivery of protein-based immunogens has been investigated for several decades. The development of an intranasal vaccination regimen offers two main hypothetical advantages. First, from a public health perspective, the ease of needle-free administration and associated sociological benefits is attractive. Second, there may be favourable immunological outcomes induced by mucosal delivery of antigen; this includes mucosal lymphocyte homing and favourable immunoglobin (Ig) isotype switching profiles (Barackman et al., 1999; Ranasinghe and Ramshaw, 2009). Collectively, these may aid in the protection of the upper respiratory tract to control and eliminate respiratory pathogens such as SARS-CoV-2 and influenza A virus (IAV).

One challenge to the development of viable intranasal vaccination is the limited repertoire of mucosal adjuvants available, which are

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important when using modestly immunogenic protein-based immunogens (Reed et al., 2013; Zeng, 2016). A primary function of adjuvants is to promote the recruitment and activation of antigen presenting cells (APCs), as reviewed elsewhere (Pulendran et al., 2021). This, in turn, bolsters downstream activation of helper T (Th) cells. Given that Th cells coordinate both cellular and humoral arms of the adaptive immune system, adjuvants have important implications for vaccine efficacy. Moreover, adjuvants may sequester antigen, enhancing presentation to APCs in a particulate form which is favourable for uptake and presentation (Awate et al., 2013; Gnatic and Bhardwaj, 2013; Tizard, 2021).

However, some mucosal adjuvant candidates tested to date have had a high reactogenicity penalty, characterised by respiratory distress and/or inflammation of the olfactory bulb (Zeng, 2016). Thus, there is substantial motivation in investigating novel mucosal adjuvants that a) offer potent immunopotentiating properties, b) have a favourable safety profile, and c) are cheap and producible at scale (Aoshii, 2017; Lavelle and Ward, 2021; Zeng, 2016). Our laboratory previously showed that PEI - the organic polycation used routinely for nucleic acid transfection in vitro and gene/DNA vaccine delivery in vivo - displays potent mucosal (Wegmann et al., 2012) and systemic (Sheppard et al., 2014) adjuvanticity that acts, at least in part, via release of host dsDNA and downstream Ifn-3 activation (Wegmann et al., 2012). In mice, PEI was well-tolerated and offered potent non-proinflammatory responses, inviting further investigation as a mucosal adjuvant for potential human use.

However, our previous study (Wegmann et al., 2012) and those of others were based on intranasal administration to anaesthetised mice, and often of relatively large volumes (Miller et al., 2012). Not only is the use of anaesthetic irrelevant to human use, but excess inoculum may enter the lung and therefore represents intra-pulmonary immunisation (Miller et al., 2012). We therefore set out to develop a small-volume liquid intranasal immunisation that does not require anaesthesia and results predominantly in local nasal-associated lymphoid tissue (NALT) antigen-specific antibody responses.

Here we provide proof of concept based on the SARS-CoV-2 S glycoprotein formulated with the potent experimental mucosal adjuvant PEI. We show that the intranasal administration of SARS-CoV-2 S formulated with PEI elicits superior antibody outcomes in both systemic and mucosal compartments compared with S alone, or formulated with the established mucosal adjuvants CpG (TLR-9-mediated) (Iho et al., 2015) or chitosan (cGAS/STING-mediated) (Riteau and Sher, 2016). Antigen-specific serum IgG was associated with modest neutralisation activity, indicating that small volume intranasal immunisation elicits functional antibody outcomes.

2. Materials and methods

2.1. Protein production, purification and use

Recombinant SARS-CoV-2 Spike (S) (“Victoria” strain) with C-terminal His tag was synthesised in-house. Freestyle 293-F cells were cultured in Freestyle expression media (Life Technologies) (37°C, 8% CO₂, 115 rpm orbital shaking). Cells were transfected at a density of 1 x 10⁹ cells/L. The expression vector (300 μg/L) and polyethyleneimine (PEI) MAX (Polysciences) (900 μg/L) were pre-incubated in serum-free OPTI-MEM media (Life Technologies) for 30 mins at 25°C, and the mixture added to the cells. After 5 days, supernatant was harvested after centrifuging the cell culture (1500 x g, 20 mins, 4°C) and supernatant passed through a sterile 0.22 μm filter. Protein was purified via immobilised metal chromatography (matrix and manufacturer), and protein yield was quantified via a bicinchoninic acid (BCA) assay (Life Technologies). Yield was ~800 μg/L supernatant. RBD-Fc was synthesised similarly, with a yield of >10 mg/L supernatant. The S-specific neutralising monoclonal antibody CR3022 (ter Meulen et al., 2006) and the ACE-2-Fc (Barton et al., 2021) were obtained as purified proteins and used at concentrations as described in the figure legends.

2.1.1. Endotoxin assay

To check the immunogen preparations for endotoxin, a reporter line was used: HEK 293 T cells transfected with TLR4-CD14-MD2 (Invitrogen) that were cultured according to the manufacturer’s instructions. To test for lipopolysaccharide (LPS) contamination, cells were plated at 50000 cells/well in a 96-well flat-bottom plate. Protein samples were added at 50 μg/mL and left for 12 h. PBS and Ultrapure-LPS (Invivogen) controls were also set up. Supernatant was collected and the human IL-8 concentration was measured via ELISA (Thermo Fisher) according to the manufacturer’s instructions as a readout of TLR-4 activation.

2.2. Native and reducing SDS PAGE

To determine protein purity and S protein integrity, reducing SDS was run according to the manufacturer’s instructions (Life Technologies). Briefly, for SDS-PAGE, 5 μg of S was loaded into the wells of a NuPAGE 4–12% gradient Bis-Tris Gel (Life Technologies) and run in the presence of 1 x MES Running Buffer (Life Technologies) for 90 mins at 125 V.

2.3. Mice, immunisation regimens and sampling

Wild-type pathogen-free female 6-week-old BALB/c mice were purchased from Charles River. Animals were monitored daily and provided standard chow and water ad libitum. Mice were immunised with 10 μg of recombinant SARS-CoV-2 S in PBS or in formulation with adjuvants: PEI, CpG (immunostimulatory CpG 1826, Invivogen) or chitosan (Pacific Gene Technology). These immunogens were prepared in sterile, endotoxin-free PBS and were administered intranasally (i.n.) without anaesthetic in a total volume of 10 μL (5 μL administered to each nostril) according to the prime-boost regimen described in the Results section (weeks 0 and 4). Animals were sacrificed via a rising CO₂ gradient and subsequent cervical dislocation schedule 1 procedure. Serum samples were obtained via peripheral tail vein bleeding and prepared following whole blood clotting and centrifugation. Nasal cavity wash, bronchoalveolar lavage and vaginal lavage fluids were also collected. Mouse experiments were based on a block design with cages containing one animal receiving each of the treatment regimens to avoid cage effects. Animal experiments were conducted in two independent experiments, each containing 4 animals per group (total n = 8).

2.4. ELISA

Samples were serially-diluted and incubated on antigen-coated and blocked SpectraPlate-96 HB (PerkinElmer) plates. Bound antibodies were detected with the following secondary antibodies: anti-mouse IgG-HRP (STAR120P, Bio-Rad), anti-mouse IgA-HRP (STAR137P, Bio-Rad), anti-mouse IgG1-HRP (STAR132P, Bio-Rad) or anti-mouse IgG2a-HRP (STAR133P, Bio-Rad). ELISAs were developed using 1-Step Ultra TMB ELISA substrate (Life Technologies) and the reaction terminated with 0.5 M H₂SO₄. Endpoint titres were determined by calculating the maximum dilution in which OD₄50–450 > 0.01 above background. Competition ELISAs were performed using competing serum and ACE-2 against S. S-coated and blocked plates were pre-incubated with serially diluted serum for 1 h before an ACE-2 huloAg Fc fusion protein (Absolute Antibody) was added at the EC₅₀ concentration (20 ng/mL) and incubated for 1 h. ACE-2 fusion protein binding was detected using anti-human IgA (STAR141P, Bio-Rad).

2.5. SARS-CoV-2 production

Viruses were produced in Vero E6-TMPRSS2 cells. Cells were propagated in T175 flasks, in cDMEM (10% FBS, 1% P/S). Once approximately 70% confluent, propagation medium was removed and cells infected with 1 mL of medium (DMEM, 1% FBS, P/S) containing virus at
a final MOI of 0.001. Flasks were incubated for 10 mins at RT before addition of a further 19 mL of DMEM (1% FBS, 1% P/S). Cells were incubated at 37 °C and periodically inspected for cytopathic effect (CPE). Once wide scale CPE was observed across the culture flask (~72 h), medium was collected, cell debris pelleted, and supernatant harvested as the fresh virus stocks.

2.6. Neutralisation assay

Neutralisation assays were performed as described elsewhere (Skelly et al., 2021). Briefly, test sera were initially diluted 1:20 into DMEM (1% FBS, 1% P/S), then further diluted serially to produce 7 concentrations at half log10 intervals. Dilutions for each test sample were transferred into quadruplicate wells of an assay plate (20 μL per well). Diluent medium was used in control wells. Virus stock (VIC/01/2020) was diluted to ~7500 focus-forming units per mL (FFU/mL) (Caly et al., 2020). 20 μL of virus solution was added to each plate well, such that each well contained ~150 FFU. The test concentration of the serum was calculated at this stage; therefore, the top concentration was 1:40. Plates were left at RT for 30 mins before addition of 100 μL of Vero-CCL81 cells (at 4.5 × 10^5/mL), and incubated at 37 °C for 2 h. An overlay of 100 μL 1.5% carboxymethyl cellulose (CMC) solution was added to each well then plates returned to 37 °C for a further 20 h. The assay was stopped by removal of all media, washing with PBS and fixation with 4% paraformaldehyde solution. After fixing, cell monolayers were stained with human antibody EY2A (Huang et al., 2021)—which recognises SARS-CoV-2 N protein—and subsequently with anti-human IgG HRP, TrueBlue peroxidase substrate to produce foci demarcating infected cells. These foci were counted in an automated process by an AID ELI Spot reader (AID GmbH). Focus counts were normalised to serum-free control wells and plotted in GraphPad Prism Version 8. Neutralisation titre 50% (NT50) values were calculated using Prism’s in-built ECAnything equation, with ECF set to 50 and bottom constrained to 0.

2.7. Statistics

ELISA endpoint titre values were log-transformed. All datasets were deemed to be not normally distributed, according to a Kolmogorov-Smirnov test. Hence, data were analysed using a Kruskal-Wallis test, with a significance limit p < 0.05. The relationship between endpoint titre values and neutralisation titre values was conducted via Spearman rank correlation (non-parametric).

3. Results

3.1. Expression and purification of recombinant SARS-CoV-2 spike

Wild-type (“Victoria” strain) recombinant soluble C-terminally His-tagged SARS-CoV-2 Spike (S) trimer with a deleted S1-S2 furin cleavage site was expressed via transient transfection of HEK 293F cells and purified using an immobilised nickel column. Purified S was evaluated via SDS-PAGE, which revealed a high-purity product, with intact S1-S2 protomers accounting for >80% of total protein (Fig. 1a). To ensure preservation of the trimeric immunogen, stocks were stored aliquoted at ~−80 °C until administration. Antigenicity screening was conducted using the RBD-specific neutralising antibody CR3022 and an ACE-2-Fc fusion protein, which yielded EC50 values of 0.084 nM and 0.24 nM respectively (Fig. 1b). These data are consistent with published affinities for these reagents (Liu et al., 2021; Yuan et al., 2020) and indicate proper S folding and retention of critical RBD “up-exposed” surfaces, the site at which a cluster of potent neutralising epitopes are found (Dejnirattisai et al., 2021; Leach et al., 2021; Linling et al., 2022). Endotoxin analysis revealed essentially undetectable (<0.125 ng/mL) lipopolysaccharide (LPS) (Fig. 1c).

3.2. Intranasal administration of S with PEI promotes systemic antibody responses

Naïve female wild-type 6-week-old BALB/c mice were immunised with 10 μg of recombinant S in formulation with adjuvant as follows: i) 50 μg CpG; ii) 5% CA; iii) 100 μg PEI (Fig. 2a) (Wegmann et al., 2012). We further tested a heterologous RBD-Fc boost in formulation with PEI to evaluate whether this might focus immune responses towards the RBD, the main target of antibody-mediated neutralisation (Dejnirattisai et al., 2021). The formulation was administered in a total volume of 10 μL (5 μL to each nostril) to un-anaesthetised animals to mimic conscious intra-nasal liquid-jet immunisations that could hypothetically be received by a human. Thus, the bulk of the immunisation volume would most likely remain in the nasal cavity and stimulate the NALT rather than enter the lung. Animals were primed and boosted 4 weeks apart. To evaluate formulation toxicity animals were weighed daily following the prime (Fig. 2b). Changes to weight with respect to time post-prime was evaluated; compared with the unadjuvanted control, neither the homologous S + PEI or heterologous S/RBD-Fc + PEI groups saw significantly different changes to weight gain (P = 0.882 and P = 0.1428, respectively; Kolmogorov-Smirnov test). No breathing abnormalities or other complications were observed in any mouse given any formulation and no reactogenicity events were reported.

Since our previous study demonstrated induction of robust antigen-
specific serum IgG responses after intranasal immunisation under anaesthesia (Wegmann et al., 2012), we evaluated anti-S IgG endpoint titres following the prime and boost. The serum S-specific IgG titres measured 3-weeks post-prime revealed that most animals that had received a PEI-formulated immunisation gave detectable titres (9/16), with responding mice exhibiting endpoint titres in the $10^4$–$10^5$ range. No responses were detected in the unadjuvanted or CpG- or chitosan-adjuvanted groups (Fig. 2c). Endpoint titres in the adjuvanted groups increased following the boost and at the terminal timepoint, CA- and CpG-adjuvanted groups had median endpoint titres of $10^3$–$10^4$, which was not significantly greater than the unadjuvanted control ($P = 0.4963$ and $P > 0.9999$, respectively). PEI adjuvanted animals reached IgG endpoint titres $10^6$, which, despite considerable variation in the titres, were significantly greater than unadjuvanted S (homologous regimen, $P = 0.0005$; heterologous, $P = 0.0011$) (Fig. 2d). Although the RBD-Fc boost increased S-specific IgG titres, indicating successful recall of relevant memory B cells, the titres were comparable to the homologous regimen ($P = 0.382$, Mann-Whitney test).

Next, anti-Spike IgG1 and IgG2a were evaluated as an indicator of T cell help bias. It was observed that IgG1 was the predominant subisotype among the responding mice (Fig. 2e). PEI was shown to induce some IgG2a, although the median endpoint titres were roughly two orders of magnitude less than that for IgG1 (Fig. 2f). IgG2a was detected in all responding mice in the CpG-adjuvanted group in-line with the known Th1-biasing effects of the TLR9-dependent adjuvant.

We further evaluated systemic IgA, although the median titre was undetectable in all groups (Fig. 2g).

### 3.3. PEI induces mucosal IgA production following intranasal S-based immunisation

Since mucosal immunisation is aimed at eliciting responses at mucosal surfaces, we screened various lavage fluids for antigen-specific IgA. Nasal cavity wash fluid was collected upon termination at 5-weeks post-boost, and yielded greater S-specific IgA in the in the animals that received PEI compared to the other adjuvants (Fig. 3a). A weak but significant correlation between the serum IgG and mucosal IgA titres was observed ($\rho = 0.61$, $P < 0.0001$, Spearman correlation) (Fig. 3b) reflecting the adjuvanticity of PEI in both the systemic and mucosal compartments. We checked for evidence of systemic contamination in the nasal wash samples by evaluating S-specific IgG titres (Fig. 3c). IgA titres were also measured in the bronchioloalveolar lavage (BAL) and vaginal lavage, with trends largely reflecting those observed in the nasal wash (Fig. 3d, e). Interestingly, titres in the BAL were weaker and only half of the mice had a detectable titre, probably resulting from limited drainage of the small volume inoculum into the lungs. Thus, in the homologous S-PEI group, 4/8 mice responded in the BAL, whereas 8/8 mice responded in the nasal wash titre.

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**Fig. 2.** Evaluation of systemic antibody responses following i.n. immunisation. (a) Female 6-week-old WT BALB/c mice were primed and boosted 4 weeks apart via the i.n. route with 10 μg of recombinant SARS-CoV-2 S formulated with adjuvant. Animals were bled periodically. (b) Weight of animals was monitored daily for the first week post-prime. S-specific IgG endpoint titres (EPT) in serum were evaluated at (c) week 3 and (d) week 9 via direct ELISA. At the terminal timepoint, serum S-specific (e) IgG1, (f) IgG2a and (g) IgA were measured. ELISA data are represented as median, with dots representing biological replicates ($n = 8$; data are pooled from two independent experiments, as denoted by circle and square dots). Statistical significance was determined via Kruskal-Wallis test combined with Dunn’s multiple comparison test. $p$-value denotations: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. 

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3.4. PEI enhances neutralising antibody responses

Next, we evaluated the functional antibody responses by first evaluating RBD-specific endpoint titres—the region of S that delivers the most potently neutralising antibodies (Fig. 4a). The observed trends largely reflect that of the S-specific antibody responses, which is interesting given that the RBD-Fc boost would selectively engage RBD-specific B cells rather than other S regions (e.g. the N-terminal and S2 domains). The fact that there is no significant difference between the homologous and heterologous regimens may reflect RBD immunodominance in S.

Given that SARS-CoV-2 neutralisation is primarily mediated by antibodies inhibiting host cell attachment by occupying or disrupting the RBD (Baum et al., 2020a, 2020b; Zost et al., 2020), we performed a competition ELISA measuring the ability of sera to inhibit soluble dimeric ACE-2 binding to RBD on the solid phase. These data showed a greater IC₅₀ value in animals that received a PEI-formulated immunisation compared with the unadjuvanted, CpG-or CA-adjuvanted groups (P < 0.01 for all) (Fig. 4b). We further conducted a serum neutralisation assay against the autologous wild-type/Wuhan SARS-CoV-2 (Fig. 4c), serum from animals that received PEI displayed superior neutralisation titres compared with the unadjuvanted or alternatively adjuvanted groups, and this reached significance when pairwise comparing PEI with other groups (P < 0.01 for all). This pattern is consistent with that observed in the competition assay. Interestingly, there were no significant differences observed between the homologous S or heterologous S/RBD-Fc regimens, indicating no functional benefit to the RBD-Fc boost. We compared the S-specific IgG titres against the competition ELISA IC₅₀ values, which revealed a weak correlation (r = 0.33, P < 0.038, Spearman correlation) (Fig. 4d). Unfortunately, we did not perform a neutralisation assay on the nasal wash given the limited volume available.

4. Discussion

The ongoing SARS-CoV-2 pandemic has re-initiated interest in mucosal vaccine delivery, a function of both the ease of administration and hypothetical downstream immunological payoffs that may better protect the upper respiratory tract and potentially also yield systemic protection (Mouro and Fischer, 2022). Here, we tested the experimental mucosal adjuvant, PEI (Sheppard et al., 2014; Wegmann et al., 2012) in formulation with recombinant S in mice. We identified that intra-nasal administration of PEI using a small-volume without anaesthetic, produces significantly better mucosal and systemic anti-S and anti-RBD IgG titres and neutralisation titres compared to CpG and chitosan, implying that PEI offers more potent adjuvanticity via this route. The superior adjuvanticity offered by PEI may reflect its reported Irf3-activating properties (Sheppard et al., 2014; Wegmann et al., 2012). Irf3 is an effective augmenter of follicular helper T cell (Tfh) responses that bolsters B cell proliferation, affinity maturation and isotype switching.
Our data suggest that protein-based, low-volume upper respiratory immunisation still facilitates classical secretory antibody responses typically found in the lower respiratory tract (Helfrifsch and Scherlieβ, 2019). This is reflected by IgA titres measured not only at the vaccination site (that is, the nasal wash fluid) but also in BAL and vaginal lavage, demonstrating that the antibody response is reaching distant mucosal surfaces. Thus, intranasal immunisation using PEI might have utility for immunisation against sexually transmitted infections as has been shown in other model systems (Persson et al., 2016). Unfortunately, the limited volume of nasal lavage prevented us from carrying out competition and neutralisation assays, and we had to rely on serum. Therefore, whether local antibody titres in the upper respiratory tract would be sufficient to reduce or prevent SARS-CoV-2 infection would have to be tested in a challenge model which is beyond the scope of this study.

Intra-nasal immunisations often result in a Th2-biased immune response (Ranasinghe et al., 2014). This is in part a function of programmed skewing in the cytokine sensitivity of mucosally-homed immune cells (Deimel et al., 2021; Ranasinghe et al., 2007; Roy et al., 2020) but may also be a function of inflammasome activation by PEI which may impart a Th2 bias (Wegmann et al., 2012). Accordingly, in homologous S-based regimens tested in this study, IgG1 was the predominant isotype detected. Interestingly, boosting with RBD-Fc + PEI did not boost the IgG2a titres compared with the S + PEI homologous regimen. This is surprising, since it was expected that the RBD-Fc construct would facilitate a Th1-biasing effect based on FcR interactions promoting pro-inflammatory Th1-biasing cytokine/chemokine production via neutrophil recruitment (Bruhns and Jönsson, 2015; Wang and Jönsson, 2019). While we did not explicitly evaluate the mucosal vaccine-specific T cell responses in this study, the effects of both the sequential immunogen schedule and the role of PEI in biasing the T cell outcome warrants further investigation.

The rationale behind the heterologous S/RBD-Fc immunisation regimen was to selectively recall RBD-specific memory B cells in the boost, the B cells associated with the greatest neutralisation breadth and potency (Dejirnattisai et al., 2021). Whilst we have not directly demonstrated selective recall here, which would require a longitudinal evaluation of clonal B cell responses, we did observe a dramatic bolstering in antibody titres following RBD-Fc immunisation, as evidenced by the gain in systemic IgG compared with the post-prime titres. Interestingly, however, the RBD-specific IgG titres were roughly the same in the homologous and heterologous regimens, indicating that a second S immunisation equivalently generates an effective boost response against the RBD.

Our data indicate that a small-volume intranasal inoculum using antigen formulated with PEI is a promising mucosal adjuvant that provides robust systemic and mucosal isotype-switched antibodies against SARS-CoV-2 S with modest neutralisation activity detected in the serum. Systemic neutralisation titres were overall pretty low, even in the PEI-adjuvanted groups, and thus it is unclear whether these responses are functionally significant in providing, for example, sterilising immunity. However, we envisage further development of this adjuvant, for example, evaluating the early innate response to the adjuvant and the histological effects of the adjuvant at the vaccination site. In line with our expectations of the small-volume, no anaesthetic model, antigen-specific titres were highest in the NALT and weaker in the BA. This murine model of mucosal delivery of protein immunogens may assist in generating novel vaccine approaches against SARS-COV-2 infection and disease.

Author contributions

QJS and LPD conceived the study. LPD, LX, JG-J, SL, WSJ and QJS designed and performed experiments. LPD and QJS wrote the
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