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1 **Respiratory mucosal vaccination of peptide-poloxamine-DNA nanoparticles**
2 **provides complete protection against lethal SARS-CoV-2 challenge**

3
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33 **Abstract:** The ongoing SARS-CoV-2 pandemic represents a brutal reminder of the
34 continual threat of mucosal infectious diseases. Mucosal immunity may provide robust
35 protection at the predominant sites of SARS-CoV-2 infection. However, it remains unclear
36 whether respiratory mucosal administration of DNA vaccines could confer protective immune
37 responses against SARS-CoV-2 challenge due to the insurmountable barriers posed by the
38 airway. Here, we applied self-assembled peptide-poloxamine nanoparticles with mucus-
39 penetrating properties for pulmonary inoculation of a COVID-19 DNA vaccine (pSpike/PP-
40 sNp). Not only displays the pSpike/PP-sNp superior gene-transfection and favorable
41 biocompatibility in the mouse airway, but pSpike/PP-sNp promotes a tripartite immunity
42 consisting of systemic, cellular and mucosal immune responses that are characterized by
43 mucosal IgA secretion, high levels of neutralizing antibodies, and resident memory phenotype
44 T-cell responses in the lungs of mice. Most importantly, pSpike/PP-sNp completely
45 eliminates SARS-CoV-2 infection in both upper and lower respiratory tracts and enables
46 100% survival rate of mice following lethal SARS-CoV-2 challenge. Our findings indicate
47 PP-sNp might be a promising platform in mediating DNA vaccines to elicit all-around
48 mucosal immunity against SARS-CoV-2.

49

50 **Key words:** mucosal vaccine, COVID-19, SARS-CoV-2, DNA vaccine, nanoparticle,
51 pulmonary delivery.

52

53

54 **Introduction**

55 The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory
56 syndrome coronavirus 2 (SARS-CoV-2) has posed a huge and continual threat to the world.
57 Although there are already several authorized COVID-19 vaccines, all these vaccines are
58 inoculated via parenteral route, which predominantly elicit systemic immunity dominated by
59 serum IgG antibodies without conferring mucosal immunity¹. SARS-CoV-2, which invades
60 the host through the mucosa of the respiratory tract, may seed in the initial reservoir and
61 continue to spread between individuals². Recent studies have demonstrated that respiratory
62 mucosal vaccinated adenoviral vaccines could overcome the drawbacks of parenterally
63 administered counterpart via the stimulation of broad local immune responses in the airways
64 that in turn can block both infection and spread from this reservoir³⁻⁶. Secretory
65 immunoglobulin A (sIgA) and pulmonary resident memory T cells (T_{RM}) are suggested to be
66 key components in this first-line of defense⁷⁻⁹. Compelling evidences have indicated that sIgA
67 in the respiratory mucosa contributed to SARS-CoV-2 neutralization to a greater extent than
68 IgG equivalents^{10, 11}, and lung-T_{RM} cells are critical in mediating protection against
69 respiratory pathogens¹². Since mucosal route of immunization is considered by the research
70 community as the most straightforward approach to induce potent mucosal immunity¹³, safe
71 and efficient delivery of vaccine to the respiratory mucosa would be needed in order to
72 reliably stimulate sIgA secretion and engender protective mucosal immunity against SARS-
73 CoV-2¹⁴.

74

75 Despite the great potential of mucosal immunity, the major advances seen with parenterally
76 administered vaccines (such as adjuvanted subunit antigens, DNA and, more recently, RNA
77 vaccines) have not been translated into licensed mucosal vaccines. There continues to be a
78 dearth of safe and effective respiratory mucosal vaccine platforms despite decades of
79 investigations¹⁵. Up to date, the only successful approach is virus-based mucosal vaccines. Of
80 the nine mucosal vaccines approved for use in humans (one intranasal and eight oral) all are
81 either live attenuated or whole-cell inactivated vaccines¹⁶. The dichotomy in parenteral and
82 mucosal approaches is, in part, due to the existence of multiple barriers (e.g., the mucus layer
83 that traps exogenously inhaled substances) and natural defense mechanism (such as
84 mucociliary clearance) keeping the foreign substances out¹³. Viruses have evolved to
85 proficiently overcome these barriers, this explains why the vast majority of investigations
86 achieving protection against SARS-CoV-2 using airway route of immunization exclusively
87 utilized virus-based vaccines, such as adenoviruses^{6, 17-19}. Nevertheless, these virus vaccines

88 are often compromised by preexisting immunity and safety issues, some adenovirus-based
89 COVID-19 vaccines may cause cerebral venous sinus thrombosis²⁰, rendering the US Food
90 and Drug Administration puts strict limits on this type of COVID-19 vaccines. Therefore, it is
91 difficult to strike a delicate balance between immune efficacy and safety of these virus
92 vaccines. On the other hand, cutting-edge advances and research into the nucleic acid-based
93 vaccine technologies provide alternative options to solve the unmet needs for safe and
94 efficient non-viral based mucosal vaccines.

95

96 DNA vaccines, which proved to be helpful in controlling the pandemic²¹, would offer
97 substantial advantages over virus-based vaccines, including safe profiles^{22, 23}, ease of
98 manufacture with low costs, and stability at room temperature²¹. However, DNA vaccines
99 generally display limited immunogenicity in the airways because the most commonly used
100 electroporation device is not applicable and consequently require potent delivery systems to
101 overcome the barriers within the respiratory tract²⁴. Traditional DNA delivery vectors, such as
102 cationic polymers and even lipid-based formulations that were tested in clinical trials, have
103 turned out to be inefficient owing to the poor airway mucus penetrating properties^{25, 26}.
104 Therefore, the pulmonary administration of conventional DNA nanoparticles is unlikely to
105 shuttle DNA cargos efficiently to initiate robust immune responses in the respiratory tract. As
106 revealed by a previous study, the intranasal COVID-19 DNA vaccination in mice only led to a
107 modest induction of local T-cells secreting IFN- γ , without eliciting mucosal sIgA and
108 circulating IgG against SARS-CoV-2 even after prime and two boost doses²⁷. To the best of
109 our knowledge, whether a COVID-19 DNA vaccine delivered via the airway could confer
110 protective efficacy against SARS-CoV-2 challenge was still unknown.

111

112 In a previous study, we developed a self-assembled peptide-polyamine nanoparticle (PP-
113 sNp) delivery system that is specifically designed for efficient delivery of plasmid DNA
114 across the mucus layer of the respiratory tract²⁸. PP-sNp carrying a *Sleeping Beauty*
115 transposon system successfully enabled the genomic integration of *CFTR* gene in the airway
116 epithelia of cystic fibrosis mice with a safe integration profile²⁸. These results suggest that PP-
117 sNp would be promising in mediating DNA vaccines to stimulate potent mucosal immune
118 responses against SARS-CoV-2. To this end, we integrated an intercellular adhesion
119 molecule-1 (ICAM-1/CD54, which is abundantly expressed on the epithelial and immune
120 cells²⁹) targeting moiety into the PP-sNp with the purpose of enhancing the specific cellular
121 uptake of pulmonary dendritic cells and airway epithelial cells. These cells have been

122 suggested to play important roles in both initiating potent respiratory immune responses and
123 orchestrating innate immunity to maintain normal airway architecture^{30, 31}. A DNA vaccine
124 encoding the wild-type spike protein of SARS-CoV-2 (pSpike, which has been tested in
125 clinical trials²²) was formulated in PP-sNp (pSpike/PP-sNp) for mucosal vaccine applications.
126 Three vaccine doses of pSpike/PP-sNp via respiratory tract induced comprehensive and broad
127 protective immune responses, including mucosal immunity (SARS-CoV-2 specific sIgA in
128 the airways as well as lung-T_{RM} cells) and systemic immunity (neutralizing serum IgG). All
129 these merits conferred virtually full protection of vaccinated mice against lethal SARS-CoV-2
130 challenge and completely removed the virus in both the turbinates and lungs of mice. Thus,
131 our study may serve as the first proof-of-concept revealing that respiratory mucosal
132 immunization of DNA vaccines holds the potential to elicit robust protective immunity
133 against SARS-CoV-2 infection in the upper and lower respiratory tracts.

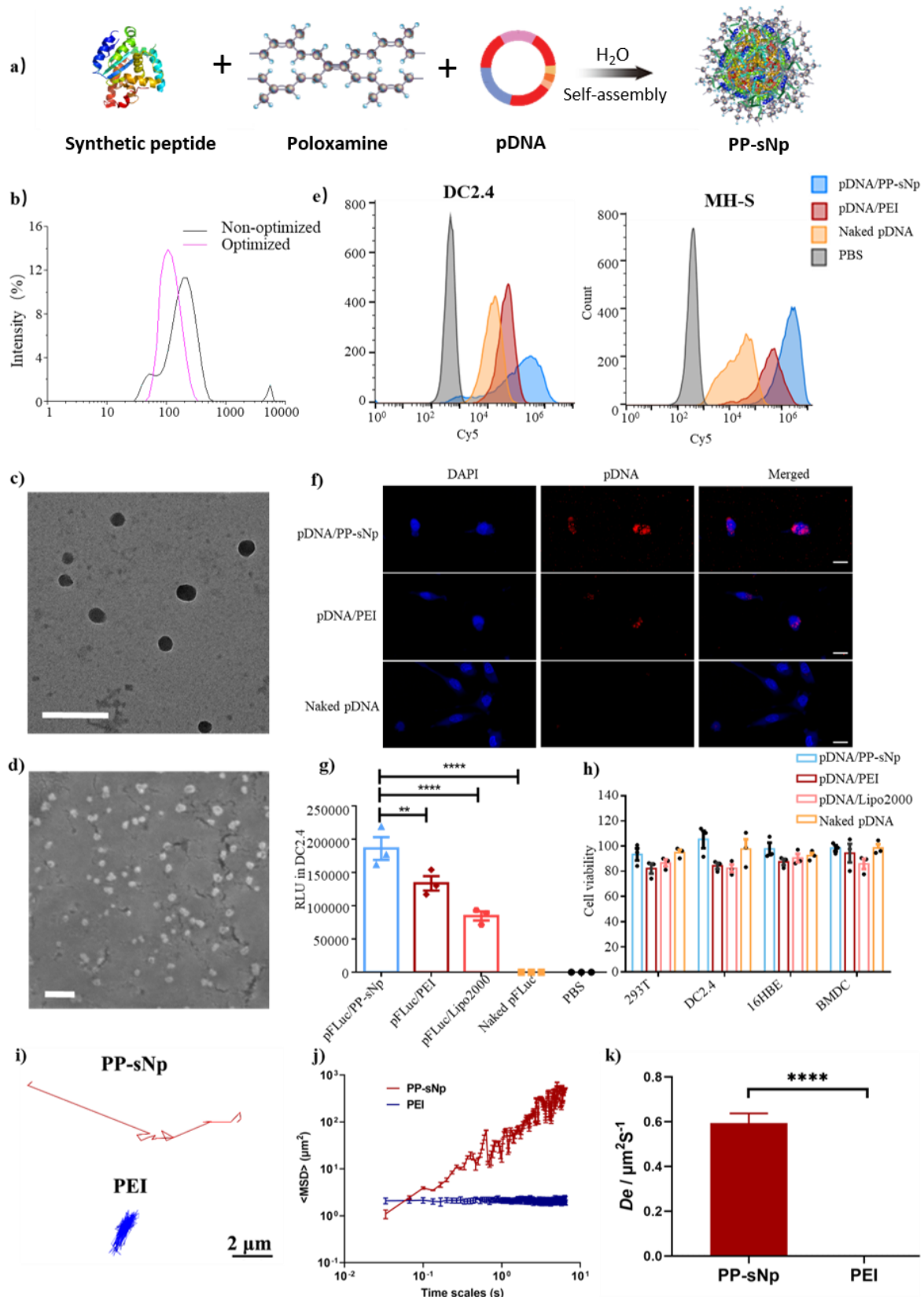
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135 **Results**

136 **The characterization and in vitro investigation of peptide-ploxamine 137 nanoparticle (PP-sNp) containing plasmid DNA**

138 The PP-sNp formulation is formed by a simple self-assembly of multi-modular peptide
139 (Supplementary Table 1), poloxamine 704 (a block copolymer proven to be capable of
140 mediating efficient DNA transfection in the airways to a level that is significantly better than
141 "gold-standard" lipid-based GL67A formulation that utilized in clinical trials^{26, 32, 33}) and
142 pDNA components (Fig. 1a). We engineered small and monodisperse PP-sNp nanoparticles
143 with negative charge (ζ potential: -34.7 ± 0.5 mV) via an optimized procedure in order to deal
144 with the small volumes and high concentrations required in vivo (Fig. 1b, Supplementary
145 Table 2). The electron microscopy showed that PP-sNp had a spherical morphology with
146 uniform size distribution (Fig. 1c and 1d). PP-sNp can be stored at room or high temperatures
147 and remains stable (Supplementary Fig. 1). We then evaluated the uptake efficiency of PP-
148 sNp in DC2.4 and MH-S cell lines, the results indicate an enhanced cellular uptake of PP-sNp
149 containing Cy5 labelled pDNA (Cy5-pDNA) by both cell lines compared to the 25 kDa
150 branched polyethyleneimine (PEI, a representative polymer based delivery vehicle in DNA
151 mucosal vaccines³⁴⁻³⁶)-based and naked Cy5-pDNA-based controls (Fig. 1e, Supplementary
152 Fig. 2). Confocal microscopy revealed that the majority of Cy5-fluorescence was observed in
153 the nucleus of Cy5-pDNA/PP-sNp transfected DC2.4 cells with dispersed patterns, while the
154 Cy5-fluorescence signal within the nucleus of cells transfected by Cy5-pDNA/PEI and naked
155 Cy5-pDNA was very limited (Fig. 1f). Afterwards, pDNA encoding firefly luciferase (pFLuc)

156 was applied as a reporter to evaluate their transfection efficiency. As shown in Fig. 1g and
157 Supplementary Fig. 3, PP-sNp displayed the most efficient transfection in several cell lines
158 (DC2.4, Calu-3, 16HBE and BEAS-2B) compared to control formulations, such as
159 lipofectamine2000 (lipo2000)-based lipoplex, PEI-based polyplex and naked-pFLuc.
160 Meanwhile, we evaluated the cytotoxicity of these formulations and found PP-sNp based
161 formulation did not provoke significant cytotoxicity on the viability of different cell lines,
162 whereas Lipo2000-based and PEI-based counterparts did (Fig. 1h). Additionally, multiple
163 particle tracking (MPT) assays were performed to evaluate the mucus penetrating ability of
164 PP-sNp. The trajectories of the PP-sNp-based and PEI-based particle motions were captured
165 (Supplementary Video 1 and 2), and representative trajectories were mapped in Fig. 1i. PEI
166 nanoparticles were almost trapped by the mucus network. In contrast, PP-sNp was able to
167 move freely in a large area, displaying a better diffusion pattern. The mean square
168 displacement ($\langle \text{MSD} \rangle$) of PP-sNp was about 1000-fold higher than PEI counterpart at 10 s
169 (Fig. 1j). Consistently, the effective diffusivities (D_{eff}) of PP-sNp were significantly higher
170 than that of PEI (Fig. 1k). The distinct movement patterns suggest that PP-sNp hold the
171 capability of efficient mucus penetrating.



172

173 **Fig. 1 | Characterization and in vitro evaluation of pDNA/PP-sNp.** a) Schematic showing the formation
 174 of pDNA/PP-sNp through a self-assembly process in aqueous solution. b) The dynamic laser scattering
 175 results measured for particle sizes of pDNA/PP-sNp prepared by different methods. c) The transmission
 176 electron microscopy (TEM) micrograph and d) the scanning electron microscope (SEM) micrograph of

177 pDNA/PP-sNp. Scale bar: 200 nm. **e**) Cellular uptake of Cy5-pDNA/PP-sNp, Cy5-pDNA/PEI and naked
178 Cy5-pDNA in DC2.4 and MH-S cells. Cy5-fluorescence intensity within the cells was measured by flow
179 cytometry. **f**) Subcellular fate of the Cy5-pDNA/PP-sNp, Cy5-pDNA/PEI and naked Cy5-pDNA after 4 h
180 incubation with DC2.4 cells detected by confocal laser scanning microscopy. Blue channel, nuclei stained
181 by DAPI; Red channel, Cy5-pDNA; Merged, combination of the aforementioned channels. Scale bars: 20
182 μm . **g**) Transfection efficiency of pFLuc/PP-sNp in DC2.4 cells. DC2.4 cells were incubated with
183 pFLuc/PP-sNp, pFLuc/PEI, pFLuc/lipo2000 or naked-pFLuc for 4 h, then subjected to detection of
184 bioluminescence in cell lysate 48 h post transfection. Statistical significance was calculated by one-way
185 ANOVA with Dunnett's multiple comparisons tests (** $P < 0.01$, **** $P < 0.0001$). **h**) CCK-8 cell viability
186 assay of pDNA/PP-sNp, pDNA/PEI, pDNA/lipo2000 or naked-pDNA after 4 h incubation with different
187 cell lines (all formulations were prepared under conditions showing the highest transgene expression). Data
188 in **g** and **h** represent mean \pm SEM ($n = 3$ independent experiments). Multiple particle tracking (MPT)
189 studies were carried out to investigate the movement of PP-sNp in mucus mimicking gel, the motion of PEI
190 nanoparticles was quantified for comparison as a control. **i**) Representative trajectories for particles in
191 mucin solution (3%, w/v) during the 15-s movies. **j**) The mean square displacements ($\langle \text{MSD} \rangle$) of
192 individual nanoparticle as a function of the time scale. **k**) The effective diffusivities (D_{eff}) of individual
193 nanoparticle ($n > 100$ nanoparticles per experiment). Data in **j** and **k** represent mean \pm SEM of three
194 independent experiments. A two-tailed unpaired t -test was used to determine the significance of the
195 indicated comparisons (**** $P < 0.0001$).

196

197 **In vivo evaluation of the transfection properties and biocompatibility of PP-** 198 **sNp**

199 Intratracheal application of PP-sNp led to strong bioluminescence signals in the lungs of mice
200 24 h later, the signal reached peak 48 h post-dosing and declined to the background level 7
201 days afterwards (Fig. 2a and Supplementary Fig. 4). Bioluminescence signals from live
202 animals and excised organs reveal intratracheally administered PP-sNp exhibited superior
203 efficacy compared to pFLuc/PEI or naked-pFLuc counterparts, and the lung was the most
204 abundant fLuc-expressing site and no signals were detected in other organs (e.g., heart, liver,
205 spleen, kidneys and small intestinal) after the PP-sNp transfection (Fig. 2b). Based on these
206 results, we further evaluated the capability of PP-sNp in intratracheal delivery of a plasmid
207 encoding the full-length spike protein of SARS-CoV-2 (pSpike)²². Considerable levels of
208 spike gene-specific mRNA were detected in the lungs of mice transfected by pSpike/PP-sNp
209 compared to pSpike/PEI or naked-pSpike counterparts (Fig. 2c). Histopathological analysis
210 demonstrated that the lungs and other major organs (including heart, liver, spleen and kidney)
211 from pSpike/PP-sNp treated mice were indistinguishable from naked-pSpike and phosphate-
212 buffered saline (PBS) controls, without significant histopathological signs of inflammation
213 (Supplementary Fig. 5). In contrast, administration of pSpike/PEI counterpart resulted in signs

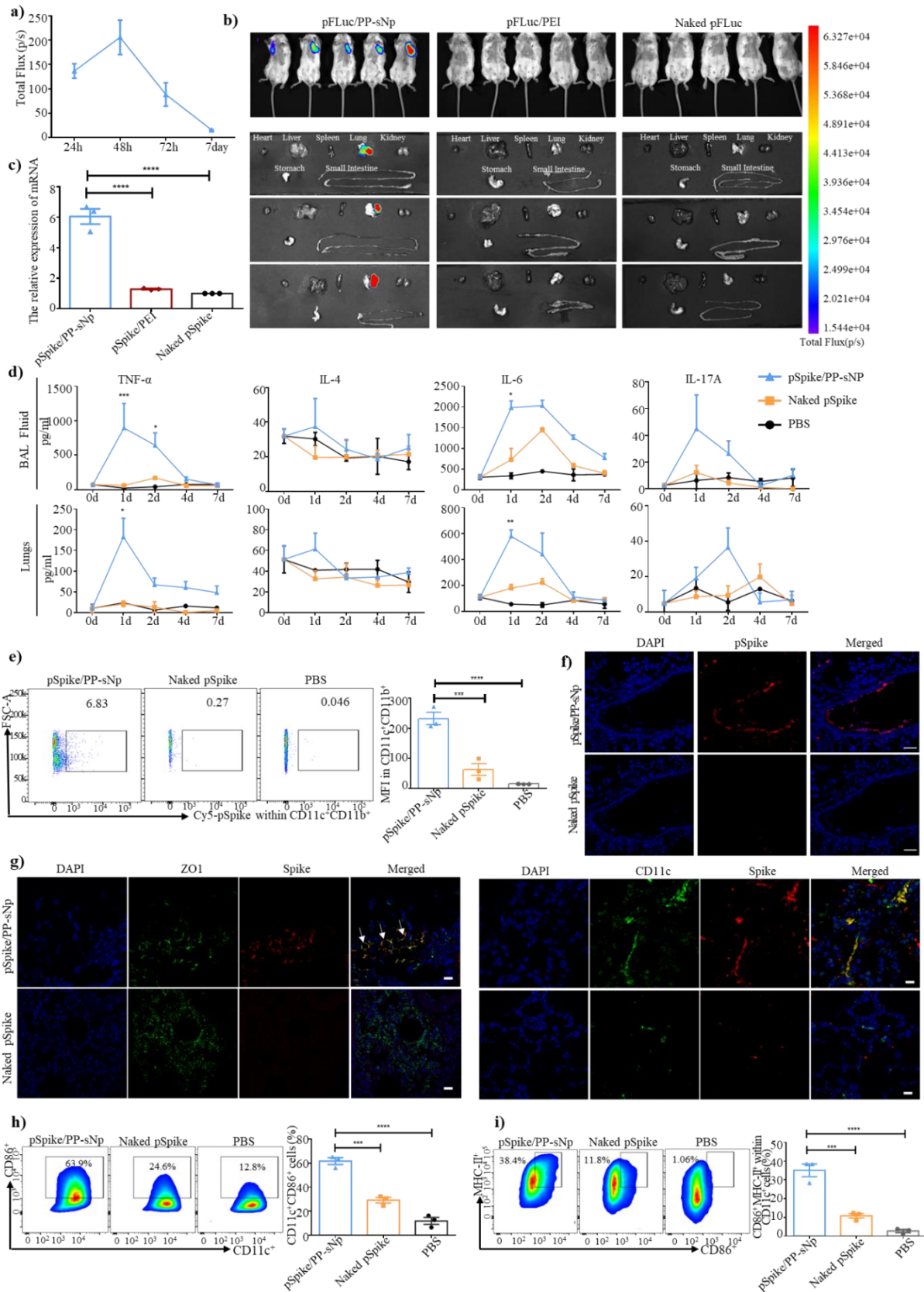
214 of interstitial oedema, damage to the epithelial barrier, and overt infiltration of inflammatory
215 cells in the lung (Supplementary Fig. 5), which is in consistent with previous findings
216 indicating that the highly positively charged and non-degradable nature of PEI based
217 formulation tends to provoke the activation of genes with apoptosis, stress responses, and
218 oncogenesis³⁷. PEI-based formulation was excluded in the following studies due to its poor
219 transfection efficiency and toxic profiles. Although there was a lack of overt lung
220 inflammation observed with histology, pSpike/PP-sNp may rapidly and robustly, but only
221 transiently, activate innate immunity. As shown in Fig. 2d, tumor necrosis factor- α (TNF- α)
222 and interleukin-6 (IL-6) expression within bronchoalveolar lavage fluid (BALF) and
223 supernatants of lung homogenates peaked during 1-2 days after administration then
224 immediately resolved to background levels. However, no significant increase of cytokines
225 was found in the spleen-based and serum-based counterparts (Supplementary Fig. 6).

226

227 Mature DCs are powerful antigen-presenting cells (APCs) and play key roles in initiating
228 antigen-specific immune responses. We therefore evaluated whether the pulmonary DCs
229 (CD11c⁺CD11b⁺) can efficiently uptake the pSpike/PP-sNp. Comparative analysis showed
230 that 6.15 \pm 0.65% of pulmonary DCs internalized Cy5-pSpike/PP-sNp with a mean
231 fluorescence intensity (MFI) of 232.0 \pm 20.60, which was significantly higher than naked Cy5-
232 pSpike counterpart with a 1.23 \pm 0.64% uptake rate (MFI: 62.5 \pm 19.90) (Fig. 2e, Supplementary
233 Fig. 7). Meanwhile, we confirmed a deep penetration and widespread distribution of the
234 intratracheally administered Cy5-pSpike/PP-sNp in the mucus-covered respiratory tract in
235 vivo (Fig. 2f). On the contrary, identically administered naked-pSpike was almost
236 undetectable (Fig. 2f), demonstrating the superior in vivo mucus penetrating ability of PP-sNp.
237 Immunofluorescence sections of airway region suggest that considerable pSpike expression
238 mediated by PP-sNp could be clearly observed in pulmonary DCs and airway epithelial cells
239 (AECs) with a widespread distribution pattern, while the spike protein signal within naked-
240 pSpike treated group was very limited and neither co-localize with pulmonary DCs nor AECs
241 (Fig. 2g). To our surprise, we could not detect transgene expression mediated by pSpike/PP-
242 sNp in alveolar macrophages (Supplementary Fig. 8), which are representative airway APCs
243 being supposed to engulf most of the exogenous antigens³⁸. We further examined the ability
244 of pSpike/PP-sNp in promoting DC maturation. Compared to naked-pSpike, pSpike/PP-sNp
245 could induce two-fold expressions of CD11c⁺CD86⁺ on bone marrow derived cells (BMDCs)
246 (Fig. 2h and Supplementary Fig. 9). The expressions of CD86⁺MHC-II⁺ on CD11c⁺ cells were
247 significantly elevated by more than 300% in pSpike/PP-sNp treated group compared to

248 naked-pSpike control (Fig. 2i and Supplementary Fig. 9).

249



250

251 **Fig. 2 | In vivo transfection profiles and biocompatibility of pDNA/PP-sNp. a)** Kinetics of transgene

252 expression mediated by intratracheally administered pFLuc/PP-sNp over time in mice. Data represent mean

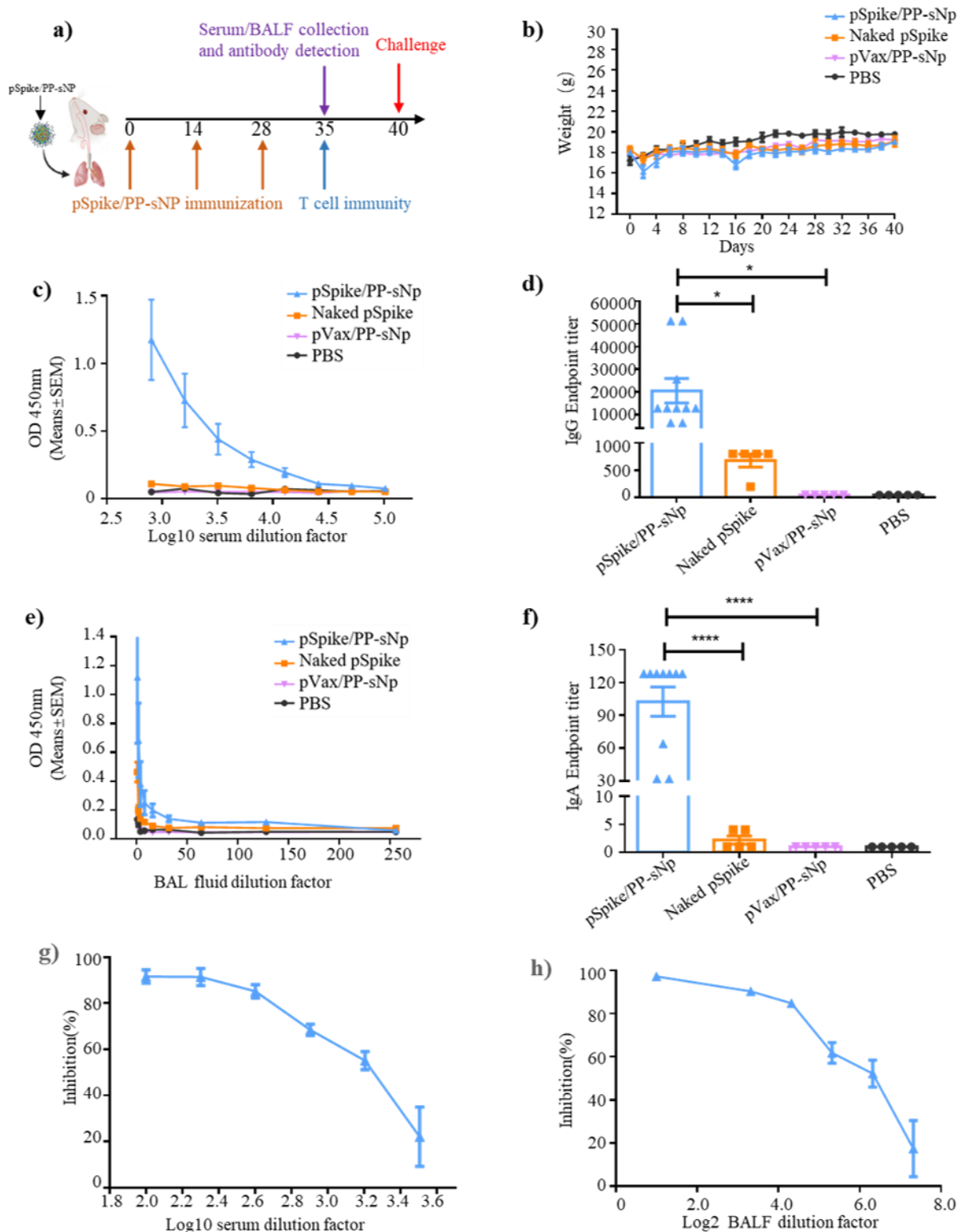
253 \pm SEM (n = 5 biologically independent samples). **b)** Images of in vivo bioluminescence induced by
254 pFLuc/PP-sNP, pFLuc/PEI and naked-pFLuc 48 h after intratracheal dosing in mice. **c)** The expression of
255 the SARS-CoV-2 spike gene specific-mRNA in lung of mice intratracheally administered pSpike/PP-sNP,
256 pSpike/PEI and naked-pSpike, measured by RT-qPCR 48 h after transfection. **d)** Supernatants of lung
257 homogenates and BALF samples were collected at indicated time points and were analyzed for cytokine
258 levels via enzyme-linked immunosorbent assay (ELISA). Data represent mean \pm SEM (n = 3 biologically
259 independent samples). **e)** In vivo DC uptake of pSpike/PP-sNP and naked-pSpike 15 h following
260 intratracheal administration. Mice were sacrificed to detect the fluorescence intensity of Cy5 in pulmonary
261 DC cells via flow cytometry. **f)** Representative confocal images demonstrating the mucus-penetration
262 properties of Cy5-pSpike/PP-sNP and naked Cy5-pSpike through airway mucus of mice 15 h after the
263 administration. Blue channel, nuclei stained by DAPI; Red channel, Cy5-pSpike Scale bars: 50 μ m. **g)**
264 Immunohistochemical staining of the spike protein (red channel) and CD11c (green channel) or ZO1
265 (zonula occludes protein 1, which indicates the epithelium, green channel) in lung of mice treated by
266 pSpike/PP-sNP and naked-pSpike via pulmonary route. Scale bars: 25 μ m. **h)** Expression of CD11c⁺CD86⁺
267 or **i)** CD86⁺MHC-II⁺ (gated on CD11c⁺) on BMDCs after 24 h incubation with pSpike/PP-sNP, naked-
268 pSpike and phosphate buffered saline (PBS) (n = 3 biologically independent samples). Each symbol in the
269 bar chart of **c**, **e**, **h**, and **i** represents one sample from a biologically independent mouse. Data in **c**, **e**, **h** and **i**
270 are shown as mean \pm SEM. One-way ANOVA with Dunnett's multiple comparisons tests was used to
271 determine significance (* P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001).

272

273 **pSpike/PP-sNP induces robust mucosal and humoral immunity**

274 To determine the immunogenicity of mucosal vaccination, mice were immunized via
275 intratracheal route with pSpike/PP-sNP, naked-pSpike, an empty vector pVax loaded PP-sNP
276 (pVax/PP-sNP, served as Mock control) or PBS (Fig. 3a). We observed the body weight of
277 mice treated by pSpike/PP-sNP and naked-pSpike transiently decreased during 2-3 days post
278 the prime and 1st boost dosing, which progressively recovered to the levels displayed by other
279 control groups (Fig. 3b). We also confirmed the necessity of the 2nd boost dose by detection of
280 IgG in serum and sIgA in BALF at pre-determined time points (Supplementary Fig. 10).
281 Intratracheal immunization of pSpike/PP-sNP, but neither pVax/PP-sNP, naked-pSpike nor
282 PBS controls, induced high levels of spike-specific IgG antibodies in serum samples (Fig. 3c).
283 The highest IgG titer within serum from pSpike/PP-sNP group reached 1/51200 on day 35
284 (Fig. 3d). The titer ratio of IgG2a/IgG1 implies that pSpike/PP-sNP tends to induce a Type 1
285 T helper cell (Th1)-biased immune response (Supplementary Fig. 11). Most importantly, only
286 pSpike/PP-sNP induced high levels of spike-specific sIgA antibody in BALF with most
287 samples could reach an endpoint titer of 1/128 (Fig. 3e and Fig. 3f). Furthermore, distal
288 mucosal immune responses were also induced by pSpike/PP-sNP as it proved by the presence
289 of SARS-CoV-2-specific sIgA antibody in vaginal lavage fluid (Supplementary Fig. 12). The

290 neutralization titer (ND_{50}) of serum and BALF samples from pSpike/PP-sNp group
291 approached $\sim 1/1875$ (Fig. 3g) and $\sim 1/73$ (Fig. 3h), respectively. Whereas no neutralizing
292 antibodies (NAb) could be detected in samples from other groups (naked-pSpike, pVax/PP-
293 sNp and PBS). Besides, we successfully detected antibody-secreting plasma cells producing
294 IgA or IgG against SARS-CoV-2 in the spleen after intratracheal immunization with
295 pSpike/PP-sNp (Supplementary Fig. 13).
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Fig. 3 | Humoral immune responses after intratracheal immunization of pSpike/PP-sNp in mice. a) Schematic diagram of immunization, sample collection and challenge schedule. Mice were immunized on day 0 and boosted with the same dose on day 14 and day 28, respectively. **b)** Animal weights were recorded during the whole period of immunization. Data represent mean \pm SEM (n= 10 mice/group). **c)** OD450 nm values of SARS-CoV-2 spike (S1 + S2) protein-specific IgG in serial diluted serum samples collected 35 days after initial vaccination. Data represent mean \pm SEM (n= 10 biologically independent samples). **d)** The SARS-CoV-2 spike (S1 + S2) protein-specific IgG antibody titer in serum samples

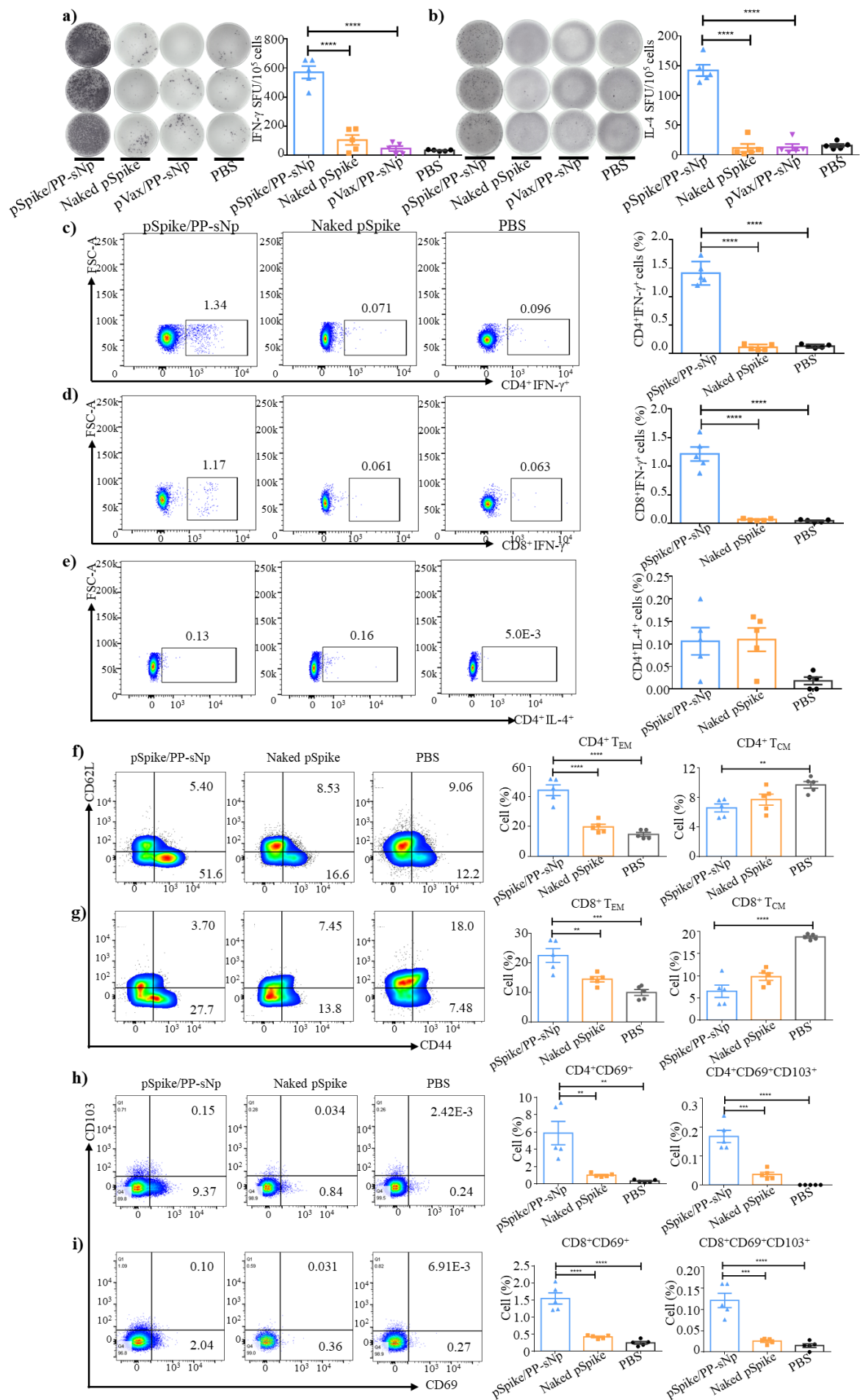
305 collected 35 days after initial vaccination. Each symbol represents one sample from a biologically
306 independent mouse. **e**) OD450 nm values of SARS-CoV-2 spike (S1 + S2) protein-specific sIgA in serial
307 diluted BALF samples collected 35 days after priming. Data represent mean \pm SEM (n= 10 biologically
308 independent samples). **f**) The SARS-CoV-2 spike (S1 + S2) protein-specific sIgA antibody titer in BALF
309 samples collected 35 days after initial vaccination. Each symbol represents one sample from a biologically
310 independent mouse. Pseudovirus neutralizing antibody titer in **g**) serum samples and **h**) BALF samples
311 collected 35 days after priming. Data in **g** and **h** are shown as mean \pm SEM of samples collected from three
312 mice. One-way ANOVA with Dunnett's post-hoc test was used to determine significance within d and
313 f (* P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001).

314

315 **pSpike/PP-sNp elicits potent T cell responses and memory-biased immunity**

316 In order to investigate cellular immune responses activated via mucosal immunization, we
317 assessed the production of interferon- γ (IFN- γ) and interleukin-4 (IL-4) in pulmonary
318 lymphocytes or splenocytes using ELISpot assay. A significantly higher level of IFN- γ and
319 IL-4 secretion was detected in pulmonary lymphocytes from pSpike/PP-sNp group, while
320 negligible levels of both cytokines were detected in samples from other groups (Fig. 4a and
321 4b). A similar trend was also observed in splenocyte samples (Supplementary Fig. 14-15). We
322 next evaluated the intracellular IFN- γ and IL-4 production within CD4⁺ and CD8⁺ T cells
323 after *ex vivo* re-stimulation. Flow cytometry analysis showed that pSpike/PP-sNp led to a
324 significant secretion of IFN- γ ⁺ by CD4⁺ (1.412 \pm 0.077)% (Fig. 4c) and CD8⁺ T cells
325 (1.212 \pm 0.102)% (Fig. 4d) within pulmonary lymphocytes. However, there was no significant
326 difference in IL-4 secretion by CD4⁺ T cells between pSpike/PP-sNp immunized mice and
327 naked-pSpike treated ones (Fig. 4e). We also failed to detect significant secretion of IFN- γ or
328 IL-4 in splenic CD8⁺ T and CD4⁺ T cells (Supplementary Fig. 16-17). These data reveal a
329 comprehensive SARS-CoV-2-specific Th1 and cytotoxic T cells activation in the lung of
330 pSpike/PP-sNp-treated mice. To determine whether memory T cell responses within
331 pulmonary mucosal sites were elicited by pSpike/PP-sNp, the effector memory T (T_{EM}) cells
332 or central memory T (T_{CM}) cells, identified as CD44^{hi}CD62L^{lo} and CD44^{hi}CD62L^{hi}, were
333 detected by flow cytometry. We found both CD4⁺ T_{EM} and CD8⁺ T_{EM} cells in lung, but not
334 splenic T_{EM} cells or T_{CM} cells, were significantly activated via the immunization of
335 pSpike/PP-sNp (Fig. 4f and 4g, Supplementary Fig. 18-19). Recent studies have suggested
336 that tissue-resident memory T (T_{RM}) cells play crucial roles in maintaining long-term
337 protective immunity against mucosal pathogens^{39, 40}. We investigated the expression of the
338 tissue-retention markers CD69 and CD103 on total CD4⁺ or CD8⁺ T cells isolated from the
339 lung. As shown in Fig. 4h, CD4⁺CD69⁺ cells and CD8⁺CD69⁺ cells in pSpike/PP-sNp

340 immunized group displayed 5.6- and 3.3-fold increase than naked-pSpike counterpart,
341 respectively. Although the activation of CD103 marker was relatively limited, the presence of
342 CD4⁺CD69⁺CD103⁺ cells and CD8⁺CD69⁺CD103⁺ cells within pSpike/PP-sNp immunized
343 group could be identified and was significantly higher than other controls (Fig. 4i). These
344 results denote the tremendous potential of pSpike/PP-sNp in inducing comprehensive mucosal
345 immune responses and immune memory to impart a powerful anti-SARS-CoV-2 protection.
346



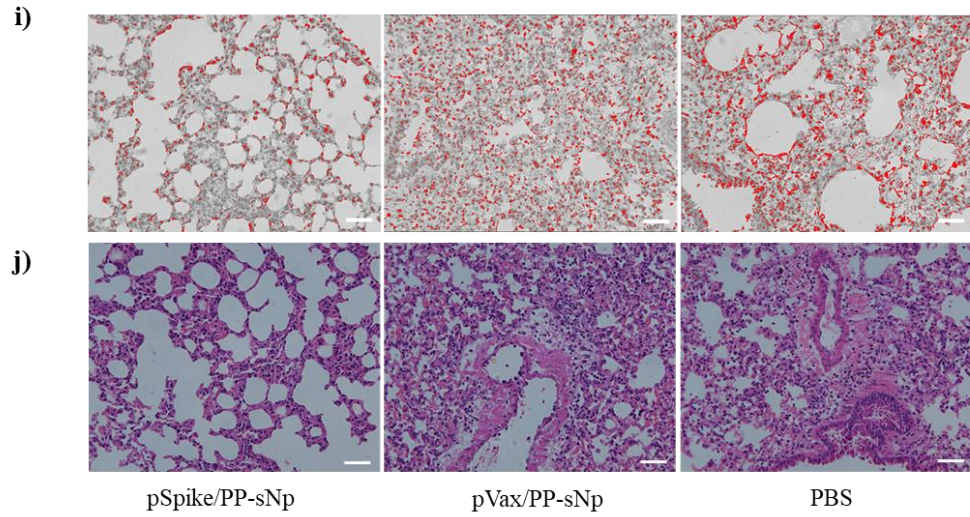
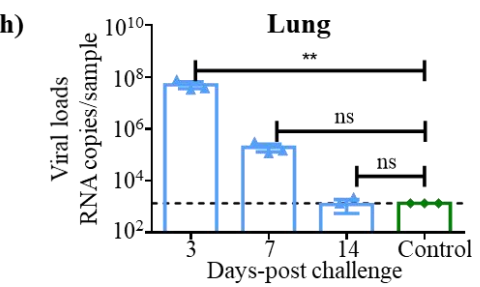
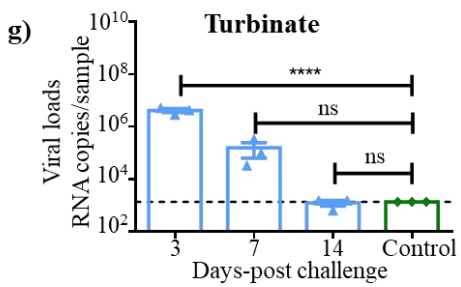
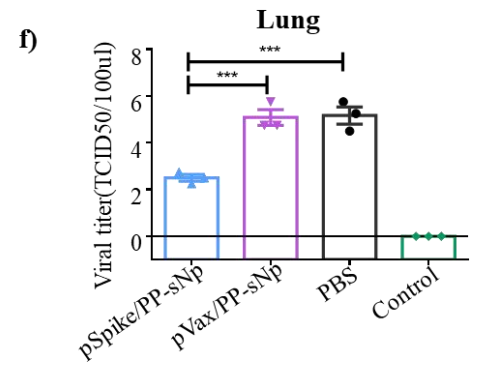
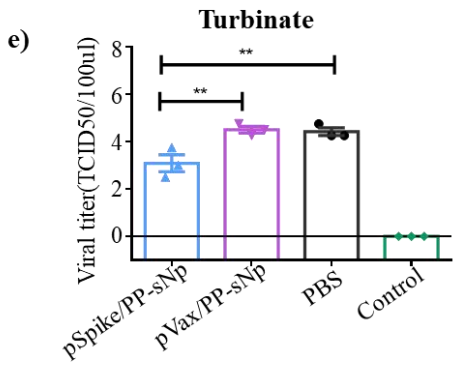
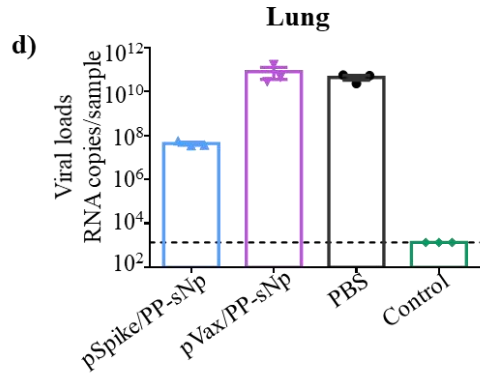
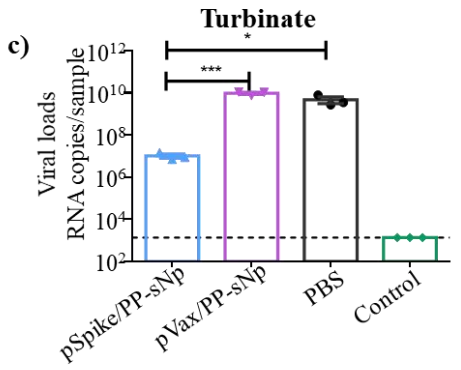
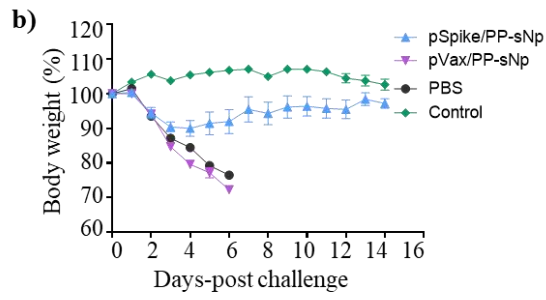
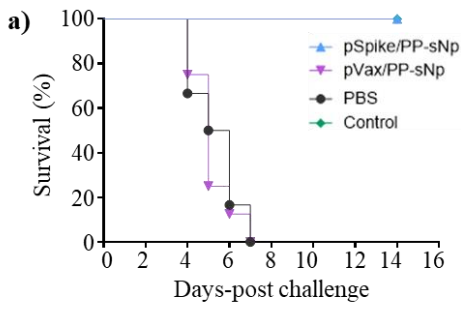
348 **Fig. 4 | SARS-CoV-2-specific T cell immune responses and memory-biased immunity in pSpike/PP-**
349 **sNP vaccinated mice.** Enzyme-linked immunospot (ELISpot) analyses of **a)** IFN- γ and **b)** IL-4 spot-
350 forming cells in pulmonary lymphocyte after re-stimulation with peptide pools of 14-mer overlapping
351 peptides spanning the SARS-CoV-2 receptor binding domain (RBD) region. **c)** CD4⁺ T cells and **d)** CD8⁺
352 T cells in the lung were assayed for IFN- γ ⁺ expression by flow cytometry after re-stimulation with the
353 SARS-CoV-2 RBD peptide pool. **e)** CD4⁺ T cells in the lung were analyzed for IL-4⁺ expression via flow
354 cytometry in the same way as describe above. **f)** Percentage of CD4⁺ effector memory T cells (T_{EM}) co-
355 expressing CD44^{hi} and CD62L^{lo} and central memory T cells (T_{CM}) co-expressing CD44^{hi} and CD62L^{hi} in
356 the lung of mice 35 days after priming. **g)** Percentage of CD8⁺ T_{EM} and T_{CM} in lung of mice 35 days after
357 priming. **h)** Percentage of CD4⁺ T_{RM} and **i)** CD8⁺ T_{RM} in lung of mice 35 days after initial vaccination.
358 Data in **a-i** represent mean \pm SEM (n=5 biologically independent samples). One-way ANOVA with
359 Dunnett's post-hoc test was used to determine significance (** P < 0.01, *** P < 0.001, **** P < 0.0001).
360

361 **Immunization with pSpike/PP-sNp completely prevents lethal SARS-CoV-2** 362 **infection in the upper and lower respiratory tracts**

363 The data described above prompted us to confirm if sufficient protection against SARS-CoV-
364 2 could be achieved via mucosal immunization of pSpike/PP-sNp. To this end, a SARS-CoV-
365 2 C57MA14 strain (which causes severe respiratory symptoms and mortality in mice) was
366 applied in the challenge study. The pSpike/PP-sNp vaccine demonstrated remarkable
367 protective efficacy as evidenced by an 100% survival rate at the predetermined end (14 days
368 post-challenge), while all mice from control groups were deceased during 4-7 days after
369 challenge (Fig. 5a). The body weights of pSpike/PP-sNp vaccinated mice decreased slightly
370 (<10%) in the first 3 days post-challenge but gradually recovered to a level displayed by the
371 control group (without challenge). In contrast, pVax/PP-sNp and PBS groups led to a
372 dramatic decrease in body weight until 6 days after challenge when all animals died (Fig. 5b).
373 Some mice were randomly euthanized to analysis the viral RNA loads in the airways 3 days
374 post-challenge. Significant lower levels of viral RNA were detected in the turbinates (Fig. 5c
375 and 5e) and the lungs (Fig. 5d and 5f) of pSpike/PP-sNp vaccinated mice compared to control
376 counterparts. Moreover, the viral RNA loads in turbinates and lungs of pSpike/PP-sNp treated
377 mice decreased with time and were almost undetectable 14 days post-challenge (Fig. 5g and
378 5h), indicating pSpike/PP-sNp not only prevents virus infection but eventually eliminates the
379 virus in respiratory tract completely. Additionally, lung sections were subjected to an
380 immunohistochemistry assay aiming to explore the replication of SARS-CoV-2 by detecting
381 its SARS-CoV-2 N protein expression in the lung 3 days post-challenge. Less SARS-CoV-2
382 N protein in lung sections from pSpike/PP-sNp immunized mice was detected (Fig. 5i). On

383 the other hand, mice treated by pVax/PP-sNp or PBS not only been detected with obvious
384 SARS-CoV-2 N protein (Fig. 5i), but also developed typical lung lesions characterized by
385 denatured epithelial tissues, thickened alveolar septa, and activated inflammatory cell
386 infiltration according to the histopathological assays (Fig. 5j). Whereas the pathological
387 changes significantly alleviated in lung sections from pSpike/PP-sNp vaccinated mice (Fig.
388 5j). We further evaluated the potential roles of NAb and T_{RM} cells in the process of
389 eliminating SARS-CoV-2. As depicted in Supplementary Fig. 20, significant numbers of
390 CD4⁺T_{RM} cells were identified exclusively in pulmonary sections of pSpike/PP-sNp treated
391 mice after challenge, and the NAb titers against SARS-CoV-2 C57MA14 strain in serum of
392 pSpike/PP-sNp immunized mice were elevated more than 5 times 14 days after challenge
393 (Supplementary Fig. 21), implying NAb and T_{RM} mediated protective effects were probably
394 involved in the protection of mice from lung lesions and the complete elimination of infected
395 SARS-CoV-2 in the whole respiratory tract.

396



398 **Fig. 5 | Pulmonary immunization with pSpike/PP-sNp confers complete protection against lethal**
399 **SARS-CoV-2 challenge in mice.** Forty days after the initial immunization, mice were challenged with a
400 lethal dose (50 LD₅₀) of SARS-CoV-2 C57MA14 strain via intranasal instillation, and the indicated tissues
401 were collected at indicated time points after challenge to detect viral loads and the lung pathology. **a)** The
402 survival rate of mice (n= 10 mice/group) intratracheally immunized with pSpike/PP-sNp, pVax/PP-sNp
403 (Mock control) and PBS after the SARS-CoV-2 challenge. Untreated mice without challenge were served
404 as a control (Control) **b)** The body weight changes of mice (n= 10 mice/group) intratracheally inoculated
405 with pSpike/PP-sNp, pVax/PP-sNp and PBS after the SARS-CoV-2 challenging. Untreated mice without
406 challenge were served as a control (Control, marked in green). Data represent mean ± SEM. Viral RNA
407 loads in **c)** the turbinates and **d)** the lungs of mice treated by pSpike/PP-sNp, pVax/PP-sNp and PBS as
408 described above and that in untreated counterpart (Control) 3 days after the SARS-CoV-2 challenge. Viral
409 titers in **e)** the turbinates and **f)** the lungs of mice treated by indicated formulations as described above and
410 that in untreated counterpart (Control) 3 days after the SARS-CoV-2 challenging. The viral loads in **g)** the
411 turbinates and **h)** the lungs of mice treated by intratracheally vaccinated pSpike/PP-sNp 3, 7, 14 days post-
412 challenge. **i)** Immunohistochemistry assay for SARS-CoV-2 N protein 3 days post-challenge. Scale bar,
413 100 µm. Positive signals are shown in red. **j)** Representative hematoxylin-eosin staining (H&E) staining of
414 lung pathology 3 days after the SARS-CoV-2 challenge. Scale bar, 100 µm. Data in **c-h)** represent mean ±
415 SEM (n= 3 biologically independent samples). One-way ANOVA with Dunnett's post-hoc test was used to
416 determine significance (ns represent not significant, **P*< 0.05, ***P*< 0.01, ****P*< 0.001, *****P*< 0.0001).

417

418 **Discussion**

419 The development of mucosal COVID-19 vaccines that impart protective immunity would be
420 highly desirable. However, apart from extensive investigations using adenovirus-based
421 vaccines^{17, 18, 41}, whether DNA vaccines could achieve this goal remains unclear. In this study,
422 we demonstrate a COVID-19 DNA vaccine (pSpike/PP-sNp) delivered via pulmonary route
423 could induce a robust protective immunity consisting of mucosal, humoral and cellular
424 immune responses, which is sufficient to completely protect the upper and lower respiratory
425 tracts against lethal SARS-CoV-2 challenge in mice. Early studies suggested that DNA
426 vaccines are poorly immunogenic in the airways with low levels of antigen expression owing
427 to the barriers posed by the respiratory tract. This could be reflected by the previous study
428 indicating intranasal COVID-19 DNA vaccination were not able to elicit mucosal sIgA and
429 circulating IgG against SARS-CoV-2 even after three times of repeated dosing²⁷. Similar
430 observations have been reported in another study showing that the serum neutralization IC₅₀
431 of a COVID-19 DNA vaccine just reached 1/83.8 against SARS-CoV-2 pseudoviruses after
432 seven-times repeated intranasal vaccinations⁴². Although the previous studies reveal the
433 intranasal administered DNA vaccines could trigger a certain degree of SARS-CoV-2 specific

434 immune responses^{27, 42}, none of them has disclosed critical data regarding the mucosal
435 application of COVID-19 DNA vaccine, including sIgA levels in BALF samples, details in
436 the subsets of effector T cells and memory T cells, and the protection efficiency in a SARS-
437 CoV-2 challenge assay.

438

439 In order to potentiate the mucosal COVID-19 DNA vaccine, we adopted the PP-sNp gene
440 delivery system which is specifically developed for pulmonary DNA transfection^{28, 32}.
441 Previous studies from both our group and others have clearly demonstrated that poloxamine-
442 based delivery system mediates significantly better levels of DNA transfection and lower
443 levels of associated inflammatory response in the airways of mouse and pig models than were
444 achieved with the cutting-edge lipid-based formulations^{26, 32, 33}. PEI was adopted as a control
445 formulation because it is one of the most well-studied polymer-based gene carrier and has
446 been applied in many mucosal DNA vaccine applications⁴³. Although PEI appears to be
447 promising in delivery of mucosal DNA vaccines encoding antigens of Influenza, HIV and
448 SARS-CoV⁴³, we found it fails to mediate efficient DNA transfection in the mouse airway,
449 perhaps owing to its poor mucus penetrating properties. We applied an improved method of
450 preparing nanoparticles without aggregation to enhance the utility of PP-sNp for in vivo
451 applications⁴⁴. The optimized nanocomplexes appeared as evenly distributed spherical
452 nanoparticles with sizes being smaller than mucus mesh pores ($\sim 140\pm 50$ nm)⁴⁵. The unique
453 high mobility in mucus gel facilitates deep penetration and widely spread of PP-sNp through
454 the airway mucus layer (Fig. 6a), thereby improving the probability of DNA payloads
455 encounter and uptake by target cells. Subsequently, the targeting moiety within PP-sNp
456 (which binds the ICAM-1/CD54) provides the possibility to specifically deliver the DNA
457 cargos via receptor-mediated uptake, concurrent with efficient endosome escape and nucleus
458 localization in these cells²⁸. By virtue of these merits, PP-sNp efficiently mediates the
459 transfection of antigen-expressing plasmid in pulmonary DCs and AECs (Fig. 6b). Being the
460 most powerful APCs, pulmonary DCs are imperative in shaping antigen-specific immune
461 responses⁴⁶. The enhanced DNA vaccine uptake by pulmonary DCs and subsequent DC
462 differentiation may lead to more profound and durable antigen presentation to T-cells⁴⁷. Our
463 finding is also in agreement with previous evidences demonstrating a positive correlation
464 between the ratio of antigen-activated DCs and the magnitude of T_{EM}-biased response⁴⁸.
465 Meanwhile, AECs appear to be essential for determining the potency of mucosal immune
466 response and IgA production. Several studies have suggested that AECs provides a constant
467 supply of cytokines (such as IL-6) which are essential for B cell proliferation, IgA isotype

468 switch and differentiation into IgA producing plasma cells^{49, 50}. The close proximity of AECs
469 to DCs and B cells situated in respiratory mucosa probably ensures efficient antigen cross-
470 presentation which directs B cells isotype switch towards IgA₁ and IgA₂ with the help of
471 cytokines produced by AECs⁵⁰. AECs also hold critical roles in orchestrating innate and
472 adaptive immune responses in the respiratory system during viral infection^{51, 52}. As an RNA
473 sequencing analysis reveals, pSpike/PP-sNp successfully activated innate and adaptive anti-
474 virus pathways in vaccinated mice (Supplementary Fig. 22, more details are reported in the
475 Supplementary Results), which is in consistent with previous findings suggesting the
476 transiently activated innate immunity is sufficient to augment subsequent adapted immune
477 responses⁵³.

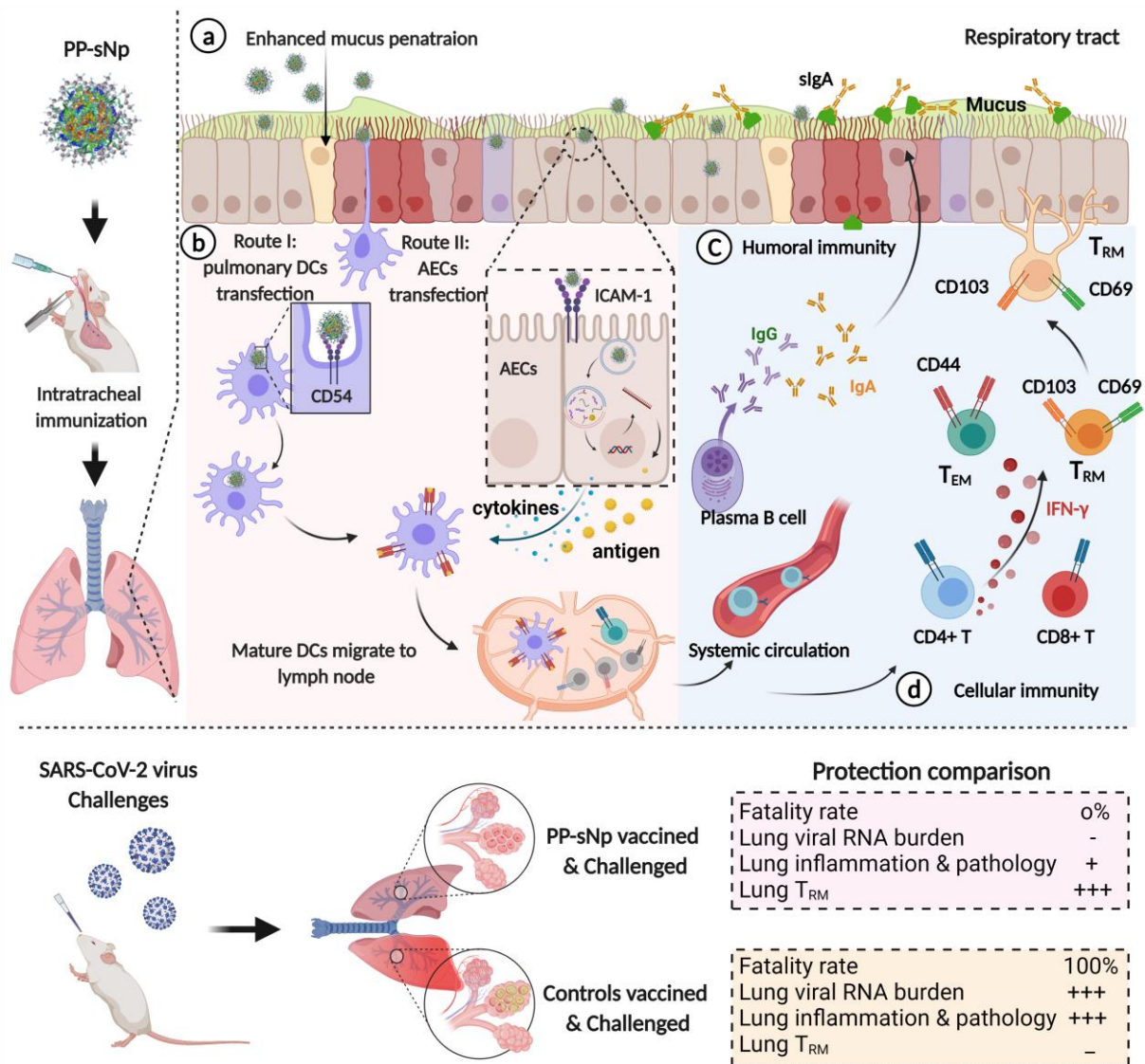
478

479 The complete protection against lethal SARS-CoV-2 challenge was largely due to the ability
480 of pSpike/PP-sNp in stimulating comprehensive mucosal, humoral and cellular immunity,
481 characterized by robust secretion of sIgA and NAb as well as potent T cell responses
482 (especially T_{RM} cells) in the respiratory system. pSpike/PP-sNp efficiently activated systemic
483 immune response, concurrent with significant levels (ND₅₀ at ~1/1875) of NAb in serum.
484 Previous findings suggest a serum NAb titer >300 was generally associated with protection
485 against SARS-CoV-2⁵⁴. It is worth noting that pSpike/PP-sNp vaccinated mice displayed B
486 cells secreting IgA and high levels of SARS-CoV-2-specific sIgA antibody titer (with a mean
487 dilution titer of 1/102.4) in the BALF samples (Fig. 6c), which are even higher than those
488 induced by intranasal vaccination of adjuvanted subunit vaccines according to previous
489 publications⁵⁵⁻⁵⁷. Although the SARS-CoV-2-specific sIgA antibody titer in the BALF of
490 intranasally vaccinated mice using adenovirus-vector is insurmountable when compared to
491 non-viral vaccine induced counterpart, substantial NAb titers could be detected in BALF
492 samples of pSpike/PP-sNp vaccinated mice with a level (~1/73) that is comparable to the
493 adenovirus-based counterpart (~1/100)¹⁹.

494

495 Additionally, it has been long recognized that high-quality antigen-specific T cell responses
496 are pivotal in combating various types of coronavirus infection⁹. pSpike/PP-sNp successfully
497 mediated the presence of SARS-CoV-2 specific CD8⁺IFN- γ T cells (cytotoxic T cells) and
498 elicited a Th1-biased cellular immune responses (Fig. 6d), both of which would be
499 advantageous in eliminating coronavirus without adverse effects, since Th2 cell responses are
500 suggested to be associated with enhancement of lung diseases⁵⁸. pSpike/PP-sNp also
501 generated a robust memory-based cellular immunity in the airways, including the significantly

502 activated T_{EM} cells and T_{RM} cells with a resident memory phenotype (Fig. 6d). T_{EM} -biased
 503 response created by pSpike/PP-sNp, which could immediately act on and rapidly removes
 504 respiratory pathogens, is particularly pronounced in the lung. But we failed to observe
 505 increased ratio of T_{CM} cells, which is in line with previous findings indicating that T_{CM} -biased
 506 response generally induced by conventional route of vaccination (e.g., electroporation)⁵⁹.
 507 Accumulating evidences demonstrate that pulmonary $CD4^+T_{RM}$ and $CD8^+T_{RM}$ cells play
 508 crucial roles in resisting respiratory pathogen infections, including MERS-CoV, SARS-CoV
 509 and SARS-CoV-2^{3, 60}. The airway T_{RM} cells, which are exclusively generated through the
 510 respiratory route vaccination/infection, reside in the lung and does not recirculate⁶¹, so that
 511 they can instantly recognize invading pathogens in the airway and efficiently prevent virus
 512 replication at the early stage of infection/re-infection.
 513



514

515 **Figure 6 | Schematic of the SARS-CoV-2 specific immune responses and subsequent protection**

516 **established by pSpike/PP-sNp vaccine inoculated via respiratory route. a) After pulmonary inoculation,**

517 pSpike/PP-sNp is able to penetrate efficiently through the physical and biological barriers at the airway
518 mucosal site. **b)** With the help of targeting moiety (ICAM-1/CD54 ligand) in PP-sNp, pSpike/PP-sNp is
519 internalized and captured by airway epithelial cells (AECs) or pulmonary dendritic cells (DCs). The
520 cationic moiety within PP-sNp mediates endosome escape and nucleus localization of pSpike, followed by
521 the transcription and translation process. The SARS-CoV-2 derived antigens expressed in AECs are
522 subsequently presented to other immune cells such as pulmonary DCs. AECs simultaneously orchestrate
523 the adaptive immune responses via the transient secretion of cytokines. Mature pulmonary DCs then
524 migrate to the bronchial-associated lymphoid tissues (e.g., mediastinal lymph node) and present antigens to
525 naïve T cells and B cells for humoral and cellular immune responses. **c)** Activated B cells proliferate and
526 differentiate into antibody-secreting plasma cells to generate secretory IgA (sIgA) and systemic IgG
527 antibodies, the former of which efficiently neutralizes invading SARS-CoV-2 within the upper and lower
528 respiratory tracts. **d)** Meanwhile, a portion of T cells obtains a tissue resident memory phenotype (T_{RM}),
529 enabling them to reside in the airway and respond rapidly when encountering SARS-CoV-2 virus. Other
530 activated $CD4^+$ or $CD8^+$ T cells, including $CD44^{hi}CD62L^{lo}$ T cells, $CD4^+IFN-\gamma$ T cells and $CD8^+IFN-\gamma$ T
531 cells, take part in the process of eliminating SARS-CoV-2 infections as well. The robust and
532 comprehensive immunity conferred by pulmonary vaccination of pSpike/PP-sNp probably controls SARS-
533 CoV-2 replication and removes the viruses at the initial sites of infection, thus protects the vaccinated mice
534 from lung lesions and death.

535

536 Despite the promising results we observed in the current study, pSpike/PP-sNp is still far
537 from successful clinical translation and more in-depth investigations are necessary. There are
538 several limitations that we did not address in this study and will be useful topics for future
539 studies, including the absence of data on the neutralization and protection efficiency elicited
540 by pSpike/PP-sNp against emerging SARS-CoV-2 variants of concern. Similar to those cases
541 of authorized COVID-19 vaccines⁶², the neutralizing activity of NAb induced by the
542 pSpike/PP-sNp vaccine may suffer a significant decrease within several months/years after
543 vaccination, more boost doses may be necessary. Besides, immunization and challenge
544 studies with larger animals such as non-human primates should be carried out to confirm the
545 extent of protective mucosal immunity conferred by pSpike/PP-sNp. Another limitation
546 relates to the intratracheal dosing which is not appropriate to be applied in humans when
547 considering its poor compliance. Most of the relevant studies chose the intranasal inoculation
548 because of its noninvasive and convenient features, but there are still huge concerns and
549 uncertainties regarding intranasal route of vaccination. For example, negative perception for
550 nasal vaccines was generated from reported cases of Bell's palsy after intranasal dosing of
551 influenza vaccines^{63, 64}. Alternatively, the noninvasive nebulized formulations seem to be one
552 of the most appropriate approaches in delivering mucosal vaccines to the human airway.
553 However, the nebulized DNA formulations still face many challenges as indicated by a

554 previous study showing that as little as 10% of the DNA payload in a nebulization device
555 chamber could be successfully emitted⁶⁵, it thus requires advanced nebulization strategies and
556 further optimization on the formulations to ensure the transfection efficiency⁶⁶. Finally, the
557 mechanism and pathways that involved in the pSpike/PP-sNp induced immune responses will
558 be explored in details with the help of cutting-edge technologies such as the single-cell RNA
559 sequencing in order to further improve the vector/platform design and our understanding of
560 DNA-based mucosal vaccines.

561

562 In summary, our dataset reveals that pSpike/PP-sNp with pulmonary mucus penetrating
563 properties was capable of inducing comprehensive mucosal, humoral and cellular immune
564 responses to provide complete protection against SARS-CoV-2 infection. The safe profile and
565 ability to potentiate DNA vaccines for strong mucosal immunity make PP-sNp a promising
566 platform for COVID-19 mucosal vaccines if its efficacy can be shown in large animal models
567 and clinical trials, since robust protection at the respiratory mucosal site would be, at least
568 theoretically, one of the most effective means to prevent the infection of SARS-CoV-2.

569

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732

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742

743 AUTHOR CONTRIBUTIONS:

744 S.G. and Q.Z. conceived and directed the project. Y.D., D.L., W.Z., P.L., P.C., B.P. and J.R.
745 contributed experimental materials. S.S., J.T., Q.Z., L.C., C.X., C.S., Y.O., C.L., H.L., and
746 Y.D. performed experiments and analyzed data. E.L., Y.G., C.T. and Y.L. designed and
747 performed the challenge study and relevant end-point investigations. C.L. was responsible for
748 the bioinformatics and RNA sequencing data analysis. S.G., G.Z., B.W., Y.L. and Q.Z.
749 designed and supervised the research. S.G. and S.S. wrote the manuscripts with help and
750 comments from all authors.

751

752 COMPETING FINANCIAL INTERESTS:

753 S.G., S.S., Q.Z. and C.L. have applied for patents related to this study. B.W. is a scientific co-
754 founder of the biotechnology company Advaccine Biopharmaceuticals (Suzhou, China),
755 which focuses on the development of DNA vaccines. G.Z., C.S. and Y.D. are employees of
756 Advaccine Biopharmaceuticals Co., Ltd. B.P. is a scientific co-founder of In-Cell-Art (Nantes,
757 France) and owns stock of In-Cell-Art, which commercializes tetra-functional block
758 copolymers for DNA vaccines. The other authors declare that they have no competing
759 financial interests.

760

761 **Methods**

762 **Reagents.** Poloxamine 704 (T704) was kindly provided by InCellArt (Nantes, France). All
763 synthetic peptides were manually synthesized by Chinese Peptide Company (Hangzhou,
764 China) with purity > 95%. Branched PEI (average molecular weight at 25 kDa), Cell
765 Counting Kit-8 (CA1210) and D-Luciferin (L6882) were all purchased from Sigma-Aldrich
766 (Saint Louis, MO, USA). Lipofectamine2000 was purchased from Invitrogen (11668019,
767 Carlsbad, USA). pGL4.51-Luciferase Reporter Vectors (pFLuc, E1320) was purchased from
768 Promega (Madison, USA). Plasmids encoding SARS-CoV-2 S protein (pSpike) and pVax
769 were kindly provided by Advaccine Biopharmaceuticals Co., Ltd (Suzhou, China). Purified
770 full length S1 + S2 ECD spike protein of SARS-CoV-2 was purchased from Sino Biologics
771 (40589-v08B1, Beijing, China). For in vivo uptake and flow cytometric analysis, plasmid was
772 fluorescently labeled with the Cy5 fluorophores using the Mirus Label IT[®] tracker
773 intracellular nucleic acid localization kit (MIR 7021, Mirus Bio, Madison, USA) according to
774 the manufacturer's instruction. Other reagents were obtained from Sigma-Aldrich (Saint
775 Louis, MO, USA) as analytical grade or better.

776

777 **Cell lines.** MH-S cell line (Mice alveolar macrophages cells), DC2.4 cell line (Mouse bone
778 marrow-derived dendritic cells), BEAS-2B (human bronchial epithelial cells) cell line and
779 Calu-3 (human lung cancer cells) cell line were obtained from ATCC (Manassas, VA, USA).
780 16HBE (human bronchial epithelial cells) cell line was generously provided by Prof. Dr.
781 Dieter C. Gruenert (University of California at San Francisco, CA, USA). ACE2-293T cells
782 (ACE2-expressing cell line, constructed by hygromycin B screening) were purchased from
783 PackGene (LV-2058, Guangzhou, China). Cells were maintained in medium DMEM (Gibco,
784 USA) supplemented with 10% fetal bovine serum (Gibco, USA), penicillin (100 units/mL)
785 and streptomycin (100 µg/mL) (complete medium) at 37 °C in 5% CO₂. All cell lines used in
786 current study were obtained from original providers who authenticated the cell lines using
787 morphology, karyotyping and PCR-based approaches. No additional authentication has been
788 performed. All cell lines tested negative for mycoplasma contamination. All experiments were
789 performed on cells in the logarithmic growth phase.

790

791 **Preparation and characterization of pDNA/PP-sNp formulations.** The pDNA/PP-sNp
792 formulations were prepared via a simple self-assembly process as described previously²⁸. For
793 in vivo applications, T704 stock solution (10 mg/mL) prepared in nuclease-free water was
794 mixed with an equal volume of peptide solution (0.667 mg/mL) using a self-designed

795 microfluidic mixer. pDNA solution (0.6 mg/mL) in double volumes were mixed with the
796 above T704/peptide solution using the same mixer. The complex was incubated for 20 min at
797 room temperature before further use. For in vitro study, T704 solution at a certain
798 concentration (defined as the w/w ratio between T704 and pDNA) and peptide solution at a
799 specific concentration (defined as the N/P ratio; namely, the ratio between nitrogen residues
800 in peptide and nucleic acid phosphate groups) were applied using similar methods. For PEI
801 based formulation, brPEI (25 kDa, Sigma) at an optimum N/P ratio of 10 was mixed with
802 pDNA solution in nuclease-free water, the resulting complex was incubated at room
803 temperature for 20 min and served as a polyplex control. the Lipofectamine 2000 complex,
804 serving as a lipoplex control, was prepared according to the manufacturer's instructions using
805 the optimum concentration. Size measurements were performed using dynamic light
806 scattering (DLS) on a Malvern Zetasizer Nano-ZS (Malvern). The morphology of pDNA/PP-
807 sNp formulations were investigated by transmission electron microscope and scanning
808 electron microscope (JEOL Ltd).

809

810 **Cellular uptake and in vitro transfection.** For cellular uptake investigation, the Cy5
811 conjugated pDNA was prepared according to manual instructions. Cells were incubated with
812 Cy5-pDNA/PP-sNp or naked Cy5-pDNA for 4 h in Opti-MEM I Reduced Serum Medium
813 (31985062, Invitrogen). The cells were further collected for the analysis of mean fluorescence
814 intensity (MFI) by flow cytometry. To evaluate the in vivo transfection of PP-sNp, pDNA/PP-
815 sNp complexes encoding firefly luciferase (i.e., pFLuc/PP-sNp) were prepared. pFLuc/PP-
816 sNp and naked-pFLuc were incubated with cells for 4 h in Opti-MEM I Reduced Serum
817 Medium, then were replaced by fresh complete medium. The protein expression of firefly
818 luciferase in cells was observed by bioluminescence imaging at 48 h by Firefly Luciferase
819 Reporter Gene Assay Kit (RG005, Beyotime).

820

821 **Optical video recording of PP-sNp and the mean-squared displacement (MSD) analysis.**

822 Leica SP8 microscope equipped with a 40× water objective was used to record the motion of
823 PP-sNp. Cy5-DNA-containing PP-sNp were prepared as described above. PP-sNp (20 μL, 1.3
824 μg/μL) were mixed with mucin gel (400 μL, Mucin II solution 3%, w/v) mimicking mucus,
825 followed by a 30 min incubation at 37°C. DNA/PEI nanoparticles were also added as control.
826 Fifteen-second Movies (Frame rate 23 fps) were captured using the LAS4.5 software (Leica).
827 The trajectories of the nanoparticles were precisely quantified from the videos by software
828 (TrackMate plugin in FIJI (ImageJ)), then the trajectory data was used to calculate the MSD

829 and the corresponding diffusion coefficients (De) in MATLAB through the following
830 equations, as implemented in MSD Analyzer.

$$831 \quad \text{MSD}(\Delta t) = \langle [r(t+\Delta t) - r(t)]^2 \rangle$$

$$832 \quad De = 0.25 \text{ MSD} / \Delta t$$

833 where De represents the effective diffusion coefficient and Δt represents the time interval.

834

835 **Animals.** 6–8 weeks old specific pathogen-free (SPF) female BALB/c mice were purchased
836 from the Beijing HFK Bioscience Co., Ltd (Beijing, China). All animal studies were approved
837 by the Laboratory Animal Welfare and Ethics Committee of Third Military Medical
838 University and were performed in accordance with the institutional and national policies and
839 guidelines for the use of laboratory animals. The mice were kept and vaccinated in SPF
840 facilities, and provided with free access to sterile food and water. Animals were randomly
841 divided into groups and conceded an adaption time of at least 7 days before the beginning of
842 the experiments. For intratracheal dosing, the mice were sedated with isoflurane and received
843 the formulations via a self-improved micro-injector.

844

845 **Bioluminescence imaging.** Mice were anaesthetized with isoflurane, followed by an injection
846 of the substrate D-Luciferin (150 mg/kg) intraperitoneally. Bioluminescence was measured 10
847 min later using a Lumina Series III In Vivo Imaging System (PerkinElmer). To evaluate the
848 in vivo delivery capability and visualize protein expression and tissue distribution of
849 pDNA/PP-sNp formulations, pDNA encoding a firefly luciferase (pFLuc) was incorporated.
850 The formulations were administered to mice via intratracheal (i.t.) route. The protein
851 expression of firefly luciferase mediated by pFLuc/PP-sNp was observed by bioluminescence
852 imaging at 24 h, 48 h, 72 h and 7 days post-dosing, respectively. The pFLuc/PEI (at N/P ratios
853 of 10) and naked pFLuc plasmid counterparts were also injected via i.t. route with same dose
854 of pFLuc to evaluate the bioluminescence in vivo.

855

856 **Bone marrow derived dendritic cells (BMDC) maturation study.** Bone marrow cells were
857 isolated from the femurs of female BALB/c mice and cultured in RPMI 1640 complete
858 medium (Gibco, USA) supplemented with 10% FBS, 1% penicillin/streptomycin, 10 ng/mL
859 of Interleukin-4 (IL-4) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF).
860 The culture media was replaced with fresh media on day 2 and 5 to remove the non-adherent
861 and loosely adherent cells. The remaining cells continued to culture for another 2 days. To
862 examine the maturation of BMDCs in vitro, BMDCs ($1 \times 10^6 \text{ mL}^{-1}$) were co-cultured with

863 pSpike/PP-sNp and naked-pSpike only for 24 h, respectively. Subsequently, FITC anti-mouse
864 CD11c (117305, Biolegend), PE-Cy7 anti-mouse MHC-II (25-5321-82, Invitrogen), and APC
865 anti-mouse CD86 (105011, Biolegend) were used to stain the cells in flow cytometry staining
866 (FACS) buffer for 30 min at 4 °C before being washed and analyzed by BD FACS Array
867 software™ on a BD FACS Array flow cytometer (BD Biosciences, USA).

868

869 **Mouse vaccination and challenge experiments.** Mice were immunized on day 0 and boosted
870 with the same dose on day 14 and 28, respectively. Each anesthetized mouse intratracheally
871 received 50 µL of pSpike/PP-sNp formulation containing 15 µg pSpike. pVax/PP-sNp and
872 phosphate buffered saline (PBS) was adopted as a mock control and a negative control,
873 respectively. Mice were sacrificed on day 35 for assessing respiratory mucosal immune
874 response, cellular immune response and memory establishment. Relevant tissues (lung and
875 spleen) were harvested and processed for flow cytometry analysis. Bronchoalveolar lavage
876 fluid (BALF) was collected by washing the lungs of euthanized mice with 500 µL of ice-cold
877 PBS containing 0.05% Tween-20. Collected BALF was stored at -80°C for further use.

878

879 The SARS-CoV-2 challenge model was based on a novel mouse-adapted SARS-CoV-2 strain,
880 C57MA14 (NCBI GenBank number: OL913104.1, details can be found in:
881 <https://www.ncbi.nlm.nih.gov/nuccore/2167992552>), that causes severe respiratory symptoms,
882 and mortality to BALB/c mice. Immunized BALB/c mice were challenged intranasally with
883 50 LD₅₀ SARS-CoV-2 C57MA14 on day 40 post initial immunization. On day 3 post
884 challenge, 3 animals/group were sacrificed, and the lung and trachea tissues were collected for
885 subsequent viral loads detection.

886

887 **Quantification of viral by quantitative RT-PCR and TCID₅₀ in challenged mouse**
888 **tissues.** Viral RNA in lung and turbinate tissues from challenged mice was detected by
889 quantitative reverse transcription PCR (RT-qPCR). Briefly, tissue samples were homogenized
890 with stainless steel beads in a TissueLyser-24 (Shanghai jingxin Industrial Development CO.,
891 LTD) in 500 uL of DMEM. Viral RNA in tissues were divided into two parts. One part was
892 extracted using the QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's
893 protocol. SARS-CoV-2 RNA quantification was performed by RT-qPCR targeting the S gene
894 of SARS-CoV-2 using One Step PrimeScript RT-PCR Kit (Takara) with the following SARS-
895 CoV-2 specific primers and probes: CoV-F3 (5'-TCCTGGTGATTCTTCTTCAGGT-30),
896 CoV-R3 (5'-TCTGAGAGAGGGTCAAGTGC-30), and CoV-P3 (5'-FAM-

897 AGCTGCAGCAC CAGCTGTCCA-BHQ1-30). Another part was serially diluted in DMEM
898 and added into Vero E6 cells in 96-well plates. The plates were incubated 1 hour at 37°C with
899 5% CO₂, the inoculation was replaced with DMEM containing 2% FBS and 1% penicillin-
900 streptomycin. After incubating for 72 h, the median tissue culture infective dose (TCID₅₀)
901 was detected by the cytopathic effect (CPE).

902

903 **Enzyme linked immunosorbent assay (ELISA).** SARS-CoV-2 S protein specific antibodies
904 in mouse serum and BALF were measured by ELISA. Briefly, polystyrene microtiter 96-well
905 plates were coated with full length SARS-CoV-2 spike protein (3µg/mL in carbonate buffer,
906 pH=9.6) and incubated overnight at 4 °C. After blocking with 1% bovine albumin (BSA) in
907 PBS, 100 µl/well pre-diluted samples were added into the plates with 1 h incubation at 37 °C.
908 After three-times washes with PBST (PBS with 0.05% Tween-20), plates were added with
909 horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Ab231712), IgA (Ab97235),
910 IgG1 (ab97240), or IgG2a (Ab97245) (1:10000, Abcam, UK) and incubated for 40 min at
911 37 °C. Plates were then washed three-times and added with peroxidase substrate (Ab171522,
912 Abcam, UK), the reaction was terminated by stop solution (Ab171529, Abcam, UK) and the
913 absorbance at 450 nm was read using a microplate reader (AID iSpot, Germany).

914

915 **SARS-CoV-2 pseudovirus neutralization assay.** Mouse serum samples from pSpike/PP-
916 sNp vaccinated mice were serially diluted double fold starting at a 1:100 dilution with DMEM
917 contain 2% FBS for the assay. Serum or BALF were incubated with 10 µl of Luc-SARS-Cov-
918 2 pseudotyped virus (LV-2058, PackGene, China) for 60 min, then added to the HEK293T
919 cells stably expressing ACE2 to incubate in a standard incubator (37°C, 5% CO₂) for 72 h.
920 Post infection, cells were lysed and detected using a luminescence reporter gene assay system
921 (RG006, Beyotime Biotechnology, China). The Luciferase activity was measured using the
922 Promega GloMax Navigator Detection System (GloMax, Promega, USA) and expressed as
923 relative light units (RLU). The 50% neutralization titers (NT₅₀) were calculated as the serum
924 dilution at which RLU were reduced by 50% compared with RLU in virus control wells after
925 subtraction of background RLU in cell control wells.

926

927 **Tissue processing and flow cytometry.** Single cell suspensions of splenic and pulmonary
928 lymphocytes were prepared from resected spleens and lungs of mice. The lung tissues were
929 cut into scraps and digested with collagenase II (0.5 mg/mL, C8150, Solarbio, China) in
930 calcium chloride (1mM) and magnesium chloride (1mM) solution at 37 °C for 1 h with

931 shaking at 220 rpm. Next, the samples were filtered through a 75 mm cell strainer to obtain a
932 single cell suspension. The immune cells were obtained by density gradient centrifugation
933 with Percoll (17-0891-09, GE Healthcare, USA) according to the manufacture's instruction.
934 Splenic lymphocytes were collected by grinding spleen in PBS then passed through a 75 mm
935 cell strainer, cell pellets were re-suspended in 5 mL of red blood cell lysis buffer (RT122-02,
936 TIANGEN, China) for 5 min at RT for remove the red blood cell. PBS was added to wash the
937 cells twice, then centrifuged at $1500 \times g$ for 5 min, the cell pellets were eventually re-
938 suspended in RPMI1640 media supplemented with 10% FBS and 1% penicillin/streptomycin.

939

940 For flow cytometric study, cells were first stained with the LIVE/DEAD fixable cell stains kit
941 (65-0865-14, Invitrogen, USA) according to the manufacturer's protocol. For surface markers,
942 the cells were incubated with anti-mouse CD4 (11-0041-82, Invitrogen), anti-mouse CD8a
943 (45-0081-82, Invitrogen), For intracellular cytokine staining, cells were stimulated with the
944 overlapping peptide pool spanning of 14-mer peptides overlapping by nine amino acids from
945 the SARS-CoV-2 RBD proteins (see Supplementary Notes) for 6 h at 37 °C, 5% CO₂. Then
946 the cells were incubated with anti-mouse IL-4 (17-7041-82, Invitrogen) and anti-mouse IFN- γ
947 (12-7311-82, Invitrogen) after processing with the Cytofix/Cytoperm
948 Fixation/Permeabilization Kit (554714, BD Biosciences,) according to the manufacturer's
949 instructions. In order to detect the T_{EM}/T_{CM} and T_{RM} cells, the cell samples were stained with
950 the following indicated antibodies in FACS buffer: anti-CD62L (161204, BioLegend), anti-
951 CD44 (25-0441-82, BioLegend), anti-CD69 (104506, BioLegend) and anti-CD103 (121416,
952 BioLegend). All the samples were measured on a BD FACS Array flow cytometer (BD
953 Biosciences). Data are analyzed with FlowJo software V10.

954

955 **Enzyme linked immunospot (ELISpot) assay.** Cellular immune responses in mice were
956 performed using mouse IFN- γ /IL-4 ELISPOT PLUS plates (3321-4AST-2/3311-4APW-2,
957 MABTECH, Sweden). 96-well ELISPOT plates were pre-treated as the manufacturer's
958 instructions. 5×10^5 mouse splenocytes or pulmonary lymphocytes were plated into each well
959 and stimulated with the above-mentioned peptide pools at a final concentration of 1 μ g of
960 each peptide per well. Additionally, PMA/Ionomycin were added as a positive control and
961 RPMI 1640 media was used as a negative control. After incubation at 37 °C, 5% CO₂ for 24 h,
962 the plates were washed with PBS and incubation with biotinylated anti-mouse IFN- γ or IL-4
963 antibody for 2 h at RT. Finally, TMB substrate solution were added to visualize the spots.

964 Spots were scanned and quantified by an ImmunoSpot CTL (Bio-Rad) reader. Spot-forming
965 unit (SFU) per million cells was calculated by subtracting the negative control wells.

966

967 **Statistics and analysis.** Statistical analyses were performed using the GraphPad Prism 8
968 (GraphPad Software, USA). Unless otherwise specified, data were expressed as mean \pm
969 Standard Error of Mean (SEM). Dual comparisons were made using Welch's t-test, and
970 comparisons between multiple conditions were analyzed using analysis of variance (ANOVA)
971 followed by the appropriate post-hoc tests. All the tests were two tailed. Differences were
972 considered statistically significant when $P < 0.05$. All of the experiments were successfully
973 repeated at least twice with three or more biological replicates to ensure the reproducibility of
974 the data.