Saponin Nanoparticle Adjuvants Incorporating Toll-Like Receptor Agonists Improve Vaccine Immunomodulation

Ben S. Ou[‡,1], Julie Baillet[‡,2], Maria V. Filsinger Interrante[3,4,5], Julia Z. Adamska[6], Xueting Zhou[1], Olivia M. Saouaf[2], Jerry Yan[1], John H. Klich[1], Carolyn Jons[2], Emily L. Meany[1], Adian S. Valdez[7,8], Lauren Carter[7,8], Bali Pulendran[6,9,10], Neil P. King[7,8], Eric A. Appel[1,2,3,6,11,12]*

1. Department of Bioengineering, Stanford University, Stanford, CA 94305, USA

2. Department of Materials Science & Engineering, Stanford University, Stanford, CA 94305, USA

3. Stanford ChEM-H, Stanford University, Stanford, CA 94305, USA

4. Stanford Biophysics Program, Stanford University School of Medicine, Stanford University, Stanford, CA 94305, USA

5. Stanford Medical Scientist Training Program, Stanford University School of Medicine, Stanford University, Stanford, CA 94305, USA

6. Institute for Immunity, Transplantation and Infection, Stanford University School of Medicine, Stanford University, Stanford, CA 94305, USA

7. Department of Biochemistry, University of Washington, Seattle, WA 98109, USA

8. Institute for Protein Design, University of Washington, Seattle, WA 98195, USA

9. Department of Microbiology & Immunology, Stanford University School of Medicine, Stanford University, Stanford, CA 94305, USA

10. Department of Pathology, Stanford University School of Medicine, Stanford University, Stanford, CA 94305, USA

11. Department of Pediatrics - Endocrinology, Stanford University School of Medicine, Stanford University, Stanford, CA 94305, USA

12. Woods Institute for the Environment, Stanford University, Stanford CA 94305, USA

‡ These authors contributed equally to this work.

* Corresponding Author: eappel@stanford.edu

Abstract

Over the past few decades, the development of potent and safe immune-activating adjuvant technologies has become the heart of intensive research in the constant fight against highly mutative and immune evasive viruses such as influenza, SARS-CoV-2, and HIV. Herein, we developed a modular saponin-based nanoparticle platform incorporating toll-like receptor agonists (TLRas) such as TLR1/2a, TLR4a, TLR7/8a, or a mixture of TLR4a and TLR7/8a adjuvants and denoted them as TLR1/2a-SNP, TLR4a-SNP, TLR7/8a-SNP, and TLR4a-TLR7/8a-SNP respectively. These TLRa-SNPs greatly improved the potency, durability, breadth, and neutralization of both COVID-19 and HIV vaccine candidates, suggesting the potential broad application of this newly designed adjuvant technology to a range of different antigens. More importantly, in addition to their potency, different formulations of TLRa-SNPs induced unique acute cytokine and immune-signaling profiles, leading to specific Th-responses that could be of interest depending on the target disease for prevention. Overall, this work demonstrates a modular TLRa-SNP adjuvant platform which could have a major impact on modern vaccine indications.

Introduction

The development of protective vaccines has been at the heart of intensive scientific research for the last hundred years, but the emergence of global pandemics such as SARS-CoV-2, HIV, and influenza have further catalyzed their development.[1] Yet, many commercial vaccines are still traditional live-attenuated or inactivated virus vaccines, both of which suffer from complex manufacturing processes and potential safety hazards.[2] Over the last few decades, protein-based subunit vaccine approaches have been shown to offer desirable characteristics in terms of safety, cost effectiveness, scalability, and manufacturability. Subunit vaccines leverage the use of viral protein antigens paired with immune stimulating molecules, also known as adjuvants, that are essential in promoting the magnitude and durability of the immune response.

To date, only a handful of adjuvants are clinically licensed in vaccine formulations and approved by the Food and Drug Administration (FDA), including the more traditional insoluble aluminum salt Alum and oil-in-water emulsions such as MF59 and AS03.[3] There are other adjuvant candidates in development, many of which offer promising improvements in the overall potency of subunit vaccines. A specific class of adjuvants that has been gaining attention is toll-like receptor agonists (TLRas).[4,5] They include TLR1/2a Pam3CSK4 (Pam3CysSerLys4), TLR3a Poly(I:C) (Polyinosinic:polycytidylic acid), TLR4a MPL (Monophosphoryl lipid), TLR7/8a Resiquimod or 3M-052 (imidazoquinoline molecules), and TLR9a CpG-ODN (synthetic single-stranded DNA). TLRas differ in physical and chemical properties, and can include lipids, small molecules, single-stranded DNA, and double-stranded RNA. Nonetheless, they all bind to toll-like receptors (TLRs), commonly expressed by antigen-presenting cells (APCs), to activate immune signaling pathways. Recent FDA approvals of vaccines comprising CpG-ODN 1018 (Dynavax),

AS04 (GSK; MPL/Alum), AS01 (GSK; liposomes comprising MPL) and the numerous ongoing clinical trials using 3M-052 (3M/AAHI) and other TLRas reinforce their emergence as promising adjuvants for both vaccines and cancer therapeutics.

Although adjuvants can be delivered in their soluble form, intensive research has demonstrated the advantage of formulating adjuvants as nanoparticles, improving their stability, solubility, cellular uptake, and immunogenicity, ultimately augmenting vaccine immune responses in favorable ways.[6-12] For example, saponins such as Quil-A (natural triterpene glycosides from Quillaja which are a leading non-TLRa class of adjuvant molecules) can be toxic when delivered alone but have been shown to be non-toxic when delivered in nanostructures such as the well-known honeycomb-like structure commonly known as ISCOMATRIX.[13-19] These well-defined nanostructures are 30-70 nm in size and are generated by spontaneous self-assembly of the Quil-A saponins with cholesterol and phospholipids. These adjuvants have been used for decades in vaccine formulations and have been demonstrated to be highly potent and safe. Indeed, the nanoparticulate adjuvant Matrix-M licensed by Novavax, which is a comparable self-assembled saponin-based nanostructure adjuvant, was approved by the FDA in October 2022 for its use in Novavax COVID-19 vaccine. While the mechanism of immune stimulation of these self-assembled saponin-based nanostructure adjuvants has not yet been fully elucidated, recent reports suggest they increase lymph flow and lymph node permeability to enhance antigen acquisition by B cells in draining lymph nodes (dLNs).[14,19]

Few attempts have been made to combine both TLRas and saponin-class adjuvants into the same nanostructure.[14,20-24] GSK uses the liposome AS01 containing MPLA and QS-21 saponin in its

licensed Shingrix and Mosquirix vaccines.[21] Similarly, Irvine and coworkers recently designed ISCOMATRIX incorporating TLRa MPLA and saponins (SMNP).[14] Despite the potential synergistic effect associated with combining two classes of adjuvants such as TLRas and saponins, no literature has yet shown the incorporation of other TLRas into saponin nanoparticles. Here, we report a platform of saponin nanoparticles incorporating toll-like receptor agonists (TLRa-SNPs). We created four formulations of TLRa-SNPs incorporating Pam3CSK4 (TLR1/2a-SNP), MPLA (TLR4a-SNP), an imidazoquinoline derivative (TLR7/8a-SNP), as well as a mixture of both MPLA and imidazoquinoline derivative (TLR4a-TLR7/8a-SNP). Compared to traditional ISCOMATRIX (plain saponin nanoparticle, SNP), all four TLRa-SNPs elicited improved humoral immune responses when used as adjuvants for SARS-CoV-2 and HIV vaccines in mice. TLRa-SNPs led to more potent antibody titers, improved antibody durability, enhanced breadth against variants of concern (VOCs), and induced better neutralizing responses when compared to SNPs or a clinically relevant control adjuvant CpG/Alum. Notably, TLRa-SNPs induced unique acute cytokine profiles, leading to distinct Th-skewed responses depending on the nature of the TLRa adjuvant incorporated. Overall, we have generated a potent and modular TLRa-saponin nanoparticle platform that improves humoral immune responses and generates tunable long-term Th-skewed responses, a pertinent feature in preventing diseases for which different Th-skewed responses lead to better clinical outcomes.

Results

Formulation and characterization of TLRa-SNPs

We sought to create a platform of lipid-based saponin nanoparticles incorporating various TLRa adjuvants. The formulation of plain saponin nanoparticles (SNPs), traditionally known as

ISCOMATRIX, has been widely reported in literature: mixing Quil-A saponin, dipalmitoylphosphatidylcholine (DPPC), and cholesterol at a molar ratio of 10:10:5 in aqueous medium triggers their spontaneous self-assembly into honeycomb-like nanoparticles 30-70 nm in diameter.[13,15,16] We hypothesized that lipid-based TLRas could be readily incorporated with SNPs at a specific stoichiometry via hydrophobic effects, while non-lipidated TLRas could be conjugated with a cholesterol motif to specifically interact with saponins. In this way, we successfully incorporated TLR1/2a Pam3CSK4, TLR4a synthetic MPLA (MPLAs), and cholesteryl TLR7/8a imidazoquinoline derivative with SNPs by mixing the desired TLRa adjuvant, Quil-A saponin, DPPC, and total cholesterol content at a molar ratio of 1:10:2.5:10 across all formulations (Figure 1a, Table S1, Figure S1-3, and SI for synthesis). The resulting nanoparticles showed monodisperse populations of hydrodynamic diameters between 40 and 60 nm and negative surface charges equal to or below -30 mV (Figures 1b, 1c). Cryo-electron microscopy confirmed all nanoparticles conserved the signature honeycomb-like structure after introduction of TLRa adjuvants (Figure 1d). Moreover, TLRa-SNPs maintained their colloidal stability for at least 6 weeks when stored at 4 ºC and for several months when stored at -20 ºC (Figure S4).

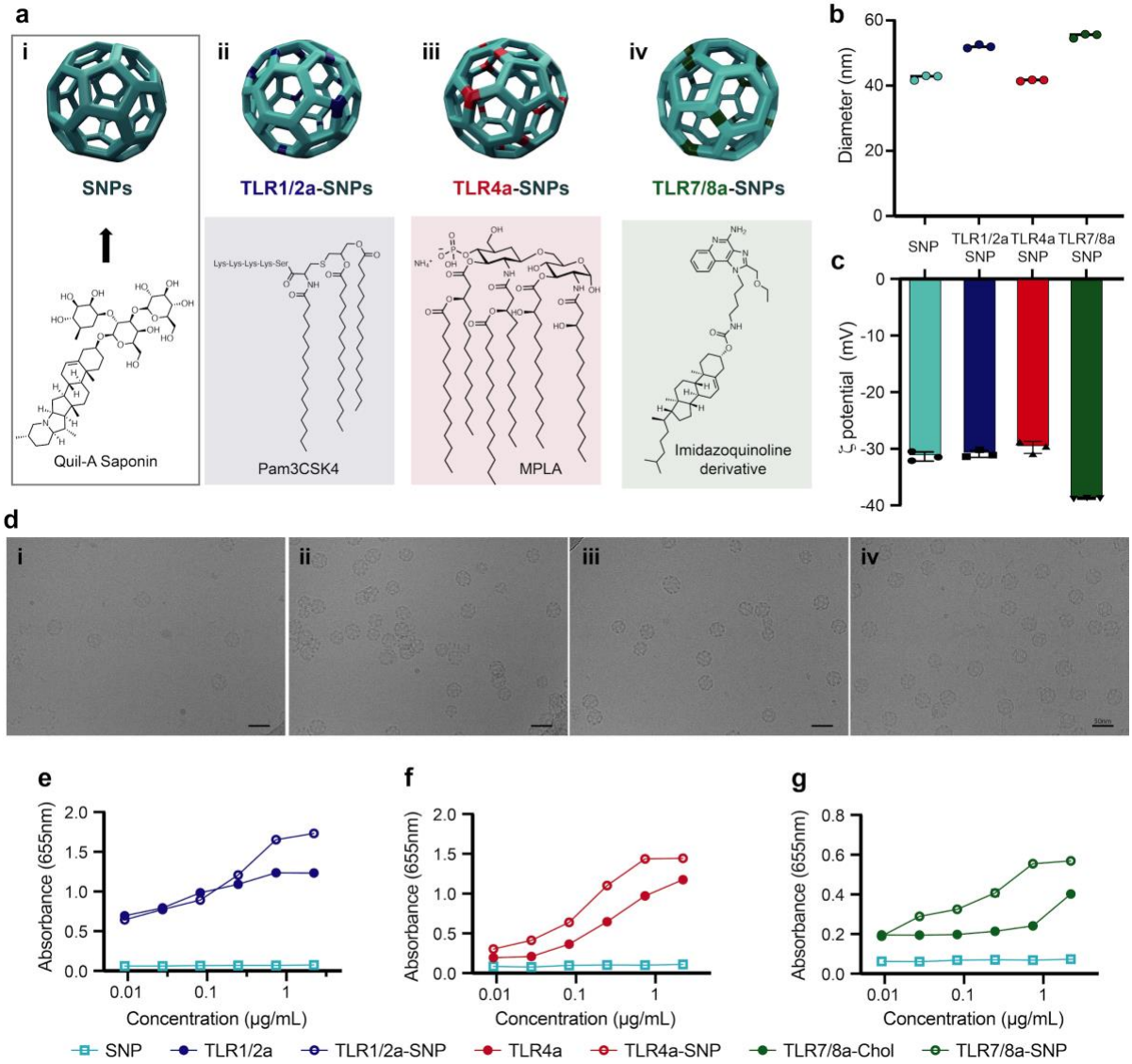


Figure 1: Design and characterization of TLRa-SNPs. **(a)** Schematic representation of SNP and different formulation of TLRa-SNPs: TLR1/2a-SNP incorporating Pam3CSK4, TLR4a-SNP incorporating MPLA, and TLR7/8a-SNP incorporating imidazoquinoline derivative. Hydrodynamic diameter **(b)** and surface charge **(c)** of SNP and TLRa-SNPs measured by Dynamic Light Scattering and Zetasizer. **(d)** Cryo-Electron Microscopy of **(i)** SNP, **(ii)** TLR1/2a-SNP, **(iii)** TLR4a-SNP, and **(iv)** TLR7/8a-SNP, demonstrating the maintenance of SNP structure after introduction of the TLRas. Scale bars: 50 nm. RAW-Blue macrophage cells were incubated with saponin-nanoparticle (SNP) or TLRa incorporated SNPs (TLRa-SNPs). Activation curves of **(e)** soluble TLR1/2a and TLR1/2a-SNP, **(f)** soluble TLR4a and TLR4a-SNP, and **(g)** soluble cholesteryl-modified TLR7/8a and TLR7/8a-SNP across a range of TLRa concentrations (0.0091-2.22 μg/mL) with 100,000 RAW-Blue cells (n = 3).

***In vitro* activation of TLRa-SNPs**

To further confirm that TLRas had been incorporated with SNPs and maintained their bioactivities, we performed a RAW-Blue transgenic mouse macrophage *in vitro* cell assay to confirm TLR activation. RAW-Blue cells were incubated with different formulations of TLRa-SNPs (SNPs, TLR1/2a-SNPs, TLR4a-SNPs, or TLR7/8a-SNPs) or the corresponding soluble TLRa adjuvants. Activation of the TLRs by the TLRas would lead to downstream activation of NF-κB and AP-1 pathways, which can be assessed with QUANTI-Blue for a colorometric output. Concentration-dependent NF-κB and AP-1 activation curves were generated by stimulating the cells with a range of soluble TLRa or TLRa-SNP concentrations (n=3; equivalent TLRas concentration of 2.22-0.0091 μg/mL; Figure 1e-1g, Figure S5). No activation with plain SNPs or relevant buffer controls was observed. In contrast, all TLRa-SNPs generated concentration-dependent NF-κB and AP-1 activation. Interestingly, TLRa-SNPs produced more potent activation compared to their soluble counterparts, most likely due to the ability of the nanoparticle structure to enhance cellular uptake. These data show that all three different TLRas could be incorporated into SNPs and maintain their bioactivity *in vitro*, demonstrating the success of this platform approach.

RBD-NP vaccines adjuvanted with TLRa-SNPs generate potent and broad humoral responses

The immune responses generated by different TLRa-SNPs were investigated using SARS-CoV-2 vaccines formulated with receptor-binding domain nanoparticles (RBD-NP) antigen and TLRa-SNPs as nanoparticle adjuvants. The RBD-NP used in this study, also called RBD-16GS-I53-50, has been extensively studied in mice, nonhuman primates (NHPs), and human clinical trials.[25-29] It has been shown to generate potent neutralizing humoral responses when formulated with AS03

or CpG/Alum adjuvants, leading to licensure in several countries of RBD-NP adjuvanted with AS03 as a COVID-19 vaccine for adults 18 years or older.[30] Following the same immunization regime as previous studies,[27,28] 7-8 week old C57BL/6 mice (n = 5) were subcutaneously (s.c.) immunized with vaccines comprising 1.5 μg RBD-NP and 10 μg of TLRa-SNP formulations (SNP, or TLR1/2a-SNP, or TLR4a-SNP, or TLR7/8a-SNP). For comparison, a clinical control containing CpG/Alum (20 μg and 100 μg, respectively) was evaluated. Mice were immunized at Week 0 (prime) followed by a boost at Week 3, and sera were collected from Week 0 to Week 11 (Figure 2a).

We first evaluated anti-RBD total IgG titers over time (Figure 2b). At Week 3, prior to boosting, mice immunized with RBD-NP vaccines adjuvanted with CpG/Alum or SNP elicited inconsistent antibody titers with around half the mice's anti-RBD IgG endpoint below the limit of detection. In contrast, on Week 3, all mice immunized with vaccines adjuvanted with TLRa-SNPs (TLR1/2a-SNP, TLR4a-SNP, and TLR7/8a-SNP) seroconverted and generated antibody titers significantly higher than the CpG/Alum and SNP controls (*p* values in Table S3). Two weeks post-boost (Week 5), no significant difference in titers was observed between the two controls CpG/Alum and SNP, with an average endpoint titer of 2.1×10^5 and 9.6×10^4, respectively. However, TLRa-SNPs induced significantly higher titers for which the average endpoint titers were 9.1×10^5 (TLR1/2a-SNP), 5.2×10^5 (TLR4a-SNP), and 4.3×10^5 (TLR7/8a-SNP). Additionally, TLRa-SNP vaccinated mice maintained significantly higher anti-RBD IgG endpoint titers at all timepoints post-boost and produced robust titers with little deviations across animals (*p* values in Table S3). In contrast, mice vaccinated with CpG/Alum resulted in variable titer responses spanning up to two orders of

magnitude, leading to a standard error of mean (SEM) measured to be at least twice that of the rest of the treatments after boosting.

We calculated the area under the curves (AUCs) of the endpoint titers over the 11-week period to estimate the mean IgG production. No significant difference in AUC titers between CpG/Alum and SNP was observed (Figure 2c; *p* values in Table S4). While all TLRa-SNPs resulted in significantly higher AUCs than CpG/Alum, only TLR1/2a-SNP produced a significantly higher AUC than SNP. EC_{50} titers (half-maximal binding dilution) were also measured two weeks post-boost (Week 5) to ensure the endpoint titers reported correlated with the functional potency of the antibody (Figure 2d). Similar to comparisons of endpoint titers, CpG/Alum and SNP induced low and variable EC_{50} titers compared to TLRa-SNPs, which all generated significantly higher average EC_{50} (*p* values in Table S5). Furthermore, we assessed the endpoint titers of the vaccinated mice a year after immunization (D365, Figure 2e). While the average endpoint titers from Week 11 to D365 drastically dropped by 24-fold for CpG/Alum (2.6×10^3) and 13-fold for SNP (3.9×10^3), TLRa-SNPs' endpoint titers remained high with only a 7-fold (6.7×10^4) and 5-fold (5.4×10^4) decrease for TLR1/2a-SNP and TLR4a-SNP, respectively. TLR7/8a-SNP vaccinated mice were excluded from the comparison due to an insufficient sample size that remained (n=2 at D365 due to murine dermatitis leading to euthanasia criteria; statistical power calculated using Mead's Resource Equation).[31,32] Overall, the titers measured at D365 from mice receiving vaccines adjuvanted with TLRa-SNPs were significantly higher than mice that received vaccines adjuvanted with either SNP or CpG/Alum controls. More importantly, these titers were at the same order of magnitude as the endpoint titers measured for the SNP and CpG/Alum controls on Week 11,

demonstrating the durability of RBD-NP vaccines adjuvanted with TLRa-SNPs (Figure S6, *p* values in Table S6).

We further determined anti-Spike antibody titers to confirm the antibodies produced from RBD-NP adjuvanted with TLRa-SNP vaccines could bind to native spike proteins. Moreover, the breath of the antibody response was assessed by measuring the endpoint titers against different SARS-CoV-2 VOCs including Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) spike proteins two weeks post-boost (Week 5) compared to CpG/Alum (Figure 2f). While no differences in endpoint titers were observed between SNP and CpG/Alum regardless of variants (*p* values in Table S7), TLR1/2a-SNP led to significantly higher titers against all variants. Overall improved titers against variants were also observed for TLR4a-SNP and TLR7/8a-SNP. Notably, the ratio of titers against different variants compared to wild-type spike titer (percent drop) showed more consistent and higher breadth for TLRa-SNPs compared to CpG/Alum and SNP controls, for which more variable and larger interquartile ranges were observed (Figure S7). Overall, these findings suggested that SNP as an adjuvant did not perform better than the clinical control CpG/Alum in generating humoral responses. Only TLRa-containing SNPs elicited more potent and durable titer responses with better breadth.

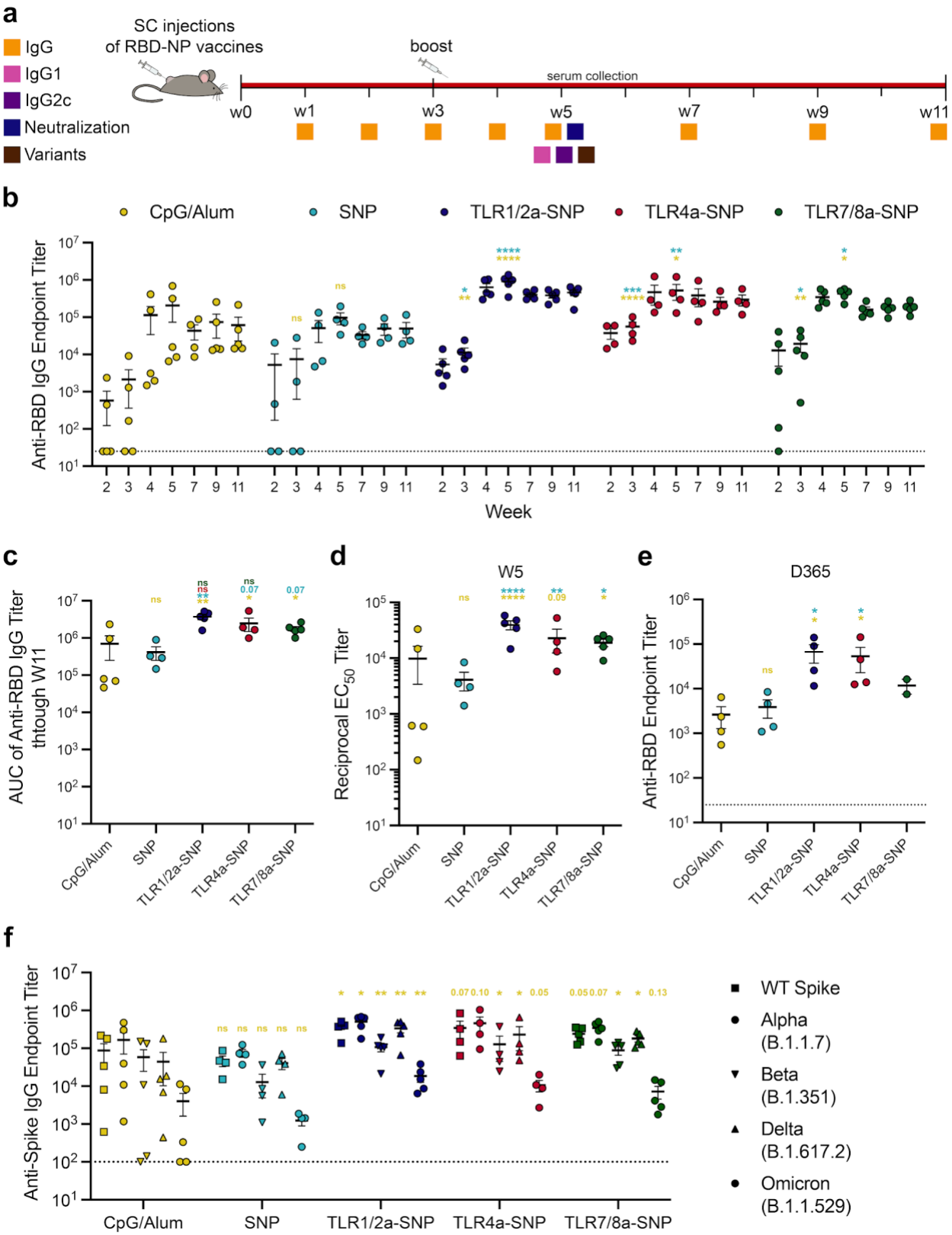


Figure 2: In vivo humoral response to RBD-NP vaccines adjuvanted with TLRa-SNPs. **(a)** Timeline of immunization and blood collection to determine IgG titers. Mice were immunized on

Week 0 and boosted on Week 3 with RBD-NP vaccines adjuvanted with CpG/Alum, SNP, or TLRa-SNPs. IgG1, IgG2c, neutralization, and variants titers were determined on Week 5. **(b)** Anti-RBD IgG binding endpoint titers of RBD-NP vaccines adjuvanted with CpG/Alum, SNP, or TLRa-SNPs. **(c)** Area under the curves (AUCs) of anti-RBD IgG endpoint antibody titers from Week 0 to Week 11 of the different RBD-NP vaccines. **(d)** Half-maximal binding dilution (EC_{50}) of anti-RBD IgG endpoint titers on Week 5 of the different RBD-NP vaccines **(e)** Anti-RBD IgG binding endpoint titers a year (D365) after immunization **(f)** Anti-spike IgG binding endpoint titers from sera collected on Week 5 after the initial immunization. Titers were determined for wildtype WT spike as well as Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) variants of the spike protein. Data (n = 4-5) are shown as mean +/- SEM. *p* values were determined using the genearal linear model followed by Tukey's HSD comparison procedure on the logged titer values for IgG titer comparisons. Complete *p* values for comparisons are shown in Table S3-S7. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

<u>RBD-NP vaccines adjuvanted with TLRa-SNPs produce strong neutralizing antibodies</u>

Since mice immunized with RBD-NP vaccines adjuvanted with TLRa-SNPs generated potent, durable, and broad antibody responses, we sought to measure the neutralizing activities of the sera. Week 5 sera neutralization was evaluated by utilizing a SARS-CoV-2 spike pseudotyped lentivirus to measure serum-mediated inhibition of viral entry into HeLa cells overexpressing ACE2 and TMPRSS2 (Figure 2a). Serum neutralization ID_{50} was measured through neutralizing activities of a range of sera concentrations to determine the half-maximal inhibition of infectivity (ID_{50}) (Figure 3a-f). Consistent with previous findings,[29] mice immunized with RBD-NP adjuvanted with CpG/Alum resulted in highly variable neutralizing responses, with two mice having serum neutralizing activity below the limit of detection. In contrast to CpG/Alum, RBD-NP vaccines adjuvanted with SNP and TLRa-SNPs induced significantly higher neutralizing ID_{50} (*p* values in Table S8). Notably, TLRa-SNPs elicited higher ID_{50} than the "high titer" classification according to FDA's recommendation ($ID_{50} \sim 10^{2.4}$).[33] Moreover, mice sera neutralization activities were compared with human patients' convalescent plasma by testing the pseudoviruses' infectivity at the lowest sera dilution (1:100 dilution; Figure 3g). Variable results were observed for SNP and

CpG/Alum controls, where not all mice receiving vaccines with these two adjuvants reached 0% infectivity. Mice immunized with vaccine adjuvanted with CpG/Alum resulted in significantly higher infectivity than those immunized with vaccines adjuvanted with TLRa-SNPs, with an average comparable to mice that received non-adjuvanted RBD-NP vaccine (*p* values in Table S9). On the contrary, we measured 0% infectivity from all mice receiving vaccines adjuvanted with TLRa-SNPs, indicating neutralization of all lentivirus and prevention of their entry into and infection of HeLa cells. Notably, the sera were even more neutralizing than the human patients' convalescent plasma, for which the average infectivity was 15%. Overall, TLRa-SNPs as vaccine adjuvants generated superior humoral responses in potency, durability, breadth, and neutralization compared to both SNP and CpG/Alum controls.

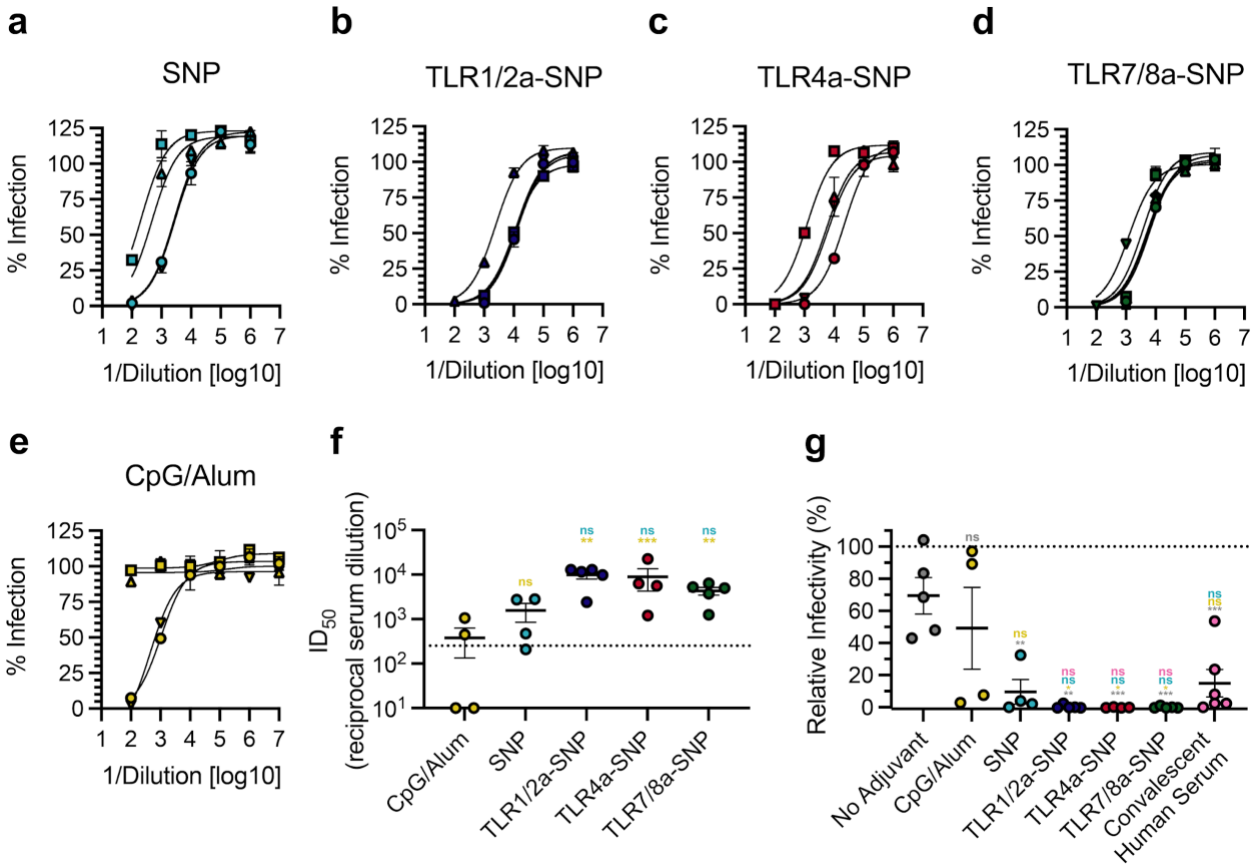


Figure 3: Neutralizing antibodies in mice post RBD-NP vaccines adjuvanted with TLRa-SNPs. **(a-e)** Percent infectivity for all vaccine treatments at a range of Week 5 serum dilutions as determined by a SARS-CoV-2 spike-pseudotyped viral neutralization assay. **(f)** Comparison of ID_{50} values determined from neutralization curves. Dotted line denotes the threshold for which the FDA considers as "high titer".[33] **(g)** Relative percent infectivity of all vaccine formulations compared to convalescent human serum at 1:100 dilution. Data (n = 4-5) are shown as mean +/- SEM. *p* values were determined using the genearal linear model followed by Tukey's HSD multiple comparisons ptocedure on the logged neutralization ID_{50} values or relative infectivity comparisons. Complete *p* values for comparisons are shown in Table S8-S9. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

TLRa-SNPs elicit tunable Th-skewed responses

After assessing the potency of TLRa-SNPs in inducing durable and broad antibody responses, we further evaluated whether the nature of TLRa adjuvant affected the immune response of the TLRa-SNPs, since TLRas have been shown to generate unique immune signaling and T-helper responses.[34,35] We hypothesized that different TLRa-SNPs could elicit unique levels of IgG1 and IgG2c titers, as these isotypes are respectively strong indicators of Th2- and Th1-skewed

responses. IgG isotypes were therefore measured across the different vaccine treatments. Indeed, while all TLRa-SNPs led to improved total IgG antibody responses compared to SNP and CpG/Alum controls, they generated different levels of IgG isotypes. While TLR4a-SNP and TLR7/8a-SNP generated similar IgG1 responses compared to SNP and CpG/Alum, TLR1/2a-SNP induced significantly higher IgG1 titers compared to all other groups, with average titers an order of magnitude higher compared to those generated by SNPs (Figure 4a, *p* values in Table S10). On the other hand, all TLRa-SNPs elicited higher IgG2c titers, with at least a 5-fold increase in average titers compared to the controls (Figure 4b, *p* values in Table S11). The differences in IgG1 and IgG2c titers suggested that different TLRa-SNPs resulted in different "flavors" of antibody responses, which we measured by calculating the ratio of IgG2c to IgG1 titers (Figure 4c). TLR1/2a-SNP elicited a Th2-skewed response whereas TLR4a-SNP and TLR7/8a-SNP induced a Th1-skewed response. These results are drastically different from the balanced Th1/Th2 responses generated by SNP and CpG/Alum controls. The tailored Th-responses induced from the different TLRas thereby position TLRa-SNPs as a potent and modular adjuvant platform that could be of interest for inducing immunity against other challenging viruses.

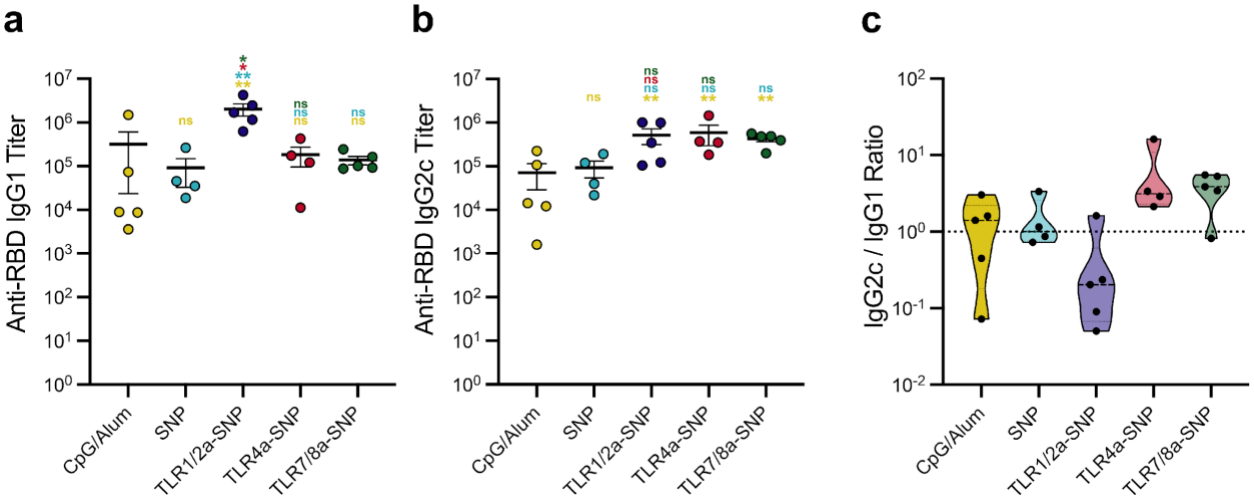


Figure 4: Antibody subtype response to RBD-NP vaccines adjuvanted with TLRa-SNPs. **(a)** Anti-RBD IgG1 and **(b)** IgG2c titers from sera collected on Week 5, 2 weeks after boost. **(c)** Ratio of

Anti-RBD IgG2c to IgG1 titers. Lower values (below 1) suggest a Th2 response or humoral response, and higher values (above 1) imply a Th1 response or cellular response. Data (n = 4-5) are shown as mean +/- SEM. *p* values listed were using the genearal linear model followed by Tukey's HSD multiple comparison procedure on the logged titer values. Complete p values for comparisons are shown in Table S10-S11. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Different TLRa-SNPs induced unique acute cytokine induction in the draining lymph node and robust germinal center responses

We hypothesized that the differences in Th-skewed responses from different TLRa-SNP formulations were due to different TLRas leading to differences in immune signaling. To measure the acute immune response post-vaccination, we assessed dLN cytokine profiles 1-day (24 hours) post-immunization through a Luminex assay (Figure 5a). Instead of reporting the results in pg/mL, which can suffer from plate/batch/lot inconsistencies, nonspecific binding, differences between standards and real biological samples, and other experimental artifacts, we analyzed the raw fluorescence signal by correcting for nonspecific binding as a covariate in the regression analysis to better infer the true biological patterns in Luminex data.[36-39] Sufficient levels of cytokines in the dLN are necessary to mount a successful vaccine response, and different cytokines induced can result in different Th responses. Comparing to mice adjuvanted with SNP, those that received TLRa-SNPs (TLR1/2a-SNP, TLR4a-SNP, or TLR7/8a-SNP) all induced significantly higher median fluorescence intensities (MFIs) of IFN-γ, IP10 (CXCL10), IL3, and IL15 (Figure 5b, *p* values in Table S12). These are critical proinflammatory cytokines for initiating strong immune responses and overall improved Th response.[40] Elevated levels of IL6, IL25, and IL31 observed with TLR1/2a-SNP adjuvanted vaccine could explain the observed Th-2 skewed response. On the other hand, elevated levels of cytokines such as IFN-γ, IP10, IL18, IL27, and GMCSF observed with TLR4a-SNP could be the cause of the observed Th1-skewed response. Notably, of the 48

cytokines measured in the Luminex assay, only 11 did not show significant differences between SNP and at least one of the TLRa-SNPs (Figure S8). Thus, to better understand the overall cytokine induction profile of each adjuvant treatment, we performed a dimensional reduction analysis by creating a penalized supervised star plot (PSS, Figure 5c). We were successful in generating distinct and separate cluster for each adjuvant. Not only did TLRa-SNPs induce higher effector cytokine production in the dLNs, but different formulations of TLRa-SNPs also led to unique acute cytokine profiles post-vaccination.

We also assessed germinal center activity in the dLN 12 days after immunizing with RBD-NP adjuvanted with TLRa-SNPs (Figure 5a). While mice treated with CpG/Alum adjuvanted vaccines had the highest count of germinal center B cells (GCBCs), they also had the lowest percent of GCBCs of total B cells, indicating CpG/Alum's inability to convert B cells to GCBCs (Figure 5d-e, *p* values in Table S13-S14). On the contrary, mice immunized with vaccines adjuvanted with TLRa-SNPs resulted in higher percent of GCBCs as well as higher total GCBC counts compared to mice receiving vaccines adjuvanted with SNP, suggesting robust germinal center activities. Since robust germinal center activity is important for generating better neutralizing and higher affinity antibodies, these data are consistent with the vaccination results that demonstrated increased magnitude and higher neutralizing antibodies with the TLRa-SNP groups. These experiments suggest TLRa-SNPs are superior in generating unique early proinflammatory responses and robust germinal center activity compared to SNP and CpG/Alum, resulting in an overall improved potency, durability, and breadth of humoral responses.

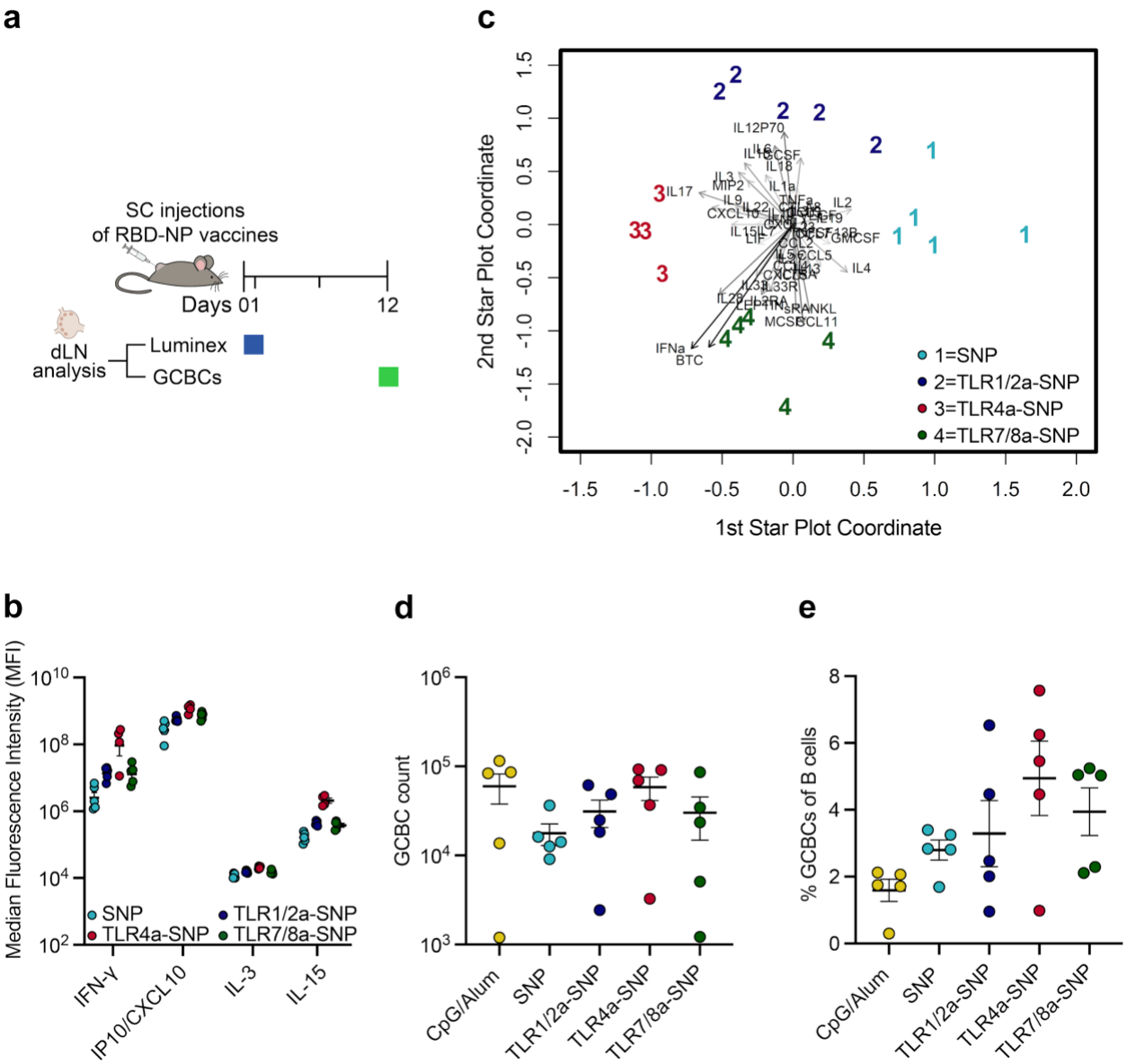


Figure 5: Draining lymph nodes (dLNs) analysis post-immunization with RBD-NP vaccines adjuvanted with SNP or TLRa-SNPs. **(a)** Timeline of immunization and dLN analysis. Luminex analysis was performed 1 day (24 hours) after immunization and germinal center B cells (GCBCs) were measured 12 days after immunization. **(b)** All three TLRa-SNPs induced significantly higher beneficial proinflammatory cytokine levels of IFN-γ, IP10, IL03, and IL-15 in the dLNs compared to SNP, reported in median fluorescence intensity (MFI). **(c)** Dimensional reduction analysis of the full murine Luminex 48-plexed cytokine assay (Figure S8) by performing a penalized supervised star (PSS) plot of the RBD-NP vaccines. Result shows clear separation of vaccines containing different adjuvants, with each number signifying an individual sample from the treatment groups. Vectors are the projection coefficients for each individual cytokine. **(d)** Total GCBC count from RBD-NP vaccines and **(e)** Frequency of GCBC from all B cells. Data (n = 4-5) are shown as mean +/- SEM. *p* values for the Luminex assay were from a generalized maximum entropy estimation regression adjusted to control the false discovery rate and *p* values for FACS were determined using the genearal linear model followed by Tukey's HSD comparison procedure on the logged values. Complete *p* values for comparisons are shown in Table S12-S14.

Saponin nanoparticle incorporating both TLR4a and TLR7/8a generated synergistic effect

Prior research has shown that the combination of certain TLRas such as TLR4a and TLR7/8a can synergistically activate the immune system.[5,41] We therefore created a new formulation of TLRa-SNP, TLR4a-TLR7/8a-SNP, in which we incorporated equimolar amounts of TLR4a MPLA and cholesteryl TLR7/8a imidazoquinoline derivative in SNPs (Figure 6a). The total molar ratio of TLRa to saponin was kept identical to the previously described TLRa-SNP formulations (Table S1). The average hydrodynamic radius of TLR4a-TLR7/8a-SNP, 47.9 nm, is consistent to the radii measured from SNPs and other TLRa-SNPs (Figure 1b, Figure S10a-b). To confirm whether co-incorporating TLR4a and TLR7/8a in the same SNP is synergistic, we immunized C57BL/6 mice (n=5) with vaccines formulated with 1.5 μg of RBD-NP adjuvanted with either 10 μg of TLR4a-TLR7/8a-SNP or a 1:1 mixture of TLR4a-SNP and TLR7/8a-SNP (5 μg each, referred as TLR4a-SNP + TLR7/8a-SNP). Mice were vaccinated on Week 0 and boosted on Week 3 matching the previous schedule. Binding titers were measured to compare with those from SNP, TLR4a-SNP, and TLR7/8a-SNP formulations (Figure 6b). We observed no significant difference of anti-RBD total IgG titers over time in mice adjuvanted with TLR4a-SNP + TLR7/8a-SNP compared to mice adjuvanted with either TLR4a-SNP or TLR7/8a-SNP (Figure 6c, *p* values in Table S15). In contrast, mice adjuvanted with TLR4a-TLR7/8a-SNP resulted in overall higher titers than all other groups, with a significant increase observed after the boost. The co-incorporation of both TLR4a and TLR7/8a on the same SNP is therefore pivotal for their synergistic effect. Consistent with other TLRa-SNP formulations, mice receiving vaccines adjuvanted with either TLR4a-SNP + TLR7/8a SNPs or TLR4a-TLR7/8a-SNP maintained robust binding titers against wild-type spike and different SARS-CoV-2 variant of concerns (Figure S10c-d). Finally, both the TLR4a-SNP + TLR7/8a-SNP and TLR4a-TLR7/8a-SNP formulations maintained similar IgG1 and IgG2c titers

compared to mice adjuvanted with either TLR4a-SNP or TLR7/8a-SNP, resulting in overall equally Th1-skewed responses (Figure 6d-f).

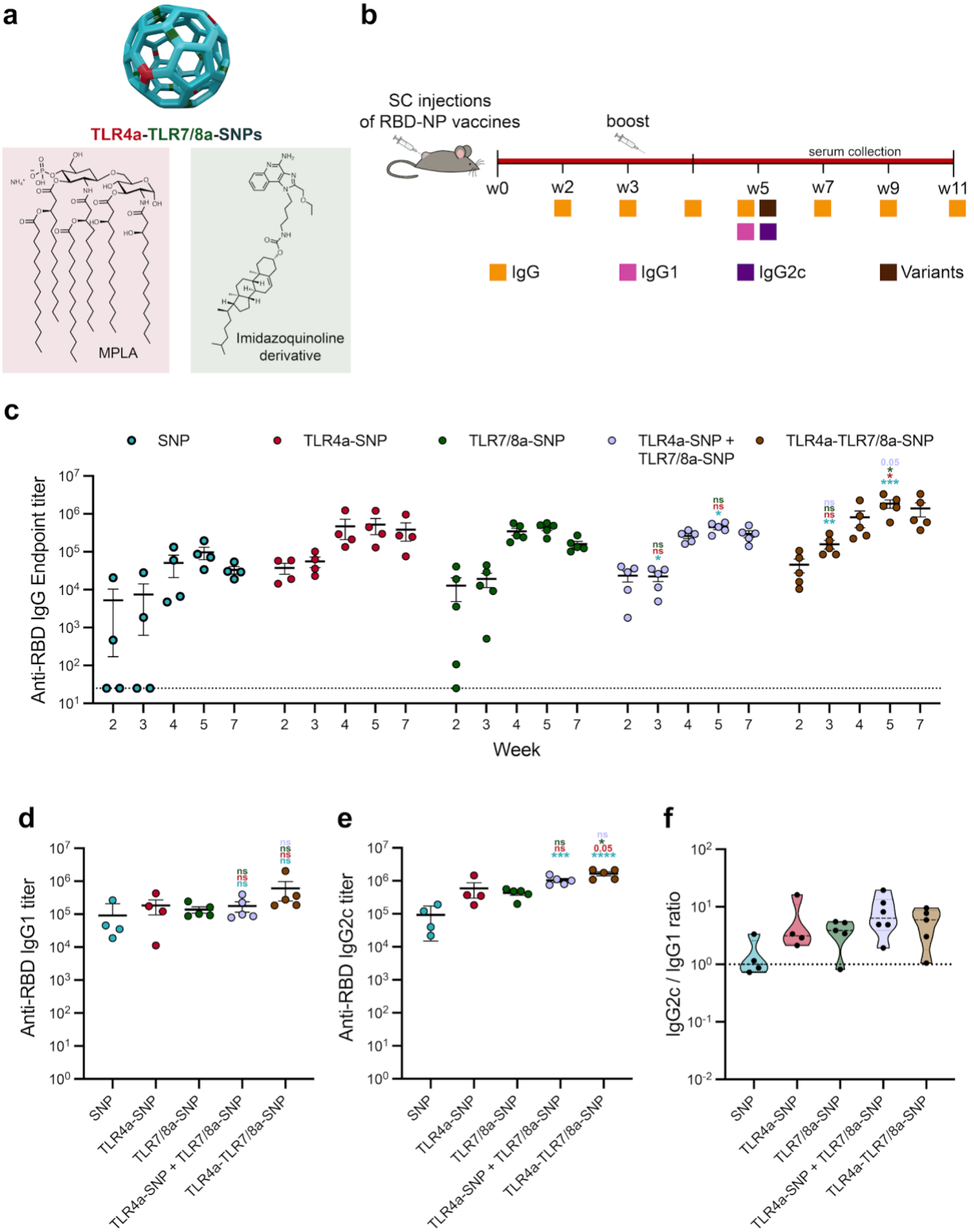


Figure 6: TLRa-SNP incorporating both TLR4a and TLR7/8a generated synergistic effect. **(a)** Schematic representation of TLR4a-TLR7/8a-SNP for which SNPs co-incorporate TLR4a MPLA and TLR7/8a imidazoquinoline derivative. **(b)** Timeline of RBD-NP vaccines immunization and

blood collection to determine IgG titers. Mice were immunized on Week 0 and boosted on Week 3 with RBD-NP vaccines adjuvanted with a 1:1 mixture of TLR4a-SNP and TLR7/8a-SNP or TLR4a-TLR7/8a-SNP. IgG1, IgG2c, and variants titers were measured on Week 5. **(c)** Anti-RBD IgG binding endpoint titers of RBD-NP vaccines. **(d**) IgG1 titers and **(e)** IgG2c titers. **(f)** Ratio of Anti-RBD IgG2c to IgG1 titers. Lower values (below 1) suggest a Th2 response or humoral response, and higher values (above 1) imply a Th1 response or cellular response. Data (n = 4-5) are shown as mean +/- SEM. *p* values were determined using the genearal linear model followed by Tukey's HSD comparison procedure on the logged titer values for IgG titer comparisons. Complete *p* values for comparisons are shown in Table S15-S17. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

<u>TLRa-SNPs as gp120 vaccine adjuvants induce robust humoral responses</u>

Finally, to confirm that TLRa-SNPs can elicit robust immune responses against other antigens, we vaccinated C57BL/6 mice (n=5) with HIV gp120 antigen adjuvanted with TLR1/2a-SNP, TLR4a-SNP, and TLR4a-TLR7/8a-SNP, the three formulations eliciting the highest antibody titers from RBD-NP vaccines. Titer responses were compared to the two following controls, gp120 vaccines adjuvanted with SNP or Alum. Mice were immunized on Week 0 and boosted on Week 4 and sera were collected from Week 0 to Week 6 to assess total anti-gp120 IgG and subtype titers (Figure 7a). Before boosting, most mice that received Alum or SNP adjuvanted vaccines did not have detectable titers, but all mice that received vaccines adjuvanted with TLR1/2a-SNP, TLR4a-SNP, or TLR4a-TLR7/8a-SNP seroconverted and elicited significantly higher titers (Figure 7b, *p* values in Table S18). Two weeks post-boost (Week 6), vaccines containing TLRa-SNP adjuvants generated significantly higher antibody titers compared to those with Alum, with average endpoint titers around 2000-fold higher. Notably, mice adjuvanted with TLR4a-TLR7/8a-SNP elicited the strongest response. Moreover, TLRa-SNPs also led to over 3-fold higher titers than SNP. Similarly, we observed significantly higher overall antibody production from mice adjuvanted with TLRa-SNPs compared to the controls by determining the AUCs of titers over the 6-week period (Figure 7c, *p* values in Table S19).

To confirm that the unique Th-skewed responses observed from different TLRa-SNPs in RBD-NP vaccines were due to influences of TLRas in immune signaling, we measured Week 6 IgG1 and IgG2c titers from mice immunized with gp120 vaccines. Consistent with our previous observations, mice adjuvanted with TLR1/2a-SNP and TLR4a-SNP both induced robust IgG2c responses while only TLR1/2a-SNP generated significantly higher IgG1 titers compared to SNP (Figure 7d-e, *p* values in Table S20-21). On the other hand, TLR4a-TLR7/8a-SNP elicited significantly higher IgG1 and IgG2c titers compared to SNP. This resulted in a more Th2-skewed response from mice adjuvanted with TLR1/2a-SNP and a more Th1-skewed response from mice adjuvanted with TLR4a-SNP (Figure 7f). Overall, these results confirmed TLRa-SNPs' ability to generate potent and robust antibody responses with distinctive Th-skewed responses regardless of the antigen.

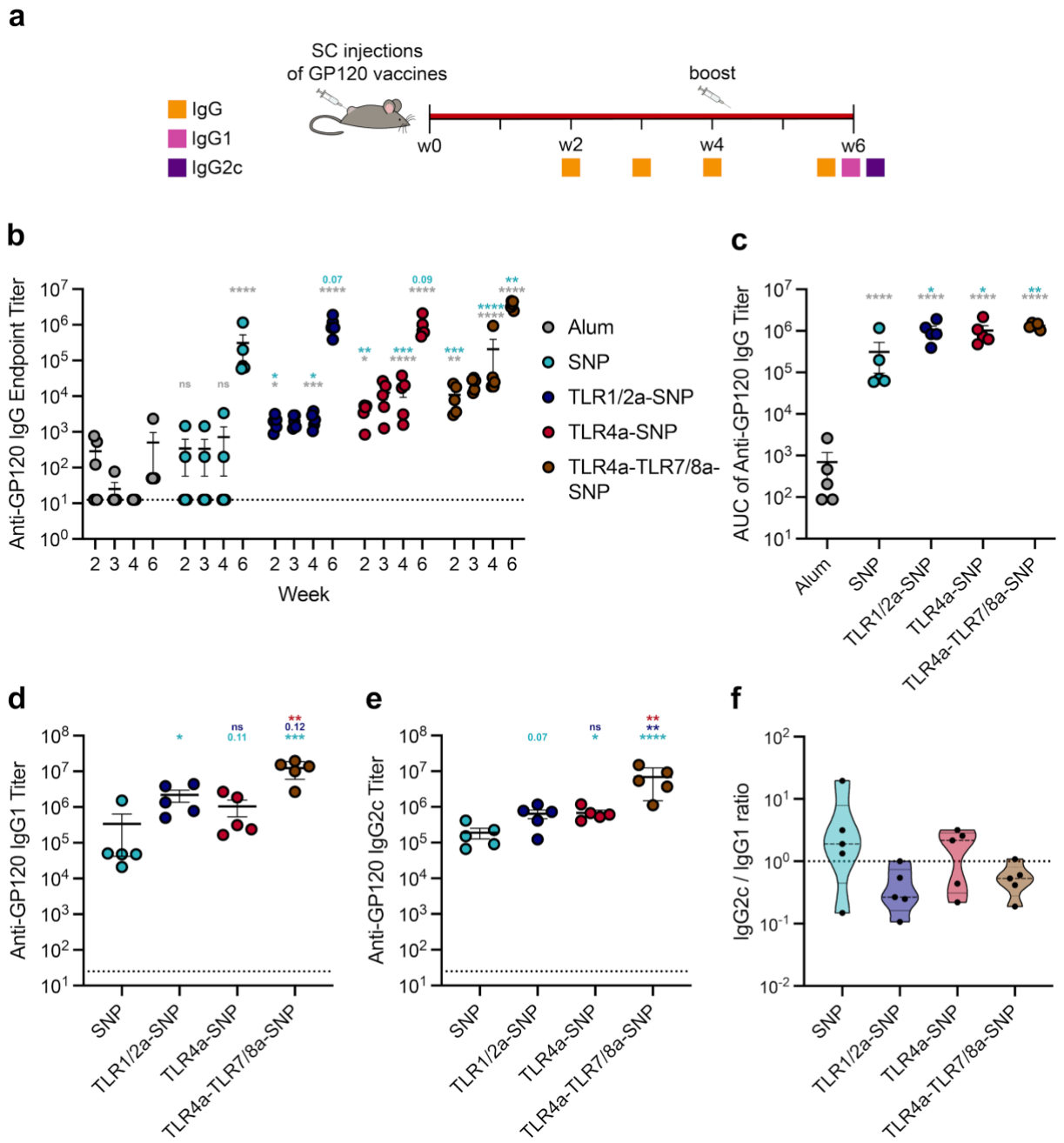


Figure 7: In vivo humoral response to HIV GP120 vaccines adjuvanted with TLRa-SNPs. **(a)** Timeline of immunization and blood collection to measure IgG titers. Mice were immunized on Week 0 and boosted on Week 4 with GP120 vaccines adjuvanted with Alum, SNP, or TLRa-SNPs. IgG1 and IgG2c titers were determined on Week 6. **(b)** Anti-GP120 IgG binding endpoint titers of GP120 vaccines adjuvanted with Alum, SNP, or TLRa-SNPs. **(c)** Area under the curves (AUCs) of anti-GP120 IgG endpoint antibody titers from Week 0 to Week 6 of different GP120 vaccines. **(d)** Anti-GP120 IgG1 and **(e)** IgG2c titers from sera collected on Week 6, 2 weeks after boost. **(f)** Ratio of Anti-GP120 IgG2c to IgG1 titers. Lower values (below 1) suggest a Th2 response or humoral response, and higher values (above 1) imply a Th1 response or cellular response. Data (n = 5) are shown as mean +/- SEM. *p* values were determined using the genearal linear model followed by Tukey's HSD comparison procedure on the logged titer values for IgG titer comparisons. Complete *p* values for comparisons are shown in Table S18-S21. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

Discussion

Over the past few decades, modern vaccinology has moved towards the use of subunit protein-based vaccines over more traditional vaccine formats on account of their increased affordability, ease of manufacturing, and high safety profile.[2] Unfortunately, the poor immunogenicity of many protein antigens reinforces the need for developing more potent and modular adjuvants. In this study, we designed an adjuvant platform combining three approaches in adjuvant design and selection: (i) molecular TLRa adjuvants resembling pathogen-associated molecular patterns found on bacteria, viruses, and other foreign invaders our immune system is trained to recognize; (ii) saponin adjuvants from natural sources that improve lymphatic flow; and (iii) particulate design displaying both adjuvants together with improved trafficking kinetics and cellular uptake. Previous studies have reported the design of combined TLR4a MPLA and saponins into nanoparticle constructs, such as AS01 and SMNP. Nonetheless, a tunable platform approach must consider incorporating other clinically relevant TLRas. In this regard, we demonstrated that lipid TLRas, such as MPLA and Pam3CSK4, could be readily incorporated with clinically relevant stoichiometric ratios due to the hydrophobic nature of SNPs. Non-lipid TLRas, such as TLR7/8a Resiquimod, could be incorporated by conjugating a cholesterol motif that hydrophobically interacts with SNPs. The facile assembly and modularity of our SNP platform also allow the design and investigation of SNPs containing multiple TLRas in a same particle such as TLR4a and TLR7/8a (TLR4a-TLR7/8a-SNP). In this regard, consistent with previous reports suggesting TLR4a and TLR7/8a synergistically activate the innate immune system,[5,41] we observed improved antibody responses from mice receiving vaccines adjuvanted with TLR4a-TLR7/8a-SNP.

A recent report by Irvine and coworkers provided detailed mechanistic analyses of the improved humoral response induced by ISCOMATRIX and SMNP, similar to SNP and TLR4a-SNP in the present study.[14] Mainly, both adjuvants enhance lymph flow in a mast cell-dependent manner, which promotes antigen delivery into the dLNs. Since this mechanism was determined to be saponin-dependent, we assumed all TLRa-SNPs improved vaccine immune responses in a similar manner. Further, since mast cells were shown to be essential in saponins' ability to improve lymph flow and are effector cells of Th-2 response, we hypothesized that TLR1/2a-SNP conferred superior humoral responses due to its ability to generate a Th-2 skewed response. Future investigation could determine other synergistic effects of different TLRas with SNPs. Moreover, no significant elevation in proinflammatory cytokines or chemokines in the dLNs was observed when mice received soluble MPLA mixed with ISCOMATRIX compared to mice receiving ISCOMATRIX alone, suggesting it is necessary to deliver both adjuvants in the same particle for their synergistic effects. In addition, previous reports also suggested that delivering soluble TLRas can lead to potentially toxic levels of systemic inflammation due to their rapid clearance into the bloodstream.[14,42,43] Hence, delivering soluble TLRas without carriers such as Alum or various relevant nanoparticle constructs poses safety risks that may hinder clinical translation.[44] As such, soluble TLRas mixed with SNP matching TLRa-SNP formulations were not tested as controls in this study. Furthermore, Irvine and coworkers reported no signs of toxicity of their SMNP design. The present study includes the use of clinically safe TLRas dosed in mice in lower masses than previously reported.[14,45-47] We therefore assumed the TLRa-SNPs were non-toxic as well, an assumption supported by the absence of any toxicity signs in mice such as weight loss or injection site reactogenicity.

Numerous preclinical and clinical studies have utilized the RBD-NP antigen RBD-16GS-I53-50 developed by King and coworkers.[25-28] In this study, the same RBD-NP antigen was used in a dose- and schedule-matched study design as in previous reports, allowing for a direct comparison of the potency of TLRa-SNPs with previously screened adjuvants.[25-28] From those studies, AS03 and CpG/Alum have been reported to induce comparable humoral responses such as robust neutralizing responses, and both outperformed other clinically relevant adjuvants such as AS37, AddaVax, and Essai O/W 1849101.[27] The use of CpG/Alum as a control in the present study highlights the superiority of all TLRa-SNPs (i.e., TLR1/2a-SNP, TLR4a-SNP, TLR7/8a-SNP, and TLR4a-TLR7/8a-SNP) in generating potent, durable, broad, and neutralizing antibody responses compared to previously tested adjuvants.[27] This observation is even more striking considering that the dosage of CpG/Alum was doubled compared to that used in literature.[27] Additionally, mice receiving vaccines adjuvanted with TLRa-SNPs demonstrated complete neutralization in pseudovirion neutralization assays, in contrast to convalescent human plasma which resulted in a quantifiable level of infectivity. This observation is particularly striking considering all human patients had previously received up to date COVID-19 vaccines, including an original prime and boost regimen plus an additional booster of either Moderna or Pfizer COVID-19 mRNA vaccines prior contracting COVID-19 only 8-12 weeks before sample collection. Previous reports have demonstrated that patients with such hybrid immunity have superior neutralizing antibodies than patients who had been infected but not previously vaccinated.[48-52] These data show great promise for further investigation of the TLRa-SNPs adjuvant technology in larger animal models.

Additionally, in an NHP study, treatment groups receiving AS03-adjuvanted RBD-NP antigen vaccines had extended antibody titers and more durable Omicron variant protection when

compared to values previously reported for commercial Pfizer-BioNTech and Moderna mRNA vaccines.[25] Nonetheless, antibody titers waned to pre-boost magnitudes by 6 months post-boost, necessitating additional boosters to maintain a robust protection.[25] In contrast, we showed that mice receiving TLRa-SNPs adjuvanted RBD-NP vaccines maintained antibody endpoint titers for a full year following vaccination that were of similar magnitude to animals receiving vaccines adjuvanted with SNP or CpG/Alum at Week 11 following vaccination. These astonishing results were further emphasized by the rapid seroconversion of all mice after a single immunization (i.e., the prime immunization), thereby demonstrating the potential of single-immunization vaccines adjuvanted with these TLRa-SNPs. The rapid and complete seroconversion was further verified with HIV gp120 protein antigen, suggesting the superiority and potential broad application of TLRa-SNPs compared to SNP and clinically-relevant control adjuvants. Notably, we did not observe any substantial differences between the two clinical controls, CpG/Alum (mimicking Dynanvax's CpG1018) and SNP (mimicking Novavax's Matrix M). Although the adjuvant potencies remained similar for these two control adjuvants, the differences in their immune-stimulating mechanism might create synergistic effects when combined, illustrated by the potent, durable, broad, and neutralizing humoral responses induced by TLRa-SNPs, which take advantage of multiple immune-stimulating mechanisms. Overall, TLRa-SNPs adjuvants can rapidly lead to protective levels of neutralizing antibodies and prolong the durability of neutralizing responses, therefore strongly decreasing the need for costly booster shots, which is crucial for vaccines capable of rapidly and broadly protecting worldwide populations during a pandemic.

The unique acute cytokine induction profiles and Th-skewed responses observed in this study for each TLRa-SNP formulation are consistent with prior literature.[3,5,53] TLR1/2a-SNP, TLR4a-SNP,

and TLR7/8a-SNP generated robust IgG2c titers, thereby suggesting a strong Th1 response. Indeed, all three TLRas can induce Th1 response by activating the NF-κB pathway. Elevated levels of IL-12 and IL1β, hallmark cytokines of NF-κB activation, in the Luminex assay further verify the incorporation of these three TLRas and their impact on immune signaling.[3] Additionally, only TLR1/2a-SNP elicited higher IgG1 titer, indicating an overall Th2-skewed response. The activation of TLR1 and TLR2 triggers the ERK1 and ERK2 pathways, which could result in higher Th2 responses.[3] Similarly, the initiation of endosomal TLRs such as TLR7 and TLR8 activates the IRF-pathway, which in turn induces strong type I interferon response such as the secretion of IFN-α and IFN-β. The PSS plot clearly indicates a stronger expression of IFN-α (demonstrated by the vector) in the TLR7/8a-SNP cluster (cluster 4, Figure 5c). While the Luminex panel did not include IFN-β, we nonetheless observed other strong interferon responses, such as high IFN-γ, IP10, and IL28 production from mice adjuvanted with TLR7/8a-SNP. The overall balanced but slightly Th-1 skewed response is consistent with prior findings.[3,43,53] Likewise, the Th1-skewed response observed with TLR4a-SNP is consistent with the previously reported SMNP, for which only IgG2a titers, but not IgG1, were significantly higher than the SNP control.[14] Overall, the Luminex assay helped to further verify the success of TLRas incorporation to the SNP as well as the unique Th responses induced from the library of TLRa-containing saponin-based nanoparticles. In this regard, literature has shown that while Th2-skewed responses resulted in more favorable clinical outcomes for some infectious diseases such as Rabies virus,[54,55] Th1-skewed responses were favorable for diseases such as COVID-19.[56-59] Thus, having a library of TLRa-SNPs with tailored Th-skewed responses may have profound clinical implications for which a specific adjuvant formulation could be selected during the early stage of vaccine development depending on the disease the vaccine is designed to prevent.

Protein-based vaccines have been previously reported to not induce strong cellular immunity. RBD-NP, the primary antigen used in this study, was found to not generate a significant CD8+ T cell response.[26] We observed comparable results when assessing antigen-specific IFN-γ+CD8+ T cells by ELISpot following activation of splenocytes two weeks post-boost with pan-RBD peptides (Figure S11). While most clinical vaccines focus on inducing humoral responses,[60] further insights on TLRa-SNPs' ability to generate cellular-responses could make them promising candidates as T cell or cancer vaccines. In addition, other than generating different Th-skewed responses, different TLRas have also been found to induce differences in other immune cell populations, such as different levels of long-lived plasma cells or memory cells. Studying the activation and induction of innate cells and effector cells by different TLRa-SNPs could be of interest for developing a comprehensive understanding of the immune-stimulating nature of these adjuvant formulations.

Conclusion

Following our efforts to develop broadly applicable vaccine technologies, the newly designed toll-like receptor agonist containing saponin-nanoparticles (TLRa-SNPs) showed great promise in improving the potency, durability, breadth, and neutralization of vaccines with the potential of preventing infection by complex immune evasive viruses such as SARS-CoV-2 and HIV. Notably, the different adjuvant formulations induced unique acute cytokine and immune-signaling profiles, leading to different Th-responses. This robust and tunable adjuvant library reinforces the global effort of the Coalition for Epidemic Preparedness Innovations and the World Health Organization to develop and manufacture vaccines within 100 days in response to "Disease X," an infectious agent currently unknown to cause human disease.

Materials and Methods

Materials

Cholesterol (ovine wool, > 98%) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids. Quil-A adjuvant, Monophosphoryl Lipid A (from *S. minnesota R595*, MPLA-SM VacciGrade) and Pam3CSK4 (VacciGrade) were purchased from Invivogen. Cholesteryl imidazoquinoline derivative was synthetized in a one-step reaction from 4-amino-2-(ethoxy methyl)-1H-imidazo[4, 5-C]quinoline-1-butylamine (Career Henan Chemical Co., Henan Province, China; see supporting information). SARS-CoV-2 proteins were purchased from Sino Biological including SARS-CoV-2 RBD protein (40592-V08H), SARS-CoV-2 spike protein (40589-V08H4), Alpha B.1.1.7 spike (40591-V08H10), Beta B.1.351 spike (40591-V08H12), Delta B.1.617.2 spike (40591-V08H23), and Omicron B.1.1.529 spike (Sino Biological 40591-V08H41). HIV antigen GP120 was purchased from Immune Technology Corporation (IT-001-022p). CpG1826 (Vac-1826) and Alum (Alhydrogel 2%, vac-alu) were purchased from Invivogen. Maxisorp plates were purchased from Thermofisher. Goat anti-mouse IgG Fc secondary antibody (A16084) HRP (Horseradish peroxidase) was purchased from Invitrogen. Goat anti-mouse IgG1 and IgG2c Fc secondary antibodies (ab97250, ab97255) HRP and 3,3',5,5'-Tetramethylbenzidine (TMB) high sensitivity ELISA Substrate were purchased from Abcam. Single color mouse IFN-γ ELISPOT kit was purchased from Cellular Technology Limited. Antigen-specific cell stimulating peptides (Pan-SARS-CoV-2 S-RBD peptides) were purchased from JPT (PM-WCPV-S-RBD-1).

Formulation of SNPs and TLRa-SNPs

The formulation of lipid-based nanoparticles has been previously reported.[13-18] Briefly, cholesterol and DPPC (20 mg/mL) were each dissolved in a solution of 20% (w/v) of MEGA-10 detergent in Milli-Q water. Quil-A saponin (20 mg/mL) was dissolved in pure Milli-Q water. All solutions were heated at 50 °C until complete dissolution before use. When required, Cholesteryl imidazoquinoline, MPLA, and Pam3CSK4 (2 mg/mL) were each dissolved in a solution of 20% (w/v) of MEGA-10 detergent in Milli-Q water and heated at 70 °C under sonication until dissolution. All formulations were obtained after quickly mixing the appropriate volume of each compound at 60 °C without stirring in this order: Cholesterol/DPPC/Adjuvant/Quil-A saponin followed by its dilution with PBS 1X to reach a final concentration of 1 mg/mL in cholesterol (Table S1). Solutions were left for equilibration overnight at room temperature and dialyzed against PBS 1X (MWCO 10 kDa) for 5 days. Solutions were then filtered using 0.2 µm Acrodisc® Syringe Filters and concentrated using Centricon spin filters (MWCO 50 kDa, 3000 RCF, 40 mins) in stock solutions of 5 mg/ml in Quil-A Saponin.

Dynamic Light Scattering and Zeta Potential

The hydrodynamic diameter and surface charge of the NPs were respectively measured on a DynaPro II plate reader (Wyatt Technology) and a Zetasizer Nano Zs (Malvern Instruments). Three independent measurements were performed for each sample.

CryoEM and cryoET sample preparation and data collection

CryoEM and cryoET samples were prepared using Vitrobot Mark IV. First, 1.2/1.3 Quantifoil grids (Ted Pella inc., 658-200-CU-100) were glow discharged using PELCO easiGlow™ for 30 s.

For cryoET, 6 nm gold fiducial was mixed with the samples in a 1:10 volume ratio. Then, 2 mg/mL sample was applied to the glow discharged gird (Electron Microscopy Sciences, 25486), followed by removing the extra sample amount using filter paper and quickly plunging into liquid ethane. CryoEM and cryoET data were acquired by Titan Krios G3 electron microscope equipped with a K3 direct detector and energy filter with an accelerated voltage of 300 kV using SerialEM.[61] CryoEM micrographs were collected on a pixel size of 1.13A/pixel with and -2 μm defocus. CryoET data was collected from -60 °C to +60 °C with a 2 °C increment. The total electron dose was 80 e^-/A^2 with -3 μm defocus, and the pixel size was 1.13A. Reconstruction was done by IMOD[62] and visualized by chimera.[63]

In vitro RAW-Blue reporter assay

RAW-Blue (NF-κB-SEAP) reporter cell line (Invivogen, raw-sp) was used to prove the proper incorporation of TLRas with the SNPs and their potencies compared to soluble TLRas. RAW-blue cells were cultured at 37 °C with 5% CO_2 in DMEM supplemented with L-glutamine (2 mM), D-glucose (4.5 g/L), 10% HI-FBS, and penicillin (100 U/mL)/streptomycin (100 µg). Every other passage, zeocin (100 µg/mL) was added to the culture medium. Serial dilutions of solvent controls, soluble SNP components (cholesterol, DPPC, saponin), soluble TLRas (TLR1/2 Pam3CSK4, TLR4 MPLAs, and TLR7/8 cholesteryl imidazoquinoline derivative), SNP, and TLRa-SNPs were added to a 96-well tissue culture treated plate to achieve final concentrations between 40 and 0.01 µg/mL of TLRas (or equivalent concentration of individual component based on TLRa-SNPs added for the negative controls). About 100,000 cells were added to each well in 180 µL of medium and were incubated for 24 h at 37 °C in a 5% CO_2 incubator. Manufacturer instructions were followed for SEAP quantification, and absorbance levels were detected at 655 nm after 2 h

incubation with QUANTI-Blue Solution (Invivogen). Nonlinear regression fits were estimated using the "Log(agonist) vs. response – EC_{50}" function in GraphPad Prism 8.4 software.

Preparation of RBD-NPs

The nanoparticle immunogen (RBD-NP) components and the nanoparticle formulation methodology have been previously described.[28] RBD-NP was in the following buffer conditions: 50 mM Tris pH 8, 150 mM NaCl, 100 mM l-arginine and 5% sucrose.

Vaccine Formulation

The model vaccines included an antigen of either 1.5 μg dose of RBD-NP (University of Washington) or 10 μg dose of gp120 (SIV/mac239, Immune Technology Corporation). Depending on the formulations, vaccines also contain an adjuvant of CpG1826/Alum (20 μg + 100 μg, respectfully, Invivogen), SNP, or one of the four TLRa-SNPs with an equivalent dose of 10 μg of Quil-A saponins (TLR1/2a-SNP, TLR4a-SNP, or TLR7/8a-SNP). An additional vaccine was formulated with an equivalent dose of 5 μg saponins of TLR4a-SNP and 5 μg saponins of TLR7/8a-SNP to test their synergistic effects. CpG/Alum group adjuvanted with RBD-NP was prepared similarly as described previously by Pulendran and coworkers as the clinical control,[26,27] although both the CpG and Alum dosages were doubled in the present study. Vaccines were prepared in PBS 1X to a volume of 100 μl per dose and loaded into syringes with a 26-gauge needle for subcutaneous injection. Mice were boosted on Week 3 for RBD-NP vaccines or Week 4 for GP120 vaccines.

Mice and Vaccination

Six-to-seven week old female C57BL/6 (B6) mice were purchased from Charles River and housed in the animal facility at Stanford University. Several days before vaccine administration, mice were shaved on their right flank. On the day of injection, 100 µL of soluble vaccines were subcutaneously injected on the right side of their flank under brief isoflurane anesthesia. Mouse blood was collected from the tail vein following the schematic shown in Figure 3. The inguinal dLNs were collected for GC analysis as well as Luminex after euthanasia and spleens were collected for splenocyte ELISpot analysis after euthanasia.

Mouse serum ELISAs and antibody concentration

Serum Anti-RBD or Anti-GP120 IgG antibody endpoint titers were measured using an endpoint ELISA. Maxisorp plates were coated with SARS-CoV-2 RBD protein, spike protein, a spike protein variant (B.1.1.7, B.1.351, B.1.617.2, or B.1.529), or GP120 at 2 µg/mL in PBS overnight at 4 °C and subsequently blocked with PBS 1X containing 1 wt% BSA for 1 h at 25 °C. Plates were washed 5x in between each step with PBS 1X containing 0.05% of Tween-20. Serum samples were diluted in diluent buffer (PBS 1X containing 1% BSA) starting at 1:100 followed by a 4-fold serial dilution and incubated in the coated plates for 2 h at 25 °C. Thεν, goat-anti-mouse IgG Fc-HRP (1:10,000), IgG1 Fc-HRP (1:10,000), or IgG2c Fc-HRP (1:10,000) was incubated for 1h at 25 °C. Plates were developed with TMB substrate for 6 mins and the reaction was stopped with 1 N HCl aqueous solution. Plates were analyzed using a Synergy H1 microplate reader (BioTek Instruments) at 450 nm in absorbance. Total IgG, subtypes, and variants were imported into GraphPad Prism 8.4.1 to determine serum titers by fitting the curves (absorbance vs dilution) with a three-parameter non-linear logistic regression (baseline constrained to 0.054, the negative control

average). The dilution titer value at which the endpoint threshold (0.2 and 0.1 for RBD-NP and GP120 vaccines, respectively) was crossed for each curve was reported as endpoint titer. Samples failing to meet endpoint threshold at a 1:100 dilution were set to below the limit quantitation for the assay (which we plotted as 1:25 dilution).

SARS-CoV-2 Spike-pseudotyped Viral Neutralization Assay

Neutralization assays were conducted as described previously.[64] Briefly, SARS-CoV-2 spike-pseudotyped lentivirus was produced in HEK239T cells. Six million cells were seeded one day prior to transfection. A five plasmid system[65] and BioT (Bioland Scientific) were used for viral production per manufacturer's protocol with the full-length wild-type spike sequence from the Wuhan-Hu-1 strain of SARS-CoV-2. Virus-containing culture supernatants were harvested ≈72 hours after transfection by centrifugation and filtered through a 0.45 µm syringe filter. Stocks were stored at −80 °C prior to use. For the neutralization assay, ACE2/HeLa cells were plated 1–2 days prior to infection.[66] Mouse and human sera were heat inactivated at 56°C for 30 min prior to use. Sera and viruses were diluted in cell culture medium and supplemented with a polybrene at a final concentration of 5µg/mL. Serum/virus dilutions were incubated at 37°C for 1 h. After incubation, media was removed from cells and replaced with serum/virus dilutions and incubated at 37 °C for 2 days. Cells were then lysed using BriteLite (Perkin Elmer) luciferase readout reagent, and luminescence was measured with a BioTek plate reader. Each plate was normalized by wells with cells or virus only and curves were fit with a three-parameter non-linear logistic regression inhibitor curve to obtain IC50 values. Serum dilution curves display mean infectivity ± SEM for each individual mouse at each serum dilution. Normalized values were fit with a three-parameter

non-linear logistic regression inhibitor curve in GraphPad Prism 8.4.1 to obtain IC50 values. Fits were constrained to have a value of 0% at the bottom of the fit.

Luminex Cytokine Assay

Mice were immunized with RBD-NP and different TLRa-SNPs as described in the immunization section. 24 hours after injection, mice were euthanized and the injection draining inguinal lymph nodes were excised and processed for analysis.[67] Briefly, the dLNs were weighted and 10 mL of lysis buffer (Cell Extraction Buffer FNN0011 with 1x Halt protease inhibitor, Themo Fisher) was added per 1 g of tissue. Tissues were then grinded using a Dounce Tissue Grinder and centrifuged at 10,000 RCF for 5 min using protein lo-bind Eppendorf tubes to pellet tissue debris. Supernatants were collected into new protein lo-bind Eppendorf tubes. Meanwhile, a Pierce BCA assay (Thermo Fisher) was set up to analyze the total protein content of the samples. When possible, samples were diluted and normalized to 2 mg/mL of total sample protein content per sample using additional lysis buffer. Tissues/homogenates were kept on ice throughout processing and normalized samples were stored at -80 °C. Injection dLN homogenates were provided to the HIMC for analysis by a 48-plex murine Luminex cytokine panel.

Dimensional Reduction Analysis via Penalized Supervised Star Plot

Cytokine MFI data were detrended for cage and nonspecific binding effects using ordinary least squares.[68] Detrended data were used to construct a penalized supervised star plot.[69] Analyses were conducted in SAS® v.9.4 (SAS® Institute, Cary, North Carolina, USA) and R (www.r-project.org). Star plots were constructed with R packages maptools,[70] matrixcalc,[71] plotrix,[72] JPEN,[73] and VCA.[74]

Immunophenotyping in Lymph Nodes by Flow Cytometry

According to previously reported methodology,[75] mice were euthanized using CO_2 12 days post immunization. Inguinal lymph nodes were collected and dissociated into single cell suspensions. Cells were stained for viability using Ghost Dye Violet 510 (Tonbo Biosciences, Cat: 13-0870-T100) for 5 min on ice and washed with FACS buffer (PBS with 2% FBS, 1mM EDTA). Fc receptor was blocked using anti-CD16/CD38 (clone: 2.4G2, BD Biosciences, cat: 553142) for 5 min on ice and then stained with fluorochrome conjugated antibodies: CD19 (PerCP-Cy5.5, clone: 1D3, BioLegend, cat: 152406) CD95 (PE-Cy7, clone: Jo2, BD Biosciences, cat: 557653), CD38 (BUV395, clone: 90, BD Biosciences, Cat: 740245), CXCR4 (BV421, clone: L276F12 , BioLegend, cat: 146511), CD86 (BV785, clone: GL1 , BioLegend, cat: 105043), GL7 (AF488, clone: GL7, BioLegend, cat: 144613), CD3 (AF700, clone: 17A2, BioLegend, cat: 100216), CD4 (BV650, clone: GK1.5, BioLegend, cat: 100469), CXCR5 (BV711, clone: L138D7, BioLegend, cat: 145529) and PD1 (PE-Dazzle™594, clone: 29F.1A12, BioLegend, cat: 135228) for 30 mins on ice (Table S2). Cells were washed, fixed with 4% PFA on ice, washed again and analyzed on an LSRII flow cytometer (BD Biosciences). Data were analyzed with FlowJ 10 (FlowJo, LLC, representative gating scheme in Figure S9).

ELISpot

The number of antigen-specific IFN-γ producing splenocytes were determined to correlate with antigen-specific IFN-γ producing CD8+ T cells using a Mouse IFN-γ Single-Color ELISPOT kit (CTL ImmunoSpot). Spleen cells were harvested 2 weeks post-boost (D35) following the immunization outlined in Figure S11a. 800,000 splenocytes per sample were pipetted into the well

and were stimulated with 1 μg/mL Pan-SARS-CoV-2-RBD peptides (JPT) for 24 hours at 37°C. Spots were then developed following manufacturer's instruction (Immunospot S6 Ultra M2).

Animal Protocol

Mice were cared for following the Institutional Animal Care and Use guidelines. All animal studies were performed in accordance with the National Institutes of Health guidelines and the approval of Stanford Administrative Panel on Laboratory Animal Care (Protocol APLAC-32109).

Collection of Serum from Human Patients

Convalescent COVID-19 blood was collected from donors 8–12 weeks after the onset of symptoms, all donors had previously been immunized with COVID vaccines authorized by the FDA (total of three immunizations, excluding the omicron bi-valent booster). Blood was collected in microtubes with serum gel for clotting (Starstedt), centrifuged for 5 min at 10,000 g and then serum was stored at −80 °C until used. Blood collection was performed by finger-prick in accordance with National Institutes of Health guidelines, the approval of the Stanford Human Subjects Research and IRB Compliance Office (IRB-58511) and the consent of the individuals.

Statistical Analysis

For *in vivo* experiments, animals were caged blocked. Mead's Resource Equation was used to calculate the sample size above which additional subjects (n) will not have significant impact on power.[31,32] For most experiments, a sample size of $n = 5$ per group was used. A few mice died due to reasons external to the treatment effects as evaluated by the veterinarian staff. Groups resulting

with $n = 4$ still had enough power for statistical analyses using Mead's Resource Equation. No animals were considered outliers.

All results except for Luminex are expressed as mean ± standard error of mean (SEM). Unless otherwise specified, comparison between multiple groups and p values were conducted with the general linear model (GLM) followed by Tukey HSD procedure test in JMP and we accounted and adjusted for unequal variances, cage blocking, and multiple comparison. Statistical significance was considered as $p < 0.05$. Selected p values are shown in the text and the figures and remaining p values are in the supporting information.

For the Luminex assay, statistical analyses were provided by the Statistical Consultation Service of the Human Immune Monitoring Center at the Institute for Immunity, Transplantation and Infection, Stanford University School of Medicine. The logarithm of median fluorescence intensity (MFI) was regressed on treatment group, cage, and logarithm of nonspecific binding MFI using generalized maximum entropy estimation regression.[76] Within each treatment group, p values were adjusted to control the false discovery rate[77,78] at 5% across all cytokines. Analyses and plotting were done in SAS® (SAS® Institute, Cary, North Carolina, USA).

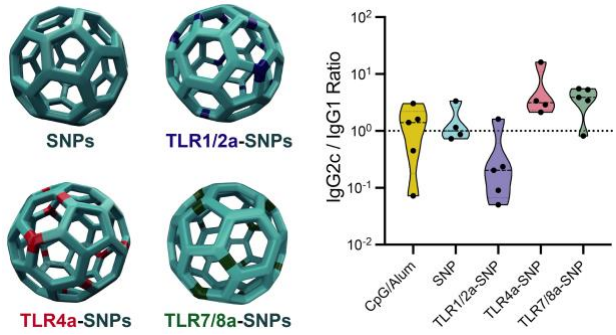
Data Availability

The data supporting the findings of this study are available upon reasonable request from the corresponding author.

Safety Statement

No unexpected or unusually high safety hazards were encountered.

TABLE OF CONTENTS (TOC) GRAPHIC



SYNOPSIS

Saponin nanoparticles adjuvants containing Toll-like receptor agonists improved the potency, durability, breadth, and neutralization of both COVID-19 and HIV vaccines with unique Th1/Th2 responses.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Additional tables and figures of synthesis, purification, and characterization of SNP NPs, and additional immune response data and figures. (PDF)

AUTHOR INFORMATION

Corresponding Author

Eric A. Appel

Department of Materials Science & Engineering, Stanford University, Stanford CA 94305, USA

E: eappel@stanford.edu

Author Contributions

BSO, JB, and EAA designed the broad concepts and research studies. MVFI, JZA, and XZ designed specific experiments. BSO, JB, MVFI, JZA, XZ, OMS, JY, ELM, JHK, CJ, AV, and LC performed research and experiments. BSO, JB, and EAA wrote the paper. OMS, JY, ELM, JHK, CJ, and NPK edited the paper. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Conflict of Interest Statement

E.A.A, B.S.O, and J.B. are listed as inventors on a pending patent application. N.P.K. is a cofounder, shareholder, paid consultant, and chair of the scientific advisory board of Icosavax, Inc. The King lab has received unrelated sponsored research agreements from Pfizer and GSK. All other authors declare no conflicts of interest.

ACKNOWLEDGMENT

This work was financially supported by the Center for Human Systems Immunology with the Bill & Melinda Gates Foundation (OPP1113682; OPP1211043; INV027411; INV-010680) and the National Institute of Allergy and Infectious Disease (P01AI167966). B.S.O. is grateful for an Eastman Kodak Fellowship. M.V.F.I. is thankful for the support by the National Institutes of Health under award numbers T32GM007365 (MVFI), F30AI152943 (MVFI). X.Z. is supported by a Stanford ChEM-H COVID-19 Drug and Vaccine Prototyping seed grant. O.M.S., J.Y., and C.J. are thankful for a National Science Foundation Graduate Research Fellowship. O.M.S. is also thankful for Hancock Fellowship of the Stanford Graduate Fellowship in Science and Engineering.

E.L.M. is grateful for the NIH Biotechnology Training Program (T32 GM008412). The authors would like to thank former and current members of the Appel lab for their technical expertise and scientific discussion, especially Dr. Celine S. Liong, Dr. Catherine M. Kasse and Anahita Nejatfard for their help in animal work and advice. We acknowledge Dr. Wah Chiu and Dr. Peter Kim for their assistance and advice. We extend our gratitude to Dr. Tyson H. Holmes and Dr. Yael Rosenberg-Hasson at the Stanford Human Immune Monitoring Center as well as Duo Xu for his invaluable advice in implementing the spike-pseudotyped lentiviral neutralization assay (to MVFI). We also would like to thank the human patients for donating their blood, the staff of the BioE/ChemE Animal Facility who cared for the mice and the Stanford Core Facilities that were central to the completion of this work. Specifically, we thank the Vincent Coates Foundation Mass Spectrometry Laboratory (Stanford University Mass Spectrometry - RRID:SCR_017801), and the Department of Chemistry Nuclear Magnetic Resonance facility (Stanford University, NIH High End Instrumentation grant 1 S10 OD028697-01). Cryo-Electron Microscopy was performed at the Stanford-SLAC facility (NIH shared instrumentation grant S10OD021600). FACS sorting was performed on an instrument in the Shared FACS Facility obtained using NIH S10 Shared Instrument Grant (S10RR027431-01).

REFERENCES

(1) Mascola, J. R.; Fauci, A. S. Novel vaccine technologies for the 21st century. *Nature Reviews Immunology* **2020,** *20* (2), 87.

(2) Moyle, P. M.; Toth, I. Modern Subunit Vaccines: Development, Components, and Research Opportunities. *ChemMedChem* **2013,** *8* (3), 360.

(3) Pulendran, B.; S. Arunachalam, P.; O'Hagan, D. T. Emerging concepts in the science of vaccine adjuvants. *Nature Reviews Drug Discovery* **2021**, DOI:10.1038/s41573-021-00163-y 10.1038/s41573-021-00163-y.

(4) Maisonneuve, C.; Bertholet, S.; Philpott, D. J.; De Gregorio, E. Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants. *Proceedings of the National Academy of Sciences* **2014,** *111* (34), 12294.

(5) Duthie, M. S.; Windish, H. P.; Fox, C. B.; Reed, S. G. Use of defined TLR ligands as adjuvants within human vaccines. *Immunological Reviews* **2011,** *239* (1), 178.

(6) Ledesma, F.; Ozcan, B.; Sun, X.; Medina, S. M.; Landry, M. P. Nanomaterial Strategies for Delivery of Therapeutic Cargoes. *Advanced Functional Materials* **2022,** *32* (4), 2107174.

(7) Wu, S.; Xia, Y.; Hu, Y.; Ma, G. Bio-mimic particles for the enhanced vaccinations: Lessons learnt from the natural traits and pathogenic invasion. *Advanced Drug Delivery Reviews* **2021,** *176*, 113871.

(8) Ou, B. S.; Picece, V. C. T. M.; Baillet, J.; Gale, E. C.; Powell, A. E.; Saouaf, O. M.; Yan, J.; Lopez Hernandez, H.; Appel, E.; Cold Spring Harbor Laboratory, 2023, DOI:10.1101/2023.01.02.522505 10.1101/2023.01.02.522505.

(9) Ou, B. S.; Saouaf, O. M.; Baillet, J.; Appel, E. A. Sustained delivery approaches to improving adaptive immune responses. *Advanced Drug Delivery Reviews* **2022,** *187*, 114401.

(10) Sun, B.; Xia, T. Nanomaterial-based vaccine adjuvants. *Journal of Materials Chemistry B* **2016,** *4* (33), 5496.

(11) Irvine, D. J.; Aung, A.; Silva, M. Controlling timing and location in vaccines. *Advanced Drug Delivery Reviews* **2020,** *158*, 91.

(12) Irvine, D. J.; Hanson, M. C.; Rakhra, K.; Tokatlian, T. Synthetic Nanoparticles for Vaccines and Immunotherapy. *Chem Rev* **2015,** *115* (19), 11109.

(13) Morein, B.; Sundquist, B.; Höglund, S.; Dalsgaard, K.; Osterhaus, A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature* **1984,** *308* (5958), 457.

(14) Silva, M.; Kato, Y.; Melo, M. B.; Phung, I.; Freeman, B. L.; Li, Z.; Roh, K.; Van Wijnbergen, J. W.; Watkins, H.; Enemuo, C. A.et al. A particulate saponin/TLR agonist vaccine adjuvant alters lymph flow and modulates adaptive immunity. *Sci Immunol* **2021,** *6* (66), eabf1152.

(15) Lövgren-Bengtsson, K.; Morein, B. *Vaccine Adjuvants: Preparation Methods and Research Protocols*; Humana Press: Humana Totowa, NJ, 2000.

(16) Pedersen, S., Jan; Oliveira, L. P., Cristiano; Hübschmann, B., Henriette; Arleth, L.; Manniche, S.; Kirkby, N.; Nielsen, M., Hanne. Structure of Immune Stimulating Complex Matrices and Immune Stimulating Complexes in Suspension Determined by Small-Angle X-Ray Scattering. *Biophysical Journal* **2012,** *102* (10), 2372.

(17) Sanders, M. T.; Brown, L. E.; Deliyannis, G.; Pearse, M. J. ISCOM-based vaccines: The second decade. *Immunology & Cell Biology* **2005,** *83* (2), 119.

(18) Pearse, M. J.; Drane, D. ISCOMATRIX adjuvant for antigen delivery. *Adv Drug Deliv Rev* **2005,** *57* (3), 465.

(19) Maraskovsky, E.; Schnurr, M.; Wilson, N. S.; Robson, N. C.; Boyle, J.; Drane, D. Development of prophylactic and therapeutic vaccines using the ISCOMATRIX adjuvant. *Immunology & Cell Biology* **2009,** *87* (5), 371.

(20) Van Hoeven, N.; Wiley, S.; Gage, E.; Fiore-Gartland, A.; Granger, B.; Gray, S.; Fox, C.; Clements, D. E.; Parks, D. E.; Winram, S.et al. A combination of TLR-4 agonist and saponin adjuvants increases antibody diversity and protective efficacy of a recombinant West Nile Virus antigen. *npj Vaccines* **2018,** *3* (1).

(21) Didierlaurent, A. M.; Laupèze, B.; Di Pasquale, A.; Hergli, N.; Collignon, C.; Garçon, N. Adjuvant system AS01: helping to overcome the challenges of modern vaccines. *Expert Review of Vaccines* **2017,** *16* (1), 55.

(22) Silva, A.; Mount, A.; Krstevska, K.; Pejoski, D.; Hardy, M. P.; Owczarek, C.; Scotney, P.; Maraskovsky, E.; Baz Morelli, A. The combination of ISCOMATRIX adjuvant and TLR agonists induces regression of established solid tumors in vivo. *J Immunol* **2015,** *194* (5), 2199.

(23) Martinez, P.; Sundling, C.; O'Dell, S.; Mascola, J. R.; Wyatt, R. T.; Karlsson Hedestam, G. B. Primate immune responses to HIV-1 Env formulated in the saponin-based adjuvant AbISCO-100 in the presence or absence of TLR9 co-stimulation. *Scientific Reports* **2015,** *5* (1), 8925.

(24) Raaijmakers, T. K.; Van Den Bijgaart, R. J. E.; Den Brok, M. H.; Wassink, M.; De Graaf, A.; Wagenaars, J. A.; Nierkens, S.; Ansems, M.; Scheffer, G. J.; Adema, G. J. Tumor ablation plus co-administration of CpG and saponin adjuvants affects IL-1 production and multifunctional T cell numbers in tumor draining lymph nodes. *Journal for ImmunoTherapy of Cancer* **2020,** *8* (1), e000649.

(25) Arunachalam, P. S.; Feng, Y.; Ashraf, U.; Hu, M.; Walls, A. C.; Edara, V. V.; Zarnitsyna, V. I.; Aye, P. P.; Golden, N.; Miranda, M. C.et al. Durable protection against the SARS-CoV-2 Omicron variant is induced by an adjuvanted subunit vaccine. *Science Translational Medicine* **2022,** *14* (658), eabq4130.

(26) Arunachalam, P. S.; Walls, A. C.; Golden, N.; Atyeo, C.; Fischinger, S.; Li, C.; Aye, P.; Navarro, M. J.; Lai, L.; Edara, V. V.et al. Adjuvanting a subunit COVID-19 vaccine to induce protective immunity. *Nature* **2021,** *594* (7862), 253.

(27) Grigoryan, L.; Lee, A.; Walls, A. C.; Lai, L.; Franco, B.; Arunachalam, P. S.; Feng, Y.; Luo, W.; Vanderheiden, A.; Floyd, K.et al. Adjuvanting a subunit SARS-CoV-2 vaccine with clinically relevant adjuvants induces durable protection in mice. *npj Vaccines* **2022,** *7* (1).

(28) Walls, A. C.; Fiala, B.; Schäfer, A.; Wrenn, S.; Pham, M. N.; Murphy, M.; Tse, L. V.; Shehata, L.; O'Connor, M. A.; Chen, C.et al. Elicitation of Potent Neutralizing Antibody Responses by Designed Protein Nanoparticle Vaccines for SARS-CoV-2. *Cell* **2020,** *183* (5), 1367.

(29) Ou, B. S.; Saouaf, O. M.; Yan, J.; Bruun, T. U. J.; Baillet, J.; Zhou, X.; King, N. P.; Appel, E. A. Broad and Durable Humoral Responses Following Single Hydrogel Immunization of SARS-CoV-2 Subunit Vaccine. *Advanced Healthcare Materials* **2023,** *n/a* (n/a), 2301495.

(30) SK bioscience's COVID-19 vaccine approved for use by Republic of Korea. **2022**.

(31) Charan, J.; Kantharia, N. D. How to calculate sample size in animal studies? *J Pharmacol Pharmacother* **2013,** *4* (4), 303.

(32) Arifin, W. N.; Zahiruddin, W. M. Sample Size Calculation in Animal Studies Using Resource Equation Approach. *Malays J Med Sci* **2017,** *24* (5), 101.

(33) Hinton, D. Convalescent plasma EUA letter of authorization. **2021**.

(34) Fornefett, J.; Krause, J.; Klose, K.; Fingas, F.; Hassert, R.; Benga, L.; Grunwald, T.; Muller, U.; Schrodl, W.; Baums, C. G. Comparative analysis of humoral immune responses and pathologies of BALB/c and C57BL/6 wildtype mice experimentally infected with a highly virulent Rodentibacter pneumotropicus (Pasteurella pneumotropica) strain. *BMC Microbiol* **2018,** *18* (1), 45.

(35) Coffman, R. L.; Sher, A.; Seder, R. A. Vaccine adjuvants: putting innate immunity to work. *Immunity* **2010,** *33* (4), 492.

(36) Rosenberg-Hasson, Y.; Hansmann, L.; Liedtke, M.; Herschmann, I.; Maecker, H. T. Effects of serum and plasma matrices on multiplex immunoassays. *Immunologic Research* **2014,** *58* (2-3), 224.

(37) Breen, E. J.; Tan, W.; Khan, A. The Statistical Value of Raw Fluorescence Signal in Luminex xMAP Based Multiplex Immunoassays. *Scientific Reports* **2016,** *6* (1), 26996.

(38) Phillips, D. J.; League, S. C.; Weinstein, P.; Hooper, W. C. Interference in microsphere flow cytometric multiplexed immunoassays for human cytokine estimation. *Cytokine* **2006,** *36* (3-4), 180.

(39) Maecker, H. T.; Rosenberg-Hasson, Y.; Kolstad, K. D.; Steen, V. D.; Chung, L. S. A Novel Utility to Correct for Plate/Batch/Lot and Nonspecific Binding Artifacts in Luminex Data. *J Immunol* **2020,** *204* (12), 3425.

(40) Mosmann, T. R.; Sad, S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* **1996,** *17* (3), 138.

(41) Kasturi, S. P.; Skountzou, I.; Albrecht, R. A.; Koutsonanos, D.; Hua, T.; Nakaya, H. I.; Ravindran, R.; Stewart, S.; Alam, M.; Kwissa, M.et al. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* **2011,** *470* (7335), 543.

(42) Liu, H.; Moynihan, K. D.; Zheng, Y.; Szeto, G. L.; Li, A. V.; Huang, B.; Van Egeren, D. S.; Park, C.; Irvine, D. J. Structure-based programming of lymph-node targeting in molecular vaccines. *Nature* **2014,** *507* (7493), 519.

(43) Smirnov, D.; Schmidt, J. J.; Capecchi, J. T.; Wightman, P. D. Vaccine adjuvant activity of 3M-052: an imidazoquinoline designed for local activity without systemic cytokine induction. *Vaccine* **2011,** *29* (33), 5434.

(44) Wu, T. Y.; Singh, M.; Miller, A. T.; De Gregorio, E.; Doro, F.; D'Oro, U.; Skibinski, D. A.; Mbow, M. L.; Bufali, S.; Herman, A. E.et al. Rational design of small molecules as vaccine adjuvants. *Sci Transl Med* **2014,** *6* (263), 263ra160.

(45) Ou, B. S.; Saouaf, O. M.; Yan, J.; Bruun, T. U. J.; Baillet, J.; King, N. P.; Appel, E. A.; Cold Spring Harbor Laboratory, 2022, DOI:10.1101/2022.12.12.520166 10.1101/2022.12.12.520166.

(46) Gale, E. C.; Powell, A. E.; Roth, G. A.; Meany, E. L.; Yan, J.; Ou, B. S.; Grosskopf, A. K.; Adamska, J.; Picece, V. C. T. M.; D'Aquino, A. I.et al. Hydrogel-Based Slow Release of a Receptor-Binding Domain Subunit Vaccine Elicits Neutralizing Antibody Responses Against SARS-CoV-2. *Advanced Materials* **2021,** *33* (51), 2104362.

(47) Lynn, G. M.; Laga, R.; Darrah, P. A.; Ishizuka, A. S.; Balaci, A. J.; Dulcey, A. E.; Pechar, M.; Pola, R.; Gerner, M. Y.; Yamamoto, A.et al. In vivo characterization of the physicochemical properties of polymer-linked TLR agonists that enhance vaccine immunogenicity. *Nat Biotechnol* **2015,** *33* (11), 1201.

(48) Oh, S. J.; O, S. W.; Choi, Y. J.; Kim, J. M.; Kim, D.; Kim, I. H.; Park, A. K.; Kim, H. M.; Rhee, J. E.; Jang, Y. R.et al. Neutralizing antibody responses in vaccinated and unvaccinated individuals infected with Omicron BA.1 variant. *J Clin Virol* **2022,** *155*, 105253.

(49) Bates, T. A.; McBride, S. K.; Leier, H. C.; Guzman, G.; Lyski, Z. L.; Schoen, D.; Winders, B.; Lee, J.-Y.; Lee, D. X.; Messer, W. B.et al. Vaccination before or after SARS-CoV-2 infection leads to robust humoral response and antibodies that effectively neutralize variants. *Science Immunology* **2022,** *7* (68), eabn8014.

(50) Yu, Y.; Esposito, D.; Kang, Z.; Lu, J.; Remaley, A. T.; De Giorgi, V.; Chen, L. N.; West, K.; Cao, L. mRNA vaccine-induced antibodies more effective than natural immunity in neutralizing SARS-CoV-2 and its high affinity variants. *Scientific Reports* **2022,** *12* (1).

(51) Jeong, H. W.; Kim, S.-M.; Jung, M. K.; Noh, J. Y.; Yoo, J.-S.; Kim, E.-H.; Kim, Y.-I.; Yu, K.; Jang, S.-G.; Gil, J.et al. Enhanced antibody responses in fully vaccinated individuals against pan-SARS-CoV-2 variants following Omicron breakthrough infection. *Cell Reports Medicine* **2022,** *3* (10), 100764.

(52) Lechmere, T.; Snell, L. B.; Graham, C.; Seow, J.; Shalim, Z. A.; Charalampous, T.; Alcolea-Medina, A.; Batra, R.; Nebbia, G.; Edgeworth, J. D.et al. Broad Neutralization of SARS-CoV-2 Variants, Including Omicron, following Breakthrough Infection with Delta in COVID-19-Vaccinated Individuals. *mBio* **2022,** *13* (2), e0379821.

(53) Kwissa, M.; Nakaya, H. I.; Oluoch, H.; Pulendran, B. Distinct TLR adjuvants differentially stimulate systemic and local innate immune responses in nonhuman primates. *Blood* **2012,** *119* (9), 2044.

(54) Moore, S. M.; Wilkerson, M. J.; Davis, R. D.; Wyatt, C. R.; Briggs, D. J. Detection of cellular immunity to rabies antigens in human vaccinees. *J Clin Immunol* **2006,** *26* (6), 533.

(55) Garenne, M.; Lafon, M. Sexist diseases. *Perspect Biol Med* **1998,** *41* (2), 176.

(56) Gil-Etayo, F. J.; Garcinuno, S.; Utrero-Rico, A.; Cabrera-Marante, O.; Arroyo-Sanchez, D.; Mancebo, E.; Pleguezuelo, D. E.; Rodriguez-Frias, E.; Allende, L. M.; Morales-Perez, P.et al. An Early Th1 Response Is a Key Factor for a Favorable COVID-19 Evolution. *Biomedicines* **2022,** *10* (2).

(57) Gil-Etayo, F. J.; Suarez-Fernandez, P.; Cabrera-Marante, O.; Arroyo, D.; Garcinuno, S.; Naranjo, L.; Pleguezuelo, D. E.; Allende, L. M.; Mancebo, E.; Lalueza, A.et al. T-Helper Cell Subset Response Is a Determining Factor in COVID-19 Progression. *Front Cell Infect Microbiol* **2021,** *11*, 624483.

(58) Howard, F. H. N.; Kwan, A.; Winder, N.; Mughal, A.; Collado-Rojas, C.; Muthana, M. Understanding Immune Responses to Viruses—Do Underlying Th1/Th2 Cell Biases Predict Outcome? *Viruses* **2022,** *14* (7), 1493.

(59) Infante-Duarte, C.; Kamradt, T. Th1/Th2 balance in infection. *Springer Seminars in Immunopathology* **1999,** *21* (3), 317.

(60) Plotkin, S. A. Correlates of protection induced by vaccination. *Clin Vaccine Immunol* **2010,** *17* (7), 1055.

(61) Mastronarde, D. N. Automated electron microscope tomography using robust prediction of specimen movements. *Journal of Structural Biology* **2005,** *152* (1), 36.

(62) Kremer, J. R.; Mastronarde, D. N.; McIntosh, J. R. Computer Visualization of Three-Dimensional Image Data Using IMOD. *Journal of Structural Biology* **1996,** *116* (1), 71.

(63) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **2004,** *25* (13), 1605.

(64) Powell, A. E.; Zhang, K.; Sanyal, M.; Tang, S.; Weidenbacher, P. A.; Li, S.; Pham, T. D.; Pak, J. E.; Chiu, W.; Kim, P. S. A Single Immunization with Spike-Functionalized Ferritin Vaccines Elicits Neutralizing Antibody Responses against SARS-CoV-2 in Mice. *ACS Central Science* **2021,** *7* (1), 183.

(65) Crawford, K. H. D.; Eguia, R.; Dingens, A. S.; Loes, A. N.; Malone, K. D.; Wolf, C. R.; Chu, H. Y.; Tortorici, M. A.; Veesler, D.; Murphy, M.et al. Protocol and Reagents for Pseudotyping Lentiviral Particles with SARS-CoV-2 Spike Protein for Neutralization Assays. *Viruses* **2020,** *12* (5), 513.

(66) Rogers, T. F.; Zhao, F.; Huang, D.; Beutler, N.; Burns, A.; He, W. T.; Limbo, O.; Smith, C.; Song, G.; Woehl, J.et al. Isolation of potent SARS-CoV-2 neutralizing antibodies and protection from disease in a small animal model. *Science* **2020,** *369* (6506), 956.

(67) Correa, S.; Meany, E. L.; Gale, E. C.; Klich, J. H.; Saouaf, O. M.; Mayer, A. T.; Xiao, Z.; Liong, C. S.; Brown, R. A.; Maikawa, C. L.et al. Injectable Nanoparticle-Based Hydrogels Enable the Safe and Effective Deployment of Immunostimulatory CD40 Agonist Antibodies. *Advanced Science* **2022**, DOI:10.1002/advs.202103677 10.1002/advs.202103677, 2103677.

(68) Blais, J. A. R. Least Squares for Practitioners. *Mathematical Problems in Engineering* **2010,** *2010*, 1.

(69) Holmes, T. H.; Subrahmanyam, P. B.; Wang, W.; Maecker, H. T. Penalized Supervised Star Plots: Example Application in Influenza-Specific CD4+ T Cells. *Viral Immunol* **2019,** *32* (2), 102.

(70) Bivand, R.; Lewin-Koh, N. maptools: Tools for handling spatial objects. R package version 1.1-4. . **2022**. https://CRAN.R-project.org/package=maptools

(71) Novomestky, F. matrixcalc: Collection of functions for matrix calculations. R package version 1.0-5. **2021**. https://CRAN.R-project.org/package=matrixcalc

(72) Lemon, J. Plotrix: a package in the red light district of R. *R-News* **2006,** *6* (4), 8.

(73) Maurya, A. JPEN: Covariance and inverse covariance matrix estimation using joint penalty. R package version 1.0. **2015**. https://CRAN.R-project.org/package=JPEN

(74) Schuetzenmeister, A.; Dufey, F. VCA: Variance component analysis. R package version 1.4.4. **2022**. https://CRAN.R-project.org/package=VCA

(75) Roth, G. A.; Gale, E. C.; Alcántara-Hernández, M.; Luo, W.; Axpe, E.; Verma, R.; Yin, Q.; Yu, A. C.; Lopez Hernandez, H.; Maikawa, C. L.et al. Injectable Hydrogels for Sustained Codelivery of Subunit Vaccines Enhance Humoral Immunity. *ACS Central Science* **2020,** *6* (10), 1800.

(76) Golan, A.; Judge, G.; Miller, D. J. *Maximum entropy econometrics: robust estimation with limited data*, 1996.

(77) Benjamini, Y.; Krieger, A. M.; Yekutieli, D. Adaptive linear step-up procedures that control the false discovery rate. *Biometrika* **2006,** *93* (3), 491.

(78) Kim, K. I.; Van De Wiel, M. A. Effects of dependence in high-dimensional multiple testing problems. *BMC Bioinformatics* **2008,** *9* (1), 114.