Title

Enhancing antibody responses by multivalent antigen display on thymus-independent DNA origami scaffolds

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Abstract

Multivalent antigen display is a well-established design principle to enhance humoral immunity elicited by subunit vaccines. Protein-based virus-like particles (VLPs) are an important vaccine platform that implements this principle but also contain thymus-dependent off-target epitopes, thereby generating neutralizing and defocused antibody responses against the scaffold itself. Here, we present DNA origami as an alternative platform to display the receptor binding domain (RBD) of SARS-CoV-2. DNA-based scaffolds provide nanoscale control over antigen organization and, as thymus-independent antigens, are expected to induce only extrafollicular B-cell responses. Our icosahedral DNA-based VLPs elicited valency-dependent BCR signaling in two reporter B-cell lines, with corresponding increases in RBD-specific antibody responses following sequential immunization in mice. Mouse sera also neutralized the Wuhan strain of SARS-CoV-2—but did not contain boosted, DNA-specific antibodies. Thus, multivalent display using DNA origami can enhance immunogenicity of protein antigens without generating scaffold-directed immunological memory and may prove useful for rational vaccine design.

Introduction

The multivalent display of antigens at the nanoscale has been demonstrated to improve the immunogenicity of subunit vaccines[1-3]. Nanoparticulate vaccines with diameters between 20 and 200 nm ensure efficient trafficking to secondary lymphoid organs[4]. In secondary lymphoid organs, high valency and avidity promote B-cell receptor (BCR) crosslinking and signaling as well as BCR-mediated antigen uptake, thereby driving early B-cell activation and humoral immunity[5-12]. The importance of BCR signaling for antibody responses was initially recognized for thymus-independent (TI) antigens, particularly of the TI-2 class[13-15]. The multivalent display of these non-protein antigens induces BCR crosslinking in the absence of T-cell help, ensuring that antibody responses proceed through extrafollicular B-cell pathways and thereby limiting germinal center (GC) reactions, affinity maturation and induction of B-cell memory[16-17]. Multivalent antigen display also enhances the BCR-mediated response to thymus-dependent (TD) antigens including proteins[7-8]. In this context, follicular T-cell help enables GC reactions to generate affinity-matured B-cell memory that can be boosted or recalled upon antigen reexposure[18-20]. Consequently, the nanoscale organization of antigens represents a well-established vaccine design principle, not only for TI antigens, but also to elicit humoral immunity through the TD pathway[1-3].

Leveraging this design principle, native and engineered protein-based virus-like particles (P-VLPs) have emerged as an important platform for multivalent subunit vaccines[21-36]. P-VLPs enable the rigid display of antigens and have recently been used to investigate the impact of valency on B-cell activation *in vivo* to greater detail, suggesting differential regulation of affinity maturation and enhanced breadth of antibody responses at high valency[7-9]. However, valency control remains limited by the number of distinct protein components used for VLP assembly or by statistical functionalization with antigens—and is typically dependent on scaffold size and geometry. Notably, protein-based scaffolds are also TD antigens that elicit humoral immunity, including both T- and B-cell memory[36-37]. These scaffolds contain, and multivalently display, off-target epitopes that can defocus antibody responses, and such defocusing competes with the principles of rational vaccine design[38-39]. Scaffold-directed immunological memory can further complicate sequential or diversified immunizations with a given P-VLP, resulting in antibody-dependent clearance of the vaccine platform[40-41].

We sought to address these limitations by combining rigid, multivalent antigen display with scaffolds composed of TI antigens. We hypothesized that such nanoscale organization could promote TD antibody responses against protein antigens but confine scaffold-directed B-cell responses to the non-boostable, extrafollicular pathway devoid of immunological memory. Wireframe DNA origami provides access to designer VLPs of controlled geometry and size at the 20 to 200 nm scale with independently programmable geometry, valency and stoichiometry of antigen display[42-46]. We and others recently leveraged this platform to probe the nanoscale parameters of IgM recognition and of BCR signaling in reporter B-cell lines, suggesting that increased antigen spacing up to 30 nm promotes early B-cell activation[47-48]. However, these nanoscale design rules remain to be validated *in vivo*. While the utility of DNA-based VLPs (DNA-VLPs) to enhance antibody responses has not been demonstrated, DNA origami has been successfully employed to deliver therapeutic cargo to tumors in mice[49-50]. Other examples of *in vivo* delivery include the co-formulation of antigens and adjuvants to elicit T-cell immunity[51]. Importantly, and in contrast to P-VLPs, DNA-based scaffolds constitute TI antigens and should therefore be excluded from the boostable follicular pathway[51-52].

As proof-of-concept, we report on the fabrication of DNA-VLPs functionalized with the SARS-CoV-2 receptor binding domain (RBD) derived from the spike glycoprotein, a key target for eliciting neutralizing antibodies against the virus[53-56]. Our nanoparticulate vaccine displayed enhanced binding to ACE2-expressing cells and induced valency-dependent BCR signaling *in vitro*. Following sequential immunization in mice, we observed corresponding valency-dependent enhancement of RBD-specific antibody responses and B-cell memory recall. Mouse sera also efficiently neutralized the Wuhan strain of SARS-CoV-2 for DNA-VLPs compared with monomeric RBD—but did not contain boosted, DNA-specific antibodies. Taken together, our findings suggest that DNA origami can be leveraged for multivalent antigen display without eliciting TD B-cell responses against the DNA-based scaffold, rendering this platform useful for rational vaccine design.

Results and Discussion

The spherical SARS-CoV-2 virion is approximately 100 nm in diameter and displays approximately 100 trimeric spike glycoproteins[57]. Each monomer contains the RBD which is essential for engaging the ACE2 receptor and viral uptake, rendering it a key target of neutralizing antibody responses[53-56]. We adapted our previous DAEDALUS design, an icosahedral DNA-VLP with 50 potential conjugation sites and approximately 34 nm in diameter, to display the RBD and investigate impact of nanoscale antigen organization by DNA origami on B-cell activation[48]. A covalent *in situ* functionalization strategy employing strain-promoted azide-alkyne cycloaddition (SPAAC) chemistry was used for antigen attachment (**Figure 1A**)[44]. Towards this end, we synthesized 30 oligonucleotide staples bearing triethylene glycol (TEG)-DBCO groups at their 5' ends to assembly DNA-VLPs symmetrically displaying 1x, 6x or 30x DBCO groups on their exterior (**Figure S1**, **Table S1 to S3**). Employing a reoxidation strategy, the RBD was selectively modified at an engineered C-terminal Cys with a SMCC-TEG-azide linker and subsequently incubated with DBCO-bearing DNA origami to fabricate **I52-1x-, 6x-, 30x-RBD** (**Figures 1B and S2**, **Note S1**). The optimization of reaction conditions yielded near-quantitative conversion and coverage of more than 80% of conjugation sites on average as determined by denaturing, reversed-phase HPLC and Trp fluorescence (**Figures 1C and S3**). Notably, conversion was dependent on maximum DBCO concentrations and we obtained only up to 30% coverage for **I52-1x-RBD**. The monodispersity of purified DNA-VLPs was validated by dynamic light scattering (DLS) (**Figure 1D**). Analysis of **I52-30x-RBD** via negative-strain transmission electron microscopy (TEM) validated structural integrity of the DNA origami (**Figure 1E and S4**). While the icosahedral geometry could not be fully resolved, presumably due to accumulation of uranyl formate in the interior of the DNA origami, antigens were clearly visible and organized symmetrically.

To investigate the binding activity of RBD-Az before and after conjugation to DNA-VLPs, we conducted flow cytometry experiments with ACE2-expressing HEK293 cells (**Figure 2A**). Initially, monovalent binding of wild-type RBD and fluorophore-labeled RBD-Cy5, obtained by selectively labeling the azide, was compared (**Figure 2B and C**). The RBD constructs were incubated at 200 nM with the HEK293 cells and bound antigen was detected using the previously described anti-RBD antibody CR3022[56]. These experiments revealed comparable binding between the two constructs, demonstrating preservation of the receptor binding motif (RBM) and the viability of the reoxidation strategy for selective labeling of the terminal Cys (**Figure S2, Note S1**). Next, we explored whether multivalent RBD display using DNA-VLPs would result in increased avidity. Two additional fluorophore-labeled VLPs, **I52-30x-RBD-5x-Cy5** and **I52-5x-Cy5**, were synthesized to allow for direct detection of binding (**Figure 1B and S1**). Indeed, binding

of the RBD-functionalized VLPs was significantly enhanced compared to monomeric RBD-Cy5, while no binding was observed for the **I52-5x-Cy5** (**Figure 2D and E**). When correcting for Cy5 brightness per RBD, **I52-30x-RBD-5x-Cy5** displayed approximately one order of magnitude higher median fluorescence intensity compared with monomeric RBD-Cy5, indicative of avidity effects for VLP recognition**.**

We then evaluated the capacity of RBD-functionalized DNA-VLPs to induce BCR signaling using a previously described Ca^{2+} flux assay (**Figure 2A**)[58]. Specifically, Ramos B-cell lines expressing the somatic CR3022 or B38 antibodies were established[56, 59]. BCR signaling was initially validated by incubation with an anti-IgM antibody. At 30 nM antigen, monomeric wild-type RBD did not elicit B-cell activation *in vitro* (**Figure 2F and G**). By contrast, incubation of the Ramos B cells with multivalent DNA-VLPs at the same antigen concentration resulted in efficient BCR signaling. We further observed valency-dependent increases in total Ca^{2+} flux for both cell lines with **I52-30x-RBD** being more potent than **I52-6x-RBD**. CR3022 (K_D = 0.27 μM, **Figure 2F**) and B38 (K_D = 1.00 μM, **Figure 2G**) bind distinct RBD epitopes with moderate monovalent affinity as reported for the corresponding Fab fragments[60]. Despite this 4-fold difference in affinity, we observed comparable total BCR signaling relative to the IgM control for all functionalized DNA-VLPs, consistent with previously described avidity effects at the B-cell surface[61]. We concluded that our DNA-VLPs efficiently interacted with and induced signaling by RBD-specific BCRs, analogous to previous studies using similar assays to evaluate multivalent subunit vaccines[58, 62-68]. The increased B-cell activation for **I52-30x-RBD** contrasts our previous findings for HIV antigens for which total Ca^{2+} flux saturated beyond a valency of 10x[48]. Notably, the antigen-BCR systems differ with respect to affinity and mode of antigen attachment: The affinity of the HIV antigen was substantially higher (eOD-GT8, K_D = 30 pM) and the antigens were non-covalently attached to DNA origami using rigid DNA-PNA duplexes.

Next, we investigated whether RBD-functionalized DNA-VLPs could activate B-cells *in vivo* and induce antibody responses. C57BL/6 mice were sequentially immunized with monomeric wild-type RBD, **I52-6x-RBD** and **I52-30x-RBD** at doses equivalent to 7.5 μg RBD (**Figure 3A and S3**). IgG responses against the RBD were monitored throughout this regimen using ELISA and correlated with our *in vitro* BCR signaling findings (**Figure 3B and S5**). Post-boost 1, we observed an approximately 130-fold increase in endpoint dilutions for the 30-valent DNA-VLP over monomeric RBD. **I52-6x-RBD** did not enhance the B-cell response and elicited comparable antibody titers to monomeric RBD, both post-boost 1 and 2. Overall, endpoint dilutions were further increased post-boost 2 but converged between the groups. Earlier and stronger boosting of IgG titers and efficient B-cell memory recall is a hallmark of multivalent versus monomeric subunit vaccines[22-23]. Our findings are further consistent with enhanced IgG titers elicited by P-VLPs of increasing the valency[7-9]. Notably, we did not observe boosting of DNA-specific IgG titers against the scaffold, indicating an absence of B-cell memory for the DNA-VLP (**Figure 3C and S6**). While this finding was expected for TI antigens such as DNA, it was also established that TD antibody responses can be generated against TI antigens by covalent attachment to protein antigens[69-70]. The inverse case does not appear to be the default—scaffolding protein antigens with TI antigens does not direct the B-cell response to the extrafollicular pathway. By contrast, we observed valency-dependent TD antibody responses to the RBD, akin to virosomal and ISCOM-based vaccine design principles in which protein antigens are multivalently displayed by TI antigen-composed matrices[71-74]. The valency-dependent enhancement of RBD-specific antibody responses was further reflected in the efficient neutralization of the wild-type, Wuhan strain of

SARS-CoV-2 (**Figure 3D**)[75-76]. These findings suggest that immunization with **I52-30x-RBD** not only resulted in increased IgG titers but also induced functionally improved humoral immunity.

Conclusions

Here, we report on the use of wireframe DNA origami to program the display of SARS-CoV-2 antigens. RBD-functionalized DNA-VLPs efficiently bound to the ACE2 receptor and activated B cells *in vitro*. BCR signaling increased with DNA-VLP valency and no saturation effects were observed for up to 30x RBD antigens. We further demonstrate the utility of DNA-VLPs as an *in vivo* platform for rational vaccine design. In particular, we provide proof-of-concept that multivalent DNA-VLPs can enhance TD antigen-specific humoral immunity in mice, but, as TI scaffolds, do not generate boostable B-cell memory against the vaccine platform itself. Because DNA origami also offers independent control over VLP size and geometry versus multivalent antigen display, DNA-based scaffolds may prove particularly useful if epitope focusing and nanoscale control are desired. By contrast, several P-VLPs explored as multivalent subunit vaccines against SARS-CoV-2 and other viruses elicit scaffold-directed humoral immunity[30-31, 36-37]—and the defocusing of RBD-specific antibody responses has been shown to reduce cross-neutralization of SARS-CoV-2 variants[37].

Maintaining antigen display in B-cell follicles over time has been shown to promote GC reactions and humoral immunity[77-78]. While our findings suggest that non-protected, covalently functionalized DNA-VLPs are sufficiently stable to enhance antibody responses, it will thus be important to investigate to what extent multivalent antigen display is maintained in secondary lymphoid organs in the presence of nuclease degradation[79-80]. Trafficking to secondary lymphoid organs and B-cell activation might also be enhanced by varying DNA-VLP size and valency, for example to mimic SARS-CoV-2 virions[57]. Beyond vaccine design, our findings are of potential importance to gene therapy by addressing antibody-dependent clearance[40-41]—with DNA origami emerging as an alternative delivery platform[81].

Methods

Methods are described in the **Supporting Information**.

Acknowledgments

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Figures

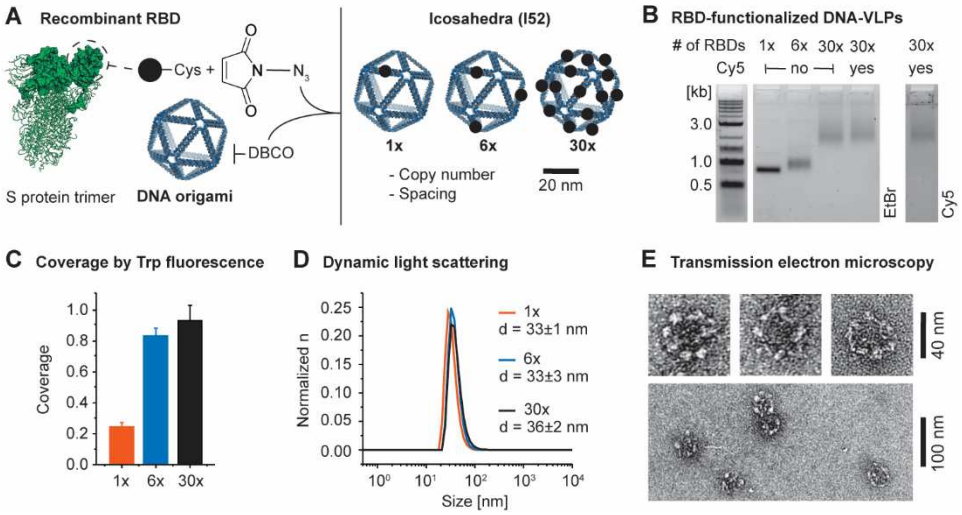

Figure 1. Design and synthesis of DNA-VLPs covalently displaying the SARS-CoV-2 RBD.
(A) Recombinant RBD bearing an additional Cys residue at the C-terminus was expressed. The C-terminal Cys was selectively labeled with and SMCC-TEG-azide linker and subsequently conjugated to DBCO-bearing DNA-VLPs. The icosahedral DNA origami objects of approximately 50 nm diameter displaying 1, 6 and 30 copies of the RBD were fabricated. **(B)** Agarose gel electrophoresis (AGE) shows the gel shift due to increasing RBD copy number as well as low polydispersity of the VLPs samples after purification. An additional VLP bearing 5 copies of Cy5 was produced for ACE2-binding flow cytometry experiments. **(C)** The coverage of the DNA-VLPs with RBD was quantified via Trp fluorescence. **(D)** Dynamic light scattering (DLS) was used to assess the dispersity of functionalized VLP samples. Representative histograms are shown**. (E)** Transmission electron micrographs (TEM) of **I52-30x-RBD** were obtained by negative staining using 2% uranyl formate and validate the symmetric nanoscale organization of antigens. Coverage values were determined from n = 3 biological replicates for **I52-1x-RBD** and from n = 6 biological replicates for **I52-6x** and **30x-RBD**. Diameters were determined from 3 technical replicates.

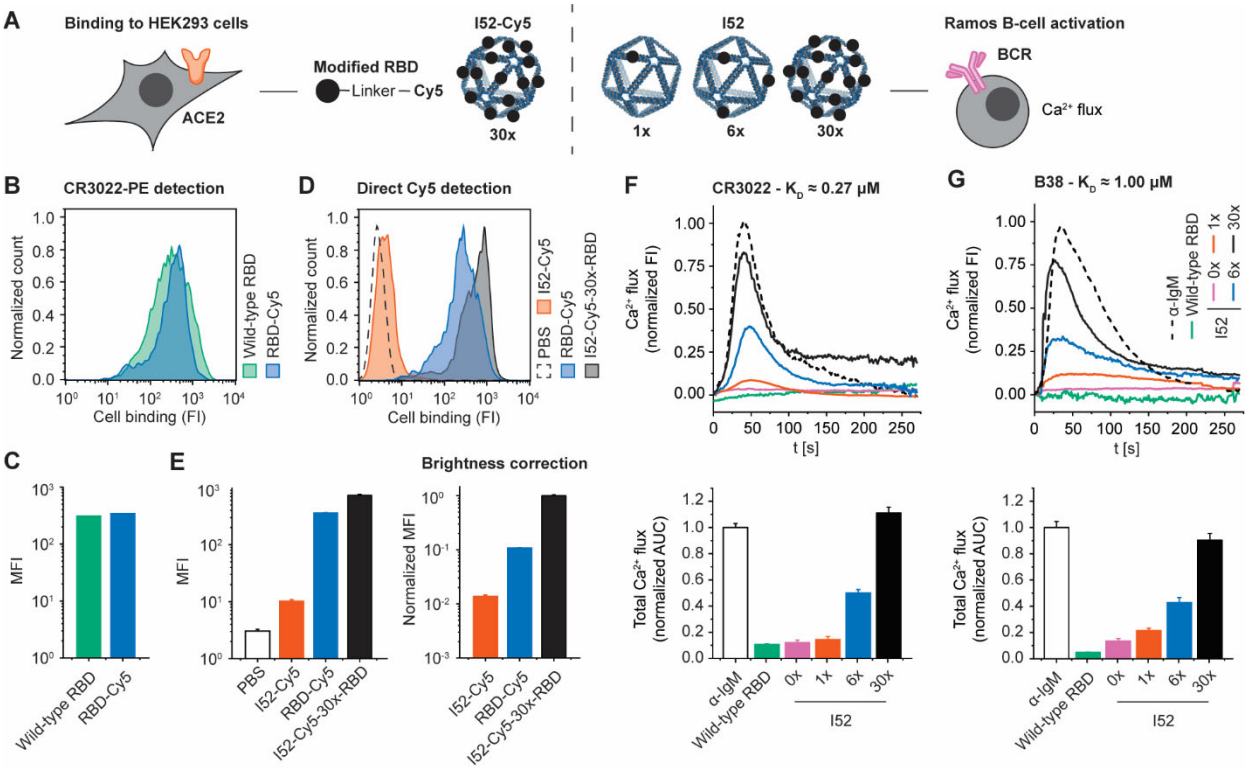

Figure 2. In vitro activity of RBD-functionalized DNA-VLPs.
(A) An overview of the in vitro activity assays and corresponding DNA-VLPs is shown. **(B and C)** ACE2-expressing HEK293 cells were incubated with 200 nM RBD. Binding was detected in flow cytometry experiments using PE-labeled CR3022 and a PE-labeled secondary antibody, demonstrating preserved binding activity for chemically modified RBD-Cy5 compared to wild-type RBD. **(D and E)** Incubation with Cy5-labeled **I52-30x-RBD** at 100 nM RBD revealed enhanced binding compared to RBD-Cy5 due to multivalency effects. No unspecific binding for non-functionalized **I52** was observed. The brightness of Cy5-labeled **I52-30x-RBD** (5 Cy5 per 30 RBDs) and RBD-Cy5 (1 Cy5 per 1 RBD) was quantified experimentally (**Figure S4**) and MFI values were corrected accordingly. **(F and G)** Ramos B cells expressing the BCRs C3022 and B38 were incubated with α-IgM, wild-type RBD or RBD-functionalized DNA-VLPs at 30 nM RBD. Ca^{2+} flux in response to RBD incubation was assayed using Fura Red. Representative fluorescence intensity curves are shown (top). Total Ca^{2+} flux was quantified via the normalized AUC, revealing robust activation of BCR-expressing Ramos B cells by functionalized DNA-VLPs (bottom). No stimulation was observed for wild-type RBD or for non-functionalized **I52**. Representative histograms are shown for ACE2 binding assays and MFI values were determined from n = 3 biological replicates. Normalized AUC values were determined from n = 3 biological replicates.

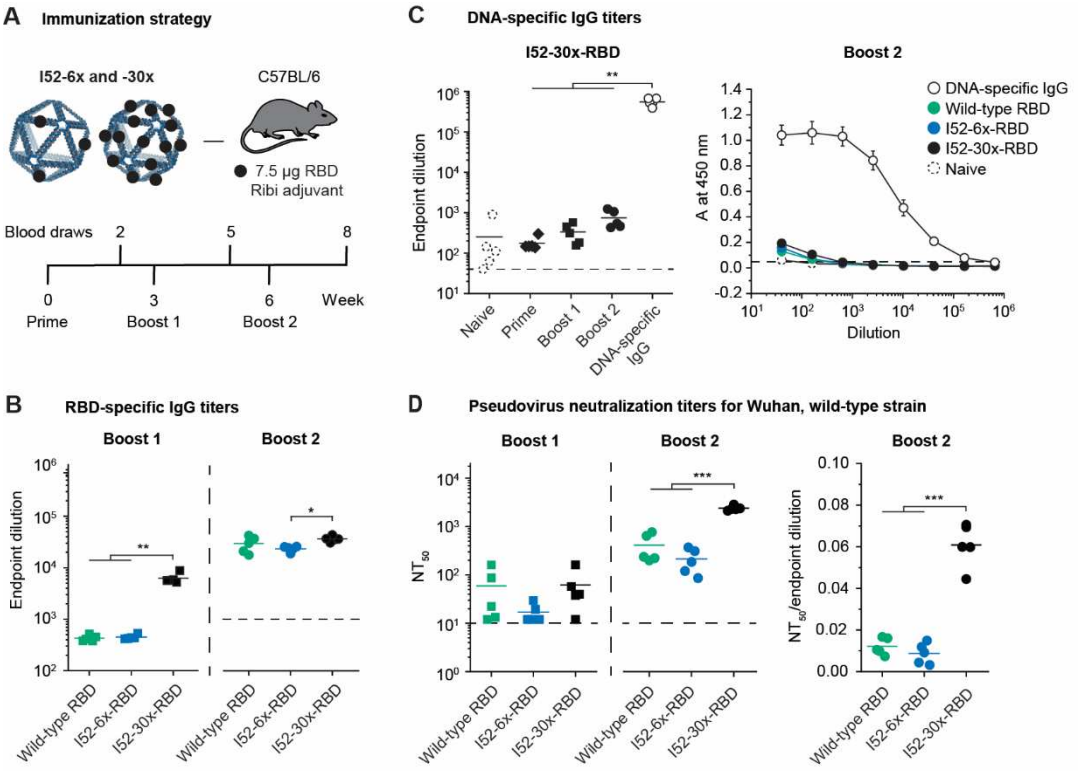

Figure 3. Antibody responses to RBD-functionalized DNA-VLPs.

(A) Mice were immunized intraperitoneally with monomeric RBD and RBD-functionalized DNA-VLPs of varying copy number following a prime-boost-boost regimen. **(B)** RBD-specific IgG endpoint dilutions were determined via ELISA, revealing enhanced antibody responses for **I52-30x-RBD** compared to both monomeric RBD and **I52-6x-RBD. (C)** DNA-VLPs did not elicit enhanced DNA-specific IgG titers compared to monomeric RBD as measured by ELISA. Importantly, DNA-specific IgG were not increased after boost immunizations with DNA-VLPs. DNA-specific IgG was diluted from 10 µg/ml. **(D)** Serum neutralization titers expressed as NT_{50} values against pseudoviruses modeling the wild-type, Wuhan strain were determined. We observed enhanced, valency-dependent neutralization efficiency for **I52-30x-RBD.** Mice were immunized with 7.5 ug RBD. IgG titers, RBD-specific IgG B cell fractions and NT_{50} values were determined from n = 5 biological replicates. One-way ANOVA was performed followed by Dunnett's T3 multiple comparison test at $\alpha = 0.05$.

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