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A spike-trimer protein-based tetravalent COVID-19 vaccine elicits enhanced breadth of neutralization against SARS-CoV-2 Omicron subvariants and other variants

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Multivalent vaccines combining crucial mutations from phylogenetically divergent variants could be an effective approach to defend against existing and future SARS-CoV-2 variants. In this study, we developed a tetravalent COVID-19 vaccine SCTV01E, based on the trimeric Spike protein of SARS-CoV-2 variants Alpha, Beta, Delta, and Omicron BA.1, with a squalene-based oil-in-water adjuvant SCT-VA02B. In the immunogenicity studies in naïve BALB/c and C57BL/6J mice, SCTV01E exhibited the most favorable immunogenic characteristics to induce balanced and broad-spectrum neutralizing potencies against pre-Omicron variants (D614G, Alpha, Beta, and Delta) and newly emerging Omicron subvariants (BA.1, BA.1.1, BA.2, BA.3, and BA.4/5). Booster studies in C57BL/6J mice previously immunized with D614G monovalent vaccine demonstrated superior neutralizing capacities of SCTV01E against Omicron subvariants, compared with the D614G booster regimen. Furthermore, SCTV01E vaccination elicited naïve and central memory T cell responses to SARS-CoV-2 ancestral strain and Omicron spike peptides. Together, our comprehensive immunogenicity evaluation results indicate that SCTV01E could become an important COVID-19 vaccine platform to combat surging infections caused by the highly immune evasive BA.4/5 variants. SCTV01E is currently being studied in a head-to-head immunogenicity comparison phase 3 clinical study with inactivated and mRNA vaccines (NCT05323461).

tetravalent, SARS-CoV-2 vaccine, Omicron subvariants, broad neutralization

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INTRODUCTION

The unending surge in coronavirus disease 2019 (COVID-19) cases is propelled by the continuous rapid emergence of SARS-CoV-2 variants. The recently emerging SARS-CoV-2 variant named Omicron (B.1.1.529 BA.1) was first identified in Botswana and South Africa on November 9, 2021 and has rapidly spread across more than 20 countries (Bai et al., 2022; https://www.gisaid.org/hcov19-variants). To make matters worse, the newly emerging Omicron variant and its subvariants (BA.1.1, BA.2, BA.3, BA.4, and BA.5) (Cao et al., 2022; Desingu et al., 2022; Khan et al., 2022) can break past immune defenses even in people fully vaccinated with ancestral strain-based first-generation COVID-19 vaccines, as well as protection mounted against previous SARS-CoV-2 variants infection (Carreño et al.,

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2022; Chen et al., 2022; Qu et al., 2022; Schubert et al., 2022), thus posing a significant challenge in our quest to curb the pandemic.

While first-generation vaccines, such as BNT162b2, AZD1222, mRNA-1273, BBIBP-CorV, CoronaVac, and Ad26.COV2.S, may still offer some protection against infection from new variants, their protective effectiveness wanes rapidly as have been frequently reported (Cameroni et al., 2022; Dejnirattisai et al., 2022; Ikemura et al., 2021; Lu et al., 2022; Wang et al., 2022a; Zhao et al., 2022). For example, BNT162Bb2, an mRNA vaccine targeting full Spike protein of SARS-CoV-2 with D614G mutation, showed a 21-fold and 6.5-fold decrease in neutralization titer against Omicron (B.1.1.529) BA.1 variant compared with D614G in 2-dose and 3-dose vaccine recipients, respectively (Lu et al., 2022). Similarly, Omicron BA.1-specific 50% maximal neutralizing titer (NT_{50}) elicited by mRNA-1273 was 26.6 times lower than that against D614G (Zeng et al., 2022). In addition, NT_{50} against Omicron BA.1 decreased by 36 times or 39 times in convalescent sera from SARS-CoV-2 or Delta variant infected patients, when compared with D614G-specific NT_{50} (Zhang et al., 2021). The recently identified Omicron subvariants BA.4 and BA.5 have become the dominant variants in many countries due to their ultra-immune evasion capabilities against antibodies elicited by ancestral strain-based vaccines and BA.1 infection (Cao et al., 2022; Qu et al., 2022; Tuekprakhon et al., 2022b). Antigenic changes, especially the L452R and F486V mutations in the receptor-binding domain (RBD) of BA.4 and BA.5, are key players in substantially impairing the neutralizing capacity of sera from vaccinated or BA.1-infected individuals (Tuekprakhon et al., 2022b).

Mutations shared by different variant strains (such as D614G, E484K, N501Y, K417N, L452R, and P681R) and unique mutations of a specific variant strain work together to confer increased neutralization resistance (Park and Hwang, 2021; Saxena et al., 2022). Meanwhile, inter-variant recombination might generate more virulent chimeric strains (Haddad et al., 2021; He et al., 2022; Jackson et al., 2021; Ou et al., 2022; Vatteroni et al., 2022). Therefore, multivalent vaccines combining various crucial mutations from phylogenetically divergent variant strains might provide an effective approach to defend against existing and future variants.

In this study, we generated a tetravalent COVID-19 vaccine candidate named SCTV01E, based on the Spike-trimer protein of four SARS-CoV-2 variants (Alpha, Beta, Delta, and Omicron BA.1) with a squalene-based oil-in-water adjuvant SCT-VA02B. The immunogenicity of SCTV01E was evaluated in naïve mice or as a booster shot in mice previously vaccinated with a D614G-matched vaccine mimicking first-generation vaccines, in comparison with a D614G vaccine booster regimen. Assessments of antigen-specific T cell activation elicited by SCTV01E were also conducted. SCTV01E is currently in a phase 3 immunogenicity clinical trial in a head-to-head comparison with inactivated and mRNA vaccines as controls (NCT05323461).

RESULTS

Characterization of SCTV01E antigens

SCTV01E antigens are based on trimeric spike protein extracellular domain (S-ECD) (1–1,208 aa) of SARS-CoV-2 variants Alpha, Beta, Delta, and Omicron BA.1, with a squalene-based oil-in-water adjuvant SCT-VA02B (Figure 1A). The four antigens were all produced with stable CHO cell lines using a platform technology and purified with multiple chromatographic steps to high purities. The purified antigens were formulated with the adjuvant into single vials as vaccine product. Transmission electron microscope (TEM) analysis demonstrated a trimeric pre-fusion conformation with a size of 12 nm×20 nm (Figure 1B). Apparent molecular weight of each antigen was estimated to be 150 kD, as indicated by reduced SDS-PAGE analysis (Figure 1C), consistent with the theoretical value. Binding affinity (K_D) between SCTV01E antigens and human angiotensin converting enzyme-2 (hACE2) was 3.42, 2.90, 3.78, and 3.10 nmol L^{-1} for trimeric S-ECD of Alpha, Beta, Delta, and Omicron BA.1, respectively (Figure 1D; Table S1 in Supporting Information). SCTV01E antigens exhibited enhanced binding affinities to hACE2 when compared with the trimeric D614G S-ECD protein (6.00 nmol L^{-1}) (Table S1 in Supporting Information).

Humoral immune responses elicited by monovalent vaccines based on SARS-CoV-2 variants Alpha, Beta, Delta, or Omicron BA.1 in mice

To evaluate the immunogenicity of monovalent vaccines based on each antigen of SCTV01E, C57BL/6J mice and BALB/c mice were intramuscularly injected with 1 μg of Alpha-, Beta-, Delta-, or Omicron BA.1-matched vaccine adjuvanted with SCT-VA02B, on Day 0 and Day 14. Mouse sera were collected on Day 21 for measurement of neutralizing antibody titers (NTs) against the corresponding pseudoviruses (PsVs) as shown in Figure 2A. In C57BL/6J mice, each monovalent vaccine induced the highest NT_{50} against antigen-matched PsV, reaching a geometric mean titer (GMT) of 10,533 for B.1.1.7, 14,469 for B.1.351, 14,882 for B.1.617.2, and 10,832 for B.1.1.529, respectively (Figure 2B–E). The results indicated that monovalent vaccines targeting pre-omicron variants (Alpha, Beta, and Delta), to some extent, showed cross-reactivity against antigen-mismatched PsVs, which was similar to our previous find-

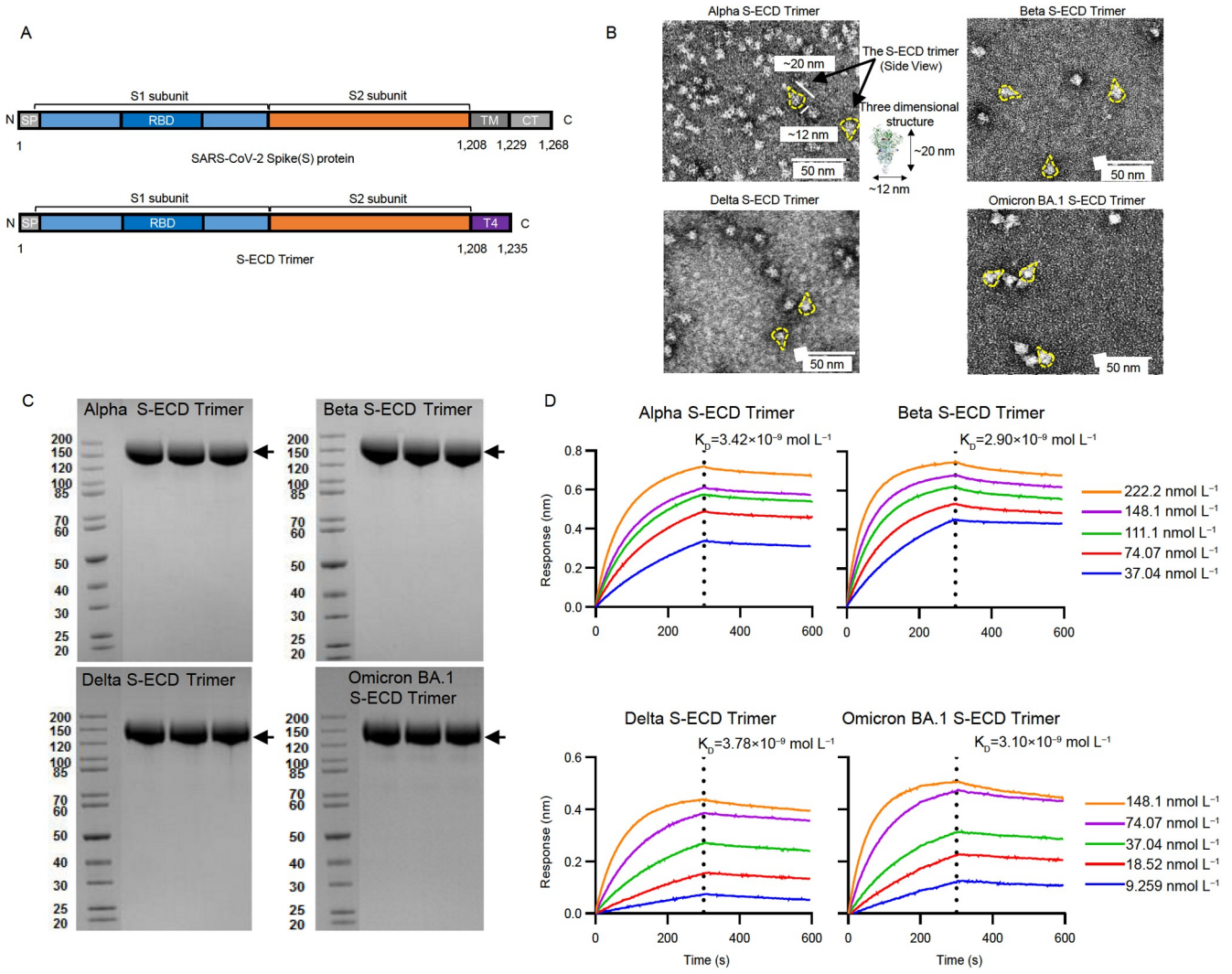

Figure 1 Characterization of SCTV01E antigens. A, Schematic diagram of SCTV01E antigens. B, TEM image of SCTV01E antigens. Scale bar: 50 nm. C, SDS-PAGE analysis of SCTV01E antigens. D, Binding affinity between SCTV01E antigens and human ACE2 protein characterized by Bio-Layer Interferometry (BLI) analysis.

ings (Wang et al., 2022b). A drastic reduction in NT_{50} against Omicron BA.1 was observed in Alpha, Beta, or Delta monovalent vaccine sera (Figure 2B–D). Notably, the Omicron BA.1 monovalent vaccine was unable to elicit neutralizing antibody responses against pre-omicron variants, with an NT_{50} GMT of 70 for B.1.1.7, 87 for B.1.351, and 87 for B.1.617.2, suggesting limited cross-neutralizing potency against earlier variants (Figure 2E). These findings were further confirmed in BALB/c mice (Figure S1 in Supporting Information)

Optimization of antigen proportions of SCTV01E in mice

Data from a study for optimization of antigen proportions of SCTV01E in C57BL/6J mice demonstrated that a balanced neutralizing capacity against the four antigen-matched variants can be achieved when Alpha, Beta, Delta, and Omicron BA.1 S-ECD were mixed at a ratio of 1:1:1:3, as indicated by comparable neutralizing antibody titers against Alpha, Beta, and Delta variants (less than 2-fold variation in NT_{50}) and superior neutralizing potency against the recently emerging variant Omicron BA.1 (3.4-fold increase in NT_{50}) (Figure 3). Balanced humoral immune responses against antigen-matched PsVs were also observed in BALB/c mice intramuscularly immunized with SCTV01E twice (on Day 0 and Day 14) at 4 dose levels (0.75, 1.5, 3, and 6 μg). Comparable NT_{50} GMT against Alpha, Beta, Delta, and Omicron BA.1 PsVs were observed at each dose level, with less than 3-fold variation between the minimal and maximal NT_{50} GMT values (Figure S2 in Supporting Information). Overall, SCTV01E showed broad-spectrum neutralizing activities against vaccine-matched variants.

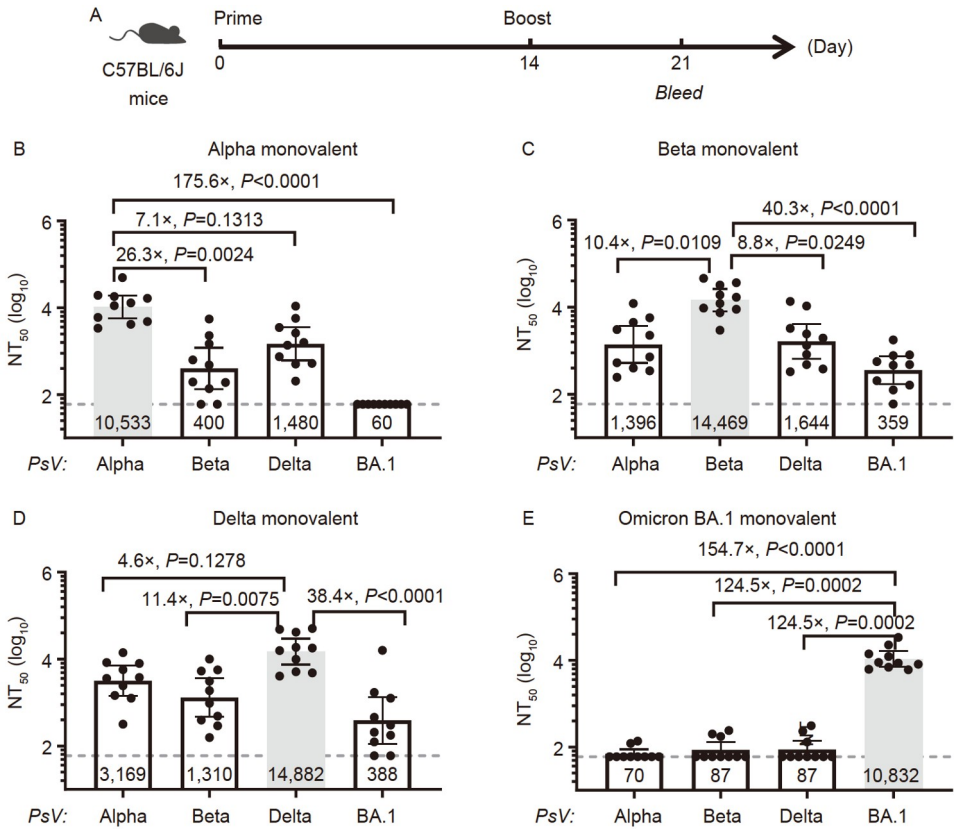

Figure 2 Humoral immune responses induced by monovalent vaccines based on each antigen of SCTV01E with a prime-boost regimen in C57BL/6J mice. A, Scheme of immunization and serum collection. C57BL/6J mice (n=10/group) were intramuscularly immunized with 1 μg of Alpha, Beta, Delta, or Omicron BA.1 monovalent vaccine on Day 0 and Day 14. Mouse sera were collected on Day 21. Neutralizing tilters (NTs) of Alpha (B), Beta (C), Delta (D), or Omicron BA.1 monovalent vaccine (E). Immunized mouse sera were detected on Day 21. Data are shown as mean±SD of $\log_{10}$ transformed values. SD, standard deviation. Data were analyzed by Kruskal-Wallis test (nonparametric one-way ANOVA).

Cross-neutralization capacity of SCTV01E vaccinated mouse sera

C57BL/6J mice were intramuscularly injected with either D614G-matched monovalent vaccine, Omicron BA.1-matched monovalent vaccine, or SCTV01E (1 μg/dose) twice with a 2-week interval. The NTs against PsVs based on multiple SARS-CoV-2 variants were measured 1 week after the second vaccination. As shown in Figure 4A, in D614-matched vaccinated mouse sera, there was no neutralizing activity against Omicron BA.1.1, BA.2, BA.2.12.1, and BA.3. Compared with the D614-matched monovalent vaccine, a significant increase in NTs against these four variants was detected in mice vaccinated with either Omicron BA.1-matched vaccine or SCTV01E. Moreover, the tetravalent SCTV01E vaccine induced much higher specific NTs against Omicron BA.2.12.1 than the Omicron BA.1-matched vaccine.

We further compared the neutralizing potency of D614G-matched monovalent vaccine, Omicron BA.1-matched monovalent vaccine or SCTV01E against earlier strains among different vaccination groups. We found no neutralizing activity against D614G, Gamma, Lambda, and Mu strains in Omicron BA.1-immunized sera. D614-matched monovalent vaccine induced the highest NTs against D614G (GMT=8,583) and Lambda (GMT=1,492). Compared with the Omicron BA.1-matched vaccine, SCTV01E induced a significant increase in NTs against these four variants. A significant increase in NTs against the Mu strain was induced in SCTV01E-vaccinated mice compared with D614G-immunized mice (Figure 4B).

The most recent Omicron subvariants BA.4 and BA.5, more resistant to vaccines, is causing the present wave of SARS-CoV-2 infection (Hachmann et al., 2022; Tuekprakhon et al., 2022a; Yamasoba et al., 2022). A sharp decrease in neutralizing capacity against BA.4 elicited by convalescent sera from BA.1-infected population, previously vaccinated or not, has been reported (Cao et al., 2022; Khan et al., 2022). In the present study, we further investigated the cross-reactivity of BA.1 monovalent vaccine or SCTV01E against Omicron BA.4/5. When C57BL/6J mice (n=8/group) were immunized with 1 μg/dose BA.1 monovalent vaccine or SCTV01E in a prime-boost regimen, neutralizing responses could only be detected in 1 out of 8 Omicron BA.1 vaccine sera, while 3 out of 8 SCTV01E vaccine sera showed detectable neutralizing potency against BA.4/5 (Figure 4C).

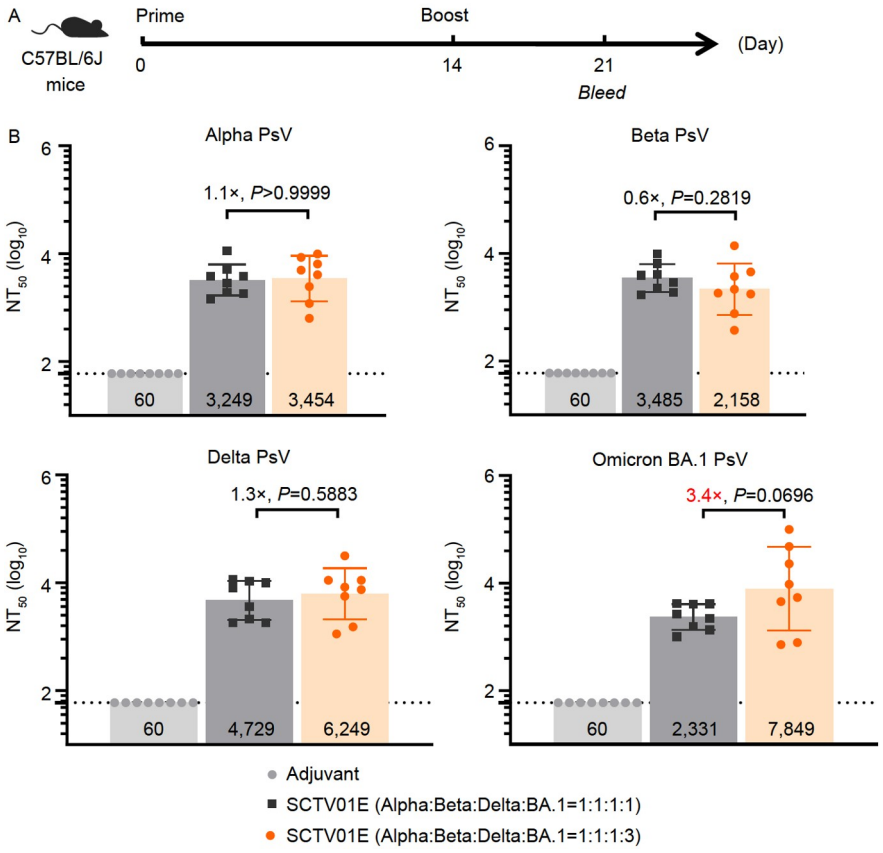

Figure 3 Optimization of SCTV01E antigen proportions. A, Scheme of immunization and serum collection. B, C57BL/6J mice (n=8/group) were intramuscularly injected twice with adjuvant or SCTV01E on Day 0 and Day 14. After 7 d, mouse sera were collected for measurement of neutralizing antibody titers (NT_{50}) against multiple SARS-CoV-2 variant pseudoviruses. Data are shown as mean±SD of $\log_{10}$ transformed values. SD, standard deviation. Data were analyzed by Kruskal-Wallis test (nonparametric one-way ANOVA).

An increased dosage of Omicron BA.1 monovalent vaccine or SCTV01E to 6 μg/dose potentiated the neutralizing capacity against BA.4. The neutralizing antibody titer in SCTV01E group (NT_{50} GMT: 816) was significantly higher than that elicited by Omicron BA.1 vaccine sera (NT_{50} GMT: 137) (P=0.0377) (Figure 4C). Overall, SCTV01E exhibits the broadest and the most potent neutralizing activities against pre-Omicron variants and newly emerging Omicron subvariants.

Humoral immune responses elicited by SCTV01E as booster shots in mice

Next, we investigated the humoral immune responses of SCTV01E as booster shots in C57BL/6J mice. In brief, mice were intramuscularly primed with the D614G-specific vaccine twice (on Day 0 and Day 14), mimicking the population previously received first-generation COVID-19 vaccines. Mice received a D614G-specific vaccine, Omicron BA.1-specific vaccine, or SCTV01E as a third shot (on Day 70), followed by injection of the corresponding vaccine as a fourth shot (on Day 182). Mouse sera were collected at defined time points for the measurements of neutralizing capacity against Omicron subvariants (Figure 5A).

We found that the NTs against tested Omicron variants induced by two doses of D614G-matched vaccine were considerably low, the NT_{50} GMT was 105 for BA.1, 115 for BA.2, 69 for BA.3, and 67 for BA.4/5. After the third shot of SCTV01E in mice, the neutralizing potency against Omicron variants was significantly increased by 22.0-fold, 11.2-fold, 17.4-fold, and 13.5-fold in GMT of NT_{50} against BA.1, BA.2, BA.3, and BA.4/5 compared with NT_{50} before the first booster. Furthermore, a substantial increase in NTs (GMT) against four Omicron variants induced by the fourth dose of SCTV01E was detected in contrast to neutralizing activities induced by a prime-boost regimen of D614G-matched vaccine (BA.1 GMT: 14,872 *vs*. 105, P<0.0001; BA.2 GMT: 6,897 *vs*. 115, P=0.0010; BA.3 GMT: 8,768 *vs*. 69, P<0.0001; BA.4/5 GMT: 1,136 *vs*. 67, P=0.0014) (Figure 5A).

The immunogenicity of SCTV01E as booster shots was further evaluated in comparison with the D614G-matched vaccine and Omicron BA.1 vaccine in mice. The third dose of SCTV01E induced a 2.7-fold, 3.3-fold, 2.2-fold, and 3.3-

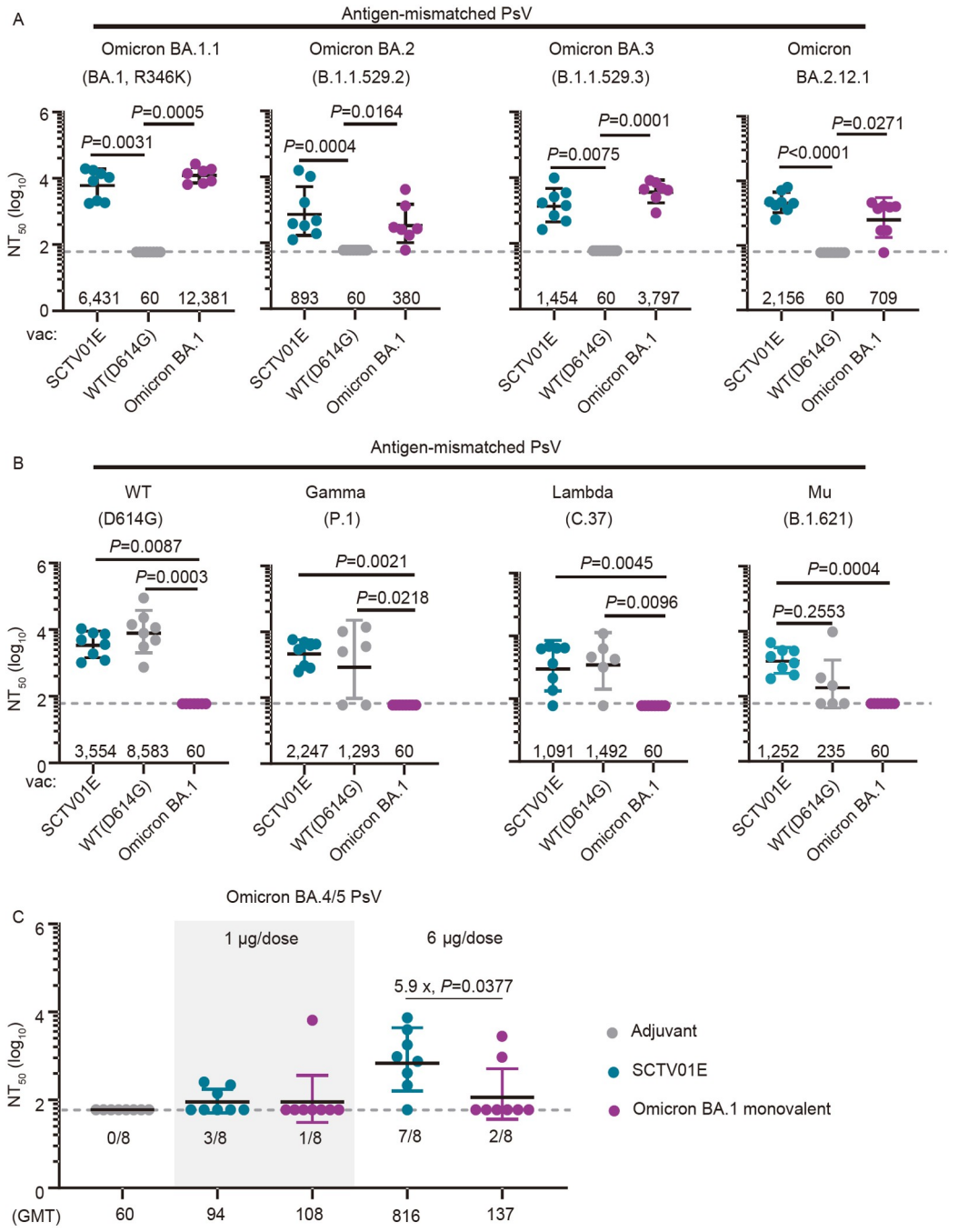

Figure 4 Cross-neutralization capacity of SCTV01E against earlier variants and newly emerging Omicron subvariants in C57BL/6J mice. A and B, C57BL/6J mice (*n*=8/group) were intramuscularly immunized with D614G monovalent vaccine, Omicron BA.1 monovalent vaccine, or SCTV01E (1 μg/dose) twice (on Day 0 and Day 14). Seven days after the second immunization, sera were used to detect NTs against antigen-mismatched PsVs based on Omicron BA.1.1, BA.2, BA.3, and BA.2.12.1 (A) and antigen-mismatched PsVs based on prior variants D614G, P.1, C.37, and B.1.621 (B). C, C57BL/6J mice (*n*=8/group) were intramuscularly injected with Omicron BA.1 monovalent vaccine or SCTV01E (1 μg/dose, 6 μg/dose) twice (on Day 0 and Day 14). 7 d after the second immunization, sera were used to detect NTs against Omicron BA.4/5 PsV. Data are shown as GMT mean±SD of $\log_{10}$ transformed values. SD, standard deviation. Data were analyzed by Kruskal-Wallis test (nonparametric one-way ANOVA).

fold increase in NTs against BA.1, BA.2, BA.3, and BA.4/5, respectively, compared with D614G-matched vaccine (Figure 5B). Moreover, a booster shot with SCTV01E remarkably elevated the NTs against antigen-matched pre-Omicron variants (Alpha, Beta, Delta), superior to a homologous booster shot with D614G-matched vaccine (2.1 to 4.3-fold increase in NT_{50}), and comparable to a heterologous booster shot with Omicron BA.1-matched vaccine regarding NT_{50} against Alpha and Delta (1.3 and 1.1-fold variation in NT_{50}, respectively), except for neutralizing activity against the Beta variant (2.7-fold increase in NT_{50}) (Figure S3 in Supporting Information). The fourth dose of SCTV01E

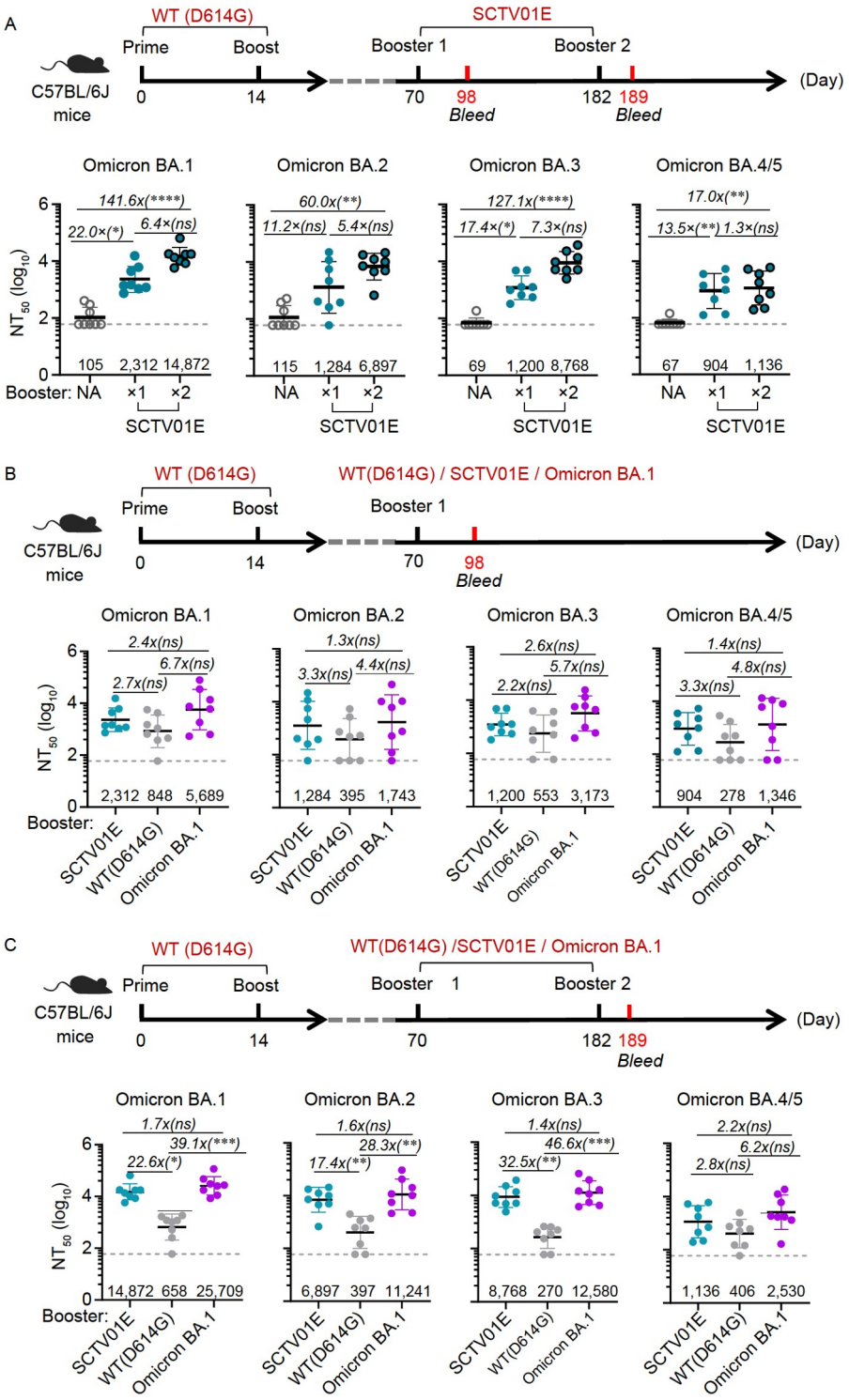

Figure 5 Neutralizing potency of SCTV01E as booster shots against Omicron subvariants in C57BL/6J mice. A, C57BL/6J mice (n=8/group) were immunized with D614G monovalent vaccine on Day 0 and Day 14 (1 μg/dose) and boosted with SCTV01E (1 μg/dose) on Day 70 and Day 182. Mouse serum collection was performed at defined time points (upper panel). Neutralizing activities against PsV based on Omicron BA.1, BA.2, BA.3, and BA.4/5 were analyzed (lower panel). B, C57BL/6J mice (n=8/group) were immunized with D614G monovalent vaccine on Day 0 and Day 14 (1 μg/dose) and boosted with SCTV01E, D614G, or Omicron BA.1 monovalent vaccine (1 μg/dose) on Day 70. Mouse sera collection was performed on Day 98 (upper panel). Neutralizing activities against PsV based on Omicron BA.1, BA.2, BA.3, and BA.4/5 were analyzed (lower panel). C, C57BL/6J mice (n=8/group) were immunized with D614G monovalent vaccine on Day 0 and Day 14 (1 μg/dose) and boosted with D614G-matched monovalent vaccine, Omicron BA.1-matched monovalent vaccine, or SCTV01E (1 μg/dose) on Day 70 and Day 182. Neutralizing activities against PsV based on Omicron BA.1, BA.2, BA.3, and BA.4/5 were analyzed using mouse sera on Day 189. Bars show Log 10 transformed GMT±SD. Data were $\log_{10}$ transformed. Data were analyzed by Kruskal-Wallis test (nonparametric one-way ANOVA). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; "ns" means $P\geqslant$0.05.

significantly increased the BA.1-, BA.2-, and BA.3-specific NTs compared with D614G-matched vaccine (BA.1 GMT: 14,872 *vs*. 658, P=0.0100; BA.2 GMT: 6,897 *vs*. 397, P=0.0088; BA.3 GMT: 8,768 *vs*. 270, P=0.0049) by induction of a 22.6-fold, 17.4-fold, and 32.5-fold increase in NTs against BA.1, BA.2, and BA.3 (Figure 5C). The neutralizing potency against tested Omicron variants induced by booster doses of SCTV01E seemed to be comparable with Omicron BA.1-matched vaccine (Figure 5B and C).

These data suggest that additional booster doses of SCTV01E vaccine would augment the protective effect against tested Omicron subvariants. SCTV01E or Omicron BA.1-matched vaccine as booster doses is superior to D614G-matched vaccine in neutralizing potency against these four Omicron subvariants.

Antigen-specific T cell activation elicited by SCTV01E

We further investigated the T-cell immune responses induced by SCTV01E in C57BL/6J mice. Splenocytes from immunized mice were stimulated with either SARS-CoV-2 Spike peptides (WT S peptides) or Omicron Spike peptides (Omicron S peptides). Antigen-specific T cell activation ($CD137^+$ $CD134^+$ $CD4^+$ T cells, $CD137^+$ $CD69^+$ $CD8^+$ T cells) (Grifoni et al., 2020; Liu et al., 2022; Tarke et al., 2021) and central memory T cells ($CD44^+$ $CD62L^+$ $CD4^+$ T cells, $CD44^+$ $CD62L^+$ $CD8^+$ T cells) (Bi et al., 2021; Fisher et al., 2021; Luan et al., 2022) were evaluated by FACS. Assessment of antigen-specific Th1 (IFN-γ^+) or Th2 (IL-4^+) responses was conducted by ELISpot assay *in vitro*. A significant increase in the percentage of $CD137^+$ $CD134^+$ $CD4^+$ T cells and $CD137^+$ $CD69^+$ $CD8^+$ T cells in splenocytes upon stimulation with WT S peptides was observed in SCTV01E vaccinated mice. Likewise, when Omicron S peptides were used for stimulation, the percentage of $CD137^+$ $CD69^+$ $CD8^+$ T cells increased significantly, and a modest increase in the percentage of $CD137^+$ $CD134^+$ $CD4^+$ T cells was observed (P=0.0310) (Figure 6A).

Upon vaccination with SCTV01E, the percentage of both wild-type (WT) S peptides and Omicron S peptides-specific central memory $CD4^+$ T or $CD8^+$ T cells significantly increased. There was no significant difference in the number of central memory $CD4^+$ T or $CD8^+$ T cells regardless of differences in stimuli (Figure 6B). Finally, we evaluated the Th1/Th2 immune responses induced by SCTV01E. As shown in Figure 6C, no significant change in IFN-γ^+ or IL-4^+ spot-forming cells (SFCs) was observed in SCTV01E-vaccinated mouse splenocytes treated with either WT S peptides or Omicron S peptides. Collectively, these data indicate that vaccination with SCTV01E in mice induces antigen-specific T cell activation, evokes central memory T cells, and triggers balanced Th1/Th2 immune responses.

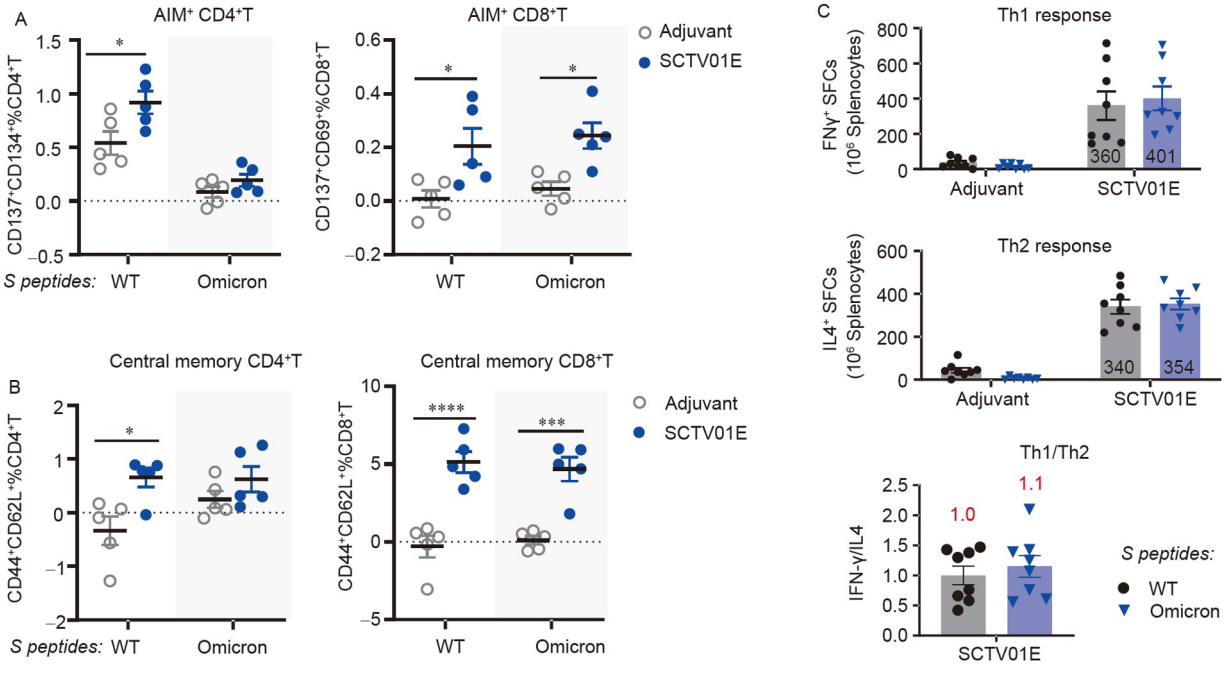

Figure 6 Cellular immune responses induced by SCTV01E in C57BL/6J mice. C57BL/6J mice (n=8/group) were immunized with adjuvant or SCTV01E on Day 0, Day 14, and Day 28. The splenocytes were isolated and stimulated with WT SARS-CoV-2 or Omicron variant S peptides (2 μg mL^{-1}) for 20 h on Day 47. A, The S peptides-induced activation of $CD134^+$ $CD137^+$ $CD4^+$ T cells and $CD69^+$ $CD137^+$ $CD8^+$ T was determined using AIM assay. B, $CD44^+$ $CD62L^+$ $CD4^+$ and $CD44^+$ $CD62L^+$ $CD8^+$ T cells were determined by FACs. C, Quantification of IFN-γ or IL-4 SFCs in splenocytes of vaccinated mice when treated with wild-type SARS-CoV-2 or Omicron S peptides using ELISpot assays. Data are shown as mean±SD. SD, standard deviation. AIM, activation-induced marker. Significance was calculated using two-way ANOVA with Sidak's multiple comparisons tests. *, P<0.05; ***, P<0.001; ****, P<0.0001.

DISCUSSION

Several first-generation COVID-19 vaccines, based on the SARS-CoV-2 ancestral strain, have been authorized for emergency use or received conditional approval (WHO, 2022). Nevertheless, long-term surveillance is warranted to monitor vaccine candidate's safety profiles and protection efficacy, especially efficacy against newly emerging SARS-CoV-2 variants, in different subpopulations (Nohynek and Wilder-Smith, 2022).

Variant-based monovalent vaccines carry mutational sites of a specific variant, which might lead to limited cross-neutralization capacity against evolving variants, as confirmed by previous studies based on human convalescent sera (Bekliz et al., 2022; Lechmere et al., 2022; Rössler et al., 2022b) or mouse immune sera (Amanat et al., 2021; Lee et al., 2022). Cross-neutralization capacity of monovalent vaccine based on pre-omicron variants against Omicron BA.1 sharply diminished, and vice versa, similar to previously published reports (Rössler et al., 2022a; Richardson et al., 2022; Suryawanshi et al., 2022). In addition, our data demonstrated limited cross-neutralization activities of variants Alpha, Beta, Delta, or Omicron BA.1 based monovalent vaccine against antigen-mismatched variants (Figure 2; Figure S1 in Supporting Information).

Our study provides a comprehensive and unbiased evidence in support of multivalent vaccines as an effective approach to cope with future outbreaks since multi-variant combined vaccines might provide broad coverage for the crucial mutations with high prevalence (Li et al., 2022). We have previously reported that SCTV01C, a bivalent protein subunit vaccine candidate targeting Alpha and Beta Spike (S) proteins, not only induces potent cellular immune responses but also promotes the cross-neutralizing activities against multiple SARS-CoV-2 variants in mice (Wang et al., 2022b). Bivalent vaccines based on ancestral strain and Omicron BA.1 variant showed a more comprehensive and potent neutralizing potency against tested variants, including Omicron BA.1 and BA.2 subvariants, in contrast to monovalent vaccine (Du et al., 2022; Su et al., 2022).

The antigens of SCTV01E were developed on the basis of our first-generation bivalent vaccine SCTV01C by adding the newly emerging Delta and Omicron variants on top of the Alpha and Beta variants in SCTV01C. SCTV01C has been shown to induce superior cross-immune responses against 14 VOC, VOI and VUM variants to either Alpha or Beta monovalent vaccine in mouse immunogenicity studies (Wang et al., 2022b). The inclusion of Delta and Omicron variants in SCTV01E covers more mutated neutralizing epitopes and could potentially provide better cross-neutralizing activities against a broad range of future emerging variants. In the present study, the Alpha, Beta, Delta, and Omicron BA.1 variants-based tetravalent vaccine SCTV01E exhibits the most favorable immunogenic characteristics to induce broad-spectrum neutralizing potency against multiple SARS-CoV-2 variants-based pseudoviruses in rodents (Figures 3 and 4; Figure S2 in Supporting Information). Likewise, Zhang et al. (2022) demonstrated that SARS-CoV-2 variant-based multivalent inactivated vaccine led to optimal neutralizing potency against tested variants, including pre-Omicron variants and Omicron BA.1. The cross-neutralizing activity of SCTV01E against newly emerging Omicron subvariants BA.1.1, BA.2, or BA.3 modestly decreased (3 to 4-fold reduction), which was consistent with existing data based on BA.1-infected convalescent sera (Willett et al., 2022; Zou et al., 2022). In alignment with the findings from previous reports (Cao et al., 2022; Khan et al., 2022), the most recent Omicron subvariants BA.4 and BA.5 showed drastic resistance to BA.1 monovalent vaccine. On the contrary, a relatively modest resistance of BA.4/5 to the tetravalent vaccine SCTV01E was observed, when compared with the BA.1 monovalent vaccine (Figure 4C). The superior cross-reactivity of SCTV01E against BA.4/5 PsV might be attributed to more broad coverage of mutations from divergent variants since BA.4/5 is antigenically distinct from BA.1 (Willett et al., 2022), which needs further elucidation.

The recent data released from Pfizer-BioNTech revealed that the third dose of BA.1 monovalent vaccine or BA.1/WT bivalent vaccine in mice enhances the neutralizing efficacy against Omicron BA.1 and recently prevailing BA.4/5 variants, superior to the ancestral strain-based monovalent vaccine (FDA, 2022). Preliminary clinical data released by Moderna demonstrated that Omicron BA.1-containing bivalent vaccines as a fourth dose induced higher levels of neutralizing antibody titers against Omicron BA.1 when compared with the fourth dose of prototype vaccine (Branche et al., 2022). Neutralizing antibody titers against BA.4/5 sharply decreased when compared with BA.1 in both studies (Branche et al., 2022; FDA, 2022). Booster shots with SCTV01E remarkably elevated the neutralizing capacity against Omicron subvariants (Figure 5A), superior to a homologous booster shot with a D614G-matched vaccine (Figure 5B and C). The superiority of SCTV01E over BA.1 monovalent vaccine in neutralizing potency against BA.4/5, as demonstrated in naïve mice, was not observed in mice previously vaccinated with a 2-dose regimen of D614G-matched vaccine. Comparable neutralizing antibody titers against BA.4/5 were also observed when BA.1 monovalent vaccine or BA.1\WT bivalent vaccine was used as a booster shot in mice (NT_{50} GMT: 1,064 in BA.1 monovalent group, 1,477 in BA.1/WT bivalent group) (FDA, 2022). Further research conducted at different dose levels is warranted. Meanwhile, the influence of previous vaccination or infection on immune responses evoked by booster shots needs further elaboration, as implicated by a recent report concerning the impact of prior immune imprinting on immune

boosting by Omicron BA.1 infection (Reynolds et al., 2022).

Due to limitations in Biosafety Level-3 laboratory, data from animal challenge study or *in vitro* assessment of neutralizing potency based on live viruses are currently not available. These studies might be carried out in the future to validate the findings of this study, and to further demonstrate the potential superiority of SCTV01E in protective efficacy against SARS-CoV-2 variants, including Omicron subvariants.

In summary, the current study elaborated the humoral and cellular immune responses induced by SCTV01E, which elicited broad-spectrum neutralizing activities against Omicron subvariants and antigen-specific T cell responses. These data support further evaluation of SCTV01E in clinical trials.

MATERIALS AND METHODS

Characterization of vaccine antigens

SARS-CoV-2 spike protein extracellular domain (S-ECD) was fused to a T4 bacteriophage fibritin motif (i.e., T4 Foldon), the natural trimerization domain of T4 fibritin (Meier et al., 2004), to stabilize the conformation of the trimeric protein. SCTV01E antigens were produced and purified as previously reported (Wang et al., 2022b). SCTV01E contains a mixture of the trimeric S-ECD antigens based on Alpha (GISAID ID: EPI_ISL_764238), Beta (GISAID ID: EPI_ISL_736940), Delta (GISAID ID: EPI_ISL_1999775), and Omicron BA.1 (GISAID ID: EPI_ISL_6640917) variants.

TEM analysis of SCTV01E antigens (0.3 mg mL^{-1}) was carried out with an electron microscope (Hitachi JEM1200) at 200 kV (JEM1200, JEOL, Japan). After being loaded to the copper net, samples were stained with uranyl acetate for 90 s, and subjected to image acquisition. For analysis by SDS-PAGE under reducing conditions, SCTV01E antigens were incubated in 70°C for 10 min and further analyzed on a 7.5% NuPAGE® gel (Thermo Fisher Scientific, USA). Binding kinetics of SCTV01E antigens, serially diluted to a range of 9.26–222.2 nmol L^{-1}, to hACE2 (hACE2-Fc, 10 μg mL^{-1}) (10108-H02H, Sino Biological, Beijing, China) were evaluated by Bio-Layer Interferometry (BLI) with Octet® RED96e (Forte Biosciences, Inc., USA) and data analysis were performed with Octet® Data Analysis V11.1 software (Forte Biosciences, Inc.).

Animal immunization scheme

Mice were housed in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures were in accordance with Chinese animal use guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC). Female BALB/c and C57BL/6J mice aged 6–8 weeks were obtained from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). Alpha, Beta, Delta, and Omicron BA.1 S-ECD were mixed at a ratio of 1:1:1:3 as antigens for SCTV01E. An antigen ratio of 1:1:1:1 was used in a comparative study for optimization of antigen proportions.

For the prime-boost immunization studies, C57BL/6J mice (n=8–10/group) were immunized with different vaccines twice (on Day 0 and Day 14). Sera were collected on Day 21 for the detection of PsV neutralizing activities against multiple SARS-CoV-2 variants. BALB/c mice (n=8–10/group) were immunized with the corresponding vaccines twice (on Day 0 and Day 21). Sera were collected for the analysis of neutralizing titers against multiple SARS-CoV-2 variants on Day 42.

For the booster immunization study, C57BL/6J mice (n=8/group) were primed with 2 doses of D614G-matched vaccine on Day 0 and Day 14. Mice received a booster shot with SCTV01E, D614G or Omicron BA.1 monovalent vaccine on Day 70 and Day 182, respectively. The serological tests were performed on Day 98 and Day 189. The dose levels of each experiment are described in the figure legends.

Pseudovirus neutralization assay

All the SARS-CoV-2 pseudoviruses carrying the firefly luciferase genes were manufactured by Sinocelltech Ltd (Beijing, China) (Table S2 in Supporting Information). Both Luciferase Assay System (E1501) and Passive Lysis 5x Buffer (E1941) were purchased from Promega (USA). Mouse sera after heat-inactivated at 56°C for 30 min were serially diluted in DMEM+10% FBS, followed by incubation with different pseudotyped SARS-CoV-2 variants (200 $TCID_{50}$) in 96-well plates at 37°C for 1 h, respectively. Afterward, the mixture was co-cultured with 2×10^4 Huh-7 cells (Chinese Culture Tissue Collection Center, China) at 37°C for 20 h. The bioluminescence signal was determined by CentroXS3 LB 960 Microplate Luminometer. The 50% inhibitory dilution (IC_{50}) was calculated using the Reed-Muench method (Reed and Muench, 1938).

ELISpot assay

IFN-γ and IL-4 ELISpot assays were performed according to manufactory protocol (MabTech, 3321-4AST-2 & 3311-4APW-2) to determine antigen-specific T-cell responses. The peptide pools, 15-mer peptides with 11 overlapping amino acids covering the entire SARS-CoV-2 spike protein, were used as stimuli (SciLight Biotechnology, Beijing, China) because 90% of the $CD4^+$ T cell epitopes and 97% of the $CD8^+$ T cell epitopes are conserved in the Alpha and Beta variants compared with the ancestral strain (Tarke et al.,

2021). Briefly, the splenocytes (2×10^5 cells/100 μL per well) were cultured into the plate. Then, the Spike S peptide pools (2 μg mL^{-1}) were added. Unstimulated cells were used as a negative control. After incubation for about 20 h, IFN-γ or IL-4-positive cells were measured using the Enzyme-Linked Spot Analyzer (ImmunoSpot® S6, CTL, USA). The negative control value was subtracted from the sample value. Values below zero were presented as zero. ELISpot analysis was performed as previously described (Wang et al., 2022b).

Flow cytometry

The spleens from different antigen-immunized mice were prepared for the single splenocyte suspension, which was further stimulated with either SARS-CoV-2 wild-type or Omicron S peptides (SciLight Biotechnology), at 37°C for 20 h in a humidified incubator with 5% CO_2. Afterwards, cells were washed with PBS and centrifuged at 200×*g* for 5 min. Then, the supernatant was discarded and the cells were re-suspended in PBS. For the detection of T cell activation, splenocytes were stained with BV510 anti-mouse CD3e antibody (BioLegend, USA), FITC-conjugated rabbit anti-mouse CD4 antibody (Sino Biological), APC-conjugated rabbit anti-mouse CD8a antibody (Sino Biological), BV650 hamster anti-mouse CD69 (BioLegend), PE-conjugated rat anti-mouse CD137 antibody (BD Biosciences, USA), and Brilliant Violet 421TM anti-mouse CD134 antibody (BioLegend), BV650 Rat Anti-Mouse CD44 (BD Biosciences), and BV421 Rat Anti-Mouse CD62L (BD Biosciences) at 4°C for 20 min in the dark. After staining, the cells were rinsed with PBS. Flow cytometry was performed using BD FACS Celesta™ Cell Analyzer (BD Biosciences). The analysis was performed using FlowJo software.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism™ (version 8.0). Two-tailed Student's *t*-test was used to compare two independent experiment groups. Comparisons among multiple groups were performed using Kruskal-Wallis test (nonparametric One-Way ANOVA). *P* values<0.05 were considered significant.

Compliance and ethics *The author(s) are employees of Sinocelltech Ltd. and have an ownership or the potential stock option of the company.*

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SUPPORTING INFORMATION

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