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Three exposures to the spike protein of SARS-CoV-2 by either infection or vaccination elicit superior neutralizing immunity to all variants of concern

Paul R. Wratil[1,2], Marcel Stern[1], Alina Priller[3], Annika Willmann[4], Giovanni Almanzar[5], Emanuel Vogel[4], Martin Feuerherd[4], Cho-Chin Cheng[4], Sarah Yazici[3], Catharina Christa[4], Samuel Jeske[4], Gaia Lupoli[1], Tim Vogt[5], Manuel Albanese[1,#], Ernesto Mejías-Pérez[1], Stefan Bauernfried[6], Natalia Graf[4], Hrvoje Mijocevic[4], Martin Vu[4], Kathrin Tinnefeld[4], Jochen Wettengel[2,4], Dieter Hoffmann[2,4], Maximilian Muenchhoff[1,2], Christopher Daechert[1], Helga Mairhofer[1], Stefan Krebs[7], Volker Fingerle[8], Alexander Graf[7], Philipp Steininger[9], Helmut Blum[7], Veit Hornung[6], Bernhard Liebl[8], Klaus Überla[9], Martina Prelog[5], Percy Knolle[3], Oliver T. Keppler[1,2], Ulrike Protzer[2,4]

These authors contributed equally: Paul R. Wratil[1,2], Marcel Stern[1], Alina Priller[3]

[1]Max von Pettenkofer Institute and Gene Center, Virology, National Reference Center for Retroviruses, Faculty of Medicine, LMU München, Munich, Germany

[2]German Centre for Infection Research (DZIF), Partner Site, Munich, Germany

[3]Institute of Molecular Immunology and Experimental Oncology, University Hospital rechts der Isar, Technical University of Munich (TUM), School of Medicine, Munich, Germany

[4]Institute of Virology, Helmholtz Center Munich / Technical University of Munich (TUM), School of Medicine, Munich, Germany

[5]Pediatric Rheumatology / Special Immunology, Pediatrics Department, University Hospital Würzburg, Würzburg, Germany.

[6]Gene Center and Department of Biochemistry, LMU München, Munich, Germany

[7]Laboratory for Functional Genome Analysis, Gene Center, LMU München, Munich, Germany

[8]Bavarian Health and Food Safety Authority (LGL (LGL), Oberschleißheim, Germany

[9]Institute of Clinical and Molecular Virology, University Hospital Erlangen, Friedrich-Alexander Universität Erlangen-Nürnberg, Erlangen, Germany

[#] Present address: National Institute of Molecular Genetics (INGM), Milano, Italy

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***Corresponding authors:**

Oliver T. Keppler, M.D., Max von Pettenkofer Institute, Virology, LMU München, Feodor-Lynen-Straße 23, 81377 Munich, Germany. Phone: +49 (0)89 - 218072901, E-mail: keppler@mvp.uni-muenchen.de

Ulrike Protzer, M.D., Institute of Virology, Technical University of Munich / Helmholtz Zentrum München, Trogerstr. 30, 81675 Munich, Germany. Phone: +49-(0)89-41406886, E-mail: protzer@tum.de; protzer@helmholtz-muenchen.de; Twitter: #UlrikeProtzer

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Abstract

Infection-neutralizing antibody responses after SARS-CoV-2 infection or COVID-19 vaccination are an essential component of antiviral immunity. Antibody-mediated protection is challenged by the emergence of SARS-CoV-2 variants of concern (VoCs) with immune escape properties, such as omicron (B.1.1.529) that is rapidly spreading worldwide. Here, we report neutralizing antibody dynamics in a longitudinal cohort of COVID-19 convalescent and infection-naive individuals vaccinated with mRNA BNT162b2 by quantifying anti-SARS-CoV-2-spike antibodies and determining their avidity and neutralization capacity in serum. Using live-virus neutralization assays, we show that a superior infection-neutralizing capacity against all VoCs, including omicron, developed after either two vaccinations in convalescents or after a third vaccination or breakthrough infection of twice-vaccinated, naive individuals. These three consecutive spike antigen exposures resulted in an increasing neutralization capacity per anti-spike antibody unit and were paralleled by stepwise increases in antibody avidity. We conclude that an infection-plus-vaccination-induced hybrid immunity or a triple immunization can induce high-quality antibodies with superior neutralization capacity against VoCs, including omicron.

Keywords

SARS-CoV-2; COVID-19; variant of concern; omicron; neutralizing antibodies; vaccination; immunity; breakthrough infection; antibody avidity

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Main Text

The World Health Organization classified B.1.1.529 (omicron) on November 26, 2021 as a SARS-CoV-2 variant of concern (VoC). Omicron has since become the dominant VoC in most countries[1]. Earlier VoCs showed either an enhanced ability for transmission (VoCs alpha (B.1.1.7) and delta (B.1.617.2)) or a partial immune escape with variable effects on neutralization by polyclonal serum antibodies (VoCs beta (B.1.351), gamma (P.1/B.1.1.28)) and delta)[2-7]. A striking characteristic of the VoC omicron, that apparently developed independently, is the large number of amino acid substitutions, insertions and deletions in the viral spike protein - 32 compared to the original Wuhan-hu-1 virus[8] - that likely contribute to its extraordinarily rapid spread in the population. Since the number of epitopes in the spike protein, which are relevant for neutralization and are targeted by polyclonal antibody responses in COVID-19 convalescent or vaccinated naive individuals, is an important determinant of the genetic barrier to viral escape from humoral immunity[6,9], physician-scientists anticipated early on omicron's potential for a pronounced immune escape.

Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection[10]. Affinity maturation of neutralizing antibodies can markedly alter their capacity to control SARS-CoV-2 variants[11]. In general, somatic hypermutations in variable regions of antibodies increase their binding affinity depending on type and duration of antigen exposure[6,12]. Affinity maturation can markedly expand the breadth and efficiency of neutralizing antibodies against SARS-CoV-2[13]. This may even enable the neutralization of emerging virus variants that have evolved to escape neutralization by ancestral antibodies.

In this study, we characterized the antibody response in a longitudinal cohort of 98 convalescent individuals, infected with SARS-CoV-2 during the first pandemic wave in spring 2020, and 73 infection-naive individuals matched for sex, age, working conditions and risk factors[14]. We quantified anti-spike IgG titers, IgG antibody avidity and infection-neutralizing capacity in serum samples from these two groups collected after the first, second and third vaccination with the mRNA BNT162b2 COVID-19 vaccine. The aim of the study was to characterize the dynamics of infection neutralization against SARS-CoV-2 and its VoCs after different timely spaced infection events and vaccinations.

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Results

Convalescents develop a higher neutralization capacity against all SARS-CoV-2 VoCs than naive individuals after vaccination

We established a cohort of 98 convalescents from mild COVID-19 (for details see **Suppl. Table 1, Extended Data Fig. 1** and Koerber et al.[14]), of which 6 were excluded because of suspected SARS-CoV-2 re-exposure and 62 were followed up after vaccination. 73 infection-naive individuals were randomly matched for age, sex and infection exposure risk. These individuals were continuously followed since the first wave of the COVID-19 pandemic in spring 2020, through their initial COVID-19 vaccinations with mRNA BNT162b2 in early 2021 and after a third vaccination during the last quarter of 2021, with a total of 486 serum samples collected. In this cohort, we determined the dynamics of anti-SARS-CoV-2 spike antibodies and serum neutralization capacity against the early clinical SARS-CoV-2 isolate B.1.177 (EU1) and all five VoCs: B.1.1.7 (alpha), B.1.351 (beta), P.1/B.1.1.28.1 (gamma), B.1617.2 (delta) as well as B.1.1.529 (omicron) (**Extended Data Fig. 1**). The first (#1) and second (#2) COVID-19 vaccination were given three weeks apart, and the third vaccination dose (#3) was applied 9 months later.

To quantify infection neutralization, we employed a novel, high-throughput live virus neutralization assay comprising all known VoCs that were isolated from COVID-19 patients. Hereby, immortalized human MDA-MB-231 cells expressing the angiotensin-converting enzyme 2 (hACE2) receptor (MDA-MB-231-hACE2 cells)[15,16], which are highly susceptible to SARS-CoV-2 infection and display a strong cytopathic response to infection, allowed for the rapid quantification of neutralizing activities against SARS-CoV-2. Sera from COVID-19 convalescents collected approx. 9 months after infection showed a low-level infection-neutralization capacity against the early 2020 SARS-CoV-2 variant EU1 and against all VoCs (**Fig. 1a**). After a first vaccination (#1) with mRNA BNT162b2, serum neutralization titers of convalescents showed a 63-fold increase on average, while titers in infection-naive vaccinees remained close to background (**Fig. 1b**). Neutralization titers in naive individuals markedly increased after vaccination #2, still remaining significantly lower than those of convalescents (**Fig. 1c**). Interestingly, even at 4 and 7 months after vaccination #2, no significant difference in neutralization capacity was detected comparing convalescents vaccinated once or twice within a three-week interval (**Fig. 1d, Extended Data Fig. 2**). Although in naive individuals the infection-neutralization capacity after vaccination #2 was significantly lower than that of vaccinated convalescents (**Fig. 1a-d**), the relative ability of

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individual VoCs to escape neutralization relative to EU1 at 7 months after vaccination #2 was similar for convalescent and naive individuals (**Fig. 1e, Extended Data Fig. 3**). Overall, the infection-neutralization capacity for omicron and, albeit less pronounced, for beta was lower than for the other SARS-CoV-2 variants confirming the immune escape properties of these two VoCs (**Fig. 1a-e**, **Extended Data 2,3**). 40.6% (95% confidence interval: 29.4 – 52.9 %) of naive individuals, but only 4.0% (95% confidence interval: 1.1 – 13.5 %) of convalescents showed no neutralization activity against omicron 7 months after the initial vaccinations.

Strikingly, after COVID-19 vaccination #3, administered 9 months after vaccinations #1 and #2, the infection-neutralization capacity against all VoCs, including omicron, reached high levels in both naive and convalescent individuals (**Fig. 1f**). Again, infection-neutralization capacity remained significantly higher in vaccinated convalescents, and there was no difference whether convalescents had received one or two vaccine doses (**Fig. 1f**). **Fig. 1g** summarizes neutralization of VoCs compared to that of EU1, highlighting both the prominent immune escape properties of omicron and the impact of a third vaccination in naive individuals that was able to partially counteract this pathogen's evolution.

Overall, COVID-19 convalescents showed a higher neutralization capacity against all SARS-CoV-2 VoCs compared to infection-naive individuals, even after three vaccinations in the latter. The omicron VoC is characterized by an unprecedented escape from antibody neutralization in serum samples from convalescents and naive individuals at all time-points of this study.

Increased infection-neutralization capacity is associated with higher antibody avidity

The higher neutralization capacity of convalescents in light of the immune escape properties of the omicron VoC prompted us to investigate the longitudinal dynamics of infection-neutralization and compare these to binding antibody titers against the S1 domain and polyclonal antibody-binding strength to the S1 and S2 ectodomains of the spike protein of the original Wuhan SARS-CoV-2 strain. Serum anti-spike IgG levels reached their maximum in convalescents after one vaccine dose, and in naive individuals after two vaccinations (**Fig. 2a**). Subsequently, IgG levels declined in both groups at 4 months and even more so at 7 months after vaccination #2, albeit more rapidly in naive individuals (**Fig. 2a**). After vaccination #3, serum anti-spike IgG levels increased markedly compared to 7

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months after the initial vaccinations, on average by a factor of 2.7 and 9.6 for vaccinated convalescent and naive individuals, respectively (**Fig. 2a**).

The marked decline in serum anti-spike IgG levels in both study groups following vaccination #2 (**Fig. 2a**) was contrasted by a substantial infection-neutralization capacity of convalescents against all VoCs (**Fig. 1d**). This lack of a direct correlation between antibody titers and infection-neutralization capacity led us to re-analyse the data from our cohort for the dynamics of neutralization activity against the different VoCs over time (**Extended Data Fig. 4**). We found that neutralization capacity in infection-naive individuals, which was particularly low against omicron, significantly increased after vaccination #3 (**Fig. 2b,c**). In convalescents, vaccination #3 further increased their capacity to neutralize EU1 as well as alpha, gamma and omicron, and less pronounced beta or delta VoCs (**Fig. 2b,c, Extended Data Fig. 4, 5).** Specifically, the neutralization capacity against delta, reflected by the IC_{50} value, showed an 8.1-fold increase in naive individuals, but only a 4.6-fold increase in convalescents (**Fig. 2d**). Against omicron, a >42-fold increase in naive individuals and a >14-fold increase in convalescents, respectively, were observed (**Fig. 2e**), indicating the particular relevance of a third vaccination to be able to neutralize this VoC.

To better assess the relative efficacy of serum antibodies for virus neutralization we determined the ratio between the IC_{50} neutralization and anti-spike IgG titers. Notably, we observed a high neutralization capacity per antibody unit in sera of convalescents against EU1 and all VoCs, including omicron, that slightly increased after vaccination #2 and became more pronounced after vaccination #3 (**Fig. 2f,g, Extended Data Fig. 6**). For naive individuals, in contrast, this ratio was low after vaccination #1 and #2, increased over time (m4 and m7) and further after vaccination #3, reaching levels comparable to those seen in convalescents (**Fig. 2f,g, Extended Data Fig. 6**).

Collectively, these results suggest a maturation of antibody responses over time and after each encounter with the SARS-CoV-2 spike protein. Conceptually, this could be due to either an increased breadth of the polyclonal neutralizing antibody repertoire directed against the spike protein or an increase of their strength of binding to the spike protein. To experimentally address the latter, we quantified the avidity of serum IgG binding the S1/S2 SARS-CoV-2 spike protein ectodomain of the original Wuhan-hu-1 SARS-CoV-2 strain. In convalescents we detected a step-increase in antibody avidity after a single vaccine dose, which remained largely stable over the following 7 months and did not further increase after vaccination #3 (**Fig. 2h**). In convalescents, this is consistent with a maturation of spike-

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specific antibodies that have been reported after SARS-CoV-2 infection[17,18] and which required only a single vaccination to reach maximal avidity. Hereby, the long time period of nine months after infection may have supported a matured antibody response. In naive individuals, however, spike protein-specific antibody avidity only increased 7 months after vaccination #2, and vaccination #3 was required to increase the avidity to levels comparable to those in vaccinated convalescents (**Fig. 2f**). Taken together, these results suggest that an increase in antibody avidity may be critical for a highly potent infection-neutralization, and provide mechanistic insight into the exceptional benefit of a third vaccination in infection-naive individuals or two timely-spaced vaccination in convalescents to counteract VoCs with immune escape potential such as omicron.

Delta and omicron breakthrough infections in twice-vaccinated, naive individuals boost neutralizing responses comparably to a third vaccination

To explore the applicability of the findings in our longitudinal cohort of the high immune-protective benefit of three separate exposures to SARS-CoV-2 spike antigen – either from vaccination alone or from infection and vaccination - in a real-world scenario, we investigated a second cohort of 31 individuals with 16 delta and 15 omicron breakthrough infections. Of these, 30 individuals had received two vaccine doses and one person had been vaccinated with a single dose of Ad26.COV2.S, on average 5 months earlier (**Suppl. Table 2**). In this second cohort, we determined infection-neutralization titers on average seven days after PCR-based diagnosis of a breakthrough infection. Remarkably, neutralization titers were significantly higher among these 31 individuals than among twice-vaccinated naive study participants of the first cohort and comparable to those detected in twice-vaccinated convalescent and triple-vaccinated naive individuals of the first cohort two weeks after the last vaccination (**Fig. 3a**). We did not detect significant differences in the infection-neutralization capacity against the different VoCs, including omicron, between individuals with either delta or omicron breakthrough infections (**Fig. 3a**). Although not statistically significant, individuals seven days after delta breakthrough infection seemed to neutralize the omicron VoC less well. Findings were similar when analysing only individuals of the second cohort vaccinated twice with mRNA BNT162b2 (**Extended Data Fig. 7**). This observation corresponded well to the increased antibody avidity to the Wuhan-hu-1 spike protein after a delta or omicron breakthrough infection (**Fig. 3b**). Interestingly, we detected

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increasing antibody avidity in single individuals over time in a longitudinal analysis following delta breakthrough infection (**Fig. 3c**) that did, however, not reach statistical significance.

Together, the results obtained in this independent cohort of vaccinated individuals with newly diagnosed SARS-CoV-2 breakthrough infections corroborated the findings from the longitudinal analysis in the first cohort: both for vaccinated naive and for convalescent individuals a total of three timely spaced challenges of the immune system with SARS-CoV-2 spike protein, irrespective of the type of exposure, led to superior infection-neutralization capacity.

Discussion

Using a rapid and sensitive high-throughput infection-neutralization assay with replication-competent, clinical isolates of all known SARS-CoV-2 VoCs, we quantified and compared the serum-neutralization capacity in a longitudinal cohort of COVID-19 convalescents and matched infection-naive individuals before and after vaccination. This allowed us to determine the distinct dynamics of infection-neutralization capacity associated with the type and order of antigen exposure in the form of vaccination or infection. Comparison to a second cohort of vaccinated individuals with recent delta and omicron breakthrough infections identified three timely spaced encounters with SARS-CoV-2 spike protein as the common determinant to reach a superior neutralization capacity against all SARS-CoV-2 VoCs, including the emergent omicron VoC that shows the ability to escape immunity.

We here report four key findings: First, in a direct comparison with all other VoCs, omicron displays the most pronounced humoral immune escape evading antibody neutralization at early and late time points after vaccination. Second, a “hybrid immunity” in convalescents after one mRNA vaccination is not further enhanced by a second vaccination after a short time frame of three weeks. In contrast, a timely spaced, second vaccination after several months further increases neutralization capacity to combat VoCs such as omicron with an unprecedented ability of immune escape. Third, in a longitudinal analysis there is no direct association between anti-spike IgG titers and the infection-neutralization capacity. A stepwise increase in the avidity of SARS-CoV-2 spike-specific antibodies after the first vaccination in convalescents and after the second and third vaccination in naive individuals was noted, consistent with the reported occurrence of affinity-matured memory B cells up to

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6 months after infection[19], highlighting that the quality rather than the mere quantity of antibodies is important. Fourth, triple-vaccinated naive individuals reach almost the same level of neutralization capacity against the immune escape VoC omicron as vaccinated convalescents, as well as individuals who experienced a break-through infection with either the delta or the omicron VoC. Thus, the more rapid induction of high-avidity antibodies in convalescents after vaccination can be compensated for by three mRNA vaccinations in infection-naive individuals, and also develops after a breakthrough infection in twice-vaccinated individuals.

"Hybrid immunity" was achieved either after two mRNA vaccinations in convalescents (first cohort) or after a SARS-CoV-2 breakthrough infection in naive individuals, who had received a two-dose COVID-19 vaccination regimen (second cohort), both resulting in superior infection-neutralizing immune responses against SARS-CoV-2 VoCs including omicron. Of note, a robust neutralization response in convalescents was seen already after a single vaccine dose, and a second shot only increased the response if given with a delay. An alternative path towards a comparably high neutralizing immunity is reported here for individuals triple-vaccinated with BNT162b2, consistent with similar observations by others[20-25].

From our data we conclude that a superior infection-neutralization capacity against SARS-CoV-2 VoCs - including those with immune escape properties - needs to develop over time following a total of three spike antigen exposures. Our results support the notion that a single infection with SARS-CoV-2 does not provide a similar level of protection as the combination of infection and vaccination. Importantly, the dynamics by which the infection-neutralization capacity increased were paralleled by an enhanced avidity of SARS-CoV-2 spike-binding antibodies providing a critical refinement for predicting the efficacy of protective humoral responses against a range of different VoCs.

Further studies will be required to analyse the breadth of the spike-specific antibody repertoire after repeated vaccinations in naive and convalescent individuals, and to characterize the avidity of spike-specific antibodies generated after infection or vaccination specifically to current and future VoCs. While a superior infection-neutralization capacity against immune escape VoCs is induced by repeated exposure to the original SARS-CoV-2 spike protein as encoded by the BNT162b2mRNA vaccine, a boosting and refinement of immunity through VoC-specific vaccines may provide higher and long-lasting protection from infection.

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It should be noted that this study focussed on determining serum infection-neutralization capacity following infection and vaccination as a correlate of protection and identified antibody avidity as an important factor. We, however, lack the information on how the antibody repertoire may evolve over time and did not analyse antibody levels and neutralizing capacity at timepoints shortly before the third vaccination. The study also does neither provide insights into the breadth of antibody responses nor into antibody avidity against the spike of the different VoCs.

Notwithstanding our finding of a superior infection-neutralization capacity after three mRNA vaccinations, protection from severe COVID-19 may already be achieved after two antigen encounters in particular in children and young adults[26]. In this context, cell-mediated immunity elicited by infection or by vaccination likely contributes to protection from severe COVID-19 ref.[27]. In our study, however, we did neither directly assess the protective efficacy of two versus three antigen doses against severe disease nor address the protective effect of T-cell responses. Although the development of infection-neutralization capacity mediated by spike-specific antibodies and antiviral T cell immunity has been shown to develop in parallel[14], further studies are required to elucidate whether three timely spaced encounters with spike antigen also go along with a quantitative and qualitative increase in protective T cell immunity.

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Author contributions

These authors equally share responsibility for this work: P.A.K, O.T.K. and U.P.

Conceptualization: P.A.K., O.T.K., U.P.

Methodology: P.R.W., M.S., A.P., A.W., E.V., N.G., H.M., M.F., C-C.C., G.L., M.A., E.M-P., M.M., S.K., H.M., C.C., J.W., D.H., S.K., G.A., T.V., M.P., O.T.K.

Resources: S.B., P.S., V.F.

Formal Analysis: C.D., M.M., A.G., S.J., N.G., H.M., M.W., E.V., S.Y., K.T., C.K.

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Writing - Original Draft: P.R.W., A.P., P.A.K., O.T.K., U.P.

Writing - Review & Editing: all authors.

Visualization: P.R.W., A.P, P.K., O.T.K., U.P.

Supervision: H.B., V.H., B.L., K.U., P.A.K., O.T.K., U.P.

Funding Acquisition: B.L., K.U., P.A.K., O.T.K., U.P.

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Declaration of interests

The authors declare to have no conflict of interest concerning the study content. Outside of the study they declare the following interests: UP is co-founder, share-holder and board member of SCG Cell therapy, member of the scientific advisory board of Leukocare and member of topic-specific scientific advisory boards of Sanofi-Pasteur, GILEAD and GSK and ad hoc advisor for BioNTech (without remuneration).

Data availability

All primary data that was used to generate the results obtained in this study are available in the source data of this manuscript.

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Figure Legends

Figure 1 | Kinetics and comparison of infection-neutralization activities for SARS-CoV-2 VoCs in naive individuals and convalescents after BNT162b2 vaccination

COVID-19 convalescents (orange), convalescents who received only vaccinations #1 and #3 (red), and naive individuals (blue) at indicated time points before and after BNT162b2 vaccination, convalescents with only vaccination #1 and #3 (red). **a-d**,**f**, serum IC_{50} values for infection-neutralization capacity of SARS-CoV-2 strain EU1 and VoCs alpha, beta, gamma, delta and omicron normalized to 10^7 viral RNA copies shown as box plots with median, bounds between upper and lower quartiles, and whiskers between the 10^{th} and 90^{th} percentiles. Numbers of serum samples analysed are indicated in the following, those against omicron in brackets. **a**, 51 (50) SARS-CoV-2 convalescents at approx. 9 months post infection and 34 (29) SARS-CoV-2 naives prior to vaccination (pre), naives vs. convalescents for omicron $^{**}P$=0.0033, beta $^{***}P$=0.0002, all other VoCs $^{****}P$<0.0001, for all variants $^{****}P$<0.0001. **b**, 59 (56) convalescents and 48 (42) naives at 2 weeks after vaccination #1 (w2). **c**, 23 (22) convalescents and 47 (42) naives at 2 weeks after vaccination #2. **d**, 16 (16) convalescents and 65 (64) naives at 7 months (m7) after vaccination #2 and 34 (34) convalescents having received only vaccination #1, naives vs. twice vaccinated convalescents for all variants $^{****}P$<0.0001, and vs. once vaccinated convalescents for EU1 $^{**}P$ = 0.0011, alpha $^{**}P$=0.0054, beta $^{***}P$=0.0004, gamma $^{**}P$=0.0031, delta $^{****}P$<0.0001, omicron $^{**}P$=0.0034. **e**, fold-reduction of IC_{50} values comparing neutralization of EU1 with that of VoCs depicted as box plots with median, bounds between the upper and lower quartiles, and whiskers between the 10^{th} and 90^{th} percentiles in 50 convalescents and 64 naives (blue) at m7; numbers above boxes indicate average (avg.) fold changes comparing EU1 and VoCs; in convalescents comparing EU1 to alpha $^{**}P$=0.0017, delta $^{***}P$=0.0005 all other VoCs $^{****}P$<0.0001, and in naives comparing EU1 and alpha $^{***}P$=0.0002, all other VoCs $^{****}P$<0.0001. **f**, 14 convalescents and 59 naives at 2 weeks after vaccination #3 and 22 convalescents who received only vaccination #1 and #3; naives vs. twice vaccinated convalescents for gamma $^{**}P$ = 0.0064, delta $^{**}P$ = 0.0025, omicron $^{**}P$=0.0069, and vs. three-times vaccinated convalescents for alpha $^{*}P$ = 0.0307, beta $^{*}P$=0.0155, gamma $^{*}P$=0.0342, delta $^{*}P$=0.0115, omicron $^{**}P$=0.0089. **g**, heatmap illustrating avg. fold-reduction of IC_{50} values for VoCs compared to IC_{50} values for EU1 in convalescent (conv.) and naive participants. Connecting lines indicate statistically significant differences between groups. Absence of connecting lines or asterisks indicates absence of significance. Statistics were done using Mann-Whitney test (a-c), Kruskal-Wallis-test with Dunn's multiple testing correction (d, f) and

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two-sided Friedman test with Dunn's multiple testing correction (e). Abbreviations, pre: prior to first vaccination; #1 – first vaccination; #2 – second vaccination; #3 – third vaccination; w2 – two weeks after respective vaccination; m4 – 4 months after vaccination #2.

Figure 2 | Longitudinal analysis of serum antibody titers, infection neutralization of delta and omicron VoCs and antibody avidity following mRNA BNT162b2 vaccination

a, anti-spike S1 domain IgG titres in 274 sera from 62 convalescents, and 304 sera from 73 naive participants as binding arbitrary units (BAU)/mL, convalescent $^{***}P$=0.0004, naive pre-vaccination (pre) vs. w2 after vaccination (vacc.) #1 $^{***}P$=0.0002, w2 after vacc. #1 vs. m4 after vacc. #2 $^{*}P$=0.0181, m4 after vacc. #2 vs. w2 after vacc. #3 $^{*}P$=0.0123, convalescent m7 after vacc. #2 vs. w2 vacc. #3 $^{***}P$=0.0005, naive w2 after vacc. #1 vs. m7 vacc. #2 $^{***}P$ = 0.0003. **b**,**c**, serum IC_{50} values for infection-neutralization capacity normalized to 10^7 viral RNA copies of SARS-CoV-2 VoCs delta in 266 / 296 (**b**) and omicron and 261 / 279 (**c**) sera from 62 convalescents / 73 naives, respectively; convalescent w2 vacc. #1 vs. m7 vacc. #2 $^{*}P$=0.0357, and vs. w2 vacc. #3 $^{**}P$=0.0043, w2 vacc. #2 vs. m4 vacc. #2 $^{**}P$=0.0049, naive pre vs. m4 vacc. #2 $^{*}P$=0.0197, and vs. m7 vacc. #2 $^{*}P$=0.0376, w2 vacc. #1 vs. m4 vacc. #2 $^{*}P$=0.0236, and vs. m7 vacc #2 $^{*}P$=0.0043. **d**,**e**, heatmaps showing average fold-changes in IC_{50} values for delta (**d**) and omicron (**e**) between the respective time points for convalescent and naive individuals. **f**,**g**, ratios between infection-neutralization IC_{50} values and anti-spike S1 domain antibody titers for (**f**) delta in 263 / 295; convalescent pre vs. m4 vacc #2 $^{**}P$=0.0030, vs. m7 vacc. #2 $^{**}P$=0.0052, and vs. w2 vacc. #3 $^{***}P$=0.0005, w2 vacc. #2 vs. m7 vacc. #2 $^{***}P$=0.0003, and vs. m7 vacc. #2 $^{***}P$=0.0005, naive w2 vacc. #1 vs. m7 vacc. #2 $^{**}P$=0.0027, and vs. w2 vacc. #3 $^{**}P$=0.0032; and (**g**) omicron in 258 / 278 sera from 62 convalescents / 73 naives; convalescent pre vs. m4 vacc. #2 $^{*}P$=0.0340, naive w2 vacc #2 vs. m4 vacc. #2 $^{**}P$=0.0077, and vs. m7 vacc. #2 $^{**}P$=0.0011. **h**, IgG-type anti-spike antibody avidity in 288 sera from 90 convalescents, and 150 sera from 47 naives, convalescent pre vs. m4 vacc. #2 $^{*}P$ = 0.0340, naive w2 vacc. #2 vs. m4 vacc. #2 $^{**}P$=0.0077, and vs. m7 vacc. #2 $^{**}P$=0.0011. **a**-**c**,**h**, box plots with median, bounds between upper and lower quartiles, and whiskers between the 10th and 90th percentiles, SARS-CoV-2 convalescents (orange) and naive participants (blue). **d,e**, medians (lines) and interquartile ranges (error bars). Differences between time points analysed for statistical significance using the Kruskal-Wallis test with Dunn's multiple testing correction, (**a**-**c**,**f**-**h**) $^{****}P$<0.0001. Connecting lines indicate statistically significant differences between groups. Absence of connecting lines or asterisks indicates absence of significance. Abbreviations, inf: after infection; pre: prior to first vaccination; #1 – first vaccination; #2 – second vaccination; #3 – third vaccination; w2 – two

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weeks after respective vaccination; m4 – 4 months after vaccination; m5 – 5 months after infection; m7 – 7 months after vaccination; m8 – 8 months after infection.

Figure 3 | Infection neutralization capacity for SARS-CoV-2 VoCs after breakthrough infection with delta and omicron in vaccinated individuals.

a, serum IC_{50} values for infection-neutralization capacity normalized to 10^7 viral RNA copies of SARS-CoV-2 VoCs in 47 naives (42 for omicron) 2 weeks after vaccination #2 (dark blue), 59 naives (light blue) and 36 convalescents 2 weeks after vaccination #3, as well as 16 and 15 vaccinated individuals on average 7 days after PCR-confirmed breakthrough infections with delta (green) or omicron (purple), respectively; naives 2w after vaccination (vacc.) #2 vs. naives and convalescents 2w after vacc. #3 ****P<0.0001 for all variants, vs. delta breakthrough infection for EU1 $^{***}P$=0.0007, alpha $^{****}P$<0.0001, beta $^{***}P$=0.0010, gamma $^{***}P$=0.0007, delta $^{***}P$=0.0006, omicron $^{***}P$=0.0002, and vs. omicron breakthrough for EU1 $^{*}P$=0.0251, alpha $^{***}P$=0.0003, beta $^{**}P$=0.0024, gamma $^{**}P$=0.0016, delta $^{**}P$=0.0022, omicron $^{****}P$<0.0001. **b**, IgG-type anti-spike antibody avidities in 44 naïve participants 2 weeks (2w) after vaccination #2 (dark blue), 19 naive (light blue) and 18 convalescent participants 2w after vaccination #3, as well as 13 and 13 vaccinated individuals on average 7 days after PCR-confirmed breakthrough infections with delta (green) or omicron (purple), respectively; $^{****}P$<0.0001. **c**, IgG-type anti-spike antibody avidity in vaccinated individuals on average 7 days (n=13), 2 weeks (n=14), 3 weeks (n=10), and 4 weeks (n=11) after PCR-confirmed breakthrough infections with delta. Data are shown as Box plots with median, bounds between upper and lower quartiles, and whiskers between the 10^{th} and 90^{th} percentiles. Differences between groups were analysed for their statistical significance using the Kruskal-Wallis test with Dunn's multiple testing correction. Connecting lines indicate statistically significant differences between groups. Absence of connecting lines or asterisks indicates absence of significance. Abbreviations, inf: after infection; #2 – second vaccination; #3 – third vaccination; w1 – 7 days after infection; w2 – 2 weeks after respective vaccination/infection; w3 – 3 weeks after infection; w4 – 4 weeks after infection.

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Methods

Study participants and sample collection

In a screening effort, 4554 health care workers were tested for SARS-CoV-2 nucleocapsid-specific antibodies with a commercial chemiluminescence immunoassay (iFlash CLIA, YHLO Biotechnology, China) (Erber et al, in press 2022). Convalescents from SARS-CoV-2 infection in the first pandemic wave in March-April 2020 were identified either by positive PCR or by two to four independent serological assays (specificity of ≥98% for each assay results in a specificity of ≥99.96% for the convalescent cohort)[14]. Naive individuals tested negative in at least two different SARS-CoV-2 nucleocapsid-specific IgG assays. 171 (98 convalescent and 73 naive) individuals were enrolled into a follow-up study that was conducted from April 2020 onwards at the University Hospital rechts der Isar of the Technical University of Munich (**Suppl. Table 1**). The study scheme is depicted in **Extended Data Fig. 8**. No statistical methods were used to pre-determine sample sizes but our sample sizes increase those reported in previous publications[20-25]. Studies were approved by the local ethics committee (ethics vote 476/20 and 26/21S-SR) and participants gave written informed consent to study participation and biobanking.

68 convalescents gave written informed consent for further analyses after their COVID-19 vaccination. 73 SARS-CoV-2 naive individuals were matched by sex, age, working conditions and risk factors present in the convalescent cohort. Median age was 36 (interquartile range [IQR] 29 to 53) years in naive and 40 (IQR 29 to 54) years in convalescent participants. 65.8% naive and 57.6% convalescent participants were female. All naive and 25/68 convalescent individuals continuously followed-up received two doses of BNT162b2 mRNA-vaccine (Comirnaty™, Biontech/Pfizer) as immunization. The interval between the two vaccinations was on average 22 and 21 days for naive and convalescent individuals, respectively. Due to a change in the national guidelines in March 2021, the remaining 43/68 convalescents from the first wave were only vaccinated once with BNT162b2 until mid 2021 assuming that the prior infection substitutes for one vaccination[28]. For all analyses, six convalescent individuals were excluded because they showed a ≥4- and ≥8-fold increase in a surrogate neutralization and in IC_{50} value for neutralization, respectively, independent of vaccination indicating SARS-CoV-2 re-exposure[14].

Sera from 34 naive and 51 convalescent participants were analysed prior to vaccination, from 48 naive and 59 convalescent participants two weeks after the first

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vaccination and from 47 naive and 23 convalescent participants two weeks after the second vaccination. 45 and 72 naive and 51 and 56 convalescent participants were tested four and seven months after their basic immunization, respectively, including 31 and 37 of convalescents who did not receive a second vaccine dose. Finally, sera from 59 naive participants and 36 convalescents were evaluated 2 weeks after receiving an additional BNT162b shot as third immunization after on average 9 months (**Extended Data Fig. 1**).

Additionally, a second cohort of 31 individuals with PCR-confirmed breakthrough infections with SARS-CoV-2 delta or omicron VoC ≥14 days after vaccination #2 were included (cohort 2, **Suppl. Table 2**). This study was approved by the local ethics committee (ethics-vote 229/21) and all participants gave written informed consent. Median age was 35 (IQR 31 to 38) years in delta- and 41 (IQR 28 to 49) years in omicron-infected participants. Specimens were collected on average 7 days (V1), 2 weeks (V2), 3 weeks (V3) and 4 weeks (V4) after the first positive PCR result proving breakthrough infection. VoC-specific PCR and/or whole genome sequencing identified delta (B.1.617.2) in respiratory samples of 16/31 and omicron (B.1.1.529) in respiratory samples of 15/31 individuals. In this cohort, 26/31 participants (84%) had received two doses of an mRNA vaccine (22 BNT162b2, 4 mRNA-1273). 5/31 had received a first vaccination with an adenoviral vector vaccine, two of which subsequently received the same vaccine and two were vaccinated with BNT162b2 (**Suppl. Table 2**). Median time span between first positive PCR result and a complete vaccination cycle was 141 (IQR 99 to 242) days in delta-infected and 166 (IQR 146 to 194) days in omicron-infected individuals.

Antibody detection and avidity assays

IgG-type antibody responses to the Wuhan-hu-1 strain S1 domain of SARS-CoV-2 spike antigen were quantified in 10-fold diluted serum specimens using the commercial Anti-SARS-CoV-2 QuantiVac-ELISA (IgG) (EuroImmun, Germany). Binding strength of the SARS-Cov-2 IgG antibodies was determined by adaptation of the commercial IgG agile SARS-CoV-2 ELISA (Virion/Serion, Germany) using ammonium thiocyanate (NH_4SCN) (Roth, Germany) as chaotropic agent as described previously[29]. Briefly, serum samples were measured using the IgG agile SARS-CoV-2 ELISA and adjusted to 100 BAU/mL according to the standard curve provided by the manufacturer to exclude an influence of variable antibody concentrations. Then, serum samples were incubated in the plates pre-coated with Wuhan SARS-CoV-2-spike-ectodomain S1, S2 and RBD recombinant antigens for 1h at 37°C in a

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humid chamber. After washing, antigen-antibody complexes were incubated in the presence of 1.0 M ammonium thiocyanate or PBS as control for 10 min at room temperature. After washing to remove antibodies bound with low-avidity, the ELISA was completed according to the manufacturer‘s instructions. The relative avidity index was calculated as follows: IgG concentrations (NH4SCN) / IgG concentrations (PBS) x 100 and is given in percent[29,30].

SARS-CoV-2 neutralization assay

High-titer virus stocks were generated by infection of Vero-E6 cells (American Type Culture Collection, ATCC, USA) grown in virus expansion medium (Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum, 100 U/mL penicillin-streptomycin). Cells were incubated with clinical isolates of different SARS-CoV-2 variants (GISAID EPI ISL: 2450298 [EU1/B.1.177], 2095258 [alpha/B.1.1.7], 1752394 [beta/B.1.351], 2095178 [gamma/P.1/B.1.1.28.1], 2772700 [delta/B.1.617.2], 7808190 [omicron/B.1.1.529]). EU1 and the omicron VoC were isolated from nasopharyngeal swabs of COVID-19 patients. Virus stocks were expanded by two passages before harvest and stored at -80 °C. All virus stocks were only used for infection experiments after sequencing of the complete viral genomes. Virus stocks were characterized by rRT-PCR as reported previously[31].

For each individual SARS-CoV-2 VoC, the tissue culture infectious dose resulting in 90% loss of target cell viability ($TCID_{90}$) 48h after infection was determined using a dilution series of the virus stock on MDA-MB-231 cells (ATCC) overexpressing the human angiotensin-converting enzyme 2 receptor (MDA-MB-231-hACE2). For infection neutralization, cells were cultured and infected in 384-well plates (7,500 cells/well). The respective $TCID_{90}$ of each virus stock was incubated for 2 h with different concentrations of each serum to be tested. Subsequently, 10 µL of the virus-serum mixtures were added to 20 µL medium and added to MDA-MB-231-hACE2 cells. 48 h post infection, cytopathic effects were recorded by addition of 10 µL CellTiter-Glo 2.0 reagent (Promega, Wisconsin, USA) and subsequent measurement of bioluminescence signals (0.5 s integration time, no filter) to quantify virus-mediated killing of target cells.

Statistical analysis

Data and statistical analyses were performed in Prism 9 (GraphPad Software, California, USA). $TCID_{90}$ values for tissue culture infectious doses and IC_{50} values for neutralization

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were calculated after normalized, sigmoidal dose response curve approximation of the respective data.

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Figure 1

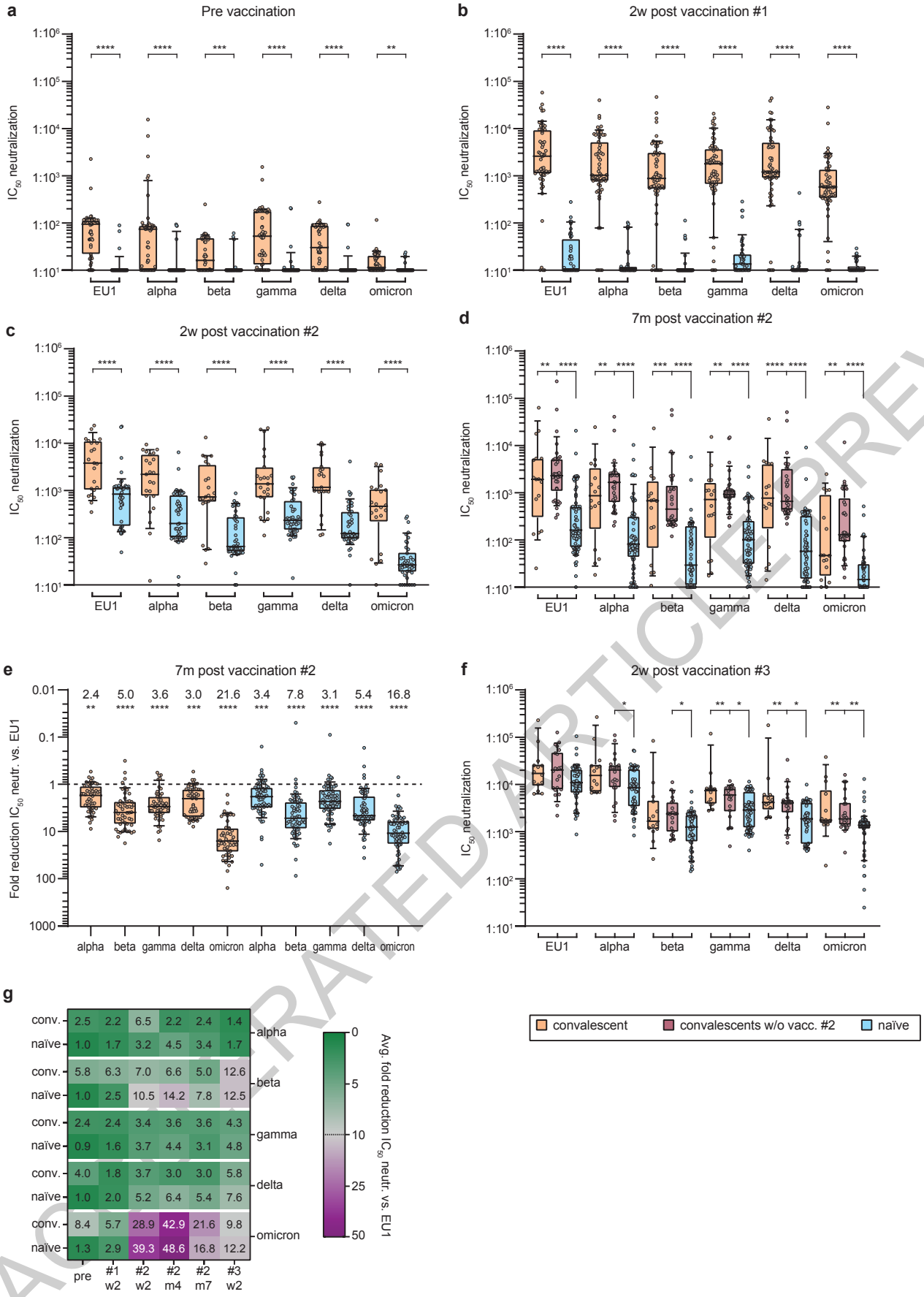

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Figure 2

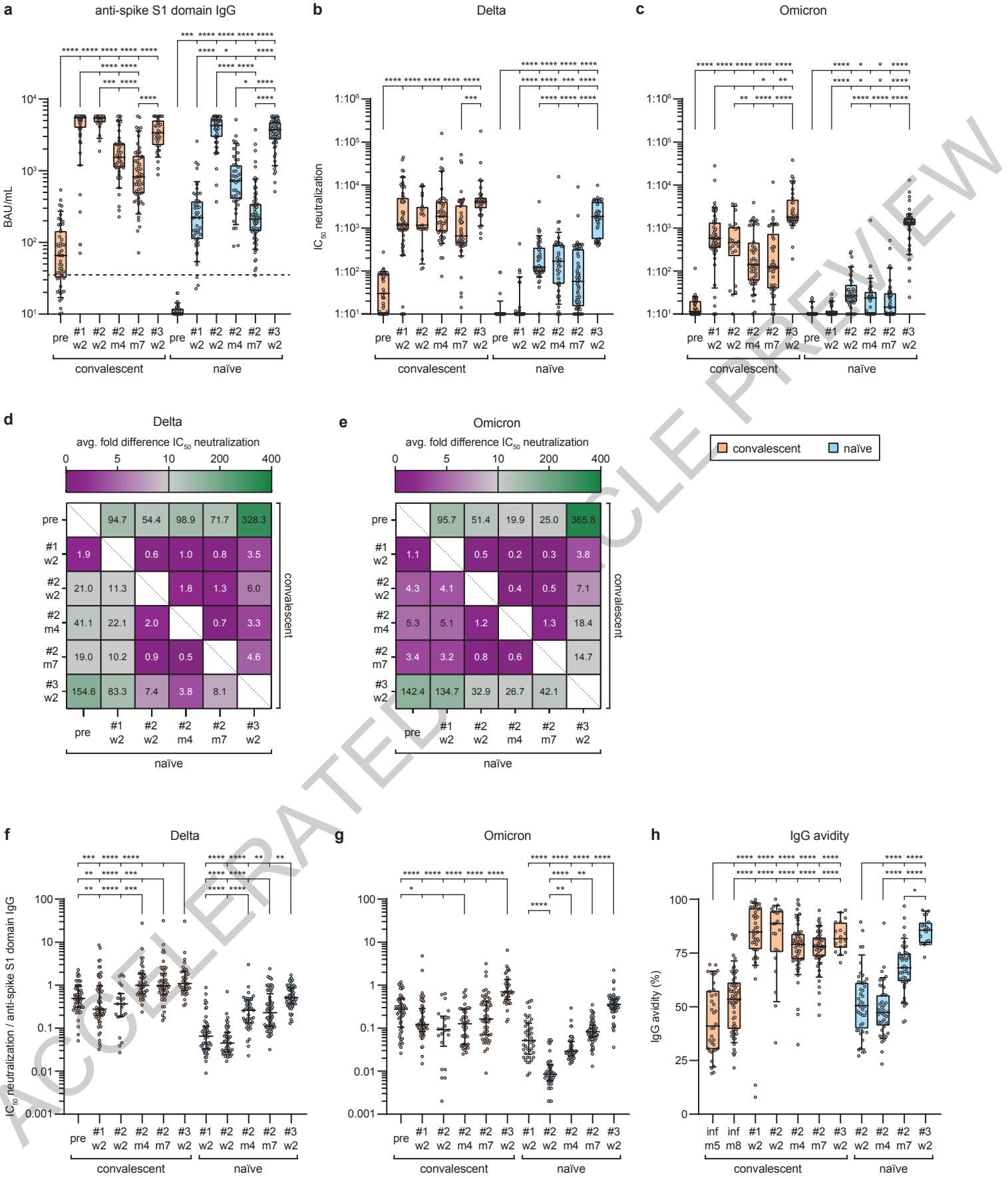

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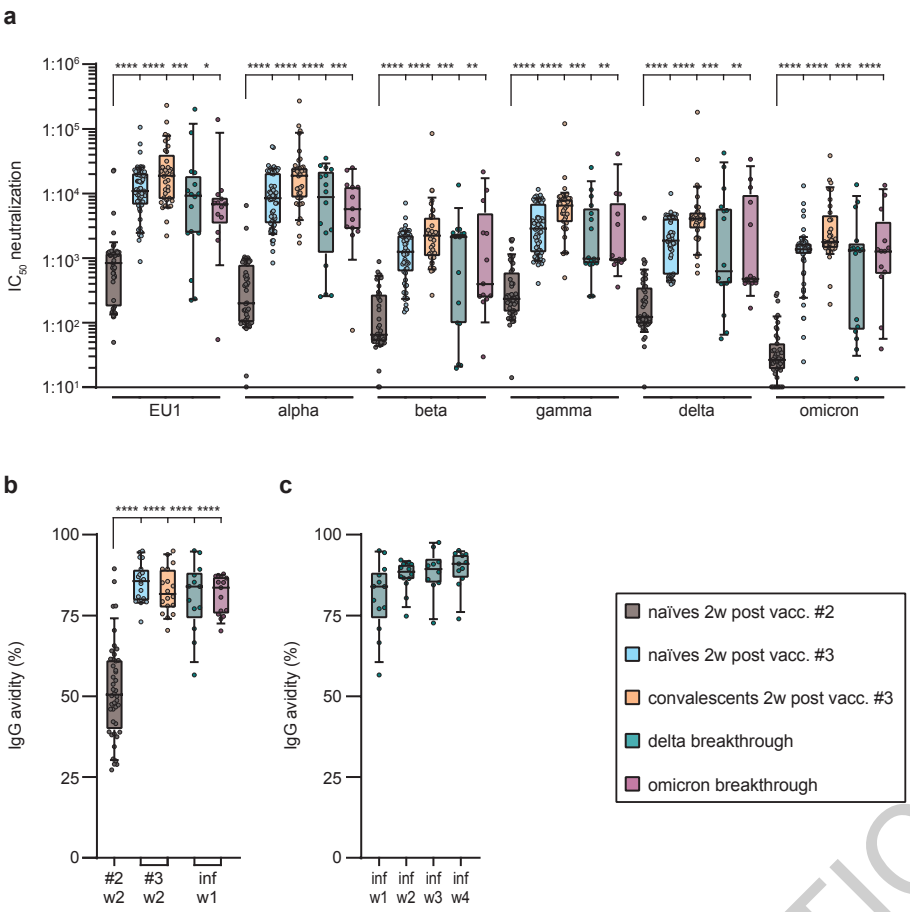
Figure 3



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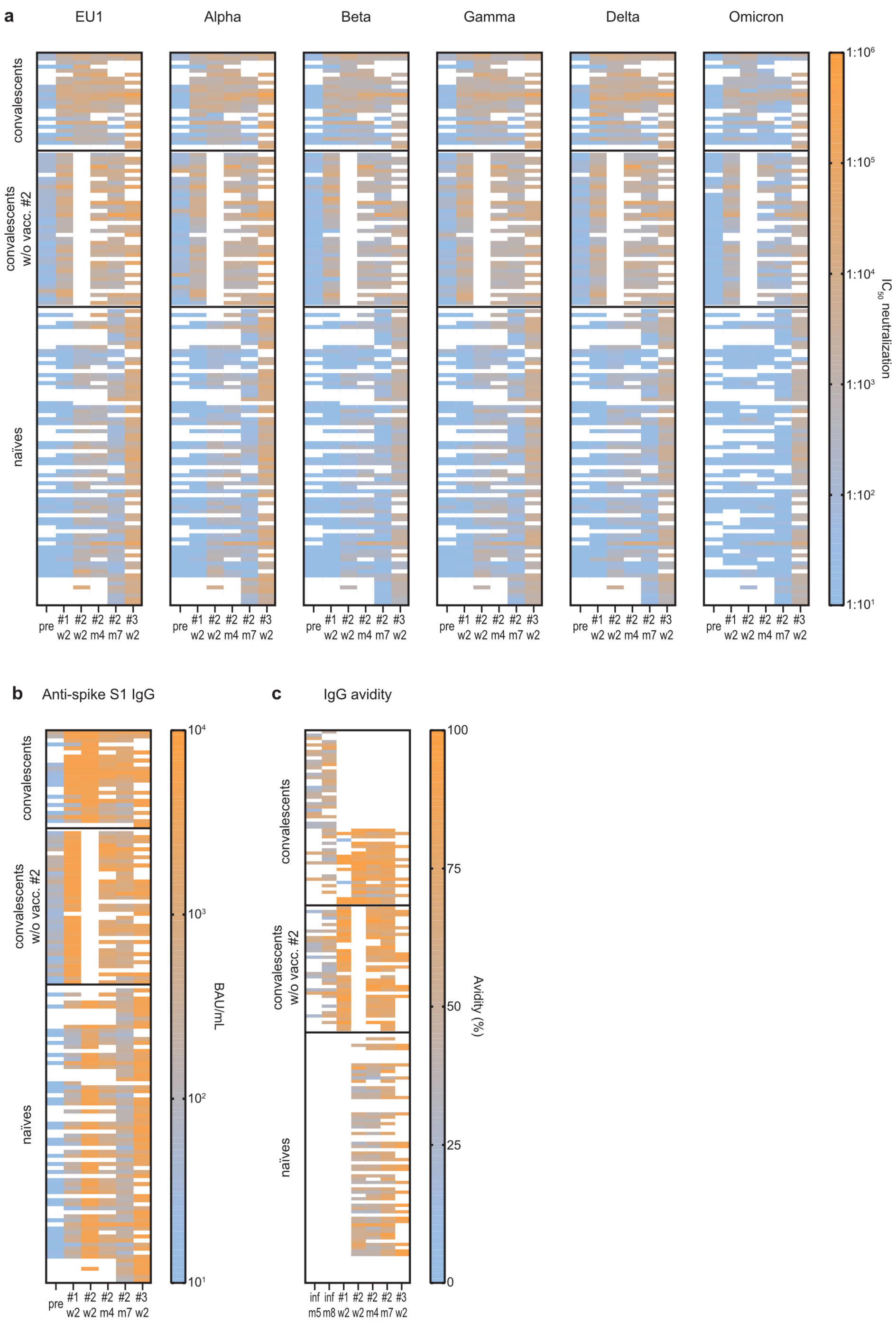
Extended Data Figure 1



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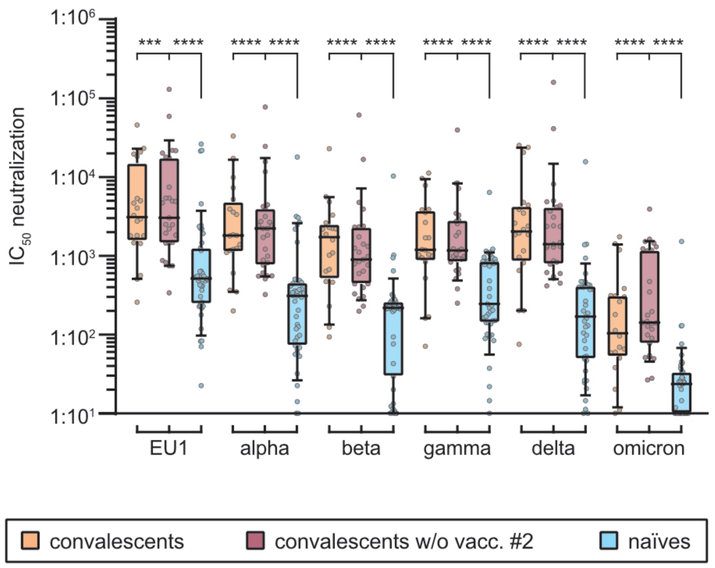

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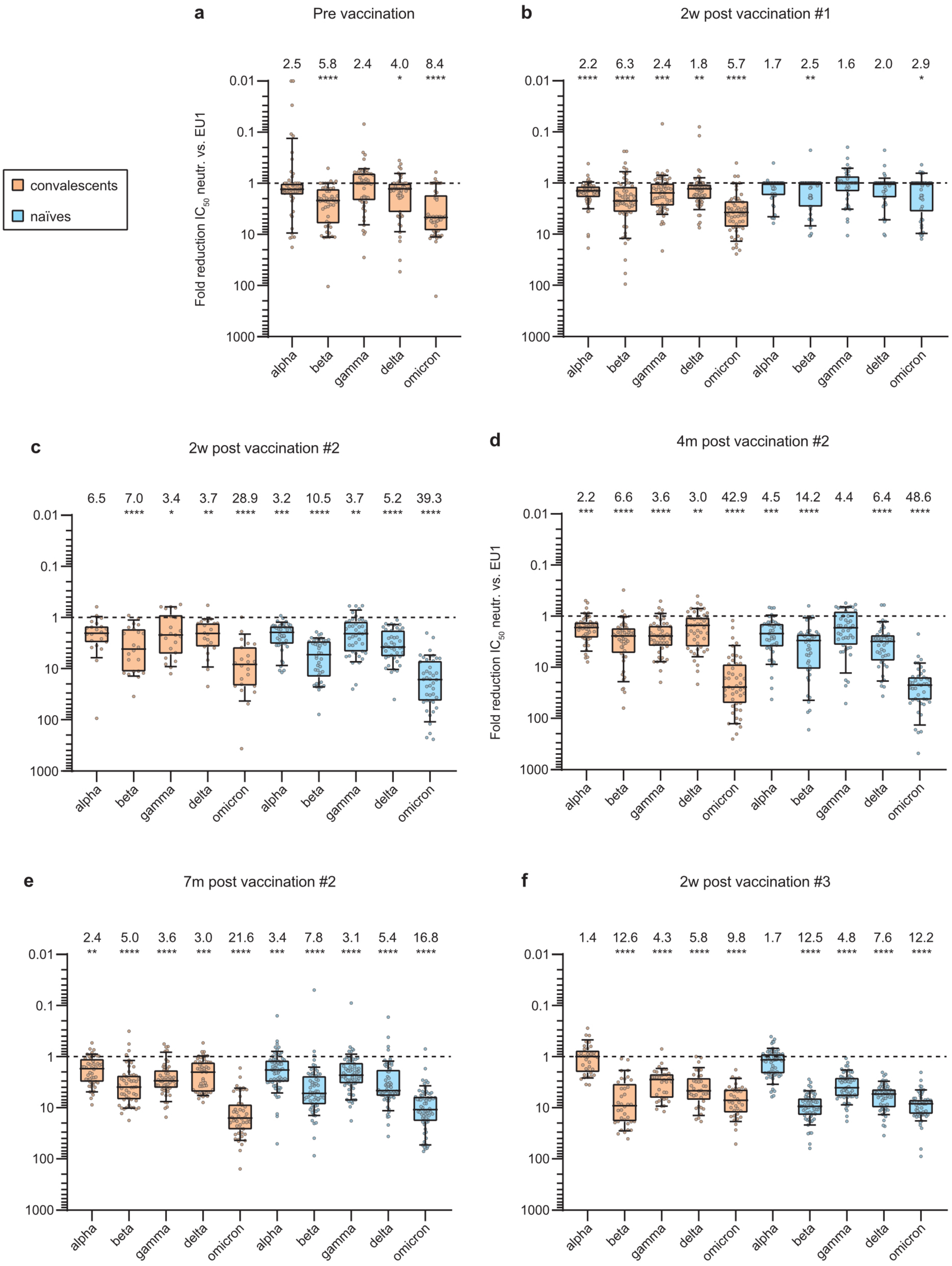

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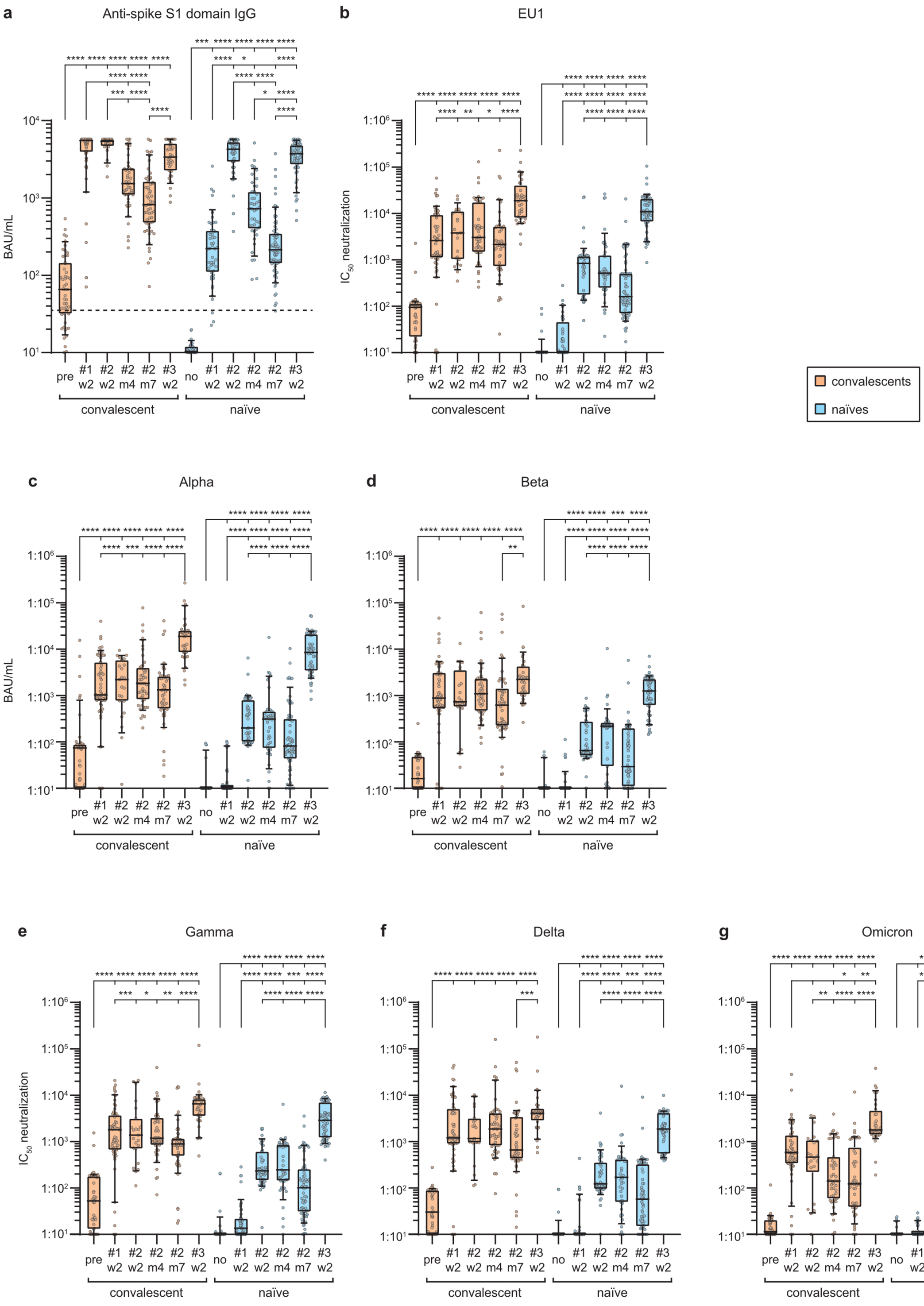

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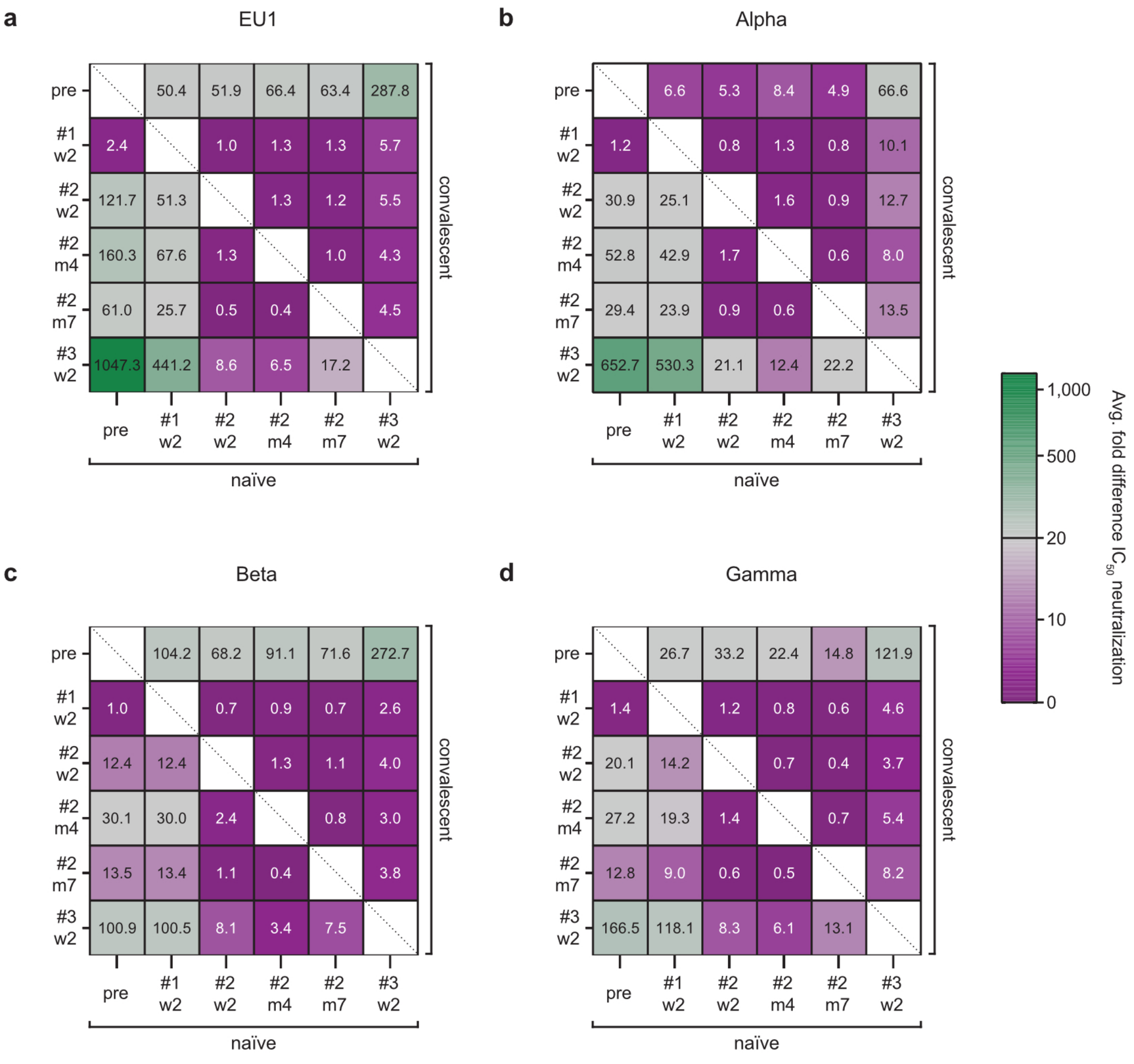

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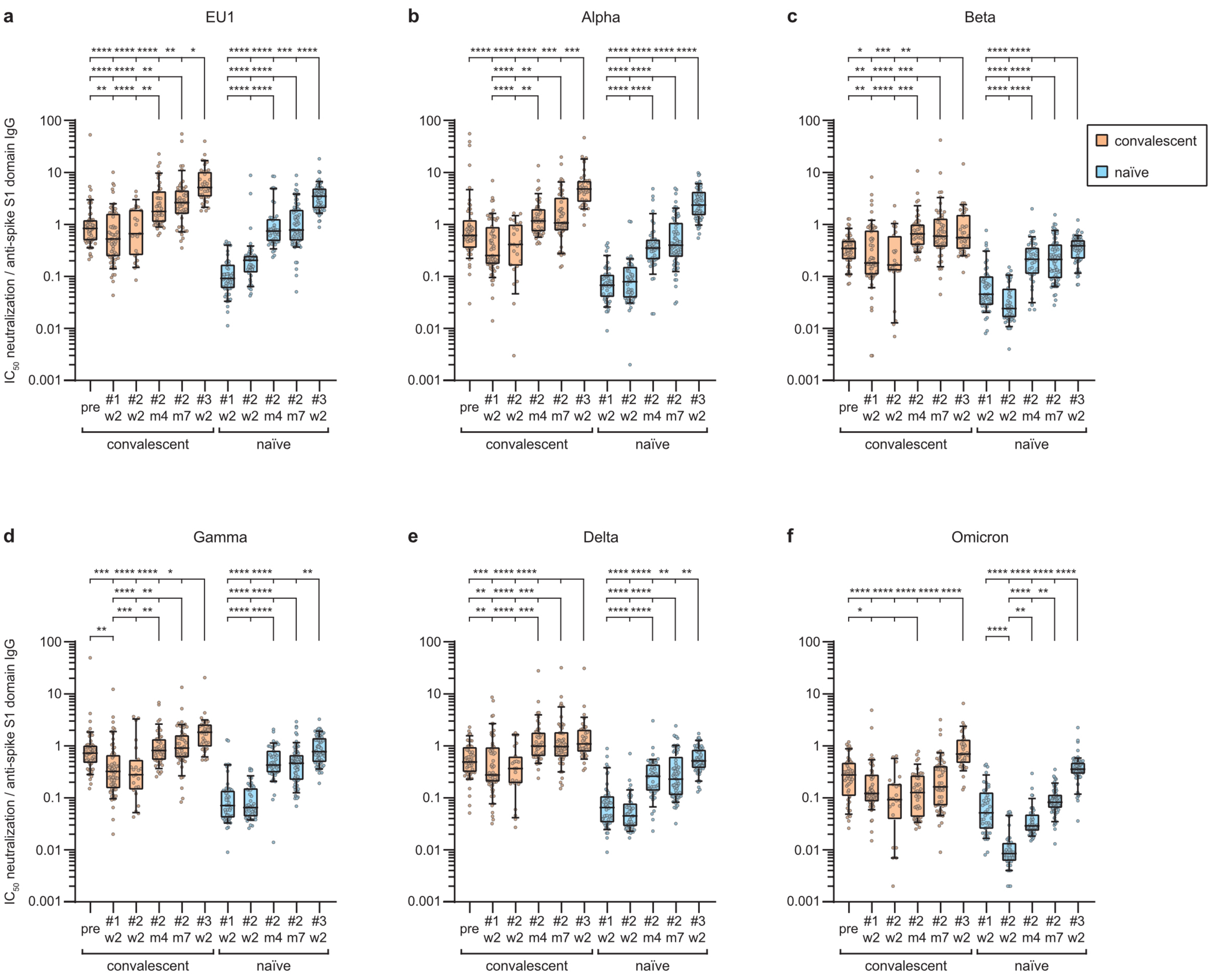

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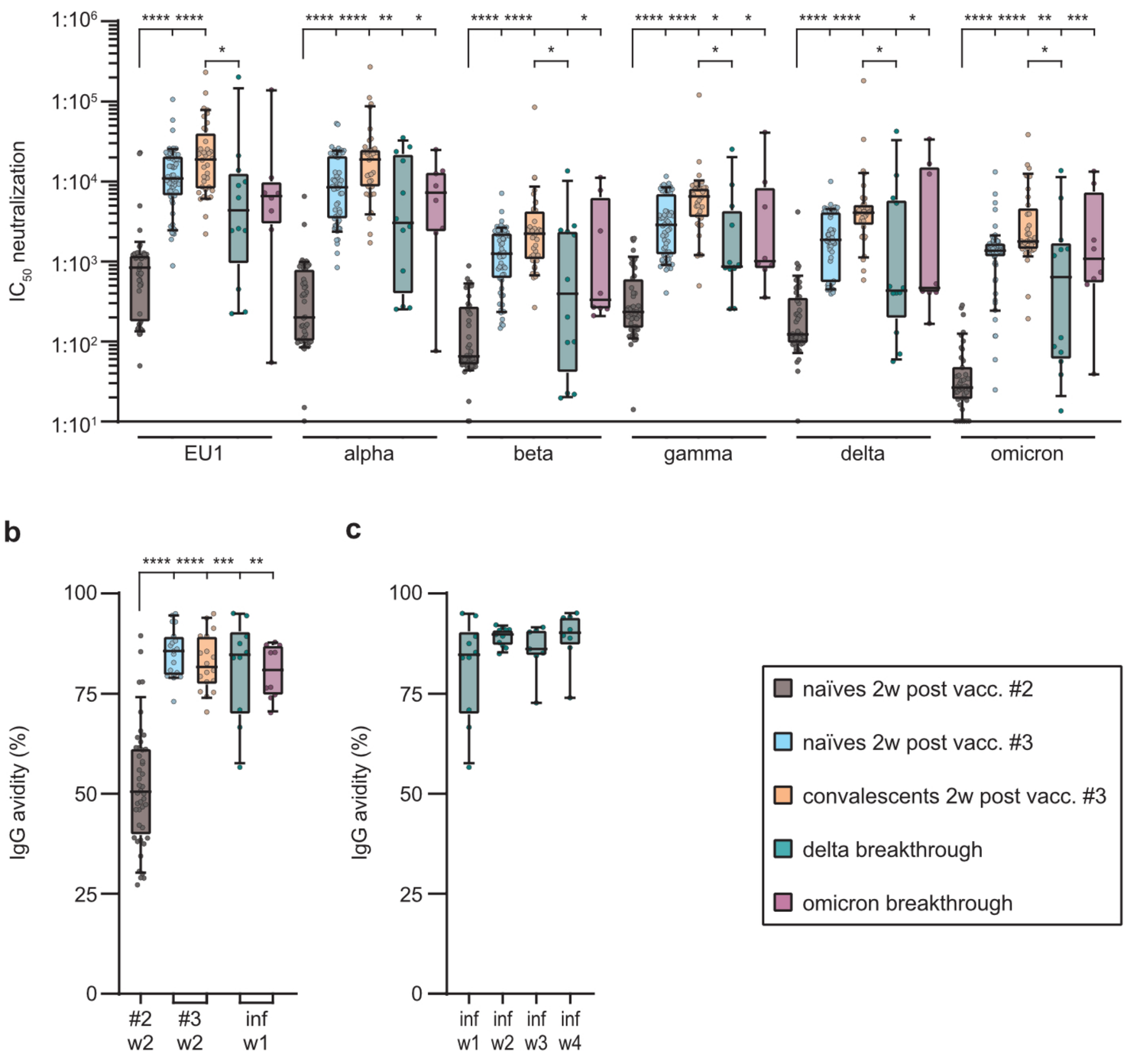
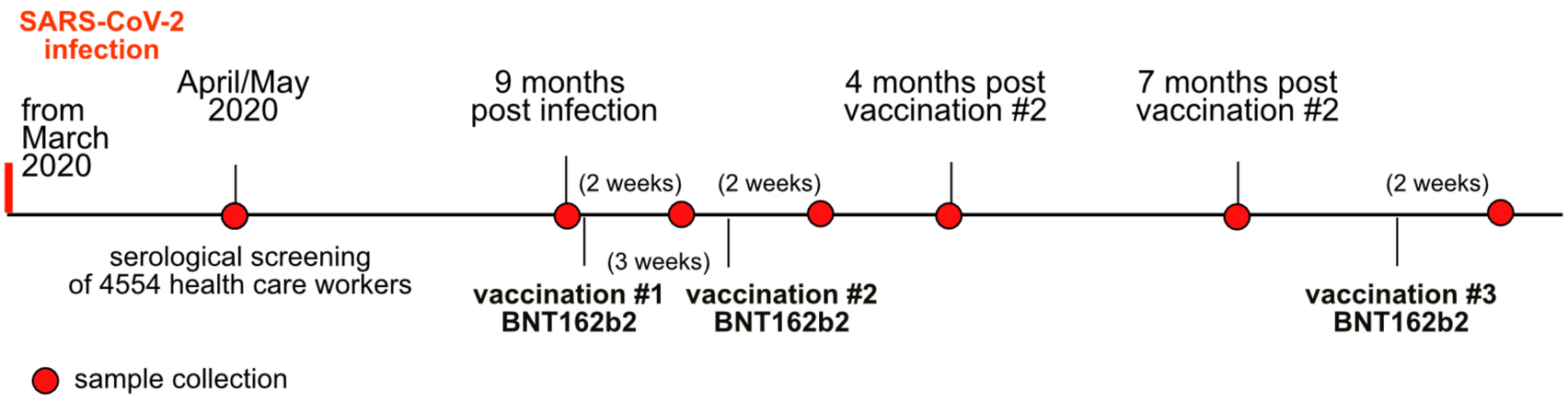


Extended Data Figure 8



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Corresponding author(s): Oliver Keppler and Ulrike Protzer

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☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	All data from participants were obtained after informed written consent. Clinical data from participants were collected in DIS (digital information system, University Hospital rechts der Isar, Technical University of Munich, Germany) that assures anonymization of clinical and laboratory data.
Data analysis	Data was analyzed using Prism 9.3.1. (GraphPad Software, USA)

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All primary raw data that was used to generate the results obtained in this study are available in the source data of this manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	All healthcare workers of a quaternary care hospital were invited using different modes of communication to participate in the study irrespective of their work environment. 4,554 were screened for SARS-CoV-2 infection after giving written informed consent. All COVID-19 convalescent individuals identified were invited to be followed up, of whom 98 agreed and were enrolled in this study. A sex-, age-, working conditions- and risk factor-matched cohort of 73 infection-naive individuals was established from the seronegative participants of the study. In total, 486 serum samples were longitudinally collected from the convalescent and naïve individuals within this cohort. In addition from a second cohort, in which we studied breakthrough infections in vaccinated individuals, sera from 15 vaccinated patients infected with SARS-CoV-2 VoC omicron, and 51 sera from 16 vaccinated patients infected with SARS-CoV-2 VoC delta were analyzed. The number of participants was tested to be suffiecient to allow a statistically significant comparison of the immune response to vaccination in convalescents vs infection-naive inidividuals by the institutional biostatistician.
Data exclusions	Six convalescent individuals were excluded because they showed a ≥8-fold increase in a surrogate assay and in IC50 neutralization, respectively, independent of vaccination indicating a recent SARS-CoV-2 re-exposure.
Replication	The assay to determine binding antibody titers was performed using a commercial, diagnostical assays that is well-validated and makes use of plate-wise calibrators, negative and positive controls. Titers were determined according to WHO standard binding units (BAU) assuring high standardization. Binding antibody titers were confirmed in a second, independent commercial assay before avidity testing. Experiments to determine antibody avidity were performed in duplicates showing low variance between results. The neutralization assay was validated previously showing low variance between results of independent experiments. Furthermore, each sample was tested in the neutralization assay at six different concentrations. Because of the low sample volumes available, experiments to determine neutralization titers were not replicated.
Randomization	4554 health care workers were screened for sub-acute/resolved COVID-19. 98 COVID-19 convalsescent participants were followed up. Naive individuals were randomly matched to the convalescent cohort according to sex, age, working conditions and other risk factors.
Blinding	Laboratory experiments and data evaluation were performed with blinded samples. De-blinding of cohorts was performed after the evaluation of all raw data.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	MDA-MB-231 (German collection of Microorganisms and Cell Cultures, Germany), Vero-E6 (American Type Culture Collection, USA)
Authentication	Cells were authenticated by short tandem repeat (STR) analysis.

Mycoplasma contamination	All cell lines tested negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used in this study.

Human research participants

Policy information about studies involving human research participants

Population characteristics	Median age was 36 (interquartile range (IQR) 29 to 53) years in naïve and 38 (IQR 29 to 53) years in convalescent participants. 65.8% naïve and 54.1% convalescent participants were female. Median age was 35 (IQR 31 to 38) years in delta- and 42 (IQR 28 to 52) years in omicron-infected participants.
Recruitment	All healthcare workers of a quaternary care hospital were invited to join an antibody testing study. 4,554 participants were recruited using E-mails, handouts and via personal communication without selection bias. Convalescents were identified to be SARS-CoV-2 antibody positive from this large-scale antibody screening. All convalescents were invited to participate in the follow-up study and all individuals who agreed to participate were included. Individuals with a possible re-exposure to SARS-CoV-2 were excluded. Naive individuals were randomly matched from the original 4,554 individuals cohort. Study participants did not receive any compension.
Ethics oversight	The study protocol was approved by the ethics committee of the Technical University Munich (TUM) (protocols 476/20, 26/21S-SR, 229/21).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	Ethics protocols of follow-up studies are: 476/20, 26/21S-SR, 229/21; no clinical trial was performed.
Study protocol	The ethics study protocols are available upon reasonable request.
Data collection	Serum samples were collected between April 2020 and December 2021 at the University Hospital rechts der Isar of the Technical University of Munich.
Outcomes	Primary and secondary outcome measures are describedin the manuscript.