European Journal of Immunology

New Technology

Basic

Research Article

A surrogate cell-based SARS-CoV-2 spike blocking assay

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To monitor infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and successful vaccination against coronavirus disease 2019 (COVID-19), the kinetics of neutralizing or blocking anti-SARS-CoV-2 antibody titers need to be assessed. Here, we report the development of a quick and inexpensive *s*urrogate SARS-CoV-2 *b*locking *a*ssay (SUBA) using immobilized recombinant human angiotensin-converting enzyme 2 (hACE2) and human cells expressing the native form of surface SARS-CoV-2 spike protein. Spike protein-expressing cells bound to hACE2 in the absence or presence of blocking antibodies were quantified by measuring the optical density of cell-associated crystal violet in a spectrophotometer. The advantages are that SUBA is a fast and inexpensive assay, which does not require biosafety level 2- or 3-approved laboratories. Most importantly, SUBA detects blocking antibodies against the native trimeric cell-bound SARS-CoV-2 spike protein and can be rapidly adjusted to quickly pre-screen already approved therapeutic antibodies or sera from vaccinated individuals for their ACE2 blocking activities against any emerging SARS-CoV-2 variants.

Keywords: Surrogate blocking assay · COVID-19 · SARS-CoV-2 · Spike protein · hACE2

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Coronavirus disease 19 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly infectious disease with variable outcome. Typical symptoms are fever, sore throat, cough, myalgia, as well as neurological and gastrointestinal symptoms [1, 2], especially advanced age, high blood pressure, diabetes, or obesity are risk factors for severe disease. A fraction of patients develops severe pneumonia requiring intensive care. If lung function is severely compromised, those patients require artificial ventilation or, at worst, extra corporal blood

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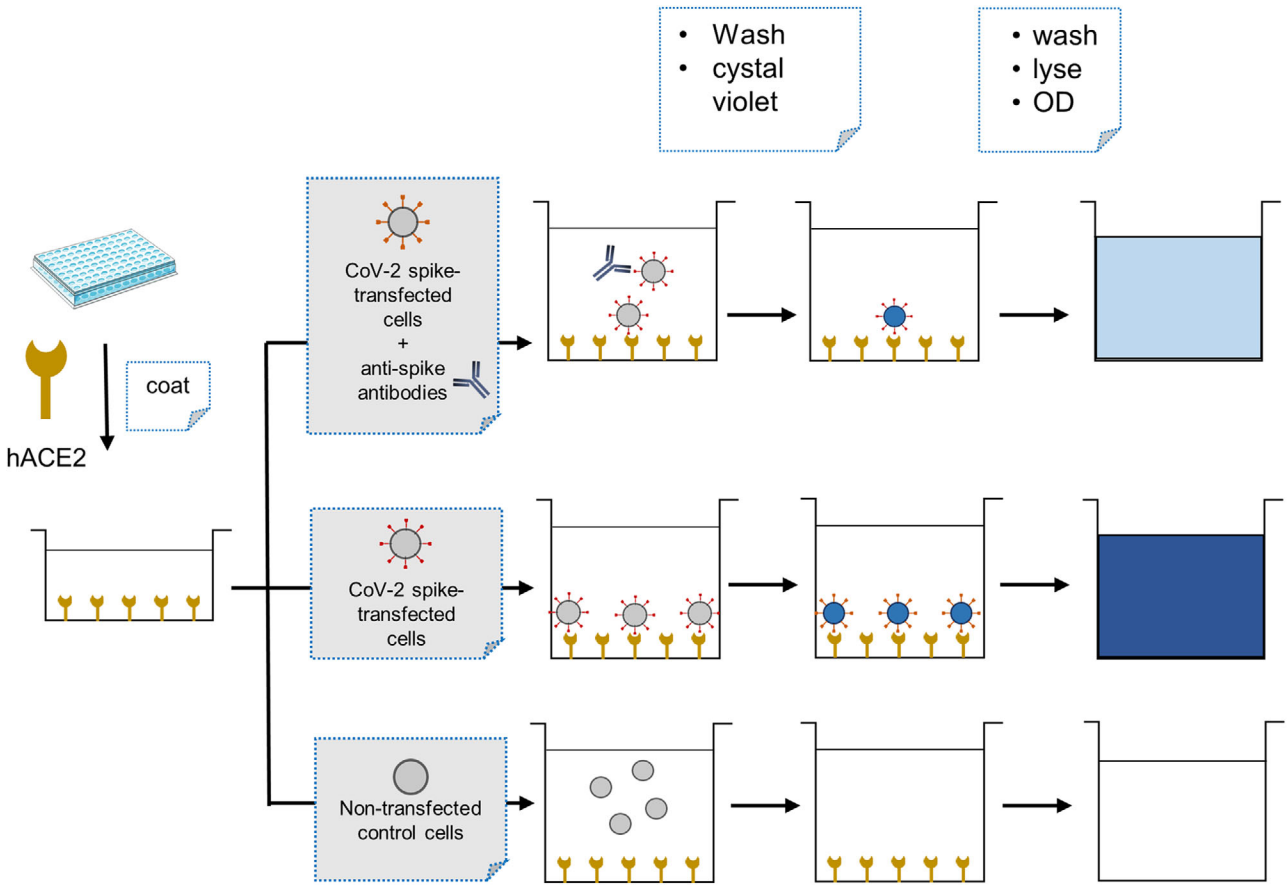

Figure 1. Design of the *s*urrogate SARS-CoV-2 spike *b*locking *a*ssay (SUBA). Note that 96-well plates are coated with soluble recombinant human ACE2. Ramos B cells or HEK293T cells expressing constitutively or tetracycline-inducibly the SARS-CoV-2 spike protein are attached to the well in the absence or presence of anti-spike protein antibodies. Nonbound cells are removed, bound cells are fixed and stained with crystal violet. Fixed crystal violet is solubilized and quantified in a spectrophotometer.

oxygenation [1–3]. SARS-CoV-2 infection as well as mRNA-, DNA vector- and protein-based vaccinations induce neutralizing antibodies and antigen-specific T cells [4–14]. Both, vaccination and infection induce memory B and T cells [15–17]. Most—but not all—neutralizing antibodies interfere with the interaction of the receptor binding domain (RBD) of the trimeric SARS-CoV-2 spike protein with angiotensin-converting enzyme (ACE2), its cognate host receptor [18–21].

To monitor SARS-CoV-2 infection and successful treatments and vaccination strategies, the serum titers of neutralizing or blocking anti-SARS-CoV-2 antibodies must be assessed and quantified. The gold standards are virus neutralization assays with pseudotyped or infectious SARS-CoV-2 using Vero cells [22] or plaque-reduction neutralization assays [23]. These assays require not only the infectious replication-competent SARS-CoV-2, pseudotyped retroviruses, or vesicular stomatitis virus expressing the SARS-CoV-2 spike protein [24] but also biosafety level 3 or 2 [25], respectively, and the precise titration of the multiplicity of infection (MOI) for each viral preparation [14]. SARS-CoV-2 blocking antibodies can also be quantified by competitive ELISA assays using recombinant and labeled trimeric ecto spike protein (S_{ecto}) or RBD proteins [26, 27]. However, commercial assays using immobilized recombinant trimeric SARS-CoV-2 spike protein are expensive and the industrial production of recombinant proteins to detect emerging SARS-CoV-2 variants is time consuming. Besides, a recombinant protein barely reflects the spike protein's configuration on the virus and could be denatured during noncompliant storage. Most importantly, existing test systems to assay blocking antibodies should be quickly adaptable to escape mutants that appear to be more infectious and are suspected to worsen the disease [14, 28–30].

To address these points and to track and characterize antibodies blocking the binding of SARS-CoV-2 spike protein to its cognate ACE2 receptor, we developed a simple, inexpensive, and reproducible *s*urrogate cell-based SARS-CoV-2 spike protein *b*locking *a*ssay (SUBA). SUBA can be performed under standard biosafety level 1 conditions in any molecular biology laboratory, and most importantly, it utilizes the native, membrane-bound form of the SARS-CoV-2 spike protein.

Results

Human cells expressing the SARS-CoV-2 spike protein on their surface should mimic the native conformation of the

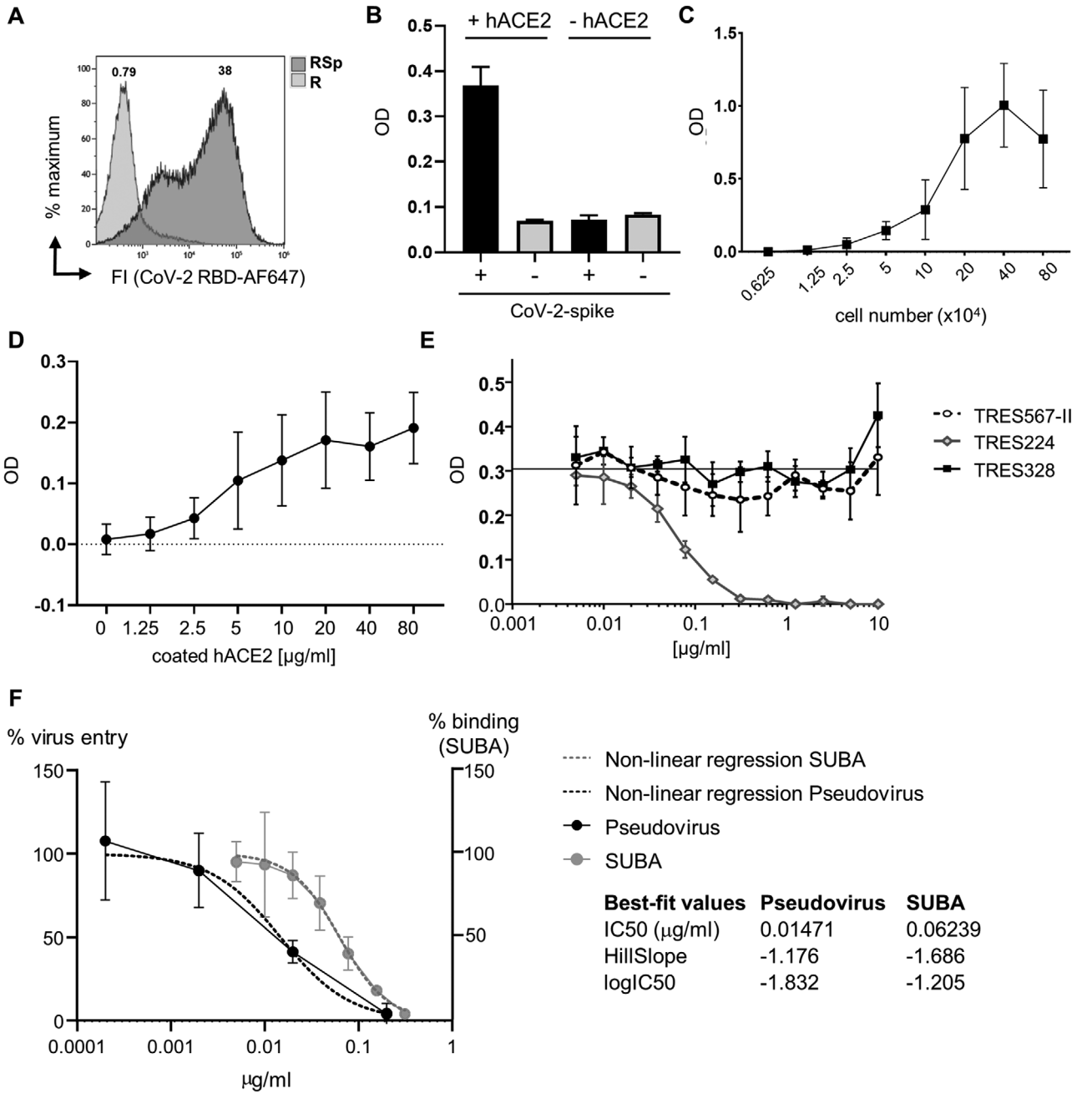

Figure 2. Determination of SUBA parameters. (A) Stably spike-expressing Ramos-null B cells (RSp) or Ramos-null B cells (R) were stained with the recombinant anti-SARS CoV-2-spike-RBD binding antibody TRES224, followed by secondary AF647-conjugated anti-human IgG antibody and analyzed by flow cytometry. Live cells were pregated based on FSC/SSC characteristics. Numbers indicate the mean fluorescence intensity ($\times 10^3$). (B) 10^5 stably spike-expressing Ramos-null B cells (RSp) or Ramos-null B cells (R) were allowed to attach to hACE2- or noncoated plates, fixed and stained with crystal violet. Crystal violet was solubilized and OD_{570nm} was measured. Data are depicted as mean ± SD of one experiment with three technical replicates. Representative of at least ten experiments. (C) Titration of the cell number per 96 well with stably SARS-CoV-2-spike expressing Ramos-null B cells (RSp) and background (Ramos-null B cells) subtracted. Data are presented as mean ± SD of three experiments performed with each with three technical replicates. (D) Titration of the hACE2 coating concentration (µg/mL) with stably SARS-CoV-2-spike-expressing Ramos-null B cells (RSp; 10^5/well) and background (Ramos-null B cells) subtracted. Data are presented as mean ± SD of three experiments performed with each three technical replicates. (E) Blockage of RSp cell binding to hACE2 with the RBD-specific TRES224 antibody. 10^5 RSp cells were allowed to attach to hACE2-coated plates in the presence of increasing concentrations of the anti-RBD neutralizing antibody TRES224, an anti-SARS-CoV-2-spike N-terminal domain (NTD) antibody (TRES328), and an isotype-matched control antibody (TRES567-II), fixed and stained with crystal violet. Crystal violet was solubilized and OD_{570nm} was measured. The black line represents the control (without antibody). Data are represented as mean ± SD of four measurements from two experiments with two technical replicates/experiments. (F) The anti-RBD neutralizing antibody TRES224 was tested in a Pseudovirus-neutralization assay (mean ± SD of four replicates) and in SUBA (two experiments performed with each three technical replicates, mean ± SD). Data were fitted by nonlinear regression and IC50 values as well as Hill slopes are depicted.

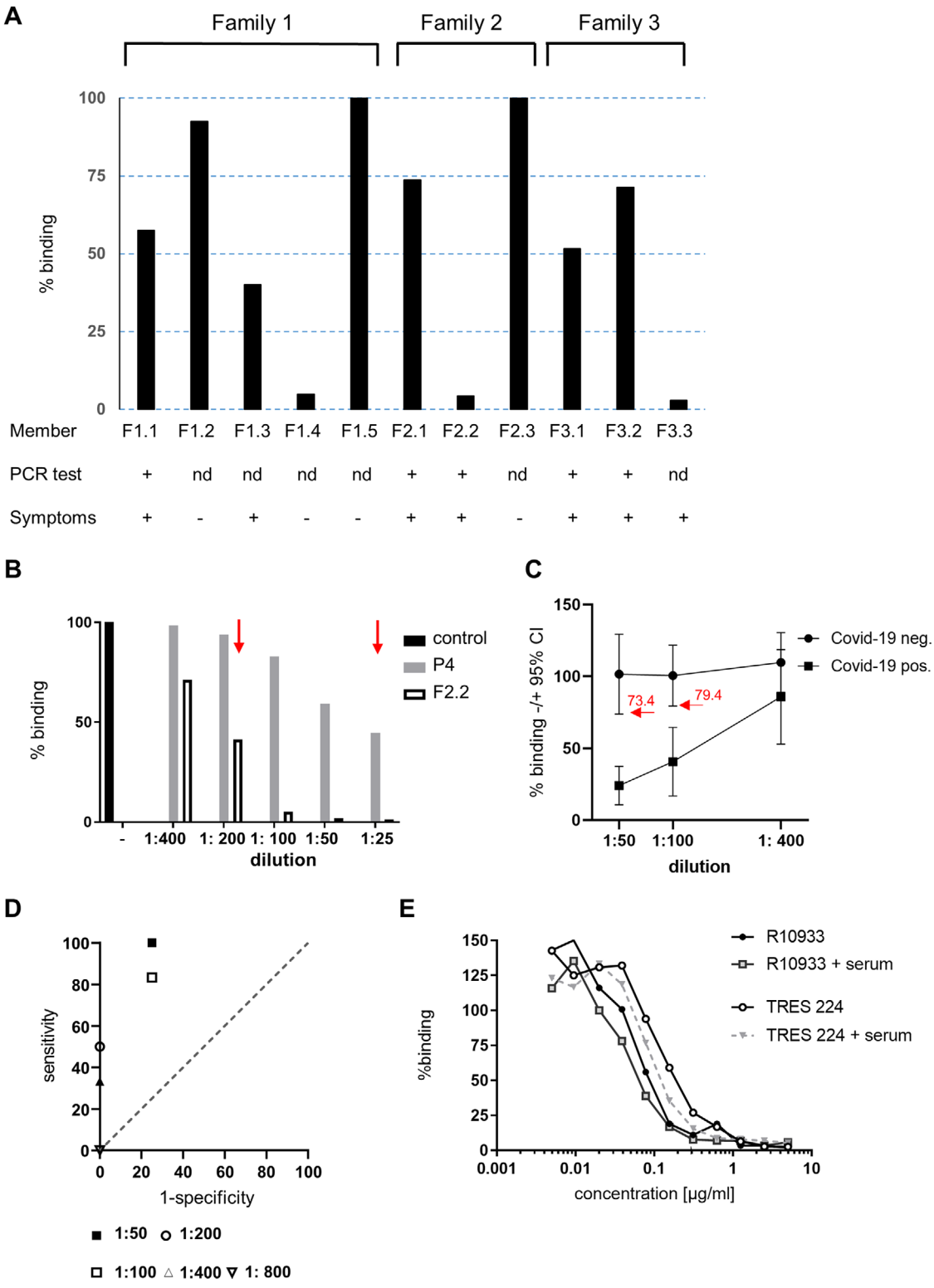

Figure 3. Analysis of spike-blocking activity in human sera. (A) Spike-expressing Ramos-null B cells (RSp; 10^5/well) were allowed to attach to hACE2-coated wells in the presence or absence of sera of purified SARS-CoV-2 blocking antibodies or sera from SARS-CoV-2-infected individuals, with (+) or without (–) symptoms, from three families (F1–F3) with at least one SARS-CoV-2 PCR-positive family member (PCR test: +). Plates were washed, fixed, stained with crystal violet, solubilized, and OD was measured. The background (Ramos-null B cells) was subtracted. Data are presented as mean % binding relative to control (RSp cells in the absence of serum or blocking antibodies) of three measurements performed with three technical replicates each. nd: not determined. (B) The binding assay was performed with increasing dilutions of sera with no (control), weak (serum P4), and strong blocking activity (serum F2.2). Spike-expressing Ramos-null B cells (RSp; 10^5/well) were allowed to attach to hACE2-coated

SARS-CoV-2 spike protein on the viral surface. In contrast to the soluble ectodomain of the SARS-CoV-2 spike protein or only parts of it, such as the RBD, the complete native SARS-CoV-2-spike protein should have a conformation similar to that on the viral surface. Moreover, recombinant and soluble SARS-CoV-2 spike protein could lack conformational or glycosylated epitopes that are recognized by neutralizing antibodies on the native SARS-CoV-2 [31]. Surface expression of SARS-CoV-2 spike protein on a mammalian cell should, therefore, allow rigorous testing of an antibody's blocking activity against SARS-CoV-2 spike protein presented on coronaviruses without using recombinant SARS-CoV-2 spike proteins or pseudotyped or infectious SARS-CoV-2 (Fig. 1). To test this idea, we attached SARS-CoV-2 spike protein-expressing cells to plates coated with recombinant and soluble human angiotensin-converting enzyme 2 (hACE2) in the absence or presence of antibodies that block the binding of SARS-CoV-2 spike protein to hACE2 (Fig. 1). Cells bound to hACE2 were fixed, washed, and nuclei were stained with crystal violet. The fixed crystal violet was solubilized and its optical density (OD) was measured in a standard photometer. We used two SARS-CoV-2 spike protein-expressing cell lines: First, mutant Ramos-Ig null B cells lacking expression of the B-cell antigen receptor [32] and stably transduced with a SARS-CoV-2 spike protein-encoding retrovirus (RSp, Fig. 2A), and second, HEK293T cells, stably transfected with a bidirectional doxycycline-controllable promoter [33] SARS-CoV-2 spike expression construct (HEK-Dox-Spike). Doxycycline treatment induced green fluorescent protein (GFP) as well as spike protein on the cell surface (Supporting Information Fig. S1A). Only Ramos-null cells expressing the SARS-CoV-2 spike (RSp) but not Ramos-null cells (R) could bind an AF647-labelled anti-RBD antibody as shown by flow cytometry (Fig. 2A). Most importantly, only spike-positive RSp cells but not spike-negative R cells bound efficiently to hACE2-coated plates (Fig. 2B). Similar results were obtained with HEK293T cells that were either stably transfected with a doxycycline-inducible gene for SARS-CoV-2 spike protein (Supporting Information Fig. S1B), or transiently expressed SARS-CoV-2 spike protein (data not shown). Titration of the cell number in 100 μl volume at a fixed coating concentration of hACE2 in the 96-well plate-format showed a saturation starting at approximately 2×10^5 RSp cells/well (Fig. 2C). Note that 10^5 cells per well were still in the linear range and, therefore, this number was used in the following quantitative experiments. Similar results were obtained with HEK293T cells treated with different concentrations and at different time points with two doxycycline derivates (Supporting Information Fig. S1C–F). Titration of the hACE2 concentration with a fixed RSp cell number (10^5 cells/well) revealed a binding saturation at 20 μg/ml of coated hACE2 (Fig. 2D). Hence, the following experiments were conducted in 100 μl with 10^5 cells per well of a 96-well plate with 20 μg/ml of coated hACE2.

While the assay worked well with the SARS-CoV-2 spike-expressing HEK293T cells (Supporting Information Fig. S1), we preferred RSp cells because they grew faster and did not require trypsinization. To test whether anti-SARS-CoV-2 RBD antibodies can indeed block the binding of Rsp cells to hACE2, we incubated RSp cells with increasing concentrations of a fully human recombinant anti-RBD neutralizing antibody (TRES224) that was established in our lab [34, 35]. In contrast, neither TRES328, a recombinant antibody recognizing the N-terminal domain (NTD) of the SARS-CoV-2 spike protein, nor the isotype-matched control IgG1 control antibody (TRES 567.II) showed a specific blocking activity (Fig. 2E). We found that TRES224 blocked the binding of SARS-CoV-2 spike-expressing RSp cells with an IC50 of ∼62 ng/ml (Fig. 2F), while the IC50 of TRES224 in a Pseudovirus-neutralization assay was 15 ng/mL (Fig. 2F). Albeit the IC50 of TRES224 in the virus neutralization was ∼4× lower compared to SUBA, the Hill slope of the fitted dose response curve was very similar (Fig. 2F). On the basis of these findings, we conclude that our SUBA system allows the specific detection of antibodies that block the binding of hACE2 to its cognate native SARS-CoV-2 spike protein ligand. The sensitivity of SUBA is lower than that of a virus neutralization assay, but the general characteristics of SUBA are similar to a virus neutralization assay.

To determine whether the SUBA assay allows the quantification of SARS-CoV-2 spike blocking antibodies in human sera, we analyzed sera with a dilution of 1:100 from families with at least one confirmed SARS-CoV-2-infected family member (SARS-CoV-2 PCR-positive) and observed a high heterogeneity in their blocking activities. We found different blocking activities resulting in 0–25%, 25–50%, 50–75%, and 75–100% binding (Fig. 3A). To confirm that SUBA detects differences of SARS-CoV2 spike-blocking antibody titers, we titrated sera of individuals that were tested positive for SARS-CoV-2 by PCR and had either a low (serum P4; see Fig. 4) or high (serum F2.2, Fig. 3A) blocking activity with a 1:100 serum dilution (Fig. 3B). We found that the titer of blocking antibodies in those sera differed by a factor of 8 (see red arrows in Fig. 3B). To estimate the upper and lower limits of anti-SARS-CoV-2 blocking activities in SUBA, we diluted randomly picked sera from noninfected [8] or infected [6] individuals (Fig. 3C). Data are plotted as mean ± 95% CI, showing clear blocking activities at 1:50 and 1:100 dilutions of sera from SARS-CoV-2-infected

wells in the presence or absence of different dilutions or sera with previously defined weak or strong blocking activity. Data are presented as mean % binding relative to control (RSp cells in the absence of serum or blocking antibodies) performed in three technical replicates in one experiment. Red arrows indicate the dilutions where similar blocking activity was detected. (C) Sera from eight COVID-19 negative and six COVID-19 positive donors were subjected to SUBA in two technical replicates in one experiment. Data are depicted as mean ± 95% CI. Red arrows and numbers depict the lower limit of COVID-19 negative sera. (D) *Receiver operating characteristic* (ROC) analysis of SUBA using the data obtained in (C) and additional serum dilutions. (E) Spike-expressing Ramos-null B cells (RSp; 10^5/well) were allowed to attach to hACE2-coated wells in the presence of increasing concentrations of the monoclonal antibodies TRES224 or Regeneron R10933 diluted either with the assay medium or a human serum from an individual tested negative for anti-SARS-CoV-2 antibodies. Data are presented as mean % binding relative to control (RSp cells in the absence of serum or blocking antibodies) performed in three technical replicates. Representative of two experiments.

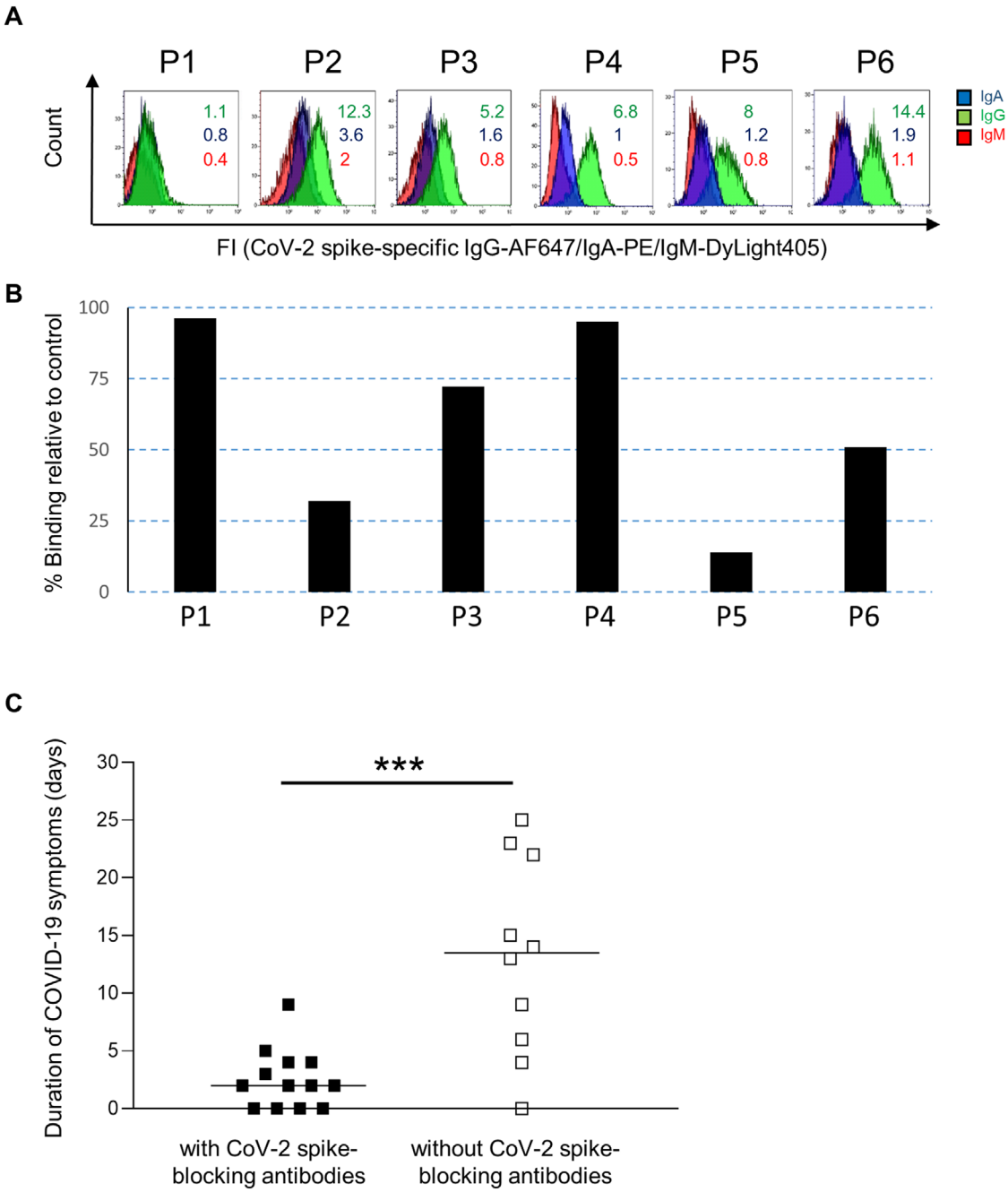

Figure 4. Analysis of SARS-CoV-2 spike-binding and -blocking antibodies. (A) Flow cytometric analysis of SARS-CoV-2 spike-binding IgM, IgA, and IgG serum antibodies in patients (P1–P6) from different families with at least one PCR-positive case of SARS-CoV-2 infection. P1 served as a negative control and did not contain serum antibodies recognizing the SARS-CoV-2 spike protein. HEK293T cells were transiently transfected with a plasmid encoding SARS-CoV-2 spike protein and stained with sera as described in Fig. S2. Numbers indicate the mean fluorescence intensities (MFI) of SARS-CoV-2-Spike IgG (green), IgA (blue), and IgM (red). (B) SUBA assay to detect SARS-CoV-2-blocking serum antibodies in sera from patients P1–P6. Data are presented as mean % RSp binding relative to control (RSp cells in the absence of serum or blocking antibodies) of three measurements performed in three technical replicates each. (C) Correlation between COVID-19 symptoms and the presence of SARS-CoV-2 spike-blocking antibodies in family members with at least one reported PCR-positive SARS-CoV-2 infection. Typical symptoms were fever, head and body ache, diarrhea, exhaustion, cough, shortness of breath, and chest tightness. $p = 0.0003$ (unpaired t test).

donors (Fig. 3C). At a 1:100 dilution of COVID-19 naive sera, the lower limit of the 95% CI has been determined to be 79.4% and at a 1:50 dilution, it was 73.4%. Next, we performed a *receiver operating characteristic* (ROC) analysis by plotting the sensitivity of the assay (defined as the identification rate of sera known to contain anti-SARS-CoV-2 antibodies) against the false-positive rate (defined as the rate of SARS-CoV-2 negative sera that contain blocking anti-SARS-CoV-2 antibodies) (Fig. 3D). The ROC analy-

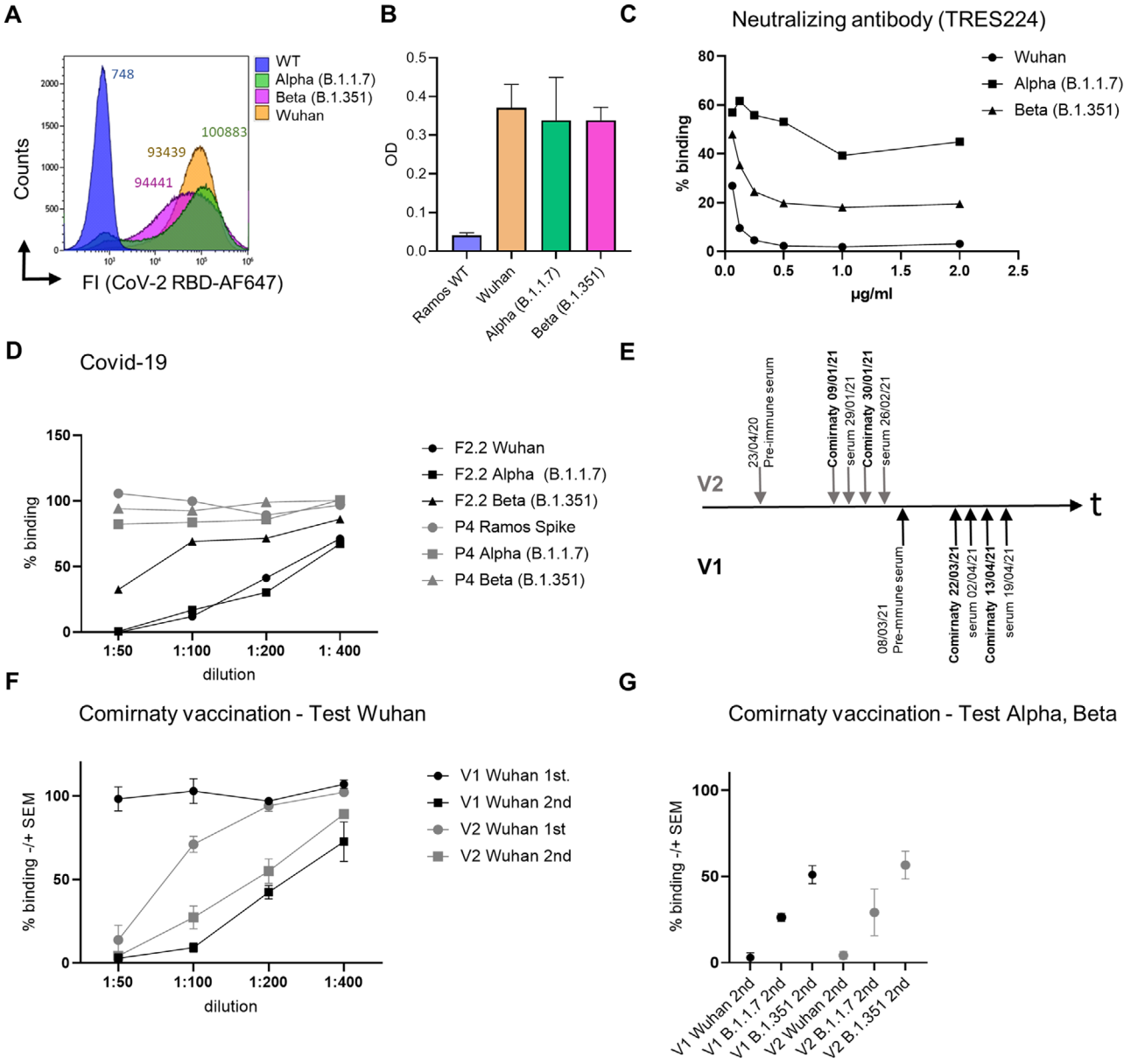

Figure 5. COVID-19 and vaccination responses against SARS-CoV-2 mutants measured by SUBA. (A) Flow cytometric analysis of Ramos cells expressing SARS-CoV-2 spike wild-type (Wuhan), Alpha (B.1.1.7) or Beta (B.1.351) mutants with anti-RBD Ab TRES224 and secondary AF647-labeled anti-human IgG antibody. Live cells were pregated based on FSC/SSC characteristics. Representative of three experiments with one sample per experiment. Numbers indicate the mean fluorescence intensities (MFI). (B) SUBA assay for Ramos cells expressing SARS-CoV-2 spike wild-type (Wuhan, $n = 14$), Alpha (B.1.1.7, $n = 11$), or Beta (B.1.351, $n = 11$) mutants. Data are shown as mean ± SD from three experiments. (C) Wuhan spike-expressing Ramos-null B cells or Ramos-null B cells expressing the Alpha (B.1.1.7) or Beta (B.1.351) spike mutants (each 10^5/well) were allowed to attach to hACE2-coated wells in the presence of increasing concentrations of the monoclonal antibody TRES224 diluted with the assay medium. Data are presented as mean % binding relative to control (RSp cells in the absence of serum or blocking antibodies) performed in one experiment with two technical replicates. (D) Wuhan spike-expressing Ramos-null B cells or Ramos-null B cells expressing the Alpha (B.1.1.7) or Beta (B.1.351) spike mutants (each 10^5/well) were allowed to attach to hACE2-coated wells in the presence of decreasing dilutions of sera from COVID-19 positive (F2.2) or negative (P1) donors and SUBA was performed. Data are presented as mean % binding relative to control (cells in the absence of serum or blocking antibodies) performed in two technical replicates. Representative of two experiments. (E) Timeline for blood sampling before and after Comirnaty vaccination from two volunteers (V1 and V2). (F and G) Wuhan spike-expressing Ramos-null B cells or Ramos-null B cells expressing the Alpha (B.1.1.7) or Beta (B.1.351) spike mutants (each 10^5/well) were allowed to attach to hACE2-coated wells in the presence of decreasing dilutions of sera from V1 and V2 donors and SUBA was performed. Data are presented as mean % binding ± SEM, relative to control (cells in the absence of serum or blocking antibodies) from two experiments performed with each two technical replicates, with values from the preimmune sera subtracted.

sis showed that 1:50–1:100 dilutions of sera are suitable for SUBA because they hit the sweet spot between sensitivity and specificity.

To directly show that the SUBA assay can distinguish between two antibodies that differ in their RBD-blocking strength, we titrated the monoclonal anti-RBD antibody TRES224 [34, 35] and the clinically tested anti-RBD antibody R10933 [19] (Fig. 3E) either diluted in PBS or serum from an SARS-CoV-2-negative serum donor. We found that R10933 and TRES224 showed a 60% blocking activity, which represents the activity we used to distinguish between the two sera in Fig. 3B, at ∼65 ng/mL and ∼120 ng/mL, respectively (Fig. 3E). Therefore, SUBA can quantitatively discriminate between the blocking activities of antibodies.

The absence of blocking activity in human serum could simply indicate the absence of any antibodies that bind to the anti-SARS-CoV-2 spike protein. To test this hypothesis, we used a flow cytometry-based assay to detect IgA, IgG, or IgM serum antibodies recognizing the anti-SARS-CoV-2 spike protein on transiently transfected HEK293T cells (Supporting Information Fig. S2). Although anti-SARS-CoV-2 spike IgA and IgG antibodies were detectable by flow cytometry in five infected donors with similar mean fluorescence intensities (P2–P6) (Fig. 4A), only two donors (P2 and P5) showed a good hACE2-blocking activity (Fig. 4B). This confirms that SUBA can indeed differentiate between different amounts or affinities of serum anti-SARS-CoV-2 spike antibodies, some of which may exhibit a delayed development of neutralizing antibodies [23]. These results are in accordance with the finding that different antibody classes and isotypes to different epitopes are elicited during SARS-CoV-2 infections [8, 14, 36] and strongly suggest that the mere presence of SARS-CoV-2 spike-binding antibodies as detected by flow cytometry does not provide information about their virus neutralization activity.

We next asked whether the presence of blocking anti-SARS-CoV-2 spike antibodies would correlate with the duration of the symptomatic phase in COVID-19 patients and the titer of Spike-binding antibodies. We found that the presence of anti-SARS-CoV-2 spike antibodies with a >50% blocking activity in the SUBA assay correlated inversely with the duration of disease symptoms, but not with the titer of spike-binding antibodies, which supports previous reports [37, 38] (Fig. 4C). This suggests that antibody quality is decisive.

To evaluate SUBA for its use with SARS-CoV-2 spike mutants, we generated Ramos-null B cells expressing the B.1.1.7 (Alpha; https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/) and B.1.351 (Beta; https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/) variants of the spike protein on their cell surface. Flow cytometric analysis in Fig. 5A demonstrates similar surface expression of the wild-type (Wuhan) spike as well as B.1.1.7 and B.1.351 spike mutant proteins on RSp, RSp.1.1.7 or RSp.1.351 cells, respectively. In accordance with equal spike surface expression, RSp.1.1.7 and RSp.1.351 cells attached as well as RSp cells to hACE2.

Next, we addressed the effect of the Alpha and Beta mutations on the blocking activity of the fully humanized RBD-binding Ab TRES224 [35]. TRES224 showed a reduced blocking activity toward B.1.1.7 while, interestingly, maintaining a potent blocking activity toward spike B.1.351 (Fig. 5C). These results are consistent with virus neutralization assays [35], thereby, confirming the specificity of SUBA for the use of SARS-CoV-2 spike mutations.

Moreover, we analyzed the serum blocking activities from a COVID-19-diseased donor (F2.2; Fig. 3). While there was no significant blocking activity in the control serum (P4; see Fig. 4), F2.2 blocked B.1.1.7 spike comparably to the Wuhan-spike (Fig. 5D). The B.1.351 mutant was blocked less effectively but considering a threshold of ∼79% binding (compare Fig. 3C), binding was still clearly blocked.

To monitor the development of specific blocking activities in sera after vaccination, we followed the SARS-CoV-2 specific immune response elicited by Comirnaty (BNT162b2, Biontech/Pfizer) vaccination in two volunteers (V1 and V2) (see timeline in Fig. 5E). Because there may be pre-existing neutralizing humoral activity due to previous SARS-CoV-2 infections or immune cross-reactivity among seasonally spreading human coronaviruses (HCoVs) [39], we subtracted the background activity of the preimmune sera from the vaccine-elicited anti-spike blocking activity to measure specifically the SARS-CoV-2-directed blocking activity. Both volunteers developed a potent Wuhan-spike-neutralizing activity after the second vaccination (∼95%), with V2 showing already a response after only one dose (Fig. 5F). Comirnaty vaccination also elicited a response toward the spike B.1.1.7 mutant detected at 1:50 dilutions (∼75%) (Fig. 5G). Compared to the B.1.1.7 mutant, we detected a lower anti-spike-B.1.351 blocking activity (∼50%) after the second Comirnaty vaccination in both subjects, V1 and V2 (Fig. 5G).

Discussion

Here, we introduce a SUBA by expressing the SARS-CoV-2 spike protein (Wuhan, Alpha or Beta) on the surface of Ramos or HEK293T cells, allowing the cells to attach to hACE2. By blocking this interaction specifically with RBD-directed Ab, but not with NTD-directed Ab, as well as by reconvalescent or immune serum, we demonstrate the specificity and sensitivity of this assay setup. The binding curves obtained with SUBA are comparable to a Pseudovirus-neutralization assay albeit SUBA is less sensitive.

While also antibodies recognizing the NTD of SARS-CoV-2 can neutralize the virus in vivo [35], SUBA can only specifically measure the disruption of the hACE2-RBD interaction. Nevertheless, in summary, we propose that SUBA is a fast, inexpensive, reproducible, and valid method to monitor the COVID-19 vaccination efficacy and the recovery from COVID-19. Our SUBA assay can be easily adapted to test variants of SARS-CoV-2 and, thus, represents a facile tool to investigate and assess the kinetics of SARS-CoV-2 immune responses in the vaccinated population against newly emerging SARS-CoV-2 variants, like the Delta variant. The results obtained with two vaccinated volunteers showed that Comirnaty vaccination elicited a blocking activity against SARS-CoV-2 spike that was quantifiable by SUBA, and that was lower for the Alpha and Beta variants compared to the

Wuhan variant. These results are congruent with studies of large cohorts, showing an efficacy of Comirnaty in preventing SARS-CoV-2 infection of 95% for the Wuhan SARS-CoV-2 strain, 89.5% for the B.1.1.7 (Alpha) variant, and 75% for the B.1.351 (Beta) variant [40]. Therefore, we suggest that SUBA can be applied to test sera from reconvalescent or from immunized donors for the presence of spike-blocking antibodies. We would like to emphasize that our analyses of vaccinated donors were only designed as examples to establish the feasibility of SUBA in principle, and not to perform epidemiologic or efficacy studies. Future studies may use SUBA to quantify the prevalence of anti-SARS-CoV-2 (mutant) blocking activity in serum or plasma from larger cohorts of reconvalescent patients (to normalize it for passive immunization), or from individuals immunized successively with different vaccines.

While SUBA is only a surrogate assay for virus neutralization, it does employ the complete, native SARS-CoV-2 spike protein. For this reason and for its simplicity and economy, the SUBA system constitutes a complementary alternative to systems using recombinant parts of the SARS-CoV-2 spike protein. Importantly, results generated by SUBA at different locations are directly comparable because error-prone purification steps, enzymatic reactions, or infection procedures are not involved. This will speed up basic and clinical research toward SARS-CoV-2.

Materials and methods

Antibodies and flow cytometry

The anti-SARS-CoV-2 RBD antibody TRES224 (human IgGκ) [34, 35] has been described previously and was produced by Celltheon (San Francisco, CA). TRES328 (human IgG1κ, NTD binder), TRES480, and TRES567 (human IgG1κ, nonbinders) were generated during the same immunization and purified from transfected HEK293T cells according to standard procedures [35]. For flow cytometric analyses, anti-human IgG FITC antibody and DyLight405-conjugated anti-human IgM were purchased from Jackson (Dianova, Hamburg), PE-conjugated anti-human IgA, and AF647- and Cy5-conjugated anti-human IgG were obtained from Southern Biotech (Birmingham, AL). Anti-SARS-CoV-2 RBD antibody R10933 (Regeneron) [19] was produced by Sino Biological (Eschborn, Germany). Flow cytometric analysis of surface SARS-CoV-2 spike protein on Ramos-null spike cells (RSp and mutants) and transfected HEK293T cells (HEK-Dox-Spike) was performed by staining cells with anti-RBD-binding antibody TRES224 in FACS buffer (PBS/2% FCS/0.05 % sodium azide) for 15 min on ice. Next, cells were washed with FACS buffer and then stained with AF647- or Cy5-conjugated anti-human IgG antibodies in FACS buffer for 15 min on ice. After washing with FACS buffer, cells were analyzed by using a Gallios or Cytoflex flow cytometer (Beckman Coulter) and Kaluza flow cytometry software (Beckman Coulter).

Plasmids

To generate an inducible vector for SARS-CoV-2 spike protein expression, the open reading frame of SARS-CoV-2 spike was amplified from pCG1_CoV_2019S (encoding the Wuhan-Hu-1 Spike protein (QHD43416.1, AA1-1273, position 21580–25400 from Genbank NC_045512)) by polymerase chain reaction (PCR) with the primers fw_NheI (5′-ctggctagcgccaccatgtttctgctgaccacc-3′) and rev_StuI (5′-tcaaggcctttaggtgtagtgcagtttcacgccc-3′) for 30 cycles at 65°C annealing temperature. The PCR product was purified, digested with *Nhe*I and *Stu*I and cloned into *Nhe*I and *Eco*RV digested pWHE469 [33], followed by sequencing.

Cell lines

Ecotropic Ramos-null cells have been described [32] and were cultured in fully supplemented RPMI1640 medium (Gibco) containing 10% fetal calf serum (FCS) at 5% CO_2 in a fully humidified incubator at 37°C. Ramos-null cells were infected with retroviral particles derived from pMIG (https://www.addgene.org/9044/) encoding the Wuhan-Hu-1 Spike protein (QHD43416.1, AA1-1273, position 21580–25400 from Genbank NC_045512) and then sorted for GFP expression to obtain Ramos-null spike (RSp) cells. The B.1.1.7 (α) and B.1.351 (β) spike mutants were generated by site-directed mutagenesis and Ramos-null B cells expressing the mutants were generated as described above. HEK293T cells were cultured in fully supplemented DMEM medium (Gibco) containing 10% FCS at 7.5% CO_2 in a fully humidified incubator at 37°C. HEK293T cells were transfected with the linearized plasmid pWHE469-SARS-CoV-2 spike by lipofectamine (Gibco) and selected with puromycin (2 μg/mL; Gibco).

Recombinant proteins

The plasmid encoding the ectodomain of human ACE2 (hACE2) (AA18-738, NP_001358344.1) with an IgLκ signal peptide fused to a human IgG1 Fc part followed by Myc and His-Tag was cloned in pCEP4 (Thermo Fisher). Recombinant hACE2 was produced with the FreeStyle 293 Expression System (Fisher Scientific) by transfecting FreeStyle 293-F cells kept in Freestyle 293 Expression Medium with the pCEP4-hACE2 plasmid. hACE2 was affinity-purified from filtered cultured supernatant on a High-Trap Protein G column (GE Healthcare, Chicago, USA), immediately neutralized, dialyzed against phosphate buffered saline and stored in 50% glycerol at −20°C. The quality of protein purity was assessed by SDS-PAGE Coomassie staining. Protein concentrations were determined by OD at 280 nm and verified by a Bradford assay (Thermo Fisher Scientific, Waltham, USA).

Serum samples

Serum samples were analyzed in the context of a longitudinal study of 56 households with at least one member who had COVID-19 to investigate the course of illness, immune responses,

and long-term consequences of SARS-CoV-2 infection in patients with ectodermal dysplasia and control subjects of the same age group (ClinicalTrials.gov identifier: NCT04741412). This study was approved by the ethics committee of the University Erlangen-Nürnberg and conducted in accordance with the principles of the declaration of Helsinki. All individuals provided written informed consent to participate.

Cell-based SUBA

Flat bottom ELISA plates were coated with 50 μL/well of 20 μg/mL hACE2 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) overnight at 4°C. Plates were washed three times with phosphate-buffered saline (PBS) and blocked with 100 μL of 10 % bovine serum albumin (BSA) in PBS for 1 h at room temperature. Plates were washed twice with PBS and 10^5 cells in 100 μl full medium containing the desired serum or antibody were plated per well (complement inactivation of human serum for 30 min at 56°C may be necessary if serum containing B cell depleting antibodies is analyzed). Plates were incubated for 1 h at 37°C in an incubator and then emptied into the sink with momentum. Plates were then placed on ice and wash twice with 200 μL/well of cold PBS by gently tapping the plate on paper towels. Cells were then fixed cells on ice for 10 min with 200 μL/well of ice-cold pure methanol, methanol was emptied with vigor, and plates were tapped twice gently on paper towel. The plate was then stained at room temperature for 10 min with 50 μL/well of 0.5% crystal violet solution in 25% methanol and washed three times with de-ionized water in a large container by submersion. The plate was gently tapped twice on a paper towel and 100 μL/well of a 1% SDS solution were added. (http://www2.kumc.edu/soalab/LabLinks/protocols/cvassay.htm). Using a multichannel pipette, the fixed crystal violet was dissolved to homogeneity and plates were analyzed in an ELISA reader at 570 nm. IC50 values were calculated by plotting the Rsp-binding activity in percent against the antibody concentrations using the normalized response versus inhibitor equation (variable slope) of GraphPad Prism 7.02.

Flow cytometry-based analyses of SARS-CoV-2 spike-specific antibodies in the serum of patients

HEK293T cells were transiently transfected using the PEI method with the SARS-CoV-2-spike-encoding plasmid pCG1_CoV_2019S [41] in combination with a GFP encoding plasmid. HEK293T cells transfected only with a GFP-encoding plasmid served as a negative control. Two days after transfection, SARS-CoV-2 spike/GFP-co-transfected and GFP-only transfected HEK293T cells were stained with serum samples (1:100 dilution) from SARS-CoV-2-infected patients and noninfected controls followed by staining with a secondary antibody mixture (2° antibody mix) consisting of PE-conjugated anti-human IgA (Southern Biotech, Birmingham, AL), AF647-conjugated anti-human IgG (Southern Biotech), and DyLight405-conjugated anti-human IgM (Jackson Immuno Research, Dianova, Hamburg) antibodies. Cells were analyzed using a Gallios flow cytometer (Beckman Coulter) or a Cytoflex flow cytometer (Beckman Coulter) and Kaluza flow cytometry software (Beckman Coulter).

Pseudovirus neutralization assay*

Neutralizing activities of antibodies were assessed in a pseudovirus neutralization assay as described [42]. Briefly, 1 × 104 Vero cells were seeded per well of a 96-flat bottom plate 1 day before the infection. Antibodies were pre-diluted in cell culture medium and pre-incubated for 30 min with vesicular stomatitis virus-based pseudoviruses bearing the SARS-CoV-2 spike protein in a final volume of 100 μl per replicate (four replicates per experiment) before being inoculated on Vero cells. Pseudoviruses incubated in culture medium without antibody served as control (100% cell entry). At 16–18h postinoculation, the culture medium was aspirated and pseudovirus cell entry was analyzed by measuring the activity of virus-encoded firefly luciferase in cell lysates using a commercial substrate (Beetle-Juice, PJK GmbH) and a Hidex Sense plate luminometer (Hidex). IC50 values were calculated by plotting the virus entry in percent against the antibody concentrations and using the normalized response vs. inhibitor equation (variable slope) of GraphPad Prism 7.02.

Acknowledgements: This work was in part supported by the Deutsche Forschungsgemeinschaft (DFG) by TRR130/P09 to HMJ and WS, TRR130/P28 to HMJ, TRR130/P03 to DM, TRR130/P02 to MR, the BMBF (COVIM and 01KI2043A, both to HMJ), the Bayerische Forschungsstiftung (CORAcd to HMJ), and the Bavarian Ministry of Science and Art (to HMJ). This work was done in partial fulfillment for the bachelor's degree of LB and SK.

Conflict of interest: DM and HMJ have filed a patent application for SUBA. All other authors have no commercial or financial conflict of interests.

Author contributions: DM and WS designed the study, acquired and analyzed data, interpreted data, and wrote the manuscript. HMJ designed the study, analyzed and interpreted the data, and wrote the manuscript. LB, TS, SRS, LW, ER, MHa, SK, PR, MR, and MHo acquired and analyzed data. HS provided human serum samples and interpreted data. MR and NV generated the Ramos Ig-null cell lines expressing SARS-CoV-2 spike proteins (RSp).

*Correction added on 01/11/2021 after first online publication: Section Pseudovirus neutralization assay has been corrected.

Data availability statement: The data that supports the findings of this study are available in the supplementary material of this article or from the author upon reasonable request.

Peer review: The peer review history for this article is available at https://publons.com/publon/10.1002/eji.202149302.

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Abbreviations: **COVID-19**: coronavirus disease 2019 · **hACE2**: human angiotensin-converting enzyme 2 · **NTD**: N-terminal domain · **RBD**: receptor binding domain · **SARS-CoV-2**: severe acute respiratory syndrome coronavirus 2 · **SUBA**: *su*rrogate cell-based SARS-CoV-2 spike surrogate *b*locking *a*ssay

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Received: 13/4/2021
Revised: 29/7/2021
Accepted: 17/9/2021
Accepted article online: 21/9/2021