The nuts and bolts of SARS-CoV-2 Spike Receptor Binding Domain heterologous expression

Mariano Maffei^{1*}, Linda Celeste Montemiglio⁴, Grazia Vitagliano², Luigi Fedele^{2†}, Shaila Sellathurai², Federica Bucci², Mirco Compagnone³, Valerio Chiarini², Cécile Exertier⁵, Alessia Muzi², Giuseppe Roscilli^{1,2}, Beatrice Vallone⁵, Emanuele Marra^{1,2*}

1. Evvivax Biotech, Rome, Italy

2. Takis Biotech, Rome, Italy

3. Neomatrix Biotech, Rome, Italy

4. Institute of Molecular Biology and Pathology (IBPM), National Research Council, Rome, Italy

5. Department of Biochemical Sciences “A. Rossi Fanelli” University of Rome, Sapienza, Rome, Italy

†Present address: Luigi Fedele, InnovaVector s.r.l., Pozzuoli, Italy

***Correspondence to:**

maffei@takisbiotech.it

marra@takisbiotech.it

Abstract

COVID-19 is a highly infectious disease caused by a newly emerged coronavirus (SARS-CoV-2) that has rapidly progressed into a pandemic. This unprecedented emergency has stressed the significance of developing effective therapeutics to fight current and future outbreaks. The receptor-binding domain (RBD) of the SARS-CoV-2 surface Spike protein is the main target for vaccines and represents a helpful “tool” to produce neutralizing antibodies or diagnostic kits. In this work, we provide a detailed characterization of the native RBD produced in three major model systems: *Escherichia coli*, insect and HEK-293 cells. Circular dichroism, gel filtration chromatography and thermal denaturation experiments indicated that recombinant SARS-CoV-2 RBD proteins are stable and correctly folded. In addition, their functionality and receptor-binding ability were further evaluated through ELISA, flow cytometry assays and bio-layer interferometry.

Keywords

SARS-CoV-2; Receptor-Binding Domain; COVID-19; Spike protein; heterologous expression; protein production.

Introduction

At the end of 2019, a novel respiratory pathogen responsible for the COVID-19 disease, namely severe acute respiratory syndrome-related coronavirus (SARS-CoV-2), emerged in Wuhan, China¹. Only three months later, the virus spread worldwide causing one of largest outbreak of the century that rapidly progressed into pandemic with more than 222 million of confirmed cases and 4,59 million deaths by September 9th ². In response to this exceptional situation, an enormous effort has been made by the scientific community to study and characterize the pathogen and to quickly develop safe and effective prophylactic and therapeutic drugs.

The SARS-CoV-2 is an enveloped virus whose surface is decorated with an integral membrane protein (M), an envelope protein (E), a surface spike protein (S), and an additional unexposed structural nucleocapsid protein (N)^{3,4}. Among those, the Spike protein is critical to recognize the host-cell receptors and for mediating viral entry, therefore it represents the most studied viral component and the best candidate for drug target ^{5,6}. The 140 kDa SARS-CoV-2 S protein is organized into two major subunits (S1 and S2) connected by a furin-cleavage site⁷. The S1 subunit contains the receptor-binding domain (RBD; aa 319-541), a 25 kDa domain that is directly involved in the interaction with the Angiotensin-converting enzyme 2 (ACE2)^{8,9}. RBD contains nine cysteines, including eight that form disulfide-bridges involved in the RBD fold. In addition, the domain displays two N-glycosylation sites (Asn₃₃₁ and Asn₃₄₃) known to participate to folding, stability, and function^{10–12}. Mutations occurring within this domain are constantly monitored to predict the emergence of novel variants that could be naturally selected and quickly spread, such as the recent isolated alpha (B.1.1.7), beta (B.1.351), gamma (P.1), and delta (B.1.617.2) variants of concern^{13–16}.

RBD as isolated protein is broadly used in different types of clinical and medical applications (serological tests, vaccine formulation etc.)^{17–20} and therefore its *in-vitro* production is of paramount importance. Mammalian and insect cells are the model systems of election used for the heterologous expression of SARS-CoV-2 RBD due to its intrinsic structural complexity. Attempts have also been made using other systems such as *Pichia pastoris*²¹ or *Nicotiana benthamiana*²². Although *Escherichia coli* (*E. coli*) represents the most common organism employed for the expression of recombinant proteins, its usage is not recommended for challenging targets that require complex folding and/or post-translational modifications such as RBD. Nevertheless, *E. coli* gathers many technical and practical advantages (low-cost, easy handling) compared to other model systems that could be beneficial both for research-scale and large industrial production²³.

In this study, we present a structural and functional comparison of the native RBD of SARS-CoV-2, recombinantly produced in the three major and most frequently used expression systems (*E. coli*, insect and mammalian HEK-293 cells). The characterization of recombinant RBD proteins is of the utmost relevance in drug design to tackle the Covid-19 pandemic.

Abbreviations

ACE2, angiotensin-converting enzyme 2; BLI, bio-layer interferometry; CD, circular dichroism; COVID-19, coronavirus disease-2019; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; HEK, human embryonic kidney; IgG, immunoglobulin G; IMAC, immobilized metal affinity chromatography; MALDI-MS, matrix-assisted laser desorption/ionization- mass spectrometry; MFI, mean fluorescence intensity/geometric mean; MS, mass spectrometry; MW, molecular weight; MWCO, molecular weight cut-off; OD, optical density; PMF, peptide mass fingerprint; PMTV, photomultiplier voltage;

POI, protein of interest; RBD, receptor binding domain; *E. coli*-RBD, receptor binding domain produced in *E. coli*; HEK-293-RBD, receptor binding domain produced in HEK293 cells; insect-RBD, receptor binding domain produced in insect cells; RP-UHPLC-MS, reversed-phase - ultra-high performance liquid chromatography - mass spectrometry; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SEC, size exclusion chromatography; T_m, melting temperature; UHPLC, ultra-high performance liquid chromatography; WHO, World Health Organization;

Results

Design, expression, and purification of SARS-CoV-2 RBD in E. coli

The RBD protein (Figure 1(a)) was recombinantly expressed with a C-terminal 6x-His purification tag both in BL-21 *Star* and Lemo21 cells. The Lemo21 bacterial strain allows to express challenging targets such as toxic, highly insoluble, and membrane proteins by reducing inclusion body formation and potential inhibitory effects on cells growth, thus resulting in an increased level of properly folded products. However, only negligible amount of RBD was found in the soluble fraction, even exploring alternative growing conditions including lower temperature, distinct induction times, and increasing concentrations of L-Rhamnose (data not shown). The target protein was totally recovered from inclusion bodies with yields representing 5.2% (*Star*) and 8.1% (Lemo21) of the total protein extract (Figure 1(b), Suppl. Figure 1 (a)). Protein purification was carried out in the presence of denaturing agents (6 M urea) followed by a slow refolding-process through an over-night dialysis against buffer containing the redox pair of oxidized and reduced glutathione to induce proper disulfide bond formation (Figure 1(b), Suppl. Figure 1(b)). As shown in Figure 1(c), the purified *E. coli*-RBD protein shows a high degree of purity (>90%) and migrated as a single-smear band at the expected height on 4-12% SDS-PAGE (theoretical mass, 26052 Da).

Moreover, Western blot analysis indicated that the protein was efficiently recognized by anti-His and anti-SARS-CoV-2 Spike S1 subunit antibodies (Figure 1(c)). Approximately 1.25 mg of purified RBD was obtained starting from 0.5 L of bacterial culture (final yield ~2.5 mg/L). Among distinct batches, concentration ranged from 0.1 mg/mL (3.8 μ M) to 0.3 mg/mL (11.5 μ M). Concentrations higher than 0.3 mg/mL led to protein precipitation. Finally, the molecular weight and primary aminoacidic sequence of SARS-CoV-2 RBD purified from *E. coli* were further validated by Mass-spec analysis (Suppl. Figure 1(c),(d)).

Design, expression, and purification of SARS-CoV-2 RBD in insect and mammalian cells

RBD fragment with a C-terminal 8x-His purification tag and was cloned downstream of the gp64 for expression in insect cells (Figure 1(a)). Generation and amplification of recombinant baculovirus were carried out in Sf21 cells, while protein expression was performed in Hi-5 infected insect cells (Figure 1(b)). The soluble protein of interest (POI) was secreted into culture media and purified through Immobilized Metal Affinity Chromatography (IMAC) using a Ni-Nta resin (Figure 1(b), Suppl. Figure 2(a)). Isolated insect-RBD migrated as a single band slightly higher than 25 kDa on 4-12% SDS-PAGE (theoretical mass, 26266 Da) exhibiting a high level of purity (>95%), and it was clearly detected by immunoblotting (anti-His and anti- SARS-CoV-2 Spike S1 subunit) (Figure 1(d)). At a laboratory-scale, final yields were around 6.5 mg RBD per Liter of insect cells with batch concentrations ranging from 0.25 mg/mL (9.5 μ M) to 0.5 mg/mL (19 μ M). The experimental mass (28936 Da) of the recombinant insect-RBD determined by MALDI mass-spectrometry analysis (Suppl. Figure 2(b),(c)) was higher than the theoretical one based on the amino acid composition, thus suggesting the presence of glycosylations²⁴.

Regarding RBD expression in mammalian cells, SARS-CoV-2 RBD flanked by a C-terminal 6x-His tag was cloned downstream the Ig Kappa chain-signal peptide responsible

137 for protein secretion (Figure 1(a)). Cells were transfected with DNA and left under stirring
 138 and controlled CO₂ atmosphere for 1 week expressing the POI. The RBD-containing
 139 medium was filtrated and the POI was purified by affinity chromatography (Figure 1(b),
 140 Suppl. Figure 2(d)). The eluted protein migrates as a single-slightly diffuse band below 37
 141 kDa, indicating that RBD (theoretical mass, 26135 Da) contains glycosylations. Indeed,
 142 experimental mass obtained from MALDI-MS analysis was 31453 Da, confirming the
 143 presence of post-translation modifications as previously reported^{21,25,26} (Suppl. Figure
 144 2(e),(f)). Additionally, the eluted protein was efficiently detected by anti-His and anti-S1
 145 subunit of SARS-CoV-2 Spike antibodies (Figure 1(d)). Around 800 mL of transfected cells
 146 yielded 58.8 mg of pure purified protein, with batch concentrations reaching up to 1.8 mg/mL
 147 (69 µM).

148

149 **Figure 1. SARS-CoV-2 RBD production in *E. coli*, insect and mammalian cells.** (a)
150 Schematic representation of the RBD protein constructs expressed in *E. coli* (left), insect
151 cells (middle), and mammalian HEK-293 cells (right). (b) Diagram summarizing the RBD
152 recombinant expression from *E. coli* (left), insect cells (middle), and mammalian HEK-293
153 cells (right) and the subsequent purification. (c) SDS-PAGE (left panel) and Western blot
154 analysis (right panels) of *E. coli*-purified RBD protein. (d) SDS-PAGE (left panel) and
155 Western blot analysis (right panels) of RBD fragment produced in Hi-5 insect cells and
156 mammalian HEK-293. L = molecular weight ladder (e) Theoretical molecular masses

calculated according to RBD amino acid composition (above) and RBD production yields (below).

Biochemical characterization of RBD

RBD produced in HEK-293, insect cells and *E. coli* were analyzed by size exclusion chromatography (SEC) (Figure 2(a)). *E. coli*-RBD elutes as a single and narrow peak centered at 18.7 mL whereas RBD proteins produced in HEK-293 and insect cells display elution peaks shifted to lower retention volumes owing to the presence of glycosylations. In fact, HEK-293-RBD elutes as a main peak centered at 15.3 mL, while RBD produced in insect cells elutes as a major one centered at 16 mL and a minor one at 14.6 mL. The presence of two peaks in the insect-RBD elution profile suggests the existence of at least two populations of the protein showing alternative glycosylation patterns and differing from the one of HEK-293-RBD. The lack of glycosylation of *E. coli*-RBD shifts the retention volumes to higher values. Altogether, all the elution peaks observed are all consistent with a ~30 kDa protein.

RBD proteins were analyzed by far-UV CD spectroscopy. The spectral profiles of HEK-293-RBD and insect-RBD reported in Figure 2(b) are by-an-large identical, both displaying a single minimum at ~206 nm and a maximum at ~230 nm. Conversely, the far-UV CD spectrum of *E. coli*-RBD differs from those of the eukaryotic counterparts, as also observed by Mycroft-West et al²⁷. However, the analysis of the secondary structure composition returned an overall similar distribution (Figure 2(c)).

The conformational stability of RBDs was investigated by means of temperature denaturation experiments: we followed the variations in far-UV CD ellipticity at 222 nm upon increase of temperature from 290 K to 360 K (Figure 2(d)). The change in ellipticity, monitored for each construct, followed a sigmoidal dependence upon temperature increase,

suggesting that the RBDs reversibly unfold. Differently to what expected for a typical folded-to-unfolded transition followed by far-UV CD, the ellipticity values decrease with raising temperature (no loss of CD signal).

The observed denaturation curves could be well fitted to a 2-state transition according to equation (1). The resulting T_m values are identical within experimental error, yielding a mean value of 323.1 ± 0.6 K for HEK-293-RBD, 323.1 ± 1.4 K for insect-RBD, and 322.7 ± 1.3 K for *E. coli*-RBD over three independent experiments. The estimated T_m values are consistent to what previously reported for RBD produced in eukaryotic cells in similar ionic strength conditions²¹. It is worth mentioning that the denaturation curve of *E. coli*-RBD shows a biphasic behaviour, indicating the existence of an initial unfolding event preceding the main one and taking place around 305 K (data not shown). We surmise that this initial phase is likely due to a minor portion of the protein that failed refolding during sample purification from inclusion bodies, probably owing to the absence of glycosylations.

196

197 **Figure 2. Biochemical characterization of recombinant RBD.** (a) Gel filtration
198 chromatographic profiles. Protein separation was performed at room temperature using a
199 Superdex 200 Increase 10/300 GL with 40 μ g of RBD produced in HEK-293 (black), 47 μ g
200 of RBD produced in insect (blue), 40 μ g of RBD produced in *E. coli* (green), each in 50 mM
201 Tris·HCl, 150 mM NaCl, pH 8.3. (b) Far-UV CD spectra of RBD produced in HEK-293
202 (black), insect (blue) and *E. coli* (green) cells. All spectra were collected at 20°C, using a 0.1
203 cm path length quartz cuvette. (c) The histogram reports the distribution of the secondary
204 structure content determined for the RBD proteins (at least three independent CD
205 experiments (mean $\pm$ standard deviation)), in comparison with the secondary structure
206 composition of RBD reported by Lan et al. (dark grey bars)²⁸. (d) Thermal denaturation
207 profiles of RBD *E. coli* (green), insect (blue), and HEK-293 (black) continuously monitored
208 by far-UV CD at 222 nm over the range 290–370 K. Data were fitted using a two-state model.

209

210 *ELISA assays*

211 The functionality of the RBD protein was determined through ELISA assays using
212 plates coated with RBD produced either in *E. coli*, insect or in HEK-293 cells. First, to test
213 coating conditions, 50 ng/well of RBD proteins were used for coating in 50 μ L of phosphate-
214 buffered saline (PBS) or carbonate buffer. Serially diluted (1:1000, 1:10000, 1:50000) rat
215 sera (immunized with COVID-eVax vaccine²⁹) were used to detect the optical density (OD)
216 associated with antibody-RBD interaction under distinct conditions. Significant differences
217 were observed between plates, suggesting that PBS buffer is the most efficient buffer for
218 coating (data not shown).

219 Subsequently, plates were coated with increasing RBD protein concentrations
220 (ranging between 1 and 5 μ g/mL) and serum from rats previously immunized with COVID-
221 eVax²⁹ vaccine was applied to each plate for RBD protein binding. Of note, independently
222 from protein concentration (1, 3 and 5 μ g/mL) both insect-RBD and HEK-293-RBD were
223 efficiently recognized by rat IgG, whereas rat IgG-*E. coli*-RBD interaction was much lower
224 (Figure 3(a)). The observed differences between RBD produced in *E. coli* and in insect or
225 mammalian counterparts are probably due to the major affinity of the latter ones to the IgG
226 produced in rats.

227 We also monitored the interaction between RBD and a commercial antibody against
228 the S1 subunit of SARS-CoV-2 Spike. As shown in Figure 3(b), the observed OD signal of
229 insect-RBD was not markedly different from that of HEK-293-RBD although at
230 concentrations < 1 μ g/mL, RBD from insect showed a slightly higher binding ability
231 compared to HEK-293-RBD (Suppl. Figure 3(a),(b)). In contrast, *E. coli*-RBD showed a lower
232 binding compared to HEK-293 and insect RBDs. We hypothesized that this lower binding

Figure 3. Indirect ELISA assays (a) Serum from immunized rat with COVID-eVax was used to compare different concentrations (1, 3, 5 µg/mL) of RBD expressed in *E. coli* (green), insect (blue) and HEK-293 cells (black). The y-axis represents the optical density (OD) measured at 405 nm while the x-axis accounts for RBD concentrations and serum dilution factors (1:1000, 1:10000, 1:50000). Bars indicate standard deviations. (b) Commercial antibody against the S1 subunit of SARS-CoV-2 Spike was used to compare different concentrations (1, 3, 5 µg/mL) of RBD produced in *E. coli* (green), insect (blue) and HEK-

243 293 cells (black). Optical density (OD) was measured at 450 nm and bars indicate standard
244 deviations.

245

246 *Flow cytometry assay*

247 The receptor binding ability and functionality of RBDs produced in the three presented
248 model systems were further investigated through flow cytometry. Vero E6 cells have been
249 shown to express ACE2 receptor on their apical membrane and to be susceptible to SARS-
250 CoV-2 infection^{30,31}. Thus, we tested RBD-ACE2 binding by incubating recombinant RBD
251 proteins with cultured Vero E6 cells. Figure 4 shows that all the three studied RBDs were
252 able to efficiently bind Vero E6 cells, while no signal was observed when cells were
253 incubated only with antibodies (Suppl. Figure 4). This result suggests that recombinant RBD
254 proteins are efficient in recognizing ACE2.

	Geometric Mean (MFI)
Vero E6	37617.1
IgG	40232
<i>E. coli</i>	115218
Insect	109547.3
HEK-293	115657.9

255

Figure 4. Flow cytometry assays. RBD-Vero E6 cells binding experiment. (a) Gray curve: Vero E6 cells alone; Red curve: Vero E6 cells incubated with only secondary antibody; Green curve: Vero E6 cells incubated with *E. coli*-RBD; Blue curve: Vero E6 cells incubated with Insect-RBD; Black Curve: Vero E6 cells incubated with HEK-293-RBD. Incubation with RBD was followed by anti-RBD primary antibody and secondary antibody. Table represents the intensity of the staining measured as geometric mean (median fluorescence intensity, MFI) value.

Bio-Layer interferometry binding assay

Finally, the binding affinity to ACE-2 receptor of the RBD produced in *E. coli*, insect and HEK-293 cells was evaluated using bio-layer interferometry (BLI). ACE2-hFc fusion protein was immobilized onto anti-human Fc biosensor and different concentrations of RBD proteins (range 150 nM – 9.8 nM) were tested to obtain association curves. After fitting, the dissociation constant (K_d) of ACE2-hFc to insect-RBD and to HEK-293-RBD was determined to be $7.49 \cdot 10^{-9}$ M and $5.34 \cdot 10^{-10}$ M, respectively, while much lower binding affinity was observed for *E. coli*-RBD ($K_d = 1.21 \cdot 10^{-6}$ M) (Figure 5(a),(b) and Suppl. Figure 5).

Model System	K_d (M)
Insect cells	$7,49 \times 10^{-9}$
HEK-293	$5,34 \times 10^{-10}$

272

273 **Figure 5. BLI measurements.** (a) BLI profiles accounting for the binding of insect-RBD and
 274 (b) HEK-293-RBD to ACE2-hFc. After a baseline, the sensorgram starts with the association
 275 (0 - 300 s) of RBD to the ACE2 loaded sensor, followed by the dissociation phase (900 s).

276

277 Discussion

278 The emergence of the novel SARS-CoV-2 pathogen at the end of 2019, which has
 279 quickly degenerated into a pandemic, has underlined the importance of immediate and
 280 responsive actions from the local governments, health authorities, and the world scientific
 281 community in order to tackle this situation that probably represents the biggest challenge
 282 that modern society has faced. As a result, during the last two years, several vaccines have
 283 been developed and many drugs are currently under screening or evaluation in clinical
 284 trials^{32–34}. Most of those therapeutics targets the Spike protein and more specifically its
 285 receptor-binding domain, which is exposed on the viral envelope and that is directly involved
 286 in receptor-binding and cell-entry. Moreover, both full-length Spike and RBD are widely used
 287 as viral antigens for diagnostic tests representing a critical tool for a fast response to the
 288 pandemic.

289 In this study, we provide technical insights into the heterologous expression,
 290 purification, and characterization of the native SARS-CoV-2 RBD produced in *E. coli*, insect
 291 and HEK-293 model systems. Bacterial RBD production was achieved by recovering the
 292 protein from the insoluble fraction and through a careful process of refolding. Efforts to
 293 increase its solubility by using Lemo21 *E. coli* strain failed in agreement with previous
 294 attempts to produce this protein, or its ancestor (SARS-CoV RBD), in *E. coli* in its native
 295 soluble form^{21,35,36}. After refolding, isolated *E. coli*-RBD showed a good degree of purity and
 296 was efficiently recognized by commercial antibodies. Considering the challenging target,
 297 final obtained yields were not high, but enough to carry out most of the lab-scale downstream
 298 applications. In contrast, production in insect and HEK-293 cells resulted in more soluble,
 299 highly glycosylated RBD proteins with yields up to 60 mg/L. The presence of post-
 300 translational modifications (glycosylations) in the latter samples was indirectly observed by
 301 SDS-PAGE, mass-spectrometry analysis, and size-exclusion chromatography. The nature

and the type of glycosylation was not further investigated. Of note, although the far-UV-CD spectrum profile of the RBD from *E. coli* appears different from the one observed for HEK-293-RBD and insect, the overall distribution of the secondary structure composition and the measured T_m were similar among the three samples, suggesting that most of the RBD expressed in bacteria recovers the proper fold, homogeneity, and conformational stability even though lacking glycosylations.

RBD functionality was demonstrated *in-vitro* using Enzyme-linked immunosorbent assay (ELISA). RBD produced in *E. coli* displayed a weak binding affinity to IgG produced in rats and to commercially available antibodies while efficient response was observed for both insect and mammalian derived RBDs. Remarkably, at lower concentration ($< 1 \mu\text{g/mL}$), insect-RBD gave a slightly better signal than HEK-293-RBD. We also investigated the capability of isolated RBDs to bind the ACE2 receptor. All the RBDs produced in this work efficiently bind to Vero E6 cells as confirmed by FACS assay. ACE2-RBD binding was further confirmed and quantified by bio-layer interferometry, with the bacterial-RBD displaying again the lowest binding efficiency. Our data suggest that the absence of glycosylations could partially affect ACE-2 binding *in-vitro* as also previously observed¹⁰. In addition to this, we must consider that the presence of a sub-population of protein that failed refolding, as indicated by circular dichroism and ELISA assays, might also contribute to the observed lower binding efficiency of the bacterial RBD.

To summarize, this work offers a technical and practical overview of RBD production using the three most widely used expression systems, highlighting the main advantages and drawbacks, reported in Table 1. RBD obtained from both eukaryotic systems resulted in a high-quality final bioproduct potentially eligible for diverse downstream applications (vaccine design, diagnostic kits, drug screening etc.). However, the high costs (resources), the time-consuming production, the requirement of specific equipment, and access to dedicated

facilities could be a limitation for many laboratories or for the industrial production. By contrast, the bacterial-derived RBD offers a low production cost, a broader availability, and easy handling as main advantages, which make it more accessible. However, limitations in the quality of the produced sample include the absence of glycosylation that partially affects protein stability and efficiency, the presence of heterogeneous folded populations, and the relative low production yields which may result in a final product not eligible for some clinical and medical applications. Overall, all the recombinantly produced RBDs represent valuable tools for research purposes against the pandemic. Recently, expression and purification strategies described in this article have been also proved to be successful in the production of mutants of RBD corresponding to the variants of concern.

Model System	Cost	Production time	Yields	Glycosylation	Folding	ELISA	FACS	ACE-2 receptor binding
E. Coli	Low	≤ 1 week	+	No	++	+	++	+
Insect	Medium	2-3 weeks	++	Yes ++	+++	+++	+++	+++
HEK-293	High	2-3 weeks	+++	Yes +++	+++	+++	+++	+++

Table 1. Overview of the main aspects of RBD produced in the three major model systems.

Material and Methods

RBD protein production in E. coli

The SARS-CoV-2 Spike Receptor Binding Domain sequence (aa 319-541, Uniprot ID P0DTC2) was cloned with a C-terminal 6x-His tag into a pET-21a(+) plasmid. *E. coli* BL21 StarTM (DE3) (genotype: F⁻*ompT hsdS_B* (r^B⁻, m^B⁻) *gal dcmrne131*) competent cells, and *E. coli* Lemo21 (DE3) (genotype: *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS/ pLemo(Cam^R)*) competent cells were transformed with 100 ng of plasmid of interest. A single colony was incubated in 15 mL starter culture (LB) with Ampicillin (BL21 Star strain) or

349 Ampicillin/Chloramphenicol (Lemo21 strain), grown at 37°C on agitation over-night. Starter
 350 culture was successively inoculated in 500 mL (LB) with antibiotics incubated at 37°C until
 351 mid-log phase ($OD_{600} = 0.6$ to 0.8). Protein expression was induced with 0.5 mM isopropyl
 352 β -D-1-thiogalactopyranoside (IPTG) at 30°C or 37°C for 4 hours on agitation. Cells were
 353 harvested at 6'000 rpm for 10 minutes, washed once with 50 mM Tris·HCl (pH 8.0) and then
 354 further centrifuged. Pellet was resuspended in a solution containing 50 mM Tris·HCl, 500
 355 mM NaCl (pH 8.0) also containing a protease inhibitor cocktail (Roche 11836170001) prior
 356 sonication. The suspension was then centrifuged at 11'000 rpm for 45 minutes to separate
 357 soluble and insoluble fractions. The pellet containing the RBD target protein was
 358 resolubilized in extraction buffer containing 50 mM Tris·HCl, 500 mM NaCl, 20% glycerol,
 359 10 mM β -mercaptoethanol, 8 M urea (pH 8.0). The washed inclusion bodies were shortly
 360 sonicated and left 1 h at room temperature (RT) or O/N at 4°C on agitation. Protein was
 361 purified using IMAC (His-Trap, Cytiva) under denaturing conditions (Elution buffer: 50 mM
 362 Tris·HCl, 500 mM NaCl, 20% Glycerol, 10 mM β -Mercaptoethanol, 6 M urea, 300 mM
 363 imidazole pH 8.0). Eluted fractions were analysed by SDS-PAGE and firstly dialyzed over-
 364 night against buffer containing 50 mM Tris·HCl, 500 mM NaCl, 20% Glycerol, GSH-GSSG
 365 (3 mM : 1 mM), 2 M urea (pH 8.0) with slow agitation. The day after, protein solution was
 366 dialyzed against 50 mM Tris·HCl, 500 mM NaCl, 1 mM TCEP (pH 8.0) or PBS 1x (pH 7.4)
 367 for 4 hours. The purified *E. coli*-RBD sample protein was quantified by UV-visible
 368 spectroscopy, aliquoted, and stored at -80°C.

369

370 *RBD protein production in insect cells*

371 The SARS-CoV-2 Spike Receptor Binding Domain sequence (aa 319-541, Uniprot
 372 ID P0DTC2) was cloned into a pFAST-bac1 plasmid downstream of the gp64 signal
 373 sequence to promote secretion, along with a C-terminal 8x-His tag for affinity purification.

100 ng of plasmid was transformed into DH10Bac competent cells (MAX Efficiency™ DH10Bac Competent Cells, Gibco #10361012) for bacmid DNA production. Each bacmid, extracted from 3 mL of an O/N colony culture, was diluted in a final volume of 220 µL of Sf900 III medium and then combined with a mix of 10 µL of XtremeGene (Cellfectin™ II Reagent, Gibco #10362100) in 100 µL Sf900 III medium. This solution was left 15 minutes at room temperature to allow complex formation, according to the manufacturer's protocol. For transfection, the latter solution was added dropwise onto Sf21 cells (Gibco #11497013) previously plated on a 6-well plate at 1.0×10^6 cells/well confluency. 60 hours post-transfection, supernatant containing the first generation of recombinant baculovirus (V_0) was harvested and amplified to obtain a high titer of virus. Hi-5 cells (BTI-TN-5B1-4) (Gibco #B85502) were cultured in Express Five™ SFM (Serum-Free Media) medium (Gibco #B85502 Expression Systems) at a cell density of 0.5×10^6 cells/mL and infected with recombinant virus. Cells were kept at 27°C and 130 rpm for protein expression. After 72 hours, supernatant containing secreted RBD was collected and subjected to IMAC (His-Trap Excel, Cytiva). RBD was eluted using 50 mM Tris·HCl, 150 mM NaCl, 300 mM imidazole, pH 8.0). Eluted fractions were analysed on 4-12% SDS-PAGE and dialyzed over-night against 50 mM Tris·HCl, 150 mM NaCl, pH 8.0 with slow agitation. Purified RBD protein was quantified by UV-visible spectroscopy, aliquoted, and stored at -80°C.

RBD protein production in HEK-293 cells

C-terminal 6x-His tagged SARS-CoV-2 RBD fragment (aa 319-541, Uniprot ID P0DTC2) was cloned downstream of the Ig Kappa chain-signal peptide for expression as secreted protein in mammalian cells (Expi293). Cells were transfected at a concentration of $\sim 3 \times 10^6$ /mL with 1 µg of DNA per milliliter of cell culture. Feed enhancers and PEN-STREP were added to the cells after 20 and 24 hours, respectively. Cells were left in agitation at

37°C for 1 week before clarification by centrifugation at 12700 rcf. After filtration, the RBD-containing supernatant was purified by affinity chromatography on a 1 mL INDIGO column (Cube Biotech). Sample was diluted with binding buffer (20 mM NaPi, pH 7.4, 500 mM NaCl) and loaded at 1 mL/min flowrate. Elution was carried out in the same conditions, with a single step of 250 mM imidazole (20 mM NaPi, pH 7.4, 500 mM NaCl, 250 mM Imidazole). Eluted protein was readily dialyzed against DPBS 1x. Isolated RBD protein was quantified by UV-visible spectroscopy, aliquoted, and stored at -80°C.

Mass-spectrometry analysis

SARS-CoV-2 RBD recombinant protein(s) molecular weight and primary aminoacidic sequence were determined by MALDI-MS and by peptide mass fingerprint (PMF), respectively. Determination of the molecular weight was achieved by MALDI mass spectrometry analysis on a MALDI Ultraextreme (Bruker, GmbH) in positive linear mode. 30 µL of sample were desalted by diafiltration using Amicon filters with 3.5 kDa MWCO or by Zip Tip C18 (Millipore) and 2 µL of sample were mixed with a solution of the matrix superDHB. A volume of 2 µL of the resulting solution were deposited on the target plate and left dry in the air.

In order to acquire information on the primary aminoacidic sequence, an aliquot of each sample was reduced, alkylated, digested with trypsin, and analysed by RP-UHPLC-MS/MS. RP-UHPLC-MS analysis was performed on a Q-Exactive HF-X (ThermoFisher Scientific) mass spectrometer coupled with an UHPLC Ultimate 3000 RSLCnano System (ThermoFisher Scientific). A volume of 1 µL of the resulting peptide mixtures were injected on a column EasySpray PepMap RSLC C18 100 Å 2 µm, 75 µm x 15 cm (Thermo Fisher Scientific). The column oven was maintained at 35°C, the analysis was carried using a gradient elution (phase A: 0.1% formic acid in water; phase B: 0.1% formic acid in

acetonitrile). The flow rate was maintained at 300 nL/min. The mass spectra were acquired using a “data dependent scan”, able to acquire both the full mass spectra in high resolution and to “isolate and fragment” the twelve ions with highest intensity present in the mass spectrum. Raw data were analyzed using the Biopharma Finder 2.1 software from ThermoFisher Scientific.

SDS-PAGE and Western blot

Purified proteins (500 ng) were analyzed on 4-12% NuPAGE Bis-Tris gels (Life Technologies) under reducing conditions followed by Coomassie Brilliant Blue staining (Invitrogen LC6060). For Western blot analysis, gels were electroblotted onto nitrocellulose membranes (Bio Rad). Blots were incubated with primary antibodies in 5% non-fat dry milk in PBS plus 0.1% Tween20 overnight at 4°C. Detection was achieved using horseradish peroxidase-conjugate secondary antibody anti-rabbit and anti-mouse (Bio Rad #1706516, #1706515) and visualized with ECL (Cytiva RPN2232). Images were acquired by using a ChemiDoc™ Touch Imaging System (Bio Rad) and analyzed by Image Lab software (Bio Rad).

Antibodies

The primary antibodies used in this study are: rabbit anti-SARS-CoV-2 Spike S1 Subunit (Sino Biological, 40150-T62), mouse anti-His Tag (Invitrogen MA1-21315). Secondary antibody used are: horseradish peroxidase-conjugate anti-rabbit and anti-mouse (Bio Rad #1706516, #1706515).

447 *Densitometric analysis*

448 Intensities of bands corresponding to RBD proteins were measured using Gel Doc
449 2000 and Image Lab software (Bio-Rad, Hercules, CA, USA) in order to measure protein
450 expression levels. Briefly, blots were acquired using the Gel Doc 2000 apparatus; images
451 were imported into the Image Lab software; contrast was adjusted such that the bands were
452 clearly visible on the blot image; area around each band was selected; background intensity
453 was subtracted from the blot image; bands were then selected by drawing a tight boundary
454 around them; intensities of the selected bands were exported in excel format file which was
455 used to perform further analysis.

456

457 *ELISA assay*

458 ELISA plates were coated with different concentrations of *E. coli*-RBD, Insect-RBD
459 and HEK-293-RBD proteins. After washing and blockade of free protein-binding sites with
460 PBS – 0.05% Tween20 - 3% BSA, different concentrations of rat serum (immunized with
461 COVID-eVax vaccine) or anti-SARS-CoV-2 Spike S1 Subunit antibody (Sino Biological,
462 40150-T62) were added to each well and incubated overnight at 4°C in PBS – 0.05%
463 Tween20 - 1% BSA. After washing, AP-conjugated goat anti-rat IgG antibody (SIGMA
464 A8438) or AP-conjugated goat anti-rabbit IgG antibody (SIGMA A8025) was added, and the
465 plates were further incubated for 1 hour at RT. Finally, 3,3',5,5'-Tetramethylbenzidine (TMB)
466 Liquid Substrate System (Sigma T8665) or alkaline Phosphatase Yellow (pNPP) Liquid
467 Substrate System for Elisa (Sigma P7998) were added as a substrate. After 30 minutes the
468 TMB reaction was stopped with the stop reagent for TMB substrate (Sigma S5814) and the
469 absorbance was measured at 450 nm, while the pNPP reaction was measured at 405nm at
470 different time points.

471

472 FACS

473 Vero E6 cells were incubated with RBD protein (0.45 µg/mL, final concentration)
474 followed by incubation with human anti-RBD antibody (primary antibody) (40150-D003, Sino
475 Biological) and goat anti-human IgG AF488-conjugated antibody (secondary antibody) (A-
476 11013, Thermo Fisher Scientific). Staining with only secondary antibody was used to
477 determine the level of background due to non-specific antibody binding. Each staining step
478 was performed at 4°C for 20 minutes in FACS buffer. Samples were run on a CytoFlex flow
479 cytometer (Beckman Coulter). Analysis was performed using the CytExpert software
480 (Beckman Coulter).

481

482 CD static spectra and thermal denaturation

483 Far-UV (200-250 nm) circular dichroism (CD) spectra of the SARS-CoV-2 HEK-293-
484 RBD and (insect) were recorded using 0.6 mg/mL protein solution in PBS pH 7.4 and 0.3
485 mg/mL protein solution in 50 mM Tris·HCl, 150 mM NaCl, respectively. CD spectra of the *E.*
486 *coli*-RBD were monitored at 0.5 mg/mL protein concentration in 50 mM Tris·HCl, 150 mM
487 NaCl, 1 mM TCEP, 20% glycerol, pH 8. All CD static spectra were collected at 20°C,
488 scanning at 50 nm/min, using a 0.1 cm path length quartz cuvette (Hellma, Plainview, NY)
489 and a JASCO-815 spectropolarimeter equipped with a Jasco programmable Peltier element
490 (Jasco, Easton, MD, USA). For each sample five scans were averaged and scans
491 corresponding to buffer solution were averaged and subtracted from the sample spectra.
492 Results are expressed as the molar ellipticity ([θ]). The formula used to calculate the molar
493 ellipticity is: $[\theta] = (\theta \times MW)/(C \times L \times 10)$, where [θ] is the molar ellipticity, θ the experimental
494 ellipticity in mdeg, MW the molecular weight of the protein in Daltons, C the protein

concentration in mg/mL, and L the path length of the cuvette in cm. Secondary structure composition was assessed using the BeStSel analysis server^{37,38}.

CD thermal denaturation experiments were followed at 222 nm, heating from 20 to 90°C at a rate of 1 °C min⁻¹ controlled by a Jasco programmable Peltier element (Jasco, Easton, MD, USA). The dichroic activity at 222 nm and the photomultiplier voltage (PMTV) were continuously monitored in parallel every 1.0 °C³⁹. Data were fitted to a standard two-state denaturation⁴⁰, according to the equation (1):

$$\Delta G_{D-N} = \Delta H_m \left(1 - \frac{T}{T_m}\right) + \Delta C_p [T - T_m - \left(T \ln \frac{T}{T_m}\right)]$$

where ΔG_{D-N} is the free energy of the unfolding process, T_m is the melting temperature that corresponds to midpoint of the thermal denaturation, ΔH_m is the enthalpy of denaturation at the transition midpoint, and ΔC_p is the change of heat capacity of denaturation. The latter parameter is related to the amount of hydrophobic area that becomes exposed to solvent upon unfolding and it is constant for a given protein. To a first approximation, the melting temperature (T_m) of unfolding has been estimated using the ΔC_p value reported for a α -chymotrypsin (241 amino acids)⁴¹. All denaturation experiments were performed in triplicate.

Size exclusion chromatography

Analytical gel filtration chromatography was performed using a Superdex 200 Increase 10/300 GL SEC column (Cytiva, USA) coupled to an HPLC system (Azura System, Knauer- Berlin, Ge) equipped with a UV-vis absorbance detector (Smartline 2520, Knauer- Berlin, Ge). The column was equilibrated with 50 mM Tris·HCl pH 8.0, containing 150 mM NaCl. A total of 40 µg of HEK-293-RBD, 47 µg of Insect-RBD and 40 µg of *E. coli*-RBD were injected into the column and eluted at a flow rate of 0.75 mL/min in isocratic mode. Elution profile was followed at 280 nm at room temperature. The shape of the elution profiles and

the difference between HEK-293-RBD, Insect-RBD, and RBD (*E. coli*) were observed reproducibly in three independent experiments.

Bio-layer interferometry (BLI)

Binding studies were carried out using the Octet Red system (Forte Bio). All steps were performed at 25°C with shaking at 600 rpm in a 96-well plate (microplate 96 well, F-bottom, black, 655209, from Greiner bio-one) containing 200 µL of solution in each well. Kinetics buffer 1x (cat. No.18-1105, Forte Bio) was used throughout this study for samples dilution and for sensors washing.

Kinetic assays were performed by first capturing ACE2-hFc using anti-human Fc Octet biosensors (Anti-human IgG Fc Capture Biosensors, cat. No. 18-5060, FORTEBIO). Biosensors were soaked for 10 min in 1x kinetic buffer followed by a baseline signal measurement for 60 s and then loaded with ACE2-hFc recombinant protein (10 µg/mL) for 300 s (until the biosensor was fully saturated). After a wash step in 1× kinetic buffer for 120 s, the ACE2-Fc-captured biosensor tips were then submerged for 300 s in wells containing different concentrations of antigen (RBD *E. coli*, insect, and HEK-293) to evaluate association curves, followed by 900 s of dissociation time in kinetic buffer. The ACE2-hFc captured biosensor tips were also dipped in wells containing kinetic buffer to allow single reference subtraction to compensate for the natural dissociation of captured ACE2-hFc. Biosensor tips were used without regeneration.

The binding curve data were collected and then analysed using data analysis software version 12.0 (FORTEBIO). Binding sensorgrams were first aligned at the last 5 seconds of the baseline step average. The single reference subtraction binding sensorgrams were globally fit to a 1:1 Langmuir binding model to calculate K_d values.

543

544 **Conflict of interest**

545 Mariano Maffei, Grazia Vitagliano, Shaila Sellathurai, Federica Bucci, Alessia Muzi and
546 Valerio Chiarini are employees of Takis and Evvivax companies. Mirco Compagnone is
547 employee of NeoMatrix. Luigi Fedele is a former employee of Takis company. Giuseppe
548 Roscilli and Emanuele Marra are co-founders of Takis and Evvivax companies.

549 The other authors declare no conflict of interest.

550

551 **Author Contributions**

552 **Mariano Maffei:** Conceptualization; Investigation; Supervision; writing-original draft; writing-
553 review and editing. **Linda Celeste Montemiglio:** Investigation; writing-review and editing.
554 **Grazia Vitagliano:** Investigation; editing. **Luigi Fedele:** Investigation. **Shaila Sellathurai:**
555 Investigation. **Federica Bucci:** Investigation. **Mirco Compagnone:** Investigation. **Valerio**
556 **Chiarini:** Investigation. **Cécile Exertier:** Investigation. **Alessia Muzi:** Investigation.
557 **Giuseppe Roscilli:** Resources; writing-review and editing. **Beatrice Vallone:** Resources,
558 writing-review and editing. **Emanuele Marra:** Conceptualization; Supervision; resources,
559 writing-review and editing.

560

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713

714 **Supporting information**

715 Additional supporting information may be found online in the Supporting Information section.

716