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R&D STUDY REPORT No. R-20-0085

COVID-19: IMMUNOGENICITY STUDY OF THE LNP-FORMULATED MODRNA ENCODING THE VIRAL S PROTEIN-V9

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Date: 23 NOV 2020

Reported by (b) (6)

Test item: BNT162b2 (animal trial material)
Key words: Coronavirus, COVID-19, modRNA, ATM, mouse, immunogenicity

This R&D report consists of 93 pages.

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LIST OF ABBREVIATIONS

AH-1	Irrelevant peptide derived from endogenous retroviral gene product envelope glycoprotein 70
ATM	Animal trial material
BCS	Body Conditioning Score
BNT162	BioNTech's SARS-CoV-2 vaccine candidate
CD	Cluster of differentiation
ConA	Concanavalin A
COVID-19	Coronavirus disease emerged 2019
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
Hsopt10	Nucleoside optimization protocol 10 based on *Homo sapiens* databank
ICS	Intracellular cytokine staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.m.	Intramuscularly
K_D	Binding affinity
k_{off}	Dissociation rate constant (off-rate)
k_{on}	Association rate constant (on-rate)
LNP	Lipid nanoparticle
LLOQ	Lower limit of quantification
MACS	Magnetic cell separation
modRNA	Nucleoside-modified mRNA
nAb	Neutralizing antibody
No.	Number
OD	Optical density
PBS	Phosphate-buffered saline
PMA	Phorbol 12-myristate 13-acetate
pVNT	Pseudovirus-based neutralization test
RBD	Receptor-binding domain
RNA	Ribonucleic acid
S protein	Spike protein
S1	Subdomain 1 of the S protein
S2	Subdomain 2 of the S protein
saRNA	Self-amplifying mRNA
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
SPR	Surface plasmon resonance
T_H1/T_H2	Type 1/2 helper T cells
TNF	Tumor necrosis factor
ULOQ	Upper limit of quantification
uRNA	Uridine-containing mRNA
V	Variant
VSV	Vesicular stomatitis virus

RESPONSIBILITIES

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	BioNTech SE	Date

Meaning of the signatures:

Person responsible for the study: I am responsible for the content of the R&D report and confirm that it represents an accurate record of the results. This study was performed according to the SOPs and methods as well as the rules and regulations described in the report.

Author: I am the author of this document.

Reviewer: I reviewed the R&D report and confirm that this document complies with the scientific and technical standards and requirements.

QA representative: I confirm that this document complies with the relevant quality assurance requirements.

The author gave approval to this document via e-mail according to CC-20-0081 (see attachment

1 SUMMARY

BioNTech is developing RNA-based vaccines designed to protect against the novel coronavirus disease that emerged in 2019 (COVID-19). The project involves testing three RNA platforms which are under development at BioNTech with the surface or spike protein (S protein) of the novel coronavirus (SARS-CoV-2) as the viral antigen.

In the present study, the immunogenicity of a nucleoside-modified mRNA (modRNA) encoding the antigen variant 9 (V9) of the generated variants of the S protein, BNT162b2, was investigated. Four groups of eight female BALB/c mice were immunized on day 0 with doses of 0.2 µg, 1 µg, or 5 µg per animal of the modRNA encapsulated in lipid nanoparticles (LNPs), or with the buffer alone (control group), by intramuscular injection. Blood was collected on days 7, 14, 21, and 28 after immunization to analyze the antibody immune response by ELISA and pseudovirus-based neutralization test (pVNT). On day 28, spleens were collected for splenocyte isolation and analysis of T-cell responses using interferon γ (IFN-γ) -specific ELISpot assays. Luminex assays and intracellular cytokine staining (ICS) and were performed to assess cytokine responses.

The vaccine candidate was highly immunogenic; treatment with all tested BNT162b2 doses induced a strong immune response across the observation period of 28 days. Total IgG ELISA showed that the construct induced a strong, dose-dependent generation of antibodies against the S1 antigen and the receptor-binding domain (RBD). Vaccine-elicited IgG had a strong binding affinity for S1 and the RBD, both had low off-rates, as detected by surface plasmon resonance spectroscopy (SPR). In pVNT analysis, all mice developed functional neutralizing antibodies starting at 14 days after immunization and increasing up to final study day. The summary of antibody titers on day 28 is as follows:

	BNT162b2 0.2 µg	BNT162b2 1 µg	BNT162b2 5 µg
Anti-S1 protein total IgG [µg/mL]	73.0 ± 10.4	205.9 ± 21.0	392.7 ± 28.9
Anti-RBD protein total IgG [µg/mL]	83.1 ± 12.3	241.7 ± 17.2	448.6 ± 28.6
pVN_{50} titer [reciprocal dilution]	33.0 ± 9.8	192.0 ± 31.4	312.0 ±35.1

By profiling the IgG subtypes, a balanced IgG2a/IgG1 response was detected for the higher doses, while the low dose induced a response with higher IgG1 than IgG2 levels. Cellular assays and cytokine profiling revealed that in addition to a cytotoxic $CD8^+$ T-cell response, a proinflammatory, T_H1-specific response was activated after peptide stimulation. Therefore, BNT162b2 is a promising candidate for further testing in clinical trial.

(b) (6)

BioNTech RNA Pharmaceuticals GmbH

23NOV20

Date

2 GENERAL INFORMATION

Sponsor

BioNTech RNA Pharmaceuticals GmbH
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Test Facility

BioNTech SE
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2.1 Participating Personnel

Responsible person: (as defined in SOP-100-024)	(b) (6) An der Goldgrube 12 55131 Mainz
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2.2 Study Dates

Start of experiments: 31 MAR 2020

Completion of experiments: 17 SEP 2020

2.3 Guidelines and Regulations

All experiments are executed in accordance with the existing standard operating procedures and described processes from BioNTech SE. Applicable documents are listed below.

- Animal test application approval number: G18-12-100, amendment from 18.02.2020 (approved 20 FEB 2020)
- SOP-010-017 Brutschränke - Biolytics
- SOP-010-028 Vi-Cell XR
- SOP-010-045 Brutschrank HERAcell 150i
- SOP-010-047 Zentrifuge Eppendorf 5810/5810R
- SOP-010-051 Tiefkühlschränke -80 °C
- SOP-010-058 Sicherheitswerkbank Klasse II
- SOP-010-086 Zentrifuge Thermo Scientific Heraeus Pico und Fresco 17
- SOP-010-099 CTL ELISPOT Reader
- SOP-020-009 Ansetzen von Medien und Zusätzen für die Zellkultur
- SOP-030-043 Kryokonservierung von Zellen
- SOP-030-071 Abtöten von Mäusen
- SOP-030-072 Fixiergriff und Ohrmarkierung bei Mäusen
- SOP-030-073 Betäubung bei Mäusen
- SOP-030-074 Blutentnahme bei Mäusen
- SOP-030-078 Isolierung muriner Splenozyten
- SOP-030-079 Intramuskuläre Applikation bei Mäusen
- SOP-030-110 IFNγ ELISpot (murin)
- SOP-030-112 Durchführung eines virusprotein-spezifischen ELISA
- SOP-090-013 Biological safety in laboratories
- SOP-110-022 Entsorgung von Biostoffabfällen

2.4 Changes and Deviations

This R&D study was conducted according to R&D plan P-20-0085.

A change occurred in the pVNT. It was planned to perform this analysis with an external partner, (b) (4) . However, the CRO had no pVNT or VNT in place when samples were ready to analyze. Therefore, an internal assay was developed using the VSV-based pseudovirus to analyze for neutralizing antibodies.

Furthermore vaccine-induced SARS-CoV-2 specific antibodies were analyzed for their affinity toward recombinant SARS-CoV-2 S and RBD protein via surface plasmon resonance (SPR) spectroscopy. Affinity measurements were only conducted with day 28 sera of the 5 µg BNT162b2 dose cohort.

Another change occurred in the protocol for murine ELISpot, described within SOP-030-110. The described change resulted in faster dryness of the ELISpot plate and thus its readiness for the subsequent protocol step; analysis of spot numbers per well via ImmunoSpot® S6 Core Analyzer, CTL. This change has no impact on performance of the protocol.

Furthermore, in a first run with fresh splenocytes a miscalculation of cells in the group immunized with 5 µg modRNA occurred. Therefore, a second ELISpot run was included with frozen splenocytes.

Because the utilized major histocompatibility complex (MHC) I/II blockade was not effective in determining T-cell subtypes, an additional ELISpot analysis was performed after separation of $CD4^+$ and $CD8^+$ cells by MACS isolation to identify the responding T-cell subtype (group 4 only).

Cytokine concentrations in supernatants of re-stimulated splenocytes were determined using a bead-based, T_H1/T_H2 mouse ProcartaPlex immunoassay. An intracellular cytokine staining was added for T_H1/T_H2 cytokine analysis.

2.5 Documentation and Archive

Study plans and reports are stored and archived according to SOP-100-003 Archiving of Paper-Based Documents.

Raw data and evaluated data are saved at:

- P:\BioNTechRNA\RN9391R00_CoV-VAC\04_Preclinic\00_Pharmacology\ mCorVAC#11_modRNA-V9
- Animal Models & Facility: Lab book No. 1893
- Infectious Disease Vaccines (ELISA): Lab book No. 1858, 1978
- Infectious Disease Vaccines (ICS): Lab book No. 1937
- Immunomodulators: Lab book No. 1935, 1936
- Cancer Vaccines: Lab book No. 1934
- New Scaffolds: Lab book No. 2009

3 INTRODUCTION

3.1 Background

In December 2019, an outbreak of pneumonia of unknown cause in Wuhan, Hubei province in China was reported. The disease spread rapidly and in January 2020, the agent was identified. By 21 June 2020, infection with the novel coronavirus (SARS-CoV-2) was confirmed in over 8,700,000 people with more than 460,000 casualties[1]. A vaccine is urgently needed and BioNTech decided to develop a rapid vaccine project based on the surface or spike protein (S protein) of the virus as the viral antigen. The S protein is a trimer and during viral egress, the precursor protein is cleaved in S1 and S2 (Figure 1). While the S1 domain recognizes the host receptor, the S2 domain is essential for the membrane fusion of viral envelope and endosomal membrane. To initiate the membrane fusion, the S2 domain undergoes a conformational change within the central helix domain.

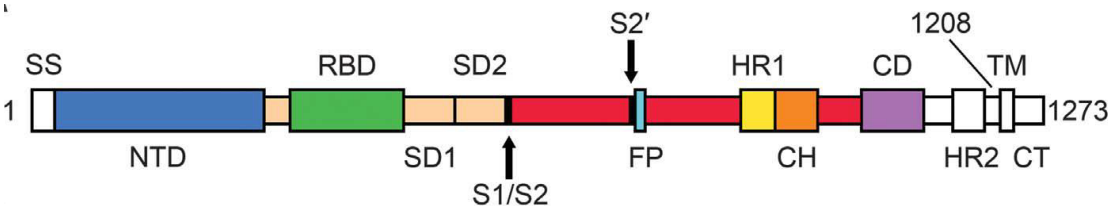

Figure 1: Schematic overview of the S protein organization of the SARS-CoV-2 S protein.

The sequence within the S1 subunit consists of the signal sequence (SS) and the receptor-binding domain (RBD) which is the key subunit within the S protein which is relevant for binding to the human cellular receptor ACE2. The S2 subunit contains the S2 protease cleavage site (S2') followed by a fusion peptide (FP) for membrane fusion, heptad repeats (HR1 and HR2) with a central helix (CH) domain, the transmembrane domain (TM) and a cytoplasmic tail (CT); source: modified from (Wrapp et al. 2020).

Based on these features, the S protein is the target of the neutralizing antibody (nAb) that binds dominantly to the RBD of the S protein. Vaccine candidates selected for non-clinical testing include the following vaccine antigens:

- A secreted variant of the RBD of the SARS-CoV-2 S protein (called V5) (Kirchdoerfer et al. 2018)
- Membrane-tethered full-length S protein with two point mutations within the central helix domain (called V8/V9). Mutation of the two amino acids to proline, (KV286-287PP) retains the S protein in an antigenically optimal prefusion conformation (called V8 or V9) (Wrapp et al. 2020, Pallesen et al. 2017)

[1] Coronavirus disease (COVID-2019) situation report 153, World Health Organization; www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports

The development of *in vitro* transcribed RNA as an active platform for the use in infectious disease vaccines is based on the extensive knowledge of the company in RNA technology, which has been gained over the last decade. The core innovation is based on *in vivo* delivery of a pharmacologically optimized, antigen-coding RNA vaccine to induce robust neutralizing antibodies and accompanying/concomitant T cell response to achieve protective immunization with minimal vaccine doses (Vogel et al. 2017, Moyo et al. 2018, Pardi et al. 2017).

At BioNTech, there are three different RNA platforms under development, namely non-modified uridine-containing mRNA (uRNA), nucleoside-modified mRNA (modRNA), and self-amplifying RNA (saRNA). It is unknown today which RNA vaccine platform performs best in terms of activation and duration of a potent immune response. Therefore, BioNTech has developed a project plan that is based on testing GMP-produced, available material that has already been tested in clinical trials. The three vaccine platforms will be tested for each antigen construct in non-clinical mouse studies and tested for their virus-neutralizing response and the total amount of IgG antibodies developed against the S protein. Candidates that induce a high fraction of nAb within the total IgG population are desired. This report covers a mouse study testing modRNA encoding the antigen variant 9 (V9) of the generated variants of the S protein.

3.2 Objectives

In this study, the primary objective was to understand the immunogenicity of the designed construct. For this purpose, a dose titration in BALB/c mice was performed with the LNP-formulated modRNA encoding the antigen variant 9 (V9) of the generated variants of the S protein (V9 main characteristics: S protein full-length with two point mutations, opt1 sequence optimization that increases the GC-content of the coding sequence). The immune response was analyzed focusing on the antibody immune response and included the analysis of the IFN-γ release of splenocytes at the end of study as well as assessment of cytokine/chemokine responses.

3.3 Study Design

Four groups of eight female BALB/c mice were immunized once (on day 0) with BNT162b2 at three different doses, or with the buffer alone (control group). Immunizations were given intramuscularly (i.m.) in a dose volume of 20 µL. Blood was collected once weekly for three weeks (days 7, 14, and 21) to analyze the antibody immune response by ELISA and pseudovirus-based neutralization assay (pVNT). At the end of the study (on day 28), blood was collected for ELISA and pVNT analyses (all samples), as well as for affinity measurements of vaccine-induced antibodies toward recombinant SARS-CoV-2 S and RBD via SPR (high-dose cohort samples only). Animals were then euthanized for spleen collection and additional analysis of the T-cell response in splenocytes by ELISpot, Luminex assay, and ICS (see Table 1).

Table 1: Study design

Group no.	No. of animals	Vaccine/ batch	Concentration of active component [µg/animal]	Immunization day	Dose volume [µL] / route	Blood collection day	End of study day
1	8	Buffer	-	0	20 / i.m.	7, 14, 21, 28	28
2	8	BNT162b2 /RBP020.2	0.2	0	20 / i.m.	7, 14, 21, 28	28
3	8	BNT162b2 /RBP020.2	1	0	20 / i.m.	7, 14, 21, 28	28
4	8	BNT162b2 /RBP020.2	5	0	20 / i.m.	7, 14, 21, 28	28

4 MATERIALS AND METHODS

4.1 Test Item

BNT162b2, animal trial material (ATM): For CoAs see Appendix 2: Certificates of Analysis.

- RNA batch: RNA-RF200321-06
- Polymun batch RBP020.2 LNP with the lot: CoVVAC/270320

4.2 Control Item

- PBS+300 mM sucrose (from Polymun)

4.3 Test System

- 32 female BALB/c mice at an age of 9 weeks at study start.

4.4 Materials

Table 2: Materials

Product name	Application/ specification	Article no.	Working dilution	Provider
15 mL/50 mL tube	Conical bottom, PP, 30/115 MM, CELLSTAR®	188271/ 227261	N/A	Greiner Bio-One GmbH
2 mL tube	CRYO.S, round bottom	122278	N/A	Greiner Bio-One GmbH
2-Mercaptoethanol	50 mM	31350-010	N/A	Gibco
8-channel manifold	Polypropylene	BR704526-1EA	N/A	Sigma-Aldrich Chemie GmbH
96-well flat-bottom plate	pVNT	655160	N/A	Greiner
96-well microplate	Clear round bottom TC-treated microplate, with lid, sterile	3799	N/A	Corning Holding GmbH
96-well V-bottom plate	pVNT	651180	N/A	Greiner
AffiniPure goat anti-mouse IgG	SPR	115-005-071	N/A	Jackson ImmunoResearch
Alexa Fluor® 488 anti-mouse TNF-α antibody, clone MP6-XT22	ICS	506313	1:100	BioLegend
Amine coupling kit	SPR	BR100050	N/A	GE Healthcare
Ammonium chloride	NH_4Cl	A0988,5000	N/A	AppliChem GmbH
Anti-rat/hamster Ig, κ/negative control (FBS*)	Compensation Particles Set	552845 component no. 51-90-9000949	1 drop	BD

Product name	Application/ specification	Article no.	Working dilution	Provider
Anti-VSV-G antibody	Clone 8G5F11	EB0010	N/A	Kerafast
APC anti-mouse IL-2 antibody	ICS	503810	1:100	BioLegend
BD Pharmingen™ purified rat anti-mouse CD16/CD32	Mouse BD Fc Block™ (2.4G2)	553142	1:100	BD
Blocker™ bovine serum albumin (BSA) in PBS (10×)	ICS	7011150	1×	ThermoFisher
Brilliant Violet 510™ anti-mouse CD4 antibody	ICS	100559	1:200	BioLegend
Brilliant Violet 711™ anti-mouse IL-4 antibody	ICS	504133	1:200	BioLegend
Brilliant Violet 785™ anti-mouse CD25 antibody PC61	ICS	102051	1:200	BioLegend
BV421 rat anti-mouse CD8a antibody	ICS	100753	1:200	BioLegend
Capillary pipettes	minicaps®, blood sampling, 4 µL/10 µL, not heparinized	9000104/ 9000110	N/A	Hirschmann Laborgeräte GmbH & Co.KG
Casein blocking buffer 10×	ELISA	B6429-500ml	N/A	Sigma-Aldrich Chemie GmbH
CM5 sensor chip	SPR	BR100012	N/A	GE Healthcare
Combitips advanced®	Biopur®, 50 mL	0030089693	N/A	Eppendorf Vertrieb Deutschland GmbH
Concanavalin A	From *Canavalia ensiformis* (Jack bean, 5 mg),Type IV-S, lyophilized	C0412-5MG	N/A	Sigma-Aldrich Chemie GmbH
Cover films	ELISA	RATI6018410	N/A	VWR International GmbH
Dimethyl sulfoxide (DMSO)	For cell culture	A3672,0100	N/A	AppliChem GmbH
DPBS	No calcium, no magnesium	14190-094	1 ×	Thermo Fisher Scientific
Easystrainer 70 µm	For 50 mL tubes	542070	N/A	Greiner Bio-One GmbH
eBioscience™ Fixable Viability Dye eFluor™ 780	ICS	65-0865-18	1:1,000	ThermoFisher
Eppendorf safe-lock tubes	0.5 mL/ 1.5 mL/ 2.0 mL/ 5.0 mL, Eppendorf Quality™	0030121023/0030120086/0030120094/0030119401	N/A	Eppendorf Vertrieb Deutschland GmbH

Product name	Application/ specification	Article no.	Working dilution	Provider
Ethylenediaminetetraacetic acid solution	EDTA	03690-100ML	N/A	Sigma-Aldrich Chemie GmbH
Fetal bovine serum (FBS)	Non-USA origin, sterile-filtered	F7524	N/A	Sigma-Aldrich Chemie GmbH
Filtration unit for medium flasks	High Performance, PES, 0.45 µm, 1,000 mL	514-0301	N/A	VWR International GmbH
Goat anti-mouse IgG (POX)	Whole IgG Fc y fragment, secondary antibody, IgG isotype-specific ELISA	115-035-071	1:15,000	Jackson ImmunoResearch via Dianova
Goat anti-mouse IgG HRP	ELISA	115-035-071	1:15,000	Jackson ImmunoResearch
Goat anti-mouse IgG1 (HRP)	IgG1 Fc y subtype-specific, secondary antibody, IgG isotype-specific ELISA	115-035-205	1:5,000	Jackson ImmunoResearch via Dianova
Goat anti-mouse IgG2a (HRP)	IgG2a Fc y subtype-specific secondary antibody, IgG isotype-specific ELISA	115-035-206	1:5,000	Jackson ImmunoResearch via Dianova
Goat anti-rabbit IgG HRP	ELISA	A0545-1ml	1:10,000	Sigma-Aldrich
GolgiPlug	ICS	555029	1:1,000	BD
GolgiStop	ICS	554724	1:1,500	BD
HBS-EP+ buffer 10×	SPR	BR100669	N/A	GE Healthcare
HEPES	1 M	15630-056	N/A	Gibco
Human SARS coronavirus spike S1 subunit antibody Anti-COVID-19-S1 Isotype: rabbit IgG	ELISA	40150-RP01	S1: 1:1,000 RBD: 1:2,000	Sino Biological
Insulin syringes	BD Micro-Fine™+, 30 G, 0.3 mL	324826	N/A	Becton Dickinson GmbH
Ionomycin	ICS	I9657	1 µg/mL	Sigma
Isoflurane	Anesthesia	9714675	N/A	Piramal Critical Care
Isotonic saline	Injection solution	06173569	N/A	Fresenius Kabi Deutschland GmbH
Lipofectamine® LTX & PLUS™	Transfection reagent	15338-100	N/A	Invitrogen
MACS LS columns	MACS	130-042-401	N/A	Miltenyi Biotec
MACS® MicroBeads	CD8a (Ly-2)/CD4 (L3T4)	130-117-044/130-117-043	N/A	Miltenyi Biotec
MaxiSorp plate	ELISA	439454	N/A	Thermo Scientific
MEM non-essential amino acids (NEAA) solution	100×	11140-035	1×	Gibco

Product name	Application/ specification	Article no.	Working dilution	Provider
Mouse IFN-γ ELISpot^{PLUS} kit	Kit for enumeration of cells secreting mouse IFN-γ	3321-4APT-2	N/A	Mabtech
Mouse IgG1-BIOT	Clone 15H6, isotype control for IgG-specific ELISA	0102-08	1:100	Southern Biotech via Biozol
Mouse IgG2a-BIOT	Clone HOPC-1, isotype control for IgG-specific ELISA	0103-08	1:100	Southern Biotech via Biozol
Mouse IgG-UNLB	ELISA	0107-01	Starting dilution 1:300	Southern Biotech
PBS powder	No calcium, no magnesium	L182-10	N/A	Merck KGaA
pcDNA3.1-derived expression plasmid	VSV vector production	V79020	N/A	Invitrogen
PE hamster anti-mouse CD3e clone 145-2C11	ICS	553064	1:200	BD
PE/Cy7 anti-mouse IFN-γ antibody, clone XMG1.2	ICS	505826	1:500	BioLegend
Penicillin-streptomycin	10,000 U/mL	15140-122	N/A	Gibco
Phosphate-buffered saline (PBS), powdered	ELISA	0780-10L	N/A	VWR International GmbH
Pipette tips	ep Dualfilter T.I.P.S.®, PCR clean und sterile, 0.1–10 µL/2–100 µL/50–1,000 µL/50–1,250 µL/0.1–5 mL	0030077512/0030077547/0030077555/0030077927/0030077750/0030078616	N/A	Eppendorf Vertrieb Deutschland GmbH
Phorbol 12-myristate 13-acetate (PMA)	ICS	P1585	0.5 µg/mL	Sigma
Potassium bicarbonate	$KHCO_3$	A2375,1000	N/A	AppliChem GmbH
ProcartaPlex assay	Bead-based, 11-plex T_H1/T_H2 mouse immunoassay	EPX110-20820-901	N/A	Thermo Fisher Scientific
Recombinant RBD protein SARS-CoV-2 (2019-nCoV) spike protein (RBD, Fc Tag)	ELISA	40592-V02H	100 ng/ 100 µL	SinoBiological
Reservoir	25 mL, 100 mL	613-1174/613-1171	N/A	VWR International GmbH
Roti Histofix, 4% formaldehyde	ICS	P087.4	2%	Carl Roth GmbH & Co. KG

Product name	Application/ specification	Article no.	Working dilution	Provider
RRPMI 1640 medium	GlutaMAX™ supplement	61870-010	N/A	Gibco
SARS-CoV-2 (2019-nCoV) spike antibody, rabbit Mab	ELISA	40150-R007	S1: 1:500 RBD: 1:1,000	Sino Biological
SARS-CoV-2 (2019-nCoV) spike RBD-Fc recombinant protein	ELISA	40592-V02H	100 ng/ 100 µL	Sino Biological
SARS-CoV-2 (2019-nCoV) spike RBD-His recombinant protein	SPR	40592-V08B	N/A	Sino Biological
SARS-CoV-2 (2019-nCoV) spike S1-His recombinant protein	ELISA, SPR	40591-V08H	N/A	Sino Biological
Serological pipettes	5 mL, 10 mL, 25 mL, 50 mL	606180/607 180/601180/ 768180	N/A	Greiner Bio-One GmbH
Single-use syringe	Injekt® Solo 5 mL	4606051V	N/A	B. Braun Melsungen AG
Sodium bicarbonate	ELISA	S5761	N/A	Sigma-Aldrich Chemie GmbH
Sodium carbonate	ELISA	S7795	N/A	Sigma-Aldrich Chemie GmbH
Sodium pyruvate	100 mM	11360-039	N/A	Gibco
Sterile filters	0.45 µm	514-4123	N/A	VWR International
Sulfuric acid 25% EMSURE®	ELISA	1007161000	N/A	VWR International GmbH
TMB One (3,3',5,5'-Tetramethylbenzidine) ready-to-use-solution	ELISA	4380A	N/A	Biotrend Chemikalien GmbH
Tween 20	ELISA	9127.1	N/A	Carl Roth GmbH & Co. KG
Vero-76 cells	Pseudovirus titration	CRL-1587	N/A	ATCC
Vi-CELL™ XR Quad Pak	For Vi-CELL™ XR Cell Viability Analyzer	383722	N/A	Beckman Coulter GmbH
VSV-ΔG-GFP vector	VSV vector production	EH1004	N/A	Kerafast

Table 3: Equipment

Product name	Application	Provider
Biacore T200	SPR analysis	Cytiva
Vi-CELL™ XR Cell Viability Analyzer	Splenocyte count	Beckman Coulter GmbH
CTL ImmunoSpot S6 Core Analyzer	ELISpot plate reader	Cellular Technology Ltd.
BioTek Epoch reader	ELISA plate reader	BioTek
IncuCyte Live Cell Analysis system	pVNT	Sartorius
Celesta	Flow cytometry analysis (ICS)	BD

Table 4: Software

Product name	Application	Provider
Biacore T200 Evaluation Software 3.1	SPR analysis	Cytiva
Excel	Animal monitoring, raw data	Microsoft Corp.
GraphPad Prism 8	Analysis of ELISpot, ELISA, and pVNT	GraphPad Software Inc.
Gen5 software 3.0.9	ELISA plate read out	BioTek
ImmunoCapture 7.0.7.0	ELISpot analysis	Cellular Technology Ltd.
ImmunoSpot® analysis software version 57.0.17.0	ELISpot analysis	Cellular Technology Ltd.
IncuCyte Live Cell Analysis system	pVNT	Sartorius
BD FACSDiva software version 8.0.1.1	Flow cytometry analysis (ICS)	BD

Table 5: Peptide pools for stimulation of splenocytes for ELISpot assays

S protein-specific peptides	
Name	**Sequence**
2019-nCoV S.wt With a total of 315 overlapping peptides (Format 15/11) GenBank: QHD43416.1 Batch: 43000LHB-1 and 43000LHB-2	MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQ DLFLPFFSNVTWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNIIRGWI FGTTLDSKTQSLLIVNNATNVVIKVCEFQFCNDPFLGVYYHKNNKSWMESEF RVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDGYFKIYSKHTPI NLVRDLPQGFSALEPLVDLPIGINITRFQTLLALHRSYLTPGDSSSGWTAGAA AYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLKSFTVEKGIYQTS NFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLY NSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIADYN YKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQA GSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCG PKKSTNLVKNKCVNFNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRD PQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQDVNCTEVPVAIHADQLTPT WRVYSTGSNVFQTRAGCLIGAEHVNNSYECDIPIGAGICASYQTQTNSPRRA RSVASQSIIAYTMSLGAENSVAYSNNSIAIPTNFTISVTTEILPVSMTKTSVDCT MYICGDSTECSNLLLQYGSFCTQLNRALTGIAVEQDKNTQEVFAQVKQIYKTP PIKDFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDCLGDIAA RDLICAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAM QMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVVN QNAQALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLITGRLQSLQTY VTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQSAPHG VVFLHVTYVPAQEKNFTTAPAICHDGKAHFPREGVFVSNGTHWFVTQRNFYE PQIITTDNTFVSGNCDVVIGIVNNTVYDPLQPELDSFKEELDKYFKNHTSPDVD LGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYIKWPWYIWLG FIAGLIAIVMVTIMLCCMTSCCSCLKGCCSCGSCCKFDEDDSEPVLKGVKLHY T
RBD-specific peptides	
Name	**Sequence**
2019-nCoV RBD With a total of 48 overlapping peptides (Format 15/11)	VRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKC YGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGC VIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVE GFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPK
Irrelevant peptide control	
Name	**Sequence**
AH-1	SPSYVYHQF

4.5 Methods

4.5.1 Animal Care

4.5.1.1 General Information

BALB/c mice were delivered at the age of at least six weeks. Delivered mice were used for experiments after approximately one week of acclimatization. All experiments and protocols were approved by the local authorities (local animal welfare committee), conducted according to the Federation of European Laboratory Animal Science Associations (FELASA) recommendations and in compliance with the German animal welfare act and Directive 2010/63/EU. Only animals with an unobjectionable health status were selected for testing procedures.

All animals were registered upon arrival in the lab animal colony management system PyRAT (Scionics Computer Innovation GmbH, Dresden, Germany) and tracked until death. Each cage was labeled with a cage card indicating the mouse strain, sex, date of birth, and number of animals per cage. At the start of an experiment additional information was added such as the project and license number, the start of the experiment and details on interventions. Where necessary for identification, animals were arbitrarily numbered with earmarks.

4.5.1.2 Housing Conditions and Husbandry

Mice were housed at BioNTech SE's animal facility (An der Goldgrube 12, 55131 Mainz) under barrier and specific-pathogen-free (SPF) conditions in individually ventilated cages (Sealsafe GM500 IVC Green Line, TECNIPLAST, Hohenpeißenberg, Germany; 500 cm²) with a maximum of five animals per cage. The temperature and relative humidity in the cages and animal unit were kept at 20-24°C and 45-55%, respectively, and the air change (AC) rate in the cages was 75 AC/h. Cages contained dust-free bedding made of debarked chopped aspen wood (Abedd LAB & VET Service GmbH, Vienna, Austria, product code: LTE E-001) and additional nesting material was changed weekly. Autoclaved ssniff M-Z food (sniff Spezialdiäten GmbH, Soest, Germany; product code: V1124) and autoclaved tap water were provided *ad libitum* and changed at least once weekly. All materials were autoclaved prior to use.

4.5.2 Animal Monitoring

Routine animal monitoring was carried out daily and included inspection for dead mice and control of food and water supplies. The health of each mouse was closely assessed at least once weekly and the results documented in health monitoring sheets (see Appendix 1: Animal Monitoring - Observations). The general physical condition of the mice was assessed according to the following parameters:

- Body weight change
- Macroscopic assessment of activity level/behavior
- Macroscopic assessment of general discomfort: drop in body temperature determined by touch and by visual inspection of ears and paws (ears and paws appear pink in a healthy mouse, white in a mouse with discomfort indicates reduced blood circulation)
- Macroscopic assessment of fur condition and appearance of eyes, inspection of body cavities/fluids
- Macroscopic assessment of irregularities in breathing ability
- Indication of pain
- Macroscopic assessment for signs of automutilation and/or fighting

Details on animal monitoring criteria are shown in Appendix 1: Animal Monitoring - Observations, Table 9.

4.5.3 Animal Treatment

4.5.3.1 Treatment Schedule, Route of Administration, and Dose

The test compound was administered i.m. once at three different doses (0.2 µg, 1 µg, or 5 µg per animal) to the three test groups of mice on day 0. The control group was treated with buffer alone.

4.5.3.2 Immunization

Following anesthesia by inhalation of 2.5% isoflurane in oxygen, the injection site on the hind leg of the mouse was shaved for immunization. Buffer or dissolved test item was applied i.m. into the *musculus gastrocnemius* in a volume of 20 µL. After immunization and a short recovery phase from anesthesia, the mice were observed for any immediate signs of discomfort due to the immunization procedure.

4.5.3.3 Blood Sampling via the Retro-Orbital Venous Plexus or *Vena Facialis*

Blood was sampled via the retro-orbital venous plexus according to SOP-030-074. In short, mice were anesthetized by inhalation of 2.5% isoflurane in oxygen and tightly held for blood collection. A thin glass capillary (29 G) was inserted gently through the retro-orbital sinus membrane and blood was collected into an appropriate plastic tube (Sarstedt, Z-gel included for clotting activation). After careful removal of the glass capillary, the restraining hold on the mouse was loosened. Alternatively, blood collection took place via the *vena facialis* according to SOP-030-074. In short, without prior anesthesia, mice were tightly held for blood collection, and the *vena facialis* was punctured using a lancet in a precise and short movement. Blood was collected into an appropriate plastic tube (Sarstedt, Z-gel included for clotting activation), and then

the restraining hold on the mouse was loosened. Blood samples were centrifuged at 10,000 ×g and RT for 5 min and serum transferred to a pre-labeled 0.5 mL reagent tube for use in subsequent downstream assays or storage at -20°C.

4.5.4 Endpoint of Experiment/Termination Criteria

Animals were euthanized in accordance with §4 of the German animal welfare act and the recommendation of the German Society of Laboratory Animal Science (GV-SOLAS) by cervical di location or by e po ure to carbon dio ide Additionally, termination criteria were applied according to the specification within the respective animal test approval as listed below. Body weight losses exceeding 20%, or a high severity level in any of the parameters found in Section 4.5.2 were on their own sufficient reason for immediate euthanasia.

4.5.4.1 Dissection of Animals and Organ Collection

Following euthanasia, mice were disinfected with 70% ethanol and the dissection was performed starting with an abdominal incision. The spleen was collected and stored in DPBS on ice for subsequent splenocyte preparation.

4.5.5 ELISA

Serum samples were tested in 96-well plates for their S-specific antibody concentration based on SOP-030-112 (with minor modifications as described below). Briefly, for the time points 7, 14, and 21 days after immunization, a screening analysis was performed and for day 14 and 28, serum samples were analyzed by endpoint titration.

1. Coat each well of a MaxiSorp plate with 100 ng/100 µL recombinant protein per well or isotype controls according to plate layout.
   - Coating buffer: 50 mM sodium carbonate buffer (1.696 g Na_2CO_3 + 2.856 g $NaHCO_3$, top up to 1 L distilled H_2O, pH 9.6 (pH adjustment not needed))
2. Cover plates and incubate at 4°C o/n.
3. Wash three times with 300 µL/well PBS with Tween (PBS-T).
4. Block all wells with 1xBB, 250 µL/well.
5. Incubate at 37°C for 1 h on shaker.
6. Wash three times with 300 µL/well PBS-T.
7. Dilute primary antibodies (samples and positive control) according to schedule.
8. Incubate at 37°C for 1 h on shaker.
9. Wash three times with 300 µL/well PBS-T.
10. Dilute the secondary antibodies according to calculations.
11. Incubate at 37°C for 45 min on shaker.
12. Wash three times with 300 µL/well PBS-T.
13. Add 100 µL/well TMB substrate.
14. Incubate 8 min at RT (clear->blue).

15. Stop the reaction with 100 µL 25% sulfuric acid. (blue -> yellow).
16. Read on plate reader (450 nm, reference: 620 nm).

For concentration analysis, the signal of the specific samples was correlated to the isotype control. For analysis of IgG subtypes, the mean ΔOD 450-620 nm per group was calculated and the ratio of IgG2a:IgG1 ratio was calculated.

For reciprocal serum endpoint titer, the serum dilution that emitted the OD exceeding 4-fold background was used. The background was defined as the OD signal given by the recombinant protein incubated with the secondary detection anti-mouse IgG antibody only.

4.5.6 Surface Plasmon Resonance Spectroscopy

Binding kinetics of murine S1- and RBD-specific serum IgGs was determined using a Biacore T200 device with HBS-EP running buffer at 25°C. Carboxyl groups on the CM5 sensor chip matrix were activated with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC) and N-hydroxysuccinimide (NHS) to form active esters for the reaction with amine groups. Anti-mouse-Fc-antibody was diluted in 10 mM sodium acetate buffer pH 5 (30 µg/mL) for covalent coupling to immobilization level of ~10,000 response units (RU). Free NHS esters on the sensor surface were deactivated with ethanolamine.

Mouse serum was diluted 1:50 in HBS-EP buffer and applied at 10 µL/min for 30 seconds to the active flow cell for capture by immobilized antibody, while the reference flow cell was treated with buffer. Binding analysis of captured murine IgG antibodies to S1-His or RBD-His was performed using a multi-cycle kinetic method with concentrations ranging from 25 to 400 nM or 1.5625 to 50 nM, respectively. An association period of 180 seconds was followed by a dissociation period of 600 seconds with a constant flow rate of 40 µL/min and a final regeneration step. Binding kinetics were calculated using a 1:1 Langmuir global kinetic fit model.

4.5.7 Pseudovirus-based Neutralization Test

For analyzing the amount of functional nAbs in the serum samples, pVNTs were performed.

4.5.7.1 Production of SARS-CoV-2-S Pseudotyped VSV Vector

Replication-deficient vesicular stomatitis virus (VSV) that lacks the genetic information for the VSV envelope glycoprotein VSV-G but contains an open reading frame (ORF) for green fluorescent protein (GFP) was used for SARS-CoV-2-S pseudovirus generation. VSV pseudotypes were generated according to a published protocol (Hoffmann et al. 2020).

In brief, HEK293T/17 cells cultured in DMEM supplemented with 10% FBS were transfected with a pcDNA3.1-derived expression plasmid (Invitrogen) coding for the SARS-CoV-2 spike protein (GenBank ID: QHD43416.1) with shortened cytoplasmic tail, i.e., pSARS-CoV-2-S-CΔ19, using Lipofectamine® LTX & PLUS™ Reagent (Invitrogen) following the manufacturer's instructions. The cytoplasmic tail was truncated for the 19 C-terminal amino acids to facilitate a more efficient integration of SARS-CoV-2-S into VSV virions analogous to SARS-CoV-2-S pseudotyped VSV (Fukushi et al. 2005). At 24 h post transfection, cells were inoculated with VSV-G transcomplemented VSV-ΔG-GFP vector (Indiana strain, *de novo* generated by reverse genetics from plasmid (Lawson et al. 1995)) at a multiplicity of infection (MOI) of three and incubated for 2 h at 37°C and 5% CO_2. Next, the inoculum was removed, cells were washed with PBS, and standard culture medium which contained 0.5 µg/mL anti-VSV-G antibody (clone 8G5F11) was added to neutralize residual input virus. Twenty-four hours after infection, VSV/SARS-CoV-2-S pseudovirus-containing supernatants were harvested, filtered (0.45 µm) and stored at -80°C in aliquots until further use.

4.5.7.2 Titration of VSV/SARS-CoV-2-S Pseudovirus

For titration of VSV/SARS-CoV-2-S pseudovirus, Vero-76 cells (ATCC) were thawed according to SOP-030-041, diluted to 2.67×10^5 cells/mL in assay medium (DMEM and 10% FBS) and seeded in 96-well flat-bottom plates at 4×10^4 cells per well. Cells were incubated for 4 to 6 h at 37°C and 7.5% CO_2. Meanwhile, two-fold, eight-step serial dilutions were prepared in 96-well V-bottom plates beginning with undiluted pseudovirus supernatant. Vero-76 wells were inoculated with 50 µL of the diluted pseudovirus supernatant and incubated for 16 to 24 h at 37°C and 7.5% CO_2. Each dilution was tested in duplicate wells. After the incubation, the cell culture plates were removed from the incubator, placed in an IncuCyte Live Cell Analysis system (Essen Bioscience) and equilibrated for 30 min prior to the analysis. Whole well scanning for brightfield and GFP fluorescence was performed using a 4× objective. The number of infected GFP-fluorescent cells per well was plotted as a function of pseudovirus supernatant dilution using GraphPad Prism. Data (x = logx) were fitted with linear regression and the derived slope and y-intercept used to calculate the amount of viral supernatant needed to obtain 144 infected cells/96-well (20% excess for virus neutralization test included).

4.5.7.3 Pseudovirus-based Neutralization Test

Sera from blood samples collected 14, 21 and 28 days after immunization were tested using the VSV/SARS-CoV-2-S pseudovirus neutralization test (pVNT). For the pVNT assay, Vero-76 cells were thawed according to SOP-030-041, diluted to 2.67×10^5 cells/mL in assay medium (DMEM and 10% FBS) and seeded in 96-well flat-bottom plates at 4×10^4 cells per well. Cells were incubated for 4 to 6 h at 37°C and 7.5% CO_2. Initial dilutions of mouse serum samples were prepared by adding 10 µL of serum

to 50 µL assay medium in a 96-well V-bottom plate. Seven additional dilutions were subsequently prepared in two-fold dilution steps, by iteratively transferring 30 µL of diluted sera to wells containing 30 µL assay medium. VSV/SARS-CoV-2 pseudovirus was thawed and diluted to obtain 120 infected cells/25 µL (4.8×10^3 infectious units [IU]/mL). 30 µL of diluted pseudovirus (corresponds to 144 infected cells; see Section 4.5.7.2) was added to the wells containing the serum dilution series. Pseudovirus/serum dilution mix was incubated for 5 min at RT on a microplate shaker at 750 rpm, and additional 5 min at RT without agitation. Pseudovirus/serum dilution mix was then added to the seeded Vero-76 cells (50 µL mix per well, MOI:0.003), followed by incubation for 16 to 24 h at 37°C and 5% CO_2. Each dilution of serum samples was tested in duplicate wells. Vero-76 cells incubated with pseudovirus in the absence of mouse sera were used as positive controls. Vero-76 cells incubated without pseudovirus were used as negative controls. After the incubation, the cell culture plates were removed from the incubator, placed in an IncuCyte Live Cell Analysis system and incubated for 30 min prior to the analysis. Whole well scanning for brightfield and GFP fluorescence was performed using a 4× objective. To calculate the neutralizing titer, infected GFP-positive cell number per well was compared with the no-serum pseudovirus positive control. Mean values of the no-serum pseudovirus positive control multiplied by 0.5 represent the pseudovirus neutralization 50% (pVN_{50}); mean values of the no-serum pseudovirus positive control multiplied by 0.1 represent the pseudovirus neutralization 90%. Serum samples with mean values below this cut-off exhibit >50% or >90% virus neutralization activity, respectively.

4.5.8 Preparation of Splenocytes

The single cell suspensions from collected spleens were prepared according to SOP-030-078. To this end, the spleens were squeezed through 70 µm cell meshes using the plunger of a syringe to release the splenocytes into a tube. Splenocytes were washed with an excess volume of DPBS followed by centrifugation at 300 × g for 6 min at RT and discarding the supernatants. Erythrocytes were then lysed with erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 5 min at RT. The reaction was stopped with an excess volume of DPBS. After another washing step, cells were resuspended in medium (10% FBS, 1% NEAA, 1% sodium pyruvate, 0.5% penicillin/streptomycin), passed through a 70 µm cell mesh again, counted according to SOP-010-028, and stored short-term at 37°C for use on the same day or frozen in liquid nitrogen, resuspended in 1 mL FBS/10% DMSO. For the use of frozen splenocytes in ELISpot analysis after thawing, the amount of cells per well was doubled (1×10^6 cells). Immediately after thawing, pre-warmed (RT) PBS was added to splenocytes. Two washing steps using pre-warmed PBS to remove DMSO from freezing process were performed and splenocytes were counted according to SOP-010-028. Splenocytes were stored short-term at 37°C for further use.

4.5.9 ELISpot Assay

ELISpot assays with fresh or frozen splenocytes were performed according to SOP-030-110 (with minor modifications as described below) using the mouse IFN-γ ELISpot^{PLUS} kit. Briefly, 96-well ELISpot plates were washed with PBS and blocked with medium for at least 30 min at 37°C. 100 µL of the splenocyte solution (fresh cells: 5×10^5 cells; frozen cells: 1×10^6 cells) were transferred to the respective well of the 96-well ELISpot plate. Another 100 µL of overlapping peptide pools or controls were added in the following concentrations:

- overlapping peptide mix PepMix™ against SARS-CoV-2 S.wt: 0.1 µg/mL final concentration per peptide
- overlapping peptide mix PepMix™ against SARS-CoV-2 RBD: 0.1 µg/mL final concentration per peptide
- irrelevant peptide (AH-1): 4 µg/mL
- Concanavalin A (ConA): 2 µg/mL

For positive control, the splenocytes were stimulated with ConA, for a non-stimulation control only medium was added and as a negative control to detect unspecific background signals, the irrelevant peptide was added (AH-1). Plates were incubated overnight in a 37°C humidified incubator with 5% CO_2 and after approximately 18 h, cells were removed from the plates and the detection protocol of spots was initiated. To this end, the detection antibody, Streptavidin-ALP, and the ready-to-use substrate were added to the wells according to the manufacturer's protocol. After plate drying for 2–3 h under the laminar flow, an ELISpot plate reader (ImmunoSpot® S6 Core Analyzer, CTL) was used to count and analyze spot numbers per well.

4.5.9.1 Subtyping of $CD8^+$ versus $CD4^+$ T-cell Responses

This method was performed with fresh splenocytes (non-frozen). $CD8^+$ or $CD4^+$ T cells were isolated from splenocyte cell suspensions using MACS® MicroBeads (CD8a (Ly-2) or CD4 (L3T4)) according to the manufacturer's instructions. Labeled cells were eluted from MACS LS columns, centrifuged (5 min at 460 ×g) and taken up at a concentration of 1×10^6 cells/mL in medium. 100 µL of $CD8^+$ or $CD4^+$ T cells were subsequently re-stimulated by addition of 50 µL peptide solution (control peptide AH-1 (2 µg/mL), RBD peptide mix (0.1 µg/mL per peptide) or S peptide mix (0.1 µg/mL per peptide)) and 50 µL of bone marrow-derived dendritic cells (1×10^6 cells/mL, cells were frozen at -80°C prior use and prepared from BALB/c mice according to SOP-030-080) in an IFN-γ ELISpot assay (SOP-030-110). Each condition was tested in duplicate.

4.5.10 Luminex Assay

1×10^6 previously frozen splenocytes in 100 µL DC medium (part of SOP-030-110) were transferred to a 96-well flat-bottom cell culture plates. 100 µL of an overlapping peptide pool or controls were added in the following concentrations:

- overlapping peptide mix PepMix™ against SARS-CoV-2 S.wt: 0.1 or 0.03 µg/mL final concentration per peptide (equal to 31.5 or 9.6 µg/mL total peptide)
- overlapping peptide mix PepMix™ against SARS-CoV-2 RBD: 0.66 or 0.2 µg/mL final concentration per peptide (equal to 31.5 or 9.6 µg/mL total peptide)
- PMA: 1 µg/mL and ionomycin: 2 µg/mL

The plates were incubated for 48 h and supernatant thereafter was harvested for cytokine profiling. Cytokine concentrations in supernatants of the re-stimulated splenocytes were determined using a bead-based, 11-plex T_H1/T_H2 mouse ProcartaPlex immunoassay according to the manufacturer's instructions. Fluorescence was measured with the Bioplex200 System (Bio-Rad) and analyzed with ProcartaPlex Analyst 1.0 software (Thermo Fisher Scientific). The following analytes were measured: IFN-γ, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-6, TNF-α, GM-CSF, and IL-18.

4.5.11 Intracellular Cytokine Staining

Briefly, 5×10^5 fresh splenocytes in 100 µL DC medium (part of SOP-030-110) were transferred to 96-well flat-bottom cell culture plates. Finally, 100 µL of an overlapping peptide pool or controls were added in the following concentrations:

- overlapping peptide mix PepMix™ against SARS-CoV-2 S.wt: 0.1 µg/mL final concentration per peptide (equal to 31.5 µg/mL total peptide)
- overlapping peptide mix PepMix™ against SARS-CoV-2 RBD: 0.1 µg/mL final concentration per peptide (equal to 4.8 µg/mL total peptide)
- PMA: 1 µg/mL and ionomycin: 2 µg/mL

As a non-stimulation control, only medium was added to detect unspecific background signals. Plates were incubated for 1 h in a 37°C humidified incubator with 5% CO_2 before adding a GolgiStop+GolgiPlug. After another 4 h, cells were harvested and transferred to a 96-well, V-bottom plate for flow cytometry staining. After the staining procedure, cells were solved in 100 µL FACS buffer (PBS + 0.1% BSA) for flow cytometry analysis using a FACS Celesta (BD).

A detailed protocol is presented in Appendix 5: Detailed ICS Protocol.

4.5.12 Statistical Analysis

GraphPad Prism 8 Software (La Jolla, USA) was used for statistical analysis and figure generation. All test groups were compared to the buffer control group by a one-way analysis of variance (ANOVA) on each measurement day as described in the respective results section. For Luminex assays, statistical significance was assessed by mixed-effects analysis/Sidak's comparison.

5 RESULTS

5.1 ELISA

5.1.1 Whole IgG ELISA

IgGs against recombinant S1 protein or RBD were detected by ELISA analysis in serum samples obtained on study days 7, 14, and 21. Statistical significance was assessed by one-way ANOVA and Dunnett's multiple comparisons test.

Before immunization, no S1 protein- or RBD-specific IgGs were detected (Pre-treatment, Figure 2, Figure 3). Treatment with BNT162b2 induced the formation of IgGs specific for S1 protein and RBD, while these antibodies were not detected in samples from buffer control animals independent of the day of sample collection. A dose-dependent increase in S1-specific IgGs was observed on all study days (Figure 2), with statistically significant differences between the treatment groups and the buffer control group ($p < 0.0001$ for all doses and test days).

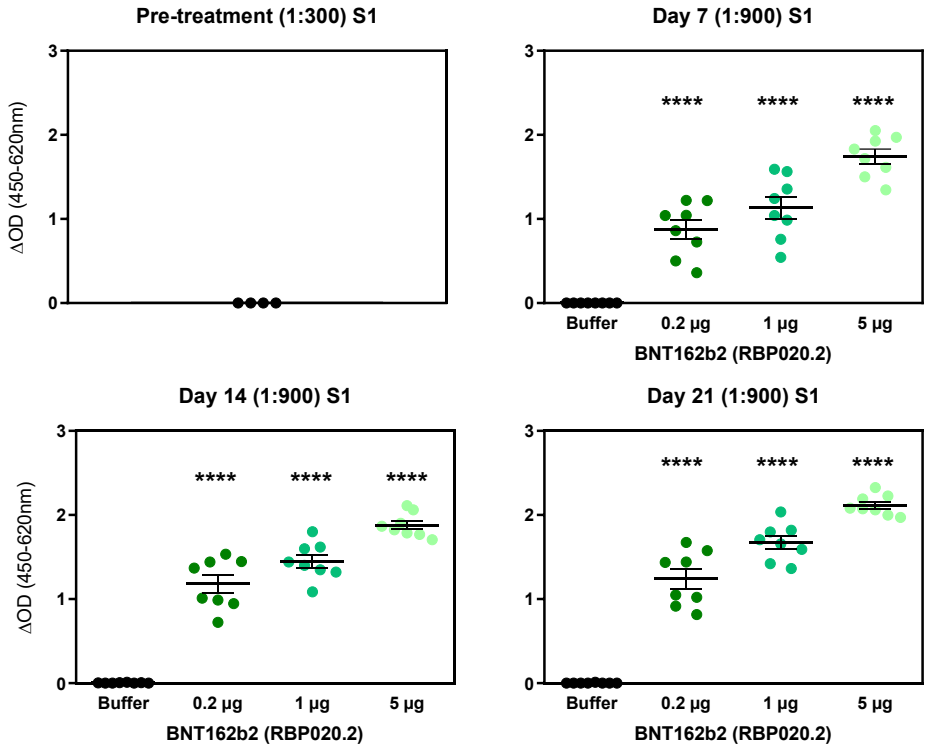

Figure 2: ELISA screening analysis on days 7, 14, and 21 against the recombinant S1 protein

ELISA was performed using serum samples collected on days 7, 14, and 21 after immunization to analyze antibody responses. The serum samples were tested against the S1 protein. Individual ΔOD values for each mouse (measured in duplicates) are shown by dots; group mean values are indicated by horizontal bars (±SEM). **** $p < 0.0001$.

All test groups showed a statistically significant increase in RBD-specific IgGs compared to buffer control (Figure 3; $p < 0.0001$ for all doses and test days).

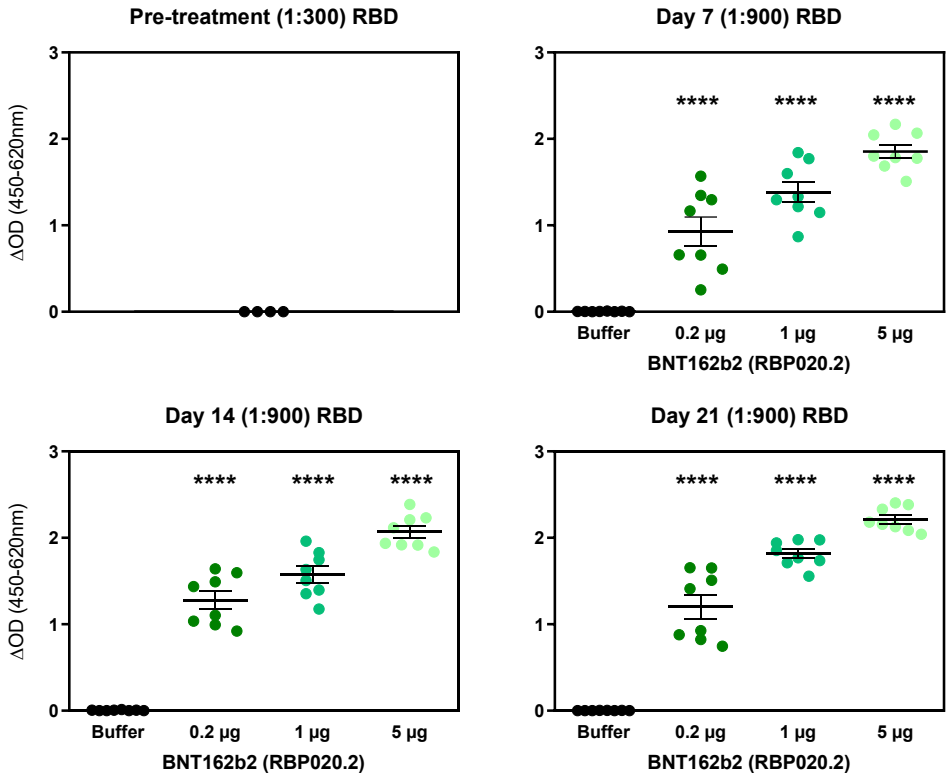

Figure 3: ELISA screening analysis on days 7, 14, and 21 against the recombinant RBD

ELISA was performed using serum samples collected on days 7, 14, and 21 after immunization to analyze antibody responses. The serum samples were tested against the receptor-binding domain (RBD). Individual ΔOD values for each mouse (measured in duplicates) are shown by dots; group mean values are indicated by horizontal bars (±SEM). **** $p < 0.0001$.

ELISA endpoint titration was performed on day 28 after immunization to analyze antibody responses (Figure 4A, B).

Antibody concentrations in the serum samples were calculated for the individual sampling days and the kinetics of IgGs against S1 and RBD proteins is shown in Figure 5. Antibody concentrations against S1 (Figure 5A) and RBD (Figure 5B) increased in a dose-dependent manner over time in the test groups. Statistical significance of the differences in IgG concentrations between the test groups and the control group was assessed by one-way ANOVA with Dunnett's multiple comparison post-test on day 28.

The differences in concentrations of IgGs against S1 and RBD in the test groups compared to the buffer control group were statistically significant (S1: $p = 0.0259$ for 0.2 µg, $p < 0.0001$ for 1 µg and 5 µg; RBD: $p = 0.0072$ for 0.2 µg, $p < 0.0001$ for 1 µg and 5 µg) on day 28.

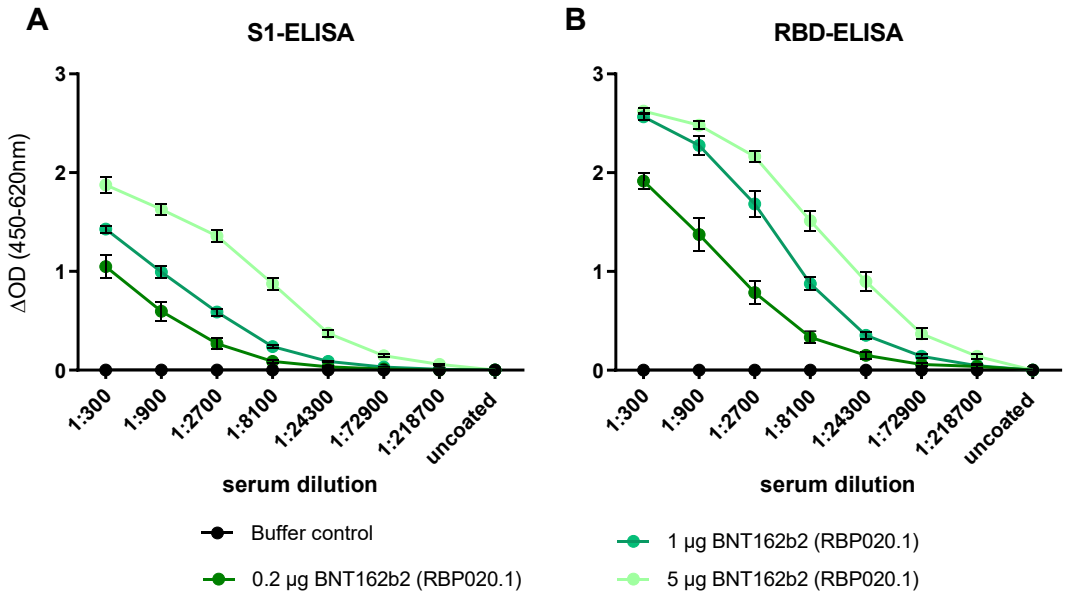

Figure 4: ELISA endpoint titration on day 28

Endpoint titration was performed on day 28 after immunization to analyze antibody responses. The serum samples were tested against the S1 protein (A) and RBD (B). Group mean values (±SEM) are shown.

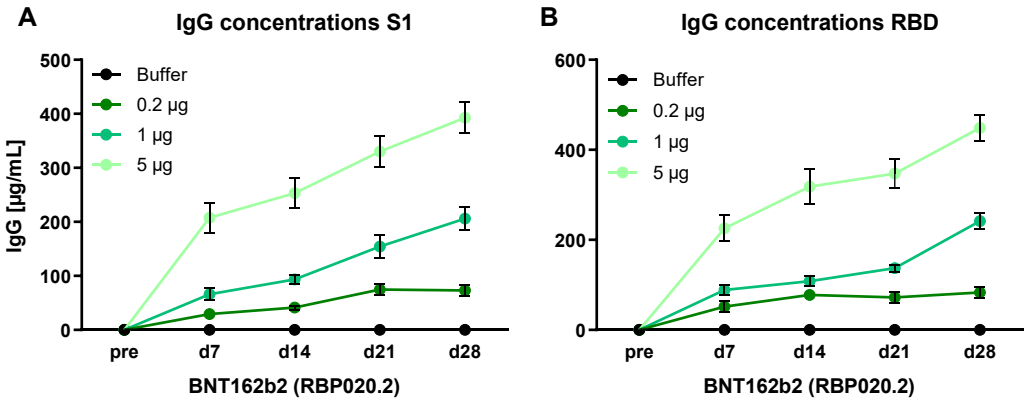

Figure 5: Kinetics of the antibody concentration against the viral antigen

For individual ΔOD values, the antibody concentrations in the serum samples were calculated. The serum samples were tested against (A) the S1 protein and (B) RBD. Group mean antibody concentrations are shown (±SEM).

Furthermore, to calculate the reciprocal serum endpoint titer of antibodies we performed an endpoint titration for day 14 and 28 samples after immunization exceeding the previously shown dilution steps (Figure 6A, B).

The reciprocal serum endpoint titer was defined as the first highest dilution step which emitted an OD exceeding the background signal four-times as shown. Reciprocal serum endpoint titer against S1 (Figure 7A) and RBD (Figure 7B) were high already 14 days after immunization and increased in a dose-dependent manner over time in the test groups. Statistical significance of the differences in IgG concentrations between the test groups and the control group was assessed by a one-way ANOVA with Tukey's multiple comparison post-test.

The differences in titers of IgGs against S1 and RBD in the test groups compared to the buffer control group were statistically significant (S1, day 28: $p = 0.0082$ for 1 µg, $p < 0.0001$ for 5 µg; RBD, day 14: $p < 0.0001$ for 5 µg and day 28: $p = 0.0109$ for 1 µg, $p < 0.0001$ for 5 µg).

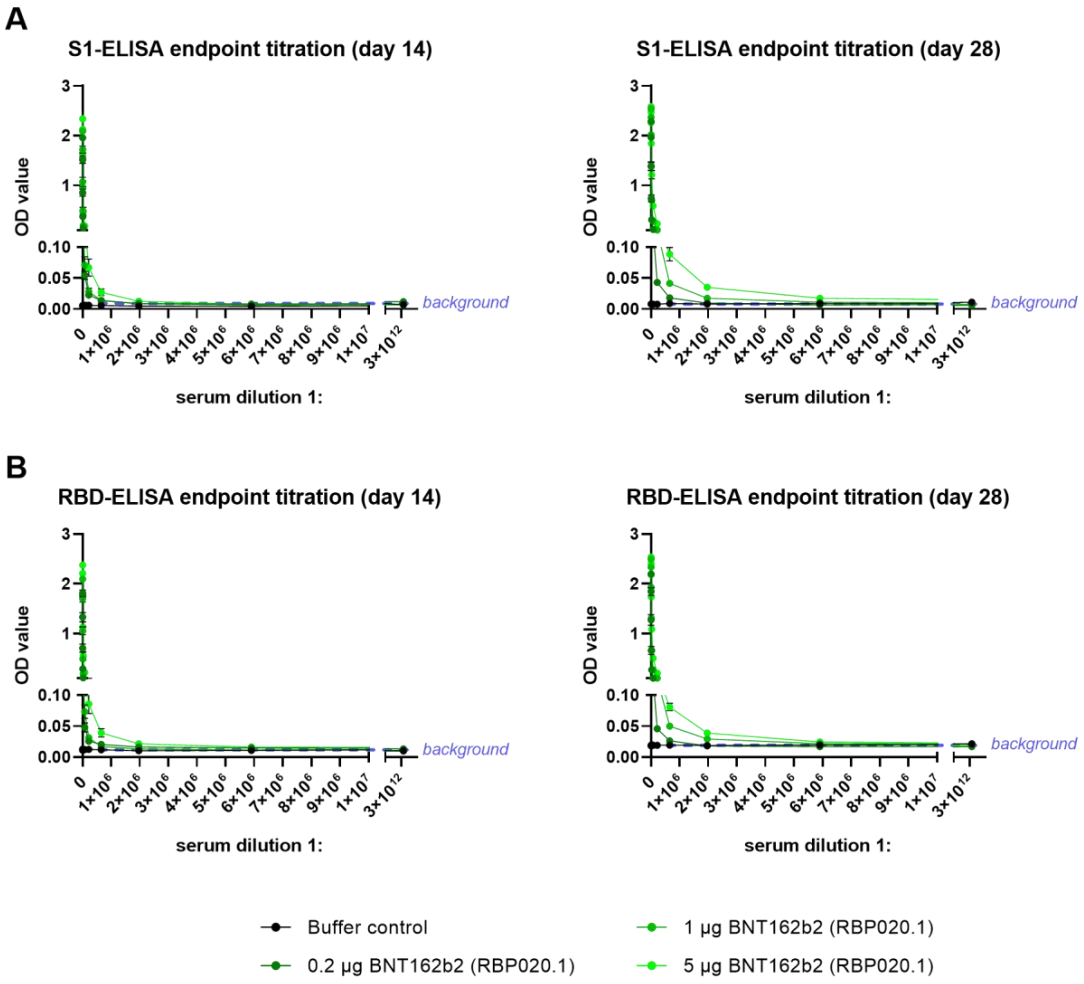

Figure 6: ELISA endpoint titration (long titration)

Endpoint titration against the S1 protein (A) and RBD (B) was performed on day 14 (left) and 28 (right) after immunization to analyze reciprocal serum endpoint titer of antibodies. Group mean values (±SEM) are shown; samples were measured in duplicates. Background was defined as the OD value of the recombinant protein incubated with the secondary anti-mouse IgG detection antibody only and included in the graphs (blue dotted line; n = 8).

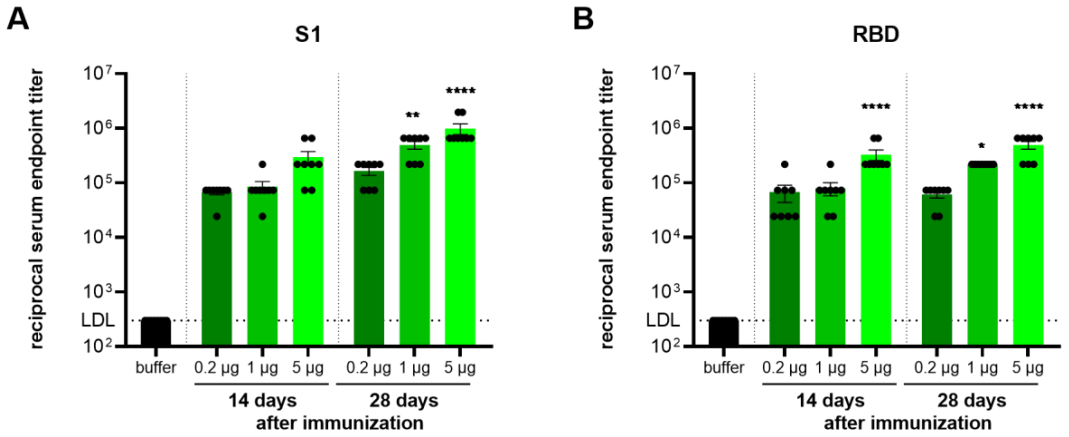

Figure 7: Reciprocal serum endpoint titer at day 14 and 28 after immunization

For individual OD values, the reciprocal serum endpoint titer was calculated. The serum samples were tested against (A) the S1 protein and (B) RBD. Group mean titer are shown (±SEM). Significance compared to buffer control is included, * $p \leq 0.05$, ** $p \leq 0.01$, **** $p < 0.0001$; LDL = lower detection limit.

5.1.2 IgG Subtype-specific ELISA

IgG1 and IgG2a subtypes of anti-S1 antibodies were analyzed by IgG subtype-specific ELISA in serum samples obtained on study day 28. Statistical significance was assessed by one-way ANOVA followed by a Dunnett's multiple comparison post-test.

Treatment with BNT162b2 induced the formation of IgG1 and IgG2a specific for S1 protein, while these antibodies were not detected in samples from buffer control animals independent of the day of sample collection (Figure 8).

On day 28, all dose groups displayed significantly higher group mean ΔOD values for IgG1 and IgG2 antibodies than the control animals (IgG1: $p < 0.0001$ for all doses, IgG2a: $p = 0.0020$ for 0.2 µg, $p < 0.0001$ for 1 µg and 5 µg).

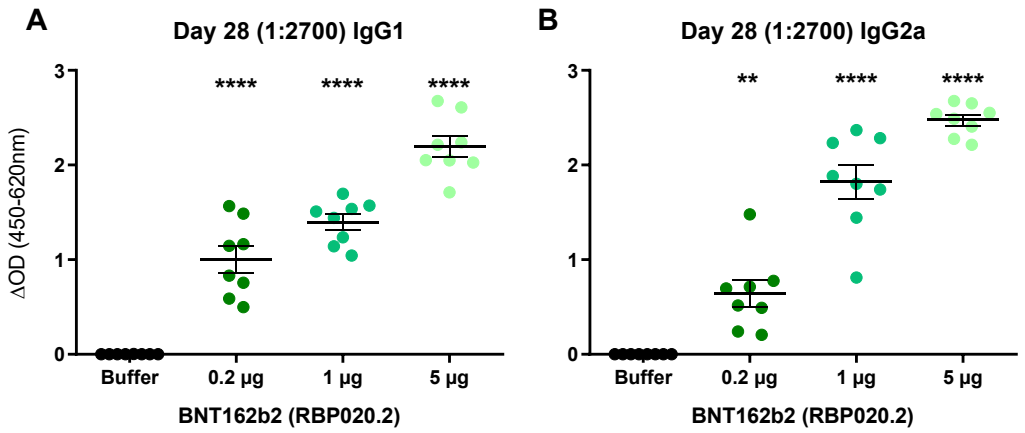

Figure 8: IgG subtype-specific ELISA on day 28

ELISA was performed using serum samples collected on day 28 after immunization to analyze (A) IgG1 and (B) IgG2a responses. The serum samples were tested against theS1 protein. Individual ΔOD values for each mouse (measured in duplicates) are shown by dots; group mean values are indicated by horizontal bars (±SEM). ** $p \leq 0.01$, **** $p < 0.0001$.

ELISA endpoint titration was performed on day 28 after immunization to analyze IgG1 and IgG2a responses (Figure 9A, B).

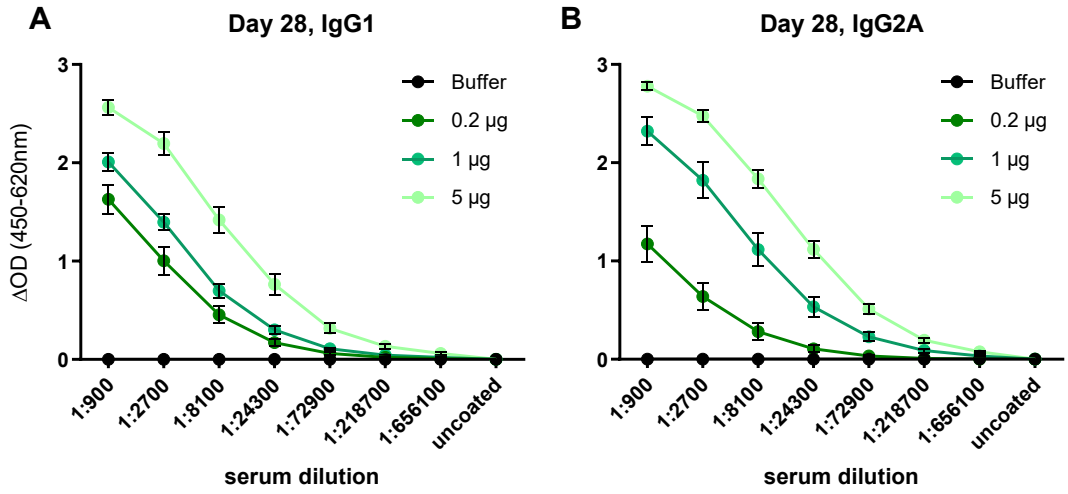

Figure 9: ELISA endpoint titration on day 28 (IgG subtypes)

Endpoint titration was performed on day 28 after immunization to analyze IgG1 (A) and IgG2a (B) responses. Group mean values (±SEM) are shown.

5.1.3 IgG2a/IgG1 Ratio

To analyze the ratio between the two IgG subtypes, the ΔOD values were used. Antibody ratios in the serum samples were calculated for day 28 (Figure 10). Statistical significance was assessed by one-way ANOVA followed by Tukey's multiple comparison post-test to compare all test groups with each other.

While the two higher doses induced a balanced IgG2a/IgG1 response, the lowest dose induced a higher ignal for IgG1 than IgG2a The difference between the group treated with 0.2 µg and the groups treated with 1 µg and 5 µg were statistically significant (p = 0.0004 for 0.2 µg vs 1 µg, p = 0.0041 for 0.2 µg vs 5 µg).

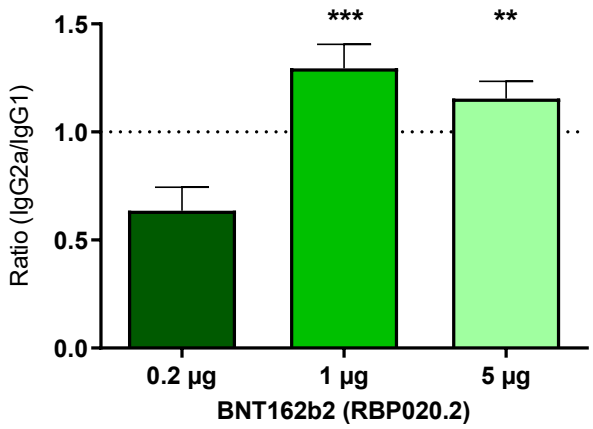

Figure 10: IgG2a/IgG1 subtype ratio on day 28

Based on the 1:2,700 dilution step (see Figure 9), the ΔOD the ΔOD for every single sample were used to calculate the ratio of IgG2a and IgG1. For this purpose, the ΔOD value of IgG2a was divided by the ΔOD values of IgG1 per mouse. Group mean values (±SEM) are shown. The value of "1" in the graph would give the equal signal between the two subtypes while ratio > 1 mirror a higher IgG2a subtype detection. ** p ≤ 0.01, *** p ≤ 0.001.

5.2 Binding Kinetics of Antigen-specific IgGs Using SPR

To obtain kinetic and affinity information about the binding of vaccine-elicited IgG to SARS-CoV-2 S1 fragment and RBD, SPR spectroscopy was conducted. Whole IgG from sera (n = 8) generated at 28 days after immunization with 5 µg BNT162b2 was captured by high-affinity anti-IgG antibody immobilized on the sensor chip surface. Binding analysis of captured murine IgG antibodies to recombinant S1-His or RBD-His protein was performed using a multi-cycle kinetic method with concentrations ranging from 25 to 400 nM S1-His or 1.5625 to 50 nM RBD-His. Kinetic parameters were calculated by fitting the sensorgram curves with a 1:1 Langmuir global kinetic fit model.

At day 28 after immunization, vaccine-elicited IgG had a strong binding affinity for S1-His (geometric mean K_D = 12 nM), with affinities ranging from 8.06 nM to 34.5 nM across the 8 serum samples tested (Figure 11A, Table 6). Somewhat higher binding affinity was detected for RBD-His (geometric mean K_D = 0.99 nM), with affinities ranging from 0.48 nM to 2.78 nM (Figure 11A, Table 7). Binding to S1-His and RBD-His can be characterized by a comparable low dissociation rate constant (geometric mean $k_{off} = 4\times10^{-4}$ s^{-1} vs. 5.97×10^{-4} s^{-1}). However, association of RBD-His to captured IgG was approximately 20-fold faster (geometric mean $k_{on} = 6.02\times10^{5}$ $M^{-1}s^{-1}$ vs. 3.33×10^{4} $M^{-1}s^{-1}$).

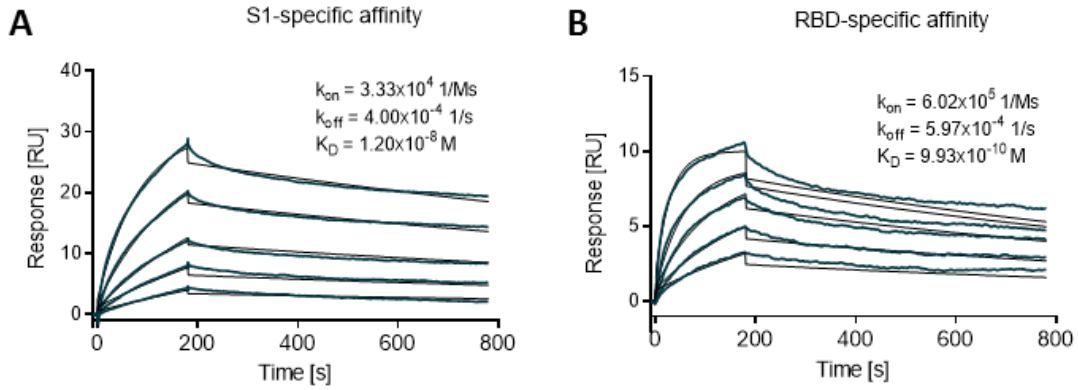

Figure 11: Binding affinities of vaccine-elicited IgG for S1-His and RBD-His protein

Representative SPR sensorgram of the binding kinetics of recombinant S1-His (A) and RBD-His protein (B) to immobilized mouse IgG from serum 28 days after immunization with 5 µg BNT162b2 (n=8). Actual binding (dark blue) and the best fit of the data to a 1:1 binding model (thin line in black) is shown.

Table 6: Summary of binding kinetic parameters of vaccine-elicited IgG for S1-His

Animal no.	k_{on} [1/Ms]	k_{off} [1/s]	K_D [nM]
4-1	2.62E+04	9.05E-04	34.5
4-2	3.36E+04	5.70E-04	17.0
4-3	2.91E+04	4.92E-04	16.9
4-4	4.78E+04	5.95E-04	12.5
4-5	2.94E+04	4.54E-04	15.4
4-6	2.45E+04	2.95E-04	12.0
4-7	2.91E+04	3.16E-04	10.9
4-8	3.36E+04	2.71E-04	8.06
Geometric mean	3.33E+04	4.00E-04	12.0

Table 7: Summary of binding kinetic parameters of vaccine-elicited IgG for RBD-His

Animal no.	k_{on} [1/Ms]	k_{off} [1/s]	K_D [nM]
4-1	4.35E+05	6.79E-04	1.56
4-2	2.89E+05	8.04E-04	2.78
4-3	6.64E+05	7.23E-04	1.09
4-4	4.82E+05	5.82E-04	1.21
4-5	4.64E+05	6.26E-04	1.35
4-6	8.46E+05	4.07E-04	0.481
4-7	6.36E+05	5.55E-04	0.873
4-8	1.06E+06	5.42E-04	0.512
Geometric mean	6.02E+05	5.97E-04	0.993

5.3 Pseudovirus-based Neutralization Test

Virus-neutralizing antibodies in serum samples obtained on study days 14, 21, and 28 were detected by pVNT. Statistical significance was assessed by one-way ANOVA with Dunnett's multiple comparison post-test.

Treatment with all tested uRNA doses induced the formation of virus-neutralizing antibodies with temporally increasing pVN_{50} titers (Figure 12). On day 14, several samples from animals treated with 0.2 µg modRNA displayed pVN_{50} titers that were below the lower limit of quantification. Significantly higher pVN_{50} titers were measured in samples from animals treated with the high dose of 5 µg RNA than in buffer control samples ($p = 0.0010$). On days 21 and 28, the differences of the groups treated with 1 µg and 5 µg BNT162b2 compared to the buffer control group were statistically significant (day 21: $p = 0.0036$ for 1 µg, $p < 0.0001$ for 5 µg; day 28: $p < 0.0001$ for 1 µg and 5 µg).

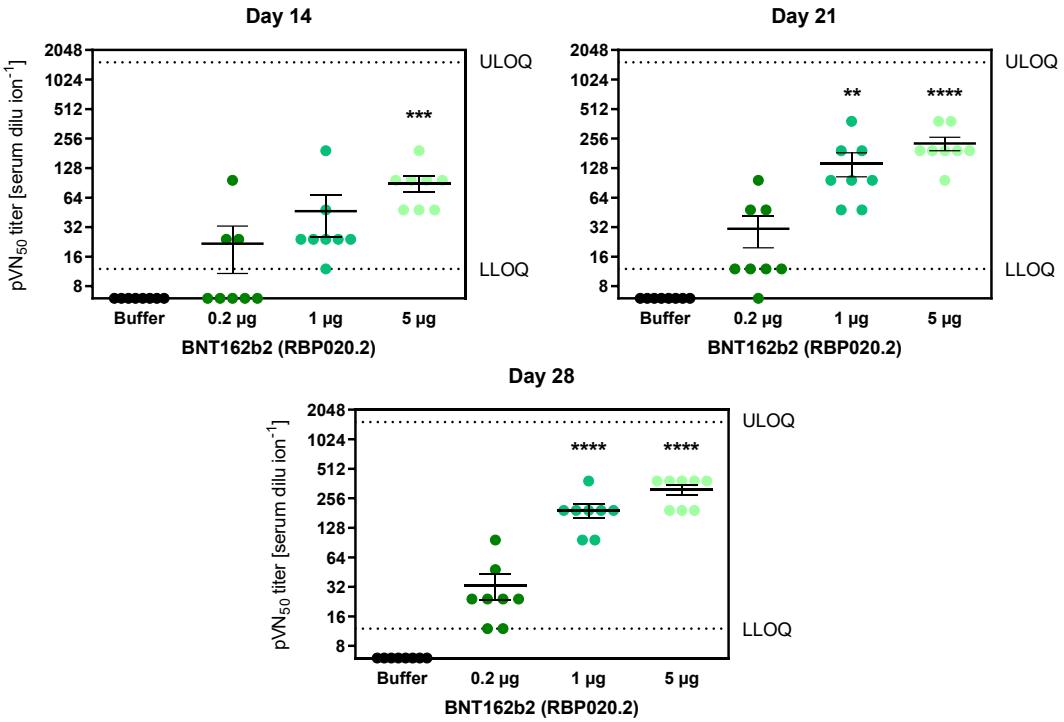

Figure 12: Titers of neutralizing antibodies on days 14, 21, and 28

Serum samples were collected on days 14, 21, and 28 after immunizations and titers of virus-neutralizing ant bodies were determined by pseudovirus-based neutralization test (pVNT). Individual VNT titers are shown by dots; group mean values are indicated by horizontal bars (±SEM, standard error of the mean). ULOQ: Upper limit of quantification, LLOQ: Lower limit of quantification. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$.

5.4 ELISpot Analysis

Mice were euthanized on day 28 and splenocytes were isolated to assess T-cell responses by ELISpot analysis. Splenocytes were stimulated with S1- and RBD-specific overlapping peptide pools (Table 5) and IFN-γ secretion was detected. Statistical significance was assessed by one-way ANOVA with Dunnett's multiple comparison post-test. Control measurements were performed using an irrelevant peptide pool, medium only or Concanavalin A (ConA, for exemplary controls for the assay performed with frozen splenocytes see Appendix 3: Controls for ELISpot Analysis, Figure 21).

Stimulation of fresh splenocytes with an S protein- or RBD-specific overlapping peptide pool induced IFN-γ responses in T cells of immunized animals (Figure 13). After stimulation with either the S protein-specific or RBD peptide pool, splenocytes of the groups treated with modRNA displayed significantly higher spot numbers than buffer

control splenocytes (for S protein: p = 0.0001 for 0.2 µg, p < 0.0001 for 1 µg; RBD: p = 0.0094 for 0.2 µg and p < 0.0001 for 1 µg).

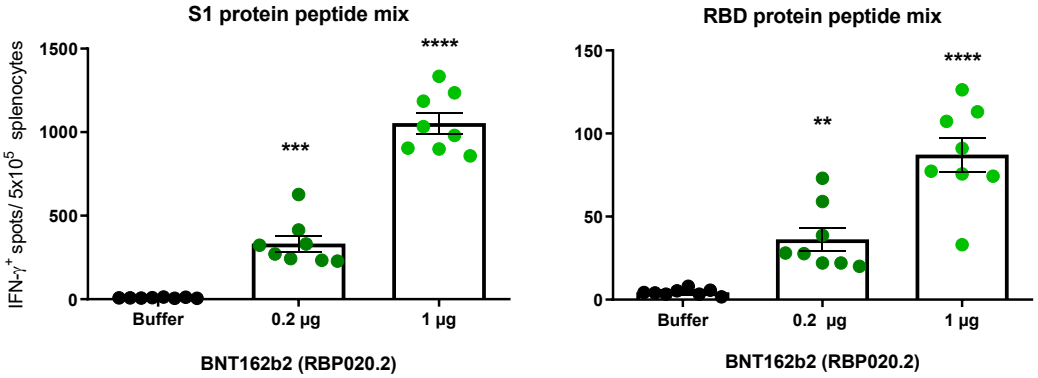

Figure 13: ELISpot analysis using fresh splenocytes on day 28

ELISpot assay was performed using splenocytes isolated on day 28 after immunization. Splenocytes were stimulated with S protein- or RBD-specific overlapping peptide pools and IFN-γ secretion was measured to assess T-cell responses. Individual spot counts are shown by dots; group mean values are indicated by bars (±SEM). ** p ≤ 0.01, **** p < 0.0001. Note that for the 5 µg group, a miscalculated cell number was used in the assay, therefore this group is not included in the graph.

In the assay with fresh splenocytes, a miscalculation of cells in the group immunized with 5 µg modRNA occurred. Therefore, a second ELISpot run was included with frozen splenocytes.

Stimulation of frozen splenocytes with an S protein- or RBD-specific overlapping peptide pool induced IFN-γ responses in T cells of immunized animals (Figure 14). Frozen splenocytes of the groups treated with modRNA displayed significantly higher spot numbers than buffer control splenocytes (p = 0.0087 for 0.2 µg, p < 0.0001 for 1 µg and 5 µg) after stimulation with the S protein-specific peptide pool. Stimulation with the RBD-specific peptide pool induced significantly higher spot numbers in the groups treated with 1 µg and 5 µg modRNA compared to the buffer control group (p = 0.0001 for 1 µg, p = 0.0015 for 5 µg).

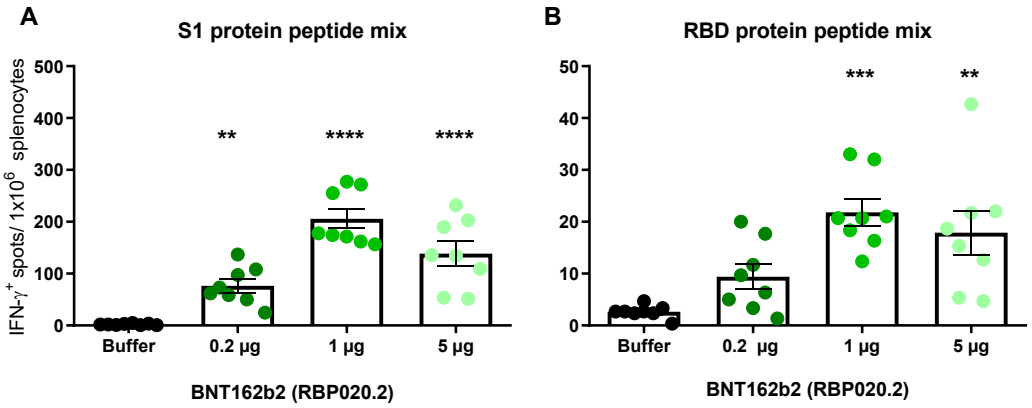

Figure 14: ELISpot analysis using frozen splenocytes on day 28

ELISpot assay was performed using previously frozen splenocytes isolated on day 28 after immunization. Splenocytes were stimulated with S protein- or RBD-specific overlapping peptide pools and IFN-γ secretion was measured to assess T-cell responses. Individual spot counts are shown by dots; group mean values are indicated by bars (±SEM). ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$.

To identify the responding T-cell subtype, an additional ELISpot analysis was performed after separation of fresh $CD4^+$ and $CD8^+$ cells by MACS isolation using splenocytes isolated from the group treated with 5 µg RNA. Statistical significance was assessed by one-way ANOVA with Dunnett's multiple comparison post-test comparing cells stimulated with RBD- and S protein-specific peptide pools to cells stimulated with an irrelevant AH-1-specific peptide pool.

After stimulation with an S protein-specific peptide pool, but not after stimulation with irrelevant AH-1, both $CD4^+$ and $CD8^+$ cells displayed IFN-γ responses (Figure 15). The differences between cells stimulated with the S protein-specific peptide pool and the cells stimulated with the AH-1-specific peptide pool were statistically significant ($p < 0.0001$ for $CD4^+$ and $CD8^+$ cells). No significant increase in spot numbers was detected in $CD4^+$ and $CD8^+$ cells after stimulation with an RBD-specific peptide pool.

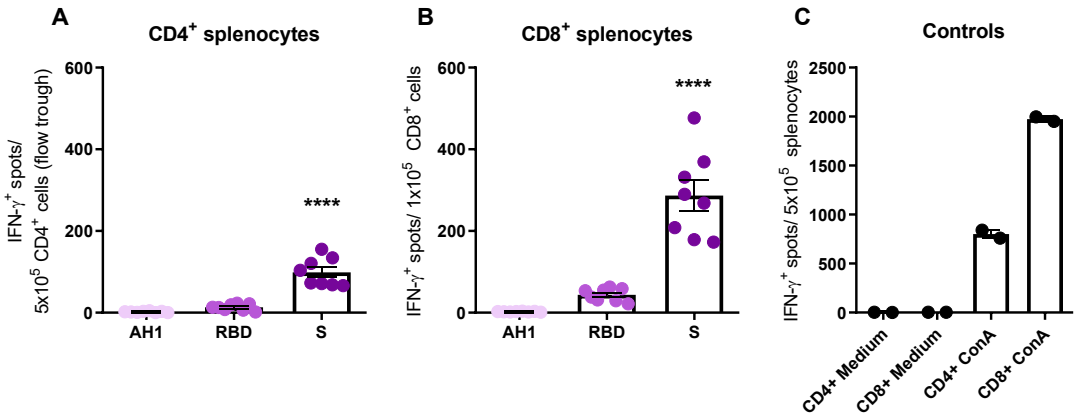

Figure 15: ELISpot analysis using splenocytes of 5 µg BNT162b2 (RBP020.2) immunized mice on day 28 after MACS cell separation

ELISpot assay was performed using splenocytes isolated on day 28 after immunization from group 4 after magnetic cell separation MACS. $CD4^+$ splenocytes (A) or $CD8^+$ splenocytes (B) were stimulated with an RBD- or S protein-specific overlapping peptide pool and IFN-γ secretion was measured to assess T-cell responses. (C) Splenocytes were stimulated with an irrelevant peptide or with medium alone or Concanavalin A. IFN-γ secretion was measured to assess T-cell responses. Mean values ±SEM are shown. **** $p < 0.0001$.

5.5 Luminex Assay

Cytokine concentrations in supernatants of re-stimulated splenocytes were determined using a bead-based, 11-plex T_H1/T_H2 mouse ProcartaPlex immunoassay (Table 8).

Table 8: Chemokines and cytokines included for multiplex measurement

T-cell population	Analytes
T_H1	IFN-γ, GM-CSF, TNF-α, IL-1β, IL-6, IL-12p70, IL-18
T_H2	IL-4, IL-5, IL-13
T_{eff}	IL-2

For cytokine analysis, frozen splenocytes from immunized animals were stimulated with either medium, PMA and ionomycin, or the S- or RBD-overlapping peptide mix.

Immunization with BNT162b2 induced an increased level of T_H1-specific and proinflammatory analytes. Stimulation of splenocytes with 0.1 µg/mL per peptide (total peptide concentration = 31.5 µg/mL) of the S-specific overlapping peptide pool induced a stronger increase in cytokine concentrations than 0.66 µg/mL per peptide (total peptide concentration = 31.5 µg/mL) of the RBD-specific overlapping peptide pool.

Several values were below the lower level of quantification. Therefore, statistical analysis was assessed by mixed-effects analysis/Sidak's comparison. Taking the background of the buffer group, medium control signal into account, a stimulation of

IFN-γ, TNF-α, GM-CSF, IL-6, IL-18, and IL-2 was observed for the groups immunized with 1 µg BNT162b2 (Figure 16). Due to the missing values, statistical significance was not reached or could in some cases not be calculated for the cytokines shown in Figure 16 , even though a clear trend was observed. Therefore, statistical significance is not depicted, but only shown in Appendix 6: Statistical Analysis.

A more detailed summary of the results including PMA controls is shown in Appendix 4: Summary of Luminex Assay.

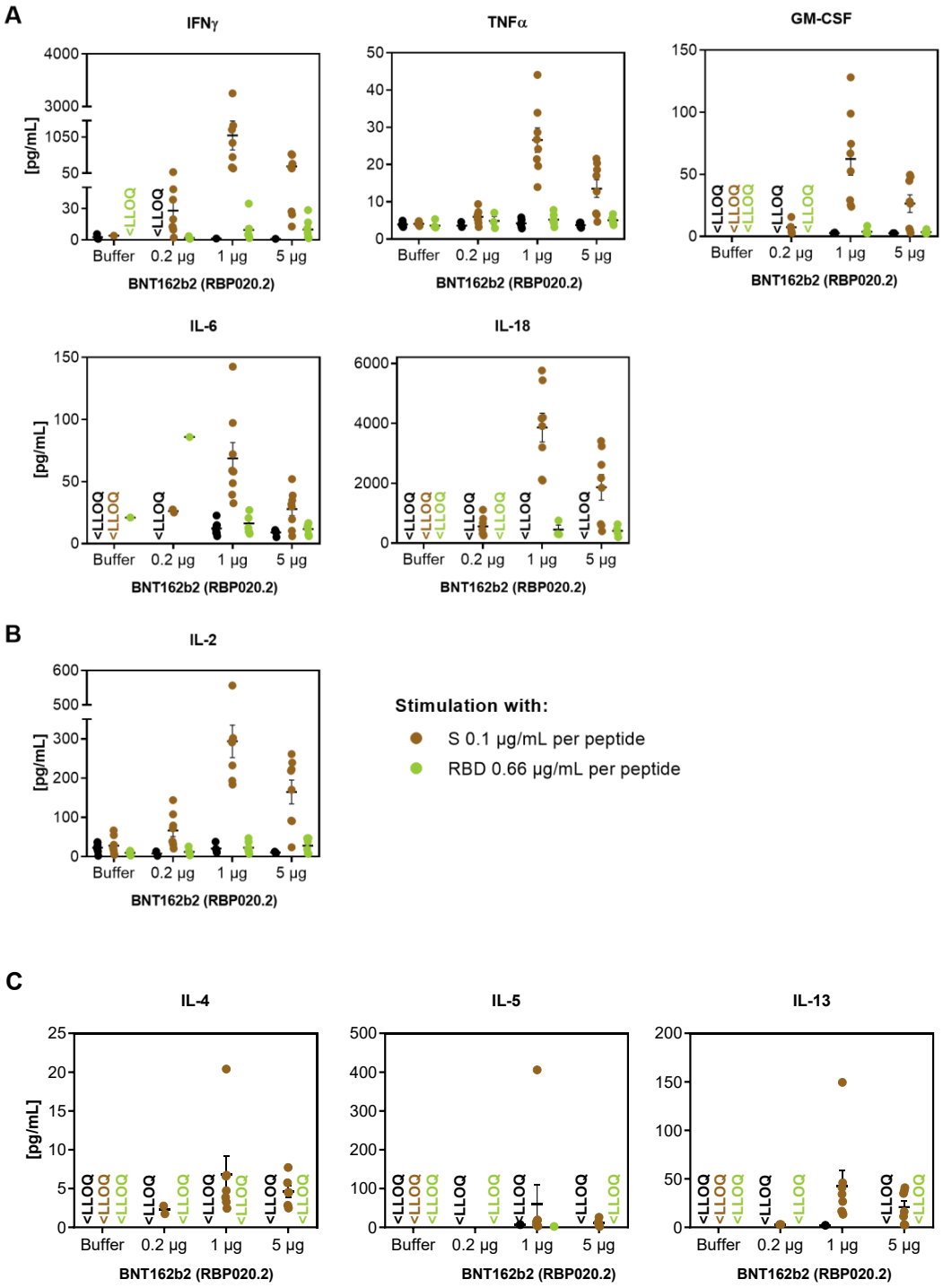

Figure 16: Cytokine concentrations in supernatants of re-stimulated splenocytes 28 days after immunization

Luminex assay was performed using frozen splenocytes isolated on day 28 after RNA injection to assess concentrations of the indicated chemokines/cytokines. After 48 h of stimulation with S- or RBD-overlapping peptide mix, supernatant was collected and secretion of different A) T_H1-specific and proinflammatory, B) T_{eff}-specific and

C) T_H2 cytokines was determined. Values for individual animals are shown by dots. Although all animals within the groups were tested, several values were excluded as they were below the lower level of quantification (LLOQ) and out of standard range. If all values within one group were <LLOQ, this is marked in the graph. Mean values ±SEM are shown.

5.6 Intracellular Cytokine Staining

ICS was performed after stimulation of splenocytes with an overlapping peptide pool of the S protein or controls (Figure 17). Statistical significance was assessed by one-way ANOVA with Dunnett' multiple compari on po t te t

Due to a miscalculation of cells in the group immunized with 5 µg modRNA, ICS results are only shown for buffer control and the groups treated with 0.2 µg and 1 µg BNT162b2.

A peptide-specific stimulation was observed for specific cytokines. The fraction of IFN-γ-expressing $CD4^+$ T cells was significantly higher for animals immunized with 0.2 µg and 1 µg BNT162c2 ($p = 0.0002$ for 0.2 µg, $p < 0.0001$ for 1 µg, Figure 17A) than for buffer control animals. No statistically significant increase was observed for IL-4 after BNT162c2 treatment in comparison to buffer control (Figure 17B). The fraction of TNF-α-expressing $CD4^+$ T cells was significantly higher for animals immunized with 1 µg modRNA ($p < 0.0001$, Figure 17C) than for animals treated with buffer control. For IL-2 expression, the $CD4^+$ T-cell fractions were significantly higher in both treatment groups than in the buffer control group ($p = 0.0015$ for 0.2 µg, $p = 0.0001$ for 1 µg, Figure 17D).

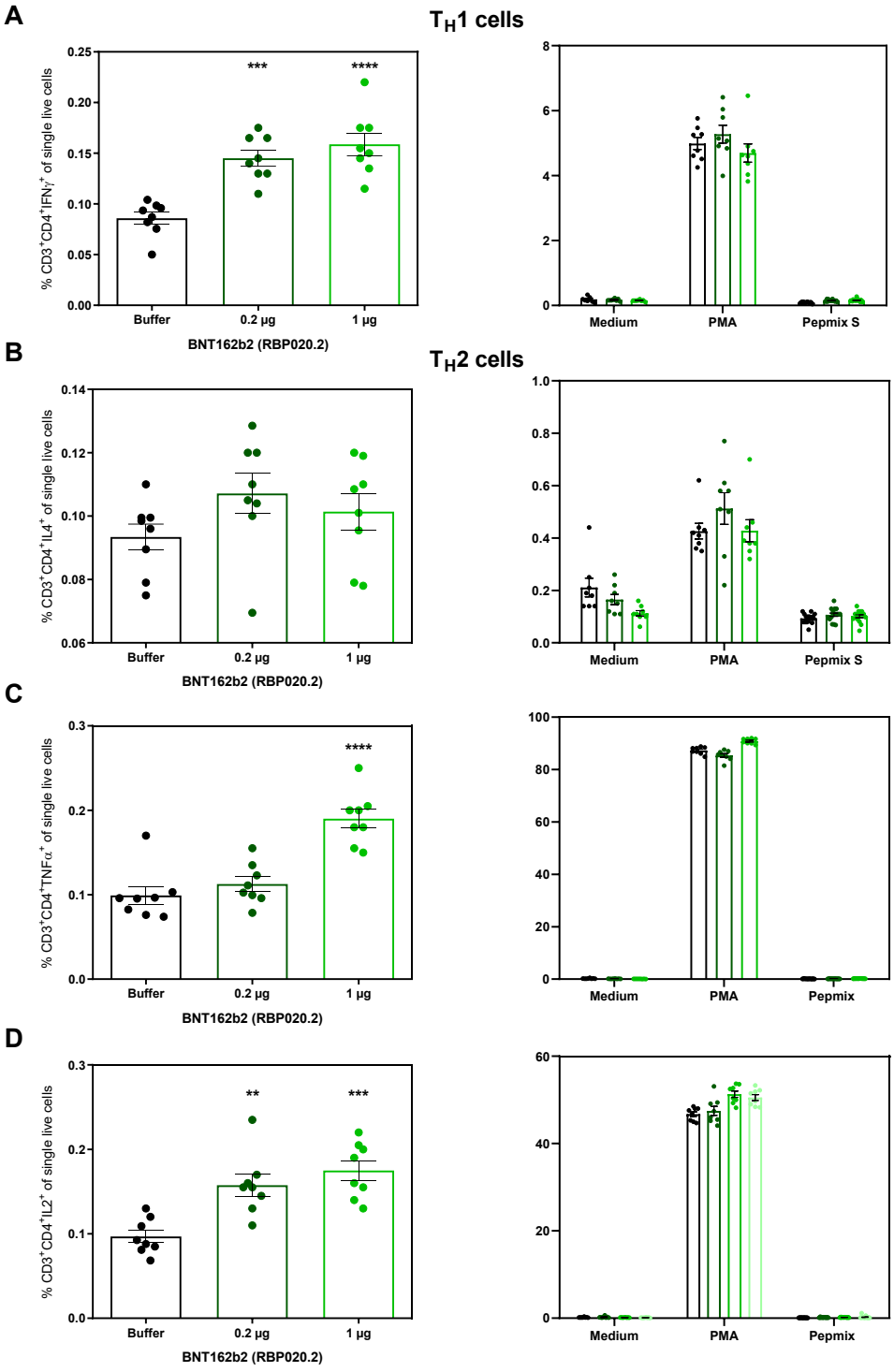

Figure 17: $CD4^+$ T cell intracellular cytokine staining 28 days after immunization

On day 28 after RNA injection, isolated splenocytes were stimulated with either buffer, PMA or an S protein-overlapping peptide mix (Pepmix) to assess the detailed T-cell response via flow cytometry. The intracellular cytokine expression of $CD4^+$ T cells expressing (A) IFN-γ, (B) IL-4, (C) TNF-α, or (D) IL-2 was analyzed. The left

graph in each subfigure shows the fraction after peptide stimulation (measured in duplicates), the right graph shows each single value for all stimulation conditions (buffer and PMA stimulation was measured in single replicates, the Pepmix is the same data as in the left graph but with all single values). Mean values ±SEM are shown. ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

For $CD8^+$ T cells, a statistically significant induction of IFN-γ, TNF-α, and IL-2 was detectable after peptide stimulation in the groups immunized with 0.2 µg and 1 µg RNA compared to buffer control (IFN-γ: $p = 0.0002$ for 0.2 µg, $p < 0.0001$ for 1 µg; TNF-α: $p = 0.0013$ for 0.2 µg, $p < 0.0001$ for 1 µg; IL-2: $p = 0.0003$ for 0.2 µg, $p < 0.0001$ for 1 µg; Figure 18A, B, and C).

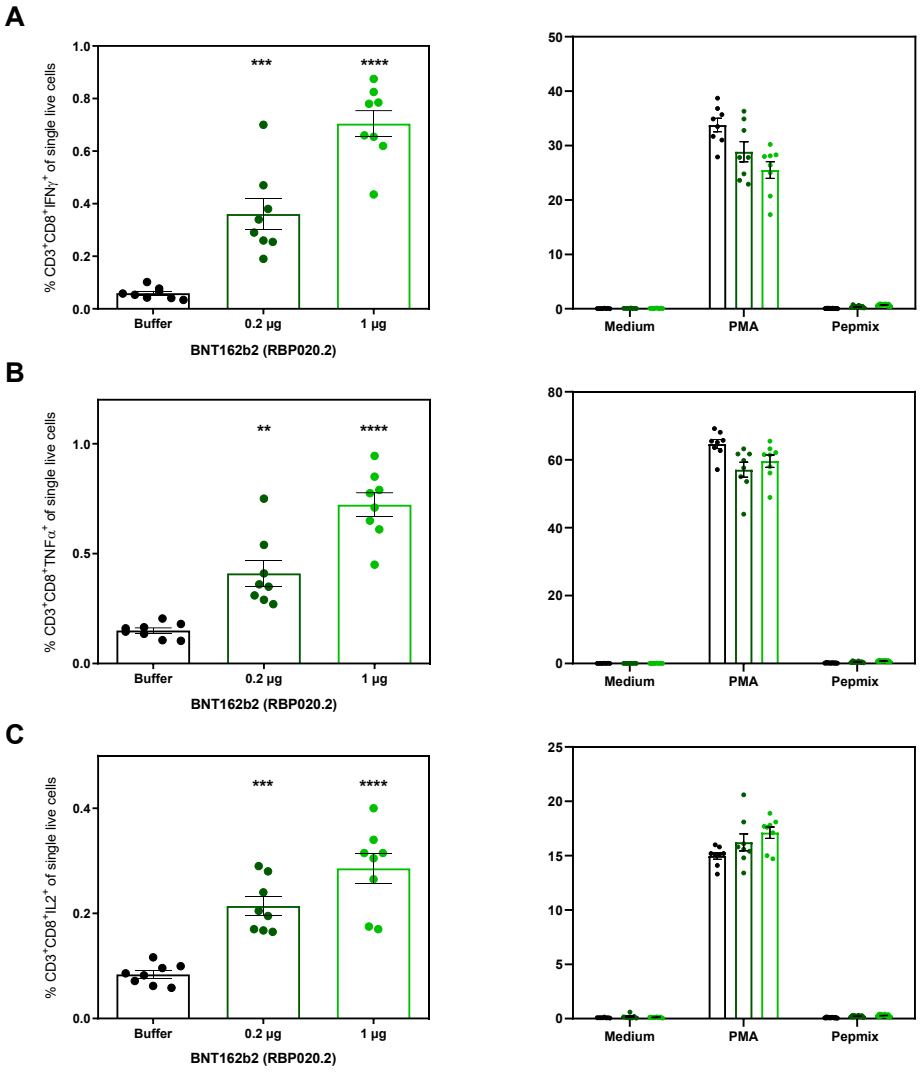

Figure 18: $CD8^+$ T cell intracellular cytokine staining 28 days after immunization

On day 28 after RNA injection, isolated splenocytes were stimulated with either buffer, PMA or an S protein-overlapping peptide mix (Pepmix) to assess the detailed T-cell response via flow cytometry. The intracellular

cytokine expression of $CD8^+$ T cells expressing (A) IFN-γ, (B) TNF-α, or (C) IL-2 was analyzed. The left graph in each subfigure shows the fraction after peptide stimulation (measured in duplicates), the right graph shows each single value for all stimulation conditions (buffer and PMA stimulation was measured in single replicates, the Pepmix is the same data as in the left graph but with all single values). Mean values ±SEM are shown. ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

5.7 Animal Monitoring

The animals' body weight as well as observations regarding fur appearance and injection site reactions are shown in Figure 19 and Figure 20.

The group mean body weights of animals treated with RNA displayed a development comparable to the buffer control group (Figure 19). Over the course of the study, a slight increase in body weights was observed for all groups.

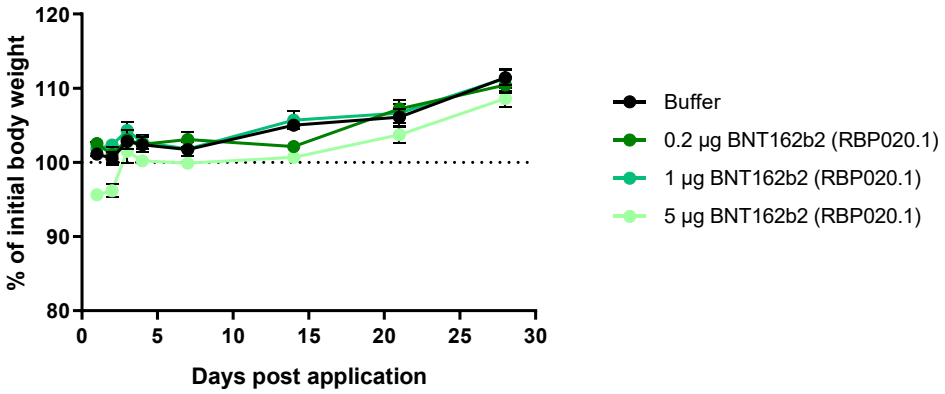

Figure 19: Body weights of experimental mice during study

Experimental animals were weighed at study start and at indicated days, and the change in body weight was calculated as a percentage of the initial weight of the individual mouse. Group mean values (±SEM) are shown.

No changes in fur appearance (i.e., fur defects, neglected grooming, ruffled) were observed in animals treated with BNT162b2 (Figure 20A).

Slight to distinct observations at the injected muscle (i.e., edema formation seen as swollen muscle without flinching in reaction to touch, indicating absence of pain) in comparison to the non-injected hind leg were made in animals treated with 1 µg and 5 µg of BNT162b2 (Figure 20B). By day 2 (1 µg group) or latest by day 4 (5 µg group), the injection site reactions had fully resolved.

Additional animal monitoring details are shown in Appendix 1: Animal Monitoring - Observations.

A

Treatment	Mouse ID	dpi
		1
Buffer	BIO-IJ26	0
	BIO-IJ27	0
	BIO-IJ28	0
	BIO-IJ29	0
	BIO-IJ30	0
	BIO-IJ31	0
	BIO-IJ32	0
	BIO-IJ33	0
0.2 µg BNT162b2 (RBP020.2)	BIO-IJ34	0
	BIO-IJ35	0
	BIO-IJ36	0
	BIO-IJ37	0
	BIO-IJ38	0
	BIO-IJ39	0
	BIO-IJ40	0
	BIO-IJ41	0
1 µg BNT162b2 (RBP020.2)	BIO-IJ42	0
	BIO-IJ43	0
	BIO-IJ44	0
	BIO-IJ45	0
	BIO-IJ46	0
	BIO-IJ47	0
	BIO-IJ48	0
	BIO-IJ49	0
5 µg BNT162b2 (RBP020.2)	BIO-IJ50	0
	BIO-IJ51	0
	BIO-IJ52	0
	BIO-IJ53	0
	BIO-IJ54	0
	BIO-IJ55	0
	BIO-IJ56	0
	BIO-IJ57	0

B

Treatment	Mouse ID	Days post application			
		1	2	3	4
Buffer	BIO-IJ26	0	0	0	0
	BIO-IJ27	0	0	0	0
	BIO-IJ28	0	0	0	0
	BIO-IJ29	0	0	0	0
	BIO-IJ30	0	0	0	0
	BIO-IJ31	0	0	0	0
	BIO-IJ32	0	0	0	0
	BIO-IJ33	0	0	0	0
0.2 µg BNT162b2 (RBP020.2)	BIO-IJ34	0	0	0	0
	BIO-IJ35	0	0	0	0
	BIO-IJ36	0	0	0	0
	BIO-IJ37	0	0	0	0
	BIO-IJ38	0	0	0	0
	BIO-IJ39	0	0	0	0
	BIO-IJ40	0	0	0	0
	BIO-IJ41	0	0	0	0
1 µg BNT162b2 (RBP020.2)	BIO-IJ42	0	0	0	0
	BIO-IJ43	+	0	0	0
	BIO-IJ44	+	0	0	0
	BIO-IJ45	+	0	0	0
	BIO-IJ46	+	0	0	0
	BIO-IJ47	+	0	0	0
	BIO-IJ48	+	0	0	0
	BIO-IJ49	+	0	0	0
5 µg BNT162b2 (RBP020.2)	BIO-IJ50	+++	++	+	0
	BIO-IJ51	++	++	+	0
	BIO-IJ52	++	++	+	0
	BIO-IJ53	++	+	0	0
	BIO-IJ54	++	++	+	0
	BIO-IJ55	+++	++	+	0
	BIO-IJ56	++	+	0	0
	BIO-IJ57	++	+	0	0

Figure 20: Summary of observations made during study's concomitant animal monitoring

Shown are deviations to normal appearance in (A) fur condition and (B) observations at the injection site (edema formation) of each mouse. Severity of observations is graded with 0, none; +, slight; ++, moderate; and +++, distinct.

6 CONCLUSION

Treatment with all tested BNT162b2 doses, namely 0.2, 1 and 5 µg, induced a strong immune response across the observation period of 28 days after vaccination with a safe profile in terms of animal monitoring.

Total IgG ELISA showed that the construct is immunogenic and induced a strong, dose-dependent generation of antibodies against the S1 antigen and the receptor-binding domain. First detection of IgG antibodies was possible 7 days after immunization for all animals throughout the groups with an increase of total antibody amount until day 28. At day 28 after immunization, vaccine-elicited IgG had a strong binding affinity for S1 (geometric mean K_D = 12 nM) and the RBD (geometric mean K_D = 0.99 nM), both had low off-rates.

Profiling the IgG subtypes, a balanced IgG2a/IgG1 response was detected for the two higher doses, while the low dose induced a response with higher IgG1 than IgG2 levels. In pVNT analysis, starting 14 days after immunization, a development of functional neutralizing antibodies was shown for all animals and the titers increased until the final study day. The summary of antibody titers at day 28 is as follows:

	BNT162b2 0.2 µg	**BNT162b2 1 µg**	**BNT162b2 5 µg**
Anti-S1 protein total IgG [µg/mL]	73.0 ± 10.4	205.9 ± 21.0	392.7 ± 28.9
Anti-RBD protein total IgG [µg/mL]	83.1 ± 12.3	241.7 ± 17.2	448.6 ± 28.6
pVN_{50} titer [reciprocal dilution]	33.0 ± 9.8	192.0 ± 31.4	312.0 ±35.1

The ELISpot assay confirmed a strong T-cell activation with the dose of 1 µg resulting in the strongest reactivity. An additional ELISpot with $CD8^+$- and $CD4^+$-separated T cells showed both a reactive $CD8^+$ and $CD4^+$ T-cell response. In Luminex analysis, chemokines and cytokine production after peptide stimulation was confirmed for the group dosed with 1 µg for analytes that indicate a T_H1-driven and proinflammatory immune response in line with the ELISpot. Similarly, reactive IFN-γ-, TNF-α-, and IL-2-secreting $CD4^+$ as well as $CD8^+$ T cells were detected after peptide stimulation in ICS. Taken together, the cellular analysis revealed that in addition to a cytotoxic $CD8^+$ T-cell response, a T_H1-specific response was activated after peptide stimulation.

In summary, the vaccine candidate was highly immunogenic and induced high IgG and neutralizing antibody titers against the antigen as well as a desired T_H1-driven T-cell response including a strong cytotoxic T-cell response. Therefore, BNT162b2 is a promising candidate for further testing in clinical trial.

7 DOCUMENT HISTORY

Reasons for changes compared to previous version:

Minor editorial changes, such as the correction of typing errors, are not specifically listed.

Sections	Version 01	Version 02	Reason for change
1 2.4 3.3 4.5.6 5.2 6	-	Further experimental information added	Added SPR measurements of binding affinities of BNT162b2 vaccine-induced SARS-CoV-2-specific antibodies toward recombinant SARS-CoV-2 S and RBD proteins.
4.4	-	Further equipment and software information added	Equipment table was added and software table was updated.

Sections	Version 02	Version 03	Reason for change
List of Abbreviations	-	Update of list	Additional abbreviations included.
2.4 4.5.5 5.1.1	-	Further experimental information added	Reciprocal endpoint serum titer added for day 14 and day 28 serum samples.
4.5.10 5.5 9	-	Luminex	The used peptide concentration was corrected and the CoA of the ProcartaPlex was included.
4.5.11	-	ICS	The used peptide concentration was corrected.
5.2	-	SPR	Table corrected.

Sections	Version 03	Version 04	Reason for change
4.5.3.1	-	Information for 3 doses of BNT162b2 modified	Doses corrected

8 REFERENCES

Fukushi S, Mizutani T, Saijo M, Matsuyama S, Miyajima N, Taguchi F et al. Vesicular Stomatitis Virus Pseudotyped with severe acute respiratory syndrome coronavirus spike protein. J Gen Virol. 2005;86(Pt 8):2269-2274.

Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor Cell. 2020;181(2):271-280.

Kirchdoerfer RN, Wang N, Pallesen J, Wrapp D, Turner HL, Cottrell CA et al. Stabilized coronavirus spikes are resistant to conformational changes induced by receptor recognition or proteolysis. Sci Rep. 2018;8(1):15701.

Lawson ND, Stillman EA, Whitt MA, Rose JK. Recombinant vesicular stomatitis viruses from DNA. Proc Natl Acad Sci U S A. 1995;92(10):4477-81.

Moyo N, Vogel AB, Buus S, Erbar S, Wee EG, Sahin U et al. Efficient Induction of T Cells against Conserved HIV-1 Regions by Mosaic Vaccines Delivered as Self-Amplifying mRNA. Molecular therapy. Methods & clinical development. 2018;12, 32-46.

Pallesen J, Wang N, Corbett KS, Wrapp D, Kirchdoerfer RN, Turner HL et al. Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen. Proc Natl Acad Sci USA. 2017;114(35):E7348-E7357.

Pardi N, Hogan MJ, Pelc RS, Muramasu H, Andersen H, DeMaso CR et al. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. Nature. 2017;543 (7644), 248-251.

Ullman-Culleré MH, Foltz CJ. Body condition scoring: a rapid and accurate method for assessing health status in mice. Lab Anim Sci. 1999;49(3):319-23.

Vogel AB, Lambert L, Kinnear E, Busse D, Erbar S, Reuter KC et al. Self-amplifying RNA vaccines give equivalent protection against influenza to mRNA vaccines but at much lower doses. Molecular therapy: the journal of the American Society of Gene Therapy. 2017;26 (2), 446-455.

Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020;367(6483):1260-1263.

9 APPENDIX

Appendix 1: Animal Monitoring - Observations

Table 9: Parameters for experimental animal monitoring (single mouse assessment)

The table is separated in immediate euthanasia criteria (end of experiment) and criteria which solitarily observed do not lead to an immediate termination, but result in shorter monitoring frequency (re-assessment).

		Observation (if applicable, categorize [a]):	
Code	**Parameter**	**Renew assessment within < 24 h. Attention: evaluate cumulation**	**Immediate euthanasia criteria**
1	Bodyweight [b]. Take into account Body Conditioning Score (BCS) [c]	Body weight loss > 5 – 10% or BCS transition 3 → 2	Bodyweight loss > 15 - 20% or BCS 2
2	Activity	Moderate deviation from normal or unusual behavior (e.g., limited, reduced, or hyperactive movements)	Immobility, very slow movements (high grade of lethargy), self-isolation
3	Appearance (condition) of fur & eyes	Fur defects/ grooming malfunction (reduced or exaggerated grooming). Moderate orbital tightening.	Distinct scruffy fur, strongly neglected grooming. Eyes lids narrowed, eyes closed and sticky.
4	Body cavities & body fluids	slight - moderate damp & sticky cavities	Clinical signs of disease (diarrhea, distinct sticky)
5	Body temperature & blood circulation ears	-	Animal's body temperature low, ears appear white, and hardly noticeable blood vessels
6	Posture	Moderate deviation of normal physiological posture i.e., short pause in hunched posture	Abnormal posture, hunched, abnormally stretched (belly touches ground) or cramps
7	Reaction to stimulus [d]	Delayed reaction to unconditioned stimulus, moderate deviation from normal behavior (e.g., slight - moderate apathy)	Abnormal (distinct delayed reaction to unconditioned stimulus). Winding and enduring sound utterance ("pain"), aggressiveness at touch
8	Automutilation	-	Burden for the animal noticeable i.e., missing extremities, continuous nibbling, biting and gnawing, open wounds

		Observation (if applicable, categorize [a]):	
Code	**Parameter**	**Renew assessment within < 24 h. Attention: evaluate cumulation**	**Immediate euthanasia criteria**
9	Bites (tail, vibrissae, reproductive organs), other wounds	Open and bleeding wounds (take care of wounds and separate animal)	Burden for the animal noticeable i.e., inflamed wounds
10	Respiration frequency	Moderate deviation of spontaneous breathing (normal respiration frequency)	High frequency, any sign of dyspnea, gasping, flat stretched posture in combination with strongly retracting flanks
11	Motor function	Weak, loose grip (cage grid)	Staggering, circular movement, missing grasp
12	Other abnormalities [e]	-	-

a Categories: NAD, no abnormality detected; +, slight; ++, moderate; +++, distinct.

b Calculate ratio bodyweight start of experiment/ bodyweight monitoring day.

c According to Ullman-Culleré and Foltz 1999.

d Unconditioned = Stimulus to force a reaction e.g., normal background noise, tapping the cage, and normal handling procedure e.g., tilt and turns of the cage.

e Description of abnormality (or abnormalities) on monitoring sheet.

Table 10: Record of body weights of experimental mice during study

						Bodyweight (grams)								
Cage	Mouse ID	Strain	Gender	Date of birth	Treatment	Day 0	Day 1	Day 2	Day 3	Day 4	Day 7	Day 14	Day 21	Day 28
SBIO-15164	BIO-LJ26	BALB/cJRj	f	28.01 20	1	21.4	21.6	21.6	22 0	21.9	21.7	22.1	22.2	24.4
SBIO-15164	BIO-LJ27	BALB/cJRj	f	28.01 20	1	22.0	22.4	21.6	22 2	23.9	22.2	23.1	22.9	24.2
SBIO-15164	BIO-LJ28	BALB/cJRj	f	28.01 20	1	19.9	20.8	20.7	21.1	20.7	20.3	21.3	20.9	22.7
SBIO-15164	BIO-LJ29	BALB/cJRj	f	28.01 20	1	22.3	22.8	23.1	23.4	22.3	22.7	23.2	24.4	24.2
SBIO-15165	BIO-LJ30	BALB/cJRj	f	28.01 20	1	21.6	21.2	20.9	21 5	21.4	21.2	23.4	22.4	23.9
SBIO-15165	BIO-LJ31	BALB/cJRj	f	28.01 20	1	20.8	20.8	20.8	21 2	21.2	21.7	21.9	23.4	24.0
SBIO-15165	BIO-LJ32	BALB/cJRj	f	28.01 20	1	21.8	22.1	22.2	22 8	22.1	22.6	22.8	23.4	24.2
SBIO-15165	BIO-LJ33	BALB/cJRj	f	28.01 20	1	22.1	22.1	21.9	22 5	22.5	22.5	22.7	22.8	23.8
SBIO-15166	BIO-LJ34	BALB/cJRj	f	28.01 20	2	20.1	20.6	19.9	20 3	20.0	20.6	20.5	21.6	21.8
SBIO-15166	BIO-LJ35	BALB/cJRj	f	28.01 20	2	20.6	20.6	19.8	19.7	20.1	20.1	20.3	20.7	21.8
SBIO-15166	BIO-LJ36	BALB/cJRj	f	28.01 20	2	19.7	20.7	20.3	20 5	20.0	20.3	20.1	20.7	21.3
SBIO-15166	BIO-LJ37	BALB/cJRj	f	28.01 20	2	20.3	20.2	20.2	20 5	20.7	21.9	21.0	22.5	22.8
SBIO-15167	BIO-LJ38	BALB/cJRj	f	28.01 20	2	21.2	21.7	21.6	22.7	21.8	21.6	21.9	23.3	24.1
SBIO-15167	BIO-LJ39	BALB/cJRj	f	28.01 20	2	21.2	21.7	21.3	22.1	22.3	21.7	21.8	23.1	23.5
SBIO-15167	BIO-LJ40	BALB/cJRj	f	28.01 20	2	19.6	20.8	20.9	20 8	20.7	20.6	20.6	21.4	22.6
SBIO-15167	BIO-LJ41	BALB/cJRj	f	28.01 20	2	21.0	21.5	21.4	22.1	22.1	21.9	21.0	22.2	22.9
SBIO-15168	BIO-LJ42	BALB/cJRj	f	28.01 20	3	19.7	19.7	19.7	19 5	19.6	19.6	19.9	21.1	20.8
SBIO-15168	BIO-LJ43	BALB/cJRj	f	28.01 20	3	21.2	22.5	22.0	22 6	23.1	22.2	22.7	23.3	23.5
SBIO-15168	BIO-LJ44	BALB/cJRj	f	28.01 20	3	21.7	21.5	21.8	22 5	22.4	20.9	23.6	21.9	24.4
SBIO-15168	BIO-LJ45	BALB/cJRj	f	28.01 20	3	20.8	21.1	21.1	21 8	21.4	21.6	22.6	23.5	23.5
SBIO-15169	BIO-LJ46	BALB/cJRj	f	28.01 20	3	20.5	20.6	21.0	20 8	20.7	20.9	21.4	22.0	22.6
SBIO-15169	BIO-LJ47	BALB/cJRj	f	28.01 20	3	20.2	20.4	20.5	21 6	20.6	20.5	20.7	21.2	22.8
SBIO-15169	BIO-LJ48	BALB/cJRj	f	28.01 20	3	19.8	20.9	20.9	21 5	20.1	20.7	21.9	20.9	23.3
SBIO-15169	BIO-LJ49	BALB/cJRj	f	28.01 20	3	20.5	20.7	21.2	21 2	21.1	20.9	21.0	21.4	22.2
SBIO-15170	BIO-LJ50	BALB/cJRj	f	28.01 20	4	22.6	22.1	22.9	23 6	23.6	22.7	23.2	24.5	24.9
SBIO-15170	BIO-LJ51	BALB/cJRj	f	28.01 20	4	22.4	21.5	21.5	22.1	22.3	22.1	23.0	22.8	23.9
SBIO-15170	BIO-LJ52	BALB/cJRj	f	28.01 20	4	21.2	20.9	20.3	20 8	21.2	21.6	21.0	21.7	23.2
SBIO-15170	BIO-LJ53	BALB/cJRj	f	28.01 20	4	21.8	20.5	21.2	21 8	22.1	22.1	22.0	22.9	25.1
SBIO-15171	BIO-LJ54	BALB/cJRj	f	28.01 20	4	20.3	19.7	19.5	20 2	20.1	20.0	20.5	20.8	22
SBIO-15171	BIO-LJ55	BALB/cJRj	f	28.01 20	4	20.5	19.2	19.1	22 9	20	20.8	20.4	20.2	22
SBIO-15171	BIO-LJ56	BALB/cJRj	f	28.01 20	4	22	20.8	21.2	21 8	22.2	22.3	22.1	22.9	23.4
SBIO-15171	BIO-LJ57	BALB/cJRj	f	28.01 20	4	22.8	21.3	21.3	22 9	22.5	21.8	22.6	24.4	24

Table 11: Record of animal monitoring for each mouse during study

						Animal Monitoring - Observations								
Cage	Mouse ID	Strain	Gender	Date of birth	Treatment	Day 0	Day 1	Day 2	Day 3	Day 4	Day 7	Day 14	Day 21	Day 28
SBIO-15164	BIO-LJ26	BALB/cJRj	f	28.01.20	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15164	BIO-LJ27	BALB/cJRj	f	28.01.20	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15164	BIO-LJ28	BALB/cJRj	f	28.01.20	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15164	BIO-LJ29	BALB/cJRj	f	28.01.20	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15165	BIO-LJ30	BALB/cJRj	f	28.01.20	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15165	BIO-LJ31	BALB/cJRj	f	28.01.20	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15165	BIO-LJ32	BALB/cJRj	f	28.01.20	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15165	BIO-LJ33	BALB/cJRj	f	28.01.20	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15166	BIO-LJ34	BALB/cJRj	f	28.01.20	2	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15166	BIO-LJ35	BALB/cJRj	f	28.01.20	2	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15166	BIO-LJ36	BALB/cJRj	f	28.01.20	2	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15166	BIO-LJ37	BALB/cJRj	f	28.01.20	2	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15167	BIO-LJ38	BALB/cJRj	f	28.01.20	2	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15167	BIO-LJ39	BALB/cJRj	f	28.01.20	2	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15167	BIO-LJ40	BALB/cJRj	f	28.01.20	2	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15167	BIO-LJ41	BALB/cJRj	f	28.01.20	2	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15168	BIO-LJ42	BALB/cJRj	f	28.01.20	3	NAD	12+	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15168	BIO-LJ43	BALB/cJRj	f	28.01.20	3	NAD	12+	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15168	BIO-LJ44	BALB/cJRj	f	28.01.20	3	NAD	12+	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15168	BIO-LJ45	BALB/cJRj	f	28.01.20	3	NAD	12+	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15169	BIO-LJ46	BALB/cJRj	f	28.01.20	3	NAD	12+	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15169	BIO-LJ47	BALB/cJRj	f	28.01.20	3	NAD	12+	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15169	BIO-LJ48	BALB/cJRj	f	28.01.20	3	NAD	12+	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15169	BIO-LJ49	BALB/cJRj	f	28.01.20	3	NAD	12+	NAD	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15170	BIO-LJ50	BALB/cJRj	f	28.01.20	4	NAD	12+++	12++	12+	NAD	NAD	NAD	NAD	NAD
SBIO-15170	BIO-LJ51	BALB/cJRj	f	28.01.20	4	NAD	12++	12++	12+	NAD	NAD	NAD	NAD	NAD
SBIO-15170	BIO-LJ52	BALB/cJRj	f	28.01.20	4	NAD	12++	12++	12+	NAD	NAD	NAD	NAD	NAD
SBIO-15170	BIO-LJ53	BALB/cJRj	f	28.01.20	4	NAD	12++	12+	ok	NAD	NAD	NAD	NAD	NAD
SBIO-15171	BIO-LJ54	BALB/cJRj	f	28.01.20	4	NAD	12++	12++	12+	NAD	NAD	NAD	NAD	NAD
SBIO-15171	BIO-LJ55	BALB/cJRj	f	28.01.20	4	NAD	12+++	12++	12+	NAD	NAD	NAD	NAD	NAD
SBIO-15171	BIO-LJ56	BALB/cJRj	f	28.01.20	4	NAD	12++	12+	NAD	NAD	NAD	NAD	NAD	NAD
SBIO-15171	BIO-LJ57	BALB/cJRj	f	28.01.20	4	NAD	12++	12+	NAD	NAD	NAD	NAD	NAD	NAD

Appendix 2: Certificates of Analysis

BioNTech RNA Pharmaceuticals GmbH

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BIONTECH
RNA Pharmaceuticals

Report of Results
In vitro transcribed mRNA

Product:	***In vitro* transcribed mRNA RBP020.2 (ATM batch modRNAv09)**
Lot/Batch No.:	RNA-RF200321-06
RNA length:	4283 nt
Media and additives:	10 mM HEPES/0.10 mM EDTA (pH 7.0)
Production date:	19 Mar 2020 (produced by BioNTech RNA Pharmaceuticals GmbH)
Storage:	-30 °C to -15 °C

Test	Result
Content (RNA concentration) Ultraviolet Absorption Spectrophotometry; A_{260}	(b) (4)
Identity (RNA length) Denaturing Agarose Gel Electrophoresis	
RNA integrity Capillary Electrophoresis (Fragment Analyzer, Advanced Analytical)	
Potency *In vitro* translation followed by gel electrophoresis	
pH Potentiometric Determination of pH	
Bacterial Endotoxins LAL-test (Ph. Eur. 2.6.14)	
Residual DNA template Quantitative PCR	
Residual dsRNA Antibody-based limit test	
Osmolality Measurement of depression of freezing point	
Bioburden Microbial examination of non sterile products (Ph. Eur. 2.6.12)	

Remarks:

None.

(b) (6) (b) (6)

Report of Results_ RBP020.2 (ATM batch modRNAv09); Version 01 1/1

POLYMUN SCIENTIFIC
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http://www.polymun.com

Non-GMP CoA

Material not for human use
Version 3

Product: **CoVVAC**
Batch: RBP020.2LNP
Lot: CoVVAC/270320

Test	Method	Result
Appearance	Visual Inspection (224/SOP/011)	(b) (4)
RNA identity	CE (223/SOP/016)	
RNA integrity	CE (223/SOP/016)	
RNA content	Ribogreen Assay (221/SOP/018)	
RNA encapsulation	Ribogreen assay +/- LNP disruption (221/SOP/018)	
ALC-0315 content	HPLC-CAD (222/SOP/044)	
ALC-0159 content	HPLC-CAD (222/SOP/044)	
DSPC content	HPLC-CAD (222/SOP/044)	
Cholesterol content	HPLC-CAD (222/SOP/044)	
Particle size (Z_{ave})	Dynamic light scattering (224/SOP/002)	
Polydispersity index (PDI)	Dynamic light scattering (224/SOP/002)	
pH	pH (224/SOP/016)	
Osmolality	Freezing point depression (224/SOP/009)	
Endotoxins/Pyrogens	Turbidimetric, kinetic LAL assay (Ph.Eur. 2.6.14/ USP<85>)	
Bioburden	Membrane filtration method 225/SOP/001	

Store at: -70°C

Date: 09.04.20 (b) (6)

Date: 09.04.20

CarVac # 09

ThermoFisher SCIENTIFIC

Bender MedSystems GmbH
Campus Vienna Biocenter 2
A-1030 Vienna, Austria
www.thermofisher.com

Certificate of analysis

ProcartaPlex

Cat. number: **EPX110-20820-901**
96 tests/11 analytes

Name: **ProcartaPlex Mouse Th1/Th2 Cytokine Panel 11plex**

Lot number: **232634-004**

Expiry date: **2022-04**

Components		Quantity	Lot	Store at
BK20820EX	det. antibody Mix B	1 x 70µl (50x)	202902000	2-8°C
B20820EX	Bead Mix B	1 x 5ml (1x)	202901000	2-8°C
S26088EX	Standard Mix A	2 each	220399-001	2-8°C
DDBMEX/4	Detection Ab Diluent	1 x 3ml	205752000	2-8°C
RBEX/46	Reading Buffer	1 x 40ml	19127887	2-8°C
WBEX/28	10x Wash Buffer	1 x 25ml	19127883	2-8°C
SA-PE	Streptavidin-PE	1 x 5ml	233434-000	2-8°C
UABEX/11	Universal Assay Buffer 1x	1 x 10ml	20018141	2-8°C
SVM104	Black Microplate Lid	1 each		2-8°C
SVM16	Plate Covers	8 each		2-8°C
SVM182	Flat bottom Plate (black)	1 each		2-8°C
SVM183	PCR 8-Tube Strip	2 each		2-8°C

Bead Mix B Lot#202901000

Target Name	Bead Number	Std1 Concentration pg/ml	Standard
GM-CSF	42	9950	Standard Mix A
IFN gamma	38	4800	Standard Mix A
IL-1 beta	19	4350	Standard Mix A
IL-12p70	39	6550	Standard Mix A
IL-13	35	8650	Standard Mix A
IL-18	66	207000	Standard Mix A
IL-2	20	5250	Standard Mix A
IL-4	26	4950	Standard Mix A
IL-5	27	8000	Standard Mix A
IL-6	28	19500	Standard Mix A
TNF alpha	45	11700	Standard Mix A

Standard Mix A Lot#220399-001

Provided below is a table of Standard 1 (Std1) value for each analyte in each tube when prepared according to the "Preparing Standard" procedure of the Manual.

Analyte	Std1 Concentration (pg/ml)	ULOQ / LLOQ (pg/ml) Determined in cell culture medium
Eotaxin (CCL11)	2550	2550 / 0,62
GM-CSF	9950	9950 / 2,43
GRO alpha (CXCL1)	5950	5950 / 1,45
IFN gamma	4800	4800 / 1,17
TNF alpha	11700	11700 / 2,86
IL-10	8400	8400 / 2,05
IL-12p70	6550	1638 / 1,60
IL-13	8650	8650 / 2,11
IL-17A (CTLA-8)	5750	5750 / 1,40
IL-18	207000	51750 / 51
IL-1 beta	4350	4350 / 1,06
IL-2	5250	5250 / 1,28
IL-22	40400	40400 / 9,86
IL-23	34500	34500 / 8,42
IL-27	8350	8350 / 2,04
IL-4	4950	4950 / 1,21
IL-5	8000	8000 / 1,95
IL-6	19500	19500 / 4,76
IL-9	66900	66900 / 16
IP-10 (CXCL10)	2250	2250 / 0,55
MCP-1 (CCL2)	28300	28300 / 6,91
MCP-3 (CCL7)	900	900 / 0,22
MIP-1 alpha (CCL3)	1400	1400 / 0,34
MIP-1 beta (CCL4)	6200	1550 / 1,51
MIP-2 alpha (CXCL2)	3350	838 / 3,27
RANTES (CCL5)	10800	2700 / 2,64

Analytical information:

This product has been tested by Quality Control and passed internal specifications.

Quality control: (b) (6)

For Research Use Only. Not for use in diagnostic procedures. If you have any further questions about this Certificate of Analysis, please contact Technical Services at 1-800-955-6288 (US and Canada) or 1-760-603-7200, x2 (all other countries).
For inquiries, contact us at "thermofisher.com/askaquestion"

Appendix 3: Controls for ELISpot Analysis

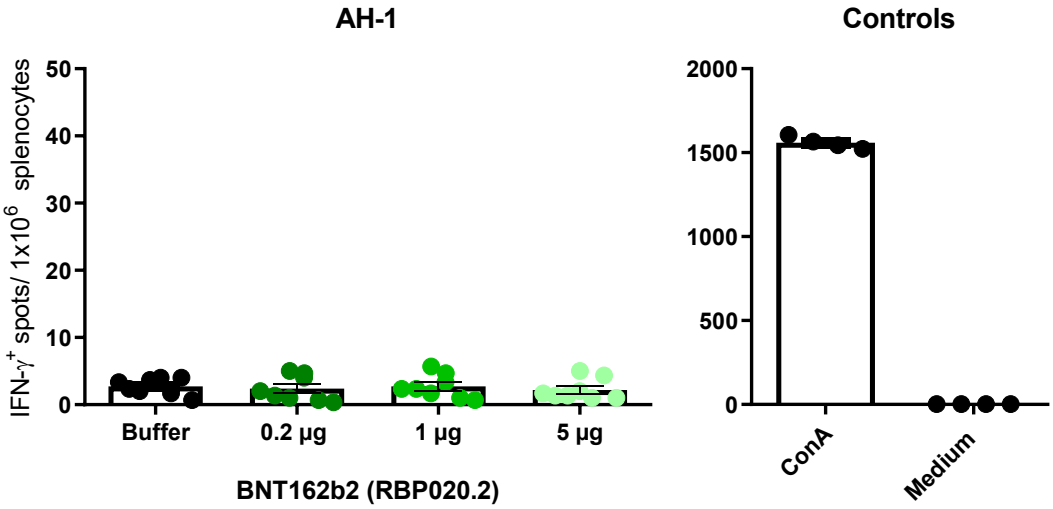

Figure 21: Controls for ELISpot analysis using splenocytes on day 28

ELISpot assay was performed using splenocytes isolated on day 28 after immunization. Splenocytes were stimulated with the irrelevant peptide AH-1 (left), or with Concanavalin A or medium alone (right). IFN-γ secretion was measured to assess T-cell responses. Mean values ±SEM are shown.

Appendix 4: Summary of Luminex Assay Data

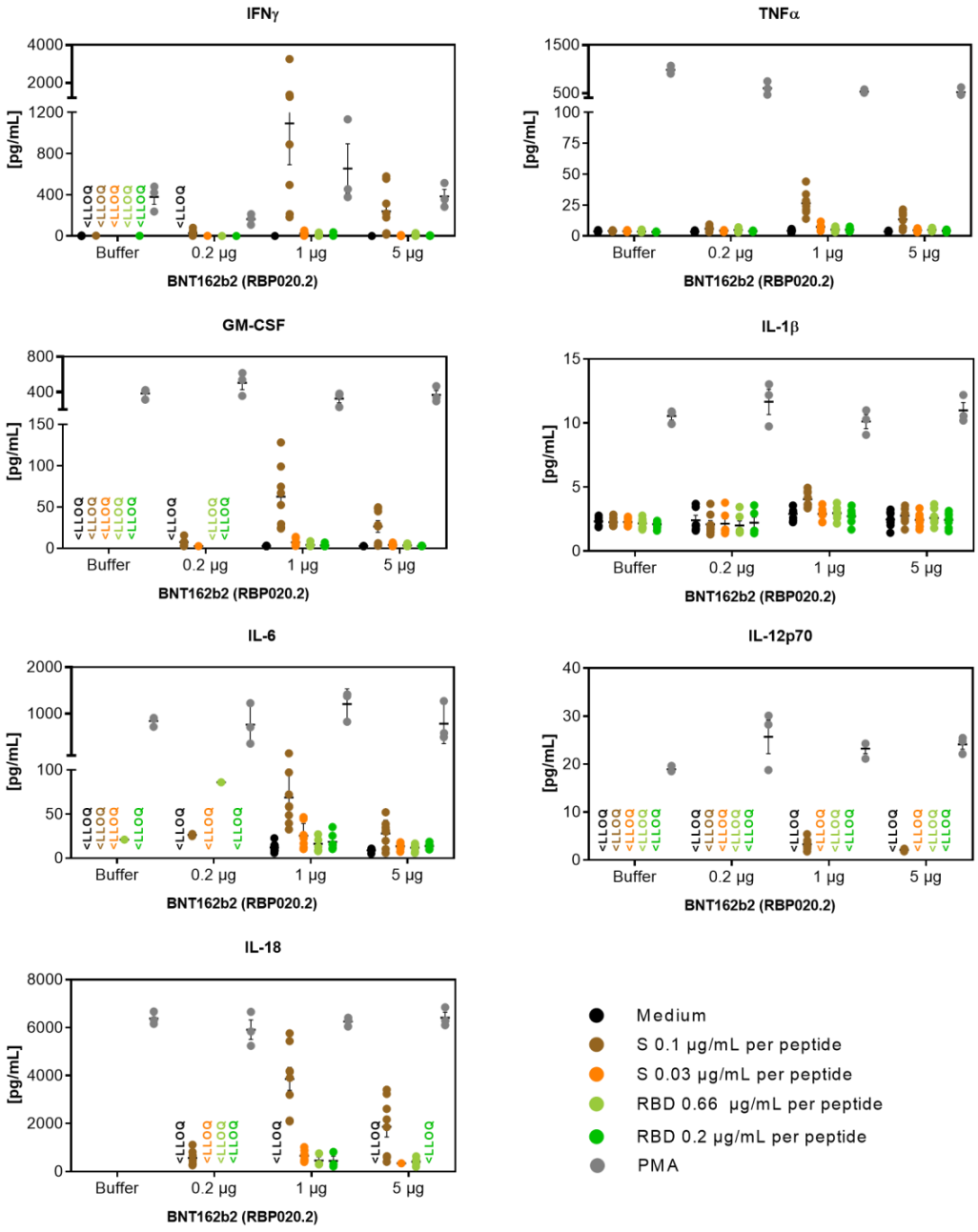

Figure 22: T_H1 and proinflammatory cytokine concentrations in supernatants of re-stimulated splenocytes 28 days after immunization

Luminex assay was performed using frozen mouse splenocytes isolated on day 28 after RNA injection to assess concentrations of the indicated chemokines/cytokines. After 48 h of stimulation with medium, PMA plus ionomycin (PMA), or the S-/RBD-overlapping peptide mix, supernatant was used for the analysis of the secretion of different analytes. Values for individual animals are shown by dots; group mean values are indicated by lines (±SEM). Several values were excluded, as they were below the LLOD or out of the standard range (<LLOQ) or upper the limit of quantification (ULOQ). Therefore, no statistical information was included in the figure (for calculation, see Appendix 6: Statistical Analysis). Medium, S 0.1 µg/mL and RBD 0.66 µg/mL per peptide are shown in Figure 16.

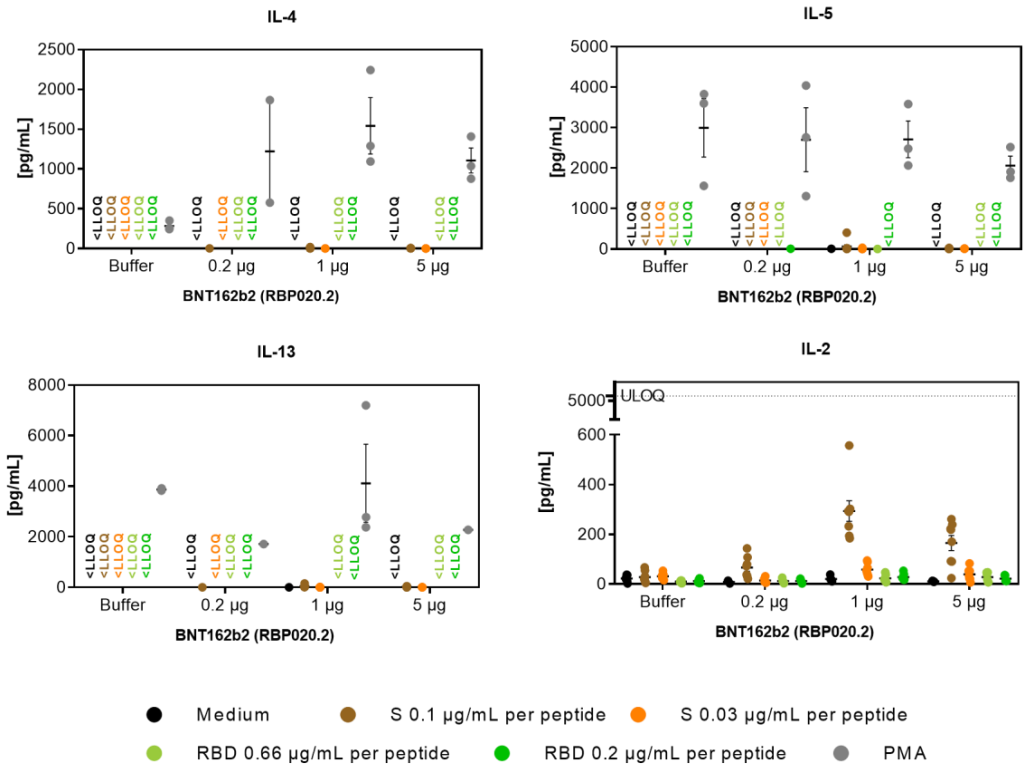

Figure 23: T_H2 cytokine and IL-2 concentrations in supernatants of re-stimulated splenocytes 28 days after immunization

Luminex assay was performed using frozen mouse splenocytes isolated on day 28 after RNA injection to assess concentrations of the indicated chemokines/cytokines. After 48 h of stimulation with medium, PMA plus ionomycin (PMA), or the S-/RBD-overlapping peptide mix, supernatant was used for the analysis of the secretion of different analytes. Values for individual animals are shown by dots; group mean values are indicated by lines (±SEM). The RBD peptide mix was not tested for animals treated with 0.2 µg and 1 µg RNA. Several values were excluded, as they were below the LLOD or out of the standard range (<LLOQ). Therefore, no statistical information was included in the figure (for calculation, see Appendix 6: Statistical Analysis). Medium, S 0.1 µg/mL and RBD 0.66 µg/mL per peptide are shown in Figure 16. Note after PMA stimulation, all IL-2 concentrations were above the upper limit of quantification (ULOQ).

Appendix 5: Detailed ICS Protocol

FACS panel (FACS Celesta)

	Wavelengths	Markers	µL per 50 µL	Clones	Colors	Name	Company	Catalog no.
1	450/50	CD8a	0.25	53-6.7	BV421	Brilliant Violet 421™ anti-mouse CD8a antibody	BioLegend	100753
2	525/50	CD4	0.25	RM4-5	BV510	Brilliant Violet 510™ anti-mouse CD4 antibody	BioLegend	100559
	610/20		no		BV605			
3	710/50	IL-4	0.25	11B11	BV711	Brilliant Violet 711™ anti-mouse IL-4 antibody	BioLegend	504133
4	780/60	CD25	0.25	PC61	BV786	Brilliant Violet 785™ anti-mouse CD25 antibody PC61	BioLegend	102051
5	530/30	TNF-α	0.5	MP6-XT22	Alexa 488	Alexa Fluor® 488 anti-mouse TNF-α ant body, clone MP6-XT22	BioLegend	506313
6	575/25	CD3	0.25	145-2C11	PE	PE Hamster anti-mouse CD3e clone 145-2C11	BD	553064
7	695/40		no		PerCP-Cy5.5			
8	780/60	IFN-γ	0.1	XMG1.2	Pe-Cy7	PE/Cy7 anti-mouse IFN-γ antibody, clone XMG1.2	BioLegend	505826
9	670/30	IL-2	0.5	JES6-5H4	APC	APC anti-mouse IL-2 antibody	BioLegend	503810
10	730/45		no		APC-R700			
11	780/60	dead	0.05-0.03		eFluor780	eBioscience™ Fixable Viability Dye eFluor™ 780	ThermoFisher	65-0865-14

96-well plates:

Plate 1: against S protein

	1	2	3	4	5	6	7	8	9	10	11	12
A	pool*[1]	pool*[1]	pool*[1]	pool*[1]		1-1	1-1	1-1	1-1			
B	pool*[2]	pool*[2]	pool*[2]	pool*[2]		1-2	1-2	1-2	1-2			
C	pool*[3]	pool*[3]	pool*[3]	pool*[3]		1-3	1-3	1-3	1-3			
D						1-4	1-4	1-4	1-4			
E						1-5	1-5	1-5	1-5			
F						1-6	1-6	1-6	1-6			
G						1-7	1-7	1-7	1-7			
H						1-8	1-8	1-8	1-8			

Plate 2: against S protein

	1	2	3	4	5	6	7	8	9	10	11	12
A	2-1	2-1	2-1	2-1	3-1	3-1	3-1	3-1	4-1	4-1	4-1	4-1
B	2-2	2-2	2-2	2-2	3-2	3-2	3-2	3-2	4-2	4-2	4-2	4-2
C	2-3	2-3	2-3	2-3	3-3	3-3	3-3	3-3	4-3	4-3	4-3	4-3
D	2-4	2-4	2-4	2-4	3-4	3-4	3-4	3-4	4-4	4-4	4-4	4-4
E	2-5	2-5	2-5	2-5	3-5	3-5	3-5	3-5	4-5	4-5	4-5	4-5
F	2-6	2-6	2-6	2-6	3-6	3-6	3-6	3-6	4-6	4-6	4-6	4-6
G	2-7	2-7	2-7	2-7	3-7	3-7	3-7	3-7	4-7	4-7	4-7	4-7
H	2-8	2-8	2-8	2-8	3-8	3-8	3-8	3-8	4-8	4-8	4-8	4-8

Plate 3: against RBD

	1	2	4	5	5	6	7	8	9	10	11	12
A	1-1	1-1		2-1	2-1		3-1	3-1		4-1	4-1	
B	1-2	1-2		2-2	2-2		3-2	3-2		4-2	4-2	
C	1-3	1-3		2-3	2-3		3-3	3-3		4-3	4-3	
D	1-4	1-4		2-4	2-4		3-4	3-4		4-4	4-4	
E	1-5	1-5		2-5	2-5		3-5	3-5		4-5	4-5	
F	1-6	1-6		2-6	2-6		3-6	3-6		4-6	4-6	
G	1-7	1-7		2-7	2-7		3-7	3-7		4-7	4-7	
H	1-8	1-8		2-8	2-8		3-8	3-8		4-8	4-8	

	* controls	[1]No Ab	[2]CD3+ L/D	[3]LD + CD3 + CD4 + CD8+ L/D
	Medium only	Positive stimulus	S protein	
			RBD	

Mastermixes for stimulation

	Stimulus	concentration stock (mg/mL)	concentration needed (mg/mL)	concentration needed (mg/mL) * 2	Dilution factor	total wells	total volume needed (mL; 100µl/well; incl 10% spare)	Volume stimulus (µL)	Volume DC medium (µL)
Medium only	-	-	-	-		32	3,52	**0**	**3520**
Positive stimulus	PMA	1	0,0005	0,001	1000	32	3,52	**3,52**	**3515,78**
	Ionomycin	10	0,001	0,002	5000	32	3,52	**0,70**	
S protein peptide mix 1	158 peptide	15,8	0,0315	0,062	254,84	64,00	7,04	**27,63**	**6984,57**
S protein peptide mix 2	157 peptide	15,7			253,23	64,00	7,04	**27,80**	
RBD peptides	48 peptides	**1,2**	0,0048	0,0096	125	64	7,04	**56,32**	**6983,68**
the S peptide stocks has a concentration of 100µg/mL per peptide; RBD has 25µg/mL									

Mastermix for blocking reagents

Blocking reagent	Volume needed/well [µL]	Volume needed/mL [µL]	Total wells	Total volume needed (ml; 10 µL/well; incl 10% spare)	Volume blocking (µL)	Volume DC medium (µL)
GolgiStop	0,13	13	192	2,112	**27,46**	**2042,30**
GolgiPlug	0,2	20	192	2,112	**42,24**	

working concentration Stop (1:1500), Plug (1:1000)

10 µL per well

Stimulation protocol:

1. Prepare a 96-well tissue culture (F-well)
2. Add 100 µL of stimulus (or medium) to the according well; “pool” on plates are FACS controls – 100 µL medium is sufficient
3. Add 500,000 cells in DC medium per well (100 µL)
4. Incubate plate for 1 h @ 37°C in 5% CO_2
5. Add 10 µL of blocking reagents
6. Swing plates (5× 8-moves)
7. Incubate for 5 h @37°C in 5% CO_2
8. Proceed with staining protocol or put plates in 4°C o/n

Mastermix for L/D reagents

	Live-dead reagent	Volume needed/well [µL]	Volume needed/mL [µL]	Total wells	Total volume needed (ml; 50 µL/well; incl 5% spare)	Volume L/D (µL)	Volume PBS (µL)
eFluor780	L/D	0,05	1	200	11	**11,00**	**10989,00**
	working concentration Stop is 1:1000						
	50 µL per well						

Antibody mix control 1 mastermix

	Markers (extracellular)	µL per reaction (50 µL)	Total wells	Volume Ab (µL)	Volume FACS buffer	
BV510	CD4	0,25	4	**1,05**	**207,9**	includes 5% spare volume
BV421	CD8	0,25	4	**1,05**		

Antibody mix 1 mastermix

	Markers (extracellular)	µL per reaction (50 µL)	Total wells	Volume Ab (µL)	Volume FACS buffer (µL)	
BV421	CD8a	0,25	192	**50,40**	**9928,8**	includes 5% spare volume
BV510	CD4	0,25	192	**50,40**		
BV786	CD25	0,25	192	**50,40**		

Staining protocol:

Note: work with a 4°C cooled centrifuge

9. Mix cells by pipetting 3× up and down and transfer total volume to 'v' bottom plate
10. Centrifuge at 350 ×g, 5 min
11. Wash cells once with 150-200 µL cold PBS
12. Centrifuge at 350 ×g, 5 min. Discard supernatant
    a. Vortex cells carefully; snap against the wells to support pellet dissolving
13. Stain with L/D reagent 50 µL to each well in PBS at 4°C for 15 min
14. Add 100 µL PBS
15. Centrifuge at 350 ×g, 5 min. Discard supernatant; vortex/snap
16. Add antibody master mix 1/antibody mix 1 controls/FACS buffer to each well 50 µL
17. Incubate 30 min at 4°C
18. Add 100 µL FACS buffer
19. Centrifuge at 350 ×g, 5 min. Discard supernatant; vortex/snap
20. Add 2% Histofix
21. Incubate following protocol a, b, or c:
    a. keep it in 4°C for overnight (up to ~16 h) if not proceeding for intracellular staining immediately
    b. incubate for at least 1 h at 4°C and
        i. proceed for intracellular staining or
    c. incubate for at least 1 h at 4°C

i. add 100 µL PBS
ii. centrifuge at 400 ×g, 5 min. Discard supernatant; vortex/snap
iii. add 100 µL PBS and keep it in 4°C until further usage (within ~36 h)
iv. proceed with intracellular staining protocol

Fc Block mastermix

Fc Block	µL per reaction (25 µL)	Total wells	Volume Ab (µL)	Volume perm buffer (µL)	
CD16/CD32	0,5	200	**105**	**4935**	includes 5% spare volume

Antibody mix control 2 mastermix

	Markers (extracellular)	µL per reaction (50 µL)	Total wells	Volume Ab (µL)	Volume perm buffer (µL)	
PE	CD3	0,25	4	**1,05**	**208,95**	includes 5% spare volume

Antibody mix 2 mastermix

	Markers (intracellular)	µL per reaction (25 µL)	Total wells	Volume Ab (µL)	Volume perm buffer (µL)	
BV711	IL-4	0,25	192	**50,40**	**4717,44**	includes 5% spare volume
PE	CD3	0,25	192	**50,4**		
Alexa 488	TNF-α	0,5	192	**100,80**		
Pe-Cy7	IFN-γ	0,1	192	**20,16**		
APC	IL-2	0,5	192	**100,80**		

Intracellular staining protocol:

1. Centrifuge at 400 ×g, 5 min, 4°C. Discard supernatant, vortex/snap
2. Suspend cells with perm buffer, 150 µL each well
3. Centrifuge at 400 ×g, 5 min, 4°C. Discard supernatant, vortex/snap
4. Add Fc block 25 µL amount, to each well, incubate for 10 min
5. Add 25 µL of the antibody master mix 2/antibody mix 2 controls/FACS buffer to the cells, mix carefully and incubate for another 45 min, 2-8°C
6. Add 100 µL 1× Perm buffer
7. Centrifuge at 400 ×g, 5 min, 4°C. Discard supernatant, vortex/snap
8. Add 200 µL 1× Perm buffer

9. Centrifuge at 400 ×g, 5 min, 4°C. Discard supernatant, vortex/snap
10. Discard the supernatant carefully
11. Resuspend the cells carefully in 100 µL FACS buffer or FACS flow
12. Mix cells by pipetting 3× up and down and transfer total volume to ‘U’ bottom plate for FACS Celesta/HTS acquire
13. Store plate at 4°C until time point of measurement/flow cytometry
    a. Note that the plates should be acquired the same day as the intracellular staining may decrease in signal.

Appendix 6: Statistical Analysis

ELISA

Descriptive statistics, ELISA screening analysis, day 7, S1

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	0,362	0,544	1,35
Maximum	0,00100	1,22	1,59	2,05
Range	0,00100	0,858	1,05	0,704
Mean	0,000125	0,872	1,14	1,74
SD	0,000354	0,321	0,373	0,245
SEM	0,000125	0,113	0,132	0,0867

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ELISA screening analysis, day 14, S1

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	0,724	1,09	1,71
Maximum	0,0100	1,53	1,80	2,11
Range	0,0100	0,809	0,713	0,407
Mean	0,00325	1,18	1,45	1,88
SD	0,00388	0,299	0,218	0,143
SEM	0,00137	0,106	0,0772	0,0504

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ELISA screening analysis, day 21, S1

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	0,815	1,36	1,97
Maximum	0,0100	1,67	2,04	2,33
Range	0,0100	0,858	0,672	0,357
Mean	0,00138	1,24	1,67	2,12
SD	0,00350	0,327	0,219	0,122
SEM	0,00124	0,116	0,0775	0,0430

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, ELISA screening analysis, day 7, S1

ANOVA summary	
F	55,55
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,8562

Please note that commas are used as decimal separators.

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-0,8714	-1,213 to -0,5301	Yes	****	<0,0001
Buffer vs. 1 µg	-1,135	-1,477 to -0,7940	Yes	****	<0,0001
Buffer vs. 5 µg	-1,745	-2,086 to -1,403	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ELISA screening analysis, day 14, S1

ANOVA summary	
F	131,1
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9335

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-1,179	-1,425 to -0,9326	Yes	****	<0,0001
Buffer vs. 1 µg	-1,449	-1,695 to -1,202	Yes	****	<0,0001
Buffer vs. 5 µg	-1,875	-2,121 to -1,629	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ELISA screening analysis, day 21, S1

ANOVA summary	
F	156,3
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9436

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-1,240	-1,496 to -0,9841	Yes	****	<0,0001
Buffer vs. 1 µg	-1,672	-1,928 to -1,417	Yes	****	<0,0001
Buffer vs. 5 µg	-2,115	-2,370 to -1,859	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Descriptive statistics, ELISA screening analysis, day 7, RBD

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	0,253	0,869	1,51
Maximum	0,00800	1,57	1,84	2,17
Range	0,00800	1,32	0,971	0,661
Mean	0,00288	0,930	1,38	1,85
SD	0,00270	0,473	0,330	0,221
SEM	0,000953	0,167	0,117	0,0783

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ELISA screening analysis, day 14, RBD

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	0,920	1,18	1,84
Maximum	0,0130	1,64	1,96	2,39
Range	0,0130	0,720	0,785	0,550
Mean	0,00388	1,28	1,57	2,07
SD	0,00476	0,293	0,265	0,195
SEM	0,00168	0,104	0,0936	0,0690

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ELISA screening analysis, day 21, RBD

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00100	0,747	1,56	2,04
Maximum	0,00400	1,65	1,98	2,40
Range	0,00300	0,907	0,424	0,362
Mean	0,00250	1,20	1,82	2,21
SD	0,00107	0,392	0,150	0,140
SEM	0,000378	0,138	0,0529	0,0493

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, ELISA screening analysis, day 7, RBD

ANOVA summary	
F	52,18
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,8483

Please note that commas are used as decimal separators.

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-0,9270	-1,311 to -0,5432	Yes	****	<0,0001
Buffer vs. 1 µg	-1,381	-1,765 to -0,9975	Yes	****	<0,0001
Buffer vs. 5 µg	-1,852	-2,236 to -1,468	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ELISA screening analysis, day 14, RBD

ANOVA summary	
F	128,0
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9320

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-1,274	-1,547 to -1,000	Yes	****	<0,0001
Buffer vs. 1 µg	-1,571	-1,844 to -1,297	Yes	****	<0,0001
Buffer vs. 5 µg	-2,065	-2,338 to -1,791	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ELISA screening analysis, day 21, RBD

ANOVA summary	
F	152,6
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9424

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-1,198	-1,472 to -0,9232	Yes	****	<0,0001
Buffer vs. 1 µg	-1,813	-2,087 to -1,539	Yes	****	<0,0001
Buffer vs. 5 µg	-2,211	-2,485 to -1,937	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Descriptive statistics, ELISA, IgG concentrations, day 28, S1

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	35,8	117	312
Maximum	0,00	117	289	536
Range	0,00	81,5	172	224
Mean	0,00	73,0	206	393
SD	0,00	29,3	59,3	81,7
SEM	0,00	10,4	21,0	28,9

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ELISA, IgG concentrations, day 28, RBD

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	36,7	161	345
Maximum	0,00	129	298	583
Range	0,00	92,8	137	238
Mean	0,00	83,1	242	449
SD	0,00	34,9	48,5	80,9
SEM	0,00	12,3	17,2	28,6

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, ELISA, IgG concentrations, day 28, S1

ANOVA summary	
F	86,02
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9021

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-72,97	-138,3 to -7,684	Yes	*	0,0259
Buffer vs. 1 µg	-205,9	-271,2 to -140,6	Yes	****	<0,0001
Buffer vs. 5 µg	-392,7	-458,0 to -327,4	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ELISA, IgG concentrations, day 28, RBD

ANOVA summary	
F	123,4
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9297

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-83,10	-145,5 to -20,66	Yes	**	0,0072
Buffer vs. 1 µg	-241,7	-304,2 to -179,3	Yes	****	<0,0001
Buffer vs. 5 µg	-448,6	-511,0 to -386,1	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Tukey's multiple comparisons post-test, ELISA, reciprocal serum endpoint titer, S1

ANOVA summary	
F	13,76
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,6276

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg (day 14)	-66525	-470716 to 337666	No	ns	0,9987
Buffer vs. 1 µg (day 14)	-84750	-488941 to 319441	No	ns	0,9949
Buffer vs. 5 µg (day 14)	-291300	-695491 to 112891	No	ns	0,3063
Buffer vs. 0.2 µg (day 28)	-163725	-567916 to 240466	No	ns	0,8727
Buffer vs. 1 µg (day 28)	-491775	-895966 to -87584	Yes	**	0,0082
Buffer vs. 5 µg (day 28)	-983850	-1388041 to -579659	Yes	****	<0,0001
0.2 µg (day 14) vs. 1 µg (day 14)	-18225	-422416 to 385966	No	ns	>0,9999
0.2 µg (day 14) vs. 5 µg (day 14)	-224775	-628966 to 179416	No	ns	0,6131
0.2 µg (day 14) vs. 0.2 µg (day 28)	-97200	-501391 to 306991	No	ns	0,9893
0.2 µg (day 14) vs. 1 µg (day 28)	-425250	-829441 to -21059	Yes	*	0,0332
0.2 µg (day 14) vs. 5 µg (day 28)	-917325	-1321516 to -513134	Yes	****	<0,0001
1 µg (day 14) vs. 5 µg (day 14)	-206550	-610741 to 197641	No	ns	0,7009
1 µg (day 14) vs. 0.2 µg (day 28)	-78975	-483166 to 325216	No	ns	0,9965
1 µg (day 14) vs. 1 µg (day 28)	-407025	-811216 to -2834	Yes	*	0,0474
1 µg (day 14) vs. 5 µg (day 28)	-899100	-1303291 to -494909	Yes	****	<0,0001

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
5 µg (day 14) vs. 0.2 µg (day 28)	127575	-276616 to 531766	No	ns	0,9580
5 µg (day 14) vs. 1 µg (day 28)	-200475	-604666 to 203716	No	ns	0,7289
5 µg (day 14) vs. 5 µg (day 28)	-692550	-1096741 to -288359	Yes	****	<0,0001
0.2 µg (day 28) vs. 1 µg (day 28)	-328050	-732241 to 76141	No	ns	0,1837
0.2 µg (day 28) vs. 5 µg (day 28)	-820125	-1224316 to -415934	Yes	****	<0,0001
1 µg (day 28) vs. 5 µg (day 28)	-492075	-896266 to -87884	Yes	**	0,0081

One-way ANOVA with Tukey's multiple comparisons post-test, ELISA, reciprocal serum endpoint titer, RBD

ANOVA summary	
F	17,63
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,6834

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg (day 14)	-66525	-250933 to 117883	No	ns	0,9224
Buffer vs. 1 µg (day 14)	-78675	-263083 to 105733	No	ns	0,8433
Buffer vs. 5 µg (day 14)	-327750	-512158 to -143342	Yes	****	<0,0001
Buffer vs. 0.2 µg (day 28)	-60450	-244858 to 123958	No	ns	0,9498
Buffer vs. 1 µg (day 28)	-218400	-402808 to -33992	Yes	*	0,0109
Buffer vs. 5 µg (day 28)	-491775	-676183 to -307367	Yes	****	<0,0001
0.2 µg (day 14) vs. 1 µg (day 14)	-12150	-196558 to 172258	No	ns	>0,9999
0.2 µg (day 14) vs. 5 µg (day 14)	-261225	-445633 to -76817	Yes	**	0,0012
0.2 µg (day 14) vs. 0.2 µg (day 28)	6075	-178333 to 190483	No	ns	>0,9999
0.2 µg (day 14) vs. 1 µg (day 28)	-151875	-336283 to 32533	No	ns	0,1707
0.2 µg (day 14) vs. 5 µg (day 28)	-425250	-609658 to -240842	Yes	****	<0,0001
1 µg (day 14) vs. 5 µg (day 14)	-249075	-433483 to -64667	Yes	**	0,0024

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
1 µg (day 14) vs. 0.2 µg (day 28)	18225	-166183 to 202633	No	ns	>0,9999
1 µg (day 14) vs. 1 µg (day 28)	-139725	-324133 to 44683	No	ns	0,2513
1 µg (day 14) vs. 5 µg (day 28)	-413100	-597508 to -228692	Yes	****	<0,0001
5 µg (day 14) vs. 0.2 µg (day 28)	267300	82892 to 451708	Yes	***	0,0009
5 µg (day 14) vs. 1 µg (day 28)	109350	-75058 to 293758	No	ns	0,5396
5 µg (day 14) vs. 5 µg (day 28)	-164025	-348433 to 20383	No	ns	0,1112
0.2 µg (day 28) vs. 1 µg (day 28)	-157950	-342358 to 26458	No	ns	0,1385
0.2 µg (day 28) vs. 5 µg (day 28)	-431325	-615733 to -246917	Yes	****	<0,0001
1 µg (day 28) vs. 5 µg (day 28)	-273375	-457783 to -88967	Yes	***	0,0007

Descriptive statistics, IgG subtype-specific ELISA, day 28, IgG1

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	0,498	1,04	1,71
Maximum	0,00300	1,57	1,70	2,68
Range	0,00300	1,07	0,651	0,966
Mean	0,000375	1,00	1,40	2,20
SD	0,00106	0,399	0,229	0,319
SEM	0,000375	0,141	0,0811	0,113

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, IgG subtype-specific ELISA, day 28, IgG2a

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,00	0,206	0,811	2,21
Maximum	0,00100	1,48	2,37	2,68
Range	0,00100	1,27	1,56	0,462
Mean	0,000125	0,640	1,82	2,48
SD	0,000354	0,399	0,515	0,167
SEM	0,000125	0,141	0,182	0,0589

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, IgG subtype-specific ELISA, day 28, IgG1

ANOVA summary	
F	85,19
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9013

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-1,004	-1,351 to -0,6567	Yes	****	<0,0001
Buffer vs. 1 µg	-1,396	-1,743 to -1,049	Yes	****	<0,0001
Buffer vs. 5 µg	-2,197	-2,544 to -1,849	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, IgG subtype-specific ELISA, day 28, IgG2a

ANOVA summary	
F	88,60
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9047

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-0,6398	-1,057 to -0,2221	Yes	**	0,0020
Buffer vs. 1 µg	-1,821	-2,238 to -1,403	Yes	****	<0,0001
Buffer vs. 5 µg	-2,475	-2,893 to -2,057	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Descriptive statistics, ELISA, IgG2a/IgG1 ratio, day 28

	0.2 µg	1 µg	5 µg
Number of values	8	8	8
Minimum	0,272	0,777	0,827
Maximum	1,27	1,81	1,56
Range	0,998	1,03	0,737
Mean	0,635	1,29	1,15
SD	0,307	0,311	0,227
SEM	0,109	0,110	0,0804

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Tukey's multiple comparisons post-test ELISA, IgG2a/IgG1 ratio, day 28

ANOVA summary	
F	11,92
P value	0,0003
P value summary	***
Significant diff. among means (P < 0.05)?	Yes
R square	0,5317

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
0.2 µg vs. 1 µg	-0,6595	-1,018 to -0,3009	Yes	***	0,0004
0.2 µg vs. 5 µg	-0,5186	-0,8771 to -0,1600	Yes	**	0,0041
1 µg vs. 5 µg	0,1409	-0,2177 to 0,4995	No	ns	0,5907

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Pseudovirus-based neutralization test

Descriptive statistics, pVNT, day 14

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	6,00	6,00	12,0	48,0
Maximum	6,00	96,0	192	192
Range	0,00	90,0	180	144
Mean	6,00	21,8	46,5	90,0
SD	0,00	31,1	59,6	47,6
SEM	0,00	11,0	21,1	16,8

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, pVNT, day 21

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	6,00	6,00	48,0	96,0
Maximum	6,00	96,0	384	384
Range	0,00	90,0	336	288
Mean	6,00	30,8	144	228
SD	0,00	31,3	112	102
SEM	0,00	11,1	39,5	36,0

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, pVNT, day 28

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	6,00	12,0	96,0	192
Maximum	6,00	96,0	384	384
Range	0,00	84,0	288	192
Mean	6,00	33,0	192	312
SD	0,00	27,8	88,9	99,4
SEM	0,00	9,82	31,4	35,1

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, pVNT, day 14

ANOVA summary	
F	6,330
P value	0,0021
P value summary	**
Significant diff. among means (P < 0.05)?	Yes
R square	0,4041

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-15,75	-66,89 to 35,39	No	ns	0,7862
Buffer vs. 1 µg	-40,50	-91,64 to 10,64	No	ns	0,1440
Buffer vs. 5 µg	-84,00	-135,1 to -32,86	Yes	***	0,0010

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, pVNT, day 21

ANOVA summary	
F	14,28
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,6047

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-24,75	-120,6 to 71,14	No	ns	0,8582
Buffer vs. 1 µg	-138,0	-233,9 to -42,11	Yes	**	0,0036
Buffer vs. 5 µg	-222,0	-317,9 to -126,1	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, pVNT, day 28

ANOVA summary	
F	35,44
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,7916

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-27,00	-111,5 to 57,54	No	ns	0,7684
Buffer vs. 1 µg	-186,0	-270,5 to -101,5	Yes	****	<0,0001
Buffer vs. 5 µg	-306,0	-390,5 to -221,5	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

ELISpot analysis

Descriptive statistics, day 28, fresh splenocytes, S protein

	Buffer control	0.2 µg	1 µg
Number of values	8	8	8
Minimum	6,00	228	858
Maximum	13,7	626	1334
Range	7,67	399	477
Mean	8,54	334	1054
SD	2,72	134	177
SEM	0,961	47,4	62,6

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, day 28, fresh splenocytes, RBD

	Buffer control	0.2 µg	1 µg
Number of values	8	8	8
Minimum	1,67	20,0	33,0
Maximum	8,00	73,0	126
Range	6,33	53,0	93,3
Mean	4,46	36,3	87,3
SD	1,90	19,6	29,2
SEM	0,672	6,93	10,3

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, day 28, fresh splenocytes, S protein

ANOVA summary	
F	139,2
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9299

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-325,0	-477,0 to -173,0	Yes	***	0,0001
Buffer vs. 1 µg	-1045	-1197 to -893,3	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, day 28, fresh splenocytes, RBD

ANOVA summary	
F	33,83
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,7631

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-31,83	-55,90 to -7,763	Yes	**	0,0094
Buffer vs. 1 µg	-82,79	-106,9 to -58,72	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Descriptive statistics, day 28, frozen splenocytes, S protein

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,667	24,7	157	51,7
Maximum	4,67	137	277	232
Range	4,00	112	121	180
Mean	2,13	76,4	206	139
SD	1,49	35,9	52,3	66,7
SEM	0,527	12,7	18,5	23,6

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, day 28, frozen splenocytes, RBD

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,333	1,33	12,3	4,67
Maximum	4,67	20,0	33,0	42,7
Range	4,33	18,7	20,7	38,0
Mean	2,63	9,38	21,8	17,9
SD	1,20	6,72	7,21	12,0
SEM	0,425	2,38	2,55	4,25

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, day 28, frozen splenocytes, S protein

ANOVA summary	
F	28,56
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,7537

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-74,25	-131,4 to -17,11	Yes	**	0,0087
Buffer vs. 1 µg	-203,6	-260,8 to -146,5	Yes	****	<0,0001
Buffer vs. 5 µg	-136,5	-193,6 to -79,32	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, day 28, frozen splenocytes, RBD

ANOVA summary	
F	9,726
P value	0,0001
P value summary	***
Significant diff. among means (P < 0.05)?	Yes
R square	0,5103

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-6,750	-16,43 to 2,933	No	ns	0,2210
Buffer vs. 1 µg	-19,17	-28,85 to -9,484	Yes	***	0,0001
Buffer vs. 5 µg	-15,25	-24,93 to -5,567	Yes	**	0,0015

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Descriptive statistics, day 28, ELISpot after MACS

	CD4			CD8		
	AH-1	RBD	S	AH-1	RBD	S
Number of values	8	8	8	8	8	8
Minimum	0,00	1,50	66,0	1,00	21,5	173
Maximum	4,50	23,0	155	3,50	62,5	477
Range	4,50	21,5	89,0	2,50	41,0	304
Mean	1,81	13,0	98,8	2,19	43,6	287
SD	1,62	7,75	34,6	0,843	15,6	104
SEM	0,574	2,74	12,2	0,298	5,51	36,9

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, day 28, ELISpot after MACS, $CD4^+$

ANOVA summary	
F	53,66
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,8363

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
AH-1 vs. RBD	-11,19	-35,47 to 13,10	No	ns	0,4568
AH-1 vs. S	-97,00	-121,3 to -72,71	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, day 28, ELISpot after MACS, $CD8^+$

ANOVA summary	
F	51,01
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,8293

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
AH-1 vs. RBD	-41,44	-113,6 to 30,70	No	ns	0,3119
AH-1 vs. S	-284,5	-356,6 to -212,4	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Intracellular cytokine staining

Descriptive statistics, ICS, $CD4^+$, IFN-γ

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,0500	0,110	0,115	0,0730
Maximum	0,104	0,175	0,220	0,240
Range	0,0540	0,0650	0,105	0,167
Mean	0,0858	0,145	0,159	0,119
SD	0,0172	0,0220	0,0317	0,0550
SEM	0,00606	0,00779	0,0112	0,0194

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ICS, $CD4^+$, IL-4

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,0750	0,0695	0,0780	0,0590
Maximum	0,110	0,129	0,120	0,184
Range	0,0350	0,0590	0,0420	0,125
Mean	0,0934	0,107	0,101	0,0968
SD	0,0116	0,0180	0,0163	0,0471
SEM	0,00411	0,00638	0,00577	0,0166

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ICS, $CD4^+$, TNF-α

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,0740	0,0785	0,150	0,130
Maximum	0,170	0,155	0,250	0,265
Range	0,0960	0,0765	0,100	0,135
Mean	0,0992	0,113	0,190	0,193
SD	0,0305	0,0243	0,0317	0,0514
SEM	0,0108	0,00858	0,0112	0,0182

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ICS, $CD4^+$, IL-2

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,0685	0,110	0,130	0,0995
Maximum	0,130	0,235	0,220	0,660
Range	0,0615	0,125	0,0900	0,561
Mean	0,0968	0,158	0,175	0,223
SD	0,0210	0,0365	0,0331	0,195
SEM	0,00741	0,0129	0,0117	0,0689

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, ICS, $CD4^+$, IFN-γ

ANOVA summary	
F	20
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,66

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-0,059	-0,088 to -0,030	Yes	***	0,0002
Buffer vs. 1 µg	-0,073	-0,10 to -0,044	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ICS, CD4+, IL-4

ANOVA summary	
F	1,6
P value	0,2304
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R square	0,13

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. ns: Not significant.

No post-test for insignificant main test.

One-way ANOVA with Dunnett's multiple comparisons post-test, ICS, CD4+, TNF-α

ANOVA summary	
F	23
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,69

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-0,013	-0,048 to 0,021	No	ns	0,5633
Buffer vs. 1 µg	-0,091	-0,13 to -0,056	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ICS, CD4+, IL-2

ANOVA summary	
F	14
P value	0,0001
P value summary	***
Significant diff. among means (P < 0.05)?	Yes
R square	0,57

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-0,061	-0,097 to -0,024	Yes	**	0,0015
Buffer vs. 1 µg	-0,078	-0,11 to -0,042	Yes	***	0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Descriptive statistics, ICS, CD8+, IFN-γ

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,0340	0,190	0,435	0,210
Maximum	0,102	0,700	0,875	0,645
Range	0,0680	0,510	0,440	0,435
Mean	0,0594	0,361	0,704	0,463
SD	0,0223	0,162	0,141	0,134
SEM	0,00789	0,0572	0,0500	0,0472

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ICS, CD8+, TNF-α

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,104	0,270	0,450	0,270
Maximum	0,205	0,750	0,945	0,670
Range	0,102	0,480	0,495	0,400
Mean	0,150	0,410	0,723	0,524
SD	0,0350	0,162	0,154	0,134
SEM	0,0124	0,0571	0,0543	0,0475

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

Descriptive statistics, ICS, CD8+, IL-2

	Buffer control	0.2 µg	1 µg	5 µg
Number of values	8	8	8	8
Minimum	0,0585	0,165	0,170	0,135
Maximum	0,117	0,290	0,400	0,555
Range	0,0580	0,125	0,230	0,420
Mean	0,0840	0,214	0,286	0,268
SD	0,0198	0,0504	0,0794	0,172
SEM	0,00699	0,0178	0,0281	0,0608

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Dunnett's multiple comparisons post-test, ICS, $CD8^+$, IFN-γ

ANOVA summary	
F	54
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,84

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-0,30	-0,45 to -0,15	Yes	***	0,0002
Buffer vs. 1 µg	-0,64	-0,79 to -0,50	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ICS, $CD8^+$, TNF-α

ANOVA summary	
F	39
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,79

Dunnett's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
Buffer vs. 0.2 µg	-0,26	-0,41 to -0,11	Yes	**	0,0013
Buffer vs. 1 µg	-0,57	-0,73 to -0,42	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

One-way ANOVA with Dunnett's multiple comparisons post-test, ICS, $CD8^+$, IL-2

ANOVA summary	
F	27
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,72

Dunnett's multiple comparisons test	**Mean diff.**	**95,00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P value**
Buffer vs. 0.2 µg	-0,13	-0,20 to -0,064	Yes	***	0,0003
Buffer vs. 1 µg	-0,20	-0,27 to -0,14	Yes	****	<0,0001

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

(b) (6)

Von: (b) (6)
Gesendet: Montag, 23. November 2020 14:17
An: (b) (6)
Cc: (b) (6)
Betreff: Final R-20-0085 version 4.0 report, with signatures
Anlagen: R-20-0085 modRNA V9_Report_V4.0_signatures.pdf

Kennzeichnung: Zur Nachverfolgung
Kennzeichnungsstatus: Gekennzeichnet

Hi (b) (6),

Attached here is the final updated PDF for R-20-0085 version 4. With this email, I'm giving my approval as Author. I'll wet-ink sign this document when I'm next in Mainz.

Best,
(b) (6)

(b) (6)
BioNTech SE
(b) (6)