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R&D STUDY REPORT No. R-20-0112

CHARACTERIZING THE IMMUNOPHENOTYPE IN SPLEEN AND LYMPH NODE OF MICE TREATED WITH SARS-COV-2 VACCINE CANDIDATES

Version 01
Date: 13 AUG 2020

Reported by (b) (6)

Test item: BNT162a1, BNT162b1, BNT162b2, BNT162c2
Key words: Covid-19, SARS-CoV-2, Vaccine, BALB/c mice, immunophenotyping

This R&D report consists of 105 pages.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ab	Antibody
CP	Cytoplasmic domain
dLNs	Draining lymph nodes
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FM	Fluorescence minus
FP	Fusion peptide
GC	Germinal center
HR1, HR2	Heptad repeats 1 and 2
i.m.	Intramuscular
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL	Iliac
IN	Inguinal
LD	LiveDead viability dye
LN	Lymph node
LNPs	Lipid nanoparticles
Lot	Lot number
MM	Master mix
modRNA	Nucleoside modified mRNA
NEAA	Non-essential amino acids
PO	Popliteal
RBD	Receptor binding domain
RBM	Receptor binding motif
S	Spike protein
saRNA	Self-amplifying RNA
SP	Signal peptide
T_{FH}	Follicular helper T cells
T_H	T helper cells
TM	Transmembrane domain
TNF	Tumor necrosis factor
uRNA	Non-modified uridine-containing mRNA

RESPONSIBILITIES

Person responsible for the study:	(b) (6)	27 Aug 2020
	(b) (6) BioNTech RNA Pharmaceuticals	Date
Author:	(b) (6)	27 Aug 2020
	(b) (6) BioNTech RNA Pharmaceuticals	Date
Reviewer:	(b) (6)	27 AUG 2020
	BioNTech RNA Pharmaceuticals	Date
QA representative	(b) (6)	27AUG2020
	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.

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 (S) protein of the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the viral antigen.

In the present study, T- and B-cell responses as well as the ability of $CD8^+$ T cells to kill viral antigen-presenting cells induced by four clinical SARS-CoV-2 vaccine candidates were characterized.

The study was divided into two parts, with the first part characterizing the vaccine candidates BNT162a1 and BNT162b1, and the second part characterizing BNT162b2 and BNT162c2. For each part, eight BALB/c mice per group were vaccinated with 5 µg of RNA encapsulated in lipid nanoparticles (LNPs) or buffer control on day 0 by intramuscular injection. T and B cells were analyzed seven days after vaccination in the blood. Serum for optional determination of SARS-CoV-2 specific IgG responses was stored, spleen and the draining lymph nodes (dLNs) were analyzed after 12 days (BNT162a1, BNT162b1 and BNT162b2), or 27 days (BNT162c2). Splenocytes were used for IFNγ ELISpot assay and xCELLigence cytotoxicity assay, and cell suspensions prepared from dLNs and spleen were analyzed by flow cytometry. Cytokines produced by restimulated dLN and spleen cells were analyzed by ProcartaPlex cytokine multiplex assay.

IFNγ ELISpot revealed a strong S protein specific T-cell responses particularly in BNT162b2, BNT162b1 and BNT162c2 and to a lesser extent in BNT162a1 treated groups. In line, $CD8^+$ and $CD4^+$ T cells in dLNs were significantly increased after BNT162b2 treatment, the former already detectable at day 7 in the blood. A trend for increased T cell numbers was detected in the BNT162b1 and BNT162a1 groups. Particular BNT162b1 and BNT162b2 treatment resulted in T cell activation (CD44, CD38, PD1 and ICOS expression of T cells in blood) and antigen specific secretion of cytokines by splenocytes. In those groups, a predominant T_H1 phenotype was detected with increased numbers of T-bet^+ $CD4^+$ T cells, high secretion of T_H1 type cytokines (IFNγ, IL-2, TNF) and low secretion of T_H2 type cytokines (IL-4, IL-5). In all analyzed compartments BNT162b1, BNT162b2 and BNT162c2 treatment mediated the increase and activation of T_{FH} cells, a cell type known for its crucial support of B cell responses. B cell numbers in dLNs were significantly elevated after BNT162b1 and BNT162b2 treatment with higher numbers of antibody producing plasma B cells, class switched and germinal center B cells essential for affinity maturation of antibodies.

Due to the prominent induction of both T and B cell responses, these results particularly support further clinical evaluation of the SARS-CoV-2 vaccine candidates BNT162b1 and BNT162b2.

(b) (6)	27 Aug 2020
BioNTech RNA Pharmaceuticals	Date

2 GENERAL INFORMATION

2.1 Sponsor and Test Facilities

Sponsor

BioNTech RNA Pharmaceuticals GmbH
An der Goldgrube 12
55131 Mainz
Germany

Test Facility

BioNTech SE
An der Goldgrube 12
55131 Mainz
Germany

2.2 Participating Pers

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Experimenter:	(b) (6) BioNTech RNA Pharmaceuticals GmbH

2.3 Study Dates

Start of experiments: 06 MAY 2020

Completion of experiments: 04 JUN 2020

2.4 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 24.04.2020 (approved 30.04.2020).
- SOP-010-015 Pipetten und Dispenser
- 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-010-128 FACSCelesta
- SOP-020-009 Ansetzen von Medien und Zusätzen für die Zellkultur
- SOP-030-038 Standardisierte Kultivierung von Zellen
- SOP-030-041 Auftauen von Zellen
- SOP-030-050 Elektroporation von Zellen
- SOP-030-051 Selektion mit MACS MicroBeads
- SOP-030-054 Extrazelluläre Färbung für Durchflusszytometrie
- 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-090-013 Biological safety in laboratories
- SOP-110-022 Entsorgung von Biostoffabfällen

2.5 Changes and Deviations

This R&D study was conducted according to R&D plan P-20-0112. Table 1 summarizes all changes and deviations to the R&D plan.

Table 1: Changes and deviations to R&D study plan

Experiment	Assay	Plan	Change/Deviation	Reason for change/deviation	Implications
mCorVAC#15	xCELLigence cytotoxicity assay	Duplicates for all samples	Duplicates only for some samples	Not enough cells per mouse	Some samples were assayed in singlicates
mCorVAC#15	Flow cytometry	Functional and phenotypic T cell analysis, dLN: 2×10^6 cells/well	Functional and phenotypic T cell analysis, dLN: 1×10^6 cells/well	Not enough cells per mouse	Lower sample numbers for this assay
mCorVAC#15	Flow cytometry	Phenotypic T cell analysis, SP: 2×10^6/well	Phenotypic T cell analysis, SP: 4×10^6/well	Improve the quality of the results	None.
mCorVAC#15	Flow cytometry	B cell analysis, dLN, SP: 2×10^6/well	B cell analysis, dLN: 2.5×10^5/well. SP: 1×10^6/well	Not enough cells per mouse	Lower sample numbers for this assay
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mCorVAC#15, mCorVAC#16	Flow cytometry	Analysis of SP and dLNs on day 12 (day 27)	Analysis of blood 7 days after immunization	Gain further information on the T and B cell immunophenotype early after immunization	Blood phenotyping data available for 7 days after immunization.
mCorVAC#15, mCorVAC#16	ELISpot	Use MultiScreenHTS plates (Millipore)	Precoated ELISpot plates were used (Mabtech)	bring in line with previous mCorVac experiments	None.
mCorVAC#15, mCorVAC#16	Flow cytometry, cytokine multiplex assay	Restimulation with S peptide mixes at 0.1 µg/mL per peptide	Restimulation with S peptide mixes at 0.2 µg/mL per peptide (mCorVAC#15 and mCorVAC#16) and at 0.5 µg/mL per peptide (flow cytometry, mCorVAC#16)	Increase peptide concentration to increase sensitivity and pick up low responses	Direct quantitative comparison of cytokine profiles derived from multiplex assay of mCorVac#15 (BNT162a1 and BNT162b1) and mCorVac#16 (BNT162b2 and BNT162c2) no longer advisable
mCorVAC#15, mCorVAC#16	Flow cytometry (functional T cell analysis), cytokine	dLN: All samples	dLN: Not all samples included in assay	Not enough cells per mouse	Lower sample numbers for this assay

Experiment	Assay	Plan	Change/Deviation	Reason for change/deviation	Implications
	multiplex analysis				
mCorVAC#15, mCorVAC#16	Flow cytometry	Brefeldin A and GolgiStop in medium were added to a final concentration of 10 µg/mL brefeldin A and a dilution of GolgiStop of 1:500.	GolgiStop and GolgiPlug in medium were added to a final dilution of GolgiStop of 1:1,500 and GolgiPlug of 1:1,000.	GolgiPlug contains brefeldin A and replaced brefeldin A solution. Dilution of GolgiStop was lowered as this lower dilution was found to be sufficient for its purpose.	None.
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mCorVAC#15, mCorVAC#16	Cytokine multiplex assay	dLN: 5×10^5/well	dLN: 4×10^5/well	Not enough cells per mouse	Lower sample numbers for this assay
mCorVAC#16	Flow cytometry	Phenotypic T cell analysis, dLN: 2×10^6 cells/well.	Phenotypic T cell analysis, dLN: 1.5×10^6 cells/well	Not enough cells per mouse.	Lower sample numbers for this assay
mCorVAC#16	Flow cytometry	Phenotypic T cell analysis, SP: 2×10^6/well	Phenotypic T cell analysis, SP: 4×10^6/well	Improve the quality of the results.	None.
mCorVAC#16	Flow cytometry	B cell analysis, dLN, SP: 2×10^6/well	B cell analysis, dLN: 2.5×10^5/well. SP: 1×10^6/well	Not enough cells per mouse.	Lower sample numbers for this assay
mCorVAC#16	xCELLigence cytotoxicity assay	Using CT26 cells electroporated	S RNA electroporated CT26 cells were loaded with S peptide mix after electroporation	Improve the quality of the results	None.

Experiment	Assay	Plan	Change/Deviation	Reason for change/deviation	Implications
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mCorVAC#15, mCorVAC#16	Cytokine multiplex assay	dLN: 5×10^5/well	dLN: 4×10^5/well	Not enough cells per mouse	Lower sample numbers for this assay

2.6 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#15_modRNA_uRNA_V5_dLN_SP

- P:\BioNTechRNA\RN9391R00_CoV-VAC\04_Preclinic\00_Pharmacology\mCorVac#16_saRNAV9_modRNAV9_dLN_SP
- Lab book #1934, page 16-80

3 INTRODUCTION

3.1 Background

In December 2019, an outbreak of pneumonia of unknown cause in Wuhan, Hubei province in China, started. The disease spread rapidly and in January 2020, the agent was identified. By July 27th 2020, infection with the novel Coronavirus SARS-CoV-2 was confirmed in approximately 16,100,000 people with more than 640,000 casualties[1]. A vaccine is urgently needed against the elicited coronavirus disease 19 (COVID-19) and BioNTech decided to initiate a rapid vaccine project based on the surface or spike (S) protein of the virus as the viral antigen. The S protein is a trimer and during viral egress, the precursor protein is cleaved into S1 and S2 (Figure 1). While the S1 domain recognizes the host receptor, the S2 domain is essential for membrane fusion of the viral envelope and the endosomal membrane. To initiate membrane fusion, the S2 domain undergoes a conformational change within the central helix domain.

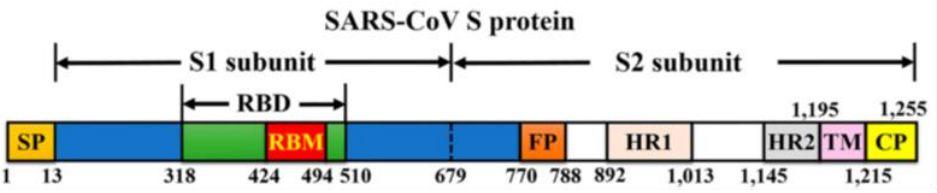

Figure 1: Schematic overview of the S protein structure of the SARS-CoV S protein

The sequence within the S1 subunit consists of the signal peptide (SP) and the receptor binding domain (RBD) with its receptor binding motif (RBM). The S2 subunit contains the fusion peptide (FP) for membrane fusion, heptad repeats (HR1 and HR2), the transmembrane domain (TM) and a cytoplasmic domain (CP). Source: modified from Song et al. 2019.

Based on these features, the S protein is the target of neutralizing antibodies that bind predominantly the receptor-binding domain (RBD) of the S protein.

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 concomitant T-cell responses to achieve protective immunization with minimal vaccine doses (Vogel et al. 2018, Pardi et al. 2017, Moyo et al. 2019).

At BioNTech, there are three different RNA platforms under development, which are non-modified uridine containing mRNA (uRNA), nucleoside modified mRNA (modRNA) and self-amplifying RNA (saRNA). It is unknown today which RNA vaccine

[1] Coronavirus disease (COVID-2019) situation report 189, World Health Organization; https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports

platform performs best in terms of activation and duration of a potent immune response. Initial studies in mice demonstrated the induction of T-cell responses as well as SARS-CoV-2-specific (neutralizing) IgG antibodies with vaccine candidates of all platforms. Four of these candidates are currently tested in clinical trials (Table 2).

The BNT162 vaccine candidate RNA is encapsulated into lipid nanoparticles (LNPs), which protect the RNA from degradation and enable transfection of host cells after intramuscular (i.m.) injection. For all of the BNT162 vaccine candidates, the same LNP formulation is used.

Table 2: Clinical stage SARS-CoV-2 vaccine candidates developed at BioNTech

ID	RNA platform	Antigen
BNT162a1	uRNA	RBD of S1S2 protein (V5)
BNT162b1	modRNA	RBD of S1S2 protein (V5)
BNT162b2	modRNA	S1S2 full-length protein, sequence variant (V9)
BNT162c2	saRNA	S1S2 full-length protein, sequence variant (V9)

This report covers a mouse study characterizing the immunophenotype in the blood, spleen and lymph nodes of mice treated with these four SARS-CoV-2 vaccine candidates.

3.2 Objectives

The objective of this study was to further characterize the four clinical SARS-CoV-2 vaccine candidates to support fast clinical development and approval. In particular, the goal of this study was to:

- Characterize T- and B-cell responses in the spleen, lymph nodes and blood. Analysis included a thorough phenotypic and functional (cytokine secretion on the cellular level) characterization of cells by ELISPot and flow cytometry, and definition of the cytokine profile by multiplex protein quantitation. In particular, the subtype of SARS-CoV-2-specific $CD4^+$ T cells (T_H1, T_H2, T_{FH}) and the abundance of plasma and germinal center (GC) B cells were of interest. Characterize changes in the myeloid cell compartment.
- Determine the ability of $CD8^+$ T cells to kill cells presenting the vaccine-encoded antigen.
- Collect serum of mice to determine (neutralizing) antibody responses (collection was performed, analysis of samples may be performed in the future, if required).

3.3 Study Design

The study was separated into two parts characterizing the vaccine candidates BNT162a1 and BNT162b1 (mCorVac#15, Figure 2) and BNT162b2 and BNT162c2 (mCorVac#16, Figure 3). Each part compared the effects of vaccinated mice to a

control group receiving buffer only. Eight BALB/c mice per group were vaccinated once (day 0) and blood analyzed 7 days later. Serum and tissues were analyzed 12 days later. Since T-cell responses of mice vaccinated with saRNA (BNT162c2) take longer to develop, the analysis time point for serum and tissues was postponed to day 27 after vaccination.

Blood, spleen and draining lymph nodes (dLNs) were harvested from mice. Figure 4 shows an overview of the subsequent analytical methods including sample allocation to the respective assays.

- Serum was obtained from blood and stored frozen for optional determination of SARS-CoV-2 specific IgG responses.
- Splenocytes were tested for recognition of an S protein-specific peptide mix or S RNA-electroporated CT26 cells by secretion of IFNγ (IFNγ ELISpot assay).
- A fraction of splenocytes (N=3 only for the control group, N=8 for treatment groups) was restimulated overnight with an S protein-specific peptide mix and recombinant IL-2, and isolated $CD8^+$ T cells were challenged on the next day for killing of S RNA-electroporated CT26 colon carcinoma cells (xCELLigence cytotoxicity assay).
- Splenocytes and dLN (popliteal, iliac and inguinal, pooled) cells were analyzed for T- ($CD4^+$ T_H1, T_H2, T_{FH}, $CD8^+$ T cells) and B-cell phenotype (GC, plasma, memory B cells), T-cell cytokine secretion after restimulation with an S protein-specific peptide mix, and myeloid cell subsets (flow cytometry).
- dLN and spleen cells were restimulated for 48 h with an S protein-specific peptide mix to analyze T-cell secreted cytokines in the supernatant (ProcartaPlex cytokine multiplex assay).

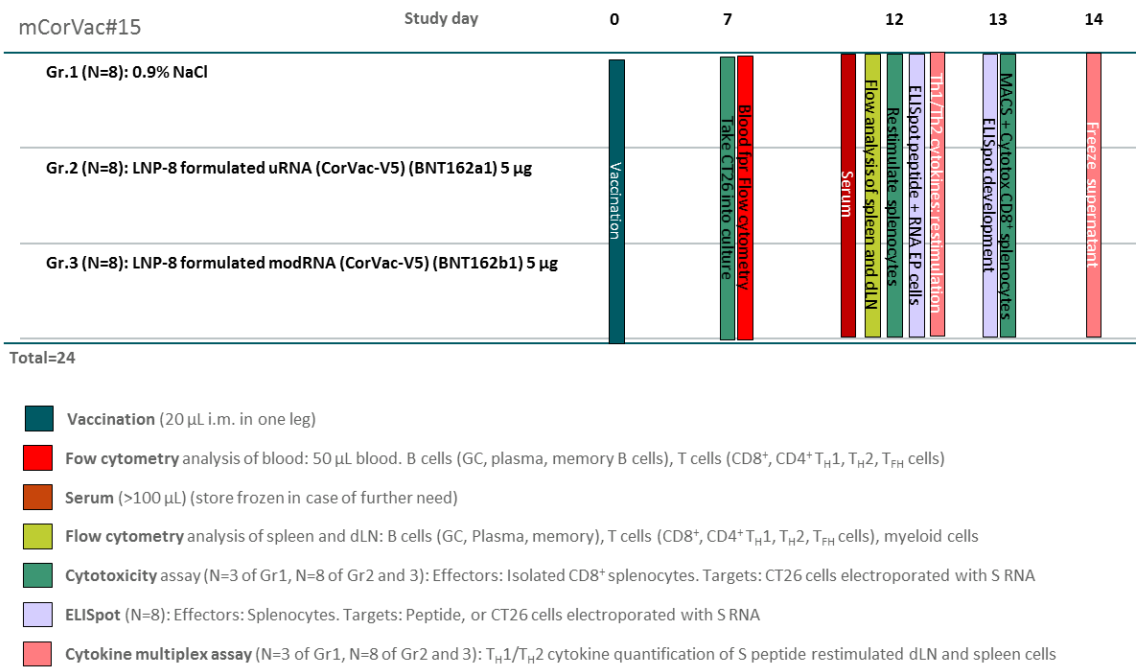

Figure 2: Workflow of part 1 of the study (mCorVac#15)

dLN, draining lymph node. GC, germinal center. Gr, group. N, number of mice per group. T_H, T helper cells. T_{FH}, follicular T helper cells.

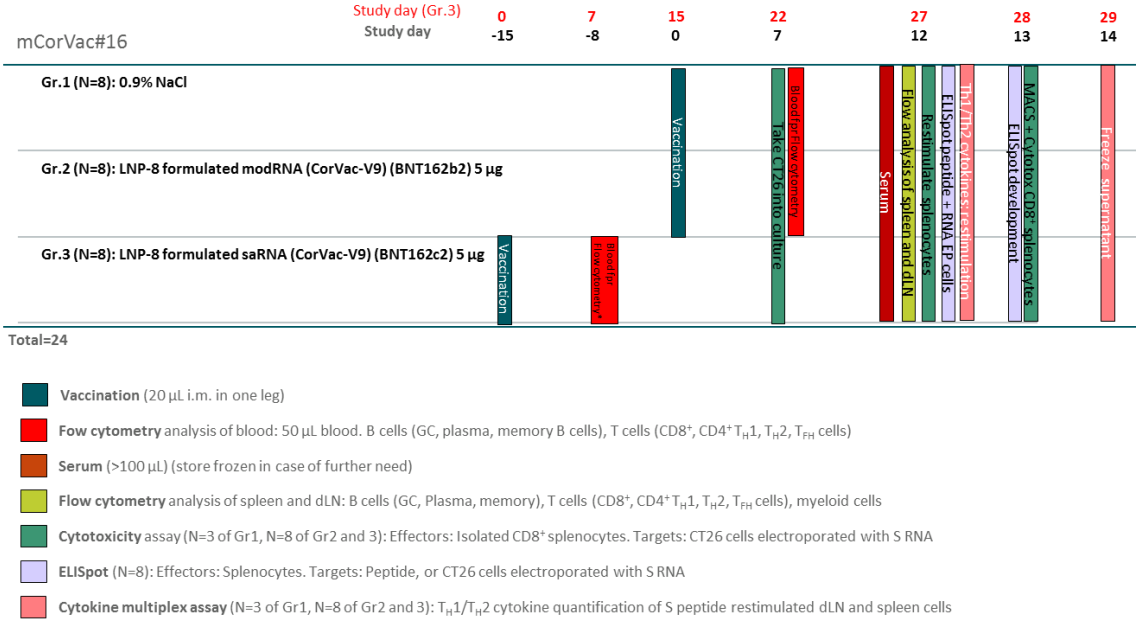

Figure 3: Workflow of part 2 of the study (mCorVac#16)

Study dates for group 3 are depicted in red. dLN, draining lymph node. GC, germinal center. Gr, group. N, number of mice per group. T_H, T helper cells. T_{FH}, follicular T helper cells.

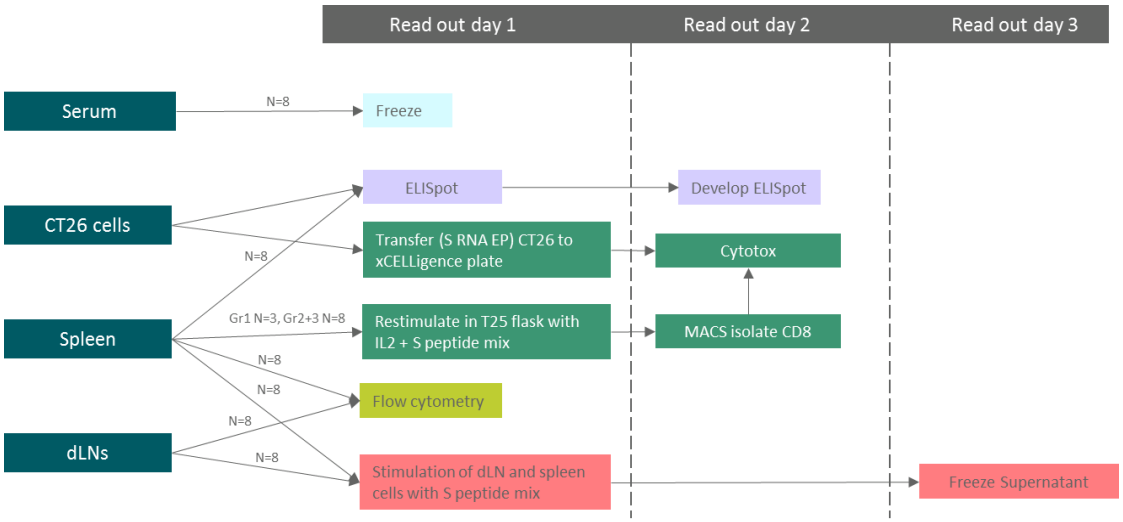

Figure 4: Analysis and assay overview

Schematic depiction of sample allocation to different analysis methods and their timing during analysis days 1 to 3. dLN, draining lymph node. EP, electroporated.

4 MATERIALS AND METHODS

4.1 Test Item

BNT162a1 (ATM): For CoAs see Appendix 9.6

- RNA batch: RNA-SK200305-01
- Polymun batch RBL063.3 LNP with the lot: CoVVAC/090320
- Dilution buffer: 0.9% NaCl
- Concentration: 0.5 mg/mL

BNT162b1 (ATM): For CoAs see Appendix 9.6.

- RNA batch: RNA-RF200304-03
- Polymun batch RBP020.3 LNP with the lot: CoVVAC/100320
- Dilution buffer: 0.9% NaCl
- Concentration: 0.5 mg/mL

BNT162b2 (ATM): For CoAs see Appendix 9.8.

- RNA batch: RNA-RF200321-06
- Polymun batch RBP20.2 LNP with the lot: CoVVAC/270320
- Dilution buffer: 0.9% NaCl
- Concentration: 0.5 mg/mL

BNT162c2 (ATM): For CoAs see Appendix 9.9.

- RNA batch: RNA-RF200310-01
- Polymun batch RBS004.2 LNP with the lot: CoVVAC/170320
- Dilution buffer: 0.9% NaCl
- Concentration: 0.3 mg/mL

Test items are diluted to 0.25 mg/mL with sterile 0.9% NaCl before administration.

4.2 Control Item

- 0.9% NaCl

4.3 Test System

- 48 female BALB/c mice with approximately nine weeks of age at study start

4.4 Materials

For antibodies used in flow cytometry, refer to Section 4.5.11.

Table 3: 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
4mL Sample Cup	Cell counting	NC9756824	N/A	Beckman Coulter GmbH
8-channel manifold	Polypropylene	BR704526-1EA	N/A	Sigma-Aldrich Chemie GmbH
96-well Microplate	Clear round bottom TC-treated microplate, with lid, sterile	3799	N/A	Corning Holding GmbH
ACK lysis buffer	Flow cytometry (blood)	A10492-01	1x	Gibco
Ammonium chloride	NH_4Cl	A0988,5000	N/A	AppliChem GmbH
Brilliant Stain Buffer	Flow cytometry	563794	N/A	BD Bioscience
Brilliant Stain Buffer Plus	Flow cytometry	566385	N/A	BD Biosciences
Capillary pipettes	minicaps®, blood sampling, 4 µL/ 10 µL, not heparinized	9000104/ 9000110	N/A	Hirschmann Laborgeräte GmbH & Co.KG
Cell culture flask 250 ML, 75 cm^2	Cell culture	658175	N/A	Greiner Bio-One GmbH
CD8a (Ly-2) MicroBeads	CD8 T cell purification	130-117-044	N/A	Miltenyi Biotec
Collagenase D	Lymphnode preparation	11088866001	1 mg/ml	Merck KGaA
Combitips advanced®	Biopur®, 50 mL	0030089693	N/A	Eppendorf Vertrieb Deutschland GmbH
Concanavalin A	from Canavalia ensiformis (Jack bean, 5mg),Type IV-S, lyophilized	C0412-5MG	2 µg/mL	Sigma-Aldrich Chemie GmbH
Dimethyl sulfoxide	Cell culture	A3672,0100	N/A	AppliChem GmbH

Product name	Application/specification	Article no.	Working dilution	Provider
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
Electroporation cuvette	Electroporation	732-1137	N/A	VWR International GmbH
E-Plate VIEW 96 PET	xCelligence	300600910	N/A	ACEA Biosciences
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
Ethylenediaminetetraacetic acid solution	EDTA	03690-100ML	N/A	Sigma-Aldrich Chemie GmbH
Fetal Bovine Serum	Non-USA origin, sterile-filtered	F7524	N/A	Sigma-Aldrich Chemie GmbH
Filtration unit for medium flasks	High Performance, PES, 0.45 µm, 1000 mL	514-0301	N/A	VWR International GmbH
FoxP3/Transcription Factor Staining Buffer Set	Flow cytometry	00-5523-00	N/A	Thermo Fisher Scientific
GolgiStop	Flow cytometry (Restimulation)	554724	1:1,500	BD Biosciences
GolgiPlug	Flow cytometry (Restimulation)	555029	1:1,000	BD Biosciences
Heparin Tubes	Flow cytometry (Blood)	20.1309	N/A	Sarstedt AG & Co.
HEPES	1 M	15630-056	N/A	Gibco
Insulin syringes	BD Micro-Fine™+, 30 G, 0.3 mL	324826	N/A	Becton Dickinson GmbH
Ionomycin, 10 µg/µL	Flow cytometry (Restimulation)	I9657	1 µg/mL	Sigma
Isoflurane	Anesthesia	9714675	N/A	Piramal Critical Care
Isotonic saline	Injection solution	06173569	N/A	Fresenius Kabi Deutschland GmbH
LS columns	CD8 T cell purification	130-042-401	N/A	Miltenyi Biotec

Product name	Application/specification	Article no.	Working dilution	Provider
MEM Non-Essential Amino Acids Solution (100X)	Cell culture	11140-035	1X	Gibco
Mouse IFN-γ ELISpot^{PLUS} kit	Kit for enumeration of cells secreting mouse IFN-γ	3321-4APT-2	N/A	Mabtech
PBS dry substance	No calcium, no magnesium	L182-10	N/A	Merck KGaA
Penicillin-Streptomycin	10,000 U/mL	15140-122	N/A	Gibco
PepMix™ against RBD	ELISpot	N/A (customized)	0.0625mg per peptide/ vial	JPT
Pipette tips	ep Dualfilter T.I.P.S.®, PCR clean und sterile, 0.1–10 µL/2–100 µL/50–1000 µL/50–1250 µL/0.1–5 mL	0030077512/ 0030077547/ 0030077555/ 0030077792/ 0030077750/ 0030078616	N/A	Eppendorf Vertrieb Deutschland GmbH
PMA, 1 µg/µL	Flow cytometry (Restimulation)	P1585	0.5 µg/mL	Sigma
Potassium bicarbonate	$KHCO_3$	A2375,1000	N/A	AppliChem GmbH
ProcartaPlex mouse T_H1/T_H2 cytokine 11-plex kit	Cytokine multiplex assay Lot. No. 232634-004	EPX110-20820-901	N/A	Thermo Fisher Scientific
Proleukin S	Cell culture	N/A	100 U/ml	Clinigen
Reservoir	25 mL, 100 mL	613-1174/613-1171	N/A	VWR International GmbH
RotiHistofix	Flow cytometry	P087.1	2%	Roth
Round bottom 5-mL tubes	Flow cytometry (blood)	10579511	N/A	Thermo Fisher Scientific
RPMI 1640 Medium	GlutaMAX™ Supplement	61870-010	N/A	Gibco
Serological pipettes	5 mL, 10 mL, 25 mL, 50 mL	606180/607180/601180/768180	N/A	Greiner Bio-One GmbH
Serum Tubes	Serum preparation	20.1344	N/A	Sarstedt AG & Co.
Single-use syringe	Injekt® Solo 5 mL	4606051V	N/A	B. Braun Melsungen AG

Product name	Application/specification	Article no.	Working dilution	Provider
Sodium acide (10%)	Flow cytometry (blood)	13553.00100	0.01%	Morphisto
Sodium Pyruvate	100 mM	11360-039	N/A	Gibco
StemPro™ Accutase™ Cell dissociation reagent	Cell culture	A1110501	N/A	Gibco
Sterile filters	0.45 µm	514-4123	N/A	VWR International
Vi-CELL™ XR Quad Pak	For Vi-CELL™ XR Cell Viability Analyzer	383722	N/A	Beckman Coulter GmbH
X-VIVO 15, serum-free	Electroporation	BE02-060Q	N/A	Lonza Group Ltd

Table 4: Peptide pool for restimulation of splenocytes and dLN cells for ELISpot assays, flow cytometry and cytokine multiplex assay

S protein-specific peptides	
Name	**Sequence**
2019-nCoV S.wt With a total of 315 overlapping peptides (15mers overlapping by 11 amino acids) 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

Table 5: RNAs used for CT26 electroporation

Name	Sequence (open reading frame)
S RNA (Full construct name: JR206_mRNA_pST4_m1ψ_wtsec_SARS-CoV-2-S (Sino CDS) CΔ19)	atgtttgtgttcctggtgctgctgccactggtgtccagccagtgtgtgaacctgaccaccaggacccaacttcctcctgcctaca ccaactccttcaccaggggagtctactaccctgacaaggtgttcaggtcctctgtgctgcacagcacccaggacctgttcctg ccattcttcagcaatgtgacctggttccatgccatccatgtgtctggcaccaatggcaccaagaggtttgacaaccctgtgctgc cattcaatgatggagtctactttgccagcacagagaagagcaacatcatcaggggctggatttttggcaccaccctggacag caagacccagtccctgctgattgtgaacaatgccaccaatgtggtgattaaggtgtgtgagttccagttctgtaatgacccattc ctgggagtctactaccacaagaacaacaagtcctggatggagtctgagttcagggtctactcctctgccaacaactgtaccttt gaatatgtgagccaaccattcctgatggacttggagggcaagcagggcaacttcaagaacctgagggagtttgtgttcaag aacattgatggctacttcaagatttacagcaaacacacaccaatcaacctggtgagggacctgccacagggcttctctgcctt ggaaccactggtggacctgccaattggcatcaacatcaccaggttccagaccctgctggctctgcacaggtcctacctgaca cctggagactcctcctctggctggacagcaggagcagcagcctactatgtgggctacctccaaccaaggaccttcctgctga aatacaatgagaatggcaccatcacagatgctgtggactgtgccctggacccactgtctgagaccaagtgtaccctgaaatc cttcacagtggagaagggcatctaccagaccagcaacttcagggtccaaccaacagagagcattgtgaggtttccaaacat caccaacctgtgtccatttggagaggtgttcaatgccaccaggtttgcctctgtctatgcctggaacaggaagaggattagca actgtgtggctgactactctgtgctctacaactctgcctccttcagcaccttcaagtgttatggagtgagcccaaccaaactgaa tgacctgtgtttcaccaatgtctatgctgactcctttgtgattaggggagatgaggtgagacagattgcccctggacaaacagg caagattgctgactacaactacaaactgcctgatgacttcacaggctgtgtgattgcctggaacagcaacaacctggacagc aaggtgggaggcaactacaactacctctacagactgttcaggaagagcaacctgaaaccatttgagagggacatcagca cagagatttaccaggctggcagcacaccatgtaatggagtggagggcttcaactgttactttccacctccaatcctatggcttcc aaccaaccaatggagtgggctaccaaccatacagggtggtggtgctgtcctttgaactgctccatgcccctgccacagtgtgt ggaccaaagaagagcaccaacctggtgaagaacaagtgtgtgaacttcaacttcaatggactgacaggcacaggagtgc tgacagagagcaacaagaagttcctgccattccaacagtttggcagggacattgctgacaccacagatgctgtgagggac ccacagaccttggagattctggacatcacaccatgttcctttggaggagtgtctgtgattacacctggcaccaacaccagcaa ccaggtggctgtgctctaccaggatgtgaactgtactgaggtgcctgtggctatccatgctgaccaacttacaccaacctgga gggtctacagcacaggcagcaatgtgttccagaccagggctggctgtctgattggagcagagcatgtgaacaactcctatg agtgtgacatcccaattggagcaggcatctgtgcctcctaccagacccagaccaacagcccaaggagggcaaggtctgtg gcaagccagagcatcattgcctacacaatgagtctgggagcagagaactctgtggcttacagcaacaacagcattgccatc ccaaccaacttcaccatctctgtgaccacagagattctgcctgtgagtatgaccaagacctctgtggactgtacaatgtatatct gtggagacagcacagagtgtagcaacctgctgctccaatatggctccttctgtacccaacttaacagggctctgacaggcatt gctgtggaacaggacaagaacacccaggaggtgtttgcccaggtgaagcagatttacaagacacctccaatcaaggactt tggaggcttcaacttcagccagattctgcctgacccaagcaagccaagcaagaggtccttcattgaggacctgctgttcaac aaggtgaccctggctgatgctggcttcatcaagcaatatggagactgtctgggagacattgctgccagggacctgatttgtgc ccagaagttcaatggactgacagtgctgcctccactgctgacagatgagatgattgcccaatacacctctgccctgctggctg gcaccatcacctctggctggacctttggagcaggagcagccctccaaatcccatttgctatgcagatggcttacaggttcaat ggcattggagtgacccagaatgtgctctatgagaaccagaaactgattgccaaccagttcaactctgccattggcaagattc aggactccctgtccagcacagcctctgccctgggcaaactccaagatgtggtgaaccagaatgcccaggctctgaacacc ctggtgaagcaactttccagcaactttggagccatctcctctgtgctgaatgacatcctgagcagactggacaaggtggaggc tgaggtccagattgacagactgattacaggcagactccaatccctccaaacctatgtgacccaacaacttatcagggctgct gagattagggcatctgccaacctggctgccaccaagatgagtgagtgtgtgctgggacaaagcaagagggtggacttctgt ggcaagggctaccacctgatgagttttccacagtctgcccctcatggagtggtgttcctgcatgtgacctatgtgcctgcccag gagaagaacttcaccacagcccctgccatctgccatgatggcaaggctcactttccaagggagggagtgtttgtgagcaatg gcacccactggtttgtgacccagaggaacttctatgaaccacagattatcaccacagacaacaccttttgtgtctggcaactgt gatgtggtgattggcattgtgaacaacacagtctatgacccactccaacctgaactggactccttcaaggaggaactggaca aatacttcaagaaccacaccagccctgatgtggacctgggagacatctctggcatcaatgcctctgtggtgaacatccagaa ggagattgacagactgaatgaggtggctaagaacctgaatgagtccctgattgacctccaagaactgggcaaatatgaac aatacatcaagtggccatggtacatctggctgggcttcattgctggactgattgccattgtgatggtgaccataatgctgtgttgta tgacctcctgttgttcctgtctgaaaggctgttgttcctgtggctcctgttgttgatga
Irrelevant RNA (Batch: RNA-KG200106-06c)	atgggcgccatggcccctagaacattgctcctgctgctggccgctgccctggcccctacacagacaagagctggacctggc ggctctggaggaggcggctccggaggcggaggatccggtggtggcggcagcggcggcgtgatcgtggctgtgctgggcg tgctgggagccatggccatcattggagccgtggtggccttcgtgatgaagcggagaagaaacaccggcggcaagggcgg cgattacgctctggctcctggcagccagtccagcgagatgagcctgagagactgcaaggcctagtaa

Table 6: Software

Product name	Application	Provider
BD FACSDiva software version 9.1 and 8.0.1.1	Flow cytometry	BD Biosciences
Excel	Raw data	Microsoft Corp.
FlowJo software version 10.6	Flow cytometry	FlowJo LLC, BD Biosciences
GraphPad Prism software version 8	Statistical analysis	GraphPad Software Inc.
ImmunoCapture 7.0.7.0	ELISpot assay	Cellular Technology Ltd
ImmunoSpot® analysis software version 57.0.17.0	ELISpot assay	Cellular Technology Ltd
ProcartaPley Analyst software version 1	Cytokine multiplex assay	Thermo Fisher Scientific
RTCA Data analysis software	xCELLigence cytotoxicity assay	ACEA Biosciences
xCELLigence RTCA Software Pro	xCELLigence cytotoxicity assay	ACEA Biosciences

Table 7: Machines

Product name	Application	Provider
BD Symphony A3	Flow cytometry	BD Biosciences
BD Celesta	Flow cytometry	BD Biosciences
Bioplex200 system	Cytokine multiplex assay	Bio-Rad
Centrifuges	Centrifugation	Eppendorf
CTL ELISPOT reader ImmunoSpot® S6 Core Analyzer	ELISpot assay	Cellular Technology Limited
Electroporation system	Electroporation	BTX
Vi-CELL XR	Cell counting	Beckman Coulter GmbH
xCELLigence RTCA MP	xCELLigence cytotoxicity assay	ACEA Biosciences

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 welfare committee), conducted according to the 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 labelled with a cage card indicating the mouse strain, gender, 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 Condition and Husbandry

Mice were housed at BioNTech SE's animal facility under barrier and SPF conditions (An der Goldgrube 12, 55131 Mainz) 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 was kept at 20-24°C and 45-55%, respectively, and the air change (AC) rate in the cages at 75 AC/h. The cages with 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 were changed weekly. Autoclaved ssniff M-Z food (sniff Spezialdiäten GmbH, Soest, Germany; product code: V1124) and autoclaved water (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 animals and control of food and water supplies. Each animal's health was closely assessed at least once weekly and the results documented in health monitoring sheets. The general physical condition was assessed with regard 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 indicated by 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

4.5.3 Animal Treatment

4.5.3.1 Treatment Schedule, Route of Administration, and Dose

The test compounds were administered i.m. once at a dose of 5 µg (see Figure 2 and Figure 3). The control group was treated with buffer only.

4.5.3.2 Immunization

For immunization, prior anesthesia by inhalation of 2.5% isoflurane in oxygen, the injection site (hind leg) was shaved. 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 animals were observed for any immediate signs of discomfort following 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 held tightly. A thin glass capillary (29 G) was inserted gently through the retro-orbital sinus membrane and blood was collected into either Serum tubes (serum preparation) or Heparin tubes (flow cytometry analysis). After careful removal of the glass capillary, the restraining grip was loosened. Alternatively, blood collection was performed via the *vena facialis* according to SOP-030-074. In short, without prior anesthesia, mice were held tightly and using a lancet, the *vena facialis* was punctured in a precise and short movement. Blood was collected into either Serum tubes (serum preparation) or Heparin tubes (flow cytometry analysis), and the restraining grip was loosened. Blood samples were centrifuged at 10,000 x g and ambient temperature for 5 min and serum transferred to a pre-labeled 0.5 mL reagent tube, to be stored 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 GV-SOLAS by cervical dislocation or by exposure to carbon dioxide. Additionally, termination criteria 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 and dLNs (popliteal (PO), iliac (IL) and inguinal (IN), see Figure 5) were collected, pooled and stored in PBS on ice for subsequent single cell preparations.

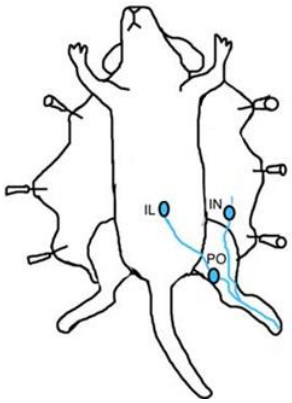

Figure 5: Draining lymph nodes resection for subsequent analysis

Depicted are the predicted draining lymph nodes after i.m. injection into the gastrocnemius muscle used for further analysis. Figure adopted according to **Harrell et al. 2008**. IL, iliac. IN, inguinal. PO, popliteal.

4.5.5 Preparation of Splenocyte Single Cell Suspensions

Single cell suspensions from collected spleens were prepared according to SOP-030-078. To this end, spleens were squeezed through 70 µm cell strainers using the plunger of a syringe to release the splenocytes into a 50 mL tube. Splenocytes were washed with an excess volume of PBS followed by centrifugation at 300 x g for 6 min at ambient temperature and discarding the supernatants. Erythrocytes were lysed with erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 5 min at ambient temperature. The reaction was stopped with an excess volume of PBS. After another washing step, cells were resuspended in DC medium (RPMI medium1640 (1x) + GlutaMAX-I [Life Technologies], 10% FBS, 1% NEAA, 1% sodium-pyruvate, 0.5% penicillin/streptomycin, 50 µM 2-mercaptoethanol), passed through a 70 µm cell strainer again, counted according to SOP-010-028, and stored at 4°C until further use.

4.5.6 Preparation of Lymph Node Single Cell Suspensions

The popliteal, iliac and inguinal dLNs (Figure 5) were stored together in a plastic tube containing 450 µL PBS at ambient temperature in the dark until single cell preparation. 50 µL collagenase D (10 mg/mL) were added to yield a final concentration of 1 mg/mL, the dLNs were thoroughly cut into pieces using forceps or scissors, and incubated for 10 min at 37°C. Cells were passed through a 70 µm cell strainer placed on a 50 mL plastic tube and minced using the plunger of a 5 mL syringe. The cell strainer is subsequently rinsed using 5 mL of PBS and the cell solution counted according to SOP-010-028.

4.5.7 RNA Electroporation

CT26 colon carcinoma cells (ATCC) were washed once with 10 mL of serum-free X-Vivo 15 medium, centrifuged (300 ×g, 6 min, ambient temperature), taken up in 1–2 mL of X-Vivo 15 medium, counted (SOP-010-028), and diluted to a concentration of 25 × 10^6 cells/mL. S Protein encoding modRNA or irrelevant modRNA (10 µg in 40 µL of X-Vivo 15 medium each) was carefully placed at the bottom of a 4 mm electroporation cuvette, topped up with 200 µL of cells (corresponding to 5 × 10^6 cells) and shortly mixed by pipetting up and down. Electroporation was then performed with a BTX™ ECM™ 830 Square Wave Electroporator applying one 300 V pulse for 15 ms. Immediately after electroporation, cells were transferred to a 15 mL tube containing 1–2 mL of DC medium, counted, and diluted to 4 × 10^5 cells/mL for the cytotoxicity assay, and 5 × 10^5 cells/mL for the IFNγ ELISpot assay (Section 4.5.8).

4.5.8 ELISpot Assay

IFNγ ELISpot assay was 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 serum-containing medium (DC medium) for at least 30 min at 37°C. After blocking, 100 µl of the splenocyte solution (5 x 10^5 cells) as well as 100 µl electroporated CT26 cells (5 x 10^4 cells) or 100 µl S peptide mix (final concentration per well: 0.1 µg/ml) were added yielding a final volume per well of 200 µL. No peptide or irrelevant RNA transfected cells were used as controls. Plates were incubated overnight in a 37°C humidified incubator with 5% CO_2. After approximately 18 h cells were discarded and a second biotinylated anti-mouse IFN-γ antibody incubated for 2 h at ambient temperature. The plate was then developed by addition of Streptavidin-ALP for 1 h at ambient temperature in the dark followed by addition of BCIP®/NBT substrate for 5–7 min at ambient temperature in the dark. Spots were counted on a CTL ELISPOT reader ImmunoSpot® S6 Core Analyzer according to SOP-010-099.

4.5.9 xCELLigence Cytotoxicity Assay

Preparation of targets:

Of DC medium, 50 µL per well were added to a 96-well PET E-plate to perform a blank measurement at an xCELLigence RTCA MP, Real Time Cell Analyzer. Tumor cells (50 µL of a 4 × 10^5 cells/mL suspension, corresponding to 2 × 10^4 cells) electroporated with S RNA or irrelevant RNA (10 µg each) were subsequently added to the E-plate. After allowing the cell suspension to settle down for 30 min at ambient temperature, the E-plate was transferred to the xCELLigence device and measurement was continued.

Peptide loading of targets:

In mCorVac#16, 100 µl S peptide mix (final concentration per well: 0.1 µg/ml) was added to S RNA electroporated tumor cells one hour prior T cell addition. After one hour of incubation, the medium was carefully aspirated and the wells were washed with PBS twice. Before adding the effector cells, 100 µl of DC medium was dispensed per well.

Addition of effectors:

On the same day, splenocytes were transferred to a T25 cell culture flask at a density of 1.5–2 × 10^6 cells/cm^2. S peptide mixes and recombinant IL-2 (Proleukin) were added to yield a final concentration of 0.1 µg/mL and 100 U/mL, respectively, and the cell suspension was kept at 37°C, 5% CO_2 overnight. On the day after, restimulated splenocytes were transferred to a 15 mL plastic tube, the T25 flask was rinsed with 5 mL of MACS buffer and added to the same tube. Subsequently, $CD8^+$ cells were isolated from restimulated splenocytes using CD8a (Ly-2) MACS® MicroBeads according to the manufacturer's instructions. Labeled cells were eluted from MACS LS columns, centrifuged (5 min at 460 ×g), taken up in 1–2 mL of warm (approximately 37°C) DC medium, counted (SOP-010-028) and diluted with DC medium to a concentration of 6 × 10^6 cells/mL. $CD8^+$ cells (100 µL), DC medium or Staurosporin (4 µM final concentration) were added in duplicate to the targets in the E-plate and the xCELLigence measurement was continued for at least three days. RTCA Data analysis software or xCELLigence RTCA Software Pro (both ACEA Biosciences) were used for data analysis.

4.5.10 Cytokine Multiplex Protein Quantification

Cytokine concentrations were determined in supernatants derived from *ex vivo* restimulated splenocytes and dLN cells. 5 × 10^5 splenocytes or dLN cells in 100 µL medium/well were transferred to a 96-well U-bottom plate, and 100 µL medium supplemented with S peptide mixes to a final concentration of 0.2 µg/mL/peptide/well, or cell culture medium only (negative control) were added and mixed. For each group, three samples were treated with 100 µL PMA and ionomycin to a final concentration of

0.5 µg/mL and 1 µg/mL/well, respectively (positive controls). Cells were incubated for 48 h at 37°C, 5% CO_2. Supernatants were harvested and stored at -20°C for the cytokine multiplex assay.

Cytokine concentrations in supernatants of restimulated splenocytes and dLN cells were determined from thawed cell culture supernatants using a bead-based, 11-plex Th1/Th2 mouse ProcartaPlex immunoassay according to the manufacturer's instructions. Analytes included in the assay were IFNγ, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-6, TNF, GM-CSF, and IL-18.

Fluorescence was measured with the Bioplex200 system and analyzed with ProcartaPlex Analyst 1.0 software (Thermo Fisher Scientific).

4.5.11 Flow Cytometry

All flow cytometric data were acquired on a BD Symphony A3 or BD Celesta (B cell analysis) flow cytometer using BD FACSDiva software version 9.1 or 8.0.1.1, respectively, and analyzed with FlowJo 10.6 (FlowJo LLC, BD Biosciences).

4.5.11.1 Restimulation of T cells for functional T cell analysis in the spleen and dLN

For functional analysis, splenocytes and dLN cells were *ex vivo* restimulated. 4×10^6 splenocytes and 1×10^6 (mCorVAC#15) or 2×10^6 (mCorVAC#16) dLN cells in 100 µL DC medium/well were transferred to a 96-well U-bottom plate. To each well, 50 µL medium were added, supplemented with either S peptide mixes to a final concentration of 0.2 µg/mL/peptide/well (mCorVAC#15) or 0.5 µg/mL/peptide/well (mCorVAC#16), or medium only (negative controls), and mixed. To one sample per group, 50 µL PMA and ionomycin to a final concentration of 0.5 µg/mL and 1 µg/mL/well, respectively, were added (positive controls). Three additional wells of any group were added as unstained controls.

Cells were quickly spun down (30 s, 460 × g) and incubated for 1 h at 37°C, 5% CO_2. To each well, 50 µL GolgiStop and GolgiPlug in medium were added to a final dilution of GolgiStop of 1:1,500 and GolgiPlug of 1:1,000, mixed, and cells were further incubated for 4 h at 37°C, 5% CO_2.

4.5.11.2 Functional T cell analysis in the spleen and dLN

For mouse functional T cell analysis, restimulated cells (see 4.5.11.1) were centrifuged (5 min, 300 × g) and supernatants discarded. Flow cytometry master mixes (MM) for functional T cell analysis are depicted in Table 8 and Table 9.

Cells were stained with fixable viability dye and extracellularly with antibodies against CD3, CD4, CD8α, CD44, PD-1, CD40L, CD62L and CXCR5 (mCorVAC#15, MM1a), or CD4, CD8α, CD44, CD45, PD-1, CD40L, CD62L and CXCR5 mCorVAC#16, MM1b)