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CLINICAL TRIAL REPORT

A phase I/IIa study of the mRNA-based cancer immunotherapy CV9201 in patients with stage IIIB/IV non-small cell lung cancer

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Abstract

CV9201 is an RNActive®-based cancer immunotherapy encoding five non-small cell lung cancer-antigens: New York esophageal squamous cell carcinoma-1, melanoma antigen family C1/C2, survivin, and trophoblast glycoprotein. In a phase I/IIa dose-escalation trial, 46 patients with locally advanced ($n=7$) or metastatic ($n=39$) NSCLC and at least stable disease after first-line treatment received five intradermal CV9201 injections (400–1600 µg of mRNA). The primary objective of the trial was to assess safety. Secondary objectives included assessment of antibody and ex vivo T cell responses against the five antigens, and changes in immune cell populations. All CV9201 dose levels were well-tolerated and the recommended dose for phase IIa was 1600 µg. Most AEs were mild-to-moderate injection site reactions and flu-like symptoms. Three (7%) patients had grade 3 related AEs. No related grade 4/5 or related serious AEs occurred. In phase IIa, antigen-specific immune responses against ≥ 1 antigen were detected in 63% of evaluable patients after treatment. The frequency of activated $IgD^{+}CD38^{hi}$ B cells increased > twofold in 18/30 (60%) evaluable patients. 9/29 (31%) evaluable patients in phase IIa had stable disease and 20/29 (69%) had progressive disease. Median progression-free and overall survival were 5.0 months (95% CI 1.8–6.3) and 10.8 months (8.1–16.7) from first administration, respectively. Two- and 3-year survival rates were 26.7% and 20.7%, respectively. CV9201 was well-tolerated and immune responses could be detected after treatment supporting further clinical investigation.

Keywords Active cancer immunotherapy · mRNA · Non-small cell lung cancer · Immunomonitoring · Clinical trial · CV9201

Abbreviations

ANA	Antinuclear antibody
DLT	Dose-limiting toxicity
LNP	Lipid nanoparticles
MAGE-C1/2	Melanoma antigen family C1/C2
NCI-CTCAE	National Cancer Institute Common Terminology Criteria for Adverse Events
NY-ESO-1	New York esophageal squamous cell carcinoma-1
RP2D	Recommended phase II dose
TSH	Thyroid stimulating hormone
5T4	Trophoblast glycoprotein

Introduction

Cancer immunotherapy has dramatically expanded the therapeutic options available to patients with NSCLC. In recent years, immune checkpoint inhibitors such as anti-PD-1 mAbs nivolumab and pembrolizumab have demonstrated unprecedented clinical efficacy and durable responses in

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certain NSCLC patients [1–4]. Despite these advances, the number of unselected NSCLC patients responding to PD-1 blockade alone irrespective of PD-L1 expression remains low (~20% for nivolumab [1–3]) and the addition of chemotherapy or ipilimumab adds toxicity [4].

Apart from checkpoint blockade strategies which are broadly applied for different indications, antigen-specific vaccination approaches have been also considered and tested as tumor-specific therapeutic options. One of these approaches is based on the administration of antigen-specific mRNA which aims at generation of long lasting, protective humoral and cellular immune responses against multiple tumor-associated antigens without adding substantial toxicity. Throughout the years, several different mRNA formulations have been used in preclinical and clinical studies. In the first phase I/II trial where mRNA was directly injected into melanoma patients, autologous amplified total tumor RNA adjuvanted with GM-CSF was administered intradermally [5]. This approach quickly evolved to using defined and selected tumor antigen mRNA rather than complete tumor cRNA [6, 7]. The mRNA technology was further improved by mixing protamine-complexed mRNA with uncomplexed mRNA molecules to enable immune activation via TLR 7/8 and secure high antigen expression. Administration in preclinical models resulted in antigen-specific $CD4^+$ and $CD8^+$ T cell as well as B cell responses, which were associated with tumor regression and long-term anti-tumor immunity [8–11]. A protamine-complexed mRNA immunotherapy encoding melanoma-associated antigens was well tolerated in patients with metastatic melanoma and increased frequencies of antigen-specific T cells but not Tregs post-treatment [12].

Antigen-specific immune responses against all four encoded antigens were described after administration of the mRNA-based immunotherapy CV9103 in a phase I/IIa trial in patients with castration-resistant prostate cancer, with a favorable safety profile [13]. Similar to CV9103, the RNActive® (CureVac AG, Germany) antigen-specific immunotherapy (CV9201) employed in the phase I/IIa NSCLC study reported here comprises free and protamine-complexed full-length mRNAs engineered with chemically unmodified, natural nucleotides. It encodes multiple, indication-specific, NSCLC TAAs. The antigens in CV9201 were selected for their role in NSCLC oncogenesis, differential expression between malignant and normal tissues, and ability to induce cytotoxic lymphocytes and/or antigen-specific antibodies. These comprise the cancer/testis antigen 1B (New York esophageal squamous cell carcinoma, NY-ESO-1), melanoma antigen family C1 (MAGE-C1) and C2 (MAGE-C2), baculoviral inhibitor of apoptosis repeat-containing five (survivin), and trophoblast glycoprotein (5T4). NY-ESO-1, MAGE-C1, and MAGE-C2 are only expressed in male germ cells in healthy individuals, but are frequently detected in tumor cells, including NSCLC [14–17]. Gene expression analysis of 928 NSCLC tissues revealed that at least one of the CV9201 antigens was expressed in 99.6% of samples (CureVac AG, data on file). While NY-ESO-1 expression is a poor prognostic factor in many tumor types, including NSCLC [18], patients with humoral or T cell responses against NY-ESO-1 have improved survival [19, 20]. Survivin is an apoptosis inhibitor that is absent from terminally differentiated cells but detectable and immunogenic in most tumors [21, 22]. Survivin expression predicts poor survival in advanced NSCLC [23]. Finally, expression of the oncofetal antigen 5T4 in adults is restricted to tumor-initiating cells and is associated with worse clinical outcome in NSCLC [24].

In this first-in-human, multi-center, phase-I/IIa study, CV9201 was administered intradermally at different dose levels to evaluate safety and tolerability in patients with advanced NSCLC. Immune responses and clinical outcomes were also assessed.

Materials and methods

Patients

Eligible patients were ≥ 18 years old with advanced (stage IIIB/IV according to UICC 6.0 criteria) NSCLC and at least stable disease according to RECIST criteria (v1.0) [25] after first-line treatment (chemotherapy, chemo-radiotherapy) for advanced, unresectable disease. Patients had a life expectancy > 6 months, Eastern Cooperative Oncology Group (ECOG) performance status ≤ 1, and adequate renal, hepatic, cardiac, and bone marrow function. Between June 2009 and January 2011, 55 patients with advanced NSCLC were screened at 12 centers in Germany and Switzerland (only 12 of 14 centers enrolled at least one patient). Nine patients failed screening (Supplementary Table 1) and 46 were treated with up to five administrations of CV9201 (Supplementary Tables 2 and 3). No included patient received concomitant chemotherapy. Baseline patient characteristics are shown in Table 1.

Study design

This was a prospective, multicenter, open-label, uncontrolled phase I/IIa trial. Dose escalation of CV9201 (phase I) was conducted according to a classical 3 + 3 design. Dose-limiting toxicities were defined as National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) grade 3/4 neutropenia with fever and/or infection, grade 3/4 non-hematological toxicity, grade ≥ 2 autoimmunity/allergy, or dosing delay > 48 h due to toxicity experienced prior to the week 5 visit. The primary objective of

Table 1 Baseline characteristics of the patient population

Characteristic	Treatment group				Total $N=46$
	Cohort I (400 µg) $N=3$	Cohort II (800 µg) $N=3$	Cohort III (1600 µg) $N=3$	Phase IIa (1600 µg) $N=37$	
Mean age, years (SD)	75.0 (12.2)	62.7 (8.3)	62.3 (9.1)	64.2 (10.1)	64.7 (10.2)
Male sex, *n* (%)	3 (100)	3 (100)	1 (33)	22 (59)	29 (63)
NSCLC stage, *n* (%)					
Stage IIIB	1 (33)	1 (33)	–	5 (14)	7 (15)
Stage IV	2 (67)	2 (67)	3 (100)	32 (86)	39 (85)
Histology, *n* (%)					
Adenocarcinoma	2 (67)	2 (67)	1 (33)	22 (61)	27 (60)
Squamous	1 (33)	1 (33)	1 (33)	10 (28)	13 (29)
Large cell	–	–	–	3 (8)	3 (7)
Mixed	–	–	1 (33)	1 (3)	2 (4)
Missing[a]	–	–	–	1 (3)	1 (1)
Mean time since diagnosis, weeks (SD)	200.3 (223.0)	73.7 (73.8)	21.9 (4.8)	59.5 (91.3)	67.2 (102.5)
Prior therapy, *n* (%)					
Surgery	2 (67)	3 (100)	2 (67)	29 (78)	36 (78)
Radiotherapy	2 (67)	3 (100)	1 (33)	9 (24)	15 (33)
Chemotherapy	3 (100)	3 (100)	3 (100)	36 (97)	45 (98)
Platinum-based	3 (100)	3 (100)	3 (100)	33 (92%)[b]	42 (93)[b]
Radiotherapy and chemotherapy	2 (67)	3 (100)	1 (33)	9 (24)	15 (33)
ECOG, *n* (%)					
0	2 (67)	1 (33)	3 (100)	20 (54)	26 (57)
1	1 (33)	2 (67)	–	16 (43)	19 (41)
2[c]	–	–	–	1 (3)	1 (2)

ECOG Eastern Cooperative Oncology Group

[a]Percentages are out of the number of patients with data

[b]Chemotherapy data missing for one patient

[c]One patient had an ECOG score of 2 due to neuropathy. A waiver was granted for this patient

phase I was to determine the recommended phase II dose (RP2D), defined as the highest dose level in which a dose-limiting toxicity (DLT) was observed in no more than one of six evaluable patients before the week 5 visit. The primary objective of the extension cohort (phase IIa) was to determine the safety profile of CV9201; secondary objectives were to evaluate anti-tumor efficacy and immune responses.

Treatment

CV9201 was generated using proprietary RNActive® technology [9, 13]. The mRNAs encoding the five antigens of CV9201 were formulated separately and administered at doses of 80, 160, or 320 µg mRNA (total dose 400, 800, or 1600 µg mRNA, respectively). Each CV9201 component was administered individually at up to 160 µg per injection to the thighs and upper arms. The 320 µg dose was split into two injections resulting in up to 10 injections per administration visit due to technical restrictions to 200 µL per injection with a maximal concentration of 0.8 µg/µL mRNA (reproducibility of intradermal application in man is difficult to achieve with greater volumes). Individual components were administered to the same body sides at each visit during phase I and to the opposite body side at different visits in phase IIa.

During phase I, CV9201 was administered at weeks 1, 3, 7, 11, and 15 and at weeks 1, 2, 3, 5, and 7 during phase IIa since early tumor progressions were observed during phase I preventing the administration of all five scheduled injections.

In case of tumor progression, concomitant chemotherapy was allowed during phase IIa at the investigator's discretion.

Safety assessments

Treatment-emergent AEs were graded according to NCI-CTCAE (v3.0) at each visit from the first dose until 30 days after the last dose of CV9201. Biochemistry and hematology assessments were conducted at screening, each administration visit, end-of-treatment, and week 26 follow-up.

Levels of antinuclear antibody (ANA), thyroid stimulating hormone (TSH), antithyroglobulin, rheumatoid factor, and anti-smooth muscle antibodies were measured at screening, week 5, end-of-treatment, and week 26 visits to monitor for the potential induction of autoimmunity. DLTs occurring before the week 5 visit were assessed during the dose-escalation part of the trial.

Immune response assessment

Serum antibody responses and antigen-specific $CD4^+$ and $CD8^+$ T cell responses to CV9201-encoded antigens were determined in peripheral blood taken at weeks 5, 9, and 17 (phase I) and weeks 5 and 9 (phase IIa) and compared with baseline values. All patients who received ≥ 3 CV9201 treatments were evaluated. Methodology for humoral and cellular immune response assessment is described previously [13], and was adapted for the CV9201 antigens.

Humoral responses

Humoral IgG and IgM responses were assessed by ELISA using four serial dilutions of patient plasma or pooled plasma (healthy volunteers) in a MaxiSorb plate coated with MAGE-C1, MAGE-C2, survivin, and 5T4 peptides (IBA GmbH, Germany) and NY-ESO-1 (Ludwig Cancer Institute, New York, USA) recombinant proteins. Antigen-specific antibodies were detected using HRP-labeled anti-IgM or anti-IgG antibodies with tetramethylbenzidine as substrate. Primary antibodies for these antigens were used as a positive control: pan-MAGE (Santa Cruz), survivin (Thermo Scientific), NY-ESO-1 (SIGMA), and 5T4 (Abnova). Antibody responses were considered positive if ELISA readings were greater than the baseline values plus three times the standard deviation in at least three dilutions (Supplementary Fig. 1).

Cellular responses

Antigen-specific $CD4^+$ and $CD8^+$ T lymphocyte responses were assessed ex vivo by ELISPOT assay and ICS in patient-derived PBMCs prepared using a dedicated kit (Interlab GmbH, Germany). After incubating PBMCs for 24 h with a pool of HLA-type-specific class-I/II (short or long) peptides covering epitopes of the five CV9201 antigens (Supplementary Table 4), the number of IFN-γ-producing cells was measured by ELISPOT according to recommendations of the Cancer Immunotherapy Consortium and the Association for Cancer Immunotherapy [26–28]. PBMCs cultured in the presence of peptides for HIV or PMA / ionomycin were used as negative and positive controls, respectively. An antigen-specific T cell response was considered positive if spot counts were at least twice the negative control and baseline values, exhibited at least five spots, were measured at least in duplicate, and had non-overlapping error bars.

Flow cytometry was used to measure intracellular accumulation of IFN-γ, TNF-α, and IL-2 within the $CD8^+$ and $CD4^+$ T cell populations after ex vivo stimulation with predicted HLA class-I/II epitopes for CV9201-specific antigens in the presence of anti-CD28 antibody (Becton Dickinson GmbH, Heidelberg, Germany) for 6 h [13]. The frequency of cytokine-producing cells within the $CD8^+$ and $CD4^+$ T cell populations was determined using a gating strategy shown in Supplementary Fig. 2. Antigen-specific T cell responses were considered positive if frequencies of cytokine-producing T cells were at least twice the negative control and baseline values and at least 0.02% of the respective T cell population. Flow cytometry plots were evaluated by two independent raters to exclude artifacts.

Phenotypic analyses by flow cytometry

Subpopulations or maturation/activation markers on NK cells (CD3/CD4/CD16/CD25/CD27/CD56/CD69), B cells (CD3/CD19/CD20/CD27/CD38/CD86/IgD), and T cells (CD3/CD4/CD8/CD27/CD28/CD45RA/CCR7) were assessed using flow cytometry [29].

A lymphocyte proliferation assay was performed exemplarily in one IgM-responding patient who did not show a cellular immune response. Patient-derived PBMCs were rested overnight at 37 °C and 5% CO_2 in cell X-Vivo-15 culture medium and labeled with 0.5 µM CFSE division tracking dye. Labeled cells were incubated in culture medium in a microtiter plate coated with human recombinant NY-ESO-1, MAGE-C1, MAGE-C2, survivin, or 5T4 proteins (see ELISA) for 5 days and stained for CD3, CD19, CD27, CD38, and IgD on Day 6.

Cytometric measurements were performed on a calibrated BD FACSCanto II (4-2-2) cytometer using the mAbs listed in Supplementary Table 5.

Anti-cancer efficacy

Tumor response was assessed according to RECIST (v1.0) [25]. Patients were evaluated by the investigators at baseline and reassessed according to local practice up to week 52 to determine objective disease response and PFS. Patients who provided separate informed consent were followed for survival up to 3 years.

Statistical analysis

Sample size was primarily based on clinical and safety considerations; a formal power calculation was not performed. The number of DLTs and patients with early discontinuation determined the number of enrolled patients. It was expected

to enroll 9–18 patients for determination of RP2D and 24 patients for evaluation of immune response for the phase IIa cohort. Continuous and categorical data were summarized with descriptive statistics. Survival data was estimated from the time of treatment initiation using the Kaplan–Meier method. SAS and GraphPad Prism 6.05 were used for statistical analysis of immune data. Clinical analyses and immune analysis were confirmed by the International Drug Development Institute S.A (Louvain-la-Neuve, Belgium).

Results

Dose escalation and safety

Nine patients received CV9201 during the dose-escalation phase of the study (three patients each at total doses of 400, 800, and 1600 µg mRNA per administration). The median (range) number of doses of CV9201 was 3 (2–5), 3 (3–5), and 4 (3–5) in the 400 µg, 800 µg, and 1600 µg cohorts, respectively (Supplementary Table 2). No patient experienced a DLT; therefore, the maximum tolerated dose was not reached. Consequently, 1600 µg was selected as the RP2D and 37 patients were treated at this dose in phase IIa.

Overall, 45/46 (98%) patients received at least two treatments and 33/46 (72%) patients received all five planned doses of CV9201. Eleven (24%) patients discontinued treatment due to disease progression, one (2%) patient due to a treatment-related AE (grade 3 asthma attack in a patient with preexisting chronic obstructive pulmonary disease), and one (2%) patient was withdrawn from treatment due to a protocol violation (patient had brain metastasis as identified on the MRI after initial vaccination). All 46 patients were included in the safety analysis.

All patients experienced at least one AE during study participation (Table 2). Most AEs were grade 1 (85%) or 2 (13%) intensity. Forty-four (96%) patients experienced a treatment-related AE, predominantly injection site reactions including injection site erythema (37 patients; 80%), pruritus (nine patients; 20%), discoloration of the injection site (six

Table 2 Adverse events occurring in ≥ 10% of all patients by preferred term (PT) and all serious AEs

Adverse event (PT), n[a] (%)	Cohort I (400 µg) $N=3$	Cohort II (800 µg) $N=3$	Cohort III (1600 µg) $N=3$	Phase IIa (1600 µg) $N=37$	Total $N=46$
Total patients with at least one AE	3 (100)	3 (100)	3 (100)	37 (100)	46 (100)
Injection site erythema	3 (100)	3 (100)	3 (100)	27 (73)	36 (78)
Fatigue	1 (33)	1 (33)	1 (33)	11 (30)	14 (30)
Pyrexia	–	–	1 (33)	9 (24)	10 (22)
Injection site pruritus	1 (33)	–	–	8 (22)	9 (20)
Nausea	1 (33)	–	–	8 (22)	9 (20)
Chills	–	–	–	7 (19)	7 (15)
Injection site discoloration	–	–	–	6 (16)	6 (13)
Headache	–	–	1 (33)	5 (14)	6 (13)
Vertigo	1 (33)	–	–	5 (14)	6 (13)
Cough	–	1 (33)	1 (33)	3 (8)	5 (11)
Myalgia	–	1 (33)	–	4 (11)	5 (11)
Nasopharyngitis	–	1 (33)	–	4 (11)	5 (11)
Serious AEs[b]	–	1 (33)	–	6 (16)	7 (15)
Atrial tachycardia	–	–	–	1 (3)	1 (2)
Disease progression	–	–	–	2 (5)	2 (4)
Pneumonia	–	–	–	2 (5)	2 (4)
Septic shock	–	–	–	1 (3)	1 (2)
Neutropenic infection	–	–	–	1 (3)	1 (2)
Femur fracture	–	1 (33)	–	–	1 (2)
Bone pain	–	–	–	1 (3)	1 (2)
Pleural effusion	–	–	–	2 (5)	2 (4)
Pulmonary embolism	–	–	–	1 (3)	1 (2)

PT preferred term

[a]Multiple occurrences of the same AE are counted once

[b]None of the serious AEs were considered related to treatment

patients; 13%) and pain (four patients; 9%). Other treatment-related AEs experienced by ≥ 5% of patients were pyrexia and fatigue (nine patients each; 20%), chills (six patients; 13%), myalgia and nausea (both four patients; 9%), and headache, increased ANA, and chills (all three patients; 7%).

Fifteen (33%) patients had grade 3 AEs; in three of these patients the AE was considered to be drug-related (fatigue, injection site pustule, and asthma). Two grade 4 (neutropenic infection and pleural effusion) and three grade 5 (disease progression, pulmonary embolism, and septic shock) AEs occurred; none were considered drug-related. None of the 12 serious AEs in seven (15%) patients (Table 2) were drug-related.

A shift from normal to abnormal values of laboratory parameters, potentially indicating autoimmunity, occurred for TSH in nine patients, ANA in five patients, rheumatoid factor in four patients, and antithyroglobulin in one patient. No cases of clinically significant autoimmune disease were observed.

Antigen-specific cellular and humoral responses

In phase I, there was no clear dose–response relationship regarding antigen-specific immune responses; 2/2, 2/3, and 0/3 patients showed immune responses in the 400, 800, and 1600 µg cohorts, respectively. In phase IIa (RP2D of 1600 µg CV9201), 19/30 (63%) evaluable patients had at least one cellular or humoral antigen-specific response to at least one of the five antigens. Eight (27%) patients had an antigen-specific T-cell response as measured by ex vivo ICS, including six (20%) patients with $CD4^+$ T cell responses or with $CD8^+$ T cell responses, respectively (Fig. 1a). Five (17%) patients exhibited a T cell response determined by ex vivo IFN-γ ELISPOT with two (7%) patients showing an immune response against a pool of long, HLA class-II peptides and three patients (10%) against a pool of short, HLA class I peptides. Fourteen (47%) patients had antigen-specific humoral responses, with more than twice as many patients exhibiting IgM (12 [40%]) than IgG (four [13%]) responses. Increases in antigen-specific T cell frequencies by ICS and ELISPOT or antibody levels were observed for all five antigens in the combined patient population (Fig. 1b), however, T cell responses were generally very rare. Two (7%), two (7%), and six (20%) of the patients had an immunological response to two or more antigens by ICS, ELISPOT, or ELISA, respectively, with the majority of patients only responding to one of the CV9201-encoded antigens (Fig. 1c).

The strength of the T cell response detected after CV9201 treatment varied, with some patients showing very distinct responses (Fig. 2a, b). In two patients corresponding CD4 and CD8 T cell responses against one antigen were observed by ICS (patient 12 NY-ESO-1, patient 30 Survivin). However, the majority of the T cell responses detected by ICS were mono-functional and modest in nature and incongruent with the ELISPOT results. In one case, having sufficient amounts of residual patient's PBMCs, we conducted an additional exploratory analysis. We used recombinant TAA proteins to stimulate PBMCs derived from a single patient with undetectable ex vivo immune responses to assess antigen-specific lymphocyte proliferation in a CFSE dilution assay. After 5 days of cell culture, proliferating T cells were detected in response to MAGE-C2 and NY-ESO-1 (Supplementary Fig. 3), suggesting the possibility that ex vivo immune assays may not be sensitive enough to detect TAA-specific lymphocytes.

CV9201 treatment increased activated IgD^+CD38^{hi} B cell levels

Post-treatment increases of activated IgD^+CD38^{hi} B cells were detected (Fig. 3a). Activated IgD^+CD38^{hi} B cells counts increased significantly at both assessments compared with baseline (Fig. 3b, c). Frequencies of activated IgD^+CD38^{hi} B cells in untreated healthy controls remained low and comparable with patient baseline values (data not shown). Eighteen (60%) of the 30 patients evaluable in phase IIa had a ≥ twofold increase in the frequency of activated IgD^+CD38^{hi} B cells compared with baseline (Fig. 3d). One patient had a 13-fold expansion of the activated IgD^+CD38^{hi} B cells. We also analyzed NK cells and various T cell subsets in peripheral blood but were unable to detect substantial changes in these lymphocyte subsets post treatment (data not shown).

Anti-cancer efficacy

Tumor response up to week 52 was evaluable in 29 patients. Nine patients had stable disease and 20 had progressive disease as best overall response. No objective responses were seen. Median PFS in the total population ($n = 46$; 85% with stage IV disease) was 2.7 (95% CI 1.9–5.8) months; 6- and 12-month PFS rates were 35.6% and 15.6%, respectively. In patients treated at the RP2D in phase IIa, ($n = 37$; 86% with stage IV disease) the median PFS was 5.0 (1.8–6.3) months and the 6- and 12-month PFS rates were 38.9% and 16.7%, respectively (Fig. 4a). Median OS was 11.5 (8.5–18.8) months in the total population. Two patients did not consent to prolonged survival follow-up beyond week 52 and were censored. The 1-, 2- and 3-year OS rates were 48.9%, 30.9%, and 23.2%, respectively. For the 37 patients treated in phase IIa, median OS was 10.8 (8.1–16.7) months and the 1, 2 and 3-year OS rates were 44.4%, 26.7%, and 20.7%, respectively (Fig. 4b).

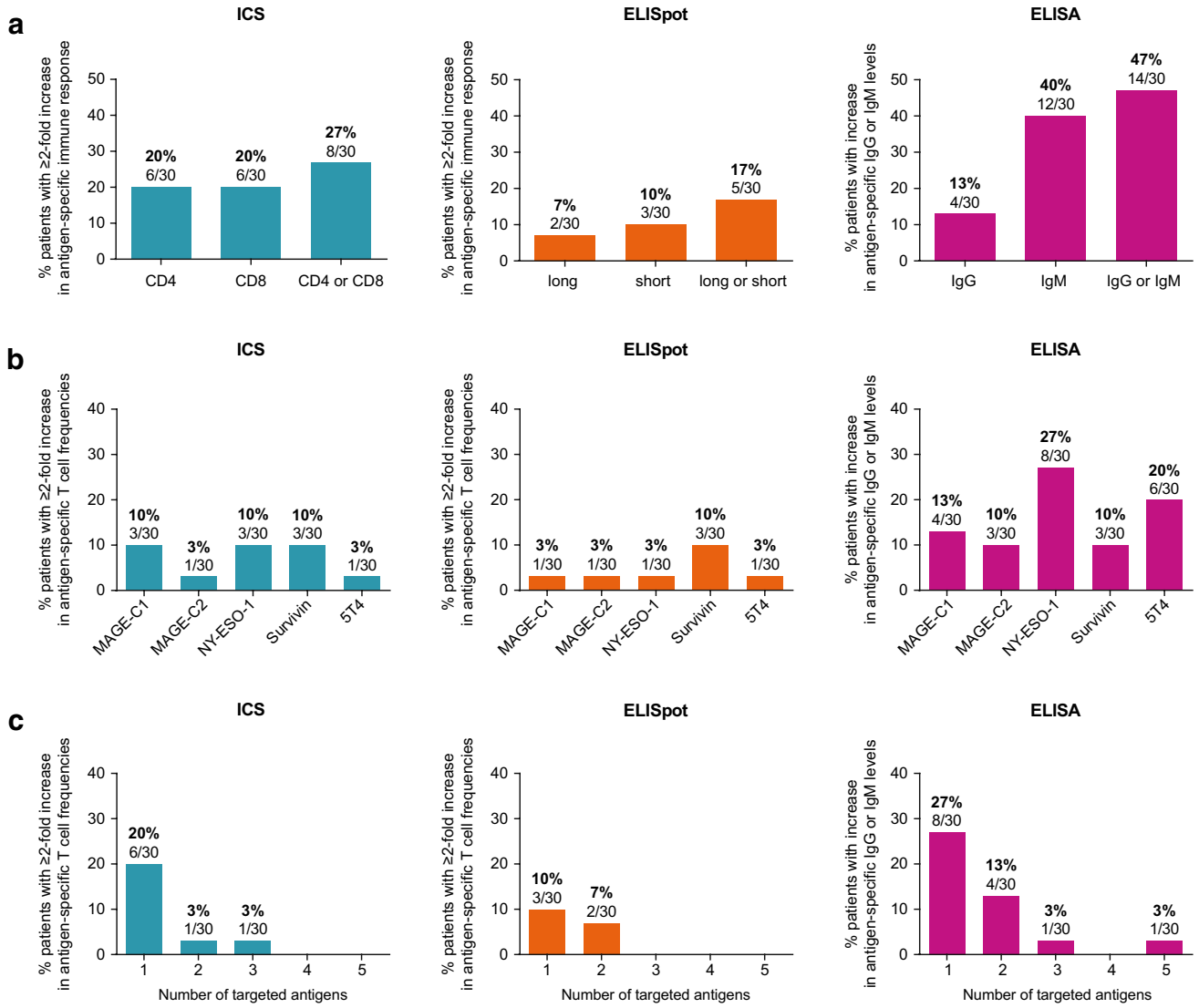

Fig. 1 Frequencies of patients with an at least twofold increase in antigen-specific immune reactions following CV9201 treatment in phase IIa of the study ($N=30$ evaluable patients). Values displayed above the bars indicate the percentages and actual number of patients with increase in immune responses. **a** Frequencies of evaluable patients with antigen-specific cellular and humoral immune responses against one or more CV9201 antigens at one or more time points. Cellular responses were detected ex vivo by ICS in the $CD4^+$ and $CD8^+$ cell population and by IFN-γ ELISPOT using a pool of either short or long peptide epitopes which were predicted for the five CV9201 antigens (see Supplementary Table 4). Humoral responses (IgG or IgM levels against the five CV9201 antigens) were measured by ELISA. **b** Frequencies of patients with a cellular (determined by ICS or ELISPOT) or humoral response (determined by ELISA) to each of the antigens encoded by CV9201. **c** Frequencies of patients with antigen-specific T cells or antibodies increase compared to baseline against one or more antigens encoded by CV9201; at any post-baseline time point. *5T4* trophoblast glycoprotein, *MAGE* melanoma antigen family, *NY-ESO-1* New York esophageal squamous cell carcinoma-1

Discussion

We investigated an antigen-specific mRNA-based immunotherapy in patients with stage IIIB/IV NSCLC with at least stable disease after completion of first-line treatment with chemotherapy or chemoradiation. Our data show that active immunotherapy with full-length mRNAs encoding TAAs complexed with protamine is feasible and well-tolerated. The AE profile of CV9201 was similar to that of the related prostate cancer-specific RNActive® immunotherapy, CV9103 [13], despite a higher overall mRNA dose per visit with CV9201 (1600 µg versus 1280 µg for CV9103). Mild-to-moderate injection site reactions and flu-like symptoms were common (consistent with other mRNA/peptide-based cancer vaccines and intradermal vaccines in general [30–34]) and resolved without intervention.

Despite some patients exhibiting increases in TSH, ANA, rheumatoid factor, and anti-thyroglobulin, no cases

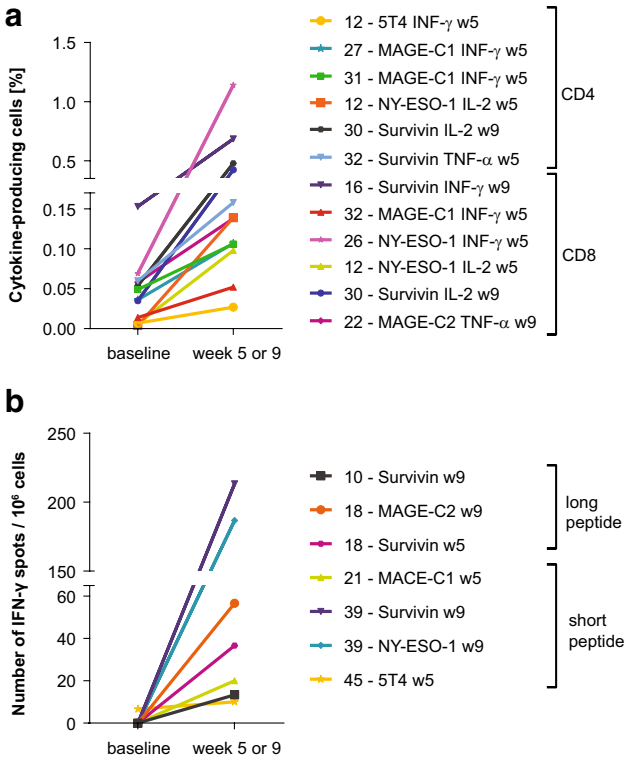

Fig. 2 Magnitude of T cell responses as measured by (**a**) ICS or (**b**) IFN-γ ELISPOT of patients with an at least twofold increase in antigen-specific immune reactions following CV9201 treatment in phase IIa of the study. Values are shown as the percentage of positive cells gated on both $CD4^+$ or $CD8^+$ populations for ICS. ELISPOT results are plotted as number of spots per 1 million PBMCs after background subtraction (HIV negative control of corresponding week). Legends indicate patient ID, antigen, measured cytokine (only for ICS) and time point. *5T4* trophoblast glycoprotein, *MAGE* melanoma antigen family; *NY-ESO-1* New York esophageal squamous cell carcinoma-1, *w* week

of clinically apparent autoimmune disease were observed. The clinical relevance of this finding requires further investigation, particularly since changes in these parameters are not uncommon in the elderly population and in patients with advanced NSCLC [35].

No objective tumor responses were seen with CV9201. The observed PFS and OS are consistent with those reported for maintenance chemotherapy in NSCLC patients not progressing after first-line chemotherapy [36]. Tumor responses, assessed according to classical criteria, are rarely observed after administration of cancer vaccines as monotherapy in patients with advanced solid tumors; however, prolongation of OS has been suggested with some vaccination approaches [34, 37] but so far no cancer vaccine approach targeting cancer-associated self-antigens has shown a survival benefit in phase III [38–40]. Hence, combination treatment with checkpoint inhibitors to overcome the tumor environment immunosuppression or targeting of neoantigens that may be less prone to tolerance are approaches which could help to achieve clinical benefit from vaccination. RNActive®-based immunotherapies combined with low doses of anti-CTLA-4 or anti-PD-1 showed synergistic effect, resulting in complete tumor rejection in some murine models [11]. Consequently, a modified version of CV9201 [expanded to include the Muc-1 antigen, CV9202 (BI1361849)] which was previously investigated in combination with radiotherapy in advanced NSCLC [41], is being tested in an ongoing trial in combination with checkpoint inhibitors (LUD2014-012-VAC).

We observed increases in activated IgD^+CD38^{hi} B cells post mRNA-based immunotherapy. The increase of activated IgD^+CD38^{hi} B cells was not related to antigen-specific immune responses. The precise role and ontology of these cells remain elusive. These cells might represent an intermediary step in the differentiation of naïve B cells to antibody-producing plasma cells [42] or represent a TLR-mediated unspecific B cell response [43]. Alternatively, these cells could represent transitional, immature B cells [44] or regulatory B cells [45]. Further studies are required to elucidate their functional role and to investigate whether the expansion of these cells is associated with mRNA therapy or NSCLC disease progression.

In the phase I dose escalation part of our trial we noticed nominally higher frequencies of immune responses in patients treated with lower mRNA dose levels, which need to be interpreted with caution due to the low number of patients per cohort. Decreases in immune responses with increasing RNActive doses were neither observed in animal models nor in a previous RNActive-based immunotherapy trial in prostate cancer patients [13]. The phase IIa part of the trial was initiated with the recommended high dose of 1600 µg. Both cellular and humoral immune responses were detected against all antigens, albeit the responses were modest and revealed high inter-patient variability. Antigen-specific T cell immunity was detectable after treatment in peripheral blood in 27% and 17% of the patients by ICS and ELISPOT, respectively. Based on these results it appears unlikely that activation-induced cell death in T lymphocytes, which has been described upon over-stimulation with antigens/peptides [46], was the reason for the higher immune response frequency in the low dose cohort of the phase I part.

Noteworthy, these cellular responses were evaluated ex vivo, either after just 6 (ICS) or 24 (ELISPOT) hours stimulation using a limited number of predicted and/or literature-based peptides. Prolonged in vitro re-stimulation in the range of several days is often applied to detect immune responses in response to cancer antigen immunization. We decided not to rely on lengthy in vitro stimulation since it can also result in artificial generation and expansion of antigen-specific and unspecific T cells in culture (own observations during assay implementation, data not shown). In addition, the limited number of CV9201 antigen-derived

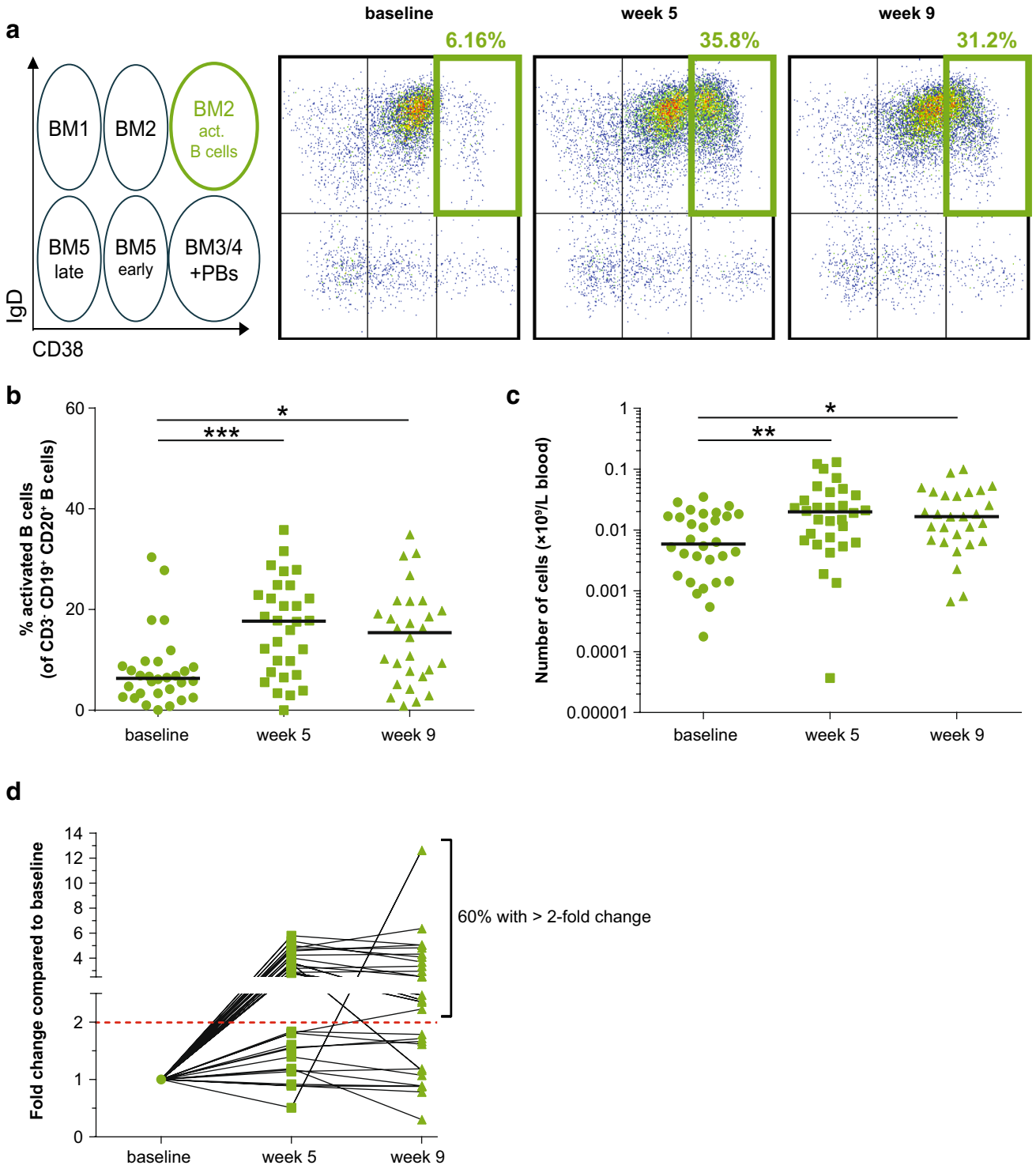

Fig. 3 Effect of CV9201 treatment on other cell populations of interest ($N=30$ evaluable patients). **a** Frequency of B cells at different developmental stages determined by FACS analysis of IgD and CD38 staining showing an increase of activated $IgD^{+}CD38^{hi}$ B cells in week 5 and week 9 following treatment with CV9201. **b** Frequency of activated $IgD^{+}CD38^{hi}$ B cells in 30 evaluable patients of the phase IIa extension cohort at baseline, week 5 and week 9. Statistical significance was determined by the Kruskal–Wallis test followed by Dunn's multiple testing. $***p<0.001$; $*p<0.05$. **c** Corresponding absolute number of activated $IgD^{+}CD38^{hi}$ B cells. Statistical significance was determined by the Kruskal–Wallis test followed by Dunn's multiple testing. $**p<0.01$; $*p<0.05$. **d** Fold-change in activated $IgD^{+}CD38^{hi}$ B cell numbers at week 5 and week 9 compared with baseline ($N=30$). *BM* bone marrow, *PB* plasma blasts

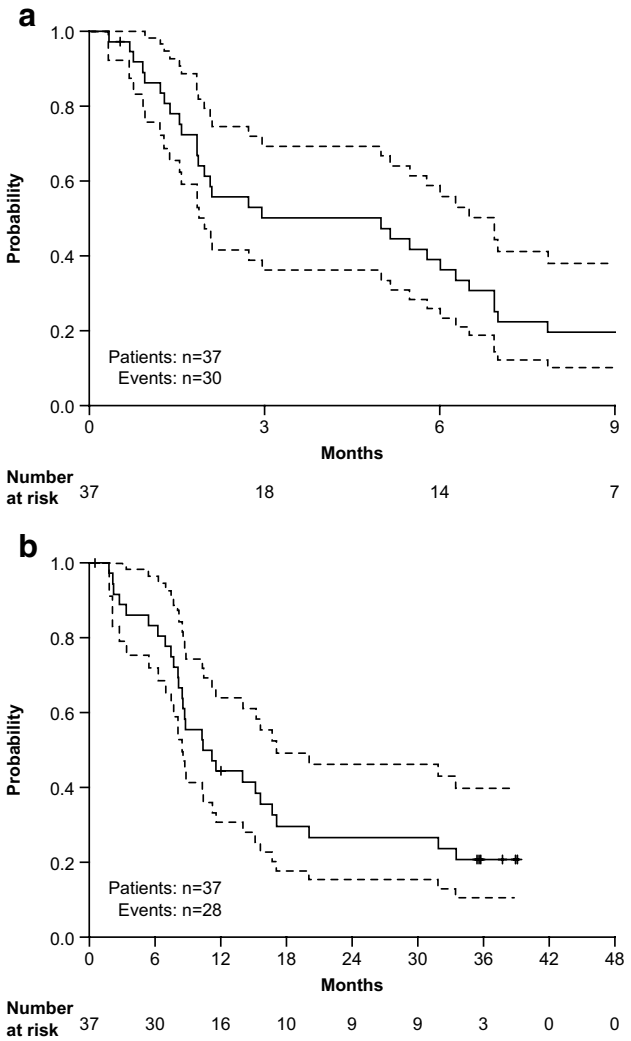

Fig. 4 Kaplan–Meier **a** PFS and **b** overall survival (OS) curves from initiation of treatment for the phase IIa cohort ($N=37$; 86% with stage IV disease). Dashed lines represent 95% CI. Median PFS was 5.0 months (95% CI 1.8–6.3 months) and the 6- and 12-month PFS rates were 38.9% and 16.7%, respectively. Median OS was 10.8 months (95% CI 8.1–16.7 months) and survival rates at 1, 2, and 3 years were 44.4%, 26.7%, and 20.7%, respectively

T cell epitopes (peptides) selected for use in the analyses of cellular responses is likely to under-represent all immunogenic T cell epitopes from every patient's HLA. Thus, methodological reasons could account for a putative underestimation of the cellular response rates.

Nonetheless, the detected immune responses were rather rare (e.g. against single antigens), monofunctional (T cells secreting single cytokine type), not persistent in nature (e.g. detectable at both post-vaccine time points) strongly suggesting that the immunogenicity of the tested mRNA-immunotherapeutic needs to be improved. We have recently conducted a prophylactic vaccine trial using protamine-formulated mRNA encoding the rabies glycoprotein [47]. This study demonstrated that the use of needle-free injection devices was essential to induce antiviral immune response levels considered to be protective. In contrast to syringe–needle administration, needle-free injection devices could lead to broader dispersion patterns of the RNA in the skin resulting in improved cellular uptake and antigen expression and consequently improved immunogenicity. Similarly, the choice of administration method (needle-injection) may have contributed to the rather weak immunogenicity of CV9201 in this trial. Based on this experience the successor vaccine CV9202 (BI1361849) targeting 6 antigens is being administered via needle-free jet injection in the ongoing phase I trial testing combination with checkpoint blocking antibodies (LUD2014-012-VAC).

Another key factor for developing a potent and efficacious mRNA immunotherapy, is to adjust and improve the mRNA formulation for the given indication and application. A promising approach is the use of lipid nanoparticles (LNP) as mRNA carriers, which may enhance immunogenicity as well as cellular uptake of mRNA and depending on the design and lipid composition may improve biodistribution. We have recently shown that using sequence optimized, chemically unmodified mRNA formulated in optimized LNPs strong and long-lasting immune responses can be elicited in non-human primates after a single intramuscular injection [48]. Similarly, Kranz et al. demonstrated that by adjusting the net charge of the so-called RNA-lipoplexes, the RNA can be targeted to antigen presenting cells in spleen, lymph nodes and bone marrow [49]. First results of an LNP-formulated RNA influenza vaccine revealed acceptable tolerability as well as robust prophylactic immunity in an ongoing phase I trial [50]. Additionally, work done by Sahin et al. highlighted the successful use of RNA neo-epitope vaccines as individually tailored medicines [51] proving that choosing the right RNA design and formulation is crucial for its success in immunotherapy and should be carefully considered for future clinical trials.

In conclusion, the protamine-formulated mRNA immunotherapy CV9201 showed an acceptable tolerability profile and evidence of immune activation. The successor vaccine CV9202 is being investigated in combination with checkpoint blocking antibodies using needle-free administration technique, which has been shown to improve the immunogenicity of other protamine formulated mRNA vaccines.

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Author contributions MS, BS, JP, TL, UG-V, K-JK, IH, and MF-M conceived, designed and supervised the study. MS, LB, AZ, FM, MR, DA, MT, FS, JS, HB, AG, and AK recruited patients and provided clinical samples. AS, BS, AM, HSH, TS, VW, FM and SDK acquired and analyzed the data. AS, BS, AM, HSH, and SDK drafted the manuscript. All authors read and approved the final version of the paper.

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Compliance with ethical standards

Conflict of interest Martin Sebastian reports personal fees from Lilly and Roche during the conduct of the study, and personal fees from Boehringer-Ingelheim, Pfizer, Astra-Zeneca, Bristol-Myers Squibb (BMS), Merck Sharp & Dohme (MSD), and Novartis, outside the submitted work. Alfred Zippelius reports grants from Roche, Actelion, Piqur, Secarna, and Beyondsprings, and personal fees from BMS, MSD, and NBE Therapeutics outside the submitted work. Martin Reck reports personal fees from Roche, Lilly, Boehringer-Ingelheim, BMS, AstraZeneca, MSD, Novartis, Pfizer, and Celgene, outside the submitted work. Alexander Knuth reports a former scientific advisory role for CureVac AG and is co-inventor on multiple patents of Ludwig Institute for Cancer Research (LICR) related to NY-ESO-1 and MAGE, partly licensed to multiple companies including CureVac AG. Birgit Scheel, Anke Muth, Tanja Strack, Volker Wiegand, Ulrike Gnad-Vogt, Ingmar Hoerr, Florian von der Muelbe and Mariola Fotin-Mleczek are employees of CureVac AG. Thomas Lander, Andreas Schröder, Henoch S. Hong, Jochen Probst, Karl-Josef Kallen and Sven D. Koch were employees of CureVac GmbH/AG. Thomas Lander was a clinical consultant to CureVac GmbH until 2012. Thomas Lander, Jochen Probst, and Ingmar Hoerr jointly hold a patent related to the use of mRNA vaccines for treating lung cancer (WO2009/046974) filed in several jurisdictions (issued in some and pending in others). Ulrike Gnad-Vogt, Karl-Josef Kallen, and Mariola Fotin-Mleczek jointly hold a patent related to the use of mRNA vaccines for treating lung cancer (WO2015/024666) filed in several jurisdictions (still pending). All other authors declare no conflicts of interest.

Ethical approval and ethical standards The protocol was approved by the regulatory authorities, a central ethics committee for the 12 participating centers in Germany (Ethikkommission bei der Landesärztekammer Hessen, Frankfurt am Main, Germany - Approval Number: FF 2/2009) and two local ethics committees for the two participating centers in Switzerland (Spezial-Unterkommission (SPUK) Innere Medizin, Kantonale Ethikkommission UniversitätsSpital Zürich, Zürich, Switzerland - Approval Number: EK-1639, and Ethikkommission beider Basel (EKBB), Universitätsspital Basel, Basel, Switzerland - Approval Number: 20/09). The study was conducted in accordance with Good Clinical Practice guidelines (EudraCT No.: 2008-007785-39). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent on being treated as well as on the use of generated data for research purposes and publication was obtained from all individual participants included in the study.

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