Clinical Cancer Research

Targeting Carcinoembryonic Antigen with DNA Vaccination: On-Target Adverse Events Link with Immunologic and Clinical Outcomes

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Abstract

Purpose: We have clinically evaluated a DNA fusion vaccine to target the HLA-A*0201–binding peptide CAP-1 from carcinoembryonic antigen ($CEA_{605–613}$) linked to an immunostimulatory domain (DOM) from fragment C of tetanus toxin.

Experimental Design: Twenty-seven patients with CEA-expressing carcinomas were recruited: 15 patients with measurable disease (arm-I) and 12 patients without radiological evidence of disease (arm-II). Six intramuscular vaccinations of naked DNA (1 mg/dose) were administered up to week 12. Clinical and immunologic follow-up was up to week 64 or clinical/radiological disease.

Results: DOM-specific immune responses demonstrated successful vaccine delivery. All patients without measurable disease compared with 60% with advanced disease responded immunologically, while 58% and 20% expanded anti-CAP-1 $CD8^+$ T cells, respectively. CAP-1–specific T cells were only detectable in the blood postvaccination but could also be identified in previously resected cancer tissue. The gastrointestinal adverse event diarrhea was reported by 48% of patients and linked to more frequent decreases in CEA ($P < 0.001$) and improved global immunologic responses [anti-DOM responses of greater magnitude ($P < 0.001$), frequency ($P = 0.004$), and duration] compared with patients without diarrhea. In advanced disease patients, decreases in CEA were associated with better overall survival (HR = 0.14, $P = 0.017$). CAP-1 peptide was detectable on MHC class I of normal bowel mucosa and primary colorectal cancer tissue by mass spectrometry, offering a mechanistic explanation for diarrhea through $CD8^+$ T-cell attack.

Conclusions: Our data suggest that DNA vaccination is able to overcome peripheral tolerance in normal and tumor tissue and warrants testing in combination studies, for example, by vaccinating in parallel to treatment with an anti-PD1 antibody. *Clin Cancer Res; 22(19); 4827–36. ©2016 AACR.*

Introduction

The goal of harnessing the immune system and provoking antitumor immunity is now beginning to be realized, and recent regulatory approval of the first immunotherapeutics for prostate cancer (1) and melanoma (2, 3) heralds a new and exciting phase in the field. A number of vaccines appear to confer clinical benefit in early-phase studies (4–7), with many agents now in phase III testing. Checkpoint blockade is taking its place in the mainstream management of cancer, but after enormous excitement, it is also becoming clear that only a subgroup of patients benefit (8). If immunologic visibility of the tumor is reflected in the presence of tumor-infiltrating lymphocytes and links to survival (9, 10), activating preexisting antigen-specific T-cell responses could be an effective approach to improving clinical outcomes. Exploiting antigen-specific responses to tumor antigens that are shared between different patients and cancer types is one option, provided that antigen remains visible to immune attack and is not eliminated by selective pressures within the tumor.

Carcinoembryonic antigen (CEA) is an immunoglobulin-like molecule involved in cell adhesion (11). In healthy adults, CEA is primarily found at low levels on the surface of colonic mucosa. Following transformation, CEA is expressed in 70% of all human cancers (12), making it an attractive target for immunotherapy (13). Different immunotherapeutic strategies targeting CEA have

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Translational Relevance

We report on a phase I/II clinical trial of an anti-CEA DNA fusion vaccine in patients with carcinoembryonic antigen (CEA)–expressing cancers. We have linked the HLA-A*0201–binding peptide CAP-1 to an immunogenic domain from fragment C of tetanus toxin to exploit the nontolerized $CD4^+$ T-cell repertoire for tetanus and to help stimulate $CD8^+$ T-cell immune responses against the CAP-1 peptide. Using MHC I peptide elution, we demonstrate that CAP-1 is expressed on human cancer cells and nonmalignant tissue, confirming it can be targeted for immunotherapy. We show that DNA vaccination can expand preexisting intratumoral immune responses and overcome tolerance. On-target, off-tumor immune-related effects in the form of the gastrointestinal adverse event diarrhea correlate with measurable antivaccine immune responses. Better immune responses identify patients with advanced disease who live longer. Our data suggest that immunotherapy against nonmutated, cancer-associated antigens should be pursued and should be considered as a backbone to combination immunotherapies.

been tested clinically; vaccines using peptides (14, 15), dendritic cells (DC; refs. 16–18), plasmid (19, 20) or viral vector delivery (21–26), and engineered T cells (27) have been assessed in phase I/II clinical trials and demonstrate varying clinical efficacy.

Our approach has been to develop DNA vaccines to stimulate antitumor immunity. To overcome the weak immunogenicity of tumor-associated antigens, we have linked an MHC class I–restricted peptide to a potently immunogenic domain (DOM) from fragment C (FrC) of tetanus toxin (28). By exploiting the nontolerized $CD4^+$ T-cell repertoire for tetanus, DOM stimulates $CD8^+$ T-cell immune responses via linked T-cell help with induction of cytotoxic $CD8^+$ T cells against the vaccine-encoded MHC class I–restricted peptide in wild-type and HLA-A2 transgenic mice (28). The fusion vaccines are able to stimulate $CD8^+$ T cells in tolerant mice, induce T-cell memory, and protect against tumor challenge, with early clinical data supporting the translation into humans (29).

In this exploratory phase I/II study, we use DNA fusion vaccination to target the HLA-A*0201–restricted CAP-1 peptide from CEA (30) in patients with CEA^+ malignancies.

Patients and Methods

Study design

In this multicenter, nonrandomized, two-arm phase I/II study we examined the safety, immunogenicity, and clinical effects of our DNA vaccine encoding the DOM–CAP-1 fusion gene (28) in patients with CEA-expressing cancers (EudraCT number: 2004-001932-21; Cancer Research UK protocol PH1/099). Regulatory and ethical approval was obtained from the UK Medicines and Healthcare Regulatory Authority, the national ethics committee responsible for the conduct of studies with genetic vaccines (GTAC), and the local research ethics committee at each center. Written informed consent was provided by each patient prior to any study-specific interventions. The vaccine was produced to GMP by the NHS Blood and Transplant, Clinical Biotechnology Centre, University of Bristol (Bristol, United Kingdom). The study was sponsored by Cancer Research UK.

Patients

Eligibility criteria included a CEA-expressing malignancy confirmed by central immunohistochemical review of archived tumor; ≥18 years of age; HLA-A*0201 positivity; World Health Organization (WHO) performance status of ≤1; completion of oncological treatments >8 weeks prior to consent; lymphocyte count of $>1.0 \times 10^9$/L; platelet count of $>50 \times 10^9$/L, with normal clotting; no clinical or immunologic signs of autoimmunity, specifically inflammatory bowel disease; absence of systemic immunosuppressants, such as steroids; and adequate contraception in place. Patients with radiologically measurable metastatic disease after exhaustion of standard treatment options and in progression were entered into arm-I. Patients in radiological complete remission after surgical clearance with or without adjuvant chemotherapy, but at a high risk of recurrence (estimated at recruitment to be ≥50% at 5 years), were recruited to arm-II. Patients receiving ≥3 or ≥1 dose(s) of vaccine were evaluable for immunogenicity and toxicity, respectively.

Study procedures

DNA vaccine (1 mg/dose) was delivered intramuscularly at weeks 0, 1, 2, 4, 8, and 12 (29, 31); on-study evaluation was up to week 64 or clinical/radiological disease. At WHO grade ≥3 toxicity, vaccination ceased; at grade 2 toxicity, further vaccination was delayed until recovery to ≤1 (CTC v2.0). Imaging by CT was at baseline, weeks 16, 24 (arm-I only), and 64/off-study visit, or as clinically required, with assessment using RECIST v1. Regular blood collections for full blood count, biochemistry, serum CEA, and immunologic evaluation were performed (baseline, weeks 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 40, 52, and 64). Autoimmune profiles were measured at 3 monthly intervals. Clinical assessment and co-medications were documented at each visit. Time to progression (TTP) and overall survival (OS) were recorded from date of consent to date of event or censor (December 31, 2012).

CAP-1 presentation on human tissue

CAP-1 peptide presentation was assessed on HLA-A*0201^+ renal control, normal colonic, and malignant colorectal tissue (6). HLA peptide pools from shock-frozen tissue samples were immunoprecipitated with BB7.2 antibody (32). Peptides were analyzed by online nano liquid chromatography: nanoAcquity UPLC system with 25-cm capillary columns (internal diameter 75 μm) filled with 1.7 μm BEH130 C18 particles (Waters). The acetonitrile (in 0.1% formic acid) gradient consisted of 1%–13% for 10 minutes, 13%–26.5% for 140 minutes, and 26.5%–34.5% for 40 minutes, with a 300 nL/minute flow rate. Analysis was on an LTQ-Orbitrap Velos with a nanoelectrospray ion source (Thermo Fisher Scientific). CAP-1 peptide detection was performed by data independent acquisition in CID mode, including an isotope ($^{13}C_6/^{15}N$)–labeled CAP-1 peptide. Resolution was 30,000 for the Orbitrap; fragment spectra were recorded at low resolution in the LTQ. Data analysis was performed using Skyline (33).

Immunologic evaluation

Immunologic responses were assessed using validated assays (34–36); T-cell responses were reported to MIATA guidelines (http://www.miataproject.org; Supplementary Table S1; ref. 37).

Antivaccine humoral responses were measured in the serum of patients (triplicate) by separate ELISAs against recombinant DOM and FrC protein (31, 34). A response to the DOM helper sequence was assigned when a significant ($P < 0.05$) increase over

Table 1. Summary of patient demographics and immune responses

Patient ID (F;M/age)	Tumor: Primary tumor	Tumor: Best response	CEA: Baseline CEA (μg/L)	Anti-DOM: Humoral (ELISA)	Anti-DOM: Cellular: *ex-vivo* (ELISPOT)	Anti-CAP-1: Cellular: *ex vivo* ELISPOT	Anti-CAP-1: Cellular: *ex vivo* Tetramer	Anti-CAP-1: Cellular: cultured ELISPOT	Anti-CAP-1: Cellular: cultured Tetramer
Arm-I									
101 (F/61)	CRC	SD	36.9	++	++	-	-	-	nt
102 (M/66)	CRC	PD	7.2	-	-	-	-	-	nt
103 (M/66)	CRC	PD	32.3	++	-	-	-	-	nt
104 (M/69)	Stomach	PD	118.0	-	-	-	nmc	-	nmc
105 (M/69)	CRC	PD	2,318.0	-	-	-	nmc	-	nmc
106 (M/68)	CRC	SD	53.1	++	++	-	-	-	nt
107 (F/58)	CRC	PD	25.6	+	-	-	-	-	-
108 (M/62)	Lung	SD	0.8	++	-	++	++	++	++
109 (F/63)	CRC	PD	426.0	++	+	-	-	++	-
110 (F/68)	Pancreas	NE	62.0	nt	-	-	-	-	nt
111 (F/63)	CRC	NE	625.0	nt	-	-	-	-	nt
112 (F/68)	CRC	PD	6.7	++	+	-	-	-	-
113 (M/69)	CRC	PD	7.9	++	+	-	-	-	-
114 (M/61)	CRC	PD	2,385.0	-	-	-	+	+	+
115 (M/76)	CRC	PD	3.0	++	++	-	-	-	nt
			36.9 (median)		60%			20%	
Arm-II									
201 (F/57)	Lung	N/A	12.0	++	++	-	-	+	-
202 (F/63)	Breast	N/A	1.0	++	+	-	-	+	+
203 (F/59)	Lung	N/A	1.4	++	++	-	+	-	+
205 (F/49)	CRC	N/A	1.6	++	++	-	-	+	-
206 (F/51)	CRC	N/A	ND	+	-	-	-	+	-
207 (M/70)	Lung	N/A	4.4	++	++	-	-	-	nt
209 (M/54)	Liver	N/A	ND	++	-	-	-	-	nt
210 (F/63)	CRC	N/A	ND	-	++	-	-	-	nt
211 (M/60)	CRC	N/A	ND	++	-	-	-	-	nt
212 (F/58)	CRC	N/A	ND	++	+	-	-	-	-
213 (F/50)	Lung	N/A	53.3	++	++	-	+	++	+
214 (M/56)	CRC	N/A	1.5	++	-	-	-	+	-
			1.6 (median)		100%			58%	

NOTE: Patients with diarrhea are shaded.

Abbreviations: CRC, colorectal cancer; N/A, not applicable; ND, not detectable; NE, not evaluable; nmc, no more PBMCs for analysis; nt, not tested; PD, progressive disease; SD, stable disease; ++, antigen-specific immune response, multiple time points; +, antigen-specific immune response, single time point; -, no immune response.

prevaccination baseline (week 0) was detected at single [+] or multiple [++] time points; an increase of $\geq$ 2-fold over baseline was not mandated.

Antivaccine cellular responses were determined by *ex vivo* IFNγ ELISPOT assay (34–36); PBMCs (4×10^5) were stimulated (triplicate) with recombinant FrC protein (20 μg/mL; ref. 34), CAP-1 peptide (YLSGANLNL, 10 μg/mL; Protein Peptide Research UK) or control for 20 hours at 37°C and 5% CO_2 (Supplementary Table S1). PBMCs were also cultured *in vitro* for 8 days with CAP-1 peptide (10 μg/mL) or control in the presence of IL2 (20 IU/mL; day 3 and 6); IFNγ secretion was measured by ELISPOT following restimulation with peptide (10 μg/mL; Supplementary Table S1; refs. 28, 38).

CAP-1–specific $CD8^+$ T cells were identified using CAP-1 HLA-A*0201 tetramer (5 μg/mL; Protein Core Facility, University of Southampton, Southampton, United Kingdom) plus LIVE/DEAD Aqua dye (Invitrogen) and anti-CD3/CD8/CD4 staining at selected time points (Supplementary Table S1).

CAP-1–specific TCR rescue

CAP-1–specific T cells from patient #108 were expanded *in vitro* for 8 days in the presence of CAP-1 peptide (5 μg/mL), IL2 (10 IU/mL), and IL7 (5 μg/L), stained with specific tetramer or control (CMV-pp65/A2; Beckman Coulter), and single cell sorted into a 96-well V-bottom plate containing NIH-3T3 carrier cells on a BD FACSAria Flow Cytometer (BD Biosciences; ref. 39). RNA was isolated from single T cells, reverse transcribed to cDNA, and amplified by SMART-based 5'-RACE PCR. Full-length T-cell receptor (TCR) V(D)J regions were amplified with degenerate primers covering all functional Vα and Vβ genes in combination with Cα- and Cβ-specific primers (39) and cloned into pST1-TCRα/β-2βgUTR-A(120) for *in vitro* transcription.

CAP-1–specific T cells in primary tumor tissue

Genomic DNA was extracted from 10-μm paraffin sections after dewaxing and proteinase K digestion using a Qiagen DNA Extraction Kit (Qiagen). DNA (100 ng) was amplified by PCR (duplicate) with TCR Vβ29-1 CDR3-specific primers (forward: 5'-CTGCTCCTTCTCCTGGGACTAGGCT-3', reverse: 5'-TGGAGGGGTAAACCGTCCCTGTCC-3') for 37 cycles at 94°C/30 seconds, 64°C/30 seconds, and 72°C/60 seconds; amplification products were TA-cloned and sequenced.

Ethical standard

This study was conducted in accordance with the principles expressed in the 1964 Declaration of Helsinki and reviewed and approved by the Medicines and Healthcare Regulatory Authority, the Gene Therapy Advisory Committee, and the Local Research Ethics Committee. Written informed consent was obtained from all individual participants included in the study.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software, v6.0a (GraphPad Software, Inc.) and IBM SPSS Statistics,

v22.0 (IBM Corp). Univariate and multivariate analyses were performed in SPSS. Significance was assessed by two-sided, nonparametric Wilcoxon signed-rank test or Mann–Whitney test. Spearman rank correlation coefficient was used to test the association between two ranked variables. Distributions of time to event data were estimated using the Kaplan–Meier method and compared using Mantel–Cox log-rank testing and Cox regression analysis.

Results

Patient demographics and adverse events

Twenty-seven patients were recruited and evaluable for toxicity and immunologic responses; 15 patients had advanced disease (arm-I), and 12 patients were in radiological complete remission (arm-II; Table 1). Ten patients completed the study; 17 patients progressed prior to week 64 (Fig. 1A). The vaccine was well tolerated; toxicities were mainly grade 1 and resolved without intervention. A high frequency of diarrhea was observed with 23 episodes reported for 13 patients (48%); of these, 6 patients were in arm-I and 7 patients in arm-II. Diarrhea was reported in patients with bowel, lung, and breast cancer (Table 1), most frequently during the initial 8 weeks on study (Fig. 1B). Onset following nearest vaccination [median 12 (1–194) days] and duration [4.5 (1–95) days] were variable.

Clinical outcome

No RECIST responses were observed; patient #101 had a 50% decrease at a single metastatic site at week 16. All patients with advanced disease progressed and died during the study/follow-up period, with median TTP 119 (56–392) days and median OS 391 (62–1,058) days (Fig. 1A). Outcome was significantly better in patients without measurable disease at trial entry (arm-II, log-rank $P < 0.001$ for progression and survival); 4 patients progressed [median TTP 501 (235–1441) days] and 3 died [median follow-up 1,696 (766–2,393) days for all patients].

Serum CEA

Serum CEA was detectable in 22 (81%) patients at baseline, including all patients with advanced disease (arm-I) and 7 of 12 patients without measureable disease (arm-II). Median CEA was significantly greater in the serum of patients from arm-I at baseline [36.9 (0.8–2,385) μg/L] compared with those from arm-II [1.6 (1–53.3) μg/L, $P = 0.021$; Table 1]. Baseline CEA was elevated (>9 μg/L) in 12 patients and became elevated in 4 further patients while on study. Transient decreases in CEA were observed in 11 of 22 (50%) patients from both study arms (Table 2).

Anti-DOM immune responses

Humoral and cellular responses to DOM from FrC demonstrated successful vaccine delivery with an overall response rate of 60% (arm-I) and 100% (arm-II; Table 1). In patients with advanced disease, DOM responders had a better outcome than nonresponders (log-rank $P = 0.012$). Six patients that did not respond to the DOM helper sequence had significantly higher baseline CEA [median 371.5 (7.2–2,385) μg/L] than DOM responders [median 25.6 (0.8–426) μg/L, $P = 0.026$; Table 1]. DOM-specific antibody responses peaked between weeks 8 to 16 (Fig. 2A), while cellular response kinetics were variable (Fig. 2B). Mean anti-DOM humoral responses were significantly higher in patients from arm-II ($P = 0.020$; Fig. 2C). The difference in magnitude of cellular anti-DOM responses between study arms was not significant (Fig. 2D).

Presentation of CAP-1 on MHC class I

We confirmed CAP-1 presentation by mass spectrometry on frozen samples from HLA-A*0201$^+$ normal colon ($n = 7$), primary colorectal tumor ($n = 9$), and metastatic colorectal tumor ($n = 4$), but not on normal kidney tissues ($n = 4$, negative control; Fig. 3A–C).

Anti-CAP-1 immune responses

Overall, CAP-1–specific T-cell responses were detected in 10 (37%) patients postvaccination, including 3 of 15 (20%) patients with advanced disease (arm-I) and 7 of 12 (58%) patients without measureable disease at trial entry (arm-II). Low levels of IFNγ-secreting cells (<50 spots forming cells/10^6 PBMCs) were observed by *ex vivo* ELISPOT in 1 of 27 patients (#108: weeks

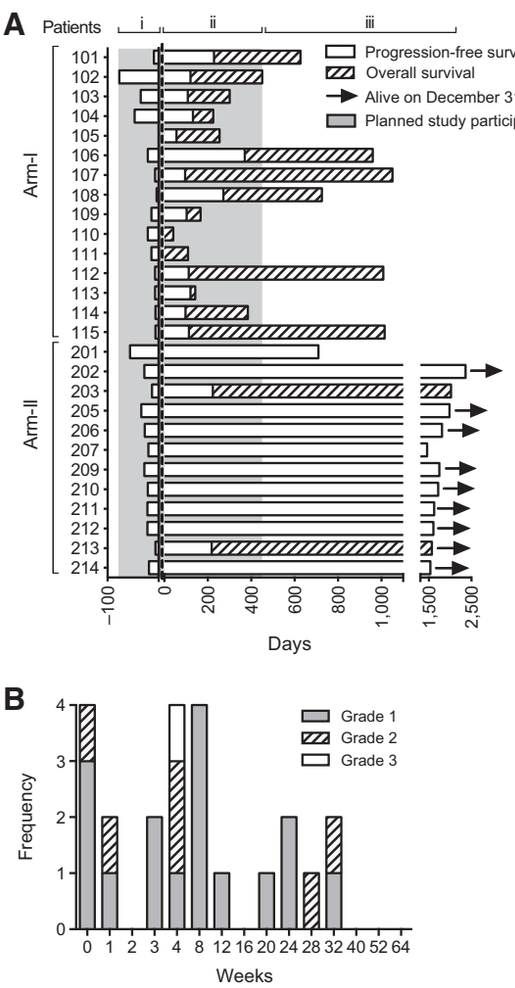

Figure 1.
Clinical effects of DNA vaccination. **A,** TTP and OS were recorded for each patient to event or censor date; data were frozen on December 31, 2012. Black dashed line, first vaccination at week 0; black arrow indicates that the patient was alive at censor date. The date of disease progression was not documented for patients #201 and #207. i, consent period; ii, monitored period; iii, off-study. **B,** frequency, timing, and grading of gastrointestinal adverse events (diarrhea) were recorded.

Table 2. Changes in serum CEA

	Patient ID	Onset of diarrhea[a]	Transient decrease in CEA (from baseline) Week (%)	Serum CEA status[b] Baseline	End of trial
Patients with diarrhea					
Arm-I	101	1	2 (−20.6) 4 (−1.6)	Elevated	Elevated
	103	0, 1, 8		Elevated	Elevated
	106	3	2 (−11.5) 4 (−10.4)	Elevated	Elevated
	107	0	2 (−9.8) 4 (−4.3)	Elevated	Elevated
	113	8	2 (−1.3)	Normal	Elevated
	115	24	28 (−3.3) 32 (−13.3) 40 (−30.0)	Normal	Normal
Arm-II	201	0	40 (−0.8) 64 (−10.0)	Elevated	Elevated
	202	0, 3	8 (−20.0) 12 (−10.0) 32 (−10.0) 52 (−20.0)	Normal	Normal
	205	4, 8, 24, 32	2 (−6.3) 4 (−12.5) 8 (−12.5) 12 (−12.5) 16 (−6.3)	Normal	Normal
	210	32		Normal	Normal
	212	1, 4, 8, 12, 20, 28		Normal	Normal
	213	4	2 (−13.7) 4 (−7.3)	Elevated	Elevated
	214	4	4 (−6.7) 16 (−6.7) 64 (−13.3)	Normal	Normal
% Patients with transient decreases in CEA: 77%					
Patients without diarrhea					
Arm-I	102	N/A		Normal	Elevated
	104	N/A		Elevated	Elevated
	105	N/A		Elevated	Elevated
	108	N/A		Normal	Normal
	109	N/A		Elevated	Elevated
	110	N/A		Elevated	Elevated
	111	N/A		Elevated	Elevated
	112	N/A	2 (−23.9)	Normal	Elevated
	114	N/A		Elevated	Elevated
Arm-II	203	N/A		Normal	Elevated
	206	N/A		Normal	Normal
	207	N/A		Normal	Normal
	209	N/A		Normal	Normal
	211	N/A		Normal	Normal
% Patients with transient decreases in CEA: 7%					

NOTE: Patients showing transient decreases in serum CEA are shaded.
[a]The week(s) on study when diarrhea symptoms were reported.
[b]Normal and elevated CEA are defined as <9 μg/L and >9 μg/L, respectively.

12, 20, 24, 28, and 32), increasing to 9 of 27 (33%) patients after *in vitro* culture (Table 1). Responses were of varying magnitude and were observed more frequently in patients from arm-II; 3 of 15 (20%) patients with advanced disease generated CAP-1–specific T-cell responses detectable by postculture ELISPOT compared with 6 of 12 (50%) patients without measureable disease. *Ex vivo* tetramer staining confirmed CAP-1–specific T cells postvaccination in patient #108 (Supplementary Fig. S1A) as well as patients #114, #203, and #213; CAP-1–specific responses could also be detected postculture by ELISPOT and/or tetramer staining in all cases (Table 1). CAP-1–specific T cells expanded (6.5- to 35.6-fold) *in vitro* with a good correlation between cultured ELISPOT and *ex vivo* tetramer data (Spearman rank $r = 0.735$, $P = 0.003$; Supplementary Fig. S1B). For patient #202, CAP-1 tetramer$^+$ T cells were evident following culture but could not be detected directly *ex vivo*.

Of the 18 patients that progressed and died during the study/follow-up period, 5 patients generated a CAP-1–specific response. We observed a median OS for CAP-1 responders of 730 (181–2,035) days compared with 528 (62–1,479) days in nonresponders (Supplementary Fig. S2); this difference did not reach significance.

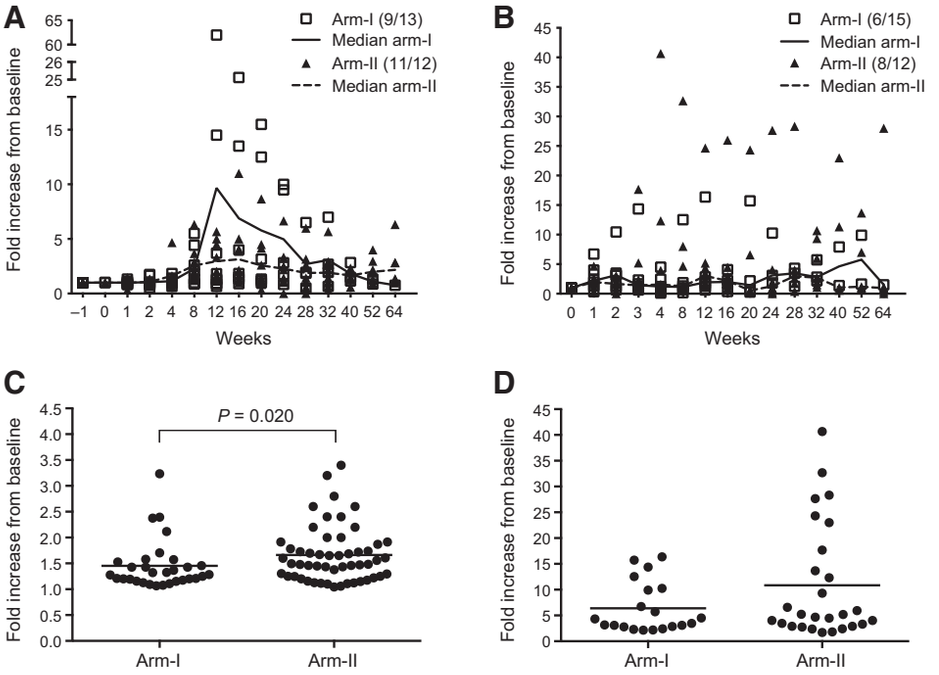

Figure 2.
Kinetics of DOM-specific immune responses and effect of tumor load. **A,** humoral responses to DOM were measured in the sera of vaccinated patients by ELISA. **B,** cellular responses to DOM were detected in PBMCs from vaccinated patients by *ex vivo* IFNγ ELISPOT. In both **A** and **B,** a fold increase from baseline was calculated for each subsequent visit (week) based on relative antibody units (humoral responses) and spot-forming cells/10^6 PBMC (cellular responses). Black line, median fold increase for all responding patients from arm-I (full) and arm-II (dashed). The proportion of responding patients for each study arm is indicated in brackets. **C,** the magnitude (fold increase from baseline) of DOM-specific antibody responses in patients from arm-I ($n = 31$ positive time points) and arm-II ($n = 53$ positive time points). **D,** the magnitude (fold increase from baseline) of DOM-specific cellular responses in patients from arm-I ($n = 20$ positive time points) and arm-II ($n = 27$ positive time points). Black horizontal line, mean (**C** and **D**).

Effect of tumor load on vaccine-induced immune responses

Globally, vaccine-specific immune responses were significantly more frequent in patients without measureable disease (arm-II) compared with patients with advanced disease (arm-I; $P = 0.037$; Supplementary Fig. S3). This was also true for CAP-1–specific immune responses ($P = 0.049$; Supplementary Fig. S3).

Vaccine-induced expansion of preexisting clonal CAP-1–specific T cells

We rescued paired TCRα/β chains from 24 single CAP-1 tetramer$^+$ CD8$^+$ T cells from patient #108 after antigen-specific *in vitro* expansion (Supplementary Fig. S4A). Eleven TCRs belonged to 2 different clonotypes (Supplementary Table S2); Vα12-2−J28−C paired with Vβ29-1−D1−J1-6−C1 represented the dominant clonotype ($n = 10$). CAP-1 specificity was confirmed by ELISPOT following transfection of primary CD8$^+$ T cells with full-length TCRα/β–encoding *in vitro*–transcribed mRNA (Supplementary Fig. S4B). Insufficient material was available for testing of expanded, patient-derived CAP-1–specific T cells against CEA$^+$ HLA-A*0201$^+$ tumor cells. The CDR3β sequence of the dominant TCR clonotype was detectable by RT-PCR in the blood at CAP-1 tetramer$^+$ time points, as well as from genomic DNA isolated from paraffin-embedded primary tissue resected 18 months prior to vaccination (data not shown).

Correlation of autoimmune effects with clinical outcome

Of the 18 patients who progressed and died during the study/follow-up period, 7 patients reported diarrhea. We observed a longer median OS for patients experiencing diarrhea [766 (149–1,058) days] compared with those that did not [391 (62–2,035) days; Supplementary Fig. S5]; this difference did not reach significance. For patients with advanced disease, median OS was 809 (149–1,058) days if the patient experienced diarrhea and 272 (62–1,016) days if diarrhea was not reported (Fig. 3D). In arm-II, 1 patient with diarrhea and 2 without progressed and died (Supplementary Fig. S6A). Multivariate analyses did not add any further information.

The median baseline CEA level in patients experiencing diarrhea [12 (1–53.3) μg/L for 11/13 patients with detectable CEA at baseline] was not significantly different to that of patients who did not report diarrhea [62 (0.8–2,385) μg/L for 11/14 patients; Table 1]. Diarrhea was associated with transient drops in CEA to below baseline levels (Table 2); decreases in CEA were significantly more frequent in patients with diarrhea (26 events, $n = 10$) compared with patients without (1 events, $n = 1$, $P < 0.001$) and identified a group of patients with advanced disease (arm-I) with a better OS [log-rank $P = 0.008$; HR = 0.14; 95% CI, 0.03–0.71; $P = 0.017$; Fig. 3E). Three patients who were in remission at trial entry (arm-II) subsequently progressed and died during the follow-up period, and of these, 1 patient showed transient drops in CEA (Supplementary Fig. S6B). Diarrhea and transient decreases in CEA were significantly associated (Spearman rank $r = 0.710$, $P < 0.001$; $r = 0.722$, $P = 0.002$, arm-I vs. $r = 0.714$, $P = 0.009$, arm-II).

Correlation of autoimmune effects with DOM-specific immune responses

All 13 patients who reported diarrhea responded immunologically. Diarrhea was observed in 13 of 22 (59%) patients with antivaccine responses, including 6 patients from arm-I and 7 from arm-II (Table 1). Anti-DOM cellular responses were of significantly greater magnitude ($P < 0.001$; Fig. 4A) and frequency ($P = 0.004$; Fig. 4B and C) in patients with diarrhea; no effect on humoral responses was observed.

Discussion

We conducted an exploratory phase I/II trial to test the safety and efficacy of an anti-CEA DNA fusion vaccine encoding

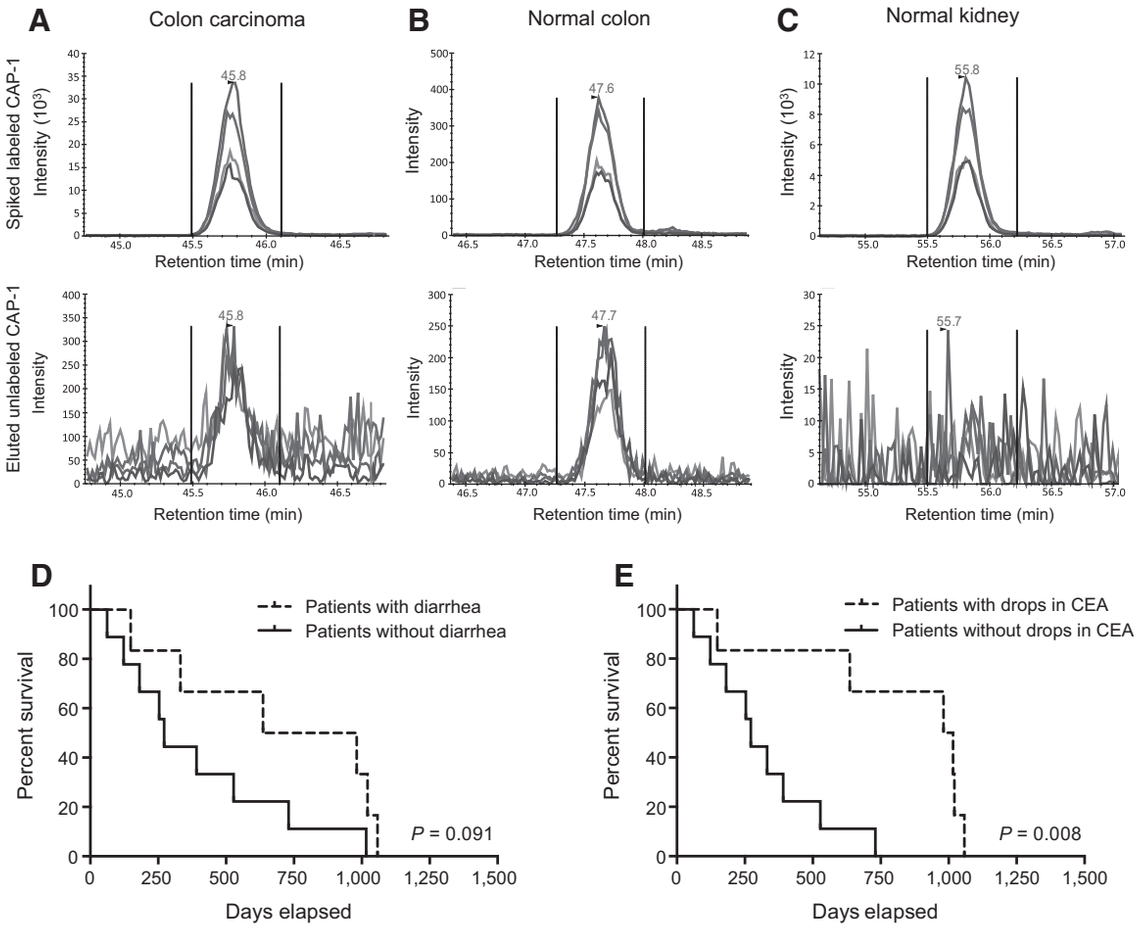

Figure 3.

Presentation of CAP-1 peptide on MHC class I and the effect of diarrhea and decreases in CEA on patient survival. **A–C,** the presentation of CAP-1 on MHC class I was assessed by peptide elution from colon carcinoma, normal colon, and normal kidney tissue, followed by targeted mass spectrometry: spiked isotope-labeled synthetic CAP-1 peptide, transitions from single-charged parent m/z = 971.527 (top); coelution of CAP-1 peptide, m/z = 964.5098^{+}, detected in colon carcinoma and normal tissue, but not in normal kidney (bottom). **D,** OS of patients with measurable disease (arm-I) with ($n = 6$) or without ($n = 9$) incidences of diarrhea during the time on study was assessed. **E,** OS of patients with measurable disease (arm-I) with ($n = 6$) or without ($n = 9$) transient drops in CEA to below that of baseline was assessed. **D** and **E,** OS is defined as the time (days) from consent to death.

DOM-CAP-1 in patients with CEA-expressing cancers. The vaccine was safe and well tolerated. Mild bowel-related toxicity was observed in 48% of patients and was associated with better clinical and immunologic outcomes. Diarrhea was significantly associated with transient drops in CEA and identified a group of patients with advanced disease with an 86% reduction in risk of death; decreases in CEA also appeared to link to improved survival for patients without measureable disease at trial entry, but with only three events the difference did not reach significance.

TCR rescue from CAP-1–specific T cells showed that CAP-1 can be recognized in the tissue spontaneously and that CAP-1–specific T cells can be expanded by DNA vaccination and detected in the circulation. Although we do not directly show effector function in the bowel mucosa, the demonstration of CAP-1 peptide presentation in both benign and malignant tissue makes it plausible that antigen-specific tissue recognition underpins our observations of both diarrhea and CEA drops as vaccine-mediated, on-target effects. In support, when targeting prostate-specific membrane antigen in 30 patients using an otherwise identical vaccine design, no diarrhea was reported (29, 31). As gastrointestinal effects were not restricted to patients with colorectal cancer, it seems unlikely that diarrhea is a reflection of previous surgical or cytotoxic manipulation of the bowel.

Dose-limiting gastrointestinal autoimmune pathology has been reported previously in association with effective CEA-targeted immunotherapy in preclinical (40) and human (27, 41) studies, demonstrating that CEA must be targeted with caution. Other trials of CEA-targeted vaccines have reported gastrointestinal side effects, however, without relating diarrhea events to clinical benefit (14, 16, 19), or interpretation was confounded by coadministration of chemotherapy (15). Conversely, some CEA vaccine trials do not report bowel-related adverse events (17, 18, 20–24), and this may reflect differences in vaccine potency.

Our vaccine was developed to exploit T-cell help from an undeleted repertoire against the foreign antigen DOM from tetanus (28, 42) to overcome tolerance to tumor-associated antigens; this can be achieved preclinically (28). We demonstrate here that if tolerance against CAP-1 exists in patients, it can be

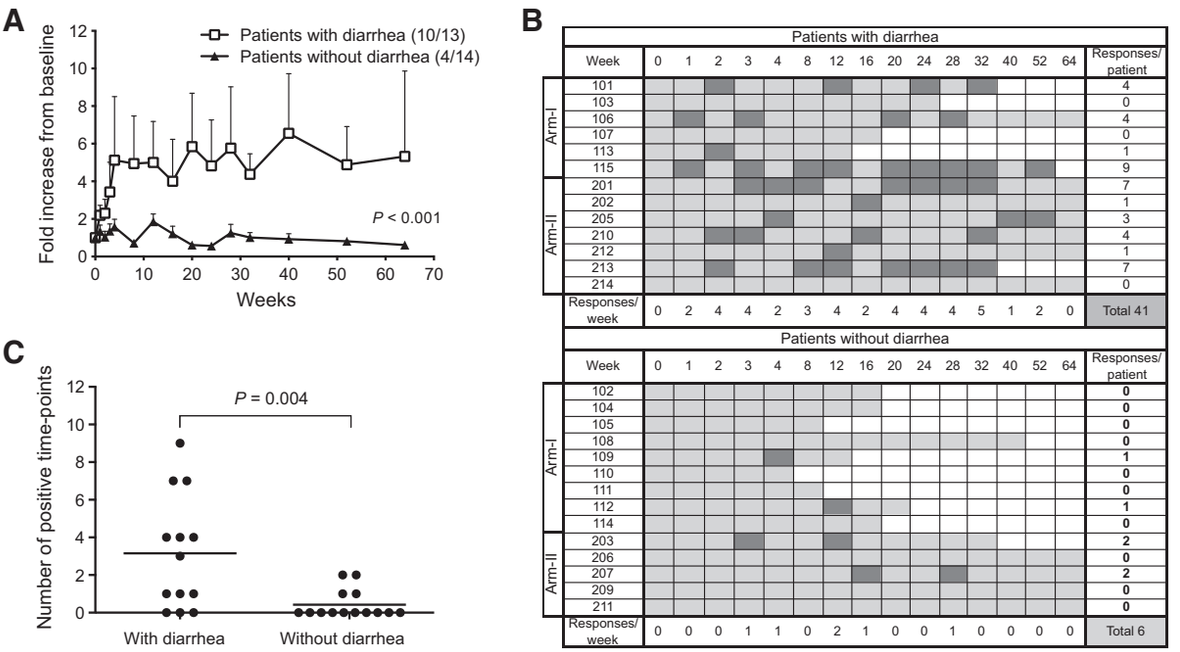

Figure 4.

Effect of diarrhea on DOM-specific immune responses. **A,** cellular responses to DOM were assessed by *ex vivo* IFNγ ELISPOT in PBMCs from vaccinated patients with ($n = 13$) or without ($n = 14$) diarrhea. Data, mean + SEM fold increase from baseline for all responding patients. The proportion of responding patients is indicated in brackets. **B,** the frequency and timing of anti-DOM cellular immune responses (black) in patients with (top) or without (bottom) diarrhea; where no anti-DOM response was detected at a given time point or no blood sample was collected for evaluation, time-points are indicated in gray or remain unshaded, respectively. **C,** the number of time points with a DOM-specific cellular response for each patient, with ($n = 41$ positive time points) and without ($n = 6$ positive time points) diarrhea. Black horizontal line, mean.

overcome relatively easily by vaccination. Conditioning of the vaccine site through tetanus-derived T-cell help has recently been assessed in a study in patients with glioblastoma (43) with previous exposure to tetanus where it was associated with improved outcomes. We are using linked T-cell help, which may be more efficient than separate antigens. Of note, in the glioblastoma study, the intended vaccine target pp65 from CMV is a xenogeneic sequence, and the bar for induction of anti-pp65 T-cell responses may be lower than for CEA (43).

Our data further demonstrate that clinical context affects vaccination outcome. Where previous studies were conducted in patients with advanced disease, tumor load may have adversely impacted measurable effects through systemic and local immunosuppression. This also implies that adverse immune effects, which must be linked to an intact immune effector function, could be missed (44–47). We demonstrate CAP-1- and DOM-specific responses of significantly lower frequency and magnitude in arm-I, the latter offering a measurable insight into the global loss of immune competence in patients with advanced epithelial cancers. It is uncertain whether this would be further confounded by ongoing or previous cytotoxic treatment (48). The observed immunologic differences were not due to differential presence of regulatory T cells in the blood, although other suppressive populations were not assessed. Functional assessment also helps to evaluate the immunologic context in which a particular vaccine approach is tested, and this should be considered in future vaccine studies.

Our vaccine was able to evoke CAP-1–specific T-cell responses in the blood of 37% of patients, with a good correlation between *ex vivo* CAP-1 tetramer$^+$ CD8$^+$ T cells and CAP-1–specific IFNγ-producing cells in ELISPOT postculture, linking specificity to functionality. Our data are consistent with previous studies, where IFNγ secretion by CAP-1–specific T cells *in vitro* correlated with clinical benefit (16, 20, 21, 24). A recent study (49) argued that CAP-1 is not efficiently processed and presented by CEA-expressing tumor cells. We demonstrate CAP-1 presentation on HLA-A*0201$^+$ MHC class I in both CEA-expressing tumors and normal bowel mucosa, supporting CAP-1 as a suitable target for immunotherapy. Our data further suggest that the assessment of diarrhea offers a more sensitive predictor of outcome than measuring circulating CAP-1–specific immune cells. Diarrhea was more frequent (48%) than the presence of CAP-1–specific T cells in the peripheral blood and may result from the homing of CAP-1–specific T cells to the site of antigen expression. It is tempting to speculate that the circulating immune cells are not the same as those with effector function in the tissue, as has been observed in patients with melanoma (50).

In summary, we demonstrate a link between tumor load and the ability to respond to vaccine-specific immune stimulation, that is, immune competence. Where vaccination induced CAP-1 responses, we observed longer TTP and OS, although this did not reach significance. More than half of the patients experienced diarrhea. This in turn linked to immunologic and clinical outcomes, in particular, to transient decreases in CEA that allowed the identification of a group of patients with measurable disease who fared much better. Our data suggest a breaking of peripheral tolerance to the self-antigen CAP-1 by expansion of antigen-specific CD8$^+$ T cells and further argue that these T cells can home to the tissue where the antigen is located and visible to T-cell

attack. Furthermore, our data argue that shared tumor-associated antigens can contribute to successful immune attack and should continue to be considered as important targets for immunotherapy. In this particular clinical context, diarrhea can be used as a biomarker predicting clinical and immunologic efficacy of CEA-targeting therapies and offers an extra dimension to the assessment of circulating immune cells. A larger, ideally randomized, study is required to confirm our observations. Combination studies to increase vaccine potency, for example by using anti-PD1 antibodies, are in development.

Disclosure of Potential Conflicts of Interest

T. Weinschenk has ownership interests (including patents) in Immatics Biotechnologies. P. Simon is listed as a co-inventor on a patent on a method for providing antigen-specific T-cell receptors that is owned by BioNTech Cell & Gene GmbH and TRON, Translational Oncology at the University Medical Center of the Johannes Gutenberg University. U. Sahin is an employee of and has ownership interests (including patents) in BioNTech. No potential conflicts of interest were disclosed by the other authors.

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