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

Running Title: Anti-CEA DNA Fusion Vaccination

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Key words: Immunotherapy; DNA vaccination; carcinoembryonic antigen; biomarker; colorectal cancer

Financial Support: This study was supported by a clinical trials grant from Cancer Research UK and in part by the NIHR Experimental Cancer Medicine Centre, Southampton, and by the Southampton Welcome Trust Clinical Research Facility.

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Conflict of Interest: TW is an employee and shareholder of immatics biotechnologies GmbH. PS and US are employees of BioNtech and Tron, respectively. The other authors declare no related conflicts of interest.

Author contributions: AM set up the assay validation, undertook immune evaluation and data analysis and wrote the manuscript. AC and KJM undertook assay validation, immune evaluation and data analysis and wrote the manuscript. LC and JS undertook immune evaluation. ST evaluated TCR of CAP-1 specific T-cells in the blood and tissue. AK made and pre-clinically evaluated the vaccine construct and co-developed the clinical trial protocol. PLE led vaccine production in the GMP facility. EB and CE contributed to the trial design, supervised study conduct for the sponsor and reviewed and monitored data. SH acted as the sponsor's medical advisor. AB, AO, SC, DAA and DIJ set up the study in the respective centres, recruited and followed patients. SC wrote the manuscript. TW undertook and evaluated the peptide presentation of CAP-1 and wrote the manuscript. PS and US undertook and evaluated the TCR rescue. GJT undertook the central pathology review and data analysis and wrote the manuscript. FKS developed the vaccine concept and contributed to the trial design and data interpretation. CHO designed and led the study, the data evaluation and writing of the manuscript. All authors reviewed, commented on and approved the final manuscript.

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.

Trial registration ID: EudraCT number: 2004-001932-21; Cancer Research UK protocol PH1/099

This study has been presented in part at the meeting of the American Society of Clinical Oncology, Abstract 2579, 2010, Chicago, Illinois, USA.

Translational Relevance

We report on a Phase I/II clinical trial of an anti-CEA DNA fusion vaccine in patients with 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 non-tolerized $CD4^+$ 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 non-malignant tissue, confirming it can be targeted for immunotherapy. We show that DNA vaccination can expand pre-existing 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 anti-vaccine immune responses. Better immune responses identify patients with advanced disease who live longer. Our data suggest that immunotherapy against non-mutated, cancer-associated antigens should be pursued and should be considered as a backbone to combination immunotherapies.

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 (1mg/dose) were administered up to week 12. Clinical and immunological follow-up was to week 64 or clinical/radiological disease.

Results: DOM-specific immune responses demonstrated successful vaccine delivery. All patients without measurable disease compared to 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 post-vaccination, 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 immunological responses (anti-DOM responses of greater magnitude ($p<0.001$), frequency ($p=0.004$) and duration) compared to 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.

Introduction

The goal of harnessing the immune system and provoking anti-tumor 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 immunological visibility of the tumor is reflected in the presence of tumor-infiltrating lymphocytes and links to survival (9, 10), activating pre-existing 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 been tested clinically; vaccines using peptides (14, 15), dendritic cells (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 anti-tumor 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 non-tolerized $CD4^+$ 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 multi-center, non-randomized, 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. Regulatory and ethical approval was obtained from the UK Medicines and Healthcare Regulatory Authority (MHRA), 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, UK. The study was sponsored by Cancer Research UK.

Patients

Eligiblity criteria included: a CEA-expressing malignancy confirmed by central immunohistochemical review of archived tumor; ≥18 years of age; HLA-A*0201 positivity; WHO performance status of ≤1; completion of oncological treatments >8 weeks prior to consent; lymphocyte count of $>1.0x10^9$/L; platelet count of $>50x10^9$/L, with normal clotting; no clinical or immunological signs of autoimmunity, specifically inflammatory bowel disease; absence of systemic immunosuppressants, such as steriods; 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 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 (1mg/dose) was delivered intramuscularly at weeks 0, 1, 2, 4, 8 and 12 (29, 31); on-study evaluation was 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 computer tomography was at baseline, weeks 16, 24 (Arm-I only) and 64/off-study visit, or as clinically required, with assessment using Response Evaluation Criteria in Solid Tumors (RECIST), v1. Regular blood collections for full blood count, biochemistry, serum CEA and immunological 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 (31st Dec 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 immune-precipitated with BB7.2 antibody (32). Peptides were analyzed by on-line nano-liquid-chromatography: nanoAcquity UPLC system with 25cm capillary columns (internal diameter 75μm) filled with 1.7μm BEH130 C18 particles (Waters, Eschborn, Germany). The acetonitrile (in 0.1% formic acid) gradient consisted of 1-13% for 10 min, 13-26.5% for 140 min and 26.5-34.5% for 40 min, with a 300nL/min flow rate. Analysis was on an LTQ-Orbitrap Velos with a nanoelectrospray ion source (Thermo Fisher Scientific, Bremen, Germany). CAP-1 peptide detection was performed by data independent acquisition in CID mode including an isotope(^{13}C6/^{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).

Immunological evaluation

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

Anti-vaccine 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 pre-vaccination baseline (week 0) was detected at single [+] or multiple [++] time-points; an increase of ≥ 2-fold over baseline was not mandated.

Anti-vaccine cellular responses were determined by *ex-vivo* IFN-γ ELISPOT assay (34-36); PBMCs ($4x10^5$) were stimulated (triplicate) with recombinant FrC protein (20μg/mL) (34), CAP-1 peptide (YLSGANLNL, 10μg/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 (10μg/mL) or control in the presence of IL-2 (20 IU/mL; day 3 and 6); IFN-γ secretion was measured by ELISPOT following re-stimulation with peptide (10μg/mL) (Supplementary Table S1) (28, 38).

CAP-1-specific $CD8^+$ T-cells were identified using CAP-1 HLA-A*0201 tetramer (5μg/mL; Protein Core Facility, University of Southampton) 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 (5μg/mL), IL-2 (10 IU/mL) and IL-7 (5μg/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 FACS Aria flow cytometer (BD Biosciences) (39). RNA was isolated from single T-cells, reverse transcribed to cDNA and amplified by SMART-based 5`-RACE PCR. Full-length 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 10μm paraffin sections after de-waxing and proteinase K digestion using a Qiagen DNA extraction kit (Qiagen, Hilden, Germany). DNA (100ng) 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/30s, 64°C/30s and 72°C/60s; amplification products were TA-cloned and sequenced.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software, v6.0a (GraphPad Software, Inc., La Jolla, USA) and IBM SPSS Statistics, v22.0 (IBM Corp., Armonk, USA). Univariate and multivariate analyses were performed in SPSS. Significance was assessed by two-sided, non-parametric Wilcoxon signed-rank test or Mann-Whitney test. Spearman's rank correlation coefficient was used to test the association between 2 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 immunological 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 I 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 (62-1058) 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 1696 (766-2393) 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 7/12 patients without measureable disease (Arm-II). Median CEA was significantly greater in the serum of patients from Arm-I at baseline (36.9 (0.8-2385) μg/L) compared to those from Arm-II (1.6 (1-53.3) μg/L, $p=0.021$; Table 1). Baseline CEA was elevated (>9μg/L) in 12 patients and became elevated in 4 further

patients while on study. Transient decreases in CEA were observed in 11/22 (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 non-responders (log-rank p=0.012). Six patients that did not respond to the DOM helper sequence had significantly higher baseline CEA (median 371.5 (7.2-2385) 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 8-16 (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, 3B and 3C).

Anti-CAP-1 immune responses

Overall, CAP-1-specific T-cell responses were detected in 10 (37%) patients post-vaccination, including 3/15 (20%) patients with advanced disease (Arm-I) and 7/12 (58%) patients without measureable disease at trial entry (Arm-II). Low levels of IFN-γ-secreting cells (<50 spots forming cells/10^6 PBMCs) were observed by *ex-vivo* ELISPOT in 1/27 patients (#108: weeks 12, 20, 24, 28 and 32), increasing to 9/27 (33%) patients after *in-vitro* culture (Table 1). Responses were of varying magnitude and were observed more frequently

in patients from Arm-II; 3/15 (20%) patients with advanced disease generated CAP-1-specific T-cell responses detectable by post-culture ELISPOT compared to 6/12 (50%) patients without measureable disease. *Ex-vivo* tetramer staining confirmed CAP-1-specific T-cells post-vaccination in patient #108 (Supplementary Fig. S1A) as well as patients #114, #203 and #213; CAP-1-specific responses could also be detected post-culture by ELISPOT and/or tetramer staining in all cases (Table 1). CAP-1-specific T-cells expanded (6.5-35.6-fold) *in-vitro* with a good correlation between cultured ELISPOT and *ex-vivo* tetramer data (Spearman's 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 (181-2035) days compared to 528 (62-1479) days in non-responders (Supplementary Fig. S2); this difference did not reach significance.

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 to 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 pre-existing 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$^+$ tumour 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 that progressed and died during the study/follow-up period, 7 patients reported diarrhea. We observed a longer median OS for patients experiencing diarrhea (766 (149-1058) days) compared to those that did not (391 (62-2035) days; Supplementary Fig. S5); this difference did not reach significance. For patients with advanced disease, median OS was 809 (149-1058) days if the patient experienced diarrhea and 272 (62-1016) 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 (0.8-2385) 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 to 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 that 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's 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 13/22 (59%) patients with anti-vaccine 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 4C) 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 pDOM-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 immunological 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). Since 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 pre-clinical (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 co-administration 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 pre-clinically (28). We demonstrate here that if tolerance against CAP-1 exists in patients, it can be 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 immune-suppression. 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 if this would be further confounded by ongoing or previous cytotoxic treatment (48). The observed immunological 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 immunological 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 IFN-γ-producing cells in ELISPOT post-culture, linking specificity to functionality. Our data are consistent with previous studies where IFN-γ 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 as well as normal bowel mucosa supporting CAP-1 as a suitable target for immunotherapy. Our data further suggests 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, i.e., immune competence. Where vaccination induced CAP-1 responses, we observed longer TTP and OS, although this did not reach significance. Over half of the patients experienced diarrhea. This in turn linked to immunological 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 immunological 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.

Acknowledgments

We thank Mr Scott Harris of the Medical Statistics Department, University of Southampton, for advice on the statistical evaluation and interpretation of this study.

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Table 1 Summary of patient demographics and immune responses

	Patient ID (F;M/Age)	Tumor		CEA	Anti-DOM		Anti-CAP-1			
		Primary tumor	Best response	Baseline CEA (µg/L)	Humoral (ELISA)	Cellular: *ex-vivo* (ELISPOT)	Cellular: *ex-vivo*		Cellular: cultured	
							ELISPOT	Tetramer	ELISPOT	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	2318.0	-	-	-	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	2385.0	-	-	-	+	+	+
	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: F, female; M, male; CRC, colorectal cancer; SD, stable disease; PD, progressive disease; NE, not evaluable; ND, not detectable; N/A, not applicable; ++, antigen-specific immune response, multiple time-points; +, antigen-specific immune response, single time-point; -, no immune response; nmc, no more PBMCs for analysis; nt, not tested.

Table 2 Changes in serum CEA

		Patient ID	Onset of diarrhea[a]	Transient decrease in CEA (from baseline)		Serum CEA status[b]	
				Week	%	Baseline	End of trial
Patients with diarrhea	Arm-I	**101**	1	2 4	-20.6 -1.6	Elevated	Elevated
		103	0, 1, 8			Elevated	Elevated
		106	3	2 4	-11.5 -10.4	Elevated	Elevated
		107	0	2 4	-9.8 -4.3	Elevated	Elevated
		113	8	2	-1.3	Normal	Elevated
		115	24	28 32 40	-3.3 -13.3 -30.0	Normal	Normal
	Arm-II	**201**	0	40 64	-0.8 -10.0	Elevated	Elevated
		202	0, 3	8 12 32 52	-20.0 -10.0 -10.0 -20.0	Normal	Normal
		205	4, 8, 24, 32	2 4 8 12 16	-6.3 -12.5 -12.5 -12.5 -6.3	Normal	Normal
		210	32			Normal	Normal
		212	1, 4, 8, 12, 20, 28			Normal	Normal
		213	4	2 4	-13.7 -7.3	Elevated	Elevated
		214	4	4 16 64	-6.7 -6.7 -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 <9μg/L and >9μg/L, respectively

Figure Legends

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 31st Dec 2012. The *black dashed line* indicates the first vaccination at week 0. A *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, the frequency, timing and grading of gastrointestinal adverse events (diarrhea) was recorded.

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* IFN-γ 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). A *black line* represents the 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). In both C and D a *black horizontal line* represents the mean.

Figure 3

Presentation of CAP-1 peptide on MHC class I and the effect of diarrhea and decreases in CEA on patient survival. A, B, and 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 panel); Co-elution of CAP-1 peptide, m/z = 964.5098^+, detected in colon carcinoma and normal

tissue, but not in normal kidney (bottom panel). 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. In D and E OS was defined as the time (days) from consent to death.

Figure 4

Effect of diarrhea on DOM-specific immune responses. A, cellular responses to DOM were assessed by *ex-vivo* IFN-γ ELISPOT in PBMCs from vaccinated patients with (n=13) or without (n=14) diarrhea. The data are presented as 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 panel) or without (bottom panel) 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 *grey* 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. A *black horizontal line* represents the mean.