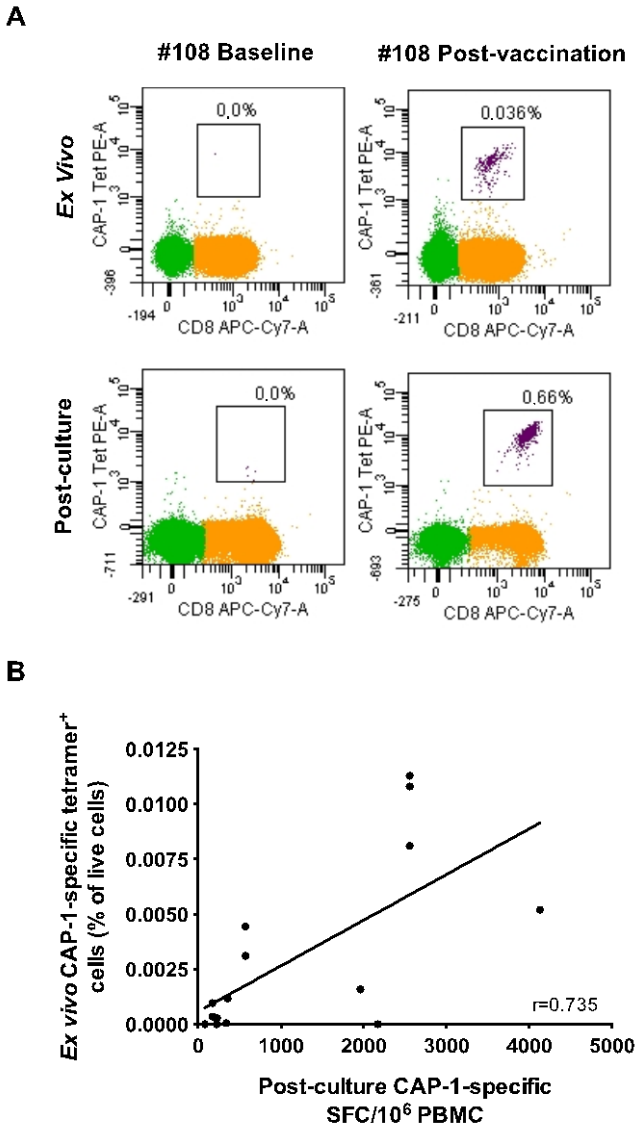
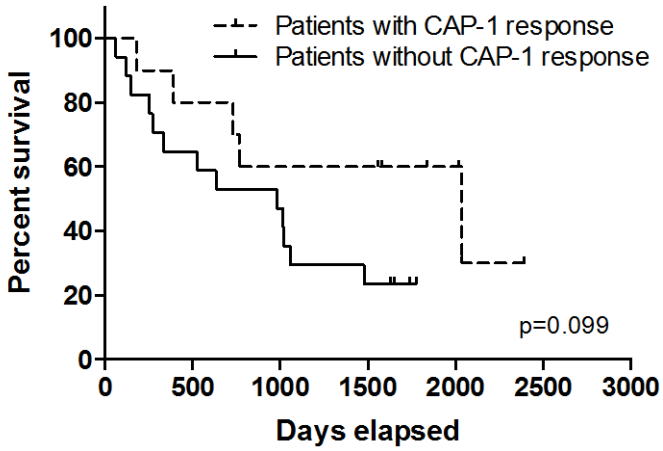


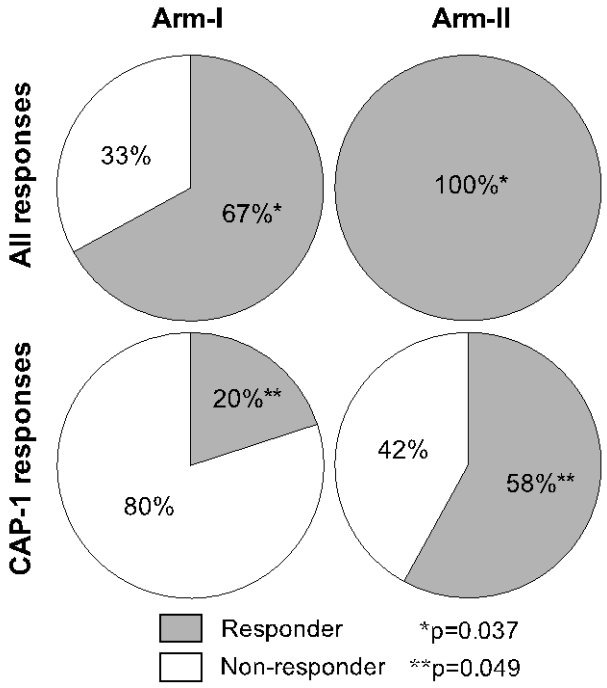
Supplementary Figure S1

Detection of CAP-1-specific T-cells. A, the frequency of CAP-1-specific T-cells was assessed in PBMCs of patient #108 at baseline (week 0) and post-vaccination (week 16) by tetramer staining, both *ex-vivo* and post-culture. CAP-1-specific T-cells were identified as CAP-1 tetramer$^+$ cells (*boxed*) within a live/CD3$^+$CD8$^+$ gate. B, the correlation between CAP-1-specific T-cells detected by cultured ELISPOT and *ex-vivo* tetramer staining. The percentage of CAP-1 tetramer$^+$ cells (of live cells) was plotted against the number of CAP-1-specific spot forming cells/10^6 PBMCs for any patient with a CAP-1-specific response detectable at any given time-point post-vaccination (n=15 CAP-1 tetramer$^+$ time-points).



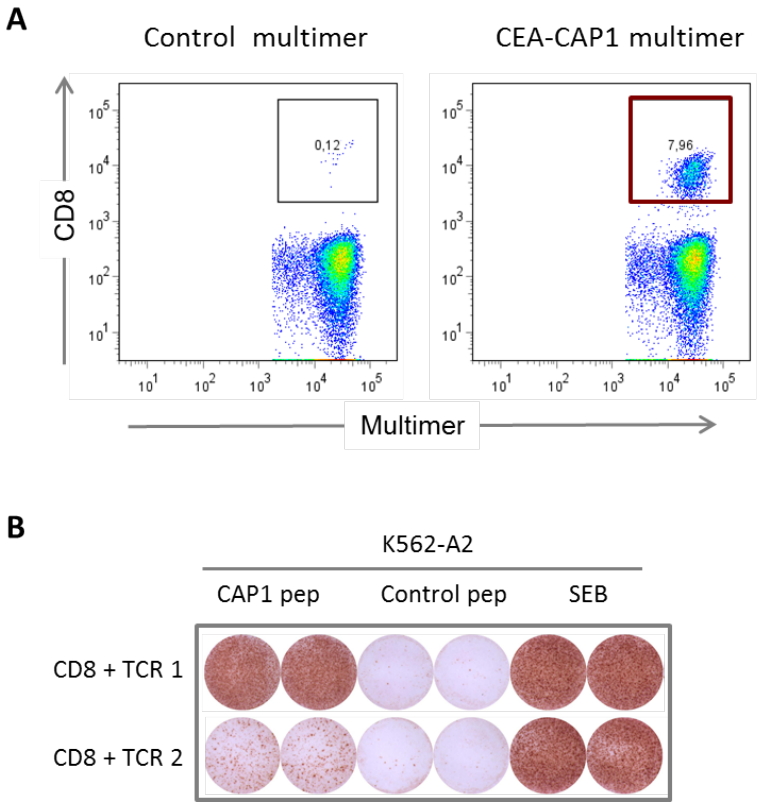
Supplementary Figure S2

Effect of CAP-1-specific immune responses on OS. OS of all patients from Arm-I and Arm-II with (n=10) or without (n=17) a CAP-1-specific immune response was assessed as the time (days) from consent to death or censor date; data were frozen on 31st Dec 2012 for analysis.



Supplementary Figure S3

Effect of tumor load on vaccine-induced immune responses. The proportion of patients from Arm-I (n=15) and Arm-II (n=12) with (*grey*) or without (*white*) vaccine-induced immune responses (humoral or cellular); responses to all components of the vaccine (DOM and/or CAP-1) and to CAP-1 alone are represented.

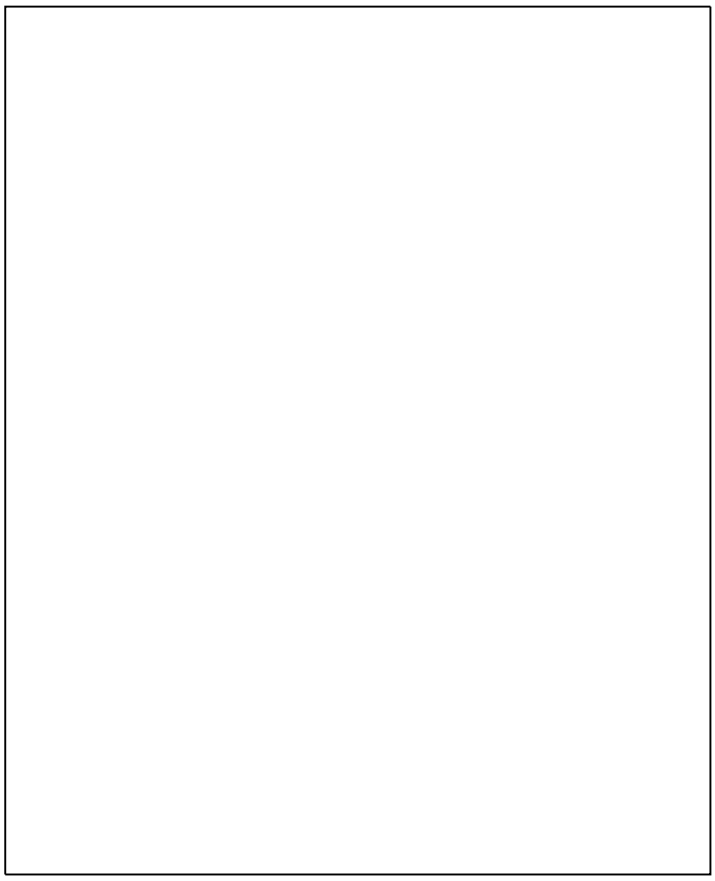


Supplementary Figure S4

Discovery of CAP1-specific TCRs from patient #108, A, CAP-1-specific $CD8^+$ T-cells were isolated from patient #108 after 8 days of *in-vitro* expansion in the presence of CAP-1 peptide, PBMCs were stained with CAP-1 tetramer or a control tetramer (CMV-pp65/A2). Cells were negatively gated on CD14/CD19/CD4 and CAP-1-specific T-cells were identified as CAP-1 $tetramer^+$ cells (*boxed*) within a live/$CD3^+CD8^+$ gate and sorted in a multi-well plate containing NIH-3T3 carrier cells for TCR cloning. B, the specificity of TCRs isolated from CAP-1 $tetramer^+/CD3^+CD8^+$ T-cells was assessed by IFN-γ ELISPOT. $CD8^+$ T-cells of an HLA-A*0201$^+$ healthy donor were transfected with TCR-α/β chain RNAs and tested for recognition of K562-A*0201 pulsed with CAP-1 peptide; an irrelevant peptide (PLAC1-31-39) and SEB served as a negative and positive control, respectively.

Supplementary Figure S5

Effect of diarrhea on OS. OS of all patients from Arm-I and Arm-II with (n=13) or without (n=14) episodes of diarrhea during the time on study was assessed as the time (days) from consent to death or censor date; data were frozen on 31st Dec 2012 for analysis.



Supplementary Figure S6

Effect of diarrhea and CEA drops on OS of patients without measurable disease. A, OS of patients from Arm-II with (n=7) or without (n=5) episodes of diarrhea during the time on study was assessed. B, OS of patients from Arm-II with (n=5) or without (n=7) transient drops in CEA during the time on study was assessed. In both A and B OS was defined as the time (days) from consent to death or censor date; data were frozen on 31st Dec 2012 for analysis.

Supplementary Data
McCann *et al.*, Targeting Carcinoembryonic Antigen with DNA Vaccination: On-Target Adverse Events Link with Immunological and Clinical Outcomes

Supplementary Table S1 MIATA − minimal information about T-cell assays

MODULE 1: MINIMAL INFORMATION ON THE SAMPLE
IA: DONOR
Patient demographics are reported in Table 1.
1B: SOURCE
At each study visit and for each of the vaccinated patients blood was collected in Lithium/Heparin tubes. The time between drawing and processing blood was <4 hrs. PBMCs were purified by density gradient centrifugation using Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway), counted and viability assessed prior to freezing (see section 2A).
1C: CRYOPORESERVATION AND STORAGE
PBMCs were frozen at a concentration of 5-10 x10^6 per vial in 50% human AB serum (Sigma-Aldrich Company Ltd., batch tested), 40% complete RPMI 1640 (RPMI plus sodium pyruvate, penicillin, streptomycin and L-glutamine; see section 2B) and 10% DMSO. Freezing additives were added at room temperature. Freezing followed a step-wise drop in temperature to -80°C using a Nalgene® Mr Frosty® (Thermo Fisher Scientific, Roskilde, Denmark). After ~24 hours, PBMCs were transferred to Liquid N_2 where they were stored until the end of follow-up (week 64) and prior to assay. PBMCs were stored in Liquid N_2 for a mean period of 22 months (median 20, range 4-51) prior to thawing and assay -duration was calculated based on baseline (week 0) PBMCs: For *ex-vivo* assays, mean 18 months (median 20, range 4-24); for cultured assays, mean 26 months (median 23, range 4-51).
1D: QUALITY OF CELL MATERIAL
Mean recovery of PBMCs after thawing was 97% (median 89, range 13-392), expressed as a percentage of initial PBMC input before freezing. Mean viability of PBMCs after thawing was 93% (median 95, range 36-100).
MODULE 2: MINIMAL INFORMATION ON THE ASSAY
2A: CELL COUNTING
Manual cell counting/viability assessment used a haemocytometer and 0.4% trypan blue stain (Sigma-Aldrich Company Ltd., Gillingham, UK). See also section 1D.
2B: MEDIUM/SERUM
Ex-vivo ELISPOT used RPMI 1640 (PAA Laboratories GmbH, Pasching, Austria) containing 1mM sodium pyruvate (Invitrogen Ltd., Paisley, UK), 100U/mL Penicillin – 0.1mg/mL streptomicin and 2mM L-glutamine (all Sigma-Aldrich Company Ltd.) plus 10% human AB Serum (Sigma-Aldrich Company Ltd., decomplemented and batch tested). Cultured ELISPOT used X-Vivo 15 (Lonza Group Ltd., Basel, Switzerland) plus 5% human AB serum (Sigma-Aldrich Company Ltd., decomplemented and batch tested).
2C: THE ASSAY
All assays were performed using cryopreserved PBMCs. *Ex-vivo* ELISPOT: Clear Multiscreen 96-well ELISPOT plates (MAIPS4510; Millipore (UK) Ltd., Watford, UK) were pre-coated with 10µg/mL anti-human IFN-γ antibody (mAb 1-D1K; MABTECH AB, Nacka Strand, Sweden) overnight at 4°C. PBMCs (4x10^5/well) were incubated in triplicate with medium only, test antigen and a negative and positive control for 20 hrs (peptides) or 40 hrs (proteins) at 37ºC in 5% CO_2. Test antigen was 10µg/mL of peptide: CAP-1, CEA 571-579, (YLSGANLNL); IMI, CEA 691-699, (IMIGVLVGV); a pool of four viral peptides comprising of cytomegalovirus (CMV), CMV pp65 493–499, (NLVPMVAVT), influenza A, Matrix 1 58–66, (GILCFVFTL), Epstein Barr virus (EBV), BMLFI 259–217 (GLCTLVAML) and measles, non-structural C protein 84–92 (KLWESPQEI), each at 2.5µg/mL. HLA-A0201-binding peptides were obtained with certificates of 95% purity from Peptide Protein Research Ltd (Fareham, UK). Test antigen was recombinant FrC protein at 20µg/mL: recombinant FrC was tagged with constant kappa and generated in house using the mammalian FreeStyle™ 293 expression system (Invitrogen), purification used a column of polyclonal sheep anti-human free constant kappa linked to Sepharose 4B

beads; protein quality was confirmed by SDS gel and western blot, endotoxin levels were confirmed to be < 1.0EU/mL.

Control antigens were HIV peptide, IV9 RT 476–484 (ILKEPVHGV), at 10µg/mL (negative) and Phytohemagglutinin (PHA; Sigma Aldrich Company Ltd.) at 5µg/mL (positive).

IFN-γ secreting memory T-cells specific for the antigen were detected as spots using 1µg/mL biotinylated anti-human IFN-γ antibody (mAb 7B61 biotin, MABTECH AB) followed by 1µg/mL streptavidin-alkaline phosphatise (MABTECH AB) followed by BCIP/NBT detection kit (Zymed GmbH, Rennbahnweg, Vienna).

Cultured ELISPOT: On day 0, cells were cultured at a concentration of $2x10^6$ per well with 10µg/mL of peptide and in a final volume of 2mL X-Vivo 15 medium plus 5% human AB serum in a 12-well, flat-bottomed plate. In test wells, cells were cultured with CAP-1 (YLSGANLNL) and HIV (ILKEPVHGV) peptides, the latter which served as a negative control, together in the same well and each at 10µg/mL. In positive control wells, cells were cultured with a pool of viral peptides (as detailed above). The plates were incubated at 37ºC in 5% CO_2. On days 3 and 6, cultures were fed with 20IU/mL IL-2 (R&D systems Inc., Minneapolis, MN, USA) delivered in 1mL X-Vivo plus 5% human AB serum, retaining a final volume of 2mL. On day 8, the cells were harvested, washed 3 times and rested overnight in complete RPMI plus 10% human AB serum (see section 2B) at 37C, 5% CO_2. The following day the cells were counted and viability assessed (see section 2A) and added at a concentration of $5x10^4$ per well (or $5x10^3$ per well for cells stimulated in culture with viral antigens) to ELISPOT plates previously coated with 10µg/mL anti-human IFN-γ antibody (mAb 1-D1K; MABTECH AB). The cells were stimulated in the plate overnight with medium only or with 10µg/mL of peptide matched to the peptide they were stimulated with in culture: CAP-1, HIV or viral pool. IFN-γ secreting memory T-cells specific for the antigen were detected as detailed above.

Tetramer staining: For those patients with surplus PBMCs after ELISPOT analysis, tetramer staining was performed on the pre-vaccination time-point and one or two pre-selected post-vaccination time-points where a CAP-1-specific response was indicated by cultured ELISPOT. For each time-point, $4x10^6$ PBMCs were stained with Aqua Live/Dead Fixable Dead Cell Staining kit (Invitrogen Ltd.) according to the manufacturer's instructions prior to CAP-1-PE tetramer staining at 5µg/mL in 50% FCS in FACS Buffer (0.5% BSA (w/v) and 0.1% NaN_3 (w/v) in PBS) for 30 mins at room temperature and in the dark. The diluted tetramer was pre-spun at 13,000rpm for 5 mins to pellet aggregates before adding to the cells. (For tetramer production see section 2E). Following tetramer staining the cells were washed once then stained with pre-optimized CD3-HV450 (UCHT1), CD4-PerCP.Cy5.5 (SK3) and CD8-APC-H7 (SK1) (all BD Biosciences, Oxford, UK) for 25 mins at 4°C and in the dark prior to flow cytometric analysis.

2D: CONTROLS

Ex-vivo ELISPOT: A negative control of media only and HIV peptide (10µg/mL) and a positive control of PHA (5µg/mL; Sigma Aldrich Company Ltd.) were used.

Cultured ELISPOT: A pool of viral peptides (10µg/mL) was used as a positive control for the *in-vitro* culture (see section 2C). In the final ELISPOT, cells were tested against media only and HIV peptide (10µg/mL) as a negative control and viral pool (10µg/mL) as a positive control.

Tetramer staining: PBMCs from patient #108 post-vaccination were stained with the CAP-1-PE tetramer and served as a positive control. Each patient and time-point was stained with an irrelevant tetramer as a negative control: WT1-37-PE (VLDFAPPGA) or WT1-126-PE (RMFPNAPYL).

2E: TETRAMER PRODUCTION

All tetramers were made in-house by the ECMC protein core facility, University of Southampton, UK.

HLA-monomer production and storage:

Peptide sequence (source)	YLSGANLNL (Peptide Protein Research Ltd)
Method	Altman *et. al.*, Science 1996
Production buffer	20mM Tris, pH 8
Final concentration	500µg/mL
Storage buffer	16% glycerol and 0.01% NaN_3 in PBS
Storage temperature	-80°C

HLA-tetramer production and storage:

Streptavidin-flouorochrome/HLA-monomer ratio	4:1
Purification method	Size exclusion chromatography
HLA-tetramer freezing buffer	16% glycerol, 0.01% NaN_3 and 0.5% BSA in PBS
Final concentration	45µg/mL
Storage temperature	-80°C

MODULE 3: DATA ACQUISITION

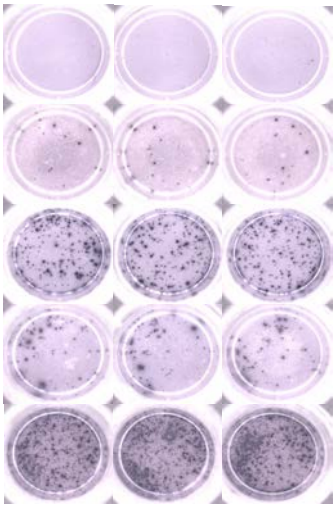
3A: EQUIPMENT AND SOFTWARE

ELISPOT: Spot forming cells (SFC) were counted using the AID ELISpot reader System ELR04 and AID ELISpot software version 4.0 (AutoImmun Diagnostika GmbH, Strassberg, Germany). Universal spot count settings, including spot intensity, size and gradient, were pre-defined and used as standard for all patients on study; T-cell responses of buffy coat donors to the common viral antigens FLU, EBV and CMV were used to define count settings.

Tetramer staining/flow cytometry: Stained PBMCs were analyzed on a BD FACSCanto II flow cytometer using FACSDiva™ software version 6.1.3 (BD Biosciences).

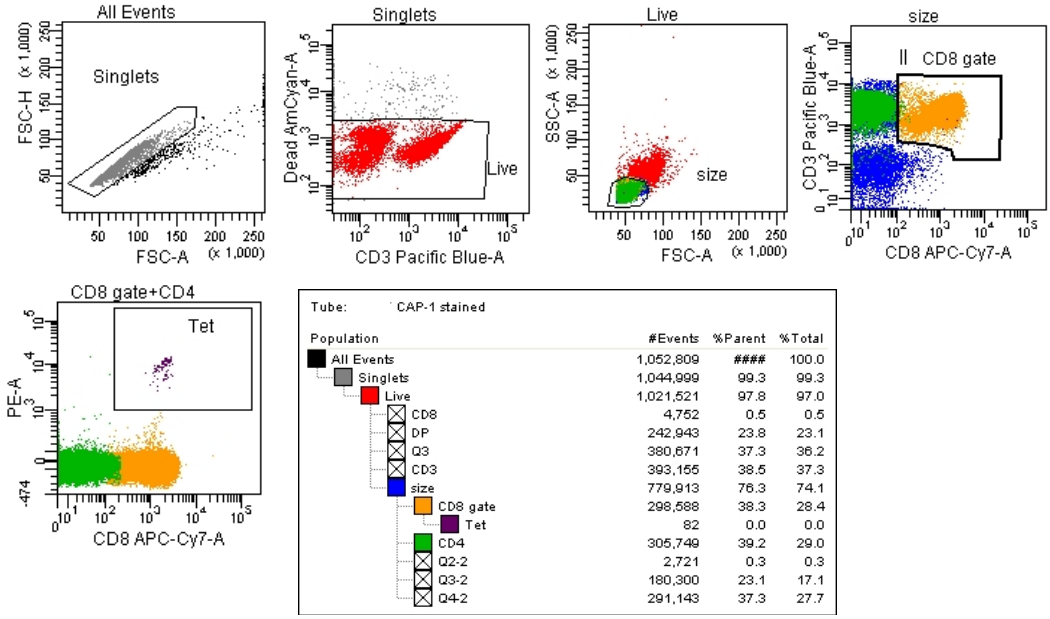
3B: ACQUISITION STRATEGY

ELISPOT:



Example of raw data: representative wells taken from *ex-vivo* and cultured ELISPOT assays.

Tetramer flow cytometry:



Tetramer gating strategy: CAP-1 tetramer$^+$ cells were identified using gating strategy above: singlets, live, lymphocytes by size, CD3$^+$CD8$^+$, tetramer$^+$. Where possible, a minimum of 100,000 CD3$^+$CD8$^+$CD4$^-$ cells were acquired per test.

MODULE 4: THE INTERPREATION OF RESULTS

4A: RAW DATA

ELISPOT: Plates were read (see section 3A) and data calculated as follows:

- SFC/well recalculated to SFC/million
- Mean of medium only control triplicate subtracted from antigen-specific wells
- Mean and SDEV calculated for each triplicate for antigen-specific responses

Data exclusion applied on a maximum of three wells per plate e.g. an outlier could be removed from a triplicate.

Below is a table of IFN-γ SFC/million from all patients at all time-points following stimulation with media only or antigen (CAP-1 or FrC).

		All samples			Baseline			Weeks 2-64		
		Median	**Mean**	**Range**	**Median**	**Mean**	**Range**	**Median**	**Mean**	**Range**
ex-vivo	**Background** (medium only)	11	20	0-175	13	18	0-70	11	20	0-175
	CAP-1-specific response	0	4	0-74	0	2	0-7	1	4	0-74
	Background (medium only)	7	13	0-284	7	15	0-76	7	13	0-284
	FrC-specific response	50	65	0-502	38	54	0-193	51	66	0-502
Cultured	**Background** (medium only)	47	275	0-8643	52	407	0-5333	47	256	0-8643
	(HIV)	0	37	0-753	0	11	0-67	0	41	0-753
	CAP-1-specific response	7	179	0-4120	0	74	0-1320	13	195	0-4120

Raw data can be provided on request.

Tetramer Flow cytometry: The stained cells were acquired on the flow cytometer and tetramer$^+$ cells detected as illustrated in the gating strategy above (see section 3A and B). Below is a table of tetramer$^+$ cells identified *ex-vivo* from patient's PBMCs before and after vaccination; the selection of a post-vaccination time-point for tetramer staining analysis was guided by cultured ELISPOT data. Where availability allowed, PBMCs were assayed on more than one occasion to monitor consistency. No irrelevant tetramer$^+$ events (WT1-37 or WT1-126) were detected.

	Baseline (week 0)		Post-vaccination	
Patient	**CAP-1 tetramer$^+$ (% of CD8 gate)**	**Tet$^+$ events /CD8$^+$ events**	**CAP-1 tetramer$^+$ (% of CD8 gate)**	**Tet$^+$ events /CD8$^+$ events**
#101	0.0000	0/326,090	0.0000 (week 12)	0/304,485
#102	0.0000	0/214,628	0.0000 (week 4)	0/99,730
#103	Not done	Not done	0.0000 (week 24)	0/115,222
#106	0.0000	0/69,138	0.0000 (week 4)	0/53,157
#107	0.0013	3/226,993	0.0000 (week 2)	0/218,382
#108	0.0007	5/519,323	0.0291 (week 12) 0.0232 (week 12) 0.0341 (week 12)	84/288,915 212/582,038 118/346,184
#109	0.0000	0/15,438	0.0000 (week 4)	0/27,893
#110	0.0016	4/244,863	0.0020 (week 4)	1/49,506
#111	0.0025	5/201,745	0.0022 (week 4)	3/134,502
#112	0.0000	0/354,336	0.0000 (week 3)	0/137,712

#113	0.0000	0/131,039	0.0042 (week 4)	4/96,251
#114	0.0006	1/170,293	0.0126 (week 4)	21/166,321
#115	0.0000	0/355,529	0.0000 (week 16)	0/319,684
#201	0.0003	2/284,387	0.0009 (week 32)	4/152,723
#202	0.0011 0.0003	3/268,823 2/284,387	0.0056 (week 16) 0.0009 (week 32)	3/53,787 4/152,723
#203	0.0009	1/110,160	0.0367 (week 12)	60/163,564
#205	0.0000	0/55,819	0.0000 (week 8)	0/63,276
#206	0.0000	0/255,983	0.0004 (week 16)	1/242,131
#207	0.0000	0/109,254	0.0019 (week 3)	1/51,422
#209	0.0000	0/360,959	0.0003 (week 3)	1/385,317
#210	0.0017	3/172,648	0.0005 (week 2)	1/188,510
#211	0.0006	1/167,833	0.0000 (week 4)	0/168,855
#212	0.0000	0/164,534	0.0024 (week 2)	4/164,256
#213	0.0014	1/73,206	0.0016 (week 3) 0.0146 (week 32)	2/125,406 7/48,109
#214	0.0012	1/82,693	0.0000 (week 4)	0/114,356

Raw data can be provided upon request.

4B: RESPONSE DETERMINATION, STATISTICAL TESTS AND EMPIRICAL RULES

ELISPOT: A response to an antigen at any given time-point was defined as mean SFC minus mean SFC of pre-vaccination baseline (wk 0) =

- greater than 25 or 100 SFC/million for *ex-vivo* and cultured ELISPOT, respectively
- greater than 2 SDEV above medium only wells
- Statistically significant ($p<0.05$); Statistical analyses were performed with the GraphPad Prism software program using a student t-test.

The definition criteria for an antigen-specific response was defined before the study commenced.

- A patient showing no response has zero time-points that meet the above criteria
- A responder [+] has a single time-point that meets the above criteria
- A responder [++] has more than one time-point that meets the above criteria

Tetramer Flow cytometry: An antigen-specific response was assigned when the percentage of CAP-1-specific $CD8^+$ T-cells was 2-fold or more greater than that of pre-vaccination baseline and above the lowest detection limit for the CAP-1 tetramer (0.0025% of $CD8^+$ cells determined by staining x10 HLA-$A2^-$ donors). Qualitative evaluation for CAP-1-specific $CD8^+$ T-cells was additionally undertaken by 3 blinded scientists experienced in the interpretation of tetramer analyses.

MODULE 5: THE LABORATORY ENVIRONMENT

5A: GENERAL LABORATORY OPERATION

This study was conducted in a laboratory that operates under GCP for laboratories principles.

5B: LABORATORY PROCEDURE STANDARDIZATION

This study was performed using standard operating procedures.

5C: STATUS OF ASSAY QUALIFICATION AND VALIDATION

Supplementary Data
McCann *et al.*, Targeting Carcinoembryonic Antigen with DNA Vaccination: On-Target Adverse Events Link with Immunological and Clinical Outcomes

This study was performed using validated assays.

Supplementary Table S2

Dominant TCR alpha and beta chains from CAP-1-specific T-cells

TCR	Alpha Chain	CDR3 alpha	Beta Chain	CDR3 beta
1	Vα12-2−J28−C	CAVQLTGAGSYQLTF	Vβ29-1−D1−J1-6−C1	CSVMDRDGLPLHF
2	Vα14*02−J9−C	CAMREVNTGGFKTIF	Vβ9−D2−J2-5−C2	CASSVELAGAETQYF