Therapeutic vaccination of Epstein-Barr Virus (EBV) Malignancies: phase 1 trial of recombinant Modified Vaccinia Ankara encoding EBV-encoded tumour antigens in nasopharyngeal carcinoma patients

Methods – additional information

Vaccine manufacture

The MVA-EL vaccine was supplied as a frozen aqueous suspension of 8.51 x 10^8 pfu/ml in 10mM Tris buffer containing 140mM NaCl, pH 7.7, manufactured to GMP grade by Impfstoffwerk Dessau-Tornau (Germany).

Blood sampling

Whole blood samples (typically 40ml) were collected into lithium heparin vacutainers during screening, on day 1 and 8 of each cycle and at week 10, week 11, week 14, month 6 and month 12. Blood was diluted with an equal amount of RPMI medium, layered onto a cushion of Ficoll density medium and centrifuged according to manufacturer's recommendations. Aliquots of cell-free plasma were collected before harvesting peripheral blood mononuclear cells (PBMC) from the top of the ficoll cushion. Plasma samples were frozen and PBMCs cryo-preserved by controlled cooling in medium containing fetal calf serum and DMSO prior to long-term storage in the vapor phase of liquid nitrogen. Samples were transported in liquid nitrogen to the School for Cancer Sciences, University of Birmingham for analysis. An additional 4ml EDTA whole blood sample was also collected for HLA class I and II typing by the National Blood Service, Vincent Drive, Edgbaston, Birmingham. HLA A2 subtyping was performed by Dr Martin Barnardo, Transplant Immunology, Oxford Transplant Centre, Churchill Hospital, Oxford, UK.

ELIspot Assays

ELIspot assays (Mabtech, Stockholm, Sweden) were performed to detect IFNγ-secreting epitope-specific T cells. For each patient, samples taken from across the whole vaccine course were thawed, rested overnight and then tested in the same assay. Cell input was 3x10^5 cells/well in all assays with one exception (patient HK0517 with 1x10^5 cells/well used in the assay utilizing defined epitope peptides; results were adjusted accordingly). In most assays cell numbers permitted the use of mean readings from two or three identical wells. Patient's cells from each time point were always tested with an appropriate negative control as well as the T cell mitogen phytohaemagglutin to confirm the cells were functional in the assay. Standardized controls included in the assays were PBMC from healthy volunteers (buffy coats from the National Blood Service, Birmingham) harvested in large numbers and cryopreserved in multiple small aliquots that had known performance properties in ELIspot assays. After assay completion, individual cytokine-producing cells visualized as dark spots (AP color development kit, Biorad, London, UK) were counted using an automated reader (AID GmbH, Strasberg, Germany). ELIspot data was accepted provided there was complete documentation of the procedure, noting

any deviations. Recorded operator error would have resulted in affected wells being excluded from analysis. Results of the standardized control samples confirmed assays had been undertaken correctly.

Two types of ELIspot assays were undertaken for the study. The first used pepmixes, pools of 15-mer peptides overlapping by 11 residues that span an antigen's entire primary sequence (JPT Peptide Technologies GmbH, Berlin, Germany). The pepmixes used included EBNA1 and LMP2 as well as the non-vaccine sequences EBNA 3A and combined influenza matrix and nucleoprotein (termed "FLU"); actin pepmix served as the negative control. PBMC from three time-points (pre-vaccination, post cycle 2 and post cycle3) were tested. For each PBMC sample, the readings for antigen recognition were adjusted by subtracting the readings of background IFN-γ release in control wells tested against actin peptides. The second type of ELIspot assay used peptides corresponding to defined HLA I- and HLA II-restricted T cell epitopes (Figure 1 and listed in Supplementary Table 1) to analyze the T cell response in finer detail. Peptides (synthesized by Alta Bioscience, Birmingham UK) were selected for use in each assay on the basis of the patient's HLA type and were used in the assay individually or, in cases where cell numbers were limiting, in pools of two or three peptides (when used in pools the concentration of each peptide was maintained). DMSO solvent, added to wells at the same concentration, served as the negative control. For each PBMC sample, the readings for peptide recognition were adjusted by subtracting the readings of background IFN-γ release in control wells tested against DMSO.

PBMC from screening, day 1 and 8 on each cycle, cycle 3 day 22 and 29, week 14, and at 6 and 12 months were tested in this ELIspot assay format. Complete per-patient data on ELIspot were presented graphically. In addition, the mean of adjusted readings across multiple time points was calculated: before vaccination (screening and C1D1); during vaccination (C1D8, C2D1, C2D8 and C3D1) and post vaccination (C3D8, C3D22 and C3D29).

Multiparametric flow cytometry

PBMC were recovered, washed twice in cold phosphate buffered saline (PBS) and stained on ice for 30 minutes with the following monoclonal antibodies (mAbs): v450-conjugated anti-CD3 (eBioscience, San Diego, California), APC-conjugated anti-CD4 (Becton Dickinson, San Jose, California), PE-Cy7-conjugated anti-CD25 (Becton Dickinson), FITC-conjugated anti-CD127 (Becton Dickinson). LIVE/DEAD fixable dead cell stain (Invitrogen, Carlsbad, California) was included so dead cells could be excluded from the subsequent data analysis. After staining with fluorochrome-conjugated mAbs, cells were washed twice then fixed and permeabilized using a Fixation/Permeabilization kit specifically designed for FoxP3 detection (eBioscience). After blocking with normal rat serum (eBioscience) cells were divided into two aliquots and stained with either PE-conjugated anti-FoxP3 mAb (clone PCH101, eBioscience) or the corresponding PE-conjugated isotype control mAb. Cells were washed twice with permeabilization buffer then analyzed on an LSRII flow cytometer (Becton Dickinson). Compensation was performed using compensation beads (Invitrogen) stained with each of the above fluorochrome-conjugated mAbs; beads stained with an ECD-conjugated mAb were used as a compensation control for the LIVE/DEAD stain. Data was processed using FACSDiva software (Becton

Dickinson). Forward scatter versus side scatter was used to identify PBMCs. Dead cells and CD3 negative cells were then excluded from the analysis by appropriate gating. Regulatory T cells were identified as either CD4+ CD25hi CD127lo or CD4+ CD25hi FoxP3+ cells and are expressed as a percentage of CD4+ T cells.

EBV genome levels

EBV genome levels in plasma were measured by real time quantitative PCR as described (Gallagher et al, Int J Cancer **84**, 442-448, 1999) in the Health Protection Agency Laboratory, Birmingham Heartlands Hospital, UK

Anti-EBNA1 Antibody Levels

EBNA1 specific IgG antibodies were measured using a commercial diagnostic ELISA (Diamedix, Miami FL) modifying the manufacturer's protocol to allow changes in antibody levels to be detected. Thus, an initial experiment was first performed in which a dilution series of each patient's pre-vaccination plasma sample was tested in the ELISA. This identified, for each patient, a plasma dilution that elicited an ELISA absorbance value in the midpoint of the assay's linear range. All plasma sample from a single patient were then diluted the same and tested in the ELISA – since the pre-vaccine sample is in the middle of the linear range this allowed any increase or decrease in antibody levels to be detected. An increase in ELISA absorbance > 25% of the pre-vaccine level was used to identify a response.

Anti-MVA immunoglobulin levels

Total anti-vaccinia virus antibody titer was measured by ELISA against vaccinia strain WR-infected cell lysates, inactivated by UV, as described (Johnson et al. J Gen Virol **92**, 2405-2410, 2011). IgG end-point titers were defined as the reciprocal serum dilution giving twice the average optical density obtained from BSA. A control serum from an individual vaccinated multiple times was used to normalize end-point titers between plates (anti-vaccinia titer: 1:12747). Serum samples from eight independent vaccinia-naïve individuals were used to calculate cut-off titers for seropositivity, defined as three times the geometric mean titer (GMT) for these samples; this cut off titer was 1:183.

Table S1. Related adverse events.*

Dose level	1			2			3			4			5		
Vaccine Dose	$5x10^7$ pfu			10^8 pfu			$2x10^8$ pfu			$3.3x10^8$ pfu			$5x10^8$ pfu		
Number of patients	3			3			3			3			6		
Toxicity grade	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Injection site reaction	2	1		3			1	1	1	3			6		
Flu-like (chills, fever)	1						2			1	1		3		
Fatigue	1	1		1	1		1			3			4	1	
Arthralgia	2	1												1	
Myalgia										2			2	1	
CNS (headaches, dizziness)	2						1						1		
Hepatotoxicity					1										

*The worst adverse event grading is listed per patient. Injection site reactions were graded using protocol specific criteria; Grade 1 – A reaction, including pain and itching, having a minimal effect on activities of daily living, erythema (<50% arm circumference) or swelling (<25% arm circumference), induration (<15mm) or ulceration (fluid vesicle or ulcer <10mm); Grade 2 – A reaction restricting activities of daily living, erythema (>50% arm circumference), swelling (<50% arm circumference), induration (15 to 30mm), ulceration (blood vesicle or ulcer 10 to 20mm, or healing $\leq$2 weeks), or phlebitis; Grade 3 - any reaction that prevents activities of daily living, swelling (>50% arm circumference), induration (>30mm) or ulceration (blood vesicle or ulcer >20mm, or healing >2 weeks); Grade 4 – any reaction requiring surgery. All other adverse events were graded using CTCAE version 3.0. (CNS, central nervous system).

Table S2. EBV epitope peptides used in defined epitope ELIspot assays.

Antigen	MHC	co-ordinates[1]	Restriction[2]	peptide	Vaccine sequence[3]
EBNA1	class I	407-415	B*35:01	HPV	HPVG**D**ADYFEY
	class I	574-582	A*02:03	VLK	VLKDAIKDL
	class II	475-489		NPKF	NPKFENIAEGLR**V**LL
	class II	485-499		LRVL	LR**V**LLARSHVERTT**E**
	class II	515-528		TSLY	TSLYNLRRGI**A**LA
	class II	529-543		PQCR	PQCR**I**TPLSRLPHGM
	class II	563-577	DRB1*15	MVFL	MVFLQTHIFAEVLKD
	class II	564-583	DPB1*05	VFLQ	VFLQTHIFAEVLKDAIKDLV
LMP2	class I	131-139	A*23:01/ A*24:02	PYL	PYLFWLAAI
	class I	200-208	B*40:01	IED	IEDPPFNS**I**
	class I	329-337	A*02:01	LLW	LLWTLVVLL
	class I	331--338	B*15:16	WTL	WTLVVLLI
	class I	340-350	A*11:01	SSC	SSCSSCPLSK**V**
	class I	356-364	A*02:01	FLY	FLYALALLL
	class I	419-427	A*24:02	TYG	TYGPVFM**S**L
	class I	426-434	A*02:01	SLG	**S**LGGLLTM**A**
	class I	447-455	A*02:03	LLS	LLSAWILTA
	class I	453-461	A*02:06	LTA	LTAGFLIFL

[1] Epitope co-ordinates are given as amino acids numbered according to the B95-8 reference strain of EBV. [2] Note that for some class II epitopes within EBNA1 the restricting allele is not fully defined. [3] Epitope sequences show the B95.8 reference sequence and polymorphisms identified from a typical Chinese strain of EBV. Epitope peptides used in ELIspot assays corresponded to the Chinese EBV sequence.

Table S3 Overview of epitope-specific responses.

Patient (HLA class I)	Epitopes tested		Epitopes recognized (frequency of responding cells, recalculated as responders / million PBMC)				
			Pre-vaccination response	Amplified response following vaccination			
				After cycle 1	After cycle 3	week 14	6 months
0101 (A2:03, A24, B13, B40)	LMP2:	LLW, FLY, SLG, LLS, LTAG, TYG, IED				not done	not done
	EBNA1:	VLK, PQCR				not done	not done
0102 (A2:03, A11, B15, B48)	LMP2:	SLG, [FLY+SSC], [LLW+LLS+LTAG]		[FLY+SSC] (108)	[FLY+SSC*] (221)	[FLY+SSC] (130)	
	EBNA1:	[LRVL+MVFL], VLK	VLK (1166)	[LRVL+MVFL] (161)	[LRVL*+ MVFL*] (138)		
0103 (A2:01, A11, B15, B54)	LMP2:	SLG, FLY, LLW, [LLS+LTA], SSC			SSC (107)		[LLS+LTA] (128)
	EBNA1:	VLK, [PQCR+VFLQ]				[PQCR+VFLQ] (54)	
0204 (A2:07, B46)	LMP2:	FLY, LLW, SLG					
	EBNA1:	VLK, [MVFL+VFLQ]					
0205 (A2:03, A3, B38)	LMP2:	FLY+LLW, LLS+LTA					not done
	EBNA1:	[VLK+SLG (LMP2)], [LRVL+VFLQ]				[VLK+SLG] (168)	not done
0206 (A2:03, A11, B38, B51)	LMP2:	SLG, [FLY+SSC], LLW, LLS		[FLY+SSC*] (45)	[FLY+SSC] (96), LLS (43)	[FLY+SSC] (62)	not done
	EBNA1:	VLK, VFLQ, [LRVL+TSLY], [NPKF+TSLY]		VLK (103), VFLQ (70)	VLK (201), VFLQ (142)	VLK (104), VFLQ (89), [LRVL+TSLY] (34)	not done

0307	LMP2:	[SLG+FLY], LLW, [LLS+LTA]					not done
(A2:07, A33, B13, B51)	EBNA1:	VLK, VFLQ, [LRVL+PQCR], [NPKF+TSLY]					not done
0308	LMP2:	SLG, [FLY+TYG], [LLW+PYL], [LLS+LTA]	[LLW+PYL] (143)	SLG (217)	SLG (236)	[FLY+TYG] (108)	[LLS+LTA] (206)
(A2:07, A24, B35, B46)	EBNA1:	VLK, HPV, [VFLQ+LRVL], [NPKF+TSLY+PQCR]					[VFLQ+LRVL] (36)
0309	LMP2:	SLG, FLY, LLW, [LLS+LTA]				FLY (152)	
(A2:07, A33, B46, B58)	EBNA1:	VLK, VFLQ, [LRVL+PQCR], [TSLY+NPKF]				VFLQ (147)	
0410	LMP2:	PYL, TYG, SSC					
(A11, A24, B38, B55)	EBNA1:	VFLQ, [TSLY+NPKF], [LRVL+PQCR]					
0411	LMP2:	SSC			SSC (110)	SSC (147)	not done
(A11, A33, B15, B58)	EBNA1:	VFLQ, [TSLY+NPKF], [LRVL+PQCR]	VFLQ (37), [TSLY+NPKF] (59)	VFLQ (147), [TSLY+NPKF] (135), [LRVL+PQCR] (51)	VFLQ (150), [TSLY+NPKF] (153), [LRVL+PQCR] (70)	VFLQ (117), [LRVL+PQCR] (90)	not done
0412	LMP2:	FLY, [SLG+IED], [LLW+TYG], [LLS+LTA]					not done
(A2:06, A24, B15, B40)	EBNA1:	VLK, VFLQ, [LRVL+PQCR]. [TSLY+NPKF]					not done
0513	LMP2:	[PYL+SLG], [FLY+TYG], [LLW+LLS+LTA]	[PYL+SLG] (99), [FLY+TYG] (105)	[PYL*+SLG] (304), [FLY+TYG*] (268), [LLW+LLS+LTA] (53)	[PYL+SLG] (333), [FLY+TYG] (263), [LLW+LLS+LTA] (93)	[PYL+SLG] (198)	

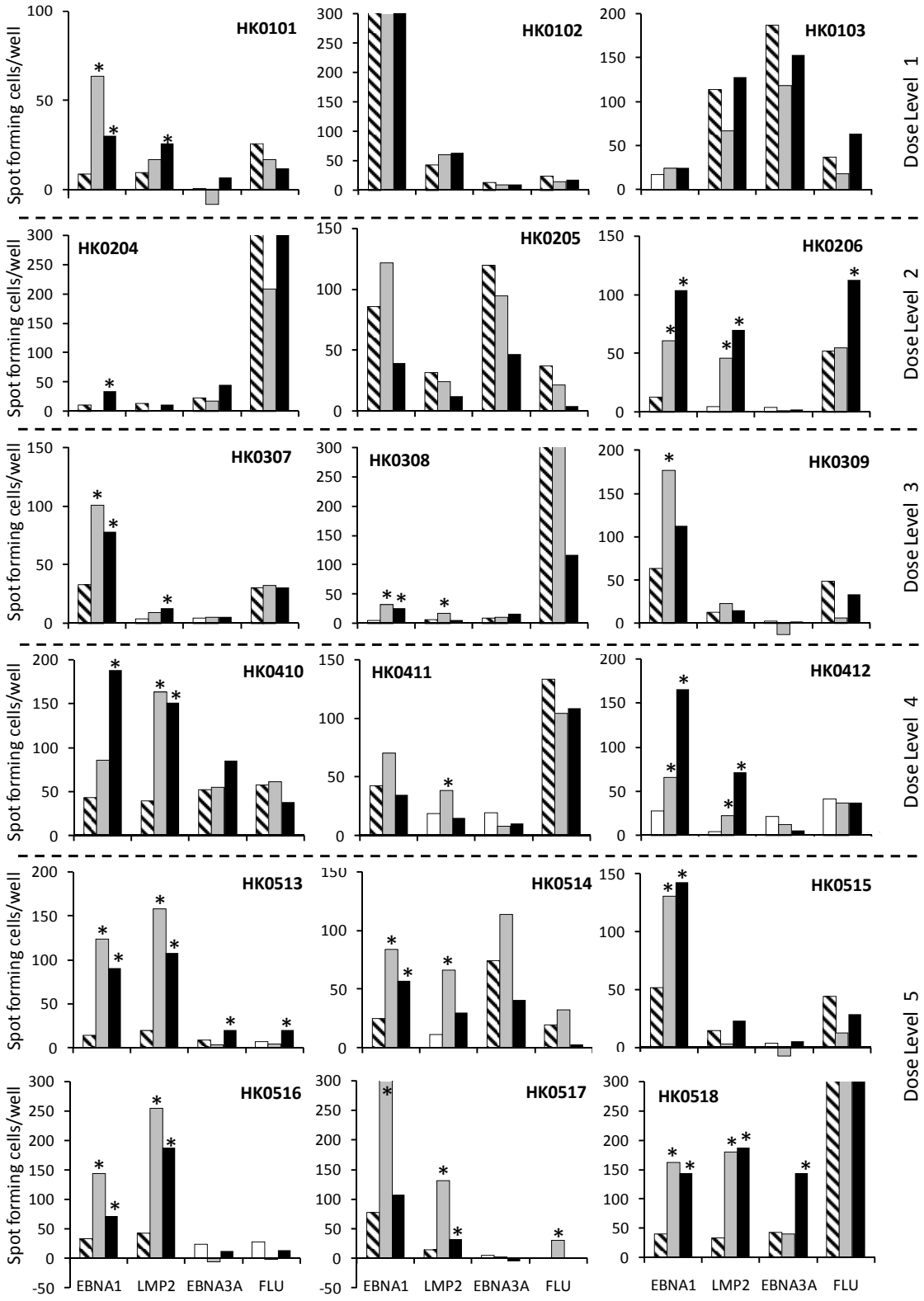
(A2:07, A24, B13, B46)	EBNA1:	VLK, [TSLY+VFLQ], [LRVL+PQCR]	VLK (108), [TSLY+VFLQ] (97), [LRVL+PQCR] (155)	VLK (242), [TSLY+VFLQ] (201)			
0514	LMP2:	[SLG+FLY], [LLW+LLS+LTA],					
(A2:07, A11, B15, B46)	EBNA1:	VLK, [TSLY+NPKF+VFLQ], [LRVL+PQCR]	[TSLY+NPKF+VFLQ] (110)	VLK (162), [TSLY+NPKF+VFLQ] (268)	VLK (87), [LRVL+PQCR] (62)		
0515	LMP2:	FLY, SLG, LLW, [LLS+LTA]					
(A2:03, A2:07, B38, B46)	EBNA1:	VLK, [TSLY+NPKF+VFLQ], [LRVL+PQCR]	VLK (133), [TSLY+NPKF+VFLQ] (76)	VLK (404), [TSLY*+NPKF+VFLQ*] (425)	VLK (474), [TSLY+NPKF+VFLQ] (515)	VLK (291), [TSLY+NPKF+VFLQ] (237)	VLK (315), [TSLY+NPKF+VFLQ] (278)
0516	LMP2:	PYL, TYG		PYL (152)	PYL (253)		PYL (226), TYG (151)
(A11, A24, B15, B54)	EBNA1:	VFLQ, [TSLY+NPKF], [LRVL+PQCR]	VFLQ (81)	VFLQ (401), [TSLY*+NPKF*] (176), [LRVL*+PQCR*] (166)	VFLQ (359), [TSLY+NPKF] (210)	VFLQ (241)	VFLQ (381), [TSLY+NPKF] (187), [LRVL+PQCR] (221)
0517	LMP2:	[WTL+IED+FLY], [LLW+LLS+LTA]					
(A2:06, B15, B40)	EBNA1:	[VLK+SLG] (LMP2), [TSLY+VFLQ], [PQCR+LRVL+NPKF]		[PQCR+LRVL+NPKF] (135)	[PQCR+LRVL+NPKF] (88)		
0518	LMP2:	PYL, TYG, IED				TYG (123)	
(A11, A24, B15, B40)	EBNA1:	VFLQ, [TSLY+NPKF], [LRVL+PQCR]		[TSLY+NPKF*] (450)	[TSLY+NPKF] (381)	[TSYL+NPKF] (218)	[TSLY+NPKF] (189)

Cryo-preserved PBMC from multiple time points were thawed and tested in ELIspot assays at $3x10^5$ /well against single peptides or groups of peptides (marked in square brackets) or against the DMSO solvent control. MHC class I and II epitopes are denoted by 3 and 4 residue codes respectively. Adjusted readings were the difference between the spot forming cells / well of the test and control wells. The mean of adjusted readings across multiple time points was calculated: before vaccination (screening and cycle 1 day 1 - C1D1); after cycle 1 (C1D8, C2D1, C2D8 and C3D1) after cycle 3 (C3D8, C3D22 and C3D29) and during follow-up (week 14 and 6 months post cycle 1). Measurements were also available for HK0513 and HK0514 1 year post cycle 1 vaccination, neither

demonstrating epitope recognition. Antigen recognition is defined as an adjusted reading (difference between test and control wells) exceeding 10sfc/300,000 PBMC and ratio of test versus control wells ≥2.0. Amplification of response during or post vaccination or during follow up is defined as antigen recognition in which the ratio compared to the pre-vaccination adjusted reading is ≥2.0. Frequencies, converted in this table to responders/million PBMC, are shown only for antigen recognition detected before vaccination or for amplified responses during or post vaccination or during follow up.

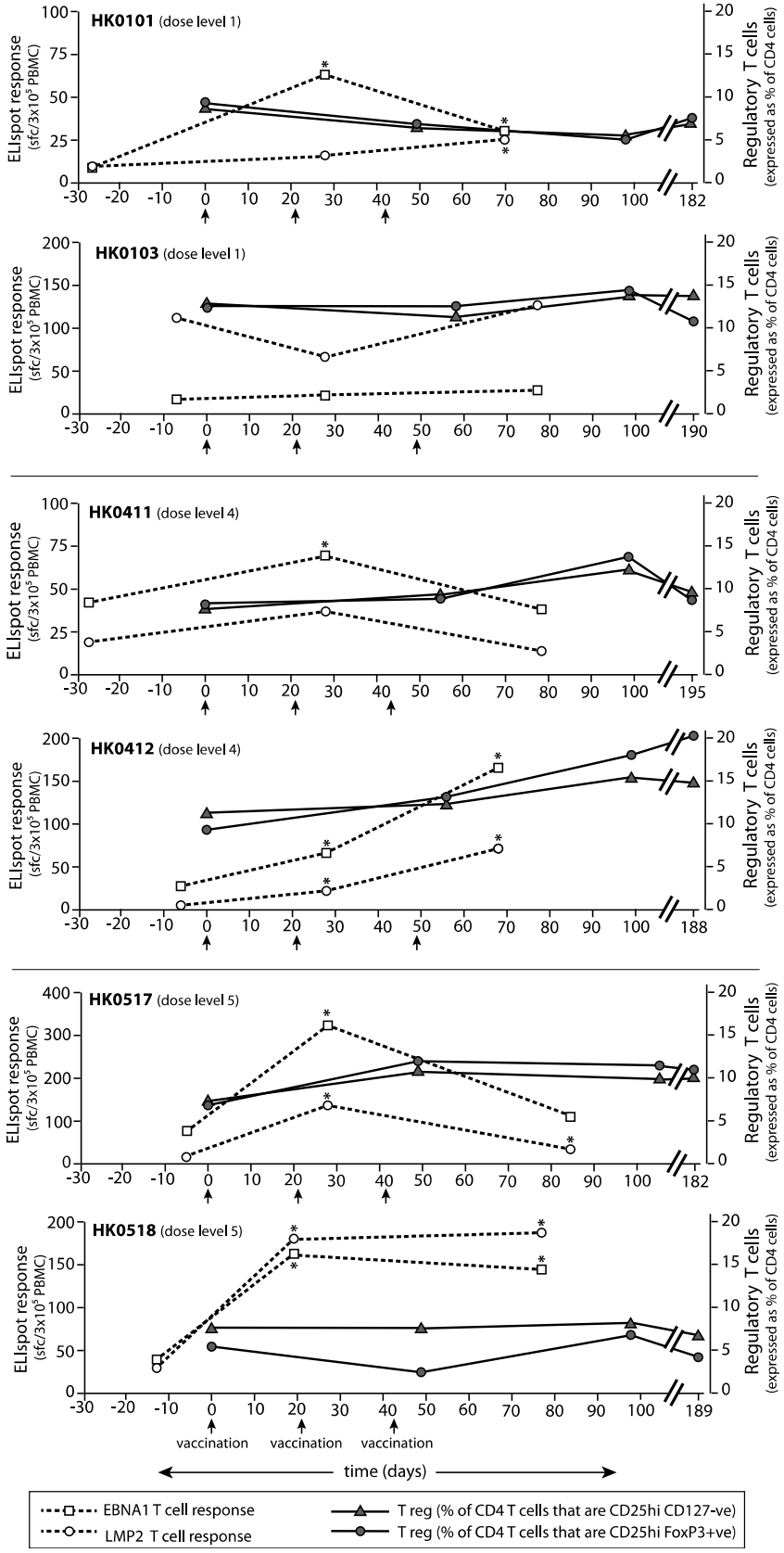
* Where responses were detected against a pool of peptides, it was possible for some timepoints to repeat the assay testing each peptide from the pool separately. In such cases the peptide(s) in the pool that elicited a T cell response on subsequent retesting are marked with an asterisk. Pools that are not marked by an asterisk were not retested due to sample unavailability.

Figure S1. Recognition of EBNA 1 and LMP2 by PBMC from vaccines.



Cryo-preserved PBMC from three time points were thawed and tested in ELIspot assays against overlapping peptides covering the whole sequence of Actin, EBNA1, LMP2, EBNA3A and influenza antigen (FLU). The cell input number was $3x10^5$ PBMC. The adjusted spot count / well is shown as the difference between the spot count /well for the test antigen and that for the self-antigen, actin. The median reading for actin was 12 spots / well, with 75% readings below 25 spots / well and a maximum reading of 87 spots / well (HK0516 C2D8). The upper limit of accuracy for measuring readings was 300 spots / well: the bars for readings exceeding this are truncated. Recognition of antigen was defined arbitrarily as a reading ≥10 spots / well and ≥2-fold greater than the reading for actin. The first sample had been taken before vaccination except for HK0102 in which the C1D8 sample is shown. Presence (hatched boxes) or absence (open boxes) of pre-existing recognition is shown. Subsequent samples were taken during vaccination on C2D8 (grey) and post-vaccination C3D22 or C3D29 (black). A change in immune recognition across the vaccine cycles was defined arbitrarily as meeting an additional criterion, the adjusted spot count against antigen at the during-vaccination or post-vaccination time points was ≥2-fold greater than the adjusted reading before vaccination (asterisk).

Figure S2. Regulatory T cell frequencies in patients before, during and after vaccination.



For six representative patients cryo-preserved PBMC from four time points were thawed and analyzed by multiparametric flow cytometry. These six patients include those that had small or large increases in EBNA1 or LMP2 T cell response following vaccination and one patient for whom no increase was observed (HK0103). The x-axis shows time (in days) with day 0 being when the first vaccination took place; the days when the second and third vaccinations took place are also indicated by arrows below the x-axis of each graph. The percentages of CD4+ T cells with a T reg phenotype (either CD25hi CD127lo or CD25hi FoxP3+) are shown as filled symbols. In normal individuals the frequency of CD25hi CD127lo T reg cells has been reported to be up to 7-8% of CD4+ T cells (Liu, Putnam, Xu-yu *et al*. Journal of Experimental Medicine **203:**1701-11). Also shown are the adjusted spot counts (sfc/3x10^5 PBMC) obtained by ELIspot assays using overlapping peptides covering EBNA1 or LMP2 (open symbols). Asterisks indicate where a doubling of the post-vaccination EBNA1 or LMP2 T cell frequency compared to the pre-vaccination value occurred, which is used as an arbitrary measure of immune response (see Supplementary Figure 1).