Phase 1 trial of recombinant Modified Vaccinia Ankara (MVA) encoding Epstein-Barr viral tumor antigens in nasopharyngeal carcinoma patients

Edwin P Hui *1, Graham S Taylor *2, Hui Jia 2, Brigette BY Ma 1, Stephen L Chan 1, Rosalie Ho 1, WL Wong 1, Steven Wilson 3, Benjamin F Johnson 4, Ceri Edwards 5, Deborah D Stocken 2, Alan B Rickinson 2, Neil M Steven #2, Anthony TC Chan #1

* EPH and GST contributed equally as first authors; # NMS and ATTC are joint corresponding authors.

1 State Key Laboratory in Oncology in South China, Sir YK Pao Center for Cancer, Hong Kong Cancer Institute and Li Ka Shing Institute for Health Sciences, Department of Clinical Oncology, The Chinese University of Hong Kong, Shatin, Hong Kong

2 Cancer Research UK Centre, School of Cancer Sciences, University of Birmingham, Birmingham, B15 2TA, United Kingdom

3 Health Protection Agency, West Midlands Public Health Laboratory, Heart of England Foundation Trust, Bordesley Green East, Birmingham, B9 5SS, United Kingdom

4 Section of Virology, Imperial College, London, W2 1PG, United Kingdom


Financial Support: Supported by grants from Cancer Research UK, Research Grant Council of Hong Kong (CUHK 460708) and Hong Kong Cancer Fund

Running title: Therapeutic vaccination of Epstein-Barr Virus malignancies

Contact: Dr Neil Steven. School of Cancer Sciences, University of Birmingham, Vincent Drive, Birmingham, B15 2TT, UK. n.m.steven@bham.ac.uk. Prof Anthony Chan, The Chinese University of Hong Kong, Hong Kong. anthony@clo.cuhk.edu.hk

Conflict of Interest Statement: The authors declare no conflict of interest

Manuscript Word Count: 5248 words. 5 Figures 2 Tables.
ABSTRACT

Epstein-Barr virus (EBV) is associated with several malignancies including nasopharyngeal carcinoma (NPC), a high incidence tumor in Chinese populations, in which tumor cells express the two EBV antigens EBNA1 and LMP2. Here we report the Phase I trial of a recombinant vaccinia virus, MVA-EL, which encodes an EBNA1/LMP2 fusion protein designed to boost T cell immunity to these antigens. The vaccine was delivered to Hong Kong NPC patients to determine a safe and immunogenic dose. The patients, all in remission >12 weeks after primary therapy, received 3 intradermal MVA-EL vaccinations at 3 weekly intervals, using 5 escalating dose levels between 5x10^7 and 5x10^8 pfu. Blood samples were taken during pre-screening, immediately before vaccination, 1 week afterward and at intervals up to one year later. Immunogenicity was tested by interferon-gamma ELIspot assays using complete EBNA1 and LMP2 15-mer peptide mixes and known epitope peptides relevant to patient MHC type.

Eighteen patients were treated, three per dose level 1-4 and six at the highest dose, without dose-limiting toxicity. T cell responses to one or both vaccine antigens were increased in 15/18 patients and, in many cases, were mapped to known CD4 and CD8 epitopes in EBNA1 and/or LMP2. The range of these responses suggested a direct relationship with vaccine dose, with all 6 patients at the highest dose level giving strong EBNA1/LMP2 responses. We concluded that MVA-EL is both safe and immunogenic, allowing the highest dose to be forwarded to Phase II studies examining clinical benefit.

Precis: Vaccination of nasopharyngeal carcinoma patients after they have completed chemo/radiotherapy produces potent immune responses targeting two viral antigens expressed in the tumor cells.
INTRODUCTION

Epstein-Barr virus (EBV) persists in most individuals as a life-long asymptomatic infection, with both lytic virus replication in the oropharynx and latent growth-transforming infections in the B lymphoid system being kept in check by immune T cell surveillance (1). Despite the usually asymptomatic nature of EBV carriage, the virus has potent growth-transforming ability in vitro and oncogenic potential in vivo, causing fatal B-lymphoproliferative disease lesions in the immunosuppressed and being strongly linked to several malignancies; these include endemic Burkitt Lymphoma, a subset of Hodgkin Lymphomas, certain aggressive T/NK cell lymphomas and an epithelial tumor, nasopharyngeal carcinoma (NPC) (2). Of these, NPC is the most important in world health terms, occurring worldwide but at particularly high incidence throughout South East Asia, especially among Chinese people (3); thus the Hong Kong Cancer Registry 2009 data report a male ASI of 14.6/100000, ranking NPC the 6th most common cancer in men in that region. Despite radiotherapy and (in meta-analyses) additional chemotherapy improving outcomes, 2 and 5-year disease-free survival rates are still only 63% and 52% respectively, and distant metastases account for over 40% of recurrences (4), at which point the prognosis is very poor (5, 6). New treatment modalities are therefore needed in both the adjuvant and salvage settings (7), particularly approaches designed for populations at highest risk.

The consistent presence of EBV in all NPC cells opens up the possibility of an immunotherapeutic approach, exploiting the potential of the immune T cell system to recognize tumor cells through their expression of viral antigens. In that regard, most NPCs express just two latent cycle proteins, the EB nuclear antigen 1 (EBNA1), a sequence-specific DNA binding protein involved in maintenance of the episomal virus genome, and latent membrane protein 2 (LMP2), a membrane signaling protein with growth-promoting activity in epithelial cells (2, 8, 9). From the study of healthy individuals, these proteins do not constitute dominant targets of the T cell response induced by natural EBV infection; nevertheless they do contain a number of sub-dominant CD8+ target
epitopes (mainly in LMP2) and several CD4 epitopes (mainly in EBNA1), presented by individual HLA I and HLA II alleles respectively (1). Indeed there is already some preliminary evidence both from a dendritic cell-based vaccine trial (10) and from subsequent adoptive T cell therapy trials (11-15) that boosting CD8 immunity to LMP2 epitopes may be of clinical benefit in NPC. The present approach is distinct from the above and is based on vaccination with a Modified Vaccinia Ankara (MVA) recombinant vector expressing the tumor-associated viral antigens; unlike the above cell-based protocols, such a procedure is not limited to specialized centers but has the potential for delivery on a large scale. The vaccine virus, MVA-EL, was constructed using sequences cloned from a typical Chinese EBV strain and encodes a functionally inactive fusion protein containing the C-terminal half of EBNA1 and full length LMP2A (16). Note that the C-terminal EBNA1 fragment includes almost all known epitopes in this protein (1) but lacks the gly-ala repeat domain that partly impairs presentation of cis-linked sequences to CD8+ T cells (17-19).

To date MVA or similar poxvirus-based therapeutic vaccines expressing cellular target antigens have been tested in certain non-virus-associated cancers; these trials almost all involve Caucasian patient cohorts and most focus only on CD8+ T cell responses, indeed often on responses restricted through just one common Caucasian HLA I allele, HLA-A*02:01 (20, 21). By contrast, the present trial has several distinct features. Firstly, the vaccine antigens are viral proteins against which patients were likely to have some (albeit low) pre-existing immunity (22, 23) Secondly, the analysis focuses on both CD4 and CD8 responses to these antigens, since both arms of the T cell system are likely to be important for effective tumor control in the longer term (24-26). Thirdly, the vaccine is specifically designed for use in the high-risk Chinese population, with its own distinctive array of HLA I and HLA II alleles that will determine target epitope choice (23, 27-29). Thus testing MVA-EL in NPC patients is important not only because it addresses an urgent clinical need but also because it has the potential to provide lessons of general relevance for tumor vaccination strategies.
Here we report the results of a first trial of MVA-EL vaccination in Chinese NPC patients, focusing on patients who were in remission following standard therapy. The objectives were firstly to determine tolerability of the vaccine across a range of doses, and secondly to determine its capacity to induce EBNA1 and/or LMP2 specific T cell responses, using natural fluctuations in immunity against irrelevant viral targets as an internal control. In the absence of safety concerns, the scale, specificity and diversity of vaccine responses were the critical factors determining dose selection and whether to proceed to further investigation.
METHODS

Patients

This was a phase I dose-escalation trial of MVA-EL vaccination for patients with EBV-associated NPC. Eligible patients had histologically poorly differentiated NPC, either confirmed as EBV-positive by in situ hybridisation for the non-coding EBER RNAs or with characteristically raised serum IgA to EBV capsid antigen. All patients were ≥12 weeks following completion of first line treatment, were in remission and satisfied the following inclusion criteria: ≥18 years; free from toxicities ≥grade 1; using adequate birth control; performance status 0-1; life expectancy >4 months; alkaline phosphatase and alanine aminotransferase (ALT) <1.5xULN; creatinine clearance >50ml/min, hemoglobin >10.0g/dl; lymphocytes >1.0x10^9/L; neutrophils ≥1.5x10^9/L; platelets ≥100x10^9/L; lacking known active hepatitis B, hepatitis C or human immunodeficiency virus infection, autoimmune or skin disease requiring therapy, active infection, severe egg allergy, splenic dysfunction, previous myeloablative or current immunosuppressive therapy.

The protocol (clinicaltrials.gov NCT01256853) was approved by the institutional review board. Conduct was ICH-GCP-compliant and all patients gave written informed consent.

Procedures

Patients received three intra-dermal vaccinations of MVA-EL (Impfstoffwerk Dessau-Tornau, Germany) delivered at 3 week intervals. Sequential cohorts of three patients received doses of either 5x10^7, 1x10^8, 2x10^8 or 3.3x10^8 plaque forming units (pfu) per vaccination (dose levels 1 to 5 respectively). To gain additional immune data, the highest dose cohort, dose level 05 receiving 5x10^8 pfu per vaccination, comprised 6 patients. Throughout the text patients are identified by the code xxyy, where xx is the vaccine dose level from 01 to 05 and yy is the patient number from 01 to 18. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events.
(CTCAE) Version 3.0 and using a protocol specific grading of local injection site reactions. Blood was collected at pre-screening, immediately before each vaccination (day 1, cycle 1, 2 or 3), 7 days after each vaccination (day 8, cycle 1, 2 or 3) and at weeks 10, 11, 14, and 6 and 12 months following the start of the vaccine course. Peripheral blood mononuclear cell (PBMC) and plasma samples were cryopreserved on each occasion, and T cell response assays on an individual patient were conducted on simultaneously thawed PBMC samples. ELIspot assays of T cell responses were conducted either against overlapping 15-mer peptides pools (pepmixes) spanning an antigen’s primary sequence or against defined epitopes peptides. Antigen-specific recognition was defined as numbers of spot forming cells (sfc) against a viral antigen ≥10sfc/well and ≥2-fold greater than that against a negative control (actin) pepmix, epitope-specific recognition as a reading ≥10sfc/well and ≥2-fold greater than against the DMSO solvent control. Mean spot counts for negative control wells (actin or DMSO) were subtracted from those for test wells (viral antigen pepmix or peptides respectively) to generate mean adjusted readings. Vaccine responses were defined as antigen or epitope recognition for which the adjusted counts post-vaccine cycle 2 or 3 were ≥2-fold higher than for pre-vaccination samples. EBV DNA loads in plasma were assayed by Q-PCR; EBNA1 and vaccinia-specific antibodies were measured before and after vaccination using standard assays. Full details of all procedures are provided in Supplementary information.

Design and statistical analysis

The primary objective was (i) to determine safety and to characterize the toxicity profile of MVA-EL vaccine and (ii) to describe changes in the frequency of functional T-cell responses to EBNA1 and LMP2. The secondary objective was to assess changes in levels of EBV genomes in plasma.

In all ELIspot assays, criteria to define antigen recognition and vaccine response were pre-determined and systematically applied. Exploratory analysis of pepmix assay readings was undertaken across all patients using repeated measures ANOVA to test whether mean adjusted readings at the three time-points differed
significantly. For antigens in which mean adjusted readings at the three time-points were significantly different, adjusted readings at each later time-point were compared to those for pre-vaccination samples using Dunnett’s multiple comparison test. The relationship between size of response and vaccine dose was analyzed by linear regression. All statistical testing was undertaken using GraphPad Prism 5.
RESULTS

Table 1 gives the clinical history of the 18 Chinese NPC patients (15 male, 3 female) who participated in the trial between November 2006 and April 2009. All had received prior radiotherapy and 14/18 also chemotherapy for their disease. The median duration from last treatment to first vaccination was 20 weeks (range 14-42 weeks) and all patients were clinically disease-free on entry into the trial. Each of these 18 individuals completed their three vaccinations at the planned dose and the trial proceeded through the five levels of increasing vaccine dose (5x10^7 to 5x10^8pfu/vaccination) with no evidence of dose-limiting toxicity. As detailed in Supplementary Table 1, related adverse events mainly involved injection site reactions, seen at grade 1 in most patients but extending to grade 2 in two cases and, transiently, to grade 3 in one individual. Fatigue, flu-like symptoms and arthralgia were observed occasionally at all vaccine doses whereas myalgia was only reported for patients on dose level 4-5.

The vaccine-coded EBNA1/LMP2 fusion protein, shown diagrammatically in Figure 1A, contains a 280 amino acid sequence from the EBNA1 C-terminus and the full 497 amino acids of LMP2A. To look for vaccine responses, PBMC samples cryopreserved from individual patients before vaccination, 1 week after the second vaccination (cycle 2, day 8) and within 4 weeks after the third vaccination (cycle 3, usually day 22) were tested in ELIspot assays against separate pepmixes encompassing the primary sequences of EBNA1 and LMP2. As controls, these assays tested the same PBMCs against separate pepmixes covering the sequences of actin (a self-antigen used to assess assay background), of EBNA3A (an EBV latent protein not encoded by the vaccine) and of the influenza matrix and nucleoproteins (FLU, an unrelated virus against which patients are expected to have pre-existing immunity). Note that these assays on total PBMCs detect the sum of both CD4+ and CD8+ T cell responses to epitope peptides within each pepmix. Figure 1B shows examples of the results. Patients 0516 and 0410 were typical of many vaccinees in that the pre-vaccine sample revealed low but detectable pre-existing memory responses to both EBNA1 and LMP2, as well as responses to EBNA3A and FLU. Importantly, the responses to
EBNA1 and LMP2 were clearly increased after the second and third vaccinations, whereas those to the control antigens were not. By contrast, patient 0206 was one of a minority in which we were unable to detect any significant pre-vaccine response to EBNA1 or LMP2 (nor, in this case, to EBNA3A). Here vaccination again induced a response to both EBNA1 and LMP2 but not to the EBNA3A control; interestingly, this patient also provided a rare example where the pre-existing FLU response was coincidentally raised in one of the two post-vaccine samples.

The numbers of patients showing significant increases in responsiveness following vaccination at the different dose levels are shown in Table 2 (shaded areas). Responses to EBNA1 were seen in 12 patients after two and three vaccine cycles, whereas LMP2 responses were seen in 10 patients after two and in 9 patients after three cycles. By contrast, only 4/18 patients showed a coincident increase in responses to one or other of the control antigens (EBNA3A or FLU) and in all but one case the increase was only seen in one of the two post-vaccine samples. This clear difference between vaccine-coded and control antigen responses was observed despite the fact that, before vaccination (see hatched columns, Supplementary Figure 1), the incidence of patients with low level pre-existing immunity to EBNA1 (15/18) and LMP2 (12/18) was not dissimilar to the numbers with pre-existing immunity to EBNA3A (9/18) or FLU (14/18). The individual pepmix assay results from all 18 patients are shown as histograms in Supplementary Figure 1, those responses that are significantly raised above pre-vaccine levels after two (grey columns) or three (black columns) being identified by asterisks. Vaccination was associated with increased responses to both EBNA1 and LMP2 in 11 patients, to EBNA1 only in 3 patients, and to LMP2 only in one case. Overall therefore, 15/18 patients responded to one or other vaccine-coded antigen, in most cases with responses being apparent both after two and after three vaccine cycles.

As to the absolute size of these vaccine-induced changes, Figure 2A combines data from all 18 patients and shows, for each pepmix antigen, the mean difference (+/- 95% confidence intervals) in Elispot counts per well after two
and three vaccine cycles and the corresponding pre-vaccine value. Clearly there were statistically significant increases in reactivity to the vaccine-coded EBNA1 and LMP2 antigens but no significant change in reactivity to the control EBNA3A and FLU antigens. It is also instructive to express these increases relative to the pre-existing levels of EBNA1-specific and LMP2-specific immunity seen in the pre-vaccine pepmix counts. Again combining data from all 18 patients, reactivity to EBNA1 was increased by means of 3.5-fold (range 0.0 to 8.8) and 3.2-fold (0.5 to 8.2) after two and three vaccine cycles respectively, and reactivity to LMP2 by means of 3.7-fold (0.0 to 10.5) and 3.9-fold (0.4 to 16.6) over the same period. It is also apparent from Table 2 that the proportion of patients mounting significant responses to EBNA1 and/or LMP2 was greater at dose level 5 than at the lower dose levels 1-4, implying a dose-response relationship. This was further explored by plotting response size (the difference in adjusted readings between pre- and post-vaccination levels) against vaccine dose. The results, shown in Figure 2B, indeed suggested a linear relationship for both EBNA1 and LMP2 responses following cycle 2 and for LMP2 responses following cycle 3 (Figure 2B).

While vaccine dose clearly appeared to influence both the incidence and size of responses to the MVA-EL vaccine, we considered two other possible factors that could be involved. The first factor was regulatory T (T reg) cells. Multi-parametric flow cytometric analysis of PBMCs from six representative patients showed the vaccine-induced increases in EBNA1- and/or LMP2-specific T cell responses seen in ELispot assays occurred in patients regardless of whether T reg cell frequencies were normal or elevated in their peripheral blood (Supplementary Figure 2). These data also showed that for 3/6 patients T reg cell frequency was unchanged by vaccination; a small increase was observed in two patients and a small decrease occurred in another. Focusing on two patients, 0517 and 0518, for whom long-term (six month) immune data was available and who both received the same vaccine dose we noted it was patient 0518, who had the lower frequency of T reg cells, in whom the EBNA1- and LMP2-specific T cell responses were sustained. Indeed, the EBNA1-specific response in this patient was readily detectable six months after vaccination (Supplementary Table 3).
The second factor was the prior status of the patient with respect to vaccinia exposure from smallpox vaccination. We therefore screened all 18 patients and identified 4 individuals who were negative for anti-vaccinia antibodies; these included patients receiving the MVA-EL vaccine across a range of doses from level 1 (patient 0101), through 3 (patient 0308) to level 5 (patients 0513, 0514). Figure 3A shows the size of vaccine-induced T cell responses to EBNA1 and LMP2 pepmixes in these 4 patients versus the 14 patients with serologic evidence of prior vaccinia exposure. No significant differences were apparent. Note that non-replicating MVA-based vaccines such as MVA-EL are primarily designed to induce T cell rather than antibody responses to the vaccine-coded antigen; nevertheless such vaccines would be expected to induce a serologic response to structural proteins of the MVA virion. Indeed we confirmed that anti-vaccinia antibodies were induced in the 4 previously vector-naïve recipients and were boosted in those with pre-existing immunity (data not shown).

Interestingly, as shown in Figure 3B, we also noted that the mean anti-EBNA1 antibody titer in the vaccine recipients was raised slightly above the pre-vaccine mean, although the increase only achieved statistical significance in the sample taken soon after completing the vaccine course, and not 6 or 12 months later. Again, there was no apparent difference in the antibody response to EBNA1 in vaccinia-naïve and vaccinia-immune individuals.

We then sought to further analyze the vaccine-induced T cell responses seen in the pepmix assays. Previous work has identified a number of peptide epitopes within the primary sequences of EBNA1 and LMP2 recognized by CD4+ and CD8+ T cells (1, 23, 27-29). Figure 1A shows the location of 6 known CD4 epitopes and 12 known CD8 epitopes lying within the EBNA1-LMP2 vaccine antigen; their coordinates, amino acid sequences and, where available, their HLA restrictions are detailed in Supplementary Table 2. Additional PBMC aliquots were tested in Elispot assays against epitope peptides relevant to their HLA I and HLA II types, either using the peptides individually or, where cell numbers were limiting, against small pools of relevant CD4 or CD8 epitopes.
Figure 4A shows the epitope-specific Elispot assay plates from one such patient, 0206 (HLA-A*11:01, A*02:03 and HLA-DP5-positive), who had already shown evidence of vaccine-induced EBNA1 and LMP2 responses in the pepmix assays (see Figure 1 and Supplementary Figure 1). In line with that LMP2 pepmix response, this patient gave evidence of responses to two known LMP2-derived CD8 epitopes, SSC and LLS, that are restricted by the A*11:01 and A*02:03 alleles respectively; likewise, in line with the EBNA1 pepmix response, this patient responded to a CD8 epitope, VLK/A*02:03, and a CD4 epitope, VFLQ/DP5, both derived from EBNA1. These results, and corresponding data from epitope peptide screening on two further vaccine recipients, patients 0513 and 0516, are shown as graphs in Figure 4B, below histograms of the same patients’ pepmix responses. Patients 0513 and 0516 had been identified as responding to LMP2 in pepmix assays; both individuals were HLA-A*24:02 positive and indeed both responded to two LMP2-derived CD8 epitopes restricted by this allele, PYL and TYG. Likewise both patients had shown an EBNA1 pepmix response and epitope peptide screening confirmed that both responded to one or more relevant EBNA1-derived CD4 epitopes.

The detailed results obtained from all patients screened in epitope peptide assays are recorded in Supplementary Table 3. Overall 11/18 patients gave evidence of epitope responses appropriate to their HLA type in the period during the vaccine course (up to, but not including, week 14). Responses were again seen most consistently in individuals receiving the highest vaccine dose. Six-month follow up samples were available for 12 patients who had an EBNA1 and/or LMP2 response during vaccination allowing the durability of these responses to be measured. Persistence of previously identified vaccine responses was not observed for the six patients vaccinated at dose levels 1-4 but were identified in 3/6 patients at the highest dose level.

All patients were also screened by Q-PCR for the presence of EBV DNA in plasma; this not only provides a surrogate measure of tumor burden in NPC (30) but also shows a transient increase as tumor cells lyse in vivo immediately following radio/chemo-therapy (31). In this trial all patients were clinically disease-free at
the time of vaccination and so, not surprisingly, most patients’ plasma samples proved to be EBV DNA-negative both before and one month after vaccination. However, one patient (0517) did have low but detectable pre-vaccination levels of plasma EBV DNA. Interestingly, as shown in Figure 5, this patient made a significant T cell response to both EBNA1 and LMP2 pepmixes (and to at least one EBNA1 CD4 epitope) during the course of vaccination, though this appeared to fall subsequently. Immediately following the peak of this immunologic response, EBV genome levels in plasma rose to a peak at 10 weeks and then fell to undetectable levels in the 6 and 12 month samples. After 19 months, however, this patient experienced nodal relapse, with plasma EBV DNA again detectable.
DISCUSSION

Virus-associated malignancies offer an important model for the development of T cell-based cancer therapies, and the example of NPC is particularly interesting in several respects. On the one hand, NPC cells consistently carry the EBV genome and in most cases are also positive for both HLA I and HLA II molecules at the cell surface (29, 32, 33), implying visibility to both CD4+ and CD8+ T cells. On the other hand, virus antigen expression in NPC cells is limited to EBNA1 and LMP2 (and in a minority of cases, also LMP1) and none of these proteins are dominant targets of the natural virus-induced T cell response (1). Nevertheless most NPC patients do have low levels of CD4 and CD8 memory T cells specific for EBNA1 and/or LMP2 in the circulation (22, 23), and indeed low numbers of CD8+ T cells with relevant reactivities have been detected in at least some tumor biopsies (29, 34). Furthermore adoptive transfer of autologous T cells, expanded in vitro to enrich for EBNA1/LMP2-specific reactivities, has given early indications that NPC may be accessible to attack by appropriately activated, antigen-specific cells (11-15). The present work set out to explore whether one might be able to reactivate patients’ immunity to the EBNA1 and LMP2 antigens by vaccination rather than by adoptive T cell transfer, thereby opening up the possibility of a second immunotherapeutic strategy for NPC, indeed one that would be deliverable to large numbers of patients without requiring specialist laboratory support.

The results of this first vaccine trial carried out on NPC patients in disease remission following conventional therapy, show that the MVA-EL vaccine is both safe and immunogenic. On the issue of safety, vaccine doses up to 5x10⁸ pfu, given intra-dermally on three successive occasions at three weekly intervals, were well tolerated even in patients who were recovering from potentially immunosuppressive multicycle chemotherapy 4-10 months previously. Adverse events were limited to mainly mild injection site and transient systemic reactions, similar to reported patterns for other recombinant MVA vaccines up to 10⁹ pfu (21, 35-37) (Supplementary Table 1). This safety profile bodes well for
future development of the MVA-EL-based vaccine approach as an immunotherapy for NPC.

As to immunogenicity, from the evidence of pepmix assays the vaccine specifically increased circulating T cell responses to EBNA1 and/or LMP2 in 15/18 patients. In most cases, responses to these antigens were detectable pre-vaccination, albeit at low levels, indicating that the vaccine was largely boosting rather than priming immunity. There were occasional examples of EBNA1 and/or LMP2 responses in the apparent absence of significant pre-existing immunity (see patients 0308 and 0412, Supplementary Fig.1), but it may well be that memory cell numbers in these individuals before vaccination were simply below the level of detection of the pepmix assay. Combining data from all 18 patients, mean levels of both EBNA1- and LMP2-specific T cell numbers in the blood were increased 3- to 4-fold by vaccination. This effect was specific since idiosyncratic rises in pre-existing immunity to control antigens (EBNA3A and FLU) were observed only occasionally, in 4/18 patients, and overall there was no significant change in their mean response levels. As to the absolute size of responses to the vaccine-coded antigens, the mean increases in EBNA1 and LMP2 pepmix counts (Figure 2A), recalculated as 230 and 156 spot-forming cells/106 PBMC respectively, compare well against those observed with other MVA recombinant vaccines, being similar to those for the oncofetal antigen 5T4 in colorectal cancer patients with concurrent chemotherapy (38) and exceeding those seen for other MVA cancer vaccines (21, 39). Higher frequency responses have been observed with MVA vaccines against malaria or tuberculosis; however those studies used primed healthy volunteers and focused on recall responses to some of those organism’s most immunogenic proteins (40, 41), not (as in the present work) recent chemotherapy recipients responding to naturally sub-dominant EBV antigens. Perhaps more importantly, the size of MVA-EL-induced responses in the blood was similar or higher than measured for LMP2-specific effector frequencies following adoptive transfer of in vitro-expanded T cell preparations into NPC and Hodgkin Lymphoma patients (11, 14, 42).
An important trial objective was to determine the dose for subsequent efficacy trials. Selection of $5 \times 10^8$ pfu is supported by several pieces of evidence. Thus a greater proportion of responders in pepmix assays were observed at the highest than the lower four doses and the size of response also increased with dose (Table 2, Figure 2B). Likewise defined epitope-specific responses were detected in only 5/12 patients receiving dose levels 1-4 but were present, typically at higher levels, in all 6 patients at the highest dose (Supplementary Table 3).

Although the small sample prevented multi-variate analysis for all possible factors influencing responsiveness (e.g. HLA type, age, NPC treatment and pre-vaccine immunity), univariate regression modeling also suggested a relationship between vaccine dose and immune response. Interestingly this dose/response relationship was more apparent after 2 rather than 3 vaccine cycles, raising the possibility that vaccine dose may be less critical with repeated exposure. It is still a question of debate whether pre-existing antibodies to the vaccinia vector itself, particularly virus-neutralizing antibodies, might limit the effectiveness of MVA-based vaccination. Our data on this point are limited since only 4/18 vaccine recipients were anti-vaccinia antibody-negative. However, we saw no evidence that immune responses to the vaccine-encoded EBNA1 and LMP2 antigens were significantly different in vector-naive versus vector-immune individuals (Figure 3).

The pepmix assays provide an important overall measure of T cell immunity to a specific antigen but do not discriminate between CD4+ and CD8+ responses and give no information on specific epitope choice. In a second set of assays, therefore, we took advantage of existing epitope maps of the EBNA1 and LMP2 antigens (1) to re-examine the vaccine-induced responses in Elispot assays using defined epitope peptides relevant to patients’ HLA types. This showed that overall responses detected in pepmix assays often included reactivities to known CD8 epitopes in LMP2 or EBNA1 epitopes plus known CD4 epitopes in EBNA1. This ability to stimulate both CD4+ and CD8+ T cells may reflect the EBNA1/LMP2 fusion protein’s ability to access both HLA class I and class II processing pathways within MVA-EL-infected antigen-presenting cells (16). Certainly this co-induction of CD4 and CD8 responses by the vaccine is
encouraging since both T cell subsets are likely to be required for effective vaccine-induced tumor surveillance (24-26). In this regard, we noted that varying response kinetics were observed for different epitopes; these included (i) stable responses post-cycle 1, (ii) early responses diminishing immediately before cycle 2 and boosted on cycle 2 and 3 and (iii) responses rising and falling with successive vaccinations (Figure 4). These different patterns in the blood may reflect the complex interplay between boosting responses, mobilization of T lymphocytes from the circulation into tissue, and possibly effector cell exhaustion, all factors that remain to be dissected in the context of cancer vaccination. It was also interesting to note occasional examples of patients with EBNA1 and/or LMP2 pepmix responses that could not be mapped in the epitope peptide assays (see patient 0101 for both antigens, patient 0514 for LMP2); this might be because not all potentially relevant epitopes could be included in all assays, but may equally well indicate that new target epitopes remain to be discovered in these proteins. Combining both pepmix and defined epitope screenings would therefore appear to be the best strategy for therapeutic vaccine trials, particularly where these involve patients not selected by HLA type and/or not of Caucasian origin. Variation in HLA alleles and sub-alleles, both within and between human populations, can dictate novel patterns of epitope choice that are distinct from previously mapped reactivities. Using as an example the different distribution of A2 sub-alleles prevalent in Chinese versus Caucasian populations, in the present work vaccine-induced reactivities were more often seen against recently identified HLA-A*02:03-restricted peptides in EBNA1 (VLK) and LMP2 (LLS) than to the better known LMP2 epitopes presented by the A*02:01 allele.

As to the therapeutic potential of this vaccine, we would stress that patients in the current trial were in remission and it was not the purpose to measure clinical effect. However it was interesting to observe that the one patient who had a detectable load of EBV DNA in plasma as a marker of tumor burden before vaccination (patient 0517) showed a transient spike in EBV levels following the development of a vaccine-induced T cell response (Figure 5). A similar observation was made in an NPC patient treated with EBV-specific T cells (11),
and transient increases in EBV DNA are well recognized as an indicator of tumor
cell killing in patients receiving conventional therapies (31). The example of
patient 0517 is also interesting because this patient suffered a late recurrence of
tumor some time after the vaccine-induced T cell response had subsided. In fact
only 3/6 patients given the highest vaccine dose (patients 0515, 0516, 0518)
showed a sustained rise in circulating EBNA1 and/or LMP2-specific memory cell
numbers present in the post-vaccine bleeds at 6 months. While an immediate
boosting of tumor-specific immunity by MVA-EL vaccination may be valuable in a
therapeutic context, achieving a durable increase in these responses is an
important long-term goal. Extending the vaccine course, either using the same
vector or alternating with a heterologous vector expressing the EBNA1/LMP2
fusion antigen, would be one approach in that regard. Another possible factor
determining long-term vaccine-induced immunity could be T reg cells. In
agreement with a previous study (44) we found the frequency of T reg cells was
raised in the blood of some NPC patients. Interestingly, upon comparing two
patients for whom T reg cell frequencies and long-term immune data was
available (and who received the same dose of vaccine) we found differences in
the durability of the vaccine-stimulated immune response inversely correlated
with T reg cell frequency. EBNA1- and LMP2-specific T cell responses were
sustained in patient 0518 (with a vaccine-boosted EBNA1-specific response
being detectable six months after vaccination) but were transient in patient 0517
in whom the frequency of T reg cells was higher. However, confirming whether T
reg cells are indeed responsible for the differences in durability of the vaccine-
induced T cell response we observed will require a larger number of patients to
be analyzed.

In summary these findings, and another trial of MVA-EL in UK patients
(manuscript in preparation), clearly demonstrate the immunogenicity of the
vaccine and point to its potential as an adjuvant treatment for NPC when
combined with conventional therapies. The work has also allowed the highest
and most consistently immunogenic vaccine dose, 5x10⁸ pfu, to be chosen for an
ongoing phase II trial determining the clinical response rate to MVA-EL in the
setting of relapsed or metastatic disease. Demonstration of the vaccine’s
immunogenicity in the context of NPC opens up its potential applicability to a wider range of EBV-associated tumors. Thus EBV-positive Hodgkin Lymphoma expresses both EBNA1 and LMP2 (as well as LMP1) (2) while EBV-positive T/NK lymphoma, another malignancy seen most commonly in S.E.Asia, expresses EBNA1 and a shortened LMP2 which, though lacking N-terminal sequences, retains all of the currently known T cell target epitopes (43). In each of these settings the vaccine might be used to maintain remission following primary treatment or as treatment for relapsed disease, either alone or in combination with adoptive T cell therapy or other modalities.
FIGURE LEGENDS

Figure 1. T cell responses in NPC patients stimulated by MVA-EL vaccination.

(A) Design of the EBNA1 LMP2 chimeric gene insert in the MVA-EL vaccine. DNA encoding the carboxy terminal half of EBNA1 (dark grey) was fused to the full length LMP2 gene (light grey) to generate the fusion gene inserted into MVA. Locations of defined CD8 and CD4 T cell epitopes studied in ELIspot assays are shown as black or white lines respectively. Two tyrosine residues within the LMP2 part of the fusion were mutated to phenylalanine to abrogate LMP2 signaling function.

(B) Representative results from three patients using the whole antigen pepmix ELIspot assay. PBMC samples taken at three different timepoints (screening, C2D8 and C3D22) were each stimulated with EBNA1 and LMP2 pepmixes in triplicate wells. Actin pepmix serves as the negative control indicating background T cell activity in the assay. Cells were also tested with EBNA3A and Influenza pepmixes; these antigens are not present in the MVA-EBNA1/LMP2 vaccine and therefore measure non-specific amplification of T cell responses. In all three patients the T cell response to EBNA1 and LMP2 is increased at C2D8 following two cycles of vaccine. These responses increased further at C3D22 for patients receiving lower doses of vaccine (patients 0206 and 0410) whereas no further amplification was seen for the patient receiving the highest dose (patient 0513). Note that for patient 0206 the influenza response is increased at C3D22 but is unchanged at the earlier timepoint, possibly indicating seasonal infection with influenza. Across the trial only two patients had altered influenza T cell response compared to fifteen patients with increased EBNA1/LMP2 responses.

Figure 2. Summary of whole antigen pepmix responses for all patients in the trial.

(A) The mean change in ELIspot readings for all patients that occurred after two (diamond) or three (cross) cycles of vaccination are shown along with the 95% confidence intervals. Results are expressed as sfc/3x10^5 PBMC. First, a repeated measures ANOVA was used to test whether the mean pepmix ELISpot readings
(adjusted by subtracting background counts against actin) at the three time-points were significantly different from each other: EBNA 1 p=0.0001; LMP2 p=0.002; EBNA 3A p=0.40; FLU p=0.27. Using Dunnett’s multiple comparison test, statistically significant increases (p<0.05 as shown) were observed for vaccine-encoded antigens EBNA1 and LMP2 but not for control antigens EBNA3A or FLU.

(B) Higher vaccine doses elicit stronger EBNA1 and LMP2 T cell responses. Responses obtained in whole antigen pepmix assays from all 18 vaccinated patients are plotted against vaccine dose. The change in the adjusted spot count/3x10^5 PBMC in the pre-vaccination samples versus the samples after cycle 2 and cycle 3 respectively is plotted against the vaccine dose given in pfu. The linear regression lines with 95% confidence intervals are shown with an estimate of goodness of fit of the line to the data (R^2) and the p-value testing the null hypothesis that the slope of the line does not differ significantly from zero.

**Figure 3. Pre-existing immunity to the vector does not affect the size of immune response to the vaccine-coded EBV antigens.**

(A) The difference between whole antigen pepmix response measured by ELIspot after two cycles of vaccination and that pre-vaccination is plotted against seropositivity for vaccinia specific immunoglobulin prior to MVA-EL vaccination. The means are shown as horizontal bars and are expressed as sfc/3x10^5 PBMC.

(B) EBNA1 antibody responses before and after MVA-EL vaccination. Using data from all patients, pre-vaccination anti-EBNA1 IgG titres were compared to those measured using samples from C3 D29, 6 and 12 months post vaccination. Patients who were vaccinia seronegative prior to MVA-EL vaccination are represented by open symbols. The mean values (horizontal bars) were significantly different (p=0.025 repeated measures ANOVA). Compared to the pre-vaccination control sample, the mean difference in reading was significant at the 5% level only for the samples taken at C3 D29 but not at 6 and 12 months (Dunnett’s multiple comparison test).

**Figure 4. MVA-EL stimulates CD8 and CD4 immune responses to multiple EBNA1 and LMP2 epitopes within an individual.**
(A) Shown are results obtained testing PBMCs from multiple timepoints from a single patient (0206) in an ELIspot assay using defined EBNA and LMP2 epitope peptides. Epitope sequences are denoted as being restricted through MHC class I (three residue codes) and MHC class II (four residue codes). DMSO solvent was included as a negative control and PHA as a positive control. For most timepoints sufficient cells were available for testing with each peptide or control in triplicate, to aid clarity only one representative well is shown in the figure. Following vaccination, epitope specific T cell responses can be detected within eight days and increase in magnitude, peaking eight days after the third dose of vaccine (C3D8) and still detectable up to a further 50 days later (C3D51). Note that the EBNA1 and LMP2-specific T cell responses detected in the whole antigen ELIspot assay (see Figure 1A) map to known CD8 epitopes in LMP2 and to known CD8 and CD4 epitopes in EBNA1.

(B) MVA-EL stimulates broad immune responses in multiple patients. Top panels show responses observed for three patients to whole antigens (pepmix); actin control (open boxes), EBNA1 (grey) and LMP2 (black) using samples obtained at three timepoints. The remaining panels demonstrate the results of ELIspot assays against epitope peptides within LMP2 and EBNA1 respectively across a wider range of timepoints. These panels show the adjusted readings, i.e. the difference between the epitope peptide and DMSO control spot counts at the same time points and are expressed as sfc/3x10^5 PBMC. For patients 0206 and 0513 pools of two LMP2 peptides were used (Table S3); subsequent assays assigned T cell responses to the individual epitope peptides indicated on the figure.

**Figure 5. Time course of EBV genomes in plasma in relation to immune responses for patient 0517.**

Events during screening, on and after trial participation are shown. Upper graph: Whole antigen pepmix ELIspot data showing sfc/3x10^5 PBMC recognizing EBNA1 (open triangles) and LMP2 (closed triangles) pepmixes. Middle graph: ELIspot data showing sfc/3x10^5 PBMC recognizing a pool of three EBNA1 CD4+ T cell epitopes (PQCR+LRVL+NPKF). Lower graph: EBV genome copies/ml.
plasma, the lower limit of detection of the assay is 500 EBV genomes per ml plasma (shown as horizontal broken line). Times of vaccination are shown as arrows below the x axis and the timescale shown on this axis applies to all three graphs. Low but stable levels of EBV DNA in the patient’s plasma then rose to a peak immediately after vaccination before decreasing to undetectable levels. This peak was preceded by vaccine stimulated increases in EBNA1 and LMP2 immune responses. Much later (67 weeks) this patient experienced a parotid lymph node relapse (arrow). At time of relapse, the EBV level was 1637 copies/ml but is not plotted because it was measured in a different center using a different assay.
REFERENCES


Table 1. Patients, diagnoses and previous treatments

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Stage*</th>
<th>Radiotherapy</th>
<th>Chemotherapy**</th>
<th>Time since treatment (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0101</td>
<td>41</td>
<td>M</td>
<td>I T1N0M0</td>
<td>6600 cGy in 33 fractions, (NP boost by intubation) 1800 cGy in 4 fractions</td>
<td>NA</td>
<td>15</td>
</tr>
<tr>
<td>0102</td>
<td>57</td>
<td>F</td>
<td>II T2bN0M0</td>
<td>6600 cGy in 33 fraction, (Right PP boost) 1400 cGy in 7 fractions</td>
<td>NA</td>
<td>16</td>
</tr>
<tr>
<td>0103</td>
<td>55</td>
<td>M</td>
<td>III T2bN2M0</td>
<td>6600 cGy in 33 fractions, (Bilateral PP boost) 1000 cGy in 5 fractions, (Left PP boost) 400 cGy in 2 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>15</td>
</tr>
<tr>
<td>0204</td>
<td>59</td>
<td>F</td>
<td>II T2bN1M0</td>
<td>6600 cGy in 33 fractions, (NP boost) 800 cGy in 4 fractions</td>
<td>NA</td>
<td>22</td>
</tr>
<tr>
<td>0205</td>
<td>46</td>
<td>M</td>
<td>III T2bN2M0</td>
<td>6600 cGy in 33 fractions, (NP boost) 800 cGy in 4 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>19</td>
</tr>
<tr>
<td>0206</td>
<td>46</td>
<td>M</td>
<td>IV T2bN3M0</td>
<td>6600 cGy in 33 fractions, (Left PP boost) 1400 cGy in 7 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>27</td>
</tr>
<tr>
<td>0307</td>
<td>53</td>
<td>M</td>
<td>III T3N2M0</td>
<td>6600 cGy in 33 fractions, (NP boost) 4 fractions 800 cGy</td>
<td>Cisplatin (7 cycles)</td>
<td>30</td>
</tr>
<tr>
<td>0308</td>
<td>40</td>
<td>M</td>
<td>III T2bN2M0</td>
<td>6600 cGy in 33 fractions, (NP boost) 800 cGy in 4 fractions, (LN Boost) 750 cGy in 2 fractions</td>
<td>Cisplatin (7 cycles)</td>
<td>33</td>
</tr>
<tr>
<td>0309</td>
<td>47</td>
<td>M</td>
<td>IV T4N1M0</td>
<td>7000 cGy in 35 fractions</td>
<td>Induction Cisplatin+ Carboplatin+ Paclitaxel (2 cycles), cisplatin, (7 cycles)</td>
<td>22</td>
</tr>
<tr>
<td>0410</td>
<td>50</td>
<td>M</td>
<td>IV T4N0M0</td>
<td>7000 cGy in 35 fractions</td>
<td>Induction Cisplatin +Gemcitabine (2 cycles), cisplatin, (6 cycles)</td>
<td>39</td>
</tr>
<tr>
<td>0411</td>
<td>57</td>
<td>F</td>
<td>III T3N0M0</td>
<td>7000 cGy in 35 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>17</td>
</tr>
<tr>
<td>0412</td>
<td>57</td>
<td>M</td>
<td>III T3N0 M0</td>
<td>7000 cGy in 35 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>14</td>
</tr>
<tr>
<td>0513</td>
<td>62</td>
<td>M</td>
<td>II T2bN1M0</td>
<td>7000 cGy in 35 fractions</td>
<td>Cisplatin (5 cycles)</td>
<td>14</td>
</tr>
<tr>
<td>0514</td>
<td>42</td>
<td>M</td>
<td>IV T4N1M0</td>
<td>7000 cGy in 35 fractions, (LN boost) 750 cGy in 2 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>15</td>
</tr>
<tr>
<td>0515</td>
<td>55</td>
<td>M</td>
<td>II T2bN1M0</td>
<td>7000 cGy in 35 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>21</td>
</tr>
<tr>
<td>0516</td>
<td>36</td>
<td>M</td>
<td>II T2bN0M0</td>
<td>7000 cGy in 35 fractions</td>
<td>NA</td>
<td>18</td>
</tr>
<tr>
<td>0517</td>
<td>63</td>
<td>M</td>
<td>IV T4N1M0</td>
<td>7000 cGy in 35 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>42</td>
</tr>
<tr>
<td>0518</td>
<td>55</td>
<td>M</td>
<td>II T2bN1M0</td>
<td>7000 cGy in 35 fractions</td>
<td>Cisplatin (6 cycles)</td>
<td>27</td>
</tr>
</tbody>
</table>
Patients identifiers are denoted by the prefix xx-yy, where xx is the dose level from 01 to 05, and yy is the patient number from 01 to 18. *According to UICC/AJCC 1997 cancer staging manual. **Cisplatin 40 mg/m² was given weekly concurrently with RT (except as induction chemotherapy). NP, nasopharynx; PP, parapharynx; LN, lymph node.
Figure 1

A

<table>
<thead>
<tr>
<th>GA repeat</th>
<th>EBNA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>363</td>
</tr>
<tr>
<td></td>
<td>642</td>
</tr>
<tr>
<td>EBNA1/LMP2</td>
<td></td>
</tr>
</tbody>
</table>

B

Patient 0516

<table>
<thead>
<tr>
<th>pre-vaccination</th>
<th>C2D8</th>
<th>C3D22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin (-ve control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA3A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLU</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Patient 0410

<table>
<thead>
<tr>
<th>pre-vaccination</th>
<th>C2D8</th>
<th>C3D22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin (-ve control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA3A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLU</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Patient 0206

<table>
<thead>
<tr>
<th>pre-vaccination</th>
<th>C2D8</th>
<th>C3D22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin (-ve control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA3A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLU</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2. Numbers of patients with ≥2-fold increases in antigen-specific responders across vaccination according to vaccine dose level.

<table>
<thead>
<tr>
<th></th>
<th>Following cycle 2 versus pre-vaccination</th>
<th>Following cycle 3 versus pre-vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EBNA1</td>
<td>LMP2</td>
</tr>
<tr>
<td>Dose level 1</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Dose level 2</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>Dose level 3</td>
<td>3/3</td>
<td>1/3</td>
</tr>
<tr>
<td>Dose level 4</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Dose level 5</td>
<td>6/6</td>
<td>5/6</td>
</tr>
<tr>
<td>All patients</td>
<td>12/18</td>
<td>10/18</td>
</tr>
</tbody>
</table>

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, EBNA 3 and influenza antigen (FLU) at 3x10^5/well. Adjusted spot counts were the spot counts for the viral antigens minus the spot count for actin. Post vaccination, adjusted readings ≥2-fold higher than for pre-vaccination samples defined an immune response.
Figure 2

A

Change in ELLspot readings from pre-vaccination samples

p<0.05  p<0.05  p<0.05  p<0.05

EBNA 1  LMP2  EBNA 3A  FLU

B

Cycle 2 Day 8

EBNA1

R² = 0.35
p = 0.01

LMP2

R² = 0.48
p = 0.001

Cycle 3 Day 22-29

EBNA1

R² = 0.12
p = 0.16

LMP2

R² = 0.26
p = 0.03
Figure 3

A

EBNA 1

LMP 2

ELISPOT response to MVA-EL

Pre-vaccination vaccinia status

seronegative seropositive

seronegative seropositive

B

P = <0.05

EBNA1 antibody level (o.d. units)

C1 D1 C3 D29 6 m 12 m

Plasma sample time

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 4

A  Patient 0206

<table>
<thead>
<tr>
<th></th>
<th>Pre-vaccination</th>
<th>During vaccination</th>
<th>Post-vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>screening</td>
<td>C1D8</td>
<td>C2D1</td>
</tr>
<tr>
<td>DMSO (-ve control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSC (MHC I/LMP2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLS (MHC I/LMP2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLK (MHC II/EBNA1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VFLQ (MHC II/EBNA1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA (+ve control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>-14</th>
<th>1</th>
<th>8</th>
<th>21</th>
<th>28</th>
<th>49</th>
<th>56</th>
<th>77</th>
<th>84</th>
<th>106</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vaccination</td>
<td>vaccination</td>
<td>vaccination</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B  Patient 0206  Patient 0513  Patient 0516

Whole antigen

LMP2 epitopes

EBNA1 epitopes

<table>
<thead>
<tr>
<th></th>
<th>Patient 0206</th>
<th>Patient 0513</th>
<th>Patient 0516</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSC (A24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLS (A2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLK (A2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VFLQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FI</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>vacc</th>
<th>vacc</th>
<th>vacc</th>
<th>vacc</th>
<th>vacc</th>
<th>vacc</th>
<th>vacc</th>
<th>vacc</th>
</tr>
</thead>
</table>

Downloaded from cancerres.aacrjournals.org on October 30, 2017. © 2013 American Association for Cancer Research.
Figure 5

Graph 1: Comparison of EBNA1 pepmix and LMP2 pepmix.

Graph 2: EBNA1 CD4 peptides.

Graph 3: EBV genomes per ml plasma, with lymph node relapse and raised EBV levels.

Vaccinations and weeks post vaccine cycle 1 are indicated on the x-axis.
Phase 1 trial of recombinant Modified Vaccinia Ankara (MVA) encoding Epstein-Barr viral tumor antigens in nasopharyngeal carcinoma patients


Cancer Res  Published OnlineFirst January 24, 2013.