Review Article

Therapeutic vaccination strategies to treat nasopharyngeal carcinoma

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Abstract: Epstein-Barr virus (EBV) infects most people worldwide. EBV has oncogenic potential and is strongly associated with several lymphomas and carcinomas, including nasopharyngeal carcinoma (NPC), that together total 200,000 cases of cancer each year. All EBV-associated cancers express viral proteins that allow highly selective immunotherapeutic targeting of the malignant cells. A number of therapeutic EBV vaccines have been tested in clinical trials with evidence of immune boosting and clinical responses in NPC patients. Therapeutic vaccination could be used after adoptive T-cell transfer to increase and sustain the number of infused T-cells or combined with immunotherapies acting at different stages of the cancer immunity cycle to increase efficacy. The therapeutic EBV vaccines tested to date have been well tolerated with minimal off-target toxicity. A safe therapeutic vaccine that was also able to be mass produced could, in principle, be used to vaccinate large numbers of patients after first line therapy to reduce recurrence.

Keywords: Immunotherapy; Protein Death receptor 1 (PD1); PD-L1; tumor

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Epstein-Barr virus (EBV): co-existence and disease

EBV, one of eight human herpesviruses, infects over 95% of people worldwide (1). EBV is transmitted orally and is often acquired during childhood, the infection going unnoticed or at least not standing out from the usual minor infections that occur in children. In more socioeconomically developed communities infection with EBV is often delayed until adolescence. Here infection may also pass unnoticed although some people develop infectious mononucleosis, a transient but debilitating condition characterised by symptoms of sore throat, lymphoadenopathy, fever and fatigue (2). In both cases EBV infection is countered by a robust immune response comprising natural killer cells, CD8+ cytotoxic T-cells and CD4+ helper T-cells (3). This response can be extremely strong and the symptoms of infectious mononucleosis are thought to stem from an over-exuberant immune response to infection. Nevertheless EBV establishes permanent colonisation of a subset of the host's B lymphocytes, evading anti-viral immunity by silencing viral protein expression. This strategy enables EBV to persist for the lifetime of the host as a latent infection, a hallmark of the herpesvirus family. In order to be transmitted to new hosts, some of the B cells carrying EBV undergo reactivation, producing new progeny virus in the oropharynx for transmission to susceptible individuals (4).

The fine balance that exists between EBV and its host means that most people suffer no long-term health effects from this infection. Although relatively benign in most people, EBV has powerful growth transforming potential and is classified as a group I carcinogen by the World Health Organisation (5). It is aetiologically linked to

Table 1 EBV-associated malignancies and the viral proteins in the tumour cells

Tumour	EBV proteins expressed
Post-transplant lymphoproliferative disease	EBNA1, 2, 3A, 3B, 3C, LP, LMP1, LMP2
Hodgkin lymphoma	EBNA1, LMP1, LMP2
Diffuse large B lymphoma	EBNA1, LMP1, LMP2[a]
Burkitt lymphoma	EBNA1[b]
Extranodal T/NK lymphoma	EBNA1, LMP2[c]
Gastric carcinoma	EBNA1, LMP2[d]
Nasopharyngeal carcinoma	EBNA1, (LMP1[e]), LMP2[d]

[a], some DLBCL tumours express a wider range of latency proteins; [b], 10–15% of endemic Burkitt lymphomas express EBNA-1, -3A, -3B -3C, -LP and BHRF-1; [c], a shortened version of LMP2 is expressed in extranodal T/NK lymphoma; [d], BARF1 is reported to be expressed in a proportion of EBV-positive carcinomas; [e], LMP1 is expressed in a subset of NPC tumours at apparently low levels. EBNA, Epstein-Barr Nuclear Antigen; LMP, Latent Membrane Protein; EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma.

several distinct lymphomas: Burkitt lymphoma, Hodgkin lymphoma, extranodal T/NK lymphoma (ENKTL) and an estimated 10% of diffuse large B cell lymphomas (5). EBV is also a problem in the transplant setting, where iatrogenic immunosuppression of normal anti-viral immunity may result in posttransplant lymphoproliferative disease (PTLD) (3,4). The largest burden of disease, however, stems from EBV's ability to infect and transform epithelial cells. EBV is associated with 10% of gastric carcinomas and almost all cases of the non-keratinising subtype of nasopharyngeal carcinoma (NPC) (5). This subtype represents most cases (>95%) of NPC in geographical regions where the disease is endemic. Taken together EBV is associated with an estimated 200,000 cases of cancer each year, representing 1% of all cancers worldwide (6).

All EBV-associated malignancies express viral proteins although the number of proteins varies for different diseases (*Table 1*). Post-transplant lymphomas, particularly those that arise in the first year following transplantation when immunosuppression is greatest, express all eight EBV latent cycle proteins. These comprise six Epstein-Barr Nuclear Antigens (EBNAs) and two Latent Membrane Proteins (LMPs). Notably the EBNA 3A, 3B and 3C proteins, which are particularly good CD8+ T-cell targets, are expressed (4). In contrast, a more limited range of less immunogenic EBV proteins are expressed in the other EBV-associated malignancies, possibly reflecting their origin in people who are not overtly immunosuppressed.

Several clinical trials of immunotherapies targeting these EBV proteins have been conducted worldwide. Most work to date has focussed on adoptive T-cell therapy, with T-cell effectors being prepared *in vitro* for infusion into patients (7-12). This approach has been applied mostly to PTL although several clinical trials have extended it to other EBV-associated lymphomas (13-15) and to NPC (16-23). A smaller number of trials have employed a different strategy, using therapeutic vaccines to boost relevant T-cell responses in the patient themselves (24-27). Finally, interim results have recently been released for a clinical trial in NPC patients of an immune checkpoint antagonist targeting Protein Death receptor 1 (PD1) (28), an approach that could potentially be used by itself but with considerable potential for synergy when combined with the aforementioned therapies.

Tumor associated antigens as targets for immunotherapy

A large number of tumor-associated antigens (TAAs) have been identified. They can be divided into two broad groups based on their expression pattern (29). Some have low tumoral specificity. Overexpressed TAAs, for example, are present at high levels in cancerous cells but lower levels in healthy tissue. Others have much higher tumoral specificity making them more attractive targets for immunotherapy. In this latter group are neoantigens, altered proteins generated through cancer-specific somatic mutation. These are exquisitely tumor specific allowing the cancer to be targeted with minimal risk of damage to normal tissues (30). Because they arise after later in life, after the immune system has developed, neoantigens represent foreign immune targets. Neoantigen-specific T-cells are therefore highly avid because they have not been subjected to central tolerance (30). Neoantigen generation is a stochastic process requiring a mutation that: (I) is located in the open reading frame of a gene expressed by the cancer cell; (II) generates an altered peptide capable of being presented by one of the patient's HLA molecules; and (III) is able to stimulate a cognate T-cell response (31,32). These requirements mean that neoantigens are more likely to occur in cancers with high mutational load, such as melanoma and smoking-associated lung cancer, and less likely in others (33-35).

The mutational load of the EBV-associated malignancies

is relatively low, reducing the probability of exploitable neoantigens being present in a patient's tumor (36-38). In principle, however, the virus-encoded proteins expressed in the malignant cells of the EBV associated cancers might also serve as neoantigens. Immune responses to these EBV-encoded antigens are likely to be tumor selective because the viral proteins are otherwise expressed in a very limited number of EBV-infected B cells. It is also reasonable to expect that EBV-specific T-cells have not undergone central tolerance; the high avidities exhibited by EBV-specific T-cell clones *in vitro* suggests this is indeed the case (39-45). Unlike many mutational neoantigens, which are 'private' and limited to only a single patient's cancer, all EBV-positive tumors express one or more viral proteins and immune therapies targeting them therefore have wide applicability.

Epstein-Barr virus (EBV)-encoded immunotherapeutic targets in nasopharyngeal carcinoma (NPC)

Two EBV proteins are consistently expressed in NPC. The first is EBNA1, a protein of critical importance to the virus as it maintains the viral DNA in dividing cells. EBNA1 also regulates the expression of viral and cellular genes, modulating a range of cellular pathways (46). EBNA1 contains a large glycine/alanine repeat domain that interferes with the protein's processing and presentation by the HLA-class I antigen processing pathway. Although this reduces EBNA1's visibility to CD8+ T-cells, it is important to note that EBNA1-positive cells can still be recognised by these effectors (47-49). EBNA1 possesses many more CD4 T-cell epitopes and T-cell responses to these are frequently detected in the population (50,51). EBNA1 was the first viral protein identified as being processed by macroautophagy and some, but not all, CD4+ T-cell epitopes are generated by this pathway (52-54). Such endogenous processing is important because it could potentially allow EBNA1-specific CD4 T-cells to act as direct effectors against HLA-class II positive cells and in this respect it is interesting to note that NPC tumor cells are often HLA-class II positive (55,56). Although EBNA1's nuclear localisation decreases its processing by macroautophagy and consequently visibility to CD4+ T-cells (53), EBNA1-specific CD4+ T-cells suppressed tumor growth in an animal model of Burkitt Lymphoma (57). The recent report of clinical responses in PTL patients treated with EBNA1-stimulated T-cell lines (7) also supports harnessing EBNA1 as an immunotherapeutic target.

The second protein consistently expressed in NPC is the transmembrane protein LMP2. Although its principle function is to negatively regulate B-cell receptor signalling, LMP2 exhibits a range of activities and is required for the outgrowth of epithelial cells *in vitro* (58). LMP2 mRNA transcripts are detected in more than 98% of NPC biopsies and protein can be detected by immunohistochemistry in >50%; this discrepancy likely being due to the lower sensitivity of the latter technique (59). LMP2 contains many CD8+ T-cell epitopes (4,39,41,42,60) and is therefore considered the best CD8+ T-cell target expressed in NPC. A smaller number of CD4 T-cell epitopes have also been identified in LMP2 and there is evidence that these epitopes are displayed by LMP2-positive cells as well (43). In LCLs LMP2 reduces expression of the co-activatory ligand NKG2D, decreasing T-cell recognition (61); it is not known if LMP2 has a similar effect in NPC cells.

Another membrane protein, LMP1, is present in a minority of NPC biopsies and appears to be expressed at lower levels in the tumor cells (62). LMP1 functions as a constitutively active member of the TNFR superfamily to provide growth and differentiation signals to B cell but exerts a range of effects in cells including the upregulation of anti-apoptotic proteins and is considered to be the main transforming protein of EBV (58). Although several CD8+ T-cell epitopes have been identified in LMP1 (40,60), this protein's tendency to self-aggregate limits HLA-class I restricted LMP1 epitope generation (63). Several LMP1-specific CD4+ T-cell epitopes have been defined (43,44,51) and, as is the case for EBNA1 and LMP2, some of these epitopes are displayed on the surface of LMP1-positive cells (43). The antigen processing pathways involved in generating HLA-class II restricted LMP1 and LMP2 epitopes are currently unknown.

Although not expressed in lymphoid malignancies, the BARF1 protein is consistently detected in most EBV-positive NPC and gastric tumors (64,65). BARF1 is reported to have transforming activity, inhibit apoptosis and act as a colony stimulating factor 1 decoy receptor. Relatively little attention has been given to BARF1 as a T-cell target. Both CD8 and CD4 T-cell responses were detected in the blood of NPC patients and were present at lower levels in healthy donors (66). A number of HLA-A2 restricted CD8+T-cell epitopes have been defined (66) and, given the above result, it is likely that more remain to

be discovered.

The immune status of nasopharyngeal carcinoma (NPC) patients

Given that NPC occurs in people who are not undergoing immunosuppressive therapy, an obvious but important question is whether they have suffered selective loss of immune responses to the EBV proteins present in the tumor. A comprehensive screen of newly diagnosed untreated NPC patients found that, with the exception of a HLA-B*4001 restricted LMP2-specific response, CD8+ and CD4+ T-cells responses against the EBNA1, LMP1 or LMP2 proteins were similar to those in healthy control donors (67). This work examined T-cell responses using *ex vivo* ELIspot assays that detect T-cells with immediate effector function, typically the effector memory subset. Studies using methods that detect both effector and central memory T-cells differ in their conclusions. Analysis by tetramer staining shows that newly diagnosed NPC patients still possess LMP1 and LMP2-specific T-cell responses although at lower frequencies compared to healthy donors (68). One study using *in vitro* culture methods reported that LMP2-specific T-cell responses appear normal at the time of diagnosis (69) whereas others report that LMP2 and EBNA1-specific CD8+ T-cells are decreased in frequency (70,71). Removing regulatory T-cells from the cultures increased the number of EBNA1 and LMP2-specific T-cells detected suggesting that regulatory T-cells may limit EBV-specific immunity (71). In this respect it is interesting to note that the frequency of regulatory CD4+ T-cells is raised in the blood of newly-diagnosed patients and those who have completed first-line chemoradiotherapy (24,72). Adjuvant chemotherapy with cisplatin has been reported to increase the frequency of regulatory T-cells in head and neck squamous cell carcinoma patients (73). It is not known whether this agent, the standard first-line chemotherapy for NPC, might similarly affect regulatory T-cell numbers in NPC patients.

Nasopharyngeal carcinoma (NPC) as a target for immune responses

The fact that many NPC patients still possess relevant EBV specific T-cell responses raises the question of whether these T-cells are capable of acting in the tumor itself. Early studies found that NPC cell lines express HLA class I molecules, possess normal antigen processing activity and, when tested *in vitro*, can be recognised and killed by T-cells (69,74). Analysis of NPC tumor biopsies shows that HLA class I is expressed in 63%, reduced in 22% and lost in 15% of cases (56). HLA class II is also frequently detected in tumours (55,56), raising the possibility that CD4+ T-cells may be able to play a direct anti-tumor role: indeed, such cells may be the only effective T-cell population in cases that have lost HLA class I expression.

The fact that HLA loss is uncommon in NPC may reflect the presence of other immune evasion mechanisms. The tumor cells themselves express multiple immunomodulatory molecules. These include HLA-G, an inhibitor of T and NK cell function that is associated with decreased survival (75) and galectin-9, which is secreted in exosomes and inhibits T-cell function (76). NPC is also characterised by a heavy infiltrate of lymphoid cells that may also exert suppressive effects. The infiltrating cells are predominantly CD3+ T-cells with smaller numbers of NK cells, B cells, dendritic cells (DCs) and monocytes detected (77). Regulatory T-cells are enriched in the tumor compared with adjacent normal nasopharyngeal tissue (72,78). Indoleamine 2,3-dioxygenase, which indirectly suppresses T-cell activity via tryptophan depletion, is present in 75% of tumor biopsies although it is currently unclear whether this represents expression by infiltrating myeloid derived suppressor cells or by the tumor cells (79). Although present in high numbers, the functional capacity of CD3+ T-cells in the tumor remains unclear. In 20% of NPC tumors they show reduced expression of the T-cell receptor zeta chain required for T-cell activation following target recognition (78). The fact that functional T-cells can be cultured from NPC tumors suggests that any impairment in T-cell function is potentially reversible (23,69).

The negative immune regulator PD-L1 is expressed in 89–95% of NPC tumors (28,80,81). This frequent expression provides an important therapeutic opportunity as several antibody-based therapies that can disable this inhibitory pathway are now licensed or in the advanced stages of clinical development (82). In some cases PD-L1 expression in the tumor is induced by inflammatory cytokines released by infiltrating immune cells and its expression here is thought to counter an anti-tumor immune response (83). Such cases may respond more favourably when PD-1 or PD-L1 inhibitors are used as monotherapy (84) as anti-tumor effectors are already present. An alternative, but not necessarily mutually exclusive, mechanism responsible for PD-L1 expression is intrinsic resistance. Here PD-L1 upregulation is the result of alterations in signalling pathways within the malignant

cells or genetic amplification of the PD-L1 locus: the latter frequently occurs in EBV-associated gastric cancer (38). It is conceivable that such cases might be more likely to benefit from PD1 or PD-L1 inhibition when it is combined with immunotherapies capable of generating the tumor-specific immune responses that would otherwise be absent.

For NPC it is currently unclear which of these two mechanisms is responsible for the frequent expression of PD-L1. LMP1-mediated up-regulation of the AP-1, STAT3 or NF-kB pathways has been suggested to be responsible (80,81) and in this respect it is interesting to note that BL tumors, which lack LMP1, do not express PD-L1 (80). Conversely, the heavy lymphoid infiltrate is consistent with a role for adaptive immune resistance and this is supported by *in vitro* data that show interferon-gamma increases PD-L1 expression by NPC cell lines (81). Although the underlying mechanism of PD-L1 expression is unclear, interim data from KEYNOTE-028, a non-randomised Phase IB trial (NCT02054806) of the PD-1 inhibitor pembrolizumab in solid tumors, have provided a signal of efficacy for checkpoint blockade monotherapy in NPC. Of the 27 patients with advanced unresectable or metastatic NPC treated with pembrolizumab, one experienced a complete response, six experienced partial responses and 14 had stable disease giving an overall response rate of 25.9% (28). These preliminary data are encouraging and, given that PD1 inhibitors are generally well tolerated, there is clearly potential for them to be used in combination with other agents to increase response rates.

Lessons from infusional T-cell therapy for Epstein-Barr virus (EBV)-positive cancers

In the laboratory EBV readily infects and transforms B cells into permanently growing lymphoblastoid cell lines (LCLs). These B-cell lines, like PTL tumors, express all eight latent cycle proteins, high levels of HLA class I and class II molecules and they efficiently activate and expand EBV-specific T-cells *in vitro*. The specificity of the resulting T-cell lines tend to focus on the immunodominant EBNA3A, 3B and 3C proteins. Because PTLD tumors express these proteins infusion of such T-cell lines has proven highly successful as prophylaxis and therapy for PTLD in the haematological transplant setting (8) with evidence of efficacy for PTLD following solid organ transplantation (9). LCLs have also been used to establish T-cell banks from third party donors and these have also shown efficacy when used to treat partly HLA-matched PTLD patients (10). Subsequent studies have extended adoptive T-cell therapy to patients with other EBV-associated lymphomas (85). The T-cell lines used in these trials were initially generated using LCLs, with more recent trials employing a range of techniques to focus the immune response onto the smaller number of EBV antigens present in the malignant cells of Hodgin Lymphoma and ENKTL (13-15). Detailed discussion of these trials is beyond the scope of this review, and we will instead highlight four key insights from this work relevant for NPC immunotherapy.

First, focusing the EBV-specific immune response onto a limited repertoire of epitopes runs the risk of the tumor escaping this narrow immunological pressure. A PTLD patient's lack of response to T-cell therapy was discovered to be caused by the tumor, which expressed a truncated EBNA3B protein, being poorly recognised by the infused T-cells. The T-cell line, prepared using LCLs as the antigenic stimulus, was dominated by T-cells specific for two epitopes located in the region of EBNA3B that was lost from the tumor cells (86). This incident may represent a special case, with high level immunosuppression allowing the proliferation of tumor cells carrying this unusual mutation, but it clearly illustrates the dangers inherent in relying on a highly focussed immune response which could become a single point of failure. Using a diverse range of T-cell responses to provide redundancy and targeting proteins the malignant cells needs for cellular growth or survival will both help minimise the risk of treatment failure.

Second, LCL-stimulated T-cell lines containing high numbers of CD4 T-cells yielded significantly better clinical responses in PTLD patients (87). Whether this is due to these cells acting as effectors in their own right, providing help to CD8 T-cells or a combination of the two is unknown, but it adds further support for using a broad range of EBV-specific effectors in patients.

Third, EBV specific T-cells are able to induce clinical responses in patients with Hodgkin Lymphoma, a disease with a complex immunosuppressive tumor microenvironment and, in clinical trials, T-cell preparations enriched for LMP2-specific T-cells are more effective (13-15).

Fourth, EBNA1-specific T-cells yielded clinical responses in seven of ten PTLD patients treated with them. These clinical responses were associated with expansion of the infused EBNA1-specific T-cells *in vivo* (7). Increases in LMP2-specific T-cell responses were also detected in three donors and although these could indicate contaminating cells in the infused T-cell product, they could equally

represent antigen spreading stimulated by the release of antigen from lysed tumor cells. Overall, this study suggests EBNA1 should be given serious consideration as a potential target in future therapeutic strategies.

Treating NPC with infusional T-cell therapy represents a much greater challenge because of the more limited range of EBV targets in the tumor cells and, as described above, local and systemic immunosuppressive mechanisms need to be overcome. Adoptive T-cell therapy for NPC is discussed elsewhere in this special issue of *Chinese Clinical Oncology* and we will therefore discuss this work only briefly. A number of groups have used LCL-stimulated autologous T-cell lines to treat NPC (16-20,88,89) and some studies have reported clinical responses. In a Phase I trial of ten patients with stage IV NPC in progression after chemotherapy and radiotherapy, two partial responses and four cases of stable disease were observed after T-cell infusion (18). A separate Phase I/II clinical study observed complete responses in three of four patients with locoregional disease but only one of eleven patients with metastatic disease (19,42). Administering lymphodepleting chemotherapy or antibodies prior to T-cell therapy, to promote greater T-cell expansion *in vivo*, did not markedly increase the number of clinical responses observed (16,20). These trials each recruited a mixed cohort of patients, many of whom had undergone multiple lines of treatment. To explore the efficacy of adoptive T-cell therapy as first line therapy, 35 newly diagnosed NPC patients were treated with four cycles of gemcitabine and carboplatin before receiving autologous LCL-stimulated T-cells (21). After T-cell infusion thirteen patients had a further partial response and seven had stable disease.

Two groups have used alternative methods to prepare therapeutic T-cells for infusion. To focus the immune response against the subset of EBV antigens expressed in NPC, Smith and colleagues prepared T-cell lines using as the antigen stimulus an adenovirus expressing a fusion protein of EBNA1 (lacking the glycine/alanine repeat domain) and a polyepitope string of defined LMP1 and LMP2 peptides (22). Although clinical responses were not observed, median overall survival for the patients treated with T-cells, who had locoregional recurrence or distant metastases, was greater than a historical patient cohort. In a different study, patients were treated with a single dose of autologous TILs one week after completing chemoradiotherapy (23). The TILs had a high frequency of CD4+ T-cells and consistently responded to EBNA1 peptides when tested *in vitro*. Following TIL infusion, increases in LMP1, LMP2 and EBNA1-specific T-cells could be detected in the blood of some patients. The near-contemporaneous use of chemotherapy and TILs in this trial makes it difficult to determine whether T-cell infusion resulted in improved outcomes compared to chemotherapy alone. This question may be addressed by a Phase II trial that is reported to be underway (23).

Autologous dendritic cell vaccination for nasopharyngeal carcinoma (NPC)

The first therapeutic vaccination trial for NPC consisted of four cycles of autologous monocyte-derived DCs loaded with LMP2 CD8+ T-cell epitope peptides (26). All 16 patients had residual disease when recruited to the study and LMP2-specific T-cell responses were very low or undetectable in *ex vivo* ELIspot assays. Vaccination boosted LMP2-specific T-cells in nine patients and these increases were sustained for three months before declining. Seven patients failed to make a T-cell response of whom four had persistent leukopaenia and immune impairment on trial entry. Partial clinical responses were detected in two patients coincident with increases in circulating LMP2-specific T-cell frequency.

A trial of similar design, again using autologous DCs loaded with LMP2 CD8+ T-cell epitope peptides, showed vaccination increased circulating LMP2-specific T-cells in 7 of 16 patients treated (23). Clinical responses were not described but a small decrease in serum EBV DNA levels, a surrogate marker of tumor burden, was noted. This decrease did not correlate with immune responses in the blood but instead correlated with the presence of a delayed type hypersensitivity response to the LMP2 peptides used to immunize each patient.

Both of the above studies used a small number of defined LMP2 CD8+ T-cell epitope peptides selected on the basis of the patients HLA type. An alternative approach by Chia and colleagues used autologous DCs transduced with an adenovirus expressing truncated LMP1 and full length LMP2 protein (27). This method has the advantage of potentially boosting a wider range of T-cell specificities including those that are currently undefined or are presented through rare HLA alleles. Patients recruited to the study had been heavily pre-treated and on trial

entry only one had a detectable T-cell response to LMP1, LMP2 or EBNA1 in *ex vivo* ELIspot assays. Following vaccination, 9 of 12 patients showed increased delayed type hypersensitivity reactions to transduced DCs. No increases in T-cell responses were detected by *ex vivo* ELIspot assays but, nevertheless, one partial clinical response and two instances of stable disease were achieved.

Non-cellular therapeutic vaccination for nasopharyngeal carcinoma (NPC)

The above studies are important as they show vaccination can overcome any systemic immunosuppression that may exist in NPC patients to boost therapeutically relevant T-cell responses. Customized patient-specific DC vaccines, however, require highly trained staff and specialized facilities presenting a significant challenge to their widespread use. A vaccine that could be mass-produced would be far better suited to widespread use, particularly in countries with limited health resources. The economic case for such an 'off-the-shelf' vaccine is compelling. A low marginal cost of production means it would benefit from economies of scale, with the cost per dose decreasing as larger amounts of vaccine are made. This fundamental economic concept led us to develop an off-the-shelf therapeutic vaccine to treat NPC or indeed any other EBV-associated cancer. Based on the modified vaccinia Ankara (MVA) vector, which is highly attenuated and has an excellent clinical safety record (90), MVA-EBNA1/LMP2 encodes a fusion protein consisting of the carboxy terminal half of EBNA1 fused to full length LMP2 (91). This design retains almost all known CD4 T-cell epitopes and removes the EBNA1 glycine/alanine repeat domain that would otherwise interfere with protein expression and antigenic processing. All known LMP2 CD8 and CD4 T-cell epitopes are included in the construct and its antigenicity is enhanced by the LMP2 protein sequence redirecting EBNA1 into the HLA class II processing pathway (91).

Two dose escalation Phase IA trials of MVA-EBNA1/LMP2, conducted in NPC patients in Hong Kong and the United Kingdom, have demonstrated the vaccine is safe and well tolerated (25). Side effects were predominantly grade 1, with seven instances of grade 2 and one transient grade 3 in the 34 patients who were vaccinated in these trials. Before vaccination, low-level T-cell responses specific for EBNA1 and LMP2 were detectable in most patients in *ex vivo* ELIspot assays. Compared to previous studies this level of pre-existing T-cell response is much higher and likely reflects the fact that most patients had undergone only one line of therapy before trial recruitment. Of the 27 patients for whom data were available, 18 and 12 showed a post-vaccination increase in T-cells specific for EBNA1 and LMP2 respectively. There was clear evidence of a dose effect and all patients who received dose level three (2×10^8 pfu at three-weekly intervals) responded to EBNA1, LMP2 or both. Analysis of the fine detail of these antigen-specific responses using a panel of epitope peptides revealed that vaccination boosted a broad range of CD8+ and CD4+ T-cell responses against LMP2 and EBNA1 respectively in patients of Chinese and European descent (24). The vaccine was therefore immunogenic across the natural variation that exists for HLA alleles and circulating EBV strains in different populations.

The size of the immune responses stimulated by the vaccine was sufficient to allow T-cell phenotyping to be performed by *ex vivo* flow cytometry. This revealed several key insights. First, assays measuring interferon gamma, the method most commonly used to monitor immune responses in NPC immunotherapy trials, will underestimate the true size of the immune response because only a minority of EBNA1- and LMP2-specific T-cells produce this cytokine.

Second, the quality of the patient's EBNA1- and LMP2-specific T-cells, determined by the number of effector functions they exhibited, was lower compared with T-cells specific for the non-tumor EBV protein EBNA3A (24). These differences in immune quality may represent a wider phenomenon as they have also been observed in some, but not all, studies performed in healthy donors (92,93). When tested *in vitro*, galectin-1 suppressed the activity of low quality LMP-specific T-cells whereas high quality T-cells were resistant (92). Vaccination-induced increases in immune quality may therefore allow T-cells to overcome the array of immunosuppressive mechanisms present in NPC tumors.

Third, as might be expected LMP2-specific CD8+ T-cells and EBNA1-specific CD4+ T-cells possessed distinct functional properties. The former degranulated upon antigen stimulation, consistent with perforin-mediated cytotoxicity. Importantly, vaccination greatly increased the number of LMP2-specific CD8+ T-cells capable of degranulation when exposed to antigen. By contrast, EBNA1-specific CD4+ T-cells did not degranulate in response to stimulation. Note that this does not necessarily mean these cells lack cytotoxicity as they have reported

to utilise the FAS/FAS-Ligand pathway (94). Many of the CD4+ T-cells were able to produce interleukin-2, a property the almost all LMP2-specific CD8 T-cells lacked. Taken together, these functional differences between the two immune subsets support our strategy of including both antigens in the vaccine.

Fourth, treating patients with three cycles of vaccination did not drive EBNA1 and LMP2-specific effectors to terminal differentiation, suggesting additional cycles of vaccination could be used to maintain or further boost these responses over time. Repeated monthly vaccinations have been used for a different poxvirus-based vaccine to maintain long-term control of pancreatic and rectal cancer (95).

These results have led to two subsequent clinical trials of MVA-EBNA1/LMP2. In the United Kingdom a Phase IB trial (NCT01800071) in NPC patients in remission or with current disease is examining vaccine immunogenicity in finer detail and testing the potential boosting effects of using a fourth vaccine cycle. In Hong Kong a Phase II trial (NCT01094405) is treating patients with persistent, recurrent or metastatic NPC to determine the clinical efficacy of the vaccine.

Looking to the future: increasing the efficacy of nasopharyngeal carcinoma (NPC) immunotherapy

As others and we have shown, it is possible to boost therapeutically relevant T-cells in NPC patients through adoptive T-cell therapy or vaccination. The challenge now is to improve the clinical response rate in NPC patients. One way to do this is to combine therapies. Some positive steps have already been taken in this direction (16,20,21) and there is enormous potential for further research in this area.

How would a therapeutic EBV vaccine help in this endeavour? First, a vaccine could complement adoptive T-cell NPC therapies of NPC. Vaccination following T-cell infusion could potentially boost the number of T-cells in the patient and sustain these increases for longer periods of time. Arguing against this strategy is the fact that the presence of a detectable T-cell response in the blood often does not correlate with clinical responses in NPC patients (19,22,89). It is important to note, however, that this lack of correlation does not mean that increasing the magnitude of the T-cell response is pointless. Rather, it may simply reflect the fact that only 2% of T-cells are present in the blood (96), the key effectors migrating to relevant tissues. Although chemokine receptors have been suggested as being involved in T-cell homing to NPC tumors (97,98), surprisingly little attention has been given to the homing properties of the T-cells used to treat NPC, whether generated *in vitro* for infusion or boosted *in vivo* via vaccination. With this in mind, one could envisage administering the vaccine via a route that would boost the number of T-cells but also encourage them to home to the relevant anatomical sites (99). In this respect it is interesting to note that an MVA tuberculosis vaccine (the same vector as MVA-EBNA1/LMP2) can be safely administered as an inhaled aerosol and this method of delivery boosted mucosal immunity (100). Modifying the vaccination route could be a simple way of achieving the best balance of circulating and tissue resident CD4+ and CD8+ T-cells to achieve control of locoregional and disseminated disease.

Second, there is increasing awareness that conventional cancer therapies may exert immune modifying effects. Radiotherapy can stimulate antigen processing and presentation pathways (101) and some chemotherapies and targeted therapies can affect the number and function of regulatory immune cells or alter the tumor microenvironment (102,103). Furthermore, an increasing number of small molecules (104) and biologic drugs (82) that manipulate the immune system are being developed or are already in clinical use. The cancer immunity cycle provides a useful conceptual framework for considering which agents to combine together (105). The induction of an effective anti-tumor immune response requires all of the rate limiting steps in the cycle to be overcome. For NPC the frequent expression of PD-L1 is likely to represents a fundamental rate-limiting step at a late stage in the cycle. The deficit in EBNA1 and LMP2-specific T-cells in NPC patients represents a second rate-limiting step operating at an early stage of the cycle (70,71). Contemporaneously removing both rate-limiting steps, by combining anti-PD1 with vaccination, could substantially increase clinical response in NPC patients as is the case for other settings (106-108). An important caveat here is that additional immunosuppressive mechanisms may also need to be addressed and these could potentially vary for different patients and stages of disease (109).

Third, despite radiotherapy and chemoradiotherapy some 5% to 15% of NPC patients develop local failure and 15% to 30% experience failure at distant sites with poor prognosis (110). An 'off-the-shelf' therapeutic vaccine, benefitting from economies of scale and therefore suitable for widespread use, could conceivably be used to vaccinate large numbers of patients following first line therapy

to reduce the risk of recurrence. The advantage of this approach is two-fold. First, it would avoid the toxicities that have so far hampered the use of additional chemotherapies in this way (111). Second, it would utilise vaccination when it is likely to be most effective, in less heavily treated patients with low volume disease (112). NPC presents an ideal situation to test this strategy because (I) the same target EBV antigens are expressed in all tumors and (II) there is a simple and reliable way to stratify patients into risk groups. Patients who have elevated EBV plasma DNA after first line therapy have almost 12 fold greater risk of recurrence (113). If successful in these high risk patients, one could envisage vaccination being extended to all patients to eliminate occult micrometastases, resulting in further decreases in recurrence rates.

This review has focussed on the prospects for a therapeutic EBV vaccine that would benefit NPC patients in the immediate future. The success of prophylactic vaccines against hepatitis B virus and, more recently, oncogenic strains of human papillomavirus has stimulated interest in the possibility of primary prevention of the EBV-associated cancers (114). A small Phase II trial of a candidate EBV vaccine, based on the viral envelope glycoprotein gp350, reported a decrease in infectious mononucleosis but no overall protection against EBV acquisition. Although encouraging, a better immune response clearly needs to be induced to achieve complete protection. Multimeric forms of gp350 have now been developed and these have much stronger immunogenicity in animals compared to the monomeric gp350 used in the Phase II trial (115-117). Vaccine efficacy could also be improved by incorporating additional EBV glycoproteins and harnessing appropriate cellular immune responses (118,119). A safe, effective prophylactic EBV vaccine would be a major advance in human health. Its development is an important long-term goal, but it is also important to realize that the enormous number of people that already carry the virus, and for whom a preventative vaccine is already too late, means that NPC incidence will not decrease until years after a preventative vaccination campaign has started. Developing better ways to treat NPC will therefore remain an important area of research both now and in the future.

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Footnote

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