# Immunization with Epstein-Barr Virus (EBV) Peptide-pulsed Dendritic Cells Induces Functional CD8+ T-Cell Immunity and May Lead to Tumor Regression in Patients with EBV-positive Nasopharyngeal Carcinoma<sup>1</sup>

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# ABSTRACT

Nasopharyngeal carcinoma (NPC), a common neoplasm in Southeast Asia, is EBV-positive and expresses a limited number of antigens, including latent membrane protein 2. In this study, autologous monocytederived dendritic cells were cultured from patients with advanced NPC, matured with cytokine, pulsed with HLA-A1101-, A2402-, or B40011restricted epitope peptides from EBV latent membrane protein 2 and injected into inguinal lymph nodes. Sixteen patients with local recurrence or distant metastasis after conventional therapies received four injections at weekly intervals. Epitope-specific CD8+ T-cell responses were elicited or boosted in 9 patients receiving HLA-A1101- or A2402-restricted peptides, with stronger responses seen to the A1101 peptide. Furthermore, epitope-specific cytotoxicity was detectable in peripheral blood T cells harvested at 3-months after vaccination from A1101-responsive patients, and in 2 patients, this coincided with partial tumor reduction. Approaches leading to stronger and more sustained EBV-specific T-cell responses, therefore, may have therapeutic potential in the context of NPC.

# **INTRODUCTION**

NPC<sup>3</sup> is a common neoplasm throughout Southeast Asia, especially in southern Chinese (1-4). Radiotherapy is effective against earlystage NPC, and combination chemotherapy with cisplatinum and 5-fluorouracil usually can achieve a brief remission in metastatic disease (5, 6). After disease recurs or progresses, no additional effective therapy is available, and nearly 85% of patients die in 1 year and virtually all in 3 years (1, 7).

EBV is a ubiquitous  $\gamma$ -herpes virus that is associated with several malignant diseases, including NPC (8–10). The vast majority of people in all populations harbor EBV as a lifelong infection (8), and CD8+ CTLs are involved in maintaining such a virus-host balance (11). EBV latent antigen-specific CTLs from the blood of healthy carriers can be reactivated by *in vitro* stimulation with autologous virus-transformed lymphoblastoid cell lines (12, 13) and infusion of reactivated EBV-specific CTLs into immunosuppressed transplant patients can eradicate lesions associated with EBV-driven B-cell lymphoproliferative disorders (14–17). Enhancing the response to

appropriate EBV latent antigens therefore may be exploited to combat other EBV-related malignancies.

In contrast to the above lymphoproliferative lesions where the full spectrum of EBV latent proteins, including the immunodominant EBV nuclear antigen 3 proteins, are expressed, NPC cells express only one of the nuclear antigens EBNA1, as well as LMP1 (in a subset of tumors) and LMP2 (18-20). Of these, LMP2 is the most frequently recognized protein by CD8+ CTLs, although often as a subdominant target (13). Many Chinese healthy EBV carriers mount a detectable CTL response to LMP2, and NPC tumor cells can present LMP2 epitopes from endogenously expressed antigens in vitro (21). Several CD8+ T-cell epitopes have been identified in LMP2 that are restricted through HLA alleles, A1101, A2402 and B40011 (22), which are particularly common in the southern Chinese population (23, 24). DCs play a central role in initiation of T-cell responses (25), including those against tumor antigens in animal models and more recently in human trials (26-30). Here, we present results of a Phase I study of immunotherapy of patients with advanced EBV-associated NPC using DCs pulsed with LMP2 epitopes.

### MATERIALS AND METHODS

**Patients.** Patient characteristics are shown in Table 1. All patients had histologically proven NPC detectable with at least one detectable lesion because of local recurrence or metastatic diseases and expressed HLA-A1101, A2402, or B40011 by HLA typing. All were >18 years old, gave written consent, and had an expected survival of at least 4 months. Complete medical examinations were performed before entry of this trial. Female patients had a negative pregnancy test. All treatment, including herbal drug intake, was stopped at least 2 months before trial.

Treatment Protocol. The treatment protocol was approved by the local medical ethics committee, and informed consents were obtained according to the local regulations. PBMCs and autologous plasma were harvested after 150 ml of leukaphoresis, and most were cryopreserved at -180°C in aliquots until use. PBMCs  $(1-2 \times 10^8)$  were used to generate each of the four batches of mature DCs in vitro by culture for 7 days in granulocyte macrophage colonystimulating factor and IL-4 as previously described (31), followed by maturation in TNF- $\alpha$  (50 ng/ml) for 3 days. DCs were analyzed for purity on day 9 by immunofluorescence staining for CD80, CD83, and CD86. On day 10, DCs  $(5-10 \times 10^5 \text{ cells/ml})$  were incubated for 6-8 h with KLH (20  $\mu$ g/ml; Boehringer Mannheim, Mannheim, Germany) and one of the following LMP2 peptides (diluted to 100  $\mu$ g/ml from stock solution in DMSO): SSCSSCPLSK (HLA-A1101-restricted, LMP2 aa340-aa349); TYGPVFMSL (HLA-A2402restricted, LMP2 aa 419-aa427); and IEDPPFNSI (HLA-B-40011-restricted, LMP2 aa 200-aa208; Ref. 26), as appropriate for the patient's HLA type. DCs were then resuspended in saline containing 1% autologous serum (total volume = 0.5 ml) and delivered immediately by ultrasound-guided intranodal injection into one inguinal lymph node. To monitor immunological responses, patients were bled 2 weeks and 1 month after the first injection, and in selected patients, blood was also taken at 3, 6, and 10 months for analysis.

**ELISPOT Assay.** ELISPOT assays to detect LMP2 epitope-specific, IFN- $\gamma$ -producing cells were performed as described previously (32). Briefly, in the

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<sup>&</sup>lt;sup>3</sup> The abbreviations used are: NPC, nasopharyngeal carcinoma; CTL, cytotoxic T lymphocyte; LMP, latent membrane protein; DC, dendritic cell; PBMC, peripheral blood mononuclear cell; IL, interleukin; TNF, tumor necrosis factor; KLH, keyhole limpet hemocyanin; ELISPOT, enzyme-linked immunospot; DTH, delayed-type hypersensitivity.

#### Table 1 Patient characteristics

Sixteen patients were enrolled, all with local recurrence or distant metastasis after conventional therapies. Patients 1, 5, 11, and 12 had persistent leucopenia and a negative delayed-type hypersensitivity test to KLH tested 1 month after the first injection.

Patient	HLA	Age/sex	Clinical status	KLH response
1	A2402	36/M	Primary recurrence	-
2	A1101	43/F	Vertebral metastasis	+
3	A1101	45/M	Lung metastasis	+
4	A2402	47/M	Primary recurrence	+
5	A1101	46/M	Liver metastasis, bilateral neck lymph node metastasis	-
6	A1101	48/M	Primary recurrence	+
7	B40011	42/F	Bone metastasis	+
8	A1101	56/M	Primary recurrence	+
9	A2402	48/M	Neck and axillary lymph node metastasis	+
10	B40011	57/M	Primary recurrence	+
11	B40011	46/M	Lung metastasis	-
12	A1101	50/M	Lung metastasis	_
13	A2402	48/F	Primary recurrence	+
14	A1101	50/M	Primary recurrence	+
15	B40011	47/M	Primary recurrence	+
16	A2402	53/M	Primary recurrence	+

presence of 2 µg/ml of the relevant peptides, PBMCs  $(1 \times 10^5 \text{ and } 5 \times 10^5/\text{ well})$  were plated overnight in 96-well polyvinylidene difluoride-backed plates (Millipore, Bedford, MA) precoated with an anti-IFN- $\gamma$  monoclonal antibody (15 µg/ml; Endogen, Woburn, MA). Cells were then removed, and the wells incubated with biotinylated anti-IFN- $\gamma$  monoclonal antibody (1 µg/ml; Endogen) for 2–3 h, followed by streptavidin-conjugated alkaline phosphatase (Serotec) for 2 h and developed using an alkaline phosphatase-conjugated substrate kit (Sigma). Spots were counted under a dissection microscope. Specific T-cell responses were calculated after subtracting control values from cells exposed to DMSO solvent alone.

Flow Cytometric Analysis of CD8+ T Cells Producing Intracellular IFN- $\gamma$ . To determine the frequency of IFN- $\gamma$  producing CD8+ T cells in peripheral blood, PBMCs were harvested from patients before vaccination, 3 and 6 months after the first injection and were incubated with IL-2 (10 units/ml) and the relevant peptide epitopes (100 µg/ml) for 24 h followed by Brefeldin A (1 µg/ml; Sigma) for 2 h to prevent additional cytokine secretion. HLA-mismatched epitope peptides were used as controls. PBMCs (1 × 10<sup>5</sup>) were stained with intracellular anti-IFN- $\gamma$  antibody (Peprotech, Rocky Hill, NJ) and FITC-conjugated secondary antibody (Dako, Glostrup, Denmark) with IntraPrep permeabilization reagent (Immunotech) according to manufacturer's instructions. Surface CD8 expression was determined by anti-CD8 antibody (Peprotech) followed by PE-conjugated secondary antibody (Dako, Hamburger, Germany). Cells were analyzed by flow cytometry (FACS; Becton Dickinson, Palo Alto, CA).

**Cytotoxicity Assay.** Frozen PBMCs  $(1 \times 10^6)$  were thawed and incubated with IL-2 (10 units/ml) and the relevant epitope peptides (100  $\mu$ g/ml) for 24 h. As effector cells, T cells were enriched either before or after activation by nylon wool passage of the PBMCs (33, 34), followed by negative depletion of CD16+ and CD56+ cells with magnetic beads. As targets, PHA blasts were prepared from frozen PBMCs harvested before vaccination by culture in 10  $\mu$ g/ml phytohemagglutinin for 3 days and pulsing with epitope peptides or HLA-mismatched epitopes (100  $\mu$ g/ml) for 8 h. They were labeled with [<sup>51</sup>Cr]O<sub>4</sub> and incubated in 96-well V-bottomed plates at an E:T ratio of 20:1. The percentage of specific lysis (means of triplicate wells) was calculated after deducting the spontaneous lysis.

**Clinical Evaluation.** Patients were evaluated for overall tumor response every 3 months after the fourth injection, compared with the image studies taken before vaccination. All lesions were measured by the same method. Clinical response was defined as complete response, partial response, stable disease, and progressive disease, according to the standard clinical criteria.

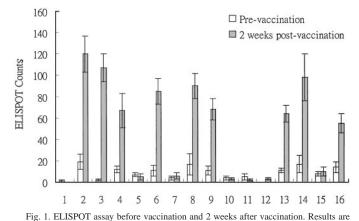
# RESULTS

**Patient Characteristics.** Sixteen patients (median age, 47.6 years; range, 36–57 years) were enrolled between September 1999 to October 2000, as detailed in Table 1. Four patients (patients 1, 5, 11, and 12) had persistent leucopenia (<2500/mm<sup>3</sup>) before leukapheresis and

had a negative DTH test to KLH at 1 month after the first injection. The other 12 patients had no signs of defective cellular immunity, as demonstrated by a positive DTH test to KLH.

Adverse Effects. All patients tolerated the treatment without serious side effects. Minor adverse events occurred in four patients: patient 1 and 4 subjectively experienced local rigor and swelling at the lesion side of the neck on the night of the first injection, which persisted for 24–48 h and subsided without specific treatment; patient 6 had local swelling at the injection site because of extravasation of the injection; and patient 10 developed low grade fever and mild fatigue 2 days after the first injection, which subsided without specific management.

**Immune T-Cell Responses Assayed by IFN-\gamma Production.** Before vaccination, levels of epitope-specific IFN- $\gamma$ -producing T cells in the blood were very low or undetectable in all patients (<20/million PBMCs; Fig. 1). After two vaccinations, *i.e.*, 2 weeks after the first injection, 5 of 7 patients immunized with the *A1101* peptide and 4 of 5 patients receiving *A2402* peptide made a response. The frequency of epitope-specific, IFN- $\gamma$ -producing T cells increased in the peripheral blood to 90–120/10<sup>6</sup> PBMCs in *A1101* patients (patients 2, 3, 6, 8, and 14) and to 60–70 PBMCs in *A2402* patients (patient 4, 9, 13, 16; Fig. 1). All 9 responsive patients were among the subset of 12 of 16 patients who were relatively immunocompetent as measured by a detectable response to KLH (Table 1). By contrast, none of the 4



expressed as the numbers of spot-forming (*i.e.*, IFN- $\gamma$ -producing) cells/10<sup>6</sup> PBMCs and as the mean  $\pm$  SD of triplicate well assays. Background numbers of spot-forming cells (as seen in wells stimulated with DMSO solvent control) were always low and have been subtracted from peptide-stimulated values.

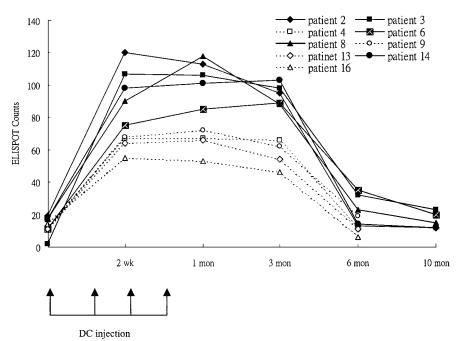


Fig. 2. ELISPOT assays of epitope peptide-specific T-cell responses, expressed as mean of triplicate wells, in PBMCs sampled before vaccination and at various times after vaccination from immunoresponsive patients (*i.e.*, patients 2, 3, 4, 6, 8, 9, 13, 14, and 16). PBMCs from HLA-A1101 patients (patients 2, 3, 6, 8, and 14) were also examined 10 months after vaccination. DC vaccination schedule was indicated as *arrows*.

patients receiving the *B40011* peptide gave a response, including 2 who were immunocompetent as judged by KLH responses (patients 7 and 10). Those who were leukopenic before vaccination and had a negative DTH to KLH (patients 1, 5, 11, and 12) had virtually no increase in the frequency of EBV LMP2 epitope-specific, IFN- $\gamma$ -producing T cells.

As shown in Fig. 2, in all 9 responsive patients, the increased frequency of epitope-specific, IFN- $\gamma$ -producing T cells was maintained for at least 3 months but declined almost to prevaccination level after 6 months. Stronger responses were observed in patients responsive to the HLA-*A1101* epitope peptide (patients 2, 3, 6, 8, and 14) than to the *A2402* peptide (patients 4, 9, 13, and 16). The T-cell frequency was also examined at 10 months after vaccination in 5 *A1101*-responsive patients, and the frequency of epitope-specific T cells was noted to be as low as that at 6 months.

To determine whether the IFN-y-producing T-cell population shown by ELISPOT involved CD8+ T cells, double immunostaining of peptide-stimulated PBMCs was performed with intracellular staining of IFN- $\gamma$  and surface staining of CD8. Fig. 3A shows data from the PBMCs of patient 2 examined for the response to the A1101 peptide before and 3 months after vaccination. A population of CD8+ cells that produced IFN- $\gamma$  in response to the A1101 peptide was clearly detectable by double staining after vaccination. In line with ELISPOT results of Fig. 2, in all immunoresponsive patients, the frequency of epitope-specific, IFN- $\gamma$ -producing CD8(+) cells was high at 3 months but declined to almost prevaccination level at 6 months after vaccination (Fig. 3B). Again by this assay, the A1101-responsive patients had a higher frequency of epitope-specific functional CD8+ T cells at 3 months after vaccination than A2402-responsive patients (Fig. 3C; P < 0.005 by Student t test). None of the other 7 patients who either received B40011 peptide injection or were immunosuppressed had a postvaccination increase in double-positive cells in this assay (data not shown).

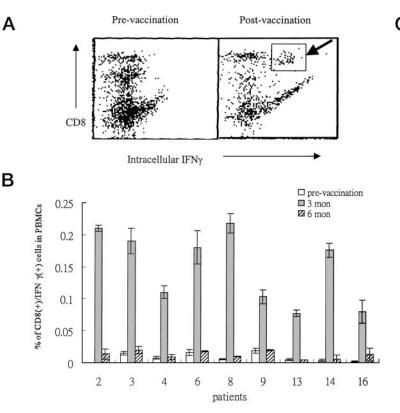
**Immune T-Cell Responses Assayed by Cytotoxic Capacity.** To test if the epitope-specific CD8+ T cells generated after DC immunotherapy possessed lytic activity, PBMCs (enriched for T cells and exposed to IL-2 plus peptide for 24 h *ex vivo*) were used as effectors in a chromium release assay on autologous peptide-loaded targets. Postvaccination cells from HLA-*A1101*-responsive patients (patients

2, 3, 6, 8, and 14) showed significant epitope-specific killing above background (range, 16-25%) compared with cells harvested from the same patient before vaccination (Fig. 4). This effect was observable at both 1 and 3 months after the first injection. However, no obvious lytic capacity of cells from *A2402* patients (patients 4, 9, 13, and 16) was detectable at either 1 or 3 months after vaccination using this approach.

Two A1101-responsive Patients Had Tumor Regression 3 Months after Vaccination. All patients were followed up for >1year, and their postvaccination clinical status are summarized in Table 2 alongside the records of their immunological responses. Patients 2 and 3, both of whom had given strong immunological responses to vaccination with the A1101 peptide, had clear indication of a clinical response, albeit partial. Patient 2 had bone metastasis at the third lumbar vertebrae body (Fig. 5A, arrow), presenting as severe lower back pain and requiring daily morphine intake for pain relief. Three months after the first injection, a decrease in both the size and density of sclerotic change was noted on the image (Fig. 5B, arrow), accompanied by subjective improvement of the symptoms and a reduction of the need for narcotics. This patient's disease remains stable after 1 year. Patient 3 had metastatic tumors in lungs and mediastinal lymph nodes (Fig. 5C, arrows). Three months after the treatment, diminished mediastinal lymph nodes and resolution of lung masses were noticed (Fig. 5D). Respiratory distress also improved significantly. However, regrowth of the metastatic tumor in the lungs was noted 10 months after the treatment and chemotherapy was therefore reinitiated for palliation. No clinical response could be observed in the other patients.

# DISCUSSION

In this study, autologous DCs were pulsed with HLA-restricted LMP2 epitope peptides and injected into inguinal lymph nodes of patients with advanced NPC. Matured DCs were used in our trial for peptide loading because they secrete IL-12 and express a variety of costimulatory and adhesion molecules at the cell surface, which facilitate T-cell priming (25); also their high surface MHC class I expression may increase the numbers of immunogenic MHC-peptide complexes and enhance the efficacy of CD8+ T-cell activation (35).



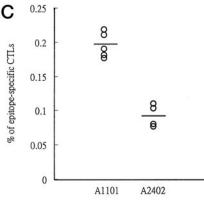


Fig. 3. Double immunostaining of PBMCs in ELISPOT-responsive patients before vaccination, 3 and 6 months after the first vaccination. Cells were in vitro stimulated with peptides and IL-2 for 24 h and stained with surface CD8 and intracellular IFN- $\gamma$ . A clear population of cells doubly stained for CD8 and intracellular IFN- $\gamma$  (*A*, arrow) in patient 2 was demonstrated at 3 months after vaccination. The percentage of CD8+/IFN- $\gamma$ + cells months) from 9 ELISPOT-responsive patients is shown in (*B*); results expressed as the mean  $\pm$  SD of three separate experiments. The frequency of CTLs in A1101-responsive patients is higher than that from A2402-responsive patients (*C*).

By contrast, repetitive stimulation with immature, rather than mature, DCs may induce IL-10-producing, nonproliferating T cells with regulatory properties (36), which might counteract the generation of the desired antitumor Th1 immunity. Various routes of administration of DCs have been used in previous studies (37) but, at least in a mouse model, intranodal injection of DCs can induce more potent Th1 antitumor immunity than i.v. or s.c. injection (38). Our study revealed that intranodal injection of EBV LMP2 epitope peptide-pulsed DCs into one of the inguinal lymph nodes is a well-tolerated procedure with negligible side effects in patients.

LMP2 was chosen as the target antigen because it appears to be consistently expressed in NPC (39) and is the most likely target for CD8+ T-cell recognition among the few EBV proteins present in the tumor (21). Interestingly, although naturally occurring LMP2 epitopespecific responses tend to be weak, they include responses restricted through HLA alleles (A1101, A2402, and B40011) that are unusually prevalent in Southeast Asian populations (23, 24). Memory T cells specific for these epitopes have been detected in healthy EBVinfected Chinese people (21, 40). Indeed the HLA-A1101 allele appears to be associated with a reduced risk of developing NPC in Chinese [41.8% of Chinese NPC patients versus 58.7% controls are A1101-positive (41)], and it has been postulated that such a protective role may be mediated by the A1101-restricted LMP2-specific CD8+ T-cell response (22). Therefore, immunotherapeutic approaches based on the above LMP2-derived epitopes could have wide applicability in areas with a high NPC incidence.

In this study, we showed that vaccination with epitope-loaded DCs significantly boosted LMP2 epitope-specific cell frequencies in 9 of 16 NPC patients. The incidence of positive responses is even higher if one allows for the fact that 4 of 7 nonresponders were clearly immunologically suppressed, as reflected by their leucopenia and inability to respond to such a strong foreign antigen as KLH. Moreover the LMP2-epitope-specific cells induced in our patients were functional in that they were detected by the capacity to synthesize

IFN- $\gamma$  in response to brief peptide stimulation *in vitro*, as measured either in ELISPOT assay (Fig. 1), and were confirmed to be CD8+ T cells by intracellular cytokine staining (Fig. 2). Such rapid cytokine production after epitope exposure *in vitro* is a typical property of "effector memory" CD8+ T cells (42). Furthermore, in the case of *A1101* (but not *A2402*) epitope-vaccinated patients, *ex vivo* cytotoxicity assays confirmed that the responding cells had epitope-specific cytotoxic activity (Fig. 4). Such cytotoxic function is also detectable in *ex vivo* PBMC or T-cell preparations from healthy EBV-immune donors, providing the frequency of epitope-specific CD8+ T cells is sufficiently high (43). Our inability to detect epitope-specific lysis in the blood of patients receiving the *A2402* peptide vaccine therefore probably reflects the lower level of reactive cells induced in these patients.

Previous studies have shown that T-cell responses to LMP2 epitopes are stable over periods of at least 3–6 months in the blood of healthy EBV-immune individuals,<sup>4</sup> and so the increase observed in patients in this study seems likely to be a response to epitope peptide vaccination. It is important to note from the ELISPOT assays that the frequencies of *A1101* and *A2402* epitope-specific, IFN- $\gamma$ -producing cells induced in the blood by vaccination were in the region of 60–120/10<sup>6</sup> PBMCs; for the *A1101* allele, in particular, such frequencies are at least as high as those seen in healthy *A1101*-positive Chinese individuals (40).<sup>5</sup> However, the *B40011* epitope peptide did not appear to be immunogenic when presented in the context of DC vaccination, and the reason for this requires additional investigation.

The vaccine-induced responses in our patients, which were detected as early as 2 weeks after the first injection and sustained for the first 3 months, later declined and returned almost to prevaccination levels by 6 months. In most DC-based immunotherapy trials, multiple injections were administered to generate antitumor responses (26–30),

<sup>&</sup>lt;sup>4</sup> N. M. Gudgeon and A. B. Rickinson, unpublished observations.

<sup>&</sup>lt;sup>5</sup> R. S. Midgley and A. B. Rickinson, unpublished results.

#### DENDRITIC CELL IMMUNOTHERAPY AND NASOPHARYNGEAL CARCINOMA

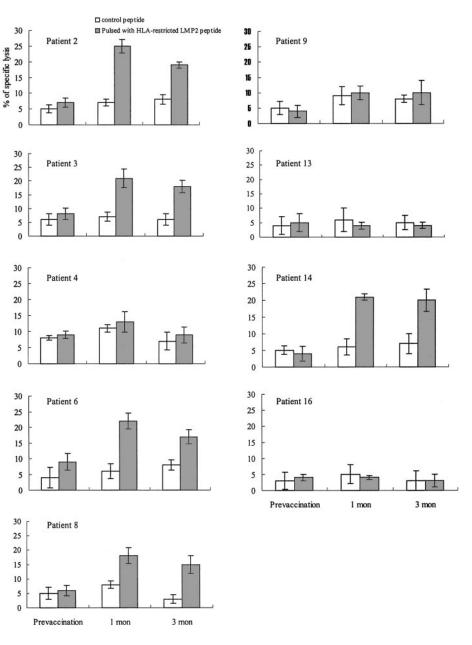


Fig. 4. Cytotoxic assays of the T cells sampled from patients 2, 3, 4, 6, 8, 9, 13, 14, and 16 at the indicated times before and after the first injection. Results are expressed as mean  $\pm$  SD. At both 1 and 3 months after the first vaccination, cytotoxic T cell lytic capacity was detected in A1101 patients (patients 2, 3, 6, 8, and 14) but not in A2402 patients (patients 4, 9, 13, and 16).

but it is still unclear how the injection schedule may impact on the timing and magnitude of T-cell responses in humans. In mice, it has been shown that when tested 24-48 h after weekly injection, the activity of T cells in lymph nodes peaked after the third but diminished after the sixth or seventh injection (44). Here, we found that the numbers of epitope-specific CTLs in peripheral blood peaked within a week of the second intranodal injection and were sustained at these levels until about 2 months after the final (fourth) injection. The subsequent fall in epitope-specific T-cell numbers in peripheral blood of our patients is more likely because of the natural life span of the T cell than to a change in their migratory patterns. However, the factors determining the fate of CD8+ T cells in the effector memory compartment are not yet understood. In a clinical trial involving the adoptive transfer of gene-marked EBV-specific T-cell preparations (usually dominated by CD8+ T cells reactive against the immunodominant EBV nuclear antigen 3 family of EBV latent antigens), there was evidence of their persistence in vivo for at least 18 months after infusion (16). Similar infusions into patients with EBV-positive

Table 2 Summary of postvaccination immunological assays and clinical responses

Patient	HLA	ELISPOT	CD8+/IFN+	Cytotoxicity	Clinical response <sup>a</sup> (1-year follow-up)
1	A2402	_ <i>b</i>	_	_	PD
2	A1101	+	+	+	PR
3	A1101	+	+	+	PR-PD
4	A2402	+	+	_	PD
5	A1101	_	_	_	PD
6	A1101	+	+	+	PD
7	B40011	_	_	_	PD
8	A1101	+	+	+	PD
9	A2402	+	+	_	PD
10	B40011	_	_	_	PD
11	B40011	_	_	_	PD
12	A1101	_	_	_	PD
13	A2402	+	+	_	PD
14	A1101	+	+	+	PD
15	B40011	_	_	_	PD
16	A2402	+	+	-	PD

<sup>*a*</sup> PR, partial response; decrease of tumor size by at least half of the sum of the products of the largest perpendicular diameters of all measurable lesions in the case of bimentionally measurable disease;  $\geq$ 25% increase in the size of at least one bidimentionally or unidimentionally measurable lesion or appearance of a new lesion.

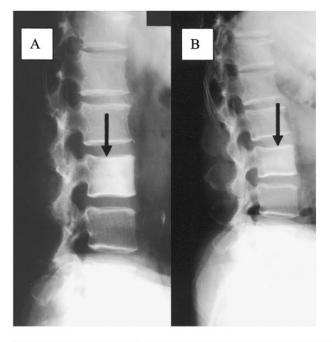


Fig. 5. Follow-up image studies of patients 2 and 3. Patient 2 had metastatic tumor at the third lumbar vertebral body before vaccination (A, arrow). Three months after vaccination, a reduction in both the size and density of the sclerotic change at the third lumbar vertebral body was noted (B, arrow). Patient 3 had tumor metastasis at the mediastinum and lung before vaccination (C, arrows). Three months after vaccination, there was diminished mediastinal lymphadenopathy and resolution of lung masses (D).

Hodgkin's Disease have shown persistence of the EBV-specific CTLs for 13 weeks in selected cases (45).

It is interesting, and may be significant, that the 2 patients (patients 2 and 3) exhibiting a partial clinical response after vaccination were both strong responders to the A1101 peptides in the CTL assays. The clinical responses involved regression of metastatic tumors in bone and lung (Table 2 and Fig. 5), although it is still unclear if the vaccine-induced epitope-specific effectors detectable in the blood were infiltrating and/or active at the tumor sites. By contrast, 3 other immunological responders to A1101 epitope vaccination (patients 6, 8, and 14) gave no clinical response. In these patients, there was tumor regrowth at the previously irradiated primary site in the nasopharynx. It is therefore possible that local immunosuppression at the primary site, resulting either from the irradiation or from factors secreted by the primary tumor itself as has been postulated in the case of EBVpositive Hodgkin's Disease (46), may have influenced the clinical outcome in these particular patients. Another factor may well be the time-scale in that the recurrence of the metastatic lung tumors occurred 10 months after vaccination, some time after the fall in epitopespecific T-cell responses in the blood had occurred. In that regard, future vaccine strategies must aim to sustain the LMP2 epitopespecific responses for longer periods. Extending the immunization schedule is one obvious possibility. Another factor may well be the need for a source of EBV-specific CD4+ T-cell help because there is increasing evidence that CD4+ T cells play a crucial role in the maintenance of CD8+ T-cell memory (47). In that context, it is now clear that EBNA1, another of the EBV antigens expressed in NPC cells, is a strong immunogen for CD4+ T-cell responses in healthy EBV carriers (48). Using EBNA1-derived CD4+ T-cell epitopes in conjunction with LMP2-derived CD8+ epitopes could be a future strategy worth exploring.

In conclusion, vaccination with LMP2 peptide-pulsed DCs elicited or boosted epitope-specific CD8+ T-cell responses detectable in the peripheral blood of NPC patients and in selected patients, a partial clinical response could be documented. We believe that our data are sufficiently encouraging to explore ways of developing more effective vaccine strategies that can induce sustained T-cell responses to EBV epitopes expressed in NPC.

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