Therapeutic LMP1 Polyepitope Vaccine for EBV-Associated Hodgkin’s Disease and Nasopharyngeal Carcinoma

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Running title: Immunotherapeutic Vaccine for HD and NPC

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ABSTRACT

Development of an epitope-based vaccination strategy designed to enhance Epstein-Barr virus (EBV)-specific CD8+ cytotoxic T lymphocytes is increasingly being considered as a preferred approach for the treatment of EBV-associated relapsed Hodgkin’s disease (HD) and Nasopharyngeal Carcinoma (NPC). EBV encoded latent membrane proteins, LMP1 and LMP2 are the only target antigens available for therapeutic augmentation of CTL responses in patients with HD and NPC. Here we describe preclinical studies using a recombinant poxvirus vaccine that encodes a polyepitope protein comprising of six HLA A2-restricted epitopes derived from LMP1. Human cells infected with this recombinant polyepitope construct were efficiently recognized by LMP1-specific CTL lines from HLA A2 healthy individuals. Furthermore, immunization of HLA A2/Kb mice with this polyepitope vaccine consistently generated strong LMP1-specific CTL responses to five of the six epitopes which were readily detected by both ex vivo and in vitro assays. More importantly, this polyepitope vaccine successfully reversed the outgrowth of LMP1 expressing tumours in HLA A2/Kb mice. These studies provide an important platform for the development of an LMP-based polyepitope vaccine as an immunotherapeutic tool for the treatment of EBV-associated HD and NPC.
Introduction

The concept of a role for the immune system in the control and elimination of virus-infected malignant cells has existed for many years, giving rise to the theory of immunological surveillance against tumours. More recently, it has been established that tumour rejection is mediated by lymphocytes and most notably by cytotoxic T lymphocytes (CTL). This concept is based mainly on the assumption that, like normal virus-infected cells, tumour cells can present virus-specific epitopes on their surface in conjunction with MHC molecules, which can be recognized by CTLs. Indeed, Epstein-Barr virus associated Hodgkin’s disease (HD) and nasopharyngeal carcinoma (NPC) represent two of the classic examples of virus-infected malignancies which are characterized by the expression of EBV nuclear antigen 1 (EBNA1) and latent membrane proteins (LMP) 1 and 2. Molecular analysis of both NPC and HD have shown that unlike other EBV-associated malignancies such as Burkitt’s lymphoma, in vitro established tumour cell lines from these malignancies are highly susceptible to lysis by EBV-specific CTLs and express normal levels of antigen-processing genes (TAP-1 and TAP-2).

In spite of the highly immunogenic phenotypes associated with these malignancies, tumour cells in NPC and HD can escape CTL-mediated immune control in vivo. A number of possible mechanisms have been proposed to explain this immune evasion strategy. Immunological and biochemical analysis of LMP1 sequences associated with NPC and HD have shown that these sequences are not only highly oncogenic but seem to be poorly immunogenic in murine models when compared with the LMP1 sequences derived from normal EBV-infected B cells. Moreover, independent studies by different groups have also shown that Reed-Sternberg cells in HD secrete anti-inflammatory cytokines (such as IL10 and TGFβ), which may interfere with the activation of LMP-specific CTLs.
It has been proposed that strategies designed to augment LMP1- and/or LMP2-specific CTL responses in HD and NPC patients may provide a therapeutic benefit for these malignancies. Indeed, adoptive transfer of polyclonal EBV-specific autologous CTLs into patients with advanced HD has recently been reported. In this study, all patients who received this adoptive therapy had incidental improvement, including reduction of high virus load, increase in virus-specific CTL precursor frequency, resolution of some symptoms and stabilization of the disease. Unfortunately, all of these patients failed to recover from the advanced stages of HD. One of the major limitations of this approach was that the CTLs transferred in this study were expanded by stimulating the peripheral blood lymphocytes with autologous EBV transformed lymphoblastoid cell lines (LCLs) which are known to preferentially stimulate T cells specific for EBNA antigens rather than LMP1 and LMP2. In spite of these limitations, it was encouraging to see a short-term therapeutic effect and further improvement of the CTL activation strategy may allow selective expansion of T cells that are specific for a limited range of viral antigens expressed in HD and NPC. In the present study, we have developed a novel strategy based on an LMP1 polyepitope vaccine, which allows an efficient activation of LMP1-specific CTL responses in vivo and these CTLs in turn provide a strong therapeutic benefit against LMP1-expressing tumours.
Materials and Methods

Construction of a Recombinant LMP Polyepitope Expression Vector

The amino acid sequences of six contiguous minimal LMP1 CTL epitopes (Figure 1) were translated to DNA using mammalian frequency codon usage. The resulting LMP polyepitope insert was constructed using four overlapping synthetic oligonucleotides: LMP-A 5’-CGCGGATCCCTGCAGGCCACCATGTACCTGCTGGAGATGCTGTGGAGGCTGTACCTCCAGCAGAACTGGT-3’, LMP-B 5’-CCGGTCGACCAGCACAGCAGCAGCAGGTTCCACAGGGCGATGACCAGCAGCAGGGTCCACCAGTTCTGCTGAGGTA-3’, LMP-C 5’ CCGGTCGACCTGCTGTGGCTGCTGACCCTGCTGGTGGACTGCTGTGGCTGAGGCTGG-3’, LMP-D 5’-CCCAAGCTTTCTAGACTACAGCTGCCAGATGGTGGCGCCCAGCCTCAGCCACAGCAGG-3’ (Sigma Genosys). The synthetic fragment included Bam H1 and Pst 1 restriction sites at the 5’end, a Kozak consensus sequence, ATG start codon, six contiguous minimal LMP1 CTL epitopes, a stop codon and Xba1 and Hind III restriction sites. These oligonucleotides were designed such that both LMP-A and LMP-B, and LMP-C and LMP-D included a complimentary 20 base pair overlap as indicated in figure1. The oligonucleotide pairs were annealed and extended using Pfu polymerase (Stratagene). The resulting fragments were cloned sequentially into the Bam H1/ Sal 1 and Sal 1/Hind III sites of pUC19 respectively. The resulting full length LMP polyepitope was subcloned into the Bam H1 and Hind III sites of the 7.37 Kb vaccinia virus shuttle vector pTK-7.5A and used to create recombinant vaccinia virus designated as Vacc.polyLMP. Construction of a TK- recombinant virus (Vacc. TK-) using marker rescue recombination as described previously 10.

Establishment and Maintenance of Human and Mouse Cell Lines

EBV-transformed LCLs were established from seropositive donors by exogenous virus transformation of peripheral B cells using the B95.8 virus isolate.
These cell lines were routinely maintained in RPMI 1640 (Gibco Invitrogen Corp., Carlsbad, CA) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin plus 10% FCS (growth medium). In addition, a mouse thymoma tumour cell line EL4 expressing a HLA A2/Kb chimeric molecule (referred to as EL4-A2/ Kb) was also used in this study. These cells were maintained in growth medium supplemented with G418 (500µg/ml). EL4-A2/ Kb cells were transfected with an expression vector encoding full-length genomic B95.8 LMP1 (pSG5-LMP1; referred to as EL4-A2/ Kb -LMP1) and cultured in growth medium supplemented with G418 (500µg/ml) for 4-6 weeks. Expression of LMP1 protein in EL4-A2/ Kb -LMP1 cells was confirmed by immunofluorescence and western blotting using an LMP1-specific CS1-4 antibody (Dako, Botany, Australia).

**Immunization of HLA A2/Kb Transgenic Mice with an LMP Polyepitope Vaccine**

HLA A2/Kb transgenic mice used in this study have been described elsewhere11 (a kind gift from Dr. L. Sherman, Scripps Research Institute). These mice express a chimeric class I molecule composed of the alpha 1 & 2 domains of the human A*0201 allele and the alpha 3 domains of the mouse H-2Kb class I molecules. These animals were vaccinated intraperitoneally with 5 × 10⁷ PFU of recombinant vaccinia encoding the LMP polyepitope. After 3 weeks, splenocytes were harvested and 4 × 10⁶ splenocytes from each mouse were restimulated with syngeneic irradiated (2000rad) 1 × 10⁶ lipopolysaccharide (LPS) blasts12. The LPS blasts were sensitized with peptide (10 µg/ml for 1h at 37°C) and washed twice prior to use. Cells were cultured in growth medium supplemented with 5 × 10⁶M mercaptoethanol (Sigma). On day 6 the cultures were used as effectors in standard 5h ⁵¹Cr release assays against EL4-A2/Kb target cells (32), which were sensitized with the indicated peptide (10 µg/ml) at the same time as being radiolabelled and washed twice prior to use. The
remaining CTL cultures were restimulated as described above on day 6 and tested for CTL activity again on day 10.

Synthesis of Peptides

Peptides, synthesized by the Merrifield solid phase method, were purchased from Chiron Mimotopes (Melbourne, Australia), dissolved in dimethyl sulphoxide, and diluted in serum-free RPMI 1640 medium for use in standard CTL assays. Purity of these peptides were tested by mass spectrometry and showed >90% purity.

Assessment of T cell Responses by IFN-γ ELISPOT Assay

This assay is based on a modification of the IFN-γ ELISPOT method described previously. Ninety six-well mixed cellulose ester membrane plates (Millipore, Bedford, USA) were coated overnight at 4°C with 75µL/well of 8µg/ml anti-IFN-γ, capture mAb (PharMingen, clone R4-6A2) in freshly prepared filter-sterilized 0.1M NaHCO₃, pH 8.4. The plates were washed six times with PBS, prior to blocking for 1h at 37°C with PBS containing 5% FCS. Splenocytes were harvested into growth medium supplemented with 5 × 10⁵ M 2-mercaptoethanol. These cells were plated in ELISPOT plates (10⁶/well) and then synthetic peptide epitopes were added into the wells at a final 1µg/ml concentration. For negative controls, splenocytes were incubated without peptide. The plates were incubated at 37°C with 5% CO₂ overnight (about 16-20 hr), then washed three times with PBST (0.05% Tween-20 in PBS) and three times with PBS before addition of 75µL/well of 1µg/ml anti-IFN-γ-biotin (PharMingen, clone XMG1.2) and incubated at room temperature for further 4hr. After incubation, plates were washed again three times each with PBST and PBS, and 100µL/well of 1µg/ml Streptavidin-alkaline phosphatase conjugate was added and incubated at room temperature for 2 hr. After a final PBS wash, BCIP/NBT developing/substrate solution (Sigma) was added at 100µL/well and kept at room
temperature until individual IFN-γ-producing cells were detected as dark spots (3-5 min). Color development was stopped by thoroughly washing the plates in tap water, prior to drying. Spots were counted automatically using an image analysis software (ImagePro)\textsuperscript{15}, and were expressed as spot forming cells (SFC) per 10\textsuperscript{6} PBMC. The number of IFN-γ-secreting T cells were calculated by first correcting for the background by the subtraction of the negative control SFC.

**Establishment of Human Polyclonal CTL Lines and LMP1-Specific CTL Clones**

Polyclonal CTL lines and LMP1-specific CTL clones were established according to previously published methods \textsuperscript{3}. Briefly, 2 × 10\textsuperscript{6} PBMCs from two HLA A2-positive healthy virus carriers (referred to as SB and LL) were stimulated with 1 × 10\textsuperscript{6} autologous lymphocytes (responder to stimulator ratio of 2:1) pulsed with 10μM peptide for 1h. After 3 days, growth medium with IL-2 (10U/ml) was added and the cells were further expanded. These lymphocytes were restimulated on day 7 with γ-irradiated (8000 rad) autologous LCLs. After 10 days in culture medium, the cells were used as polyclonal effectors in a standard \textsuperscript{51}Cr-release assay against peptide-sensitized autologous PHA blasts.

To generate peripheral blood CTL clones specific for the LMP1 derived peptides, PBMCs (2 × 10\textsuperscript{6}) of healthy donors were reactivated with peptide-sensitized (10μg of peptide/ml) autologous lymphocytes (1 × 10\textsuperscript{6}) in 2ml wells of a 24-well plate in growth medium. After 3 days, the cells were seeded onto 0.35% low melting gel agarose and maintained in T cell growth medium containing rIL2 (50 IU/ml). After another 3 days, growing clones were transferred to 96-well round-bottom tissue culture plates (Life Technologies) and cultured in T cell growth medium containing rIL2 (50-100 IU/ml).
**In Vitro Cytotoxicity Assays**

Target cells pre-sensitized with synthetic peptide epitopes or infected with recombinant vaccinia encoding the LMP polyepitope were incubated with $^{51}$Cr for 90 min. Following incubation, these cells were washed in growth medium and used as targets in standard 5 h $^{51}$Cr-release assays.

**In vivo cytotoxicity assays**

LMP1 epitope-specific in vivo CTL activity was assessed by using CFSE dye (Molecular Probes, Eugene, OR) labelled target cells\(^{16}\). Briefly, HLA A2/Kb splenocyte cell suspensions were divided into two populations following red cell lysis. One population was pulsed with an LMP1 epitope (1 µg/ml) for 90 min at 37°C, washed in PBS, and labelled with a high concentration (5 µM) of CFSE. Control, uncoated target cells were labelled with a low concentration of CFSE (0.5 µM). Cells ($10^7$) of each population were mixed in 200 µl of PBS and injected intravenously into Vacc.polyLMP or Vacc.TK- immunized mice. Specific in vivo cytotoxicity was determined by collecting the cells from spleen from recipient mice 18h after injection, and the number of cells in each target cell population was determined by flow cytometry. The ratio between the percentages of uncoated vs LMP1 peptide-coated (CFSE\(^{\text{low}}$/CFSE\(^{\text{high}}\)) cells was calculated to obtain a numerical value of cytotoxicity.

**Tumour Challenge and Polyepitope Immunization**

Two different vaccination strategies were used to assess the efficacy of the LMP polyepitope vaccine. In the first set of experiments, HLA A2/K\(^b\) mice were immunized intraperitonealy with either Vacc.polyLMP or Vacc.TK- (10\(^7\) PFU/mouse). Three weeks after the immunization, these mice were challenged subcutaneously with live 10\(^7\) EL4-A2/K\(^b\)-LMP1 cells. Following challenge, these animals were regularly monitored for 21 days and the tumour size measured by a calliper. In the second set of experiments, HLA A2/K\(^b\) mice were first challenged with EL4-A2/K\(^b\)-LMP1 (10\(^7\))
cells/mouse) tumour cells. Twelve days after the challenge, when the tumour size was approximately 0.4 cm in diameter, these mice were immunized with either Vacc.polyLMP or Vacc.TK-. The therapeutic efficacy of the LMP polyepitope vaccine was assessed by regular monitoring of tumour regression. Any mice showing tumour size >1.0 cm in diameter were sacrificed according to the guidelines of the institute animal ethics committee.
Results

LMP1-specific CTL Lines Efficiently Recognize Target Cells Infected With a Recombinant LMP Polyepitope

A recombinant LMP polyepitope vaccinia virus (Vacc.polyLMP) encoding six different HLA A2-restricted epitopes (Table 1) was derived using homologous recombination. To test whether the LMP1 epitopes encoded by this polyepitope were endogenously processed, target cells infected with Vacc.polyLMP were exposed to LMP1-specific CTL polyclonal/clonal lines specific for YLLEMLWRL (referred to as YLL) and YLQQNWWTL (referred to as YLQ) epitopes. These CTL lines were generated from healthy virus carriers and their specificity was confirmed by their ability to lyse HLA A2-positive target cells coated with respective peptide epitopes (Fig. 2; panel A). HLA A2-positive fibroblast infected with Vacc.polyLMP were also efficiently recognized by YLL- and YLQ-specific CTL lines (Fig. 2; panel B). These results clearly show that HLA class I-restricted CTL epitopes included in the LMP polyepitope are efficiently processed and presented to the target cells.

Generation of LMP1-specific CTL Responses in HLA A2/Kb Mice Vaccinated with a LMP Polyepitope

To determine whether the LMP polyepitope construct was capable of raising CTL responses in vivo, HLA A2/Kb transgenic mice were vaccinated with either the Vacc.polyLMP or Vacc.TK- and 21 days following immunization, CTL responses to each of the six epitopes were assessed. Three different methods were used to assess T cell responses. In the first set of experiments, ex vivo T cell reactivity to each of the peptide epitopes was assessed by ELISOPOT technology. A minimum of six animals were assessed in each group. Splenocytes from these mice were used as responder cells for the detection of epitope responsive T cells. Data presented in figure 3 clearly show that all mice consistently responded to five (YLL, YLQ, TLL, LLV and LLL) of the
six LMP1 CTL epitopes included in the polyepitope vaccine. On the other hand, low levels of T cell response to RLG epitope were detected (Fig. 3).

In the next set of experiments, these precursor T cells were stimulated *in vitro* to determine whether epitope specific CTL effectors could be expanded following immunization with the LMP polyepitope. Representative data from a group of six animals is shown in figure 4. Following a single stimulation with peptide sensitized LPS blasts, strong CTL responses to two epitopes (YLL and TLL) were detected, while a low to moderate response to the LLV epitope was detected. No epitope-specific lysis was detected for LLL and YLQ epitopes. However, a significant increase in the levels of CTL lysis was observed following secondary stimulation with peptide-sensitized LPS blasts. It is important to mention here that no CTL effectors were expanded following stimulation with the RLG peptide. These observations are consistent with our ELISPOT data presented in figure 3.

Finally, LMP1 epitope-specific CTL activity was observed in Vacc.polyLMP immunized mice using an *in vivo* cytotoxicity assay to monitor depletion of target cells labeled with immunogenic peptide and CFSE dye. Vacc.polyLMP and Vacc.TK- mice were inoculated with CFSE-labeled target cells coated with LMP epitopes (1µg/ml) and clearance of these cells was compared to that of targets labeled with CFSE but no peptide. The ratio (r) of the percentage of unlabelled cells and the percentage of epitope-coated cells represents the relative cytotoxic CD8+ CTL activities. Data presented in figure 5 clearly demonstrate that Vacc.polyLMP immunized mice cleared the great majority of peptide coated target cells, while uncoated cells were not lysed. On the other hand, Vacc.TK- immunized mice showed minimal difference in the clearance of peptide coated and uncoated target cells. Taken together these analyses clearly demonstrate that immunization with the Vacc.polyLMP vaccine can efficiently
generate CTL responses to LMP1 epitopes and that these T cells display strong lytic activity against peptide sensitized HLA A2-positive target cells.

**Immunization with Vacc.polyLMP Affords Protection Against LMP1-expressing Tumours**

To test whether the Vacc.polyLMP vaccine induced T cell responses can afford protection against LMP1-expressing tumour cells, two groups of HLA A2/Kb mice (10 mice in each group) were first immunized with Vacc.polyLMP or Vacc.TK- and then challenged with EL4-A2/Kb-LMP1 cells. These mice were regularly monitored for tumour outgrowth. Although both groups of animals developed tumours, the tumour outgrowth in Vacc.TK- was highly aggressive and showed no evidence of protection from tumour challenge (Figure 6). On the other hand, these tumours grew much less aggressively in animals immunized with Vacc.polyLMP and this outgrowth was completely resolved in 90% of the animals by the end of the observation period. By day 24, the average tumour load in Vacc.polyLMP-immunized mice was 30 - 33 fold lower when compared to Vacc.TK- immunized mice (Figure 6). It is important to mention here that animals immunized with Vacc.TK- or Vacc.polyLMP showed no protection against challenge with EL4/A2Kb cells indicating that the epitope-specific immune response was critical for this protection (data not shown).

The use of the LMP1 polyepitope vaccine as a therapeutic tool for the treatment of an actively proliferating tumour is one of the major challenges for its translation for human use. To explore the therapeutic efficacy of the polyepitope vaccine, a group of 40 HLA A2/Kb mice were challenged subcutaneously with 1x10^7 EL4-A2/Kb-LMP1 cells and monitored for tumour load. On day 12, when the tumour size was approximately 0.4cm in diameter, mice were divided into two groups and immunized with Vacc.TK- (20 mice) or Vacc.polyLMP (20 mice). Following immunization, the tumour size was regularly measured and any mice showing a
tumour size >1.0cm in diameter were sacrificed. Representative data from one such therapeutic vaccination is shown in figure 7. The tumour size progressively increased in almost all animals immunized with Vacc.TK- and by day 20, post immunization (day 32 post tumour challenge), almost 100% were dead. In contrast, a dramatic reduction in the tumour load was observed in mice immunized with Vacc.polyLMP and >60% of the mice were completely tumour free by day 20 post immunization and showed long-term protection. Moreover, the average tumour load in Vacc.polyLMP treated mice was significantly lower when compared to the Vacc.TK- vaccinated mice (data not shown).

**Comparative Analysis of T cell Responses to LMP1 Epitopes in HLA A2/Kb Mice Following Tumour Challenge**

To determine whether the CTL responses *in vivo* correlated with the tumour protection, LMP1-specific T cell responses were assessed following tumour challenge. Data from one such analysis is presented in figure 8. All animals immunized with Vacc.polyLMP vaccine and showing complete protection from tumour challenge demonstrated strong *ex vivo* LMP1 epitope-specific CTL responses. One of the interesting aspects of this result was that a significant increase in the T cell response to the RLGATIWQL epitope was observed following tumour challenge. Our initial studies (see figure 3) had indicated that very low levels of CTL responses are generated to RLGATIWQL epitope following immunization with Vacc.polyLMP vaccine. Furthermore, the levels of T cell responses to the LMP1 epitopes in individual mice showed strong correlation with the tumour load on day 21. Those mice with higher precursor frequency for LMP1 epitopes showed complete resolution of tumour, while small tumours were evident in those mice which had lower precursor frequency. These observations clearly indicate that a strong T cell response to LMP1 is crucial for a successful rejection of the LMP-expressing tumours. This contention is further
supported by lack of tumour protection in Vacc.TK- immunized mice, which showed 2-2.5 fold lower LMP1 epitope-specific T cell responses following tumour challenge.
Discussion

Over the past several decades, the goal of curing the majority of patients with primary HD and NPC has been reached. In the past three decades, two powerful tools, irradiation and multi-agent chemotherapy have emerged as the mainstays of modern treatment (reviewed in 17). Although both these therapeutic strategies are most efficient in eradicating a proportion of tumors, the non-specific nature of these treatments often results in significant side effects including long-term toxicities, development of secondary cancers and infectious complications18. Moreover, a small but significant proportion of HD and NPC patients relapse following chemo- and radio-therapy and also fail to respond to conventional therapeutic salvage strategies. More recently, there has been an increasing emphasis on the development of novel therapeutic strategies which are specifically designed to prime the patient’s own immune system to recognise EBV antigens expressed in malignant cells of HD and NPC and to specifically destroy these cells with minimal or no associated toxicities19,20.

The present study illustrates the possibility of using multiple HLA class I-restricted LMP1 CTL epitopes as a polyepitope vaccine for the treatment of EBV-associated HD and NPC. The LMP polyepitope vaccine induced LMP1 epitope-specific CTLs of multiple specificities in HLA-A2/Kb transgenic mice and the epitopes encoded by the recombinant virus were recognized by CTL lines from HLA A2-positive healthy seropositive donors. These observations indicate that five of the six epitopes encoded by the polyepitope construct are efficiently processed and presented by antigen presenting cells. It is important to stress here that the polyepitope vaccine technology also overcomes the potential limitation of the use of LMP1 protein as a vaccine for the induction of antigen-specific T cells. It is now firmly established that expression of LMP1 protein alone can transform normal cells and initiate the oncogenic process. Moreover, the transmembrane localization of LMP1 restricts its
accessibility to the cytosolic degradation pathways and thus limits its presentation through the classic class I pathway. Previous studies have shown that a polyepitope protein is highly unstable and is rapidly degraded through the proteasome pathway thus allowing more efficient presentation of all the epitopes encoded within this recombinant protein.

The therapeutic efficacy of the polyepitope vaccine was assessed using a quasi HD/NPC tumour model in HLA A2/Kb transgenic mice. This tumour model was based on the EL4-A2/Kb cells, which express the LMP1 oncogene as a transgene. Subcutaneous injection of these tumour cells (1x10^7 cell/mice) consistently resulted in tumour outgrowth in >95% of the HLA A2/Kb mice. Prior immunization of HLA A2/Kb mice with the LMP polyepitope vaccine provided a high degree of protection and the tumour outgrowth was significantly reduced when compared to the TK- mice. Ex vivo CTL analysis indicated that this protection was coincident with the generation of strong LMP1 epitope-specific responses. More importantly, the LMP1 polyepitope vaccine was not only efficient as a prophylactic therapy but was successfully used to reverse the outgrowth of pre-existing tumours. Immunization of tumour bearing HLA A2/Kb mice dramatically arrested the tumour outgrowth and by day 32 post-tumour challenge, more than 60% of the mice showed complete resolution of tumour outgrowth. On the other hand, immunization with Vacc.TK- failed to arrest the tumour outgrowth and 100% mice were dead by day 32 post-tumour challenge. These results further confirmed the efficacy of the LMP polyepitope vaccine for the treatment of LMP1-expressing tumours. It is important to stress here that although the tumour model used in this study displays immunological phenotype similar to that seen in HD and NPC, a proper validation of polyepitope vaccine approach will require a formal clinical trial in human patients.
The data presented here provide an important platform for the future development of immunotherapeutic strategies for the treatment of EBV-associated relapsed HD and NPC. Although HLA A2 is one of most common HLA class I alleles, the wider application of LMP-based polyepitope technology will require the inclusion of CTL epitopes restricted through other HLA class I alleles prevalent in NPC endemic regions of the world (HLA A11, A24, B27 and B57). Moreover, inclusion of additional epitopes from LMP1 and LMP2 will also allow the targeting of both antigens. We anticipate that the translation of polyepitope vaccine technology for human application will require other delivery modalities which are more likely to be approved by safety and human ethics committees. These include replication deficient adenovirus, poxvirus (Modified Vaccinia Ankara) vectors, naked DNA, or transduced autologous dendritic cells. Considering the highly immunosuppressive nature of malignant cells of HD, it is possible that LMP1 and LMP2 CTL induction might need to be facilitated by co-delivery of cytokines and/or prime boost strategies.
Figure Legends:

Figure 1: Schematic description of the construction of a recombinant vaccinia virus that expresses a synthetic DNA encoding for a polyepitope protein which contains six HLA A2-restricted LMP1 epitopes (see box and Table 1). Each of the alternate epitope sequences are underlined. The DNA sequence encoding this polyepitope protein was constructed using epitope sequence specific primers (referred to as LMP-A, LMP-B, LMP-C and LMP-D) and a technique based on mutual priming and overlap extension as described in the “Material and Methods” section. The nucleic acid sequence of the fragment coded (from the 5’ end) a BamHI and PstI restriction sites, a Kozak sequence, a methionine start codon, six contiguous minimal LMP1 CTL epitopes, a stop codon, and Xba I and HindIII restriction sites at the 3’ end. This DNA insert was subcloned behind the vaccinia p7.5 promoter in the plasmid shuttle vector pTK-7.5A using specific restriction enzymes for the construction of recombinant vaccinia virus.

Figure 2: Endogenous processing of LMP1 CTL epitopes encoded by Vacc.polyLMP. A: LMP1 epitope-specific lysis by YLL and YLQ-specific CTL lines derived from two healthy virus carriers (SB and LL). Peptide sensitized and uncoated HLA A2-positive PHA blasts were used as target cells in the CTL assay. B: HLA A2-positive fibroblasts were infected with either Vacc.polyLMP or Vacc.TK- for 18h and then exposed to YLL and YLQ-specific CTL lines from donors SB and LL. An effector:target ratio of 10:1 was used for both assays.

Figure 3: Ex vivo functional analysis of LMP1 epitope-specific T cells following immunization with Vacc.polyLMP. HLA A2/Kb mice were immunized i/p with $10^7$ PFU/mouse (Vacc.polyLMP or Vacc.TK-) and 21 days post immunization, LMP1
epitope-specific reactivity was assessed in the splenocytes by ELISPOT assays as described in the “Material and Methods” section. A minimum of six mice from each group were assessed for LMP1-specific T cell reactivity. The results are expressed as Mean ± SE of spot forming cells (SFC) per 10^6 splenocytes.

**Figure 4:** Activation of LMP1 epitope-specific CTL lines from HLA A2/K^b^ mice immunized with Vacc.ployLMP. CTL reactivity specific for LMP epitopes (YLQ, YLL, LLL, LLV and TLL) was assessed in splenocytes of Vacc.polyLMP immunized mice following primary (1°) and secondary (2°) stimulation with indicated peptide epitope sensitized LPS blasts. These T cell lines were used as effectors against EL4 A2/K^b^ cells sensitized with the same peptide (■) or no peptide (□). Mean lysis values (+SE) for each group of mice are presented. A minimum of six animals were assessed for each epitope.

**Figure 5:** Vacc.polyLMP immunization generates strong CTL activity in vivo. Vacc.polyLMP or Vacc.TK- immunized mice were inoculated with CFSE-labeled uncoated and peptide sensitized (YLL or YLQ) splenocytes at 21 days after immunization. The clearance of these cells was monitored 18h post inoculation by FACScalibur. Ratios in the top right corner of each plot represent the percentage of peptide-pulsed, high CFSE-labeled cells to the percentage of low CFSE-labeled uncoated splenocytes retrieved from inoculated mice. A ratio of 1 indicates little or no clearance of peptide-labeled cells. Figures are representative values from individual mouse from a group of four mice. Data are representative of two independent experiments.
Figure 6: Immunization with Vacc.polyLMP affords protection against LMP1 expressing EL4-A2/K\textsuperscript{b} tumour cells. Two groups of ten mice were immunized with Vacc.polyLMP or Vacc.TK- (10\textsuperscript{7} PFU/mouse) respectively. Twenty-one days post immunization, mice were challenged subcutaneously with EL4-A2/K\textsuperscript{b}-LMP1 cells (10\textsuperscript{7} cell/mouse) and monitored for tumour size for 24 days post challenge. Data is presented as mean ± SE of tumour size.

Figure 7: Regression of LMP1 expressing tumours in HLA A2/K\textsuperscript{b} mice following therapeutic immunization with Vacc.polyLMP. HLA A2/K\textsuperscript{b} mice were challenged with 10\textsuperscript{7} EL4-A2/K\textsuperscript{b}-LMP1 tumour cells. Twelve days after the challenge, when the tumour size was approximately 0.4cm in diameter, these mice were immunized with either immunized with Vacc.polyLMP or Vacc.TK-. The therapeutic efficacy of the LMP polyepitope vaccine was assessed by regular monitoring of tumour regression. Any mice showing tumour size >1.0cm in diameter were sacrificed according to the guidelines of the ethics committee. Data is presented as percentage of mice surviving after immunization with Vacc.polyLMP or Vacc.TK-.

Figure 8: Comparative analysis of T cell responses to LMP1 epitopes in HLA A2/K\textsuperscript{b} mice following tumour challenge. LMP1 epitope-specific reactivity was assessed in the splenocytes by ELISPOT assays as described in the “Material and Methods” section. A minimum of six mice from each group were assessed for LMP1-specific T cell reactivity. The results are expressed as Mean ± SE of spot forming cells (SFC) per 10\textsuperscript{6} splenocytes.
Table 1: List HLA A2-restricted LMP1 epitopes included in the polyepitope vaccine

<table>
<thead>
<tr>
<th>Epitope Sequence</th>
<th>Epitope code</th>
<th>EBV antigen</th>
<th>LMP1 Localization</th>
<th>HLA restriction</th>
<th>Reference</th>
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<td>YLLEMLWRL</td>
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Reference List


Fig. 1
Figure 2

Panel A: Comparison of PHA blast and PHA blast + peptide on % Specific Lysis.

Panel B: Comparison of vacc.TK- and vacc.polyLMP on % Specific Lysis.
Fig. 3
Fig. 4
Fig. 5

YLL

$\text{CFSE}^{r=1.2}$

$\text{Vacc.TK}^{r=0.34}$

YLQ

$\text{CFSE}^{r=1.1}$

$\text{Vacc.polyLMP}^{r=0.31}$
Fig. 7

Days Post Tumour Challenge

% Survival

- Vacc.polyLMP
- Vacc.TK-

Tumour Challenge
Vacc.polyLMP Vaccination

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Fig. 8
Therapeutic LMP1 Polyepitope Vaccine for EBV-Associated Hodgkin’s Disease and Nasopharyngeal Carcinoma

Jai Duraiswamy, Martina Sherritt, Scott Thomson, Judy Tellam, Leanne Cooper, Geoff Conolly, Mandvi Bharadwaj and Rajiv Khanna