Induction of Therapeutic T-Cell Responses to Subdominant Tumor-associated Viral Oncogene after Immunization with Replication-incompetent Polyepitope Adenovirus Vaccine

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ABSTRACT

The EBV-encoded latent membrane proteins (LMP1 and LMP2), which are expressed in various EBV-associated malignancies have been proposed as a potential target for CTL-based therapy. However, the precursor frequency for LMP-specific CTL is generally low, and immunotherapy based on these antigens is often compromised by the poor immunogenicity and potential threat from their oncogenic potential. Here we have developed a replication-incompetent adenoviral vaccine that encodes multiple HLA class I-restricted CTL epitopes from LMP1 and LMP2 as a polyepitope. Immunization with this polyepitope vaccine consistently generated strong LMP-specific CTL responses in HLA A2/Kb mice, which can be readily detected by both ex vivo and in vivo T-cell assays. Furthermore, a human CTL response to LMP antigens can be rapidly expanded after stimulation with this recombinant polyepitope vector. These expanded T cells displayed strong lysis of autologous target cells sensitized with LMP1 and/or LMP2 CTL epitopes. More importantly, this adenoviral vaccine was also successfully used to reverse the outgrowth of LMP1-expressing tumors in HLA A2/Kb mice. These studies demonstrate that a replication-incompetent adenovirus polyepitope vaccine is an excellent tool for the induction of a protective CTL response directed toward multiple LMP CTL epitopes restricted through 14 different HLA class I alleles prevalent in different ethnic groups where EBV-associated malignancies are endemic.

INTRODUCTION

Of the interventions designed to treat human malignancies, immunotherapy with CTLs is increasingly being recognized as the most efficient strategy with minimal side effects (1–4). One key factor in the development of a CTL-based therapy is the characterization of the immune correlates and the delineation of the specific portions of the tumor-associated antigens that elicit this response. CD8+ CTLs are considered important elements for protection against various virus-associated malignancies and have emerged as the major element that regulates the immune control of malignant cells (5). These findings provided an impetus to test the hypothesis that adoptive transfer of EBV-specific CTLs could protect humans against virus-associated malignancies (6, 7). Indeed, a number of studies have been published recently that have shown that adoptive immunotherapy can be successfully used to reverse the outgrowth of polyclonal B-cell lymphomas in transplant patients (8, 9).

Extension of a similar strategy to other EBV-associated malignancies, such as Hodgkin’s lymphoma (HL) and nasopharyngeal carcinoma (NPC), has been attempted recently; however, a limited long-term therapeutic benefit was observed (10). It has been argued that the expansion strategies used for generating EBV-specific CTLs in these studies were dominated by a T-cell repertoire specific for EBV nuclear antigens (EBNAs), which are not expressed in the malignant cells of HL or NPC. Hence, there is considerable interest in the possibility of targeting the virus-specific immune response to the viral antigens, which are expressed in these malignancies. These antigens include EBNA1, BARF0, LMP1, and LMP2 (11). Of these, LMP1 and LMP2 are the only potential targets, because EBNA1 and BARF0 are poorly processed and presented by virus-infected cells through the MHC class I pathway. Recent studies from our laboratory have shown a low CTL precursor frequency to epitopes within LMP1 in healthy virus carriers (12), suggesting that reconstitution of both LMP1- and LMP2-specific CTL responses may be necessary for a long-term therapeutic benefit of NPC and HL patients. The use of full-length LMP antigens in a clinical setting is unlikely to be approved because these proteins can independently initiate an oncogenic process in normal cells (13).

Recently, a recombinant vaccinia virus-based LMP1 polyepitope vaccine was successfully used to immunize mice, demonstrating the potency of these formulations in inducing therapeutic immunity against LMP1-expressing tumors (14). However, the potential risks associated with vaccinia virus, including encephalopathy and post-vaccine encephalitis, limits the large-scale application of recombinant vaccinia vaccines in humans (15, 16). To overcome these potential limitations, we have developed a novel approach of generating LMP-specific CTL responses with a replication-incompetent adenovirus encoding multiple epitopes from LMP1 and LMP2. This multi-epitope formulation includes 13 CTL epitopes restricted through 14 different HLA class I alleles, which span >90% of the population in different ethnic groups. Attractive features of adenovirus-based vaccines are their well-characterized genetic arrangement and function, as well as their extensive and safe usage in North American army recruits without inducing adverse side effects (17, 18). We have used an E1/E3-deleted recombinant adenovirus composed of a chimeric Ad5/F35 vector that has been engineered to substitute the shorter-shafted fiber protein from Ad35 strain. This expression system provides an advantage over previous Ad5 vectors with respect to efficiency of expression of recombinant protein in hematopoietic stem cells and dendritic cells (19). Here we show that an LMP polyepitope sequence expressed by the recombinant adenovirus is highly efficient in generating CTL responses to LMP1 and LMP2 epitopes in HLA A2/Kb transgenic mice and at recalling memory T-cell responses from healthy virus carriers. These CTLs not only recognized target cells sensitized with synthetic LMP epitopes in vitro and in vivo but also reversed the outgrowth of LMP1-expressing tumors in HLA A2/Kb mice.

MATERIALS AND METHODS

Construction of a Recombinant LMP Polyepitope Insert. The DNA sequence coding for the polyepitope amino acid sequence was designed by

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using the human universal codon usage. Five long oligonucleotides (primers 1–5 in the range of 74–100 mer) overlapping by 20 bp, representing the polyepitope DNA sequence, were annealed together by using Splicing by Overlap Extension and stepwise asymmetric PCR (Ref 20; Fig. 1). Briefly, polyepitope sequence-specific primers LMP-A and LMP-B were amplified in a hot-start PCR reaction (94°C for 1 min) for five cycles in a 20-μl reaction volume containing Elongase enzyme mix, PCR buffers, and deoxynucleotide triphosphates. At the end of five cycles, the PCR program was paused at 72°C, 2 μl of reaction was transferred to a new 20-μl reaction already at 72°C, and subjected to a further five cycles with primer LMPC and the polyepitope sequence-specific forward primer. This stepwise PCR was repeated until all oligonucleotides were joined. In the final step, 2 μl of the last reaction were amplified for 25 cycles using polyepitope sequence-specific forward and reverse primers. The nucleic acid sequence of the final fragment coded for a BamHI restriction site, a start codon, 13 contiguous minimal LMP2 and LMP1 CTL epitopes, a stop codon, followed by an HI restriction site, a methionine start codon, 13 contiguous minimal LMP1 and LMP2 CTL epitopes, a stop codon, and a 2 domain of the human A2/Kb mice were vaccinated with various doses of plaque-forming units (PFUs) of Ad5F35-LMPpoly vaccine. These primers were designed in such a way that the amplified product contained XbaI and KpnI restriction sites at the 5′ and 3′ ends, respectively (see the box in Fig. 1).

(b) After amplification in Escherichia coli, the expression cassette from pShuttle was excised and ligated into an Ad5F35 expression vector and confirmed by DNA sequence analysis.

The recombinant Ad5F35 vector was transfected into human embryonic kidney (HEK) 293 cells, and recombinant adenosivirus (referred to as Ad5F35-LMPpoly) was harvested from the transfected cells by successive freeze-thawing cycles.

Establishment and Maintenance Cell Lines. EBV-transformed lymphoblastoid cell lines (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 mML -glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin plus 10% FCS (referred to as growth medium). In addition, the HEK 293 cell line (23) was maintained in DMEM containing 10% FCS.

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

Immunization of HLA A2/Kb Transgenic Mice with Ad5F35-LMPPoly Vector. The HLA A2/Kb transgenic mice used in this study have been described elsewhere (Ref 24; a kind gift from Dr. L. Sherman, Scripps Research Institute, La Jolla, CA). These mice express a chimeric class I molecule composed of the α 1 and 2 domains of the human A*0201 allele and the α3 domain of the mouse H-2Kb class I molecules. To assess the efficacy of the Ad5F35-LMPPoly vaccine, HLA A2/Kb mice were vaccinated with various doses of plaque-forming units (PFUs) of recombinant virus. In addition, the efficacy of this vaccine following different routes of immunization (i.e., i.p., i.m., s.c. and i.v.) was also assessed. After 3 weeks, splenocytes were harvested and tested for epitope-specific T-cell responses assessed using ELISPOT and in vivo CTL assays (13).

ELISPOT Assay. The ELISPOT assay was used to detect LMP epitope-specific T cells after stimulation with synthetic peptide(s) as described previously (25). Briefly, responding cells were incubated in triplicate with each peptide epitope (10 μg/ml) for 18 to 20 h in 96-well multiscreen HA filtration plates (MAHA S4150; Millipore, Bedford, MA) coated with anti-IFN-γ monoclonal antibody (Mabtech AB, Nacka, Sweden). After incubation, the plates were extensively washed with PBS with 0.5% Tween 20 and incubated with a second biotinylated anti-IFN-γ monoclonal antibody, followed by the addition of streptavidin-conjugated alkaline phosphatase. Cytokine-producing cells were detected as purple spots after a 30-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Spots were counted automatically using image analysis software. T-cell precursor frequencies for each peptide epitope were based on the total number of cells and the number of spot-forming cells per well (average of

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**Table 1 List of HLA class I-restricted LMP1 and LMP2 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>
</tr>
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<tbody>
<tr>
<td>YLLELMWRL</td>
<td>YLL</td>
<td>LMP1</td>
<td>aai215-233</td>
<td>HLA A2, A68 &amp; A69</td>
<td>3</td>
</tr>
<tr>
<td>YLQQNWTL</td>
<td>YLQ</td>
<td>LMP1</td>
<td>aai159-167</td>
<td>3</td>
<td></td>
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<tr>
<td>ALLVLQSF</td>
<td>ALL</td>
<td>LMP1</td>
<td>aai51-60</td>
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</tr>
<tr>
<td>ILYLQQNW</td>
<td>IAL</td>
<td>LMP1</td>
<td>aai156-164</td>
<td>B57 &amp; B58</td>
<td>35</td>
</tr>
<tr>
<td>SSCSCPL2</td>
<td>SSCC</td>
<td>LMP2</td>
<td>aai348-361</td>
<td>HLA A11</td>
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</tr>
<tr>
<td>SKI</td>
<td>SKI</td>
<td>LMP2</td>
<td>aai340-350</td>
<td>HLA A11</td>
<td>36</td>
</tr>
<tr>
<td>PLYFLWAAI</td>
<td>PLYL</td>
<td>LMP2</td>
<td>aai313-129</td>
<td>A23, A24 &amp; A30</td>
<td>37</td>
</tr>
<tr>
<td>TYGPVFMC</td>
<td>TYG</td>
<td>LMP2</td>
<td>aai419-427</td>
<td>HLA A24</td>
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<td>RRRWRLTV</td>
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<td>LMP2</td>
<td>aai264-276</td>
<td>B27</td>
<td>36</td>
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<td>LLSS</td>
<td>LMP2</td>
<td>aai447-465</td>
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<td>LTAGGLFL</td>
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<td>HLA A25</td>
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<td>B40</td>
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<td>CLG</td>
<td>LMP2</td>
<td>aai268-276</td>
<td>HLA A21</td>
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**Fig. 1. Schematic representation of the construction of a recombinant adenovirus that expresses a synthetic DNA encoding for a polyepitope protein that contains 13 HLA class I-restricted LMP1 and LMP2 epitopes (see box and Table 1). Each of the alternate epitope sequences is underlined. The DNA sequence encoding this polyepitope protein was constructed using epitope sequence-specific primers (referred to as LMP-A, LMP-B, LMP-C, LMP-D, and LMP-E) and a technique based on mutual priming and overlap extension as described in “Materials and Methods.” This synthetic insert was first cloned into a pcDNA3 expression vector and was subsequently amplified and subcloned into a pShuttle vector. This amplified nucleic acid sequence of the fragment encoded a polyepitope DNA sequence, were annealed together by using Splicing by Overlap Extension and stepwise asymmetric PCR (Ref 20; Fig. 1):**
three wells). LMP epitope-specific spots were calculated after subtraction of the number of spots in control wells consisting of peripheral blood mononuclear cells (PBMCs) without added peptide (average of six wells).

**In Vivo Cytotoxicity Assays.** LMP1 epitope-specific in vivo CTL activity was assessed using carboxyfluorescein (CFSE) dye (Molecular Probes, Eugene, OR) labeled target cells (26). Briefly, HLA A2/Kb splenocyte cell suspensions were divided into two populations after red cell lysis. One population was pulsed with an LMP1 epitope (1 μg/ml) for 90 min at 37°C, washed in PBS, and labeled with a high concentration (5 μM) of CFSE. Control, uncoated target cells were labeled with a low concentration of CFSE (0.5 μM). Cells (10⁶) from each population were mixed in 200 μl of PBS and injected i.v. into Ad5F35-LMPpoly or control adenovirus immunized mice. Specific in vivo cytototoxicity was determined by calculating the spleenocytes from recipient mice 18 h after injection, and the number of cells in each target cell population was determined by flow cytometry. The ratio between the percentages of uncoated versus LMP1 peptide-coated (CFSE<sup>-</sup>):CFSE<sup>+</sup>) cells was calculated to obtain a numerical value of cytototoxicity.

**Establishment of Human Polyclonal CTL Lines Specific for LMP Epitopes.** Polyclonal CTL lines specific for LMP1 and LMP2 epitopes were established according to methods published previously (3). Briefly, 2 × 10⁶ PBMCs from healthy virus carriers were stimulated with 1 × 10⁶ autologous lymphocytes (responder to stimulator ratio of 2:1) and pulsed with 10 μM peptide for 1 h. After 3 days, growth medium with IL-2 (10 units/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 51Cr-release assay against peptide-sensitized autologous phytohemagglutinin antigen (PHA) blasts.

**In Vitro Cytotoxicity Assays.** Target cells were either presensitized with synthetic peptide epitopes or infected with recombinant adenovirus (at a multiplicity of infection (MOI) of 50:1) encoding the LMP polyepitope. The adenovirus infection of target cells was assessed using anti-Hexon antibody according to the manufacturer’s recommendations (BD Biosciences, Palo Alto, CA). Target cells infected with recombinant adenovirus were incubated for 14–16 h at 37°C, washed in growth medium, labeled with 51Cr, and used as targets in standard 5-h 51Cr-release assays.

**Expansion of LMP-specific CTLs Using Ad5F35-LMPpoly.** LMP-specific CTL lines were generated from a panel of three healthy virus carriers by stimulating PBMCs with Ad5F35-LMPpoly. For adenovirus polyepitope stimulation, 2 × 10⁶ PBMCs from healthy virus carriers were cocultured with autologous PBMCs (1 × 10⁶) infected with Ad5F35-LMPpoly (MOI, 10:1) at a responder:stimulator ratio of 2:1 for 7 days. On day 7, these cultures were restimulated with adenovirus-infected PBMCs as described above, and the growth medium was supplemented with recombinant interleukin 2 (10–20 units/ml). LMP-specific T-cell reactivity was sequentially assessed on day 20 using in vitro cytotoxicity assays.

**Tumor 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<sub>b</sub> mice were immunized intraperitoneally with either Ad5F35-LMPpoly or control adenovirus (10⁶ PFUs/mouse). Three weeks after the immunization, these mice were challenged s.c. with live 10⁷ EL4-A2/K<sub>b</sub>-LMP1 cells. After challenge, these animals were regularly monitored for 21 days, and the tumor size was measured by calipers. In the second set of experiments, HLA A2/K<sub>b</sub> mice were first challenged with EL4-A2/K<sub>b</sub>-LMP1 (10⁴ cells/mouse) tumor cells. Ten days after the challenge, when the tumor size was ~0.2 cm in diameter, mice were immunized i.p. with either Ad5F35-LMPpoly or control adenovirus. The therapeutic efficacy of the LMP polyepitope vaccine was assessed by regular monitoring of tumor regression. Mice showing a tumor size >1.0 cm in diameter were sacrificed according to the guidelines of the institute’s animal ethics committee.

**Statistical Analysis.** The therapeutic efficacy of the polyepitope vaccine was analyzed by Kaplan-Meier test.

**RESULTS**

**Endogenous Processing and CTL Recognition of CTL Epitopes Encoded by Recombinant Adenovirus LMP Polyepitope.** A recombinant adenovirus LMP polyepitope (Ad5F35-LMPpoly) encoding 13 different HLA class I-restricted epitopes (Table 1) was derived using the protocol described in “Materials and Methods.” To test whether the LMP epitopes encoded by this polyepitope were endogenously processed, target cells (fibroblasts or LCLs) infected with Ad5F35-LMPpoly (MOI, 50:1) were exposed to LMP1- or LMP2-specific CTL polyclonal lines specific for various epitopes. The adenovirus infection of target cells was confirmed with anti-Hexon antibody (Fig. 2A). Both LCLs and fibroblasts infected with the control Ad5F35 virus and Ad5F35-LMPpoly showed strong expression of Hexon protein, although the level of adenovirus infectivity was higher in fibroblasts when compared with LCLs. LMP-specific CTL lines were generated from healthy virus carriers, and their specificity was confirmed by their ability to lyse HLA-matched target cells coated with respective peptide epitopes (Fig. 2B). HLA matched fibroblasts or LCLs infected with Ad5F35-LMPpoly were also efficiently recognized by individual LMP-specific CTL lines, whereas target cells infected with control Ad5F35 virus were not recognized (Fig. 2C). This CTL recognition of LMP polyepitope was highly specific because the MHC-matched LCLs infected with Ad5F35-LMPpoly or presensitized with peptide epitope
were recognized, whereas the MHC-mismatched target cells infected with Ad5F35-LMPpoly or control adenovirus were not recognized by these T cells. Representative data for an LMP2 epitope (IED, HLA B40-restricted) and an LMP1 epitope (YLL, HLA A2-restricted) are presented in Fig. 3. These results demonstrate that HLA class I-restricted CTL epitopes included in the adenosine LMP polyepitope are efficiently processed and presented to the target cells.

In the next set of experiments, the adenoviral LMP polyepitope vaccine was used to restimulate a secondary CTL response in vitro from PBMCs obtained from healthy EBV-seropositive individuals. The resulting polyclonal cultures were used as effectors against autologous PHA blasts sensitized with LMP1 or LMP2 peptide epitopes. Data presented in Fig. 4 show that the LMP polyepitope was highly efficient in recalling multiple CTL responses, which were specific for the epitopes restricted by the HLA alleles expressed by each donor. For instance, the LMP polyepitope stimulated a strong T-cell response to the epitopes YLL (HLA A2-restricted, LMP1), CLG (HLA A2-restricted, LMP2), and IED (HLA B40-restricted, LMP2) from PBMCs derived from donor SE (HLA A2, A29, B40, B44). Similarly, multiple epitope-specific CTL responses were also detected from other donors (Fig. 4).

Assessment of Immunogenicity of Ad5F35-LMPpoly Vector in HLA A2/Kb Mice. To determine whether the adenovirus LMP polyepitope construct was capable of stimulating CTL responses in vivo, HLA A2/Kb transgenic mice were vaccinated with either the Ad5F35-LMPpoly or control adenovirus, and 21 days after immunization, CTL responses to five different HLA A2-restricted epitopes were assessed. In the first set of experiments, HLA A2/Kb mice were immunized i.p. with various doses (1 × 10^7, 5 × 10^7, and 1 × 10^8 PFUs/mouse) of the LMP polyepitope vaccine, and e vivo T-cell reactivity to each of the peptide epitopes was assessed by ELISPOT technology. Splenocytes were used as responder cells for the detection of epitope-specific responsive T cells. Data presented in Fig. 6 show that all mice immunized with 10^8 PFUs of Ad5F35-LMPpoly consistently responded to all HLA A2-restricted LMP1 (YLL, YLQ, and ALL) and LMP2 (CLG and LTA) CTL epitopes included in the polyepitope vaccine. Low to undetectable levels of T-cell responses were observed in mice immunized with 10^7 and 5 × 10^7 PFUs of the LMP polyepitope vaccine. No LMP epitope-specific reactivity was observed in mice immunized with the control adenovirus (Fig. 6). In the next set of experiments, the efficacy of the LMP polyepitope vaccine was assessed by immunizing HLA A2/Kb mice using different routes. Four different groups of animals were immunized i.p., i.m., i.v., or s.c. routes with the LMP polyepitope vaccine (10^6 PFUs/mouse), and 21 days after immunization, LMP-specific T-cell responses were assessed by ELISPOT assays. Data presented in Fig. 7 show that all four different modes of immunizations showed comparable levels of T-cell activation.
Ad5F35-LMPpoly or control adenovirus twice at 14-day intervals and subsequently challenged with EL4-A2/Kb-LMP1 cells. These mice were regularly monitored for tumor outgrowth. Although both groups of animals developed tumors, the tumor outgrowth in control adenovirus-immunized mice was highly aggressive and showed no evidence of protection from tumor challenge (Fig. 9). On the other hand, although these tumors initially grew quite aggressively in animals immunized with Ad5F35-LMPpoly, outgrowth was completely resolved in 90% of the animals after a booster immunization on day 14 after tumor challenge. By day 24, the average tumor load in Ad5F35-LMPpoly-immunized mice was 10-fold lower when compared with control adenovirus immunized mice (Fig. 9). It is important to mention here that animals immunized with control adenovirus or Ad5F35-LMPpoly showed no protection against challenge with EL4-A2/Kb cells, indicating that the epitope-specific immune response was critical for this protection (data not shown).

To explore the therapeutic efficacy of the adenoviral polyepitope vaccine, a group of 12 HLA A2/Kb mice were challenged s.c. with $1\times10^7$ EL4-A2/Kb-LMP1 cells and monitored for tumor load. On day 8, when the tumor size was $\sim0.2$ cm in diameter, mice were divided into two groups and immunized with control adenovirus (six mice) or Ad5F35-LMPpoly (six mice). After immunization, the tumor size was regularly measured, and mice showing a tumor size $>1.0$ cm in diameter were sacrificed. Representative data from one such therapeutic vaccination are shown in Fig. 10. The tumor size progressively increased in almost all animals immunized with control adenovirus, and by day 8 after immunization (day 16 after tumor challenge), all mice had tumor sizes $>1$ cm (Fig. 10). In contrast, a dramatic reduction in the tumor load was observed in mice immunized with Ad5F35-LMPpoly, and 70% of the mice were completely tumor free by day 48 after immunization (day 40 after tumor challenge) and showed long-term protection (Fig. 10). Moreover, the average tumor diameter in Ad5F35-LMPpoly-treated mice (0.189 cm) was significantly lower ($P<0.001$) when compared with the control adenovirus (0.7561 cm) vaccinated mice (data not shown).

**DISCUSSION**

The data presented in this study provide a highly efficient immunotherapeutic strategy for the treatment of human malignancies where the tumor-associated antigens are poorly immunogenic and/or carry a cleared the majority of peptide-coated target cells, whereas uncoated cells were not lysed. On the other hand, mice immunized with control adenovirus showed a minimal difference in the clearance of peptide-coated and uncoated target cells. Taken together, these analyses demonstrate that immunization with the Ad5F35-LMPpoly vector can efficiently generate CTL responses to both LMP1 and LMP2 epitopes and that these T cells display strong lytic activity against peptide-sensitized HLA A2-positive target cells.

**Immunization with Ad5F35-LMPpoly Vaccine Reverses Outgrowth of LMP1-expressing Tumors.** To test whether the adenoviral LMP polyepitope vaccine induced T-cell responses that afford protection against LMP1-expressing tumor cells, two groups of HLA A2/Kb mice (10 mice in each group) were first immunized i.p. with Ad5F35-LMPpoly or control adenovirus twice at 14-day intervals and

Fig. 5. MHC-specific recognition by Ad5F35-LMPpoly-stimulated CTLs. PBMCs from donor SE were cocultured with autologous PBMCs infected with Ad5F35-LMPpoly (MOI, 10:1) at a responder to stimulator ratio of 2:1, and a CTL line was generated as described in "Materials and Methods." These CTL effectors were tested against autologous PHA blasts (referred to as SE PHA) and allogeneic PHA blasts presensitized with synthetic peptide epitopes or left uncoated. The peptide epitopes used in these assay were YLL (HLA A2-restricted, LMP1), CLG (HLA A2- restricted, LMP2), B40 (HLA B40-restricted, LMP2), ALL (HLA A2-restricted, LMP1), D, and ALL (HLA A2-restricted, LMP1-D). These CTL effectors were tested at different E:T ratios.

Fig. 6. Ex vivo functional analysis of LMP1 epitope-specific T cells after immunization with various dosages of an LMP polyepitope vaccine. Four different groups of HLA A2/Kb mice were immunized with either Ad5F35-LMPpoly ($1\times10^7,5\times10^7$, or $1\times10^8$ PFUs) or control adenovirus ($1\times10^7$ PFUs). Twenty-one days after immunization, LMP1 and LMP2 epitope-specific reactivity was assessed in the splenocytes by ELISPOT assays as described in "Materials and Methods." The epitopes tested for T-cell reactivity were YLL, YLQ, and ALL from LMP1, CLG (HLA A2-restricted, LMP2), B40 (HLA B40-restricted, LMP2), ALL (HLA A2-restricted, LMP1-D), and ALL (HLA A2-restricted, LMP1-D). These CTL effectors were tested at different E:T ratios.

Fig. 7. Influence of different immunization routes on the generation of an LMP-specific memory T-cell response. Four different groups of HLA A2/Kb mice were immunized i.v., i.m., s.c., or i.p. with Ad5F35-LMPpoly vaccine ($10^7$ PFUs/mouse). Twenty-one days after immunization, LMP1 and LMP2 epitope-specific reactivity was assessed in the splenocytes by ELISPOT assays as described in "Materials and Methods." The epitopes tested for T-cell reactivity were YLL, YLQ, and ALL from LMP1, CLG and LTA from LMP2. A minimum of six mice from each group were assessed for LMP-specific T-cell reactivity. The results are expressed as means of spot-forming cells (SFC) per $10^8$ splenocytes from six individually tested mice; bars, SE.
potential oncogenic threat if used as immunogens in a therapeutic vaccine. EBV-associated malignancies such as HL and NPC are the classic examples of human cancers where there is an urgent need to develop more efficient therapeutic strategies for the management of relapsed disease (7, 27). One of the major limiting factors toward the development of an efficient therapeutic strategy is the viral gene expression, which is often restricted to only a few latent antigens (i.e., EBNA1 and LMP1 and LMP2), which are not only poorly immunogenic but also in some cases have the potential to initiate an independent neoplastic process in normal cells (28, 29). Thus, a strategy that can overcome both of these potential limitations is very likely to provide a safe and long-term therapeutic benefit to cancer patients. One such strategy involves the delivery of immunogenic determinants from LMP1 and LMP2 as a polyepitope vaccine. Indeed, recent studies from our laboratory have shown that multiple HLA class A2-restricted EBNA1 and LMP1 and LMP2, which are not only poorly immunogenic but also recalled memory CTL responses specific for these epitopes are not only efficiently processed endogenously by the human cells but also optimally selected MHC class I-restricted epitopes would include A20, 20%, B57, 36%, A11, 56%, A24, 27%, B40, 28%, particularly where NPC is endemic. Hence, we have developed a new LMP polypeptide therapeutic vaccine using a replication-incompetent adenovirus containing both LMP1 and LMP2 epitopes restricted through A11, A24, and B40 are of particular interest because these alleles are very common in the Southern Chinese population (A11, 56%; A24, 27%; B40, 28%), particularly where NPC is endemic. These vectors are also increasingly being used for cancer immunotherapy (30–32). Two of the most promising recent reports were studies in non-human primate models of the Ebola virus and HIV (33, 34). In each study, an immunization regimen that included priming with plasmid DNA, followed by boosting with adenovirus vector particles, showed the induction of effective CTL responses when compared with the plasmid DNA alone (33). In the present study, we have shown that vaccination with an adenovirus vector expressing the LMP polypeptide protein composed of a series of contiguous minimal LMP1 and LMP2 CTL epitopes is capable of inducing multiple, independent MHC-restricted CTL responses. These epitopes are not only efficiently processed endogenously by the human cells but also recalled memory CTL responses specific for

Fig. 8. Adenoviral LMP polyepitope vaccine generates strong CTL activity in vivo. Ad5F35-LMPpoly or control adenovirus immunized mice were inoculated with CFSE-labeled uncoated (referred to as No peptide) and peptide sensitized (referred to as + peptide: YLL or YLQ) splenocytes at 21 days after immunization. The clearance of these cells was monitored 18 h after inoculation by FACSScalibur. Rats (r) in the top right corner of each plot represents the percentage of peptide-pulsed, high CFSE-labeled cells divided by the percentage of low CFSE-labeled uncoated splenocytes retrieved from inoculated mice. Figures are representative values from an individual mouse from a group of four mice. Data are representative of three independent experiments.

Fig. 9. Immunization with an adenoviral LMP polyepitope vaccine affords protection against LMP1-expressing EL4-A2/Kb tumor cells. Two groups of 10 mice were immunized with Ad5F35-LMPpoly or control adenovirus (108 PFUs/mouse) on days −28 and −14, respectively. At day 0 (28 days after immunization), mice were challenged s.c. with EL4-A2/Kb-LMP1 cells (105 cells/mouse) and monitored for tumor size. Both of these groups of mice were given another booster dose of immunization on day 14 after tumor challenge. Data are presented as means of tumor size, bars, SE.

Fig. 10. Regression of LMP1-expressing tumors in HLA A2/Kb mice after therapeutic immunization with an adenoviral LMP polyepitope vaccine. HLA A2/Kb mice were challenged with 105 EL4-A2/Kb-LMP1 tumor cells. Eight days after the challenge, when the tumor size was ~0.2 cm in diameter, these mice were immunized with either Ad5F35-LMPpoly or control adenovirus (Adcontrol; 1 × 108 PFUs/mouse). The therapeutic efficacy of the LMP polyepitope vaccine was assessed by regular monitoring of tumor regression. Mice showing a tumor size >1.0 cm in diameter were sacrificed according to the guidelines of the ethics committee. Data are presented as a percentage of mice surviving after immunization with Ad5F35-LMPpoly or Adcontrol.
LMP antigens in healthy virus carriers. Furthermore, the adenoviral polyepitope vaccine is capable of inducing a primary T-cell response, which was shown to be therapeutic in a tumor challenge system. It is important to mention here that the adenovector (Ad5FS5) used in this study provides an advantage over traditional Ad5 vectors with respect to efficiency of expression of recombinant protein in hematopoietic stem cells and dendritic cells (19). This efficient expression of recombinant proteins in professional antigen-presenting cells may provide an improvement over the traditional vaccine vectors for priming an efficient antitumor immunity in vivo.

It is important to stress here that a polyepitope-based vaccine for HL and NPC has a number of advantages over the traditionally proposed vaccines, which are based on full-length LMP antigens. Previous studies from our laboratory have indicated that polyepitope proteins are extremely unstable and may be rapidly degraded within the cytoplasm as a result of their limited secondary and tertiary structure. On the other hand, the full-length LMP antigens are unlikely to be degraded rapidly and may initiate various intracellular signaling events leading to the development of secondary cancers at the site of injection. Another important advantage includes the ability of polyepitope vaccine to induce long-term protective CTL responses against a large number of CTL epitopes using a relatively small construct without any obvious need for a cognate help. It is important to stress here that the possibility of non-cognate help provided by the viral vector itself cannot be ruled out fully. Finally, the polyepitope-based vaccine is also likely to overcome any potential problem with the prevalence of LMP1 genetic variants in different ethnic groups of the world.

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Induction of Therapeutic T-Cell Responses to Subdominant Tumor-associated Viral Oncogene after Immunization with Replication-incompetent Polyepitope Adenovirus Vaccine

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