B Cells Transduced with HPV16 E6/E7-expressing Adenoviral Vector Can Efficiently Induce CTL-dependent Anti-Tumor Immunity

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ABSTRACT

Background: Human papillomavirus (HPV) infection is responsible for cervical cancer, a common cancer in women. Since HPV infection and cancer development are controlled by the host immune system, immunotherapy against HPV can be helpful in preventing or treating HPV-associated cervical cancer. Two oncoproteins of HPV16, E6 and E7, are promising targets for immunotherapy against cervical cancer, because they are constitutively expressed in cervical cancer. **Methods:** Since cellular vaccines using B cells as well as dendritic cells offer an efficient approach to cancer immunotherapy, we opted to use B cells. We evaluated the immunogenicity and anti-tumor effects of a B cell vaccine transduced with HPV16 E6/E7-expressing adenovirus. **Results:** Vaccination with HPV16 E6/E7-transduced B cells induced E6/E7-specific $CD8^+$ T cell-dependent immune responses and generated anti-tumor effects against E6/E7-expressing TC-1 tumor. The anti-tumor effect induced by this B cell vaccine was similar to that elicited by DC vaccine, showing that B cells can be used as an alternative to dendritic cells for cellular vaccines. **Conclusion:** Thisstudy has shown the feasibility of using B cells as immunogenic APCs and the potential for developing prophylactic and therapeutic vaccines against HPV-associated cervical cancer using a B cell vaccine transduced with adenovirus expressing HPV16 E6/E7. **(Immune Network 2007;7(3):109-116)**

Key Words: HPV16 E6/E7, adenoviral vector, B cells, anti-tumor immunity

Introduction

High-risk human papillomavirus (HPV) infection, particularly by HPV16, is responsible for development of cervical cancer (1-4). The importance of the immune system in controlling HPV infection and preventing cancer development can be deduced from the increased incidence of persistent infection and progression to cervical cancer in patients whose immune system is defective. Moreover, humoral and cellular immune responses against HPV were detected in patients whose pre-cancerous lesions spontaneously regressed (5,6). Therefore, immunotherapy, which potentiates host immune responses against HPV, can be helpful in preventing or treating HPV-associated cervical cancer. Previous reports have demonstrated that immunotherapy using HPV viral antigens can elicit anti-tumor immunity against HPV-related tumors in animal models (7-11).

Prophylactic vaccines using the HPV structural proteins L1 and/or L2 have been developed and approved by the FDA (2,4). However, because L1 and L2 are not expressed in cervical cancer, vaccines using them cannot be expected to have any therapeutic effect and

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have indeed been used only in those who have not been exposed to HPV. A great need still exists for the development of more effective prophylactic and therapeutic vaccines for HPV-associated cervical cancer. Two oncoproteins of HPV, E6 and E7, offer potential targets for cancer immunotherapy, because they are constitutively expressed in cervical cancer and cause cellular transformation, as well as maintain malignant phenotype (2).

Cellular vaccines using antigen-presenting cells (APC) such as dendritic cells (DCs) efficiently activate antigen-specific $CD8^+$ and $CD4^+$ T cells (12). Dendritic cells are able to stimulate diverse effector cells by capturing, processing, and presenting antigens with co-stimulatory signals (13). Despite the strong immunogenicity of DCs, obstacles exist to the clinical development of DC vaccines, namely the difficulty in obtaining large numbers of DCs in a homogeneous state (14). Thus, alternative APCs should be considered as potential sources of cellular vaccines. B cells offer an attractive alternative in that large numbers of them can be obtained from a small volume of blood with high purity (14,15). Although poorly immunogenic and tending to induce T cell tolerance due to lack of co-stimulation (16-18), activated B cells have been reported by several groups to generate antigen-specific immune responses by priming both $CD4^+$ and $CD8^+$ T cells (19-22).

Recombinant adenoviral vectors can be used to transduce tumor antigens into APCs because they are able to infect both resting and proliferating cells and to induce both humoral and cellular immune responses (23,24). Moreover, adenovirus transduction in itself could induce DC differentiation and maturation (25,26).

In an effort to develop more efficient vaccines for the prevention and treatment of HPV-associated cervical cancer, we generated B cell vaccine by transducing B cells with adenovirus expressing HPV16 E6/E7 (AdE6E7). This B cell vaccine induced E6/E7-specific cell-mediated immune responses and anti-tumor effects against E6/E7-producing TC-1 tumors as efficiently as did DC vaccines.

Materials and Methods

Mice. Female C57BL/6 mice (The Charles River, Seoul, Korea) were purchased and kept under specific pathogen-free conditions in the Animal Center for Pharmaceutical Research at Seoul National University. All mice were used at the age of 6∼8 weeks. The experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University.

Cell lines. The E6/E7-expressing TC-1 cell line ($H\text{-}2^b$), which was obtained from the American Type Culture Collection (Manassas, VA), was maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum and penicillin streptomycin.

Antibodies. Antibodies from the hybridomas GK1.5 (anti-CD4) and 2.43 (anti-CD8) were obtained from nude mice and intraperitoneally (i.p.) injected (200 μl/mouse) to deplete the respective lymphocyte subsets in vivo. FITC-labeled anti-CD19 antibody; PE-labeled anti-CD80, CD86, CD40, I/A-I/E and CD8 antibodies; and APC-labeled anti-IFN-γ antibody (all from Biolegend, USA) were used for flow cytometric analysis.

Construction of replication-defective adenoviruses expressing HPV16 E6E7. The synthetic HPV16 E6E7 gene was engineered by changing the viral codons into the human consensus sequence, based on a codon usage database (Genescript Co., USA). To reduce the risk of oncogenicity, mutagenesis of E6E7 gene was simultaneously introduced in the codons at positions Cys_{63} and Cys_{106} of E6, and Cys_{24} and Glu_{26} of E7 that became translated into Gly. Recombinant replication- defective adenovirus (Ad) encoding HPV16 E6E7 was generated according to AdEasy^{TM} Vector System (Q. Biogene) as described earlier (27). Briefly, the complementary DNA of E6E7 was sub-cloned into the *Kpn I/Xba I* sites of the adenoviral shuttle vector (pShuttleCMV). pShuttleCMV/E6E7 construct was co-transformed with adenoviral backbone vector, pAdEasy, into Escherichia coli BJ5183 by electroporation to achieve homologous recombination. The recombinant obtained was transfected into 293 cells by the conventional calcium phosphate co-precipitation method. Recombinant adenovirus was isolated from a single plaque, expanded in 293 cells, and purified by double cesium chloride ultracentrifugation. Purified viruses were extensively dialyzed against 10 mM Tris, 5% sucrose, 2 mM $MgCl_2$ and stored in aliquots at -80°C. Titer of adenovirus was determined by Tissue culture infectious dose 50 ($TCID_{50}$) and by plaque assays in 293 cells.

Generation of B cell and DC vaccines. B cells were magnetically isolated from spleen using anti-B220 microbeads (Miltenyi Biotec, USA), Dendritic cells were generated by culturing bone marrow cells in media containing GM-CSF (20ng/ml) for 6 days. B cells and DCs were transduced with 100 multiplicity of infection (MOI) of AdE6E7 for 24 hours.

RT-PCR. Total RNAs from B/AdE6E7 and DC/AdE6E7 were extracted with trizol. Reverse transcription was performed with 1.2 μg of RNAs, and PCR was run with 5 μl of the cDNA using the E7-specific primer (F5'-ACGAGTACATGCTGGATCTG-3', R5'-GATGTCTACGTGGGTGCTCT-3).

Prophylactic and therapeutic tumor models. To test the therapeutic effect of the vaccine, TC-1 tumor cells were challenged subcutaneously. One or three days after challenge, manipulated B cells were administered to tumor-bearing mice. In the prophylactic model, naive mice were immunized with B cell vaccines 7 days before subcutaneous transfer of tumor cells. Tumor size was measured using calipers thrice weekly.

In vitro cytotoxicity assay. Splenocytes from vaccinated mice were cultured *in vitro* with irradiated TC-1 cells for 6 days. Stimulated splenocytes (effectors) were then harvested and co-incubated with Cr-51-labeled TC-1 cells (targets) at the Effectors:Targets (E:T) ratios of 30:1, 10:1, 3:1, and 1:1. After an additional 4 hours, the supernatants were harvested and Cr-51 radioactivity was determined in a Wallac 1470 Wizard ™ automatic γ-counter. Specific lysis (%) was calculated as [(experimental release - spontaneous release)/(maximal release - spontaneous release)] $\times$100.

Intracellular cytokine-staining (ICS) assay. Splenocytes from vaccinated mice were cultured in vitro with HPV16 E7 (H-2D^b) peptide (RAHYNIVTF, $E7_{49-57}$) for 24 hours. Golgistop™, a protein transport inhibitor (BD biosciences, USA), was added for the last 6 hours. Stimulated cells were stained with PE-labeled anti-CD8 and APC-labeled anti-IFN-γ and analyzed using flow cytometry.

Statistical analysis. The Student t test was used to compare the differences between the two groups using SPSS (SPSS, Chicago, Illinois). Values of $p<0.05$ were considered significant at a 95% confidence interval.

Results

Generation of B cell vaccine. To make B cell vaccine, we isolated resting B cells from spleen using anti-B220 beads and analyzed surface molecules using flow cytometry. The B cells were CD19-positive (>99%) and expressed high levels of MHC class II, intermediate levels of CD86 and CD40 and low levels of

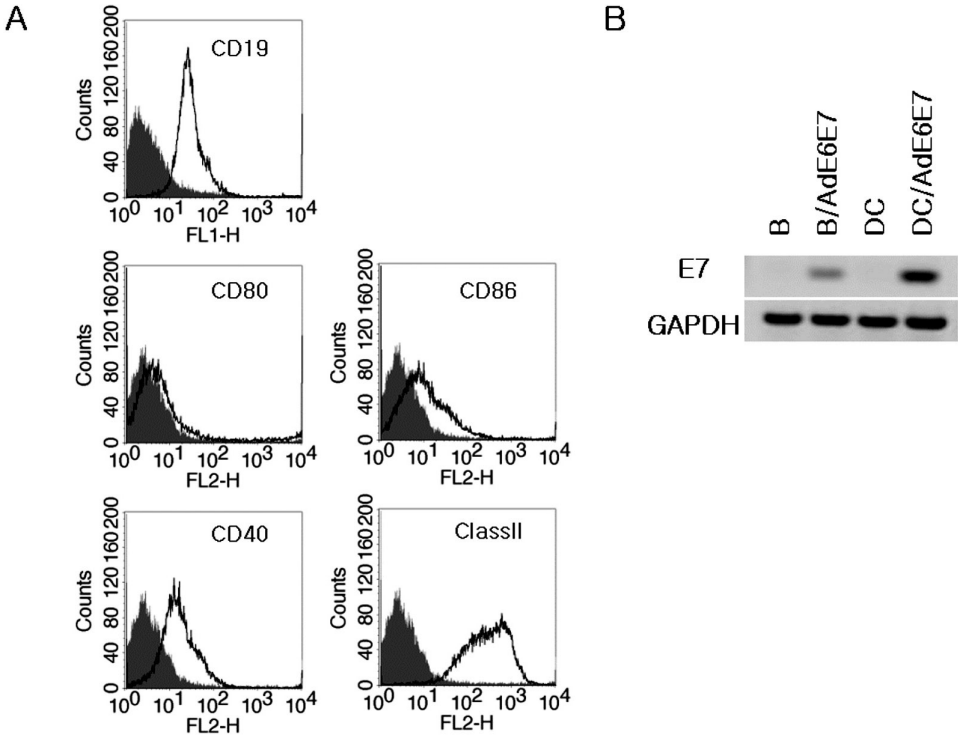

Figure 1. Generation of the B cell vaccine. (A) B cells were isolated from splenocytes of C57BL/6 mice using anti-B220 microbeads. Surface CD molecules were stained by antibodies (anti-CD19, anti-CD80, anti-CD86, anti-CD40, and anti-I-A/I-E) and analyzed by flow cytometry. The solid lines represent the indicated antibodies; the filled areas represent the control antibody. (B) B cells and bone marrow-derived DCs were transduced with 100 MOI of AdE6E7 (B/AdE6E7, DC/AdE6E7). RT-PCR demonstrated the expression of the E7 gene in B/AdE6E7 and DC/AdE6E7.

CD80 (Fig. 1A). The expression level of these surface molecules were not changed after transduction with E6/E7-expressing adenovirus (AdE6E7), at least within 24 hours (data not shown). To confirm antigen expression after AdE6E7 transduction, we performed RT-PCR using E7-specific primers. We detected the expression of HPV16 E7 on AdE6E7-transduced B cells, although the expression level was relatively low compared to that of AdE6E7-transduced DCs (Fig. 1B).

B cells transduced with AdE6E7 generate therapeutic anti-tumor immunity against E6/E7-expressing tumor as efficiently as DCs. To test whether B cells transduced with AdE6E7 (B/AdE6E7) could elicit anti-tumor immunity, we established a therapeutic tumor model using TC-1 tumor cells. Mice were subcutaneously injected with 1×10^5 of TC-1 tumor cells on the left flank. One day later, mice were vaccinated with 2×10^6 of B/AdE6E7 or DC/AdE6E7. Vaccination with B/AdE6E7 prevented tumor growth, and the anti-tumor effect induced by B/AdE6E7 was comparable to that induced by DC/AdE6E7 (Fig. 1A). Next, we tested anti-tumor effects under different conditions. Mice were subcutaneously challenged with 5×10^4 of TC-1 tumor cells and then 3 days later immunized with B/AdE6E6 or DC/AdE6E7. The anti-tumor effects against TC-1 challenge elicited by vaccination with DC/AdE6E7 were immediate, while those induced by B/AdE6E7 vaccination were delayed for approximately 2～3 days. Although requiring time to induce anti-tumor immunity, B/AdE6E7 eventually eradicated the tumor cells (Fig. 2B). Through these experiments, we confirmed that an AdE6E7-transduced B cell vaccine could generate anti-tumor effects against E6/E7- expressing tumor cells, resulting in the elimination of tumor cells.

B/AdE6E7 vaccination generates prophylactic anti-tumor immunity. For the evaluation of prophylactic anti-tumor effect induced by B/AdE6E7 vaccination, naive mice were injected intravenously with B/AdE6E7. Seven days later, mice were challenged with 1×10^6 of TC-1 tumor cells. The anti-tumor immunity elicited by B/AdE6E7 was so potent that no tumor growth was seen (Fig. 3). The tumor mass remained impalpable until the end of the experiment.

E7-specific $CD8^+$ T cell responses are induced by B/AdE6E7 vaccination. To better understand how B/AdE6E7 vaccination elicited anti-tumor effects against TC-1 tu-

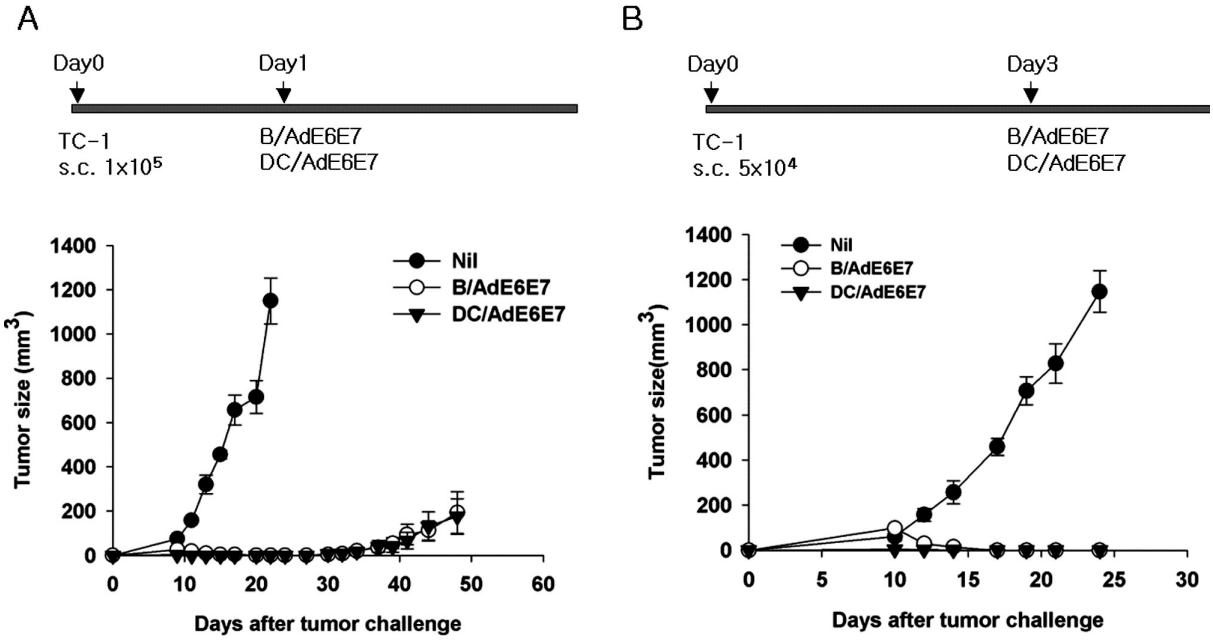

Figure 2. B cells transduced with AdE6E7 generated anti-tumor responses against E6/E7-expressing tumor. To evaluate therapeutic effects, 2×10^6 of B/AdE6E7 or DC/AdE6E7 were injected intravenously after tumor challenge (n=5). (A) One day before vaccination, mice were subcutaneously inoculated with 1×10^5 of TC-1 tumor cells in the left flank. (B) Therapeutic effects in the 3-day therapeutic model. Three days before vaccination, mice were inoculated with 5×10^4 of TC-1 tumor cells. Tumor size was measured three times a week. Nil; not immunized with cell-based vaccines.

mor, we evaluated antigen-specific immune responses. Because E6 and E7 are cytoplasmic proteins, MHC class I-dependent $CD8^+$ T cell responses are known to be important in eliminating E6/E7-producing cells (28). Therefore we assessed $CD8^+$ T cell dependent-immune responses. Splenocytes from vaccinated mice were cultured *in vitro* with irradiated TC-1 cells for 6 days to restimulate CTLs. Stimulated splenocytes were then harvested and CTL activity was determined using a Cr-51 release assay (Fig. 4). Splenocytes from B/AdE 6E7-immunized mice lysed TC-1 tumor cells (41.0%±9.3% at E:T ratio of 10:1). The cytotoxicity induced by B/AdE6E7 vaccination was weaker than that induced by DC/AdE6E7 vaccination (72.9% ±9.6% at E:T ratio of 10:1). IFN-γ production was

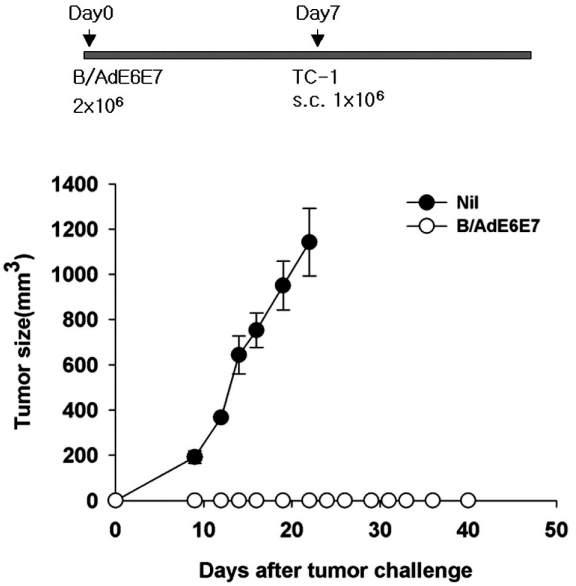

Figure 3. B/AdE6E7 vaccination generates prophylactic anti-tumor immunity. C57BL/6 mice were vaccinated with 2×10^6 of B/AdE6E7 (n=5). To analyze the prophylactic effects induced by the B cell vaccine, mice were subcutaneously challenged 7 days after vaccination with 1×10^6 of TC-1 tumor cells. Tumor size was measured 3 times a week. Nil; not immunized with cell-based vaccines.

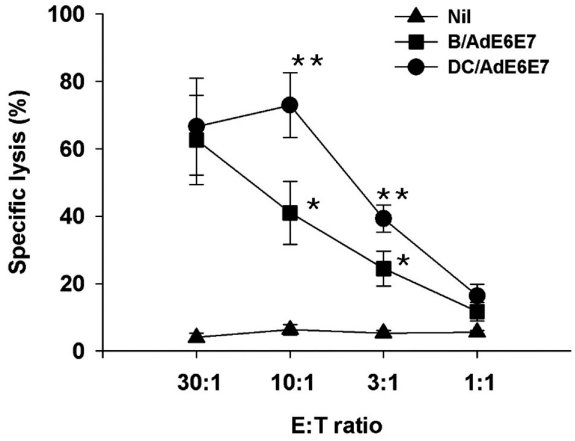

Figure 4. CTL responses were elicited by B/AdE6E7. To estimate CTL responses, mice (n=3) were vaccinated with 2×10^6 of B/AdE6E7 or DC/AdE6E7. Ten days after vaccination, splenocytes were isolated and cultured with irradiated TC-1 tumor cells. Six days later, stimulated effecter cells were harvested, and CTL activity was assessed by measuring TC-1 lysis using a Cr-51 release assay. *$p<0.05$, **$p<0.005$ compared to nil. Nil; not immunized with cell-based vaccines.

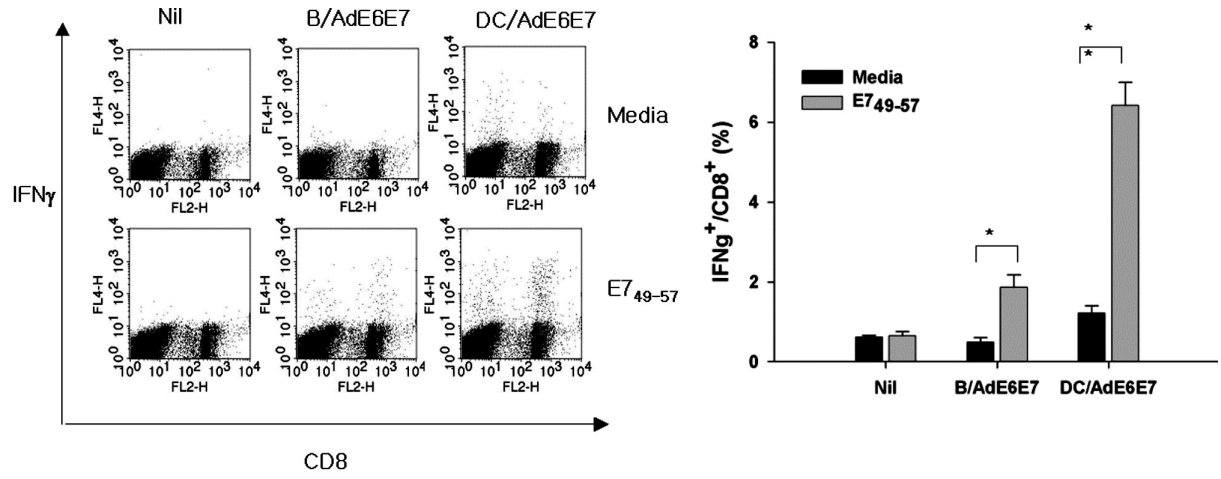

Figure 5. B/AdE6E7 activates $CD8^+$T cells to produce IFN-γ. To estimate IFN-γ production in $CD8^+$ T cells, mice (n=3) were vaccinated with 2×10^6 of B/AdE6E7 or DC/AdE6E7. Ten days after vaccination, splenocytes were isolated and stimulated with or without E7 peptide (H-$2D^b$) for 24 hours. Golgistop™ was added for the final 6 hours. Stimulated cells were stained with anti-CD8 PE and anti-IFN-γ APC before being analyzed by flow cytometry. *$p<0.05$, **$p<0.005$. Nil; not immunized with cell-based vaccines.

also assessed using an intracellular cytokine staining (ICS) assay (Fig. 5). Spleen cells were stimulated with MHC class I - restricted peptide ($E7_{49-57}$) *in vitro* for 24 hours. B/AdE6E7 elicited E7-specific IFN-γ -secreting $CD8^+$ T cells in immunized mice, though fewer than DC/AdE6E7 (Nil 0.65%±0.10%; B/AdE 6E7, 1.86% ±0.31%; DC/ AdE6E7, 6.41%±0.57%). Collectively, these findings show that vaccination with B/ AdE6E7 activated $CD8^+$ T cells to secrete IFN-γ and induced cytotoxicity against E6/E7-expressing TC-1 cells.

$CD8^+$ T cells are major effector cells for the eradication of TC-1 tumor cells. To identify the crucial immune cell subset for TC-1 tumor elimination, we removed $CD8^+$ or $CD4^+$ T cells using depleting antibodies. Mice were inoculated with 5×10^4 of TC-1 tumor cells in the left flank and then immunized with B/AdE6E7 3 days later. Depletion of $CD8^+$ or $CD4^+$ T cells was achieved by injecting anti-CD4 antibody (GK1.5) and anti-CD8 antibody (2.43) three times at three-day intervals. $CD8^+$ T cell deficiency promoted tumor growth, making evident that $CD8^+$ T cells are the main effector cells for the eradication of tumor cells (Fig. 6). In contrast, $CD4^+$ T cells seem to act as helper rather than as the main effector cells. When $CD4^+$ T cells were depleted, tumor growth was rapid; tumor size paralleled that of the control group in early stages but later decreased. Although responses were delayed, B/AdE6E7 vaccination did succeed in inducing E6/E7-specific anti-tumor responses in the absence of $CD4^+$ help. Collectively, the data indicate that B/AdE6E7 vaccination induces $CD8^+$ T cell-dependent anti-tumor immunity and that $CD4^+$ T cell help is necessary for the efficient eradication of E6/E7-expressing tumor.

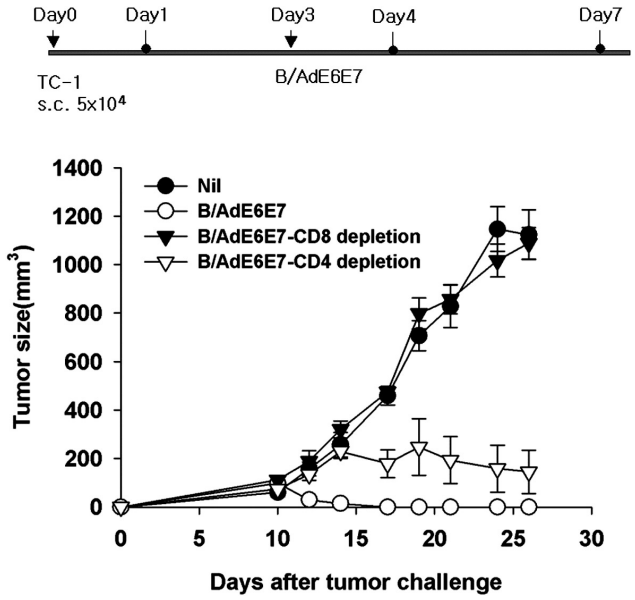

Figure 6. $CD8^+$ T cells are the major effector cells for the elimination of TC-1 tumor. Mice (n=5) were subcutaneously challenged with 5×10^4 of TC-1 tumor cells on day 0. At day 3, B/AdE6E7 was administered to tumor-bearing mice. GK1.5 (anti-CD4) and 2.43 (anti-CD8) were injected intraperitoneally (200 μl/mouse) to deplete the respective lymphocyte subsets at day 1, 3 and 7. The right panel indicates the ratio of IFN-γ producing cells in $CD8^+$ cells. Nil; not immunized with cell-based vaccines.

Discussion

Human papillomavirus infection is the main cause of cervical cancer, a common cancer in women (1-3). Immune defects have been shown to lead to persistent infection and progression to cervical cancer. Therefore, immunotherapy to potentiate the host immune system against HPV can be effective for preventing and treating HPV-associated cervical cancer (2,4). Although prophylactic vaccines have been developed using the viral capsid proteins L1 and/or L2, their application is restricted to non-HPV-exposed people. Because these proteins are absent in cervical cancer, no therapeutic effect can be expected. In contrast, E6 and E7, HPV viral oncoproteins, are constitutively expressed in precancerous lesions and cervical cancer and so are well known to be promising targets for HPV vaccines (2,4,7-10).

To develop an efficient cellular vaccine against HPV16 E6/E7, we used B cells as APCs. Although B cells have been regarded as poorly immunogenic APCs (16-18), activated B cells have been shown to act as immunogenic APCs capable of inducing antigen- specific immune responses (19-22). We generated B cell vaccines by transducing B cells with HPV16 E6/ E7-expressing adenovirus (B/AdE6E7). By using an adenoviral vector encoding full-length antigen as an antigen-delivery tool, we were able to overcome the problems related to HLA restriction and occurrence of tumor antigen variants. We also expect to achieve diverse immunities against multiple epitopes.

We observed that vaccination with B/AdE6E7 activated $CD8^+$ T cells to secrete IFN-γ, induced CTL activity, and finally elicited anti-tumor effects against

E6/E7-expressing TC-1 tumor. We also found that although $CD8^+$ T cells are the major effector cells for the elimination of TC-1 tumor cells, $CD4^+$ T cell help is necessary to elicit efficient therapeutic effect. As shown in Figure 6, delayed anti-tumor effects were observed when $CD4^+$ T cells were depleted. However, even in the absence of $CD4^+$ help, B/AdE6E7 is able to elicit anti-tumor effects. Therefore, we assume that B/AdE6E7 vaccination may be effective in AIDS patients and transplant recipients who are at high risk of developing HPV-associated cervical cancer because of low levels of $CD4^+$ T cells (29).

We compared the immune response and anti-tumor effects induced by the B cell vaccine with those induced by DC vaccines, which have generally been used for cellular vaccines (12,13). Immunization with B/AdE6E7 induced weaker immune responses against HPV16 E6/E7 than did DC/AdE6E7. These results may reflect the small size, the low antigen-expression level and the weak co-stimulatory signal of B cells compared to DCs. Although B/AdE6E7 generated weaker antigen-specific immune responses than DC/AdE6E7, the anti-tumor effect elicited by B/AdE6E7 was sufficient to eradicate E6/E7-producing TC-1 tumor as effectively as DC/AdE6E7.

Moreover, the B cell vaccine offers a number of clear advantages over DC vaccines for clinical application: 1) large numbers of highly pure B cells can be obtained from a small volume of blood; 2) the process for generating B cell vaccines is relatively simple, consisting of only two steps, i.e., purification of B cells from blood and transduction with adenovirus; and 3) B cell vaccines require less time and expense to produce than DC vaccines. In contrast, DCs, which are differentiated from precursor cells, require periods of culture with cytokines.

Further studies are necessary to improve the anti-tumor effect of the B cell vaccine and to test its efficacy in clinical trials. One method for enhancing the anti-tumor effect of the B cell vaccine is the use of a bicistronic adenoviral vector co-expressing antigen and a cytokine such as IL-12 that potentiates anti-tumor immunity (7,30). Enhancement of the co-stimulatory signal can also augment the immunogenicity of B cells. Hence, CD40 ligation has been used to activate B cells and to enhance their antigen-presentation capacities (19-21). The activation of B cells with NKT cell help could help induce antigen-specific cytotoxic anti-tumor immunity (22).

In summary, our study has shown the feasibility of using B cells as immunogenic APCs and the potential for developing prophylactic and therapeutic vaccines against HPV-associated cervical cancer using a B cell vaccine transduced with adenovirus expressing HPV16 E6/E7. The B/AdE6E7 vaccine could be more easily, inexpensively, and quickly produced than DC vaccines and should be ready for clinical application in the near future.

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