# Modification of Professional Antigen-Presenting Cells with Small Interfering RNA *In vivo* to Enhance Cancer Vaccine Potency

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## Abstract

**RNA interference using small interfering RNA (siRNA) is an effective means of silencing gene expression in cells. Intradermal administration of nucleic acids via gene gun represents an efficient method for delivering nucleic acids to professional antigen-presenting cells *in vivo*. In this study, we show that the coadministration of DNA vaccines encoding human papillomavirus type 16 E7 with siRNA targeting key proapoptotic proteins Bak and Bax prolongs the lives of antigen-expressing dendritic cells in the draining lymph nodes, enhances antigen-specific $CD8^+$ T-cell responses, and elicits potent antitumor effects against an E7-expressing tumor model in vaccinated mice. Our data indicate that intradermal administration of siRNA to manipulate gene expression represents a plausible strategy for modification of the properties of professional antigen-presenting cells *in vivo* to enhance cancer vaccine potency.** (Cancer Res 2005; 65(1): 309-16)

## Introduction

Intradermal administration of DNA vaccines via gene gun can efficiently deliver genes of interest into professional antigen-presenting cells (APC) *in vivo* (1). The skin contains numerous bone marrow–derived APCs (called Langerhans cells) that are able to move through the lymphatic system from the site of injection to draining lymph nodes, where they can prime antigen-specific T cells (2). Gene gun immunization therefore provides the opportunity to test vaccine strategies that require direct delivery of DNA or RNA to APCs. We previously used this approach to test several intracellular targeting strategies, including *Mycobacterium tuberculosis* heat shock protein 70 (HSP70; ref. 3), calreticulin (CRT; 4), or the sorting signal of the lysosome-associated membrane protein 1 (LAMP-1; ref. 5), which are able to route model antigen, human papillomavirus type 16 (HPV-16) E7, to desired subcellular compartments and enhance antigen processing and presentation to T cells. Therefore, direct delivery of DNA vaccines into dendritic cells via gene gun provides an opportunity to modify the quality and quantity of DNA-transfected dendritic cells and influence vaccine potency.

Recently, we found that a variety of antiapoptotic factors can enhance dendritic cell survival and E7-specific $CD8^+$ T-cell immune responses when coadministered with E7 DNA (6). Because intracellular targeting and antiapoptotic strategies modify dendritic cells via different mechanisms, we have combined antiapoptotic strategies for prolonging dendritic cell life with intracellular targeting strategies to improve DNA vaccine potency (7). Although coadministration of DNA-encoding antigen with DNA-encoding antiapoptotic proteins can significantly enhance DNA vaccine potency, the use of DNA-encoding antiapoptotic proteins, such as Bcl-$x_L$, raises significant concerns related to oncogenicity. RNA interference (RNAi) using small interfering RNA (siRNA) targeting proapoptotic proteins may provide similar effects while alleviating oncogenic concerns. Concerns for oncogenicity are alleviated due to the transient nature of the silencing of target genes by siRNA in mammalian cells (for review, see ref. 8) and due to the fact that RNA-based strategies carry no concerns for integration and permanent genetic change.

RNAi is an effective technique for silencing gene expression. This is accomplished by using siRNA to induce sequence-specific degradation of mRNA or by inhibiting translation of its complementary mRNA (for review, see ref. 8). It may thus be possible to prolong dendritic cell life by targeting key proapoptotic proteins with the appropriate siRNAs. Bax and Bak are members of the Bcl-2 family and play key gatekeeping roles in the intrinsic apoptotic pathway (for review, see ref. 9). Thus, Bax and Bak represent potentially ideal targets for down-regulation by siRNA to inhibit apoptosis. We hypothesized that intradermal delivery of BAK and/or BAX siRNA to antigen-expressing dendritic cells would prolong the lives of transfected dendritic cells and lead to enhanced antigen-specific T-cell–mediated immune responses *in vivo*.

In this study, we tested our hypothesis using intradermal coadministration of DNA vaccines encoding HPV-16 E7 antigen combined with BAK and/or BAX siRNA. We chose HPV-16 E7 as a model antigen because HPVs, particularly HPV-16, are associated with most cervical cancers and E7 is essential for the induction and maintenance of cellular transformation. Effective vaccines against E7 can potentially be used for the control of HPV infections and HPV-associated lesions. By examining E7-specific immune responses, antitumor effects, and survival of DNA-transfected dendritic cells, we show that coadministration of antigen-containing DNA with BAK and/or BAX siRNA represents an innovative strategy to enhance DNA vaccine potency while alleviating concerns for oncogenicity. These encouraging results suggest a potential for clinical translation of our siRNA strategy and make possible the development of future siRNA-based strategies to manipulate the functions of dendritic cells *in vivo*.

## Materials and Methods

**Plasmid DNA Constructs and DNA Preparation.** The generation of pcDNA3-E7 (3) pcDNA3-Sig/E7/LAMP-1 (6), pcDNA3-E7/HSP70 (3), pcDNA3-CRT/E7 (4), pDNA3-E7/GFP (10), and pcDNA3-OVA (6) has been described previously. The plasmid containing influenza hemagglutinin (HA),

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pcDNA3-HA, was kindly provided by Dr. Drew Pardoll (Johns Hopkins School of Medicine). The accuracy of these constructs was confirmed by DNA sequencing. DNA was amplified in *Escherichia coli* DH5 and purified as described previously (3).

**Preparation of siRNAs.** siRNAs were synthesized using 2′-*O*-ACE-RNA phosphoramides (Dharmacon, Lafayette, CO). The sense and antisense strands of siRNA were *Bak*, beginning at nucleotide 310, 5′ P-UGCCUACGAA-CUCUUCACCdTdT-3′ (sense) and 5′ P-GGUGAAGAGUUCGUAGGCAdTdT-3′ (antisense), and *Bax*, beginning at nucleotide 217, 5′ P-UAUGGA-GCUGCAGAGGAUGdTdT-3′ (sense) and 5′ P-CAUCCUCUGCAGCUCCAUA-dTdT-3′ (antisense); P represents 5′ phosphate. RNAs were deprotected and annealed according to the manufacturer's instruction. Nonspecific control siRNA (target 5′-NNATTGTATGCGATCGCAGAC-3′) was acquired from Dharmacon.

**Cells.** The HPV-16 E7-expressing murine tumor model, TC-1, has been described previously (11). In brief, HPV-16 E6 and E7 and the *ras* oncogene were used to transform primary C57BL/6 mouse lung epithelial cells to generate TC-1. DC-1 cells were generated from the dendritic cell line (12) provided by Dr. Kenneth Rock (University of Massachusetts, Boston, MA). With continued passage, we have generated subclones of dendritic cells (DC-1) that can be easily transfected (13). All cells were maintained in RPMI (Invitrogen, Carlsbad, CA) supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 20 mmol/L HEPES, 50 μmol/L β-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA).

**Western Blot Analysis.** DC-1 cells ($2 \times 10^5$) were transfected with 300 pmol of the synthesized BAK + BAX siRNA or control siRNA in a final volume of 2 mL using OligofectAMINE (Invitrogen) according to the vendor's manual. We used FITC-labeled siRNA to assess the transfection efficiency of the DC-1 cells by flow cytometry analysis. Virtually 100% of DC-1 cells were successfully transfected with siRNA (data not shown). The expression of BAK and BAX proapoptotic proteins in DC-1 cells transfected with BAK and/or BAX siRNA was characterized by Western blot analysis. Western blot analysis was done with 50 μg of the cell lysate from the transfected DC-1 cells and anti-BAK and/or BAX mouse monoclonal antibody (Cell Signaling Technology, Inc., Beverly, MA) using a protocol similar to what has been described previously (10).

**Determination of Apoptotic Cells.** DC-1 cells ($2 \times 10^5$) were transfected with BAK + BAX siRNA or control siRNA as described above. Two days after transfection, the DC-1 cells were pulsed with 10 μg/mL E7 peptide (RAHYNIVTF) or HA peptide (IYSTVASSL) for 2 hours and subsequently incubated with an E7-specific $CD8^+$ T-cell line (14) at different E:T ratios (5:1, 1:1, 0.5:1, or 0.1:1) for 4 or 20 hours. Detection of apoptotic cells in the DC-1 cells was done using phycoerythrin-conjugated rabbit anti-active caspase-3 monoclonal antibody (BD PharMingen, San Diego, CA) according to the vendor's protocol. Briefly, cells were harvested and stained with FITC-conjugated anti-CD8 antibody as described previously (3). The cells were subsequently fixed and permeabilized using the Cytofix/Cytoperm kit (BD PharMingen) for 20 minutes at room temperature and stained with phycoerythrin-conjugated rabbit anti-active caspase-3 monoclonal antibody using 20 μl per $1 \times 10^6$ cells for 60 minutes at room temperature. Following incubation with the antibodies, the cells were washed, resuspended and analyzed by flow cytometry analysis. Analysis was done on a Becton Dickinson FACScan with CellQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA). We gated CD8 cells and analyzed active caspase-3-positive DC-1 cells to determine the percentage of apoptotic DC-1 cells.

**Mice.** Six- to 8-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and kept in the oncology animal facility of the Johns Hopkins Medical Institutions. All animal procedures were done according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

**DNA/siRNA Vaccination.** Gene gun particle-mediated DNA/siRNA vaccination was done using a helium-driven gene gun (Bio-Rad, Hercules, CA) according to the protocol for RNA vaccination provided by the manufacturer, with a slight modification. Briefly, DNA/siRNA-coated gold particles were prepared by combining 25 mg of 1.6 μm gold microcarriers (Bio-Rad), 50 μg of plasmid DNA (50 μL), 5 μg of siRNA (50 μL), and 10 μL of 3 mol/L sodium acetate. Isopropyl alcohol (200 μL) was added to the mixture drop-wise while mixing by vortex. This mixture was allowed to precipitate at room temperature for 10 minutes. The microcarrier/DNA/siRNA suspension was then centrifuged (10,000 rpm for 30 seconds) and washed thrice in fresh absolute ethanol before resuspending in 3 mL polyvinylpyrrolidone (0.1 mg/mL, Bio-Rad) in absolute ethanol. The solution was then loaded into 2.5 ft of gold-coated tube (Bio-Rad) and allowed to settle for 10 minutes. The ethanol was gently removed, and the microcarrier/DNA/siRNA suspension was evenly attached to the inside surface of the tube by rotation. The tube was then dried by 0.4 L/min of flowing nitrogen gas. The dried tube coated with microcarrier/DNA/siRNA was then cut to 0.5 in. cartridges and stored in a capped dry bottle at 4°C. The DNA/siRNA-coated gold particles (1 μg DNA and 0.1 μg siRNA per bullet) were delivered to the shaved abdominal region of the mice using a helium-driven gene gun (Bio-Rad) with a discharge pressure of 400 p.s.i. C57BL/6 mice were immunized with 2 μg of the pcDNA3 plasmid encoding E7, Sig/E7/LAMP-1, E7/HSP70, CRT/E7 HA, or OVA and mixed with 0.2 μg BAK + BAX siRNA or control siRNA. The mice received a booster with the same dose 1 week later.

To determine the effect of BAK + BAX siRNA and/or control siRNA administered during the priming and/or boosting phases, mice were first vaccinated with 2 μg pcDNA3-Sig/E7/LAMP-1 coadministered with 0.2 μg BAK + BAX siRNA or with control siRNA. Mice were then boosted with 2 μg pcDNA3-Sig/E7/LAMP-1 coadministered with 0.2 μg BAK + BAX siRNA or control siRNA.

**Intracellular Cytokine Staining and Flow Cytometry Analysis.** Splenocytes were harvested from mice 1 week after the last vaccination. Prior to intracellular cytokine staining, $4 \times 10^6$ pooled splenocytes from each vaccination group were incubated for 16 hours with 1 μg/mL E7 (RAHYNIVTF), HA (IYSTVASSL), or OVA peptide (SIINFEKL) peptide containing a major histocompatibility class I epitope for detecting antigen-specific $CD8^+$ T-cell precursors or 1 μg/mL E7 peptide containing a major histocompatibility class II epitope (amino acids 30 to 67) for detecting antigen-specific $CD4^+$ T-cell precursors. Intracellular interleukin-4 and IFN-γ staining and flow cytometry analysis were done as described previously (3).

***In vivo* Tumor Protection and Tumor Treatment Experiments.** For the tumor protection experiment, C57BL/6 mice (five per group) were s.c. challenged with $5 \times 10^4$ TC-1 tumor cells per mouse in the right leg 1 week after the last vaccination. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week. To study the subsets of lymphocytes that are important for the antitumor effects, an *in vivo* antibody depletion experiment was done using the method described previously (11).

For the tumor treatment experiment, mice were challenged with $1 \times 10^4$ TC-1 tumor cells per mouse in the tail vein to simulate hematogenous spread of tumors (5). Mice were treated with DNA mixed with siRNA 3 days after tumor challenge. Mice were sacrificed on day 42 after the last vaccination. The mean number of pulmonary nodules in each mouse was evaluated by experimenters blinded to sample identity. *In vivo* tumor protection, antibody depletion, and tumor treatment experiments were done at least twice to generate reproducible data.

**Preparation of $CD11c^+$ Cells in the Inguinal Lymph Nodes from Vaccinated Mice.** C57BL/6 mice (five per group) were first primed with pcDNA3-Sig/E7/LAMP-1 or pcDNA3 DNA via gene gun at a dose of 2 μg per mouse. Seven days later, the mice received 16 inoculations of non-overlapping intradermal administration via gene gun on the abdominal region. Gold particles used for each inoculation were coated with 1 μg pcDNA3-E7/GFP DNA mixed with 0.1 μg BAK + BAX siRNA or control siRNA. pcDNA3 mixed with BAK + BAX siRNA was used as a negative control. Inguinal lymph nodes were harvested from vaccinated mice 2 or 5 days after pcDNA3-E7/GFP vaccination. $CD11c^+$ cells were enriched from a single cell suspension of isolated inguinal lymph nodes using CD11c (N418) microbeads (Miltenyi Biotec, Auburn, CA). Enriched $CD11c^+$ cells were analyzed by forward and side scatter and gated around a population of cells with size and granular characteristics of dendritic cells. $GFP^+$ cells were analyzed by flow cytometry analysis using a protocol described

previously (15). Data are expressed as percentage of $GFP^+$ $CD11c^+$ cells among gated monocytes. The percentage of $GFP^+$ cells among the gated $CD11c^+$ cells was analyzed using flow cytometry analysis.

An antibody depletion experiment was done similar to what has been described previously (16). Depletions were initiated 5 days after priming. Depletion was terminated at time of lymph node harvest.

**Statistical Analysis.** All data expressed as means ± SE are representative of at least two different experiments. Data for intracellular cytokine staining with flow cytometry analysis and tumor treatment experiments were evaluated by ANOVA. Comparisons between individual data points were made using a Student's *t* test. In the tumor protection experiment, the principal outcome of interest was time to tumor development. The event time distributions for different mice were compared using the method of Kaplan and Meier and the log-rank statistic. $P < 0.05$ was considered significant.

## Results

**Transfection with BAK and/or BAX siRNA Leads to Down-Regulation of BAK and BAX as Well as Resistance to Apoptotic Cell Death.** To determine if the expression of Bak and/or Bax was down-regulated in a dendritic cell line (DC-1) transfected with BAK and/or BAX siRNA, we did Western blot analysis using cell lysate from DC-1 cells transfected with the various siRNAs. As shown in Fig. 1*A*, the expression of Bak and/or Bax proteins was undetectable in DC-1 cells transfected with BAK and/or BAX siRNA. In contrast, expression of Bak and Bax proteins was detected in DC-1 cells after transfection with control siRNA, and the levels of expression were similar to the expression of Bak and Bax proteins by non-siRNA-transfected DC-1 cells (data not shown). The expression of β-actin protein was consistent among all DC-1 cell groups transfected with the various siRNAs. We also examined the kinetics of inhibition of Bak and Bax protein expression by DC-1 cells transfected with BAK + BAX siRNA. As shown in Fig. 1*B*, significant down-regulation of Bax and Bak expression was observed by day 1 after transfection. No expression of Bak or Bax was detected at days 3, 5, and 7 after transfection. Expression of Bak and Bax was detected at below normal levels by day 9, and expression returned to normal levels by day 11 after transfection.

To determine if DC-1 cells transfected with BAK and/or BAX siRNA could resist CTL-induced apoptosis, we incubated E7 peptide-loaded, siRNA-transfected DC-1 cells with an E7-specific $CD8^+$ T-cell line and determined the percentages of apoptotic cells. As shown in Fig. 1*C*, 80% to 90% of E7 peptide-loaded DC-1 cells transfected with control siRNA were apoptotic after 20 hours of incubation. In comparison, DC-1 cells transfected with BAK + BAX siRNA generated significantly lower percentages of apoptotic cells, particularly at low E:T ratios.

Taken together, these data suggest that transfection of DC-1 cells with BAK and/or BAX siRNA leads to down-regulation of BAK and BAX protein expression, resulting in resistance to apoptosis induced by antigen-specific $CD8^+$ T cells.

**Coadministration of BAX + BAK siRNA with Antigen-Specific DNA Vaccines Can Significantly Enhance Numbers of Antigen-Specific $CD8^+$ T-Cell Precursors in Vaccinated Mice.** To determine if the antiapoptotic function of BAK + BAX siRNA observed in dendritic cells *in vitro* can be translated into an *in vivo* system, we coadministered BAK + BAX siRNA with pcDNA3-E7 intradermally via gene gun. As shown in Fig. 2*A* and *B*, the coadministration of pcDNA3-E7 with BAK and/or BAX siRNA significantly enhanced the E7-specific $CD8^+$ T-cell response (by at least 10-fold) in vaccinated mice compared with coadministration of pcDNA3-E7 with control siRNA.

To determine if the same strategy can be applied to other antigens, we coadministered pcDNA3-HA and pcDNA3-OVA with BAK + BAX siRNA. As shown in Fig. 2*C* and *D*, the coadministration of pcDNA3-HA or pcDNA-OVA with BAK + BAX siRNA significantly enhanced the antigen-specific $CD8^+$ T-cell response in vaccinated mice compared with coadministration with control siRNA. Thus, our data indicate that BAK + BAX siRNA can significantly enhance

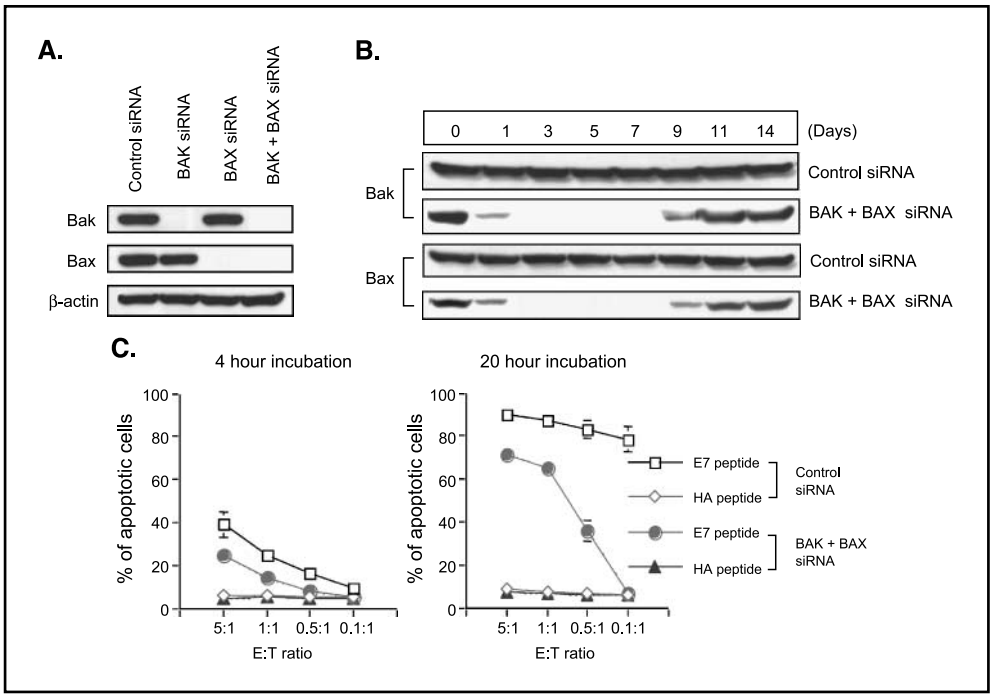


**Figure 1.** Detection of Bak and Bax expression and evaluation of resistance to apoptotic cell death after delivery of BAK and/or BAX siRNA. *A*, Western blot analysis of the expression of Bak and/or Bax protein in transfected cells. *B*, Western blot analysis of the kinetics of Bak and Bax expression in siRNA-transfected DC-1 cells. β-actin was used as an internal control for quantification of protein expression. *C*, percentage of apoptotic cells in E7 peptide (amino acids 49-57, RAHYNIVTF)–pulsed DC-1 cells transfected with BAK + BAX siRNA or control siRNA after 4 or 20 hours of incubation with an E7-specific $CD8^+$ T-cell line. DC-1 pulsed with HA peptide (IYSTVASSL) was used as a control.

antigen-specific CD8$^+$ T-cell–mediated immune responses when coadministered with antigen-specific DNA vaccines in mice.

**Coadministration of BAK + BAX siRNA with a HPV-16 E7-Specific DNA Vaccine Can Significantly Enhance Antitumor Effects against an E7-Expressing Tumor Cell Line in Vaccinated Mice.** To determine if the observed enhancement of E7-specific CD8$^+$ T-cell–mediated immunity leads to a significant improvement of the E7-specific antitumor effect, we did an *in vivo* tumor protection experiment using TC-1 tumor cells (11). As shown in Fig. 3*A*, 100% of mice receiving E7 DNA mixed with BAK + BAX siRNA remained tumor free 35 days after TC-1 challenge. In contrast, all of the mice receiving E7 DNA combined with control siRNA or pcDNA3 combined with BAK + BAX siRNA developed tumors by day 10.

We did an *in vivo* antibody depletion experiment to determine the subsets of lymphocytes important for the antitumor effects. As shown in Fig. 3*B*, 100% of the mice depleted of CD8$^+$ T cells grew tumors within 10 days after TC-1 challenge. In contrast, 100% of the mice depleted of CD4$^+$ T cells or natural killer cells remained tumor free 35 days after TC-1 challenge. These data indicated that CD8$^+$ T cells are important for the antitumor effect generated by the DNA vaccine combined with BAK + BAX siRNA.

We also did an *in vivo* tumor treatment experiment using a hematogenous spread pulmonary tumor model (5). As shown in Fig. 3*C*, mice immunized with E7 DNA mixed with BAK + BAX siRNA exhibited the fewest pulmonary tumor nodules ($P < 0.005$) compared with mice vaccinated with E7 DNA mixed with control siRNA or pcDNA3 mixed with BAK + BAX siRNA. Taken together, these results indicate that vaccination with E7 DNA mixed with BAK + BAX siRNA leads to potent protective and therapeutic effects against E7-expressing TC-1 tumor cells.

**The Combined Application of Antiapoptotic BAK + BAX siRNA and an Intracellular Targeting Strategy Further Enhances Antigen-Specific T-Cell–Mediated Immune Responses.** To assess the effect of coadministration of BAK + BAX siRNA with DNA-encoding E7 linked to an intracellular targeting molecule, mice were vaccinated with Sig/E7/LAMP-1 DNA, HSP70/E7 DNA, or CRT/E7 DNA mixed with BAK + BAX

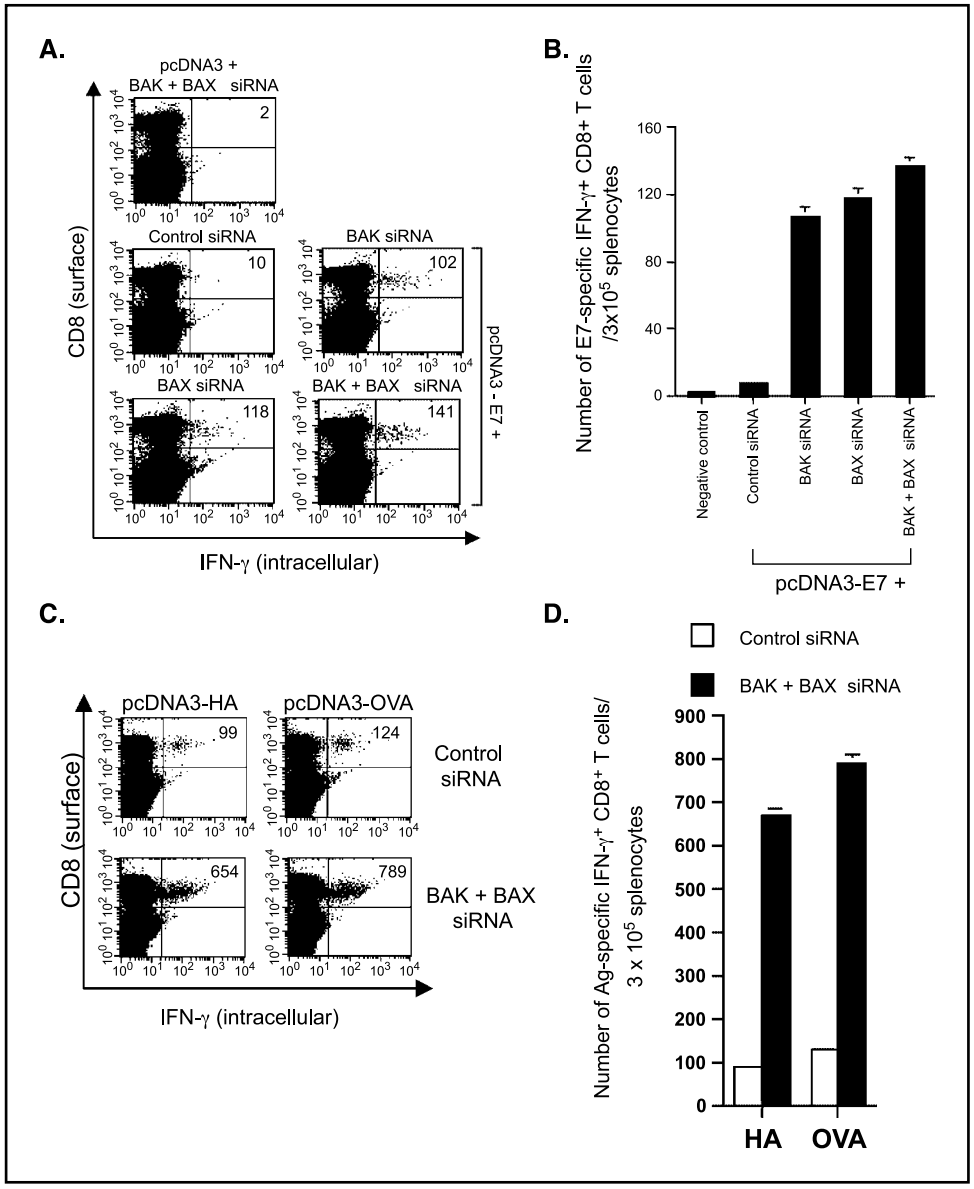


**Figure 2.** Intracellular cytokine staining and flow cytometry analysis to determine the antigen-specific CD8$^+$ T-cell response in mice to a DNA vaccine coadministered with control or BAX and/or BAK siRNA. *A* and *B*, mice were vaccinated with pcDNA3-E7. *C* and *D*, mice were vaccinated with pcDNA3-HA or pcDNA3-OVA. pcDNA3 with BAK + BAX siRNA was used as a negative control. *A*, representative flow cytometry data. *B*, number of IFN-γ-expressing E7-specific CD8$^+$ T cells in splenocytes from vaccinated mice. *C*, representative flow cytometry data. *D*, number of IFN-γ-expressing HA- or OVA-specific CD8$^+$ T cells in splenocytes from vaccinated mice.

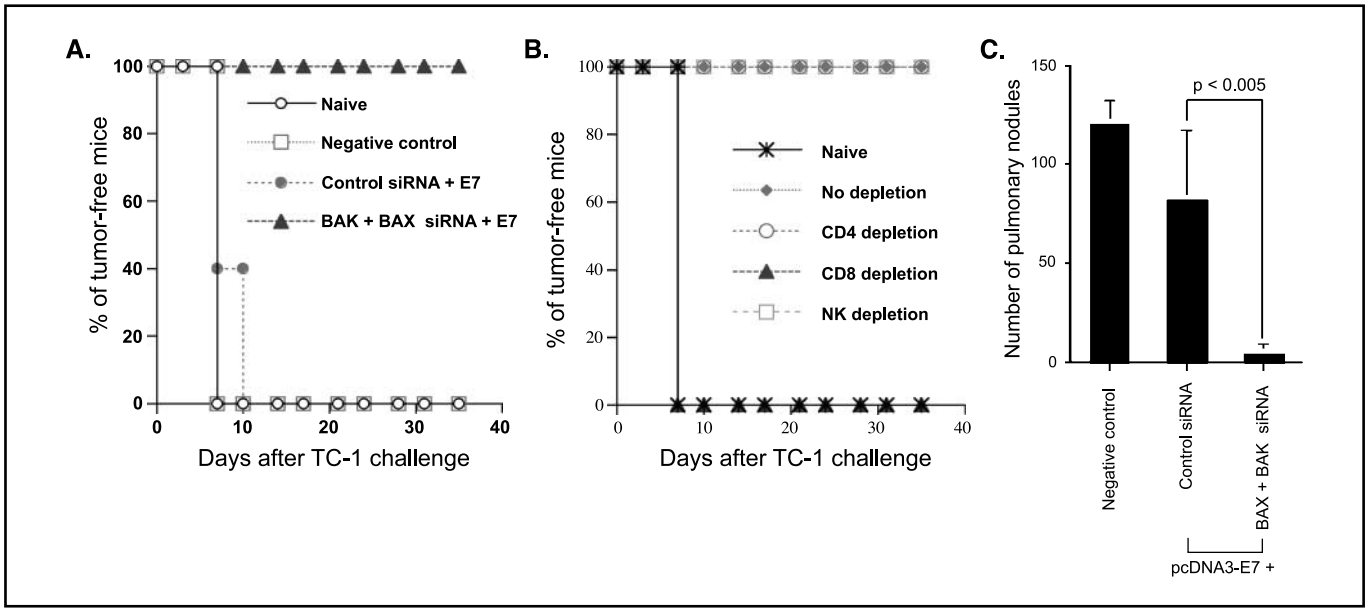


**Figure 3.** *In vivo* tumor protection and treatment experiments using E7-expressing TC-1 tumor cells. *A, in vivo* tumor protection experiment. pcDNA3 with BAK + BAX siRNA was used as a negative control. *B, in vivo* antibody depletion experiments to determine the contribution of subsets of lymphocytes to the observed protective antitumor effect. *C, in vivo* tumor treatment experiment using the hematogenous spread lung model. pcDNA3 with BAK + BAX siRNA was used as a negative control.

siRNA or control siRNA. As shown in Fig. 4*A* and *B*, coadministration of BAK + BAX siRNA with pcDNA3 encoding Sig/E7/LAMP-1, HSP70/E7, or CRT/E7 resulted in an increase in IFN-γ-expressing, E7-specific $CD8^+$ T-cell precursors compared with coadministration of these three constructs with control siRNA in vaccinated mice. Among these intracellular targeting strategies, mice vaccinated with pcDNA3-Sig/E7/LAMP-1 mixed with BAK + BAX siRNA displayed the greatest fold increase in the number of IFN-γ-expressing E7-specific $CD8^+$ T-cell precursors (~19-fold increase). Our data indicate that administration of BAK + BAX siRNA can be used in combination with any of the intracellular targeting strategies to further enhance DNA vaccine potency and that coadministration of BAK + BAX siRNA with pcDNA3-Sig/E7/LAMP-1 displays the greatest fold increase in the E7-specific $CD8^+$ T-cell immune response.

The ability of the Sig/E7/LAMP-1 targeting strategy to enhance antigen presentation to $CD4^+$ T lymphocytes is achieved through targeting of expressed antigen to endosomal/lysosomal compartments, important loci for the major histocompatibility class II antigen presentation pathway (17). As shown in Fig. 4*C* and *D*, vaccination with pcDNA3-Sig/E7/LAMP-1 mixed with BAK + BAX siRNA generated significantly more E7-specific $CD4^+$ Th1 cells and similar numbers of E7-specific $CD4^+$ Th2 cells when compared with vaccination with pcDNA3-Sig/E7/LAMP-1 mixed with control siRNA. These data suggest that coadministration of Sig/E7/LAMP-1 DNA with BAK + BAX siRNA may elicit a predominant E7-specific $CD4^+$ Th1 cell response.

**Coadministration with Antiapoptotic BAX + BAK siRNA Improves Survival of DNA-Transfected Dendritic Cells in Inguinal Lymph Nodes of Mice Vaccinated with E7/GFP DNA.** Mice were primed with pcDNA3-Sig/E7/LAMP-1 to generate sufficient E7-specific $CD8^+$ T cells for testing of the antiapoptotic ability of BAK + BAX siRNA in E7/GFP-expressing dendritic cells. pcDNA3 was used as a negative control. One week later, mice received pcDNA3-E7/GFP DNA with BAX + BAK siRNA or control siRNA via gene gun. As shown in Fig. 5*A* and *B*, at day 2 after vaccination in mice primed with pcDNA3, there was no significant difference between the percentages of $GFP^+$ $CD11c^+$ dendritic cells in mice given BAK + BAX siRNA and in mice given control siRNA. In comparison, in mice primed with pcDNA3-Sig/E7/LAMP-1, we detected a significant decrease in the percentage of $GFP^+$ $CD11c^+$ dendritic cells in mice given control siRNA compared with the percentage of $GFP^+$ $CD11c^+$ dendritic cells in mice given BAK + BAX siRNA. At day 5 after vaccination with pcDNA3-E7/GFP, we observed a similar trend in mice primed with Sig/E7/LAMP-1, except to a much lesser degree. Furthermore, we assayed for apoptotic cells in $GFP^+$ $CD11c^+$ dendritic cells by staining for activated caspase-3 followed by flow cytometry analysis. More than 90% of $GFP^+$ $CD11c^+$ dendritic cells were caspase-3 negative, indicating that these cells were not apoptotic (data not shown). Thus, our data suggest that coadministration of E7/GFP DNA with antiapoptotic BAK + BAX siRNA may protect DNA-transfected dendritic cells from killing by E7-specific $CD8^+$ T cells generated by priming with pcDNA3-Sig/E7/LAMP-1.

We did an antibody depletion experiment to confirm that $CD8^+$ T cells were responsible for the induction of apoptosis in $GFP^+$ $CD11c^+$ dendritic cells. As shown in Fig. 5*C*, the percentages of $GFP^+$ $CD11^+$ dendritic cells in the inguinal lymph nodes of mice depleted of $CD8^+$ T cells were similar for mice administered BAK + BAX siRNA or for mice administered control siRNA. In comparison, percentages of $GFP^+$ $CD11^+$ cells in the inguinal lymph nodes of mice depleted of $CD4^+$ T cells and natural killer cells or mice without depletion were significantly lower in mice administered control siRNA than in mice given BAK + BAX siRNA ($P < 0.005$). These data indicate that $CD8^+$ T cells are responsible for the induction of apoptosis in antigen-expressing dendritic cells in the draining lymph nodes of vaccinated mice.

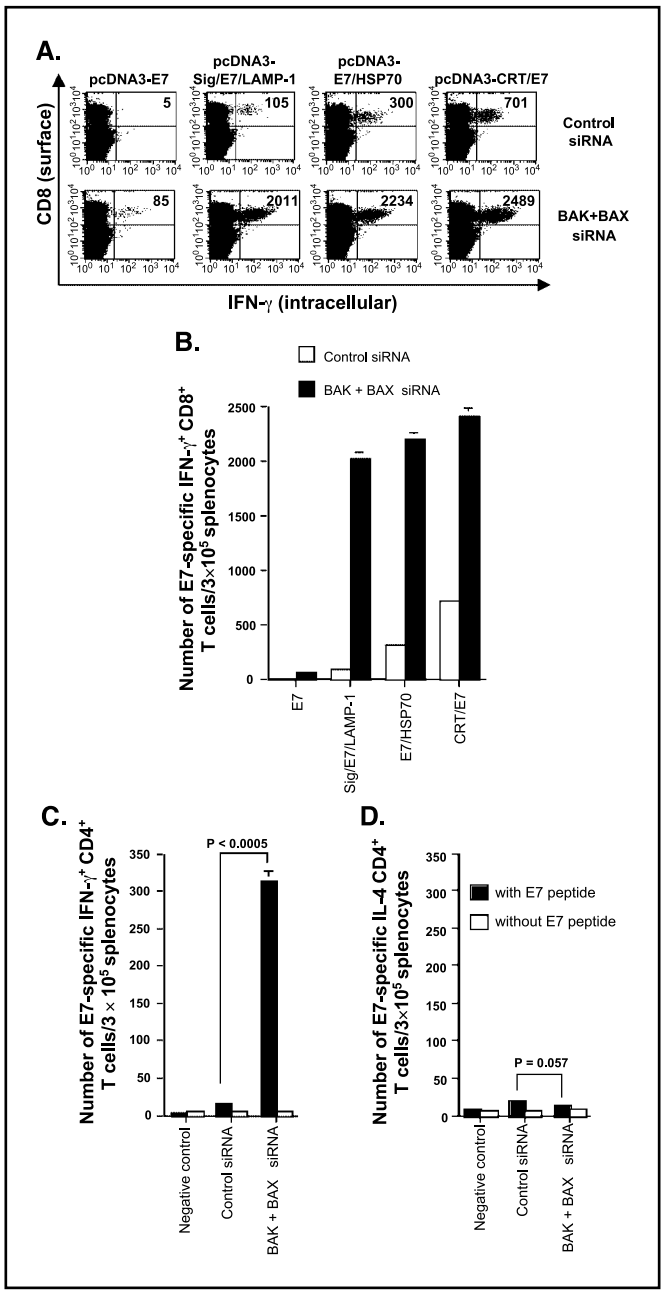


**Figure 4.** Intracellular cytokine staining and flow cytometry analysis to determine E7-specific $CD4^+$ or $CD8^+$ T-cell responses in mice vaccinated with DNA vaccine employing intracellular targeting strategies combined with siRNA. *A* and *B,* mice were vaccinated with pcDNA3-E7, pcDNA3-Sig/E7/LAMP-1, pcDNA3-E7/HSP70, or pcDNA3-CRT/E7 combined with BAK + BAX siRNA or control siRNA. *C* and *D,* mice were vaccinated with pcDNA3-Sig/E7/LAMP-1 combined with BAK + BAX siRNA or control siRNA. *A,* representative flow cytometry data. *B,* number of IFN-γ-expressing E7-specific $CD8^+$ T cells in splenocytes from vaccinated mice. *C,* number of IFN-γ-expressing E7-specific $CD4^+$ Th1 cells in splenocytes from vaccinated mice. *D,* number of interleukin-4-expressing E7-specific $CD4^+$ Th2 cells in splenocytes from vaccinated mice.

**Coadministration of BAK + BAX siRNA with DNA Vaccines during the Boosting Phase Can Elicit a Significantly Stronger Antigen-Specific $CD8^+$ T-Cell Response than Coadministration during the Priming Phase.** As shown in Fig. 5, our data suggest that the antiapoptotic siRNA strategy is most crucial for prolonging dendritic cell life when there is a preexisting antigen-specific $CD8^+$ T-cell population, as occurs in the boosting phase of DNA vaccination.

To determine whether prolonging the life of antigen-expressing dendritic cells is more important during the priming or the boosting phases of vaccination, we coadministered pcDNA3-Sig/E7/LAMP-1 with BAK + BAX siRNA or control siRNA during the priming or boosting phases. As shown in Fig. 6, mice given BAK + BAX siRNA during the priming and boosting phases generated the greatest number of E7-specific $CD8^+$ T-cell precursors when compared with the other vaccination groups. Furthermore, mice given BAK + BAX siRNA during the boosting phase generated a significantly higher number of E7-specific $CD8^+$ T cells than mice given BAK + BAX siRNA during the priming phase ($P = 0.002$). These data suggest that prolonging the life of antigen-expressing dendritic cells via siRNA during the boosting phase is most important for the clonal expansion of antigen-specific T cells.

## Discussion

*In vivo* delivery of siRNA to target cells represents a significant challenge. Considerable endeavors have been devoted to efficient delivery of siRNA to specific cell types or organs *in vivo* (18). Thus far, these endeavors have met with only limited success (for review, see ref. 19). We have shown that intradermal delivery to APCs via gene gun is an effective system for delivery of siRNA into professional APCs, allowing us to evaluate siRNA-based strategies to modify dendritic cells. Thus, our study pioneers the use of intradermal delivery of siRNA to dendritic cells and represents a system to investigate the properties of antigen-expressing dendritic cells *in vivo*.

The encouraging results from this study suggest that modifying the function of dendritic cells *in vivo* using siRNA technology targeting other key proapoptotic proteins, such as caspase-3, -6, -7, -8, or -9, may also lead to enhanced DNA vaccine potency. Furthermore, a combination of siRNAs targeting key proapoptotic proteins within the extrinsic and intrinsic apoptotic pathways will likely result in stronger resistance to apoptotic stress in transfected DC-1 cells and a greater number of antigen-expressing dendritic cells in the draining lymph nodes of effectively primed mice. In addition, surface molecules, such as PD-L1 and PD-L2 (for review, see refs. 20, 21), and/or cytokines, such as interleukin-4 and interleukin-10 (for review, see refs. 22, 23), expressed by dendritic cells have been shown to suppress T-cell responses. Expression of these molecules can potentially be silenced by the siRNA technology to enhance antigen-specific immune responses and antitumor effects.

We have observed a significant increase in the number of $GFP^+$ dendritic cells in the draining lymph nodes of vaccinated mice after coadministration of pcDNA3-E7/GFP with BAK + BAX siRNA compared with coadministration of pcDNA3-E7/GFP and control siRNA (see Fig. 5*A*). This increase is likely due to enhanced dendritic cell survival mediated by BAK + BAX siRNA rather than an influence on migration of dendritic cells mediated by nonspecific siRNA effects, because coadministration of pcDNA3-E7/GFP with control siRNA did not generate similar effects. Our previous observations using DNA-encoded antiapoptotic proteins also support such a notion. Previously, we have used coadministration of DNA vaccines encoding antigen with DNA-encoding Bcl-$x_L$ to prolong the lives of transfected dendritic cells (6). Whereas coadministration of DNA-encoding antigen with DNA-encoding Bcl-$x_L$ led to an increased number of antigen-expressing dendritic cells in the draining lymph nodes of vaccinated mice, coadministration of DNA-encoding antigen with DNA-encoding mutant Bcl-$x_L$ (which has minimal mutations in a region critical to antiapoptotic function) failed to

lead to such an increase (6). Thus, the increase in $GFP^+$ dendritic cells in the draining lymph nodes after coadministration of BAK + BAX siRNA is likely due to changes in survival of dendritic cells rather than migration.

The increase in the number of antigen-expressing dendritic cells in the lymph nodes following the coadministration of BAK + BAX siRNA may contribute to increased numbers of E7-specific $CD8^+$ T cells in vaccinated mice through multiple mechanisms. Not only do antigen-expressing dendritic cells provide signals to trigger proliferation and expansion of antigen-specific T cells, but they also may provide necessary signals resulting in the reduction of T-cell apoptotic death. Normally, dendritic cell death leads to decreasing interaction between APCs and lymphocytes, causing T cells to down-regulate antiapoptotic molecules and potentially up-regulate proapoptotic molecules ( for review, see ref. 9). This process would naturally lead to a decline in numbers of activated antigen-specific $CD8^+$ T cells. The continued survival of antigen-expressing dendritic cells as a result of siRNA-mediated silencing of proapoptotic molecules would provide the necessary signals to prevent this decline. Furthermore, there may be other explanations for enhanced T-cell responses, such as qualitative changes in antigen-expressing dendritic cells as a result of vaccination with BAK + BAX siRNA. We have observed that antigen-expressing dendritic cells transfected with BAK + BAX siRNA could activate antigen-specific $CD8^+$ T cells more efficiently than dendritic cells transfected with control siRNA (data not shown). Thus, it is possible that the antiapoptotic function mediated by BAK + BAX siRNA may modify the quantity and quality of dendritic cells, leading to enhanced T-cell activation.

Our data indicated that prolonging the life of antigen-expressing dendritic cells during the boosting phase is important for the clonal expansion of antigen-specific T cells (see Fig. 6). Killing of antigen-expressing dendritic cells is a natural process for regulating the clonal expansion of antigen-specific $CD8^+$ T cells. Preexisting antigen-specific $CD8^+$ T cells in draining lymph nodes can lyse antigen-expressing dendritic cells, limiting clonal expansion (24, 25). This $CD8^+$ T-cell – mediated lysis of dendritic cells is more significant during the boosting phase of vaccination than during the priming phase due to the increased number of antigen-specific $CD8^+$ T cells elicited by the priming vaccination. Therefore, while prolonging the lives of antigen-expressing dendritic cells during priming and boosting leads to the strongest clonal expansion of antigen-specific $CD8^+$ T cells, it is during the boosting phase that the prolongation of the lives of dendritic cells contributes most to this expansion.

The BAK and BAX siRNA technology can also be extended to the preparation of dendritic cells *ex vivo*. We have shown that E7 peptide-pulsed DC-1 cells transfected with BAK + BAX siRNA



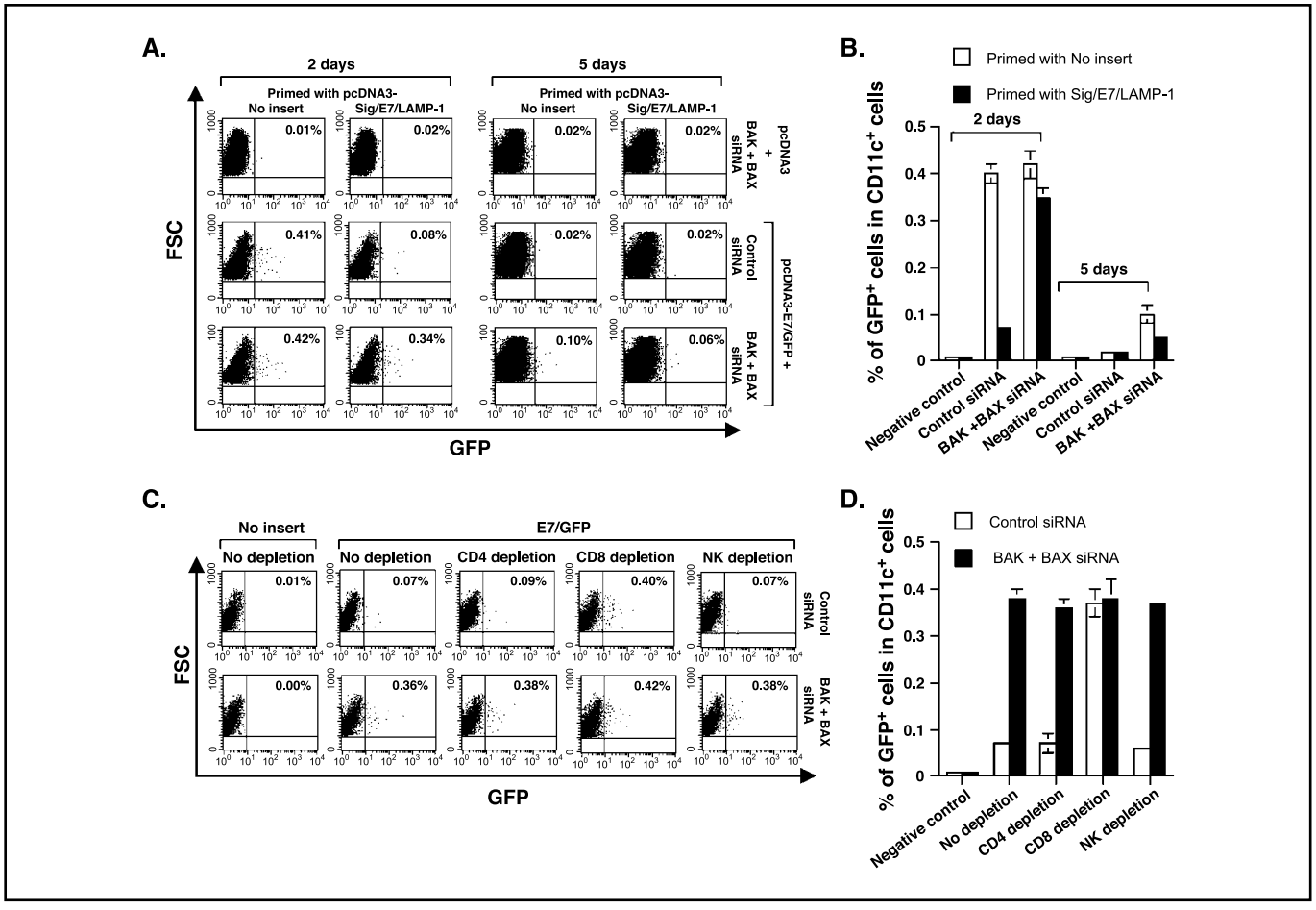


**Figure 5.** Flow cytometry analysis to detect GFP-expressing dendritic cells in draining lymph nodes of mice vaccinated with E7/GFP DNA combined with BAK + BAX siRNA. *A,* representative flow cytometry data at 2 and 5 days after intradermal administration of pcDNA3-E7/GFP; percentages of GFP-expressing cells out of total $CD11c^+$ cells. *B,* percentages of GFP-expressing cells out of total $CD11c^+$ cells. *C,* representative *in vivo* antibody depletion experiment. *D,* percentages of $GFP^+$ cells out of total $CD11c^+$ cells after antibody depletion.

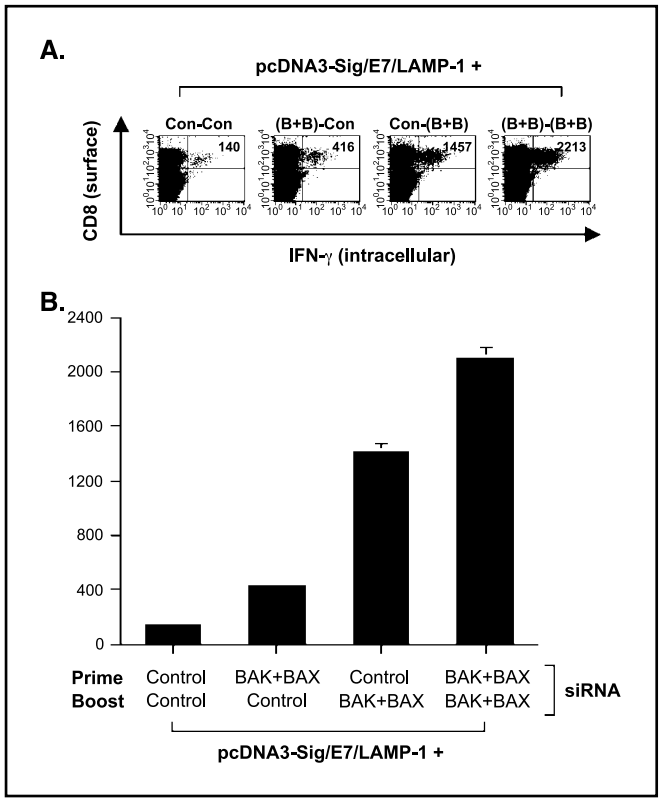


**Figure 6.** Intracellular cytokine staining with flow cytometry analysis to determine the effect of coadministration with BAK + BAX siRNA during the priming and/or boosting phases. Mice were vaccinated with pcDNA3-Sig/E7/LAMP-1 combined with BAK + BAX siRNA (*B + B*) and/or control siRNA (*Con*) in the priming and/or boosting phases. *A,* representative flow cytometry data. *B,* numbers of IFN-γ-expressing E7-specific $CD8^+$ T cells in splenocytes from vaccinated mice.

were more capable of resisting killing by E7-specific $CD8^+$ T cells compared with DC-1 cells transfected with control siRNA (Fig. 1*C*). Furthermore, we have recently found that vaccination with E7 peptide-pulsed DC-1 cells transfected with BAK + BAX siRNA led to significantly higher numbers of E7-specific $CD8^+$ T cells in vaccinated mice compared with vaccination with E7 peptide-pulsed DC-1 cells transfected with control siRNA (data not shown). These data suggest that the potency of dendritic cell–based vaccines prepared *ex vivo* can be further enhanced with siRNA targeting key proapoptotic proteins, such as Bak and Bax.

In summary, the targeting of BAK + BAX siRNA with antigen-encoding DNA vaccines to dendritic cells *in vivo* represents an innovative approach to enhance DNA vaccine potency. In addition, the use of siRNA alleviates safety concerns associated with the use of DNA vaccines encoding antiapoptotic proteins (6, 7). Not only does gene gun delivery of siRNA to dendritic cells result in prolonged dendritic cell life, but it also does not carry with it the concerns for oncogenicity associated with DNA-encoding antiapoptotic proteins. Furthermore, we did not observe any gross or histologic changes in the vital organs of vaccinated mice compared with nonvaccinated mice, alleviating concern for autoimmunity induced by prolonging the lives of dendritic cells. Thus, this BAK + BAX siRNA strategy shows potential for eventual adaptation to the clinical arena to further enhance DNA vaccine potency for the control of cancer and infectious disease.

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