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Vaccination with Dendritic Cells Transfected with BAK and BAX siRNA Enhances Antigen-Specific Immune Responses by Prolonging Dendritic Cell Life

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Abstract

Dendritic cell-based vaccines have become an important approach for the treatment of malignancies. Numerous techniques have recently been designed to optimize dendritic cell activation, tumor antigen delivery to dendritic cells, and induction of tumor-specific immune responses *in vivo*. Dendritic cells (DCs), however, have a limited life span because they are subject to apoptotic cell death mediated by T cells, hindering their long-term ability to prime antigen-specific T cells. Small interfering RNA targeting Bak and Bax antiapoptotic proteins can be used to allow transfected DCs to resist killing by T cells *in vivo*. In this study, we show that human papillomavirus E7-loaded dendritic cells transfected with BAK/BAX siRNA downregulate Bak and Bax protein expression and become resistant to killing by T cells, leading to enhanced E7-specific CD8⁺ T cell activation and antitumor effects *in vivo*. More importantly, we found that vaccination with E7-loaded DCs transfected with BAK/BAX siRNA was capable of generating a strong therapeutic effect in vaccinated mice, compared with DCs transfected with control siRNA. Our data indicate that transfection of dendritic cells with BAK/BAX siRNA represents a plausible strategy for enhancing dendritic cell-based vaccine potency.

OVERVIEW SUMMARY

Dendritic cells (DCs) have a limited life span, which hinders their ability to prime antigen-specific T cells. In this study, we showed that peptide-loaded dendritic cells transfected with BAK/BAX siRNA downregulated Bak and Bax protein expression and became resistant to killing by antigen-specific T cells *in vivo*. Furthermore, we found that vaccination with DCs transfected with BAK/BAX siRNA was capable of generating a stronger therapeutic antitumor effect in vaccinated mice compared with DCs transfected with control siRNA. Thus, transfection of DCs with BAK/BAX siRNA may represent a plausible strategy for enhancing DC-based vaccine potency.

INTRODUCTION

Antigen presentation by dendritic cells (DCs) is a critical element for the induction of the cellular immune responses necessary for tumor immunotherapy. Several clinical trials have demonstrated that immunizations with tumor antigen-pulsed DCs could break the tolerance of the immune system against antigens expressed by tumor cells and in some cases generate appreciable clinical responses. Thus, DC-based vaccines represent a promising method for the treatment of malignancies (Gunzer and Grabbe, 2001; Engleman, 2003; Schuler *et al.*, 2003; Cerundolo *et al.*, 2004; Figdor *et al.*, 2004; Markiewicz and Kast, 2004; Turtle and Hart, 2004).

DCs, however, have a limited life span that hinders their long-term ability to prime antigen-specific T cells (Ronchese and Hermans, 2001). A principal contributor to the short life of DCs is CTL-induced apoptosis. After activation by DCs, CTLs can recognize antigens and kill the cells expressing them. Because DCs express MHC I: antigen peptide complexes, newly primed CTLs can kill the very DCs that activated them (Medema *et al.*, 2001). Thus, DC-based vaccination could be enhanced by employing an approach to inhibit apoptosis and prolong the survival of antigen-expressing DCs *in vivo* (Kim *et al.*, 2003a,b).

T cell-mediated apoptotic cell death can occur through two major pathways, the intrinsic and the extrinsic pathways (Russell and Ley, 2002). The intrinsic pathway (granzyme B/perforin-mediated apoptosis) has been shown to be important for induction of T cell-mediated apoptotic death of DCs. The pore-forming protein perforin and the serine protease granzyme B secreted into cells by antigen-specific CD8⁺ T cells induce the release of a number of proapoptotic factors, such as cytochrome c, from the mitochondria (Russell and Ley, 2002). Of particular note is that the release of cytochrome c is likely controlled by two members of the Bcl-2 family, the gatekeeper proteins Bak and Bax, and leads to the activation of another initiator caspase, caspase-9 (Jacotot *et al.*, 1999; Korsmeyer *et al.*, 2000; Degli Esposti and Dive, 2003; Opferman and Korsmeyer, 2003). Activated caspase-9 ultimately results in the activation of effector caspases (such as caspase-3, -6, and -7) in a protein complex called the apoptosome (Johnson and Jarvis, 2004), eventually leading to proteolysis of a cascade of substrates that results in apoptotic death. Therefore, Bak and Bax represent potentially ideal targets for the inhibition of DC apoptosis (Opferman and Korsmeyer, 2003).

In the current study, we investigated the use of RNA interference (RNAi) technology to downregulate the expression of Bak and Bax in order to inhibit the T cell-mediated apoptotic cell death of dendritic cells. We confirmed this by demonstrating that dendritic cells transfected with BAK/BAX siRNA abolish Bak and Bax protein expression. Furthermore, antigenic peptide-pulsed DCs transfected with BAK and BAX siRNA are capable of generating stronger antigen-specific CD8⁺ T cell immune responses and antitumor effects in vaccinated mice, compared with antigenic peptide-pulsed DCs transfected with control siRNA. Our data also suggest that BAK/BAX siRNA-transfected DCs survive longer *in vivo* than antigenic peptide-loaded DCs transfected with control siRNA in mice adoptively transferred with antigen-specific CD8⁺T cells. 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 DC *ex vivo*.

MATERIALS AND METHODS

SiRNA synthesis and transfection

siRNAs were synthesized using 2'-O-ACE-RNA phosphoramidates (Dharmacon, Lafayette, CO). The sense and antisense strands of siRNA were: *Bak*, beginning at nt 310, 5'-UGCCUACGAACUCUUCACCTdT-3' (sense), 5'-GGUGAAGAGUUCGUAGGCAdTdT-3' (antisense); *Bax*, beginning at nt 217, 5'-UAUGGAGCUGCAGAGGAUGdTdT-3' (sense), 5'-CAUCCUCUGCAGCUCCAUAAdTdT-3' (antisense) (P, 5' phosphate). RNAs were deprotected and annealed according to the manufacturer's instructions. Nonspecific Control siRNA (target: 5'-NNATTGTATGCGATCGCAGAC-3') was acquired from Dharmacon. DC-1 cells or bone marrow-derived DCs (BM-DCs) incubated for 6 days were transfected with BAK/BAX siRNA or control siRNA using Oligofectamine (Invitrogen, Carlsbad, CA). The transfected cells were used for subsequent experiments 24 to 48 hours later.

Peptides

The H-2D^b-restricted HPV-16 E7 peptide, RAHYNIVTF (E7 aa49-57) (Feltkamp *et al.*, 1993), was synthesized by Macro-molecular Resources (Denver, CO) at a purity of $\geq 70\%$.

Cells

TC-1 cells were generated as described previously (Lin *et al.*, 1996), and were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 100 μ M nonessential amino acids, and 0.4 mg/ml of G418. DC-1 cells were generated from the dendritic cell line (Shen *et al.*, 1997) provided by Dr. Kenneth Rock at the University of Massachusetts. With continued passage, we have generated subclones of DCs (DC-1) that can be easily transfected (Kim *et al.*, 2004). The cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 μ M nonessential amino acids, 5×10^{-5} M β -mercaptoethanol, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal bovine serum. An H-2D^b-restricted HPV-16 E7-specific T cell line has also been described previously (Wang *et al.*, 2000). The T cell line was stimulated with irradiated TC-1 and 20 U/ml murine recombinant IL-2 weekly.

Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BM-DCs) were generated from bone marrow progenitor cells as described by Inaba and colleagues (Inaba *et al.*, 1992) with a modification. Briefly, bone marrow cells were flushed from the femurs and tibiae of 5- to 8-week-old C57BL/6 mice. Cells were washed twice with RPMI-1640 after lysis of red blood cells and resuspended at a density of 1×10^6 /ml in RPMI-1640 medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 μ M nonessential amino acids, 5×10^{-5} M β -mercaptoethanol, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 5% fetal bovine serum, and 20 ng/ml recombinant murine GM-CSF (PeproTech, Rock Hill, NJ). The cells were then cultured in a 24-well plate (1 ml/well) at 37°C in 5% humidified CO₂. The wells were replenished with fresh medium supplemented with 20 ng/ml recombinant murine GM-CSF on days 2 and 4. The cells were harvested after 6 days and used for siRNA transfection.

Western blot analysis

2×10^5 DC-1 cells were transfected with 300 pmol of the synthesized BAK/BAX siRNA or control siRNA in a final volume of 2 ml using Oligofectamine, 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 performed with 50 μ g of the cell lysate from the transfected DC-1 cells and anti-BAK and/or BAX mouse monoclonal antibody (Cell Signaling Technology, Beverly, MA), using a protocol similar to that described previously (Hung *et al.*, 2001).

Mice

Six- to 8-week-old C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). All animals were maintained under specific pathogen-free conditions, and all procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DC immunization

DC-1 cells or BM-DCs were transfected with the synthesized BAK/BAX siRNA or control siRNA as described above. Two days after transfection, the DC-1 cells or BM-DCs transfected with BAK/BAX siRNA or control siRNA were pulsed with HPV-16 E7 aa49-57 peptide (aa49-57, RAHYNIVTF) (10 μ g/ml) at 37°C for 2 hours. The cells were then washed with RPMI-1640, supplemented with 10% FCS and HBBS, and re-suspended in HBBS at the final concentration of 5×10^6 /ml (for DC-1 cells) or 2×10^6 /ml (for BM-DCs). 100 μ l/mouse of DC-1 cells or BM-DCs were injected into mice via footpad injection. One week later, the mice were boosted once with the same dose and immunization regimen.

Intracellular cytokine staining and flow cytometry analysis

Before intracellular cytokine staining, 3.5×10^5 pooled splenocytes from each vaccination group were incubated overnight with 1 μ g/ml of the HPV-16 E7 aa49-57 peptide. GolgiPlug (BD Pharmingen, San Diego, CA) was added to the culture and incubated at 37°C overnight. Cells were then washed once with FACSscan buffer and stained with phycoerythrin-conjugated monoclonal rat antimouse CD8a (clone 53.6.7). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Intracellular IFN- γ was stained with FITC-conjugated rat antimouse IFN- γ .

Analysis of surface markers of nontransfected or siRNA-transfected DCs was performed on FACS Calibur and analyzed using the CellQuest software (BD Biosciences, San Jose, CA). FITC-conjugated antimouse monoclonal antibodies against the surface markers CD11c, CD40, CD86, I-A^b, or H-2K^b/D^b (all from BD Pharmingen) were used in the study. Flow cytometry analysis was performed using FACS Calibur with CellQuest software.

In vivo tumor protection and treatment experiments

For the tumor protection experiment, C57BL/6 mice (five per group) were immunized with E7-pulsed DC-1 cells as indicated and boosted once after 1 week with the same dose. One week after the booster, each mouse was challenged with 5×10^4 TC-1 tumor cells subcutaneously in the right leg and then monitored twice a week.

For the tumor treatment experiment, the mice were challenged with 5×10^4 TC-1 tumor cells per mouse in the tail vein to simulate hematogenous spread of tumors (Ji *et al.*, 1999). Mice were immunized with 5×10^5 E7 peptide-pulsed siRNA-transfected DC-1 cells 3 days after tumor challenge and boosted once after 1 week. Mice were sacrificed on day 28 after the last immunization. The mean number of pulmonary nodules in each mouse was evaluated by experimenters blinded to sample identity.

Adoptive transfer of T cells and rapid DC elimination assay

To create two distinctly carboxyfluorescein (CFSE)-labeled E7 peptide-loaded BM-DCs transfected with different siRNA, we first prepared E7 peptide-loaded BM-DCs transfected with either BAX/BAX siRNA or control siRNA using methods as described above. The E7 peptide-loaded BM-DCs transfected with control siRNA were labeled with 5 μ M CFSE, while BAK/BAX siRNA transfected DCs were labeled with 0.5 μ M CFSE. We then injected a mixture consisting of 2.5×10^5 low CFSE-labeled E7 peptide-loaded BM-DCs and 2.5×10^5 high CFSE-labeled E7 peptide-loaded BM-DCs into C57BL/6 mice 3 days after adoptive transfer of 1×10^6 E7-specific T cells into the mice via tail vein injection. Sixteen hours later, single cell suspensions from the lung and spleen were prepared and analyzed for CFSE positivity by flow cytometry.

Statistical analysis

All data expressed as means $\pm$ standard error (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 analysis of variance (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. All *p* values of <0.05 were considered significant.

RESULTS

Dendritic cells transfected with BAK/BAX siRNA abolish the expression of Bak and Bax proteins

We performed Western blot analysis to determine whether DC-1 cells (a murine dendritic cell line) transfected with BAK/BAX siRNA will influence the expression of Bak and Bax proteins in transfected cells. As shown in Figure 1, lysates from DC-1 cells transfected with BAK/BAX siRNA showed significant reduction in the expression of Bak and Bax proteins 24 and 48 hours after transfection. In contrast, the expression of Bak and Bax in lysates from DC-1 cells transfected with control siRNA was comparable to lysates from DC-1 cells without transfection 24 and 48 hours after transfection. We also analyzed β -actin expression in transfected DCs to demonstrate that equal amounts of cell lysates were loaded for Western blot analysis. These results indicate that DC-1 cells transfected with BAK/BAX siRNA abolish Bak and Bax protein expression during the period of time examined.

Recently, we demonstrated that DC-1 cells transfected with BAK and/or BAX siRNA can resist CTL-induced apoptosis. We incubated E7 peptide-loaded, siRNA-transfected DC-1 cells with an E7-specific CD8⁺ T cell line. Our results showed that E7 peptide pulsed DC-1 cells transfected with BAK/BAX siRNA resisted killing by E7-specific CD8⁺ T cells *in vitro* (Kim *et al.*, 2005). Taken together, our data suggest that transfection of DC-1 cells with BAK and/or BAX siRNA leads to downregulation of BAK and BAX protein expression, resulting in resistance to apoptosis induced by antigen-specific CD8⁺ T cells.

Vaccination with E7 peptide-loaded DCs transfected with BAK/BAX siRNA leads to a significant increase in E7-specific IFN- γ ⁺ CD8⁺ T cell precursors in vaccinated mice

To determine whether vaccination with E7 peptide-loaded DCs transfected with BAK/BAX siRNA could enhance the generation of E7-specific IFN- γ ⁺ CD8⁺ T cell precursors in mice, we performed intracellular cytokine staining and flow cytometry analysis using the splenocytes from mice vaccinated with the various DC-1 cells. As shown in Figure 2, mice vaccinated with E7 peptide-loaded DCs transfected with BAK/BAX siRNA exhibited an ~5.4-fold increase in the number of E7-specific IFN- γ ⁺ CD8⁺ T cells (655 ± 21.21)

compared to mice vaccinated with E7-loaded DCs transfected with control siRNA (121 ± 5.66). E7-loaded DC-1 cells transfected with control siRNA generated similar numbers of E7-specific CD8⁺ T cells as E7-loaded DC-1 cells without transfection. Our results demonstrate that immunization with E7 peptide-loaded DCs transfected with BAK/BAX siRNA can significantly increase the number of E7-specific IFN- γ ⁺ CD8⁺ T cells generated in vaccinated mice.

Vaccination with E7 peptide-loaded BM-DCs transfected with BAK/BAX siRNA leads to a significant increase in E7-specific IFN- γ ⁺ CD8⁺ T cell precursors in vaccinated mice

It is important to determine if the BAK/BAX siRNA technology applies to BM-DCs as well as DC-1 cells, as BM-DCs would be a more appropriate source of dendritic cells for clinical translation of DC-based vaccines than would immortalized dendritic cell lines. Therefore, we used E7 peptide-loaded BM-DCs transfected with either BAK/BAX siRNA or control siRNA for our study. To determine E7-specific CD8⁺ T cell precursors in vaccinated mice, we performed intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 3, mice vaccinated with E7 peptide-loaded BM-DCs transfected with BAK/BAX siRNA exhibited an ~2.2-fold increase in the number of E7-specific IFN- γ ⁺ CD8⁺ T cells (4706 ± 78.5) compared with mice vaccinated with E7 peptide-loaded DCs transfected with control siRNA (2210 ± 134.3) ($p = 0.00194$). Our data indicate that the BAK/BAX siRNA technology can also be applied to BM-DCs to enhance DC-based vaccine potency.

Vaccination with E7-loaded DCs transfected with BAK/BAX siRNA generates better antitumor effects than vaccination with E7-loaded DCs transfected with control siRNA

To determine whether the observed increase in the number of E7-specific CD8⁺ T cell precursors translated into a better E7-specific antitumor effect, we performed an *in vivo* tumor protection experiment using a previously characterized E7-expressing tumor model, TC-1 (Lin *et al.*, 1996). As shown in Figure 4A, 100% of mice receiving E7 peptide-loaded DCs transfected with either control siRNA or BAK/BAX siRNA remained tumor-free 30 days after a subcutaneous TC-1 challenge, whereas the nonvaccinated mice developed tumors within 10 days after tumor challenge. Therefore, vaccination with E7 peptide-loaded DC-1 transfected with BAK/BAX siRNA or transfected with control siRNA is able to elicit protective antitumor immunity against challenge by an E7-expressing tumor and the *in vivo* tumor protection model fails to distinguish between the antitumor effects generated by the two groups of siRNA-transfected DC-1 cells.

To further compare the antitumor effects generated by vaccination with E7 peptide-loaded DCs transfected with BAK/BAX siRNA or control siRNA, we performed an *in vivo* tumor treatment experiment in a more stringent lung tumor metastasis model using TC-1 tumor cells (Ji *et al.*, 1998). Mice were first challenged with the TC-1 tumor cells via tail vein, followed by treatment with E7 peptide-loaded DC-1 cells transfected with BAK/BAX siRNA or control siRNA. Mice were sacrificed 28 days after the tumor challenge, and the growth of pulmonary tumor nodules was examined. As shown in Figure 4B, mice treated with E7 peptide-loaded DCs transfected with BAK/BAX siRNA demonstrated the lowest number of pulmonary nodules (2.2 ± 0.84) compared with mice treated with E7 peptide-loaded DCs transfected with control siRNA (24.8 ± 5.89) or the naïve control group (103 ± 12.29 ; t test, $p < 0.001$). These results show that vaccination with E7-loaded DCs transfected with BAK/BAX siRNA generates a significantly better therapeutic antitumor effect than vaccination with E7-loaded DCs transfected with control siRNA.

E7 peptide-loaded DCs transfected with BAK/BAX siRNA survive longer in vivo than E7 peptide-loaded DCs transfected with control siRNA

To determine if transfection with BAK/BAX siRNA improves the survival of E7 peptide-loaded DCs *in vivo*, we first created two distinctly CFSE-labeled E7 peptide-loaded groups of BM-DCs transfected with different siRNAs. E7 peptide-loaded BM-DCs transfected with control siRNA were labeled with a higher concentration of CFSE (5 μ M), while BAK/BAX-transfected BM-DCs were labeled with a lower concentration of CFSE (0.5 μ M). The relative levels of CFSE in these two distinctly CFSE-labeled E7 peptide-loaded BM-DCs were characterized by flow cytometry analysis (Fig. 5A). Mice were then challenged with 1×10^6 E7-specific T cells per mouse via tail vein injection. Three days later, we injected a mixture consisting of 2.5×10^5 low CFSE-labeled BM-DCs per mouse and 2.5×10^5 of high CFSE-labeled BM-DCs per mouse into the challenged mice intravenously. Sixteen hours after injection with CFSE-labeled BM-DCs, we performed flow cytometry analysis to characterize the ratio of low CFSE-labeled BM-DCs to high CFSE-labeled BM-DCs using cells collected from the spleen and lungs of challenged mice. As shown in Figure 5B, a significantly higher number of low CFSE-labeled BM-DCs was observed (~3.7-fold), compared with the number of high CFSE-labeled BM-DCs. These results suggest that transfection of E7 peptide-loaded BM-DCs with BAK/BAX siRNA may prolong DC life *in vivo*, resulting in a higher number of E7 peptide-loaded BM-DCs.

E7 peptide-loaded DC-1 cells transfected with BAK/BAX or control siRNA express similar levels of CD11c, CD40, CD86, MHC I, and MHC II

The significant therapeutic effect generated by vaccination with E7 peptide-loaded DCs transfected with BAK/BAX siRNA might be due to changes in the expression of molecules important for antigen presentation in DCs, such as CD11c, CD40, CD86, MHC I, and MHC II. We therefore performed flow cytometry analysis to determine the expression levels of these molecules in an E7 peptide-loaded DC-1 cell line transfected with BAK/BAX siRNA or control siRNA or in non-transfected DC-1 cells. As shown in Figure 6, there was no significant change in the expression of the tested molecules among the E7 peptide-loaded DC-1 cells. We also characterized the expression levels of these molecules in E7 peptide-loaded BM-DCs transfected with BAK/BAX siRNA or control siRNA or in nontransfected BM-DCs. We did not observe significant changes in the expression of these molecules among the E7 peptide-loaded BM-DCs transfected with the various siRNA constructs (data not shown). Taken together, our data indicated that the surface expression of CD11c, CD40, CD86, MHC I, and MHC II on the E7 peptide-loaded murine DC-1 cell line or BM-DCs are not affected by transfection with BAK/BAX or control siRNAs.

DISCUSSION

In this study, we demonstrated that vaccination with E7 peptide-loaded DCs transfected with BAK/BAX siRNA generated enhanced E7-specific T cell-mediated immune responses and antitumor effects *in vivo*. Transfection of DCs with BAK/BAX siRNA likely inhibited apoptotic cell death of DCs mediated by T cells, leading to prolongation of DC survival and resulting in an improved DC-based vaccine.

Previous studies have shown that DC survival can be efficiently prolonged *in vivo* through transfection of DCs with DNA encoding antiapoptotic proteins (Kim *et al.*, 2003b). This technique, however, has raised significant concerns regarding the potential for oncogenic transformation as a result of overexpression of these antiapoptotic proteins. Antiapoptotic proteins, such as the Bcl-2 family, are known to be overexpressed in some cancers and therefore have been implicated as contributors to cellular immortalization (Lebedeva *et al.*, 2000). The modification of dendritic cells using siRNA targeting Bak and Bax proteins

alleviates many of these concerns for oncogenicity. Due to the transient nature of siRNA-mediated silencing of target genes as well as the fact that RNA-based strategies carry no concerns for integration and permanent genetic change, transfection of DCs with BAK/BAX siRNA may represent a potentially safe and effective method for enhancing DC-based vaccine potency by prolonging DC life without risk of DC immortalization.

We have recently demonstrated that a dendritic cell line (DC-1) transfected with BAK/BAX siRNA was capable of resisting killing by antigen-specific CD8⁺ T cells *in vitro* (Kim *et al.*, 2005). In the current work, we used BM-DCs for the CFSE durability assays to demonstrate the resistance of BM-DCs to killing by antigen-specific CD8⁺ T cells *in vivo*. While there is some decrease of the CFSE level intensity in each CFSE-labeled population (probably due to continuous replication of the BM-DCs), we were able to distinguish the high- and low-level CFSE intensity groups after collecting cells from the spleen and lungs of the challenged mice. We observed a significantly higher number of BAK/BAX siRNA-transfected BM-DCs (~3.7-fold), compared with the number of control siRNA-transfected BM-DCs (Fig. 5). These results suggest that transfection of E7 peptide-loaded BM-DCs with BAK/BAX siRNA may prolong DC life *in vivo*, resulting in a higher number of E7 peptide-loaded BM-DCs.

The data from our dendritic cell-based vaccine prepared *ex vivo* using siRNA technology targeting Bak and Bax are consistent with our data from modification of dendritic cells using BAK/BAX siRNA vaccination *in vivo*. We have recently used intradermal gene-gun coadministration of DNA-encoding antigen with BAK/BAX siRNA to prolong the life of antigen-expressing DCs *in vivo*. Our data showed that mice vaccinated with DNA coadministered with BAK/BAX siRNA results in significantly enhanced antigen-specific CD8⁺ T cell-mediated immune responses and antitumor effects compared with mice vaccinated with DNA coadministered with control siRNA (Kim *et al.*, 2005). Taken together, these data indicate that siRNA technology can be used to modify dendritic cells *ex vivo* or *in vivo* to improve vaccine potency.

The encouraging results from this study suggest that the modification of a DC-based vaccine with BAK/BAX siRNA, as well as siRNA targeting other key proapoptotic proteins may further enhance DC-based vaccine potency. Since BAK/BAX siRNA only affects the intrinsic granzyme B/perforin-mediated apoptotic pathway, a combination of siRNAs targeting key proapoptotic proteins in the intrinsic granzyme B/perforin pathway along with siRNAs targeting key proapoptotic proteins in the extrinsic Fas-mediated apoptotic pathway will likely result in stronger resistance to killing of the transfected DCs by T cells *in vivo*. One possible protein to target for RNA interference along the extrinsic pathway is caspase-8, a caspase that induces the proteolysis of a cascade of effector caspases leading to apoptotic cell death. Other caspases involved in cell apoptosis that could serve as potential targets for siRNA include caspases 3, 6, and 7. Thus, a DC-based vaccination strategy employing siRNAs targeting key proapoptotic proteins in both the intrinsic and extrinsic apoptotic pathways (e.g., antigen-loaded DCs transfected with BAK/BAX siRNA and caspase-8 siRNA) may generate an even greater enhancement of DC resistance against killing by T cells, possibly resulting in an improved T cell immune response and antitumor effects *in vivo*.

In this study, antigen was loaded onto DCs by pulsing DCs with antigenic peptides. This BAK/BAX siRNA technology could also be applied to DCs prepared through other antigen-loading strategies, including viral vector-mediated, protein-mediated, RNA-mediated, and DNA-mediated transfection strategies. These various approaches have different advantages and disadvantages. For example, viral vector-mediated strategies produce highly efficient transfection of DCs, but have a limited life expectancy, whereas DNA-mediated strategies

are easily prepared but have a lower transfection efficiency in DCs. In particular, both viral vector-mediated and DNA-mediated strategies could benefit from the use of BAK/BAX siRNA technology. For example, it would be possible to further enhance the potency of DC-based vaccines through the combined use of BAK/BAX siRNA technology as an antiapoptotic strategy with other vaccine enhancement strategies, such as the intracellular targeting of antigen inside DCs for more efficient intracellular processing. DNA-mediated strategies of DC-based vaccination could employ DCs transfected with BAK/BAX siRNA coad-ministered with DNA plasmids encoding an antigen peptide linked to an intracellular targeting molecule, such as heat shock protein 70. The intracellular targeting molecule would target the antigen for intracellular processing within the DCs, thereby resulting in increased expression of the antigen on the surface of the DCs, while transfection by BAK/BAX siRNA would prolong the life of the DCs; these two factors would likely generate increased T cell activation and an enhanced antigen-specific immune response.

In the future, further studies employing BM-DCs should be conducted *in vitro* in order to validate the data generated using the BAK/BAX siRNA-transfected immortalized murine DC-1 cell line. This is particularly important for future clinical translation, since only BM-DCs, not immortalized dendritic cell lines, can be used for clinical application. Furthermore, it would also be worthwhile to conduct studies to determine the optimal dose and regimen for RNAi-based modification of the properties of dendritic cells and the length of time over which such a DC-based vaccine strategy is effective at prolonging DC survival. Thus, a possible schedule could be developed to determine dosing times for vaccine administration.

In summary, antigen-loaded DCs transfected with BAK/BAX siRNA as a DC-based vaccine strategy offers an effective and potentially safer approach for prolonging the life of DCs and increasing the potency of DC-based vaccines than does transfection of DCs *in vivo* with DNA-encoding antiapoptotic proteins. Our findings indicate that administering antigen peptide-loaded DCs transfected with BAK/BAX siRNA prolongs the life of transfected DCs and enhances antigen-specific CD8⁺ T cell activity, as well as elicits strong antitumor effects *in vivo*. Thus, a DC-based vaccine strategy incorporating antigen-loaded DCs transfected with BAK/BAX siRNA shows potential for eventual adaptation to the clinical arena to further enhance DC-based vaccine potency for the control of cancer and infectious disease.

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FIG. 1.

Western blot analysis to detect the expression of Bak and Bax protein in DC-1 cells transfected with the various siRNA constructs. DC-1 cells were transfected with either BAK/BAX siRNA or control siRNA. Western blot analysis was performed with 50 μ g of cell lysates 24 and 48 hours after transfection. β -actin was used as a control to indicate that equal amounts of cell lysates were loaded. Untransfected DC-1 cells were used as a negative control.

FIG. 2.

Intracellular cytokine staining and flow cytometry analysis to determine the number of IFN- γ -producing E7-specific CD8⁺ T cells in mice after immunization with E7 peptide-pulsed DCs transfected with the various siRNA constructs. Mice (five per group) were vaccinated with E7 peptide-loaded DCs transfected with BAK/BAX siRNA or control siRNA. Mice vaccinated with E7 peptide-loaded DCs without transfection were used as an additional control. **(A)** Representative flow cytometry data for pooled splenocytes harvested from the vaccinated mice and stimulated with E7 aa49-57 peptide or without peptide stimulation. **(B)** Bar graph depicting the number of IFN- γ -secreting E7-specific CD8⁺ T cell precursors/ 3×10^5 splenocytes from mice after immunization with E7 peptide-loaded DCs transfected with control siRNA or BAK/BAX siRNA or from nontransfected mice (mean $\pm$ SE; t test, $p < 0.001$).

FIG. 3. Intracellular cytokine staining and flow cytometry analysis to determine the number of IFN- γ -producing E7-specific CD8⁺ T cells in mice after immunization with E7 peptide-pulsed BM-DCs transfected with the various siRNA constructs. Mice (five per group) were vaccinated with E7 peptide-loaded BM-DCs transfected with BAK/BAX siRNA or control siRNA. The bar graph depicts the number of IFN- γ -secreting E7-specific CD8⁺ T cell precursors/ 3×10^5 splenocytes from mice after immunization with E7 peptide-loaded BM-DCs transfected with control siRNA or BAK/BAX siRNA or from nonimmunized mice (mean $\pm$ SE; *t* test, $p < 0.002$).

FIG. 4.

In vivo tumor protection and treatment experiments. **(A)** Tumor protection experiment. Mice (five per group) were immunized with E7 peptide-loaded DCs transfected with either control siRNA or BAK/BAX siRNA and boosted after 1 week. Seven days after the last immunization, the mice were challenged with 5×10^4 TC-1 tumor cells per mouse as described in the Materials and Methods section. The tumors were monitored twice a week. Mice without vaccinations were used as a negative control. **(B)** *In vivo* tumor treatment experiment. E7 peptide-loaded DCs transfected with BAK/BAX siRNA or control siRNA were administered 3 days after TC-1 tumor challenge (5×10^4 TC-1 cells/mouse). Mice were boosted with the same dose and regimen of E7 peptide-loaded DCs 1 week later. Mice were sacrificed 28 days after tumor challenge to examine the growth of pulmonary nodules. Data are expressed as the mean number of lung nodules $\pm$ SE (*t* test, $p < 0.001$).

FIG. 5.

Determination of the survival of E7 peptide-loaded BM-DCs transfected with BAK/BAX siRNA or control siRNA after administration of E7-specific CD8⁺ T cells *in vivo*. **(A)** Flow cytometry analysis to demonstrate the different level of CFSE-labeled E7 peptide-loaded BM-DCs transfected with either BAK/BAX siRNA (low CFSE) or control siRNA (high CFSE). BAK/BAX-transfected BM-DCs were labeled with 0.5 μ M CFSE, while BM-DCs transfected with Control siRNA were labeled with 5 μ M CFSE. The representative graph shows the presence of similar numbers of low CFSE-labeled E7 peptide-loaded BM-DCs transfected with BAK/BAX siRNA and high CFSE-labeled E7 peptide-loaded BM-DCs transfected with control siRNA before tail vein injection. **(B)** Flow cytometry analysis to demonstrate the ratio of low CFSE to high CFSE-labeled E7 peptide-loaded BM-DCs in the spleen and lungs of mice 16 hours after injection with a mixture containing equal numbers (2.5×10^5 /mouse) of low CFSE-labeled E7 peptide-loaded BM-DCs transfected with BAK/BAX siRNA and high CFSE-labeled E7 peptide-loaded BM-DCs transfected with control siRNA. These CFSE-labeled BM-DCs were injected into mice 3 days after the administration of 1×10^6 E7-specific T cells per mouse. Note that the number of low CFSE-labeled cells was significantly higher than the number of high CFSE-labeled cells.

FIG. 6.

Characterization of the surface molecules of E7 peptide-loaded DCs after transfection with BAK/BAX siRNA or control siRNA. Flow cytometry analysis was performed to determine the level of expression of CD11c, CD40, CD86, MHC I, and MHC II molecules in E7 peptide-loaded murine DC-1 cells transfected with BAK/BAX siRNA or control siRNA. E7 peptide-loaded DCs without transfection with siRNA were used as a negative control.