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Enhancing DNA Vaccine Potency by Combining a Strategy to Prolong Dendritic Cell Life with Intracellular Targeting Strategies

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We have recently shown that intradermal coadministration of DNA encoding Ag with DNA encoding inhibitors of apoptosis, including Bcl-xL, prolongs dendritic cell (DC) life and thereby enhances the potency of DNA vaccines in vivo. We have also demonstrated that DNA vaccines targeting Ag to subcellular compartments, using proteins such as Mycobacterium tuberculosis heat shock protein 70, calreticulin, or the sorting signal of the lysosome-associated membrane protein type 1 (LAMP-1), enhanced DNA vaccine potency. In this study, we reasoned that the combination of a strategy to prolong DC life with intracellular targeting strategies might produce a more effective DNA vaccine against human papillomavirus E7. We showed that coadministration of DNA encoding Bcl-xL with DNA encoding E7/heat shock protein 70, calreticulin/E7, or Sig/E7/LAMP-1 resulted in further enhancement of the E7-specific CD8+ T cell response for all three constructs. Of these strategies, mice vaccinated with Sig/E7/LAMP-1 DNA mixed with Bcl-xL DNA showed the greatest increase in E7-specific CD8+ T cells (~13-fold increase). This combination of strategies resulted in increased CD8+ T cell functional avidity, an increased E7-specific CD4+ Th1 cell response, enhanced tumor treatment ability, and stronger long-term tumor protection when compared with mice vaccinated without Bcl-xL DNA. Therefore, DNA vaccines that combine strategies to enhance intracellular Ag processing and prolong DC life have potential clinical implications for control of viral infection and neoplasia. The Journal of Immunology, 2003, 171: 2970–2976.

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4 Abbreviations used in this paper: DC, dendritic cell; HPV, human papillomavirus; HSP70, heat shock protein 70; CRT, calreticulin; LAMP-1, lysosome-associated membrane protein 1; CD4KO, CD4 knockout; mt, mutant.
Bcl-xL with various DNA constructs incorporating different intracellular targeting strategies with E7. We have found that these combinations enhance the E7-specific immune response in vaccinated mice to a greater degree than either antiapoptotic or intracellular targeting strategies alone. This combination strategy also results in greater enhancement of the CD8+ immune response in CD4 knockout (CD44KO) mice than vaccination with intracellular targeting strategies alone in wild-type mice. These results suggest that a vaccination strategy combining intracellular targeting with a strategy to prolong DC life may further enhance DNA vaccine potency and may also have significant future clinical implications.

Materials and Methods

Plasmid DNA constructs and DNA preparation

The generation of pcDNA3, pcDNA3-E7, pcDNA3-Sig/E7/LAMP-1 (7), pcDNA3-CRT/E7 (6), and pcDNA3-HSP70/E7 (5) has been described previously. pcSG5 plasmids encoding Bcl-xL or mt 7 (our mtBcl-xL) were generated as described previously (9). The DNA was amplified and purified as described previously (5).

Mice

Six- to 8-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, Maryland). Six- to 8-week old C57BL/6 CD4KO female mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DNA vaccination

DNA-coated gold particles were prepared according to a previously described protocol (5). DNA-coated gold particles were delivered to the shaved abdominal region of mice using a helium-driven gene gun (Bio-Rad, Hercules, CA) with a discharge pressure of 400 psi. C57BL/6 mice were immunized with 2 μg of pcDNA3 encoding E7, CRT/E7, E7/HSP70, or Sig/E7/LAMP-1 mixed with 2 μg of pcSG5 or pcSG5-Bcl-xL. The mice received a booster with the same dose 1 wk later.

Intracellular cytokine staining and flow cytometry analysis

Splenocytes were harvested from mice (five per group) 1 or 7 wk (for memory T cells) after the last vaccination. Before intracellular cytokine staining, 4 x 10^6 pooled splenocytes from each vaccination group were incubated overnight with 1 μg/ml E7 (RAHYNNIVTF) peptide containing an MHC class I epitope (aa 49–57) for detecting E7-specific CD8+ T cell precursors or 1 μg/ml E7 peptide containing an MHC class II epitope (aa 30–40) for detecting E7-specific CD4+ T cell precursors. Intracellular IL-4 and IFN-γ staining and flow cytometry analysis were performed as described previously (5). Analysis was performed on a FACSscan with CellQuest software (BD Biosciences, Mountain View, CA).

For the determination of the avidity of E7-specific CD8+ T cells, mice were vaccinated with pcDNA3-Sig/E7/LAMP-1 with pcSG5-no insert, with pcSG5-Bcl-xL, or with pcSG5-mtBcl-xL. Mice were boosted with the same vaccine 1 wk after the first vaccination. Splenocytes were collected and pooled 1 wk after the booster. The pooled splenocytes were incubated with different concentrations of E7 peptide (aa 49–57), 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} μg/ml overnight. The number of E7-specific IFN-γ-secreting CD8+ T cells was determined using intracellular cytokine staining and FACScan analysis as described above.

In vivo tumor treatment and long-term tumor protection

The HPV-16 E7-expressing murine tumor model, TC-1, has been described previously (10). In brief, HPV-16 E6, E7, and ras oncogene were used to transform primary C57BL/6 mice lung epithelial cells to generate TC-1. The mean number of pulmonary nodules in each mouse was evaluated by experimenters blinded to sample identity.

For the tumor treatment experiments, mice (five per group) were challenged with 1 x 10^4 TC-1 tumor cells/mouse in the tail vein to simulate hematogenous spread of tumors (7). Mice were treated with DNA 3 days after tumor challenge. Mice were monitored twice per week and sacrificed on day 42 after tumor challenge.

To study the subsets of lymphocytes that are important for the antitumor effects, a tumor protection experiment was performed, coupled with in vivo Ab depletion using a protocol similar to one previously described (11).

Briefly, mice were vaccinated, boosted 1 wk later, and challenged with 1 x 10^4 TC-1 tumor cells 1 wk after boosting. Ab depletion was initiated concurrently with tumor challenge and continued until sacrifice. mAb GK1.5 was used for CD4 depletion. mAb 2A3 was used for CD8 depletion. mAb PK136 was used for NK depletion. Mice were monitored twice per week and sacrificed on day 42 after tumor challenge.

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 Student's t test. In the tumor protection experiment, the principal outcome of interest was time to development of tumor. The event time distributions for different mice were compared by Kaplan and Meier and by log-rank analyses.

Results

Combined antiapoptotic and intracellular targeting strategies can further enhance E7-specific CD8+ T cell responses in vaccinated mice

To explore whether DNA encoding Bcl-xL is capable of enhancing DNA vaccines using the various intracellular targeting strategies, we coadministered Bcl-xL with E7 linked to HSP70, CRT, or LAMP-1. As shown in Fig. 1, A and B, coadministration of Bcl-xL with any of the three intracellular targeting strategies resulted in an increase in IFN-γ-secreting E7-specific CD8+ T cell precursors compared with coadministration with pcSG5 empty vector. CRT/E7 mixed with Bcl-xL produced the strongest response, but Sig/E7/LAMP-1 mixed with Bcl-xL displayed the greatest fold increase in the number of IFN-γ-secreting E7-specific CD8+ T cell precursors (at least a 10-fold increase). Our data indicate that coadministration with Bcl-xL can be used in combination with any of the intracellular targeting strategies to further enhance DNA vaccine potency, and that coadministration of Bcl-xL DNA with Sig/E7/LAMP-1 DNA displays the greatest fold increase of the E7-specific CD8+ T cell immune response.

Coadministration of pcDNA3-Sig/E7/LAMP-1 with pcSG5-Bcl-xL increases the avidity of the E7-specific CD8+ T lymphocyte immune response

Prior studies have shown that high-avidity CTL provide better protection against viral infection (12) and tumor challenge (13) than low-avidity CTL. In addition, duration of DC and T cell interaction has been implicated to be important for the generation of high-avidity T cells (14). Thus, we performed a functional avidity assay to determine the avidity of E7-specific CD8+ T cells generated by vaccination with Sig/E7/LAMP-1 mixed with Bcl-xL, mtBcl-xL, or empty vector. We defined the number of IFN-γ-secreting CD8+ T cells stimulated by 1 μg/ml E7 peptide (aa 49–57) as a maximum response and compared the functional avidity of T cells from mice vaccinated with Bcl-xL or empty vector at 50% of the maximum. We found that the concentration of E7 peptide required to achieve 50% of the maximum IFN-γ CD8+ T cell response was ~4 x 10^{-5} μg/ml for mice vaccinated with Sig/E7/LAMP-1 with Bcl-xL, and ~3 x 10^{-3} μg/ml for mice vaccinated with Sig/E7/LAMP-1 mixed with empty vector or mtBcl-xL (Fig. 2). Therefore, coadministration of Sig/E7/LAMP-1 with Bcl-xL generates higher-avidity E7-specific CD8+ T cells than coadministration of Sig/E7/LAMP-1 with empty vector or mtBcl-xL in vaccinated mice. Furthermore, because the functional avidity of E7-specific CD8+ T cells elicited by vaccination with mtBcl-xL was nearly identical with that elicited by vaccination with empty vector, it is likely that
the antiapoptotic function of Bcl-xL was responsible for the observed increase in functional avidity.

Vaccination with pcDNA3-Sig/E7/LAMP-1 mixed with pSG5-Bcl-xL generated an enhanced Th1 and a reduced Th2 T cell response in mice vaccinated with pcDNA3-Sig/E7/LAMP-1 mixed with pSG5 or pSG5-Bcl-xL. Mice were immunized with pcDNA3-Sig/E7/LAMP-1 mixed with pSG5-Bcl-xL, pSG5-mtBcl-xL, or pSG5. Splenocytes were collected 1 wk after vaccination and incubated with different concentrations of E7 peptide (aa 49–57) for 20 h. pcDNA3 mixed with pSG5 encoding Bcl-xL was used as a negative control. Data were acquired for comparison of concentrations of peptide needed to generate 50% of maximum CD8+ T cell response in mice vaccinated with DNA encoding Bcl-xL, mtBcl-xL, or empty vector.

10^5 splenocytes) and fewer E7-specific Th2 CD4+ lymphocytes (43.4 ± 3.8 vs 65.2 ± 6.4/3 × 10^5 splenocytes) than vaccination with Sig/E7/LAMP-1 mixed with empty vector. These data suggest that coadministration with DNA encoding Bcl-xL may contribute to an enhanced E7-specific CD4+ Th1 cell response and a reduced E7-specific CD4+ Th2 cell response.

Vaccination with pcDNA3-Sig/E7/LAMP-1 mixed with pSG5-Bcl-xL results in a stronger E7-specific CD8+ T cell response in CD4KO mice than vaccination with pcDNA3-Sig/E7/LAMP-1 mixed with pSG5 in wild-type mice. These data suggest that coadministration of Bcl-xL DNA with pcDNA3-Sig/E7/LAMP-1 mixed with pSG5-Bcl-xL was used as a negative control. Data were acquired for comparison of concentrations of peptide needed to generate 50% of maximum CD8+ T cell response in mice vaccinated with DNA encoding Bcl-xL, mtBcl-xL, or empty vector.

Although there was a considerable decrease in the number of E7-specific CD8+ T cells generated in CD4KO mice compared with the number of E7-specific CD8+ T cells generated in wild-type mice vaccinated with the same regimen, our data demonstrated that the coadministration of Bcl-xL DNA with Sig/E7/LAMP-1 DNA in CD4KO mice was able to generate ~2-fold more E7-specific CD8+ T cells than coadministration of Sig/E7/LAMP-1 DNA with pSG5 in wild-type mice. These data suggest
that a DNA vaccine combining an antiapoptotic strategy with an intracellular targeting strategy may be able to generate better CD8\textsuperscript{+} T cell-mediated immune responses in a CD4-depleted host than those generated by a DNA vaccine using only an intracellular targeting strategy in an immunocompetent host.

Coadministration of pcDNA3-Sig/E7/LAMP-1 with pSG5-Bcl-x\textsubscript{L} generates better antitumor treatment effects than coadministration of pcDNA3-Sig/E7/LAMP-1 with pSG5.

A factor vital to the success of an HPV therapeutic vaccine is the ability to treat patients with HPV infection or HPV-related cancers. To determine the therapeutic effectiveness of our strategy, we tested the ability of Sig/E7/LAMP-1 mixed with Bcl-x\textsubscript{L} or empty vector to treat TC-1, an E7-expressing tumor cell line, using a previously described hematogenous spread model (7). As shown in Fig. 5A, mice treated with Sig/E7/LAMP-1 mixed with Bcl-x\textsubscript{L} developed a significantly lower number of tumor nodules than mice treated with Sig/E7/LAMP-1 mixed with empty vector, the control, or the naive mice. This indicates that coadministration with Bcl-x\textsubscript{L} generates better tumor treatment ability than coadministration with empty vector in a mouse model.

We also performed a tumor protection experiment with Ab depletion to determine the subset of T lymphocytes responsible for the antitumor response. Mice were vaccinated with Sig/E7/LAMP-1 mixed with Bcl-x\textsubscript{L} and were subsequently challenged with TC-1. We initiated depletion of subsets of lymphocytes concurrent with tumor challenge. In Fig. 5B, we see that mice depleted of CD8\textsuperscript{+} T cells display nearly the same degree of tumor growth as naive mice, and mice depleted of CD4\textsuperscript{+} T cells display slightly increased tumor growth compared with nondepleted mice. Mice depleted of NK cells did not generate a significantly different number of tumor nodules compared with mice with no depletion. These data suggest that CD8\textsuperscript{+} T cells are essential for the antitumor effect, and that CD4\textsuperscript{+} T cells may also contribute to the observed antitumor effect, although not as strongly as CD8\textsuperscript{+} T cells.

**Immunization with pcDNA3-Sig/E7/LAMP-1 mixed with pSG5-Bcl-x\textsubscript{L} generates significantly enhanced long-term immune response and antitumor protection in vaccinated mice**

A successful vaccine must be capable of generating an effective long-term protective immune response. To assess the ability of our vaccination strategy to generate long-term E7-specific CD8\textsuperscript{+} T cell immune responses and protective antitumor effects against TC-1, we compared vaccination with Sig/E7/LAMP-1 mixed with Bcl-x\textsubscript{L} to vaccination with Sig/E7/LAMP-1 mixed with empty vector. Intracellular cytokine staining followed by flow cytometry analysis to determine E7-specific CD8\textsuperscript{+} T cells was performed 1 and 7 wk after immunization. As shown in Fig. 6A, Sig/E7/
LAMP-1 mixed with Bcl-xL generated an ∼7-fold higher E7-specific IFN-γ CD8⁺ T lymphocyte response at 7 wk postimmunization than Sig/E7/LAMP-1 mixed with empty vector. Thus, coadministration of the Sig/E7/LAMP-1 vaccine construct with Bcl-xL generates a stronger long-term immune response than coadministration of Sig/E7/LAMP-1 with empty vector.

To determine the long-term tumor protection ability of our vaccination strategy, we challenged vaccinated mice with 1 × 10⁴ TC-1 tumor cells 7 wk after the final immunization. As shown in Fig. 6B, mice vaccinated with Sig/E7/LAMP-1 mixed with Bcl-xL exhibited no pulmonary tumor nodules, whereas mice vaccinated with Sig/E7/LAMP-1 mixed with empty vector exhibited 1.6 ± 2.3 tumor nodules 42 days after TC-1 challenge. Therefore, coadministration of Sig/E7/LAMP-1 with Bcl-xL completely prevented tumor nodule formation 7 wk after vaccination. Taken together, our data indicate that a DNA vaccine combining an intracellular targeting strategy with a strategy that prolongs DC life generates a strong long-term E7-specific CD8⁺ T cell immune response and excellent long-term protective antitumor effects.

**Discussion**

Our results support our hypothesis that a DNA vaccination strategy combining intracellular targeting with a strategy to prolong DC life is capable of enhancing Ag-specific immune responses to a greater degree than a DNA vaccination strategy using intracellular targeting strategies alone. We found this combination strategy to be effective with three vaccine strategies using intracellular targeting: HSP70/E7 (5), CRT/E7 (6), and Sig/E7/LAMP-1 (7), resulting in strong E7-specific CD8⁺ T cell responses as well as long-term tumor protection in vaccinated mice. These results may be attributed to prolonged DC life via inhibition of apoptosis by Bcl-xL, resulting in an increased quantity of longer-lived DCs in the draining lymph nodes (8), as well as to enhanced processing of Ag as a result of expression of CRT, LAMP-1, or HSP70 linked to E7.
resulting in increased MHC:peptide presentation to T lymphocytes (5–7). Thus, our data indicate that it is possible to modify DCs simultaneously via two different mechanisms to further enhance DNA vaccine potency.

Of the targeting strategies tested, we observed that Sig/E7/LAMP-1 displayed the greatest enhancement of the E7-specific CD8⁺ T cell immune response when coadministered with Bcl-xL DNA (Fig. 1B). A possible explanation for this may be related to an increase in CD4 Th cells. The Sig/E7/LAMP-1 construct is the only one that targets Ag to the MHC class II processing pathway (15), activating Ag-specific CD4⁺ T cells more effectively than other constructs. Furthermore, in our study using CD4KO mice, we observed a significantly lower number of E7-specific CD8⁺ T cells in CD4KO mice than in wild-type mice. Thus, CD4⁺ T cells are important for the observed enhancement of the E7-specific CD8⁺ T cell response to vaccination with Sig/E7/LAMP-1.

Although CD4KO mice exhibited an overall reduced CD8⁺ T cell immune response when compared with wild-type mice vaccinated with the same regimen (Fig. 4, A and B), the coadministration of Bcl-xL DNA with Sig/E7/LAMP-1 DNA can enhance the E7-specific CD8⁺ T cell immune response in CD4KO mice when compared with CD4KO mice vaccinated with Sig/E7/LAMP-1 DNA with empty vector. Moreover, we showed that the coadministration of Bcl-xL DNA with Sig/E7/LAMP-1 DNA in CD4KO mice is able to generate more E7-specific CD8⁺ T cells than coadministration of Sig/E7/LAMP-1 DNA with pSG5 in wild-type mice. These results suggest that a DNA vaccination strategy combining intracellular targeting with antiapoptotic proteins is capable of enhancing the E7-specific CD8⁺ T cell response in individuals with a significant reduction in CD4⁺ T cell levels. This depletion is a likely cause of the increased severity of HPV infection and associated lesions in HIV-positive subjects (for review, see Refs. 16 and 17). Thus, this combination strategy might prove useful for the control of HPV infection and HPV-associated lesions in CD4-depleted human subjects.

Coadministration of DNA encoding Bcl-xL resulted in the generation of higher-avidity E7-specific CD8⁺ T cells (Fig. 2). The antiapoptotic function of Bcl-xL was likely essential for this effect, because coadministration of mtBcl-xL DNA elicited E7-specific CD8⁺ T cells with a functional avidity nearly identical with those elicited by coadministration with empty vector. The ability of Bcl-xL to extend DC life span might lead to prolonged DC and T cell interaction in draining lymph nodes, and the duration of DC and T cell interaction has been implicated to be important for generating high-avidity Ag-specific T cells (14). Thus, prolonged DC life due to coadministration with Bcl-xL DNA may contribute directly to increased E7-specific CD8⁺ T cell avidity.

Ag-specific CD8⁺ T cells with high avidity are known to result in better qualitative Ag-specific immune protection and antitumor effects in vaccinated mice than low-avidity CD8⁺ T cells (18). High-avidity CD8⁺ T cells enhance protection by recognizing low Ag densities and killing infected cells sooner than low-avidity CD8⁺ T cells (12). Prior studies have shown that higher-avidity CTLs may produce a stronger antitumor effect in vaccinated mice (13, 19). Selective expansion of high-avidity Ag-specific T cells and memory T cell precursors may in turn enhance antitumor immunity. Thus, higher CD8⁺ T cell avidity may contribute to the strong antitumor effect that we observed in mice vaccinated with Sig/E7/LAMP-1 DNA mixed with Bcl-xL DNA.

As HPV vaccine research has moved into the clinical arena, it has become relevant to discuss the clinical implications of newly developed HPV vaccines in anticipation of potential future clinical application. Safety is a major consideration for the clinical application of any new vaccination strategy and is especially important in this case, because increased Bcl-xL expression could be considered potentially hazardous to human subjects. This is due to the concern that the antiapoptotic effects of Bcl-xL could interfere with the normal regulation of DC function, which could result in autoimmunity. However, no evidence of autoimmunity was observed in any of the mice used in the study, based on histology of immunized mice (data not shown).

Oncoogenesis is another relevant concern, because Bcl-xL has been implicated in oncogenic transformation of healthy cells (20). One strategy to improve safety is to transfect DCs with DNA encoding factors that may indirectly enhance DC survival with a reduced concern for oncoGenesis, such as TNF-related activation-induced cytokine (21), CD40 ligand (22), IL-12 (23), and IL-15 (24), and serine protease inhibitor 6 (25). Among these molecules, CD40 ligand (26), IL-12 (27), and IL-15 (28) have been tested to enhance DNA vaccine potency. It would be interesting to evaluate the role that apoptotic inhibition plays in enhancing the Ag-specific immune responses mediated by these molecules in DNA vaccines.

In summary, we have shown that coadministration of DNA encoding an antiapoptotic protein with DNA encoding intracellular targeting strategies can further enhance the potency of DNA vaccines against an HPV-16 E7-expressing tumor line. This vaccine strategy led to an increased CD8⁺ T cell response, higher CD8⁺ T cell functional avidity, stronger immune memory, and enhanced antitumor effects in vaccinated mice when compared with vaccines using only intracellular targeting strategies. In addition, a DNA vaccine combining an antiapoptotic strategy with an intracellular targeting strategy may be able to generate better CD8⁺ T cell-mediated immune responses in a CD4-depleted host than those generated by DNA vaccination using only the intracellular targeting strategy in an immunocompetent host. These results encourage the application of antiapoptotic proteins in combination with other vaccine enhancement strategies for future development of therapeutically DNA vaccines to combat HPV infection and cervical cancer in the clinical realm.

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