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Design of Immunogenic and Effective Multi-Epitope DNA Vaccines for Melanoma

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Abstract

Plasmid DNA vaccination is an attractive way to elicit T cell responses against infectious agents and tumor cells. DNA constructs can be designed to contain multiple T cell epitopes to generate a diverse immune response to incorporate numerous antigens and to reduce limitations due to MHC restriction into a single entity. We have prepared cDNA plasmid constructs containing several mouse T cell epitopes connected by either furin-sensitive or resistant linkers, and studied the effects of a cationic cell penetrating sequence from HIV-tat. Significant CD8 T cell responses were obtained with multi-epitope DNA vaccines followed by *in vivo* electroporation regardless of the type of linker used and whether the construct had the HIV-tat sequence. The magnitude of immune responses was very similar to all CD8 T cell epitopes contained within each vaccine construct indicating the absence of immunodominance. Incorporating a T helper epitope into the constructs increased the T cell responses. Prophylactic and therapeutic anti-tumor responses against B16 melanoma were obtained using a construct containing epitopes from melanosomal proteins, indicating that this vaccination was successful in generating responses to self-antigens that potentially may be subjected to immune tolerance. These findings are useful for designing DNA vaccines for a multitude of diseases where T lymphocytes play a protective or therapeutic role.

Keywords

Multi-epitope; DNA vaccine; Melanoma; Tumor immunity

Introduction

There is little doubt that CD8 and CD4 T cells can play a determinant role in the prevention, control and resolution of many types of infections and malignant disorders. Thus, the design of vaccines capable of inducing effective antigen-specific T cell responses constitutes a top area research area. The definition of the minimal antigenic units (epitopes) recognized by T cells as small peptides derived from protein antigens that are presented by MHC molecules has resulted in the development of epitope-based vaccines [1-3]. Our group has worked for a

long time in the use of synthetic peptides representing defined T cell epitopes in mouse models as vaccine components to induce immune responses capable of displaying anti-tumor effects [4-8]. Being cognizant that vaccines targeting a single MHC-restricted T cell epitope will not offer broad population coverage due to the limitations imposed by MHC restriction, and may become ineffective by the emergence of antigen escape variants, we have devoted some efforts to the development of multi-epitope vaccines. Initially utilizing a synthetic peptide approach, we described the development of Trojan Antigens, which contained several CD8 and one CD4 T cell epitopes joined via furin-sensitive linkers [9]. In addition, the Trojan Antigens contained a highly cationic cell penetrating amino acid sequence (usually from HIV-tat), which allowed the construct to enter the MHC-I processing pathway in a TAP-independent manner [10]. Although the peptide-based Trojan Antigens induced potent CD8 and CD4 T cell responses, the synthesis and purification of these constructs was challenging and costly due to their large size (~40 amino acids), making it difficult to take this approach into the clinic. In view of the above-mentioned limitations, we now explore the use of cDNA-based vaccination using multi-epitope plasmid constructs in order to induce simultaneous T cell responses in a simpler, practical and more cost effective manner. We present here results indicating that intramuscular DNA injections followed by *in vivo* electroporation (IVE) with multi-epitope plasmids induced strong and effective anti-tumor CD8 and CD4 T cell responses. Contrary to our findings with peptide Trojan Antigens, the multi-epitope DNA vaccines (MEDVs) did not require cationic cell-penetrating sequences or furin-sensitive linkers, suggesting a different mode of action. These results generate a new opportunity to the development of multi-epitope T cell vaccines for the treatment/prevention of malignant disorders and infectious diseases.

Materials and Methods

Mice and cell lines

Six- to eight-week-old female C57BL/6 and (BALB/c X B6)F1 mice were obtained through the National Cancer Institute/Charles River program (Wilmington, MA). Mice were allowed to acclimate to our animal facility for 1 wk before beginning experiments in a pathogen-free environment. All animal care and experiments were conducted according to committee guidelines of our facility. B16-F10 melanoma (H-2^b), EL4 thymoma (H-2^b), P815 mastocytoma (H-2^d), and B cell lymphoma LB27.4, which express mouse MHC class II (I-Ab) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). B16-KbLo, a variant of B16-F10 was established in our laboratory by transfecting H-2Kb into a B16 subline that lost expression of this MHC-I [11]. All cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Grand Island, NY).

Peptides and antibodies

The peptide sequences used in this study are listed in Table 1. All synthetic peptides were purchased from A&A Labs (San Diego, CA) and the purity (>95%) and identity of the peptides was determined by analytic high-performance liquid chromatography and mass spectrometry analysis. Anti-mouse programmed death Peptides and antibodies ligand-1 mAb

(PD-L1; 10F.9G2) was purchased from BioXCell (West Lebanon, NH). All fluorescent antibodies for flow cytometry were purchased from eBioscience (San Diego, CA).

Construction of multi-epitope DNA vaccines

The template DNA sequences coding several T cell epitopes were designed on the basis of mouse codon usage and were obtained from GenScript (Piscataway, NJ). The plasmids for DNA vaccination were constructed by PCR-based cloning from the DNA templates into the pCI expression vector (Promega, Madison, WI). The constructs contained combinations of various CD8 T cell epitopes (Ova₂₅₇, SIY, LCMV₃₃, PyCSP₂₈₀, LLO₉₁, Trp1₁₈₀ and hgp100₂₅) linked with either furin-resistant (VRVV) or -sensitive (RVKR) spacers. In addition, some of the constructs contained the cell penetrating sequence, HIV-tat₄₉ and a T helper epitope (Ova₃₂₃). The schematics of the constructs are presented in Fig. 1 and the sequences of the epitopes in Table 1. Control cDNA vaccines were a pcDNA3 vector coding for chicken ovalbumin (pOva) or an empty pCI vector backbone (pCI). All constructs were confirmed by DNA sequencing and DNA was purified for vaccination using EndoFree Plasmid Mega Kits (Qiagen, Valencia, CA).

Immunizations

Mice were vaccinated with a total of 100 µg of plasmid DNA resuspended in normal saline, injected into both quadriceps femoris muscles. Immunization was followed immediately by electroporation of the injected area (95 V, 4 X 65 ms pulses with repoling) using an Electro Square Porator device (TX830; BTX, San Diego, CA). In some experiments, the mice were given identical booster immunization two weeks later. Immune responses were typically measured 8 days after the last immunization.

Evaluation of immune responses

Numbers of antigen-specific T cells were measured with EliSpot interferon γ (IFN γ)-release assays, performed as described [4] using purified CD8 or CD4 T cells from spleens (Miltenyi Biotec; Auburn, CA). Briefly, responder CD8 T cells were incubated together with peptide-pulsed or unpulsed EL4 cells or with B16 melanoma cells at various responder to stimulator cell ratios in EliSpot plates. Responder CD4 T cells were incubated with peptide-pulsed, or unpulsed LB274 cells, in a similar manner as described for the CD8 assays. Cultures were incubated at 37 °C for 20 h and spots were developed as described by the EliSpot kit manufacturer (Mabtech, Inc., Mariemont, OH). Spot counting was done with an AID EliSpot Reader System (Autoimmun Diagnostika GmbH, Strassberg, Germany). For intracellular staining, splenocytes from individual mice were incubated with 5 µg/ml equivalent for 6 h at 37°C and subsequently stained for intracellular IFN γ following the directions provided by vendor (BD Bioscience, San Diego, CA) using FITC-conjugated MHC class II, PerCP Cy5.5-conjugated CD8a, and PE-conjugated anti-IFN γ Abs (eBioscience; San Diego, CA).

Antitumor effects

In the prophylactic setting, previously immunized mice were challenged with 1×10^5 B16-F10 cells injected through the tail vein 7 d after the booster vaccination. To evaluate the

therapeutic effects of vaccination, mice first received an intravenous inoculation of 1×10^5 B16-F10 cells, and 3 and 18 days later, the mice received their cDNA immunizations. Antitumor effects were evaluated by the enumeration of lung tumor nodules and measurement of lung weights ~4 wk after tumor injections (when the mice in the non-vaccinated control group became ill). In some experiments, the antitumor effects were evaluated by survival measurements (time to euthanasia). In addition, a subcutaneous tumor therapeutic model was used, where mice first received a subcutaneous B16 tumor inoculation (3×10^5 or 1×10^5 cells, as indicated), followed 4 days later by the first immunization. 12 to 14 d later the mice were given an identical booster. In some experiments, 200 μ g of anti-PD-L1 mAb was administered i.p. on days 3, 5, 7, and 9 after each DNA vaccination. Antitumor effects were evaluated by the measurements of tumor areas. Tumor growth was monitored every 2 to 4 d in individually tagged mice by measuring two opposing diameters with a set of callipers. Results are presented as the mean tumor size (area in mm^2) $\pm$ SD for every treatment group at various time points until the termination of the experiment.

Statistical analyses

Statistical significance to assess the numbers of antigen-specific CD8 T cells (EliSpot) and absolute number of lung tumor nodules were determined by unpaired Student *t* tests. Tumor sizes between 2 populations throughout time were analyzed for significance using 2-way ANOVA. Statistical significance for survival was established by Kaplan-Mayer curves using log-rank tests. All analyses and graphics were done using Prism 5.01 software (GraphPad).

Results

To assess the immunogenicity of MEDVs, and determine if these vaccines functioned in a similar manner as our previously described Trojan Antigens [9], we prepared several cDNA plasmidconstructs containing various defined mouse CD8 T cell epitopes, with and without a CD4 T helper epitope, connected with either furin-sensitive (RVKR) or -resistant (VRVV) linkers, and possessing or not a cell penetrating cationic sequence from HIV-tat (Fig. 1). We first evaluated the immunogenicity of 4 constructs prepared with the LCMV₃₃ (H-2D^b-restricted), SIY (H-2K^b-restricted) and Ova₂₅₇ (H-2K^b-restricted) CD8 T cell epitopes joined to each other with either furin-sensitive or furin-resistant linkers. C57BL/6 mice (B6) received a single i.m. DNA injection immediately followed by electroporation as described in “Materials and Methods” and 12 days later the numbers of functional antigen-specific CD8 and CD4 T cells in spleens were enumerated using EliSpot assays. The results in Fig. 2a indicate that all 4 constructs induced substantial CD8 T cell responses to all 3 epitopes, regardless of whether or not they contained HIV-tat sequence, furin-sensitive or -resistant linkers. Notably, the magnitude of the immune responses was very similar amongst the 3 epitopes indicating the absence of an overt immune dominance, which could occur due to the position (order) that an epitope occupies within a construct that may affect its production during antigen processing. Nevertheless, it is possible that the immunogenic potency of the various constructs could differ amongst themselves when used at lower doses.

Next, we assessed whether the inclusion of an MHC-II-binding peptide sequence (OVA₃₂₃) into an MEDV construct (pME-5, Fig. 1) would result in the induction of CD4 T cell responses. In addition, we determined whether the magnitude of the CD8 T cell responses would be increased due to the simultaneous stimulation of CD4 T helper cells. The results in Fig. 2b show that the pME-5 MEDV construct containing the OVA CD4 T cell epitope at the carboxyl terminus end was efficient in generating antigen-specific CD4 T cell responses. In addition, a significant increase (40-60%) in the magnitude of the CD8 T cell responses was observed with OVA₃₂₃ containing MEDV construct, as compared to the pME-3 construct, which only contained the 3 CD8 epitopes. For comparison, we tested the immunogenicity of a cDNA construct encoding the entire ovalbumin protein (pOva). Notably, the immune responses to both the CD8 and CD4 Ova T cell epitopes generated by the pME-5 MEDV, expressing the T helper Ova₃₂₃ sequence were approximately 25% higher than those generated with the pOva construct.

The results presented so far indicate that plasmids containing up to 3 CD8 T cell epitopes (and a CD4 epitope) are efficient in generating immune responses of similar magnitude to all the components. Next, we determined whether the inclusion of 2 additional CD8 T cell epitopes, in this case restricted by a different MHC-I allele (H-2^d) would continue to elicit responses of equal intensity to all the epitopes. As shown in Fig. 2c, (BALB/c X B6)F1 mice immunized with the pMEMEDV, containing 5 CD8 T cell epitopes, 3 restricted by H-2^b and 2 by H-2^d elicited T cell responses of approximately similar magnitude to all the 5 epitopes. In addition, CD4 T cell responses to the Ova₃₂₃ epitope were maintained at similar levels as those previously observed with the pME-5 construct (data not shown).

To evaluate whether the CD8 T cell responses invoked by MEDVs exhibit any activity in a disease setting, we prepared a cDNA construct containing 3 CD8 T cell epitopes derived from melanosomal proteins (Trp1, Trp2 and gp100) expressed by the transplantable B16 melanoma cell line (pME-7, Fig. 1). Since these antigens are also expressed by normal melanocytes, an effective vaccination must be able to overcome potential immune tolerance. Immunization of B6 mice following the same protocol as with the previous experiments (single *i.m.* immunization followed by electroporation) resulted in CD8 T cell responses that were somewhat lower (~2-fold) as those generated using MEDVs composed of foreign antigens (data not shown). In view of this, we administered two sequential immunizations (prime-boost, 2-weeks apart). Substantial immune responses were clearly evident to all 3 melanoma epitopes as measured by intracellular cytokine staining (Fig. 3a). Again, the magnitude of the responses amongst the 3 epitopes was of a similar range. These results indicate that the melanosomal MEDV was effective in overcoming any potential immune tolerance to antigens that are expressed by normal tissues. However, it would be expected that CD8 T cells reactive with the melanosomal antigens would be recruited from low to intermediate avidity precursors that have escaped central and peripheral tolerance and will only respond to high levels of antigen and may not be effective in recognizing the melanoma tumor cells. Nonetheless, as demonstrated in Fig. 3b, CD8 T cells from spleens of vaccinated mice were effective in recognizing not only the peptide-pulsed APCs (EL4 cells), but also B16 melanoma cells.

Having shown that the melanosomal MEDV construct was immunogenic, capable of inducing similar levels of immune responses as those observed with constructs containing T cell epitopes from foreign antigens, we proceeded to study the *in vivo* anti-tumor effects of this vaccination strategy. In the prophylactic setting (tumor challenge after vaccination), the MEDV was completely effective in preventing the establishment of lung tumor colonies, when B16 cells were injected intravenously 7 days after the second immunization (Fig. 4a). In a more realistic tumor model, therapeutic vaccination 3 days post-tumor inoculation, the MEDV showed a significant benefit as compared to the control vaccine, reducing the number of tumors by approximately 50% (Fig. 4b). In a survival experiment, therapeutic vaccination increased medium survival time by approximately 12 days in this very aggressive tumor model where all control mice perished by day 28 post-tumor inoculation (Fig. 4c).

We have recently reported that IFN γ has a negative effect in the efficacy of peptide vaccination in the B16 melanoma model [11]. We observed that CD8 T cells from peptide-vaccinated mice poorly recognized B16 cells treated with IFN γ , although the levels of MHC-I on the tumor cells were enhanced by the cytokine. The results from these studies suggested that B16 cells exposed to IFN γ evade CD8 T cells via 2 separate mechanisms: 1) enhanced expression of non-cognate (irrelevant) MHC-I molecules, which could sequester CD8 co-receptors outside of the immune synapse, limiting T cell activation; and 2) induction of the expression of PD-L1 (also known as B7-H1) inhibitory molecules. Thus, when a B16 tumor subline (B16-Kb^{Lo}) that does not overexpress MHC-I when treated with IFN γ was used in combination with PD1 blockade, peptide vaccination was able to completely eradicate large established subcutaneous tumors [11]. These observations suggest that limiting the negative effects of IFN may increase the efficacy of MEDVs against B16 melanoma. We first determined whether IFN γ -treatment of B16 cells would decrease the recognition by CD8 T cells from the melanosomal MEDV immunized mice. As shown in Fig. 5a, the IFN γ -treated B16 cells were recognized less effectively (~66%) as compared to the untreated cells. In view of this, we evaluated the effectiveness of the melanosomal MEDV against the B16-Kb^{Lo} cell line in a therapeutic subcutaneous model both in the presence and absence of PD-1 blockade. The results presented in Fig. 5b illustrate that the MEDV administered by itself had a minimal, but significant therapeutic effect in limiting B16-Kb^{Lo} tumor progression. On the other hand, MEDV administered in combination with anti-PD-L1 antibodies had a dramatic therapeutic effect. In a similar therapeutic vaccination experiment, but using conventional B16 cells (which express high levels of MHC-I after IFN γ treatment) showed that MEDV alone had no significant effect on tumor progression, but that vaccination with PD-1 blockade demonstrated a small, but significant effect (Fig. 5c). These results indicate that as with peptide vaccination, IFN γ exerts a negative effect in the therapeutic efficacy of MEDCs against established tumors in the B16 melanoma system. However, significant therapeutic effects can be obtained in circumstances when these negative effects are overcome.

Discussion

In this report, we have presented evidence that DNA vaccination with constructs containing multiple epitopes results in the induction of strong CD8 and CD4 T cell responses to all the

components of the vaccines. It remains to be determined whether this approach will function with constructs containing more than 5 CD8 T cell epitopes and if an upper limit in the number of epitopes exists before antigenic competition becomes evident. Nevertheless, this approach allows the simultaneous induction of T cell responses to numerous epitopes using a single entity, which is essential to offer broad population coverage, due to extensive polymorphic nature of the MHC. In addition, by targeting more than one T cell epitope, the probability of the emergence of escape variants should hopefully be reduced.

Our original intent was to design MEDVs to produce immunogens that would function in a similar manner as the peptide-based multi-epitope Trojan Antigens that we previously studied, which generate strong T cell responses [9]. The Trojan Antigens relied on a cell penetrating cationic sequence to enter the antigen processing pathway in a TAP-independent manner and required furin-sensitive linkers to produce individual MHC-I binding peptides [9,10]. We were somewhat surprised by the results that the presence of the cationic HIV-tat sequence and furin-sensitive linkers was not required for the MEDVs to exhibit immunogenicity (Fig.2a). Moreover, the presence of HIV-tat and furin-sensitive linkers did not significantly increase the level of CD8 T cell responses. These results suggest that the mode of action of immunogenicity of the MEDVs differs substantially from that of the peptidic Trojan Antigens. Intramuscular injection of plasmid DNA followed by electroporation results in high levels of the expression of the gene product [12,13]. However, our constructs do not include a leader sequence, so it would not be expected that the transduced muscle cells would secrete the translation products. Most likely, as with other DNA-based vaccines that are administered intramuscularly, many of the muscle cells may be damaged by the subsequent electroporation intended to increase DNA uptake, and later die providing cell debris rich in antigen and danger/adjuvant signals [14-16], leading to the activation of professional APCs and initiating the T cell immune response. There are other reports of DNA-based vaccination approaches using constructs containing multiple T cell epitopes [17-21]. In a report by Livingston, *et al.*, [20] DNA constructs containing up to 20 CD8 T cell epitopes described that the magnitude of the T cell responses varied enormously (in some cases more than 90%) from epitope to epitope and ascribed this to differences in antigen processing of the cDNA products. This group also reported that a basic (K, R), amide (Q, N) or small (C, G, A, T, S) residue in linkers flanking the C-terminal end of the epitope improved the immunogenicity. Our studies showed that the furin-sensitive and resistant linkers, which place an R or a V (considered bulky) residue, respectively in this position worked equally well in generating strong immune responses. At this time we do not know whether the 2 linkers we used are important or not for the generation of the individual epitopes through antigen processing, and whether other specialized linker types such as AAY [21], or simply the blunt joining of the epitopes to each other will suffice. The disparity between our results and those of Livingston could be due to one or more differences of the systems in addition to the type of linkers used, such as numbers of epitopes per construct, presence of leader sequence (in their constructs), lack of electroporation (in their vaccine).

These results demonstrate that little, if any antigenic competition was observed with the MEDV constructs allowing the induction of T cell responses to up to 5 distinct CD8 T cell

epitopes, without any evidence of immunodominance, since all the responses were basically of equivalent intensity. It has been reported that naïve T cell precursor frequencies and peptide MHC-I binding affinities dictate to a large extent the immunodominance hierarchy of T cell responses to antigens or pathogens containing multiple epitopes [22]. Other factors involved in antigen processing (proteasomal cleavage, transport to the ER), which determine the amount of peptide epitopes produced may also affect the degree of immunodominance [23]. It is likely that all 5 foreign epitopes in pME-6 and the 3 melanosomal epitopes in pME-7 bind to their respective MHC-I molecules with various degrees of affinity and that the naïve T cell precursor frequencies will vary substantially between these epitopes. Thus, we find it quite remarkable that the MEDVs generated T cell responses of similar magnitude to all epitopes within each construct. We believe that the plasmid constructs used in our experiments are effective in producing all the peptide epitopes within their sequence in sufficient amounts, allowing sustained antigen presentation resulting in successful T cell activation and clonal expansion, regardless of some differences in precursor frequency and MHC-I binding affinities. Nevertheless, it is certain that a specific threshold in the MHC binding strength of a peptide must be met in order for the epitope within a construct to generate a T cell response. Regardless of the exact mode of action, the MEDVs described here proved to be effective in generating T cell responses in mice. It remains to be determined whether this approach will function in primates, where DNA vaccination in general has been more challenging.

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References

1. Bijker MS, Melief CJ, Offringa R, van der Burg SH. Design and development of synthetic peptide vaccines: past, present and future. *Expert Rev Vaccines*. 2007; 6(4):591–603. [PubMed: 17669012]
2. Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. *Nat Rev Drug Discov*. 2007; 6(5):404–414. [PubMed: 17473845]
3. De Groot AS. Immunomics: discovering new targets for vaccines and therapeutics. *Drug Discov Today*. 2006; 11(5-6):203–209. [PubMed: 16580597]
4. Cho HI, Celis E. Optimized peptide vaccines eliciting extensive CD8 T-cell responses with therapeutic antitumor effects. *Cancer Res*. 2009; 69(23):9012–9019. [PubMed: 19903852]
5. Assudani D, Cho HI, DeVito N, Bradley N, Celis E. In vivo expansion, persistence, and function of peptide vaccine-induced CD8 T cells occur independently of CD4 T cells. *Cancer Res*. 2008; 68(23):9892–9899. [PubMed: 19047170]
6. Nava-Parada P, Forni G, Knutson KL, Pease LR, Celis E. Peptide vaccine given with a Toll-like receptor agonist is effective for the treatment and prevention of spontaneous breast tumors. *Cancer Res*. 2007; 67(3):1326–1334. [PubMed: 17283170]
7. Davila E, Kennedy R, Celis E. Generation of antitumor immunity by cytotoxic T lymphocyte epitope peptide vaccination, CpG-oligodeoxynucleotide adjuvant, and CTLA-4 blockade. *Cancer Res*. 2003; 63(12):3281–3288. [PubMed: 12810660]
8. Itoh T, Celis E. Transcutaneous immunization with cytotoxic T-cell peptide epitopes provides effective antitumor immunity in mice. *J Immunother*. 2005; 28(5):430–437. [PubMed: 16113599]
9. Lu J, Higashimoto Y, Appella E, Celis E. Multiepitope Trojan antigen peptide vaccines for the induction of antitumor CTL and Th immune responses. *J Immuno*. 2004; 172(7):4575–4582.

10. Lu J, Wettstein PJ, Higashimoto Y, Appella E, Celis E. TAP-independent presentation of CTL epitopes by Trojan antigens. *J Immuno*. 2001; 166(12):7063–7071.
11. Cho HI, Lee YR, Celis E. Interferon gamma limits the effectiveness of melanoma peptide vaccines. *Blood*. 2011; 117:135–144. [PubMed: 20889921]
12. Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, Delaere P, Branellec D, Schwartz B, Scherman D. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc Natl Acad Sci USA*. 1999; 96(8):4262–4267. [PubMed: 10200250]
13. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. *Nat Biotechnol*. 1998; 16(9):867–870. [PubMed: 9743122]
14. Buchan S, Gronevik E, Mathiesen I, King CA, Stevenson FK, Rice J. Electroporation as a “prime/boost” strategy for naked DNA vaccination against a tumor antigen. *J Immuno*. 2005; 174(10): 6292–6298.
15. Gronevik E, Mathiesen I, Lomo T. Early events of electroporation-mediated intramuscular DNA vaccination potentiate Th1-directed immune responses. *J Gene Med*. 2005; 7(9):1246–1254. [PubMed: 15822067]
16. Prud'homme GJ. DNA vaccination against tumors. *J Gene Med*. 2005; 7(1):3–17. [PubMed: 15538731]
17. Suhrbier A. Multi-epitope DNA vaccines. *Immunol Cell Biol*. 1997; 75(4):402–408. [PubMed: 9315485]
18. Doan T, Herd K, Ramshaw I, Thomson S, Tindle RW. A polytope DNA vaccine elicits multiple effector and memory CTL responses and protects against human papillomavirus 16 E7-expressing tumour. *Cancer Immunol Immunother*. 2005; 54(2):157–171. [PubMed: 15480657]
19. Velders MP, Weijzen S, Eiben GL, Elmishad AG, Kloetzel PM, Higgins T, Ciccarelli RB, Evans M, Man S, Smith L, Kast WM. Defined flanking spacers and enhanced proteolysis is essential for eradication of established tumors by an epitope string DNA vaccine. *J Immunol*. 2001; 166(9): 5366–5373. [PubMed: 11313372]
20. Livingston BD, Newman M, Crimi C, McKinney D, Chesnut R, Sette A. Optimization of epitope processing enhances immunogenicity of multiepitope DNA vaccines. *Vaccine*. 2001; 19(32): 4652–4660. [PubMed: 11535313]
21. Hanke T, Neumann VC, Blanchard TJ, Sweeney P, Hill AV, Smith GL, McMichael A. Effective induction of HIV-specific CTL by multi-epitope using gene gun in a combined vaccination regime. *Vaccine*. 1999; 17(6):589–596. [PubMed: 10075166]
22. Kotturi MF, Scott I, Wolfe T, Peters B, Sidney J, Cheroutre H, von Herrath MG, Buchmeier MJ, Grey H, Sette A. Naive precursor frequencies and MHC binding rather than the degree of epitope diversity shape CD8+ T cell immunodominance. *J Immunol*. 2008; 181(3):2124–2133. [PubMed: 18641351]
23. Tenzer S, Wee E, Burgevin A, Stewart-Jones G, Friis L, Lamberth K, Chang CH, Harndahl M, Weimershaus M, Gerstoft J, Akkad N, Klenerman P, Fugger L, Jones EY, McMichael AJ, Buus S, Schild H, van Endert P, Iversen AK. Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. *Nat Immunol*. 2009; 10(6):636–646. [PubMed: 19412183]

Fig. 1.

Multi-epitope plasmid constructs used in this study. pME-1 and pME-2, plasmids coding 3 well-known CD8 T cell epitopes prepared with 2 types of linkers, furin-sensitive (RVKR) or furin-resistant (VRVV) containing the HIV-tat₄₉ (RKKRRQRRRR) cationic cell penetrating sequence. pME-3 and pME-4, similar constructs to pME-1 and pME-2 lacking HIV-tat₄₉. pME-5, similar construct to pME-3 but containing a CD4 T cell helper epitope Ova₃₂₃. pME-6, plasmid coding 5 distinct CD8 T cell epitopes and a CD4 T cell helper epitope Ova₃₂₃. pME-7, plasmid coding 3 CD8 T cell epitopes for melanosomal antigens and a CD4 T cell helper epitope Ova₃₂₃. Sequences for all epitopes and HIV-tat₄₉ are shown in Table 1.

Fig. 2.

Immunogenicity of MEDV constructs encoding foreign CD8 T cell epitopes. Mice (4 per group) were vaccinated *i.m.* with various DNA plasmid constructs (as indicated) followed by electroporation as described in “Materials and Methods”. On day 12, CD8 and CD4 T cells were purified from pooled splenocytes, and antigen-induced IFN γ secretion to the individual epitopes was evaluated in EliSpot assays using peptide-pulsed APCs. **a** Role of epitope linkers and cationic cell penetrating sequence (HIV-tat₄₉) in the immunogenicity of MEDV constructs. **b** Immunogenicity of a CD4 T helper epitope (Ova₃₂₃) and potentiating effect in the CD8 T cell responses of DNA vaccination. Numbers above columns represent the percentage response of the pME-5-immunized mice compared with that of the pME-3-immunized mice. A plasmid containing the ovalbumin cDNA (pOva) was used for comparison. *P* values were calculated using the two-way ANOVA test between the pME-5-immunized and the pME-3-immunized group. **c** CD8 T cell responses to vaccination using a MEDV construct containing 5 distinct CD8 T cell epitopes in CB6F1 mice. EL4 (H-2^b) and P815 (H-2^d) cells pulsed with the corresponding peptides were used as APCs to evaluate the CD8 T cell responses and LB27.4 (I-A_b) cells were used to evaluate the CD4 T cell response to Ova₃₂₃. Results represent the average number of spots from triplicate wells with SD (error bars) of the means. Representative results of data obtained from two different experiments.

Fig. 3.

Evaluation of a MEDV construct containing melanoma-relevant CD8 T cell epitopes. B6 mice (4 per group) were vaccinated *i.m.* on days 0 and 14 with plasmid pME-7, which is coding 3 melanosomal antigen-derived CD8 T cell epitopes (Fig. 1 and Table 1), followed by *in vivo* electroporation. **a** The presence of epitope-specific CD8 T cells in splenocytes was measured by intracellular cytokine (IFN γ) staining gating on CD8 positive cells. No Tx, cells incubated without peptide. Numbers in each rectangular gate represent the % positive cells of all CD8 T cells. **b** Freshly isolated CD8 T cells from pooled splenocytes were evaluated for their capacity to recognize individual peptides (Trp₁₄₅₅, Trp₂₁₈₀, and mgp100₂₅) pulsed onto APCs (EL4) and B16 melanoma cells using EliSpot assay. Results represent the average number of spots from triplicate wells with SD (*error bars*) of the means. Representative results of data obtained from two different experiments.

Fig. 4.

DNA vaccination coding multiple melanoma-specific CD8 T cell epitopes results in strong anti-tumor effects. **a** Prophylactic antitumor effect of MEDV immunization. B6 mice (4 per group) were immunized on days -25 and -7 with either pME-7 or control backbone plasmid pB, followed by electroporation. Seven days after last immunization, the mice received 1×10^5 B16 melanoma cells administered *i.v.* and on day 28 (when the control mice started to appear ill), the presence of B16 pulmonary nodules was evaluated. **b** Therapeutic antitumor effect of DNA vaccination. B6 mice (4 per group) received 1×10^5 B16 cells *i.v.* and 3 and 18 days later, the mice were vaccinated with either pME-7 or pB, followed by electroporation. On day 26 (when the control mice started to appear ill), the presence of B16 pulmonary tumors was evaluated. Results for **a** and **b** are presented as “Number of lung tumors” and “Lung weights” for individual mice. When lung foci were too numerous to count, they are shown as >300. Each *data point* represents the number of lung foci and its lung weight in mgs for each individual mouse and *horizontal line* represents the average value of the group. Representative photographs of lungs of 2 mice from each group are shown. *P* values were calculated using unpaired Student's *t* test. **c** Therapeutic MEDV immunization results in increased survival advantage. Using the same experimental protocol presented in panel **b**, mice (8 per group) were studied for their survival (time to euthanasia). Graph represents the percentage of mice in each group that survived overtime after the tumor injection. *P* value was determined by log-rank test.

Fig. 5.

Blocking the negative effects of IFN γ increases the efficacy of MEDV against advanced subcutaneous B16 tumors. **a** Treatment of B16 cells with IFN γ reduces their capacity to be recognized by CD8 T cells from DNA vaccinated mice. CD8 T cells from B6 mice (4 animals) immunized with pME-7 (prepared in the same manner as in Fig. 3) were tested for their ability to recognize B16 cells treated (100 U/ml; 48 h) or not with IFN γ . Results represent the average number of spots from triplicate wells with SD (error bars) of the means. **b** Mice (4 per group) were inoculated subcutaneously with B16-Kb Lo , a B16 variant that expresses low levels of H-2K b , and were vaccinated with plasmid pME-7 followed by electroporation on days 4 and 18. Anti-PD-L1 mAb was administered *i.p.* on days 3, 5, 7, and 9 after each DNA vaccination. Control plasmid pCI-vaccinated mice inoculated with the same number of tumor cells were included as controls. Tumor sizes were determined in individual mice by measurements of 2 opposing diameters and are presented as tumor areas in mm 2 . Points, mean for each group of mice; gray bars, period of anti-PD-L1 mAb treatment; and bars, SD. *P* value was calculated using two-way ANOVA test. These experiments were repeated twice with similar results. **c** Similar experiment as presented in panel **b**, but using unmodified B16 cells that express normal levels of H-2K b , which can be increased by IFN γ .

Table 1

Peptide sequences incorporated into multi-epitope constructs

Epitope	Sequence	Source	MHC Restriction
Ova ₂₅₇	SIINFEKL	Ovalbumin	H-2K ^b
Ova ₃₂₃	ISQAVHAAHAEINEAGR	Ovalbumin	I-A ^d /I-A ^b
SIY	SIYRYYYGL	N/A	H-2K ^b
LCMV ₃₃	KAVYNFATM	lymphocytic choriomeningitis virus glycoprotein 33	H-2D ^b
HIV-Tat ₄₉	RKKRRQRRRR	HIV Trans-activator of transcription	N/A
PyCSP ₂₈₀	SYVPSAEQI	<i>P. yoelii</i> circumsporozoite protein	H-2K ^d
LLO ₉₁	GYKDGNEYI	<i>Listeria monocytogenes</i>	H-2K ^d
Trp ₁₄₅₅	TAPDNLGVM	Tyrosinase-related protein 1	H-2D ^b
Trp ₂₁₈₀	SVYDFFVWL	Tyrosinase-related protein 2	H-2K ^b
hgp100 ₂₅	KVPRNQDWL	Human (melanoma), glycoprotein 100	H-2D ^b
mgp100 ₂₅	EGSRNQDWL	Mouse (melanoma), glycoprotein 100	H-2D ^b

N/A = not applicable