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REVIEW

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DNA vaccines for cancer too

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Introduction

Vaccines based on DNA encoding the antigens, rather than antigenic proteins or peptides, is a recently developed approach that has the potential to elicit strong and long-lasting immunity while also having several practical advantages over other vaccines [1]. DNA vaccines can be conveniently and cheaply produced and purified. They do not require special handling or storage conditions. Also, unlike viral vectors that can elicit immune responses to the vector proteins as well as to the immunizing DNA, naked DNA vaccines elicit immune responses only to the encoded antigen of interest. This allows their repeated use to boost immune responses without loosing immunogenicity by provoking an immune attack against the vector-encoded proteins [2].

DNA vaccines usually consist of the gene encoding the antigen of interest inserted into bacterial plasmid DNA containing promoter/enhancer elements of choice to drive antigen expression. Following delivery, the host cell that has taken up the DNA synthesizes the encoded protein and it drains or is transported to the nearest lymph node where it can induce specific immunity. The development of new technologies for antigen discovery, such as DNA gene microarray and proteomics, together with further refinement of recombinant DNA technology, continue to expand the repertoire of antigens and vectors, candidates for inclusion in DNA vaccines. Research in DNA vaccines is thus still actively ongoing and their real potential, while clearly promising, has not yet been fully validated. This review is intended to serve as an update on an ever-expanding volume of information coming from studies in animal models as well as from clinical trials of DNA cancer vaccines.

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From tumor lysates to defined tumor antigens to whole tumor cells as vaccines: are DNA vaccines a further improvement?

After more than 100 years of study, the optimal methodology for vaccinating patients against their established tumors has not yet been found. Early tumor vaccines were comprised of whole tumor cells, fragments of tumor cells and protein lysates from tumor cells. It was first shown that tumor lysate vaccine had the advantage of enhanced antigen presentation [3, 4]. Later whole irradiated or inactivated tumor cells were used as tumor vaccines to stimulate immune responses. The immunization strategy was successful in animal models. However, some intrinsic weaknesses of whole tumor cell vaccines could not be overcome, such as weak immunogenicity, lack of co-stimulatory molecules, and potential to provoke autoimmunity [3, 5]. In the past two decades, considerable efforts have been made to discover new tumor antigens. Tumor antigen-based vaccines are expected to elicit tumor-specific immune responses and long-term memory without danger of autoimmunity. Vaccines based on shared tumor antigens can be developed for use in a large number of patients. Recently, however, development of cancer vaccines has focused again on the use of whole tumor cells or tumor cell lysates. The reasons given are that these complex mixtures may contain, in addition to several known shared tumor antigens, unknown unique tumor antigens that are expressed only by individual tumors. A vaccine based on defined shared tumor antigens, however, could elicit not only specific anti-tumor responses predicted by the antigens included in the vaccine, but also responses to other antigens on the tumor through a process known as "epitope spreading" [6–8] or "provoked immunity" [9]. This is important considering that tumors are very heterogeneous in molecules that they express. Even in the

same patient, different sub-populations of tumor cells express different combinations of tumor antigens and in different amounts, especially in metastatic sites. Furthermore, tumor cells can develop immune escape mechanisms by generation of antigen loss variants. It would thus be ideal to immunize not only with one or two but with a number of different antigens simultaneously in order to reduce the chance of tumor escape. DNA vaccines can easily incorporate more than one gene of interest into a suitable expression vector or be administered as mixtures of multiple vectors. By stimulating immune responses to different antigens expressed by tumor cells, more of the heterogeneous tumor may be destroyed.

Role of dendritic cells (DC) in DNA vaccination

Dendritic cells are professional antigen-presenting cells that play a crucial role in priming immune responses for effective elimination of tumors and long-term tumor-specific memory. In DNA vaccination, DC are involved in two major pathways of antigen presentation, the direct pathway [10, 11] and the cross-presentation pathway [12]. Direct targeting of DC with the plasmid DNA encoding the gene(s) of interest leads to endogenous synthesis of the protein and processing and presentation of antigen through the endogenous pathway. Endogenously synthesized proteins are processed into peptides and primarily presented on MHC class I molecules. This is the most efficient way to generate CD8+ T cells effective in direct tumor elimination. During the most common administration of DNA vaccines, by intramuscular injection, DNA ends up predominantly in keratinocytes and myocytes. The protein made and released by these cells is taken up as exogenous antigen by DC and other antigen-presenting cells (APC) and processed through the exogenous antigen-presenting pathway. Exogenous antigen is degraded in lysosomes into peptides and presented primarily on MHC class II molecules. CD4+ T cells that are important helper cells recognize the MHC class II-peptide complex. They may secrete cytokines that directly lead to tumor destruction, promote expansion of CTL (Th1 type helper T cells) or help B-cell antibody responses (Th2 type helper T cells) [13]. Thus, in DNA vaccination settings, both MHC class I and class II pathways of APC are utilized to trigger T-cell immunity [14, 15], leading to a very comprehensive immune response.

Since only a small number of DC are targeted by DNA vaccination, the full potential of every single DC to deliver the antigenic signal needs to be further explored. There are many ongoing studies to further optimize the function of APC, especially DC in DNA vaccination. One approach is to prolong the life of the transfected DC for long-term presentation of the antigen. To prolong survival, DC have been co-transfected with DNA encoding antigen of interest and DNA encoding inhibitors of apoptosis. Effects of anti-apoptotic proteins or pro-apoptotic proteins in enhancing anti-tumor immunity of DNA vaccines are not yet understood and the interpretations of the data have been diverse. Wu et al. [16] demonstrated that co-administration of antigen DNA with DNA encoding anti-apoptotic proteins such as Bcl-xL, enhances antigen-specific CD8+ T cell-mediated immune responses and increases the survival of DC in the draining lymph nodes. In contrast, other studies demonstrated that DNA vaccines encoding antigen co-expressed with pro-apoptotic agents such as Fas [17] or mutant caspases with altered active sites [18], or suicide DNA encoding antigen, are able to enhance antigen-specific T-cell immune responses [19]. Many factors may contribute to the apparent discrepancy of the results. Among those, the route of administration likely plays an important role. When administering apoptosis-inducing DNA vaccine via intramuscular immunization, targeted cells are myocytes that are not ideal professional APCs. Transfection of these cells with DNA encoding pro-apoptotic factors leads to their apoptosis or necrosis, resulting in increased uptake of antigen by professional APC through an exogenous cross-priming pathway. A similar outcome can be achieved by intradermal instead of intramuscular injection, which can directly target antigen to Langerhans cells, and anti-apoptotic administration can facilitate direct presentation of antigen to T cells by longer lived DNA-transfected DC [16].

Another approach towards better immunogenicity of DNA vaccines is through HSP70-mediated antigen cross presentation. When administered exogenously to DC, the peptides transported by HSP are known to be very efficiently presented by MHC class I molecules. Furthermore, recombinant HSP-antigen fusion proteins are able to elicit CD4-independent CD8+ cytotoxic T-cell responses. Hauser et al. [20] designed a DNA vaccine in which synthetic HSP70 was used as a DC binding molecule to deliver human papilloma virus type-16 E7 antigen. A leader peptide was exploited to re-route the fusion protein HPV-E7-HSP into the endoplasmic reticulum for secretion. The synthesized fusion protein was targeted to and then captured by DC via the interaction of HSP70 with its surface receptors, bringing the antigen into MHC class I pathway. Enhanced antigen presentation and CTL responses were shown [20, 21]. Ye et al. [21] also showed that MAGE-1-HSP70 fusion gene vaccine enhanced the frequency of MAGE-1-specific cytotoxic T-cell responses, thus turning a less effective MAGE-1 DNA vaccine into one with significant potency against established MAGE-1–expressing tumors.

Immune responses elicited by DNA vaccines

The major advantage of DNA vaccines is that both cellular (including CD4+ and CD8+ T cells) and humoral immune responses can be induced because the encoded antigen is processed through both endogenous and exogenous pathways, and peptide epitopes are

presented by both MHC Class I and Class II complexes [22].

Cellular responses

In the case of intradermal administration, directly transfected cells (Langerhans cells, DC and other APC) can synthesize antigen of interest. The endogenously produced protein is processed into peptides by the proteasome. Membrane-associated transporters of antigenic peptides (TAP) move these peptides into the endoplasmatic reticulum where they can be presented on MHC class I molecules. With the presence of co-stimulatory molecules, CD8+ T-cell response could be induced. In the case of intramuscular administration, transfected myocytes release synthesized protein when lysed. These proteins are then taken up, processed and presented by DC through cross-priming to stimulate CD4+ T cells. It has also been shown that exogenous proteins can be taken up by specialized DC to be presented in the MHC class I pathway [23–25].

In a study employing a DNA vaccine to induce HER-2–specific tumor immunity, the anti-tumor effect relied on both CD4+ and CD8+ T cells. IFN-γ, and to a lesser extent IL-4, showed to be crucial cytokines during tumor rejection [26, 27]. Only 10% of vaccinated IFN-/- mice rejected the tumors, whereas 90% of wild-type mice remained tumor-free. The importance of IFN-γ in tumor rejection supports the use of DNA vaccines, as they appear to more reproducibly elicit IFN-γ-based Th1 type responses. Report from Amici et al. [27] showed a Th1 phenotype skewing by DNA vaccination in a transgenic FVB/neu mouse model. The dependency on IL-4 in Her-2/neu tumor rejection seen in this study, may be related to previous reports demonstrating that CTL+ and Th1-associated tumor immunity required IL-4 during the priming phase [28].

Tumor-specific CD4+ cells provide help for the induction of specific CD8+ CTL and can also activate macrophages and eosinophils to produce nitric oxide and superoxides, which participate in the destruction of tumor cells. Since neither macrophages nor eosinophils have an intrinsic capacity for tumor specificity, the tumor specificity of these effectors is based on their activation by neighboring tumor-specific CD4+ helper cells. In addition, CD4+ helper cells may provide help to activate B-cell antibody production. Because of this, even though most tumors are MHC class II negative and may also display antigen-processing defects, ability to stimulate CD4+ remains an important requirement for cancer vaccines. DNA vaccination approach has reproducibly led to the induction of antigen-specific CD4+ T cells.

The precise role of CD4+ help in the generation of CD8+ CTL is, however, still not fully understood. For example, anti-HIV vaccines administered to CD4-/- mice showed that the CTL memory was dramatically reduced and the vaccine much less effective. At the same time, mice lacking CD4+ T cell retain largely intact primary CTL responses [29]. In another study, CTL activity was associated with an enhanced antibody response after DNA immunization [30]. C57BL/6 mice generated a stronger antibody response to OVA DNA immunization than congenic bm1 mice in which the immunodominant CTL epitope of OVA was no longer presented. Furthermore, pre-existing OVA-specific CTL increased the antibody response to a second unrelated antigen (β-galactosidase) co-administered with OVA. One postulated mechanism was that CTL might release antigen from DNA-transfected cells by killing or damaging them, and this freed antigen is then accessible to DC and B cells [31, 32].

Humoral responses

DNA vaccines are generally not as efficient as protein vaccines in inducing antibody responses. This might not only be because of the tendency to drive a Th1 dominant response, but could also be due to the low levels of antigen produced. Release and uptake of intact protein is required for the induction of antibodies. It is likely, in intramuscular administration, that muscle cells transfected with the gene of interest are recognized by CTL and lysed to release soluble antigen for B-cell recognition. Destruction of muscle fibers is detected after DNA immunization, but the role of muscle cells in inducing antibody responses is still unclear [33]. To increase antigen levels by increasing transfection efficiency, several physical methods such as electroporation or delivery of DNA on microparticles, have been used

It was shown that in vivo electroporation is able to yield powerful humoral and cellular responses in various species, including nonhuman primates [34]. Otten et al. [35] used in vivo electroporation to enhance the potency of an HIV DNA vaccine in rhesus macaques. Results showed increased onset, magnitude and duration of antibody and cell-mediated immune responses, demonstrating the utility of the electroporation technology for use in large animals.

Microparticle delivery is also used to enhance humoral responses elicited by DNA vaccines, mostly in mucosal immunization routes. A cationic microparticle-adsorbed plasmid DNA was shown to be able to induce higher titers of antibodies when compared to those induced by the naked DNA [36].

DNA vaccines for cancer

Defined tumor antigens

An extensive list of well-defined tumor antigens is available [37] and more are being discovered through the use of new technologies [38]. Tumor antigens can be categorized roughly into four groups (a) antigens

unique to an individual patient's tumor; (b) antigens common to a histologically similar group of tumors; (c) tissue-differentiation antigens; and (d) ubiquitous antigens expressed by normal and malignant cells [39]. In general, any gene expressed differentially in tumor cells compared to normal cells could be considered to encode a potential tumor antigen and could thus be a candidate for DNA vaccines. There are some important factors to consider, however, when evaluating tumor antigens as candidates for DNA vaccines. For example, tumor antigens such as ras [40], p53 [41], and BCR-ABL [42] are mutated in tumor cells and thus, potentially, the mutated gene or a segment of that gene could be incorporated into a DNA vaccine. It is difficult to predict, however, whether the unique amino acid sequence encoding the mutation or the fusion breakpoint will be either processed or presented by most common HLA molecules. Additional concerns exist for genes encoding whole proteins that are either overexpressed in tumors, such as carcinoembryonic antigen (CEA) and MUC1 [43, 44], aberrantly expressed, such as non-mutated p53, [45] Her-2/neu [46], and cyclin B1 [47], or uniquely expressed in normal expendable tissues as well as tumors, such as melanocytes/melanoma antigens gp100, MAGE-1, MAGE-3, prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) [48]. Cells producing these molecules as a result of transfection with the vaccine DNA, such as myocytes, keratinocytes or DC, would be expected to produce these molecules in the form that is expressed on normal cells rather than the form produced by tumor cells and thus promote autoimmunity rather than tumor immunity. This is especially critical for molecules that undergo different posttranslational modifications in tumors compared to normal cells. Several of these molecules are being tested in DNA vaccines now and these concerns will be best resolved in individual tumor antigen bases.

Chang et al. [46] have already shown that plasmid encoding the transmembrane and the extracellular domains of Her-2/neu was capable of inducing anti-tumor immunity in animal models. Furthermore, it was shown that the anti-tumor effects were improved with bicistronic plasmids co-expressing Her-2/neu antigen and cytokines, compared to co-administration of individual plasmids. In particular, co-expression of IL-18 or GM-CSF increased anti-tumor immunity in both prophylactic and therapeutic settings.

CEA-based DNA vaccines have also been explored by several groups [49]. Most recently, Zhou et al. [50] used a CEA-A2Kb double transgenic mouse model for preclinical screening and critical evaluation of human CEA vaccine efficacy in vivo. It was demonstrated that an oral DNA minigene vaccine could induce effective HLA-A2-restricted, CEA-specific anti-tumor CTL responses. The anti-tumor effects of plasmid DNA vaccines encoding human CEA fused to mouse GM-CSF were also examined. Tumor growth delay was shown in mice immunized with the CEA-GM-CSF fusion plasmids [49, 51]. In a phase z safety study of a human CEA DNA vaccine, low-grade transient toxicity was observed [52]. While no CEA-specific antibodies were detected, some patients (4 of 17) showed induction of lymphoproliferative responses to CEA after vaccination. There was no relationship between objective tumor regression and sustained declines in circulating CEA, nor correlation between a lymphoproliferative response and a stable disease. In another recent clinical trial, cDNA encoding human gp100 was used as the vaccine. The results did not demonstrate clinical or immunological responses to the vaccine [53].

Both overexpression and aberrant glycosylation in many carcinomas make MUC-1 a good candidate as a cancer vaccine [54]. Kontani et al. [44] immunized C57BL/6 mice with a DNA vaccine encoding MUC1 polypeptide. These mice were able to reject a challenge with MUC1-transfected syngeneic tumor cells. There was no efficacy when the vaccine was used to treat tumor-bearing mice. Co-administration of ex vivo manipulated DC with the DNA vaccine was used to further enhance anti-tumor cellular immunity, which resulted in suppression of tumor growth [44, 55].

Vaccination against PSA using a DNA vaccine has been investigated as immunotherapy treatment of prostate cancer. In animal studies, Pavlenko et al. [56] demonstrated that vaccination with plasmid vector carrying the PSA gene results in PSA-specific cellular responses and protection against tumor challenge. A phase I trial of a PSA DNA vaccine was undertaken in patients with hormone-refractory prostate cancer. Among eight patients, a PSA-specific cellular immune response, measured by IFN-γ production against recombinant PSA protein, and a rise in anti-PSA IgG were detected in two of three patients after vaccination in the highest dose cohort. A decrease in the slope of PSA was observed in two patients who exhibited IFN-γ production in response to PSA.

Gp100, a melanoma-associated antigen, is expressed in melanocytes and in highly tumorigenic B16 melanoma cells. Since mouse gp100 (mgp100) is poorly immunogenic in mice, Nawrath et al. [57] used a xenoimmunization approach and vaccinated mice with the human gp100 (hgp100). Mouse and human gp100 are highly homologous (80%). The vaccine consisted of hgp100 DNA combined with three synthetic peptides corresponding to putative cytotoxic T-cell epitopes of hgp100. Anti-tumor immunity was induced, which was effective against a challenge with poorly immunogenic B16-F0 malignant melanoma cells expressing mgp100. Similar results were obtained by Hawkins et al. [58] who showed that immunization of mice with hgp100 breaks "tolerance" to mouse gp100 to generate anti-tumor immunity.

Tyrosinase-related proteins-1 and 2 (TRP-1 and TRP-2) are melanosomal membrane glycoproteins and candidate tumor antigens. Mouse TRP-1 and TRP-2 are highly homologous to their human counterparts with TRP-1 having higher homology (93%) than

TRP-2 (84%). Furthermore, there is 52% identity at the amino acid level in these two tumor antigens, allowing for the possibility that a common determinant may be presented in the subset of animals. Immunization with TRP-2 DNA vaccine led to a potent induction of CD8+ T cells and required both CD4+ and CD8+ effectors for tumor protection [59]. There was no dependence on antibodies or NK1.1+ cells in this case. In related studies, O et al. [60] showed that mice vaccinated with a DNA vaccine expressing TRP-2 were partially protected against subcutaneous, intravenous, or intracerebral challenge with glioblastoma cells. This protection required both CD4+ and CD8+ T cells. Vaccine efficacy was enhanced upon addition of IL-12 as a genetic adjuvant. Srinivasan et al. [61] observed immunity against mouse TRP-1 or TRP-2 when mice were immunized with human TRP-1 or TRP-2 DNA vaccines. A subset of mice immunized with human TRP-2 developed antibody responses to TRP-1. Wolchok et al. [62] showed that DNA vaccine composed of human TRP-1 induced antibody-dependent tumor immunity in mice and autoimmune depigmentation without evidence of T-cell involvement, while xenogeneic TRP-2 DNA immunization induced immunity in the mice mediated by CD8+ T-cells. The role of IFN-γ in the generation of tumor immunity and autoimmune depigmentation in these two models was further investigated. No tumor protection and only minimal depigmentation was observed after immunization with human TRP-2 DNA in the mice deficient in IFN-γ. Repletion with recombinant murine IFN-γ restored anti-tumor immunity. Experiments using IL-4-deficient mice demonstrated that tumor immunity was unaffected but that autoimmune depigmentation was potentially accelerated, consistent with down-modulation of autoimmunity against TRP-2 by IL-4. In contrast, IFN-γ was not required for the generation of immunity to TRP-1. In fact, exogenous IFN-γ ablated autoantibody responses against TRP-1 after xenogeneic DNA immunization, consistent with a downregulatory effect of IFN-γ. Results showed that immunity to TRP-2 following DNA immunization uses an IFN-γ-dependent Th1 pathway, but immunity to TRP-1 is downregulated by IFN-γ.

Recently, B-cell differentiation antigens such as murine CD20 and human CD20, were used as DNA vaccines for immunotherapy of B-cell lymphoma [63]. Results showed that mice immunized with DNA encoding the human extracellular domain of CD20 mounted a T-cell mediated immune response against mouse CD20 and a small but significant survival advantage in a tumor challenge model, compared with mice immunized with full length and truncated mouse CD20. It is postulated that human CD20 mini-gene expresses one or more heteroclitic epitopes that are more efficiently processed and/or presented than the corresponding mouse peptides.

Another set of heavily studied antigens as candidates for DNA vaccines are idiotypic immunoglobulins (Id Ig) of B-cell tumors and the clonotypic T-cell receptors of T-cell tumors. Idiotype antigens are of specific interest because they are clonally expressed and therefore tumor-specific. For B-cell tumors, the encoding VH and VL immunoglobulin sequences can be readily identified and isolated in a variety of molecular forms. Timmerman et al. [64] included the two chains of the Id Ig molecule within one expression cassette. The constant region sequences were derived from xenogeneic Ig to provide some foreign protein to better stimulate the immune response. The construct was effective in animal models, but only yielded modest evidence for induction of immunity in clinical trials, even when GM-CSF was co-injected.

Stevenson et al. [65] assembled the Id antigen as a single chain Fv (scFv). In preclinical models, scFv alone was poor at inducing immunity. The fragment C (FrC) of tetanus toxin was then incorporated as a stronger antigen that can serve as an adjuvant to better activate immunity. The vaccine based on the fusion scFv–FrC was tested in animal models of lymphoma and myeloma. Anti-Id responses were elicited and were able to protect against tumor challenge [65, 66]. A clinical trial with 25 patients with low-grade B-cell NHL showed consistent increases in anti-FrC responses in terms of peripheral blood mononuclear cell proliferation and IFN-γ production by ELISPOT in about 60% of patients. A similar vaccine was also tested in myeloma patients. Anti-FrC responses were detected, both cellular and humoral, as well as a strong and durable induction of anti-Id T cell response. Antibodies were induced and were strikingly specific for each patient's immunoglobulin with little cross-reactivity between patients, even when similar VH or VL gene were involved [67]. Since most patients had been previously immunized against tetanus toxoid, there was a possibility that the anti-tetanus immune response would prevent induction of anti-Id response. To avoid such a problem, instead of the tetanus toxoid, the sequence encoding a viral coat protein from potato virus X (PVXCP) was selected [68]. Following fusion to the scFv sequence, the PVXCP was also highly effective in amplifying anti-Id response in lymphoma and myeloma models with an increasing tendency to activate a Th1-dominant response. Other DNA Id vaccines used fusions with genes encoding chemokines [69], defensins [69], a nonapeptide derived from IL-1β [70]. In those cases, high level of T-cell help was required to activate immunity and to break tolerance.

DNA vaccines directed against oncogenic viruses

Oncogenic viruses, to name a few, such as human papillomavirus (HPV), various hepatitis viruses, Epstein Barr Virus, are the cause of transformation in a number of tumors. DNA vaccines directed against viral antigens are good candidates for prevention or

therapy of these cancers. It is well known that most cervical cancers are associated with the infection of HPV16 and HPV18, in particular two oncogenic proteins E6 and E7. De Marco et al. [71] constructed several E7-encoding DNA vaccines including unmodified E7 or E7 fused to ubiquitin or to the invariant chain, in order to increase the presentation of E7-derived peptides by MHC class I or II molecules. These vaccines were administered intramuscularly, and immunized mice challenged with E7-positive tumor cell lines expressing different levels of MHC class I molecules. Results showed that engineering the intracellular pathway for antigen presentation is able to produce a valid therapeutic response even against tumors with downregulated MHC class I.

Another study assessed the capacity of a plasmid DNA that expresses the L1 gene of HPV type 16 to induce a protective immune response. Animals that received the DNA vaccine intramuscularly, subcutaneously and orally developed systemic anti-L1 IgG antibodies. Specific IgA antibodies were also found in vaginal washes from immunized mice. Either oral, intramuscular, or subcutaneous administration was able to induce the production of local IgA antibodies. Mice immunized subcutaneously showed the highest IgA titer. Both systemic and local antibodies proved effective in a surrogate neutralization assay. CTL activity mediated by CD8+ cells was also achieved. Challenged with a melanoma cell line engineered to express the HPV16-L1 protein, mice showed slower tumor growth rate and a longer survival time [72].

Other vaccines encoding E6 and E7 epitopes or whole proteins have been used successfully prophylactically and therapeutically in animal models [73, 74]. Recently, a therapeutic HPV16 DNA vaccine, ZYC101, was tested in a phase I trial against anal dysplasia [75]. This DNA vaccine encoded four multiple HLA-A*0201 epitopes from the E7 protein encapsulated in biodegradable polymer microparticles. Ten of twelve subjects demonstrated increased immune responses to the relevant epitopes. A more comprehensive DNA vaccine ZYC101a is currently being tested in a phase II clinical trial for cervical dysplasia. This vaccine is composed of 60 epitopes from the HPV16 and 18 E6 and E7 proteins.

Another vaccine trial reported by de Jong tested the HPV 16 L2, E6 and E7 genes encoding a single fusion protein (TA-CIN). In this phase I placebo-controlled study, both IgG antibodies and proliferative responses against TA-CIN were elicited in healthy volunteers. T-cell immunity against the HPV16 E6 and E7 oncoproteins was also detected in ELISPOT assays from 8 of 11 subjects. Continued boosting was used but this approach appears sub-optimal since responses tended to decrease after subsequent vaccinations. Tumors associated with EBV infections such as Burkitt's lymphoma, nasopharyngeal carcinoma and AIDS-associated lymphoma might also benefit from DNA vaccination and are currently under investigation.

DNA vaccines for prevention of angiogenesis

Another way to utilize DNA vaccines for cancer treatment is to induce immune responses that can target molecules on tumor blood vessels. Inhibition of angiogenesis is a physiological mechanism that is unlikely to be subject to mutagenesis and immune escape. Furthermore, each tumor capillary supplies nourishment to hundreds of tumor cells and thus targeting tumor vasculature can induce anti-tumor effects by starving the tumor. Niethammer et al. [76] targeted tumor vasculature by using the upregulated cell membrane receptor tyrosine kinases FLK-1 (fetal liver kinase, also designated vascular-endothelial growth factor receptor 2) of proliferating endothelial cells as an antigen. The FLK-1 vaccine effectively protected mice from lethal challenges with melanoma, colon carcinoma and lung carcinoma, and reduced the growth of established metastases in a therapeutic setting. CTL-mediated killing of endothelial cells expressing FLK-1 indicated breaking of peripheral immune tolerance against this self antigen, resulting in markedly reduced dissemination of spontaneous and experimental pulmonary metastases.

Considerations for the design and delivery of DNA vaccines

Design and selection of vectors is of considerable importance for the outcome of vaccination. DNA from tumor antigens can be either used in a plasmid as "naked" DNA-vaccine or can be used in constructed recombinant viral vectors. In contrast to naked DNA, vaccines based on viral vectors, most often adenovirus or vaccinia virus, have the advantage of efficient delivery of genes of interest to target cells and activation of innate immunity. However, they may generate immune responses against viral antigens, which make booster immunizations less likely to be effective.

Vaccines delivering the same gene through different vectors may generate different qualities of immune responses and anti-tumor effects [77]. A head-to-head comparison of different vector delivery systems for HPV vaccine, naked DNA versus vaccinia containing the same gene Sig/E7/LAMP-1, showed that naked DNA generated immunity against a high dose of TC-1 tumor cells as well as against TC-1 P2 tumor cells that were resistant to the Vaccinia-Sig/E7/LAMP-1 vaccine. IL-4 knockout mice vaccinated with the vaccinia-based vaccine exhibited a more potent anti-tumor effect than wild-type mice, suggesting that IL-4 may play a detrimental role in the anti-tumor effect mediated by vaccinia-based vaccines. Clearly in this specific case, naked DNA vaccines are a better choice.

DNA vaccines are usually administered by intramuscular or intradermal injection, but other routes and methods such as transdermal delivery, in vivo electroporation and mucosal delivery utilizing different microparticles are also being tested. DNA vaccines can be

applied by a gene gun (also known as ballistic delivery) using gold particles coated with DNA and delivered into the target tissue through acceleration by helium gas under high pressure. Gene gun delivery is highly effective, atraumatic and offers the advantage of using much smaller amounts of DNA compared with intramuscular injection. Gene gun immunization results in 10–100 times higher expression of the delivered gene product compared with intramuscular application [78]. The reason is that ballistic delivery introduces DNA directly into dermal APC, which subsequently migrate into local lymph nodes and prime immune responses [10, 11]. It is of interest that gold particles without plasmid can also have a modest effect on tumor regression, hypothesized to be related to both the local and regional accumulation of APC observed in skin samples and lymph nodes of animals treated by gene gun [79].

The application of DNA by in vivo electropermeabilization (EP) is a promising new method for increasing transfection efficacy by DNA vaccines. Drabick et al. [80] showed in a porcine model that in vivo skin EP may be used to increase transgene expression up to an average of 83-fold relative to DNA injection. Transfected cells were principally located in the dermis and included adipocytes, fibroblasts, endothelial cells, and numerous mononuclear cells with dendritic processes. Transfected cells were also observed in lymph nodes draining electropermeabilized sites. Analysis of humoral immune responses, including immunoglobulin subclass profiles, revealed strong enhancement of EP-mediated vaccination compared to DNA injection. Mendiratta et al. [81] explored a novel strategy of nonviral genetic vaccination coupled with muscle electroporation. Electroporation-enhanced immunization with plasmids encoding either human gp100 or mouse TRP-2 antigens induced only partial rejection of B16 melanoma challenge while combination of these two antigens caused tumor rejection in 100% of the immunized mice.

In view of the importance of mucosal and regional lymph node immunity in the control of infectious disease and cancer, several groups have developed immunization strategies that target plasmid DNA to a variety of mucosal surfaces [82]. Even though all the protocols so far have only been used to deliver plasmid to control infectious disease, they might be important in the delivery of cancer vaccines as well, especially for prevention of tumors that arise at epithelial surfaces. Gene expression in mucosal epithelia was seen to be extremely inefficient after delivery of naked plasmid DNA. The reason might be the significant physical and chemical barriers that impede transfection, such as mucins, endonucleases, mucolytic enzymes, low PH, turbulence and rapid removal by ciliated epithelium. To increase plasmid DNA uptake and transfection efficiency, several approaches have been taken such as condensing the naked plasmid with cationic acid [83], liposomes [84], or biodegradable microsphere, such as poly DL-lactide-co-glycolide (PLG) [85] or chitosan [86]. Cationic lipids (cytofectins) have been shown to be highly effective in increasing the transfection efficiency of plasmid DNA in vitro. Several groups have reported that complexing plasmid DNA with cationic lipids (cytofectins) enhanced the expression of plasmid DNA-encoded protein in the respiratory tract and increased the induction of specific secretory IgA and IgG and cytotoxic T-lymphocytes [83, 87, 88]. Compared with naked DNA, these plasmid DNA-cytofectin complexes have a longer half-life protecting the DNA from nuclease degradation in vivo. Cytofectins may also exhibit synergystic activity with the CpG in the plasmid to activate the innate responses and augment T-cell priming [89].

Poly DL-lactide-co-glycolide is a biocompatible and biodegradable polymer. The development of PLG microparticles has been directed towards oral delivery of antigens. The uptake and transport of PLG microparticles are dependent on particle size and their hydrophobicity. Microspheres between 5 μm and 10 μm in diameter are taken up from the lumen of the gut and transported to underlying APCs [90]. The rate of plasmid DNA release from PLG microparticles can vary from several days to months depending on the percentage of lactic and glycolic acids in the polymer.

Chitosan is a cationic polysaccharide that is widely used as a drug delivery vehicle. There is evidence to suggest that chitosan increases transcellular and parcellular transportation across epithelia, which may prove useful in the delivery of encapsulated plasmid DNA to APC within the submucosa [91].

Adjuvants

Efficient vaccines consist of specific antigenic determinants and a nonspecific moiety–adjuvant components that increase vaccine immunogenicity [92–94]. It is important to design and develop adjuvants that can promote development of not only humoral responses but also cellular responses.

CpG

A useful feature of a DNA vaccine is that it already carries its own adjuvant in the form of immunostimulatory sequence, unmethylated cytidine phosphate guanosine motifs (CpG), residing in the plasmid portion of the expression vector. The intracellular receptor for CpG oligonucleotides is Toll-like receptor (TLR) 9, which is expressed by B cells, DC and other cells in the innate immune system. Thus, the CpG motif is capable of causing maturation and activation of APC. Activation leads to an inflammatory response, with the production of multiple cytokines, including interferon (IFN)-$\alpha/\beta/\gamma$, IL-6, IL-12, IL-18 and tumor necrosis factor alpha (TNF-α) [95]. These cytokines are supportive of generation of Th1 type immune responses that have been shown to be responsible for tumor rejection. Other sequences, known as neutralizing motifs, which

can counteract stimulating CpG motifs, may be also found in DNA vaccines. Thus, the efficacy of a DNA vaccine might be improved through the removal of neutralizing motifs and addition of species-specific stimulatory CpG motifs [95].

Extrapolation from murine studies to humans is difficult since not all receptors are expressed by the same cell population in each species. For example, the critical TRL9 molecule is expressed by monocyte-derived DC in mice, but only by lymphoid-derived plasmacytoid DC in humans. The consequences that would be faced while designing vaccines for human subjects are difficult to predict [96].

Heat-shock proteins (HSP)

Heat-shock proteins are among the most highly conserved molecules of the biosphere. They are found in eukaryotes, in prokaryotes and even in plants. Owing to their chaperon function, they participate in the assembly of antibody molecules, in the stabilization of MHC Class I and Class II molecules and can stimulate the synthesis of cytokines [97]. HSP-peptides complexes may form inside the cells allowing the transport of peptides resulting from the proteolytic cleavage of endogenous proteins. Specialized receptors on DC allow the cross presentation of the HSP-peptide complexes [98, 99]. Amigorena et al. [100] showed that more IFN-γ-producing CD8+ T cells can be elicited by a DNA vaccine that encodes HSP73-binding antigen than by a DNA vaccine that encoded non-HSP-binding antigen. This was attributed to the observation that exosomes that selectively accumulate HSP73 were taken up into the lumen of endocytic vesicles of DC, entered an endosome-to-cytosol transport, and gained access to the cytosolic Ag-processing machinery and the conventional MHC class I Ag presentation pathway. Along the same line, Kammerer et al. [101] showed in vitro and in vivo evidence that antigenic material from tumor cells taken up by DC can more efficiently cross-stimulate or cross-prime CTL when it is expressed in association with HSP.

Biological adjuvants

To modulate the immune responses elicited by DNA immunization, studies of co-administration of biological adjuvants such as GM-CSF and IL-12, or the inclusion of genes encoding co-stimulatory molecules such as B7.1, B7.2, CD40, have been conducted.

GM-CSF is thought to enhance the initiation of immune responses by recruiting APC to the site where antigen is expressed. GM-CSF stimulates the proliferation and the activity of DC, induces differentiation from immature DC to mature DC and increases the expression of MHC Class II molecules on DC [102–104]. Another cytokine that is important for the generation of DC and augmenting their function and quantity is fms-like tyrosine kinase 3 (Flt3) ligand. Studies showed that fusion of a gene encoding extracellular domain of Flt3-ligand to an antigen gene can greatly enhance the potency of DNA vaccines [105].

B7 signals are required to activate both CD4+ and CD8+ T cells and several models have demonstrated that their use counteracts immune escape mechanisms of tumors and thus increases the protection from subsequent tumor challenge and induction of CTL [106, 107]. It has been shown that vaccination of animals with plasmids encoding antigen and B7.1, but not B7.2, can induce immune responses against transfected malignant tumor expressing that antigen [108].

New technologies that support further development of DNA vaccines

DNA microarray technology is a new and powerful tool that can simultaneously analyze a large number of differentially expressed genes in a rapid and efficient way. The advantage of microarry technology is that it can assist researchers to better define and understand the expression profile of a given genotype associated with disease, and to better understand or predict immune responses to specific antigens. DNA microarray technology has been applied to the comprehensive analysis of multiple gene mutations and expressed sequences and information used for new drug design, understanding of host-pathogen interactions and the design of new vaccines. The opportunity to compare the expression of thousands of genes in varied pathophysiological conditions allows the identification of virulence factor (malignancy factors in the case of tumors) that can be useful in vaccine design [109].

In 2000, Villaret et al. [110] combined cDNA subtractive methodology with microarray technology to identify unique genes specific for squamous cell carcinoma of the head and neck. A subtractive library was made from two head and neck squamous cell cancers and six normal tissues. Of all the genes searched, 107 showed differential expression between tumor and normal tissues. Among those, 13 independent genes were found to be overexpressed in tumor tissues. Of these 13 genes, 9 were previously known while 4 had not yet been identified. The previously identified genes included Keratins K6 and K16, laminin-5, plakophilin-1, matrix metalloproteinase-2 (MMP), vascular endothelial growth factor, connexin 26, 14-3-3 sigma, and tumor antigen CaN19. These genes, as well as the four newly identified ones have the potential to be developed as tumor markers, drug targets or cancer vaccines.

Sturniolo et al. [111] were the first to systematically identify potential tumor antigens by combining computational algorithms with DNA microarrays. Virtual matrices of HLA-DR that represent the majority of human HLA-DR peptide-binding specificity were established. The matrices were incorporated into computer software (TEPITOPE) capable of predicting

promiscuous HLA Class II ligands. DNA microarrays were used to reveal genes that are specifically expressed or upregulated in colon cancer. Beginning with nearly 20,000 genes, a database of 130 colon cancer-specific and promiscuous T-cell epitopes was compiled. Antibodies for some of the microarray-selected candidate antigens were detected in patients, lending support to further exploration of these molecules. Clearly, having DNA sequence information makes them easier to evaluate as candidates for DNA vaccines.

Alizadeh and Staudt [112] created a specialized microarray, ''lymphochip'', to study selected genes in normal and malignant lymphocyte settings. The ''lymphochip'' cDNA microarry provides two complementary genomic-scale views of normal and malignant lymphocytes, and generates insights and hypotheses that are related to known cellular pathways. This too provides a plethora of potential targets to be incorporated into DNA vaccines against lymphoid malignancies. Antibody microarray has also been used to screen and identify potential biomarkers. Miller et al. [113] developed a practical strategy for serum protein profiling using antibody microarrays and applied the method to the identification of potential biomarkers in prostate cancer serum. A statistical filter based on the correlation of data from "reverse-labeled" experiment sets accurately predicted the agreement between the microarray measurements and enzyme-linked immunosorbent assay measurements. Five proteins, such as von Willebrand Factor, immunoglobulin M, α1-antichymotrypsin, villin and IgG were identified, which had significantly different levels between the prostate cancer samples and the controls.

Continuous efforts have been made to bridge microarray discoveries to real tumor antigens. A combination of cDNA subtraction and microarray analysis identified genes preferentially over-expressed in lung squamous cell carcinoma. Several aberrantly expressed genes, such as L530S, L531S [114], L552S (an alternatively spliced isoform of SAGE-1) [115], L523S (an RNA-binding protein within the KOC family [116] were reported. In 2003, Wang et al. [116] further reported expression of L523S in lung cancer by real-time PCR, Western blot and immunohistochemistry staining. Also, anti-L523S antibody was detected in eight of 17 lung pleural effusion from lung cancer patients.

On the basis of cDNA microarray analysis of 23,040 genes, Nakatsura et al. identified glypican-3 (GPC3) overexpressed specifically in human hepatocellular carcinoma. This oncofetal protein is highly immunogenic in mice and can elicit effective anti-tumor immunity with no evidence of autoimmunity.

Concluding remarks

The goal of cancer vaccines is to induce antigen-specific immunity that would be able to provide an effective means to prevent cancer occurrence or treat cancer before its progression or recurrence. In order to be effective, cancer vaccines need to elicit a robust immune response, both cellular and humoral, and a long-term immune memory. In addition, effective cancer vaccines should be affordable and practical to be delivered to millions of cancer patients. To that end, DNA vaccines have been shown to be able to elicit both cellular and humoral immunity, as well as to be simple to produce and distribute. With the further development of new technologies, DNA vaccines are becoming a promising agent in the fight against cancer.

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References

1. Haupt K, Roggendorf M, Mann K (2002) The potential of DNA vaccination against tumor-associated antigens for antitumor therapy. Exp Biol Med (Maywood) 227:227–237
2. Liu MA (1998) Vaccine developments. Nat Med 4:515–519
3. Lewis JD, Reilly BD, Bright RK (2003) Tumor-associated antigens: from discovery to immunity. Int Rev Immunol 22:81–112
4. Sinkovics JG, Horvath JC (2000) Vaccination against human cancers (review). Int J Oncol 16:81–96
5. Yannelli JR, Wroblewski JM (2004) On the road to a tumor cell vaccine: 20 years of cellular immunotherapy. Vaccine 23:97–113
6. Disis ML, Shiota FM, Cheever MA (1998) Human HER-2/neu protein immunization circumvents tolerance to rat neu: a vaccine strategy for 'self' tumour antigens. Immunology 93:192–199
7. Ranieri E, Kierstead LS, Zarour H, Kirkwood JM, Lotze MT, Whiteside T, Storkus WJ (2000) Dendritic cell/peptide cancer vaccines: clinical responsiveness and epitope spreading. Immunol Invest 29:121–125
8. Butterfield LH, Ribas A, Dissette VB, Amarnani SN, Vu HT, Oseguera D, Wang HJ, Elashoff RM, McBride WH, Mukherji B, Cochran AJ, Glaspy JA, Economou JS (2003) Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. Clin Cancer Res 9:998–1008
9. Henderson RA, Finn OJ (1996) Human tumor antigens are ready to fly. Adv Immunol 62:217–256
10. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN (1998) Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. J Exp Med 188:1075–1082
11. Akbari O, Panjwani N, Garcia S, Tascon R, Lowrie D, Stockinger B (1999) DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J Exp Med 189:169–178
12. Doe B, Selby M, Barnett S, Baenziger J, Walker CM (1996) Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. Proc Natl Acad Sci U S A 93:8578–8583
13. Ridge JP, Di Rosa F, Matzinger P (1998) A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 393:474–478
14. Corr M, Lee DJ, Carson DA, Tighe H (1996) Gene vaccination with naked plasmid DNA: mechanism of CTL priming. J Exp Med 184:1555–1560
15. Fu TM, Ulmer JB, Caulfield MJ, Deck RR, Friedman A, Wang S, Liu X, Donnelly JJ, Liu MA (1997) Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. Mol Med 3:362–371

16. Kim TW, Hung CF, Ling M, Juang J, He L, Hardwick JM, Kumar S, Wu TC (2003) Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. J Clin Invest 112:109–117
17. Chattergoon MA, Kim JJ, Yang JS, Robinson TM, Lee DJ, Dentchev T, Wilson DM, Ayyavoo V, Weiner DB (2000) Targeted antigen delivery to antigen-presenting cells including dendritic cells by engineered Fas-mediated apoptosis. Nat Biotechnol 18:974–979
18. Sasaki S, Amara RR, Oran AE, Smith JM, Robinson HL (2001) Apoptosis-mediated enhancement of DNA-raised immune responses by mutant caspases. Nat Biotechnol 19:543–547
19. Leitner WW, Ying H, Driver DA, Dubensky TW, Restifo NP (2000) Enhancement of tumor-specific immune response with plasmid DNA replicon vectors. Cancer Res 60:51–55
20. Hauser H, Chen SY (2003) Augmentation of DNA vaccine potency through secretory heat shock protein-mediated antigen targeting. Methods 31:225–231
21. Ye J, Chen GS, Song HP, Li ZS, Huang YY, Qu P, Sun YJ, Zhang XM, Sui YF (2004) Heat shock protein 70/MAGE-1 tumor vaccine can enhance the potency of MAGE-1-specific cellular immune responses in vivo. Cancer Immunol Immunother 53:825–834
22. Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A (1992) Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Hum Mol Genet 1:363–369
23. Rock KL, Clark K (1996) Analysis of the role of MHC class II presentation in the stimulation of cytotoxic T lymphocytes by antigens targeted into the exogenous antigen-MHC class I presentation pathway. J Immunol 156:3721–3726
24. York IA, Rock KL (1996) Antigen processing and presentation by the class I major histocompatibility complex. Annu Rev Immunol 14:369–396
25. Rock KL, Gamble S, Rothstein L (1990) Presentation of exogenous antigen with class I major histocompatibility complex molecules. Science 249:918–921
26. Lindencrona JA, Preiss S, Kammertoens T, Schuler T, Piechocki M, Wei WZ, Seliger B, Blankenstein T, Kiessling R (2004) CD4+ T cell-mediated HER-2/neu-specific tumor rejection in the absence of B cells. Int J Cancer 109:259–264
27. Amici A, Venanzi FM, Concetti A (1998) Genetic immunization against neu/erbB2 transgenic breast cancer. Cancer Immunol Immunother 47:183–190
28. Schuler T, Qin Z, Ibe S, Noben-Trauth N, Blankenstein T (1999) T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. J Exp Med 189:803–810
29. von Herrath MG, Yokoyama M, Dockter J, Oldstone MB, Whitton JL (1996) CD4-deficient mice have reduced levels of memory cytotoxic T lymphocytes after immunization and show diminished resistance to subsequent virus challenge. J Virol 70:1072–1079
30. Dyer CM, Zhan Y, Brady JL, Carbone FR, Smyth MJ, Lew AM (2004) Unexpectedly, induction of cytotoxic T lymphocytes enhances the humoral response after DNA immunization. Blood 103:3073–3075
31. Albert ML, Sauter B, Bhardwaj N (1998) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 392:86–89
32. Heath WR, Carbone FR (2001) Cross-presentation in viral immunity and self-tolerance. Nat Rev Immunol 1:126–134
33. Davis HL, Millan CL, Watkins SC (1997) Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. Gene Ther 4:181–188
34. Zhang L, Widera G, Bleecher S, Zaharoff DA, Mossop B, Rabussay D (2003) Accelerated immune response to DNA vaccines. DNA Cell Biol 22:815–822
35. Otten G, Schaefer M, Doe B, Liu H, Srivastava I, zur Megede J, O'Hagan D, Donnelly J, Widera G, Rabussay D, Lewis MG, Barnett S, Ulmer JB (2004) Enhancement of DNA vaccine potency in rhesus macaques by electroporation. Vaccine 22:2489–2493
36. Kaur R, Rauthan M, Vrati S (2004) Immunogenicity in mice of a cationic microparticle-adsorbed plasmid DNA encoding Japanese encephalitis virus envelope protein. Vaccine 22:2776–2782
37. Renkvist N, Castelli C, Robbins PF, Parmiani G (2001) A listing of human tumor antigens recognized by T cells. Cancer Immunol Immunother 50:3–15
38. Novellino L, Castelli C, Parmiani G (2005) A listing of human tumor antigens recognized by T cells: March 2004 update. Cancer Immunol Immunother 54:187–207
39. Berzofsky JA, Terabe M, Oh S, Belyakov IM, Ahlers JD, Janik JE, Morris JC (2004) Progress on new vaccine strategies for the immunotherapy and prevention of cancer. J Clin Invest 113:1515–1525
40. Gjertsen MK, Bakka A, Breivik J, Saeterdal I, Solheim BG, Soreide O, Thorsby E, Gaudernack G (1995) Vaccination with mutant ras peptides and induction of T-cell responsiveness in pancreatic carcinoma patients carrying the corresponding RAS mutation. Lancet 346:1399–1400
41. Yanuck M, Carbone DP, Pendleton CD, Tsukui T, Winter SF, Minna JD, Berzofsky JA (1993) A mutant p53 tumor suppressor protein is a target for peptide-induced CD8+ cytotoxic T-cells. Cancer Res 53:3257–3261
42. Pinilla-Ibarz J, Cathcart K, Korontsvit T, Soignet S, Bocchia M, Caggiano J, Lai L, Jimenez J, Kolitz J, Scheinberg DA (2000) Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. Blood 95:1781–1787
43. Zaremba S, Barzaga E, Zhu M, Soares N, Tsang KY, Schlom J (1997) Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen. Cancer Res 57:4570–4577
44. Kontani K, Taguchi O, Ozaki Y, Hanaoka J, Tezuka N, Sawai S, Inoue S, Fujino S, Maeda T, Itoh Y, Ogasawara K, Sato H, Ohkubo I, Kudo T (2002) Novel vaccination protocol consisting of injecting MUC1 DNA and nonprimed dendritic cells at the same region greatly enhanced MUC1-specific antitumor immunity in a murine model. Cancer Gene Ther 9:330–337
45. Theobald M, Biggs J, Dittmer D, Levine AJ, Sherman LA (1995) Targeting p53 as a general tumor antigen. Proc Natl Acad Sci U S A 92:11993–11997
46. Chang SY, Lee KC, Ko SY, Ko HJ, Kang CY (2004) Enhanced efficacy of DNA vaccination against Her-2/neu tumor antigen by genetic adjuvants. Int J Cancer 111:86–95
47. Kao H, Marto JA, Hoffmann TK, Shabanowitz J, Finkelstein SD, Whiteside TL, Hunt DF, Finn OJ (2001) Identification of cyclin B1 as a shared human epithelial tumor-associated antigen recognized by T cells. J Exp Med 194:1313–1323
48. Correale P, Walmsley K, Nieroda C, Zaremba S, Zhu M, Schlom J, Tsang KY (1997) In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. J Natl Cancer Inst 89:293–300
49. Hodge JW (1996) Carcinoembryonic antigen as a target for cancer vaccines. Cancer Immunol Immunother 43:127–134
50. Zhou H, Luo Y, Mizutani M, Mizutani N, Becker JC, Primus FJ, Xiang R, Reisfeld RA (2004) A novel transgenic mouse model for immunological evaluation of carcinoembryonic antigen-based DNA minigene vaccines. J Clin Invest 113:1792–1798
51. Lima J, Jenkins C, Guerrero A, Triozzi PL, Shaw DR, Strong TV (2005) A DNA vaccine encoding genetic fusions of carcinoembryonic antigen (CEA) and granulocyte/macrophage colony-stimulating factor (GM-CSF). Vaccine 23:1273–1283

52. Conry RM, Curiel DT, Strong TV, Moore SE, Allen KO, Barlow DL, Shaw DR, LoBuglio AF (2002) Safety and immunogenicity of a DNA vaccine encoding carcinoembryonic antigen and hepatitis B surface antigen in colorectal carcinoma patients. Clin Cancer Res 8:2782–2787
53. Rosenberg SA, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, Restifo NP, Haworth LR, Seipp CA, Freezer LJ, Morton KE, Mavroukakis SA, White DE (2003) Inability to immunize patients with metastatic melanoma using plasmid DNA encoding the gp100 melanoma-melanocyte antigen. Hum Gene Ther 14:709–714
54. Vlad AM, Kettel JC, Alajez NM, Carlos CA, Finn OJ (2004) MUC1 immunobiology: from discovery to clinical applications. Adv Immunol 82:249–293
55. Graham RA, Burchell JM, Taylor-Papadimitriou J (1996) The polymorphic epithelial mucin: potential as an immunogen for a cancer vaccine. Cancer Immunol Immunother 42:71–80
56. Pavlenko M, Roos AK, Lundqvist A, Palmborg A, Miller AM, Ozenci V, Bergman B, Egevad L, Hellstrom M, Kiessling R, Masucci G, Wersall P, Nilsson S, Pisa P (2004) A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. Br J Cancer 91:688–694
57. Nawrath M, Pavlovic J, Moelling K (2001) Synergistic effect of a combined DNA and peptide vaccine against gp100 in a malignant melanoma mouse model. J Mol Med 79:133–142
58. Hawkins WG, Gold JS, Dyall R, Wolchok JD, Hoos A, Bowne WB, Srinivasan R, Houghton AN, Lewis JJ (2000) Immunization with DNA coding for gp100 results in CD4 T-cell independent antitumor immunity. Surgery 128:273–280
59. Bowne WB, Srinivasan R, Wolchok JD, Hawkins WG, Blachere NE, Dyall R, Lewis JJ, Houghton AN (1999) Coupling and uncoupling of tumor immunity and autoimmunity. J Exp Med 190:1717–1722
60. O I, Blaszczyk-Thurin M, Shen CT, Ertl HC (2003) A DNA vaccine expressing tyrosinase-related protein-2 induces T-cell-mediated protection against mouse glioblastoma. Cancer Gene Ther 10:678–688
61. Srinivasan R, Houghton AN, Wolchok JD (2002) Induction of autoantibodies against tyrosinase-related proteins following DNA vaccination: unexpected reactivity to a protein paralogue. Cancer Immun 2:8
62. Wolchok JD, Srinivasan R, Perales MA, Houghton AN, Bowne WB, Blachere NE (2001) Alternative roles for interferon-gamma in the immune response to DNA vaccines encoding related melanosomal antigens. Cancer Immun 1:9
63. Palomba ML, Roberts WK, Dao T, Manukian G, Guevara-Patino JA, Wolchok JD, Scheinberg DA, Houghton AN (2005) CD8+ T-Cell-Dependent Immunity Following Xenogeneic DNA Immunization against CD20 in a Tumor Challenge Model of B-Cell Lymphoma. Clin Cancer Res 11:370–379
64. Timmerman JM, Singh G, Hermanson G, Hobart P, Czerwinski DK, Taidi B, Rajapaksa R, Caspar CB, Van Beckhoven A, Levy R (2002) Immunogenicity of a plasmid DNA vaccine encoding chimeric idiotype in patients with B-cell lymphoma. Cancer Res 62:5845–5852
65. Spellerberg MB, Zhu D, Thompsett A, King CA, Hamblin TJ, Stevenson FK (1997) DNA vaccines against lymphoma: promotion of anti-idiotypic antibody responses induced by single chain Fv genes by fusion to tetanus toxin fragment C. J Immunol 159:1885–1892
66. King CA, Spellerberg MB, Zhu D, Rice J, Sahota SS, Thompsett AR, Hamblin TJ, Radl J, Stevenson FK (1998) DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. Nat Med 4:1281–1286
67. Forconi F, King CA, Sahota SS, Kennaway CK, Russell NH, Stevenson FK (2002) Insight into the potential for DNA idiotypic fusion vaccines designed for patients by analysing xenogeneic anti-idiotypic antibody responses. Immunology 107:39–45
68. Savelyeva N, Munday R, Spellerberg MB, Lomonossoff GP, Stevenson FK (2001) Plant viral genes in DNA idiotypic vaccines activate linked CD4+ T-cell mediated immunity against B-cell malignancies. Nat Biotechnol 19:760–764
69. Ruffini PA, Neelapu SS, Kwak LW, Biragyn A (2002) Idiotypic vaccination for B-cell malignancies as a model for therapeutic cancer vaccines: from prototype protein to second generation vaccines. Haematologica 87:989–1001
70. Hakim I, Levy S, Levy R (1996) A nine-amino acid peptide from IL-1beta augments antitumor immune responses induced by protein and DNA vaccines. J Immunol 157:5503–5511
71. De Marco F, Hallez S, Brulet JM, Gesche F, Marzano P, Flamini S, Marcante ML, Venuti A (2003) DNA vaccines against HPV-16 E7-expressing tumour cells. Anticancer Res 23:1449–1454
72. Rocha-Zavaleta L, Alejandre JE, Garcia-Carranca A (2002) Parenteral and oral immunization with a plasmid DNA expressing the human papillomavirus 16-L1 gene induces systemic and mucosal antibodies and cytotoxic T lymphocyte responses. J Med Virol 66:86–95
73. Chu NR, Wu HB, Wu TC, Boux LJ, Mizzen LA, Siegel MI (2000) Immunotherapy of a human papillomavirus type 16 E7-expressing tumor by administration of fusion protein comprised of Mycobacterium bovis BCG Hsp65 and HPV16 E7. Cell Stress Chaperones 5:401–540
74. Eiben GL, da Silva DM, Fausch SC, Le Poole IC, Nishimura MI, Kast WM (2003) Cervical cancer vaccines: recent advances in HPV research. Viral Immunol 16:111–121
75. Klencke B, Matijevic M, Urban RG, Lathey JL, Hedley ML, Berry M, Thatcher J, Weinberg V, Wilson J, Darragh T, Jay N, Da Costa M, Palefsky JM (2002) Encapsulated plasmid DNA treatment for human papillomavirus 16-associated anal dysplasia: a Phase I study of ZYC101. Clin Cancer Res 8:1028–1037
76. Niethammer AG, Xiang R, Becker JC, Wodrich H, Pertl U, Karsten G, Eliceiri BP, Reisfeld RA (2002) A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. Nat Med 8:1369–1375
77. Chen CH, Wang TL, Ji H, Hung CF, Pardoll DM, Cheng WF, Ling M, Wu TC (2001) Recombinant DNA vaccines protect against tumors that are resistant to recombinant vaccinia vaccines containing the same gene. Gene Ther 8:128–138
78. Cheng L, Ziegelhoffer PR, Yang NS (1993) In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. Proc Natl Acad Sci U S A 90:4455–4459
79. Tanigawa K, Yu H, Sun R, Nickoloff BJ, Chang AE (2000) Gene gun application in the generation of effector T cells for adoptive immunotherapy. Cancer Immunol Immunother 48:635–643
80. Drabick JJ, Glasspool-Malone J, King A, Malone RW (2001) Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization. Mol Ther 3:249–255
81. Mendiratta SK, Thai G, Eslahi NK, Thull NM, Matar M, Bronte V, Pericle F (2001) Therapeutic tumor immunity induced by polyimmunization with melanoma antigens gp100 and TRP-2. Cancer Res 61:859–863
82. Hobson P, Barnfield C, Barnes A, Klavinskis LS (2003) Mucosal immunization with DNA vaccines. Methods 31:217–224
83. Klavinskis LS, Barnfield C, Gao L, Parker S (1999) Intranasal immunization with plasmid DNA-lipid complexes elicits mucosal immunity in the female genital and rectal tracts. J Immunol 162:254–262
84. Perrie Y, Frederik PM, Gregoriadis G (2001) Liposome-mediated DNA vaccination: the effect of vesicle composition. Vaccine 19:3301–3310

85. Chen SC, Jones DH, Fynan EF, Farrar GH, Clegg JC, Greenberg HB, Herrmann JE (1998) Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. J Virol 72:5757–5761
86. Roy K, Mao HQ, Huang SK, Leong KW (1999) Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nat Med 5:387–391
87. Freimark BD, Blezinger HP, Florack VJ, Nordstrom JL, Long SD, Deshpande D.S, Nochumson S, Petrak KL (1998) Cationic lipids enhance cytokine and cell influx levels in the lung following administration of plasmid: cationic lipid complexes. J Immunol 160:4580–4586
88. Wheeler CJ, Felgner PL, Tsai YJ, Marshall J, Sukhu L, Doh SG, Hartikka J, Nietupski J, Manthorpe M, Nichols M, Plewe M, Liang X, Norman J, Smith A, Cheng SH (1996) A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. Proc Natl Acad Sci U S A 93:11454–11459
89. Barnfield C, Brew R, Tilling R, Rae A, Wheeler C, Klavinskis LS (2000) The cellular basis of immune induction at mucosal surfaces by DNA vaccination. Dev Biol (Basel) 104:159–164
90. Eldridge JH, Staas JK, Meulbroek JA, McGhee JR, Tice TR, Gilley RM (1991) Biodegradable microspheres as a vaccine delivery system. Mol Immunol 28:287–294
91. Iqbal M, Lin W, Jabbal-Gill I, Davis SS, Steward MW, Illum L (2003) Nasal delivery of chitosan-DNA plasmid expressing epitopes of respiratory syncytial virus (RSV) induces protective CTL responses in BALB/c mice. Vaccine 21:1478–1485
92. Toka FN, Pack CD, Rouse BT (2004) Molecular adjuvants for mucosal immunity. Immunol Rev 199:100–112
93. Klinman DM (2003) CpG DNA as a vaccine adjuvant. Expert Rev Vaccines 2:305–315
94. Audibert F (2003) Adjuvants for vaccines a quest. Int Immunopharmacol 3:1187–1193
95. Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, Wagner H, Lipford GB (2001) Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U S A 98:9237–9242
96. Krieg AM (2004) Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. Curr Oncol Rep 6:88–95
97. More SH, Breloer M, von Bonin A (2001) Eukaryotic heat shock proteins as molecular links in innate and adaptive immune responses: Hsp60-mediated activation of cytotoxic T cells. Int Immunol 13:1121–1127
98. Binder RJ, Han DK, Srivastava PK (2000) CD91: a receptor for heat shock protein gp96. Nat Immunol 1:151–155
99. Li Z, Menoret A, Srivastava P (2002) Roles of heat-shock proteins in antigen presentation and cross-presentation. Curr Opin Immunol 14:45–51
100. Amigorena S (1998) Anti-tumour immunotherapy using dendritic-cell-derived exosomes. Res Immunol 149:661–662
101. Kammerer R, Stober D, Riedl P, Oehninger C, Schirmbeck R, Reimann J (2002) Noncovalent association with stress protein facilitates cross-priming of CD8+ T cells to tumor cell antigens by dendritic cells. J Immunol 168:108–117
102. Xiang Z, Ertl HC (1995) Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. Immunity 2:129–135
103. Tazi A, Bouchonnet F, Grandsaigne M, Boumsell L, Hance AJ, Soler P (1993) Evidence that granulocyte macrophage-colony-stimulating factor regulates the distribution and differentiated state of dendritic cells/Langerhans cells in human lung and lung cancers. J Clin Invest 91:566–576
104. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–252
105. Hung CF, Hsu KF, Cheng WF, Chai CY, He L, Ling M, Wu TC (2001) Enhancement of DNA vaccine potency by linkage of antigen gene to a gene encoding the extracellular domain of Fms-like tyrosine kinase 3-ligand. Cancer Res 61:1080–1088
106. Sin JI, Kim J, Chattergoon M, Ayyavoo V, McCallus D, Ugen KE, Boyer JD, Weiner DB (2000) Engineering of DNA vaccines using molecular adjuvant plasmids. Dev Biol (Basel) 104:187–198
107. Hurwitz AA, Kwon ED, van Elsas A (2000) Costimulatory wars: the tumor menace. Curr Opin Immunol 12:589–596
108. Corr M, Tighe H, Lee D, Dudler J, Trieu M, Brinson DC, Carson DA (1997) Costimulation provided by DNA immunization enhances antitumor immunity. J Immunol 159:4999–5004
109. Dhiman N, Bonilla R, O'Kane DJ, Poland GA (2001) Gene expression microarrays: a 21st century tool for directed vaccine design. Vaccine 20:22–30
110. Villaret DB, Wang T, Dillon D, Xu J, Sivam D, Cheever MA, Reed SG (2000) Identification of genes overexpressed in head and neck squamous cell carcinoma using a combination of complementary DNA subtraction and microarray analysis. Laryngoscope 110:374–381
111. Sturniolo T, Bono E, Ding J, Raddrizzani L, Tuereci O, Sahin U, Braxenthaler M, Gallazzi F, Protti MP, Sinigaglia F, Hammer J (1999) Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. Nat Biotechnol 17:555–561
112. Alizadeh AA, Staudt LM (2000) Genomic-scale gene expression profiling of normal and malignant immune cells. Curr Opin Immunol 12:219–225
113. Miller JC, Zhou H, Kwekel J, Cavallo R, Burke J, Butler EB, Teh BS, Haab BB (2003) Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. Proteomics 3:56–63
114. Wang T, Hopkins DA, Fan L, Fanger G.R, Houghton R, Vedvick TS, Repasky E, Reed SG (2001) A p53 homologue and a novel serine proteinase inhibitor are over-expressed in lung squamous cell carcinoma. Lung Cancer 34:363–374
115. Wang T, Fan L, Watanabe Y, McNeill P, Fanger GR, Persing DH, Reed SG (2001) L552S, an alternatively spliced isoform of XAGE-1:is over-expressed in lung adenocarcinoma. Oncogene 20:7699–7709
116. Wang T, Fan L, Watanabe Y, McNeill PD, Moulton GG, Bangur C, Fanger GR, Okada M, Inoue Y, Persing DH, Reed SG (2003) L523S, an RNA-binding protein as a potential therapeutic target for lung cancer. Br J Cancer 88:887–894