Translational nanoparticle engineering for cancer vaccines

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ABSTRACT
Conventional cancer treatments remain insufficient to treat many therapy-resistant tumors.4 Cancer vaccines attempt to overcome this resistance by activating the patient’s immune system to eliminate tumor cells without the toxicity of systemic chemotherapy and radiation. Nanoparticles (NPs) are promising as customizable, immunostimulatory carriers to protect and deliver antigen. Although many NP vaccines have been investigated in preclinical settings, a few have advanced into clinical application, and still fewer have demonstrated clinical benefit. This review incorporates observations from NP vaccines that have been evaluated in early phase clinical trials to make recommendations for the next generation of NP-based cancer vaccines.

Introduction
Despite advances in chemotherapy, radiation, and surgical resection, cancer is the second leading cause of death in the United States.5 Cancer immunotherapy has recently produced significant advancements in treatment and is widely recognized as one of the major recent breakthroughs in clinical oncology.6 Adaptive cellular therapies, involving the transfer of \textit{ex vivo} expanded tumor-reactive lymphocytes, have produced substantial clinical responses in humans, but are complex modalities that require extensive cell preparation \textit{ex vivo}, resulting in high cost, limited availability, and significant regulatory hurdles to clinical approval.5 Vaccine therapies hold the promise of engendering long-lasting immunity by inducing innate cells including dendritic cells (DCs) to prime and activate T cells \textit{in situ}. However, to date, cancer vaccines have failed to achieve meaningful and durable responses in the majority of treated patients. Early vaccine development focused on the use of peptides as antigens. However, peptide-based vaccines require immunogenic carriers to activate DCs and are often limited to presentation by specific HLA alleles.5 Nucleic acid vaccines have been proposed as “universal” vaccines that bypass HLA restriction, but these require protection from proteases and entry into cytoplasm and/or nuclear membrane for translation into immunogenic peptides. NPs have been used to protect cargo from degradation, permit entry into cells, and stimulate DC maturation.5 As a result, immunostimulatory NP vaccines have been proposed as “off the shelf” vehicles to deliver antigen directly to antigen presenting cells (APCs) \textit{in vivo}.6,7 Although preclinical work has produced a multitude of NPs capable of delivering antigen to APCs \textit{in vitro} and in mouse models, only a few have been approved for investigational use in humans. Those that have been investigated have largely failed to produce significant clinical benefit in late phase clinical trials.8–10

This trend is consistent across NP disciplines. Despite massive increases in the number of articles published each year with the search term “nanoparticle” in the biomedical literature, a few applications have progressed into clinical evaluation.11 Analysis of the traits that facilitate rapid translation could inform development of nanotherapeutics likely to reach clinical application and ultimately improve patient outcomes. This review provides a critical analysis of NPs that have been used as tumor vaccines in humans. Clinical and preclinical literature are synthesized to make recommendations on NP engineering and trial design criteria for optimal antitumor efficacy and translation. Application of the insights gained in this review to early NP development may lower regulatory barriers and hasten the development of effective NP vaccines for cancer treatment.

Overview of nanoparticle vaccines investigated in humans
Of 1,564 clinical trials listed on ClinicalTrials.gov with “(nanoparticle OR liposome) AND cancer” as search terms, only 76 utilize delivery of antigen for cancer treatment. Within this group, clinical trial results are reported in PubMed for only nine nanoparticle products (Table 1). Although this sample size is small, the similarities of these NPs may be useful to develop treatments of rapid clinical use. This review begins with a description of each vaccine and its use in clinical trials. Critical analysis of all of these NPs is then used to develop design criteria for future NP vaccines for cancer therapy.
Table 1. NP-based cancer vaccines for antigen delivery currently being used in humans.

| NP composition | Adjuvant | Antigen | Size (nm) | Disease | Trials | Ref. |
|----------------|----------|---------|-----------|---------|--------|------|
| Tecemotide (L-BLP25, StimuVax) | Cholesterol, DMPG, DPPC | BLP25, Monophosphoryl Lipid A | MUC1 | 150–580 | Breast cancer, NSCLC, prostate cancer, CRC | 14 |
| AS15 | Monophosphoryl Lipid A, QS-21 | CpG7909 | MAGE-A3, dHER2 | ND | Metastatic melanoma, NSCLC, breast cancer | 24 |
| Lipovaxin-MM | POPC, 3NTA-DTDA, NISO4 | IFN-γ | Recombinant proteins from MM200 cells | 240 | Stage IV melanoma | 1 |
| DepoVax | Phosphatidyl choline: Cholesterol 10:1 | Montanide ISA 51, tetanus toxoid | 7 HLA-A2 restricted TAA or survivin peptides | 120 | Breast, ovarian, and prostate cancer | 6 |
| RNA-LPX | DOTMA, DOPE | None | MAGEA3, tyrosinase, NY-ESO1, TPTE mRNA | 200–400 | Stage IIIb–IV melanoma | 1 |
| OncoVax – Id/ IL-2 | DMPC | IL-2 | Autologous idiotype protein | NF | Follicular lymphoma | 50 |
| CHP | Pullulan, cholesterol isocyanate | +/- GMCSF or OK-432 | Recombinant MAGE-A4, truncated 164HER2, or NY-ESO-1 protein | 20–50 | Breast, esophageal, stomach, lung cancer | 4 |
| ISCOMATRIX | Cholesterol, phospholipid | Quillaja saponin | Recombinant NY-ESO-1 protein | 40–50 | NY-ESO-1 expressing tumors | 2 |
| VLP | Qβ bacteriophage | A-type CpG | Melan-A/MART-1 peptides | 30 | Stage I–IV melanoma | 5 |

Composition, vaccine design, size, disease targeted, and number of trials registered on ClinicalTrials.gov evaluating use in cancer patients for each of the nine NP vaccines. DMPG, dimyristoyl-phosphatidylglycerol; DPPC, dipalmitoyl phosphatidylcholine; DOPE, 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTMA, 1, 2-di-O-octadecenyl-3-trimethylammonium propane; ND, not determined; POPC, α-palmitoyl-β-oleoyl-phosphatidylcholine; 3NTA-DTDA, 3(nitriilotriacetic acid)-ditetradecylamine.

**Tecemotide**

**Vaccine design**

Tecemotide (L-BLP25, StimuVax) is a 150–580 nm liposome composed of cholesterol, dimyristoyl phosphatidylglycerol (DMPG), and dipalmitoyl phosphatidylcholine (DPPC). Tecemotide delivers MUC1 glycoprotein, which is overexpressed on the apical surfaces of epithelia in many mucosal cancers, in the presence of the immunostimulatory lipid BLP25 and TLR4 agonist Monophosphoryl Lipid A, which is known to induce a shift toward Th1 polarization and CD8+ T-cell response.

**Clinical studies**

Tecemotide is safe, immunogenic, and may provide clinical benefit for subsets of patients with non-small cell lung cancer (NSCLC) (Table 2). Although phase II and III trials failed to demonstrate significant survival benefit for patients with NSCLC, subgroup analyses found significant survival benefit in patients with stage IIIb locoregional disease and patients treated concurrently with cyclophosphamide. However, a subsequent study to evaluate Tecemotide with concurrent cyclophosphamide treatment was stopped prematurely after a fatal encephalitis that may have been caused by multiple repeated doses of cyclophosphamide and Tecemotide. Nevertheless, Tecemotide is currently being investigated in an ongoing multinational phase III trial of colorectal cancer following curative resection of hepatic metastases and as maintenance therapy for patients with phase III NSCLC.

**AS15**

**Vaccine design**

AS15 is an “immunostimulatory lipid” that is co-delivered with Melanoma Associated Antigen 3 (MAGE-A3) protein as a treatment of metastatic melanoma and NSCLC or recombinant HER2 protein (dHER2) as treatment of breast cancer. MAGE-A3 is a tumor-associated cancer/testes antigen that is expressed in 24% of patients with NSCLC and is associated with poor prognosis. Human Epidermal Growth Factor Receptor 2 (HER2) is overexpressed on surfaces of some malignant breast cancer cells. AS15 contains the saponin QS21 that activates the inflammasome in APCs when co-administered with Monophosphoryl Lipid A. AS15 has been combined with CpG analogs to further potentiate response. CpG7909 is of particular interest as a TLR9 agonist used to stimulate plasmacytoid DCs to produce robust CD8+ T-cell immunity.

**Clinical trials**

AS15 trended toward benefit to overall survival in Phase II and III studies and produced complete responses in 3/36 patients with stage III or IV metastatic melanoma. Treated patients demonstrated increased antibody production and CD4+ T-cell activation. AS15 induced significantly increased CD4+ and CD8+ T-cell responses in patients with unresected stage IB-III MAGE-A3 positive NSCLC, suggesting that tumor tissue may serve to augment immunologic response. AS15 also demonstrated safety, antibody responses, and a trend toward increased disease-free survival using HER2 peptide as antigen in a Phase I trial. However, a large, randomized, double blind Phase III trial to evaluate use of AS15 in NSCLC patients failed to improve disease-free survival.

**DepoVax**

**Vaccine design**

DepoVax (DPX) is a 120-nm liposomal vaccine composed of a 10:1 mix of Phosphatidyl Choline:Cholesterol that is used in multiple formulations. DPX-0907 delivers seven tumor-associated antigens (TAAs) that are expressed by MHC I on an ovarian cancer cell line. DPX-Survivac delivers survivin, an inhibitor of apoptosis protein that is overexpressed in a variety of cancers and expressed on normal tissues at only low levels. Both DPX vaccines are co-delivered with tetanus toxoid, a treatment of breast cancer.
| Vaccine          | Phase | Design   | Patients | Control treatment | Endpoint          | Result (T cell/Ab) | Immune response | Immune response | Disease            | Combinatorial treatments | Ref. |
|------------------|-------|----------|----------|-------------------|-------------------|--------------------|-----------------|-----------------|-------------------|-------------------------|------|
| Tecemotide       | II    | R, 2 arm | 17       | NUC               | Safety/Immunogenicity | pos/pos            | neg/neg         | Stage IIIB/IV NSCLC | Cyclophosphamide: single IV dose | 13   |
|                  | III   | R, NB, 2 arm, ITT | 171       | BSC               | Median survival     | neg/g/pos          | neg/neg         | Stage IIIB/IV NSCLC | Cyclophosphamide: single IV dose | 12   |
|                  | III   | R, DB, 2 arm, MITT | 1513      | Placebo           | Overall survival    | neg/pos            | pos/NR          | Stage III NSCLC     | Cyclophosphamide: single IV dose | 8    |
|                  | II    | R, NB, 2 arm | 34        | NUC               | Antigen-specific T-cell response | pos/pos            | pos/NR          | Multiple myeloma, stage II/III untreated or stable II/III | Cyclophosphamide: single IV dose or metronomic low doses | 17   |
|                  | II    | NR, NB    | 22        | NUC               | Safety              | pos/NR/NR          | Unresectable stage III NSCLC | Cyclophosphamide: single IV dose 3 d before treatment | 83   |
|                  | I/II  | NR, NB    | 6         | NUC               | Safety              | pos/NR/NR          | Unresectable stage III NSCLC | Cyclophosphamide: single IV dose 3 d before treatment | 19   |
|                  | Pilot study | NR, NB, single arm | 16      | NUC               | Safety              | pos/NR/NR          | Prostate cancer   | Cyclophosphamide: single IV dose 3 d before treatment | 84   |
|                  | AS15  | Phase II  | 75        | AS02              | Survival/Safety     | neg/pos            | Stage IIIB/IV melanoma |                     | 10   |
|                  |       | Phase III | R, NB, 2 arms, T | 2312 | Placebo           | Disease-free survival | neg                | Stage II, II, IIIA NSCLC | With or without cisplatin | 9.21 |
|                  |       | Phase 0   | R, 2 arms | 25        | NUC               | Safety/T-cell response | pos/pos     | Stage IIIB/IV NSCLC | Concurrrent with, after, or without cisplatin and vinorelbine | 77   |
|                  |       | Phase I/II | 4 arms, NR, T | 67 | NUC               | Safety/T-cell response | pos/pos     | Stage IIIB/IV NSCLC | Laptatinib               | 28   |
| CHP-MAGE-A4      | Phase I | 4 arms, NR, DE | 61        | NUC               | Safety/Humoral response | pos/NR/NR         | Stage II–III HER-2+ breast cancer |                     | 20   |
|                  | Phase I | NR, NB    | 12        | NUC               | Safety/Humoral immunity | pos/neg            | HER-2+ breast cancer |                     | 85   |
|                  | Phase I | NR, NB    | 9         | NUC               | Humoral immunity    | pos              | Esophageal cancer, prostate cancer, and melanoma |                     | 33   |
|                  | Phase I | 2 Arm NR, DE, T | 25      | NUC               | Safety/Humoral immunity/overall survival | pos/ND/pos | Stage IV esophageal cancer |                     | 34   |
| CHP-NY-ESO-1     | Phase I | NR, NB, single arm | 8        | NUC               | Safety/Immunogenicity | pos/ND/pos       | NY-ESO-1 “HER-2” esophageal cancer | OK-432               | 36   |
| CHP-NY-ESO-1 and CHP-HER2 | Phase I | NR, NB, single arm | 8        | NUC               | Safety/Immunogenicity | pos/ND/pos       | NY-ESO-1 “HER-2” esophageal cancer |                     | 31   |
| CHP-HER2         | Phase I | NR, NB, 2 arm | 9         | NUC               | Safety/T-cell response | pos/ND            | HER-2+ cancers | GMCSF or OK-482    |                     | 32   |
| ISCOMATRIX       | Phase I | NR, NB, 2 arms | 15        | NUC               | Safety/Humoral immunity | pos/pos          | HER-2+ cancers | GMCSF (75 μg × 5 d) |                     | 42   |
|                  | Phase I | NR, DB, DE, 2 arms | 46       | Placebo           | Safety/Immunogenicity | Pos/pos           | NY-ESO-1+ tumors (melanoma, bladder, rectal, breast) |                     | 43   |
|                  | Phase I | R, B, DE   | 31        | Placebo           | Safety/Immunogenicity | Pos/pos           | Cervical intraepithelial neoplasia | Cyclophosphamide: single IV dose or LAGE-1+ melanoma | 40.5,7 |
|                  | Phase I | R, B, 2 arms | 46        | NUC               | Safety/Immunogenicity | pos/pos           | Stage IV or unresectable stage III NY-ESO-1 or LAGE-1+ melanoma |                     | 44   |
| DPX-0007         | Phase I | NR, NB, 2 doses | 23        | NUC               | Safety              | pos/ND            | Resected melanoma patients vaccinated with NY-ESO-1 ISCOMATRIX | Fowlpox virus containing recombinant full length NY-ESO-1 | 7    |
| DPX-Survivin     | Phase I | NR, NB, 3 arm | 19        | NUC               | Safety/Immunogenicity | pos/ND            | Breast, ovarian, and prostate cancer | Concurrent metronomic cyclophosphamide or single dose | 29   |
| OncoVAX-id/IL-2  | Phase I | NR, NB, 1 arm | 10        | NUC               | Safety/Immunogenicity | pos/ND            | Ovarian cancer in 1st or 2nd remission |                     | 50   |
| Lipo-MERIT       | Phase I | NR, NB, DE | 3         | NUC               | Safety              | ND/pos            | Follicular lymphoma | >6 cycles of PACE regimen completed before treatment | 6    |
| VLP              | Phase I | R/NR, NB, 4 arm | 22        | NUC               | Safety/Immunogenicity | pos/pos          | Stage IIIB/IV melanoma |                     | 47   |
| Lipo-MERIT       | Phase II | 4 arm | 21        | NUC               | Immunogenicity/Safety | pos/pos          | Stage IIIB/IV melanoma |                     | 48   |

Design and outcome information for each nanoparticle vaccine with published clinical trial results. "Results" represents the outcome of the primary endpoint for the general treated population. Therefore, a negative result does not preclude effect in subgroup analysis.

BSC, best supportive care; DB, double blind; DE, dose escalation; ITT, intention to treat analysis; MITT, modified ITT; NB, nonblinded; ND, not determined; neg, no statistically significant improvement or an increase in less than 50% of treated patients if no control cohort is available for comparison; NR, non-randomized; NUC, no untreated control; pos, statistically significant improvement or increase in greater than 50% of patients if no control cohort is available for comparison; R, randomized; T, only treated patients considered in statistical analysis. "T-cell response" includes both flow cytometry to quantify numbers of increased antigen-specific T cells and ELISAs of peripheral blood to evaluate IFN-γ-release after restimulation with tumor antigen.
proprietary TLR9 agonist, and Montanide ISA 51, which is a Freund’s Adjuvant called Montanide ISA 51, and a proprietary TLR9 agonist.29

**Clinical trials**

DPX-0907 induced persistent antigen-specific T-cell responses in 39% of patients with breast, ovarian, or prostate cancer in a Phase 1 trial.29 Interestingly, individual patients responded to unique sets of antigens.29 A trial using DPX-Survivac to treat patients with ovarian cancer included a metronomic cyclophosphamide regimen to selectively eliminate regulatory T cells (TReg) while allowing the proliferation of effector cells targeted against multiple survivin peptides.29 Although no patients demonstrated evidence of objective clinical response, all patients in this trial generated antigen-specific CD8+ T-cell responses in peripheral blood after three subcutaneous vaccines.29

**CHP**

**Vaccine design**

Cholesteryl pullulan (CHP) nanogels deliver peptides of MAGE-A4 and NY-ESO-1 or recombinant proteins of HER2.31-34 Preclinical models suggest that these “immunologically stealth” particles act mainly via medullary macrophages in lymph nodes after subcutaneous injection.35 CHP-HER2 NPs are sometimes combined with GMCSF or OK-432, a killed, low virulence strain of Streptococcus pyogenes that activates TLR-4.31,36

**Clinical trials**

CHP-NY-ESO-1 demonstrated widespread T-cell and antibody responses that correlated with PSA stabilization in prostate cancer patients and tumor regression in esophageal cancer and melanoma patients whose tumors expressed NY-ESO-1 protein.37-39 Subsequent studies found enhanced survival benefit in esophageal cancer patients who received 200 µg dose compared with 100 µg.40 CHP-MAGE-A4 induced substantial antibody responses that correlated with prolonged progression free and overall survival among esophageal cancer patients whose tumors highly expressed MAGE-A4 protein.33 However, treatment also resulted in increased prevalence of CD4+FoxP3+CD45RA+ regulatory T cells in peripheral blood.33 Likewise, CHP-HER2 induced antibody responses in almost all patients but was associated with loss of tumor antigen, presence of CD68+ macrophages, and reduced CD4+ and CD8+ infiltration in tumor after treatment.32,34

**ISCOMATRIX**

**Vaccine design**

ISCOMATRIX is a 50-nm particle containing saponin, cholesteryl, and phospholipid designed to increase cross-presentation of MHC class I restricted epitopes.41 ISCOMATRIX has been used to deliver recombinant E6/E7 peptide to treat cervical intraepithelial neoplasia (CIN) or NY-ESO-1 protein as treatment of NY-ESO-1 positive melanoma, bladder, rectal, and breast cancer.42,43

**Clinical trials**

ISCOMATRIX originally garnered enthusiasm after a Phase I trial demonstrated dose-dependent antibody responses and reduced risk of relapse for patients with NY-ESO-1 positive tumors.45 However, Phase II trials failed to demonstrate CD8+ T-cell responses in patients with advanced disease and instead revealed production of an antigen-specific TReg population in vaccinated patients.44-46 A current search on ClinicalTrials.gov revealed two terminated studies and one suspended study with this drug for cancer treatment (NCT01341496, NCT02054104, and NCT01258868).

**VLPs**

**Vaccine design**

Virus-like nanoparticles (VLPs), also known as CYT004-MelQbG10, are generated from the coat protein of bacteriophage Qb and loaded with the CpG “G10”.47 This A-type CpG is known to stimulate IFN-α production by plasmacytoid dendritic cells (pDCs) to a greater degree than the B-Type CpG found in Montanide via stimulation of TLR9, but was previously unable to be used in humans due to rapid degradation by DNase.47 VLPs deliver peptides of melanoma-associated antigens Melan-A/MART-1.47,48

**Clinical trials**

A randomized Phase I trial found that VLPs safely generate T-cell responses in most patients with Stage II–IV melanoma.47 Comparisons of multiple vaccination routes demonstrated increased production of antigen-specific T cells and effector memory and central memory subsets of antigen-specific T cells with intradermal or subcutaneous injection compared with intranodal injection.48

**OncoVAX-Id/IL-2**

**Vaccine design**

OncoVAX-Id/IL-2 was designed to generate more robust responses and a more homogeneous product after a protein conjugate vaccine demonstrated efficacy in follicular lymphoma patients in complete remission.49 OncoVAX-Id/IL-2 incorporates protein for autologous lymphoma idiotypes and recombinant IL-2 as adjuvant inside a dimyristoylphosphatidylcholine (DMPC) liposome.50

**Clinical trials**

A Phase I trial with 10 patients found that OncoVAX-Id/IL-2 safely induced T-cell responses in 100% of patients and antibody responses in 40% of follicular lymphoma patients in clinical remission.50
Lipovaxin

**Vaccine design**

Lipovaxin is a 240-nm liposomal NP that delivers recombinant protein antigen and IFNγ intravenously (IV) to treat Stage IV melanoma. This vaccine is composed of membrane vesicles from MM200 melanoma cells fused to POPC (α-palmitoyl-β-oleoyl-phosphatidylcholine) liposomes for stability and an anti-DC SIGN (DC-specific intercellular adhesion molecule 3 grabbing nonintegrin) immunoglobulin single variable domain to increase DC uptake.

**Clinical trials**

A Phase I trial evaluating Lipovaxin was completed in 2012, but no results have been reported (NCT01052142).

Lipo-MERIT

**Vaccine design**

Lipo-MERIT is a 200-nm liposomal vaccine that delivers mRNA encoding four common melanoma antigens (NY-ESO-1, MAGE-A3, tyrosinase, and TPTE) to APCs. The 200–400 nm particle contains DOTMA (1,2-di-O-octadecenyl-3-tri-methylammonium propane) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and is targeted to lymphoid organs after systemic injection by modifying lipid:RNA ratios, so that the net particle charge is negative.

**Clinical trials**

Lipo-MERIT is currently in a Phase I trial to assess safety and dosing for patients with stage IV melanoma (NCT02410733). Although final results have not been reported, the first three patients developed flu-like symptoms, released IFN-α and IP-10, and increased levels of antigen-specific CD4+ or CD8+ T cells.

**Critical analysis of clinical outcomes**

These trials and NPs have many similarities. While all studies report either antibody or T-cell response, no trials demonstrated statistically significant survival benefit in primary analysis. The lack of clinical benefit despite immune outputs indicates that responses are either of the wrong character, insufficient magnitude, or too brief in duration. These insufficient outcomes may result from poor biodistribution or immunosuppression via tumor-induced mechanisms such as immune checkpoint activation. Understanding the impact of particle characteristics, combination therapies, and study design may inform development of successful therapeutics.

**Study design**

**Outcome variable**

Primary endpoints for each trial are included in Table 2. Although T cells are intended mediators of antitumor responses, their activation and proliferation is often not a direct predictor of clinical response. As an example, clinical responders to AS15 did not have increased rates of CD4+ or CD8+ T-cell responses. Similarly, although proliferative response of antigen-specific T cells in the blood correlated with increased median survival among all patients vaccinated with Tecemotide (n = 88), the subset of patients that showed a survival benefit compared with controls (n = 35) included only two patients with proliferative T-cell responses. Both analyses are limited by sample size and tepid responses to treatment, but this combined evidence may indicate that detectable levels of antigen-specific T cells are neither necessary nor sufficient to produce clinical response to vaccination.

Another possibility is that T-cell characterization as CD4+ and CD8+ is insufficient to predict outcome. Instead, a tipping of the balance between effector, memory, and regulatory T cells may be necessary for rational vaccine design to induce clinical antitumor immunity. This effect was evaluated in Tecemotide, in which immunologic responders generated reduced effector and effector memory CD4+ T cells. In another approach, Berinstein and colleagues differentiated T cells functionally and found that DepoVax induced increased production of polyfunctional central and effector memory T cells. Both analyses were applied to evaluate responses to VLPs, which induce production of multifunctional effector memory and central memory antigen-specific T cells.

Furthermore, regulatory responses may counteract effector T cells. CHP-MAGE-A4 nanogels and NY-ESO-1/ISCOMATRIX both induced significant formation of regulatory T cells in many patients. In one patient with metastatic melanoma, CHP-NY-ESO-1 induced systemic humoral and cellular responses but also high levels of CD4+CD25+Foxp3+ regulatory T cells and macrophages at the tumor sites. This patient ultimately succumbed to metastatic pulmonary infiltrates. Likewise, histologic analysis of tumor from a small number of patients treated with CHP-HER2 revealed increased CD68+ macrophages, reduced CD4+ and CD8+ infiltration, and loss of antigen expression after treatment. Interestingly, clinical responses to CHP-MAGE-A4 correlated with antibody production.

Studies to corroborate the relationship between these immunological classifications and clinical outcomes will likely demonstrate roles for CD8+ T-cell phenotype, regulatory cells, and CD4+ T cells in evaluation of vaccine efficacy.

**Combinatorial treatments**

Synergy between antigen-specific T-cell activation and chemotherapy has significant implications on future trial design. Combination chemotherapy improved quality or quantity of T cell responses generated by AS15, Tecemotide, ISCOMATRIX, and DepoVax. One proposed explanation is that cyclophosphamide selectively reduces the regulatory T-cell compartment. Additionally, the lymphodepletion induced by chemotherapy may induce homeostatic proliferative responses that benefit vaccine-induced T cells. Although somewhat counterintuitive, we also observed enhancing effects of lymphodepletive chemotherapy in vaccinated patients with glioblastoma. Murine experiments suggest that lymphodepletion is followed by a surge of IL-7 that stimulates lymphocyte proliferation. In the setting of vaccination, IL-7 can be...
co-opted to dramatically enhance expansion of tumor-reactive T cells.\textsuperscript{62,64}

Combination therapy with other vaccine approaches may also avoid barriers to efficacy. ISCOMATRIX vaccine demonstrated this principle in showing increased generation of CD8\textsuperscript{+} T-cell responses when paired with a fowlpox virus vaccine bearing the same antigen.\textsuperscript{44} Although unexplored for NP-based vaccines, treatment with immune checkpoints may also potentiate vaccine efficacy by reducing the effect of vaccine-generated T\textsubscript{Reg} and potentially overcoming tumor-induced immunosuppression.

Vaccine design

Antigen selection

Choice of antigen may also explain low treatment efficacy. None of the described trials targeted tumor-specific neoantigens. Instead, these studies generated responses against overexpressed antigens (Her2, survivin, and telomerase), lineage-restricted antigens (tyrosinase), and cancer-testis antigens (MAGE and NY-ESO). The low immunogenicity of overexpressed peptide antigens may explain the presence of antibody and CD4\textsuperscript{+} T-cell responses without clinical response.\textsuperscript{3,8,9,12,20,28} Selection of appropriate peptides within a protein antigen may also improve vaccinations. The importance of peptide selection was demonstrated with ISCOMATRIX, in which some epitopes stimulated antitumor T-cell responses to cryptic antigens and others generated functional T\textsubscript{Reg} specific for tumor antigens.\textsuperscript{43,65}

Many vaccines contained only a single cancer antigen. However, this strategy allows tumor cells to escape immune detection upon loss of the selected vaccine target.\textsuperscript{66} This "antigen loss" was recorded after use of both VLRs and CHP nanogels.\textsuperscript{34,48} Efforts to reduce this risk focus on the use of multiple antigens. Individual patients vaccinated with the seven TAA’s encoded within DPX-0907 responded to unique subsets of these antigens.\textsuperscript{7} The same was true of responses to Lipo-MERIT, which went further by using a variety of types of cancer antigens.\textsuperscript{6} Although sample size is small, each of the three patients responded to a unique set of two of the four antigens in the vaccine.\textsuperscript{8} However, studies with CHP showed that vaccination with multiple antigens may reduce the magnitude of humoral responses to specific antigens compared with single antigen vaccination.\textsuperscript{36} Therefore, multiple antigens are likely needed to induce clinically significant responses, but further studies are needed to understand how distinct cancer antigens can be combined effectively.

Antigen form

Peptides bind avidly to MHC molecules but are limited to patients who express certain HLA molecules. Recombinant protein allows presentation of additional MHC I and MHC II epitopes, but requires a carrier that facilitates antigen presentation on both MHC molecules.\textsuperscript{45} While protein and peptide vaccines have consistently achieved measurable immunologic responses, low clinical efficacy and HLA restriction encouraged the development of nucleic acid based vaccination strategies.\textsuperscript{6,7,10} Although the bulk of work with nucleic acid vaccines uses DNA, mRNA is attractive for NP delivery because it is intranatly immunogenic and does not require entry into the nuclear membrane. LipoMERIT was the first human trial to evaluate the systemic administration of mRNA liposomes as vaccines in humans.\textsuperscript{6} While preliminary data are promising, survival benefit will be necessary to determine the utility of this development.

Adjuvant

Multiple adjuvants have been used to bolster clinical responses. These include IFA, which increased numbers of antigen-specific and effector memory CCR7\textsuperscript{+}CD45RA\textsuperscript{−} T cells in response to VLPs, GMCSF, which accelerated antibody production after vaccination with CHP-HER2, and CPG7909, which trended toward providing survival benefit when combined with AS15.\textsuperscript{10,32,48} Other adjuvants produced mixed results, such as the TLR7 agonist Imiquimod, which reduced percentages of antigen-specific T cells but increased CCR7\textsuperscript{+}/CD45RA\textsuperscript{−} central memory phenotype and CD127\textsuperscript{+} T cells.\textsuperscript{48}

Effects of NP composition on DC activation

While insufficient information on characteristics of the available NP constructs makes clinical comparisons difficult, preclinical evidence suggests that NP composition can significantly alter DC response. Many NPs, including Lipo-MERIT, are known to be taken up by DCs via multiple endocytosis pathways including macropinocytosis, but definitive analysis of the best pathway for subsequent antigen presentation remains elusive.\textsuperscript{6,66} Studies in other cell types have suggested that addition of cationic lipids to otherwise neutral NPs changed the intracellular destination of NPs.\textsuperscript{68} Notwithstanding the lack of mechanistic explanation, cationic NPs have generally emerged as more effective than neutral or anionic NPs for stimulating DC activation \textit{in vitro}.\textsuperscript{69,70} Soema et al. recently explored multiple variables with a Design of Experiment model to determine that optimal DC activation occurs at a NP charge of +30 mV.\textsuperscript{71} Other groups found similar benefits of cationic NPs with diverse starting materials using elegant screening methods \textit{in vitro}.\textsuperscript{72,73} However, \textit{in vitro} studies are likely not sufficient to draw conclusions on translational potential since the characteristics that determine particle uptake are also thought to govern NP localization after injection.

Delivery method

Effect of composition on localization

Clinical trials included subcutaneous (SC), intramuscular (IM), intradermal (ID), and intravenous (IV) vaccine administration (Table 3). Differences in trial outcomes may be reflected by the different cell populations targeted with each method. ID and SC administration are thought to lead to NP uptake by immature DC subsets including Langerhans cells and CD14\textsuperscript{+} dermal DCs that migrate to lymph nodes and stimulate CD8\textsuperscript{+} T-cell responses.\textsuperscript{74} However, CD8\textsuperscript{+} T-cell response may be more readily generated with transfection of multiple DC subtypes including those deep within lymph nodes.\textsuperscript{75,76} IV vaccines, on the other hand, are thought to circulate before transfecting APCs in lymphoid organs.\textsuperscript{8} Consideration of the DC subsets that will be activated by each injection route should influence selection of injection method. Although CD8\textsuperscript{+} T-cell responses were seen with IM administration of AS15, a recently reported human study of AS15 found that while no routes induced
robust CD8⁺ T-cell responses, ID and SC administration tended toward increased CD4⁺ T-cell responses compared with IM vaccination.⁷⁷ Studies with VLP corroborate a lack of difference between ID and SC injection and demonstrate reduced antigen-specific, effector, and memory T cells after direct intranodal injection.⁴⁸ Other injection methods have been explored preclinically, including intranasal vaccination, which has been shown to generate robust DC accumulation in pulmonary LNs and produce CD8⁺ T-cell and antibody responses in peripheral blood in mice.⁷⁸ Future human studies should consider all available forms of vaccination and select the most optimal based on NP design and desired response.

Once a delivery method is decided, simple modifications can be made to enhance delivery to target organs. None of the clinical studies track NP fate, but lessons from preclinical work may be extrapolated to understand results of clinical trials. Although cationic liposomes increase DC function and activation in culture, positively charged particles accumulate als. Although cationic liposomes increase DC function and work may be extrapolated to understand results of clinical trials, particle localization in humans will require consistent reporting of size and charge in ongoing trials. Despite the clear importance of particle localization, none of the evaluated trials track particle fate in humans. Future particle development will require adoption of clinically relevant methods to determine effects of particle characteristics on particle localization in humans.

### Future trial design

Although promising, nanoparticle vaccines face many challenges in practice. Tolerance induced by long-term exposure to tumor antigens and the immunoregulatory tumor microenvironment may contribute significantly to this underwhelming clinical benefit. However, combination with immune checkpoint inhibitors may overcome these barriers. By "turning off the brakes" on the immune system, anti-CTLA-4, anti-PDL-1, and anti-PD-1 antibodies prevent tolerance induction and allow unfettered T-cell activation. Although combination therapies are more challenging to evaluate in clinical trials, the clinical approval and acceptance of ipilimumab (anti-CTLA-4), pembrolizumab (anti-PD-1), and nivolumab (anti-PD-1) for a variety of advanced malignancies has paved the way for the initiation of a myriad of combinatorial therapeutic trials. Therefore, to unleash the full therapeutic potential of nanoparticle vaccines, future trials will likely be designed in combination with agents that remodel the intratumoral microenvironment.

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**Table 3. Injection methods and vaccination schedule for NP vaccines in clinical trials.**

| NP | Vaccination schedule | Vaccination method | References |
|----|----------------------|--------------------|------------|
| Tecmotide | 8 doses weekly, then continued doses every 6 weeks until progression | 4 SC sites | 8,12,17,83,84 |
| AS15 | Biweekly for 12 weeks, triweekly for 18 weeks, every 6 weeks for 4 mo, quarterly for 1 y, and biannually for 3 y | IM | 10 |
| DepoVax | 3 doses triweekly | IM or ID/SC | 10 |
| Lipovaxin | ND | IV | 54 |
| Lipo-MERIT | Weekly vaccinations with increasing dose | IV | 6 |
| OncoVAX - Id/IL-2 | Months 0, 1, 2, 3, and 5 | 4 SC sites | 31,33,36,40 |
| CHP-NY-ESO-1 or CHP-HER2 | 6 doses biweekly | SC | 31 |
| | 14 biweekly doses | SC | 34 |
| | 3 doses biweekly | SC | 31 |
| | 4 doses biweekly, then regularly up to 12 vaccinations | SC | 32,39 |
| ISCOMATRIX | 3–6 doses monthly | IM | 42,44,51 |
| | Weeks 1, 3, 5, 9, then 3 monthly, then 1 every 12 weeks until progression | IM | 46,57 |
| | 1–3 doses weekly | IM | 43,57 |
| VLP | Weekly or daily for 14 weeks | SC or IM | 47 |
| | 3 weekly, then 3 monthly | SC, ID, or intranodal | 48 |

ID, intradermal; IM, intramuscular; SC, subcutaneous; triweekly, every third week.
Conclusions
NP vaccines that have been investigated in humans have many similarities. Most contain components that were approved for use in other products and are co-delivered with previously approved immunostimulatory adjuvants. Analysis of these early studies indicates the need for multiple, tumor-specific antigens, rational selection of combinatorial treatments, and NP design specific to route of administration. Future studies should consider the impact of NP characteristics on particle localization, determine immune correlates that accurately predict clinical outcomes, and consider combination therapy with immune checkpoint inhibitors. Consistent reporting of NP dict clinical outcomes, and consider combination therapy with resected MAGE-A3-Positive non-small-cell lung cancer (NSCLC). Ann Oncol 2014; 25:409-16; PMID:24368400; https://doi.org/10.1093/annonc/mdu089.

Disclosure of potential conflicts of interest
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