Progress on new vaccine strategies for the immunotherapy and prevention of cancer

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In recent years, great strides in understanding and regulating the immune system have led to new hope for harnessing its exquisite specificity to destroy cancer cells without affecting normal tissues. This review examines the fundamental immunologic advances and the novel vaccine strategies arising from these advances, as well as the early clinical trials studying new approaches to treat or prevent cancer.

Despite multiple approaches to therapy and prevention, cancer remains a major cause of death worldwide. Most nonsurgical approaches targeting rapidly dividing cells, using radiotherapy or chemotherapy, also affect normal cells and result in side effects that limit treatment. In principle, the exquisite specificity of the immune system could be marshaled to precisely target cancer cells without harming normal cells. This hope has motivated much research over several decades but has met with only limited success to date. However, the rapid increase in knowledge of the immune system and its regulation have led to a resurgence of interest in immunologic approaches to target and eliminate cancer (1–7).

A major difference between microbial pathogens and tumors as potential vaccine targets is that cancer cells are derived from the host, and most of their macromolecules are normal self-antigens present in normal cells. To take advantage of the immune system's specificity, one must find antigens that clearly mark the cancer cells as different from host cells (1, 2), limiting the number of antigens available. Additionally, many potential tumor antigens are not expressed on the surface of tumor cells and thus are inaccessible to antibodies.

The immune system has evolved a solution to this problem: the MHC antigens (HLA molecules in humans) that act as an internal surveillance system to detect foreign or abnormal proteins made inside the cell (Figure 1) (8, 9). A sampling of all proteins synthesized in the cell is cleaved by proteasomes into short fragments (peptides) that are transported into the endoplasmic reticulum. There, the peptides are loaded onto newly synthesized class I MHC molecules, such as HLA-A, -B, and -C. The peptide-MHC complexes are transported to the cell surface for recognition by the T cell receptors (TCRs) of CD8+ T lymphocytes, such as CTLs. Thus, CTLs recognize short peptides, 8–10 amino acid residues in length, arising from the proteasomal degradation of intracellular proteins and able to bind to class I HLA molecules. For this reason, CTLs are not limited to tumor antigens expressed intact on the cell surface but can detect any abnormal protein synthesized in the cell, greatly expanding the range of tumor antigens detectable by the immune system. Furthermore, CTLs play an important role in the rejection of transplanted organs and tissues (10), analogous to tumors as foreign or abnormal human cells invading the host. Thus, although monoclonal antibodies have clearly shown therapeutic efficacy in certain cancers (e.g., trastuzumab, rituximab, alemtuzumab) (11), most cancer vaccine strategies have focused on induction of CTLs that lyse tumor cells. Recent understanding of the mechanisms of activation and regulation of CD8+ T cells has given new life to tumor immunology. Notwithstanding the critical role of CD8+ T cells, induction of tumor-specific CD4+ T cells is also important not only to help CD8+ responses, but also to mediate antitumor effector functions through induction of eosinophils and macrophages to produce superoxide and nitric oxide (12).

For naive CD8+ T lymphocytes to be activated initially, or "primed," they generally require presentation of antigens by professional APCs, such as DCs (13). DCs express high levels of costimulatory molecules, such as CD80 and CD86, which can make the difference between turning off the CTL precursor and activating it. DCs also secrete critical cytokines such as IL-12 and IL-15 that contribute to CTL activation and memory. In addition, a number of regulatory mechanisms that dampen the immune response are exploited by tumors to escape immunosurveillance. These mechanisms include the inhibitory receptor CTLA-4 on the T cells themselves (14) and negative regulatory cells such as the CD25+CD4+ regulatory T cell (15–17) and also certain types of CD4+ natural killer T (NKT) cells that inhibit tumor immunosurveillance (4, 17–19).

Major hurdles in developing cancer vaccines include: identification of antigens that focus the exquisite specificity of the immune system on cancer cells without harming normal cells; development of methods to induce an immune response sufficient to eradicate the tumor, in the face of self-tolerance to many tumor antigens; and overcoming mechanisms by which tumors evade the host immune response.

Types of tumor antigens

An extensive listing of the known tumor-associated antigens is available, and more are being discovered (20). Tumor antigens can be categorized 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. These cat-
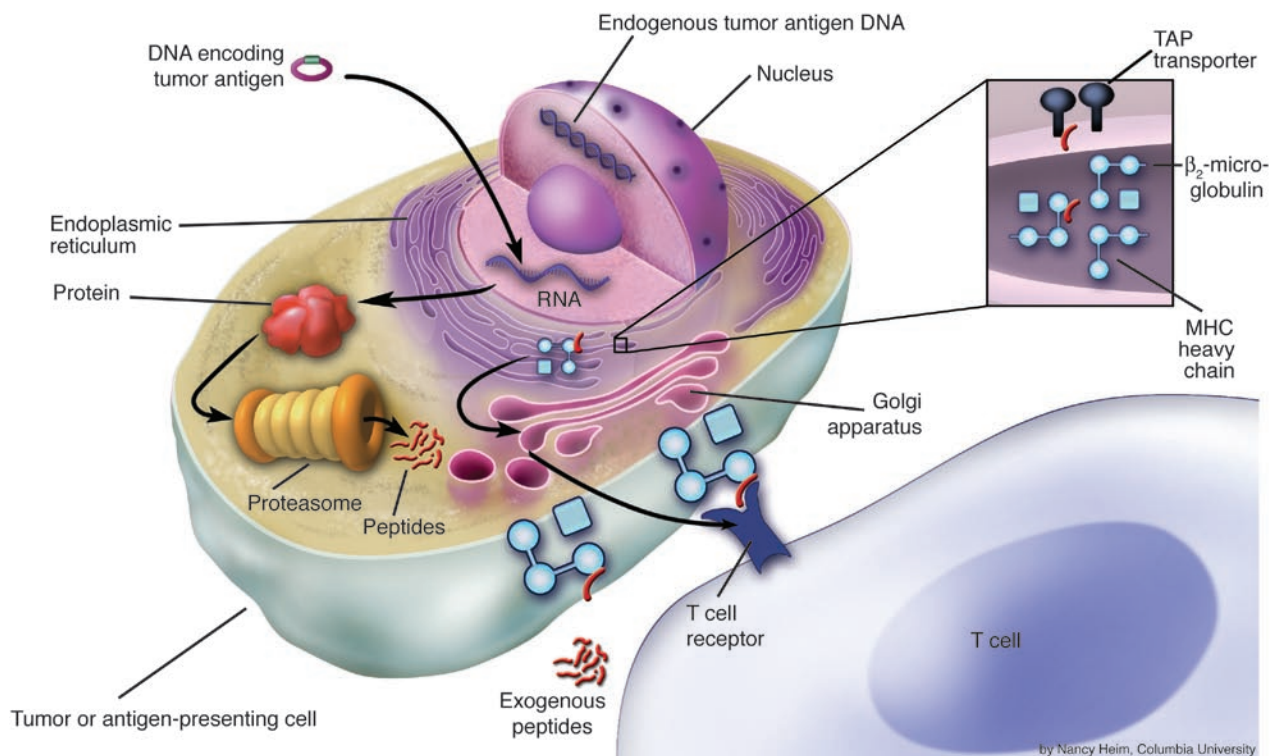
Figure 1
The class I MHC antigen processing pathway acting as an internal surveillance mechanism to detect any abnormal or foreign protein synthesized in the cell. Tumor antigens encoded in the endogenous DNA of the tumor cell, or encoded in a DNA plasmid or viral vector vaccine taken up by an APC, are synthesized and cleaved by the 26S proteasome into fragments that are transported by TAP, the transporter associated with antigen processing, into the endoplasmic reticulum, where they are loaded onto newly synthesized class I MHC molecules that transport them to the cell surface for recognition by the T cell receptor.

egories and the two major strategies used to identify tumor antigens are described in *Approaches to tumor antigen discovery*.

To specifically target tumors, antigens must be expressed only in tumor cells. Such unique tumor antigens (21) include mutated proteins, such as mutated oncoprotein ras (22) and mutated tumor suppressor proteins p53 (21, 23) and von Hippel Lindau; and fusion proteins created by chromosomal translocations, such as BCR-ABL in chronic myelocytic leukemia (24), PAX-FHKR in alveolar rhabdomyosarcoma (25), EWS-FLI1 in Ewing sarcoma (26), and SYT-SSX in synovial sarcoma (27). K-*ras* mutations occur in 30–40% of colorectal carcinomas (28), and mutations of p53 are found in 60–70% of all human cancers (29). Peptides derived from common *ras* mutations bind to specific MHC molecules and can generate tumor-specific immune responses (30, 31). Because the mutations are necessary for generation and maintenance of the neoplastic phenotype, they are expressed by all of the tumor cells and cannot be lost. However, only a short segment of the amino acid sequence encompassing the mutation or fusion breakpoint is actually unique, and this region may not be presented by many common HLA molecules. Another type of tumor antigen unique to cancer cells are antigens with tumor-specific posttranslational modifications, exemplified by MUC1, which shows altered glycosylation in cancer cells, creating neoantigenic sites by exposing protein sequences normally masked by glycosylation (1, 7).

To overcome this problem, investigators have searched for whole proteins either not highly expressed in adult tissues, such as carcinoembryonic antigen (CEA) (32, 33); overexpressed in can-

cer cells, such as nonmutated portions of p53 (34); or uniquely expressed in expendable tissues. The latter include melanocyte antigens gp100, MART1, or tyrosinase in melanoma (3) and prostate-specific antigen (PSA) or prostate-specific membrane antigen (PSMA) in prostate cancer (35). The disadvantage is that self-tolerance may limit responses to these normal host proteins. Expression of these proteins can also be lost if they are not essential for malignancy, allowing tumor escape. The most foreign tumor antigens are viral proteins, such as human papillomavirus-16/18 (HPV-16/18) E6 and E7 oncoproteins in cervical cancer (7) or EBV proteins in certain B cell malignancies (36). In the case of HPV, these E6/E7 proteins are essential for malignant transformation and so cannot be lost to escape the immune response.

Cancer vaccine modalities
The vaccine strategies used against cancer depend on how well defined the target antigens are and whether there are conserved antigens that are shared among tumors of the same type in many individuals. We will discuss the rationale for, and experience with, some of the most widely studied approaches (Table 1).

Modified tumor cell vaccines
The richest source of rejection antigens is the tumor itself. However, use of autologous tumor cell vaccines is cumbersome and not amenable to large-scale vaccine production, and tumor samples are often unavailable. Approaches using allogeneic or generic cell lines as vaccines are more widely applicable.

Approaches to tumor antigen discovery

1. Based on existing host response   **Examples**

Identify tumor-reactive T cells or antibodies from patients; use to screen tumor libraries
T cells may be tumor-infiltrating lymphocytes (TILs) or peripheral blood T cells   gp100, MAGE, MART-1

Advantages: Antigen known to be immunogenic
Disadvantages: Limited to tumors that are immunogenic
                  Limited to antigens that induce a response without active immunization
                  Not always possible to identify the antigen
                  Antigen may not be unique to tumor

2. Based on tumor characterization (what is different)   **Examples**
Overexpressed antigens   HER-2/neu, CEA
Altered antigens   p53, Ras, fusion proteins
Tissue-specific antigens   tyrosinase, PSA
Novel antigens (in adult)
                  Fetal   CEA
                  Viral   HPV E6, E7
                  Clonal   Idiotype
Advantages: Can select antigen that is unique to tumor and essential for tumorigenicity
Disadvantages: Must demonstrate immunogenicity

Tumor cells engineered to secrete a number of different cytokines have been shown to protect mice from challenge with the same tumor type (37). Of the cytokines studied, GM-CSF appeared most effective. Local expression of GM-CSF increases DCs and other APCs at the site of injection of vaccination. These acquire, process, and present antigen to T cells. A number of genetically modified autologous or allogeneic tumor cell vaccines have been tested in clinical trials (38–41). Studies in patients with advanced prostate cancer (38) and metastatic malignant melanoma (39) used irradiated autologous tumor cells transduced with a retroviral vector expressing GM-CSF, resulting in one partial response in 21 melanoma patients, although extensive inflammatory infiltrate with necrosis and fibrosis of tumor was seen in 11 of 16 melanoma patients biopsied (39). In another phase I trial, among 14 patients with resectable pancreatic cancer vaccinated with GM-CSF–transduced allogeneic pancreatic cancer cell lines after surgery (40), three patients remained disease free at 23 months. A number of other genetically altered autologous and allogeneic tumor cell vaccines expressing IL-2, IL-4, B7.1, and α-(1,3) galactosyltransferase are currently in clinical trials (42–44).

Peptide vaccines
Elucidation of the crystal structure of the MHC and of the peptides bound to it (45) and discovery of anchor-residue sequence motifs accounting for binding specificity of peptides to MHC molecules (46) has provided the visual and mechanistic answer to how T cells recognize antigens in the form of short peptides. The observation that short peptide segments (8–10 amino acids) fit into a groove in the MHC molecule, combined with knowledge of the amino acid sequences of tumor epitopes, prompted the use of peptides as therapeutic agents in the treatment of cancer. These observations were followed by cloning of the first human tumor-associated antigen and identification of its nonamer peptide sequence (47). Several strategies have been developed both to improve immunogenicity and to steer the immune system toward desired types of responses. Also, peptides have been loaded onto autologous DCs for administration, as described below. However, individual peptides will be useful only in patients with appropriate HLA molecules capable of presenting that peptide.

Epitope enhancement. Modification of the amino acid sequence of epitopes, commonly referred to as epitope enhancement, can improve the efficacy of vaccines through several means: (a) increasing affinity of peptide for MHC molecules (4, 48, 49); (b) increasing TCR triggering (32, 33, 50); or (c) inhibiting proteolysis of the peptide by serum peptidases (4, 6, 51). Whenever the peptide sequence is altered, it is important to demonstrate that the T cells induced still recognize the native peptide sequence. There is precedent for epitope-enhanced peptides showing greater efficacy in clinical trials (49) (see below).

Cytokines, chemokines, and costimulatory molecules as adjuvants. Another approach is to deliver the peptide with adjuvants containing cytokines, chemokines, costimulatory molecules, or other immunomodulators that amplify and direct the immune response (4). Th1 CD4+ T cells and CD8+ cytotoxic T cells crucial for antitumor immunity are induced more efficiently by professional APCs, such as DCs. Based on this knowledge, a recent strategy for the development of peptide vaccines uses a synergistic combination of cytokines that induce DC recruitment (GM-CSF) and costimulatory molecules that induce DC maturation (CD40L or agonistic anti-CD40) (52) in combination with other Th1/CTL-supporting cytokines such as IL-12 and IL-15. Other synergistic combinations have been described in animal models (4). Another adjuvant, a CpG-oligodeoxynucleotide (CpG-ODN), characteristic of bacterial DNA, is used to elicit a broad range of immune cells (53). Differences between humans and mice in the response to cytokines such as GM-CSF and expression of the toll-like receptor 9 (TLR9) for CpG-ODN on DCs will require confirmation during the testing of these approaches in clinical trials. Another approach to target DCs is to use ex vivo–generated DCs that have been pre-incubated (pulsed) with the peptide of interest. Using peptides with broad MHC class II binding such as pan–HLA-DR–binding peptide (PADRE) (54), endogenous helper epitopes (55, 56), or enhanced helper epitopes (57) to stimulate CD4+ T cell help also results in increased CD8+ T cell responses.

Clinical trials of peptide vaccines. The best-studied clinical model of peptide vaccination is malignant melanoma. The various immunologic monitoring methods employed are listed in Table 2. Each

has advantages and disadvantages, but none has been shown to provide a surrogate marker for tumor prevention or regression.

Rosenberg and colleagues evaluated vaccination with native gp100 peptide 209–217 and found that it produced only low levels of T cell reactivity in two of eight melanoma patients analyzed, whereas an epitope-enhanced gp100 (g209-2M) peptide generated strong T cell reactivity in 10 of 11 patients immunized (49). Nevertheless, only a single objective clinical response was reported. Immunization with g209-2M, combined with high dose IL-2 treatment, produced anti-tumor responses in 42% of patients, although T cell reactivity was observed in less than 10% of patients. In the adjuvant setting, Smith et al. (58) found a vaccination frequency of every two or three weeks resulted in a median frequency of CD8+ cells binding g209-2M tetramers of 0.34% after 6 months, compared with 0.02% before vac-

cination, whereas less frequent vaccination gave substantially lower responses of 0.03%. Addition of either IL-12 (59) or GM-CSF (60) increased this percentage slightly. The impact of age was striking (58). In patients under 60 years old, the median number of tetramer-positive CD8+ cells induced by vaccination was 0.64%, whereas in those over 60 it was 0.08%.

Immunization with tyrosinase peptide has been significantly less effective despite epitope enhancement. Immunization with the tyrosinase 370D peptide in incomplete Freund's adjuvant (IFA) with or without cytokines, including IL-12 (59) or GM-CSF (60), only rarely resulted in low numbers of tetramer-positive CD8 cells or cytokine production. The levels of 370D-responsive cells detected by enzyme-linked immunoabsorbent spot (ELISPOT) assay have been low (0.01–0.03% of input cells) (61). The use of

Table 1

Antitumor vaccines in clinical trials

Vaccine	Advantages	Disadvantages
Whole tumor cell	1. Studied extensively 2. Can be processed to enhance antigen presentation (e.g., irradiated tumor cells or tumor lysates); 3. Can be administered with adjuvants (e.g., BCG, KLH, viruses, etc.); 4. Likely to express the relevant tumor antigens; 5. Antigens need not be defined	1. Requires availability of autologous tumor or an allogeneic cell line sharing the relevant tumor antigens; 2. Poor ability to stimulate immune responses; 3. Few responses and little benefit reported when used adjuvantly in randomized clinical trials
Gene-modified tumor cells	1. Likely to express the relevant tumor antigens; 2. Antigens need not be defined; 3. Often engineered to coexpress immunostimulatory molecules and cytokines (e.g., GM-CSF, IL-2); 4. Use of allogeneic tumor cell lines and fibroblasts are under investigation as an approach to accelerate vaccine production; 5. Some immunological and clinical responses reported	1. Requires availability of autologous tumor or an allogeneic cell line expressing the relevant tumor antigens; 2. Weak antigen presentation by many tumors; 3. Long manufacturing time; 4. Need for ex vivo cell culture; 5. Cost, time, and labor intensive
Plasmid (naked) DNA	1. Constructed to express the relevant tumor antigen; 2. Easy to produce and stable; 3. Can be administered as a direct injection or biolistically ("gene gun")	1. Requires detailed knowledge of the antigen DNA sequence; 2. Low immunological potency for self (tumor) antigens; 3. Response may be Th2 skewed; 4. High doses of plasmid DNA are required to generate immune responses
Peptides	1. Can limit immune response to epitopes distinct from the wild type (e.g., point mutations or breakpoint-fusion genes); 2. Epitopes can be enhanced; 3. Easy to produce and stable; 4. Can be combined as cocktails of peptides; 5. Some immunological and clinical responses reported	1. Requires knowledge of the specific epitope; 2. Immunogenicity restricted to a limited number of MHC molecules; 3. Usually requires the addition of an adjuvant for immunogenicity
Viral gene transfer vectors	1. Engineered to express the relevant tumor antigen; 2. Can be engineered to coexpress immunostimulatory molecules and cytokines; 3. Wide variety of available vectors (e.g., adenovirus, pox viruses, lentiviruses, etc.); 4. Some cellular immune responses reported	1. Immunodominance of viral antigens over tumor antigens; 2. Weak antitumor responses seen with most viral vectors; 3. Preexisting immunity against viral vectors may attenuate the antitumor response; 4. Risk of toxicity with "live" viruses
Antigen-modified DCs	1. Use of powerful APCs; 2. Techniques available to generate large numbers of clinical grade DCs; 3. Target antigens may be defined or uncharacterized; 4. Multiple antigen loading techniques (e.g., peptide, lysates, whole protein, RNA transfection, viral vectors, etc.) are available; 5. Some immunological and clinical responses reported	1. Need for ex vivo cell culture; 2. Cost, time, and labor intensive; 3. Optimal technique for antigen loading remains undefined; 4. Possibility of tolerization by immature DCs; 5. Lack of criteria for standardization of final product

BCG, bacille Calmette-Guérin; KLH, keyhole limpet hemocyanin.

Table 2
Immune response monitoring for clinical trials

Assay	Advantages	Disadvantages	Sensitivity
Proliferation	Technically simple	Unable to enumerate specific cells; measures predominantly CD4, not CD8, T cells	
Tetramer staining	Quantitative cell number; subset analysis possible	Requires synthesis of specific tetramers; not a measure of functional activity; limited to single epitopes	$1{:}10^4$
Cytokine flow cytometry	Quantitative cell number; functional assay; subset analysis possible	Requires incubation; unable to obtain live cells after assay; technically complicated	$1{:}5 \times 10^4$
ELISA	Functional assay	No phenotypic information on responding cells; unable to enumerate specific cells; bystander activation	
ELISPOT	Quantitative cell number; functional assay	No phenotypic information on responding cells; bystander activation	$1{:}10^5$
Cytotoxicity	Functional assay; relative cell quantitation	Requires autologous tumor/targets; in vitro stimulations required; unable to enumerate specific cells	
RT-PCR	Universal reagents; indirect measure of function	Measures mRNA, not protein; unable to enumerate specific cells; requires several steps (time consuming)	$1{:}10^5$
Limiting dilution	Quantitative cell number; functional assay	Labor intensive; requires multiple stimulations; detects only cells that can proliferate	$1{:}10^5$

peptide vaccines may be additionally complicated by the choice of adjuvants. Most studies used IFA, but in the study by Schaed et al. (61), patients showed no response with 370D peptide in IFA, whereas almost 50% of patients showed low-level ELISPOT responses with QS21 or GM-CSF as adjuvants.

The importance of epitope enhancement is supported by the promising results of vaccination with the epitope-enhanced carcinoembryonic antigen (CEA) peptide (33). FLT3 ligand–expanded DCs were pulsed with this peptide to immunize patients with advanced colorectal cancer. Five of 12 patients immunized developed greater than 1% tetramer-positive CD8+ T cells, and two showed clinical responses.

Despite the small sample sizes and the variable populations treated, some principles emerge. Immunization with native peptide sequences is often insufficient to generate reactive T cells and clinical responses in most patients. Epitope-enhanced peptides can generate T cell responses but not always clinical tumor responses. Adjuvants, including cytokines and costimulatory molecules, improve the immunogenicity of peptide vaccination. Paradoxically, combining peptide vaccination with IL-2 significantly reduced detection of specific T cells in blood, but nearly half the patients showed objective cancer regressions (49), possibly due to IL-2–induced innate immunity combined with vaccine immunity. In a new approach, blockade of the negative regulatory molecule CTLA-4 showed promise when combined with vaccination with g209-2M in melanoma patients. Three of 14 patients treated had objective tumor regressions although at the cost of development of autoimmune disease, including bowel, liver, and pituitary dysfunction (62). Blockade of other negative regulatory pathways has shown promise in animal models (4, 16, 18).

Recombinant viral vectors

A number of trials utilizing recombinant viruses expressing tumor antigens such as CEA or PSA, some with immunostimulatory cytokines, have been reported or are in progress (63, 64). Adenovirus, vaccinia, and avipox vectors have been used. The high prevalence of antiviral neutralizing antibodies may limit use of these vectors, especially for multiple doses, except for fowlpoxes (e.g., the canarypox virus vector ALVAC) that do not appear to induce neutralizing antibodies. Possible resistance due to prior systemic immunity to poxviruses can potentially be overcome by mucosal immunization, because systemic immunization is poor at inducing mucosal immunity, but mucosal immunization can induce both systemic and mucosal immunity (65). Immunodominance is also problematic. Stronger immune responses may be induced against viral vector antigens than against weaker tumor antigens. The potency of these vectors may be enhanced by the addition of genes for immunostimulatory molecules or cytokines (66, 67). Such vectors are entering clinical trials. These vectors can also be used to express antigens in DCs, as described below.

DNA vaccines

Intramuscular injections of naked DNA expression plasmids have been shown to generate immune responses (68, 69). Such DNA vaccines introduce tumor antigen genes into DCs for endogenous processing and presentation to CTLs in draining lymph nodes or into other cells for cross-presentation by DCs, without the need for a viral vector (Figure 2). Thus, problems of competition from viral vector epitopes, reduced efficacy due to prior immunity to the viral vector, and potential dangers associated with a live virus are avoided. Constitutive, tissue, or tumor-specific promoters may be used for selective expression.

The results of a number of plasmid DNA vaccine trials have been reported. Among 12 patients with follicular lymphoma vaccinated with plasmids encoding tumor-specific idiotypes (70), four mounted a humoral anti-idiotype or a specific anti-idiotype T cell proliferative response. In another trial, among 17 patients with metastatic colorectal carcinoma vaccinated with a plasmid encoding both CEA and hepatitis B surface antigen (HBs) as a control (71), six developed protective levels of anti-HBs antibody, but none developed antibody to CEA, although 4 of 17 developed a lymphoproliferative response to CEA. Thus, DNA vaccines have not yet shown much promise for antitumor vaccination.
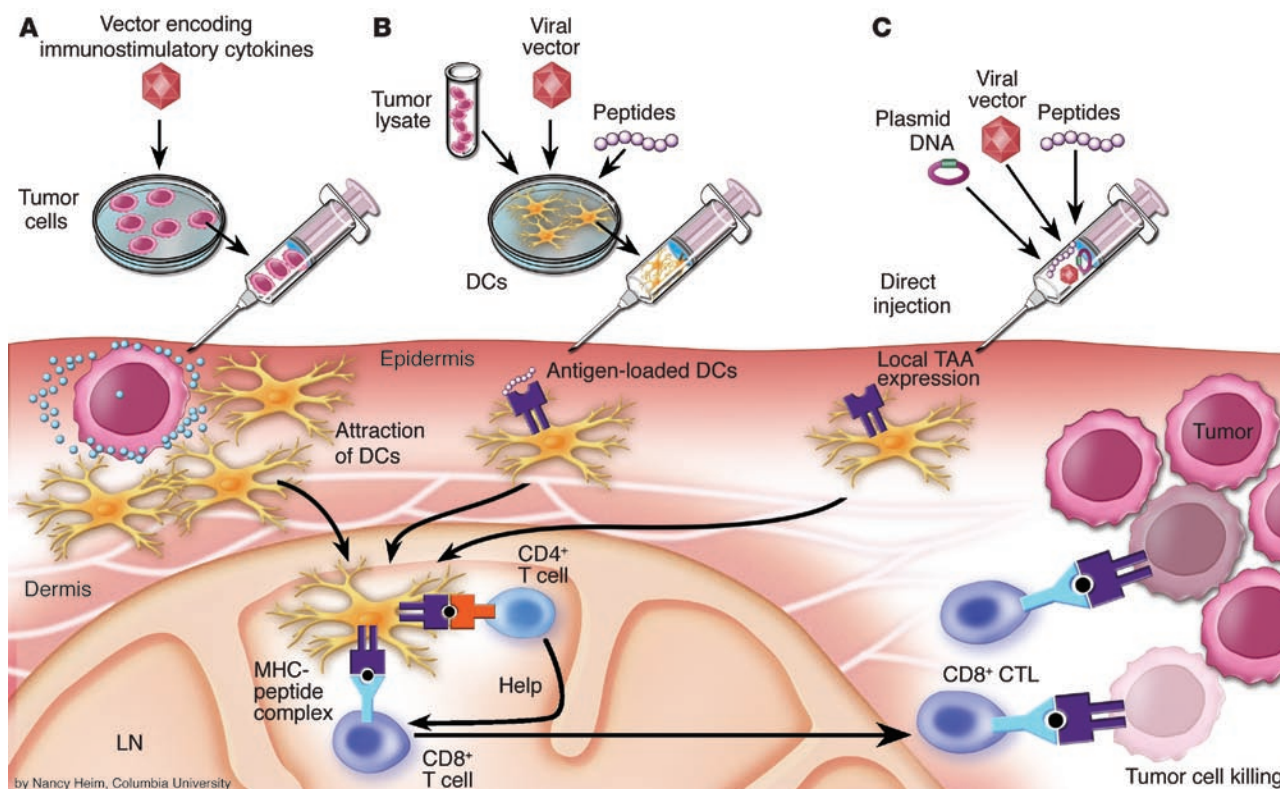
Figure 2
Approaches to antitumor vaccination. (**A**) Irradiated tumor cells transduced with a viral gene transfer vector encoding a cytokine such as GM-CSF attract APCs (DCs) that acquire, process, and present tumor-associated antigens (TAAs) encoded by the vector in the context of MHC. (**B**) DCs can be directly loaded by incubation with tumor protein lysates or peptides with sequences based on expressed tumor antigens, or by viral gene transfer vectors expressing TAAs. (**C**) TAAs can be locally supplied to DCs by the direct injection of peptides, viral gene expression vectors, or naked DNA expression plasmids. DCs migrate to secondary lymphoid tissues where they present the antigen epitopes to T cells to generate an antitumor cytolytic T cell response.

Dendritic cell vaccines
DCs are professional APCs and are the most powerful stimulators of naive T cells (13, 72, 73). Immature DCs sample the antigenic environment through phagocytosis, micropinocytosis, receptor- and lectin-mediated endocytosis and are more effective at processing antigen. When DCs encounter inflammatory mediators such as bacterial LPS, or TNF-α, they mature. Helper T cells also induce DC maturation via CD40 ligand interaction with CD40. As they mature, DCs downregulate their antigen uptake and processing machinery; express CD83; upregulate MHC, costimulatory molecules (CD80 and CD86), and the chemokine receptor CCR7; and travel to lymph nodes where they activate antigen-reactive T cells.

Demonstration of acquired defects in DC maturation and function in tumor-bearing animals and cancer patients suggests a rationale for using ex vivo–generated DCs as antitumor vaccines. Gabrilovich and colleagues reported ineffective CTL induction in a murine mutant p53 fibrosarcoma model associated with defects in DC function (74). Supernatants from tumor cells suppressed DC maturation, ultimately attributed to an effect of VEGF (75). In contrast, DCs generated in vitro from marrow progenitors stimulated allogeneic T cells and induced mutant p53 peptide–specific T cell responses (74, 76, 77). Immunization with mutant p53-peptide–pulsed DCs inhibited the growth of established tumors in the mice.

DCs pulsed with tumor lysates (78), tumor protein extracts (79–81), synthetic peptide tumor epitopes (74, 82), or DCs fused with irradiated tumor cells (83) could generate protective immunity to subsequent tumor challenge. Transfer of nucleic acids encoding tumor antigens into DCs using plasmid transfection (84), retroviral vectors (85), recombinant adenoviruses (86), lentiviruses (87), or electroporation of tumor RNA (81) has been effective. Antigens can also be targeted to DCs by coupling to DC-specific antibodies (88). Transfer of genes encoding costimulatory molecules (B7) and cytokines (IL-12) into DCs has also enhanced antitumor vaccine efficacy (80).

Clinical trials of DC vaccines have depended on development of techniques for obtaining large numbers of clinical grade human DCs (89, 90). Currently, two general approaches are used: (a) purification of immature DC precursors from peripheral blood (72); and (b) ex vivo differentiation of DCs from CD34+ hematopoietic progenitor cells (91, 92) or peripheral blood monocytes (90), commonly by culture of monocytes with GM-CSF and IL-4 (Figure 3). Immature DCs can be matured with CD40 ligand, LPS, or TNF-α (93). In mice, CD40 ligand–mediated maturation was the most effective approach for DC vaccine preparation (94).

In humans, the finding that two healthy volunteers receiving immature DCs pulsed with influenza matrix peptide (FMP) had a reduction in FMP-specific CD8+ T cell activity (95) raised concerns
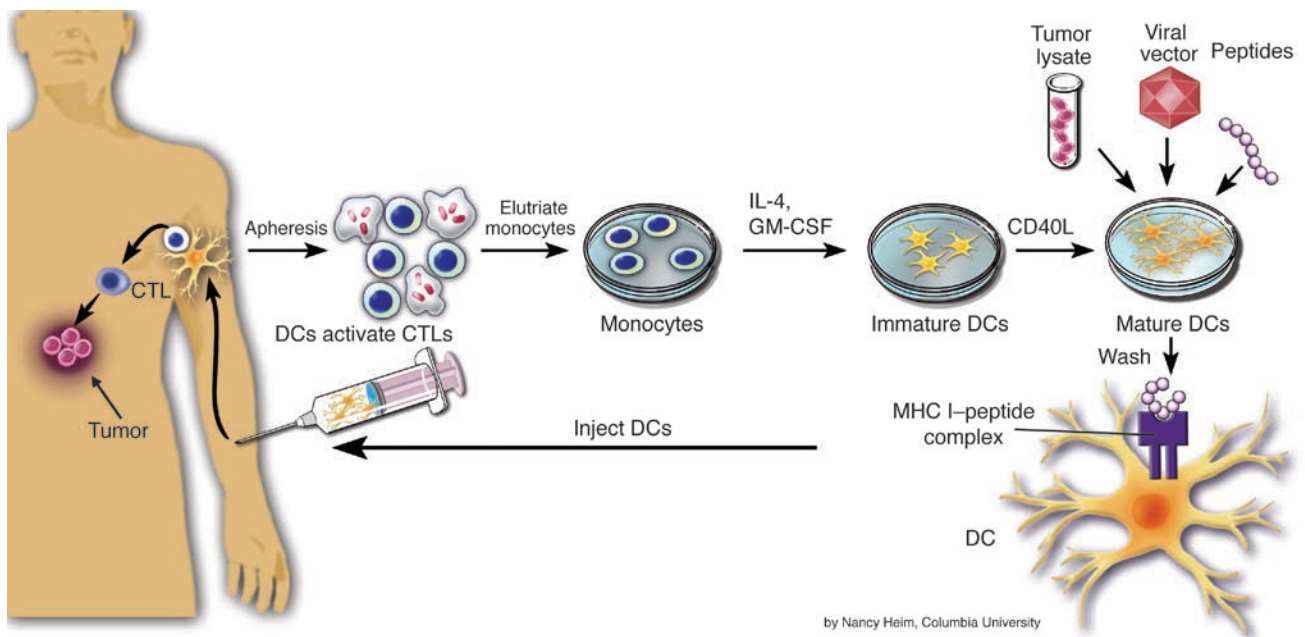
Figure 3
Generation of antitumor DC vaccines from peripheral blood monocytes. Elutriated monocytes from a leukapheresis are cultured with GM-CSF and IL-4 to produce DCs, which are then matured with CD40 ligand (CD40L) or other agents, pulsed with peptide or tumor lysate, or transduced with an expression vector and then injected into the patient as an autologous DC vaccine to induce a T cell immune response against the tumor.

that immature DCs might induce tolerance to antigens (96, 97). The recent trend has been to use DCs matured using TNF-α, CD40 ligand, monocyte-conditioned media, or cytokine cocktails (98, 99).

Clinical trials of anticancer DC vaccines. A number of DC cancer vaccine trials have been reported. Hsu et al. reported the first DC vaccine trial for treatment of cancer in patients with follicular B cell lymphomas (100), which express a unique clonal B cell receptor (idiotype, Id) that can distinguish lymphoma cells from nonmalignant lymphocytes. Initially, ten patients were treated with peripheral blood DCs pulsed with a tumor-specific Id protein. Eight developed Id-specific cellular proliferative responses, and one developed a specific CTL response. Two patients had complete responses (CRs), one a partial response (PR), and another a complete molecular response. A follow-up study with an additional 25 patients found that 15 of 23 generated T cell and humoral anti-Id responses (101).

Multiple myeloma (plasma) cells also express unique clonal immunoglobulin idiotypes (102–104) but do not express Id protein on the cell surface, instead producing large amounts for secretion. Myeloma cells can still be detected by CTLs that recognize Id peptides presented by HLA molecules. Of 26 patients treated with Id-pulsed DCs after high-dose chemotherapy and stem cell transplantation, four developed Id-specific T cell proliferative responses. Although the stem cell transplant itself produced 5 CRs and 21 PRs, 8 patients with PRs had further declines in their serum monoclonal spike after the DC vaccination, and of the four patients that developed an immune response, two remained in CR at 35 and 28 months after transplantation (103). In another trial (104), patients with residual low-volume myeloma after transplant were immunized with DCs pulsed with autologous serum as a source of Id. Of the 13 evaluable patients, three achieved a CR and 3 a PR.

DC vaccines have also been studied in patients with solid tumors. Of 21 patients with recurrent or metastatic prostate cancer and elevated serum prostatic acid phosphatase (PAP) treated with DCs

pulsed with rodent PAP, ten developed T cell–proliferative responses to PAP (105). Among 16 patients with metastatic melanoma receiving peptide- or tumor lysate-pulsed DCs injected directly into lymph nodes, eleven developed delayed-type hypersensitivity responses to peptide-pulsed DCs, and two had durable CRs (106). Among 11 melanoma patients receiving monocyte-derived DCs pulsed with HLA-A1–restricted MAGE-3 melanoma peptide, eight developed a CTL response, and some minor tumor regressions were observed (107). Recently, 20 patients with advanced pancreatic, hepatocellular, cholangiocarcinoma, or medullary thyroid carcinoma were treated with monocyte-derived DCs pulsed with tumor lysates, matured with TNF-α, and administered along with daily subcutaneous IL-2 (108). Although 18 of 20 patients developed DTH responses and 8 had a decline in serum tumor markers, no CRs or PRs were seen. A small, uncontrolled clinical trial in metastatic renal cell carcinoma with tumor RNA-loaded DCs reported an unusually low mortality from tumor in the follow-up period (109).

Inconsistency in trial results may relate to several variables, including the type and quality of DCs that vary with method of generation and maturation, the technique used for epitope loading, and the dose, route, and frequency of vaccination. Animal models indicate benefit of multiple vaccinations compared to a single dose (74) and of subcutaneous and intradermal administration (110). Thus, while the use of DCs as a vehicle for therapeutic cancer vaccination holds promise to circumvent some strategies tumors use to evade the immune system, there are still many technical issues to be resolved in this approach, which needs to be compared directly with other vaccine modalities.

Future strategies to enhance cancer vaccine efficacy
It is becoming clear that the immune response may be hindered by the hurdles created by tumors to evade the immune system. As noted, VEGF and other tumor-derived factors can inhibit DC

maturation, and maturation of DCs ex vivo can circumvent this roadblock. We also discussed under "Peptide vaccines" (above) the use of cytokines, chemokines, and costimulatory molecules to activate and direct the immune response toward the appropriate type, as well as epitope enhancement to improve binding of tumor antigen epitopes to MHC molecules or TCRs.

Another area generating much recent interest is the possibility of overcoming mechanisms that downregulate or attenuate the immune response (Figure 4). Such mechanisms may have evolved to reduce inflammation and immunopathology or to prevent autoimmunity. Tumors have co-opted these mechanisms to evade immunosurveillance. T cells themselves express inhibitory receptors, the best studied being CTLA-4 (111, 112). This binds costimulatory molecules CD80 and CD86, but, instead of activating the T cell, dampens its response. Blockade of CTLA-4 has been shown to improve tumor immunosurveillance and amplify effects of cancer vaccines in animals (14, 111). Recently, monoclonal anti–CTLA-4 antibodies have been studied in clinical trials alone or in conjunction with cancer vaccines (62). A substantial number of objective responses were found in a melanoma trial, but not without a number of autoimmune side effects, all reversed when therapy was stopped.

Another regulatory mechanism is the $CD25^+CD4^+$ regulatory T cell (15, 17, 113). Such cells are induced by antigens, especially in the presence of high IL-2 levels, but their effector activity is not antigen-specific (113). Blockade or elimination of these cells has been shown to enhance tumor immunosurveillance and efficacy of antitumor vaccines (15). Furthermore, concurrent blockade of $CD25^+CD4^+$ regulatory T cells and of CTLA-4 was synergistic in augmenting efficacy of a cancer vaccine (16).

Another novel immunoregulatory cell is the $CD4^+$ NKT cell. These are T lymphocytes that share certain markers with NK cells but also express normal CD3 and $\alpha\beta$ TCRs. However, the TCRs used are limited to a few types that predominantly recognize glycolipids presented by a nonclassical class I MHC molecule, CD1d (114). These cells play a role in regulating immune responses causing autoimmune diseases, such as diabetes (115). Recently, we found that $CD4^+$ NKT cells also can inhibit tumor immunosurveillance in a mouse fibrosarcoma model (17–19) and an orthotopic breast cancer model (116), and have now extended these findings to a non-regressor colon cancer model (19) (Park et al., unpublished results) (Figure 4). Elimination of NKT cells, or blockade of their effector mechanisms, such as IL-13 or downstream TGF-β, prevented tumor recurrence (17–19) and improved CTL responses and antiviral efficacy of a peptide AIDS vaccine in mice (52). Whereas regulatory NKT cells express predominantly Th2 cytokines, other NKT cells making IFN-γ can actually contribute to tumor immunosurveillance (17). Factors influencing these opposing roles of NKT cells are under study. Thus, we propose that blockade of NKT cell negative regulatory cytokines can be used as a strategy to increase the efficacy of anticancer vaccines (17).

A complementary approach is to selectively induce high avidity CTLs (4, 117), which were first shown to be more effective at clearing virus infections (118) and then also to be more effective at killing tumor cells and eradicating tumors (119, 120). We recently found that increasing levels of costimulation, using a triad of costimulatory molecules — CD80, ICAM-1 and LFA-3 — in conjunction with a vaccine, can selectively induce CTLs skewed toward higher avidity and more effective at killing tumor cells (67). More-
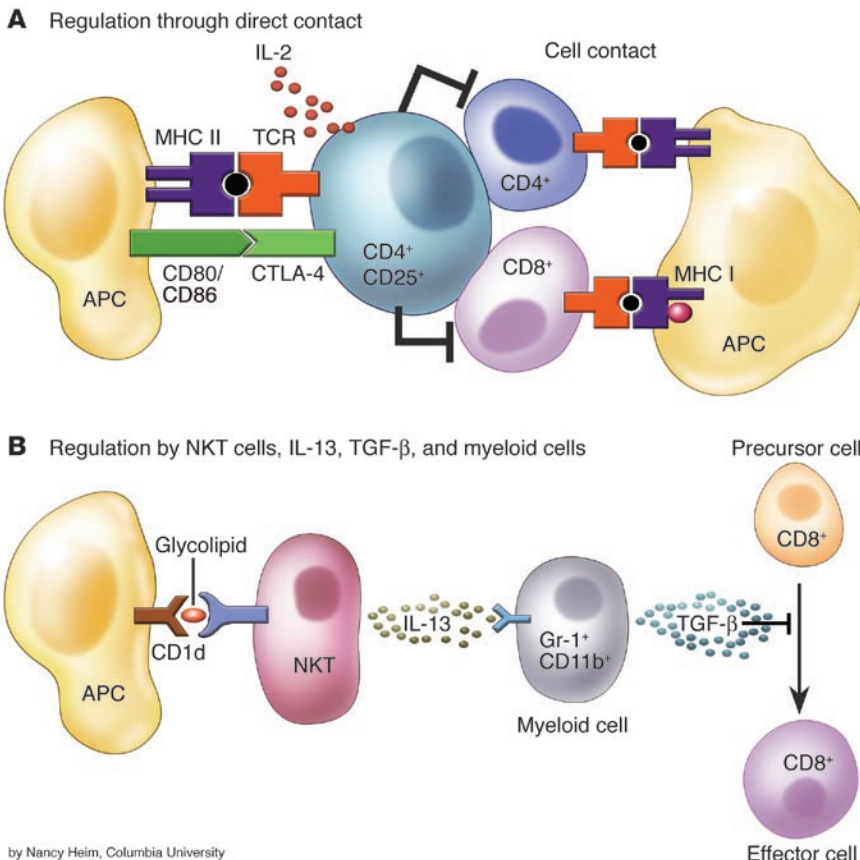

Figure 4
Negative regulation of tumor immunosurveillance and antitumor immune responses. (**A**) $CD4^+CD25^+$ T regulatory cells, induced by peptide presented by class II MHC molecules in the presence of IL-2, may inhibit induction of effector $CD4^+$ or $CD8^+$ T cells by a contact-dependent or cytokine-dependent mechanism, possibly involving cell surface and/or secreted TGF-β. (**B**) $CD4^+$ NKT cells may be induced by tumor glycolipid presented by CD1d to secrete IL-13, which stimulates $Gr-1^+CD11b^+$ myeloid cells to produce TGF-β, which inhibits induction of $CD8^+$ CTLs mediating tumor immunosurveillance. TGF-β may also inhibit $CD4^+$ T cells (not shown). Blockade of either mechanism can improve immunosurveillance and the response to vaccines. Other suppressor or negative regulatory cells have been described in other contexts but not as well studied in the context of cancer.

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over, IL-15 expressed by a vaccine has also been found to selectively induce longer-lived CTLs that may be more effective (121) and also a higher average avidity of CTLs (Oh et al., unpublished results). Indeed, we suggest that the recently discussed role of CD4+ T cell help and signal strength in inducing long-lived memory CD8+ CTLs (122–125) may be mediated in part by helper cell stimulation of DCs to produce IL-15 when they present antigen to CTLs, as we have mimicked with IL-15 in the vaccine (121). These strategies may be useful in optimizing anticancer vaccines.

Conclusion

As prophylaxis against acute infectious diseases, vaccines have been among the most cost-effective agents, saving many millions of lives. However, for treatment of chronic infections and cancer, vaccines have yet to achieve widespread success. Increased understanding of the immune system has raised new hope of harnessing the exqui-site specificity of the immune system to attack cancer and has led to novel second-generation vaccine approaches that hold promise to control or cure cancer. The pace of identification of new tumor antigens has accelerated. New strategies are being developed to make more potent vaccines against inherently weak tumor antigens, to selectively induce high avidity CTLs more effective at clearing tumors, and to overcome negative regulatory mechanisms that inhibit tumor immunosurveillance and immune responses to antitumor vaccines. A number of promising new cancer vaccine strategies have entered clinical trials, and we eagerly await their findings.

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