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EXPERT REVIEW

Cancer Immunotherapy and Nanomedicine

Wei-Yun Sheng • Leaf Huang

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ABSTRACT The immune system has the ability to recognize and kill pre-cancer and cancer cells. However, with the immune system's surveillance, the survival tumor cells learn how to escape the immune system after immunoselection. Cancer immunotherapy develops strategies to overcome these problems. Nanomedicine applications in cancer immunotherapy include the nanodiagnostics and nanobiopharmaceuticals. In cancer nanodiagnostics, it looks for specific "molecular signatures" in cancer cells or their microenvironment by using genomics and proteomics. Nanobiopharmaceuticals is the field that studies nanotechnology-based therapeutic agents and drug carriers. DNA, RNA, peptides, proteins and small molecules can all be used as cancer therapies when formulated in nanocarriers. Currently, cancer vaccines are applied in treatments with existing cancer or to prevent the development of cancer in certain high risk individuals. Most of the non-specific immune activation agents include adjuvants which enhance immunogenicity and accelerate and prolong the response of cancer vaccines. The carriers of vaccines, such as viruses and nanoparticles, have also been in clinical studies for many years. This review will discuss the relationships between the tumor and the immune system, and also will include topics covering the strategies used in eliminating tumors by using nanomedicine.

KEY WORDS adjuvant · cancer immunotherapy · cancer vaccine · nano · tumor immunoediting

W.-Y. Sheng • L. Huang (✉)
Division of Molecular Pharmaceutics, Eshelman School of Pharmacy
University of North Carolina at Chapel Hill
1319 Kerr Hall
Chapel Hill, North Carolina 27599, USA
e-mail: leafh@unc.edu

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen-presenting cell
bAb	bispecific antibody
CIK	cytokine-induced killer cells
CT	Chlorella toxin
CTL	cytotoxicity T-lymphocytes
CTLA-4	cytotoxic T-Lymphocyte Antigen 4
DC	dendritic cells
DLN	draining lymph node
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate
EPR	enhanced permeability and retention effects
GM-CSF	granulocyte macrophage colony-stimulating factor
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule-1
iDC	immature DC
IFA	incomplete Freud's adjuvant
IFN	interferon
IL	interleukin
LFA-1	lymphocyte function-associated antigen-1
LPD	lipid-polycation-DNA
LT	labile enterotoxin
mDC	mature dendritic cells
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
NK-cell	natural killer cell
NP	nanoparticle
ODN	oligodeoxynucleotide
PD-1	programmed death-1
PEG	ethylene glycol
PLGA	poly(lactic-co-glycolic acid)
ROS	reactive oxygen species
Tc-cell	cytolytic T-cell
TCR	T-cell receptor

TGF-β	transforming growth factor β
Th-cell	helper T-cell
TLR	toll-like receptor
TNFα	tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
Treg-cell	regulatory T-lymphocyte
TT	Tetanus toxoid
VEGF	vascular endothelial growth factor

INTRODUCTION

The definition of *nanomedicine* as given by NIH is "an offshoot of nanotechnology, refers to highly specific medical interventions at the molecular scale for curing disease or repairing damaged tissues" (http://nihroadmap.nih.gov/nanomedicine/). It can be subdivided into five fields by the European Science Foundation (ESF): analytical tools, nanoimaging, nanomaterials and nanodevices, novel therapeutics and drug delivery systems, and clinical, regulatory, and toxicological issues. This review will introduce how tumors escape from the immune system and how nanomedicine can be applied for helping the immune system to recognize and eliminate cancer cells.

Cancer has many causes, such as viral infection (EBV, HBV and HPV), bacterial infection (*Helipbacter pylori*), carcinogen, ultraviolet (UV) radiation exposure, and genetic abnormalities. However, the immune system can recognize, eliminate, and protect the body from viral, bacterial infections, and the transformed cells (pre-cancer cell) extension. For example, Rag2-/- mice, which lack B- and T- lymphocytes, develop spontaneous malignancies in multiple organs including intestine and lung (35% and 15%, respectively) (1). Therefore, the immune system can identify cancer and pre-cancer cells on the basis of tumor-specific antigens expressed on tumor cells or molecules induced by cellular stress. The process preventing and eliminating the development and growth of tumors is called *immune surveillance*. Various immune cells, including B and T-lymphocytes, NK-cells, dendritic cells (DC), macrophages, and polymorphonuclear leukocytes, are recruited to the tumor (2). However, the tumor can still evade the immune surveillance.

The concept of tumor escape, which was first described in 2001, is called *immunoediting* (1). Three phases of cancer immunoediting were described by Schreiber (3): elimination, equilibrium, and escape. The elimination phase is the process of tumor immune surveillance when a tumor is detected and eradicated by innate and adaptive immunity, such as the secretion of IFN-γ, IFN-α/β, perforin, NKG2D and TRAIL. When the elimination process is complete, tumor cells are cleared. If it is incomplete, surviving tumor cells will enter into the equilibrium phase. During this stage, the tumor cells may continue, chronically or immunologically sculpted by genetic instability and/or immune selection, to produce new populations. Theses populations may escape from the immune system by multiple mechanisms (3,4), including loss of MHC-I, loss of adhesion molecules, generation of regulatory T (Treg)-lymphocyte, expansion of Myeloid-derived suppressor cells ($CD11b^+$ $Gr\text{-}1^+$ cells, MDSCs), immunosuppression, blocking of NKG2D-mediated activation, and apoptosis induction of anti-tumor effector cells (5–7). The immunoedited tumor is more difficult to treat. Thus, there are many opportunities for nanomedicine to overcome these problems in tumor immunotherapy.

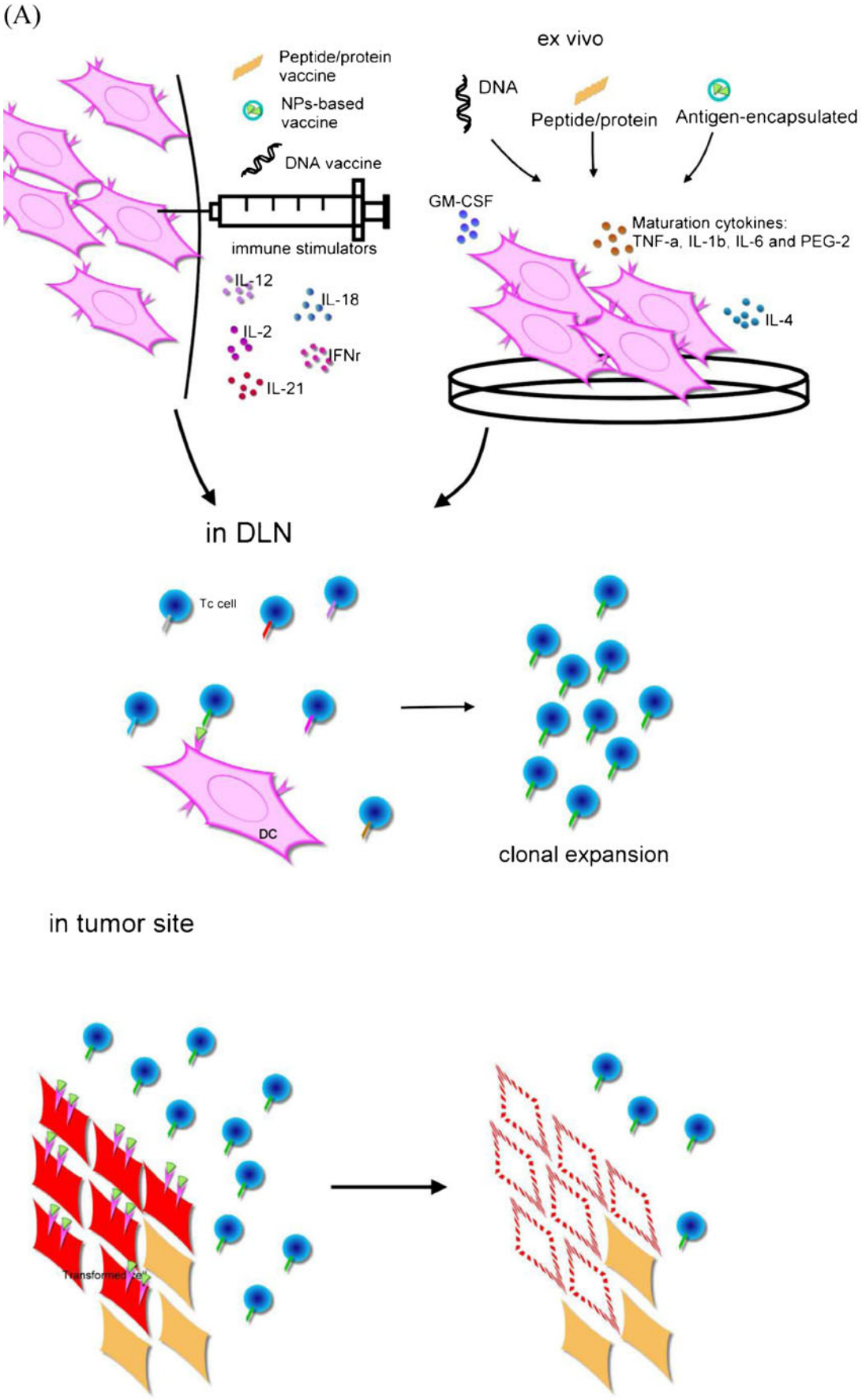
STRATEGIES OF CANCER IMMUNOTHERAPY

Based on the cancer immunoediting, two strategies have been applied in cancer immunotherapy: non-specific immune activation and tumor-specific immune activation. These two strategies also work together for eliminating tumor cells by increasing tumor antigen presentation and inducing specific CTL activity, guiding T-cells to the tumor and down-regulating tumor Treg-cell or MDSCs (Fig. 1).

Non-specific Immune Activation

The non-specific immune activation strategy includes cytokines, interferons, or Toll-like receptors (TLRs) agonist treatment. These all are used in the fight against the tumor microenvironment and help with immune system activation. A number of studies using non-specific immune activation in various cancers have been reported to date.

Fig. 1 The immunotherapies of a tumor. (**A**) Increasing antigens present and induce specific CTL activity. Two types of strategies are applied in this therapy. The first type (*upper*, *left*) is one in which there is an injection of tumor antigen vaccines such as peptide, protein, DNA or tumor lysate vaccine or NP-based vaccines by intramuscular (i.m.) subcutaneous (s.c.) and intraperitoneal (i.p.) injections. The second type (*upper*, *right*) is *ex vivo* cultured autologous DCs treated with cytokines and antigen vaccines. Then, the tumor antigen presented-mDCs are injected back into the host *via* s.c. or i.p. routes. Both strategies generate tumor antigen-presented mDCs which migrate to DLN. In DLN, the Th- or Tc-cells which interact with tumor antigen-presented mDCs proliferate (clonal expansion). These T-cells are tumor antigen-specific T-cells. They will migrate into the tumor, target the tumor cells and perform CTL response. (**B**) Guide T-cells to the tumor. Since some tumors lose MHC or adhesion molecules, immune cells are weak and less likely to recognize or interact with tumor cells. bAbs serve as mediators between an immune cells and a tumor target. One site can recognize the tumor, the other can recognize the immune cells, thus bringing the killer to the target. (**C**) The down-regulation of Treg-cell, or MDSCs ($CD11b^+$ $Gr\text{-}1^+$ cells). Administration of antibodies which are specific for Treg-cell, such as CD25 and CTLA4, and cytokines such as TGF-β and IL-10, will neutralize and lower the activity of inhibitory Treg-cells. Antibody specific for MDSCs, such as Bv8, will reduce the number of tumor-associated $CD11b^+$ $Gr\text{-}1^+$ cells.


(A)
Peptide/protein vaccine
NPs-based vaccine
DNA vaccine
immune stimulators
IL-12
IL-18
IL-2
IFNr
IL-21
ex vivo
DNA
Peptide/protein
Antigen-encapsulated
GM-CSF
Maturation cytokines: TNF-a, IL-1b, IL-6 and PEG-2
IL-4
in DLN
Tc cell
DC
clonal expansion
in tumor site

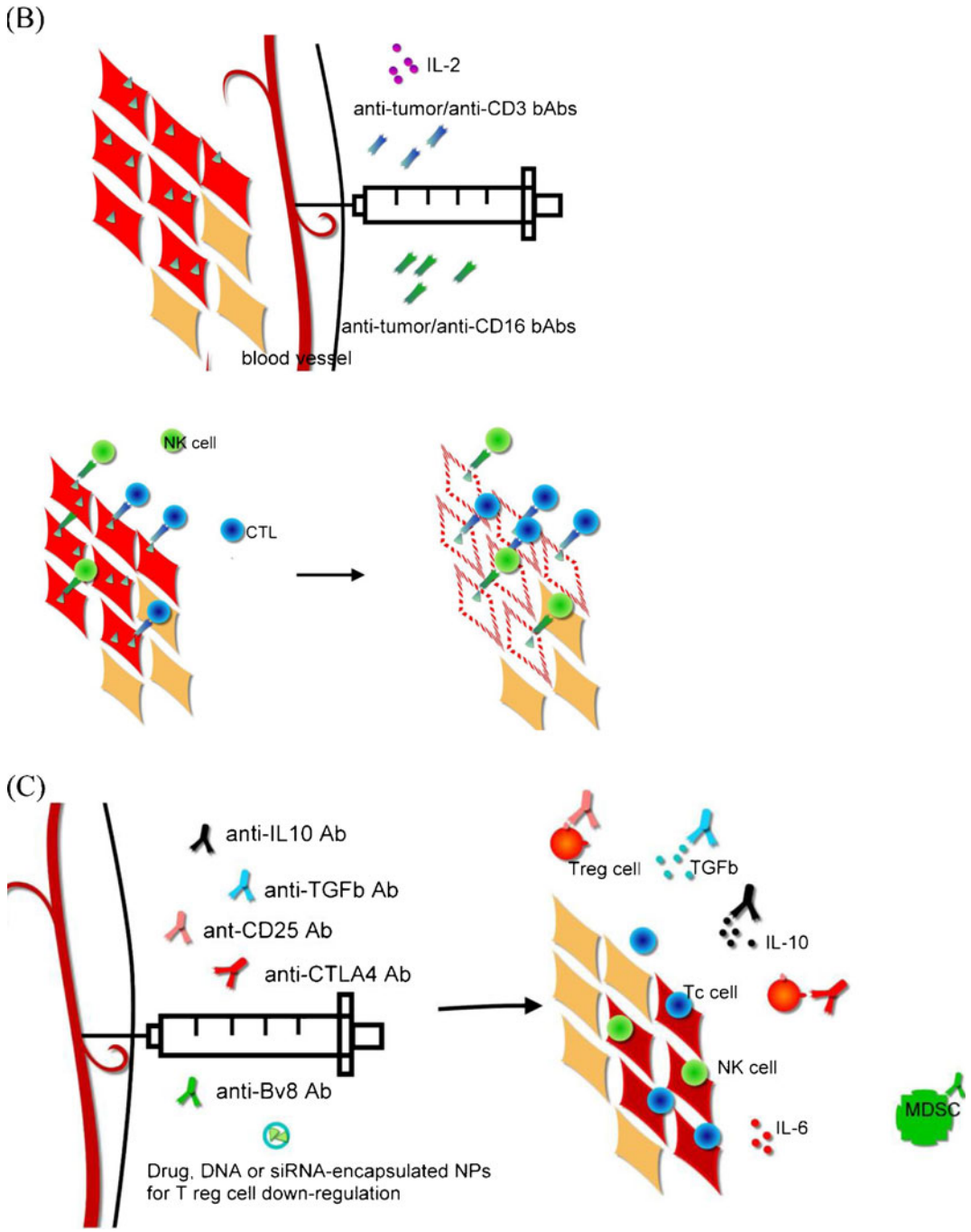


Fig. 1 (continued).

Cytokines

IL-2 promotes proliferation and enhances the cytotoxicity of effector immune cells (8), and also restores the immune response following suppression by a negative regulatory receptor such as programmed death-1 (PD-1) (9). Some investigations showed IL-2 can activate cytokine-induced killer cells (CIKs), which are non-major histocompatibility complex (MHC)-restricted cytotoxic lymphocytes, which possess anti-tumor activity (10–12). In animals, and in some human studies, systemic administration of IL-2 suppresses tumor growth, and it has shown clinical efficacy in malignant melanoma and renal carcinoma (13–15). Moreover, it has also been used to enhance the efficiency of immune therapy such as vaccine therapy or adoptive immune therapy (16).

However, IL-2 is a positive regulator for Treg-cells. Wei's study showed increasing numbers of Treg-cells accumulated in the tumor site after IL-2 treatment, but dropped in IL-2-treated ovarian cancer patients after IL-2 cessation (17). In view of the negative effect of the Treg cells, one must be careful in using IL-2 as a therapeutic strategy. In addition, IL-2 therapy still causes significant dose-related morbidity, since IL-2 toxicity occurs in most organ systems, including heart, lungs, kidneys, and central nervous system. Therefore, managing toxicity is important for successful IL-2 therapy (18).

Other cytokines, such as IL-21 and IL-18, which have been chosen instead of IL-2, activate effector cells, but not Treg. IL-21, an important regulator of both innate and adaptive immune activations, activates $CD4^+$ T-cells, $CD8^+$ T-cells, NK-cells, and B-cells and suppresses Treg-cells. IL-21 also greatly enhanced the production of IFN-γ, IL-2, tumor necrosis factor α (TNFα), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1β and IL-6 by activating T-cells. Moreover, treatment of IL-21 combined with anti-DR5 antibody therapy, promoted the tumor-specific CTL activity, suppressed TRAIL-sensitive tumor metastases, and enhanced memory responses to tumor rechallenge (19). Administration of IL-21 alone was associated with anti-tumor activity in patients with metastatic melanoma and renal cell carcinoma activity in a phase-I clinical trial study (20).

IL-18 has recently emerged as an immunostimulatory cytokine with the capacity to augment anti-tumor therapy with IFN-γ, IL-2, TNF-α, GM-CSF and IL-1α induction, effector T-cell activation, and NK-cell cytotoxicity enhancement (21). IL-18 also promotes protection against tumor challenges in mice (21). In a phase-I clinical study, IL-18 was safely administered as monotherapy to 28 patients with solid tumors, and no dose-limiting toxicities were observed. Even with weekly administration or five consecutive daily administrations, repeated every 28 days, the toxicity of IL-18 was generally mild to moderate, and a maximum tolerated dose has not been reached to date (22,23). Moreover, IL-18 also has been used for the study of combination therapy with liposomal doxorubicin. This combination therapy (22% of the mice remained tumor-free for 6 months) significantly suppresses ID8 ovarian tumor growth compared with either monotherapy (0% 6-month survival) *in vivo* (24).

Interferons

Type I interferons (IFNα and β) possess anti-tumor activity and enhance activity of NK-cell (25), increase expression of Fcγ receptors (26), and inhibit the generation of allospecific suppressor T-cells (27). IFNα/β markedly inhibits the growth of a wide variety of transplantable tumors in mice (28,29), and also pulmonary metastases (30). Type I interferon clinical trials have shown the efficacy in treatment of leukemia, melanoma and renal-cell carcinoma (31–35). However, there was no significant effect in recurrent, platinum-resistant ovarian cancer (36). Moreover, an adenovirus-mediated IFN-β gene therapy in a phase I clinical trial generated anti-tumor immune responses at high rates in malignant pleural mesothelioma and metastatic pleural effusions after a single dose (37). Therefore, it is evident that IFN-β can serve as a potent anticancer agent.

Type II interferons (IFNγ) are secreted by NK-cells and effector T-cells in response to targeting and recognition. In *in vitro* and *in vivo* studies, IFNγ induces apoptosis and upregulates HLA-I and HLA-II and antigen presentation in cancer cells and antiangiogenic effects (38–42). Furthermore, some cancer cell lines with MHC I deficiencies, such as NCI-H146, NCI-H1092 and IMR-32, can be restored to the MHC I expression by treatment with IFNγ *in vitro* (42,43). Research also proved that IFNγ mediated tumor rejection in adoptive tumor therapy (44). The tumor-specific CD8 cells were isolated from CT26-immunized mice and stimulated with or without anti-TCR/CD28 antibodies for 4 or 6 h to induce expression of IFNγ *in vitro*, and then transferred to three-day CT26 tumor-inoculated mice. The results showed that adoptive enriched $IFN\gamma^+$ $CD8^+$ cell therapy showed significant tumor rejection in 60% of the mice and delayed tumor growth in the remaining mice. Neither rejection nor substantial growth delay was observed after transfer $IFN\gamma^-CD8^+$ cells (44). Currently, IFNγ has been shown clinical activity in combination therapy in ovarian cancer, and in a prospective randomized phase III trail. IFNγ, in combination with cisplatin and cyclophosphamide, leads to a significant improvement in progression-free survival at 3 years (45–48). Moreover, IFNγ expression is demonstrated to correlate with a predictor of prognostic factor and cancer survival (49,50)

Toll-Like Receptor (TLR) Agonist

TLR's engagement alerts the immune system and leads to activation of innate and adaptive immune responses. TLRs trigger DC maturation, stimulate proliferation of $CD4^+$ and $CD8^+$ T-cells and modulate the suppressive function of Treg-cells (51–53). Several clinical trials have demonstrated that administration of TLR7 and/or 9 agonists can enhance the activity of cancer vaccines in several malignancies (54–57). Preclinical data showed *Salmonella choleraesuis* up-regulates IFNγ, CXCL9 (MIG) and CXCL10 (IP10) and induces TLR4-mediated anti-tumor responses in melanoma-bearing C3H/HeN mice (58). However, some studies also showed TLR4 agonists promote tumor cell survival, growth and paclitaxel resistance in proportion with

ovarian cancer cells (59,60). Thus, the choice of TLR agonists could be important for cancer therapy.

Recently, synthetic oligodeoxynucleotides (ODNs) that contain CpG motifs trigger immunomodulatory effects through TLR9. CpG ODN promotes Th1 polarization, is safe for use in human, and has been suggested for use as a vaccine adjuvant in many studies (61,62). Moreover, a study of CpG ODN lipid nanoparticles (LNPs) has shown anti-tumor activities in preclinical studies. G3139, a CpG ODN against Bcl-2, encapsulated by LNPs which contained DC-Chol/egg PC/mPEG$_{2000}$-DSPE and protamine, effectively enhanced by about four-fold of IFN-γ, IL-2, IL-4 and IL-10 and significantly enlarged the spleen size as compared to free G3139 and empty LNP. The G3139-LNP effectively inhibited tumor growth (>50%) and prolonged host survival by 245% (63).

Moreover, cytokines, interferons and TLR agonists are not only applied for non-specific immune activation, but are also commonly used in assisting with specific immune activation (see next section). Despite many advantages of these non-specific immune agents, there are shortcomings that must be considered, including short half-life in the circulation and systemic toxicities. Therefore, many methods, such as gene delivery vectors, nanoparticle (NP) delivery systems and tumor targeting, are used for resolving these problems. For example, NPs have been applied for immunogenic agents delivering to improve immune reactions, such as GM-CSF genes, or siRNA delivering to inhibit the expression of immune suppression genes in tumor microenvironments, such as TGF-β (64). Both could improve the efficiency of immunotherapy.

Tumor-Specific Immune Activation

Tumor-specific immune responses are focused on activated adaptive immune systems when they encounter tumor cells. This strategy in cancer therapy is teaching the immune cells to recognize tumor cells specifically.

B-cells secrete antigen-specific antibodies which recognize, bind and help destroy targets. However, B-cells need help from $CD4^+$ cells, since the $CD4^+$ T-cells recognize the antigens presented by MHC II molecules, and then stimulate B-cells to produce antibodies to that specific antigen. The antigen-specific antibodies recognize and bind to the specific antigens on the targeted cells, and then antibody-coated cancer cells are recognized and killed by NK-cells, macrophage and activated monocytes (65,66). This is called *antibody-dependent cell-mediated cytotoxicity (ADCC)*. Currently, most prophylactic vaccines depend on this kind of response. Examples are HBV (FDA approved in 1981) and HPV (FDA approved in 2006) vaccines. Both viruses can cause cancer in human.

Tc-cells are capable of killing targets by releasing perforin and granzymes when their T-cell receptors (TCR) specifically recognize and interact with antigen-MHC I complex on the tumor surface. However, training Tc-cells to recognize targets requires the help of DCs or APCs. Therefore, most of the research in cancer vaccines tries to drive DC cells to present tumor antigens to Tc-cells. These include peptide vaccines, DNA vaccines, DC vaccines and nanoparticle-based vaccines (Fig. 1A). How these vaccines work will be introduced in the next section.

Since MHC loss is one of the mechanisms for tumor escape from immune surveillance, bispecific antibodies (bAb) are designed for serving as mediators (adaptors) between an effector and a tumor target. With bAbs, one end targets tumor-associated antigen and the other targets immune cells, guided effector cells (such as Tc-cell and NK-cell) to tumors and induced tumor-specific immune responses (Fig. 1B). However, bAbs do not work until the host is given an immune stimulator such as IL-2 (67). Currently, there is one bispecific tandem scFv molecule (MT103) directed against CD19 (tumor antigen of Non-Hodgkin's Lymphoma) and CD3 (T-cell) in a clinical phase I trial. This antibody was very potent in destroying CD19-expressing tumor cells *in vitro* and *in vivo* in a T-cell co-stimulation independent manner (68).

On the other hand, administration of antibodies, siRNA or drugs also applies for directly inhibiting the immune suppressor cells. Some examples are down-regulation of Treg-cells, MDSCs and immunosuppressive cytokines (Fig. 1C). The anti-CD25 antibody and anti-CTLA4 antibody can internalize Treg-cells, and the anti-Bv8 antibody treatment can reduce the number of tumor-associated $CD11b^+Gr\text{-}1^+$ cells which might regulate their homing to the tumor site (5). Gemcitabine and 5-Flurouracil also can reduce the number of $CD11b^+Gr\text{-}1^+$ cells and so on (5). This therapy can adjust the tumor microenvironment and enhance tumor-specific immune activation.

THERAPEUTIC CANCER VACCINE

Cancer vaccines are applied for treating existing cancer or preventing the development of cancer in certain high risk individuals. Usually, the components of vaccines include tumor-specific antigens, carriers or delivery systems and adjuvants. Tumor-specific antigens usually come from cancer cells—including proteins, carbohydrates, glycoproteins or glycopeptides and gangliosides, or gene (DNA or RNA) encoding cancer-associated antigens. A few examples of this can be seen in the E7 protein of HPV 16 being a protein-based vaccine (Phase I/II clinical trial), the E7 peptide (11–20) of HPV16 being a peptide-based vaccine (phase I trial) and the DNA encoding E7 epitope (aa 83-95) being a DNA-based vaccine (Phase I trial) (69–71). However, the choice of tumor antigen needs to follow the

rules of MHC I and II presentation, except for the protein vaccine. The effector $CD8^+$ T-cells recognize short peptides, 8–10 amino acid residues in length, which present through MHC I (HLA-A, -B, -C); the $CD4^+$ T-cell recognizes long peptides, 15 amino acid residues in length, which present though MHC II (HLA-DP, -DM, -DOA, -DOB, -DQ and -DR). When $CD4^+$ cells recognize the peptide-MHC II complex, it stimulates antibody-producing B-cells to produce antibodies to that specific antigen. The MHC I and II binding peptides can be predicted in some websites (72–74).

To look for specific targets of tumors, antigens must be expressed only in tumor cells; however, some mutated proteins and tumor-specific posttranslational modified proteins cannot be good targets, since the mutant region may not be presented by MHC molecules or the region is masked by modification such as glycosylation. Therefore, the proteins which are highly expressed in tumor cells and normal/low expressed in normal tissue are chosen for tumor targets, such as tyrosinase in melanoma (75). Moreover, these antigens are often essential for tumor survival or transformation and will not be likely to escape the immune surveillance. For example, the viral proteins E6 and E7 of HPV16 are important for malignant transformation, and they are good candidates in virus-induced cervical cancer (76).

When the antigen was chosen, the carrier or deliver system served as cargo vehicles to carry and deliver the antigen to the appropriate immune cells and to the appropriate compartments within those cells. The materials of these systems include oil-in-water emulsion, mineral salts, aluminum compound, microsphere (for example, chitosan), NPs, attenuated viruses, cells and so on. The advantages of NPs applied in vaccine carrier and delivery systems are derived from their size. The nano-sized particles, generally less than 1 μm in diameter, are ideal for the induction of systemic immunity because they are internalized efficiently by DCs as well as by macrophages. The particle sizes in the range of 20–50 nm are small enough to facilitate rapid transport through the lymphatics and large enough to prevent leakage into blood vessels (77,78). Studies also suggest that nanomaterials (< 100 nm) provide enhanced immunogenicity compared to larger systems (79; therefore, NP-based vaccines may also provide the adjuvant effects. On the other hand, the capacity of antigen loading, immune potentiation, targeting and transporting the loaded antigens are seeing great improvement in NP-based vaccines recently. The materials of NP included poly (lactic-co-glycolic acid (PLGA) (80,81), magnetic (82), liposomes, chitosan (83), poly(glutamic acid) (PGA) (84,85) and so on, and have been studied in antigen delivery and immunity elicitation. The practice and design of NP-based vaccines improve the development of peptide/protein vaccines, DNA vaccines and DC vaccines, which will be introduced in the next section.

Type of Vaccine

According to the type of antigens and carriers, cancer vaccines can be divided into several types: peptide/protein vaccines, DNA vaccines, DC-based vaccines, tumor-based vaccines, and NP-based vaccines. However, DCs play a very important role of the antigen-presenting cells (APC) in the immune system. The principle of most vaccines is based on antigen delivery and presenting onto the APCs (Fig. 1A).

Peptide/Protein Vaccine

The sequence of a peptide vaccine follows the basic rule mentioned above such that it can be loaded and presented by MHC I or II molecules on the surface of APC. A protein vaccine is taken up by DCs directly and processed and presented by MHC I and MHC II molecules without MHC restriction. Both peptide and protein vaccines are locally supplied to DCs by direct injection. In some studies, protein vaccines elicit better antibody response, whereas peptide vaccines elicit better cytotoxicity T-lymphocyte (CTL) response. These vaccines are safe, with limited immune response only to the epitopes delivered. They are also stable and can be combined with other peptides. Currently, peptide and protein vaccines are studied in clinical trials, such as cervical cancer, breast tumors, nasopharyngeal tumors and melanoma (69,70,86–88). However, peptide/protein vaccines usually show low immunogenicity and require the addition of adjuvants or cytokines for increased immunogenicity (89).

On the other hand, the modified peptides are used for inducing specific CTL and increasing immunogenicity, for example, lipopeptide acting as a self-adjuvanting vaccine, in which a lipid was attached to the end of the HLA-epitope (90,91). It has been shown that lipopeptide is a ligand of TLR 1 and 2, and also that the lipopeptide-pulsed human DCs also secrete IL-12 and induce functional stimulation of $CD8^+$ T-lymphocytes specific for the epitopes (90,92,93). Some constructs of lipopeptide contain two peptides, one for presenting MHC I and the other for presenting MHC II (94,95). For example, Le Gal and coworker linked lipid tails to universal tetanus toxoid (TT 830-843) epitopes of Th cells that was itself linked to the HLA-A2 restricted MART 27–35 CTL epitope (lipid - K-GR - (Th-cells epitope) - RGR - (CTL epitope)). This lipopeptides vaccine proved to increase immunogenicity (by lipid tail), induce strong and long-lasting antigen-specific CTL responses (by TT830-843) and elicit CTL response (by MART 27-35) (94). Therefore, lipopeptides can be considered an effective vaccine for cancer immunotherapy.

DNA Vaccine

The DNA vaccines simply use plasmid DNA, which contains a DNA sequence of tumor antigen and a promoter for gene expression in the mammalian cell. In 1990, Felgner published the result that simple plasmids directly enter mammalian cells, and the encoded protein was expressed after injection into the muscle of a mouse (96). Moreover, intramuscular injections of naked DNA plasmid have been shown to generate immune response (97,98). It was demonstrated that DNA vaccine introduces an antigen gene into DC or APC and produces the antigen as an endogenous protein for processing and presentation to the Tc-cells in DLNs. It also can produce the antigen in other cells (such as myocyte), and the antigen is taken up and presented by DCs or APCs (99). Theoretically, DNA vaccines do not require formulation or viral vector for delivery. Naked DNA is safe and stable and can be used to sustain the expression of antigen in cells for longer periods of time than RNA or protein vaccines. Furthermore, it has been proven that DNA vaccine can induce antibody responses and CTL responses (100,101). However, some concerns are noted: for example, vaccination of oncogene, such as E6, may transform normal cells into abnormal cells. Moreover, DNA vaccines cannot amplify by themselves and have weak immunogenicity. Repeated vaccination and/or high dose administration are necessary. Nowadays, some strategies are used for enhancing the efficacy of DNA vaccines, such as encoded protein fused with calreticulin (enhance MHC I antigen presentation), fused cytokine for increasing immunogenicity or encapsulating DNA in nanoparticles to protect the DNA from degradation, enhancing the uptake into APCs and/or increasing immunogenicity (102–104). These strategies are successful in improving the immune response of DNA vaccines.

DC Vaccine

DCs are professional APCs for processing and presenting antigens. Immature DCs (iDCs) take up antigens through phagocytosis, micropinocytosis, receptors and lectin-mediated endocytosis. When iDCs encounter inflammatory mediators, such as TNF-α, they start to mature (mDCs). In the meantime, the antigen uptake and processing are down-regulated, and the expression of MHC is up-regulated. In addition, mDCs travel to DLNs, where they present the antigen to T-cells. The MHC I and II molecules in DC can be physically loaded with antigen *ex vivo*. The loading can be accomplished by pulsing DCs with antigenic peptide or protein, tumor lysate, fusing DCs with irradiated tumor cells, or transfecting DCs with DNA or RNA encoding tumor antigens which can be carried by themselves, nanoparticles or virus (such as adenovirus) (105–107). Finally, antigen-loaded mDCs are injected into the patient as an autologous DC vaccine to induce T-cell immune responses against the tumor.

Currently, DC vaccines are not only studied in solid tumors, but also non-solid tumors. Examples can be seen in B-cell lymphoma, where 15 of 23 patients induced T-cell and humoral anti-Id response (108). However, 20 patients with solid tumors (advanced pancreatic, hepatocellular, cholangiocarcinoma, or medualy thyroid carcinoma) were treated with tumor lysate-pulsed DCs, and none of them were able to meet the formal criteria for complete or partial response (109). A phase III clinical trial in stage IV melanoma showed it was not as effective as chemotherapy (110). The clinical trials in DC-based vaccines have been disappointing. However, an exciting DC-based vaccine (Sipuleucel-T, Provenge®, Dendreon Corp.) was just approved by the FDA in April 2010. In the phase III clinical trial, researchers cultured the autologous DCs from advanced prostate cancer patients with prostatic acid phosphatase (PAP)-GM-CSF fusion proteins for 36–44 h, and then infused them back into patients. The results showed the Sipuleucel-T group ($n=341$) significantly extended the median survival of patients with metastatic, castrate-resistant prostate cancer for an average of 4.1 months longer than the placebo group ($n=171$) in a randomized, double-blind study. This is encouraging because Sipuleucel-T is the first approved cancer therapeutic vaccine in the world.

Nanoparticle-Based Vaccine

The design of NP-based vaccines can be simply divided into three parts: antigen, targeting ligand and delivery materials (Fig. 2). As mentioned previously, an antigen can be a protein, peptide or piece of DNA encoding the tumor antigen. Targeting ligands, such as DC-specific antibodies (anti-lectin DEC-205 antibody) or TLR ligands (monophosphoryl lipid A), can be conjugated with NPs to facilitate intracellular transport (111,112). Delivery materials such as multiple emulsions, liposomes and polymeric NPs (such as PLGA) are currently studied as vaccine formulations. They are also effective in triggering mucosal and systemic immune responses (113).

Biodegradable polymers have been reported as promising antigen-delivery systems for different vaccine applications. One of the most widely studied is PLGA. PLGA has adjuvant effects that elevate in cellular and humoral immune responses and in the induction of immunological memory (114). Moreover, an oral PLGA-based vaccine yielded a long-term protection that was equivalent to three doses of the injected antigen (115,116). PLGA NPs are efficiently phagocytosed by the DCs *in vitro*, and also show up-regulation of surface expression of MHC class II and CD86 molecules (80). NP containing MUC-1 peptide-

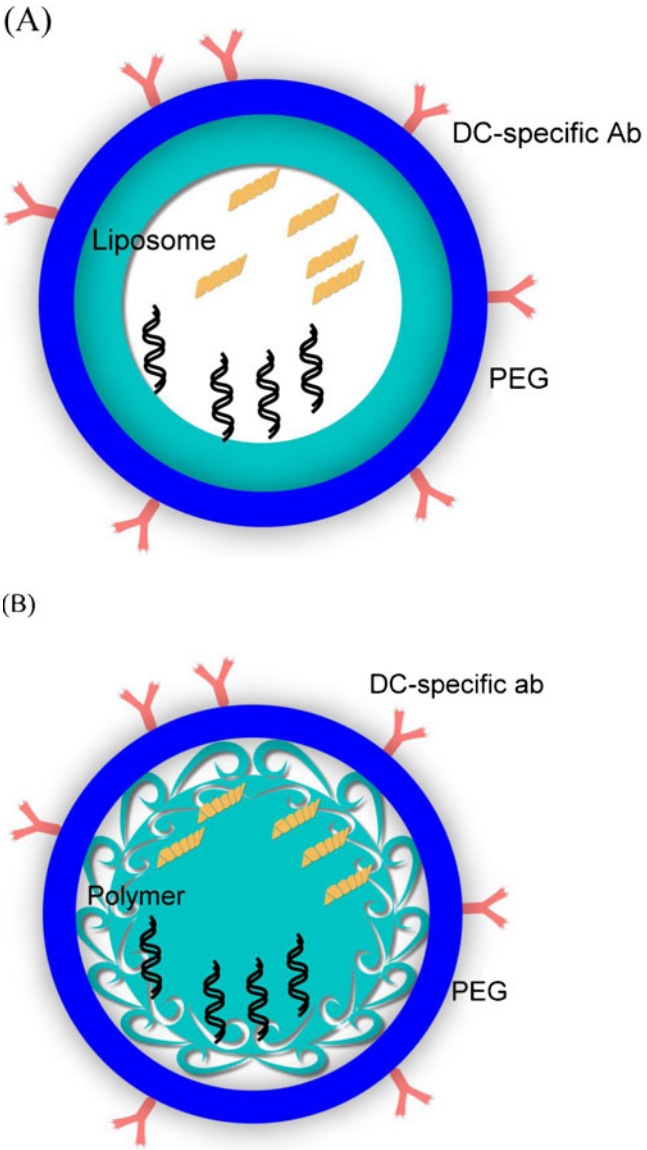


Fig. 2 Ideal nanoparticle in immunotherapy—target, carrier, antigen and protector. Examples are (**A**) liposome and (**B**) PGLA NP. Target: Targeting ligands, such as DC-specific antibodies or TLR ligands, can be conjugated with NPs to facilitate intracellular uptake. Carrier: these include multiple emulsions, liposomes (**A**) and polymeric nanoparticles (**B**), etc. Antigens include protein, peptide and DNA of tumor antigens for increasing antigen expression on DCs or antibodies for neutralizing the tumor microenvironment and improving immune responses. Protector: for example, PEG is employed to conjugate onto the surface of NPs, which reduces the number of NPs taken up by macrophages and increases the half-life of NPs in the blood circulation.

encapsulated PLGA is capable of eliciting specific Th1 responses *in vivo* (117). These results strongly suggest that PLGA NPs provide an efficient vaccine delivery system for targeting DCs and the development of DC-based cellular vaccines (80). Moreover, in order to improve the pharmacokinetics, polymers have also been applied to modify NPs. For example, poly (ethylene glycol) (PEG) polymer is grafted onto the surface of NPs, which improves their pharmacokinetics since PEG reduces the number of NPs taken up by macrophage and increases the half-life in circulation for many different types of NPs (118)

Liposome, a self-assembled, closed structure composed of lipid bilayers and an aqueous interior has been used to encapsulate protein and DNA for delivery *in vitro* and *in vivo*. It exerts immunomodulatory effects when introduced as a vaccine adjuvant. Proteins and DNA can attach to the outer surface of the liposome, or can be encapsulated in the inner space, or both. After introduction into the host, the vaccine is taken up and delivered into APCs for antigen presentation *via* MHC I or II pathway. Eventually, it generates the antigen-specific immune response (119–124). Our previous studies demonstrated that the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) and lipid-polycation-DNA (LPD) plays the role of vaccine formulation for delivery and adjuvant towards anti-tumor activity *in vivo* (121,125,126). DOTAP helping the antigen presentation to MHC I *in vivo* had been demonstrated in the early 1990s (127,128). LPDs, which is composed of two lipid bilayers and a compact core, are prepared by mixing cationic liposomes, a polycationic peptide (protamine), and nucleic acids at an optimized ratio (129). The advantage of LPD is the cationic lipid-DNA complex enhances the efficiency of transfection and protects the DNA from attack by DNA-degrading enzymes (130,131). Upon the administration of LPD, levels of TNF-α, IL-12, and IFN-γ increase rapidly (132). LDP was taken up by ~50% of DCs, ~50% of NK-cells and ~30% of macrophages in popliteal lymph nodes after subcutaneous footpad injection. The E7 peptide-encapsulated LPD (LPD/E7) generated the antigen-specific CTL responses and caused complete tumor regression in the treated mice (tumor injected 6 days before the onset of treatment) (125,133). LPD has been used in a clinical trial to treat children with the Canavan's disease (134). However, due to high level of TNF-α induced, LPD may cause potential systemic toxicity.

On the contrary, E7 peptide-encapsulated DOTAP liposomes (DOTAP/E7) showed low expression of TNF-α (125,135,136), but still induce the migration of activated DCs to the DLNs, generate good antigen-specific CTL activity, and regress tumor growth (121,135). DOTAP/E7 treatment increased the population of $CD4^+$ T-cells and decreased Treg-cells in tumor-bearing mice (121). Thus, DOTAP itself could be a potent adjuvant to enhance vaccine activity with little or no unwanted toxicity. In addition, DOTAP does not present the problem of recombination, virulence, or pre-existing immunity as viral-based vaccines do. However, overdose of DOTAP induced massive reactive oxygen species (ROS) production and apoptosis of DC in DLN, which led to diminished anti-cancer immunity (121).

Nowadays, Stimuvax®, a MUC-1-peptide-encapsulated liposome-based cancer vaccine product, was in clinical trials for treatment of non-small-cell lung cancer and breast cancer. However, the trials were temporarily closed in 2010, since there was an unexpected serious adverse reaction in a patient with multiple myeloma. Currently, studies are still being done to see what is the mechanism affecting the response to this vaccine.

Adjuvant

Adjuvant is an agent that stimulates immune response and increases, accelerates and prolongs the response of a vaccine and remains non-toxic and safe to the host. Many different kinds of adjuvant have been developed over the years: a) mineral salt—aluminum hydroxide (alum) and aluminum phosphate; b) oil emulsions—oil-in-water emulsion (such as MF59); c) particulate adjuvant—DOTAP, virosomes (viral membrane proteins incorporated in the bilayer membrane), immunostimulating complexes (ISCOMS); d) microbial derivatives—monophosphoryl lipid A (MPL), bacille Calmette-Guérin (BCG), heat labile enterotoxin (LT), chlorella toxin (CT), CpG oligonucleotides (CpG ODN; TLR9 agonist); e) plant derivatives—purified saponin (such as QS21); f) cytokines—GM-CSF, IL-12 and IL-2 (137). These adjuvants elicit their effects *via* different immune responses. The Th1 immune response, which is responsible for the cellular immune response such as antigen-specific CTL activation, is induced by DOTAP (138,139), CpG ODN (140), or IL-12, whereas the Th2 immune response, which is responsible for the humoral immune response and enhances antigen-specific antibody generation, is promoted by alum, LT and CT (141,142). Therefore, the choice of adjuvant is an important part in the process of designing a cancer vaccine.

Presently, for reasons of serious restrictions for adjuvant safety issues, alum and AS04 are the only approved adjuvants for human in the USA, whereas incomplete Freud's adjuvant (IFA) is used in humans in some countries, and AS04 and MF59 is licensed in Europe (143,144). The formulation of Gardasil (Merck & Co. of Rahway, New Jersey) contains alum as adjuvant; Cervarix (GlaxoSmithKline (GSK) Biologicals of Rixwnsart, Belgium) uses AS04, which contains aluminum and MPL, as adjuvant. Both have shown to be safe and effective in phase III trials of cervical cancer and are in use in many countries, including the U.S.

DRAWBACKS OF NANOMEDICINE IN IMMUNE RESPONSE AND IMMUNOTHERAPY

Cancer vaccines can be powerful therapeutic methods for cancer therapy; however, some potential disadvantages should be noted. Protein and DNA vaccines may cause cell transformation. Viral-based vaccines contain the risk of genetic recombination, chromosome integration, virulence, and pre-existing immunity. NP-based vaccines also present potential toxicity. Anti-liposome or PEG antibody responses against the drug delivery system have been found (145,146).

Since cancer vaccines stimulate specific immune responses and direct them against the targets, side effects of cancer vaccines are observed in patients. They include flu-like symptoms, including fever, chills, dizziness, nausea and vomiting, and inflammation, such as pain, swelling, itchiness, and rash. More serious symptoms, such as asthma, autoimmune disease and severe hypersensitivity, have also been found in a few cases. Therefore, not only should the cytotoxicity of nanomaterials be a safety concern, but the body immune system and biological effects should also be considered before the treatment. Accurate diagnosis is a very important requirement before personalized therapy can begin.

CURRENT AND FUTURE DEVELOPMENTS

Enhancing vaccine efficacy and overcoming the immunoediting of tumors are important issues for future studies. As we already know, the loss of MHC molecules, increasing the level of Treg-cells and MDSCs, up-regulation of TGF-β, and so on, in the tumor microenvironment help the tumor to survive. The efficacy of a cancer vaccine may be offset by changes in the tumor microenvironment. Attempts to block or down-regulate Treg-cells, such as treatment with monoclonal anti-CTLA-4 antibodies, have been studied in clinical trials as an agent alone or in combination with cancer vaccines (147). Increasing levels of co-stimulation, such as CD80, ICAM-1 and LFA-3, or cytokines, such as IL-15, combined with vaccine therapy can selectively induce longer-living CTLs and be more effective in killing tumor cells (148,149).

Moreover, combination therapies, such as vaccine therapy with chemotherapy, have also been studied. The immunomodulation of chemotherapy has been demonstrated, since cyclophosphamide, doxorubicin and paclitaxel increase the number and function of antigen-specific T-cells and thus enhance anti-tumor immune responses (150). The mechanisms of immunomodulatory effects caused by chemotherapy are a) induction of immunogenic tumor cell death, which greatly increased tumor antigen uptake by DCs (151,152); b) direct activation of APC and effector mechanisms, such as activation and maturation of DCs (153,154); c) suppression of immune inhibitory cells which increase anti-tumor immune responses (150,155,156). Thus, it is believed the combination of these chemo drugs

with cancer vaccines may produce more significant clinical results.

Some NPs can pass the blood-brain barrier to the central nervous system with a measurable pharmacological consequence (157). Most new therapeutic studies of brain tumor are performed by thermotherapy using magnetic NPs (158,159) or vaccine (158). In a study of combination therapy, Schneider (64) delivered TGF-β antisense oligonucleotides by polybutyl cyanoacrylate NPs which apparently passed the blood brain barrier, and combined with a vaccine therapy after five days post-glioblastoma cell inoculating intracerebrally in a rat model. The results showed rats treated with the combined therapy survived longer than those treated with vaccine alone or not treated. However, the improvement in the survival was marginal at best.

There are an increasing number of specific and multifunctional drugs and delivery systems being developed and targeting tumors by using the features of the tumor, including high proliferation, oncogene expression, and the well-known enhanced permeability and retention (EPR) effect in the tumor microenvironment (160–162). However, some of these features also occur in normal cells. Features such as the EPR effect are also occurring in inflamed sites (163). In the past, many drug delivery systems have tried to overcome the drug resistance mechanisms, kill tumor stem cells or specific targets to tumors and metastatic lesions. Taken together, one of the simplest ways to resolve these problems is using the application of combination therapy. In this manner, the deficiency of one therapy can be made up by another. However, the addition of a second therapy may cause more side effects. Testing and optimizing these combinations will maximize efficacy and decrease toxicity which are important issues for future consideration.

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