Nanomedicine to modulate immunotherapy in cutaneous melanoma (Review)

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Abstract. Cancer immunotherapy has shifted the paradigm in cancer treatment in recent years. Immune checkpoint blockage (ICB), the active cancer vaccination and chimeric antigen receptor (CAR) for T-cell-based adoptive cell transfer represent the main developments, achieving a surprising increased survival in patients included in clinical trials. In spite of these results, the current state-of-the-art immunotherapy has its limitations in efficacy. The existence of an interdisciplinary interface involving current knowledge in biology, immunology, bioengineering and materials science represents important progress in increasing the effectiveness of immunotherapy in cancer. Cutaneous melanoma remains a difficult cancer to treat, in which immunotherapy is a major therapeutic option. In fact, enhancing immunotherapy is possible using sophisticated biomedical nanotechnology platforms of organic or inorganic materials or engineering various immune cells to enhance the immune system. In addition, biological devices have developed, changing the approach to and treatment results in melanoma. In this review, we present different modalities to modulate the immune system, as well as opportunities and challenges in melanoma treatment.

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Contents

1. Introduction

Cutaneous melanoma is the most aggressive form of skin cancer and its incidence is on the increase worldwide. The most promising strategy for treating melanoma is represented by the therapeutic manipulation of the immune system. The key challenge for the clinical implementation of immunotherapies is controlling the mechanisms of the immune system, with high responses and low side effects. This modulation requires administration of the most appropriate immunomodulator dose during the right time at the most suitable tissular, cellular and intracellular location (1).

Biomedical nanotechnologies represent a promising choice through engineering biomaterials, drug-delivery systems, even immune cells for targeting the immune system in a controlled manner. Improving the ability of immunomodulatory molecules to reach disease tissues, immune cells, or their intracellular compartments, and manipulating immune cells to kill cancer cells remain two essential objectives for every immuno-nanotechnology platform. Several categories of

NPs or biologic devices that enhance the efficacy of immunotherapy are cancer vaccines, immune checkpoint inhibitors and nano-immunostrategies that can be employed to reprogram the tumor microenvironment.

2. Immune system in cancer

The composition of the immune system includes immune cells and immune organs, strongly related to other non-immunological cells and organs, with the purpose of protecting the host from foreign microorganisms and their bodies. This protective function is performed simultaneously with the maintenance and tolerance of self-antigens. Innate and an adaptive immune responses are described. Innate immunity includes macrophages, natural killer cells (NKs) and dendritic cells (DCs) that are responsible for the first barrier against non-selfs. DCs and macrophages trigger a response manifested by inflammation, which is followed by innate and adaptive cell alerting.

In cancer, the immune cells from innate and adaptive immunity constitute the tumor immune microenvironment (TIME). T cells from TME are represented by tumor-infiltrating lymphocytes (TILs), which play a major role in tumor initiation and progression (2) and exert both protumoral and antitumoral activity. Normally, for the inhibition of tumor growth, $CD4^+$ T helper 1 (Th1) and T helper 2 (Th2), $CD8^+$ T cells and natural killer (NK) T cells produce interferon-gamma (IFN-γ), which activates macrophages for cancer cell phagocytosis. Macrophages are involved in interleukin 2 (IL-2) synthesis, which enhances Th1 cell differentiation (3). The balance between Th1 and Th2 cells is critical in the antitumor immune response. Th1 cells stimulate IL-2 and IFN-γ production, which trigger the induction of cellular immunity by eradicating the tumor mass, whereas Th2 cells are essential in stimulating the humoral immunity by inducing tumor necrosis (4). IFN-γ is responsible for the stimulation of the antigen-presenting cells (APC) that activate cytotoxic $CD8^+$ T cells, which recognize the peptide antigens presented by MHC class I molecules from the tumor and promote tumor cell lysis. Most tumors are positive for MHC class I and negative for MHC class II. Th2 release IL-10, IL-13, IL-5 and IL-4, enhancing T-regulatory (Treg) cells that inhibit the $CD4^+$ and $CD8^+$ synthesis (5,6). Immature myelomonocytic cells are represented by myeloid-derived suppressive cells (MDSCs) that improve the immunosuppressive activity on T cells. MDSCs produce arginase-1 (ARG-1) and indoleamine 2,3 dioxygenase (IDO) that generates inefficient T-cell receptor complex expression on Ag-activated T cells (7,8) and are involved in expressing reactive oxygen species, IL-10, TGFβ and nitric oxide, responsible for the suppression of anti-tumoral immunity (9). Macrophages are other major players in cancer progression and various types of activation of these macrophages are described, related with different signals: i) The classical activation of macrophages (M1), associated with the production of proinflammatory cytokines, that produces reactive oxygen species that causes cytolysis in cancer cells (10), and ii) alternative activation of macrophages (M2), which generate anti-inflammatory cytokines that enhance tissue repair and angiogenesis, favoring tumor progression (11). IFN-γ promotes M1 and IL-4 enhances M2, leading to the description of a bipolar axis. Prostaglandin, free fatty acids, IL-10 or high-density lipoprotein are other factors involved in macrophage activation alongside the bipolar M1/M2 axis (12,13). The immune checkpoint proteins as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and Programmed Death-1 (PD-1), both expressed by activated T cells, can also enhance the immunosuppression of TIME. PD-1 blocks the effector T-cell activation, preventing the interaction with its ligands PD-L1 or PD-L2. In addition, CTLA-4 binds to the APCs surface with CD80 and CD86, producing the inhibition of T cells (14).

The response of the immune system to tumor growth is represented by immunoediting, a dynamic process consisting of three distinct phases: Elimination, equilibrium and escape. In the elimination phase, the cells and molecules of innate and adaptive immunity work together in order to find the presence of the tumor and eliminate it. In some cases, variants of tumor cells may not be completely destroyed, but enter the following phase, namely the equilibrium phase, where the immune system controls the tumor cell growth. In the equilibrium phase, the innate immune system cannot totally eliminate cancer cells, but keeps them in a state of immune-mediated tumor dormancy. The escape phase can be considered as a failure of the immune system to eliminate or control cancer cells, enabling the survival of cell variants, in an unrestricted manner. Related to these dynamic phases, one must take into account the described immune phenotypes of tumor microenvironment (TME): i) The immune-desert phenotype is characterized by immunological tolerance (not the response to antigen presentation), ignorance (lack of antigen) and lack of T-cell priming; ii) The immune-excluded phenotype, where the immune cells from the periphery of the tumor or stroma are impeded by extravascular stroma and immature vessels; iii) The inflamed-phenotype, where pro-inflammatory cytokines are expressed by T cells from parenchyma, representing a failure of antitumor immune response. Of note, the TME has various compositions in different cancer types, different patients with the same cancer and also different tumor sites within the same patient (15).

3. Immunotherapy in melanoma

Immunotherapy aims to manipulate the immune system and is used for treating diseases. This targeting has two main goals: The inhibition or enhancement of the immune system, depending on the intended effect. The general classification related to therapy includes active and passive immunotherapy (16). This classification takes into account how immunotherapy stimulates or inhibits the immune system. Active immunotherapy is composed of treatments aiming to enhance the immune response against antigens. The checkpoint inhibitors and vaccines are included in this category. These are immune active and act only in close relationship with the host immune system. Conversely, passive immunotherapy is based on an intrinsic immune response, mediated by molecules, such as antibodies and cytokines that stimulates the immune response. Examples of passive immunotherapy are monoclonal therapeutic antibodies and adoptive cell transfer therapy (17).

Three main strategies in which immunotherapies for cancer are classified were described, related to active or passive therapies. The first strategy is represented by nanoparticles (NPs) that represent delivery systems for stimulating molecules or antigens and show promising results in treating melanoma. NPs are nanocarriers that trigger specific receptors and target the immune system activation or inhibition. Furthermore, if the molecule carried by nanoparticles is an antigen, they are known as nano-vaccines and the purpose is to migrate into the lymph nodes (LNs) in order to trigger the T lymphocytes and generate a specific cytotoxic response against the tumor (18). In addition, specific nanoparticles can target dendritic cells from the tumor microenvironment using the same mechanism of presenting antigens to stimulate cytotoxic T cells. Adoptive cell transfer therapy is the second strategy in which the immune cells are collected from the patient, trained *ex vivo*, and reinfused into the patient (19). The third strategy, with promising results in melanoma treatment, is represented by the delivery of therapeutics to the immune tumor microenvironment area. These therapeutics can target cancer cells, but also elements of the immune system that are present in the tumor immune microenvironment including DCs, tumor-associated macrophages, cytokines (IFNs, TGF-β, IL-2) and enzymes involved in the metabolic pathways of cancer cells. The development of biologic devices as viral therapies in the last decades opened new routes to modulate the immune system against tumor cells (Fig. 1). Currently, the main available medical immunotherapies of melanoma are represented by tumor vaccines, gene therapy, checkpoint inhibition immunotherapies, T-cell directed therapies and non-specific approaches including cytotoxic chemotherapy, photodynamic therapy, photothermal therapy, and radiotherapy, recommended only in subsets of patients carefully selected for the clinical benefit (20).

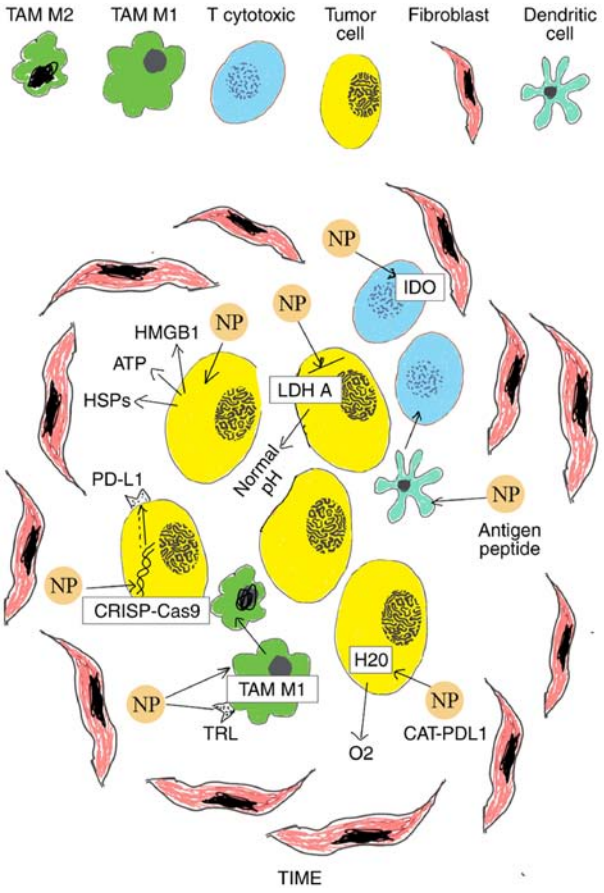

Figure 1. Schematic of various nanomedicines applied in cancer immunotherapy of cutaneous melanoma. i) Multifunctional CAT-PDL1 liposomes includes CAT that decreases tumor hypoxia decomposing H_2O_2 into O_2 and PDL1 which improves immunotherapeutic effects, promoting $CD4^+$and $CD8^+$ T cells; ii) PLGA NPs deliver antigenic peptides that target dendritic cells to promote cytotoxic T lymphocyte responses; iii) NPs inhibit IDO and block tryptophan metabolism of cancer cells; iv) NPs block LDH A in tumor cells leading to normal pH; v) NPs cause the translocation of calrecutin and lead to the release of ATP, HMGB1 and HSPs in extracellular environment, inducing ICD of cancer cells; vi) co-polymer NPs aPBAE knock down Cdk5 and cause PD-L1 downregulation via CRISPR-Cas9 genome editing; vii) aCD47@CaCO3 NPs increase the macrophage polarization to M1 phenotype and block the 'don't eat me' signal in cancer cells. NPs,nanoparticles;PLGA,poly-lactic-co-glycolicacid;TIME,TumorImmune Microenvironment; IDO, indoleamine 2,3-dioxygenase; CAT, encapsulated catalase; ICD, Immunogenic cell death; ATP, adenosine triphosphate; HMGB1, high mobility group box 1 protein; HSPc, heat shock proteins.

4. Classification of nanotechnologies for cancer immunotherapy

Nanomedicine represents the medical application of nanotechnology and includes medical applications of nanomaterials, biological devices and applications of molecular technologies. The main issues of nanomedicine are related to toxicity and environmental impact of nanoscale materials. Specifically, nanomaterials and biological devices used for enhancing cancer immunotherapy are classified into polymeric nanoparticles, lipid nanocarriers, metal nanoparticles, inorganic non-metallic nanoparticles, exosome and engineered viruses (21-25).

Polymeric nanoparticles. Polymeric NPs are represented by poly-lactic-co-glycolic acid (PLGA), dendrimers and micelles and have been used in many drug delivery systems. Advantages of polymeric nanoparticles are the versatility in size, morphology, high loading of therapeutic drugs and surface functionalization. The disadvantages are represented by the synthesis of proinflammatory molecules and the inconstant degradation and inactivation of the therapeutic pay load in the preparation process.

PLGA. PLGA is FDA approved and represents the most commonly used polymer which is biocompatible, biodegradable and of low toxicity. Microspheres of PLGA target the pathways for MHC class I and II molecules and cause an increase in the maturation of DCs (26). PLGA delivery systems were designed for cytokine agonists, siRNAs or CpG-coated tumor antigen transportation to promote the internalization of antigens by DC and the generation of immune responses stimulating CTL ($CD8^+$) and Th ($CD4^+$) (27-29). In addition, PLGA nanoparticles seem to be more suitable to target DCs than PLGA microparticles are, with a 10- to 100-fold higher efficiency in delivery of hD1 for nanoparticles (30).

Dendrimers. Dendrimers are branched macromolecules, composed of a core and cavities to entrap drugs, suitable for modified drug delivery due to water solubility, polyvalency and well-defined chemical structure (31), being described as a direct interaction between immune cells and dendrimers. The

surface of dendrimers shows many groups with the possibility to be functionalized.

Lipid nanocarriers. Lipid nanocarriers are represented by liposomes, solid-lipid NPs and phospholipids micelles. Liposomes are vesicles with high biocompatibility, including synthetic or natural phospholipids, with a cell membrane-like structure such as hydrophobic tails of phospholipids cluster associated with hydrophilic heads. The existence of hydrophilic and hydrophobic compartments makes it possible to be encapsulated and released by different compound mechanisms, without the influence of intracellular mechanisms (32).

Micelles. Micelles are vesicular particles generated by spontaneous aggregation of amphiphilic molecules with many applications in cancer treatment as carriers for imaging, radiotherapy, chemotherapy and immunotherapy. Compared with other nanocarriers, the synthesis of micelles is almost easier. Micelles can deliver intracytoplasmatic, are biodegradable and non-toxic (33). Ovalbumin (OVA) and metabolism-related enzymes such as IR780 are involved in IDO metabolism can be transported also by micelles (34).

Metal NPs

Gold nanoparticles. Gold nanoparticles (AuNPs) are carriers for antigenic proteins and gene oligonucleotides to specific sites. Covalent and non-covalent interactions with various biomolecules were described, such as peptides, DNA and antibodies on the surface of Au NPs (35). AuNPs interact with selected subcellular organelles in tumor cells, being related to cancer cell survival, growth, proliferation and death. Combining AuNPs with photothermal ablation is a promising concept, investigated in various trials (36). In addition, AuNPs are used in delivering CgP oligonucleotides that enhance the migration of macrophages and DCs in the tumor microenvironment (TME) (37). Different sized and shaped gold nanoparticles, such as nanoshells, nanostars and nanorods were designed for immunotherapeutic delivery adjuvants as OVA or CpG (38).

Iron oxide nanoparticles. Iron oxide nanoparticles are promising carriers for vaccine delivery, polarizing immune cells, such as DCs and macrophages, and increasing immune response. They can also carry adjuvants such as OVA to potentiate the immune system (39).

Inorganic non-metallic NPs

Mesoporous silica NPs. Mesoporous silica NPs (MSNs) is a honeycomb-like porous structure including hundreds of empty mesopores that absorb large amounts of bioactive molecules (40). Materials from mesoporous silica interact with biosystems and biodistribution, cellular uptake, biodegradation, toxicity, and interaction with immune cells are related with specific physical and chemical properties including particle shape, size, porosity, and surface functionality of the materials (41). Mesoporous silica materials are degradable in physiological conditions via hydrolysis in the silica matrix, being related to the stability and release profile of guest molecules, particle size, surface functionality, concentration, porosity, morphology, degree of condensation, and the type of degradation medium. Mesoporous silica can be released to body tissues and excreted via renal clearance, being non-toxic (42). Larger particles of mesoporous silica and higher concentrations are more effective on monocyte-derived dendritic cells (MDDC) than small particles in low concentrations, suggesting the use of mesoporous silica as a component of cancer vaccines (43). An example was designed, a complete vaccine formulation using mesoporous silica (XLMSNs + OVA + CpG-ODN). This vaccine induces dendritic cell (DC) maturation with high levels of CD86 expression, and increases the secretion of pro-inflammatory cytokines, especially IL-12 and TNF-α (44). Other MSNs utility was found for transportation of drugs together with siRNAs which were co-delivered into the body, inducing the secretion of cytokines (45).

Carbon nanotubes. Carbon nanotubes (CNTs) are cylindrical multi-walled carbon nanotubes (MWNTs) that have various potential roles as tumor antigen nanocarriers, represented by ovalbumin (OVA) and cytosine-phosphate-guanine oligodeoxynucleotide (CpG), which are delivered to antigen presenting cells (APCs) (24). Single-walled carbon nanotubes combined with photothermal ablation of primary tumors and with anti-CTLA-4 antibody therapy aiming to trigger adaptive immune responses and prevent the metastatic process were also investigated (46).

Exosomes. Exosomes (EXOs) are extracellular vesicles released by the majority of cell types, with the size of 30-100 nm, with functions of intercellular transporters for lipids, proteins and nucleic acids among cells and organs that play different roles in various physiological and pathological processes in the immune system, including mediators, modulators and activators. Exosomes are generated via plasma membrane invagination initially as endosomes, which migrate to the center of the cell, resulting in the generation of multivesicular bodies (MVBs) that carry DNA, mRNA and non-coding RNA species or protein. The secretion of exosomes is produced mainly in lymphoid and myeloid lineages but also in many types of cells involving TME and cancer cell so-called tumor-derived exosomes containing growth factors and microRNAs (47,48). Exosomes can also inhibit tumors by delivering chemical drugs avoiding phagocytosis by macrophages. The potential benefits of using exosomes as a therapeutic approach to promote melanoma immunotherapy for inducing strong and lasting responses is ongoing.

Engineered viruses

Virus-like particles. Virus-like particles (VLPs) are 20-100 nm in size and are artificial nanostructures containing viruses without the possibility to replicate. The functions of VLPs are to stimulate immune responses, being immunogenic, and target immune cells as an engineered vaccine.

A VLP-based vaccine was designed, using a plant virus, *cowpea mosaic* virus, in an empty CPMV (eCPMV) VLP system, RNA-free and non-infectious. It was reported that eCPMV nanoparticles have strong immunotherapeutic efficacy and modulate the tumor immune environment. VLPs are involved in specifically targeting TME cells and tumor

cells and can be used as a nanocarrier for tumor antigens and drugs (49).

Oncolytic viruses. Oncolytic viruses are engineered viruses that infect tumor cells selectively, followed by tumor cell death. The aim during delivery consists in generating systemic and local immune response against tumor cells with a minimum of collateral effects on normal cells. Oncolytic viruses are engineered to act selectively on tumor cells in order to achieve this purpose. Viral replication is followed by lysis, release of antigens, damage-associated molecular patterns, and cytokines, promoting the immunogenic reaction and modelling the antitumor immune system (50). Consequently, in melanoma the immune-suppressed microenvironment is transformed into an immune-inflamed microenvironment. For a successful oncolytic virus, several conditions are required: It must target and replicate in tumor cells, it must have *in vivo* stability and it must not be integrated into tumor chromosomes (51).

5. Factors that modulate the efficacy of nanoparticles

Microbiome modulation. Gut microbiota constitute a variety of essential and opportunistic microorganisms hosted in the gastrointestinal tract as bacteria, viruses, protozoa, fungi, phages and archaea. Heterogeneity of the immune treatment effect can be explained by gut microbiota composition. Gut microbiota has a regulatory effect on the immune system suggesting that a large number of microorganisms can influence the functions of immune cells, especially Tregs, $CD4^+$ and $CD8^+$ T cells. It has been shown that human commensal *Bacteroides fragilis* enhance the transformation of $CD4^+$ naive T cells into Treg and stimulate the production of anti-inflammatory cytokines (52). The thymus-derived Tregs from the colon recognize the antigenic materials from *Clostridiales, Lactobacillus* and *Bacteroides* and could preserve tolerance to these bacteria. Antibiotics decreasing mainly the members of Clostridium family in gut microbiota composition can decrease the number of colonic Tregs (53). It has been demonstrated that some commensals such as *Escherichia coli* can improve the pro-inflammatory gut immunity in a 'love-hate' relationship (54-56). In addition, increased gut *Faecalibacterium* is correlated with elevated $CD4^+$ or $CD8^+$ T cells (57). The efficacy of anti-PD-1 treatment in metastatic melanoma patients is influenced by gut microbiota. The presence of some bacteria, such as *Bifidobacterium longum, Bifidobacterium adolescentis, Enterococcus faecium, Klebsiella pneumoniae, Collinsella aerofaciens, Parabacteroides merdae, Veillonella parvula* and *Lactobacillus* species is related with the response to anti-PD-1 treatment through various mechanisms, such as elevating the secretion of IFN-γ, enhancing DCs and increasing $CD8^+$ tumor-infiltrating T cells in contrast to *Roseburia intestinalis* and *Ruminococcus obeum* that were found enriched in nonresponders (58,59).

EPR effect. The enhanced permeability and retention (EPR) effect was first described in studies of inflammation (60). The enhanced permeability and retention (EPR) effect represent a unique phenomenon found in solid tumors, strongly correlated with anatomical and physiological characteristics. These features can be represented by the inadequate architecture of the vessels, large branches among endothelial cells in blood vessels, vascular mediators in excess and defective lymphatic drainage, followed by the significant extravasation of components of plasma and nanomedicines. The EPR effect determined the accelerated development of macromolecular antitumoral drugs, known as nanomedicines (61,62). It has been noted that a different EPR effect was observed in various tumors or different areas of the same tumor, especially in large tumors. In addition, the EPR effect is a dynamic phenomenon involving pathophysiological factors, biological events inside the body, tumoral growth, and inflammatory processes. EPR effect is the basic concept of tumor targeting with nanomedicines and it is related with the size, biocompatibility and conformation of macromolecules. Surface of charge and half-time in circulation are another critical point for the tumor-targeting nanomedicines (63-65). The concept of EPR-based tumor targeting was investigated in recent studies and it has described the potential possibilities of investigating transcytosis for tumor targeting by nanomedicines.

The nanomedicine effectiveness related to the EPR effect can be enhanced by pharmacological and physical co-treatments designed to prime the tumor microenvironment. Improvement of the EPR effect can be obtained by adding supplementary strategies related to molecular targeting, and physical or physiological modulation of the tumor microenvironment (66).

Protein corona. The interactions between nanoparticles and biological fluids is important to be understood to anticipate the fate of injected NPs. This interaction is the consequence of several factors related to nanoparticles, such as shape, size, charge, or coating agents. These are critical and related to features of biological fluids including protein concentration, ionic strength, temperature and pH (67-72). NPs are in contact with biological fluids and have interactions with active biological molecules (nucleic acids, lipids, proteins). Consequently, there is an inappropriate absorption of proteins on the surface of NPs, with protein corona (PC) formation, a different biological identity being generated in comparison to normal NPs. PC can have two roles in biomolecular recognition. Firstly, in a process defined as 'immune-blinding', the PC covers the surface of NPs and hides the antigen or biomolecule carried by NPs from the interaction with its specific receptor. Secondly, in some cases, proteins included in PC can link to the receptors of immune cells promoting unwanted immune responses. Nanoparticle-based immunotherapy can fail because of PC formation, inducing two types of responses: A non-response and an uncontrolled response. The immune-blinding response (non-response) may be promoted by partially or totally covering the antigens or stimulating molecules present on the surface of the nanoparticles, and consequently, the specific stimulation will be retarded, with the absence of the immune response. Additionally, in some cases, PC can express an altered structure during the PC formation on the surface of the NPs that can bind to scavenger receptors from monocytes and macrophages and induce phagocytosis. In this situation, recognition of the stimulating molecules expressed on the surface of NPs is avoided.

In an uncontrolled response, aggregation of NPs triggers toxic effects by strange-body recognition via the immune system (73-78).

6. Nanomedicine to enhance the immunotherapy in melanoma

Cancer vaccines. Cancer vaccines administered for enhancing the treatment of tumors has generated greater interest as an attractive type of cancer immunotherapy strategy. These vaccines can be classified into several classes: Neoantigen, dendritic cell, nucleic acid, and whole tumor cell vaccines (79,80). A challenge in promoting T-cell responses to eradicate tumor cells after vaccination is the ability in presenting the antigens to dendritic cells (DCs). A polyamidoamine dendrimer modified with guanidinobenzoic acid (DGBA) was found to represent an efficient cargo for some proteins such as ovalbumin (OVA) representing antigen, and unmethylated cytosine-guanine dinucleotides (CpG) representing the adjuvant, followed by an effective antigen cross-presentation by DCs. This DGBA-OVA-CpG nano-vaccine can promote powerful antigen-specific cellular immunities and has demonstrated prophylactic efficacy against B16-OVA melanoma. Combining anti-PD-1 treatment with DGBA-OVA-CpG nano-vaccine, an increased percentage of the tumor-infiltrating $CD3^+CD8^+$ and $CD3^+CD4^+$ T-cells among $CD3^+$ T-cells in tumors was observed. Conversely, vaccination with only DGBA-OVACpG or anti-PD-1 treatment is followed by a low infiltration of $CD8^+$ (79). It is well known that tumors with higher tumor mutational burden (TMB), such as cutaneous melanoma, produce more neoantigen liable to activate the immune system for recognizing tumors, in relation with two main elements: The number and the type of mutations. Neoantigens are specific non-autologous proteins, produced by non-synonymous mutations in the tumor cell genome antigens. They are characterized by strong and specific immunogenicity, higher affinity towards MHC, and lack of expression in normal tissues; due to these properties, they can virtually eliminate the risk of off-target side effects, while reinforcing the immune response to destroy cancer cells (80). Classical tumor-associated antigens (TAA) are present both in tumor and normal tissues, being highly enhanced in tumor cells expressing HER2, MART-1, MUC1, and MAGE. Neoantigens express stronger immunogenicity and higher affinity towards MHC than TAAs, not being able to be affected by central immunological tolerance. The first step in neoantigen identification is the fast comparison of the DNA sequences of tumor cells and normal cells. The majority of neoantigens are actually identified using several software applications based on whole-exon sequencing technology (81-84). Neoantigens activate T cells and stimulate the production of highly active T cells with strong affinity towards MHC-neoantigen-peptide complexes, avoiding recognition by the central immune system (85). The recently developed bioinformatics algorithms were combined with sequencing technology; it is now possible to accurately identify tumor neoantigens and predict their MHC affinity and immunogenicity. Neoantigen vaccination can enhance pre-existing neoantigen-specific T-cell populations and promote an extensive collection of new T-cell specificities in cancer patients, changing the intratumoral balance in favor of enhanced tumor control. Some antigens from melanoma tumors express four peptide sequence epitopes, similar to the pathogen and more easily recognized by T cells, with sustained clinical responses to immunosuppressive agents (86).

Anti-tumor therapy is a complex process, where a neoantigen vaccine initially presents the antigen to be recognized by T cells, and subsequently attacks the tumor. These processes act on various targets and are difficult to obtain with a single drug. In a recent study, a three-in-one immunotherapy nano-platform was designed, where aPD-L1@HC/PM NPs combining Chlorin e6 (Ce6)-conjugated hyaluronic acid (HC), dextro-1-methyl tryptophan (1-mt)-conjugated polylysine (PM) with anti-PD-L1 monoclonal antibodies (aPD-L1) was prepared. A comparison of melanoma mice model radiotherapy with aPD-L1@HC/PM NPs treatment showed that the tumor volume in mice receiving radiotherapy was reduced, while the tumor volume of the mice treated with aPD-L1@HC/PM NPs had disappeared almost completely (87).

Although neoantigens were considered optimal targets for an anti-tumor immune response, their discovery and evaluation became possible only by frequently using parallel sequencing and machine learning approaches for detecting the mutations within tumors and to predict mutated peptides with high affinity that bind autologous human leukocyte antigen (HLA) molecules. In a clinical study on six patients with advanced melanoma, personalized vaccines including 20 different fragments of peptide containing neoantigen and PolyIC:LC as the immune adjuvant were prepared (83). It has been demonstrated that 60% of the peptides developed a T-cell immune response in the patients, and out of six treated patients, four patients presented stable disease 25 months after vaccination and two patients presented recurrence treated with PD-1 antibody treatment with complete remission (83).

Aiming to increase the therapeutic effect of PD-1 inhibitors, a vaccine combined with a PD-1 inhibitor was designed, which also improved the effective response of PD-1 inhibitor. The antigen-specific vaccine stimulates the immune system to produce PD-1 positive T-cells that interact and work together with PD-1 inhibitor, sustaining a double attack against the tumor. This personalized vaccine can accelerate the immune response and remove the obstacles from the PD-1 inhibitors, reducing the recurrence and incidence of metastasis (88). Nucleic acid vaccines were also designed, which include mRNA or DNA encoding neoantigens delivered to intracellular (mRNA) or intranuclear (DNA) APCs (89).

Antigens are presented to T-lymphocytes, which destroy tumor cells expressing antigens with the same epitope. RNA vaccines have the advantage that they can bypass integration into host cell genome. Many clinical trials of DNA and RNA vaccines have failed to actually demonstrate the efficacy due to the delivery barriers and immunogenicity, but recent promising studies are ongoing (90). A new strategy for enhancing immune check-point blockade could be to promote the antitumor immune response using a liposomal RNA vaccine intravenously administered, currently under development (FixVac), which targets four non-mutated, tumor-associated antigens. In an exploratory analysis of clinical activity from a phase I dose-escalation trial of FixVac alone or combined with anti-PD-1 in patients with stage IIIB, IIIC, or IV melanoma (Lipo-MERIT trial, ClinicalTrials.gov

identifier NCT 02410733) in 50 patients, the IFNγ-ELISpot assay showed immune responses in more than 75% of patients. Regarding the clinical responses in 42 patients with stage IV melanoma, FixVac monotherapy obtained 12% partial responses and 28% stable disease and a combination of FixVac with check-point inhibitors revealed partial response in 35% of the 17 patients (91).

Tumor cell lysate-derived vaccines are also included in cancer immunotherapies and are classified into autologous cancer vaccines and allogeneic cancer vaccines. Autologous vaccines are with tumor cell lysate derived from the patient and allogeneic cancer vaccination are with another member of the same species. Tumor cell lysates are presented by MHC (major histocompatibility complex) molecules to trigger immune responses. It is well known that the NY-ESO-1 cancer/testis antigen is expressed in 25% of patients with melanoma. In a study on 11 patients with melanoma, tumors expressing NY-ESO-1 that received autologous TCR-transduced T cells plus interleukin-2 reported objective clinical responses in five patients, representing the first demonstration of the successful treatment of a non-melanoma tumor using TCR-transduced T cells (92).

A biomaterial-based vaccination system using an encapsulated GM-CSF that enhances DCs activity and cytosine-phosphodiester-guanine oligodeoxynucleotide (CpG ODN), a specific toll-like receptor (TLR) agonist which activates DCs, into sponge-like macroporous cryogels was designed. The cryogels were administered subcutaneously to mice in a melanoma model in order to deliver immunomodulatory factors (GM-CSF and CpG ODN) in a controlled manner. This vaccine caused local infiltrates consisting of DCs that induce a potent, durable, and specific anti-tumor T-cell response, indicating the potential for cryogels to be used as a platform for cancer cell vaccinations (93).

Another target in immune therapy of melanoma can be cancer stem cells (CSC). CSCs have been identified in melanoma, where their extensive proliferation is responsible for metastasis and recurrence of the tumor, but how to target and eliminate CSCs *in vivo* remains a major issue. Synthetic high-density lipoprotein nanodiscs represent a novel approach to reduce the aldehyde dehydrogenase (ALDH), a marker for isolating CSCs. This vaccine is designed against CSCs that are highly enriched in ALDH, increasing antigen trafficking to lymph nodes and generating robust ALDH-specific T-cell responses (94).

A vaccine targeting ALDH highly enriched CSCs targeting dendritic cells (CSC-DC vaccine) in combination with anti-PD-L1 and anti-CTLA-4 was designed and it was suggested that this combination could manipulate T-cell functions and induce the activation and proliferation of T cells in a B16-F10 murine melanoma tumor model (95).

Targeting immune checkpoint inhibitors to improve immunotherapy. The immune check-point blockade treatment in melanoma is related with adverse events (AEs), with a global incidence of 26.8% (all grades) in a meta-analysis of 46 studies including 12,808 cancer patients treated with PD1/PD-L1 inhibitors (96). Enhancing the efficacy of check-point inhibitors can be obtained by escalation of the doses for enhancing the efficacy of check-point inhibitors, but it is hampered by the appearance of AEs and represent an emergent issue in cancer immunotherapy. Delivery systems containing biomaterials were experimented such as hydrogels, nanoparticles (NPs) and microneedle patch-assisted delivery. Celecoxib and an anti-PD-1 monoclonal antibody (PD-1mAb) were locally delivered by way of a designed alginate hydrogel system for treating a B16-F10 melanoma model. The alginate hydrogel delivery system was found to significantly enhance the antitumor activities of celecoxib (CXB), PD-1mAb, or combination of both. This hydrogel system synergistically improved the accumulation of $CT4^+$ and $CD8^+$ T cells within the tumor (97). Another nanocarrier was designed as a self-degradable microneedle patch containing biocompatible hyaluronic acid integrated with dextran nanoparticles that encapsulate aPD1 and glucose oxidase, for the delivery of an anti-PD1 antibody (aPD1). A single intratumoral injection of microneedle patch in a B16F10 mouse melanoma was demonstrated to induce robust immune responses (98). The delivery of anti-PD-1 antibodies was also examined using encapsulated PLGA nanoparticles (anti-PD-1 NPs) into the spleen in a B16-F10 murine melanoma model, demonstrating the enhancement of the antitumor effect of this agent. Administration of a high dose of anti-PD-1 NPs can develop significantly higher mortality compared with administration of free anti-PD-1 antibodies, due to the hyperexpression of T cells. By contrast, administration of anti-PD-1 NPs to splenectomized mice has produced a decreased mortality and showed the importance of secondary lymphoid tissues in mediating the toxicity of anti-PD-1 antibodies. It has also been demonstrated that anti-PD-1 NPs stimulate internalization by DCs in the spleen, followed by the maturation and activation of T cells (99). (CTLA-4)-siRNA ($NP_{siCTLA-4}$) is another platform biomaterial-based delivering cytotoxic lymphocyte-associated molecule-4 employed in a mouse model bearing B16 melanoma, the results of which showed an increase of cell activation and proliferation of $CD4^+$ and $CD8^+$ T cells, following $NP_{siCTLA-4}$ *in vitro* treatment (100).

Specific chemotherapeutics (such as oxaliplatin, doxorubicin) have the competence to induce immunogenic cell death (ICD) of cancer cells by inducing various signals that release ATP, CXCL10, calreticulin (CALR) and high mobility group box 1 (HMGB1) and stimulate the immune system. The combinatory therapy between chemotherapy agents and check-point inhibitors is another paradigm in cancer treatment. In a recent study, an anti-CTLA-4 was combined with chemotherapy (liposomal doxorubicin) encapsulated in a PEGylated liposome, in order to increase the efficiency of treatment and decrease the SEs of anti-CTLA-4. In a B16 mouse melanoma model, the liposomal anti-CTLA-4 produced a reduction in the size of tumors and increased survival in comparison with non-liposomal anti-CTLA-4 (101).

Nano-immunostrategies to reprogram the tumor microenvironment

Targeting TME conditions. Tumor-infiltrating cytotoxic T lymphocytes have a major role in controlling tumor development and it has been observed that they retard their functions in an acidic tumor microenvironment. Targeting tumor acidity is a promising concept for the reversal of the anergic state of T cells and the improvement of T cell-associated immunotherapy. A

concept of RNAi nanoparticles that reversed tumor acidity and rendered T cells for enhancing the checkpoint blockade therapy functional was developed. Following this concept, the *in vivo* use in melanoma tumor models of an optimized vesicular cationic lipid-assisted nanoparticle to mediate systematic blocking of lactate dehydrogenase A (LDH A) in tumor cells has been reported. The treatment was followed by reduction of lactate production, neutralization of tumor pH and enhancing of infiltration with $CD8^+$ T and NK cells with the result in slowing down tumor growth. The restored tumoral pH stimulated checkpoint inhibition therapy using the antibody of PD-1 (102).

Hypoxia is also a major component of the tumor-suppressive microenvironment and it has been demonstrated to have a negative regulatory effect on the activation of T cells. A multifunctional immunoliposome was developed, known as CAT@aPDL1-SSL, which contains modified aPDL1s on the surface for improving the immunotherapeutic effects against the tumor and an encapsulated catalase (CAT). It was suggested that the CAT-encapsulated liposomes decreased tumor hypoxia through the activity of CAT, which decomposes endogenous H_2O_2 into O_2. Furthermore, these immunoliposomes promoted the infiltration of $CD4^+$and $CD8^+$ T cells in tumor tissues and stimulate the blocking of the PD-1/PD-L1 pathway (103).

Targeting cancer cells. Immunogenic cell death (ICD) is an umbrella term containing some cell death modalities, including apoptosis, necroptosis and immunogenic apoptosis. Generally, ICD is represented by the production of damage-associated molecular patterns (DAMPs), cytokines, chemokines, leading to the initiation of enhanced anti-tumor immune responses. ICD can be induced by radiotherapy, chemotherapy (e.g., oxaliplatin, cyclophosphamide), magnetic fluid hyperthermia, photodynamic therapy or other stimuli. New experimental data indicate that the immunogenicity of dying cancer cells can be enhanced by the use of biomaterials, so-called '*in situ* tumor vaccines' and constitute a new modality that makes immunotherapy more efficient by combining with ICD-inducing modalities (104). In the ICD process, the translocation of calreticulin (CRT) is produced on the cell surface and adenosine triphosphate (ATP), the HMGB1 protein together with heat shock proteins (HSPs) are released into the extracellular environment. The immune system reacts by activating APCs and cytotoxic T cells, which eradicate tumors and metastases. ATP recruit APCs by chemo-attraction, and CRT generates an 'eat-me' signal in order to stimulate the APCs to capture the dying tumor cells and their debris. Concomitantly, HMGB-1 and HSPs enhance antigen presentation to T cells (105). In addition, ICD induces the release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β converting an immunosuppressive TIME to an immunogenic TIME (106). Antigen-capturing nanoparticles (AC-NPs) were engineered to sequester TAAs and to present them to APCs. It has been demonstrated that AC-NPs promoted the proliferation of $CD8^+$ and $CD8^+$ cytotoxic T cells, improving the efficacy of anti-PD-1 treatment on the B16F10 melanoma model with up to a 20% cure rate compared to 0% without AC-NPs (107). Another study demonstrated the synergy between low-doses paclitaxel and a toll-like receptor-7 (TLR-7) agonist-imiquimod administrated in a co-delivery system for treatment of B16F10 melanoma (108). The researchers observed an improved proliferation (250%) of DCs and secretion of pro-inflammatory and Th1 cytokines with an inhibition of tumor growth, eventually leading to 70% survival as compared to individual components with 0% survival at day 41 (108). Cationic copolymer aPBAE for delivering CRISPR-Cas9 genome editing system was designed to retard the PD-L1 expression on tumor cells *in vivo*. The expression of PD-L1 on tumor cells was significantly attenuated by knocking out cyclin-dependent kinase 5 (Cdk5), followed by effective tumor growth inhibition in murine melanoma. It has been demonstrated that aPBAE/Cas9-Cdk5 treatment stimulate strong T cell-mediated immune responses in tumor microenvironment, thereby stimulating the increasing of CD8 T cells and the decreasing of Tregs (109).

A biological platform for delivery of nanoparticles comprising biodegradable materials that can genetically reprogram cancer cells and their microenvironment *in situ* was also designed. The reprogrammed cancer cells mimic tumor-associated antigen-presenting cells (tAPCs) by inducing the expression of an immunostimulatory cytokine (IL-12) and a costimulatory molecule (4-1BBL). The nanoparticles combined with checkpoint blockade significantly retarded tumor growth in B16-F10 melanoma model. *In vitro* and *in vivo* analyses showed that tAPC-reprogramming nanoparticles locally delivered produce an enhanced cell-mediated cytotoxic immune response, with systemically translated effects (110). In another study, a plasmid DNA expressing small hairpin RNA of PD-L1 (shPD-L1) was loaded in dual-rebound nanoparticles (shPD-L1@NPs) that were pH-dependent to silence the PD-L1 gene and decrease the PD-L1/PD-1 interactions between T cells and tumors. Overexpressed hyaluronic acid (HA) was degraded by Hyaluronidase (HAase) in the extracellular matrix (ECM) of the tumor tissues to increase the penetration of the shPD-L1-loaded nanoparticles in tumors. An enhanced tumor inhibitory effect was reported by the combination treatment of HAase and shPD-L1@NPs in a malignant melanoma mouse tumor model (111).

Targeting the tumor immune microenvironment

Targeting antigen-presenting and dendritic cells. In a study using the vaccination of mice with melanoma B16 tumors with PLGA nanoparticles (NPs), which contained encapsulated poorly immunogenic melanoma antigen, tyrosinase-related protein 2 (TRP2) and Toll-like receptor (TLR) ligand covered by TLR4 agonist (7-acyl-lipidA) were evaluated. It has been shown that this vaccine can induce therapeutic anti-tumor effect by interferon-gamma production in lymph nodes and spleens of the vaccinated mice and an enhanced level of cytokines was demonstrated compared to the control group (112). Another poly(d,l-lactide-co-glycolide) nanoparticle (PLGA-NP) was designed to deliver antigenic peptides to induce cytotoxic T-lymphocyte responses against tumor-associated self-antigens in C57BL/6 melanoma mouse models. Vaccination with PLGA-NP carrying both $TRP2_{180-188}$ and monophosphoryl lipid A (a toll-like receptor 4 agonist) slowed down the growth of subcutaneously inoculated B16 melanoma cells. This anti-tumor potential of the peptide-loaded DC vaccine was further enhanced when it was administered in combination with IFN-γ to suppress tumor escape (113).

Targeting tumor-associated macrophages. Nanomedicines are first collected in tumors through passive or active targeting mechanisms and are then involved in local tumor immunosuppression mediated by MDSC, targeting tumor-associated macrophages (TAM), and soluble inhibitors, reducing the immunosuppression in the TIME with the increase of infiltration, maturation, proliferation, survival, and activity of effector immune cells. TAM is a major population of immune cells with an M2-like phenotype in tumors, which have pro-tumoral functions, reducing the infiltration of effector T cells (114,115). In a recent study, cyclodextrin nanoparticles were designed that target a small molecule toll-like receptor 7/8 agonist to macrophages from the TIME, stimulating M2 to M1 polarization and increasing the efficacy of checkpoint-inhibiting immunotherapy in anti-PD-1 unresponsive tumors (116). $CaCO_3$ nanoparticles combined with anti-CD47 antibodies also increase the macrophages polarization towards an M1 phenotype followed by improving the outcome of checkpoint blockade therapy. $CaCO_3$ nanoparticles were locally administrated as hydrogel during tumor surgery and an interaction between $CaCO_3$ and the protons in the TIME was demonstrated. The embedded anti-CD47 antibodies have the function to block the 'don't eat me' signal on tumor cells, increasing phagocytosis of cancer cells by macrophages (117).

Tumor-targeted delivery systems can also increase the antitumor efficacy of statins. A long-circulating liposome that encapsulates simvastatin (LCL-SIM) was compared with free SIM in B16.F10 murine melanoma-bearing mice as antitumor activity. It has been previously demonstrated that B16.F10 melanoma growth was strongly inhibited by LCL-SIM (by 85%), whereas free SIM has no antitumor activity. The efficacy of LC-SIM was related with the reduction of the TAM-mediated oxidative stress as well as of the production of the hypoxia-inducible factor 1 α (HIF-1 α) in tumors, concluding that the tumor-targeting property of the liposome formulation is correlated with the presence of TAM in tumor tissue (118).

Other designed carriers are M2-like TAM dual-targeting nanoparticles (M2NPs). By loading anti-colony stimulating factor-1 receptor (anti-CSF-1R) small-interfering RNA (siRNA) on the M2NPs, a molecular-targeted immunotherapeutic approach was designed that blocks the survival signal of M2-like TAMs, reducing them from melanoma tumors. After administration to tumor-bearing mice, a notable elimination of M2-like TAMs (52%) was reported, with a tumor size decrease (87%) and prolonged survival. In addition, M2NP-based siRNA delivery system inhibited the IL-10 and TGF-β production and increased the cytokine (IL-12 and IFN-γ) expression and $CD8^+$ T-cell infiltration in the TME and retardation of the expression of PD-1 and Tim-3 on infiltrating $CD8^+$ T cells, restoring the T-cell immune function (119).

Targeting indoleamine 2,3-dioxygenase (IDO1). Indolamine-2,3-dioxygenase 1 (IDO1) is a cytosolic enzyme with a heme prosthetic group secreted by DCs that converts tryptophan (Trp) from the tumor microenvironment to kynurenine (Kyn). IDO1 is overexpressed in more than 50% of tumors that use the mechanisms of IDO1 to enhance their spread and survival (120). In the 'elimination' phase, IDO1 is produced at low levels within the TME and inhibits tumor proliferation. During the degradation of IDO1, tolerogenic dendritic cells (DCs) are converted into immunogenic cells (121). In the 'equilibrium' phase, surviving tumor cells become 'edited' by the permanent attack of the immune system and accumulate mutations. In the 'escape' phase, high IDO1 level is described, produced by tumor cells and tolerogenic immune cells (DCs, MDSCs, TAMs). Trp depletion and Kyn accumulation inhibit the effector T cell and NK cell functions, switching DCs to and stimulating regulatory T cells (122). Small molecules of IDO inhibitors incorporated in nanomedicine formulations were tested in preclinical and clinical trials (123).

A three-in-one immunotherapy nanoplatform involved in the three phases of cancer immunity cycle (elimination, equilibrium and escape) was reported. An aPD-L1@HC/PM NPs platform (Ce6-conjugated hyaluronic acid, dextro-1-methyl tryptophan-conjugated polylysine and aPD-L1) was designed against tumor metastasis relapses and postsurgical regrowth. A bilateral mouse tumor model of B16F10 melanoma was also developed to verify the abscopal effect of aPD-L1@HC/PM NPs. Through the simultaneous collaboration of the enhancing tumor antigen for DC maturation followed by lymphocyte activation (elimination), the suppression of the IDO pathway (equilibrium), and the blocking of the PD-1/PD-L1 pathway for supporting tumor elimination (escape), all three phases of cancer immunity cycle were efficiently manipulated to enhance the immune response and immune memory (88). Peptide-based nanoparticles were also designed to promote a dual function: IDO inhibitor by blocking tryptophan metabolism and antagonist of programmed cell death-ligand 1 (PD-L1). This NP creates an environment, which enhances the survival and activation of cytotoxic T lymphocytes and effectively inhibits melanoma growth in mice by stimulating anticancer immunity (124). A synergistic immunotherapy strategy was also developed; it targets the immunoinhibitory receptor programmed cell death protein 1 (PD1) and immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) into the TIME for the treatment of melanoma through an embedded immunotherapeutic nanocapsule microneedle-based transcutaneous delivery approach (125).

Targeting TGF-β. TGF-β, a pleiotropic cytokine, is a key signal produced in the tumor microenvironment promoting tumor evasion from the immune response. Transforming the signaling of growth factor-β (TGF-β) is an important mechanism of immune suppression in the tumor microenvironment, but systemic blockade of TGF-β signaling pathway may induce multifocal inflammation, autoimmune disease and significant cardiac toxicities in animal models (126). Current nanoparticle designs have an inefficient accumulation in tumors after systemic administration due to slow passage through vascular barriers in tumors and rapid clearance of particles by the reticuloendothelial system. A PEGylated liposomal form antibody-targeted of TGF-βI that inhibits TGF-β signaling in primary T-cells was synthesized, maintaining T-cell proliferation and cytotoxicity in B16F10 melanoma tumors. The liposomal delivery of TGF-βI that targets an internalizing receptor (CD90, or Thy1) was also compared with a TGF-βI that targets non-internalizing receptor (CD45) and it was demonstrated that T-cells pre-loaded *ex vivo* with liposomes that target CD45-infiltrated tumors are more efficient (127). NPs coated with a T-cell membrane (TCMNPs) that represents T-cell-mimicking nanoparticles were developed. TCMNPs

can eliminate tumors due to T-cell membrane-derived proteins on TCMNPs. In addition, TCMNPs can release anticancer drugs and stimulate the suppressed CTLs by inhibition of TGF-β1 and PD-L1. In combination with dacarbazine, TCMNP produced a higher reduction of tumor growth in a B16F10 melanoma model, increasing the percentages of $CD8^+$ Granzyme B^+ and $CD8^+$ IFN-γ^+ T cells in tumors. In current cancer immunotherapies, TCMNPs have potential advantages of being cost-effective and less time-consuming than adoptive T-cell transfer therapy, as they are prepared from T-cell lines and synthetic polymers within 2 days (128).

Targeting the peripheral immune system. Immune compartments situated outside of tumors represented by the peripheral immune system have aroused increased interest in nanomedicine in recent years. The secondary lymphoid organs, such as lymph nodes and the spleen are parts of the peripheral immune system where antigen presentation and cytotoxic T-cell generation occurs. These compartments are often affected in terms of cancer occurrence and progression. Restoration of the functions of the peripheral immune system can lead to potentiation of antigen presentation by engineering T-cells (129). Findings of a previous study (130) showed that the administration of tumor-draining lymph nodes (TDLN)-targeting NPs, which contain tumor-associated antigen TRP-2 or CpG oligonucleotide to B16-F10 melanoma cancer model is followed by the induction of strong cytotoxic lymphocyte (CTL) responses. It has been demonstrated that this strategy could significantly slow down immunosuppressive cells and enhance antitumor immune cells in TDLN. The antigen-adjuvant combination in NPs could promote the delivery to DCs from TLDN and induce anti-tumor T-cell responses. Generally, oncolytic viruses mediate anti-tumor activity expressing a dual mechanism of selective replication and lysis within infected cancer cells and inducing host anti-tumor immunity. Talimogene Laherparepvec (T-VEC) is a type I herpes simplex virus (HSV-1) genetically modified which is preferentially replicated in tumor cells and induces a systemic antitumor immunity capable of eradicating tumor at a distance. T-VEC was engineered by deleting the neurovirulence genes responsible for fever development and deleting a viral gene that blocks antigen presentation. T-VEC was further modified to enhance antigen presentation and T-cell priming by deleting the ICP47 viral gene, human GM-CSF being incorporated into the virus design. It was demonstrated that T-VEC selectively replicates in tumor cells through oncogenic disruption of the PKR pathway. Locally, T-VEC acts on an immunosuppressive tumor microenvironment, producing the local release of interferons, chemokines, pathogen-associated molecular pattern (PAMP) and danger-associated molecular pattern (DAMP) factors. It has been observed that Toll-like receptor agonists help reverse the suppressed tumor milieu into a more pro-immunogenic environment capable of enhancing anti-tumor immune responses. Local GM-CSF expression promoted by T-VEC enhances migration and maturation of dendritic cells, which form phagocyte soluble tumor antigens and apoptotic tumor cells. The dendritic cells then migrate to regional lymph nodes where they present antigens to specific CD4 C helper and CD8 C cytotoxic T-cells, initiating a systemic immune response. The T-VEC generates a higher immune response in injected tumor compared to the response rate of distant metastases as compared to distant metastasis, due to an inadequate effector T-cell expansion and/or inability of circulating effectors to defeat the immunosuppressive tumor microenvironment at distant sites. This is the reason of combinatory therapy with TVEC and immune checkpoint blockade, suggesting its efficacy in retarding the progression of melanoma (131-133).

T-VEC is the first viral oncolytic immunotherapy, FDA approved in 2015 for the local treatment of unresectable, cutaneous, subcutaneous and nodal lesions in patients with melanoma recurrent after initial surgery based on data from OPTiM, a randomized phase III open-label trial. OPTiM trial comparing T-VEC vs. GM-CSF have demonstrated a 4.4-month longer median overall survival (OS) in patients receiving T-VEC than GM-CSF, with estimated 5-year survival for the T-VEC arm of 33.4%, reaching 48.9% in patients with early metastatic melanoma (stage IIIB-IVM1a), with an acceptable safety profile (134). T-VEC combined with check-point inhibitors in melanoma has shown improved efficacy vs. CPIs alone. A phase III trial of T-VEC/placebo plus pembrolizumab is underway in unresectable stage IIIB-IVM1c melanoma (MASTERKEY 265; NCT02263508) (135). Another phase IB trial of ipilimumab C TVEC (NCT01740297) in 19 patients with advanced melanoma showed tolerability of standard dose TVEC combined with ipilimumab (136). TVEC plus ipilimumab vs. ipilimumab were compared in a randomized, open-label phase II trial in 198 patients with advanced melanoma and an improved overall response rate (ORR) was demonstrated with the combination (39 vs. 18%, P=0.002) (135). A phase II randomized trial on resectable stage III B/C or IV melanoma in 150 patients treated with immediate surgical resection versus 12 weeks of neoadjuvant intratumoral TVEC followed by surgery (NCT02211131) was also carried out. Most recently, results of the interim 1-year analysis of recurrence-free survival results demonstrated that in the T-VEC treatment group, a great percentage of patients remained recurrence-free (33.5 vs. 21.9%, P=0.05) and overall survival after 1 year was higher in patients treated with T-VEC prior to surgery (95.9 vs. 85.8%) (137-139).

7. Conclusions and future directions

Current immunotherapy for melanoma has reached a limit of clinical responses. New methods are needed to increase the effectiveness of the treatments. One of the major ways to improve clinical responses is represented by nanomedicine modalities to manipulate the immune responses. The major challenge for nanomedicine-based immunotherapy remains the optimization of tumor targeting, drug delivery vs. clearance and control of toxicity (140-143).

In order to achieve a sustained and efficient anti-tumor immune response, a controlled release of immunostimulating substances, together with antineoplastic drugs combined with specific targeting are needed. In addition to classical well-known check-point inhibitors PD-1, PD-L1 and CRLA-4, other potential molecular targets in immunomodulatory therapy of melanoma can be represented by newly identified small-molecule immune checkpoint co-stimulators (GITR, OX40 with their ligands), inhibitors (VISTA, LAG-3, TIM-3, TIGIT) (143-145). The immune system can also be modulated by

small molecules that enhance cellular immunity, such as IDO/TDO, STING agonists, TLR agonists, GSK-3 inhibitors. The tumor microenvironment modulators including CSF-1R inhibitors, TGF-β or CXCR antagonists and epigenetic regulators of immune response as HDAC inhibitors, BET, EZH2 inhibitors are also promising for the enhancement of immunotherapy.

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Authors' contributions

CV supervised the study. CV, SV, SN, CRS, CCV contributed equally to the conception and design of the study and wrote the original draft. CL, DS, BMC, CS, CG, IA, CLU edited and critically revised the manuscript, read and approved the final manuscript.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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