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ARTICLE TYPE

Nanoparticles: Properties and Applications in Cancer Immunotherapy

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Abstract:

Background: Tumours are no longer regarded as isolated masses of aberrantly proliferating epithelial cells. Rather, their properties depend on complex interactions between epithelial cancer cells and the surrounding stromal compartment within the tumour microenvironment. In particular, leukocyte infiltration plays a role in controlling tumour development and is now considered one of the hallmarks of cancer. Thus, in the last few years immunotherapy has become a promising strategy to fight cancer, as its goal is to reprogram or activate anti-tumour immunity to kill tumour cells, without damaging the normal cells and provide long-lasting results where other therapies fail. However, the immune-related adverse events due to the low specificity in tumour cell targeting, strongly limit immunotherapy efficacy. In this regard, nanomedicine offers a platform for the delivery of different immunotherapeutic agents specifically to the tumour site, thus increasing efficacy and reducing toxicity. Indeed, playing with different material types, several nanoparticles can be formulated with different shape, charge, size and surface chemical modifications making them the most promising platform for biomedical applications.

Aim: In this review, we will summarize the different types of cancer immunotherapy currently in clinical trials or already approved for cancer treatment. Then, we will focus on the most recent promising strategies to deliver immunotherapies directly to the tumour site using nanoparticles.

Conclusions: Nanomedicine seems to be a promising approach to improve the efficacy of cancer immunotherapy. However, additional investigations are needed to minimize the variables in the production processes in order to make nanoparticles suitable for clinical use.

Keywords: Nanoparticles, Immunotherapy, Cancer, Nanomedicine, Immunity, Tumour microenvironment.

1. INTRODUCTION

Bidirectional communication between cells and their microenvironment is critical for both normal tissue homeostasis and tumour growth. In particular, growing evidence has supported the idea that the immune system plays a crucial role in initiation, progression and tumour development and the presence of immune cells within the tumour microenvironment (TME) is now considered as a hallmark of cancer [1,2].

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Thus, cancer is a complex disease that often requires multiple traditional treatments that target tumour cells, however in the last few decades there has been a surge in therapies that target or activate the immune response in cancer [3]. Indeed, the goal of cancer immunotherapy is to engage the immune system to attack and kill tumour cells specifically, without damaging the normal cells [2]. Different types of immunotherapy have been developed in the last few years [3,4]: for instance, the genetic reprogramming of immune cells in order to kill cancer cells, i.e. chimeric antigen receptor (CAR)-T cell therapy, the use of cytokines to modulate the immune response or the use of

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monoclonal antibodies (mAbs) against specific antigens (Ags) expressed on tumour surfaces, such as anti-PD-L1 mAbs [3-5]. In addition, oncolytic virotherapy is an emerging strategy traditionally known for its oncolytic action on tumour cells, and it is increasingly being recognised for its ability to boost the immune response against cancer [6,7]. Despite these advancements, cancer immunotherapy still has some challenges and full potential is not being achieved with respect to efficacy and immune-related adverse events (IRAEs) [8]. Thus, the current efforts are focusing on further developments to enhance specificity, effectiveness and reduce toxicity. A promising strategy for increasing the specificity of tumour immunotherapy is nanomedicine. Indeed, due to their small sizes, nanoparticles (NPs) can cross cell and tissue barriers, protect bioactive molecules and overcome poor drug distribution and lack of selectivity, making them a favourable material for biomedical applications [9,10]. In this review article, we will provide a general overview on the different types of immunotherapies and then we will focus on the most promising strategies to deliver immunotherapeutic agents directly to the tumour site through the use of nanomedicine.

2. Immune cells in cancer

The presence of leukocytes within tumours, observed in the 19th century by Rudolf Virchow, provided the first indication of a possible link between inflammation and cancer [11]. Nowadays, a role for inflammation in tumourigenesis is generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumours, including some in which a direct causal relationship with inflammation is not yet proven. The hallmarks of cancer-related inflammation include the presence of inflammatory cells and inflammatory mediators (for example, chemokines, cytokines and prostaglandins) in tumour tissues, tissue remodelling and angiogenesis, similar to those seen in chronic inflammatory responses and tissue repair [12]. Immune cells in the TME include both innate and adaptive immune cells. For instance, T cells have been found in the TME of various tumour types, they are called tumour-infiltrating lymphocytes (TILs) and are considered to play a decisive role in tumour initiation and progression [13]. TILs can have both pro- and anti-tumour activity: indeed, for example, CD4+ Thelper (Th)1, CD8+ T and natural killer (NK) T cells inhibit tumour growth by producing interferon gamma (IFNγ) that in turn activates tumouricidal macrophages for the phagocytosis of cancer cells. Activated macrophages also produce interleukin (IL)-12 that promotes Th1 cell differentiation, establishing a tumour-suppressing feedback loop [14]. T cell-released IFNγ is also responsible for stimulating the antigen presenting cells (APCs) that then activate the CD8+ cytotoxic T cells in order to promote tumour cells lysis. Moreover, most tumours are positive for MHC class I, but negative for MHC class II and cytotoxic CD8+ cells are able to induce tumour killing upon direct recognition of peptide antigens, presented by the tumour's MHC class I molecules [15]. In contrast, some studies show that Th2 and T regulatory (Treg) cells have a pro-tumoural function. For instance, Th2 cells release IL-4, IL-5, IL-10, and IL-13 that induce anergy of T-cells and loss of cytotoxicity [16,17]. Since Tregs inhibit the activation of both CD4+ and CD8+ T cells, within the TME they are considered to have a pro-tumoural activity by suppressing anti-cancer cell immunity. Indeed, transforming growth factor beta (TGFβ) secreted by Tregs may suppress IFNγ production by Th1 and CD8+ T cells, while also promoting the generation of Foxp3+ Tregs and differentiation of Th17 cells, which, when taken together, likely favours the growth and progression of cancer [15-17]. In addition, myeloid derived suppressive cells (MDSCs) are a heterogeneous group of immature myelomonocytic cells that are functionally defined by their potent immunosuppressive activity on T cells. Indeed, they express several enzymes such as arginase-1 (ARG-1) and indoleamine 2,3 dioxygenase (IDO) causing arginine, tryptophan and cysteine depletion in the TME. This situation causes inefficient T cell receptor complex formation and then proliferative arrest of Ag-activated T cells [18-20]. Moreover, MDSCs produce reactive oxygen species (ROS), nitric oxide (NO), TGFβ and IL-10 that suppress anti-tumoural immunity [21,22]. Macrophages are other crucial players in cancer progression: in response to different signals, they undergo polarized activation: the 'classically' activated (M1) and the 'alternatively' activated (M2) macrophages. M1 macrophages have tumouricidal activity as they produce high amounts of inflammatory cytokines, ROS and activate the immune response [23,24]. On the other hand, M2 macrophages promote tissue repair and angiogenesis, and favour tumour progression [25-27]. Although tumor-associated macrophages (TAMs) are generally described as an M2-like population, evidence suggests that M1/M2 classification is blurred as, especially in vivo, M1-like phenotype often overlaps with inflammatory and immunosuppressive features [28,29].

Other molecules that take part to the immunosuppression observed in the TME are the immune checkpoint proteins cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and Programmed Death 1 (PD-1) that are both expressed by activated T cells. CTLA-4 binds CD80 and CD86 (on the surface of APCs) with greater affinity and avidity than CD28 and transmits an inhibitory signal to T cells [30]. Similar to CTLA-4, PD-1 has been shown to block effector T cell activation and functions, upon the interaction with its ligands PD-L1 or PD-L2 [30]. Accordingly, overexpression of PD-L1 and CTLA-4 has been observed on the surface of tumour cells in several types of cancer [31]. At present it is generally accepted that immune response can inhibit tumour growth in the early stage of cancer development through a process called immune-editing. However, in advanced state of tumour progression, cancer cells promote an immunosuppressive microenvironment that inhibits anti-tumour immunity. Indeed, studies in murine models from the lab of Robert Schreiber have suggested the "Three Es of cancer immune-editing": the interplay between tumour and the immune system goes through three phases characterized by immune-elimination (of cancer cells), immune-equilibrium (between cancer cells and cells of the immune system) and immune-escape (by cancer cells) (Figure 1) [32,33].

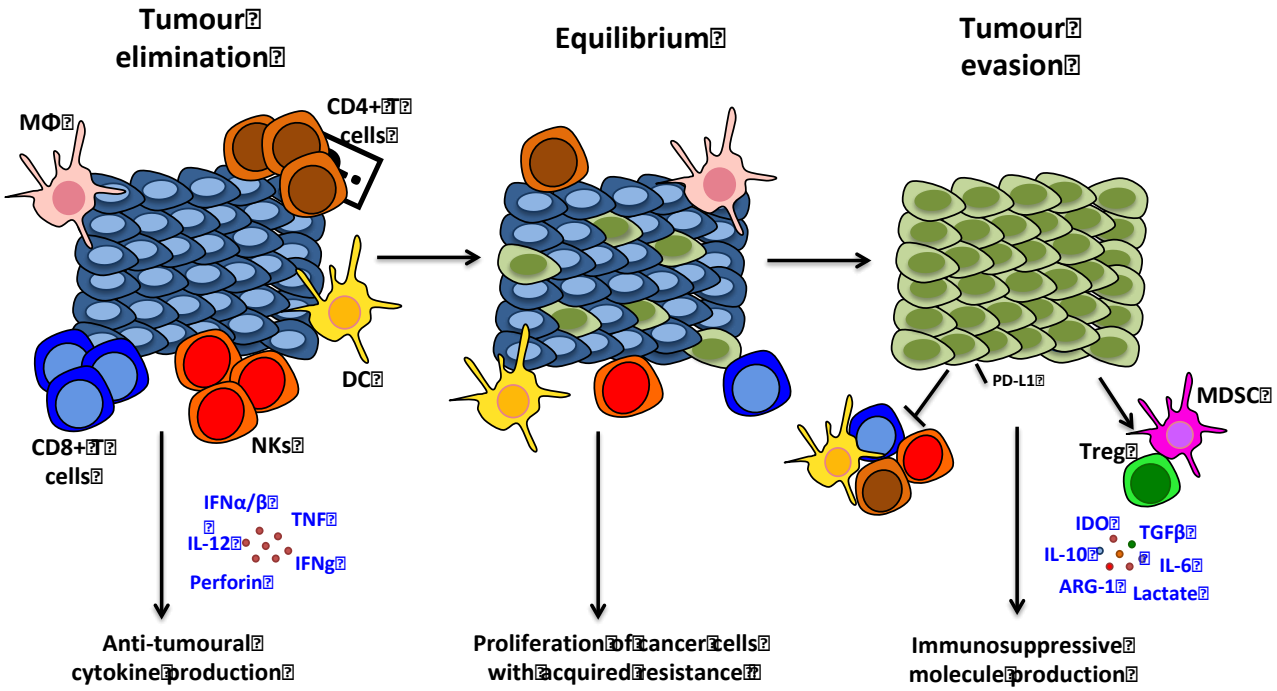

Figure 1. The immune-editing process – elimination, equilibrium, and escape. The interplay between tumour and the immune system goes through three phases characterized by immune-elimination (of cancer cells), immune equilibrium (between cancer cells and cells of the immune system) and immune escape (by cancer cells).

During the elimination phase, cells of the innate immune system recognize the presence of a growing tumour that has undergone stromal remodelling, causing local tissue damage. This is followed by the induction of inflammatory signals essential for recruiting cells of the innate immune system (e.g. NK cells, NKT cells, macrophages and dendritic cells (DCs)) to the tumour site. During this phase, the infiltrating lymphocytes are stimulated to produce IFNγ promoting cancer cell death. Tumour cell debris is then ingested by DCs, followed by the migration of these cells to the draining lymph nodes. The recruitment of more immune cells also occurs and it is mediated by the chemokines produced during the inflammatory process. In the draining lymph nodes, tumour-loaded DCs trigger the differentiation of Th1 cells that in turn facilitates the development of cytotoxic CD8+ T cells. In the final phase of elimination, tumour-specific CD4+ and CD8+ T cells home to the tumour site and the cytotoxic T lymphocytes then destroy the Ag-bearing tumour cells. Tumour cell variants that have survived the elimination phase enter the equilibrium phase. In this phase, lymphocytes and IFNγ exert a selection pressure on tumour cells that are genetically unstable and rapidly mutating. Tumour cells that have acquired resistance to elimination continue to grow and expand in an uncontrolled manner and modulate the TME promoting the activity of immunosuppressive cells in order to evade the immune response and thus, leading to malignancy [32,34]. Since some cancers progress faster and reach the escape phase earlier than others, it could be speculated that the quality and quantity of the immune response induction may be influenced by numerous factors in the TME: for instance, cytokines and chemokines determine the cell types attracted, but also the availability of oxygen and nutrients could influence the immune response and functions [34].

3. Cancer Immunotherapy

As mentioned above, inflammation is a hallmark of cancer and even if the immune system acts against cancer cells, tumours can escape from immune attack [32-34]. For this reason, in the last few years, many studies have been conducted with the aim of trying to enhance the activity and functions of anti-tumoural immune cells, paving the way to the development of cancer immunotherapy [3-5]. This is the modulation and use of the patient's own immune system to target cancer cells. In that manner, cancer immunotherapy focuses on developing agents that activate or enhance the immune system's recognition and killing of the tumour cells [35,36]. This section will summarize the main strategies and the most recent advances in the field of immunotherapy for the treatment of tumours, in particular, highlighting the immunotherapeutic agents already approved for clinical use by the Food and Drug Administration (FDA) or in clinical trials (Table 1).

3.1 Cell-based immunotherapy and vaccines

Cellular immunotherapy is an innovative treatment approach that harnesses the body's own immune system to fight cancer. It consists of manipulation or stimulation of autologous immune cells that specifically recognize tumour

cells and in the past few years it has attracted much research and clinical trial interest [36]. In particular, many studies have focused on the role of DCs as they have the unique function to act as APCs for T cell activation and thus, for the stimulation of the immune response against a specific Ag, providing a crucial link between both innate and adaptive immune responses. These features allow DCs to be considered as a target for the development of therapies that can induce immune responses and anti-tumour activity [37-39]. DC-based immunotherapy approaches can be employed in a couple of ways: i) the direct in vivo DC targeting to enhance their anti-cancer phenotype and ii) the ex vivo DC stimulation in order to infuse them back into the host for carrying out anti-cancer effector function [40]. Several strategies for the in vivo DC targeting have been developed in the last few years [41,42]. For instance, it has been demonstrated that tumour Ag-conjugated antibodies (Abs) against a variety of molecules primarily expressed on DCs such as DEC-205, CD11c and Clec9A could prevent or inhibit tumour growth in a mouse model of melanoma and breast cancer [42,43]. Indeed, immunization with tumour-Ag anti-DEC-205 or anti-Clec9A monoclonal Abs (mAbs) induced delayed cancer growth in melanoma-bearing mice [44-47]; while a decreased in tumour development was observed in mice immunized with anti-CD11c mAb in a model of murine breast cancer [48]. Although these results are promising, most of these mAbs are still in preclinical phase with only the anti-DEC-205 Ab undergoing a phase I clinical trial. Indeed, in this study, the anti-DEC-205-NY-ESO-1 fusion protein, CDX-1401, has been given intravenously in combination with decitabine and nivolumab in patients with myelodysplastic syndrome (MDS) or low blast count acute myeloid leukaemia (AML) in order to help the immune system to specifically target and kill cancer cells (https://clinicaltrials.gov/ct2/show/NCT03358719).

Alternatively, as circulating DCs constitute only about 1% of peripheral blood mononuclear cells (PBMCs), several ways to generate them from precursors have been investigated for DC vaccination purposes [15]. Indeed, DCs can be generated from CD34+ progenitor cells or monocytes by stimulation with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Then, DCs are loaded with tumour-associated Ags and administered to the patient [39,49]. Recently, a DC-based vaccine (Sipuleucel-T; Dendreon) was approved for the treatment of advanced prostate cancer [50,51]. The development of Sipuleucel-T is based on the concept that patient's isolated DCs are incubated ex vivo with recombinant fusion protein Ag, which contains both the antigen prostatic acid phosphatase (PAP), an enzyme highly expressed by prostate cancer cells, and GM–CSF. Once activated, Ag-loaded DCs are infused back into the patient and are able to fight prostate cancer cells by activating an immune response against them [49,52]. As Sipuleucel-T was tested in phase III trials and induced a survival benefit [53-55], DC-based vaccines seem to be a promising solution for effective cancer therapy. Other promising results have been obtained by the use of CAR-T cell therapy. This a new strategy that consists of the transduction of patient's isolated T cells with chimeric antigen receptors (CARs) that bind to specific ligands on cancer cells. These modified T cells are then amplified and infused back into the patient where they can attack cancer cells [37,56]. One of the first clinical trials using CAR-T cells was reported by Kalos and colleagues, showing that CAR-T cells targeting B cells in chronic lymphocytic leukaemia (CLL) were able to induce complete remission in two of three patients included in the study [57]. Recently, many clinical trials of CAR-T cells have been completed for the treatment of B-cells malignancies, such as Kymriah (tisagenlecleucel; Novartis) and Yescarta (axicabtagene ciloleucel; Kite Pharma) that were approved by the FDA in August and October 2017, respectively [59-61]. However, CAR-T cell therapy against solid cancers have shown less specificity and efficacy compared to liquid tumours, probably due to the lack of a specific target and promising results have been obtained only in neuroblastoma and sarcoma patients [57,60,61]. Moreover, several studies have indicated that NK cells are frequently deficient or dysfunctional in patients with malignancy, indicating that this may be a key factor in cancer immune-evasion and progression. In addition, their rapid killing and broad cytotoxicity make NK cells uniquely primed for use in adoptive therapy. For these reasons, potentiating NKs against tumour cells is an encouraging approach in cancer immunotherapy [62]. In this regard, several strategies for the ex vivo NK cells expansion have been evaluated in the last few years: for instance, the company Miltenyi Biotec has developed a protocol to expand peripheral NK cells from irradiated autologous PBMCs, used as feeder cells, in the presence of NKs activating molecules, such as the anti-CD16 mAb, in order to provide a suitable environment for the NK cells expansion. [63-65], while the company Glycostem enabled a system to expand NKs from umbilical cord blood stem cells and they have been shown to be effective and safe in a phase I clinical trial in elderly AML patients [63,66]. However, these approaches are very personalized and expensive and thus, it may not be possible to apply them to most of the patients.

3.2 Immune checkpoints inhibitors

Unfortunately, the immunosuppressive TME often compromise the therapeutic effects of cell-based immunotherapy, as cancer cells are able to evade the immune system by inducing nutrient depletion and activating negative immune regulatory pathways, thus impairing anti-tumour immune cell functions and promoting the activity of immunosuppressive Tregs and MDSCs [22,67]. For instance, it has been demonstrated that molecules usually expressed on activated T cells, such as the immune checkpoint proteins CTLA-4 and PD-1 play a crucial role in the immunosuppression observed in the TME [60-70]. As mentioned above, PD-1 has been shown to suppress effector T cell activation and functions, upon interaction with its ligands, PD-L1 or PD-L2. Likewise, CTLA-4 binding to CD80 and CD86, expressed on APCs, transmits an inhibitory signal to T cells [30]. Recent evidence has shown PD-L1 and CTLA-4 upregulation on the surface of tumour cells in several types of cancer [31] and for this reason, in the last few years, mAbs targeting CTLA-4, PD-1, PD-L1 or PD-L2 have been developed and tested in clinical trials for the treatment of several types of cancers [67,70-72]. Among the mAbs against CTLA-4, Ipilimumab (Yervoy; Bristol-Myers

Table 1. Main immunotherapeutic agents approved by the FDA or in clinical trial for cancer treatment.

Immunotherapy	Drug name	Mechanism	Phase	Tumour type	References
DC-based Vaccine	Sipuleucel-T	Patients' APCs activated by PAP and GM-CSF	Approved by FDA	Advanced prostate cancer	[50,51]
CAR-T cell therapy	Kymriah	Patient's T cells are engineered to target a protein called CD19	Approved by FDA	B-cell acute lymphoblastic leukaemia	[57,59]
CAR-T cell therapy	Yescarta	Patient's T cells are engineered to target a protein called CD19	Approved by FDA	Large B-cell lymphoma	[58,59]
NK cell therapy	oNKord	NKs generated *ex vivo* from umbilical cord blood progenitor cells.	Phase I clinical trial	Acute myeloid leukaemia	[63-66]
ICI	Ipilimumab	Anti-CTLA-4 mAb	Approved by FDA	Unresectable or MM	[72-79]
ICI	Nivolumab	Anti-PD-1 mAb	Approved by FDA	NSCLC, MM, HL, SCCHN, MUC	[80,82,84,86]
ICI	Pembrolizumab	Anti-PD-1 mAb	Approved by FDA	NSCLC, MM, HL, SCCHN, MUC	[81,83,85,87]
ICI	Durvalumab	Anti-PD-L1 mAb	Approved by FDA	MUC	[89]
ICI	Avelumab	Anti-PD-L1 mAb	Approved by FDA	Metastatic merkel carcinoma	[90]
ICI	Atezolimumab	Anti-PD-L1 mAb	Approved by FDA	NSCLC, MUC	[88]
ICI	CA-170	Anti-PD-L1/PD-L2 and VISTA mAbs	Phase I clinical trial	Lymphomas and solid cancers	[72,93]
Cytokine	Proleukin	IL-2	Approved by FDA	Metastatic melanoma, RCC	[100,101]
Cytokine	Roferon-A	IFNα2a	Approved by FDA	HCL, CML	[100,101]
Cytokine	Intron-A	IFNα2b	Approved by FDA	AIDS-related Kaposi's sarcoma, melanoma, FL, multiple myeloma, HCL, CIN	[100,101]
Oncolytic virus	T-Vec (Herpes simplex virus)	Tumour oncolysis and since virus is armed with GM-CSF this leads to recruitment of APCs	Approved by FDA	Advanced melanoma	[38,103,108]
Oncolytic virus	JX-594 (vaccinia virus)	Tumour oncolysis and GM-CSF expression	Phase I clinical trial	Melanoma, HCC	[113,114]
Oncolytic virus	CG0070 (Adenovirus)	Tumour oncolysis (viral replication under the control of Rb)	Phase I clinical trial	Non-muscle invasive urothelial cancer	[115-117]
Oncolytic virus	Reolysin (Reovirus)	Tumour oncolysis (viral replication under the control of Ras)	Approved by FDA	Malignant glioma, metastatic breast cancer	[121,122]

ICI= Immune checkpoint inhibitor; NSCLC=non-Small Cell Lung Carcinoma; MM= Metastatic Melanoma; HL= Hodgkin Lymphoma; SCCHN= Squamous Cell Carcinoma of Head and Neck; MUC= Metastatic Urothelial Carcinoma; FL= Follicular Lymphoma; HLC= Hairy Cell Lymphoma; CIN= Cervical Intraepithelial Neoplasms; HCC= Hepatocellular Carcinoma.

Squibb) is worthy of a mention as it was one of the first immunotherapies to be approved by the FDA for the treatment of unresectable or metastatic melanoma (https://www.cancer.gov/about-cancer/treatment/drugs/fda-ipilimumab) [72-79]. Since that time, several mAbs have been tested in clinical trials or have been approved for the treatment of different malignancies: i.e. both Nivolumab (Opdivo; Bristol-Myers Squibb) and Pembrolizumab (Keytruda; Merck and Co.) target PD-1 and have been approved for the treatment of metastatic non-small cell lung carcinoma (NSCLC), metastatic melanoma, classical Hodgkin lymphoma, squamous cell carcinoma of head and neck (SCCHN) and metastatic urothelial carcinoma [80-87]. Moreover, Durvalumab (Imfinzi; AstraZeneca UK Limited), Avelumab (Bavencio; EMD Serono, Inc.) and Atezolizumab (Tecentriq; Genentech Oncology), all of which target PD-L1, have been approved for the treatment of metastatic urothelial carcinoma, metastatic Merkel cell carcinoma and metastatic NSCLC, respectively [88-90]. However, only a low fraction of patients respond to immune checkpoint inhibitors due to primary or acquired resistance mechanisms, as recently reviewed by Vanpouille-Box C. and colleagues [72]. Thus, in the last few years, considerable attention has been attracted by mAbs that target co-inhibitory receptors other than CTLA-4 and PD-1, including V-set immunoregulatory receptor (VSIR, best known as B7-H5 or VISTA) with the aim of co-targeting different immune checkpoint molecules [91,92]. Indeed, the biotech company Curis is now testing in a phase 1 clinical trial a novel oral small molecule, also known as CA-170, dual inhibitor of immune checkpoints PD-L1/PD-L2 and VISTA for the treatment of advanced solid tumours or lymphomas (https://clinicaltrials.gov/ct2/show/NCT02812875) [72,93].

3.3 Cytokines therapy

As mentioned above, cancer cells are able to communicate with the other components of the TME, shaping and manipulating the surrounding microenvironment to produce cytokines, growth factors and metabolites to meet their own needs [2,94,95]. In particular, deregulated cytokines production and aberrant cytokine signalling can lead to altered cell growth, differentiation and apoptosis as well as the secretion of factors that foster cancer progression and immune evasion [96,97]. Indeed, cytokines are major regulators of the innate and adaptive immune system allowing cells to communicate over short distances in a paracrine and autocrine manner and controlling leukocyte proliferation, differentiation, effector functions and survival [98]. Thus, recently, cytokines have been explored in the treatment of cancer in order to enhance anti-tumoural immunity [99,100]. However, only three cytokines have been approved so far by the FDA for use in cancer patients: recombinant interleukin IL-2 (Proleukin; Chiron) and two variants of recombinant interferon alpha 2 called IFNα2a (Roferon-A; Roche) and IFNα2b (Intron-A; Merk & Co). IL-2 acts as a T- cell growth factor during the initiation of the immune response, and has a crucial role in terminating T-cell responses for the maintenance of self-tolerance, while IFNα belongs to the family of type I IFNs and, even if it has anti-viral properties, recently it has also been linked to the pathogenesis of infectious disease, inflammatory disorders, autoimmune diseases and cancers [100]. Proleukin has been approved for the treatment of metastatic melanoma and renal cell carcinoma (RCC), Roferon-A for the treatment of hairy cell lymphoma (HCL) and chronic myelogenous leukaemia (CML), while Intron-A has been approved for the use in patients with AIDS-related Kaposi's sarcoma, melanoma, follicular lymphoma (FL), multiple myeloma MM, HCL and cervical intraepithelial neoplasms (CIN) [100,101]. In addition, several clinical trials have been carried out to evaluate the use of IL-15, IL-12, IL-21 or GM-CSF for the treatment of melanoma and renal cell cancer. However, regardless of their promising results in murine models, these cytokines showed inconsistent results in humans together with several side effects and thus require further clinical evaluation [100,102].

3.4 Oncolytic viruses

Oncolytic viruses (OVs) represent a new class of therapeutic agents that promote anti-tumour responses through a dual mechanism of action that is dependent on selective tumour cell killing and the induction of systemic anti-tumour immunity. Specifically, viral replication in cancer cells, followed by cancer cell lysis, results in the release of more viruses, as well as tumour Ags. Consequently, Ag uptake by APCs indirectly promotes both innate and adaptive immune responses [38, 103-105]. In addition to these important properties, OVs are considered very flexible agents as they can be genetically engineered to further enhance anti-tumour activity and, thus, to maximize their features. Indeed, for instance, the capsid of OVs that do not have an innate tropism for cancers can be engineered to enhance virus binding to tumour cell receptors [103,106,107]. Moreover, other strategies to improve selective replication in cancer cells are the deletion/insertion of tissue- or cell type- specific promoters to induce gene expression in tumour cells [108]; or the placement of viral genes under the control of tissue specific elements. For instance, Ad[I/PPT-E1A] is a new OV that specifically replicates in prostate cancer cells as the adenovirus early region 1 (E1A) is under the control of three prostate specific elements, such as the prostate-specific antigen (PSA) enhancer, the prostate-specific membrane antigen (PSMA) enhancer and the T-cell receptor gamma-chain alternate reading frame protein (TARP) promoter [108]. Therefore, these features allow OVs to be considered as a promising strategy for cancer immunotherapy. Consequently, several OVs are currently being tested in clinical trials as anti-cancer agents and in 2015 the Talimogene laherparepvec (T-Vec; Amgen Inc.) was approved by the FDA as the first OV for the treatment of advanced melanoma [38,109]. This attenuated herpes simplex virus (HSV) is engineered to preferentially replicate in cancer cells and to express the immune-modulatory cytokine GM-CSF which in turn, promotes APCs recruitment to the TME, thereby inducing the anti-tumour immunity [38,103,108]. In the phase III OPTiM clinical trial for treating advanced melanoma, T-Vec treatment showed extended durable response rates and prolonged median overall survival compared with GM-CSF alone, with minimal adverse effects, highlighting the fact that these

agents can be safely used in a clinical setting [110-112]. Based on these promising results, several OVs are currently being tested in clinical trials: i.e. in early phase I studies, JX-594, a genetically engineered thymidine kinase-mutant/GM-CSF expressing Vaccinia virus (VAC), demonstrated highly promising results in patients with melanoma [113] and hepatocellular carcinoma (HCC) [114]. In addition, CG0070 is a GM-CSF expressing adenovirus where the E1A gene is under the control of E2F, a retinoblastoma (Rb)-dependent transcription factor, providing selectivity for cells with an abnormal p16/Rb pathway [115]. CG0070 has selective replication, cytotoxicity, production of GM-CSF and anti-tumour efficacy in urothelial carcinoma and a human phase I trial of intravesical administration of CG0070 included 35 patients with non-muscle invasive urothelial cancer [116] showed that the therapy was well tolerated; however, the cohort of patients was too small for definitive conclusions [117]. Among reoviruses (RVs), Reolysin (Pelareorep; Oncolytics Biotech Inc.) has been extensively evaluated in preclinical models and clinical studies for the treatment of solid tumours and haematological malignancies [118,119]. Reolysin replicates in and eventually kills Ras-activated tumour cells, and as cell death occurs, progeny virus particles are then free to infect surrounding cancer cells until all tumour cells carrying an activated Ras pathway are destroyed [120]. In April 2015, the FDA granted orphan drug designation to Reolysin for malignant glioma [121] and in May 2017, the FDA granted fast track designation for Reolysin in metastatic breast cancer [122].

3.5 Immunotherapy challenges

Despite all the advances described above, obstacles still exist in cancer immunotherapy and the efficacy of this treatment has been limited. Indeed, immunotherapy seems to work only for some patients and types of tumours, and some patients acquire resistance and relapse after a period of response [123]. Therefore, there is the need to develop new strategies to overcome resistance and to boost the immune system against cancers, especially for the patients with little or no infiltrating immune cells [4,124]. Moreover, one of the main problems of cancer immunotherapy is that it is often associated with mild-to-severe toxicities and autoimmune diseases due to anomalous and general immune activation [125]. Indeed, several studies have shown that cancer immunotherapy is associated with IRAEs such as, inflammatory skin disorders, thyroid dysfunctions, hypophysitis, type 1 diabetes mellitus, hepatotoxicity, gastrointestinal, neurological, cardiac, rheumatologic and renal toxicities. These problems are often due to the non-specific delivery of the immunotherapeutic agents that can target also healthy tissues, resulting in adverse events with low drug delivery to the tumour site [126-128]. Moreover, the presence in the TME of immunosuppressive cells, such as MDSCs and Tregs, results in the increased production of immunosuppressive cytokines (e.g., IL-10, TGFβ) that together with the low immunogenicity of tumour Ags strongly limits the anti-tumour response elicited by immunotherapies [127]. Therefore, to overcome these problems we need to develop new strategies for delivering an optimal amount of immunotherapy to a specific site, with appropriate kinetics and dosing schedule, without inducing deleterious side effects. A possibility could be the combination of different therapies to obtain differential targeting within the TME, such as the concomitant increase of Ag presentation by APCs and T-cell activation, together with the induction of cancer cell death or the targeting of other stromal components of the TME [128]. However, obtaining these achievements is complicated and an easier and more promising approach could be the use of nanomedicine. In fact, in the case of systemic administration of drugs, NPs can better protect the therapeutic agent from its clearance, thus offering a platform that can help the specific delivery of immunotherapeutic agents to the tumour site, thus reducing their adverse events. Indeed, NPs can protect bioactive molecules and can be engineered to modify their size and shape, immunogenicity, biodistribution, site-specific targeting, therapeutic loading and detectability by medical imaging [10,129]. All these features allow NPs to become promising candidates for the delivery of cancer immunotherapies and will be discussed in the following sections.

4. Nanoparticles properties

In general, NPs are divided into different categories based on their physical and chemical properties such as material type, size, shape, charge and surface chemical modifications, and it is now well accepted that all these features play an important role in their kinetics of internalization, bio-distribution, cellular uptake, immunogenicity and loading efficiency [10,127]. For instance, various materials have been experimented for biomedical applications including lipids, metals, polymers, carbon structures and inorganic materials that can be used for NP formulation and all of them have different features. In particular, polymers such as poly(lactic-co-glycolicacid) (PLGA) or chitosan have been shown to increase NP immunogenicity by supporting DC maturation and thus enhancing immune responses [10,131]. When considering immunogenicity, a component that is now generally used for NP formulation is the polyethylene glycol (PEG) polymer coating. In fact, PEG is well known for immune-camouflage properties as it reduces NP immunogenicity: PEGylation is generally believed to decrease complement activation responses and evade clearance by the immune system, thereby enabling longer NP circulation time [130]. Moreover, according to a general definition, NPs are defined as having dimensions below 100nm; however, especially in the area of drug delivery, relatively larger NPs (size >100 nm) may be needed for loading a sufficient amount of drug onto the particles: for instance big oncolytic viruses, such as adenoviruses with a size around 90-100nm, have to be encapsulated inside larger NPs [9,129,130]. On the other hand, modifying the NP size could critically affect their clearance. For instance, it has been shown that NPs with a diameter smaller than 10 nm are generally cleared in the kidneys, while bigger NPs are usually cleared by the spleen and the liver [127,132]. In addition, NP size can interfere with their transport into the blood and their extravasation to a tumour or an inflammatory site. Indeed, at the tumour site, the large fenestrations present in the tumour vasculature could allow the extravasation also

of large NPs. This is due to the rapid cancer cell proliferation that promotes the formation of neovasculature for their nutritional and oxygen supply. These newly formed tumour vessels are usually abnormal in forms and architecture with a lack of smooth muscle layer and large fenestrations, facilitating NP accumulation within the tumours. This process is also known as the enhanced permeability and retention (EPR) effect and it is exploited for improving nanomedicine delivery to the tumour site [132-135]. However, the EPR effect can be limited by the presence of cancer cells and dense collagen fibres that cause extracellular matrix (ECM) stiffness and high interstitial fluid pressure. These circumstances act all together to trap NPs and obstruct their extravasation [132,133]. In this context, together with NP material type and size, also the shape can be a crucial factor for NP tissue permeation. For instance, Chauahn and colleagues showed that NP-tumour penetration is dependent on their shape. Indeed, comparing nanorods and nanospheres the authors found that nanorods exhibited superior transport and distribution into mammary tumours in vivo versus nanospheres of similar plasma half-life [136]. Moreover, it seems that cylindrical hydrogel NPs are more efficiently internalized by APCs, compared to rods and spherical NPs, therefore eliciting a stronger T cell activation and proliferation when injected subcutaneously in mice [137]. In addition, the nanodrug surface charge can affect NP cellular uptake and tissue permeation. For instance it has been shown that cationic NPs are easily taken up by DCs and macrophages, but can also be trapped by the negatively charged ECM reducing their tissue permeation compared with neutral and anionic carriers [10,138-140]. However, exploiting the EPR effect, the specific NP properties and the composition of the TME is not enough to deliver a sufficient drug amount at the tumour site. Therefore, passive nano-targeting is not the best approach for drug delivery. A possible solution could be an active nano-targeting achieved by chemical modifications of the NP surface: this approach could allow a specific NP delivery to tumours together with a low nanocarries distribution in healthy tissues [141]. Indeed, targeting molecules can be linked to the NP surface with the aim of binding receptors expressed by tumour cells or by the surrounding microenvironment. The internalization of the complexes occurs by receptor-mediated endocytosis through the formation of the endosome and the subsequent fusion with the lysosome where the acidic pH allows the release of the drug inside the cell [141]. In this context, promising examples of receptors that can be actively targeted are the folate receptor, the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptors (VEGFR-1/2), integrins and matrix metalloproteinases (MMPs) that are usually overexpressed by cancer cells or by the surrounding stroma [141]. Therefore, NPs are a promising strategy to deliver immunotherapeutic agents and in the last few years many goals have been achieved in this field. Indeed, several immunotherapies such as monoclonal Abs, cancer vaccines, OVs, cytokines and peptides have been successfully conjugated to different types of NPs in order to stimulate anti-tumour immunity (Figure 2). Thus, in the next sections we will describe the most recent advancements in the nanodelivery of cancer immunotherapies.

4.1 Lipid nanocarriers

Examples of lipid nanocarriers are liposomes, solid-lipid NPs and phospholipids micelles. In particular, among them, liposomes are considered an ideal drug delivery system as they consist of spherical vesicles having one or more lipid layers containing an aqueous core, so that they can entrap both hydrophilic and lipophilic agents in the lipid layers or in the internal compartment, respectively [142,143]. Therefore, it is not surprising that in the last few years many studies have focused on the delivery of liposomes-encapsulated drugs. The first success in this context has been the liposome-encapsulated form of Doxorubicin (Doxil) approved by the FDA in 1995 for the treatment of ovarian cancer and AIDS-related Kaposi's sarcoma [142-144]. This success paved the way for the use of liposomes for the delivery of several different drugs/agents also in field of cancer immunotherapy. For instance, in 2004 van Broekhoven et al. tried to target DCs through anti-DEC-205 or anti-CD11c mAbs located on the surface of liposomes containing tumour Ags (B16 melanoma Ags or lipopolysaccharide), thus inducing potent anti-tumour immunity both in vitro and in vivo [145,146]. Moreover, lipid NPs have been used to improve CAR-T cell therapy against solid tumours. Indeed, as mentioned above, one of the main challenges of CAR-T cell therapy success in solid cancers is the presence of an immunosuppressive TME that can decrease the treatment efficacy [127] and in this context, the use of NPs could limit this obstacle. In fact, a recent study from Zhang and colleagues has shown promising results in a model of murine breast cancer. By the infusions of lipid NPs coated with the tumour-targeting peptide iRGD and loaded with a combination of a PI3K inhibitor to block immunosuppressive tumour cells activity and α-GalCer (an iNKT cell activator), the authors were able to switch the TME from immune- suppressive to stimulatory. This treatment created a therapeutic window of 2 weeks, enabling tumour-specific CAR-T cells to home to the tumour, undergo robust expansion and trigger tumour regression [147]. In addition, liposomes have been also used for improving cytokine therapy that is often associated with severe toxicities and failure in reaching the tumour site [100]. For instance, it has been demonstrated that, in a model of metastatic melanoma, the liposomal delivery of a stimulator of interferon genes (STING) agonist improves IFNγ production by tumour-associated APCs, leading to anti-tumour immunity enhancement and cancer regression compared to the free drug [148]. Another promising strategy that is now in a phase I/II clinical trial for advanced melanoma treatment is the use of lipoplexes (LPX). They protect a specific RNA from extracellular ribonucleases and allow its efficient uptake and expression of the encoded Ag by DCs and macrophages in various lymphoid compartments. The complexes trigger IFNα release by plasmacytoid DCs and macrophages promoting DC maturation in situ and anti-tumoural immune responses

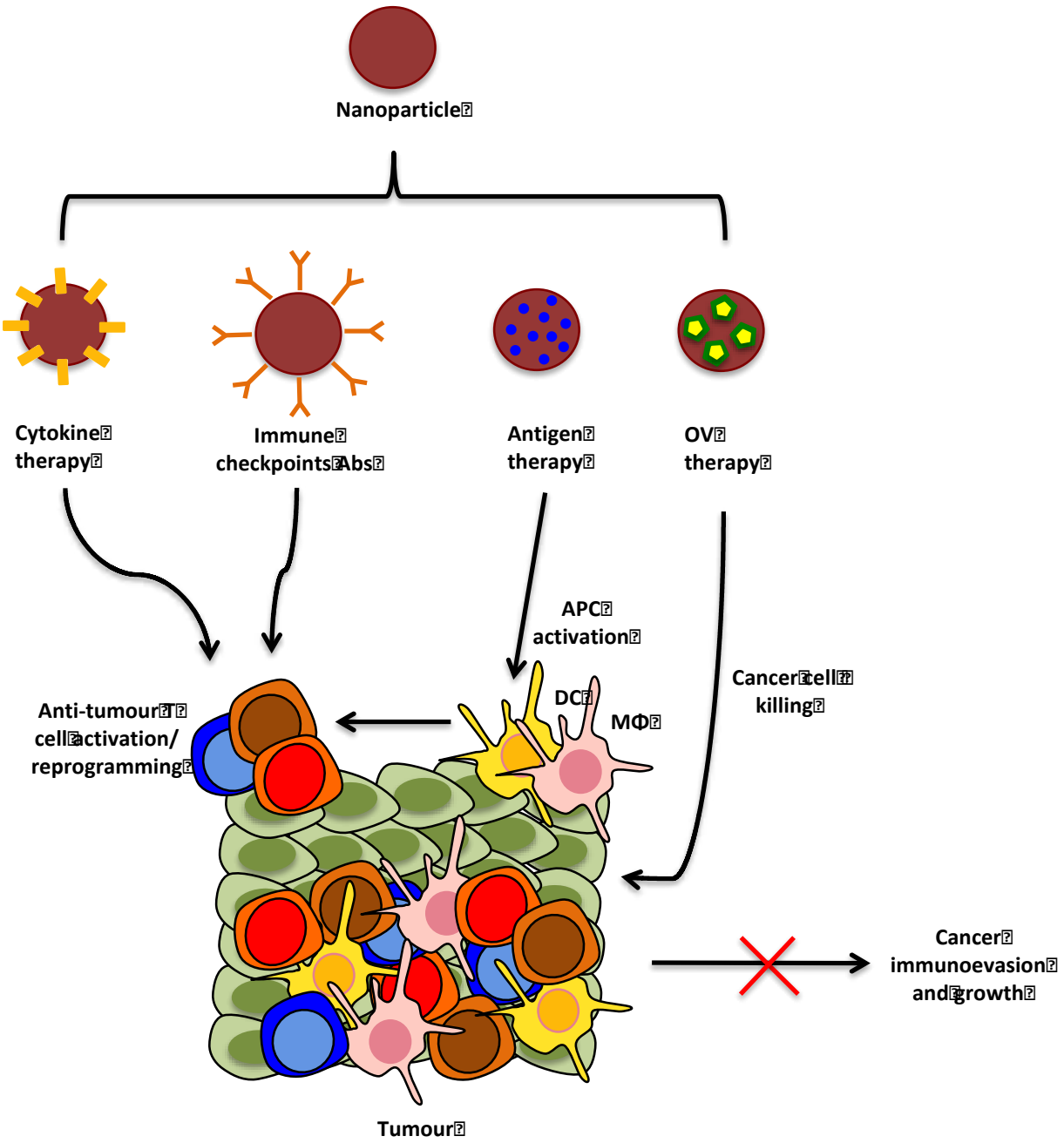

Figure 2. NPs design strategy in cancer immunotherapy. NPs can be formulated and designed for potentiating immunotherapy efficacy, improving their specific delivery to the tumour site. They can be coated with immune checkpoints Abs, peptides and cytokines or can be loaded with OVs and Ags in order to boost the anti-tumour T cells for fighting cancer and escape from immunosuppressive mechanisms that could limit immunotherapy benefits.

demonstrated that anchoring anti-CD137 Ab and an engineered IL-2Fc fusion protein to the surfaces of PEGylated liposomes avoided toxicities and treated the majority of established primary tumours in the B16F10 melanoma model compared to the equivalent intratumoural doses of soluble immunotherapeutic agent [151]. In addition, recently, a micelleplex made of an acid-activatable cationic micelle, a photosensitizer and a small interfering RNA (siRNA) was developed against PD-L1 in order to treat a B16-F10 murine melanoma tumour model. The micelleplex was activated only upon internalization in the acidic endocytic vesicles of tumour cells allowing the combined therapy to inhibit tumour growth and distant metastasis formation compared to photothermal therapy alone. These promising results strengthen the idea that the use of therapy combination together with NPs as delivery systems could strongly increase the efficacy of cancer immunotherapy [67,152]. Finally, also OV encapsulation inside lipid NPs is another strategy that needs to be mentioned as it has been shown encouraging results in the last few years. Indeed, Chen and colleagues have demonstrated that encapsulating the ZD55-IL-24 oncolytic adenovirus inside lipid NPs

reduces the OV neutralization by pre-existing Abs upon its systemic administration. Moreover, this complex efficiently inhibits HCC cell proliferation, induces apoptosis and enhances anti-tumour immunity in vivo [153]. In addition, in another study the authors showed that liposome-encapsulated plasmid DNA of telomerase-specific oncolytic adenovirus (TelomeScan) reduced production of adenovirus-neutralizing Abs upon intravenous administration into immune-competent mice compared to the naked virus and had potent anti-tumour effects on colon carcinoma cells both in vitro and in vivo [154].

4.2 Polymeric nanoparticles

Polymeric NPs are one of the most studied organic strategies for nanomedicine and some of them have been recently used for in vivo studies and clinical trials [67,155]. This is due to the fact that polymers are flexible and lightweight materials that can be produced at low cost. Moreover, they can be moulded for creating the desired structure and many of them are biodegradable and thus, less toxic [67,156]. For instance, PLGA is one of the most successfully used biodegradable polymer as its hydrolysis produces lactic acid and glycolic acid that can be easy metabolized by the cells through the Krebs cycle [157,158]. For this reasons, it is not surprising that in the last few years PLGA NPs have been extensively tested for improving cancer immunotherapy effects. Cruz and colleagues demonstrated that PLGA NPs encapsulated with tetanus toxoid (TT) peptide and ovalbumin (OVA) protein - as model Ags - in combination with the TLR7/8 ligand R848 - as adjuvant - increase the kinetics of Ag delivery, processing, presentation, DC maturation and cytokines release, highlighting the idea that polymeric nanosystems could be used for fighting cancer [159]. In the context of cancer vaccines, it is also worth mentioning WDVAX: a phase I clinical trial for the treatment of melanoma (https://clinicaltrials.gov/ct2/show/NCT01753089). In particular, WDVAX consists of PLGA scaffolds loaded with melanoma Ags, GM-CSF and CpG, in order to activate DCs [160]. In the preclinical study, these scaffolds reprogrammed DCs to activate a robust immune response against murine B16F10 melanoma tumours. The authors showed that GM-CSF enhanced DC recruitment while CpG oligonucleotides (CpG-ODNs) – a TLR9 ligand - complexed to cationic polymer polyethylenimine (PEI) promoted DC activation in situ [160,161]. In another study, Hamdy et al. used PLGA NPs co-encapsulating the poorly immunogenic melanoma Ag, tyrosinase-related protein 2 (TRP2), together with a TLR4 ligand (7-acyl lipid A). NP-vaccinated mice showed TRP2-specific CD8+ T cell responses capable of mediating therapeutic anti-tumour response. More importantly, this vaccine strategy led to the reversal of immunosuppressive milieu within the TME, as evidenced by the increased levels of pro-inflammatory Th1 related cytokines (IL-2, IL-6, IL-12 and IFNγ) and the decreased in the levels of VEGF that is required for tumour growth [162]. PLGA is not the only polymer that has been used for drugs encapsulation: i.e., Li and colleagues used PEG-block-poly(D,L-lactide) (PEG–PLA) NPs loaded with CTLA-4 siRNA (siCTLA-4) and showed direct T cell activation both in vitro and in vivo. These NPs efficiently delivered the siCTLA-4 into T cells in vitro. Furthermore, they simultaneously increased in vivo the percentage of anti-tumour CD8+ T cells and decreased Tregs cells among TILs, resulting in inhibition of tumour growth and prolonged survival time [163]. In addition, polymers have been also loaded with viruses. Indeed, it has been shown that PLGA microspheres can be used to encapsulate a PEGylated adenovirus, showing an increased stability and transfection efficiency in Hela cells compared to the naked virus, paving the way to the possibility of OV encapsulation also in smaller particles for cancer treatment [164].

4.3 Inorganic Nanoparticles

Inorganic NPs can be synthesized using metals, metal oxides and semiconductors such as silicon or ceramic. They have several advantages as they are relatively stable, easy to be synthetized, and can be easily loaded with enzymes, fluorochromes and magnets in order to facilitate their in vivo tracking [67,129]. One of the most employed type of inorganic NPs for biomedical applications are gold (Au)NPs that provide a versatile platform for the combination with oligonucleotides, Abs and proteins [165]. Therefore, it is not surprising that AuNPs have been studied for cancer treatment. For instance, recently, a nanomedicine platform called Aurimune has been developed. It consists of a colloidal gold NP covered with a PEG layer and coated with the tumour necrosis factor (TNF) molecule that selectively binds TNF receptors on blood vessel cells at the site of disease, causing blood vessel destruction and thus impairing tumour's nutritional support structures and protective barriers. A phase I clinical trial in patients with advanced solid tumours has shown that recombinant human (rh)TNF, formulated as Aurimune, can be administered systemically at doses of rhTNF that were previously shown to be toxic and a phase II clinical trial to treat pancreatic cancer patients in combination with standard of care second line therapy has been announced (https://clinicaltrials.gov/ct2/show/NCT00356980).
Moreover, other anti-cancer agents, such as IFNγ, have been successfully linked to the platform and need to be tested in clinical studies [166,167]. In the context of cell-based immunotherapy, Zhou et al. optimized a method to deliver the OVA peptide and CpG-ODNs to bone marrow derived DCs through the use of a nanoAu-cocktail consisting of both 60nm and 80nm AuNPs [168]. Intravenously transfused nanoAu-cocktail pulsed DCs showed dramatically improved in vivo homing ability to lymphoid tissues and elicited an Ag-dependent CD8+ T cell response, indicating a potential use for cancer treatment [168]. Moreover, in another interesting study, AuNPs were functionalized with a red fluorescent protein as a model Ag, and CpG-ODN as a TLR9 ligand for interaction with DCs within the lymph nodes, thereby strongly promoting a significant anti-tumour response in preclinical melanoma tumour models [169,170]. Mesoporous silica is another material used for preparing inorganic NPs, due to its high biocompatibility, and it has been shown to enhance anti-tumour immunity [67]. In this context, anti-CTLA-4 Abs have been encapsulated in functionalized mesoporous silica (FMS) NPs for the

treatment of mouse melanoma. It has been demonstrated that FMS-anti-CTLA-4 inhibited tumour growth [171]. Moreover, luminescent porous silicon NPs (LPSiNPs) have been used as carriers of FGK48, an agonistic anti-CD40 Ab, for APC stimulation and activation. These nanocomplexes had an intrinsic near infrared range (NIR) photoluminescence that served as a tracking indicator to study the interaction of the complexes with the immune system. Indeed, the authors showed that LPSiNPs successfully improved B cell activation by 30–40 fold by enhancing the expression of the activation markers CD86 and MHC II [67, 172]. Iron oxide nanoparticles (Fe3O4 NPs) have attracted broad interest due to their good biocompatibility, superparamagnetic properties and also because they have potential applications in many fields such as magnetic resonance imaging (MRI) that makes them promising for oriented delivery. Indeed, Fe3O4 NPs can be guided by an external magnetic field to concentrate in desired sites for targeted delivery and to help map their distributions [173]. In fact, a recent study from Cho and colleagues showed that Fe3O4 NPs covered with a photonic ZnO shell and loaded with carcinoembryonic Ag (CEA) are efficiently taken up by DCs within one hour and can be detected in vitro by confocal microscopy and in vivo by MRI. Furthermore, in a model of murine colon adenocarcinoma, mice immunized with DCs containing the NP-Ag complexes showed enhanced tumour Ag specific T-cell responses, delayed tumour growth and better survival than controls [174]. Cruz and colleagues equipped superparamagnetic iron oxide particles (SPIO) with PLGA NPs recognizing DC receptors (e.g. the C-type lectin receptor CD209). MRI allowed tracking DCs - targeted with NPs carrying SPIO - within the lymph nodes where the authors also found an enhanced T cell proliferation [175]. In addition, another group recently used iron-dextran NPs functionalized with T cell MHC-peptides and anti-CD28 Abs to create artificial APCs for T cell expansion and activation. Upon the application of an external magnetic field, these artificial APCs aggregated and activated T cells which, in turn, were able to inhibit B16 melanoma in vivo growth [176]. In the context of immunecheckpoint inhibitors, Chiang et al. demonstrated that fucoidan-dextran-based magnetic NPs (IO@FuDex3) conjugated with an anti-PD-L1 Ab and T-cell activators (anti-CD3 and anti-CD28) can switch the TME of a murine breast cancer model from immunosuppressive to anti-tumoural by TILs stimulation [177]. Attempts were also made to conjugate iron oxide NPs with OVs. An interesting study showed magnetic NPs conjugated with OV particles could be internalized by multiple cell lines and display a strong oncolytic activity. In addition, the complexes could be detected in vivo when administered in an intratumoural manner in an orthotopic rat hepatocellular carcinoma model [178].

4.4 Magnetosomes

Until recently, a biologically derived alternative to metallic particles, namely magnetosomes, has been relatively overlooked presumably due to the challenges associated with their biomanufacture [179,180]. Magnetosomes are functional magnetic NPs that are naturally generated by magnetotactic bacteria (MTB) and comprise a crystal of magnetite coated in a phospholipid membrane. They possess many advantages over chemically synthesized magnetic NPs including a narrow size and shape distribution and a biologically compatible surface chemistry, which prevents aggregation and provides an anchor for functional groups for biotechnology applications. Medical applications in oncology have so far exploited the magnetic properties of magnetosomes for MRI contrast agents [181] and magnetic hyperthermia [182]. Indeed, magnetic hyperthermia is showing particular promise for hard to reach tumours including glioblastoma whereby 50% of mice inoculated with the GL-261 mouse glioma cell line were fully cured 5 weeks post treatment (albeit using a subcutaneous model [183]) as well as 40% of mice bearing intracranial U87-Luc tumours [184]. Both studies observed only partial tumour coverage using nanoparticles and alluded to other mechanisms contributing to tumour cell destruction upon application of an alternating magnetic field (AMF) including tumour ischemia and/or enhancement of the antitumour immune response. Indeed, the heat shock protein HSP70 expressed during hyperthermia induced necrotic cell death using magnetite cationic liposomes has been shown to suppress T-9 rat glioma in F344 rats [185] and boosting the immune response by heating tumours is a growing area with a number of review articles [186,187] (including mathematical models [188]). Recruitment for a clinical trial (NCT03393858) is also currently underway to investigate the clinical efficacy and toxicity of anti-PD-1 monoclonal antibody plus autologous dendritic cell-cytokine induced killer cell (DC-CIK) immunotherapy combined with hyperthermia in advanced malignant mesothelioma patients. As a nanoscale drug carrier, magnetosomes are easily functionalized by utilizing their magnetosome membrane in a number of ways. Firstly, directly loading the magnetosome surface with drugs using a number of cross-linking methods has demonstrated efficacy with single compounds. Doxorubicin linked to magnetosomes using glutaraldehyde, has demonstrated in vitro and in vivo antitumour properties against hepatocellular carcinoma [189]. In addition, cytosine arabinoside (Ara-C) for acute leukemia treatment has previously been cross-linked onto magnetosomes using genipin (a naturally derived, less toxic alternative to glutaraldehyde) to reduce the side effects and increase long-term stability and release behaviours in circulation [190]. Strategies to increase the efficacy of drug-loaded magnetosomes has included co-delivery of combination therapies, successfully validating that cross-linkers can be used to couple two kinds of drugs with the magnetosome membrane. Combination of Ara-C with daunorubicin (DNR) was used to target acute myelogenous leukaemia (AML) with long-term release stability and minimized side effects [191] and more recently doxorubicin has been co-delivered with si-RNA [192]. These ample functional groups on the magnetosome membrane have also been exploited for conjugation of tumour-targeting peptides such as P75 for nanomolar affinity to EGFR and HER2 for specific detection of MDA-MB-468 and SKBR3 cells as well as xenograft tumors using MRI [193]. Secondly, the addition of extra polymeric coatings such as PLGA onto the magnetosome surface has been used to both enhance drug loading and

facilitate controlled drug release under certain conditions, for example pH [194]. Thirdly, magnetosomes can be manipulated genetically to express particular proteins on their surface including anti-HER2 affibodies (antibody mimemtics) for non-invasive imaging of tumours [195] or the expression of protein A in the membrane can conjugate antibodies directly [196]. Most promisingly, magnetosomes may help to overcome the issues surrounding nonspecific biodistribution of pharmaceuticals by utilizing their magnetic properties for magnetic targeting under the use of an external magnetic field. MTB bacteria themselves have been conjugated with drug-containing liposomes for delivery to hypoxic areas of tumours, notoriously difficult to access with conventional therapies. By utilizing the bacteria's preference for low oxygen environments together with magnetic guidance, investigators were able to show up to 55% of MC-1 bacterial cells penetrated into hypoxic regions of HCT116 colorectal xenografts following a peritumoural injection and that the superior penetration depths were associated with active directional motility through magnetotaxis and aerotaxis of the MC-1 cells [197]. This technique has been adopted for administration of DNA plasmids for the generation of antigen-specific immunity. By combining human papillomavirus type E7 and Ig-Fc fragment (pSLC-E7-Fc) with magnetosomes, Tang et al created a DNA vaccine for tumour immunotherapy generating antigen-specific cytotoxic T lymphocyte activity with resultant tumour inhibition in a murine metastatic lung model (average pulmonary metastatic tumour weight of 343.6 mg vs 58.9mg) when administered with magnetic exposure [198]. The design of artificial antigen-presenting cells mimicking the characteristics of magnetosomes are also proving versatile agents for adoptive T-cell transfer to overcome the challenges associated with T-cell expansion and in vivo targeting. Persistent T cells in a murine lymphoma model were associated with tumour suppression brought about by a 78-fold ex-vivo expansion together with their magnetic enrichment [199]. This ability of magnetosomes to deliver therapies to target sites is relatively new within the field of immunotherapy, but is a promising strategy combining low systemic toxicity with superior non-invasive targeting.

4.5 Carbon nanomaterials

As carbon is one of the most abundant elements in the known universe, and it only follows oxygen with regard to abundance in the human body, it could be considered as a suitable element for delivering medicine and other substances to the human body, especially at a cellular level. Furthermore, carbon nanomaterials have a large surface area that offers the possibility of multivalent conjugation of drugs/agents, thus explaining why several groups have recently tried to use carbon nanomaterials for improving immunotherapy efficacy [67,200]. In particular, carbon nanotubes (CNTs), that are cylindrical nanostructures made of carbon molecules, have been conjugated with tumour Ags with the aim of potentiating patients' immune response against cancer. Indeed, Meng and colleagues showed that multi-wall CNTs can be conjugated with a tumour- cell vaccine (TCV), made of inactivated cancer cells and DCs that have been exposed to tumour Ags, and can enhance the efficacy of the vaccine in a murine model of liver cancer [201]. In another approach, single-wall CNTs have been used to increase the uptake of weak immunogenic peptides into DCs and macrophages. Furthermore, in vivo administration of the complexes promoted a specific T cell response [202]. Also, the addition of pathogen-associated molecular pattern (PAMP) agonists, such as CpG-ODNs, to functionalized CNTs has been shown to increase the TCV immunostimulatory properties. Moreover, the direct stimulation of T cells has been achieved by CNTs linkage with both peptide-loaded MHC-I and the co-stimulatory anti-CD28 [200,203]. CNTs were also combined with polymeric NPs containing IL-2. The combined complex has been shown to be able to expand in vitro CD8+ T cells that in turn, upon in vivo administration, delayed tumour growth in a murine melanoma model [204]. CNTs have also been extensively studied for photothermal cancer therapy as they show strong absorbance in the NIR [67,205]. This feature allowed the Liu group to develop a combined therapy strategy: single-wall CNTs conjugation with anti-CTLA-4 Abs enabled the achievement of photothermal tumour destruction together with anti-tumoural T cells infiltration, thus inhibiting cancer growth and metastasis in both murine melanoma and breast cancer models [205]. Once again, therapy combination through the use of NPs seems to be a promising strategy in the fight against cancer.

4.6 Virus-like nanoparticles

Engineered virus-like particles (VLPs) resemble viruses, but are non-infectious because they contain no viral genetic material. The expression of viral structural proteins, such as envelope or capsid proteins, can result in the self-assembly of the VLPs. They can be engineered to display multiple epitopes or peptides in order to optimize their antigenicity and immunogenicity. Collectively, these unique characteristics of VLPs make them optimal cancer vaccine candidates [67,206]. For example, cowpea mosaic virus (CPMV), a plant virus that self-assembles into icosahedral shaped NPs, has been shown to interact with APCs. In particular, CPMV interacts with Vimentin expressed on APCs surface allowing virus entry into cells both in vitro and in vivo [207]. Furthermore, in another interesting study, Steinmentz and colleagues showed that CPMV VLPs could directly act on tumour cells by binding to Vimentin expressed on metastatic tumour cells [208]. In this context, Lizzotte et al. demonstated that the inhalation of CPMV NPs reduced established B16F10 lung melanoma metastasis and simultaneously activate neutrophils that can directly kill tumour cells or induce anti-tumour activity of T and NK cells [209]. Intratumoural administration of another kind of virus-like NPs significantly slowed down melanoma progression and prolonged survival of tumour bearing mice. Indeed, Lamarre group showed that the treatment with papaya mosaic virus (PapMV) NPs enhanced chemokine and pro-inflammatory cytokine production that, in turn, induced CD8+ T cell accumulation together with a concomitant decrease in MDSC presence within the tumour [210]. Finally, MelQbG10, a VLP loaded with A-type CpG-ODN has been tested in a phase IIa clinical trial in melanoma

patients (https://clinicaltrials.gov/ct2/show/NCT00651703) and it has been shown to trigger both effector and memory T cell responses [211].

5. Conclusions

In the last few decades immunotherapy has become an emergent and promising approach to fight cancer in addition to conventional therapies. As result, several immunotherapeutic agents have been tested in clinical trials and then approved by the FDA/EMA. However, some limitations decrease the potential benefits of cancer immunotherapy including failure in patients with low quantity of TILs and also, immunotherapy is associated with toxicities due to drug accumulation in healthy tissues that causes an anomalous immune activation. Furthermore, the immunosuppressive TME can limit the efficacy of immunotherapy [124-128]. In order to overcome these problems, nanomedicine has been exploited to obtain several important advantages, such as tumour specific targeting and drug protection from its clearance in circulation. More importantly, NPs aim to decrease IRAEs and increase immunotherapy specific delivery. Furthermore, NPs can be modified in size, shape, immunogenicity, charge, surface alterations and can also be detected for medical imaging [10,67]. For this reason, a remarkable number of studies have been conducted recently to provide clear evidence that nanomedicine could have a significant impact in cancer immunotherapy. Although NP-based immunotherapy has shown excellent and promising results in preclinical studies, supporting the idea that it has an enormous potential for fighting cancer, to date, few strategies has been tested in clinical trials and none of them have been approved yet for clinical use [10]. Therefore, there is an urgent need to better clarify the interactions between nanomaterial properties and immune cells for a better design of the nanosystems. Similarly, we need to optimize nanoformulations and delivery systems that ensure low toxicities, high specificity, bio-availability and long-lasting efficacy. To achieve this purpose, it would be important to develop new methodologies that can minimize the variables in the production process and avoid NP- heterogeneity of size and shape, aggregation, endotoxin contamination or the presence of non-encapsulated drugs. In addition, the co-targeting by the use of NPs could be another possibility to strongly increase the efficacy of anti-cancer therapies. For instance, an NP-based delivery system loaded with an immunotherapeutic agent together with chemotherapy could overcome the problem of the acquired resistance to one of the two drugs. Similarly, NP loading with both immunotherapy and a drug that targets a TME stromal component could destroy the interactions between cancer cells and the surrounding microenvironment that have been shown to play important role in cancer development. Although it is really difficult to define the ideal NP for cancer immunotherapy, playing with NP features can allow us to find certain NP characteristics that must be successfully incorporated such that the encapsulated drug shows acceptable biocompatibility, targeting capacity and efficient activity in reducing tumour growth. As mentioned above, several types of NPs exist on the base of the material used for their formulation. Among them, certainly liposomes can be considered an ideal drug delivery system as they are composed of naturally occurring phospholipids, are biologically unreactive and indistinguishably immunogenic, thus possessing a low intrinsic toxicity. They are normally used for the transport of drugs because of their unique properties. Indeed, liposome composition strictly mimics the cell membranes allowing them to be easily internalized by cells through endocytosis or plasma membrane fusion. Moreover, a liposome possesses an aqueous core to encapsulate hydrophilic solutes that could not directly cross the cell membranes and a lipid bilayer to entrap hydrophobic substances, so that liposomes can carry both hydrophilic and hydrophobic drugs [142,143]. These features allow liposomes to be considered a valid solution for loading several drugs and thus for achieving the co-targeting of different components of the TME. For instance, the efficacy of Doxil (the liposome-encapsulated form of Doxorubicin) already approved for some kind of cancers [142-144], could be enhanced by the addition on the lipid bilayer surface of ICIs such as anti-PD-L1 or anti-CTLA/4 Abs (Figure 3). Furthermore, the presence of a dye such as Rhodamine B or Fluorescein embedded in the aqueous core or in the lipid bilayer could allow the in vivo tracking of liposomes for a better understanding of liposome/drug distributions in tissues and organs. In addition, liposomes could be modified in order to contain a polymeric matrix such as PLGA to better entrap the drug and maintain liposome shape. Moreover, exploiting PLGA immunogenicity we could induce DC maturation and therefore enhance immune responses [10,131]. On the other hand, the composition of our ideal NP strongly depends on several aspects such as the route of administration, the target and the loaded drug. If the aim is to prolong NP circulation time in blood, a possible strategy could be the addition of PEG on the surface of the NP: in fact, due to its immune-camouflage properties it escapes from the clearance by immune cells [130,212]. Importantly, hybrid lipid NPs made of phospholipids, PLGA and PEG on the outer layer, have shown targeting capacity and a selective uptake by pancreatic cancer cells when coupled with an anti-CEA half-antibody compared to control NPs. [213,214]. Other important features to consider for our ideal NP are its size and charge: in fact, spherical NP diameter should be no more than 150 nm as bigger NPs activate the complement system and are easily removed from blood stream, accumulating in the spleen and the liver [214]. Defining NP ideal charge is quite difficult as this strongly depends on their route of administration: i.e. a positive charge is important to increase the attraction to the cell membrane that is negatively charged, however, cationic liposomes cannot be used for intravenous injection as they tend to aggregate and stick to each other. The ideal strategy is trying to find the best lipid formulation and ratio in order to obtain a NP able to interact with cells without generating sticky aggregates that are extremely dangerous, especially in the blood stream.

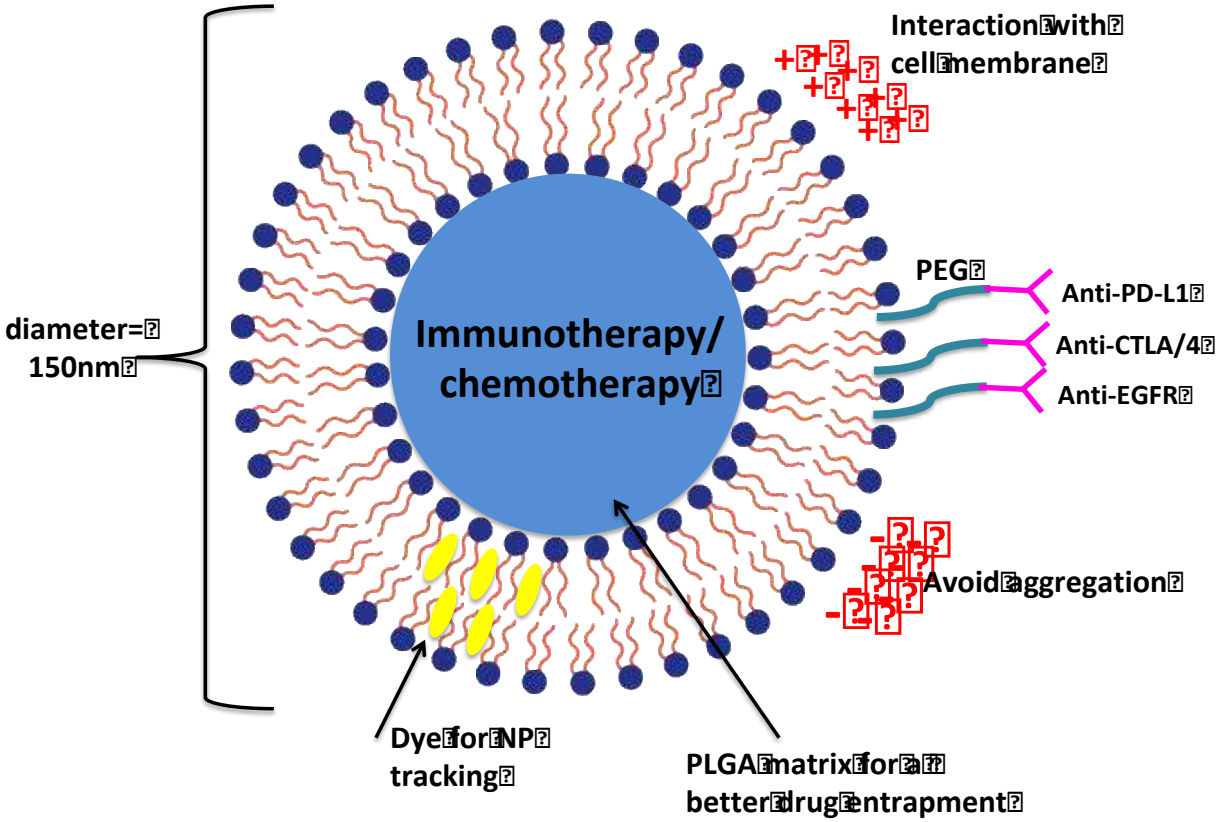

Figure 3. The ideal NP. The important components of a NP for cancer immunotherapy delivery include the choice of nanomaterial (*e.g.*, lipids), targeting molecules/antibodies, the best size for avoiding NP clearance, the net charge for promoting cellular uptake and limit NP aggregation, the incorporation of a dye for NP tracking, the presence of PEG for increasing NP circulation time, the PLGA matrix for drugs incorporation.

This "perfect" drug delivery system we have described gives only a general idea of the features that the ideal NP should have for the delivery of immunotherapeutic agents; however, as mentioned above, there is no "perfect" NP as its characteristics strongly depend on its application, route of administration, targeting tissue and the loaded drugs. Thus, only if we consider all these aspects we will be able to find the best NP features for a specific treatment and NP clinical usage could finally become a reality.

Abbreviations

Ab, antibody

Ag, antigen

AMF, alternating magnetic field

AML, acute myeloid leukaemia

APC, antigen presenting cell

Ara-C, arabinoside

Au, gold

CAF, cancer-associated fibroblast

CARs, chimeric antigen receptors

CEA, carcinoembryonic antigen

CIN, cervical intraepithelial neoplasms

CLL, chronic lymphocytic leukaemia

CML, chronic myelogenous leukaemia

CNT, carbon nanotube

CpG-ODN, CpG oligonucleotide

CPMV, cowpea mosaic virus

CTL, cytotoxic T lymphocytes

CTLA-4, cytotoxic T-lymphocyte-associated protein 4

DC, dendritic cells

DC-CIK, dendritic cell-cytokine induced killer cell

DNR, daunorubicin

ECM, extracellular matrix

EGFR, epidermal growth factor receptor

EPR, enhanced permeability and retention

FDA, food and drug administration

FL, follicular lymphoma

FMS, functionalized mesoporous silica

GM-CSF, granulocyte-macrophage colony-stimulating factor

HCC, hepatocellular carcinoma.

HL, hodgkin lymphoma

HLC, hairy cell lymphoma

HSV, herpes simplex virus

ICI, Immune checkpoint inhibitor

IDO, indoleamine 2,3 dioxygenase

IFNα, interferon alpha

IFNγ, interferon gamma

IL, interleukin

IRAEs, immune-related adverse events

LPXs, lipoplexes

MDSC, myeloid-derived suppressor cells

MM, metastatic melanoma

MMP, metalloproteinase

MRI, magnetic resonance imaging

MTB, magnetotactic bacteria

MUC, metastatic urothelial carcinoma

NK, natural killer

NO, nitric oxide

NOS, nitric oxide synthase

NP, nanoparticle

NSCLC, non-Small Cell Lung Carcinoma

OV, oncolytic virus

OVA, ovalbumin

PAMP, pathogen-associated molecular pattern

PapMV, papaya mosaic virus

PBMCs, peripheral blood mononuclear cells

PD-1, programmed death 1 receptor

PD-L1/2, programmed death ligand 1 and 2

PEG, polyethylene glycol

PEI, polyethylenimine

PLGA, poly(lactic-co-glycolicacid)

PSA, prostate-specific antigen

PSMA, prostate-specific membrane antigen

Rb, retinoblastoma

RCC, renal cell carcinoma

RV, reovirus

SCCHN, squamous cell carcinoma of head and neck

SiRNA, small interfering RNA

SPIO, superparamagnetic iron oxide particles

STING, stimulator of interferon genes

TAM, tumour associated macrophages

TARP, T-cell receptor gamma-chain alternate reading frame protein

TCV, tumour- cell vaccine

TGFβ, transforming growth factor β

Th, T helper

TILs, tumour-infiltrating lymphocytes

TME, tumour microenvironment

TNF, tumour necrosis factor

Tregs, T regulatory cells

TRP2, tyrosinase-related protein 2

TT, tetanus toxoid

VAC, Vaccinia virus

VEGF, vascular endothelial growth factor

VLP, virus-like particle

Conflict of interest

The authors declare no conflict of interests.

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