Combinatorial prospects of nanoparticle mediated immunotherapy of cancer

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COMBINATORIAL PROSPECTS OF NANO-TARGETED CHEMOIMMUNOTHERAPY

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CHAPTER 2

Abstract

Despite the significant increase in our knowledge on cancer initiation and progression, and the development of novel cancer treatments, overall patient survival rates have thus far only marginally improved. However, it can be expected that lasting tumor control will be attainable for an increasing number of cancer patients in the foreseeable future, which is likely to be achieved by combining cancer chemotherapy with anticancer immunotherapy. A plethora of new cancer chemotherapy reagents are expected to become accessible to the clinic in the coming years which can then be used for efficient tumor debulking and aid in antigen exposure to the immune system. Durable remission and the eradication of micrometastases are likely to be achieved with specialized monoclonal antibodies and therapeutic cancer vaccines that modulate the immune system to overcome immunosuppression and kill distant cancer cells. Moreover, the method of drug delivery to tumors, stromal and immune cells is expected to shift largely from conventional 'free' drug molecules to encapsulated in targeted nano-vehicles, therapeutics often referred to or considered part of "nanomedicine". Several biocompatible nano-vehicles, such as metal-nanoparticles, biodegradable-nanoparticles, liposomes or dendrimers are potential candidates for targeted drug delivery but may also serve additional purposes. A dexterous combination of nanomedicine, cancer immunotherapy and chemotherapeutic engineering are likely to become the basis for new hope in the form of targeted cancer therapies that could attack tumors early in their development. One can envision nano-vehicles that would selectively deliver effective doses of chemotherapeutic agents to cancer cells while leaving healthy cells untouched. Furthermore, given that after chemotherapeutic treatment there often remains a limited number of

chemo-resistant tumor cells, which go on to drive tumor progression, nano-vehicles could also be engineered to provoke an appropriate immune response to destroy these cells. Here, we discuss the potential of the combinatorial role of cancer chemotherapy, cancer immunotherapy and the prospective of nanotechnology for the targeted delivery of chemoimmunotherapeutic agents.

1. INTRODUCTION

Cancer chemotherapy regimens, together with surgery and radiotherapy, are currently the main means of tumor mass debulking. Unfortunately these methods of intervention are often insufficient to cure cancer patients and relapse commonly follows due to clinically undetectable micrometastases. It is tempting to speculate that a combination of cancer chemotherapy, to deplete tumor cells, combined with immunotherapy, to prevent relapses, could increase patients' outcome. In fact, some types of chemotherapies reduce the number of regulatory, immunosuppressive, T cells (Tregs) in the tumor, allowing a more immune-favorable environment to form, thereby clearing a path for an effector and memory T cell response to act in concert to destroy cancer cells.[1] There is evidence that the phenotype and function of the immune infiltrates in tumors markedly affect prognosis of the most common cancer types and patient's outcome may be predicted following cancer chemotherapy by the characteristics of the anti-cancer specific immune responses.[2] Furthermore, considering the advantages and disadvantages of existing cancer therapies, a new approach in which cancer chemotherapy and immunotherapy are rationally combined is conceivably quite more effective than either modality alone. However, drug combinations are also likely to increase treatment costs and induce systemic toxicity, an issue that will need to be carefully evaluated during pre-clinical research and clinical trials.

Although a high dose of cytotoxic chemotherapeutics is immunosuppressive, and may lead to lymphopenia, properly dosed and scheduled chemotherapy can rather facilitate, and not inhibit, an immune response against cancer cells.[3] In more recent years it has become apparent that a few specific chemotherapeutic drugs have an attribute, in addition to conventional killing of tumor cells, that is to induce a distinct –immunogenic– form of cell death or by directly having an activating effect on immune cells when provided at low doses.[4,5] Therefore, low doses of immunogenic chemotherapy may synergize with other forms of immunotherapy.

In the emerging field of nanomedicine, nano-sized tools are deployed that generally aim to improve pharmacological therapies, as well as to introduce novel modalities in disease prevention, diagnosis and treatment.[6] Moreover, nanomedicine technology may increase the efficacy, and rationally integrate distinct modalities into one potent anti-cancer treatment. A major segment in this field is the assisted delivery of drugs, commonly with the purpose to decrease bio-distribution of a drug, thereby reducing off-target side effects, whilst increasing drug exposure to target cells only. There is also a significant segment that makes use of inherent physicochemical properties of nanomaterials themselves to achieve desired biological or chemical effects. For instance, photodynamic and photothermal therapy, and nano-agents used for molecular imaging.

In this review, we will describe the immunological state of the tumor microenvironment to illustrate the complex challenges that researchers are confronted with, and how nanotechnology is currently being adopted to improve contemporary and upcoming therapies. Next, we will describe and summarize the immunogenic properties of some commonly used chemotherapies and discuss how current approaches harness, and highlight the future potential, of rationally combined immunotherapy and chemotherapy using nanotechnology.

2. NANOMEDICINE

Recent developments in the field of nanomedicine have highlighted major advantages of nano-vehicles (NVs) in anti-cancer drug delivery with the aim to reduce systemic wide chemotherapy distribution and reducing adverse effects whilst increasing treatment efficacy.[7] These vehicles, with sizes ranging from the nano to the micro scale, are versatile and highly adaptable. A manifold of NV types are currently in research, such as NVs that react to a magnetic field, certain pH levels or temperatures, or convert light to heat and radical oxygen species. A distinct class of NVs is used for transport and delivery of therapeutic compounds of which several types are currently being developed, such as dendrimers, metallic nanoparticles, liposomes (LPs) and nanoparticles (NPs). From these, both LPs and NPs are of particular interest, as they have been proven to be biocompatible, to efficiently transport and deliver antigens to antigen presenting cells (APCs), but also to protect the antigens from degradation and to gradually release the antigens, thereby prolonging half-life. It has been demonstrated that LPs are suitable carriers

of antigens for efficient delivery to APCs for a variety of pathogens.[8] Among its many advantages, LPs are absent of toxicity, low immunogenic, do not induce hypersensitivity or form granuloma at the site of administration, are simple to make and are inexpensive. LPs that are taken-up via endocytosis by APCs, such as immature dendritic cells (DCs), result in a highly concentrated amount of intracellular (cytoplasmic) antigen, which favor cross-presentation via major histocompatibility complex (MHC; HLA region in humans) class I, pivotal to mount an effector T cell response.[9,10]

Unlike LPs, the advantages of NPs, such as the poly(lactic-co-glycolic acid; PLGA) particles, are the excellent stability benefiting long-term storage, and the exceptional biodegradability and biocompatibility. The catabolic remnants of the PLGA particle in the body are lactic and glycolic acid, both natural and non-toxic metabolites and PLGA particles have been used for decades in various therapeutic applications in the clinic. PLGA-NPs are FDA approved and like LPs its physicochemical properties can be manipulated for controlled time- and location-specific release of drugs. Particularly the size and type of coating determine the blood circulation time with particle size being the main determining factor. Particles < 20 to 30 nm in size are eliminated by renal excretion while particles > 300 nm are removed by opsonization (surface modulation) and are scavenged by circulating phagocytes and macrophages or are filtered by the liver and spleen.[11,12] The NP optimum circulation time size range is 70-300 nm and may be further enhanced with a surface polyethylene glycol (PEG) coating. PEGylation of NPs is reported to extend half-life, reduce immunogenicity and not to form any additional toxic metabolites.[13,14] Conversely, PEGylation has also been reported to decrease bioavailability, enhance serum protein binding and elicit immune responses.[15] From a chemical perspective, PEGylation provides a highly flexible platform that allows the attachment of chemical residues or useful molecules to target PLGA NPs to specific cells.[16]

2.1. ACTIVE AND PASSIVE TUMOR TARGETING

In the context of anti-cancer drug delivery, NVs can target the tumor in a passive or active manner. Passive targeting is a process of accumulation of NVs in solid tumors that occur due to the enhanced permeation and retention (EPR) effect, which is caused by leaky blood vessels in tumors, originated from unregulated secretion of angiogenic factors, and decreased lymphatic drainage.[17] The aberrant vasculature

decreases the efficient exchange of molecules into the bloodstream thereby allowing the accumulation and retention of NVs. The retention time is long enough to facilitate the NV uptake by cancer cells via pinocytosis or to be exploited by the NVs that use the retention time for self-disintegration and the release of its contents in the tumor cell and its surroundings.[18] In case of absence of the EPR effect, NV extravasation into the tumor bed is unlikely and therefore access to cancer cells is challenging, although some strategies may be employed to circumvent such obstacle.[19,20]

Interestingly, although the EPR effect does not always exist or found to be pronounced enough in cancer patients, in some cases it is possible to induce or augment the EPR effect, e.g. increase systolic blood pressure via slow angiotensin II infusion or the administration of topical nitroglycerin that is converted to nitric oxide in the tumor microenvironment.[21,22]

Active or targeted delivery may enhance drug delivery by covalent coupling of ligands on the NP surface (e.g. PEG residues) that increase the affinity of NVs to specific cells and may enhance retention and specific uptake.[23] Notwithstanding, the EPR effect is still indispensable to expose the target cells to the targeted NVs in the first place. Examples of targeting moieties that could be used are specific ligands or monoclonal antibodies targeting receptors, integrins and selectins found overexpressed in cancer cells. These targeting moieties are best directed to specific or overexpressed receptors with endocytic capability, such as the folate receptor or the gonadotropin-releasing hormone receptor, which are often found overexpressed in tumors.[24–26] A graphical overview depicting the main differences between passive and active tumor targeting is given in **Figure 1**.

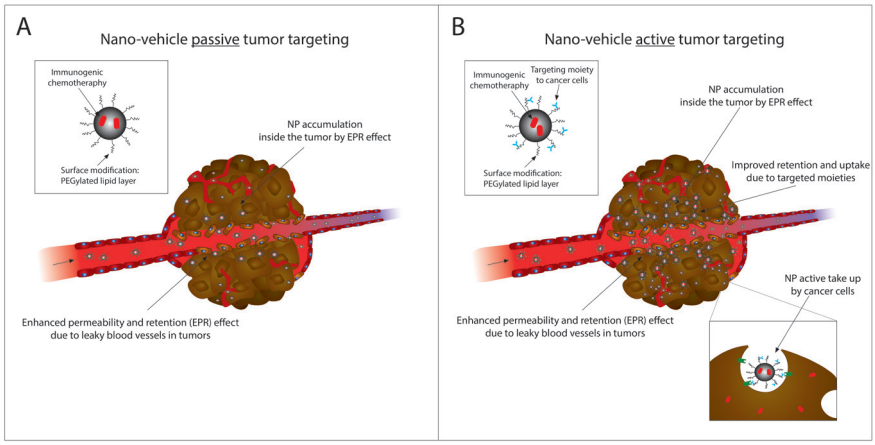


Figure 1. An overview depicting the main differences between passive and active tumor targeting using nano-vehicles. A) Nano-vehicles without targeting moieties accumulate in tumors exhibiting the EPR effect. **B)** Nano-vehicles with targeting moieties also accumulate in tumors exhibiting the EPR effect; however, the targeting moieties on the nano-vehicles enable more efficient retention and uptake of the nano-vehicles by cancer cells.
Abbreviations: EPR: enhanced permeation and retention.

To illustrate that active targeting may indeed enhance target cell specific delivery under certain circumstances, Kirpotin et al. [27] coupled monoclonal antibodies against HER2 on LPs. Although both targeted and non-targeted LPs accumulated in the tumor equally well, the targeted LPs were found to be 6 fold more concentrated inside cancer cells while the non-targeted LPs were found mostly concentrated in the stroma and inside macrophages.

3. THE TUMOR IMMUNOSUPPRESSIVE MICROENVIRONMENT

Evading immune destruction by eluding immunogenicity or exhausting the extent of immunological killing is a recognized hallmark of cancer and several methods have been proposed that explain, at least in part, how some cancerous tumors can survive in an immunocompetent system.28 A proposed hypothesis is that an immune response against cancer cells may actually have taken place before the tumor was clinically detectable and that the highly immunogenic cancer cell clones

were cleared while the weak immunogenic variants remained, a process known as immunoediting.[29] Another instance, or concurrent with immunoediting, is that the action of CD8+ cytotoxic T lymphocytes (CTLs) and natural killer cells is impaired by tumor- or tumor-stromal cells due to increased expression of negative co-stimulatory molecules, such as programmed cell death 1 receptor ligand 1 (PD-L1) or 2 (PD-L2) and the presence of high concentrations of immune inhibitory cytokines, such as the transforming growth factor beta (TGFβ) and IL10.[30–32] In addition, distinct cells with immunosuppressive traits are also often found at the tumor site, such as Tregs, suppressor macrophages and M2-like type of macrophages, and deplete myeloid-derived suppressor cells (MDSCs).[33,34] Tregs are known to significantly contribute to an immunosuppressed microenvironment by secreting high amounts of TGFβ and IL10 that inhibit CTLs and APCs anti-tumor function.[35] High expression of ectonucleotidases by Tregs also reduces the amount of extracellular ATP, secreted by dying cancer cells, thereby reducing immunogenicity and pro-inflammatory millieu.[36] In addition, Tregs were also found to exert immunosuppression by secreting exosome vesicles targeted to specific T helper and effector cells enriched in miRNAs with pro-apoptotic or anti-proliferative functions.[37]

On the other hand, suppressor macrophages in the tumor bed impede immune function through the induction of oxidative stress and secretion of immune suppressive cytokines. Oxidative stress that is induced by the secretion of reactive nitrogen and reactive oxygen intermediates, mainly disrupts the T cell receptor-CD3 complex, by interfering with the CD3 ζ-chain peptide expression, and disrupts the co-stimulatory CD3/CD28 interaction required for T cell activation and survival.[38,39] The complement of cytokines secreted by suppressor macrophages includes IL10, IL6 and tumor necrosis factor alpha (TNFα).[40] Although TNFα is a potent pro-apoptotic cytokine, cancer cells are able to subvert TNFα's effect by inducing the NF-κB-pathway. Based on the staging of tumorigenesis, some NF-κB pathway components may advocate a tumor promoter, instead of tumor suppressor, role of NF-κB pathway activation.[41] This effect is mainly achieved by subversion of apoptosis and enhancement of the production of immune suppressive cytokines, such as TGFβ, IL10, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor and vascular endothelial growth factor, effectively suppressing the innate and adaptive immunity against the early stages of tumor development.

The M2-like type of macrophages, also known as alternatively activated macrophages, is another class of macrophage differentiation often found in tumors. This class of macrophages is mostly involved in mediating tissue repair with immunosuppressive traits that produce several anti-inflammatory cytokines and modulators, including IL10, TGFβ, IL1 receptor antagonist (IL1ra), IL2rα and arginase I.[40,42]

MDSCs are composed of a heterogeneous population of suppressive or immature dendritic cells, granulocytes, and early myeloid progenitors. They are able to efficiently impede an effector T cell response against cancer cells by expressing arginase I and inducible nitric oxide synthase.[43] As arginine is a pivotal amino acid for T cells, its deficiency induces severe dysfunctional effects including impeded cell division, T cell receptor complex and ζ-chain peptide expression, as well as memory formation.[44] Additional T cell suppression is achieved through nitric oxide production by nitric oxide synthase which destabilizes IL2 mRNA and blocks the phosphorylation of Janus kinase 1 and 3, AKT, ERK, and STAT5, which are located downstream of IL2 and are regulators of T cell proliferation.[45] There is also accumulating evidence that MDSCs can mediate the recruitment and expansion of tumor-specific Tregs and actively contribute towards M2 type macrophage differentiation.[46–48]

In addition to viable cancer cells, apoptotic cancer cells also contribute to maintain an immunosuppressive microenvironment. As Sekar et al. [49] reported, priming DCs with apoptotic cancer cells prevented DCs from establishing cytotoxicity, as apoptotic cancer cells released sphingosine-1-phosphate. Sphingosine-1-phosphate induced DCs to produce IL27, which favors Treg cells thereby further contributing to tumor establishment.

Recent insights into the process on how tumors acquire an immunosuppressive environment reinforce the hypothesis that an anti-tumor effector response, such as of the CD8+ T cell response, takes place but is possibly abrogated prematurely due to a negative feedback response.[50] Despite that the precise aetiology remains unknown, the overall effect is an impaired immune system that is incapable to effectively halt cancer progression.

4. CANCER IMMUNOTHERAPY

A key strategy in tumor immunology is to simultaneously disrupt the tumor immunosuppressed microenvironment, elicit a robust effector T cell response against several tumor epitopes and induce a sustainable immunological memory against a broad repertoire of cancer epitopes. In some cases, merely mounting or re(activating) a robust effector T cell response with specific immune adjuvants may provide enough momentum to overcome the tumor immunosuppressed microenvironment. Although tumor specific T cell immunity is often found in cancer patients, it is generally silenced, suppressed or tolerized and current efforts focus on (re)activating these T cells either by nonspecific or specific means.[51–53] Nonspecific (re)activation can be induced with check point blockers derived from humanized monoclonal antibodies such as nivolumab or ipilimumab. Nivolumab blocks the ligand activation of the PD-1 receptor on activated T cells, which is highly expressed by tumor cells. Ipilimumab binds to the cytotoxic T lymphocyte antigen 4 receptor thereby interrupting its tolerizing function. Both modalities are able to reduce the negative regulation of the immunological system in a nonspecific manner, thereby possibly inducing undesired auto-immune reactions. The non-antigen specific immune modulation of the tumor microenvironment with targeted NVs also appear to hold great potential. As reported by Kwong et al. [54] that deployed local LP-anchored anti-CD137 and IL2 that induced local and systemic antitumor immunity and cured established melanoma tumors in mice, while avoiding systemic toxicity induced by potent pro-inflammatory cytokines.

Alternatively, the inherent or adapted physicochemical properties of nanomaterials themselves may be harnessed to elicit non-antigen specific immune responses against cancers. For instance, photo-thermal tumor ablation using near infrared-absorbing nanoparticles was applied to successfully eradicate established colon tumors in mice.[55] Zhou et al. [56] reported the successful tumor eradication and long-term survival in mice by using an immunologically modified single-walled carbon nanotube system that killed cancer cells when the tumors were locally irradiated by a laser. This approach also induced potent anti-cancer immune responses triggered by the release of antigen and danger signals from the dying cancer cells. On the other hand, specific (re)activation also aims to break T cell clone tolerization but to specific antigens only, preferably ones that are unique or

highly expressed by cancer cells. This specific task can be achieved with several specialized immunotherapies, such as dendritic cell vaccination or therapeutic cancer vaccines (TCV).

Early TCV clinical trials were the treatment consisted of free not successful in eradicating cancer, however, current versions have been improved and a much higher rate of therapeutic success is expected in the near future. In addition to induce a robust immunological anti-tumor attack, TCV strategies must often specifically address the cancer mechanisms of immune defense and evasion. TCVs promise to be an elegant solution for tumor control and considerable advancements have been achieved in the last decade with the discovery of specific tumor antigens and tumor associated antigens. In addition, more detailed understanding of mechanisms of immunological evasion, tumor immunological recognition and destruction are contributing to better insights on how to improve TCVs. Some tumor antigens and several tumor associated antigens have been identified, which can be classified mainly into five categories: viral antigens that are associated with cancer development, mutated antigens or neo-antigens originated by chromosomal aberrations, differentiation antigens, cancer-testis or cancer germline antigens and overexpressed antigens (which can induce danger signals, but are prone to autoimmune diseases). Tumor antigens can stimulate cellular and/or humoral immune responses in cancer patients and the epitopes contained in tumor (associated) antigens are presented at the surface of cancer cells in the MHC class I molecules to cognate CD8+ T cells.[57] Some tumor antigens also contain epitopes for the MHC class II molecules on APCs and sometimes cancer cells, which can be recognized by cognate CD4+ T cells.[58,59]

The rationale behind TCVs is to onset a potent CD8+ effector CTL and a T helper type 1 (Th1) immune response against tumor antigens. The Th1 response is very effective in the activation of CTLs, memory formation and the production of associated cytokines such as IL1**β**, interferon gamma and TNF**α**. A Th1 response can be skewed by IL12 production by APCs. The induction of a T helper type 2 (Th2) immune response is less efficient because it mainly activates the humoral immunity by targeting B cells that produce non-cytolytic antibodies and IL4.[60] In addition to inducing a strong Th1 response, an effective TCV must also be able to induce a functional CD8+ central and effector memory subtypes in order to achieve durable and persistent tumor control.[61,62]

Some predicted challenges for tumor vaccines are the limited epitopes known and to properly modulate the immune system such to mount a robust enough effector response able to counteract the tumor immunosuppressed microenvironment. Furthermore, most self-derived neo-antigens generated by mutations or translocations linked to tumor development are likely poorly immunogenic and because of the use of predetermined antigens in tumor vaccines, immunoediting may take place rather than full tumor clearance. Albeit, new target epitopes are expected to be exposed after the initial tumor attack, which may allow the generation of new effector responses against these epitopes to be mounted, thereby maintaining the anti-tumor response momentum against a broader range of epitopes.

When tumors have become clinically detectable, they have, almost by definition, already mounted mechanisms to evade immune responses. This must be taken into consideration when designing an effective and durable anti-tumor immune strategy. Another foreseeable challenge is the availability of antigen specific reactive T cells. Thymic education has left only low-avidity and functionally suboptimal T cells specific for self-antigens or tumor antigens, a challenge that will be difficult to solve and is expected to play a role in cancer patients that are non-responsive to immunotherapy.

For further insight in TCVs, please refer to the thorough review of Melero et al. [63] that also include an overview of current TCV clinical trials.

NVs have also been pushed forward as ideal candidates to improve TCV by augmenting the quantity and quality of antigen-specific CTL responses against tumors. Specifically the ability for targeted and simultaneous delivery of antigen and immune stimulators render NVs an attractive method to improve TCVs.
As most antigen in the form of protein or peptides are non-immunogenic, most current formulations should include highly immunogenic adjuvants either soluble or encapsulated, such as ligands of the Toll-like receptors (TLR).[5,64,65] TLRs are part of a broad family of pattern-recognition receptors which recognize pathogens or damage-associated molecular patterns. Upon activation, an innate and adaptive response can be initiated. The specific aimed activation of TLRs in DCs will activate the NF-κB pathway, thereby inducing the production of IL12 and increase the expression of co-stimulatory receptors such as CD40. CD40 interacts with CD40L on T cells and CD80/86 that on their turn interact with CD28 or cytotoxic T lymphocyte

antigen 4 (its inhibitory counterpart) on T cells, amongst others.[66–68] Properly activating the TLR pathway is a potent and effective method to mature and activate DCs such to be able to reverse anergic T cell clones as found in advanced cancer patients.[69,70] Moreover, some TLR agonists were able to differentiate M2 type macrophages to an M1 phenotype and Tregs to (temporarily) cease the production of immune suppressive cytokines.[69,71] When screening for suitable TLR agonists, the target DC subtype is also relevant as several different DC subtypes have been identified that express different TLRs. Some TLRs are common to all DC subtypes while others are more specific, i.e. LC/dermal and CD141+ DCs express TLR3 but the same receptor will be less expressed in the CD1c+ DCs and monocyte-derived DC subsets whereas plasmacytoid DCs are described to express higher amounts of TLR7 and TLR9.[72] Some TLR agonists, such as the TLR3 ligand poly(I:C) and the TLR9 ligand CpG, are known to be able to convert the immunosuppressed tumor microenvironment from chronic to the intended acute inflammation thereby reducing the amount of Tregs present in the tumor.[73]

It has become evident that certain immune activating elements should be included in new strategies, although there is also reason to warrant caution. In addition to tumor hormesis for anti-cancer drugs and immunotherapy [74–76], cancer cells are commonly found to escape immune attack by altering and rewiring the activated NF-κB pathway to their advantage by increasing resistance against apoptosis and allowing more metastasis to occur regardless of the acute pro-inflammatory milieu.[77,78] Moreover, several different TLRs are in fact highly expressed in many tumors warranting that certain precaution measures should be taken not to use an unfavorable TLR agonist.[79–83] Alternatively, the (co)activation of nucleotide-binding oligomerization domain-like receptors could also induce an effective anti-tumor immune response.[84,85]

Several NVs have been described to able to induce potent antigen-specific CTLs and anti-tumor responses. For instance, PLGA NPs have been reported to be successful transport and delivery agents for antigenic peptides to plasmacytoid DCs.[86] Several receptors have been described as viable targets for efficient delivery to DCs using uptake receptors such as C-type lectin DEC-205, blood DC Ag-2, CD40, CD11c, DC immunoreceptor or the FcR CD32.[87–89] Moreover, the concurrent delivery of TLR-ligands, e.g. R878 and unmethylated CpG oligonucleotides, were found to be potent pDC activators.[90,91] Moreover, a combination of antigen and

immune stimulants loaded into LPs has been shown to effectively induce antigen-specific T cell cytotoxicity and eradicate tumors.92 Varypataki et al. [93] reported that the intradermal administration of cationic LPs, containing antigen and the immune adjuvant Poly (I:C), induced a 25 fold increase of the cognate CD8 T cells in mice as compared to non-encapsulated formulation. In an another study by Hansen et al. [94], cationic LPs were deployed carrying antigen and Poly (I:C) that significantly delayed tumor growth in melanoma and a lung cancer model in mice. Jérôme et al. [95] has shown that the generation of antigen-specific T cells was possible with a 1000 fold lower concentration of antigen when presented in LPs. In addition, the inclusion of the immune stimulant CpG in the LP formulation was shown to be imperative for the protection against low-immunogenic self-peptide presenting tumors in mice. A graphical overview depicting the main methods of TCV (also NV mediated) is given in **Figure 2**.

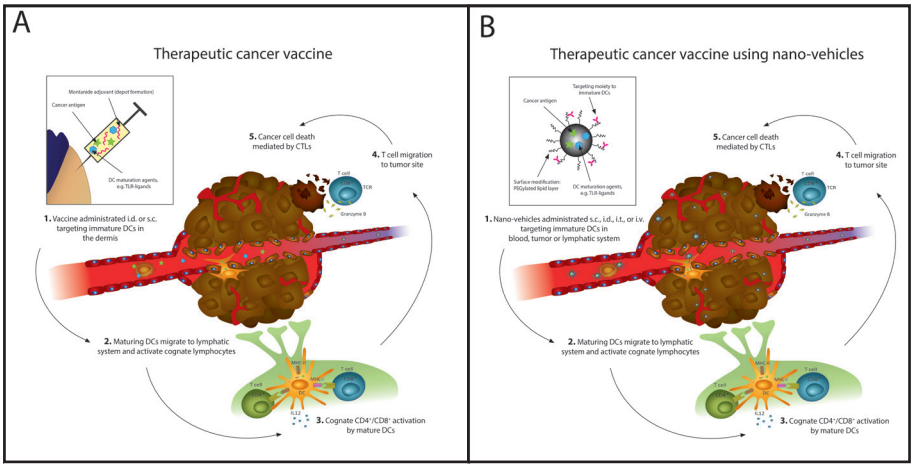


Figure 2. An overview depicting the main steps in therapeutic cancer vaccines. A) A cancer vaccine can be administered via a subcutaneous injection containing antigen and immunostimulants (e.g. TLR-ligands) in a depot forming solution. The resident antigen presenting cells, such as immature DCs, take-up the vaccine contents and migrate to lymphoid organs. Upon arrival at the lymphoid organs, the matured DCs present the antigenic peptides to, and activate, cognate lymphocytes. Specific cytotoxic T cells, such as CD8+ T cells, migrate to the tumor area and eradicate cancer cells bearing the cognate antigen peptide. **B)** Immature dendritic cell targeted nano-vehicles containing antigen and immunostimulants (e.g. TLR-ligands) are administrated either via intravenous, intratumoral, intradermal, subcutaneous or oral (pill) route. The nano-vehicles are taken-up by immature DCs circulating in the blood, the tumor or lymphatic system after which the DCs migrate to lymphoid organs. Similarly to A, upon DC arrival at the lymphoid organs, the matured DCs present the antigenic peptides to, and activate, cognate lymphocytes. Specific cytotoxic T cells, such as CD8+ T cells, migrate to the tumor area and eradicate cancer cells bearing the cognate antigen peptide. Abbreviations: CTLs: cytotoxic T lymphocytes; DCs: dendritic cells; i.d. intradermal; s.c.: subcutaneous; TLR: Toll-like receptor.

5. NANO-TARGETED CHEMOIMMUNOTHERAPY

It was become recently apparent that some chemotherapy types have a positive immunogenic effect on the tumor microenvironment.[4,5] One of these characteristics is the distinct induction of immunogenic cell death. The advantage of inducing immunogenic cell death is that the remains of the cancer cells themselves may serve as a "vaccine" and resemble the type of cell death that occurs in some other therapeutic modalities, such as photo-thermal and photodynamic therapy.[96,97]

Although the whole process of this unique form of cell death is not precisely understood, and is drug specific, some mechanisms have been described that involve the exposure or secretion of specific molecules. One of which is the pre-apoptotic exposure on the cell surface of calreticulin, an endoplasmic reticulum chaperone, or of heat-shock proteins, such as heat-shock protein 70 and 90, that are very potent phagocytosis signals to APCs.[98,99] Calreticulin is recognized by CD91 receptor on DCs while heat-shock proteins enhance cross-priming of tumor antigens to specific T cells.[100–102] Other strong cues leading to phagocytosis by APCs are the autophagy-dependent active secretion and extracellular accumulation of ATP as well as the nuclear non-histone high mobility group box 1 (HMGB1) proteins in the proximity of dying tumor cells.[103–106] ATP and HMGB1 can activate and induce maturation of DCs and stimulate the release of pro-inflammatory cytokines, such as IL1β and IL2.[105,107] Additionally to the presentation and secretion of immunogenic molecules, there are other immunogenic effects that occur in tumor cells. For example, Ramakrishnan et al. [108] recently described that paclitaxel, doxorubicin and cisplatin increased cancer cell sensitization to granzyme B, a serine protease secreted by CTLs cells, by a process that is mediated via upregulation of mannose-6-phosphate receptors on cancer cells. This process did not only take place on the cancer cells expressing the cognate antigen but also surrounding (cancer) cells that did not express the antigen. The authors hypothesized that this finding could be a possible explanation on how a limited amount of CTLs are able to mediate a potent anti-tumor effect when combined with specific types of cancer chemotherapy.
In addition to the direct immunogenic effect on cancer cells, these chemotherapies can also be combined with immune adjuvants to further boost immune responses against cancer cells. For instance, Gou et al. [109] described a potent combination of oxaliplatin with IL7 that inhibited colon cancer metastasis in mice. In another

study, Bagcchi [110] has shown that combining chlorambucil with obinutuzumab, an anti-CD20 antibody, substantially improved the progression-free and overall survival in patients with previously untreated chronic lymphocytic leukaemia. Despite that this type of immune modulation appears very promising, it is yet unclear whether these strategies are efficient and sufficient enough to overcome the tumor immunosuppressed microenvironment, cancer epitope T cell clone anergy or tolerization as often found in advanced cancer patients.2 A graphical overview of the main immunogenic effects by (low dose) chemotherapy is given in **Figure 3**.

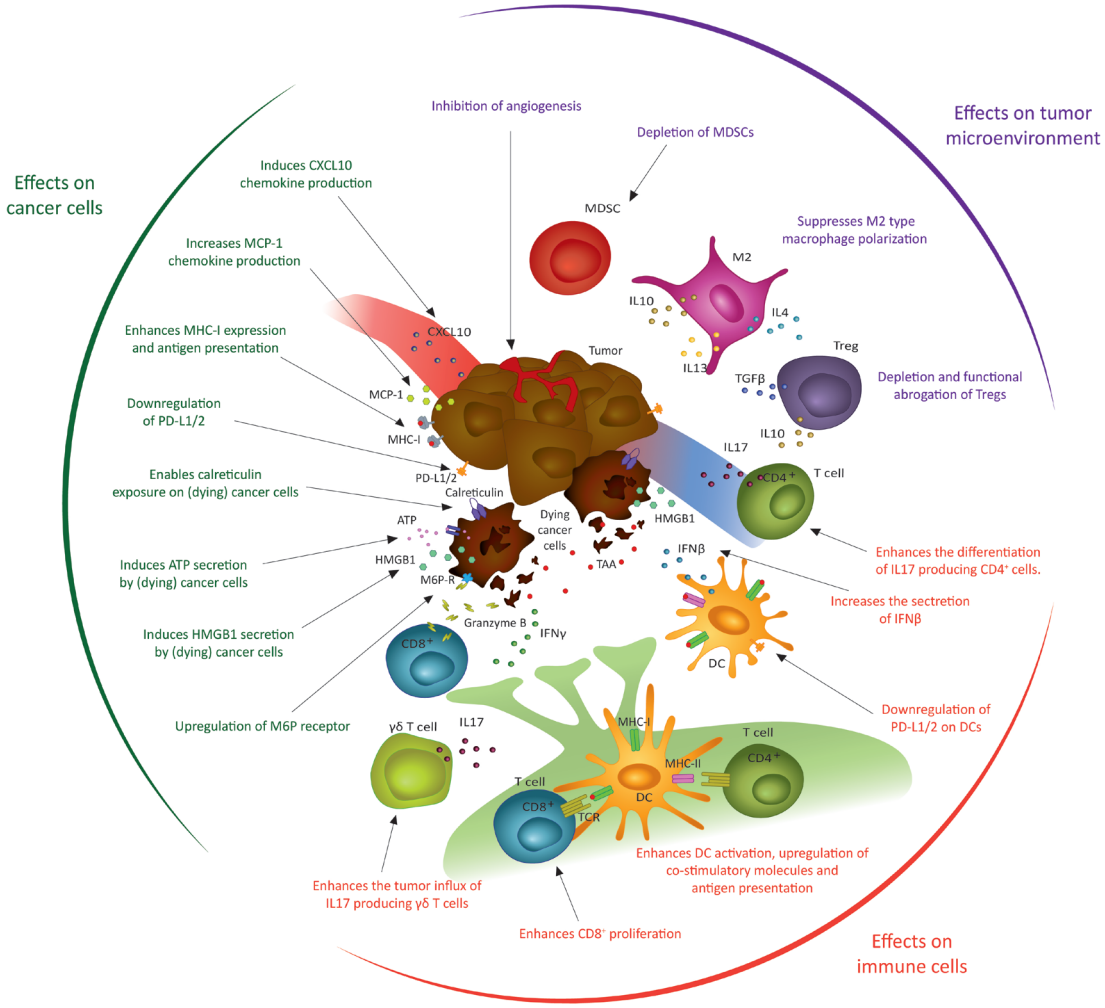


Figure 3. Illustration of the main effects of (low dose) immunogenic chemotherapy directly on cancer cells, the tumor microenvironment and immune cells elsewhere. The individual effects of each immunogenic chemotherapy are given in Table 1.

Abbreviations: HMGB1: nuclear non-histone high mobility group box 1; IFNβ: interferon beta; IFNγ: interferon gamma; M6P: mannose-6-phosphate; MCP-1: macrophage chemoattractant protein-1; MDSCs: myeloid-derived suppressor cells; MHC: major histocompatibility complex; PD-L: programmed death-ligand; Tregs: regulatory T cells.

It is noteworthy to report that some immunogenic chemotherapies have been described to have ambivalent effects, exerting simultaneous positive and negative effects on the tumor. For example, 5-Fluorouracil (5-FU) can reduce the number of immune suppressive populations in the tumor. However, at the same time intracellular inflammasomes are triggered by 5-FU, in the remaining suppressive cells, which may lead to a signaling cascade to advert angiogenesis, regain tumor growth and promote metastasis.111,112 Ambivalent function on anti-tumor immune responses has also been reported for bleomycin that enhances Treg cell proliferation, doxorubicin that upregulates the nuclear expression of CD274 conferring resistance against apoptosis and gemcitabine by a process similar to 5-FU. An overview with references of currently known chemotherapies that may aid the immune response to clear cancer cells is given in Table 1.

Table 1. List of chemotherapies reported to contribute to an immunological anti-tumor response. Table data was partially based on Galluzzi et al. [113] and was extended and updated.

Agent	**Mechanism**	**Refs.**
5-Fluorouracil	- Depletion of myeloid-derived suppressor cells.***	112,114–116
Bleomycin	- Enables calreticulin exposure on cancer cells.**/***	117
Carboplatin	- PD-L1 and PD-L2 downregulation on both human DCs and human tumor cells. - Increases macrophage chemoattractant protein-1 (MCP-1) expression by cancer cells.	118,119
Cisplatin	- PD-L2 downregulation (and PD-L1 to a lesser extent) on both human DCs and human tumor cells. - Sensitizes tumor cells to granzyme B by upregulation of the mannose-6-phosphatase receptors. - Enhances T cell proliferation by stimulating DC antigen presentation and IFNβ production. - Enhances monocyte and natural killer cell mediated cytotoxicity. - Enhances HMGB1 expression on (dying) cancer cells.** - Enhances the recruitment of macrophages and tumor-specific CD8+ T cells.*	108,118,120–125

Cyclophosphamide	- Enhances homeostatic proliferation/activation of lymphocytes and specific tumor infiltration. - Enhances the differentiation of IL17 producing CD4+ cells. - Depletion and functional abrogation of regulatory T cells.* - Depletion of myeloid-derived suppressor cells. - Suppresses M2 type macrophage polarization and associated IL4, IL10 and IL13 production accordingly. - Increases MHC-I expression on tumor cells. - Preferential expansion of CD8α+ DCs.	126–136
Daunorubicin	- Enhances antigen expression by tumor cells.	137
Docetaxel	- Enables calreticulin exposure on cancer cells.** - Depletion of myeloid-derived suppressor cells.	138,139

Doxorubicin	- Enhances antigen presentation by DCs.* - Enhances antigen presentation on cancer cells. - Sensitizes tumor cells to granzyme B by upregulation of the mannose-6-phosphatase receptors. - Enhances the tumor influx of IL17 producing γδ T cells preceding the accumulation of CTLs. - Enhances cancer antigen-specific, IFNγ producing CD8+ T cells in the tumor and stimulates CD8+ proliferation in the tumor draining lymph node. - Enables calreticulin exposure on cancer cells.** - Induces ATP secretion by dying cancer cells, which attracts inflammatory CD11c+CD11b+Ly6Chi cells into the tumor bed.** - Depletion of myeloid-derived suppressor cells. - Enhances DC activation (CD80 upregulation). - Induces a type I interferon response, including CXCL10 chemokine production. - PD-L1 downregulation on cancer cells.***	100,108, 120,140–147
Gemcitabine	- Increase HLA-I expression in tumor cells. - Enhances antigen presentation on cancer cells. - Depletion of myeloid-derived suppressor cells.*** - Depletion of regulatory T cells.	

Methotrexate	- Enhances antigen presentation by DCs.* - Enables ATP secretion by dying cancer cells, which attracts inflammatory CD11c+CD11b+Ly6Chi cells into the tumor bed.** - Enhances DC activation (CD40, CD80 & CD86 upregulation) and T cell proliferation.*	140,143,154
Mitomycin-C	- Enhances antigen presentation by DCs.* - Enhances DC activation (CD80 upregulation) and T cell proliferation.*	140,154
Mitoxantrone	- Enables calreticulin exposure on cancer cells.**	100
Oxaliplatin	- Increase HLA-I expression in tumor cells. - Sensitizes tumor cells to granzyme B by upregulation of the mannose-6-phosphatase receptors. - Enables calreticulin exposure on cancer cells.** - Induces a type I interferon response, including CXCL10 chemokine production.	118,122,128,146

Paclitaxel	- Enhances antigen presentation by DCs.* - Enhances antigen presentation on cancer cells. - Sensitizes tumor cells to granzyme B by upregulation of the mannose-6-phosphatase receptors. - Enhances DC activation (CD40, CD80 & CD86 upregulation).* - Depletion and functional abrogation of regulatory T cells. - Increases macrophage chemoattractant protein-1 (MCP-1) expression by cancer cells. - Depletion of myeloid-derived suppressor cells.* - Prevents the tolerogenic state of DCs and myeloid-derived suppressor cells in the tumor microenvironment.*	108,119,140,144, 154–159
Vinblastine	- Enhances DC activation (CD40, CD80 & CD86 upregulation).*	154
Vincristine	- Enhances DC activation (CD40 & CD86 upregulation).* - Enhances antigen presentation by DCs.*	140,154

* When subjected to low (non-cytotoxic; metronomic) chemotherapy concentrations.

** Immunogenic cancer cell death.

*** Ambivalent function described.

Although very promising, the combined treatment of immunotherapy with low dose immunogenic chemotherapy is not always favorable. For instance, the combination of alkylating chemotherapy and the induction of immune responses against neo-antigens, whereby the influence of Treg depletion is restricted, was found to be deleterious to responder lymphocytes.[160,161] However, this does not appear to be the case for self-antigens.

Moreover, most immunogenic chemotherapies appear to share the ability to deplete MDSCs from the tumor microenvironment. However, as tumor shrinkage also takes place due to cancer cells death, it is not always clear whether the reduction of MDSCs is a consequence of tumor size reduction or actually due to direct MDSCs killing by the immunogenic chemotherapy.

With the currently elucidated advantages of utilizing specific types of chemotherapy, that aid in tumor debulking and facilitate immune responses against cancer cells simultaneously, there may be additional benefit to combine these specific chemotherapies with other active immunotherapies by utilizing nanotechnology. For instance, Roy et al. [162,163] combined chemoimmunotherapy against cancer using PLGA NPs loaded with paclitaxel and the TLR4 agonist sodium salt of phthalate derivative of parent lipopolysaccharide was found more effective than any of the compounds alone. In addition, a higher number of CD4+ and CD8+ T cells, CD11c+, and CD14+ cells infiltrated the tumor and correlated to enhanced survival of mice than either standalone modalities. Another chemoimmunotherapeutic study combined doxorubicin with a carrier plasmid of unmethylated CpG oligonucleotides in an active delivery dendrimer bioconjugate, which yielded smaller tumors compared to any of the components alone.[164]

NVs can be modified with targeting moieties that increases cargo delivery specificity but are not only limited to be applied to standard cancer chemotherapeutic agents and TLR-agonists, they can be further adapted to modulate biological processes, including the immune system, in situ. As described by a study conducted by Calcinotto and colleagues [165], the authors conjugated TNF**α** to NGR, a tumor-homing peptide that recognizes an aminopeptidase N isoform that is selectively expressed by endothelial cells in tumor vessels. This TNF**α**-NGR conjugate combined with doxorubicin prolonged the survival of mice with B16OVA melanoma tumors and significantly increased the infiltration of CD8+ T cells into the tumor.

In a way, this is an elegant approach that directly addresses the finding of Motz et al. [166] that described that Fas-ligand expression by tumor endothelium aids in promoting tolerance in tumors by inducing apoptosis on activated effector T cells arriving at the tumor site.

Multi-step drug delivery of NPs has also recently been described by Sun et al. [167] that designed two distinct diblock copolymer NPs that fuse when in close proximity, such as in an endosome of a cell, but not while circulating in blood. This approach could enable novel applications in controlled release. For instance, one particle could carry an inactive form of a drug while the other NP acts as the activator of the same drug, thereby increasing target cell specificity whilst reducing drug adverse effects even further. Another considerable advantage of NPs is the prospect of drug delivery via the oral route. NPs can be formulated into a tablet or a pill carrying the drug. While the drug is protected from low pH, salts and enzymes from the stomach, the physicochemical parameters can be further adapted such to release the drug only at a specific pH thereby increasing the drug availability at the target site.168 A study performed by Bhardwaj et al. [169] compared the efficacy of orally administrated paclitaxel loaded PLGA NPs against intravenous administrated native paclitaxel and found that the uptake via de oral route was not only feasible but improved the efficacy in chemical-induced breast cancer in rats. Similar experiments were also conducted with cisplatin loaded PLGA NPs, which yielded superior results compared to native intravenous cisplatin.170,171 The prospect of cancer chemotherapy delivery as a "simple" pill, that can be taken orally, has great potential for cutting costs in the oncological health care, as patients will require less hospitalization and no intravenous administration of cancer chemotherapy, which reduces therapy burden. This method of oral administration becomes even more attractive if the application of the metronomic chemotherapy regimen, which entails the daily administration of chemotherapeutic agents at relatively low and minimally toxic doses, will become a future modality of anti-cancer therapy to delay solid tumor outgrowth.172,173

Furthermore, Morton et al. [174] described a process that used NPs for the dynamic rewiring of signaling pathways combined with cancer chemotherapy for enhanced tumor decimation. Not only did the authors combine tyrosine kinase inhibitors, such as erlotinib to rewire the apoptotic pathways, they designed their NPs in a specific

order that allowed a timed release of doxorubicin at the optimum moment when the cells were made most chemotherapy-prone.

Another aspect where NPs may be useful is in combating cancer chemotherapy resistant cancer cells. For instance, breast cancer cells are known to be initially sensitive to doxorubicin but resistance may occur when the cancer cells starts to overexpress the ABCG2 gene coding for the P-glycoprotein efflux transporter.[175] Doxorubicin enclosed in NPs is inherently less affected by efflux transporters compared to soluble doxorubicin while NPs coated with cyclosporin A, a P-glycoprotein inhibitor, were found to reduce the efflux of doxorubicin even further.[176]

Another known mechanism of doxorubicin resistance is the down-regulation of the expression of HuR, a RNA binding protein involved in the post-transcriptional regulation of a large range of mRNAs.[177] It would be compelling to unravel whether the sensitivity to doxorubicin resistant breast cancer cells could be restored, by using NPs that target both P-glycoprotein and HuR simultaneously. This may be possible by cyclosporine A coated NPs caring doxorubicin and Rottlerin, a compound known to restore HuR expression.

Marrache et al. [178] recently proposed an elegant option to overcome cisplatin resistance, by adapting a PLGA NP, carrying cisplatin and guided with a triphenylphosphonium cation, aiming for cisplatin delivery not to the cell nucleus but to mitochondria. As mitochondria lack the nucleotide excision repair mechanism, the cells are not able to repair the mitochondrial DNA damage, favoring cell death. The PLGA NP was found to be 17 times more efficient against neuroblastoma cells compared to cisplatin alone.

There is also a large untapped therapeutic potential by merging cancer immunochemotherapy modalities with NP targeted delivery of shuttle vectors or RNA-guided genome editing complexes, as well as potentially beneficial combinations that include NF-κB pathway inhibitors, such as curcumin, to overcome chemotherapy resistance induced by tumor stromal cells in the tumor microenvironment.[179]

6. FUTURE PROSPECTS

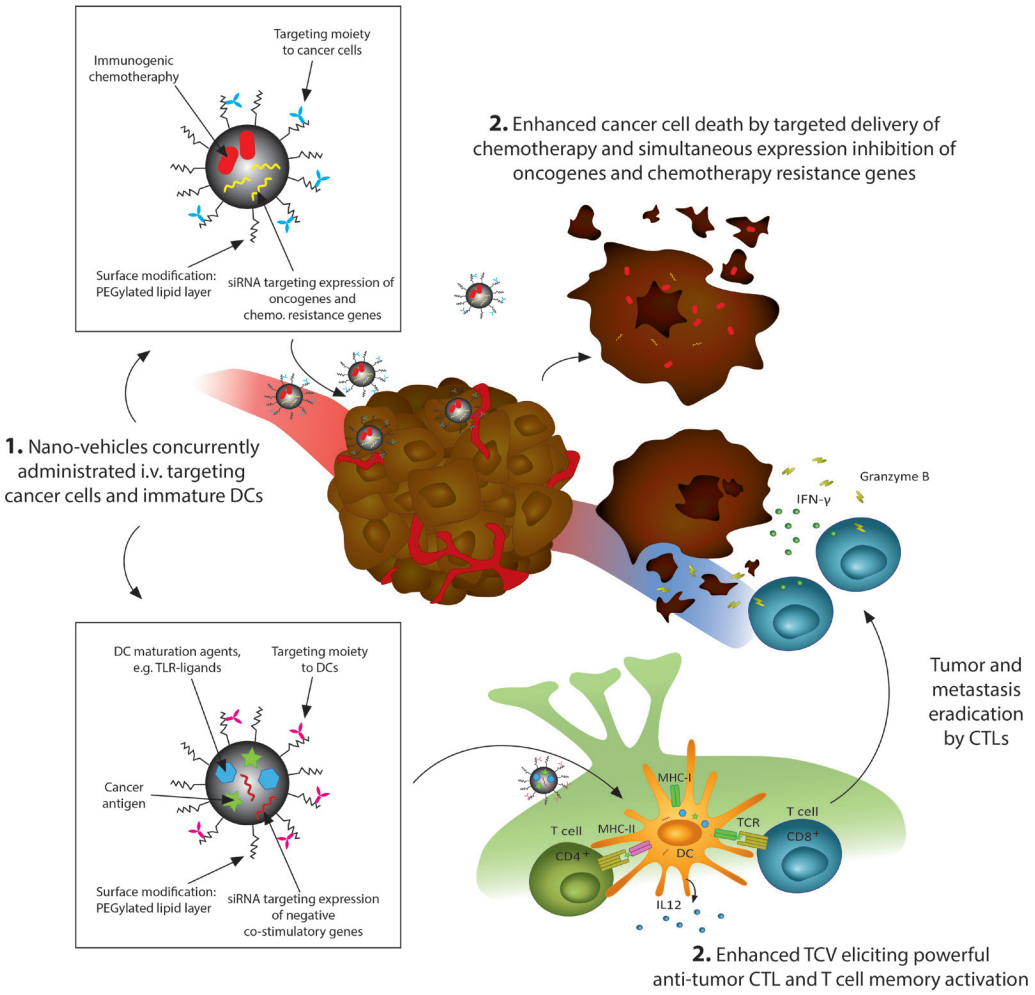
The new generation of NVs holds great promise to become the future backbone of medicine. With outstanding drug protection capabilities from the body secretion and catabolic processes, drugs previously only administrable via intravenous route may become available as NV encapsulated oral pills, potentially reducing health costs and therapy burden. Putative anticancer drugs that previously were discarded due to solubility issues may once again become potential therapeutic modalities. NVs also provide a flexible platform for novel and bold combinations, such as targeted immunogenic chemotherapy combined with local or systemic treatment with check point blockers that may yield synergistic effects and increase therapy efficacy further. Beside the possible reduction of therapy adverse effects by targeted delivery, NVs may aid in dye and contrast agent delivery to enable earlier and more accurate tumor and micrometastases detection. Moreover, NVs comprise of an untapped potential to regulate a plethora of biological processes, even in situ or organ specific, that may well reach beyond oncological therapy to cover an extent of other diseases.

To gain durable tumor control, the paradigm for cancer treatment must change from relatively nonspecific chemotherapy towards an increasingly targeted therapeutic approach. The therapy course is likely to compose targeted nano-vehicles encapsulating immunogenic cytotoxic agents combined with small molecules and immune adjuvants, aiming at vital tumor cell pathways, perturbing mechanisms of chemo resistance and immune evasion. The new generation of (nano-targeted) TCVs is coming of age and may well spark the first necessary step to halt tumor dissemination. New viable targeted modalities are impending candidates for future therapeutics in the treatment of early and advanced cancer disease.

As approximately twelve percent of human tumors are of viral aetiology, predominated by the human papillomavirus and by the hepatitis B/C virus, it would appear viable in the future to design efficient and standardized targeted TCVs against these tumors, that are likely to express unique viral antigens.[180]

Based on extensive immunological research over the last decades, we have learned how to harness, activate and modulate a suppressed immune potential to fight

cancer, enhancing cancer patients' survival and opening the doors for durable and efficient tumor control. Although considerable research is still required, there is a particular need to identify biomarkers that can predict which patients will benefit from chemoimmunotherapy from the patients that lack the necessary immune potential, such as cancer epitope T cell anergy or tolerization. Additionally, it is also currently unknown what the effect of chemoimmunotherapy is in effectively neutralizing the supporting tumor stroma, particularly in late stage cancer patients. A renewed outlook on NVs clinical prospective is likely to emerge as ideal delivery vehicles for gene therapy. In fact, a clinical trial is currently running that targets the mRNA of the M2 subunit of ribonucleotide reductase and another clinical trial that targets vascular endothelial growth factor and kinesin spindle protein, both using NPs as delivery agents.[181,182] It is tempting to speculate whether a combination of targeted NPs, one targeted to the tumor carrying chemotherapy and oncogene silencing by small interfering RNAs, and another targeting immature DCs, carrying antigen, TLR-ligands and small interfering RNAs against negative co-stimulatory mRNA molecules would yield even superior tumor clearance rates. A graphical representation of such a putative modality is given in **Figure 4**.


Targeting moiety to cancer cells
Immunogenic chemotheraphy
Surface modification: PEGylated lipid layer
siRNA targeting expression of oncogenes and chemo. resistance genes
2. Enhanced cancer cell death by targeted delivery of chemotherapy and simultaneous expression inhibition of oncogenes and chemotherapy resistance genes
1. Nano-vehicles concurrently administrated i.v. targeting cancer cells and immature DCs
Granzyme B
IFN-γ
DC maturation agents, e.g. TLR-ligands
Targeting moiety to DCs
Cancer antigen
Surface modification: PEGylated lipid layer
siRNA targeting expression of negative co-stimulatory genes
MHC-I
T cell
MHC-II
TCR
T cell
CD4+
DC
CD8+
IL12
Tumor and metastasis eradication by CTLs
2. Enhanced TCV eliciting powerful anti-tumor CTL and T cell memory activation

< **Figure 4. A putative modality for future treatment of cancer.** First, NPs targeting the overexpressed cancer cell receptors are efficiently taken-up by receptor-mediated endocytosis. The NPs contents are then released to the cytosol where the immunogenic chemotherapy promote the cancer cell death and at the same time the expression of driver oncogenes and genes mediating chemotherapy resistance are inhibited by the release of small interfering RNAs. As tumor growth is hampered, a time window is created for the immune system to mount an effective anti-tumor response and alleviate the immunosuppressive tumor microenvironment. Second, NPs targeting immature DCs are also administrated. The NPs deliver cancer antigens and immunostimulants, which activate DCs that migrate to the lymphatic system where the (matured) DCs present the antigenic peptides to, and activate, cognate lymphocytes. To improve the activation of lymphocytes further, the NPs also deliver small interfering RNAs that inhibit the expression of negative co-stimulatory receptors and cytokines. Specific cytotoxic T cells, such as CD8+ T cells, migrate to the tumor and metastasis areas and eradicate the remaining cancer cells bearing the cognate antigen peptide. Abbreviations: CTLs: cytotoxic T lymphocytes; DCs: dendritic cells; i.v.: intravenous; siRNA: small interfering RNA; TCV: therapeutic cancer vaccine; TLR: Toll-like receptor.

Finally, with the emergence of the ever more accurate RNA-guided genome editing complexes as well as improved targeted delivery agents, in situ gene repair and modulation may be within reach in the coming years as the ultimate treatment of a broad range of diseases. In addition to targeted delivery of therapeutics, targeted particulates can also be combined with highly precise nano-targeted molecular imaging compound to improve diagnostics, earlier-stage detection of disease, as well as real-time particulate tracking and visualization of therapy progression. There are a number of different probes coupled NVs reported to successfully enable molecular imaging, such as fluorocarbons, fluorescent and near-infrared dyes and 19F isotopes, amongst others.[183–186]

7. CONCLUSION

Immunogenic chemotherapy, when provided at low but adequate doses, can efficiently kill cancer cells while additionally engage and stimulate the immune system. Further synergy may be achievable by rationally combining immunogenic chemotherapy with immunotherapy. Moreover, by using nanotechnology for the targeted delivery, the therapeutic effect may be augmented while side-effects are potentially reduced. As NVs have the potential of controlled release and multi-compound encapsulation, the co-delivery of immune adjuvants and small molecules, or combined with check point blockers, antibodies, and cancer vaccines, may possess an untapped potential to favorably incline the immune balance in the tumor allowing the immune system to eradicate tumors and distant metastasis.

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