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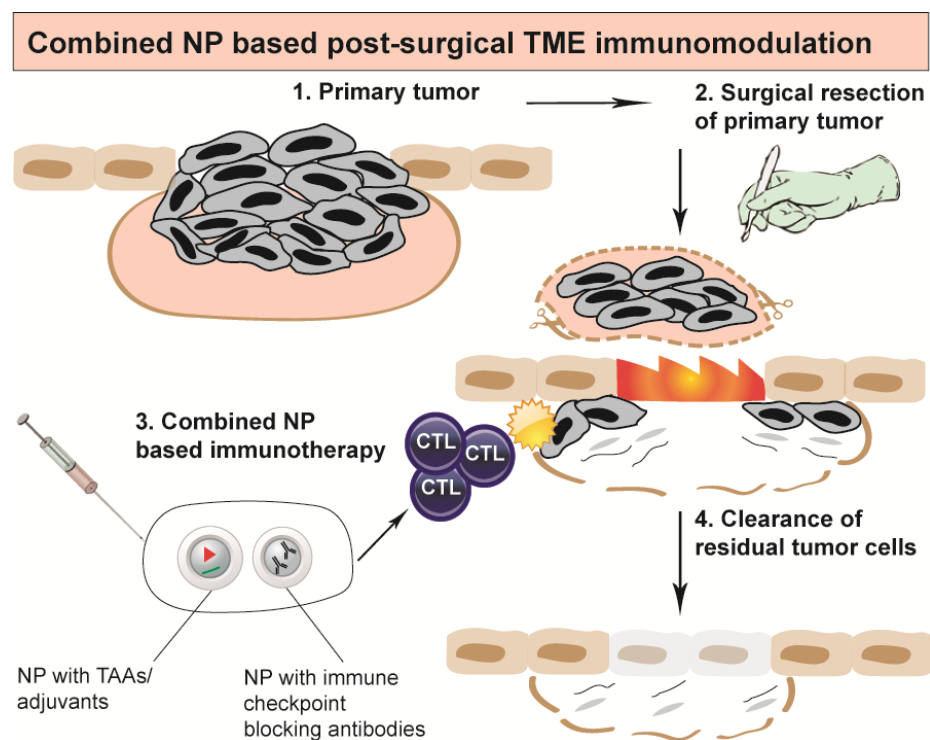[b] Percuros B.V., Zernikedreef 8, 2333 CL Leiden, The Netherlands.

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Graphical abstract

Abstract

Cancer immunotherapy is emerging as a candidate treatment modality for treating post-surgical metastasis and recurrences. Despite the great promises with therapeutic cancer vaccines and checkpoint blocking antibodies in pre-clinical studies, response rates in the clinic still remain unsatisfactory. The evaluation of immunotherapy after surgery in patients could confront significant unexpected hurdles. Surgery itself tends to cause immune suppression, while wound healing factors also stimulate tumor cell outgrowth and metastasis. Regarding the marked changes in the post-surgical tumor microenvironment, one can anticipate that better tumor growth control is attainable by combining cancer vaccines with immune checkpoint blockade. However, it is important that vaccines and checkpoint blocking antibodies are delivered efficiently to their target cells, are released sustained and locally and do not induce cytotoxic effects. The generation of effective anti-tumor immunity and durable response rates could largely depend on these parameters. In the last decade, researchers spend tremendous effort in optimizing the delivery of immunotherapeutic compounds with the use of nanomedicine. Biocompatible nanoparticle based delivery systems demonstrated intriguing results with regard to specific immune cell activation, improved drug delivery, cell targeting, limiting off target toxicity and improving treatment outcome. It therefore makes sense, to speculate on the promises of combined cancer vaccination and immune checkpoint blocking immunotherapy with the aid of nanomedicine. A powerful nanoparticle combination immunotherapy conferring durable therapeutic benefit whilst leaving healthy tissue untouched represents the base for more efficient post-surgical cancer treatment.

1. Introduction

Cancer is a worldwide leading cause of mortality following cardiovascular diseases[1]. Although surgery represents the conventional way to remove the primary tumor mass, its notorious limitation is the omission of residual and occult tumor cells. These often progress into relapses and metastasis, thereby accounting for the majority of cancer deaths[2, 3]. To prevent this from occurring, (neo)adjuvant therapies including hormone, chemo- and radiotherapy are often provided. Unfortunately, these modalities bear certain short-comings, including off-target toxicity, treatment resistance and marginal response rates[4, 5].

In meantime, therapeutic cancer vaccines (TCVs) and checkpoint blocking antibodies (CBAs) are emerging as promising immunotherapeutic treatment modalities for the prevention of post-surgical tumor recurrences. Immunotherapy explores the body's own immune system to fight cancer cells and features a promising strategy to prevent post-surgical tumor recurrences[6-8]. Unfortunately, clinical trials evaluating TCVs and CBAs for the treatment of post-surgical cancer recurrences have mostly not yielded convincing results so far. The clinical evaluation of immunotherapy with regard to surgery has confronted numerous challenges. It is apparent that the complex interplay between surgery, anti-tumor immunity and tumor pathophysiology has not been adequately considered during the design of immunotherapies. Surgery tends to exert immunosuppressive effects, while wound healing factors induce unfavorable immune phenotypical alterations in recurring tumors. Omitting these factors putatively lead to inadequate evaluation and design of (neo)adjuvant immunotherapies in the clinic. This could explain why certain clinically evaluated TCVs failed to prevent post-surgical tumor recurrences, although they could generate T cell responses[9]. The marked immunosuppressive effects of surgery and the post-surgical tumor microenvironment (TME) tend to dampen T cell function and viability. Considering these hurdles, combination immunotherapies concomitantly targeting immunosuppressive and immunostimulatory pathways are putatively more effective to halt tumor outgrowth. Combined vaccination with TCVs together with immune checkpoint blockade is deemed to harness these synergistic effects. Additional immune stimulation from CBAs that alleviate immune suppression and restore T cell function may strongly potentiate the T cell responses, which may break the immunosuppressive post-surgical TME barrier.

Whilst combination immunotherapies portray promising concepts for halting tumor progression, particular combinations could trigger systemic immune related side effects (IRAEs). Moreover, drug release control, cumbersome treatment schedules and poor therapeutic efficiency could raise treatment costs. In this regard, the field of nanomedicine

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has spent tremendous effort on optimizing nanomaterial based drug delivery systems to tackle these issues. Recent advances in nanomedicine elegantly underscored the promises of nanoparticle (NP) assisted immunomodulation. Important focus points of nanomedicine based immunotherapy encompass the improvement of drug bioavailability, extension of immune cell activation, targeting of specific (immune) cells and prevention of systemic drug delivery to healthy tissues[10-12].

This review will highlight the complex tumor-immunological changes in the post-surgical TME. Combination therapies with TCVs and CBAs can confer synergistic effects to achieve more powerful TME immunomodulation, but require further improvement. We will focus on the recent advances of NP based immunotherapy, and speculate on the benefits of combined TCV and CBA immunomodulation with the aid of nanomedicine.

2. The challenges of applying immunotherapy in combination with surgery

2.1. The role of T cell immunity in protecting against post-surgical tumor recurrence

The human immune system orchestrates pivotal functions in the defense against invading pathogens and detection and elimination of cancerous cells, a theory referred to as 'immune surveillance' by Burnet[13, 14]. Although 'immune surveillance' is accepted as a mostly T-cell mediated controlling system that protects our body for malignancies, it is obvious that this system has failed in cancer patients. In these patients it has been shown that there are tumor-specific T cells present, but these are generally in a tolerized state mainly caused by the suppressive TME and immune escape[15]. Current immunotherapies aim to re-activate these tumor-specific T cells to attack and eliminate malignant cells. There are several types of immune responses. Cytotoxic CD8 T lymphocyte (CTL) responses mainly targeted to specific intracellular epitopes presented on the MHC-I of (cancer) cells, are efficient to eliminate cancer cells. A T-helper 1 (TH1) type immunity is mediated by CD4 T helper 1 cells of the adaptive immune system and is commonly initiated by antigen presenting cells (APCs), which can orchestrate an effective CD8 T cell response. Dendritic cells (DCs) are APCs with superior capacities to process antigens and present them to (naïve) CD4 and CD8 T cells via MHC-II and I molecules, respectively[16]. Specific damage-associated molecular patterns or pathogen associated molecular patterns danger signals can trigger DCs to initiate a skew towards a TH1 type of response. These danger signals stimulate DCs to upregulate the

expression of co-stimulatory molecules including CD40, CD80 (B7.1), CD86 (B7.2) and MHC-I/II molecules and to produce TH1 cytokines, such as interleukine-12 (IL-12) and interferon gamma (IFN-γ). These TH1 polarizing signals are necessary to effectively prime naïve CD4 T cells into TH1 cells[17, 18]. TH1 cells and DCs form subsequent cognate pairs with naïve CD8 T cells to develop them into CTLs, which then enter the blood to seek and destroy cancer cells.

A TCV is regarded as a treatment modality that aims to stimulate an immune response to fight existing cancers[19-21]. Tumor cells are typically hallmarked by the expression of tumor associated antigens (TAAs) and tumor antigens. Vaccinating with adjuvants and antigen stimuli can induce DC maturation to onset tumor specific CTL responses to eliminate cancer cells[22]. Notably the cancer testis antigens (BAGE, MAGE, NY-ESO), tissue differentiation antigens (gp100, Melan-A/MART-1) and oncofetal antigens (MUC-1, CEA) have been extensively explored as targets for TCVs. Rather than delivering a single antigen, TCVs could also contain whole tumor cells. A well-known example is GVAX, that consists of whole tumor cells secreting granulocyte-macrophage colony-stimulating factor (GM-CSF). This TCV recruits and maturates DCs for effective priming of CTLs and is especially evaluated in pancreatic cancer patients[23-25]. In a comparable way, OncoVAX® consists of autologous tumor cells supplemented with Bacillus Calmette-Guerin adjuvant and has been evaluated in patients with stage II and III colorectal cancer and was notably effective in stage II patients[26].

CTLA-4 and PD-1 are inhibitory checkpoint molecules, which activation leads to T cell activity inhibition. They further tend to promote regulatory T cells (Tregs) development, albeit the exact mechanisms remain elusive[27, 28]. Their functional opposites are immunostimulatory checkpoint molecules, such as CD40, OX-40 and 4-1BB. CTLA-4 binds to CD80 and CD86 similar to CD28, although with considerably higher affinity. CTLA-4 transmits inhibitory signals in T cells and is functionally the opposite of CD28[29]. The cognate ligands for PD-1 are PD-L1 and PD-L2, which are often found overexpressed by (tumor) cells in the TME, including tumor associated macrophages (TAMs), tumor associated DCs, fibroblasts and Tregs. PD-L1 or PD-L2 binding to PD-1 induces T cell anergy or apoptosis[30-32]. Not surprisingly, inhibitory checkpoint molecules are regarded as appealing targets to restore effector T cell function[7, 33]. Ipilimumab (Yervoy®) is an anti-CTLA-4 antibody approved by the FDA in 2011 for the treatment of metastatic melanoma[34]. A PD-1 blocking antibody Nivolumab is approved for treating melanoma and non-small cell lung carcinoma since 2015[35-37]. Ipilimumab was recently evaluated as post-

surgical therapy for resectable stage III cutaneous melanoma in a double blind phase III trial and significantly prolonged relapse-free survival (RFS) and overall survival (OS)[38, 39].

At the end of the previous century, it was apparent that the immune system could reduce post-surgical cancer recurrences. Háková et al.[40] evaluated IL-2 gene therapy as post-surgical immunotherapy in mice bearing MK16 carcinomas. While tumor recurrence rate in the operated-only mice was 90%, recurrence rate was 38.5% in mice receiving also IL-2. After approximately 50 days, splenocytes were collected, cultured further in IL-2 containing medium and analyzed for splenocyte activity. Splenocyte activity was considerably higher in mice that remained free of tumor relapses. Natural killer (NK), CD4 and CD8 T cell depletion abrogated the effects of post-surgical IL-2 treatment. In their follow-up study, the authors evaluated IL-2, IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) as cytokine therapy after surgical resection of HPV16-associated tumors (TC1 and MK16)[41]. Splenocyte cytolytic activity was significantly higher in mice free of relapses, but this result was irrespective of the adjuvant treatment and tumor type.

In pre-clinical studies, immunotherapies stimulating the TH1 type response demonstrated great promises as potential cancer treatment. Various therapies were further explored in the clinical setting for different cancer types. Although some TCVs improved OS and RFS following surgical resection of melanomas[42-44], results were not convincing in other trials and cancer types[45-47]. Several reasons may underlie the discordance between pre-clinical studies and clinical trials.

2.2.   Surgery suppresses TH1 type immunity and induces profound cellular and cytokine changes in the post-surgical TME

Surgery could affect local and systemic immunity and tumor pathophysiology[48]. Whilst immunotherapies have been thoroughly evaluated in a variety of murine tumor models, these models often did not introduce any surgical variables. It may not be adequate to directly translate results obtained from non-surgical studies to the surgical clinic. Surgery is a large stressor and stimulates the hypothalamic-pituitary-adrenal (HPA) axis and the release of stress hormones, notably glucocorticoids and corticosterones, while also increasing sympathetic nervous outflow and the release of (nor)adrenaline. These 'fight-or-flight response' hormones have immunosuppressive properties. Surgical stress could further induce tumor vascularization and tumor cell dissemination, mainly via angiogenesis, with VEGF as the main regulator[49-51]. In response to surgical stress and anesthesia, cancer cells are also more prone to escape immune attack and tend to possess enhanced survival and proliferative

capacity[52]. Table 1 provides an overview of some effects that surgery could exert on immunity.

Table 1

Surgical induced stress can exert numerous effects on the immune system

Effect of surgery	Ref.
Adrenaline ↑ which in turn decreases IL-2 production	[53]
Decrease in neutrophil and macrophage numbers/mobility	[54-56]
Decrease in NK cell numbers and function	[57, 58]
Decrease in numbers of circulating T cells and proliferation	[53, 59-61]
Decrease of TH1 type of cytokine production (i.e. IL-2, IL-12, IFN-γ, TNF-α)	[62-64]
Increase in anti-inflammatory gene expression (i.e. lipocortin-6, IL-1, IL-10, neutral endopeptidase)	[64]
Anesthesia and analgesia induced general immunosuppression	[65-68]

Only a few pre-clinical studies have explicitly assessed immunotherapy in the context of tumor resection. In these studies, subcutaneous tumors were established and the entire tumor could be easily resected. However, this may not be a realistic representation of tumor recurrence in patients, since residual tumor cells are often omitted[48]. These cells reside in the post-surgical wound and may be influenced by surgical stress, wound healing and inflammatory mediators. Predina et al.[48] introduced an interesting post-surgical tumor recurrence model that for the first time introduced considerably more surgical variables representative for human surgery. The novelty in this study was that the authors left behind a small portion (margin) from subcutaneously growing tumors at the site of resection. Some studies revealed a rise of wound and inflammatory mediators, including TGF-β, COX-2,

prostaglandins and VEGF in the post-surgical TME[69, 70]. These mediators can promote tumor cell outgrowth and suppress anti-tumor immunity, i.e. by exhausting T cells, impairing T cell infiltration, and promoting the development of Tregs and myeloid-derived suppressor cells (MDSCs)[71]. Furthermore, Tregs in dormant tumor tissue may also contribute to tumor recurrence[72]. Indeed, the administration of TGF-β and COX-2 inhibitors effectively led to the cessation of tumor growth, but also affected wound healing. In their following study[73], the authors evaluated various TCVs in mice that underwent surgical resection of subcutaneous tumors. The TCV-induced T cell responses were effective to clear tumor cells from primary, non-resected tumors. However, these T cell responses were infective to halt outgrowth of tumors recurring at the surgical area. TME and tumor draining lymph nodes (TDLNs) analysis of these recurring tumors revealed higher numbers of wound healing macrophages (type 2 macrophages; M2), TAMs and Tregs, but lower numbers of tumor infiltrating T cells (TILs). This was accompanied by a decrease of IFN-γ and a rise of IL-6, IL-10 and VEGF. Besides wound healing factors, platelets covering the surgical wound could also trigger inflammation and metastasis. Interestingly, Wang et al.[74] utilized platelets as delivery system for immunotherapeutics. The authors conjugated PD-L1 blocking antibody to platelets, and the release of PD-L1 blocking antibody reduced post-surgical tumor recurrence in mice bearing B16F10 melanoma and 4T1 (murine tumor model for metastatic breast cancer) tumors. Platelets delivered the bound PD-L1 blocking antibody to the surgical area, which also reached circulating cancer cells.

Above discussion highlights the unwanted general decrease of TH1 type immunity and reveal certain cumbersome hurdles for the design of immunotherapies to treat post-surgical recurrences. Surgery affects immunity and commonly promotes the formation of an immune suppressed environment that is necessary to allow wound healing to occur. But this wound healing environment is also a favorable environment for residual cancer cells to grow and metastasize unhampered, thereby representing a large risk for developing tumor relapses (see Fig. 1).
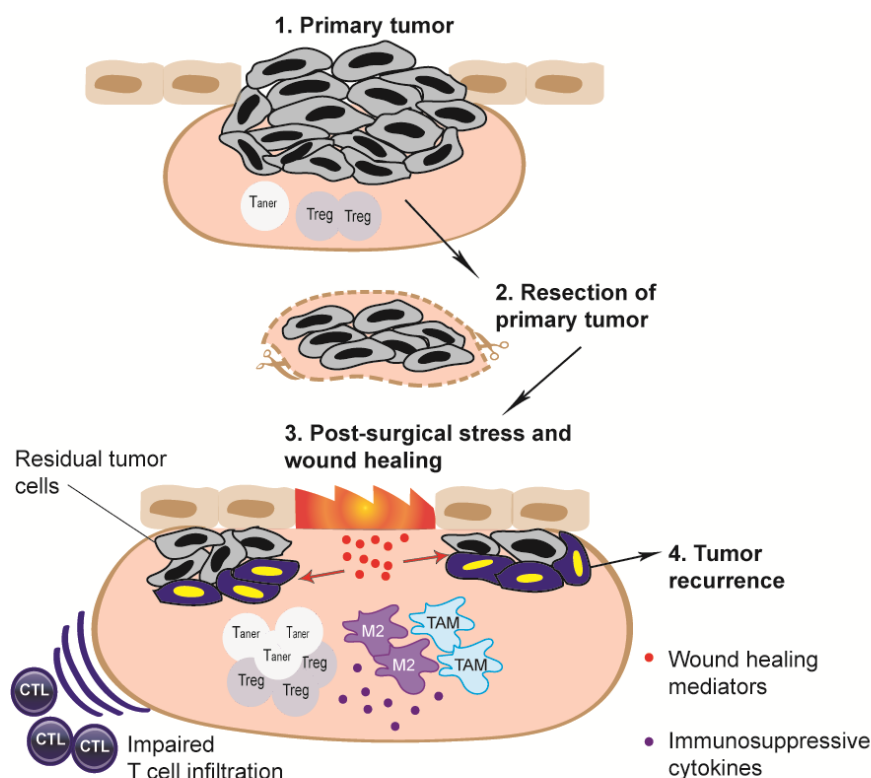
Fig. 1. Profound cytokine and cellular changes in the post-surgical TME. Surgery induces local tissue/skin damage, resulting in the production of wound healing and inflammatory factors. These factors could potentiate the outgrowth and metastasis of residual tumor cells. The post-surgical TME is furthermore enriched in Tregs, Taner, M2 and TAM type of macrophages. These profound cytokine and cellular changes typically favor the outgrowth of omitted residual cancer cells, whilst antagonizing CTL infiltration and anti-cancer TH1 type of immunity. Abbreviations: CTL: cytotoxic T lymphocyte; M2: type 2 macrophage; TAM: tumor associated macrophage; Taner: anergic T cell; TME: tumor microenvironment; Tregs: regulatory T cells.

Omitting these insights may lead to inaccurate evaluation and design of post-surgical immunotherapies in the clinic, which may – at least in part – explain the marginal outcomes in earlier clinical trials. Whilst TCVs could generate substantial CTL responses sufficient to contain the outgrowth of naïve tumors in mice, these may succumb to the profound immune dampened and wound healing post-surgical TME of patients. More effective immunomodulation is likely attainable by combining immunotherapies, that (re-)shape the TME in such way, that it favors anti-tumor immunity while leaving wound healing unhampered. There is rationale to concomitantly generate effective CTL responses while

alleviating immunosuppression on immune cells, which effect could be attained by combining TCVs and CBAs.

2.3.   Combined cancer vaccination and immune checkpoint blockade for synergistic TME immunomodulation

Early studies demonstrated how GVAX, a TCV composed of whole tumor cells genetically modified to produce the cytokine GM-CSF, synergized with CTLA-4 blocking therapy in poor immunogenic melanoma and prostate cancer[75-77]. Importantly, combined TCV and CBA treatment modulates the TME by depleting intratumoral Treg populations, while expanding T effector cell populations[78-81]. In a similar way, CTLA-4 blockade on both CTLs and Tregs confers the full therapeutic effects of CTLA-4 blockade[82]. PD-(L)1 blockade exerts comparable immunological effects[83-87]. Pancreatic tumors with weak PD-L1 expression are often resistant for PD-1 blockade. Soares et al.[86] demonstrated that GVAX enhanced PD-L1 expression in pancreatic tumor bearing mice. Combined with PD-1 blockade, GVAX elevated the number of TILs and IFN-γ concentration in the TME, while abolishing immunosuppressive pathways. Lutz et al.[88] demonstrated that TCVs sensitized a poor immunogenic murine pancreas tumor for CBA therapy. These results were extended into a clinical trial, where the GVAX vaccine together with cyclophosphamide (a Treg depleting compound) were administrated prior to surgical debulking of pancreatic ductal adenocarcinomas. Examination of resected tumors revealed a higher TIL-Treg ratio in vaccinated patients but also increased PD-L1 expression, which increased sensitivity to PD-1 blockade[89]. A noteworthy observation in patients is that CBAs are mostly effective against melanomas or non-small cell lung cancer (NSCLC). A plausible explanation for this phenomenon is that these cancer types harbor many immunogenic (point) mutations, thereby yielding neo-epitopes[90, 91]. These cancer types attract more TILs and in fact trigger a baseline endogenous anti-tumor immune response[92, 93]. It is speculated that CBAs synergize with the intrinsic T cell responses against the melanoma epitopes, while poor immunogenic tumors are often resistant to CBA therapy. In the clinic, numerous clinical trials are finished or planned that combine TCVs with ipilimumab or nivolumab for post-surgical immunomodulation (Table 2).

Table 2

Overview of trials combining ipilimumab or nivolumab with cancer vaccines

Cancer type	Regimen	Readout/analysis	Reference/trial ID
Resectable melanoma stage, IIIc/IV	Ipilimumab + gp100/Tyros/MART-1	OS, RFS TAA specific CTLs	[94]
Resectable melanoma, stage IIIc/IV	Nivolumab + NY-ESO-1 /gp100/MART-1 multi-peptide vaccine	OS, RFS TAA specific CTLs and THs	[95]
Resectable melanoma, stage IIIc/IV	Nivolumab + NY-ESO-1/gp100/Montanide	OS, RFS	NCT01176474[a]
Resectable melanoma, stage IIIc/IV	Nivolumab + GVAX + Cyclophosphamide	OS, DMFS[a]	NCT03161379[a]
Resectable pancreatic cancer	Neo/adjuvant GVAX + Nivolumab	OS, RFS	NCT02451982[a]

Abbreviations: DMFS: distant metastasis free survival; gp100: glycoprotein 100; OS: overall survival; RFS: relapse free survival; GVAX: granulocyte macrophage colony-stimulating factor (GM-CSF)-secreting pancreatic cancer vaccine.

[a] Full trial data can be retrieved at https://clinicaltrials.gov

To date, two clinical trials finished evaluating this combination therapy after resection of high-risk stage IIIc/V melanomas. In a phase II trial[94], ipilimumab was combined with a TCV after melanoma resection. The vaccine did not significantly improve the T cell responses, but increased the frequency of TH17 inducible cells, which was a marker of RFS. In the other trial[95], nivolumab and a multi-peptide vaccine (gp100, MART-1, and NY-ESO-1 with Montanide ISA 51) were provided following melanoma resection. In the treated groups, CTLA-4(+)/CD4(+), CD25(+)Treg/CD4(+), and tetramer specific CD8(+) T-cell

population were increased. In relapse free patients, there was a trend towards lower baseline MDSCs and Tregs. Other (control) groups were however not included. New trials are in meantime scheduled to evaluate nivolumab with gp100/NY-ESO-1 TCV (NCT01176474) or nivolumab with GVAX TCV (NCT03161379) following resection of melanoma and pancreatic tumors, respectively. One trial (NCT02451982) is evaluating an interesting combination with nivolumab and GVAX before as well as after surgical resection of pancreatic cancer. Altogether, pre-clinical research convincingly provides supporting evidence for the advantages of combining TCVs with CBAs. Clinical studies have not yet yielded equally convincing data, but there appears to be reasonable interest and optimism to further explore and optimize this combination therapy. While new trials are planned, certain challenges can already be anticipated with the current experimental set-up. A noteworthy aspect is that the TCVs in most trials consist of the low immunogenic 'self-antigen' TAAs, to which tolerance have been developed during T cell education[96], in contrast to neo-antigens. Neo-antigens have been postulated as better candidates for generating stronger anti-tumor immunity, since these neo-epitopes originate from genome mutations to which these non-self-peptides immune tolerance do not develop[97, 98]. However, a disadvantage with neo-antigen vaccination is antigen identification, which is a time consuming task requiring personalized vaccine design. Furthermore, classical TAA expression could be very heterogenic among patients and generalized vaccine design is often not feasible[99]. Another current concern is the inefficient drug dosing, generation of durable immune responses, patient response rates and side effect management[20-22]. A challenge with conventional TCVs is the inefficient delivery of adjuvant and antigen to DCs, which typically hampers the generation of durable and specific anti-cancer CTL responses[100]. Adjuvants delivered in 'soluble' form also spread systemically and induce pain, erythema, swelling, flu-like symptoms and potentially fatal hematological toxicities and cytokine storms[101-104]. Similarly, CBAs are conventionally (over)dosed in the systemic circulation, thereby often IRAEs, autoimmune diseases, cytokine storms or even death. Due to these IRAEs, many immune therapies can only be combined with utmost care. The need of multiple dosing can finally rises treatment costs[105-107]. In the recent years, there is increasing interest for improving local immunotherapy with the use of nanomaterial platform based delivery systems[108]. Nanomedicine assisted local immune activation bears attractive advantages that can be integrated with cancer surgery.

3. NPs as promising delivery systems for optimizing TCV and CBA immunotherapy

3.1. Foundations of NP based research

The term nanomedicine generally describes the application of nanotechnology in medicine. While the research field of nanomedicine is very broad, this review will be limited to the application of nanomaterials - in particular NPs - for cancer immunotherapy. NPs are colloidal structures ranging in size of 10 to 1000 nm and have found a broad range of application as drug delivery and imaging studies[109]. They are classified according to the material they are made up with. Polymeric NPs, lipid based NPs, micelles and gold NPs are frequently manufactured drug delivery systems. Material preference depends on various factors. Polymeric NPs manufactured with the polymer poly (lactic-co glycolic acid; PLGA) are for instance appraised for the good biocompatibility of PLGA, as PLGA hydrolysis yields the non-toxic monomers lactic acid and glycolic acid[110]. These compounds are readily metabolized with the Krebs-cycle. Liposomes are likewise popular due to their good biocompatibility. Many of the clinical approved formulations – notably for the delivery of chemotherapeutic drugs – are liposomal formulations, including Doxil, DepoCyt, DaunoXome and Onivyde[111-113]. NPs can encapsulate or be coupled with multiple compounds, including antibodies, dyes and cancer therapeutics. Such construction permits the co-delivery of multiple compounds with different functions, which could obviate the need to deliver compounds separately[114]. For instance, NPs co-delivering imaging tracking molecules (e.g. dye's, contrast agents, isotopes, etc.) and therapeutics allow *in vivo* tracking, which is the main emphasis of theranostics[115-117]. Another advantage of NPs is that they could improve drug pharmacokinetic (PK) properties, since NPs can release their cargo in a sustained manner. This not only increases drug bioavailability and therapeutic efficacy, it furthermore reduces unnecessary drug delivery to healthy tissue[118]. This potentially reduces the induction of off-target toxicity[119].

NPs size is a crucial parameter since size influences NP trafficking *in vivo*. NPs are believed to passively drain to the tumor via the hypothesized existence of the enhanced permeability retention (EPR) effect[120]. This theory is based on the leaky tumor vasculature and inefficient tumor lymphatic drainage, which permits small NPs passively draining to tumors. Caution is warranted with very small NPs (<30 nm), as these may be subjected to renal clearance[121]. Very large NPs, in contrast, may remain entrapped in the extra cellular matrix near the site of administration[122]. Noteworthy, these concepts are mainly based on murine studies, so it remains ambivalent whether the EPR effect exists in man[123]. Besides passive

delivery, NPs can be conjugated with targeting moieties, in particular antibodies, in order to specifically deliver therapeutics to the targeted cell types[124, 125]. Other characteristics of NPs that could influence particle adjuvanticity include particle charge, shape, hydrophobicity and coating with surfactants. For more detailed insights on these physico-chemical properties, please consider the review article from Silva et al.[126]. In the next sessions, we will discuss the recent milestones of NP based immunotherapy, in particular for TCVs and CBAs.

3.2.   NPs platform based TAA and adjuvant delivery induces effective anti-tumor immune responses

In the last years, various NPs have been formulated as multifunctional delivery system for TAAs and adjuvants (Toll-like receptor ligands; TLR-Ls) including TLR3-L (poly(I:C))[127, 128], TLR4-L (MLPA)[129-131], TLR-7/8 (R848)[132] and TLR-9L (CpG)[132-135]. These NPs platform based TCVs are sometimes referred to as 'nanovaccines' and have particularly found application in pre-clinical studies. Ranging in particular in sizes around 200 nm and co-delivering TLR-Ls and antigens, NPs mimic pathogens and are easily engulfed by DCs[136]. Hamdy et al.[129] showed that PLGA NPs co-encapsulating Trp2 (tyrosinase relate peptide 2) peptide and TLR4-L strongly stimulated IFN-γ secretion at the lymph nodes and spleens, which strongly potentiated tumor containment. Xu et al.[135] manufactured multifunctional lipid-calcium-phosphate NPs co-delivering Trp2 peptide and TLR9-L. These NPs had superior effects on IFN-γ production, CTL expansion and containing B16F10 tumor outgrowth, as compared to freely dosed TLR9-L and Trp2. The CTLs moreover effectively cleared distal metastasis in the lung. Ilyinskii et al.[132] also focused on immune adverse effects and demonstrated that NPs co-delivering TLR7/8-L or TLR9-L together with OVA induced strong CTL responses, without signs of systemic toxicity. In stark contrast, freely dosed adjuvants resulted in 50 to 200 folds higher serum levels IL-6 and TNF-α, a condition referred to as 'cytokine storm'[137]. These studies uniformly demonstrated that NP based TCVs induce more efficient DC and CTL activation and improved tumor containment, as opposed to free administration of adjuvants and antigens. These benefits are putatively attributed to antigen protection, more efficient delivery to DCs and extended release of antigen and adjuvant within DCs. These features are, among others, crucial for efficient DC maturation and the generation of strong and durable CTL responses[138]. Indeed, Silva et al.[139] demonstrated that a low burst release of antigens favors efficient DC activation. NPs facilitate the co-release of TLR-Ls and TAAs inside DCs, which could obviate the need to administer multiple compounds separately[140]. These benefits were clearly lost if burst

release take place outside DCs, a problem with NPs releasing their cargo prematurely. This leads to comparable problems as when adjuvants and TAAs are dosed freely[141].

Since DCs are the main targets of NP based TCVs, researchers recently designed NPs with targeting moieties to specifically target DC receptors, including CD40, DEC-205, DC-SIGN and CD11c[142-146]. These receptors mediate DC uptake or activation functions. Targeting these receptors is believed to further improve DC antigen and adjuvant delivery, while limiting delivery to other tissues. DC targeting NPs demonstrated better DC and CTL activation as compared to untargeted NPs, as shown by better DC NP internalization, increased T cell proliferation, higher IL-12 and IFN-γ production and more efficient tumor containment. Targeting moieties thus represent promising attributes to improve specific DC antigen and adjuvant delivery. In Fig. 2, the promises of NP based TCV therapy are represented.
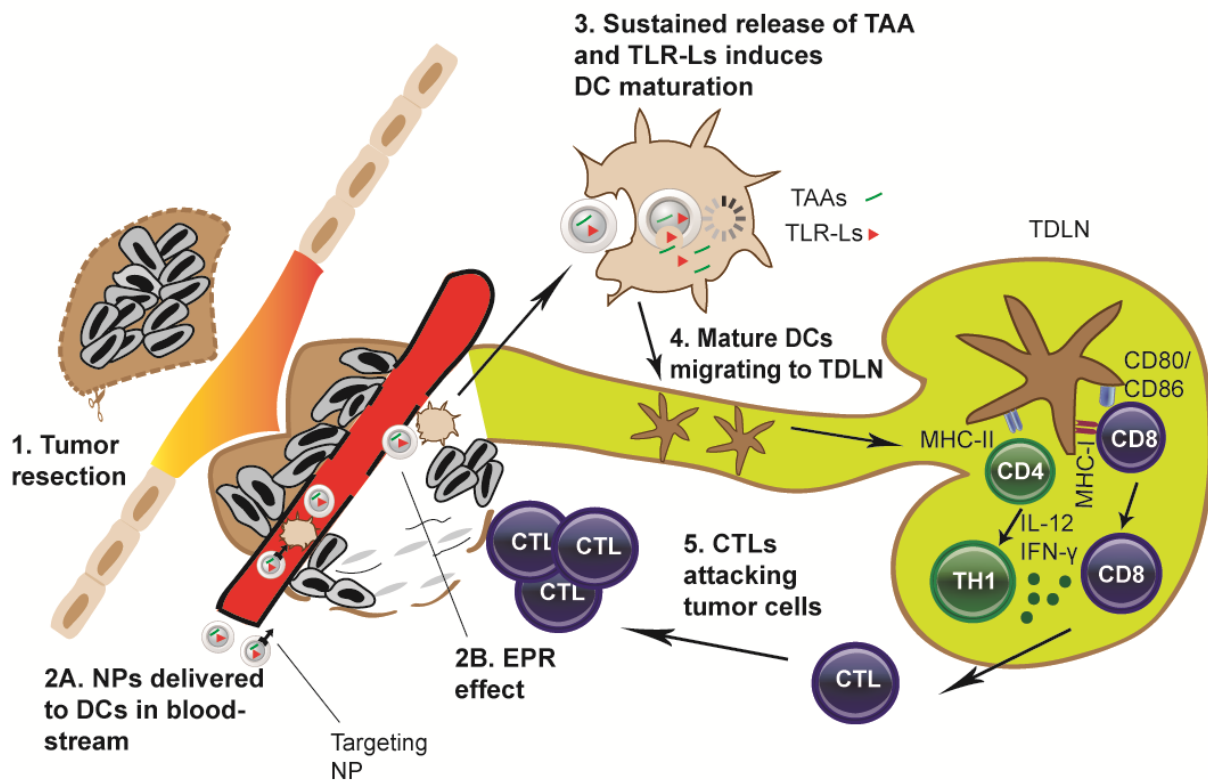

Fig. 2. Efficient DC maturation with NPs delivering TAAs and adjuvants. After tumor resection (1), vaccination with NPs could lead to DC maturation in different ways. NPs injected in bloodstream may passively reach or be actively targeted to DCs in the bloodstream (2A). NPs may furthermore passively drain to DCs residing in the tumor through the larger fenestrations in the tumor vasculature (2B). Altogether, the sustained and controlled release of TAA and TLRs initiate efficient DCs maturation (3), which migrate towards the TDLNs to efficiently prime the development of TH1 and

CTL cells (4). Activated CTLs leave the TDLN to eliminate residual tumor cells (5). Abbreviations: CTL: cytotoxic T lymphocyte; DC: dendritic cell; EPR: enhanced permeability and retention; IFN-γ: interferon gamma; IL-12: interleukine-12; MHC: major histocompatibility complex; TAA: tumor associated antigen; TDLN: tumor draining lymph node; TH1: T-helper 1 cell; TLR-L: Toll-like receptor ligand.

3.3.   NPs mediated immune checkpoint blockade for local and sustained alleviation of immunosuppression

There is increasing optimism that local and sustained CBA delivery with slow release platforms offers promising perspectives for immune checkpoint blockade therapy[147, 148]. Simmons et al.[147] generated the GM-CSF producing cell line expressing the full-length CTLA-4 monoclonal antibody gene for cellular mediated release of anti-CTLA-4 antibody. Cellular mediated antibody delivery induced potent anti-tumor responses in mice, while furthermore reduced serum antibody concentrations. This significantly reduced the induction of systemic IRAEs. Fransen et al.[149] demonstrated that local and slow delivery of the agonistic anti-CD40 antibody with Montanide ISA 51 nearby the TDLN reduced systemic autoimmunity, as compared to systemic antibody delivery. Interestingly, when antibodies were given locally, a much lower dose sufficed for effective tumor growth containment. However, mineral oil depots bear the risk of inducing chronic inflammation-associated adverse effects[150, 151]. Lei et al.[152] encapsulated anti-CTLA-4 antibodies in functionalized mesoporous silica for achieving sustained release of anti-CTLA-4 antibodies. NPs mediated CTLA-4 blockade mediated superior effects on inhibiting tumor outgrowth, as compared to free antibodies given systemically. Rahimian et al.[153] designed polymeric microparticles (MPs) with a diameter of 12-15 μm to investigate local anti-CD40 and anti-CTLA-4 antibody delivery. The rationale for larger particles is that these are putatively less prone for non-specific uptake (phagocytosis)[122, 154]. The treatment was evaluated in the immune checkpoint blockade sensitive MC-38 murine colon cancer model. Incomplete Freund's Adjuvant and MP mediated CTLA-4 and CD40 blockade were equally effective in conferring MC-38 tumor growth inhibition, albeit the MP treated mice did not develop serum antibodies against the therapeutic antibodies. MP treated mice furthermore displayed no remnants of inflamed depots at the injection site. Similarly, Fransen et al.[155] evaluated anti-CD40 blockade with dextran coated MPs. Dextran is considered as an appealing biocompatible building block for slow delivery systems[156]. Dextran coated MPs loaded

with anti-CD40 antibodies displayed slow release of anti-CD40 antibody without inducing systemic toxicity. Unfortunately, they did induce severe local inflammation without improving tumor growth inhibition. A putative explanation is that dextran contains sugar, which may have served as nutrient source for tumor cells. Polyethylene glycol (PEG) could represent an alternative for limiting non-specific uptake of NPs by phagocytes, as PEG increases the stealthness of the NPs[157, 158]. In summary, local and sustained release of CBAs could greatly improve the efficacy of immune checkpoint blocking therapies, while also reducing unnecessary spread of CBAs in the systemic circulation. Besides the reduced toxicity, it may also spare treatment costs, because of the increased CBA bioavailability and residence time (Fig. 3).
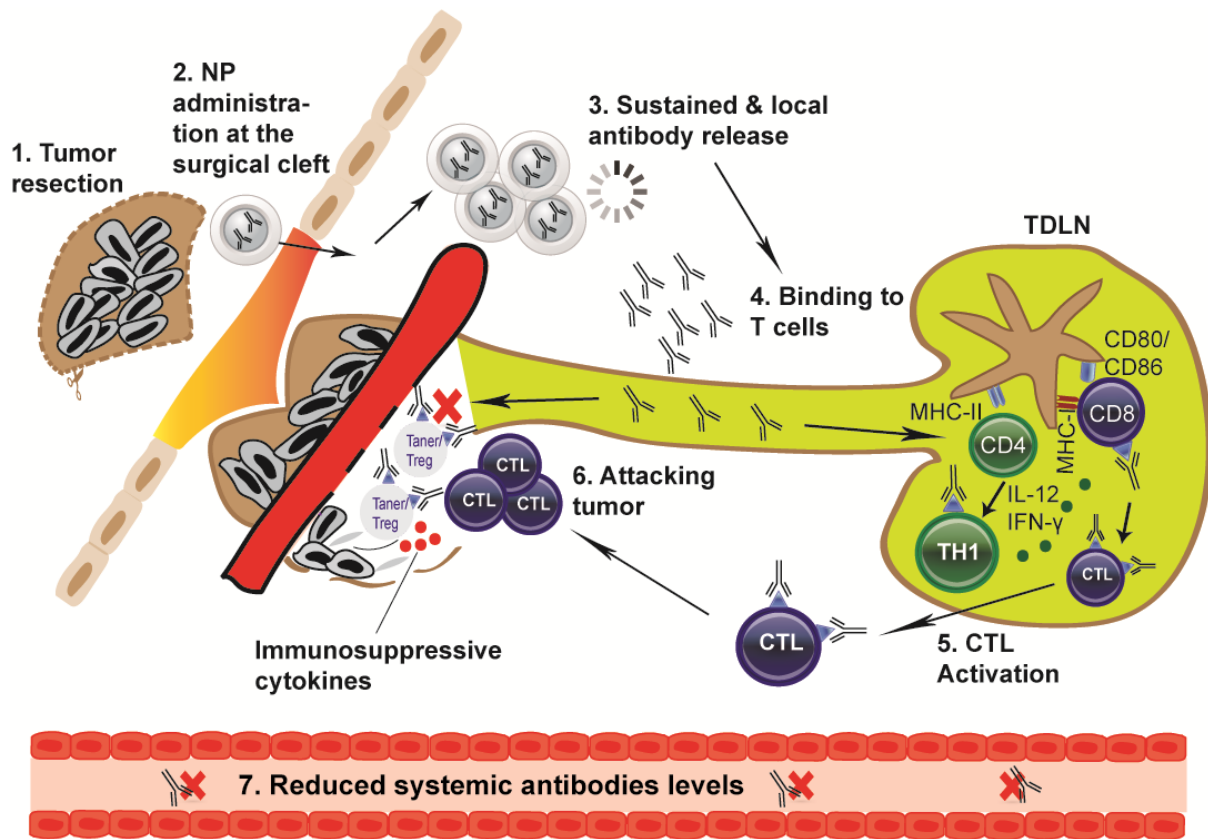

Fig. 3. NPs for sustained and local immune checkpoint blocking therapy. After tumor resection (1), the TME (left) is typically featured by the constitution of immunosuppressive Tregs/Taner cells and increased concentrations of immunosuppressive cytokines, which exert immunosuppressive effects on nearby immune cells and TDLNs. NPs administrated nearby the TME gradually release CBAs (2 and 3). Locally released CBAs then bind to inhibitory checkpoint molecules such as CTLA-4 and PD-1 (4). These alleviate immune suppression on CTLs, TH cells and stimulate their activation (5), while supposedly depleting Tregs or Taner in the TME. Activated CTLs can then effectively eliminate

tumor cells (6). Because released locally, the antibodies are mostly retained within the TME/TDLN zone, which putatively limits the spread of antibodies in systemic circulation (7). Abbreviations: CBA: checkpoint blocking antibody; CTL: cytotoxic T lymphocyte; IFN-γ: interferon gamma; IL-12: interleukine-12; MHC: major histocompatibility complex; Taner: anergic T cell; TDLN: tumor draining lymph node; TH1: T-helper 1 cell; TME: tumor microenvironment; Treg: regulatory T cell.

3.4. Post-surgical TME and TDLN as targets for local immunomodulation to inhibit post-surgical tumor outgrowth

Local and sustained immune checkpoint blockade with NPs platform based immunotherapy could keep the TME in an immunogenic state for a sustained period of time, which offers clear benefits for the generation of durable anti-tumor immune responses after surgery. For numerous reasons, TDLNs represent interesting organs of consideration when evaluating surgery with immunotherapy, as TDLNs in fact harbor the majority of relevant cells for immunotherapy[159]. As these cells tend to be immunosuppressed, it could particularly be promising to regard the (targeted) delivery of immunomodulators or checkpoint blockers to the TME to reactivate these suppressed cells. The advantage is that locally activated immune cells are capable of attacking distal metastasis, as has been shown with tumor re-challenge studies. Secondary tumors could mimic metastasis and several studies demonstrated that locally activated T cells could efficiently clear secondary tumors[160, 161]. It would therefore make sense to administer/target NPs in the proximity of TDLNs and TMEs zone. The sustained release of immunotherapeutics and prolonged immune activation is deemed to convert the tumor in a sustained 'protective auto-vaccine'[162]. A promising feature is that NPs could be administered more distantly from the tumor, but may still target local immune cells in the TME/TDLN. This is especially valuable for tumors or distal metastasis that are difficult to reach for surgeons. Fluorescent dyes coupled to NPs enable their tracking *in vivo*. Cruz et al.[163] monitored DC targeting PLGA NPs that encapsulate fluorescent and superparamagnetic iron oxide (SPIO) labeled antigen. DCs that engulfed the fluorescently and SPIO labeled NPs were traced back in the lymph nodes, which illustrates the possibility to monitor NPs and immune cell travelling *in vivo*.

The discussion about TDLNs as immune organs could raise interesting questions for surgeons, as they typically consider TDLNs as cancerous tissues. Khong et al.[164] addressed this dubious question by evaluating the agonistic anti-CD40 antibody following surgical resection of subcutaneous AB1-HA mesothelioma tumors in BALB/c mice. Recurrence was

mimicked by injecting tumor cells in the post-surgical wound. Agonistic anti-CD40 antibody stimulated DC activation and facilitated tumor specific CTL priming. Interestingly, TDLN resection did not abrogate anti-CD40 therapy efficacy. This suggests that, at least for the efficacy of anti-CD40 therapy, TDLNs were dispensable. In their subsequent study[165], anti-CD40 therapy together with TLR7 ligand imiquimod were evaluated as post-surgical immunotherapy with the same tumor model, albeit without TDLN resection. Post-surgical anti-CD40 + imiquimod therapy effectively facilitated tumor growth inhibition and expanded TILs number in the TDLN and tumor. A recent study further underscored the promises of local and sustained immune activation following surgery with the use of hydrogels scaffolds. Hydrogel scaffolds are another class of nanomaterial that could extend immunotherapeutic drug release. Engraftment of hydrogels loaded with different compounds, including TLR7/8, anti-PD-1 or anti-CTLA-4 after surgery greatly increased the numbers of DCs, NK cells and T cells, while also induced production of type I interferons. This indicates the conversion of an immunosuppressive TME into one that favors TH1 anti-tumor immunity. In line with this, the local treatment reduced development of post-surgical metastasis in mice carrying 4T1 tumors [166]. These benefits were neither observed in mice receiving the same treatment in PBS nor in those receiving the treatment systemically. While local and sustained drug release was crucial, the authors also speculated on the timing of the immunotherapy, as hydrogel scaffold were implanted directly after tumor resection (peri-operative therapy). The advantage of peri-operative immunotherapy is that the tumor is directly accessible for immunotherapeutics. Furthermore, peri-operative immunotherapy may directly counteract surgical stress induced immunosuppression. In contrast, the study from Liu et al.[167] evaluated immunotherapy before tumor resection (neo-adjuvant therapy). The authors demonstrated that the combined neo-adjuvant anti-PD-1 and anti-4-1BB therapy strongly prevents post-surgical recurrence in 4T1 tumors. In contrast, mice receiving the therapy few days later after surgery developed more metastasis and larger tumors. A rationale for neo-adjuvant is that the tumor mass is still present, which serves as a source of TAAs for T cells. Also, immunity has not been affected yet by surgery. More studies are required in order to find the optimal dosing moments of immunotherapy with respect to surgery.

4. Future perspective of combinational NP based immunotherapy

When evaluating immunotherapy in combination with surgery, it is important to consider the complex interplay between the immune system, tumor pathophysiology and the effects of

surgery on the TME. Until now, these concepts have only been partially addressed in (pre-)clinical studies, however there is increasing awareness that surgery could impact immunotherapy. The difficulty of eliminating recurring cancers with conventional TCVs calls for the exploration of stronger combination immunotherapeutic treatment regimens that target the anti-tumor immune response at multiple levels. Different strategies could be envisaged, such as reducing the amount of Tregs and TAMs, depleting immunosuppressive cytokines and stimulating immune pathways that favor anti-tumor immunity[168]. A desired outcome is the generation of durable high affinity anti-tumor immune responses that effectively clear the tumor, while exerting minimal cytotoxicity. This goal is putatively attainable by creating, or re-establishing a TME that favors the generation of anti-tumor immune responses, without affecting wound healing. Whilst combined immunotherapy represents a promising avenue of research, certain aspects need to be regarded carefully. Uncontrolled immune activation could strongly limit the evaluation of potentially strong combination therapies. Furthermore, combination treatments may interfere with each other, which should be avoided by carefully timing the administration moment of the different modalities. Particularly the type of CBA could play an important role, since they exert their (predominant) effects at different phases within the immune cycle. Rather than giving all modalities at once, it is recommended to give the different modalities in sequence, repeat the treatment by multiple drug dosing and treat at different locations. To demonstrate, anti-CTLA-4 is expected to be most efficient at the moment when T cells received a strong TCR signal from potent vaccines, and it is suggested to be more efficient at the time a second (booster) vaccine is given[169].

In this review, we addressed how recent investments in nanomedicine based immunotherapy research clearly sparked the interests of NPs as delivery system for cancer immunotherapy. Alongside the growing interest for manufacturing and optimizing NPs for TCV and CBA based immunotherapy, combined NP based immune checkpoint blockade with TCVs could emerge as a future treatment strategy for more effective post-surgical TME immunomodulation. This is putatively a pivotal step for the treatment of post-surgical recurrences, as depicted in Fig. 4.
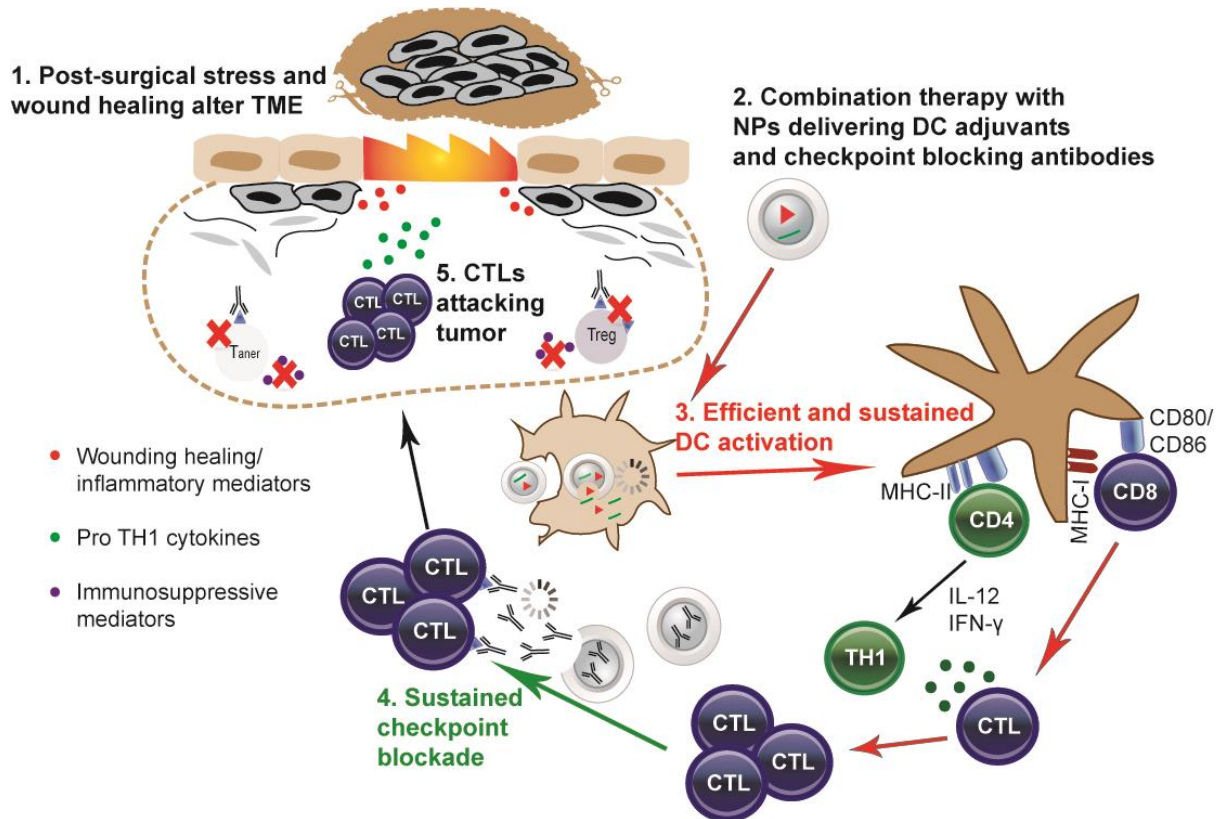
Fig. 4. The promises of synergistic NP based immunotherapy with TCVs and CBAs. Surgery triggers stress and tends to lead to aberrant alteration of the TME (1). A more effective treatment approach is putatively realized with combination immunotherapy with NPs inducing DC maturation and NPs delivering CBAs, which could mediate synergistic effects on tumor elimination (2). Sustained TAA and TLR release leads on one hand to efficient DC maturation (3; pathway shown with red arrows) and mature DCs efficiently prime CTL development. On the other hand, NPs releasing CBAs can alleviate immunosuppression on immune cells (4; pathway shown with green arrow). Proper dosing schedules and repeated dosing could further improve therapeutic outcome. The sustained bioavailability of immunotherapeutics prolong immune cell activation, which is deemed to convert the immunosuppressive post-surgical TME into one that favors CTL mediated anti-tumor immunity (5). Abbreviations: CBA: checkpoint blocking antibody; CTL: cytotoxic T lymphocyte; DC: dendritic cell; IFN-γ: interferon gamma; IL-12: interleukine-12; MHC: major histocompatibility complex; Taner: anergic T cells; TH1: T-helper 1 cell; Treg: regulatory T cells

Several studies are already under way to explore combination immunotherapies with the aid of nanomedicine. Ali et al.[170] combined PLGA NP GVAX vaccine with CTLA-4 and PD-1 blockade, which efficiently facilitated the regression of B16 melanoma tumor in mice, even without vaccine boosting. Particularly CTLA-4 blockade strongly increased CTL activity in the TDLN and at the site of vaccination, while PD-1 blockade alone increased CTL activity

in the tumor only. Chen et al.[171] assessed the synergism between CTLA-4 blockade and PLGA NPs co-delivering imiquimod with indocyanine green (photothermal compound). The NPs were used for near infrared (NIR) laser mediated photothermal therapy of primary tumors, thereby inducing immunogenic cell death and the release of TAAs. The imiquimod release in the dying tumor cells served as an endogenous vaccine, which strongly synergized with CTLA-4 blocking antibodies to clear distal metastasis. CTLA-4 blockade combined with NPs was furthermore evaluated as post-surgical therapy. The combination therapy effectively cleared primary tumors and distal metastasis, while neither mono-therapy with CTLA-4 blockade nor NP alone facilitated tumor growth inhibition. NP based combination treatments that target other (comparable) immunostimulatory and immunosuppressive pathways in the TME are also under development. Xu et al.[172] evaluated a combined NP immunotherapy, with one set of NP (vaccine NP) delivering Trp2 peptide and TLR9-L CpG to stimulate DCs and another set of lipid–polymer hybrid NPs delivering siRNA to suppress TGF-β expression in the TME. The combination therapy facilitated significant inhibition of tumor growth compared to mice receiving the NP vaccine only. Park et al.[173] formulated a combination therapy within one NP delivery system with IL-2 adjuvant and TGF-β inhibitor using nanolipogel NPs, which effectively contained outgrowth of B16 melanoma tumors. Options remain open to encapsulate various other types of immunotherapeutics, ranging from adjuvants, siRNAs, immunomodulatory antibodies etc. Regarding these developments, it is conceivable that NP based immunotherapy will find application soon in the clinic. One PLGA nanovaccine formulation (WDvax) is already being evaluated in a phase I trial for melanoma (NCT01753089).

5. Conclusion

Surgery could induce a general state of immunosuppression with the post-surgical TME often being manifested by profound cellular and cytokine changes. These changes do not favor anti-tumor immunity, whilst also promoting tumor cell outgrowth. More durable response rates are likely attainable by strategically combining immunotherapies that change the post-surgical TME into one that promotes anti-tumor immunity. Versatile combined NP based immunotherapies with TCVs and CBAs may serve well for post-surgical TME immunomodulation and yielding better response rates in patients.

Declaration of interest

AC is affiliated to Percuros B.V. as a founder. DK is affiliated to JeNaCell GmbH as a founder.

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