Nanoparticle design to induce tumor immunity and challenge the suppressive tumor microenvironment

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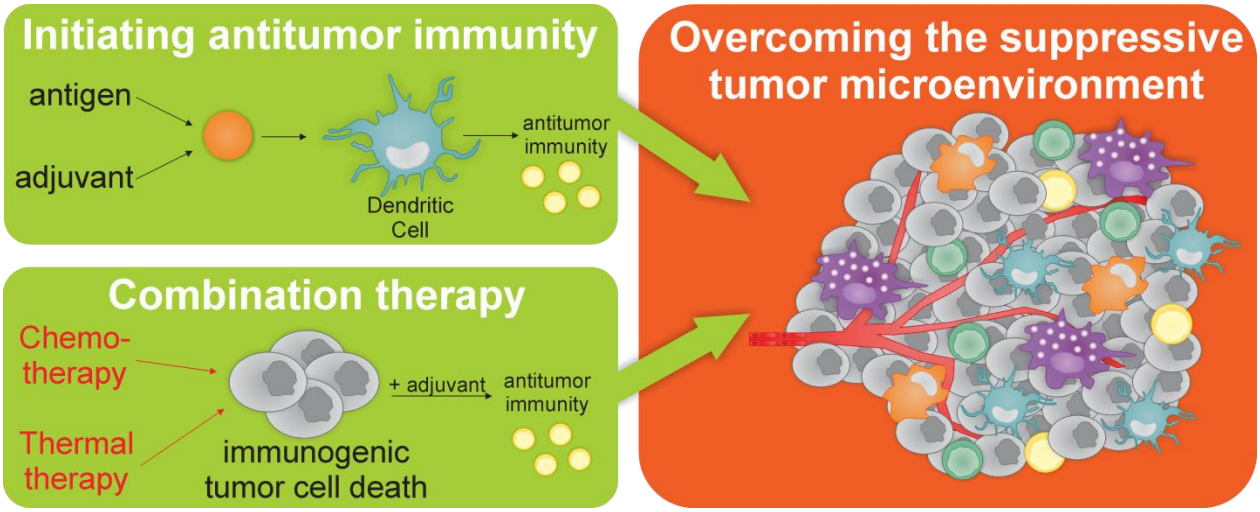
Abstract

Over the years research in the field of cancer immunotherapy has flourished, bringing about crucial breakthroughs, but at the same time revealing new and important pathways of immune suppression that put a break on the success of cancer immunotherapy. This review focuses on how nano- and micromaterials can be used to induce antitumor immune responses and what their role in overcoming immune suppression could be. It is now beyond question that this requires elegantly designed particles that can reach their target cells, deliver antigenic cargo and most importantly immune stimulants in order to provoke and sustain antitumor immunity.



1. Introduction: Cancer immunotherapy on the rise

The initial idea of exploiting the immune system to combat cancer originated in the early 1800s, when Dr. Wiliam Coley achieved (limited) antitumor effects by injecting microbe-derived toxins [1]. Since then, the field of cancer immunotherapy has evolved drastically, mainly due to a number of crucial breakthroughs in our understanding of the complex function of the immune system, a number of which are listed in **Figure 1**.

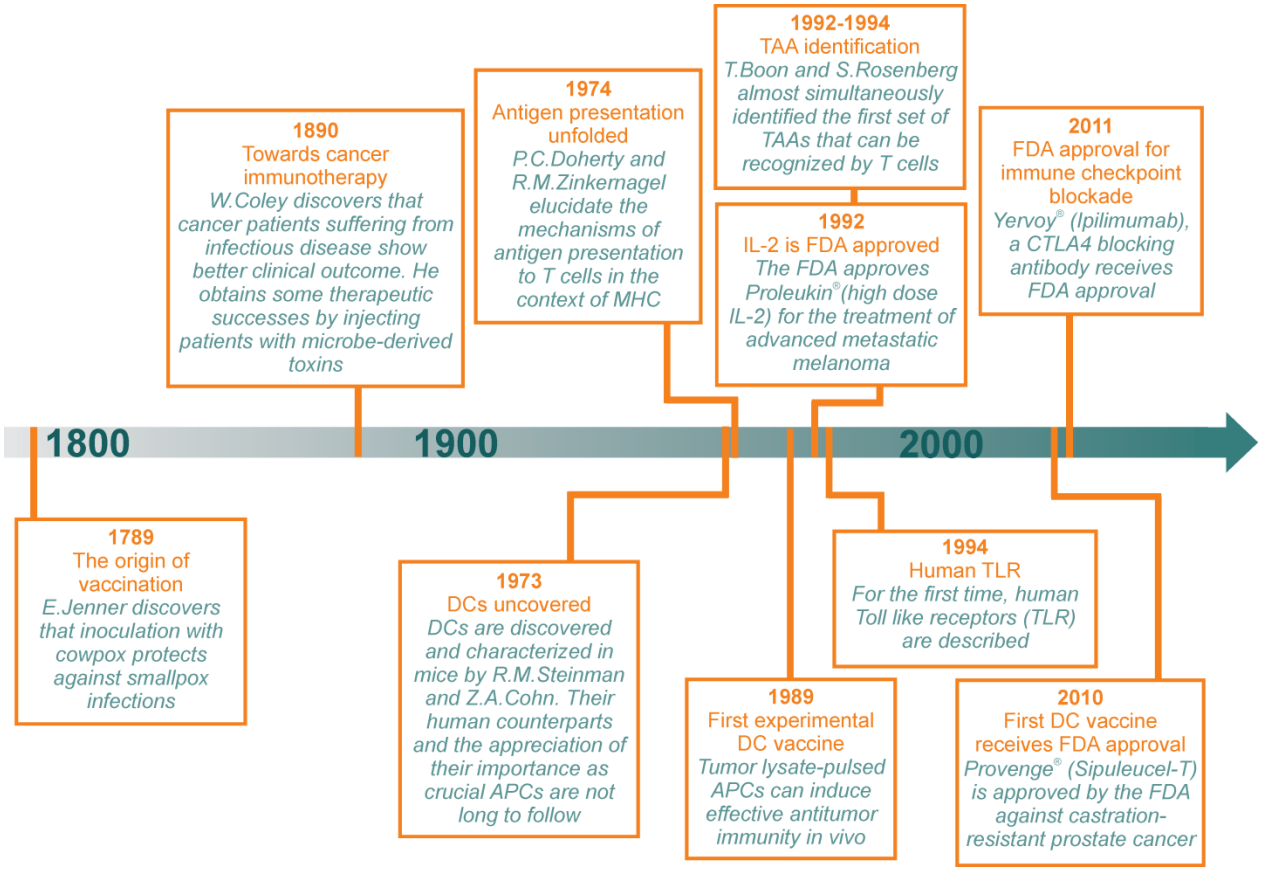


Figure 1: Timeline overview of important discoveries and milestones that have shaped cancer immunotherapy today [2-6].

An essential step forward was the identification of dendritic cells (DCs) as the initiators of immunity. These cells form a bridge between innate and adaptive immunity by capturing antigens and efficiently presenting them to T cells in specialized molecules called major histocompatibility complexes (MHCs). Depending on the intracellular location of the antigenic protein, presentation will occur either in MHC-I, for cytoplasmic proteins, or in MHC-II for

exogenous antigens, which have been endo- or phagocytosed by the cell. Once the antigen-MHC complexes are translocated to the DC surface, the presented antigen can be recognized by $CD8^+$ or $CD4^+$ T cells in case of MHC-I or MHC-II presentation, respectively. Importantly, these antigen-presenting cells (APCs) are unmatched in their capacity to activate naïve antigen-specific T cells: DCs are the only cell type that can provide the necessary co-stimulatory signals (e.g. ligation of CD86 or CD80 on the DC surface to CD28 on the T cell membrane), chemokines (e.g. IP-10 and MIG) and cytokines (e.g. type I IFN and IL-12) required for T cell stimulation. This way DCs can initiate proliferation and differentiation of antigen-specific $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs), and $CD4^+$ T cells into helper T cells (Th) [3].

In the meanwhile, researchers were looking into the Achilles' heel of tumor cells. For a long time, the existence of tumor-associated antigens (TAAs), which are selectively or preferentially expressed by tumor cells, was presumed but not proven. As early as 1905, Clowes and Baeslack reported that immunity occurred in mice that spontaneously recovered from their tumor burden [7]. This was further evidenced by Gross' experiments in 1943, who proved that somehow the immune system of mice was capable of recognizing and rejecting implanted tumors [8]. However, it was only in the early 1990s that the first sets of TAAs against which CTL responses could be mounted, were actually identified [9, 10]. A timeline review where the milestones in TAA discovery and the importance of the choice of TAA for immunotherapy were highlighted, was recently published by Coulie et al. [10]

Taken together, the soft spots of tumor cells and the strengths of our immune system were exposed. By combining these novel insights, the road to the development of new therapeutic strategies that harness the patient's immune system to selectively recognize and destroy tumor cells was paved. Of note, this review will focus on inducing antitumor immunity by targeting DCs. Excellent reviews on other promising strategies in immunotherapy, such as adoptive T cell therapy and immune checkpoint blockade, can be found in references [11-13].

2. Inducing antitumor immunity

2.1. Dendritic cell-based immunotherapy

The identification of DCs as the initiators of immunity made them interesting targets in cancer immunotherapy. Not so long after their discovery, the first report on DC-based vaccination was published [14]. In DC-based vaccination, DCs are modified to present TAAs to T cells resulting in TAA-specific CTL activation. The production of such cellular vaccines firstly requires loading of the DCs with TAAs, after which the cells need to be matured, in order to become potent APCs.

With regards to the antigen-loading of DCs, various strategies have already been explored. The earliest studies made use of tumor lysates as a source of antigen. Later on, based on the discovery of TAAs, recombinant antigens or antigen-derived peptides were produced and passively pulsed into DCs. More recently, researchers have moved on to the use of viral vectors for the delivery of DNA or RNA encoding TAAs [15, 16]. Due to safety issues associated with the use of these pathogen-based delivery systems, the use of non-viral carriers for the delivery of DNA and RNA are currently under investigation.

After antigen-loading, the DCs are exposed to maturation stimuli. These can be pathogen or danger-associated molecular patterns (PAMPs and DAMPs respectively) that can bind to the DCs' pathogen recognition receptors (PRRs) and initiate an intracellular cascade resulting in the production of pro-inflammatory cytokines, increased antigen-presentation and augmented expression of co-stimulatory molecules (e.g. CD40, CD80, CD86) and chemokine receptors (e.g. CCR7). The mature antigen-presenting DCs can then be injected as therapeutic cancer vaccines. The initial results were promising, and in 2010, years of research in this field were rewarded by the FDA approval of the first DC-based vaccine: Provenge® (Sipuleucel-T, marketed by Dendreon) [17].

Although DC vaccine research is flourishing, a number of important drawbacks hinder the applicability of current DC-based vaccines. First of all, and this might even be the most limiting factor, all the modification steps described above (i.e. antigen-loading and maturation) are performed on isolated cells. This way, the production of these vaccines is labor-intensive. In

addition, working with isolated cells makes these therapeutics patient-specific. For every other patient, cell isolation, antigen-loading and maturation needs to be repeated, which adds to the production costs of the vaccines. Thirdly, it was shown that upon subcutaneous injection of the cellular vaccine, DC survival and migration towards the lymph nodes where T cell activation occurs, is poor. Merely 5% of the transferred cells are capable of reaching the lymphatics, requiring the use of large numbers of antigen-modified cells in order to establish therapeutic effects [18]. As a result, only cells that can be isolated in sufficient numbers can be employed. For primary plasmacytoid or "conventional" DCs, this is a challenge, therefore researchers have chosen for a long time to use monocyte-derived DCs (MoDCs). These cells are obtained by isolating a patient's peripheral blood monocytes (PBMCs) and culturing them with cytokines (GM-CSF with or without IL-4) to stimulate their differentiation into MoDCs. These cells, however, do not show the same phenotypic properties as most of the lymphoid-resident DCs, but rather correspond to "emergency DCs" which are usually only detected at sites of inflammation [19]. This, together with loss of activation of the cells once injected, and insufficient cytokine production by the *in vitro* modulated cells, has sparked interest to find ways of loading antigens into various circulating DCs subsets *in vivo* [20, 21].

2.2. Advantages of particulate systems for DC vaccination *in vivo*

The search for *in vivo* antigen-delivery techniques promptly resulted in the production of nanoparticles (NPs) and microparticles (MPs) to carry antigenic material towards DCs in the skin or the lymphatics, since the use of particle structures offers many important benefits over the delivery of free antigen. Before describing the advantages of particulate vaccines it should be noted that a number of prerequisites will determine whether these particulate cancer vaccines are indeed able to induce potent immune responses *in vivo*.

A first important point is that many novel antigen-delivery systems are still entirely evaluated *in vitro* on murine or human DCs that were generated from bone marrow or blood precursor cells, respectively. Indeed, a thorough characterization of the materials with respect to their capacity to encapsulate antigen and/or adjuvant, their stability, uptake by DCs and antigen-transfer to these cells, purely *in vitro* testing comes with a number of pitfalls. Firstly, it should be noted that *in vitro* generated DCs need to be considered as models for a complex *in vivo* situation where numerous types of DCs exist [22-24]. In addition, for murine DCs, it was reported that

slight variations in cell culture protocol resulted in large variation in the phenotype of the generated cells, as well as in their capacity to respond to particulate antigen [25]. Moreover, *in vitro* experiments evaluating the particle's characteristics and uptake by DCs are often exclusively performed in serum-free media, making it even more difficult to draw conclusions on their *in vivo* behavior. Therefore, *in vitro* validation of new carriers should comprise proof of the compatibility of the carrier with biological fluids with respect to particle aggregation, size and antigen release kinetics. For example, cationic particles are often used for electrostatic binding of antigenic protein or nucleic acids encoding antigens. Without additional surface modifications, such as grafting polyethylene glycol (PEG) chains on the outer particle surface to provide the particle with a hydrophilic shield that reduces the particles' surface charge, these structures tend to quickly aggregate upon contact with serum proteins. This can result in premature antigen release and a change in particle size, which leads to different cellular uptake and antigen transfer kinetics [26, 27].

Secondly, the aim of antigen-loaded particles is to deliver the antigenic material to APCs for the induction of potent antitumor immune responses. Importantly, evidence emerged indicating that potent immune responses can only be mounted when antigens are presented to T cells by mature DCs. In fact, where mature DCs stimulate antitumor immunity, antigen-presentation by their immature counterparts will rather lead to tolerance and suppression of effector antigen-specific T cells [28, 29]. The process of DC maturation is complex, finally resulting in a complete shift of the cell's function towards antigen presentation. Thus, fully mature DCs are characterized by (a) combined up-regulation of numerous co-stimulatory molecules (e.g. CD40, CD80, CD86), (b) cytokine production (e.g. IL-12p70 and IL-6) and (c) increased migratory capacities for their translocation to the T cell areas of the lymph nodes. Only when all of these processes occur simultaneously are DCs considered as fully mature and are they expected to be able to mount potent antitumor immune responses. Thus, an *in vivo* applicable particulate system for DC vaccination should not only deliver antigen, but also exhibit immune adjuvant effects and induce complete maturation of the antigen-loaded DCs. Whether NPs and MPs as such possess intrinsic adjuvant activity is a recurring topic of discussion. Thorough comparisons of the various reports are difficult to make due to the plethora of confounding factors (e.g. particle size, surface charge, production methods, additional surface modifications) that prevent unbiased conclusion-drawing. Hence, the opinions on carrier-related auto-adjuvancy

remain divided: cationic liposomes might induce surface charge density-related DC activation [30-32], others show exactly the opposite, pointing towards enhanced adjuvancy of neutral or anionic liposomes [33]. Similar debates arose for PLGA capsules, another commonly investigated antigen-carrier. Whereas Sharp et al. pointed out that PLGA could induce significant cytokine production [34], many other researchers could not observe any PLGA-induced DC maturation or cytokine secretion [35]. For most particles, however, it is clear that stimulation of DC maturation by including adjuvants will result in the production of more effective particulate vaccines. Of note, it was demonstrated that it is crucial to deliver both antigen and adjuvant to the same intracellular compartment to obtain both $CD4^+$ and $CD8^+$ T cell activation [36]. Schlosser and colleagues investigated the effect of delivering antigenic protein and adjuvant (CpG oligodeoxynucleotides (ODNs), a toll-like receptor 9 (TLR9) agonist or Poly(I:C), a known TLR3 agonist) packaged in separate Poly(lactic-co-glycolic) acid (PLGA) microspheres, or co-formulated within the same microsphere. As expected, co-encapsulation of antigen with a TLR agonist significantly improved antigen (cross-)presentation and induction of potent CTL responses *in vivo* [37]. Similar results demonstrating the need for physical association of antigen, carrier and adjuvant were obtained for liposomes by Zaks et al. [38]. Therefore, particles should be co-formulated with both antigen and PRR agonists (often TLR agonists). In this way, a three-component system is produced, containing (a) a target antigen, which will direct the immune responses towards the cancer cells, (b) an immune potentiator to enhance immune responses against the antigen, and (c) a suitable particulate carrier to deliver antigen and adjuvant to the DCs at the site of interest.

Thirdly, choosing a potent adjuvant is crucial, and this could also become a pitfall in particle design. For this, it is first of all important to consider that not all TLRs are expressed by all DC subsets. Therefore rational design and adjuvant selection based on the subset of DCs that will be targeted by the particulate vaccine *in vivo* are key. Moreover, the cellular location of the TLR which is targeted by the adjuvant-particle composite can also play a role. This is exemplified by the work of Bal and colleagues, who evaluated liposomal formulations containing either Pam_3CSK_4 (a TLR2-1 agonist) or CpG ODNs (a TLR9 agonist). They observed that the delivery of adjuvants to endosomal TLRs, such as TLR3, 7, 8 and 9 benefit more from the liposomal delivery than adjuvants to TLRs that are located on the cell surface. As a result, liposomal encapsulation of CpG ODNs resulted in prominently better results over liposomal

Pam_3CSK_4 [39]. Furthermore, there are differences in TLR expression on mouse and human DC subsets. For instance TLR9, receptor to the popular adjuvant CpG ODNs, is absent on most human DC subtypes [40]. Careful and critical interpretation of immunological effects observed in mouse models is therefore warranted. An overview of the TLRs, their cellular location and commonly used agonists is given in **Figure 2**. Of note, also other danger-sensing pathways have been identified that could be of interest for the development of molecular adjuvants for immunotherapy. Potential targets include cytosolic sensors for DNA (e.g. STING) and RNA (e.g. RIG-I and MDA-5) and the inflammasome [41, 42].

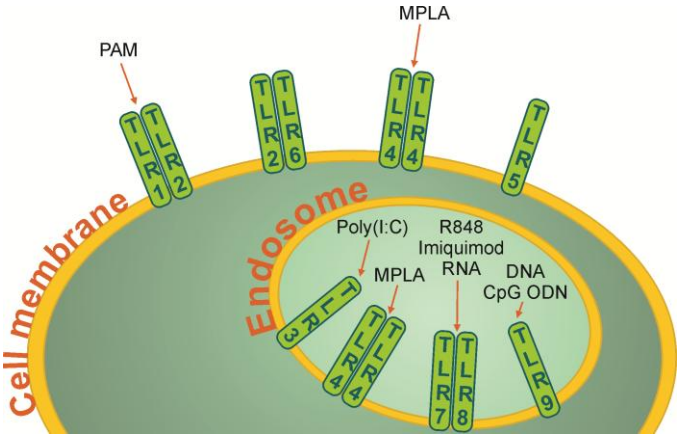


Figure 2. TLRs on the cell membrane and within the endosomes can ligate to a variety of agonists. By using these agonists as immune adjuvants, DC maturation can be induced and antigen-presentation and stimulation of antigen-specific T cells becomes possible. (TLR = toll like receptor; PAM = Pam_3CSK_4; MPLA = monophosphoryl lipid A; ODN = oligodeoxynucleotide)

Taking the pitfalls of *in vitro-in vivo* translation and the requirement for simultaneous antigen and adjuvant delivery into account, the following sections will describe the advantages of different biomaterials for vaccination purposes, and the different strategies that are used to enhance the particulate's immunogenicity. An overview of different carrier-antigen-adjuvant combinations that have been designed and evaluated is listed in **Table 1**.

Table 1: Nanoparticulate carriers for simultaneous antigen and adjuvant delivery to DCs *in vivo*.

Carrier	Particle-related advantages	Antigenic cargo	Combined immune adjuvants	References
PLGA	Antigen release rate can be modified by tweaking the ratio of glycolic acid to lactic acid	Protein	CpG ODNs Poly(I:C) Poly(I:C) combined with resiquimod (R848) R848	[37, 43, 44] [37, 43] [45] [44]
		Tumor lysate	CpG ODNs	[46]
		Peptide	MPLA CpG ODNs	[47] [48]
		pDNA	GpG + IL-10 siRNA Poly(I:C) + IL-10 siRNA	[49] [49]
Liposomes	Easy modification and production Possible auto-adjuvancy (depending on the liposome's physicochemical properties) [31, 39, 50]	Protein	MPLA non-coding DNA CpG ODNs Poly(I:C) TDB (C-type lectin ligand) PAM_3CSK_4 (PAM) Cationic lipid (3,5-didodecycloxybenzamidine, TRX)	[51] [38] [38, 39, 52, 53] [38, 53, 54] [53] [39] [32]
		Peptide	Non-coding DNA CpG ODNs Poly(I:C)	[38] [38, 55] [38]
Cubosomes	Increased encapsulation compared to liposomes	Protein	MPLA + imiquimod	[56]
Gold NPs	NP size is easy to control Tracking via computed tomography (CT) imaging	Protein	CpG ODNs	[57, 58]
Bacterial magnetic particles	Enhanced gene delivery by magnet application Auto-adjuvant effects	pDNA	*Auto-adjuvant*	[59]
Polymeric micelles	High protein loading capacity Auto-adjuvant effects	protein	*Auto-adjuvant* Poly(I:C)	[60] [60]

2.2.1. Passive DC targeting and the loophole of cross-presentation

A first advantage of formulating antigens in particles is that by packaging the antigenic material in larger constructs, antigens are passively targeted towards phagocytic APCs and can be considered for cross-presentation. In general, exogenous antigenic material that is taken up by DCs via phagocytosis or endocytosis will end up in the MHC-II presentation pathway, leading to the activation of Th cells. Cytoplasmic proteins on the other hand, can be presented in MHC-I and result the induction of CTLs. Cross-presentation acts as a loophole in these pathways, allowing exogenous antigens to be redirected towards MHC-I. How exactly phagocytosed or endocytosed antigens find their way into MHC-I presentation remains vague, although increasing evidence demonstrates the involvement of phagosomes, early endosomes and autophagosomes [61-63]. By formulating antigens in NPs and MPs, these cross-presentation-competent organelles can be targeted, resulting in activation of both CTLs (via MHC-I) and long-lasting memory T cells (via MHC-II) [64]. The role of particulate delivery systems in governing antigen-presentation can be illustrated by the research of Stano et al. They elegantly showed that the use of solid-core NPs where antigen is attached to the particle surface, preferentially leads to $CD4^+$ T cell activation, whereas administration of NPs that encapsulate the antigen within their watery core primarily results in $CD8^+$ responses. As a result, the authors propose co-administration of both antigen-loaded particles since these result in superior immune responses with both $CD4^+$ and $CD8^+$ properties [65]. Additional modifications can be made to nanoparticulate vaccines to promote cross-presentation. An example is the inclusion of pH-sensitive polymers into liposomes. After endocytic uptake, the reduced endosomal pH will activate the fusogenic potential of these polymers, causing disruption of the endosomal membrane and cytoplasmatic delivery of the antigenic material. In this way antigen presentation via the MHC-I pathway is stimulated and enhanced CTL induction could be achieved [32, 66]. Of course, this specifically applies for protein antigen delivery. When antigen-encoding DNA or RNA are delivered, this problem completely turns upside down, as the cytoplasmatic protein production upon nucleic acid delivery will favor the MHC-I presentation pathway. Here, strategies that target the produced antigens to the MHC-II pathway (e.g. via attachment of lysosome-targeting sequences) in order to call for T cell help can be applied [67].

2.2.2. Protection of antigen integrity and tuning antigen-release kinetics

Secondly, encapsulating antigens in particles protects them from premature degradation and enables modulation of the antigen delivery kinetics. Antigens that are packaged within NPs or MPs are shielded from digestive enzymes (proteases and nucleases) that are ubiquitous in blood and interstitial fluid. This is especially important for the delivery of nucleic acids (i.e. pDNA or mRNA encoding tumor antigens) to DCs, which are much more prone to premature degradation. The kinetics of antigen presentation were also shown to exert a major effect on the strength, duration and memory of the induced immune responses. For example, Johansen and colleagues showed that dose-escalating antigen contact resulted in much stronger $CD8^+$ T cell responses compared to single-shot antigen delivery [68]. Therefore, particles that result in a burst release of the antigenic material upon engulfment by DCs will induce less pronounced immune responses compared to sustained release particles. This was evidenced by Demento et al. who compared immune response induction by liposomal (fast release) versus slow release PLGA-based protein delivery. Indeed, mice vaccinated with liposomes exhibited lower and shorter-lived antibody titers and a lower frequency of memory T cells compared to PLGA-vaccinated mice. Similar observations were made when slow and fast-release PLGA particles were compared [69]. Thus, tweaking the release kinetics of the encapsulated antigen can augment antitumor immunity. As a result, clever modifications to basic polymer and liposome structures to ensure longer antigen-retention and controllable antigen release have been reported. For example, Moon et al. created covalent crosslinks between different bilayers of multilamellar liposomes in order to slow down the release of antigen [51]. Another considerable option to achieve longer-term antigen presentation by DCs is to use nucleic acid-based vaccines instead of protein or peptide vaccines. In addition to favoring MHC-I antigen presentation as discussed in 2.2.1., genetic vaccines can prolong the duration of antigen presentation and subsequently the activation of CTLs. This was demonstrated in a comparative study of Liao et al. where a sustained period of antigen presentation was measured after RNA transfection, whereas peptide pulsed DCs rapidly lost their ability to present the antigen [70]. Interestingly, the delivery of mRNA have been shown to generate more rapid and longer lasting antigen presentation compared to DNA, making mRNA an interesting source of antigen [71].

2.2.3. Multifaceted antigen-delivery vehicles

In addition, particle design is not limited to the mere creation of an antigen-delivery structure. NPs and MPs can be equipped with tracer molecules or can be constructed from materials that allow *in vivo* imaging of injected antigen-loaded particles. Imaging and tracking the migration of these multifaceted particles from the injection site to the draining lymph nodes can provide initial feedback on the vaccine efficacy. For instance, Lee et al. described the production of gold NPs that could be covalently loaded with a model protein antigen and CpG ODNs. Upon injection of the resulting 23 nm particles in the rear footpads of mice, their migration to the popliteal and inguinal nodes could be followed with CT scans [57] . To date however, not many research groups have reported on such multimodal particulate vaccines yet. The reason for this, is that up to now traceable particles have been mainly designed to determine the fate and migratory capacities of *ex vivo* modified antigen-loaded DCs. The focus on *in vivo* DC targeting is a more recent development in cancer immunotherapy, which gives us reason to believe that new multifaceted particles aiming for *in vivo* modification and migration trafficking are expected to follow.

2.2.4. Alternative biomaterial-based strategies

Another possibility is to target DCs towards the antigens rather than vice versa. For this, Ali and coworkers designed a poly-lactide-co-glycolic (PLG) matrix that releases GM-CSF in order to recruit DCs into the scaffold *in vivo*. Importantly, the authors showed that the number of DCs that could be attracted in this way, resembles the amount of *ex vivo* modified DCs that are routinely injected as a cellular vaccine. Upon DC infiltration into the scaffold, the cells can come in contact with entrapped tumor lysate as a source of antigen, and CpG ODNs as immune stimulants. By fine-tuning the GM-CSF release kinetics from the polymer matrix, the antigen-loaded mature DCs can be released from the scaffold and migrate to the lymph nodes to allow T cell induction [72, 73]. This discovery has lead to the design of alternative immune priming centers that aim to attract and *in vivo* modulate DCs [74].

3. The hurdles of tumor-induced immune suppression

However promising DC vaccination seemed in the early days, this therapeutic strategy failed to live up to the expectations in a clinical setting. Most DC vaccines could increase the overall survival by a couple of months with a number of reports describing tumor regression in about 15% of patients. Nevertheless, in a high number of patients no effective immune response can be induced despite the induction of tumor-specific T cells upon vaccination [21, 75, 76]. In part this can be explained by the advanced disease in most patients that were included in DC vaccination studies. A more important explanation, which is related to the previous one, is immune suppression. Increasing evidence indicates that during tumor development a growth-supporting micro-environment is created, harboring a plethora of immune suppressive cell types which allow the tumor to escape from immune recognition and block effector immune cells at the tumor site [77, 78]. Therefore, even if using intelligent antigen- and adjuvant-delivery strategies results in the effective proliferation of antitumor CTLs, their recruitment to the tumor or their antitumor effector function might be blocked.

3.1. Mechanisms supporting tumor progression

When taking a closer look into the tumor microenvironment, the tumor cells themselves act as the main mediators of immune suppression. Via different processes, tumors stimulate their own growth and deeper tissue invasion. A schematic overview of these mechanisms is given in **Figure 3**. In order to provide nutritional supplements, rapidly growing tumors stimulate angiogenesis by secretion of the vascular endothelial growth factor (VEGF). These newly formed vessels, however, have larger fenestrae in between the endothelial cells. This results in the enhanced permeability and retention (EPR) effect: larger molecules and NPs are capable of leaving the circulation and entering the tumor tissue where they are retained due to the reduced lymphatic drainage within the tumor tissue [79]. However, neovascularization is often too slow to keep up with the rapidly increasing tumor mass, resulting in local hypoxic regions. Especially there but even when oxygen is available the growing tumor will increase their glycolytic activity, resulting in the accumulation of lactic acid, which reduces the interstitial pH to values of generally 7.0-6.6, although values as low as 5.8 have also been reported. This phenomenon of “aerobic glycolysis” is known as the Warburg effect [80]. An additional feature of invading

tumor cells is their capacity to degrade the extracellular matrix in order to promote deeper tissue penetration by producing matrix metalloproteinases (MMPs) [81].

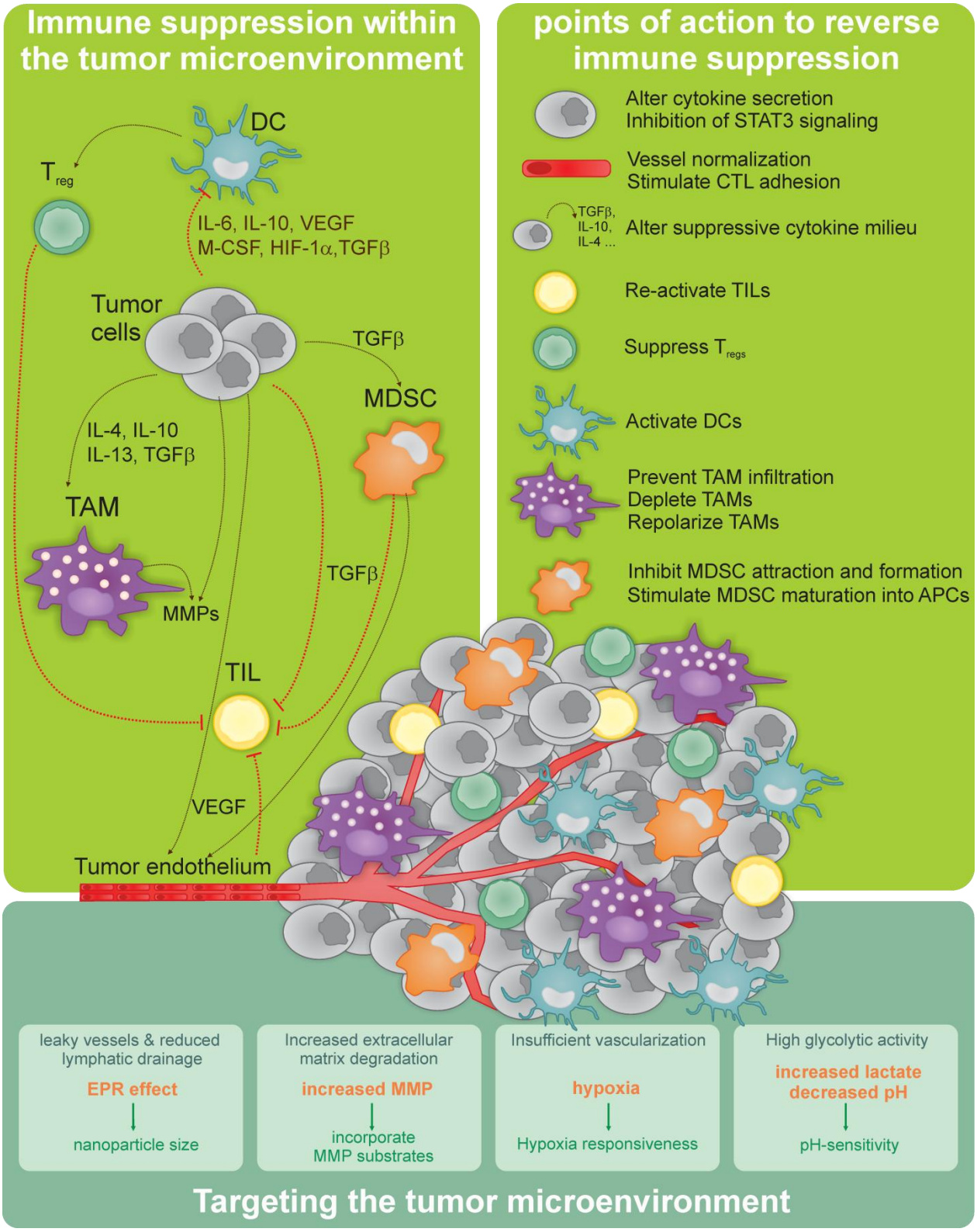


Figure 3. The tumor microenvironment. Schematic representation of the soluble and cellular mediators of the immune suppressive microenvironment (upper left panel) and how these can be addressed to overcome tumor-induced immune suppression (upper right panel). Various factors, such as cytokines, secreted by tumor cells create a local suppressive milieu in which different immune cells infiltrate and are forced towards an immunosuppressive state. These suppressive mechanisms can be generally or specifically addressed to counteract the local tumor-promoting microenvironment, and (re)activate antitumor immune responses. The lower panel explains the basic biochemical changes at the tumor site, and how these can be used to target NPs to the tumor microenvironment. (Abbreviations: DC = dendritic cell; TAM = tumor-associated macrophage; MDSC = myeloid-derived suppressor cell; T_{reg} = regulatory T cell; TIL = tumor-infiltrating lymphocyte; VEGF = vascular endothelial growth factor; M-CSF = macrophage colony-stimulating factor; HIF1α = hypoxia-inducible factor 1α; TGFβ = transforming growth factor beta; STAT3 = signal transducer and activator of transcription 3; CTL = cytotoxic T lymphocyte; APC = antigen-presenting cell; MMP = matrix metalloproteinase; EPR = enhanced permeability and retention)

Along with these biochemical effects, growing tumors create a local inflammatory environment. Already within the early stages of uncontrolled proliferation, infiltration of immune cells into the tumor mass has been observed. This can be correlated to the immune surveillance theory, which states that the immune system plays a major role in protecting against tumor growth. Indeed, the presence of tumor-infiltrating lymphocytes (TILs), mainly antitumor CTLs and natural killer (NK) cells within the tumor and in the circulation points are correlated with a better patient prognosis, providing evidence for the immune system's attempt to clear the malignant cells [10, 82]. On the other hand the tumor microenvironment is also characterized by the prevalence of other immune cells, which will rather support than suppress tumor growth. A non-limiting overview of the major mediators of tumor-induced immune suppression is presented in Figure 2. Briefly, secretion of inflammatory mediators such as transforming growth factor β (TGFβ) by the tumor cells will cause the inactivation of DCs, thus abrogating their capacity to efficiently present antigens and induce T cell activation. What is more, suppressive DCs will even induce activation of regulatory T cells (T_{regs}) that counteract CTL responses. Monocytes that are attracted into the tumor microenvironment can differentiate into macrophages which either promote or counteract tumor growth, depending on the local environment [83]. Although this is a gross generalization, M1 polarized macrophages (stimulated by INFγ) are tumoricidal, whereas M2 polarized macrophages, also known as tumor-associated macrophages (TAMs, differentiation upon encounter of TGFβ, IL-10, IL-4 and IL-13) will stimulate tumor progression. Another major mediator of tumor-induced immune suppression are the myeloid-derived suppressor cells (MDSCs). These aberrantly differentiated myeloid cells will secrete suppressive cytokines that in

their turn block the activity of TILs and induce the expansion of T_{reg} populations. More thorough reviews on the different cell types that are present within the tumor microenvironment can be found elsewhere [84-86].

All these soluble factors and suppressive cells help to create a local environment that supports tumor growth and counteracts the antitumor effects of activated CTLs and NK cells Therefore, finding ways to overcome this tumor-mediated immune suppression and to shift the balance back from immune suppression towards immune stimulation could significantly improve the immunotherapeutic outcome. Interestingly, many of the immune stimulants that can be used to promote the activity of DC vaccines, could also serve as potent adjuvants to modulate (suppressed) immunity [87-89]. However, systemic application of these immune activating factors generally leads to severe side effects due to unspecific immune activation [90, 91]. Thus a more localized approach is warranted. As a result, nano- and micromaterials have been produced to locally deliver immune-activating signals in order to shift suppressive cells to an immune stimulating phenotype and to overpower immune suppression. The next sections will provide an overview of the strategies that can be used to target the tumor microenvironment and which particle and adjuvant combinations can target and counteract these different suppressive cell types.

3.2. Particulate systems to overcome tumor-induced immune suppression.

3.2.1. Targeting the tumor microenvironment

In order to deliver immunostimulants to different target cells within the tumor microenvironment, particulate adjuvants should be able to be directed towards the tumor site, deeply penetrate into the tumor tissue and locally deliver their cargo to the target cells. This can be made possible by designing NPs that respond to the biochemical differences that exist between tumors and neighboring tissues. The different options include (a) making use of the EPR effect by controlling NP size; (b) including pH-sensitive materials into nanomaterials; (c) creating hypoxia-responsive nanoparticles or (d) including substrates for intratumoral MMPs.

The EPR effect can be utilized by packaging therapeutics into long-circulating NPs that are too large to leave the vasculature in healthy tissue, but that do pass through the larger fenestrae of

aberrant neoangiogenic tumor vasculature. Variable pore sizes in solid tumor vasculature have been reported, depending on the type of tumor and its localization, but overall, sizes range from 380 to 780 nm [92]. This means that the ideal NP size to benefit from this EPR effect is likely between 10 nm and 400 nm although the upper size range will be tumor-dependent. Indeed, different studies have shown selective uptake and drug release into the tumor based on this principle. However, the EPR effect only ensures enhanced extravasation and prolonged retention of the particles at the tumor site. In order for NPs to reach more distant regions within the dense tumor network or to ensure efficient internalization by cells in the perivascular region, additional modification strategies are needed. This has resulted in the design of NPs that can extravasate at the tumor site where they respond to pH, oxygen levels or MMP presents. As a result, NP penetration into the tumor tissue and/or cellular uptake can be promoted. Overall, the benefits of utilizing the EPR effect should be addressed with the necessary caution. The main reasons for this are the heterogeneity of the EPR effect in different tumors and the limited data that point out the advantages of using the EPR effect in humans [93].

As a first intratumoral trigger, the decreased intratumoral pH can be exploited. As an example, Hu and colleagues produced 35-65 nm cationic micelles that were shielded by PEG via a pH-sensitive linker. Thus, in the circulation the PEG corona protects the particles from unspecific uptake by phagocytes, whereas at the tumor site, the PEG corona is removed and cellular uptake is allowed [94]. It is important to note that the difference in pH between healthy tissues (pH 7.4) is usually not that drastically different from the pH in the microenvironment of solid tumors (generally around pH 6.6-7.0). Therefore, tumor-targeting particles should be designed to already respond to slight differences in pH. Materials that need larger pH differences to release their payload or change in conformation could rather serve to enhance cytoplasmic delivery upon endosomal acidification, as this occurs at lower pH values. This could then aid in avoiding further lysosomal degradation or to promote antigen cross-presentation, as previously discussed in section 2.1.1 [95].

Secondly, the hypoxic tumor microenvironment is characterized by reduced local oxygen pressures down to 5-10 mmHg [96]. By incorporating oxygen-sensitive moieties, such as 2-nitroimidazoles or azobenzene into NPs, hypoxia can be used to trigger PEG-deshielding and

enhance cellular uptake of NPs that have reached the tumor site [97] or to provoke drug release within the tumor site [98].

It is important to consider that hypoxia and decreased pH only occur more deeply within the tumor. When NPs shrink due to acidic pH or hypoxia in order to allow deeper tumor penetration, the NPs should already be able to cover a substantial distance within the tumor before they actually encounter their triggers and decrease in size. The increased MMP levels on the other hand, are detectable rapidly after extravasation, making this an interesting trigger for size-changing NPs. This was elegantly demonstrated by Wong et al, who designed 100 nm gelatin particles that can benefit from the EPR effect to be delivered at the tumor site. Immediately after extravasation, the gelatin core is degraded by MMP-2 and MMP-9 (both potent gelatinases), resulting in the release of smaller 10 nm particles that experience less hindrance during their migration through the tumor's extracellular matrix. This way, delivery deep into the tumor tissue could be achieved [99]. Combining multiple tumor-targeting approaches can significantly increase the NP's selectivity towards the tumor. For example, Huang et al. even combined three of these tumor-targeting approaches. They reported on a 100 nm polymer NP that is equipped with a MMP- and pH-sensitive masked cell penetrating peptide (CPP) for DNA delivery. Upon extravasion based on the EPR effect, the reduced intratumoral pH will loosen the electrostatic interaction between the CPP and its masking sequence that is cleaved from the CPP by MMPs. Thus, the unmasked CPP is free to facilitate cellular uptake and enhance intratumoral DNA delivery [100].

3.2.2. Picking and targeting immune suppressive players within the tumor microenvironment.

Once NPs can penetrate deeply into tumor tissue, they still need to deliver their therapeutic cargo to the target cells of interest. We have previously described which cells play a role in sustaining the immunosuppressed tumor microenvironment, and each of them can serve as a target to subvert local immune suppression (section 3.1). In the following paragraphs the development of a NP toolbox designed to target different cellular components of the suppressive microenvironment (summarized in Figure 3, upper right panel) will be highlighted.

The key initiators of immune tolerance are obviously the tumor cells themselves. They secrete immunosuppressive cytokines and a plethora of other factors to sustain their own growth

and express ligands on their surface that block the activity of TILs (e.g. programmed death ligand-1, PD-L1, which induces CTL apoptosis). Therefore, finding ways to suppress tumor cell-mediated suppressive mechanisms could tip the balance back towards immune stimulation and result in enhanced antitumor effects. An important pathway that mediates immune suppression at the tumor microenvironment, is STAT3 (signal transducer and activator of transcription 3) signaling. STAT3 mediates tumor growth by – amongst other things – promoting angiogenesis and hypoxia, increasing the expression of MMPs and by inducing the secretion of suppressive cytokines (e.g. IL-10, IL6, TGFβ) whilst reducing the production of pro-inflammatory cytokines (e.g. IL-12, IFNγ, TNF) [101, 102]. Knowledge on the important role of STAT3 has lead to the production of NPs that interfere with this pathway, e.g. by delivery of STAT3 siRNA [103]. Specific targeting of NPs to cancer cells is possible by incorporating ligands such as folate or transferrin. Overall, the number of NPs that address immunotherapeutic targets on tumor cells is limited. The reason for this is the genetic instability of transformed cells, which is even more pronounced due to the favorable microclimate within the tumor environment. Therefore, tumor cells are not such an interesting target as they are capable to rapidly adapt to changed circumstances.

Closely related to the tumor cells, tumor endothelium also acts as a major barrier for TILs to reach their target cells [104]. Targeting tumor vasculature is not only interesting to highjack the tumor's oxygen and nutrient supply by delivering anti-angiogenic agents, they can also be addressed in light of immunotherapy. An interesting example is the use of CpG ODN-containing liposomes which bind to the tumor blood vessels and create a local inflammatory environment. This was shown to result in increased numbers of infiltrating $CD4^+$ and $CD8^+$ T cells into the tumor tissue and the induction of antitumor CTLs [105]. Selectively targeting NPs to the tumor endothelium is possible by targeting tumor endothelial markers such as the vascular endothelial growth factor receptors (VEGF-R) or by using vascular-targeting peptides such as RGR or RGD [106].

As many immune suppressive mechanisms are initiated by intratumoral cytokines, which abolish antitumor immunity and promote tumor progression, locally modulating the cytokine milieu within the tumor stroma is a therapeutic strategy that can simultaneously tackle multiple immunosuppressive mechanisms. Especially TILs and T_{regs} which are respectively suppressed or

induced by the local cytokine environment, are hard-to-reach cellular targets, but their functionality can rapidly change by modulating the local cytokine situation. Therefore, packaged cytokines that can target the tumor microenvironment and in this way avoid adverse events that are typically associated with systemic cytokine delivery, could be powerful immunomodulators. Most liposomal or polymeric cytokine-releasing particles (as shown for IL-2 and IL-12) that have been designed to date indeed showed significant tumor growth slow-down, however, they did not address tumor targeting but were injected intratumorally instead [107-110]. Designing delivery systems that can target the tumor microenvironment upon systemic administration, based on the principles described in section 3.2.1, could beyond doubt enhance the particle's applicability. For instance, Park and coworkers developed nanoscale liposomal polymeric gels co-encapsulating a TGFβ inhibitor and IL-2 that were shown to be delivered to the tumor upon intravenous administration. This resulted in an increased NK cell infiltration and a higher CTL-to-T_{reg} ratio, which in turn delayed tumor growth and prolonged overall survival in a mouse melanoma model [111]. Another strategy could be to even go one step further backwards by opposing two of the driving forces of tumor-mediated immune suppression, which steer infiltrating immune cells towards a suppressive state: acidosis and hypoxia. This is exemplified by the work of Prasad et al. who developed NPs that consume intratumoral H_2O_2 while producing O_2 and increasing the local pH from 6.7 to 7.2 [112].

Recently developed NPs for tumor microenvironment modulation attempt to address specific subsets of tumor-infiltrating immune cells. The reasons why these are such interesting targets are plural: they are genetically stable, upon activation or re-purposing, their cytokine secretion profile changes, which in turn will affect other immune cells that reside in the tumor microenvironment. It should first of all be noted that most of the particles that are described below generally target the tumor microenvironment. Only in some cases are additional modifications made to reach specific cellular targets. As a result, many of these NPs could be taken up by various intratumoral immune cells. Moreover, most immunosuppressive cell types present within the tumor microenvironment can be stimulated in a similar way, more specifically: TLR triggering has beneficial effects on intratumoral DCs, TAMs as well as MDSCs, shifting their function towards immune activation and tumor growth suppression [85]. Thus, it is often difficult to exactly pinpoint the cells that mediate the observed effects, especially since a complete investigation of the effects of a single NP on all mediators of intratumoral immune

suppression, was not always performed. Keeping this in mind, NP solutions to tackle different cell subsets within the tumor microenvironment will be discussed in the following paragraphs.

A first specific subset of immune cells that can be steered towards antitumor immunity is the intratumoral DC population (**Figure 4**). Infiltrated DCs can be altered by the tumor microclimate to induce T cell anergy or even apoptosis (via PD-L1), stimulate T_{regs} and to produce immunosuppressive cytokines. Thus, there is a major role for NPs in re-educating these intratumoral DCs, and re-establishing their antigen-presenting capacities [104]. The particles that can be used for this share characteristics to particles for immune induction: they mainly incorporate TLR ligands as immune adjuvants to restore their immune activating potential. Examples are the use of PEI to complex PD-L1 siRNA to both silence PD-L1 expression and resulting CTL apoptosis by DCs while simultaneously utilizing PEI's TLR5 and TLR7 stimulatory capacity to activate the cells [113]. Alternatively, Dominguez and Lustgarten reported on a 250 nm PLA NP equipped with a tumor-targeting antibody (in this case anti-*neu* in a breast cancer model) as well as with anti-CD40. The latter can ligate to CD40 expressed by DCs, macrophages and B cells within the tumor, resulting in the significant up-regulation of pro-inflammatory cytokines, reduced intratumoral T_{reg} levels and tumor rejection in 70% of the animals [114].

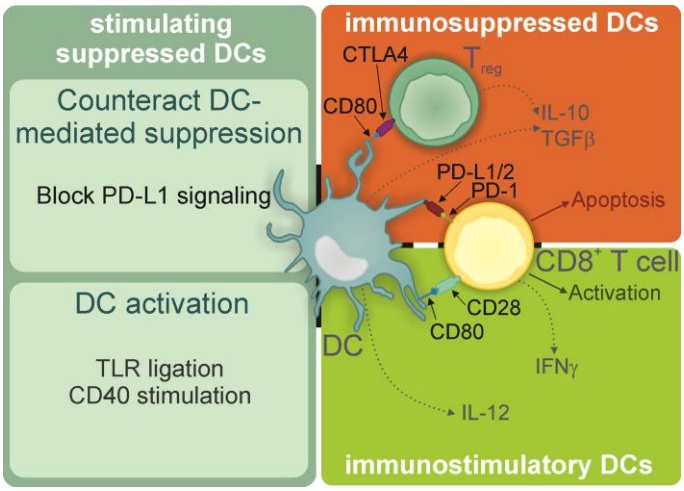


Figure 4: Dendritic cells at the tumor microenvironment. Intratumoral DCs can be forced to an immunosuppressed state by the local cytokine milieu. As a result, they block CTL activation and even induce CTL apoptosis (via PD-1 PD-L1 signaling), activate T_{regs} and secrete immunosuppressive cytokines (upper right panel).

This can be reversed by stimulating DC activation and blocking their suppressive mechanisms (left panel).
(Abbreviations: CTLA4 = cytotoxic T lymphocyte ligand A)

TAMs, which represent a rather heterogeneous population of macrophages that reside within the tumor, play a crucial part in promoting tumor growth. Most importantly, they protect tumor cells from chemotherapeutics, attract T_{regs} and induce CTL apoptosis. To deal with TAMs, there are 3 main options, which are summarized in **Figure 5** [115, 116]. Firstly, the infiltration of inflammatory monocytes and their differentiation into M2 polarized macrophages could be inhibited. For this, strategies aiming to reduce the production of chemo-attractants (e.g. macrophage colony-stimulating factor, M-CSF) or modulate the suppressive cytokines milieu responsible for TAM development (e.g. IL-10 and IL-4) can be used. Currently the number of NPs that are specifically designed to address this pathway are limited. They mainly focus on sustained release of cytokines within the tumor milieu, as previously discussed. Secondly, it is possible to deplete TAMs from the tumor microenvironment, which was shown to restore the antitumoral activity of CTLs and lead to tumor growth reduction [117]. A routinely used NP-based method to selectively deplete TAMs, is the use of liposomal formulations containing clodronate or glucocorticoids [118, 119]. Also, Leuschner et al. described a siRNA liposome-based strategy that prevents the migration of inflammatory monocytes causing a strong reduction of TAMs in the tumor site [120]. Lastly and most prominent in current NP research, is finding ways to enhance the antitumoral M1 effects of TAMs while blocking their tumor-supporting M2 effects. Inducing this macrophage switch can be achieved by delivering TLR agonists. Especially for the delivery of CpG ODNs (that ligate to TLR9), different delivery vehicles have been designed. For instance, gold NPs can be conjugated with CpG ODNs via thiol linkage and showed promising cytokine productions *in vitro* and *in vivo* [121, 122]. Other groups focused on the auto-adjuvant effects of cationic polymers, such as PEI to convert TAMs [123]. However, when targeting TAMs, it is important to consider that systemically injected NPs are often subject to unwanted clearance by normal macrophages. To avoid this, microenvironment-targeting strategies should be employed during particle design. This is exemplified by the work of Huang et al. who report on galactosylated cationic dextran particles that are PEGylated via a pH-sensitive linker to protect the particles against cellular uptake outside of the tumor microenvironment, but release them from uptake within the tumor site. There, the presence of the galactose moieties allows macrophage targeting, resulting in selective delivery of the CpG ODNs

to TAMs. As a result, a phenotypic shift towards M1, reduced neovascularization and reduced tumor outgrowth could be observed [124]. Alternative TAM-targeting moieties could be mannose and β-glucan (both substrates for the macrophage marker CD206) or folate, since the folate receptor β is considered a marker for M2 regulatory macrophages [125].

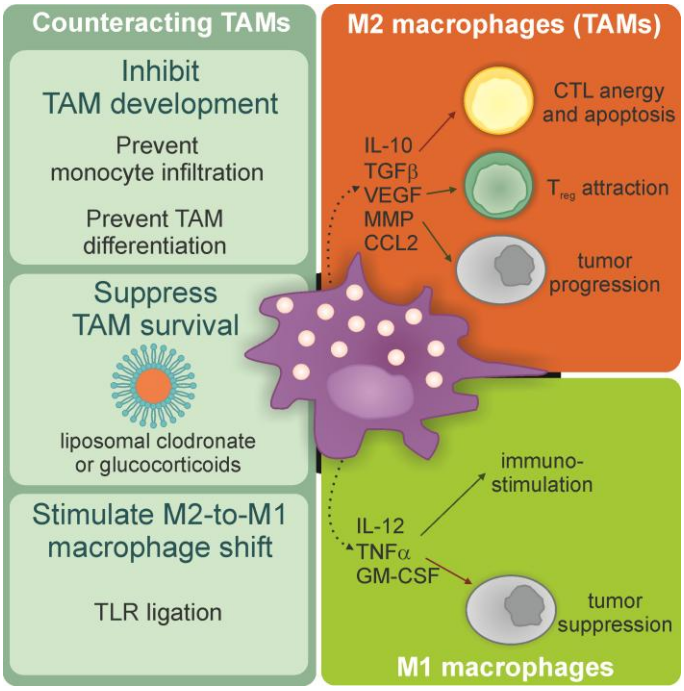


Figure 5: Tumor-associated macrophages. TAMs are M2 polarized macrophages which suppress immunity and promote tumor progression. Their pro-tumor influence can be suppressed by preventing TAM formation from infiltrating monocytes, by selectively depleting TAMs or by driving TAMs towards a tumoricidal and immunostimulatory M1 macrophage phenotype. (Abbreviations: CCL2 = chemokine (C-C motif) ligand 2; TNFα = tumor necrosis factor α; GM-CSF = granulocyte macrophage colony-stimulating factor)

During cancer development, the differentiation of myeloid cells is altered, promoting the existence of a diverse population of pathological MDSCs. Similar to their other suppressive accomplices, the main actions of MDSCs to promote tumor progression are impairing CTL migration into the tumor, reducing NK cell function, supporting TAM activity and expanding T_{reg} populations (**Figure 6**) [85]. The key to overthrowing their suppressive effects is to force their differentiation and maturation into tumor-rejecting monocytes and even functional APCs. Although research in the pathways involved in MDSC formation and maturation is still ongoing and novel potential targets to deplete MDSCs are being identified [126], successes have been obtained via cytokine therapy (local delivery of IL-12 or IFNα) or by the triggering of their TLRs

[127]. Once more, CpG ODN-delivering NPs were shown to have potential [122] as well as less known particulate adjuvants, such as very small size proteoliposomes (that combine outer membrane vesicles from *N.meningitidis* with a GM3 ganglioside) which were shown to modulate myeloid populations within the tumor environment, overcome T cell unresponsiveness and reduce tumor growth [128].

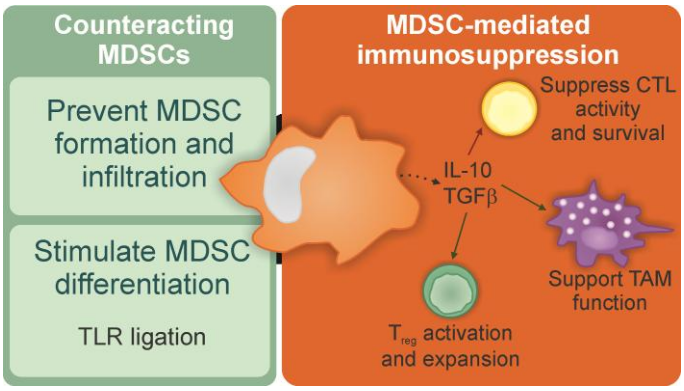


Figure 6: Myeloid-derived suppressor cells. MDSCs represent a heterogeneous population of aberrantly differentiated myeloid cells that suppress CTL activity, activate and expand T_{regs} and promote TAM function. Their effects can be counteracted by preventing their formation and their infiltration, or by inducing maturation of MDSCs into antitumoral monocytes or APCs.

Once more, we wish to stress that it is very likely that the entire tumor microenvironment benefits from modulation of a single suppressed cell subset. By reverting specific cells to a (re)activated state, they will for example start producing immunostimulatory cytokines, which will likely impact the intratumoral cytokine environment, and could aid to stimulate other immune cell subsets as well. On the other hand, and this might become one of the pitfalls in these immunomodulation strategies, it should not be forgotten that immune adjuvants can also cause adverse effects. First of all, there are the off-site adverse effects, for example when NPs are taken up by phagocytic immune cells outside of the tumor microenvironment (e.g. in the liver and spleen) or when immune adjuvants are released into the circulation. Therefore, peripheral immune activation should always be taken into consideration and delivery strategies need to be optimized to restrict the distribution of immune adjuvants to the tumor microenvironment [129]. Secondly, there are the on-site off-target adverse effects. This can be illustrated by the dichotomous role of TLRs on cancer cells and MDSCs: where short-term high-dose TLR

stimulation can result in antitumor immunity, chronic low-grade TLR ligation will rather promote tumor growth [130, 131].

Overall, tumor-mediated immune suppression is a more recently discovered phenomenon, and this also translates in the number of particulates that have been designed to address this problem. Especially in comparison to NP vaccines for immune induction, we are still lagging behind in addressing the tumor microenvironment. However, we are making important progress. As the influence of these immunological accomplices of the tumor cells are now more acknowledged, researchers are more and more taking effects of therapeutics on immune cells, instead of exclusively on the tumor cells themselves, into account. For example, when She et al. observed a drastic improvement in tumor rejection when pixantrone (a topoisomerase II inhibitor) was encapsulated within liposomes equipped with sialic acid, they hypothesized that this could very well be attributed to TAM killing, as TAMs highly express sialic acid binding receptors on their surface [132]. Whether this is indeed the case remains to be investigated, but it already points out that looking beyond expected effects on cancer cells and broadening our view to also take a look at infiltrating immune cells, could significantly improve novel particulate antitumor therapies. In this light, merging established therapies with the exploitation of our immune system could be particularly interesting and the development of combination therapies is on the rise.

4. Combination therapy

4.1. Immunogenic cell death

Since Polly Matzinger proposed the “danger model” in 1994, the view on the primary occupation of the immune system changed. Where we previously believed that the immune system’s basic occupation was to discriminate between “self” and “non-self”, she postulated that it is far more concerned with recognizing danger and signs of destruction. In this way, the immune system would become activated when it encounters signs of distress, such as cell stress or unprogrammed cell death [133, 134].

In this regard, it would be crucial for the immune system to be able to distinguish programmed cell death, which occurs under normal circumstances, e.g. during embryonic development and unprogrammed cell death, which is associated with for instance viral infections.

This was the dawn of "immunogenic cell death", a process where dying cells become recognizable to the immune system by exposing signals that are normally hidden within the cells [133, 135]. Over the years, this theory gained support and its validity was evidenced by the identification of three key mechanisms that will determine whether cell death can be considered as immunogenic [135]. Firstly, the expression of calreticulin (CRT) is normally restricted to the endoplasmatic reticulum (ER). However, when the ER experiences stress, CRT is translocated to the cell membrane and acts as an "eat-me-signal" activating DCs and macrophages to engulf stressed and dying cells. Secondly, stressed cells can release ATP, which again serves as a chemoattractant for phagocytic cells. Finally, high-mobility group box 1 (HMGB1), a chromatin protein that is expressed by all nucleated cells, can be released. These 3 hallmarks of immunogenic cell death were classified as danger-associated molecular patterns (DAMPs). Similar to PAMPs (discussed in section 2.1) these patterns will trigger changes within APCs, resulting in enhanced antigen presentation and immune stimulation. Moreover, these destroyed tumor cells can release TAAs, giving the immune system new targets to combat [136].

This would mean that inducing tumor cell death in such a way that it becomes immunogenic, dying tumor cells could in a way become auto-vaccines by (a) turning tumor cells into cellular antigens and (b) exerting adjuvant functions that will trigger the immune system to act against released danger signals. Indeed, for various established cancer treatments aiming to destroy as much of the tumor tissue as possible, DAMP exposure was detected. For instance anthracyclines such as doxorubicine as well as tumor irradiation or hyperthermia results in the occurrence of all 3 characteristics of immunogenic cell death [137, 138]. These observations paved the way for something that was long thought to be impossible: combining chemo- and immunotherapy. For years cytostatic drugs were considered to be detrimental for immunity, as toxic effects on lymphocytes are a commonly observed side-effect. Still, studies point towards the synergy between TIL reactivation by blocking PD-1 (which suppresses CTL activation) and glucocorticoid-induced TNFR related protein (GITR) (which additionally stimulates T cells) and chemotherapeutics (paclitaxel or cisplatin) [139-141]. These and other stories of enemies becoming allies were recently picked up by material scientists, who packaged these synergistic components into single NPs. It should be noted that these combinatorial NPs are still in their infancy. In most cases, evidence of their potential is based on *in vitro* characteristics and

biodistribution studies showing their tumor targeting and safety, whereas *in vivo* results are often still underway.

4.2. Chemo-immunotherapy

Combination approaches for chemo-immunotherapy both comprise co-delivery of cytostatic NPs with adjuvant NPs, as well as the generation of NPs in which both the chemotherapeutic drug and the immunostimulant are packaged. Whether or not co-packaging is beneficial depends on the nature of the immunostimulants used. In some cases, pre-treatment with either the chemotherapeutic or the adjuvant will be preferable, thus requiring the sequential delivery of both agents in separate NPs. For example, Su et al. developed TNF-α pDNA polyplexes which were delivered 48 h prior to Doxil® (i.e. commercially available stealth doxorubicin liposomes). Pre-treatment with the TNF-α pDNA resulted in an increased delivery of Doxil® into the tumor, likely due to TNF-α-mediated opening of the tumor endothelial tight junctions and enhanced tumor killing in comparison to treatment with each of these NPs alone [142]. In contrast, Roy and colleagues focused on co-encapsulating NPs. They conjugated paclitaxel to a high-molecular weight sodium phtalate salt of bacteria-derived lipopolysaccharide (SP-LPS), a potent TLR4 agonist. These conjugates self-assemble into ~200 nm NPs which upon intravenous administration increase the amount of phenotypically mature macrophages and activated CTLs into the tumor. More importantly, these conjugate NPs resulted in an improved therapeutic outcome compared to each treatment moiety alone [143]. Similar observations could be made when paclitaxel was co-encapsulated with SP-LPS into PLGA NPs [144].

4.3. Thermo-immunotherapy

In addition to combination with chemotherapeutics, immunotherapy can also be linked to thermal ablation of tumors. This therapeutic approach is based on the local cooling (cryo-ablation) or heating (radiofrequency (RF), high-intensity focused ultrasound (HIFU) or photothermal ablation) of cancer tissue. For all these different techniques, immunogenic cell death and associated release of DAMPs such as heat shock proteins (HSP) and HMGB1, as well as increased immunostimulatory cytokine secretion and infiltration of APCs into the ablated regions, was reported [145]. Here too, NPs can be designed aiming to improve the thermal effects and simultaneously boost the induced antitumor immunity. Mainly, NPs have been used as nuclei

for local heating. In this way, NPs have been developed to deliver thermal energy due to their capacity for near infrared (NIR) light absorption (e.g. carbon nanotubes and gold nanoshells) [146, 147] or magnetic properties [148]. A recent study by Ito et al. demonstrated that application of local hyperthermia using melanoma-targeted magnetite NPs and an external alternating magnetic field resulted in a clonal expansion of T cells in B16 tumors [149]. To further enhance the immunostimulatory effects of thermal therapies, NPs for photothermal therapy have been modified with glycated chitosan (GC) which serves as an immune adjuvant. Indeed, GC inclusion resulted in macrophage and DC activation and laser-mediated tumor ablation further increased the release of HSP70 and the production of IFNγ, as well as in a significant increase in overall survival in a mouse hepatoma model [150]. These first reports indicate that localized hyperthermia-based treatments could be enhanced to induce peripheral immunity. In this way both the primary tumor (via ablation) and distant metastasis (via immunostimulation) could be tackled simultaneously. However, this will require careful optimization of the temperatures that should be reached within the tumor to balance destruction of the primary tumor on the one hand, and the induction of immunological benefits on the other hand. For example, coagulative ablation strategies, where high temperatures "melt" the tumor tissue and completely cut off intratumoral blood flow might have the most destructive effect on the primary tumor, but the coagulated tumor mass will likely form a barrier for immune cell infiltration and immune induction [146]. More thorough comparative studies where multiple immunological parameters are studies are warranted.

5. Conclusions and future perspectives

Over the last few decades we have come a long way from beginning to understand how the immune system protects us from danger and how we can harness it in the battle against cancer. By the day our knowledge on effector cells, stimulatory and suppressive cytokine signaling pathways and potential therapeutic targets expands. Importantly, integrative strategies that trigger antitumor immune responses and simultaneously address tumor-mediated immune suppression are on the rise, and these could significantly improve the outcome of clinical trials. Overall, the future probably lies in the art of combining: merging established and emerging strategies could bring immunotherapy one step closer to the clinic, and it is simply impossible to ignore the rising evidence of their synergistic effects. In addition, with what we now know about immunogenic

cell death, we could modulate traditional treatment parameters to boost DAMP and antigen release, and in this way boost antitumor immune induction.

Although there still seems to be a gap between these discoveries and the NP solutions that have been developed, we are starting to bridge it, as more and more particulate adjuvants, tumor-targeting strategies and multi-modal vehicles are being designed. Still, nanoparticulate immunotherapy remains an underexplored area which could benefit from cross-pollination between immunologist and material scientists.

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