Combinatorial prospects of nanoparticle mediated immunotherapy of cancer

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1

GENERAL INTRODUCTION

CHAPTER 1

Introduction

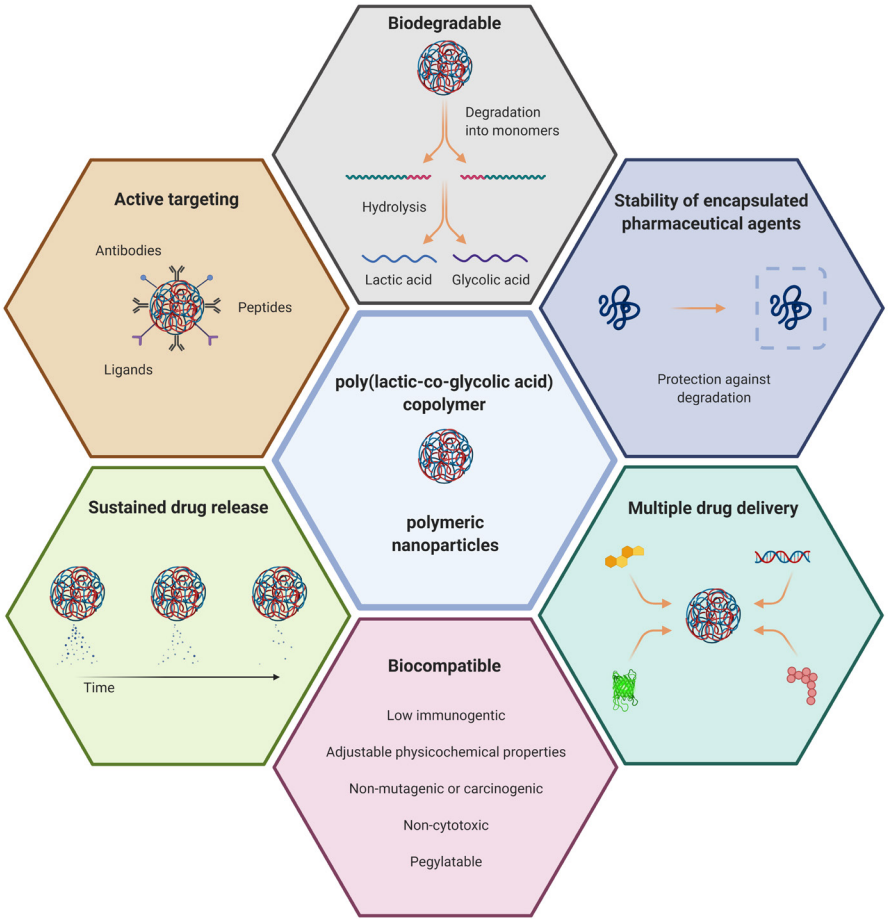
Beyond the macro- and microscale, it is there where the tangible structures that we are built of, perceive, and experience, are composed. It is matter at the nanoscale. The lecture of the American physicist Richard Feynman "There's Plenty of Room at the Bottom" [1] laid the crib in the late 1950s to what we understand nowadays as Nanotechnology; although it was the Austrian chemist Richard Zsigmondy the first to observe and perform size measurements of particles at (and coined the term) "nanometer" scale almost 100 years ago [2]. Both were formidable pioneers who laid the foundations of a new field in science and awarded righteously with the Nobel Prize in Chemistry for their accomplishments in 1925. Yet it took many years for nanotechnology to reveal its potential applications in medicine and to emerge as an ever so tiny, yet powerful, tool to join the combat against human diseases.

THE NANOMEDICINE FIELD OF RESEARCH

Nanotechnology generally refers to the field of science and study of (synthetic) molecular structures with diameters in size smaller than a micrometer, usually between 1 to 500 nanometers (nm). While this field has a wide overlapping span to many other scientific branches, pronouncedly in chemistry and physics, it is referred to as Nanomedicine when its study is attributed to the field of Medicine. There, the manipulation of matter at the nanoscale is applied to the synthetic assembly of (preferably biocompatible) materials and structures for a wide range of applications, including the delivery of drugs, with the purpose of diagnosis and therapy of human pathophysiologies. However, it is generally the application of nanotechnology for

medical purposes that defines the name rather than the operating size scale, as arguably, the fields of molecular biology and pharmacology operate at similar size scale. One of the main problems that researchers working in the field of nanomedicine try to solve is to reduce the biodistribution of drugs while maintaining effective dosage of drugs on the target site; as drugs tend to distribute in all organs, tissues and cells within the organism most particularly at higher doses [3]. The biodistribution of a drug in an organism often lies root to unwanted adverse effects due to human pharmacological intervention that is aimed to treat or ameliorate disease symptoms with drugs. After all, a drug designed to kill cancer cells should not accumulate elsewhere than in the tumor and is toxic when accumulated, for example, in the heart. To address this problem, synthetic nanoparticles were proposed as vehicles to transport, protect, and deliver drugs aimed to reduce drug biodistribution and, under certain circumstances, even enhance drug delivery to the anatomical site of interest. For this purpose, several nanoparticle types, with differing compositions and shapes, were developed during the past years that can be generally divided in two main categories: of either inorganic or organic etiology. Inorganic nanoparticles are mostly composed of colloids of silver, gold or silicon. Organic nanoparticles are mostly composed of lipids, sugars, and (biodegradable) polymers. Within the different types, one or multiple drugs can be simultaneously encapsulated, entrapped, or attached by ion bonds or covalently, in, between, or on the outside of nanoparticles. One of the most important biological aspect of the nanoparticles physicochemical properties is its size. Nanoparticles for medical application are usually assembled within the size range of 20 to 500nm, based on the balance between optimal blood circulation time to facilitate tumor accumulation versus drug loading capacity [4]. Particles smaller than 10 nm are rapidly cleared by the kidneys and bigger nanoparticles tend to be removed gradually in time from blood circulation via opsonization and clearance by the mononuclear phagocytic system and via phagocytosis in the liver [5]–[7]. Pegylation, a chemical process where polyethylene glycol groups are added to the outer layers of nanoparticles, can slow down opsonization and reduce interactions with the immune system, which increases blood circulation time [8]. The nanoparticle size range is also an important factor in a biological process known as the enhanced permeability and retention (EPR) effect. The EPR effect is hypothesized to occur within some solid tumors where the local vasculature is aberrantly formed, which includes the absence of a smooth muscle layer covering the blood vessels and the presence of wide gaps between endothelium cells [9]. Combined with ineffective lymphatic drainage in the tumor tissue, nanoparticles tend to passively accumulate more in the tumor than

in other organs [10]. When extravasated from the blood vessels to inside the tumor tissue, binding of the nanoparticles to cancer or other cell types of interest can be further enhanced by modifying the surface of nanoparticles with specific antibodies, peptides, or receptor ligands. When nanoparticles acquire higher binding capability to cells of interest, most specifically guided to receptors with endocytosis capacity, these nanoparticles are termed targeted nanoparticles [11]. Other physicochemical properties of the nanoparticles can be further adjusted for sustained, sequential, or slow drug release depending on the surrounding milieu such as temperature, specific enzymes, or pH. An overview illustration of these properties is depicted in **Figure 1** for poly(lactic-co-glycolic) (PLGA) copolymer-based nanoparticles.



< **Figure 1. An overview of the properties and applications of polymeric poly (lactic-co-glycolic) nanoparticles.** The poly (lactic-co-glycolic) (PLGA) copolymers are FDA approved for human medical applications. PLGA nanoparticles have strong biodegradable and biocompatible traits [12]. The remnants of PLGA nanoparticles are lactic and glycolic acid which are also produced by cells during normal metabolic function [13]. Additional noteworthy traits are: **1)** enhanced drug protection capabilities. For instance, genetic products (e.g. silencing RNA, proteins, mRNA, CRISPR-Cas, etc.) are protected inside the PLGA nanoparticles from direct degradation and removal from the blood. **2)** Capacity to load multiple drug types into one nanoparticle and maintaining a sustained controlled release of the drugs [14], [15]. **3)** Surface functionalization of nanoparticles for active targeting to specific cells (e.g. with antibodies, ligands, peptides, etc.) [16].

NANOMEDICINE IN CANCER RESEARCH AND THERAPY

Many nanoparticle-based nanomedicine modalities for cancer diagnosis and therapy are currently in late phase clinical trials [17]. Some were already approved decades ago, for instance Doxil®, a liposomal variant of the chemotherapy drug doxorubicin, received FDA approval in 1995. Doxil® solved a problem induced by doxorubicin (i.e. cardiotoxicity) by reducing doxorubicin exposure to the heart (i.e. reduced biodistribution) while maintaining anti-cancer efficacy [18]. Since then, Abraxane® and Onivyde® were also introduced as nanoparticle variants of paclitaxel and irinotecan, respectively. More recently, VYXEOS® and Hensify® were FDA approved for the treatment of cancer. VYXEOS® is a combination chemotherapy nanoparticle of cytarabine and daunorubicin for the treatment of myeloid leukemia [19]. Hensify® is a hafnium loaded nanoparticle that significantly enhances standard radiation oncological procedures [20]. Similar combined drug nanomedicine solutions to VYXEOS® and the emergence of nanoparticle-based combined/multiple drug combination therapy (such as the combination of chemotherapy with immunotherapy) is reviewed and discussed in chapter 2. The type of applications of nanoparticles in oncology is vast as still rapidly growing. Nanoparticle technology in nanomedicine is currently regarded as de facto delivery platform for chemotherapy and other standard anti-cancer drugs, but also for direct or indirect tumor microenvironment remodeling, systemic immune modulation or immune modulation of the tumor microenvironment and lymph nodes; as well as mediators of delivery or adjuvant therapy of immunotherapy, gene therapy, radiotherapy, therapeutic cancer vaccine therapy, photodynamic and photothermal therapy and other sub-types of anti-cancer therapies [21].

THE GENESIS OF THE IMMUNE SUPPRESSED TUMOR MICROENVIRONMENT AND THE REGULATION OF IMMUNE ACTIVATION

A tumor microenvironment which is characterized as immune suppressed is thought to be acquired after selective interactions with the immune system during tumor development. During these interactions, it is likely that cancerous cell clones which have harbored highly immunogenic mutations were eliminated, while weak immunogenic cancer cells escape immune surveillance, a process named immunoediting [22]. The process of immunoediting may continue for several cycles, where the surviving cancer cells acquire more mutations and undergo epigenetic alterations thereby selecting the clones which can proliferate and escape immune attack [23], [24]. This will lead to an immune suppressed microenvironment which increasingly becomes impregnatable for cancer cell killing immune cells [25]. At this point, even newly developed cancer cell clones with additional immunogenic mutations may not be eliminated anymore and the cancer becomes established. An important part of the process to acquire such a milieu is attained by tumor cells that produce specific chemokines **(Figure 2)**. Chemokines are small molecules that can attract specific cell types. For instance, the chemokines CCL2, CCL5, CCL17 and CCL22 are commonly found in tumors that can recruit Myeloid derived suppressor cells (MDSCs) [26], Tumor associated macrophages (TAMs) [27], and Regulatory T cells (T-regs) [28] to the tumor area. In part, the recruitment of these cells may be the product of the evolution process of the cancer cells that acquire this ability due to selection. A putative mechanism has been put forward relating chromosome instability to both immune evasion and metastasis [24]. On the other hand, the recruitment of these cells may also be part of the immune resolution process, a negative feedback mechanism that takes place after the elimination of highly immunogenic cancer clones. Which process initiates or how significantly it contributes to the immune suppressed microenvironment in different cancer types is currently not fully understood. It is very likely that not only one process is responsible but several. However, regardless of the type of process responsible for the migration of these cells to the tumor area, the consequence is the establishment of a chronically immune suppressed environment (i.e. for cancer killing immune cells) that facilitates the continuous expansion of cancer cells, as well as the recruitment of MDSCs, TAMs and T-regs. This environment is also self-maintaining by the production of cues and cytokines such as ARG1, iNOS, TGFβ, IL10, COX2 and

IDO produced by MDSCs [29]. TAMs can produce VEGF-A, TGFβ, FGFβ, IL10, CCL17, CSF1 and TREM2 as well as the PD-L1 marker [30]–[40] while T-regs can express several markers and immune suppressive factors like CTLA-4, TIM-3, LAG-3, GITR, TGFβ and IL10 [41], see figure 2. This process will continue allowing the cancer cells to grow large in numbers and acquire a tumor mass until a point it will start to induce symptoms on patients as it invades surrounding tissue and metastasize to distal parts of the organism. The production of the cues that maintain the immune suppressive microenvironment is likely to also start to affect the systemic function of the immune system and homeostasis [42], [43]. Regarding the treatment for the patient, whether the tumor and the metastases will respond to different types of therapy is dependent of several factors and mechanisms. A therapy modality that is composed of multiple drugs is most commonly required for effective clinical responses [44].

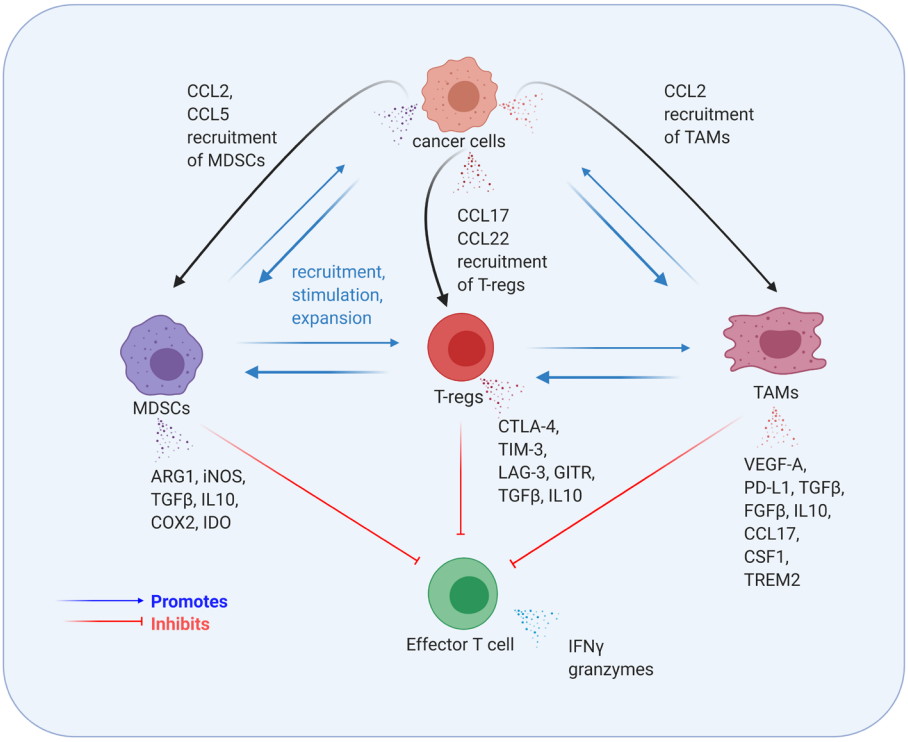


Figure 2. The role of chemokines to establish an immune suppressed tumor microenvironment. Cancer cells and other tumor-associated cells produce CCL2, CCL5, CCL17 and CCL22 that recruits Myeloid derived suppressor cells (MDSCs) [26], Tumor associated macrophages (TAMs) [27], and Regulatory T cells (T-regs) [28] to the tumor area. A chronic inflamed milieu that facilitates the continuous recruitment, stimulation, and expansion of cancer cells, MDSCs, TAMs and T-regs is maintained by the production of cues and cytokines such as ARG1, iNOS, TGFβ, IL10, COX2 and IDO produced by MDSCs [29]; VEGF-A, PD-L1, TGFβ, FGFβ, IL10, CCL17, CSF1 and TREM2 by TAMs [30]–[40]; and CTLA-4, TIM-3, LAG-3, GITR, TGFβ and IL10 by T-regs [41]. The recruitment of MDSCs, T-regs, and TAMs to the tumor area facilitates cancer cell survival, proliferation, angiogenesis, invasion and metastasis, immunosuppression, and drug resistance.

The process of immunoediting commonly starts with the recognition of cancer cells by specific T lymphocytes. This recognition is made possible as cancer cells acquire unique mutations in their DNA leading to the translation of 'altered' proteins which can be presented as small processed peptides in the major histocompatibility complex (MHC) molecules at the cell surface [45]. The MHC loaded with the antigenic peptide (i.e. neoepitope) can be recognized by the T cell receptor (TCR) of cognate T cells [46]. Activation and expansion of such T cells (i.e. that can recognize neoepitopes on cancer cells) will be initiated by antigen-presenting cells (APCs), such as dendritic cells (DCs) which can process and (cross)present cancer cell-derived antigenic peptides [47]. For their activation, T cells require 3 signals. The first signal is provided by the binding between the TCR and the MHC I and/or II, containing the neoepitope. The second signal is provided by co-stimulatory or co-inhibitory receptors. Naïve T cells require the co-stimulatory binding of CD28 with CD80/86 on APCs for their survival and proliferation. Negative feedback is also part of the regulatory process to control the magnitude of the immune response in the form of the expression of the CTLA-4 receptor which, at a certain point, will start to compete with CD28 for the co-inhibitory binding to CD80/86 on APCs. Also, other co-stimulatory interactions like CD27 with CD70 on APCs are important for activated T cells to develop effector functions. Since signal 2 provided by APCs is crucial to initiate an adaptive response, expression of these co-stimulatory molecules is tightly regulated to avoid autoimmunity. Immature APCs are poor (cross)presenters of antigen by default and display a non-activated phenotype (i.e. low MHC-I and II, low CD40, CD70, CD80 and CD86). However, APCs can acquire an activated phenotype when exposed to ligands of the pattern recognition receptors (PRRs) family which is broadly divided into two types, the damage-associated molecular pattern molecules (DAMPs) or pathogen-associated molecular pattern molecules (PAMPs). Due to the dysregulated expansion of cancer cells, some of these cells die and secrete DAMPs. These DAMPs are contained in the membranes of dead cancer cells or released by adjacent living cells in response to cell death and stress. When APCs engulf the dead cancer cells or debris, their PRRs can be activated by DAMPs and it can contribute to the activation of APCs. In turn, the activation of the APCs can incite the expression of MHC-I and MHC-II and of co-stimulatory receptors (e.g. CD40, CD70, CD80 and CD86).

The third signal is provided by cytokines which will influence the type of immune response that will be generated. The APC derived IL12 cytokine will induce a Th type 1 (Th1) immune response characterized by IFNγ and IL2 production, which will optimally

stimulate cytotoxic T lymphocytes (CTLs). The Th type 2 (Th2) immune response is characterized by IL4 cytokine production, which is aimed to stimulate extracellular immunity via antibodies. A Th1 or a Th2 response is thought to be regulated by differential activation of the type of PRRs by DAMPs and PAMPs.

After optimal initiation and activation, T cells will start to proliferate and then migrate to the tumor and will be able to kill cancer cells. However, the survival, proliferation rate, killing capacity, and activation status can still be modulated, most commonly when T cells arrive in the suppressive environment in the tumor. For instance, activated T cells can be deactivated by ligands for the Programmed cell death protein 1 (PD-1) receptor, which is expressed on activated T cells [48]. When arriving at the tumor site, cancer and non-cancer cells can express the PD-L1 or PD-L2 ligands of PD-1 receptor which effectivity renders the T cells inert. Moreover, when the T cells arrive at the immune suppressed tumor microenvironment they are likely to encounter T-regs that express the co-inhibitory cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) receptor and abundant IL10, TGFβ, and other suppressive cytokines that can also effectively abrogate T cells [49]. Besides the aforementioned examples, a plethora of similar immune checkpoints and other immune regulatory cues are already currently described and likely more are to be discovered in the future [50].

TARGETS FOR CANCER IMMUNOTHERAPY

The aim of cancer immunotherapy is to initiate de novo immune responses or to augment and/or recommence existing suppressed T cell immune responses against cancer cells. Immune checkpoint inhibitors are currently the most common form of immunotherapy where co-inhibitory receptors are targeted by blocking antibodies thereby 'releasing the breaks' from existing, but inactive tumor-specific T cells. Ipilimumab and tremelimumab are antibodies against CTLA-4 that can be administered to hinder the interaction of CD80/86 with CTLA-4 thereby facilitating the interaction of CD80/86 with CD28 which enhances T cell activation. Similarly, the interaction of the T cell inhibitory receptor PD-1 with its ligands PD-L1 and PD-L2 can be inhibited with nivolumab or pembrolizumab thereby leading to T cell activation [51]. Another type of immunotherapy is guided to modulate immune responses, often targeted to innate immune cells. Immune adjuvants (also known as immune modulators or immune stimulants) commonly aim to activate specific PRRs in APCs [52]. As previously described, during cancer development many cancer cells die and

release DAMPs that principally induce Th1 type activation. However, APC activation with DAMPs can be limited by the secretion of immune suppressive cues from the tumor microenvironment or even initiate another type of immune response other than Th1 but this is dependent on the type and concentration of DAMPs. Therapy with photo dynamic therapy, immunogenic chemotherapy or with other drugs that kills cancer cells directly can also induce the release of high concentrations of DAMPs. However, a type of PRRs in DCs that mainly recognize PAMP molecules can be exploited therapeutically to force a Th1 type immune response and induction of tumor specific CTLs. The Toll-like receptor (TLR) family is a type of PRRs that can recognize PAMPs such as microbial fragments, including viral and bacterial fragments [53]. TLRs can also be activated with immune adjuvants including synthetic viral facsimile molecules, such as poly (I:C) or resiquimod. Upon binding of the immune adjuvants to the respective TLRs, the DCs upregulate the expression of CD40, CD80, and CD86 and initiate the secretion of IL12, which differentiates naïve Th to Th1 T cells as described above. Therapeutic cancer vaccines combine the administration of defined antigens with immune adjuvants to induce a strong antigen-specific Th1 immune response and CD8 CTLs [54].

THE INTRATUMORAL ADMINISTRATION OF IMMUNE ADJUVANTS AND ABSCOPAL EFFECTS

The therapeutic potential of immune adjuvants also extends further when administered directly in the tumor [55], [56]. The direct intratumoral administration concept is certainly not new, as the American surgeon William Coley, in the beginnings of the 1890s, observed that some tumors on sarcoma patients temporarily shrank upon injection of live bacteria directly in the tumors [57]. More recently, research has provided a much better understanding of this process and developed improved and safer drugs and methods specifically tuned to harness the power of the immune system to direct it against the tumor more efficiently and with less side effects. For instance, immune adjuvants can resolve the immune suppressed phenotype of TAMs, M2-like macrophages, and MDSCs in the tumor and tumor-draining lymph nodes and abrogate T-regs from suppressive activity [58], [59]. Some immune adjuvants can reverse cytotoxic T cells in the tumor from an anergic state to an activated state and invigorate NK killing capacity [60]. Also, whether it is advantageous to activate single or multiple TLRs simultaneously has been studied [61]. For instance, it has

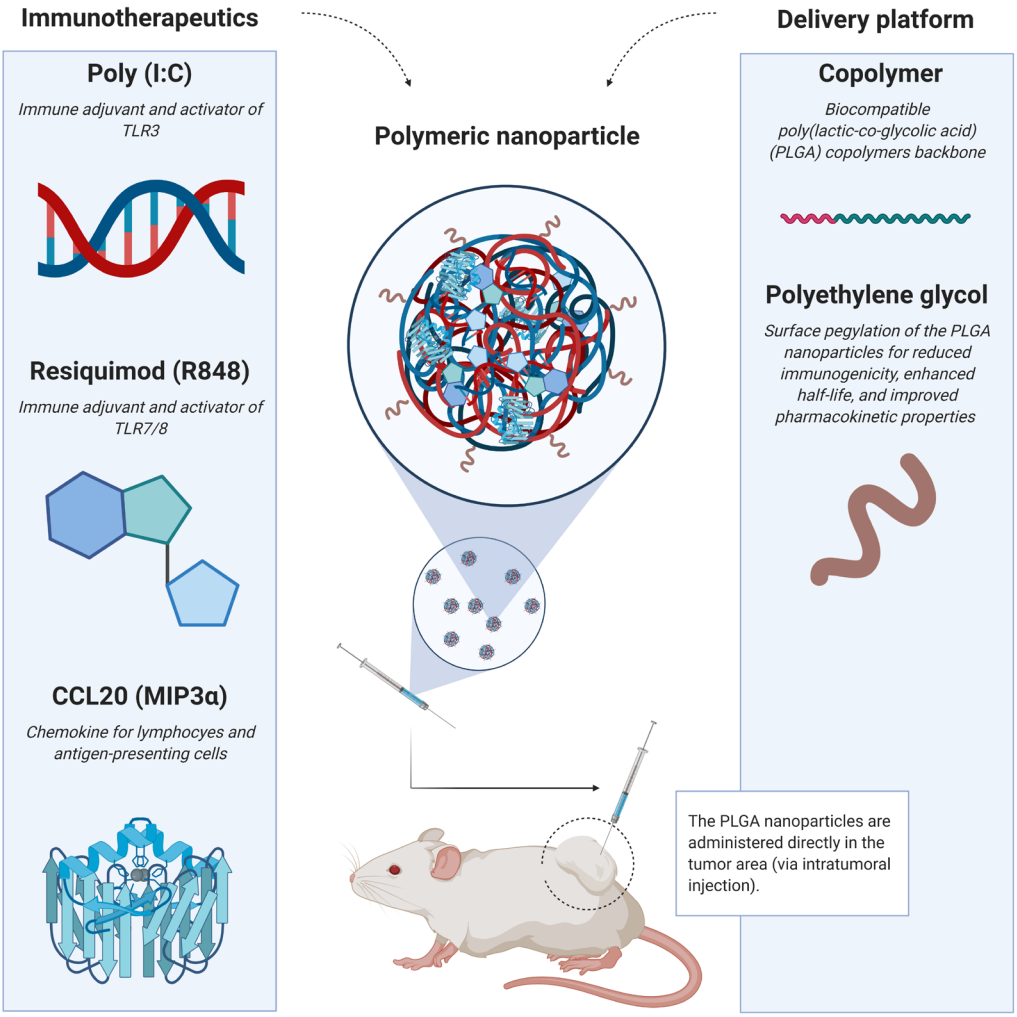


Figure 3. Design and composition of the PLGA nanoparticles that is central to this thesis. The immune adjuvants Poly (I:C) and Resiquimod, and the chemotactic CCL20, were simultaneously encapsulated into pegylated PLGA nanoparticles and injected intratumorally.

been reported that the combined activation of TLR3 and TLR7 synergizes in cytokine production in vitro [61]–[63]. The activation of TLR3 by Poly (I:C), a synthetic analog of double-stranded RNA, induces NK-κB and IRF3 activation via the TRIF-RIP1-TRAF6 axis that culminates in the production of inflammatory cytokines and type I IFNs such as IL6, IL8, TNFα, IFNα and IFNβ [64], [65].The activation of TLR7/8 by resiquimod, an imidazoquinoline compound, induces NK-κB and IRF5/7 activation via the MyD88-IRAK4 route and leads to robust production of pro-inflammatory cytokines and type I IFNs including IL1β, IL6, IL12, TNFα and IFNα [66]. Despite that both poly (I:C) and resiquimod activate the same subfamily of PAMPs, their effects exerted on the immune system and tumor response outcomes can be quite distinct [67]. While in vitro exploratory research provided indications of potential immunological additive or synergistic effects when both immune adjuvants were combined, the therapeutic effects of this combination in cancer models in vivo remains underexplored [61]–[63]. Another interesting strategy to improve immune responses against cancer cells is to recruit more immune cells to the tumor area. This can be accomplished by chemokines, such as CCL20 [68]. More drugs are administered intratumorally nowadays, following many reports showing optimal therapeutic effect with less side-effects which have been validated in the clinic for several cancer types [69]. Most importantly, the effects of local treatment commonly induces abscopal effects, which are imperative for the control of metastases [70], [71]. In addition, the administration of drugs in the region of the tumor-draining lymph node has also been recommended for less accessible tumors with similar therapeutic outcomes [72].

A STRIKE FROM MULTIPLE DIRECTIONS: CANCER CHEMOIMMUNOTHERAPY AND PHOTODYNAMIC THERAPY

Chemotherapy is an ablative class of cytotoxic and/or cytostatic drugs with the aim to eliminate as many cancer cells as possible. A tumor responsive to chemotherapy will initially display a mass shrinkage but the effects are usually only temporary due to other resistance mechanisms that emerge against the applied chemotherapy. Besides killing cancer cells directly, there are indications that chemotherapy can reverse the immune suppressed state in tumors (i.e. release of DAMPs) under certain specific circumstances and by eliminating suppressive immune cells directly. Furthermore, the systemic immune system appears less affected when chemotherapy is provided at lower doses. Research exploring the combination of (lower dose) chemotherapy with immunotherapy, either administered simultaneously or one before the other,

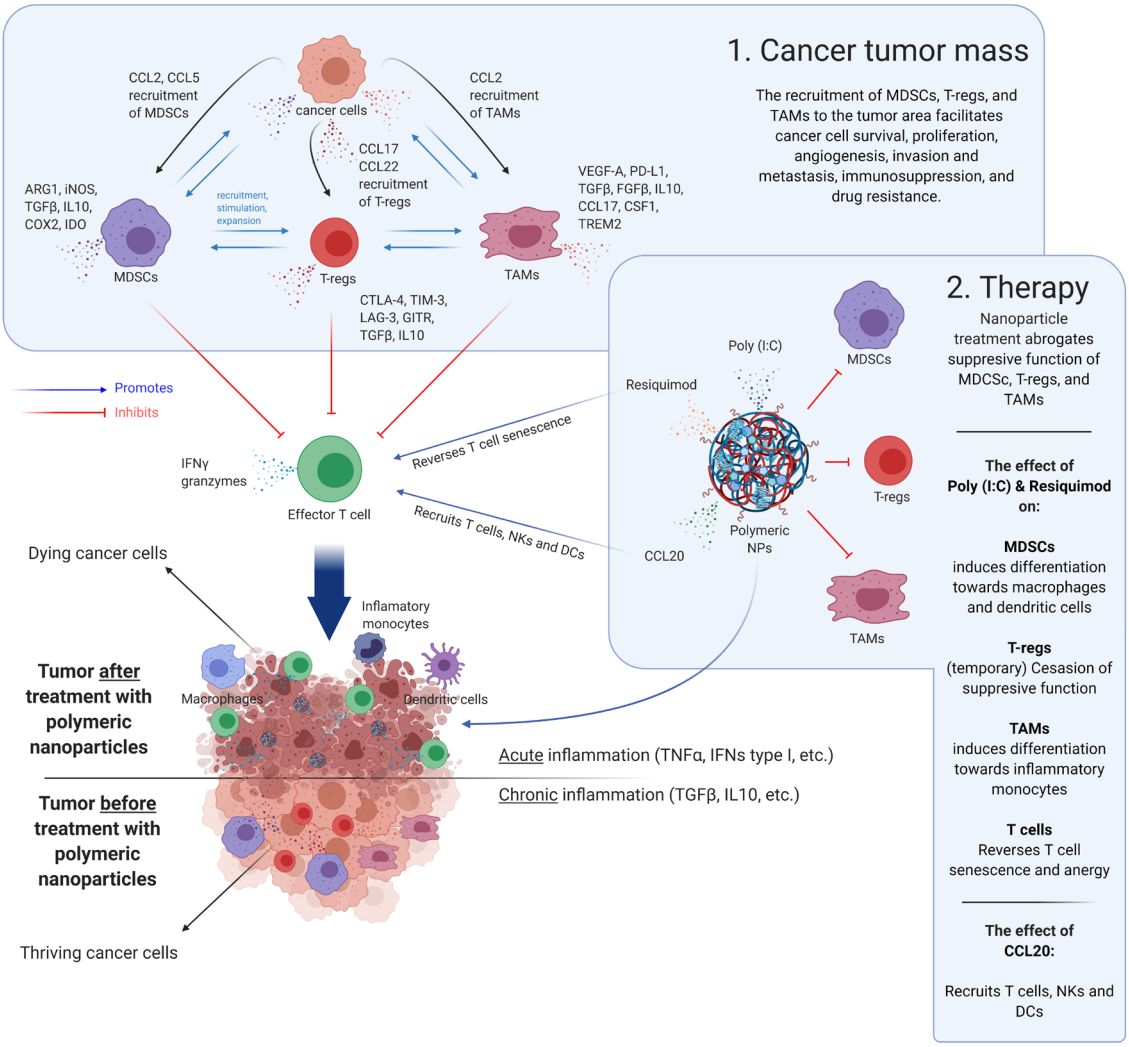
is compelling and has the potential to enhance therapeutic strategies for cancer patients in the near future. The therapeutic strategy of chemotherapy combined with a form of immunotherapy is regarded as a cancer chemoimmunotherapy.

An upcoming modality is photodynamic therapy, which ablates tumor cells by a two-step treatment. First, a photosensitizer drug is administered to the patient and allowed to accumulate in cells after which a light source (i.e. a laser) emanating light, often at a specific wavelength. Upon encountering such light, the photosensitizer will utilize the light's energy to initiate a chemical process that results in the generation of reactive oxygen species or other reactive molecules, however the type of reaction or effect can vary depending on the type of photosensitizer. In turn, the reactive oxygen species can induce severe local structural alterations to cell components and membranes, ultimately leading to cell death. It is not uncommon that a high generation of DAMPs takes place during this ablative process as well as the release as cancer antigens that can facilitate immune responses against remaining cancer cells and metastases via abscopal effects [73]. Two major advantages of photodynamic therapy are the possibility to only expose the tumor tissue to harmless visible light, and therefore induce no meaningful damage to non-exposed tissue in contrast to radiotherapy, and it is not mutagenic therapy. A disadvantage of photodynamic therapy is that it currently limited to tumor types which are easily accessible for laser illumination.

THE SCOPE OF THIS THESIS

The work presented in this thesis was the result of multi-disciplinary cooperation between the fields of Nanotechnology, Oncology, and Immunology. The aim was to study novel therapeutical concepts to improve insights and therapy responses for cancer patients in the near future. The concept of injecting bacteria or bacterial products directly in tumors is an established approach but we improved intratumoral delivery with modern nanotechnology and well-defined synthetic compounds. Since there is a population of patients that do respond to this type of therapy, there is a window of opportunity to improve therapy responses by utilizing the current knowledge on the mechanisms of immunotherapy and of nanotechnology. In this thesis, a novel therapeutic drug combination was tested. Instead of using live bacteria or bacterial products, two well defined synthetic immune adjuvants, namely poly (I:C) and resiquimod (also known as R848), were combined with the chemotactic CCL20 (also known as MIP3α) and incorporated into one nanoparticle treatment modality **(Figure 3)**.

The hypothesis is centered on the combination of poly (I:C) with resiquimod that could effectively modulate the immune system to abrogate the local immune suppressed state when injected intratumorally. Furthermore, the combination with CCL20 could yield better therapeutic results due to the capacity of CCL20 to recruit cancer fighting immune cells to the tumor [74]–[76]. To reduce rapid drug diffusion to the blood and to contain the drugs to the tumor area, poly (I:C), resiquimod, and CCL20 were simultaneously encapsulated into pegylated PLGA nanoparticles and injected intratumorally. An overview of the putative mechanisms is depicted in **Figure 4**


1. Cancer tumor mass
The recruitment of MDSCs, T-regs, and TAMs to the tumor area facilitates cancer cell survival, proliferation, angiogenesis, invasion and metastasis, immunosuppression, and drug resistance.
CCL2, CCL5 recruitment of MDSCs
cancer cells
CCL2 recruitment of TAMs
CCL17 CCL22 recruitment of T-regs
ARG1, iNOS, TGFβ, IL10, COX2, IDO
recruitment, stimulation, expansion
VEGF-A, PD-L1, TGFβ, FGFβ, IL10, CCL17, CSF1, TREM2
MDSCs
T-regs
TAMs
CTLA-4, TIM-3, LAG-3, GITR, TGFβ, IL10
Promotes
Inhibits
IFNγ granzymes
Effector T cell
Reverses T cell senescence
Recruits T cells, NKs and DCs
Dying cancer cells
Inflamatory monocytes
Tumor after treatment with polymeric nanoparticles
Macrophages
Dendritic cells
Acute inflammation (TNFα, IFNs type I, etc.)
Chronic inflammation (TGFβ, IL10, etc.)
Tumor before treatment with polymeric nanoparticles
Thriving cancer cells
2. Therapy
Nanoparticle treatment abrogates suppresive function of MDCSc, T-regs, and TAMs
Poly (I:C)
Resiquimod
Polymeric NPs
CCL20
MDSCs
T-regs
TAMs
The effect of Poly (I:C) & Resiquimod on:
MDSCs
induces differentiation towards macrophages and dendritic cells
T-regs
(temporary) Cesation of suppresive function
TAMs
induces differentiation towards inflammatory monocytes
T cells
Reverses T cell senescence and anergy
The effect of CCL20:
Recruits T cells, NKs and DCs

< **Figure 4. The putative effects and mechanisms of the PLGA nanoparticles loaded with poly (I:C), resiquimod, and CCL20, when injected in the tumor area.** The immune adjuvants Poly (I:C) and resiquimod can neutralize most of these immune suppressive generating processes. For instance, MDSCs can differentiate towards (mature, M1-like) macrophages and dendritic cells upon activation of TLR7 [77]; type I interferons produced by MDSCs and TAMs upon treatment with Poly (I:C) and/or resiquimod can directly inhibit T-regs [58], [59], [78], [79]; Activation of TLR3 in TAMs promotes differentiation towards a mature, M1-like, tumoricidal phenotype [80], [81]; Both activation of TLR3 and TLR7 in T cells have invigorating effects, such as reversal of T cells senescence and anergy [58], [82]. Finally, the artificial administration of CCL20 into the tumor area can directly repress the proliferation of myeloid progenitors [68] and actively attract cells expressing CCR6/CD196 such as dendritic cells, (memory) T cells, natural killer cells, and granulocytes [74]–[76], [83], [84]. The resulting inhibition of immune suppression is a milieu less friendly to cancer cells and more friendly to (cytotoxic) T cells and other (innate) tumoricidal cells.

In **chapter 2**, current published literature on the combinatorial prospects of nano-targeted chemoimmunotherapy is summarized, reviewed, and discussed to set the stage for studying the combination of the triple immune stimulation nano-sized modality with doxorubicin chemotherapy as described in chapter 4. In **chapter 3**, the biodistribution and the blood clearance rate was studied on tumor-bearing mice utilizing a surrogate nanoparticle loaded with a near-infrared dye upon intratumoral, subcutaneous, or intravenous administration of the nanoparticles.

In **chapter 4**, a study about the additive potential of nanoparticle mediated delivery of poly (I:C), resiquimod, CCL20, and doxorubicin to eradicate established tumors in two distinct in vivo aggressive cancer models is reported. In addition to the tumor growth inhibition and survival, in depth tumor microenvironment, circulating cancer specific T cells, and immune organ analysis is also studied. In **chapter 5**, the effect of each immune adjuvant and the chemokine encapsulated (i.e. separately but also in combination) combined with therapeutic cancer vaccines was studied and reported. The therapeutic potential of the nanoparticle-based modality was studied in combination with photodynamic therapy and the results reported in **chapter 6**. In **chapter 7**, the potential of multi-compound nanoparticles to bypass drug resistance in cancer is reviewed and discussed. A general discussion of these chapters and the potential and caveats of the nanoparticle-based modality reported here is provided in **chapter 8**.

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