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Leveraging immunotherapy with nanomedicine

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Abstract

Considerable progress has been made in the development and understanding of immunotherapy, notably with the emergence of novel chimeric antigen receptor T cells (CAR-T) which changed our perception of personalized therapy. However, cell-based immunotherapy not only lacks therapeutic efficiency in various solid cancers but also raised concerns related to important side effects. The convergence of immunotherapy and nanomedicine is timely as nanoparticles can now be easily conjugated to various antibodies and peptides enabling outstanding abilities to target specific cell populations *in vivo*. Here, we describe the state-of-the art of immuno-nano-therapy that *in vivo* activates the immune system, either by acting as vaccines or as tumor microenvironment (TME) activators. Then, we discuss the development of *ex vivo* immune-cell surface labelling strategies to indoctrinate/exploit immune cells as trojan horses, thereby improving the delivery of the therapeutics in the TME. Such strategy is likely to considerably amplify the efficacy of the immunotherapy.

Introduction

Cancer immunotherapies lead to specific and durable anti-cancer responses, overcoming traditional cancer treatment limitations. Successful immunotherapy approach aims to restore an immune response by either stimulating or suppressing the immune system^{1,2}. Thanks to the recent advancement (progress?) of novel monoclonal antibodies (mAbs) and immune cell-based therapies, the field of immunotherapy has oriented our perception of medicine towards a personalized approach.

~~Hence, different therapeutic modalities have been evaluated clinically to improve cancer immunotherapy by harnessing the immune system. These include, amongst others, the use of mAbs blocking pro-angiogenic factors or immune checkpoints inhibitors (e.g., NCT01274338, NCT02252042, NCT02125461). Targeting immune checkpoints proteins with anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and anti-programmed cell death receptor-1 (PD-1) antibodies demonstrated outstanding results when compared to cytotoxic chemotherapies or to targeted therapies^{3,4}. Although results remain modest in the majority of solid cancers, immune checkpoints inhibitors have become a standard of care in some advanced setting (e.g., melanoma, non-small-cell lung carcinoma, basal breast cancer, head and neck cancer, etc.)^{5,6}. These moderate effects could be partly explained because solid tumors exhibit low immunogenicity together with primary or acquired mechanisms of resistance⁷⁻¹⁰. In addition, the safety profile of immunotherapy remains a challenge to overcome (e.g., cytokine release syndrome)^{11,12}. Currently, administration of immunotherapeutic agents is limited by the induction of systemic autoimmunity (e.g., myocarditis, colitis, etc.), and grade-3 or grade-4 adverse events such as gastrointestinal,~~

renal or pulmonary toxicities^{13,14}. As such, a paramount need in improving efficacy as well as safety in cancer immunotherapy remains.

While monoclonal antibodies have demonstrated to be powerful therapeutic modalities, they are yet limited in efficacy and raised some safety issues (see box 1). Cell-based therapies have recently emerged (e.g., dendritic cell (DC)-based vaccines, chimeric antigen receptor (CAR) T and CAR NK cells, etc.) and lead to promising results in several clinical trials (e.g., NCT03274219, NCT02498912, NCT02408016, NCT02311621, etc.)¹⁵⁻¹⁸. DC-based vaccines use nanoparticles (NPs) to pulse cancer cell lysates, DNA or mRNA into DCs to prime tumor-specific T cells^{19,20} while CAR T and CAR NK cells act in a HLA-independent mechanism without requiring antigen presentation²¹. These recent approaches allow to overcome the low immunogenicity of tumors that might hinder the tumor-associated antigen presentation, and thus the cytotoxic response. However, the response remains heterogeneous throughout the population^{19,22}. Transition of DCs and engineered immune cells into an *in vivo* immunosuppressive environment may alter their viability and functionality and thus jeopardize their ability to induce an anti-tumor immune response. Cancer cells can also undergo antigenic modulation (*i.e.*, antigen loss or downregulation) that enables immune escape²². Moreover, the efficiency of these patient-derived cell-based therapies is strongly dependent on the patient's immune system exhaustion level when the cells are harvested²³. Finally, such therapies have limited efficacy in patients with solid tumors²².

Nanomedicine was originally developed to improve the therapeutic index of small molecules by decreasing their side effects or improve the specificity of the drug delivery into the tumor^{24,25}. Once injected in the bloodstream, the NPs were designed to passively accumulate in the tumor by using the leaky vasculature produced by the rapid tumor neoangiogenesis and impaired lymphatic vessels (also called enhanced permeability and retention (EPR) effect)²⁶. However, this passive targeting method recently raised some concerns because of the limited amount of NPs reaching the tumor cells²⁷. For this reason, improving the targeted delivery of NPs to the tumor remained a challenge.

Liposomal NPs were the first generation of drug carriers. Although drugs encapsulation into liposomes improve their pharmacokinetic and biodistribution in comparison to free drugs, no marketed liposomal therapeutic agents have yet demonstrated a significant increase in overall survival versus the standard agent²⁸. Further liposome PEGylation allows a decreased plasma clearance and results in a longer retention time in the bloodstream^{29,30}. Nevertheless, improved safety profiles justified PEG-liposomal NPs routine use in tumors where the original active ingredient has failed to provide results (e.g., liposomal doxorubicin in ovarian cancer: DOXIL[®], CAELYX[®]). Liposomal deliveries are however seriously limited by their encapsulation, their stability, and their uncontrolled drug delivery abilities (*i.e.*, dose dumping).

Second NP generation based on micelles and nanoparticles obtained from synthetic polymers (e.g., poly(d,l-lactic-co-glycolic acid)-*b*-poly(ethylene glycol); PLGA-PEG, organic-based NPs, dendrimers, *etc.*) have been developed for several biomedical applications such as drug delivery, or medical imaging^{31–34}. These long-circulating polymeric NPs demonstrated an improved therapeutic index in comparison to small molecules, but their use in the clinic remains limited, notably because of their high liver internalization and remaining toxicity concerns³⁵.

More recently, theranostic NPs (*i.e.*, carrying both imaging and therapeutic agents) have emerged. Their conjugation with near-infrared (NIR) fluorescent dyes, magnetic resonance imaging (MRI) tracers, or positron emission tomography (PET) contrast agents allow to track the NPs through non-invasive and whole-body imaging and thus facilitate triggered drug delivery to tumor site^{36,37}. This application is only at its beginning and multiple clinical trials are currently ongoing^{38,39}. Altogether, the different generations of NPs aim to improve the therapeutic window of free drugs by either reducing the toxicity, increasing the total amount of small molecules delivered to the tumor, or enabling the delivery of highly toxic molecules^{40–42}. *In vivo* tracking of NPs further provides an additional level of understanding and can even better improve their therapeutic benefit with personalized therapy^{43–45}. However, despite the advancements of nanotechnology over the last decade, a retrospective analysis demonstrated that no more than 0.9 % of the injected dose reach the tumor cells on average²⁷.

The EPR effect was questioned as to be the best route of delivery in patients. Indeed, its effect varies significantly between both patients and tumor types (and vascularization), with some differences within the same patient or tumor type over tumor progression^{46,47}. These findings also questioned the use of the appropriate animal model to better assess the NP efficacy⁴⁸. Additionally, alternatively to the known EPR effect used for the passive targeting delivery of NPs, a recent study demonstrated that NPs tend to actually predominantly enter the tumors through active transcytosis process through endothelial cells rather than using the neovasculature gaps⁴⁹. A deepened understanding of this mechanism may lead to its manipulation to an improvement of NPs accumulation in the tumor. Despite all the questioning regarding the passive uptake mechanisms, some NPs still confirm their effective tumor internalization in patients through passive targeting delivery^{50,51}.

To overcome the low yield of NPs reaching the tumor, some routes have been investigated (**Fig. 1A**); *i*) The tumor blood vessel normalization approach consists in using antiangiogenic agents to transiently normalize the tumor neovascularization to decrease the tumor hypoxia and to increase the efficacy of conventional therapies by increasing the total amount of NPs (smaller than 12 nm) or small molecules to be delivered⁵²⁻⁵⁴. *ii*) More recently, the use of an external trigger combined with NPs has been investigated. This trigger could be ultrasounds⁵⁵⁻⁵⁷, photothermal therapy (PTT)⁵⁷⁻⁵⁹, photodynamic therapy (PDT)^{57,60,61}, or even radiation therapy^{62,63}. Most often, the approach consists in targeting the tumor blood vessel using $\alpha v\beta 3$ peptide to disrupt the neovessels with the external trigger in order to improve the delivery of the drug in a second time (**Fig. 1B**). *iii*) An alternative approach consisting in priming the liver with "blanked" liposomal NPs prone to be highly accumulated in this organ. A second injection consisting in the therapeutic compound is then administrated and avoid liver retention, resulting in an increased accumulation of NPs or small molecules to be delivered at the tumor^{64,65} (**Fig. 1C**). Altogether, these novel passive targeting methods seem to alleviate the NPs tumor uptake and/or improve the drug delivery.

In parallel, novel strategies have been studied to deliver the therapeutic agent to the cancer cells. This is achieved through the design of therapeutic vaccines or the conjugation of peptides and monoclonal, bispecific, or trispecific antibodies^{66,67} to activate immune cells (both circulating and within the tumor microenvironment). While active targeting based on the use of peptides, or antibodies, conjugated at the surface of the NPs was first employed to improve the NP tumor uptake, such strategy only improved the delivery specificity of NPs to the tumor cells without improving the total amount of tumor internalization⁶⁸⁻⁷⁰. As such, utilizing nanomedicine to specifically target immune cells could hence improve the efficiency of immunotherapies or, at least, enhance their toxicity.

In this review, we will discuss how nanomedicine could foster immune cell-based therapy and overcome the usual therapeutic-limiting secondary effects by either *i*) activating *in vivo* the immune system to turn poorly immunogenic tumors into inflamed tumors, or *ii*) by modifying *ex vivo* the immune cells behavior before infusion to enable targeted drug delivery as well as immune activation in the tumor site.

1. Nanomedicine to improve immunotherapy

Rather than trying to target directly the tumor, and because of the low tumor uptake of NPs, novel strategies to specifically target circulating immune cells have emerged. The advantage of this approach is based on the fact that, once activated, the immune cells will propagate the message to turn the tumor into a 'hot tumor' and will recruit more native unactivated immune cells, resulting in a potent immunotherapeutic response. Immune cells targeting can be performed by conjugating peptides, antibodies, or ligands at the surface of the NPs (**Fig. 2**). It was demonstrated that the biodistribution and pharmacokinetics of ultrasmall NPs (< 5 nm diameter) are dictated by their ligand decoration, increasing their circulation time in the body and hence improving tumor cells accumulation⁷¹. However, because of their size, a high drug loading is difficult to achieve, orienting their use mostly for medical imaging applications^{33,34,71}. At the opposite, the biodistribution and pharmacokinetics

properties of large NPs (> 50 nm diameter) are dictated by the NP, resulting in a large accumulation in the liver and lymph nodes^{72,73}. This preferential accumulation into lymphatic vessels and lymph nodes is of interest to target immune cells for vaccination approach or for neoantigen recruitment after radiotherapy^{73,74}.

1.1. Nanoparticles for *in vivo* immune cells activation.

In vivo activation of immune cells remains the main goal of immunotherapy. While activating T cells or tumor-associated macrophages (TAMs) has resulted in great therapeutic responses, the co-stimulation of several immune cells simultaneously is a goal not yet fully achieved.

Circulating and tumor-infiltrating myeloid cells | Myeloid cells such as TAMs or immature myeloid-derived suppressor cells (MDSCs) play an important role in initiating the immunosuppressive environment that strongly suppress the function of cytotoxic immune cells, negatively impacting the immunotherapy efficacy^{75,76}. NPs-based therapies can either polarize TAMs towards a more antitumor M1 phenotype or completely neutralize or kill them (**Fig. 2A**). A prominent approach to reprogram TAM towards a M1 antitumor phenotype is to deliver TLR agonists. R848, a TLR7/8 agonist, loaded in β -cyclodextrin (β -CD) NPs potently drive TAM polarization towards the M1 phenotype, leading to efficient tumor growth control in multiple mouse models and synergize with anti-PD-1 antibodies⁷⁷. Another strategy to reeducate TAM is to interfere with intracellular mRNAs by delivering siRNA, miRNA or mRNA via mannosylated NPs due to high expression of mannose receptor 1 on TAMs surface⁷⁸⁻⁸¹. Glucan-decorated NPs allow *in vivo* delivery of specific anti-macrophage migration inhibitory siRNA which results in macrophage polarization towards an antitumor phenotype expressing pro-inflammatory cytokines, such as TNF α and IL-2, which subsequently enhanced T cell infiltration and function at the tumor site⁸⁰. More recently, mannose-modified PLGA-based NPs allow delivery of mRNA encoding the M1-polarizing interferon regulatory factor 5 transcription

factor to TAMs, which reverse the immunosuppressive pro-tumorigenic phenotype of TAMs, and reprogram them to an anti-tumor one inducing immunity and inhibiting tumor growth in models of ovarian cancer, melanoma, and glioblastoma⁸¹.

Another approach to modulate the tumor immune microenvironment myeloid compartment is to completely neutralize or kill TAMs by delivering them cytotoxic molecules through ligand-decorated NPs that target the TAMs overexpressed receptors (e.g., mannose receptor, folate receptor beta, etc.)⁸²⁻⁸⁴. Similarly, granulocyte-colony stimulating factor (G-CSF) decoration of albumin NPs promote preferential *in vivo* accumulation in MDSCs in a 4T1 metastatic triple-negative breast mouse model⁸⁵. This system could provide a cell lineage-specific delivery of indocyanine green (ICG), an effective photothermal and photosensitizing agent that can be used for MDSCs ablation in highly immunosuppressed patients^{86,87}.

In parallel, cancer cells upregulate CD47 that ligates with SIRP α present on TAMs surface and eventually inhibit their phagocytic functions. Blocking CD47 and/or SIRP α with mAbs showed interesting but limited results notably due to low bioavailability at the tumor site⁸⁸⁻⁹⁰. The co-delivery of CD47 and SIRP α sequestered on a multivalent lipid-based phagocytosis nanoenhancer (LPN) enables simultaneous engagement of TAMs and cancer cells at significantly lower concentrations of antibodies than the ones used in recent studies (2 mg/kg vs 5 mg/kg)⁹¹ (**Fig. 2A**). TAMs activation allows intra-tumoral infiltration of effector T cells and NK cells, leading to significantly enhanced tumor growth inhibition as well as increased survival in B16F10 melanoma tumor bearing mice.

However, widespread expression of some receptors led to readily uptake of these targeted drug delivery systems by normal macrophages, mononuclear phagocyte system or even liver sinusoidal endothelial cells^{92,93}.

Circulating and tumor-infiltrating T cells | In order to override the abovementioned limitations, NPs-based approaches have been designed to leverage T cells as drug carriers and demonstrated greater drug levels in the tumor than ones delivered by NPs alone⁹⁴⁻⁹⁷ (**Fig. 2A**).

This could notably be explained by the ability of the T cells to freely circulate in the body, and hence being used as trojan horse for the NPs to avoid macrophages and direct liver accumulation after their first pass in blood system⁹⁸. In one approach, immunoliposomes decorated with engineered interleukin-2 (IL-2) molecule on an Fc framework or an antibody F(ab')₂ fragment against a congenic cell surface receptor were used to effectively target *in vivo* adoptively transferred T cells (> 95%). Using F(ab')₂ fragments to decorate NPs demonstrated high target specificity and avidity along with little interactions with Fc receptors expressed by the mononuclear phagocyte system, which is a major way of NPs clearance⁹⁹. Conjugation of immunoliposomes to the surface of exogenous T cells induced repeated waves of cells expansion, improving their potency^{96,97}. Nevertheless, cell-bound NPs become diluted over cell proliferation, and adoptive cell transfer (ACT) remains a cumbersome and costly procedure. In another approach, PLGA NPs were used to encapsulate either SD-208, a TGFβR1 inhibitor, to restore T cells function, or a TLR7/TLR8 agonist to recruit lymphocytes to non-inflamed tumors⁹⁵. PLGA NPs were decorated by anti-PD-1 antibody F(ab')₂ fragments via thiol-maleimide minute-process, such that the NPs bound approx. 5% of the circulating and tumor-infiltrating CD8⁺ PD1⁺ T cells^{95,100}. Targeted delivery of a TGFβR1 inhibitor led to extended survival in a mouse model of colorectal cancer compared with free drugs at similar dosages, while TLR7/TLR8 agonist increased the proportion of tumor-infiltrating CD8⁺ T cells and sensitized tumors to anti-PD-1 therapy when compared to NPs lacking the targeting moiety or equivalent doses of the free drugs⁹⁵.

With the recent development and clinical demonstration of the efficiency of bispecific antibodies, or at the preclinical level with tri-specific antibodies, these approaches demonstrated tremendous results. While these antibodies have already been conjugated to drugs to form antibody-drug conjugates, they could now be used to ease NPs decoration for multiple targeting. Interestingly, based on a click-chemistry approach, generating dual-targeted NPs, or tri-specific NPs is a minute-process^{101,102}, allowing to quickly evaluate novel targeting conjugations. NPs platform combining immune checkpoint blockade agents along with co-stimulatory signals have been developed to overcome the autoimmune-mediated

dose-limiting toxicities of free mAbs¹⁴. These dual targeting systems redirect effector T cells to recognize cancer cells while simultaneously blocking checkpoint inhibitors^{103–106}. Various NPs types (e.g., liposome, PEG-PLGA, etc.) have been decorated with different T cells agonists (e.g., anti-4-1BB mAb, anti-OX40 mAb, etc.) and immune checkpoint blockade agents (e.g., anti-PD-L1 mAb, anti-PD-1 mAb, etc.). These approaches resulted in a marketed therapeutic activity as demonstrated by tumor regression, tumor-specific T cells expansion, and immune responses in B16 melanoma and 4T1 breast cancer models^{103–106}.

Targeting NPs-based immunotherapies to blood circulating and tumor-infiltrating immune cells rather than tumor cells directly allows efficient *in vivo* activation of immune cells with limited doses administration, and thus lower immune-mediated adverse events occurrence. These NPs-based immunotherapeutic approaches can also be used to target immune cells in the lymph node as cancer vaccination tool that elicits a potent antitumor immune response.

1.2. Vaccines with nanomedicine.

The anti-tumor vaccines directed against tumor-associated antigens (TAAs) or tumor specific antigens were amongst the first immunotherapeutic agents developed since the late 1980s^{107,108}. Cancer cell lysates, DNA or mRNA are pulsed into dendritic cells (e.g., FDA-approved Sipuleucel-T, PROVENGE® for prostate cancer) to activate T cells by presenting them the tumor antigen, leading to a cytotoxic T cells (CTL) response^{109,110}. Because DCs can be inhibited by the cancer cells or the tumor microenvironment, and because the anti-tumor vaccines are difficult to standardize due to their requirement of highly immunogenic antigen as well as potent adjuvant, this strategy shows decreased potency to eradicate tumors^{108,111}. Newly developed nanoengineered vaccines demonstrated better efficacy in cancer treatment over former anti-tumor vaccines which come with major limitations such as poor immunogenicity and off-target side effects^{112–114}.

The size of the nanomaterial has a detrimental effect in therapeutic outcome in cancer vaccination. NPs sizes ranging from 10 to 100 nm in diameter and negatively charged (-3 to -15 mV) tends to preferentially accumulate into lymphatic vessels and lymph nodes^{73,74}. The lymph nodes-targeted NPs behave as artificial antigen presenting cells (APCs) to directly stimulate CD8⁺ T cells. To achieve their activation, T cells require a T cell receptor (TCR)-Ag recognition followed by a co-stimulatory signal procured by the interaction between the CD28 from the T cells and its receptors present at the DC surface (*i.e.*, CD80 or CD86). A third signal mediated through IL-2 can enhance T cells stimulation but is not required for their activation¹¹⁵. Artificial APCs are coated with TAA and anti-CD28 antibodies recapitulate both mandatory signals for T cells activations^{116–119} (**Fig. 2B**). The other benefit for nanomaterials-based vaccines rely on their ability to co-deliver the TAAs and adjuvants simultaneously, at a controlled ratio, and at the same location (*e.g.*, CpG, Poly(I:C)), improving the ability of APCs to present up taken antigens with MHC-I molecules to CD8⁺ T cells and enhancing CTL responses^{120–122} (**Fig. 2B**). Although nanoengineered vaccines lead to a potent activation of the immune system, delivery through lymphatic draining depends on NPs composition, morphology, and surface chemistry. Alternatively, other NP designs have been sought to target specifically the spleen to activate B lymphocytes¹²³ or to recruit, through the use of hydrogels, for example, dendritic cells¹²⁴. These approaches enabled a specific targeting of immune cells without decorating the NPs with conventional peptides or antibodies.

1.3. Recruitment of neoantigens post-radiotherapy.

Radiation oncology combined with immunotherapy has gained a substantial interest due to its inherent ability to generate an abscopal effect defined as transforming "cold tumor" into "hot tumor" as defined by the total amount of tumor antigen, APC deficit, absence of T cells and impaired trafficking to the tumor mass post-radiation^{13,125}. By irradiating the tumor bed, which includes the tumor microenvironment, the radiation beams generate additional immune response inside the tumor microenvironment. It was demonstrated that the irradiation of the tumor leads to the liberation of TAAs that are endocytosed by APCs and then presented to

CD8⁺ T cells¹²⁶. This process increases the diversity of the TCR repertoire of intra-tumoral T cells and leads to a tumor-specific immune response directed against primary tumor and metastatic tumor sites^{127,128}. Unfortunately, the presence of immunosuppressive cells (for example, MDSCs and Treg cells) producing immunosuppressive cytokines (e.g., IL-10 and TGF β) in the tumor microenvironment hamper the development of robust and sustained abscopal responses even with combination approaches⁷⁻⁹. This combination therapy led only to a limited abscopal effect due to T cells exhaustion mediated by the upregulation of PD-L1 on tumor cells. As such, multiple strategies have been attempted to increase this tumor immunogenicity to boost the abscopal effect. Amongst them, targeted NPs have been used to deliver potent immunotherapeutic compounds to the tumor microenvironment and tumor draining lymph nodes in order to reverse immunosuppression, as illustrated by the use of CpG oligodeoxynucleotides conjugated to a polymer NPs¹²⁹. This compound was developed to activate DCs in the lymph node, resulting in an increased activation of CD8⁺ T cells/T_{reg} ratio¹²⁹. Activating DCs or tumor-infiltrating immune cells by immune checkpoint inhibitors or TLR agonists potentiate the abscopal effect, leading to delayed tumor growth. However, little is known about potential adverse effects emerging from combining radiotherapy with immune-checkpoint inhibition¹³⁰.

Alternatively, rather than activating the DCs by using targeted NPs, boosting the abscopal effect through the use of radiation therapy enhancer NPs made of high-atomic number atoms (gold, gadolinium, or hafnium NPs, among others) was also evaluated. The radiosensitization properties of these inorganic NPs is attributed to an increase of Auger electrons production via the photoelectric effect leading to an increased amount of reactive oxygen species in the tumor bed¹³¹⁻¹³⁸. Altogether, physical and biological boost effects induced by the presence of metallic inorganic NPs during the radiation treatment increased local DNA damage¹³⁹. The local boost of radiation dose deposition is hypothesized to enable a higher release of TAAs upon tumor cells death that potentiate the abscopal response through an increased tumor immunogenicity^{140,141}. Toward these findings, the development of antigen-capturing nanoparticles (AC-NPs) to exploit the release of TAAs upon radiotherapy in

order to boost the abscopal effect was also performed¹⁴² (**Fig. 2C**). By formulating polymeric PLGA NPs with diverse surface modifications (decoration with 1,2-dioleoyloxy-3-(trimethylammonium)propane, DOTAP; maleimide, NH₂; mPEG; or unmodified PLGA) to determine the effects of NP surface chemistry on antigen capture efficiency, it was demonstrated that NPs allow to capture efficiently tumor neoantigens post-radiation¹⁴³. Interestingly, these AC-NPs also captured a number of damage-associated molecular pattern proteins (DAMPs) that potentiated the immune response by being efficiently internalized by APCs and trafficked to lymphoid tissues. In a B16F10 melanoma bilateral tumor model, they showed that intra-tumoral injection of NPs in one tumor significantly improved the efficacy of anti-PD-1 antibodies and enhanced abscopal responses, generating a 20% cure rate compared to 0% without any NPs. The mechanistic studies revealed that AC-NPs induced an expansion of CD8⁺ cytotoxic T cells and increased both CD4⁺ T cells/T_{reg} and CD8⁺ T cells/T_{reg} ratios.

Although harnessing the immune system to enhance the abscopal effect demonstrated promising results, these approaches are still at their stammering. There is a great impetus for more cross-disciplinary research combining radiotherapy and immunotherapy with NPs in boosting the abscopal effect, thereby improving the treatment of both local and metastatic disease.

2. Immune cells-based therapy with ex vivo nanomedicine labeling

Alongside with immune checkpoints blockade, adoptively transferred autologous T cells have shown tremendous therapeutics effects. Patients-isolated T or NK cells can be genetically engineered to express a CAR that recognize tumor cells in an HLA-independent mechanism without requiring antigen presentation²¹. These CAR T cells, CAR NK cells, or more recently CAR macrophages, showed impressive results in treating acute lymphoblastic leukemias, refractory large B cell lymphomas or multiple myelomas^{15–17,144}. However, CAR cells remain costly, time consuming and only allow the addition of a single targeting moiety at the time¹⁴⁵. Moreover, the overall response to these therapies remain heterogeneous and CAR

immune cells have shown only moderate successes in treating solid cancers²². Once infused into patients, adoptively transferred cells migrate to the tumor sites and require a sustained supply of oxygen, nutrients and cytokines to support their viability, functions and proliferation. Moreover, cells have to overcome the immunosuppressive tumor microenvironment^{8,9}. To promote their anti-tumoral actions, adjuvant cytokines and immune-checkpoint blockade agents can be administered^{146–148}. However, systemic administration of such drugs requires repeated injection to maintain therapeutic levels resulting in dose-limiting toxicities^{149,150}. In order to overcome the aforementioned limitations and enhance ACT potency, cell surface bioengineering strategies have been developed¹⁵¹. Immune cells are an attractive option for cell surface bioengineering because of their natural abilities to circulate in the bloodstream and pass challenging biological barriers before to accumulate into the tumor microenvironment.

2.1. Cell surface conjugation routes

Cell surface conjugation strategies have to satisfy the following biocompatibility fundamental principles: *i)* any cell surface modifications should not have detrimental effects either on cell viability or cellular functions. *ii)* Bioengineering should further minimize alterations in membrane fluidity or bending elasticity that are critical for cell adhesion, migration and signaling. *iii)* Moreover, surface-engineered immune cells are exposed to *in vivo* shear stress and hemodynamic forces that can dissociate the NPs from the cell surface. Thus, the introduced modifications have to be compatible with the *in vivo* complex mechanical and biochemical interactions. *iv)* Finally, they should also not lead to the apparition of severe adverse effects such as thrombus formation after infusion¹⁵².

As such, to design novel and smarter immune cells, diverse bioengineering methodologies have been investigated. They can be subdivided in non-covalent physical biointeraction, and covalent chemical conjugation (**Fig. 3**).

Non-covalent non-specific biointeractions | Non-covalent conjugation of NPs can be achieved using non-specific (e.g., hydrophobic, electrostatic, etc.) interactions or specific ligand-receptors ones¹⁵¹ (**Fig. 3A**). Since the plasma membrane is composed of a hydrophobic lipid bilayer, NPs conjugated with hydrophobic moieties such as glycoinositol phospholipids (GPIs) can spontaneously be anchored into the membrane^{153,154}. Besides being hydrophobic, the plasma membrane is also negatively charged, as a result of phosphate groups of phospholipids, carboxylate groups on proteins and sialic acid termination of glycoproteins¹⁵⁵. Thus, NPs harboring many cationic sites can bind to cell surfaces via electrostatic interactions. Nevertheless, non-covalent non-specific conjugations have intrinsic tremendous drawbacks. Indeed, GPI-anchored NPs recapitulate natural membrane-associated molecules turnover rates and are thus rapidly internalized ($t_{1/2}$ between 3.8 and 20 hours)^{156,157}. Moreover, the electrostatic interactions between the negatively charged plasma membrane and the NPs positive surface can trigger local membrane depolarization and lead to cellular uptake¹⁵⁸. As such, non-specific non-covalent interactions are a rapid and easy route of conjugation but yet suffer from *in vivo* shear stress exposure in the systemic circulation or endothelial diapedesis. More specific and robust interactions have thus been designed.

Non-covalent specific biointeractions | Specific ligand-receptor interactions can be highly specific but yet pose the risk of undesired cellular responses. Cell surface expressed transmembrane receptors that can be targeted to conjugate NPs functionalized with their respective ligands^{159,160}. These ligand-receptors interactions are transient and dictated by their intrinsic binding and dissociation kinetics (K_d) which hinder stable coupling of NPs on cell surface^{161,162} (**Fig. 3A**).

Biotin-avidin interactions have also been extensively studied as specific, non-covalent cell surface interactions^{163,164}. This approach requires the introduction of a biotin group on cell surfaces. Biotin moieties can be covalently attached whether by amide bond formation with lysine residues¹⁶⁵ or aldehyde groups formation through mild oxidation of cell surface monosaccharides followed by functionalization with a hydrazide-biotin crosslinker¹⁶⁶.

However, streptavidin as an immunogenic xenoprotein can elicit neutralizing antibodies that lead to opsonization and phagocytic clearance of the engineered cell¹⁶⁷.

Sialylated carbohydrate ligands such as sialyl-lewis^X are naturally present at the surface of leukocytes and can provide other opportunities for non-covalent, specific biointeractions. As an example, E-selectin/TRAIL-coated or anti-NK1.1/TRAIL-coated liposomes were designed to interact with the sialylated carbohydrates present at the surface of the leukocytes and with the TRAIL receptors expressed by circulating tumor cells (CTCs)^{168,169}. This approach allowed CTCs elimination and prevention of lymph nodes metastasis formation in patient-derived xenograft model.

Covalent chemical conjugation | Covalent approaches were performed by (i) targeting native functional groups (e.g., thiols, amines) present on the cell surface, by (ii) chemical generation of reactive groups (e.g., aldehydes) or by (iii) using metabolic strategies to introduce non-natural functional groups (**Fig. 3B**).

Native functional groups | The naturally expressed thiol groups help to protect cells against oxygen radicals¹⁷⁰. Maleimide-functionalized NPs (e.g., liposomes, multilamellar liposomes, PLGA, etc.) have efficiently been conjugated to immune cells surface^{171–173} and their release can be triggered by the reduction potential of the tumor microenvironment (e.g., glutathione increase, etc.)¹⁷⁴. In addition to thiol groups, aldehydes have also been successfully targeted to covalently tether NPs on cell surfaces. However, aldehydes have to be generated by mild oxidation of primary alcohols contained in carbohydrates natively present on the cell surface¹⁷⁵.

Metabolic strategies | Metabolic glycoengineering strategies use bioorthogonal chemistry to modify natural oligosaccharides present on live cells. Alkyne- or azide-modified monosaccharides such as sialic acid or N-acetylmannosamine are metabolically incorporated into living cells and processed through natural biosynthetic pathways to be eventually

incorporated into the membrane as glycoengineered oligosaccharides that will react with azides or alkynes groups^{176–178} (**Fig. 3B**). Up to now, glycoengineered glycan are used for broad range of diagnostic, therapeutic or theranostic approaches^{177,179}. However, an emerging strategy using a dibenzocyclooctyne (DIBO) alkyne-decorated PAMAM dendrimer to target N-azidoacetylmannosamine-expressing macrophages demonstrated the feasibility of creating hybrid cell-NPs by bio-orthogonal chemistry to develop personalized immunotherapy¹⁸⁰. Although this approach exhibited no cell viability, intracellular signaling pathways, and motility altering, its toxicity and efficacy have to be evaluated *in vivo*. In parallel, the recent development of bio-orthogonal cleavage chemistry open new perspectives for NPs release and specific drug delivery¹⁸¹.

Receptor genetical engineering | The aforementioned approaches result in stable interactions between the NPs and the cell surface but provide only moderate control over the resulting cell surface engineering. Thus, stable and controlled cell-NPs interactions have been sought. Immune cells naturally express receptors at their surface which can be genetically engineered to provide an exclusive orthogonal ligand-receptor interaction¹⁸² (**Fig. 3B**). NK cells, T cells, and some macrophages present the NKG2D receptors on their surfaces that recognize the MHC class I polypeptide-related sequences (MIC) ligands family overexpressed on cells stressed by viral infection or cancer transformation. Leveraging the natural $\alpha 1$ - $\alpha 2$ binding domain of these ligands through protein engineering allows to develop an exclusive orthogonal ligand-receptor interaction to generate the components of a universal CAR T cell platform¹⁸². The engineered extracellular domain of the inert NKG2D receptors (iNKG2D) is fused to the intracellular 4-1BB and CD3 ζ co-signaling domains to generate the CAR while the mutant ligand domains that specifically bind to the iNKG2D are fused to intact human antibodies. Up to now, efficacy of rituximab-based convertible CAR T cells has been investigated in NSG mice bearing Burkitt lymphoma and demonstrated dose-dependent control of tumor mass. Similarly, SpyCatcher technology can be used to develop a universal

immune receptor¹⁸³. SpyCatcher fused to the intracellular 4-1BB and CD3 ζ co-signaling domains is used as an immune receptor that is armed with an antibody fused with SpyTag moieties. These new approaches can be exploited for cell surface modification. NPs can be decorated by the orthogonal ligands mutated $\alpha 1$ - $\alpha 2$ domain or SpyTag moieties to be conjugated on iNKG2D- or SpyCatcher-expressing immune cells, respectively.

Cell surface retention | As previously mentioned, cell membrane components (e.g., lipids and proteins) are continuously internalized, degraded and replaced¹⁸⁴. Therefore, means to prolong cell surface retention of NPs have been studied^{172,173,185–190}. In a thiol-maleimide approach, NPs-binding proteins have been identified by mass spectrometry¹⁷². The leukocyte common antigen CD45 is predominantly bound by maleimide-functionalized NPs. Other surface proteins such as LFA-1, CD2 or CD97 have also been identified. However, depending on the cell type, the direct targeting of a surface receptor can trigger undesired cellular responses. Consequently, other ways to increase cell surface retention have been investigated^{185–190}. Depending on their compositions and the type of interaction with the cell, NPs can be internalized by different mechanisms (e.g., clathrin- and/or caveolae-mediated endocytosis)¹⁹¹. Converging *in vitro* and *in vivo* evidences demonstrated that the antimalarial agent chloroquine effectively inhibits NPs clathrin-mediated endocytosis by depleting the phosphatidylinositol binding clathrin assembly protein^{185,186}. Similar results were found by studying the antipsychotic drug chlorpromazine^{187–189}. Nonetheless, these results have not been validated in *in vivo* models. More recent trial demonstrated that the antiemetic and antipsychotic drug prochlorperazine (PCZ) can be repurposed to reversibly inhibit dynamin-mediated endocytosis of membrane proteins targeted by therapeutic mAbs¹⁹⁰. In mouse models (squamous cell carcinoma, colon carcinoma and renal carcinoma) and in a pilot clinical study on head and neck squamous cell carcinoma, treatment with PCZ led to increased clustering of receptors on the cell surface, enhancing tumor cell-NK cells contacts, and finally driving to improve antibody-dependent cell-mediated cytotoxicity in response to approved

IgG1 mAbs, such as cetuximab (anti-EGFR mAb) and avelumab (anti-PD-L1 mAb)¹⁹⁰. However, PCZ did not affect targets that are not internalized by dynamin. Thus, this perspective might only be of interest in NPs anchoring targets with dynamin-mediated endocytosis. Although the abovementioned molecules showed promising results *in vitro* and in pilot clinical study, those molecules might have severe adverse effects *in vivo* (e.g., chloroquine: cardiac toxicities; chlorpromazine: agranulocytosis and prochlorperazine: extrapyramidal symptoms) and must thus be carefully handled.

2.2. Application of T cells backpacking

Many cell therapy protocols require adjuvant drugs to maintain transferred cells functions, phenotype and lifespan^{146–148}. However, systemic administration of such drugs is challenging due to their pleiotropic activities, leading to dose-limiting toxicities^{149,150}. NPs-targeting strategies with specific cell-targeting ligands, such as antibodies or small molecules have been investigated to deliver these molecules to the tumor site^{192,193}. Nonetheless, it has been shown that targeting ligands do not modify the overall NPs biodistribution but rather enable more efficient reaching of the tumor site by targeted NPs^{68–70}. Immune cells backpacking strategy with NPs-containing adjuvant cytokines and immune-checkpoint blockade agents have been explored to focus administration of such drugs on tumor site^{94,171–173}.

Backpacking T cells with up to 100 (\pm 20) liposomes (300 nm in diameter) did not affect key cellular functions (e.g., activation, transendothelial migration, tumor homing properties and antitumor functions) and allowed a 176-fold increase in NP accumulation into the EL4 tumor site when compared to free NP¹⁷¹. Loading interleukins 15 and 21 (IL-15 and IL-21, respectively) into the surface of multilamellar liposomes supported T cells antitumor function in an autocrine-like manner through a continuous release of bioactive interleukins over 7 days, and hence enhanced the therapeutic efficacy by efficiently preventing tumor growth up to 30 days after treatment. Moreover, the on-site drug action allowed the use of interleukins doses that are inefficient when systemically administered. Multilamellar liposomes loaded with SN-

38, the active metabolite of irinotecan, a potent topoisomerase I poison have also been used to tether T cells⁹⁴. In a Burkitt lymphoma mice model, tumor cells disseminated in lymph node were not sensitive to treatment with free SN-38 or liposomal formulation because of the drug poor pharmacokinetics due to a short half-life ($t_{1/2} = 7$ mins) and a rapid hepatic clearance¹⁹⁴ along with the lack of leaky neovasculature into the lymph node and thus, a lack of EPR effect. This lymph node homing property were exploited to deliver SN-38-loaded NPs on T cells in lymphoid organs enriched in lymphoma cells. SN-38-loaded NPs carried by T cells accumulated in lymph nodes 20h after infusion and SN-38 concentrations were 63-fold greater than free NPs and were maintained for 4 days. SN-38 released in a paracrine-like manner leading to a 60-fold reduction in tumor burden and an extended survival of mice up to 12 days at relatively low doses (7 mg/kg) when compared to the free drug. Although these approaches allowed to increase the treatment potency, the payloads (*e.g.*, IL-15, IL-21, SN-38, *etc.*) can passively leak out of the multilamellar liposomes and continually stimulate the T cells until their activation-induced depletion leading to a reduction in the effective dose of T cells trafficking to the tumor site.

To override this issue, a second generation of backpacking NPs have been designed¹⁷³. Nanogel (NG) backpacks have been engineered to transport IL-15 to tumors together with adoptively transferred T cells. ALT-803, an IL-15 superagonist molecule were aggregated into a NG with a linker including reduction-sensitive disulfide bonds that senses the reducing potential of the environment¹⁹⁵. To facilitate and prolong their cell surface retention, small quantities of anti-CD45 mAbs and poly(ethylene glycol)-*b*- poly(L-lysine) (PEG-PLL) were incorporated onto the NG surface. By engaging their cognate antigen in the tumor, activated T cells induce thiol groups emission at the cell surface, increasing their cell surface reduction potential that detaches the NGs. The release of IL-15 superagonist through this approach resulted in a 16-fold expansion of T cells in tumors, as compared with free IL-15. This approach allowed an 8-fold higher dose of cytokine to be administered without toxicity, widening its therapeutic window and enabling a significant increased tumor clearance by ACT

T cells and CAR T cells. However, stimulation of cell division will eventually lead to dilution of the backpacked NPs.

Cell-attached cargo actively transmigrate the endothelial barrier and accumulate in tumor sites, thereby enhancing their actions on transferred cells anti-tumor abilities and limiting systemic adverse effects. Given the plethora of available NPs tailored to deliver small molecules drugs, proteins, siRNA or magnetic imaging agents, T cells backpacking approaches profoundly open new perspectives for adoptive cell therapies and drug delivery that can be extended far beyond the small molecule drugs and recombinant proteins delivery aforementioned.

Conclusion

Nanomedicine is used in several ways to improve immunotherapy. Historically, NPs have been used to improve *in vivo* activation of immune cells by enhancing vaccination or radiotherapy efficacies, leading to an improved antitumor response. More recently, a deep focus on the immune cell surface bioengineering to tackle the intrinsic drawbacks of ACT of autologous immune cells or CAR cells is being investigated. These *ex vivo* immune cells labeling by NPs approaches will also allow NPs to either target the tumor microenvironment, or to use it as a trojan horse to enhance the tumor drug delivery.

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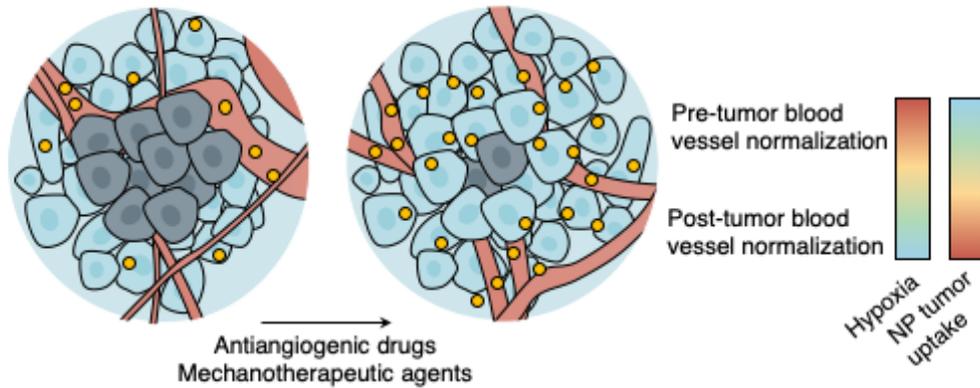
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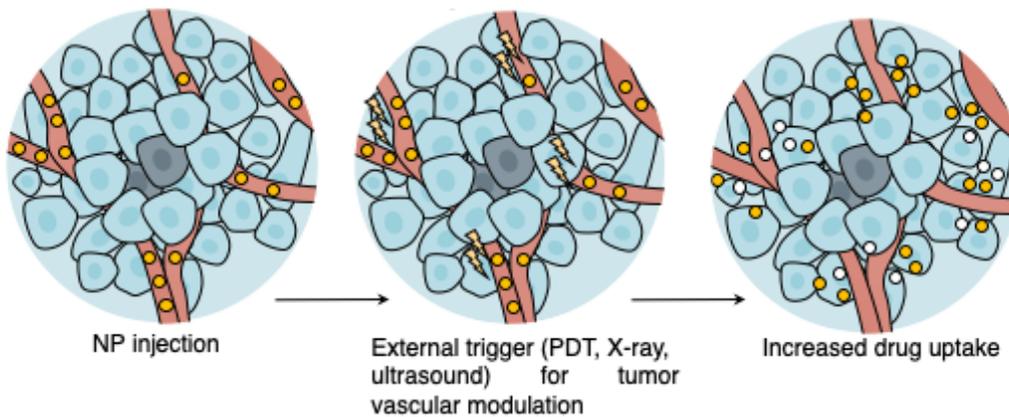
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Figures

A. Tumor blood vessel normalization



B. Tumor blood vessel disruption with external trigger



C. Liver priming

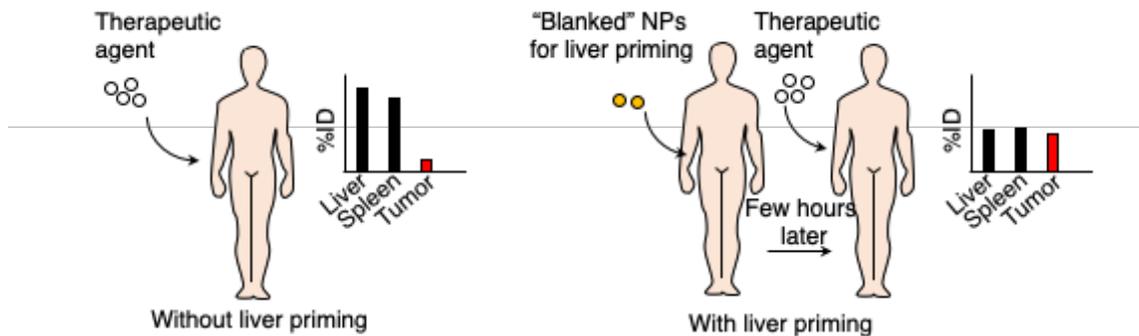


Fig. 1. Improving the passive targeting strategy. A) Tumor blood vessel normalization consists of using antiangiogenic drugs or mechanotherapeutic agents in order to decrease tumor hypoxia levels but also in normalizing the sizes of the endothelial gaps. Below 12nm diameter size, the NPs can benefit from this approach to increase their tumor uptake⁵²⁻⁵⁴. **B)** Tumor blood vessel disruption by an external element (e.g., radiation, UV, ultrasound) can dramatically improve NP uptake and drug delivery⁵⁰⁻⁵⁸. **C)** The liver priming consists in injecting blanked liposomes known into the liver followed by the therapeutics an hour later to override the hepatic metabolism and hence increase their circulation time in the body, resulting in an improved uptake in the tumor^{64,65}.

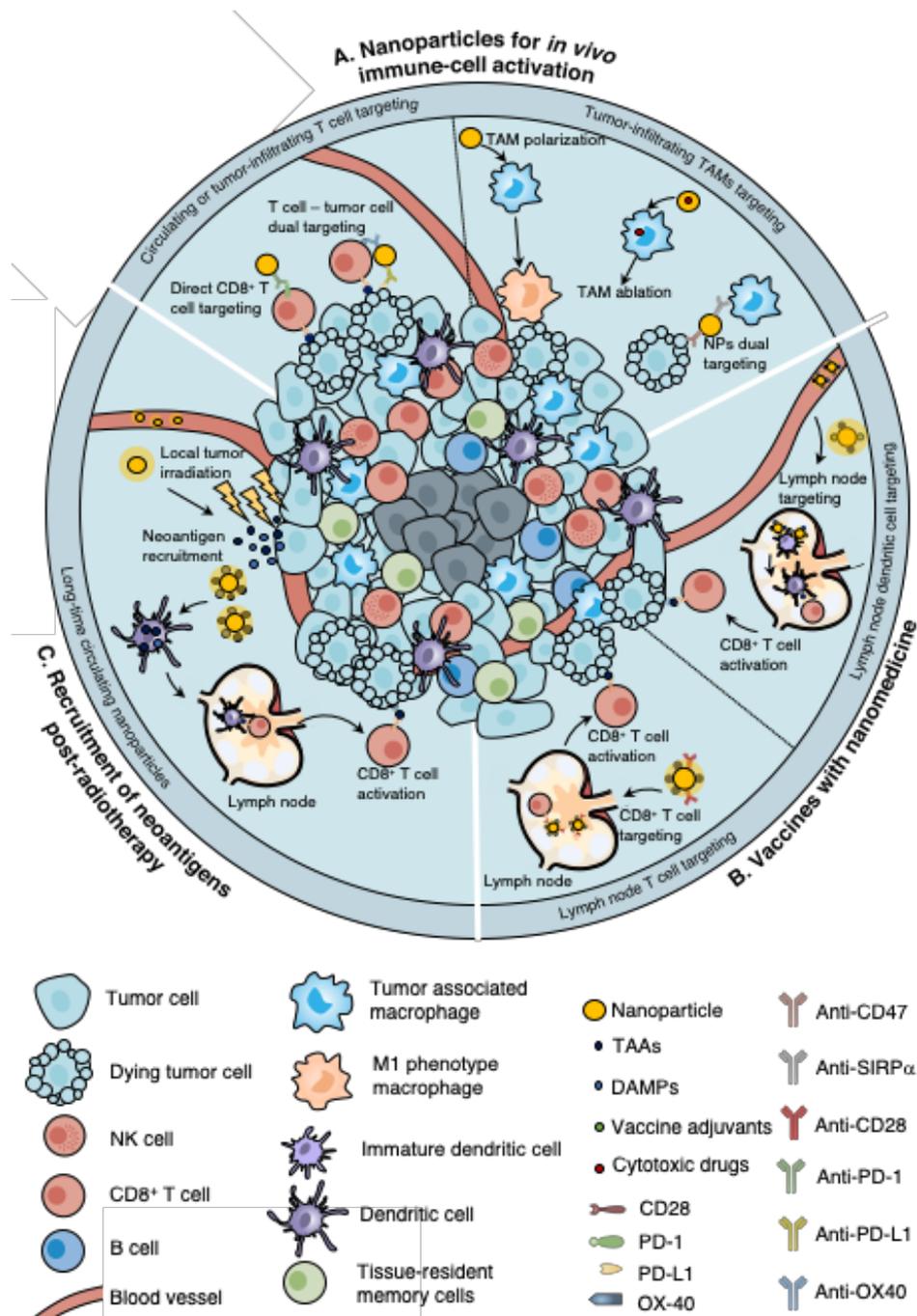


Fig. 2. Nanomedicine to improve immunotherapy. **A)** Nanoparticles (NPs) can be used for lymphocytes activation by targeting either the T- or NK cells or both immune and tumor cells. NPs can be used to target other immune cells present in the tumor microenvironment such as TAMs. They can either *i)* polarize TAMs towards a more antitumor M1 phenotype with mRNA, siRNA or TLR agonists. *ii)* Ablate them with cytotoxic drug delivery or *iii)* co-target TAMs and tumor cells to promote their phagocytosis. This activation can potentiate the delivery of small molecules that tackle the immunosuppressive tumor microenvironment^{77–84,91,94–97}. **B)** NPs can also be used as artificial antigen presenting cells to directly activate CD8⁺ T cells in the lymph node (lower panel)^{116–119} or to co-deliver tumor associated antigens (TAAs) and adjuvants (e.g., CpG, Poly(I:C)) (upper panel)^{120–122}. **C)** Radiotherapy leads to high TAAs and damage associated molecular patterns (DAMPs) release in tumor microenvironment captured by antigen-capturing NPs¹⁴². These NPs are uptake by dendritic cells that migrate to the lymph nodes to activate CD8⁺ T cells activation.

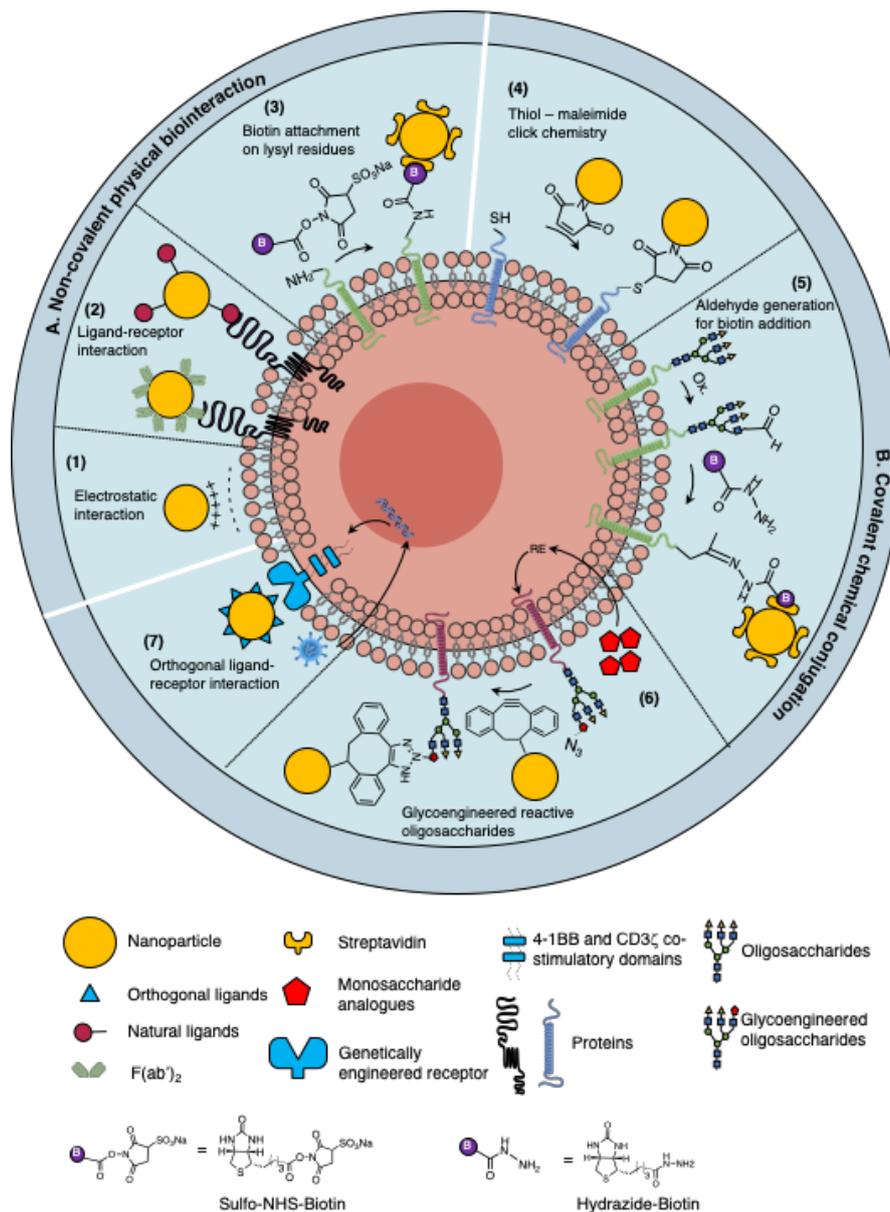


Fig. 3. Immune cells-based therapy with ex vivo nanomedicine labeling. Various biochemical strategies have been employed to conjugate antibodies or nanoparticles to yield active immune cells and can be broadly subdivided in **A)** non-covalent physical biointeractions and **B)** covalent chemical conjugation. Non-covalent conjugation of NPs to cell surface can be achieved between the negatively charged plasma membrane and NPs harboring cationic sites via electrostatic interactions¹⁵¹ (1). Cell surface also express receptors can be targeted by NPs functionalized with their respective ligands or antibodies fragments directed against them^{159,160} (2). Chemical groups existing on membrane proteins (e.g., -NH₂, -SH) can be leverage to successfully bonded biotin moieties^{163–165} (3) or maleimide-decorated therapeutic nanomaterial cargos^{171–173} (4). Biotin hydrazide mediated amidation of natural oligosaccharides can also be used to bind streptavidin functionalized NP^{163,164,166} (5) and glycoengineered oligosaccharides metabolically incorporated into living cell can be targeted with NPs presenting azides or alkynes groups at their surface^{176–178} (6). Finally, immune cells receptors can be genetically engineered to provide an exclusive orthogonal ligand-receptor interaction^{182,183} (7).