Advanced Drug Delivery Reviews 197 (2023) 114829

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/adr

Nanoparticle-based immunotherapeutics: From the properties of nanocores to the differential effects of administration routes

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ARTICLE INFO

Article history:
Received 22 February 2023
Revised 24 March 2023
Accepted 14 April 2023
Available online 28 April 2023

Keywords:
Nanoparticles
Immune system
Vaccine
Cancer
Immunotherapy
Combination therapy

ABSTRACT

The engagement with the immune system is one of the main cornerstones in the development of nanotechnologies for therapy and diagnostics. Recent advances have made possible the tuning of features like size, shape and biomolecular modifications that influence such interactions, however, the capabilities for immune modulation of nanoparticles are still not well defined and exploited. This review focuses on recent advances made in preclinical research for the application of nanoparticles to modulate immune responses, and the main features making them relevant for such applications. We review and discuss newest evidence in the field, which include *in vivo* experiments with an extensive physicochemical characterization as well as detailed study of the induced immune response. We emphasize the need of incorporating knowledge about immune response development and regulation in the design and application of nanoparticles, including the effect by parameters such as the administration route and the differential interactions with immune subsets.

Contents

Abbreviations: APC, Antigen presenting cells; BCR, B cell receptor; CTL, CD8+ Cytotoxic lymphocytes; DAMPs, Damage-associated molecular patterns; IDC, Immunogenic cell death; ICB, Immune checkpoint blockade; IA, Intra-arterial injection; ID, Intradermal injection; IM, Intramuscular injection; IN, Intranasal injection; IP, Intraperitoneal injection; IT, Intrathecal injection; IV, Intravenous injection; LNs, Lymph nodes; MHC, Major Histocompatibility Complexes; PAMPs, Pathogen Associated Molecular Patterns; PD-1, Programmed cell death 1; PRRs, Pattern recognition receptors; SC, Subcutaneous; TCR, T-cell receptor; Tex, Exhausted CD8+ T cells; Th, CD4+ T helper; TLR, Toll-like receptor; TME, Tumor microenvironment; TNF, Tumor necrosis factor.

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https://doi.org/10.1016/j.addr.2023.114829
0169-409X/

1. Introduction

Nanoparticles (NPs) have shown high versatility as tools capable of actively interacting with specific cell receptors similarly to biological entities, stimulating the development of nano-based applications in the biomedical field. At early stages, one important discovery that launched NP research was the NP capability to passively accumulate in solid tumors, phenomenon known as enhanced permeability and retention (EPR) effect, originally proposed by Y. Matsumura and H. Maeda [1]. Such size-specific accumulation was claimed to be a consequence of the defective vascularization and lymphatic drainage, promoting NP transvasation in the tumor microenvironment (TME). Although the EPR effect remains highly controversial [2], it currently remains one of the main motivations and claimed advantages over traditional drugs, to direct nano formulations into solid tumors to exert an effect. Influencing such accumulation are intrinsic physicochemical parameters of the NPs like size and shape, composition, and synthetic and biological coatings [3-6]. Collective and vast research efforts have been focused on relating such physicochemical properties of NPs with their passive targeting capacity. Along with this fact, NPs have shown certain degree of intrinsic immunogenic properties capable of activating different cascades and pathways of the immune response, trying to apply and use these effects to stimulate immunity. Owing to this, different core compositions have been tested and studied as tools to treat diseases, mostly applied to cancer [7-11]. Intrinsic immunogenicity of NPs can be produced in different ways, for example, by degradation and liberation of products to the bloodstream and accumulation in tissues, or by their interaction with biomolecules and subsequent engagement with immune cells that will be highly directed by the nature of the biomolecular surface of the NP.

Difficulties encountered to understand and control such effects by NPs have led to a body of literature reporting a broad range of effects depending on the cell type studied and the experimental set up, and unclear origin of such immunogenicity. In addition, passive targeting of NPs has the typical drawbacks of other passive drugs such as off-target effects, mediated by the formation of the so-called biomolecular corona through interactions of NPs with surrounding plasma proteins, making the NPs being rapidly eliminated from the bloodstream by the reticuloendothelial system (RES) [2,12].

NPs have been manufactured of different natures and multiple source materials (metallic, polymeric, lipidic, *etc.*), with defined surface chemistries and functionalities, and enhanced physicochemical properties [13-15]. The strength of nanoformulations resides in the stabilization and protection of therapeutic agents, as well as the capability to enhance the amount delivered to a specific site, either by encapsulation or grafting (or both) of molecules [16-19]. However, there has been relatively modest impact of, specifically, inorganic-based NPs as vehicles for vaccination or other therapeutic applications. This can be explained by the vast range of different effects that have been reported in the literature as a function of the different sizes, surface coatings and experimental set up (*i.e.*, cell and animal models, dosing, contamination, *etc.*) when trying to search for intrinsic immunomodulatory effects of inorganic NPs, focusing the attention on the NP core itself. In this way, there is a medley of potential formulations with different effects depending on their extremely highly characterized physicochemical properties and, in many cases, the outcome immune responses appear characterized in a superficial way. Recently, R. Mohammapdour and H. Ghandehari have extensively reviewed the interaction between inorganic nanomaterials and the immune system focusing on the aforementioned intrinsic properties of nanomaterials [20]. As a matter of fact, most of the approved nanomaterials correspond to soft structures like liposomes, polymeric and protein NPs, while inorganic-based NPs, have shown relatively poor advances in terms of translating such materials to clinical trials for immune related purposes and most of the approved formulations based on inorganic NPs are applied as imaging and contrast agents [7]. Feeding from the field of vaccinology, soft material-based NPs, like liposomes, polymeric and protein NPs, or virus-like particles (VLPs) have developed in close conjunction with immunology and adjuvant sciences, and reached higher levels of clinical translation and commercialization, with the most recent example being SARS-CoV-2 vaccines. Most of them rely on a more efficient delivery of antigens and adjuvants in relatively simple formulations to the lymphatic system, enhancing lymph node trafficking and processing by immune cells at specific niches, thus promoting immunity. In contrast to such inert vehicles, inorganic based NPs, like metallic or alloy NPs, possess in their nanosized version great properties that have shown promising for inducing specific effects that can trigger new or promote existing immune responses. By incorporating magnetic, plasmonic or radioactive properties to the formulation it is possible to induce effects such as the so-called immunogenic cell death (ICD), which can enhance immunity. Also, the size and the shape of inorganic NPs can be precisely controlled, which directly affect their lymphatic trafficking to lymphoid tissue and the way of engaging with resident cell membrane receptors, features that can be beneficial for specific therapies. In addition, the ease and high control over encapsulation and surface functionalization with a variety of molecules in a density-controlled way make inorganic NPs great tools with huge room for improvement towards therapeutic applications similarly to soft materials.

To enhance even more the potentiality of NPs in such areas, a better understanding of how adaptive immune responses are regulated at a high mechanistic and functional levels have highlighted

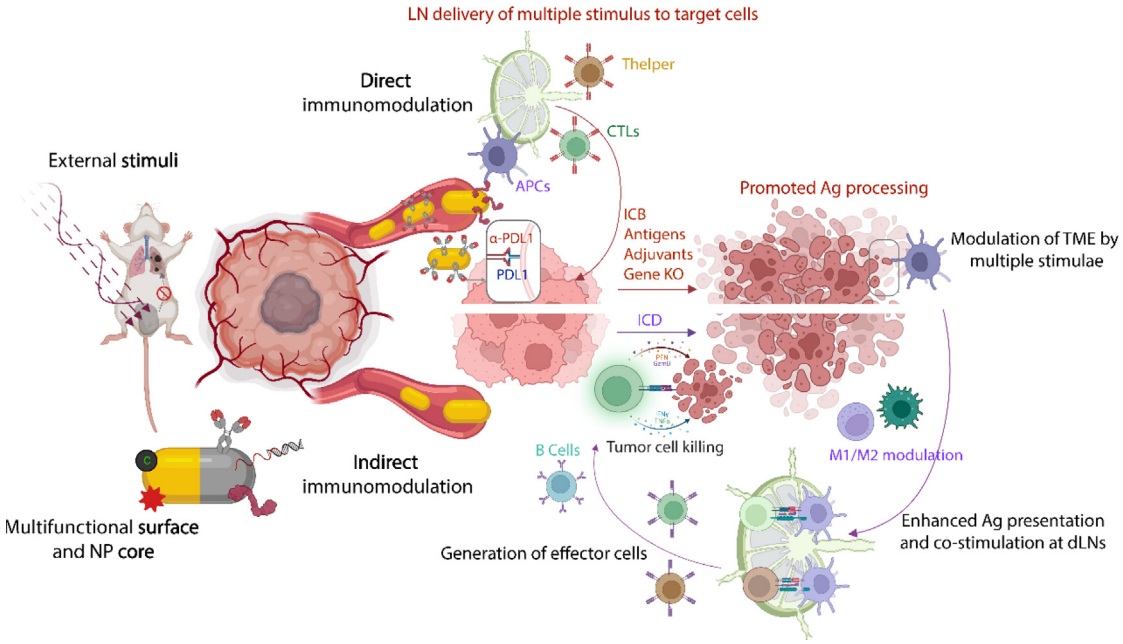

Fig. 1. Schematic representation of the multiple features and point of actions for inorganic NP based immunotherapy. Multifunctional NPs can include different levels of modulation, here named as direct or indirect. By direct modulation it is possible the delivery of active molecules such as adjuvants, ICB antibodies, nucleic acid for gene knock in or knock out (siRNA, mRNA), chemotherapeutic drugs *etc.* In addition, it is possible to take advantage of the intrinsic properties of the core materials that can respond to external stimulate (photodynamic therapy, PDT, photothermal therapy, PTT, etc.) or sense environmental conditions (pH, hypoxia, *etc.*). This results in the possibility of modulation at the immunological synapse level at target sites such as the lymph nodes (LNs), for instance by effective delivery of ICB antibodies, or repression of regulatory signals, it can be unleashed pre-existing immunity and promote de novo responses; in addition, indirect NP effects can typically result in immunogenic cell death (ICD) enhancing antigen (Ag) presentation. Together, correct processing by antigen presenting cells (APCs) and generation of immunogenic co-stimulation in secondary lymphoid organs (SLOs) promotes differential effector cell (and cell subset) development.

multiple ways to modulate them for specific outcomes, opening the era of immunotherapies [21]. It is now clear that, in order to have a significant therapeutic effect, the designed formulations should affect specific compartments of the immune landscape [22]. Current efforts in immunotherapy aim to develop specific effector cells, namely, B and T lymphocytes. One of the most promising immunotherapies nowadays correspond to immune checkpoint blockade (ICB), reverting processes such as T cell exhaustion by targeting inhibitory molecules [23-25]. The unravelling of the functions of molecules such as Cytotoxic T lymphocyte antigen 4 (CTLA-4) and Programmed cell death 1 PD-1 lead to the development of monoclonal antibodies interfere with such markers within the immune synapse, re-stablishing the functionality of the immune cells [26]. The FDA has already approved the use of immune checkpoint blockade (ICB) monoclonal antibodies to CTLA-4 (Ipilimumab) and PD-1 (Pembrolizumab and Nivolumab). B and T lymphocytes (particularly the second ones in which this review is mostly focused) correspond to the main effector cells of the adaptive immune system. Therefore, developing pathogen-specific B and T cell immunity is the main outcome sought in the search for both therapeutic and prophylactic actions.

Nanotechnology can generate smart drugs that reduce some of the drawbacks of generic drugs like chemotherapeutics and immunotherapies [27]. Most typical flaws are reduced circulating times, quick degradation, lack of specificity producing off-target and deleterious high dosing effects leading to immune pathology. NP based formulations can help in the stabilization and concentration of multiple compounds in one nano system, therefore reducing deleterious effects. Besides, the physicochemical properties of the inorganic cores (photothermal, photodynamic, magnetic, catalytic, *etc.*), in combination with modulatory molecules, can enhance their performance by being able to induce local effects. It is also relevant to mention that, opposite to the typical systemic administration of NPs that typically produces a rapid accumulation in filtering organs, there has been a shift based on knowledge mostly from vaccinology that relates to the differential route application based on the target.

In this review, we focus on a range of recently developed NP-based nano formulations, predominantly inorganic-based, that were specifically designed to perform tasks in a target organ and cell to achieve a relevant benefit in the form of immune modulation (Fig. 1). We intend to slightly shift the focus of the NP design to relevant aspects from the immunological perspective, *i.e.,* the main effector cells that currently are believed to play key roles in developing immunity, the features and functions of such cells and the different strategies. In this way, we believe that deep knowledge on the immune status, the specific cell and subpopulations target and the interplay in the context of a complex immune response to mount and trigger specific responses, is key for the correct development of the field. Besides, we show the possible benefits and current ways of applying inorganic NPs based on the contribution of the core to the constructs. Lastly, we evaluate the relevance and impact on the administration route. Accordingly, we discuss how such formulations are administrated and assess the effectiveness based on the route.

2. Current understanding of immune effects triggered by NPs

Following NP administration, complex interactions with the immune system typically arise based on the physicochemical properties of the NPs [20,28-32]. It seems critical to understand that the interaction of NPs and the immune system relies on multiple factors, including size, shape, composition, structure, charge, and hydrophilicity.

It has been proposed that different NPs cores, based on their physicochemical properties such as the material size or shape, induced differential effects in immune cells *in vitro*. Proteomic studies have revealed differential profiles for Cd, Au and Cu NPs

in terms of regulation of nuclear topoisomerases and heat-associated proteins (HSPs), as well as induced reactive oxygen species (ROS) production [33]. On the other hand, gold NPs (AuNPs) promoted the up-regulation of inflammatory cytokines (Nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB) which is mediated by the inactivation of a negative regulator of NF-κB [33]. Ag-based, have been shown to decrease the secretion of pro-inflammatory cytokines in response to bacterial lipopolysaccharide (LPS), likely because of the release of silver ions leading to an interference with Toll-like receptor (TLR) signaling [34,35].

One of the most reported and studied effects of NPs include their role in generation of ROS. ROS are free radical molecules resulting from natural metabolism, which, when excessive and unregulated, are directly correlated with the onset or remission of inflammation, one of the key processes mediating human pathologies such as cancer, neurodegeneration, and stroke, among others. Inflammation provokes the unbalance between endogenous production of free radicals and antioxidant defenses, resulting in oxidative stress [36]. Moving forward to more specific effects, NPs are being used to deliver immunosuppressive drugs, as it is the case of iron oxide NPs (IONPs), which have been proved to weaken the antigen-specific humoral response and T cell cytokine expression in ovalbumin (OVA)-challenged mice [37], or multi-walled carbon nanotubes (MWCNTs), which have been reported to suppress systemic humoral immunity in mice [38,39]. CeO_2 NPs were reported to reduce ROS and the level of inflammatory cytokines interleukin-6 (IL-6) and tumor necrotic factor (TNF)-α in murine macrophages [40], while fullerene formulations have been proved to inhibit hypersensitivity reaction to allergens both, *in vitro* and *in vivo* [31,41]. The final effect of NPs can also depend on interaction with living systems which can result in degradation process like surface ion dissolution, modifying preexisting properties. NPs that are more prone to suffer from this phenomenon such as ZnO and FeO exhibits higher toxicity whereas the less corrodible ones such as CeO_2 and TiO_2 exhibit fewer immune effects [42]. It appears obvious that physicochemical properties of NPs cannot be overruled when thinking on specific effects. Thus, the connection of such parameters to the immunological applications is discussed.

Undoubtedly, such features of NPs have an influence on the engagement with immune cells and tissues and they should be considered as subject in this review, on the other hand, as previously stated, they will not be the focus as they have been extensively reviewed before. While they will definitely affect the performance of immune targeted formulations, the main otcomes at the effector immune response level will be achieved by immunomodulatory, bioactive and targeting molecules included in the synthetic construct.

2.1. Shape

During NP-cell interaction, the different shape patterns displayed by NPs are expected to elicit a differential interaction with the cell membrane leading to the triggering of a variety of cell recognition pathways. NP shape variation could induce different stress on cells, mediated by the clustering of surface receptors or by stressing the cytoskeleton locally [43]. Cells will differentially engage with such patterns, emphasizing the need for controlling NP shape for biomedical purposes. Moreover, upon *in vivo* administration NP surface is going to be modified due to the unspecific absorption of plasma proteins (opsonins) which will affect to the downstream NP-cell interactions generated. Comparing Au nanospheres of 50 nm with Au nanourchins and Au nanostars, authors described an 2–3-fold increase of shape dependent production of immunoglobulin (Ig) G in rat serum after subcutaneous (SC) administration of Au nanourchins. In addition, the urchin-shaped NPs also contributed to the generation of a more diverse B cell repertoire [44]. Those results were in coherence with previous reports in which the transcriptome of dendritic cells (DCs) *in vitro* was differentially affected by the exposure to the urchin shaped AuNPs [44,45]. Since these NPs included no adjuvant or immunomodulator, the effect observed can be ascribed uniquely to the different shape displayed by AuNPs. NP shape is going to act as an independent modulator of the immune response, influencing processes like antigen presentation or B cell-T cell collaboration. When tested with an adjuvant on their surface, AuNPs have also shown differential immune modulation. Using Au nanospheres of 20 and 40 nm and Au nanorods of 30 and 40 nm, authors found a differential adjuvanticity enhancement for Au nanorods after intranasal administration. Using 20 and 40 nm sized Au nanospheres and 30 and 40 nm sized Au nanorods, authors found a differential adjuvanticity enhancement for Au nanorods after intranasal administration. Au nanorods induced higher level of IgA in nasal wash samples and in nasal mucosa emphasizing the differential role of size in the modulation of adaptative immune response [10].

2.2. Size

NPs can be produced in a broad range of sizes, also, once injected in bloodstream, NPs can agglomerate (a reversible phenomenon) or aggregate (irreversible) depending on their surface chemistry and on the complex environmental conditions (ionic strength, non-specific protein adsorption, *etc.*) [8]. Aggregated and big NPs (1–5 μm) are shown to be detected more effectively taken up by phagocytes in different organs, whereas NPs below 200 nm are preferentially internalized by other endocytic routes [29]. Using 20 and 40 nm sized Au nanospheres as well as 40 nm sized Au nanorods and 40 nm sized Au nanocubes of 40 nm coated with a West Nile virus protein, authors revealed a differential profile in the induced immune response. Spheres of 40 nm were proved to be the stronger inducer of specific IgG production [46]. Deepening in the analysis of the antigen presentation in LNs, authors have shed light about the optimal AuNP size range for an efficient antigen delivery to immune cells. In LNs, APCs internalize AuNPs-OVA conjugates in a size-dependent manner. NPs below 15 nm are relatively faster cleared from the LNs (48 h) whereas NPs from 50 to 100 nm remained for at least 5 weeks. This enhancement of NP retention led to a 175-fold increase in antigen presentation ability by APCs, 5-fold increase of adaptative immune response induction as revealed by the B cell generation and the specific anti OVA antibody production [28]. Those results proved that the surface area relation of NPs of various sizes and shapes is a key feature which directly influenced both the secretion of pro-inflammatory cytokines and antibody production.

2.3. Charge

Inorganic NP surface charge plays a key role during NP internalization by cells. As generally believed, positively charged NPs are more prone to interact with negatively charged cell membranes therefore enhancing the uptake of cationic NPs [6]. Regarding pulmonary therapy, cationic NPs modulated the local lung environment to promote recruitment and maturation of lung DCs, but anionic NPs were found to be immunologically inert in the lung [11]. On the other hand, neutral coatings achieved with a silica layer on polymeric NPs have been showed to exert no immune activation *in vitro*, significantly decreasing the magnitude of immune response [47]. However, this interaction could experiment variations depending on the cell type as revealed by a recent comprehensive study with SiNPs, where anionic coatings led to an

enhancement of NP uptake by intestinal epithelial cells through the interaction between NP coating with cell surface integrins [16].

2.4. Biomolecular corona and surface patterning

Surface tailoring of inorganic NPs allows incorporation of molecular features different from their inherent core material. Those modifications will influence different parameters like hydrophobicity or the arrangement of structural patterns or the biomolecules displayed on the NP surface. Regarding hydrophobicity, most pathogen, and damage-associated molecular patterns (PAMPs and DAMPs), include hydrophobic motifs which are recognized specifically by immune cells receptors triggering of innate immune response [48]. Using AuNPs of 2 nm it has been shown that NPs bearing a hydrophobic zwitterionic functionality boost inflammatory outcomes while hydrophilic zwitterionic NPs generate minimal immunological responses both *in vitro* and *in vivo*. These results demonstrate the ability of simple surface ligands to provide immunomodulatory properties [49].

The immune system has evolved to recognize patterns displayed in virus capsids and envelopes, which are characterized by repeated shapes in a structured hierarchy [50,51]. This has been highlighted to be one of the main mechanisms by which NP trigger the innate immune response for mounting immunity. For example, triggering of the complement system by Complement protein C1q, which shares a common origin with other opsonins like Igs, occurs by recognition of PAMPs. Similarly, NPs can trigger such cascade activation events by biomolecular corona formation. The newly formed biomolecular surface is going to influence the NP-cell recognition as well as the internalization by receptor-dependent endocytosis conferring a completely different biological entity to the NPs [52,53]. The biomolecular corona can promote the recognition and subsequent elimination by immune cells. One of the most studied effects is the process of opsonization as a result of binding of apolipoproteins, Igs, and complement proteins [54].

Generally, upon opsonization NPs are easily recognized by the mononuclear phagocyte system (MPS) decreasing the blood circulation time and therefore the bioavailability of NPs. From these receptors, FcRs and Complement receptors are critically involved in NP internalization through the recognition of IgG and complement components C3d/C3bi respectively [32]. Complement activation involves a set of pathways which leads to the exposure of C3 protein fragments (C3b and iC3b) in the surface of antigenic agents in the bloodstream. Those fragments are then recognized by macrophages and Kupffer cells of the RES system (through the complement receptors 1 and 3, CR1 and CR3), facilitating the removal of intravenously injected NPs. Moreover, during complement activation, several proteins are generated by proteolytic cleavage (C4a, C3a and C5a), resulting in the production of an anaphylactic reaction by the histamine release by the mast cells [9].

Dysopsonins like clustering or serum albumin can also cause the opposite effect, prolonging the circulation time in blood and enabling escape from MPS clearance [5]. Corona formation remain one of the most studied issues related to NP applications in the biomedical field and its effect has been deeply studied. In a way, most of the unwanted and non-specific immune effects of NPs relate to the formation of such layer. However, for specific applications, such as the design of immune triggering vectors, it could be beneficial the enhanced recognition by target immune cells such as antigen presenting cells (APCs) [55].

Similarly, there might be an effect of molecules introduced during the synthesis process. One typical example often underrated is the presence of LPS adsorbed to NP surface. The binding to TLR-4 on cell surface will lead to the induction of proinflammatory response involving caspase activation leading to an alteration in monocyte and DCs status [56-58]. Trace amounts of LPS if we want to avoid extra immunomodulatory effects leading to a misestimation of the experimental results [58]. Other potential microbial contaminants, like cytosolic double-stranded DNA, typically cyclic dinucleotides (which generally comes from pathogens), can activate the stimulator of interferon genes (STING) pathway leading to immune cell activation. In this regard, NPs incorporating other cyclic structures, for example cyclic lipids, that mimic double-stranded DNA, could induce STING signaling regardless of their cargo [59]. Authors have proposed that the main effectors of these reactions are not the nanomaterials themselves, but the proteins adsorbed on their surface upon administration, at least during the activation of the complement alternative pathway as it was demonstrated using dextran coated IONPs incubated with serum and plasma proteins [32]. With regards to cancer immunotherapy, complement activation could condition the efficacy of current nanomedicines. The release of the C5a protein is termed to increase the presence of immunosuppressive cells in the TME, hindering for example the infiltration of cytotoxic T cells [32].

Extensive research has been addressed to the rational modification of NPs surface to influence or modify opsonization. Current strategies addressed to the preparation of stealth nanomaterials are focused on the NP surface modification with polymers (*i.e.*, PEG) and block copolymers, zwitterionic coatings and polysaccharides (*i.e.*, dextran). In this regard, zwitterionic coatings are termed to be highly hydrophilic since they are hydrated through strong electrostatic interactions. Molecules that hydrate zwitterionic polymers are structured in the same way as in bulk water. This arrangement makes zwitterionic polymers thermodynamically unfavorable for protein adsorption because the displacement of water molecules from the surface by plasma proteins does not provide an increase in the free energy [60]. PEGylation of NP surface has been the most employed antifouling strategy during the NP design for biomedical applications due to the properties exhibited including electrical neutrality, significant spatial repulsion, and high hydrophilicity. In addition, its chemical structure offers several reactive residues for modification and further grafting on NP surface. The PEGylation degree of NPs can be flexibly changed and adjusted, and it is critical to precisely assess the coverage density and conformation of PEG on the NP surface [61]. PEGylation provides a amphipathic protective shield surrounding NPs crating a steric coating where polymer prevents from the binding of either undesired plasma proteins or complement ligands upon administration in the bloodstream and/or tissues [12]. Recently some concerns have arisen from clinical studies of the SARS-CoV-2 mRNA vaccines regarding the possibility of antibody responses against the PEG included in vaccine formulations, which might induce allergic reactions [62]. Nevertheless, these findings are still reviewed under controversy since the causality relation between immunization and anti-PEG reactions remains unclear. In contrast to the previous work, in a recent extensive study involving rodents and non-human primates, authors did not find the induction of anti-PEG antibodies up to three months after immunization with pegylated HIV peptide [63].

As mentioned before, the structural geometry and patterning of biomolecular motifs has being connected to differential recognition and processing [51]. It has been shown that Au nanorods with large aspect ratio conjugated with and anti(α)-CD3 and α-CD28 antibodies induces T cell expansion as well as cytokine releasing including IL-2, IFN-γ, and tumor necrosis factor-alpha (TNF-α). Anisotropic stimulatory ligand presentation increases CD137 expression which leads to the differentiation of naïve CD8 + T cells. High membrane tension observed in high aspect ratio Au nanorods modulates actin filament rearrangement, inducing phenotypic changes in T cells like membrane ruffle formation, cell spreading, and large T cell receptor (TCR) cluster formation [64].

Such parameters mentioned in this section have a clear impact on the behavior of the NPs once introduced in biological systems, however, in terms of the triggering and development of immune responses for therapeutic applications, there is a lack of clarity regarding the outcome responses that makes difficult to connect the features of the NP cores to any specific effect that could be beneficial. In opposition, we propose a body of literature that put focus on the rational design and administration of NP-based formulations with clearly define targets and deeper characterization of the immune repertoire.

3. Main immune cell targets for NP-enabled modulation

The immune system relies on two different kinds of responses to detect and eliminate dangerous entities known as innate and adaptive responses [65]. The innate arm of the immune system is a broad, non-specific response which recognition and triggering components are fully encoded in the genome [66]. Its primary mission is to quickly detect PAMPs and DAMPs from microbes as well as self-aberrancies (death cells, tumor development, misfolded proteins, immune complexes). Pattern recognition receptors (PRRs) located mostly in the membranes myeloid innate immune cells such as DCs, macrophages, monocytes (Mo) and neutrophils are the main responsible for detecting those structures. TLRs or scavenger receptors (such as macrophage receptor with collagenous structure or MARCO) are some of the most widely studied examples of innate receptors and they have been heavily studied as the main drivers of NP clearance [3,4,67,68]. In contrast, the adaptive immune response relies on the generation of pathogen-specific molecules though a set of recombination events of genes in the germ line. T cell receptors and B cell receptors are the membrane-anchored molecules responsible for pathogen-specific recognition by the adaptive effector cells, T and B lymphocytes respectively.

The generation of a functional immune response to an external pathogen, tumor cell or vaccine, involves a complex cascade of events requiring the interplay between innate and adaptive immunity. A basic immune cell cycle can be delineated. Innate immunity oversees recognizing the threat through their PRRs, internalize it and present antigens through their Major Histocompatibility Complexes (MHC) to cells from the adaptive response, along with other types of co-signals that will influence the outcome of the response, such as co-stimulatory and co-inhibitory receptors, and soluble factors like cytokines and chemokines [69]. That series of events will define the final immune populations. Thus, understanding the immunological landscape and the biology of the target populations should be the basis of the design. Immune responses originate at specialized anatomical locations such as secondary lymphoid organs (SLOs, LNs and spleen) and local lymphoid tissue in the site of the lesion. Therefore, they must be considered as well for specific applications when designing the route of administration of any nano-based therapeutic agent [70].

Immunotherapies are increasingly recognized to be a promising strategy to elicit systemic immune responses and establish wide-spectrum treatment regimens for a variety of tumor types, since they aim to target the immune system rather than the tumor itself. The best way to apply different sets of molecules targeting different markers is to know their biological function within the tumor immune cycle. Besides MHC-Antigen presentation by APCs, specific signaling to naïve cells is required for correct immunogenicity. For instance, co-stimulatory molecules (*e.g.*, CD80, CD86, CD40, CD137L, OX-40L) and cytokines (*e.g.*, IL-12, IL-6, IFN-γ) ensure the generation of a functional antitumor response. The application of NPs for therapeutic actions will increase the efficacy when the effects are understood at the target population level. Therefore, we have separated the different contribution aiming to highlight the main cell type or response that was pursued and define which is the outcome and how that could be used for immunotherapy.

In this section we will show several recent examples of reports focusing on the characterization of specific responses that drive the NP design, this means, selection of antigens and immunomodulatory molecules for enhancing specific cytotoxic and/or humoral responses. To do so, we have selected examples of both inorganic but also organic based formulations as we considered it instrumental to making the point that design should be closely related to specific outcome to be triggered.

3.1. Dendritic cell targeting for antigen presentation

DCs belong to the MPS, and they are present in a wide range of phenotypes (conventional DCs, plasmacytoid DCs, monocyte-derived DCs) based on their expression patterns, localization, and functions [71]. DCs are central players in the initiation and regulation of adaptive responses and immune tolerance. DCs correspond to the most effective and specialized APCs to trigger immune responses though recognition of PAMPs and DAMPs by their PRRs [65]. Once activated and maturated, DCs possess the roles of antigen processing and presentation in lymphoid tissues for effector T cell stimulation [72]. Internalized antigens are degraded in endo/lysosomal compartments by proteases and loaded into MHC class II molecules for $CD4^+$ T helper (Th) Cell stimulation and activation in classical antigen presentation. Intracellular antigens are instead loaded in MHC-I molecules (cross-presentation) for engagement with $CD8^+$ cytotoxic lymphocytes (CTL) [73]. Antigen presentation and effector cell activation takes place mainly in draining LNs, where naïve T cells are attracted by chemokines for antigen presentation, and they are selected based on their TCR specificity. The different formulations used for such task are highly diverse [11,63].

Induction of DC-based therapies have already shown certain efficacy, for instance, by generating *ex vivo* induced DCs (Sipuleucel-T) that carry tumor antigens [74]. However, the efficacy of such therapies is still low and only less than 10% of mature DC-mediated immunotherapy has been reported to be effective in clinical trials [75]. To develop effective DC-based therapies, it is important to produce delivery systems that can efficiently carry antigens to DCs to generate potent T cell immunity [76]. In addition, the target DCs should develop in the desired subpopulation and generate the right co-stimulation. Along with the antigen delivery other functionalities and/or cargoes are needed for the correct development of immunity. A broad range of NPs have now been developed for such task in different formats, both by immobilization in their surface or encapsulation of relevant molecules. Different kinds of inorganic-based materials have been heavily studied, such as silica [77-80] and gold [81], as well as organic-based such as PAMAM dendrimers [82], polymers or lipids [67,83], and combinations of materials of varying sizes and shapes [43,44,84].

The common strategy followed independently of the physicochemical properties of the material is to provide, along with the antigen of interest, molecules, or drugs in the form of adjuvants that are co-delivered by the nanovector, enhancing the capability to be recognized and internalized by DCs to induce maturation and secretion of specific cytokines. Unmethylated cytosine–guanine (CpG) oligonucleotides engage with TLR9 for activation of immature iDCs to mature mDCs and is one of the most widely used PAMP to trigger immunity. Cho and coworkers have developed a multifunctional core–shell NPs of superparamagnetic iron oxide (SPIO) core and ZnO shell and report efficient uptake by bone marrow-derived iDCs *in vivo* in draining LNs [85]. iDC activation was shown based on the levels of MHC-II^+ $CD40^+$ and $CD80/CD86^+$-

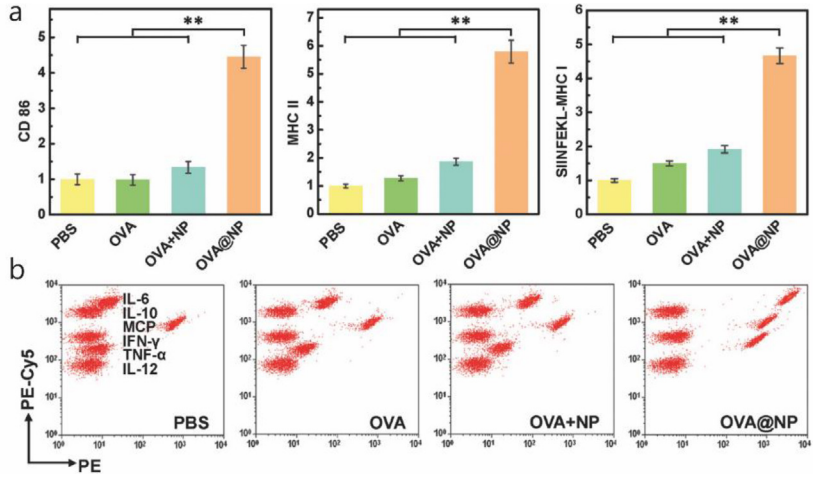

Fig. 2. $CaCO_3$ complexed with OVA efficiently delivers antigen to DCs to induce maturation, antigen presentation and cytokine secretion. a) Induction of DC maturation by OVA@NPs and increased antigen cross-presentation. b) Flow cytometry analysis of a range of cytokines induced by OVA@NP treatment. Adapted with permission from Ref. [86].

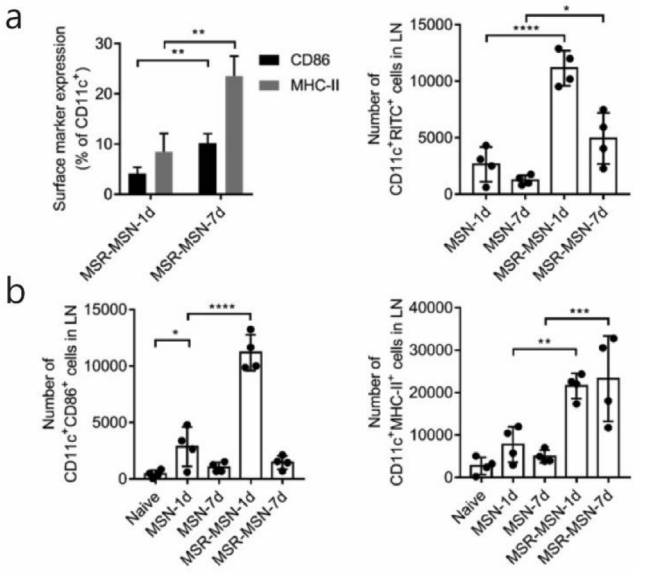

Fig. 3. Co-delivery of chemokines, antigen and TLR agonist by a 3D assembly of MSNs enhanced DC recruitment, maturation, and antigen presentation. a) Time-scale activation of $CD11c^+$ DCs by flow cytometry analysis of CD86 and MHC-II expression (Left) and increased infiltration of DCs to draining LNs (Right). b) Absolute counts of mature $CD11c^+$ $CD86^+$ and $CD11c^+$ $MHC\text{-}II^+$ DCs in DCs after treatment with multiple formulations. Adapted from Ref. [79].

cells. Such particles were designed to deliver tumor antigens (carcinoembryonic antigen, CEA) and produced certain remission of the tumor *in vivo* as well as slight increase in survival. Such effect is linked by the authors to the generation and boost of a specific antitumoral anti-CEA effector cell response.

$CaCO_3$ NPs have been widely studied as delivery vectors [86]. Wang et al. have developed a one-pot approach to prepare a $CaCO_3$ NP-based high-performance antigen delivery and cross-presentation of OVA as a model antigen to demonstrate potential for DC-based therapy (Fig. 2) [86]. The enhanced capacity of the construct to induce MHC class I-Antigen complex (MHC-I-Ag) expression and presentation is based on the capability of the NPs to burst lysosomes producing lysosomal scape of the OVA for further processing. Vaterite ($CaCO_3$) can rapidly decompose in the lysosomes inducing CO_2 generation and blasting the lysosomal membranes. The synthetic strategy is based on the templating effect of OVA promoting the production a hierarchical structure to facilitate the decomposition of the NPs therefore liberating the antigen along with the production of CO_2. The authors show induction of the maturation of DCs based on the expression levels of activation markers CD86, MHC-II and MHC-I-OVA antigen loaded complexes (Fig. 2a). In addition, increased levels of IL-6, and IFN-γ were found (Fig. 2b) Analysis of specific effector T cells show the capacity of such NPs to efficiently deliver specific antigens an induce antitumor responses. These outcomes cooperatively promote antigen cross-presentation.

Mesoporous silica NPs (MSNs) have been long studied as delivery vectors owing to their pores that provide high surface area, their easy modification in terms of physicochemical properties, easy functionalization, and biocompatibility. It has also been reported the intrinsic adjuvanticity likely due to the interaction with plasma proteins and subsequent engagement with immune cells. Given such properties, silica NPs also became popular delivery vectors for more precise responses. Lee and coworkers developed an efficient delivery structure by synthesizing hollow core extra-large pore silica NPs (H-XL-MSNs) with large mesopores, and a hollow interior void based on a single-step synthesis from core-shell MSNs (Fig. 3) [87]. The hollow cores were achieved by introducing self-assembled IONPs. The size of the core assembly was easily controlled by the polarity and the amount of NPIONPs. With the removal of the iron oxide assembly, a hollow void inside the MSNs with a large mesopore was obtained, allowing advantages of both the hollow void and large mesopore for a high loading efficiency of various model proteins with different sizes. The H-XL-MSNs were coated with an amine group (APTMS) or poly(ethyleneimine) (PEI) to provide adjuvanticity and OVA was loaded onto the particles as a model antigen. The levels of activation of dendritic cells assessed by the expression of CD86 and MHC-II amongst CD11c + cells remained similar between the NH_2 and PEI functionalized NPs. However, when looking at the effector cell compartment, mainly antigen-specific $CD8^+$ T cell measured by MHC-II-Ag tetramer staining (used to specifically stain antigen recognizing TCRs), the levels of OVA-specific $CD8^+$ T cells (level of SIINFEKL-specific CTLs in the LN) were significantly elevated for the H-XL-MSNs-$NH2^+$ OVA NPs. Similarly, the reduction on the tumor growth and the survival rates of C57BL/6 mice were higher. These data suggest the benefit of the antigen loading strategy of

such NPs for DC delivery to generate or boost antigen specific cytotoxic responses able to suppress tumor growth.

For DC activation, Nguyen et al. also report a strategy based on MSNs using larger 3D microstructures that target the LNs (Fig. 3) [79]. MSNs were loaded with an antigen (OVA) and a TLR9 agonist (CpG) was incorporated in on mesoporous silica rods (MSR) containing DC-recruiting chemokines (GM-CSF) forming the 3D structure. By doing so, large numbers of DCs are recruited and maturation was induced (Fig. 3a). By subcutaneous administration of the MSR-MSN complexes promote the recruitment and maturation of $CD11c^+$ in the LNs as shown by the expression of CD86 and MHC-II (Fig. 3b). This resulted in the increase of INF-γ secreting $CD8^+$ T cells.

Wagner et al. showed a strategy based on MSNs incorporating an adjuvant in the formulation that could be released in a pH-dependent manner, which is an interesting strategy due to the known acidic pH of the TME [87]. A spatially segregated core – shell MSNs was loaded with the synthetic TLR7/8 agonist R848 (resiquimod). Upon subcutaneous injection, the particles can be found in draining LNs and taken up in a higher amount by migratory DCs and as an outcome, enhanced proliferation of OVA specific $CD8^+$ T cells was observed after stimulation with OVA-specific peptides.

As shown by these reports, the first piece of the immunotherapeutic puzzle is the efficient delivery of antigens and maturation of DCs able to modify the TME and promote or boost the infiltration of cytotoxic cells.

3.2. T Cells mediated responses

While DCs correspond to the main orchestrator of adaptive responses as the most efficient APC, T cells are the main effector cells in many chronic conditions such as viral infections and cancer [22,73], thus, they attract a great deal of attention in this review. T lymphocytes are the main effector cells during an adaptive immune response in cancer or viral infections. CTLs and Ths correspond to the main effector cells in several diseases [73,88]. CTLs and Ths differ in their effector function after activation. CTLs are killer cells that induce apoptosis by the expression of molecules like Granzymes (Grzms) and Perforin (Perf) upon formation of the immune synapse with a tumor or infected cell. On the other hand, Th cells are involved in coordinating the response by providing a range of different signals to effector cells. $CD4^+$ Ths can differentiate in a wide range of subpopulations based on microenvironmental signals. Tay et al have recently revised the roles of CD4s in immunotherapy and we refer to such report for more detail [89]. Relevant to this review are the most widely known and characterized subtypes of Ths, namely, Th1, Th2 and T regs [90]. They differ in the expression patterns of surfaces molecules and transcription factors and will provide specific signaling. Th1s promote cytotoxic responses with CTLs as the main effector cells and are driven by the secretion of IFN-γ and TNF-α. Th2 responses promote humoral responses that lead to the production of antibodies by plasma cells (from mature B cells). Secretion of IL-4, IL-5 and IL-13 are main driver of this response. On the opposite hand, Tregs are known by their immune suppressive and tolerogenic character and are highly studied by their role mainly in cancer [90,91]. They are distinguished by the expression of the transcription factor FoxP3 and CD25 (IL-2 receptor). By secretion of cytokines such as IL-10 and TGF-β, they suppress immunogenic responses by inhibiting effector T cells and inducing tolerance [92]. Presence of Tregs in the TME is connected to poor prognosis [93]. Therefore, not only targeting CTLs have the potential to affect T cell outcomes, but modulating Th-mediated responses have the potential to change the effector landscape in disease [94].

In the search for the generation of specific responses, nanotechnology has been focusing on the capability to potentiate pre-established or generate *de novo* T cell responses to different antigens. In this sense many different strategies have been designed utilizing different particles of a wide range of materials to achieve good delivery to DCs for enhanced antigen presentation as previously shown and provide the needed co-stimulatory signaling to effector cells. Typically, studies of cytotoxicity *in vitro* are performed prior to moving to *in vivo* models, usually mice, where the particles are injected *via* different routes and the effector immune cell landscape is studied along with monitoring the tumor volumes and survival rates. Important progress has been made in the application of nanomaterials for design of immunotherapeutic formulations, many of them of inorganic origin, following the previously mentioned rationale. Bai and colleagues have designed a dual MHC-I and MHC-II antigen delivery platform for broad T cell activation [95]. Pegylated aluminum NPs were used to encapsulate peptides linked by a cleavable sequence. In combination with CpG-ODN 1826, the internalization and processing of two epitopes by DCs induced a stronger Th1 response, as observed by the level of functional ($IFN\text{-}\gamma^+CD4^+$ and $TNF\text{-}\alpha^+IFN\text{-}\gamma^+CD8^+$) T cells (Fig. 4a). The levels of Ag-specific T cells were also elevated (Fig. 4b).

The previously discussed work by Wang and co-workers showed the capability to enhance MHC-I cross-presentation [86]. Cross-presentation for $CD8^+$ T cell activation is usually reported to depend on an endosome-to-cytosol pathway, where the internalized extracellular epitopes are transported from endosomes to the cytosol for proteasome degradation and further transport to the Endoplasmic Reticulum (ER) where they are loaded onto MHC-I complexes [96]. On the other hand, an alternative route known as the vacuolar pathway, proteins transported into the lysosomes are degraded and loaded directly onto MHC-I, however, it is believed to be a less effective route as protein degradation negatively affect the number of presented peptides [96,97]. NPs internalized by cells typically end up in lysosomes where their content is degraded, therefore, to enhance cross-presentation, lysosomal escape is a promising strategy allowed by some intrinsic physicochemical properties of NPs. In the mentioned study, blasting the lysosomes allows antigen escape to the cytoplasm and promotes autophagy through the LC3/Beclin 1 pathways. Enhanced DC maturation and cross-presentation impacts the $CD8^+$ response. A stronger tumor killing capacity is observed OVA-specific $CD8^+$ T cells which show higher proliferation (CFSE staining) and killing capacity *in vitro* as indicated by the higher capacity of spleen CTLs to kill OVA-expressing E.G7 cells versus the non-expressing EL-4 cells. *In vivo* a reduction in the tumor burden and survival rates is overserved up to 100% survival after 30 days of treatment in a E.G7 cell tumor model injected in C57BL/6 mice. The authors show a potential strategy for ag-specific CTL activation with minimum levels of toxicity based on serum analysis of liver, lung and heart function and histological analysis.

Endosomal trapping and degradation by the endolysosomal pathway are some of the main current issues of NP based formulations. Similarly to the previous study, Gong et al., attempt to escape such pathway by using transforming organic-based NPs driven by protons in acidic pH such as the lysosomes, therefore promoting antigen processing and cross-presentation [98]. In addition, the show an in-depth characterization of the response at the subpopulation level. Their system is comprised of a polymer–peptide conjugate-based nano transformer (NT) loaded with antigenic peptide to create a vaccine formulation (NTV). Briefly, they synthesized p(DMAEMA22-OGEMA4)-b-p(MAVE)30 and conjugated to either a Naphthalene-conjugated d-peptide (NDP) or a pyrene-conjugated d-peptide (PDP). The amphiphilic polymer-peptide conjugates self-assembled into spherical nanostructures at physiological pH, and in acidic conditions they release the NDP or PDP

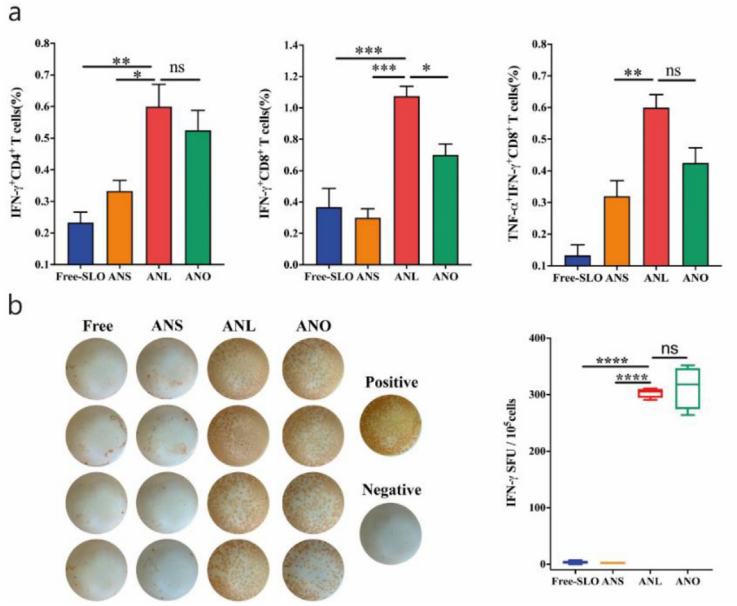

Fig. 4. Enhanced Th1 CD8 + T cell responses by vaccination with Aluminum NPs containing dual-epitope peptides and adjuvants. a) Flow cytometry analysis of the levels of cytokine secreting $CD4^+$ and $CD8^+$ splenic T cells induced by different formulations containing short peptides (ANS), long peptides (ANL) and OVA (ANO). b) ELIspot analysis of the induction of IFN-γ-secreting cells. Adapted from Ref. [95].

and reassemble into either fibers or sheets that will burst the containing vesicles. During the process, the encapsulated peptide or antigen will be then released to the cytosol and processed for presentation. The authors tried the two formulations (NTV1 and NTV2 for Naphtalene and Pyrene) for differential efficiency of presentation and $CD8^+$ Ag-specific responses. In a model mouse dendritic cell line (DC2.4) it was shown the capability of NTV2 to enhance cytosolic liberation of the cargo and increased membrane localization, suggesting enhanced presentation. In BMDCs, the levels of cytokine secretions (IL-1β and TNF-α) were increased and resulted in promoted OT-I OVA-specific T cell proliferation. The particles were localized to the LNs after SC injection and the cytotoxic capabilities are also highly increased for nanosheet transforming NPs. In a B16F10-OVA melanoma model, NTV2 showed strong inhibition of tumor growth (37.5% of animals surviving at day 62). NTV2-treated group showed higher total T cell infiltration ($CD45^+$-$CD3^+$) and the ratio $CD8^+/CD4^+$ was also increased. A deeper analysis was performed by analyzing different populations of infiltrating T cells, namely central-memory (CM, defined as $CD45^+$-$CD3^+CD4^+CD44^+CD62L^+$ or $CD45^+CD3^+CD8^+CD44^+CD62L^+$) and effector-memory (EM, defined as CD4 $5^+CD3^+CD4^+CD44^+CD62L^-$ or $CD45^+CD3^+CD8^+CD44^+$CD62L-) that have differential capacities to proliferate or kill, respectively. Both CM and EM populations were increased by NTV2 treatment suggesting a strong proliferation and infiltration into the tumor. Moreover, the immunosuppressive TME is alleviated by the decrease of Tregs ($CD4^+CD25^+FoxP3^+$). Overall, the author provides deep and comprehensive understanding, including some mechanistic data, of how the TME is regulated upon treatment and how it affects to different populations of effector cells.

Xu et al. have developed self-assembling NPs scaffolding Trp2 and Gp100 peptides capable of inducing epitope-specific CTL responses in a higher degree than the corresponding monomeric vaccines or CpG-adjuvanted peptide vaccines [99]. DNA vaccination has proven to enhance $CD8^+$ T cell responses in preclinical animal models and in clinical trials. Here, the authors apply their previously published strategy [100], using DNA to *in vivo* produce NP vaccines (DLnano-vaccines) that enhance both humoral and CTL responses (Fig. 5). Briefly, by encoding a modified form of the known eOD-GT8-60mer scaffolded with the C-terminus of the lumazine synthase (LS) from *Aquifex aeolicus*, which can self-assemble into 60 nm NPs, they could express specific antigens to modulate immune responses. A higher level of $CD11c^+MHCII^+$ DCs was observed for mice treated with DLnano_LS_GT8 in addition to electroporation. By using BATF3-KO transgenic mice that do not develop $CD8a^+$ cDCs highlight effect on priming T cells by the observed decrease in the levels of IFN-γ^+CD8^+ T cells in the spleen compared to the wild type. The authors also assessed the level of tissue damage upon vaccination as a potential source of cell death that could enhance antigen presentation. Cleaved caspase-3 was observed 4 days post injection (d.p.i.) in the muscles of mice immunized with DLnano_LS_GT8 combined with electroporation while it was not observed with protein eOD-GT8-60mer without electroporation. Also, double-stranded DNA breaks and cellular apoptosis were observed in such groups suggesting that electroporation was crucial for antigen uptake and presentation to induce CTL responses by DLnano-vaccines.

Some of the most widely used inorganic-based NPs are based on gold cores due to the ease of synthesis and functionalization, as well as colloidal and chemical stability. And this includes their use as vectors for immune activation. In a recent work by Xu et al., the chirality of inorganic nanostructures and its effect in the capability to differentially engage with the immune system generating T cell responses is studied [101]. The NPs were synthesized under circularly polarized light (CPL) in the presence of different dipeptides. By applying left or right CPL illumination at 594 nm in the presence of cysteine-phenylalanine (CYP) dipeptides, single-crystal AuNPs showed distinct chiral shapes with a size range of about 120 nm in size. *In vitro* data obtained by treating BMDCs with different formulations of the enantiomers (*i.e.*, l-P+ and d-P-) containing OVA. Levels of $CD40^+$, $CD80^+$ and $CD86^+$-DCs, and upregulation of MHC-I OVA Ag-specific and MHC-II were prominent in the L enantiomer treated groups, and ELISA showed elevated secretion of TNF-α and IL-12. Interestingly, the levels of uptake of OVA by BMDCs was comparable between the two forms. The authors conclude that the regulation of the response is clearly asymmetric and given the uptake studies, regulated by distinct intracellular processing. The next step was to test the adjuvanticity

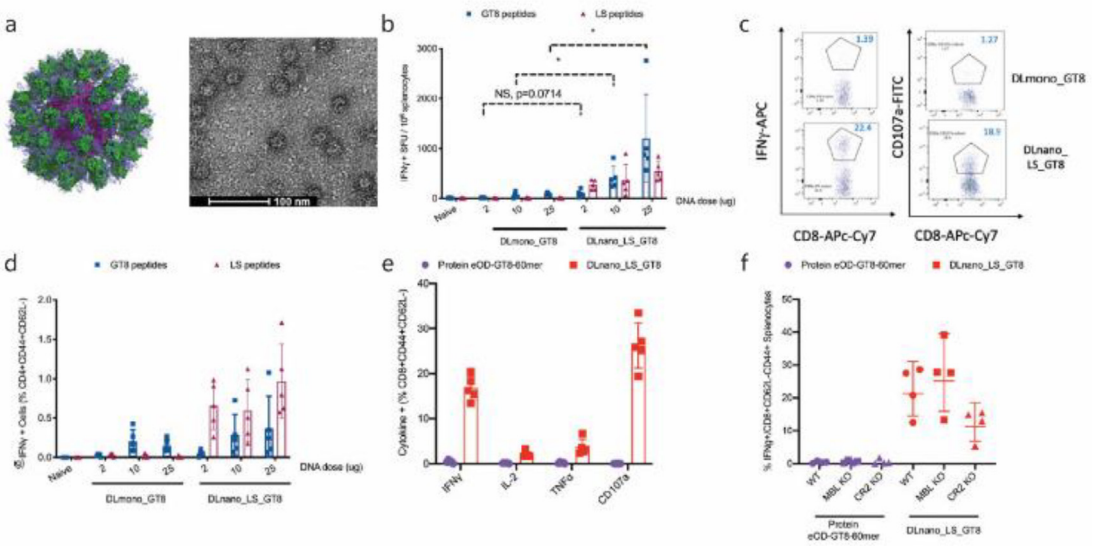

Fig. 5. ***In vivo*** **encoded self-assembled antigen-decorated NPs induce strong multifunctional cytotoxic T cell responses in intradermally vaccinated mice.** a) Schematic representation of the Ag-decorated NPs. The self-assembling lumazine synthase (LS) scaffold is shown in purple and in green the encoded antigen (GT8). To the right the electron microscopy images. b) ELIspot determination of IFN-γ dose-dependent responses induced to the GT8 peptides and the LS peptides in spleens. c) Representative flow cytometry plot of the induction of functional $CD8^+$ responses. d) Flow cytometry analysis of intracellular levels of IFN-γ in effector Effector memory $CD4^+$ T cells ($CD3^+$-$CD4^+CD44^+CD62L^-$) directed to the different antigens. e) Enhanced functional responses in the effector memory CD8 + T cell compartment by DL_nano_LS_GT8 analysed by flow cytometry of intracellularly stained cytokines (ICS). f) Levels of Effector Memory $CD8^+$ responses in KO mice for complement system receptors show attenuation by Complement Receptor 2 (CR2) blockage. Adapted from Refs. [99,100].

of l-P+ and d-P- NPs *in vivo* to validate *in vitro* results. Confirming *in vitro* results levels of CD40, CD80 and CD86 were upregulated in $CD11c^+$ DCs in the draining LNs (dLNs) while achiral or racemic particles did not produce such effect. Such behavior directly reflects on the regulation of T cell responses. Both Ths and CTLs showed increased production of IFN-γ and TNF-α and proliferation in mouse spleen after activation by l-P+ NP compared with d-P- NP. The authors conclude that NP enantiomers and their achiral homologue differ substantially in the capacity to engage and modulate immune system in a vaccine setting by co-administering chiral NPs and antigens. From a mechanistic point of view, the authors show a differential engagement with specific immune receptors by the L-P+ NPs (namely, CD97 and EMR1) and differential inflammasome activation and IL-1b secretion. The described chiral effects highlight another level of tunability of particles influencing the capability to use them as potential immunomodulators for specific responses.

T cell biology is rapidly evolving and the role of different subpopulations in health and disease is being highlighted [25,102]. In line with the previous study, some other recent reports are going further in understanding the response to NPs, and the modulatory capacity based on different parameters. It is the case of studies by Lynn et al. [103], and Knight and co-workers [104]. The first one shows the potentiality and versatility of nanoparticles by presenting a vaccine platform (SNP-7/8a) based on the self-assembly peptide–TLR-7/8 agonist. Such a strategy could be extensible to multiple peptide antigen compositions for promoting $CD8^+$ T cell responses. The MHC-I epitope from OVA SIINFEKL was used as a model antigen in two different formats, as a particle (LSP) or in solution (LSS) and in the presence or absence of the TLR-7/8a adjuvant as a flanking region (LSP-7/8a, LSS-7/8a) highlighting the relevance of the particulate system vs the soluble peptide, already known to be hardly recognized by APCs. In fact, it shows a higher uptake of LSP-7/8a by $CD11c^+$ DCs. Consequently, the level of division of antigen specific cells as well as the percentage of antigen specific $CD8^+$ T cells was prominently enhanced. To test the capability to extrapolate to different formulations and test the immunogenicity of their SNP-7/8 contruct, the authors synthesized a range of seven MC38-derived neoantigens known to bind MHC-I (Aatf, Adpgk, Cpne1, Dpagt, Irgq, Med12 and Reps1). Good stability was achieved with the different LPs and when formulated as SNP-7/8a induced strong $CD8^+$ T-cell responses as shown by analysis of IFN-γ secreting $CD8^+$ Ag-specific cells. In 5 out of 7 cases, the IFN-γ secretion was enhanced compared to typical adjuvants. The authors achieve a higher level of antigen-specific $CD8^+$ responses both in mice and rhesus macaques with high levels of polyfunctionality based on the analysis of secretion of IFN-γ, IL-2 and TNF-α.

The potential of PRR agonist as triggers of Innate immunity for potentiating APC responses is one of the most applied approaches as show by this SNP-7/8a based conjugates. Modulation of the precise response and signaling for specific $CD8^+$ T cell induction would greatly improve the applicability and translation of these formulations.

In the study by Knight et al. it is depicted the tissue-resident antigen-specific immune landscape after intranasal vaccination with polymeric pH-responsive NPs that induce MHC-I pathway cross-presentation (Fig. 6). Tissue-resident T cells are now being highly studied as one of the main effector cells in tissues, showing differential functional and phenotypic traits from those in the circulation [105]. The authors show strong antigen specific $CD8^+$ T cell responses in different anatomical localizations in comparison to the non-pH responsive control (Fig. 6a). Then, they show that dual delivery of grafted OVA-NPs + electrostatically bound CpG was key in generating potent antigen specific and functional responses based on the expression of IFN-γ and TNF-α by $CD8^+$ T cells. As mentioned before, the authors show that, among the $CD8^+CXCR3^+$ resident cells in the airways, the majority showed typical residency markers such as CD69 and CD103, demonstrating the establishment of resident memory cells.

A smart strategy to improve the compatibility of NPs by coating inorganic particle cores with a layer of cell membranes to generate biomimetic particles. It offers great benefits for vaccination by taking advantage of antigen-rich membrane coatings, combined with

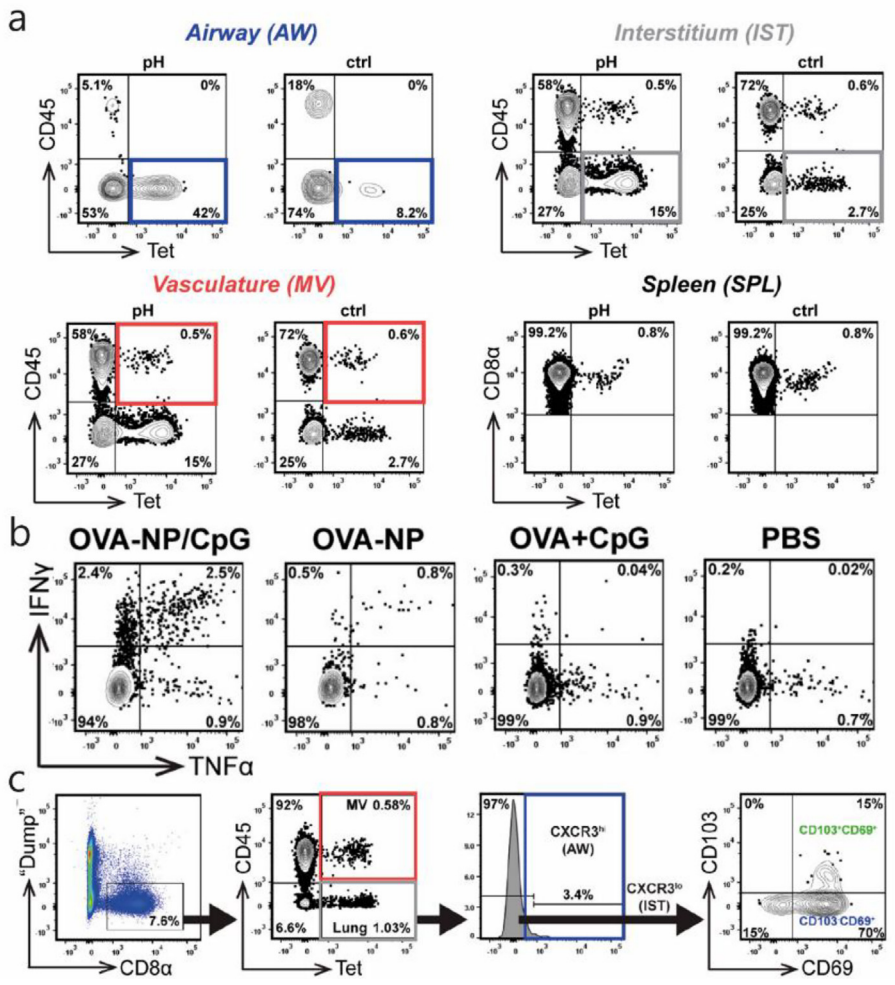

Fig. 6. Immunization with pH-responsive antigen-loaded NPs induced tissue-resident antigen-specific T cell responses. a) Intranasal vaccination enhances lung-resident CD8$^+$ T cell response as shown by the representative flow plots depicting the frequency of Tetramer$^+$CD8$^+$ T cells in different compartments. b) Co-delivery of TLR agonists and antigen induces polyfunctional memory responses by looking at the levels of IFN-γ and TNF-α secreting CD8$^+$ T cells. c) Flow cytometry shows that Tetramer$^+$-CD8$^+$ T cells expressed Tissue-resident markers (CD103, CD69) in the lung interstitium (CXCR3^{lo}). Adapted from Ref. [104].

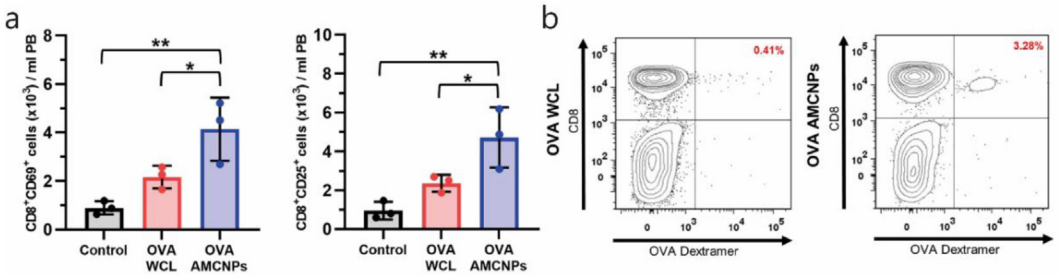

Fig. 7. Antigen-specific CD8 + T cell responses are induced by Acute myeloid leukemia cell membrane-coated nanoparticle (AMCNP) vaccination for blood tumors. a) Shows the total counts by flow cytometry analysis of activated CD8$^+$ T cells (CD8$^+$CD69$^+$) and Regulatory CD8$^+$ cells (CD8$^+$CD25$^+$). b) Representative flow cytometry plots showing enhanced antigen specific CD8$^+$ T cell frequency induced by AMCNPs. Adapted from Ref. [109].

the loading of nanoparticles for performing specific tasks [106-108]. Johnson et al. [109], developed an acute myeloid leukemia cell membrane-coated PLGA nanoparticle (AMCNP) construct for immune modulation of antigen-specific T cell responses. AMCNPs are efficiently taken up by DCs in LNs and spleen and induce maturation and antigen presentation by CD11c$^+$ DCs. Regarding T cell activation, the authors show that a higher percentage of peripheral blood CD8$^+$ T cells are activated after re-challenge by looking at the levels of CD8$^+$CD69$^+$ and CD8$^+$CD25$^+$ T cells (Fig. 7a). OVA-specific CTLs are expanded in the spleen as shown by both tetramer and dextramer staining which produce higher levels of IFN-γ. In alignment with the promotion of the proliferation of Ag-specific T cells, both CM and EM compartments are prominently increased. In an *in vivo* model of leukemia combining chemotherapy + AMCNPs vaccination, a benefit in the survival rates is observed before and after rechallenging, by applying 3 boosts of the vaccine after a cycle of chemotherapy. Overall, the authors show a potential strategy for vaccination to promote CD8$^+$ T cell responses.

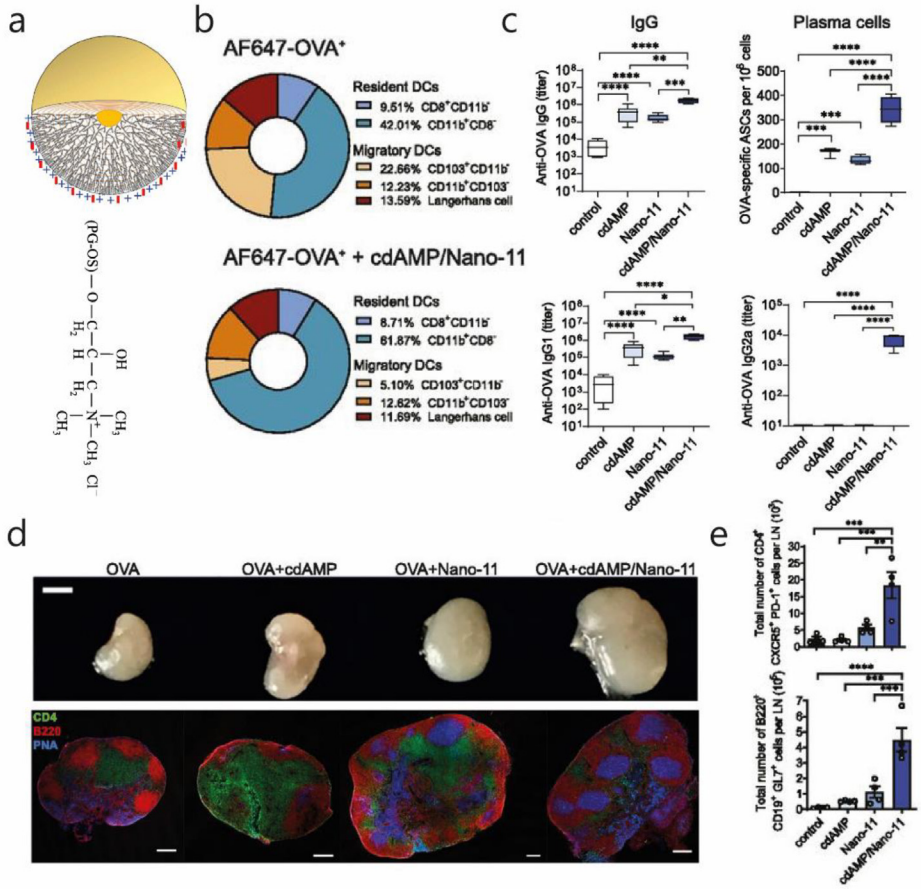

Fig. 8. Intradermal vaccination with a-D-glucan NPs (Nano-11) with cdAMP increases skin migratory and lymph node–resident DC antigen uptake and presentation and enhances humoral immune responses. a) Represetation of dendrimer-like a-D-glucan NPs. b) Differential levels of OVA-specific DC subsets after immunization with AF647-OVA alone and co-delivered with cdAMP/Nano-11. c) Levels of IgG, IgG1 and IgG2 in response to different formulations after ID injection determined by ELISA and ELIspot analysis of plasma cell levels. d) Vaccination with OVA + cdAMP/Nano-11 induces GC reactions as observed by the increased size of the LNs (Top) and GC B cells (Bottom). Anti-PNA-FITC (blue), anti-CD4-PE (green) and anti-B220-allophycocyanin (red). e) Total flow cytometry counts of Follicular Helper $CD4^+$ T cells (Top) and GC B cells (Bottom). Adapted from Refs. [114,115].

In this subsection, we showed the multiple configurations and strategies that are being tested recently, and how, in our understanding, should evolve the characterization of immune responses to NPs. This means, moving from bulk $CD8^+$ and/or $CD4^+$ T cell activation, to understanding functional profiles of differentially activated subsets at different locations that could be more relevant for translational purposes.

3.3. B cells mediated responses

B cells conform the other effector component of the adaptive immune system. Similarly to T cells, their membrane B cell receptor (BCR) contains a variable region that specifically recognize epitopes [110]. The BCR correspond to membrane-membrane anchored form of an Ig or antibody. B cells can sense such antigens presented by APCs mainly in SLOs such as the LNs through their rearranged BCRs that generate a high variability for a broad spectrum of antigens [111]. Upon engagement of the cognate BCR antigen presented by APCs, B cells undergo a process of clonal expansion, class switch recombination to the different Ig subclasses based on the nature of the stimulus (*i.e.*, IgA, IgD, IgE, IgG or IgM), and somatic hypermutation, which increases the variability and specificity of the antibody. Once activated, B cells can follow different fates, mainly, develop into antibody plasma cells, establishment as memory B cells or germinal center (GC) B cells. Induction of B cell responses are a key outcome of current vaccination processes to confer protection by generating long lasting antibody responses.

NP vaccines have now been long hypothesized and studied and they offer multiple advantages over other types of formulations. For example, enhanced lymphatic trafficking, localization, and uptake by APCs for presentation, and increased activation of B cells through receptor crosslinking. A critical step in developing and inducing humoral responses is the localization of antigens in B cell follicles and germinal centers by delivery to follicular DCs (fDCs) [112,113]. Thus, a deeper understanding of the interactions and tracking of NPs within lymphoid tissue, including which cell types are activated, can benefit the design of NPs.

Hernandez-Franco et al. show enhancement of humoral immune responses by combining the STING activator cyclic-di-AMP (cdAMP) and their previously developed plant-derived NP adjuvant Nano-11 (Fig. 8) consisting of a dendrimer-like a-D-glucan NPs (Fig. 8a) [114,115]. The authors show that both intradermal (ID) and intramuscular (IM) vaccination in mice and pigs enhances DC uptake that reflects in the development of Ag-specific responses. By depicting the DC landscape uptake, they show the main subsets being triggered by cdAMP/Nano-11-OVA. It is shown differential activation of LN–resident and migratory skin dendritic cell subpopulations, including Langerhans cells (Fig. 8b). ID immunization with cdAMP/Nano-11-Ag elicited an enhanced immune response with a significant increase of total OVA-specific IgG and antibody secreting plasma cells, and at the

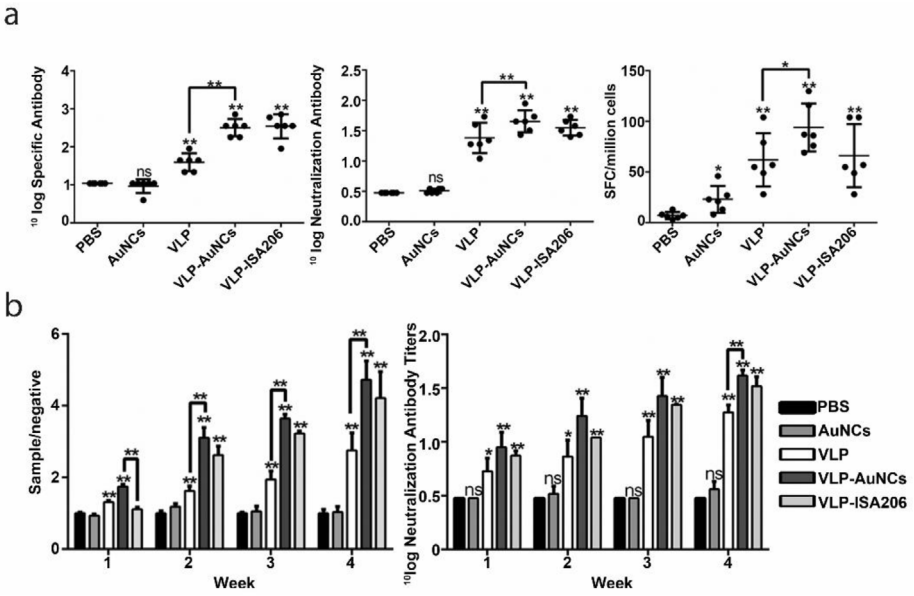

Fig. 9. Gold nanocages as carriers for foot-and-mouth disease VLPs with adjuvant properties for dual functionality. a) Serum specific antibody levels and neutralizing antibody measured by ELISA and spleen-derived cytokine secreting cells measure by ELIspot in mice. b) Time-lapse of the protective humoral response produced in guinea pigs after vaccination. Adapted from Ref. [116].

IgG subclass levels, IgG1 and IgG2a show significant increase (Fig. 8c). It is also induced GC reactions at the draining LNs, as shown by the increased size of dLNs (Fig. 8d) and the levels of germinal center B cells and follicular helper T cells (Fig. 8e). It in mice at a reduced dose compared with IM immunization. Nano-11 and cdAMP demonstrated a strong synergistic interaction, as shown in the activation of mouse, human, and porcine APC, with increased expression of costimulatory molecules and secretion of TNF-α and IL-1β.

Teng et al., designed bi-functional gold nanocages (AuNCs) as carrier and adjuvant for FMDV treatment [116]. By complexing the structural proteins of FMDV in the form of VLPs with the AuNCs, they achieved stimulation of immune cells in higher degree as the two separate components. It was achieved *in vitro* a higher production of inflammatory cytokines (IL-1β and TNF-α) and the authors hypothesize that TLR4 signaling is a major contributor of such activation by the increased expression levels of downstream proteins involved in such pathway (MyD88, TRAF6, TAK1 and TRIF), which could drive the higher uptake. In an *in vivo* model of BALB/c female mice VLP-AuNCs could more effectively induce both $CD4^+$ and $CD8^+$ lymphocyte proliferation than VLP. 14 days following the second boost, specific antibody level showed elevated in comparison to the VLP alone and certain degree of increase in the of the neutralizing antibody titer as measured by microneutralization assay, in both cases at similar levels than VLP + the adjuvant ISA206 typically co-administered in FMDV vaccination (Fig. 9a). Immunization was also performed in guinea pigs and again both specific and neutralizing antibodies were enhanced compared to the rest of the treatments (Fig. 9b). Overall, the authors show the capabilities of AuNPs to act as vectors for enhance the responses to already existing vaccine formulations, showing the capabilities to specifically engage in a more efficient way with cellular machinery to promote such responses.

Similarly, using different core materials, Hou and coworkers employ MSNs with a flower-like shape to boost the potency of VLPs for inducing a humoral response [117]. The authors rely on the concept that particles with rough, irregular, or spiked structures interact strongly with cell membrane receptors impacting recognition and immune response [10,44,45]. For the preparation of the complexes VLP-MSNs (VLP-SiNPs) a mass ratio (VLPs:SiNPs) of 1:6 showed the higher coupling and incorporation of the FMDV proteins (VP0, VP1 and VP3) and a progressive release was achieved over a time spam of 72 h. Mice were immunized intramuscularly showing a boost in Th2-type IL-4 and IL-10 secretion by splenocytes *ex vivo* after re-stimulation with VLPs. Polarization to Th2 responses would impact the production of ag-specific antibodies. Measurements of anti-FMDV IgG levels showed certain elevation for the VLP-SiNPs groups compared to the VLP alone, but not as pronounce as the VLP-ISA206 treated groups. This suggests a benefit of the NP complex vs the VLPs alone, probably due to the slower and localized release of antigens in lymphoid tissues. In fact, while the levels of IgG seem to plateau for the ISA206 formulation, VLPs-SiNPs seem to produce a sustained growth up to the fifth week. IgG isotype analysis showed certain degree of isotype switching towards IgA subclass as detected in sera, feces and intestinal mucosa, and a trend after 35 days that point out to the generation of higher levels of IgG1 by the VLP-SiNPs compared to the other treatments following intramuscular injection.

The authors explored a different inoculation route to better understand differential effects. Intranasal vaccination is being explored nowadays to generate mucosal immunity particularly against respiratory pathogens, *e.g.*, SARS-CoV-2, but also long-lasting systemic memory. To test the effects of their constructs, the authors intranasally immunized and measured the antibody levels in saliva, blood, and feces. IgA was increased in mucosa (saliva, lung fluid and intestine) and feces at 14 days post immunization, but at similar levels than VLPs alone. On the hand, no subclass switch was observed. Overall, the author shows a potential benefit to apply irregular shapes of MSNs to enhance the capabilities of antigen delivery to induce stronger humoral responses, despite the relatively mild effect observed. Further understanding of the balance between Th1/Th2 responses and APC uptake and presentation would benefit the design of such constructs, that already show certain advantage to VLPs alone.

Displaying a range of different antigens in a patterned fashion combined with a particular topological structure favors recognition and processing by immune cells. For instance, by creating multivalent NPs that can mimic the structures of naturally occurring par-

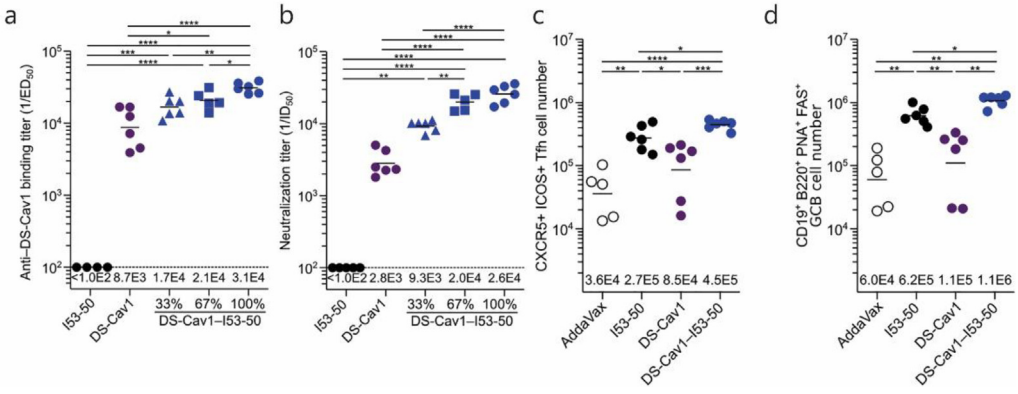

Fig. 10. NPs displaying differential spatially distributed epitopes by self-assembling scaffolds modulate the generation of strong neutralizing antibody responses. a and b) ELISA assay showing the DS-Cav1-specific and neutralizing antibody levels in serum from mice immunized with different formulations of the NP immunogens. c and d) Determination of the induction of GC reactions in the draining lymph nodes by flow cytometry. The total counts of follicular helper T cells (Tfh) ($CXCR5^+ICOS^+$) and GC B cells ($CD19^+B220^+PNA^+FAS^+$) are shown. Adapted from Ref. [118].

ticles such as viruses. Protein NPs are a paramount example of the potential of NPs, and a strategy with great potentiality due to their compatibility and self-assembling character, including the possibility to provide multivalency. As an example not consisting in inorganic based NPs, Marcandalli et al., design a self-assembling protein structure that display up to 20 copies of the respiratory syncytial virus (RSV) F prefusion protein capable of generating a potent neutralizing antibody response [118]. The F protein corresponds to a trimeric glycoprotein that is involved in the membrane fusion and the main immunogen that generates neutralizing antibodies, and many are targeted to the prefusion region. By using their previously published strategy to create self-assembling proteins with customized structures they achieve a structure that induces higher neutralizing responses [119]. To test the induction of humoral responses as a correlate of the coverage and exposure of the prefusion protein F antigen DS-Cav1 at 3 different levels of coverage (33%, 66% and 100% of the available anchoring locations) the DS-Cav1 NP responses were tested in BALB/c mice immunized subcutaneously. The anti-DS-Cav1-NP titers were directly correlated to the density of coverage and in all cases higher than the trimeric DS-Cav1, as well as the levels of neutralizing antibodies. The authors link to the icosahedral NP the 3-fold increase in antigen-specific antibody titers and 9-fold higher neutralizing antibody levels compared to the control (Fig. 10a and b). Interestingly, the ratio of binding to neutralization was also decreased as the density of the exposed antigen was higher suggesting a better quality of the response. The explanation that they proposed relates to the better topography and exposure of specific sites in the NP form. Tfh cells, key cells for B cell maturation in the GCs were increased more than 5-fold and a similar trend was observed by a slightly total GC B cells induced the immunogen (Fig. 10c and d). These data are consistent with previous studies showing that particulate immunogens enhance GC formation and Tfh expansion compared to soluble antigen in RSV-naive mice and nonhuman primates. On the other hand, anti-scaffold antibody responses were observed, which raises questions regarding the applicability of such self-assembling entities which opens a venue for the use of other kinds of scaffolds like the previously mentioned inorganic cores.

A similar example of potent induction of humoral responses taking advantage of the capability to precisely tune the geometry of antigens in NPs has been reported by Boyoglu-Barnum et al. [120]. Self-assembling proteins are used to display a multiarray of different antigens (H1, H3, B/Victoria/2/1987-like and B/Yamagata/16/1988-like from the HA protein) which are efficiently recognized by the immune system and induce robust humoral responses. The authors compared two types of formulations to a commercial quadrivalent seasonal influenza virus vaccine (QIV). The two formulations consisted of a NP containing a mosaic of the 4 antigens (qsMosaic) and a cocktail of self-assembly NPs of each individual antigen mixed (qsCocktail). It is shown that both NPs were equivalent or superior to those induced by QIV and that even un-adjuvanted inoculations generate similar results. The authors show that their constructs confer protection to historical and heterologous and protects from lethal doses in a more effective manner than the commercial vaccine.

B cells are critical cells in the development of the immune response not only for their role in producing antigen specific neutralizing antibodies but there are also involved in the generation of lymphoid tissue in situ in the lesion sites. Recently, it has been described certain structures named tertiary lymphoid structures (TLS) that are highly correlated to good prognosis in tumors [93,112]. Recent reports have been focusing on the exogenous induction of TLS by providing specific formulations that promote lymphoid tissue inducers that can attract follicular B cells that promote the formation of such tissues where the immune response can be enhanced [121]. Thus, to induce good humoral responses by using NPs, factors such as the mentioned here should be taken into account, as well as the effect of NPs on such immune landscape.

3.4. Macrophages polarization

Macrophages are a critical cell type driving immune responses in different disease contexts [71,122,123]. Macrophages appear as a diverse collection of cell types with a wide range of functional roles in homeostatic and pathological conditions. They can respond to multiple environmental cues that will define their phenotype and function. Typically, macrophages are divided in two broad subtypes, namely M1 and M2. M1 macrophages are more pro-inflammatory, and promote tumor immunity, while M2 macrophages are prone to favor cancer progression. Expression of high levels TNF, inducible nitric oxide synthase (iNOS) or MHC class II molecules are typical features of an M1 antitumor response while high levels of arginase 1 (ARG1), IL-10 or mannose receptor (CD206) are markedly pro-tumorigenic. By production of cytokines and expression of specific membrane markers, macrophages can regulate de TME affecting the functions of other cell types such as T cells. For instance, expression of iNOS induces expression of vascular cell adhesion molecule 1 (VCAM1) leading to enhanced

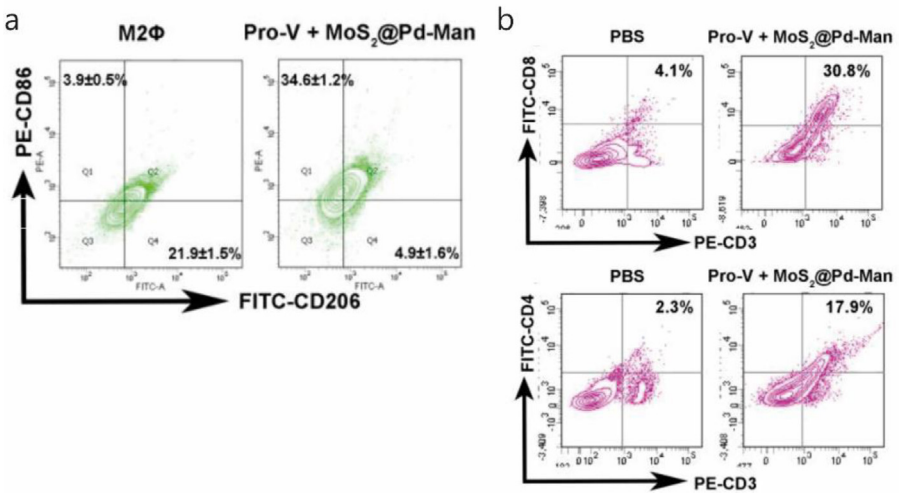

Fig. 11. Macrophage repolarization to modulate the TME by bioortogonal nanozymes composed of PdNPs as catalytic sites. a) Flow cytometry histograms showing increased levels of CD86 within the F4/80$^+$ macrophage population in tumor tissues after intratumor treatment. b) Effect at the cellular response after treatment in both CD8$^+$ and CD4$^+$ T cell populations. Adapted from [131]

recruitment of CD8$^+$ T cells [124]. Also, expression of Programmed cell death 1 ligand 1 (PD-L1) by macrophages can render T cells to a dysfunctional state. Therefore, the outcome of a response is impacted by the balance between M1/M2 macrophages and polarizing such responses are a potential way to control and reprogram immune responses. Many microenvironmental cues drive macrophages to develop into each of the different phenotypes and details on this topic can be revised elsewhere [125].

NPs can be applied in similar ways as already illustrated to induce specific polarization or change the macrophage landscape [125-130]. Typical approaches are by inducing pro-inflammatory responses by delivering TLR agonists (*e.g.*, resiquimod, imiquimod) or cytokines (*e.g.*, IL-12). Other smarter ways are now enabled by NPs, for example, Wei et al., taking advantage from biorthogonal chemistry, designed a palladium based nanozyme to reprogram TME by in situ synthesis of histone deacetylase inhibitor (HDACi) vorinostat (Vor, FDA approved) targeted to M2 macrophages [131]. Ultra-small Pd NPs (PdNPs) deposited on MoS2 nanoflowers confer the catalytic activity to generate Vor. Through modification of the surface with mannose, a higher accumulation in the target M2 macrophages that overexpress CD206 occurs, promoting an M1 polarization (Fig. 11a). In this way, this appears a great example of how multivalency through smart design of inorganic NPs can be used for developing multiple tasks. *In vitro* experiments in macrophage cell lines models (RAW267.4) proved the capability to polarize M2-induced macrophages by IL-4 to a M1 phenotype capable of increased production of iNOS and TNF-α and reduce the immunosuppressor cytokine IL-10. In an *in vivo* CT26-tumor-bearing mice model the biorthogonal chemistry approach showed capacity to modulate the TME. Increased F4/80$^+$CD86$^+$-macrophages induced by the nanozyme increased the number of T cells infiltrating the tumor (Fig. 11b), improving survival rates and tumor growth control. Therefore, such an approach constitutes a multivalent smart NP for macrophage re-polarization for TME modulation.

Kwon et al., designed a strategy for encapsulating cytokines, in this case IL-4 for M2 macrophage polarization, based on MSNs with extra-large pores (XL-MSNs), once again showing a potential application of such construct [128]. The authors modified their previously published work to achieve the large pores by employing high amounts of ethyl acetate and CTAB-stabilized iIONPs in a silica sol – gel reaction [132]. 3 different NP sizes (*i.e.*, 100, 150 and 180 nm) with similar pore size (3.6 nm) were poorly immunogenic based on the cytokine production in model mouse macrophages, and the authors focused on the 180 nm NPs. *In vivo* experiments after IV injection show that most cells with high uptake correspond to the myeloid lineage. In blood, neutrophils and monocytes showed the highest uptake while in the spleen, both macrophages and dendritic cells show high levels of uptake. This is relevant for efficient antigen presentation and effector responses, showing improved Ag incorporation. IL-4 encapsulation was performed by mixing the particles with the soluble molecule in aqueous media. The authors suggest an effect of the formulation inducing M2 polarization based on the mRNA levels of resistin like alpha (Retnla), marker for M2 macrophages. *In vivo*, following IV injection, the mRNA levels of M2 markers *Arg1* and *Chil3* were indeed significantly different between XL-MSNs IL-4 and soluble IL-4 at a dose of 100 ug per mouse (Fig. 12). No difference was observed at lower doses. Using larger pores has shown already applicability [133,134], however, more mechanistic, and deeper understanding on the immune cell landscape would be needed to further understand their potential application.

Ex vivo approaches have also being used for macrophage polarization. Different cell types have been successfully pulsed with combinations of cytokines and antigens to promote maturation and antigen presentation [135-137]. Avoiding the hurdles of *in vivo* delivery, NPs can be used *ex vivo* to enhance recognition, maturation and development of precise cell phenotypes and functions prior reinfusion. Liu et al., propose the development of a DC vaccine combined with TAM polarization agents for ameliorating immune suppression by using quantum dot (QD)-based approaches [135]. QDs were functionalized with CpG and tumor antigens to generate QD-P-CpG-A complexes. BMDCs from C57BL/6j female mice were extracted and pulsed with 50 ug/ml of NPs for 24 h. *In vitro* data suggest potent activation of DCs based on analysis of supernatant content of TNF-α and IL-12p70 and expression levels of MHC-II, CD40 and CD86 and suggested and adjuvant effect of the QDs through NLRP3-dependent inflammasome activation which is likely induced by the lysosomal disruption induced by the NPs. Combination of the QD pulsed DC vaccines with chloroquine (CQ), an FDA-proven anti-malarial drug which has been recently reported as an efficient immunomodulator of TAM polarization, enables amplified combination immunotherapy in a lung metastatic B16–F10 model (Fig. 13). This approach showed to ameliorates immunosuppressive TME via resetting TAMs to M1 macrophages and reducing the immunosup-

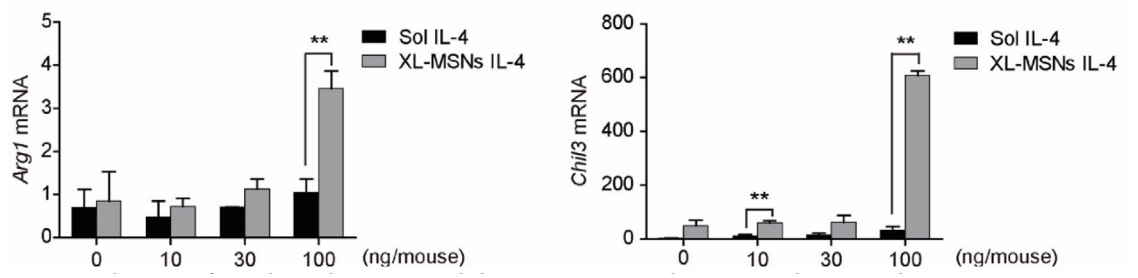

Fig. 12. Delivering of Cytokines by MSNs with large pores can induce macrophage repolarization. Dose-dependent induction of *Arg1* and *Chil3* mRNA production. Adapted from Ref. [128].

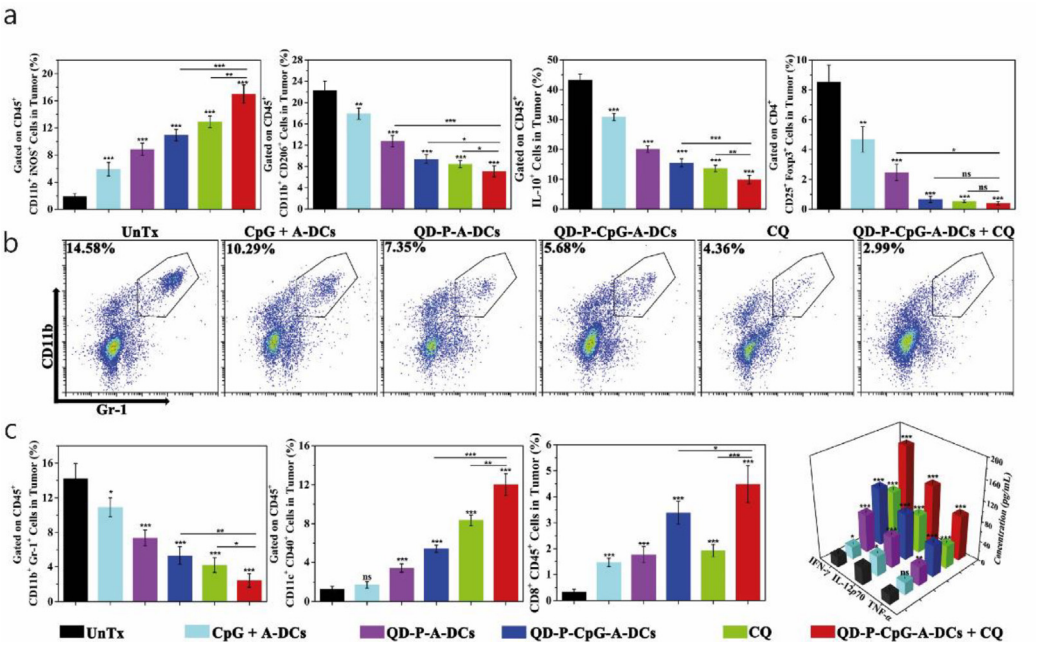

Fig. 13. Ex-vivo co delivery of Antigen/adjuvant complexed with QDs to DCs induces macrophage polarization to an M1-like, less immunosuppressive TME after re-infusion in combination with chloroquine (CQ). a) Flow cytometry analysis of the immune landscape showing the levels of M1 macrophages ($CD45^+CD11b^+iNOS^+$), M2 macrophages ($CD45^+CD11b^+CD206^+$), IL-10 expressing regulatory cells ($CD45^+IL\text{-}10^+$) and Tregs ($CD4^+CD25^+Foxp3^+$) after treatment. b) Representative flow plots of MDSCs ($CD45^+CD11b^+Gr\text{-}1^+$) frequency after infusion of DCs treated with the different formulations. c) Flow cytometry analysis of the changes in the immune cell composition in the tumor at the myeloid and lymphoid level. ELISA determination of serum levels of secreted cytokines. Adapted from Ref. [135].

pressive environment (Fig. 13 a and b), thereby boosting the therapeutic efficacy. Additionally, the authors showed, along with the effect on the myeloid compartment, the effect on the total $CD8^+$ T cell infiltration induced by the different combination treatments, as well as the induction of inflammatory responses. (Fig. 13c). If not strictly ligated to the effect of the NPs as vehicles, it is true that this approach shows how the combination therapies that could easily be incorporated in the NPs can have synergistic effects to improve the efficacy of NPs.

Macrophages are key regulators of immune responses and have strong capability to modulate immune microenvironments. Targeting and modifying the balance between different macrophage subtypes is a viable way to produce exhert immune modulation. In this sense, delivering agents that directly affect macrophage maturation and polarization like cytokines seems a smart strategy.

4. Nanotechnology for cancer immunotherapy

While NPs can be used for a wide range of applications, most of the research is done in the context of cancer, where immunotherapies have the potential to have a huge impact. Also, chronic infectious diseases such as HIV can benefit from its application. Thus, NP-based immunotherapies in cancer are the focus of this review, however, such advances could be translated to other disease contexts.

To develop more efficient therapies, the immune landscape and status of the disease must be considered [22,138,139]. Cancer formation, progression and spreading is a multifactorial process combining, (I) intrinsic features of tumor cells, such as low immunogenicity due to downregulation of MHC molecules, expression of inhibitory or "do not eat me" molecules like PD-L1 or CD47; and (II) immune-derived cancer promoting events, like incomplete maturation of DCs, improper co-stimulation of naïve T cells and abundance of regulatory cells (*e.g.* Tregs, M2-Macrophages, myeloid-derived suppressors cells) [88,139]. Such processes have an impact on effector cell functions and phenotypes, particularly on CTLs, leading them to a state known as exhausted. Tackling several of such issues at once will have a high impact in better prognosis and multifunctional NPs can be designed for that, in addition, they offer the possibility to move forward from the classical view of "one fits all" cancer therapeutics [7].

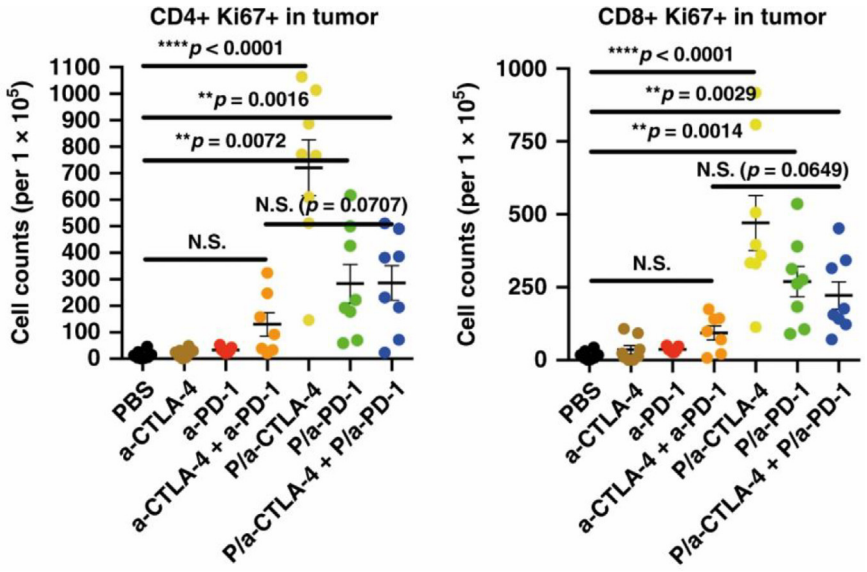

Fig. 14. Multifunctional NPs containing ICB and TfR antibodies promote T cell infiltration in tumor and enhanced survival rates in mice following IV administration. Total flow cytometry counts of intra tumoral $CD4^+$ and $CD8^+$ proliferating ($Ki67^+$) cells. Adapted from Ref. [145].

Exhaustion is directly linked tumor progression and immune deficiency in chronic viruses by the loss of functionality of effector CTLs [140]. Exhausted $CD8^+$ T cells (Tex) have a particular epigenetic program and show high expression of inhibitory receptors (IRs) like PD-1 or CTLA-4 that act as immune checkpoints. Such cells have shown impaired capabilities to produce cytolytic molecules and cytokines [141]. The discovery of exhaustion and key molecular regulator of such process have promoted the introduction of ICB molecules, able to revert inhibitory stimuli and recover the functional capabilities of T cells [142]. Such knowledge is of critical relevance when designing nanomaterials based on the immune landscape, in fact, targeting such molecules and modifying the interaction with ligands is being extensively incorporated in NP research nowadays [143,144].

NPs offer the capability to combine multiple moieties and active molecules in a way that synergistic effects can be applied in a way that modifies the responses in specific contexts, creating cascades of responses that promote the generation of broad and strong humoral and cellular responses. Therefore, the next section will comment on some distinguished strategies in the direction of combining synthetic chemistry with basic and fundamental immunology to achieve the best combinatorial set of effects based on the disease's context.

4.1. Nps for ICB in cancer

ICB is one of the most promising therapeutic interventions nowadays to unleash pre-existing suppressed immunity. Incorporating in NPs ligands to IRs and co-administration of different functionalities for synergic action is one of the most studies approaches. A good example of multifunctionality incorporating ICB antibodies is shown by Luo et al. They used biodegradable PLGA NPs for encapsulation of anti-PD-1 peptide (APP) and hollow gold nanoshells (HAuNS) to build a therapeutic formulation that combines PD-1 blocking with photothermal ablation for malignant tumors (APP- and HAuNS-loaded PLGA NPs, AA@PN) [144]. In the format of AA@PN, APP is slowly released but under near infrared (NIR) illumination it is accelerated and enhances the therapeutic effect in tumor models. The elimination of most primary tumors compared with other treatments, and significant inhibition of the growth of the distant non-injected primary tumors, similarly to free APP with frequent injections. This kind of strategy combining the different properties of inorganic components with other functional molecules will be described more in detail in following sections.

Galstyan and co-workers also developed a promising NP-based immunotherapeutic device in an example of multifunctionality [145]. ICB antibodies were covalently bound to the surface of multifunctional NPs (Fig. 14). Along with anti-PD-1 and anti-CTLA-4 antibodies, other proteins are co-delivered to enhanced Blood Brain Barrier (BBB) penetration like transferrin (Tf) and angiopep-2 (AP-2). It is shown how the labelled antibodies alone do not penetrate the BBB while they localize in the tumor area when incorporated to the NPs. In this model, the P/a-CTLA-4 penetrates more than the P/a-PD-1 or the combination of both. Following a similar trend, the infiltration of activated $CD4^+$ and $CD8^+$ T cells is promoted (Fig. 14a), by looking at a typical proliferation marker such as Ki67. The authors characterize in detail the tumor microenvironment in terms of immune populations, showing a decrease in the immunosuppressive environment.

Metal-Organic Frameworks (MOF) are being introduced in the field of immunotherapy [146]. The structure of MOFs makes them great candidates for ultrahigh loading of molecules and even other NPs for controlled release. Li et al, developed a MOFs-based multifunctional vector by encapsulating MSNPs for pH-dependent co-delivery of antigen and adjuvants [147]. OVA and the TLR3 agonist PolyIC are administrated along with ICB antibodies promoting antigen specific $CD8^+$ T cell responses that are visualized by tetramer staining. The authors show a set of comparative strategies for co-delivering several functional molecules in a mice lymphoma model and compare the effect of lowering the dosing by enhanced delivery. First, they show that cross-presentation of antigenic peptides in an MHC-I context is achieved by endo/lysosomal pathway scape, due to pH-responsive nanoadjuvants. After subcutaneous injection, the encapsulated antigen (MS@(A647-OVAinMOF)) shows enhanced accumulation in the draining LNs and reduced off-target delivery to other organs. By co-delivering OVA and ICB CTLA-4 antibodies the production of OVA-specific $CD8^+$ T cells in both the tumor and in spleen is enhanced, obtaining higher percentages by encapsulating the antigen in the MS core and both the ICB + adjuvant within the MOF, (MS@OVAinMOF)@(anti-CTLA4inMOF). Also, highest cytokine production in the spleen (IFN-γ

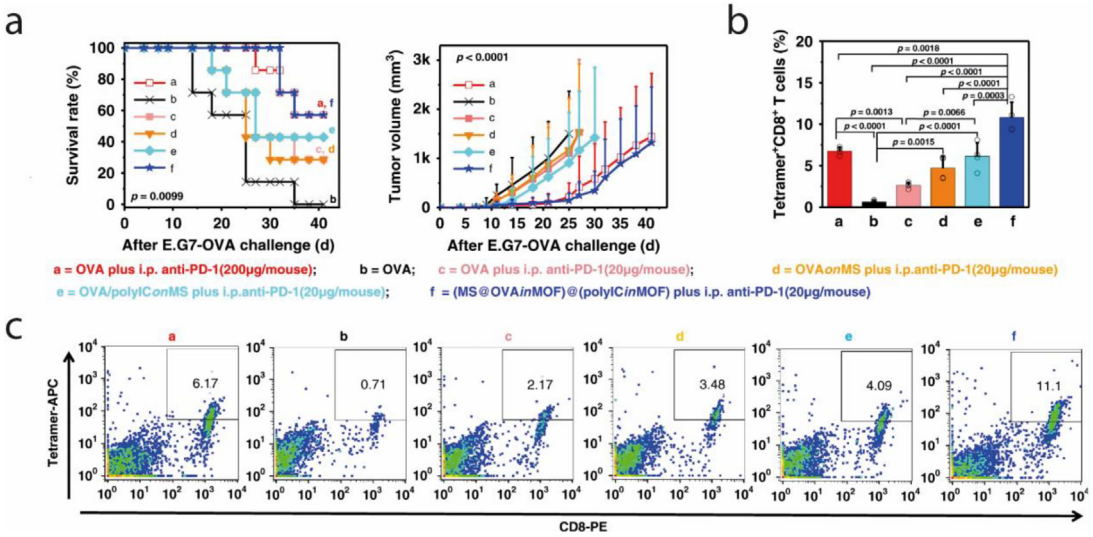

Fig. 15. Co-delivery of antigen and adjuvant encapsulated in MOFs coated with MS induces tumour-specific immune response in mice after subcutaneous injection. a) Survival curves and tumor volume after the different treatments and the levels of splenic antigen specific $CD8^+$ T cells. b and c) Representative flow cytometry plots of $tetramer^+CD8^+$ and $CD4^+$ T cells at the endpoint. Adapted from Ref. [147].

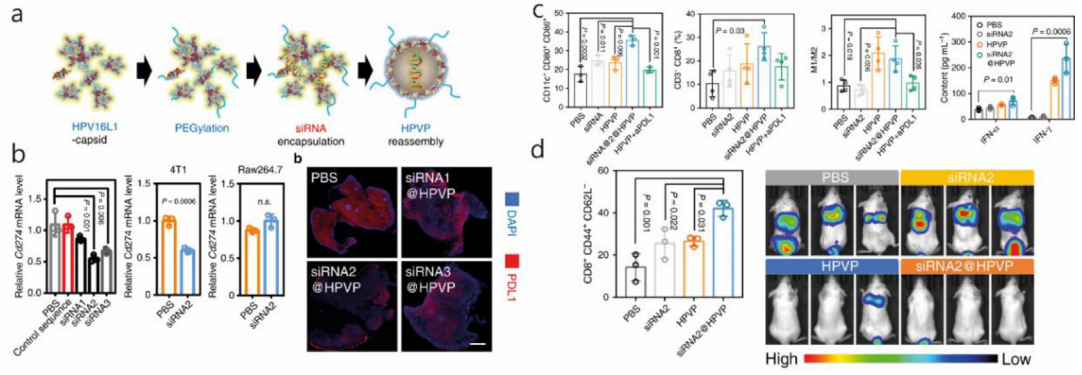

Fig. 16. HPV-derived NPs for siRNA encapsulation induces tumor immunity by engaging with the innate immune system through TLRs. a) HPV capsid is dissembled, Pegylated and reassembled for encapsulation of siRNA molecules. b) Differential efficiency in the silencing of PDL1 is shown by different siRNA molecules to the target. c) Effect on myeloid and lymphoid immune cells measure by flow cytometry after IV injections in mice showing differential induction of mature DCs ($CD11c^+CD80^+CD86^+$) in the LNs (left) and cytotoxic T cells in the tumor ($CD3^+CD8^+$), intratumoral M1/M2 ratio ($CD11b^+CD206^+$ M2 macrophages and $CD11b^+CD206$- M1 macrophages), and serum levels of IFN-α and IFN-γ measure by ELISA. d) Flow cytometry shows differential frequencies of TEM cells ($CD8^+CD44^+CD62L$-) in spleen after 40 days. E) anti-metastasis effect of the different treatments. Adapted from Ref. [152].

and TNF-α) is achieved. Similar antitumor effect is obtained by the application of (MS@OVAinMOF)@(polyICinMOF) in combination with a low dose of anti-PD-1 (20 ug/mouse) compared to that of OVA + high anti-PD-1 dose (200 ug/mouse) in terms of survival and tumor growth (Fig. 15a) while certain benefit is obtained looking at the $tetramer^+$ T cells (Fig. 15b and c). This is relevant to reduce the potential deleterious effects of high doses of immunotherapeutics. Overall, certain degree of characterization of the immune landscape generated by such constructs is reported by the authors based on flow cytometry analysis of bone marrow dendritic cells upon incubation with the different materials, reporting a higher level of activated DCs based on the expression of chemokine receptor CCR7 and co-stimulatory molecules CD80 and CD40. Such features imply the enhanced capability of antigen presentation and trafficking to secondary lymphoid organs to mount $CD8^+$ responses. These kinds of phenotypic studies are more and more relevant due to the existence of different functional subpopulations playing a role in tumors rather than generic activation of $CD8^+$ (or $CD4^+$) responses.

4.2. Modification of immune synapses by controlling protein expression

ICB consists in applying antibodies to block the interaction between specific membrane molecules at the APCs-T cells synapse, leading to immune inhibition. On the other hand, modification of the expression levels of proteins involved in signaling events at the immune synapses is an alternative strategy to bypass immune checkpoints. Delivery of target-specific RNA molecules, like siRNA corresponds to another recently exploited approach in which NPs also prove to be valuable [148-151]. Zheng et al. carried out the encapsulation of siRNA for the PD-1 ligand PD-L1 (CD274) expressed in APCs, for blocking its expression and promote cellular anti-tumor immunity (Fig. 16a) [152]. To do that, they used an already successful vaccine approach based on papillomavirus (HPV) L1 protein (HPV16 L1). They tested 3 different siRNAs and explored the capabilities to induce DC and macrophage maturation and cytokine production. siRNA2 showed higher capability to reduce CD274 transcript *in vitro* (Fig. 16b). The three formulations

showed similar levels of $CD11c^+$ DC maturation based on the expression of CD80 and CD86. Whereas differential profile at the macrophage compartment was associated to each of the three formulations, being the siRNA2 the one promoting higher levels of cytokine production. When tested *in vivo*, siRNA2@HPVP showed lower off-targeting to other organs as indicated by imaging and a prolonged tumor free stage as compared to the controls and the aPD-L1 control. The treatment promoted $CD11c^+$ DCs and $CD8^+$ T cell infiltration and higher M1/M2 ratio and secretion of IFN-α and IFN-γ (Fig. 16c), which correspond to good prognostic indicators. TLR7 was shown to play a major role in siRNA2@HPVP-mediated APCs activation, promoting Th1 immunity based on recruitment of $CD4^+$T-bet^+ cells.

The authors report an increase in effector memory T cells ($CD8^+CD44^+CD62L^-$) in the spleen (Fig. 16d) and argued the generation of immunological memory to explain that siRNA2@HPVP-treated mice did not present relapse or metastasis (except from 1 of the mice) (Fig. 16d). The levels of splenic TEM cells in the siRNA2@HPVP group (42%) were threefold higher than the PBS-treated. Blocking PD-L1 expression by siRNA in combination with Paclitaxel proved to be slightly more effective than blocking with ICB antibodies (anti-PD-1). This shows an example of the potentiality and effectiveness of NPs for RNA delivery, an increasingly interesting approach in the era of RNA vaccines.

Knock out of inhibitory molecules by siRNA delivery is a promising approach being adopted by different authors. Liu et al., have developed a multifunctional construct by encapsulating a photoactive molecule (Iondocyanune green, ICG) in a shell of $CaCO_3$ coating a MnO_2 core that acted as scaffold [153]. In the surface, PD-L1 siRNA molecules were immobilized electrostatically to form the final nanoplatform. By IV injection, EPR makes the NPs accumulate in the tumor, but at similar levels of other organs such as the lung. Delivery of siRNA increases the levels of IFN-γ and frequency of T cell infiltration (Fig. 17a). The application of $Mn@CaCO_3$/ICG@siRNA proves to reduce tumor growth and increase survival for the length of the experiments (40 days) (Fig. 17b). The effect is due to a combination with other NP-intrinsic functionalities dependent on laser irradiation.

4.3. Modification of signaling pathways

Targeting molecular inhibitory motifs at the immunological synapse is one of the main strategies that is proving some results as demonstrated by ICB/siRNA KO and where NPs could also help in enhancing delivery [7,34,154-156]. Administration of drugs targeting components of specific signaling pathways increasing the dose control and targeting is critical in the application of NPs. The Wnt/β-catenin signaling pathway at tumor sites has been shown to play an important role in determining tumor-infiltrating T cell levels. However, due to toxicity issues related to dosing drug like Wnt inhibitors has not been approved for clinical use. By applying dynamic combinatorial chemistry (DCC), Liu and coworkers developed a metal–organic NP (MOICP) incorporating hypoxia and pH responsive PEG polymers for reducing non-specific delivery outside the tumor microenvironment [157]. The triggering of this effect by such conditions is relevant since systemic administration once again generates higher off target accumulation, particularly in the liver. Gene enrichment analysis shows strong regulation of the Wnt and β-catenin pathways by the MOICP (Fig. 18a). *In vivo*, the tumor volumes are reduced in the MOICP (about 50% reduction) similarly to and anti-PD-1 treatment and a synergistic or combinatorial effect is achieved by the combination therapy (Fig. 18b). Such an effect could be due to an increase activation and infiltration of T cells in the tumor. In fact, the authors attribute that to the elevated levels of IFN-γ and granzymes that are significantly higher in the combination therapy. Using cargoes for intracellular delivery and inhibition of intracellu-

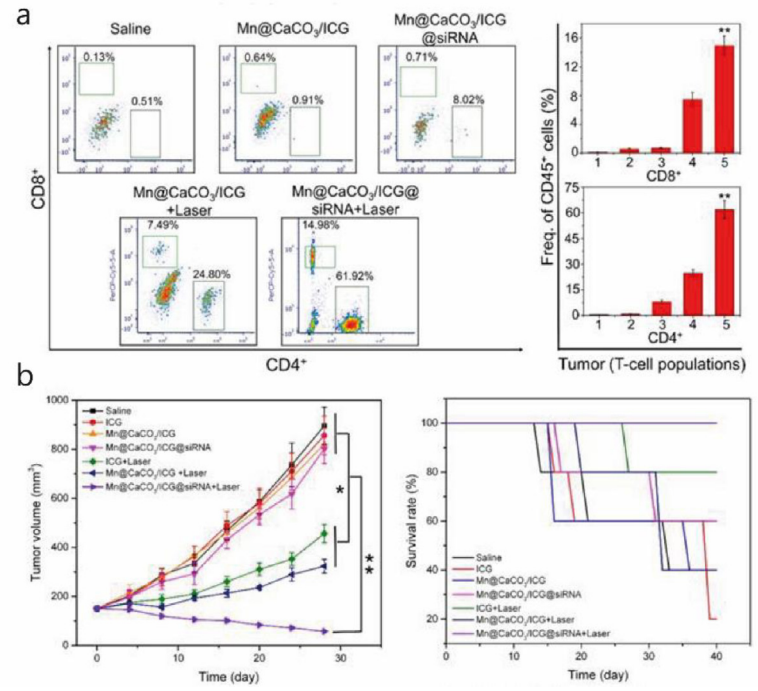

Fig. 17. Biodegradable CaCO3-based NPs are applied for targeted irradiation-driven delivery of siRNA to block PD-L1 expression locally and modulate the TME. a) Representative flow cytometry plots of lymphocyte infiltration frequencies in tumor-bearing mice 4 days after laser irradiation. b) Tumor volume and survival rates are depicted in the different groups after laser irradiation. Adapted from Ref. [153].

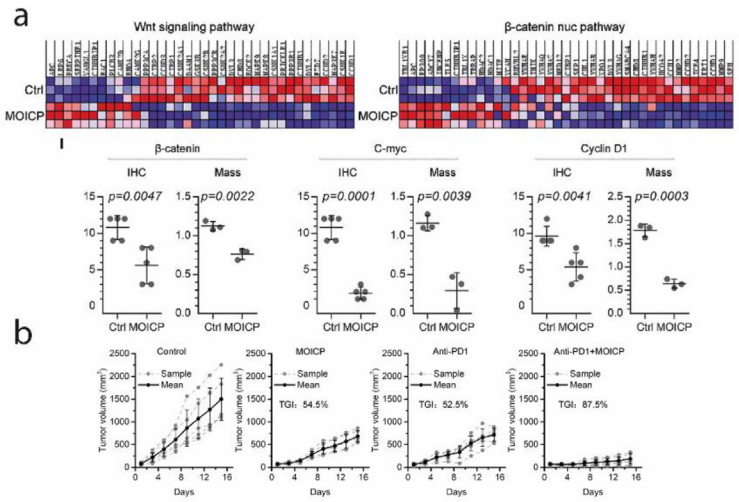

Fig. 18. Metal-organic-NPs pH and hypoxia responsive accumulate in the TME and promote the Wnt/b-catenin inhibition. a) Suppression of Wnt and b-catenin signaling gene expression (Top) and protein quantification by of key proteins in the pathway. b) Tumor volumes after combinatorial therapy with PD-1. Adapted from Ref. [157].

lar pathways is one of the most exploded and pursued ways to redirect and modulate immune responses as shown in such article.

4.4. Ex vivo "training"

Another type of recent immunotherapy is based on the infusion of *ex vivo* modified cells. Chimeric Antigen Receptor T cells (CAR-T) are cells extracted from the patient and genetically modified to express specific TCRs for tumor antigens. After modification, cells are again infused into the patient. Immune cells can be extracted from the patient and treated to increase their level of activation and effector capabilities in specific conditions. Nanotechnology offers the possibility as well to be used *ex vivo* to promote antigen processing, presentation, and activation of immune cells to then be reinfused. This approach is readily being used by many researchers by applying different materials combining the delivery of innate immune activators and adjuvants (TLR ligands) cytotoxic drugs such as doxorubicin or to directly load the cells with NPs for exerting specific functions once reinfused [158-161]. In a recent study, Chen et al. designed a dendrimer-entrapped AuNP to *ex vivo* activate T cells for adoptive cell therapy that could trigger potent T cell responses [162]. Briefly, PEGylated G5 PAMAM dendrimers were used as templates to synthesize Au DENPs with a 25:1 Au atom/dendrimer molar ratio. The developed NPs were used to concentrate the amount of TLR agonist for DC activation that will later promote T cell immunity. BMDCs treated with the functional Pegylated dendrimers showed an increased level of uptake of the TLR ligand than in its soluble form. Increased expression levels of MHC-II, CD80 and CD86 indicate that the BMDCs were induced to be mature BMDCs (mBMDCs). Also increased levels of cytokines were detected in the culture supernatants (TNF-α, IL-12p70 and IL-6) independently of the NP dose (from 50 to 100 ug/ml). Experiments in tumor models both *in vitro* and *in vivo* showed the capabilities to enhance the antitumor capacity in a trans-well setting by co-culturing activated and non-activated T cells by BMDCs with B16 cells (in different chambers) observing a decrease in the viability of B16 cells in the culture where T cells where previously cultured with mBMDCs. On the other hand, C57/BL6 mice injected with B16 cells were treated intravenously and intratumorally with mBMDC-activated or inactivated T cells. Cytokine secretion (IL-2 and IFN-γ) showed significant differences between the two treatments and injection routes, while moderate effect in the tumor remission was observed after 20 days. Overall, the authors claim that maturation *ex vivo* of DCs can be a good strategy for specifically induce the maturation of T cells to effector phenotypes for adoptive cell therapies [86]. The authors show the effect of mDCs in inducing T cell activation and how reinfusion of such activated T cells have an antitumor effect. Despite that the article uses non-specific activation without a specificity towards any particular antigen, it serves as a proof of concept study that *ex vivo* maturation is a way to go in NP-based immunotherapy to explode enhanced activation of immune cells prior to reinfusion [162].

An interesting use of NPs for immunotherapy based on adoptive transfer technologies are the ones that involve the magnetically driven targeting of NK cells to the tumor. In a recent paper by Sanz-Ortega *et al.*, 3 aminopropyl triethoxysilane (APS) coated MNPs are directly attached to $CD8^+$ T cell membranes, targeting them magnetically to accumulate in the tumour [158]. The authors show that MNP attachment does not influence the phenotypic and functional profile of the OT-I specific T cells by looking at classical activation (CD69, CD25, CD27, CD44) and subpopulation (CD62L, CD127) markers. The degranulation capacity of MNP-conjugated OT-I T cells was tested against the EG7-OVA cells line, rendering a comparative level of degranulation and cytotoxicity as well as production of IFN-γ, thus not affecting the performance of the cells towards their target. Magnetic targeting of adoptively transferred tumour-specific NP-loaded $CD8^+$ T cells does not improve their tumour infiltration in a mouse model of cancer but promotes the retention of these cells in tumour-draining LNs. The analysis of the infiltration into the tumour-draining LN showed that the group treated with APS-MNPloaded OT-I $CD8^+$ T cells and exposed to EMF presented greater infiltration in the tumour-draining LNs of $CD8^+$-$V\alpha2/V\beta5^+$ T cells with an activated profile (Fig. 19). This indicates that a fraction of the transferred APS-MNP-loaded OT-I $CD8^+$ T cells infiltrated the tumour-draining LN and remained there 14 days after cell transfer which could hold certain potential to deliver specific antigens in a different setting, such as by APCs magnetically labelled, or to target metastatic cells that are primarily migrating to draining LNs.

The previous examples represent a step ahead in the design of approaches involving NPs, and the multiple potential targets and points of intervention that could be tackled by NPs. Despite of the promising results, most reports still lack depth of functional and phenotypical understanding of the cellular responses elicited

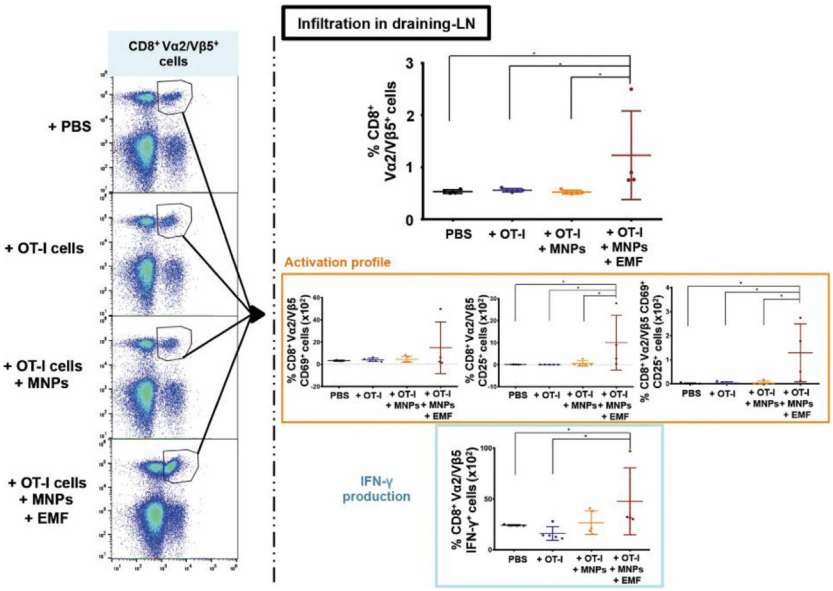

Fig. 19. Tumour–specific T cells modified with magnetic NPs improve tumor accumulation the expansion and retention of tumor-specific cells in the tumour–draining LNs. Percentage, activation levels ($CD69^+$ cells) and IFN-γ production appear increased in the draining lymphnodes of treated mice exposed to EMF.

and the evolution overtime in different compartments such as the circulation and the tumor. Thus, there is big room for improvement.

5. Inorganic contribution of inorganic materials for modulating immune responses

Inorganic materials such as gold, iron or silica among others have been extensively used to synthesize nanostructured materials for various biomedical applications. Inorganic NPs are can be engineered to have a wide variety of sizes, structures, and geometries as it the case of AuNPs, which are synthetized in a broad range of forms such as nanospheres, nanorods, nanostars, nanoshells and nanocages [163]. Additionally, inorganic NPs have unique physical, electrical, magnetic, catalytic and optical features, due to the inherent size-dependent properties of the core material. For example, the optical (plasmonic) properties of AuNPs are size and shape dependent, providing them with photothermal properties when excited with light matching the corresponding plasmonic band. AuNPs are also easily functionalized, granting them additional properties and delivery capabilities [164]. IONPs are attractive for clinical use because of their stable, biocompatible, colloidal suspensions, they can be conjugated with a range of ligands and molecules. In addition, they present superparamagnetic properties. This combination of features has resulted in applications including imaging, sensing, targeted drug delivery, therapeutic heating (magnetic hyperthermia), and mechanical stimulation [165]. IONPs make up most FDA-approved inorganic-based nanomedicines. Magnetic IONPs, composed of magnetite (Fe_3O_4) and/or maghemite (Fe_2O_3), possess superparamagnetic (non-permanent magnets) properties bellow certain sizes, and have shown success as contrast agents, drug delivery vehicles and thermal-based therapeutics [166]. Other common inorganic-based NPs include calcium phosphate and mesoporous silica NPs [167,168], which have both been used successfully for gene and drug delivery. QDs, typically made of semiconducting materials such as silicon, are unique NPs used primarily for *in vitro* imaging applications, but they show promise for *in vivo* diagnostics [169].

Due to their magnetic, radioactive and plasmonic properties, inorganic NPs are uniquely qualified for applications such as diagnostics, imaging and therapeutic actions. In general, most have good biocompatibility and stability, and fill niche applications that require properties unattainable by organic materials. However, they are limited in their clinical application by low solubility and toxicity concerns, especially in formulations using heavy metals [170].

Such inorganic properties arising from the nanosized dimension of the NPs are relevant to local and controlled tumor cell death leading to the so-called ICD. Dying cells stimulate innate immune responses against newly exposed tumor antigens and molecules. The molecular mechanisms underlying ICD includes expression of calreticulin (CRT) on the membrane of dying cancer cells, release of adenosine triphosphate (ATP) in conjunction with other endogenous adjuvants such as high mobility group box 1 (HMGB1), heat shock proteins (HSPs) and uric acid [171]. Macrophages and DCs are recruited to tumor sites and DCs migrate to draining LNs to present antigens to T cells. Induction of ICD seems a critical approach for a combinatorial strategy promoting the immune system to target primary tumors as well as secondary distant metastasis.

Many of the current anticancer approaches using nanomedicines induces cell stress-related processes such as DNA synthesis inhibition or ER stress through the effect of an antineoplastic drug (*i.e.*, doxorubicin, cisplatin, *etc.*). ROS-dependent ER stress strategy plays a major role in ICD. The ER, responds to oxidative stress induced after the overproduction of ROS by activating signaling pathways that further disturbs homeostasis in the ER, eventually contributing to the induction of ICD [172]**.** In addition, combinatorial approaches using inorganic NPs could provide the substrate for the energy transformations (*i.e.*, ROS generation, hyperthermia *etc.*) which eventually could contribute to the induction of ICD.

Radical-based approaches include sonodynamic therapy (SDT), photodynamic therapy (PDT), and chemodynamic therapy (CDT) addressed to the generation of ROS for damaging cancer cells after being subjected to an external energy stimulus, inducing similar innate immune effects [173]. In SDT and PDT, NPs bearing sonosensitizer or photosensitizers are accumulated in tumors which are then stimulated with ultrasonic waves or photons respectively for generating cytotoxic singlet oxygen ($^1O_2$) from endogenous O_2. In addition, inorganic NPs have received extensive attention

in CDT due to their ability to generate long-lasting, light- and oxygen- independent chemical reactions with application for promoting cell death. Typically, metal ions such as Fe, Co, Ni, and Cu catalyze the transformation of endogenous intratumoral hydrogen peroxide (H_2O_2) into –OH radicals through Fenton reactions in the TME [174]. As a highly toxic ROS, –OH can induce significant damage and inhibit tumor growth. However, the therapeutic effect of CDT is limited due to the scavenging of –OH species by glutathione (GSH) and by the low local concentration of H_2O_2. By inducing acute local inflammation, ROS-mediated dynamic therapies are known to be strongly immunogenic [175]. However, the efficacy of radical therapies is diminished by many factors in the TME, such as high intracellular concentrations of reducing thiol species and hypoxia, among others [176]. With the evolution of nanotechnology, strategies such as pH-responsive sequential decomposition or the use of reducing agents to tailor the TME have also been explored to increase the availability of the needed substrates expanding the application of ROS-mediated dynamic therapies.

We show herein a comprehensive set of examples of smart formulations taking advantage of the properties of inorganic NPs for immunomodulation in conjunction with external stimuli. A detailed table including a selection of the most relevant works about this topic can be found in supplementary materials (Table 1).

5.1. Magnetic hyperthermia

The main principle behind the use of magnetic hyperthermia is the local generation of heat (up to 46 °C) in a desired body region (tumor) after the stimulation with an alternating magnetic field (AMF). Despite the higher capacity to induce tumor cell death, and the higher susceptibility of cancer cells to hyperthermia compared with normal cells [177], this technique can also affect and damage surrounding healthy tissues. When using magnetic NPs, the generation of heat is originated through the energy conversion achieved by the coupling of the magnetic moments of the NPs with an applied AMF [178]. In addition, the generated hyperthermia could contribute to hemodynamics changes, increasing blood flow and therefore alleviating the hypoxic conditions in the tumor region as well as facilitating the accumulation of other intravenously administrated drugs. Intense efforts have been focused on tailoring NP size, shape, composition, and surface chemistry to enhance the thermal conversion efficiency [179].

Magnetic hyperthermia can also contribute to the generation of an immune response against tumor. Cell damage mediated by heat is termed to elicit the expression and release of damage-associated molecular patterns, specifically HSPs. Those proteins participate in numerous signaling pathways with immune system cells, favoring the antigen presentation in APCs by the MHC complex, enhancing tumor recognition, and triggering anti-tumor T cell responses [180].

Using dextran-coated IONPs directly injected in GL261 tumors, authors recently confirmed the consistent immune response induced by magnetic hyperthermia. Tumor regions in mice were subjected to a 30 min AMF, which led to a temperature increase of 9 °C with the consequent significative reduction in tumor volume. Deepening into immune mechanisms behind this effect, authors found an increased proportion of $CD8^+$ T cells, with a corresponding reduction in the proportion of Tregs in tumors treated with IONPS and AMF. Additionally, $CD8^+$ TILs extracted from treated tumors were found to exhibit a significant increase in granzyme B marker expression, indicative of a higher proportion of cytotoxic activity [181].

Doping effect on IONPs has been recently explored by Pan et al. [182]. Authors prepared 15 nm Zn^{2+}-doped core − shell NPs with enhanced specific loss power features. Once tested in a mice model of liver cancer, core–shell NPs led to an increase of liver region up to 44°C and the analysis of tumors revealed a significant elevation in the percentage of NK cells as well as of it related cytokines (TNF-α and IFN-γ). Altogether, these results provide evidence of the potential antitumor immune triggering of IONPs-driven magnetic hyperthermia.

5.2. Photothermal therapy

Photothermal therapy (PTT) relies in the use of photothermal effects for the generation of localized heat in the tumor region after light excitation of absorber NPs (such as plasmonic or carbon-based nanomaterials) in biological windows (700–1700 nm). The hyperthermia generated by the photothermal effect may lead to a local temperature increase, damaging cancer cell membranes and impairing cytoskeleton and cell basic metabolism like DNA synthesis [183]. As a result, those mechanisms can cause tumor cell apoptosis and/or necrosis. During this process, several tumor associated antigens (TAAs) can be released as a result of ICD leading to T cell priming and antitumor response activation [184]. In addition, it has been proposed that cancerous tissues are more prone to be affected by hyperthermia than the healthy ones due to the hypoxic and acidic conditions found in the tumor microenvironment [185]. In conjunction, all these features proved the suitability of this technique for a simple, non-invasive, and specific phototherapy.

There are several organic photothermal transduction agents that have been classically studied including indocyanine green (ICG) and IR780; polydopamine (pD), polyaniline (PANI) and polypyrrole NPs which despite their biocompatibility (ICG has long been approved by the FDA) and all the advantages derived from the use of light in the NIR window, still suffer from some drawbacks such as degradation and aggregation issues as well as a photobleaching and reduced blood circulation time [186]. Mild temperature increase have shown to induce upregulation of MHC-I and MHC-II molecules, as well as CD80 and CD86 in DCs, promotes migration to draining LNs, and further enhances the ability to cross-present antigens to T cells [187]. In addition, local heat can increase blood flow, vascular permeability, and interstitial pressure to facilitate T cell infiltration into tumors [188].

Nanomaterial-based PTT has garnered increased attention by modifying them for enhanced blood circulation time, and by incorporation of active targeting ligands (antibodies and peptides) increasing the therapeutic efficacy and reducing off-target. In addition, anti-neoplastic drugs and immune adjuvants could also be incorporated in nanosystems, contributing to the anticancer effect directly or through the triggering of the immune response. AuNPs, for example, due to their surface plasmon resonance properties, exhibit a strong photothermal conversion capacity [188]. Also carbon-based materials, such as single-walled carbon nanotubes (SWCNTs) and carbon dots (CD); sulfide metallic NPs like CuS and MnS_2 or black phosphorus NPs have been applied for PTT [50,189,190].

Lin and colleagues explored the features described above by proposing a nanosystem based in a ∼ 150 nm- SiO_2 mesoporous NPs loading CuS into the porous cavities and delivering an IL-12 encoding plasmid [151]. Such NPs exhibit absorption in the so-called NIR-II light window (1000–1700 nm), which represents a significant extension in comparison with classical NIR-I (700–1300 nm) window agents. NIR II implies a noticeable enhancement of the penetration depth (3–5 cm) due to the higher wavelengths employed [191]. The authors, using a melanoma mice model, described a robust antitumor and antimetastatic effect of CuS–SiO_2-IL-12 NPs after NIR laser irradiation. Mice irradiated after intratumorally administration of the nanoformulation exhibited a higher antitumor response compared with the NPs with no laser exposure indicating that the PTT-derived ICD contributes signifi-

cantly to triggering the response. Indeed, the authors report an increase of proinflammatory cytokines IFN-γ and TNF-α and granzyme B in the tumor microenvironment. An increased infiltration of mature DCs in the LNs is found in the PTT groups, which is consistent with the proliferation and infiltration levels of $CD8^+$ T cells. This immune response associated to PTT was also found when tested in bilateral tumor model, proving the anticancer effect of CuS–SiO_2-IL-12 NPs in distant tumor inhibition. Silica mesoporous NPs has been widely used for PTT due to the high and versatile loading capacities as proposed by in another melanoma mice model [192]. In this case using a TLR7 agonist as immune effector, authors found a significant changes in ICD after PTT and the robust triggering of immune response as showed by the level of mature DCs and the increment of $CD3^+$ and $CD8^+$ T cells.

PTT-based approaches have also been explored coupling adjuvants to NPs aiming to elicit a stronger immune response. This strategy has been explored successfully using bidimensional graphene oxide nanosheets loaded with CpG-oligodeoxynucleotide. TLR9 activation by CpG leads to the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-12. As for other DNA delivery approaches *in vivo*, CpG suffer from a low ability for being internalized by cells because of the negatively charged phosphate groups. Authors addressing this issue used graphene oxide NPs as delivery platform for PTT due to their high photothermal conversion capability [193]. After intratumoral administration in a mice model of subcutaneous colon carcinoma, irradiated graphene oxide@CPG NPs led to a tumor growth inhibition of ∼ 91% which is a significant increase compared with the group of study without laser irradiation (∼38%). This synergistic effect between PTT and immunomodulation through CpG has also been explored by Han et al., in a combinatorial approach designing a system for PTT in pancreatic and breast cancer mice model [83]. They proposed a Cu_2S-based photothermal agent stabilized with thermally-sensitive lipid shell consisted in 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine poly (ethylene glycol) 2000 (DSPE–PEG2k), which could disintegrate above 41 °C (the phase transition temperature of DPPC). Moreover, the nanosystem incorporated the CpG oligonucleotides and JQ1, a PD-L1 inhibitor. After NIR-II laser irradiation (1064 nm), the temperature increment achieved led to NP disintegration and therefore to the release of both drugs. The effect of CpG in TLR9 and JQ1 in PD-L1 combined with the photothermal ablation for tumor growth inhibition in after intravenous administration, as well as for avoiding the emergence of new distant tumors. CuS effect allowed to achieve a local temperature of 54.2 °C in the tumor region, inducing ICD markers release and promoting the maturation of DCs in tumor-draining LNs which synergizes with the effect of CpG on TLR9. The levels of $CD8^+$ infiltrating T cells in tumors were significantly increased due to the combinatorial contribution of ICD and the effect of JQ1 inhibiting PD-L1.

The use of cell-derived biomimetic coatings has also been explored. Ye et al. developed an anticancer vaccination strategy for PTT enhancement in a breast cancer model where black phosphorous quantum dots of ∼ 1–3 nm were coated with cell membranes derived from surgically removed 4 T1 breast tumors and embedded in a hydrogel for subcutaneous injection [194]. Results showed a local temperature increase up to 45 °C in mice treated with Gel-BPQD-CCNVs and irradiated with NIR. The heat generated contributed to an increase of DC maturation in LNs as revealed by expression of CD11c and MHC-II. In addition, the levels, and the proliferation of cytotoxic $CD4^+$ T cell and $CD8^+$ T cells were also found elevated in the irradiated group in both LNs and spleen. Gel-BPQD-CCNVs provided a strong immune response which led to the inhibition of distant tumor metastasis. Moreover, authors showed a further significant increment in all the previous parameters due to the combination of Gel-BPQD-CCNVs with anti-PD-L1 antibody.

ICB still has a modest response rate in some cases due to low tumor immunogenicity and immunosuppressive TME, still suffering from treatment resistance, systemic side effects and elevated production costs. Therefore, just a fraction of the entire patients can benefit from these innovations. This motivates current research efforts looking for combination of strategies which could help to overcome these limitations. Altogether, the evidence described above proved PTT as a successful strategy for combining hyperthermia-derived ICD and immune agents for achieving robust antitumor immune activation.

Despite all the potential applications exhibited in these studies, PTT still lacks from common clinical application due to the limitations of NIR light in tissue penetration. Most of the irradiation remains in the surface of the tissues and just a small percentage 1–10% is able to reach depths of 2.4 to 3.5 mm in lungs and mammary tissue, respectively, as revealed in a recent work using 835 nm laser irradiation [195]. Given that, most of the studies are limited to subcutaneous cancer models, which are not faithfully representing the real environment of a tumor. Nevertheless, a continuous improvement in the development of enhanced PTT agents with greater performance in the NIR and even in the NIR-II window continuously develops, aiming moving for clinical implementation.

5.3. Photodynamic therapy

Photodynamic therapy (PDT) relies in the use of photo-excitable agents (photosensitizers, PS) absorbing the energy from light, which leads to the formation of $^1O_2$ after reacting with molecular oxygen. Then the ROS generated can intracellularly react with biomolecules causing cytotoxicity process such as cell apoptosis, necrosis, autophagy and eventually tumor ablation [196]. Cell damage generated after PDT may lead to the induction of ICD, causing the release of damage associated ligands which can activate specific immunogenicity and improve the therapeutic effect of PDT (Fig. 20). In oncology, this mechanism has been explored for non-invasive accumulation of PSs in the tumor region and selectively destroy cancer tissue without interfering with healthy areas. However, PS usually typically suffer from aggregation in biological fluids leading to quenching and limiting optical properties [197]. Moreover, tumors are complex tissues in which we can differentiate a central core region, characterized by deficient blood irrigation and exhibiting low O_2 concentration low pH and high lactate levels, where cells are progressively suffered from hypoxia-driven physiologically impairments. The role of hypoxia in cancer has been linked to angiogenesis activation and thereby with metastasis transformations and also with tumor survival and anti-tumor immunity suppression [198]. Moreover, tumor cells are growing radially forming a network where the main cancerous cells, stem cells, vascular cells, macrophages, and fibroblasts, among others, stablish a dynamic crosstalk. Several factors including the dense extracellular matrix secreted in the tumor microenvironment, the tissular complexity and the hypoxic conditions contributes to hindering nanomedicines performance, therefore decreasing their overall efficacy [199,200].

Since PDT need from O_2 supply, the consumption of O_2 for ROS generation will increment the hypoxic conditions causing a detrimental feedback cycle. NP-PSs conjugates has been studied aimed to the enhancement of photophysical properties of PSs in PDT. Given the low light absorption exhibited by most of the PSs in the biological window (above 650 nm), researchers have made use of different nanosystems for boosting photodynamic mechanisms. This is the case of upconverting NPs (UCNPs) coupled to Zn based PS, where UCNPs can efficiently transfer energy from

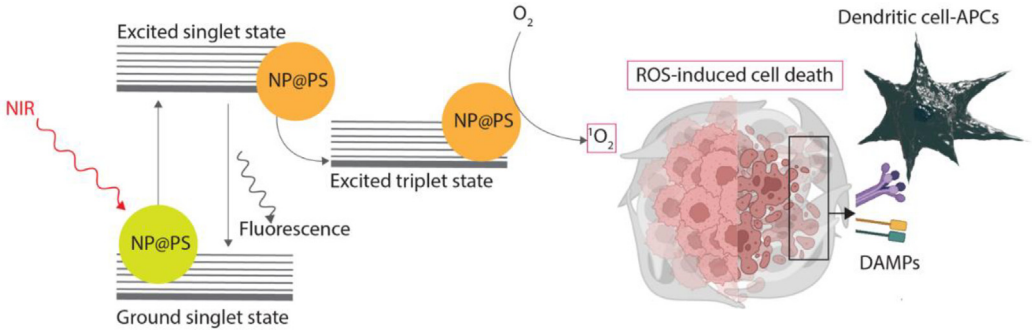

Fig. 20. Mechanism underlying PDT. Photosensitizers present in NPs absorbs the energy from light which leads to the formation of $^1O_2$ after reacting with molecular oxygen. Then the cell death induced by ROS generated led to the release of damage associated ligands which can be recognized by APCs triggering the immune response.

NIR to the acceptor Zn-PS. UCNPs can emit visible light in the PS-absorbing spectral regions, triggering the enhancement of PDT by transferring the upconverted energy to PSs [197,201]. Self-generation of appropriate substrate for NP-mediated ROS generation has proved efficient. Since tumor microenvironment exhibit a high H_2O_2 concentration, Song et al. tried to use this excess of H_2O_2 as fuel for MnO_2 NPs which can react with of H_2O_2 converting it to O_2. Therefore, the generation of O_2 can alleviate hypoxic conditions, contributing to restructure tumor cell microenvironment and revert tumor progression. Moreover Mn^{2+} can act as T_1 contrast agent enhancer, providing contrast for a follow up of the tumor by magnetic resonance imaging (MRi) [202].

Following this principle, Yang et colleagues proposed a TME responsive and TME modulating strategy using MnO_2 NPs [203]. The design described a pegylated hollow MnO_2 nanosheets, which can incorporate both chlorin6 as photosensitizer and doxorubicin as antineoplastic drug in the mesoporous spaces. On the one hand, authors took advantage of the acidic pH found in TME for stimuli responsive release of chemotherapy drug and for T_1 weighted MRi of tumors provided by Mn^{2+} ions generated at low pH. On the other hand, they benefit from the H_2O_2 conversion to O_2 for alleviate hypoxia and providing a substrate for PDT. As a result, mice injected with H-MnO_2-PEG/C&D formulations exhibited effective hypoxia reduction inside tumors. In addition, mice treated with H-MnO_2-PEG/C&D and subsequentially irradiated at 660 nm exhibited the slowest tumor growth speed and greatest tumor size reduction at the end of follow up with negligible toxic side effects in the healthy tissues. The significant antitumor effect in irradiated groups reflected the role of PDT-driven immune response. Regarding these immunomodulatory mechanisms, authors found a notable increase of macrophage infiltration with reduced M2 load. This polarization was further confirmed by the cytokine secretion profile which was characterized by an increment in IL-12 and a decrease in IL-10 production. Mice injected with H-MnO2-PEG/C&D plus irradiation exhibited a significant increase in CTLs as well as in TNF-α production compared with non-irradiated mice. This approach proved the effect of H-MnO2-PEG/C&D as cancer suppressor both directedly and indirectly after inducing immunogenic cell death and the consequent activation of DCs and effector T cells. In addition to primary tumors, PDT has been also proved effective to suppress distant metastasis. Liang and collaborators used core–shell AuNC@MnO_2 for the treatment of metastasis in a mice model of triple negative breast cancer [204]. AuNPs exhibit high NIR absorbance which make them suitable for PDT purposes (Fig. 21). Moreover, recent works have demonstrated how AuNPs can produce ROS under laser irradiation matching plasmonic bands. Continuous or pulsed laser irradiation act through the directly excited primary hot electrons leading to the production of $^1O_2$ [205]. The photodynamic properties are enhanced by the catalytic process of TME H_2O_2 by the MnO_2 shell as described previously, generating more oxygen substrate for ROS production. Irradiation with 808 nm laser in mice injected with AuNC@MnO_2 NPs elicit a robust $CD8^+$, $CD4^+$ and NK cells mediated by ICD which ultimate results in the suppression of both the primary beast rumor as well as the lung metastatic transformations.

MnO_2 NPs constitutes a vastly explored strategy for cancer PDT, but it has also been proved to contribute to sepsis management in a mice model of *S. aureus* infection. Pegylated MnO_2 NPs loaded with chlorine e6 as PS (Ce6-MnO_2-PEG) benefits from TME conditions for the catalytic processing of the H_2O_2 produced by *S. aureus* in the skin abscesses with the consequent O_2 production which can be used for chlorine-mediated ROS generation. In addition to the infection symptoms alleviation, the effect of Ce6-MnO_2-PEG NPs was further proved by the strong immunological response against recurrent infections. The ICD induced after irradiation with a 661 nm laser, generated DCs migration and activation resulting in the antigen presentation and memory B cells production responsible for the prolonged immune response [206].

5.4. Sonodynamic therapy

Sonodynamic therapy (SDT) take advantage from a safe and noninvasive technique widely used in clinical practice such as ultrasounds (US). US-based treatment and diagnosis have opened a broad new application framework for deep tumor treatment due to the high tissular penetration capacity [207]. SDT-based strategies rely in highly toxic ROS production through the action of sonosensitizer agents. Those can induce apoptosis and eventually cancer cell death after ultrasound stimulation, promoting ICD and generating tumor antigens which are able to elicit antitumor response [208]. NP-driven SDT has been attracting attention since the proved efficacy of biocompatible and stimuli-responsive nano systems. Numerous works have been described successful approaches in cancer treatment using lipidic and inorganic NPs describing outstanding tumor inhibition properties [209,210]. Recent works have tried to elicit insights about the interplay between SDT and immune system deepening in the immunomodulatory-immunostimulatory mechanisms.

Zhu and colleagues have proved the immune role of a system based in Zn^{2+} nanosheets coupled with tetrakis(4-carboxyphenyl) porphyrin (TCPP) as sonosensitizer and CpG as adjuvant [211]. After US application, Zn-TCPP/CpG NPs + US promoted a solid primary and distant tumor inhibition, avoiding cancer recurrence and increasing mice survival up to 60 days. Authors showed the key role of the system promoting ICD which led to an increase of DC maturation (about a 70%) in draining LNs in the groups with US compared with the nanoformulation alone. Furthermore, an increase of CD8 + infiltrating T cells and a notable ultrasound effect

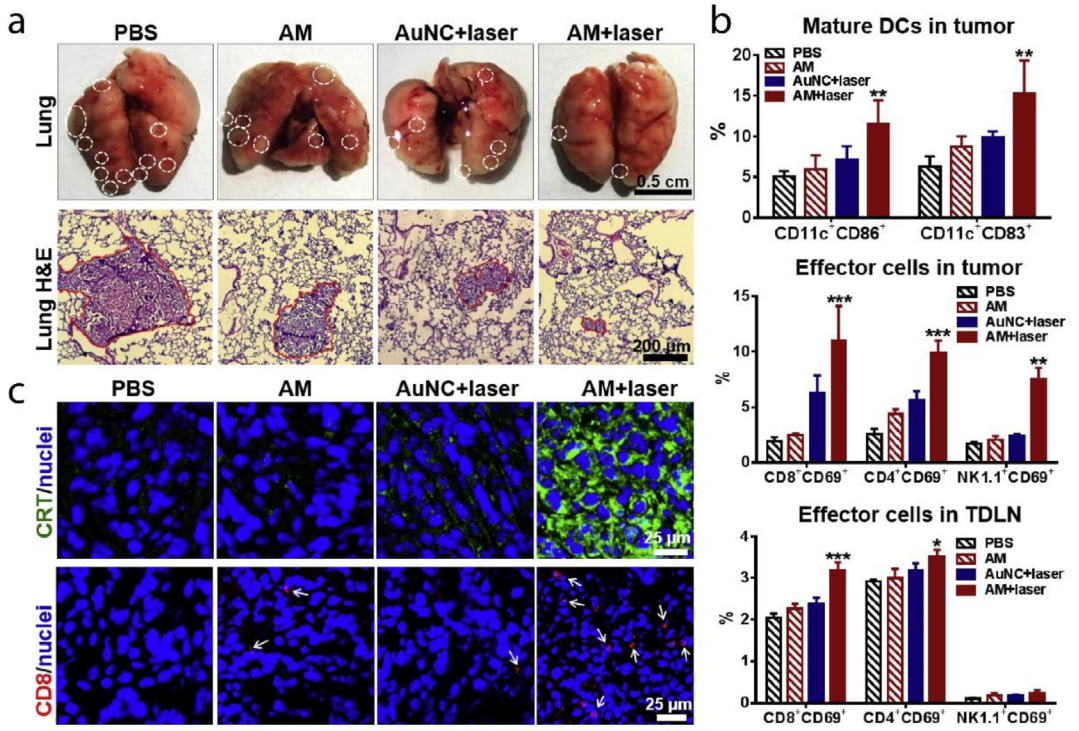

Fig. 21. Example of inorganic NP-boosted immunogenic PDT for the treatment of tumor and metastases. a) PDT induced antitumor immune response and tumor regression. Treatment with core–shell gold nanocages subjected to laser irradiation led to a reduction in tumor volume as well as in metastases manifestations. b) Anticancer effect was mediated by immune cells both in tumor and LNs. c) Laser irradiation promotes ICD-driven immunomodulation. Adapted from Ref. [204].

reducing the percentage of Tregs ($CD3^+CD4^+Foxp3^+$) present in the tumor were reported. Authors hypothesized that US induced the downregulation of CTLA-4 with the consequent increment of T-effs cells ($CD3^+CD4^+Foxp3^-$) differentiation after the engaging of CD28 with CD80/CD86 on DCs. When tested in a model with a secondary distant tumor, Zn-TCPP/CpG + US based SDT treatment on the first tumor exhibited a remarkable increase (25%) in the $CD8^+$ T cells infiltration in the new tumors where Treg cells levels were also reduced. Furthermore, the levels of effector memory T cells ($CD3^+CD8^+CD62L^-CD44^+$) in peripheral blood was proved to be higher in this group, indicated robust memory immune response. Altogether these results showed the suitability of the SDT-driven anti-tumor immune effect of Zn^{2+} nanosheets.

Motivated by the inherent catalytic properties of Mn^{2+}, Zhan and collaborators employed Mn-based MOF, a hybrid coordination nanostructure, for enhance SDT in a melanoma mice model [212]. The NPs proposed consisted in a SDT inducer and nanovaccine biomimetic system, with the Mn-MOFs bearing CpG oligodeoxynucleotide as adjuvant and coated with cell membranes obtained from murine melanoma B16 cells overexpressing OVA antigen. Mn-based MOFs were the responsible of ROS production enhancement after SDT and the membrane provided higher bioavailability due to preferential cancer cell uptake and the increased blood circulation time. Mn-MOF@CM of 125 nm was proved to be suitable for ROS generation *in vitro* even during hypoxia, and when tested *in vivo*, US application 24 h after injection led to a strong tumor growth inhibition, increasing mice lifespan to 37 days, and providing memory immunity against recurrent tumors. Authors described the increase in mature DCs and activated $CD8^+CD69^+$ T cells, as well as $CD8^+IFN\text{-}\gamma^+$ T cells in both tumor and spleen. Effector memory T cells ($CD8^+CD44^+CD62L^-$) were found in elevated levels after SDT in spleen and LNs, demonstrating the ability to generate memory immune response. The biomimetic coating has also been exploited for SDT using IONPs and hollow mesoporous TiO_2 NPs [213].

5.5. Radiotherapy

As one of the most stablished strategies in oncological daily practice for decades (more than 60% of cancer patients undergo at some point), radiotherapy (RT) has also exploited the benefits due to the enhancing properties offered by nanomaterials since the use radiotherapy still generates major concerns regarding the radiation side effects in healthy tissues or the inadequacy for metastasis transformations [214].

X-rays can decompose in indifferent types of radiation after interacting with a material. The Compton scattering describes how the irradiating photon transfer part of their energy to the electrons, releasing them from the atom. The photoelectric effect also explains the production of electrons after X-radiation where part of the irradiation is transferred in the form of Auger electrons which due to their low energy are responsible of the ionization of a limited range [215]. Both types (Compton and photoelectronic-derived) of electrons can interfere with cellular metabolism impairing DNA as well as generating reactive oxygen species during radiotherapy (Fig. 22). The damage of DNA and the production of ROS can trigger apoptosis pathways therefore contributing to tumor destruction.

The use of a material as radiosensitizer relies in the employment of elements with higher atomic number since the photoelectric effect of X-ray radiation is proportional to the function $(Z/E)^3$. As higher is the atomic number higher is the enhancement derived from a given radiation dose since higher is the chance for elemental nucleus-radiation interaction. Radio-sensitization effect is based on the strong absorption of photons by high-Z atoms in comparison with surrounding areas because of the contrast exhibited between radio sensitizers (high atomic number) and body tissues (C, H, O has lower Z numbers) which leads to an increase in the local dose deposition [217]. UCNPs, bismuth nanoplates and tungsten nanodots have been already exploited as nano-radiosensitizers following this principle. AuNPs has been largely

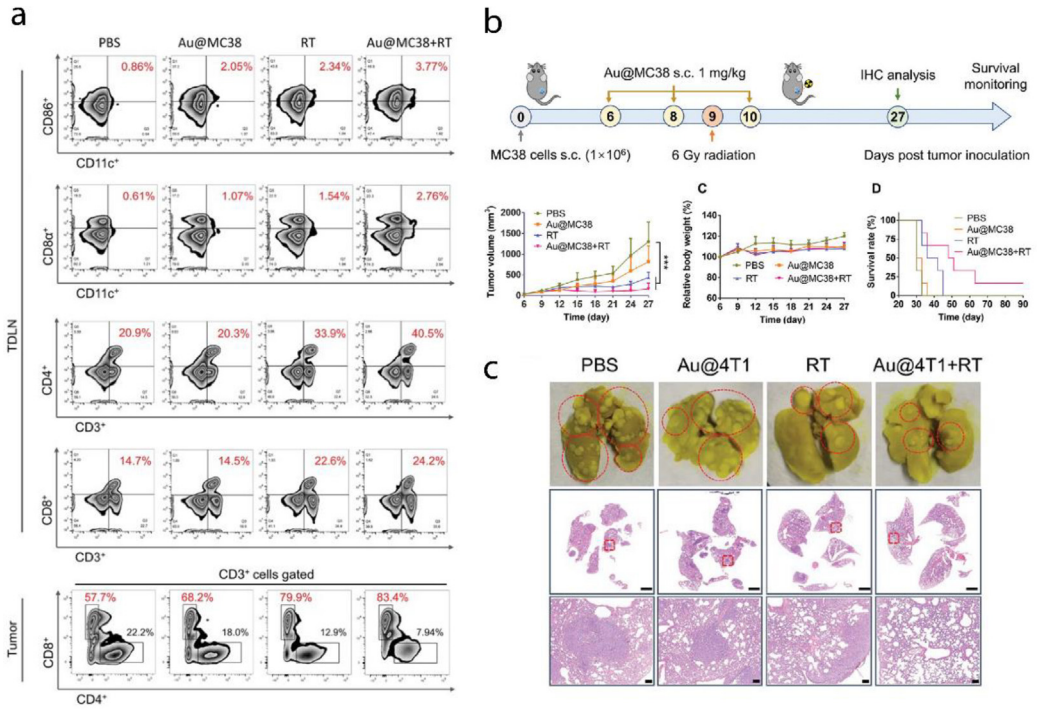

Fig. 22. Example of the use of inorganic NP for sensitizing radiotherapy and enhance immune response against cancer. a) Flow cytometry analysis of TDLN and tumor tissues revealing an increase in DC levels as well as an increase in CD8 T cell populations int the groups of treated with radiation and Au@MC38. b) Tumor volume was significantly reduced after combinatory treatment, leading to an increase in the survival rate of treated mice. c) Radio-sensitization of Au@4T1 on orthotopic breast cancer model led to a reduction of lung tissue metastases. Adapted from Ref. [216].

studied as radiosensitizer due to the radiation enhancement derived from the high photoelectric absorption of Au [218]. In a very innovative approach Qin et colleagues achieved an outstanding enhancement of immune response by using AuNPs synthetized and secreted *ad hoc* by cells. Authors used cells as bioreactor for the synthesis of AuNPs from the precursors $HAuCl_4$ and Au^{3+} (Fig. 22). The obtained NPs allowed to increase the accumulation of radiosensitizer in the tumor through the homologous targeting provided by cell membranes. Upon X-ray radiation, AuNPs elicit a robust ICD through the production of ROS which led to the inhibition of both primary and metastasis transformations in a mice model of colon carcinoma. Interestingly, the anti-tumor efficiency of AuNPs + RT was further combined and compared with an α-PD-1 as ICB. Notably neither α-PD-1 monotherapy nor AuNPs + α-PD-1 combination could elicit a significant tumor growth inhibition suggesting the weak "cold" response to α-PD-1 immune checkpoint blockade therapy. Those experiments showed the prominent antitumor role of RT in these types of cancer as revealed by the increment of mature DCs in LNs as well as by the increased levels of $CD8^+$ T cells found (in both LNs and tumors) in the mice subjected to RT [216].

5.6. Chemodynamic therapy

Chemodynamic therapy (CDT) is a recently developed therapeutic approach in cancer which take advantage of Fenton reactions for the generation of highly deleterious reactive oxygen species. Usually, transition metals like Fe, Co, Mn, Ni and Cu can take part in this reaction producing ROS from H_2O_2. For instance, following the oxidation of Fe triggered by cellular H_2O_2, highly reactive hydroxyl radicals are produced, leading to impairment of lipidic metabolism and DNA biosynthesis. Despite being in an initial stage of development, approaches based in CDT have garnered increased attention since the underlying mechanisms are light and O_2 independent thereby avoiding issues related to stimuli penetration depth [219].

The use of metallic NPs has been shown suitable for providing the necessary substrates for Fenton reaction, accelerating and enhancing the ROS generation, constituting a promising anticancer strategy minimizing systemic toxicity, as recently revealed using pegylated FePt-NPs IONPs loaded in ZIF-8 MOFs and Cu based NPs [220-223].

Going further into immunomodulatory mechanisms, He and collaborators prepared a nanosystem for enhanced CDT based on polyethyleneimine (PEI) and copper ions nanocomplexes ($PLNP^{Cu}$). The NPs were loaded with lactate oxidase, an enzyme responsible to metabolize lactate. This metabolic process yields H_2O_2 as subproduct, providing a substrate for enhancing Cu-mediated Fenton reaction. As consequence, the proposed nanosystem was able to promote ROS-induced ICD leading to an 88% tumor growth inhibition in a mice model of breast cancer. Weather the antitumor effect was promoted by the activation of the immune response was further elucidated evaluating the cytotoxic immune profile of tumors and draining LNs. Authors found that mice treated with $PLNP^{Cu}$ displayed higher levels of cytotoxic $CD8^+$ T cells and interferon-γ in tumors. In addition, M1 polarized macrophages were also increased while Treg cells were found decreased in tumor tissues. Regarding systemic response, the analysis of the spleens revealed an increment of $CD8^+$ T cells as well as a shift to mature $CD80^+$ profile in LN DCs [224].

In another recent work, Su et collaborators fabricated a 140 nm manganese-alginate nanogel where the Mn^{2+} ions were responsible for reacting with H_2O_2 to produce OH radicals through a Fenton-like reaction. Indeed, flow cytometry analysis revealed the expression of CRT in tumor cell membranes as ICD indicator. The remarkably high tumor growth inhibition (93.6%) found in groups treated with Mn nanogel was associated to a notable increase of mature DC in LNs as well as an increase in infiltrating

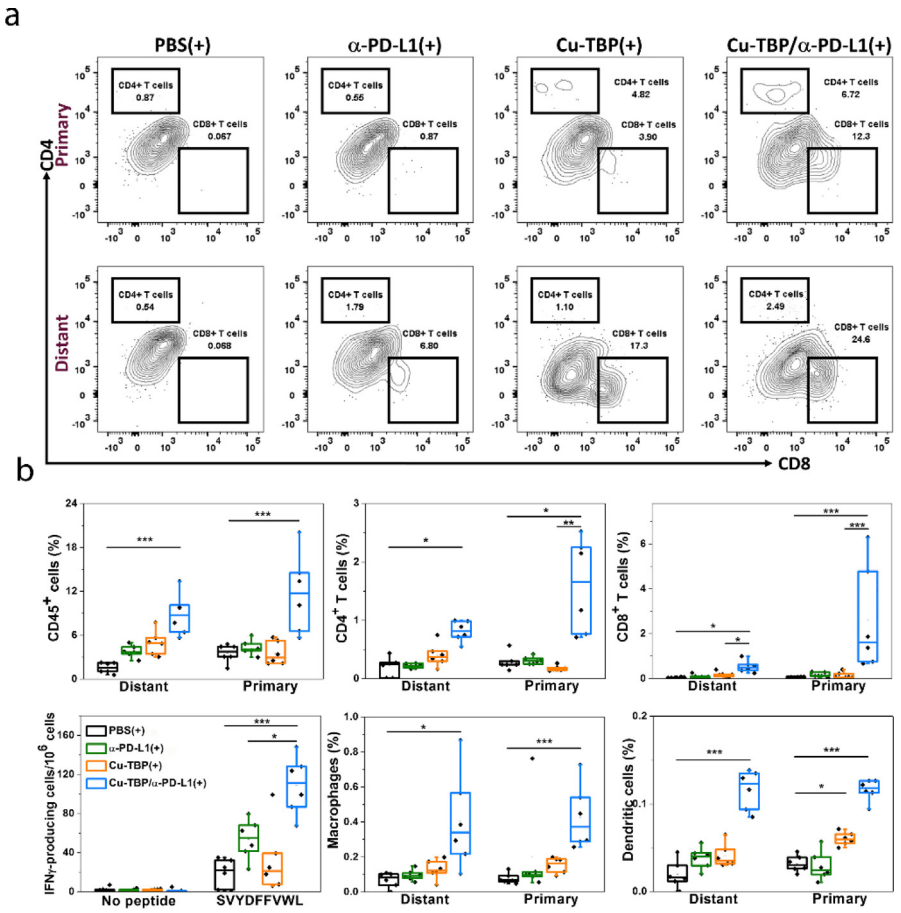

Fig. 23. Example of the use of inorganic NP in a synergistic approach between MOF-assisted chemodynamic therapy, light-driven PDT and ICB in a melanoma mouse model. a) Flow cytometry analysis of specific immune effect elicited after synergic approach. Data showing the increase in both primary and distant tumors of $CD4^+$ and $CD8^+$ T cell populations. b) Increase in DC and macrophages in both tumors as well as an increase in IFN-γ producing T cell levels in splenocytes [229].

$CD8^+$ T cells in tumors. Moreover, no systemic toxicity was found, which was in agreement with the progressive tumor accumulation of Mn-nanogel as revealed by T_1-weighted MRi due to Mn-derived contrast [225].

5.7. Synergic approaches

The implementation of cancer immunotherapies has constituted a revolution regarding the improvement of life expectancy in cancer patients, allowing to educate the own immune system for long-lasting tumor elimination. However, these therapies have an elevated cost, requires a complex manufacturing procedure, and suffers from immunological side effects as well as inefficacy in solid tumors (as it's the case of CAR-T cells). Furthermore, in "cold" tumors exhibit an immune suppressive microenvironment, diminishing T cell function, immunotherapies failed to effectively induce cell death in tumors of a significant subset of patients [88]. Given that, nanoengineered materials has emerged providing a new landscape where the combination of classical monotherapies with novel therapeutic modalities cooperates in an innovative and efficient strategy. NP-mediated therapies as PTT, PDT, RD, CDT *etc.*, allowed more precise, personalized, and effective approaches combining for example the benefits derived from the NP-induced ICD with the already proved efficacy of ICB as well as the advantages provided by the inorganic nature of NPs for imaging and monitoring the therapeutic performance [226]. Moreover, since the combination of therapies usually implies a mixture of different agents with different pharmacokinetics and biodistribution, NPs constitute a platform where the inherent properties of the materials are displayed in conjunction with the molecules of interest in the same structure providing a synergistic therapeutic effect [227]. A good example of that is the work of Chiang and colleagues using IONPs for coupling multiple antibodies; an α-PD-L1 antibody and two T cell activators, α-CD3 and α-CD28. The resultant nanostructure of 140 nm was comprised of a hollow core with an outer shell with 5 nm IONPs embedded. Authors were able to enhance the targeting of breast and colon cancer tumors under magnetic guidance. As a result, checkpoint inhibition and T cell proliferation was achieved simultaneously as revealed by the increase of infiltrating $CD4^+$ and $CD8^+$ T cells together with the subsequent change in the $CD8^+$ T cells/Treg ratio as well as the increase of specific IFN-γ^+CD44^+ T cells in tumors. This approach proved the efficacy of a nanoplatform for combine multiple therapeutic features with further benefits from its superparamagnetic nature [228].

Ni et al. compared the efficacy of a α-PD-L1 treatment with and without synergic chemo-and photodynamic therapy in bilateral melanoma mice model using Cu-TBP (TBP = 5,10,15,20-tetraben zoatoporphyrin) MOFs (Fig. 23) [229]. Cu^{2+} ions released from the MOF structure catalyzed the generation of OH and O_2^- radicals whereas the photosensitizer H_4TBP converted oxygen to cytotoxic $^1O_2$ after 650 nm laser irradiation effectively generating ICD. As a result, combination of CDT, PTT and sequential ICB extend the treated mice survival time to 31 days which is significant more than the 12 days achieved with the ICB. Indeed, immunological evaluation revealed increased levels of IFN-γ^+ T cells in spleens of tumor-bearing mice treated with Cu-TBP MOFs and a-PD-L1. In addition,

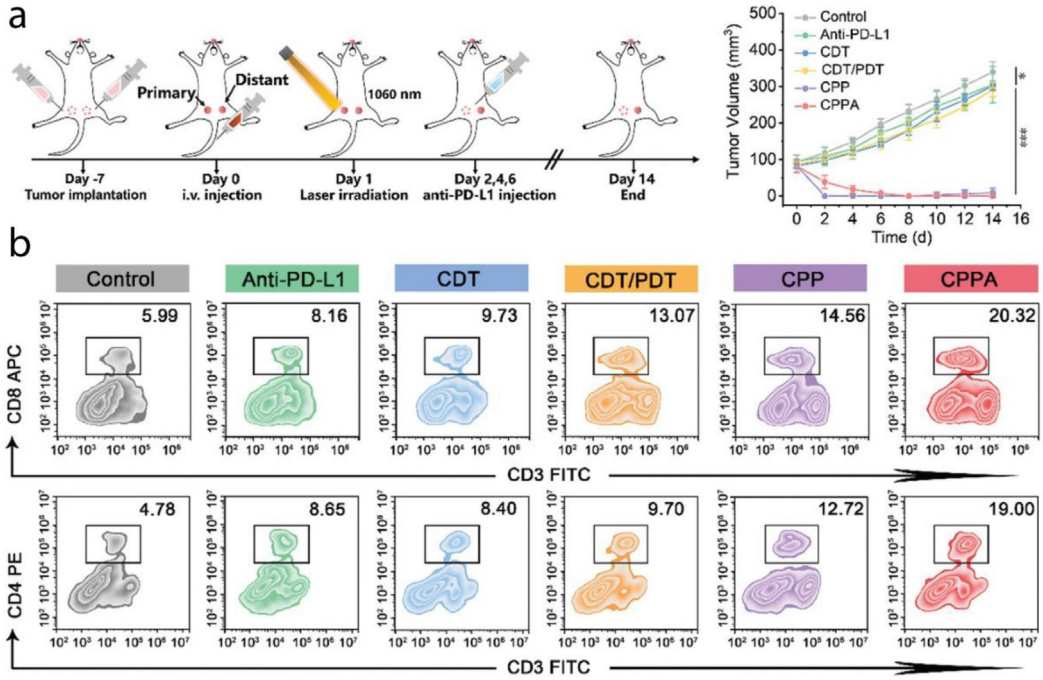

Fig. 24. Example of the use of inorganic NP in a synergistic approach between Near-Infrared II NP-driven CDT, PDT and PTT combined with ICB to mediate antitumor imune response. a) Schematic illustration of the FWO-PEG treatment for primary tumor on female BALB/c-nude mice. IR thermal images with temperature changes at tumor regions after combinatorial treatments which led to an noticeable reduction in tumor volume reaching the complet tumor removal. b) Immune effect of combninatorial treatment. APC and $CD4^+$ T cells levels were increased in distant tumors after subjecting mice to the combination of the four treatments leading to a significant reduction in distant tumor volume [231].

analysis of primary and distant tumors showed a remarkable elevation in the percentage of $CD4^+$ (6.7%) and $CD8^+$ T cells (12.3%) after synergic therapy compared with α-PD-L1 treatment alone in primary and in distant tumors. Results proved the strong tumor-specific T cell response induced by the synergic treatment in comparison with ICB monotherapy.

The synergistic effect of inorganic NP-driven ICD and PD-L1 blockade to enhance antitumor immunity has also been proved recently by Xiang et al., in an innovative approach combining CDT, PDT and PTT using pegylated iron tungsten oxide nanosheets (FeWO-NS) in a mice model of breast cancer. On the one hand FeWO-NS trigger ROS production through the generation of ·OH by Fenton reactions catalyzed by Fe ions. Then, FeWO-NS can efficiently absorb NIR light in the NIR-II window (1060 nm), providing a local temperature increase of 48.2 °C, which is optimal for PTT [230]. Furthermore, due to this remarkably NIR absorption, FeWO-NS can participate in PDT mediating the conversion of O_2 into ROS. On the other hand, the Fe and W components allowed the signal enhancement in MRi and CT, which in conjunction with NIR-derived photoacoustic (PA) imaging, provides multimodal imaging for a non-invasive follow up of the tumor evolution (Fig. 24). The synergic approach proved to elicit a strongest antitumor response than ICB alone as demonstrated by the increased levels of infiltrating CD4 + and $CD8^+$ T cells as well as by the production of immunostimulatory IL-6, IL-12, TNF-α and IFN-γ [231].

6. Relevance and differential effects of route of administration

6.1. The application defines the route

NP-mediated targeted drug delivery research has evolved exponentially in the last decades, however, despite all the progress, their implementation has suffered from several drawback during translation to the clinics and currently just a minority of the nano compounds tested in trials have reached the clinical use. The aforementioned changes that NP suffer in contact with biological fluids once inoculated in addition to the numerous physiological barriers and environment present in tissues and blood limit their efficacy. For example, the blood brain barrier for delivery to the central nervous system, extreme acidity and enzymatic activity in the gastrointestinal tract after oral delivery, the low pH exhibited in the TME for cancer nanotherapeutics or mucus and pulmonary surfactants constitute some of the main factors that can affect delivery [232,233]. In addition, NPs distribute in tissues in a size-dependent manner, with the liver and spleen exhibiting the highest degree of accumulation due to the aforementioned MPS retention when injected IV The rational design of NPs has allowed researchers to target challenging locations by modifying NP features *ad hoc*. Specific targeting could be achieved through the conjugation of different ligands for specific tissue receptors and the NP size, shape and surface charge and hydrophobicity could be addressed accordingly to the desired application. These features will be then affected differentially as function of the exposure to different body microenvironments therefore modifying their biodistribution. Given that, it seems critical to analyze the implications of the administration route on the biotransformations suffered by the NPs in the body, therefore the efficacy and limitations of various administration strategies [234].

Despite extensive work characterizing the NP physicochemical properties, primarily size, charge, and surface modifications, a lack in comprehensive and systematic studies evaluating the impact and influence of nanomaterials upon *in vivo* administration remains. Differential administration routes, including intraperitoneal (IP), intradermal (ID), intrathecal (IT), intranasal (IN), intravenous (IV), intra-arterial (IA) injection, and oral, will imply differential interactions with and the body fluids and immune system, critically influencing pharmacokinetic and eventually influ-

ence both the tissular fate and the main immune cell subsets exposed to NPs, as well as the organs primarily exposed, considering if NPs will drain to the lymph or leak to the bloodstream.

Dogra et al. compared SiNPs with the same structure and composition but varying in their size from 25, 50 and 150 nm during IV and IP administration. Results showed IP administrated SiNPs being accumulated in abdominal and thoracic LNs at short periods of time regardless of their size. Interestingly, SiNPs appeared to be preferentially pooled in a size dependent manner over time, being founded mainly in axillary LNs and spleen except for the smallest (25 nm) which ended up in inguinal LNs. IV administration showed a completely different biodistribution with smaller NPs remaining in circulation at shorter periods of time whereas 150 nm SiNPs appeared accumulated in liver and spleen just from the outset. In all cases blood half-life decreased as function of size and despite being quite similar for those of 25 and 50 nm, SiNPs of 150 nm injected IP remained in circulation almost 2 more hours (4.97 vs 3.19 h) than the equivalent nanoformulations administrated IV [235].

The administration of therapeutics in the tumor tissue has been vastly employed since the advantages obtained from direct injection of NPs in the target regions. SiNPs of 340 nm with a zeta potential of −30 mV loaded with a TLR7 agonist were used by Seth et al. in a mice model of melanoma subjected to PTT. These SiNPs elicit a 2-fold increase in $CD8^+$ T cells of as well as a 3.1-fold increase of mature DCs compared to control (PBS) group. These results were in concordance to those found by Lin et al. in the same mice model using SiNPs of 144 nm and + 23 mV loaded with IL-12 encoding plasmid. In this case authors showed a 2.4-fold increase in $CD8^+$ T cells and 3-fold increase in mature DCs after PTT [151]. Interestingly, a recent study of Zhang et al. showed a ∼ 10-fold increase in $CD8^+$ T cells using SiNPs of 120 nm and −25 mV conjugated with α-CD47 and a pro-phagocytic molecule. These results emphasized the role of macrophages in TME. Reversing the "don't eat me" message from the tumor cells and therefore increasing the antigen presentation by APCs elicit a remarkably T cell response in comparation with the previous approaches even with no external stimuli like PTT [236].

The recent impact of mRNA vaccines in our daily life has shown the relevance of the nanomaterial-based vaccination. In the last decades vaccines evolved exponentially, using the technology from advanced drug delivery strategies, and stablishing new solid evidence for the use of subunit antigens (proteins, peptides, or nucleic acids) as immunomodulator agent. Nanomaterial-based vaccines have been proposed as a promising approach improving vaccine stability, blood half-life and therefore safety and tolerability [237]. Despite the massive employment of lipid-based and polymeric NPs as vaccine vehicle, inorganic NPs have also shown promising results in this field. Unlike lipid-based, inorganic core structures are less prone to degrade due to temperature/storage conditions. Formulation designed for vaccination approaches should be able to elicit a robust both cellular responses and humoral responses, thus, reaching SLOs is a must for effective response triggering, also avoiding the systemic circulation that may cause off-target effects, like systemic inflammation, leading to insufficient lymphatic and spleen accumulation and therefore to an eventual immune unresponsiveness [238].

Most vaccines, either from classical formulations or NP-based, has been administrated through IM injections since it constituted a feasible, well tolerated, and non-risky administration procedure. However, in recent years SC-ID administration have garnered attention due to the more efficient targeting and activation of APCs in the injection area compared with IM administration [239]. Recent studies in rhesus macaques using glycoprotein vaccines, showed a differential fate of the vaccine after SC or IM administration. IM or SC injection lead to different tissular targeting. SC administration elicited the accumulation of antigen in LN close to the injection site while IM injected was preferentially found in distal LNs. However, despite those anatomic differences, several clinical trials have revealed no significant differences between SC and IM vaccination in eliciting effective immune responses which is consistent with the findings within this study regarding IgG avidity, IgA titers, memory B cells in blood, and $CD4^+$ T cells in circulation [240].

IM and SC administration relies in lymphatic drainage of APCs after antigen uptake for both, amplifying immune response as well as generating immunological memory. Antigens administrated SC and IM will persist longer at the injection site and drain to the lymphatic system more efficiently compared with those administrated IV. Despite of that, SC immunization elicits $CD8^+$ T cell activation, both systemically and in SLOs appear significantly higher in comparison with IV injections. Interestingly, it has been reported a connection between IV administration and $CD8^+$ T cell differentiation towards a TCF-1^+ stem cell-like phenotype providing a pool of self-renewing cells to react against a new infection/antigen exposure [70]. Such studies, emphasize the impact of the administration route not only in the magnitude of the immune response, but also in the differentiation profile of the cellular response. A direct comparison between studies is not always completely feasible since the eventual fate and function of a NP upon administration *in vivo* is going to depend on a variety of factors. Size, surface modifications and coupled/loaded immunoligands are going to affect NP biodistribution and pharmacokinetics.

Despite the lack of uniformity in studies as such, there are good examples of a comprehensive comparison of the differential effect of the administration route of inorganic NPs. Using AuNPs coupled to immunostimulatory nucleic acids, authors compared SC and IV administrations reporting a differential effect. Intradermal administrated nanoconjugates elicited a remarkably increase in proinflammatory cytokines (IL-12 and TNF-α) in draining LNs, with no observable effect on serum cytokine levels. Interestingly, IV administration led to a significant increase of IL-12 levels in serum at the same time points, providing evidence of the impact of IV administrated nanoconjugates in systemic immune activation, which could be attribute to the higher levels of activated DCs in spleen after IV administration of nanoconjugates [241].

Nanovaccines based on hybrid NPs of 150–200 nm SiNPs encapsulated in liposomes and loaded with two melanoma-derived antigenic peptides and a TLR4 inducer as adjuvant injected SC induced a ∼ 2-fold increase in total $CD8^+$ T cells compared with soluble vaccination without SiNPs [17]**.** In a similar approach authors employed SiNPs of 77 nm and 15 mV, loaded with OVA peptide and CpG oligonucleotides (TLR9 agonist) in mice thymoma model. In this case the antigen specific T cell found in peripheral blood was 10 times higher than the OVA-CPG injected control [242]. In addition, the percentage of $CD11c^+$ DCs was found similar (26% vs 28% respectively) in both studies. These results were different from what would be expected based on NPs charge. Negative or neutral NPs are termed to be more efficient for SC route since positive NPs are more prone to stablish interactions with extracellular matrix components remained constricted to injection site and therefore decreasing the antigen presentation to immune cells in LNs. Altogether these studies emphasized the specific role of adjuvants and antigen functionalities in NPs, which could determine the vaccination efficacy beyond the assumptions based on their physicochemical properties.

IV administration has also been explored using inorganic NPs for generating effective T cell response. This is the case of SiNPs of 87 nm and 21 mV loaded with CpG oligonucleotides as adjuvant and a tumor neoantigen in a colon cancer mice model subjected to PDT [243]. The analysis of systemic immune response revealed a 23.5 and 85-fold increase of specific $CD8^+$ T cells for the tumor

antigen loaded in the SiNPs after priming and boosting IV injections respectively. Regarding tumor analysis, more than 20-fold increase in specific $CD8^+$ T cells was found in tumor tissue and in all cases the combination of SiNPs vaccine with PDT remarkably enhanced the immune response. When tested in a more aggressive tumor as a B16/F10 bilateral melanoma mice model, SiNPs elicit a ~ 3.5-fold increase in mature DCs and approximately 10 to 20-fold increase in $CD8^+$ cells in the tumor. In all the cases PDT treatment improved the vaccination immune response suggesting that the synergy between the PDT-induced ICD with the SiNPs for antigen presentation (~1.75-fold increase in DCs level due to PDT) contributed to the enhancement of T cell response, both locally and systemically.

In a recent study, Wang et al conducted a study comparing three different administration strategies (IV, IP and IT) for alleviating respiratory inflammation. Authors used AuNPs of 13 nm coated with a hexapeptide able to inhibit TLR signaling pathways and repolarize macrophages to M2 phenotype. At higher doses, AuNPs@hpep had a similar behavior in the alleviation of inflammatory symptomatology regardless of the administration route. Interestingly, when the dose was reduced, only IT administration proved to have a therapeutic effect. Authors found that IV and IP administration lead to a predominant accumulation in liver and spleen while IT administration significantly increase the targeting of lung macrophages enhancing the bioavailability of the AuNPs in the lungs and therefore improving their therapeutic effect [244]. These conclusions evinced the need of adapting the administration route for the demanded outcome. IV and IP administration could easily lead to an accumulation of the NPs in LNs and spleen and therefore constituting an efficient route for activate immune cells in the main lymphoid tissues. On the other hand, local administration of NPs could be a more effective approach in that cases that allows a direct NP administration in the tissue of interest.

In addition to systemic (IV) and canonical (SC, IM) administration methods, local administration of nanoconjugates in tumors has also been explored. The fate of intravenously administered IONPs during MRi and iron replacement therapies is well understood, they are rapidly taken up by Kupffer cells in the liver and other cells of the mononuclear phagocyte system like the spleen, after which they are metabolized and regulated by normal physiological iron homeostatic mechanisms. In case of local administration, and even though constituting a direct way for targeting tumors, there has been proposed that after intratumoral administration of ferumoxytol in a mice model of glioma, the 90% of the injected materials are recognized and phagocytized by tumor-associated microglia and macrophages which lead to the eventual accumulation in the liver at days 14 and 21 after administration [245]. In addition, cationic NPs are termed to adhere to the negatively charged ECM, hindering tumor penetration.

In the case of breast cancer patients, it has been shown that locally injected IONPs are rapidly accumulated in sinuses and the subcapsular space of sentinel LNs. Researchers have evaluated the impact of locally administrated NPs in the first events during the triggering of immune response. Authors proposed a mechanical transport pathway where NPs are primary transported during the first hours from the tumor to the draining LNs by the regular mechanical drainage instead through dendritic cell-mediated pathways. The size and properties of particles can determine the rate at which they transit by mechanical means to LNs, ranging from seconds to a day, with optimal transport via lymphatics achieved in particles between 20 and 60 nm in diameter. In addition, authors evaluated the long-term impact of the local administration in the functionality of immune cells. Results revealed no long-term changes neither in macrophage phenotype nor in macrophages functionality [246]. There is still controversy regarding the mechanism of antigen trafficking to LN after SC injection. It has been proposed that the direct draining of antigens to LN observed in most of the studies could be attributed to the experimental design itself [247]. Injecting a high volume of sample intradermally in a constrained space could led to a hydrostatic pressure-driven forced transport of antigens into the lymphatic system. The same authors proposed a completely different mechanism depending on DCs for active antigen transport to LNs under injections performed IP or SC (but in areas less compacted than footpads).

Many infections (*i.e.*, HIV, SARS-CoV-2, influenza, rotavirus, and cholera) are triggered by pathogens that colonize and invade the host at mucosal sites since there are the places more directedly exposed to the environment. The immune system responds to this threat though the secretion of IgA (SIgA) at the site of infection. IgA-mediated response is the main humoral defense at mucosal tissue sites for achieving a immune removal of pathogen and toxins in the onset of the infection and plays a critical role providing protection through mechanisms such as immune exclusion, inhibition of transcytosis, and direct neutralization of pathogens [248]. Immunization strategies should provide both systemic antibody responses and humoral immunity at mucosal sites, combining the production of IgG and IgA antibodies, for acute and long-lasting response against the mucosal infections [230]. Many current vaccines used in clinics are administrated fail to induce strong pathogen-specific mucosal immunity at the infection site. Parenteral vaccines have historically exhibited poor immunogenicity and short-lived responses when applied to mucosal barriers, due, in large part, to challenges of delivery and poor uptake. Delivery of vaccine components across mucosal barriers has been a major challenge for mucosal vaccine development. Vaccine uptake into the underlying mucosal immune compartment is impeded by multiple factors, including potential rapid antigen loss due to degradation by proteolytic enzymes and acidic conditions at mucosal surfaces, high rates of mucociliary clearance, and the lack of diffusive uptake across the tight junctions of the epithelial monolayer.

6.2. Oral administration

In opposition to the previously mentioned strategies, researchers have explored less invasive approaches as could be oral and nasal administration. Oral vaccine formulations could potentially avoid the cold chain for storage, which would benefit their use in the developing world. These strategies are termed to elicit local and systemic immune response minimizing off-target side effects, but as counterpart, they need to overcome the biological barriers constituted by the acidic stomach pH (1–3), the mucus produced by the goblet cells in gastrointestinal tract, as well as the presence of a broad range of degradative enzymes. Moreover, these conditions can undergo variations under pathology state, differing from the regular physiology and therefore adding extra complexity to NP delivery [249].

Most inorganic NPs are unstable at acidic pH limiting their gastrointestinal absorption, thus there is the need of tailoring their size, surface charge or hydrophobicity for increasing the efficiency of epithelial uptake. However, SiNPs usually remains stable at low pH protecting their cargos from premature degradation. It has been proposed that small anionic SiNPs can enhance intestinal permeability through the interactions between the negatively charged SiNPs and the integrins of intestinal epithelium [250]. Strategies are focused on reinforcing NP colloidal stability with protective functionalization as it is the case of polymeric coatings with polyanionic polymers [251]. AuNPs of 40 nm coated with chitosan has been proved as effective nanoplatform for oral-mediated induction of systemic and local immune response against tetanus toxoid. CsAuNPs protected antigens against gastric degradation

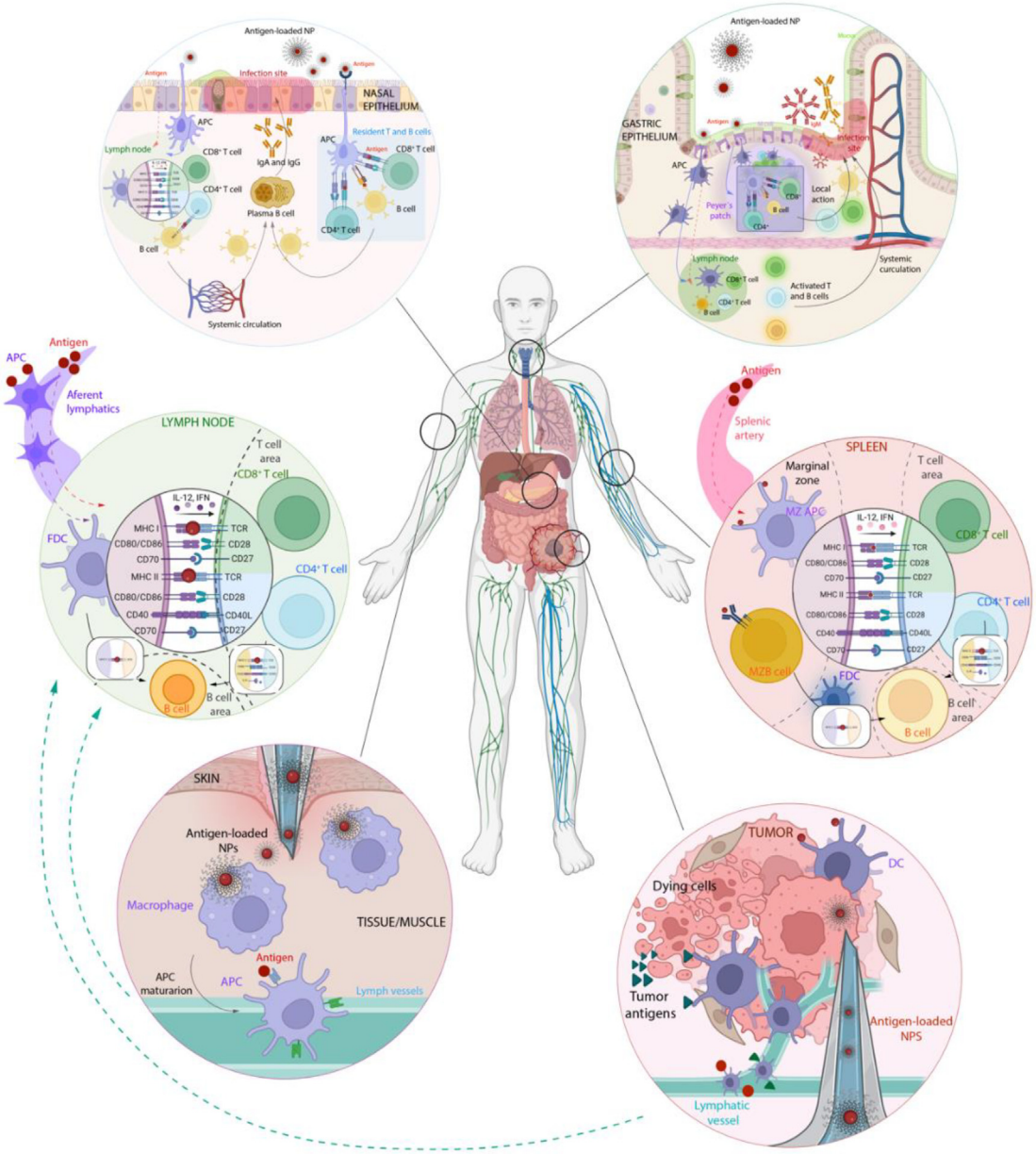

Fig. 25. Immune effects of NPs following differential administration routes. After IN administration, NPs are taken up by APC (exceptionally could passively diffuse to LN) which process the antigens and can be translocated to the proximal LN for present the antigen to $CD8^+$, $CD4^+$ T cells and B cells. Then activated immune cells are concentrated in the infection site at the nasal epithelium where they carried out cytotoxic activity and IgA secretion respectively. Additionally, APCs could present antigen directly to tissue resident T cells eliciting a fast local response. After oral administration, NPs should face the limiting environment caused by the acidic pH and the mucus present in int gastric epithelium. APCs internalizes the NPs, often trough a specific cell called M cells. Once processed, antigen presentation could take place in mesenteric lymph node or directly in in the Peyeŕs patch where CD4, CD8 and B cells are activated triggering IgA and IgM local production in the infection site. When administrate IV, NPs reach the spleen via the splenic artery. Then the APCs in the marginal zone internalize NPs and process the antigens. APCs migrate to the T cell area where they present antigen to $CD4^+$ and $CD8^+$ T cells. In addition, B cells from the marginal zone could also take the antigens and present them to follicular DCs. Eventually, CD4 + and follicular DC present the antigens in the B cell region to immature B cells triggering the immune response. IM/SC administration constitutes the canonical immunization strategy. NPs are injected under the skin or in muscular tissues where macrophages take up them. Then macrophages become mature APC which migrate to LN via the afferent lymphatic vessels. Occasionally antigens can also diffuse through those vessels and reach the LNs. There, FDCs present the antigen to B and T cells in their respective regions, activating $CD8^+$ and $CD4^+$ T cells through the signaling of MHC I and II over T cell receptors. IT administration has garnered attention as a straightforward approach for cancer treatment where NP are processed similarly than in the previous case. In addition, this strategy cools enhance antitumor immune response by triggering ICD and providing new antigens for DCs which is termed to trigger the activations of cytotoxic infiltrating T cells in the tumor.

and promoted systemic and local immune responses compared with control vaccines which reinforce the need for establishing feedbacks between innate and adaptive immunity [252]. Further functionalization with ligands for enterocytes receptors such as vitamin B12 or nenonatal Fc receptors (FcRn), have been proved for an effective intestinal targeting enhancing antigen uptake and transepithelial trafficking [253]. FcRn expressed by mucosal epithelial cells has received attention as a "mucosal gateway" for improving drug uptake across the mucosal epithelium in nasopharyngeal, pulmonary, and gastrointestinal tissues [254]. Special interest for vaccine development is focused on the microfold (M) cells from the Peyeŕs patches which are responsible of processing the infectious antigens. Those cells internalize the viral or bacterial antigens through endocytosis making them accessible to APCs for

triggering the immune response. Mesoporous carbon NPs of 470 nm loaded with BSA as antigen were proved to increase the mucosal retention time and more efficient M cell uptake, resulting in the enhanced cellular and mucosal immune responses via Th1 and Th2 mediated pathways following oral immunization [255].

6.3. Nasal administration

IN has gathered enormous attention as an emergency need for COVID-19 pandemic as it is emphasized by the current 12 nasal vaccines involved in clinical trials [256]. From a practical point of view, this strategy implies a lesser invasive administration compared with IM vaccines, which can be an important advantage regarding mass vaccination during the infancy. Moreover, this route is not dependent of systemic circulation, which allows the avoidance of hepatic first-pass metabolism where most of the inoculated NPs are retained in the liver.

Nasal immunization is expected to elicit mucosal immunity, through the production of IgA immunoglobulins in respiratory tract but also contributing to systemic immunity, preventing rapid acute infection and transmission, and providing long lasting protection. Nasal vaccination led to the elevation of IgA levels in both serum and respiratory fluids, whereas classical intramuscular vaccines mainly induce the production of serum IgG [257]. Focusing on respiratory diseases, infection takes place initially in the upper respiratory mucosa, which is not easily accessible for IgG generated during classical IM vaccination. On the contrary, mucosa resident B Cell and T Cell induction after IN immunization elicit a faster response in the primary sites of infection being crucial for clearing viral or bacterial agents before systemic spreading [63]. In this regard, identifying the cells involved in the first steps of antibody response generation will significantly influence vaccination strategy for eliciting an effective immune response. It has been recently proposed that SC antigen transport is mediated by cDC2s DC subset while IN involved both both, cDC1s and cDC2s in antigen processing. In both cases (SC and IN) migratory DCs instead of resident DCs are the responsible for antigen presentation, but interestingly since cDC2 subset are implicated in Th cell priming for B cell activation and cDC1 are directedly associated to T cell activation, SC or IN nanoimmunization is termed to trigger differential immune response [247].

Nevertheless, effective vaccination strategies need not be restricted to a single route. Indeed, IN boosting after IM vaccine in mice have been proved to generate higher levels of tissue T resident memory cells and B resident memory cells secreting IgA at the respiratory mucosa which are critical against future reinfections. Given that, the best approach could include a combination of IM and IN strategies. Combining the systemic IgG response generated during IM vaccination, and an intranasal booster that recruits memory B and T cells to the nasal passages for mucosal protection, including IgA secretion and tissue-resident memory cells in the respiratory tract.

Intranasal vaccines could be designed displaying the same formulation than IM counterparts or adapting their design for suitable nebulization for example. Even though most of the preclinical studies involved polymeric or lipid-based vaccines, inorganic NPs have also been explored for IN administration. Tazaki et colleagues compared the effect of AuNPs loaded with the RNA analog poly(I:C) as influenza vaccine through IN or SC inoculation [10]. Authors found that among all the NP formulations, Au rods of 30 and 40 nm proved to be the more efficient in reducing virus titer in nasal wash samples regardless of the administration route but interestingly, in addition to the production of IgA in mucosa, IN inoculation also lead to a noticeable production of systemic IgG. This stands in coherence with trends showing that nanorods are internalized into epithelial cells more efficiently than spheres are. In all cases, the smallest Au spheres, and rods, 20 and 30 nm respectively were found to induce the highest degree of immune response. These results emphasized the need for optimization in the shape and size of the nanoconjugate accordingly to the vaccine administration route (Fig. 25).

7. Outlook

NPs have shown extreme potential for biological applications and increased control and knowledge over critical intrinsic parameters influencing biodistribution and pharmacokinetics in preclinical phases may favor their development to clinical trials. In what concerns the triggering of immune responses, the complexity of its mounting and regulation in different diseases must be considered for all therapeutic actions that aim to produce immunomodulation. This means, as it is the objective to highlight in this review, considering different anatomic and physiological aspects in the design of NPs and the following experimental settings, as well as the clear scope of the studies. We have emphasized studies that propose a clear immunological effect based on a rational design of the NPs for a specific outcome, and that such outcome is properly studied and followed. In this way, by combining a proper design with the appropriate administration route, better targeted therapies with enhanced performance, along with effects derived from the intrinsic properties of inorganic-based NPs, such as PDT, PTT, SDT *etc*., will promote the incorporation of inorganic NPs to clinical trials.

Some IV administrated nanomaterials have successfully reached the clinics, however, attending to the immune cycles and processes, other routes have been recently explored in trials studying specific immunological outcomes [258]. This is the case of a recent work involved six patients suffering from glioblastoma at different rates of malignancy recurrence [259], in which they were subjected to thermotherapy for magnetic hyperthermia generation after intra-tumoral application after tumor resection, promoting CTL and macrophage infiltration, and a Th1 polarized response. Thus, the combination of the appropriate material with the most suitable administration strategy is expected to precisely induce immunomodulation as well as provide novel synergic approaches with clinically stablished treatments like chemotherapy and immunotherapy.

While most of the shown examples and included clinical trials involving inorganic NPs are administered IV or locally, we foresee that, considering such multidisciplinary knowledge and incorporating the study of relevant immune parameters will reflect on the number of inorganic NP-based immunotherapeutics.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the financial support of the European Research Council (starting grant #950421), the European Union (INTERREG V-A Spain–Portugal #0624_2IQBIONEURO_6_E, NextGeneration EU/PRTR and ERDF; H2020-FET-Open grant agreement No. 899612), the MCIN/AEI (PID2020-119206RB-I00, PID2020-119479RA-I00, PID2019-111218RB-I00, RYC-2017-

23457, RYC-2019-028238-I and RYC2021-034576-I), and the Xunta de Galicia (ED431F 2021/02, 2021-CP090, ED431C 2022/018, and Centro Singular De Investigación de Galicia Accreditation 2019–2022 #ED431G 2019/03). This project was also supported by the ISCIII, under the framework of EuroNanoMed III_2020 (AC20/00041, PLATMED).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.addr.2023.114829.

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