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RESEARCH: Review

RNAi nanomaterials targeting immune cells as an anti-tumor therapy: the missing link in cancer treatment?

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siRNA delivery targeting tumor cells and cancer-associated immune cells has been gaining momentum in the last few years. A combinatorial approach for silencing crucial factors essential for tumor progression in cancer-associated immune cells and in cancer cells simultaneously can effectively shift the tumor microenvironment from pro-oncogenic to anti-tumoral. Gene-therapy using RNAi nanomaterials can help shift this balance; however, fully utilizing the potential of RNAi relies on effective and specific delivery. RNAi nanomaterials can act as a Trojan horse which delivers siRNAs against immunosuppressive factors and reverses the regulatory activity of tumor immune cells residing in the tumor microenvironment. Here we review potential RNAi targets, means to activate and control the immune response, as well as ways to design delivery nanovehicles for successful RNAi immunotherapy.

Introduction

The archetype for cancer treatment is slowly changing from relatively nonspecific cytotoxic agents to selective mechanism-based therapeutics. The combination of immune-targeted gene silencing and other cancer therapeutics represents an untapped opportunity in cancer therapy and requires a deeper understanding of specific tumor mechanisms.

Tumor cells induce the infiltration of other cell types and instruct them (fibroblasts, endothelial cells and immune cells) in a cell-contact dependent (paracrine, receptor-mediated) as well as contact independent manner (endocrine, cytokines and other signaling molecules) to establish a self-promoting and mutually self-reinforcing tumor microenvironment (TME) that promotes tumor progression [1]. Tumor associated immune cells are major contributors to the TME as well as tumor growth and development, and their levels can be correlated to patient prognosis [2]. Modulation of this microenvironment represents the key for controlling tumor growth, as well as the development of metastasis. The enormous potential of targeting the immune system for improved cancer therapies was recognized as the 'Science breakthrough of the Year 2013' [3].

Several approaches to target tumor immunity are being explored, including (1) cancer vaccines [4,5], (2) immune cell checkpoint inhibitors and (3) specific immune cell depletion [6]. Cancer immunotherapy can be employed as a single therapy or in combination with therapeutics directly targeting tumor cells [7–9]. Targeting the immune system for anti-tumor responses has several advantages over therapies targeting tumor cells alone and especially over broad chemotherapeutic agents. In contrast to chemotherapeutics with dose-limiting toxicities and potential drug resistance in patients, re-programming cancer-associated immune cells to combat tumorigenesis is highly specific and able to induce a long lasting memory response [10].

RNAi technology, such as short interfering RNA (siRNA), has already been shown to modulate specific gene expression in cancer cells with subsequent tumor regression. Therefore, we believe that this technology should be extended to target immune cells, individually or as a combination therapy [11]. Despite their high potential, using naked siRNA molecules presents several

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challenges, such as extremely short half-lives (i.e. minutes), weak protection against nucleases poor chemical stability, and dissociation from the vectors used [12]. Therefore it is imperative to pursue appropriate design and construction of nanoparticles for safe and efficient siRNA delivery.

Nanotechnology offers versatile, targeted delivery platforms for RNAi therapeutics [13–16]. In the last decade the use of inorganic (quantum dots, gold, silica and magnetic nanoparticles) and organic nanoparticles (liposomes, lipids, dendrimers, micelles) as siRNAs delivery agents has been extensively described [17–23]. Therefore, using RNAi in conjunction with nanomaterials is a valuable tool to target immune cells for cancer treatments (Fig. 1). Despite its therapeutic potential, the application of RNAi to orchestrate immune responses has so far been overlooked, but likely represents a highly valuable tool to combat tumors and shift the tumor microenvironment from pro-oncogenic to anti-tumoral.

This review article focuses on cancer immunomodulation and therefore only includes studies in which RNAi nanomaterials were used to target cancer associated immune cells or both the tumor and immune cells simultaneously.

RNAi delivery: promises and challenges

RNAi (e.g. siRNA) is a ubiquitous, highly specific, endogenous and evolutionarily conserved mechanism used to modify gene expression and is increasingly being used for therapeutic applications. siRNAs are 21–23 nucleotide (nt), double stranded molecules (dsRNA), with symmetric 2–3 nt 3′ overhangs and 5′-phosphate and 3′-hydroxyl groups, that mediate the cleavage and subsequent degradation of complementary mRNA sequences and thus regulate gene expression [24,25]. The RNA silencing pathway begins when long dsRNA precursors are processed to siRNA duplexes by the RNase-III-like enzyme Dicer. These short dsRNAs are subsequently unwound and assembled into an effector complex, called the RNA Induced Silencing Complex (RISC) which can direct RNA cleavage, mediate translational repression or induce chromatin modifications [26,27]. The theoretical approach seems relatively simple; however, several hurdles have to be overcome to successfully introduce functional siRNAs into the target cell. Initially, the siRNA has to be transported to the desired tissue then penetrate a specific cell and finally be released into the cytoplasm.

The use of nanoparticles as carriers for siRNA has expanded dramatically during the last decade, revealing these materials as excellent candidates for gene therapy. Nanoparticles have the potential to replace viral vectors and their inherent disadvantages, such as toxicity and lack of specificity [28]. Extensive reviews about the toxicity of viral vectors can be found elsewhere [29–31].

Nanoparticles (NPs) have the ability to carry the siRNA to the target tissue and induce transfection. The intracellular delivery of nanoparticles remains challenging, however, extensive reviews of the progress made in this field can be found elsewhere [32–34]. Following successful internalization into immune cells, the siRNA can selectively modify immune responses dependent on the therapeutic target, the siRNA payload, or the route of administration [35]. Nanomaterials can be applied either systemically or locally; the route of administration plays a pivotal role in dictating the efficiency of these materials. In order to improve the efficacy of immunotherapies based on RNAi nanomaterials the route of administration must be carefully chosen and optimized (Fig. 2).

Systemic administration

Most therapeutic NPs are administered intravenously, which is generally associated with a strong immune response [11]. Intravenously injected nanomaterials are exposed to an extremely complex microenvironment of blood components immediately upon injection, which determines their biodistribution and ultimately their therapeutic efficacy. This route could potentially be used to

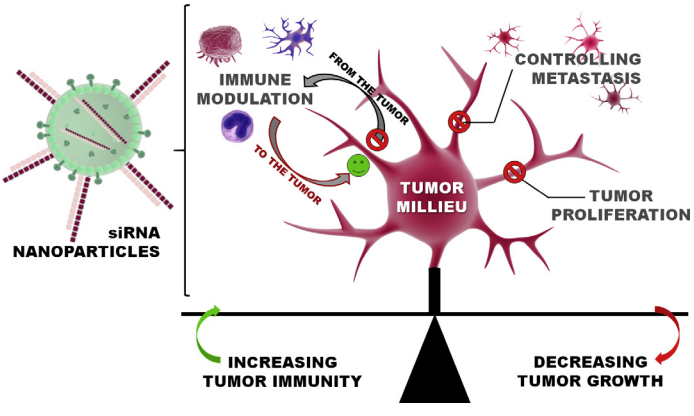

FIGURE 1

Tumors create a heterogeneous environment to promote their progression and suppress tumor immunity. Tumors are associated with infiltrating innate and adaptive immune cells. These cells aid tumor growth by creating an environment that disables immunogenic responses and assists in angiogenesis. Modulation of this microenvironment constitutes the key for controlling tumor immunity and tumor growth, as well as the development of metastasis. The delivery of RNAi nanomaterials is a promising new therapeutic to regulate immune responses and restore tumorigenic mechanisms.

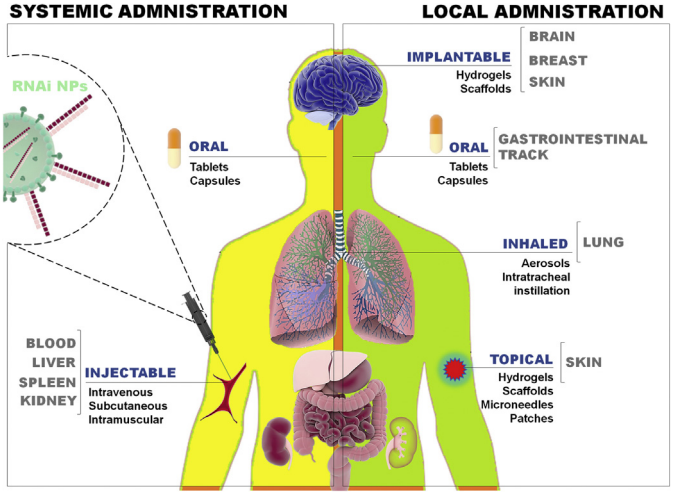

FIGURE 2

Routes of administration and potential target organs for RNAi nanomaterials. RNAi nanoparticles have to be applied in an organ dependent manner to reach the target tissue and consequently interact with the local immune or tumor cells.

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deliver RNAi NPs to cells in the blood stream (e.g. monocytes, NK cells, B and T lymphocytes) thereby directly targeting the immune system, however due to the flow rate in blood the uptake of nanoparticles is challenging. Systemically injected RNAi NPs are rapidly cleared from the blood stream and accumulate in kidney, spleen, liver and lymph nodes, where they are taken up by scavenging dendritic cells and macrophages [16]. This mechanism can be exploited in case of kidney or liver pathologies to target these immune cells and to regulate the local microenvironment. In contrast, the delivery to other organs is more challenging. This difficulty can be overcome by using a local delivery system, reducing cellular barriers, or by developing new ways to exploit nanoparticles' extravasation into specific target sites [36]. Within an intact vasculature the extravasation and diffusion of nanoparticles to target tissues is difficult [37]. This however is an advantage for the delivery to tumors, which present defective (leaky) vasculature and poor lymphatic drainage, resulting in an enhanced permeability and retention (EPR) effect that facilitates the delivery of therapeutic particles to these regions [38,39]. In fact, it has been shown that nanoparticles $\leq$ 400 nm in diameter extravasate through tumor vessels and specifically accumulate at tumor sites [40–42]. Also, tumors with defective blood capillaries allow nanoparticles to passively accumulate. Alternatively, NPs can be actively anchored to specific biomarkers (over-)expressed by tumor cells through targeting moieties [37]. Nanoparticles for *in vivo* siRNA delivery (~100 nm diameter) can be further modified to increase steric stabilization and active targeting via ligands, such as antibodies, peptides and aptamers [43–48]. Despite these strategies, homing of systemically administered nanoparticles to specific target organs followed by tissue extravasation remains challenging [36].

Local administration

Nanoparticles used in conjunction with local delivery system encounter fewer cellular barriers. The local application of nanoparticles at or near the target sites might be the method of choice for the multitude of pathologies. In fact, local delivery can prevent most of the systemic toxicity while simultaneously enabling effective concentration and retention of the therapeutic agent. Continuous release of siRNA into the local cell/tissue microenvironment can be accomplished by formulating siRNA into biocompatible, biodegradable and immunologically inert matrices, like hydrogels [49,50]. These hydrogels can be further engineered to interact with specific tissues and to control the release kinetics and stability of the nanoparticles. Intelligent hydrogel-based matrix designs would enable the programming of nanoparticles' release kinetics to occur in response to extrinsic factors such as light, temperature or pH. This can be combined with the ability to track the release using optical imaging, making these hydrogels ideal candidates for the local delivery of siRNAs [50–52].

Implantable or locally injectable NP-embedded hydrogels can be exploited to deliver siRNAs to target tissues. As an example, the skin is a very attractive organ to deliver engineered RNAi NPs. Nanoparticles can be delivered subcutaneously by intradermal injection [53], epidermal electroporation [54] or via microneedles [55] but also as topical applications using hydrogel scaffolds or patches [50].

Inhalation and ingestion

Nasal and oral routes can be exploited for future RNAi applications in cancer immunotherapy for lung or gastric delivery, although oral administration has a lower bioavailability in contrast to the systemic route. The delivery of NPs via nasal routes can target the mucosa-associated lymphoid tissues and residing macrophages or lymphocytes, with a concomitant passage of immune cells/products to the circulation and other body tissues. In fact, the delivery by intratracheal instillation is very efficient as the therapeutic material is directly delivered to bronchial airways, where they can efficiently target immune cells and tumor cells [22]. This non-surgical technique is simpler than inhalation exposure procedures, permitting the introduction of a wide dose range to the lungs within a short time [56].

Taken together, nanomaterial-based delivery of siRNA provides a strategy for innovative targeting of specific cell populations for therapeutic purposes [57]. In fact, in the last years nanoparticles have been gaining momentum as robust system for the effective delivery of therapeutic siRNA owing to their inherent properties, chemical stability and physical constancy, high purity via reproducible synthetic protocols allowing for adjustable size and morphology control, ease of surface modification for improved siRNA binding and targeted delivery [58]. In the past decade, the use of inorganic and organic nanoparticles as siRNAs delivery agents has been extensively investigated and described especially for novel cancer therapeutics [13,16]. Recent advances in the development of nanomaterials (liposomes and lipid-based [59–61], metallic nanoparticles [22,23], RNA nanoparticles [62–66], and polymers/dendrimers [50,67–69]) for siRNA delivery in cancer therapies enabled the production of highly potent, specific and biocompatible nanoparticle delivery vehicles. These nanomaterials have already shown promising results in tumor targeting and may represent a valuable role in targeting immune cells in the tumor microenvironment. Although such nanomaterials were successfully used for targeting tumor cells, siRNA platforms targeting the immune system for cancer therapy are still under development.

Immune cells and cancer: knocking down barriers in therapy

The immune system is paramount for homeostatic functions, clearance of debris, tissue repair and defense during infections. In particular, immune cells have the unique ability to recognize aberrant/cancerous cells and induce their elimination (Table 1). This task is mainly performed by macrophages, neutrophils and natural killer (NK) cells (innate cells) as a first line of defense and is mediated through their cytotoxic mechanisms [70]. As a second line of defense, macrophages and dendritic cells present tumor-associated antigens (TAAs) to T cells and induce a potent adaptive immune response, a mechanism exploited for cancer vaccines [71]. Despite these powerful immune mechanisms, tumors develop mechanisms to evade the immune system on several levels, such as changing the expression of surface molecules to become invisible to immune cells as well as actively suppressing tumor-directed immunogenic responses to induce immunotolerance, a process referred to as immunoediting [72]. Hence, immune cells associated with tumors are modified from tumoricidal toward

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TABLE 1

Immune cells and their functions for homeostasis and in the tumor environment.

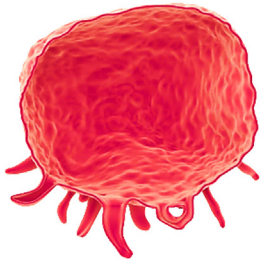
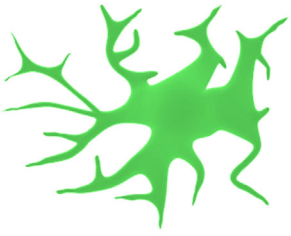
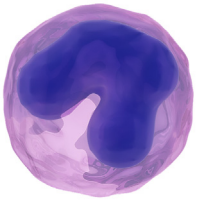
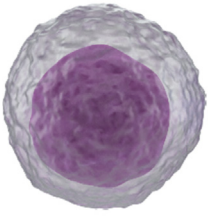
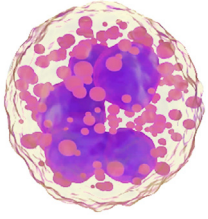
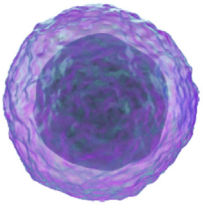
Immune cell		Function in tumor microenvironment	Refs
Macrophages • Important phagocytes • Maintain tissue homeostasis • Distinct activation phenotypes show characteristic functions for bacterial and viral infections (M1), parasitic infections, tissue repair (M2), immune-regulation and suppression (M2-like)		**Tumor associated macrophages (TAMs)** • Similar to M2 phenotype • Suppress anti-tumor immunity via contact dependent (receptor expression) and independent (cytokines) mechanisms • Aid in neoangiogenesis (VEGF, EGF)	[77]
Dendritic cells (DCs) • Most potent antigen-presenting cells (APC) • Phagocytose material, home to lymph nodes and activate CD4 and CD8 T cells to induce adaptive immune response • Determine the type of immune response (Th1, Th2 or T-reg) • Cross-presentation of extracellular antigens to CD8 T cells important for viral infections and tumors		**Tumor associated regulatory Dendritic cells** • Functions silenced by tumor cells • Skewing of T cells toward Th2 and/or IL-10-secreting T cells	[78–80]
Monocytes • Immature circulating precursors of macrophages and DCs		**Myeloid-derived suppressor cell (MDSCs)** • Heterogeneous cell population sharing markers of DC and macrophages • Inhibit T cell proliferation • Show inverse relationship to DC in tumors	[81–83]
CD4+ T lymphocytes • Mediate strong Antigen-dependent immune response • Type of T cell is important for the type of target: Th1 viral and bacterial infection, Th2 parasitic, Th17 bacterial infections • Regulatory T cells (T-reg) limit overzealous responses and collateral damage		**Regulatory T cells (T-regs)** • Suppress tumorigenic Th1 responses • Secrete high levels of IL-10 and TGF-β	[84,85]
Neutrophils • Short-lived phagocytes • First line of defense against microbial infections • Contain high levels of serine proteinases, antimicrobial peptides, ROS		**Tumor-associated neutrophils (TANs)** • Mediate tumor associated inflammation and angiogenesis • Secretion of immunosuppressive cytokines	[86,87]
Natural Killer (NK) cells • Innate cytolytic cells (perforin, granzyme) • Target aberrant cells, MHC1-independent but through alternative receptors, for example, NKG2D • Aid in the induction of T cell mediated immunity through cytokine secretion		**Tumor infiltrating NK cells** • Tumors shed soluble NKG2D ligands to evade NK cell lysis	[88,89]

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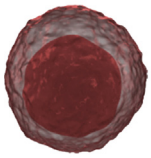
TABLE 1 (*Continued*)

Immune cell		Function in tumor microenvironment	Refs
Cytotoxic T cell (CD8) • Can kill virus infected and cancer cells • MHC-1 dependent		**Tumor-specific cytotoxic T cell** • Insufficient activation by DCs leads to T cell exhaustion	[85,90]

tumorigenic and further implement the immunosuppressive environment through the secretion of cytokines, chemokines and metabolic mediators as well as through cell-contact dependent signaling mechanisms. The role of tumor-associated immune cells and their contribution to tumor progression have been extensively reported elsewhere [6,73–76]. A deeper understanding of the changes of immune cell phenotype and their signaling networks is important in order to identify targets to reset and modulate the immune response.

How to activate an anti-tumoral immune response and sensitize to immune-mediated destruction

The immune system comprises multiple distinct cell types that work in concert to maintain tissue homeostasis and to orchestrate the immune response. Their activation is dependent on local environmental stimuli that trigger specific signaling loops thus determining the type of immune response generated. The activation factors for immune cells for activation of immune cells can be classified into 4 categories; chemokine and cytokines, metabolites, cell surface receptors and intracellular signaling mediators (Fig. 3).

In a simplified point-of-view, the cytokines IL-12, IFNγ, IL-1β and IL-23 are key pro-inflammatory factors that promote tumoricidal immune functions; these are associated with M1-type macrophages, mature DCs, CD4+-Th1 and cytotoxic CD8+ T cells. Activated macrophages and DCs present increased levels of NF-κB and STAT1 as well as the co-stimulatory molecules CD80/86, MHCI and MHCII. Together, these factors are essential during infections, while in cancer they indicate a potent anti-tumor response [91–93].

The tumor microenvironment (TME) however alters immune cell phenotypes, which typically leads to elevated levels of IL-6, TGF-β, PGE2, COX2, MCP-1, M-CSF, IL-4 and IL-10. These factors are involved in immunosuppression (tumor promoting inflammation and angiogenesis) and are linked to tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T-reg) (Fig. 3) [74,94]. The TME is further characterized by metabolites that play an important role in immune cell functions; these are, for example, specific amino acids important for tumoricidal T cell activation (e.g. Tryptophan, depleted by Indoleamine (IDO); Arginine, depleted by Arginase), while their alternate degradation products (L-Kynurenine, ornithine) favor immunosuppressive regulatory T cells [95]. Surface receptors, such as CTLA-4 and PD-1 mediate suppression through silencing tumor-specific T cells. Intracellular signaling molecules including transcription factors (e.g. STATs, NF-κB, HIF) and their accessory molecules (e.g. SOCS, iκB, IKKβ) are paramount in determining the activation state of immune cells [96–99]. They dictate transcriptional programs that drive the immune response and, in the case of cancer, reinforce the immunosuppressive tumor environment, while simultaneously inhibiting a tumoricidal response (Fig. 3) [6].

Due to the concomitant regulatory mechanisms within the TME, many factors have redundant functions, therefore targeting specific combinations rather than a single target might be necessary to tip the balance from immunosuppression to immunogenicity.

Tumor-associated myeloid cells (TAMs, DCs and MDSCs) are potent mediators of the suppressive TME through the abovementioned factors and are able to either activate or suppress immune responses which make them a prime target for immunotherapy [100]. Their intracellular signaling pathways are crucial to these functions; therefore, the regulation of these factors is one potential approach to convert their phenotype. Additionally, new therapeutics that target immunosuppressive T cells show promising results for cancer. As an example, antibodies blocking the CTLA-4 (ipilimumab) or PD-1 (nivolumab) receptor on T cells show improved tumor responses [101]. The best approach to target individual or multiple factors still needs to be carefully evaluated.

RNAi is ideally suited to target immune cells; it allows the targeting of individual or multiple targets and can be tailored to target a specific cell type. In contrast to antibody blockade [102], RNAi can be used to directly down-modulate gene expression in immune cells in order to regulate signaling molecules (e.g. CTLA-4, PD-1, STATs, NF-κB) and ultimately their phenotype, to drive tumoricidal responses. In the past decades a multitude of factors that are responsible for immunosuppression in tumor-associated immune cells have been identified and can be targeted to control immunosuppression. Some of these factors are already being successfully targeted, in particular, STAT3 (via RNAi or small molecule inhibitors) showing a shift toward a potent anti-tumor response [103–105]. Other potential targets remain to be investigated (e.g. non-canonical NF-κB pathway to inhibit MDSC-suppression) [106]. RNAi offers the possibility to control the expression of any desired mediator, independent on the availability of pharmacologically active inhibitors. Consequently, combining RNAi immunotherapy with new advances in nanomaterial technology is a great opportunity to improve cancer treatments.

RNAi nanomaterials design for the modulation of an immune response in cancer

Modulating *in vivo* immune response using RNAi nanomaterials can be divided into two categories: inhibition of immune suppression or enhancement of the immune response. To achieve these goals proper design of RNAi nanomaterials must be fulfilled in order to attain a successful immune response. The antitumoral effect of specific RNAi treatment should not be dependent exclusively on the inhibitory effect of siRNA, but should also be combined with RNAi inducing immunostimulatory effects

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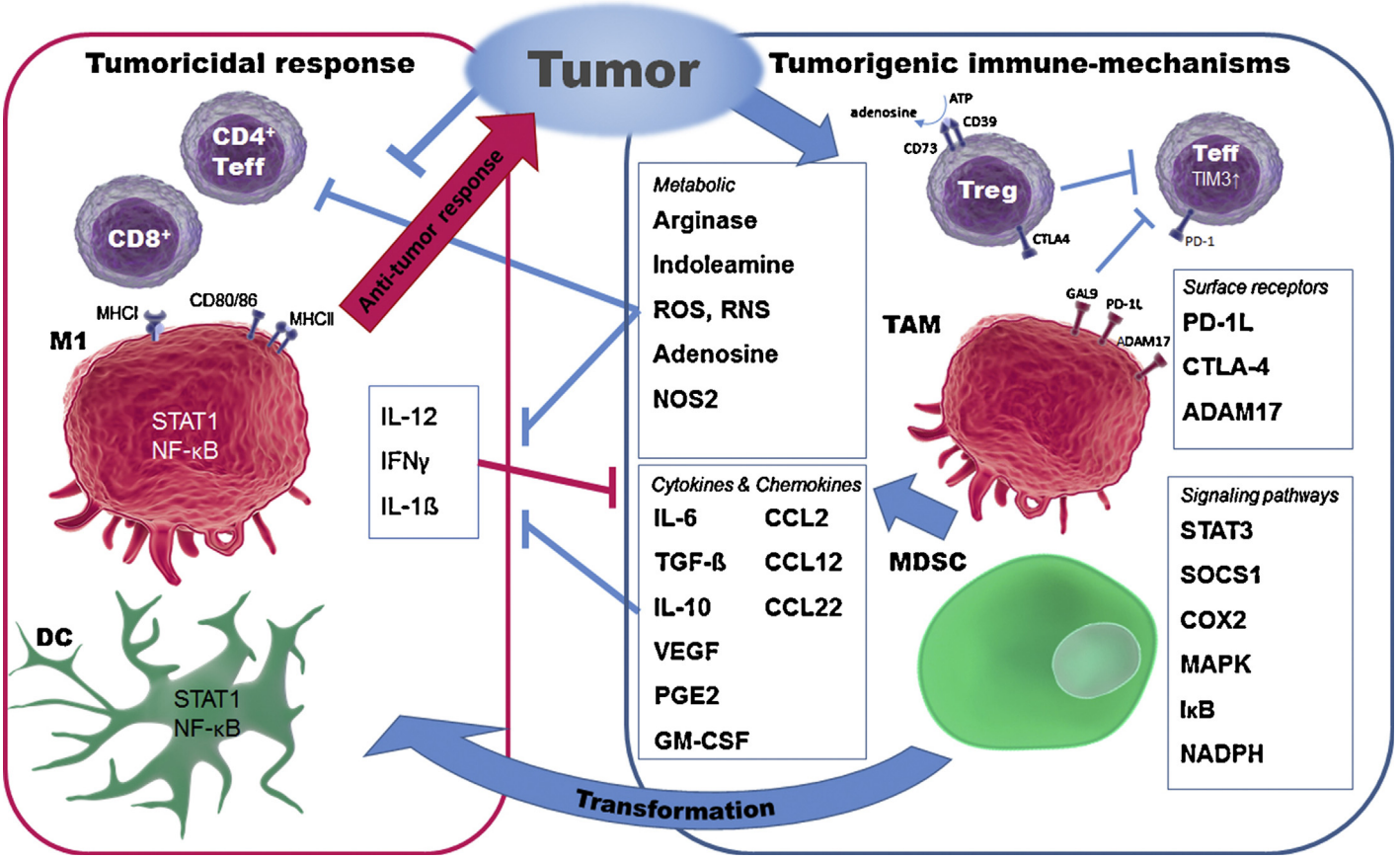

FIGURE 3

Potential RNAi targets for immunotherapy. Immune cells in the tumor milieu adapt their phenotype based on the cues they receive. Many of these cues activate immunosuppressive pathways and inhibit a tumoricidal response. Myeloid cells are promising as potential targets as they moderate the TME, suppress tumoricidal functions and control adaptive immune responses. During an immune response, myeloid cells show activation of NF-κB and STAT1 as well as increased expression of co-stimulatory molecules CD80/86, MHCI, MHCII and pro-inflammatory cytokines (IL-12, IFNγ, IL-1β, IL-23). This enables them to efficiently activate and polarize T cells and to raise a potent tumoricidal immune response. The tumor environment however alters immune cell phenotypes. The cytokines TGF-β and IL-10, among others, induce myeloid-derived cells (monocytes/macrophages, DCs) to become immunosuppressive. These cells are described as TAM (tumor-associated macrophages) or MDSC (myeloid-derived suppressor cells) and are characterized by secretion of the same cytokines that induced their polarization while also secreting other mediators that promote angiogenesis (VEGF), and tumor-associated inflammation, including recruitment and metastasis. Secretion of soluble mediators can be controlled by targeting their individual gene expression or their upstream signaling modalities (e.g. STAT3, SOCS1). Targeting these signaling molecules can be used to control not only the secreted mediators but also the myeloid cell activation phenotype [93,98] and also the expression of receptors that mediate suppression of effector T cells (T-eff) and/or induction of regulatory T cells (T-reg) including CTLA-4, PD-1L, Gal9 and ADAM17.

(e.g. TLR-activation), providing gene silencing with a stimulatory immune response in order to destroy tumors [107].

The success of RNAi therapy is highly dependent on the effective conjugation of siRNA to the nanoparticles, but also on several factors that affect RNAi efficiency, such as route of administration, circulation time and stability, tissue extravasation, targeting and cell internalization and endosomal escape, as delineated below.

Preventing nonspecific immune activation

Systemic delivery of therapeutic agents is the most convenient application route that can potentially reach any target site non-invasively. However, systemic application in particular is the most challenging route for the agents' delivery to specific organs other than liver and kidney. To begin with, systemically applied naked siRNAs can induce nonspecific activation of the immune system through the Toll-like receptor 7 (TLR7) pathways [36,108]. Recent studies have reported positive therapeutic effects of *in vivo* delivery of siRNAs in clinical trials to the nonspecific activation of TLR7 [108] or TLR3 [109]. Although this intrinsic immunostimulatory function may raise some apprehensions about the efficacy or specificity in gene silencing using siRNAs, it also opens new opportunities for reversing immunosuppressive mechanisms commonly regulated by tumors while silencing, at the same time, critical immunosuppressive factors [110]. Smart design of siRNA sequences and their carriers is necessary to overcome these non-specific immune responses, the extremely short half-lives and subsequent accumulation in the liver and kidney in the case of systemic application [12,36,111]. For example, the incorporation of chemical siRNA backbone modifications such as 2′-O-methyl or 2′-fluoro into the sugar structure of selected nucleotides in sense and antisense strands may avoid recognition by the innate immune system and protect siRNA from degradation [11,112].

Circulation time and stability

Nanomaterials can be modified to protect and shield the siRNAs from endogenous clearance mechanisms. siRNA can be conjugated

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to the surface of nanoparticles or entrapped in nanovesicles, which act to protect the siRNA from serum nucleases and to increase their chemical stability. Compared to conventional transfection agents, nanoparticle-conjugated siRNAs have been shown to be less susceptible to degradation by nuclease activity, to exhibit greater cellular uptake and to have a higher siRNA effective concentration, all of which have accelerated siRNA research into this delivery method over the past few years. Unfortunately, linking the siRNA to a nanoparticle alone does not protect it from clearance. Blood serum components interact with siRNA/nanomaterials and mark them for uptake via the mononuclear phagocyte system (MPS), especially by Kupffer cells in the liver [12,111]. In fact, the stimulatory or anti-stimulatory action is typically due to binding of proteins in the blood, which influence the uptake of nanoparticles by cells [12]. The use of nanomaterials composed of hydrophilic polymers including acrylic acid, acrylamide, and maleic anhydride polymers and copolymers, as well as allylamine, ethyleneimine, oxazoline (for example, Polyethylene glycol, Polyethylenimine, Poly(acrylic acid), Poly(vinyl alcohol), Poly(*N*-isopropylacrylamide)) inhibits serum protein binding and clearance by immune cells which thereby drastically increases their circulation time [12,113].

Tissue extravasation

Particle size is critical for efficient tissue delivery. Nanoparticles in the size range of 10–100 nm are generally accepted as efficient delivery agents, determined by *in vivo* clearance, biodistribution and toxicity. Particles of less than 10 nm are subject to renal clearance, while larger particles >15 μm are removed from the circulation by the reticulo-endothelial system (RES) in the liver and spleen [58,59]. The RES (also known as macrophage or mononuclear phagocyte systems), is a network of cells located throughout the body that support the elimination of small particles, also involved in the identification of foreign substances in blood and tissues [114].

Owing to the effective elimination of NPs by the RES, the optimal delivery of NPs to target sites through intravascular delivery constitutes a challenge. NPs' uptake, mainly by macrophages, is dependent on size, charge and other surface modifications, which influence the lifetime and the diffusion of nanoparticles to certain cells/tissues/organs. Therefore, the size of nanoparticles and their payload should be large enough to prevent rapid leakage in blood capillaries but at the same time small enough to escape from the scavenge of macrophages in the RES, such as the liver and spleen or being cleared out by the kidneys. Appropriately sized nanoparticles can be chemically modified to increase their retention time in the circulatory system, using cationic polymers as described [115,116] or directly engineered to target phagocytic cells to increase uptake and antigen presentation [117].

Targeting and cell internalization

Once the siRNA is inside the target tissue it has to reach the target cells while excluding healthy cells. Nanoparticles can be functionalized with cell-specific ligands that allow receptor-mediated uptake into target cells, for example markers which are overexpressed on tumor or immune cells. Additionally, the surface charge on the nanoparticle is a crucial factor that affects cellular internalization (by both normal/cancer cells and immune cells) and also determines potential *in vivo* circulation. Positively charged particles have been shown to exhibit increased internalization not only by tumor cells but also by macrophages and DCs compared to neutral or negatively charged nanoparticles. In general, positively charged particles are also phagocytosed by macrophages but are more efficiently taken up because of electrostatic interactions between the positively charged particle surface and the negatively charged cellular membrane. Conversely, nanoparticles with negative surface charges typically exhibit low cellular internalization; however these nanoparticles can circulate longer *in vivo* and thus, better accumulate at tumor sites [118,119].

Endosomal escape

In order to activate the RNAi pathway, siRNAs must be successfully delivered into the cytoplasm, a process challenged by the large size and hydrophilicity of the nanoparticles, limiting their ability to cross the cell membrane in the absence of a transfection agent. Moreover, the cellular uptake of nanoparticles (endocytosis) as well as their subsequent discharge (exocytosis) is affected by their shape, size and charge. Generally, small (<200 nm) positively charged nanoparticles adsorb to the negatively charged plasma membrane, followed by clathrin-dependent endocytosis. In contrast, larger particles (>200 nm) enter the cell by receptor- and clathrin-independent endocytosis [120]. The uptake pathway can greatly influence the interaction/effect of particles on cellular responses. The cell types that have been studied most extensively for distinct uptake mechanisms are DCs and macrophages, where several studies show that the route of uptake as well as the particle size significantly changes their responses. While DCs generally internalize (pinocytosis) smaller particles and show the highest antigen-presentation capacity; macrophages efficiently phagocytize larger particles (<10 μm) but are less potent antigen-presenters [121,122].

Once the nanoparticle is taken up by the target cells via endocytosis, its release from the endosome into the cytoplasm is the next challenge. Many of the described systems/vehicles get trapped in the endosome, which fuses with lysosomes (i.e. endolysosome) thereby destroying the siRNAs. Specific targeting via fusogenic peptides (i.e. Influenza hemagglutinin – HA) [123], lysosomotropic compounds/surfactants (i.e. Chloroquine, Quinacrine, Tilorone, Suramine) [124] or PEI polymer [125] is necessary to enable the endosomal/endolysosomal escape and allow the formation of the RISC complex (Fig. 4).

The design criteria described above are dependent on the particular subsets of immune cells to be targeted. A multitude of immune targets are possible and the choice depends largely on the location and type of cancer. This will certainly offer an extensive diversity of targets for RNAi.

Current anti-tumor immunotherapies using RNAi nanomaterials

It has been well established that myeloid cells are key contributors of the suppressive TME and a hallmark for cancer progression. These cells include the tumor-polarized DCs, TAMs as well as newly recruited monocytes and MDSCs, which become polarized within the TME. Targeting this specific component of the immune system is an essential tool and can be used to abrogate monocyte infiltration, down-modulate immunosuppressive mediators and induce tumoricidal responses.

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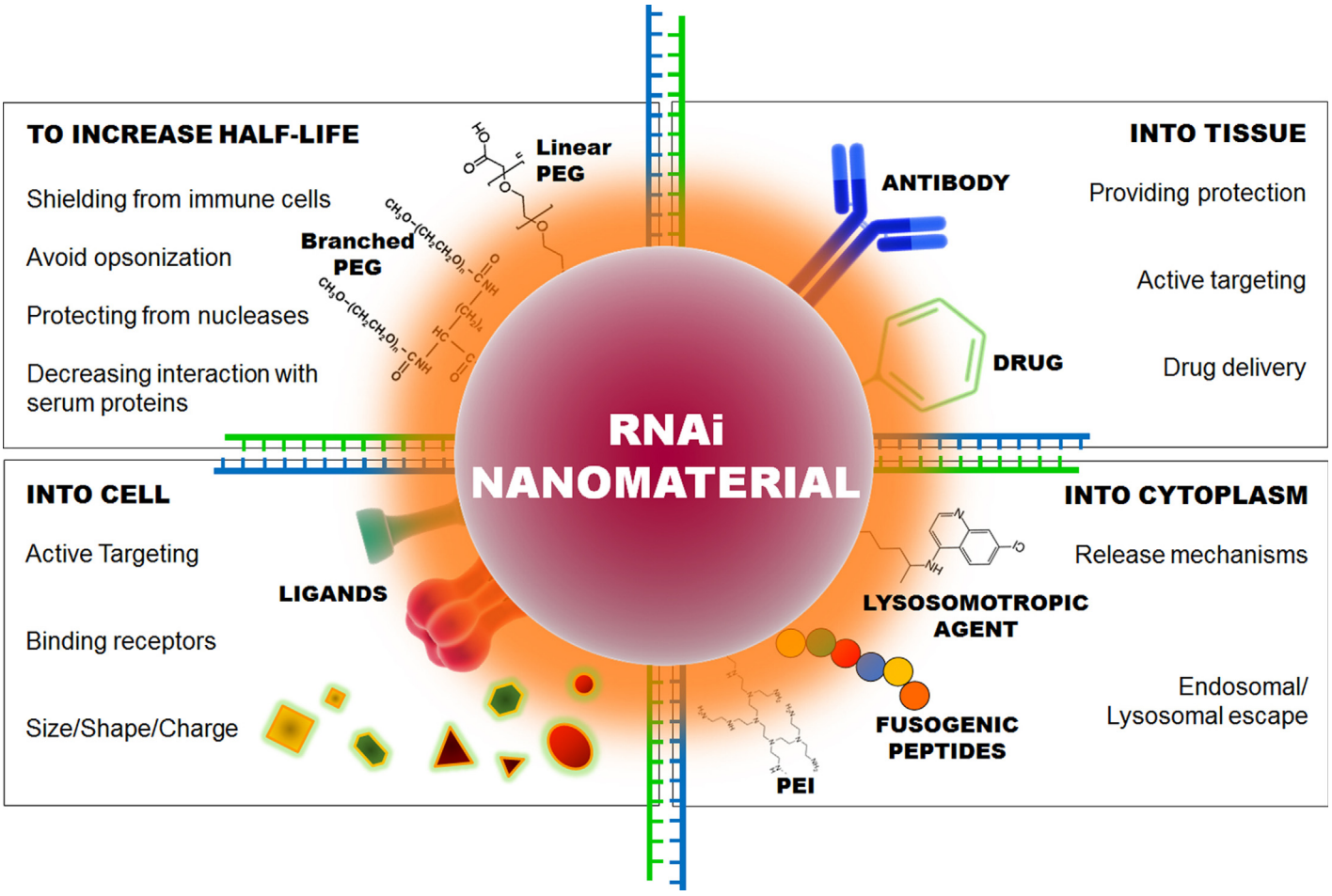

FIGURE 4

Designing delivery materials for RNAi therapeutics: promises and challenges. RNAi NPs can be engineered to increase their half-life using polymers (i.e. PEG – linear, branched). RNAi NPs can also be modularly assembled from different materials with differing size, charge, shape and composition with different physical and chemical properties and functionalized with a myriad of ligands for biological targeting, specific intracellular applications, release mechanisms and drug delivery. Endosomal/lysosomal escape can be achieved using fusogenic peptides, lysosomotropic compounds/surfactants or PEI polymer.

Interestingly, only few studies have been performed using RNAi nanomaterials for immunotherapy in cancer. The targeting of an important sub-set of the populations of immune cells in combination with cancer cells could shift the tumor microenvironment from pro-oncogenic to anti-tumoral. Table 2 summarizes all the RNAi-nanoparticle devices to target and modulate immune cells in cancer reported to date.

Concerning gene targets in immune cells using RNAi nanomaterials very little has been presented so far for cells other than DCs. In fact, more than 70% of the reported studies concerning RNAi nanomaterials for cancer immunotherapy target DCs and all of the nanomaterials were administered systemically (Table 2). In the last 4 years several studies have demonstrated the importance of silencing essential genes (PD-L1, SOCS1, STAT3) in DCs and their function in tumor immunity, because of their potent contribution to immune responses.

The majority of RNAi nanoparticles for immunotherapy in cancer are targeting dendritic cells (DCs) for cancer vaccine strategies. This approach through 'nano-vaccination' can provide antigens together with an adjuvant to elicit a therapeutic T cell response *in vivo*. DCs are considered an excellent candidate as they are crucial mediators of immune responses and able to regulate immunity as well as tolerance and therefore are an essential target in the efforts to produce and control tumor immunity, being a therapeutic key against cancer [78,122].

The most abundant material used to modify surface properties to provide better interaction with biological materials are polymer-based nanoparticles, especially the copolymer PLGA (poly(lactide-*co*-glycolic acid)) which is FDA approved and used to increase circulation times *in vivo* [139]. Moreover, PLGA nanoparticles have also been explored as vaccine formulations because they provide sustained release, protect encapsulated antigens from harsh environments and enzymatic degradation, allow for targeted delivery with the attachment of ligands; and may have additional adjuvant effects [140].

Heo et al. reported the development of PLGA programmed nanoparticles (pNPs) that can modify the immunotherapeutic function of primary bone marrow-derived dendritic cells (BMDCs) by their *ex vivo* manipulation prior to *in vivo* injection (Fig. 5). In this particular case, DCs promoted the induction of potent tumoricidal functions and subsequent tumor rejection. The authors reported the use of two types of NPs: (1) PLGA NPs functionalized with a siRNA anti-STAT3 (signal transducer and activator of transcription-3, an immune-suppressor gene) and an immune

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TABLE 2

Summary of all anti-tumor immunotherapies with RNAi nanomaterials described up to date.

Targeted immune cells	Surface modification	Type of nanomaterial	Targeted gene	Cell/Animal model	Observations/Results	Refs
Dendritic cells	siRNA pool	Lipid-based	Hck, MR, DC-SIGN, Dectin-1	*In vitro*	Protocol enables efficient gene knockdown in human primary mononuclear phagocytes	[126]
	siRNAs; pH-dependent fusogenic peptide (GALA)	Stearylated octaarginine-lipid-based	SOCS1	*In vitro*; *ex vivo*	Knockdown of SOCS1, a negative-feedback regulator of the immune response in BMDCs resulted in an enhanced phosphorylation of STAT1, and the production of proinflammatory cytokines, suppressing tumor growth	[127]
	PEI polymer; siRNA	PEI-based	PD-L1	*In vitro* (HEK293 cells); *in vivo* (ovarian cancer mice – intraperitoneal injection)	Intrinsic TLR5 and TLR7 stimulation of siRNA-PEI NPs in combination with gene silencing to transform tumor-infiltrating regulatory DCs into DCs capable of promoting therapeutic antitumor immunity	[128]
	Tumor antigen; siRNA	PLGA-based	SOCS1	*In vitro*	Potent strategy to enhance immunotherapeutic effects in BMDC-based cancer therapy via NPs that can deliver both tumor antigen and immunosuppressive gene siRNA; NPs increased level of pro-inflammatory cytokines (TNF-α, IL-6, IL-12, IL-2)	[129]
	siRNA; immune response modifier (imiquimod, R837); tumor model antigen (ovalbumin); NIR dye (ICG)	PLGA-based	STAT3	*In vivo* (lymphoma mice, subcutaneous injection)	Immunization of mice NPs-treated DCs induced ovalbumin-specific cytotoxic T lymphocytes activity against the tumor model, inhibiting tumor growth	[130]
	PEI polymers; siRNA	PLGA-based	STAT3	*In vitro*	STAT3 silencing restored DC maturation and functionality via the upregulation of CD86 expression, high secretion of TNF-α and significant allogenic T cell proliferation	[131]
	miRNA mimics	PEI-based	miR-155	*In vivo* (ovarian cancer mice, intraperitoneal injection)	Increased miR-155 activity and silencing of immunosuppressive mediators; decreased tumor growth by restoring ability of DCs to mediate a CTL response	[132]
	siRNA; PEI-StA	PEI-based	STAT3	*In vivo* (melanoma B16 mice, intratumoral injection)	STAT3 down-regulation associated with restored ability of DCs to mediate a CTL response; decreased tumor growth	[133]
	siRNA; cholesterol; PEG	Lipid-based	PD-L1 PD-L2	*In vitro*; *ex vivo*	Efficient and specific knockdown of PD-L expression on human monocyte derived DCs; siRNA-NP transfection combined with target antigen peptide loading and mRNA electroporation	[134]
	siRNA; pH-dependent fusogenic peptide (GALA)	Lipid envelope-type	A20	*In vitro*	Significant A20 knockdown effect, with an enhanced production of proinflammatory molecules, after lipopolysaccharide (TLR4 ligand) stimulation	[135]
Monocytes	siRNA pool	Lipid-based	Hck, MR, DC-SIGN, Dectin-1	*In vitro*	Protocol enables efficient gene knockdown in human primary mononuclear phagocytes	[126]
	siRNA	Lipid-based	CCR2	*In vivo* (lymphoma graft, tail vein injection)	Efficient degradation of CCR2 mRNA in monocytes prevents their accumulation in sites of inflammation, and results in reduced tumor volumes and lower numbers of monocytes	[136]

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TABLE 2 (*Continued*)

Targeted immune cells	Surface modification	Type of nanomaterial	Targeted gene	Cell/Animal model	Observations/Results	Refs
Macrophages TAMs	siRNA pool	Lipid-based	Hck, MR, DC-SIGN, Dectin-1	*In vitro*	Protocol enables efficient gene knockdown in human primary mononuclear phagocytes	[126]
	Copolymers (AzEMA, DMAEMA, BMA-*co*-PAA-*co*-DMAEMA); siRNAs	Mannosylated Polymeric Micelles	CD206 (mannose receptor)	*In vitro*	pH responsive micelles improved the delivery of siRNA into primary macrophages by fourfold and induce ~90% knockdown	[137]
	siRNA; PEG; M2 peptide	Gold NPs	VEGF	*In vitro*; *in vivo* (lung cancer mice, intratracheal instillation)	Treatment with low doses of siRNA (ED_{50} 0.0025–0.01 mg/kg) in a multi and long-term dosing system substantially reduces the recruitment of TAMs in lung tumor tissue, reduces tumor size (±95%), and increases animal survival (±75%) in mice	[138]

A20, negative regulator of the toll-like receptor and TNF receptor signaling pathways; AzEMA, 2-azidoethyl methacrylate; DMAEMA, 2-dimethylaminoethyl methacrylate; BMA, butyl methacrylate; BMDCs, bone marrow-derived dendritic cells; CCR2, Chemokine (C–C motif) receptor 2; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, also known as CD209 cluster of differentiation 209; Dectin-1, natural killer-cell-receptor-like C-type lectin; Hck, hemopoietic cell kinase; ICG, indocyanine green; MR, major histocompatibility complex; PAA, 2-propylacrylic acid; PEI, polyethylenimine; PEI-StA, stearic acid-modified polyethylenimine; PD-L1, programmed cell death 1-ligand 1; PLGA, poly(lactide-*co*-glycolic acid); SOCS1, suppressor of cytokine signaling 1; STAT3, signal transducer and activator of transcription-3; VEGF, vascular endothelial growth factor.

response modifier (imiquimod, R837) for the activation of DCs via toll-like receptor 7 (TLR7) and (2) PLGA NPs containing tumor model antigen (ovalbumin, OVA) and near-infrared (NIR) dyes (indocyanine green, ICG), to deliver tumor-antigen-specific information to DCs *ex vivo* and track the migration of DCs *in vivo*. These innovative pNPs were internalized into DCs, induced TLR activation, silenced immunosuppressive genes, led to cross-presentation, and inhibited tumor growth significantly. With a huge potential for efficient DC-based cancer therapy, these pNPs can tailor the function of immunotherapeutic cells and monitor their migration *in vivo* [130].

In another key report, *Cubillos-Ruiz* et al. described a new system to activate TLRs using polyethylenimine (PEI)-based nanoparticles. PEI nanoparticles may be very useful as carriers to target immune cells. Every macromolecule of PEI can be protonated and catalyzed into a polymer with high-density cationic potential and a highly branched network [141], which allows PEI to disrupt the endosomal membrane by osmotic imbalance, also known as the 'proton-sponge effect'. This effect occurs when unprotonated species can absorb protons as they are pumped into the lysosome. This results in an increased influx of Cl^- ions and water that causes swelling and rupture of the lysosomal membrane with subsequent release of its contents into the cytoplasm [142]. Moreover, PEI by itself is able to trigger robust TLR5 activation in wild-type mice, but not in Tlr5−/− littermates, and can further stimulate APCs within the tumor microenvironment when combined with the encapsulated immunostimulatory siRNA [128].

The authors also showed that in the absence of targeting, PEI-complexed siRNA oligonucleotides elicit the stimulation of TLR3 and TLR7 and that the nonspecific activation of multiple TLRs (specifically, TLR5 and TLR7) reversed the immunosuppressive phenotype of human and mouse ovarian tumor-associated DCs. Further, linear PEI-based nanoparticles encapsulating siRNA were preferentially and enthusiastically scavenged by regulatory DCs expressing CD11c and programmed cell death 1-ligand 1 (PD-L1) in ovarian cancer mice. These NPs modified the immunosuppressive phenotype of ovarian tumor-associated DCs (human and mouse) to an immunostimulatory phenotype which resulted in increased antigen presentation and increased numbers of tumor-specific cytotoxic CD8+ T cells in the tumor microenvironment. Mice treated with PEI-siRNA NPs showed improved survival and superior anti-tumor immunity as compared to non-targeting siRNA-PEI nanocomplexes. These NPs have the capacity to transform tumor-infiltrating regulatory DCs into DCs able to enhance anti-tumor immunity. Taking advantage of the intrinsic immunostimulatory capacity of siRNA may activate TLR pathways. The authors demonstrate that the inherent capacity of siRNA-PEI nanocomplexes to activate innate immune mechanisms offers a major opportunity to reverse tumor-induced immunosuppression while simultaneously silencing specific TLR genes [128].

In contrast to targeting dendritic cells (or other tumor-associated immune cells), a promising approach by *Anderson* and co-workers investigates the inhibition of inflammatory monocyte infiltration by blocking CCR2, an essential monocyte homing factor. The authors focused on CCR2 because the recruitment of inflammatory monocytes intimately depends on the chemokine/chemokine receptor pair MCP-1/CCR2. This study reports the optimization of lipid nanoparticles containing CCR2-silencing short interfering RNA are able to localize with monocytes, with considerable accumulation in spleen and bone marrow with rapid clearance from the blood, when administered systemically in mice [136]. Due to the physical properties of cationic lipids used, in the efficient encapsulation of oligonucleotides, this study shows a lipid nanoparticle system can be used for *in vivo* delivery of siRNA to immune cells, Efficient degradation of CCR2 mRNA in monocytes prevents their accumulation in sites of inflammation, specifically reducing tumor volumes and accumulation of inflammatory monocytes in the tumor (Fig. 6). This study may also be extended to induce tumoricidal functions in monocytes prior to their migration to tumor sites but the primary focus was on inflammatory diseases where infiltration of inflammatory monocytes is part of the pathophysiology. This study definitely opens a new translational avenue to approach the many diseases driven by recruitment of inflammatory monocytes [136]. However, no targeting moiety was used to achieve active targeting of the studied immune cells.

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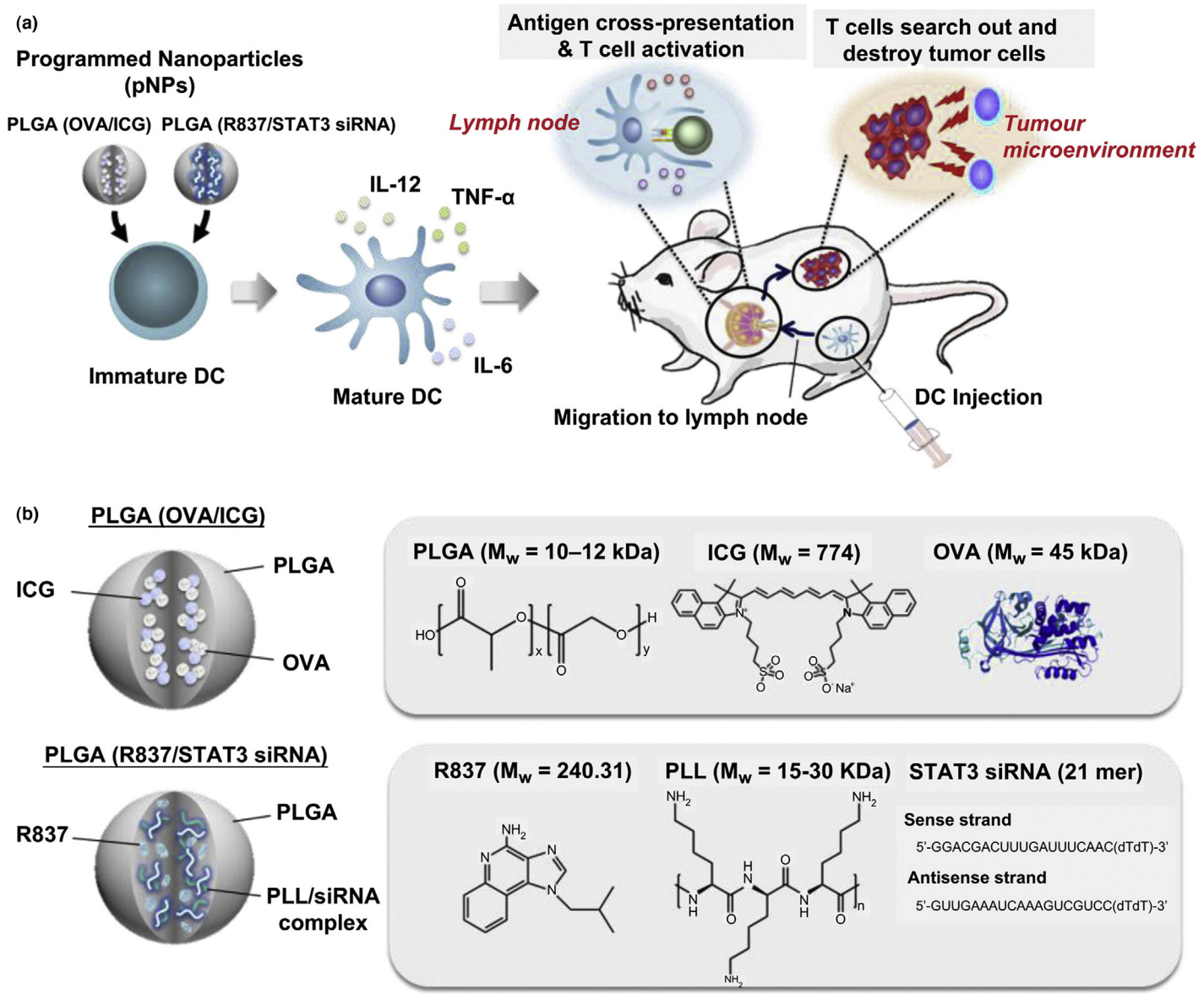

FIGURE 5

Programmed nanoparticles (pNPs) for immune cell-based cancer therapy. (a) Activated dendritic cells (DCs) by pNPs migrate to lymph node, induce antigen specific immune responses, and lead to activation of cytotoxic T cells, which can destroy tumor cells. (b) Synthesis of pNPs: PLGA (OVA/ICG); antigen presentation (ovalbumin; OVA) and monitoring DCs (indocyanine green; ICG), PLGA (R837/STAT3 siRNA); combined immunomodulation with R837 (for activation of TLR7) and STAT3 siRNA (for silencing of immunosuppressive genes, STAT3). Reproduced and adapted with permission [130]. Copyright 2015, Elsevier.

Using metallic instead of polymeric nanoparticles, Conde et al. reported highly potent and selective anti-vascular endothelial growth factor (VEGF) siRNA-M2pep gold nanoparticles that when administered via intratracheal instillation in a lung cancer murine model, are rapidly distributed in tumor-associated macrophages (TAMs) (see Fig. 7). The authors prove that gene silencing can be achieved in cancer cells using regular RNAi NPs as well as in TAMs when M2 peptide-based nanoparticles were used to achieve active targeting of TAMs. Their data show that treatment with low doses of siRNA (ED_{50} 0.0025–0.01 mg/kg) substantially reduced the accumulation of inflammatory TAMs in lung tumor tissue, reducing tumor size (~95%) and increasing animal survival (~75%) [138]. Synergistic VEGF-silencing in TAMs and cancer cells led to potent and long-lasting VEGF inhibition without signs of toxicity/inflammation and demonstrating immune modulation of the tumor milieu combined with tumor suppression. VEGF is a key angiogenic factor secreted primarily by TAMs and well known to mediate neoangiogenesis as well as promoting cancer progression and metastasis, therefore VEGF has been correlated with the presence of macrophages within tumors. The main goal was to target TAMs specifically to modulate the tumor microenvironment and thereby inhibiting TAM accumulation and consequently their tumor promoting functions [138].

Future immunotherapy targets using RNAi nanomaterials: How to move forward?

Malignant solid tumors are known to contain an abundant population of macrophages within the infiltrating leukocytes. TAMs

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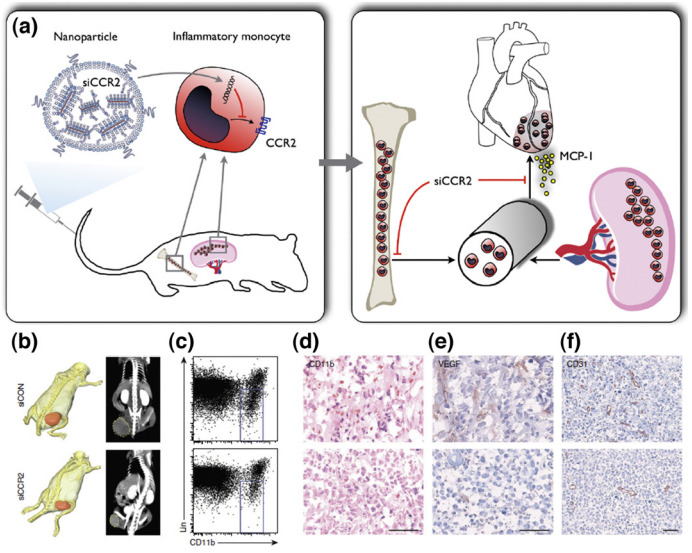

FIGURE 6

Therapeutic siRNA silencing in inflammatory monocytes in mice. (a) Schematic outline of *in vivo* siRNA CCR2 silencing in inflammatory monocytes. (b) Tumor size measured by X-ray CT. Representative images from mice on day 10 after implantation of EL4-tumors ($n = 7$ per group). Top row displays cohorts treated with control siRNA (siCON), bottom row shows siCCR2 treatment. The three-dimensional CT reconstruction shows tumor in red pseudocolor. (c) FACS analysis of tumors with monocyte/macrophage gate (blue box) after treatment with control siRNA (top) or siCCR2 ($n = 5$ per group). (d–f) Immunohistochemical evaluation of tumors for myeloid cells ((c), CD11b), vascular endothelial growth factor ((d), VEGF) and vessel density ((f), CD31). Reproduced and adapted with permission [136]. Copyright 2015, Nature Publishing Group.

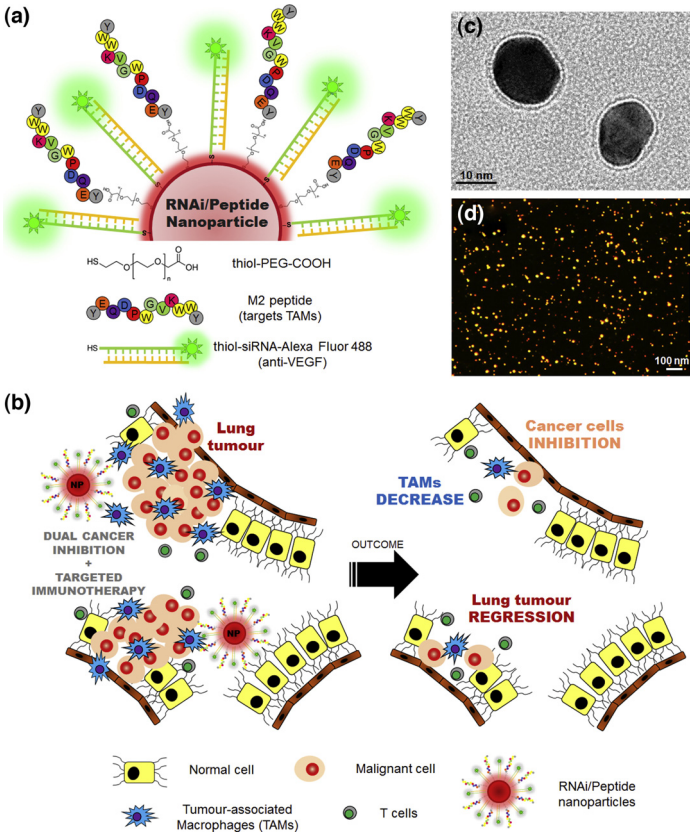

FIGURE 7

Nanoparticle-based strategy to deliver RNAi for VEGF silencing specifically to tumor-associated macrophages (TAMs). (a) Gold nanoparticles (AuNPs, ~15 nm) functionalized with thiolated-PEG-COOH conjugated to TAMs-targeting peptide (M2pep) and thiolated anti-VEGF siRNA labeled with Alexa Fluor 488 (b) Schematic of the outcome of the proposed therapy *in vivo* (siRNA VEGF silencing in TAMs in lung cancer via highly specific and potent nanoparticles administered directly to bronchial airways. Transmission electron microscopy (TEM) with negative staining (c) and dark-field light scattering microscopy (d) images of RNAi-M2pep NPs. Reproduced and adapted with permission [138]. Copyright 2015, Wiley.

play a crucial role in many aspects of tumor growth and development with similar and overlapping functions as DCs/MDSCs. In fact, TAMs play an important role in determining cancer progression, metastasis and resistance to therapy [143]. TAMs therefore represent an attractive target for cancer immunotherapy with important target genes comprising chemokines and pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α), as well as other pro-angiogenic factors. Some of these potential future RNAi targets to be used in cancer immunotherapy are depicted in Fig. 3. Restoring the expression of immunogenic cytokines such as IL-2, IL-15 and IL-12 and simultaneous down-regulation of immunosuppressive cytokines (e.g. IL-10, TGF-β) may be able to support T cell proliferation and function. An additional subset of immune cells often associated with tumors are myeloid-derived suppressor cells (MDSC), which are a heterogeneous population of immature myeloid cells that show overlapping phenotypes with macrophages and DCs. All three myeloid-derived cell types (DCs, TAMs and MDSCs) are insufficiently characterized; however they show similar/overlapping immunosuppressive functions in the TME and employ similar signaling pathways. Due to their endocytic potential, these cells can be targeted by the introduction of particles that favor endocytosis (e.g. liposomes). In addition to the prominent role of myeloid cells in the TME, regulatory T cells are major contributors to the immunosuppressive environment and are a promising target for RNAi [144]. While some methods aim to eradicate certain immune cells from the TME, a superior approach may be to convert their phenotype from their tumorigenic role toward raising a potent anti-tumor response, including the activation of T cell responses (Th1, CD8).

There is an urgent need for new therapeutics to restore the specific tumoricidal immune response to minimize collateral damage. The majority of conventional drugs with positive clinical outcomes lack cellular specificity and affect surrounding healthy tissue, culminating in undesired side effects. Some work has been done concerning antigen delivery and adjuvants serving to enhance cancer vaccines. Lipidic, polymeric particles, and virus-like particles have been used to enable long-term release of tumor-antigens, as have oriented antigen and/or adjuvant presentation, multivalent presentation, and DC targeting [145]. Extensive research has led us to exploit engineered biomolecules, such as proteins, peptides, antibodies and oligonucleotides designed to enhance immune-based mechanisms, thereby re-shaping the future of immunotherapeutic outcomes. Numerous clinical investigations in the last years have demonstrated that the biological effect of these compounds depends on their ability to attain their transport across biological barriers. The use of nanoscale devices has the potential to overcome the delivery, efficacy and safety

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TABLE 3

Potential strategies for the intracellular delivery of RNAi nanomaterials to immune cells: From function to modification using specific payloads. Adapted from [33,43,147].

Function	Modification	Action	Common payloads
Circulation time and stability	Polymers	Reduces RES uptake and increases circulation time	PEG Poly(acrylic acid) Poly(vinyl alcohol) Poly(*N*-isopropylacrylamide)
Active targeting	Antibodies	Cell surface targeting agents	Cetuximab HER2
		Highly specific binding regions	EGFR CD19
	Peptides Proteins	Biologically active	RGD EGF
		Possible biosensor action	Transferrin NGF
		Possible therapeutic effect	Cholera toxin B
	Aptamers		T-cell specific
	Vitamins		B12 Biotin
	Carbohydrates		Glucose Mannose Lacto-*N*-fucopentaose III
	Small molecules		Folic acid LHRH
Intracellular uptake	Cationic coatings	Interact strongly with negatively charged cell plasma membrane to induce membrane permeability	Cationic liposomes Polypeptides Amine-containing Polymers Cholesterol PEI
	Cell penetrating peptides	Facilitate translocation of cargoes across the plasma membrane and to specific organelles within the cell	TAT (HIV-derived) Penetratin Transportan Polyarginine Pep-1
Endosomal/lysosomal escape	Lysosomotropic compounds/ surfactants	Accumulate in lysosomes, inhibit autophagy by increasing pH and blocking the fusion of autophagosomes with lysosomes	Chloroquine Quinacrine Tilorone Suramine
	Fusogenic peptides	Destabilizes the endosomal membrane promoting endosomal escape by a pH-responsive mechanism	HA (Influenza-derived) GALA

EGF, epidermal growth factor; HA, hemagglutinin; HER2, human epidermal growth factor receptor; LHRH, luteinizing hormone-releasing hormone; NGF, nerve growth factor; PEG, poly(ethylene glycol); PEI, polyethylenimine; RGD, Arg-Gly-Asp; TAT, transactivator of transcription.

issues associated with these biological blockades. Nanoparticles offer further advantages for efficient targeting of immune cells, such as pathogen-like size/appearance beneficial for increasing cellular uptake by phagocytic cells, and a capacity to carry high levels of therapeutic payloads, such as siRNAs. However, the development of clinical nanoformulations capable of selectively delivering siRNA to all immune cells remains challenging but not impossible [146]. Some of the most promising modifications extensively used in the past to only target tumor cells can now also be applied to target immune cells this includes specific functionalities and payloads (Table 3).

Using the valuable knowledge that we have acquired in the last 30 years about nanomaterial synthesis and payload functionalization [33,43], silencing specific key mediators of immunosuppressive signaling using engineered RNAi nanomaterials represents an efficient strategy for tumor therapeutics to tip the balance of the TME from immunosuppressive to tumoricidal. The use of RNAi in particular allows us to regulate specific and even multiple targets simultaneously.

Future perspective: combining immunotherapy and targeted therapies in cancer?

Despite significant advances in how we view and understand cancer mechanisms, the survival rate for patients with the most aggressive tumors has scarcely improved in the last 40 years. Most of the aggressive types of cancer escape from adaptive immune mechanisms and subsequently use the body's inflammatory machinery to establish cancer progression. Several strategies can be applied to use the immune system in our favor. Selective inhibition of immune cell infiltration represents only one initial approach; this can be combined with conventional therapies to effectively eliminate cancer cells. In a different

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approach, endogenous cells can be modified with RNAi nanoparticles *ex vivo* and then be used as a Trojan horse to reverse the regulatory activity of tumor immune cells while targeting cancer cells.

Importantly, tumors are frequently associated with the overexpression of immunosuppressive genes in order to evade immunocytotoxicity. The use of RNAi nanomaterials *in vivo* to silence specific genes will be an invaluable tool for future approaches in cancer therapies. We propose using specific RNAi nanomaterials for cancer immunotherapy to eliminate the immunosuppressive function of tumor-associated immune cells and at the same time to raise potent anti-tumor immune responses. Despite the discussed difficulties, RNAi nanoparticles are ideally suited to pursue new avenues of cancer immunotherapies due to their versatility and specificity. Further, the use of intelligent nanoparticles can be efficiently combined with available successful therapies to increase their efficacy. Cell-targeted therapies have the capacity to inhibit molecular pathways that are crucial for tumor growth and maintenance and minimize collateral damage in healthy cells. Some targeted therapies such as drug-coated nanoparticles elicit dramatic tumor regressions, but generally only demonstrate a short-lived response thus limiting their overall clinical benefit. Immunotherapy stimulates host immune responses that potentially results in long-lived tumor inhibition, anti-tumor immune memory and, consequently, improved clinical outcomes. This suggests that the two approaches might have complementary roles and that combinatorial therapy can have an important synergistic effect in cancer treatment [8,9]. RNAi nanomaterials used to target and inhibit the immunosuppressive nature of the tumor microenvironment are slowly gaining attention. However, the development of efficient delivery vehicles for *in vivo* applications, especially when systemic delivery to immune cells is sought, has remained a challenge due to lack of specificity, selectivity, and targeting [12,36]. We believe that nanomaterials used for RNAi immunotherapy have a huge potential to overcome some of these drawbacks and provide a ray of hope for more efficacious future cancer therapies [36].

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