

Recent advances in development of nano-carriers for immunogene therapy in various complex disorders

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ABSTRACT

Immunotherapy is a novel preference for the treatment of various complex diseases. Considering the application of varying agents for suppression or activation of the immune system, immunogene therapy was confirmed to stand as a proper alternative for other immunotherapeutic strategies due to its capability in targeting cells with more specificity that leads to controlling the expression of therapeutic genes. This method facilitates the local and single-dose application of most gene therapies that result in the usage of high therapeutic doses with a low risk of systemic side effects while being cost-efficient in long-term administrations. However, the existing barriers between the administration site and cell nucleus limited the clinical uses of genetic materials. These challenges can be overcome through the promising method of exerting non-carriers with high stability, low toxicity/immunogenicity, and simple modifications. In this study, we attempted to review the potential of nanoparticle application throughout the immunogene therapy of different diseases including cancer, microbial diseases, allergies, inflammatory bowel disease, rheumatoid arthritis, and respiratory infections. We included the outline of some challenges and opportunities in regards to the delivery of genetic materials that are based on nano-systems through immunotherapy of these disorders. Next to the promising future of these vectors, more detailed analyses are required to overcome the current limitations in clinical approaches.

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Introduction

Immunotherapy is an effective strategy for preventing and treating various disorders including viral infections, allergies, cancer, and autoimmune diseases (1). The application of numerous agents was trialed for suppression or activation of the immune system such as modified dendritic cells, monoclonal antibodies, stem cells, peptides, nucleic acids (NAs), and nanoparticles (NPs) (2).

The procedure of gene therapy attempted to introduce exogenous NAs into specific cells for the modulation of gene expression, which could also counteract or replace a malfunctioning gene (3). Immunogene therapy is a novel preference for the treatment of various complex disorders and a proper alternative for other immunotherapeutic strategies. This method is capable of targeting cells with more specificity, which leads to controlling the expression of therapeutic genes. Consequently, this route facilitates the local and single-dose application of most gene therapies that result in the usage of high therapeutic doses with a low risk of systemic side effects, while being cost-efficient in long-term administration.

Therapeutic genetic materials are categorized into DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid) types with limited clinical uses due to the existing barriers between the administration site and cell nucleus.

The delivery efficiency of genetic materials was attempted to be improved through the development of various gene carriers, which are required to have the potential of overcoming certain obstacles such as containing the ability to circulate in the bloodstream, remain undetected by the immune system, survive in the extracellular environment, successfully cross the cell membrane, protect the NAs from nuclease degradation, and release the functional form of NAs in the nucleus (4, 5).

The application of viral vectors with high transfection efficiency is extensively considered for gene delivery purposes. However, there are disadvantages to this approach such as irregular cytotoxicity and immune system stimulation, limited targeting of specific cell types, low DNA carrying capacity, inability to infect non-dividing cells (6), mutagenesis (7, 8), high risk of wild-type virus reversion (9, 10), and inflammation (11, 12). Therefore, nano-carriers (13) attracted the focus of many owing to their high stability, low toxicity, decreased immune responses, flexible designs, and simple modifications (14-16). On the other hand, the main challenge of nano-delivery systems is to develop a delivery vehicle with high cellular uptake efficiency and the ability to promote endosomal escape, inhibit its transference to lysosomes, and ensure its localization in appropriate cellular compartments (17).

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NPs proved to stand as a promising method for overcoming the mentioned challenges (NPs) due to offering notable advantages such as the ability to prevent degradation, deliver high concentrations of the payload for targeting the desired site, and function as a controlled release system (18-20). All of the nano-carriers that were exerted in immunogene therapy, including polymeric NPs, lipid-based NPs, liposomes, carbon nanotubes, and gold NPs (3, 21, 22), could be designed to produce efficient long-term immune responses (23).

The objective of this study was to review the applications of nano-carriers, which are based on immunogene therapy, for the treatment of various diseases.

Genetic materials used in immunotherapy

Various genetic materials were applied in immunogene therapy including synthetic oligonucleotide (ODN), plasmid DNA, and RNA interference (RNAi).

Oligonucleotides

ODNs are known as short, single-stranded synthetic DNA molecules and according to several studies, they contain unmethylated CpG (Cytosine-Phosphate-Guanine) motifs that are capable of arranging potent immune systems, which consequently improve the obtained responses to vaccines, reduce allergic responses, and provide protective effects in immunotherapy and cancer therapy (24). Figure 1 depicts the action mechanisms of ODNs throughout the immune system.

Recognized by toll-like receptor 9 (TLR9) (25) in antigen-presenting cells (APCs), the activation of CpG ODN oligonucleotides requires the Myeloid differentiation primary response 88(MYD88)-IRF7 (interferon regulatory

transcription factor) signaling, which leads to up-regulation of IRF and nuclear factor- κ B (NF- κ B). The activated APCs are able to stimulate the secretion of immunomodulatory cytokines, including interleukin-6 (IL-6), IL-1, IL-2, IL-10, IL-12, IL-18, Tumor necrosis factor- α (TNF α), and Interferon- α/β (IFN α/β) (26), that consequently activate the innate immune effector cells (e.g., natural killer cells [NK]). These cytokines provide the production of other immune-stimulatory cytokines and chemokines, as well as improve the adoptive immunity through the induction of Ag-specific T cells (27-29) and increase neutrophil migration, monocyte, and macrophage activation, and acute-phase protein production (30, 31). Another capability of CpG ODNs is to initiate the activation of B cells as an APC in order to produce IgM and cytokines such as IL-6 and IL-10. Additionally, IL-6 can facilitate the activation and differentiation of long-lived plasma cells for secretion of other kinds of antibodies such as IgG (32).

According to recent studies, CpG formulations could inhibit immune responses through enzyme up-regulation of indoleamine-pyrrole 2, 3-dioxygenase (IDO), and T regulatory cell (Treg)-mediated suppression (33). The induction of general immune responses stands as the main difficulty of naked CpGs, which is caused by the release of inflammatory cytokines and systemic activation of lymphocytes (33). However, this issue can be surpassed by exertion of specific vehicles designed for carrying CpGs.

Plasmid DNA

Another candidate for immunogene therapy is plasmid DNA (pDNA) due to its ability in expressing particular antigens (34). The produced antigens can increase the antigen expression of traveling APCs towards draining lymph nodes that present the antigenic peptide-MHC complexes for T cell stimulation, which results in the generation of long-term cellular immunity. Furthermore, humoral immunity can be generated through this process since it initiates the antibody production cascade (35-38). The sequences of CpG throughout bacterial plasmid were exerted as potent adjuvants in pDNA-mediated vaccination (25, 39, 40). In addition to adaptive immunity, the unmethylated CpG oligonucleotide sequences can turn the functionality of pDNA into a potent innate immunity inducer. Figure 2 displays the immune cell modulation that involves the internalization of NPs, which carry the plasmids throughout the suppression of allergic reactions.

The internalized NPs, which carry plasmid DNA into the targeted APCs end up engulfed by endosomes and the plasmid DNA is translated to the intended protein to result in the activation of APCs. The activated APCs induce T helper cell (Th1) differentiation to suppress the Th2 cells through IFN- γ secretion and decrease the allergic cytokine secretions (IL-4 and IL-5). Moreover, these APCs can create Treg cells with the ability to suppress Th2 cells, as well as arrange the secretion of IgG2a and IgA from B cells through inhibitory cytokines, suppress mucus production, and inhibit other allergic immune cells such as basophils, mast cells, and eosinophils (41, 42).

RNA interference (RNAi)

RNA interference (RNAi) mediated by short interfering RNA (siRNA), short hairpin RNA (shRNA), and microRNA (miRNA) (43) is a natural cellular process that acts as a gene silencer throughout the eukaryotic system at transcriptional, posttranscriptional, or translational levels (44-46). In this process, siRNA molecules function as the key mediators for inhibiting target gene expressions. Owing to their high

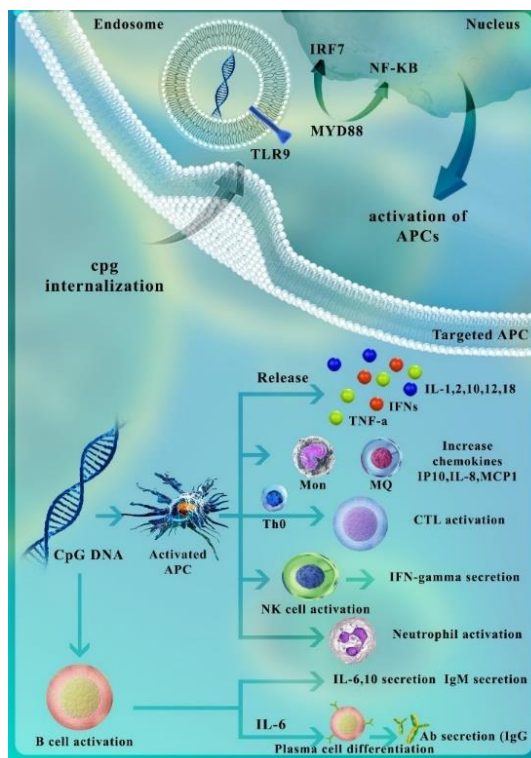


Figure 1. Mechanism of effects of ODNs on immune system
As CpG ODN oligonucleotides are internalized in APCs, activated APCs release various cytokines and chemokines, causing effector immune cell differentiation and activation; TLR: toll-like receptor, IRF: interferon regulatory transcription factor, NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells, Mon: monocytes, MQ: macrophage

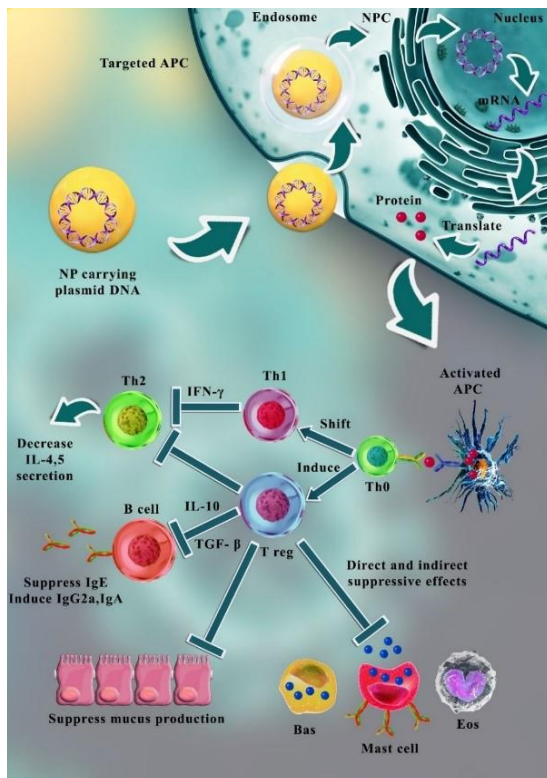


Figure 2. Immune cell modulation through internalization of NP carrying plasmids in suppression of allergic reactions

efficiency, these molecules are considered promising gene silencers and biotherapeutic agents for a wide array of diseases. In addition, siRNA can serve as the guide sequence for creating the cleavage of target-specific Messenger RNA (mRNA) through activation of highly conserved regulatory mechanisms (47). It has been strongly proven that siRNA can be recognized by endosomal (e.g., TLR 3, 7, 8) and cytoplasmic pathways such as retinoic acid-inducible gene I, melanoma differentiation-associated antigen 5, and RNA-activated protein kinase. This process leads to activation of various transcription factors, including NF-κB, interferon regulatory factor 3 (IRF-3), and IRF-7, and consequently result in IFN secretion. Furthermore, T cells, NK cells, and DCs can be also activated through this process (48).

Figure 3 demonstrates the silencing mechanism of programmed death-ligand 1 (PDL1) by PDL-1 siRNA which is a model for reduction of tumor cell survival. Accordingly, long double-stranded RNA (dsRNA) was cleaved through the dicer enzyme (endoribonuclease) next to the production of siRNA, which enabled the cells to assemble the RNA-induced silencing complex (RISC). siRNA becomes unwound upon incorporation of siRNA into RISC and forms a single-strand siRNA that binds to its PDL1 complementary mRNA and creates mRNA cleavage; considerably, this type of mRNA cannot be translated to PDL1 protein (49). Due to lack of PDL1 expression on tumor cell surfaces, immune cells with PD1 on their surface cause a reduction in tumor cell survival, which include cytotoxic T cells (CTL), macrophages (MQ), NK cells, and invariant natural killer T cells (iNKT) (50, 51).

Nano-carriers in immunogene therapy

Nowadays, various biodegradable and biocompatible NPs are exerted in the form of gene delivery vehicles. In addition to their applications as protective genetic materials against

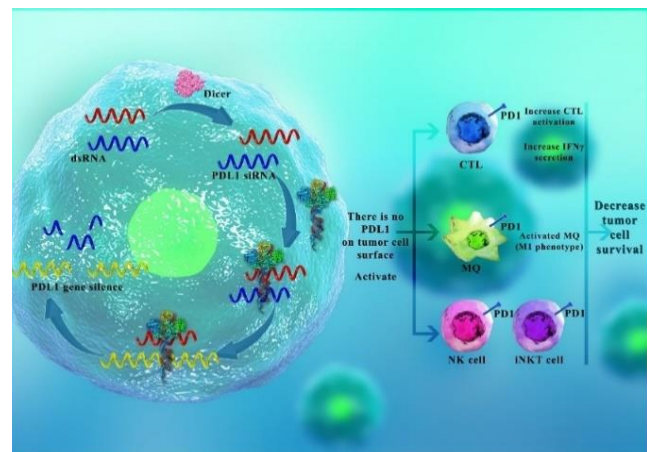


Figure 3. Silencing of programmed death-ligand 1 (PDL1) to decrease tumor cell survival

enzymatic degradation, nano-carriers can efficiently deliver therapeutic agents to the targeted cells. In the following section, we discussed the potential of nano-carriers in immunogene therapy of various diseases including cancer, microbial diseases, allergies, inflammatory bowel disease, rheumatoid arthritis, and respiratory infections.

Cancer

According to recent findings, the applied nano-carriers for gene delivery are capable of modulating immune responses and affecting the tumor microenvironment (TME). In this regard, several studies attempted to focus on this method for treatment of colon and ovarian cancers and melanoma. Figure 4 exhibits the immunomodulatory effects of NPs on cancer. It can be observed that the entrance of NPs containing various immune modulatory genes into the tumor environment leads to the occurrence of changes including the minimized infiltration of tumor-associated macrophages (TAM), modulation of cellular immunity cells (e.g., CTL, Treg, and B lymphocytes), innate immune cell modulation (e.g., NK cells), and modulated secretion of soluble mediators from immune cells (e.g., cytokines and chemokines). All of these alterations may result in to tumor cell necrosis or apoptosis.

Table 1 displays some of the exerted nano-carriers for forming a complex with various genetic materials for cancer immunotherapy.

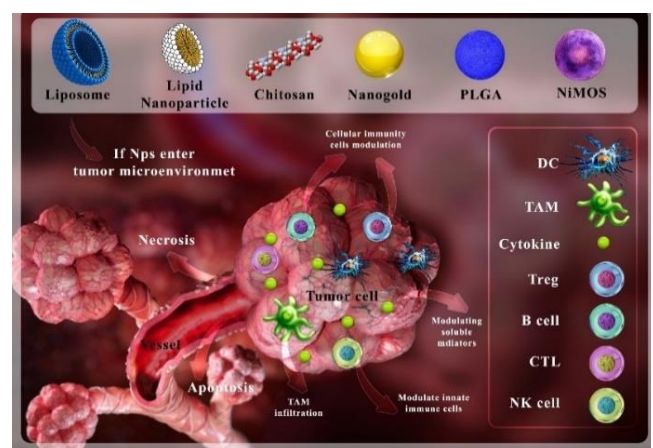


Figure 4. Immune modulatory effects of NPs in the tumor microenvironment

Melanoma

Melanoma has yielded numerous immunogenic epitopes such as tyrosinase-related protein (TRP-2) in the form of a

tumor-associated antigens (52) and low levels of MHC-I (75). Application of MHC-I-dependent CTLs proved to be essential for suppression of tumor growth and inhibition

Table 1. Nanoparticles (NPs)-based immunogene delivery used in cancer treatment

Cancer type	Gene therapeutic agents	NPs	Functional mechanisms	Ref.
Melanoma	Tlr9 agonist	Liposome	High-activity CTL generation	(52)
Melanoma	Trp2 peptide	Lipid-calcium-phosphate (LCP)	DC Activation	(53)
Melanoma	Tlr9 agonist		Modest CTL response	
Melanoma	Trp2 peptide	Gelatinase NP	Moderate DC activation	(33)
Melanoma	Tlr9 agonist		T Cell activation	
Melanoma	Hsp-70 gene	Magnetic cationic liposomes (MCL)	Immune response amplification	(54)
Melanoma	C-myc Antisense	Liposome	CTL activation	(26)
Melanoma			Elicit Pro-inflammatory cytokines	
Melanoma	Ovalbumin	Lipid NP	MHC-I Up-regulation	(55)
Melanoma			Immune Cell activation	
Melanoma	Ovalbumin-TLR9 ligand	MgAl layered double hydroxide (MgAl-LDH)	Increased CD69,86 expression	(56)
Melanoma			Increased CD8 ⁺ T Cells	
Leukemia	PDL1-siRNA	Lipid NP	Increased IFN- γ , IgG1, and IgG2a secretion	(57)
Leukemia	β -galactosidase	Chitosan	Induction of humoral immune responses	(58)
Leukemia	IL-12	Chitosan	Induction of balance between Th 1 and Th 2	(59)
Leukemia	IL-12	LNP (lipid nanoparticles)	Breaking self-antigen tolerance	(60)
Ovarian cancer	PDL1-siRNA	Polyethylenimine (PEI)	Increased CD8 ⁺ T Cell levels	(50)
Ovarian cancer	PDL1-siRNA	PEI	Increased innate immune system pattern Recognition receptors	(51)
Ovarian cancer	miR-155 ds-RNA duplexes	PEI	Type I IFN up-regulation	(61)
Ovarian cancer	IL-12	Liposome	Increased IgG2a/IgG1 ratio	(62)
Lung cancer	Anti-VEGF siRNA	Gold NP	Induced Th 1/Th 2 balance	(63)
Lung cancer	IL-12	PEI-DNA NP	Increased CD8 ⁺ T Cell recruitment	(64)
Lung cancer	IL-12	tumor-targeted micelleplexes	Increased CD8 ⁺ T Cell proliferation	(65)
Hepatocellular carcinoma	IL-12	PLGA DOTAP	CTL degranulation upon Ag re-stimulation	(66)
Hepatocellular carcinoma	PDL-1	Tumor targeted lipid-dendrimer-calcium-phosphate (TT-LDCP NP)	Increased MMP-9 levels	(67)
Hepatocellular carcinoma	IL-2		No Pro-inflammatory and IL-10 changes	
Hepatocellular carcinoma	IL-12	PAMAM	APC activation	(68)
Pancreatic cancer	HIF α siRNA	Lipid polymer hybrid NP	Increased IL-12 and IFN- γ levels	(69)
Colon cancer	IL-12	DMP micelle	Modulated immune system	(70)
Colon cancer	IL-12	Cholesterol-conjugated PEGylated PAMAM	Increased IL-12 and IFN- γ levels	(71)
Colorectal cancer	CCL19	Folate-modified nanoparticle	Increased IL-12 and IFN- γ levels	(72)
Colon cancer	IL-15	Protamine-liposome system (CLPP)	Induced immune responses	(73)
Brain cancer	TGF β	Polybutyl cyanoacrylate nanoparticles	NK cell activation	(74)
			Increased IL-2 level	
			Decreased PD-L1 level	
			Increased T CD8 ⁺ activation and infiltration to TME	
			Increased IL-12 level	
			Decreased IL-6 and IFN- γ Levels	
			T cell infiltration to the tumor microenvironment	
			Increased TNF- α levels	
			Stimulated TH1 differentiation	
			Increased TNF- α and IFN- γ secretion	
			Activated T cells	
			Decreased T regulatory cells	
			Decreased M2 macrophages	
			Decreased MDSCs	
			Increased NK cells	
			Activated TCD8 ⁺ cells	
			Increased TNF- α secretion	
			Increased T CD25 ⁺ cells	
			Decreased TGF β 2 levels	

of tumor recurrence. Down-regulation of MHC-I is a fundamental mechanism for the escape of tumor cells from T-cell-mediated anti-tumor immunity (76, 77).

Xu *et al.* reported the usage of TRP-2-loaded lipid-calcium-phosphate (LCP) NPs in conjugation with CpG ODN (TLR-9 agonist). Accordingly, the surface of NPs was modified with mannose due to expression of its receptor on DCs. The prepared formulation consisted of 50 µg of TRP-2 antigen, and 20 µg of CpG was administered to melanoma-bearing female C57BL/6 mice. In comparison with the other groups, the obtained results were indicative of modest CTL response, mild DC activation signals, and modest antitumor activity (53).

In another study, the melanoma-bearing C57BL/6 mice were pretreated with Heat shock proteins (HSP-70) gene (total amount: 20 µg/mouse), which was combined with induced hyperthermia by magnetic cationic liposomes (MCLs), and succeeded in strongly arresting tumor growth; presumably, activation of CTL through MHC-I up-regulation could be the reason behind this achievement (54). HSP-70 is also capable of acting as an extracellular protein with regulatory effects on monocytes eliciting proinflammatory cytokines for providing innate immunity (78).

According to the findings of Oberli *et al.* the administration of a lipid NP complex with unmodified mRNA-encoding ovalbumin (10 µg of mRNA/mouse) to melanoma-bearing C57BL/6 mice was resulted in blocking tumor growth through the induction of IFN-I up-regulation comparing with control group. IFN-I is an essential factor for CD8⁺ T cell protective immunity. This data could be transfected to various immune cells such as DCs, MQs, Neutrophil (NEU), and B cells due to resulting in strong activation of CD 8⁺ T cells. In this regard, the gathered evidence confirmed the effectiveness of this formulation as an immune system activator and modulator (55).

Another related research was performed and in which the exertion of MgAl-layered double hydroxide (LDH) with a similar chemical composition to Alum was applied in conjugation with TLR-9 ligand CpG and ovalbumin (OVA) for the treatment of melanoma in female C57BL/6 mice. The findings indicated that this nanof ormulation induced an approximately 6-fold higher IgG2a/IgG1 ratio than Alum-CpG-OVA ($P<0.01$), which can strongly direct the immune responses towards Th1 (56). LDH is capable of effectively causing immune responses through several mechanisms including the efficient loading and protection of OVA and CpG, as well as increased uptake of these cargos by APCs and endosomal processing, in which the released CpG interacts with TLR-9 receptor on the surface of endosomes (79). The proposed formulation could also increase the recruitment of CD8⁺ T-cell to TME and consequently block tumor growth. In the mentioned study, the application of MgAl-LDH with immune modulation can efficiently deliver Ag and CpG to APCs in order to provoke the immune system (56).

Li *et al.* reported that the administration of self-replicated IL-12 RNA along with lipid nanoparticles (LNP) to C57BL/6/J and BALB/C mice (10 µg/mice) was lead to a higher IL-12 and IFN γ expression and recruited monocytes, as well as observed the presence of NK cells and T cells throughout the tumor microenvironment and reported the inducement of STING and MYD88 signaling pathway. Furthermore, the outcomes of *in vitro* assays on B16F10 tumor cells indicated the occurrence of innate immunity activation caused by the upregulation of TLR3, MDA5 and IFN1 along with an

increase in IL-12 gene expression. In total, this therapy could modulate tumor microenvironment and increase the rate of immunogenic cell death (60).

Leukemia

Leukemia is a malignant disease that involves the release of an increased number of immature or abnormal leukocytes into the bloodstream or bone marrow. DC vaccines function as T-cell immune response enhancers and stand among the optimal treatment options for leukemia.

A group of researchers conducted an study on human DC vaccine with an improved immunogenic potential by forcing siRNAs to target the incorporated PD-L genes into the lipid NPs (LNPs) in conjugation with Minor histocompatibility antigen (MiHA, a key Ag in the antitumor responses expressed by malignant cells) and mRNA electroporation. Moreover, Immature DCs (iDCs) were generated by culturing the isolated monocytes from peripheral blood mononuclear cells. Their findings pointed out the enhanced proliferation of Ag-specific CD8⁺ T cells in comparison with the control group. However, these specialized DCs were not capable of expressing PDL-1, while the activated T cells remained in highly activated states. In addition, the expanded MiHA-specific CD8⁺ T cells could be efficiently de-granulated upon Ag re-stimulation. In the mentioned study, LNPs were exerted due to their ability to efficiently encapsulate and deliver siRNA through the cell membrane (57). Moreover, considering the common usage of monocytes for the generation of DCs (79), NPs were covered by Polyethylene glycol (PEG) in order to take advantage of its transfection efficiency for human monocyte-derived DCs (57).

CAR-T (chimeric antigen receptor) cells are T lymphocytes engineered in laboratories to express the artificial receptors that would be targeted towards specific antigens in tumor cells.

According to another findings, the delivery of PDL-1-siRNA by the combination of PEI NPs with anti-PDL-1 antibody was results in knockdown of PDL-1 on epithelial ovarian cancer (EOC) cells, which consequently increases the functionality of chimeric CAR-T through higher IFN- γ secretion ($P<0.01$) and CD107a expression on the surface of CAR-T cells ($P<0.05$). CD107a is a lysosomal marker that involves the degranulation activity of CAR-T cells (51). However, CAR-T cell therapy has been undermined by various immunosuppressive mechanisms that are based on EOC cells, as well as the interaction between PD-1 and its ligand that leads to the hyporesponsiveness of T-cells (80-83). Therefore, this method can modulate the immune system through the blockage of PDL-1 on ovarian cancer cells.

Previous findings confirmed the essentiality of miRNAs for regulation of every immune cell type (84, 85). MiR-155 is basally expressed at low levels in B cells (85, 86), T cells (87), MQs (88), and DCs (85) and stands as a perfect example of miRNA functionality throughout the immune system (61). On the other hand, MiR-155 is expressed at high levels in human cancer cells (61). Signals activation (e.g., Ags) can rapidly increase the expression of miR-155 in leukocyte subsets that contain bone marrow DCs and MQs (85-87).

In a study in 2012, the encapsulated miR-155 ds-RNA duplexes (50 µg/mice) by PEI-based NPs were transmitted to C57BL/6 mice that suffered from ovarian cancer. Active miRNA can be bound to the miRNA recognition element on multiple mRNAs, resulting in simultaneously silencing

multiple target genes. Therefore, it is indicated that the treatment can reverse the tolerance activity of ovarian cancer-associated DCs and promote their potential to enhance antitumor immunity through enhancement of robust Ag presentation, production of Th1 cytokines ($P < 0.01$), and expansion of tumor-reactive T cells. According to the mentioned study, the duplexes (double-stranded molecules of nucleic acid) were processed by tumor-associated DCs in order to generate mature miR-155 (61).

Lung cancer

Bronchogenic carcinoma is currently a worldwide highly prevalent malignant disease (64). The infiltrating leukocytes of malignant solid tumors are abundantly filled with MQs (63). The inflammatory and regulatory phenotypes of these tumor-associated MQs (TAM) are exhibited through their M2 markers (89). In fact, TAMs have been recently identified as the key mediators in cancer progression, metastasis, and resistance to therapy (90, 91).

M2 peptide was coupled with gold NPs (AuNPs) and used to deliver anti-VEGF siRNA (0.05 mg/kg) in the sample of BALB/c mice with lung cancer. According to the findings, this hybrid approach could reduce the number of existing MQs in lung tissues when compared with the results of the treated group with solitary AuNPs and siRNA. However, there were no changes observed in the cytokines at the mRNA level along with the lack of any TLR activation. It is notable that the mentioned study succeeded in targeting an angiogenic factor (VEGF) that was produced by inflammatory MQs and lung cancer cells and modulated the recruitment of TAMs (63). Therefore, these observations proved the capability of this therapy in modulating the immune cell infiltration into inflammatory sites.

It was shown that the combination therapy of cisplatin (CDDP) and pIL-12 (plasmid encoding IL-12) lead to achieving a higher rate of antitumor activity and cancer cells apoptosis. Besides, the delivery of this formulation to C57 mice bearing lung cancer cells ((50 µg/mice) resulted in a higher IL-12 expression, increased immune effector cells (T4+, T8+, and NK cells), conversion of M2 MQs into M1 MQs, and higher levels of IFN γ and TNF α . Therefore, this formulation proved to be capable of enhancing the immune responses in the tumor microenvironment (65).

Colon cancer

Men *et al.* introduced the application of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)- modified mPEG-PCL micelles (DMP) for the delivery of plasmid encoding IL-12 gene to colon carcinoma-bearing female BALB/c mice, which increased the level of IL-12 in treated mice in comparison with the control group ($P < 0.001$) (70). IL-12 is an immunomodulatory cytokine with the ability to regulate the immune system through induction of immune cell proliferation (92). According to the obtained results, the proposed method could provide a strong T cell infiltration into the tumor microenvironment and increase the rate of TNF- α secretion from immune cells such as macrophages and neutrophils (70). Thus, it is indicated that successful expression of IL-12 in the tumor microenvironment leads to activation of immune cells and causes inducement of strong antitumor immune responses.

In conformity to another study, the delivery of cholesterol-conjugated PEGylated Polyamidoamine (PAMAM) complexed with plasmid encoding IL-12 into

HT29 cells was resulted in increasing the production of IL-12 when compared with the control group (71). Doubtlessly, IL-12 initiated an immunomodulatory effect in this method through stimulation of TH1 differentiation, which augmented the antitumor activity of the immune system (93).

Considering the findings of Liu *et al.* the administration of folate-modified NPs containing Chemokine (C-C motif ligand) (CCL19) gene to female BALB/c mice with colorectal cancer caused a higher expression of CCL19 when compared with the control group ($P < 0.05$). A higher level of CCL19 can intensify the expressions of IFN- γ ($P < 0.05$) and TNF- α ($P < 0.05$), activate lymphocyte T, decrease regulatory T cells, reduce M2 macrophage (CD45⁺CD11b⁺F4/80⁺CD206^{high}) polarization, and decrease the number of myeloid-derived suppressor cells (MDSCs) (Gr1⁺CD11b⁺) (72). In conclusion, this combination therapy can induce antitumoral activities by activating immune responses.

In another study, an experiment was conducted on the delivery of protamine-liposome system (CLPP)/IL-15 mRNA complex (20 µg protamine /20 µg liposome /10 µg IL-15 mRNA per mouse) to female BALB/c mice bearing colon tumor and resulted in increasing level of IL-15 when compared with the control group ($P < 0.05$). This outcome can modulate immune responses by increasing the population of NK cells and activating T CD8⁺, as well as inducing a high level of TNF- α , IFN- γ , and IL-12 ($P < 0.05$) (73). Undoubtedly, this method is capable of inhibiting tumor cell growth through immunomodulation.

Other cancer types

According to recent studies, other types of tumors can be also modulated by the performance of immunogene therapy.

Hypoxia-inducible factor 1- α (HIF-1 α) is a key transcription factor in tumor development, which is also known to play a fundamental role in hypoxia-mediated apoptosis (94). In a related study, Zhao *et al.* considered the exertion considered the exertion of lipid-polymer hybrid NPs for the co-delivery of HIF-1 α siRNA (1.33 mg/kg) and gemcitabine (4 mg/kg) in female BALB/c mice with pancreatic cancer. Their findings indicated that the levels of IL-6 and IFN- γ were decreased to normal limits when compared with the group treated with free siRNA (69), which signified the possible modulation of immune responses that consequently reduced the inflammatory effects.

In another research, lipid NPs and chemokine receptor type 2 (CCR2) siRNA (0.5 mg/kg/day siCCR2) was administered for the treatment of colon cancer in a C57BL/6 and BALB/C mice, which resulted in silencing the CCR2 miRNA in inflammatory monocytes that consequently inhibited their migration to the inflammatory site. Moreover, it can be inferred that this method modulates the immune cell inducers of inflammation due to the existing CD14⁺CD16⁻ phenotype in the inflammatory monocytes, which can cause a rise in classical MQs that exacerbate inflammation (95).

A dual-targeted immune-gene therapy system was designed based on tumor-targeted lipid-dendrimer-calcium-phosphate (TT-LDCP) NPs for the *in vitro* and *in vivo* delivery of siRNA against immune checkpoint ligand PD-L1 and plasmid DNA encoding immunostimulatory IL-2 to hepatocellular carcinoma cell (HCC). This co-

delivery system resulted in significantly decreasing PD-L1 and increasing IL-2 expression in liver tumor cell lines in comparison with the performed treatment with solitary PD-L1 siRNA or IL-2 pDNA in TT-LDCP NPs. Furthermore, the observations indicated that the significant enhancement of tumoral CD8+ T cell infiltration and activation led to suppression of HCC progression (67).

According to another finding, administration of a TGF- β 2 antisense oligonucleotides and a CMV- β -gal plasmid attaching to polybutyl cyanoacrylate nanoparticles (9.34 nmol/Fisher rat) combined with vaccination with F98 cells infected by New Castle Virus in rats bearing brain tumor, was lead to elevated levels of T CD25+ cells, this indicates the proliferation of activated IL-2 receptor-positive lymphocytes. The TGF- β 2 levels were also significantly reduced (74). This evidence could confirm that this method may increase the survival rate.

It was reported that the delivery of plasmid encoding IL-12 gene in combination with PAMAM into mesenchymal stem cells (MSC), which resulted in increasing the expression of IL-12 from MSCs (68); increased levels of IL-12 can cause inducement of stronger tumor responses (96).

Viral and microbial diseases

According to the literature, immunogene therapy-based nano-carriers can be effective in the treatment of infectious diseases. Table 2 demonstrates the application of nano-carriers against microbial and viral diseases in immunogene therapy.

Piglet paratyphoid is recognized as one of the most severe epidemics caused by *Salmonella* infection (170). Plasmid-encoding IL-2 (6 pmol/mouse) were encapsulated in chitosan NPs for being exerted for the paratyphoid treatment of female BALB/c mice. According to the findings, the applied method increased the levels of IL-2, 4, 6 in peripheral blood when compared with the controls ($P < 0.05$) (32). In addition, IL-2 was able to stimulate T cell proliferation and its clonal expansion (102), while the activated T cells secreted Granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ , and IL-4, which leads

to APC proliferation and recruitment of more specific T cells to the infection site. On the other hand, IL-2 proved to be capable of activating the proliferation of Th2 cells which causes the releasing of IL-4, 5, 6 and stimulated B cell differentiation into mature antibody-producing plasma cells and memory cells, which also promotes the production of Ig-G, A, M (32). Therefore, these data confirmed the effectiveness of this method in significantly promoting cellular and humoral immunity.

Streptococcus pneumoniae is considered a leading cause of invasive pneumonia. Pneumococcal surface antigen A (PsaA) is a lipoprotein that is highly conserved across its serotype (103). In a study, conducted in (2010), chitosan NPs encapsulated with PsaA DNA (50 μ g/mouse) were used for the pneumococcal treatment of Specific-pathogen-free (SPF) female BALB/c mice. According to the results, the ratio of IgG1/IgG2a was balanced and a significant increase was induced in the expression of IL-17A and IFN- γ ($P < 0.01$) (97). Recent related studies reported that IL-17 is mainly released from Th17 cells and has the ability to promote host immunity through the recruitment of neutrophils to mucosal sites and provoking their bactericidal potential (104, 105). Therefore, this method can be applied to efficiently modulate the immune system.

The liver maintains a tolerogenic environment against persistent viral infections, such as the *hepatitis C virus*, and often takes advantage of hepatic tolerance. PDL1-siRNA-cationic lipid NPs (containing 0.5 mg of siRNA/kg) were exerte for the treatment of hepatic disease in C57BL/6 mice, which resulted in the occurrence of PDL-1 surface expression knockdown. This inducement increased the effector function of CD8+ T cells and caused the release of granzyme B (GrB) from these cells that consequently improves their virus clearance potential. Furthermore, GrB secretion from the NK cells was enhanced without causing any changes in effector function (98). In viral hepatic infections, the high expression of PDL-1 can be observed on Kupffer cells and T cells, as well as NK, DC, and liver sinusoidal cells (106, 107).

Table 2. Nanoparticle-based immunogene delivery used in treatment of viral and microbial diseases

Microbial disease	Gene therapeutic agents	Nanoparticles	Functional mechanisms	Ref.
Paratyphoid	IL-12	Chitosan	Increased IL-2,4,6 T cell activation, and recruitment to the disease site Th2 cell proliferation Increased APC proliferation Increased GM-CSF and IFN- γ secretion Stimulated B cell proliferation Increased Ig G, A, M secretion	(32)
Pneumococcal disease	PsaA	Chitosan	Induced IgG1/IgG2a balance Increased IL-17A and IFN- γ expression	(97)
Hepatic disease	PDL1 siRNA	Lipid NP	PDL1 knockdown Increased CD8+ effector T cells Increased granzyme B release from NK and CTLs	(98)
<i>Mycobacterium Tuberculosis</i>	HLA	Chitosan	No NK effector function changes Human DC maturation Increased CD 86,83,80 expression Increased IFN- γ secretion	(99)
RSV infection	NS1	Chitosan	Decreased goblet cell hyperplasia Decreased inflammatory cell infiltration Decreased IFN- α and β levels Increased IL-4 and IFN- γ secretion Increased cellular immunity	(100)
RSV infection	RSV antigen	Chitosan	Increased IRF-1 and STAT1 expression Increased IgG Increased nasal IgA Increased IFN- γ levels in lung High CTL generation	(101)

Table 3. Nanoparticle-based immunogene delivery used in allergy treatment

Allergy	Gene therapeutic agents	Nanoparticles	Functional mechanisms	Ref.
House dust mite allergy	Derp 1	Chitosan	Increased immune response	(41)
Allergic asthma	IFN γ	Chitosan	Increased specific IgG2a and IgA	(42)
			Decreased AHR symptoms	
			Decreased eosinophils in BAL fluid	
			Decreased mucosal metaplasia	
House dust mite allergy	Derp1	Chitosan	Decreased airway cellular infiltration	(115)
			Decreased IL-4,5 and IgE secretion	
			Increased IFN- γ	
Peanut allergy	PCMV Arah2	Chitosan	Activated STAT4 signaling pathway	(116)
			Moderate increase in IgG2a levels	
			Suppressed IgE secretion	
asthma	Thymulin	Poly β -Amino Ester (PBAE NP)	Decreased IL-4 and increased IFN- γ secretion	(117)
			Decreased IgE secretion	
			Decreased anaphylaxis symptoms	
			Increased IgG2a and IgA secretion	
			Decreased histamine levels	
Decreased CCL11 and CXCL1 levels	(117)			
Increased CCL17 level				
Decreased IL-4 and IL-13 levels				
			Decreased TGF- β level	
			Increased M1 macrophages	

In another study, the application of DNA plasmid encoding 8 HLA Mycobacterium tuberculosis (Mtb) epitopes, which were formulated in chitosan NPs (containing 25 μ g of DNA per mouse), was considered for the treatment of HLA-A*0201/Kb female transgenic mice infected with *M. tuberculosis*. According to the findings, this method caused an increase in the surface levels of CD86, CD83, and CD80 which led to maturing of human DC (99). It was indicated by other studies that CD80 and CD86 are labeled as T cell costimulatory molecules, while CD83 has the potential of affecting the capacity of DC for stimulating T cells (108). In comparison with the outcomes of the intramuscular route, the application of this formulation was observed to increase the secretion of IFN- γ (99). Therefore, the capability of the proposed treatment method in modulating and activating immune responses was confirmed.

Respiratory syncytial virus (RSV) stands as the most common cause of lower respiratory tract viral infection that can result in inducement of bronchiolitis and otitis (109). According to the literature, nonstructural (NS) proteins, as well as F and G RSV proteins, have a fundamental role in disease progression (110, 111).

Plasmid NS1 siRNA gene complex with chitosan NPs was used for the treatment of BALB/c mice with RSV infection. In the course of the disease, NS1 was observed to suppress STAT1 ($P < 0.05$), IRF-1 activation ($P < 0.01$), and type I IFN secretion from the DCs ($P < 0.05$), while NS1 proteins down-regulated the signaling of the IFN system by inactivating IRF-1 and STAT1. It can be concluded that this technique can decrease the goblet cell hyperplasia of bronchi and reduce the number of infiltrating inflammatory cells throughout the interstitial regions, while increasing IRF-1, STAT1, IFN- β , and IFN- α expression and causing the release of IL-4 and IFN- γ from CD4⁺ and CD8⁺ T cells; thereby, these occurrences result in enhancing the cellular immunity when compared with the control groups (100). In the mentioned study, the nano-formulation was observed to modulate the host immunity.

Chitosan nanospheres containing plasmid encoding all RSV antigen cDNAs were used for the treatment of BALB/c mice with RSV infection, which also resulted in increasing the neutralization of specific IgG and nasal IgA secretion, while increasing IFN- γ secretion in the pulmonary tissues and generating powerful CTL responses when compared with the controls ($P < 0.01$) (101). The literature confirmed

the role of RSV proteins as suitable targets for CTLs along with affirming the direct antiviral effects of IFN- γ , which are essential for stimulating the cytolytic activity of NK cells and CTLs (112, 113). These findings signify the potential of this method for being applicable in the treatment of RSV infections.

Allergies

Allergic diseases or type I hypersensitivity refer to the illnesses that are mediated by the IgE antibody that is produced by the atopic individual after being exposed to safe environmental allergens (114). The oral route is considered as a common track of gene delivery in allergy treatments (41, 115, 116), which provides poor efficacy due to the acidic pH of stomach and gastrointestinal tract enzymes (41). However, specific nano-carriers, such as cationic polymers, were utilized in an attempt to overcome these obstacles. Table 3 presents data on the application of immunomodulatory nano-formulation that contained genetic materials.

In the work of Chew *et al.* plasmid DNA containing Derp1 cDNA (50 μ g) was encapsulated in chitosan NPs to be orally administered to female BALB/c mice that were allergic to house dust mite (Derp1). According to their outcomes, the immune responses against Derp1, as well as the production of specific IgG2a and IgA throughout the systemic circulation, was increased in the sample animals. They also reported the ability of chitosan to facilitate immunity induction by crossing the mucosal epithelial barrier to cause the occurrence of chitosan-DNA complex uptake by M cells and provide translocation of molecules to the underlying lymphoid organs in which APCs are located (41). Therefore, the effectiveness of this method in oral gene delivery was confirmed.

In another study, researchers administered chitosan-IFN- γ pDNA NPs (CIN) (25 μ g/mouse) to allergic BALB/c mice. Their results included the observance of attenuated airway hyper-responsiveness (AHR) ($P < 0.01$), significant reduction in the number of eosinophils in the Bronchoalveolar lavage fluid ($P < 0.01$), decreased mucus cell metaplasia, cellular infiltration into the airways, and significant reduction in IL-4, 5 and IgE ($P < 0.05$). However, the experiment also caused an increase in the secretion of IFN- γ , while the induced immune modulation via the CIN involved the Signal transducer and activator of transcription (STAT) 4-dependent (42). Moreover, the uptake of CIN

occurred through the means of MQs and epithelial cells, both of which play a fundamental role in asthma immunomodulation (118). CIN therapy can facilitate the induction of IFN- γ expression in epithelial cells and lead to immunomodulation. It is also notable that other Th1 cytokines (e.g., IL-12) were also involved in this process and displayed the ability to activate STAT4 (42). Based on these findings, it is inferred that mucosal therapy can decrease the allergen that is induced and established by AHR.

In another research, chitosan NPs that containing plasmid DNA and encoded house dust mite allergen Derp1 (100 μ g/mouse) was conducted in allergic BALB/c mice. Their findings reported the inducement of a moderate increase in IgG2a levels ($P<0.01$), along with the suppression of IgE responses ($P<0.05$), a decrease in IL-4 ($P<0.01$), and an increase in IFN- γ ($P<0.01$) when compared with the groups treated with the allergen alone (only NPs and phosphate-buffered saline). IgG2a and IgE isotypes reflect the type of T cell responses (115) and in conformity to previous discoveries, high levels of IgG2a in conjugation with low levels of IgE can propose the shift of immune responses from Th2 toward Th1 (119). Therefore, it is indicated that this method is effective for the modulation of immune responses towards allergies.

On the same note, chitosan NPs that were composed of plasmid DNA and encoded dominant peanut allergen gene (pCMVArah2; 50 μ g of DNA per mouse) for the treatment of peanut allergy in AKR/J mice. According to their results, the IgE levels and anaphylaxis symptoms were decreased, while IgG2a and IgA levels faced an increase when compared with the control group. In addition, they observed a reduction in the levels of histamine plasma. This method can activate mucosal immunity and systemic immunity (116) since the generation of mucosal immunity can protect the antigens' entry to the systemic immune system and also lead to unresponsive behaviors toward food allergens (120). Considering these data, the proposed method could be presumed as an effective technique for the modulation of anaphylactic responses (116).

Thymic nonapeptide (thymulin or serum thymus factor) is capable of preventing the cascades of inflammatory and fibrotic responses in a mouse model of allergic asthma (121). In this regard, PBAE NPs (Poly (ethylene oxide)-modified poly (beta-amino ester), consisted of thymulin expressing plasmids (50 μ l of NP that contained 50 μ g of plasmid per mouse) were applied for the treatment of asthmatic female BALB/c mice (117). In comparison with the control, the results of the target group displayed a decrease in the levels of CCL11 and CXCL1, which are responsible for recruitment of eosinophil and neutrophil ($P<0.05$) (122). An increase in the level of CCL17 ($P<0.05$) as a fundamental factor in T regulatory transmigration (123) causes a decrease in IL-4 and IL-13 ($P<0.05$) levels that involves the suppression of

Th2 responses, a decrease in TGF- β level ($P<0.05$), and an increase in M1 macrophages ($P<0.05$) (117). Therefore, this method can effectively suppress allergic responses through immunomodulation.

Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is an inflammatory condition caused by dysregulation of mucosal immunity to the luminal antigens derived from the intestinal microflora (124, 125). IBD has two main phenotypes including Crohn's disease (CD) and ulcerative colitis (UC). The condition of CD mostly affects the end of the small intestine and the beginning of the colon, while UC only affects the colon (126). Table 4 exhibits the application of nano-carriers against IBD in immunogene therapy.

In a related study, the application of nanoparticles-in-microsphere oral system (NiMOS) was reported for the delivery of pDNA-encoding IL-10 (100 μ g/mouse) throughout the treatment of UC in BALB/c mice. The obtained results were indicative of increased IL-10 levels ($P<0.05$) and decreased TNF α , IL-12, IL-1 β , and IFN- γ . Furthermore, colitis inflammation was detected in histological examinations when compared with the control group. About IBD, the levels of Th1 cytokines (e.g., TNF α , IL-12, IL-1 β , and IFN- γ) were up-regulated in order to enable IL-10 to modulate Th1-derived cytokines. Their other findings reported reduced levels of monocyte chemoattractant protein-1 (MCP-1) and Macrophage inflammatory protein-1 α (MIP-1 α) without mentioning any observance of direct action in between these chemokines and IL-10 (127). Therefore, it can be inferred that this transfection and oral gene delivery is an effective option for IBD treatments.

In another study, the conjugation of NiMOS NPs with TNF- α siRNA were utilized for the treatment of UC in BALB/c mice. The obtained results were indicative of a decrease in TNF- α when compared with the control group ($P<0.05$) (126). According to related evidence, this cytokine has a fundamental functionality throughout modulation of inflammatory conditions in IBD (130). Furthermore, this study reported achievement of a lower expression of GM-CSF, MIP-1 α , and MCP-1, which are accountable for regulating cell infiltration into the disease site and seem to be directly correlated with inflammation. Moreover, the solitary usage of NiMOS can cause immunomodulation effects and moderately down-regulate IL-1 β ($P<0.001$), IL-5 ($P<0.001$), MIP-1 α , and GMCSF (126). Based on these data, this formulation can be effective and applicable for the treatment of IBD.

Relatively, TNF- α siRNA-polyethyleneimine noncomplex (100 μ g/kg) was used in C57BL/6 mice with IBD. The obtained results included the inducement of a

Table 4. Nanoparticle-based immunogene delivery used in IBD treatment

IBD	Gene therapeutic agents	Nanoparticles	Functional mechanisms	Ref.
IBD	IL-10	NiMOS	Decreased IL-10,12,1 β and TNF α and IFN- γ secretion Decreased colitis inflammation	(127)
IBD	TNF- α siRNA	NiMOS	Decreased MCP-1 and MIP-1 α expression Decreased TNF- α secretion	(126)
IBD	TNF- α siRNA	PEI	Decreased GM-CSF, MIP-1 α , and MCP-1 expression Down-regulated IL-1 β and IL-5 secretion Regulated cell infiltration to the inflammatory site	(128)
IBD	TNF- α siRNA	PLGA	Decreased TNF- α levels	(129)

Table 5. Nanoparticle-based immunogene delivery used in RA treatment

Rheumatoid arthritis	Gene therapeutic agents	Nanoparticles	Functional mechanisms	Ref.
RA	Anti-TNF α dicer substrate siRNA	Chitosan	Decreased TNF- α and IFN-I release from MQs Non-induction of innate immune response Decreased inflammatory cell infiltration	(135)
RA	TNF- α siRNA	Liposome	Decreased TNF- α secretion Decreased IL-6 and MCP-1 locally Increased IL-10 secretion Induced balance between Th1 and Th2 through increased IL-4 and decreased IFN- γ	(136)

significant decrease in TNF- α levels when compared with the control group ($P < 0.01$) (128), and also displayed the ability of nano-complex in mediating inflammation in IBD.

In another study, researchers loaded TNF- α siRNA into the galactosylated PLGA NPs and co-delivered this formulation with IL-22, which was embedded in chitosan/alginate hydrogel (20 $\mu\text{g}/\text{kg}$ of siTNF and 50 $\mu\text{g}/\text{kg}$ of IL-22), to the FVB male mice with UC. The essential functionality of IL-22 in mucosal healing is undeniable. Considering the induced decrease in TNF- α expression as a result of this procedure ($P < 0.05$), it is evident that the blockade of TNF- α expression leads to inhibition of IL-22 production (129), which signifies the ability of this co-administration to facilitate the recovery of UC through immunomodulation.

Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is associated with joint destruction caused by the synovial infiltration of Th1 cells (131). As an important cytokine in RA, TNF- α is involved in inflammatory conditions (132) and therefore, its targeting at the RNA level can stand as an effective therapeutic approach for the treatment of RA (133, 134). Table 5 represents the application of immunogene therapy by the usage of various nano-formulations in RA treatment.

In a related study, chitosan NPs that contained the unmodified anti-TNF- α dicer substrate siRNA (DsiRNA) (2.5 μg) were transfected to DBA/mouse with RA. The results of this method included a decrease in TNF- α ($P < 0.01$) and IFN type I ($P < 0.05$) from the MQs when compared with the untreated groups, which led to observing the lack of innate immune responses (135). During the acute and chronic phases of arthritis, the activated peritoneal MQs release inflammatory cytokines such as IL-1,6 and TNF- α (137). In this regard, the proposed method can be considered as a novel strategy for RA treatment due to succeeding in reducing joint destruction and inflammatory cell infiltration (135).

In conformity to another study, the application of cationic liposome, containing TNF- α siRNA, in DBA/1 mice with RA caused a decrease in TNF- α release throughout the knee joint and led to the local reduction of IL-6 and MCP-1 while increasing the secretion of anti-inflammatory cytokines (e.g., IL-10) when compared with the control group. Therefore, this method was confirmed to be effective in RA treatment since TNF- α suppression can significantly balance the induced changes between Th1 and Th2 in the draining lymph node by causing an increase in IL-4 secretion while decreasing the IFN- γ levels (136).

Conclusion and future perspective

Immunogene therapy is potentially considered an effective approach for the treatment of various complex disorders due to being capable of targeting cells more specifically and providing control over the expression of

therapeutic genes. On the other hand, the local and single-dose application of most gene therapies results in the exertion of high therapeutic doses with a low risk of systemic side effects. In this case, the U.S. Food and Drug Administration (FDA) has reviewed and confirmed all comments about clinical trial of immunogene therapy in combination with AstraZeneca's Tagrisso[®] in patients with late-stage non-small cell lung cancer (NSCLC) whose disease progressed after treatment with Tagrisso in Genprex, Inc. (138). Various gene delivery systems have been developed to improve the delivery efficiency of genetic materials. Most of the applied carriers in human clinical trial phases for immunogene therapy include different types of viral vectors. The combination of AdCD40L (an adenovirus carrying CD40 ligand gene, a key activator of adaptive immunity) in phase I/II study (NCT01455259) with cyclophosphamide chemotherapy was exerted for patients with metastatic malignant melanoma (139). In addition, clinical-grade chimeric antigen receptor (CAR) T cells were engineered by viral vectors and established to mediate tumor rejection. There are important limitations due to uncontrolled responses as a consequence of constitutive expression of the CAR molecules on the surface of T cells (140). So, more standard manufacturing processes are required for immunogene T-cell therapies (141).

Viral vectors were also exerted for the development of clinical trials with critical inflammatory cytokine gene therapies for cancer treatments.

There are reports on the application of nano-systems in clinical or preclinical studies for the delivery of therapeutic genes. Nano-carriers offer prominent advantages such as safety, high targeting potential, low toxicity, cost-efficiency, and easy preparation. However, several factors can possibly affect the immune responses of NPs such as the composition, size, surface chemistry, transfection efficiency, cytotoxicity, and exposure route of carriers.

Most of the immunogene therapy studies by the usage of NPs were conducted with animal models, which provide a strong preclinical basis for human clinical approaches. Therefore, further clinical trials are required in order to clarify the therapeutic efficacy and possible toxic effects of exerted nanosystems in immunogene therapy.

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Authors' Contributions

MH Supervised the research; SSH and FO Draft manuscript preparation; SSH and EH Critically revised the paper. SSH, FO, EH and MH Read and approved the the final version to be published.

Conflicts of Interest

The authors declare that there are no relevant financial or

non-financial competing interests to report.

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