Chapter 10
Polymer-Based DNA Delivery Systems for Cancer Immunotherapy

Ayelet David and Adi Golani-Armon

Abstract The use of gene delivery systems for the expression of antigenic proteins is an established means for activating a patient's own immune system against the cancer they carry. Since tumor cells are poor antigen-presenting cells, cross-presentation of tumor antigens by dendritic cells (DCs) is essential for the generation of tumor-specific cytotoxic T-lymphocyte responses. A number of polymer-based nanomedicines have been developed to deliver genes into DCs, primarily by incorporating tumor-specific, antigen-encoding plasmid DNA with polycationic molecules to facilitate DNA loading and intracellular trafficking. Direct in vivo targeting of plasmid DNA to DC surface receptors can induce high transfection efficiency and long-term gene expression, essential for antigen loading onto major histocompatibility complex molecules and stimulation of T-cell responses. This chapter summarizes the physicochemical properties and biological information on polymer-based non-viral vectors used for targeting DCs, and discusses the main challenges for successful in vivo gene transfer into DCs.

Keywords Antigen presenting cells • Cancer immunotherapy • Cationic polymers • Chitosan • Dendrimers • Dendritic cells • DNA vaccine • Immunization • Gene delivery • Polyamidoamine • Polyethylenimine • Poly(lactic acid) • Poly(lactide-co-glycolide) • Poly(glycolic acid) • Poly(lysine)

Abbreviations

Ag	Antigen
APC	Antigen-presenting cell
BMDCs	Bone marrow-derived dentritic cells
CRD	Carbohydrate recognition domain
CLR	C-type lectin receptor

A. David (✉) • A. Golani-Armon
Department of Clinical Biochemistry and Pharmacology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
e-mail: ayeletda@bgu.ac.il

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CTL	Cytotoxic T-lymphocytes
DCs	Dendritic cells
ER	Endoplasmic reticulum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
iDCs	Immature DCs
MΦs	Macrophages
MHC	Major histocompatibility complex
MR	Mannose receptor
MRD	Minimal residual disease
NK	Natural killer
NLS	Nuclear localization signal
OVA	Ovalbumin
PAMAM	Polyamidoamine
PEG	Poly(ethylene glycol)
PEI	Poly(ethylenimine)
PIC	Polyion complexes
PLGA	Poly(lactide-co-glycolide)
PLL	Poly(L-lysine)
PS	Polystyrene
SiRNA	Small interfering RNA
TAA	Tumor-associated antigen
TAP	Transporter associated with antigen presentation
WTC	Whole tumor cells

10.1 Introduction

Cancer has long represented a major burden on health and longevity [1]. The high prevalence of the disease has made it the basis of a major research focus, with the disease being investigated in many contexts. Intensive research has yielded a large body of information that has served to uncover biological, biochemical and pathophysiological aspects of the disease, as well as their underlying mechanisms. Based on the information gathered by the efforts of numerous researchers, novel nanosized medicines (nanomedicines), that can be effectively localized to tumors and actively taken up by cancer cells have been developed, and are expected to replace traditional chemotherapy and possibly irradiation due to increased efficiencies and attenuated adverse side-effects [2].

Unfortunately, recent years have seen only minor improvements in cancer morbidity and mortality rates, with the most commonly used cancer therapies still being conventional chemotherapy, despite their serious side-effects. This has encouraged researchers to seek answers in the field of immunotherapy [3]. Whereas exogenous anti-cancer agents have failed to generate strong and effective responses against tumors and induce regression, the endogenous activity of the immune

system was hoped to elicit specific anti-tumor immunity that would result in tumor regression and elimination. In particularly, dendritic cell (DC) vaccinations were expected to induce immunological memory that would enable recognition and destruction of residual tumor cells that evaded earlier treatment, thereby avoiding recurrence of the disease [4]. Previous studies employing mouse models indicated that the establishment of anti-tumor immunity required the presentation of tumor-associated antigens (TAA) by DCs [5, 6]. Two unique properties make DCs the most potent antigen-presenting cells (APCs), namely their ability to present exogenous antigens on major histocompatibility complex (MHC)-I molecules to prime $CD8^+$ T-cells (i.e., cross-presentation), and their capability to initiate, activate and modulate the various arms of the immune system in a coordinated manner. Indeed, in vivo targeting of tumor antigens into DCs was shown to elicit strong TAA-specific $DC4^+$ and $CD8^+$ immune responses [7]. Therefore, DCs loaded with TAA or tumor antigen-encoding plasmid DNA may facilitate the development of new immunotherapies for cancer treatment. This chapter highlights the repertoire of non-viral, nanosized polymeric DNA delivery systems (polyplexes) available to achieve efficient gene transfer into DCs for immunotherapeutic applications in cancer therapy. The physicochemical characteristics and surface properties of polyplexes required for efficient gene transfer and gene expression in DCs are discussed. The influence of the ligand valency on the targeting of DCs via dendritic cell surface receptors is also described.

10.1.1 Cancer Immunotherapy

The field of immunotherapy aims at activating a patient's own immune system against a host disease. Immunotherapy includes the investigation of different mechanisms leading to activation of the immune response, and the development of methods to control such responses in a desired manner so as to combat disease, either as a single strategy or in combination with other treatments. In the specific case of cancer immunotherapy, it is speculated that the in vivo activity of endogenous immune cells would be far more sensitive and specific than would any exogenous treatment [8]. Anti-cancer vaccines have, thus been designed to induce both tumor-specific effector T cells and tumor-specific memory T-cells [4]. Such anti-cancer vaccination, it is hoped, will induce regression of established tumors, as well as prevent the onset of secondary tumors and metastases, possibly by addressing the issue of minimal residual disease (MRD). In this case, sparse cancerous cells that evaded irradiation or chemotherapy will be detected and destroyed by the immune system cells that, unlike the temporary exogenous treatment, are present in the body at all times. A major requirement for an efficient anti-cancer vaccination is the generation of a cytotoxic T-lymphocyte (CTL) response, but manipulation of the other arms of the immune response, mainly $CD4^+$ T helper cells, is critical for a robust and long lasting CTL response [9–11]. CTL response generation depends on antigen (Ag) presentation on MHC to naïve $DC8^+$ T cells. The poor Ag presentation

activity demonstrated by tumor cells [12] highlights the need for professional APCs to generate the desired CTL response. Among the several types of APCs in the body, DCs are the most suitable for this purpose.

10.2 Dendritic Cells

10.2.1 Dendritic Cell Biology

In 2011, Ralph Steinman was awarded the Nobel Prize for his 1973 discovery of DCs. DCs are a family of professional APCs. As the most powerful APCs, they are equipped with specialized machinery that enables them to regulate the initiation of a primary immune response [13–15]. Originally derived from bone marrow, DCs are seeded in all tissues, where they sample their environment in an attempt to detect tissue damage, pathogen entry, inflammation and malignantly transformed cells by taking up particles and molecules, processing them into short peptides and presenting the resulting peptides on MHC molecules. DCs use different routes to capture Ags, including phagocytosis, micro- and macropinocytosis, and receptor-mediated endocytosis [9, 15, 16]. Importantly, receptor-mediated endocytosis can be exploited for the targeting of TAAs into DCs.

Immature DCs in peripheral tissues are characterized by high Ag capture activity, low surface MHC levels and co-stimulatory molecules, as well as a tendency to induce tolerance to self-antigens. Upon recognition of "danger" signals, DCs undergo a maturation process characterized by a reduction of Ag capture activity and an increase of MHC and co-stimulatory molecule expression on the cell surface [17]. Mature DCs migrate to adjacent lymph nodes, where they present antigenic peptides to naïve T-cells [4]. Ag presentation, co-stimulatory molecules expression, and the secretion of an appropriate set of cytokines and chemokines by DCs facilitate the differentiation of naïve $CD4^+$ and $CD8^+$ T-cells into effector T-helper or T-cytotoxic cells, respectively [18, 19]. DCs can, thus, simultaneously activate both a CTL response to directly kill pathogens or affected cells and a T-helper response to recruit and enhance the activity of other immune cells in the right context. This unique ability of DCs to activate the various arms of the immune system to achieve a powerful and efficient immune response makes them the most promising candidates for immunotherapeutic manipulations.

10.2.2 Antigen Presentation Pathways in Dendritic Cells

Three distinct pathways are used by different cell types for the presentation of antigens, namely MHC class I and class II antigen presentation, and cross-presentation.

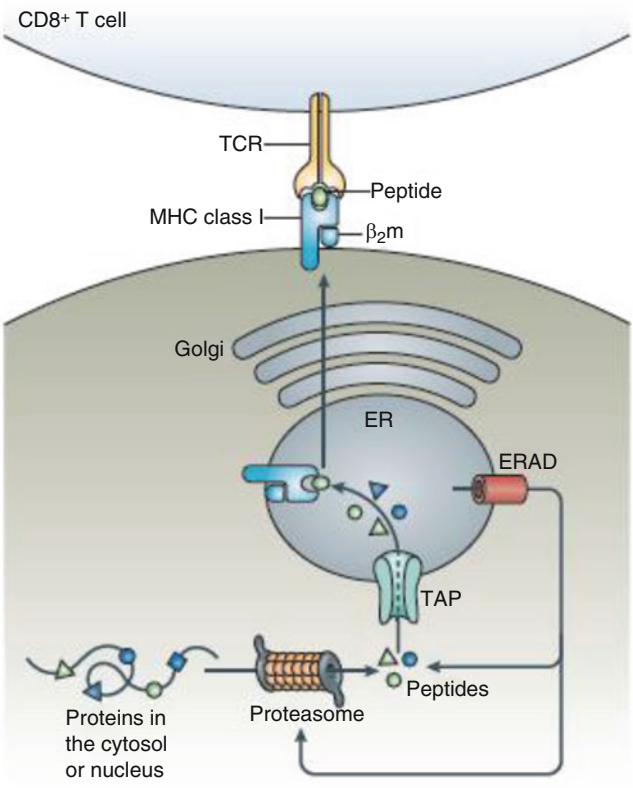

Fig. 10.1 MHC class I processing and presentation pathway. Taken with permission from Ref. 18

10.2.2.1 MHC Class I Antigen Presentation

This pathway is present in all nucleated cells, and is used to present endogenous antigens derived from the nucleus or the cytoplasm. MHC class I antigen presentation can, therefore, report intracellular bacterial or viral infections, as well as malignant transformation in the presenting cell. The resulting intracellular products are degraded in the cytoplasm by the proteasome, translocated into the endoplasmic reticulum (ER) via the transporter associated with antigen presentation (TAP), and loaded on newly formed MHC class I molecules. The MHC class I/peptide complexes are then exported along the constitutive secretory pathway to the cell membrane, where they are presented to naïve $CD8^+$ T-cells. The interaction between MHC class I/peptide complexes on DCs and T-cell receptors (TCR) on $CD8^+$ T-cells induce the differentiation of naïve $CD8^+$ T-cells into cytotoxic T-cells that are capable of killing the infected or transformed cells (Fig. 10.1) [14, 18, 20].

10.2.2.2 MHC Class II Antigen Presentation

This pathway is restricted to APCs, including macrophages (MΦs), DCs and B lymphocytes, and is used to present exogenous antigens. The exogenous antigens are taken up into these cells by endocytosis, where they are found in early endosomes, a mildly acidic cell compartment containing a small amount of proteases. Early endosomes develop into late endosomes that are more acidic and present higher proteolytic activity, which process the captured Ag into short peptides suitable for MHC presentation [21]. The α and β chains of the MHC class II molecule are assembled in the ER and are associated with the Invariant chain (Ii) that

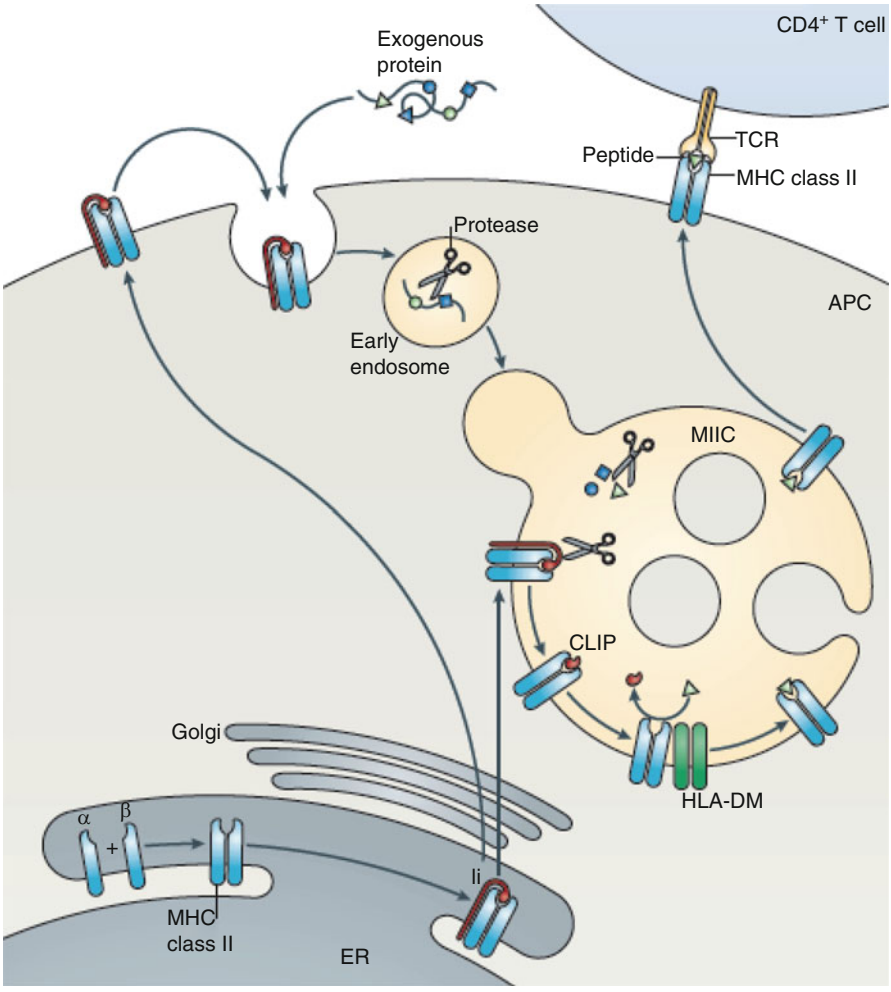

Fig. 10.2 MHC class II processing and presentation pathway. Taken with permission from Ref. 18

occupies the peptide-binding groove of the complex. The MHC class II molecule is then transported to the late endosome, also referred to as the MHC class II compartment, where, following partial digestion of Ii by cathepsins, it finally binds the exogenous peptide. The MHC class II/peptide complexes are transported to the cell membrane for peptide presentation to $CD4^+$ T-cells, and induction of T-helper response to support and enhance the activity of other immune cells (Fig. 10.2) [18].

10.2.2.3 Cross-Presentation

The generation of a CTL response against virally infected or transformed cells is possible through the MHC class I pathway described above. However, other kinds of threats can be addressed by the immune system APCs through the MHC class II pathway, but this pathway exclusively generates a T-helper response. As it is obvious that not every viral infection or malignant transformation involves APCs, and that many pathogens can impair antigen presentation by their host cells, an alternative mechanism must exist that would enable APCs to generate a cytotoxic T-cell response against the various exogenous threats they encounter [22]. Such a pathway indeed exists and is a unique property of DCs. In addition to the MHC class I and MHC class II antigen presentation pathways, DCs can present exogenous antigens on MHC class I molecules to $CD8^+$ T-cells. This pathway is termed cross-presentation, implying that it involves mechanisms from both the MHC class I and the MHC class II pathways [23]. Although first described more than three decades ago, the details of this pathway remain only poorly understood, with two main theories attempting to explain the mechanisms leading to MHC class I loading of endocytosed exogenous antigens. The cytosolic track suggests that the antigen is translocated from the endosome into the cytosol, where it enters the cytosolic processing pathway. The endocytic track, on the other hand, suggest that MHC I molecules are recycled from the cell membrane to the endosome, where they are loaded with antigenic peptides processed by endosomal proteases [21, 24].

10.2.3 The Role of DCs in Anti-Tumor CTL-Response Generation

As mentioned earlier, anti-cancer immunity requires the generation of a specific anti-tumor CTL response that, in turn, requires proper antigen presentation. As part of the many techniques tumor cells use to avoid or weaken the immune system, they can substantially reduce the expression of MHC class I/peptide complexes on their cell surface. Since tumor cells are, thus, poor Ag-presenting cells, cross-presentation of tumor antigens by DCs is essential for the generation of tumor-specific CTL responses [4]. In addition, given the unique ability of DCs to initiate and orchestrate

the various arms of the immune system, including recruitment and activation of macrophages, natural killer (NK) cells, T-helper cells and B-cells, a strong and comprehensive anti-cancer immune response can hopefully be achieved by inducing such cells to present an appropriate tumor antigen [10].

10.3 Dendritic Cell-Based Cancer Vaccination

10.3.1 DC Immunization

DC immunization requires that an appropriate antigen be presented to DCs in a proper manner and in the right immune context so as to allow Ag uptake, processing and presentation on MHC molecules, parallel to DC maturation and T-cell priming. The first method developed for DC immunization involved the use of whole tumor cells (WTC) or tumor cell lysates, either alone, mixed with an adjuvant, or genetically modified to express an adjuvant. The major advantage of this method is that no Ag identification is required, and multiple Ags are being delivered simultaneously. Nevertheless, all WTC vaccine methods showed limited efficiency in clinical trials, probably due to insufficient interaction between the tumor antigens and the DCs [25]. Pulsing DCs with antigenic proteins or peptides corresponds to another approach. Protein-pulsed DCs are capable of presenting Ags on both MHC class I and MHC class II molecules, with a long half-life of MHC class I presentation and no HLA restriction (i.e., no need for patient selection), but they require an appropriate processing of the protein by DCs, which may be difficult to achieve. Peptide-pulsed DCs, on the other hand, require no processing. Still, they show limited MHC class II presentation, a short half-life of MHC class I presentation, and a HLA restriction. Specific or total tumor mRNA can be processed and presented on both MHC class I and class II molecules. This, however, requires mRNA extraction from a tumor sample and is, thus, patient-specific [15, 26]. Lately, the delivery of tumor Ag-encoding plasmid DNA has emerged as a promising method for DC immunization [19, 27].

10.3.2 DNA Vaccines

DNA vaccines are DCs that were genetically modified to express TAAs. DNA vaccines are advantageous for several reasons. They enable the presentation of multiple epitopes of full-length TAAs on MHC class I and class II molecules, and since processing occurs within the cell, they are not HLA-restricted. In addition, efficient gene transfer allows for a continuous supply of peptides in the modified DC [10]. Genetic modification of DCs can be achieved either by ex vivo gene delivery methods, including use of a gene gun, electroporation, ultrasound and microinjection, or by in vivo approaches, including naked DNA delivery, and viral or synthetic

vectors [8, 27]. Ex vivo delivery offers many advantages. The extracted cells are cultured and undergo maturation under controlled conditions and in the absence of inhibitory signals provided by the tumor cells, such that maturation status can be determined before re-administration. Since transfection occurs in culture, high specificity is achieved as only the selected cells are transfected. Nevertheless, ex vivo gene delivery carries substantial limitations. The process is laborious, costly and time-consuming [12]. Patients must undergo cytopheresis, followed by culturing and maturation of the acquired cells, steps that must be performed for each patient separately. Reproducibility is very low and different quality control methods are used. Thus, only a limited number of patients would be expected to benefit from this approach. Hence, ex vivo gene delivery is not likely to become widely marketable. Moreover, transfected mature DCs can show poor distribution from the injection site and ex vivo maturation can impair DC trafficking to lymph nodes, a critical requirement for cross-priming [28, 29]. In contrast, in vivo gene delivery may be expected to become "off the shelf" therapy. As a single product suitable for all patients, in vivo gene delivery can be produced on a large scale with lower costs. Simple and uniform manufacturing and product control procedures will enable reproducibility and control over product quality. In addition, DCs can be targeted at different sites and in their natural environment, thereby not impairing their natural course of maturation and activation [25, 29]. For these reasons, in vivo gene delivery is considered by many to be the best strategy for Ag delivery into DCs [8, 10, 19].

10.3.3 In Vivo Gene Delivery

In vivo gene carriers must meet important requirements. First, they should be able to incorporate their plasmid DNA cargo into the core of the nanoparticle and be stable enough to carry such cargo in the circulation and protect it from degradation. Second, they should be able to selectively target the desired cell type and be properly internalized. Third, they must facilitate escape from the endosome, cytoplasm trafficking, nuclear transport and DNA unpacking [30]. To date, the most efficient gene delivery systems are viral vectors. Using small amounts of DNA, viral vectors can induce high transfection efficiency and stable, long-term gene expression. Unfortunately, viral vectors possess some serious safety issues, including toxicity, immunogenicity and oncogenicity, with numerous clinical trials having been terminated because of this [15, 31, 32]. Restricted gene size is another major limitation of viral vectors. Synthetic vectors correspond to cationic lipids or cationic polymers, respectively termed lipoplexes or polyplexes, which electrostatically bind the negatively charged DNA. The main drawback of such vectors is their low transfection efficiency, as compared to viral vectors. Yet, synthetic vectors are simple, safe, easy to manufacture on a large scale and can carry plasmids of unrestricted size. Moreover, they can be easily modified to possess desirable properties, including targetability, serum stability, reactivity to external signals and an ability to stimulate

an immune response. In fact, cationic polymers themselves may provide immune stimulation and mimic nuclear localization signals (NLS), facilitating nuclear transport of their cargo [9, 30].

10.4 Polymer-Based Systems for DC-Targeted Gene Delivery

Various polymeric carriers for DNA and RNA delivery have been developed in the last decade as alternatives to viral vectors. Cationic polymers with free primary, secondary and/or tetiary amines were the earliest DNA carriers investigated as transfection reagents in mammalian cells. Such polymers can condense large genes into smaller structures, protect DNA from enzymatic degradation, and can mask the negative charges of DNA, a prerequisite for successful transfection of most types of cells. Some cationic polymers, such as poly-(L-lysine) (PLL), L-polyethylenimine (L-PEI) and chitosan, are linear, while others, like B-PEI and polyamidoamine (PAMAM) dendrimers, are highly branched chains [33]. Branched cationic polymers, such as PEI and PAMAM, exhibit high transfection efficiency in mammalian tissues. Micro- and nanoparticulate systems which adsorb or encapsulate oligonucleotides or genes, based on, for example, poly(lactide-co-glycolide) (PLGA), polycyanoacrylate, polyorthoesters, gelatin, alginate or chitosan, are also under investigation as sustained release matrices for genetic drugs. Some of the most important polymer-based systems utilized for DNA delivery into APCs are discussed below.

10.4.1 Poly(L-Lysine)

Poly(L-lysine) (PLL) was the first cationic polymer developed for gene delivery. PLL is a linear polypeptide presenting L-lysine residues in repeat units (Fig. 10.3c). This first generation cationic polymer bears ε-amino groups that electrostatically bind the negatively charged nucleic acids, and interact with the negatively charged cell membrane. The large number of active functional amine groups allows for easy modification with targeting ligands [34]. However, the transfection efficiency of PLL is very low because it cannot mediate escape from the endosomal compartment and release into the cytosol. To overcome this barrier, PLL is usually used in combination with chloroquine, although use of this agent is limited due to its cytotoxicity [35]. Another disadvantage of PLL polyplexes is that transfection efficiency is significantly influenced by serum, probably due to the rapid binding to negatively-charged serum components [36]. Furthermore, in vivo applications of PLL polyplexes are complicated by a high level of cytotoxicity [37] and lack of in vivo stability [38, 39]. Because of low transfection efficiency, imidazole groups (pKa ~ 6.5) were introduced into PLL to improve buffer capacity, thereby, enhancing transfection efficiency [40]. PLL with high imidazole content mediated high

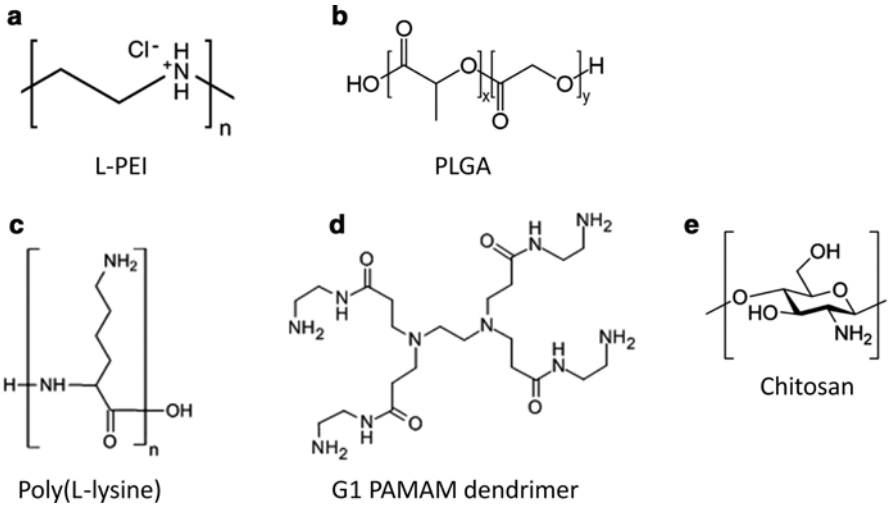

Fig. 10.3 Structures of (**a**) linear PEI, (**b**) PLGA, (**c**) poly(L-lysine), (**d**) first generation (G1) PAMAM dendrimer and (**e**) chitosan

gene transfection efficiency, with gene expression levels close to those of PEI (discussed below), and exhibited low cytotoxicity. The PEGylation of PLL can also improve its cytotoxicity profile [36].

Finally, whereas it has been found that PLL/DNA complexes can be taken up by DCs, PLL/DNA did not alter DC phenotype through surface marker expression [41]. Complexation of plasmid DNA encoding for chicken egg ovalbumin (OVA) with PLL-coated polystyrene (PS) particles induced high levels of CD8+ T-cells as well as OVA-specific antibodies in C57BL/6 mice, and further inhibited tumor growth after challenge with OVA expressing tumor cells [42]. PLL-based microspheres displaying mannan or mannoside-modified surfaces (for targeting the mannose receptor, discussed below) were readily phagocytosed by both DCs and macrophages, however neither surface-assembled mannan- nor mannoside-modified microspheres could stimulate DC maturation [43]. Thus, despite the promising results shown in early studies, PLL is not likely to find clinical applications [30].

10.4.2 Poly(ethylenimine)

Poly(ethylenimine) (PEI) is a second generation and one of the most useful polycations for gene delivery [44]. Linear PEI has only secondary amino groups that are almost all protonated under physiological conditions. Branched PEI presents not only primary and secondary amines but also tertiary amines. As such, only about

two-thirds of the amino groups in branched PEI are protonable under physiological conditions. The transfection efficiency of PEIs depends on the molecule weight, the PEI nitrogen/DNA phosphate charge ratio (N/P) and the cell type. As every third position on the polymer backbone is occupied by a protonable amino group (Fig. 10.3a), its cationic charge density is very high, allowing for condensation of the negatively charged DNA. Under physiological conditions, only about 20 % of the PEI nitrogen atoms are protonated, leaving the other 80 % to facilitate the important step of endosomal escape by the so-called "proton sponge effect" [30, 45, 46]. Thus, PEI is the most popular and most effective polymeric transfection reagent cited to date [47]. However, the positive charge of PEI/DNA polyplexes cause some serious problems, including adsorption to cells and negatively charged blood components, recognition by the immune system components, resulting in rapid clearance from the circulation, and cytotoxicity to non-target cells [44, 48]. To overcome these limitations, PEI has been conjugated to hydrophilic polymers (i.e., poly(ethylene glycol) (PEG) [49], or hyaluronic acid (HA)) of different molecular weights [50]. PEG is wildly used in drug and gene delivery systems to shield charged, immunogenic or toxic segments, resulting in less toxic "stealth" particles that can evade the immune system and, thus, avoid rapid clearance from the circulation [51, 52]. The conjugation of PEG to PEI was previously shown to increase polyplex solubility and serum stability and reduce cytotoxicity by shielding the high positive charge of PEI [49]. PEGylated PEI, however, showed lower transfection efficiency, as compared to PEI/DNA complexes, and also lacked cell-specificity [53]. Ligation of PEI/DNA complexes to molecules targeting DC uptake receptors can significantly increase transfection efficiency.

Approaches for targeting DC receptors have generally involved either natural receptor ligands or the use of antibodies raised against specific receptors. Targeting DC cell surface receptors may also provide cell activation signals [4, 54]. Different DC receptors have been used to facilitate targeted delivery of gene and drug carriers into DCs, including β-integrins (CD11b and CD11c) [55], CD40 [56] and the Fc receptor [57], but the most studied proteins for this purpose are members of the C-type lectin receptor (CLR) family [58]. CLR is a family of receptors sharing structural homology in their carbohydrate recognition domain (CRD), where specific sugar residues are bound in a calcium-dependent manner. One of the CLRs, the mannose receptor (MR, CD206), is the most widely used receptor for targeting DCs, using both mannose and mannan [59]. While several studies demonstrated that mannosylated-PEI conjugates are effective in gene delivery via MR [60–62], their transfection potential for primary human and mouse DC was found to be rather low [60]. This might be due to the low affinity of carbohydrate ligands to their receptors, which is usually in the low millimolar range [63], thus, limiting in vivo transfection efficiency. The eight adjacent CRDs in MR may help to increase the binding affinity and specificity of polyplexes containing mannosylated glycans in a multivalent display [59, 64]. We have recently described the design of multivalent mannosylated PEI/DNA complexes bearing mono- and trivalent mannose as a ligand for targeting MR-positive DCs [65]. Complexes bearing mono- and trivalent mannose (Man-PEG-b-PEI/DNA and Man_3-PEG-b-PEI/DNA, respectively) were safe and demonstrated significantly higher in vitro transfection efficiency in DCs. The mannosylated

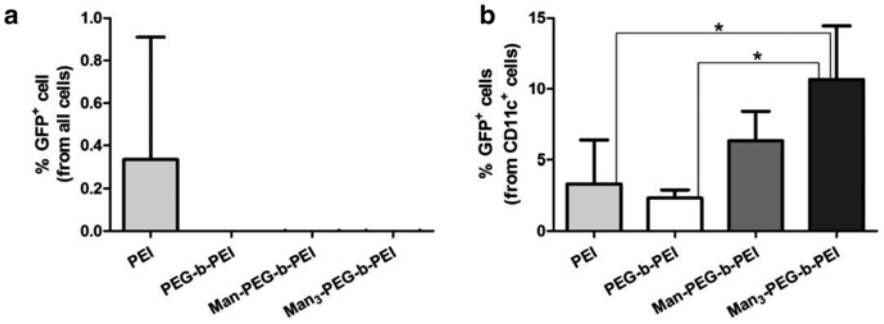

Fig. 10.4 In vivo uptake of mannosylated-PEI/DNA complexes by cells in the draining lymph nodes of C57/BL6 mice 24 h after subcutaneous injection. Results represent the percentage of GFP+ cells in the entire cell population (**a**) and in the CD11c+ cell population (**b**). Taken from Ref. 65

complexes were injected into the tail base of C57/BL6 mice, and 24 h post-injection, the percentages of GFP positive cells in the entire cell population and in the CD11c + cells taken from the inguinal lymph nodes were measured. When examining the entire cells population extracted from the lymph node, only PEI/DNA complexes showed detectable GFP activity, but no significant change was observed between the treatments (Fig. 10.4a). However, Man_3-PEG-*b*-PEI/DNA was significantly more efficient in transfecting CD11c+ DCs collected from inguinal lymph nodes, as compared to polyplexes prepared with PEG-b-PEI/DNA or PEI/DNA (Fig. 10.4b).

One challenge of targeting the MR, as holds true for all of the other above-mentioned receptors, is that they are not exclusively expressed on DCs, and are also on several other cell types [57]. MR, for example, is also expressed on monocytes, subsets of endothelial cells and tumor-associated macrophages [9]. Furthermore, the synthesis of carbohydrate ligands and analogs, especially multivalent or complex carbohydrates, often requires many time-consuming, low yielding steps [66]. The stereochemistry of such carbohydrate ligands is difficult to control, and the products are difficult to purify. A frequently used alternative for carbohydrate ligands are antibodies raised against DC receptors (i.e., integrin CD11c/CD18 [55], Fc receptors [67], DEC-205 [7], DC-SIGN [68] and MR [69]), but these, even when humanized, may still elicit adverse immune responses that decrease the efficiency of treatment and induce auto-immune side-effects [70].

The use of peptide ligands remains, nonetheless, a promising method to target DC receptors. Such ligands are easily synthesized, do not possess immunogenicity, and show high selectivity to DCs (Table 10.1). Accordingly, peptide ligands that could serve as DC-targeting moieties have been sought.

A DC3-nona-arginine fusion peptide (DC3-9dR) that binds nucleic acids by electrostatic interactions was previously exploited for the specific delivery of siRNA to DCs in vitro and in vivo [78] and was also used to silence immunosuppressive molecules in DCs so as to induce strong human T-cell immune responses [79]. The DC3 targeting peptide (Table 10.1) was recently studied by our group to mediate the specific delivery of PEGylated-PEI/DNA polyplexes into DCs [80]. Polyplexes

Table 10.1 Examples for peptide ligands used for targeting DCs

Peptide name	Amino acid sequence	Characteristics	Reference
C-GRWSGWPADL-C	C-GRWSGWPADL-C	A circular peptide that binds to human CD11c/CD18, which shares homology with the D4 domain of intracellular adhesion molecule (ICAM)-1	[71]
APEDNGRSFS	APEDNGRSFS	Derived from ICAM-1, and shares homology with C-GRWSGWPADL-C	[72]
DC3	FYPSYHSTPQRP	Identified using a phage display peptide library. Binds specifically to CD11C+ cells but not monocytes, T and B lymphocytes, or NK, endothelial or fibroblast cells. The cognate receptor for DC3 on the DC surface is unknown	[73]
P-D2	VTLTYEFAAGPRD	Derived from the Ig-like domain 2 of intercellular adhesion molecule 4 (ICAM-4)	[74]
TP	TPAFRYS	Identified by phage display method. The counterpart receptor for TP on BMDC surface is unknown	[75]
NW	NWYLPWLGTNDW	Identified by phage libraries on monocyte-derived immature DCs (iDCs)	[76]
Pan HLA DR-binding peptide	PADRE	MHC class II-binding pan-DR peptide, acts directly on iDCs to induce differentiation into mature DCs, with potent T cell-stimulating capacity	[77]

show significant transfection efficiency in DCs when decorated with DC3 peptide but not in endothelial cells. The transfection efficiency observed was higher than that of PEI/DNA, signifying the potential use of DC3-bearing polyplexes for immunotherapy via DCs.

10.4.3 Polyamidoamine (PAMAM) Dendrimers

Polyamidoamine (PAMAM) dendrimers are hyper-branched, symmetrical, flexible and monodisperse polymeric molecules (Fig. 10.3d). Szoka et al. firstly investigated PAMAM cascade polymers as non-viral gene delivery vectors [81]. These polymers have an ammonia initiator core and amido-amine repeat units of different

generations (G2–G10). Their stepwise synthesis results in products that are uniform in terms of structure and size [82] and bear a well-defined number of primary and tertiary amines. The surface primary amines are protonated, resulting in an extremely positively charged surface that enables DNA binding in stable complexes, and interaction with the cell membrane. The tertiary amines in the interior of the polyplexes are protonable, endowing the dendrimers with pH buffering capacity that enables endosomal escape of the polyplexes following cellular uptake [83]. All these properties, in addition to their non-immunogenic nature, make PAMAM dendrimers an alternative to the highly efficient and highly immunogenic viral vectors. However, a major drawback of PAMAM dendrimers is that low generation dendrimers (G5 or lower) show poor transfection efficiencies, while dendrimers of high generation (G6 and higher) are efficient transfection agents, yet possess serious cytotoxicity [82]. In addition, synthesis of high generation dendrimers is a high cost, labor-consuming process that last several days, making them less likely to be designed for large scale production [83].

Mannosylated G4-PAMAM dendrimers conjugated to OVA specifically targeted DCs and induced cross-presentation in vivo [84]. Moreover, pre-immunization with mannosylated PAMAM-OVA leads to delayed onset of B16-OVA melanoma development, slower kinetics of tumor growth and increased survival of OVA-immunized mice. G4-PAMAM dendrimers bearing DC-SIGN ligand in multivalent presentation also achieved efficient DC targeting properties, however, did not affect DCs maturation [20]. It has been postulated that mannosylated dendrimers, as opposed to DC-SIGN-modified dendrimers, may trigger not only DC-SIGN, but also other mannose-specific CLRs that contributes to DC maturation and activation. G5-PAMAM dendrimers conjugated to MHC class II-targeting peptide (PADRE, Table 10.1) and surface-loaded DNA have been shown to effectively transfect murine and human APCs in vitro [85]. When applied subcutaneously, this conjugate preferentially transfected DCs in draining lymph nodes, promoted generation of high affinity T-cells, and elicited rejection of established B16 tumors.

10.4.4 Chitosan

Chitosan is a linear polysaccharide (Fig. 10.3e) obtained by deacetylation of its parent polymer chitin, a compound that is widely distributed in nature. Chitosan is composed of randomly distributed ß-(1-4)-linked D-glucosamines (deacetylated units) and N-acetyl-D-glucosamines (acetylated units) and has an apparent pKa value of 6.5 [86]. Chitosan is a biodegradable and biocompatible polymer, with low or no immunogenicity and antibacterial activity [86, 87]. It was previously shown to be non-toxic in both test animals [88] and humans [89]. Due to these properties, chitosan is attractive for drug and gene delivery. The high density of positive charges along the polymeric chain contributes to the condensation of the negatively charged DNA, and more frequently, siRNA molecules, into compact structures, thus protecting them from degradation by blood nucleases, promoting

their cellular uptake [90]. Chitosan also possess adhesive properties by interacting with glycoproteins in the mucus [91], and is thus used in muco-adhesive drug delivery systems as an adsorption enhancer [92]. DNA plasmid incorporated into chitosan nanoparticles was able to induce DCs maturation and increase IFN-γ secretion from T cells after pulmonary mucosal immunization [93]. However, unless modified, chitosan has low solubility at physiological pH, which significantly limits its use in many applications [87, 92]. Chemical modifications of chitosan have been performed to improve its solubility at physiological pH, including PEGylation or quaternization of the amine groups of chitosan. However, the transfection efficiency of chitosan-based derivatives reported so far is generally not superior to that of PEI [94].

Various DC-targeted chitosan-based DNA delivery formulations has been designed to overcome the major obstacles facing the clinical development of chitosan, namely, the lack of cell-specificity and low transfection efficiency. Mannosylated chitosan/DNA complexes were more efficient in transfecting DCs, as compared to water-soluble chitosan/DNA, and induced better INF-γ production from DCs [95]. Mannosylated-chitosan-entrapping PEI/HBV-DNA complexes induced significantly enhanced serum antibody production and CTL levels after intramuscular immunization [96]. Biotinylated chitosan nanoparticles were modified with bifunctional fusion protein (bfFp) vectors for achieving DC-selective targeting. bfFp is a recombinant fusion protein consisting of truncated core-streptavidin fused to an anti-DEC-205 single chain antibody (scFv). Intranasal administration of plasmid DNA-loaded bfFp/chitosan nanoparticles, along with anti-CD40 DC maturation stimuli, enhanced the amount of mucosal IgAs, as well as systemic IgGs, against nucleocapsid (N) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) [97]. Finally, to improve low transfection efficiency, chitosan-linked-PEI/DNA complexes have been designed, and showed high transfection efficiency and low cytotoxicity towards DCs [98]. Vaccination with DCs transfected with chitosan-linked-PEI/DNA encoding gp100 (melanoma-associated antigen) slightly improved resistance to the B16BL6 melanoma challenge.

10.4.5 Micro- and Nano-Particulate DNA Delivery Vectors

Polymeric particulates have been shown to be efficient in delivering plasmid DNA into APCs (reviewed in [99–101]). A key advantage of particulate vectors relative to other non-viral gene delivery systems is their superior in vivo stability. The principal types of polymers studied in this context include those made of poly(lactide) (PLA; reviewed in [99]), poly(lactide-co-glycolide) (PLGA; reviewed in [99, 100, 102]), polyorthoesters [103], polystyrene (PS) [104, 105] and poly(ε-caprolactone) [106]. Of these, PLGA has been studied most extensively in terms of its capacity to stimulate APCs.

10.4.5.1 Poly(lactide-co-glycolide)

Poly(lactide-co-glycolide) (Fig. 10.3b) is an FDA- and European Medicine Agency-approved polymer for drug delivery systems for parenteral administration [107]. This biocompatible and biodegradable polymer slowly degrades in vivo by hydrolysis, and its byproducts (lactic and glycolic acid) are easily metabolized and excreted. DCs and macrophages appear to have high affinity for PLGA particles, as a high level of internalization of antigen-loaded particles has been demonstrated in both in vitro and in vivo settings [108, 109]. PLGA microspheres carrying protein antigens or antigen-encoding plasmid DNA are capable of eliciting potent antigen-specific immune responses [100, 110, 111]. PLGA also affected expression maturation markers and cytokine production in DCs [112]. This capacity appears to be driven by unique physical features of PLGA particles (i.e., surface charge, shape and the rate of polymer degradation [100]). The mechanisms leading to maturation induction in DCs, however, remain unclear. With respect to their stability in PLGA particles, antigen-encoding plasmid DNA offers advantages over protein-based immuno-modulators, which can lose their biological activity in response to small changes in their tertiary and quaternary structures during formulation.

DNA delivery into DCs can be improved by using ligand-decorated PLGA particles. Mannose-grafted PLGA nanoparticles lead to a significant enhancement of OVA accumulation in the inflamed colon compared to the healthy one, underlining the benefit of active targeting of macrophages and dendritic cells in diseased tissues [113]. Mannan-decorated OVA-loaded PLGA nanoparticles simultaneously enhanced antigen-specific CD4+ and CD8+ T-cell responses in vaccinated mice [114]. Modification of PLGA micro-particles with P-D2 peptide (Table 10.1) significantly improved DC antigen presentation in vitro, and increased the rate and extent of microsphere translocation by DCs and macrophages in vivo [110].

PLGA has been also utilized in combination with other cationic polymers for gene expression in DCs, with [115] or without targeting ligands [116, 117]. PLGA scaffolds encapsulating PEI/DNA and granulocyte-macrophage colony-stimulating factor (GM-CSF) led to a significant increase in gene expression, and high levels of expression that persisted for a period of time [116]. Yet, since PLGA nanoparticles are hydrophobic in nature, they tend to form aggregates that reduce the efficiency of the system [108]. In addition, PLGA particles are opsonized by the immune system components, and degraded before reaching their destination. Another drawback of PLGA for delivery of genetic vaccines stems from their sustained release property [102]. PLGA particles release their cargo very slowly, over days or even weeks, but in vivo they may be exocytosed from the cell or degraded in the lysosome over much shorter periods of time, before sufficient amounts of cargo are released [35]. Moreover, most DCs die within 7 days after activation and migration to draining lymph nodes [118], hence even fast degrading PLGA systems, which fully release the encapsulated DNA within few weeks, cannot meet with the rapid release kinetic criteria and thus fail to induce high levels of target gene expression [108]. Finally, hydrolysis of PLGA leads to low pH within the particle and thus to DNA degradation [102]. For these reasons, despite the promising results obtained with animal models [114] and clinical trials [119, 120], PLGA has been of limited use in this sense [121].

10.5 Conclusion

The delivery of antigens into DCs carries tremendous potential for immune modulation, and specifically, for cancer immunotherapy manipulations. Various polymeric nanomedicines for DNA delivery, ranging from linear homopolymers to block copolymers, branched polymers, as well as combinations of different types of polymers, selected to reduce cytotoxicity and facilitate endocytosis of particles into DCs, are now been routinely examined for their ability to modulate DCs and macrophages. Ultimately, DNA delivery systems that can also induce DC maturation and activation would be advantageous. The main challenges for successful in vivo gene transfer into DCs are the cytotoxicity associated with many cationic polymer gene carriers, the lack of cell specificity and relatively low transfection efficiency, when compared to viral vectors. Together with the fact that DCs compose only 1–3 % of cells in peripheral tissues, such gene systems will have limited success unless targeted. Given the number of targeting molecules, immune-modulatory agents, chemokines, growth factors and antigens that can be considered for DC-specific delivery, large numbers of potentially useful formulations for DC manipulation are available. Targeting DCs in vivo with tumor antigen-encoding plasmid DNA can elicit effective and long-lasting tumor antigen-specific immunity, with minimal inconvenience to the patient. Furthermore, DNA delivery formulations that are stable for extended periods of time and which can enhance antigen presentation on both MHC-I and MHC-II molecules are preferable. With respect to clinical translation, efficacious non-viral gene delivery into DCs will depend on the combination of intelligent material design, the appropriate tumor specific antigen-encoding DNA and immuno-stimulatory molecules to promote DC maturation and activation.

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