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Immune Responses to PEGylated Lipoplexes, a Review

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Abstract

Gene therapy is the "products that mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered microorganisms. The products may be used to modify cells *in-vivo* or transferred to cells *ex-vivo* prior to administration to the recipient". Generally, gene therapies must retain two main criteria: (a) they contain an active material consists of nucleic acids such as deoxyribonucleic acid (DNA) or small interfering ribonucleic acid (siRNA). These macro molecules have the ability to regulate, repair, replace, add, or delete a genetic sequence; (b) the therapeutic, prophylactic, or diagnostic effect of these molecules are related directly to its gene sequence they contain or to the product of gene expression of this sequence. Gene therapies are directed mainly to treat multiple incurables, debilitating, and genetic diseases which have never treated by small active ingredients. Comparing data of cancer, genetic diseases, and autoimmune diseases with other diseases or to the actual number in the last decade reflects the obvious failure of conventional therapies in the treatment or controlling these diseases. However, the therapeutic effect of these macro molecules is hampered by higher sensitivity, lower stability, non-specific biodistribution, and lower cell permeability. PEGylated lipoplexes are the most common gene delivery non-viral based systems for transferring macromolecules such as DNA. Whatever the immune responses to PEGylated lipoplexes could limit their efficient contribution. In this review, immune responses to PEGylated lipoplexes were studied in detail.

Keywords
PEGylated lipoplexes, Immune responses, ABC phenomenon, Anti-PEG IgM, Nucleic acids, B cells

1. Introduction

Therapeutic nucleic acids (TNAs) are nucleic acids or closely related molecules used for the treatment of specific diseases[1]. TNAs are complexed, high molecular weight, and charged macromolecules with physicochemical properties different from small active molecules. There are several TNAs available and under clinical trials. But it can be divided into two broad classes. Firstly, DNA-based therapeutics such as antisense oligonucleotides (ASOs), DNA aptamers, plasmid DNA (pDNA), and gene therapy. Secondly, RNA-based therapeutics such as micro RNAs (miRNA), small interfering RNA (siRNA), messenger RNA (mRNA), ribozymes, and circular RNA.

The main principles of TNAs are based on the type of the delivered nucleic acids. One approach through the binding of ASOs to a specific mRNA sequence associated with a diseased state via Watson and Crick base pairing. This binding process consequently inhibits the translational event and finally a detrimental and/ or altered protein is produced (antisense). The second approach through inhibition of translational process via targeting duplex DNA, particularly through Hoogsteen base pairing and formation of a triple helix (antigene). The third approach through using plasmid DNA which targets biosynthesis of new healthy proteins. The final approach through targeting the synthesized proteins via DNA or RNA aptamers or using siRNA for gene silencing (**Fig. 1**)[2].

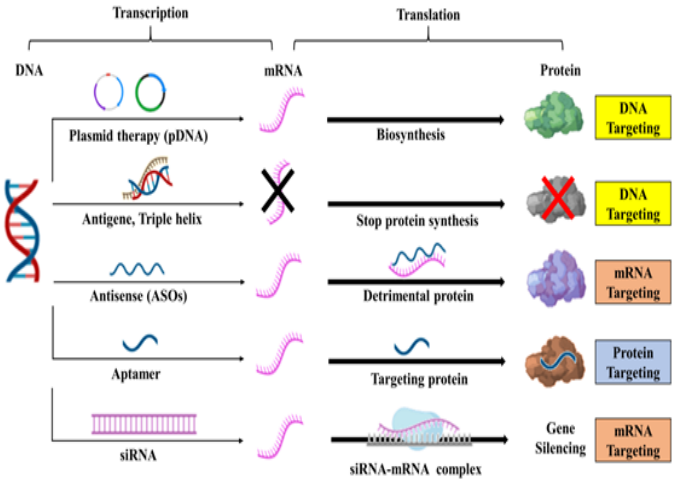

Figure 1: Categories of nucleic acid therapies and targeted sites

2. Nucleic acids drug delivery systems

A plethora of nucleic acid-based therapeutics are available and can be used for human, but unfortunately the clinically approved therapeutics are limited with poor successes. The main drawbacks of nucleic acids are lower stability, shorter lifetime, unpredictable pharmacokinetics, enzymatic degradation, poor cellular uptake, and off target toxicity[3]. However, nucleic-acid

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delivery systems should deserve some ideal properties such as easy to formulate, higher transfection efficiency, lower toxicity and immunogenicity, and pharmaceutically stable[3]. Nucleic acid-based delivery systems include various types, but the most commonly is vector-assisted delivery (viral based and non-viral based systems).

2.1. Viral-based delivery systems

In terms of viral-delivery systems, attenuated nonpathogenic viruses can be used to transfer specific DNA molecules such as pDNA[4, 5]. Viruses can easily transfer DNA to the cells as they are infective agents in nature. The most commonly used viruses are adenoviruses, lentiviruses, parvoviruses, and retroviruses[6,7]. The most advantage of viruses as gene delivery systems is higher transfection ability even for cells that extremely difficult to be transfected such as smooth muscles and human endothelial cells[8, 9]. Virus-based delivery systems occupy about 70% of clinical trials in gene delivery worldwide[10]. The only available and approved gene delivery drug is Gendicine® which is a transgene pDNA delivered using a recombinant adenovirus vector for the treatment of head and neck squamous cell carcinoma[11]. Unfortunately, the higher transfection ability of viruses is hampered by the higher immunogenic responses[12]. Lethal immune responses have been developed in animal models and in all clinical trials. Higher mortality rates, 1999 deaths of a patient participating in FDA-adenovirus gene delivery system, lead to suspension of all clinical trials in the USA[13]. In addition, potential risk of mutations, lacking of targeting, and have a limited length of nucleic acids are common drawbacks and need to be resolved[14].

2.2. Non-viral nucleic acid delivery systems

Higher immunogenicity of viral vectors promotes the emergence of new TNAs delivery systems. Non-viral based systems are gradually inclined to be the mainstream of gene delivery during the last decade. Non-viral systems are classified into two main classes: cationic lipoplexes, and cationic polyplexes, the former is concern of the present study[14]. Alongside, many other systems are available such as dendrimers, cationic polymers, and nanoparticles[15].

3. Cationic lipoplexes

Cationic lipoplexes or cationic liposome-nucleic acid complexes are the most studied non-viral vectors for delivery of nucleic acids[16]. These complexes are composed of cationic lipids, which are preferable for interacting with negatively charged DNA or siRNA (**Fig. 2**), as well as with neutral lipids and cholesterol[17]. Cationic lipids also facilitate the interaction of lipoplexes with cell membranes via electrostatic interaction and trigger internalization of the lipoplex mainly via endocytosis[18]. Lipoplexes prepared via mixing of equal volumes of the genetic materials (DNA or siRNA) and cationic liposomes at room temperature. Cationic liposomal system is generally composed of three main components: cholesterol, neutral lipids, and cationic lipids.

Cholesterol (**Fig. 3**) is the most common lipid component used for the preparation of all different types of liposomes. Cholesterol is particularly acting as a stabilizer for the liposomal system via different mechanisms. The steroid can control the tenacity and/ or stoutness of the liposomal system via increasing the backing of phospholipids[19], improving vesicle resistance to aggregation[20], reducing liposomal permeability to electrolyte and non-electrolyte solutes[21], and increasing system rigidity via affecting liposomal fluidity[22]. Our most important goal of lipoplexes is achieving higher transfection efficiency (TE). Some data explained the role of cholesterol in increasing the TE of lipoplexes. Cholesterol acting mainly via decreasing the hydration layer of lipoplexes. Consequently, minimizing the hydration repulsion with the anionic membrane of endosomes, and finally enhancing the endosomal release of lipoplexes[23]. Unfortunately, increasing cholesterol content to a certain limit results in a decreased encapsulation efficiency[24], liposomal structure deformation, and anaphylactic reactions due to autoantibodies against cholesterol[25].

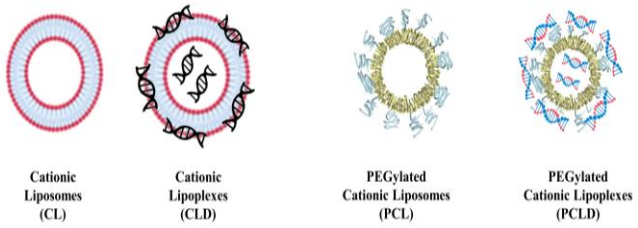

Figure 2: Cationic liposomes (CL), lipoplexes (CLD), PEGylated cationic liposomes (PCL), and PEGylated lipoplexes structure (PCLD).

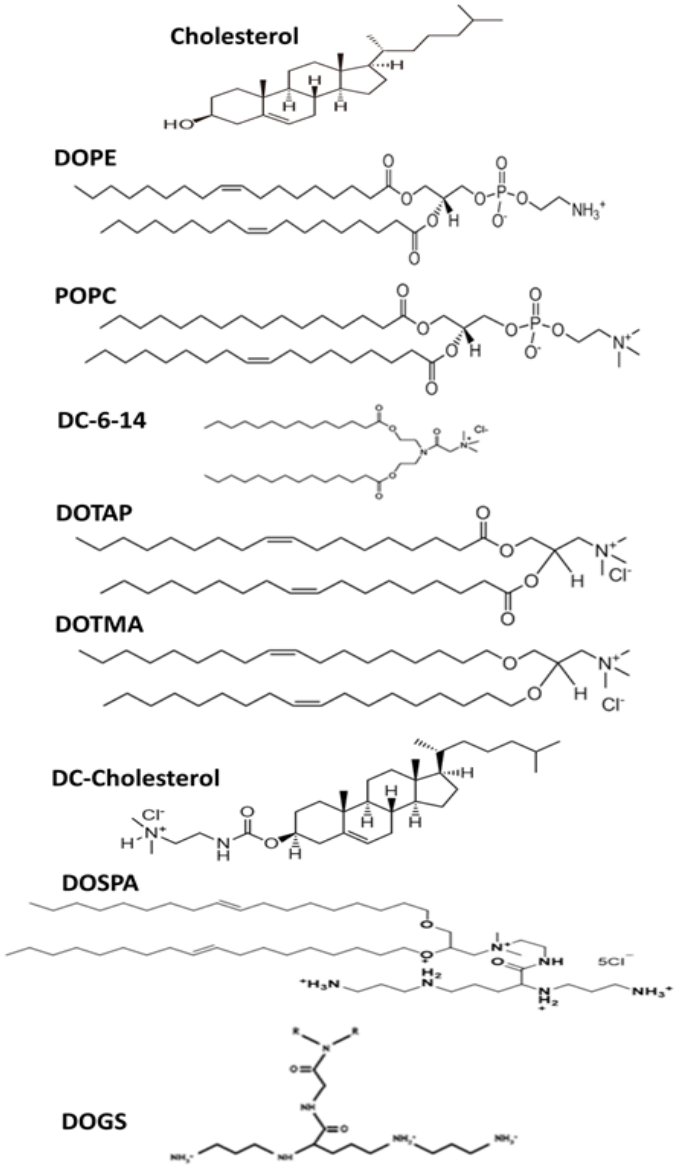

Figure 3: Chemical structure of most commonly lipids used in the preparation of cationic liposomes and lipoplexes.

Neutral lipids or helper lipids affects the lipoplexes action as they improve and enhance TE to a significant extent[15]. Inclusion of helper lipids affects the lipoplexes electrostatics, lipid hydration, and the way of lipids self-assemblies[26]. Two commonly neutral lipids (**Fig. 3**) are 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)[27]. The enhanced transfection efficiency is thought to be due to the conformational changes of the liposomal structure at low pH. Neutral lipids allow conformational changes in the liposomal structure to hexagonal conformation. It is well established that the hexagonal conformation allows for the efficient escape of lipoplexes from the endosomal compartment via membrane destabilization. Also, DOPE stabilizes the DNA complex via forming a salt bridge between the positively charged head groups of cationic lipids and the phosphate groups of DOPE molecules[15].

Cationic lipids (**Fig. 3**) are positively charged head groups followed by hydrophobic tails with different compositions[28]. To ensure efficient TE, shorter, and unsaturated hydrocarbon chains are used. There are a plethora of cationic lipids available for liposomal preparation include: monovalent cationic lipids, multivalent lipids, and ionisable lipids[29]. The most commonly used are O,O-ditetradecanoyl-n-(α-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14), and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt, DOTAP). Utilization of DOTAP or DC-6-14 alone for the preparation of lipoplexes is not preferred due to the higher charge density that requires higher energy to separate DNA from the complex inside the cell[15]. Other types of cationic lipids are available such as 1,2-di-o-octadecenyl-3-trimethylammonium propane (DOTMA), and Dc-cholesterol[16, 17]. Multivalent cationic lipids also available such as 2,3-dioleyloxy-n-[2-(sperminecarboxamido) ethyl]-n,n-dimethyl-l-propanaminium trifluoroacetate (DOSPA) which commercially available in a combination with DOPE (3:1 molar ratio) as Lipofectamine®. Another type called Di-octadecyl-amido-glycyl-spermine (DOGS), and commercially available as Transfectam®[30].

4. PEGylated cationic lipoplexes (PCLD)

Conventional lipoplexes, unfortunately, showed lower stability and shorter blood circulation due to the rapid aggregation with blood cells or rapid opsonization of cationic lipoplexes, and in turn recognition with the cells of mononuclear phagocyte system [27, 31]. PEGylation has been one of the attractive solutions to increase lipoplex stability and prolong their circulation half-life [32, 33]. It is well known that PEG acts through increasing the surface hydrophilicity of the coated particles via the formation of hydrogen bonding with water molecules. The formed hydration layer in tandem with the flexible chains of PEG forming a steric barrier against serum proteins. Also, PEG molecules significantly increase the hydrodynamic size of nanoparticles and affect their physicochemical properties such as conformation, electrostatic binding, and steric hindrance[34]. Regarding the multiple chemical and physical changes, PEGylated lipoplexes show longer blood circulation and higher stability via decreasing protein opsonization, macrophage uptake, and stability against enzyme degradation [35, 36].

5. Barriers against PCLD

Several barriers are facing the TE of PEGylated cationic lipoplexes (PCLD). Barriers could be classified as follows: extracellular barriers, and intracellular barriers[37]. Extracellular barriers including serum proteins as most of them are negatively charged proteins that bind to PCLD. The binding of proteins and opsonins to PCLD leads to rapid clearance from blood circulation with lower therapeutic efficiency. The PEGylation process could decrease protein binding to a certain extent, but the remaining positive charge of PCLD can attract some of the circulating proteins. Binding of serum proteins forming PCLD-protein complex called "protein corona" and specifically can bind to phagocytic cells like macrophages and dendritic cells[37, 38]. Intracellular barriers could be summarized in the cellular uptake and both lysosomal and endosomal uptake of lipoplexes. The harish acidic environment of endosomes could degrade the lipoplexes with loss of TE[38, 39]. The most important point in the present study is the immune responses of the injected PCLD after the intravenous (IV) dose as discussed in the next sections.

6. PEG and DNA as antigens

PEGylated lipoplexes are thought to be non-immunogenic delivery systems and can be used instead of viral vectors that acquire higher immunogenic properties[40]. Unfortunately, studies show an immune response directed against PEGylated liposomes and PEGylated lipoplexes with loss of therapeutic efficiency in repeated dose administration. The elicited immune response is directed mainly against two main components of PCLD namely: PEG chains, and the incorporated nucleic acids (DNA, siRNA). Most surprisingly that both PEG and DNA are non-immunogenic molecules in nature[40-42].

For better understanding the mechanisms of immune responses directed toward non-immunogenic particles (PEG and/ or DNA), the state of the art should be applied. Macfarlane Burnet[43] declared in his theory "clonal selection theory" that B cells with receptor specificity to a particular antigen pre-exist in an organism, even before they encounter this antigen[44]. However, not all antigens can induce an immune response as many factors controlling the antigen such as origin, composition, size, and repetitive units. So, the presence of simple cognate antigens cannot induce an immune response successfully. Finally, an additional signal is required to stimulate the immune system and activate B cells. Activation of B cells results in clonal expansion, and B cell differentiation into antibody-secreting plasma cells[45]. Immune response not only includes antibody production, which is the main component, but also includes cytokines, and complement stimulation.

There are two main mechanisms for antibody production: T-dependent pathway (TD), and T-independent pathway (TI)[44]. The first one, TD pathway in which the antibody production is mainly dependent on the help of T-cells. Briefly, TD antigens are recognized via phagocytic cells such as dendritic cells (DCs)[46]. Activation of DCs results in cytokines production that subsequently activates T-helper cells (CD4+ cells). T-helper cells are capable of recognizing the antigen on the surface of phagocytic cells (antigen-presenting cells, APCs) in the context of major histocompatibility complex class II (MHC II). At the same time, B cells recognize the antigen via B cell receptor (BCR). BCR-antigen interaction results in a plethora of transduction signals which activate B cells[47]. Activated B cells internalize and process the antigen on its surface with MHC II. Finally, an interaction between the activated T-helper cells that present the antigen in the context of MHC II with the activated B cells occurring at the germinal center (GC). The interaction between T and B cells stimulates B cell proliferation (clonal expansion) and induce antibody production (**Fig. 4**). Another signal is provided through the released cytokines and toll-like receptors (TLR) activation[48]. TD response is characterized by the co-operation between T-cells and B cells (mainly follicular zone, FOB cells), production of high-affinity antibodies, isotype

class switching, and the generation of immunological memory cells[49, 50]. TD antigens are mainly proteins such as botulinum toxin, ova albumin (OVA), and anti-coagulant factor VIII (FVIII)[44].

Thymus-independent or T-independent antigens (TI-antigens) are non-proteins that activate B cells in the absence of T-helper cells[51-53]. TI-antigens require only one or two signals to activate B cell proliferation and induce antibody production. TI-antigens could be classified into two main categories: T-independent antigens class-1 (TI-1 antigens) and T-independent antigens class-2 (TI-2 antigens). The classification is based on their immunogenicity on mouse strains that possess an X-linked defect in B cell function[49]. TI-1 antigens such as bacterial liposaccharide (LPS), and nucleic acids (DNA, siRNA) are still immunogenic in these strains and can elicit both non-antigen and antigen-specific antibodies[49, 54]. TI-2 antigens are polymeric molecules with highly repetitive units such as polysaccharides and PEG molecules[55]. TI-2 antigens cannot elicit an immune response in X-linked defect B cell function mouse strains[54]. Taken together, TI-1 antigens are capable of activating B cells without any help from T-cells or other immune cells. TI-1 antigens require only one signal to activate B cells and generate antibodies[56]. Generally, this signal is mediated through the activation of B cell-intrinsic TLRs (DNA can stimulate TLR-9, siRNA can stimulate TLR-7, and LPS can stimulate TLR-4)[33, 57, 58]. Finally, TI-1 antigens at higher concentrations are considered a B cell mitogen or a polyclonal B cell activator[44, 59, 60]. However, TI-2 antigens can induce B cell proliferation only in the presence of a second signal. The first signal is produced via the cross-linking of the antigenic epitopes with multiple BCR, and the second one is provided via APCs such as macrophages[61, 62]. TI-immune response is characterized by low-affinity antibodies, mainly IgM isotype, little isotype class switching, no or little memory cells production, and marginal zone B cells (MZB) or B-1 cells are the main components of TI-immune responses[49, 63].

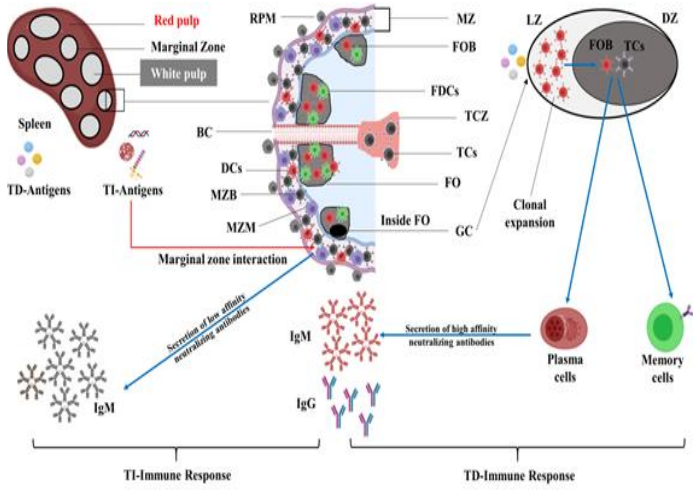

Figure 4: Microanatomical structure of mouse spleen with TD and TI-immune response mechanisms.

7. Immunogenicity of PEGylated lipoplexes

The lymphoid organs are the major sites controlling the immune responses against foreign particles. The main sites for immune recognition and immune responses include spleen, peritoneal cavity, lymphnodes, bone marrow, and circulating immune cells in the blood. The present study focus on three major sites that control the immune response toward TI-antigens: spleen, peritoneal cavity, and bone marrow.

Spleen is the most important lymphoid organ capable of responding to both TD and TI-antigens[64]. The microanatomical structure of the spleen (**Fig. 4**) showed two distinct zones namely: red pulp (RP) and white pulp (WP)[65]. Splenic RP extracts aged, toxic, antigenic, and opsonized materials from the blood. The most important cell component in RP which involved in the immunological response is red pulp macrophages (RPM) which defined by F4/80+ marker[64, 66].

The innate immune response starts at the splenic white pulp (WP). Mouse splenic WP is divided into two main zones with no boundaries separating them from the splenic RP[64]. The first zone is the marginal zone (MZ) which a dynamic complex structure builds up from MZ B cells (MZB)[67], marginal metallophilic macrophages (MMM), marginal zone macrophages (MZM), and marginal zone dendritic cells (MDCs)[68]. Marginal zone B cells reside at a steady state for both innate and adaptive immune responses[69]. The T-independent immune response is mainly localized in this area (TI-1 and TI-2 antigens). Marginal zone B cells are B-1 similar cells characterized by CD21+CD23-IgM+IgDlo markers[70, 71] and co-operate in bidirectional pathways with the MZM (CD209+ or DC-SIGN+ cells)[66] and MMM (CD169+ cells)[72, 73]. Marginal zone B cells upregulate the expression of certain molecules on the surface of MZM called specific intercellular adhesion molecule-grabbing nonintegrin receptor 1 (SIGN-R1) which is very important for antigen capture and/or antigen presentation[74, 75]. In the same way, MZMs control the retention and trafficking of MZB cells in the marginal zone via the interaction between a collagenase structure expressed in MZM called macrophage receptor with collagenous structure (MARCO) and an unknown receptor on MZB cells[76]. Finally, MZB cells express highly autoreactive BCR that responds rapidly to pathogens and produce higher levels of low affinity neutralizing antibodies[70]. Also, MZB cells express most types of TLRs (TLR-2, TLR-4, TLR-7, and TLR-9)[77]. TI-2 antigens require the presence of both MZB and MZM. Meanwhile, TI-1 antigens require MZB cells only with TLR expression[56]. It should be mentioned that the TD-antigens require the co-operation between the activated T-cells in the T-cell zone (TCZ) and activated B-cells in the follicular zone (FO). The clonal expansion starts in the light zone (LZ) of the germinal center and is followed by the interaction between T-cells and B-cells in the dark zone (DZ). The immune response includes secretion of high-affinity antibodies, plasma cell proliferation, and generation of memory cells[78].

Peritoneal cavity immune cells are mainly divided into three main categories: B cells (50-60%), macrophages (30%), and T-cells (5-10%)[79]. The most important in the present study is the B cells. Peritoneal B cells are divided into three subclasses include B-1a cells (defined by B220+CD23-CD5+), B-1b cells (defined by B220+CD23-CD5-), and B-2 cells (defined by B220+CD23+CD5-)[80, 81]. For deep insight, the focus will be mainly on B-1 cells which are characterized by higher IgM expression and CD5hi expression and home predominantly in the peritoneal cavity and pleural cavities[53]. B-1 cells have unique characters such as longer half-life time[82], respond mainly to T-independent antigens[83], produce higher amounts of autoantibodies preferably IgM isotype[84], recognize a wide range of self-antigens such as phosphatidyl choline, DNA, Ig, and membrane proteins[85]. Also, B-1 cells express most of TLR (TLR-2, TLR-4, TLR-7, and TLR-9) and respond rapidly to lipopolysaccharide (LPS) stimulation[86]. B-1 cells also present in the spleen, blood, and lymph nodes[87]. As previously

reported, the B-1 cells in the spleen are responsible for the majority of resting IgM autoantibodies (80-90%) in serum more than B-1 cells in the peritoneal cavity and bone marrow[88, 89]. Autoantibodies produced via B-1 cells are polyreactive and secreted spontaneously[90].

The most important cells in the bone marrow are plasma cells which defined by CD138+. Long-lived plasma cells secrete antibodies naturally without any stimulation (most isotypes IgM, IgG, IgA, and IgE). New data states that long-lived plasma cells are induced in the early response to T-independent antigens in T-cells defect mice model (BALB/c nu/nu mice)[91].

8. PEGylated lipoplexes and immune system interplay

Liposomes alone can interact with many immune cells depending on their physicochemical properties such as size, composition, surface charge, and surface chemistry[92]. Cationic liposomes (CL) have more impact on immune cells than conventional liposomes due to the positive charge that interacts with negatively charged phospholipids of the cell membrane[93]. Decoration of liposomes with a hydrophilic polymer such as PEG has a great promise in altering pharmacokinetic (PK) properties via decreasing surface charge and interaction between liposomes and cells[34]. Unfortunately, both liposomal surface decoration with PEG and DNA incorporation result in higher immunogenic responses. The most apparent immunogenic response is the "accelerated blood clearance (ABC) phenomenon".

8.1. Accelerated blood clearance (ABC) phenomenon.

The ABC phenomenon was introduced by Dams *et al.*[94] in 2000 when they showed that the injected first dose of PEGylated liposomes in rats, leads in rapid blood clearance for the second dose. To date, the ABC phenomenon has not had serious problems with the clinically approved PEGylated products such as Doxil®[95]. The higher consumption of market products containing polyethylene glycol, the higher number of patients exhibited circulated pre-existing anti-PEG IgM antibodies (now ~ 50%) could affect PEGylated products in the future[96, 97].

8.1.1. Mechanism of the ABC phenomenon

According to Dams *et al.*, the first dose of PEGylated liposomes caused a rapid clearance for the second dose injected within 5-21 days after the first dose. In addition, the transfusion of serum from mice pretreated with PEGylated liposomes into normal mice elicited the ABC phenomenon. Dams *et al*., mentioned that the ABC phenomenon was totally abolished when the serum was preheated at 56°C for 30 min prior to transfusion. This treatment inactivates the complement system. Also, ABC phenomenon could be achieved via removal of the antibodies from the serum by other means[94].

8.1.2. Induction phase of ABC phenomenon

The exact mechanism of the ABC phenomenon is unknown and under investigation[98]. Extensive research data showed that the ABC phenomenon was elicited in various animal models such as mice[99], rats[99], minipigs[100], rabbits, and beagle dogs[101]. The proposed mechanism could be described as follow: injection of the first dose of PEGylated liposomes resulted in stimulation of MZB cells in the spleen in a T-independent manner as previously described[61, 102]. It is well established that PEG itself is a non-immunogenic molecule[34, 103, 104]. The PEG molecules induce anti-PEG antibodies only upon conjugation with lipid and/ or protein carriers. This phenomenon is hypothesized by the haptogenic manner of PEG conjugates[105-107]. Marginal zone is the first lymphoid compartment where B-cells meet and can respond to blood-borne antigens[103]. Stimulation of B cells resulted in a rapid proliferation of PEG-specific B cells and the production of massive amounts of neutralizing anti-PEG IgM antibodies "induction phase"[108-111] (**Fig. 5**). The ABC phenomenon was elicited in both B and T cells-abundant mice model (BALB/c mice) and T cells-deficient mice model (BALB/c nu/nu), but not in B and T cells-deficient mice model (SCID mice model) which confirming the T-independent pathway[107, 112]. Similarly, Cheng and co-workers[113, 114] have reported that the injection of PEGylated β-glucuronide resulted in the production of anti-PEG IgM which recognizes the repeated -(O-CH2-CH2)n- subunit (16 units) of PEG and confirming the assumption that PEG acts as TI-2 antigen. PEG molecules shared most characters of T-independent antigens such as higher molecular weight and highly repetitive structure[115-117]. Many reports stated that splenectomy or MZB cells depletion using an intraperitoneal injection (IP) of cyclophosphamide reduced the magnitude of the ABC phenomenon, but not completely reverse the phenomenon. Induction of the ABC phenomenon in splenectomized mice suggests the presence of other serum factors and/or other cells contribute to the ABC phenomenon[103, 110, 118]. Additionally, there are some assumptions claimed that the MZB cells respond in a strong manner to membrane conjugated antigens more than soluble antigens[119].

8.1.3. Characters of anti-PEG IgM antibodies

The produced anti-PEG antibodies are mainly IgM isotype[99, 102, 107, 114] and characterized by the wave pattern, meaning that after the first dose injection of PEGylated liposomes, anti-PEG IgM titers start to increase at day 3, peaked at day 5, started to decrease at day 7, and gradually decrease in serum until undetectable at day 28[118, 120-122]. The exact specificity of anti-PEG IgM is still unknown as reported by most studies which used ELISA as a detection method and mPEG-DSPE as antigen[96]. However, Armstrong reported that the antigenic determinant of anti-PEG IgM antibodies collected from healthy volunteers (25%) was directed toward PEG chains[123]. From published studies, the accepted minimum epitope of recognition by anti-PEG IgM is 4-5 repeated units of oxyethylene[123]. There is an assumption that the anti-PEG IgM antibodies are classified into two types. The first is terminal methoxy-specific antibodies that bind to the methoxy group rather than the PEG diol. The second one is backbone-specific antibodies that bind to both PEG diol and methoxy group with no difference[124]. A study by Huckaby *et al.* confirmed the strategy of how anti-PEG IgM antibodies bind to a highly flexible repeated structure without fixed conformation (PEG) which is considered a non-immunogenic molecule[98]. Finally, the anti-PEG IgM antibodies are characterized by the inverse relationship with the amount of injected first dose. The lower the dose of PEGylated liposomes, the higher the anti-PEG IgM production[121, 122, 125]. The inverse dose manner is a character of T-independent antigens. The proposed explanation states that at a low antigen dose, an optimal amount of antigen can cross-link with BCR and induce a stronger immunological response[126, 127]. However, at a higher dose, PEGylated therapeutics exhibit a longer circulation which subsequently increased the contact with MZB cells. The longer contact with MZB cells resulted in B cell anergy or immune tolerance[128].

8.1.4. Role of nucleic acids in ABC induction phase

Incorporation of nucleic acids (pDNA, ODN, siRNA, and RNA ribozyme) in PEGylated liposomal systems resulted in significant

ABC phenomenon with rapid clearance for second dose, failure of gene expression, and higher mortality rates[58, 60]. Immunogenicity of incorporated nucleic acids depends on many factors such as methylation, and nucleic acid sequence[129-132]. The most influential one is the unmethylated CpG motifs content. CpG motifs are defined as Cytosine triphosphate deoxynucleotide (C), Guanine triphosphate deoxynucleotide (G), and the phosphodiester link between the consecutive nucleotide (p). The presence of higher content of unmethylated CpG motifs increases the stimulatory and the immunogenicity of nucleic acid-cationic liposome complexes. Many mechanisms involved in the nucleic acid effect, the most important is TLRs activation pathway[133]. There are many subsets of TLR (TLRs 1-13), the first ten TLRs are expressed in both human and mice cells. But mice express TLRs 11, 12, and 13[134]. The most important are TLR-7 and TLR-9. Both TLR-7 and TLR-9 are present in the cell compartment (not in the outer cell surface) and are mainly located in the endosomal compartment[135]. Toll-like receptors are categorized as pattern-recognition receptors (PRR) which recognize many microbial structures called Pathogen Associated Molecular Patterns (PAMPs)[136, 137]. The signaling pathway of most TLRs including TLR-7 and TLR-9 are mediated via Myeloid differentiation factor-88 protein (MyD88)[134]. Toll-like receptor-7 recognizes siRNA; however, TLR-9 recognizes DNA that contains higher unmethylated CpG motifs (Bacterial DNA or pDNA) rather than mammalian DNA that contains higher methylated CpG motifs[138, 139]. Both types are expressed in many categories of immune cells such as phagocytic cells (macrophages, monocytes, and dendritic cells) and B cells[140, 141]. Activation of TLRs results in the production of higher levels of cytokines such as IL-6, IL-12, INF-1[141]. Finally, activated TLRs on B cells results in B cells differentiation and proliferation, and enhanced antibody production[77]. In addition to TLRs, There are a plethora of nucleic acid-machinery sensors that recognize DNA and/or siRNA and induce potent immune response. Nucleic acids can be recognized via RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and DNA sensing proteins like stimulator of interferons gene-1 (STING-1 protein)[57]. Taken together, the incorporation of nucleic acids in PEGylated lipoplexes enhances the immune response and induce a potent antibody production via complexed pathways.

8.1.5. Effectuation phase of ABC phenomenon

The delayed effectuation phase of the ABC phenomenon is manifested from day 5 to day 21 after the injection of the first dose[95]. This period is the time course of IgM production which is peaked at day 5 and finally diminished after 21 days. At this period, a subsequent dose of PEGylated liposomes or lipoplexes are rapidly opsonized via complement protein (C3 fragments) and rapidly cleared from blood circulation via Kupffer cells in the liver and/or phagocytic cells in the spleen (**Fig. 5**). The rapid clearance is depending mainly on the magnitude of IgM antibodies and the coordination of the complement system[95]. The binding of IgM antibodies to the second dose resulted in the deformation of the pentameric IgM shape which consequently activates the complement classical pathway via binding with C1q fragments[142]. The activated complement system resulted in various immune responses include: opsonization of the second dose, rapid clearance via phagocytic cells, transport of the second dose to the splenic follicular zone, and hypersensitivity reactions (HSRs) or complement activation-related pseudoallergy (CARPA, **Fig. 5**)[95, 103, 143]. Wang *et al.* reported that the degree of the ABC phenomenon is not entirely correlated to the degree of complement inhibition prior to the treatment, which means that there are other contributors for the ABC phenomenon rather than complement proteins[144].

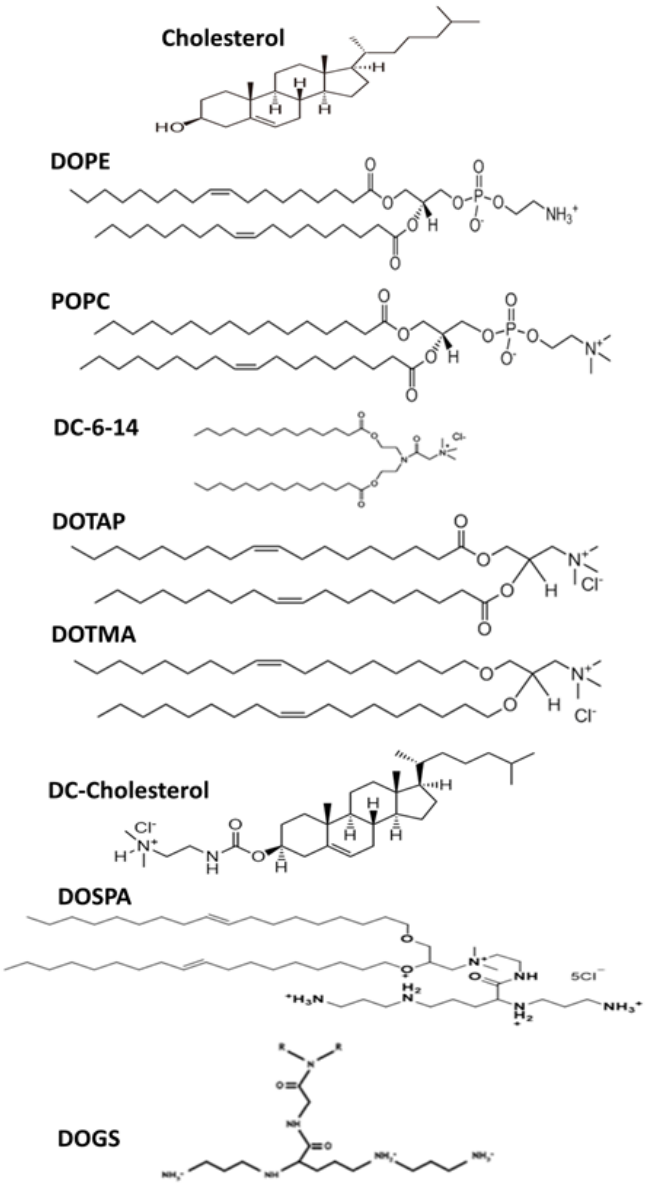

Figure 5: Mechanism and sequence events of induced ABC phenomenon and CARPA phenomenon.

9. Interaction of DNA with non-B cells (accessory cells)

The injected lipoplexes are able to interact with variable types of non-B cells such as phagocytic cells, natural killer cells, and T-cells[59]. Nucleic acids with higher un methylated CpG motifs interact with dendritic cells (DCs) and especially plasmacytoid dendritic cells (pDCs) via the activation of TLR-9[145, 146]. Direct activation of TLR-9 on pDCs resulted in higher expression of MHC II marker, and increased cytokines secretion (IL-6, TNF-α, and IL-10)[145, 146]. In mouse model, DNA can directly activate both macrophages and monocytes. Interaction with macrophages resulted in the activation of Nuclear factor kappa B pathway (NFκB pathway) and subsequently resulted in cytokines expression especially TNF-α[147, 148]. However, the CpG containing DNA is not directly stimulate natural killer cells (NK), but it seems that the DNA acts as a co-stimulatory signal. Also, activation of NK cells requires the presence of other adherent cells. The final product obtained after NK activation is the

production of INF-γ as shown in studies of mouse models[149, 150]. Finally, CpG DNA can interact with T-cells but in a similar way as NK cells. Nucleic acids with CpG motifs have not direct stimulatory effect on T-cells. The type-1 interferon produced via other adherent cells stimulates T-cells[151, 152]. At the end, CpG DNA can interact with other cells even non-immune cells[59]. Briefly, the immunogenicity of DNA is more complexed and requires more intensive future work to solve the immune system/ lipoplexes interplay as a main tool for enhancing gene delivery using cationic lipoplexes.

10. Conclusion

The immune responses to PEGylated lipoplexes are more complexed and involved many cellular and humoral pathways. At the end, more intensive work is required to understand the different immunogenic pathways as an important tool for enhancing immunogenic action of lipoplexes as a vaccine delivery system. also, as a main way to avoid severe immune responses in gene delivery systems.

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