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## Development of Nonviral DNA Delivery Systems

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Hans E. J. Hofland, Frank L. Sorgi, and Edward G. Spack

### 1. Introduction

DNA delivery holds great therapeutic potential, but several barriers have frustrated many creative approaches over the last decade. The presence of an established anti-viral immunity in many patients and the rapid induction of an adaptive immune response in naïve patients continues to block many attempts to introduce DNA by viral vectors. Nonviral delivery strategies avoid the problems of viral-coat proteins, but encountered new challenges of low and transient expression related to physiological and innate immune barriers. Naked DNA must overcome serum nucleases, conserved immune receptors, nonspecific clearance, cellular membrane barriers, endosomal degradation, and intracellular trafficking to ensure optimal localization and expression (Fig. 1). This chapter reviews the obstacles to nonviral DNA delivery and highlights current formulation strategies designed to ensure efficient localization and expression of therapeutic genes.

### 2. Overcoming Barriers to DNA Delivery

#### 2.1. Physiological Barriers

##### 2.1.1. DNA Protection

Systemic gene delivery faces formidable biological barriers. Following injection, DNA is subjected to immediate degradation by nucleases in the blood stream. Approximately 70% of the DNA is cleared from the circulation within the first minute after injection, thereby completely eliminating any chance for gene expression (1). In order to avoid this rapid degradation, the DNA must be protected from nuclease attack. Nonviral gene-delivery systems, including cationic lipids and polymers, have been shown to protect DNA effectively (2–4). These complexes are formed by the charge–charge interaction between the cationic lipid/polymer and the anionic DNA. This electrostatic interaction produces an uncontrolled aggregation and subsequent particle formation (5). The resulting lipid/DNA complexes or polymer/DNA complexes must meet stringent size criteria, such that the particles are sufficiently small for filter-sterilization and intravenous injection. This limits the final particle size to less than 0.2  $\mu\text{m}$ . Therefore, in addition to shielding, the DNA packaging process must include DNA compaction. Compounds that are very effective in both protection and compaction include

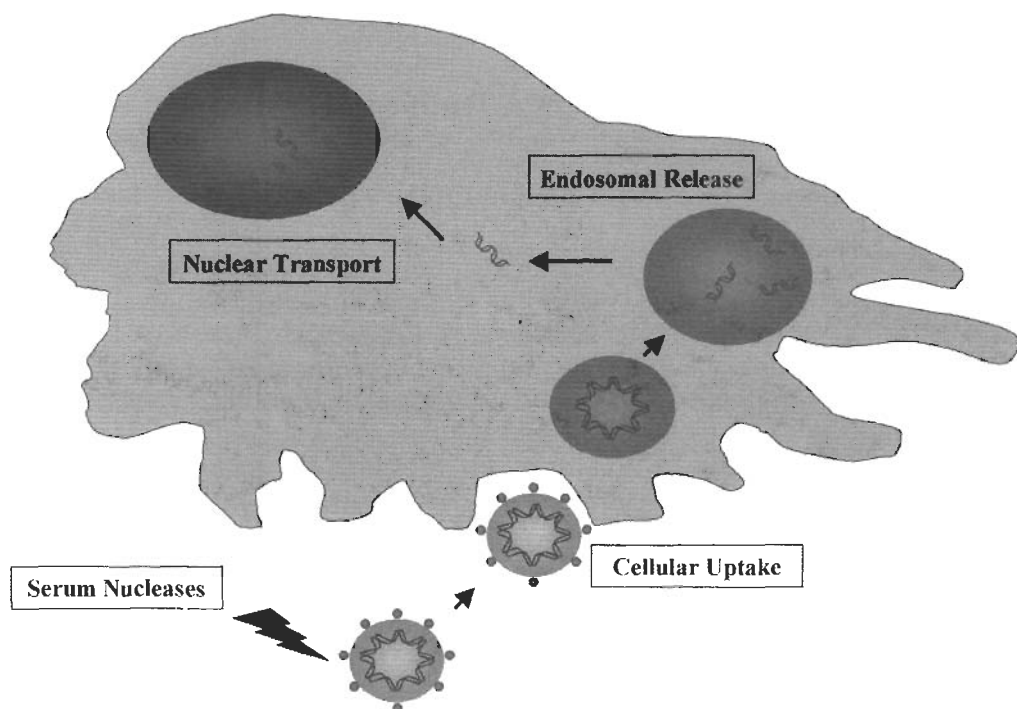


Fig. 1. Potential barriers to nonviral DNA delivery. Plasmids are vulnerable to serum nucleases and endosomal degradation. Various strategies are designed to protect DNA, improve targeting to specific cells or regions, and increase the efficiency of cellular uptake and nuclear transport.

polycationic lipids (6–10), polymers (e.g., starburst polymers, polyethyleneimine REF), peptides (11,12), or a combination of these components (13,14).

### 2.1.2. Biodistribution (Delivery to Target Cells)

Compacted DNA particles injected into the circulation face the daunting task of avoiding immediate phagocytosis by mature macrophages residing in the tissues, i.e., the reticulo- endothelial system (RES). Both the size and charge of these particles determine how rapidly they are scavenged from the circulation (15–17). Changing the zeta-potential of the polycation/DNA complex from negative to positive results in a sharp decrease in lung accumulation, accompanied by an increase in spleen accumulation (18). However, the effect of charge on biodistribution can be clouded by the effect of size of the complex. Large particles (>400 nm) will simply embolize in the capillary beds, which is the probable mechanism for complex accumulation in the lung (19,20).

Analogous to what has been described in the liposome literature, coating of DNA complexes with polyethylene glycol (PEG) may help polycation/DNA complexes avoid RES uptake and prolong their circulation time (21). Tumors are known to have ill-defined, leaky vasculature (22). This allows particles to extravasate into the tumor tissue, where they are retained owing to a lack of lymphatic drainage, i.e., enhanced permeation and retention (EPR). Several groups have tried to exploit this phenomenon in order to achieve systemic tumor targeting (23–26). However, targeting to the tumor

site does not necessarily result in subsequent expression within the tumor cell. Often, the pegylation (of DNA or the lipid/polymer) provides a substantial hindrance to cellular uptake.

The systemic biodistribution of gene expression is also dependent on the route of administration. Following intravenous administration of lipid/DNA complexes, the highest levels of gene expression can be found in the lungs (20), possibly resulting from a physical embolism mechanism. Relative to this expression level, 78% was found in the spleen and 63% in the liver. Following intraperitoneal (ip) injection, however, the highest expression was found in the spleen, and only 25% of this level was found in the liver and 18% in the lungs. Expression in the spleen was also the highest following subcutaneous (sc) injection. Respective liver and lung expression were 28% and 72%.

The most direct means to avoid biodistribution problems and assist the DNA in reaching its target cells is by local administration: simply placing the formulation in the proximity of the target cells. Thus far, most clinical applications in nonviral gene therapy have been limited to local therapies: intratracheal administration for the treatment of cystic fibrosis (CF) (27–29); direct intratumor injections of suicide genes (30,31), apoptosis pathway genes (32), or immune-stimulating genes (33); stereotactic/intracranial injections aimed to treat localized disease in the brain such as Parkinson's disease (PD) (34) and Canavan's disease (35); intramuscular injections (36) or vaccination (37) to induce angiogenesis in peripheral artery (38) or coronary artery diseases (39); and direct intra-arterial instillation to treat restenosis (40).

Although local administration has obvious advantages based on reduced exposure to potential toxic materials and the elimination of biodistribution problems, its use will likely be limited. A "next generation" of gene-delivery vehicles is needed that is designed for systemic administration and targeting to specific tissues.

## **2.2. Cellular Barriers**

### **2.2.1. Cell Entry**

Cellular uptake of nonviral DNA-delivery systems is a nonspecific process. Because of the excess positive charge of the complex, it will bind nonspecifically to a negatively charged cell membrane and is subsequently taken up by endocytosis or membrane fusion (41). Electron micrographs have shown that both lipid/DNA complexes (42) and lipopoly(L-lysine)/DNA complexes (43) are taken up predominantly by endocytosis. These studies contradict earlier suggestions that the main mechanism of cell entry is by membrane fusion (44,45). Other studies have demonstrated that the effect of lysosomotropic agents is dependent on both the formulation and cell type, suggesting that either mechanism may occur and is dictated by the cell type and formulation used (46,47).

Although the mechanisms behind cellular uptake of DNA (with or without a carrier system) are still unclear, the current belief is that, at least in an *in vivo* situation, DNA enters the cell by endocytosis (47,42,43). The specificity of gene expression can be increased and potential toxicity can be reduced by coating the particles with PEG. Unfortunately, although PEG coating renders these particles invisible to the scavenging RES, it also reduces uptake by target cells. Therefore, cell-specific uptake mechanisms must be incorporated into the formulation.

Current targeting ligands under study for oncology applications include the use of the folate receptor (23,48), transferrin (49), endothelial growth factor (EGF) receptor (50), c-erbB-2 receptor (51), and angiogenic-specific receptors (52). Coating lipid/DNA

complexes with PEG dramatically reduced gene-transfer to all tissues. However, the attachment of folic acid at the distal end of the PEG-lipid restored gene-transfer activity in specific tumor tissue only. The gene transfer activity in lungs was reduced by 50–100-fold compared with nontargeted lipid/DNA complexes without PEG. This approach demonstrates in vivo proof-of-concept necessary to achieve targeted tumor gene delivery (23).

Strategies for other disease applications include targeting to inflamed tissues by using selectins such as asialo-LewisX. Targeting to the lungs has been pursued by using the polymeric immunoglobulin receptor (53) or specific antibodies (54). Liver hepatocyte targeting has been demonstrated by galactosylated delivery systems (55,56). Using proteins, such as asialoglycoprotein or antibodies as targeting ligands, is inherently associated with the possibility of evoking an immune response (57). However, reducing the size of antibodies to Fab fragments or smaller may prevent an immune response against these molecules (58).

Next-generation gene-delivery systems are becoming increasingly complex. Recent innovations include the combination of both nonviral DNA and viral elements into a single gene-delivery system (59). Ultimately, the success of nonviral gene therapy will greatly depend on the ability to design systems with reduced toxicity, increased stability in serum, and specific tissue targeting.

### 2.2.2. Endosomal Release

Once the DNA reaches the cells, the degree of cellular uptake is most likely not the cause of the low transfection efficiency of nonviral vectors (42,46,60). In vitro studies demonstrated that 6 h after adding lipid/DNA complex at a dose of 2  $\mu$ g DNA to  $2 \times 10^5$  COS cells, each cell contained an average of  $3 \times 10^5$  copies of the plasmid. However, only less than 50% of these cells actually expressed the transgene. This suggests that the success of nonviral delivery of DNA could be owing to a mass action effect.

Zabner (42) showed that following endocytosis, the DNA containing particles are retained in perinuclear vesicles, or in the lysosomes, where it will be degraded. Escape from these vesicles is thought to be a major barrier for transfection. Therefore, two distinctly different strategies are followed to enhance endosomal release. First, fusogenic lipids or peptides are used to disrupt membranes (61–63). Fusogenic lipids form hexagonal structures or pores in membranes. Enhanced expression of genes transferred by pH-sensitive liposomes is attributed to the instability of these formulations to environments of lower pH (64). The pH-sensitive liposomes will destabilize the endosome, allowing the plasmid to escape into the cytoplasm, before lysosomal degradation can take place. Others have also described the use of fusogenic delivery systems. Plasmid DNA was encapsulated into liposomes containing the nuclear protein high mobility group 1, and fused with inactivated Hemagglutinating Virus of Japan (HVJ). Expression of various genes was achieved with this fusion hybrid of liposome and virus (65–69). In vitro levels of transfection found with this system were similar or slightly better than Lipofectin<sup>®</sup>-mediated transfection, depending on the cell type (70). Unfortunately, these fusogenic liposome formulations are not applicable for iv delivery because their particle size is well over 1  $\mu$ m (71). These large liposomes are readily taken up by the reticuloendothelial system. The circulation half-life could be improved by size reduction, but this would result in a substantial reduction in the encapsulation efficiency of the DNA. Cationic lipid/DNA complexes generally contain the fusogenic helper lipid DOPE (72,73). Upon adhesion of the cationic lipids to the negatively

charged lipids in the cellular membrane, it is believed that phase separation occurs. This results in the presence of DOPE-rich regions, which induce hexagonal phases and therefore, membrane destabilization.

A second strategy involves the use of DNA delivery systems that possesses a high buffering capacity. This will prevent acidification of the endosome and subsequently leads to the rupture of the endosomal membrane (74). The rationale that polymer/DNA complexes are taken up by cells via endocytosis, and that endosomes can be ruptured if the pH drop of the late endosomes is inhibited by the buffering capacity of the formulation, led to the use of polyethylenimine as a DNA-delivery polymer (75). However, concerns of cellular toxicity may become an issue as a result of the emptying of the endosomal contents into the cell following the rupture of the endosome.

### 2.2.3. Nuclear Localization

Following endosome escape, transport of DNA to the nucleus is required for expression. Some reports indicate that it is not the release from the endosome, nor the uncoating of the DNA, but the transport of the DNA from the cytoplasm into the nucleus (76) that is the rate-limiting step for expression to occur (77,78). The DNA in a cationic liposome/DNA complex can be displaced from the complex upon the addition of anionic liposomes. This DNA release may be owing to the multivalent nature of the anionic lipid surface and the collaborative effects of electrostatic interactions and hydrophilic-hydrophobic interactions of the lipids. Lipid mixing results in neutralization of the charges, which allows for diffusion of the cationic lipids away from the DNA. Thus, the anionic lipids in the liposomes will compete with the DNA for binding to cationic lipids. Likewise, it is suggested that the anionic lipids present in the endosome membrane can displace the DNA from cationic lipid/DNA complex. This study shows that DNA release by competition is a very efficient process. Equal moles of anionic lipid added to the cationic lipid/DNA complex leads to 80% release of the DNA.

Micro-injection studies also revealed that nuclear localization is an inefficient process (79). DNA injected into the nucleus of a cell led to expression of the transgene, whereas micro-injection of the same DNA into the cytoplasm did not. Micro-injection studies also revealed that the DNA must be uncoated before it enters the nucleus, because lipid/DNA complexes that were directly injected into the nucleus did not lead to gene expression. This is in agreement with the studies of Zabner et al. (76) who observed that even at a 100-fold charge excess of DNA, neither tRNA, ATP, poly(glutamic acid), spermine, spermidine, nor histones were able to displace DNA from the cationic lipid/DNA complex.

One strategy to circumvent the requirement for nuclear transport employs a powerful cytoplasmic expression system utilizing the T7 promoter. Purified T7 RNA polymerase could be co-delivered with the DNA using DC-Chol liposomes (80,81). This system is particularly suitable for expression of short RNA molecules, such as antisense oligonucleotides or ribozymes.

### 2.2.4. Gene Expression

After conquering all the hurdles described earlier, the DNA finally reaches the nucleus where the transgene can be expressed. Zabner et al. (42) showed that upon micro-injection of the monocationic lipid DMRIE/DNA complex into the nucleus, the transfection efficiency is dependent on the lipid to DNA ratio. At ratios where the lipid/DNA has been optimized for in vitro cellular transfection, the expression of the "opti-

mized" complexes were less than that of naked DNA following micro-injection into the nucleus. At suboptimal lipid/DNA ratios however, the expression was increased. This suggests that gene expression may be inhibited owing to lack of dissociation of the lipid from the DNA. Remy et al. (74) proposed an explanation for the increased transfection efficiencies of polyamine containing delivery systems lies in the high affinity of the polyamines for DNA that leads to competitive uncoating of the plasmid DNA by the chromosomal DNA. This facilitation in lipid uncoating may be the cause of the increased levels of expression.

When gene delivery is no longer the limiting factor to reaching therapeutic levels of the transgene, the problem of how to regulate gene expression will have to be addressed. Tissue-specific promoters, or even inducible promoters, would be an elegant means to keep the expression of the desired transgene within the therapeutic window. In one example, pH-sensitive anionic immunoliposomes delivered the Herpes Tyrosine Kinase gene under the rat promoter for the phosphoenolpyruvate carboxykinase gene (82). This promoter can be regulated by cAMP drugs (83). Another example of inducible gene expression is via the tetracycline (tet)-regulated transactivation system (84). This system is based on the transcriptional transactivators that interact specifically with bacterial *cis*-regulatory elements, and tet that modulate the binding of the transactivators. The gene will be switched on by giving the patient low, nontoxic doses of the antibiotic.

### **2.3. Immunological Barriers**

Maintaining genomic integrity is the biological prime directive, and strategies to prevent the insertion of foreign genes by pathogens such as viruses date far back in the timescale of evolution. Within the past decade, immunologists have realized that vertebrate immune defense is a balance of innate and adaptive systems. The adaptive system, controlled by T and B lymphocytes, is characterized by receptors (T-cell receptors and immunoglobulins, respectively) whose sequence diversity is generated by a recombination of germline sequences and transcripts. This system provides a broad set of receptors for surveillance against pathogens. If one of these receptors binds to a pathogen, the cell bearing the receptor is activated to expand clonally and produce effector T cells and antibodies that will neutralize the pathogen and prevent replication or incorporation of pathogen genes. Anti-viral antibodies and cytotoxic T lymphocytes that react to viral coat proteins remain a major barrier to viral-delivery systems.

The innate immune system is a more ancient defense. Ironically, whereas the discovery of adaptive immunity dates back over a century to the work of Pasteur and Ehrlich and many others, the role of innate immunity gained general recognition within the last decade. As a result, several barriers to nonviral DNA delivery were encountered before innate immunity was recognized. In contrast to adaptive immunity, the receptors that mediate innate immunity are invariant, encoded in the germline, and bind to conserved structures common to many pathogens, referred to generally as pathogen-associated molecular patterns (PAMPs) (84a). PAMPs are conserved, essential microbial structures including mannose (yeast), lipopolysaccharides (gram-negative bacteria), bacterial lipoprotein, lipoteichoic acid, and peptidoglycan (85). The first PAMP receptor, Toll, was discovered in *Drosophila* as a key receptor in antifungal responses. Homologs termed Toll-like receptors (TLRs) were subsequently identified in mammals (86).

Work in several labs demonstrated that bacterial DNA also contains PAMPs. Microbial DNA, but not vertebrate DNA, activates the innate immune system (87). The

dinucleotide combination CpG is suppressed in mammalian DNA, and in 75% of mammalian CpG pairs the cytosine is methylated to 5-methylcytosine. In contrast, bacterial DNA sequences contain abundant, unmethylated CpG pairs (88). These unmethylated DNA sequences are recognized as PAMPs and trigger the mammalian innate immune system. Synthetic, unmethylated oligodeoxynucleotides (ODNs) containing CpG motifs are also immunostimulatory (89), enabling the identification of PAMP sequences. Different CpG sequences are immunostimulatory in mice and man. In mice, the core PAMP sequence consists of an unmethylated CpG flanked by two 5' purines and two 3' pyrimidines, e.g., GACGTT (89,90). These sequences are relatively inactive when added to human peripheral blood mononuclear cells (PBMCs) (91). Instead, two distinct subclasses of CpG sequences stimulate different sets of human immune cells (92,93). Sequences classified as "CpG-A" or "CpG-D" by different laboratories activate natural killer (NK) cells and plasmacytoid dendritic cells (PDCs), and induce monocytes to differentiate into DCs. CpG-A/CpG-D sequences contain a purine/pyrimidine/CpGpurine/pyrimidine motif flanked by three to four self-complementary sequences (e.g., GGTGCATCGATGCAGGGGG) (94). A different sequence, variously dubbed "CpG-B" or "CpG-K," activates B cells and induces monocyte proliferation. These sequences contain one or more unmethylated CpG dinucleotide with a thymidine immediately 5' of the pair and a TpT or ApT on the 3' side (e.g., TCGTTTCGTTCTC) (94).

Several lines of evidence recently demonstrated that TLR9 is the cellular receptor for unmethylated CpG PAMPs. TLR9-deficient mice do not respond to CpG DNA (95). Conversely, transfection of human cells with TLR9 renders them responsive to CpG DNA (96). TLR9 is expressed by all CpG DNA-responsive cells, including the PDC subset, whereas myeloid dendritic cells (MDC) express TLR4 and respond to lipopolysaccharide (LPS) (97). All 10 members of the TLR family contain extracellular leucine-rich repeats and a cytoplasmic Toll/IL-1R homology domain. Binding of a PAMP to a TLR (e.g., CpG DNA:TLR9; LPS:TLR4) initiates signaling by recruitment of the adaptor molecule MyD88, which in turn engages the IL-1R associated kinases (IRAK) and oligomerizes the adaptor TRAF (Fig. 2). This leads to activation of a trio of signal transducers commonly involved in inflammatory stimuli: Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and nuclear factor  $\kappa$ B (NF- $\kappa$ B) (98). Most TLRs are expressed on the cell surface, but TLR9 is located in the lysosomal compartment. Internalization of CpG DNA and endosomal maturation are required for TLR9 activation (99). This makes evolutionary sense, because ordinarily a host cell would not encounter bacterial DNA until the bacteria was phagocytosed and the cell wall was digested. As discussed earlier, a significant proportion of plasmid DNA delivered in gene-therapy applications is also routed to endosomes, where CpG sequences within the plasmid vector and encoded gene(s) trigger TLR9. The result of this signal transduction through TLR9 is the activation of genes encoding inflammatory cytokines (e.g., interleukin-12 [IL-12], IL-18, interferon- $\gamma$  [IFN- $\gamma$ ], tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ]).

The strong induction of inflammatory cytokines by CpG DNA can in extreme cases induce systemic toxicity. Because TLRs signal by a common pathway, described earlier, it is not surprising that bacterial DNA can induce septic shock comparable to that caused by the binding of LPS to TLR4 (100). Complexing of bacterial plasmid DNA with cationic lipids and other agents may in some cases exacerbate these toxic effects. Furthermore, inflammatory cytokines inhibit the expression of plasmid DNA that is successfully delivered to target cells. Most plasmid expression vectors designed for

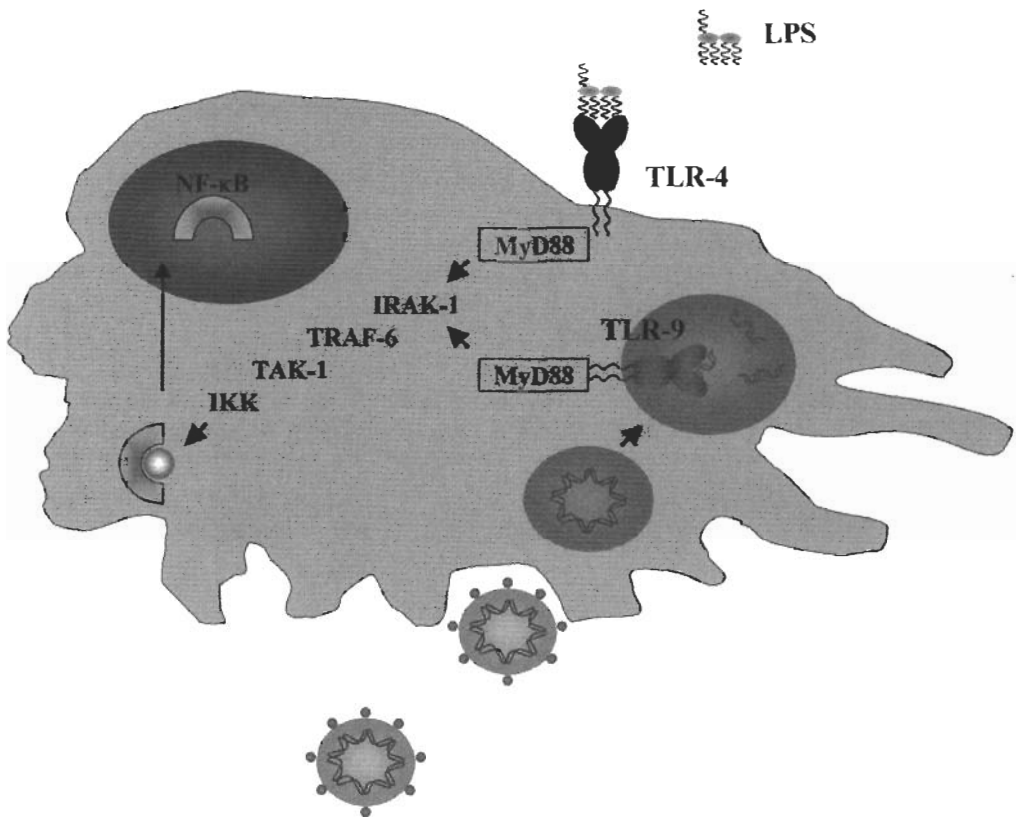


Fig. 2. Plasmid DNA activates the innate immune system. Unmethylated CpG sequences in bacterial DNA bind to the Toll-like receptor 9 (TLR9) in the endosomal compartment. This recruits the adaptor molecule MyD88, which in turn triggers an intracellular signaling cascade (e.g., IRAK-1, TRAF-1, TAK-1), culminating in the activation of transcription factors (e.g., NF- $\kappa$ B, JNK). TLR9 activates a subset of the genes activated by TLR4 (the cell surface receptor for lipopolysaccharide, LPS), including inflammatory cytokines (e.g., TNF- $\alpha$ , IFN- $\gamma$ ).

gene delivery use viral promoters derived from cytomegalovirus (CMV), Simian virus 40 (SV40), Moloney murine leukemia virus (MMLV), or Rous sarcoma virus (RSV) (101). These promoters drive high transcription rates *in vitro*, but CpG sequences in the plasmid trigger inflammatory cytokines *in vivo* that repress the transcription of viral promoters (102,103). Viral promoters are particularly susceptible to suppression by IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$ , inflammatory cytokines produced by the triggering of TLR9 in PDCs and NK cells. Rising levels of inflammatory cytokines limit the level and duration of expression by genes encoded in naked or formulated DNA plasmids. Furthermore, the persistence of these cytokines blocks attempts to re-dose with plasmid DNA for several days until their level drops below inhibitory levels. In summary, the innate immune response induced by binding of unmethylated CpG sequences to TLR9 in the endosomes of phagocytic cells severely limits the expression of genes encoded within these plasmids.

### 3. Conclusion

DNA delivery to target cells faces several formidable barriers. Viral vectors, despite substantial elimination of viral components and the use of helper cells, trigger antibodies and cytotoxic T lymphocytes that limit transfection and expression. Nonviral delivery systems also encounter immunological barriers. Plasmid DNA is synthesized in bacterial hosts, and therefore contains unmethylated CpG sequences that bind to TLR9, a receptor in the endosomes of NK and DCs. The binding of DNA with TLR9 triggers a signaling pathway leading to the secretion of inflammatory cytokines that suppress viral promoters. To overcome this innate immune response, new strategies may be required, including antagonists of TLR9 and suppression of stimulatory CpG sequences through deletion or methylation. A better understanding of the role different carrier lipids play in the stimulation of TLR9 may also aid better expression and persistence. Although the cytokine storm induced by nonviral DNA delivery limits many applications, it may prove an ally in vaccines and tumor therapy. In these cases, the lipid component of lipid:DNA formulations may be optimized to provide improved adjuvants.

The physiological and cellular barriers to successful DNA delivery and expression are also formidable. There are several options for route of delivery, each with its challenges. Intramuscular delivery often produces transfection limited to the injection track. Subcutaneous injections by syringe or ballistic microspheres can improve local expression, but can also transfect Langerhans cells in the skin, triggering migration to regional lymph nodes and initiating a systemic immune response. Many cationic lipids are toxic when administered intravenously, and some particles may cause pulmonary embolisms when trapped in lung capillaries. The enzymes found in serum and lung fluid also pose a danger to the integrity of DNA enroute to the target cells. When lipid:DNA complexes successfully reach target cells, they must negotiate an equally perilous pathway through uptake and nuclear translocation. Many new strategies are evolving for efficient cellular and intracellular targeting, often including ligands and protective lipids. In some cases, the complexities of these solutions may pose practical challenges for manufacturing cost and reproducibility.

The development of monoclonal antibody (MAb) therapy may provide a useful parable for the development of effective nonviral DNA delivery. Initial attempts to treat human diseases with mouse MAbs met with failure owing to the development of immune responses to the foreign mouse sequences. Following this initial disappointment came a new wave of strategies to construct chimeric antibodies, to predict and remove immunostimulatory sequences, and to induce fully human antibodies in immunoglobulin-transgenic mice. Several MAbs now receive FDA approval each year, and the pipeline of these molecules is among the fastest growing sectors of biotechnology. Physiological and immunological barriers to nonviral DNA delivery have dampened initial enthusiasm for long-term gene replacement and required a reassessment that is reminiscent of the early days of MAb development. As the limits and opportunities of DNA delivery are better recognized, there is reason to hope that some of the approaches discussed in this review will survive the perilous journey from the bench to the clinic.

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