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Reducing the immunostimulatory activity of CpG-containing plasmid DNA vectors for non-viral gene therapy

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The mammalian innate immune system has the ability to recognise and direct a response against incoming foreign DNA. The primary signal that triggers this response is unmethylated CpG motifs present in the DNA sequence of various disease-causing pathogens. These motifs are rare in vertebrate DNA, but abundant in bacterial and some viral DNAs. Because gene therapy generally involves the delivery of DNA from either plasmids of bacterial origin or recombinant viruses, an acute inflammatory response of variable severity inevitably results. The response is most serious for non-viral gene delivery vectors composed of cationic lipid–DNA complexes, producing adverse effects at lower doses and lethality at higher doses of complex. This review examines the role of immunostimulatory CpG motifs in the acute inflammatory response to non-viral gene therapy vectors. Strategies to neutralise or eliminate CpG motifs within plasmid DNA vectors, and the existing limitations of CpG reduction on improving the safety profile of non-viral vectors, will be discussed.

Keywords: CpG motifs, gene therapy, inflammation, innate immune response, non-viral vectors, plasmid DNA

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1. Introduction

The delivery of very large polyanionic polymers of nucleic acids into cells is not a naturally-occurring process, especially when complexed with cationic lipids or other macromolecular conjugates. These reagents deposit numerous copies of plasmid DNA into the cytoplasm of cells, usually without discrimination as to cell type. Thus, dendritic cells (DCs), macrophages and other antigen-presenting cells (APCs) take up the DNA complexes as well as the target cells. In humans, B cells and plasmacytoid DCs are activated by unmethylated cytosine–phosphate–guanine (CpG) motifs present in DNA, provoking the maturation, differentiation and proliferation of monocytes, macrophages, T cells and natural killer (NK) cells [1]. These cells secrete a T helper (Th)1-type spectrum of pro-inflammatory cytokines and chemokines that is favourable for generating an adaptive immune response to a given antigen [2-4]. Such a response is the basis for using plasmid DNA as an adjuvant to co-administered vaccines, or as a vaccine itself when the plasmid encodes an antigen [5]. CpG-containing plasmids and oligodeoxynucleotides are also being evaluated to prevent allergy (by redirecting a Th2- to a Th1-type immune response), and as an immunotherapy for cancer. These therapeutic applications have been reviewed recently [6,7] and will not be covered here. Rather, this review will focus on applications for which the CpG response is problematic, the possible ways to mitigate the effect of CpGs, and the benefits and limitations that result.

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It was Tokunaga and his colleagues [8,9], while studying the antitumour activity of a bacterial toxin, who first determined that bacterial, but not vertebrate, DNA, is the key component involved in activating NK cells and inhibiting tumour growth. Krieg *et al.* [10] then identified unmethylated CpG motifs as being the critical immunostimulatory sequences, providing an explanation for the phenomenon of CpG suppression found in mammalian and other vertebrate genomes. The frequency of CG dinucleotides in vertebrate DNA is a third to a quarter that of the expected mathematical frequency of 1 out of 16 bases. Moreover, ~ 80% of the CpGs are methylated. In contrast, bacterial DNA is unmethylated and not CpG-suppressed, and, therefore, has a significantly higher frequency of unmethylated CpG motifs. Bacterial DNA and CpG-containing oligodeoxynucleotides are highly stimulatory to human and murine leucocytes *in vitro*, inducing B cell proliferation and immunoglobulin secretion, DC and macrophage activation, and NK cell lytic activity [3,10-12].

Unmethylated CpG motifs are recognised by Toll-like receptor (TLR)9, a member of a family of receptors that recognise specific pathogen-associated molecular patterns that are present on micro-organisms [13,14]. For example, TLR4 recognises lipopolysaccharide, an integral component of Gram-negative bacteria, and TLR5 recognises flagellin [15,16]. Like other TLRs, TLR9 is a transmembrane protein with a large extracellular domain that contains several leucine-rich repeats [17]. TLR9 also has a conserved cytoplasmic sequence, known as the Toll receptor/IL-1 receptor (TIR) domain. TLR9 is not present on the cell surface, but is instead located intracellularly. Confocal microscopy studies by Latz *et al.* [18] show that the receptor is initially localised to the endoplasmic reticulum of macrophages and DCs. DNA containing unmethylated CpGs enters the cell and moves first into early endosomes and then into a highly motile tubular lysosomal compartment. TLR9 concomitantly translocates from the endoplasmic reticulum to where the CpG-containing DNA is located. Ligand-binding experiments and surface plasmon resonance both indicate that CpG-containing DNA binds directly to TLR9, although the existence of accessory binding proteins cannot be ruled out [18-20].

On binding of plasmid DNA to TLR9, signalling is initiated by the cytoplasmic TIR domain, which associates with myeloid differentiation primary-response protein 88 (MyD88). MyD88 then recruits IL-1 receptor associated kinase (IRAK)4, which phosphorylates IRAK1. Signalling then continues through several other kinases and adapter proteins that include tumour necrosis factor-associated factor (TRAF)6, transforming growth factor β-activated kinase (TAK1), TAK1-binding protein (TAB)1 and TAB2 [21]. Both MAPKs and a variety of transcription factors such as NFκB are then activated that promote the subsequent expression of several pro-inflammatory chemokine and cytokine genes.

2. CpG and autoimmunity

In addition to acute inflammation, the long-term consequences of delivering CpG-containing DNA should also be considered. One possible hazard is the development of autoimmune disease. Studies by Marshak-Rothstein and colleagues have demonstrated a crucial role for TLR9 in autoimmunity [22,23]. They observed in the blood of autoimmune mice complexes of a particular subclass of self-immunoglobulin (IgG2a) and DNA. These complexes activate particular B cells that secrete rheumatoid factors (anti-immunoglobulin antibodies) through simultaneously activating the B cell antigen receptor and TLR9, thus triggering proliferation. The results demonstrate that TLR9 signalling can activate B cells without T cell help. Hypomethylated DNA is required [24], and consistent with this observation is that the levels of DNA methylation are reduced in mice and humans with autoimmune disease [25,26]. In addition, several reports show that CpG oligonucleotides can induce the activation and expansion of autoreactive T cells [27-29].

However, more than one signalling event is required for autoimmune disease to develop, and there are multiple active mechanisms for maintaining self tolerance [30]. Repeated immunisation of normal mice with either plasmid DNA or bacterial DNA induces significant quantities of anti-DNA antibodies, but the mice do not develop autoimmune disease [31,32]. Plasmid DNA vaccines have also been administered to a number of human patients without significant adverse reactions [33-35]. Nevertheless, one may speculate that there may be a slight increased risk to susceptible individuals on exposure to CpG DNA. Waldner *et al.* [36] conducted studies with a strain of transgenic mice that express a T cell receptor specific for an encephalitogenic antigen (myelin proteolipid protein). Although these mice have a high frequency of self-reactive T cells, they are relatively resistant to developing experimental autoimmune encephalomyelitis. Activation of APCs in these mice through either TLR4 or -9 signalling breaks self-tolerance and induces autoimmune disease. However, results in mice cannot predict the response in humans, and the degree of risk should not be overstated. Those working in the field of DNA vaccines, anti-allergen therapy and cancer immunotherapy are aware of the potential issues, and additional safety data from ongoing clinical trials will improve our ability to gauge the potential for CpG-mediated autoimmunity in the context of gene therapy applications.

3. Role of CpG motifs in the toxicity of cationic lipid–DNA complexes

An acute, dose-dependent inflammatory response is observed after administering cationic lipid–DNA complexes either into the lumen of the lung or systemically into the bloodstream. The inflammation in the lung after instillation of complex is characterised by an influx of neutrophils, multifocal lesions in the alveolar region, and induction of the pro-inflammatory

cytokines TNF-α, IFN-γ and IL-12 [37]. Cationic lipid alone or plasmid DNA alone does not induce a significant cytokine response, indicating that the cationic lipid–DNA complex is the causative agent [38]. Aerosol delivery of polyethylenimine-DNA complexes also generates elevated TNF-α and IL-1β levels in the bronchoalveolar lavage fluid [39]. The inflammatory response has been observed not only in mice, but also in sheep [40] and humans [41,42]. In a clinical trial involving nasal and aerosol delivery of a cationic lipid–DNA complex into individuals with cystic fibrosis, seven of eight patients that received complex, but not those that received cationic lipid alone, developed mild flu-like symptoms that resolved within 36 h [41].

The evidence that unmethylated CpGs are responsible for much of the inflammatory response in the lung came initially from studies using complexes containing methylated DNA, which produced significantly lower levels of cytokines compared with unmethylated DNA complexes [38,43]. In addition, when complexes were formed with a plasmid DNA vector that had been depleted of ~ 50% of its CpG motifs, the levels of pro-inflammatory cytokines induced after instillation were markedly decreased [44]. These results strongly imply that unmethylated CpGs are responsible for the vast majority of the cytokine response in the lung.

A similar response occurs after systemic administration of cationic lipid–DNA complexes. Within 24 h of receiving intravenous injection of complex, mice appear scruffy and lethargic, the extent of which is dose dependent. These symptoms usually resolve over time, but higher doses of complex can be lethal. Elevated levels of the pro-inflammatory cytokines TNF-α, IL-12, IL-6 and IFN-γ are found in the serum [45], and CD69, a very early activation antigen, is upregulated on $CD8^+$ and $CD4^+$ T cells, NK cells, B cells, and macrophages in the spleen [46]. There is also activation of complement [47], accumulation and activation of NK cells and apoptosis in the lung [48], and elevations of serum transaminases and acute phase proteins, which are indicative of hepatocellular necrosis [45,49,50].

As was seen with lung delivery, intravenous delivery of complexes containing CpG-methylated plasmid DNA resulted in significant reductions of pro-inflammatory cytokines [48]. Methylation has also been reported to decrease alanine aminotransferase and aspartate aminotransferase levels [49], although this has not been observed in all cases [45]. Methylation also had no effect on the acute depletion of white blood cells and platelets from the serum after intravenous administration [51]. However, a highly CpG-depleted vector (pGZB) was able to not only decrease the elevation of IL-12 and IFN-γ, but also reduced the loss of leucocytes and platelets, and decreased the elevations of alanine aminotransferase and aspartate aminotransferase in the blood [52]. The results reinforce the dominant role of unmethylated CpGs in many of the acute toxicities that occur after intravenous delivery. The data, however, also suggest that methylating CpG motifs and eliminating CpGs are not entirely equivalent. Whether methylated CpG motifs in the plasmid stimulate a TLR9-independent pathway remains to be determined.

4. CpG-mediated toxicity from naked DNA vectors

The inflammatory response from naked DNA delivery is considerably less than that observed with cationic lipid–DNA complexes. However, for many strategies plasmid DNA is injected into muscle, which is a common target tissue for inducing an antibody response to a given transgene. Although suitable for DNA vaccines, such a response is deleterious for any gene therapy requiring extended expression. Direct injection of plasmid DNA into skeletal muscle induces infiltration of mononuclear cells, mainly macrophages and $CD4^+$ and $CD8^+$ T cells [53]. The unmethylated CpG motifs in the plasmid also induce the expression of the chemokine monocyte chemotactic protein-1 and major histocompatibility complex (MHC) class II molecules on myocytes [54]. Injection of methylated plasmid DNA or a highly CpG-reduced vector results in substantially reduced mononuclear infiltration, with fewer regenerating fibres indicative of decreased muscle damage [53]. Methylating the plasmid vector also abolishes the antibody response to an expressed viral glycoprotein [55]. Although the inflammation observed with naked DNA delivery into muscle is relatively mild and transient, the potent adjuvant effect of unmethylated CpG motifs is still of concern, especially when expressing a foreign protein or the normal version of a mutant protein, for example, dystropin for the treatment of Duchenne muscular dystrophy [56].

5. Modifying and eliminating CpG motifs from plasmid DNA vectors

The *in vivo* studies highlight the beneficial effects of methylating or eliminating CpGs. One additional approach is to add so-called neutralising or suppressive motifs to counter the effects of immunostimulatory CpG motifs [57]. If one is to determine how best to generate a less inflammatory plasmid DNA vector, it is perhaps useful to discuss the merits, limitations and practical considerations of these three strategies.

The results described above indicate that methylation can reduce many, but not all, CpG-mediated effects. Methylation of plasmid DNA is accomplished easily *in vitro* using purified CpG methylase (M.Sss I), which methylates all cytosine residues at the C5-position within the sequence 5′-CG-3′ [58]. Plasmid vectors can also be methylated within a strain of *Escherichia coli* that harbours a second plasmid encoding the M.Sss I gene [59]. Methylated CpG DNA does not activate TLR9 signalling in spleen cell cultures or various cell lines *in vitro*, fails to induce the spectrum of pro-inflammatory cytokines normally observed using unmethylated plasmid DNA, and has no Th1-type adjuvant effect [17,59]. In fact, co-administering methylated CpG DNA with unmethylated plasmid DNA can reduce the inflammatory response [60]. Nevertheless, methylated CpG DNA is not equivalent to non-CpG DNA, because methylated DNA still induces some of the adverse hematological changes (e.g., lymphopenia, thrombocytopenia) that were observed with unmethylated DNA complexes [51].

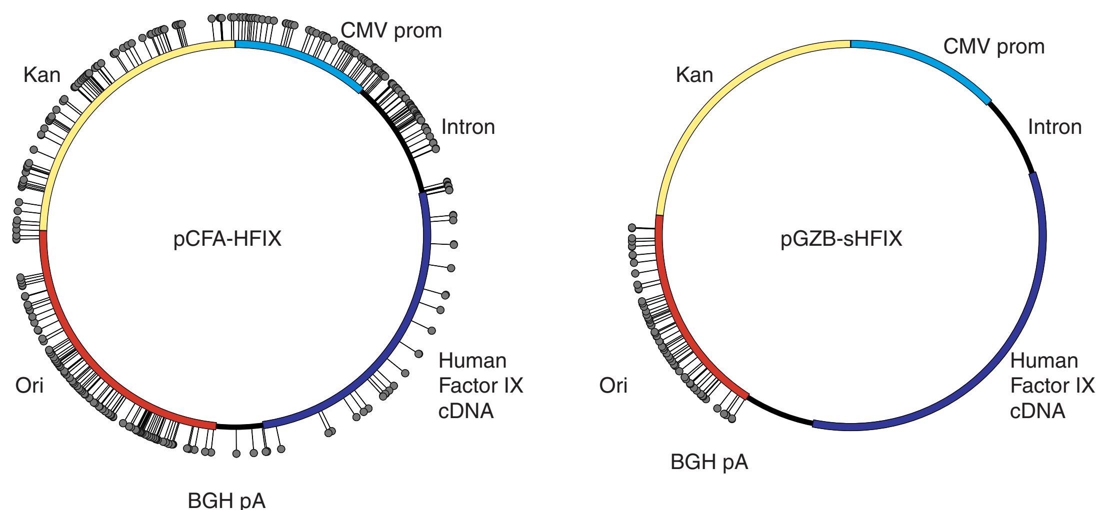

Figure 1. Unmodified plasmid vector (pCFA-HFIX) and corresponding CpG-reduced vector (pGZB-sHFIX) expressing human Factor IX. Each symbol represents one CpG (CpGs from both strands of the double-stranded DNA).
BGH pA: Bovine growth hormone polyadenylation signal; CMV prom: Cytomegalovirus enhancer-promoter; Kan: Kanamycin resistance gene; Ori: Plasmid replication origin.

An additional major limitation of globally methylating the plasmid is that it inhibits the transcriptional activity of most promoters [61]. A methylated promoter may retain only a few per cent of its original activity, although a few promoters, such as the mouse mammary tumour virus long terminal repeat or simian virus 40 early promoter, are relatively insensitive to methylation [62]. However, these promoters possess relatively weak transcriptional activity. Furthermore, methylation of non-promoter sequences in a given plasmid also inhibits gene expression, probably through the formation of an inactive chromatin structure [63-66].

A second possible approach to reduce the CpG-mediated response to plasmid DNA vectors is to incorporate sequence motifs that suppress or neutralise immunostimulatory CpG motifs. The existence of such motifs is supported by the finding that vertebrate DNA that is completely unmethylated still fails to induce an immune response. Krieg *et al.* [67] first recognised that certain sequence motifs consisting of direct repeats or clusters of C and G could block stimulatory CpGs. These suppressive motifs are over-represented in serotype 2 adenoviral DNA, which is non-stimulatory, and are also over-represented in the human genome. Removing 52 of 134 of these neutralising motifs from a plasmid DNA enhanced its ability to stimulate a Th1-like response *in vivo* [67]. Experiments using oligonucleotides containing stimulatory and neutralising motifs suggest that both the spacing and relative position (5′ or 3′) of a neutralising to a stimulatory motif affect neutralising activity [68,69]. More recent studies suggest that the ability of a given sequence to form a tetrad structure is critical for its suppressive activity [70]. Mammalian telomeres contain numerous single-stranded hexanucleotide repeats with the sequence TTAGGG. This sequence has neutralising activity that correlates with its ability to form G-tetrads. Stacey *et al.* [71] also found that G-rich sequences such as GGAGGG appear at a higher frequency in the mouse genome than in *E. coli* DNA, and were also potently inhibitory.

However, applying these observations to building plasmid DNA vectors may not be so straightforward. Both the effective number and positioning of the suppressive motifs still need to be determined. In addition, if many motifs need to be incorporated to be effective, the increase in plasmid size would be undesirable.

Therefore, given the limitations of methylation and the complexities of suppression, the simplest approach to reduce the CpG response is to eliminate CpGs from the plasmid vector (**Figure 1**). As there are > 200 – 300 CpGs in a typical plasmid, extensive site-directed mutagenesis would be too laborious. Instead, plasmids can be assembled using chemically synthesised DNA that can be designed to any given sequence. The CpGs in coding regions of the vector, such as the transgene and antibiotic resistance gene, can be eliminated by exploiting the degeneracy of the genetic code. With the exceptions of methionine and tryptophan, all the amino acids are encoded by more than one codon. Through the appropriate choice of codons, all CpGs can be removed without altering the amino acid sequence. The CpGs in any introns present in the vector can also be modified so long as functional splice donor, splice acceptor, and lariat branch point sites are preserved. The few CpGs in most polyadenylation signal sequences can also be removed readily.

The CpGs within the promoter sequence can be removed, but with unpredictable effects on promoter activity. Eliminating CpGs from the cellular ubiquitin B gene promoter markedly reduces its activity (data not shown). However, eliminating CpGs from the human cytomegalovirus (CMV) immediate-early gene promoter reduces activity by < 50% [52].

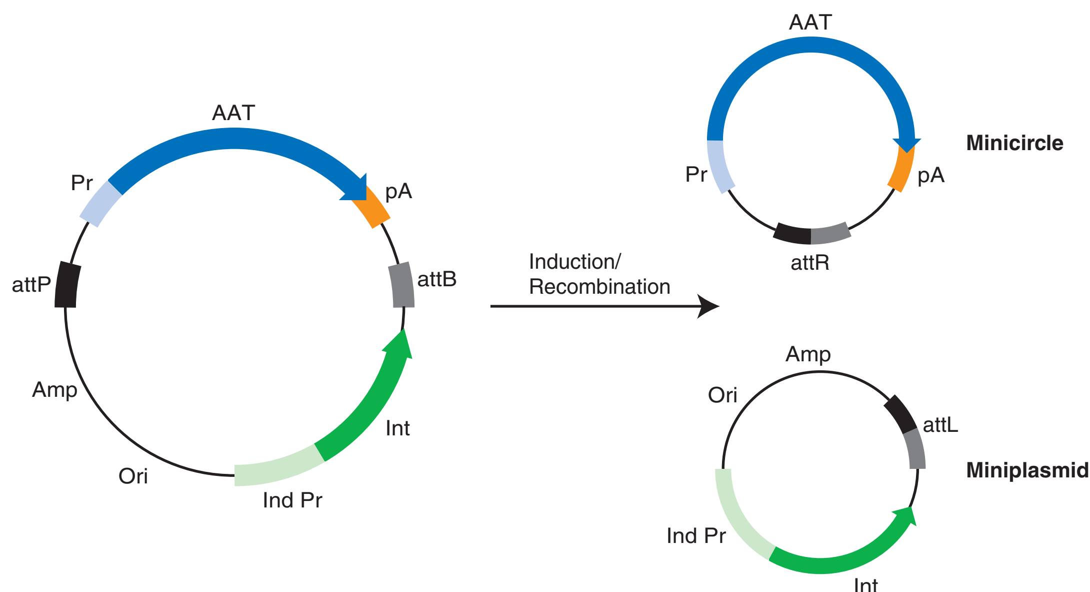

Figure 2. Generation of minimal plasmid vectors by site-specific recombination. Induction of ØC31 integrase expression and subsequent intramolecular recombination generates a minicircle vector containing the functional expression cassette and a miniplasmid containing the vector backbone. Reprinted from CHEN ZY, HE CY, EHRHARDT A, KAY MA, Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression *in vivo*. *Molecular Therapy* **8**(3):495-500, Copyright © (2003), with permission from Elsevier [83].

AAT: α_1-Antitrypsin cDNA; attB: Bacterial attachment site; attL: Left hybrid sequence; attP: Phage attachment site; attR: Right hybrid sequence; Amp: Ampicillin resistance gene; Ind Pr: Arabinose inducible promoter; Int: ØC31 integrase gene; Ori: Bacterial replication origin; pA: Polyadenylation signal sequence; Pr: Promoter.

This strong viral promoter contains multiply reiterated copies of many transcription factor binding sites that likely make it more resistant to inactivation [72]. The murine CMV promoter has extensive regions of non-CpG sequence within its long complex enhancer and so requires less modification to become CpG free [73]. A non-CpG murine CMV promoter has been incorporated into a non-CpG plasmid vector [101].

The CpGs within a plasmid replication origin are the most difficult to eliminate. Most commercially available plasmid vectors use a ColE1-derived replication origin that encodes a 553 nucleotide RNA transcript, RNA II, which primes replication [74]. RNA II has a complex secondary structure with loop domains that are important for regulating replication [75]. Nevertheless, from mutagenesis experiments it is possible to eliminate ~ 60% of the CpGs within RNA II and still preserve replicative function (data not shown). However, further attempts to eliminate the remaining CpGs have been unsuccessful, and RNA-regulated plasmids are unlikely to be made completely CpG-free. Therefore, plasmids using other types of replication control have been investigated. For plasmids such as pSC101, P1, F, and R6K, a protein regulates replication initiation. The γ-origin of replication from R6K has been made completely CpG free and incorporated into a plasmid that is also devoid of CpGs [101]. This plasmid is propagated in an *E. coli* strain in which a high copy mutant *pir* gene has been integrated. The *pir* gene expresses the π-protein that is required for initiation of replication [76]. This vector is commercially available [101].

6. Minimal plasmid vectors

Prior to these efforts in extensively mutating the plasmid vector to remove CpGs, other investigators found ways to excise the vector backbone completely, using either restriction endonuclease digestion or site-specific recombination. The impetus was not directed specifically toward CpGs, but rather to remove unwanted bacterial DNA and to make the vector smaller, and thus, possibly more efficient in transducing cells.

Linear DNA molecules consisting of only the expression cassette (promoter–intron–transgene–poly A signal) have been generated by polymerase chain reaction amplification, or by restriction endonuclease digestion and, optionally, purification of the expression cassette fragment [77,78]. These linear DNAs have exposed ends that are susceptible to exonuclease digestion, but nevertheless appear sufficiently stable to provide long-term expression after hydrodynamic delivery into the liver [78]. One group has ligated stem-loop-forming oligonucleotides to the ends of the linear cassettes, resulting in capped linear DNA molecules, termed 'minimalistic immunogenically defined gene expression' vectors [79].

Other groups have generated minimal plasmids by site-specific recombination. Darquet *et al.* [80,81] utilised the activity of bacteriophage λ integrase, which normally mediates integration between the attP site in the phage genome and the attB site in the *E. coli* chromosome. A plasmid was constructed containing the attP and attB sites flanking an expression cassette. The plasmid was propagated in an *E. coli* strain

that contained a thermoinducible λ integrase. Induction of integrase expression generated two supercoiled molecules, one consisting of the vector backbone and the other the minicircle consisting of the expression cassette. Bigger *et al.* [82] used a similar approach, employing the bacteriophage P1 Cre recombinase to induce site-specific recombination between *lox*P sites flanking an expression cassette. A third integrase, the *Streptomyces* phage integrase ØC31 integrase has been placed under the control of an arabinose inducible promoter, with minimal *att*P and *att*B sites in the plasmid vector (**Figure 2**) [83]. All three systems create supercoiled minicircles, although the unidirectional reaction of the phage integrases may be more efficient in generating minicircles compared with using the bidirectional Cre recombinase [83].

Large-scale production and purification of linear DNA cassettes may be problematic. Purification of supercoiled minicircles also may be an issue, as the minicircles must be separated from the recombined vector backbone and possibly small amounts of unrecombined plasmid. However, a clever strategy that linearises and digests the unwanted plasmid forms has recently enabled the purification of > 1 mg of minicircle DNA from 1 l of culture using conventional affinity chromatography [84].

7. Duration of transgene expression from CpG-depleted vectors

A second consequence of CpG depletion was an unexpected dramatic increase in the persistence of transgene expression from the highly CpG-reduced vector pGZB. In this vector, most of the CpGs have been eliminated (80%), including those within the CMV enhancer-promoter region, with the exception of those CpGs residing in the replication origin. After intravenous injection of cationic lipid–pGZB DNA complexes into BALB/c mice, expression of the CAT reporter protein declined initially, but then increased steadily over time. By 7 weeks postinjection CAT levels often exceeded day 1 levels by several-fold [52]. This was also observed using a pGZB vector expressing α-galactosidase A, achieving minimally therapeutic levels of enzyme in a transgenic disease model of α-galactosidase A deficiency (Fabry disease) [85]. After hydrodynamic delivery of naked pGZB DNA expressing human Factor IX or α-galactosidase into the mouse liver, expression declined initially by ~ 1 log over the first 2 weeks, but was then fairly stable over the next 5 months (**Figure 3**) [86]. Similar expression profiles were observed in C57BL/6, BALB/c or CD1 mice, indicating that this effect of CpG depletion is independent of mouse strain.

The mechanism underlying the sustained expression from pGZB is unknown. One possibility is that the non-CpG CMV promoter is insensitive to its normal downregulation, as a result of mutating one or more repressor binding sites, or eliminating its potential to be inactivated by methylation [87,88]. Although resistance to promoter methylation would be the simplest explanation, there is evidence that the reduction

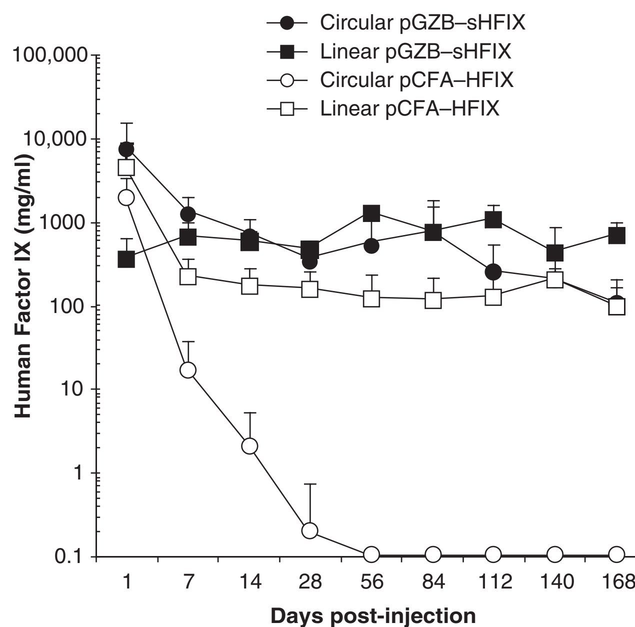

Figure. 3. Effect of CpG-depletion and linearisation of a plasmid DNA vector on the duration of transgene expression. BALB/c mice were injected hydrodynamically with supercoiled (circles) or linear (squares) pCFA-HFIX (open symbols), a CpG-replete CMV promoter vector expressing human Factor IX, or pGZB-sHFIX (filled symbols), a CpG-reduced version of pCFA-HFIX (see **Figure 1**). Plasma was collected at different time points and assayed for Factor IX. CpG-depletion and linearisation independently increase expression persistence. Reprinted from HODGES BL, TAYLOR KM, JOSEPH MF, BOURGEOIS SA, SCHEULE RK: Long-term transgene expression from plasmid DNA gene therapy vectors is negatively affected by CpG dinucleotides. *Molecular Therapy* (2004) **10**(2):269-278, Copyright © (2004), with permission from Elsevier [86].

in CpG content of the overall vector and not just the promoter has an important influence on the duration of transgene expression. Vectors containing an unmodified CMV promoter in a CpG-depleted backbone exhibit increased persistence of expression [52]. In addition, whereas expression from a plasmid containing the non-CpG CMV promoter in the context of an unmodified, CpG-replete backbone is more persistent than from a completely unmodified vector, expression is less persistent than when the non-CpG promoter is in the context of the extensively CpG-depleted backbone [52]. These results suggest that the CpG motifs in the vector backbone negatively affect extended transgene expression.

Extensive studies by M Kay and colleagues demonstrate that the bacterial DNA backbone is responsible for silencing of the vector [78,89]. They separated the transgene-containing expression cassette from the bacterial DNA backbone by restriction endonuclease digestion. These linearised DNA molecules exhibited significantly higher levels of extended transgene expression (10- to 100-fold increase in α_1-antitrypsin levels compared with closed circular DNA) when

injected hydrodynamically into the liver [78]. The linearised DNAs were found to form large, unintegrated concatamers. Additional studies confirmed that the covalent linkage of the bacterial DNA backbone to the expression cassette was the critical determinant for silencing [89]. Methylation of the CpGs in the backbone is the favoured theory for inactivating expression. Kass *et al.* [66] methylated discrete regions of sequence within a given plasmid and showed that these methylated patches within non-promoter sequences led to transcriptional inhibition. They propose that an inactive chromatin structure spreads from these methylated patches. The results with the pGZB vectors could also be explained using this theory, as pGZB has potentially fewer available sites in the backbone to be methylated. Although there is some evidence of *de novo* methylation occurring in transfected cells, additional *in vivo* studies are needed to examine the methylation state of plasmid DNA in tissues over time. Other unknowns are the chromatin structure, condensation state, and involvement of methylated CpG binding proteins with the transduced plasmid DNAs.

8. Non-CpG responses to non-viral gene delivery

Although one may hope that a minimal, CpG-free plasmid DNA vector will be the panacea for non-viral gene delivery, the data indicate that such a vector will not eliminate all the toxic responses, at least when administered as a cationic lipid–DNA complex. Studies conducted in transgenic mice lacking TLR9 have delineated the extent to which toxicity can be reduced in the complete absence of TLR9-mediated signalling. $TLR9^{-/-}$ mice injected intravenously with cationic lipid–DNA complex exhibit a greatly reduced acute inflammatory response, with minimal to no induction of TNF-α, IL-1, IL-12, RANTES and IFN-γ [90]. The $TLR9^{-/-}$ mice also have significantly decreased levels of liver transaminases compared with wild-type mice. Perhaps most importantly, these mice display an improved tolerance to higher doses of complex, and survive at doses that are lethal in normal mice [90].

However, a pronounced loss of lymphocytes and platelets is observed in $TLR9^{-/-}$ mice that received complex, indicating that not all the toxic responses have been eliminated. A few cytokines, such as granulocyte-colony stimulating factor, continue to be induced. Although complexes containing non-CpG oligonucleotides administered into normal mice are well tolerated, they still induce very high levels of amino alanine transferase at higher doses [90]. Although single-stranded oligonucleotides are not equivalent to plasmids, one can predict that a non-CpG plasmid would elicit a similar response. One possible cause for these non-CpG effects is due to the propensity of cationic lipid–DNA complexes to form aggregates following exposure to serum, and the engulfment of these aggregates by various cells, including APCs, may trigger their activation. Another cause may be the ability of sequences other than unmethylated CpGs to activate an immune response. Double-stranded DNA has been reported to induce MHC class I expression irrespective of the DNA sequence; and there appear to be CpG-independent pathways for activating B cells and neutrophils [91-93]. A better understanding of these non-CpG responses will be necessary to fully solve the problem of cationic lipid–DNA-mediated toxicity.

9. Expert opinion and conclusion

The problems posed by immunostimulatory CpGs become apparent as one uses increasing doses of non-viral vectors *in vivo* to achieve therapeutic levels of transgene expression. These higher doses are necessitated by the inefficiency of current non-viral systems, as only a small fraction of the input DNA is translocated into nuclei and actively transcribed. The innate immune system recognises and responds to the presence of this mass of prokaryotic DNA by activating TLR9 and initiating an acute inflammatory response. CpG-depleted vectors can eliminate most, but not all, of the acute toxicities observed after intrapulmonary or intravenous administration of cationic lipid–DNA complexes. In addition, the vectors exhibit greatly increased persistence of expression through an unknown mechanism, which may involve preventing methylation and formation of an inactive chromatin configuration.

There are now no perceived technical obstacles to creating minimal-sized, CpG-reduced or totally non-CpG plasmid DNA vectors consisting only of an expression cassette without any bacterial sequences. With some developmental effort these vectors could be produced in large amounts, and their several advantageous features should eventually lead to their widespread use, replacing existing conventional plasmid vectors. Minor challenges will remain, such as creating robust, non-CpG, tissue-specific promoters and enhancers, which will be important for reducing presentation of an antigenic transgene product to the immune system, and in turn reducing the antibody response [94]. However, increasing the efficiency of delivering these improved vectors is paramount. Given the inherent toxicity of conventional cationic lipid–DNA complexes, either novel delivery agents or naked DNA delivery may be the solution. Pressure-mediated delivery or catheter-delivery methods are being developed to transduce naked DNA into tissues with minimal toxicity [95-97]. Although many aspects of CpG signalling remain unknown, improved delivery methods and use of non-CpG vectors should largely negate the impact of immunostimulatory CpG motifs on non-viral gene delivery systems.

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