

Cationic Microparticles and Emulsions As Effective Delivery Systems for Immune Stimulatory CpG DNA

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1. Introduction: The Use of Microparticles and Emulsions for Vaccine Delivery

The adjuvant effect achieved as a consequence of the linking of antigens to synthetic particles has been known for many years and has been reviewed previously (1). However, the particles used in early studies were nondegradable and therefore, were not appropriate for development as vaccine adjuvants for human use. In addition, antigens were often chemically conjugated to the nondegradable particles, adding an extra level of complexity and making commercial development even less likely (2). Particulate delivery systems present multiple copies of antigens to the immune system and promote trapping and retention of antigens in local lymph nodes. The biodegradable and biocompatible polyesters, the polylactide-co-glycolides (PLG) are the primary candidates for the development of microparticles as adjuvants, because they have been used in humans for many years as resorbable suture material and as controlled release drug-delivery systems (3,4). The adjuvant effect achieved through the encapsulation of antigens into PLG microparticles was first demonstrated by several groups in the early 1990s (5-7). Microparticles were shown to be very potent for the induction of enhanced antibody responses and in contrast to alum, were also shown to be effective for the induction of cytotoxic T lymphocyte (CTL) responses in rodents (8,9).

The mechanism of action for microparticles as adjuvants is predominantly through "antigen delivery" and is largely a consequence of the uptake of microparticles ($<5\text{ }\mu\text{m}$) by antigen-presenting cells (APC), which has been demonstrated on many occasions both *in vitro* and *in vivo*. In an early paper, Kanke et al. (10) described the uptake of microparticles ($1\text{--}3\text{ }\mu\text{m}$) by macrophages, but showed that microparticles of $12\text{ }\mu\text{m}$ were not taken up. Similarly, Tabata and Ikada showed that maximal uptake of microparticles into macrophages occurred with particles of $<2\text{ }\mu\text{m}$ (11,12). In addition, surface charge and hydrophobicity of the microparticles was shown to modify uptake (12). It has also been reported that macrophages that carry microparticles to lymph nodes can mature into dendritic cells (DC) (13). In addition, uptake of PLG microparticles into DC *in vitro* (14) and *in vivo* has been demonstrated (15). It is assumed that the uptake of microparticles into APC underpins the ability of the particles to perform as vaccine-delivery systems/adjuvants.

Emulsions have also been used as vaccine adjuvants for many years, but early studies were undertaken with nondegradable oils, which often tended to induce significant adverse effects, making these approaches unsuitable for commercial use (16). Nevertheless, a potent oil-in-water (o/w) adjuvant, the syntex adjuvant formulation (SAF) (17) was developed using a biodegradable oil (squalane) in the 1980s, as a replacement for Freund's adjuvants. Freund's adjuvants are potent, but toxic water in mineral oil adjuvants, which may also contain killed mycobacteria (16). However, SAF contained a bacterial cell wall-based synthetic adjuvant, threonyl muramyl dipeptide (MDP), and a nonionic surfactant, poloxamer L121, and proved too toxic for widespread use in humans (18). Therefore, a squalene o/w emulsion was developed (MF59), without the presence of additional immunostimulatory adjuvants, which proved to be a potent adjuvant with an acceptable safety profile (19,20). Uptake of the adjuvant into macrophages *in vivo* appears to be an important component of the mechanism of action of MF59 (21). The safety and immunogenicity of MF59 adjuvanted influenza vaccine (FLUAD™) has been demonstrated in elderly subjects in clinical trials (22) and this data allowed the approval of this product for licensure in Europe in 1997. In summary, MF59 is a safe and well-tolerated vaccine adjuvant in humans and is effective for the induction of potent antibody responses. In many subsequent studies, emulsions similar to MF59 have been used as delivery systems for immunostimulatory adjuvants, including MPL and QS21.

2. Mechanistic Interpretation of the Activity of CpG DNA

2.1. Stimulatory CpG Oligonucleotides

The immunostimulatory effects of bacterial DNA have been known since the mid-1980s and, in retrospect, possibly since the time of William Coley, who used bacterial extracts to treat cancer patients more than 100 years ago. Tokunaga and colleagues first demonstrated that DNA, isolated from mycobacteria, could activate natural killer (NK) cells and elicit antitumor effects in animal models (23). They further showed the importance of a CpG element within active DNA sequences (24). Pisetsky et al. then reported that bacterial DNA and synthetic poly C-G oligonucleotides had strong B-cell mitogenic properties, inducing them to proliferate and produce immunoglobulins (25). They also demonstrated the need for unmethylated cytosines in active oligonucleotides (26). Through a series of studies to define the basic CpG motif, Krieg and colleagues identified purine-purine-C-G-pyrimidine-pyrimidine as generally the most active (27). The potency and nature of immune stimulation by CpG oligonucleotides was subsequently shown to depend on the size, number, spacing, and base context of the CpG motifs, the nature of the oligonucleotide backbone, and the species from which the activated cells were derived (for review *see ref. [28]*). There appears to be two basic classes of active CpG oligonucleotides based on their physical nature and the type of activity induced (29,30). CpG-A oligonucleotides activate NK and plasmacytoid dendritic cells (pDC) to secrete interferon- α (IFN- α) and typically have phosphodiester backbones. CpG-B oligonucleotides have phosphorothioate backbones and strong activity on B cells. A third type of CpG oligonucleotide has counteracting effects on active sequences and has been termed CpG-N (31).

2.2. Mode of Action of CpG Oligonucleotides

Active CpG oligonucleotides can exert their effects, either directly or indirectly, on many cell types. CpG-B act directly on B cells to induce proliferation, secrete cytokines

and immunoglobulins, and upregulate the cell-surface expression of Fc receptors, major histocompatibility complex (MHC) class II, and costimulatory molecules (27,32). These effects can be potentiated by an anti-apoptotic effect of CpG on B cells mediated by downregulation of Fas (33). pDC are also affected directly by CpG, resulting in cell-surface expression of activation markers, maturation, and secretion of cytokines (such as interleukin-6 [IL-6], tumor necrosis factor- α [TNF- α], IFN- α , IFN- γ , IL-12) and chemokines (34). CpG have little or no effect on myeloid DC (mDC). CpG has indirect activating effects on monocytes, macrophages, NK cells and T cells in a paracrine fashion mediated by cytokines secreted by other cells. It has recently been shown that CpG acts specifically through Toll-like receptor 9 (TLR9) (35). The distribution of TLR9 on various cells is consistent with direct activation of B cells and pDC, but not mDC, NK cells, or T cells.

The mode of action of CpG oligonucleotides involves a rapid (within minutes) signal-transduction cascade leading to upregulation of mitogen-activated protein kinases (MAPKs), NF- κ B, and transcription factors controlling the expression of cytokines, chemokines and costimulatory molecules. TLR9 is both necessary and sufficient for these effects (36). Thus, CpG oligonucleotides are recognized by the innate immune system as pathogen-associated molecular patterns (PAMPs), in a manner similar to other PAMPs such as lipopolysaccharide (LPS), dsRNA, and other microbial signatures.

2.3. Utility of CpG Oligonucleotides

CpG oligonucleotides have been shown to enhance or modulate the innate and adaptive immune systems. Innate immune protection has been demonstrated by pretreatment with CpG in animal models of *Listeria monocytogenes* (37), *Francisella tularensis* (38), *Leishmania major* (39), and Ebola virus (40). In some cases, amelioration of disease was noted with therapeutic application after onset of disease. CpG has been widely used as a vaccine adjuvant to increase adaptive immune responses. CpG as an adjuvant has a strong tendency to induce a Th-1 type of helper T-cell response, as seen by IFN- γ production by CD4 and CD8 T cells and CTL activity. Antibody responses are also increased, owing to enhanced T-cell help and/or direct activation of B cells. These effects have been reported for many antigens in animal models of infectious disease (for review, see ref. 41), and in human clinical trials. CpG has also been used extensively in cancer immunotherapy. It has been effective as a stand-alone treatment to suppress tumor growth via cytokines and activated lymphocytes (42), as an adjunct to monoclonal antibody (MAb) therapy via antibody-dependent cellular toxicity (43), and as a cancer vaccine adjuvant to enhance immune responses against tumor antigens (44). Finally, the strong Th1-type of responses induced by CpG has been used effectively to modulate the negative effects of Th2-type responses in allergy and asthma (45).

3. Barriers to Cellular Uptake of CpG

Toll-like receptor 9 (TLR9) is a transmembrane receptor that, unlike certain other TLRs, appears to reside in endosomes, rather than on the plasma membrane (35). Studies on the uptake of CpG by cells have shown it to similarly localize in endosomes (46). There does not appear to be a specific "DNA receptor" on the cell surface, hence this uptake is probably mediated by fluid-phase endocytosis. Activity of CpG requires cellular uptake (27) and endosomal acidification (47). Taken together, these data suggest

that the site of CpG-TLR9 interaction and initiation of the signal-transduction cascade occur in endosomes. However, means to disrupt endosomal membranes or directly transfect CpG oligonucleotides into cells enhances activity and results in an accumulation in the nucleus (48,49), suggesting an additional effect of CpG downstream of TLR9. With respect to efficient use of CpG as a vaccine adjuvant, there are several possible important considerations, including delivery of CpG to the appropriate cells (e.g., B cells and pDC), achieving a sufficient critical mass of CpG molecules locally (i.e., in endosomes), and temporal and spatial association with the antigen for optimal induction of antigen-specific immune responses.

4. Delivery Systems for CpG DNA

Recently, we described a novel approach designed to improve the potency of CpG as an adjuvant, through the use of cationic microparticles, which were able to adsorb the CpG onto the surface (50). This approach had previously been shown to be successful as a delivery systems for DNA vaccines, which were also adsorbed onto the cationic surface (51,52). The objective of this work was to ensure that both the CpG adjuvant and the antigen of interest would be taken up into the same population of APC. To achieve this, both the CpG and the antigen were adsorbed onto charged PLG microparticles, either cationic for the CpG, or anionic for the antigen (53). We had previously shown that the cationic microparticles with adsorbed DNA were able to transfect DCs in culture, whereas naked DNA was unable to transfect DC (54). Moreover, the cationic microparticles were able to efficiently adsorb DNA at high loading levels and were able to offer protection against degradation *in vitro* and *in vivo* (55). The ability of cationic microparticles to efficiently adsorb and deliver DNA and CpG is a consequence of the use of the cationic surfactant cetyl trimethylammonium bromide in the microparticle preparation process (51,55,56). A similar approach was also described recently in which we used cationic emulsions to adsorb the CpG and ensure effective delivery into APC (57). The potency of cationic microparticles and emulsions for delivery of adjuvant active CpG will be described in more detail in Subheadings 5 and 6.

Several groups have recently described the use of liposomes as delivery systems for CpG (58–61). The approaches used varied from co-encapsulating the antigen and CpG in cationic liposomes (58), the encapsulation of CpG alone in novel lipid vesicles (59), the encapsulation of CpG in large multilamellar vesicles (61), and encapsulation in haptenized liposomes (60). However, the principal observation was consistent and showed that the potency of CpG as an adjuvant was enhanced by its encapsulation in liposomes. In addition to liposomes, nanoparticles prepared from PLG have also been used as a delivery system for CpG, in which the antigen was also entrapped (62). This work followed on from earlier work, which had showed that the delivery of antisense oligonucleotides to macrophages *in vitro* could be improved by microencapsulation in PLG (63). Similarly, the work on liposomal delivery of CpG was preceded by the use of liposomes as delivery systems for antisense oligonucleotides (64).

In addition to the use of synthetic particulate formulations as delivery systems for CpG, several groups have also described the use of “natural” particulates as delivery systems. The potency of hepatitis B core antigen (HBcAg) was significantly improved when it was expressed with a C-terminal cationic peptide region, which was able to bind small amounts of CpG (65). Similarly, Storni et al. (66) showed that the potency of HBcAg was improved by its administration in conjunction with CpG. In a recent study undertaken to evaluate the use of CpG in combination with conventional adju-

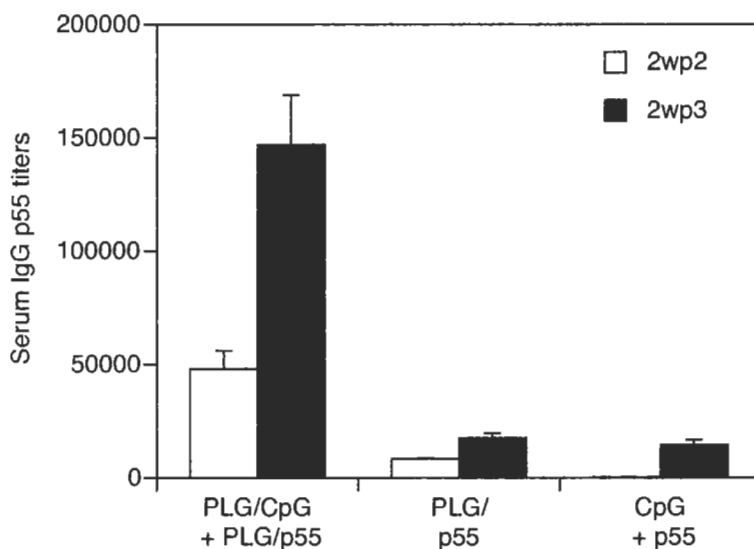


Fig. 1. The adjuvant effect of CpG for the induction of antibody responses against p55 gag. Both CpG and antigen were evaluated as soluble components, and adsorbed to PLG microparticles.

vants, it was clear that the use of CpG in conjunction with a variety of emulsion formulations was capable of inducing potent immune responses (67).

5. Cationic PLG Microparticles for CpG Delivery

As described earlier, we have used novel cationic PLG microparticles as delivery systems for CpG, which have been used in combination with antigens adsorbed onto similar anionic PLG microparticles (50). CpG alone did not appear to be a potent adjuvant for a recombinant HIV-1 p55 gag protein antigen, because the responses induced were only comparable to those induced by the protein adsorbed to PLG (Fig. 1). The CpG + p55 gag protein combination did induce an enhanced response in comparison to immunization with a control inactive oligonucleotide. However, when the CpG was adsorbed to the cationic PLG microparticles, there was a clear and significant adjuvant effect at 2 wk post second immunization (2wp2) and at 2 wk post third immunization (2wp3), in comparison to the alternative groups. In addition, the adsorption of CpG to PLG microparticles induced a dramatic switch in the ratio of IgG2a/IgG1, which was not achieved when CpG was simply added to the p55 gag antigen (Fig. 2). Hence, the optimal mouse CpG sequence (68) was only potent as an adjuvant for p55 gag, when it was used in combination with PLG microparticles.

Subsequent studies evaluating the antibody responses with CpG and a recombinant env gp120 from HIV-1 showed similar responses to those described above for p55 gag (Fig. 3). CpG alone was an ineffective adjuvant for gp120, but was very effective when adsorbed to cationic PLG microparticles. Notably, PLG/gp120 in combination with PLG/CpG induced significantly enhanced antibody responses in comparison to MF59 + gp120, a well-established potent adjuvant formulation. Hence for the induction of enhanced responses to env gp120, it appeared that both the antigen and the CpG adjuvant needed to be presented to the immune system on the surface of PLG microparticles.

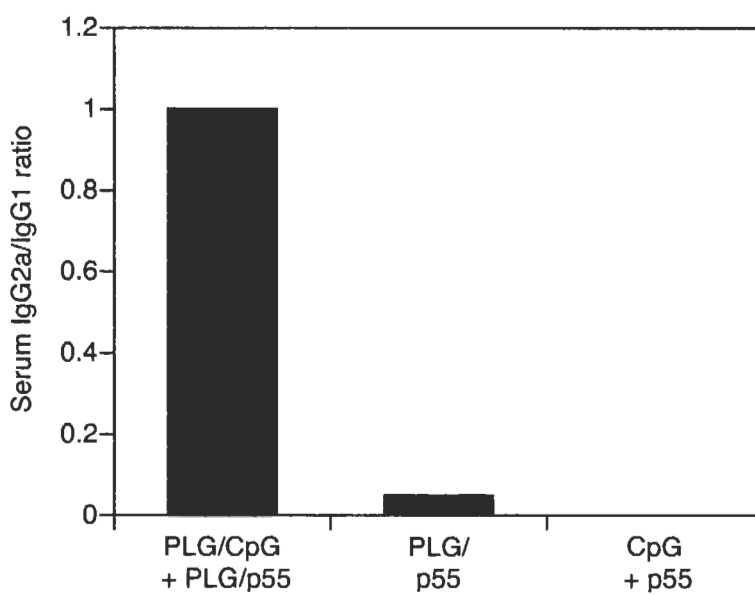


Fig. 2. Induction of higher IgG2a titers with the PLG microparticle formulation with adsorbed p55 gag antigen and CpG adjuvant. Both CpG and antigen were also evaluated as soluble components.

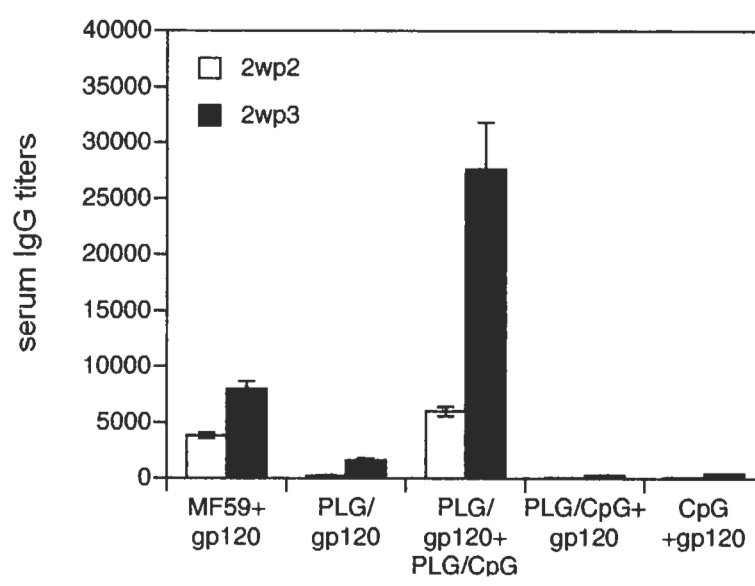


Fig. 3. The adjuvant effect of CpG for the induction of antibody responses against gp120. Both CpG and gp120 were evaluated as soluble components, and adsorbed to PLG microparticles.

The observations on antibody induction with CpG were reproduced in studies to evaluate the induction of CTL responses to an epitope in p55 gag. CpG was effective for CTL induction against p55 gag only when adsorbed onto cationic PLG microparticles (Fig. 4). In contrast, p55 + CpG was not effective for CTL induction. Remarkably, the

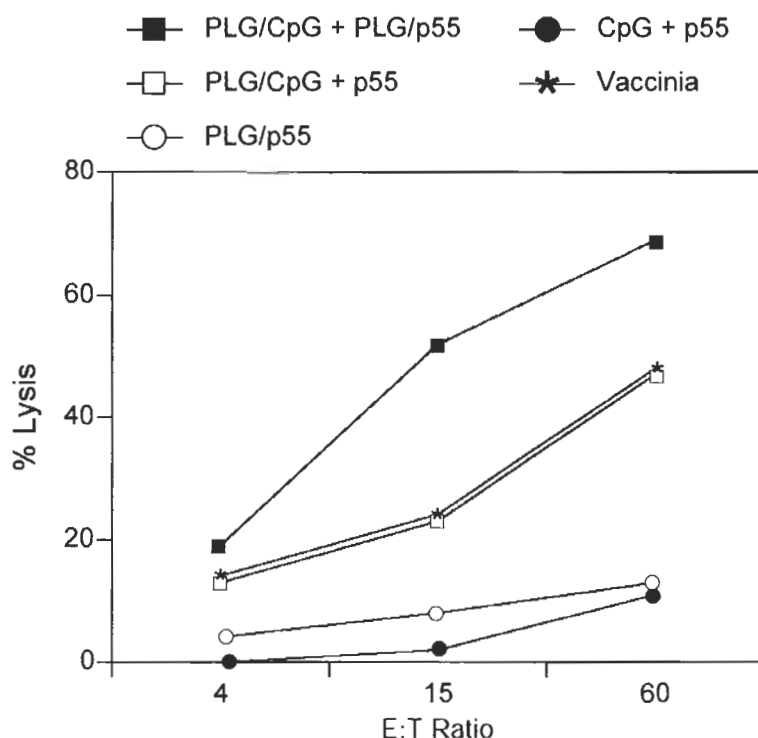


Fig. 4. The adjuvant effect of CpG for the induction of CTL responses against p55 gag. Both CpG and antigen were evaluated as soluble components, and adsorbed to PLG microparticles. Infection with vaccinia gag-pol was used as a positive control.

PLG/p55 + PLG/CpG group, which had both antigen and adjuvant adsorbed to separate PLG microparticles, was more potent than the positive control, involving intraperitoneal (ip) infection with vaccinia virus.

Hence, although it was clear that CpG DNA was capable of performing as a vaccine adjuvant for the HIV-1 proteins evaluated, it was ineffective when used as an adjuvant in the absence of microparticles. The cationic microparticles described in the current studies may offer an attractive, practical, and potent approach for the delivery of adjuvant active CpG DNA sequences. However, the range of antigens for which microparticle formulations are necessary to ensure the potency of CpG adjuvants remains to be determined.

6. Cationic Emulsion for CpG Delivery

In addition to charged microparticles, we also developed cationic submicron emulsions for CpG delivery (57), which were prepared in the presence of DOTAP (1,2-Dioleoyl-3-Trimethylammonium-propane) or DDA (dimethyl dioctadecyl ammonium) as cationic agents and used squalene as the oil phase, as used previously for MF59 (20). Similar to the work described earlier on microparticles, CpG was adsorbed onto the cationic emulsion droplets to ensure optimal delivery into APC and the formulations were used in conjunction with a recombinant p55 gag antigen from HIV-1 (Fig. 5). The simple combination of MF59 and CpG resulted in the induction of a significantly greater antibody response than either component alone. However, adsorbing the CpG to the cationic emulsion did not result in an increase in antibody titer over what was

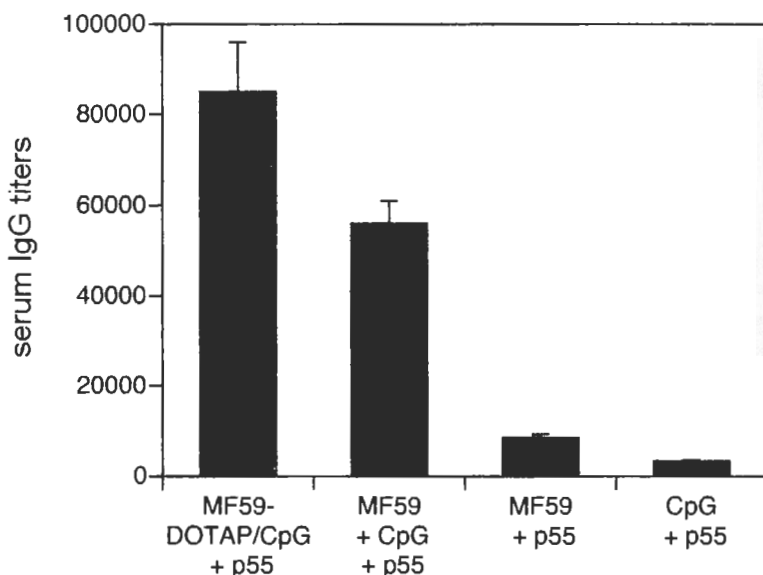


Fig. 5. The adjuvant effect of CpG for the induction of antibody responses against p55 gag. Both CpG and antigen were evaluated as soluble components and adsorbed to MF59/DOTAP emulsion.

achieved with the simple combination. Nevertheless, the cationic emulsions with adsorbed CpG were able to induce CTL responses against p55 gag, while simple combinations of MF59 and CpG were not (69). Hence, the studies described here demonstrated that the potency of CpG as a vaccine adjuvant for HIV-1 antigens was enhanced by the presentation of the CpG adsorbed onto the surface of cationic emulsions.

The hypothesis that led to development of cationic emulsions was that direct delivery of CpG to the endosomal compartment of DC would enhance activity, because MF59 emulsion has previously been shown to be taken up into endosomes of phagocytic cells after intramuscular injection. (70). In addition, cationic PLG particles with similar zeta potential to the emulsions, and ability to adsorb CpG and DNA have been shown to be able to deliver adsorbed DNA into DCs (54). Recently, we have also shown that cationic emulsions are an effective delivery systems for DNA vaccines (71).

7. Conclusions and Future Directions

In summary, the studies we have undertaken and described here have clearly demonstrated that the potency of CpG as a vaccine adjuvant for HIV-1 antigens can be significantly enhanced by its presentation on the surface of cationic microparticles or emulsions. These results are entirely consistent with similar work done elsewhere with alternative antigens, which have described the use of various particulate-delivery systems, including liposomes, emulsions, and nanoparticles, to improve the potency of CpG as an adjuvant. It is postulated that the CpG adjuvant is more potent when used in conjunction with delivery systems, largely as a consequence of its improved delivery into the endosomes of APC, where it can more easily interact with TLR9. However, other factors may also contribute to the enhanced potency of CpG when used in conjunction with delivery systems, including the ability of the various particulate carriers

to protect CpG against degradation *in vivo*. In addition, the detergents used in the preparation of the cationic microparticles and emulsions described here may help to disrupt endosomes, allowing release of CpG into the cytoplasm and enhancing nuclear delivery. However, this represents a mere speculation, which needs to be proved experimentally in dedicated studies.

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