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Innovative strategies for co-delivering antigens and CpG oligonucleotides

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Abstract

Cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG ODN) is a recent class of immunostimulatory adjuvants that includes unmethylated CpG dinucleotide sequences similar to those commonly found in bacterial DNA. CpG ODN specifically triggers toll like receptor 9 (TLR9), which is found within phagoendosomes of antigen presenting cells (APCs) such as dendritic cells (DCs). CpG ODN triggers activation and maturation of DCs and helps to increase expression of antigens. CpG ODN can be used to induce polarized Th1 type immune responses. Several studies have shown that antigens and CpG ODN must be co-localized in the same APC to generate the most potent therapeutic antigen-specific immune responses. Delivery vehicles can be utilized to ensure co-delivery of antigens and CpG ODN to the same APCs and to significantly increase uptake by APCs. These strategies can result in antigen-specific immune responses that are 5 to 500-fold greater than administration of antigen alone. In this review, we discuss several recent and innovative strategies to co-delivering antigens and CpG ODN adjuvants to APCs. These approaches include the utilization of conjugate molecules, multi-component nanorods, liposomes, biodegradable microparticles, pulsatile release chips and cell-microparticle hybrids.

Keywords

delivery systems; antigen; CpG ODN; adjuvant; vaccine; controlled release

1. Introduction

Immunization with antigens alone produces poor immunity. By co-administrating the antigen with an adjuvant, better immune responses can be generated [1-3]. This occurs because the adjuvants act as immunostimulatory agents. The severe inflammation induced by many adjuvants, such as killed bacteria, negates their potential for human application [4]. Those adjuvants that are currently approved by the FDA, such as alum, are not very efficient. Technologies that incorporate adjuvants to increase the immunogenicity of antigens has been recently cited as one of the top ten technologies that will significantly impact global health [5]. Although the mechanism of adjuvants is not fully understood, it is believed that they promote specific types of immune responses, such as antigen-specific CD8+ cytotoxic T lymphocytes (CTLs) [6]. Cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG ODN), when used as an adjuvant, results in much stronger CD8+ responses than antigen delivery alone [3,7-10].

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2. CpG ODN as an adjuvant

CpG ODN with sequence patterns like those found in bacterial DNA activate natural killer cells to secrete interferon- γ (IFN- γ) and generate a cell-mediated immune response [11]. The specific sequence motif present in bacterial DNA that is responsible for triggering these immune responses is the unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines [12,13]. CpG ODN are taken up by cells via adsorptive endocytosis and bind to the toll-like receptor 9 (TLR9) present within the endolysosomes in the intracellular compartment of B cells and plasmacytoid dendritic cells (pDCs) [14-16]. The binding triggers cell signaling pathways that induce leukocyte gene expression and cytokine secretion. CpG ODN triggers an immunostimulatory cascade inducing the maturation, differentiation and proliferation of multiple immune cells including B and T lymphocytes, macrophages, natural killer (NK) cells and monocytes/macrophages that produce interleukin (IL)- 1, 6, 12 and 18, interferon- γ (INF- γ) and tumor necrosis factor- α (TNF- α) (Figure 1) [17-19]. Rapid induction of these immune responses and production of Th1 related cytokines is a critical step to controlling the early spread of pathogens [5].

3. Rationale and need for devices that co-deliver antigens and CpG ODN

While CpG ODN exhibits potent immunostimulatory effects, the rapid degradation and ineffective delivery into the intracellular compartments of APCs are major obstacles to improving its efficacy [20]. When antigen is administered alone, it elicits strong Th2 type immune responses. A significant shift in the isotype of antibody response can be achieved by co-administering antigen and CpG ODN [7,12,21,22]. Addition of CpG ODN has been reported to result in a significant increase in production of IgG2a antibody, increasing the IgG2a: IgG1 ratio over nine-fold [12]. This enhanced Th1 type immune response is essential for counteracting intracellular pathogens including choriomeningitis virus, hepatitis B virus and tetanus toxoid [12,23-25]. For example, co-administration of CpG ODN and hepatitis B surface antigen (HBsAg) vaccine to the same site of the muscle significantly enhanced the antibody response [5]. In contrast, when CpG ODN was administered separately following the administration of the vaccine, it did not induce any significant improvement in immunostimulatory effects over the administration of vaccine alone [5]. These studies highlight the importance of delivery devices that protect CpG ODN from enzymatic degradation, improve targeting of CpG ODN to the endolysosomes, ensure that both CpG ODN and antigen are co-delivered to the same APC, and provide long-term immunity. Here, we review several different delivery systems that are being developed for CpG ODN to meet some of these challenges.

4. Approaches for co-delivering antigens and CpG ODN

We and others have shown that CpG ODN and antigens can be delivered to the same APCs using a number of different strategies. These include the utilization of multicomponent metallic nanorods [1], biodegradable microparticles and nanoparticles [26,27] including cationic microparticles [28-30], pulsatile releasing drug delivery chips [31], and cell-microparticle hybrids [32] (Figure 2). Perhaps the simplest approach to co-delivering antigens and CpG ODN is to chemically conjugate them together thereby ensuring that both components will enter the same cell [12,33-36].

4.1 CpG ODN and antigen conjugates

One approach to crosslinking antigens and CpG ODN has been to utilize the biotin-avidin interaction to link the two molecules together. The cross-linked molecule elicited a ten-fold increase in immune responses when compared to the use of antigen alone [12]. Furthermore, the effect was eliminated upon treating the complex with DNase. This confirmed that the

increase in immune response was due to the presence of the ODN bearing the CpG motif [12]. The conjugate exhibited a higher preferential uptake by APCs over the unconjugated mixture of antigen and ODN. This was attributed to the CpG ODN binding to receptors expressed by the APCs that enabled a significant increase in endocytosis of the conjugated antigen. Conjugation of the CpG ODN to antigen is also reported to enhance cross-presentation [37,38]. Cross-presentation refers to the ability of certain APC's to process and present extracellular pathogens to cytotoxic T cells (CD8 T cells). CpG ODN-antigen conjugate molecules demonstrate much higher efficacy than mixtures of antigen and CpG ODN at equivalent concentrations [35,39].

We have synthesized CpG ODN-antigen conjugate molecules by covalently linking CpG ODN to the model antigen ovalbumin (OVA) [39]. Figure 3 shows the schematic for the synthesis of the conjugate molecule. In this approach, the phosphothiolated sulfhydryl modified ODN containing the CpG motif was linked to the amino terminal of the ϵ - lysine residues on OVA using N-hydroxysuccinimide (NHS) chemistry. In addition to providing the aforementioned advantages of a conjugate molecule, chemical modification of the ODN backbone by phosphothiolation as used in this study has been shown to significantly reduce degradation by nuclease enzymes and hence increase the half life *in-vivo* [40]. Additionally, the chemically modified form of CpG ODN has been shown to retain excellent immunostimulatory activity on APCs. We measured IFN- γ production by pulsing DCs generated from bone marrow of wild C57BL mice with CpG ODN-OVA conjugate molecules, OVA/alum or OVA and CpG ODN in solution. Splenocytes harvested from naive transgenic C57BL/6 OT-1 mice were co-cultured with the treated DCs for a further 24 hours and the supernatant from the co-culture was analyzed for the presence of IFN- γ by ELISA. The CpG ODN-OVA conjugate molecule generated a 16-fold higher IFN- γ response in comparison to co-cultures that were pulsed with CpG ODN and OVA in solution (Table I).

A chemical conjugate of CpG ODN to an allergen, Amb a1, for the prophylactic treatment of allergic rhinitis and asthma was synthesized by Tighe *et al.* [41]. Figure 4 shows the IgG2a levels in mice after intradermal injection of the conjugate or a physical mixture of the two. Table II represents the cytokine profile of the activated spleen cells of mice immunized by the antigen alone, the conjugate molecule and a mixture of Amb a1 and alum. Figure 4 and Table II show that conjugation of Amb a1 to CpG ODN induced the strongest Th1 type immune response and induction of a 10-fold higher IFN- γ response. The enhanced immunogenicity of Amb a1-CpG ODN conjugates was even more pronounced in rabbits and monkeys. These species formed high titers of IgG antibodies to the Amb a1-CpG ODN conjugate but failed to respond to the antigen alone [41]. CpG ODN facilitates a higher uptake of the conjugate molecule by APCs and produced a polarized T_H1 type response by translocation of the conjugate to lysosomal-associated membrane protein (Lamp-1) positive endosomal-lysosomal compartments. Another factor that may be contributing to the increased efficacy is that conjugating CpG ODN to antigen shifts antigen uptake from inefficient fluid phase pinocytosis to efficient DNA receptor-mediated endocytosis. Even non-stimulatory ODNs linked to antigen, enhanced uptake although the presence of the stimulatory CpG ODN was essential for triggering maturation of the DCs [38]. For example, conjugation of CpG ODN to OVA was shown to increase OVA uptake in B cells by 40-fold, which in turn, led to upregulation of co-stimulatory molecules and cytokines such as IL-12 [36]. CpG ODN-antigen conjugates have significant potential for inducing immune responses to weak antigens or lower doses of antigen used in prophylactic allergen immunotherapeutic applications [38,42].

4.2 Multicomponent nanorods

Ballistic delivery of antigens to the sub-dermal layers (that contain an abundance of APCs such as Langerhans cells) using the gene gun can stimulate strong antigen-specific immune

responses [43-45]. The gene gun has been used to primarily deliver single functional gold particles. We developed a multi-component nanorod that could deliver CpG ODN and antigens to the same cells when bombarded into the skin using the gene gun. The nanorods were fabricated by electrodeposition into alumina templates with an array of cylindrical pores [46]. An evaporated silver film on one side of the template served as the working electrode in a three-electrode configuration [1,46-48]. A thin layer of silver was electrodeposited into the template to ensure easy release of the nanorods from the template. Gold segments were deposited prior to nickel segments to prevent erosion of the nickel layers during silver removal. The silver layers were dissolved using nitric acid and the alumina template was then dissolved using potassium hydroxide.

Nanorod length could be controlled by the length and strength of the potential applied and the composition could be controlled by the metal ion solution used to deposit the metal segments [1,46-48]. Using chemical moieties that bind selectively to either gold or nickel, we attached plasmid DNA (pDNA) constructs bearing the unmethylated CpG sequence or the antigen OVA to the different segments [1]. The antigens and DNA molecules were bound to the same nanorod in spatially defined regions [46]. This was achieved by converting a small proportion of the primary amine groups of the model antigen OVA into sulfhydryl groups. The OVA was then bound to the gold segments of the nanorods through a thiolate linkage. Electrostatic interactions were used to bind DNA to the nickel segments by suspending the dual component nanorods in a 0.1 M solution of 3-[(2-aminoethyl)dithio]-propionic acid (AEDP). The carboxylic acid terminus of AEDP binds to the native oxide on the nickel segments. This results in the surface presentation of primary amine groups spaced by a reducible disulfide linkage. In the reducing environment of the cell, the disulfide linkage between the plasmid/CpG and the nanowire is cleavable to enhance release of the plasmid/CpG. When both OVA and CpG motifs were bound to the same nanorod, we observed a 10-fold increase in the CD8+ T-cell response in comparison to OVA delivery on nanorods alone (Figure 5). These nanorods are expected to have significant potential for co-delivery of CpG ODN and antigens using ballistic delivery methods such as the gene gun.

4.3 Liposomes

Liposomes were amongst the first strategies developed for co-delivering antigens and CpG ODN [49,50]. Liposomes are artificial closed vesicles composed of concentric lipid bilayers that are separated by aqueous regions and have been utilized as delivery systems for several anti-cancer drugs, proteins and DNA. Liposomal delivery systems have been used as carriers for immunoadjuvants to induce immune responses against a variety of antigens [49,50]. Like biodegradable polyesters including poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and polylactic-co-glycolic acid (PLGA), liposomes are safe and biodegradable making them attractive vehicles for co-delivery of CpG ODN and antigens [49,50]. Liposomal vehicles have been used to deliver CpG ODN alone and to co-deliver CpG ODN and antigens for therapy of diseases such as leishmaniasis [51,52].

Current therapy against leishmaniasis shows limited effect and the available drugs bear a high toxicity potential. Studies show that recovery and protection against further infection are mainly dependent on the induction of Th1 type immune responses and hence a strategy co-delivering CpG ODN with antigen would be a promising vaccine against all forms of human leishmaniasis [52]. Liposomes co-encapsulating CpG ODN and antigens have been prepared by a dehydration-rehydration vesicle (DRV) method [53]. This method involves the lipid phase consisting of distearoylphosphatidylcholine (DSPC) and cholesterol dissolving in a chloroform-methanol mixture. The lipid film formed upon solvent evaporation is then hydrated to form multilamellar vesicles (MLV). The rLmSTII antigen and CpG ODN were added to empty vessels and converted to 100 nm unilamellar vesicles using high pressure extrusion. The

liposomes were then lyophilized and rehydrated. The Th1 biased antigen-specific antibody response and the ratio of IgG2a/IgG1 response in mice sera is shown in Figure 6A and 6B, respectively. The co-delivery approach resulted in the highest levels of IgG2a antibodies and the highest ratio of IgG2a/IgG1 antibodies in comparison to the groups immunized with a solution form of the components or the antigen and CpG ODN administered alone.

Additionally, the number of viable parasites (*L. major*) was quantified in spleen of different groups of mice after challenge with different formulations (Figure 7). The lowest number of live parasites was seen in the group of mice immunized with the liposomal formulation co-encapsulating antigen and CpG ODN. These results show that liposomal delivery of CpG ODN with antigen may be a suitable strategy to enhance Th1 type immune responses and induce protection against several pathogens [51,54,55].

4.4 Biodegradable microparticles

Biodegradable microparticles fabricated from FDA approved polymers such as PLGA have shown great potential for protein, peptide and DNA delivery over the last two decades [27]. Microparticle-based vaccines have been shown to stimulate strong immune responses and this therapeutic approach has been applied for the prevention and treatment of a variety of diseases including tetanus, influenza, hepatitis and cancer [27,56-58]. One of the most attractive advantages of using PLGA microparticle systems in the area of vaccine delivery is the ability to develop a single-shot vaccine [59,60]. For example, a three-injection schedule for prevention of hepatitis B infection has been converted into a single shot therapy by employing PLA and PGA microparticles encapsulating hepatitis B surface antigen (HBsAg). In addition, microparticles have also been shown to enhance the immunogenicity of encapsulated antigens due to their particulate nature [61]. Antigen-loaded PLGA microparticles generate a strong Th1 type immune response even against poor immunogens [27]. We have shown that incubating microparticles with DCs triggers activation and maturation, which are key steps in initiating the immune response against target antigens [26].

Microparticles are fabricated from biodegradable polymers like PLA, PGA and PLGA using techniques such as emulsification/solvent evaporation [62-64]. Several studies have shown that careful control over the formulation parameters such as surfactant concentration and stirring speed can be used to optimize loading levels and particle sizes [26,65-69]. These systems can be delivered orally or to mucosal membranes (e.g. nasal and vaginal) and can protect CpG ODN from enzymatic degradation by nucleases [26]. Additionally, studies have shown that particles up to 10 μm in size are preferentially internalized by APCs thus functioning as suitable delivery vehicles for the co-delivery of antigen and CpG ODN [26]. This efficient internalization by APCs is attributed to the fact that microparticle carriers have comparable dimensions to the pathogens that the immune system has evolved to combat [70]. We and other have adopted these desirable properties to target APCs, co-deliver antigen and CpG ODN to the same APC, and generate stronger Th1 type antigen-specific immune responses with reduced toxicity [26,71-73].

For example, we have developed a PLGA-based microparticle delivery system that co-entraps OVA as the model antigen and CpG ODN as the adjuvant [26]. The PLGA microparticles were formed using a double emulsion solvent evaporation technique. In this approach, a solution of OVA and CpG ODN is dissolved in a 1% w/v aqueous solution of poly (vinyl alcohol) (PVA) and is rapidly emulsified using ultrasonication into an external oily phase comprising of dichloromethane (DCM) to form the first emulsion. This O/W emulsion is subsequently added to an external aqueous phase consisting of 1% w/v PVA to form the second emulsion. Following evaporation of DCM, the microparticles are collected by centrifugation and lyophilized. The microparticles exhibited an average size of 1.4 μm with a loading efficiency of 23 % for the antigen and 33- 35 % for CpG ODN.

Particle uptake by dendritic cells was assessed by flow cytometry using rhodamine-loaded microparticles. Figure 8 shows the efficient uptake of the microparticles by DCs with 92% of DCs having internalized particles by the end of 16 hours. Additionally, the microparticle system entrapping the antigen and CpG ODN generated a 20-fold higher INF- γ production by T-cells in comparison to the aqueous solution vehicle for co-delivery of OVA and CpG ODN.

We studied the IgG levels using ELISA and plotted the response over a 10 week course (Figure 9). Microparticles co-entrapping OVA and CpG ODN generated significant increases in antigen-specific antibody responses in comparison to empty microparticles or CpG ODN and OVA co-administered in an aqueous solution (Figure 9). These results highlight the substantial improvement in the immune stimulatory effects that can be achieved when the antigen and adjuvant are co-delivered to the same cell at the same time in a microparticle carrier.

4.4.1 Alginate Microparticles—Rebelatto *et al.* have demonstrated that intranasal immunization is the most effective way to induce mucosal immunity as antibodies resulting from parenteral administration do not effectively reach mucosal surfaces, which is the primary entry point of infectious agents into the host body [74]. Antigens loaded into particles are more effective than soluble antigens for intranasal delivery because of greater endocytosis of the antigen by the mucosal-associated lymphoid tissue (MALT). Additionally, alginate when used as the particulate carrier has been shown to act as an adjuvant in animal species [74].

Tafaghodi and co-workers have coupled the advantages of alginate as the particulate carrier for tetanus toxoid (TT) and CpG ODN as adjuvant by fabricating alginate microparticles co-encapsulating these two components [75]. The microparticles prepared by an emulsion solvent precipitation technique were tested for their immunostimulatory effects by intranasal administration in white albino rabbits. Figure 10 shows the serum anti-TT IgG titres up to 12 weeks stimulated by antigen and CpG ODN in solution, antigen loaded microparticles, antigen and CpG ODN co-encapsulated microparticles and blank microparticles. Co-encapsulation of CpG ODN and antigen greatly enhanced IgG A titers when compared to microparticles loaded with antigen alone. Figure 11 shows the serum anti-TT antitoxin titers from these formulations. Intranasal administration of these microparticles in four human volunteers did not result in nasal irritation and did not result in hemolytic effects on the erythrocytes.

Co-encapsulation of CpG ODN with TT generates larger sized particles in comparison to the microparticles loaded with antigen alone. These larger microparticles resist translocation to the regional lymph nodes and are retained in the MALT. This enhances the uptake of the microparticles by the cells in the MALT, thereby inducing potent mucosal immunostimulatory responses.

In a modification to the above approach, hepatitis B surface antigen (HBsAg) was adsorbed on alginate-coated chitosan nanoparticles and co-administered with CpG ODN-loaded nanoparticles. This combination generated the highest humoral mucosal immune response and the highest IFN- γ activity relative to all control groups. In this approach, chitosan acts as the core for the carrier system and alginate is used to tailor a desired release profile for the antigen [76]. These studies highlight the potential of alginate-based microparticles for co-delivering CpG ODN and antigen. This is an approach that has shown particular promise for mucosal routes of administration.

4.4.2 Cationic microparticles—A derivation of the biodegradable microparticle delivery approach is to prepare cationic microparticles that can bind CpG ODN to the surface of the microparticle. The adsorption is facilitated by an electrostatic interaction between the negative charge on the ODN backbone and a positive charge on the cationic microparticle surface [77]. For example, anthrax vaccine (AVA) formulated in alum was used as a model antigen

and co-administered with CpG ODN adsorbed onto the surface of cationic PLGA microparticles and compared with co-delivery with the free form of CpG ODN. Figures 12A and 12B show the IgG2a levels and toxin neutralizing activity (TNA) titers, respectively, over a period of 16 weeks in mice. The microparticle system with adsorbed CpG ODN co-delivered with AVA showed a 50-fold higher IgG2a anti-PA specific antibody activity and the highest TNA response. Figure 13 shows the survival rate of the mice vaccinated with various formulations. The mice immunized with the microparticles with adsorbed CpG ODN co-delivered with AVA showed greater than 80% survival even at the most susceptible stage of infection (2 weeks). In contrast, other formulations including the antigen alone and the antigen administered with the free form of CpG ODN failed to elicit a protective immune response and resulted in a mortality rate of 90%. Dissociation of the free CpG ODN from the antigen due to free diffusion from a solution form is inhibited by adsorption on the surface of PLGA microparticles ensuring localized delivery of the antigen and adjuvant to the APCs.

Other cationic microparticles that can potentially be used to deliver CpG ODN and antigens include biodegradable microparticles that have been functionalized with cetyltrimethylammoniumbromide (CTAB) [78], cetyldimethylethylammonium bromide (CDAB), dimethyl dioctadecyl ammonium bromide (DDAB) [79], 1,2-dioleoyl-1,3-trimethylammonio propane (DOTAP), cationic DDAB [79], poly(L-lysine) (PLL) [80-83], polyamidoamine (PAMAM) dendrimers [28], polyethylenimine [72,84-95] and chitosan [96]. The advantage of these cationic microparticles is that they also have the potential to provide sequential release of antigen and CpG ODN, which could be used to enhance the immune response further [28]. Another approach that can be used to provide sequential release of CpG ODN and antigens is pulsatile release systems.

4.5 Pulsatile Delivery Systems

Pulsatile delivery systems release drugs in bursts or periodic intervals, separated by time intervals of little or no drug release [97,98]. For certain active agents, such as hormones, it has been well established that pulsatile release offers advantages over sustained release because pulsatile release profiles more strongly mimic the body's natural release profiles [97,98].

Vaccines are traditionally administered as an initial single shot of the antigen and/or adjuvant followed by repeated booster shots to optimize protective immunity [99]. Pulsatile release systems could offer the possibility of single shot vaccines if the initial and booster release of the antigen can be achieved from the same system. Medlicott *et al.* and Powell *et al.* have advocated the need to develop a delivery system that can carry both the antigen and adjuvant in the same delivery vehicle and yet provide differential release of the antigen and adjuvant to maintain high antibody titers for prolonged periods of time for protective immunity [98,99]. Additional features of such an "autoboost" vehicle would ideally be reduced toxicity and maintenance of antigen and CpG ODN integrity until final release so as to elicit maximal antigen-specific immune responses [99].

Several systems that provide pulsatile release of drugs and hormones have been investigated. These include delivery systems that respond to changes in pH [100], temperature [101], electric [102] and magnetic fields [103] or exposure to triggers such as ultrasound [104,105], enzymes [106] or light [107]. More recently, promising approaches for pulsatile release of drugs have been developed using silicon-based microchips with wells that can provide release of single or multiple chemicals in response to an electrical stimulus [108-110]. We have developed a poly(dimethylsiloxane) (PDMS) chip with multiple reservoir wells that are covered with biodegradable seals that can provide pulsatile release of CpG ODN and antigens [31].

We selected PDMS as the reservoir component as it is a highly flexible and robust material that can be effectively molded. PLGA polymer films of varying composition and thickness

were used as seals to the wells. The composition, molecular weight and thickness of the PLGA films were all parameters used to control the degradation rate of the seals. For example, thicker films degraded faster than thinner films. It was found that the film degradation rate can be used to fine-tune the release of CpG ODN over day length periods [31,111].

Figure 14 shows the pulsatile release profile from a chip incorporating two doses of a model antigen OVA and CpG ODN. CpG ODN and OVA could be delivered in sequential pulses 4-6 days apart and the entire sequence repeated after 18 days providing the necessary booster dose to maintain high antibody titers [31]. The ability to provide repeated sequential release of CpG oligonucleotides and antigens, suggests significant potential for this device in vaccinations or applications that require defined complex release patterns.

4.6 Cell-microparticle Hybrids

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a cytokine that functions as white blood cell growth factor [112]. GM-CSF has been shown to stimulate the production of white blood cells following chemotherapy. GM-CSF has a critical role in maturation and functioning of APCs like dendritic cells and converts Langerhans cells of the skin to immunostimulatory APCs [113,114]. Co-delivery of the free form of CpG ODN and soluble GM-CSF as an aqueous solution triggers an enhanced production of antigen specific antibodies [115]. In comparison to soluble antigen, whole tumor cells serve as effective vaccine vehicles because they carry a complete complement of tumor cell antigens that can overcome resistant mutations and tumor cell variants [2]. Irradiated tumor cells engineered to secrete GM-CSF are safe and stimulate potent antitumor immunity in mice [116]. As described in the earlier sections, an approach that can deliver CpG ODN localized with the antigen in a non-solution type vector ensures both components are delivered to the same APC and this can significantly amplify the antigen-specific immune response [12].

We have recently developed a novel hybrid system that conjugates whole tumor cells with microparticles entrapping CpG ODN using the biotin-avidin binding mechanism (Figure 15) [117]. Native sialic acid residues on the cell surface are converted to non-native aldehydes using periodate treatment [118]. The aldehyde groups are then conjugated to biotin hydrazide via an amide linkage to produce biotin-functionalized cell surfaces. Particles prepared from polylactic acid-polyethylene glycol-biotin [119] or chitosan (unpublished data) are then assembled with the biotin presenting cells using avidin as a bridging molecule. Figure 16A shows a light microscopy image of multiple cell-microparticle hybrids cultured on a well plate and Figure 16B shows a scanning electron microscopy (SEM) image of a single hybrid [117]. In order to ensure that transfection of tumor cells to express GM-CSF does not inhibit their ability to form a hybrid, we carried out the assembly process using rhodamine-loaded microparticles with cells that had been previously transfected to express the model reporter green fluorescent protein (GFP). Figure 16C shows that prior transfection does not diminish the ability of cells to form a hybrid. Such a co-delivery approach to deliver irradiated tumor cells expressing GM-CSF with microparticles encapsulating CpG ODN in a localized manner to APCs would be a potential mechanism to augment anti-tumor activity and achieve a significant increase in the antigen-specific immune response. We are currently investigating this system for effective vaccination against solid tumors.

5. Conclusions and future challenges

The strategies for co-delivering antigens and CpG ODN described in this review have the potential to improve the efficacy of CpG ODN as an adjuvant against a wide range of diseases. These include asthma [120], anthrax [77], neuroblastoma [2], lymphoma [121], and prostate cancer amongst others [122,123]. Future approaches to enhancing the efficacy of CpG ODN include co-administering with agonists for different toll-like receptors and delivering CpG

ODN as an adjuvant to other vaccines currently in development [124]. For example, CpG ODN has been shown to enhance the efficacy of adenovirus-based prostate cancer vaccines and vaccines based on irradiated neuroblastoma cells that express GM-CSF [2,122,123]. In these studies, delivery vehicles are being utilized that further improve the enhancements to the antigen-specific immune response generated by CpG ODN.

Approaches that ensure co-delivery of CpG ODN and antigen to the same APC result in stronger immunogenic responses including enhancement in speed and duration of immune response, modulation of the isotype of antigen-specific antibody response and increase in the immunogenicity of weak antigens. Delivery systems described in this review have demonstrated targeted delivery of CpG ODN to the intracellular compartments in APCs. The exact vehicle used for delivery of CpG ODN is dependent on the application and the route of administration. For example, multi-component metallic nanorods are uniquely suited to ballistic delivery [1,46].

Future studies are expected to continue to develop delivery vehicles that are tailored to the application, dosing profile required and route of administration. Ongoing clinical studies have demonstrated safety and activity of CpG ODN in humans [125] but some of the areas that still need to be addressed are a more precise understanding of how different classes of ODN regulate the immunostimulatory cascade, better monitoring of the long-term safety of CpG ODN, identification of the optimal dose and duration of vaccine therapy relative to the type of vehicle being used and blocking or reducing immunosuppressive responses. Several recent studies have shown that the choice of material used for the delivery vehicle can also modulate the immune response. This is a potential parameter that could be used to further enhance the efficacy of the CpG ODN class of immunological adjuvants.

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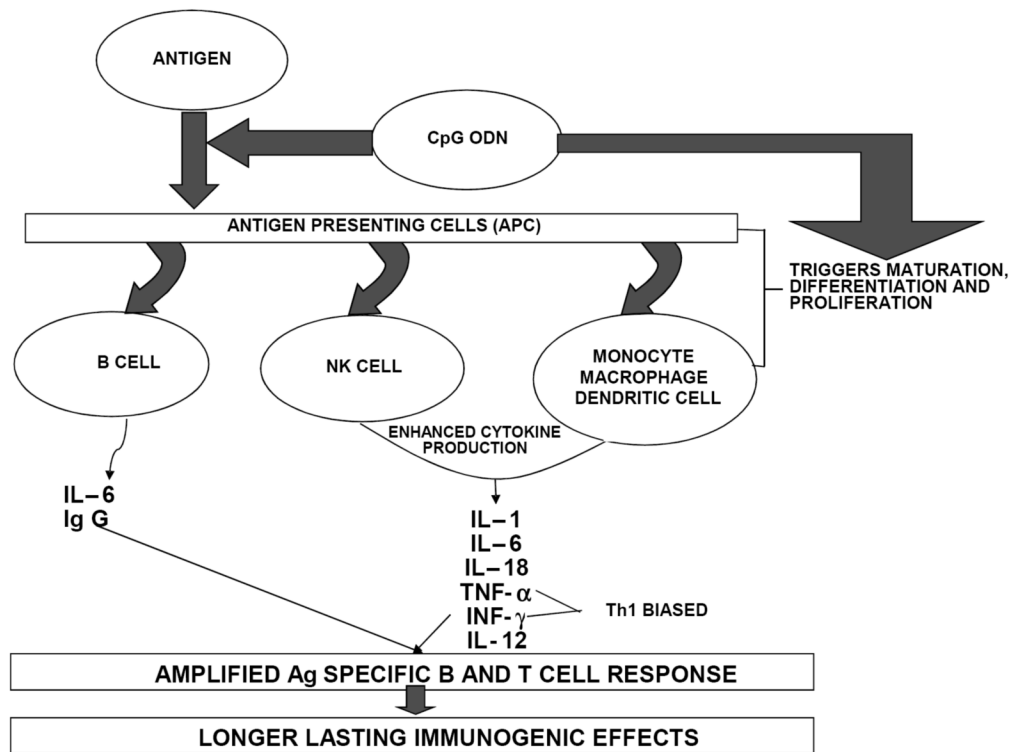


Figure 1. Schematic showing immunostimulatory cascade triggered by co-delivery of antigen and CpG ODN.

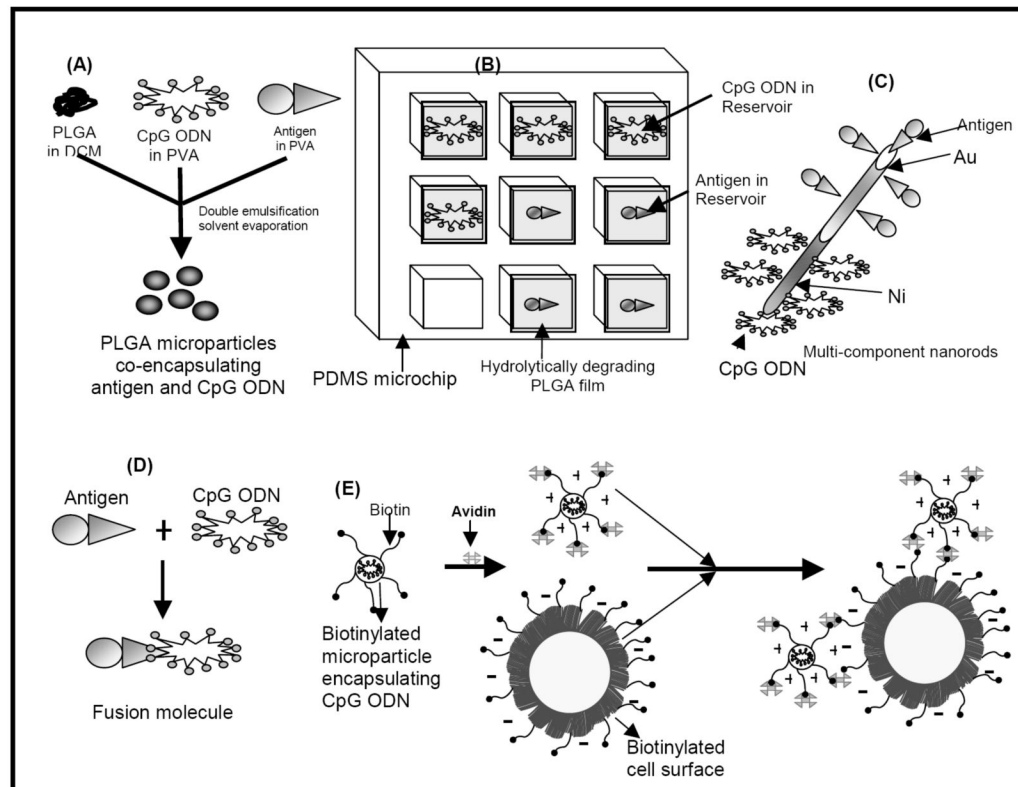


Figure 2. Schematic showing some recent innovative strategies to co-delivering antigen and CpG-ODN. These include (A) PLGA microparticles co-entrapping antigen and CpG ODN, (B) pulsatile delivery of antigen and CpG ODN from PDMS microchips with biodegradable PLGA seals, (C) multi-component nanorods functionalized with antigen and CpG ODN in spatially-defined regions, (D) antigen-CpG ODN conjugate molecules and (E) Cell-microparticle hybrids.

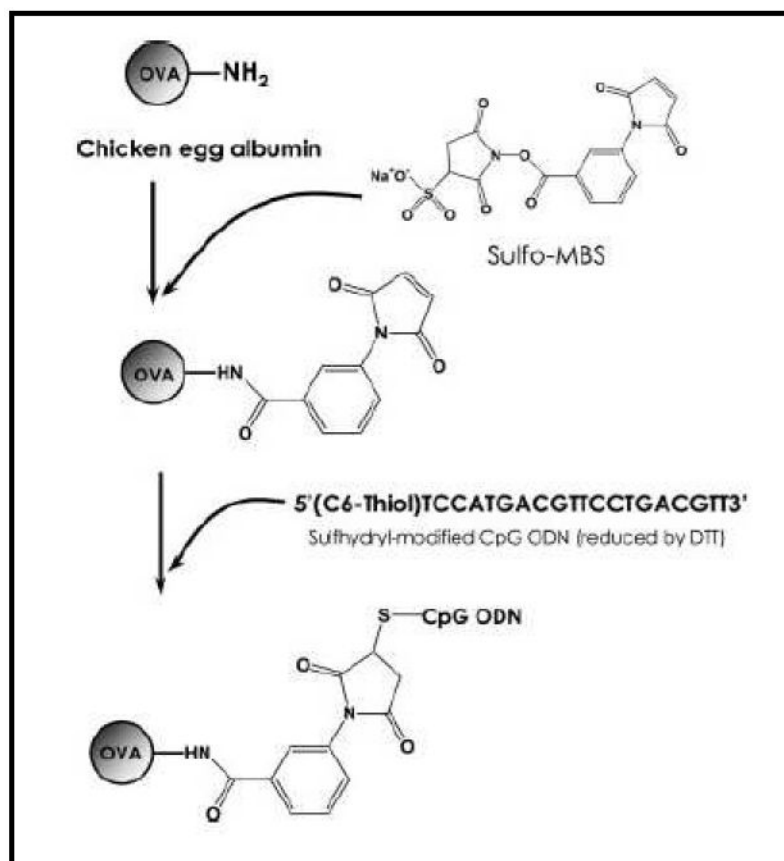


Figure 3. Schematic showing synthesis of the OVA-CpG ODN conjugate molecule. Reproduced with permission from [39]. Copyright © Wiley-VCH Verlag GmbH & Co. KGaA.

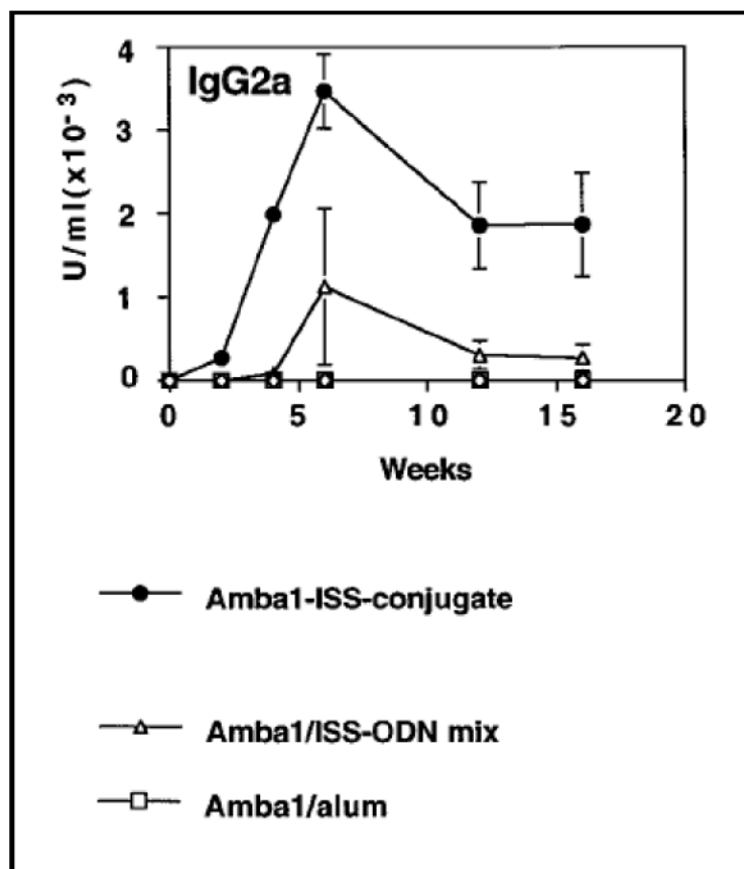


Figure 4. Graph showing IgG2a antigen (Amb a1) specific IgG2a profile generated by antigen-immunostimulatory ODN (ISS ODN) conjugate, antigen-ISS ODN free solution mixture and antigen-alum mixture. Reproduced with permission from [41]. Copyright © Elsevier B.V.

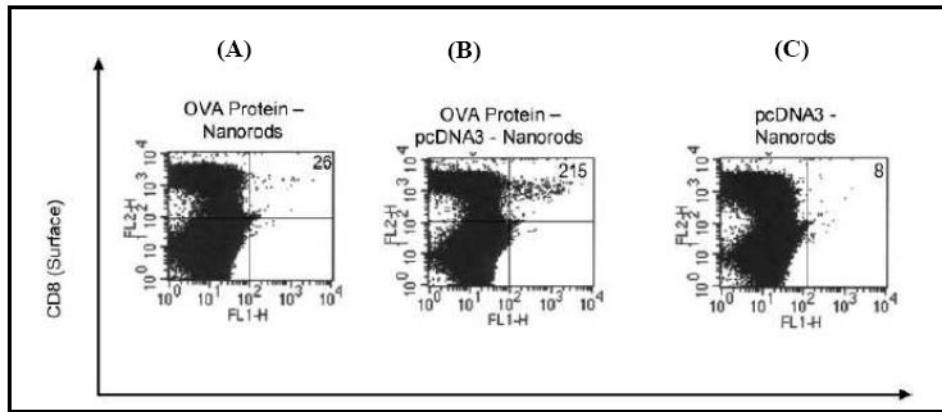


Figure 5.

Ten-fold increase in ovalbumin-specific CD8⁺ responses are observed in C57BL/6mice immunized with nanorods co-functionalized with OVA and CpG motif **(B)** in comparison to nanorods functionalized only with OVA **(A)** or CpG motif **(C)** alone. Reproduced and adapted with permission from [1]. Copyright © IOP.

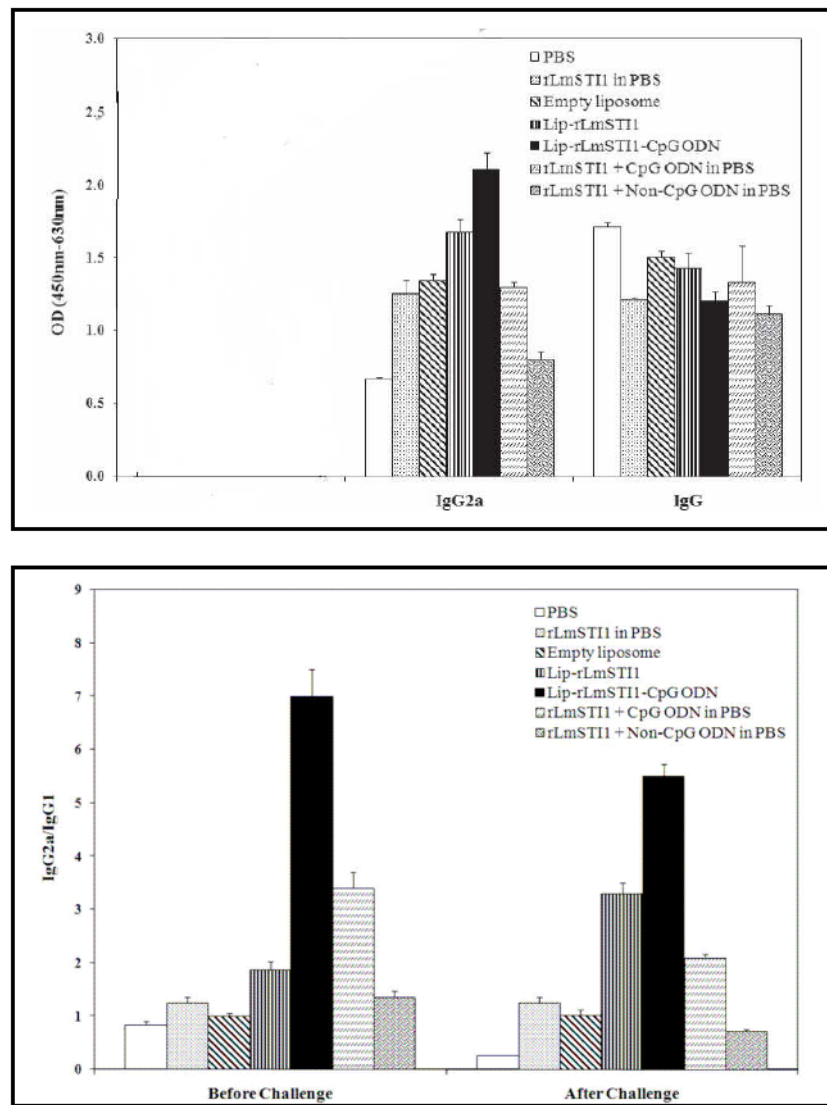


Figure 6. Bar charts showing IgG2a and IgG levels (**A**) and ratio of IgG2a/IgG1 (**B**) in sera of immunized mice at the end of 14 weeks. The mice were immunized with antigen alone (rLMSTII in PBS), empty liposomes, liposomes encapsulating antigen alone (Lip-rLMSTII), liposomes co-encapsulating both antigen and CpG ODN (Lip-rLMSTII-CpG ODN), both antigen and CpG ODN in solution (rLMSTII + CpG ODN in PBS) or liposomes entrapping antigen alone and co-administering CpG ODN in solution (rLMSTII + Non-CpG ODN in PBS). Reproduced with permission from [52]. Copyright © The American Society for Microbiology.

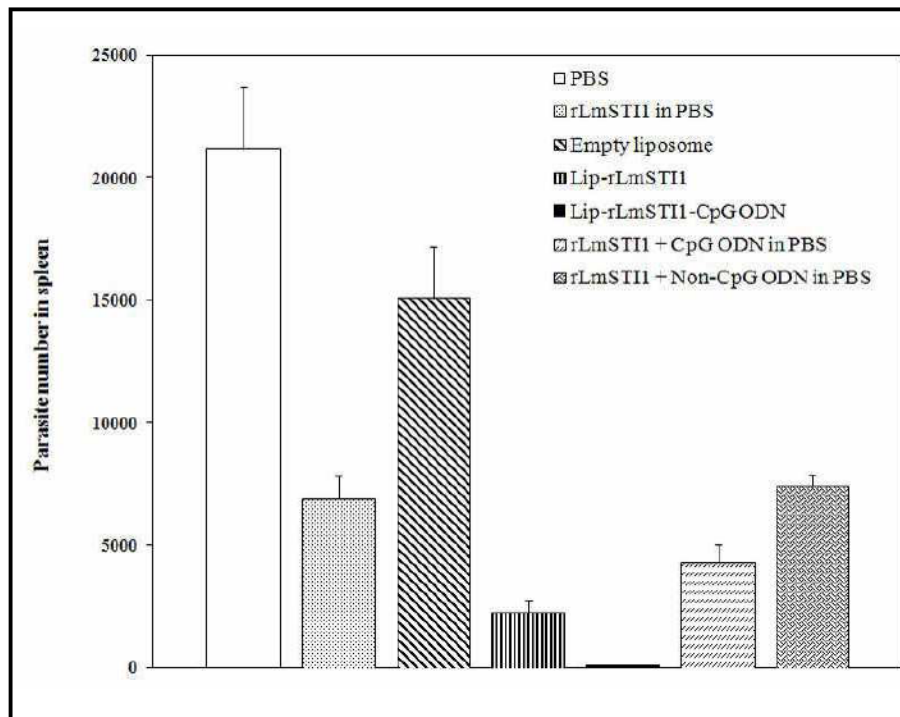


Figure 7. Viable parasite (*L. major*) in spleen of different groups of mice immunized with antigen alone (rLMSTII in PBS), empty liposomes, liposomes encapsulating antigen alone (Lip-rLMSTII), liposomes co-encapsulating both antigen and CpG ODN (Lip-rLMSTII-CpG ODN), antigen and CpG ODN in solution (rLMSTII + CpG ODN in PBS) or liposomes entrapping antigen alone and co-administering CpG ODN in solution (rLmSTII + Non-CpG ODN in PBS). Reproduced with permission from [52]. Copyright © The American Society for Microbiology.

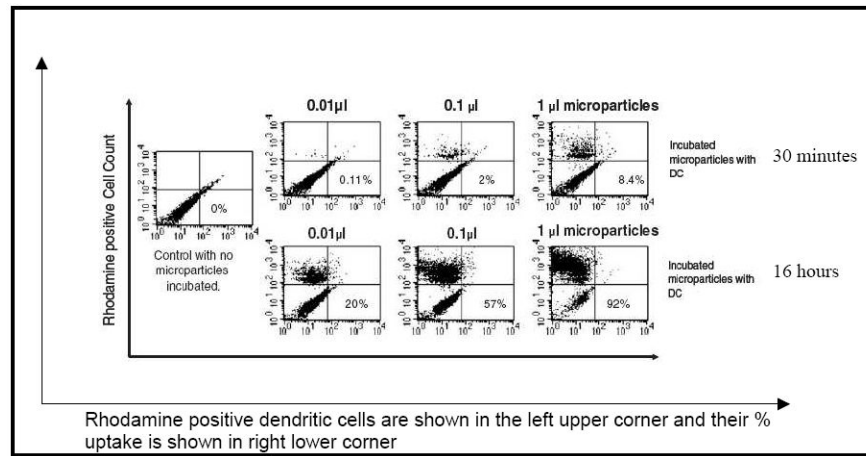
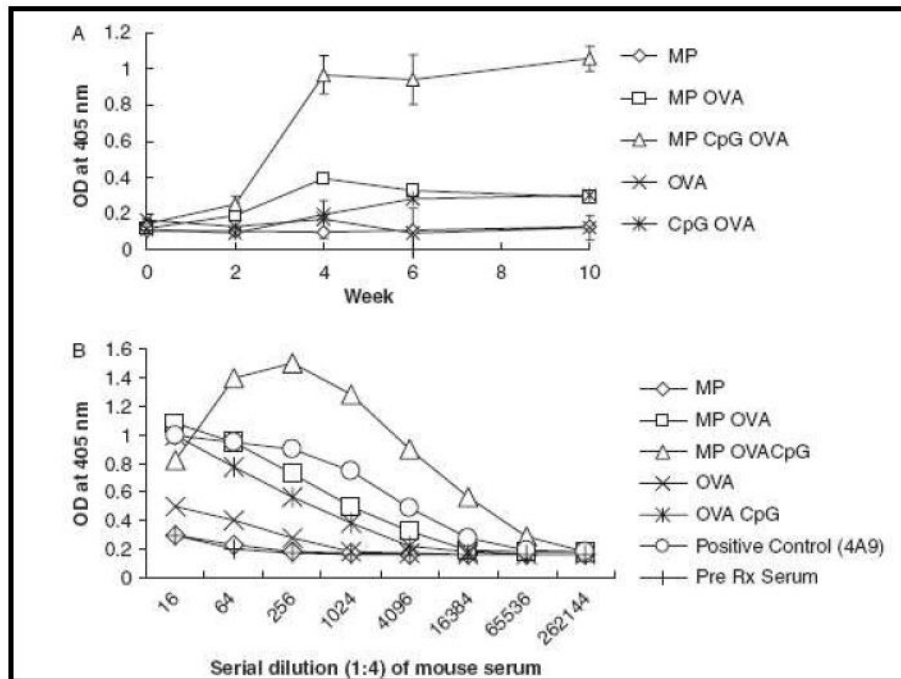


Figure 8. Uptake of rhodamine-loaded PLGA microparticles by DC2.4 dendritic cells (DCs) at the end of 30 minutes and 16 hours as measured by flow cytometry. Reproduced with permission from [26]. Copyright © Lippincott Williams & Wilkins.

**Figure 9.**

Graphs showing data from (A) ELISA assay of anti-OVA IgG response in mouse serum. Mice were inoculated twice on day 1 (week 0) and 14 (week 2) with either empty microparticles (MP), microparticle entrapping OVA alone (MP OVA), or microparticle co-encapsulating OVA and CpG ODN (MP CpG OVA), or with OVA administered as solution) or OVA and CpG co-administered in solution and (B) serial dilution in phosphate buffered saline of week 4 serum from mice immunized at week 0 and week 2. The positive control is a monoclonal anti-Ova antibody (4A9). The negative control is pretreated mouse serum. Reproduced with permission from [26]. Copyright © Lippincott Williams & Wilkins.

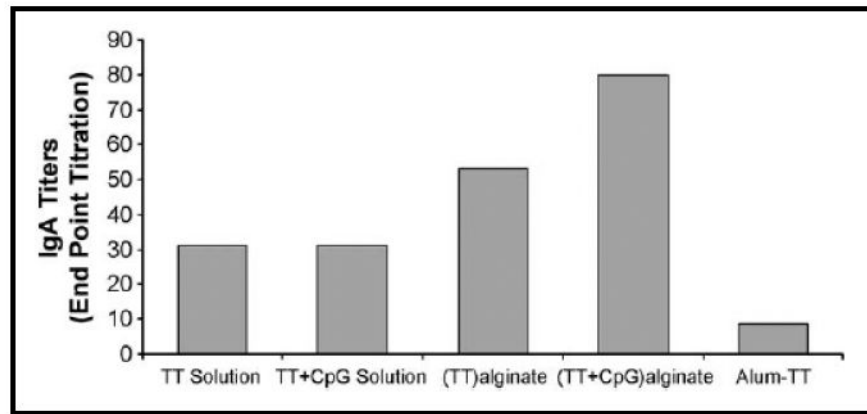


Figure 10.

Nasal Lavage anti-IgG A titers in rabbits immunized with intranasal formulations of antigen and CpG ODN in solution, alginate microparticles loaded with antigen alone, alginate microparticles co-encapsulating antigen and CpG ODN or antigen co-delivered with the alum as adjuvant. Reproduced with permission from [75]. Copyright © Elsevier B.V.

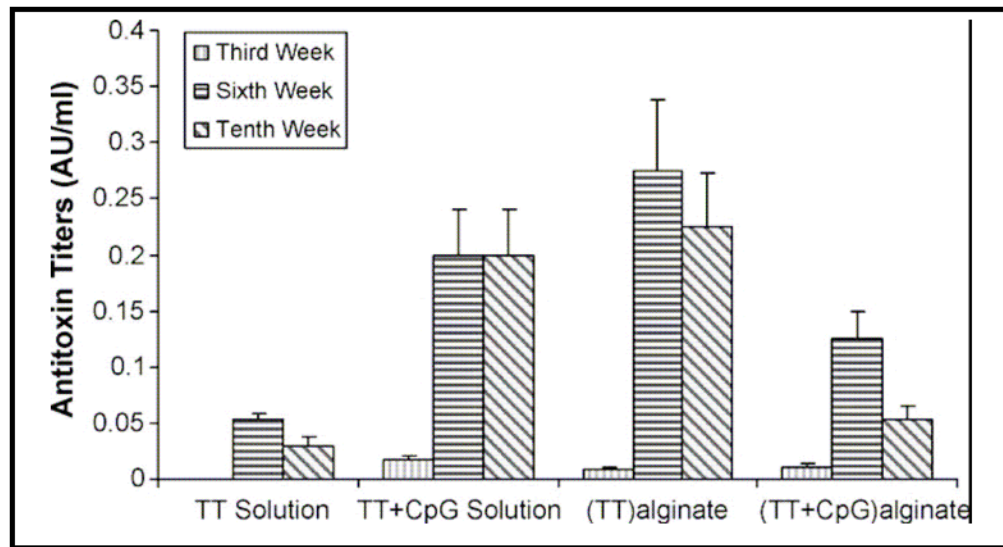


Figure 11.

Serum antitoxin titers in rabbits immunized with intranasal formulations of antigen with or without CpG ODN in solution, alginate microparticles loaded with antigen alone, or alginate microparticles co-encapsulating antigen and CpG ODN [75]. Copyright © Elsevier B.V.

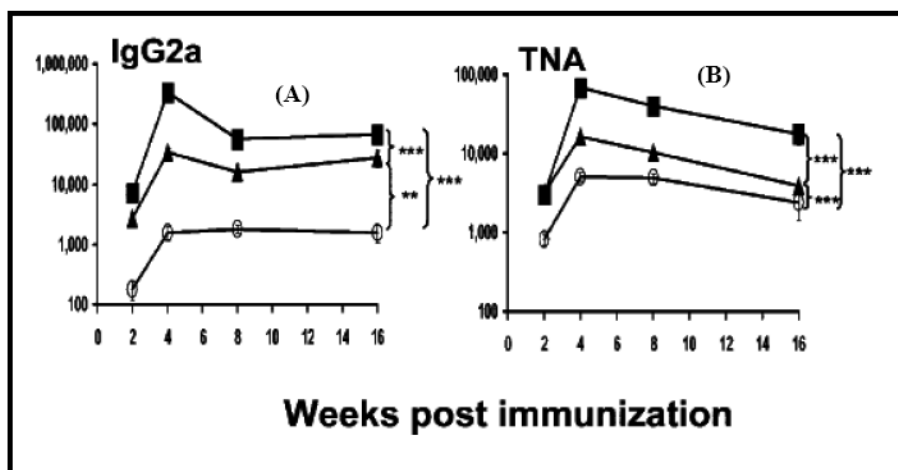


Figure 12. Graph showing IgG2a anti-PA antibody titers (A), and toxin neutralizing activity (TNA) (B), in anthrax-vaccinated mice. Male A/J mice were immunized intraperitoneally with AVA (O) with free CpG ODN (\blacktriangle) or PLGA-adsorbed CpG ODN (\blacksquare). Reproduced with permission from [77]. Copyright © The American Center for Microbiology.

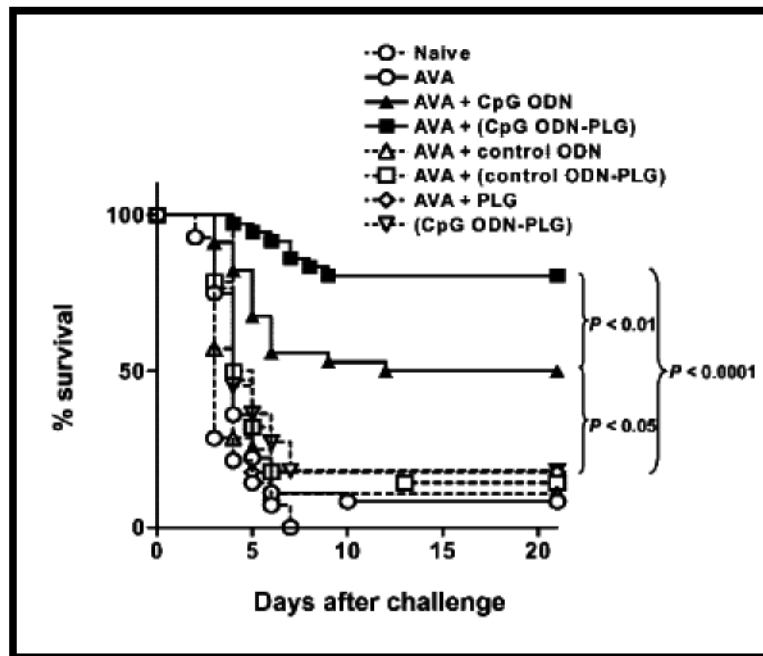


Figure 13.

Graph showing survival of vaccinated mice. A/J mice were immunized intraperitoneally with anthrax vaccine (AVA) plus free CpG ODN (AVA + CpG ODN), AVA and PLGA-adsorbed CpG ODN (AVA + CpG ODN-PLG), AVA alone (AVA) or PLGA. Reproduced with permission from [77]. Copyright © The American Center for Microbiology.

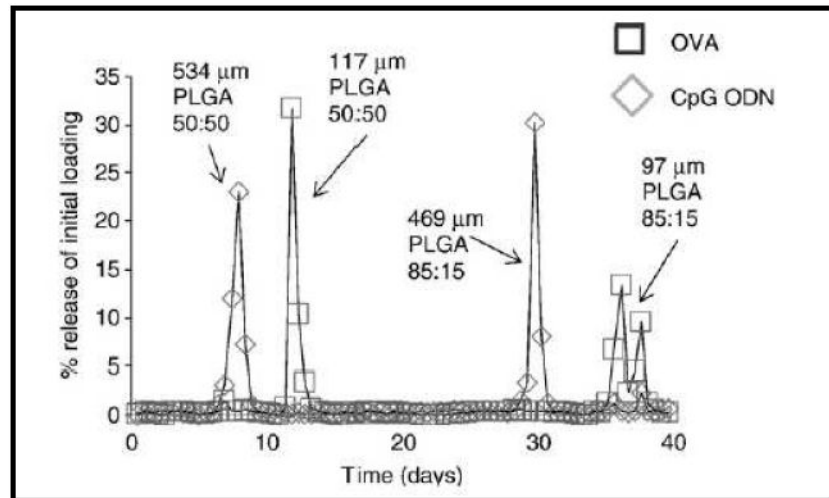


Figure 14. Graph showing pulsatile release of OVA and CpG ODN from PDMS microchips with hydrolytically degrading PLGA seals. Reproduced with permission from [31]. Copyright © Elsevier B.V.

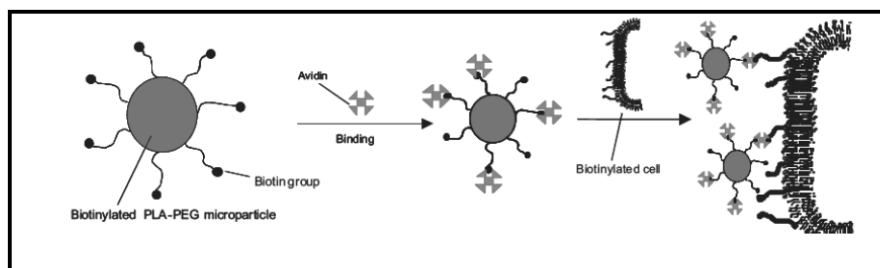


Figure 15. Schematic showing the avidin-biotin mediated self-assembly of hybrid cell-microparticle carrier systems for potential co-delivery of antigen and CpG ODN. Reproduced with permission from [117]. Copyright © Wiley-VCH Verlag GmbH & Co. KGaA.

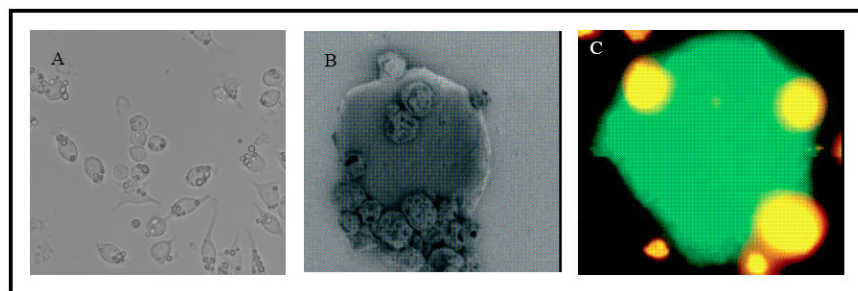


Figure 16. (A) Light microscopy image of multiple cell-microparticle hybrids cultured on a well plate, (B) SEM image of a cell-microparticle hybrid and (C) rhodamine-loaded microparticles immobilized on the surface of a cell expressing green fluorescent protein. Reproduced with permission from [117]. Copyright © Wiley-VCH Verlag GmbH & Co. KGaA.

Table I

IFN- γ measurements of DCs pulsed with antigen-CpG ODN conjugates as co-delivery vehicle and solution as co-delivery vehicle of CpG ODN and OVA. Reproduced with permission from [39]. Copyright © Wiley-VCH Verlag CmbH & Co. KgaA.

DC + OT-1 Cells	IFN- γ \pm SE (pg/mL)
OVA-CpG fusion	862 \pm 21
OVA and Alum	None detected
OVA + CpG	53 \pm 13
Untreated DCs	None detected

Table II

Cytokine profile of antigen (Amb a1) activated spleen cells with and without adjuvant immunostimulatory ODN (ISS ODN) as a conjugate molecule and mixture forms. Reproduced with permission from [41]. Copyright © Elsevier B.V.

Antigen used for immunization	IFN- γ (pg/mL)	IL-5 (pg/mL)
Amb a 1	<10	630 \pm 150
Amb a 1-ISS conjugate	8340 \pm 2170*	<10*
Amb a 1-mODN conjugate	<10	500 \pm 150
Amb a 1/ISS-ODN mix	170 \pm 110	610 \pm 280
Amb a 1/alum	490 \pm 240	4410 \pm 880
Saline (naive)	<10	<10