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Approaches to enhancing immune responses stimulated by CpG oligodeoxynucleotides [☆]

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ABSTRACT

CpG oligodeoxynucleotides (ODN) activate the immune system and are promising immunotherapeutic agents against infectious diseases, allergy/asthma and cancer. It has become apparent that while CpG ODN are potent immune activators in mice, their immune stimulatory effects are often less dramatic in humans and large animals. This disparity between rodents and mammals has been attributed to the differences in TLR9 expression in different species. This along with the sometimes transient activity of ODN may limit its potential immunotherapeutic applications. Several approaches to enhance the activity of CpG ODN have been explored including formulation of ODN in depot-forming adjuvants, and more recently, coadministration with polyphosphazenes, inhibitors of cytokines that downregulate TLR9 activation, and simultaneous activation with multiple TLR agonists. We will discuss these approaches and the mechanisms involved, with emphasis on what we have learned from large animal models.

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1. Introduction

CpG ODN have been shown to be a very strong adjuvant in mice, and promote Th1-type immune responses, often performing better than complete Freund's adjuvant (CFA) which is the gold-standard for inducing cell-mediated immune responses in rodents. CpG ODN have the advantage in that they do not appear to cause severe local inflammatory reactions associated with CFA. CpG can have even greater

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adjuvant activity in mice if formulated or coadministered with other adjuvants [1,2]. This is consistent with an emerging paradigm in vaccinology that multiple adjuvants in combination may be more potent in enhancing immune responses to vaccines than individual adjuvants. This is a deviation from the past where investigators have conventionally tested vaccine preparations containing a single adjuvant. Indeed, evidence is accumulating that multiple adjuvants used in combination have tremendous potential in enhancing the efficacy of experimental vaccines that far surpasses what can be achieved with individual adjuvants [3–5].

Many conventional adjuvants induce good Th2-type immune responses but are not effective at promoting Th1 type immune responses. This is a major limitation in vaccines against pathogens for which Th1 responses are required for protection. Since CpG is predominantly a Th1 adjuvant, it has been of interest to determine whether CpG can modulate immune responses induced by such vaccines. In this regard, addition of CpG ODN to vaccine preparations containing some conventional adjuvants did not compromise the efficacy of these vaccines. Rather, CpG ODN complemented these conventional adjuvants resulting in not only improved magnitude, but also the quality of immune responses [1,3,5]. These include a wide range of substances with known adjuvant activity such as particulates, mineral salts, saponins, liposomes, cationic peptides, polysaccharides and bacterial toxins [2]. The mechanisms which mediate these synergistic responses between CpG and other adjuvants are not fully understood, but several factors may contribute to this synergy including; protection of CpG from enzymatic degradation, a depot effect whereby the CpG is slowly released, and possibly by increasing uptake of ODN by antigen presenting cells (APC) such as dendritic cells (DC).

Enhancing the activity of CpG ODN is even more important in humans and large animals where CpG by itself is not as potent as in rodents. This has created a need to explore ways to potentiate the activity of CpG ODN. The search for ways to enhance the activity of CpG ODN has extended beyond substances conventionally known for their adjuvant activity and now includes novel substances such as synthetic biodegradable polymers, inhibitors of immunoregulatory cytokines and stimulation with multiple TLR agonists.

2. Approaches to enhance immune activity of CpG ODN

2.1. Co-formulation of CpG ODN in depot-forming substances

2.1.1. Adaptive immunity

Early studies by Davis and colleagues [1] revealed that addition of CpG to alum containing Hepatitis B vaccine resulted in synergistic antibody response in mice. We compared a number of depot-forming adjuvants, including non-oil, metabolizable oil and mineral oil based ones, for their ability to enhance adjuvant activity of CpG ODN when formulated with a truncated secreted form of glycoprotein D (tgD) of bovine herpesvirus-1 (BHV-1). The antibody responses induced in mice by tgD and the adjuvant combinations with CpG ODN were significantly higher than those elicited by tgD formulated with CpG ODN alone. An important observation was that while CpG ODN elicited a Th1-type response and all other tested adjuvants induced a Th2-biased immune response, the combinations of CpG ODN and several of the co-adjuvants, including Quil A, Emulsigen and Emulsigen (EMULSIGEN™, MVP Laboratories, Inc., Ralston, NE, USA) resulted in a relatively balanced Th1/Th2 immune responses [3]. A similar enhancement of immune responses by combining CpG ODN with Emulsigen was observed in rabbits [6] and sheep [3], confirming that the enhancement was not limited to mice.

Even though there is no linear correlation between the animal size and CpG ODN dose, it is obvious that higher doses are needed for CpG ODNs to be effective in larger species. For example, in cattle, the amount of CpG ODN required to induce a similar immune response to one of our established mineral oil-based adjuvants, VSA3, was between 10 and 50 mg [7]. Although the cost of CpG ODN may not be a major factor for

human vaccines, such high doses of CpG ODN would be uneconomical in animal vaccine. This makes it even more important to formulate CpG ODN with a co-adjuvant as this may reduce the dose of CpG required to induce immune responses. We observed that formulation of tgD with CpG ODN and an oil-in-water adjuvant, Emulsigen, significantly enhanced serum neutralizing antibody responses and protection from BHV-1 challenge in calves when compared to formulation with CpG ODN only; indeed, even if the CpG ODN were formulated with Emulsigen at a low dose of 250 µg, the immune responses and protection were still stronger than with 25 mg CpG ODN alone [8]. This demonstrates that in addition to improvements in the immune responses and protection induced, formulation of CpG ODN with a co-adjuvant leads to a reduction in the dose and hence the cost of CpG ODN. Furthermore, formulation of tgD with both Emulsigen and CpG ODN leads to “antigen sparing”, as a reduced tgD dose elicited equivalent immune responses when compared to the antigen with Emulsigen alone (unpublished observations). Antigen sparing can be an important factor in vaccines such as influenza where large doses may be required within a short period of time. In this regard, Cooper et al. [9] demonstrated in human subjects that addition of CpG 7909 to a flu vaccine may allow the use of reduced doses of the flu vaccine with no detrimental effect on the immunogenicity.

We have also used CpG ODN as an adjuvant to develop vaccination strategies for respiratory syncytial virus (RSV), which causes upper and lower respiratory tract infections in humans of all age groups, but primarily targets infants and young children. In fact, RSV is the most common viral pathogen causing lower respiratory infections in infancy and childhood. Although RSV infection usually leads to T cell responses and viral clearance, under certain conditions, RSV can create a Th2 cytokine microenvironment that supports an immunopathologic IgE response. Consequently, an effective RSV vaccine should induce balanced immune responses, as well as local immunity at the mucosal surfaces of the respiratory tract. This is a challenge in newborns, who have difficulty developing a cell-mediated immune response. Additionally, maternal antibodies may interfere with vaccination in this age group.

Since bovine RSV (BRSV), one of the major respiratory pathogens in calves, causes very similar disease symptoms as human RSV, we chose to use BRSV to test vaccine formulations. This allowed us to first use the mouse model and then transfer promising formulations to calves. Previously, a formalin (FI)-inactivated RSV vaccine was tested in young children and did not induce protection, but instead resulted in enhanced pathology in children subsequently infected with RSV [10–13]. This was attributed to a Th2-biased immune response, and influx of granulocytes into the lung, resulting in bronchiolitis and pneumonia. Thus, it became very clear that a Th1-biased or balanced immune response is critical for induction of protective immunity to RSV. We have demonstrated that, while FI-BRSV with Emulsigen elicited a Th2-biased immune response in mice, FI-BRSV formulated with CpG ODN, or both Emulsigen and CpG ODN, induced a Th1-type or balanced immune response, characterized by enhanced IgG2a and IFN-γ production. Furthermore, mice immunized with FI-BRSV formulated with Emulsigen produced eotaxin and IL-5, as well as eosinophils in the lungs, which is indicative of an immunopathologic response, while the animals immunized with FI-BRSV and CpG ODN or Emulsigen and CpG ODN did not [14]. The amount of virus produced in the lungs upon challenge with BRSV was most dramatically reduced in the mice that received the CpG ODN formulated vaccines. Importantly, the ability of CpG ODN to balance the immune response to FI-BRSV formulated with Emulsigen, and enhance protection from viral challenge, was confirmed in newborn calves [15], strongly suggesting that the beneficial effect of CpG formulation is not restricted to mice.

2.1.2. Innate immunity

We explored whether Emulsigen would have any impact on the innate immune responses. Like in humans and mice, CpG is a potent inducer of 2'5'A synthetase in sheep and this is a reliable biomarker for

CpG-induced innate immune response in this animal model [16–18]. Subcutaneous injection of sheep with CpG 2007 formulated in 30% Emulsigen resulted in induction of peak levels of 2'5'A synthetase [16]. Newborn lambs injected with CpG2007 at a dose of 100 µg/kg body weight formulated in Emulsigen resulted in elevated 2'5'A synthetase activity and a significant reduction in viral shedding in BHV-1 [17] and bovine parainfluenza virus-3 (Mutwiri et al., unpublished observation) experimental infections. Although Emulsigen is not routinely used as a component of mucosal vaccines, we tested whether it would enhance the activity of CpG when given via the respiratory route. Interestingly, CpG plus emulsigen administered by intrapulmonary infusion induced peak serum 2'5'A synthetase responses [19]. Also, the ODN dose required to induce significant responses was reduced by 80% when the CpG was formulated in Emulsigen [19]. This was significant given that much higher doses of CpG are required to activate innate immunity compared to what is normally required for an adjuvant effect. This makes sense because innate immunity is rapidly activated within hours and may last up several days. In contrast, adaptive immunity may take weeks, which allows amplification of immune responses through clonal expansion of lymphocytes activated (as a consequence of innate immune activation). However, local tissue reactions were not assessed after intrapulmonary infusion of the Emulsigen+CpG formulation, but no undesirable side effects were observed in the animals.

2.1.3. Mechanisms

How Emulsigen enhances the activity of CpG ODN has not been fully defined. Emulsigen is an oil-in-water substance and this class of adjuvants is a component of numerous veterinary vaccines [20]. Adjuvant activity in oil-based adjuvants is generally thought to be mediated through formation of a depot, and therefore may enhance CpG ODN effect by preventing rapid degradation of the ODN and slowly releasing it from the site of injection. However, these emulsions are not inert substances and are known to cause tissue damage at the site of injection. It is now known that tissue inflammation and cell death can provide “danger” signals which may contribute to adjuvant activity [21,22]. Subcutaneous injection of emulsigen in sheep induced a mild diffuse subcutaneous infiltration of mononuclear cells at the site of injection while injection of CpG ODN alone induced an organized perivascular infiltration of leukocyte infiltration [16]. However, formulation of CpG in emulsigen changed the intensity and nature of the cellular infiltration as indicated by massive recruitment of mononuclear cells and eosinophils at the site of injection [16]. Interestingly, the majority of cells recruited by CpG were MHCII+ cells, and this was a CpG-specific phenomenon since nonCpG ODN did not induce any cell infiltration [16]. These observations suggest that Emulsigen may enhance the effect of CpG ODN possibly by recruiting immune cells to the site of injection, and these cells are then locally activated by CpG ODN.

Similarly, the mechanism by which alum enhances the adjuvant activity of CpG is not known. In fact, the mechanisms which mediate the adjuvant activity of alum are not fully understood. For many years it was thought that the adjuvant activity of alum was due to formation of a depot at the site of injection, but evidence is accumulating that other mechanisms may be involved. Alum has been shown to increase antigen uptake by DC in vitro [23]. Also, alum has immunostimulatory activity as indicated by its ability to promote cell recruitment to the site of injection [24,25] and to activate monocytes and macrophages [26,27]. Injection of alum induced recruitment of monocytes to draining lymph nodes and their subsequent differentiation into DC [28]. However, whether any of these actions of alum contributes to its synergy with CpG remains unknown. A novel mechanism has been proposed whereby alum activates a NOD-like receptor, Nlrp3 (also known as Nalp3) inflammasome which mediates the adjuvant activity of alum, and synergizes with TLR4 agonist LPS resulting in increased cytokine responses [29–31]. However, it remains to be determined whether this mechanism contributes to the synergy of alum and CpG combination in the context of adaptive immune responses.

2.2. Polyphosphazene polyelectrolytes enhance immune responses to CpG

2.2.1. Adaptive immunity: parenteral immunization

Polyphosphazenes are synthetic water soluble, biodegradable polymers that have great promise as vaccine adjuvants and drug delivery vehicles. One of the most investigated polyphosphazenes polyelectrolyte, PCPP (poly[di(sodium carboxylatophenoxy)phosphazene]), has previously been shown to have adjuvant activity in mice with a variety of viral and bacterial antigens [32–34]. Recent investigations in our laboratory have revealed that a new generation polyphosphazene, PCEP (poly[di(sodium carboxylatoethylphenoxy)phosphazene]), is an even more powerful adjuvant with influenza virus X:31 antigen and Hepatitis B virus surface antigen (HBsAg) [4,35]. We evaluated whether co-administration of CpG ODN with this class of novel adjuvants, (polyphosphazenes), would result in further enhancement of immune responses above levels achieved by the individual adjuvants. Mice immunized with a single SC injection of HBsAg plus a combination of CpG and each of the two polyphosphazenes resulted in dramatic enhancement of HBsAg-specific antibody responses to levels that could not be achieved by either adjuvant alone. Perhaps of greater significance was the observation that the combination of CpG ODN+polyphosphazenes modulated the quality of the immune responses (as indicated by Th1 versus Th2 profile) often induced by CpG ODN alone. In this regard, CpG ODN, as expected, induced a predominantly Th1-type immune response profile as indicated by high IgG2a and low IgG1 antibody responses. In contrast, PCEP induced mixed Th1/Th2 type immune responses which were associated with high levels of IgG2a and IgG1 antibody titers, while PCPP induced predominantly Th2-type immune responses as indicated by relatively low IgG2a and high IgG1 levels [4]. The combination of CpG ODN+PCEP or CpG ODN+PCPP resulted in a very strong IgG2a antibody response that was at least two-fold higher than the response seen with any of the three adjuvants individually, indicative of a synergistic rather than an additive response. Similarly, the combination of CpG ODN+PCPP resulted in synergy with respect to IgG1 antibody titers though not as strong as the IgG2a response. Thus the use of the CpG+polyphosphazene combination resulted in very strong mixed Th1/Th2 immune responses that are more likely to provide broader protection in situations where such responses contribute to disease protection. Although it is difficult to compare between different immunization studies, it appears that polyphosphazenes in combination with CpG is quite a potent adjuvant formulation. Alum and CpG combination was reported to enhance total IgG by 35-fold but no significant enhancement of IgG2a and IgG1 responses was observed [1]. In contrast, upon immunization with the polyphosphazene+CpG combination, IgG2a was increased 100-fold as mentioned above [4]. However, when we examined the effect of CpG+polyphosphazene combination on the HBsAg-specific cytokine responses in splenocytes of immunized mice, we found that there was no synergistic response with respect to IFN γ and IL-4 cytokine profiles of immunized mice. This is likely due to the fact that the cytokine responses were assessed four months after a single immunization. Perhaps assessing cytokine responses at an earlier time point, such as a few weeks after immunization, may have yielded different results.

2.2.2. Adaptive immunity: mucosal immunization

Many infectious agents target the mucosal surfaces of the respiratory or intestinal tract. Consequently, vaccines against such pathogens should preferably induce mucosal immune responses, in addition to systemic immunity. This presents additional challenges for vaccinologists as mucosal vaccine formulations will need to resist degradation in the nasal or oral cavity. Furthermore, in the case of oral immunization, a vaccine should not induce a tolerogenic response.

CpG ODNs have shown efficacy as an adjuvant for intranasal immunization. For instance, after intranasal immunization with

formalin-inactivated influenza virus and CpG ODN, influenza-specific antibodies were detected in the sera, saliva and genital tract [36]. Furthermore, intranasal immunization of mice with *Streptococcus pyogenes* M6 protein with CpG ODN resulted in production of serum IgG and lung IgA [37]. Intranasal delivery of a CpG ODN-adjuvanted *Pseudomonas aeruginosa* conjugate vaccine also induced systemic and mucosal immunity in mice [38]. CpG ODN enhanced IgG2a and IFN- γ production, while inducing equivalent nasal IgA, serum IgG and clearance, in comparison with cholera toxin, in mice immunized with *Haemophilus influenzae* P6 protein [39]. In a recent report, *Trypanosoma cruzi* trans-sialidase antigen formulated with CpG ODN elicited mucosal and systemic protective immunity in mice [40].

Since candidate vaccine formulations ultimately need to be effective in the target species, including humans, efficient translation to clinical applications is a critical step, possibly requiring additional animal models. CpG ODN have been shown to be effective as a mucosal adjuvant in piglets, which are promising. When inactivated porcine reproductive and respiratory syndrome (PRRS) was combined with CpG ODN and administered intranasally to piglets, serum IgG, lymphocyte proliferation, and IgA in feces, nasal and oral secretions were enhanced [41].

As was observed for parenteral use of CpG ODN, there also is a need to co-adjuvant CpG ODN, as well as possibly protect the CpG ODN and the vaccine antigens, from degradation when used for intranasal immunization. To address this issue, several approaches have been explored. Cholera toxin (CT) is a classical mucosal adjuvant, which has been used as co-adjuvant with CpG ODN as well. McCluskie and Davis demonstrated that intranasal immunization of mice with hepatitis B surface antigen (HBsAg) and CpG ODN elicited humoral and cell-mediated systemic immune responses, as well as a mucosal (IgA) response in the lungs. These responses were superior to those induced with CT as adjuvant, while the combination of CpG ODN and CT induced a synergistic response [42]. In contrast, generally no synergy was found for CpG ODN and heat-labile enterotoxin (LT) [43]. In another trial the combination of CpG ODN and CT administered intranasally with *Helicobacter felis* resulted in induction of sterile immunity, while the protection levels were lower when only one of the two adjuvants was used [44]. However, CT is toxic for humans and therefore of limited value with regard to clinical application. CpG ODN have also been encapsulated into liposomes and delivered intranasally to mice; which resulted in significant improvement of serum and mucosal IgG2a and IgA, proliferative responses, cytotoxicity and IFN- γ production to influenza haemagglutinin and neuraminidase, as well as HBsAg [45]. Incorporation of CpG into alginate microspheres also resulted in higher mucosal IgA in nasal lavage, but not in higher serum IgG after intranasal delivery in rabbits [46]. However, these types of formulations can be expensive or difficult to scale up.

Mucosal immunization is not only expected to induce local immune responses, but also to improve the cellular component of the immune response, as well as avoid vaccine inactivation by circulating maternal antibodies. The ability of CpG ODN to promote a Th1-biased or balanced immune response and act as a mucosal adjuvant makes it very attractive to be included in a mucosal RSV vaccine. The potential to induce protection from RSV infection by intranasal immunization has been described. For example, recombinant fusion (F) protein adjuvanted with CpG ODN [47], cholera toxin [48,49], or caprylic/capric glycerides and polyoxyethylene-20-sorbitan monolaurate [50], live viral vectors [51–53] and bacterial vectors [54,55] expressing RSV proteins or peptides can protect rodents from RSV challenge.

Since Emulsigen is unsuitable for intranasal delivery, we evaluated polyphosphazene (PP) for formulation of CpG ODN in a formalin-inactivated (FI)-BRSV vaccine preparation [56]. Mice were immunized intranasally with FI-BRSV formulated with CpG ODN, PP or both CpG ODN and PP, and then challenged with BRSV. The mice immunized with FI-BRSV formulated with CpG ODN and PP (FI-BRSV+CpG+PP) developed stronger humoral and cell-mediated immune responses than mice immunized with FI-BRSV in PBS, or with PP or CpG ODN alone. Production of BRSV-specific serum IgG was enhanced, while in

vitro re-stimulated splenocytes produced increased IFN- γ and decreased IL-5. The addition of CpG ODN to either FI-BRSV or FI-BRSV+PP shifted the response from Th2-biased, characterized by high levels of IL-5 secretion, to a Th1-type response, as was evident from high levels of IFN- γ production. Furthermore, mucosal IgG and IgA levels were also higher in the FI-BRSV+CpG+PP group than in all other groups. The FI-BRSV+CpG+PP formulation also elicited better protection as was evident from a significant reduction in viral replication upon BRSV challenge. These results suggest that CpG ODN and PP act synergistically when delivered mucosally. Furthermore, a direct comparison of delivery routes demonstrated that intranasal administration resulted in superior systemic humoral and cell-mediated, as well as mucosal immune responses than subcutaneous delivery regardless of whether FI-BRSV was formulated with CpG ODN, PP or both CpG ODN and PP (unpublished observations).

Polyphosphazenes offer some advantages as a co-adjuvant for CpG ODN. Most reports indicate a high safety profile in animals, but more studies are required especially in humans where there is only anecdotal evidence of their safety in clinical trials. In addition, polyphosphazenes are generally inexpensive to synthesize and can be stable for several years at room temperature when stored away from light.

2.2.3. Innate immunity

CpG ODN activate innate immunity and protect chicken against mortality in experimental infections with *Escherichia coli* and *Salmonella enteritidis* [57–60]. Three formulations (polyphosphazenes, Emulsigen and liposomes) were evaluated for their capacity to enhance the protective effects of CpG ODN in a chicken model of *E. coli* infection. Addition of PCPP and PCEP to CpG ODN resulted in an enhancement of the protective effects of CpG ODN as indicated by a reduction in mortality [61]. However, neither formulation prolonged the duration of protection. Interestingly, PCEP alone did confer some protection, an observation that was not very surprising given that this polymer can activate innate immunity in mice [4]. More studies are required to determine whether polyphosphazenes can be equally beneficial in other species. Surprisingly, neither Emulsigen nor liposomes had any effect in enhancing the activity of CpG ODN in chickens. The reason for this unexpected observation remains unknown since both formulations have clearly been shown to enhance CpG ODN activity in rodents and mammals [3,8,45,62].

2.2.4. Proposed mechanisms

The mechanisms which mediate the synergy between polyphosphazenes and CpG ODN have not been defined. Actually, even the mechanisms which mediate the adjuvant activity of polyphosphazenes are not fully understood. While formation of a depot at the site of injection may be a contributing factor in the synergistic response when CpG and mineral oils are used in combination [2], depot formation may not play a significant role in the adjuvant activity of polyphosphazenes as suggested by Payne et al. [33]. Andrianov et al. have recently suggested that the adjuvant activity of polyphosphazenes is a result of the physical association between the polymer and the antigen [63]. However, our observations that PCEP and PCPP induced distinct Th1 and Th2 type immune response profiles [4,35] suggested that in addition to the polymer–antigen interaction proposed by Andrianov [63], other mechanisms may be involved in mediating the immune effects of polyphosphazenes. Cytokines produced during the innate phase of immune activation play a crucial role in directing the ensuing adaptive immune responses. For example, proinflammatory cytokines such IL-12 induced by CpG ODN are thought to contribute to its potent Th1 promoting adjuvant activity [1,64]. We have recently reported that PCEP or PCPP (in the absence of antigen) stimulated production of IL-4 and IL-12, but only PCEP induced significant IFN γ production [4]. These observations provided evidence that PCEP and PCPP can stimulate innate cytokine production, suggesting a potential mechanism by which polyphosphazenes achieve their potent adjuvant effects. Additionally,

using IL-12 knockout mice, we showed that IL-12 was not required for the PCEP-induced IFN γ response, but as expected, CpG-induced IFN γ production was dependent on the presence of IL-12. Thus, polyphosphazenes and CpG both induce IFN γ production by different mechanisms which involve IL-12 dependent and independent pathways. Given the importance of IFN γ in immune responses, these observations may explain why when used in combination they induce a synergistic enhancement of immune responses.

2.3. Inhibition of regulatory molecules

It is now clear that one of the main consequences of TLR9 activation is induction of a pro-inflammatory cytokine cascade. Considering that proinflammatory cytokines can have beneficial as well as undesirable effects, the host has evolved mechanisms to appropriately downregulate TLR-induced effector responses to prevent severe inflammation and collateral tissue damage [65,66]. In this regard, it has been observed that activation of immune cells through TLR9, as well as through other TLRs also leads to induction of IL-10 [67–69], an immunoregulatory cytokine with anti-inflammatory activities and the ability to suppress potentially harmful immune responses by regulating the Th1/Th2 balance [70–73]. IL-10 is also a growth factor for regulatory T cells and induces the development of IL-10-secreting regulatory T cells (T_{regs}) [74–76]. Early in vitro studies demonstrated that CpG ODN activated B cells to produce IL-10 [77,78], and it was later demonstrated that IL-10 limits CpG responses in vitro [79]. Indeed, TLR agonists including CpG ODN act through p38 MAPK to induce IL-10 production by DC [67,69], and the IL-10 in turn promotes the development of IL-10-secreting T_{regs} [67]. Inhibitors of p38 suppressed CpG-induced IL-10 and PGE $_2$, but also enhanced IL-12 production in mouse pDC [67]. Interestingly, incubation of antigen-pulsed CpG-stimulated DC with a p38 inhibitor suppressed their ability to generate T_{reg} cells, while enhancing the generation of Th1 cells [67]. Also, inhibition of p38 enhanced the antitumor therapeutic efficacy of DC pulsed with antigen and CpG, and this was associated with an enhanced frequency of IFN γ -secreting T cells and a reduction in Foxp3 $^{+}$ T_{regs} infiltrating tumors [67]. It has also been known for some time that pathogens can suppress protective immune responses by inducing T_{regs} [75] and depletion of T_{regs} can enhance development of protective CMI during chronic infection [80]. The importance of IL-10 secreting T_{regs} in the downregulation of immune responses and that of IL-12 in promoting Th1 responses suggested that IL-10 may limit the adjuvant activity of CpG ODN. Therefore, inhibition of IL-10 may be an approach to enhance the adjuvant activity of CpG ODN. In this regard, immunization of mice with a vaccine against *Bordetella pertussis* containing CpG ODN and p38 inhibitor resulted in significant enhancement of immune responses and protection against challenge with *B. pertussis* [67]. This encouraging observation will need further investigation to determine whether similar beneficial effects can be obtained in humans or large animals, given the rather significant differences in the activity of CpG ODN in rodents versus mammals. In vitro studies in our laboratory suggest that IL-10 does limit the capacity of B cells from sheep to respond to CpG stimulation. Inhibition of IL-10 either using a neutralizing mouse anti-bovine IL-10 antibody or using a p38 inhibitor resulted in significant increases in CpG-induced IFN α production in sheep (Mutwiri et al., unpublished observation). However, in vivo studies are required to confirm whether this approach will lead to improved CpG-induced immune responses in large animals.

It also needs to be determined whether there are any undesirable consequences of inhibiting p38 in vivo, which is required for other cellular functions in addition to IL-10 production. Granted, inhibition of T_{regs} through IL-10 to enhance the efficacy of CpG ODN as an adjuvant is a novel approach that requires further investigation.

2.4. Simultaneous activation of multiple TLRs

Most investigations on the immune effects of CpG and indeed other TLR agonists have focused on activation of an individual TLR at a time.

Given that microbial pathogens express many TLR and non-TLR agonists, it seems unlikely that microbial pathogens stimulate a single TLR at any given time. It is more likely that they simultaneously or sequentially activate multiple TLRs (and other recognition receptors), thus the immune responses detected are most likely a net effect of synergistic or inhibitory signals in immune cells. For example, simultaneous activation of DC with TLR9 and TLR4 agonists CpG ODN and LPS resulted in additive effects on IL-12 production [81]. Similarly, TLR3 and TLR8, or TLR4 and TLR8 stimulation resulted in synergy as indicated by high levels of IL-12, while no synergy was observed with ligands for TLR3 and TLR4 [82]. In contrast, simultaneous activation with ligands for TLR7/8 and TLR9 resulted in inhibition of IFN α responses [83,84]. The requirement for multiple ligands to induce potent responses has been proposed as a mechanism by which the immune system exerts a stringent “combinatorial security code” whereby at least two microbial products are required to stimulate a strong immune response in response to pathogen invasion [82]. Such synergistic effects would result in robust immune responses and lead to better protection against infection. For example, using double gene knockout mice (TLR2 $^{-/-}$ TLR9 $^{-/-}$), it was shown that TLR2 and TLR9 cooperate in the control of pathogen replication following *Mycobacterium tuberculosis* and *T. cruzi* infections [85,86]. However, while cooperation among TLR during infection may result in more robust immune responses and protection, if not properly controlled, these strong responses would result in immunopathology. Therefore, inhibitory effects at the appropriate time would be necessary to prevent tissue injury.

A profound understanding of the cellular events triggered by single or combinations of TLRs will be valuable in the rational design of more successful TLR-based immunotherapies and vaccination strategies.

In vitro, murine bone marrow-derived DC (BMDC) stimulated with TLR3 and TLR9 agonists resulted in a marked synergistic response with respect to IL-12 production and polarization of antigen-specific CD4 $^{+}$ T cells towards IFN- γ -producing Th1 phenotype [87]. Mice vaccinated with BMDC loaded with B16 melanoma cells stimulated with poly I:C+ CpG ODN were able to slow the growth of B16 tumors [87]. Similarly, BMDC stimulated with the same TLR agonist combination (poly I:C+CpG ODN) resulted in synergistic responses as indicated by increased IL-6 and IL-12, and enhanced antitumor effect in an experimental model of pulmonary metastasis [88]. BMDC activated with combinations of TLR3 and TLR7 agonist combinations resulted in a more rapid and sustained production of IL-6 and IL-12, and expression of costimulatory molecules [89]. Furthermore, peptide-loaded BMDC stimulated with these TLR agonists (for TLR3 and TLR7) combinations resulted in substantial increase in CTL effector functions in mice in vivo [89].

It appears that these effects of simultaneous activation of multiple TLRs may not be restricted to mice. We have observed that costimulation of sheep lymph node cells with poly I:C (agonist for TLR3) and CpG ODN resulted in an additive increase in IFN γ responses, while the agonist for TLR7/8, gardiquimod (InvivoGen, San Diego, USA) plus CpG ODN enhanced CpG-induced proliferation responses in bovine blood mononuclear cells (Mutwiri et al., unpublished observation). The mechanism of this enhancement remains to be determined but in some cases costimulation has resulted in upregulation of TLR mRNA. However, costimulation of bovine and ovine cells with TLR7/8 and TLR9 agonists resulted in downregulation of the IFN α response, an observation which is consistent with reports in mice [84]. It appears that even in immune cells from large animals, with the exception of the proliferation response, an additive or synergistic response is likely to occur when MyD88-dependent and -independent TLRs are simultaneously activated as has been suggested by others [89,90]. Accordingly, simultaneous or sequential activation of MyD88-dependent and -independent pathways results in synergy or cooperation, while simultaneous activation through the same pathway would result in tolerance or inhibition. This likely explains why agonists for TLR7/8 tend to inhibit IFN α responses normally induced by CpG ODN, all three (TLR7/8/9) are MyD88-dependent. It appears that this synergy can also be achieved

with non-TLR agonists. It was shown that human DC stimulated with a combination of CpG ODN and agonists for NOD-1 and NOD-2 resulted in synergistic IL-12 and IFN γ responses [91].

3. Future prospects

CpG ODN are promising immunotherapeutic agent and vaccine adjuvant. Their potential in the control of infectious and non-infectious diseases can be more fully realized in humans and large animals if certain limitations to its application can be addressed. Ideally, CpG ODN need to be directed to the appropriate cell types, at the correct concentration and for reasonable duration to achieve optimal effects. This is a challenge that may require exploring a variety of approaches to identify the most feasible for each of the specific applications. The most straightforward of these approaches is the formulation and delivery of CpG ODN which has yielded positive results. A better understanding of how the immune system responds to microbial molecules and how these responses are regulated has certainly opened new opportunities which are now being explored. As this is a rapidly evolving field, we anticipate additional opportunities will arise that will lead to realization of the full potential of CpG.

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