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Review Article

The potential of CpG oligodeoxynucleotides in the development of dendritic cell vaccines

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CONTENTS

Abstract

Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) capable of initiating a primary immune response, leading to the development of $CD8^+$ cytotoxic T-cells (CTLs) and $CD4^+$ T helper (Th) cells. These characteristics make them promising as vaccine carriers. However, despite the great potential demonstrated in murine models, DC-based vaccines have only been moderately effective in human clinical trials. The efficacy of DC-based vaccination or therapy depends on a number of variables, including antigen characteristics, DC lineage and level of maturation, vaccination route, frequency and interval, and host status. Many of these parameters need to be further optimized and/or understood before DC vaccines reach their full potential. Toll-like receptor (TLR) agonists are capable of activating DCs in response to pathogens and have potential as activation and maturation factors for DCs. The immunostimulatory properties of CpG oligodeoxynucleotides (ODNs), which signal through TLR9, have been extensively studied. The concept of using CpG ODNs stimulation of murine bone marrow-derived DCs (BM-DCs) has been proven mostly in tumor challenge models. According to several published reports, TLR9 is present on human plasmacytoid DCs and B-cells, but not on monocytes, which are the most common source of DC vaccines. However, a recent study demonstrated expression of TLR9 on human monocyte-derived DCs, which suggests that CpG ODNs might be useful as activation and maturation factors. Furthermore, TLR7 and TLR8 activation was found to have a similar mode of action to TLR9. TLR8 is expressed on human monocytes and monocyte-derived DCs, and TLR8 agonists may therefore also have potential in the generation of DC vaccines.

Introduction

Dendritic cell (DC)-based vaccines are highly effective inducers of both $CD8^+$ cytotoxic T-lymphocytes (CTLs) and $CD4^+$ T helper (Th) cells, and are thus promising as prophylactic or therapeutic treatments for cancer and chronic infections. Indeed, numerous reports have provided evidence that, at least in animal models, DCs can be superior to other vaccination strategies, specifically as cancer vaccines. However, although many DC vaccine candidates have been tested in human clinical trials, they were only moderately effective. This suggests that a number of parameters need to be further optimized, including antigen loading and maturation. Since only mature DCs induce the generation of robust $CD8^+$ T-cells and interferon gamma (IFN-γ)-producing $CD4^+$ T-cells, efficient maturation of the DCs is of utmost importance, although it is not yet clear what the optimal maturation stage is. The use of Toll-like receptor (TLR) ligands, which are capable of inducing maturation of DCs in response to pathogens, is emerging as an attractive strategy. The focus of this review is to discuss the potential of TLR7, 8 and 9 agonists, in particular the TLR9 ligand CpG oligodeoxynucleotide (ODN), to enhance the efficacy of antigen-pulsed DCs as vaccines.

Dendritic cell vaccines

Bone marrow-derived DCs (BM-DCs) are present throughout nonlymphoid tissues and constitute powerful antigen-presenting cells (APCs). These DCs continuously sample and acquire antigen, followed by processing and migration via lymph vessels or blood to the T-cell areas of regional secondary lymphoid organs. Provided the correct co-stimulatory factors are present, the DCs then present MHC class I- and II-restricted peptides to naïve T-cells (1, 2). These characteristics make DCs particularly suitable for antigen delivery.

DC-based vaccines have been demonstrated to have therapeutic potential in numerous murine tumor models. Furthermore, over 100 human clinical studies have demonstrated that this type of vaccination results in infrequent and mild adverse effects (3-5); unfortunately, DC-based vaccination has only been moderately effective in human clinical trials (6). Recently, DCs have attracted

interest as immune therapy and prophylactics for chronic infections, in particular when a strong cell-mediated immune response correlates with protection. Examples include malaria (7), simian immunodeficiency virus (SIV) (8), human immunodeficiency virus type 1 (HIV-1) (9), *Candida albicans* (10), *Leishmania major* (11, 12) and hepatitis C virus (HCV) (13-15).

The efficiency of antigen delivery and loading into DCs is critical for the optimal induction of T-cell-mediated immune responses. DCs can be pulsed with synthetic peptides (16-19) or full-length proteins (12, 20, 21), transduced with recombinant viruses (22) or transfected with DNA, RNA or self-replicating RNA (14, 23, 24). Transfection with RNA is particularly effective in comparison to other methods (25, 26).

In addition to the antigen properties and method of antigen loading, there are a number of other critical variables, including DC lineage and level of maturation, number of DCs per vaccination, vaccination route, frequency and interval, and host status. Since circulating DCs represent less than 1% of white blood cells (1), BM-DCs are most frequently used for murine studies, whereas blood monocytes are the most commonly used source of DCs for human vaccination. When the monocytes are cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, this leads to differentiation to immature DCs. Immature DCs lack co-stimulatory molecules, so presentation of antigen by these immature DCs may result in deletion or anergy of the T-cells. To become efficient APCs, immature DCs need to achieve a certain level of maturation. Indeed, mature DCs are capable of eliciting Th1-biased responses characterized by IFN-γ-secreting $CD4^+$ T-cells and robust $CD8^+$ T-cell responses**.** Although several maturation factors, such as tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS) or cocktails including TNF-α, IL-1β, IL-6 and prostaglandin E_2 (PGE_2), may achieve this**,** there is evidence that maturation with TLR agonists may lead to superior immune responses.

TLR agonists

TLRs recognize and bind pathogen-associated molecular patterns (PAMPs) or TLR ligands (27-29). In mammals, 11 TLRs have been identified. Although different TLR family members bind unique pathogen products, TLRs act through conserved, common signaling pathways that involve the adaptor protein MyD88 and result in the activation of NF-κB and activating protein-1 (AP-1). This culminates in the induction of inflammatory cytokines, such as TNF-α, IL-6, IL-1 and IL-12, type I interferons and chemokines (27-29). TLRs can also signal through alternative pathways that induce appropriate effector responses against pathogens (30-33). Importantly, TLR signaling induces upregulation of co-stimulatory molecules on DCs, leading to DC maturation, which is essential for the induction of pathogen-specific adaptive immune responses.

TLR4, TLR9 and to some extent TLR7/8 agonists are the most extensively studied as immune stimulators (29). In comparison to TLR4 agonists, which are very strong immunostimulants but also highly toxic, TLR7, 8 and 9 agonists have low toxicity and can be chemically synthesized and obtained in high purity, and thus have potential for *ex vivo* and *in vivo* stimulation of DCs as a method to enhance DC vaccine efficacy.

TLR9 agonists

DNA sequence motifs containing unmethylated CpG (CpG motifs) are present in high amounts in bacterial DNA and are recognized by the mammalian immune system via TLR9 (34, 35). Bacterial DNA or synthetic ODNs with one or more CpG motifs have a direct stimulatory effect on murine B-cells, myeloid DCs (MDCs) and plasmacytoid DCs (PDCs), and on human B-cells and PDCs (Table I) (36, 37)**.** In humans, binding of CpG motifs to TLR9 on B-cells and PDCs results in increased expression of co-stimulatory molecules, resistance to apoptosis, upregulation of the chemokine receptor CCR7 and secretion of monocyte inflammatory protein-1 (MIP-1) and IFN-γ-inducible gene products, including the 10-kDa protein interferon-inducible protein 10 (IP-10) (38). B-cell functions are stimulated, including enhanced expression of co-stimulatory molecules, production of IL-6 and IL-10, and differentiation into plasma cells and antibody production (38). CpG ODNs induce maturation of PDCs (37), as well as enhancing IFN-α/β and TNF-α production. This is followed by enhanced lytic activity and IFN-γ production by natural killer (NK) cells (39), as well as T-cell receptor (TCR)-triggered activation of human T-cells in an APC-dependent manner (40). Other indirect effects include IgE inhibition (41) and monocyte activation (42).

A CpG motif consists of a hexamer, a CpG dinucleotide and two flanking bases on the 5'- and 3'-sides (Table I) (43). For mice, the optimal motif is GACGTT (43-45), whereas for humans and other species it was found to be GTCGTT (36, 44, 46). In addition to the CpG motif, the number and spacing of CpG motifs, as well as flanking sequences, poly(G) sequences and the ODN backbone influence the activity of an ODN (38). For therapeutic purposes it is desirable to have a completely or partially phosphorothioate-modified backbone, which is more resistant to nucleases, instead of a phosphodiester backbone.

Three classes of CpG ODNs have now been identified with different structural and functional properties (Table I). CpG-A (D-type) ODNs activate PDCs to undergo maturation and secrete cytokines, including high levels of IFN-α, and are specifically strong NK cell activators (47-49). In contrast, CpG-B (K-type) ODNs induce B-cell proliferation, IL-6 production and IgM secretion and the induction of PDC maturation, with little or no effect on IFN-α production by PDCs. The more recently discovered CpG ODN, called CpG-C, has properties characteristic of both CpG-A and CpG-B ODNs (47, 50, 51).

All CpG classes elicit strong Th1-biased cellular and humoral immune responses to co-administered antigens. However, a study on CpG ODN classes in rhesus mon-

Table I: TLR9 agonists.

Class	Example	Sequence	Palindrome	Ref.
CpG-A (D)	ODN 2216	5'-GGGGGACGATCGTCGGGGGG-3'	Yes	38, 48
CpG-B (K)	ODN 2006	5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'	No	38, 48
CpG-C	ODN 2395	5'-TCGTCGTTTTCGGCGCGCGCCG-3'	Yes	38, 48

Capitals: phosphorothioate backbone; small caps: phosphodiester linkage; underlined: CpG; double underlined: palindrome. CpG-A ODN: strong stimulation of PDC to produce IFN-α; potent NK cell activator; moderate induction of PDC maturation; no B-cell activation (38, 47, 48). CpG-B ODN: strong stimulation of B-cell proliferation; strong induction of PDC maturation; weak induction of IFN-α (38, 47, 48). CpG-C ODN: stimulation of B-cell proliferation; stimulation of human PDC to produce IFN-α; induction of PDC maturation (38, 47, 48).

keys suggested that the three classes induce different post-TLR9 signaling pathways and thus may have different clinical applications. CpG-A ODNs were most effective at inducing IFN-α/β genes, which are antiviral effector molecules, whereas CpG-B ODNs were potent in inducing the production of proinflammatory and immunoregulatory cytokines associated with the adaptive immune response. CpG-C ODNs stimulated both IFN-α/β and proinflammatory cytokine gene expression (52).

Due to the ability of CpG ODNs to stimulate both innate and adaptive immune responses, they have many potential applications, *i.e.,* as adjuvants for vaccines, therapeutics for infectious disease, in cancer therapy and for the treatment of asthma and allergy (reviewed in 38). CpG ODNs have excellent adjuvant properties with a variety of vaccine antigens, including peptides, proteins, live and killed vaccines, and DC vaccines. CpG ODNs are particularly superior at inducing Th1-type immune responses, or even switching from a Th2- to a Th1-biased response (53-58). Furthermore, the efficacy of CpG ODNs as adjuvants can be enhanced by co-formulation with a variety of other adjuvants, including Freund's adjuvant, alum, Quil A, Emulsigen or particles, and this is particularly important when used in larger species (54, 58-60). Several clinical trials have demonstrated that CpG-B ODNs enhance vaccine efficacy against infectious diseases, for example hepatitis B (61, 62), anthrax (38) and influenza (63). CpG ODNs have also been used as adjuvants in allergy vaccines, which leads to re-direction of the immune response bias (64), and in cancer vaccines, which resulted in strong $CD8^+$ T-cell responses (65).

Furthermore, CpG ODNs are promising in the treatment of cancer as monotherapy or combination therapy (reviewed in 66), and as monotherapy against infectious diseases and allergy. CpG ODNs have not only been extensively studied in mice, but have also been evaluated in several clinical trials in humans as vaccines or monotherapy for infectious diseases, vaccines, monotherapy or combination therapy for cancer, and monotherapy or vaccines for allergy and asthma (38). Furthermore, a good safety profile has been seen in humans at CpG ODN doses ranging from 0.0025 to 0.81 mg/kg. Adverse reactions consisted mostly of local injection-site reactions and systemic flu-like symptoms (38).

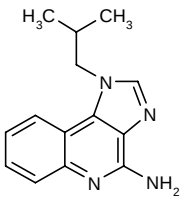
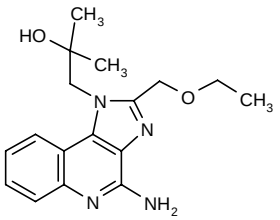
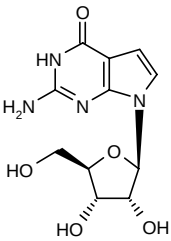
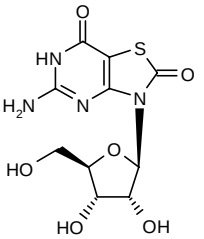
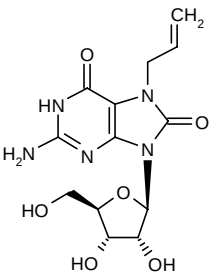
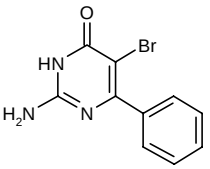
Most evidence suggests that CpG ODNs do not directly activate monocytes. However, Klinman *et al.* observed that CpG-A (D-type) and CpG-B (K-type) ODNs differentially activate human monocytes. Although monocytes bound both class A and B ODNs, CpG-B ODNs stimulated the $CD14^+$ monocytes to proliferate and secrete IL-6, whereas CpG-A ODNs activated the monocytes to mature into $CD83^+/CD86^+$ DCs at physiological concentrations (67). Recently, monocyte-derived DCs (mo-DCs) were shown to express TLR9 and respond to CpG-A ODNs, but not to CpG-B ODNs. Although freshly isolated, nonactivated human monocytes did not express TLR9 protein, differentiation of these monocytes with GM-CSF and IL-4 induced intracellular TLR9 expression, as shown by RT-PCR and flow cytometry using a new TLR9-specific antibody. CpG-A ODNs, but not CpG-B ODNs, triggered maturation of these DCs, characterized by enhanced CD83, CD86 and HLA-DR expression, and stimulation of allogeneic T-cell proliferation and IFN-γ production; furthermore, increased IFN-α production, but no increase in IL-12p70, IL-6 or TNF-α, was observed (68).

TLR7/8 agonists

The mode of TLR7 and TLR8 activation resembles that of TLR9 activation by CpG DNA (69). The natural ligand for TLR7/8 is guanidine- and uridine-rich single-stranded RNA (ssRNA; Table II) (70-72). Imidazoquinolines (imiquimod and resiquimod) and C8-/N7,C8-substituted guanine ribonucleosides, also referred to as immune response modifiers (IRMs), signal through TLR7 and have immunostimulatory activities (27, 73), inducing secretion of proinflammatory cytokines, including TNF-α, IFN-α and IL-12**.** Resiquimod also signals through human TLR8 (74). Imidazoquinolines activate both human PDCs and MDCs (27), have potent antiviral and antitumor properties (27, 75) and proven efficacy and safety (76). Imidazoquinolines also stimulate B-cells in a similar manner to CD154 (77). Furthermore, human BM $CD34^+$ progenitor cells constitutively express TLR7/TLR8, and TLR7/8 signaling induces differentiation into cells with macrophage/DC morphology (78). Imiquimod is currently approved for the topical treatment of warts and other virus-associated and cancerous dermatological lesions (75).

Similar to CpG ODNs, imidazoquinolines enhanced the production of the Th1 cytokines IFN-γ and IL-12 and decreased the Th2 cytokines IL-4 and IL-5 in mouse and

Table II: TLR7/8 agonists.

Compound	Structure	Ref.
Imiquimod		27, 48, 72
Resiquimod		27
7-Deazaguanosine		73
Isatoribine		27, 73
Loxoribine		27, 73
Bropirimine		27
ssRNA	GU-rich CpG	69-72

ssRNA and resiquimod signal through TLR7 and TLR8; imiquimod and and guanosine analogues activate TLR7 (27, 69-72). Imidazoquinolines activate both PDCs and MDCs in humans, thereby inducing secretion of proinflammatory cytokines, including TNF-α, IFN-α and IL-12 (27)**.** Guanine ribonucleosides activate human PBMCs to produce TNF-α, IL-12 and type I and II IFNs, upregulate co-stimulatory molecules and MHC class I and II expression, and enhance NK cell activity and B-cell proliferation (27, 73). ssRNA stimulates human $CD14^+CD11^+$ monocytes (72).

human cultures (79), and were shown to have adjuvant activity. When administered with antigen, resiquimod and imiquimod enhanced IgG_{2a} and reduced IgE levels in mice (80). However, CpG ODNs were shown to be superior to resiquimod as adjuvants for hepatitis B virus (HBV) surface antigen (HBsAg) in augmenting humoral and cell-mediated immune responses in mice (81), and as topical immunoprophylaxis against herpes simplex virus type 2 (HSV-2) (82).

Recently, distinct functions and agonists for TLR7 and TLR8 have been demonstrated in a number of studies. In one study, human monocytes were shown to express TLR4 and TLR8, but not TLR3 or TLR7. Combined TLR4 and TLR8 signaling resulted in T-cell-independent IL-12p70 production (83). Furthermore, although both TLR7 and TLR8 agonists were shown to induce NK cell cytotoxicity and the production of IFN-γ in an indirect manner, this was mediated by synthetic and natural TLR8 ligands through IL-18 and IL-12p70, whereas type I IFN was required for induction of CD69 on NK cells by TLR7 (84).

In another study, small-molecule agonists selective for either TLR7 or TLR8 were used to differentiate the functions of human TLR7 and TLR8. TLR7 agonists directly activated PDCs, and to a lesser degree monocytes, whereas TLR8 agonists directly activated MDCs, monocytes and mo-DCs (85). In other experiments, TLR2, 3, 4 and 8 agonists induced maturation of immature mo-DCs, based on increased CD80, CD86 and CD83 expression. However, while DCs primed with IFN-γ or resiquimod, followed by maturation with LPS or CD40L, expressed both IL-12 p35 and p40, this was not observed with the other TLR agonists. Resiquimod also induced IL-6 secretion by immature DCs, and TNF-α production when used with LPS. Interestingly, in contrast to poly I:C, which induced the generation of IL-4-producing $CD4^+$ T-cells, resiquimod-primed DCs stimulated Th1 cytokine-producing $CD4^+$ T-cells and induced $CD8^+$ T-cells with enhanced tumor cell recognition (86). These studies suggest that TLR7 and TLR8 agonists have different target cells and cytokine profiles and that TLR8 agonists, but probably not TLR7 agonists, might have potential for enhancing DC vaccines.

Oligoribonucleotides (ORNs) containing unmethylated CpG motifs and a poly(G) run at the end directly stimulate $CD14^+CD11^+$ monocytes, which express TLR8, but not DCs or B-cells. Stimulation resulted in upregulation of co-stimulatory molecules and IL-6 and IL-12, but not IFN-α production. However, activation was not mediated through TLR3, TLR7 or TLR9 (72). CpG RNA may be useful for differentiation into immature DCs, although the molecular mechanism of cell type-specific recognition needs to be studied in more detail.

TLR agonists and dendritic cell vaccines

Due to their ability to stimulate innate immunity, and in particular to induce maturation of DCs, TLR agonists have great potential to enhance the efficacy of DC vaccines. The ability of CpG ODNs to stimulate antigen-loaded DCs for adoptive therapy has been evaluated in a number of studies in mice, with very encouraging results. Until recently, human mo-DCs, which are most commonly used as DC vaccines, did not appear to express TLR9, which suggests either that alternative sources of DCs such as blood or BM $CD34^+$ progenitor cells need to be used to achieve stimulatory effects of CpG ODNs, or that the CpG ODN should be co-administered *in vivo*. However, in view of the fact that monocytes are by far the best source for generation of large numbers of DCs, a recent study that demonstrated TLR9 expression on mo-DCs, as well as maturation of mo-DCs by CpG ODNs, is very promising. Furthermore, since monocytes express TLR8, treatment of mo-DCs with imidazoquinolines such as resiquimod may be an alternative approach. Finally, based on increasing evidence suggesting that TLRs cooperate in DC activation, for example TLR3 and 4 with TLR7, 8 and 9 (87, 88), treatment with a combination of several TLR agonists may ultimately be optimal.

Ex vivo stimulation of murine BM-DCs with CpG ODNs

CpG ODNs have been used for *ex vivo* stimulation of murine BM-DCs, mostly in tumor challenge models. In one study, murine BM-DCs were matured with GM-CSF and IL-4 alone or together with TNF-α or CpG ODN 1826, which is a B-class ODN with strong immunostimulatory activity in mice. *In vitro* analyses demonstrated that, based on MHC class II expression, alloreactive T-cell proliferation and IL-12 production levels, DC maturation was more effectively stimulated by ODN 1826 than by TNF-α. Furthermore, CpG-stimulated DCs co-cultured with tumor cells induced protection from colon carcinoma tumor challenge in mice, both prophylactically and therapeutically (89).

In a murine renal cell carcinoma (RENCA) model, DCs pulsed with RENCA and treated with CpG ODN 1826 *ex vivo* significantly reduced tumor growth when injected on days –4 and +6 relative to tumor injection. Furthermore, the mice were resistant to a second tumor challenge and adoptive transfer confirmed the development of memory T-cells (90). Protection from human papillomavirus (HPV) 16 E7-associated tumor challenge was also enhanced by DCs stimulated with HPV E7 protein and CpG ODN 1826 in comparison to stimulation with E7 alone. This was associated with enhanced IL-12 production *in vitro* and increased production of IFN-γ from $CD4^+$ and, in particular, $CD8^+$ T-cells. It was further shown that antitumor protection was mediated by $CD8^+$ T-cells (91).

In a recent communication, the effect of CpG ODNs was shown to be dependent on the type of DC vaccine. Syngeneic BM-DCs pulsed with C1498 tumor cells or electrically fused to irradiated C1498 tumor cells were administered i.v. to mice, with or without co-treatment with the class B CpG ODN 7909. Although all vaccines induced splenic tumor-reactive T-cells and significantly improved survival of mice in comparison to untreated controls, CpG ODN 7909 further improved the survival only in mice that received the fused DCs (92).

Ex vivo activation by CpG ODNs is also an effective approach for DC therapy of infectious diseases. Interestingly, antigen-pulsed murine BM-DCs activated by TNF-α or CD40 ligation did not produce protection from leishmaniasis, whereas antigen-pulsed BM-DCs activated with CpG ODN 1668 induced a strong Th1-type response and complete protection from *Leishmania major* challenge, both in susceptible BALB/c and resistant C57BL/6 mice. Protective immunity lasted for at least 16 weeks. Significant IL-12 production was only observed *in vitro* after stimulation with CpG ODN. However, IL-12 produced by donor DCs did not seem to be critical for protection, whereas IL-12 availability in the recipient mice was required (12).

We recently evaluated the efficiency of adoptive transfer of DCs transduced *ex vivo* with HCV NS3 protein. BM-DCs were pulsed with NS3 protein and stimulated with CpG ODN 1826, or left untreated. The CpG ODN treatment induced phenotypic maturation and increased CD40 expression when compared to untreated DCs. The CpG ODN-matured DCs also produced higher IL-12 levels and a stronger allogeneic T-cell response. Finally, the NS3-pulsed DCs matured with CpG ODN induced stronger cellular immune responses and higher levels of protection compared to the untreated NS3-pulsed DCs upon challenge with a recombinant vaccinia virus expressing NS3 (15).

In vivo stimulation of BM-DC-vaccinated mice with CpG ODNs

Co-delivery of CpG ODN 1826 to mice injected with BM-DCs stimulated with RENCA cells was tested as a method to treat large established tumors. In contrast to treatment with either antigen-stimulated BM-DCs or CpG ODN, injection of both resulted in transient control of tumor growth. Interestingly, rejection of large tumors and long-term cure were only achieved by simultaneous injection of both antigen-stimulated DCs and CpG ODN at a distant site and peritumoral CpG ODN; this was dependent on $CD8^+$ T-cells (93). In another protection model, mice vaccinated with either antigen-pulsed immature or CpG ODN-matured DCs were unable to reject a lethal B16 melanoma challenge. However, mice vaccinated with both antigen-pulsed immature DCs and CpG ODN developed enhanced antigen-specific T-cell responses and long-term protective immunity. When mice with subcutaneous B16 melanoma were treated intratumorally with CpG ODN and B16 lysate-pulsed DCs, a reduced tumor burden and prolonged survival were achieved. In a similar model, only treatment with both tyrosine-related protein-2 (TRP-2) peptide-pulsed DCs and CpG ODN resulted in tumor regression, indicating that immunization with both antigen-pulsed DCs and CpG ODN can lead to effective therapy (94). Similarly, co-administration of CpG ODN with fused cells generated from DCs and tumor cells resulted in significant protection from lethal tumor challenge and spontaneous lung metastasis, as well as tumor re-challenge, compared to immunization with either fused cells or CpG ODN. This suggests that this approach leads to long-term tumor-specific immunity (95). Since in these studies the CpG ODN may affect both the administered DCs and local immune cells, *in vivo* treatment of human tumors with both CpG ODN and antigen-pulsed DCs might be a promising approach.

Thus far, there are very few reports on the use of TLR7/8 agonists as an approach to enhance DC therapy. In one study, central nervous system (CNS) tumor-bearing mice were treated both with human DCs pulsed with human gp100 + TRP-2 melanoma-associated antigen (MAA) peptide and with a 5% cream containing imiquimod, before and after vaccination. This treatment resulted in synergistic reduction of tumor growth in comparison to vaccination with MAA peptide-pulsed DCs alone (96). This correlated with the ability of imiquimod to enhance DC persistence and trafficking into the local draining lymph node, as well as accumulation of $CD8^+$ T -cells in the spleen and draining lymph nodes.

Ex vivo stimulation of human DCs with TLR ligands

Monocytes isolated from peripheral blood by magnetic bead selection are the most frequently used source of DCs for vaccination or adoptive therapy. The monocytes differentiate into immature DCs when cultured with GM-CSF and IL-4, and can be turned into mature DCs after the addition of various maturation cocktails, such as TNF-α, IL-1β, IL-6 and PGE_2. Until recently, most reports concluded that mo-DCs do not respond to CpG ODNs. However, a recent report by Hoene *et al*. (68) demonstrating activation and maturation of human mo-DCs by a CpG-A ODN is very promising. Even though CpG ODN stimulation of the mo-DCs was not as potent as LPS, the use of a CpG-A ODN as a maturation factor is likely to be safer and could be sufficient for DC therapy, or alternatively the CpG-A ODN could be used in combination with other TLR agonists. Since CpG-A ODNs are not easy to synthesize, it will be interesting to evaluate CpG-C ODNs for their stimulatory activity on mo-DCs. Furthermore, in addition to monocytes, BM hematopoietic $CD34^+$ progenitor cells are also sometimes used for the treatment of cancer patients, and peripheral blood DCs have been loaded with protein and used as vaccines, for example in patients with follicular B-cell lymphoma (97) or prostate cancer (98), in which case *ex vivo* treatment with TLR8 agonists or CpG ODNs may be beneficial.

TLR7/8 agonists resemble CpG ODNs in their mode of action, but activate mo-DCs, MDCs and PDCs. Thus, *ex vivo* stimulation with TLR7/8 ligands might also be a feasible approach to stimulating DCs for vaccination or therapy. *In vitro* studies on human PDCs, MDCs, B-cells and monocytes demonstrated that PDCs responded to treatment with CpG ODNs, imiquimod or resiquimod with upregulation of CD40, CD80, CD86, CD83 and MHC class II, which correlated with TLR9 and TLR7/8 expression. In contrast, MDCs responded to poly I:C, LPS or resiquimod, correlating with expression of TLR3, TLR4 and TLR7/8. In this study, monocytes and B-cells showed

very little response to TLR ligands. PDCs or MDCs that were optimally stimulated with TLR ligands and exposed to cytomegalovirus (CMV) or human HIV antigens enhanced autologous CMV- and HIV-1-specific memory T-cell responses (99).

Contradictory results have been reported with respect to the ability of TLR8 ligands to stimulate maturation of mo-DCs. A recent study suggested that the TLR7/8 agonists resiquimod and ssRNA impaired monocyte differentiation to DC, both phenotypically and functionally (100), indicating that when monocytes are used as a source of DCs, TLR7/8 agonists may not be useful for *ex vivo* treatment of DCs for vaccination and therapy. In contrast, in another recent report, resiquimod treatment increased the expression of TLR8 in immature mo-DCs, and treatment with both LPS and resiquimod induced high levels of IL-12p70, followed by Th1 cytokine production by $CD4^+$ T-cells, induction of $CD8^+$ T-cells and enhanced tumor cell recognition (86). These results indicate a role for resiquimod in the development of DC vaccines. More research on the effects of TLR7/8 ligands on DCs is needed to further evaluate the potential of these compounds in DC vaccines.

Conclusions

In mouse models, both *ex vivo* treatment of BM-DCs and *in vivo* immunization with CpG ODNs have proved to be very promising as approaches to enhancing the efficacy of DC vaccines. According to several published reports, TLR9 is present on human plasmacytoid DCs and B-cells, but not monocytes, which are the most common source of DC vaccines. However, a recent study demonstrated expression of TLR9 on human mo-DCs, which suggests that CpG ODNs might be used for both *ex vivo* and *in vivo* stimulation of DC vaccines. Furthermore, since TLR8 signals in a very similar manner to TLR9 and is expressed on monocytes and mo-DCs, TLR8 agonists might have potential in the generation of DC vaccines. In conclusion, TLR agonists such as CpG ODNs, or possibly combinations of TLR agonists, might have great potential for improving DC vaccines.

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