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In vivo cancer vaccination: which dendritic cells to target and how?

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Abstract

The field of cancer immunotherapy has been revolutionized with the use of immune checkpoint blockade antibodies such as anti-programmed cell death 1 protein (PD-1) and chimeric antigen receptor T cells. Significant clinical benefits are observed in different cancer types with these treatments. While considerable efforts are made in augmenting tumor-specific T cell responses with these therapies, other immunotherapies that actively stimulate endogenous anti-tumor T cells and generating long-term memory have received less attention. Given the high cost of cancer immunotherapies especially with chimeric antigen receptor T cells, not many patients will have access to such treatments. The next-generation of cancer immunotherapy could entail *in vivo* cancer vaccination to activate both the innate and adaptive anti-tumor responses. This could potentially be achieved via *in vivo* targeting of dendritic cells which are an indispensable link between the innate and adaptive immunities. Dendritic cells highly expressed toll-like receptors for recognizing and eliminating pathogens. Synthetic toll-like receptors agonists could be synthesized at a low cost and have shown promise in preclinical and clinical trials. As different subsets of human dendritic cells exist in the immune system, activation with different toll-like receptor agonists could exert profound effects on the quality and magnitude of anti-tumor T cell responses. Here, we reviewed the different subsets of human dendritic cells. Using published preclinical and clinical cancers studies available on PubMed, we discussed the use of clinically approved and emerging toll-like receptor agonists to activate dendritic cells *in vivo* for cancer immunotherapy. Finally, we searched www.clinicaltrials.gov and summarized the active cancer trials evaluating toll-like receptor agonists as an adjuvant.

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Conflict of interest statement

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Introduction

Compelling evidences now demonstrated that the human immune system plays an important role in tumor surveillance and suppression. Dendritic cells (DCs) are the first-line of defense against invading pathogens and actively participate in tumor surveillance by removing damaged tissues in the microenvironment. Being potent antigen-presenting cells (APCs), DCs can initiate and regulate immune responses including anti-tumor responses. DCs follows two major developmental pathways from $CD34^+$ hematopoietic progenitor cells to become either lymphoid-derived plasmacytoid DCs (pDCs) or myeloid-derived conventional DCs [1, 2]. The myeloid-derived DCs are further classified based on their tissue location, phenotype, cytokine and chemokine production, and the immune responses they elicit. (Figure 1). Myeloid monocyte-derived DCs are most widely used for tumor immunotherapy. They are differentiated *ex vivo* from peripheral monocytes with recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-4. They are highly efficient in antigen phagocytosis and production of IL-12 [3, 4], as well as eliciting anti-tumor T cell responses. However, their *ex vivo* production is laborious and costly. An attractive alternative is to target DCs *in vivo* with appropriate tumor antigens and activating them to produce proinflammatory cytokines.

Toll-like receptors (TLRs) are an integral part of the innate immunity for recognizing and eliminating invading pathogens [5–7]. They are predominantly expressed by immune cells such as DCs, macrophages and monocytes. Activation of TLRs by their corresponding ligands (e.g. natural conserved pathogen-associated molecular patterns [PAMPs] or synthetic) leads to inflammatory cytokines and chemokines productions that exert multiple effects on both innate and adaptive immunities [8, 9]. Thus, TLR activation in the context of cancer could potentially influence the activation, magnitude and quality of anti-tumor T cell responses. Ten different TLRs have been characterized in the human immune system – TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface, whereas TLR7, 8 and 9 are expressed in endosomal/lysosomal membranes of the cell. TLR3 is expressed in different cell types including immune cells [10–14]. It is interesting to note that some of the most effective vaccines, such as the live-attenuated yellow fever vaccine 17D (YF-17D), owe their effectiveness through simultaneous activation of different DC subsets to produce proinflammatory cytokines including IL-12, IL-6 and interferon (P)-α that stimulate T helper (Th) 1 and 2 responses [15, 16]. Table 1 summarizes a selection of TLR agonists that have been used for *in vivo* DC targeting.

Lymphoid-derived plasmacytoid DCs

Lymphoid-derived plasmacytoid DCs (tns) is a unique subset that localize in the lymph nodes (LNs), spleen, blood, mucosal-associated tissues, thymus and liver in normal physiological condition. Under pathological conditions including lymphoid hyperplasia of the skin [17], cutaneous systemic lupus erythematosus (SLE), psoriasis vulgaris, contact dermatitis and allergic mucosa [18], pDCs are also found in the skin. Unlike their myeloid counterparts, pDCs does not depend on GM-CSF for differentiation. Instead, they follow a distinct developmental pathway that uses IL-3 and Fms-like tyrosine kinase 3 ligand (Flt3L) [19]. pDCs play an important role against viral infections. They are the main producers of

Type I IFN-α and -β against invading viruses, and modulate different aspects of innate, adaptive and humoral immune responses via these cytokines [20]. The IFN-α/β secreted by pDCs could exert numerous effects including promoting pDC survival, inducing the differentiation of myeloid-derived DCs [21], facilitating crosspresentation and crosspriming of DCs [22], shaping both T helper (Th) 1 and $CD8^+$ T cell responses, activating natural killer (NK) cells, and inducing the generation of plasma cells and primary antibody responses against viruses [23, 24]. pDCs also efficiently expanded antigen-specific $CD8^+$ T effector cells, demonstrating their importance in adaptive immunity against subsequent viral infections [25].

In cancers, pDCs are shown to play contrasting roles. In murine B16 melanoma, pDCs were activated by TLR agonists to express tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), granzymes B and C for potential tumor killing. The activated pDCs also produced IFN-α to promote tumor cell lysis by cytotoxic T cells (CTLs) and NK cells [26–28]. In a murine orthotropic mammary tumor model, pDCs were stimulated *in vivo* via TLR7 agonist administration to elicit anti-tumor responses [29]. In a melanoma trial, treated subjects showed tumor-specific $CD4^+$ and $CD8^+$ T cell responses after receiving *ex vivo* generated blood-derived pDCs that were loaded with tumor-associated antigens (TAAs) [30]. On the other hand, some studies indirectly showed that tumor-infiltrating pDCs were defective in Type I IFN secretion [31–35], and produced immunosuppressive factors that assisted in tumor progression [36]. Also, tumor-infiltrating pDCs were significantly impaired in their TLR9 expression and responsiveness [34, 37, 38]. Moreover, tumor-infiltrating pDCs could be polarized by immunosuppressive indoleamine 2,3-dioxygenase (IDO) in the tumor microenvironment to secret IL-10 for T regulatory (Treg) cell proliferation [39–42].

Targeting pDCs with TLR7/8 agonists

Human pDCs distinctly express blood-derived cell antigen (BDCA)-2, BDCA-4 (CD304/Neuropilin), ILT-7 and IL-3Rα chain (CD123) [43]. They also express TLR7 and 9 for recognizing viral nucleic acid and bacterial DNA with unmethylated cytosine-phosphateguanine (CpG) sequences, respectively [44–46]. Imiquimod (R-837), a US Food and Drug Administration (FDA)-approved synthetic TLR7 agonist, has been used successfully as a topical treatment for superficial basal cell carcinomas [47–49], human papilloma virus (HPV)-mediated external genital warts and actinic keratosis [50–53]. It effectively induces strong infiltrations of IFN-α producing pDCs, T and NK cells in the treated carcinomas [54, 55]. In metastatic melanoma, Imiquimod given topically with intradermal injection of NY-ESO-1 protein induced activation of pDCs, NK cells, and elicited IFN-γ secretion from $CD4^+$ T cells [56, 57]. In vulvar intraepithelial neoplasia, Imiquimod given topically elicited $CD8^+$ T cells and NK cells responses and 35% of the subjects remained disease-free for more than a year [58]. In metastatic breast cancer, clinical responses were observed in subjects who applied Imiquimod topically 5 days per week for 8 weeks to their chest wall [59]. Six out of 8 subjects showed partial responses or stable diseases, and tumor regression was associated with the presence of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells [59]. In a Phase II breast cancer trial, 15 subjects applied Imiquimod topically to targeted lesions on 4 consecutive days per week for 12 weeks [60]. Albumin

bound paclitaxel (100mg/m^2) was also given intravenously once weekly for 3 weeks and repeated every 28 days for 12 weeks. Ten subjects experienced complete and partial responses [60]. The clinical response was short-lived, suggesting that longer course of treatment was needed. Nevertheless, these results demonstrated that Imiquimod is well-suited as a topical agent to activate pDCs and T cell responses via TLR7. Two clinical trials are currently evaluating the use of Imiquimod with radiotherapy and/or chemotherapy (Table 2).

852A (3M-001), like Imiquimod, is a small-molecule imidazoquinoline and synthetic analogue of DNA/RNA oligonucleotide designed to bind specifically to the TLR7 of pDCs. This binding mimics the recognition of single-stranded (ss) RNA in viral infection leading to the production of IFN-α by pDCs, and eventual priming of specific immune responses [61–64]. Compared to Imiquimod, 852A is several-folds more potent for TLR7 and almost 40 times more water-soluble at physiologic pH. Hence, 852A is well-suited for intravenous administration as it is less extensively metabolized and eliminated than Imiquimod [65]. Oral, subcutaneous and intravenous have been investigated for 852A administration in humans. However, the oral route showed very little efficacy compared to other routes and was not discussed here. In a Phase I trial, 852A was given subcutaneously twice a week for 12 weeks to 15 subjects with recurrent ovarian, cervical and breast cancers. Increased sera IP-10 and IL-1 receptor antagonist (IL-1RA) were observed, and one ovarian cancer subject experienced stable disease and a cervical cancer subject remained disease-free for 18 months [65]. In a Phase I trial of refractory metastatic melanoma, 852A was given intravenously 3 times a week for 12 weeks. Four out of 21 subjects experienced disease stabilization for more than 3 months [66]. Three clinical trials evaluating the use of 852A in refractory solid cancers (NCT00095160) and melanoma (NCT00189332, NCT00091689) have been completed, and the results are pending.

Resiquimod (R-848; S-28463) interacts with TLR7 and 8 to induce secretions of IFN-α, IL-6, −8, −12, and TNF-α from pDCs, and stimulating Th1 responses [67]. In a Phase I trial of early-stage cutaneous T cell lymphoma (CTCL), 9 out of 12 subjects treated with 0.03% or 0.06% topical Resiquimod gel showed improved or complete lesion clearance [68]. T cell receptor sequencing revealed a reduction or complete elimination of the malignant T cells in the responders. Significantly more IFN-γ and TNF-α producing CD4$^+$ T cells were detected in the high responders compared to the low responders [68]. In melanoma, subjects were given full-length NY-ESO-1 protein emulsified in Montanide (100μg) subcutaneously and topical application of 1g of 0.2% Resiquimod gel 3 times a week [69]. All the subjects showed humoral responses against NY-ESO-1, and 16 out of the 20 evaluable subjects showed CD4$^+$ T cells responses. In another study, 90% of the subjects with actinic keratosis had complete clearance of their lesions after topical Resiquimod treatment [70]. These results demonstrated the feasibility of using topical Resiquimod to activate pDCs for cancer therapy. Resiquimod is being assessed as an adjuvant in melanoma-derived peptide vaccinations and in autologous tumor lysate-pulsed DC vaccination in brain tumors [Table 2 and 4]. Given the promising results of Imiquimod and Resiquimod, one could envisage using them topically to activate pDCs in the skin and mucosa-associated tissues to elicit humoral and cellular anti-tumor responses. As more results become available attesting the effectiveness of 852A, one could envisage giving it subcutaneously or intravenously to target

pDCs residing in the blood, spleen and LNs. An emerging TLR7 agonist, Gardiquimod which is structurally similar to Imiqumod and Resiquimod, could exert anti-tumor effects in preclinical studies including inhibiting tumor cell growth [71] and triggering their apoptosis [72], and together with Imiquimod augmented tumor-lysate loaded DC therapy in B16 murine melanoma [71]. Other novel TLR7/8 agonists including DSP-0509, MEDI9197, NKTR-262 and LHC165 are currently being investigated in combination with immune checkpoint inhibitors, radiotherapy and/or chemotherapy (Table 2).

Targeting pDCs with TLR9 agonists

pDCs express TLR9 for specific recognition of unmethylated CpG motifs in bacteria and DNA viruses [73–77]. Upon stimulation with CpG, pDCs upregulated CD40, CD54, CD80, CD86 and MHC class II [78–80], and produced IFN-α, IL-1, IL-6, IL-12, IL-18 and TNF-α [81] to activate other immune cells. pDCs also differentiated into potent APCs and increased their resistance to IL-4-induced apoptosis [82]. As CpG has powerful effects on pDCs and subsequent Th1 responses, it shows promise as a cancer adjuvant [21, 83–86]. Four classes of synthetic CpG-oligodeoxynucleotides (CpG ODNs) ligands, i.e. Class A, B, C and P, are generated to mimic the immunostimulatory activity of CpG on pDCs [76, 87]. They differ in nucleotide sequences and lengths, and exhibit different functional properties. Class A (D-type) enters the lysosome compartments of pDCs to stimulate strong IFN-α production, as well as activate NK cells via its palindromic structure. Class B (K-type) traffics to the endosomal compartments of pDCs to stimulate their maturation and antigen-presentation and induces upregulation of costimulatory molecules on B cells. However, it elicits poor IFN-α production due to its nonpalindromic sequences [87–89]. Class C exhibits stimulatory properties of both Class A and B CpG ODNs [90, 91]. Class P contains two palindromic sequences and thus has greater ability than Class C in inducing IFN-α secretion [92, 93]. All of them elicit strong Th1 responses [94].

In IGROV-1 human ovarian adenocarcinoma, tumor-bearing athymic mice receiving repeated intraperitoneal injections of ODN1826 (Class B) showed ascites regression and increased survival [95]. Mice treated intraperitoneally survived significantly longer than mice treated subcutaneously or intravenously with the same CpG ODNs [96]. Moreover, mice receiving CpG ODNs intraperitoneally showed higher level of sera and tissue C-X-C motif chemokine ligand 1 (CXCL1), and increased circulating NK cells and neutrophils [96]. Subcutaneous administration of CpG ODN 7909 (Class B; Agatolimod; PF-3512676; ProMune®) in healthy volunteers elicited strong IFN-α, IL-12p40, IL-6 and IP-10 secretions at the injection site and draining LNs for 2 weeks. Interestingly, intravenous administration of CpG ODN 7909 even at 128-fold higher dose did not induce any immune responses [97]. In a Phase I melanoma trial, increased circulating MART-1 specific $CD8^+$ effector memory T cells was detected in the subjects after 4 monthly subcutaneous vaccinations of CpG ODN 7909, MART-1 peptide and incomplete Freund's adjuvant [98]. Similarly, CpG ODN 7909 co-administered with NY-ESO-1 peptides/proteins and Montanide ISA-51 subcutaneously led to an overall increased in antigen-specific $CD8^+$ T cells [57, 99, 100] that persisted for 3 years after vaccinations [100]. Furthermore, administrating CpG ODN 7909 subcutaneously activated both pDCs and myeloid DCs and priming Th1-related cytokines and anti-tumor $CD8^+$ T cell responses [97, 101]. In a Phase

I/II B cell lymphoma trial, 13 subjects treated intratumorally with SDS-101 (Class C CpG ODN) and low-dose radiation showed decreased tumor size and increased $CD8^+$ T cell in the treated lesions (NCT02254772). SDS-101 is currently being investigated in cancer trials in combination with anti-TNF receptor superfamily member 4 (OX40) antibody, pembrolizumab and ibrutinib [Table 3]. Clinical evaluation is also warranted for intraperitoneal administration of CpG ODNs as this route has shown promise in preclinical tumor models.

IMO-2125, a novel synthetic TLR9 agonist, was shown to suppress murine A20 lymphoma and CT26 colon carcinoma [102]. An increased in $CD3^+$ tumor-infiltrating T cells (TILs) was observed following intratumoral IMO-2125 administration, and further studies showed that $CD8^+$ T cells were required for tumor control. Increased immune checkpoint gene expressions including programmed cell death protein (PD)-1, PD-ligand 1 (PD-L1), OX40 and OX40 ligand, and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) were observed following IMO-2125 treatment, suggesting that immune checkpoint therapies could be combined with IMO-2125 for improved efficacy. Currently, 3 clinical trials are assessing the efficacy of IM0-2125 with ipilimumab and/or pembrolizumab in metastatic melanoma [Table 3]. Numerous novel TLR9 agonists, including MGN1703 (lefitolimod; DNA-based agonist), DUK-CPG-001 (synthetic CpG-rich oligonucleotide), DV281 (aerosolized synthetic C-class ODN for lung cancer treatment), and CMP-001 (virus-like particle-encapsulated agonist with unmethylated CpG motif-rich G10 oligonucleotides) are being tested in cancer trials (Table 3).

CpG ODNs could synergize with other TLR agonists to increase their overall potencies through activation of multiple DC subsets. CpG ODNs synergized with flagellin, a TLR5 agonist that binds to TLR5 on dermal DCs, to induce anti-tumor immunity in mice [103]. CpG synergized with Poly I:C (polyinosinic-polycytidylic acid), a TLR3 agonist recognized by TLR3 on Langerhans cells (LCs), to induce IL-6 and IL-12 productions from the DCs and promote anti-tumor responses [104]. The adjuvant AS15, which contains a TLR4 agonist monophosphoryl lipid A (MPLA) and CpG ODN 7909, is used to boost anti-tumor responses elicited by full-length recombinant PRAME (PReferentially Expressed Antigen of Melanoma) in non-small cell lung carcinoma (NSCLC) [NCT01853878].

Langerhans cells

Langerhan cells (LCs) reside in the basal and supra-basal layers of the epidermis, where they form a dense network of first-line defense against invading pathogens [105]. LCs and dermal DCs are the two major DC subsets found in the human skin. Although they develop from a common bone-marrow myeloid precursor, these DC subsets express distinct phenotypic markers and induce predominantly different types of immune responses. LCs express Langerin (CD207), Birbeck granules, DEC-205 (CD205), DCIR (DC immunoreceptor)/ CLEC4A and TLR1, 2, 3, 6 and 10 [106]. LCs seem to regulate cellular immunity, while dermal DCs distinctly regulate the humoral immunity. This notion was supported by murine studies showing that activated dermal DCs migrated into the outer paracortex beneath the B cell follicles and LCs entered the T cell-rich inner paracortex [107]. In human, LCs pulsed with tumor or viral peptides were superior to $CD14^+$ dermal DCs in priming high-avidity

antigen-specific $CD8^+$ CTLs that expressed higher levels of granzymes and perforin [108]. This might be due to LCs preferentially produce strong level of IL-15 that is known to enhance naïve $CD8^+$ T cell proliferation and stimulate memory T cell development [109]. Interestingly, the presence of LCs in tumor lesions was positively correlated with better prognosis in gastric carcinoma [110]. Similarly, more LCs were found in low-stage breast and uterine carcinomas than in advanced-stage cancers [111, 112]. These results suggested that human LCs play a role in tumor immunity.

Targeting LCs with TLR3 agonists

Poly I:C, a synthetic double-stranded (ds) RNA that binds to TLR3, can be used to target LCs as well as BDCA-3^+CD141^+ myeloid DCs in the blood, LNs and other secondary lymphoid organs via their TLR3. Poly I:C can mature these DCs leading to productions of IL-12p70, IFN-α, IFN-β, and IFN-inducible chemokines, including RANTES (CCL5) and IL-6 [113]. As Poly I:C causes serious side-effects including shock, renal failure, and hypersensitivity reactions [114], Poly(I:C12U) [Ampligen®, Hemispherx] is developed by introducing unpaired bases (uracil and guanine) to create regions of mismatches for accelerated hydrolysis. Poly(I:C12U) shows markedly reduced systemic toxicity as it binds only to TLR3, while Poly I:C binds to both TLR3 and melanoma differentiation-association antigen-5 [115]. Poly(I:C12U) was as effective as Poly I:C in stimulating IL-12 secretion in mature DCs *ex vivo* [116]. In a randomized placebo-controlled double-blinded trial, HIV-positive subjects were given Poly(I:C12U) intravenously with minimal side-effects [117]. Significantly, fewer treated subjects showed disease progression compared to the placebo group [117]. Poly(I:C12U) was given intravenously to treat chronic fatigue syndrome in a randomized, placebo-controlled, double-blind study. It improved the symptoms of the treated subjects [118, 119].

Poly-ICLC, a RNAse-resistant Poly I:C stabilized with poly-lysine (Hiltonol®, Oncovir), could upregulate type I and II IFNs, and activate the complement system and inflammasome signaling [120]. In malignant glioma, 38 subjects were given 2 or 3 low doses of poly-ICLC (~1–2mg) intramuscularly for 56 months with little toxicity [121]. Twenty subjects experienced tumor regression or stabilization, with the median survival of 8 years and 19 months for subjects who had anaplastic astrocytoma and malignant gliomas, respectively. In an open-label, single-arm Phase II trial, 55 subjects with recurrent supratentorial anaplastic glioma treated with Poly-ICLC (20μg/kg/intramuscularly) thrice weekly in a 4-week cycle showed a partial objective radiographic response (5 subjects) and stable diseases (18 subjects) [122]. In a Phase I/II trial, subjects with glioblastoma multiforme, anaplastic astrocytoma, oligodendroglioma and oligoastrocytoma were treated with peptide-pulsed myeloid monocyte-derived DCs [1 or 3 million/dose at 2-week intervals] and Poly-ICLC (20μg/kg/intramuscularly twice weekly) [123]. Significant upregulation of type-1 cytokines and chemokines, including IFN-α and CXCL10 was observed, and 9 subjects achieved progression-free status lasting 12 months. In a Phase I trial of 45 subjects with advanced malignancies, CDX-1401 (monoclonal anti-DEC-205 fused to full-length NY-ESO-1 injected intracutaneously to the dermal and subcutaneous layers) together with Resiquimod and/or Poly-ICLC given topically or subcutaneously [124]. Humoral and cellular responses to NY-ESO-1 were detected in the subjects, and 13 subjects had disease stabilization of a

median duration of 6.7 months. Six out of 8 subjects who received anti-CTLA-4 within 3 months after CDX-1401 treatment showed objective tumor regression. Currently, 23 active cancer trials are evaluating Poly-ICLC (www.clinicaltrials.gov) and a selection was listed in Table 4.

Dermal DCs

Dermal (also called interstitial) DCs are found in the papillary dermis layer of the skin (120). Three different subsets existed – 1) BDCA-1$^+$(CD1c$^+$)CD11c$^+$CD14$^-$; 2) BDCA-1$^+$(CD1c$^+$)CD11c$^+$CD14$^+$ ([125]); and 3) BDCA-3$^+$(CD141$^+$)XCR1$^+$ [126]. The CD14$^+$ dermal DC subset expresses many C-type lectins including DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing non-integrin), LOX-1 (lectin-like oxidized LDL receptor-1), CLEC-6 (C-type lectin domain family 1 member-6), dectin-1, DCIR /CLEC4A, and TLR2, 4, 5, 6, 8 and 10 [106, 127]. They distinctly regulate B cell differentiation via the secretion of IL-12 that activates CD4$^+$ T cells. The activated CD4$^+$ T cells would secrete IL-21 to stimulate B cell differentiation and antibody class switching from IgM to IgG and IgA [128, 129] and produce CXCL13 to promote B cell homing to the follicular center [108]. Compare to LCs, CD14$^+$ dermal DCs produce various cytokines including IL-1β, IL-6, IL-8, IL-10, IL-12, GM-CSF, monocyte chemoattractant protein (MCP) and transforming growth factor (TGF)-β after CD40 stimulation [108]. The recently identified BDCA-3$^+$(CD141$^+$)XCR1$^+$ DCs might play a critical role in maintaining tissue homeostasis, inducing immune tolerance, and mounting responses against invading pathogens [126, 130, 131]. They could present self-antigens, produce high levels of IL-10 to activate Treg cells [126], and crosspresent soluble antigens [130, 132].

Targeting dermal DCs with TLR2, 4 and 6 agonists

Monophosphoryl lipid A (MPLA) is a chemically modified, detoxified version of the lipopolysaccharides (LPS) derived from the outer membrane of the Gram-negative *Salmonella Minnesota* [133–135]. Both MPLA and LPS interact with the TLR2 and 4 on CD14$^+$ dermal DCs to induce Th1-priming cytokine secretions including IL-12p70 and IFN-γ-inducible protein-10 (IP-10). MPLA is the first and only TLR agonist used in licensed human vaccines, e.g. Cervarix® a prophylactic vaccine against HPV types 16 and 18. GlaxoSmithKline has developed a series of adjuvant systems (i.e. AS01–04) for evaluation against malaria, tuberculosis, leishmania, HIV, vesicular stomatitis virus and cancers [136]. AS01 is formulated with liposomes and MPLA for inducing antibody and CTL responses, while AS02 is an oil-in-water emulsion containing MPLA and QS-21 (a purified component of Quil-A) to stimulate strong antibody and Th1 responses. AS04 is an aqueous formulation of MPLA and alum for eliciting higher specific antibody titers with fewer injections [136]. A TLR4 agonist, amino alkyl glucosamimide phosphates, has been licensed for use in a Hepatitis B Virus vaccine (Dynavax®). Emerging TLR4 agonist, synthetic glycolipid GSK1795091 (CRX-601), is being evaluated in healthy volunteers and advanced solid tumors [Table 5]. Another TLR4 agonist, glucopyranosyl lipid A (GLA), is a synthetic derivative of the LPS lipid A tail that shows limited toxicity. GLA-stable emulsion (GLA-SE; G100), is being evaluated for intramuscular injections with NY-ESO-1 recombinant protein in metastatic cancers (NCT02015416) and MART-1 antigen peptide in resected

melanoma (NCT02320305) [136]. It is also tested intratumorally in combination with radiotherapy in soft tissue sarcomas, and pembrolizumab in Follicular non-Hodgkin's lymphoma [Table 5]. Using a human skin explant model, intradermal injection of GLA-SE or LPS enhanced the emigration of LCs and induced the maturation of both LCs and $CD14^{-}$ dermal DCs [137]. These activated DCs in turn stimulated a strong T cell proliferation. Hence, more studies are warranted to evaluate the use of GLA-SE intradermally in cancer treatment.

Mycobacterium bovis Bacillus Calmette-Guérin (BCG), a bacterium closely related to the bacterial strain that causes tuberculosis in human, could activate DCs via TLR2, 4 and nucleotide binding oligomerization domain (NOD)-like receptor (NLR)-2 through interactions with its peptidoglycan cell wall skeleton [138]. BCG has been approved by FDA for treating bladder cancer for more than 30 years [139], as wells as an adjuvant with MPLA in an allogeneic cell-based vaccine (Melacine®) for melanoma [140]. In Phase III colorectal carcinoma trials, subjects were treated successfully with an autologous whole tumor lysate vaccine and BCG as adjuvant [141–143]. In the largest study, 412 subjects received 3 intradermal vaccinations (1×10^7 irradiated tumor cells/dose/week) 4 weeks after surgery together with 1×10^7 BCG in the first two vaccines. The magnitude of the DTH response was correlated with improved prognosis [144]. Meta-analysis of these trials showed that the treatment provided significant clinical benefits in a subset of subjects with stage II colorectal carcinoma [143]. In contrast, renal cell carcinoma subjects treated the same way developed severe reactions and skin ulceration at the injection sites [145]. The 5-year overall survival between the treated (69%) and control (78%) subjects did not reach statistical significance. No clinical benefits were observed in a Phase III renal cell carcinoma trial using the same treatment [141]. Instead, several Phase III renal cell carcinoma trials using autologous tumor cell lysate vaccine alone showed promising results [146–148]. In melanoma, subjects receiving both BCG and Imiquimod showed encouraging clinical responses [149]. These results suggested that BCG might exert different effects in different cancer types and should be investigated carefully.

Macrophage-activating lipopeptide (MALP)-2, a synthetic lipopeptide analogue of the mycoplasma cell wall lipoprotein M161Ag, could bind to both TLR2 and 6 to activate NF-κ and cytokines and chemokines synthesis in mouse [150] and human DCs [151], as well as stimulating DC maturation [152]. In a Phase I/II pancreatic carcinoma trial, 10 subjects treated with MALP-2 (20 to 30μg/intratumorally/dose) and post-operative gemcitabine showed a mean survival of 17.1±4.2 months and 2 patients remained alive 31 months post-treatment [153]. Another dual TLR2 and 6 agonist, CBLB612, is a synthetic lipoprotein that has been evaluated in a Phase II trial for protection against neutropenia while on doxorubicin and cyclophosphamide treatments in breast cancer (NCT02778763). Its anti-tumor property was not investigated in the trial. CADI-05, a novel TLR2 agonist targeting desmocollin-3 (DSC3)-expressing cancer cells, was assessed in preclinical and clinical studies. In preclinical models, CADI-05 effectively suppressed small DSC3-expressing tumors, and synergized with chemotherapy and immune checkpoint inhibitors to increase IFN-γ secreting TILs and macrophages [154, 155]. In clinical studies, CADI-05 given together with chemotherapy \in NSCLC led to improved outcome [156]. In a randomized, multicenter trial, CADI-05 was evaluated for synergistic effects with cisplatin and paclitaxel in NSCLC

[157]. No significant survival benefit was observed between subjects receiving chemotherapy and chemotherapy plus CADI-05 [208 versus 196 days; hazard ratio (HR)=0.86; 95% confidence interval (CI) 0.63–1.19; P=0.3804]. However, subgroup analysis showed that squamous NSCC subjects receiving chemotherapy plus CADI-05 experienced a slight improvement in median survival by 127 days (HR=0.55; 95% CI 0.32–0.95; P=0.046). It has been shown that TLR2 ligation could promote tumor-infiltrating Treg cells and IL-10 production [158], thus raising concerns of using TLR2 agonists as cancer adjuvants. More preclinical studies are needed to clarify this observation.

BDCA-1$^+$ and BDCA-3$^+$ blood DCs, and targeting them with TLR8 agonists

BDCA-1$^+$ DCs constitute the majority of the myeloid-derived DCs in the LNs, and express CD1a, CD11c, CD11b and CD172a (signal-regulatory protein [SIRP]-α). The BDCA-3$^+$ DCs are present as a smaller population and uniquely express CLEC9A which is a C-type lectin with ITAM-like motif [159], CADM1 (cellular adhesion molecule-1; NECL2), XCR1, CD1a and CD11c. They also show distinct TLR expressions, i.e. BDCA-1$^+$ DC express TLR1 and 6, while BDCA-3$^+$ DCs express TLR3 and 8. The BDCA-3$^+$ DCs could be activated by TLR3 stimulation to secrete IL-12 [131], and efficiently crosspresented antigens to CD8$^+$ T cells [130]. Due to their low frequencies, the functions of these DCs have not been well-characterized. Nevertheless, based on their TLR expressions, they might specialize in mounting immune responses against different types of pathogens. BDCA-1$^+$ DCs could be targeted with TLR6 agonists, while BDCA-3$^+$ DCs could be targeted with TLR3 and 8 agonists.

A novel small molecule TLR8 agonist, VTX-2337 (Motolimod; VentiRx Pharmaceuticals, USA), could specifically stimulate TNF-α and IL-12 secretions from human myeloid DCs and monocytes. It also induced NK cells to produce IFN-γ and augmented antibody dependent cell-mediated cytotoxicity (ADCC) by rituximab and trastuzumab [160]. In a Phase I open-label trial, 38 subjects with advanced solid tumors and lymphoma were treated with ascending dose of VTX-2337 (0.1–3.9mg/m^2/subcutaneously on Day 1, 5 and 8 on each 28-day cycle) [161]. Subjects who received doses of ≥0.4mg/m^2 showed elevated plasma granulocyte-colony stimulating factor (G-CSF), MCP-1, macrophage inflammatory protein (MIP)-1β and TNF-α. Eight subjects experienced stable diseases (median duration of 54.5 days). VTX-2337 was also evaluated in combination with cetuximab in a Phase Ib, open-label, dose-escalation study of squamous cell carcinoma of the head and neck (SCCHN) [162]. Thirteen subjects with recurrent or metastatic SCCHN were given VTX-2337 at 2.5, 3.0 or 3.5 mg/m^2 on day 1, 8 and 15 together with a fixed weekly dose of cetuximab in 28-day cycles. The maximum tolerated dose of VT-2337 was determined to be at 3.0 mg/m^2. Two subjects achieved partial responses, and 5 subjects experienced stable diseases. An increased in the frequency of activated circulating NK cells were also detected. A Phase II, randomized double-blind trial was conducted to further evaluate the anti-tumor effects of VTX-2337 in a cohort of 195 SCCHN subjects [163]. The subjects received 6 chemotherapy cycles consisting of platinum (carboplatin or cisplatin), fluorouracil, cetuximab, and either placebo or VTX-2337 intravenously every 3 weeks. Then, each subject continued to receive weekly cetuximab with either placebo or VTX-2337 every 4 weeks. There were no significant differences in the progression-free survival (PFS) [6.1

versus 5.9 months; HR=0.99; 1-sided 90% CI, 0.00–1.22; P=0.47] and overall survival (OS) [13.5 versus 11.3 months; HR=0.95; 1-sided 90% CI, 0.00–1.22; P=0.4] between the VTX-2337 and the placebo-treated groups. However, a subgroup analysis revealed that HPV-positive subjects treated with VTX-2337 and not placebo showed a significantly longer PFS (7.8 versus 5.9 months; P=0.046) and OS (15.2 versus 12.6 months; P=0.03).

VTX-2337 was also being evaluated in combination with pegylated liposomal doxorubicin (PLD) in ovarian carcinomas (NCT01666444 and Table 2). In a Phase II randomized, double-blind trial, 297 subjects with recurrent epithelial ovarian carcinoma were treated with PLD (for inducing immunogenic tumor cell death) in combination with either VTX-2337 or placebo for 28-day cycles until disease progression. [164]. It was shown that PLD and VTX-2337 combination did not significantly improve the PFS [4.8 versus 5.2 months in the PLD plus placebo; log rank one-sided P=0.943, HR=1.21] and OS [18.1 versus 18.9 months in PLD plus placebo; log rank one-sided P=0.923, HR=1.22] of the treated subjects. Evaluation of TILs, TLR8 single-nucleotide polymorphisms, autoantibody biomarkers, BRCA and DNA repair gene mutation status did not correlate with the PFS and OS of the subjects. Interestingly, significantly longer OS was observed in subjects who experienced an injection site reaction (ISR) after VTX-2337 treatment compared to the subjects who did not experience an ISR after VTX-2337 treatment (19.8 versus 13.3 months; P=0.067). Further analysis also revealed that subjects who had higher baseline responses of IFN-γ (P=0.049), TNF-α (P=0.041), or IL-12p40 (P=0.024) to *ex vivo* VTX-2337 stimulation of their PBMCs prior to VTX-2337 treatment survived longer than subjects who had low baseline responses of these cytokines prior to VTX-2337 treatment. These data suggested that the immune fitness of the cancer subjects (e.g. the presence of a strong pre-existing immunity against the tumor) might be important in the VTX-2337 treatment outcome. Additionally, viral infection in cancer might create a proinflammatory milieu that enhances VTX-2337 treatment (e.g. prolonged PFS of HPV-positive SCCHN subjects as described earlier). These data underscore the complexity of the immune responses of cancer patients, and screening methods could be tailored to help select suitable subjects for future VTX-2337 cancer studies. Another novel benzazepine TLR8 agonist, VTX-294, was found to be more potent than MPLA, R848 or CL075 (a synthetic TLR7/8 agonist) in inducing TNF-α and IL-1β production from newborn cord blood and upregulated HLA-DR and CD86 in newborn monocyte-derived DCs [165]. Production of these cytokines was further enhanced when VTX-294 was combined with MPLA, thus suggesting that VTX-294 is suitable as a vaccine adjuvant in neonates.

Conclusions

In vivo cancer vaccination could be achieved via *in vivo* targeting of DCs with different synthetic TLR agonists in different tissue compartments to elicit the type of immune response desired. Several synthetic TLR agonists such as Imiquimod, CpG ODNs, MPLA and BCG have been evaluated in the clinics and showed promising results against cancers. Several novel TLR agonists have also been developed, and are currently being tested in cancer clinical trials. Different TLR agonists could potentially synergized to increase the overall efficacy of the treatment. *In vivo* targeting of DCs with TLR agonists could serve as a low-cost alternative or even complement existing immunotherapies such as immune

checkpoint blockade antibodies. Indeed, several ongoing clinical trials are evaluating different TLR agonists in combination with anti-PD-1, anti-CTLA-4 and anti-PD-L1 for synergistic anti-tumor effects.

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Highlights

- The next-generation of cancer immunotherapy could entail *in vivo* cancer vaccination to elicit both innate and adaptive anti-tumor responses.
- This could be achieved via *in vivo* targeting of dendritic cells (DCs) that serve as an indispensable link between the innate and adaptive immunities.
- Toll-like receptors (TLRs) expressed on human DCs play an important role in recognizing and eliminating pathogens. *In vivo* targeting of DCs with TLR agonists such as Imiquimod, CpG-oligodeoxynucleotides (CpG ODNs), polyinosinic-polycytidylic acid (Poly I:C) and monophosphoryl lipid A (MPLA) have shown promise in preclinical and clinical cancer studies.
- Different TLR agonists could synergize to exert profound effects on the quality and magnitude of anti-tumor T cell responses.
- Synthetic TLR agonists could be synthesized at a low cost and could potentially be a cost effective stand-alone treatment or complimentary to immune checkpoint blockade therapy.

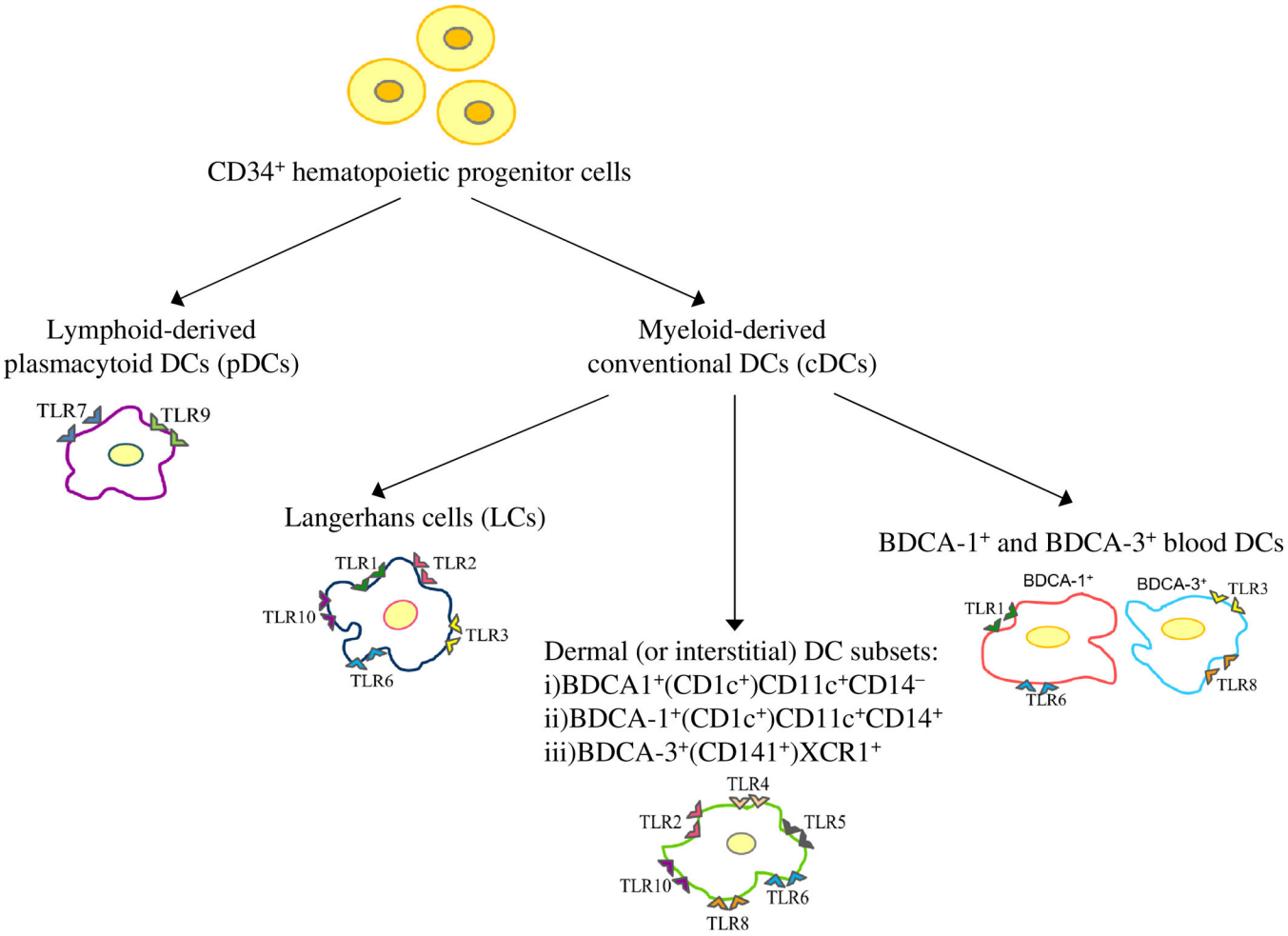

Figure 1.
Schematic diagram showing the major human DC subsets.

Table 1

Selected toll-like receptor (TLR) agonists for potential *in vivo* targeting of dendritic cells (DCs).

TLR	Synthetic agonists tested In clinics	Potential targeted DC subset(s)	References
2	LPS, MPLA (in AS01–04 adjuvant systems), amino alkyl glucosamlmide phosphates (In Dynavax®), GLA, BCG (used together with MPLA in Melacine®), MALP-2	LCs, Dermal DCs	136, 137, 139–149, 153
3	Poly I:C, Poly(I:C12U) [Ampligen®], Poly-ICLC [Hiltonol®]	LCs, BDCA-3$^+$ blood DCs	117–124
4	LPS, MPLA, amino alkyl glucosamlmide phosphates, GLA, BCG	Dermal DCs	136, 137, 139–149, 153
5	Flagellln	Dermal DCs	103
6	MALP-2	LCs, dermal DCs, BDCA-1$^+$ blood DCs	153, 154
7	Imlquimod (R-837), 852A (3M-001), Reslquimod (R-848; S-28463), Gardlqulmod	pDCs	47–72
8	Resiqulmod (R-848, S-28463), VTX-2337 (Motollmod), VTX-294	Dermal DCs, BDCA-3$^+$ blood DCs	68–72, 157, 158
9	CpG ODN 7909 (Agatollmod; PF-3512676; Promune®), SD-101	pDCs	97–102

Abbreviations: LC (Langerhans cells), pDCS (plasmacytod dendritic cells), LPS (lipopolysaccharides); MPLA (monophosphoryl lipid A; GLA (glucopyranosyl lipid A); BCG (*Mycobacterium bovis* bacillus Calmette-Guérin); MALP-2 (macrophage activating lipopeptide-2); VTX-2237 and VTX-294 (VentiRx Pharmaceuticals, USA); CpG ODN (CpG-oligodeoxynucleotides).

Table 2

Use of TLR7 agonists as adjuvant in active clinical trials (*www.clinicaltrials.gov).

Number	*NCT number and title	Cancer type(number of subjects to enroll)	Intervention(s)	Trial type	Status	Sponsor/collaborators
1	NCT01421017 Toll-like Receptor(TLR)7Agonist, Cyclophosphamide, and Radiotherapy for Breast Cancer With Skin Metastases	Metastatic and recurrent breast cancer (55)	• TLR7 agonist-Imiquimod • Radiotherapy • Cyclophosphamide	• Phase I/II, non-randomized and open-labeled	• Active, not recruiting • Start date: Aug 2011 • Completion date: Feb 2019	• New York University School of Medicine • National Cancer Institute (NCI)
2	NCT03196180 Topical Fluorouracil and Imiquimod in Treating Patients With High-Grade Cervical Intraepithelial Neoplasia	High grade cervical intraepithelial neoplasia, and p16-positive neoplastic cells present (34)	• TLR7 agonist-Imiquimod • Topical fluorouracil	• Phase I, single group assignment and open-label.	• Active, not recruiting • Start date: 30th Sept 2018 • Completion date: 31st Jan 2021	• NCI
3	NCT03416335 A Study of DSP-0509 in Patients With Advanced Solid Tumors to Determine the Safety and the Pharmacokinetic Profile	Neoplasms (44)	• TLR7 agonist-DSP-0509	• Phase I, single group assignment and open-label.	• Recruiting. • Start date: 1st June 2018 • Completion date: July 2020	• Boston Biomedical, Inc • Syneos Health
4	NCT00960752 Tumor and Vaccine Site With a Toll Like Receptor (TLR) Agonist	Melanoma (71)	• TLR7/8 agonist-Resiquimod (R 848)gel • Gp100 and MAGE-3 peptides	• Phase II, randomized factorial assignment and open-label.	• Active, not recruiting • Start date: May 2010 • Completion date: May 2019	• M.D. Anderson Cancer Center
5	NCT02556463 A Study of MEDI9197 in Subjects With Solid Tumors or CTC Landin Combination With Durvalumab and/ or Palliative Radiation in Subjects With Solid Tumors	Solid Tumors and early-stage cutaneous T Cell lymphoma [CTCL] (135)	• TLR7/8 agonist-MEDI9197 • Durvalumab (Fc optimized anti programmed cell death-ligand 1 [PD-L1] monoclonal antibody) • Radiation	• Phase I, non-randomized and open-labeled	• Recruiting. • Study Start date: 4th Nov 2015 • Completion date: 19th Aug 2020	• MedImmune LLC
6	NCT03435640 A Study of NKTR-262 in Combination With NKTR 214 and With NKTR-214 Plus Nivolumab in Patients With Locally Advanced or Metastatic Solid Tumor	Melanoma, Merkel cell carcinoma, triple Negative breast cancer, ovarian carcinoma, renal cell carcinoma, colorectal cancer, urothelial carcinoma and sarcoma(393)	• TLR7/8 agonist-NKTR-262 • CD122-biased agonist-NKTR-214 • Nivolumab (anti-PD-L1 monoclonal antibody)	• Phase I/II, non-randomized and open-label	• Recruiting • Start date: 15th Mar 2018 • Completion date: Dec 2019	• Nektar Therapeutics
7	NCT03301896 Study of the Safety and Efficacy of LHC165 Single	Solid tumors (200)	• TLR7/8 agonist-LHC165 • PDR001 (PD-1 checkpoint inhibitor)	• Phase I, non-randomized and open-labeled.	• Recruiting • Start date: 31st Jan 2018 • Completion date:	• Novartis

Number	*NCT number and title	Cancer type(number of subjects to enroll)	Intervention(s)	Trial type	Status	Sponsor/collaborators
	Agent and in Combination With PDR001 in Patients With Advanced Malignancies				20th Dec 2021	
8	NCT02431559 A Phase 1/2 Study of Motolimod (VTX-2337)and MED14736 in Subjects With Recurrent, Platinum Resistant Ovarian Cancer for Whom Pegylated Liposomal Doxorubicin (PLD) is Indicated	Ovarian carcinoma (53)	• TLR8 agonist-VTX2337 • Durvalumab • Pegylated liposomal doxorubicin (PLD)	• Phase I/II, non-randomized and open-label.	• Active, not recruiting • Start date: Nov 2015 • Completion date: Mar 2019	• Ludwig Institute for Cancer Research • MedImmune LLC • VentiRx Pharmaceuticals Inc. • Cancer Research Institute, New York City

Table 3

Use of TLR9 agonists as adjuvant in registered clinical trials (*information taken from Clinicaltrials.gov).

Number	*NCT number and tide	Cancer type (number of subjects to enroll)	Intervention(s)	Trial type	Status	Sponsor/collaborators
1	NCT02668770 Ipilimumab (Immunotherapy) and MGN1703 (TLR Agonist) in Patients With Advanced Solid Malignancies	Advanced solid cancers including melanoma (60)	• TLR9 agonist - MGN1703 • Ipilimumab (recombinant human IgGl anti-CTLA-4 monoclonal antibody)	• Phase II, non-randomized and open-label.	• Recruiting • Start date: 11 th May 2016 • Completion date: May 2020	• M.D. Anderson Cancer Center • Mologen AG
2	NCT02452697 Ph2 NK Cell Enriched DCls vv/vvo RI.R9 Agonist, DUKCPG-001 From Donors Following Allogeneic SCT	Myeloid and lymphoid malignancies (100)	• TI-R9 agonist - DUK-CPG-001 • NK Cell enriched-DLI	• Phase II, randomized and open-label.	• Recruiting • Start date: 8th June 2016 • Completion date: July 2023	• David Rizzieri, MD • Agilent Technologies, Inc. • Duke University
3	NCT03326752 Phase lb DV281 With an AntiPD-1 Inhibitor in NSCLC	Advanced NSCLC (80)	• TLR9 agonist - DV281 (synthetic, aerosolized C-class CpG ODN; RP2D) • Anti-PD-1 Inhibitor	• Phase I, non-randomized and open-label.	• Recruiting • Start date: 20th Sept 2017 • Completion date: 30th Aug 2020	• Dynavax Technologies Corporation
4	NCT03618641 CMP-001 in Combo With Nivolumab in Stage 1IIB/C/D Melanoma Patients With Clinically Apparent Lymph Node Disease	Melanoma and lymph node cancer(20)	• TLR9 agonist - CMP-001 (Virus-like particle-encapsulated TLR9 agonist) • Nivolumab	• Phase II, sequential assignment and open- label.	• Recruiting • Start date: 8th Aug 2018 • Completion date: 15th July 2023	• Diwakar Davar • Checkmate Pharmaceuticals • University of Pittsburgh
5	NCT03507699 Combined Immunotherapy and Radiosurgery for Metastatic Colorectal Cancer	Colorectal carcinoma and liver metastases (19)	• TI-R9 agonist - CMP-001 • Liver radiotherapy • Nivolumab • Ipilimumab	• Phase I, non-randomized and open-label.	• Active, not yet recruiting • Star date: 1st Jul 2018 • Completion date: 31st May 2021	• Sheba Medical Center • Checkmate Pharmaceuticals • Bristol-Myers Squibb
6	NCT03445533 A Study of IMO-2125 in Combination With Ipilimumab Versus Ipilimumab Alone in Subjects With Anti-PD-1 Refractory Melanoma (ILLUMINATE-301)	Metastatic melanoma (308)	• TLR9 agonist - IMO-2125 • Ipilimumab	• Phase III, randomized and open-label.	• Recruiting • Start date: 28th Feb 2018 • Completion date: 1st Jul 2020	• Idera Pharmaceuticals, Inc. • Bristol-Myers Squibb
7	NCT03410901 TLR9 Agonist SDS-101, Anti-OX40 Antibody BMS 986178, and radiotherapy in Treating Patients With Low Grade B-Cell Non-Hodgkin Lymphomas	B-cell Non-Hodgkin lymphoma (15)	• TLR9 Agonist - SDS-101 • Anti-necrosis factor receptor superfamily member 4 (0X40) antibody [BMS 986178) • radiotherapy	• Phase I, single group assignment and open- label.	• Recruiting • Start date: 9th April 2018 • Completion date: 9th Oct 2020	• Ronald Levy • NCI • Stanford University
8	NCT03007732 Pembrolizumab in Combination With Intratumoral SDS-	Prostate cancer (42)	• TLR9 agonist - SDS-101 • Pembrolizumab	• Phase I, randomized and open-label.	• Recuiting • Start date: 27th April 2017	• Lawrence Fong • University of California, San Francisco

Number	*NCT number and tide	Cancer type (number of subjects to enroll)	Intervention(s)	Trial type	Status	Sponsor/collaborators
	101 Therapy		• Leuprolide acetate and abiraterone acetate • Prednisone (synthetic glucocorticoid) • Stereotactic body radiotherapy		• Completion date: April 2022	
9	NCT02927964 TLR9 Agonist SDS-101, Ibrutinib, and radiotherapy in Treating Patients With Relapsed or Refractory Grade 1-3A Follicular Lymphoma	Follicular lymphoma including recurrent and recurrent forms (30)	• TLR9 agonist - SDS-101 • Ibrutinib (small-molecule inhibitor of Bruton's tyrosine kinase) • radiotherapy	• Phase I/II, single group assignment and open-label.	• Recruiting • Start date: Nov 2016 • nov.16 • Completion date: Nov 2021	• Robert Lowsky • NCI • Stanford University

Table 4

Use of TLR3 agonists as adjuvant in a selection of active clinical trials (*www.clinicaltrials.gov).

Number	*NCT number and title	Cancer type(number of subjects to enroll)	Intervention(s)	Trial type	Status	Sponsor/collaborators
1	NCT02643303 A Phase 1/2 Study of In Situ Vaccination With Tremelimumab and IV Durvalumab Plus Poly-ICLC in Subjects With Advanced, Measurable, Biopsy-accessibleCancers	Head and neck squamous cell carcinoma, Breast, prostate, renal and bladder carcinomas, melanoma sarcoma, Merkel cell and cutaneous T cell lymphoma (102)	• TLR3 agonist-Poly-ICLC • Durvalumab • Tremelimumab (human IgG2 anti-cytotoxic T-lymphocyte - associated antigen 4 [CTLA-4] monoclonal antibody)	• Phase I/II, non-randomized and open-label.	• Recruiting. • Start date: 28th Dec 2016 • Completion date: Aug 2022	• Ludwig Institute for Cancer Research • MedImmune LLC • Cancer Research Institute, New York City
2	NCT03162562 The Safety and Antitumor Activity of the Combination of Oregovomab and Hiltonol in Recurrent Advanced Ovarian Cancer	Stage III-IV and recurrent ovarian carcinoma (10)	• TLR3 agonist-Poly-ICLC • Oregovomab (murineanti-CA125 monoclonal antibody)	• Phase I, single group and open-label	• Recruiting. • Start date: 30th May 2017 • Completion date: Sept 2021	• OncoQuest Inc.
3	NCT02834052 Pembrolizumab + Poly-ICLC in MRP Colon Cancer	Metastatic colorectal carcinoma (42)	• TLR3 agonist-Poly-ICLC • Pembrolizumab	• Phase I/II, non-randomized single group assignment and open-label.	• Recruiting. • Start date: 10th Jan 2018 • Completion date: Jan 2021	• Asha Nayak • Oncovir, Inc. • Merck Sharp & Dohme Corp. • Augusta University
4	NCT02126579 Phase I/II Trial of a Long Peptide Vaccine (LPV7) Plus TLR Agonists	Melanoma (58)	• TLR3 agonist - Poly-ICLC and TLR7/8 agonist - Resiquimod • Peptide vaccine (LPV7) + Tetanus peptide • Incomplete Freund's adjuvant (IFA)	• Phase I/II, randomized and open-label	• Recruiting. • Study Start: April 2014 • Completion date: Dec 2018	• Craig L Slingluff, Jr • University of Virginia
5	NCT01976585 In Situ Vaccine for Low-Grade Lymphoma: Combination of Intratumoral Flt3L and Poly-ICLC With Low-Dose Radiotherapy	Low-Grade B-cell Lymphoma (30)	• TLR3 agonist - Poly-ICLC • Recombinant human (rhu) Flt3 ligand (CDX-301)	• Phase I/II, single group assignment and open-label.	• Recruiting. • Start date: Dec 2013 • Completion date: Nov 2018	• Joshua Brody • Icahn School of Medicine at Mount Sinai
6	NCT01720836 Study of the Immune Response of MUC1 (Mucin1) Peptide Vaccine for Non-small Cell Lung Cancer	Non-small cell lung cancer (NSCLC) (30)	• TLR3 agonist - Poly-ICLC (Hiltonol®) • Mucin 1 (MUC1) peptide	• Phase I/II, non-randomized and open-label	• Recruiting • Start date: Nov 2012 • Completion date: Sept 2020	• Olivera Finn • University of Pittsburgh
7	NCT03262103 Neoadjuvant Hiltonol® (PolyICLC) for Prostate Cancer	Prostate cancer (24)	• TLR3 agonist - Poly-ICLC (Hiltonol®)given intratumorally (0.5 mg or 1.0 mg) or intramuscularly • Radical prostatectomy	• Phase I, non-randomized and open-label	• Recruiting • Start date: May 2017 • Completion date: May 2020	• Ashutosh Kumar Tewari • Oncovir, Inc. • Icahn School of Medicine at Mount Sinai
8	NCT02721043	Solid tumors (20)	• TLR3 agonist - Poly-ICLC (Hiltolon®)	• Phase I, single • group assignment and	• Recruiting • Start date: April	• Nina Bhardwaj • Icahn School of Medicine

Number	*NCT number and title	Cancer type(number of subjects to enroll)	Intervention(s)	Trial type	Status	Sponsor/collaborators
	Safety and Immunogenicity of Personalized Genomic Vaccine to Treat Malignancies		• Synthetic peptides • Lenalidomide (a thalidomide analog)	open-label.	2016 • Completion date: July 2020	at Mount Sinai
9	NCT02061449 PolyICLC, Radiation, and Romidepsin for Advanced Cutaneous T Cell Lymphoma	Cutaneous T-cell Lymphoma (3)	• TLR3 agonist - Poly-ICLC (Hiltonol®) • Romidepsin (antibiotic) • Focal lesional radiation	• Phase I/II, non-randomized and open-labeled	• Active, not recruiting. • Start date: Mar 2014 • Completion date: June 2018	• New York University, School of Medicine • Ludwig Institute for Cancer Research
10	NCT01204684 Dendritic Cell Vaccine for Patients With Brain Tumors	Glioma, glioblastoma, anaplastic astrocytoma and Astrooligodendro-glioma (60)	• Autologous tumor lysate-pulsed DC vaccination in combination with either • TLR3 agonist - Poly-ICLC (Hiltonol®), or • 0.2% Resiquimod	• Phase II, randomized and open-labeled	• Active, not recruiting. • Start date:8th Oct 2010 • Completion date: Oct 2019	• Jonsson Comprehensive Cancer Center

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Table 5

Use of TLR4 agonist as adjuvant in active clinical trials (*www.clinicaltrials.gov).

Number	*NCT number and title	Cancer type(number Of subjects to enroll)	Intervention	Trial ype	Status	Sponsor/collaborators
1	NCT02798978 A Phase I, 2-part (Part 1 Being a Single Dose Escalation and Part2, a Parallel Group) Study of Toll-like Receptor (TLR4) Agonist (GSK1795091) in Healthy Subjects	Neoplasms (42)	• TLR4 agonist - GSK1795091 (CRX-601)	• Phase I, randomized and double-blind	• Completed, no results posted. • Sart date:10th Jan 2017 • Completion date: 13th Oct 2017	• GlaxoSmithKline
2	NCT03447314 Study of a Combination of GSK1795091 and Immunotherapies in Subjects With Advanced Solid Tumors	Neoplasms (54)	• TLR4 agonist-GSK1795091 • Humanized IgG1 anti-OX40 agonistic monoclonal antibody -GSK3174998	• Phase I, non-randomized, sequential assignment and open-label.	• Recruiting. • Sart date: 26th Mar 2018 • Completion date: 14th Feb 2020	• GlaxoSmithKline • IQVIA
3	NCT02180698 TLR4 Agonist GLA-SE and radiotherapy in Treating Patients With Soft Tissue Sarcoma That Is Metastatic or Cannot Be Removed by Surgery	Stage III and IV adult Soft tissues arcoma (16)	• TLR4 agonist–glucopyranosyl lipid A stable emulsion (GLA-SE; G100) • radiotherapy	• Phase I, single group assignment	• Active, not recruiting. No results posted. • Start date: 17th Nov 2014 • Completion date: 1st Mar 2018	• Fred Hutchinson Cancer Research Center • NCI
4	NCT02320305 MART-1 Antigen With or Without TLR4 Agonist GLA-SE in Treating Patients With Stage II-IV Melanoma That Has Been Removed by Surgery	Stage II-IV melanoma (23)	• TLR4 Agonist – GLA-SE • MART-1 antigen peptide	• Pilottrial. Randomized and open-label	• Active, not recruiting. • Start date: 27th Jan 2015 • Completion date: 1st Jan 2019	• Mayo Clinic • NCI
5	NCT02501473 Study of Intratumoral G100 With Or Without Pembrolizumab In Patients With Follicular Non-Hodgkin's Lymphoma	Follicular non-Hodgkin'slymphoma (51)	• TLR4 agonist – GLA-SE (G100) • Pembrolizumab (humanized IgG4 anti-PD-1 monoclonal antibody)	• Phase I/II, randomized and open-label	• Active, not recruiting. • Start date: 1st June 2015 • Completion date: Mar 2019	• Immune Design • Merck Sharp & Dohme Corp

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