HUMAN VACCINES & IMMUNOTHERAPEUTICS
2024, VOL. 20, NO. 1, 2297453
https://doi.org/10.1080/21645515.2023.2297453

Taylor & Francis
Taylor & Francis Group

REVIEW

Toll-like receptor agonists as cancer vaccine adjuvants

Donghwan Jeon[a], Ethan Hill[b], and Douglas G. McNeel[b]

[a]Department of Oncology, University of Wisconsin Carbone Cancer Center, Madison, WI, USA; [b]Department of Medicine, University of Wisconsin Carbone Cancer Center, Madison, WI, USA

ABSTRACT

Cancer immunotherapy has emerged as a promising strategy to treat cancer patients. Among the wide range of immunological approaches, cancer vaccines have been investigated to activate and expand tumor-reactive T cells. However, most cancer vaccines have not shown significant clinical benefit as monotherapies. This is likely due to the antigen targets of vaccines, "self" proteins to which there is tolerance, as well as to the immunosuppressive tumor microenvironment. To help circumvent immune tolerance and generate effective immune responses, adjuvants for cancer vaccines are necessary. One representative adjuvant family is Toll-Like receptor (TLR) agonists, synthetic molecules that stimulate TLRs. TLRs are the largest family of pattern recognition receptors (PRRs) that serve as the sensors of pathogens or cellular damage. They recognize conserved foreign molecules from pathogens or internal molecules from cellular damage and propel innate immune responses. When used with vaccines, activation of TLRs signals an innate damage response that can facilitate the development of a strong adaptive immune response against the target antigen. The ability of TLR agonists to modulate innate immune responses has positioned them to serve as adjuvants for vaccines targeting infectious diseases and cancers. This review provides a summary of various TLRs, including their expression patterns, their functions in the immune system, as well as their ligands and synthetic molecules developed as TLR agonists. In addition, it presents a comprehensive overview of recent strategies employing different TLR agonists as adjuvants in cancer vaccine development, both in pre-clinical models and ongoing clinical trials.

ARTICLE HISTORY
Received 4 October 2023
Revised 1 December 2023
Accepted 16 December 2023

KEYWORDS
Toll-like receptors; cancer vaccines; vaccine adjuvants

Introduction

Cancer immunotherapy has emerged as a promising strategy to treat cancer patients by engaging different parts of the immune system to recognize and eliminate cancer cells.[1] It encompasses a wide range of immunological approaches, including immune checkpoint blockade, adoptive cell transfer, cytokine therapies, and cancer vaccines. Vaccines are classified as active immunotherapies which require the host to mount a desired immune response, as opposed to passive immunotherapies that supply the immune cells or factors directly to the host. Vaccines as cancer treatment have been investigated for many decades using autologous inactivated tumor cells, subcomponents of tumors delivered as a protein/peptide or encoded within a viral or nucleic acid construct, or by delivery of tumor antigen loaded onto professional antigen-presenting cells. While one cancer vaccine, sipuleucel-T,[2] has been FDA-approved, most anti-tumor vaccines have not shown significant clinical benefit when used as monotherapies. This is in part due to the fact that the targets of cancer vaccines are "self" antigens, molecules to which the immune system should be tolerant. In addition, the tumor microenvironment may contain multiple regulatory immune populations and mechanisms to avoid detection by tumor-specific T cells that might be activated by vaccination.[3–5] Consequently, successful cancer vaccine strategies will need to take into account means to more effectively activate T cells that can recognize "self" tumor antigens, and use strategies that target tumor-associated mechanisms of immune avoidance.

One approach being used to improve the efficacy of cancer vaccines is to target tumor-specific antigens, such as mutations specific to an individual person and tumor, rather than tumor-associated antigens that are shared by multiple individuals with the same tumor type.[6,7] The concept is that there should be less tolerance to mutation-associated neo-antigens than normal "self" antigens. To date, however, while there is much enthusiasm for this approach, there are practical issues in identifying founder mutations rather than branch mutations, and whether targeting a specific mutation-derived neoantigen will lead to T cells with anti-tumor efficacy. Moreover, to date there is no clinical evidence that this approach is superior to targeting shared antigens, at least when vaccines are used as monotherapies. However, it has been clear that targeting tumor-associated mechanisms of resistance can improve the efficacy of tumor vaccines. For example, we demonstrated that vaccines can activate tumor antigen-specific CD8+ T cells, leading to increased PD-1 expression on these T cells.[8] Expression of PD-L1 in the tumor microenvironment led to inactivation of the anti-tumor efficacy of these cells, and blockade of PD-1 or PD-L1 with vaccination led to superior anti-tumor efficacy in murine models,[8,9] and in patients with advanced prostate cancer.[10,11] The recent decision by FDA to grant breakthrough designation to a personalized mutation-

CONTACT Douglas G. McNeel dm3@medicine.wisc.edu Department of Medicine, 7007 Wisconsin Institutes for Medical Research, 1111 Highland Avenue, Madison, WI 53705, USA.

associated neo-antigen mRNA vaccine, mRNA-4157-P201 given with pembrolizumab, based on the demonstration of prolonged time to disease progression in a randomized clinical trial (NCT03897881) in patients with high-risk melanoma, further supports and highlights the efficacy of this general approach of giving PD-1 blockade with anti-cancer vaccines.[12] Other approaches include using agents to target immune regulatory populations, such as myeloid-derived suppressor cells or CD4 T regulatory cells, or immune regulatory cytokines within the tumor microenvironment, in combination with anti-tumor vaccines.[13–15]

Another approach to improve the efficacy of cancer vaccines is to use these with agents that affect the function of T cells that are activated by vaccination. Adjuvants encompass substances that are delivered with vaccines to support the vaccine-mediated immune response. Based on their functions, adjuvants are generally classified into two categories: depot adjuvants and immunostimulatory adjuvants.[16] Depot adjuvants, such as mineral salts, prolong antigen availability at the injection site while immunostimulatory adjuvants, such as cytokines, function to activate innate immunity. Because most tumor antigens are self-antigens to which the frequency of reactive T cells may be low or ineffective, adjuvants for cancer vaccines have typically focused on the use of immunostimulatory adjuvants to potentiate immune responsiveness and direct the immune response toward T helper type-1 (Th1) response with fully activated CD8+ T cells.[17]

Recognition of pathogens by the mammalian immune system involves recognition of foreign molecules (pathogen-associated molecular patterns, PAMPs) that engage the innate immune system for rapid response. Ultimately, an adaptive immune response is elicited to provide specificity to eradicate a pathogen and provide immunological memory. The innate immune system also recognizes features associated with cellular damage, so called damage-associated molecular patterns (DAMPs).[18,19] Studies over the last several decades have established that activation of innate immunity is crucial to drive adaptive immune responses. Cancer vaccines have consequently taken advantage of agents that activate PAMPs or DAMPs as adjuvants to activate innate immune responses to help direct the development of immune response to the antigen targets. Among these agents are compounds that activate innate immunity receptors, such as toll-like receptors (TLRs).[20,21] TLRs are the largest family of pattern recognition receptors (PRRs) that serve as the sensors of danger from pathogens or cellular damage. The discovery of TLRs stems from the identification of the *Toll* gene in fruit flies with its crucial role in the *Drosophila* immune system.[22] In 1998, the first mammalian homologues of the *Toll* gene were identified, and these receptors were named Toll-like receptors.[23] These receptors recognize conserved PAMPs or internal molecules from damaged cells (DAMPs), and activate innate immune responses.[24] Diverse TLRs have been identified in both humans and mice (TLR1–10 for humans, TLR1–9 and 11–13 for mice). Each TLR shows distinct cellular expression patterns and cytokine induction based on its primary role in recognizing different types of pathogens.[25] Conceptually, activation of different TLRs may activate different innate signals and thus could be useful as vaccine adjuvants to optimally augment or skew the resulting immune response to the target antigen.[26]

The ability of TLR agonists to modulate innate immune response has positioned them to serve as adjuvants for vaccines targeting various infectious diseases. This has also led to their study as adjuvants for cancer vaccines.[27–30] Certain TLR agonists have also been approved as anti-cancer therapies due to their direct effects on tumor cells or the tumor immune microenvironment, aspects that have been reviewed elsewhere.[31,32] In this article, we review the immunological functions of each TLR with their ligands/agonists (Table 1) and update the recent approaches using TLR agonists as vaccine adjuvants, specifically for cancer vaccines, in both preclinical models and clinical trials.

Introduction to toll-like receptors

TLR2

TLR2 was identified in 1998 as one of the first membrane proteins found structurally related to *Drosophila Toll*.[23] It is expressed on the plasma membrane of various immune cells including macrophages, dendritic cells, B cells, and T cells, as well as endothelial and epithelial cells.[33,34] TLR2 can be activated by a broad repertoire of molecules with diacyl and triacylglycerol moieties, proteins, and polysaccharides. The major TLR2 ligands are lipoproteins (LPs), which are ubiquitous in the outer membrane of most bacteria. TLR2 has been described to form both homodimers as well as heterodimers with other TLR, notably TLR2/TLR1 and TLR2/TLR6.[35] Most functional studies have evaluated these heterodimers, however, it is known that homodimers recognize lipoteichoic acid, bacterial peptidoglycan, lipoprotein, porins, viral glycoproteins, and hemagglutinin.[36] The exact PAMP repertoire required for activating TLR2 is determined by the partnering TLR. For example, TLR2/TLR1 heterodimer recognizes triacyl LPs, while TLR2/TLR6 senses diacyl LPs.[37] Upon the recognition of PAMPs, two intracellular Toll/Interleukin-1 receptor (TIR) domains from each TLR recruit the universal TLR adaptor, Myeloid differentiation primary response-88 (MyD88).[38] This results in the activation of NFκB and downstream production of proinflammatory cytokines and chemokines to activate the innate immune system. The most well-characterized chemokines upregulated by TLR2 are CCL2 and CXCL8 from dendritic cells and macrophages, which recruit other immune cells including neutrophils or macrophages.[39,40] In addition, TLR2-stimulated dendritic cells have been shown to secrete high levels of IL-1ß, IL-6, IL-12, or TNFα and express co-stimulatory molecules such as CD80 and CD86, which are critical for priming T cells.[39,41–43]

The biochemical diversity of the ligand structures of TLR2 led to the development of a wide variety of synthetic TLR2 agonists. Among them, Pam2CSK4 and Pam3CSK4, synthetic lipopeptides which mimic the ones found in bacterial cell membranes, are the two most commonly used TLR2 agonists.[44] Both agonists have a cysteine residue at the C-terminus of di- or triacyl lipopeptides, along with two or three amino acids (Pam2Cys or Pam3Cys) that activate TLR2/

Table 1. The summary of toll-like receptors.

TLR	Cellular expression	Receptor complex	Natural Ligands	Synthetic Agonists	Signaling adaptor	Downstream effects
TLR2	Plasma membrane	2–1 2–6 2–10	Lipopeptides Peptidoglycan Lipoteichoic acid Zymosan Lipoarabinomannans	Pam2Cys Pam3Cys	MyD88	inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12) CD80, CD86 upregulation
TLR3	Endolysosome	3–3	dsRNA	PolyI:C PolyIC12U PolyICLC ARNAX RGC100	TRIF	IFNβ CXCL10 CD80, CD86 upregulation
TLR4	Plasma membrane	4-MD2	LPS	MPLA GLA ONO-4007	MyD88 TRIF	inflammatory cytokines (TNFα,IL-1β,IL-6) IFNβ CXCL10 CD80, CD86 upregulation
TLR5	Plasma membrane	5–5	Bacterial flagellin	Flagellin	MyD88	inflammatory cytokines (TNFα,IL-1β,IL-6,IL-8) CD80, CD86 upregulation
TLR7	Endolysosome	7–7	ssRNA	Resiquimod Imiquimod Vesatolimod	MyD88	Type 1 IFN CD80, CD86 upregulation
TLR8	Endolysosome	8–8	ssRNA	Resiquimod Selgantolimod Motolimod	MyD88	inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12) CD80, CD86 upregulation
TLR9	Endolysosome	9–9	CpG-DNA	ODN-2216(Class A) ODN-2006(Class B) ODN-2429(Class C) ODN-21798(Class P) IMO-2025 IMO-2125 MGN-1703	MyD88	Type 1 IFN CD80, CD86 upregulation
TLR10	Plasma membrane	10–1 10–2 10–6 10–10	Triacylated lipopeptides	N/A	Unknown	IL-1Ra
TLR11	Endolysosome	11–11 11–12	Profilin Flagellin	N/A	MyD88	IL-12
TLR12	Endolysosome	12–11 12–12	Profilin	N/A	MyD88	IL-12
TLR13	Endolysosome	13–13	Bacterial 23S rRNA	ORN Sa19	MyD88	IL-12

6 or TLR1/2 heterodimers, respectively. Other types of TLR2 agonists also exist: lipoteichoic acids (LTAs) and peptidoglycans (PGNs), found in bacterial cell walls, or Zymosan, found in yeast cell walls.[45–47] In addition, glycosylphosphatidylinositol (GPI) anchors from *Plasmodium falciparum*, or lipoarabinomannans (LAMs) from mycobacteria, also have been shown to serve as TLR2 agonists.[48,49]

TLR3

TLR3 was the first antiviral TLR identified in 2001.[50] TLR3 is broadly expressed in both immune cells and nonimmune cells, including DCs, T cells, NK cells, epithelial cells, and fibroblasts.[51–55] TLR3 is localized in intracellular organelles, such as endosomal/lysosomal compartments and endoplasmic reticulum (ER). TLR3 responds to double-stranded (ds) RNA, typically associated with viral RNA.[56] Binding of RNA to the TLR3 ectodomain triggers dimerization, and clustered TIR domains utilize adapter proteins to promote downstream signaling. Instead of MyD88, however, TLR3 uses TIR-domain-containing-adapter-inducing interferon-beta (TRIF), which results in the secretion of cytokines or chemokines including type-1 interferons (IFNs).[57] TLR3 has been shown to promote antigen presentation in antigen-presenting cells and trigger cross-presentation of the antigen, which can support a robust CD8+ T-cell response.[58] Chemokines induced by TLR3 are also known to trigger a Th1 response, which is important for T-cell-mediated anti-tumor immunity.[59]

As dsRNA serves as a PAMP recognized by TLR3, a number of TLR3 agonists have been designed by mimicking the structure of dsRNA. An example of a synthetic dsRNA TLR agonist is Polyinosinic-polycytidylic acid (Poly(I:C)).[60] Chemical modification of the PolyI:C structure also led to the development of other TLR3 agonists such as poly-IC12U and poly-ICLC. Poly-IC12U is modified from poly(I:C) with a U mismatch at every 12th base of the C strand. This generates a mismatched double-stranded RNA to increase the specificity for TLR3.[61] Poly-ICLC mimics viral genetic material with the inclusion of poly-L-lysine and carboxy-methylcellulose in poly (I:C) strands. This increases the stability of Poly-ICLC by increasing its resistance to endonucleases.[62,63] In addition to poly(I:C)-based agonists, DNA-RNA hybrids such as ARNAX and RGC100 have also been recently developed as they show improved stability and solubility. ARNAX acquires stability by capping its RNA with DNA (phosphorothioated GpC), which provides resistance to nucleases.[64] RGC100 has unique structural characteristics, such as relatively short length (100bp), low molecular weight (64.9KDa), and 100% GC content. These structural properties tightly stack bases in the dsRNA structure, thereby improving its resistance to nucleases and stability.[65–67]

TLR4

TLR4 is another TLR member first discovered as a human homolog of Toll protein in *Drosophila*.[23] TLR4 is expressed on the plasma membrane, predominantly on myeloid lineage cells, but is also expressed by epithelial or endothelial cells.[68] The natural ligands for TLR4 are lipopolysaccharides (LPS), the outer membrane components of gram-negative bacteria.[69] TLR4 activation is mediated by two co-receptors, CD14, and myeloid differentiation factor-2 (MD-2).[70] LPS first binds to CD14, and then the LPS-CD14 complex is recognized by MD-2. MD-2 has a hydrophobic binding socket for the acyl chain of LPS, and this socket facilitates the interaction between LPS and TLR4. Activation of TLR4 upregulates the secretion of a wide range of proinflammatory cytokines and chemokines, including IL-1β and IL-6, via both MyD88-dependent and -independent pathways.[71,72] One of the key molecules induced by TLR4 activation is TNFα, which is necessary for local and systemic inflammation, DC maturation, and DC migration. This promotes the activation and proliferation of Th1 lymphocytes.[73]

Although LPS serves as the primary natural ligand for TLR4, it is composed of three distinct parts: a glycan polymer, an oligosaccharide, and lipid A.[74,75] Among these, lipid A has been suggested to be the primary moiety necessary to activate TLR4 because the acyl-chain binding socket of MD-2 is specific to lipid A for TLR4 activation.[76] This finding has led to the development of diverse TLR4 agonists based on the structure of lipid A, including monophosphoryl lipid A (MPLA), glucopyranosyl lipid A (GLA), and ONO-4007. MPLA is a derivative of lipid A from *Salmonella Minnesota*, which lacks the *R*-(3) hydroxytetrade canoyl and 1-phosphate group.[77] GLA is a hexa-acylated lipid A analog which does not have sugar residues on a hydroxyl connection site.[78] ONO-4007 is a synthetic analog of lipid A which has a lower molecular weight compared to the parent LPS.[79]

TLR5

TLR5 was also first identified in 1998 with other TLRs as a human homolog of *Drosophila* Toll protein.[23] TLR5 recognizes flagellin, a monomer subunit which polymerizes into bacterial flagella.[80] TLR5 is expressed on the plasma membrane of various immune cells including macrophages and dendritic cells, but also on epithelial cells lining the respiratory and gastrointestinal tracts. This expression pattern permits rapid response to bacterial infections of the lungs or gut.[81,82] Upon flagellin binding, TLR5 receptors homodimerize and recruit MyD88 with TIR domain adaptor protein.[83] This MyD88-dependent signaling upregulates the secretion of IL-8 from epithelial cells and proinflammatory cytokines such as IL-6 or TNFα from immune cells such as monocytes or dendritic cells.[84,85]

Flagellin binds very specifically to TLR5, but the activation of TLR5 by flagellin has been reported to induce excessive immune responses including systemic inflammation and acquisition of unwanted effects such as shock and loss of vascular contractility.[86,87] In addition, flagellin itself can also serve as an immune target, and the induction of flagellin-specific antibodies can interfere with the function of flagellin as a TLR5 agonist.[88] To date, these findings have made TLR5 less desirable as a vaccine adjuvant. Therefore, recent work to develop TLR5 agonists has focused on the interaction between TLR5 and flagellin to precisely modulate the immune response triggered by flagellin. It has been shown that the D1 domain of flagellin, where the tertiary structure is highly conserved,

serves as the TLR5 recognition site. A distorted tertiary structure generated by altering a single amino acid can ablate the entire functionality of flagellin to activate TLR5.[89,90] Other investigators have shown that deleting the B cell epitope region from flagellin alleviated the generation of antibodies.[91] More investigation is necessary to develop TLR5 agonists as potential vaccine adjuvants.

TLR7/8

TLR7 and TLR8 were first discovered in 2000 as parts of an intracellular recognition system that responds to purine-rich ssRNA.[92,93] Although these two receptors are phylogenetically similar in humans, they have distinct characteristics.[94,95] Both receptors respond to guanosine-rich oligoribonucleotides (ORNs), but TLR8 is additionally activated by adenosine- and uridine-rich ORNs.[96–98] Both receptors are found in monocytes, macrophages, and T cells, but TLR7 is predominantly expressed by plasmacytoid DCs (pDCs) whereas TLR8 is primarily expressed by myeloid DCs.[94,95] While TLR7 forms a homodimer once the ligand binds, TLR8 exists as a weak homodimer which tightens its conformation upon ligand binding.[99] Both TLR7 and TLR8 utilize MyD88-dependent signaling pathway upon activation, but TLR7 mainly induces IRF7-mediated production of type1 interferons while TLR8 activates NFkB signaling to upregulate proinflammatory cytokines such as IL-12.[100,101]

Over the past few decades, a number of heterocyclic scaffolds have been described as TLR7/8 agonists, including imidazoquinolines (IMQs), benzimidazoles, and pteridinones.[102] Many studies have focused on derivatives of imidazoquinolines, such as resiquimod or imiquimod, since their small molecular size and hydrophobicity allow easy penetration of the epidermal barrier. This makes them ideally suited for delivery as vaccine adjuvants.[103–105] Most IMQs are able to stimulate both TLR7 and TLR8, but a slight modification of the chemical structure can alter the selectivity of the agonist. As an example, resiquimod can activate both TLR7 and TLR8, but a resiquimod-derivative known as imiquimod shows selectivity toward TLR7 despite its structural similarity to adenosine nucleosides.[106]

TLR9

TLR9 was also identified in 2000 as another intracellular receptor located in the endosomal membrane.[92] TLR9 detects single-stranded DNA.[107] Specifically, this receptor detects unmethylated CpG motifs.[108–110] While these motifs are more frequent in bacterial or viral genomes, self-DNA can also be recognized by TLR9, a finding that has been associated with autoimmunity.[111,112] TLR9 shows a narrower expression pattern compared to the other members; it is constitutively expressed in only pDCs and B cells, but the expression can be upregulated in other immune cells including neutrophils and monocytes upon their activation.[21,100,113,114] Similar to the other TLRs, TLR9 also uses a MYD88-dependent signaling pathway to upregulate the expression of proinflammatory cytokines such as type 1 IFN or co-stimulatory molecules CD80 and CD86.[115] Reports have shown that TLR9 stimulation can also facilitate the maturation of pDCs, NK cells, and B cells.[116–118]

Based on the chemical structure, synthetic TLR9 agonists are classified into four different groups: class A, B, C, and P.[119,120] Class A are oligonucleotides that possess poly-G nucleotides at both 5' and 3' ends, generating a secondary structure called a G-quadruplex.[117] Class B agonists consist of the conventional linear structured single-stranded oligonucleotides. Class C agonists display both linear and dimeric structures as they are composed of two polynucleotides that partially dimerize to each other.[117,121] Class P agonists have palindromic sequences at the 3' end which form a distinct secondary structure.[120] These differences in chemical structures result in distinct physiological activities.[122,123] For example, Class B agonists are transported faster into the late endosome compartment because of their single-stranded and linear structures, and induce a stronger activation of B cells and NK cells than dendritic cells compared to the Class A agonists, thereby acting as a less potent IFNα inducer.[117,121] The secondary structure of class P agonists, generated by the palindromic sequences, stabilizes these agonists from degradation by nucleases.[120] Recent studies of TLR9 agonists have focused on improving both the stability and efficacy of the agonists. For example, immunomodulatory oligodeoxynucleotides (IMO)-type CpG ODNs are modified versions of CpG generated by conjugating 3' ends of two CpG nucleotides to a non-nucleotide linker. This triggers stronger immune responses compared to traditional CpG nucleotides.[124,125] MGN1703, considered a third generation of CpG-ODN, has a distinct dumbbell-like structure with two single-stranded loops containing multiple TLR9 recognition sites connected by a double-stranded chain to delay nuclease-mediated degradation.[121,126]

TLR10

TLR10 is the latest TLR which was first reported in 2001.[127] Functional TLR10 is expressed in humans while a pseudogene exists in mice, a fact that has limited studies of TLR10 as a vaccine adjuvant. Unlike other TLRs, the expression of TLR10 varies significantly among tissues, and is mostly expressed in secondary lymphoid organs such as lymph nodes, spleens, and tonsils.[128,129] While TLR10 is closely related to TLR1 and TLR6,[127,130] the exact ligands and function of TLR10 are still debated. Analysis of the ligand-binding pocket suggests that TLR10 may share the ligands with TLR2.[129] Studies have shown that TLR10 can form either homodimers or heterodimers with other TLRs. While the function of heterodimerized TLR10 remains unclear, homodimerization of TLR10 is known to induce the production of the anti-inflammatory cytokine IL-1Ra via the PI3K/Akt pathway.[131]

TLR11, TLR12, and TLR13

TLR11, TLR12, and TLR13 are additional members of the TLR family that have been found in mice, but do not have known human homologues. As such, there has been less study of these receptors and no studies of their use as cancer vaccine adjuvants.

Investigations to date have shown that these three receptors are predominantly expressed in intracellular organelles in immune cells, such as macrophages and DCs.[132–134] All three receptors form homodimers, and a heterodimer between TLR11/TLR12 has been reported.[133–135] Profilin serves as a major ligand for TLR11 and TLR12, but flagellin also has reported to activate TLR11.[133,136] Activation of TLR13 requires an unmethylated motif present in the large ribosomal subunit of bacterial RNA (23S rRNA).[137] Similar to other TLRs, all three receptors recruit MyD88 upon activation, which results in activation of DCs and production of IL-12.[135,138,139]

Preclinical approaches using TLR agonists as cancer vaccine adjuvants

TLR2

Animal model investigations have shown that TLR2 agonists can improve the efficacy of cancer vaccines. In a study conducted by Zhang et al., mice were intracranially implanted with GL261 glioma cells and received adoptive transfer of tumor-specific T cells followed by four injections of a TLR2 agonist, bacterial lipoprotein (BLP).[140] Compared to the control group treated with tumor-specific T cells alone, the inclusion of BLP significantly improved the survival with complete tumor regression observed in 6 of 18 mice. Tumor analysis identified an increased CD8+ T-cell infiltration as well as an increased ratio of IFNγ+ CD8+ T cells with BLP treatment. In addition, the mice that survived had acquired anti-tumor immunity as evidenced by the absence of tumor development when re-challenged with the same tumor cells. In a separate study by Zahm et al., Pam3CSK4, a TLR2 agonist, was used as an adjuvant in an ovalbumin-expressing E.G7 tumor model.[141] OT-1 CD8+ T cells were adoptively transferred to E.G7 tumor-bearing mice, and the mice were vaccinated with subcutaneous injection of SIINFEKL peptide and co-administered Pam3CSK4. The addition of Pam3CSK4 significantly suppressed tumor growth compared to the group treated with SIINKEFL alone. The analysis of tumor-infiltrating lymphocytes identified increased CD8+ T-cell infiltration in response to Pam3CSK4.

To further improve the efficacy of cancer vaccines with TLR2 agonists, more recent studies have focused on the modification and bioconjugation of TLR2 agonists to vaccine antigens. For example, Renaudet et al. developed two different cancer vaccines, HER-GLP-1 and – 2, by conjugating a HER2-specific peptide and a TLR2 agonist, PAM.[142] Both conjugates showed effective uptake by DCs, resulting in the upregulation of MHC-II, CD80, and CD86 expression as well as IL-12 and TNFα secretion by the DCs, compared to the group stimulated with the peptide alone. Weekly subcutaneous immunization of the conjugates in NT2 tumor-bearing mice decreased the tumor volume and improved the survival rate compared to the control groups. In a similar study by Shi et al., 12 different vaccine-TLR2 conjugates were developed with a MAGE-A1-specific peptide vaccine and TLR2 agonist (N-Ac PamCS-M-1 ~ 12).[143] All conjugates were more effectively taken up by dendritic cells and upregulated the secretion of IL-12, IL-6, and TNFα compared to groups where DCs were incubated with the peptides and TLR2 agonist. Two conjugates, N-Ac PamCS-M-6 and N-Ac PamCS-M-7, were able to increase the expression of CD86 and CD11c in DCs compared to the group stimulated with the mixture of peptide and TLR2 agonist. Treatment of MCF-7 tumor-bearing nude mice showed that the N-Ac PamCS-M-6 conjugate significantly suppressed tumor growth when the mice were subcutaneously immunized with a DC-T-cell mixture preconditioned with the conjugate, compared to groups with the mixture of cells pre-conditioned only with the peptide.

It is clear from these studies that TLR2 agonists can improve vaccine-mediated anti-tumor immunity. In addition, the conjugation of TLR2 agonist with peptide may be a promising strategy to enhance the immunogenicity of peptide antigens. However, more studies are necessary to investigate the possible synergy using TLR2 agonists with other vaccine approaches, such as protein-, DNA-, and RNA-based cancer vaccines.

TLR3

Since TLR3 stimulation induces type-1 interferon responses, several TLR3 agonists have been investigated as promising adjuvants for cancer vaccines. In one study, vaccination with tumor lysate and poly-IC12U promoted the maturation of DCs with increased IL-12 secretion which was demonstrated to expand tumor-specific CD8+ T cells.[144] In a study reported by Zhu et al., poly-ICLC promoted a vaccine-mediated anti-tumor response in a murine glioma model.[145] Specifically, GL261 tumor-bearing mice were subcutaneously immunized with three different peptide epitopes (mEphA2, hgp100, mTRP-2), along with intramuscular injection of poly-ICLC. The combination of vaccines with poly-ICLC significantly improved the infiltration of antigen-specific CD8+ T cells in CNS tumor sites compared to vaccine-alone or poly-ICLC-alone groups. The addition of poly-ICLC improved survival (9 of 15 mice), compared to groups immunized with peptides alone (3 of 10 mice) or poly-ICLC (0 of 10) alone.

ARNAX, a mimic for double-stranded RNA, has also been shown to suppress tumor growth when used as an adjuvant in ovalbumin-expressing murine tumor models E.G7 and MO5.[146] Tumor-bearing mice were immunized with whole ovalbumin protein along with ARNAX, and the combination showed significant suppression of tumor growth in both tumor models. In the E.G7-OVA model, 40% of the mice treated with ovalbumin + ARNAX showed complete regression. Additionally, when surviving mice were re-challenged with the same tumor cell line, tumors failed to develop and an increase in effector memory CD8+ T cells was detected in the spleens, suggesting the development of a lasting anti-tumor immunity.[146]

In addition to the diverse modifications of TLR3 agonists, recent studies have also focused on delivering traditional TLR3 agonists such as poly(I:C) with different biomaterials. For example, Song et al. developed a polypeptide-hydrogel-based vaccine for melanoma by combining tumor cell lysates, poly(I:C), and self-assembled poly-valine hydrogel.[147] The inclusion of poly(I:C) further increased the ratio of DCs expressing CD80, CD86, and MHC-II

compared to the hydrogel vaccine without TLR3 agonist. In the B16 melanoma tumor model, mice subcutaneously immunized with poly(I:C)-containing hydrogel vaccine suppressed tumor growth rates and tumor weights compared to the group treated with the vaccine alone. In a similar study by Wang et al., poly(I:C) was incorporated into a cationic liposome (DOTAP)-based vaccine with tumor cell lysates to generate a PIC-DOTAP liposome complex (PDLC).[148] Splenocytes from healthy mice immunized with PDLC showed a 150-fold increase in the production of IFNγ upon *in vitro* re-stimulation with the peptides, compared to the group immunized with poly(I:C) or DOTAP alone. The complex also showed potent anti-tumor immunity in the Hepa1–6 murine tumor model; subcutaneous immunization of tumor-bearing mice with PDLC showed better tumor suppression with increased tumor infiltration by CD8+ T cells and NK cells, compared to the groups immunized with poly(I:C) or DOTAP alone.[148]

These studies demonstrate that TLR3 agonists can potentiate vaccine-mediated anti-tumor responses. In addition, improved delivery methods may further improve the efficacy of TLR3 agonists as vaccine adjuvants.

TLR4

TLR4 agonists have been extensively studied as cancer vaccine adjuvants. For example, Vindevogel et al. treated ovarian ID8 tumor-bearing mice with a vaccine composed of DCs and tumor cell lysates, delivered with an intraperitoneal injection of LPS.[149] While inclusion of LPS failed to suppress tumor growth or improve the survival of mice, the presence of LPS facilitated the infiltration of tumors by NK cells and suppressed Treg infiltration. Davis et al. formulated a GM-CSF-secreting whole tumor cell vaccines (GVAX) with LPS, and tested its therapeutic efficacy in three different murine tumor models (B16, SCCFVII/SF, and CT26).[150] In comparison with the GVAX-treated groups, LPS-GVAX significantly suppressed tumor progression in all models. Moreover, 40% of CT26 tumor-bearing mice treated with LPS-GVAX showed significant tumor regression, and mice re-challenged with the tumor cells proved to be immune to the tumors. In a separate study by Shi et al., a thermosensitive hydrogel was utilized to deliver a vaccine comprised of LPS and a truncated fibroblast growth factor peptide (tbFGF).[151] The complex enhanced both antibody- and cell-mediated immune responses toward the peptide. Increased serum bFGF-specific antibodies were detected in mice immunized with the complex, and isolated CD8+ T cells from immunized mice slightly improved *in vitro* tumor cell lysis with the upregulation of IFNγ and IL-4 secretion.

Recent studies have also reported the efficacy of synthetic TLR4 agonists as vaccine adjuvants, such as MPLA, by incorporating the agonists in nanostructure-based cancer vaccines.[152,153] In a study published by Hamdy et al., poly (lactic-co-glycolic acid) (PLGA) nanoparticles were formulated with a melanoma antigen, a peptide derived from tyrosinase-related protein 2 (TRP2), and TLR4 agonist, 7-acyl lipid A.[152] Although TRP2 alone was a poorly immunogenic antigen, the formulated nanoparticle helped overcome tolerance to this tumor-associated antigen and induced robust anti-tumor effects compared to control groups. Activated TRP2-specific CD8+ T cells also showed elevated expression of IFNγ. In a separate study, Cheng et al. developed a biohybrid liposome which served as a cancer vaccine, named vacosome, by reconstructing plasma membranes from 4T1 cells, MPLA, and a lipid matrix consisting of egg phosphatidylcholine (EPC), DSPE-PEG-2000 and cholesterol.[153] Subcutaneous immunization with the vacosome enhanced the maturation of bone marrow-derived dendritic cells (BMDCs), along with upregulation of serum level of IL-12 and TNFα in mice. Furthermore, mice previously immunized with vacosome and then challenged with 4T1 tumor cells showed significantly slower tumor growth with increased numbers of effector memory CD8+ T cells, compared to groups treated with either MPLA or the antigen-liposome complex alone.

TLR7/8

Imiquimod is a TLR7 agonist that has been approved as a topical monotherapy for basal cell and squamous cell carcinoma *in situ*.[154] Studies have demonstrated that imiquimod can have direct effects on tumor cells leading to apoptosis, as well as activate favorable dermal cellular immunity. Several preclinical studies have shown that TLR7/8 agonists can also improve the efficacy of cancer vaccines. Ma et al. examined the contribution of TLR7 agonists, imiquimod and gardiquimod, to DC-based anticancer vaccines.[155] Specifically, a DC vaccine was developed by co-incubation of DCs and B16 tumor cell lysates for 16 h, and B16 melanoma-bearing mice were immunized with intravenous infusion of DCs, along with peritumoral injection of the TLR7 agonists. The inclusion of imiquimod showed similar tumor suppression as the DC vaccine alone, but gardiquimod significantly improved vaccine-mediated suppression of tumor growth. In a separate study by Stickdorn et al., a vaccine was formulated using a pH-degradable nanogel with ovalbumin protein and a TLR7/8 agonist, IMDQ.[156] BMDCs pulsed with this nanogel facilitated the proliferation of OT-1 T cells, and increased serum level of TNFα and IFNγ, compared to the group where DCs were pulsed with the antigen and IMDQ-nanogel separately. In addition, vaccination of ovalbumin-expressing MC38 or B16-F10 tumor-bearing mice with intravenous injection of IMDQ-containing nanogel showed antigen-specific anti-tumor responses. Tumor growth of ovalbumin-expressing MC38 or B16-F10 tumors was significantly suppressed compared to native tumors (not expressing ovalbumin).

Similar to TLR2, recent studies with TLR7/8 agonists have also focused on the modification or bioconjugation of TLR7/8 agonists to vaccine antigens to enhance the vaccine-mediated anti-tumor response. For example, Chi et al. synthesized TLR7 agonist SZU-106 and developed a whole tumor cell vaccine, Aza-BFcell-106, by chemically linking B16-F10 melanoma cells to SZU-106.[157] Compared to SZU-106, *in vitro* stimulation of BMDCs with Aza-BFcell-106 showed upregulation of IL-6 and TNFα secretion. Aza-BFcell-106 stimulation also increased the expression of CD80 and CD86 on BMDCs compared to the whole-cell vaccine without SZU-106. In a similar study by

Song et al., a nanoparticle vaccine termed TLR7/8a-epitope was developed by conjugating imiquimod with peptide antigen epitopes derived from Sur, MAGE-1, and gp100.[158] Conjugation of peptide vaccine with imiquimod showed improved retention of the vaccine inside lymph nodes and significantly increased the number of CD8+ T cells in the lymph node, compared to mice immunized with a mixture of the antigen and imiquimod. Subcutaneous immunization of B16 melanoma tumor-bearing mice with the TLR7/8a-epitope showed significant tumor suppression and an increase in CD8 + T-cell infiltration, compared to a group immunized with the mixture of antigen and imiquimod.[158] Lynn et al. also developed a peptide-based vaccine, termed SNP-7/8a, by conjugating peptides from a large pool of neoantigens with TLR7/8 agonists, chemically programmed to self-assemble as nanoparticles.[159] Subcutaneous immunization of mice and non-human primates with SNP-7/8a was able to generate and sustain long-lasting antigen-specific CD8+ T cells with increased IFNγ expression. Mice inoculated with TC-1 lung cancer or B16-F10 melanoma showed suppressed tumor growth following immunization with SNP-7/8a, compared to groups immunized with the conjugate without antigen.[159]

Taken together, these studies have shown that TLR7/8 agonists are able to improve the efficacy of cancer vaccines, likely by inducing the activation and maturation of DCs. The efficacy of these TLR7/8 agonists may be further improved with novel conjugate and delivery approaches.

TLR9

TLR9 activation has also been shown to activate cytotoxic T cells and improve vaccine-mediated anti-tumor immunity. In a study by Kim et al., splenocytes from mice vaccinated with MUC1- and GD3-derived peptides were re-stimulated with 19 different adjuvants.[160] TLR9 agonist CpG ODN induced the greatest Th1-biased immune response along with the highest IFNγ secretion compared to the other adjuvants. In a separate study, Sin et al. co-immunized mice with 20 μg of the TLR9 agonist ODN1826 and 20 μg of $Trp2_{180–188}$ peptide.[161] Immunizations were given subcutaneously to B16 melanoma-bearing mice on a weekly basis, up to 3 weeks. The combination of Trp2 peptides and CpG-ODN showed significant tumor growth inhibition over the time points measured following treatment, compared to mice treated with either Trp2 peptides or CpG-ODN alone.

Like other TLR agonist adjuvants, recent studies of TLR9 agonists as cancer vaccine adjuvants have focused on novel delivery strategies to further improve the uptake of the agonists. For example, Shi et al. formulated nano-complex vaccines with self-assembling peptide-cationic R8 epitope conjugates with CpG ODN1826.[162] All nano-complexes with the OVA MHC I-restricted epitope (SIINFEKL) robustly activated purified BMDCs with increased expression of CD86, CD40, and MHC-antigen complex *in vitro*. Moreover, weekly immunization of mice bearing B16 tumors expressing ovalbumin with nano-complexes containing CpG showed significant suppression of tumor growth and increased infiltration of CD4 + and CD8+ T cells, compared to mice immunized with the nano-complex assembled without CpG.[162] In a similar study published by Chen et al., a vaccine was designed to target stem-cell-like cancer cells by chemically conjugating an OCT4-specific peptide with carrier protein keyhole limpet hemocyanin (KLH) and a TLR9 agonist.[163] Vaccination of F9 teratocarcinoma-bearing mice led to slower tumor growth and longer survival, compared to mice treated with TLR9 agonist or OCT4-KLH alone. Analysis of the tumor showed increased infiltration of CD8+ T cells, and secretion of pro-inflammatory cytokines such as IL-2, IL-6, and IL-12. In a separate study reported by Zaks et al., the addition of TLR9 agonists to liposome-based vaccines similarly improved vaccine-mediated anti-tumor responses.[164] Peptide or protein antigens (Ovalbumin, trp2, or gp61) and TLR9 agonists (CpG or pMB75.6 non-coding plasmid) were loaded within cationic liposomes. C57BL/6 mice receiving intraperitoneal delivery of vaccines formulated with TLR9 agonists showed increased splenic antigen-specific CD4+ and CD8+ T cells. Vaccines containing TLR9 agonists also improved cross-priming of the CD8+ T-cell response, independent of CD4+ T cells, and generated long-lived effector memory T cells which persisted for 3 months after vaccination. Immunization of B16 tumor-bearing mice with the trp2-specific, CpG-containing vaccine, by either subcutaneous or intraperitoneal vaccination routes, slowed the growth of tumors compared to groups immunized with the trp2 peptide-complexed liposomes alone or trp2-pulsed DCs.[164]

Summary

Pre-clinical studies have demonstrated that TLR agonists can potentiate vaccine-mediated anti-tumor immune responses. Many of these studies showed that TLR agonists can induce maturation of DCs along with secretion of proinflammatory cytokines, resulting in robust activation of T cells that can mediate anti-tumor activity. Consequently, more recent studies of TLR agonists as vaccine adjuvants focused on the co-delivery of TLR agonists and vaccines together by diverse methods, including conjugation with the vaccine antigen, or formulation with liposomes or other nano-scale structures. Given the results from multiple recent studies with these additional delivery strategies, co-delivery of vaccine and TLR agonists seems advantageous to improve the vaccine-mediated anti-tumor immune responses. However, certain aspects still have not been fully explored in preclinical studies. Notably, there is a lack of studies comparing the downstream effects of activating different TLRs, and even different agonists acting on a defined TLR (such as different classes of TLR9 agonists), on the downstream function of antigen-specific T cells. For example, we have previously found that activation of specific TLR, notably TLR1/2, TLR7/8 and TLR9 at the time of CD8+ T cell activation leads to a reduction in PD-1 expression on the CD8 + T cells and greater anti-tumor activity of these CD8+ T cells.[141] However, the effects of TLR activation on various aspects of downstream T cell function or memory development have not been well characterized. Further such studies could enable the development of vaccines in which CD8+ T cells, or CD4+ T cells, with different function or persistence might be desirable. In addition, this could lead to the use of specific combinations of TLR agonists as adjuvants, based on

Table 2. Summary of clinical trials with TLR agonists as cancer vaccine adjuvants.

TLR	Agonist	Vaccine description	Vaccine type	Target Cancer	No. of Clinical Trial	Phase	Response	References
TLR3,7	PolyICLC IMQ	Tumor lysate and DC vaccine	DC	Glioblastoma	NCT00068510	Phase I	Activation of DCs and tumor antigen specific T cells	[166]
TLR3,7	PolyICLC Resquimod	Tumor lysate-pulsed DCs	DC	Brain tumors	NCT01204684	Phase II	Ongoing study	N/A
TLR3	PolyICLC	NY-ESO-1 vaccine	Protein	Ovarian cancer	NCT00616941	Phase I	Increased antigen specific antibodies, CD4+ and CD8+ T cells	[167]
TLR3	PolyICLC	NY-ESO-1 vaccine	Protein	Melanoma	N/A	Phase I/II	robust NY-ESO-1 specific humoral and cellular immune responses	[168]
TLR3	PolyICLC	Melanoma specific neoantigen peptide vaccine	Peptide	Melanoma	NCT04364230	Phase I/II	Ongoing study	N/A
TLR3	PolyICLC	Multipeptide vaccine	Peptide	Breast cancer	NCT01532960	Phase I	Moderate induction of antigen specific CD8+ T cells	[169]
TLR3,4	PolyICLC LPS	Melanoma peptide vaccines	Peptide	Melanoma	NCT01585350	Unknown (Phase I or II)	Induction of CD8+ T-cell immune response with high OS/DFS	[170]
TLR4	GLA	Recombinant NY-ESO-1 vaccine	Protein	Solid tumors expressing NY-ESO-1	NCT02015416	Phase I	Increased antigen specific antibodies, CD4+ and CD8+ T-cell responses	[171]
TLR4	MPLA	Recombinant baculovirus-derived KSA (Ep-CAM) vaccine	Protein	Colorectal cancer	N/A	Phase I	Induction of KSA specific antibodies	[172]
TLR4	GLA	Melanoma-associated peptide(MART-1a) vaccine	Peptide	Melanoma	NCT02320305	Phase I	Increased MART-1a specific T cells	[173]
TLR7	IMQ	Flt3L and tumor antigen vaccine	Peptide	Metastatic melanoma	N/A	Phase I	Increased peptide-specific CD8+ T-cell response	[174]
TLR7	IMQ	Recombinant NY-ESO-1 vaccine	Protein	Melanoma	NCT00142454	Phase I	Increased humoral and cellular immune response specific to NY-ESO-1	[175]
TLR7	IMQ	Multipeptide cancer vaccine	Peptide	Melanoma	NCT01264731	Phase I	Activation and tumor-infiltration of CD8+ T cells and NK cells	[176]
TLR7	Resquimod	NY-ESO-1 vaccine	Protein	Melanoma	NCT00821652	Phase I	Induction of antigen specific antibodies and CD4+ T-cell responses	[177]
TLR7	Resquimod	gp100 and MAGE-3 peptide vaccine	Peptide	Melanoma	NCT00960752	Phase II	Activation of pDCs and regression of in-transit melanoma metastasis	[178]
TLR9	CpG7909	Melanoma antigen vaccine	Peptide	Melanoma	N/A	Phase I	Induction of NY-ESO-1 specific T-cell responses	[179]
TLR9	CpG7909	URLC210–177 and TTK567 peptide vaccine	Peptide	Esophageal squamous cell carcinoma	NCT00669292	Phase I/II	Induction of antigen specific CD8+ T-cell responses	[180]

their different actions. For example, we have recently found that combining TLR agonists, notably TLR3 and TLR9 agonists, could act on professional antigen-presenting cells to secrete both type I IFN and IL-12, thus improving the anti-tumor function of activated CD8+ T cells.[165] Further studies using TLR agonist combinations, and delivery approaches using these different TLR agonists, are needed.

Clinical studies using TLR agonists as cancer vaccine adjuvants

Numerous clinical studies using TLR agonists as cancer vaccine adjuvants have been completed, or are currently underway, as indicated in Table 2. Collectively, these have demonstrated that TLR agonists are safe and promising agents as cancer vaccine adjuvants using different types of vaccines.

Many cancer vaccine clinical trials using TLR agonists have used protein-based vaccines, and notably vaccines targeting New York esophageal squamous cell carcinoma-1 (NY-ESO-1) protein. In a study by Pavlick et al., patients with high-risk resected melanoma were treated with a vaccine composed of NY-ESO-1 and poly-ICLC.[168] Patients received subcutaneous vaccination of 100 μg NY-ESO-1 protein with 1.4 mg poly-ICLC, and with or without montanide (oil-based depot adjuvant). Vaccine cycles were repeated every 3 weeks for a total of 4 cycles. The vaccine was well-tolerated without any treatment-related grade 3/4 adverse events. After vaccination, the number of patients whose NY-ESO-1-specific CD4+ T cells were detected in PBMCs had increased (83%) compared to pre-immunization (41%), without additional montanide. CD8 + T cell responses were also detectable after immunization. Another clinical study was reported by Mahipal et al., who investigated the effect of a TLR4 agonist, GLA-SE, as a cancer vaccine adjuvant.[181] Twelve patients with solid tumors expressing NY-ESO-1 were treated with 250 μg of G305, a recombinant NY-ESO-1 protein vaccine combined with GLA-SE (0 to 10 μg). The vaccine was administered intramuscularly on days 0, 21, and 42. The vaccine regime was well-tolerated and only grade 1 or 2 adverse effects were detected, including soreness at the injection site. With the vaccination, 75% of patients developed NY-ESO-1-specific antibodies. Overall, T-cell responses were augmented, with CD4+ T-cell responses detectable in 44.4% of patients (4 of 9) and CD8+ T-cell responses in 36.9% of patients (4 of 11). A similar vaccination study with the NY-ESO-1 antigen was conducted by Adams et al. using imiquimod (IMQ, TLR7 agonist) as a vaccine adjuvant.[175] Recombinant, full-length NY-ESO-1 protein was administered to melanoma patients intradermally into an IMQ-preconditioned skin site with additional topical application of IMQ. Cycles were repeated every three weeks for a total of 4 injections. Vaccine regimens were well tolerated in all patients. The IMQ and NY-ESO-1 combination activated APC populations and improved their infiltration at the vaccination site. This was associated with activation of humoral and cell-mediated immunity. Specifically, PBMCs collected from patients after vaccination showed increased CD4+ T-cell responses compared to pre-vaccination (7 of 9 vs. 0 of 9 patients). Analysis of vaccine site skin biopsies demonstrated an increased number of IFNγ-secreting NY-ESO-1-specific CD4+ T cells, monocytes, macrophages, mDCs, and NK cells than in untreated control skin biopsies from the same patients. Sabado et al. similarly examined topical application of the TLR7/8 agonist resiquimod, combined with subcutaneous administration of 100 μg of full-length NY-ESO-1 plus montanide, in patients with high-risk melanoma.[182] Patients were given 100 μg NY-ESO-1 protein emulsified in 1.25 mL montanide (day 1) subcutaneously, followed by topical application of placebo gel or 1000 mg of 0.2% Resiquimod gel on days 1, 3, and 5. The vaccine-induced NY-ESO-1 specific humoral responses with high titers of antigen-specific antibodies, and increased CD4+ T-cell responses, in the majority of patients compared to placebo gel (83% vs. 73%). In addition, CD8+ T-cell responses were only detected with the inclusion of resiquimod (3 of 12), while none of the patients immunized with the NY-ESO-1 vaccine alone showed CD8+ responses (0 of 8).[182] These protein-based cancer vaccine studies, focused on NY-ESO-1 as the tumor antigen, highlight the use of TLR agonists as adjuvants. Collectively, these studies suggest that the inclusion of TLR agonists can improve antigen-specific CD8+ T-cell responses and CD4+ T-cell responses, without severe side effects.

Along with protein-based vaccines, many reports have also demonstrated that TLR agonists can improve the efficacy of peptide-based vaccines. In a study by Sabbatini et al., the immunogenicity of overlapping long peptides (OLP) from NY-ESO-1 was examined in combination with montanide and poly-ICLC.[183] Twenty-eight patients with advanced ovarian cancer were enrolled to three cohorts: Cohort 1 received NY-ESO-1 OLP only (1 mg), Cohort 2 received 1 mg NY-ESO-1 OLP with 0.5 mL montanide-ISA-51, and Cohort 3 received NY-ESO-1 OLP (1 mg), montanide-ISA-51 VG (1 mL), and Poly-ICLC (1.4 mg). Vaccines were subcutaneously administered on weeks 1, 4, 7, 10, and 13 with the final study safety assessment on week 16. The vaccine was well-tolerated and NY-ESO-1–specific CD4+ T cells were detected in all patients. Antigen-specific CD8+ T cells were detected in 8 of 13 (62%) patients vaccinated with OLP and montanide, and in 10 of 11 (91%) patients vaccinated with OLP, montanide, and ploy-ICLC. The frequency and polyclonality of NY-ESO-1–specific antibodies in patients vaccinated with OLP, montanide, and poly-ICLC were higher than in the group of patients treated with OLP and montanide (91% vs. 46%). In a similar vaccination study using imiquimod as an immune modulating agent, Mauldin et al. treated 4 patients with metastatic melanoma with topical IMQ delivered to sites melanoma plus subcutaneous and intradermal injection of a multi-peptide cancer vaccine (100 μg each of 12 melanoma-specific peptides and a tetanus toxoid-derived helper peptide).[184] Vaccines were administered in two cycles: days 1, 8, 15 for the first cycle, then days 36, 57, 78 for the second cycle. 5% imiquimod cream was applied once a day, 7 days per week for 12 weeks, to selected superficial skin metastases. The vaccine regimen was well tolerated with mild adverse effects and without any dose-limiting toxicities, but the study was too small for meaningful clinical assessments. Three of four patients developed *ex vivo* T-cell responses with increased IFNγ secretion after vaccination and IMQ treatment. The number of antigen-specific CD8 + T cells was also increased in 2 of 4 patients upon treatment.

Gene expression analysis of tumors treated with the vaccine and IMQ identified up-regulation of genes related to adaptive immunity (TCR, IFNγ, and IL-2 signaling) and T-cell/NK cell recruitment (IL2RG, IL2RB, CD3G, CD2, SLAMF6, and CD38). In another peptide-based vaccine trial, Speiser et al. reported the use of CpG7909, one of the first developed TLR9 agonists, as a vaccine adjuvant.[179] Eight melanoma patients received 4 vaccinations subcutaneously in monthly intervals with CpG7909 (500 μg), melanoma antigen A (Melan-A) peptide (100 μg), and incomplete Freund's adjuvant (IFA, 300 ul). Vaccination with CpG increased the frequency of antigen-specific T cells, to 0.07–3.00% of circulating CD8+ T cells, approximately ten times higher than before vaccination ($p < 0.01$). A control group of 8 melanoma patients treated similarly without CpG demonstrated significantly ($p < 0.01$) lower post-vaccination T-cell frequencies (0.13% ± 0.11%). Expanded T cells were mostly effector memory cells, and demonstrated antigen-specific secretion of IFNγ, granzyme B, and perforin.[179] While these were all small, pilot clinical trials, these peptide vaccine-based trials demonstrated similar findings to protein-based vaccines, suggesting that the immunological efficacy of these vaccines could similarly be augmented when combined with TLR agonists.

On the other hand, while the pilot studies above focused on the safety and immune response of vaccines delivered with TLR adjuvants, several clinical studies have reported low clinical efficacy, and/or low immunogenicity, when TLR agonists were combined with protein- or peptide-based vaccines. In a phase I clinical study conducted by Dillon et al., a vaccine containing nine different breast-cancer-associated peptides (100 μg each of peptides derived from MAGE-A1, -A3, and -A10, CEA, NY-ESO-1, and HER2/neu) was combined with a TLR3 agonist (Poly-ICLC, 1 mg) and a helper peptide derived from tetanus toxin (200 μg).[169] Twelve patients with breast cancer were vaccinated both intramuscularly and intradermally on days 1, 8, 15, 36, 57, 78. Only grade 1 or 2 toxicities were detected, including mild injection site reactions or flu-like symptoms with fatigue, and there was no dose-limiting toxicity found. However, antigen-specific CD8+ T-cell responses were detected in only 2 of 11 (18%) patients treated. In another phase I clinical study by Neidhart et al., a combination of recombinant baculovirus-derived epithelial cell adhesion molecule (Ep-CAM, KSA) vaccine with MPLA adjuvant in a liposomal emulsion was evaluated for the treatment of metastatic colorectal cancer (mCRC) patients.[172] The vaccine was formulated with 100 μg of KSA and 200 μg of MPLA in a 1.0 ml liposomal oil-in-water emulsion and administered, with or without granulocyte-macrophage colony-stimulating factor (GM-CSF), to patients subcutaneously every 4 weeks. This KSA vaccine with MPLA was shown to be safe and tolerable, and elicited significant KSA-specific humoral immune responses (7 of 11). However, none of the patients in this trial showed a clinical response. Similarly in a trial reported by Grewal et al., 23 HLA-A2-expressing patients with stage II-IV melanoma received a peptide vaccine derived from a melanoma antigen (MART-1a, 0.5 mg) and with or without a TLR4 agonist (GLA-SE, 5 μg). Vaccines were administered as intramuscular injections with 21-day cycles and up to three vaccinations. Antigen-specific immune responses were monitored by the number of antigen-specific T cells in PBMCs. There was a trend toward increased immune response to MART-1a with the inclusion of TLR4 agonist (70% vs. 63.6%), but it did not reach statistical significance. Furthermore, there was no difference in immune response to the wild-type MART-1 with TLR4 agonists (40%), compared to the control group (54.5%). Finally, in a clinical study conducted by Ishikawa et al., vaccines using peptides derived from two cancer-testis antigens (LY6K and TTK) were combined with the TLR9 agonist CpG7909 for the treatment of patients with metastatic esophageal squamous cell carcinoma.[180] Nine patients were vaccinated on days 1, 8, 15, and 22 with peptide vaccines LY6K–177(1 mg), TTK-567 (1 mg), and CpG7909 adjuvant (0.02 or 0.1 mg/kg). Patients who received CpG7909 had increased serum levels of IFNα and higher frequencies of peptide-specific CD8+ T cells in the peripheral blood. However, no patient in the trial showed a partial or complete response.[180]

In summary, published clinical studies demonstrate that TLR agonists can exert immunostimulatory effects in patients. In general, clinical trials combining TLR agonists with protein- or peptide-based vaccines demonstrated increased antigen-specific CD4+ and CD8+ T cell immune responses. However, these have been small studies, not designed to evaluate clinical efficacy, and hence larger studies are needed. Certainly, results from other vaccine trials have demonstrated that increased T-cell response is not necessarily associated with greater anti-tumor efficacy,[185] and hence trials to evaluate clinical endpoints are needed. In addition, most of the trials to date have been conducted in patients with melanoma. While this has been useful to enable some comparisons across clinical trials, and in many cases using the same antigens (e.g., NY-ESO-1), ultimately it will be important to evaluate the use of TLR agonists as adjuvants for other types of cancers, and in combination with agents that target the immunosuppressive tumor immune microenvironment. As previously highlighted, it is widely believed that better activation of T cells by vaccination alone may be insufficient for optimal tumor control, and that combination approaches are needed that can concurrently modify the immunosuppressive tumor microenvironment. For example, trials using vaccines with PD-1 blockade have demonstrated more efficacy, and this approach has not yet been explored with the addition of TLR adjuvants in preclinical or clinical models. Moreover, the choice of vaccine target may be important. For example, the E75 peptide vaccine targeting HER-2/neu for patients with breast cancer has demonstrated immunogenicity and clinical effects in larger clinical trials.[186] And the sipuleucel-T vaccine targeting prostatic acid phosphatase has been FDA-approved as a treatment for advanced prostate cancer.[2] However, there have been no trials to date using TLR agonist adjuvants for these diseases or vaccine targets for which modest clinical responses have been observed following anti-tumor vaccine monotherapy treatments, or using these advanced stage vaccines. Lastly, given the rapid development and deployment of mRNA vaccines for infectious diseases, and early results suggesting their efficacy as anti-cancer vaccines,[187] it will be important to evaluate the use of TLR agonists with other vaccine platforms, notably mRNA and DNA-based vaccine platforms. RNA and DNA are known

to activate specific TLR, and it has been proposed that part of the mechanism for their immunogenicity as vaccines is by activating different TLR.[188] Hence, nucleic acid vaccines might be strategically combined with specific TLR agonists. Collectively, these combined studies will provide a much broader understanding to select optimal TLR agonists to use as cancer vaccine adjuvants, along with agents targeting the tumor immunosuppressive microenvironment, for more effective therapies.

Concluding remarks

Cancer vaccines have great potential to improve the treatment of cancer patients. However, cancer vaccines used alone have demonstrated modest outcomes, suggesting the need to combine these with other agents, including adjuvants that can modulate the function of the resulting immune response. Numerous pre-clinical studies have shown that TLR agonists can facilitate the vaccine-mediated anti-tumor response by supporting the activation of antigen-specific CD8+ T cells in various tumor models. This is mediated by the maturation of various immune cells, including dendritic cells, and facilitated expression of co-stimulatory molecules and inflammatory cytokines. Recent pre-clinical approaches using TLR agonists has focused on further improving the functionality of vaccines with TLR agonists, by modifying their chemical structures or combining them with novel delivery strategies. Positive results from recent studies with these novel delivery methods indicate the importance of co-delivery of vaccine and TLR agonists to improve vaccine-mediated anti-tumor responses. Nonetheless, further research is required to compare the impact of distinct TLR activation when used with different vaccine approaches, as well as to assess the effects of different TLR agonists when used alone or in combination on the downstream function and memory of CD4+ and CD8+ T cells.

Clinical trials have shown the safety of TLR agonists as vaccine adjuvants, as well as their ability to augment antigen-specific T cells. However, clinical studies to date have been small trials with limited evaluation of clinical endpoints. Larger studies using different vaccine approaches, in combination with agents targeting the immunosuppressive tumor microenvironment, and used in different disease settings, will be necessary. Nevertheless, TLR agonists are viable adjuvants for cancer vaccines, and we expect that improvements in cancer vaccine efficacy will be achieved with a further evaluation of TLRs and TLR agonists.

Disclosure statement

DGM has ownership interest, has received research support, and serves as consultant to Madison Vaccines, Inc which has licensed material described in this manuscript. The other authors have no relevant potential conflicts of interest.

Funding

Grant support was provided by the NIH [P50 CA269011 and P01 CA250972]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

ORCID

Douglas G. McNeel http://orcid.org/0000-0003-1471-6723

References

1. Esfahani K, Roudaia L, Buhlaiga N, Del Rincon SV, Papneja N, Miller WH. A review of cancer immunotherapy: from the past, to the present, to the future. Curr Oncol. 2020;27(12):87–97. doi:10.3747/co.27.5223.
2. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med. 2010;363(5):411–22. doi:10.1056/NEJMoa1001294.
3. Nakamura K, Smyth MJ. Myeloid immunosuppression and immune checkpoints in the tumor microenvironment. Cell Mol Immunol. 2020;17(1):1–12. doi:10.1038/s41423-019-0306-1.
4. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol. 2007;25(1):267–96. doi:10.1146/annurev.immunol.25.022106.141609.
5. Gajewski TF, Meng Y, Blank C, Brown I, Kacha A, Kline J, Harlin H. Immune resistance orchestrated by the tumor microenvironment. Immunol Rev. 2006;213:131–45. doi:10.1111/j.1600-065X.2006.00442.x.
6. Puig-Saus C, Sennino B, Peng S, Wang CL, Pan Z, Yuen B, Purandare B, An D, Quach BB, Nguyen D, et al. Neoantigen-targeted CD8+ T cell responses with PD-1 blockade therapy. Nature. 2023;615(7953):697–704. doi:10.1038/s41586-023-05787-1.
7. Bobisse S, Foukas PG, Coukos G, Harari A. Neoantigen-based cancer immunotherapy. Ann Transl Med. 2016;4(14):262. doi:10.21037/atm.2016.06.17.
8. Zahm CD, Colluru VT, McNeel DG. Vaccination with high-affinity epitopes impairs antitumor efficacy by increasing PD-1 expression on CD8(+) T cells. Cancer Immunol Res. 2017;5(8):630–41. doi:10.1158/2326-6066.CIR-16-0374.
9. Rekoske BT, Olson BM, McNeel DG. Antitumor vaccination of prostate cancer patients elicits PD-1/PD-L1 regulated antigen-specific immune responses. Oncoimmunology. 2016;5(6):e1165377. doi:10.1080/2162402X.2016.1165377.
10. McNeel DG, Eickhoff JC, Wargowski E, Zahm C, Staab MJ, Straus J, Liu G. Concurrent, but not sequential, PD-1 blockade with a DNA vaccine elicits anti-tumor responses in patients with metastatic, castration-resistant prostate cancer. Oncotarget. 2018;9(39):25586–96. doi:10.18632/oncotarget.25387.
11. McNeel DG, Eickhoff JC, Wargowski E, Johnson LE, Kyriakopoulos CE, Emamekhoo H, Lang JM, Brennan MJ, Liu G. Phase 2 trial of T-cell activation using MVI-816 and pembrolizumab in patients with metastatic, castration-resistant prostate cancer (mCRPC). J Immunother Cancer. 2022;10(3):10. doi:10.1136/jitc-2021-004198.
12. Carvalho T. Personalized anti-cancer vaccine combining mRNA and immunotherapy tested in melanoma trial. Nat Med. 2023;29:2379–80.
13. Iclozan C, Antonia S, Chiappori A, Chen DT, Gabrilovich D. Therapeutic regulation of myeloid-derived suppressor cells and immune response to cancer vaccine in patients with extensive stage small cell lung cancer. Cancer Immunol Immunother. 2013;62(5):909–18. doi:10.1007/s00262-013-1396-8.
14. Brezar V, Godot V, Cheng L, Su L, Lévy Y, Seddiki N. T-Regulatory cells and vaccination "pay attention and do not neglect them": lessons from HIV and cancer vaccine trials. Vaccines (Basel). 2016;4(3):30. doi:10.3390/vaccines4030030.
15. Llopiz D, Ruiz M, Silva L, Sarobe P. Enhancement of antitumor vaccination by targeting dendritic cell-related IL-10. Front Immunol. 2018;9. doi:10.3389/fimmu.2018.01923.
16. Marciani DJ. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. Drug Discov Today. 2003;8(20):934–43. doi:10.1016/S1359-6446(03)02864-2.

17. Dubensky TW, Reed SG. Adjuvants for cancer vaccines. Semin Immunol. 2010;22(3):155–61. doi:10.1016/j.smim.2010.04.007.
18. Zindel J, Kubes P. DAMPs, PAMPs , and LAMPs in immunity and sterile inflammation. Annu Rev Pathol. 2020;15(1):493–518. doi:10.1146/annurev-pathmechdis-012419-032847.
19. Srikrishna G, Freeze HH. Endogenous damage-associated molecular pattern molecules at the crossroads of inflammation and cancer. Neoplasia. 2009;11(7):615–28. doi:10.1593/neo.09284.
20. Janeway CA Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today. 1992;13(1):11–6. doi:10.1016/0167-5699(92)90198-G.
21. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637–50. doi:10.1016/j.immuni.2011.05.006.
22. O'Neill LAJ, Golenbock D, Bowie AG. The history of toll-like receptors — redefining innate immunity. Nat Rev Immunol. 2013;13(6):453–60. doi:10.1038/nri3446.
23. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to *drosophila* Toll. Proc Natl Acad Sci. 1998;95(2):588–93. doi:10.1073/pnas.95.2.588.
24. Takeda K, Akira S. Toll-like receptors. Curr Protoc Immunol. 2015;109(1):14.2.1–.2.0. doi:10.1002/0471142735.im1412s109.
25. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003;21(1):335–76. doi:10.1146/annurev.immunol.21.120601.141126.
26. Tomai MA, Vasilakos JP. TLR agonists as vaccine adjuvants. In: Baschieri S, editor. Innovation in vaccinology: from design, through to delivery and testing. Dordrecht: Springer; 2012. pp. 205–28.
27. Lou Y, Liu C, Lizée G, Peng W, Xu C, Ye Y, Rabinovich BA, Hailemichael Y, Gelbard A, Zhou D. et al. Antitumor activity mediated by CpG: the route of administration is critical. J Immunother. 2011;34(3):279–88. doi:10.1097/CJI.0b013e31820d2a05.
28. Gableh F, Saeidi M, Hemati S, Hamdi K, Soleimanjahi H, Gorji A, Ghaemi A. Combination of the toll like receptor agonist and α-galactosylceramide as an efficient adjuvant for cancer vaccine. J Biomed Sci. 2016;23(1):16. doi:10.1186/s12929-016-0238-3.
29. Sajadian A, Tabarraei A, Soleimanjahi H, Fotouhi F, Gorji A, Ghaemi A. Comparing the effect of toll-like receptor agonist adjuvants on the efficiency of a DNA vaccine. Arch Virol. 2014;159(8):1951–60. doi:10.1007/s00705-014-2024-4.
30. Domingos-Pereira S, Decrausaz L, Derré L, Bobst M, Romero P, Schiller JT, Jichlinski P, Nardelli-Haefliger D. Intravaginal TLR agonists increase local vaccine-specific CD8 T cells and human papillomavirus-associated genital-tumor regression in mice. Mucosal Immunol. 2013;6(2):393–404. doi:10.1038/mi.2012.83.
31. Dunne A, Marshall NA, Mills KH. TLR based therapeutics. Curr Opin Pharmacol. 2011;11(4):404–11. doi:10.1016/j.coph.2011.03.004.
32. Yang Y, Li H, Fotopoulou C, Cunnea P, Zhao X. Toll-like receptor-targeted anti-tumor therapies: advances and challenges. Front Immunol. 2022;13:1049340. doi:10.3389/fimmu.2022.1049340.
33. Flo TH, Halaas O, Torp S, Ryan L, Lien E, Dybdahl B, Sundan A, Espevik T. Differential expression of toll-like receptor 2 in human cells. J Leukoc Biol. 2001;69(3):474–81. doi:10.1189/jlb.69.3.474.
34. Brzezińska-Błaszczyk E, Wierzbicki M. Mast cell toll-like receptors (TLRs). Postepy Hig Med Dosw (Online). 2010;64:11–21.
35. Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, Buwitt-Beckmann U, Röschmann K, Jung G, Wiesmüller K-H. et al. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. J Leukoc Biol. 2007;83(3):692–701. doi:10.1189/jlb.0807586.
36. de Oliviera Nascimento L, Massari P, Wetzler L. The role of TLR2 in infection and immunity. Front Immunol. 2012;3:3. doi:10.3389/fimmu.2012.00079.
37. Beutler B, Jiang Z, Georgel P, Crozat K, Croker B, Rutschmann S, Du X, Hoebe K. Genetic analysis of host resistance: toll-like receptor signaling and immunity at large. Annu Rev Immunol. 2006;24(1):353–89. doi:10.1146/annurev.immunol.24.021605.090552.
38. Xu Y, Tao X, Shen B, Horng T, Medzhitov R, Manley JL, Tong L. Structural basis for signal transduction by the toll/interleukin-1 receptor domains. Nature. 2000;408(6808):111–15. doi:10.1038/35040600.
39. Won K, Kim SM, Lee SA, Rhim BY, Eo SK, Kim K. Multiple signaling molecules are involved in expression of CCL2 and IL-1β in response to FSL-1, a toll-like receptor 6 agonist, in macrophages. Korean J Physiol Pharmacol. 2012;16(6):447–53. doi:10.4196/kjpp.2012.16.6.447.
40. De Buck M, Berghmans N, Pörtner N, Vanbrabant L, Cockx M, Struyf S, Opdenakker G, Proost P, Van Damme J, Gouwy M. et al. Serum amyloid A1α induces paracrine IL-8/CXCL8 via TLR2 and directly synergizes with this chemokine via CXCR2 and formyl peptide receptor 2 to recruit neutrophils. J Leukoc Biol. 2015;98(6):1049–60. doi:10.1189/jlb.3A0315-085R.
41. Need AC, Goldstein DB. Next generation disparities in human genomics: concerns and remedies. Trends Genet. 2009;25(11):489–94. doi:10.1016/j.tig.2009.09.012.
42. Chang YL, Chen TH, Wu YH, Chen GA, Weng TH, Tseng PH, Hsieh SL, Fu SL, Lin CH, Chen CJt et al. A novel TLR2-triggered signalling crosstalk synergistically intensifies TNF-mediated IL-6 induction. J Cell Mol Med. 2014;18(7):1344–57. doi:10.1111/jcmm.12294.
43. Flynn CM, Garbers Y, Lokau J, Wesch D, Schulte DM, Laudes M, Lieb W, Aparicio-Siegmund S, Garbers C. Activation of toll-like receptor 2 (TLR2) induces interleukin-6 trans-signaling. Sci Rep. 2019;9(1):7306. doi:10.1038/s41598-019-43617-5.
44. Kaur A, Kaushik D, Piplani S, Mehta SK, Petrovsky N, Salunke DB. TLR2 agonistic small molecules: detailed structure–activity relationship, applications, and future prospects. J Med Chem. 2021;64(1):233–78. doi:10.1021/acs.jmedchem.0c01627.
45. Knapp S, von Aulock S, Leendertse M, Haslinger I, Draing C, Golenbock DT, van der Poll T. Lipoteichoic acid-induced lung inflammation depends on TLR2 and the concerted action of TLR4 and the platelet-activating factor receptor. J Immunol. 2008;180(5):3478–84. doi:10.4049/jimmunol.180.5.3478.
46. Müller-Anstett MA, Müller P, Albrecht T, Nega M, Wagener J, Gao Q, Kaesler S, Schaller M, Biedermann T, Götz Fp et al. Staphylococcal peptidoglycan co-localizes with Nod2 and TLR2 and activates innate immune response via both receptors in primary murine keratinocytes. PLOS ONE. 2010;5(10):e13153. doi:10.1371/journal.pone.0013153.
47. Frasnelli ME, Tarussio D, Chobaz-Péclat V, Busso N, So A. TLR2 modulates inflammation in zymosan-induced arthritis in mice. Arthritis Res Ther. 2005;7(2):R370. doi:10.1186/ar1494.
48. Campos MAS, Almeida IC, Takeuchi O, Akira S, Valente EP, Procópio DO, Travassos LR, Smith JA, Golenbock DT, Gazzinelli RT, et al. Activation of toll-like receptor-2 by Glycosylphosphatidylinositol anchors from a protozoan Parasite1. J Immunol. 2001;167(1):416–23. doi:10.4049/jimmunol.167.1.416.
49. Tapping RI, Tobias PS. Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. J Endotoxin Res. 2003;9(4):264–8. doi:10.1177/09680519030090040801.
50. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-κB by toll-like receptor 3. Nature. 2001;413(6857):732–8. doi:10.1038/35099560.
51. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu Y-J. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med. 2001;194(6):863–9. doi:10.1084/jem.194.6.863.
52. Tabiasco J, Devêvre E, Rufer N, Salaun B, Cerottini JC, Speiser D, Romero P. Human effector CD8+ T lymphocytes express TLR3 as a functional coreceptor. J Immunol. 2006;177(12):8708–13. doi:10.4049/jimmunol.177.12.8708.

53. Schmidt KN, Leung B, Kwong M, Zarember KA, Satyal S, Navas TA, Wang F, Godowski PJ. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. J Immunol. 2004;172(1):138–43. doi:10.4049/jimmunol.172.1.138.
54. Cario E, Podolsky DK, Clements JD. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun. 2000;68(12):7010–7. doi:10.1128/IAI.68.12.7010-7017.2000.
55. Fang F, Ooka K, Sun X, Shah R, Bhattacharyya S, Wei J, Varga J. A synthetic TLR3 ligand mitigates profibrotic fibroblast responses by inducing autocrine IFN signaling. J Immunol. 2013;191(6):2956–66. doi:10.4049/jimmunol.1300376.
56. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol. 2003;171(6):3154–62. doi:10.4049/jimmunol.171.6.3154.
57. Jiang Z, Mak TW, Sen G, Li X. Toll-like receptor 3-mediated activation of NF-κB and IRF3 diverges at toll-IL-1 receptor domain-containing adapter inducing IFN-β. Proc Natl Acad Sci USA. 2004;101(10):3533–8. doi:10.1073/pnas.0308496101.
58. Schulz O, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, Azuma YT, Flavell RA, Liljeström P, Reis e Sousa C. et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature. 2005;433(7028):887–92. doi:10.1038/nature03326.
59. Lebre MC, Antons JC, Kalinski P, Schuitemaker JH, van Capel TM, Kapsenberg ML, de Jong EC. Double-stranded RNA-Exposed human keratinocytes promote Th1 responses by inducing a type-1 Polarized Phenotype in dendritic cells: role of Keratinocyte-derived tumor Necrosis factor α, type I interferons, and interleukin-18. J Invest Dermatol. 2003;120(6):990–7. doi:10.1046/j.1523-1747.2003.12245.x.
60. Jiang Z, Zamanian-Daryoush M, Nie H, Silva AM, Williams BR, Li X. Poly(dI·dC)-induced toll-like receptor 3 (TLR3)-mediated activation of NFκB and MAP kinase is through an Interleukin-1 receptor-associated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. J Biol Chem. 2003;278(19):16713–19. doi:10.1074/jbc.M300562200.
61. Padalko E, Nuyens D, De Palma A, Verbeken E, Aerts JL, De Clercq E, Carmeliet P, Neyts J. The interferon inducer ampligen [poly(I)-poly(C12U)] markedly protects mice against coxsackie B3 virus-induced myocarditis. Antimicrob Agents Chemother. 2004;48(1):267–74. doi:10.1128/AAC.48.1.267-274.2004.
62. Levy HB, Baer G, Baron S, Buckler CE, Gibbs CJ, Iadarola MJ, London WT, Rice J. A modified polyriboinosinic-polyribocytidylic acid complex that induces interferon in primates. J Infect Dis. 1975;132(4):434–9. doi:10.1093/infdis/132.4.434.
63. Levy HB, Riley FL, Lvovsky E, Stephen EE. Interferon induction in primates by stabilized polyriboinosinic acid-polyribocytidylic acid: effect of component size. Infect Immun. 1981;34(2):416–21. doi:10.1128/iai.34.2.416-421.1981.
64. Matsumoto M, Tatematsu M, Nishikawa F, Azuma M, Ishii N, Morii-Sakai A, Shime H, Seya T. Defined TLR3-specific adjuvant that induces NK and CTL activation without significant cytokine production in vivo. Nat Commun. 2015;6(1):6280. doi:10.1038/ncomms7280.
65. Jelinek I, Leonard JN, Price GE, Brown KN, Meyer-Manlapat A, Goldsmith PK, Wang Y, Venzon D, Epstein SL, Segal DM. et al. TLR3-Specific double-stranded RNA Oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. J Immunol. 2011;186(4):2422–9. doi:10.4049/jimmunol.1002845.
66. Conte MR, Conn GL, Brown T, Lane AN. Conformational properties and thermodynamics of the RNA duplex r(CGCAAAUUUGCG) 2: comparison with the DNA analogue d(CGCAAATTTGCG) 2. Nucleic Acids Res. 1997;25(13):2627–34. doi:10.1093/nar/25.13.2627.
67. Elmén J, Thonberg H, Ljungberg K, Frieden M, Westergaard M, Xu Y, Wahren B, Liang Z, Ørum H, Koch T. et al, Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. Nucleic Acids Res. 2005;33(1):439–47. doi:10.1093/nar/gki193.
68. Shetab Boushehri MA, Lamprecht A. TLR4-based Immunotherapeutics in cancer: a review of the achievements and shortcomings. Mol Pharm. 2018;15(11):4777–800. doi:10.1021/acs.molpharmaceut.8b00691.
69. Andreasen AS, Krabbe KS, Krogh-Madsen R, Taudorf S, Pedersen BK, Møller K. Human endotoxemia as a model of systemic inflammation. Curr Med Chem. 2008;15(17):1697–705. doi:10.2174/092986708784872393.
70. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008;42(2):145–51. doi:10.1016/j.cyto.2008.01.006.
71. Pereira M, Durso DF, Bryant CE, Kurt-Jones EA, Silverman N, Golenbock DT, Gazzinelli RT. The IRAK4 scaffold integrates TLR4-driven TRIF and MYD88 signaling pathways. Cell Rep. 2022;40(7):111225. doi:10.1016/j.celrep.2022.111225.
72. Ling GS, Bennett J, Woollard KJ, Szajna M, Fossati-Jimack L, Taylor PR, Scott D, Franzoso G, Cook HT, Botto M. et al. Integrin CD11b positively regulates TLR4-induced signalling pathways in dendritic cells but not in macrophages. Nat Commun. 2014;5(1):3039. doi:10.1038/ncomms4039.
73. Jang DI, Lee AH, Shin HY, Song HR, Park JH, Kang TB, Lee SR, Yang SH. The role of tumor necrosis factor alpha (TNF-α) in Autoimmune disease and current TNF-α inhibitors in therapeutics. Int J Mol Sci. 2021;22(5):22. doi:10.3390/ijms22052719.
74. Raetz CRH, Whitfield C. Lipopolysaccharide Endotoxins. Annu Rev Biochem. 2002;71(1):635–700. doi:10.1146/annurev.biochem.71.110601.135414.
75. Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. Semin Immunol. 2007;19(1):3–10. doi:10.1016/j.smim.2006.12.002.
76. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. MD-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4. J Exp Med. 1999;189(11):1777–82. doi:10.1084/jem.189.11.1777.
77. Schülke S, Flaczyk A, Vogel L, Gaudenzio N, Angers I, Löschner B, Wolfheimer S, Spreitzer I, Qureshi S, Tsai M. et al. MPLA shows attenuated pro-inflammatory properties and diminished capacity to activate mast cells in comparison with LPS. Allergy. 2015;70(10):1259–68. doi:10.1111/all.12675.
78. Arias MA, Van Roey GA, Tregoning JS, Moutaftsi M, Coler RN, Windish HP, Reed SG, Carter D, Shattock RJ. Glucopyranosyl lipid adjuvant (GLA), a synthetic TLR4 Agonist, promotes potent systemic and mucosal responses to intranasal immunization with HIVgp140. PLoS ONE. 2012;7(7):e41144. doi:10.1371/journal.pone.0041144.
79. Kuramitsu Y, Nishibe M, Ohiro Y, Matsushita K, Yuan L, Obara M, Kobayashi M, Hosokawa M. A new synthetic lipid a analog, ONO-4007, stimulates the production of tumor necrosis factor-α in tumor tissues, resulting in the rejection of transplanted rat hepatoma cells. Anticancer Drugs. 1997;8(5):500–8. doi:10.1097/00001813-199706000-00013.
80. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001;410(6832):1099–103. doi:10.1038/35074106.
81. Yu S, Gao N. Compartmentalizing intestinal epithelial cell toll-like receptors for immune surveillance. Cell Mol Life Sci. 2015;72(17):3343–53. doi:10.1007/s00018-015-1931-1.
82. Hamonic G, Pasternak JA, Wilson HL. Recognizing conserved non-canonical localization patterns of toll-like receptors in tissues and across species. Cell Tissue Res. 2018;372(1):1–11. doi:10.1007/s00441-017-2767-9.
83. Fitzgerald KA, Kagan JC. Toll-like receptors and the control of immunity. Cell. 2020;180(6):1044–66. doi:10.1016/j.cell.2020.02.041.

84. McDermott PF, Ciacci-Woolwine F, Snipes JA, Mizel SB, Moore RN. High-affinity interaction between gram-negative flagellin and a cell surface polypeptide results in human monocyte activation. Infect Immun. 2000;68(10):5525–9. doi:10.1128/IAI.68.10.5525-5529.2000.
85. Means TK, Hayashi F, Smith KD, Aderem A, Luster AD. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol. 2003;170(10):5165–75. doi:10.4049/jimmunol.170.10.5165.
86. Cui B, Liu X, Fang Y, Zhou P, Zhang Y, Wang Y. Flagellin as a vaccine adjuvant. Expert Rev Vaccines. 2018;17(4):335–49. doi:10.1080/14760584.2018.1457443.
87. Rhee JH, Khim K, Puth S, Choi Y, Lee SE. Deimmunization of flagellin adjuvant for clinical application. Curr Opin Virol. 2023;60:101330. doi:10.1016/j.coviro.2023.101330.
88. Zinsli LV, Stierlin N, Loessner MJ, Schmelcher M. Deimmunization of protein therapeutics – recent advances in experimental and computational epitope prediction and deletion. Comput Struct Biotechnol J. 2021;19:315–29. doi:10.1016/j.csbj.2020.12.024.
89. S-I Y, Kurnasov O, Natarajan V, Hong M, Gudkov AV, Osterman AL, Wilson IA. Structural basis of TLR5-flagellin recognition and signaling. Sci. 2012;335(6070):859–64. doi:10.1126/science.1215584.
90. Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, Cookson BT, Aderem A. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol. 2003;4(12):1247–53. doi:10.1038/ni1011.
91. Khim K, Bang YJ, Puth S, Choi Y, Lee YS, Jeong K, Lee SE, Rhee JH. Deimmunization of flagellin for repeated administration as a vaccine adjuvant. NPJ Vaccines. 2021;6(1):116. doi:10.1038/s41541-021-00379-4.
92. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw. 2000;11:362–71.
93. Lee J, Chuang TH, Redecke V, She L, Pitha PM, Carson DA, Raz E, Cottam HB. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. Proc Natl Acad Sci. 2003;100(11):6646–51. doi:10.1073/pnas.0631696100.
94. Cervantes JL, Weinerman B, Basole C, Salazar JC. TLR8: the forgotten relative revindicated. Cell Mol Immunol. 2012;9(6):434–8. doi:10.1038/cmi.2012.38.
95. Vasilakos JP, Tomai MA. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. Expert Rev Vaccines. 2013;12(7):809–19. doi:10.1586/14760584.2013.811208.
96. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Sci. 2004;303:1529–31. doi:10.1126/science.1093616.
97. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S. et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88–dependent signaling pathway. Nat Immunol. 2002;3(2):196–200. doi:10.1038/ni758.
98. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci. 2004;101(15):5598–603. doi:10.1073/pnas.0400937101.
99. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Sci. 2004;303(5663):1526–9. doi:10.1126/science.1093620.
100. Blasius AL, Beutler B. Intracellular toll-like receptors. Immunity. 2010;32(3):305–15. doi:10.1016/j.immuni.2010.03.012.
101. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol. 2002;3(6):499. doi:10.1038/ni0602-499.
102. Bhagchandani S, Johnson JA, Irvine DJ. Evolution of Toll-like receptor 7/8 agonist therapeutics and their delivery approaches: from antiviral formulations to vaccine adjuvants. Adv Drug Deliv Rev. 2021;175:113803. doi:10.1016/j.addr.2021.05.013.
103. Philbin VJ, Dowling DJ, Gallington LC, Cortés G, Tan Z, Suter EE, Chi KW, Shuckett A, Stoler-Barak L, Tomai M. et al. Imidazoquinoline Toll-like receptor 8 agonists activate human newborn monocytes and dendritic cells through adenosine-refractory and caspase-1–dependent pathways. J Allergy Clin Immunol. 2012;130(1):195–204. e9 doi:10.1016/j.jaci.2012.02.042.
104. Levy O, Suter EE, Miller RL, Wessels MR. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. Blood. 2006;108(4):1284–90. doi:10.1182/blood-2005-12-4821.
105. Levy O, Zarember KA, Roy RM, Cywes C, Godowski PJ, Wessels MR. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-α induction by bacterial lipopeptides, lipopolysaccharide, and Imiquimod, but preserves the response to R-848. J Immunol. 2004;173(7):4627–34. doi:10.4049/jimmunol.173.7.4627.
106. Kaushik D, Kaur A, Petrovsky N, Salunke DB. Structural evolution of toll-like receptor 7/8 agonists from imidazoquinolines to imidazoles. RSC Med Chem. 2021;12(7):1065–120. doi:10.1039/D1MD00031D.
107. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K. et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000;408(6813):740–5. doi:10.1038/35047123.
108. Kumagai Y, Takeuchi O, Akira S. TLR9 as a key receptor for the recognition of DNA. Adv Drug Deliv Rev. 2008;60(7):795–804. doi:10.1016/j.addr.2007.12.004.
109. Babenko VN, Chadaeva IV, Orlov YL. Genomic landscape of CpG rich elements in human. BMC Evol Biol. 2017;17:19. doi:10.1186/s12862-016-0864-0.
110. Wagner H. The sweetness of the DNA backbone drives Toll-like receptor 9. Curr Opin Immunol. 2008;20(4):396–400. doi:10.1016/j.coi.2008.06.013.
111. Coch C, Busch N, Wimmenauer V, Hartmann E, Janke M, Abdel-Mottaleb MM, Lamprecht A, Ludwig J, Barchet W, Schlee M. et al. Higher activation of TLR9 in plasmacytoid dendritic cells by microbial DNA compared with self-DNA based on CpG-specific recognition of phosphodiester DNA. J Leukoc Biol. 2009;86(3):663–70. doi:10.1189/jlb.0509314.
112. Lamphier MS, Sirois CM, Verma A, Golenbock DT, Latz E. TLR9 and the recognition of self and non-self nucleic acids. Ann N Y Acad Sci. 2006;1082(1):31–43. doi:10.1196/annals.1348.005.
113. Casanova JL, Abel L, Quintana-Murci L. Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. Annu Rev Immunol. 2011;29(1):447–91. doi:10.1146/annurev-immunol-030409-101335.
114. Schneberger D, Caldwell S, Kanthan R, Singh B. Expression of Toll-like receptor 9 in mouse and human lungs. J Anat. 2013;222(5):495–503. doi:10.1111/joa.12039.
115. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11(5):373–84. doi:10.1038/ni.1863.
116. Guiducci C, Ott G, Chan JH, Damon E, Calacsan C, Matray T, Lee K-D, Coffman RL, Barrat FJ. Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. J Exp Med. 2006;203(8):1999–2008. doi:10.1084/jem.20060401.
117. Vollmer J, Weeratna R, Payette P, Jurk M, Schetter C, Laucht M, Wader T, Tluk S, Liu M, Davis H. et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur J Immunol. 2004;34(1):251–62. doi:10.1002/eji.200324032.
118. Jiang W, Lederman MM, Harding CV, Rodriguez B, Mohner RJ, Sieg SF. TLR9 stimulation drives naïve B cells to proliferate and to attain enhanced antigen presenting function. Eur J Immunol. 2007;37(8):2205–13. doi:10.1002/eji.200636984.

119. Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov. 2006;5(6):471–84. doi:10.1038/nrd2059.
120. Donhauser N, Helm M, Pritschet K, Schuster P, Ries M, Korn K, Vollmer J, Schmidt B. Differential effects of P-class versus other CpG oligodeoxynucleotide classes on the impaired innate immunity of plasmacytoid dendritic cells in HIV type 1 infection. AIDS Res Hum Retroviruses. 2010;26(2):161–71. doi:10.1089/aid.2008.0278.
121. Wittig B, Schmidt M, Scheithauer W, Schmoll HJ. MGN1703, an immunomodulator and toll-like receptor 9 (TLR-9) agonist: from bench to bedside. Crit Rev Oncol Hematol. 2015;94(1):31–44. doi:10.1016/j.critrevonc.2014.12.002.
122. Holtick U, Scheulen ME, von Bergwelt-Baildon MS, Weihrauch MR. Toll-like receptor 9 agonists as cancer therapeutics. Expert Opin Investig Drugs. 2011;20(3):361–72. doi:10.1517/13543784.2011.553187.
123. Yu D, Putta MR, Bhagat L, Dai M, Wang D, Trombino AF, Sullivan T, Kandimalla ER, Agrawal S. Impact of secondary structure of toll-like receptor 9 agonists on interferon alpha induction. Antimicrob Agents Chemother. 2008;52(12):4320–5. doi:10.1128/AAC.00701-08.
124. Agrawal S, Kandimalla ER. Synthetic agonists of Toll-like receptors 7, 8 and 9. Biochem Soc Trans. 2007;35(6):1461–7. doi:10.1042/BST0351461.
125. Wang D, Zhu F, DiMuzio J, Agrawal S. Abstract B196: intratumoral administration of IMO-2125, a novel TLR9 agonist, modulates tumor microenvironment and potentiates antitumor activity of anti-PD-1 mAb in a murine colon carcinoma model. Mol Cancer Ther. 2015;14(12_Supplement_2):B196–B. doi:10.1158/1535-7163.TARG-15-B196.
126. Schmidt M, Hagner N, Marco A, König-Merediz SA, Schroff M, Wittig B. Design and structural requirements of the potent and safe TLR-9 agonistic immunomodulator MGN1703. Nucleic Acid Ther. 2015;25(3):130–40. doi:10.1089/nat.2015.0533.
127. Chuang TH, Ulevitch RJ. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. Biochimica Et Biophysica Acta (BBA) - Gene Struct And Expression. 2001;1518(1–2):157–61. doi:10.1016/S0167-4781(00)00289-X.
128. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, Endres S, Hartmann G. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG Oligodeoxynucleotides. J Immunol. 2002;168(9):4531–7. doi:10.4049/jimmunol.168.9.4531.
129. Govindaraj RG, Manavalan B, Lee G, Choi S, Selvarajoo K. Molecular modeling-based evaluation of hTLR10 and identification of potential ligands in Toll-like receptor signaling. PLOS ONE. 2010;5(9):e12713. doi:10.1371/journal.pone.0012713.
130. Sindhu S, Akhter N, Kochumon S, Thomas R, Wilson A, Shenouda S, Tuomilehto J, Ahmad R. Increased expression of the innate immune receptor TLR10 in obesity and type-2 diabetes: association with ROS-mediated oxidative stress. Cell Physiol Biochem. 2018;45(2):572–90. doi:10.1159/000487034.
131. Oosting M, Cheng SC, Bolscher JM, Vestering-Stenger R, Plantinga TS, Verschueren IC, Arts P, Garritsen A, van Eenennaam H, Sturm P. et al. Human TLR10 is an anti-inflammatory pattern-recognition receptor. Proc Natl Acad Sci. 2014;111(42):E4478–E84. doi:10.1073/pnas.1410293111.
132. Lauw FN, Caffrey DR, Golenbock DT. Of mice and man: TLR11 (finally) finds profilin. Trends Immunol. 2005;26(10):509–11. doi:10.1016/j.it.2005.08.006.
133. Koblansky AA, Jankovic D, Oh H, Hieny S, Sungnak W, Mathur R, Hayden M, Akira S, Sher A, Ghosh S. et al. Recognition of profilin by Toll-like receptor 12 is critical for host resistance to Toxoplasma gondii. Immunity. 2013;38(1):119–30. doi:10.1016/j.immuni.2012.09.016.
134. Shi Z, Cai Z, Sanchez A, Zhang T, Wen S, Wang J, Yang J, Fu S, Zhang D. A novel Toll-like receptor that recognizes vesicular stomatitis virus. J Biol Chem. 2011;286(6):4517–24. doi:10.1074/jbc.M110.159590.
135. Raetz M, Kibardin A, Sturge CR, Pifer R, Li H, Burstein E, Ozato K, Larin S, Yarovinsky F. Cooperation of TLR12 and TLR11 in the IRF8-dependent IL-12 response to Toxoplasma gondii profilin. J Immunol. 2013;191(9):4818–27. doi:10.4049/jimmunol.1301301.
136. Hatai H, Lepelley A, Zeng W, Hayden MS, Ghosh S, Blader IJ. Toll-like receptor 11 (TLR11) interacts with Flagellin and profilin through disparate mechanisms. PLoS ONE. 2016;11(2):e0148987. doi:10.1371/journal.pone.0148987.
137. Hochrein H, Kirschning CJ. Bacteria evade immune recognition via TLR13 and binding of their 23S rRNA by MLS antibiotics by the same mechanisms. Oncoimmunology. 2013;2(3):e23141. doi:10.4161/onci.23141.
138. Yarovinsky F, Hieny S, Sher A. Recognition of Toxoplasma gondii by TLR11 prevents parasite-induced immunopathology. J Immunol. 2008;181(12):8478–84. doi:10.4049/jimmunol.181.12.8478.
139. Hidmark A, von Saint Paul A, Dalpke AH. Cutting edge: TLR13 is a receptor for bacterial RNA. J Immunol. 2012;189(6):2717–21. doi:10.4049/jimmunol.1200898.
140. Zhang Y, Luo F, Li A, Qian J, Yao Z, Feng X, Chu Y. Systemic injection of TLR1/2 agonist improves adoptive antigen-specific T cell therapy in glioma-bearing mice. Clin Immunol. 2014;154(1):26–36. doi:10.1016/j.clim.2014.06.004.
141. Zahm CD, Colluru VT, McIlwain SJ, Ong IM, McNeel DG. TLR stimulation during T-cell activation lowers PD-1 expression on CD8(+) T cells. Cancer Immunol Res. 2018;6(11):1364–74. doi:10.1158/2326-6066.CIR-18-0243.
142. Renaudet O, Dasgupta G, Bettahi I, Shi A, Nesburn AB, Dumy P, BenMohamed L. Linear and branched Glyco-Lipopeptide vaccines follow distinct cross-presentation pathways and generate different magnitudes of antitumor immunity. PLOS ONE. 2010;5(6):e11216. doi:10.1371/journal.pone.0011216.
143. Shi W, Tong Z, Chen S, Qiu Q, Zhou J, Qian H. Development of novel self-assembled vaccines based on tumour-specific antigenic peptide and TLR2 agonist for effective breast cancer immunotherapy via activating CD8+ T cells and enhancing their function. Immunology. 2023;169(4):454–66. doi:10.1111/imm.13643.
144. Adams M, Navabi H, Jasani B, Man S, Fiander A, Evans AS, Donninger C, Mason M. Dendritic cell (DC) based therapy for cervical cancer: use of DC pulsed with tumour lysate and matured with a novel synthetic clinically non-toxic double stranded RNA analogue poly [I]: poly [C12U] (Ampligen®). Vaccine. 2003;21(7–8):787–90. doi:10.1016/S0264-410X(02)00599-6.
145. Zhu X, Nishimura F, Sasaki K, Fujita M, Dusak JE, Eguchi J, Fellows-Mayle W, Storkus WJ, Walker PR, Salazar AM. et al. Toll like receptor-3 ligand poly-ICLC promotes the efficacy of peripheral vaccinations with tumor antigen-derived peptide epitopes in murine CNS tumor models. J Transl Med. 2007;5(1):10. doi:10.1186/1479-5876-5-10.
146. Takeda Y, Yoshida S, Takashima K, Ishii-Mugikura N, Shime H, Seya T, Matsumoto M. Vaccine immunotherapy with ARNAX induces tumor-specific memory T cells and durable anti-tumor immunity in mouse models. Cancer Sci. 2018;109(7):2119–29. doi:10.1111/cas.13649.
147. Song H, Huang P, Niu J, Shi G, Zhang C, Kong D, Wang W. Injectable polypeptide hydrogel for dual-delivery of antigen and TLR3 agonist to modulate dendritic cells in vivo and enhance potent cytotoxic T-lymphocyte response against melanoma. Biomaterials. 2018;159:119–29. doi:10.1016/j.biomaterials.2018.01.004.
148. Wang C, Zhuang Y, Zhang Y, Luo Z, Gao N, Li P, Pan H, Cai L, Ma Y. Toll-like receptor 3 agonist complexed with cationic liposome augments vaccine-elicited antitumor immunity by enhancing TLR3–IRF3 signaling and type I interferons in dendritic cells. Vaccine. 2012;30(32):4790–9. doi:10.1016/j.vaccine.2012.05.027.
149. Vindevogel E, Baert T, Hoylandt AV, Verbist G, Velde GV, Garg AD, Agostinis P, Vergote I, Coosemans AN. The use of toll-like receptor 4 agonist to reshape the immune signature in ovarian cancer. Anticancer Res. 2016;36(11):5781–92. doi:10.21873/anticanres.11162.

150. Davis MB, Vasquez-Dunddel D, Fu J, Albesiano E, Pardoll D, Kim YJ. Intratumoral administration of TLR4 agonist absorbed into a cellular vector improves antitumor responses. Clin Cancer Res. 2011;17(12):3984–92. doi:10.1158/1078-0432.CCR-10-3262.
151. Shi HS, Gong CY, Zhang Hl, Wang YS, Zhang J, Luo ZC, Qian ZY, Wei YQ, Yang L. Novel vaccine adjuvant LPS-Hydrogel for truncated basic fibroblast growth factor to induce antitumor immunity. Carbohyd Polym. 2012;89(4):1101–9. doi:10.1016/j.carbpol.2012.03.073.
152. Hamdy S, Molavi O, Ma Z, Haddadi A, Alshamsan A, Gobti Z, Elhasi S, Samuel J, Lavasanifar A. Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent CD8+ T cell-mediated anti-tumor immunity. Vaccine. 2008;26(39):5046–57. doi:10.1016/j.vaccine.2008.07.035.
153. Cheng R, Fontana F, Xiao J, Liu Z, Figueiredo P, Shahbazi MA, Wang S, Jin J, Torrieri G, Hirvonen JT. et al. Recombination monophosphoryl lipid A-derived vacosome for the development of preventive cancer vaccines. ACS Appl Mater Interfaces. 2020;12(40):44554–62. doi:10.1021/acsami.0c15057.
154. Bubna AK. Imiquimod - Its role in the treatment of cutaneous malignancies. Indian J Pharmacol. 2015;47(4):354–9. doi:10.4103/0253-7613.161249.
155. Ma F, Zhang J, Zhang J, Zhang C. The TLR7 agonists imiquimod and gardiquimod improve DC-based immunotherapy for melanoma in mice. Cell Mol Immunol. 2010;7(5):381–8. doi:10.1038/cmi.2010.30.
156. Stickdorn J, Stein L, Arnold-Schild D, Hahlbrock J, Medina-Montano C, Bartneck J, Ziß T, Montermann E, Kappel C, Hobernik D. et al. Systemically administered TLR7/8 agonist and antigen-conjugated nanogels govern immune responses against tumors. Acs Nano. 2022;16(3):4426–43. doi:10.1021/acsnano.1c10709.
157. Chi H, Hao Y, Wang X, Tang L, Deng Y, Chen X, Gao F, Sha O, Jin G. A therapeutic whole-tumor-cell vaccine covalently conjugated with a TLR7 agonist. Cells. 2022;11(13):1986. doi:10.3390/cells11131986.
158. Song H, Su Q, Shi W, Huang P, Zhang C, Zhang C, Zhang C, Liu Q, Wang W. Antigen epitope-TLR7/8a conjugate as self-assembled carrier-free nanovaccine for personalized immunotherapy. Acta Biomater. 2022;141:398–407. doi:10.1016/j.actbio.2022.01.004.
159. Lynn GM, Sedlik C, Baharom F, Zhu Y, Ramirez-Valdez RA, Coble VL, Tobin K, Nichols SR, Itzkowitz Y, Zaidi N. et al. Peptide–TLR-7/8a conjugate vaccines chemically programmed for nanoparticle self-assembly enhance CD8 T-cell immunity to tumor antigens. Nat Biotechnol. 2020;38(3):320–32. doi:10.1038/s41587-019-0390-x.
160. Kim SK, Ragupathi G, Musselli C, Choi SJ, Park YS, Livingston PO. Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. Vaccine. 1999;18(7–8):597–603. doi:10.1016/S0264-410X(99)00316-3.
161. Sin JI, Kim H, Ahn E, Jeon YH, Park WS, Lee S-Y, Kwon B. Combined stimulation of TLR9 and 4.1BB augments Trp2 peptide vaccine-mediated melanoma rejection by increasing Ag-specific CTL activity and infiltration into tumor sites. Cancer Lett. 2013;330(2):190–9. doi:10.1016/j.canlet.2012.11.045.
162. Shi X, Song H, Wang C, Zhang C, Huang P, Kong D, Zhang J, Wang W. Co-assembled and self-delivered epitope/CpG nanocomplex vaccine augments peptide immunogenicity for cancer immunotherapy. Chem Eng J. 2020;399:125854. doi:10.1016/j.cej.2020.125854.
163. Chen T, Liu K, Xu J, Zhan T, Liu M, Li L, Yang Z, Yuan S, Zou W, Lin G. et al. Synthetic and immunological studies on the OCT4 immunodominant motif antigen-based anti-cancer vaccine. Cancer Biol Med. 2020;17(1):132–41. doi:10.20892/j.issn.2095-3941.2019.0224.
164. Zaks K, Jordan M, Guth A, Sellins K, Kedl R, Izzo A, Bosio C, Dow S. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic Liposomes1. J Immunol. 2006;176(12):7335–45. doi:10.4049/jimmunol.176.12.7335.
165. Jeon D, McNeel DG. Toll-like receptor agonist combinations augment mouse T-cell anti-tumor immunity via IL-12- and interferon ß-mediated suppression of immune checkpoint receptor expression. Oncoimmunology. 2022;11(1):2054758. doi:10.1080/2162402X.2022.2054758.
166. Prins RM, Soto H, Konkankit V, Odesa SK, Eskin A, Yong WH, Nelson SF, Liau LM. Gene expression profile correlates with T-cell infiltration and relative survival in glioblastoma patients vaccinated with dendritic cell immunotherapy. Clin Cancer Res. 2011;17(6):1603–15. doi:10.1158/1078-0432.CCR-10-2563.
167. Tsuji T, Sabbatini P, Jungbluth AA, Ritter E, Pan L, Ritter G, Ferran L, Spriggs D, Salazar AM, Gnjatic S. et al. Effect of Montanide and poly-ICLC adjuvant on human self/tumor antigen-specific CD4+ T cells in phase I overlapping long peptide vaccine trial. Cancer Immunol Res. 2013;1(5):340–50. doi:10.1158/2326-6066.CIR-13-0089.
168. Pavlick A, Blazquez AB, Meseck M, Lattanzi M, Ott PA, Marron TU, Holman RM, Mandeli J, Salazar AM, McClain CB. et al. Combined vaccination with NY-ESO-1 protein, poly-ICLC, and montanide improves humoral and cellular immune responses in patients with high-risk melanoma. Cancer Immunol Res. 2020;8(1):70–80. doi:10.1158/2326-6066.CIR-19-0545.
169. Dillon PM, Petroni GR, Smolkin ME, Brenin DR, Chianese-Bullock KA, Smith KT, Olson WC, Fanous IS, Nail CJ, Brenin CM. et al. A pilot study of the immunogenicity of a 9-peptide breast cancer vaccine plus poly-ICLC in early stage breast cancer. J Immunother Cancer. 2017;5(1):92. doi:10.1186/s40425-017-0295-5.
170. Melssen MM, Petroni GR, Chianese-Bullock KA, Wages NA, Grosh WW, Varhegyi N, Smolkin ME, Smith KT, Galeassi NV, Deacon DH. et al. A multipeptide vaccine plus toll-like receptor agonists LPS or polyICLC in combination with incomplete Freund's adjuvant in melanoma patients. J Immunother Cancer. 2019;7(1):163. doi:10.1186/s40425-019-0625-x.
171. Mahipal A, Odunsi K, Gnjatic S, Kim-Schulze S, Kenney RT, Ejadi S. A first-in-human phase 1 dose-escalating trial of G305 in patients with solid tumors expressing NY-ESO-1. J Clin Oncol. 2015;33(15_suppl):3073–. doi:10.1200/jco.2015.33.15_suppl.3073.
172. Neidhart J, Allen KO, Barlow DL, Carpenter M, Shaw DR, Triozzi PL, Conry RM. Immunization of colorectal cancer patients with recombinant baculovirus-derived KSA (Ep-CAM) formulated with monophosphoryl lipid a in liposomal emulsion, with and without granulocyte-macrophage colony-stimulating factor. Vaccine. 2004;22(5–6):773–80. doi:10.1016/j.vaccine.2003.08.021.
173. Grewal EP, Erskine CL, Nevala WK, Allred JB, Strand CA, Kottschade LA, McWilliams RR, Dronca RS, Yakovich AJ, Markovic SN. et al. Peptide vaccine with glucopyranosyl lipid A–stable oil-in-water emulsion for patients with resected melanoma. Immunotherapy. 2020;12(13):983–95. doi:10.2217/imt-2020-0085.
174. Shackleton M, Davis ID, Hopkins W, Jackson H, Dimopoulos N, Tai T, Chen Q, Parente P, Jefford M, Masterman KA. et al. The impact of imiquimod, a Toll-like receptor-7 ligand (TLR7L), on the immunogenicity of melanoma peptide vaccination with adjuvant Flt3 ligand. Cancer Immun. 2004;4:9.
175. Adams S, O'Neill DW, Nonaka D, Hardin E, Chiriboga L, Siu K, Cruz CM, Angiulli A, Angiulli F, Ritter E. et al. Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. J Immunol. 2008;181(1):776–84. doi:10.4049/jimmunol.181.1.776.
176. Mauldin IS, Wages NA, Stowman AM, Wang E, Smolkin ME, Olson WC, Deacon DH, Smith KT, Galeassi N, Teague JE. et al. Pilot clinical trials testing the safety and effects on the metastatic melanoma microenvironment of intratumoral interferon-gamma or imiquimod, plus a multipeptide melanoma vaccine. J Immuno Ther Cancer. 2015;3:139. doi:10.1186/2051-1426-3-S2-P139.
177. Sabado RL, Pavlick AC, Gnjatic S, Cruz CM, Vengco I, Hasan F, Darvishian F, Chiriboga L, Holman RM, Escalon J. et al. Phase I/II

study of resiquimod as an immunologic adjuvant for NY-ESO-1 protein vaccination in patients with melanoma. J Clin Oncol. 2014;32:9086–. doi:10.1200/jco.2014.32.15_suppl.9086.
178. Royal RE, Vence LM, Wray T, Cormier JN, Lee JE, Gershenwald JE, Ross MI, Wargo JA, Amaria RN, Davies MA. et al. A toll-like receptor agonist to drive melanoma regression as a vaccination adjuvant or by direct tumor application. J Clin Oncol. 2017;35:9582–. doi:10.1200/JCO.2017.35.15_suppl.9582.
179. Speiser DE, Liénard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, Krieg AM, Cerottini J-C, Romero P. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest. 2005;115 (3):739–46. doi:10.1172/JCI23373.
180. Iwahashi M, Katsuda M, Nakamori M, Nakamura M, Naka T, Ojima T, Iida T, Yamaue H. Vaccination with peptides derived from cancer-testis antigens in combination with CpG-7909 elicits strong specific CD8+ T cell response in patients with metastatic esophageal squamous cell carcinoma. Cancer Sci. 2010;101 (12):2510–7. doi:10.1111/j.1349-7006.2010.01732.x.
181. Mahipal A, Ejadi S, Gnjatic S, Kim-Schulze S, Lu H, Ter Meulen JH, Kenney R, Odunsi K. First-in-human phase 1 dose-escalating trial of G305 in patients with advanced solid tumors expressing NY-ESO-1. Cancer Immunol Immunother. 2019;68(7):1211–22. doi:10.1007/s00262-019-02331-x.
182. Sabado RL, Pavlick A, Gnjatic S, Cruz CM, Vengco I, Hasan F, Spadaccia M, Darvishian F, Chiriboga L, Holman RM. et al. Resiquimod as an immunologic adjuvant for NY-ESO-1 protein vaccination in patients with high-risk melanoma. Cancer Immunol Res. 2015;3(3):278–87. doi:10.1158/2326-6066.CIR-14-0202.
183. Sabbatini P, Tsuji T, Ferran L, Ritter E, Sedrak C, Tuballes K, Jungbluth AA, Ritter G, Aghajanian C, Bell-McGuinn K. et al. Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. Clin Cancer Res. 2012;18(23):6497–508. doi:10.1158/1078-0432.CCR-12-2189.
184. Mauldin IS, Wages NA, Stowman AM, Wang E, Olson WC, Deacon DH, Smith KT, Galeassi N, Teague JE, Smolkin ME. et al. Topical treatment of melanoma metastases with imiquimod, plus administration of a cancer vaccine, promotes immune signatures in the metastases. Cancer Immunol Immunother. 2016;65 (10):1201–12. doi:10.1007/s00262-016-1880-z.
185. Noguchi M, Arai G, Egawa S, Ohyama C, Naito S, Matsumoto K, Uemura H, Nakagawa M, Nasu Y, Eto M. et al. Mixed 20-peptide cancer vaccine in combination with docetaxel and dexamethasone for castration-resistant prostate cancer: a randomized phase II trial. Cancer Immunol Immunother. 2020;69(5):847–57. doi:10.1007/s00262-020-02498-8.
186. Clifton GT, Peoples GE, Mittendorf EA. The development and use of the E75 (HER2 369–377) peptide vaccine. Future Oncol. 2016;12(11):1321–9. doi:10.2217/fon-2015-0054.
187. Carvalho T. Personalized anti-cancer vaccine combining mRNA and immunotherapy tested in melanoma trial. Nat Med. 2023;29 (10):2379–2380. doi:10.1038/d41591-023-00072-0.
188. Pascolo S. Vaccination with messenger RNA. Methods Mol Med. 2006;127:23–40.