

Toll-Like Receptor 9 Agonists in Cancer

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Abstract: Toll-like receptor 9 (TLR9) is a pattern recognition receptor that is predominantly located intracellularly in immune cells, including dendritic cells, macrophages, natural killer cells, and other antigen-presenting cells (APC). The primary ligands for TLR9 receptors are unmethylated cytidine phosphate guanosine (CpG) oligonucleotides (ODN). TLR9 agonists induce inflammatory processes that result in the enhanced uptake and killing of microorganisms and cancer cells as well as the generation of adaptive immune responses. Preclinical studies of TLR9 agonists suggested efficacy both as monotherapy and in combination with several agents, which led to clinical trials in patients with advanced cancer. In these studies, intravenous, intratumoral, and subcutaneous routes of administration have been tested; with anti-tumor responses in both treated and untreated metastatic sites. TLR9 agonist monotherapy is safe, although efficacy is minimal in advanced cancer patients; conversely, combinations appear to be more promising. Several ongoing phase I and II clinical trials are evaluating TLR9 agonists in combination with a variety of agents including chemotherapy, radiotherapy, targeted therapy, and immunotherapy agents. In this review article, we describe the distribution, structure and signaling of TLR9; discuss the results of preclinical studies of TLR9 agonists; and review ongoing clinical trials of TLR9 agonists singly and in combination in patients with advanced solid tumors.

Keywords: toll-like receptor, TLR, TLR9, CpG, ODN, innate immunity, innate agonist, cancer, cancer immunotherapy, dendritic cell

Introduction

The advent of monoclonal antibodies (mAb) targeting inhibitory immune checkpoints such as programmed cell death 1 (PD-1) or –ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have transformed the management of advanced cancers. Anti-PD(L)1 immune checkpoint inhibitors (ICI) singly or in combination with anti-CTLA-4 or other agents are approved across multiple indications in sixteen separate diseases including a histology-agnostic indication in patients with microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) solid tumors.^{1,2} The hallmark of ICI therapy is the durability of responses in a subset of patients as evidenced by progression-free survival (PFS) rates of 21–29% in melanoma and 22% in non-small cell lung cancer (NSCLC) with anti-PD(L)1 singly,^{3–7} and up to 36% with anti-PD-1/anti-CTLA-4 dual ICI in melanoma.⁸ However, the majority of patients do relapse and the question of how to improve outcomes in these patients remains a vexing problem for the field.

Biomarkers associated with an improved outcome to ICI therapy include CD8 T cell infiltrate (TIL),^{9,10} interferon (IFN)- γ gene expression signature,^{11,12} tumor mutation burden (TMB),^{12–14} and PD-L1 expression.^{15–18} However, not all T cell-inflamed tumors respond, and not all tumors are inflamed, underscoring the

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importance of therapeutic strategies directed at improving responses in T cell-uninflamed tumors.^{18–20} T cell-inflamed tumors are characterized by the presence of abundant CD8+ T cells, CXCR3-binding chemokines (such as CXCL9 and CXCL10) and to a lesser extent, B cells and plasma cells – collectively represented by IFN- γ gene expression signature.^{10,11,21,22} Conversely, the tumor microenvironment (TME) of T cell-uninflamed tumors is characterized by immuno-suppressive cells including regulatory T cells (T reg), myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM), vascular endothelial cells and cancer-associated fibroblasts (CAF).^{23–26} It remains unclear in individual tumors whether the lack of anti-tumor immunity is related to defects at the level of T cell priming/trafficking, other mechanisms mediating tumor-intrinsic mechanisms of immune exclusion in TME, or a combination of both factors.

Adaptive immune recognition of a diverse array of antigens is mediated by the structural diversity of B and T cell receptors – attributable to somatic hypermutation in V-region genes, and junctional and combinatorial diversity generated during gene rearrangement in B and T cells, respectively.²⁷ Conversely, the innate immune system comprising complement system, dendritic cells (DC), natural killer (NK) cells, macrophages, leucocytes and $\gamma\delta$ T cells, is an older, evolutionarily conserved system that serves to prevent the spread and movement of foreign pathogens.

The concept of microbial pattern recognition was first proposed by Charles Janeway Jr. and describes the two cardinal features of innate immunity: the ability to distinguish non-self infectious molecules conserved across microbial species – termed pathogen-associated molecular patterns (PAMPs) – from self, and the ability to elicit adaptive immune responses against non-self PAMPs.²⁸ Since the initial description in 1989, multiple pattern recognition receptors (PRR) have been described that play critical roles in innate and adaptive immune responses including Toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I)-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, and C-type lectin receptors (CLR) recognize and are critical to the activity of the innate immune system.^{29,30}

The various PRRs recognize different PAMPs, are differentially expressed on immune cells and localize to different cellular localities (see Figure 1).³¹ TLRs mediate innate immune responses to a wide variety of distinct PAMPs derived from various microorganisms including viruses, intra and extra cellular bacteria, and fungi; and are expressed

on a broad array of immune cells.³¹ RIG-I-like receptors (RLRs) RIG-I, MDA5, and LGP2 are broadly expressed in most tissues where they sense viral RNA ligands in the cytoplasm and trigger inflammatory and anti-viral innate immune responses.^{32,33} CLRs are expressed by multiple innate immune cells particularly DCs and myeloid cells and recognize a variety of glycans on pathogens and hence play an essential role in anti-fungal immune responses.³⁴ NLRs including NOD1 and NOD2 are found in the cytoplasm of lymphocytes, macrophages and DCs where they recognize peptidoglycan motifs from bacterial cells and cooperate with TLR to activate nuclear factor-kappaB (NF- κ B) signaling and mediate anti-bacterial immune responses.^{35,36} Stimulator of interferon genes (STING) is located in the endoplasmic reticulum of APCs, endothelial, and epithelial cells and is activated when plasma membrane-bound cGAS recognizes cytosolic double-stranded DNA. cGAS-STING signaling phosphorylates IRF3 leading to IFN- β transcription and type I IFN signaling.^{37,38}

Type I and II IFNs are involved in innate and adaptive immunity and play an essential role in mediating anti-tumor immunity. Type I IFNs – IFN- α and IFN- β – are expressed in nucleated cells and enhance tumor antigen expression, stimulate dendritic cells (DCs), facilitate CD8+ cell expansion and differentiation to memory type cells, apoptosis, and regulate T cell activity resulting in tumor regression.^{39,40} Type II IFN (IFN- γ) is mainly expressed in T_H1 cells, CD8+ T cells, and natural killer (NK) cells. IFN- γ decreases the activity of T reg cells, increases MHC I and II expression on DCs, enhances antigen presentation, activates proliferation of CD4+ and CD8+ T cells, and M1 macrophages.^{41–43} Through a downstream signaling cascade that begins with type I IFN induction, type I IFNs potently and rapidly translate signals from TLRs into effects on a broad array of tissues.⁴⁴

Compelling preclinical data suggest that intra-tumoral administration of TLR9 agonists improves APC activation, in particular DCs in tumor-draining lymph nodes resulting in proinflammatory cytokine release, increased expression of type I IFN genes and T cell priming converting T cell uninflamed TMEs to T cell inflamed ones.⁴⁵ Given the importance of type I IFN and CD8+ T cells in mediating response to ICI, there has been compelling interest in evaluating combinations of innate agonists, particularly TLR9 agonists, to augment response to ICI.^{10,46} The efficacy of TLR9 agonists with ICI therapy has been investigated in preclinical studies and is currently being tested in clinical trials; and represent a promising therapeutic strategy to overcome ICI resistance,

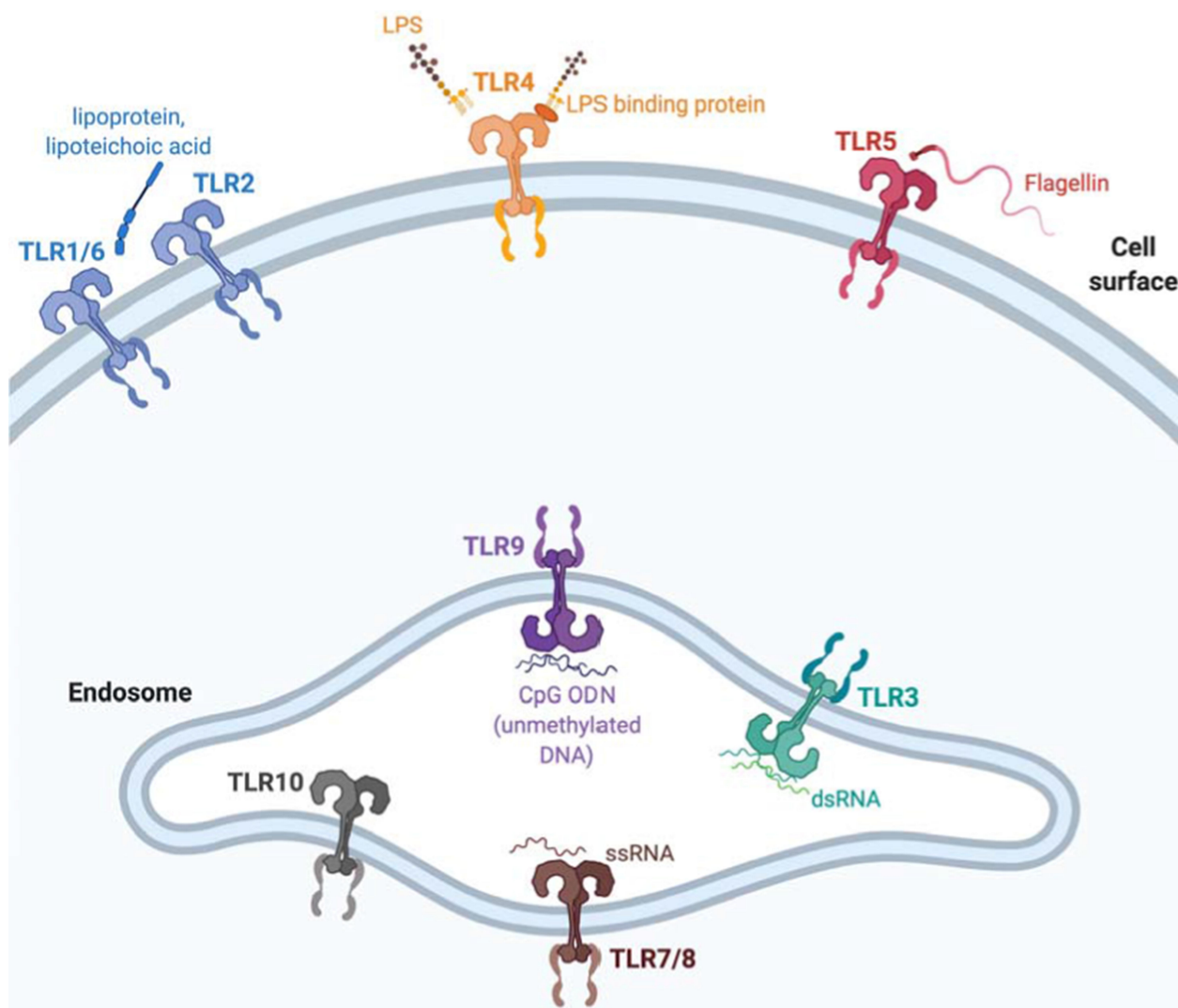


Figure 1 Cellular distribution of various TLR and respective ligands in humans

Notes: Created with BioRender.com.

Abbreviations: CpG, cytidine phosphate guanosine; dsRNA, double stranded RNA; LPS, lipopolysaccharide; ODN, oligonucleotide; ssRNA, single stranded RNA; TLR, Toll-like receptors.

particularly for T cell uninflamed tumors.^{47,48} In this review article, we describe TLR9 receptor distribution and function, describe TLR9 signaling and delineate the role of TLR9 agonists in cancer immunotherapy with a focus on ongoing clinical trials.

TLR9 Overview: Type and Distribution, Structure and Ligands, Activation and Signaling Pathways

TLR9 Type and Distribution

TLRs are homologous to the Toll receptor which was first identified in *Drosophila melanogaster* where it forms a complex with human nerve growth factor-like cysteine

knot protein Spätzle – the Toll-Spätzle complex – which is critical to both embryonic development and the generation of immune responses against fungi.^{49–51} Although structurally related to *Drosophila* TLR, molecular phylogenetic analyses have clarified that vertebrate TLR are highly conserved across various species and can be subclassified into six major families based on general class of PAMP recognized: TLR1 family which includes TLR1, TLR2, TLR6 and TLR10 (lipopeptide); TLR3 family [double stranded RNA (dsRNA)]; TLR4 family (lipopolysaccharide); TLR5 family (flagellin); TLR7–9 families [TLR7/8 – single stranded RNA (ssRNA) and TLR9 – double stranded DNA (dsDNA) or heme motifs]. The sixth remaining family – which includes TLR11-13 and

TLR21-23 subfamilies – is represented in humans only as a pseudogene.^{52,53} Following synthesis within endoplasmic reticulum, traffic to Golgi, and proper folding, TLRs are either recruited to the cell surface (cell surface TLR) or intracellularly to endosomes (endosomal TLR) – a distribution that reflects the likelihood of exposure to the particular viral and/or bacterial nucleic acids that the TLRs sense as illustrated in [Figure 1](#) (see [Figure 1](#)).

Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, whereas intracellular TLRs are localized in the endosome and include TLR3, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, and TLR13 – although TLR11, TLR12, and TLR13 are not expressed in human tissues.⁵⁴ Of the endosomal TLRs, TLR3 recognizes dsRNA, TLR7 and TLR8 recognize ssRNA while TLR9 recognizes dsDNA.^{55–57} Expression patterns of the endosomal TLRs further determine likelihood of encountering and responding to various PAMPs: TLR3 is expressed ubiquitously; TLR7 is expressed in lung, placenta and spleen; TLR8 is preferentially expressed in peripheral immune cells and lung tissue.⁵⁸ In humans, TLR9s are predominantly expressed by antigen presenting cells (APC) particularly B cells, T cells and DCs within immune-cell-rich tissues including spleen, lymph node, and bone marrow.^{59–62} In humans, TLR10 is primarily expressed within endosomes although its function remains puzzling. Compelling data suggest that TLR10 exerts both pro-inflammatory^{63,64} and anti-inflammatory⁶⁵ effects – former observed in the setting of viral infection, while the latter is mediated through a B cell-intrinsic process through antibody-mediated cross-linking. Overall, the ligand specificity and function of TLR10 remain less well characterized.

TLR7, TLR8 and TLR9 are primarily located in intracellular vesicles within the endoplasmic reticulum (ER) and translocate to endosomes upon stimulation by ligands.⁶⁶ The release of TLR9 from the ER is controlled by several mechanisms including tyrosine-based motifs in TLR9 cytoplasmic tail,^{67–70} and phosphorylation of TLR9.⁷¹ Key proteins required for TLR9 traffic from ER to endosomal compartments include glycoprotein 96 (gp96),⁷² UNC93B1,^{73,74} adapter protein 3 (AP-3),^{75,76} a protein associated with TLR4 (PRAT4A),⁷⁷ and Slc15a4.⁷⁵ The unique localization and trafficking requirements of the nucleic acid-sensing TLRs 7–9 serve as a regulatory mechanism to limit immune responses to host nucleic acids; and indeed, artificial localization of TLR9 to cell surface causes autoimmune manifestations.^{78,79} After leaving the ER, TLR9 and

other nucleic acid-sensing TLRs traffic to the endosomal compartment where they are proteolytically processed.

TLR9 Structure and Ligands

Structurally, TLRs are type I transmembrane glycoproteins comprising an extracellular N-terminal ligand recognition domain, a single transmembrane helix, and an intracellular C-terminal cytoplasmic signaling domain.⁵⁰ TLR extracellular domains (ECD) comprise repeated leucine-rich repeat (LRR) modules that bind PAMPs depending on TLR subtype as delineated in [TLR9 Type and Distribution](#).^{28,80} Each LRR module is 20–43 amino acids long and comprises a variable part and a highly conserved “LxxLxLxxN” motif where “L” is leucine, isoleucine, valine or phenylalanine and “N” is asparagine, threonine, serine or cysteine.^{81,82} LRR modules form one or two horseshoe domains wherein the “LxxLxLxxN” motifs are located in inner concave surfaces, while the variable parts form the outer convex surface. TLR receptor structure is characterized by constituent LRR motifs, repeat numbers and is flanked by two cysteine clusters including 2–4 cysteine residues across each TLR subtype as described in [TLR9 Type and Distribution](#).⁸³ The TLR intracellular domain (ICD) comprises ~150 amino acids and shares sequence homology with the signaling domains of IL-1R super-family, and hence is termed Toll/interleukin-1 receptor (TIR).^{84,85} The tertiary structures of TLR1-6 have been determined and have previously been summarized in other reviews on the topic.^{83,86}

Although TLRs 7–9 bear functional similarity to TLR3 in that they are localized intracellularly to endosomes and recognize nucleic acid PAMPs (see [TLR9 Type and Distribution](#)), the structures of TLRs 7–9 are markedly different from TLR3.^{83,87–90} The ECDs of TLRs 7–9 comprise 25 LRR modules, are heavily glycosylated, contain large insertions in LRRs 2, 5 and 8, and contain stretches of ~40 amino acid residues between LRRs 14 and 15.⁸⁶ The insertions arise from the glycan-free ECD surface involved in dimerization and hence, likely give rise to structures involved in dimerization; while the ~40 residue stretches show a high degree of species variability and hence, are unlikely involved in dimerization. The TLR9 (and TLR7 and 8) ECD contains a Z-loop or hinge region between LRR14 and LRR15 where proteolytic cleavage by a cysteine lysosomal protease occurs to form proteolytically cleaved TLR9 (amino acids 471–1032) that maintains the horseshoe shape of the protomer.^{90–92} Both full length and proteolytically cleaved TLR9 (amino acids 471–1032) are predominantly monomeric in the absence

of unmethylated cytidine phosphate guanosine (CpG). However, following ligand binding, proteolytically cleaved TLR9 dimerizes forming a homodimer.^{90,91,93}

TLR9 preferentially detects unmethylated CpG oligonucleotides (ODN) with a species-specific preference for hexamer CpG motifs (human 5'-GTCGTT-3 vs. murine 5'-GACGTT-3) that are less common in vertebrate DNA.⁹⁴ There are three major classes of CpG ODNs based on different backbones and sequence motifs. Type A CpG are characterized by: poly G sequence at 5' end, 3' end or both; internal palindromic sequence containing GC nucleotides; and a partial phosphorothioate (PS) modified backbone.⁹⁵ Type A CpG preferentially activate plasmacytoid DCs (pDC) and NK cells and induce significant IFN- α production by pDCs.^{96–98} Type B CpG are generally 18–28 nucleotides in length; have a complete PS-modified backbone; and contain one or more 6mer CpG motifs (5'-PuPyCGPyPu-3') with the most potent ODNs in this class containing three 6mer sequences.⁶¹ Type B CpG preferentially activate B cells and less so NK cells with no effect on DCs.^{99,100} Type C CpG have features of both classes A and B: a complete PS-modified backbone and an internal palindromic motif.¹⁰¹ Consequently, the effects of type C CpG comprise features of both classes: strong direct B cell stimulation, IFN- α production by pDC, APC activation and maturation, and indirect NK cell activation.¹⁰²

Besides species-specific ODN sequence preference for TLR9 activation, other factors including number and position of CpG motifs, nucleotides adjacent to CG dinucleotide, and ODN secondary structure influence ODN potency for TLR9 activation in humans.^{103–106} Separately, when considering the effects of TLR9 agonists in primates as compared to mice, it is important to consider the differential distribution and localization of TLR9 receptors in humans and mice.¹⁰⁷ Synthetic TLR9 agonists, which are being used in clinical trials, have structural differences, which makes them nuclease resistant and also increases their half-life.¹⁰⁸ A comprehensive list of synthetic TLR9 agonists categorized by type, structure and current status of clinical evaluation is provided in [Table 1](#).

TLR9 Activation and Signaling Pathways

Activation of TLR9 signaling requires two CpG ODN to symmetrically bind to the C-terminal fragment of one TLR9 protomer and the CpG-binding groove in the N-terminal fragment of another, creating a homodimer.^{90,91,93} ODN that bind only to the N-terminal fragment are

inhibitory,¹⁰⁹ while methylated single stranded DNA (ssDNA) and dsDNA have a lower affinity for TLR9 and induce less TLR9 dimerization.^{110–112} Given the differential immunostimulatory activity of unmethylated and methylated CpG ODN, it was initially thought that methylation status of CpG ODN represented a means by which vertebrate TLR9 distinguished between pathogenic bacterial (unmethylated) and eukaryotic (methylated) DNA.¹¹² However, it has since been shown that while TLR9 can recognize both unmethylated and methylated DNA; the greater immunostimulatory activity of unmethylated CpG ODN is secondary to an upstream process wherein unmethylated (but not methylated) CpG ODN induces TLR9 mobilization to the late endosomal compartment in a src-family kinase (SFK)-mediated signaling process, that permits co-localization irrespective of methylation status.^{113,114} Besides CpG ODN, TLR9 may be activated by endogenous ligands including heat shock protein (HSP), surfactant protein A (SP-A), fibronectin, high mobility group box 1 (HMGB1), in addition to numerous synthetic ligands.⁶⁰

TLRs differentially recruit specific combinations of one or more among four TIR domain-containing adaptors that mediate downstream signaling: myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor-inducing IFN- β (TRIF), TIR-containing adaptor protein/MyD88-adaptor-like (TIRAP/MAL), or TRIF-related adaptor molecule (TRAM).¹¹⁵ All TLR signaling pathways culminate in activation of the transcription factor NF- κ B – either through MyD88-dependent or MyD88-independent (or TRIF-dependent) mechanisms.¹¹⁵ MyD88 consists of an N-terminal death domain (DD) and a C-terminal TIR domain.¹¹⁶ Following TLR activation, MyD88 DD interacts with DDs of IL-1 receptor-associated kinase (IRAK) family of protein kinases, particularly IRAK1 and IRAK4.^{117,118} Following sequential phosphorylation of IRAK1 and IRAK4, these dissociate from MyD88 and interact with TNF receptor-associated factor 6 (TRAF6), a RING-type ubiquitin E3 ligase.¹¹⁹ TRAF6 promotes ubiquitination of target proteins, including TRAF6 itself and NF- κ B essential modifier (NEMO) along with the ubiquitin-conjugating enzyme complex Uev1A-Ubc13.^{120,121} Ubiquitinated NEMO and TRAF6 recruit transforming growth factor- β -activated kinase-1 (TAK1) and TAK1-binding proteins (TABs) to activate either mitogen-activated protein kinase (MAPK) (ERK, JNK, p38) pathway or the inhibitor of nuclear factor- κ B (I κ B) kinase (IKK) complex which leads to NF- κ B signaling.¹²² Separate from the MyD88-dependent pathway, TLR3 and TLR4 in particular can activate NF- κ B

Table 1 Toll-Like Receptor 9 Agonists Recently or Currently in Development

| TLR9 Agonist | Structure | CpG Type | ROA | Tumor Types Being Evaluated |
|---|--|-------------|------------------------|--|
| Cavrotolimod/ AST-008 (Exicure) | SNA with two components: (1) densely packed shell of CpG ODN radially oriented around (2) a core nanoparticle, which may be solid or hollow which facilitates increased cellular uptake | Undisclosed | IT | Melanoma; MCC; HNSCC; cSCC; advanced solid tumors |
| CMP-001 (Checkmate) | G10 CpG ODN which forms G-quadruplex that mimics retroviral DNA encapsulated within 30 nm VLP comprised of capsid proteins derived from Q β bacteriophage known as G10 | A | SC IT | Melanoma; NSCLC; CRC |
| CpG-28 (University of Paris) no longer in development) | 5'-TAAACGTTATAACGTTATGACGTCAT-3' sequence with 3 CpG motifs and a fully phosphorothioate backbone | B | Intracerebral infusion | Glioma; glioblastoma |
| EnanDIM (Mologen AG) (no longer in development) | Member of the dSLIM family exhibiting a double stem of 28 base pairs and two loops with 30 nucleotides – each containing three CG motifs – that has a linear structure | Undisclosed | SC IT | Preclinical development |
| IMO-2055 (Idera) (no longer in development) | Phosphorothioate sequence with two CpG ODN, synthetic immunostimulatory motifs and two 5' ends for increased metabolic stability | B | SC IT | Melanoma; NSCLC; CRC; HNSCC; and refractory solid tumors |
| IMO-2125/ tilsotolimod) (Idera) | Phosphorothioate sequence with three CpG ODN | B | IT | Melanoma; CRC |
| MGN1703/ Lefitolimod (Mologen AG) (no longer in development) | Member of the dSLIM family exhibiting a double stem of 28 base pairs and two loops with 30 nucleotides – each containing three CG motifs – that is dumbbell-shaped and covalently-closed | Undisclosed | SC IT | Melanoma; CRC; RCC; advanced solid tumors |
| NZ-TLR9 (LIDDS) | Undisclosed TLR9 structure encapsulated within a calcium sulfate excipient (NanoZolid) that permits slow release depot | Undisclosed | Undisclosed | Preclinical development |
| PF-3512676 (Pfizer) (no longer in development) | Phosphorothioate backbone with one or more CpG dinucleotides | B | SC IV IT | Melanoma; NSCLC; MF; CLL; CTCL; NHL |
| SD-101 (Dynavax) (no longer in development) | 30 nucleotide phosphorothioate sequence with multiple CpG motifs and internal palindromic sequences | C | SC IT | Melanoma; lymphoma; HNSCC; prostate cancer; breast cancer; refractory solid tumors |
| S-540956 (Shionogi) | Undisclosed | Undisclosed | Undisclosed | Preclinical development |

Abbreviations: CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; cSCC, cutaneous squamous cell carcinoma; CTCL, cutaneous T cell lymphoma; dSLIM, double-stem loop immunomodulator; HNSCC, head and neck squamous cell cancer; IT, intra tumoral; IV, intravenous; MCC, Merkel cell carcinoma; MF, mycosis fungoides; N/A, not applicable; NHL, non-Hodgkin Lymphoma; NSCLC, non-small cell lung cancer; ODN, oligodeoxynucleotide; RCC, renal cell carcinoma; ROA, route of administration; SC, subcutaneous; SNA, spherical nucleic acid; VLP, virus-like particle.

signaling through a TRIF-dependent pathway mediated through either TRAF6 or receptor interacting protein 1 (RIP1).^{123–125} TRIF directly binds to TRAF6 via its TRAF6-binding motifs.^{124,126} TRAF6 then activates NF- κ B signaling via a TAK1-dependent mechanism similar to what occurs in the MyD88-dependent pathway.¹²⁴ TAK1-deficient mice demonstrate impaired NF- κ B and MAPK activation in response to multiple TLR ligands underscoring the importance of TAK1 in mediating NF- κ B and MAPK activation through either MyD88-dependent or TRIF-dependent pathways.^{127,128}

TLR signaling is homeostatically regulated by a number of mechanisms, failure of which results in autoimmunity and/or inflammatory diseases. The MyD88-dependent pathway can be inhibited by ST2825, SOCS1, and Cbl-b; while the TRIF-dependent pathway is suppressed by SARM and TAG.^{129,130} Aside from these, other molecules can directly inhibit binding of MyD88 or TRIF with TLRs or downstream molecules including TRAF3, TRAF6 and TAK1 – summarized in detail elsewhere.¹³¹ Finally, NF- κ B signaling can directly be directly suppressed by cI-3, I κ BNS, Nurr1, ATF3, and/or PDLIM2.¹³²

B cells and pDC constitutively express TLR7 and TLR9 and produce large amounts of type I IFNs following exposure to cognate ligands ssRNA and CpG ODN, respectively. TLR9 (along with TLR7) signal via the MyD88-dependent pathway. While activation of MAPK and NF- κ B signaling via a TAK1-dependent process, type I IFN production is mediated by IRF7 that is phosphorylated in the cytoplasm but subsequently translocates into the nucleus.^{133,134} The critical role played by IRF7 in response to TLR7/TLR9 signaling is underscored by the inability of IRF7-deficient (but not IRF3-deficient) mice to produce normal amounts of IFN- α in response to TLR7/TLR9 ligands.¹³⁵ The TLR9 signaling pathway and pDC activation are illustrated in [Figure 2](#).

TLR9 activation on DCs and pDCs, in particular, prompts the secretion of large quantities of type I IFNs, which has direct (tumor cell inhibition) and indirect (anti-tumor immune responses) effects on cancer cells, most pronounced in the early stages of the anti-tumor immune response. Type I IFN production in the tumor facilitates antigen cross-presentation by DC, which subsequently migrates to the tumor-draining lymph nodes where they cross-prime naive CD8⁺ T cells, further amplifying anti-tumor immune responses.^{136,137} This results in DC

maturation (with expression of CD80 and CD86), increased MHC class I expression, and secretion of IL-12 leading to a T_h1 skew.^{46,138,139}

Preclinical and Clinical Studies of TLR9 Agonists

Preclinical Studies of TLR9 Agonists

The systemic and local use of TLR9 agonists as monotherapy and in combination with chemotherapy, radiation, targeted therapy, and immunotherapy have been explored in several preclinical models with promising therapeutic potential. In syngeneic models in multiple tumors including breast cancer, lung cancer, melanoma, colon, cervical, pancreatic cancer and lymphoma, TLR9 agonists inhibited tumor growth singly and in combination with chemotherapy, and targeted therapy including EGFR and Her-2/neu directed therapies.^{140–143} Across these various studies, TLR9 agonists were administered topically, subcutaneously, intratumorally or systemically; although subcutaneously and intra-tumoral administration resulted in greater tumor retention compared to intravenous administration. Intra-tumoral injections typically induced tumor rejection, and in some instances, potent abscopal effects in distant non-treated tumors were also observed.

Given the immunomodulatory role of radiation therapy (RT), several preclinical studies have evaluated the combination of TLR9 agonists and RT and demonstrated synergy in immunocompetent murine tumor models.^{144,145} In these studies, mice were able to reject tumor rechallenge demonstrating evidence of immunological memory. The anti-tumor effects of RT and TLR9 agonist combination therapy were abrogated in nude mice suggest that immune cells mediated these effects.^{146,147} Using a LLC model, Zhang et al demonstrated that RT/TLR9 combination led to a more potent tumor-specific humoral response compared to either TLR9 or RT singly.¹⁴⁶ Further, the treatment was still effective in B cell-deficient mice although less so than in non-deficient mice, suggesting that although not critical, B cells were at least partly responsible for the anti-tumor effects observed.¹⁴⁶

A major barrier limiting the effect of anti-PD(L)1 and/or anti-CTLA-4 ICI in tumors is the lack of spontaneous tumor-infiltrating T cells and defective IFN- α production in the tumor microenvironment (TME) in non-inflamed tumors.^{12,46} Preclinically, TLR9 agonists are potent adjuvants of cancer vaccines with strong immunostimulatory

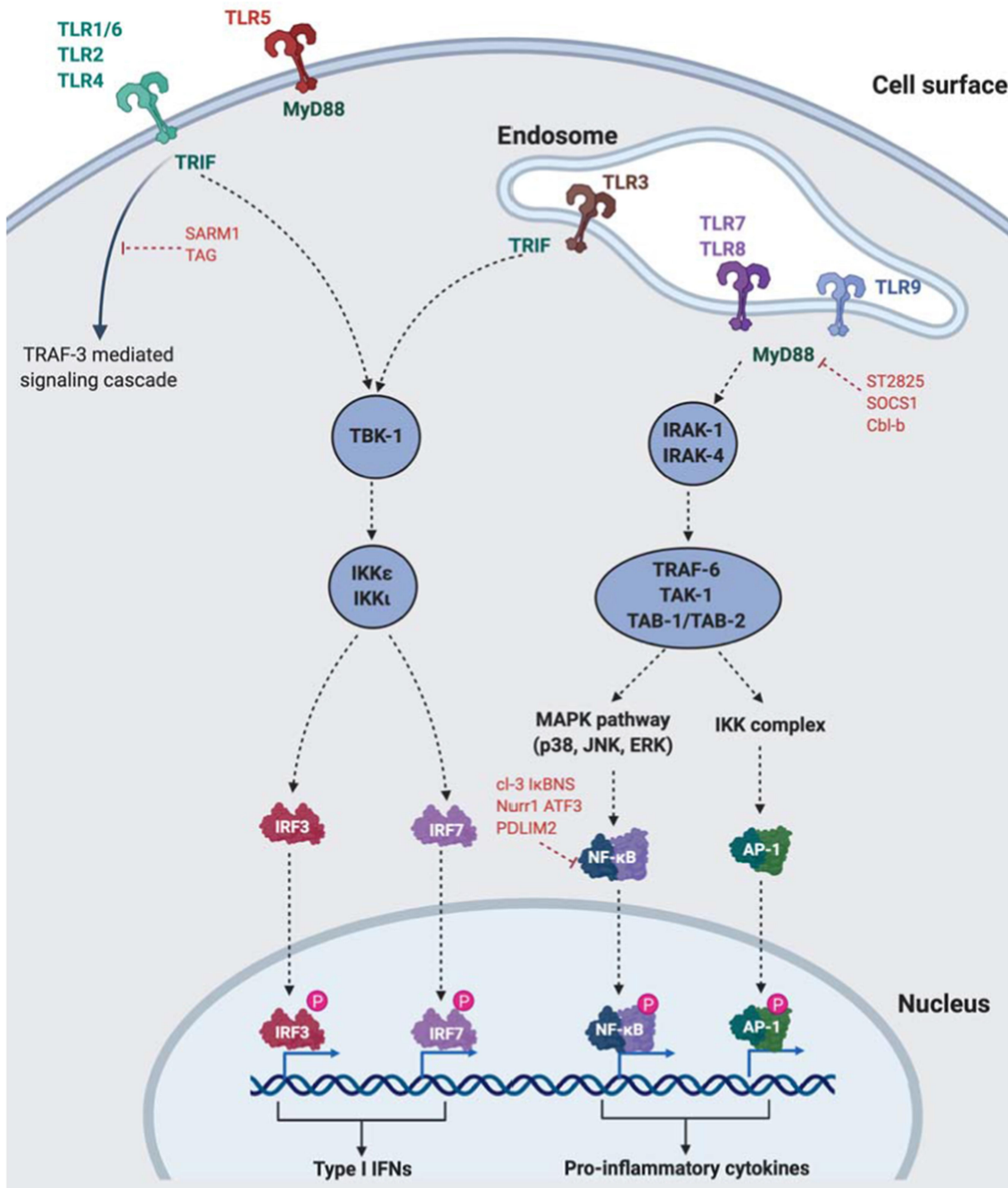


Figure 2 TLR and TLR9 cellular location and downstream signaling

Notes: Created with BioRender.com.

Abbreviations: AP-1, activator protein 1; IRF, interferon regulatory factor; IKK, IκB kinase; MAPK, mitogen activated protein kinase; MyD88, myeloid differentiation primary response 88; NFκB, nuclear factor kappa beta; SARM1, sterile α and TIR motif containing 1; TAK1, transforming growth factor-β-activated kinase-1; TLR, toll-like receptor; TRIF, TIR domain-containing adaptor-inducing IFN, beta; TRAF6, TNF receptor-associated factor 6 (TRAF6); TRAM, TRIF-related adaptor molecule; TIRAP/MAL, TIR-containing adaptor protein/MyD88-adaptor-like.

effects. TLR9 agonists in combination with peptide vaccines increase antigen-specific T cells in multiple tumors including melanoma, NSCLC, breast cancer, and sarcoma.^{148–151} These antigen-specific T cells are detectable ex vivo, produce IFN-γ, and are lytic.¹⁴⁸ Several lines of evidence suggest that TLR9 agonists in combination with ICI may enhance anti-

tumor T cell responses and augment clinical benefits as compared with either agent singly. Firstly, TLR9 agonists expand antigen-specific CD8+ T cells that upregulate PD-1 expression.^{152,153} Secondly, TLR9 agonists in combination with anti-PD-1 ICI transform the TME, increasing CD8+ T cells, NK cells, DCs, and B cells even in PD-1 non-

responding tumors.^{153,154} Thus, TLR9 agonists, particularly in combination with ICI, offer a promising therapeutic agent to circumvent the lack of IFN- α production observed in uninfamed tumors, which are poorly T cell-infiltrated and often fail to respond to immune checkpoint blockade.

TLR9 agonists have demonstrated potent synergy with anti-PD-1 and/or anti-CTLA-4 ICI in multiple models including melanoma,^{153,155} lung cancer,¹⁵⁶ HPV positive oropharyngeal cancer,¹⁵⁷ head and neck squamous cell carcinoma,¹⁵⁸ breast cancer,¹⁵³ colorectal cancer,¹⁵³ and lymphoma.¹⁵⁹ Using two separate melanoma models of diametric immunogenicity, Reilley et al demonstrated the importance of intra-tumoral therapy and TME-centric immune activation in mediating responses to TLR9 agonist and anti-PD-1/anti-CTLA-4 ICI.¹⁵⁵ In immunogenic B16/OVA melanoma, TLR9 agonist monotherapy resulted in tumor regression in treated and untreated tumors associated with increased infiltration of tumor antigen-specific T cells, reduced T regs and proinflammatory cytokines.¹⁵⁵ Conversely, in non-immunogenic B16/F10 melanoma, while intra-tumoral TLR9 agonist combined with anti-PD-1 or anti-CTLA-4 ICI resulted in regression of treated tumors, untreated tumors did not respond; although this was overcome partially by a more potent anti-CTLA-4 antibody and TLR9 agonist, underscoring the importance of the relative potency of these agents in mediating their effects. The antitumor effects of CpG/IL-10 combination

were abrogated in germ-free (GF) or antibiotic-treated mice as a result of the failure of tumor-infiltrating myeloid cells to produce inflammatory cytokines such as TNF and IL12 – linking intestinal microbiota composition to the outcome of TLR9 agonism, an adding the composition of the gut microbiome as another dimension affecting the outcome of TLR9 agonism and ICI.^{160,161}

In summary, TLR9 agonists result in secretion of proinflammatory cytokines, chemokines such as monocyte chemoattractant protein-1 (MCP-1), IFN- γ -inducible 10 protein (IP-10), activation of pDCs, NK cells and T_H1 polarization. TLR9 agonists have demonstrated single agent activity and immunological memory in immunocompetent murine models, primarily with intra-tumoral rather than systemic/subcutaneous drug administration. Synergies observed with RT and with immunotherapies in particular anti-PD-1 and/or anti-CTLA-4 ICI argued for further studies in humans. Below we summarize the safety and efficacy data for TLR9 agonists in advanced solid malignancies.

Clinical Trials Evaluating TLR9 Agonists in Solid Tumors: Safety

TLR9 agonists have been evaluated in a plethora of clinical trials singly and in combination with chemotherapy, radiotherapy, and immunotherapy. Select monotherapy and

Table 2 Clinical Trials of TLR9 Agonists as Monotherapy

| TLR9 Agonist | Study Phase | Histology | ROA | Safety | Efficacy |
|--------------------------------------|-------------|------------------------------|--|----------------------------------|---|
| PF-3512676 (CPG 7909) ¹⁶³ | Phase I | Advanced BCC or melanoma | SC 0.01 to 2.5 mg, every 2 weeks | 1 grade 3 event | ORR (BCC): 1 CR (1/5, 20%), 4 PR (80%) ORR (melanoma): 1 CR (1/5, 20%) |
| PF-3512676 (CPG 7909) ¹⁶⁴ | Phase I | CTCL | SC 0.08–0.36mg/kg, weekly | 9/28 (32%) grade ≥ 3 events | 32% per CAILS |
| PF-3512676 (CPG 7909) ¹⁶⁵ | Phase I | Advanced RCC | SC 0.08–0.81mg/kg, weekly | 6/40 (15%) grade ≥ 3 events | ORR: 5% (2/39) |
| MGN1703 ¹⁶⁶ | Phase I | All solid tumors, testing | SC 0.25–60mg, twice weekly | 2 grade 3 events | ORR: 0% 25% (6/24) SD |
| CMP-001 ¹⁶⁷ | Phase I | Advanced metastatic melanoma | IT 5–10mg weekly for 7 weeks, then q3 weekly | Not reported | ORR 22% (5/23) |
| PF-3512676 (CPG 7909) ¹⁶⁸ | Phase II | Advanced metastatic melanoma | SC 0.08–0.81mg/kg, weekly | 5/20 (20%) grade ≥ 3 events | ORR: 10% (2/20) SD rate: 15% (3/20) |

Abbreviations: BCC, basal cell carcinoma; CAILS, Composite Assessment of Index Lesion Severity; CTCL, cutaneous T cell lymphoma; CR, complete response; ORR, overall response rate; PR, partial response; ROA, route of administration; RCC, renal cell carcinoma; SD, stable disease.

Table 3 Clinical Trials of TLR9 Agonists in Combination Trials

| TLR9 Agonist | Study Phase | Histology | Combination Agent | ROA | Efficacy |
|--------------------------------------|-------------|--|---|---------------------------|---|
| PF-3512676 (CPG 7909) ¹⁷³ | II | Advanced metastatic melanoma | MART-1 (26–35, 27L), gp100 (209–217, 210M), and tyrosinase (368–376, 370D) vaccine with GM-CSF | SC 1.8mg every 2 weeks | ORR 9% (2/22) SD rate 36% (8/22) |
| PF-3512676 (CPG 7909) ¹⁷⁶ | II/III | Advanced metastatic melanoma | PF-3512676 ± DTIC 850mg/m ² | SC 10mg vs 40mg weekly | ORR: PF-3512676 10mg 2% PF-3512676 40mg 0% PF-3512676 40mg + DTIC 16% DTIC 8% |
| PF-3512676 (CPG 7909) ¹⁷⁷ | III | Advanced chemotherapy-naïve NSCLC | Carboplatin AUC6 with paclitaxel 200mg/m ² ± PF-3512676 | SC 0.2mg/kg every 2 weeks | ORR: Carboplatin/paclitaxel 23% Carboplatin/paclitaxel + PF-3512676 28% Median OS: Carboplatin/paclitaxel 9.8 months Carboplatin/paclitaxel + PF-3512676 10.0 months |
| PF-3512676 (CPG 7909) ¹⁷⁸ | II | Advanced chemotherapy-naïve NSCLC | Carboplatin AUC 6 or cisplatin 75mg/m ² with paclitaxel 175mg/m ² or docetaxel 75mg/m ² ± PF-3512676 | SC 0.2mg/kg every 2 weeks | ORR: Chemotherapy 11% Chemotherapy + PF-3512676 19% Median OS: Chemotherapy 6.8 months Chemotherapy + PF-3512676 12.3 months |
| IMO-2055 ¹⁷⁹ | IB | Advanced chemotherapy-refractory NSCLC | Erlotinib 150mg daily + bevacizumab 15mg/kg q3 + IMO-2055 | SC 0.08–0.48 mg/kg weekly | ORR 15% (5/33) |

(Continued)

Table 3 (Continued).

| TLR9 Agonist | Study Phase | Histology | Combination Agent | ROA | Efficacy |
|--------------------------------------|-------------|---|--|----------------------------------|--|
| PF-3512676 (CPG 7909) ¹⁸⁰ | II | Advanced recurrent <i>EGFR</i> mutant NSCLC | Erlotinib 150mg daily ± PF-3512676 | SC 0.2mg/kg weekly | ORR: Erlotinib 5% (1/21) Erlotinib + PF-3512676 10% (2/22) Median PFS: Erlotinib 1.7 months Erlotinib + PF-3512676 1.6 months Median OS: Erlotinib 4.7 months Erlotinib + PF-3512676 6.4 months |
| PF-3512676 (CPG 7909) ¹⁸¹ | I | Advanced chemotherapy-naïve NSCLC Japanese patients | Carboplatin AUC6 with paclitaxel 200mg/m ² and PF-3512676 | SC 0.1 vs 0.2mg/kg every 2 weeks | ORR 8% (1/12) SD rate 25% (3/12) |
| PF-3512676 (CPG 7909) ¹⁸² | III | Advanced chemotherapy-naïve NSCLC | Cisplatin 75mg/m ² with gemcitabine 1250mg/m ² ± PF-3512676 | SC 0.2mg/kg weekly | ORR: Chemotherapy 31% Chemotherapy + PF-3512676 32% Median PFS: Chemotherapy 5.1 months Chemotherapy + PF-3512676 5.1 months Median OS: Chemotherapy 10.7 months Chemotherapy + PF-3512676 11.0 months |
| IMO-2055 ¹⁸³ | IB | Advanced chemotherapy-refractory CRC | FOLFIRI/cetuximab with escalating doses of IMO-2055 | SC 0.16–0.48mg/kg weekly | ORR 14% (2/14) |
| PF-3512676 (CPG 7909) ¹⁸⁶ | I | Advanced melanoma and other solid tumors | Tremelimumab 6.0, 10.0, or 15.0 mg/kg every 12 weeks with escalating doses of PF-3512676 | SC 0.05–0.15mg/kg weekly | ORR 12.5% (2/16) |

(Continued)

Table 3 (Continued).

| TLR9 Agonist | Study Phase | Histology | Combination Agent | ROA | Efficacy |
|--------------------------------------|--------------------------------|--|--|--|---|
| Lefitolimod (MGNI703) ¹⁸⁹ | II open-label | ES SCLC following response (CR/PR) to 1L platinum-based chemotherapy | Cycle 5 and 6 of platinum-based chemotherapy ± lefitolimod (MGNI703) | SC 60mg twice weekly | ORR: Chemotherapy 8% Chemotherapy + MGNI703 12% Median PFS: Chemotherapy 4.0 months Chemotherapy + MGNI703 3.2 months Median OS: Chemotherapy 9.7 months Chemotherapy + MGNI703 10.0 months |
| Lefitolimod (MGNI703) ¹⁹⁰ | II blinded, placebo-controlled | CRC following disease control (CR/PR/SD) to 1L platinum-based chemotherapy ± bevacizumab | Lefitolimod (MGNI703) or placebo | SC 60mg twice weekly | Median PFS: Placebo 2.6 months MGNI703 2.8 months Median OS: Placebo 15.1 months MGNI703 22.6 months |
| SD-101 ¹⁵⁴ | Phase Ib | Advanced metastatic PD-I refractory and PD-I naïve melanoma | Pembrolizumab 200mg q3w with IT SD-101 | IT 1, 2, 4, 8mg weekly | ORR (PD-I naïve) 100% (7/7) ORR (PD-I refractory) 17% (2/12) |
| CMP-001 ¹⁶⁷ | Phase Ib | Advanced metastatic PD-I refractory melanoma | Pembrolizumab 200mg q3w with IT CMP-001 | IT 5 or 10mg weekly for 7 weeks then q3w | ORR (CMP-001 dose-escalation) 23% (10/44) ORR (1st expansion) 15% (10/69) ORR (2nd expansion) 26% (8/31) |

Abbreviations: AUC, area under curve; DTIC, dacarbazine; ES SCLC, extensive-stage small cell lung cancer; IT, intratumoral; PFS, progression-free survival; ORR, overall response rate; OS, overall survival; Q3W, every 3 weeks; SC, subcutaneous; SD, stable disease.

combination trials are summarized in Tables 2 and 3 respectively (see Tables 2 and 3). Of note, in dose-escalation studies of TLR9 agonists, incidence of adverse events (AE) did not increase in a dose-dependent fashion; and maximal tolerated

dose (MTD) was typically not achieved. Class-related AEs included flu-like symptoms, were generally of low-severity, with a low incidence (3–10%) of grade 3/4 events and tended to peak between third and sixth doses.

Cytokine release syndrome (CRS) is a severe AE that can occur after TLR9 agonist injections and is related to IFN-induced cytokine and chemokine release. CRS, as observed with TLR9 agonists, is graded and managed similarly as chimeric antigen receptor-T cell therapy-related CRS. As CRS symptoms can appear rapidly, patients treated in TLR9 agonist studies are typically monitored for 4–6 hours following TLR9 agonist administration. CRS prophylaxis with low-dose corticosteroids is not typically mandated; although corticosteroid prophylaxis is recommended for patients who experienced Grade 3 and 4 toxicity with prior injections or with concomitant adrenal insufficiency. Other class-specific AEs include injection site reactions with subcutaneous and intra-tumoral injection of drugs.

Studies of TLR9 agonists in combination with chemotherapy, targeted therapy, radiation, vaccines and other immunotherapy agents have generally not been associated with higher than expected AEs with one exception. IMO-2055 was studied in combination with 5FU/Cisplatin/Cetuximab in recurrent/metastatic HNSCC in a phase IB trial that was terminated early due to the higher rate of observed toxicities.¹⁶² All patients in the study experienced at least one adverse effect at any grade, and 92% had Grade 3 and greater AEs, with 31% considered related to IMO-2055 including injection site reactions, QT prolongation, bacteremia, and sepsis. TLR9 agonists have been studied in combination with various ICI including anti-PD-(L)1 and/or anti-CTLA-4 inhibitors with no significant additional AEs aside from class-specific flu-like symptoms and injection site reactions. Of note, TLR9/ICI combinations do not appear to be associated with increased frequency of immune-related adverse events (irAEs) in comparison to ICI monotherapy.

Clinical Trials Evaluating TLR9 Agonists in Solid Tumors: Efficacy

When used as monotherapy in advanced solid tumors, TLR9 agonists including PF-3512676 (CPG 7909) and MGN1703 did not reveal any clinically meaningful efficacy results in advanced solid tumors and chronic leukemias with CMP-001 being a rare exception albeit in a small number of patients (see Table 2).^{163–168} CpG-28 is a CpG ODN administered via intra-cerebral infusion to facilitate maximal brain penetration that was evaluated in treatment-refractory high-grade gliomas and glioblastomas (GBM) with minimal toxicity and preliminary evidence of efficacy in a difficult patient population in phase I and II

trials.^{169,170} However, in a randomized trial of standard chemoradiotherapy with or without CpG-28 injected into surgical cavity post tumor removal, the addition of CpG-28 did not improve survival.¹⁷¹ Phase I single agent data for motolimod (VTX2337), lefitolimod (MGN1703), tilso-tolimod (IMO-2125), and SD-101 have not been published.

Intra-tumoral vaccination with CpG in combination with peptide vaccination resulted in increased tumor-antigen specific CD8+ T cells in multiple tumors including melanoma, NSCLC, breast cancer and sarcoma.^{172–174} These tumor-antigen specific CD8+ T cells were detectable *ex vivo*, produced IFN- γ and were lytic; and CpG administration expanded tumor-antigen specific clonotypes and primed non-TA-reactive CD8+ T cells resulting in expansion of novel clonotypes.^{148,175} However, while results of local CpG in combination with peptide vaccination treatments were promising in melanoma,¹⁷³ systemic administration of CpG in combination with chemotherapy did not result in clinical responses in melanoma (PF-3512676),¹⁷⁶ NSCLC (PF-3512676 and IMO-2055),^{177–182} and colorectal cancer (IMO-2055).¹⁸³

Combinations with radiation have been evaluated in indolent lymphomas and mycosis fungoides.^{172,184,185} In mycosis fungoides, PF-3512676 combined with *in situ* vaccination with RT produced meaningful responses in distant lesions along with significant reductions in T regs intratumorally.¹⁸⁴ In patients with treatment-naive indolent lymphomas, intra-tumoral SD-101 in combination with low-dose RT was well tolerated. Responses were observed in both treated and untreated lesions, and were associated with increases in CD8+ and CD4+ effector T cells.¹⁸⁵

While it is clear that CpG administration strongly induces tumor-specific CD8+ T cell responses, objective responses are rare and T cell responses are not sustained – suggesting that augmentation of T cell trafficking with anti-PD-1 and/or anti-CTLA-4 ICI may be additive. As delineated above, in preclinical models, intra-tumoral (rather than systemic) TLR9/ICI combinations are efficacious with evidence of increased tumor-specific CD8+ T cells and reduced T regs. A phase I study of PF-3512676 with CTLA-4 inhibitor tremelimumab demonstrated few responses (2/17, 6%) in heavily pre-treated melanoma albeit with a high rate of toxicity that precluded further development.¹⁸⁶

Below we describe the current state of development for some of the TLR9 agonists in advanced testing, with

a focus on combinations with ICIs in various solid tumors, summarized in Table 3 (see Table 3).

Lefitolimod (MGN1703)

Lefitolimod (MGN1703) is a synthetic type C TLR9 agonist of the double-stem loop immunomodulators (dSLIM) family comprising a covalently closed dumbbell-shaped structure with a middle 28 base pair section flanked by two 30 nucleotides single-stranded loops at either end.^{187,188} Following a promising phase I study in advanced malignancies, lefitolimod (MGN1703) was rapidly advanced into the maintenance setting in two phase II trials in extensive-stage small cell lung cancer (IMPULSE) and metastatic colorectal cancer after front line chemotherapy.^{189,190} While primary endpoint of improved overall/progression-free survival was not met in the intent-to-treat arm in either study, improved pharmacodynamic and efficacy signals in subgroup analyses prompted a phase III trial of subcutaneous lefitolimod (MGN1703) as maintenance therapy compared with standard maintenance regimens in patients with metastatic CRC who had achieved tumor reduction with induction therapy (IMPALA, NCT02077868). Negative results have been reported and the further development of this agent is uncertain at this time.

Tilsotolimod (IMO-2125)

Tilsotolimod (IMO-2125) is a type B TLR9 agonist containing a PS backbone with the sequence 5'-TCG*AACG*TTCG*-X-G*CT TG*CAAG*CT-5' where G* represents 2'-deoxy-7-deaza-guanosine and X is a glycerol linker.¹⁹¹ Tilsotolimod (IMO-2125) is being evaluated in multiple solid tumors with the development in advanced melanoma being furthest along. ILLUMINATE-204 is a multi-center, phase 1/2 trial in patients with anti-PD-1 refractory advanced melanoma wherein escalating doses of tilsotolimod (IMO-2125) was studied with either ipilimumab or pembrolizumab in the phase I portion; after which tilsotolimod (IMO-2125) 8mg in combination with ipilimumab was studied in phase II portion. In 49 evaluable anti-PD1 refractory melanoma patients, investigators reported objective response rates (ORR) of 22% with 71% disease control rate and 48% grade 3/4 treatment-related adverse events (TRAE), although the frequency of irAE was not significantly increased.¹⁹² The combination of tilsotolimod (IMO-2125) and ipilimumab is being evaluated in a pivotal phase III trial (ILLUMINATE-301) in anti-PD-1 refractory advanced melanoma compared to

ipilimumab; while a separate study (ILLUMINATE-206) is studying tilsotolimod (IMO-2125) with ipilimumab/nivolumab in microsatellite-stable colorectal cancer.

SD-101

SD-101 is a type C TLR9 agonist that induces large quantities of type I IFNs.¹⁵³ In combination with RT in treatment-naïve B cell lymphoma, SD-101 was well tolerated and demonstrated abscopal responses.¹⁸⁵ When studied in PD-1 naïve and PD-1 relapsed/refractory melanoma in combination with anti-PD-1 ICI pembrolizumab, ORR with SD-101/pembrolizumab was 78% in treatment-naïve and 15% in PD-1 relapsed/refractory melanoma; while induction of IFN-responsive genes and significant increases of inflammatory cells including CD8+ T cells were observed in circulating leucocytes and in tumors, respectively.¹⁵⁴

CMP-001

CMP-001 is a type A CpG composed of (i) a virus-like particle (VLP) comprising capsid proteins derived from bacteriophage Qb, which encapsulate (ii) CpG ODN G10 which is a 30-nucleotide strand, flanked by 10 guanines on either end designed to induce high levels of type I IFN production. CMP-001 was designed to be taken up by pDC via anti-Qb antibodies that bind to FcR γ on pDC facilitating uptake and enabling antigen cross-presentation to T cells and other effector cells. Following CMP-001 injection intratumorally, anti-Qb antibodies are rapidly generated. These anti-Qb antibodies facilitate opsonization of CMP-001 by pDC in a FcR γ -dependent fashion that promotes pDC uptake, IFN- α induction and are critical to anti-tumor efficacy.¹⁵⁹ Although subcutaneous dosing of CMP-001 is being explored in several studies, it is unclear whether this mode of drug delivery results in anti-Qb antibody generation and CMP-001 opsonization to the degree observed with intra-tumoral therapy. In the CMP-001-001 study in PD-1 relapsed/refractory melanoma, intra-tumoral CMP-001 with pembrolizumab resulted in ORR of 25%. Side effects were class-specific including flu-like symptoms (7%), hypotension (6%), hypertension (5%), AST/ALT elevation (2–3%), injection site reactions (27%); and majority were grade 1–2. Median duration of response was not reached (>17 months) with similar responses in treated and non-treated sites.¹⁹³ The preliminary results of CMP-001 combination with atezolizumab and radiation therapy showed no responses in advanced NSCLC.¹⁹⁴

Pre-clinically, compelling data suggest that neoadjuvant immunotherapy (compared with adjuvant immunotherapy)

results in greater tumor-specific immune responses and improved eradication of distant metastases following primary tumor resection in orthotopic murine models of melanoma and breast carcinoma.^{195,196} Separately, in a sub-analysis of patients treated in CMP-001-001 study, high fraction of baseline lymph node tumor burden was a favorable feature associated with longer PFS.¹⁹⁷ Compared to post-operative adjuvant therapy, neoadjuvant therapy permits an in vivo assessment of tumor biology, and represents the ideal scenario for studying predictive biomarkers and intermediate end points that might predict long-term clinical outcomes. These observations prompted evaluation of neoadjuvant CMP-001 with nivolumab in high-risk stage III melanoma prior to definitive surgery; wherein the primary endpoint of the study was the rate of major pathologic response (MPR) as assessed using recently developed criteria.^{198–200} In this study, patients received intra-tumoral CMP-001 and nivolumab for 7 weeks pre-operatively. Following definitive surgery, patients continued nivolumab and subcutaneous CMP-001 every 4 weeks post-operatively for 12 months. In a cohort of 20 patients, 75% MPR rate was observed – significantly better than what has been reported with neoadjuvant pembrolizumab monotherapy and similar to what has been reported with neoadjuvant ipilimumab/nivolumab.^{201–205} This has prompted a randomized phase II study of the combination compared to pembrolizumab monotherapy (EA6194) and a phase II/III study of the combination against anti-PD(L)1 ICI in PD-1 naïve metastatic melanoma (CMP-001-010).

NZ-TLR

NanoZolid[®] technology represents a novel approach of creating controlled-release products using cold isostatic pressing to encapsulate active substances in a highly dense calcium sulfate microstructure. This technology has been used to generate depot-formulations of anti-androgens for use in prostate cancer.²⁰⁶ NZ-TLR is a TLR9 agonist with an undisclosed TLR9 structure encapsulated within a calcium sulfate excipient (NanoZolid) that permits slow release depot formulation following intra-tumoral dosing obviating the need for frequent dosing. Preclinical data in syngeneic tumor models have been reported but not published with plans for first-in-human clinical trials in 2021.

Cavrotolimod (AST-008)

Spherical nucleic acids (SNAs) are a unique class of nanomaterial comprised of an inorganic nanoparticle

core that acts as a scaffold for the assembly and orientation of an outer nucleic acid shell. SNA technology confers properties that distinguish SNAs from linear DNA or RNA counterparts including increased cellular uptake, improved pharmacokinetics, and biodistribution.²⁰⁷ Cavrotolimod (AST-008) is a novel SNA configuration of TLR9 with undisclosed structure that is currently being tested singly and in combination with pembrolizumab in patients with advanced Merkel cell carcinoma and cutaneous squamous cell carcinoma. Preclinical data suggested that intra-tumoral cavrotolimod (AST-008) activated immune cells and elicited a T_h1 type cytokine response. Early data from a phase I study suggested that cavrotolimod (AST-008) was safe and well tolerated with no dose-limiting AEs noted. Dose-dependent increases in NK cells and CD8 T cells peripherally were observed intra-tumoral cavrotolimod (AST-008) singly with pembrolizumab.²⁰⁷

Conclusions

TLR9 agonists are well tolerated as monotherapy and do not appear to increase the toxicity of chemotherapeutic, targeted, radiation, and other immunotherapy agents as part of combination therapy. The main adverse effects are injection site reactions and flu-like symptoms, of mild to moderate severity and typically well managed with symptomatic treatment – and have resulted in treatment discontinuation in only a small number of patients in clinical trials.

In preclinical studies, TLR9 agonists demonstrated efficacy singly and in combination with a variety of agents. Intra-tumoral and subcutaneous routes of administration showed better local and distant responses in comparison to intravenous routes. In clinical trials, single-agent TLR9 agonist therapy for advanced solid tumors did not demonstrate significant efficacy, especially with an intravenous route of administration. While combinations with chemotherapy and targeted therapy yielded disappointing results, combinations with RT in indolent lymphomas have been promising. However, the greatest excitement has been reserved for the combinations of TLR9 agonists with ICI. Tilsotolimod (IMO-2125), SD-101 and CMP-001 all demonstrated efficacy in PD-1 relapsed/refractory melanoma in small non-randomized phase II studies. Pivotal trials testing these agents against active comparators in advanced PD-1 relapsed/refractory melanoma are either ongoing (tilsotolimod/IMO-2125 – ILLUMINATE-301) or planned (CMP-001 - CMP-001-010).

Separately, the unusual response rate observed with CMP-001 in combination with nivolumab in a small phase II neoadjuvant trial raises several questions: do neoadjuvant pathological responses translate into durable relapse-free survival; is the response rate in this setting predictive of what may be seen in PD-1 naïve metastatic melanoma; and is the neoadjuvant setting with its limited tumor burden and nodal involvement the ideal setting wherein to evaluate innate agonists in melanoma and other cancers?

TLR9 agonists augment antigen presentation to APC, and promote T cell trafficking to create a T cell inflamed TME resulting in an increased response rate of ICI therapy. However, several questions remain. What is the ideal ICI partner for augmenting the benefit of innate agonists in general and TLR9 agonists in particular: anti-PD-1, anti-CTLA-4, combination of anti-PD-1/anti-CTLA-4 or other? Are TLR9 agonists enough to convert T cell uninflamed tumors to inflamed tumors; or are combinations with other innate immunostimulants such as RIG-I, STING agonists required? While accessibility of tumors has limited the scope of intra-tumoral injections, could recent advancements in image-guided procedures provide an opportunity to target visceral metastatic lesions? Could advances in structural chemistry permit depot formulations that maintain efficacy with fewer injections? How best can TLR9 agonists be combined with RT, and possibly newer modalities including alpha-emitters?²⁰⁸ Ongoing clinical trials will help to answer some questions while elucidating biomarkers of resistance and response with the eventual goal of broadening the use of TLR9 agonists in solid tumors.

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