Recent clinical trends in Toll-like receptor targeting therapeutics

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Abstract
Toll-like receptors (TLRs) are germline-encoded receptors that are central to innate and adaptive immune responses. Owing to their vital role in inflammation, TLRs are rational targets in clinics; thus, many ligands and biologics have been reported to overcome the progression of various inflammatory and malignant conditions and support the immune system. For each TLR, at least one, and often many, drug formulations are being evaluated. Ligands reported as stand-alone drugs may also be reported based on their use in combinatorial therapeutics as adjuvants. Despite their profound efficacy in TLR-modulation in preclinical studies, multiple drugs have been terminated at different stages of clinical trials. Here, TLR modulating drugs that have been evaluated in clinical trials are discussed, along with their mode of action, suggestive failure reasons, and ways to improve the clinical outcomes. This review presents recent advances in TLR-targeting drugs and provides directions for more successful immune system manipulation.

KEYWORDS
adjuvant, clinical trial, drug, innate immunity, Toll-like receptor

1 | INTRODUCTION

Toll-like receptors (TLRs) are integral membrane bound receptors that are vital for innate immunity and help to shape the adaptive immune response. These receptors are triggered by a variety of pathogen-associated molecular patterns (PAMPs), and danger-associated molecular patterns (DAMPs). PAMPs are parts of pathogens such as lipoproteins,
TABLE 1  Natural and synthetic ligands for TLRs

| TLR     | Expression       | PAMPs                                      | DAMPs                           | Nature of molecules investigated in clinical trials |
|---------|------------------|--------------------------------------------|---------------------------------|---------------------------------------------------|
| TLR2 (1/6) | B, Mo, Mac, DCs, Plt N, MyDCs, Mc | Lipoproteins, zymosan, peptidoglycan | HSPs, HMGB1, hyaluronan, HDL (modified) | Lipopeptide, recombinant protein, antibody         |
| TLR3    | DC, B, Plt       | Viral dsRNA                                 | Self dsRNA                      | Polynosinic-polycytidylic acid (polyIC, polyIC12U), anti-TLR3 antibody |
| TLR4    | Mo, Mac, N, MyDCs, Mc, B, IE, Plt | Lipopolysaccharide                         | HSPs, fibrinogen, heparin sulfate, fibronectin, HA, HMGB1, hyaluronan, oxidized LDL, ANG II | Lipid A derivates (glycolipids), anti-TLR4 antibody, polysaccharide |
| TLR5    | Mo, Mac, DC, IE  | Flagellin                                   | HMGB1                           | Flagella and flagella based molecules              |
| TLR7    | Mo, Mac, pDC, B, Plt | Viral ssRNA                                | Self ssRNA                      | SM                                                |
| TLR8    | Mo, Mac, DC, Mc  | Viral ssRNA                                 | Self ssRNA                      | SM                                                |
| TLR9    | Mo, Mac, pDC, B, Plt | Bacterial and viral CpG DNA                | Self DNA                        | DNA based, synthetic ssDNA molecules               |
| TLR10   | LN, Mo, S, B, L  | NA                                         | NA                              | NA                                                |

Abbreviations: ANG II, angiotensin II; B, B cell; DAPM, danger-associated molecular pattern; DC, dendritic cell; dsRNA, double-stranded RNA; HA, hyaluronic acid; HDL, high-density lipoprotein; HMGB1, high mobility group box 1; HSPs, heat shock proteins; IE, intestinal epithelium; L, lung; LC, liver cell; LDL, low-density lipoprotein; LN, lymph node; Mac, macrophage; Mc, mast cell; Mo, monocyte; MyDC, myeloid dendritic cell; N, neutrophil; NA, not available; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid DC; Plt, platelet; S, spleen; SM, small molecule; ssRNA, single-stranded RNA; TLR, Toll-like receptor.
lipopeptides, and flagella, as well as nucleic acids (either single-stranded or double-stranded DNA or RNA), while DAMPs are self-molecules that include multiple heat shock proteins, S100, and high mobility group box 1 (HMGB1) that are released in response to injury or any other anomaly in the cells. DAMPs or PAMPs can engage a variety of TLRs; those situated on the cell surface primarily engage TLRs that function on the cell membrane, whereas intracellularly localized TLRs are activated by nucleic acid components, which are made available after pathogen endocytosis or replication. Given their functions, TLRs are considered as the first line in immune defense.

The number of functional TLRs can vary in mammals; however, they all have conserved functions, namely the activation of inflammatory mediators. Humans have 10 TLRs; TLR2 (can heterodimerize with TLR1 or TLR6), TLR4, TLR5, and TLR10 are present at the cell membrane, whereas TLR3, TLR7, TLR8, and TLR9 are functionally localized to endosomes (Table 1). These receptors invariably work as homodimers or heterodimers, and several studies have suggested that they exhibit unusual dimer characteristics. All TLRs form homo- and heterodimers, except for TLR3 and TLR5, which are currently considered strictly homodimeric, in the absence of empirical evidence to the contrary.

TLRs are composed of three distinct domains, an extracellular domain (ECD) that senses the ligand, a transmembrane domain (TM) that anchors the TLR within membranes, and Toll/interleukin-1 (IL-1) receptor (TIR) domain that interacts with other TIR-containing adapters to initiate signaling (Figure 1A). TLRs signaling depends on its dimer assembly, and in the absence of any ligand, TLRs exist either in monomeric forms or weakly dimeric forms that are unable to initiate the signaling. While ligand binding confers dimer stability and induces a conformational change that reorients the TIR domain and initiates signaling. TLRs are ideal targets for immune modulation strategies, since they have both known modulators and proven therapeutic potential (Table 1).

Therefore, it is rational to harness their potential for improving vaccines efficacy, to treat cancers (breast and bladder cancers), to inhibit their activity in inflammatory diseases (for instance; rheumatoid arthritis (RA) and multiple sclerosis), to modulate them in autoimmune diseases such as systemic lupus erythematosus (SLE), to fine tune them to generate specific responses (humoral vs cell-mediated immune responses) and to curb the menopausal osteoporosis. Other than these, there are numerous diseases where TLRs play important roles, for that, the interested reviewers are encouraged to consult recent reviews. Given their extensive potential benefits, they are the target of choice for many therapeutic endeavors, and these efforts are bearing fruit, with many compounds that target TLRs are currently at various stages of evaluation in clinical trials.

We have therefore revised and gathered relevant data and wish to present it to the scientific community to guide them in their future investigations. The data in this paper has been collected from ongoing clinical trials that either target TLRs or use them to induce improved responses. Data have been organized in a reader-friendly manner, focusing on the clinical condition, the type of TLR being targeted, the failures and successes of drugs in different phases of clinical trials, and the synergistic efficacy of TLR ligands as adjuvants.

2 | TLR SIGNALING

In induction of inflammatory responses, TLRs primarily act via the myeloid differentiation response protein 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF)-mediated pathways. On sensing PAMPs or DAMPs, TLRs dimerize and reorient their TIR domains, which allow docking of the TIR containing proteins, MyD88 and MyD88 adapter-like (MAL). MAL is a bridging adapter frequently involved in TLR4, and to a lesser extent in TLR2, signaling pathways, and it interacts with MyD88 through TIR-TIR interaction. In addition to the TIR domain, MyD88 contains a death domain that facilitates its interaction with interleukin-1 receptor (IL1R)-associated kinase 4 (IRAK4), which can both auto-phosphorylate and trans-phosphorylate IRAK2/1. This inter-domain interaction results in a large multimeric molecule, referred to as myddosome, the phosphorylation of which leads to the activation and dimerization of tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6). TRAF6, an E3 ligase that is activated via autoubiquitination in an sequestosome 1 (SQSTM1/p62)-dependent manner, mediates the ubiquitination of transforming growth factor-β–activated kinase 1 (TAK1). TAK1 belongs to the mitogen-activated protein kinase kinase kinase
FIGURE 1  Generalized structure and signaling mechanisms of Toll-like receptors. A, A typical TLR is composed of three distinct domains, an ECD, a TM domain, and a TIR domain. B, Conventionally, TLRs are divided into two categories; cell surface functional, and endosomally functional TLRs. Endosomal TLRs are mainly activated through nucleic acids, while cell surface–expressed TLRs are activated by a variety of ligands, including proteins and lipoproteins. Upon sensing PAMPs or DAMPs, TLRs dimerize and reorient their TIR domains, allowing the docking of intracellularly localized TIR-containing proteins, including MAL, MyD88, TRIF, and TRAM. The majority of TLRs convey downstream signals through MyD88; however, TLR3 can signal only through TRIF. Exceptionally, TLR4 can transmit signals through both the MyD88 and TRIF adapter proteins. Therapeutics targeting immune-related diseases mediated by TLRs are reported to modulate these signaling mechanisms. There are many internal mechanisms that come into action to regulate TLR-mediated inflammation. These act at all levels starting from cell surface interaction to dent the TLR dimerization, and cytoplasmic interactions to block adapter molecules, alter the posttranslational modification state, and finally in the nucleus to counter overexpression of various interleukins and cytokines. There are many microRNAs that reduce the mRNA stability of different cytokines. All these mechanisms ensure a balanced response toward the invading pathogen or DAMP that unbalance the homeostasis.

ABIN3, A20 binding and inhibitor of NFκB-3; AhR, aryl hydrocarbon receptor; AP1, activated protein 1; ATF3, activating transcription factor 3; Bcl-3, B-cell lymphoma 3-encoded protein; CYLD, cylindromatosis; DOK, downstream of tyrosine kinases; DUSP, dual specificity phosphatases; ECD, extracellular domain; ERK, extracellular-regulated kinase; IFN, interferon; IκB, inhibitor of xB; IKK, inhibitor of xB kinase; IL, interleukin; IRF, interferon response factor; IRAK-M, interleukin receptor-associated kinase M; JNK, c-Jun N-terminal kinase; MAL, MyD88 adapter like; MD2, myeloid differentiation factor 2; MIR, microRNA; MKK, mitogen-activated protein kinase kinase; mRNA, messenger RNA; MyD88, myeloid differentiation primary response 88; MyD88s, myeloid differentiation primary response 88 short; NEMO, NFκB essential modulator; NFKBID, NFκB inhibitor δ; NF-xB, nuclear factor xB; Nurr1, nuclear receptor related 1 protein; p38, protein 38; PDLIM2, PDZ and LIM domain 2; PTP1B, protein tyrosine phosphatase-1B; Reg-1, regnase-1; RIPK-1, receptor interacting protein kinase 1; RP105, radioprotective 105 kDa protein; SARM, sterile α and armadillo-motif containing protein; SHP-1, Src homology region 2 domain-containing phosphatase-1; SIGGR, single immunoglobulin IL1R-related molecule; SOCS, suppressor of cytokine signaling; ST2, suppression of tumorigenity 2; ST2L, membrane bound ST2; STAT, signal transducers and activators of transcription; sTLRs, soluble Toll-like receptor; TAB, TAK-1-binding protein; TAK1, transforming growth factor β-activated kinase 1; TANK, TRAF-associated NF-xB activator; TBK1, TANK-binding kinase 1; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor; TM, transmembrane domain; TNF-α, tumor necrosis factor α; TRAF, tumor necrosis factor receptor (TNF-R)-associated factor; TRAM, TRIF-related adapter molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β; TRIM38, tripartite motif 38; TTP, tristetraprolin; USP4, ubiquitin-specific protease 4 [Color figure can be viewed at wileyonlinelibrary.com]
MAPKKKK family and forms a complex with the TAK1 binding proteins, TAB1–3. TAK1 deficiency reduces inflammatory signaling across TLRs; however, no such difference has been observed in response to a deficiency of TAB proteins. TAK1/TABs signaling then branches into two arms: activation of nuclear factor κB (NF-κB) and MAPK. NF-κB is held inactive in the cytoplasm by inhibitor of κB (IκB), which is phosphorylated by IκB kinase α (IKKα) and IKKβ, and degraded via ubiquitin mediated-proteasomal degradation, exposing a nuclear localization signal in NF-κB, and subsequently translocating to the nucleus as reviewed by Kawai and Akira. NFκB is a hub molecule for inflammatory signals and it induces the expression of a wide array of molecules that cause inflammation, alteration in cell surface receptors, expression of pro- and anti-cancerous molecules, and perturbation in cell motility, among other responses. TAK1 also activates MAPK family members, including MKK7 and/or MKK6/3, resulting in the phosphorylation of p38 and JNK, and culminating in the activation of activated protein 1 (AP1) family transcription factors and messenger RNA (mRNA) stabilization of various genes involved in the regulation of inflammation (Figure 1B).

TRIF-dependent signaling is a separate arm of TLR signaling perpetuated only by TLR3 and TLR4, where TRIF interacts with TRAF3 and TRAF6. TRAF6 interacts with receptor interacting protein kinase 1 (RIPK-1), which transduces the signal by activating TAK1, a crucial branch point in the TLR signaling pathway. TRAF3 activates IKK-related kinases, such as TANK-binding kinase 1 (TBK1) and IKKα, along with NEMO, and the transduced signals culminate in interferon (IFN)-regulatory factor 3 (IRF3) phosphorylation, which translocates into the nucleus after dimerization, inducing expression of type I IFN genes. The production of IFNs is the prominent outcome of TLR3 and TLR4 pathways to counter viral infections, which is turn regulated by IRF3. Recently, it has been shown that phosphatidylinositol 5-phosphate (PtdIns5P) can regulate IRF3 activation. This inositol lipid can bind to and facilitate complex formation between IRF3 and TBK1, leading to the IRF3 phosphorylation by TBK1, situated proximally. Furthermore, during viral infection, production of the inositol lipid, PtdIns5P, could be observed by evaluation of PIKfyve activity.

3 | ENDOGENOUS REGULATION OF TLR SIGNALING

Regulation of TLR signaling is achieved through various molecules that restrict it to an appropriate level to avoid any detrimental consequences in the form of autoimmune or inflammatory diseases. These regulatory molecules bind to key components of TLR signaling and quench their activities as reviewed elsewhere. The MyD88-dependent pathway can be suppressed by spleen tyrosine kinase, Cbl-b, and suppressor of cellular signaling 1 (SOCS1), while the TRIF arm is negatively regulated by sterile α- and armadillo-motif-containing protein (SARM) and TRAM adapter with Golgi dynamics domain (TAG). Similarly, SOCS3 and deubiquitinating enzyme A (DUBA) negatively regulate TRAF3 while A20, cylindromatosis, TANK, tripartite motif 38 (TRIM38), ubiquitin-specific protease 4, and small heterodimer partner can negatively influence TRAF6 (Figure 1B).

TLRs are involved in a wide spectrum of diseases that either directly or indirectly exacerbate the conditions. In recent years, many endeavors have been dedicated to delineate this relationship and compile data regarding the
TLR involvement in various diseases. Here, we would like to present a brief overview of how TLRs influence the pathobiology of inflammatory, autoimmune, and cancerous diseases.

Sepsis is the worst outcome of host-pathogen interaction and is the leading cause of death in the United States. The infection by Gram-positive and Gram-negative bacteria equally contribute to the development of sepsis where exaggerated immune response lead to multiorgan failure and septic shock. These bacteria harbor ligands that trigger TLR2 and TLR4; particularly, the presence of LPS significantly contributes into sepsis development. The septic shock is due to the body immune response rather than infection itself. For sepsis management, various TLR inhibitors are evaluated clinically, and new modalities are being devised recently.

Chronic pulmonary obstructive disease (COPD) is characterized by the poor reversible air flow and bronchial inflammation. This condition can also be worsened when TLRs react in response to viral infections. It has been observed that the COPD patients exhibit higher inflammatory cytokines, TNF-α and CCL5 in infections. Among various treatments, the inhibition of TLRs can also be an approach to curb the COPD.

The involvement of TLRs in RA, an inflammatory disease, is well known. The exact mechanism of RA initiation is yet debatable; however, it is believed that the PAMPs from commensal flora is crucial for RA initiation. After initial insult, an autocrine loop perpetuates that increases matrix metalloproteinases (MMP) and worsen the damage. Moreover, the DNA and peptidoglycan from intestinal bacteria have also been observed in RA joints. This result in damaged cells that will release DAMPs such as RNAs, HMGB1, S100-A8; the presence of such molecules activate TLRs that over-inflame the situation.

SLE is an autoimmune disease that featured autoantibodies against double-stranded DNA and nucleic acid-bound proteins that served both as diagnostic and prognostic markers; however, the initial events are still a mystery. SLE patients manifest deficiency in clearing apoptotic cells that promote the formation of the immune complex (IC), and these ICs can trigger the endosomal TLRs. The role of TLR7 (inflammatory) and TLR9 (protective) in SLE can be different due to variation in study samples among different studies; nevertheless, TLR9−/− murine models displayed higher TLR7-mediated inflammation concluding a regulatory role of TLR9.

An autoimmune disease where the immune system destroys the fluid secreting glands, for instance, the salivary gland, has a potential TLR involvement and is known as Sjogren’s syndrome (SS). The patients with SS exhibit higher TLR expression, with increased expression of inflammatory cytokines in response to TLR7 and TLR9 activation.

TLRs participation in cancers act as double-edge swords; their activation can regress the tumor growth or conversely promote the tumor cells. The accumulating data strongly advocate both aspects. Furthermore, it is now well-acknowledged that the inflammation and cancer are strongly correlated in various diseases. Similarly, organs with higher PAMPs density such as gastrointestinal tract and skin are prone to TLR-mediated oncogenesis along with the organs that expose to indirect TLR agonist such as the liver. The dual role of TLRs in cancers has a significant correlation with the length and amplitude of receptor activation. TLR4 has been reported to promote colon cancer, and its deficiency can alleviate the inflammation as well as tumor burden. The liver cancer has also been related to TLR4 activation; however, its role may be context dependent in skin cancer. Similarly, TLRs are also critical for the cellular transformation in breast cancers, as reviewed before, can critically modulate the metabolism in the tumor microenvironment, and can regulate other signaling networks to favor pro- or anti-tumor outcomes.

5 TLR LIGANDS: ADJUVANTS VS DRUGS

TLR signaling activates innate immunity and assists in shaping adaptive immunity. Hence, TLR ligands are attractive for use in immunotherapy and are primarily exploited as adjuvants to specifically trigger humoral and/or cell-mediated responses as reviewed elsewhere. They can also magnify the immune response toward certain poorly antigenic targets. Therefore, in the majority of clinical trials, TLR ligands are evaluated as adjuvants.

The number of trials that involve TLR ligands as adjuvants (64%) are double than those considering TLR ligands as drugs (35%). This highlights the immune-therapy role of TLRs in various diseases and their potential utilization...
for further exploration for immunomodulation therapy. Additionally, TLR activation can also alter other signaling pathways and it is desirable to cotarget multiple pathways with the aim of achieving improved treatment efficacy. Apart from many ongoing trials, Food and Drug Administration approved TLR ligands, MPLA (TLR4 agonist), and imiquimod (TLR7 agonist) could be highlighted to address adjuvant or drug roles. MPLA has been used in various vaccine formulations, for instance, Fendrix (Hepatitis B vaccine, GSK), as an adjuvant and imiquimod is famously used to cure viral diseases as a drug. The majority of TLRs produce redundant responses (inflammatory vs antiviral); however, there are slight, but distinct, differences in outcomes. These differences are largely attributable to the relative roles of ligands and tissue-dependent TLRs expression.

**FIGURE 2**  TLRs targeting ligands with respect to their relative clinical trials and disease conditions. A, The total number of clinical trials, activators (including agonists) and inhibitors (including antagonists), and the diversity of ligands are presented. The majority of ligands have been extensively pursued in different diseases, making it difficult to determine their exact numbers. The data indicate that total number of clinical trials exceeds the number of active drugs, suggesting the use of single drugs in multiple clinical trials. B, Clinical trial data showing the current status of drugs targeting TLRs from the disease perspectives. TLR ligands have been evaluated in multiple diseases including cancers, immune disorders, and viral and bacterial diseases. The largest proportion of clinical trials focuses on cancers, followed by immune disorders. "Mixed" indicates those cases where cancer and immune disease have been targeted simultaneously. The category “general” covers vaccination, clinical trials involving healthy volunteers, and those that are not covered by prior instances. This data was gathered from the clinical trials website (clinicaltrials.gov) using various keywords (cancers, immune disorders, TLR, TLRs, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and adjuvant) from June 2017 to Jan 2018. TLR, Toll-like receptor.
Given their vital roles in pathogen clearance, inflammation induction, and cancer pathogenesis, TLRs are attractive targets for manipulation of the immune system in favor of the patients. Therefore, many research centers and pharmaceutical companies are attempting to develop TLRs modulators (Figure 2A). Scaffolds of naturally occurring modulators are ideal candidates for targeting these receptors; thus, these have been heavily investigated in clinical studies and are emerging as a fruitful approach in clinical trials. An exhaustive search of the literature also supports this notion.

6.1 TLR2 (TLR1/TLR6)

TLR2, in combination with TLR1 or TLR6, detects the lipoproteins, diacyl lipid, or triacyl lipid, respectively, which makes it unique in forming functional heterodimers with other TLRs. Further, TLR2 interacts with modified proteins such as glyco- and lipoproteins, peptidoglycan, and zymosan, allowing it to detect a variety of PAMPs.87 This heterogeneity in TLR2 PAMP detection ranges across all types of pathogens, including viruses, bacteria, fungi, and parasites. The TLR2 expression has been detected in immune, endothelial, and epithelial cells,88 indicating that it is a functionally ubiquitous molecule. The homodimerization of TLR2 has been reported; however, further studies are required to confirm these findings.8,12,89 The ubiquitous nature and pivotal role of TLR2 make it an attractive drug target for various diseases; consequently, many clinical trials have been initiated to evaluate the efficacy of various lipopeptide derivates. Compounds being evaluated in clinical trials include lipopeptides, lipoproteins, oxidized low-density lipoproteins, and TLR2 specific humanized IgG4 antibody, either alone or in various combinations (Table 2).

The most recent ligands, such as CBLB612 (synthetic lipopeptide TLR2 agonist), ISA-201 (peptide agonist for TLR2), OPN-305 (TLR2 antagonizing IgG4 monoclonal antibody), are in phase 2 in clinical trials primarily for oncogenic therapy, and act both as drugs and as adjuvants (Figure 2B).90,91 The chemical constituents of these molecules are either lipoprotein or protein derivates, indicating that TLR2 can be targeted using mimetics of its natural ligands. This is not an absolute requirement; however, it is useful to note the existing therapeutic trend while targeting TLR2. Moreover, other than OPN-305, the majority of molecules in phase 2 trials are agonists of TLR2, highlighting the importance of TLR2 activation in the context of malignancies. Small molecule-based therapeutics have potential side effects that can be overcome by the application of biologics, including monoclonal antibodies (OPN-305).90 The inhibition of TLR2 overactivation using OPN-305 has potential applications in the treatment of inflammatory diseases.

TLR2 in TLR2/1 or TLR2/6 complexes exhibits a cavity on the binding junction of its convex side that allows the docking of Pam3CSK4 and other TLR2-modulating ligands.12,92 Pam3CSK4 has two esters and one amide bound lipid chains. The ester chains interact with TLR2, while the amide bound lipid chain can be accommodated into the hydrophobic cavity provided by TLR1 (Figure 3).12,93 The TLR2/1 complex can further be stabilized by interprotein hydrogen bonding and hydrophobic interactions.12 The hydrophobic cavity in TLR1 has been mutated with bulky amino acids (Met338 and Leu360 to Phe338/360) to make binding of any lipid chain unfavorable, explaining the diacyl requirement for TLR2/6 complex formation.

6.2 TLR3

TLR3 forms homodimer and signals in an exclusively TRIF-dependent manner in response to viral infections (double-stranded RNA [dsRNA]) and stimulates the production of IFNs. The only known agonist for this TLR is poly-ICLC (and its derivatives), which is being investigated in various clinical trials.94,95 The success and ubiquitous nature of poly-ICLC led to the belief that this was the only realistic possibility for targeting this TLR; however, recent studies have identified other small molecules that can either inhibit or activate TLR3.96,97 These alternatives will not be available for clinical trials for a considerable period of time. There are a few clinical trials that involve anti-TLR3 antibody to evaluate its efficacy in healthy individuals and asthmatic patients.98 The success of these
| Ligand                  | Phase  | Application                      | Target TLR adjuvant/drug | Sponsor/collaborators       | NCT number   | Type                               | Purpose                                      |
|------------------------|--------|----------------------------------|--------------------------|-----------------------------|--------------|------------------------------------|----------------------------------------------|
| CBLB612                | Phase 2| Breast cancer                    | TLR2 agonist/drug        | Cleveland BioLabs, Inc      | NCT02778763 | Synthetic lipopeptide              | Neutropenia/recover blood cells count        |
| SV-283 (NY-ESO-1)      | Phase 1| Cancer                           | TLR2 agonist/adjuvant    | SapVax LLC                  | NA           | Combination of peptide and SM     | Immune stimulation                           |
| ISA-201                | Phase 2| Head and neck tumor              | TLR2/1 agonist/adjuvant  | ISA Pharmaceuticals BV      | NCT02821494 | Combination of peptide and SM, peptide | DC maturation                                |
| OPN-305-110            | Phases 1 and 2| Second-line and first line lower risk myelodysplastic syndrome | TLR2 antagonist/drug     | Opsona Therapeutics Ltd     | NCT03337451 | Monoclonal antibody               | Anti-inflammation                            |
| OPN-305                | Phase 2| Myelodysplastic syndrome, inflammatory disease, pancreas tumor, kidney transplant rejection | TLR2 antagonist/drug     | Opsona Therapeutics Ltd     | NCT02363491 | Monoclonal antibody               | Anti-inflammation                            |

Abbreviations: DC, dendritic cells; NA, not available; NCT, national clinical trial; SM, small molecule; TLR, Toll-like receptor.
proof-of-concept studies will lay the foundation of antibody-based endosomal TLRs targeting in various diseases. However, in rhinoviral infection, the antibody could not demonstrate any improvement in asthmatic condition.99

Targeting of TLR3 is currently used as adjuvant therapy, along with other drugs or vaccines, against a variety of cancers; nonetheless, the sole activation of TLR3 to curb any disease has yet to be successfully explored (Table 3).
| Ligand                | Phase          | Application                                                                 | Target TLR adjuvant/drug | Sponsor/collaborators                                                                 | NCT number     | Type                       | Purpose                                      |
|----------------------|----------------|-----------------------------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------------|----------------|----------------------------|----------------------------------------------|
| Poly-ICLC            | Phases 1 and 2 | Low-grade B-cell lymphoma                                                   | TLR3 agonist/adjuvant    | Joshua Brody, Icahn School of Medicine                                               | NCT01976585   | Synthetic dsRNA            | DC activation                                |
| Poly-ICLC (NY-ESO-1) | Phases 1 and 2 | Melanoma                                                                   | TLR3 agonist/adjuvant    | Nina Bhardwaj, Ludwig Institute for Cancer Research, Oncovir Inc, CRI New York City, Icahn School of Medicine | NCT01079741   | Synthetic dsRNA            | Activating humoral and T cell immunity        |
| Poly-ICLC            | Phases 1 and 2 | Head and neck squamous cell carcinoma, sarcoma, Merkel cell carcinoma, cutaneous T-cell lymphoma, melanoma, renal-bladder-breast and prostate cancer | TLR3 agonist/adjuvant    | Ludwig Institute for Cancer Research, MedImmune LLC, CRI New York City               | NCT02643303   | Synthetic dsRNA            | Tumor microenvironment modulation            |
| Poly-ICLC (Hiltonol) | Unknown        | Primary ovarian cancer, fallopian tube cancer, primary peritoneal cancer    | TLR3 agonist/adjuvant    | Abramson Cancer Center of the University of Pennsylvania                            | NCT02452775   | Synthetic dsRNA, carboxymethylcellulose, poly-L-lysine | Immune stimulation                           |
| Poly-ICLC (Romidepsin) | Phase 1      | Cutaneous T-cell lymphoma                                                  | TLR3 agonist/adjuvant    | New York University School of Medicine, Ludwig Institute for Cancer Research          | NCT02061449   | Synthetic dsRNA            | Immune stimulation                           |
| Poly-ICLC (Pembrolizumab) | Phases 1 and 2 | Metastatic colon cancer, solid tumor                                       | TLR3 agonist/adjuvant    | Samir N. Khleif, Oncovir Inc, Merck Sharp and Dohme Corp, Augusta University         | NCT02804052   | Synthetic dsRNA            | Immune stimulation                           |

(Continues)
| Ligand          | Phase          | Application          | Target TLR adjuvant/drug | Sponsor/collaborators                                                                 | NCT number       | Type                  | Purpose             |
|-----------------|----------------|----------------------|--------------------------|--------------------------------------------------------------------------------------|------------------|-----------------------|---------------------|
| Poly-ICLC       | Phases 1 and 2 | Chronic HIV-1 infection | TLR3 agonist/adjuvant    | The Campbell Foundation; Oncovir, Inc, National Institutes of Health (NIH), National Institute of Allergy & Infectious Diseases (NIAID), Nina Bhardwaj, Icahn School of Medicine at Mount Sinai | NCT02071095   | Synthetic dsRNA       | Immune stimulation   |
| Poly-ICLC (DCVax-001) | Phase 1      | HIV-1 infection      | TLR3 agonist/adjuvant    | Rockefeller University                                                               | NCT01127464     | Synthetic dsRNA       | Immune stimulation   |
| PRV-300         | Phase 1       | Healthy              | TLR3 antagonist/drug     | Janssen Research & Development, LLC                                                  | NCT02008279     | Anti-TLR3 antibody    | Anti-inflammatory    |
| PRV-300         | Phases 1 and 2 | Asthma               | TLR3 antagonist/drug     | Centocor, Inc                                                                       | NCT01195207     | Anti-TLR3 antibody    | Anti-inflammatory    |

Abbreviations: DC, dendritic cells; dsRNA, double-stranded RNA; HIV, human immunodeficiency virus; NCT, national clinical trial; TLR, Toll-like receptor.
Poly-ICLC is a synthetic complex of polyinosinic-polycytidylic acid (nucleic acid mimetics and pathway intermediates), carboxymethylcellulose, and poly-L-lysine (stabilizers). As dsRNA is a natural ligand with relatively low stability, its mimetics could be an affordable means of activating this TLR. Activation of TLR3 depends on dsRNA binding at two opposite sides of its ectodomain, which favorably relocates the C-terminus of the ECD to facilitate further interactions and increased stabilization. TLR3 interacts with the nucleotide backbone, rather than nucleotide bases, which explains its activation via multiple nucleotide combinations (Figure 3).

6.3 | TLR4

TLR4 is the only TLR that can function both at the cell membrane and in the endosome, and that can signal through MyD88- and TRIF-dependent pathways. This has led to the evolution of additional precautions, such as an extensive ligand detection mechanism (cluster of differentiation 14, lipid binding protein, and coreceptor myeloid differentiation factor 2 [MD2]) and signal propagation mechanism (requirement of MAL for MyD88 and TRIF-related adapter molecule [TRAM] for TRIF signaling pathways).

Among TLRs, only TLR4 has a suitable ligand binding pocket provided by MD2, rather than by the ectodomain of TLR4. This also provides an additional means of TLR4 inactivation, since MD2 has a large hydrophobic cavity and lipid A derivatives are suitable binding molecules; however, other methods, such as disruption of MD2 binding with TLR4 or inhibition of interaction with the activating ligand, have also been explored. In case of MD2 and lipid A interaction, the lipid with six acyl chains (lipid VI-A) can fully occupy the pocket and reorient the side chain of F126 amino acid into the binding cavity. This creates a favorable environment for the other TLR4/MD2 to dock properly (Figure 3). However, in case of a lower number of acyl chains, their binding is unable to reorient the side chain that, in turns, creates a steric hindrance for other heterodimer, leading to TLR4 inhibition.

Prominent ligands that activate or inhibit TLR4 include lipid VI-A and its derivatives (lipid 4A [agonist], monophosphoryl lipid A [MPLA, a weak agonist], and glucopyranosyl lipid adjuvant [GLA, an agonist]), and recent studies have also evaluated antibody or peptide-based drugs. Among the TLRs, TLR4 has been extensively evaluated in the highest number of clinical trials and is of interest as a target for treatment of a variety of pathologies including cancers, viral infection, immune diseases, and inflammation. Both the agonistic and antagonistic aspects of TLR4 signaling pathways are being explored (Table 4). In addition to inhibition of MD2-mediated TLR4 signaling, the interaction of HMGB1 with TLR4 has also been considered in recent trials to improve the efficacy of anticancer drugs (https://clinicaltrials.gov/ct2/show/NCT02995655). Besides direct modulation, the addition of a constitutively active form of TLR4 as a vaccine substitute (https://clinicaltrials.gov/ct2/show/NCT02888756) and inhibition of dipeptidyl peptidase-4 (DPP4) that induces IL-6 expression through TLR4 are also the subjects of therapeutic evaluations.

6.4 | TLR5

TLR5 detects the bacterial monomeric flagella and mounts an immune response. It triggers the MyD88-dependent pathway in response to enterobacterial invasion and maintains intestinal homeostasis. TLR5 is expressed in almost all cell types with prominent expression in mucosal dendritic cells (DC); however, literature discussing TLR5 is scarce. From a therapeutic perspective, all clinical trials targeting TLR5 include use of recombinant flagellin protein. Additionally, small molecules to block the TLR5-flagellin interaction are being tested in preclinical studies. In the majority of therapeutic settings, ligands for TLR5 act as adjuvants rather than as stand-alone drugs, enhancing the efficacy and potency of vaccine candidates (Table 5). TLR5 can be an attractive target because it detects only protein. Short peptides derived from flagellin can be used as activators, while modified forms of such peptides can inhibit TLR5. Recently, the crystal structure of zebrafish TLR5 with flagellin was determined, providing insights into its mode of activation (Figure 3). The leucine-rich repeat 9 (LRR9) region in TLR5 has a critical role, and Arg89, Glu114, and Leu93 from flagellin form a hotspot with chemical
TABLE 4  TLR4 targeting ligands in clinical trials

| Ligand          | Phase | Applications                                      | Target TLR adjuvant/drug | Sponsor/collaborators                        | NCT number   | Type          | Purpose                                           |
|-----------------|-------|--------------------------------------------------|--------------------------|----------------------------------------------|--------------|--------------|---------------------------------------------------|
| GLA-SE (MART-1 antigen) | Phase 1 | Stage IIA–IV skin melanoma                        | TLR4 agonist/ adjuvant   | Mayo Clinic, NCI                             | NCT02320305  | Glycolipid    | Immune stimulation                                |
| LPS             | Phase 1 | Asthma                                            | TLR4 agonist/ adjuvant   | John Sundy, Duke University                  | NCT00671892  | Glycolipid    | Prospective inflammation                         |
| GLA-SE          | Phase 1 | Stage III/IV adult soft tissue sarcoma           | TLR4 agonist/ adjuvant   | Fred Hutchinson Cancer Research Center, NCI    | NCT02180698  | Glycolipid    | Immune stimulation                                |
| GSK1795091      | Phase 1 | Cancer                                            | TLR4 agonist/drug        | GSK                                          | NCT02798978  | Glycolipid    | Immune stimulation                                |
| G100            | Phases 1 and 2 | Follicular lymphoma (marginal zone allowed during dose escalation only) | TLR4 agonist/ adjuvant | Immune Design, Merck Sharp & Dohme Corp       | NCT02501473  | Glycolipid    | Tumor microenvironment alteration, DC, T and other immune cells activation |
| GLA-AF, GLA-SE  | Phase 1 | Healthy volunteers                                | TLR4 agonist/ adjuvant   | Rockefeller University, IDRI Corporation, Immune Design | NCT01397604  | Glycolipid    | Immune stimulation, DC activation                 |
| CCRE            | Phase 1 | aHealthy individual                               | TLR4 agonist/ adjuvant   | University of North Carolina, Chapel Hill, Environmental Protection Agency (EPA) | NCT02847247  | LPS          | Inflammation                                     |
| Endotoxin       | Phase 1 | Endotoxemia                                       | TLR4 agonist/ adjuvant   | Radboud University                           | NCT00184990  | SM           | Inflammation                                     |
| GLA             | Phase 1 | Hookworm infection                                | TLR4 agonist/ adjuvant   | Baylor College of Medicine, George Washington University | NCT01717950  | Glycolipid    | Immune stimulation                                |
| Eritoran        | Phase 2 | Insulin sensitivity                               | TLR4 antagonist/ drug    | The University of Texas Health Science Center at San Antonio | NCT02321111  | Glycolipid    | Lipid-induced insulin resistance                 |
| NI-0101         | Phase 2 | Rheumatoid arthritis                              | TLR4 inhibitor/ drug     | NovImmune SA                                  | NCT03241108  | Anti-TLR4 antibody, TLR4 blocker, anti-inflammatory | (Continues) |
| Ligand          | Phase  | Applications                                      | Target TLR adjuvant/drug            | Sponsor/collaborators                                      | NCT number      | Type                     | Purpose                                      |
|-----------------|--------|--------------------------------------------------|-------------------------------------|------------------------------------------------------------|-----------------|--------------------------|----------------------------------------------|
| NI-0101         | Phase 1| Healthy volunteers                               | TLR4 inhibitor/drug                 | NovImmune SA                                               | NCT01808469     | Anti-TLR4 antibody       | TLR4 blocker, anti-inflammatory             |
| CX-01           | Phase 1| Refractory Myelodysplastic Syndrome and Acute Myeloid Leukemia | TLR4 inhibitor/adjuvant             | Washington University School of Medicine, Cantex Pharmaceuticals | NCT02995655     | Polysaccharide           | Bone marrow microenvironment disruptor     |
| Eritoran        | Phase 2| Insulin sensitivity                              | TLR4 antagonist/drug                | Nicolas Musi, The University of Texas Health Science Center at San Antonio | NCT02267317     | Glycolipid               | Anti-inflammatory                           |
| MPL (Grass MATA)| Phase 3| Type 1 hypersensitivity                          | TLR4 agonist/adjuvant               | Allergy Therapeutics Plc                                  | NCT00414141     | Glycolipid               | Anti-inflammatory                           |
| MPL (Grass MATA)| Phase 2| Allergy                                          | TLR4 agonist/adjuvant               | Allergy Therapeutics Plc                                  | NCT02582073     | Glycolipid               | Anti-inflammatory                           |
| GLA-SE          | Phase 1| Merkel cell carcinoma                            | TLR4 agonist/adjuvant               | Immune Design Corp                                         | NCT02035657     | Glycolipid               | Immune stimulation                         |
| GLA (H5 VLP vaccine) | Phase 2| Influenza virus infection                        | TLR4 agonist/adjuvant               | Medicago Inc                                               | NCT01991561     | Combination of protein and SM | Immune stimulation                         |
| PEPA-10         | Phase 2| Cancer                                           | TLR4 agonist/adjuvant               | Immunovo BV                                               | NA              | SM                       | Immune stimulation                         |
| PET-lipid A (ONT-10) | Phase 1| Advanced Breast and Ovarian Carcinoma           | TLR4 agonist/adjuvant               | Cascadian Therapeutics Inc                                | NCT02270372     | Glycolipid               | Immune stimulation                         |
| PET-lipid A (ONT-10) | Phase 1| Solid tumors                                     | TLR4 agonist/adjuvant               | Cascadian Therapeutics Inc                                | NCT01556789     | Glycolipid               | Immune stimulation                         |
| PET-lipid A (ONT-10) | Phase 1| Solid tumors                                     | TLR4 agonist/adjuvant               | Cascadian Therapeutics Inc                                | NCT01978964     | Glycolipid               | Immune stimulation                         |
| Ligand | Phase | Applications | Target TLR adjuvant/drug | Sponsor/collaborators | NCT number | Type | Purpose |
|--------|-------|--------------|--------------------------|-----------------------|------------|------|---------|
| GLA (H7N9 influenza vaccine) | Phase 1 | Influenza virus A infection | TLR4 agonist/adjuvant | Medicago Inc | NCT02022163 | Glycolipid | Enhance seroprotective antibody titers |
| G-305 | Phase 1 | Cancer | TLR4 agonist/adjuvant | Immune Design Corp | NCT02015416 | Combination of protein and SM | Immune stimulation |
| Lipid A (SAR-439794) | Phase 1 | Peanut hypersensitivity | TLR4 agonist/adjuvant | Sanofi, Immune Design Corp | NCT03463135 | Glycolipid | Immune stimulation |
| GLA | Phase 1 | Schistosomiasis | TLR4 agonist/adjuvant | Immune Design Corp | NCT02337855 | Glycolipid | Immune stimulation |
| JKB-121 | Phase 2 | Nonalcoholic steatohepatitis | TLR4 antagonist/drug | TaiwanJ Pharmaceuticals | NCT02442687 | SM | Anti-inflammatory |
| GLA-SE (CMB-305) | Phase 2 | Sarcoma and its various forms | TLR4 agonist/adjuvant | Immune Design Corp | NCT02609984 | Glycolipid | Immune stimulation, DC activation |
| GLA-SE (CMB-305) | Phase 2 | Sarcoma, melanoma, nonsmall-lung cancer | TLR4 agonist/adjuvant | Immune Design Corp | NCT02387125 | Glycolipid | Immune stimulation, DC activation |
| JKB-122 | Phase 2 | Chronic hepatitis C | TLR4 antagonist/drug | TaiwanJ Pharmaceuticals | NCT02293941 | SM | Anti-inflammatory |
| JKB-122 | Phase 2 | Autoimmune hepatitis, nonalcoholic fatty liver disease | TLR4 antagonist/drug | TaiwanJ Pharmaceuticals | NCT02556372 | SM | Anti-inflammatory |
| Ibudilast (aka MN-166) | Phase 2 | Alcoholism, neuropathic pain, amphetamine and opiate dependence, glioblastoma, traumatic brain injury | TLR4 antagonist/drug | MediciNova Inc | NCT01860807 | SM | Proinflammatory cytokine suppression |
| Intralipid (20%) | NA | Diabetes, obesity | TLR4 expression | The University of Texas Health Science Center at San Antonio, NIDDK | NCT01740817 | Lipid, SM | TLR4 expression |

Abbreviations: CCRE, clinical center reference endotoxin; GLA, glycopyranosyl lipid A; GSK, GlaxoSmithKline; NA, not applicable; NCI, National Cancer Institute; NCT, national clinical trial; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; SM, small molecule; TLR, Toll-like receptor.
| Ligand                  | Phase          | Application                                           | Target TLR adjuvant/drug | Sponsor/collaborators                                                                 | NCT number   | Type     | Purpose                |
|------------------------|----------------|------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------------|--------------|----------|------------------------|
| Mobilan (M-VM3)        | Phases 1 and 2 | Prostate cancer                                      | TLR5 agonist/adjuvant    | Panacela Labs LLC                                                                     | NCT02844699 | RP       | Immune stimulation     |
| Mobilan (M-VM3)        | Phase 1        | Prostate cancer                                      | TLR5 agonist/adjuvant    | Panacela Labs LLC                                                                     | NCT02654938 | RP       | Immune stimulation     |
| Entolimod (aka CBLB502)| Phase 1        | Unspecified adult solid tumor                         | TLR5 agonist/adjuvant    | Roswell Park Cancer Institute, NCI, Cleveland BioLabs Inc                           | NCT01527136 | RP       | Immune stimulation     |
| VAX125                 | Phase 2        | Influenza                                            | TLR5 agonist/adjuvant    | VaxInnate Corporation                                                                | NCT00966238 | RP       | Immune stimulation     |
| VAX102 (flagellin, HuM2e) | Phase 1      | Influenza                                            | TLR5 agonist/adjuvant    | VaxInnate Corporation; Bill & Melinda Gates Foundation                              | NCT00603811 | RP       | Immune stimulation     |
| Entolimod (radiation therapy) | Phase 1  | Mucositis, various types of squamous cell carcinoma of various tissues | TLR5 agonist/adjuvant    | Roswell Park Cancer Institute, NCI, Cleveland BioLabs Inc                           | NCT01728480 | RP       | Immune stimulation     |

Abbreviations: NCI, National Institute of Cancer; NCT, national clinical trial; RP, recombinant protein; TLR, Toll-like receptor.
| Ligand                  | Phase       | Application                          | Target TLR                        | Sponsor/collaborators                  | NCT number       | Type | Purpose/mechanism                                      |
|------------------------|-------------|--------------------------------------|----------------------------------|----------------------------------------|------------------|------|-------------------------------------------------------|
| GSK2245035             | Phase 2     | Asthma and rhinitis                  | TLR7 agonist/drug                | GSK, PATH                              | NCT01607372     | SM   | Type 1 IFN induction                                  |
| Imiquimod (R837)       | Phases 1 and 2 | Metastatic and recurrent breast cancer | TLR7 agonist/adjuvant           | New York University School of Medicine, NCI | NCT01421017     | SM   | Adaptive immune stimulator                            |
| Imiquimod              | Phase 1     | Melanoma and metastatic cancer       | TLR7 agonist/drug                | University of Oklahoma, NCI            | NCT00453050     | SM   | Immune stimulation                                    |
| Imiquimod              | Phase 2     | Breast cancer and breast neoplasms   | TLR7 agonist/drug                | New York University School of Medicine | NCT00899574     | SM   | Immune stimulation                                    |
| 852A                   | Phase 2     | Breast, ovarian, endometrial, and cervical cancer | TLR7 agonist/drug | Masonic Cancer Center University of Minnesota, Pfizer | NCT00319748     | SM   | DC activation, IFN-α secretion                        |
| Imiquimod              | Phase 3     | Influenza viral infection            | TLR7 agonist/adjuvant           | The University of Hong Kong            | NCT02103023     | SM   | Improve vaccine immunogenicity against influenza virus |
| Imiquimod              | Phase 2     | HPV                                  | TLR7 agonist/drug                | Medical University of Vienna           | NCT00941811     | SM   | Immune stimulation                                    |
| Imiquimod (intradermal HBVv) | Phases 2 and 3 | Renal failure                        | TLR7 agonist/adjuvant           | The University of Hong Kong            | NCT02621112     | SM   | Improving vaccine immunogenicity                      |
| Imiquimod (influenza vaccine) | Unknown      | Chronic Illness                      | TLR7 agonist/adjuvant           | The University of Hong Kong            | NCT01508884     | SM   | Activation of APC                                     |
| GS-9620                | Phase 2     | Chronic hepatitis B                  | TLR7 agonist/adjuvant           | Gilead Sciences                        | NCT02166047     | SM   | Activation of pDC                                     |
| Single or multiple GS-9620 | Phase 1      | Hepatitis B                          | TLR7 agonist/adjuvant           | Gilead Sciences                        | NCT01590654     | SM   | Activation of pDC, Immune stimulation                 |
| Imiquimod              | Unknown     | Photoaged skin and normal skin       | TLR7 agonist/adjuvant           | University of Michigan                 | NCT02889159     | SM   | Immune stimulation                                    |
| RO6864018 (aka ANA773, RO-6864018) | Phase 1 | Healthy volunteer                    | TLR7 agonist/drug                | Hoffmann-La Roche                      | NCT02015715     | SM   | Immunomodulator, Immune stimulation                   |

(Continues)
| Ligand      | Phase  | Application                          | Target TLR adjuvant/drug | Sponsor/collaborators             | NCT number      | Type  | Purpose/mechanism                                                                 |
|------------|--------|--------------------------------------|--------------------------|-----------------------------------|-----------------|-------|----------------------------------------------------------------------------------|
| RO7020531  | Phase 1| Chronic Hepatitis B                  | TLR7 agonist/drug        | Hoffmann-La Roche                 | NCT02956850     | SM    | Immune stimulation, B and T cell activations                                      |
| RO6864018  | Phase 2| Chronic Hepatitis B                  | TLR7 agonist/drug        | Hoffmann-La Roche                 | NCT02391805     | SM    | Immunomodulator, Immune stimulation                                                |
| GSK2245035 | Phase 2| Mild asthma and allergic rhinitis    | TLR7 agonist/drug        | GlaxoSmithKline                   | NCT01788813     | SM    | Immune stimulation                                                                |
| GSK2445053 | Phase 1| Rhinitis, allergic                    | TLR7 agonist/drug        | GlaxoSmithKline                   | NCT01480271     | SM    | Induction of IFNα                                                                  |
| GSK2445035 | Phase 2| Allergy rhinitis, asthma, and respiratory tract allergy | TLR7 agonist/drug | GlaxoSmithKline | NCT02833974 | SM    | Immune stimulation                                                                |
| Imiquimod  | Phase 3| Actinic keratosis                     | TLR7 agonist/drug        | Graceway Pharmaceuticals, LLC     | NCT00894647     | SM    | Immune stimulation                                                                |
| Imiquimod  | Phase 4| Actinic keratosis                     | TLR7 agonist/drug        | MEDA Pharma GmbH & Co. KG         | NCT00777127     | SM    | Immune stimulation                                                                |
| Imiquimod  | Approved (China, Dec 2004) | Keratosis, mycosis fungoides, verruca vulgaris, condyloma, basal cell carcinoma, and molluscum contagiosum infection | TLR7 agonist/ adjuvant | Mochida Pharmaceutical Co Ltd, 3M Pharmaceuticals, Valeant Pharmaceuticals International Inc, iNova Pharmaceuticals Pty Ltd, Intendis GmbH, Meda AB | NCT01453179 | SM    | Immune stimulation                                                                |

Abbreviations: GSK, GlaxoSmithKline; HPV, human papilloma virus; IFN, interferon; NCI, National Cancer Institute; NCT, national clinical trial; SM, small molecule; TLR, Toll-like receptor.
and geometric complementarity. These regions on both proteins should be explored further to design novel therapeutics.

6.5 | TLR7/8

TLR7 and TLR8 are functionally active in the endosomal compartment, use MyD88 adapter molecules and are activated by single-stranded RNA (ssRNA). Majority of ligands in clinical trials that target TLR7/8 are small molecules (eg, imiquimod [R837], resiquimod, or GSK2245035), and most are derivatives of imidazoquinoline, a tricyclic organic molecule. There are functional differences between these two TLRs; for example, plasmacytoid DC and monocytes can be directly activated by TLR7 agonists; however, other than monocytes, TLR8 agonists can directly activate mDCs and monocyte-derived DCs. TLR7 agonists were more potent when compared with TLR8 agonists regarding antiviral responses in the form of IFN, I-TAC (IFN-inducible T-cell α chemoattractant), and IFN-regulated cytokines from human peripheral blood mononuclear cells (PBMC).

Proinflammatory responses, such as expression of IL-12, TNF-α, and macrophage inflammatory proteins-1α (MIP-1α) were enhanced by TLR8 agonism when compared with TLR7, leading to characteristic differential cell induction profiles.

TLR7 agonists have been actively studied in phase 1 and 2 trials aiming to curb the persistent viral load in HIV- and HBV-infected individuals. Moreover, various prodrug (pharmacologically active after metabolism) approaches have also targeted TLR7 (RO6870868 [single prodrug] or RO6864018 [double prodrug]), and use of these as TLR7 agonists was useful in treating hepatitis B infection in phase 1 clinical trials. The results of this trial were promising and paved the way for phase 2 trials (https://clinicaltrials.gov/ct2/show/NCT02015715).

TLR8 can also be activated by ssRNA as natural ligand and by VTX-2337 (motolimod), a synthetic small molecule selective for TLR8 and is being evaluated in clinical trials. TLR8 is a less studied receptor, as its roles overlap with those of TLR7, with which it shares multiple features. When treated with VTX-2337, TLR8 stimulates TNF-α and IL-12 production at lower concentrations in human PBMCs. It also induces TNF-α and IL-12 secretion from monocytes and myeloid DCs through the NF-κB pathway. IFNγ secretion was observed when NK cells were treated with VTX-2337, which can enhance the lytic capability and antibody-dependent cell-mediated cytotoxicity of NK cells. VTX-2337 also improves the efficacy of pegylated liposomal doxorubicin in treatment of ovarian cancer in a mouse model with humanized immune system that has been reconstituted with human CD34+ cells. This is the only ligand molecule that has been actively evaluated for treatment of a variety of cancers, including head and neck cancer, colorectal, pancreatic, melanoma, breast, renal cell carcinoma, nonsmall-cell lung carcinoma, and other solid neoplasms. In the majority of cases, VTX-2337 was used in combination with other drugs; however, it is also being evaluated as a stand-alone drug for treatment of lymphoma.

TLR7 and TLR8 share similar activation patterns, both have z-loops involved in ssRNA recognition, and both possess two binding sites; the first binding site binds guanosine and uridine in TLR7 and TLR8, respectively, while the second binds ssRNA in both cases (Figure 3). In TLR7 ssRNA binding primes the receptor for guanosine binding and subsequent dimerization, while synthetic molecules, such as R848, can activate TLR7 without the need for ssRNA. Importantly, TLR7 remains monomeric in the absence of any ligand and dimerizes in response to ligand binding; however, its dimer conformation is similar to TLR8 and TLR9. TLR8, on the other hand, is a naturally occurring weak dimer that undergoes conformational change upon ligand binding. The Z-loop may have an important regulatory role; when it is cleaved from TLR8, TLR8 forms a tight dimer and initiates signaling in the absence of ligand.

6.6 | TLR9

TLR9 senses CpG DNA in endosomes and induces the IFN response. TLR9 is involved in numerous diseases and has been targeted by various therapeutic approaches. All of the ligands tested in clinical trials that exclusively
| Ligand                          | Phase  | Application                                      | Target TLR adjuvant/drug | Sponsor/collaborators                        | NCT number    | Type                  | Purpose/mechanism                      |
|--------------------------------|--------|-------------------------------------------------|--------------------------|---------------------------------------------|---------------|-----------------------|----------------------------------------|
| Resiquimod, poly-ICLC          | Phase 2| Glioma, anaplastic astrocytoma, anaplastic astro-oligodendroglioma | TLR3/7/8 agonist/adjuvant | Jonsson Comprehensive Cancer Center          | NCT01204684  | SM, synthetic dsRNA   | Antitumor and antiviral immune stimulation |
| Poly-ICLC, resiquimod (R848)   | Phases 1 and 2 | Melanoma and its metastatic mucosal variants | TLR3/7/8 agonist/adjuvant | Craig L Slingluff, Jr, University of Virginia | NCT02126579  | SM, synthetic dsRNA   | Antitumor immune stimulation            |
| Resiquimod (R848)              | Phase 1 | Influenza vaccination in seniors                 | TLR7/8 agonist/adjuvant | University of British Columbia               | NCT01737580  | SM                    | Immune stimulation                      |
| MEDI9197 (durvalumab)          | Phase 1 | Solid tumors, cutaneous T cell lymphoma          | TLR7/8 agonist/adjuvant | MedImmune LLC                                | NCT02556463  | SM                    | Improving antigen presentation          |
| Resiquimod (NY-ESO-1)          | Phase 1 | Tumors                                           | TLR7/8 agonist/adjuvant | Nina Bhardwaj, CRI New York City, Icahn School of Medicine at Mount Sinai | NCT00821652  | SM                    | Immune stimulation                      |
| IMO-8400                       | Phases 1 and 2 | Waldenstrom’s macroglobulinemia                       | TLR7/8/9 antagonist/adjuvant | Idera Pharmaceuticals, Inc                   | NCT02092909  | Oligonucleotide antagonist | Immune suppression, TLR7/8/9 signaling blocker |
| IMO-8400                       | Phase 2 | Plaque psoriasis                                 | TLR7/8/9 antagonist/drug | Idera Pharmaceuticals, Inc                   | NCT01899729  | Oligonucleotide antagonist | Immune suppression                      |
| CPG-52364                      | Phase 1 | Healthy volunteers                               | TLR7/8/9 antagonist/drug | Pfizer                                       | NCT00547014  | SM                    | Immune suppression                      |
| IMO-8400                       | Phases 1 and 2 | Diffuse large B cell lymphoma                       | TLR7/8/9 antagonist/drug | Idera Pharmaceuticals, Inc                   | NCT02252146  | Oligonucleotide antagonist | Immune suppression                      |

Abbreviations: CRI, Cancer Research Institute; dsRNA, double-stranded RNA; NA, not available; NCT, national clinical trial; SLE, systemic lupus erythematosus; SM, small molecule; TLR, Toll-like receptor.
| Ligand | Phase | Application | Target TLR adjuvant/d| Sponsor/collaborators | NCT number | Type | Purpose/ mechanism |
|--------|-------|-------------|----------------------|-----------------------|-------------|------|-------------------|
| VTX-2337 (Motolimod) | Phase 1 | A variety and different stages of metastatic squamous neck cancer with occult primary squamous cell carcinoma | TLR8 agonist/adjuvant | University of Washington, NCI | NCT01334177 | SM | Immune stimulation |
| VTX-2337 | Phase 1 | For various types and stages of colorectal, pancreatic, breast, melanoma, non-small cell lung carcinoma, pancreatic, renal cell carcinoma and solid neoplasm | TLR8 agonist/adjuvant | Mayo Clinic, NCI | NCT02650635 | SM | Immune stimulation |
| VTX-2337 | Phase 1 | Various types of ovarian cancers and fallopian tube carcinoma, recurrent ovarian carcinoma | TLR8 agonist/adjuvant | Gynecologic Oncology Group, NCI | NCT01294293 | SM | Immune stimulation |
| VTX-2337 | Phase 2 | Epithelial ovarian cancer, fallopian tube cancer, primary peritoneal cancer | TLR8 agonist/adjuvant | VentiRx Pharmaceuticals Inc, Gynecologic Oncology Group | NCT01666444 | SM | Immune stimulation |
| VTX-2337 (with radiotherapy) | Phases 1 and 2 | Low grade B-Cell lymphoma | TLR8 agonist/adjuvant | VentiRx Pharmaceuticals Inc, Stanford University | NCT01289210 | SM | Immune stimulation |
| VTX-2337 | Phase 2 | Carcinoma, squamous cell of head and neck | TLR8 agonist/adjuvant | VentiRx Pharmaceuticals Inc | NCT01836029 | SM | Immune stimulation |
| VTX-2337 (with anti-PD-L1 antibody MEDI4736) | Phases 1 and 2 | Ovarian cancer | TLR8 agonist/adjuvant | Ludwig Institute for Cancer Research, MedImmune LLC, VentiRx Pharmaceuticals Inc, CRI New York City | NCT02431559 | SM | Immune stimulation |

Abbreviations: CRI, Cancer Research Institute; NCI, National Cancer Institute; NCT, national clinical trial; PD-L1, programmed death-ligand 1; SM, small molecule; TLR, Toll-like receptor.
| Ligand                  | Phase | Application                               | Target TLR adjuvant/drug | Sponsor/Collaborators                                                                 | NCT Number    | Type                              | Purpose/Mechanism                                |
|-------------------------|-------|-------------------------------------------|--------------------------|--------------------------------------------------------------------------------------|---------------|-----------------------------------|-----------------------------------------------|
| MGN1703 (Ipilimumab)    | Phase 1 | Melanoma                                  | TLR9 agonist/adjuvant    | MD Anderson Cancer Center, Mologen AG                                                | NCT02668770  | DNA-based molecule                | Antitumor immune stimulation                   |
| SD-101                  | Phases 1 and 2 | Lymphoma and its various forms                         | TLR9 agonist/adjuvant    | Robert Lowsky, NCI, Stanford University                                              | NCT02254772  | CpG-C class oligodeoxynucleotide | Antitumor immune stimulation                   |
| SD-101                  | Phases 1 and 2 | Grade 1/2/3 follicular lymphoma and recurrent and refractory follicular lymphoma | TLR9 agonist/drug        | Robert Lowsky, NCI, Stanford University                                              | NCT02927964  | CpG-C class oligodeoxynucleotide | Antitumor immune stimulation                   |
| CpG vaccine (autologous tumor cell) | Phase 1 | Colorectal neoplasms, anal, colon, and rectal cancers | TLR9 agonist/adjuvant    | Stanford University                                                                | NCT00780988  | Oligonucleotide                  | Antitumor immune stimulation                   |
| CYT003                  | Phase 2 | Moderate to severe allergic asthma         | TLR9 agonist/drug        | Cytos Biotechnology AG                                                             | NCT01673672  | Oligonucleotide                  | TH1-mediated immune response                   |
| CYT003-QbG10            | Phase 2 | Allergic Bronchial Asthma                  | TLR9 agonist/drug        | Cytos Biotechnology AG                                                             | NCT00890734  | Oligonucleotide                  | TH1-mediated immune response                   |
| DUK-CpG-001             | Phase 2 | Hodgkin lymphoma, non-Hodgkin lymphoma     | TLR9 agonist/adjuvant    | David Rizzieri, MD, Duke University                                                 | NCT02115126  | Single-stranded synthetic DNA molecules | Immune stimulation                             |
| MGN1703                 | Phases 1 and 2 | HIV                                       | TLR9 agonist/drug        | University of Aarhus                                                               | NCT02443935  | DNA-based molecule                | Antiviral immune stimulation                   |
| CpG-7909                | Phases 1 and 2 | Non-Hodgkin lymphoma, mycosis fungoides | TLR9 agonist/adjuvant    | Ronald Levy, Lymphoma Research Foundation, American Society of Clinical Oncology, Stanford University | NCT00185965  | single-stranded synthetic DNA molecules | TH1-like immune stimulator                     |
| Ligand                  | Phase       | Application               | Target TLR adjuvant/drug | Sponsor/Collaborators                                      | NCT Number  | Type                                | Purpose/ Mechanism                      |
|------------------------|-------------|---------------------------|--------------------------|------------------------------------------------------------|-------------|-------------------------------------|-----------------------------------------|
| CYT003                 | Phase 2     | Asthma                    | TLR9 agonist/drug        | Cytos Biotechnology AG                                     | NCT02087644| Oligonucleotide                      | TH1-mediated immune response            |
| CpG-7909 (pneumococcal vaccines) | Phases 1 and 2 | HIV infections            | TLR9 agonist/adjuvant    | Aarhus University Hospital                                 | NCT00562939| Oligonucleotide                      | Immune stimulator                       |
| GNKG168                | Phase 1     | Relapsed acute lymphoblastic myelogenous leukemia | TLR9 agonist/drug | Therapeutic Advances in Childhood Leukemia Consortium | NCT01743807| CpG-C class oligodeoxynucleotide    | Antitumor immune response               |
| CpG-7909               | Phase 1     | Malaria                   | TLR9 agonist/adjuvant    | Oxford University, NIAID                                    | NCT01351948| Oligonucleotide                      | Immune stimulation                      |
| EMD 1201081 (Cetuximab) | Phase 2     | Squamous cell carcinoma of the head and neck | TLR9 agonist/adjuvant    | EMD Serono                                                 | NCT01040832| Oligonucleotide                      | Immune stimulation                      |
| IMO-2125               | Phase 1     | Hepatitis C               | TLR9 agonist/adjuvant    | Idera Pharmaceuticals, Inc                                 | NCT00728936| Oligonucleotide                      | Immune stimulation                      |
| CpG 10104              | Phase 1     | Hookworm infection        | TLR9 agonist/adjuvant    | Baylor College of Medicine, George Washington University   | NCT02143518| Oligonucleotide                      | Immune stimulation                      |
| AZD1419                | Phase 2     | Asthma                    | TLR9 agonist/drug        | AstraZeneca                                                 | NCT02898662| C-type CpG oligonucleotide           | IFN induction                           |
| CpG-7909 (URLC10-177, TTK-567) | Phases 1 and 2 | Esophageal cancer      | TLR9 agonist/adjuvant    | Wakayama Medical University, Human Genome Center University of Tokyo | NCT00669292| Oligonucleotide                      | Immune stimulation                      |
| CpG-7909               | Phases 1 and 2 | Mycosis fungoides        | TLR9 agonist/adjuvant    | Stanford University, NIH                                    | NCT00226993| Oligonucleotide                      | Immune stimulation                      |
| Ligand                      | Phase | Application          | Target TLR adjuvant/drug | Sponsor/Collaborators                              | NCT Number          | Type                                      | Purpose/Mechanism                      |
|----------------------------|-------|----------------------|--------------------------|---------------------------------------------------|---------------------|------------------------------------------|----------------------------------------|
| SD-101                     | Phase 1 | Chronic hepatitis C | TLR9 agonist/adjuvant   | Dynavax Technologies Corporation, Synteract, Inc, PPD | NCT00823862         | CpG-C class oligodeoxynucleotide         | Anti-tumor immune response              |
| Mycobacterium w. (Mw)-freeze dried extract 0.5 mL | NA     | Optic neuritis       | TLR9 antagonist/adjuvant | Sudhalkar Eye Hospital                            | NCT01424735         | Bacterial mix                            | Immune suppression                      |
| Hydroxychloroquine sulfate, valsartan | Phase 4 | Primary IgA nephropathy | TLR9 inhibitor/adjuvant | Peking Union Medical College Hospital             | NCT02765594         | SM                                       | Impaired IFN-α and TNF-α secretion      |
| Hydroxychloroquine         | Phase 3 | Sjogren's syndrome   | TLR9 inhibitor/drug      | SNU Hospital                                      | NCT01601028         | SM                                       | Immune suppression                      |
| CpG-ODN (K3)               | Phase 1 | Lung tumor           | TLR9 agonist/drug        | National Institute of Biomedical Innovation, Osaka University | NA                  | Nucleotide based                         | Immune stimulation                     |

**Abbreviations:** IFN, interferon; NA, not applicable; NIAID, National Institute of Allergy & Infectious Diseases; NCI, National Cancer Institute; NCT, national clinical trial; NIH, National Institute of Health; SM, small molecule; SNU, Seoul National University; TLR, Toll-like receptor; TNF, tumor necrosis factor.
target TLR9 are either nucleotides or nucleotide derivatives. There are various types of CpG DNAs that are being evaluated in different trials for treatment of diverse conditions. AZD1419 is a C-type CpG-based inhaled TLR9 agonist for treatment of asthma and to stimulate IFNs production in lungs. This treatment was classified as well-tolerated and safe in phase 1 human trials with potential disease-modifying characteristics and is a promising new therapeutic for use in various immune diseases.\(^\text{138}\) CYT003 was initially found to be effective; however, its effects were not confirmed in phase 2 clinical trials where 35 patients were treated with varying doses of CYT003.\(^\text{139}\) Another TLR9 agonist, EMD 1201081, was evaluated in phase 2, open-label, randomized trial in patients with head and neck cancer, and was found to be ineffective in the tested dose regimen.\(^\text{140}\) GNKG168 is another CpG-based molecule that can induce CD8^+ T cell antitumor cytotoxic responses; however, it was withdrawn in clinical phase 1 because of sponsor reluctance to further support the study\(^\text{141}\) (NCT01743807) (Tables 7 and 9).

Similar to other TLRs, TLR9 forms a symmetrical complex with CpG-DNA; nonetheless, during inhibitory DNA interactions, it remains in a monomeric form. CpG-DNA binding with TLR9 is symmetric and they form a stoichiometric complex of 2:2, as DNA is recognized by both TLR9 monomers, particularly via the amino-terminal fragment (LRRNT–LRR10) from one protomer and the carboxy-terminal fragment (LRR20–LRR22) from the other.\(^\text{142}\) CpG-DNA-based TLR9 inhibition is mediated by binding to the concave surface formed by LRR2–LRR10, thereby inhibiting its signaling.

6.7 | TLR10–13

Other than TLR1–9, humans also have TLR10 and TLR11, whereas they lack TLR12 and TLR13.\(^\text{143}\) The expression of TLR10 has been confirmed in humans (spleen, lymph node, B cells, monocytes, and neutrophils)\(^\text{144}\); nonetheless, its function and specific ligand are yet to be determined. Recently, it was suggested that TLR10 may act as an anti-inflammatory TLR, rather than a conventional inflammatory receptor and that it modulates TLR2-mediated responses through the formation of heterodimers with TLR1 or TLR6.\(^\text{145}\) Humans have a pseudogene homologous to TLR11 that includes a premature stop codon, resulting in lack of protein expression.\(^\text{146}\) TLR11 and TLR12 have been studied in mouse and they have shown to detect profilin from *Toxoplasma gondii* and be capable of forming heterodimers.\(^\text{143}\)

7 | INTERDEPENDENT AND CROSS-TALK AMONG TLR PATHWAYS

Since TLRs overlap in their structures and signaling pathways, it is rational to assume that one single ligand can activate multiple TLRs; however, this is less common among plasma membrane expressed TLRs, TLR2/1, TLR2/6, TLR4, and TLR5, and there are a few ligands that can share targets, particularly for TLR2 and TLR4. This situation is very common among endosomal TLRs, partly because they are all involved in sensing nucleic acids, and endosomes have a specific pH range that is also thought to contribute to their activation. Various ligands exert their actions on multiple endosomal TLRs (eg, TLR7/8 or TLR7/8/9), which may imply a combination of multiple pathways in their activity, a common mode of activation, and, to some extent, H^+ interference of these TLRs being a common factor\(^\text{147,148}\) (Tables 7 and 10). TLR7 and TLR8 detect ssRNA, which may explain why one ligand is equally effective against both TLRs. Some studies have also explored the independent targeting for either TLR7\(^\text{149}\) or TLR8.\(^\text{150}\) The expression patterns of various TLRs differ among tissues, and the extent to which they respond to various ligands may contribute to unexpected results of clinical trials (mostly failure and toxicity issues). TLR2/TLR4 (cell surface) can be regulated by a single ligand, and there are many examples of endosomal TLRs being regulated by single ligands, suggesting that they may have similar sensing and regulatory mechanisms that could be exploited for therapeutic purposes.

TLRs may antagonize one another under certain physiological conditions. For example, TLR2 and TLR9 antagonize each other in a mouse model for oral infection with *Salmonella enterica*.\(^\text{151}\) TLR9 deficiency is manifested...


| Ligand                                                                 | Phase                  | Application                        | Mode of action | Sponsor/collaborators                                                                 | NCT number      | Type                      | Purpose/mechanism                  |
|------------------------------------------------------------------------|------------------------|------------------------------------|----------------|---------------------------------------------------------------------------------------|-----------------|---------------------------|------------------------------------|
| DC-based vaccine                                                       | Phases 1 and 2         | Melanoma                           | TLR agonist/adjuvant | Radboud University                                                                     | NCT01530698    | Biologics, SM             | DC activation                      |
| TLR agonist (Tuberculin nasal challenge, timothy grass pollen)         | NA                     | Allergic rhinitis, asthma, latent tuberculosis | TLR agonist/adjuvant | Imperial College London                                                                | NCT02090374    | Bacterial mix, SM         | Immune stimulation                 |
| Mycobacterium w                                                        | Phases 2 and 3         | Sepsis                             | TLR agonist/adjuvant | Postgraduate Institute of Medical Education & Research, PGIMS, Rohtak, St. John's National Academy of Health Sciences | NCT02330432    | Bacterial mix             | Immune stimulation                 |
| DRibbles vaccine, imiquimod, HPV vaccine                               | Phase 2                | Carcinoma, non-small-cell lung cancer | TLR2/3/4/7/9 agonists/adjuvant | UbiVac                                                                               | NCT01909752    | Combination of protein and SM | Immune stimulation                 |
| DRibbles vaccine, imiquimod, HPV vaccine                               | Phase 1                | Adenocarcinoma of the prostate      | TLR agonist/adjuvant | UbiVac                                                                               | NCT02234921    | Combination of protein and SM | Immune stimulation                 |
| MB-11040                                                               | Phase 1                | Menopause, autoimmune disease, cancer | TLR agonist/drug  | KT & G Life Sciences Corp                                                               | NA             | SM                        | Antitumor immunity                 |
| Mycobacterium w                                                        | Phases 2 and 3         | Severe sepsis, septic shock, immune modulation | TLR agonist/drug  | Postgraduate Institute of Medical Education & Research                                | NCT02025660    | Bacterial mix             | Immune stimulation                 |
| Insulin                                                                | Phase 2                | Insulin resistance                  | TLR inhibitor/drug | Kaleida Health, American Diabetes Association                                          | NCT01151605    | Biomolecule               | TLR downregulation                |
| Autologous DC vaccination                                              | Phases 1 and 2         | Melanoma                           | TLRs agonist/adjuvant | Radboud University                                                                     | NCT00940004    | SM                        | DC activation/maturation            |
| R848 (GP100, MAGE-3)                                                   | Phase 2                | Melanoma                           | TLR agonist/adjuvant | MD Anderson Cancer Center                                                              | NCT00960752    | SM                        | Immune stimulation                 |
| VB-201                                                                 | Phase 2                | Ulcerative colitis                 | TLR2-4 antagonist/drug | VBL Therapeutics                                                                     | NCT01839214    | SM                        | Immune suppression                |

(Continues)
| Ligand   | Phase | Application                                      | Mode of action adjuvant/drug | Sponsor/collaborators | NCT number       | Type | Purpose/mechanism          |
|----------|-------|--------------------------------------------------|------------------------------|-----------------------|------------------|------|--------------------------|
| PUL-042  | Phase 1 | Healthy individuals                             | TLR2/6, 9 agonist/adjuvant   | Pulmotect Inc         | NCT02124278     | SM   | Immune stimulation       |
| PUL-042  | Phase 1 | Hematologic diseases, stem cell transplants     | TLR2/6, 9 agonist/adjuvant   | Pulmotect Inc         | NCT03097796     | SM   | Immune stimulation       |

Abbreviations: DC, dendritic cell; HPV, human papilloma virus; NA, not available; NCI, National Cancer Institute; NCT, national clinical trial; PGIMS, Pandit Bhagwat Dayal Sharma; SM, small molecule; TLR, Toll-like receptor.
as reduced survival, exaggerated cytokine responses, and salmonella hepatitis, while TLR2 deficiency produces the opposite effects. Deficiency of either TLR may disrupt NK cell cytotoxicity, and IFN-γ and ROS production.\textsuperscript{151}

Synergism is very common in TLRs. When monocyte-derived DCs have been triggered with a TLR8 ligand, TLR3 or TLR4 are also activated, resulting in expression of IL-6, IL-10, IL-12, and TNF-α elevation. These results were also confirmed by increased binding of IRF and signal transducers and activators of transcription (STAT) transcription factors to their respective DNA binding sites, which was abolished when NF-κB, p38, and phosphoinositide 3-kinase (PI3K) inhibitors were used.\textsuperscript{152} These data suggest that co-operation among TLRs is perpetuated, not only at the top level but also among different signaling pathways to ensure proper and balanced expressions of target genes.

Synergy and tolerance of TLRs are long-established and are critical to the innate immune response. The coadministration of LPS (TLR4 agonist) and MALP-2 (TLR2 agonist) to mouse macrophages resulted in increased TNF-α production.\textsuperscript{153} Repeated treatment with LPS or MALP-2 resulted in a hyporespons, also termed tolerance. Intriguingly, pretreatment with any ligand results in lower responses on exposure to the second ligand.\textsuperscript{153} LPS may cause downregulation of the cell surface expression of TLR4 after the second LPS treatment; however, MALP-2-mediated reduction in responses involve modulation of downstream signaling. The acute immune tolerance and cross-tolerance between TLR4 and TLR9 have been studied,\textsuperscript{154} indicating that LPS selectively inhibits proinflammatory cytokines, while CpG suppresses both pro- and anti-inflammatory responses. IRAK-M is critical for the induction of this differential response, and its expression is modulated by IL-7.\textsuperscript{154}

The mycobacterium extract,\textsuperscript{155,156} and autophagosome-enriched cancer vaccine (DRibbles),\textsuperscript{157} which likely contain multiple biological molecules and can trigger numerous TLRs, is being evaluated in clinical trials; however, caution is required when considering the use of such substances in the clinic due to synergy and differential responsiveness of TLRs to various ligands. Moreover, DC vaccines that have been matured using TLR ligands are also therapeutically relevant, owing to the use of TLR ligands in their production.\textsuperscript{158,159}

8 | FAILED CLINICAL TRIALS

The proportion of failures of clinical trials depends on the clinical stage, as well as the type of disease; particularly, failure at phase 3 is an impediment to the development of successful therapy for various diseases and TLRs are no exception. For example, eritoran, a TLR4 antagonist, that was being evaluated for treatment of sepsis could not meet its target end-point in phase 3 when data from ~2000 patients were analyzed.\textsuperscript{160} Among the reasons of failure of eritoran, there were oversights in study design, patient population differences, improved patient care methods, and mixed bacterial infections.\textsuperscript{160} Similarly, imiquimod, a TLR7 agonist, produced a divergent result in phase 3 when evaluated for treatment of the skin disorder, molluscum contagiosum (MC) lesions, in children.\textsuperscript{161} Imiquimod was first approved by the Food and Drug Administration in 1997 for treatment of genital warts. This approval has prejudiced its subsequent off-label use as the treatment of MC in children, since it was already shown to be effective against viral-based diseases and its use is supported by several research and clinical investigations.\textsuperscript{82,83} This off-labeled use of imiquimod is still debatable.\textsuperscript{83,161} Similarly, in a meta-analysis, Qin et al (2014) has systematically analyzed the TLR9 agonist effects as observed by other clinical investigations. It has been concluded that the safety profile of TLR9 agonists is acceptable if they are not combine with immunosuppressive drugs.\textsuperscript{162}

The success rate of transition among different clinical phases (phase 1, 2, 3, and occasionally 4) is highly variable. The likelihood of a molecule passing phase 1 is 63.2%, which is the highest probability for any phase. Phase 2 has the lowest success rate (30.7%), while phase 3 has a success rate of 58.1%.\textsuperscript{163,164} Biologics have twice the final success rate (18%), compared with that of small molecules (9%). The transition failure can comprise of drugs that have not met their specific endpoints, and there are cases where particular drugs did not show any improved effect over an existing treatment of a particular condition. The situation is exacerbated when similar molecules are evaluated in multiple interventions, causing an elevated number of failed trials.
Lack of recruitment (23%) and unstated reasons (such as unknown reasons for termination, unable to begin the study, unavailability of a drug, or protocol modification resulting in cessation of a trial; 26%) comprise the majority of reasons for failure of clinical trials targeting TLRs, followed by safety issues (18%) and financial concerns (where a sponsor withdraws the drug; 15%). Moreover, 15% of trials do not show any efficacy in subsequent clinical trials. The inadequate understanding of the biology of TLRs may also contribute to drug failure, underlining the need for further studies, increased understanding of the theoretical background of disease etiology and progression, modification of protocols to address problems, and trial redesign.

While many factors contribute to a failed clinical trial, a common reason underlying failure is a lack of serious focus on biomarker discovery and implementation. There is a clear trend of success among those trials including biomarker selection (25.9%) compared with those lacking selection biomarkers (8.4%). There are several methods that can be used to reduce failure rates, such as early identification of false drug candidates, stratification of patients, development of diagnostics, proper use of pharmacogenomics through machine learning, and other analysis tools that can provide improvements and efficiencies in patient categorization. Focusing on neglected disease areas can also help to reduce the failure burden. In recent years, the Drugs for Neglected Diseases Initiative has approved six treatments within a decade, with many more in the pipeline. This is not only dramatically reducing the cost of drug development, but also providing hope for individuals affected by neglected diseases and incentivizing the pharmaceutical industry to continue their search for new drugs.

Financial and commercial reasons are also major contributors to trial failures because sponsors are "unwilling," or there are "failure to pursue" investigational drugs for commercially important diseases. This can be reduced if pharmaceutical companies focus on diseases that lack adequate therapeutic intervention, as drugs that show positive effects will soon be marketable. Additionally, if such a trial does fail, it has a lower cost impact on the company.

Drug development is a lengthy process that starts with lead molecule identification and progresses through optimization, animal modeling studies, pharmacokinetic and pharmacodynamic studies, and preclinical and clinical stage trials. Therefore, if a drug fails to show any effect or shows toxicity in clinical studies, there must have been a series of oversights during earlier experimental stages. It is hard to give a single reason for any failure and failures may encompass complex issues, such as the use of subjective, composite, or surrogate endpoints. Moreover, biases in outcome reporting and publications; underreporting of adverse events; failure to select an appropriate patient group; preference for relative outcomes, rather than absolute values; no defined core outcome sets; lack of transparency and basic science; inappropriate study population size; and lack of data integrity are among the reasons for trial failures. Finally, during clinical trials involving humans, factors that influence the drug metabolism, distribution, and secretion are diverse that predispose the pharmacokinetically and pharmacodynamically optimized drug molecules to failure.

9 | PERSPECTIVES IN TLR TARGETING

Researchers are expending extensive efforts to generate appropriate solutions for various inflammatory, autoimmune, and malignant conditions; however, the process is not straightforward, rather it is littered with unexpected events and outcomes, along with unknown obstructions that severely undermine the efforts of the research community.

In the majority of studies targeting TLRs, the investigated compounds are related to or derived from natural ligands; particularly those targeting TLR3, TLR4, TLR5, and TLR9, and somewhat those for TLR2. TLR7 and TLR8 have the benefit of being targeted by small molecules rather than ssRNA. The instability of ssRNA molecules can hinder their use for TLRs activation. However, since RNAi technology is being evaluated in more than 100 clinical trials, stability should not be an issue, rather, tuning of small molecules is far easier than tuning biologics for therapeutic purposes.

Other than molecules derived from natural ligands, it is necessary to focus on the chemical space that can be used to target TLRs. This broadening of the chemical space will provide more potent, specific, and less toxic molecules, resulting in fewer trial failures. Biologics are gaining popularity, as they have a higher ratio of success, and are comparatively safe and specific. It is estimated that the biologics will soon become the norm in
therapeutics, in addition to being responsible for the majority of revenue. For different TLRs, the therapeutic trend can vary; however, a rise in antibody-mediated TLR inhibition (TLR2, TLR3, and TLR4) and novel molecular backbone (independent of PAMPs) have been seen in recent therapeutics.

The evaluation of various drugs for similar or different conditions is also an optimal approach, which can facilitate the development of single drugs for multiple diseases. In this context, research laboratories can screen the outcomes of phase 2 failures that have been abandoned by their sponsors to evaluate them for other symptoms. Such an approach can dramatically reduce the cost, speed up the process, and will encourage pharmaceutical companies to share their data with research laboratories for application to other disease targets.

Rather than directly inhibiting TLRs, it may be more appropriate to target the transcription regulation of TLRs to suppress their expression, as described in a study where the authors used GST-21 for cytokine inhibition, which could be reversed by the janus kinase 2 inhibitor, AG490. Since the majority of TLRs regulate the similar pathway, targeting of their downstream inhibitory signaling mechanisms should also be explored to further intensify the benefits of their inhibition.

Lack of clinical data is an impediment to the development of clinical research. It is estimated that approximately half of all clinical trials are not reported in either peer-reviewed journals or clinical trial websites (clinicaltrials.gov; http://apps.who.int/trialsearch/).

It is now necessary to develop additional TLR ligands that should not mimic PAMPs, explore new biomarkers for disease progression, revise protocols, and clinical trials, target small subsets of patients, improve the understanding of the basic biology of diseases, and improve final outcomes, which must legitimately refer to the progress of the disease and the effect of the compound being applied.

10 | CONCLUSIONS

TLRs are among the ideal targets for exploitation in immunotherapy; however, their biology still needs to be better understood in the context of target diseases. These receptors are capable of inhibiting disease pathophysiology, as well as exacerbating inflammatory diseases. Given this dual role, it is imperative to fine tune their activation using a multidrug approach. Cumulative evidence suggests the participation of TLRs in almost all diseases is unique and can be exploited by including their ligands as adjuvant treatment during regular immunotherapy or as part of other therapeutic regimens.

It is vital to create superior disease models that assist in early phase evaluation of drugs, improve diagnostics and evaluation of disease progression, and facilitate identification of novel biomarkers that reliably indicate disease progression and real-time disease monitoring. Finally, the availability of clinical trial data should be ensured to guide the scientific community in their endeavors. This would also assist in the refinement of targets and lead molecules and improve the pathophysiological manifestations of diseases. Using a combination of computational power, next-generation sequencing and proteomic data, machine learning approaches, and improved availability of results, we are hopeful that a dramatic increase in new therapeutic options for various inflammatory diseases and cancers involving TLRs and a decline in clinical trial failures will be achieved.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.
AUTHOR’S CONTRIBUTION

MAA and SC participated in research design; MAA, MS, and JK performed data analysis; and MAA, MS, JK, and SC wrote or contributed to the writing of the manuscript.

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