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Review

Targeting Toll-like Receptors to Enhance Oncolytic Virotherapy: Strategies and Emerging Perspectives

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Abstract

Oncolytic virotherapy (OVT) is a promising anticancer strategy, owing to its capacity to lyse tumor cells selectively and remodel the tumor microenvironment (TME) to enhance antitumor efficacy. Despite the continuous introduction and investigation of newly engineered oncolytic viruses (OVs) in clinical trials, their clinical translation remains limited, with Toll-like receptor (TLR)-mediated host immune responses representing a critical influencing factor. TLRs constitute a family of structurally conserved transmembrane proteins distributed on the plasma membrane and endosomal membranes. They initiate innate immune responses by recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and are essential for dendritic cells (DCs) maturation, playing a pivotal regulatory role across diverse tumor therapies. However, TLRs may also restrict viral replication and spread, and some studies suggest that they could, under certain conditions, act as drivers of tumor progression. Nonetheless, the role of TLRs in OVT has not been comprehensively summarized to date. In this review, we synthesize recent advances in the application of TLRs in OVT, with particular attention to their mechanisms of action and the challenges that remain to be overcome, and consider their prospects in oncology, offering insights that may inform the clinical translation of this therapeutic approach.

Keywords: toll-like receptors; innate immunity; viral immunology; oncolytic virotherapy; tumor microenvironment

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1. Introduction

Oncolytic virotherapy (OVT) is an emerging anticancer strategy that employs genetically modified or naturally occurring viruses to selectively replicate within and lyse tumor cells while largely sparing normal tissues. The resulting tumor-restricted oncolysis releases tumor-associated antigens and danger signals, which recruit and activate the host antitumor immune response. As early as the beginning of the 20th century, clinical observations revealed that certain cancer patients experienced spontaneous tumor regression following viral infections. This phenomenon inspired scientists to propose the concept of using viruses to treat tumors. However, progress in this field remained limited for several decades due to the lack of effective methods to control viral virulence. It was not until the 1990s, with advances in viral engineering techniques, that researchers gained the

ability to precisely modify viral genomes. This breakthrough substantially enhanced the safety, targeting capability, and therapeutic efficacy of oncolytic viruses (OVs), marking a turning point in modern oncolytic virotherapy research [1]. Early studies of OVT focused primarily on the intrinsic replicative and lytic capacities of these viruses, under the assumption that the adaptive immune system would eliminate the viruses and thereby diminish therapeutic outcomes. However, accumulating evidence in recent years indicates that the immune system—particularly antitumor immune responses—plays an indispensable role in OV-mediated tumor clearance [2,3].

Toll-like receptors (TLRs), as critical pattern-recognition receptors (PRRs) of the innate immune system, play a pivotal role in OVT through their activation mechanisms. These receptors are broadly expressed on the surface of immune cells, certain tumor cells, and stromal and endothelial cells, as well as on endosomal membranes. Following OV infection and subsequent lysis of tumor cells, PAMPs such as viral nucleic acids and DAMPs such as high-mobility group box 1 (HMGB1) or heat shock protein 70 (HSP70) are released and recognized by TLRs, initiating downstream signaling cascades that drive the production of pro-inflammatory cytokines and type I interferons (IFN-I) [4]. However, TLR activation represents a double-edged sword. On one hand, it enhances antigen presentation and activates and recruits effector cells such as T cells, thereby establishing effective antitumor immunity [5]. On the other hand, an excessive IFN-I response may prematurely clear the virus, limiting its replication and spread within the tumor microenvironment (TME) and ultimately attenuating the direct oncolytic effect [6]. Moreover, studies indicate that under certain circumstances, TLR signaling may paradoxically promote tumor progression, adding further complexity to therapeutic strategies [7].

Therefore, precisely harnessing the dual nature of TLR activation is important for optimizing OVT. However, translational research bridging these two fields remains limited, leaving several key mechanistic and therapeutic questions unresolved. Consequently, this review aims at systematically elucidating the mechanistic roles of TLR signaling pathways in OVT. We focus specifically on strategies to balance immune activation and antiviral clearance within the TME through targeted modulation of TLR activity, thereby providing insights and potential directions for enhancing the clinical efficacy of OVs.

2. Characteristics of Oncolytic Viruses

OVT primarily utilizes either wild-type or genetically engineered viruses. Their design aims at targeting tumor cells specifically, leveraging abnormal gene expression or signaling pathway defects to enable selective replication, thereby achieving tumor cell killing through direct lysis and stimulation of the host's antitumor immune response [8]. A representative strategy involves modifying the regulatory effects of viral gene products on the host cell cycle. For example, through genetic engineering, the CR2 domain—essential for binding to the retinoblastoma protein (Rb)—can be deleted from the adenovirus (AdV) E1A protein. This deletion prevents infected normal cells from entering S phase, thereby disrupting the intracellular environment required for viral replication and ensuring that the engineered virus can replicate only in tumor cells capable of proliferating independently of the E1A-Rb interaction [9].

Notably, certain viruses derived from non-human hosts, such as Vesicular Stomatitis Virus (VSV) and Newcastle Disease Virus (NDV), can also serve as effective antitumor therapeutic agents. A key advantage of these viruses is that human populations typically lack high levels of pre-existing neutralizing antibodies against them, which to some extent avoids rapid antibody-mediated clearance. Combined with their capacity for selective replication in tumor cells, they may sustain immune stimulation within the TME [10,11]. However, as non-human viruses, their long-term safety in humans, the potential risk of

transmission to non-target species, and the biosafety hazards associated with viral mutation or environmental release still require evaluation through rigorous clinical trials [12].

The viral genome is the core of OVT, but the engineering logic is not uniform across virus families. Each family replicates by a fundamentally different mechanism and therefore has distinct advantages, limitations, and engineering levers. Beyond differences in mRNA synthesis defined by the Baltimore classification, viruses differ in replication site, genome size, and packaging capacity: small RNA-based OVs strongly constrain transgene insertion, whereas large DNA viruses can accommodate more complex expression cassettes. Genome integration is likewise family-dependent rather than a general property of oncolytic viruses: retroviral and lentiviral vectors can integrate, whereas the oncolytic viruses currently used in clinical practice do not rely on genome integration, so insertional mutagenesis is not a shared design concern [13,14]. Accordingly, tumor-selective replication must be engineered in a family-specific manner—for example, by targeting the E1B/Rb–p53 axis in AdV, deleting ICP34.5/ICP47 in Herpes simplex virus (HSV), attenuating thymidine kinase (TK) and vaccinia growth factor (VGF) in vaccinia virus (VACV), or exploiting defects in IFN-I signaling in NDV and VSV—making oncolytic virus engineering a family-specific optimization of selective replication, cytolysis, and immune activation [15,16].

All mature viral particles consist of at least a protein shell (the capsid) and an internal nucleic acid genome, which together form the nucleocapsid. The capsid protects the nucleic acid from degradation by external physical and chemical factors. Some viruses also possess an envelope surrounding the nucleocapsid, typically derived from the host cell membrane and embedded with virus-encoded glycoproteins (spikes). These glycoproteins specifically recognize and bind receptors on the surface of host cells, mediating viral entry [17]. In OV research, envelope glycoproteins are frequently engineered to bind tumor cells specifically. Detailed characteristics of representative oncolytic viruses are summarized in Table 1.

Table 1. Features of selected oncolytic viruses.

Virus Name	Genome Size (Type) (Packaging Capacity)	Replication Location	Enveloped	Primary Entry Receptors	Natural Human Pathogen	Main Advantages	Main Limitations
HSV [18]	152 kb (dsDNA) (30–50 kb)	Nucleus	Yes	HVEM, Nectin-1	Yes	• Large transgene capacity; • Potent oncolysis; • Availability of antiviral control (e.g., acyclovir).	• Pre-existing immunity; • Requires engineering to reduce neurovirulence.
AdV [19]	36 kb (dsDNA) (8 kb †)	Nucleus	No	CAR, CD46, DSG2, integrins	Yes	• High production titers; • Efficient transduction; • Potent oncolysis.	• Pre-existing immunity; • Potential for robust inflammatory responses.
VACV [20]	190 kb (dsDNA) (25–30 kb)	Cytoplasm	Yes	Non-specific	No *	• Extremely large cargo capacity; • Cytoplasmic replication; • Potent immunostimulator.	• Widely used as a smallpox vaccine, leading to high seroprevalence in older populations; • Pathogenic potential in immunocompromised hosts.
MV [21]	16 kb (-ssRNA) (1–2 kb)	Cytoplasm	Yes	CD46, Nectin-4	Yes	• Natural tropism for CD46 expressing tumors; • Induces syncytia; • Strong immunogenicity.	• High seroprevalence limits systemic administration efficacy.
CV [22]	7.4 kb (+ssRNA) (1 kb)	Cytoplasm	No	ICAM-1, DAF	Yes	• Natural tropism for ICAM-1 expressing tumors.	• Pre-existing immunity; • Requires attenuation for safety.
PV [23]	7.5 kb (+ssRNA) (1–1.5 kb)	Cytoplasm	No	CD155	Yes	• Strong tropism for CD155 expressing tumors; • Potent cytopathicity.	• Neurovirulence necessitates significant attenuation.
RV [24]	23 kb (Segmented dsRNA) (minimal ‡)	Cytoplasm	No	JAM-A, sialic acid	Yes	• Established favorable safety profile.	• Limited single-agent activity in most solid tumors.
VSV [10]	11 kb (-ssRNA) (1–6 kb)	Cytoplasm	Yes	LDLR	No	• Rapid, potent oncolysis.	• Potential neurotoxicity requires careful preclinical evaluation.
NDV [11]	15 kb (-ssRNA) (1–4 kb)	Cytoplasm	Yes	sialic acid	No	• Potent inducer of antitumor immunity.	• Oncolytic potency is generally moderate compared to other OVs.

Abbreviations: HSV, herpes simplex virus; AdV, adenovirus; VACV, vaccinia virus; MV, measles virus; CV, coxsackievirus; PV, poliovirus; RV, reovirus; VSV, vesicular stomatitis virus; NDV, Newcastle disease virus; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; -ssRNA, negative-sense single-stranded RNA; +ssRNA, positive-sense single-stranded RNA; HVEM, herpesvirus entry mediator; CAR, coxsackievirus and adenovirus receptor; DSG2, desmoglein-2; DAF, decay-accelerating factor; JAM-A, junctional adhesion molecule-A; LDLR, low-density lipoprotein receptor. * VACV is not a natural human pathogen but was widely propagated through smallpox vaccination. † AdV transgene capacity is ~8 kb under standard configurations; helper-dependent (gutless) adenoviral vectors can accommodate up to ~36 kb. ‡ RV is a segmented dsRNA virus with minimal conventional transgene packaging capacity; engineered systems remain limited.

3. Infection of Tumor Cells by Oncolytic Viruses

Infection of tumor cells by OVs is initiated by the binding of viral surface proteins to specific cellular receptors. Although certain entry receptors are upregulated in some tumors, OV selectivity is determined predominantly by defective IFN-I/antiviral signaling, tumor-responsive engineered promoters, deleted or attenuated viral genes, and tumor-restricted replication, with receptor abundance being only one contributory factor. For example, the CD46 molecule is upregulated in various tumors and serves as a key recognition receptor for measles virus (MV) vaccine strains and AdV serotypes of species B, for which desmoglein-2 (DSG2) is an additional protein entry receptor [25,26]. Coxsackievirus A21 (CVA21) preferentially utilizes decay-accelerating factor (DAF) and intercellular adhesion molecule 1 (ICAM-1), both highly expressed on tumor cell surfaces [27]. Additionally, AdV relies on integrin co-receptors to mediate endocytosis [28]; HSV first attaches via heparan sulfate proteoglycans (HSPGs) and subsequently triggers membrane fusion through binding of glycoprotein D (gD) to entry receptors [29]. Viral fusion mediated by enveloped viruses typically involves oligomeric transmembrane glycoproteins on the envelope, whereas non-enveloped viruses accomplish analogous functions via capsid proteins [30,31].

Receptor binding induces conformational changes in viral proteins that mediate entry. Enveloped viruses primarily enter cells through membrane fusion, a process that can occur at the plasma membrane or within endosomal membranes [32]; non-enveloped viruses often enter via endocytosis, followed by capsid reorganization within endosomes and penetration through membrane structures to release the viral genome [33]. Most RNA viruses (e.g., CVA21) replicate in the cytoplasm, whereas most DNA viruses (e.g., AdV)—with the exception of VACV—replicate within the nuclear compartment [19,20].

OVs undergo extensive replication within tumor cells, inducing cellular damage and immunogenic cell death (ICD) through mechanisms including resource depletion, spatial competition, and expression of lytic proteins [34]. However, reliance solely on the intrinsic replication capacity of wild-type viruses is often insufficient to achieve complete tumor eradication together with adequate targeting safety. To address this limitation, researchers employ genetic engineering strategies to enhance efficacy: on one hand, insertion of pro-apoptotic genes (e.g., *P53*, *BNIP3*) directly strengthens apoptotic pathways [35]; on the other hand, introduction of suicide gene systems (e.g., thymidine kinase) enables infected cells to convert non-toxic prodrugs locally into cytotoxic compounds, thereby amplifying the lytic effect [36].

Tumor cell lysis releases two critical categories of immunostimulatory signals: endogenous DAMPs are released from damaged cells, such as calreticulin (CRT), HMGB1, HSP70, and ATP; and PAMPs are generated during viral replication, including viral nucleic acids and capsid proteins [37]. Together, these molecules act to reverse the immunosuppressive state of the TME. Figure 1 illustrates the process of oncolytic virus infection and tumor cell lysis.

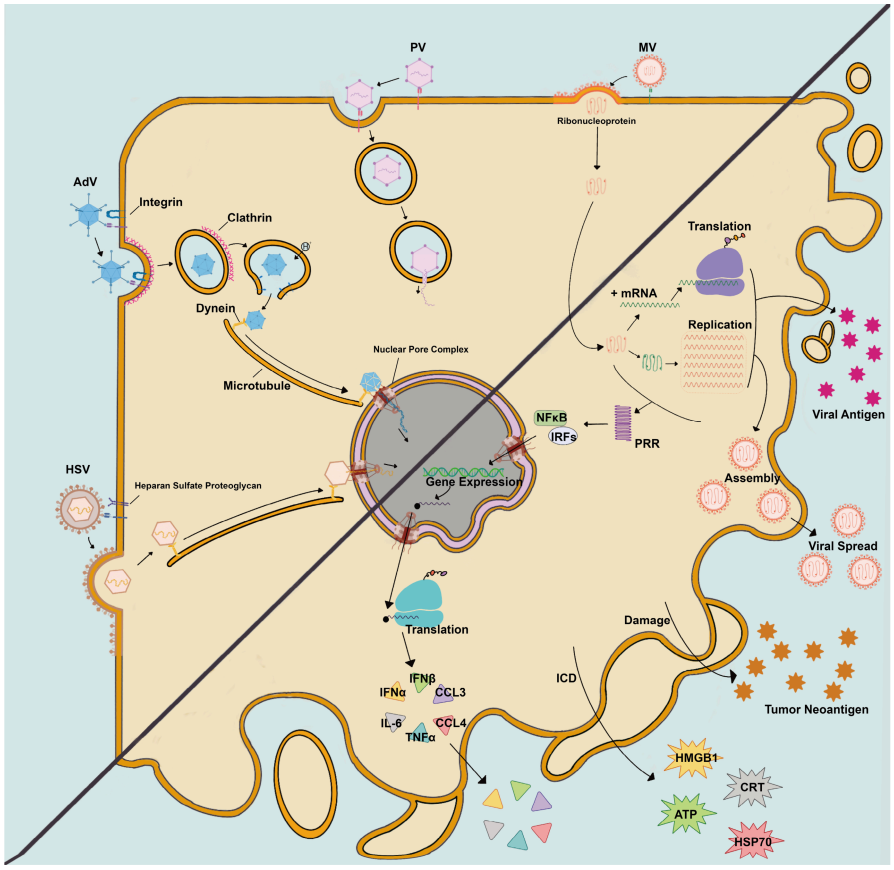


Figure 1. Schematic diagram depicting the infection process of selected oncolytic viruses and the subsequent activation of innate immunity. **Left**: Distinct viral entry strategies into tumor cells are illustrated. Herpes simplex virus (HSV) envelope fuses with the cell membrane, releasing the nucleocapsid into the cytoplasm. The nucleocapsid is then transported along microtubules by the dynein/dynactin motor complex toward the nuclear pore, where viral DNA is injected into the nucleus. Adenovirus (AdV) enters the cell via endocytosis. Endosomal acidification triggers capsid disassembly and endosomal membrane damage, allowing viral particles to escape into the cytoplasm. Subsequently, the virus undergoes microtubule-dependent trafficking toward the nuclear pore, where viral DNA is released into the nucleus. Poliovirus (PV) binds to its receptor, inducing capsid conformational changes and viral entry. Within endosomes, viral RNA is released directly into the cytoplasm through membrane-formed tubular structures. Measles virus (MV) enters via receptor-triggered, pH-independent fusion at the plasma membrane, releasing its nucleocapsid into the cytoplasm. Note that oncolytic virus (OV) tumor selectivity is multifactorial, involving not only receptor-mediated entry but also defective interferon signaling, tumor-specific promoters, and viral gene deletions that restrict replication to cancer cells. **Right**: Immune activation following successful infection is shown. Upon viral genome amplification and progeny release, infected tumor cells emit danger signals, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These signals activate pattern recognition receptors (PRRs) within the tumor cells, leading to the production of antiviral mediators, such as inflammatory cytokines, chemokines, and type I interferon (IFN-I). These mediators recruit and activate the host immune system, thereby initiating an antitumor immune response.

4. TLR Signaling and Its Crosstalk with Other Pattern-Recognition Pathways

4.1. Downstream Consequences of TLR Activation During Oncolytic Virotherapy

TLRs are type I transmembrane proteins expressed on the plasma membrane or endosomal membrane of a broad range of cell types, including dendritic cells (DCs), macrophages, and other leukocyte subsets, as well as tumor cells, stromal cells and endothelial cells. Their

structure comprises three domains: an extracellular domain rich in leucine-rich repeats (LRRs) responsible for recognizing danger signals; a transmembrane domain that anchors the receptor; and an intracellular Toll/interleukin-1 receptor (TIR) domain that mediates activation of downstream signaling pathways [38]. To date, 10 and 12 TLRs have been identified in humans and mice, respectively, with TLR1 through TLR9 highly conserved between the two species.

TLR3, TLR7, TLR8, and TLR9 are localized to endosomal membranes and primarily recognize nucleic acid components derived from OVs or tumor cells themselves. TLR3 recognizes double-stranded RNA (dsRNA) and its synthetic analog polyinosinic:polycytidylic acid (poly(I:C)); the dsRNA generated during viral genome replication can bind and activate TLR3 [39]. TLR7 and TLR8 mainly recognize viral single-stranded RNA (ssRNA) but can also recognize self-RNA released from damaged tumor cells [40]. TLR7 binds free guanosine and ssRNA, whereas TLR8 binds free uridine and ssRNA with high affinity [41,42]. Notably, TLR8 in rodent species (e.g., mice, rats) cannot be activated by human-specific ligands, a limitation that constrains the utility of mouse models in TLR8-related drug development [43]. TLR9 recognizes unmethylated 5′-C-phosphate-G-3′ (CpG) motifs enriched in viral genomes as well as synthetic CpG oligodeoxynucleotides (CpG ODNs) [44]. Moreover, self-DNA released from tumor cells can also serve as a ligand for TLR9 [40].

Transmembrane TLRs located on the plasma membrane include TLR1, TLR2, TLR4, and TLR6, which primarily recognize lipid and protein components. Although these receptors are not the primary sensors of viral nucleic acids, they play important roles in modulating immune responses during OVT. TLR2 can form heterodimers with TLR1 or TLR6 to recognize peptidoglycan, lipoteichoic acid, and other structural components; specifically, TLR2/TLR1 and TLR2/TLR6 selectively recognize triacylated and diacylated lipopeptides, respectively [45]. Additionally, TLR2 can recognize envelope proteins from various viruses as well as host-derived DAMPs. For example, the HSV glycoprotein B (gB) activates cell-surface TLR2, triggering innate immune signaling via the myeloid differentiation primary response protein 88 (MyD88) and TNF receptor-associated factor 6 (TRAF6)-dependent pathway, ultimately leading to NF-κB activation [46]. Cytomegalovirus (CMV) glycoproteins gB and gH interact directly with cell-surface TLR2/TLR1 heterodimers during viral entry, initiating signaling cascades that culminate in NF-κB activation and inflammatory cytokine secretion [47]. The H protein of wild-type MV binds TLR2 and activates innate immune responses; however, recent studies suggest that this recognition mechanism may not apply to attenuated MV vaccine strains [48]. Furthermore, self-proteins and danger signals (e.g., heat shock proteins, HSPs) released from damaged tumor cells may also activate TLR2 [40].

TLR4 primarily recognizes bacterial lipopolysaccharide (LPS). Upon binding to lipopolysaccharide-binding protein (LBP), LPS is transferred to the cell surface with the assistance of CD14, where it interacts with the TLR4-myeloid differentiation factor 2 (MD-2) complex to activate downstream signaling [49]. HMGB1, a nuclear protein released from necrotic cells, can also act as an endogenous ligand that binds TLR4 and induces NF-κB-dependent transcription [50]. Notably, the molecular mechanism by which HMGB1 activates TLR4 appears to vary, depending heavily on the redox state of HMGB1 and the presence of co-factors. Studies suggest that this signaling axis may exhibit differential dependence on MD-2 across disease models, and the detailed molecular recognition mechanism warrants further investigation [51,52]. HSPs can bind TLR4 to activate immune responses; however, some studies suggest that HSPs and TLR4 may also contribute to the maintenance of chronic inflammation and facilitate tumor cell survival [53]. The HSP70/TLR4 axis has been proposed to establish an immunosuppressive TME by recruiting immunosuppressive cells, which may antagonize T cell responses intended to be activated

by anti-PD-1 therapy, potentially contributing to therapeutic failure and unexpected disease progression [54]. Furthermore, the S100 protein family (e.g., S100A8 and S100A9) produced by stressed tumor cells has been identified as an important endogenous TLR4 ligand capable of activating and amplifying inflammatory signaling and immune cell recruitment [55]. TLR4 signals through the TIR-domain-containing adaptor inducing IFN-β (TRIF) to activate interferon regulatory factor 3 (IRF3), which induces IFN-I production.

In the TME, abundant DAMPs and PAMPs bind TLRs on antigen-presenting cells or tumor cell surfaces, triggering the recruitment of adaptor proteins via the TIR domain of TLRs to initiate signal transduction. Membrane-bound TLR2 and TLR4 first interact with the TIR-domain-containing adaptor protein (TIRAP) and recruit MyD88. Subsequently, they activate downstream TRAF6 via interleukin-1 receptor-associated kinases (IRAKs); this cascade activates the IKK–IκB pathway to enable NF-κB nuclear translocation and also triggers the MAPK pathway (e.g., p38 and JNK), ultimately promoting the transcription and release of pro-inflammatory cytokines and chemokines, including TNF-α, IL-6, IL-1β, and CXCL8/CXCL10 [38]. It should be noted that TLR4 is not confined solely to the plasma membrane; a portion of TLR4 is internalized into endosomes, where the signaling adaptor molecules switch to TIR-domain-containing adaptor molecule inducing IFN-β (TRAM) and TRIF, initiating a separate downstream pathway: one branch activates IRF3/7 via TRAF3, driving IFN-I production, while the other activates a comparatively milder, late-phase NF-κB/MAPK pathway through TRAF6, inducing cytokine production [56]. Additionally, endosomal TLR7 and TLR9 remain dependent on MyD88 and primarily activate the aforementioned signaling through rapid early-phase pathways, inducing the expression of inflammatory and co-stimulatory molecules [57]. TLR3 is generally considered the only TLR that does not rely on MyD88 and instead depends entirely on TRIF; the activation of this pathway predominantly phosphorylates IRF3 and promotes its nuclear translocation, strongly inducing IFN-I production, and can also activate NF-κB via the TRAF6 pathway, although the resulting pro-inflammatory cytokine output is typically lower than that observed with MyD88-dependent receptors. Although TLR3 canonically signals via TRIF independently of MyD88, one study in mouse bone marrow-derived macrophages showed that MyD88 partially contributes to TLR3-driven NF-κB activation and the induction of IL-6 and IFN-β following poly(I:C) stimulation, an effect weaker than that of TRIF deficiency and not shared by all cell types. These findings are consistent with a restricted, modulatory rather than general role for MyD88 in TLR3 signaling, and warrant confirmation in additional cell types [58]. The TLRs and pathways involved in activation during OVT are shown in Figure 2.

4.2. Intersection of TLR Signaling with Other Pattern-Recognition Pathways

Although TLRs occupy a central position in discussions regarding the antiviral and antitumor immunity mediated by OVT, they represent merely one branch of a vast network of pattern-recognition pathways. For actively replicating OVs, TLRs are limited by their subcellular localization and thus struggle to directly sense the substantial quantities of viral and tumor-derived ligands produced in the cytoplasm. For instance, NDV, VSV, reovirus (RV), MV, and CVA21 replicate in the cytoplasm and generate dsRNA, which is recognized by the RIG-I-like receptor (RLR) family. Specifically, retinoic acid-inducible gene I (RIG-I) primarily recognizes short RNA molecules with a 5′-triphosphate group (5′-ppp), whereas melanoma differentiation-associated gene 5 (MDA5) recognizes long dsRNA. These receptors transmit signals via the mitochondrial antiviral signaling protein (MAVS) [59]. Although the genomic replication of HSV and AdV occurs within the nucleus, leakage of replication intermediates and viral genomic DNA through the nuclear membrane exposes cytosolic dsDNA to cyclic GMP-AMP synthase (cGAS). cGAS recognizes cytosolic dsDNA

in a sequence-agnostic manner, catalyzes the synthesis of the second messenger cGAMP, and activates Stimulator of Interferon Genes (STING), thereby activating IRF3 to drive IFN-I production and NF-κB to induce pro-inflammatory cytokines and chemokines [60]. Recent studies have identified crosstalk between the cGAS-STING and RLR pathways, revealing that many RNA virus infections cause the leakage of self-DNA, such as mitochondrial DNA, into the cytoplasm, thereby activating cGAS-STING [61]. The three principal IFN-I adapter proteins—TRIF, MAVS, and STING—ultimately converge on the TBK1-IRF3 axis. Viruses may exploit these nodes of convergence across pathways to simultaneously block multiple pathways via a single protease. For example, Seneca Valley virus (SVV, an oncolytic virus) encodes a 3C protease that cleaves multiple critical nodes in host immune signaling pathways, including TRIF, MAVS, and TANK, thereby systematically suppressing IFN-I production to achieve immune evasion [62].

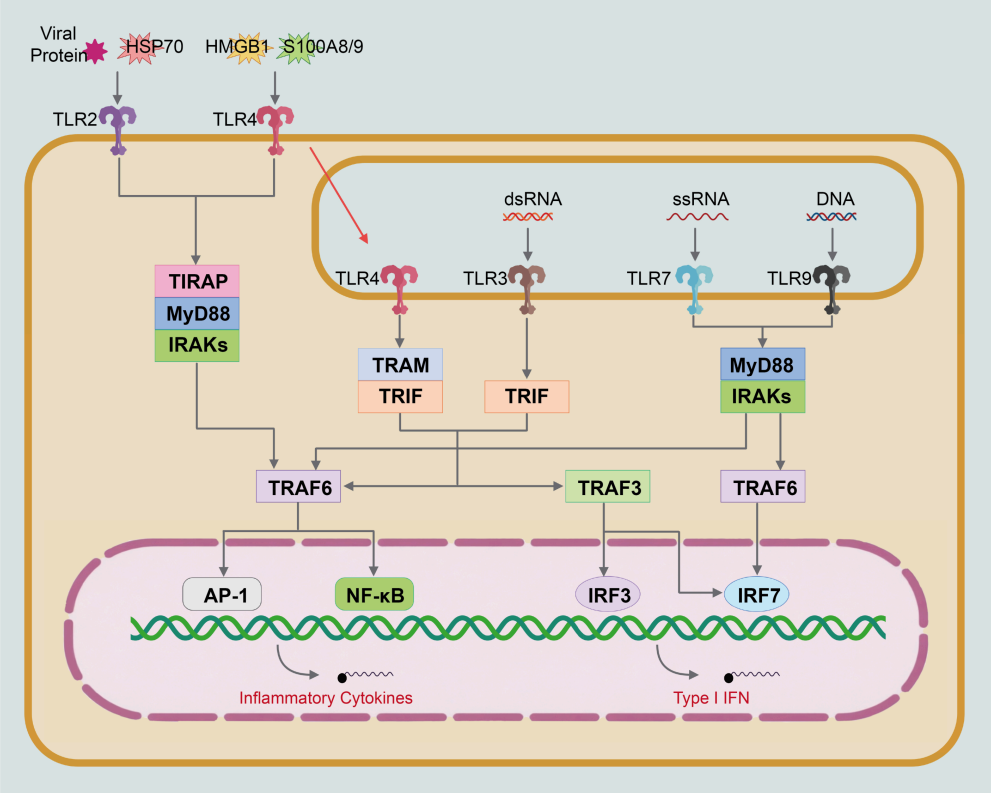


Figure 2. Sensing and signaling pathways of Toll-like receptors (TLRs). Viral structural proteins and damage-associated molecular patterns (DAMPs) are extracellularly recognized by TLR2 and TLR4, which activate nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways via myeloid differentiation primary response 88 (MyD88), leading to the production of pro-inflammatory cytokines. Within endosomes, TLR7, TLR8, and TLR9 also utilize MyD88 to generate type I interferons (IFN-I) and pro-inflammatory cytokines. TIR-domain-containing adaptor-inducing interferon-β (TRIF) is employed by TLR3 and TLR4 to activate TRIF-dependent pathways, resulting in pro-inflammatory effects and antiviral responses. Additionally, TLR7 and TLR9 signal through plasmacytoid dendritic cells (pDCs)-specific pathway, the MyD88-TRAF6 (TNF receptor-associated factor 6)-IRF7 (interferon regulatory factor 7) pathway, which facilitates the induction of IFN-I. This figure was created with MedPeer (https://www.medpeer.cn/, accessed on 1 June 2026).

Because these pathways share critical downstream molecules, experimental designs must distinguish the specific pathways contributing to the observed effects. For instance, the elevated IFN-β levels measured following poly(I:C) stimulation may result from the TLR3-TRIF pathway stimulated via the extracellular/endosomal route or from the MDA5-MAVS pathway stimulated via cytoplasmic transfection [63]. Furthermore, MyD88 serves

not only as an adapter protein for TLRs but also as a key signaling molecule for IL-1R and IL-18R [64]. Studies of VSV in the murine B16 melanoma model demonstrated that MyD88 deficiency leads to a loss of oncolytic efficacy, proving that MyD88 is an essential host factor for VSV-mediated oncolysis; however, these studies could not attribute the efficacy to any specific TLR or IL-1R upstream receptor [65]. Thus, it is important to consider the possibility of pathway crosstalk when analyzing downstream gene signatures, ensuring that conclusions regarding TLR-specific involvement are supported by adequate control of upstream receptor specificity.

Notably, NF-κB activation driven by TLR signaling can also provide critical priming signals for inflammasomes, upregulating the expression of AIM2/NLRP3 inflammasome-associated molecules and thereby licensing the activation of Caspase-1 and the maturation of IL-1β and IL-18 [66]. Thus, TLR involvement often serves as an upstream priming condition for inflammasome activation, rather than an absolute prerequisite in all contexts. Additionally, protein kinase R (PKR) is another kinase activated by double-stranded RNA, which enhances cellular antiviral states by inhibiting translation initiation through the phosphorylation of eIF2α [67]. Unlike TLR pathways, PKR functions primarily as a cytoplasmic dsRNA sensor, and its antiviral effects are largely independent of TLRs. In the context of OVT, dsRNA sensors such as PKR may contribute to TLR-independent antiviral effects, although their relative contribution weights to viral replication and antitumor immunity across various OV models remain to be further quantified and evaluated [68].

5. Strategies Linking TLR Signaling to Oncolytic Virotherapy

TLR signaling occupies a dual position in OVT. On the one hand, TLR activation bridges innate and adaptive immunity, driving DCs maturation, natural killer (NK) cell activation, and $CD8^+$ T cell priming to convert the TME toward a "hot" phenotype. On the other hand, excessive or chronic TLR activation can trigger premature viral clearance, recruit immunosuppressive myeloid populations, and in certain contexts sustain malignant progression. This tension makes TLRs both a therapeutic target and a liability. Consequently, to delineate the independent role of TLRs in OVT, we compiled research findings from the past two decades that explicitly focused on TLRs themselves, rather than merely their shared downstream signaling molecules, as summarized in Table 2.

We reorganize the field around five mechanism-based categories of TLR-centered intervention: natural sensing of OVs by endogenous TLRs; using non-replicating viruses or viral particles as TLR agonists; combining OVs with an exogenous TLR agonist; engineering OVs to enhance TLR signaling; and deliberately inhibiting TLR signaling to augment viral replication. These five categories are conceptualized in Figure 3.

Table 2. Research progress in oncolytic virus therapies associated with Toll-like receptors.

Virus Type	Tumor Type	TLR Modulation Strategies	TLR Pathway Alterations	Impact on OVT Efficacy	Study Phase	Publication Year
AdV	Breast cancer (JIMT-1)	None (natural infection)	Breast cancer initiating cells exhibit TLR2/9 defects	TLR defects block viral IFN-I induction, leading to extensive viral replication	In vitro	2010 [69]
	Lung cancer (A549), Epidermoid carcinoma (A431)	Mesenchymal Stem Cells as viral vectors	Virus activates the TLR9/NF-κB pathway in stem cells	Stem cells activate allogeneic monocytes and NK cells, enhancing anti-cancer effects	In vivo	2019 [70]
	Breast cancer (JC), Lymphoma (E.G7)	Genetically engineered virus expressing BD2	AdV-BD2 activates immune response via TLR4	AdV-BD2 induces tumor lysis and recruits pDCs	In vivo	2009 [71]
	Glioma (Primary, A172, T98G)	None (natural infection)	Virus activates the TLR9/NEAT1/STAT3 pathway in tumor cells	Induces glioma stem cell transformation	In vivo	2020 [72]
	Lung cancer (A549), Melanoma (B16-OVA)	CpG ODN insertion into viral genome	Virus enhances TLR9 stimulation	Virus exhibits superior anti-tumor efficacy	In vivo	2012 [73]
	Lung cancer (H1299, LLC), Melanoma (B16), Colon cancer (HCT116)	TLR9 or IFNAR1 knockout mice	TLR9 or IFNAR1 knockout abrogates viral NK cell recruitment	Antitumor effect was lost in IFNAR1 knockout, but partially retained in TLR9 knockout mice	In vivo	2025 [74]
	Lung cancer (CMT64)	TLR4/9 or MyD88 knockout mesenchymal stromal cells as viral vectors	Silenced cells for reduced inflammation	Silenced cells facilitate anti-tumor effect and local immune activation	In vivo	2023 [75]
HSV	Melanoma (MUM2B, 92.1, MP41)	Combined use of poly(I:C)	Combination therapy reactivates the TLR3/NF-κB pathway	Enhanced tumor cell killing, and promoted macrophage proliferation and M1 polarization	In vitro	2023 [5]
	Gastric cancer (BGC823), Colon cancer (CT26), Breast cancer (4T1)	UV-inactivated virus	UV-HSV stimulates NK cells via TLR2	Synergizes with UV-HSV and anti-NKG2A/HLA-E to exert anti-tumor effects	In vivo	2021 [76]
	AML (MN1/ND13)	UV-inactivated virus	UV-HSV stimulates NK cells via TLR2	Synergizes with UV-HSV and IL-15 to exert anti-tumor effects	In vivo	2016 [77]
	Melanoma (A375)	None (natural infection)	Virus enhances TLR2 stimulation	Promotes ICD	In vivo	2016 [78]

Table 2. *Cont.*

Virus Type	Tumor Type	TLR Modulation Strategies	TLR Pathway Alterations	Impact on OVT Efficacy	Study Phase	Publication Year
VACV	Renal cell carcinoma (Renca), Melanoma (B16)	Knockout of three viral genes: C10L, N2L, and C6L	Relief of TLR3/IRF3 pathway suppression	Significantly enhances anti-tumor T cell responses induced by the novel virus	In vivo	2021 [79]
	Colon cancer (MC38), Lung cancer (LLC)	Combined use of CpG ODN	CpG ODN activates TLR9 on host immune cells	Combination therapy simultaneously improves the TME of untreated contralateral tumors	In vivo	2020 [80]
	Renal carcinoma (Renca), Colon cancer (MC38), Breast cancer (4T1), Pancreatic cancer (Pan02)	Viral particle deglycosylation and engineered virus expressing TRIF	Virus reduces TLR2 activation yet enhances TRIF signaling after infection	Virus-induced immune redirection: shifting from Th2 to Th1/CTL responses	In vivo	2016 [81]
MV	Mesothelioma (Meso13), Melanoma (M18), Lung Cancer (A549)	None (natural infection)	Viral ssRNA can activate TLR7 in pDCs	Promotes pDC maturation and the production of IFN-I	In vitro	2013 [82]
	Lymphoma (Jurkat)	None (natural infection)	Viral ssRNA can activate TLR7 in pDCs, but not significantly in cDCs	Promotes pDC maturation and the production of IFN-I	In vitro	2017 [83]
	Synovial sarcoma (SYO-1)	Engineered virus expressing NAP Neutrophil-Activating Protein	MV-NAP activates immune response via TLR2	Promotes ICD	In vivo	2025 [84]
RV	Melanoma (B16-F10)	None (natural infection)	Viral surface protein activates TLR3 in tumor cells	Virus-induced autophagy	In vitro	2025 [85]
	Gastric cancer (Primary, AGS)	UV-inactivated virus	Viral surface protein activates TLR3 in T cells and tumor cells	T cells upregulate TRAIL expression, while tumor cells upregulate DR4/DR5 expression	In vitro	2024 [86]
	Ovarian cancer (OV-90, SKOV3)	Transfection of RV-derived dsRNA into cells	dsRNA activates TLR3	Induces apoptosis in virus-resistant tumor cells	In vitro	2021 [87]
	Lymphoma (EL4), Melanoma (B16)	None (natural infection)	dsRNA activates TLR3 in MDSCs	Suppresses MDSC activity via TLR3	In vivo	2018 [88]
	Colon cancer (HCT116)	siRNA-mediated downregulation of TLR3 expression in cancer cells	TLR3 and IFNs levels were significantly reduced	Enhances viral replication and tumor necrosis	In vivo	2017 [6]

Table 2. *Cont.*

Virus Type	Tumor Type	TLR Modulation Strategies	TLR Pathway Alterations	Impact on OVT Efficacy	Study Phase	Publication Year
VSV	Melanoma (B16)	Combined use of LPS	LPS activates TLR4	Local combination boosts efficacy, but systemic combination triggers a cytokine storm	In vivo	2013 [89]
CV	Breast cancer (4T1), Melanoma (B16F10)	Combined use of CpG ODN and Melittin	CpG ODN activates TLR9	Promotes ICD	In vivo	2023 [90]
Parvovirus	Melanoma (SK29Mel-1)	None (natural infection)	Virus stimulates DCs via TLR3/9	Promotes DC maturation and TNF-α secretion	In vitro	2013 [91]
Rotavirus	Colon cancer (HT29, Caco-2)	None (natural infection)	TLR3/7/8/9 were selectively upregulated; TLR4 showed no significant change	Induces tumor cell killing and suppresses antiviral response	In vitro	2026 [92]

Abbreviations: AdV, adenovirus; AML, acute myeloid leukemia; BD2, β-defensin-2; cDC, conventional dendritic cell; CpG ODN, CpG oligodeoxynucleotide; CTL, cytotoxic T lymphocyte; CV, coxsackievirus; DC, dendritic cell; DR4/DR5, death receptor 4/5; dsRNA, double-stranded RNA; HLA-E, human leukocyte antigen E; HSV, herpes simplex virus; ICD, immunogenic cell death; IFN, interferon; IFN-I, type I interferon; IFNAR1, interferon alpha/beta receptor 1; IL-15, interleukin-15; IRF3, interferon regulatory factor 3; LLC, Lewis lung cancer; LPS, lipopolysaccharide; MDSC, myeloid-derived suppressor cell; MV, measles virus; MyD88, myeloid differentiation primary response 88; NAP, neutrophil-activating protein; NEAT1, nuclear paraspeckle assembly transcript 1; NF-κB, nuclear factor kappa B; NKG2A, natural killer group 2 member 2A; NK, natural killer; OVT, oncolytic virus therapy; pDC, plasmacytoid dendritic cell; poly(I:C), polyinosinic:polycytidylic acid; RV, reovirus; siRNA, small interfering RNA; ssRNA, single-stranded RNA; STAT3, signal transducer and activator of transcription 3; Th1/Th2, T helper type 1/2; TLR, Toll-like receptor; TME, tumour microenvironment; TNF-α, tumour necrosis factor α; TRAIL, TNF-related apoptosis-inducing ligand; TRIF, TIR-domain-containing adapter-inducing interferon-β; UV, ultraviolet; VACV, vaccinia virus; VSV, vesicular stomatitis virus.

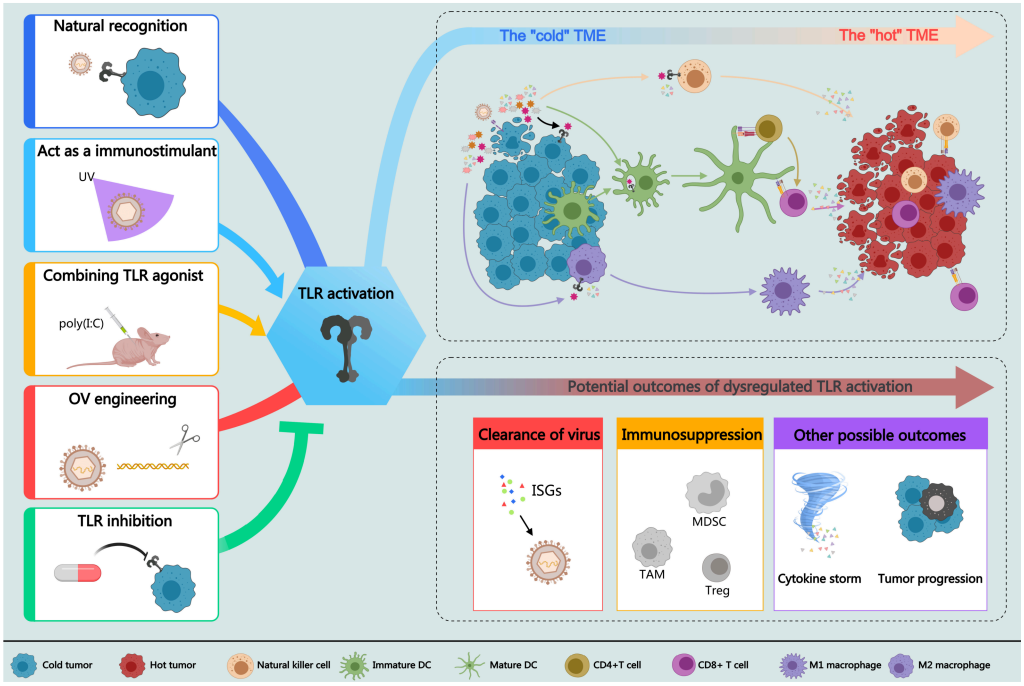


Figure 3. Mechanism-based framework of Toll-like receptor (TLR)-centered interventions in oncolytic virotherapy and the resulting immunological remodeling of the tumor microenvironment (TME). **Left**: the five categories of TLR-targeted intervention discussed in this section, comprising natural sensing of oncolytic viruses (OVs) by endogenous TLRs, the use of inactivated viruses or viral particles as TLR agonists, the combination of OVs with an exogenous TLR agonist such as poly(I:C), engineering of OVs to enhance TLR signaling, and the deliberate inhibition of TLR signaling, each converging on a central TLR signaling hub. **Upper right**: TLR-driven transition from an immunosuppressive "cold" TME to an inflamed, immunologically active "hot" TME. In the cold state (blue), TLR activation drives immature dendritic cells (DCs) to take up tumor antigens and mature; mature DCs migrate to prime naïve T cells, generating $CD4^+$ and $CD8^+$ effector T cells and NK cells that, together with M1-polarized macrophages, infiltrate the tumor bed (red) to establish effective antitumor immunity. **Lower right**: potential adverse consequences of dysregulated or excessive TLR activation, including interferon-stimulated gene (ISG)-mediated premature viral clearance, recruitment of immunosuppressive populations such as MDSCs, TAMs and Tregs, and other outcomes such as cytokine storm and malignant progression. Color-coded symbols for cold/hot tumor cells, NK cells, DC subsets, T cell subsets, and macrophage phenotypes are defined in the legend. This figure was created with MedPeer (https://www.medpeer.cn/, accessed on 4 October 2026).

5.1. Natural Sensing of OVs by Endogenous TLRs

When OVs productively infect tumors, PAMPs and DAMPs are sensed by endogenous TLRs expressed on tumor, stromal, endothelial, and immune cells, initiating the innate branch of the antitumor immune response. The consequence is highly cell-type-specific: whereas normal mammary tissue stem cells retain an intact IFN-I response that resists viral infection, breast cancer-initiating cells harbor defective IFN-I and TLR signaling, characterized by impaired TLR9/MyD88 trafficking and loss of TLR2, which renders them permissive to the capsid-modified adenovirus Ad5/3-Delta24 [69].

As key sentinel cells, DCs sit at the center of this sensing. Conventional DCs (cDCs) mature upon detecting danger signals via the NF-κB and MAPK pathways, releasing TNF-α and IL-12 and initiating T cell co-stimulation [93,94], whereas plasmacytoid DCs (pDCs) secrete large amounts of IFN-I upon TLR7/9 stimulation [95]. This discrimination is functionally decisive: when tumor cells are compared head-to-head, only MV-infected cells, rather than UV-irradiated ones, induce pDC maturation and robust IFN-α production [82]. Mechanistically, in pDCs, oncolytic measles is recognized by RIG-I-like receptors and

TLR7, a pDC-restricted sensing combination that licenses these cells to express TNF-related apoptosis-inducing ligand (TRAIL) and kill via TRAIL–DR5 (death receptors 5) engagement [83]. The resulting IFN-I, together with DC-derived IL-12, promotes the activation and expansion of NK cells [73,96]. During this IFN-I pre-stimulation phase, NK cells display enhanced cytotoxicity against targets with low or absent MHC-I, lysing them via granzyme B and perforin and secreting IFN-γ and TNF-α, thereby establishing a positive-feedback loop that amplifies antitumor immunity [97].

Crucially, this innate sensing is not an end in itself: its terminal readout is the priming of durable adaptive immunity. Beyond chemokine-mediated recruitment of $CD8^+$ T cells, mature DCs take up exogenous tumor antigens and, after lysosomal processing, present derived peptides on MHC-II to prime naïve $CD4^+$ T cells toward Th1 effector cells [98–101]. Through cross-presentation, DCs also load exogenous antigens onto MHC-I and, supported by Th1-derived cytokines, prime naïve $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs) [102,103]. Two lines of evidence illustrate this relay. DCs exposed to H-1 parvovirus-induced tumor cell lysates upregulate TLR3/TLR9 together with maturation markers such as CD86 and NF-κB/TNF-α, a phenotype consistent with enhanced cross-priming [91]. Complementary to this, pDCs phagocytosing MV-infected tumor cells have been shown to cross-present NY-ESO-1 to a specific $CD8^+$ T cell clone, confirming that OV-infected cells can license DC-mediated $CD8^+$ responses [82]. These lymphocytes then migrate to the primary tumor, expand locally, and exert potent cytotoxic effects.

5.2. Using Inactivated Viruses or Viral Particles as TLR Agonists

Unlike strategies that rely on intratumoral viral replication and direct oncolysis, some studies utilize only specific structural components of OVs or inactivated, replication-deficient viral particles as immunotherapeutic agents. Although these preparations are incapable of expressing viral genes, they fully retain virus-derived PAMPs. Consequently, they can function as natural TLR ligands to activate both innate and adaptive immunity, thereby exerting antitumor effects while avoiding replication-associated toxicity.

Regarding the TLR2 axis, studies have shown that UV-inactivated HSV-1 (UV-HSV-1) can rapidly enhance the cytotoxicity of peripheral blood mononuclear cells (PBMCs) and purified NK cells against leukemia cells within one hour of exposure. This process involves the upregulation of CD69, degranulation, and IFN-γ secretion, accompanied by metabolic reprogramming such as glycolysis. Notably, the use of TLR2-neutralizing antibodies significantly antagonizes this enhancement of cytotoxicity [77]. Similarly, UV-inactivated HSV-2 has been shown to stimulate NK cells via the TLR2-dependent NF-κB pathway, promoting IFN-γ release and enhancing antitumor activity. This effect can be further potentiated when combined with anti-NKG2A/anti-HLA-E checkpoint antibodies [76].

In the context of the TLR3 axis, RV and its UV-inactivated form (UV-RV) were found to sensitize PBMCs and $CD8^+$ tumor-infiltrating lymphocytes from gastric cancer patients. Both cell types were shown to upregulate IFN-γ and TRAIL via the TLR3/NF-κB pathway. Furthermore, the RV σC protein can engage tumor cell-associated TLR3, upregulating DR4/DR5 via the p38/p53 pathway, thereby enhancing the killing of gastric cancer cells by effector cells [86]. Additionally, transfecting ovarian cancer cells with RV-derived dsRNA was found to induce apoptosis by upregulating TLR3 and activating TBK1/IRF3 and NF-κB downstream of TRIF; this apoptosis could be reversed by using TLR3 inhibitors or TBK1/IKKε inhibitors [87].

Furthermore, although Cowpea Mosaic Virus (CPMV) does not replicate intracellularly or exert direct oncolytic effects, it may function as an in situ vaccine: its empty viral capsids activate TLR2/4, whereas intact virions additionally engage TLR7, thereby inducing IFN-I production and potentially eliciting stronger immunotherapeutic efficacy [104,105].

Collectively, the use of inactivated viruses or viral particles as TLR agonists balances safety with immune activation potency and can be combined with immune checkpoint inhibitors (ICIs), chemotherapy, and other treatments, laying the foundation for its use as a "replication-incompetent" adjuvant for cancer immunotherapy.

5.3. Combining OVs with an Exogenous TLR Agonist

Although OVs expose tumor antigens, OVs alone may fail to reverse the immunosuppressive TME. The resulting accumulation of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and M2 macrophages prevents effective antigen presentation and leads to insufficient T cell activation. TLR agonists can provide potent complementary danger signals, thereby promoting the secretion of IFN-I and pro-inflammatory cytokines. This drives the re-polarization of M2 macrophages into antitumor M1 macrophages, further enhancing T cell immune responses [106,107]. For instance, combining the TLR3 ligand poly(I:C) with HSV-1 in the treatment of uveal melanoma reactivates the virus-downregulated TLR3–NF-κB axis, promoting M1 polarization and thereby bolstering antitumor immunity [5]. Similarly, the combination of the TLR9 ligand CpG ODN (self-assembled with melittin into a CpGMel complex to enhance delivery) and coxsackievirus B3 in treating triple-negative breast cancer and melanoma leverages ICD to activate antigen presentation and T cell responses, suppressing primary tumors and delaying distant metastasis [90].

However, combination strategies may also yield unfavorable outcomes. While an "in situ vaccine" approach using a TLR9 ligand (CpG ODN) combined with a modified vaccinia virus expressing membrane-anchored IL-2 (vvDD-mIL2) successfully cleared injected tumors, prolonged survival, and induced abscopal effects with a favorable safety profile—dynamically remodeling the TME of untreated sites by increasing effector T cells, NK cells, and DCs infiltration—it also triggered a compensatory upregulation of Tregs, MDSCs, and tumor-associated macrophages (TAMs). Consequently, myeloid cell depletion or anti-PD-1 therapy is required to further amplify efficacy [80]. Furthermore, while sequential intratumoral administration of the TLR4 ligand LPS and VSVs cured 100% of tumor-bearing mice, switching to intravenous VSV followed by LPS administration caused a lethal cytokine storm characterized by a surge in TNF-α and IL-6 [89]. This serves as a stark warning regarding the narrow therapeutic window associated with systemic administration.

5.4. Engineering an OV to Enhance TLR Signaling

Unlike the strategy of administering TLR agonists exogenously, genetic engineering approaches involve directly inserting TLR agonist molecules or their coding sequences into the viral genome. This allows the OV to continuously release agonist signals in situ while replicating within the tumor. By employing a strategy of enriching TLR recognition sequences, the researchers of a previous study inserted 18 immunostimulatory CpG motifs into the AdV genome to construct the CpG-enriched virus Ad5D24-CpG. This significantly enhanced TLR9 recognition and NF-κB activation, boosting immunogenicity while fully preserving the safety profile of the parental virus. The virus attenuated the immunosuppressive function of MDSCs and increased intratumoral $CD8^+$ T cell infiltration via the TLR9 pathway, an effect confirmed to be TLR9-dependent using TLR9-knockout oligonucleotides [73].

Another strategy involves engineering the virus to express endogenous TLR agonists. For instance, an AdV was engineered to express human β-defensin-2 (BD2), an endogenous danger signal recognized by TLR4. While the virus lysed tumor cells and released antigens, BD2 recruited and promoted the maturation of pDCs, inducing tumor-specific $CD4^+$/$CD8^+$

T cell and IFN-γ responses. This generated complete protective immunity against re-challenge and inhibited natural metastasis [71]. Similarly, engineering MV to express Helicobacter pylori neutrophil-activating protein (NAP)—a TLR2 agonist-like lipoprotein—resulted in massive recruitment and activation of neutrophils in immunologically "cold" synovial sarcomas characterized by low PD-L1 expression and low T cell infiltration. This converted "cold" tumors into "hot" ones and upregulated PD-L1, creating conditions favorable for combination therapy with ICIs [84].

In addition to introducing agonist elements, another approach to enhancement is removing the virus's own innate immune antagonistic genes. One study proposed constructing a VACV that activates the TLR3–IRF3 pathway post-infection. By stepwise removal of viral genes that antagonize this pathway (C10L, N2L, and C6L), IRF3 phosphorylation and IFN-β expression were restored within tumor cells. This translated into stronger tumor antigen-specific T cell responses and therapeutic benefits in mouse models [79].

5.5. Inhibiting TLR Signaling to Modulate the Antiviral Response

The signaling cascade following TLR activation ultimately establishes a critical barrier that restricts viral replication and accelerates viral clearance. Based on this rationale, studies have sought to enhance viral replication and delivery efficiency by inhibiting or dampening TLR recognition.

At the tumor cell level, TLR3 is constitutively expressed in colonic epithelium. Knockdown of TLR3 significantly reduces host IFN-I triggering, thereby enhancing the replication of RV within tumors. This is accompanied by a marked upregulation of the viral σ1 protein and significantly enhanced virus-mediated inhibition of tumor growth compared to controls [6]. In terms of viral engineering, deglycosylation of surface proteins on VACV can weaken its binding to TLR2 and the subsequent activation of MyD88 signaling. This reduces the production of antiviral neutralizing antibodies, thereby enhancing systemic delivery of the virus to tumors following intravenous administration and preserving the capacity for repeated dosing [81]. Regarding delivery vehicles, the use of mesenchymal stem cells (MSCs) with silenced TLR4 or MyD88 as carriers for AdV can reduce systemic inflammation after systemic administration. This improves the homing and delivery efficiency of the vectors to tumors, thereby enhancing antitumor efficacy [75].

However, studies in TLR9-knockdown mice have revealed that TLR9 signaling also participates in the recruitment of NK cells to tumors, serving as a contributing factor to antitumor immunity [74]. Therefore, the activation or inhibition of TLR pathways presents a distinct "double-edged sword" effect. An ideal strategy requires striking a balance between enhancing viral replication and maintaining antitumor immunity, tailored to the specific virus type, tumor type, route of administration, and immune microenvironment.

6. Clinical Translation of TLR-Targeted Oncolytic Virotherapy

Although much of the evidence discussed above is preclinical, several OV platforms and TLR-targeted immunomodulators have reached the clinic, and their accumulated experience is essential for evaluating the translational potential of TLR-centred OVT, as summarized in Table 3.

Table 3. Clinical trials of oncolytic viruses and Toll-like receptor agonists for cancer/malignancies.

Investigational Agent	Route of Administration	Tumor Type	Combination Therapy	Key Outcome	Phase	NCT Number [Ref]
Adenovirus						
NG-350A	IV/IT	metastatic/advanced epithelial tumors	—	No objective response; both routes were well tolerated	I	NCT03852511 [108]
Ad5-IL-12	intraprostatic	recurrent prostate cancer	chemotherapy	No significant improvement in PSA/PSADT; MTD was not reached	I	NCT02555397 [109]
TILT-123	IV, then IT	advanced solid tumors	—	immunomodulation; no DLTs	I	NCT04695327 [110]
VCN-01	IT	PDAC	chemotherapy	All injected lesions achieved SD; well tolerated	I	NCT02045589 [111]
LOAd703	IT	PDAC	chemotherapy	ORR 44%, DCR 94%; mainly grade 1/2 AEs	I/II	NCT02705196 [112]
DNX-2401	IT	recurrent GBM	pembrolizumab	ORR 10.4%, CBR 56.2%; no DLTs	I/II	NCT02798406 [113]
H101	intraperitoneal	malignant ascites	—	TTRP 45 d (vs. preset control 13 d); only 2 cases of grade 3 TRAEs	II	NCT04771676 [114]
Herpes simplex virus 1						
RP1 (Tudriqev)	IT	advanced anti-PD-1-failed melanoma	nivolumab	ORR 32.9%; mainly grade 1/2 TRAEs, grade $\geq$ 3 12.9%	I/II	NCT03767348 [115]
T-VEC (Imlygic)	IT	ICI-refractory melanoma	myDCs	2 pts. durable pCR; 1 unconfirmed PR main AEs fatigue, injection pain, fever, flu-like	I	NCT03747744 [116]
	IT	advanced melanoma	pembrolizumab	ORR 19.7% (14/71); grade $\geq$ 3 TRAEs 12.7% (9/71)	II	NCT04068181 [117]
	IT	locally advanced or metastatic sarcoma	pembrolizumab	ORR 30%, DCR 70%, median PFS 17.1 w; grade $\geq$ 3 TRAEs 20%	II	NCT03069378 [118]
	intrahepatic/IT	HCC or solid tumors with liver metastases	pembrolizumab	ORR was consistently low across cohorts; 3 pts with hepatic hemorrhage	I/II	NCT02509507 [119]
	IT	advanced melanoma	pembrolizumab	not meet the primary endpoint; No significant improvement in safety	III	NCT02263508 [120]

Table 3. *Cont.*

Investigational Agent	Route of Administration	Tumor Type	Combination Therapy	Key Outcome	Phase	NCT Number [Ref]
OrienX010	IT	acral melanoma	toripalimab	CR 3.3%, pCR 14.8%; mainly grade 1/2 TRAEs, grade $\geq$ 3 13.3%	I	NCT04197882 [121]
CAN-3110	IT	recurrent GBM/HGG	—	increased OS and intratumoral T cells; no DLTs	I	NCT03152318 [122]
Vaccinia Virus						
JX-594	IT	liver cancer	—	PR 3/10, SD 6/10, PD 1/10; MTD = 1×10^9 pfu	I	NCT00629759 [123]
Measles						
MV-CEA	IT	recurrent GBM/HGG	—	median OS 11.6 mo, median PFS 3.4 mo; no DLTs	I	NCT00390299 [124]
Coxsackievirus						
V937 (CVA21)	IT	advanced melanoma	ipilimumab	ORR 30%, abscopal-like responses; no DLTs	I	NCT02307149 [125]
	IT	advanced melanoma	pembrolizumab	ORR 47%; no DLTs	I	NCT02565992 [126]
	IT	resectable stage IIIB–D melanoma	pembrolizumab	pCR 7/25, MPR 40%, ORR 32%; grade $\geq$ 3 TRAEs 7/25	I/II	NCT04303169 [127]
	IV	advanced solid tumors	pembrolizumab	No added benefit over pembro mono; grade $\geq$ 3 TRAEs 12% (0% with V937 mono)	I	NCT02043665 [128]
Poliovirus						
PVSRIPO	IT	malignant glioma	bevacizumab	median OS 12.5 mo; 1 DLTs (grade IV Intracranial Hemorrhage)	I	NCT01491893 [129]
Vesicular stomatitis virus						
VSV-IFNβ-NIS	IV	hematologic malignancies	—	all 3 OR occurred in TCL; most common grade $\geq$ 3 AEs were hematologic	I	NCT03017820 [130]
Newcastle disease virus						
MEDI5395	IV	advanced solid tumors	durvalumab	ORR 10.3%, DCR 30.8%; grade 1/2 TEAEs 69.2%, 2 DLTs	I	NCT03889275 [131]

Table 3. *Cont.*

Investigational Agent	Route of Administration	Tumor Type	Combination Therapy	Key Outcome	Phase	NCT Number [Ref]
TLR3 agonist						
Rintatolimod	IV	TNBC	chemotherapy	9 evaluable: pCR 5; No DLT; no irAEs	I	NCT04081389 [132]
TLR4 agonist						
GSK1795091	IV	Advanced solid tumors	pembrolizumab	Across all cohorts, ORR 5.6%; DCR 27.8%; TEAEs 51/54; grade $\geq$ 3 24/54	I	NCT03447314 [133]
G100	IT	NHL (FL)	pembrolizumab	ORR 6/18; abscopal regression 13/18; grade $\geq$ 3 AEs: pembro combo only	I/II	NCT02501473 [134]
TLR7 agonist						
RO7119929	PO	HCC or solid tumors with liver involvement	—	PD 54%, SD 44%. HCC durable CR 1/17; TRAEs 91%, most common: CRS 81%	I	NCT04338685 [135]
Imiquimod	Topical	OSCC	—	irMPR 60% (2 pCR); PR 40%; TRAEs 93.3%, no grade 4	I (pilot)	NCT04883645 [136]
TLR8 agonist						
Motolimod (VTX-2337)	SC	recurrent/metastatic SCCHN	Cetuximab	PFS 6.1 vs. 5.9 mo; OS 13.5 vs. 11.3 mo (n = 195, vs. placebo); most common: ISR (39% vs. 0)	II	NCT01836029 [137]
	SC	recurrent/persistent ovarian cancer	chemotherapy	irPFS 4.8 vs. 5.2; OS 18.1 vs. 18.9 mo (vs. placebo); most common: fatigue 87.8%, ISR 73.5%	II	NCT01666444 [138]
TLR9 agonist						
Vidutolimod (CMP-001)	IT; first SC	stage III melanoma	Nivolumab	31 evaluable: MPR 17; pCR 14; ORR 14; no DLTs; no grade $\geq$ 4 TRAEs	II	NCT03618641 [139]
SD-101	IT	NHL (19 FL,1 MZL)	Ibrutinib	ORR 50%; $\geq$30% distant shrinkage 70%; Mostly grade 1/2 AEs	I/II	NCT02927964 [140]
Tilsotolimod (IMO-2125)	IT	Advanced refractory melanoma	Ipilimumab	ORR 8.8% vs. 8.6%; OS 11.6 vs. 10.0 mo (vs. ipilim mono); grade $\geq$ 3 TEAEs 61.1% vs. 55.5%.	III	NCT03445533 [141]

Table 3. *Cont.*

Investigational Agent	Route of Administration	Tumor Type	Combination Therapy	Key Outcome	Phase	NCT Number [Ref]
Pixatimod (PG545)	IV	MSS mCRC or mPDAC	Nivolumab	MSS mCRC (n = 25): 3 PR, 8 SD; mPDAC (n = 18): no responders; grade $\geq$ 3 TRAEs in 12 pts (21%)	I	NCT05061017 [142]

Abbreviations: AE, adverse event; CBR, clinical benefit rate; CR, complete response; CRS, cytokine release syndrome; DCR, disease control rate; DLT, dose-limiting toxicity; FL, follicular lymphoma; GBM, glioblastoma; HCC, hepatocellular carcinoma; HGG, high-grade glioma; ICI, immune checkpoint inhibitor; irAE, immune-related adverse event; irMPR, immune-related major pathological response; irPFS, immune-related progression-free survival; ISR, injection site reaction; IT, intratumoral; IV, intravenous; MPR, major pathological response; MSS, microsatellite stable; MTD, maximum tolerated dose; mCRC, metastatic colorectal cancer; mPDAC, metastatic pancreatic ductal adenocarcinoma; myDC, monocyte-derived dendritic cell; NHL, non-Hodgkin lymphoma; OR, objective response; ORR, objective response rate; OSCC, oral squamous cell carcinoma; OS, overall survival; pCR, pathological complete response; PD, progressive disease; PDAC, pancreatic ductal adenocarcinoma; PFS, progression-free survival; PO, per os; PR, partial response; PSADT, PSA doubling time; PSA, prostate-specific antigen; SC, subcutaneous; SCCHN, squamous cell carcinoma of the head and neck; SD, stable disease; TCL, T cell lymphoma; TEAE, treatment-emergent adverse event; TRAE, treatment-related adverse event; TTRP, time to repeat paracentesis; TNBC, triple-negative breast cancer; MZL, marginal zone lymphoma.

6.1. Clinical Translation of Oncolytic Viruses

The clinical landscape of OVs has matured from first-in-class monotherapy approvals into a broad set of platform-specific and combination strategies that increasingly pair viral oncolysis with immune checkpoint blockade. Among the approved products, talimogene laherparepvec (T-VEC, Imlygic), a granulocyte-macrophage colony-stimulating factor (GM-CSF)-armed HSV-1, received FDA approval in 2015 for intralesional treatment of unresectable melanoma [143]. Most recently, in August 2026, the FDA granted accelerated approval to vusolimogene oderparepvec (TUDRIQEV, formerly RP1), a dual-engineered HSV-1 expressing the GALV fusogenic glycoprotein and GM-CSF, in combination with nivolumab for advanced cutaneous melanoma that had progressed on prior anti-PD-1 therapy [115].

Across platforms, single-agent activity remains modest and largely confined to local control, whereas clinically meaningful responses concentrate on intratumoral delivery of armed or engineered viruses, most often combined with checkpoint blockade. HSV-1 platforms typify this: RP1 plus nivolumab yielded an objective response rate (ORR) of 32.9% in anti-PD-1-refractory melanoma [115], and T-VEC plus pembrolizumab reached an ORR of 19.7% in PD-1-refractory advanced melanoma [117] and 30% in sarcoma [118], even though the confirmatory phase III T-VEC-pembrolizumab trial in PD-1-naïve melanoma missed its primary endpoints [120]. Comparable signals extend beyond HSV-1: coxsackievirus V937 plus pembrolizumab reached an ORR of 47% in advanced melanoma [126], and DNX-2401 plus pembrolizumab gave a 56.2% clinical-benefit rate in recurrent glioblastoma [113]. Conversely, poorly matched delivery or patient selection underdelivered—most clearly intravenous V937, which showed no additive benefit with pembrolizumab [128], and intrahepatic T-VEC, which produced consistently low response rates across liver-tumor cohorts [119].

The mechanistic rationale linking OVT to durable and systemic benefit is the conversion of immunologically "cold" tumors into "hot" ones, and the clinical data are broadly consistent with this model: benefit is greatest when oncolysis is delivered intratumorally and paired with checkpoint blockade, and regressions of distant, non-injected lesions have been documented with V937 combined with ipilimumab and with RP1-nivolumab [115,125].

OVT is generally well tolerated, with adverse events (AEs) predominantly being injection-site reactions (ISRs) and transient, low-grade flu-like symptoms, and treatment-related grade ≥ 3 events remaining low (12.7% with T-VEC plus pembrolizumab; 12.9% with RP1 plus nivolumab), indicating a favorable systemic safety margin [115,117]. Efficacy, by contrast, is shaped chiefly by disease setting and prior therapy exposure rather than by tolerability: in both trials, response rates diverged sharply between patients recurring in the adjuvant setting (ORR 40–47% with T-VEC plus pembrolizumab [117]; 50% with RP1 plus nivolumab) and those progressing in the metastatic setting (ORR 0–6.7% with T-VEC plus pembrolizumab) [115].

6.2. Clinical Translation of TLR-Targeted Immunomodulators

Single-agent activity of TLR agonists is generally limited. In a phase I study of RO7119929, an oral TLR7 agonist prodrug, one patient with hepatocellular carcinoma achieved a durable complete response, but overall clinical activity remained modest [135]. By contrast, imiquimod, an FDA-approved TLR7 agonist, has shown more consistent utility. It has long been used for precancerous cervical lesions, and neoadjuvant imiquimod appears safe and promising in early-stage oral cancer [136,144].

Most current developments therefore focus on combination regimens. TLR agonists are frequently paired with an ICI such as nivolumab, pembrolizumab, or ipilimumab, as well as being combined with targeted agents or chemotherapy according to tumor type. Bacillus

Calmette–Guérin (BCG), which activates TLR2, TLR4, and TLR9 pathways, illustrates a similar pattern: although it remains a cornerstone of bladder cancer immunotherapy in trials such as NCT03022825 and NCT03528694, optimal efficacy generally requires combination with additional agents [145–147].

Nevertheless, several phase II/III trials have yielded disappointing results. In anti-PD-1-refractory advanced melanoma, adding intratumoral tilsotolimod, a TLR9 agonist, to ipilimumab did not significantly improve ORR, progression-free survival (PFS), or overall survival (OS) compared with ipilimumab alone, and this strategy increased toxicity, including higher rates of grade ≥ 3 AEs and new signals of serious anaphylactic reactions [141]. Similarly, in recurrent ovarian cancer, adding the TLR8 agonist motolimod to pegylated liposomal doxorubicin did not improve OS, PFS, or response rate relative to placebo, despite confirmed immune activation [138]. Notably, motolimod-treated patients who exhibited injection-site reactions showed a substantially lower risk of death and longer OS than those who did not [138].

Systemic administration is further constrained by dose-limiting toxicity (DLT), particularly cytokine release syndrome (CRS). Oral RO7119929 induced CRS in a dose-dependent manner in 81% of patients, with treatment-related AEs of any grade occurring in 91% [135]. Intravenous GSK1795091 was associated with treatment-emergent AEs in 94% of patients [133], and intravenous pixatimod caused AEs in all treated patients [142]. Common AEs included fever, chills, fatigue, and nausea, consistent with flu-like or immune-activation symptoms. By contrast, rintatolimod, a TLR3 agonist, was relatively well tolerated after intravenous administration, with no DLTs reported [132].

Local administration combined with radiotherapy has produced the most mechanistically compelling results, particularly through induction of the abscopal effect. In patients with relapsed/refractory indolent non-Hodgkin lymphoma, treatment with intratumoral SD-101, a TLR9 agonist, combined with low-dose radiotherapy and ibrutinib induced the regression of distant, untreated tumors in most patients, with a median PFS of 11 months [140]. Similarly, following low-dose radiotherapy, intratumoral injection of G100, a TLR4 agonist, led to abscopal regression in 13 of 18 evaluable patients in the 20 mg dose cohort with follicular lymphoma [134]. These findings position TLR agonists as candidate components of an "in situ vaccination" strategy, in which localized innate immune activation generates systemic, antigen-specific adaptive immunity. This rationale also supports exploring TLR modulation as part of OVs-based immunotherapy, where local innate immune stimulation may similarly help convert an immunologically "cold" TME into one more permissive to antitumor T cell responses.

7. Discussion

This discussion synthesizes the current evidence to provide concrete translational guidance, addressing three pivotal questions that determine the clinical viability of TLR-based modulation in OVT: which receptors should be targeted or inhibited for specific viral platforms, the contexts in which such manipulation proves beneficial versus detrimental, and how antiviral and antitumor immune responses can be effectively balanced.

First, the choice of the most actionable TLR agonist depends on the genome type and replication compartment of the OV, because these features determine which ligands accumulate and where. Endosomal TLR3, signaling through TRIF, is one of the most pharmacologically accessible targets for RNA OVs that generate dsRNA—such as RV, VSV, NDV and MV—making poly(I:C) and related TLR3 agonists mechanistically compatible partners for these platforms, although RIG-I/MDA5 and TLR7/8 may also contribute to RNA sensing [39]. DNA OVs (HSV, AdV, and VACV) present unmethylated CpG motifs and release replication-derived DNA, thereby engaging TLR9 and cytosolic DNA sensors,

particularly the cGAS–STING axis, so CpG ODN and STING-directed adjuncts represent mechanistically plausible pairings [44,61]. Surface-expressed TLR2 and TLR4 mainly sense viral envelope glycoproteins and DAMPs (for example, HSV gB, MV H, HMGB1, HSP70, and S100A8/9) and are best exploited locally or with inactivated/particle preparations rather than as general drivers of productive replication [48,50]. Endosomal TLR7 and TLR8 sense ssRNA and are relevant targets for RNA-based platforms, particularly when systemic delivery or endosomal exposure is expected; however, the risk of systemic cytokine release should temper preclinical-to-clinical inference [41,42].

Second, it is the timing and anatomical context of TLR modulation, rather than its direction alone, that largely determines therapeutic benefit. Restraining the antiviral arm—as illustrated by TLR3 silencing to permit RV replication [6], VACV deglycosylation to attenuate TLR2 engagement [81], and TLR4/MyD88-silenced MSC carriers for AdV [75]—can enhance viral spread and oncolysis. This strategy appears most valuable during the early replication-and-dissemination window, particularly in IFN-competent or heavily infiltrated tumors where premature viral clearance might otherwise limit therapy. However, the same intervention may be detrimental when applied systemically or at later stages, since TLR9 signaling also supports NK cell recruitment and DC cross-priming, and indiscriminate inhibition could compromise the adaptive arm that underpins durable OVT efficacy [74,91]. A practical rule is thus to apply transient, local inhibition to protect the virus while antigen is being generated, and to favor TLR agonism after antigen release to preserve DC licensing.

Third, antiviral and antitumor immunity may be reconciled not by prioritizing one over the other, but by spatially and temporally separating these two phases. As shown above, route and sequence were decisive, and systemic OVs combined with TLR stimulation risked a lethal TNF-α/IL-6 cytokine storm, underscoring the safety advantage of intratumoral over systemic delivery [89]. Within this narrow window, pairing TLR-driven innate activation with OV-induced ICD and myeloid repolarization may help convert transient inflammation into durable CTL responses while limiting systemic toxicity [5,90]. This framework may help explain the limited single-agent activity of TLR agonists in the clinic, but remains largely preclinical and requires prospective validation.

To render these principles operational, we summarize them as a decision matrix (Table 4) that links viral genome type, dominant sensing pathways, tumor phenotype, intervention strategy, and the predicted translational benefit.

Table 4. Decision matrix for TLR-targeted oncolytic virotherapy.

Platforms (Genome Type/OVs)	Dominant Sensing Pathway	Tumor Phenotype (Favourable/Caution)	Intervention Strategy	Predicted Translational Benefit
RNA OVs sensed via dsRNA				
RV, VSV, NDV, MV	Endosomal: TLR3-TRIF, TLR7/8 Cytosolic crosstalk: RLR	Fav: immune desert Caution: IFN-I saturated "hot"	Cold: intratumoral TLR3 agonism after lysis; Hot: transient restraint during early dissemination	Cold-to-hot conversion via DC cross-priming and IFN-I-driven CTL priming
dsDNA OVs				
HSV, AdV, VACV	Endosomal: TLR9 Cytosolic crosstalk: cGAS-STING	Fav: antigen-poor, low baseline of TLR9/STING Caution: high IFN-I; cytokine-toxicity risk	intratumoral TLR9 agonism after lysis; assess systemic-dosing safety	NK recruitment and DC cross-priming for durable CTL responses

Table 4. *Cont.*

Platforms (Genome Type/OVs)	Dominant Sensing Pathway	Tumor Phenotype (Favourable/Caution)	Intervention Strategy	Predicted Translational Benefit
ssRNA picornaviruses				
CV, PV	Endosomal: TLR7/8 Cytosolic crosstalk: RLR	Fav: disseminated/multifocal tumors Caution: high systemic IFN-I; cytokine-toxicity risk	Local/systemic TLR7/8 agonism timed to antigen release; assess systemic-dosing safety; murine TLR8 blind to human ligands	Systemic stimulation of lesions unreachable by intratumoral
Envelope glycoprotein sensing				
HSV gB, MV H, Inactivated virus	Surface: TLR2, TLR4 (+DAMPs from damaged tumor)	Fav: tumors needing local DC adjuvanting Caution: chronic-inflammation/high DAMPs TME; cytokine-toxicity risk	Local use of inactivated virus preparations	Local DC adjuvant effect across OV platforms

Abbreviations: OVs, oncolytic viruses; RV, reovirus; VSV, vesicular stomatitis virus; NDV, Newcastle disease virus; MV, measles virus; HSV, herpes simplex virus; AdV, adenovirus; VACV, vaccinia virus; CV, coxsackievirus; PV, poliovirus; dsRNA, double-stranded RNA; TLR, Toll-like receptor; TRIF, TIR-domain-containing adaptor-inducing interferon-β; RLR, RIG-I-like receptor; cGAS-STING, cyclic GMP-AMP synthase–stimulator of interferon genes; IFN-I, type I interferon; DC, dendritic cell; CTL, cytotoxic T lymphocyte; DAMPs, damage-associated molecular patterns.

8. Conclusions

TLR activation in OVT functions as a genuine double-edged sword: the antiviral arm can prematurely curtail viral replication and spread, whereas the inflammatory arm licenses dendritic cells and sustains antigen-driven immunity. The balance between these opposing outcomes is shaped by the genome type and replication compartment of each viral family, which determine which ligands accumulate and where. We have organized these relationships into five mechanism-based intervention strategies, weighed them against current translational evidence, and distilled them into an actionable decision matrix. Distinguishing beneficial from detrimental TLR responses therefore remains a central challenge in harnessing innate immunity for OVT. Progress will require technologies that resolve immune responses across cellular, spatial, and temporal dimensions—single-cell multi-omics to assign TLR pathways to defined cell populations, spatial transcriptomics to recover positional information within the TME, and longitudinal sampling to define the kinetic window in which a response shifts from inflammation-sustaining to immunogenic. These approaches sharpen a practical question: Which TLR pathway, in which cell type, in which region of which tumor, and at what point during treatment, should be selectively activated or inhibited? Answering it will require converting this dichotomy into a controllable therapeutic strategy that complements rational OV engineering and checkpoint blockade. Its clinical value will depend on careful calibration of timing, dose, and route to limit systemic exposure and avoid the cytokine-driven toxicity that has constrained earlier-generation agonists, as well as reliable identification of target cell types and patient stratification based on TME heterogeneity. Future progress will hinge on reproducible biomarkers for prospective assignment of patients to TLR-pathway-dominant subgroups, standardized frameworks for serial intratumoral sampling, and trial designs that explicitly test timing, such as window-of-opportunity or adaptive scheduling studies. With these advances, TLR-targeted OVT can move from mechanistic promise toward predictable, clinically actionable practice.

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