



Targeting Cellular and Tissue HIV Reservoirs With Toll-Like Receptor Agonists

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The elimination of both cellular and tissue latent reservoirs is a challenge toward a successful HIV cure. “Shock and Kill” are among the therapeutic strategies that have been more extensively studied to target these reservoirs. These strategies are aimed toward the reactivation of the latent reservoir using a latency-reversal agent (LRA) with the subsequent killing of the reactivated cell either by the cytotoxic arm of the immune system, including NK and CD8 T cells, or by viral cytopathic mechanisms. Numerous LRAs are currently being investigated *in vitro*, *ex vivo* as well as *in vivo* for their ability to reactivate and reduce latent reservoirs. Among those, several toll-like receptor (TLR) agonists have been shown to reactivate latent HIV. In humans, there are 10 TLRs that recognize different pathogen-associated molecular patterns. TLRs are present in several cell types, including CD4 T cells, the cell compartment that harbors the majority of the latent reservoir. Besides their ability to reactivate latent HIV, TLR agonists also increase immune activation and promote an antiviral response. These combined properties make TLR agonists unique among the different LRAs characterized to date. Additionally, some of these agonists have shown promise toward finding an HIV cure in animal models. When in combination with broadly neutralizing antibodies, TLR-7 agonists have shown to impact the SIV latent reservoir and delay viral rebound. Moreover, there are FDA-approved TLR agonists that are currently being investigated for cancer therapy and other diseases. All these has prompted clinical trials using TLR agonists either alone or in combination toward HIV eradication approaches. In this review, we provide an extensive characterization of the state-of-the-art of the use of TLR agonists toward HIV eradication strategies and the mechanism behind how TLR agonists target both cellular and tissue HIV reservoirs.

Keywords: HIV, toll-like receptors, latency-reversal agents, shock and kill, reservoirs

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INTRODUCTION

HIV infection is still one of the highest causes of mortality and morbidity worldwide. The introduction of anti-retroviral therapy (ART) in 1996 decreased the mortality due to HIV infection and transformed the disease from deadly to chronic. Cure is still not attainable due to a small reservoir of infected cells that harbor the virus in a latent form and become unrecognizable by the immune system and current therapies (1–5). Several strategies have been proposed to eliminate

this latent reservoir (6). Among these strategies, the “shock and kill” approach rely on the notion that a pharmacological agent that reactivates the latent virus or latency-reversing agent (LRA) in the presence of ART will reduce the latent reservoir and could be followed by two potential outcomes. “Shock and kill” strategies can lead to complete viral eradication. In this case, the final goal is to eliminate all latent viruses. In a way, these strategies will try to echo the Berlin or the London patient, whom underwent remission due to bone marrow transplants and became undetectable for viral reservoirs (7, 8). Another outcome could be that a reduction of the latent reservoir due to the “shock and kill” strategy may be sufficient to allow the immune system control viral replication in the absence of ART (9–12). This has been termed “functional cure” and it is exemplified with the VISCONTI study. In this study, a subset of HIV-infected individuals who started ART early were able to control viremia in the absence of ART (termed Post-Treatment Controllers or PTCs) (11, 12). The authors of this study found a strong association between a low HIV reservoir in blood with the ability of the immune system to control viremia after ART treatment interruption (11).

As of today, several LRAs have been developed and some have reached clinical trials. The first generation LRAs that reached human testing include Valproic Acid (13–18), SAHA (19–21), Romidepsin (22), Panobinostat (23), Bryostat-1 (24), and Disulfiram (25, 26). However, these LRAs have resulted in limited to no clinical effect on the size of the latent reservoir (27–29). Some potential explanations for the failure of these LRAs are the following. First, the lack of or low reactivation of latent viruses with these LRAs *in vivo*. Second, the reduced killing of reactivated cells either by the low frequency or compartmentalization of HIV-specific cells on patients under ART, immune exhaustion or the presence of defective proviruses that divert the immune response from the reactivated cells carrying replication competent virus. Third, a survival advantage of latently infected cells (30–36). To that end, strategies that can efficiently reactivate latent HIV *in vivo* and also enhance immune responses against HIV may overcome these obstacles encountered by the current cure efforts.

Recently, a second generation of LRAs targeting toll-like receptors (TLRs) have reached clinical trials. TLRs are pathogen-recognition receptors (PRRs) capable of sensing small molecular motifs conserved within microbes (37, 38). In addition to their ability to reactivate latent HIV, TLR agonists also increase immune activation and promote antiviral responses (39–44). These combined properties make TLR agonists unique among the LRAs characterized to date.

In 1891, William Coley demonstrated how several bacterial components could be used to treat cancer patients (45). Since then, several TLR ligands are being investigated and in clinical trials to enhance immunity for their use in treatment of cancer (46), viral infection (47), and bacterial infection (48). Several reviews have previously focused in the development and use of TLR agonists for cancer and other diseases (49–51). Here, we provide a comprehensive literature review specifically focused on the development of TLR agonists as LRAs and their potential use of these agonists for HIV eradication purposes.

TOLL-LIKE RECEPTORS

TLRs and Their Ligands

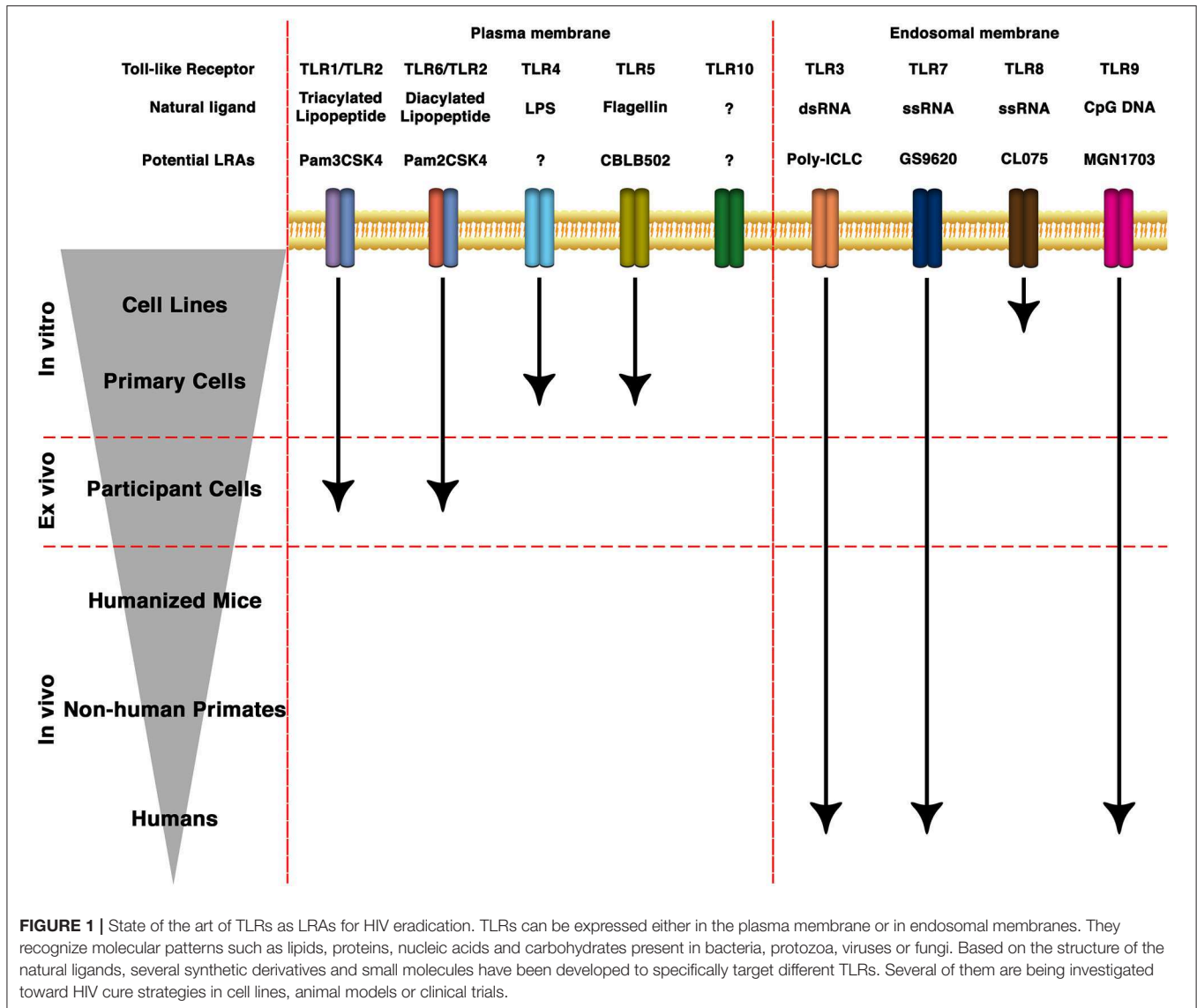
TLRs are transmembrane PRRs that recognize a plethora of molecules present in virus, bacteria, fungi or protozoa such as lipids, proteins, nucleic acids, and carbohydrates (52, 53). PRRs are germline-encoded receptors which function as first line of detection of pathogenic infections and recognize conserved molecular structures called pathogen-associated molecular patterns (PAMPs) (54). PRRs can also recognize soluble molecules released during cell death or damage. These structures are called damage-associated molecular patterns (DAMPs) (55, 56). In humans, there are 10 TLRs that differ both in their location within the cell as well as their cognate PAMP (Figure 1). TLR-1, 2, 4, 5, 6, and 10 are localized on the surface of the cells and recognize PAMPs present at the exterior of bacteria, fungi, and protozoa. On the other hand, TLR-3, 7, 8, and 9 are localized within endosomal structures and recognize nucleic acids derived from bacteria and viruses (53, 57). TLRs recognize their cognate ligand through either homodimers or heterodimers and are expressed in cells of the innate and adaptive immune system (such as dendritic cells, macrophages, granulocytes, T cells, B cells, NK cells, and mast cells) as well as epithelial and endothelial cells [reviewed in (37)].

TLRs in the Plasma Membrane

TLR-2 has a broader spectrum of ligand recognition than other TLRs due to its ability to dimerize with other receptors (58, 59). TLR-2 can recognize diacylated lipopeptides in the surface of gram-positive bacteria in conjunction with TLR-6 (60, 61). Whereas, TLR-2 can recognize triacylated lipopeptides present in gram-negative bacteria together with TLR-1 (62). TLR-2 has been shown to also induce signaling as a homodimer when recognizing lipoarabidomannan of *Mycobacterium smegmatis* (LAM-MS) and polysaccharide A of *Bacteroides fragilis* (PSA) (63, 64). Finally, TLR-2 can complex to the c-type lectin Dectin-1 to recognize zymosan, a β -glucan present in yeast cell wall (65). TLR-4 recognizes lipopolysaccharide (LPS), the principal component of gram-negative bacteria, and its truncated versions lipooligosaccharide and lipid A (66). CD14 and MD-2 are also needed for proper TLR-4 signaling (67, 68). TLR-5 recognizes flagellin, the main component of bacterial flagella (69). Lastly, TLR-10 has been an orphan receptor for a long time with no clearly defined ligand (70). TLR-10 has been shown to be involved in triacylated lipopeptides recognition by TLR-2 (71, 72). Recently, TLR-10 has been shown to sense gp41 and other HIV proteins in conjunction with TLR-1 and TLR-2 (73).

TLRs in the Endosomal Membrane

Several TLRs are present in endosomes, lysosomes and endolysosomes (74). TLR-3 recognizes double-stranded RNA (dsRNA) generated during viral infections while TLR-7 and TLR-8 both recognize single stranded RNA (ssRNA) (75, 76). Finally, TLR-9 can recognize unmethylated double-stranded DNA derived from both bacteria and viruses (77).



Synthetic Ligands

Due to their immunostimulatory properties, the discovery of small molecules that can “mimic” a TLR response is an area of active research. Over the past years, several small molecules have been developed to specifically target TLRs and are being investigated for the treatment of bacterial and viral infections, for cancer immunotherapy and to optimize vaccine efficacy [reviewed in (50, 78, 79)].

Synthetic lipopeptides have been the gold standard TLR-2 ligands. Pam2CSK4 is a synthetic diacylated lipopeptide that engages TLR-2/TLR-6 heterodimers or TLR-2 homodimers (60, 80). Pam3CSK4 is a synthetic triacylated lipopeptide that engages TLR-2/TLR-1 heterodimers (62, 81). Mono-acyl lipopeptides are the minimal structure required for TLR-2 activity (82). Guan and colleagues identified a series of compounds with a similar core structure consisting of 3-carboxylbenzothiophene linked via a carbonthioyl amino

bridge to an anilino group (83). Further structure-activity relationship (SAR) studies have yielded an optimized novel compound termed CU-T12-9 (84). This compound has shown higher efficacy than the original compound and shows a specificity toward TLR-2/TLR-1 heterodimers over TLR-2/TLR-6 heterodimers (84). Using structure-based virtual screening of over 10.5 million compounds, Chen and colleagues identified ethyl 2-(4-methylpiperazine-1-carboxamido)-5,6-dihydro-4H-cyclopenta[b]thiophene-3-carboxylate (SMU127) as a specific TLR-2/TLR-1 heterodimer ligand (85). Recently, the same group has developed 2-(1-(2-(Methylamino)-5-nitrophenyl)-1H-imidazol-4-yl)-5-(trifluoromethyl)phenol (SMU-Z1) as a specific TLR-2/TLR-1 heterodimer ligand (86). Finally, a screening in PMA-differentiated THP-1 cell line of nearly 100,000 compounds identified diprovocims as inducers of TLR-2 and TLR-1 receptor dimerization and activation in the low pM range (87).

The main synthetic ligand used for TLR-3 is polyinosinic-polycytidylic acid [poly(I:C)]. Poly(I:C) mimics dsRNA and it is formed of a strand of inosine poly(I) homopolymer annealed to a strand of cytidine poly(C) homopolymer. The antiviral and antitumoral activities of Poly(I:C) were described in the 1960's but it was not characterized as a TLR-3 agonist until the early 2000's (75). Other derivatives of Poly(I:C) have been developed such as a combination of polyinosinic-polycytidylic acid and poly-L-lysine (Poly-ICLC/Hiltonol[®]), or the introduction of uridine in the Poly(I:C) strand (poly(I:C₁₂U)/rintatolimod/Ampligen[®]), or Polyadenylic–polyuridylic acid [poly(A:U)] (88–90).

Monophosphoryl lipid A (MPL) is a detoxified form of the TLR4 agonist LPS from *Salmonella minnesota* that retains immunostimulatory properties but lacks the toxic effects of LPS (91). RC599 is a synthetic mimetic of MPL derived from aminoalkyl glucosaminide 4-phosphate (92). Both, MPL and RC599 have been shown to be efficient adjuvants and promote CD4T cell responses (93). MPL has been used in a number of complex adjuvants included in human vaccines (Supervax[®],

Cervarix, Melacine[®], Stimuvax) [reviewed in (94)]. Other lipid A mimetics (AS04, GLA-SE, GSK1795091, and OM-174) have been developed as vaccine adjuvants and are in licensed vaccines or in Phase I or II clinical trials as anticancer therapeutics [reviewed in (95)].

The polypeptide CBLB502 (Entolimod) derived from Flagellin is a potent TLR-5 ligand under extensive investigation as vaccine adjuvant, cancer and ischemia (96–99).

Imiquimod is an imidazoquinoline amine analog to guanosine that specifically activates TLR-7 (100). Several other agonists derived of imidazoquinoline have been developed that target TLR-7 such as Gardiquimod[™] and PF-04878691 [also known as 852A or 3M-001] (101, 102)], or to target TLR-7 and TLR-8 simultaneously such as CL075, CL097, or Resiquimod (also known as R-848) (103–105). In addition, guanosine analogs such as Loxoribine have been generated as specific TLR-7 ligands (106). ANA975 and ANA773, also guanosine analogs, are prodrugs derived of the TLR-7 agonist isatoribine (107, 108). CL264 and SM360320 are derivatives of 9-benzyl-8 hydroxyadenine that have been shown to stimulate TLR-7

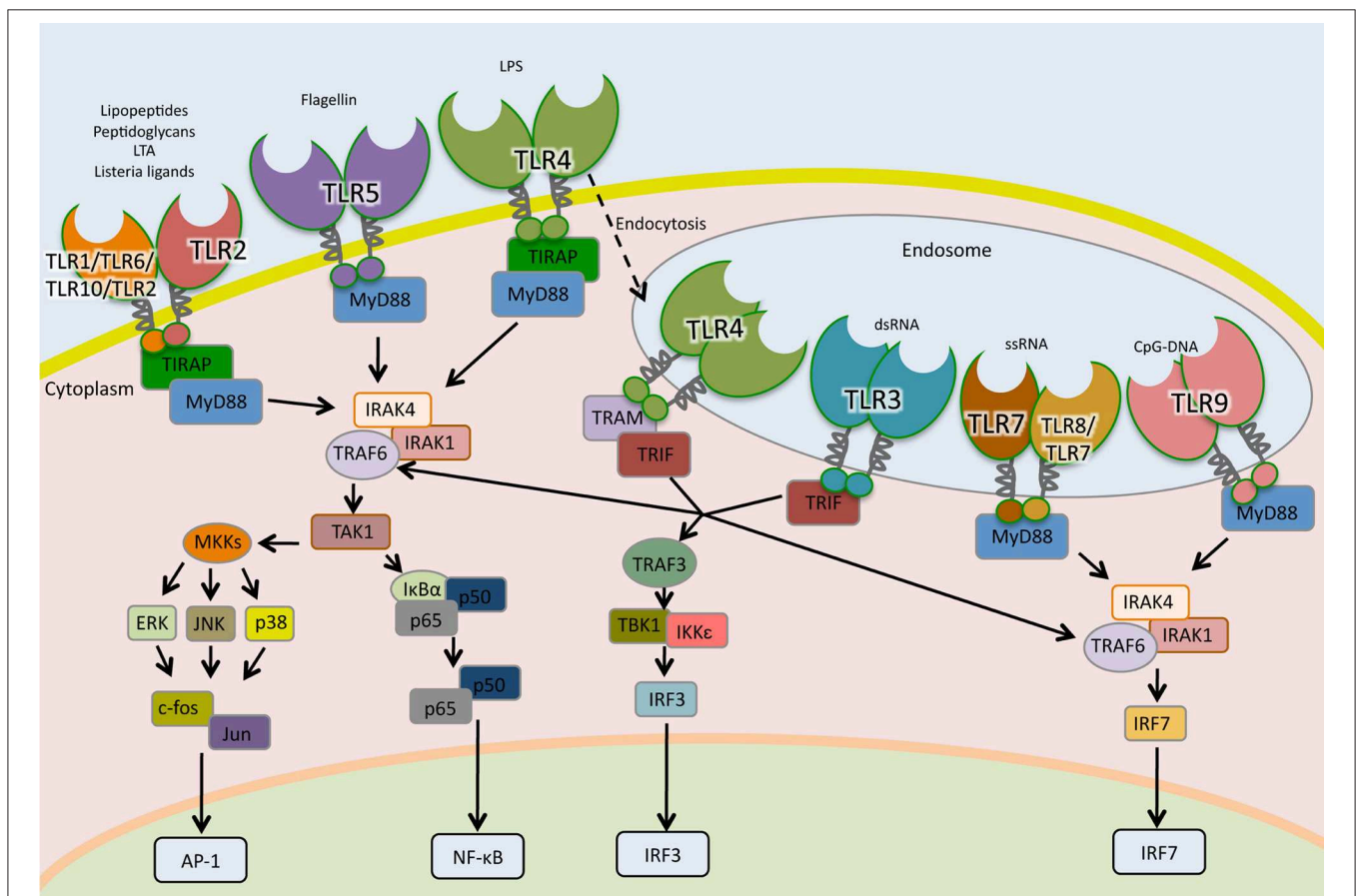


FIGURE 2 | Toll-like receptors signaling pathways. TLRs are the sentinels of host defense. The homodimers TLR5, TLR4, and TLR2 and the heterodimers TLR2-TLR1, TLR2-TLR6, and TLR2-TLR10 bind to their specific ligand at the cell surface, whereas TLR3, TLR7, TLR7-TLR8, and TLR9 localize to the endosomes, where they interact to their ligands. TLR4 following microbial detection is endocytosed into the endosome. When TLRs are activated by interaction with their ligands, adaptor molecules are recruited to stimulate downstream signaling pathways including NF- κ B, AP1, and IRFs.

(109, 110). Finally, GS-9620 (Vesatolimod) and its close analog GS-986 are also a 9-benzyl-8 hydroxyadenine derivatives that targets TLR-7 with higher activity than CL264 (41, 42, 111).

All the synthetic TLR-9 ligands generated to date are derived from CpG oligodeoxynucleotides (CpG ODNs). CpGs ODNs can be classified into three classes (A, B, or C) that differ in their structure as well as their immunostimulatory properties (112, 113). Among others, CpG ODNs include CPG10101, IMO-2125, SD-101, CpG7909 (ProMune), MGN1703 (Lefitolimod) (114–118).

Finally, a series of synthetic TLR ligands that covalently link two or more TLR ligands are under investigation. These multi-TLR ligands have been designed to enhance immune responses through the synergistic activation of two or more TLRs with different downstream pathways (42, 119).

TLR Signaling Pathway

TLRs are type I transmembrane glycoproteins composed of a leucine-rich-repeat (LRR) motifs on the extracellular or endosomal domain that mediates ligand recognition and the Toll/IL-1R (TIR) in the cytoplasmic domain responsible for signaling (120). The signaling cascades following TLR activation involves multiple steps. Adaptor proteins such as myeloid differentiation primary-response protein 88 (MyD88), TIR-domain containing adaptor protein (TIRAP, also known as MAL), TIR-domain containing adaptor protein inducing IFN- β (TRIF) and TRIF-related adaptor molecule (TRAM) are recruited to the TIR domain after interaction of PAMPs to their cognate TLR (Figure 2).

With the exception of TLR-3, all TLRs signal through the MyD88-dependent pathway. In the MyD88-dependent pathway, TLR-2 and TLR-4 require TIRAP in order to recruit MyD88 to start signal transduction while TLR-5, TLR-7, TLR-8, and TLR-9 initiate signaling using uniquely MyD88 (121, 122). After recruitment of MyD88, a complex is formed with interleukin-1 receptor-associated kinase-1 (IRAK1) and IRAK4. IRAK1 is phosphorylated and associates with TNF receptor-associated factor 6 (TRAF6), which activates transforming growth factor- β -activated kinase 1 (TAK1), also known as mitogen-activated protein kinase kinase 7 (MAP3K7). TAK1 activates, by phosphorylation, two routes; the I κ B kinase—nuclear factor kappa-light-chain-enhancer of activated B cells (IKK-NF- κ B) pathway and the mitogen-activated protein kinases (MAPK) pathway (Figure 2). In the first route, TAK-1 phosphorylates I-kappa-B- α/β (I κ B α/β). This phosphorylation leads to their degradation through the proteasome system and the release of NF- κ B, which translocates to the nucleus, binds to DNA and initiates transcription. In the second route, TAK1 activates the MAPK members extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, which lead to activation of the nuclear factor activator protein-1 (AP-1) (57, 123). In plasmacytoid dendritic cells (pDCs), TLR-7, and TLR-9 activate MyD88 signaling that leads to the phosphorylation and activation of the transcription factor interferon regulatory factor 7 (IRF7), which regulates the expression of IFN- α (124).

In the MyD88-independent pathway, TRIF is recruited to TLR-3 to initiate signaling (125). Besides TLR-3, TLR-4 can

be endocytosed and signal through TRIF using the adaptor molecule TRAM (126, 127). TRAF6 and TRAF3 are recruited to TRIF. While TRAF6 engages IKK and MAPK, leading to the activation of NF- κ B, AP-1, and IRF7; TRAF3 recruits TBK1/IKK- ϵ complex that activates IRF3 culminating in IFN- β expression (Figure 2) (128, 129).

TOLL-LIKE RECEPTOR AGONISTS AS LATENCY-REVERSING AGENTS

In vitro Studies

The importance of TLRs in the physiopathology of HIV was first postulated after observations on increased plasma viral loads seen in HIV-infected individuals exposed to vaccination regimens, suffering of opportunistic bacterial infections or sexually transmitted diseases, or had translocation of microbial products from the gut (130–138). Early studies have shown that PAMPs and their corresponding microorganisms transactivate the HIV long-terminal repeat (LTR) promoter. For example, it was described that purified protein derivative (PPD) of *Mycobacterium tuberculosis* increased viral mRNA expression in HIV infected monocytes (139). Furthermore, monocytic cell lines stimulated with live *M. tuberculosis* or lipomannan (LAM) increased p24 expression by 3-fold and enhanced HIV LTR transcription (140). Additionally, it has been shown that both *M. tuberculosis* PPD from the H37Ra strain and the mycobacterial major cell wall component mannosylated LAM (ManLAM) activated transcription of HIV in the T cell line Jurkat. ManLAM-induced HIV gene expression was mediated via protein kinases that culminated in NF- κ B nuclear translocation. Mutations in the NF- κ B binding sites in the HIV LTR abolished PDD-induced HIV expression (141, 142). These findings suggested that microbial products could be inducing HIV transcription. The latter discovery of TLRs as sensors of these microbial products suggested the idea that TLR agonists could reactivate latent HIV and could be potential LRAs.

Subsequently increasing number of reports have demonstrated the role of specific TLR agonists as latency reversing agents *in vitro*. Equils and colleagues transfected human dermal endothelial cells with an LTR luciferase construct and showed that stimulation of TLR-4 with LPS leads to NF- κ B activation and transactivation of HIV-LTR (143). However, the effect of LPS in the reactivation of the HIV promoter in T cells has been disputed by other groups (144–146). These divergent results could be explained by the use of contaminated LPS formulations with other PAMPs, like bacterial lipopeptides. Other possible reasons for the discrepancy could be variation in the cell type used in each study, since different cells respond differently to TLR agonists, the cell culture environment, which component could escalate or impair an agonists effect, and/or disparities in TLR-4 binding affinity to LPS from different bacteria. Recently, LPS has been shown to reactivate latent HIV in macrophages isolated from the urethra of patients under ART (147).

The TLR-9 agonist CpG ODNs has been shown to activate HIV replication in the chronically infected human cell lines

U1 and ACH2 in an NF- κ B dependent manner (148, 149). Furthermore, DNA from *F. nucleatum* increased HIV promoter activity through TLR-9 signaling in the THP89GFP cell line (144). Recently, the TLR9 MGN1703 has been shown to induce HIV RNA release in peripheral blood mononuclear cells (PBMCs) from aviremic HIV-infected donors on antiretroviral therapy (ART) (150).

Mycobacteria have been shown to induce HIV transcription in a TLR-2 dependent manner. Bhat et al. observed that the *M. tuberculosis* and *M. smegmatis* proline-proline-glutamic acid protein Rv1168c (PPE17) interacts with TLR-2 resulting in activation of NF- κ B and HIV transactivation in the human monocytic cell line THP1 (151). Our group has shown that the TLR-2 agonists Pam2CSK4 and Pam3CSK4 have latency reversal activity in CD4 T cells from aviremic HIV-infected participants and in a primary T_{CM} cell model of latency (42, 146, 152). Finally, the component of the *M. tuberculosis* membrane phosphatidylinositol mannoside 6 (PIM6) as well as whole bacteria in co-culture reactivated HIV in a primary T_{CM} cell model of latency. As for previous studies with *M. tuberculosis*, viral reactivation was dependent on TLR-2 (153).

R-848, a TLR-7/-8 agonist, induced p24 expression in the latently infected monocytic cell lines U1 and OM10 (154). Furthermore, a combination of the PKC agonist and LRA prostratin with a TLR-8 agonist 3M-002 was tested in a coculture of latently infected cells (J-Lat) and monocyte-derived dendritic cells (MDDCs). The combination of prostratin and 3M-002 resulted in greater reactivation of HIV latency in J-Lat than each compound alone. This synergistic interaction was dependent on TNF- α and on MDDC-T cell interactions (155).

Flagellin, the structural protein in bacterial flagella and a TLR5 agonist, has been shown to reactivate latent HIV in J-Lat, a transformed cell line latently infected with HIV derived of Jurkat (146, 156, 157). Thibault and colleagues also shown activity of flagellin in central memory T cells previously infected with a VSV-G pseudotyped NL43. However, resting CD4 T cells from aviremic patients, when challenged with flagellin, failed to elicit detectable levels of viral gene expression (157).

The TLR-3 agonist Poly(I:C) has been shown to reactivate latent HIV via NF- κ B and JNK in the monocytic cell line U38 that contains a stably integrated and silent copy of the HIV LTR promoter linked to the chloramphenicol acetyltransferase (CAT) gene (158).

The selective TLR-7 agonist GS-9620 induced extracellular HIV RNA release in the supernatants of PBMCs isolated from HIV-infected participants on ART but not in purified CD4 T cells (159). In this study, Tsai and colleagues attributed this viral reactivation to type I IFN produced by pDCs. Therefore, this last finding suggests two complementary mechanisms of latency reversal mediated by TLR agonists. One that requires a subset of immune cells to be activated with the TLR agonist and these cells release soluble factors with latency reversal activity. And a second mechanism in which the TLR agonists have a direct effect on latently infected CD4T cells. Based on this idea, we have recently characterized the mechanisms of viral reactivation mediated by the TLR-2 agonist Pam2CSK4 and the TLR-7 agonist GS-9620 and compared with that of synthetic dual

TLR-2 and TLR-7 agonists (dual TLR-2/7 agonists). We found that TLR-2 and TLR-7 agonists reactivate latency by two distinct and complementary mechanisms. TLR-2 agonists reactivate HIV by directly inducing NF- κ B activation in memory CD4 T cells, while TLR7 agonists induced the secretion of soluble factors that can reactivate latent HIV in CD4 T cells. Our results suggests that TNF- α but not type-I IFN secreted by monocytes and plasmacytoid dendritic cells (pDCs) promotes viral reactivation in CD4 T cells (42).

TLR agonists have also shown to reactivate latent HIV in other cell subsets besides CD4 T cells and monocytes/macrophages. Poly (I:C) and bacterial ribosomal RNA induced HIV reactivation through TLR-3 in a latency model using immortalized human primary microglia with simian virus-40 (SV40) large T antigen and human telomerase reverse transcriptase (160). Furthermore, stimulation with TLR-2, TLR-4, or TLR-9 agonists induced HIV replication in a primary latency model using human progenitor mast cells (161).

In vivo Studies: From Small Animals to Clinical Trials

Mouse models have been a tool to study the role of TLR agonists on the pathogenesis of HIV. Initial studies were done using a transgenic mouse model that contains intact copies of HIV proviral DNA (162). Infection of these animals with *Mycobacterium avium* or *Toxoplasma gondii* increased viral production in monocytes/macrophages (163, 164). Using *ex vivo* spleens from these transgenic mice, Equils and colleagues demonstrated that ligands for TLR-2 (soluble *Mycobacterium tuberculosis* factor or STF), TLR-4 (LPS), and TLR-9 (CpG) increased viral production and that combination of LPS with either STF or CpG increased viral production in an additive manner (165). Furthermore, Bafica and colleagues crossed this HIV transgenic mouse with either a TLR-2-deficient or a control mouse to investigate the role of TLR-2 in the activation of HIV expression. Culture filtrate proteins, phosphatidyl-inositol mannoside from *M. tuberculosis* and the synthetic lipopeptide Pam3CSK4 induced p24 expression in spleen cells from HIV transgenic mouse expressing TLR-2 but not the TLR-2 deficient mice (166).

Several studies have specifically looked at the effects of TLR agonists on the latent reservoir *in vivo* (Table 1). A study using a humanized mice model of HIV latency demonstrated that the TLR-3 agonists poly(I:C) can reactivate latent HIV *in vivo* (167). In this study, the co-administration of poly(I:C) with a vaccination regimen including recombinant anti-human CD40 antibody fused to 5 HIV peptide regions (α CD40.HIV5pep) reduced the levels of cell-associated HIV DNA and delayed 1 week viral rebound after ART interruption in these animals (167). The selective TLR-7 small-molecule agonist GS-9620 has demonstrated antiviral activity in animal models of hepatitis B virus, good safety profiles and has progressed to clinical testing (171–173). As such, TLR-7 agonists have also been tested as LRAs in SIV-infected rhesus macaques. So far, there has been 4 different studies on the administration of GS-9620 or its analog GS-986 in infected macaques under suppressive ART.

TABLE 1 | Summary of *in vivo* studies using TLR ligands as LRAs.

	Administration	Study arms	Specie	Viral blips	Effects on reservoir	References
Poly I:C (TLR3)	Two doses 2.5 weeks apart, half IP half IM	PBS Poly I:C α CD40.HIV5pep/ Poly I:C	NRG hu- mice	Yes (US/7)	Reduction on cell-associated DNA in lymphoid tissue (4/4) Delayed rebound (α CD40.HIV5pep/Poly I:C 1 week)	(167)
Poly-ICLC (TLR3)	Two consecutively daily doses, SC	Randomize (3:1) vs placebo	Human	0/12	Reduction on cell-associated DNA (0/12)	(168)
GS-986 (TLR7)	Ten doses two weeks apart, OG	Sham Ad26/MVA GS-986 GS-986/Ad26/MVA	Rhesus macaque	Sham (0/9) Ad26/MVA (0/9) GS-986 (0/9) GS-986/Ad26/MVA (1/9)	Reduction on cell-associated DNA in lymph nodes week 70 (Sham 1/9, Ad26/MVA 1/9, GS-986 4/8; GS-986/Ad26/MVA 6/8) Reduction on cell-associated DNA in PBMCs week 70 (Sham 3/9, Ad26/MVA 9/9, GS-986 4/8; GS-986/Ad26/MVA 6/8) Virologic control (Sham 0/9, Ad26/MVA 0/9, GS-986 0/8, GS-986/Ad26/MVA 3/8)	(39)
GS-9620 (TLR7)	Ten doses two weeks apart, OG	Sham PGT121 GS-9620 GS-9620/PGT121	Rhesus macaque	Sham (0/11) PGT121 (0/11) GS-9620 (0/11) GS-9620/ PGT121 (0/11)	Reduction of viral DNA in lymph nodes week 120 (Sham 4/11, PGT121 7/11, GS-9620 3/11, GS-9620/PGT121 11/11) Virologic control (Sham 0/11, PGT121 3/11, GS-9620 1/11, GS-9620/PGT121 5/11)	(40)
GS-986 (TLR7)	Dose scalation 2 weeks apart, OG	Sham GS-986	Rhesus macaque	Sham (0/3) GS-986 (4/4)	Reduction of viral DNA in memory CD4 T cells from PBMCs (3/4), LNMCs (4/4) and GMMCs (4/4)	(41)
GS-9620 (TLR7)	Ten doses 2 weeks apart, OG	Sham GS-9620 0.05 mg/kg GS-9620 0.1 mg/kg GS-9620 0.15 mg/kg	Rhesus macaque	Sham (0/3) GS-9620 0.05 mg/kg (3/3) GS-9620 0.1 mg/kg (3/3) GS-9620 0.15 mg/kg (3/3)	Reduction of viral DNA in memory CD4 T cells from PBMCs (Sham 2/2, GS-9620 0.05 mg/kg 3/3, GS-9620 0.1 mg/kg 3/3, GS-9620 0.15 mg/kg 3/3) Reduction of viral DNA in memory CD4 T cells from LNMCs (Sham 1/2, GS-9620 0.05 mg/kg 1/3, GS-9620 0.1 mg/kg 2/3, GS-9620 0.15 mg/kg 1/3) Reduction of viral DNA in memory CD4 T cells from GMMCs (Sham 0/2, GS-9620 0.05 mg/kg 2/3, GS-9620 0.1 mg/kg 3/3, GS-9620 0.15 mg/kg 3/3) Virologic control (Sham 0/2, GS-9620 0.05 mg/kg 0/3, GS-9620 0.1 mg/kg 1/3, GS-9620 0.15 mg/kg 1/3)	(41)
GS-9620 (TLR7)	First course of twelve doses 2 weeks apart, OG Second course of five dose 2 weeks apart, OG	Sham GS-9620	Rhesus macaque	Sham (0/2) GS-9620 (0/4)	No effects in viral HIV DNA	(169)
GS-9620 (TLR7)	Tablet(s) administered orally once every 2 weeks	Randomized, blinded, placebo-controlled dose-escalation study	Human	Completed trial	Completed trial	NCT02858401

(Continued)

TABLE 1 | Continued

	Administration	Study arms	Specie	Viral blips	Effects on reservoir	References
GS-9620 (TLR7)	Up to 10 doses administered as four 2 mg tablets orally every 14 days	Randomized, Double-Blind, Placebo-controlled Study	Human	Ongoing trial	Ongoing trial	NCT03060447
CpG-ODN 7909 (TLR9)	Immunized with double the standard dose of PCV7 (Pevnar [®] , Wyeth) at 0 and 3 months and with one single dose of PPV-23 (Pneumo Novum [®] , Sanofi-Pasteur MSD)	Placebo CpG-ODN 7909	Human	ND	Decrease in viral HIV DNA in PBMCs in CpG-ODN 7909 group relative to placebo control	(170)
MGN1703 (TLR9)	Eight doses twice weekly for 4 week, SC	Single-arm, open-label study	Human	6/15	No effects in viral HIV DNA in CD4 T cells	(43)
MGN1703 (TLR9)	Forty-eight doses twice weekly for 24 week, SC	Single-arm, open-label study	Human	ND	No effects in viral HIV DNA in CD4 T cells No changes in CA US HIV RNA in CD4 T cells No changes in IUPM	(44)

IP, intraperitoneally; IM, intramuscularly; SC, subcutaneously; OG, oral gavage; NRG mice, NOD.Cg-Rag1^{tm1Mom}/J2g^{tm1Wj}/SzJ mice; US, Unspecified; ND, Non-determined; CA US, cell-associated unspliced; IUPM, infectious units per million.

The first study combined the therapeutic vaccine Ad26/MVA (recombinant adenovirus serotype 26 (Ad26) prime, modified vaccinia Ankara (MVA) boost) with GS-986. They found that this combination resulted in decreased levels of viral DNA in lymph nodes and peripheral blood, and improved virologic control and delayed viral rebound following discontinuation of antiretroviral therapy (39). In another study from the same group, GS-9620 was combined with the V3 glycan-dependent bNAb PGT121 in rhesus monkeys during ART (40). This combination delayed viral rebound after ART discontinuation. Interestingly, 5 out of 11 monkeys did not show viral rebound even after CD8-depletion or adoptive transfer of PBMCs and LNMCs into naïve monkeys (40). In a third study, repeated administration of GS-9620 or its analog G-986 to SIVmac251-infected rhesus macaques under ART was associated with transient plasma viremia that peaked 24 to 48 h after dosing (41). Furthermore, TLR-7 agonists induced a reduction in cell-associated SIV DNA in sorted memory CD4 T cells obtained from peripheral blood, gastrointestinal mucosa and lymph nodes (41). Finally, a recent study also evaluated the effects of repeated doses of the TLR7 agonist GS-9620 in SIV-infected rhesus macaques receiving ART (169). The rhesus macaques that received GS-9620 during ART presented with immunologic effects due to the TLR-7 agonist, such as upregulation of IFN-stimulated genes in both blood and tissues, an increase in different plasma cytokines such as IFN- α and IL-1RA, and changes in CD8, NK and macrophage activation. In contrast with the study from Lim and colleagues, GS-9620 did not result in a measurable increase in plasma viremia or changes in viral RNA-to-viral DNA ratio in PBMCs or tissues, nor decreases in viral DNA in PBMC or tissues (169). Several differences can account for the discrepancies in these two studies. First, ART was initiated at different times after initial infection (65 vs. 13 days). Second, GS-9620 was administered at different times post-ART initiation (60 vs. 75 weeks). Third, the SIV strain used in each study were different (SIVmac251 vs. SIVmac239X). Forth, the route of inoculation was also different (intrarectally vs. intravenously). All of these differences could lead to variations in the formation of viral reservoirs and their sensitivity to TLR-7 agonists. Further studies are warranted to fully understand the discrepancies of these two studies.

TLR agonists have also reached clinical testing in ART-suppressed participants (Table 1). The TLR-9 agonist CpG-ODN 7909, a class B CpG ODN, was administered as adjuvant in HIV-infected individuals (174). Interestingly, those participants receiving the TLR9 agonist as adjuvant in the immunization protocol had some decrease in the HIV proviral reservoir compared the control group (170). This led to the idea of using TLR-9 agonists to reduce the latent reservoir. As such, two different clinical trials aimed toward HIV eradication have been done using the TLR-9 ligand MGN1703. In the first clinical trial, a single-arm, open-label study in which 15 (13 male, 2 female) virologically suppressed HIV infected individuals on ART received 60 mg MGN1703 subcutaneously twice weekly for 4 weeks (43). In this study, they characterized pDC, NK, and T-cell activation using flow cytometry on baseline and after 4 weeks of treatment. Additionally, HIV transcription was quantified by measuring plasma HIV RNA. MGN1703 treatment

increased the activation of pDCs, upregulated levels of cytokines, and enhanced activation of cytotoxic NK cells and effector CD8⁺ T cells. Furthermore, treatment with MGN1703 induced plasma HIV RNA blips up to >1,500 copies/mL in 6 of 15 participants (43). From this trial, biopsies from sigmoid colon were collected from 11 participants. Increased in solely type-1 interferon response but not a broad inflammatory response was observed in intestinal mononuclear cells. Interestingly, increased transcription of either *TLR9* or *IFNAR1* before MGN1703 administration was associated with improved efficacy in eliminating HIV DNA-containing cells in the intestine during the course of treatment (175). In the second clinical trial, the same group enrolled HIV-infected individuals on ART for an exploratory, single-arm clinical trial that tested the safety and immune enhancement effects of 24-weeks of MGN1703 (60 mg 2 weekly) therapy (44). A total of twelve individuals completed the treatment phase and nine underwent analytical treatment interruption (ATI). MGN1703 led to potent T-cell activation and increased HIV-specific T-cell responses, however there were no changes in CD4 T cells containing viral DNA nor differences in the time to rebound after ATI. In this study, a single patient was able to control viremia for 150 days after ATI. This participant had strong HIV-specific cellular and antibody-mediated immune responses, however as the study did not contain a placebo arm, the delayed viral rebound cannot exclusively be associated to MGN1703 treatment (44). The TLR-3 agonist Poly-ICLC (Hiltonol[®]) has also been tested in a randomized, placebo-controlled, double-blinded trial in ART-suppressed participants (168). In this study, participants received two daily doses of Poly-ICLC subcutaneously. Both, Poly-ICLC and placebo control were observed for adverse events, immune activation, and viral replication. As for other TLR agonists, Poly-ICLC administration lead to transient innate immune stimulation without generalized immune activation. While no effects of Poly-ICLC in reversing HIV latency or on the size of the viral reservoirs were observed; Poly-ICLC was reported safe and well-tolerated (168). Finally, the TLR7 agonist GS-9620 is currently being evaluated in clinical trials in HIV infected controllers (NCT03060447) and in those on suppressive ART (NCT02858401). These studies will provide information regarding safety and biological activity, including their impact on viral reservoirs, in HIV-infected patients.

MODULATION OF HIV-HOST IMMUNE RESPONSES BY TLR STIMULATION

Besides their potential ability to reactivate latent HIV, TLR agonists have been shown to have immunostimulatory and antiviral properties to modulate anti-HIV immune responses.

The TLR-2 agonist Pam3CSK4 has been shown to prime latently-infected CD4 T cells for CD8 T cell recognition (176). We have characterized that TLR-2 and dual TLR-2/7 agonists, besides reactivating latent HIV, they can activate NK cells and induce IL-22. We demonstrated a protective role for IL-22 in both cell-free and cell-to-cell HIV infection of CD4 T cells (42). Whether TLR-2 agonists enhance the ability of NK cells to kill HIV-infected cells has not been demonstrated yet. Furthermore,

the dual TLR-2/7 agonist PamadiFectin has been shown to enhance humoral responses in a mouse model immunized with p24 coupled to nanoparticles (119).

The TLR-3 agonist poly(I:C) enhanced the generation of HIV-specific T cell responses in BALB/c mice and in humanize mouse vaccination models (167, 177). *In vitro*, the TLR-7 agonist GS-9620 has been shown to both inhibit HIV replication in an IFN- α -mediated mechanism as well as enhance the anti-HIV activity of CD8 T and NK cells (159, 178). The TLR-7/8 agonist 3M-012, an analog of R-848, has been given as vaccine adjuvant in combination with HIV Gag antigen to non-human primates. The addition of 3M-012 to the Gag vaccine substantially enhanced Gag-specific T helper 1 and CD8 T cell responses compared to animals given the Gag protein alone (179). Furthermore, TLR-7/-8 activation through ssRNA or R-848 interfered with HIV replication cycle in lymphocyte cultures (154). In addition, The TLR-7/8 agonists 3M-002 and R-848 were able to promote HIV control *in vitro* in HIV-infected PBMCs through the activation of CD8 T cells and NK cells (180).

A randomized controlled vaccine trial conducted with 95 HIV-infected subjects investigated the impact of TLR-9 agonist as an adjuvant for pneumococcal vaccine. The trial showed that the TLR-9 agonist, CpG-ODN 7909, enhanced vaccine immunogenicity in the experimental group compared with the control group (174). *Post-hoc* analyses of the vaccine trial confirmed that patients that received TLR-9 ligand as adjuvant expressed more CD107a and macrophage inflammatory protein 1 β (MIP1 β) markers in CD8 T cells. The increase in these markers was associated with a reduction in HIV proviral load (170). Furthermore, the TLR-9 agonist CpG-ODN 2216 has been shown to activate NK cells in a pDC-mediated mechanism and enhance NK lysis of autologous HIV-infected CD4 T cells (181). In addition, TLR ligands such as Imiquimod (TLR-7), R-848 (TLR-7/8), CpG ODN (TLR-9), and Poly(I:C) (TLR-3) have been also shown to enhance the generation of HIV-specific CD8 T cells *in vitro* (182).

CONCLUDING REMARKS

The use of TLR ligands as LRAs has shown promising results in efforts toward HIV eradication either alone or in combination with other therapeutic strategies because of their ability to reactivate latent HIV, to enhance immune responses and promote antiviral responses. Several TLR agonists are under investigation both in pre-clinical models of HIV latency as well as in clinical trials (Figure 1). These preclinical and clinical studies have shown a wide variety of response even in studies using the same TLR agonists (Table 1). Why such differences are currently unknown. Based on these studies, we can speculate several factors that may be influencing the response to TLR agonists including the time of ART initiation, the length of ART treatment, the cellular composition of the latent reservoir, or the strain of SIV or HIV. There are also other factors that may influence the activity of these agonists that will need to be considered in future cure strategies. First, TLRs contain polymorphisms that influence their activity (183, 184). How these polymorphisms

affect the efficacy of different TLR ligands in HIV eradication approaches has not been characterized. Second, it is well-known that biological sex influences the responses of certain TLRs, in particular TLR-7 (185, 186). The *in vivo* evaluation of GS-9620 or other TLR-7 agonists will need to take this into account. As research progresses, emphasis also needs to be done in understanding whether these TLR agonists can reach all the different tissue compartments where HIV may hide, including lymph nodes, intestinal mucosal, and brain [reviewed in (187–191)]. Furthermore, HIV has been shown to reside latent in other cell types besides CD4 T cell (147, 192). As such, it is possible that a single TLR agonist may not be sufficient to reactivate all latent virus present in different cellular compartments as the expression of TLRs differs among different cell subsets. Additional research is warranted to fully understand which TLR agonists reactivate latent HIV in each cell compartment. Also, it will be important to address whether reactivation is due to a direct targeting of the TLR in the reservoir cell or whether other soluble factors secreted by other cells are required for efficient viral reactivation. The mechanisms involved in HIV latency are complex and involve

a plethora of cellular factors as well as epigenetic mechanisms (193). As such, TLR agonists may need to be combined with other LRAs with different mechanisms of action to efficiently reactivate all latent viruses. Finally, recent studies using animal models suggest the use of additional strategies, such as bNAbs, might be required to generate meaningful remission. Therefore, it is important to continue the investigation of TLR agonists as potential adjuvants for novel HIV cure strategies.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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