# THE ROLE OF TLR7 AND/OR TLR8 AGONISTS IN HIV CURE

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## Abstract

The latent viral reservoir is the roadblock on the way of curing HIV. Reversing latency followed by killing of cells harboring HIV, the “shock and kill” approach, is widely investigated. Toll-Like receptor (TLR) agonists, especially those targeting TLR7 and/or TLR8, are candidates in achieving HIV cure. This review analyzes and compares *in vivo* research conducted on these agonists, and provides an in-depth overview of benefits, limitations, and challenges regarding TLR7 and/or TLR8 agonist therapy for curing HIV.

PubMed was searched for relevant literature, and after screening, nine articles were included in this review.

TLR8 agonist treatment has not yet been assessed *in vivo*, but may play crucial roles in eliminating the viral reservoir in cells from monocytic origin. TLR7 agonist monotherapy using vesatolimod is thus far the only treatment investigated in HIV-infected humans, and does not affect the size of the viral reservoir, although one study reports an increase in time to rebound after ART cessation. Combination therapy of TLR7 agonists with broadly neutralizing antibodies or prime-boost immunization yields variable results regarding effects on reservoir size and time to rebound.

This review has failed to observe the ability of TLR7 agonists to achieve latency reversal, although immune modulation was observed. The latter might be a key effect of TLR7 agonists rather than the former, indicating roles for TLR agonists as adjuvants. Overall, inconsistent results, together with limited clinical relevance, and study and population heterogeneity, pose significant challenges in taking TLR7 agonists as treatment for HIV to the clinic.

## Layman's Summary

Despite being nearly four decades removed from the AIDS pandemic, the worldwide problem of HIV nowadays still poses significant challenges to public health. Although antiretroviral therapy (ART) has slowed, and even prevents progression of HIV to AIDS, it is not a solution. A reason for this is, besides the fact that not all people with HIV have access to ART, ART itself does not cure someone from HIV. To date, no clinically deployable cure for HIV has been found. This is because the virus is able to insert its genome into infected host cells, and silently reside there, causing latent infection. While on ART, this latent reservoir remains silent, unable to express itself and produce new virus particles. However, when stopping ART, HIV can reactivate and cause active infection.

Current strategies for curing HIV revolve around attempting to reverse this latency, in order for the immune system to then eliminate the latently infected cells. Able to do this are compounds that stimulate Toll-Like receptors (TLRs). TLRs are immune receptors on cells that recognize pathogens and then through signaling activate immune cells to combat the infection. Among these, TLR7 and TLR8 provide detection against RNA viruses like HIV. Stimulating TLR7 and TLR8 in HIV infection, however, induces reactivation of latent HIV, and helps the immune system in recognizing and eliminating cells harboring latent virus. This in turn may lead to a decrease in the size of the viral reservoir to such an extent that, if ART is discontinued, patients will not experience viral rebound and subsequent reinfection.

Plenty of research has been carried out on the therapeutic effects of TLR7, TLR7/8, and TLR8 stimulation in HIV-infected individuals or SIV-infected rhesus macaques, both alone or in combination with other compounds. This review aims to analyze and compare these, and give an in-depth overview of the current state of research on this subject, what benefits but also limitations it brings, and what challenges lie ahead in applying this as therapy.

Thus far, only monotherapy with TLR7 stimulant vesatolimod is assessed in both animals and humans. Results, however, vary, and it is unclear whether it truly decreases the viral reservoir and leads to an increase in time to rebound. This is similar for combination therapy using antibodies or therapeutic vaccination to enhance anti-SIV immunity in rhesus macaques, in concert with TLR7 stimulation.

Where these discrepancies come from is not fully clear, but it likely has to do with the many slight differences between the studies. For instance, different strains of HIV or SIV are used in different studies, and there are large disparities in time between infection and initiation of ART, potentially influencing the size of the viral reservoir initially already.

Aside from that, only one study reports so called reversal of latency, which has been part of the goal of stimulating TLRs. This raises questions regarding the potential of latency reversal, and whether TLR stimulation is maybe just for enhancing immunity to eliminate HIV. In addition, it seems that more than only TLR stimulation is needed to achieve clinically relevant results. Whether that is feasible in humans, especially regarding adverse effects, remains to be investigated.

# Introduction

With globally more than 37 million infections, HIV remains a substantial threat to public health[1]. Nowadays, viral replication can be suppressed with antiretroviral therapy (ART), thereby decreasing mortality of the disease and halting progression towards AIDS. However, aside from more than 25% of people living with HIV not having access to ART[1], it requires lifelong therapy and does not lead to cure. This is because the virus has the ability to latently reside in $CD4^+$ T cells by integrating its proviral DNA into the genome of the infected cell[2-4]. Cessation of ART allows for reactivation of the virus which causes viral replication and formation of new viral particles, essentially transforming chronic disease back to active HIV infection. Thus, it is the latent reservoir, considered to be established in the early phase of infection, that currently prevents curative therapy against HIV[4].

One way to target and potentially eliminate this reservoir is by reversing latency[5]. Infected cells, which are in a resting state, are activated and thereby start producing virus particles again. This reveals the hidden, latent reservoir, and makes these cells targetable again by the immune system for clearance. In addition, other compounds can aid and enhance immunity even more so that clearance is ensured. Thus, a combination of latency reversal and immune modulation, so called “shock and kill”, may be the key to eliminating the latent reservoir and opening up opportunities for curing HIV[6, 7].

Able to both induce latency reversal as well as modulate immunity are ligands that stimulate Toll-like receptors (TLRs)[8]. These are a family of pattern recognition receptors (PRRs), innate immune receptors that recognize molecular patterns associated to either pathogens or cell damage of the host. They are expressed on a wide range of immune cells, and especially prevalent on dendritic cells, the cells that form the bridge between innate and adaptive immunity. To date, ten TLRs have been discovered in humans, named TLR1 to TLR10, of which TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed on the plasma membrane of the cell. The remaining TLRs, TLR3, TLR7, TLR8, and TLR9 are expressed in intracellular compartments such as endosomes. TLRs are type-I transmembrane proteins, and upon recognition of either a damage-associated molecular pattern (DAMP) or a pathogen-associated molecular pattern (PAMP) by the TLR ligand recognition domain, downstream signaling occurs through the cytosolic tail of the receptor[9, 10].

In case of an infection with an RNA virus, such as HIV, TLR7 and TLR8 are pivotal in immune recognition and mounting an antiviral response[11, 12]. Upon recognition of the viral single-stranded RNA, adaptor protein MyD88 is recruited to the cytosolic part of the TLR, which there recruits and activates interleukin-1 receptor-associated kinases (IRAK), IRAK4 and IRAK1, and ubiquitin ligase TNF receptor-associated factor 6 (TRAF6). Then, through both the IκB kinase—nuclear factor kappa-light-chain-enhancer of activated B cells (IKK-NF-κB) pathway and the mitogen-activated protein kinases (MAPK) pathway, immune activation, inflammation, and proliferation occurs[9, 10, 12]. Additionally, in plasmacytoid dendritic cells (pDCs), MyD88 signaling causes interferon regulatory factor 7 (IRF) to be activated, inducing expression of interferon alpha, crucial in building an antiviral response[9, 10].

As aforementioned, aside from playing a crucial role in anti-HIV immunity, TLR signaling has been found to induce reactivation of latent HIV[13, 14, 15]. Over the years, several agonists of TLRs have been developed and investigated for their use in treating multiple diseases. These

agonists are being actively studied for their potential in targeting the viral reservoir in HIV infection as well[16, 17]. In this "shock and kill" approach, TLR agonists will be administered to HIV patients undergoing ART, with the intent of viral reactivation followed by killing of infected cells. This strategy can be used to reduce the size of the viral reservoir, thereby taking steps towards functional cure of HIV, in which there is sustained control of viral replication even in absence of ART. Moreover, it might even lead to sterilizing cure, with complete eradication of latently infected cells and thereby the reservoir. Regardless of the type of cure, the goal is to develop treatment that will allow for discontinuation of ART while preventing rebound from occurring.

Since TLR7 and TLR8 signaling is involved in anti-HIV-immunity[11, 12], and induces reactivation of latent HIV, agonists for these TLRs are promising candidates in achieving reduction or depletion of the HIV reservoir. To date, ample research has been conducted on the effects of multiple agonists both *in vitro* and *in vivo*, and several reviews have described studies using these among agonists to other TLRs in attempts to treat or prevent HIV infection[13, 14, 16-20]. To our knowledge, however, an in-depth overview and analysis of *in vivo* studies on the most promising TLR agonist candidates, those for TLR7 and TLR8, their benefits, limitations, and challenges for optimization, has not yet appeared. Therefore, we here address specifically the role of TLR7, TLR7/8, and TLR8 agonists in achieving HIV cure by targeting the latent HIV reservoir *in vivo*.

# Methods

## Search Strategy

To explore the role of TLR7, TLR7/8, and TLR8 agonists in curing HIV, we formulated the following research question: *"What is the role of TLR7, TLR7/8, and TLR8 agonists in depleting the viral reservoir in HIV-infected individuals undergoing ART?"*
To address this question, we searched for relevant literature in PubMed, matching the following characteristics.

## Inclusion Criteria

Population or Participants: SIV infected monkeys or HIV infected humans, undergoing ART.

Intervention: TLR7 and/or TLR8 agonists, either alone or combined with other compounds such as broadly neutralizing antibodies (bnAbs).

Controls: Sham, or at least not TLR7 and/or TLR8 agonists.

Outcomes: The primary outcome is reservoir size, and time to rebound. Secondary outcomes include viral reactivation, and immune modulation.

## Search Terms

Search terms included Toll-Like Receptor, Human Immunodeficiency Virus, Simian Immunodeficiency Virus, Acquired Immune Deficiency Syndrome, Vaccin*, Laten*, Rebound, Reservoir, Immune Modulat*, Immune Toleran*, Viremia, and Viral Load. In addition, medical subject headings were used in the query. The search was not limited by publication date, study designs, or article types. The full query used for the literature search is:
*((Toll-Like receptor[Title/Abstract]) OR (Toll-Like receptor*[Title/Abstract]) OR (Toll-Like receptor[MeSH Terms]) OR (Toll-Like receptor*[MeSH Terms]) OR (Toll Like receptor[Title/Abstract]) OR (Toll Like receptor*[Title/Abstract]) OR (Toll-Like-receptor[Title/Abstract]) OR (Toll-Like-receptor*[Title/Abstract]) OR (TLR[Title/Abstract]) OR (TLR*[Title/Abstract]))*
*AND*
*((Human Immunodeficiency Virus[Title/Abstract]) OR (Human Immunodeficiency Virus*[Title/Abstract]) OR (HIV[Title/Abstract]) OR (HIV*[Title/Abstract]) OR (HIV[MeSH Terms]) OR (HIV*[MeSH Terms]) OR (Simian Immunodeficiency Virus[Title/Abstract]) OR (Simian Immunodeficiency Virus*[Title/Abstract]) OR (Simian Immunodeficiency Virus[MeSH Terms]) OR (Simian Immunodeficiency Virus*[MeSH Terms]) OR (SIV[Title/Abstract]) OR (SIV*[Title/Abstract]) OR (Acquired Immunodeficiency Syndrome[Title/Abstract]) OR (Acquired Immunodeficiency Syndrome*[Title/Abstract]) OR (Acquired Immunodeficiency Syndrome[MeSH Terms]) OR (Acquired Immunodeficiency Syndrome*[MeSH Terms]) OR (Acquired Immune Deficiency Syndrome[Title/Abstract]) OR (Acquired Immune Deficiency Syndrome*[Title/Abstract]) OR (AIDS[Title/Abstract]) OR (AIDS*[Title/Abstract]))*
*AND*
*((Vaccin*[Title/Abstract]) OR (Vaccin*[MeSH Terms]) OR (Laten*[Title/Abstract]) OR (Laten*[MeSH Terms]) OR (Rebound[Title/Abstract]) OR (Reservoir[Title/Abstract]) OR (Reservoir*[Title/Abstract]) OR (Immune modulat*[Title/Abstract]) OR (Immune*

*toleran*[Title/Abstract]) OR (Immune toleran*[MeSH Terms]) OR (Viremia[Title/Abstract]) OR (Viremia[MeSH Terms]) OR (Viral Load[Title/Abstract]) OR (Viral Load[MeSH Terms]))*

## Study Selection

Of 453 search results on August 2nd, 2021, titles and abstracts were screened and included based on population, intervention, outcomes, and disease. Articles in languages other than English, and articles of which we could not access the full-text versions were excluded from this study. A flowchart of the search, inclusion, and exclusion process is displayed in Figure 1[21]. Study selection is performed by one researcher, SV, along with frequent discussions with supervisor KV.

## Data Extraction

From each included study at least the following was extracted: objectives, sample sizes (different intervention groups, control groups), study design, (mean) time of ART initiation, intervention characteristics (duration, dosage), outcome measures, and length of follow-up. In studies conducted in monkeys, we also extracted data on the SIV strain used for infection, and presence of alleles that enhance control of SIV infection.

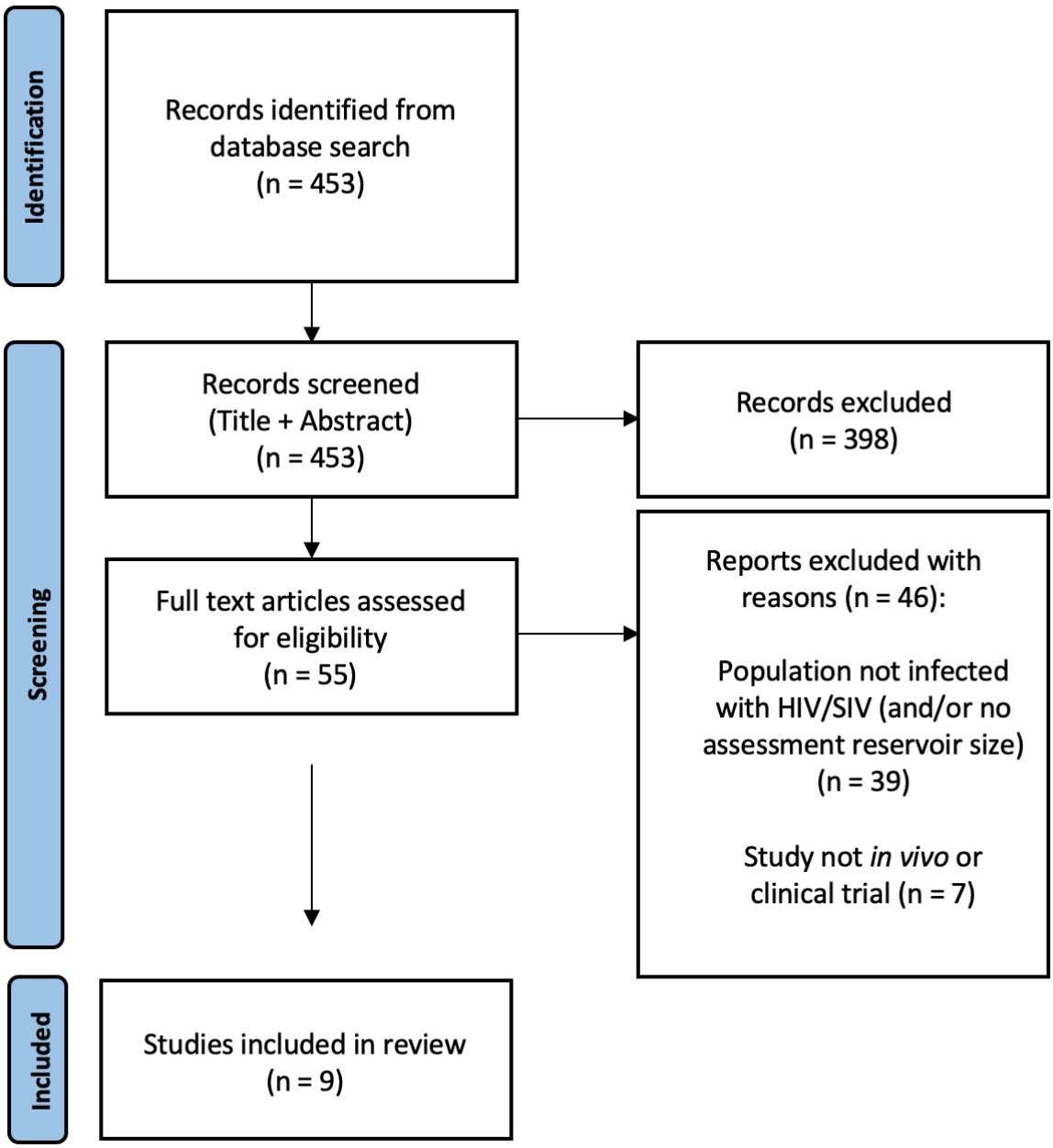


*Figure 1. PRISMA flow diagram for database search, screening, study inclusion and exclusion.*

## Results

Of all included studies (n = 9), two were carried out on humans[22, 23]. The remaining seven all explored TLR7 agonist treatment either alone or combined with other compounds on rhesus macaques[24-30]. Characteristics of included studies are summarized in Table 1. The total sample size of humans undergoing monotherapy with vesatolimod (Gilead Sciences, Foster City, CA) is 53, for rhesus macaques this is 26, and 16 for vesatolimod-analog GS-986 (Gilead Sciences, Foster City, CA). 41 animals received combination therapy, of which 16 received vesatolimod together with antibodies, 8 were treated with GS-986 plus antibodies, and a total of 17 macaques underwent treatment with GS-986 together with prime-boost immunization. For humans, 20 participants were randomized to a placebo group, whereas this number was 51 for rhesus macaques. Mainly males were included in human studies with 64 out of 73 (88%) participants being male, and only three animal studies mentioning inclusion of female monkeys[27, 29, 30], the rest all including exclusively males. Regarding other characteristics, such as dosage and intervals of treatments, SIV strains used for infection, and time between infection and start of ART, there is heterogeneity between all studies.

*Table 1. Characteristics and most important results of included studies.*

| Author, year | Population studied | Intervention(s), controls, group size | Effect on viral reservoir | Effect on time to rebound |
|---|---|---|---|---|
| **Lim *et al.*, (2018)[28] arm 1** | 10 SIVmac251-infected male rhesus macaques on ART | GS-986 (n = 4)<br>Vehicle control (n = 6) | Yes | No |
| **Lim *et al.*, (2018)[28] arm 2** | 11 SIVmac251-infected male rhesus macaques on ART | GS-986 (n = 3)<br>0.05 mg/kg vesatolimod (n = 3)<br>0.15 mg/kg vesatolimod (n = 3)<br>Vehicle control (n = 2) | Yes | Yes |
| **Del Prete *et al.*, (2019)[26]** | 6 SIVmac239X-infected male rhesus macaques on ART | Vesatolimod (n = 4)<br>Vehicle control (n = 2) | No | No |
| **Riddler *et al.*, (2020)[23]** | 48 HIV-infected humans on ART | 1 mg vesatolimod (n = 6)<br>2 mg vesatolimod (n = 6)<br>4 mg vesatolimod (n = 6)<br>6 mg vesatolimod (n = 6)<br>8 mg vesatolimod (n = 6)<br>10 mg → 12 mg vesatolimod (n = 6)<br>Placebo control (n = 12) | No | Not assessed |
| **SenGupta *et al.*, (2021)[22]** | 25 HIV-infected humans (controllers) on ART | 4 mg vesatolimod (n = 2)<br>4 mg → 6 mg vesatolimod (n = 4)<br>6 mg vesatolimod (n = 5)<br>6 mg → 8 mg vesatolimod (n = 3)<br>8 mg vesatolimod (n = 3)<br>Placebo control (n = 8) | No | Yes |
| **Bekerman *et al.*, (2019)[25]** | 20 SIVmac251-infected male rhesus macaques on ART | Vesatolimod (n = 5)<br>Anti-PD-1 Abs (n = 5)<br>Vesatolimod + anti-PD-1 Abs (n = 5)<br>Vehicle control (n = 5) | No | No |
| **Borducchi *et al.*, (2018)[27]** | 44 SHIV-SF162P3[21]-infected rhesus macaques on ART | Vesatolimod (n = 11)<br>PGT121 (n = 11)<br>Vesatolimod + PGT121 (n = 11)<br>Vehicle control (n = 11) | Yes | Yes |
| **Hsu *et al.*, (2021)[24]** | 16 SHIV-1157ipd3N4-infected male rhesus macaques on ART | GS-986 + PGT121 + N6-LS (n = 8)<br>Vehicle control (n = 8) | No | Not significant (trend observed) |
| **Borducchi *et al.*, (2016)[29]** | 36 SIVmac251- infected rhesus macaques on ART | GS-986 (n = 9)<br>Ad26/MVA (n = 9)<br>GS-986 + AD26/MVA (n = 9)<br>Vehicle control (n = 9) | Yes | Yes |
| **Bricker *et al.*, (2020)[30]** | 16 SIVmac251-infected infant rhesus macaques on ART | GS-986 + AD48/MVA (n = 8)<br>Vehicle control (n = 8) | No | No |

## TLR7 Agonist Monotherapy in Rhesus Macaques Yields Variable Results

To date, two *in vivo* studies in Indian rhesus macaques have been carried out to investigate the effects of vesatolimod in latent SIV infection. Lim *et al.*[28] conducted a dual study, assessing the therapeutic potential of TLR7 agonists vesatolimod and its analog GS-986. They infected 21 rhesus macaques, all negative for enhanced control MHC alleles, intrarectally with SIVmac251, starting ART at day 65 post infection. For the first arm of the study, four animals were treated with seven doses of GS-986, of which once 0.1 mg/kg, once 0.2 mg/kg, and five times 0.3 mg/kg, every other week. Six vehicle control animals were used, and ART was discontinued two weeks after the last dose or vehicle was received. They report no difference in time to rebound between groups, but do mention an average reservoir size reduction of 75% in GS-986 treated animals. Also, during treatment, there was transient plasma viremia and immune activation in animals in the intervention group.

The second arm of the study used three intervention groups all with three animals, and one control group with 2 macaques, to compare GS-986 to vesatolimod. All intervention groups received ten doses of either 0.1 mg/kg GS-986, 0.05 mg/kg vesatolimod, or 0.15 mg/kg vesatolimod, every other week. Additionally, after a three-month rest period, all groups but the 0.15 mg/kg vesatolimod group received nine additional doses. Again, a reduction in the viral reservoir was reported, together with transient plasma viremia, although this occurred mainly in the first phase of treatment and was highly variable. Regarding immune modulation, immune cell activation was highest in the GS-986 group, and the lowest dosage vesatolimod group showed only minimal cytokine expression after treatment. The most striking observation however in this study arm is in terms of time to rebound. Even though most animals rebounded within seven to ten days after ART cessation, two monkeys, one of the GS-986 and one of the 0.15 mg/kg vesatolimod group, did not rebound for all 700 days of follow up. After performing multiple experiments to determine the cause of this observation, the authors conclude that it is likely full clearance of replication-competent virus that occurred in the two animals[28].

Del Prete *et al*.[26] evaluated vesatolimod treatment in six rhesus macaques. They infected them intravenously with SIVmac239X and started ART on day 13 post infection. Two monkeys possessed a single MHC allele that is associated of enhanced control of HIV infection. After 75 weeks of ART, four animals orally received vesatolimod treatment, whereas two monkeys served as controls, receiving a vehicle control. vesatolimod treatment consisted of two courses, the first course consisting of two doses of 0.15 mg/kg, followed by 10 times 0.5 mg/kg, all given every two weeks. A three-week gap occurred between doses nine and ten. The second course started after a 40-41-week rest period and comprised five 0.15 mg/kg doses, of which three during ART, and the last two after ART cessation. No reduction in the viral reservoir has been observed, and all animals rebounded within four weeks of ART discontinuation, showing no improved virologic control for the duration of the 24 weeks of follow up. In addition, no increases in plasma viremia occurred during treatment, indicating absence of viral reactivation. However, positive results were yielded regarding immune modulation, with collection of peripheral blood mononuclear cells (PBMCs) showing transient upregulation of interferon-stimulated genes in the treatment group, as well as increased plasma cytokine levels. Furthermore, transient responses of multiple cell populations to vesatolimod treatment were observed, and after stopping ART, interferon (IFN) gamma, Tumor Necrosis Factor alpha (TNF-$\alpha$), and $CD8^+$ T cell responses were increased. Thus, while

vesatolimod treatment had no effect on the size of the viral reservoir, time to rebound, and viral activation, it did modulate immunity, even after ART withdrawal[26].

## Monotherapy with Vesatolimod may Promote Enhanced Control of HIV Infection in Humans

After many promising *in vitro* results on using vesatolimod to combat latent HIV infection, but inconsistent evidence in rhesus macaques, the compound has been tested in humans as well. Two clinical trials so far have reported their findings on vesatolimod as therapeutic in people living with HIV. The first is carried out by Riddler *et al.*[23], who conducted a randomized, double-blind, multicenter, placebo-controlled, dose-escalation, phase Ib clinical trial to assess the safety, virologic effects, pharmacokinetics, and immune modulatory effects of vesatolimod in people living with HIV. In six dose groups with each six participants, people received 1, 2, or 4 mg of vesatolimod every two weeks for 6 doses, 10 doses every two weeks of 6 or 8 mg, or a regime of 3 times 10 mg every two weeks followed by 7 doses of 12 mg. The placebo group consisted of 12 participants, and all participants were on ART, yet slightly fluctuating in medium time between ART initiation and HIV diagnosis. In this trial, time to rebound after ART withdrawal has not been assessed, and reservoir size did not change significantly between groups or when comparing intervention groups to the placebo group. In the same way, Riddler *et al.* state that vesatolimod had minimal effect on plasma viremia, indicating that viral reactivation as a result of vesatolimod administration did not occur. Immune activation, however, did transpire especially in intervention groups of dosages of 6 mg and higher. Here, induction of serum cytokines, and transient activation of cell populations is detected. Finally, vesatolimod was overall well tolerated, but no significant therapeutic effects in HIV patients were observed[23].

Another randomized, double-blind, placebo-controlled, phase Ib clinical trial was performed by SenGupta *et al.*[22], in which 25 people living with HIV were enrolled to assess vesatolimod. The participants were on ART at the start of the trial, however before ART, they were considered HIV controllers: people with maintained low plasma viremia even in the absence of ART. The placebo group consisted of eight participants, and five other groups acted as intervention groups: every two weeks, 2 participants received 4 mg vesatolimod, 4 received 4 mg escalated to 6 mg, 5 received 6 mg, 3 received 6 mg escalated to 8 mg, and 3 received 8 mg. 28 days after the last dose, ART was withdrawn, and a significant increase in time to rebound was observed in vesatolimod groups compared to people receiving a placebo. In regard to the size of the viral reservoir, no significant differences were observed between groups. Likewise, viral reactivation did not occur aside from in one person of the 4 mg group. Immune activation took place in all vesatolimod groups, with more activation the higher the dosage, but no differences in HIV-specific T cell responses between the intervention and control groups, indicating no effect of vesatolimod on HIV-specific immunity[22].

## Immune Checkpoint Blockade Does Not Increase Effectivity of Vesatolimod Treatment in SIV-Infected Rhesus Macaques

Programmed Death 1 (PD-1) is a receptor that when bound by its ligands inhibits T cell activation and proliferation. In HIV infection, this might be detrimental in building anti-HIV immunity to eliminate HIV-infected cells. Blocking PD-1, together with administration of vesatolimod, can therefore have beneficial effects in eradicating latent HIV. This has been investigated by Bekerman *et al.*[25], who infected 20 rhesus macaques (negative for protective

MHC alleles) intrarectally with SIVmac251, and initiated ART 70 days afterwards. After 26 months of ART, animals were equally distributed into four groups, every other week receiving either a placebo, ten doses of 0.15 mg/kg vesatolimod, four doses of 10 mg/kg anti-PD-1 (Thermo Fisher Scientific) (started after three times vesatolimod), or a combination of both treatments. Cessation of ART one month after the last vesatolimod dose caused all animals to rebound within two weeks in all groups. Besides, no reduction in size of the viral reservoir was detected. Immune activation during treatment did occur, though only in the vesatolimod and combination groups. Measurements on viral reactivation or plasma viremia after treatment are not discussed[25].

## Broadly Neutralizing Antibodies Combined with TLR7 Agonists Reduce the Viral Reservoir and Delay Rebound in Rhesus Macaques

Since bnAbs have shown therapeutic efficacy in HIV-infected people[31, 32], they might assist in immune-mediated elimination of HIV during TLR7 agonist treatment. Borducchi *et al*.[27] studied whether bnAb PGT121 (Catalent Biopharma, Madison, WI) in combination with vesatolimod is able to target and reduce the viral reservoir in rhesus macaques. 44 animals negative for alleles correlating with enhanced viral protection were intrarectally infected with SHIV-SF162P3[21] and received ART seven days after being infected. Four groups were created, all with 11 animals per group receiving treatment or placebo every two weeks: one placebo group, one group receiving 10 doses vesatolimod only at 0.15 mg/kg, one receiving 5 doses PGT121 intravenously at 10 mg/kg, and one group receiving a combination of both. Withdrawing ART 16 weeks after the last intervention dose caused all control group animals to rebound, 10 of 11 animals in the vesatolimod group, 9 of 11 animals in the PGT121, and only 6 of 11 monkeys in the combination group rebounded. Moreover, the combination group showed the highest increase in time to rebound compared with the other groups, and significant reductions in viral DNA, suggesting a reduction in the viral reservoir. During treatment, however, no viral reactivation was observed[27].

Hsu *et al*.[24] infected 16 rhesus macaques negative for protective MHC alleles intrarectally with SHIV-1157ipd3N4 and put on ART 14 days post infection. From week 14, 8 monkeys were put in the control group to receive intravenous saline as a control, and 8 animals were assigned to the active arm. This arm consisted of seven to ten oral doses of 0.1 mg/kg GS-986, and two to five doses intravenously of bnAbs PGT121 (10 mg/kg) and N6-LS (Vaccine Research Center, NIAID, NIH) (30 mg/kg), every other week, and depending on development of anti-drug antibodies. ART discontinuation four weeks after plasma bnAb levels were under a set threshold, and at week 40 in the control group, caused viral rebound in all animals, with some delay in the intervention group compared to the control arm. No reduction in the viral reservoir occurred, and no viral reactivation was detected[24].

## Therapeutic Vaccination Together with TLR7 Stimulation Generates Conflicting Results in SIV-Infected Rhesus Macaques

Another manner of boosting immunity to promote elimination of latent HIV, is by vaccination. The effect of combining TLR7 agonists for latency reversal with therapeutic vaccination for an anti-HIV response *in vivo* in macaques has first been investigated by Borducchi *et al*.[29], by using an Ad26/MVA together with GS-986. In this study, 36 rhesus macaques, negative for protective MHC alleles and intrarectally infected with SIVmac251, were put on ART seven days post infection, for 24 weeks. Afterwards, they were assigned to four groups containing

nine monkeys per group. One group served as placebo control, one group received only intramuscular vaccination twice with $3x10^{10}$ viral particles Ad26 vectors expressing SIVsmE543 Gag/Pol/Env, and were twice boosted with $10^8$ plaque-forming units MVA vectors expressing SIVsmE543 Gag/Pol/Env, all in a 12-week interval. A third group received 10 oral doses of 0.3 mg/kg GS-986 every other week, and a final group received a combination of aforementioned groups. ART withdrawal caused animals in all groups to experience viral rebound, although a slight delay occurred in the vaccination only group. Above all, the combination group significantly delayed viral rebound after ART cessation. Furhermore, reductions in the viral reservoir were detected in both groups receiving the therapeutic vaccine, as well as enhanced HIV-specific cellular immunity. Viral reactivation during all treatments was not observed[29].

Prime-boost immunization and TLR agonist treatment in SIV-infected infant macaques has been explored by Bricker *et al*.[30]. They infected 16 infant rhesus macaques, negative for protective MHC alleles, orally with SIVmac251, and started ART four weeks after infection. At week 22 and 30 post infection, 8 animals were immunized intramuscularly with Ad48 vectors expressing $3x10^{10}$ viral particles of SIVsmE543 Gag/Pol/Env. Boost immunization occurred at week 38 and 50 post infection by intramuscular injection of MVA vectors expressing $10^8$ plaque forming units of SIVsmE543 Gag/Pol/Env. In the meantime, 0.3 mg/kg GS-986 was administered orogastrically every other week from week 40 to week 60. After receiving all treatments, discontinuation of ART led to rebound in all animals within 28 days, with no observed differences in time to rebound between groups. Also, no reduction in viral reservoir size has been detected, as well as no viral reactivation during treatment. Immune modulation, however, did occur, with an increase of HIV-specific cellular immunity, and transient binding antibodies in the intervention group[30].

## Discussion

To date, ample research has been conducted on TLR7, TLR7/8, and TLR8 agonists as treatment for chronic HIV. After discovering that triggering TLRs in latent HIV infection causes viral reactivation[33], it is suggested that TLR agonists affect the viral reservoir by triggering this reactivation. In addition, they enhance anti-HIV immunity, thereby decreasing or depleting the viral reservoir[33]. This allows for either functional cure, or complete eradication of HIV from infected individuals.

*In vivo*, however, variable results are yielded in regards to therapeutic efficacy of TLR7 agonists in HIV-infected individuals. Whereas Lim *et al.*[28] detected transient viremia during treatment, and an effect on the viral reservoir in both of their studies, in only one study there was an increased time to rebound after TLR7 agonist administration. These results, however, could not be reproduced by Del Prete *et al.*[26], who detected neither viral reactivation during treatment, nor a decrease in the viral reservoir and an increase in time to rebound after ART cessation. These differences might be caused by discrepancies between the studies. That is, Lim *et al*. infected their animals with SIVmac251, while Del Prete *et al.* used SIVmac239X. In addition, Del Prete *et al.* initiated ART much faster than Lim *et al.*, with 13 days post infection *versus* 65 days post infection, respectively[26, 28]. This might have impacted formation and size of the viral reservoir in a way that the earlier one begins with ART, the smaller the viral reservoir. Targeting this smaller reservoir and moreover affecting it significantly might pose a greater challenge than in the case of a large reservoir.

In the clinical trials included in this review, vesatolimod was assessed in people living with HIV. As in the aforementioned animal studies, both trials did not observe viral reactivation during treatment, yielding little evidence for the latency-reversing capacities of vesatolimod[22, 23]. Both studies also reported no effects on the viral reservoir, yet SenGupta *et al.*[22] did see longer time to rebound in intervention groups. Important to note is that this trial was conducted in HIV controllers, which potentially either enhance or diminish therapeutic efficacy of vesatolimod[22, 23]. Additionally, one has to consider the clinical relevance of these results: an increased time to rebound is certainly beneficial and inviting to improve even more, but not enough to completely abrogate ART. For this to be possible, likely at least a large reduction of the viral reservoir is needed, something which in humans has not yet been achieved.

All in all, whether it is vesatolimod or GS-986, and in SIV-infected rhesus macaques or HIV-infected humans, few consistent results have been yielded that vow for TLR7 agonist monotherapy as HIV-curing treatment. Therefore, multiple studies have combined administration of TLR7 agonists with other compounds, addressing potential mechanisms that hamper TLR7 agonists in having therapeutic efficacy[24, 25, 27, 29, 30].

One of those can be immune checkpoints, causing HIV-infected cells to protect themselves from elimination by expressing inhibitory immune receptors. PD-1, for instance, is crucial in preventing T cell activation, and Bekerman *et al.*[25] speculated that blocking PD-1 with antibodies may increasingly enable vesatolimod to exert favorable effects in SIV-infected macaques. However, addition of PD-1 blocking antibodies during vesatolimod treatment did not enhance vesatolimod's therapeutic efficacy[25]. This might be due to reduced PD-1

expression following ART[34, 35], therefore blocking it might have limited benefits when used for improving the response to vesatolimod treatment.

Another type of combination therapy studied is addition bnAbs to GS-986 treatment. Aside from already having shown therapeutic efficacy in HIV-infected individuals, bnAbs may prevent *de novo* infection after latency reversal, thereby halting expansion of the reservoir since the goal is eliminating it. However, inconsistent results dominate also this line of research on combination therapy with TLR7 agonists in treating HIV[24, 27]. That is, Borducchi *et al.*[27] yield promising results when adding bnAb PGT121 to vesatolimod treatment. Aside from observing immune activation and reductions in the viral reservoir, just below half of the animals receiving vesatolimod + PGT121 did not rebound at all during the follow-up of 196 days[27]. Yet when Hsu *et al.*[24] explored similar combination therapy in macaques, they detected no reduction in the viral reservoir. Moreover, all animals rebounded, with only a non-significant trend towards increased time to rebound in the treatment group[24]. A reason for these differences can be the virus with which the animals were infected (SHIV-SF162P321 *versus* SHIV-1157ipd3N4). Also, whereas Borducchi *et al.* do not report development of anti-drug antibodies, Hsu *et al*. observe this as such that only one of eight monkeys in the intervention group completed the entire treatment[24, 27]. All in all, consistent therapeutic efficacy of adding bnAbs to TLR7 agonist treatment has yet to be reported.

The final two studies included in this review assessed the effects of prime-boost immunization together with TLR7 stimulation[29, 30]. An important difference, however, is that Borducchi *et al.*[29] investigated this in adult rhesus macaques, whereas Bricker *et al.*[30] researched infant macaques. Furthermore, there is a notable difference in time between infection and ART initiation, with Bricker *et al.* taking four times as much time to ART initiation than Borducchi *et al*., potentially causing a greater size of the viral reservoir before start of treatment[29, 30]. The vaccine regimen, however, aside from using different Ad vectors, is very similar. Nevertheless, uniform results lack also in these studies, with Borducchi *et al*. reporting of both a reduction in the viral reservoir and an increase in time to rebound in intervention groups, but Bricker *et al.* detecting none of this[29, 30].

In general, between all included studies, there is extensive heterogeneity in methods as well as results. This is the case especially between studies that investigate combination therapy, while for these studies to proceed to clinical trials, consistent evidence is very much needed. In addition, whereas vesatolimod is reported to be generally well tolerated[22, 23], it is necessary to consider adverse effects of combining this treatment with other treatments, potentially causing adverse effects by themselves already.

Another eye-catching characteristic among many of the studies, is the inclusion of mainly males. This might be relevant, since sex is known to play roles in infection progression, and immunity. For instance, one study reports that TLR7 stimulation in HIV-infected women induces significantly more IFN-$\alpha$ production than in men, resulting in increased immune activation[36]. This suggests that TLR7 agonists administered to women can lead to more promising results, as seen in both studies of Borducchi *et al.*[27, 29], who included female animals as opposed to studies investigating similar combinations of compounds. On the other hand, regarding TLR7/8 stimulation with R-848 (InvivoGen, San Diego, CA), men are reported to have increased responsiveness, with higher cytokine production than women[37]. Moreover, a

small nucleotide polymorphism (SNP) in TLR7, carried by 30-50% of European women, causes impaired IFN production after stimulation[38]. In addition, certain alleles can lower TLR7 expression in PBMCs of women entirely[38], potentially greatly affecting TLR7-treatment for HIV-infected carriers. This also goes for a SNP in TLR8, which is reported to impair the inflammatory response after activation, even though it did lead to increased production of TNF-$\alpha$, associated with reactivation of HIV[39]. Thus, aside from heterogeneity in the investigated studies, heterogeneity in the population as well can be of great influence on responsiveness to TLR stimulation as HIV treatment.

Maybe most interesting, though, is that not a single study aside from Lim *et al.*[28] reports observing viral reactivation, or in other words, reversal of latency. That raises questions on how true the "shock" part of "shock and kill" is, and whether beneficial effects are maybe simply because of immune modulation, causing an enhanced and improved response against HIV. Moreover, it might be this enhanced immunity that is in itself able to recognize latently infected cells, and clear them, or clear HIV effectively after ART cessation. Especially when used together with prime-boost vaccination, TLR7 agonists might as well serve as adjuvants rather than latency reversing agents[29]. Indeed, adjuvant activity for TLR7 and TLR8 agonists has been extensively studied, and desirable results have been yielded *in vivo*[40-44]. In general, TLR7 and TLR8 agonists induce a broad anti-HIV antibody response, together with T cell responses. Thus, instead of viewing TLR agonists as latency reversing agents, their use might be much greater as vaccine adjuvants for immune modulation.

Even if one continues in the direction of latency reversal, there is evidence that not only T cells carry latent virus. Indeed, HIV can latently reside in cells of monocytic origin as well[45]. Therefore, besides T cell stimulation, stimulation of these cells is needed to potentially completely eliminate the viral reservoir. This is explored using agonists for TLR7/8 and TLR8, such as R-848, 3M-001 (3M Pharmaceuticals, St. Paul, MN), and 3M-002 (3M Pharmaceuticals, St. Paul, MN)[46-48]. Studies on the effects of those compounds in HIV-infections leave good impressions, although much more research is needed to validate the therapeutic effects and safety before proceeding to *in vivo* studies.

In conclusion, TLR7, TLR8, and TLR7/8 agonists present promising approaches in targeting the viral reservoir in HIV infected individuals. However, there are many aspects still left to investigate and optimize, and whether TLR agonists alone will lead to HIV cure seems unlikely. At least a combination of TLR7 and TLR8 agonists will be needed to eliminate the latent reservoir not only from T cells, but also from cells of monocytic origin. Additionally, for prevention of *de novo* infection after viral reactivation, bnAbs, or prime-boost immunization before TLR stimulation seems a favorable strategy. Combining all these methods in humans raises questions about feasibility, and arising of adverse effects. Thus, great progress has been made in achieving HIV cure, and TLR7, TLR8, and/or TLR7/8 agonists show great potential to play a role in this. How they will, and to learn what more is needed to really battle HIV, however, still requires much more research.

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