IMMUNOTHERAPIES TOWARDS HIV CURE

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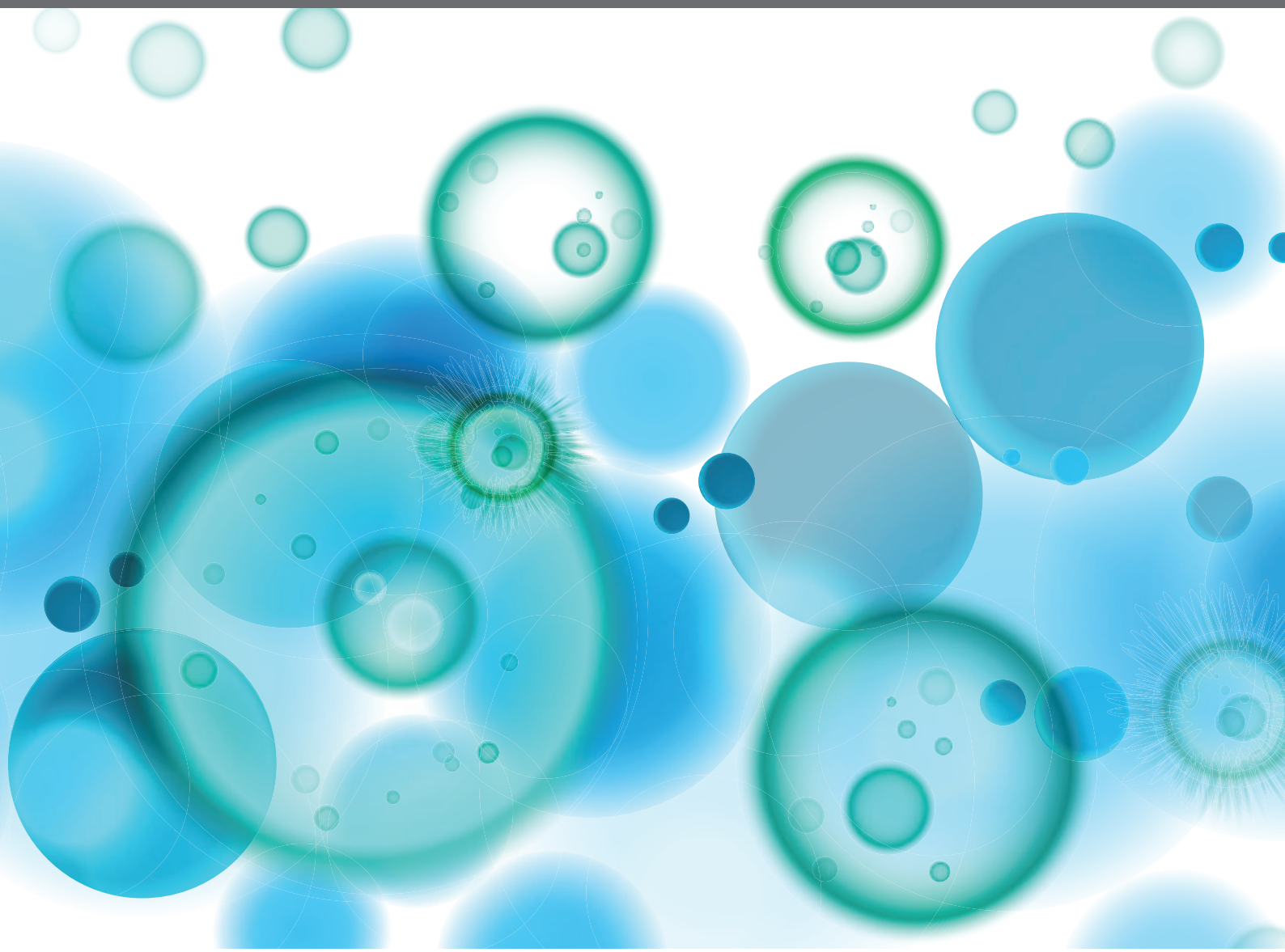

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IMMUNOTHERAPIES TOWARDS HIV CURE

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EDITORIAL
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Editorial: Immunotherapies Towards HIV Cure

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Editorial on the Research Topic

Immunotherapies Towards HIV Cure

Despite the clinical success of antiretroviral therapy (ART), there is still no cure for HIV infection. If treatment is interrupted, viremia almost inevitably rebounds, and without renewed therapy, it can lead to opportunistic infections, AIDS and death. Thus, research towards an ART free remission of HIV infection, or cure, is a high priority.

The main obstacle to achieve a cure for HIV infection rests in the early establishment of a pool of long-lived cells harboring an intact HIV genome that has entered latency. Significant advancements in the understanding of persistent infection have emerged over the last years, shedding light into possible mechanisms to tackle it. Among these mechanisms, strategies to enhance the immune system to allow the clearance of latently HIV infected cells is of special interest. Some of the ultimate immunotherapy approaches that have obtained relevant results are published in this special number.

One of the first attempts to eliminate the HIV reservoir consisted on the reactivation of the latently infected cells using epigenetic modulators (Spivak and Planelles, 2016). Although this strategy proved effective in awakening the dormant cells, it also resulted unsuccessful in reducing the size of the reservoir. Thus, it was obvious that additional strategies to clear the reactivated reservoir needed to be implemented, as well as finding more potent latency reversal agents (LRAs). One ideal solution to the problem would be the development of a drug that simultaneously reactivate the latent reservoir and stimulate the immune system. Toll-like receptor (TLR) agonists could fit this description, as reviewed by Martinsen et al. Another option would be a combined regimen including an LRA with a immune modulator, like Mothe et al. and Rosas-Umbert et al. evaluated in a clinical trial, assessing the combination of the LRA Romidepsin with a therapeutic vaccine. They observed reactivation of the reservoir to some extent and enhanced activation of HIV specific T cells, which led to a marginal reduction in the size of the reservoir. However, in the mentioned studies, immune assessment was focused on CD8 T cells, while increasing body of evidence suggest that other immune subsets are likely to contribute to viral clearance in the context of HIV eradication, such as NK cells, as demonstrated by May et al. in their *in vitro* experiments and reviewed by Mann et al. in a broader innate immune system perspective. Another alternative to enhance immune clearance of HIV is to use adoptive cell immunotherapies. In particular, the use of antigen specific T cells for other viral infections post hematopoietic stem cell transplantation (Lee et al.). Chimeric antigen receptors (CARs) are another alternative being tested to directly kill infected cells. This approach was first explored in the oncology field and now is advancing into the

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HIV arena. Hajduczki et al. demonstrate that a trispecific CD4-based CAR expressing a carbohydrate recognition domain of human mannose-binding lectin (MBL) and a third targeting moiety against a conserved Env determinant, exhibited high anti-HIV potency as well as undetectable HIV entry receptor activity. Importantly, properly detect and monitor immune responses in these trials is essential to evaluate the duration of the adopted immune response. The use of a newly developed assay named ViraFEST by Chan et al. could provide a platform to evaluate memory CD8T cells responses. Excitingly, improvement of LRA activity and reservoir clearance could be facilitated by the field of immunoengineering, as reviewed by Bowen et al.

The potential of antibodies in HIV clearance strategies is under testing and some studies have shown promising results. A step further in this concept is the development of engineered molecules to mediate viral recognition for clearance. Dual-Affinity Re-Targeting (*DART*) molecules were initially designed with an arm to engage HIV infected cells and an arm to engage T cells from HIV infected adults (Sung et al., 2015). Pollara et al. evaluate these molecules with T cells from cord blood, to investigate potential pediatric use, and furthermore, a new version of these molecules targeting CD16 expressing effector cells are examined, proving to eliminate HIV-infected cells specially after IL-15 priming. However, delivery of antibodies and other immune products remains challenging, specially thinking about long-term supply. Martinez-Navio et al. showed how adeno-associated virus (AAV) can be used as a vector to deliver anti-SIV antibodies allowing IgG detection for over 6 years, showing promise not only for antibody delivery but for other immunomodulators. Moreover, the use of antibodies for cure approaches is not restricted to HIV antibodies. For example, α4β7 ntegrin promotes homing of activated T cells to intestinal sites where they become preferentially HIV infected. Their targeting using anti-α4β7 monoclonal antibody has been proposed as means to reduce viral infection and gut inflammation, as evaluated in Rhesus macaques by Pino et al.

It is crucial to define a reliable measure of the size of the reservoir to allow quantification of the clearance strategy used. Several measures have been studied over the years, all of them presenting with limitations, and thus further development is warranted. Stuelke et al. propose a novel assay in which is combined the principle of the quantitative viral outgrowth assay (QVOA) with ultrasensitive detection, allowing the assay to be performed with minimal sample and in a reduced time than the classic assay.

In summary, immunotherapy approaches represent an area of active research towards an ART free remission or cure of HIV. The next five years will be critical to define which immunotherapy approaches have a substantial effect in the latent reservoir. As we search for an HIV cure or a long-term remission using immunotherapy approaches, we will need to ensure that implementation of such strategies will be equitable among PLWH not only in developed countries but also in developing ones. Efforts need to be done to include diversity when clinically evaluating these approaches and to reduce the costs associated with some of these strategies so it does not halt implementation in low- and middle-income countries. Ending the HIV epidemic needs to be a global endeavor and including cure strategies on the tool box against this common enemy is a scientific priority.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Long-Term Delivery of an Anti-SIV Monoclonal Antibody With AAV

José M. Martinez-Navio[1†], Sebastian P. Fuchs[1†], Desiree E. Mendes[1], Eva G. Rakasz[2], Guangping Gao[3], Jeffrey D. Lifson[4] and Ronald C. Desrosiers[1]*

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Long-term delivery of anti-HIV monoclonal antibodies using adeno-associated virus (AAV) holds promise for the prevention and treatment of HIV infection. We previously reported that after receiving a single administration of AAV vector coding for anti-SIV antibody 5L7, monkey 84-05 achieved high levels of AAV-delivered 5L7 IgG1 *in vivo* which conferred sterile protection against six successive, escalating dose, intravenous challenges with highly infectious, highly pathogenic SIVmac239, including a final challenge with 10 animal infectious doses (1). Here we report that monkey 84-05 has successfully maintained 240–350 μg/ml of anti-SIV antibody 5L7 for over 6 years. Approximately 2% of the circulating IgG in this monkey is this one monoclonal antibody. This monkey generated little or no anti-drug antibodies (ADA) to the AAV-delivered antibody for the duration of the study. Due to the nature of the high-dose challenge used and in order to rule out a potential low-level infection not detected by regular viral loads, we have used ultrasensitive techniques to detect cell-associated viral DNA and RNA in PBMCs from this animal. In addition, we have tested serum from 84-05 by ELISA against overlapping peptides spanning the whole envelope sequence for SIVmac239 (PepScan) and against recombinant p27 and gp41 proteins. No reactivity has been detected in the ELISAs indicating the absence of naturally arising anti-SIV antibodies; moreover, the ultrasensitive cell-associated viral tests yielded no positive reaction. We conclude that macaque 84-05 was effectively protected and remained uninfected. Our data show that durable, continuous antibody expression can be achieved after one single administration of AAV and support the potential for lifelong protection against HIV from a single vector administration.

Keywords: gene therapy, AAV vector, long-term expression, broadly neutralizing antibodies, HIV/SIV cure, immunotherapy, prophylaxis, rhesus monkeys

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INTRODUCTION

Gene therapy has come of age. Almost 50 years after its inception, gene therapy is now considered a promising treatment option for several human diseases including cancer, genetic disorders and infectious disease (2). Gene therapies can work by several mechanisms: replacing defective genes with healthy ones, adding new genes to help the body fight or treat disease, or deactivating problematic genes (3). Importantly, for any of these gene therapy approaches, achieving long-term delivery of the transgene remains a key, infrequently realized goal. Recombinant adeno-associated

virus (AAV) vectors have been widely used for such gene delivery applications because of their safety and cost-efficiency: a single injection can result in long-term expression of the transgene product (4). Also, recombinant AAV is ideal as a delivery vehicle in some additional respects: the only protein expressed from it comes from the inserted transgene; it can effectively transduce terminally differentiated non-dividing cells; and it shows little or no integration into host genome sequences (5–10).

One potentially important application of the AAV system is the delivery of broadly neutralizing antibodies as a gene therapy approach against HIV (1, 11–14). For that purpose, recombinant AAVs encoding neutralizing antibodies are inoculated into the host and the antibody or antibodies of interest will then be directly expressed from the transduced cells. Thus, the immune system is bypassed in the sense that no immune response to an immunogen or vaccine is needed; the desired final products (broadly neutralizing antibodies) are delivered directly to the host. This approach against HIV has been made realistically possible in recent years thanks to the isolation and characterization of a remarkable collection of potent, broadly-neutralizing, monoclonal antibodies from select individuals (15–17). These antibodies have been extensively characterized in the laboratory and some have moved to clinical trials, where they have shown activity (18–22). They have the potential to prevent infection and also serve as a therapeutic approach complementing or even substituting antiretroviral drugs (11, 14, 18, 20–25). Importantly, the use of AAV voids the need for repeated administrations of purified antibody to maintain therapeutic levels in circulation. Due to its simplicity and ease of deployment, the approach is ideal for global use (26).

One main problem has been encountered in the applicability of this approach. Host antibodies generated against the delivered antibody (generally known as anti-drug antibodies or ADAs) can reduce its functionality and concentration thus drastically reducing its effectiveness (1, 11, 24, 27–32). The large repertoire of endogenously generated antibodies present in any individual has gone through a complex checks and balances system to be allowed into circulation (33). The antibodies being made in one individual are different from the antibodies being made in another individual (34). In addition, the potent broadly-neutralizing anti-HIV antibodies that one would want to use for these applications are typically highly evolved over a prolonged period of time. Due to years of affinity maturation, they exhibit unusually high levels of somatic hypermutation in the variable domains and many have accumulated unusual structural features (35, 36) which are generally required for potent neutralization and breadth (37). This high level of mutation likely enhances the immunogenicity of these antibodies when delivered to a host other than the one in which those particular sequences originated. Interestingly, when characterizing the humoral responses to the AAV-delivered antibodies we and others have found that the variable regions were predominantly or exclusively targeted (1, 11, 28, 30). We have also reported a highly significant correlation of the magnitude of the host anti-antibody response with the distance from germline of the AAV-delivered antibody: the more mutated, the more immunogenic (28). ADAs are in fact a common problem with many gene therapy approaches (38, 39). However, if the hurdle of the ADAs can be overcome, the promise of the AAV-delivery of antibodies against HIV could be realized (12). Here, we report that monkey 84-05 has maintained 240–350 μg/ml of anti-SIV antibody 5L7 for over 6 years in the absence of detectable ADAs. Our data show that durable, continuous antibody expression can be achieved after a single administration of AAV and support the potential for lifelong protection against HIV from a single vector administration.

MATERIALS AND METHODS

Macaque 84-05

The animal used in this study is a *Mamu B*08*-neg *B*17*-neg female Indian-origin rhesus macaque (*Macaca mulatta*), originally housed at the New England Primate Research Center in a biosafety level 3 animal containment facility in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. Research was conducted according to the principles described in the *Guide for the Care and Use of Laboratory Animals* and was approved by the Harvard Medical School Animal Care and Use Committee (40). Macaque 84-05 tested negative for the presence of antibodies to HIV and AAV1 capsid prior to AAV administration. At the time this manuscript was written, the monkey was 14 years old and weighted 6.4 kg. She was administered AAV encoding for 5L7 antibody when she was 7 years old and weighted 5.5 kg (1). At week 108 (about 2 years) post-AAV administration, the monkey was transferred to and subsequently housed at the Wisconsin National Primate Research Center and cared for in accordance with the guidelines of the Weatherall Report under a protocol approved by the University of Wisconsin Graduate School Animal Care and Use Committee.

Recombinant AAV

Coding sequences were designed *in silico*, codon-optimized and gene-synthesized (GenScript). 5L7 immunoadhesin sequences (11) served as a template and full-length antibodies were constructed by adding CH1 domain and CL domain of rhesus IgG to the already known immunoadhesin sequences. 5L7 sequences originate from recombinant anti-SIV Fab sequences (347-23h) derived from the bone marrow of SIV-infected rhesus monkeys (41). The rhesus IgG1 sequence used is based on accession no. AAF14058 and AAQ57555. Rhesus kappa light chain was designed using the constant light domain sequence from AAD02577. Synthesized fragments were then cloned into the NotI site of our AAV vector plasmids (1, 42). Production of recombinant AAVs was conducted as described previously (43). In short, HEK-293 cells were transfected with a select AAV vector plasmid and two helper plasmids to allow generation of infectious AAV particles. After harvesting transfected cells and cell culture supernatant, AAV was purified by three sequential CsCl centrifugation steps. Vector genome number was assessed by Real-Time PCR, and the purity of the preparation was verified by electron microscopy and silver-stained SDS-PAGE. The AAV genomes were encapsidated with the AAV1 serotype.

Monkey 84-05 received recombinant AAV1 encoding the anti-SIV antibody 5L7, using a total dose of 1.6×10^{13} particles (2.9×10^{12} AAV vector genomes per kilogram of body weight). AAV administration was conducted by a one-time inoculation of four deep intramuscular injections (two separate 0.5 ml injections into both quadriceps).

Recombinant 5L7 Antibody

5L7 recognizes gp120 and gp140 forms of the SIVmac239 envelope glycoprotein (28) and binds conformational epitopes involving the V3-V4 region (41). HEK-293T cells were expanded and then transfected with recombinant AAV vector plasmid coding for 5L7 antibody. Cells were washed after 4 h with pre-warmed PBS and then transferred to serum-free medium (Invitrogen). Afterwards, the antibody-containing medium was harvested, precleared by centrifugation, and filtered through a 0.22 μm-pore-size membrane. Then, IgG was affinity-purified using protein A Sepharose 4 Fast Flow (GE Healthcare), concentrated and desalted, followed by protein quantification with a Nanodrop UV spectrometer (Thermo Fisher Scientific). Antibody purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Coomassie blue staining (Thermo Fisher Scientific).

In vivo 5L7 Antibody Quantification and Anti-Drug Antibody (ADA) Responses

AAV-delivered 5L7 antibody was quantitated by standard ELISA using plate bound SIVmac239 gp140 (Immune Tech) to capture the antibody from pre-diluted serum samples and then HRP-conjugated secondary anti-rhesus IgG (Southern Biotech) as the detection method. Absorbance at 450 nm was compared to a serial dilution of purified 5L7 produced in HEK 293T cells, and the amount of antibody in serum was interpolated based on the standard curve. To measure host humoral responses to the AAV-delivered 5L7 antibody, purified recombinant 5L7 was used to coat plates. Then, serum samples from monkey 84-05 were tested at a 1:20 dilution and ADA responses were detected by means of an anti-lambda HRP-conjugated secondary antibody (Southern Biotech) in a regular ELISA (28). This secondary did not cross-react with 5L7 coated on the plates since 5L7 bears a kappa light chain. This allowed us to readily detect those anti-antibodies with a lambda light chain, which have been reported to reflect humoral responses in our previous studies (1, 28). Levels of AAV-delivered 5L7, and the corresponding ADAs, were measured for a total of 340 weeks.

PepScan

PepScan or ELISA against a panel of 218 peptides overlapping the entire SIVmac239 envelope protein was used to detect antibody responses to the viral spike. Fifteen-mer-length peptides, each successive peptide overlapped by 11 amino acids, were obtained from the NIH AIDS Research and Reference Reagent Program. Peptides were properly resuspended and used to coat ELISA plates at 40 μg/ml in phosphate-buffered saline (PBS). Plates were then washed, blocked and incubated for 1 h at 37°C with a 1:20 dilution of the corresponding monkey sera or 5L7 antibody diluted to 2 μg/ml. Binding antibodies were detected with an HRP-conjugated goat anti-human IgG antibody (SouthernBiotech) diluted 1:1,000 in 5% non-fat powdered milk in PBS and developed with soluble tetramethylbenzidine (TMB) reagent (Calbiochem, Gibbstown, NJ). The reaction was stopped by the addition of 50 μl of acidic stop solution (SouthernBiotech, Birmingham, AL), and the optical density at 450 nm was measured using a Wallac Victor plate reader (Perkin-Elmer, Waltham, MA).

Antibody Responses to p27 and gp41

Antibody responses against p27 and gp41 were quantitated by regular ELISA using SIVmac239 p27 purified recombinant protein (Catalog# 13446; obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) and recombinant SIV gp41 (Catalog# 5019; ImmunoDX) to coat plates at 10 μg/ml in PBS. Plates were then washed, blocked and incubated for 1 h at 37°C with a 1:20 dilution of the corresponding monkey sera or 5L7 antibody diluted to 2 μg/ml. Antibodies binding the p27 antigen or the gp41 were detected with a goat anti-monkey secondary antibody (Catalog# 2015-05, Santa Cruz) and developed with TMB High Sensitivity Substrate Solution (Catalog# 421501, Biolegend). The reaction was stopped by the addition of 50 μl of acidic stop solution (SouthernBiotech, Birmingham, AL), and the optical density at 450 nm was measured using a Wallac Victor plate reader (Perkin-Elmer, Waltham, MA).

Viral Load Monitoring

Plasma SIV RNA was measured using a gag-targeted quantitative real-time/digital PCR method with 6 replicate reactions analyzed per extracted sample for an assay threshold of 30 SIV RNA copies/ml (44). Cell-associated SIV RNA and DNA were measured by quantitative hybrid real-time/digital RT-PCR and PCR assays (45, 46).

RESULTS

Over 6 Years of Continuous Antibody Expression *in vivo*

Monkey 84-05 received a single administration of AAV on September 6th, 2012. As we reported at that time (1), starting at week 44 post-AAV inoculation and then every 3 weeks, this animal was repeatedly challenged with highly pathogenic, highly infectious SIVmac239 for a total of six intravenous administrations (see schematics in **Figure 1A**). The first four challenges were performed with 1 animal infectious dose (AID), followed by challenge with 2 AID and then a final 10 AID challenge. Viral load measurements in plasma from 84-05 were negative (<30 copies/ml), indicating the animal successfully resisted all six challenges (1). The antibody levels achieved remarkable values of ∼270 μg/ml (1). We now report long-term follow-up on levels of 5L7 antibody and the corresponding ADAs in circulation. As shown in **Figure 1B**, macaque 84-05 has successfully maintained 240–350 μg/ml of anti-SIV antibody 5L7 for over 340 weeks (>6 years). Moreover, this animal has

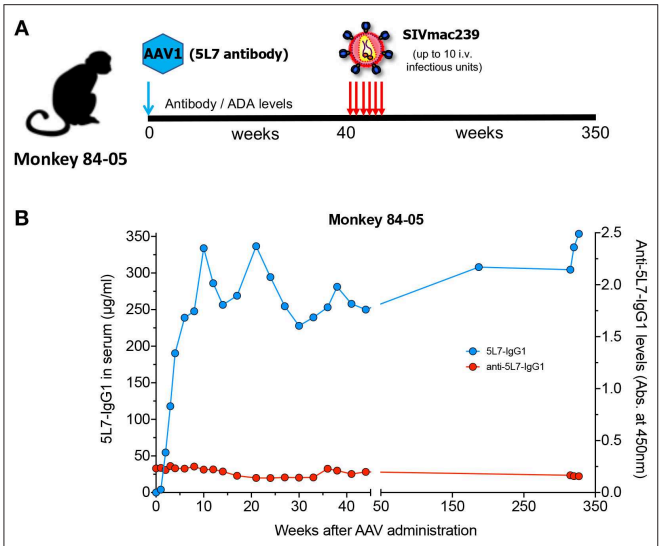

FIGURE 1 | 5L7 antibody levels and the corresponding anti-antibodies (ADAs) in serum following AAV administration. **(A)** Schematic of the experiment in which rhesus monkey 84-05 received intramuscular administration of AAV1 coding for antibody 5L7 and was subsequently challenged with SIVmac239 (six times: 4 times with a 1 animal infectious doses followed by a 2x dose challenge and a final 10x dose challenge). **(B)** 5L7 antibody levels (blue; plotted on left axis) and anti-5L7 antibody levels (red; plotted on right axis) in serum from monkey 84-05, quantified by ELISA.

shown little or no ADA responses to the AAV-delivered 5L7 antibody (**Figure 1B**).

Negative Envelope PepScan

Due to the nature of the high-dose challenge employed, and the high levels of anti-envelope reactivity found in serum of this animal, one could speculate about a potential low-level infection not detected by regular viral load measurements, which could lead to development of endogenous anti-Env responses, resulting in an overestimation of the concentration of 5L7 antibody in the anti-SIVmac239 gp140 ELISA employed (see Materials and Methods). In order to rule out this potential scenario, we tested serum from 84-05 by PepScan (ELISA against overlapping peptides spanning the whole envelope sequence for SIVmac239) (47, 48). One of the most sensitive measures of infection is seroconversion. Established immunodominant regions of the envelope, i.e., the variable loops V1–V2 and V3, the C terminus of gp120, some peptides in the ectodomain of gp41, and the highly immunogenic region (HIR) at the beginning of the cytoplasmic tail of gp41(47–49) are the regions frequently targeted by antibodies. As shown in **Figure 2A**, the serum from this animal did not detectably react to any of the peptides in the SIVmac239 envelope PepScan. Importantly, and as expected, recombinant purified antibody 5L7 (which is known to bind a conformational epitope involving the V3-V4 region of the envelope (11, 41) and should therefore not bind the linear epitopes present in the PepScan), did not detectably bind any of the tested peptides (**Figure 2B**). Conversely, when positive control serum from a SIV-infected macaque (r10028) was tested

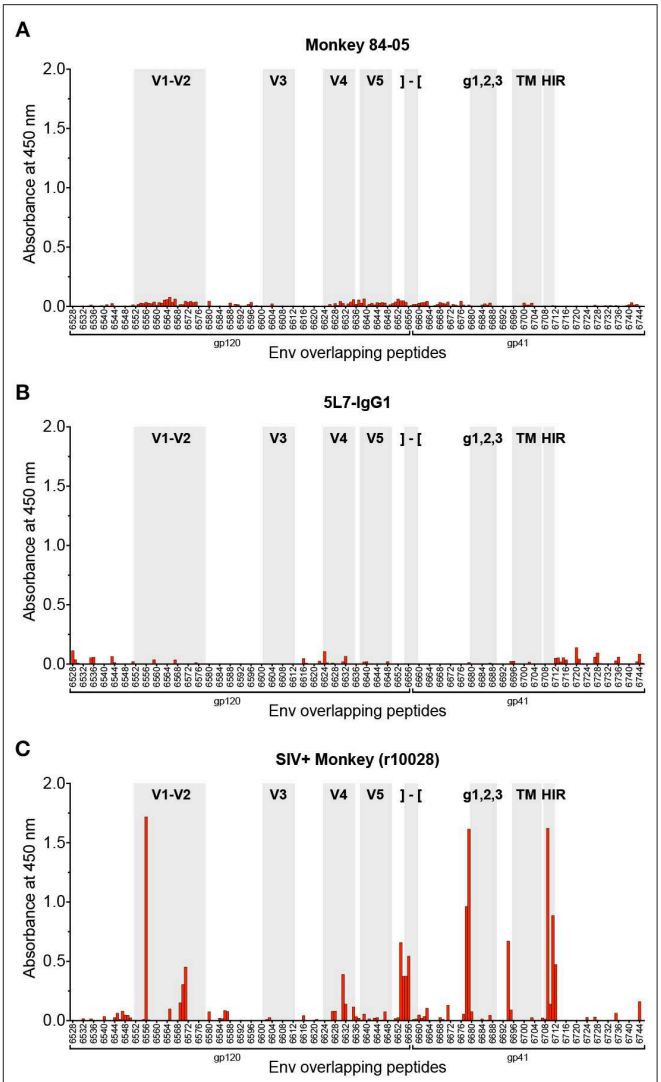

FIGURE 2 | Analysis of antibody responses by PepScan. PepScan (ELISA against overlapping peptides) spanning the whole envelope sequence for SIVmac239, performed with **(A)** serum from 84-05 (week 328 post-AAV) at a 1:20 dilution; **(B)** recombinant purified 5L7 antibody at a concentration of 2 µg/ml; and **(C)** serum from a rhesus monkey experimentally infected with SIVmac239 (week 12 post-SIV infection) at a 1:20 dilution. Note: regions of interest are shadowed in gray and indicated as follows: variable regions 1–2, 3, 4, and 5 are labeled V1–V2, V3, V4, and V5, respectively; the cleavage site and beginning of gp41 are represented by brackets; g123 indicates the location of the N-linked carbohydrate sites found in gp41; TM is the transmembrane domain of gp41; and HIR stands for highly immunogenic region. Data from SIV+ monkey in panel **(C)** is representative of many such SIV+ monkeys tested previously (47, 48).

on a PepScan in the same conditions, strong reactivity was shown to the immunodominant regions cited above (**Figure 2C**).

No Detectable Antibody Responses to p27 or gp41 by ELISA

With the high levels of 5L7 antibody consistently found in serum of 84-05, meaning high reactivity to gp120, assessing

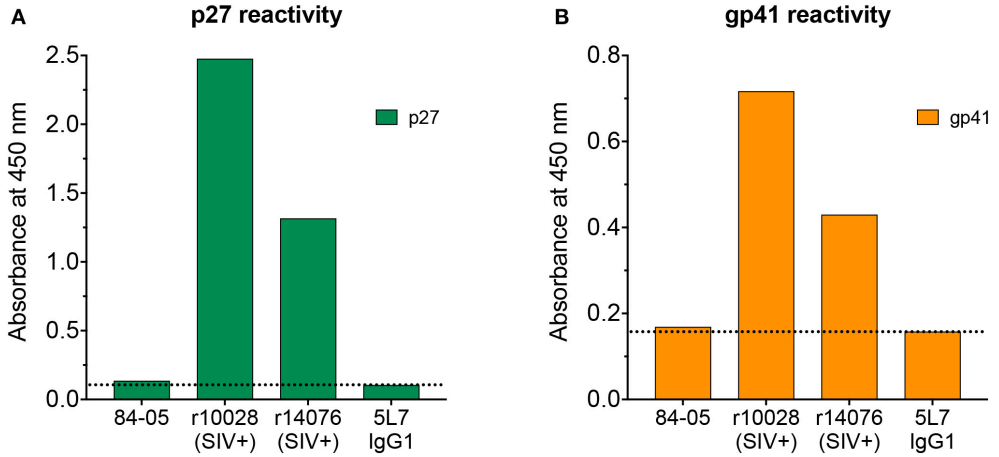

FIGURE 3 | Analysis of antibody responses to p27 and gp41. Serum from 84-05 (week 328 post-AAV) at a 1:20 dilution was tested by ELISA against recombinant purified proteins p27 **(A)** gp41 and **(B)** sera from two rhesus monkeys experimentally infected with SIVmac239 (week 12 post-SIV infection) at a 1:20 dilution were used as positive controls, and recombinant purified 5L7 as a negative.

a potential low-level infection can be complicated since that serum would definitely yield a positive result when tested against whole virus or recombinant gp120 by ELISA, the benchmark assays. An alternate method to test potential seroconversion is by measuring ELISA reactivity to p27 and gp41 recombinant proteins. Antibodies to both p27 and gp41 are readily detectable shortly after SIV infection and importantly, the 5L7 antibody present in the serum would not react to these proteins. Tests of serum from 84-05 (week 328 post-AAV) did not reveal any reactivity to p27 (**Figure 3A**), while positive control sera from two rhesus macaques experimentally infected with SIVmac239 for 12 weeks (r10028 and r14076) showed high reactivity under the same conditions. Similarly, serum from 84-05 did not detectably react against gp41, but sera from the two SIV-positive monkeys did (**Figure 3B**). Purified 5L7 antibody tested in parallel did not significantly bind to either p27 or gp41 (**Figure 3**).

Absence of Cell-Associated Viral DNA or RNA

To further rule out a potential low-level infection that could not be detected by the aforementioned ELISA-based tests, we used ultrasensitive techniques to detect cell-associated viral DNA and RNA in PBMCs from 84-05. We did not detect SIV gag RNA or DNA in the analysis of a combined total of 1.17×10^8 cell equivalents obtained over a 2-week period (week 340 and week 342; **Table 1**) with a nominal sensitivity of 1 copy per reaction.

TABLE 1 | Ultrasensitive detection of cell-associated viral DNA and RNA in PBMCs.

	Week 340[a]	Week 342[b]
Cell associated viral DNA	<1 DNA copies/10^6 cells	<1 DNA copies/10^6 cells
Cell associated viral RNA	<1 RNA copies/10^6 cells	<1 RNA copies/10^6 cells

[a] A total of 7.4×10^7 cells were analyzed for this time point and [b] a total of 4.3×10^7 cells were analyzed for this time point. Both with a nominal sensitivity of 1 copy per reaction.

DISCUSSION

Here we describe continuous, prolonged, high level delivery of an antibody to a rhesus monkey using AAV vector over 6 years of longitudinal measurements. Approximately 2% of the IgG in monkey 84-05 is derived from the vector that we inoculated more than 6 years previous. This result is consistent with the belief that muscle cells essentially do not turn over (50) and thus represent a potentially long term stable source of AAV-delivered antibody. It seems likely that monkey 84-05 will continue to produce similar levels of this antibody for the rest of its life. Importantly, this animal withstood six successive challenges with SIVmac239, including a final challenge with 10 animal infectious doses (1). By different means we show here that monkey 84-05 was effectively protected and remained uninfected: these include testing seroconversion in three different ways and the use of ultrasensitive techniques to detect cell-associated viral DNA and RNA. Due to the difficulties associated with proving curative and/or protective interventions (51), additional tests were considered such as *in vivo* CD8+ T-cell depletion and attempts at adoptive transfer of infection to naïve rhesus macaques. However, we did not want to put this precious monkey at any risk with the CD8 depletion; and, surprisingly, adoptive transfer may not be as sensitive as one would think (14).

The absence of an ADA response is almost certainly a key factor in the continuous production of the transgene product in monkey 84-05. Other examples of AAV delivery of protein to monkeys over a prolonged period have been documented in the reports of Rivera et al. (52) and Guilbaud et al. (53). Both of these reports used periodic induction of expression of an erythropoietin identical in sequence to the monkey's own erythropoietin over 5 or more years of study. Additional examples include the persistent expression of

dopamine-synthesizing enzymes in the putamen reported in one monkey for 15 years in a primate model of Parkinson's disease (54); the sustained expression of human α-L-iduronidase, an important enzyme required for the lysosomal degradation of glycosaminoglycans, reported for almost 4 years after intrathecal cervical AAV9 gene delivery in four one-month-old rhesus monkeys (55); the sustained expression of alpha-1 antitrypsin for over 5 years after one AAV vector administration in alpha-1 antitrypsin deficient patients (56); and the successful expression for 3.5 years obtained in two dogs of a dystrophin gene in a canine model for human Duchenne muscular dystrophy using AAV6 and a brief course of immunosuppressants (57), or in a similar study for over 2 years in two dogs using AAV8 in the absence of immunosuppression (58). Remarkably our animal 84-05 never received any immunosuppressant. The hemophilia gene therapy arena also has good examples of long-term delivery with AAV for up to a decade of Factor IX in hemophilic humans (59) and of Factor VIII in hemophilic dogs (60). For more examples on long-term delivery with AAV of hemophilia factors, please see the following references (61–68). The long-term delivery described in our report here is significant as the first such report for very long-term delivery of an antibody, particularly given the serious difficulties that have been encountered when AAV has been used to deliver antibodies that are significantly diverged from germ line or contain unusual features (1, 14, 28, 30, 31). The findings give hope that long-term delivery of therapeutic antibodies via AAV can be consistently achieved if the ADA problem can be overcome.

What may be responsible for the absence of ADAs in 84-05 and the continued high level of production of the transgene product over this prolonged period? Factors that have been shown to influence whether, or not, ADAs are observed following AAV-mediated expression of a transgene product include the following: the particular sequence of the transgene product (69); whether the recipient is already making a similar or identical protein (39); the serotype of AAV used (38); and targeted delivery or targeted expression at particular sites or in particular tissues or cells (54, 70–76). AAV-delivered 5L7 antibody certainly has the potential to be immunogenic in rhesus monkeys since 50% of monkeys receiving it have had robust ADA responses (1). Might there be particular genetic determinants that restrict an ADA response to the 5L7 antibody in some monkeys but not others? Might the antibody repertoire present in an individual at any one time influence to what extent the 5L7 antibody may be recognized as foreign? Might 5L7 just be on the cusp of self/non-self recognition? These questions remain unanswered at least for the time being.

Like the Miami monkey (14, 77, 78), monkey 84-05 stands out as a shining example of what is possible in the realm of AAV delivery of monoclonal antibodies in the fight against HIV. The Miami monkey has maintained high levels of two anti-HIV monoclonal antibodies and complete virologic suppression of SHIV infection for more than 4 years of follow-up without any repeated administrations and without any other antiviral therapies. It is likely that the ADA problem with AAV-delivered antibodies will need to be overcome for this approach to become a consistent reality in the context of human HIV infection. A recently-published human trial of AAV to deliver the human anti-HIV monoclonal antibody PG9 revealed readily detectable ADAs in 10 of 13 recipients in the four highest dose groups and zero measurable delivery of the PG9 antibody (31, 79). We feel that monkey modeling will need to play a key role in the development of successful strategies. Our group is currently focused on simple, easy-to-apply strategies for creating tolerance in monkeys to AAV-delivered monoclonal antibodies. If satisfactory delivery methods are found, it becomes possible to envision long-term control of viral replication in the absence of antiretroviral treatment by delivering a combination of antibodies in people, and long-lasting protection when this approach is used in a prophylactic setting. The long-term expression reported here highlights the potential of AAV-mediated antibody expression for impacting HIV-1 infections worldwide.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by The Harvard Medical School Animal Care and Use Committee and The University of Wisconsin Graduate School Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

The study was conceived and designed and the manuscript was composed by JM-N, SF, and RD. The experiments were performed by JM-N, SF, DM, ER, GG, and JL. Reagents that were used in the study were generated by JM-N, SF, DM, ER, GG, and JL. Data analysis was performed by JM-N, SF, DM, ER, GG, JL, and RD.

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BRIEF RESEARCH REPORT
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Combined Effects of HLA-B*57/5801 Elite Suppressor CD8+ T Cells and NK Cells on HIV-1 Replication

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Elite controllers or suppressors (ES) are HIV-1 infected individuals who maintain undetectable viral loads without anti-retroviral therapy. The HLA-B*57 allele is overrepresented in ES suggesting a role for HIV-specific CD8+ T cells in immune control. Natural killer (NK) cells also play a role in controlling viral replication, and genetic studies demonstrate that specific combinations of killer cell immunoglobulin-like receptor (KIR) alleles and HLA subtypes including HLA-B*57 correlate with delayed progression to AIDS. While prior studies have shown that both HIV-specific CD8+ T cells and NK cells can inhibit viral replication *in vitro*, the interaction between these two effector cells has not been studied. We performed *in vitro* suppression assays using CD8+ T cells and NK cells from HLA-B*57 ES either alone or in combination with each other. We found no evidence of antagonism or synergy between the CD8+ T cells and NK cells, suggesting that they have independent mechanisms of inhibition *in vitro*. Our data has implications for combined immunotherapy with CD8+ T cells and NK cells in HIV cure strategies.

Keywords: NK cells, CD8+ T cells, HIV-1, viral replication, viral suppression

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INTRODUCTION

Elite suppressors represent a model of a functional cure of HIV-1 infection (Walker and Yu, 2013). A substantial percentage of these subjects have protective HLA alleles (Migueles et al., 2000; Pereyra et al., 2010) and many studies have shown that CD8+ T cells in these subjects are effective at inhibiting viral replication (Migueles et al., 2002, 2008; Betts et al., 2006; Saez-Cirion et al., 2007; Hersperger et al., 2010). The role NK cells play in elite control is less clear, but studies have shown that the combination of some protective HLA- Bw4-80I alleles like HLA-B*57/5801 and certain KIR2DS1 and KIR3DL1 alleles confer more protection than either allele alone (Martin et al., 2002, 2007, 2018; Kamya et al., 2011). This suggests that NK cells may also play a role in elite control. We and others have shown that NK cells from some ES can suppress viral replication albeit generally not as effectively as CD8+ T cells (O'Connell et al., 2009; Tomescu et al., 2012; Marras et al., 2013; Walker-Sperling et al., 2017) and it has also been shown that ES have distinct NK cell profiles (Pohlmeyer et al., 2019). NK cells and CD8+ T cells respond to different signals on infected CD4+ T cells. The targeting of different signals by different effector molecules can potentially lead to synergy. However, antagonism between NK cells and CD8+ T cells has been reported for some viral infections (Su et al., 2001; Andrews et al., 2010; Lang et al., 2012; Mitrović et al., 2012). The interaction between these sets of effector cells in HIV infection will be important to understand if they are to be used together in immunotherapy. Thus, we designed experiments to interrogate how viral replication proceeds in the presence of both CD8+ T cells and NK cells. Our results have implications for HIV cure strategies.

METHODS

Study Subjects

Blood samples from HIV-positive donors were obtained with written informed consent and subsequently handled in accordance with protocols approved by the Johns Hopkins University Institutional Review Board. All ES were African American and treatment naive and maintained undetectable viral loads in the absence of ART. Viremic controller (VC) 10 maintained viral loads below 500 copies/ml in the absence of ART. The clinical characteristics of the subjects are described in **Supplementary Table 1**.

Phenotypic Studies

Blood was collected in ACD-containing tubes and incubated at room temperature overnight. The next day whole blood was stained with the following antibody panel for 15 min at 4°C: HLA-DR-PerCP-Cy5.5 and CD4-BV605 from Biolegend, and CD16-FITC, CD56-FITC, CD38-APC, CD8-APC-H7, CD3-PacBlue from BD Biosciences. Stained blood was then incubated at room temperature for 10 min in BD FACS Lysis Buffer at a 1:4 ratio of blood to buffer and then washed three times with PBS before analysis as previously described (Walker-Sperling et al., 2017).

Suppression Assay

The outline of the suppression assay is shown in **Figure 1**. Peripheral blood mononuclear cells (PBMCs) were obtained from blood by ficoll centrifugation and NK cells were purified from half of the PBMCs by negative selection with Miltenyi beads. CD8+ T cells were isolated by positive selection with Miltenyi beads from the other half of the PBMCs and CD4+ T cells were isolated from the flow-through cells by negative selection with Miltenyi beads. In all conditions, cells were cultured in RPMI media supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum and 10 U/ml IL-2. The CD4+ T cells were not stimulated to induce immune activation, rather they were infected directly after isolation by spinoculation at 100 ng of HIV-1 p24/100,000 cells with a pseudotyped virus (HIV-1-NL4-3ΔEnv–GFP) for 2 h at 1,200 × g and 37°C. HIV-1-NL4-3ΔEnv–GFP is a lab strain of HIV-1 that has env replaced with gfp. 100,000 CD4+ T cells per well were used for each assay in a 96 well round bottom plate. 25,000 NK or CD8+ T cells per well were added for the 1:4 E:T ratio experiments and 50,000 NK or CD8+ T cells/well were added for the 1:2 E:T ratio. 25,000 NK cells and 25,000 CD8+ T cells were added together for the 1:1:4 E:T ratio experiments. The final volume was 200 ul/ml. After 3 days, cells were harvested and stained with the following panel: CD3 (APC), CD8 (APC-H7), CD16 (PerCP-Cy5.5) and CD56 (PE-Cy7) (all from BD Biosciences). Viable cells were gated based on forward vs. side scatter plots for the majority of these experiments. Because of HIV mediated downregulation of surface CD4, CD4+ T cells were defined as CD3 positive, CD8 negative cells as outlined in **Figure 2**. GFP expression was used to assess the percentage of infected cells. The percentage of infected CD4+ T cells was typically 5 to10 with a median of 8. The percentage of viral suppression was calculated as [1–(%GFP + CD4+ T cells cultured with effectors)/(%GFP + CD4+ T cells without effectors)] × 100 as previously described (Pohlmeyer et al., 2013; Kwaa et al., 2019).

CD4+ T cells from ES3 were used in transwell experiments. After spinoculation, 1 million infected CD4+ T cells in 1 ml of media was placed in the outer chamber of wells in a 24 well-plate and 500,000 CD8+ T cells or NK cells in 150 ul of media was placed in the transwell for an E:T ratio of 1:2. In control experiments, 1 million infected or uninfected CD4+ T cells were cultured alone in each well or were cultured together with CD8+ T cells or NK cells at the same E:T ratio in 1.5 ml of media without transwells. Staining was performed as outlined above with the addition of Pac-Blue conjugated Annexin V.

Statistics

The parametric, one way ANOVA with Dunn's multiple comparison test was used to compare the NK cell and CD8+ T cell mediated suppression of viral replication. The Bliss independence model (Bliss, 1939) was used to predict combined suppression of NK cells and CD8+ T cells as previously described (Jilek et al., 2012; Mankowski et al., 2018).

RESULTS

The suppression assay used here has been previously described for CD8+ T cell and NK cell mediated suppression (Walker-Sperling et al., 2017; Veenhuis et al., 2018; Kwaa et al., 2019). It is based on an assay described by Saez-Cirion et al. (2007) but differs in that the CD4+ T cells are not activated prior to infection. Rather, they are infected directly after isolation with a pseudotyped virus. The absence of an *env* gene means that there is only a single cycle of infection which is very different from the exponential infection that occurs with a replication-competent virus. However, we have seen comparable levels of CD8+ T cell mediated inhibition of cells infected with pseudotyped virus and replication-competent isolates from patients (Veenhuis et al., 2018) and we may have seen even better levels of inhibition if Env epitopes were expressed on infected cells. While this assay does not measure direct killing of infected CD4+ T cells, we and others have shown that direct contact between CD8+ T cells and target CD4+ T cells is needed for suppression (Saez-Cirion et al., 2007; Veenhuis et al., 2018) and we show here that direct contact between ES3 target cells and NK and CD8+ T cells is needed to reduce viral transcription (**Supplementary Figure 1**). The frequency of Gag and Nef-specific CD8+ T cells in our subjects was not very high (combined median of 2,540 cells/million, **Supplementary Table 1**) which is consistent with the frequency of total HIV-specific CD8+ T cells found in a prior larger study (Pereyra et al., 2008). However, ES HIV-specific CD8+ T cells have been shown to proliferate in response to antigenic stimulation (Migueles et al., 2002, 2008; Pohlmeyer et al., 2018) and it is likely some level of clonal expansion occurs over the 3 day period of co-culture in our assay. Furthermore, the percentage of infected cells that express antigen is very low initially and therefore the true ratio of effectors to antigen expressing CD4+ T cells at the start of the assay is very high

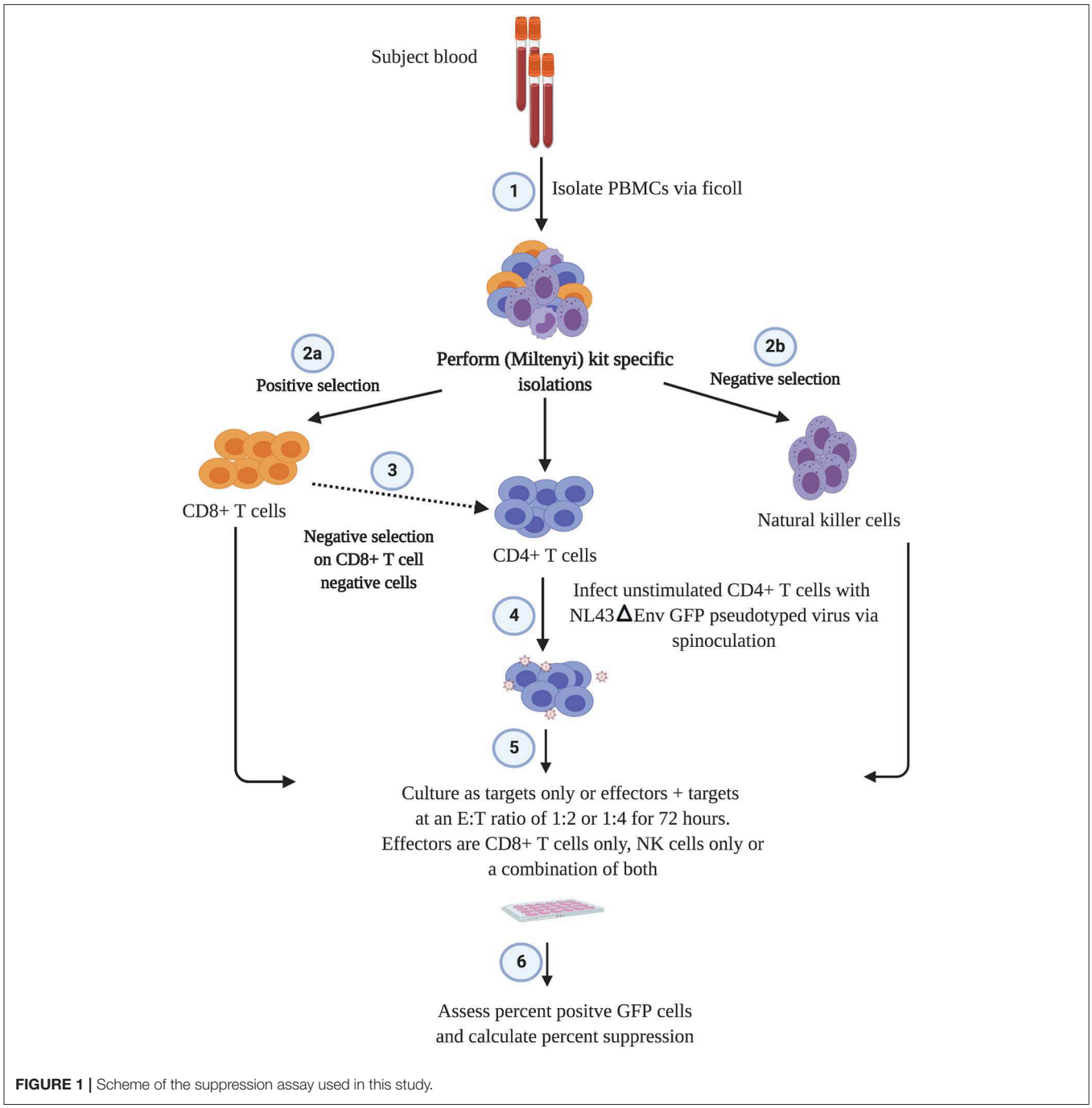

FIGURE 1 | Scheme of the suppression assay used in this study.

and changes over time as both infection and expansion of HIV-specific CD8+ T proceeds. This is in contrast to typical killing assays that use target cells that are already expressing HIV antigens. Additionally, the much shorter incubation times used in traditional killing assays means there is little chance of proliferation of effector cells so the effector to target ratio stays relatively constant.

ES CD8+ T cells have been previously shown to have high levels of immune activation (Hunt et al., 2008), and we found higher levels of HLA-DR+CD38- CD8+ T cells and NK cells in ES than in healthy donors in this study (**Supplementary Figure 2**). CD8+ T cells from all 8 ES inhibited viral replication in autologous CD4+ T cells (**Figure 3**). This suppressive capacity was specific because very little inhibition was seen when healthy donor CD8+ T cells were cultured with infected autologous CD4+ T cells (**Supplementary Figure 3**). In contrast, there was significant subject to subject variation of NK cell mediated inhibition of viral infection in both ES (**Figure 3**) and HDs (**Supplementary Figure 3**). There was no correlation between the activation status of the NK cells and

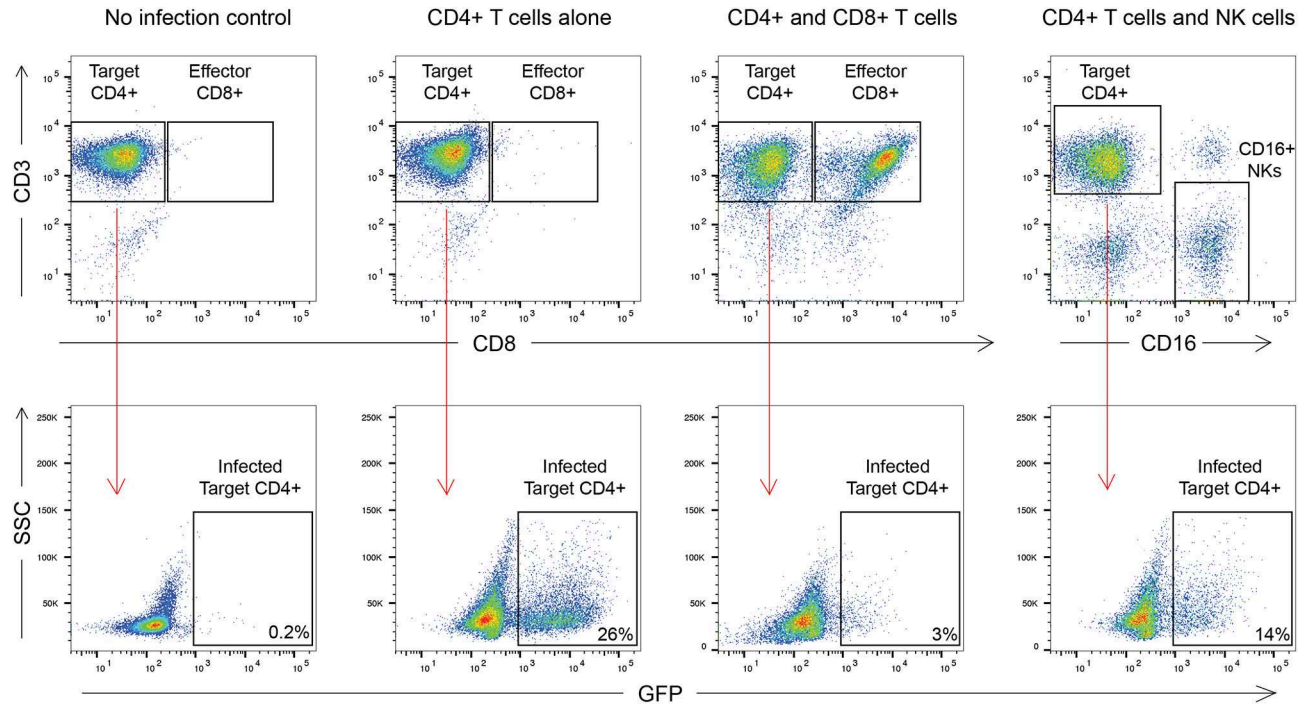

FIGURE 2 | Gating strategy used in this manuscript. CD8+ T cell suppression was calculated as [1–(%GFP + CD4+ T cells cultured with effectors)/(%GFP + CD4+ T cells without effectors)] × 100. For CD8+ T cells this was [1–3/26] × 100 or 88.4% suppression. For NK cells this was [1–14/26] × 100 or 46.2% suppression.

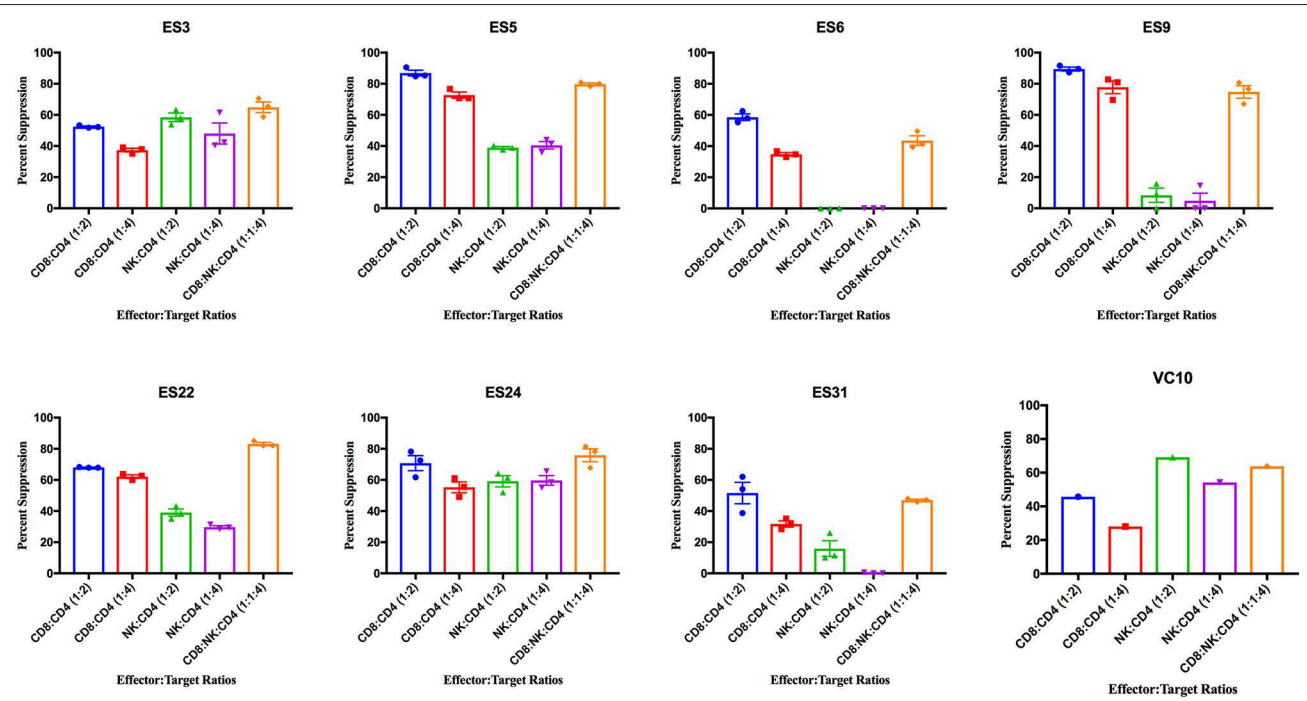

FIGURE 3 | Viral suppression induced by CD8+ T cells, NK cells, and a combination of the two effector cells at the indicated effector to target ratios for each individual. The bar is set to the mean value from 3 replicates per subject with the exception of VC10 where a single replicate was run due to limited numbers of cells. Error bars are the standard error of the mean.

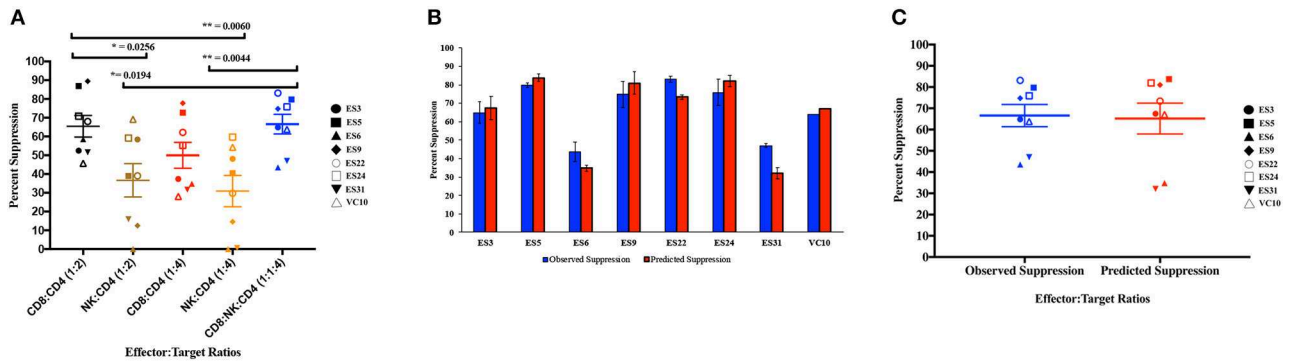

FIGURE 4 | Composite viral suppression by CD8+ T cells, NK cells, and a combination of the two effector cells **(A)**. The mean value for each subject is plotted. A comparison of the observed suppression by the combination of NK cells and CD8+ T cells (blue columns) to the degree of suppression predicted by the Bliss independence model (red columns) is shown for each individual **(B)** and for the cohort **(C)**. The bar is set to the mean value from 3 replicates per subject. Error bars represent the standard deviation from the mean.

their suppressive capacity. In subjects that are HLA-B Bw4-80I positive, a correlation between suppressive capacity and KIR3DS1 expression on NK cells has been established (Alter et al., 2007). All the ES in this study are HLA- Bw4-80I positive so we asked whether KIR3DS1 expression could explain the heterogeneous NK cell responses. All 8 subjects were KIR3DS1 negative consistent with the low frequency of this allele in African-Americans (Jiang et al., 2010). Thus, the variable NK cell suppressive responses seen here could not be explained by this allele.

As shown in **Figure 4A**, CD8+ T cell responses at the 1:2 E:T ratio were significantly more effective than NK cell responses at both E:T ratios as previously reported (Walker-Sperling et al., 2017). We combined NK cells and CD8+ T cells in a 1:1:4 ratio with infected autologous CD4+ T cells and found that while there was subject to subject variation, the combination of effector cells was significantly more effective at suppressing viral replication than NK cells alone at both the 1:4 and 1:2 E:T ratios (**Figure 4A**). In contrast, the difference between inhibition mediated by the combination of effectors cells and inhibition mediated by CD8+ T cells alone was not statistically significant. However, the presence of NK cells did not lead to a decrease in CD8+ T cell mediated suppression in any of the 8 subjects.

We next investigated the interaction between the two effector cells using the Bliss independence model (Bliss, 1939). This model can be used to identify synergy or antagonism between inhibitors under the assumption that the inhibitors have independent binding sites and independent mechanisms of action. In these subjects, the observed level of inhibition by CD8+ T cells and NK cells in combination at a 1:1:4 E:T ratio was not significantly different from the predicted inhibition for the combination calculated using the Bliss independence model equation and observed inhibition by each inhibitor individually at a 1:4 E:T ratio (**Figures 4B,C**). Thus, the data suggests that at the E:T ratio tested, the two effector cell types inhibited infection by independent mechanisms, and the inhibitory capacities of the two effector cells were neither antagonistic nor synergistic.

DISCUSSION

This is the first study to look at the direct interaction between the suppressive capacity of NK cells and CD8+ T cells in HIV infection. CD8+ T cell receptors recognize peptides presented on MHC class I molecules whereas NK cells have diverse receptors including the killer-like immunoglobulin receptors that recognize MHC proteins, C-type Lectin-like receptors that recognize stress antigens, and natural cytotoxicity receptors that recognize viral antigens. We asked whether the two different effector cells could work synergistically to inhibit viral replication since they have receptors that recognize different signals on infected CD4+ T cells. In particular, HIV-1 nef (Schwartz et al., 1996) and vpu (Apps et al., 2016) proteins have been shown to downregulate HLA proteins which leads to evasion from CD8+ T cell responses (Collins et al., 1998) but should activate NK cell responses due to the absence of ligands for inhibitory NK cell receptors. The Bliss model was used to formally assess the interaction between the cells and we found no evidence for antagonism or synergy at the E:T ratio we tested. Our study is limited by the relatively small number of subjects studied, the fact that we only looked at the combined effect of the two effector cell types at one E:T ratio, and by unexplained subject to subject variation in NK cell suppressive capacity. Interestingly, NK cells have been shown to enhance CD8+ T cell responses in CMV-infected mice (Robbins et al., 2007). In contrast, other studies have shown that NK cells can inhibit CD8+ T cell responses in LCMV-infected mice (Su et al., 2001; Lang et al., 2012) and CMV-infected mice (Andrews et al., 2010; Mitrović et al., 2012). Our *in vitro* system is incapable of capturing such complicated events that are partially mediated by third party cells. However, even with these limitations, we can conclude that NK cells and CD8+ T cell were not antagonistic at the E:T ratio we analyzed in any of the 8 subjects studied. Independent inhibition by the two effector cell types in combination is promising because HIV-specific CD8+ T cells from chronic progressors do not proliferate effectively in response to antigen (Migueles et al., 2002, 2008) so

it might be challenging to induce large numbers of effective cells with immunotherapy. Our data suggest that while NK cells do not work in synergy with CD8+ T cells *in vitro*, they also do not directly antagonize CD8+ T cell mediated inhibition of viral replication. Thus, strategies that employ both types of effector cells for immunotherapy may not result in antagonism, but this will need to be confirmed in larger cohorts of patients and with clinical trials.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Johns Hopkins IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MEM, CP, and AK performed the experiments and analyzed data. MCM analyzed data. JRB and JNB supervised the experiments and data analysis and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00113/full#supplementary-material

Supplementary Figure 1 | Effect of transwells on CD8+ T cell and NK cell mediated suppression of viral replication.

Supplementary Figure 2 | HLA-DR+CD38+ **(A)** and HLA-DR+CD38- **(B)** expression on ES, healthy donor (HD), and chronic progressor (CP) CD4+ T cells, CD8+ T cells, and NK cells.

Supplementary Figure 3 | Suppressive capacity of HD CD8+ T cells and NK cells at E:T ratios of 1:4 and 1:2.

Supplementary Table 1 | Clinical characteristics of ES used in this study.

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In vivo Effects of Romidepsin on T-Cell Activation, Apoptosis and Function in the BCN02 HIV-1 Kick&Kill Clinical Trial

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Romidepsin (RMD) is a well-characterized histone deacetylase inhibitor approved for the treatment of cutaneous T-cell lymphoma. *in vitro* and *in vivo* studies have demonstrated that it is able to induce HIV-1 gene expression in latently infected $CD4^+$ T cells from $HIV\text{-}1^+$ individuals on suppressive antiretroviral therapy. However, *in vitro* experiments suggested that RMD could also impair T-cell functionality, particularly of activated T cells. Thus, the usefulness of RMD in HIV-1 kick&kill strategies, that aim to enhance the immune system elimination of infected cells after inducing HIV-1 viral reactivation, may be limited. In order to address whether the *in vitro* observations are replicated *in vivo*, we determined the effects of RMD on the total and HIV-1-specific T-cell populations in longitudinal samples from the BCN02 kick&kill clinical trial (NCT02616874). BCN02 was a proof-of-concept study in 15 early treated $HIV\text{-}1^+$ individuals that combined MVA.HIVconsv vaccination with three weekly infusions of RMD given as a latency reversing agent. Our results show that RMD induced a transient increase in the frequency of apoptotic T cells and an enhanced activation of vaccine-induced T cells. Although RMD reduced the number of vaccine-elicited T cells secreting multiple cytokines, viral suppressive capacity of $CD8^+$ T cells was preserved over the RMD treatment. These observations have important implications for the design of effective kick&kill strategies for the HIV-1 cure.

Keywords: romidepsin, HDAC inhibitor, kick&kill strategy, therapeutic vaccine, latency reversing agent (LRA)

INTRODUCTION

Current antiretroviral therapy (ART) effectively suppresses HIV-1 replication in plasma, but it is not able to completely eliminate the virus from infected individuals. Cessation of antiretroviral treatment results in a rebound of plasma viremia within 3–4 weeks in most individuals (1). This rapid viral rebound after treatment interruption is due to the existence of a latent viral reservoir

and the inability of the immune system to effectively contain viral replication. To date, numerous strategies have been pursued to achieve a functional cure or virus eradication, including early ART initiation, ART intensification (2–6), passive administration of antibodies (7), therapeutic vaccination (8–13) and gene therapy (14, 15), among others.

HIV-1 kick&kill strategies are based on the use of latency reversing agents (LRA) to induce production of HIV-1 proteins in latently infected cells and render these cells susceptible to vaccine-induced virus-specific cytolytic T lymphocytes (CTL). The interest in LRA able to reactivate the latent provirus has increased over the past decade, with histone deacetylation inhibitors (HDACi) being some of the best characterized agents both *in vitro* and *in vivo*. The inhibitory effect of HDACi on histone deacetylation results in a higher degree of acetylated histones, causing opening of chromosomes and increased gene transcription. In *ex vivo* isolated cells from ART-suppressed HIV-1-infected individuals, exposure to HDACi resulted in reactivation of the integrated HIV-1 and led to viral protein expression by latently infected cells (16–18). Some HDACi, such as vorinostat (SAHA), panobinostat, and romidepsin (RMD) have also been tested for their *in vivo* potential to reverse HIV-1 latency (19–22). RMD, a cyclic depsipeptide naturally produced by *Chromobacterium violaceum*, is a pan-HDACi that inhibits class I HDACs. RMD was clinically developed as an anti-cancer drug and is approved for the treatment of cutaneous T-cell lymphoma (23). Furthermore, RMD has been shown to induce HIV-1 gene expression in latently infected cells *in vitro* (18, 24) and *in vivo*, when administrated alone (22) and in combination with therapeutic vaccine Vacc-4x (25) in chronically-infected ART-suppressed individuals. Although it was first thought that reactivation of the virus itself would lead to robust immune activation and control of the rebounding virus, it is generally accepted that prior stimulation of the immune effector response by a therapeutic vaccine and/or an immune checkpoint inhibitor, may be needed in order to efficiently eliminate infected cells after LRA exposure (26). Therefore, different immunotherapies are being investigated together with LRAs to test their combined effect, especially combination treatments that include T-cell vaccines.

The proof-of-concept BCN02 trial evaluated a kick&kill strategy that combined the HIVconsv T-cell vaccines with the HDACi RMD in a cohort of early-treated, HIV-1-infected individuals. Fifteen participants of BCN01 (12), who previously received simian adenovirus-vectored vaccine ChAdV63.HIVconsv and MVA.HIVconsv, were invited 2–3 years later to receive two more dosing of the MVA.HIVconsv vaccine before (MVA_1) and after (MVA_2) three weekly-doses of RMD (RMD_{1-2-3}) followed by a monitored antiretroviral pause (MAP) for a period of 32 weeks (NCT02616874). The combined strategy was proven to be safe and vaccination was highly immunogenic. RMD treatment resulted in marked increases in histone acetylation and cell-associated HIV-1 RNA levels compatible with induction of viral transcription. However, the ultimate reduction of the viral reservoir in the BCN02 trial was overall minimal (27). Aside from effects on virus reactivation, for a successful purge of the viral reservoir, it is critical that the LRA used in such strategies does not have any detrimental effects on the vaccine-induced immune cells (28, 29). Here, we assessed the *in vivo* impact of three weekly RMD doses on total and vaccine-induced T cells in longitudinal samples from the BCN02 trial (**Figure 1**).

MATERIALS AND METHODS

Study and Samples

The BCN02 clinical trial (NCT02616874) was a phase I, open-label, single-arm, multicenter study in Spain (27). The study was approved by the institutional ethical review board of the participating institutions (Reference Nr AC-15-108-R) and by the Spanish Regulatory Authorities (EudraCT 2015-002300-84) and was conducted in accordance with the principles of the Helsinki Declaration and local personal data protection law (LOPD 15/1999). Fifteen participants were immunized with MVA.HIVconsv (MVA_1, 2×10^8 pfu intramuscularly), followed by three weekly-doses of romidepsin (RMD_{1-2-3}, 5 mg/m^2 body-surface area; BSA) and a second MVA.HIVconsv boost vaccination (MVA_2, 2×10^8 pfu i.m.) before undergoing a monitored antiretroviral pause (MAP) 8 weeks later and for a maximum of 32 weeks. Cryopreserved peripheral blood

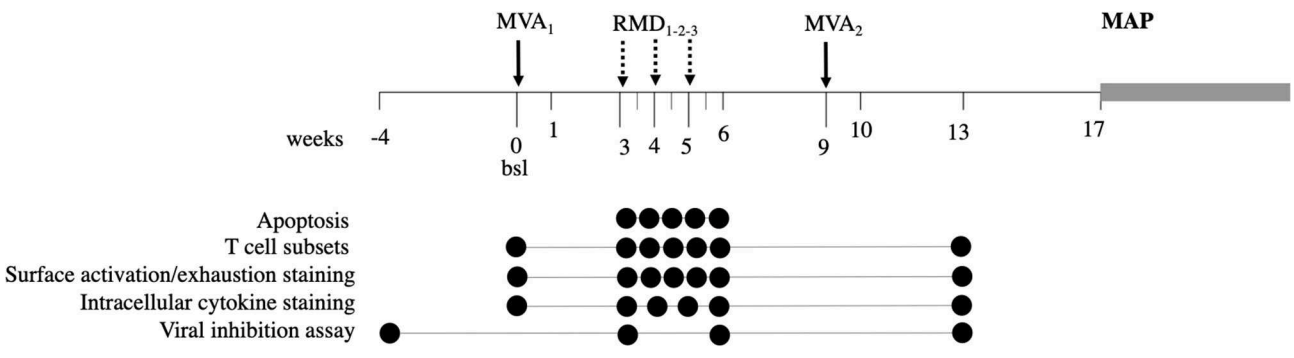

FIGURE 1 | Study design. The BCN02 study was a single arm, open label, proof-of-concept study to address safety and effect on the viral reservoir of a kick&kill strategy combining MVA.HIVconsv vaccines with the HDACi RMD. Timepoints used for the analysis presented here are indicated for each assay by filled circles.

mononuclear cells (PBMC) were stored before, at the end and after 8, 24 h (only for RMD_1), and 3 and 7 days after all RMD doses for immunological and virological studies.

Flow Cytometry

Apoptosis Measurement

PBMC viability was measured using a Pacific Blue™ Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend). Lineage surface markers (CD3, CD4, and CD8) and activation markers (HLA-DR, CD25, and CD69) were included in the staining.

Briefly, 1×10^6 of isolated PBMC were washed in PBS with 1% FBS and resuspend in 100 µl of surface staining solution (CD3, CD4, CD8, CD25, CD69, HLA-DR) and incubated for 20 min. After 2 washes with 300 µl of PBS with 1% FBS, cells were resuspended in 100 µl of Annexin V Binding Buffer with the corresponding Annexin V and 7-AAD. After 15 min of incubation, 250 µl of Binding Buffer was added to each tube and acquired on a LSRII BD cytometer. The percentages of apoptotic and live cells were analyzed using FlowJo software. The gating strategy is summarized in **Supplementary Figure 1**.

T Cells and HIVconsv-Specific T-Cell Lineage, Activation and Cytokine Detection

PBMCs were thawed and stimulated with anti-CD49d and anti-CD28 antibodies (BD) in presence/absence of three peptides pools (containing 58, 54, and 54 peptides) covering the HIVconsv immunogen protein in the presence of GolgiStop for 5 h. Cultures were then stored overnight at 4°C until staining. Cells were stained first with a viability stain (Aqua Live/Dead Fixable Dead Cell Stain kit, Invitrogen), followed by T cell lineage and maduration/activation markers (using anti-CD3-APC Cy7, anti-CD4 PECy5; anti-CD8 PerCP, anti-CCR7 B711, anti-CD45RA BV785, anti-HLA-DR BV650, anti-PD-1 BV605, anti-CD69 APC, and anti-CD25 PEDazzle594 chromogen-conjugated monoclonal antiobodies; BioLegend) and dump channel (using anti-CD19-V450 for B-cells and anti-CD14-V450 mAbs for monocytes; BioLegend) surface staining. Following the fixation and permeabilization step (Fix and Perm kit, Invitrogen), intracellular staining with conjugated antibodies specific for cytokines (IFN-γ A700; Invitrogen, IL-2 PECy7, TNF-α FITC; BiolLegend and MIP1-β PE; RD Systems) was performed. Approximately 10^5 cells were acquired on an LSRFortessa BD instrument, and analysis was performed using FlowJo 10 software. The gating strategy is summarized in **Supplementary Figure 2**.

Intracellular cytokine staining analyses were done applying boolean gates in FlowJo 10, subtracting unstimulated signals using Pestle v1.7 program and represented using SPICE v5.35 software (provided by the National Institute of Health, Mario Roeder, ImmunoTechnology Section, Vaccine Research Center, NIAID, NIH, Bethesda) (30).

Viral Inhibition Assay

$CD8^+$ T-cell mediated viral inhibition capacity was measured at 1:1 and 1:10 CD8 effector to CD4 target ratios. Cryopreserved PBMCs were obtained from timepoints before the BCN02 intervention and $CD8^+$ cells were depleted by magnetic bead separation (MACS Milteny Biotec). $CD8^+$-depleted cells ($CD4^+$-enriched fraction) were stimulated with PHA (5 µg/ml) in RPMI plus 10% fetal bovine serum (R10) and antibiotics (penicillin 100 U/mL and streptavidin 100 µg/ml). After 3 days of stimulation, the CD4-enriched fraction was infected by spinoculation with HIV-1_{BaL} and HIV-1_{IIIB} laboratory-adapted strains at a multiplicity of infection (MOI) of 0.01 as reported previously (12, 31). HIV-infected cells were cultured in duplicates or triplicates in R10 medium with 20 U/ml of IL-2 in 96-well round-bottomed plates, alone or together with unstimulated $CD8^+$ T cells obtained by positive magnetic bead separation the same day from an additional vial of frozen PBMC from screening (week −4), 3 weeks after MVA_1 (week 3, postMVA_1), 1 week after RMD_3 (week 6, postRMD_3), 4 weeks after MVA_2 (week 13, postMVA_2) timepoints. Cultures at different CD8:CD4 ratios (E:T = 1:1 and 1:10) were harvested after 6 days. Cells were stained first with Aqua Live/Dead stained for surface markers (CD3 APC-H7, BD Biosciences, CD4 PerCP, BD Biosciences, and CD8 APC, BD Biosciences), then permeabilized (FIX & PERM® Cell Permeabilization Kit, ThermoFisher). Cells were then fixed (FIX & PERM® Cell Permeabilization Kit, ThermoFisher) at room temperature and finally stained with anti-Gag p24 antibody (KC-57-FITC; Beckman Coulter). $CD8^+$ T-cell antiviral activity is expressed as % inhibition = [(fraction of $p24^+$ cells in $CD4^+$ T cells cultured alone)–(fraction of $p24^+$ cells in $CD4^+$ T cells cultured with $CD8^+$ T cells)]/(fraction of $p24^+$ in $CD4^+$ T cells cultured alone) × 100. At least 100,000 cells were collected on a LSRII BD cytometer and analysis was performed using FlowJo 10 software.

Statistical Analysis

GraphPad Prism version 7 for Windows (San Diego, CA) was used for statistical analysis. Mann-Whitney test and Wilcoxon matched paired test were used for unpaired and paired comparisons, respectively. Significant values were considered for $p < 0.05$.

RESULTS

Increased Apoptosis After RMD Exposure

Since RMD was previously described to have a toxic effect *in vitro* (28, 29), especially on activated T cells, we first measured the effect of RMD on cell viability both in total $CD8^+$ and $CD4^+$ T cells and in activated, HLA-DR^+ expressing cells in PBMC from the BCN02 participants. Viability in total T cells was assessed by flow cytometry by Annexin V/7AAD staining before and after the three RMD doses. Increases in the number of apoptotic $CD8^+$ T cells (Annexin V^+) were detected at 24 h after RMD_1 (Wilcoxon signed-rank, $p = 0.0151$) and 3 days after each RMD dose (Wilcoxon signed-rank, RMD_1 $p = 0.0413$, RMD_2 $p = 0.0181$, and RMD_3 $p = 0.0833$, respectively, **Figure 2A**). A similar pattern was observed in $CD4^+$ T cells, with apoptotic cells being significantly increased 3 days after RMD_2 and RMD_3 (Wilcoxon signed-rank, RMD_2 $p = 0.0067$ and RMD_3 $p = 0.0413$, respectively, **Figure 2B**). One week after RMD_3 the levels of apoptotic cells in both $CD8^+$ T cells and $CD4^+$ were not restored

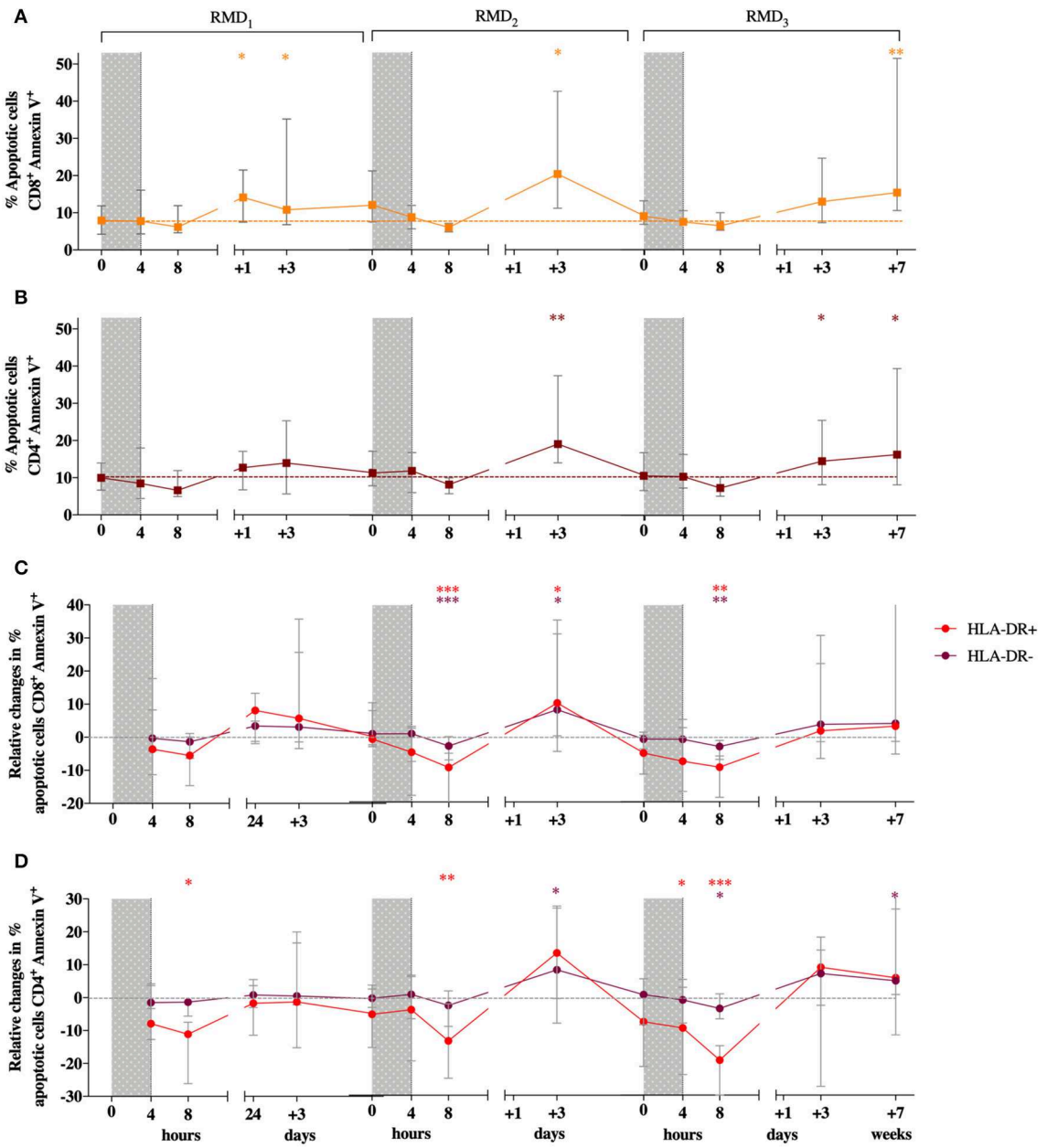

FIGURE 2 | Effect of RMD on the viability on T cells. Apoptotic cells (Annexin $V^+/7AAD^+$ and Annexin $V^+/7AAD^-$) percentages are shown for $CD8^+$ **(A)** and $CD4^+$ T cells **(B)**. Changes relative to baseline in the percentage of apoptosis in HLA-DR^+ (in red) and HLA-DR^- cells (in purple) are shown for $CD8^+$ **(C)** and $CD4^+$ **(D)** T cells. RMD administration cycles are indicated by gray bars. Sampling time points at 4 h, 8 h, 3 days, and 7 days after each RMD administration are indicated. Median with interquartile range is shown. *P*-values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) are indicative for the corresponding timepoint compared to pre-RMD_1.

to pre-RMD levels ($p = 0.0054$ for $CD8^+$ and $p = 0.0181$ for $CD4^+$), suggestive of an accumulative toxic effect of RMD.

As activated T cell have been found to be particularly sensitive to RMD exposure (28), levels of apoptosis in $CD4^+$ and $CD8^+$ T cell with high or low expression of the activation marker HLA-DR were assessed. Changes in the percentage of apoptotic cells relative to baseline during RMD exposure was comparable between HLA-DR^+ and HLA-DR^- CD8 T cells (**Figures 2C,D**). The same results were observed in CD4+ T cells. Therefore, non- activated HLA-DR^- cells had the same susceptibility to induction of apoptosis due to RMD exposure as the activated HLA-DR^+ cells.

$CD8^+$ and $CD4^+$ Naïve Populations Increase After MVA_1 and Up to 24 h After 1st Romidepsin Exposure Followed by a Shift to Memory Phenotypes

As activation status did not impact susceptibility of T cells to RMD-induced apoptosis, we next assessed whether RMD could

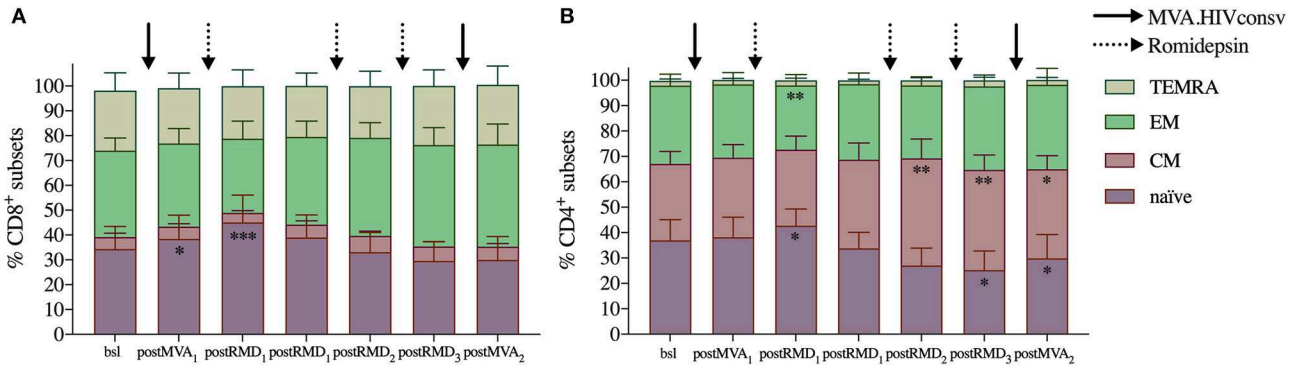

FIGURE 3 | Evolution of T-cell subsets over the course of the intervention. Averages of relative frequency of $CD8^+$ **(A)** and $CD4^+$ **(B)** T-cell subsets based on CCR7 and CD45RA expression are shown. TEMRA: highly differentiated effector cells expressing CD45RA ($CCR7^-/CD45RA^+$), EM: effector memory cells ($CCR7^-/CD45RA^-$), CM: central memory ($CCR7^+/CD45RA^-$), and naïve ($CCR7^+/CD45RA^+$). *P*-values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) are indicative for the corresponding timepoint compared with values from baseline samples prior to MVA_1.

affect the distribution of different T-cell differentiation subsets in the peripheral blood. Flow cytometry was used to measure the frequency of $CD4^+$ and $CD8^+$ differentiation subsets defined by CCR7 and CD45RA expression at each timepoint. Three weeks after MVA_1, the frequency of naïve $CD8^+$ T cells increased from median frequency of 30.5–35.2% and up to 49.6% 24 h after RMD_1 (Wilcoxon signed-rank $p = 0.0122$ and $p = 0.0005$, respectively, **Figure 3A**). Similar results were observed for naïve $CD4^+$ T cells, which increased from 33% at baseline to 35.2% 3 weeks after MVA_1 and up to 41.7% at 24 h after RMD_1 (Wilcoxon signed-rank $p = 0.0227$ and $p = 0.0110$, respectively, **Figure 3B**). The frequency of $CD8^+$ and $CD4^+$ naïve cells were not further increased after RMD_2 or RMD_3, as their frequency actually decreased after RMD_2 and RMD_3. At the same time, the median frequency of $CD8^+$ effector memory and $CD4^+$ central memory T cells progressively increased over RMD_{1-2-3}. T-cell differentiation subsets were not further changed 4 weeks after MVA_2 compared to subset population observed after RMD doses.

RMD Treatment Increases T-Cell Activation Especially of the HIVconsv-Specific T Cells

In order to assess the effect of RMD on T-cell activation and exhaustion markers, we evaluated the frequency of $CD4^+$ and $CD8^+$ T cells expressing HLA-DR, CD69, CD25, and PD-1. A peak in the frequency of $CD8^+$HLA-DR^+ T cells was observed 3 days after RMD_1 (Wilcoxon signed-rank, $p < 0.0001$) and increased levels were maintained over the course of the 3 RMD doses, as their median frequency more than doubled (4.1% at baseline to 9.6% 1 week after RMD_3, Wilcoxon signed-rank, $p = 0.0002$, **Figure 4A**). The same results were observed in $CD4^+$ T cells, with levels of HLA-DR expression increasing 3 days after RMD_1 (Wilcoxon signed-rank, $p < 0.0001$) and with twice as many HLA-DR expressing cells at day 7 after RMD_3 (median 3.1% at baseline to 6.3%, Wilcoxon signed-rank, $p = 0.0017$, **Figure 4A**) compared to baseline. The largest increase in percentage of cells expressing HLA-DR in both $CD4^+$ and $CD8^+$ T cells was observed in effector memory (EM) and highly differentiated effector cells expressing CD45RA (TEMRA) (**Supplementary Figure 3**).

Importantly, upon *in vitro* stimulation with peptides derived from the vaccine immunogen, changes in the HLA-DR^+ in expression were already detected after MVA_1. Both, HIVconsv-specific $CD8^+$ and $CD4^+$ T cells, defined upon *in vitro* antigen-specific stimulation and detection based on their ability to produce cytokines (IL-2, MIP1-β, TNF-α, IFN-γ) in response to antigen specific stimulation, showed detectable increases of HLA-DR^+ expression earlier and up to higher levels compared to the changes seen in total $CD8^+$ and $CD4^+$ T cells (**Figure 4A**). The percentage of HLA-DR^+ among HIVconsv-specific $CD8^+$ cells increased from median of 5.1% at baseline to 10.11% 3 weeks after MVA_1, and up to 21% 3 days after RMD_1 (Wilcoxon signed-rank $p = 0.0245$ and $p = 0.0002$, **Figure 4B**) and percentage of HLA-DR^+, HIVconsv-specific $CD4^+$ cells increased from median of 4.2% at baseline to 8.3% 3 weeks after MVA_1, and up to 21.8% 3 days after RMD_1 (Wilcoxon signed-rank $p = 0.5614$ and $p = 0.0023$, **Figure 4C**). High levels of activation were maintained during all RMD doses (**Figures 4C,D**) and reached the peak 3 days after RMD_3 suggestive of an additive effect of RMD on the activation of vaccine-stimulated $CD4^+$ and $CD8^+$ T cells.

As changes in *in vivo* histone acetylation and induction of viral transcription occur rapidly after RMD exposure (22, 27), we assessed the expression of early activation markers CD69 and CD25 in $CD4^+$ and $CD8^+$ T cells. Increases in the expression of CD69 in $CD4^+$ T cells were observed upon each RMD dosing, with significant increases seen after RMD_2 and RMD_3 in the CM subset (from baseline levels of 2.5 to 7.1% (RMD_2 $p = 0.0107$) and 6.7% (RMD_3 $p = 0.0479$) and in the TEMRA subset (baseline levels of 2.7 to 9% (RMD_2 $p = 0.0203$) and 6.6% (RMD_3 $p = 0.0315$), respectively (**Supplementary Figure 4B**). As with HLA-DR surface staining, expression of CD69 returned to baseline levels 4 weeks after last vaccination. No significant changes were observed in CD69 expression on $CD8^+$ T cells (**Supplementary Figure 4A**) nor in the expression of CD25 on $CD8^+$ or $CD4^+$ T cells over the course of the intervention.

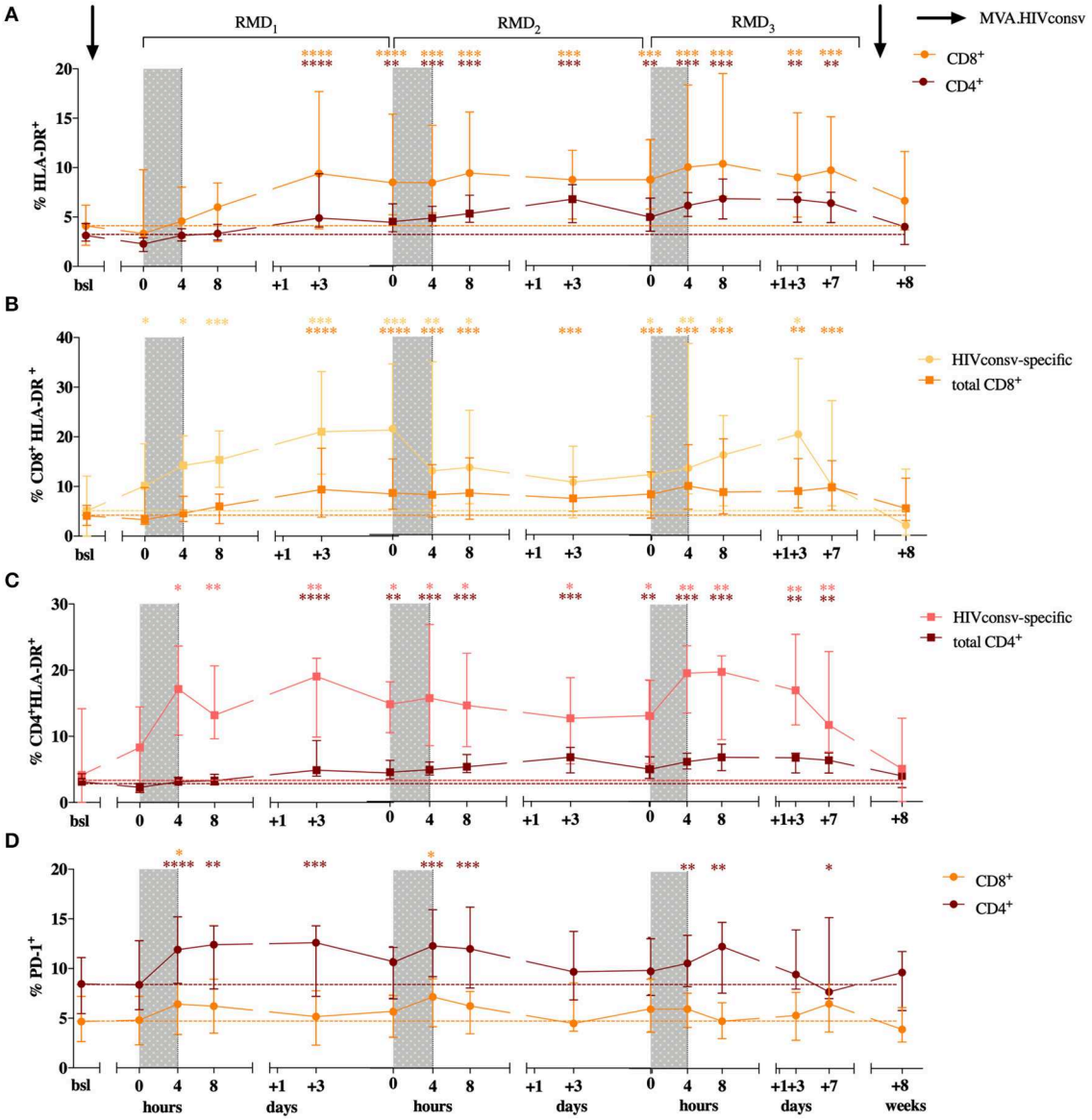

FIGURE 4 | CD4+ and CD8+ T cell activation status over the course of the intervention. Percentage of HLA-DR expression is shown for CD8+ in orange and CD4+ T cells in purple **(A)**. Levels of HLA-DR are shown for CD8+ **(B)** and CD4+ T cells **(C)** in HIVconsv specific and total T cells. HIVconsv-specific T cells were defined upon *in vitro* antigen-specific stimulation and detection based on their ability to produce cytokines (IL-2, MIP1-β, TNF-α, IFN-γ) in response to stimulation. Percentage of PD-1 is shown for CD8+ in orange and CD4+ T cells in purple **(D)**. Median with interquartile range is shown. *P*-values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) are indicative for the corresponding timepoint compared with baseline.

The expression of PD-1+ increased rapidly in CD8+ T cells from median baseline levels of 4.6–6.4, 7.2, and 6.1% at 4 h after the end of RMD_{1-2-3} treatment (Wilcoxon singed-rank, $p = 0.0215$, $p = 0.0238$, and $p = 0.0574$, respectively, **Figure 4D**). The same kinetics was observed in CD4+ T cells where the frequency of PD-1 expressing cells raised from median baseline levels of 8.5–11.9, 12.35, and 10.7% at 4 h after RMD_{1-2-3} (Wilcoxon singed-rank, $p = 0.0004$, $p = 0.0002$, and $p = 0.0046$, respectively, **Figure 4D**). However, in contrast to the additive effect of RMD_{1-2-3} on T-cell activation markers, changes in PD-1 expression in both CD8+ and CD4+ T cells were transient, and the levels of PD-1 expression were consistently restored to baseline levels within 3 days after each RMD dose.

Polyfunctionality of HIVconsv-Specific Responses Increases After MVA_1 and Decreases Over RMD Treatment

Considering the effect of RMD on the expression of markers of cell death, exhaustion and activation, we assessed the

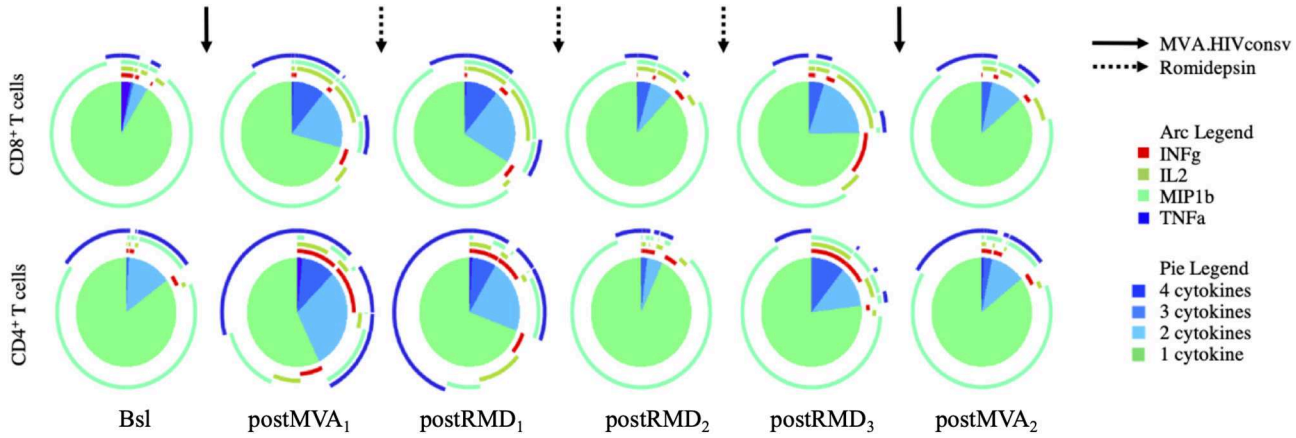

FIGURE 5 | Longitudinal assessment of the T-cell cytokine production over the course of the intervention. Polyfunctionality of $CD8^+$ and $CD4^+$ T cells was analyzed by Boolean gating. Pie charts illustrate relative proportion of each of the different subsets (cells producing 1, 2, 3, or 4 cytokines, respectively).

effect of RMD on the functionality of vaccine-elicited T cells. Polyfunctionality of HIVconsv-specific T cells was measured by stimulation of PBMC with the HIVconsv peptides and enumeration of the cells producing IFN-γ, IL-2, MIP1-β, and/or TNF-α by flow cytometry. As shown in **Figure 5**, MVA_1 vaccination increased relative polyfunctionality in both $CD4^+$ and $CD8^+$ HIVconsv-specific T cells. Three weeks after MVA_1 (postMVA_1), the highest increase in polyfunctionality was observed in vaccine immunogen-specific $CD4^+$ T cells that produced 2, 3, and 4 cytokines (post MVA_1, **Figure 5**), with a particular increase in INF-γ secreting cells. On the other hand, vaccine-specific $CD8^+$ T cells produced INF-γ, IL-2, and TNF-α and were mostly polyfunctional, producing 2 or 3 cytokines. However, during the treatment with RMD, the polyfunctionality was reduced to baseline levels in both HIVconsv-specific $CD4^+$ and $CD8^+$ T cells and the final MVA_2 vaccination was not able to re-boost functionality profiles (post MVA_2, **Figure 5** and **Supplementary Figure 5**). These results suggest that RMD treatment might have impaired functionality of vaccine-induced responses and prevented their subsequent booster vaccination effect.

$CD8^+$ Cells Maintain Antiviral Activity of After the RMD Treatment

To further study the functionality of vaccine-induced T cells during the intervention, we measured the *in vitro* antiviral capacity of $CD8^+$ T cells. The *in vitro* replication inhibition capacity of PBMC-derived $CD8^+$ T cells was measured by standard viral inhibition assay (VIA) (12, 31) using autologous $CD4^+$ T cells infected with two laboratory-adapted HIV-1 strains BaL (R5 tropic virus) and IIIB (X4 tropic virus). Virus replication was measured by flow cytometry as the percentage of HIV-1 Gag p24-positive $CD4^+$ cells. $CD8^+$ T-cell inhibitory capacity was determined at screening, 3 weeks after MVA_1 (postMVA_1), 1 week after RMD_3 (postRMD_3), and 4 weeks after MVA_2 (postMVA_2). In contrast to the changes in cytokine production/polyfunctionality detected by flow cytometry, neither MVA_1 nor RMD_{1-2-3} altered the inhibition activity, which was median of 64, 52, and 53,5% at the screening, postMVA_1 and postRMD_3 time points at the E:T ratio 1:1, respectively, against HIV-1_{BaL} and median 46, 44, and 43% at the screening, postMVA_1 and postRMD_3 time points, respectively, against HIV-1_{IIIB}. In fact, there was a weak, but statistically significant increase in the inhibitory capacity against HIV-1_{BaL} after the second MVA vaccination given 4 weeks after RMD_3 from median of 64% at baseline to 69% at postMVA_2 at the E:T ratio 1:1 and from median of 11% at baseline to 21% at postMVA_2, for the E:T ratio1:10 (Wilcoxon signed-rank, $p = 0.0200$) (**Figure 6A**) and against HIV-1_{IIIB} from median of 12% at baseline to 16% at postMVA_2, for at the 1:10 E:T ratio (Wilcoxon signed-rank, $p = 0.0156$) (**Figure 6B**). These data indicate that antiviral activity, measured by VIA with laboratory-adapted viral strains, was not negatively impacted during RMD treatment.

DISCUSSION

The BCN02 study was an HIV-1 kick&kill proof-of-concept trial combining MVA.HIVconsv vaccines with the HDACi RMD given as an LRA to the previous ChAdV63.HIVconsv-MVA.HIVconsv regimen recipients from the parental BCN01 trial (12). The immune analyses in BCN02 showed a highly significant shift of HIV-1-specific T-cell responses toward conserved regions of HIV-1 covered by the immunogen and marked increases in histone acetylation and induction of viral transcription *in vivo* by RMD. However, the reduction of the viral reservoir after these interventions was overall minimal (27). Already low baseline levels of viral reservoir in the early-treated participants enrolled in the BCN02 study likely limited latency reversal activity of RMD and/or a potential toxicity of RMD (28, 32) on vaccine-induced T cells might have precluded the capability to observe a more pronounced reduction in the size of the viral reservoir. To address the latter possibility, we here evaluated the effect that the HDACi RMD had on the T-cell viability, activation and

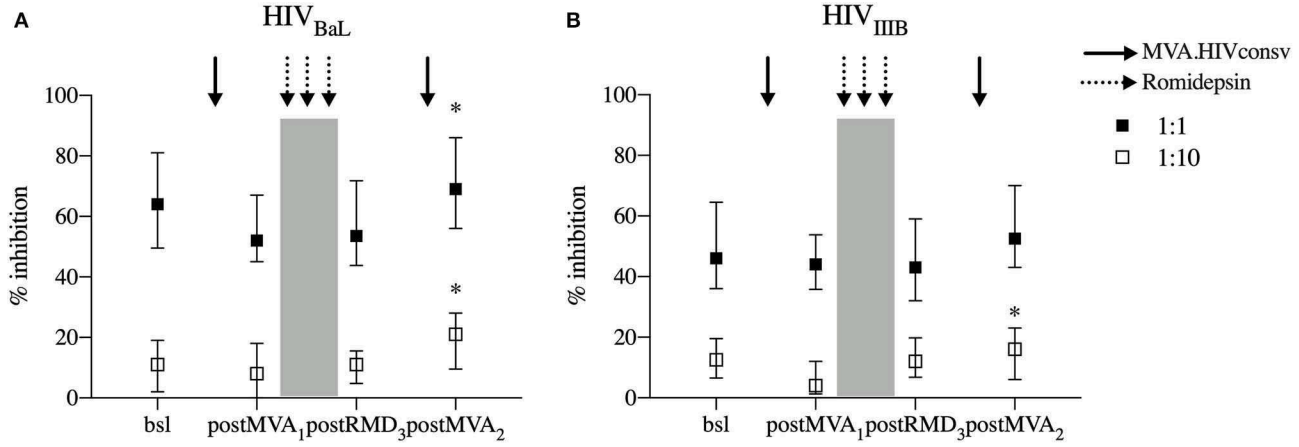

FIGURE 6 | *In vitro* viral replication inhibition capacity. Levels of $CD8^+$ viral inhibitory capacity are shown for HIV-1_{BaL} **(A)** and HIV-1_{IIIB} **(B)** at two E/T ratios (effector/target ratio) 1:1 and E/T 1:10 in individuals that underwent the intervention ($n = 14$ for HIV-1_{BaL} and $n = 15$ for HIV-1_{IIIB}). *P*-values (*$p < 0.05$) are indicative for the corresponding timepoint compared with respective bsl.

functionality throughout the intervention that included RMD given in a three weekly 5 mg/m^2 BSA regimen.

Our data document a *in vivo* toxic effect of RMD on T cells 3 days after each RMD exposure. Although these effects were transient and viability of T cells was partially recovered 7 days after RMD_1 and RMD_2, there was an additive increase in apoptosis over the full RMD regimen, which was not fully restored by 7 days after RMD_3. Jones et al. and Zhao et al. have described increases in cell death upon long term (18- and 72- h period) *in vitro* exposure to RMD, but no cytotoxic effect were observed when T cells were exposed to RMD for 4 h only, which is more similar to the *in vivo* regimen where the terminal half-life ($t_{1/2}$) of RMD is estimated to be ∼3 h (28, 32). Indeed, Clutton et al. exposed *ex vivo* PBMC to RMD for 3 h, washed them and cultivated them further in the absence of RMD for 3 days in order to mimic *in vivo* exposure, and reported that the cell viability was not changed after 3 or 24 h, but was significantly reduced at 48 and 72 h (33). These results concur with our observations showing a peak of apoptotic T cells 3 days after each RMD infusion and could explain the transient decreases in peripheral $CD4^+$ T-cell counts observed in BCN02 (27). Also, the cumulative effect on viability observed after 3 RMD doses is in line with the delayed effects of RMD suggested by Clutton.

As RMD may induce T-cell activation resulting from reservoir reactivation and viral antigen presentation, which could also drive increase in exhaustion levels prior to the induction of apoptosis, we evaluated the frequency of activated $CD4^+$ and $CD8^+$ T cells. Indeed, the additive increase in HLA-DR^+ activation markers in both $CD8^+$ and $CD4^+$ T cells over RMD treatment, especially in HIVconsv-specific T cells, was already observed after the first MVA.HIVconsv vaccination. Moreover, as the increased expression of HLA-DR was mostly observed in T effector memory cells and the fact that HIVconsv-specific T cells showed higher levels of HLA-DR expression, the data suggest that there could be a different memory distribution among HIVconsv-specific T cells compared to the total T cell population. These changes are in line with the increase in the magnitude of the immunogen-specific responses upon vaccination (27). However, the magnitudes of vaccine-specific responses were not increased further during RMD administration, suggesting that viral reactivation did not contribute in a major way to the increased levels of cell activation during RMD. In contrast, RMD treatment changed levels of CD69 and PD-1 more rapidly albeit transiently, peaking at 4 h after the end of each RMD dose. In line with our observation on CD69 longitudinal expression, an increase in $CD69^+$ expression was previously described upon RMD, panobinostat and vorinostat treatments *in vitro* (18, 24) and also *in vivo* given alone (22) or in combination with a vaccine (34). This enhanced expression of PD-1 and CD69 could be the result of a generalized increased expression of the host genes induced by the action of RMD. So far, little is known about the effect of RMD and other LRA on the host genome gene expression profiles. There is an urgent need to better understand these effects and, especially, to clarify how they can affect the antiviral immunity against HIV-1 (35) or, in fact, fueling it.

Although it was previously described that RMD can impair T-cell and NK function *in vitro* (28, 29, 36, 37), Søgaard et al. presented *in vivo* data from the REDUC trial, indicating that RMD did not alter the proportion of HIV-1-specific T cells nor inhibited T-cell cytokine production (22), at least in the peripheral blood. Nonetheless, there appears to be a trend toward decreased HIV-specific T cell responses after RMD treatment, which may have not reached statistical significance due to the limited group size. On another note, vaccine-induced responses in the REDUC trial were weaker compared to the responses induced in the BCN02 trial (12, 27), possibly due to weaker vaccine vectors/immunogens employed in the REDUC trial. In addition, REDUC did not include early-treated individuals with relatively intact immunity, and the detection of reduced polyfunctionality of vaccine-induced T-cells could have been limited. In BCN02, the first MVA.HIVconsv vaccination enhanced polyfunctionality, especially in $CD4^+$ T cells, in line

with previous reports showing that MVA-vectored vaccination can improve the polyfunctionality and T effector memory phenotype in $CD4^+$ T cells more so than in $CD8^+$ T cells (38). This increased effector function profile induced by MVA_1 was reduced upon RMD treatment but did not lead to a net reduction of the latent reservoir compared to baseline.

Our data also indicate that despite fluctuations in activation, maturation phenotypes and polyfunctionality, RMD treatment did not impair the *in vitro* antiviral capacity of $CD8^+$ T cells. While some *in vitro* studies have shown a diminished inhibitory capacity when $CD8^+$ T cells were exposed to RMD, but not to other HDACi (39, 40), such an effect was not observed *in vivo* in clinical trials using RMD. In the REDUC trial, the viral inhibition assay showed a trend toward increased inhibitory activity post-immunization that was lost after RMD exposure, but overall, antiviral capacity did not significantly change over time. Similarly, we did not see a reduction in the antiviral capacity of T cells over the course of RMD_{1-2-3}, suggesting that the preservation of the antiviral capacity may depend on the balance between the deleterious effects of RMD and the potency of the employed vaccination strategy.

Of note, our results highlight that there is no direct comparability between assays used to characterize CTL functionality, including multiparametric flow cytometry and *in vitro* inhibition assays. In particular, in our study, the polyfunctionality of $CD8^+$ T cells decreased slightly during RMD treatment, while *in vitro* VIA activity was maintained. In addition, the MVA_2 boost vaccination did not augment the proportion of cell secreting multiple cytokines, while a moderately increased *in vitro* suppressive capacity was observed. These data are consistent with other studies, which showed a disconnection between cytokine secretion and antiviral capacity (41, 42), indicating that polyfunctionality of HIV-1-specific $CD8^+$ T cells (at least as measured in standard assays) is not directly associated with viral suppression capacity (31, 43, 44). This lack of consistency between the two techniques could be due to the cytokines measured in standard protocols for intracellular cytokine assays: whilst IFN-γ, IL-2, MIP1-β, and TNF-α are used to measure functionality of HIV-1-specific T cells, evaluating secretion of granzyme B and perforin could be more accurate when assessing T-cell killing activity and may provide better concordance with VIA activity (45). However, whether antiviral capacity measured by standard *in vitro* VIA assay using laboratory-adapted viral strains will translate into effective *in vivo* killing or reactivated, HIV-1 infected cells remain to be determined as well. In addition, further work using autologous virus might be more representative of the physiological conditions in kick&kill strategies. Finally, viral suppression capacity was measured in total bulk $CD8^+$ T cells while cytokine secretion was measured in vaccine specific cells, which could have been more susceptible to *in vivo* effects by RMD.

This present study has a number of limitations, which include a small sample size and the lack of control arms, both placebo and single intervention arms to discern the effects exerted by RMD or the vaccine alone. Thus, the presented results need to be interpreted with caution. Still, the present study shows that RMD has a transient effect on T-cell viability, exhaustion and increased cell activation in an additive way over three weekly doses. Although this can result in a decrease of polyfunctionality of vaccine-induced HIVconsv-specific responses, the *in vitro* replication inhibition capacity of $CD8^+$ T cells was not impaired and should not preclude effective killing upon RMD-induced viral reactivation. As RMD increased higher levels of HIV-1 transcription on the RMD_2 and RMD_3 dose, it is tempting to speculate that increasing the number the RMD doses could result in further increased in levels of reservoir reactivation without overly inhibiting the antiviral capacity of $CD8^+$ T cells. Regardless whether this would occur *in vivo* with longer term RMD administration, the present data indicate that timing and order of LRA and T-cell immunotherapy regimens are critical in order to achieve the clearance of reactivated latently HIV-1-infected cells. Larger controlled clinical trials are needed to further investigate combinations of LRA and immune intervention in order to find the best strategy to achieve a functional cure of HIV-1.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of and approval by the Ethics Committee of the Hospital Universitari Germans Trias i Pujol (Badalona, Spain). All subjects provided their written informed consent to participate. The study was conducted according to the principles expressed in the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MR-U, MR-R, BM, and CB conceived and designed the study and drafted the manuscript. MF, MM, PC, CM, SC, JMM, BC, TH, and JM contributed to the study design. MR-U, MR-R, and MF performed the experiments. All authors revised the manuscript critically for important intellectual content and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00418/full#supplementary-material

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A T Cell Receptor Sequencing-Based Assay Identifies Cross-Reactive Recall CD8$^+$ T Cell Clonotypes Against Autologous HIV-1 Epitope Variants

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HIV-1 positive elite controllers or suppressors control viral replication without antiretroviral therapy, likely via CTL-mediated elimination of infected cells, and therefore represent a model of an HIV-1 functional cure. Efforts to cure HIV-1 accordingly rely on the existence or generation of antigen-specific cytotoxic T lymphocytes (CTL) to eradicate infected cells upon reversal of latency. Detecting and quantifying these HIV-1-specific CTL responses will be crucial for developing vaccine and T cell-based immunotherapies. A recently developed assay, called MANAFEST, uses T cell receptor (TCR) Vβ sequencing of peptide-stimulated cultures followed by a bioinformatic pipeline to identify neoantigen-specific T cells in cancer patients. This assay is more sensitive than conventional immune assays and therefore has the possibility to identify HIV-1 antigenic targets that have not been previously explored for vaccine or T cell immunotherapeutic strategies. Here we show that a modified version of the MANAFEST assay, called ViraFEST, can identify memory CD8$^+$ T cell responses against autologous HIV-1 Gag and Nef epitope variants in an elite suppressor. Nine TCR Vβ clonotypes were identified and 6 of these were cross-reactive for autologous variants or known escape variants. Our findings are a proof of principle that the ViraFEST assay can be used to detect and monitor these responses for downstream use in immunotherapeutic treatment approaches.

Keywords: HIV, cure, elite suppressors, elite controllers, clonotype, CD8 lymphocytes+

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INTRODUCTION

Antiretroviral therapy (ART) reduces viral load to undetectable levels in the majority of HIV-1-infected patients. Despite this, a persistent latent viral reservoir in tissues and blood prevents complete viral eradication and results in viral rebound upon ART cessation in the vast majority of patients (1). HIV elite suppressors (ES) are patients who control viral replication without ART (2). These patients may represent a model of a functional cure for HIV-1 since many are infected with replication-competent viruses (3, 4) and are thought to control viral replication through HIV-specific CTL (5–8). Thus it may be possible to control the rebound of viremia following the

cessation of ART in patients with progressive disease with immunotherapy. One such strategy is the "shock and kill" approach (9), whereby viral replication is induced from within latent reservoirs and endogenously- or exogenously-generated cytotoxic T lymphocytes (CTL) specific for the patient's own virus (autologous virus) kill infected cells. Several immunotherapeutic approaches have been evaluated to induce CTL killing of infected cells, including dendritic cell-based strategies (10), adoptive transfer of CAR T cells (11), and checkpoint inhibition therapy (12).

Identifying antigenic CTL epitopes and evaluating endogenous memory CTL responses will be crucial for developing these immunotherapies into effective treatments. ELISpot, intracellular cytokine staining, and multimer staining have traditionally been employed when assessing HIV-1-specific T cell responses. However, these assays can underestimate the breadth and magnitude of the response (13) and do not enable identification at the clonotypic level, which will be crucial for engineered T cell-based treatments. The mutation associated neoantigen functional expansion of specific T cells (MANAFEST) assay uses peptide-stimulated T cell cultures coupled with T cell receptor Vβ sequencing and a bioinformatic pipeline to identify neoantigen-specific $CD8^+$ T cell clonotypes (13–15). This assay has yet to be utilized to evaluate HIV-1-specific responses. This could be particularly useful given the potential for cross-reactivity of HIV-specific T-cell receptors (16–21).

Our goal was to therefore provide a proof-of-principle demonstrating the utility of the MANAFEST assay in identifying T cell responses at the clonotypic level against closely related autologous HIV-1 epitope variants. Elite suppressors are the ideal model for this analysis, owing to well-documented HIV-specific T cell responses and presumed CTL-mediated viral control in these patients. Here we demonstrate that modified use of the MANAFEST assay, called ViraFEST (viral functional expansion of specific T cells), combined with a novel analytical platform can detect cross-reactive $CD8^+$ T cell responses to autologous epitope variants in an HIV-1^+ elite suppressor (ES). This is the first report using this assay to evaluate the cross-reactive nature of T cells specific for autologous HIV-1 epitope variants. Routine use of this assay to detect and monitor T cell responses to HIV-1 antigens could reveal a broad range of immunogenic antigens not previously identified and can identify HIV-1-specific TCRs that could be exploited in vaccine or T cell-based immunotherapies.

METHODS

Study Patient

Blood samples from the study subject were obtained in 2018 and 2019 after written informed consent and subsequently handled in accordance with protocols approved by the Johns Hopkins University IRB.

HIV-1 Sequencing and Epitope Selection

Peptides corresponding to previously described autologous variants found in HLA-B*57 restricted Gag and Nef proviral, plasma, and replication competent clones were synthesized (22–25). Replication-competent isolates from 2018 were obtained as previously described (3, 4). Briefly, unfractionated $CD4^+$ T cells were isolated by negative selection with Miltenyi beads and replicates of 1 million cells were stimulated with PHA (0.5 ug/ml) and 10 million irradiated allogeneic PBMCs. Four million healthy donor lymphoblasts were added on days 2 and 9 to amplify the virus and positive cultures were identified by p24 ELISA (Perkin Elmer). gag and nef were sequenced from 2 isolates as previously described (3, 4).

T Cell Culture

A total of 25 peptides representing autologous Gag and Nef epitope variants were synthesized by GenScript with a purity of > 85% and used to stimulate T cells in a modified version of the MANAFEST assay (13–15, 26), called ViraFEST. On day 0, peripheral blood mononuclear cells (PBMC) were isolated fresh from whole blood. T cells were isolated using the EasySep Human T Cell Enrichment Kit (Stemcell Technologies). The T cell and T cell-negative fraction were washed, counted, and resuspended at 2.5×10^6/mL in IMDM supplemented with 50 μg/mL gentamicin (ThermoFisher Scientific). The T cell-negative cells were added to a 96-well plate at 100 μL per well. An equal number of T cells was added to each well. Peptide antigens representing the 25 autologous epitope variants were added to individual wells (10 μg/mL), along with a CMV, EBV, and flu peptide pool (1 μg/mL; Miltenyi), and the Ebola virus AY9 epitope, ATAAATEAY, as a negative control, and a condition without peptide to evaluate non-specific T cell expansion. Each culture condition was evaluated in triplicate, with the exception of the ISPRTLNAW peptide, which was done in duplicate, for a total of 83 T cell cultures. An aliquot of 1M T cells was saved as the uncultured baseline condition. Cells were cultured for a total of 10 days at 37°C in a 5% CO_2 atmosphere. On day 3, half of the culture media was replaced with fresh culture media supplemented with 100 IU/mL IL2, 50 ng/mL IL7, and 50 ng/mL IL15 (for final concentrations of 50 IU/mL IL2, 25 ng/mL IL7, and 25 ng/mL IL15). On day 7, half of the culture media was replaced again with fresh media supplemented with 200 IU/mL IL2, 50 ng/mL IL7, and 50 ng/mL IL15 (for final concentrations of 100 IU/mL IL2, 25 ng/mL IL7, and 25 ng/mL IL15). On day 10, cultured cells were harvested and washed, and CD8+ T cells were isolated using the EasySep Human $CD8^+$ T Cell Enrichment Kit (Stemcell Technologies).

TCR Sequencing, Bioinformatic Processing, and Assessment of Antigen-Specific Expansions

DNA was extracted from each individual cultured $CD8^+$ T cell population, as well as the uncultured baseline $CD8^+$ T cells, using the QIAamp DNA micro kit (Qiagen). TCR Vβ CDR3 sequencing was performed using the survey sequencing ImmunoSEQ platform (Adaptive Biotechnologies). Conditions with less than 5,000 productive reads after TCR sequencing were excluded from further analysis (**Supplementary Table 1**). TCR sequencing. tsv files were exported from the ImmunoSEQ analyzer and were uploaded to our publicly-available MANAFEST analysis web app (http://www.stat-apps.onc.jhmi.edu/FEST/). This package prepares TCR sequencing files for analysis, which includes alignment and trimming of nucleotide sequences to obtain only

the CDR3 region and removal of nonproductive CDR3 sequences with premature stops or frame-shifts, sequences with an amino acid length <5, and sequences not starting with "C" or ending with "F/W".

We used a modified version of our statistical criteria to identify antigen-specific T cell clonotypes. To be classified as antigen-specific, a clonotype at the nucleotide level must (1) significantly expand in at least 2 out of 3 replicates relative to the no peptide control well at an FDR of 0.01, (2) only significantly expand in other wells with peptides from the same epitope family using an FDR of 0.01, and (3) have a frequency at least 5 times higher than the frequency of the clone in wells stimulated with peptides from a different epitope family. For visualization, the mean frequency +/- standard deviation of positive and negative expansions was graphed for each well.

RESULTS

Clinical Characteristics and Viral Evolution

ES8 is a previously described elite controller who had undetectable viral loads until 5 years prior to this study when he developed persistent low level viremia. The patient's HLA haplotype is A 02/03, B-57/44.

Autologous HIV-1 *gag* and *nef* was sequenced from provirus and plasma obtained in 2004, 2005, 2007, and 2010 and from replication-competent virus cultured in 2006 and 2018 (3, 21–24) as outlined in **Table 1**. The patient initially had wild type sequence in the HLA-B*57 restricted Gag epitopes TW10 and IW9 in proviral clones and in replication-competent virus (3, 22), but he consistently had variants in both epitopes in plasma clones starting in 2004, the earliest time point studied (22). These plasma variants evolved over time (24) and by 2018, replication-competent virus also contained multiple substitutions in both epitopes. A similar discrepancy between proviral and plasma variants was seen in the non-HLA-B*57 restricted Gag epitope KK15 and in the HLA-B*57 restricted Nef epitope KF9 (22, 23). In contrast there was concordance in sequence between proviral and plasma clones in the HLA-B*57 restricted Nef epitopes HW9 and YT9 (23).

Evaluation of Memory CD8$^+$ T Cell Responses to Autologous Gag and Nef Epitope Variants

We next assessed the memory T cell responses against autologous epitope variants using a modified version of the MANAFEST assay, called ViraFEST (Viral Functional Expansion of Specific T cells). A schema of the general experimental approach is shown in **Figure 1**. Three Gag epitope families were selected for functional validation (**Table 1**). T cells isolated from PBMC were stimulated with individual peptides. When testing mutation associated neoantigens (MANAs), there is customarily only one epitope tested per mutation (there are rarely 2 nonsynonymous passenger mutations in the same codon). However, owing to the possibility of memory T cells existing against closely-related HIV-1 epitope variants (33, 34), we performed each peptide stimulation in triplicate, with the exception of the ISPRTLNAW peptide, which was evaluated in duplicate, to increase the statistical power of identifying true antigen-specific TCRs for a total of 75 HIV-1 peptide-stimulated cultures. TCR sequencing was performed on DNA extracted from each T

TABLE 1 | Characteristics of the autologous and known epitopes evaluated for CD8$^+$ T cell recognition*.

Protein	AA sequence	Description	References
GAG TW10 (240-249)	TSTLQEQIGW	Consensus epitope	
		ES8 provirus, 2005, 2007, 2010	(3, 22, 24, 27)
		ES8 replication-competent virus 2006	(28)
	TSTLTEQVAW	ES8 plasma variant 2004 and 2008	(22, 24)
	TSTLAEQVAW	ES8 plasma variant 2008	(24)
	TSTLVEQIAW	ES8 plasma variant 2008	(24)
	TSTLAEQIAW	ES8 plasma variant 2009	(24)
	TSTLSEQVAW	ES8 plasma variant 2009	(24)
	TSTLSEQIAW	ES8 plasma variant 2009	(24)
	TSTLTEQIAW	ES8 plasma variant 2009	(24)
	TSTLQEQIEW	ES plasma variant 2004	(22)
	TSTLAEQMAW	ES8 replication-competent virus 2018	
	TSNLQEQIGW	Common escape mutant	(29–31)
	TSNLQEQIAW	Common escape mutant	(30)
GAG IW9 (147-155)	ISPRTLNAW	Consensus sequence: ES8 provirus 2005	(22, 24)
		ES8 replication-competent virus 2006	
	MSPRTLNAW	ES8 plasma variant, 2004, 2009. RC virus 2018	(22, 24)
GAG KK15 (17-31)	KIRLRPGGKKKYKLK	Consensus sequence. ES8 provirus 2005	(22, 24)
	KIRLRPGGKKRYKLK	ES8 plasma variant 2004.	(22, 24)
		ES8 replication-competent virus 2006	
NEF KF9 (82-90)	KAAVDLSHF	Consensus sequence	
	KSALDLSHF	ES8 provirus variant 2005	(3, 23, 25)
	TAALDMSHF	ES8 plasma variant 2004 and 2010. RC virus 2018	(23, 25)
	KGALDLSHF	Common variant	
	KAALDLSHF	Common variant	
NEF HW9 (116-124)	HTQGYFPDW	Consensus sequence.	
		ES8 replication-competent virus 2018	
	NTQGYFPDW	Previously described escape mutation	(32)
NEF YT9 (120-128)	YFPDWQNYT	Consensus sequence.	
		ES8 replication-competent virus 2018	
	FFPDWQNYT	ES8 provirus and plasma variant (2004, 2010)	(23, 25)

*All peptides except for Gag KK15 represent HLA-B*57 restricted epitopes. Residues highlighted in red are variants of the consensus sequence.*

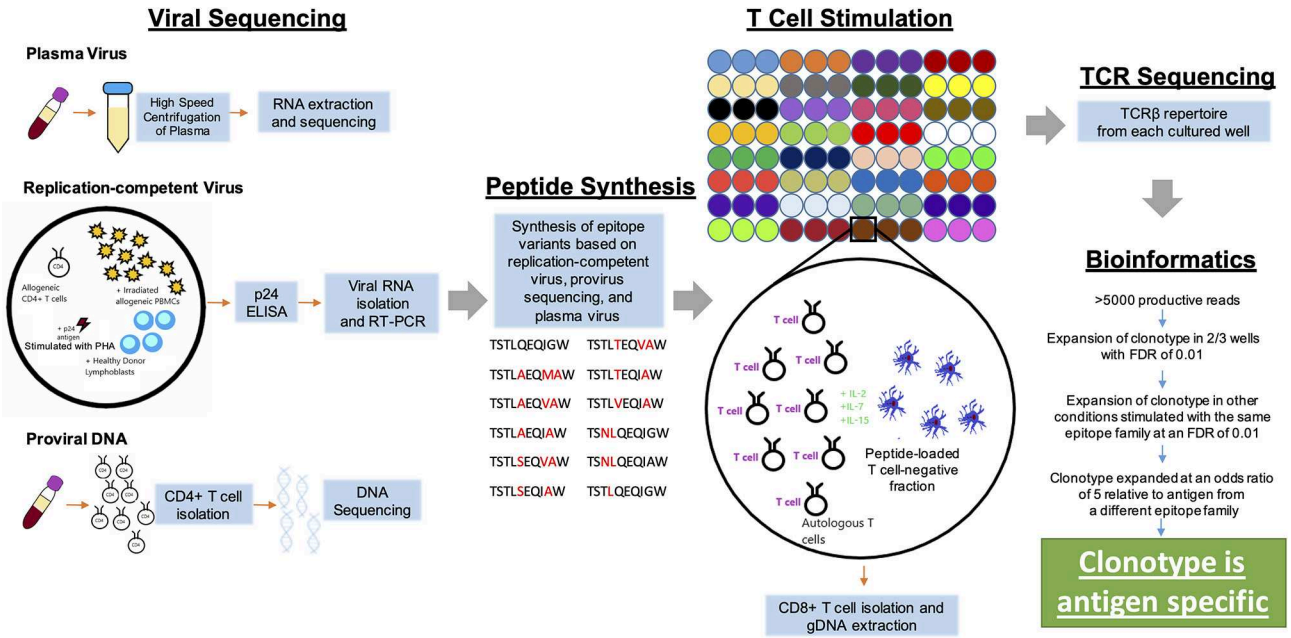

FIGURE 1 | Schematic diagram of the experimental approach. Autologous HIV-1 was sequenced from plasma, replication-competent virus, or proviral DNA. Peptides representing autologous variants of known HLA B57-restricted epitopes were synthesized and used to stimulated T cells in a modified version of the MANAFEST assay. TCR Vβ sequencing was performed on CD8+ T cells from each peptide-stimulated well, as well as uncultured and "no peptide" controls. A bioinformatic pipeline was then used to identify antigen-specific T cell clonotypes.

cell culture. After TCR sequencing, 5 of the 75 samples were removed from further analysis due to an insufficient number of productive reads (**Supplementary Table 1**). TCR sequencing files were run through a pre-processing and statistical analysis pipeline to identify antigen-specific T cell clonotypes. The conventional MANAFEST analytical platform relies on several key requirements to confirm antigen specificity, one of which is that the T cell clone must significantly expand in the relevant well relative to every other peptide-stimulated well. Because we evaluated each peptide in triplicate, and considering the potential for T cell cross-reactivity (16, 17), these criteria were modified to evaluate T cell responses to closely-related viral epitope variants where a given T cell clone could be specific for more than one epitope within an epitope family. Specifically, T cell clones should significantly expand in at least 2 out of 3 culture replicates and satisfy all additional criteria as described in the Methods to be considered antigen specific. Given that there are $\sim$1.4 x 10^6 unique TCR Vβ clonotypes in the memory T cell compartment of a given individual (35), and that we are evaluating responses to 4 different epitope families consisting of 25 unique epitopes, the probability of the same T cell clone expanding in multiple replicates of the same peptide by chance is less than 1.53×10^{-12}.

Three T cell clones specific for the Gag $p24_{235-243}$ TW10 epitope family were identified (**Figure 2**). The first clone, TGTGCCAGCAGCCCAGGGGTGGGGAACACTGAAGCTTTCTTT (CASSPGVGNTEAFF), significantly expanded at an FDR < 0.01 and an odds ratio of at least 5 in all three replicates in the T cell culture of the consensus TW10 epitope, TSTLQEQIGW, and satisfied all additional criteria to be considered antigen-specific. This consensus epitope was the predominant proviral variant seen in 2005, 2007, and 2009 (22, 24) (**Figure 2A**, **Supplementary Data 1**). Interestingly, this T cell clone was cross-reactive for the TSTLAEQIAW and TSTLSEQVAW variants that were both detected in plasma in 2009 (24) and the variant epitope TSTLAEQMAW that was present in replication-competent isolates cultured in 2018. There was no cross-reactivity with epitopes from other Gag or Nef families or the CEF- or ebola AY9-stimulated control wells. The second clone, TGTGCCAGCAGCTTAGATCCGGGGGCGAACACTGAAGCTTTCTTT (CASSLDPGANTEAFF), was also specific for the consensus TW10 epitope and was cross-reactive for the variant TSTLAEQMAW (**Figure 2B**, **Supplementary Data 1**). Although this clone significantly expanded in all three wells stimulated with the TSTLSEQVAW plasma variant from 2009, it only reached the minimum odds ratio threshold in one of the replicates and therefore cannot be considered specific for this antigen. The third clone, TGTGCCAGCAGCCCGAGACAGGCGGGTCTGGTGACCCAGTACTTC (CASSPRQAGLVTQYF) was specific for TSTLAEQMAW (**Figure 2C**; **Supplementary Data 1**) and did not demonstrate any cross-reactivity with other epitope family members.

Five T cell clones specific for the Nef_{81-90} KF9 epitope family and one clone specific for the $Nef_{116-124}$ HW9 epitope family were also identified (**Figure 3**). The first KF9-specific clone, TGTGCCAGCAGCCCTCGATGGGGGGACGCCGGGGAGCTGTTTTTT (CASSPRWGDAGELFF), recognized KAALDLSHF, a

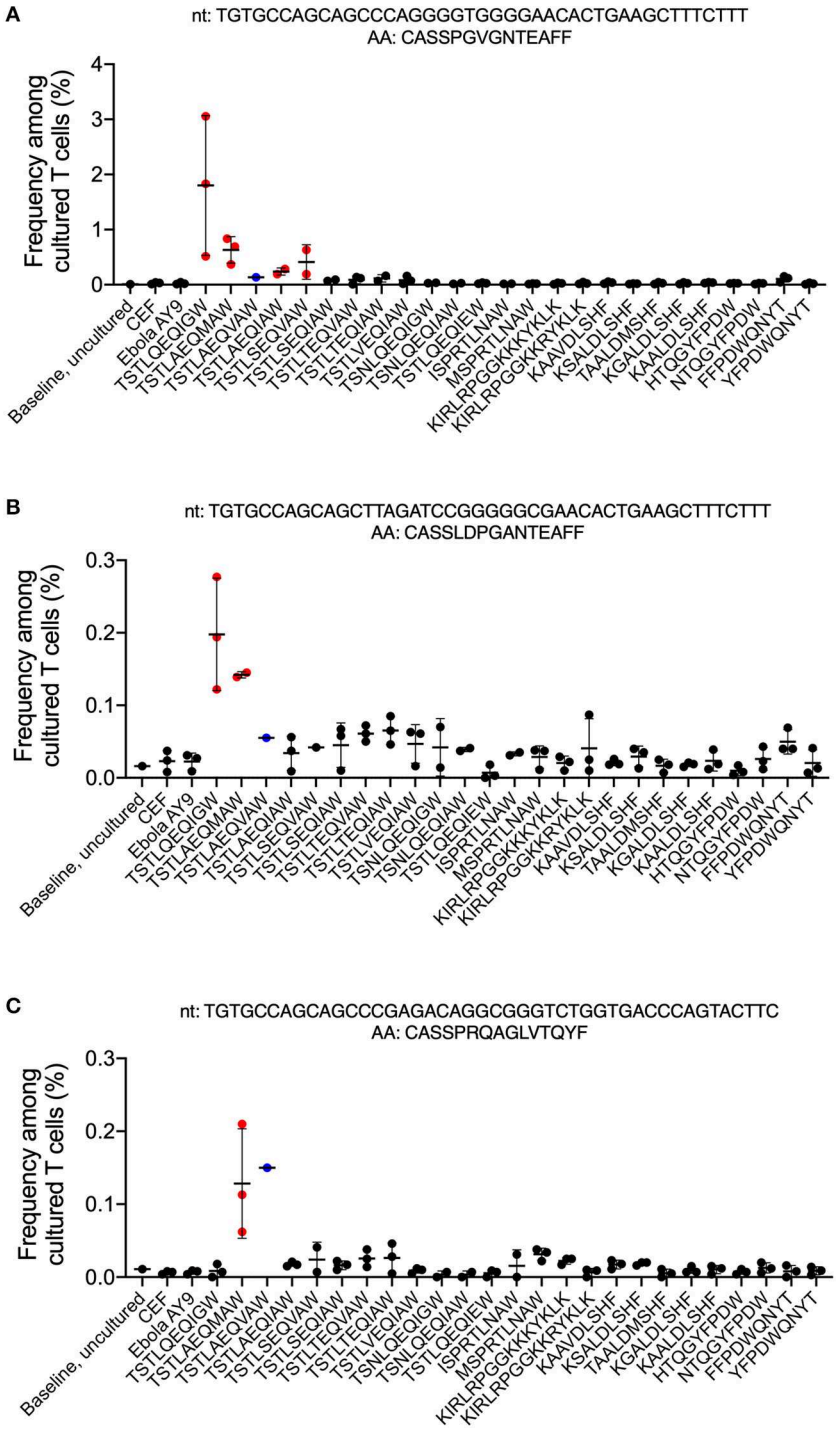

FIGURE 2 | CD8$^+$ T cell responses to autologous HIV-1 Gag epitope variants. Peptides representing autologous and known variants of the HIV-1 $Gag_{240-249}$ and Gag_{17-31} HLA-B57-restricted epitopes and the HLA mismatched epitope Gag_{17-31} epitope (**Table 1**) were synthesized and used to stimulate T cells in the ViraFEST assay. A bioinformatic pipeline that determines antigen specificity identified 3 T cell Vβ clonotypes **(A-C)** that were antigen-specific. The nucleotide (nt) and amino acid (AA) sequence for each clone is shown above their respective graph. The mean frequency (%) after culture +/- standard deviation is shown for each condition where the clonotype contributed to a positive (red) or negative (black) T cell response. In the TSTLAEQIAW-stimulated condition, two of the three triplicates were excluded from analysis, and therefore no determination of a positive or negative response could be determined (blue).

common variant of the consensus epitope and was cross-reactive for the KSALDLSHF variant which was present in proviral clones in 2005 and 2010 (23, 25) (**Figure 3A**, **Supplementary Data 1**). The second clone, TGTGCCTGGGAGACAGGGGTTAGGGA TGGCTACACCTTC (CAWETGVRDGYTF), only recognized KAALDLSHF (**Figure 3B**, **Supplementary Data 1**). The third clone, TGTGCCAGCAGCCTGTTAGCGGGAGGGAGCCTTG ATGAGCAGTTCTTC (CASSLLAGGSLDEQFF), demonstrated the most cross-reactivity, recognizing KAAVDLSHF (the consensus epitope), KAALDLSHF, and KSALDLSHF (**Figure 3C**, **Supplementary Data 1**). The fourth clone, TGCGCCAGCAG CTTGGATTTACGGACCTTTACTTACGAGCAGTACTTC (CASSLDLRTFTYEQYF), was reactive against KAALDLSHF and KSALDLSHF (**Figure 3D**, **Supplementary Data 1**) and the fifth clone, TGCGCCAGCAGCTTGGAGAGGGTGGGC TACAATGAGCAGTTCTTC (CASSLERVGYNEQFF) only recognized KAALDLSHF (**Figure 3E**, **Supplementary Data 1**). There were no T cell responses against the TAALDMSHF variant that was present in plasma in 2005 and 2010 (23, 25) and in replication-competent virus from 2018. The only clone recognizing the HW9 family, TGTGCCATCAGCCTCATGG GCACTGAAGCTTTCTTT (CAISLMGTEAFF), was specific

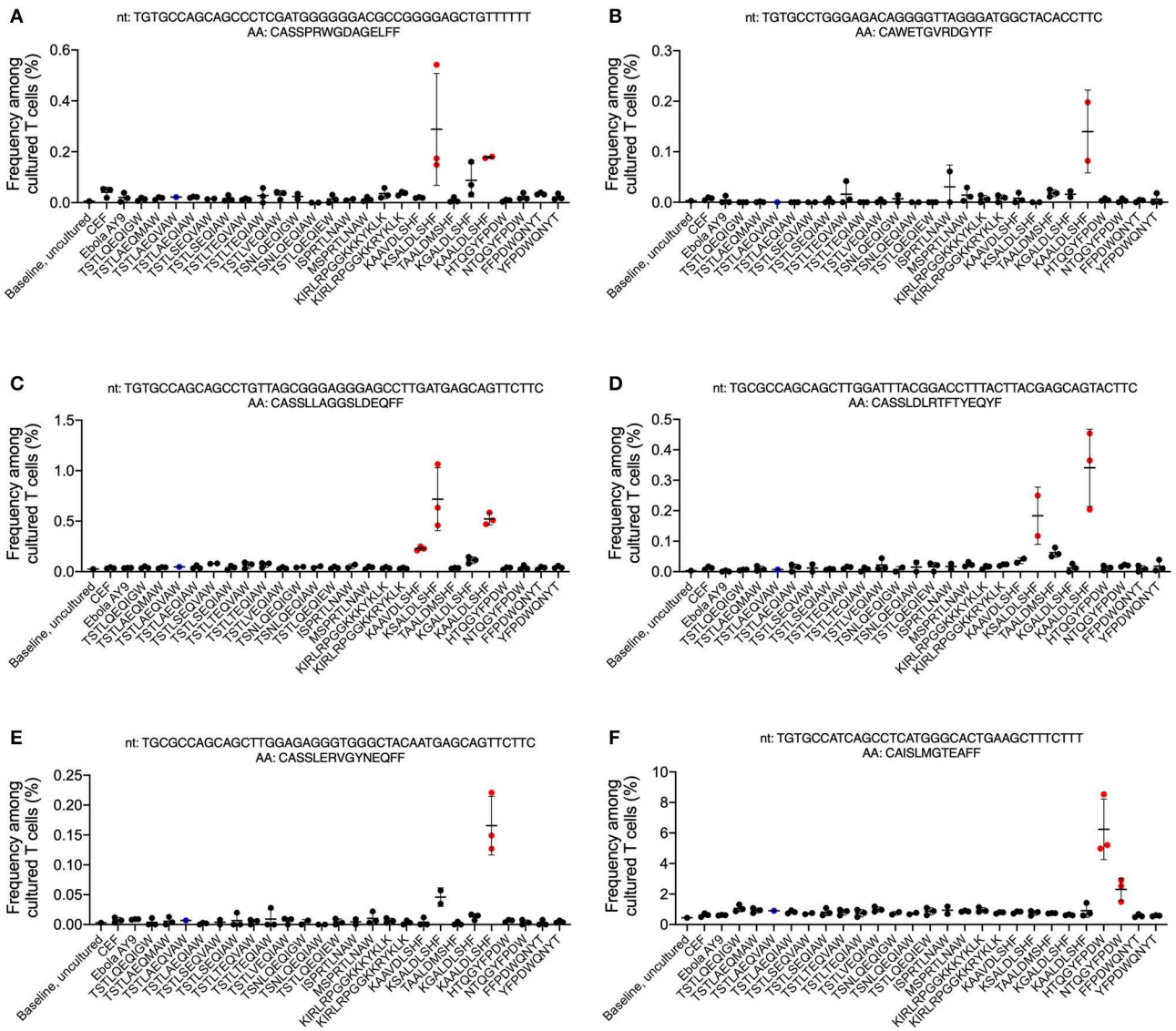

FIGURE 3 | CD8$^+$ T cell responses to autologous HIV-1 Nef epitope variants. Peptides representing autologous and known variants of the HIV-1 Nef$_{82-90}$, Nef$_{116-1204}$, and Nef$_{120-128}$ HLA-B57-restricted epitopes (**Table 1**) were synthesized and used to stimulate T cells in the ViraFEST assay. A bioinformatic pipeline that determines antigen specificity identified 6 T cell Vβ clonotypes **(A–F)** that were antigen-specific. The nucleotide (nt) and amino acid (AA) sequence for each clone is shown above their respective graph. The mean frequency (%) after culture +/- standard deviation is shown for each condition where the clonotype contributed to a positive (red) or negative (black) T cell response. In the TSTLAEQIAW-stimulated condition, two of the three triplicates were excluded from analysis, and therefore no determination of a positive or negative response could be determined (blue).

for the HTQGYFPDW consensus epitope which was present in replication-competent virus from 2018 and the NTQGYFPDW escape variant (**Figure 3F**, **Supplementary Data 1**).

Taken together, these data demonstrate the feasibility of using a modified ViraFEST analytical platform to identify cross-reactive T cell clonotypes in an ES.

DISCUSSION

This is the first use of the MANAFEST assay to evaluate T cell responses to HIV-1 antigens. This assay, which has been used to identify memory $CD8^+$ T cell responses to tumor-derived neoantigens (13–15, 26), uses TCR Vβ sequencing of peptide-stimulated T cell cultures and a bioinformatic pipeline to identify antigen-specific T cell clonotypes. In this proof of concept study, we chose to evaluate HIV-1-specific responses in ES8. ES serve as a model of a T cell-mediated functional cure of HIV. HLA-B*57 and HLA-B*27 are over-represented in ES (36, 37) and a CTL response that is focused on HLA-B*57 restricted epitopes is associated with elite control (36). In prior studies we analyzed HLA-B*57 restricted epitopes in proviral and plasma clones from ES and found that while wild-type consensus sequences were present in most proviral clones, plasma clones contained multiple substitutions in these epitopes (22). While some of these substitutions were previously well-described escape mutations such as the T242N mutation in the TW10 Gag 240-249 epitope (29–31), ES8 plasma variants had rare mutations in this epitope that were not recognized by TW10-specific $CD8^+$ T cells from other ES. However, $CD8^+$ T cells from ES8 and other ES generally recognized autologous plasma variants (22, 28), perhaps explaining why control of viral replication was maintained despite apparent virologic escape. This recognition of autologous TW10 escape variants has previously been reported (22, 27, 38–40), but the mechanism has not been identified. Specifically it's not known whether there is cross-recognition of wild type and escape variants by the same $CD8^+$ T cell clonotypes or whether distinct sets of $CD8^+$ T cell clonotypes recognize wild type and autologous escape mutants. Differentiating between the two possibilities will be important for developing T cell mediated cure strategies as escape mutants are frequently archived in the latent reservoir (32).

In this study, we identified several T cell clonotypes that recognized several wild type HLA-B*57 restricted epitopes as well as autologous variants. Two clones specific for the wild type TW10 epitope cross-reacted with variants that were found in plasma and later in replication-competent virus. A third clone recognized just the most-recent variant that was present in replication-competent virus suggesting that it was a true de novo response to a recent escape variant. In contrast, no detectable responses were made to some prior plasma variants or to epitopes containing the common T242N mutation that was never detected in ES8.

Five clones were specific for a common variant of the Nef KF9 epitope. Although this variant was never detected in any viral clones from ES8, it could have been present in the initial transmitter/founder clone and/or present at a level below the limit of detection. Three of the five $CD8^+$ T cell clones cross reacted with the proviral variant of this epitope which may represent an early escape mutation that was archived into the reservoir. Finally, the clone specific for the Nef wild type epitope HW9, cross reacted with NW9 a well characterized escape mutation (41) that was not detected in ES8.

While HIV-1-specific cross-reactivity at the clonotype level has been previously described (18), this is the first use of a TCR immunogenomic platform to identify HIV-1-specific T cell responses. As described previously (13), our MANAFEST-based approach is more sensitive than conventional IFNγ ELISpot assays and has the advantage of being able to identify oligo- and polyclonal T cell responses to antigens. Additionally, once HIV-1-specific clonotypes are identified, the TCR can be used as a molecular barcode to track these antigen-specific cells across biological compartments or in serial blood draws. This provides a benefit for immune monitoring of treatment and therapeutic interventions because the functional assay only needs to be performed once. Our approach is novel and has the potential for broad utility within and outside the HIV research community, however we recognize that this assay is costlier and more bioinformatically-intensive compared to traditional ELISpot assays. Additionally, the limitations of this approach should be considered before being implemented in routine monitoring of immune responses. First, the ViraFEST assay only identifies the Vβ chain corresponding to antigen-specific T cells. Therefore, in order to investigate engineered T cell therapy approaches additional assays would have to be performed to enumerate the Vα chain. Second, the TCR identity does not inform on function or cytokine profile, only that the clonotype is capable of proliferating specifically in response to antigenic stimulation. Although our study serves a proof-of-principle for using this assays when detecting T cell responses against autologous HIV-1 epitope variants, we only studied a single subject and additional studies should be performed to determine if clonotypic cross-reactivity is more broadly seen in ES. Furthermore, while this study was only performed on cells from one time point, the repeatability of this assay has been demonstrated previously in two separate studies using cells from the same patient obtained at different time points for viral antigens (13) and neoantigens (13, 14).

Not only does this serve as the foundation for implementing this assay during routine monitoring of HIV-1-specific responses, but it could be used to identify vaccine or T cell-based immunotherapeutic targets that were not previously identified with conventional immune assays. In this way, determining the clonotypic identity of HIV-1-specific T cells paves the way for engineered T cell therapies that could be used across many patients with common epitope variants and HLA alleles.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Adaptive Biotechnologies ImmuneACCESS Database, https://clients.adaptivebiotech.com/pub/chan-2020-fi.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Johns Hopkins IRB. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HC and CG performed the experiments described here. JZ analyzed the experimental data. AK analyzed the clinical data. JB and KS conceived of the experiments, analyzed the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00591/full#supplementary-material

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Conflict of Interest: KS has received honoraria from Illumina, Inc. and has filed for patent protection for a subset of the technology described herein (serial number 16/341,862).

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Redirection of Cord Blood T Cells and Natural Killer Cells for Elimination of Autologous HIV-1-Infected Target Cells Using Bispecific DART® Molecules

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Mother-to-child transmission of HIV-1 remains a major global health challenge. Currently, HIV-1-infected infants require strict lifelong adherence to antiretroviral therapy to prevent replication of virus from reservoirs of infected cells, and to halt progression of disease. There is a critical need for immune interventions that can be deployed shortly after infection to eliminate HIV-1-infected cells in order to promote long-term remission of viremia, or to potentially cure pediatric HIV-1-infection. Bispecific HIV × CD3 DART® molecules able to co-engage the HIV-1 envelope protein on the surface of infected cells and CD3 on cytolytic T cells have been previously shown to eliminate HIV-1 infected cells *in vitro* and are candidates for passive immunotherapy to reduce the virus reservoir. However, their potential utility as therapy for infant HIV-1 infection is unclear as the ability of these novel antibody-based molecules to work in concert with cells of the infant immune system had not been assessed. Here, we use human umbilical cord blood as a model of the naïve neonatal immune system to evaluate the ability of HIV x CD3 DART molecules to recruit and redirect neonatal effector cells for elimination of autologous $CD4^+$ T cells infected with HIV-1 encoding an envelope gene sequenced from a mother-to-child transmission event. We found that HIV × CD3 DART molecules can redirect T cells present in cord blood for elimination of HIV-infected $CD4^+$ T cells. However, we observed reduced killing by T cells isolated from cord blood when compared to cells isolated from adult peripheral blood—likely due to the absence of the memory and effector $CD8^+$ T cells that are most cytolytic when redirected by bispecific DART molecules. We also found that newly developed HIV × CD16 DART molecules were able to recruit CD16-expressing natural killer cells from cord blood to eliminate HIV-infected cells, and the activity of cord blood natural killer cells could be substantially increased

by priming with IL-15. Our results support continued development of HIV-specific DART molecules using relevant preclinical animal models to optimize strategies for effective use of this immune therapy to reduce HIV-1 infection in pediatric populations.

Keywords: pediatric HIV-1, umbilical cord blood, cytotoxic T cells, natural killer cells, redirected cytotoxicity, bispecific DART molecules

INTRODUCTION

Despite the effectiveness of perinatal and postnatal antiretroviral prophylaxis, and the relatively low transmission rates of HIV-1 *in utero*, intrapartum, and during breast-feeding, there remain over 160,000 new pediatric HIV-1 infections annually worldwide (1–4). The overwhelming majority of these infections occur perinatally, via mother-to-child transmission. Treatment is critical as infant HIV-1 peak and set-point viral loads are typically 10-fold above those observed for primary adult infection and are highly predictive of disease outcome (5–12). With proper adherence, antiretroviral therapy (ART) can successfully control viremia in infants, and if initiated shortly after birth, ART may appreciably limit the size of the latently infected cell reservoir (13, 14). However, evidence to date suggests that strict adherence to ART will need to be maintained lifelong to prevent reemergence of virus replication resulting from reactivation of latent infection. Therefore, to provide long-term control or cure of pediatric HIV infection and abrogate the need for lifelong viral suppression, early initiation of ART will need to be combined with additional strategies to eliminate HIV-1-infected cells responsible for persistence of the virus reservoir.

One promising approach to increase elimination of HIV-infected cells is passive immunotherapy with antibody-based molecules capable of recruiting and redirecting endogenous cytolytic effector cells (15, 16). Bispecific HIV × CD3 DART® molecules based on HIV-specific monoclonal antibodies (mAbs) can effectively mediate *in vitro* elimination of HIV-1 infected and reactivated latently infected cells (17, 18). Bispecific HIV × CD3 DART molecules use a monovalent HIV-targeting arm comprised of the antigen-binding region of mAbs specific for highly conserved regions of the HIV envelope protein (Env) to recognize HIV-1-infected target cells, and a monovalent CD3 binding arm for recruitment of cytolytic T cells. Only when both arms are co-engaged will polyclonal T cells be activated and redirected for cytolytic responses against Env-expressing, HIV-1-infected target cells in a major histocompatibility complex-independent manner (18, 19). As a result of these properties, HIV × CD3 DART molecule-mediated activity should be unaffected by mutations among circulating or latent viruses that confer escape from viral-specific T-cell responses, or by the low frequency and functionality of HIV-specific T cells in patients on ART (20, 21). Therefore, passive immunization with HIV × CD3 DART molecules could form the basis of a strategy for cure of HIV by combining early initiation of ART to control viral load and reduce the size of the reservoir with concurrent initiation of HIV × CD3 DART molecule immunotherapy to eliminate infected cells. Once viral load is below detection, ART and DART molecule immunotherapy would be maintained with the addition of compounds that reactivate latent virus-infected cells to generate targets for DART molecule-mediated clearance.

Newborn infant infection resulting from mother-to-child transmission of HIV-1 (MTCT) likely represents the most favorable clinical context for successful passive immunotherapy to eliminate the reservoir of HIV-infected cells. Therapy can be initiated shortly after birth, and therefore near the time of transmission events occurring late *in utero*, during labor and delivery, or via breast milk —likely prior to the establishment of large populations of latently infected cells and the onset of immune dysregulation (22–25). However, neonatal cytolytic immune cells have phenotypic and functional differences compared to the same cell populations in adults (26, 27), and the impact of these differences on the activity of cytotoxic antibody-based immunotherapeutic drugs is not known. Thus, it is crucial to first evaluate infant passive immunotherapies in a model system that recapitulates the effector cells present in newborn infants.

In this study, we used human umbilical cord blood as surrogate for neonatal peripheral blood to test the hypothesis that bispecific DART molecules could redirect neonatal effector cells for elimination of cells infected with HIV-1 encoding an envelope gene sequenced from a mother-to-child transmission event. Our data demonstrate that HIV × CD3 DART molecules can redirect cord blood T cells to kill autologous cord blood $CD4^+$ T cells infected by HIV-1 *in vitro*. However, we observed reduced killing by T cells isolated from cord blood when compared to those isolated from adult peripheral blood. We also found that HIV × CD16 DART molecules can recruit and redirect natural killer (NK) cells from cord blood to eliminate autologous HIV-infected $CD4^+$ T cells; and we determined that the redirected activity of cord blood NK cells can be substantially increased by priming with IL-15. Our data suggest that HIV-specific DART molecules combined with current ART regimens may provide a novel treatment option intended to improve virus control, promote long-term remission, or cure pediatric HIV-1 infection. Our data also indicate that strategies to optimize and enhance the cytotoxic potential of neonatal effector cells, or allowing additional time after birth for the immune system to develop, will likely be needed to maximize the therapeutic potential of HIV-specific DART molecules for use in pediatric populations.

MATERIALS AND METHODS

Study Samples

Blood and Mononuclear Cell Samples

Anonymized human umbilical cord blood donations that failed to meet the volume and/or nucleated cell count criteria required for clinical use were obtained with informed written

consent. Human peripheral venous blood was collected by leukapheresis from healthy consenting adult volunteers (28, 29). All samples were collected in accordance with the policies and regulations of the Duke Health Institutional Review Board. Cord blood mononuclear cells (CBMC) and adult peripheral blood mononuclear cells (PBMC) were processed from umbilical cord blood and human peripheral blood, respectively, using density gradient centrifugation with Ficoll-Paque plus (GE Healthcare Life Sciences, Pittsburg, PA), and were cryopreserved in 10% DMSO 90% Fetal Bovine Serum (FBS). All samples were processed within 12 h after collection.

Bispecific HIV × CD3 and HIV × CD16 DART Molecules

HIV × CD3 DART molecules were constructed with anti-HIV-1 envelope specificities based on non-neutralizing mAbs, 7B2 or A32, or an irrelevant control specificity based on the anti-respiratory syncytial virus (RSV) mAb, palivizumab, and with an anti-human CD3ε specificity based on the mAb, hXR32, as described previously (18, 30). These molecules are referred to as 7B2 × CD3, A32 × CD3, and RSV × CD3. HIV × CD16 DART molecules were constructed with anti-human CD16 specificities based on mAb h3G8 as the effector arm, which included 7B2 × CD16 and 4420 × CD16, where 4420 is an anti-fluorescein (control) specificity, and Fc-bearing ones based on mAb h5H2 as the effector arm included 7B2 × CD16, A32 × CD16, and RSV × CD16. The human immunoglobulin G1 (IgG1) Fc domain, which is incorporated to prolong circulating half-life *in vivo*, contains mutations to greatly reduce or eliminate effector function via binding to Fc-gamma receptors (FcγRs) and complement, while retaining binding to the neonatal Fc receptor (FcRn) to take advantage of the IgG salvage pathway mediated by this receptor.

Laboratory Methods

Phenotypic Characterization of T Cells

Immunophenotyping of human T cells was performed using flow cytometry analyses. Cryopreserved PBMC (5 donors) and CBMC (14 donors) were thawed and incubated overnight (18 h) in RPMI 1640 medium supplemented with 10% FBS at 37°C, 5% CO_2. The cells were then washed with buffered saline and stained with a viability marker (Fixable Aqua Dead Cell Stain Kit, Thermo Fisher Scientific, San Diego, CA) prior to surface and intracellular staining with fluorescently conjugated antibodies using standard techniques as described (31). The staining panel used to identify T cell subsets and phenotypes was based on the Optimized Multicolor Immunofluorescence Panel 22 (OMIP-22) described previously (31). Fluorescently conjugated Abs used for cell surface staining were: APC-eFluor780-CD4 (clone RPA-T4, ebioscience, San Diego, CA); Horizon V500-CD14 (clone M5E2, BD Biosciences, San Jose, CA); Horizon V500-CD19 (clone HIB19, BD Biosciences); BV785-CD45RO (clone UCHL1, Biolegend, San Diego, CA); BV605-CCR7 (clone G043H7, Biolegend); and APC-CD366 (clone F38-2E2, Biolegend). Intracellular staining was performed with PE-TR-CD3 (clone UCHT1, Beckman Coulter, Brea, CA); and Ax700-CD8 (clone HIT8a, Biolegend). Data analyses were performed using FlowJo software (v10.5.3) from BD Biosciences.

Phenotypic Characterization of NK Cells and CD16 DART-Molecule NK Cell Binding

Immunophenotyping of human NK cells was performed as previously described (32). Cryopreserved PBMC collected from 15 healthy normal adult donors, and CBMC from 15 umbilical cord blood donors, were thawed and incubated overnight (18 h) in RPMI1640 medium supplemented with 10% FBS, or in RPMI 1640 medium supplemented with 10% FBS and 10 ng/mL recombinant human IL-15 (Miltenyi Biotec, GmbH) at 37°C, 5% CO_2. The cells were then washed with buffered saline and stained with a viability marker (Fixable Aqua Dead Cell Stain Kit, Thermo Fisher Scientific) prior to surface and intracellular staining with fluorescently conjugated Abs using standard techniques as described previously (33). Fluorescently conjugated antibodies used for surface staining were based on OMIP-007 and are as follows: PE-TR-CD3 (clone S4.1, Thermo Fisher Scientific); APC-H7-CD4 (clone SK3, BD Biosciences); PE-Cy5-CD14 (clone Tuk4, Thermo Fisher Scientific); PE-Cy5-CD19 (clone SJ25-C1, Thermo Fisher Scientific); PacificBlue-CD16 (clone 3G8, BD Biosciences); PE-Cy7-CD56 (clone NCAM16.2, BD Biosciences); BV606-CD62L (clone DREG-56, Biolegend); FITC-HLA-DR (clone G46-6, BD Biosciences); APC-CD57 (clone HCD57, Biolegend); and BV785-CD69 (clone FN50, Biolegend). Intracellular staining was performed with BV711-Perforin (clone dG9, Biolegend), and PE-Granzyme B (clone GB11, BD Biosciences). Quantum™ Simply Cellular® beads (Bangs Laboratories, Inc., Fishers, Indiana) were used to determine the antibody binding capacity (ABC) of perforin and granzyme within cells according the manufacturers recommended procedure. Data analyses were performed using FlowJo software (v10.5.3).

To determine if DART molecules with CD16 targeting arms could specifically bind the surface of human NK cells, we incubated purified human NK cells with DART molecules at 1 μg/ml for 40 min at room temperature. Cells were then washed (PBS with 1% FBS) and incubated with 1 μg/ml mouse anti-EK antibody (recognizes the E/K heterodimerization region of DART molecules, MacroGenics) for 20 min at 4°C in wash buffer. Cells were then washed and incubated with PE rat-anti mouse IgG1 (BD Biosciences), at the manufactures recommended concentration, in wash buffer, for 25 min at 4°C. Finally, cells were washed, fixed, and analyzed by flow cytometry. Data are reported as mean fluorescent intensity (MFI) of PE.

Redirected T Cell Cytotoxicity Assay Against Autologous HIV-1 Env-Expressing $CD4^+$ T Cells

Redirected T cell cytotoxicity assays were performed with methods similar to those previously described (18). Cryopreserved resting CBMC and PBMC from normal healthy donors were activated for 72 h with anti-human CD3 antibody (clone OKT3, eBioscience), anti-human CD28 antibody (clone 28.2, BD Biosciences) each at 150 ng/mL, and recombinant human IL-2 (30 U/mL, Proleukin, Prometheus Therapeutics and Diagnostics) in RPMI 1640 media supplemented with 20% FBS. Next, $CD8^+$ T cells were depleted using magnetic beads (Miltenyi Biotec), leaving a $CD4^+$ T cell-enriched population that was then infected with HIV-1 infectious molecular clone virus

(HIV-1 4403bmC5) encoding a subtype C infant postnatally-transmitted/founder envelope protein (34) and Tat-inducible *Renilla* luciferase reporter gene (35), by spinoculation as described (36). Where indicated, cells were alternatively infected with an infectious molecular clone virus representing HIV-1 subtype B isolate BaL. After 48 h of infection, the $CD4^+$ T cells were incubated with $CD8^+$ T cells purified from autologous PBMC or CBMC using negative selection with magnetic beads (human $CD8^+$ T cell isolation kit, Miltenyi Biotec) at a $CD8^+$ T cell to target cell ratio of 30:1 in ½ area opaque flat bottom plates (Corning Life Sciences, Corning, NY). HIV × CD3 DART molecules were added, in duplicate, using 10-fold serial dilutions starting at 1 μg/mL, and the plates were incubated for an additional 24 h at 37°C, 5% CO_2. Control plates included only infected $CD4^+$ target cells and DART molecules without autologous $CD8^+$ T effector cells. Percent of specific killing was calculated based on the change in Relative Light Units (RLU) (ViviRen luciferase assay; Promega, Fithchburg, WI) resulting from the loss of live, intact target cells in test wells containing effector cells, target cells, and DART molecules relative to RLU in control wells containing target cells and effector cells alone (without DART molecules) according to the following formula: percent of specific killing = [(number of RLU of control well – number of RLU of test well)/number of RLU of control well] ×100. Positive responses were defined as specific killing >20%. Data were fit to a sigmoidal dose-response function for graphing and for determination of 50% effective concentrations (EC_{50}). In most cases, data are reported as positive area under the curve (pAUC), defined as the area under the log-transformed dilution curve that is above the threshold for positivity (20% specific killing), calculated with the trapezoidal method using SAS software (SAS Institute Inc., Cary, NC).

In vitro Evaluation of Strategies to Improve Redirected T Cell Cytotoxicity

Exogenous cytokines and T-cell co-stimulators were incorporated into the redirected T cell cytotoxicity assay to test their ability to improve the cytolytic activity of T cells present in CBMC and PBMC. The impact of exogenous IL-12 was evaluated by overnight incubation of the CBMC and PBMC with exogenous recombinant human IL-12 (10 ng/mL, Miltenyi Biotec), before isolation of $CD8^+$ T cells used as effector cells in the redirected cytotoxicity assay. IL-12 was maintained in the assay plates at 10 ng/mL throughout the 24-h killing assay. The impact of coreceptor signaling was assessed by incubating the CBMC and PBMC overnight with anti-CD28 (150 ng/mL, clone 28.2, BD Biosciences) or anti-CD137 (150 ng/mL, clone 4B4-1, BioLegend), prior to isolation of $CD8^+$ T cells, and maintaining the presence of the anti-coreceptor antibodies throughout the killing assay. Experiments were also performed using exogenous IL-12, anti-CD28, and anti-CD137 together, or with cells stimulated for 48 h by incubation with 150 ng/mL anti-human CD3 antibody (clone OKT3, eBioscience), anti-human CD28 antibody, and 30 U/mL recombinant human IL-2 in RPMI 1640 media supplemented with 20% FBS prior to isolation of $CD8^+$ T cells. In one set of experiments, T cells in CBMC and PBMC were activated for 72 h with anti-CD3, anti-CD28, and IL-2, before isolation of $CD8^+$ T cells, which were then allowed to rest for 72 h in absence of any stimulation before use as effector cells according to the methods described above.

Redirected NK Cell Cytotoxicity Assay Against Autologous HIV-1 Env-Expressing $CD4^+$ T Cells

Cryopreserved CBMC and PBMC samples were thawed and rested overnight (18 h) at 37°C 5% CO_2 in RPMI 1640 media supplemented with 10% FBS, or media supplemented with 10 ng/mL recombinant human IL-15 (Miltenyi Biotec), as previously described (32). Subsequently, NK cells were purified by negative selection with magnetic beads (human NK cell isolation kit, Miltenyi Biotec) and used as effector cells (NK cell to target cell ratio of 5:1) in redirected cytotoxicity assays using HIV × CD16 DART molecules, performed as described above. Positive responses were defined as specific killing >20%, and data were not fit to a dose-response function for graphing due to the presence of a prozone (37).

CD107a Degranulation Assay

Cell-surface expression of CD107a was used as a marker for T cell degranulation (38). Redirected cytotoxicity assays were performed as described above, with the following modification [as previously described in (18)]. After plating the target cells, effector cells, and DART molecules, the plate was incubated for 18 h and then FITC- or APC-Cy7-CD107a antibody (clone H4A3, BD Biosciences), brefeldin A (GolgiPlug, 1 μL/mL, BD Biosciences), and monensin (GolgiStop, 4 μL/6mL, BD Biosciences) were added to each well and the plates were incubated for an additional 6 h. Plates were then washed and stained with a viability marker and fluorescently conjugated antibodies as described above (phenotypic characterization of T cells), or with a truncated staining panel that included only antibodies specific for CD3, CD4, and CD8, using standard techniques. Data analyses were performed using FlowJo software (v10.5.3).

Statistical Methods

Kruskal Wallis tests were used to compare response magnitudes across groups. In order to assess if two groups have different response magnitudes, pairwise comparisons between groups were conducted using Wilcoxon rank sum test. Two-tailed $p < 0.05$ were considered significant. Statistical analysis was performed using SAS software (SAS Institute Inc.).

RESULTS

Cord Blood T Cells Have Modest Ability to Eliminate Autologous HIV-Infected CD4 T Cells Directed by HIV x CD3 DART Molecules *in vitro*

We and others have demonstrated that bispecific DART molecules able to co-engage HIV-1 Env on infected cells, and CD3 antigen on cytolytic T cells, can be used to eradicate acutely infected and reactivated latently-infected cells *in vitro* (17, 18). However, these initial studies were performed using immune cells from adults —the ability of these novel antibody-based molecules

to work in concert with cells present in the neonatal immune system cells had not been explored. To address this limitation, we performed redirected T cell cytotoxicity assays using CBMC as a source of neonatal $CD8^+$ T cells for use as effectors, and autologous cord blood $CD4^+$ T cells for use as targets after infection with HIV-1 *in vitro*. Target cells were infected with a subtype C HIV-1 infectious molecular clone virus that encodes the *env* gene from HIV-1 4403bmC5, a postnatally-transmitted founder virus isolated from infant plasma, and identical to a virus isolate present in matched maternal breast milk (34). Thus, this isolate is expected to be a representative of those present in the early active and latent virus reservoirs of infected infants. Assays were also performed using adult PBMC as a source of effector cells and autologous HIV-1 4403bmC5-infected target cells for comparison. We observed minimal background activity (specific killing <20%) by the negative control DART molecule, RSV × CD3, in assays performed with $CD8^+$ T cells and autologous 4403bmC5-infected $CD4^+$ cells isolated from adult PBMC (black lines and open squares, **Figure 1A**) or CBMC (blue lines and circles, **Figure 1A**). In contrast, HIV-specific DART molecules, 7B2 × CD3 and A32 × CD3, mediated specific killing activity with cells isolated from most donors (80% of CBMC samples, and 89% of PBMC samples with 7B2 × CD3; 17% of CBMC and 100% of PBMC samples with A32 × CD3) as shown in **Figures 1B,C**, respectively. However, we observed that the specific killing activity of cells from cord blood is reduced when compared to those from adult PBMC for the majority of samples, both in terms of maximum observed killing activity (**Figures 1B,C**), and EC_{50} (mean ∼100 ng/mL for 7B2 × CD3 with CBMC samples that were positive for killing, and ∼4 ng/mL with positive PBMC samples). To better compare the killing activities, we calculated the positive area under the dilution curves (**Figure 1D**), and confirmed that specific killing of HIV-1 4403bmC5-infected cells was significantly lower in assays performed with cells isolated from CBMC compared to adult PBMC ($p \leq 0.001$, Wilcoxon rank sum tests). These data demonstrate that HIV-specific DART molecules can redirect cord blood T cells to kill HIV-1 infected, Env-expressing, autologous cord blood $CD4^+$ T cells, however with activity subordinate to that mediated by adult peripheral blood T cells. Therefore, we next sought to identify characteristics of cord blood T cells that may limit their functionality when redirected by HIV-specific DART molecules.

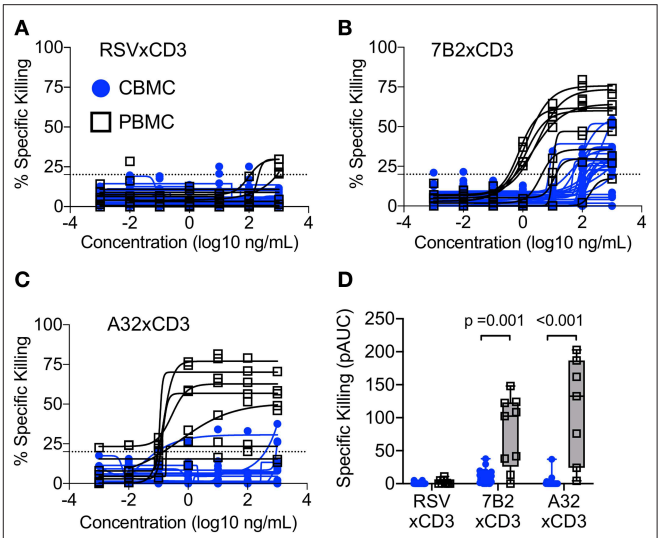

FIGURE 1 | Redirection of cytolytic T cells to autologous HIV-infected $CD4^+$ T cells by HIV × CD3 DART molecules. The ability of T cells isolated from cord blood mononuclear cells (CBMC, blue lines, and symbols) and adult peripheral blood mononuclear cells (PBMC, black lines, and open symbols) to eliminate autologous $CD4^+$ T cells infected with an HIV-1 molecular clone virus representing an infant postnatally-transmitted founder isolate (HIV-1 4403bmC5) after being redirected by **(A)** RSV × CD3 control DART molecules ($n = 25$ CBMC samples, $n = 9$ PBMC samples), **(B)** 7B2 × CD3 DART molecules ($n = 25$ CBMC samples, $n = 9$ PBMC samples), and **(C)** A32 × CD3 DART molecules ($n = 18$ CBMC samples, $n = 7$ PBMC samples). Dashed line indicates the threshold for positivity. The positive areas under the dilution curves (pAUC) are presented in **(D)** with blue circles and bars representing assays performed with cells from CBMC and black/gray squares and bars representing assays performed with cells from adult PBMC. Box plots represent the interquartile ranges, horizontal lines indicate the medians, and error bars extend to the minimum and maximum observed values. Data are from 24 h killing assays with a $CD8^+$ effector to HIV-infected autologous $CD4^+$ target cell ratio of 30:1.

Comparison of Effector and Memory T Cell Subsets in Cord Blood and Adult Peripheral Blood

We used flow cytometry immunophenotyping to compare T cell subsets present in CBMC to those present in adult PBMC. Comparisons were made using cells isolated from 14 cord blood and 5 adult peripheral blood samples. The gating strategy used to identify total T cells, and T cell subsets —naïve, effector, effector memory (T_{EM}), and central memory (T_{CM})— is indicated in **Figure 2A**. Flow plots showing representative examples of $CD4^+$ and $CD8^+$ T cell subsets among PBMC and CBMC are shown in **Figures 2B,C**, respectively. Consistent with prior observations (39, 40), we found that although CBMC contained similar frequencies of $CD4^+$ and $CD8^+$ T cells as adult PBMC, there were marked differences in the distribution of functional subsets with cord blood $CD4^+$ (**Figure 2D**) and $CD8^+$ T cells (**Figure 2E**) being predominantly naïve (naïve CD4: median 95%, interquartile range 92–97%; naïve CD8: median 89%, interquartile range 84–94%), and therefore deficient in memory and effector subsets. We hypothesized that although DART molecules are able to redirect resting T cells for killing of HIV-infected cells without need for pre-activation (17, 18), they may be more effective when redirecting effector or memory subsets. To test this, we assessed CD107a degranulation concomitant with redirected killing activity with PBMC from two healthy adult donors as a source of effector cells, and HIV-1 subtype B BaL IMC virus-infected cells as targets. We previously demonstrated high levels of HIV × CD3 DART molecule-induced $CD8^+$ T cell degranulation in the presence of $CD4^+$ cells infected by this HIV-1 isolate (18). We found that although HIV-specific DART molecules induced degranulation in all subsets of $CD8^+$ T cells during redirected cytotoxicity assays, the majority of degranulated cells were $CCR7^-$ and therefore expressed phenotypes consistent with T_{EM} and effector cells (**Figure 2F**).

These data suggest that the reduced DART molecule-mediated cytotoxicity observed in assays performed using CBMC-derived T cells is likely due in part to the predominance of naïve cells in cord blood, and the absence of memory and effector $CD8^+$ T cells, which are preferentially redirected by the HIV × CD3 DART molecules.

Evaluation of Strategies to Increase the Cytolytic Potential of T Cells in Cord Blood

We explored strategies to increase the activity of cord blood cytolytic T cells for HIV DART molecule-redirected elimination of HIV-infected cells. We first evaluated the impact of priming CBMC-derived T cells with IL-12, as this cytotoxic-T-lymphocyte-promoting cytokine is known to be poorly expressed by the naïve infant immune system (41). We found that overnight incubation with exogenous IL-12, and maintenance of IL-12 treatment throughout the redirected cytotoxicity assay, did not improve cytotoxic activity of cord blood $CD8^+$ T cells when used in combination with HIV × CD3 DART molecules (**Figure 3A**), nor was there any impact on activity of $CD8^+$ T cells from adult PBMC. Next, reasoning that the naïve $CD8^+$ T cells present in cord blood cells may require a more substantial activation signal compared to $CD8^+$ T cells present in adult PBMC, we explored if killing activity could be improved by providing additional co-stimulatory signaling via co-engagement of CD28 or CD137 receptors (42). However, the results of experiments performed using T cells isolated from CBMC and adult PBMC demonstrated that exogenous anti-CD28 or anti-CD137 co-stimulation did not significantly improve killing mediated by HIV × CD3 DART molecules (**Figure 3B**). Moreover, we found that treatment of CBMC with IL-12 combined with anti-CD28 and anti-CD137 co-stimulation also had no impact on the cytolytic activity of T cells from CBMC when used with HIV × CD3 DART molecules, but did increase activity in assays performed with adult PBMC (**Figure 3C**), although the increase did not reach statistical significance ($p = 0.064$, Wilcoxon rank sum test). We next assessed if pre-activation of cord blood T cells would increase their cytotoxic activity. Interestingly, we found that pre-activation of cord blood T cells via T cell receptor (TCR) cross linking using anti-CD3 and anti-CD28 monoclonal antibodies in combination with exogenous recombinant human IL-2 (30 U/mL) improved the ability of T cells from 5 of 5 tested cord blood samples to eliminate HIV-infected cells upon redirection by 7B2 × CD3 DART molecules, and 4 of 5 tested samples upon redirection by A32 × CD3 DART molecules (**Figure 3D**). This observation indicates that $CD8^+$ T cells present in cord blood are capable of improved cytotoxic activity when given sufficient stimulation.

We next determined if cord blood-derived $CD8^+$ cells, which had experienced prior activation and subsequently transitioned back to a resting state, were able to maintain higher cytolytic activity. To test this, we activated cord blood $CD8^+$ T cells for 72 h via TCR stimulation as described above, then allowed the cells to rest for an additional 72 h before use in the redirected killing assay. Flow cytometry analysis was used to define cell phenotypes during activation, and the return to resting state as shown in **Figures 4A–C**. As previously described, the preponderance of resting cord blood $CD8^+$ T cell presented a naïve ($CD45RO^-$, $CCR7^+$) cell surface phenotype (**Figure 4A**). CD45RO expression increased along with expression of the activation markers CD69 and HLA-DR after TCR stimulation (**Figure 4B**). Upon returning to a resting state, cell-surface expression of activation markers CD69 and HLA-DR were reduced, but the vast majority of cells maintained a cell-surface phenotype consistent with that of T_{CM} cells ($CD45RO^+$, $CCR7^+$, **Figure 4C**). Despite these changes to cell-surface phenotypes, induced during activation and maintained after returning to rest, we observed similarly low levels of cytolytic activity for resting cord blood $CD8^+$ T cells (**Figure 4D**), and $CD8^+$ T cells that were transitioned from an active to resting state *in vitro* (**Figure 4E**). Collectively, these results demonstrated that redirected killing activity of cord blood T cells mediated by HIV × CD3 DART molecules was not affected by providing exogenous IL-12 or costimulatory signals, but could be improved when given sufficient stimulation leading to activation. However, the improved cytolytic activity was not maintained when activation waned.

Cord Blood $CD4^+$ T Cells Are Recruited by HIV × CD3 DART Molecules

In parallel to the assays performed using CBMC-derived $CD8^+$ T cells as effector cells, we also evaluated whether HIV × CD3 DART molecules had anti-HIV activity when incubated with infected cord blood $CD4^+$ T cells alone. As the epitope recognized by the 7B2 × CD3 DART molecules is non-neutralizing (43) we expected minimal anti-HIV activity in absence of $CD8^+$ effector cells. In contrast, we observed 7B2 × CD3 DART molecule-dependent specific killing of HIV-infected cells at similar levels for assays performed with and without $CD8^+$ T cells from CBMC (**Figure 5A**, filled and open blue circles, respectively). We also observed 7B2 × CD3 DART molecule-dependent specific killing in assays performed without $CD8^+$ T cells from PBMC (**Figure 5A**, open black squares), however killing was higher in the presence of PBMC-derived $CD8^+$ T cells (**Figure 5A**, filled black squares). To determine if HIV × CD3 DART molecules were capable of activating effector responses from the cord blood $CD4^+$ T cells themselves, we assessed CD107a degranulation during redirected cytotoxicity assays as described previously. The gating strategy for identification of cord blood-derived $CD4^+$ T cells in this assay is shown in **Figure 5B**, with representative flow cytometry plots depicting detection of CD107a on $CD4^+$ T cells in presence of control or HIV × CD3 DART molecules. These data demonstrate that $CD4^+$ T cells from CBMC are capable of being induced to degranulate by DART molecules in redirected cytotoxicity assays. The specific killing activity observed using cord blood $CD4^+$ T cells as effectors to eliminate HIV-1 BaL-infected autologous T cells from two cord blood donations is shown in **Figure 5C**. The frequency of $CD107a^+$ $CD4^+$ T cells detected in these assays is shown in **Figure 5D**. The specific killing and concomitant degranulation responses observed across the tested HIV × CD3 DART molecule concentrations show comparable

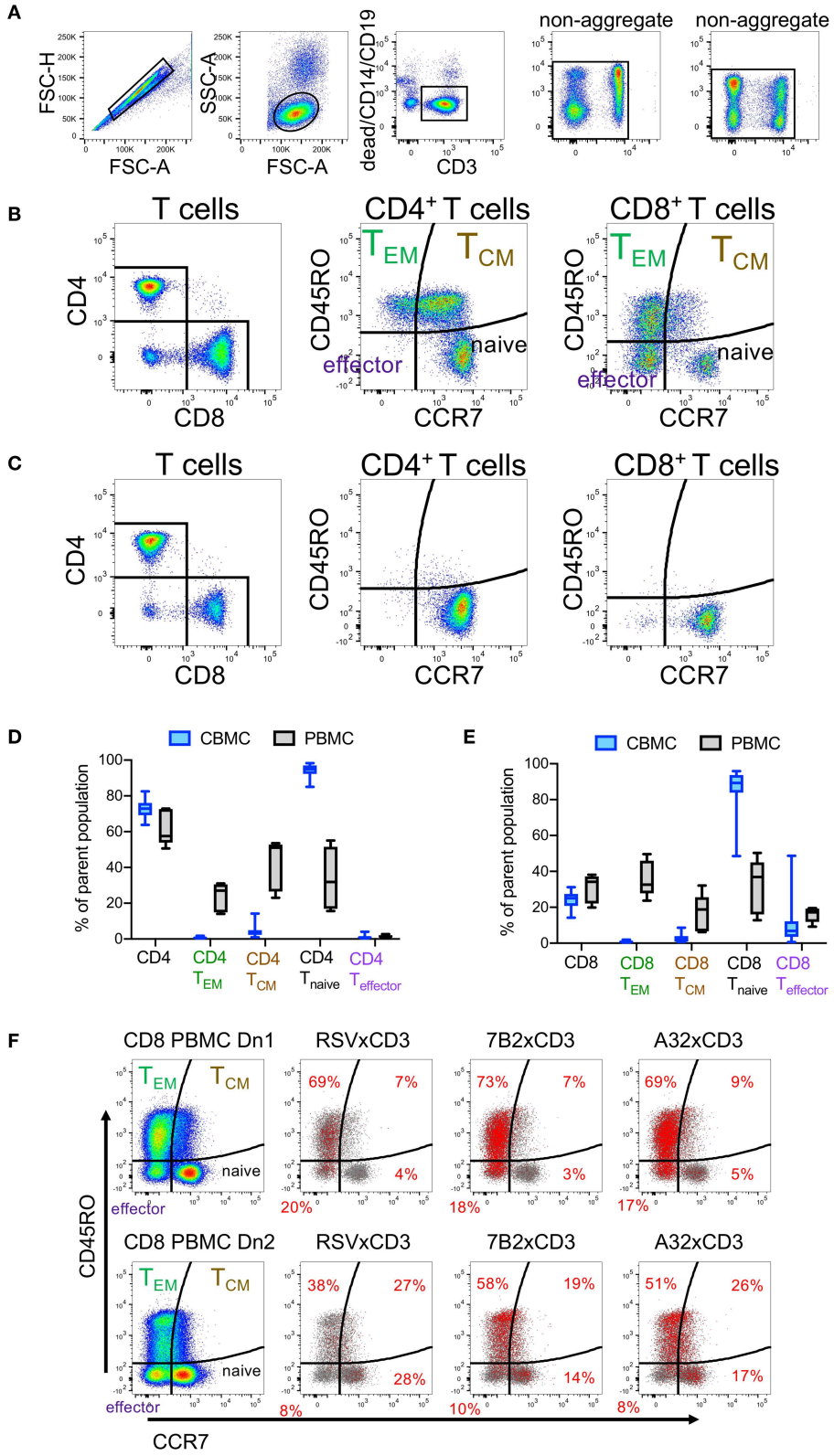

FIGURE 2 | Comparison of T cell subsets in adult peripheral blood mononuclear cells (PBMC) and cord blood mononuclear cells (CBMC) **(A)** Gating strategy. **(B)** Flow cytometry plots showing representative example of $CD4^+$ and $CD8^+$ T cell subsets among adult PBMC or **(C)** CBMC. Comparison of frequencies of **(D)** total $CD4^+$ T

(Continued)

FIGURE 2 | Cells and $CD4^+$ T cell subsets or **(E)** $CD8^+$ T cells and $CD8^+$ T cell subsets in CBMC ($n = 14$) and adult PBMC ($n = 5$). In **(D,E)**, box plots represent the interquartile ranges, horizontal lines indicate the medians, and error bars extend to the minimum and maximum observed values. **(F)** $CD8^+$ T cells subsets that degranulate concomitant with HIV × CD3 or control (RSV × CD3) DART molecule mediated cytolytic activity. Rows represent assay results using PBMC from two different adult donors, Dn1 and Dn2. Distribution of $CD8^+$ T cell subsets is shown in the first column. Red dots in the remaining columns represent CD8 T cells that have degranulated (CD107a+) in response to the indicated DART molecules (frequencies within each quadrant indicated with red text), overlaid on the total $CD8^+$ T cells (gray dots) acquired for each condition. 24 h killing assay with a $CD8^+$ effector to Bal-infected autologous $CD4^+$ target cell ratio of 30:1 and DART molecule concentration of 100 ng/mL.

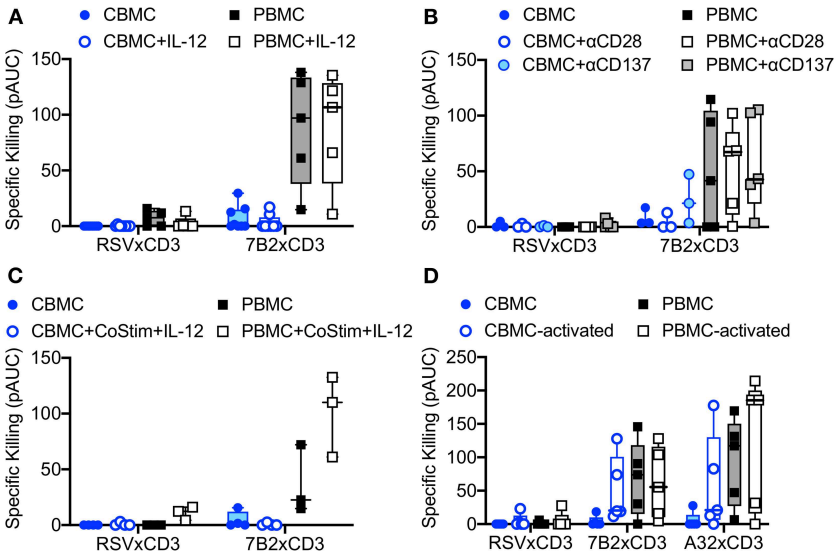

FIGURE 3 | Redirection of cord blood mononuclear cell (CBMC)-derived cytolytic T cells for elimination of autologous HIV-1 infected CD4 T cells by HIV × CD3 DART molecules is not improved by incubation with **(A)** 10 ng/mL exogenous IL-12 (CBMC $n = 8$, PBMC $n = 5$), **(B)** costimulation with anti-CD28 or anti-CD137 antibodies (CBMC $n = 3$, PBMC $n = 5$), or **(C)** a combination of costimulation with anti-CD28 and anti-CD137 (CoStim) and exogenous IL-12 (CBMC $n = 4$, PBMC $n = 3$). **(D)** HIV × CD3 DART molecule-mediated elimination of autologous HIV-1-infected $CD4^+$ T cells is improved when $CD8^+$ T cells used as effectors are activated by T cell receptor stimulation (anti-CD28, anti-CD3, and IL-2) for 48 h prior to use in the cell killing assays (CBMC and PBMC $n = 5$). Box plots represent the interquartile ranges, horizontal lines indicate the medians, and error bars extend to the minimum and maximum observed values. 24 h killing assays with a $CD8^+$ effector to HIV-1 4403bmC5-infected autologous $CD4^+$ target cell ratio of 30:1. All data reported as positive area under the dilution curve (pAUC).

trends, suggesting that degranulating $CD4^+$ T cells contribute to the *in vitro* elimination of HIV-1 infected cells when redirected by HIV × CD3 DART molecules. This finding is consistent with observations made in our prior studies performed using adult PBMC (18).

HIV × CD16 DART Molecules Can Recruit Cord Blood NK Cells for Elimination of Autologous HIV-1 Infected $CD4^+$ T Cells

Another approach for increasing HIV DART-molecule-mediated elimination of HIV-1 infected cells is to recruit additional populations of cytotoxic effector cells. We used flow cytometry immunophenotyping to compare NK cells present in CBMC and adult PBMC according to the gating strategy shown in **Figure 6A**. We found that cord blood contains more total NK cells (median 11% total NK in CBMC and 5% in adult PBMC), but similar distributions of NK cell subsets, as adult peripheral blood (**Figure 6B**). As we, and others, have previously demonstrated that pretreatment with IL-15 augments the cytotoxic potential of NK cells (32, 44–46) we compared the impact of overnight incubation with IL-15 (10 ng/mL) on the subset distribution, activation state, and intracellular abundance of cytotoxic effector molecules in NK cells present in CBMC and adult PBMC. We previously showed that IL-15 treatment increased the frequency of $CD56^{bright}$ NK cells and $CD56^{dim}CD16^{-/dim}$ NK cells, and decreased $CD56^{dim}CD16^+$ NK cells in adult PBMC [(32) and **Figure 6B**]. The impact of IL-15 on cord blood NK cell subset distributions was modest, with no significant change (Wilcoxon rank sum tests) in the frequencies of major NK cell subsets (**Figure 6B**). Importantly, the cytotoxic $CD56^{dim}CD16^+$ NK cell subset was the dominant population of NK cells in both cord blood and adult PBMC. As we previously described for IL-15 treated NK cells in adult PBMC, we found that IL-15 treatment did not impact the expression of the maturation marker CD57 on the surface of cord blood NK cells, although its expression was markedly lower than that observed on NK cells in adult PBMC ($p < 0.001$, Wilcoxon rank sum test), which is consistent with the naivety of cord blood immune cells (**Figure 6C**). We also found that fewer cord blood NK cells expressed the secondary lymphoid homing marker CD62L when compared to NK cells in adult PBMC ($p < 0.001$, Wilcoxon rank sum test), and treatment of cord blood with IL-15 further reduced the percentage of

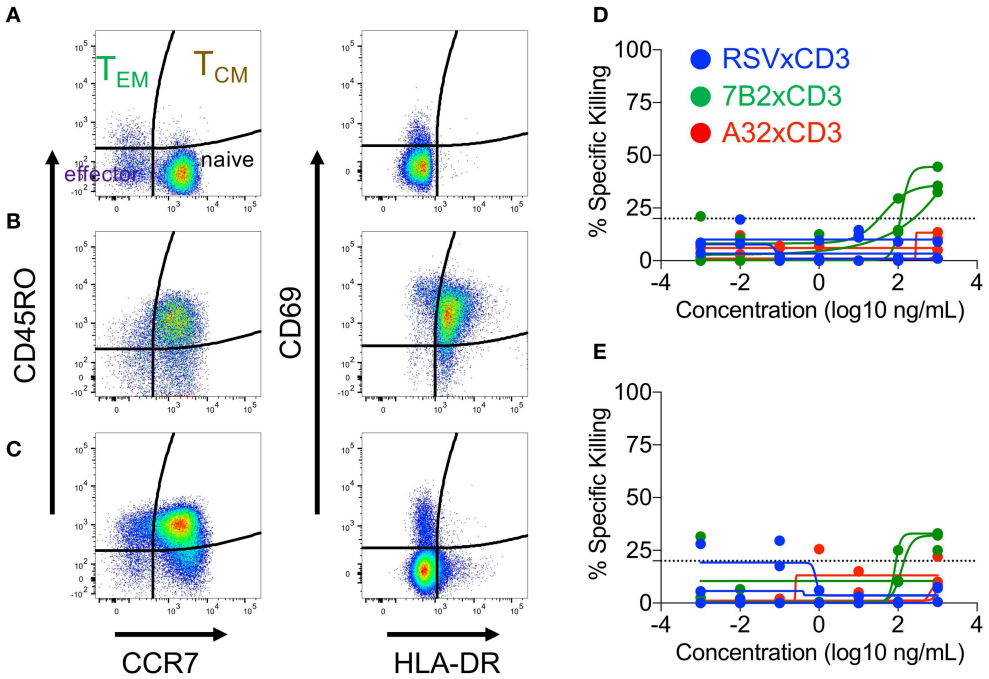

FIGURE 4 | Cord blood mononuclear cell (CBMC) CD8$^+$ T cells subset distribution (left panels) and activation state (right panels) after **(A)** overnight rest, **(B)** 3 days of T cell receptor stimulation (anti-CD3, anti-CD28, and IL-2), or **(C)** 3 day transition from activated cells back to the resting state. Representative example shown. Similar levels of DART molecule-redirected killing of autologous HIV-1 4403bmC5-infeced CD4$^+$ T cells were observed in assays performed with **(D)** CD8$^+$ T cell effectors after overnight rest, and **(E)** as they returned back to a resting state following 3 days of T cell receptor stimulation. **(D,E)** represent data from 24 h killing assays with a CD8$^+$ effector to HIV-1 4403bmC5-infected autologous CD4$^+$ target cell ratio of 30:1, performed using three different CBMC donors, each represented by individual lines.

NK cells expressing CD62L (**Figure 6D**). Moreover, and as expected, cord blood NK cells incubated with IL-15 presented a more active phenotype than untreated NK cells as evident by cell surface expression of HLA-DR (**Figure 6E**) and CD69 (**Figure 6F**), although overall, the frequency of activated NK cells was lower in CBMC compared to adult PBMC following IL-15 treatment ($p < 0.001$ for both CD69 and HLA-DR, Wilcoxon rank sum tests). Finally, using fluorescent quantitation beads to measure the intracellular content of perforin and granzyme B, we found that cord blood NK cells contained more perforin (**Figure 6G**) but less granzyme B (**Figure 6H**) than NK cells in adult PBMC, whether untreated (perforin, $p = 0.001$; granzyme B, $p < 0.001$, Wilcoxon rank sum tests) or treated with IL-15 (granzyme B, $p < 0.001$, Wilcoxon rank sum test). Collectively, these data suggest that cord blood contains similar distributions of NK cell subsets as adult peripheral blood, however the NK cells present in cord blood are less mature, less lymphoid homing, less active, and contain less of the cytotoxic effector molecule granzyme B when compared to NK cells in adult PBMC.

To recruit and redirect NK cells, DART-molecules comprised of our previously characterized HIV-1 targeting arms (18) linked to CD16 targeting arms were developed. We used flow cytometry staining to confirm that DART-molecules with CD16-targeting arms were capable of binding to the surface of human NK cells (**Figure 7A**). We next evaluated the ability of cord blood NK cells to eliminate autologous CD4$^+$ T cells infected with HIV-1 4403bmC5 virus when redirected by HIV × CD16 (clone hG38) DART molecules based on the 7B2 mAb. Similar to the data observed for cord blood CD8$^+$ T cells, we found that resting cord blood NK cells had modest ability to eliminate autologous HIV-1 infected T cells when redirected by the 7B2 × CD16 DART molecule (**Figure 7B**, filled symbols). However, overnight treatment with IL-15 significantly increased ($p = 0.007$, Wilcoxon rank sum test) the cytotoxic activity of cord blood NK cells when redirected by the HIV-specific DART molecule (**Figure 7B**, open symbols). The 7B2 × CD16 DART molecule redirected cytolytic activity of IL-15 treated CBMC NK cells was similar to that observed in assays performed with NK cells from adult PBMC (**Figure 7B**, black squares). These data demonstrate that NK cells in cord blood have potential to be effective mediators of DART molecule-redirected killing after priming with IL-15. We next explored whether combinations of HIV × CD16 DART molecules specific for different epitopes improved elimination of HIV-infected cells. For these experiments, we used HIV × CD16 DART molecules (A32 × CD16 and 7B2 × CD16) and a control molecule (RSV × CD16) that were generated using a different anti-CD16 arm (clone h5H2, **Figure 7A**). Assays were performed using NK cells from three cord blood samples that we identified as having cytolytic activity in previous assays. Using equivalent concentrations of the A32 × CD16 and 7B2 × CD16 molecules we found that activity of the combination was similar to that of the A32 × CD16 molecule alone (**Figure 7C**). Thus, there was no

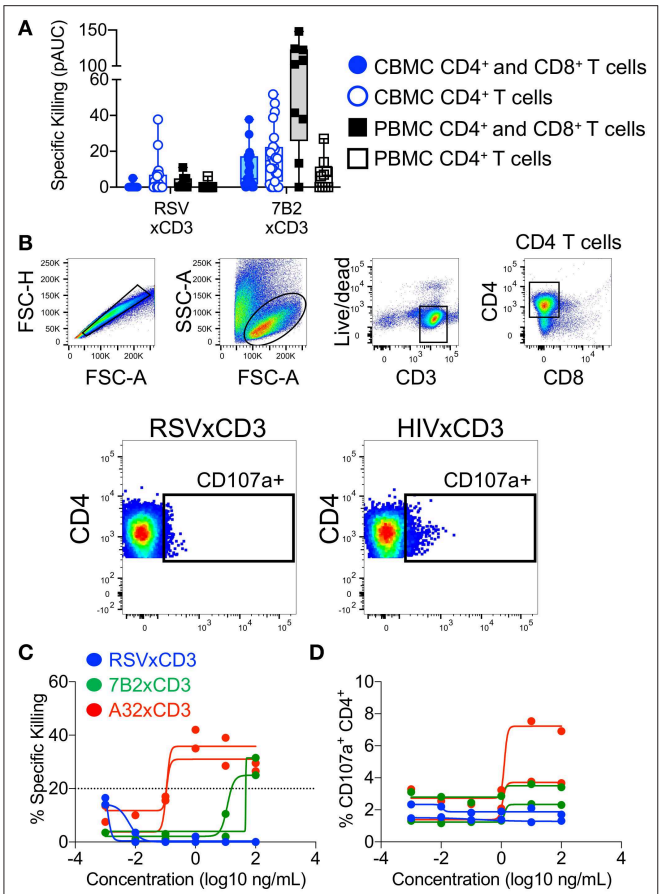

FIGURE 5 | Redirection of cord blood mononuclear cell (CBMC)-derived CD4$^+$ T cells for elimination of HIV-infected cells by HIV × CD3 or control (RSV × CD3) DART molecules. **(A)** Similar levels of HIV-1 4403bmC5 infected CBMC-derived CD4$^+$ T cell killing were observed in assays performed with autologous CD8$^+$ T cell effectors (filled blue circles and box, $n = 23$ CBMC samples) or with CD4$^+$ T cells alone, no additional effector cells (open blue circles and box). HIV-1 4403bmC5 infected PBMC-derived CD4$^+$ T cell killing observed in assays performed with autologous CD8$^+$ T cell effectors (filled black squares and box, $n = 9$ PBMC samples) or with CD4$^+$ T cells alone, no additional effector cells (open black squares and box). **(B)** Gating strategy used to identify cord blood CD4$^+$ T cells that degranulated concomitant with DART molecule mediated cytolytic activity. Redirection of CBMC-derived CD4$^+$ T cells for elimination of HIV-1 Bal-infected autologous CD4$^+$ T cells as measured by **(C)** luciferase cell killing assay, and **(D)** CD107a degranulation assay. Data are from 24 h killing assays performed using two different CBMC donors, each represented by individual lines.

evidence in these assays of an additive effect, nor of interference, when using these two HIV × CD16 DART molecules to mediate redirected killing with cord blood NK cells.

Finally, we investigated whether combinations of HIV × CD3 and HIV × CD16 DART molecules could work cooperatively for improved killing. To test this, we utilized IL-15 treated CBMC from two donors which we identified as having T cells and NK cells that supported redirected killing of autologous HIV-infected CD4$^+$ T cells. 7B2 × CD3 and 7B2 × CD16 DART-molecules were used either alone, or in combination at equivalent concentrations. RSV × CD3 and 4420 × CD16 DART molecules in equivalent combination were used as a negative control. As shown in **Figure 7D**, combinations of 7B2 × CD3 and 7B2 × CD16 DART molecules did not improve the killing of HIV-1-infected autologous CD4$^+$ T cells by cord blood cells in these assays.

DISCUSSION

Mother-to-child transmission is the principal cause of the approximate 400 new pediatric HIV-1 infections that occur each day, mostly in resource limited countries (4). Infants born into these unfortunate circumstances must strictly adhere to a lifetime of ART to control virus replication and prevent disease progression. Thus, there is a critical need for innovative therapeutic approaches enabling long-term remission or cure of infant HIV-1 infection in order to offer these children the chance to lead normal, healthy lives. One potential strategy is the use of antibody-based immunotherapy in combination with ART and latency reversing agents as a "shock and kill" approach to eliminate the virus reservoir (15, 16, 47, 48). Infants are potentially ideal candidates for immunotherapy due to the ability to initiate therapy soon after the perinatal transmission event and prior to establishment of a large virus reservoir (49, 50), and proven history of safe administration of passive antibody therapy against another infectious disease, respiratory syncytial virus (51). Here, we used human umbilical cord blood as a surrogate model of neonatal peripheral blood to test the hypothesis that HIV × CD3 DART molecules based on HIV-specific mAbs could redirect neonatal effector cells for elimination of cells infected with HIV-1. Importantly, our *in vitro* assays used autologous cord blood-derived CD4$^+$ T cells as target cells, after infecting with an HIV IMC molecular clone virus encoding an HIV-1 subtype C *env* gene sequenced from plasma of a postnatally-infected infant, and identical to an isolate found in the matched maternal breast milk (34). Thus, these assays attempt to recapitulate the effector cells and HIV-1 Env-expressing target cells expected to be present in perinatally infected infants. Our results demonstrated that T cells present in cord blood can be recruited and redirected by HIV × CD3 DART molecules to eliminate HIV-1 infected target cells. This key finding supports the continued development of HIV-specific DART molecules as immunotherapies to combat pediatric HIV infection. However, we also found that the DART molecule-dependent killing activity by T cells from cord blood is significantly lessened as compared to that by T cells from adult PBMC. We therefore explored approaches to improve the cytotoxicity of cord blood effector cells with the goal of identifying strategies to maximize the potential effectiveness of DART molecule-based immunotherapy for use in early life.

We found that cord blood T cells were largely refractory to *in vitro* cytokine treatment and co-stimulatory receptor engagement intended to increase their cytolytic activity. We observed no impact of exogenous IL-12 (41), CD28 and CD137 co-stimulatory receptor engagement via specific antibodies, nor of combined use of IL-12 and co-stimulatory receptor engagement on the ability of cord blood T cells to eliminate HIV-1 infected cells when redirected by HIV × CD3 DART

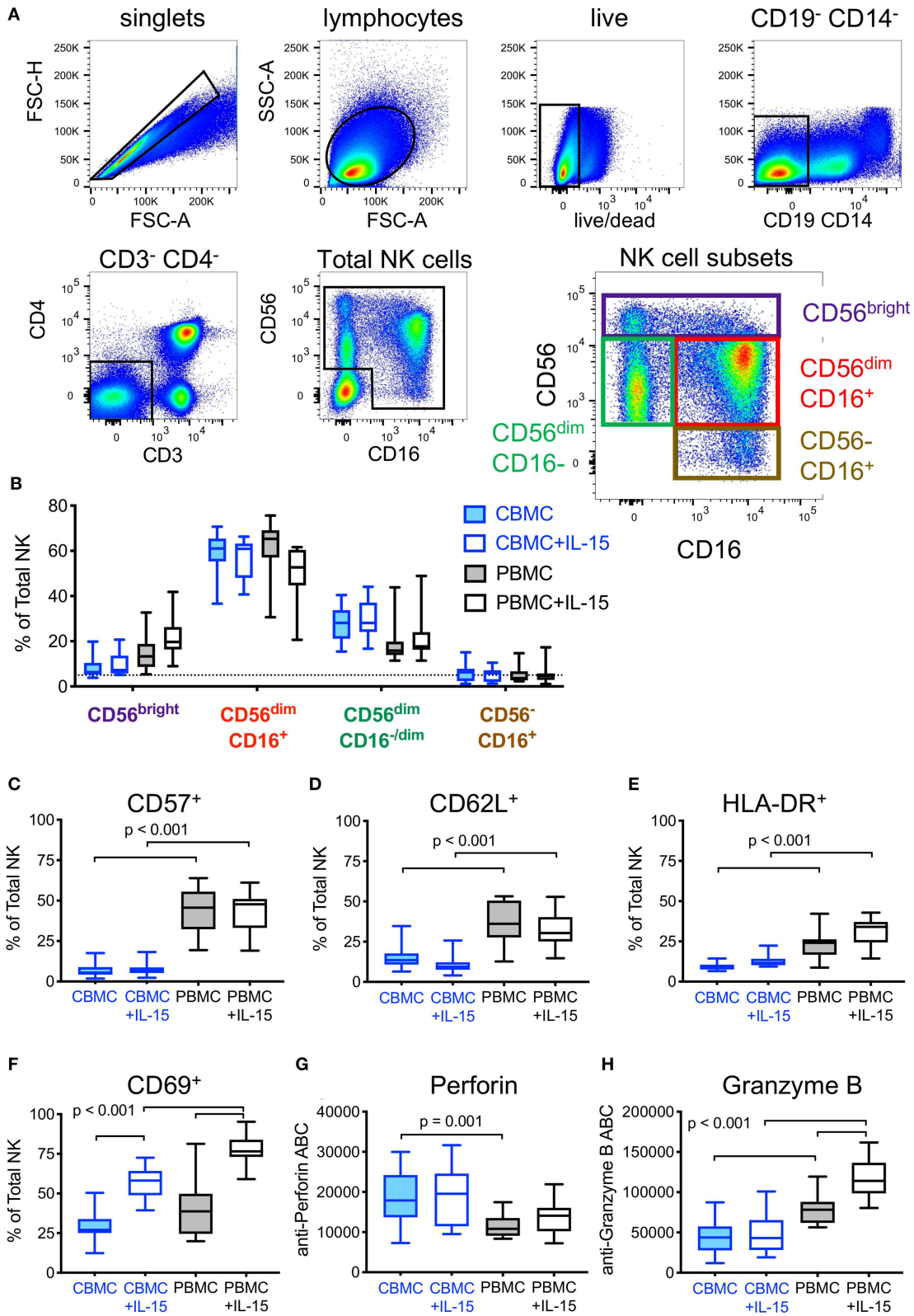

FIGURE 6 | Effect of exogenous IL-15 treatment on phenotypes of natural killer (NK) cells present in cord blood mononuclear cells (CBMC) with comparison to adult peripheral blood (PBMC). **(A)** Gating strategy used to identify total and subsets of NK cells. Data representing all CBMC samples ($n = 30$, 15 CBMC donors +/- IL-15)

(Continued)

FIGURE 6 | were combined and included in the flow cytometry plots shown. **(B)** Comparison of NK cell subset frequencies between untreated, and after overnight treatment with 10 ng/mL IL-15 ($n = 15$ CBMC and $n = 15$ PBMC samples per condition). Frequencies of total NK cells expressing **(C)** CD57, **(D)** CD62L, **(E)**, HLA-DR, and **(F)** CD69 in cells left untreated or treated overnight with 10 ng/mL IL-15. Amount of intracellular **(G)** Perforin and **(H)** Granzyme B in NK cells left untreated or after overnight treatment with 10 ng/mL IL-15, measured by antibody binding capacity (see Methods). In **(B–H)**, box plots represent the interquartile ranges, horizontal lines indicate the medians, and error bars extend to the minimum and maximum observed values.

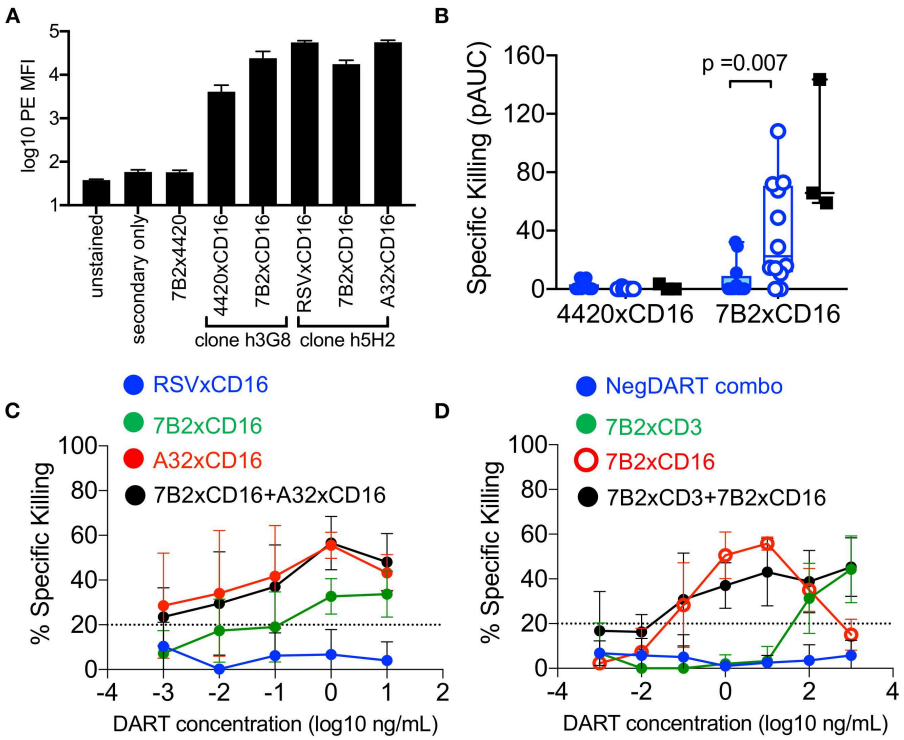

FIGURE 7 | (A) DART-molecules with CD16-targeting arms bind to the surface of human NK cells (from $n = 4$ donors) by flow cytometry analysis. Data are reported as mean fluorescent intensities (MFI), error bars represent standard deviation. **(B)** Redirection of cord blood mononuclear cell (CBMC)-derived NK cells (from $n = 12$ CBMC samples, blue circles) for elimination of HIV-1 4403bmC5-infected autologous CD4$^+$ T cells by 7B2 × CD16 or control (4420 × CD16) DART molecules in the absence of IL-15 (filled symbols) or after overnight incubation with 10 ng/mL IL-15 (open symbols). Results of assays performed with adult NK cells from three PBMC samples, in absence of IL-15, are included for comparison (black filled squares). Data are positive area under the dilution curve (pAUC) from 24 h killing assays with a NK cell to CD4$^+$ T cell ratio of 5:1. **(C)** Similar levels of HIV x CD16 DART molecule-redirected killing of autologous HIV-1 4403bmC5-infeced CD4$^+$ T cells were observed in assays performed with A32 × CD16 DART molecules and A32 × CD16 DART molecules combined with 7B2 × CD16 DART molecules ($n = 3$ CBMC-derived, IL-15 treated, NK cell samples, assayed in duplicate). **(D)** Redirection of CBMC ($n = 2$ samples, assayed in duplicate) for elimination of HIV-1 4403bmC5-infected autologous CD4$^+$ T cells by 7B2 x CD3 DART molecules, 7B2 × CD16 DART molecules, or 7B2 x CD3 and 7B2 x CD16 DART molecules used in combination. Twenty four hours killing assay with an IL-15 treated whole CBMC to CD4$^+$ T cell ratio of 30:1. Data in **(C,D)** represent mean +/− standard deviation.

molecules. However, DART molecule-mediated cytolytic activity was increased when cord blood T cells were activated by TCR stimulation in the presence of IL-2. This result suggests that neonatal T cells have the capability to be potent cytolytic effectors if given sufficient activation signals. We also found that cells transitioned *in vitro* from an activated state back to a resting state had similar cytolytic activity to cells that had not experienced activation, suggesting that transient activation did not result in a durable change in the cytolytic potential of cord blood-derived CD8$^+$ T cells. Comparative studies using cells from adult peripheral blood demonstrated that HIV × CD3 DART molecules effectively recruit and redirect memory and effector T cells —populations of cells that are poorly represented in cord blood. Thus, the limited maturation of T cell subsets in cord blood likely explains the observed reduced activity when compared to adult immune cells. However, it is important to consider that our *in vitro* cord blood-based experiments fail to fully model the diversity of T cell activation states and functional subsets present in an infant *in vivo*. Several lines of evidence suggest that T cells from pediatric peripheral blood may be more phenotypically and functionally diverse than those from cord blood. First, maternal and/or perinatal infant infections with cytomegalovirus and *Trypanosoma cruzi*, have been found to promote T cell maturation *in utero* and during infancy (52, 53). As cytomegalovirus, *T. cruzi*, and a myriad of other infectious agents and parasites are endemic in regions of high HIV-1 prevalence (54), it is likely that infants infected with HIV-1 via mother-to-child transmission have circulating T cells that differ

in maturation, activation, and functionality when compared to those in cord blood from healthy United States-based mothers, as utilized in this study. It also has been shown that there are differences between pediatric circulating T cells, as modeled by cord blood in this study, and T cells present in pediatric tissues. Seminal work by Thome and collaborators using tissues obtained from pediatric organ donors from 2 months to 2 years of age demonstrated that, although naïve cells constitute the majority of T cells in pediatric peripheral blood, spleen, and lymph nodes, T_{EM} cells comprise a large portion of the T cells in intestinal mucosal tissues (40). Moreover, they demonstrated that memory T cells from the intestine were capable of rapid secretion of the effector cytokine IFNγ in response to stimulation. Because we found that T_{EM} cells are preferentially recruited by HIV × CD3 DART molecules, the cytolytic activity of these molecules mediated by tissue-derived T cells at mucosal sites may be higher than that observed by cord blood-derived T cells *in vitro*. It is important to note that Thome and collaborators also identified higher frequencies of T regulatory cells in pediatric blood and tissues relative to adults (40). The $CD4^+$ T regulatory cells may act to either limit the cytolytic activity of infant T cell responses, or may themselves serve as effector cells for CD3 DART molecule-mediated lysis of target cells as has been previously shown (55). Thus, accurate characterization of the cytolytic activity of pediatric tissue-resident T cells when recruited by HIV × CD3 DARTs will likely require *in vivo* testing using a biologically relevant model, such as infant rhesus macaques. Finally, immune system development during early life will likely also affect the therapeutic potential of HIV × CD3 DART molecules. In fact, $CD8^+$ T cell responses may mature rather quickly, as young children (2–3 years of age) have been shown to have $CD8^+$ T cells with potent antiviral activity (56, 57). These observations suggest that the ability of HIV × CD3 DART molecules to mediate elimination of HIV-1-infected cells may improve over the first few years of life. Interestingly, we also found that cord blood $CD4^+$ T cells contributed to elimination of infected cells *in vitro*. Whether these cells would contribute to HIV × CD3 DART molecule-dependent elimination of infected cells *in vivo* is not yet known. Additional studies using blood samples collected longitudinally from HIV-infected infants and age-appropriate non-human primate models would likely help to identify relevant populations of effector cells, and to determine the optimal timing post birth for usage of HIV × CD3 DART molecules to eliminate the HIV-1 infected cell reservoir during early life. However, the presence of higher birth levels of memory T cells, and rapid expansion of T cell memory in rhesus macaques may complicate human translation of such studies (58).

Effective control and/or cure of pediatric HIV will likely require early and aggressive interventions. Thus, the likelihood of success with the HIV-specific DART molecule-based approach might be increased by recruitment of additional populations of cytolytic effector cells to further promote eradication of infected cells, and to potentially reach additional tissues and anatomical compartments. NK cells are abundant in cord blood and pediatric peripheral blood, with cytotoxic $CD56^{dim}CD16^+$ NK cells representing the dominant subset (39, 45, 59). We found that HIV × CD16 DART molecules were able to recruit and redirect neonatal NK cells present in cord blood for elimination of HIV-1 infected cells and killing activity could be substantially increased by treatment with IL-15. The ability of IL-15 to augment the cytolytic activity of cord blood derived NK cells is consistent with previously reported results from assays measuring cord blood NK cell natural cytotoxicity against sensitive target cells (45). Currently, the mechanisms by which IL-15 improves NK cell cytotoxicity are incompletely defined. We previously demonstrated that IL-15 treatment of NK cells from adult peripheral blood increases the levels of cytolytic effector molecules, perforin and granzyme B (32). By contrast, we found that cord blood NK cells contained more perforin, but less granzyme B, than NK cells in adult PBMC —and the levels of neither were impacted by IL-15 treatment. Thus, intracellular abundance of perforin and granzyme B does not explain how cord blood NK cells become more cytolytic after incubation with IL-15, which suggests involvement of other activatory or regulatory processes. Exogenous IL-15 was previously shown to increase cord blood NK cell expression of intracellular adhesion molecule 1 (ICAM-1) —a cell surface protein involved in cell-to-cell interactions including formation of NK cell immunologic synapses (60). However, this effect was only seen after extended culture for 7 days (61), not after a short overnight incubation as in our study. It has also been proposed that the higher frequency of cord blood NK cells expressing cell-surface inhibitory receptor NKG2A compared to NK cells from adult peripheral blood may contribute to the reduced activity of cord blood derived NK cells (62). In contrast, we have previously demonstrated that IL-15 promotes expression of the activating receptors NKG2D and NKp30 (46). Additional research will be required to define the impact of IL-15 on the multitude of cord blood NK inhibitory and activating receptors, and to determine how these signals may integrate with the CD16-mediated signaling that occurs when NK cells interact with HIV x CD16 DART molecules.

Having found that IL-15 stimulation is able to maximize the functionality of cord blood NK cells for DART molecule-redirected killing has generated another important question: are neonatal NK cells naturally primed by IL-15 *in vivo* during HIV-1 infection? IL-15 was transiently detected in the plasma during acute HIV-1 infection of adults, with a median time of first detection being 6 days after viremia reached detectable levels in peripheral blood plasma (63). Although a similar inflammatory cytokine response is expected in neonates, it has also been demonstrated that stimulated CBMC produce less IL-15 than adult peripheral blood (64). Whether or not sufficient levels of IL-15 to activate neonatal NK cells would be present at sites of infection will require additional research. If an absence of natural IL-15 is identified, alternative strategies including the administration of recombinant human IL-15, or engineered forms of IL-15 (65, 66), in combination with the HIV × CD16 DART molecules could be tested in the infant rhesus macaque model.

Although we found that combinations of 7B2 × CD3 and A32 × CD3 DART molecules did not result in improved cytolytic activity, it is important to note that these studies were intended to explore the cytolytic potential of neonatal effector cells, not to identify optimal combinations of DART molecules. We

used 7B2 and A32 DART molecules as an extension of our original study (18), but additional DART molecules targeting other Env epitopes have been described (17). It is probable that combinations of DART molecules targeting both broad-binding neutralizing and non-neutralizing epitopes, could be the most effective in recognizing the HIV Env diversity that results from the genetic diversity of HIV-1, and the variety of Env conformations that may be found on the surface of infected cells *in vivo* (43, 67, 68). Research aimed at identifying optimal combinations of anti-HIV antibodies using diverse HIV isolates, and patient-derived latent reservoir virus (Tuyishime et al., submitted) will likely help identify optimal combinations of DART molecules.

At present, blinatumomab, a CD19 × CD3 antibody-based bispecific molecule, is the only example of an immune cell-engaging bispecific drug that has FDA approval for the treatment of disease—B cell acute lymphoblastic leukemia (69, 70). However, there are dozens of bispecific antibody-based molecules in various stages of development, and clinical testing, against multiple types of cancers (71, 72). This includes several DART molecules being evaluated for potential use in treatment of leukemias and lymphomas (73, 74), colorectal cancer (55), and solid tumors (75). The majority of bispecifics in development are intended to recruit T cells via an anti-CD3 arm, however NK cell recruitment by anti-CD16 targeting is also being explored (76, 77). The continued development and clinical testing of DART molecules, and other antibody-based bispecifics, for use against human cancers will provide important safety, durability, and efficacy data that will help inform the best strategies for use of these types of therapies against other diseases, including HIV-1 infection.

In summary, we demonstrated that HIV × CD3 and HIV × CD16 DART molecules can recruit and redirect cytolytic immune cells present in cord blood to eliminate autologous T cells infected with HIV-1, albeit less effectively than cells present in adult peripheral blood. Our work supports the continued development of these antibody-based molecules as components of passive immunization strategies aimed at treatment and cure of pediatric HIV. However, future studies using early life blood and tissue samples, and infant or juvenile preclinical animal models, will be required to identify tenable strategies to optimize their efficacy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Anonymized human umbilical cord blood donations that failed to meet the criteria required for clinical use were obtained with informed written consent. Human peripheral venous blood was collected by leukapheresis from healthy consenting adult volunteers (28, 29). All samples were collected in accordance with the policies and regulations of the Duke Health Institutional Review Board.

AUTHOR CONTRIBUTIONS

JP conceived the study, performed experiments, analyzed and interpreted data, and wrote the manuscript. RE performed experiments and analyzed data. SJ performed statistical analyses. TH and JAP performed experiments and analyzed data. C-YL, LL, GD, and JN led the design, development, and production of the bispecific dart molecules. SP isolated and characterized the breast milk transmitted/founder HIV-1 virus. TD collected and characterized the adult leukapheresis samples. SP and GF interpreted data and contributed to design of the study. All authors commented on the manuscript and approved the final version.

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Nanoparticle-Based Immunoengineered Approaches for Combating HIV

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Highly active antiretroviral therapy (HAART) serves as an effective strategy to combat HIV infections by suppressing viral replication in patients with HIV/AIDS. However, HAART does not provide HIV/AIDS patients with a sterilizing or functional cure, and introduces several deleterious comorbidities. Moreover, the virus is able to persist within latent reservoirs, both undetected by the immune system and unaffected by HAART, increasing the risk of a viral rebound. The field of immunoengineering, which utilizes varied bioengineering approaches to interact with the immune system and potentiate its therapeutic effects against HIV, is being increasingly investigated in HIV cure research. In particular, nanoparticle-based immunoengineered approaches are especially attractive because they offer advantages including the improved delivery and functionality of classical HIV drugs such as antiretrovirals and experimental drugs such as latency-reversing agents (LRAs), among others. Here, we present and discuss the current state of the field in nanoparticle-based immunoengineering approaches for an HIV cure. Specifically, we discuss nanoparticle-based methods for improving HAART as well as latency reversal, developing vaccines, targeting viral fusion, enhancing gene editing approaches, improving adoptively transferred immune-cell mediated reservoir clearance, and other therapeutic and prevention approaches. Although nanoparticle-based immunoengineered approaches are currently at the stage of preclinical testing, the promising findings obtained in these studies demonstrate the potential of this emerging field for developing an HIV cure.

Keywords: HIV cure strategies, immunoengineering, nanoparticles, immune activation, HAART (highly active antiretroviral therapy), combination therapy for HIV, latency reversing agents

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INTRODUCTION

Approximately 37 million people worldwide are living with HIV for which there is no practical cure (1). There are two main types of the virus: HIV-1 and HIV-2. HIV-1, which is the focus of cure strategies discussed in this paper, is more prevalent and pathogenic, and primarily infects $CD4^+$ T helper cells (2). Other cell populations susceptible to HIV-1 include dendritic cells, macrophages, microglia, and astrocytes although the mechanisms of infection for these cell types are not yet fully understood (3–8). HAART is an effective treatment regimen for HIV-1 (9), however, it allows the virus to remain viable and does not provide a sterilizing or functional cure in patients. Further HAART causes several deleterious comorbidities (10, 11). Since HAART targets the HIV-1

replication cycle, HIV-1 evades targeting by undergoing latency (12, 13). Latent HIV-1 reservoirs are also able to go undetected by the immune system, which increases the risk of a viral rebound. Another confounding factor is the lack of unique surface markers on latently infected cells, which has hindered the development of strategies to generate total viral clearance or permanent latency (14–16). This challenge is supported by the fact that there have been only two well-documented cases wherein patients have experienced total HIV-1 viral clearance. This suggests that it is extremely rare for individuals to adequately control HIV-1 without a sustained antiretroviral treatment regimen (17, 18). Hence, there is an urgent need for novel HIV cure strategies.

The field of immunoengineering encompasses a broad variety of bioengineering approaches and technologies to manipulate the immune system. A notable component of these approaches involves engineered biomaterials including nanoparticles, polymeric scaffolds, and hydrogels to engage the immune system to fight disease, and represents an attractive strategy for developing a cure for HIV-1 (19–21). While numerous nanoparticle-based immunoengineered approaches have been successfully applied in the field of cancer immunotherapy (22–26), fewer studies have utilized these promising benefits for an HIV cure. The goal of this mini-review is to familiarize the reader with the field of nanoparticle-based immunoengineering approaches for an HIV cure. In particular, we focus on the use of nanoparticles to enhance HAART, latency reversal, vaccination strategies, gene editing, cell therapies, among others (**Figure 1**). We highlight both immune-mediated strategies (e.g., nanoparticles in conjunction with adoptive cell transfer) and those targeting endogenous immune cells involved in HIV (e.g., nanovaccines). For a comprehensive discussion on the use of nanoparticles for treating HIV/AIDS in the context of nanoparticles classes, formulation, and their use in drug delivery, we direct the readers to several published reviews in the literature (27–29).

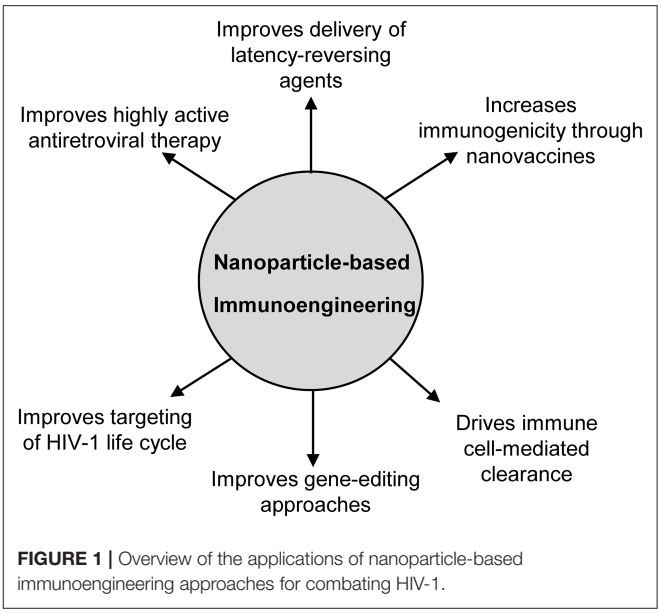

FIGURE 1 | Overview of the applications of nanoparticle-based immunoengineering approaches for combating HIV-1.

NANOPARTICLES EXHIBIT BENEFICIAL PROPERTIES FOR IMMUNOENGINEERING CURES FOR HIV-1

Nanoparticle sizes range from ∼1 to 100 nm (30). On account of their sizes, nanoparticle-based therapies can easily be administered by varied techniques (i.e., intravenously, subcutaneously, intraperitoneally) and penetrate body barriers (31). Related to their sizes and pertinent to HIV cure strategies, nanoparticles accumulate in lymphoid tissue and lymphatic organs (32), the sites of anatomical HIV reservoirs (33, 34) when parenterally injected (especially when injected intradermally, subcutaneously, and/or intramuscularly). Additionally, because nanoparticles have a large surface area-to-volume ratio, diverse molecules such as drug payloads, immunological adjuvants, and targeting ligands can be bioconjugated to their surface (35), which can then be trafficked to sites of latent HIV reservoirs. Nanoparticles can also be synthesized in the form of depots/reservoirs that encapsulate and release therapeutic drugs or immunomodulatory agents (36), allowing for their improved bioavailability and sustained release kinetics in an HIV cure setting. In the following sections, we review various examples of nanoparticles used for immunoengineering HIV cures.

Nanoparticles for Improving HAART

HAART can successfully inactivate HIV-1, however the virus is able to persist within latent lymphoid, gut, and CNS reservoirs (5–8). This requires patients to remain adherent to the HAART regimen for the duration of their lifetime to prevent viral rebound (9). Since nanoparticles facilitate the sustained release of drugs, a group of researchers have developed a long-acting slow-effective release antiretroviral therapy (termed "LASER ART"), which utilizes nanoparticles for controlled release of HAART agents, thereby improving regimen adherence (37, 38). In a recent study, the same group of researchers demonstrated that a nanocrystallized product of lamivudine, a nucleoside reverse transcriptase inhibitor (NM23TC), maintained antiretroviral activity in HIV-1-infected monocyte-derived macrophages after viral challenge for up to 30 days. NM23TC was taken up by HIV-1-infected monocyte-derived macrophages and remained in high prodrug concentration in whole blood for 30 days after a single dose. In addition, at day 28, M23TC, a metabolized version of lamivudine, was detected at high levels in the liver, lymph nodes, and spleen (39), suggesting that this nanoplatform may improve delivery of the drug to HIV-1 niches. Other groups have also developed nanoparticles for improving HAART (40–43) (**Table 1**).

Nanoparticles for Improving Latency Reversal

Latency-reversing agents (LRAs) are able to reactivate viral replication. LRAs are used in "shock and kill" treatment approaches, wherein the LRA-elicited viral replication is coupled

TABLE 1 | Nanoparticle-based immunoengineering approaches for HIV/AIDS.

Nanoparticle class	Key findings	References
NANOPARTICLES FOR IMPROVING HAART		
Poloxamer-based nanoparticles	LASER ART (also known as nanoART) nanoparticles for controlled release of HAART agents	(37–40)
Lipid nanoparticle	Lipid-drug nanoparticles exhibited 5-fold increased bioavailability of HAART drugs in lymph nodes, and markedly increased sustained release over the course of 7 days	(41)
PLGA/pluronic nanoparticle	Nanoformulation of HAART drugs demonstrate greater bioavailability in plasma and absorption in various tissues over the course of 14 days	(42)
Lactoferrin nanoparticle	First-line nanoformulated HAART drugs exhibited 4-fold increase in bioavailability and an increase in anti-HIV activity compared to soluble agents	(43)
NANOPARTICLES FOR IMPROVING LATENCY REVERSAL		
Lipid nanoparticle	LRA and protease inhibitor encapsulated within nanoparticle reversed latency and prevented HIV-1 viral spread	(44)
Lipid-coated PLGA nanoparticle	Co-administration of LRAs-individually encapsulated within nanoparticles exhibited synergistic induction of HIV-1 mRNA levels at low cytotoxicity	(45)
Iron oxide nanoparticle	LRA and vorinostat-loaded nanoparticle penetrated BBB, reversed HIV-1 latency and exhibited antiviral efficacy in astrocytes.	(46)
PLG/PEG nanoparticle	LRA and protease inhibitor encapsulated within PLGA-PEG nanoparticles reversed latency and inhibited viral spread	(47)
NANOVACCINES		
PLGA nanoparticle	Encapsulating TLR-agonist improved HIV-1 vaccine immunogenicity and decrease required dose for immunogenic effect	(48)
Lumazine synthase- and ferritin-based nanoparticles	Antigens encapsulated within nanoparticles were trafficked within germinal centers promoting a potent immunogenic response	(49)
Polyethylenimine mannose/DNA/ glucose nanoparticle	DermaVir nanoformulation delivered HIV-1 antigen to Langerhans cells, which matured into Dendritic cells, mounting an immune response	(50)
eOD-GT8 nanoparticle	Engineered outer domain (eOD)-60 mer nanoparticle exhibited sufficient precursor naïve B cell binding for bnAb production	(51)
Liposome nanoparticle	Clade C-derived trimers decorated on liposomal surface induced enhanced germinal center and bnAb responses compared to soluble trimers	(52)
Ferritin nanoparticle	Consensus-derived Env trimers conjugated to nanoparticles induced greater bnAb targeting of apex trimers of *in vivo* models	(53)
Protein nanoparticle	Nanomaterial presenting SOSIP trimer increased B-cell activation and induced greater bnAb titers against Tier-1A viral strains	(54)
Liposome nanoparticle	Vaccination with liposomes formulated with HIV envelope protein elicits bnAb targeting and neutralization	(55)
Ferritin nanoparticle	HIV antigens are presented on nanoparticles in native trimeric structure as a tool for vaccine development	(56)
NANOPARTICLES TARGETING HIV VIRAL FUSION TO IMMUNE CELLS		
Silver nanoparticle	Silver nanoparticles exert anti-HIV activity through gp120 binding in various viral strains	(57)
Poly (acrylate)-based nanoparticle	Hydrophobic nanoparticle impedes amyloid fiber structure, thereby disrupting HIV-1 trafficking to its target cell	(58)
PLGA nanoparticle	Nanoparticles coated with a T-cell membrane were able to serve as a "decoy" for HIV-1 binding, resulting in viral suppression	(59)
Extracellular vesicles	Extracellular vesicles (EVs) released by *Lactobacillus* inhibited HIV-1 viral attachment and entry to target cells	(60)
Extracellular vesicles	EVs isolated from semen inhibited HIV-1 regardless of donor infection status; EVs from ART-treated subjects inhibited HIV-1 *in vivo*	(61)
NANOPARTICLES TO ENHANCE GENE EDITING APPROACHES		
Gold nanoparticles	Au-nanoparticles can mediate CRISPR-Cas9 components to target cells with higher efficiency and lower cytotoxicity	(62)
Poloxamer-based nanoparticles	LASER ART combined with CRISPR/Cas9 eliminated HIV-1 in a small subset of mice	(63)
NANOPARTICLES TO ENHANCE CLEARANCE BY ADOPTIVELY TRANSFERRED IMMUNE CELLS		
PLGA nanoparticles	Nanoparticles encapsulating neutralizing antibody and LRA improved NK cell effector function toward J-Lat cells compared to free agents	(64)
Lipid nanoparticle	IL-15-loaded nanocapsules conjugated to HIV-1-specific CTLs improved elimination of infected cells	(65)
OTHER THERAPIES		
PEG-*b*-PR co-polymer nanoparticles	STING agonist nanoformulation reversed HIV-1 immune evasion mechanism	(66)
Quantum dots	Graphene quantum dots mediated HIV-1 viral suppression	(67)
Nanodiamonds	Efavirenz-nanodiamond conjugation improved bioavailability and blood brain barrier penetration	(68)
Gold nanoparticles	Gold conjugated with HIV integrase inhibitors could penetrate the BBB and exert antiviral efficacy in targeted HIV-1-infected microglial cells	(69)
PLGA nanoparticles	FTC-loaded nanoparticles exhibited greater bioavailability and lower IC_{50} compared to soluble agents	(70)
PLGA nanoparticles	TDF-loaded nanoparticles in thermosensitive gel conferred 100% protection from HIV-1 strains within 24 h time period and had no detectable viral levels in plasma throughout 4 weeks period	(71)
Cellulose acetate phthalate nanoparticles	DTG-loaded nanoparticles in thermosensitive gel were uptaken into vaginal epithelial cells with low cytotoxicity	(72)

to the actions of HIV-1 cell-specific cytotoxic agents or immune-mediated clearance (73). Several classes of molecules and macromolecules have been used as LRAs including protein kinase C (PKC) agonists, histone deacetylase inhibitors, and cytokines, and their mechanisms of latency reversal are well-described. For example, PKC agonists function via the NF-KB pathway. Activated PKC isoforms downregulate the inhibitor IKB, thereby releasing the transcription factor NF-KB, which translocates into the nucleus, and binds to the HIV-1 proviral long terminal repeats, thereby mediating viral transcription (74). Similar to HAART, nanoparticles have been utilized to improve the delivery of LRAs (**Table 1**). In one study, Kovochich et al. encapsulated bryostatin, a potent PKC agonist (75) and nelfinavir, an HIV-1 protease inhibitor, into nanoparticles. Their nanoplatform targeted $CD4^+$ cells in a peripheral blood mononuclear cells (PBMC) culture, activated latent virus, and inhibited viral spread (76). In a more recent study, Cao et al. synthesized hybrid lipid-coated PLGA nanocarriers that incorporated diverse LRAs. These lipid-coated nanoparticles could selectively activate $CD4^+$ T cells in nonhuman primate PBMCs as well as in murine lymph nodes with substantially reduced toxicity (44). Despite the fact that it is currently impossible to identify and target every HIV-1-infected cell in the latent reservoir (45), the ability of nanoparticles and nanocarriers to traffic and deliver LRAs to sites of latent HIV reservoirs can maximize their therapeutic benefit, and serve as an important component of successful shock and kill cure regimens. Other examples of nanoparticles for improving latency reversal appear in **Table 1** (46, 47).

Nanovaccines

Traditional vaccines for HIV-1 have been difficult to develop, and clinical trials using HIV-1 vaccines have demonstrated poor efficacy (77, 78). Vaccines fail for several reasons including poor delivery to dendritic cells, reversion of a live attenuated virus to its virulent form, or if the vaccine is too weak to facilitate an immune response (68). Consequently, an ideal vaccine should be clinically safe, stable, and capable of inducing a potent immune response (79). Nanoparticles have been shown to overcome these limitations (80) by protecting antigens from proteolytic enzymes, promoting antigen uptake and processing by antigen-presenting cells (APCs), in addition to being biocompatible and biodegradable (81). Several groups have leveraged favorable properties of nanoparticles to develop nanovaccines for HIV-1 (**Table 1**).

One effective strategy is utilizing nanovaccines to activate dendritic cells (DCs), which in turn cause T cell activation (82). To this end, Rostami et al. decorated antigens onto the surface of nanoparticles to facilitate greater interaction with the APCs due to the high surface area to volume ratio of the nanoparticles (48). Specifically, a flagellin molecule sequence derived from *Pseudomonas aeruginosa* (FLiC), a toll-like receptor 5 agonist, was conjugated to an HIV-1 p24-NeF peptide, and encapsulated within PLGA nanoparticles. The FLiC-p24-NeF-encapsulated nanoparticle elicited higher levels of lymphocyte proliferation and cytotoxic T cell activity compared to controls (48), suggesting its potential use in an HIV-1 vaccination strategy. In a more recently study by Tokatlian et al., nanoparticles encapsulating HIV-1 antigens were observe to localize to the lymph nodes more than corresponding soluble antigen counterparts, and remained localized there for up to 4 weeks (49). In another study, Lori et al. showed that their nanoplatform "DermaVir" could administer HIV-1 antigens to Langerhans cells, which resulted in a potent immunogenic response (50). DermaVir is currently undergoing a phase 3 clinical trial evaluation based on excellent responses observed in Phase I/II clinical trials (83). Together, these studies along with others summarized in **Table 1** (51–56), clearly suggest the importance of nanovaccines for treating HIV-1.

Nanoparticles Targeting HIV Viral Fusion to Immune Cells

Targeting the HIV replication cycle by inhibiting the ability of HIV-1 to fuse and/or enter a target cell has been the focus of several published studies (**Table 1**). Fusion or entry inhibition leads to inhibition of viral activity and viral cytotoxicity. In one approach, Lara et al. showed that silver nanoparticles are antiviral and prophylactic against HIV-1 fusion to target cells (57). Silver nanoparticles exert anti-HIV activity at an early stage of viral replication, likely as a virucidal agent or as an inhibitor of viral entry. Silver nanoparticles bind to gp120 in a manner that prevents CD4-dependent virion binding, fusion, and infectivity, acting as an effective virucidal agent against cell-free and cell-associated virus. Further, silver nanoparticles inhibit post-entry stages of the HIV-1 life cycle (57).

Another approach utilized semen-derived enhancer of viral infection (SEVI), which is a type of amyloid fibril present in human semen that enhances HIV-1 infection of target cells by capturing HIV-1 virions, resulting in increased viral fusion (84). SEVI serves as a mediator for HIV-1 viral attachment due to its highly cationic nature (84, 85). In their study, Sheik et al., synthesized a hydrophobic polymeric nanoparticle to reduce SEVI fibril-mediated infection (58). The hydrophobicity of the nanoparticle interferes with Aβ amyloid structure, forming amorphous aggregates, thereby disrupting the amyloid HIV-1 trafficking protein to target cells (86–88). Thus, the hydrophobic nanoparticles were able to reduce HIV-1 virion binding affinity toward their target cells (58).

Biomimicry approaches, such as plasma membrane-coated nanoparticles, represent a unique strategy to target a variety of human pathologies (89). A pivotal study showed the efficacy of coating a nanoparticle with a cell membrane to imitate and model endogenous cell activity. HIV-1 infection begins when an exposed HIV-1 surface protein, gp120, interacts with CD4 receptor and chemokine receptor type 5 (CCR5) co-receptor on target cells (90). Wei et al. coated polymeric nanoparticles with a $CD4^+$ T cell membrane, causing the modified membrane-coated nanoparticle to preferentially interact with HIV-1. This preferential binding ultimately neutralized HIV-1 viral activity in PBMCs *in vitro* (59), illustrating the potential of biomimicking nanoparticle approaches to reduce HIV-1 viral spread by blocking viral fusion to T cells.

Unlike synthetic nanoparticles, extracellular vesicles (EVs) are naturally occurring nanoscale structures that carry cargo (e.g., proteins, lipids, nucleic acids) and can be released from

both healthy and apoptotic cells (91). Recently, Palomino et al. discovered that EVs released by Lactobacillus in the healthy vaginal microbiota prevented HIV-1 attachment to target cells and thereby inhibited HIV-1 infection (60). In a recent study by Welch et al., EVs extracted from semen inhibited HIV-1 *in vitro* regardless of HIV infection status of the donor, while EVs extracted from the blood and semen of ART-treated subjected inhibited HIV-1 *in vivo* (61). These studies suggest a potential avenue for bacterial and/or EV-based treatment strategies in preventing HIV-1 viral spread.

Nanoparticles to Enhance Gene Editing Approaches

Gene therapy technologies have been explored for HIV-1 cure strategies (**Table 1**). Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9) is a gene editing platform wherein genes can be added, removed, or altered at given genetic loci (92). CRISPR-Cas9 is a faster and more efficient technique than other genetic editing platforms using other viral vectors or the Cre-Lox system, although current CRISPR-Cas9 delivery techniques use electroporation to facilitate DNA entry into living cells, which is difficult to control and can generate cytotoxicity (92, 93). Previously, gene-editing tools have knocked out CCR5 in $CD4^+$ T cells to block HIV-1 viral entry (94, 95). However, gold nanoparticles (AuNPs) have a unique ability to safely deliver CRISPR-Cas9 components to their targets (96). AuNPs have large surface area to volume ratios and are biocompatible with low toxicity (5). Shahbazi et al. developed AuNPs with layer-by-layer surface conjugation of CRISPR components (AuNP/CRISPR), targeting two locations within the hematopoietic stem and progenitor cell (HSPC) genome, CCR5 and the gamma-globin gene promoter. Genetic deficiency in CCR5 is linked to HIV-1 resistance through the elimination of viral anchoring and entry through its CCR5 co-receptor (62, 97). AuNP/CRISPR was able to penetrate into $CD34^+$ hematopoietic cell line, which is difficult to transfect. At micromolar concentrations, AuNP/CRISPR exhibited an overall low amount of gene editing and homologous directed repair (HDR) at the CCR5 and the gamma-globin promoter locus. This demonstrates that AuNP/CRISPR functioned with low efficacy. However, genetic editing and HDR via AuNP/CRISPR was higher than the electroporation-driven process. This suggests that AuNP/CRISPR could be effective in delivering gene editing for HIV-1 therapy (62).

With the promising innovations of LASER ART and CRISPR-Cas9, Dash et al. combined the two methodologies to evaluate a potentially synergistic functionality. Two of seven HIV-1-infected mice that received LASER ART followed by subsequent AAV_9-CRISPR-Cas9 treatment targeting a fragment of the HIV-1 genome were cured of viral rebound and experienced a restoration of their $CD4^+$ T cells, suggesting HIV-1 regression/elimination (63). Additionally, HIV-1 RNA levels diminished to undetectable levels in the plasma, spleen, liver, gut, and brain in the cured mice. Further, naïve humanized mice that were challenged with adoptively transferred cells isolated from the cured mice showed no detectable HIV-1 viral loads. This study demonstrates the possibility of eliminating HIV-1 in plasma and infectious tissues through this novel combination approach (63).

Nanoparticles to Enhance Clearance by Adoptively Transferred Immune Cells

Recent studies show promising effects of cell therapies for treating HIV-1 (98–100). Here, autologous or allogenic immune cells are transferred to the patient after *ex vivo* expansion and/or modification to clear HIV-1 infected cells. Nanoparticles may offer the ability to enhance the ability of immune cells to target and kill target cells in the context of HIV-1. In their study, Sweeney et al. generated a PLGA nanoplatform that co-encapsulated an LRA and a target cell-specific antibody to improve NK cell effector function in an *in vitro* cell model of latent HIV-1 (64). The nanoplatform was able to increase NK cell cytotoxicity of the target cells, thereby illustrating an example of nanoparticles enhancing immune cell function in the context of latent HIV-1 (64). In another studies, Jones et al. demonstrated that cytotoxic T lymphocytes (CTLs) were made more potent by conjugating drug-loaded lipid nanoparticles to their surface (65). HIV-1-specific CTLs were able to specifically target HIV-1-infected cells and deliver the nanoparticle-encapsulated payload (65).

Other Focus Areas

Nanoparticles to Boost Innate Immunity

HIV-1, like many other viruses, has evolved mechanisms to evade or disrupt immune surveillance. Therefore, one strategy to eliminate HIV-1 viral load is to reverse immune evasion. HIV-1 typically inhibits the cGAS-STING pathway that normally functions via cGAMP binding to STING on the endoplasmic reticulum resulting in an IFN-1-mediated antiviral response (66, 101, 102). pH-sensitive polymeric nanoparticles were engineered to deliver a STING agonist to counteract HIV-1 immune evasion via the cGAS-STING pathway. These STING agonist-nanoparticles demonstrated potent antiretroviral activity for up to 12 days (66).

Nanoparticles to Inhibit HIV-1 Reverse Transcriptase Activity

Quantum dots are biocompatible semiconductor crystal nanoparticles with low toxicity that have been used for biosensing, image contrast, and drug delivery (103, 104). These nanomaterials are attractive due to their intrinsic antiviral activity and thus, their potential as inhibitors of HIV-1. In a proof-of-concept study by Iannazzo et al., a reverse transcriptase inhibitor (RTI; CHI499) was readily conjugated onto the surface of the graphene quantum dots (GQDs) via intrinsic functional groups (67). The conjugated GQD product (GQD-CHI499) achieved remarkable anti-reverse transcriptase and cellular anti-HIV-1 activities compared to the free drug alone. This additive improvement may be the result of the GQDs' intrinsic structure, where the polycarboxylation group could mediate the inhibition of HIV-1 reverse transcriptase through viral fusion (67), suggesting the potential of GQDs in treatment for HIV-1.

Nanoparticles to Enhance Blood Brain Barrier Penetration

Aside from persistent HIV-1 in $CD4^+$ helper T cells, HIV-1 may also persist in microglial cells, which are the resident macrophages of the CNS (6). These cells may confer HAART resistance, perpetuate HIV-1 infection in peripheral tissues, and are critical in the development of HIV-1 associated neurocognitive diseases (105). The brain poses an anatomical barrier, where there is low drug penetration by virtue of the blood brain barrier (BBB). Therefore, there is a need to develop ways to penetrate the BBB to target persistent HIV-1 in microglial cells (6, 105). Nanodiamonds are ∼10 nm diamonds which are known for their inexpensive production, surface modifications, and low cytotoxic profile. In the context of HIV-1, Roy et al. complexed efavirenz (EFV), an effective non-nucleoside RTI, to a nanodiamond to effectively improve the poor bioavailability of EFV (ND-EFV) (68). ND-EFV allowed for sustained release of EFV in a BBB model *in vitro*. In addition, ND-EFV was effective in controlling HIV-1 replication for 7 days, where the EFV alone drug was able to inhibit HIV-1 for 5 days (68). Similarly, gold nanoparticles have also been used entry through the BBB. Garrido et al. showed that gold nanoparticles conjugated with HIV integrase inhibitors could penetrate the BBB with antiviral efficacy, providing another nanoplatform for targeting HIV-1-infected microglial cells (69).

Nanoparticles for Prophylactic HIV-1 Prevention

Another application of nanoparticles is in improving pre-exposure prophylaxis (PrEP), which provides a >90% effective approach to prevent HIV-1 infection but requires daily oral administration (106). PrEP comprises tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC), two nucleoside RTIs that have low half-lives and require high dosing, thereby increasing the risk of adverse effects (107). Mandal et al. encapsulated FTC within PLGA nanoparticles (FTC-NPs), and demonstrated improved bioavailability of FTC with significantly lower inhibitory concentration (IC_{50}) than free FTC *in vitro* (70). Alternatively, Destache et al. loaded TDF into PLGA nanoparticles (TDF-NPs), and subsequently incorporated them within a thermosensitive vaginal gel (71). Mice challenged with two strains of HIV-1 and treated with the TDF-NP gel were 100% protected against the virus with no detectable viral plasma load (71), suggesting the efficacy of sustained release of TDF by nanoparticles via vaginal administration to prevent HIV-1 infection. In another study, Mandal et al. encapsulated dolutegravir (DTG), an integrase strand transfer inhibitor, within nanoparticles made from cellulose acetate phthalate, a pH-sensitive polymer that intrinsically inhibits HIV-1 entry into its target cells (DTG-CAP-NPs) (72). Similarly to above, DTG-CAP-NPs were incorporated into a thermosensitive vaginal gel. Vaginal epithelial cells were able to take up DTG-CAP-NPs, where they persisted for up to 7 days with low cytotoxicity (72). These studies demonstrate the potential of nanoparticles for use in HIV-1 preventative strategies, including enhancing PrEP.

CONCLUSION

Here, we have reviewed the field of nanoparticle-based immunoengineered approaches toward an HIV-1 cure. We highlighted the potential and use of nanoparticles to facilitate and improve the delivery, bioavailability, and/or functionality of HAART, LRAs, vaccines, gene-editing approaches, and other therapeutic or preventative strategies. The innovative advances described herein demonstrate the potential of the field of nanoparticle-based immunoengineering in treating and preventing HIV-1.

AUTHOR CONTRIBUTIONS

AB, ES, and RF all participated in the writing and the preparation of the manuscript, and approved it for publication.

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HIVconsv Vaccines and Romidepsin in Early-Treated HIV-1-Infected Individuals: Safety, Immunogenicity and Effect on the Viral Reservoir (Study BCN02)

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Kick&kill strategies combining drugs aiming to reactivate the viral reservoir with therapeutic vaccines to induce effective cytotoxic immune responses hold potential to achieve a functional cure for HIV-1 infection. Here, we report on an open-label, single-arm, phase I clinical trial, enrolling 15 early-treated HIV-1-infected individuals, testing the combination of the histone deacetylase inhibitor romidepsin as a latency-reversing agent and the MVA.HIVconsv vaccine. Romidepsin treatment resulted in increased histone acetylation, cell-associated HIV-1 RNA, and T-cell activation, which were associated with a marginally significant reduction of the viral reservoir. Vaccinations boosted robust and broad HIVconsv-specific T cells, which were strongly refocused toward conserved regions of the HIV-1 proteome. During a monitored ART interruption phase using plasma viral load over 2,000 copies/ml as a criterium for ART resumption, 23% of individuals showed sustained suppression of viremia up to 32 weeks without evidence for reseeding the viral reservoir. Results from this pilot study show that the combined kick&kill intervention was safe and suggest a role for this strategy in achieving an immune-driven durable viremic control.

Keywords: romidepsin, HDAC inhibitor, kick&kill strategy, HIVconsv, early-treatment

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INTRODUCTION

Current antiretroviral therapy (ART) effectively suppresses HIV-1 replication, thus preventing disease progression. However, the infection remains chronic given that a latent HIV-1 reservoir, established early after infection, persists despite suppressive ART (1). Upon ART discontinuation, integrated replication-competent proviruses in the reservoir drive a rapid viral rebound (2). Therapeutic vaccination has been proposed as a possible approach to induce an effective immune control able to contain rebounding virus (3).

Most therapeutic vaccines tested in the past expressed one or several HIV-1 proteins, which expanded HIV-1-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses to varying levels. However, the responses were ineffective in controlling viremia after ART interruption, likely because of their suboptimal magnitude, breadth, width, specificity, and/or polyfunctionality (4–8), raising the need for novel immunogens and delivery methods to tackle HIV-1 diversity and the virus' ability to escape. New vaccines strategies are being developed to maximize the vaccine coverage of circulating viruses using multivalent mosaic immunogens designed *in silico* (9, 10). Alternatively, vaccines designs are tested that aim to focus the CTL responses toward more conserved and protective regions of the virus, which are less likely to mutate and escape the T-cell response (11–15). Among the latter, the HIVconsv immunogen is one of the most advanced vaccine candidates in clinical development. HIVconsv immunogen consists of a chimeric protein assembled from 14 highly conserved domains derived from HIV-1 genes Gag, Pol, Vif, and Env alternating, for each domain, the consensus sequence of the four major HIV-1 clades A, B, C, and D (12). Upon delivery to both HIV-1-negative and positive individuals by heterologous prime/boost regimens as DNA or in simian adenovirus of chimpanzee (ChAdV) and poxvirus MVA vectors, HIVconsv vaccines were safe and induced $CD8^+$ T cells with broad inhibitory capacity of HIV-1 *in vitro,* but showed no effect on the viral reservoir (16–23).

To overcome the limitations of therapeutic vaccines - administered alone- in targeting the viral reservoir, vaccines are combined with latency reversing agents (LRA) in the so called kick&kill strategies (24). This approach intends to activate transcription of HIV-1 using small molecules able to disrupt the viral latency and facilitate effective sensing and clearance of infected cells by vaccine-elicited HIV-1-specific CTL (25, 26). Histone deacetylase inhibitors (HDACi) have been proposed as potential HIV-1 LRA (27–31). Romidepsin (RMD; Istodax®, Celgene Ltd.) is a potent HDACi approved for the treatment of cutaneous and peripheral T-cell lymphomas, which has been shown to induce HIV-1 transcription both *in vitro* and *in vivo* (32, 33). The REDUC trial combined RMD with Vacc-4x and rhuGM-CSF in chronically suppressed HIV-1-positive individuals, resulting in a mean reduction of 39.7% in total HIV-1 DNA (34). However, this intervention failed to delay viral rebound after ART interruption, suggesting that the reservoir-purge effect was not sufficient and/or the vaccine-induced response was unable to eliminate cells actively replicating HIV-1. In fact, the increase in cell-associated HIV-1 RNA inversely correlated with time to rebound, supporting that, in the absence of an enhanced HIV-1-specific CTL response, viral reactivation might facilitate viral rebound once ART is interrupted (35).

Here, in this single-arm, open-label, phase I, proof-of-concept study, referred to as BCN02 trial (NCT02616874), we assessed the safety, tolerability, immunogenicity and effect on the viral reservoir of a kick&kill strategy consisting of the combination of HIVconsv vaccines with RMD in suppressed early-treated HIV-1-infected individuals. Participants were rolled-over from the therapeutic vaccine trial BCN01 (NCT01712425), in which individuals who started ART during acute/recent HIV-1 infection had received a prime/boost regimen of the ChAdV63.HIVconsv and MVA.HIVconsv vaccines (CM) (20). Three years after, BCN01 participants who had shown sustained viral suppression and who accepted to participate in BCN02 study were immunized with two doses of MVA.HIVconsv, before and after three weekly-doses of RMD, followed by a monitored antiretroviral pause (MAP) for a period of 32 weeks to assess the ability of the intervention to control viral rebound.

MATERIALS AND METHODS

Study Design and Interventions

The BCN02 clinical trial was an investigator initiated phase I, open-label, single-arm, multicenter, single-country study to assess the safety, tolerability and efficacy of a combined kick&kill strategy in suppressed HIV-1-infected patients that had initiated ART during acute/recent HIV-infection. Individuals were rolled over from vaccine trial BCN01 (20) and invited to participate after 3 years on suppressive ART. A complete list of inclusion/exclusion criteria is available in the Study Protocol (**Appendix**). The study took place between February 2016 and October 2017 at two HIV-1 units from universitary hospitals (Hospital Universitari Germans Trias i Pujol -HUGTIP, Badalona and Hospital Clínic, Barcelona) and a community center (BCN-Checkpoint, Barcelona). Before inclusion in the study, all participants signed an informed consent previously discussed, reviewed and approved by the Community Advisory Board of the Barcelona-based vaccine program (HIVACAT). The study was approved by the institutional ethical review board of the participating institutions (Reference Nr AC-15-108-R) and by the Spanish Regulatory Authorities; and was conducted in accordance to the principles of the Helsinki Declaration and local personal data protection law (LOPD 15/1999). The MVA.HIVconsv vaccine was GMP manufactured at IDT Biologika GmbH, Germany, and supplied for the study under an investigator initiated clinical trial contract agreement. Risk of Genetically Modified Organism release to the environment was evaluated by the Spanish Ministry of Environment (B/ES/12/09). RMD was supplied for the study by Celgene Ltd. (Couvet, Switzerland) under an investigator initiated clinical trial contract agreement.

The BCN02 trial design is summarized in **Figure 1A**. After their inclusion in the study (week 0), all participants received a first dose of 2×10^8 plaque-forming units (pfu) of MVA.HIVconsv (MVA_1) administered intramuscularly, followed

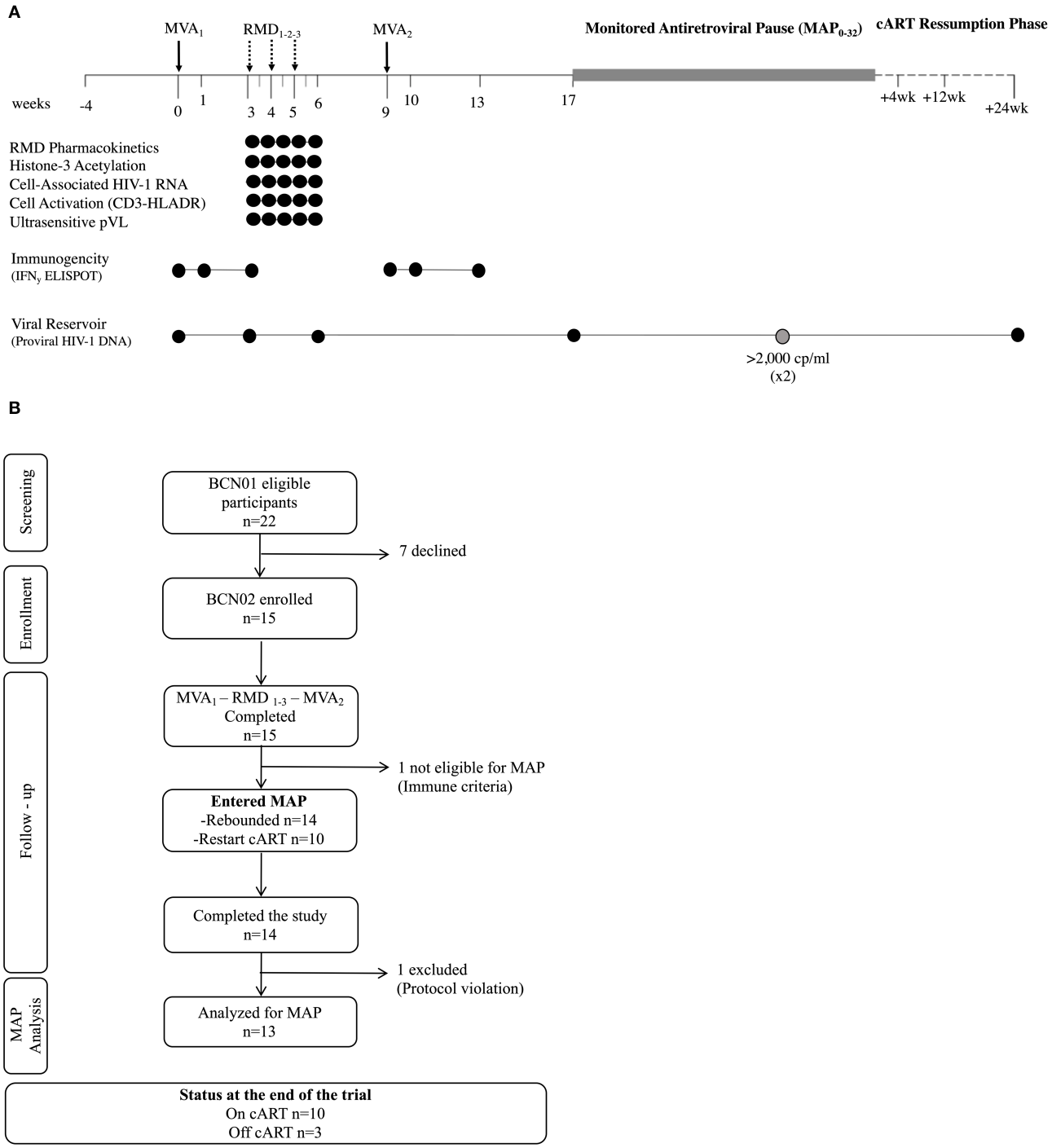

FIGURE 1 | Trial design. **(A)** Schematic study design. **(B)** Consolidated Standards of Reporting Trials (CONSORT) flow diagram for the trial. *Viral rebound during MAP was defined as pVL >20 copies/ml and #criteria for ART resumption included pVL over 2,000 copies/ml in two consecutive determinations, $CD4^+$ cell counts decrease over 50% and/or below 500 cells/mm^3 and/or development of clinical symptoms suggestive of an acute retroviral syndrome. MVA, MVA.HIVconsv vaccine; RMD, romidepsin; MAP, monitored antiretroviral pause; ART, antiretroviral therapy; pVL, plasma HIV-1 viral load.

by three weekly doses of RMD of 5 mg/m^2 BSA infused intravenously over 4 hours (RMD_{1-2-3}), and by a second dose of 2×10^8 pfu of MVA.HIVconsv (MVA_2) 4 weeks after RMD_3 to compensate for any potential impairment in the previous vaccine-induced response caused by RMD. Following RMD prescription information, participants received prophylactic

antiemetic treatment with ondasetron before and during 3 days after each RMD dose.

Eight weeks after MVA_2 (week 17), eligible participants initiated a MAP for a maximum of 32 weeks (MAP_{0-32}) or until any ART resumption criteria were met. To be eligible for the MAP, participants had to maintain undetectable HIV-1 pVL and meet the immune futility criteria, defined as showing a net increase in HIVconsv-specific immune response with MVA_2 boost measured in an *ex vivo* IFN-γ ELISPOT assay. During MAP, participants were allowed to choose either the hospital units or BCN-Checkpoint community center for the follow up visits. Symptoms suggestive of acute retroviral syndrome and sexually transmitted diseases were solicited and viral load was tested using the finger-tip Xpert HIV-1 Qual kit (Cepheid, Sunnyvale, CA, US) in all visits. When a positive result was obtained in the Xpert HIV-1 Qual, participants were called in for a confirmatory quantitative pVL within the next 24 h. If pVL was confirmed to be over 20 copies/ml, a visit was scheduled 3 days after to closely monitor viral rebound and be able to offer prompt ART resumption if required. Details on viral load management during MAP are described in the Study Protocol (**Appendix**). After ART ressumption, participants were followed at 4, 12, and 24 weeks to assure that they re-attained viral suppression.

Study Population

BCN02 participants were adult (≥18 years) HIV-1-infected individuals, who had initiated ART >6 months after estimated date of HIV-1 acquisition and who had received a prime/boost heterologous vaccination regimen using ChAdV63.HIVconsv-MVA.HIVconsv in the parental BCN01 study (20). To be eligible for BCN02, participants had to maintain optimal HIV-1 suppression during at least 3 years and $CD4^+$ cell counts ≥500 cells/mm^3 at BCN02 baseline visit. Main exclusion criteria included active hepatitis B or C, history of AIDS-defining disease, treatment for cancer or lymphoproliferative disease within 1 year before study entry or use of immunosuppressants within the 3 months prior to the screening visit. Concomitant treatment with strong CYP3A4 inhibitors was not permitted, but switching ART to a non-boosted integrase-inhibitor raltegravir- or dolutegravir-based regimen at least 4 weeks before baseline visit was allowed for those patients receiving ART containing ritonavir or cobicistat at screening.

Study Endpoints

The primary endpoint of this study was to assess the safety, tolerability and the effect on the viral reservoir size of the combined treatment with HIVconsv vaccines and RMD given as a latency reversing agent. Secondary endpoints included the extent and specificity of the CTL response and the effect of the intervention in controlling viral rebound after ART interruption. Other secondary endpoints included RMD pharmacokinetics and the effects of RMD on histone acetylation in lymphocytes, induction of viral transcription, changes in T-cell activation surface markers, and quantification of plasma viremia.

Safety and tolerability were evaluated by the development of grade ≥3 and serious adverse events (AE). Local and systemic AE were solicited prospectively for a minimum of 7 days following each immunization and RMD administration. Both local and systemic AE were graded according to the Division of AIDS (DAIDS) Table for Grading the Severity of Adult and Pediatric Adverse Events, version 2.0, November 2014, accessible online at https://rsc.niaid.nih.gov/sites/default/files/daids-ae-grading-table-v2-nov2014.pdf. AE were specified as unrelated, unlikely, probably or definitely related to the investigational products by the investigator.

Determination of RMD Pharmacokinetics

The concentration of RMD in plasma was determined, for RMD_1, at the end of the infusion (4 h) and 4.5, 5, 6, 8, 12, and 24 h after and, for RMD_2 and RMD_3, at the end of the infusion and 12 h after. RMD concentrations were measured by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), according to a validated method. A population pharmacokinetic model for RMD was developed using non-linear mixed-effects modeling with the computer program NONMEM version 7.3 (Icon Development Solution, Ellicot City, MD) (36). Bayesian estimates of the individual parameters of RMD were used to simulate individual drug concentrations, and RMD area under the concentration-time curve (AUC0-inf) was calculated for each individual on each occasion using a non-compartmental approach (Winnonlin software; Phoenix, version 7.0).

Flow Cytometry Determination of acH3 and Activation of T Cells

The levels of histone H3 acetylation in lymphocytes (based on FSC/SCC scatter) were determined by flow cytometry from samples taken before (0 h) and at the end of each RMD infusion (RMD_{1-2-3}) (4 h), at 8 and 24 h (+1 day) RMD_1, and at 72 h (+3 days) and 7 days after (RMD_{1-2-3}). Cryopreserverd PBMC were thawed 4 h before use, and 500,000 cells were blocked with 600 ul of PBS/10% FBS for 20 min and stained with polyclonal rabbit anti-acetyl histoneH3 (10 μg/ml, MerckMillipore #06–599) or normal rabbit serum (control stain, LifeTechnologies #10510) for 30 min. Cells were washed and subsequently incubated with donkey anti-rabbit IgG(H+L) (6 μg/mL, LifeTechnologies #A21206) for 30 min at room temperature in the dark. Cells were washed, re-suspended in 150 μl PBS and analyzed. ∼50,000 events were acquired per sample. The median fluorescence intensity (MFI) for each sample was calculated by substracting the background MFI (isotype control stain) from the anti-acetyl histoneH3 stain.

Activation of T cells was determined based upon HLA-DR expression on $CD3^+$ T cells. Cryopreserved PBMC were thawed, and 1,000,000 cells were stained with CD3 APC-Cy7, CD4 FITC, CD8 BV510 and HLA-DR PECy7 (BioLegend #344818, 300538, 301048, and 307616, respectively). Cells were collected on an LSRII instrument (BD), and data analyzed according to the gating criteria shown in **Supplementary Figure 1** using FlowJo 10 software.

Quantification of Cell-Associated (CA) HIV-1 RNA in CD4$^+$ T Cells

Cell-associated HIV-1 RNA was quantified in peripheral CD4$^+$ T cells by ddPCR (One-Step RT-ddPCR Advanced Kit for Probes, BioRad) from samples taken before (0 h) and at the end of each RMD infusion (4 h), at 8 and 24 h (+1 day) after RMD_1, and 72 h (+3 days) and 7 days after RMD_{1-2-3}. CA HIV-1 RNA was quantified using two different primers/probe sets annealing to the 5′LTR and GAG conserved regions of HIV-1, to circumvent potential primer mismatch in individuals' viral sequence as previously described (37). HIV-1 transcription levels were normalized to the housekeeping gene TATA-binding protein (TBP) and shown as relative to levels before RMD_1.

Ultra-Sensitive Determination of Residual Viremia

To evaluate HIV-1 RNA below 20 copies/ml, 4–8 ml of plasma samples taken before (0 h) and at the end of each RMD infusion (4 h), at 8 and 24 h (+1 day) after RMD_1, and 72 h (+3 days) and 7 days after RMD_{1-2-3} were ultracentrifugated at 170,000 g at 4°C for 30 min and viral RNA was extracted automatically using the m2000sp Abbot device. HIV-1 RNA copies were quantified using the Abbott Real-Time HIV-1 assay (Abbott Molecular Inc.) and in-house calibration curve sets as described (38). The limit of detection (2 HIV-1 RNA copies/mL) was calculated relative to the plasma volume.

Vaccine Immunogenicity

Total HIV-1 and HIVconsv-specific T cells were assessed *ex vivo* using cryopreserved PBMC obtained the day of vaccination and 1 week afterwards, 3 weeks after MVA_1, and 4 weeks after MVA_2 using an IFN-γ-detecting enzyme-linked immunoabsorbent spot assay (ELISPOT IFN-γ Mabtech kit). 15-mer peptides overlapping by 11 amino acid were combined into 6 pools of 32–33 peptides per pool corresponding to the HIVconsv vaccine insert (P1-P6, total $n = 166$ peptides, IN pools) (**Figure 3A**) and 12 pools of 39–67 peptides per pool spanning the rest of the HIV-1 viral protein sequences (OUT pools for "outside the immunogen," obtained through the NIH AIDS Reagent Program). All peptides pools were tested in duplicates. The final concentration of individual peptides in the ELISPOT assay was 1.57 μg/ml. Medium only was used as no-peptide negative control in quadruplicate wells, and PHA (50 μg/ml) and a CEF peptide pool (2 μg/ml) consisting of 23 previously defined human CD8$^+$ T-cell epitopes from cytomegalovirus, Epstein-Barr virus and influenza virus (C.T.L. OH, USA) were added as positive controls.

To address the breadth of the vaccine-induced response at the peak immunogenicity time point, an IFN-γ ELISPOT assay with *in vitro* expanded T cells was performed on stored samples from week 10 and 13 to test individual overlapping peptides covering the HIVconsv immunogen sequence ($n = 166$ OLP). Briefly, cryopreserved PBMC were thawed and incubated for 3 h at 37°C in R10 before stimulation with an anti-CD3 monoclonal antibody during 2–4 weeks in RPMI 1640 supplemented with FBS and penicillin/streptomycin with 50 U/ml of recombinant IL-2 (39). Before their use in ELISPOT assays, the expanded cells were washed twice with R10 and incubated overnight at 37 °C in the absence of IL-2 and individual OLP were added at 5 μg/ml. 100,000 cells were used per well as in the direct *ex vivo* assay.

Spots were counted using an automated Cellular Technology Limited (C.T.L., OH, USA) ELISPOT Reader Unit. The threshold for positive responses was set at ≥50 SFC/10^6 PBMC (5 spots per well), > the mean number of SFC in negative control wells plus 3 SD of the negative control wells, or > 3× the mean of negative control wells, whichever was higher. To avoid overestimating the breadth of responses, positive responses to two consecutive 15-mer overlapping peptide were counted as one response. The highest magnitude of the sequential responses was taken as the magnitude for each identified response.

Quantification of HIV-1 Reservoir

To quantify the size of the peripheral blood proviral reservoir, lysed extracts from CD4$^+$ T cells were used to measure total CA HIV-1 DNA by ddPCR. Primers and probes for the RPP30 cellular gene were used for input normalization.

Statistical Analysis

Qualitative variables were represented as mean absolute and relative frequencies, whereas quantitative variables were represented as mean or median and range. Safety endpoints are summarized by the number and percentage of participants reporting local and systemic AE and their grading. The Wilcoxon signed rank test was used to test whether the viral reservoir and the immune parameter changed as an effect of the intervention, without correction for multiple comparisons. The maximum breadth of the T-cell response per individual was estimated as the number of P1-P6 pools eliciting a positive response throughout the study and the number of individual OLP eliciting a response at peak immunogenicity time point from the mapping assay. Reservoir size and immunogenicity were analyzed using GraphPad Prism (v5.01) for Mac OS X (San Diego, CA).

To evaluate the effect of the intervention on viral rebound, a positive pre-defined efficacy signal was established if at least over 20% of patients remained with pVL below 2,000 copies/ml at week 12 of MAP, considering previous data suggesting that early treatment initiation could favor delayed viral rebound/spontaneous viral control in up to 15% of individuals (40). However, BCN02 was an exploratory pilot trial and, due to the absence of a control arm and its final small sample size, the nature of this study only allowed to detect trends in virological effects, which collectively, could be useful to design future studies. To detect possible factors associated to the viremic control observed during the MAP phase, univariate log-binomial regression models were used (41). This model uses the logarithm as a link function, and is a generalized lineal model for a binary outcome where the error terms follows a binomial distribution. The effect size measure of the model is the relative risk. Because of the low number of MAP-C ($n = 3$), multivariate log-binomial regression models were not fitted. The significance threshold for all univariate analyses was set at a two-sided $\alpha = 0.05$. The analyses were performed with R Core Team (42) (v3.0.2).

RESULTS

Participants Enrolled in the Study

Between February 29th and September 15th 2016, 15 out of the 22 eligible BCN01 participants were enrolled in BCN02. Seven declined to participate due to their inability to attend all the scheduled visits. Baseline characteristics of trial participants are summarized in **Table 1**. All 15 participants received two doses MVA.HIVconsv (MVA_{1-2}) and three doses of romidepsin (RMD_{1-2-3}) as shown in the study chronogram (**Figure 1A**), and were included in the safety, immunogenicity and reservoir analyses. One participant was not eligible for MAP due to immune futility pre-defined criteria and 14 participants underwent a MAP for a maximum of 32 weeks. Retrospective analyses of stored plasma samples obtained during MAP revealed the presence of antiretroviral drugs in some samples from one participant, whose MAP data were censored for the viral rebound kinetics analyses (**Figure 1B**).

Safety of MVA.HIVconsv and RMD Administrations

All participants reported adverse events (AE) related to both study investigational medicinal products. A total of 333 AE were recorded during the study intervention phase, 129 after MVA_{1-2} and 204 after RMD_{1-2-3}, which were mostly mild or moderate (grade 1-2) ($n = 318$, 95%). The most frequent AE related to MVA.HIVconsv, summarized in **Table 2**, were local pain at the injection site and a flu-like syndrome consisting of fatigue, headache, myalgia, and/or low-grade temperature (<38°C). Regarding AE related to RMD (**Table 3**), the most common grade 1–2 events were headache, fatigue, and gastro-intestinal symptoms. Despite prophylactic ondansetron treatment, 4 (27%) individuals vomited the days of RMD administration. One participant experienced a grade 4 AE consisting in a sepsis by *Shigella sonnei* that required hospital admission for 24 h, thus fulfilling the criteria of serious adverse event (SAE). The symptoms started within 4 h after RMD_3 and therefore, the SAE was considered as possibly related to RMD.

No laboratory abnormalities related to MVA_{1-2} were reported. All laboratory abnormalities related to RMD were grade 1-2 ($n = 22$), the most frequent being hypophosphatemia (8 events) and thrombocytopenia (5 events), except from one case of grade 4 creatinine kinase elevation with normal eGFR which resolved within 7 days without sequelae. Noteworthy, $CD4^+$ T-cell counts showed a transient decrease by a median of 248 cells/mm^3 3 days after each RMD administration which was not fully recovered by day 7 after RMD_3 (**Supplementary Figure 2**). Overall, both MVA.HIVconsv and RMD at the regimen and dose administered in this study were well-tolerated and safety profiles were consistent with data previously reported (20, 34).

During the MAP, 12 (86%) participants reported a total of 58 AE, which were all grade 1-2 and not suggestive of acute retroviral syndrome (not shown). Grade 1 anxiety was observed in one participant who repeatedly declined psychological support (same participant with protocol violation during the MAP).

RMD Pharmacokinetics and Pharmacodynamics

Pharmacokinetics of RMD was comparable to previously described data (43). A population pharmacokinetic model adequately describing RMD concentrations in plasma was developed (36). According to the individual profiles simulated using the model, each infusion was followed by a rapid and polyexponential decline in RMD concentrations in plasma, reaching nearly undetectable levels by 24 h after dosing (**Figure 2A**).

Regarding the direct effect of RMD on chromatin and induction of viral transcription, histone H3 acetylation (acH3)

TABLE 1 | Demographic, clinical, and treatment characteristics of study patients at study entry ($n = 15$).

Age (years)	43 (33–51)
Gender (M/F), *n*	14/1
MSM/HTS, *n*	14/1
Time since HIV-1 acquisition to ART (days)	92 (28–164)
Pre ART log_{10} HIV-1 RNA (copies/ml)	4.9 (3.2–5.8)
Time on ART (years)	3.23 (3.03–3.77)
ART regimen, *n (%)*	
TDF/FTC/RAL	11 (73)
ABC/3TC/RAL	2 (13)
ABC/3TC/DTG	2 (13)
$CD4^+$ T-cell counts (cells/mm^3)	728 (416–1,408)
Ratio CD4/CD8	1.37 (0.97–1.93)

Median (range) is shown unless otherwise described. M, male; F, female; MSM, men who have sex with men; HTS, heterosexual; ART, antiretroviral therapy; TDF, Tenofovir Disoproxil Fumarate; FTC, Emtricitabine; RAL, Raltegravir; ABC, Abacavir; 3TC, Lamivudine; DTG, Dolutegravir.

TABLE 2 | Summary of adverse events related to MVA.HIVconsv vaccinations ($n = 15$).

	Grade 1 *n*	Grade 2 *n*	Grade 3 *n*	Grade 4 *n*	Total *n (%)*
Injection site reaction					
Local pain	7	4	2	0	13 (87)
Redness	1	0	0	0	1 (7)
Induration	0	0	0	0	0 (0)
Systemic adverse events					
Fatigue	7	4	2	0	13 (87)
Headache	5	3	1	0	9 (60)
Myalgia	4	3	2	0	9 (60)
Fever	5	0	0	0	5 (33)
Anorexia	3	0	1	0	4 (27)
Sweating	2	2	0	0	4 (27)
Nausea	2	0	1	0	3 (20)
Abdominal pain	0	1	1	0	2 (13)
Flatulence	1	0	0	0	1 (7)
Somnolence	1	0	0	0	1 (7)

TABLE 3 | Summary of adverse events related to RMD_{1-2-3} treatment ($n = 15$).

	Grade 1 *n*	Grade 2 *n*	Grade 3 *n*	Grade 4 *n*	Total *n (%)*
Headache	9	5	0	0	14 (93)
Fatigue	9	5	0	0	14 (93)
Nausea	4	7	0	0	11 (73)
Anorexia	8	1	0	0	9 (60)
Abdominal pain	5	2	0	0	7 (47)
Metallic taste	5	1	0	0	6 (40)
Constipation	4	2	0	0	6 (40)
Abdominal distension	4	1	0	0	5 (33)
Vomits	4	0	0	0	4 (27)
Sweating	2	2	0	0	4 (27)
Palpitations	3	0	0	0	3 (20)
Myalgia	1	1	0	0	2 (13)
Rash	0	2	0	0	2 (13)
Dry mouth	1	0	0	0	1 (7)
ECG: ST-elevation	1	0	0	0	1 (7)
Anxiety	0	1	0	0	1 (7)
Libido decrease	0	1	0	0	1 (7)
Somnolence	1	0	0	0	1 (7)
Sepsis by *Shigella sonnei* (SAE)	0	0	0	1	1 (7)
Hypotension	0	1	0	0	1 (7)

increased rapidly during each RMD infusion, remained high during 4 h, and returned to baseline values 3 days after each dose (**Figure 2B**), which is consistent with previous reports (33, 34). HIV-1 transcription transiently increased in parallel, with changes more pronounced after RMD_2 and RMD_3 (**Figure 2C**). These changes were more evident without normalization for house-keeping genes (**Supplementary Figure 3**) possibly reflecting the general increase in histone acetylation levels (44) induced by RMD. Increases in T-cell activation, measured by the proportion of $CD3^+$/HLA-DR^+ cells, were observed 3 days after each RMD dose. Over the course of the three RMD doses, T-cell activation increased in a progressive manner and was maintained up to 1 week after RMD_3 (**Figure 2D**), suggesting a cumulative effect of RMD.

To evaluate changes in levels of quantifiable plasma HIV-1 RNA, an ultrasensitive single copy assay was used. Kinetics of plasma HIV-1 RNA levels did not follow a clear pattern (**Figure 2E**), despite the increasing percentage of participants with detectable low-level viremia at the end of each RMD dose (**Supplementary Figure 4**). Collectively, we reproduced effects on acH3, HIV-1 transcription and T cell activation previously reported in chronically infected individuals (34), suggesting that a lower viral reservoir level achieved by early-treatment initiation does not preclude the reactivation potential of RMD.

MVA.HIVconsv Immunogenicity

Total HIV-1 and HIVconsv-specific T cells were assessed *ex vivo* by an IFN-γ-detecting enzyme-linked immunoabsorbent spot (ELISPOT) assay using 6 peptide pools covering the HIVconsv immunogen sequence (P1-P6) on week 0 (day of MVA_1), 1, 3 (day of RMD_1), 9 (day of MVA_2), 10, and 13. A total of 90 samples were obtained, of which 3 (3%) were censored due to low positive controls and/or high background. All 15 participants showed an absolute increase in HIVconsv-specific IFN-γ-producing T cells during the study, either after MVA_1 (Wilcoxon signed-rank, $p = 0.0007$) or after MVA_2 (Wilcoxon signed-rank, $p = 0.0017$) (**Figure 3B**). Median (range) total frequencies of HIVconsv-specific T cells reached 1,965 (530-6,901) spot-forming cells (SFC)/10^6 PBMC at the peak immunogenicity time point, which represented an absolute median increase of 1,600 (300–6,621) SFC/10^6 PBMC from baseline (Wilcoxon signed-rank, $p < 0.0001$).

Over the intervention phase, participants responded to median (range) of 5 (2–6) peptide pools (**Supplementary Figure 5**). To map the maximum vaccine-induced breadth at peak immunogenicity time point (weeks 10–13), *in vitro* expanded T cells responding toward individual OLPs covering the HIVconsv immunogen were assessed. Median (range) of 8 (3–16) IFN-γ-producing responses to individual OLPs were found, with a dominance in Pol-specific T cells, consistent with the immunogen composition (**Figure 3C**).

The dominance of HIVconsv-specific responses was calculated at each time point as the percentage of HIVconsv-specific T-cell frequencies divided by the total HIV-1 proteome-specific T-cell frequencies. At the moment of HIV diagnoses, HIVconsv responses were subdominant (<10% being HIVconsv-specfic) and peaked after the CM vaccination reaching a median (range) of 58% (7%−100%) of the total anti-HIV-1 T-cell responses (BCN01 parental study) (20). In BCN02, 2 years from the last HIVconsv vaccination, the increase in the frequency of HIVconsv-specific T-cell responses after MVA_1 or MVA_2 further shifted the patterns of T-cell immuno-dominance toward HIVconsv with median (range) of 85% (54%–100%) of the total anti-HIV-1 T-cell responses at peak immunogenicity time point being HIVconsv specific (**Figure 3D**).

Effects on the HIV-1 Reservoir

All participants had detectable viral reservoirs, as measured by total $CD4^+$ T cell-associated HIV-1 DNA, throughout the study. Results from 2 samples out of a total of 60 were considered invalid and were censored. At BCN02 study entry, median (range) reservoir size was of 140 (17–752) HIV-1 DNA copies/10^6 $CD4^+$ T-cells (**Figure 4**). Proviral DNA showed a tendency to further decrease from baseline to week 17 (Wilcoxon signed-rank, $p = 0.0599$, **Figure 4**) to median (range) levels of 120 (11-680) copies/10^6 $CD4^+$ Tcells.

Monitored Antiretroviral Pause (MAP)

Participants undergoing MAP were monitored weekly for the first 12 weeks and every 2 weeks thereafter for a maximum of 32 weeks (MAP_{0-32}). Criteria for ART resumption included pVL over 2,000 copies/ml in two consecutive determinations, $CD4^+$ cell counts decrease over 50% and/or below 500 cells/mm^3 and/or development of clinical symptoms suggestive of an acute retroviral syndrome.

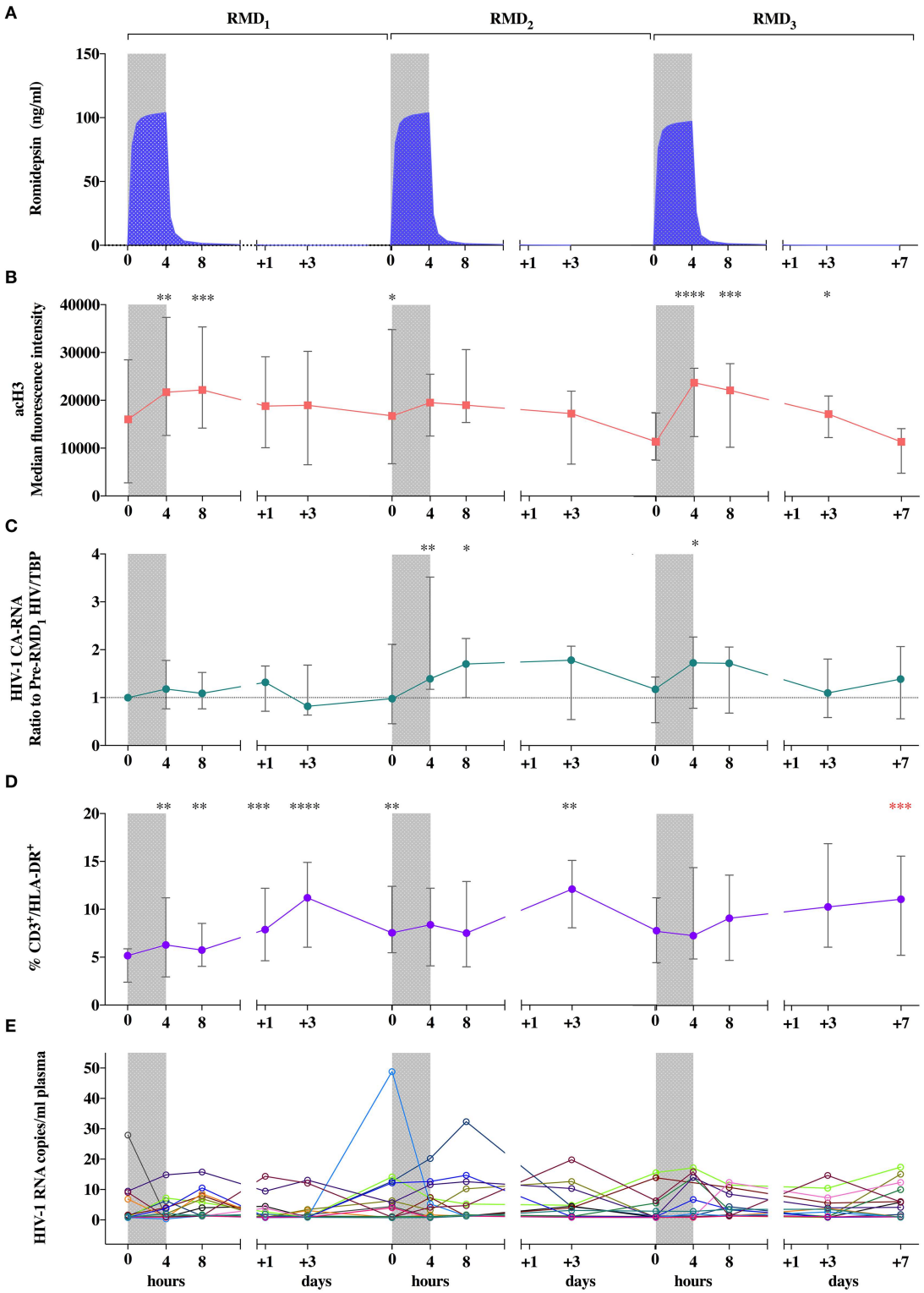

FIGURE 2 | Pharmacokinetic and pharmacodynamic effects of RMD. **(A)** Mean individual predictions of RMD plasma concentrations. **(B)** Levels of histone H3 acetylation in peripheral lymphocytes. **(C)** Viral transcription levels expressed as changes from pre-RMD_1 levels of cell-associated HIV-1 RNA in peripheral $CD4^+$ T-cells. **(D)** Levels of T-cell activation ($CD3^+$/$HLA\text{-}DR^+$ cells). **(E)** individual determinations of pVL. Median of frequencies and IQR (error bars) are represented. Wilcoxon signed-rank *p*-values compare each represented time point with the corresponding values preceding each RMD administration. $^*p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$ and $^{****}p < 0.0001$. The *p* value resulting from the comparison between the value at day 0 of RMD_1 and 7 days after RMD_3 is shown in red.

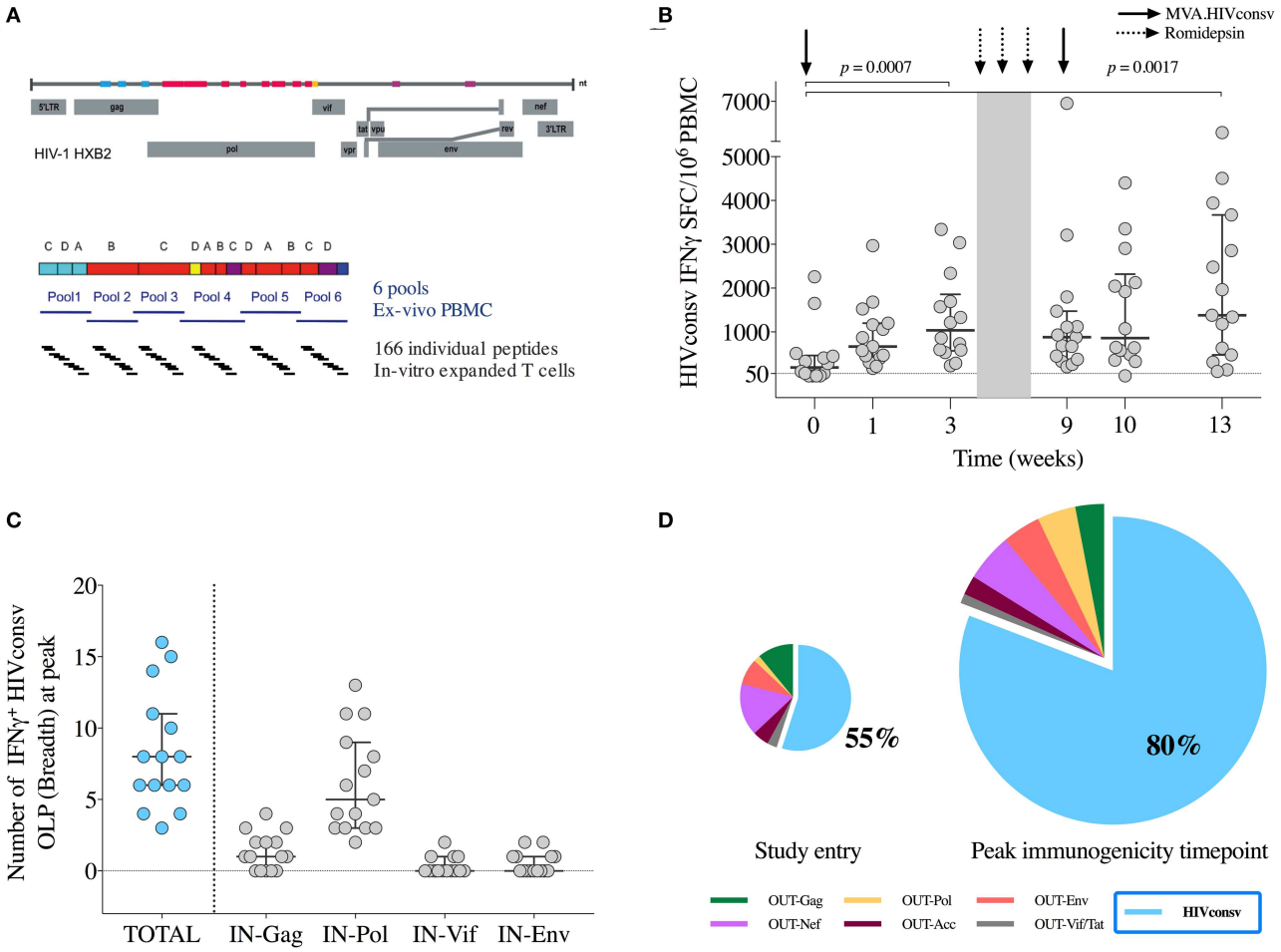

FIGURE 3 | Vaccine immunogenicity. **(A)** Schematic representation of the selected regions in the HIV-1 proteome from different clades included in the HIVconsv immunogen and distribution of 6 peptide pools (P1-P6) and individual overlapping 15-mer peptides (OLP) used in the IFN-γ ELISPOT assays. **(B)** Magnitude (sum of SFU/10^6 PBMC to pools P1-P6) of vaccine-induced responses over the BCN02 study. Horizontal and error bars represent median and IQR, respectively, and *p*-values correspond to comparisons between the indicated time points using the Wilcoxon signed-rank test. **(C)** Breadth of vaccine-elicited responses toward individual OLP included in the indicated HIVconsv regions. Horizontal and error bars represent median and IQR, respectively. **(D)** Average distribution of total HIV-1 T-cells according to their specificity at the indicated time points. HIVconsv-specific responses are shown in blue. Pie charts are scaled according to the total frequencies of responses. IN are peptide pools corresponding to the HIVconsv vaccine insert and OUT peptide pools spanning the rest of HIV-1 proteome "outside the immunogen".

All participants rebounded (detectable pVL over 20 copies/ml) during MAP (**Figure 5A**). Median (range) time to first detectable pVL was 13 (7–35) days with median (range) of first detectable pVL of 122 (28–3,410) copies/ml. Ten participants resumed ART before MAP_{12} (MAP-NC for MAP-*Non-controllers*) with median (range) time to resume ART of 28 (16–59) days. All MAP-NC resumed ART due to the viral load criteria, with median (range) pVL of 19,250 (2,900–179,000) copies/ml at the moment of ART resumption (**Figure 5B**). None of the participants resumed ART due to immune or clinical criteria. A "late-rebounder" presented the first detectable pVL 5 weeks after ART interruption (MAP_5, pVL of 59 copies/ml) and was able to maintain viral load below 2,000 for 3 more weeks, resuming ART at MAP_8. In addition to the "late-rebounder," 3 (23%) other participants remained off ART with sustained pVL <2,000 copies/ml for a total of 32 weeks (MAP-C, from MAP-*Controllers*). Highest peak pVL determination in the MAP-C was of 3,110 copies/ml at week 12 of MAP in the absence of symptoms followed by 2,460 and 1,100 three and six days later (participant decision to stay off). At week 32, two out of the three MAP-C accepted to stay off cART out of the protocol and were followed under standard of care. One MAP-C showed a late rebound after 48 weeks off cART and the other one, voluntary resumed cART after 1.5 years off cART despite sustained low-level viremia. All participants who restarted ART reached viral re-suppression within 6 months. No evidence of emergence of drug resistance was detected.

To assess re-seeding of the viral reservoir during the MAP, total HIV-1 DNA was measured at MAP_0 ($n = 13$), on the day of ART resumption ($n = 10$), and 6 months after for the 10 MAP-NC ($n = 8$ available), and at MAP_{32} for the three MAP-C. We did

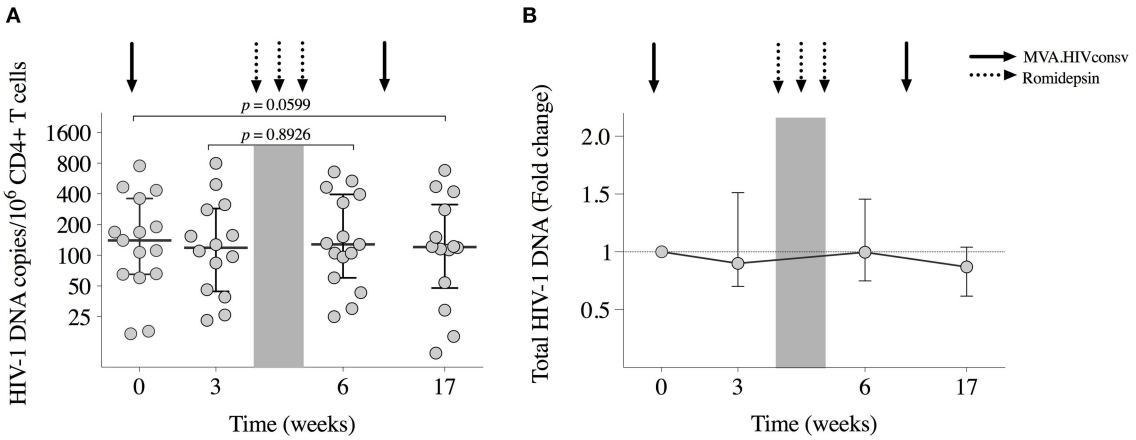

FIGURE 4 | Viral reservoir. **(A)** Total HIV-1 DNA copies/10^6 $CD4^+$ T cells for each participant are shown at study entry (week 0), week 3 (day of RMD_1), and week 6 (1 week after RMD_3) and at week 17 (8 weeks after MVA_2). Horizontal and error bars represent median and IQR, respectively, and *p*-values correspond to comparisons between the indicated time points using the Wilcoxon signed-rank test. **(B)** Changes in proviral DNA throughout the study with respect to baseline (week 0), which is considered to be 1.

not observe any significant change in total HIV-1 DNA during MAP in participants with early ART resumption (Wilcoxon signed-rank, $p = 0.5759$ for MAP-NC) and, noteworthy, nor in the three participants with sustained low level viremia for 32 weeks. Moreover, one of the three MAP-C showed a 2-fold reduction in the HIV-1 DNA (from 34 at MAP_0 to 16 copies/10^6 $CD4^+$ cells at MAP_{32}, **Figure 5C**). This was the only participant undergoing MAP carrying HLA alleles associated with natural HIV control (HLA-B*27:01/HLA-B*51:01) (45, 46).

Factors Influencing Viral Rebound Kinetics

Supplementary Table 1 shows the summary of variables explored to explain the binary outcome defined as MAP-NC vs. MAP-C. The estimated relative risks obtained from the log-binomial models for different covariates analyzed are shown in **Figure 5D**.

Univariate log-binomial regression models used to detect factors associated with virologic control during MAP revealed that pVL before ART initiation (pre-ART pVL) was the only factor statistically significantly associated with control of viral rebound after ART interruption. For each log increase on the pre-ART pVL, the probability of becoming a MAP-C decreased by 66% (RR 0.34; 95% CI 0.14, 0.79). Interestingly, albeit not statistically significant in the univariate models, the 3 MAP-C had among the lowest reservoir levels at treatment interruption time point (16, 54 and 122 copies/10^6 $CD4^+$ T cells) –consistent with lower pre-ART pVL– and showed the highest shift in CTL immunodominance pattern after vaccination (>700 HIVconsv-specific SFC/10^6 PBMCs and >75% of HIVconsv dominance at peak immunogenicity time point, **Supplementary Figure 6**).

DISCUSSION

In this single-arm, open-label, phase I, proof-of-concept trial performed in HIV-1-infected ART-suppressed individuals treated during acute/recent HIV-1 infection, we show that combination of the HIVconsv vaccines with RMD as a latency reversing agent was safe, highly immunogenic, and induced bursts of viral transcription. The combined intervention resulted in a tendency toward a reduction in proviral DNA levels and was followed by a prolonged viremic control in 23% of participants after ART interruption without evidence of reservoir re-seeding.

Early-treated individuals typically show less immune exhaustion (47) and reduced frequency of immune-escaped viral variants compared to individuals initiating ART at later stages of HIV-1 infection (48), offering a potentially favorable setting to induce a protective immune response upon therapeutic vaccinations. In the parental BCN01 trial, a prime/boost vaccine regimen with ChAdV63.HIVconsv and MVA.HIVconsv induced high frequencies of T cells with high *in vitro* suppressive capacity that markedly shifted the focus of the $CD8^+$ CTL response toward HIVconsv sequences that are subdominant during natural infection (20). In the BCN02 trial, after 3 years of viral suppression and 2 years since the last vaccination in BCN01, booster MVA.HIVconsv vaccinations were still immunogenic and further increased breadth, magnitude and immunodominance of CTL responses toward HIVconsv sequences.

Along with a strong vaccine-elicited CTL activity ("kill"), the ability to simultaneously induce reactivation of the viral reservoir ("kick") is a critical feature for the success of the kick&kill strategy (24). A 3-dose regimen of weekly RMD at 5 mg/m^2 BSA was selected based on results from previous trials (33, 34). Consistently, we observed a direct *in vivo* effect of RMD in histone 3 acetylation upon each RMD dose, which was followed by changes in cell-associated HIV-1 RNA levels. Conversely, a placebo-controlled dose-escalating trial (ACTG 5315) testing 3 RMD doses (5 mg/m^2 BSA) administered every two weeks in chronically suppressed individuals, did not show changes in viral transcription (49). The weekly administration regimen and the

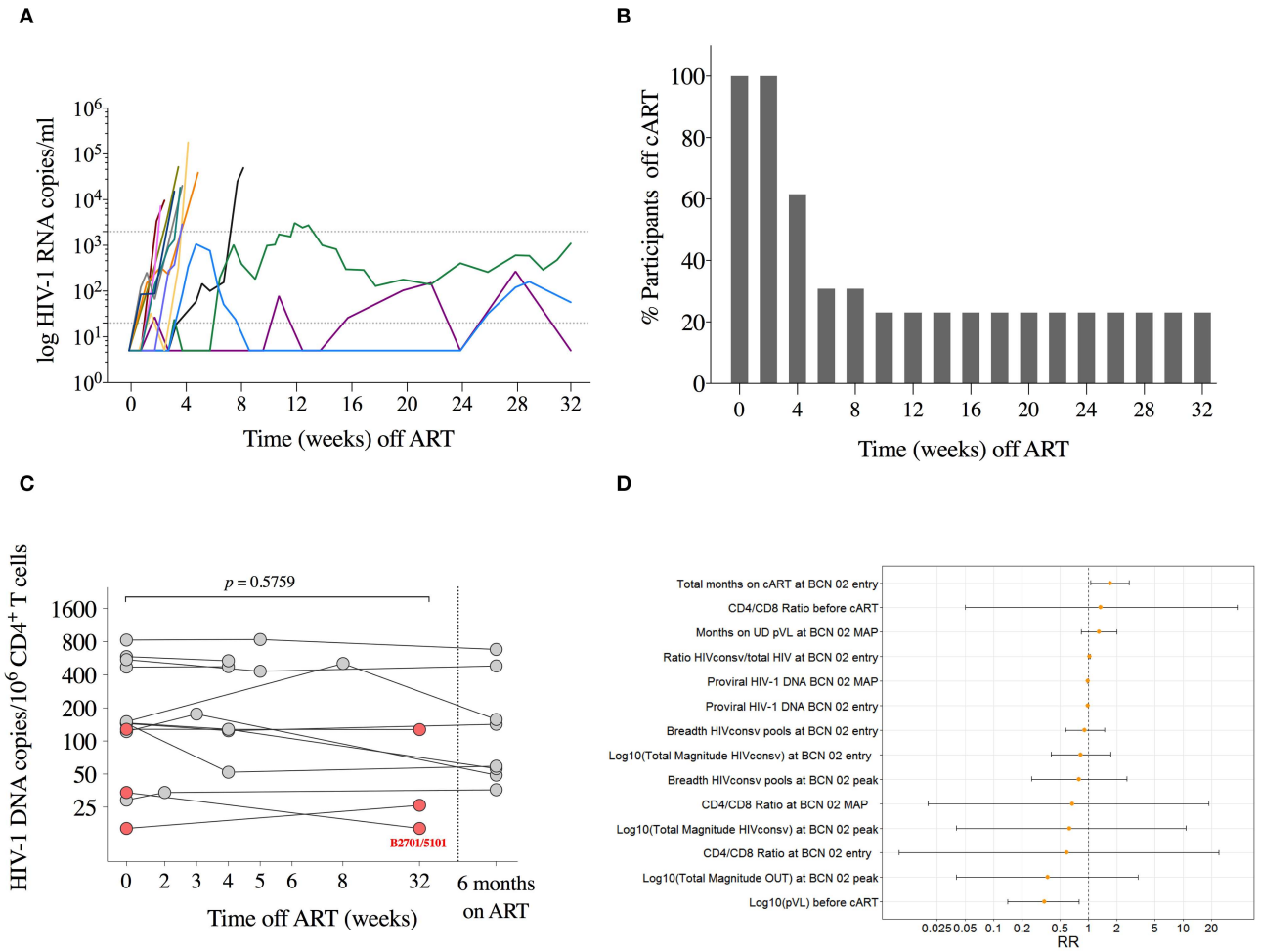

FIGURE 5 | Monitored antiretroviral pause. **(A)** Individual pVL during MAP is shown for each participant in different colors ($n = 13$). Lines are interrupted on day of ART resumption. Dotted lines represent detection limit and threshold for ART resumption (20 and 2,000 copies/ml, respectively). **(B)** Proportion of individuals remaining off ART during MAP. **(C)** Total HIV-1 DNA copies/10^6 $CD4^+$ T cells are shown for each participant at MAP initiation, at day of ART ressumption, and 24 weeks after ART. *P*-value corresponds to comparison between MAP initiation and the day of ART resumption for each individual using the Wilcoxon signed-rank test. Individuals with sustained low-level viremia over 32 weeks are shown in red. **(D)** Estimated relative risks and 95% confidence intervals for the durable control of pVL during MAP obtained from univariate log-binomial regression models.

intensive sampling after each RMD dose in our study allowed us to detect increases in CA HIV-1 RNA above 2-fold in 80% of individuals at any time point after RMD administrations. These changes were followed by consistent increases in T cell activation markers, which remarkably, were maintained one week after RMD_3, likely reflecting a direct and cumulative effect of weekly RMD dosing and an effective induction of viral transcription. Noteworthy, changes above 2.1-fold in CA-RNA have been estimated to occur in <5% of repeated measurements in an individual (50).

Despite the induction of viral transcription, the kinetics of plasma HIV-1 RNA followed an unclear pattern, similar to previous studies showing variable changes in plasma viremia following LRA administration (34, 51, 52). This variability might reflect suboptimal potency of the agents tested so far and/or the reactivation of predominantly defective proviruses. In the context of the current study, elimination of reactivated cells by vaccine-elicited T cells may have additionally blunted quantification of plasma viremia.

A critical objective of the use of LRA in a kick&kill strategy is to mobilize and ultimately reduce the viral reservoir. Our findings showed that, despite robust immunogenicity of HIVconsv vaccines and at least partial reactivation of the viral reservoir induced by RMD, the net effect on the proviral DNA levels was modest. All participants had detectable levels of HIV-1 DNA at the time of treatment interruption although a tendency toward a decrease by 19.3% from baseline to week 17 (Wilcoxon signed-rank, $p = 0.0599$) was detected. Conversely, a mean 39.7% decrease in reservoir size was observed in the REDUC trial (34). This discrepancy between REDUC and BCN02 results may be explained by the inclusion of early-treated individuals in our study, in which already baseline

levels of proviral DNA were substantially lower, challenging the quantification of the effects of the intervention on HIV-1 DNA levels. We acknowledge that the translation of HIV-1 protein expression into antigen presentation—even in case of defective proviruses (1)—upon LRA reactivation is poorly understood. Likewise, the ability of LRA-induced HIV-1 protein expression to effectively induce recognition and killing by $CD8^+$ T cells remains to be fully elucidated (53) and therefore, the potential effects of further RMD administrations on the viral reservoir are to be determined. A potential significant toxicity—suggested in *in vitro* assays (54)—on vaccine-induced T cells might also have limited our capability to observe a further reduction in the reservoir size in our study. This hypothesis is consistent with the fact that HIVconsv vaccines induced higher levels of activated T cells compared to Vacc-4x vaccination in the REDUC trial. Nevertheless, the potential toxicity of RMD *in vivo*, its relationship with RMD exposure and, ultimately, whether vaccine-induced T cells were able to sensor and remove infected cells in response to HIV-1 reactivation remains to be determined.

To attain a functional cure, a persistent immune-mediated control of residual HIV-1 might be as relevant as achieving an absolute reduction on the proviral DNA levels. In this regard, the three BCN02 MAP-C, were among the subjects with both lower viral reservoir levels at MAP and higher vaccine-induced responses. In our study, having lower pre-ART pVL was the only outstanding marker associated with viral control during MAP, which correlates with the size of the viral reservoir after ART suppression. These relationships are consistent with previous studies suggesting a role of a low viral reservoir on analytical treatment interruptions (ATI) outcomes (55, 56). Furthermore, after ART discontinuation, MAP-C did not show an initial burst in pVL followed by a fast post-peak control as described in several post treatment controllers (PTC) (8, 57). Collectively, the findings from this and other studies suggest that a small reservoir size, resulting from early ART or another intervention, may be essential to achieve sustained post-intervention control (55) but also, that a potent vaccine-induced immune pressure might contribute to prevent a peak burst of viremia and maintain suppressed viremia for a substantial period of time. This control, mediated by immune pressure, is supported by the absence of re-seeding of the viral reservoir in the BCN02 MAP-C, in contrast to reports from previous ATI trials (58, 59).

The interpretation of the outcome of kick&kill studies may be confounded by individuals controlling HIV rebound after treatment interruption without the need for a prior therapeutic intervention. The prevalence and mechanisms driving such PTC in natural HIV infection are not well-understood. A recent metanalysis (CHAMP study) including 14 interruption trials estimated a 13% rate of PTC among early-treated individuals (57). Importantly, and in contrast to the three BCN02 MAP-C who did not show a transient high burst of viremia, 32% of the 61 PTCs analyzed in the CHAMP metanalysis had peaks of viremia ranging from 1,000 to over 10,000 copies/ml within the first 24 weeks after treatment interruption. Thus, in addition to the different behavior of the 3 MAP-C (23%) with respect to the PTC, according to this metanalysis, the BCN02 trial may have missed additional MAP-C due to the conservative ART resumption criteria used (two consecutive pVL over 2,000 copies/ml).

The safety and tolerability profiles of MVA.HIVconsv and RMD were similar to those reported in previous studies (19, 20, 34). However, there was a SAE in one participant. This case highlights the need for planning intensive monitoring in this kind of pilot trials, even if not powered to detect low-frequency AE, and points toward the need for a trade-off between the number of participants potentially put at risk in well-powered controlled trials and for caution with the use of uncompletely characterized agents in large numbers of individuals. Given that natural PTC rates are considered to be up to 13% in early-treated individuals, powering trials to show viral control efficacy after an ATI becomes challenging (60). Despite frequent clinical monitoring for pVL and access to psychological support, protocol violation during MAP occurred in one individual, probably due to anxiety secondary to the antiretroviral interruption. This case warrants close psychological management in longer term ATIs.

We fully acknowledge the limitations of the small sample size and lack of a control arm in the present study. Therefore, we interpret these results with caution and regard this study only as hypothesis-generating trial for future interventions. BCN02 eligibility was restricted to vaccinated participants in the parental open-label BCN01 trial. This intrinsic restriction limited the sample size to a small number of previously vaccinated individuals and precluded the inclusion of a control arm. At the time of trial design, interventional trials including an ATI were typically small and included very conservative ART resumption criteria (58, 61). Furthermore, ATI acceptability by participants, risks of HIV-1 transmission to others in the absence of available PrEP, and potential viral re-seeding upon treatment interruption were of special concern in early-treated individuals, who had limited viral reservoirs both in size and diversity (48).

Altogether, the results from this pilot study suggest a potential role for kick&kill strategies in inducing durable immune-mediated HIV-1 control in a proportion of early-treated individuals. In view of these results, future controlled studies to identify the mechanisms underlying sustained HIV-1 suppression are warranted.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Germans Trias i Pujol and Hospital Clínic (AC-15-108-R). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BM, JM, BC, and CB conceived and designed the study. CMa, TH, JMM, JM-P, AB, PC, MM, and MV contributed to the study design. CMi, CMa, IR, JT, ACa, MR-U, and MP contributed contributed to data management. BM, MR-U, MP, ML, SM-L, AL,

JM, PC, ACa, CMi, SC, AB, and CMa performed the experiments. JM, BM, MP, MV, YA-S, GM, and KL undertook the statistical analysis. ACr and TH contributed with reagents, materials, analysis tools. BM, JM, and CB drafted the manuscript. TH, MR-U, CMa, MP, SM-L, JM-P, JMM, MV, and BC participated in study analyses and revised the manuscript critically for important intellectual content. All authors reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00823/full#supplementary-material

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REVIEW
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Boosting the Immune System for HIV Cure: A γδ T Cell Perspective

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The major barrier to HIV cure is a population of long-lived cells that harbor latent but replication-competent virus, are not eliminated by antiretroviral therapy (ART), and remain indistinguishable from uninfected cells. However, ART does not cure HIV infection, side effects to treatment still occur, and the steady global rate of new infections makes finding a sustained ART-free HIV remission or cure for HIV-seropositive individuals urgently needed. Approaches aimed to cure HIV are mostly based on the "shock and kill" method that entails the use of a drug compound to reactivate latent virus paired together with strategies to boost or supplement the existing immune system to clear reactivated latently infected cells. Traditionally, these strategies have utilized CD8+ cytotoxic lymphocytes (CTL) but have been met with a number of challenges. Enhancing innate immune cell populations, such as γδ T cells, may provide an alternative route to HIV cure. γδ T cells possess anti-viral and cytotoxic capabilities that have been shown to directly inhibit HIV infection and specifically eliminate reactivated, latently infected cells *in vitro*. Most notably, their access to immune privileged anatomical sites and MHC-independent antigen recognition may circumvent many of the challenges facing CTL-based strategies. In this review, we discuss the role of γδ T cells in normal immunity and HIV infection as well as their current use in strategies to treat cancer. We present this information as means to speculate about the utilization of γδ T cells for HIV cure strategies and highlight some of the fundamental gaps in knowledge that require investigation.

Keywords: γδ T cells, HIV latency, immunotherapy, innate immnuity, allogeneic T cell

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INTRODUCTION

Therapeutic Strategies Targeting Chronic HIV Infection

The road to developing a complete cure for HIV is fraught with potholes and dead ends. Clearing cellular and anatomical reservoirs provides a unique set of challenges. Latent virus is able to evade immune responses by integrating into the host genome of resting CD4+ T cells and entering a state of dormancy. Despite the cessation of new virion production, viral persistence is maintained by clonal expansion of HIV-infected cells (Chomont et al., 2009; Lee et al., 2020). Additional barriers include immune privileged or hard to reach anatomical sites such as the central nervous system, intestines, or secondary lymphoid organs where the virus can persist in the presence of Antiretroviral Therapy (ART) (Barton et al., 2016; Bronnimann et al., 2018; Denton et al., 2019; McManus et al., 2019). To date, HIV cure has only been achieved in two HIV-seropositive individuals who also had either acute myeloid leukemia or Hodgkin's lymphoma. Both individuals received allogeneic transplantation of stem cells carrying a homozygous mutation within the CCR5

gene (CCR5Δ32/Δ32), a chemokine receptor that facilitates viral entry (Gero Hütter et al., 2009; Gupta et al., 2019). However, it is unlikely that these practices may be feasible for wide-spread implementation. A safer, potentially more practical approach, called "shock and kill" has been the primary focus of cure research for the past 15 years (Sengupta and Siliciano, 2018). This strategy is based on the use of drug compounds, or latency reversing agents (LRAs), to reactivate viral replication followed by a treatment to enhance immune responses capable of eliminating reactivated latently HIV-infected cells (Deeks, 2012; Barton et al., 2013; Archin et al., 2014). A recent review by Kim et al. visits current LRAs under different stages of investigation (Kim et al., 2018). LRAs are classified into groups based on their primary intracellular targets. Epigenetic modifiers include histone deacetylase inhibitors (HDACi), histone methyltransferase inhibitors (HMTi), DNA methyltransferase inhibitors (DNMTi), bromodomain inhibitors (BRDi), and protein kinase C (PKC) agonists (Margolis et al., 2016). Non-epigenetic LRAs include agonists for the endosomal pattern recognition receptors TLR7, TLR8, and TLR9, which have been shown to increase both viral transcription as well as anti-HIV innate immune responses (Offersen et al., 2016; Lim et al., 2018; Meas et al., 2020). Unfortunately, within these categories, only a handful of drugs have progressed to animal studies or human clinical trials. These include the HDACis vorinostat, panobinostat, and romidepsin, the PI3K/Akt inhibitor disulfiram, PKC agonists bryostatin and ingenol, and the TLR9 agonist MGN1703. Excluding bryostatin, each of these compounds prompted an increase in detectable viral mRNA although this was not accompanied by clearance of infected cells (Kim et al., 2018). Therefore, the viability of the "shock and kill" strategy is contingent upon the discovery and development of novel LRAs with different mechanisms of action and possibly target alternative pathways.

The majority of current LRAs reactivate viral transcription through induction of the canonical NF-κB pathway. NF-κB is a host transcription factor that interacts with the HIV LTR and has been shown to be a powerful driver of the viral replication cycle (Nabel and Baltimore, 1987; Hiscott et al., 2001). This pathway is not limited to infected cells and therefore off-target toxicity via systemic immune activation remains a concern (Bratland et al., 2011). An immunosuppressive effect of LRAs on different effector immune cell subsets has also been reported, posing an additional complication when attempting to reconstitute total immune response (Garrido et al., 2016; Walker-Sperling et al., 2016). Despite these concerns, combining LRAs has been shown to produce a synergistic effect and potentially abrogate uncontrolled T cell activation. Darcis et al. demonstrated an increase in efficacy across several *in vitro* and *ex vivo* latency models when either of the PKC agonists bryostatin or ingenol are paired with the bromodomain inhibitor JQ1. Building off of this work, Albert *et al.* found both an increase in efficacy and reduction in systemic activation when bryostatin is paired with HDACis (Darcis et al., 2015; Albert et al., 2017). In addition, modulation of the non-canonical NF-κB pathway through second mitochondrial-derived activator of caspases (SMAC) mimetics could potentially be of interest for shock and kill strategies (Pache et al., 2015). Induction of this pathway leads to more persistent NF-κB-driven transcription and thereby potentially avoids the detrimental side effects observed with previous LRAs. Most recently, the SMAC mimetic AZD5582 has been shown to induce robust HIV reactivation throughout the deep anatomical reservoirs of humanized mice and non-human primates. These studies, although extremely promising, need further evaluation and testing in humans (Sampey et al., 2018; Nixon et al., 2020). These developments in latency reversal should be paired with equally innovative immunotherapies to effectively target and clear chronic HIV infection.

Cellular Based Immunotherapies for "Shock and Kill"

Harnessing the natural antiviral CTL immune response has been the most investigated strategy for the "shock and kill" approach (Borrow et al., 1994; Santra et al., 2010). This is highlighted by the development of HIV-specific *ex vivo* expanded T cells (HXTCs) capable of recognizing a variety of viral epitopes. HXTCs were shown to be safe for adoptive transfer into humans but had little effect on viral clearance in the absence of reactivation (Sung et al., 2018). Unfortunately, some LRAs including HDACis and PKC agonists may have deleterious effects on CTL function that requires further investigation (Clutton and Jones, 2018). The extent of these effects occurring *in vivo* and amongst other classes of LRAs is the subject of current clinical studies. Furthermore, CTL-based strategies continue to struggle with issues stemming from viral escape, immune exhaustion, and inaccessibility to anatomical reservoirs, including the B cell follicle (Day et al., 2006; Connick et al., 2007; Deng et al., 2015). Alternative strategies that utilize NK cells are starting to be explored, and their potential as immunotherapy in HIV infection has recently been reviewed (Desimio et al., 2019).

Additionally, the use of γδ T cells could offer a novel therapeutic avenue that may overcome some of the challenges facing traditional αβ T cell strategies. γδ T cells possess a range of antiviral function including cytolytic activity against HIV-infected cells (Wallace et al., 1996). Specifically, our group showed that Vδ2 T cells from ART-suppressed HIV-infected individuals target and kill reactivated autologous HIV-infected CD4+ T cells *in vitro*, establishing the first proof of concept of the capacity of Vδ2 T cells to be used in immunotherapeutic approaches toward an HIV cure (Garrido et al., 2018). Further proof comes from our recent work showing a correlation between γδ T cell cytotoxic capacity with a lower recovery of replication-competent HIV in cultures of resting CD4+ T cells from ART-suppressed HIV-seropositive individuals (James et al., 2020). In addition, activated γδ T cells induce adjuvant immune responses including HIV-specific T cell responses (Poccia et al., 2009). The clinical utilization of γδ T cells for HIV cure remains relatively understudied compared to the cancer field, but these initial findings paired with an examination of their basic biology warrants further investigation into their potential as an immunotherapy.

γδ T CELLS: A CRITICAL BRIDGE BETWEEN INNATE AND ADAPTIVE IMMUNITY

Characteristics of γδ T Cells

The γδ T cell lineage is a unique subset of innate-like T lymphocytes that offer an attractive alternative to conventional αβ T cells, which predominate in current cell-based immunotherapies. Since their discovery in the 1980's, γδ T cells have been shown to contribute to tumor surveillance, fighting infectious disease, and autoimmunity (Tanaka, 2006; Kabelitz, 2011; Vantourout and Hayday, 2013; Silva-Santos et al., 2015; Lawand et al., 2017). Their defining feature is a T-cell receptor (TCR) comprised of variable γ and δ chains that recognizes non-peptidic antigens in the absence of Major Histocompatibility Complex (MHC) molecules (Brenner et al., 1986; Holoshitz et al., 1993; Tanaka et al., 1995). γδ T cells develop and mature in the thymus and constitute the first T cell population to migrate and populate the periphery during fetal development. Interestingly, there is a correlation between tissue localization and the Vδ chain expression in the TCR indicating a predefined role for γδ T cells before leaving the thymus (Zhao et al., 2018). Although the specific mechanisms of γδ T cell development and differentiation are still being elucidated, the correlation between Vδ chain expression and tissue localization is well-established (Munoz-Ruiz et al., 2017).

Human γδ T cells account for 0.5–10% of circulating T cells and are classified into two major subpopulations based on the δ chain usage, Vδ1 and Vδ2 T cells. Smaller subpopulations expressing Vδ3 and Vδ5 share a degree of similar functions as Vδ1 T cells but the extent of their involvement in immunity is unknown (Takihara et al., 1989; Halary et al., 2005). The Vδ2 subpopulation is almost always paired with the Vγ9 chain (Vγ9Vδ2) and while the Vδ1 subpopulation is also capable of pairing with Vγ9, it is less frequently observed (Dimova et al., 2015). Vδ2 represents the majority of γδ T cells found in the peripheral blood whereas the Vδ1 subpopulation primarily resides in tissues such as the gut mucosa, lungs, and female reproductive system (Itohara et al., 1990; Wu et al., 2017).

Vδ1 T cells are capable of recognizing self-antigen lipids presented within the MHC-like CD1 protein family, but the specific ligand they recognize remains unknown. In contrast, Vδ2 T cell ligand has been extensively characterized. Vδ2 T cells undergo activation following recognition of low-molecular-weight phosphorylated compounds referred to as phosphoantigens (P-Ags) (Jomaa et al., 1999; Green et al., 2004; Adams et al., 2015). These P-Ags are metabolic intermediates of the isoprenoid biosynthesis pathway. Isoprenoids are a diverse class of organic compounds involved in a number of biological processes ranging from cell membrane maintenance to protein regulation. As such, isoprenoids are produced both endogenously as well as by invading microbes. The most potent, naturally occurring P-Ag commonly found in pathogenic bacteria is (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate of the MEP (non-mevalonate) pathway (Hintz et al., 2001). A second, less potent activator found in eukaryotic cells is Isopentenyl pyrophosphate (IPP), an intermediate of the mevalonate pathway that accumulates during periods of cellular stress (Gossman and Oldfield, 2002; Gober et al., 2003). Although γδ T cells were discovered more than 30 years ago, our understanding of how Vδ2 cells recognize P-Ag has been a slow process. Early on, it was suggested that presentation occurred outside of conventional MHC or known MHC-like molecules but still required cell to cell contact (Morita et al., 1995). It was only recently discovered that the ubiquitously expressed B7 family protein, CD277 (butyrophilin-3/BTN3A), acts as a mediator of P-Ag presentation to Vδ2 cells (Harly et al., 2012; Rhodes et al., 2015; Sebestyen et al., 2016; Yang et al., 2019). Furthermore, the specific antigens recognized by other γδ T cell populations remain elusive. In addition, it is unclear if BTN3A is the sole protein directly involved in presenting PAgs or if it is part of a larger mechanism involving a number of intracellular proteins or membrane bound transporters.

γδ T cells are further characterized by a diverse set of membrane bound receptors and cytokine production capability that denotes a high degree of polyfunctionality as regulatory and effector cells (Vantourout and Hayday, 2013). γδ T cells shape the total immune response by secreting a number of regulatory cytokines and interacting directly with other immune cell populations (Wu et al., 2014). Distinct subpopulations are capable of producing anti-inflammatory or pro-inflammatory signals and play a direct role in immune regulation in normal health and disease (Kuhl et al., 2009; Wakita et al., 2010; Ma et al., 2011; Coffelt et al., 2015; Peters et al., 2018). The expansive role of γδ T cells as immune modulators is underscored by their interactions with other immune cell populations. Some of these interactions are reciprocal in nature; DCs improve the antigen specific response of γδ T cells which in turn facilitate DC maturation (Conti et al., 2005; Martino et al., 2005). Activated peripheral γδ T cells can either induce anti-tumor activity of NK cells through CD137L (4-1BBL) expression or mediate NK pro-inflammatory cytokine production and DC editing through CD278 (ICOS) co-stimulation (Maniar et al., 2010; Nussbaumer et al., 2011; Cairo et al., 2014). Interestingly, activated peripheral γδ T cells also possess professional antigen-presenting cell (APC) capabilities that enable them activate naïve CD8+ αβ T cells and thus stimulate the development and differentiation of the CTL response against pathogens and tumors (Brandes et al., 2009; Khan et al., 2014).

γδ T Cells Are Potent Cytotoxic Effectors

Most γδ T cells express the NK cell receptors, DNAM-1 and NKG2D, the latter of which acts as a co-stimulatory signal upon recognition of stress markers MICA, MICB, and ULBPs. Activation through this pathway has a 2-fold effect resulting in the secretion of pro-inflammatory cytokines TNF-α and IFN-γ as well as direct cytolytic activity mediated by the release of perforins and granzymes (Wu et al., 2002; Rincon-Orozco et al., 2005; Gonzalez et al., 2008; Toutirais et al., 2009). In addition, γδ T cells can induce apoptosis through the expression of TNF-related apoptosis-inducing ligand (TRAIL) or FasL, highlighting their role in clearing tumor cells as well as activated immune cells during the resolution of inflammation (Dalton et al., 2004; Ponomarev and Dittel, 2005; Todaro et al., 2009). Effector γδ

T cell subsets can express CD16 (FcγRIII), affording them the ability to take part in ADCC of virally-infected cells as well as opsonization-aided phagocytosis of cell free pathogens (Chen and Freedman, 2008; Wu et al., 2009). As part of the first line of defense, they readily express toll-like receptors (TLR) that allow recognition and response to various microbial pathogens that invade barrier tissues (Wesch et al., 2011). Although γδ T cells exhibit many functions ascribed to innate immunity, they have a memory phenotype that can be defined by CD45RA/CD27/CD28 and CCR7 expression. This is consistent with other lymphocytes within the adaptive compartment, but collectively γδ T cells exert non-redundant functions (Dieli et al., 2003; Pitard et al., 2008; Ryan et al., 2016; Guerra-Maupome et al., 2019). Defining the complete mechanisms by which γδ T cells recognize foreign and self-ligands will require thorough investigation. Elucidating these mechanisms will further improve our understanding of γδ T cell biology as well as enable us to design efficacious therapeutic strategies. This is especially evident in the context of HIV as we currently lack a comprehensive understanding of the role of γδ T cells during HIV infection and how they recognize virally infected cells.

The Impact of HIV Infection and Antiretroviral Therapy on γδ T Cells

When assessing γδ T cells' prospect as a novel HIV cure therapeutic, it is crucial to understand the effects of both HIV pathogenesis and ART on γδ T cell populations. Pauza et al. have recently reviewed the initial studies of γδ T cell dysregulation in the context of HIV disease progression and treatment (Pauza et al., 2014). Here, we summarize those early findings in addition to key details uncovered by more recent work. The most striking effect of primary HIV infection on γδ T cells is the inversion of the frequency of Vδ2:Vδ1 subpopulations within the peripheral blood. This event occurs early on, prior to the inversion of CD4:CD8 αβ T cells, and is driven by an expansion of Vδ1 T cells and depletion of Vδ2 T cells (Autran et al., 1989; Li et al., 2014). This difference in subpopulation outcomes can be partially explained by a few subtle yet important distinctions in the receptors they express. Like most gut-associated lymphocytes, both Vδ1 and Vδ2 T cells express the integrin α4β7, but only Vδ2 T cells express the chemokine receptor CCR5. Binding of the HIV envelope glycoprotein gp120 to α4β7 and CCR5 induces cell death (Li and Pauza, 2011, 2012). In addition, although γδ T cells are typically double negative (CD4-CD8-), Vδ2 T cell activation leads to a transient upregulation of the CD4 receptor (Lusso et al., 1995; Soriano-Sarabia et al., 2015). Furthermore, *ex vivo* phenotypic analysis of CD4 and CCR5 expression on viremic individuals in the acute phase of the infection revealed a transient increase in the expression of these receptors rendering Vδ2 T cells susceptible to entry by CCR5-tropic viruses (Soriano-Sarabia et al., 2015). Typically, only a small subset of peripheral Vδ2 T cells expresses the chemokine receptor CXCR4, but an increase in expression found in individuals with chronic infection raises the possibility that Vδ2 T cell may become susceptible to CXCR4-tropic viruses after initial infection (Imlach et al., 2003). Recovery of replication-competent virus from Vδ2 T cells confirmed the possibility of direct infection, but due to their low representation within total T lymphocytes it is difficult to quantify their contribution to the viral reservoir (James et al., 2020). The surviving Vδ2 T cell population shows attenuated responsiveness to P-Ag and effector functions. Consequently, Vδ2 T cells from HIV-seropositive individuals show diminished response to *in vitro* stimulation with IPP, reduced tumor recognition, as well as a significant loss of IFN-γ and TNF-α production (Wallace et al., 1997). It remains unclear if integrated provirus plays a role in these observed defects. While γδ T cell dysfunction begins early in primary HIV infection, comparative differences in the distribution of effector phenotype and function observed during acute vs. chronic infection indicate a dynamic interplay between γδ T cells and disease progression (Kosub et al., 2008; Cimini et al., 2015). On this note, it is critical to evaluate whether ART is able to reconstitute γδ T cell numbers and functionality at each stage of infection (Juno and Eriksson, 2019).

Although the Vδ2:Vδ1 inverted frequencies are never restored, early initiation of ART has been shown to partially restore the loss of γδ T cell function in HIV-seropositive individuals. Casetti et al. found that introducing treatment during primary infection reconstitutes Vδ1 T cell direct cytotoxic capabilities but antiviral chemokine production of CCL4 (MIP-1β) remains dampened despite early intervention. Moreover, both Vδ2 T cell cytotoxic function and pro-inflammatory cytokine production appear to be negatively impacted early on and are unable to be recovered regardless of the timing of ART (Casetti et al., 2019). Interestingly, our study in HIV-seropositive individuals on suppressive ART for more than 1 year showed that the remaining Vδ2 T cells retained their ability to degranulate in the presence of reactivated latently infected CD4+ T cells (Garrido et al., 2018). Whether or not ART is able to restore antigen responsiveness and polyfunctionality is being studied further within our group. Finally, the positive correlation between CD4+ T cell count and Vδ2 T cell quality in ART-suppressed HIV-seropositive individuals underscores the potential requirement of other immune cell populations needed to maintain or reconstitute γδ T cell function after primary HIV infection (Li et al., 2008; Casetti et al., 2015).

EXPLORING THE POTENTIAL OF ALLOGENEIC γδ T CELL IMMUNOTHERAPY FOR HIV CURE

Lessons Learned From the Treatment of Cancer

In addition to altering γδ T cell frequencies, HIV infection modulates the expression of NK cell-like and potentially other receptors, directly modifying their cytotoxic capabilities (Poccia et al., 1999; Fausther-Bovendo et al., 2008; Hudspeth et al., 2012; Omi et al., 2014). We are currently missing a compelling picture of how HIV is recognized by γδ T cells, characterization of the various known receptors and mechanisms utilized to combat HIV infection reveals distinct similarities and differences between the two major γδ T cell subpopulations. Although both subpopulations display anti-HIV capabilities, investigations and

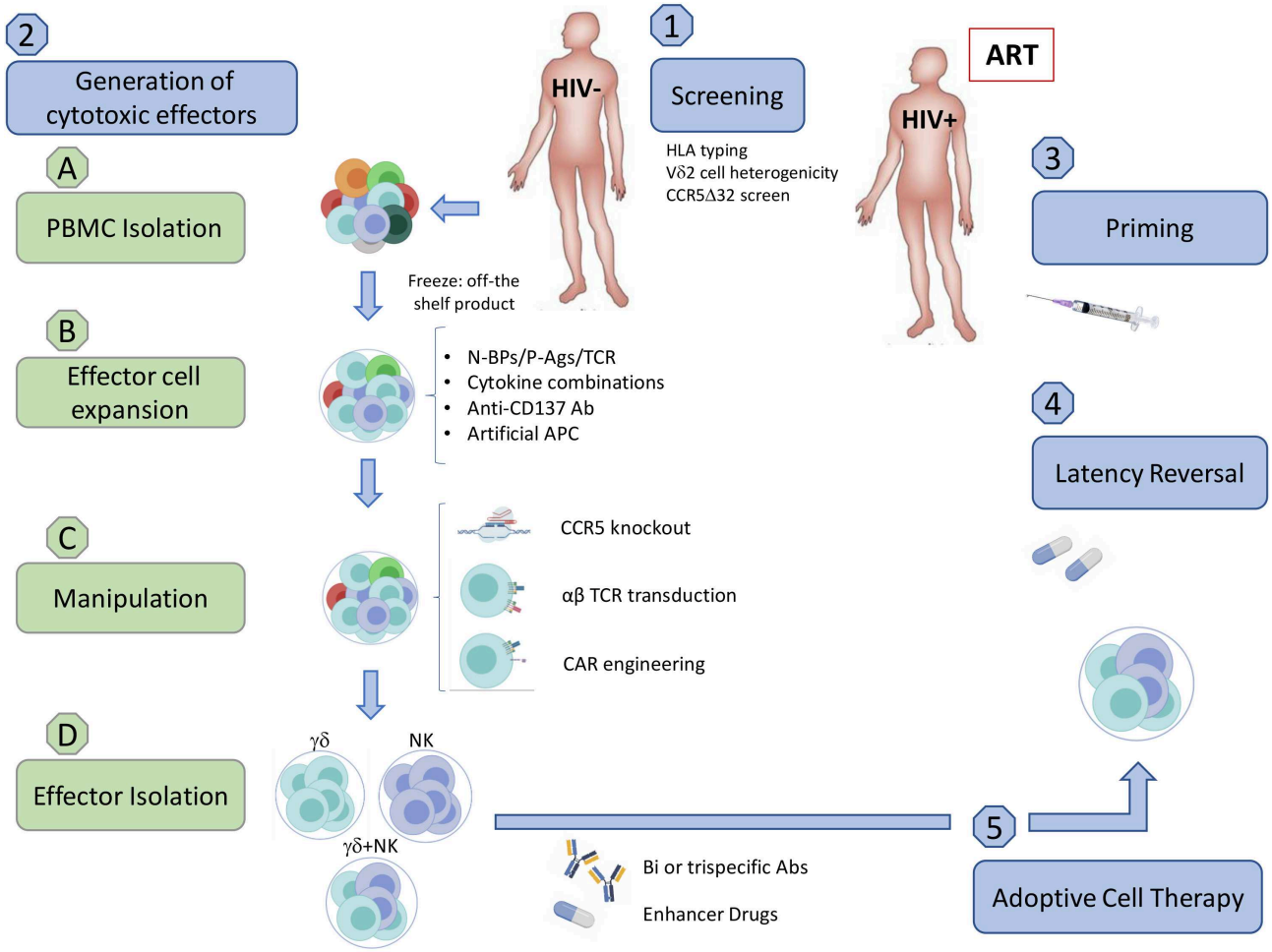

FIGURE 1 | Implementation of an allogeneic adoptive cell therapy. (1) **Pre-clinical screening**. (A) Assess donor HLA compatibility. Although allogeneic γδ T cells should not generate GVHD and were reported to be safe in some clinical trials in cancer, we need to exercise caution until further clinical testing demonstrates the safety of allogenic transfer in ART-suppressed HIV-seropositive individuals. (B) Assess Vδ2 T cell population heterogenicity (C) Ideally use a CCR5Δ32/Δ32 donor. (2) **Generation of cytotoxic effector cells from uninfected donors** (A) PBMC Isolation from uninfected donors. (a) Density gradient separation from leukapheresis product from uninfected individuals. (b) Freeze PBMC to have an off-the shelf/ready to use product. (B) Expansion. (a) Expansion of Vδ2 T cells using a cocktail of optimized cytokines and activating compounds such as N-BPs, P-Ags, or TCR crosslinkers. Addition of feeder cells can increase the number and effector function of expanded cells. These may include artificial APCs (aAPC) expressing CD40L and CMV antigen pp65 for γδ T cells, or the K562 cell line for both γδ T cells and NK cells. (b) The natural crosstalk between γδ T cells and NK cells could be exploited via co-culturing to induce binding of CD137-CD137L resulting in a boost to NK cell cytotoxic function. Co-culture may be feasible with the addition of anti-CD137 Abs and select cytokine combinations that are beneficial for both cell types, such as IL-12 and IL-15. (C) Manipulation (a) Gene-editing enzymes such as zinc-finger nucleases or CRISPR-Cas9 could be utilized to generate γδ T cell effector subsets with a CCR5-knockout, reducing the likelihood of HIV infection or CCR5-mediated cell death. (b) Transduction of αβ TCR or CAR can be included in this step to boost specificity for peptidic HIV antigens expressed on the surface of infected cells (**Figure 2**). (A) Effector cell isolation/enrichment. (a) Cell sorting of either γδ T cell or NK cell populations. (b) Enrichment of γδ cells and NK cells (c) αβ T cell depletion. While multiple methods efficiently isolate effector populations, enrichment and αβ T cell depletion carry a higher risk for inducing GVHD. (d) Ready to use product. (A) **Priming**. Vaccination may boost pre-existing anti-HIV specific responses prior to adoptive transfer. (B) **Administering Latency Reversal Agents**. Reactivate latent virus with clinically approved HDACis, PI3K/Akt inhibitor, BRDi, or TLR agonist. (C) **Adoptive cell transfer**. (a) Allogeneic adoptive cell transfer of potent cytotoxic effectors. (b) Specific killing of HIV-infected cells could be achieved through the use of bi/trispecific antibodies that would recognize CD16 on one arm, γδ TCR on the other, and HIV proteins in the last arm of the antibody. (c) Enhancer drugs like bortezomib or the use of LRAs such as BRDis may increase *in vivo* efficacy by upregulating cytotoxic receptors on effector cells or their ligands on target cell populations. Created with BioRender.com.

clinical trials have been mostly based on Vδ2 T cells. Nevertheless, Vδ1 T cells have shown potential as an immunotherapy for cancer but lacking knowledge of the specific activating ligand poses an additional barrier (Silva-Santos et al., 2019). A significant advantage of using γδ T cells for immunotherapy comes from our detailed understanding of how to manipulate the mevalonate pathway to produce isoprenoid intermediates that activate Vδ2 T cells. Aminobisphosphonates (N-BPs) are structurally similar to pyrophosphates and specifically activate Vδ2 T cells by inhibiting farnesyl pyrophosphate synthase, leading to an

accumulation of isopentenyl pyrophosphate (van Beek et al., 1999). N-BPs such as zoledronate and pamidronate are used for the treatment of low bone density-associated diseases including osteoporosis and cancer (Kunzmann et al., 1999; Berenson, 2001). Clinical trials in different malignancies first demonstrated that N-BPs combined with IL-2 strongly stimulate Vδ2 T cell activation and proliferation *in vivo*. However, some studies revealed that prolonged exposure to N-BPs leads to increased anergic Vδ2 T cells and concerns over IL-2 toxicity required refined dosing regimens (Kunzmann et al., 2000; Sicard et al., 2005; Lang et al., 2011). This led toward an adoptive transfer approach where cells could be expanded and monitored under controlled *in vitro* conditions. Despite results from these clinical trials showing mixed benefits, overall these strategies are well-tolerated and safe (Sato et al., 2005). Given the safety of these trials, we speculate that allogeneic γδ T cells from uninfected individuals have the potential to be utilized for an HIV cure and provide an overview of the clinical application of such an approach (**Figure 1**).

Ex vivo Manipulation of γδ T Cells

One of the advantages of using γδ T cells is that they can be easily manipulated *in vitro*. N-BPs and P-Ags in combination with IL-2 have been shown to be strong stimulators of Vδ2 T cell activation and proliferation in *ex vivo* human PBMCs (Kunzmann et al., 2000). Alternative methods that could be used to induce *in vitro* activation of both major human subpopulations rely on cross-linking activating surface receptors after treatment with lectin-based compounds such as concanavalin A or antibodies that target the γδ TCR or CD3 complex (Dokouhaki et al., 2010; Siegers et al., 2011; Zhou et al., 2012). γδ T cells are receptive to further possible modulation by treatment with combinations of different exogenous cytokines. IL-2 and IL-15 selectively activate resting human Vδ2 T cells in the presence of P-Ag, promoting expansion and an increase in TNF-α and IFN-γ production (Kjeldsen-Kragh et al., 1993; Garcia et al., 1998). These cytokines are compatible or in some cases have synergistic effects when combined with IL-12, IL-18, or IL-21 favoring development of an effector memory phenotype with enhanced cytolytic activity (Thedrez et al., 2009; Li et al., 2010; Domae et al., 2017). Optimizing combinations of activating compounds and cytokines constitutes one of the approaches to properly expand and maintain desired Vδ2 T cell effector phenotypes. Determining which effector subset is best suited for HIV cure strategies and optimizing an expansion protocol to generate these effectors is currently being explored by our lab. Although the activating ligand of Vδ1 T cells has yet to be characterized, successful *in vitro* expansion protocols have been published, and constitute another avenue worthy of investigation for HIV cure. Almeida et al. generated the so-called Delta one T (DOT) cells, which are Vδ1-enriched γδ T cells with overexpressed cytotoxic NK cell receptors following treatment of PBMCs from healthy human donors with an anti-CD3 antibody, IL-4 and IFN-γ. Within a mouse model, these cells effectively trafficked to malignant tissues and displayed potent anti-tumor activity (Almeida et al., 2016).

In addition to *ex vivo* expansion, γδ T cell therapies could stand to benefit from advances in gene editing or inclusion of immunomodulators such as designer antibodies or pro-cytotoxic drug compounds. Transduction of αβ TCRs could bestow γδ T cells with additional antigen recognition of intracellular peptides presented within MHC molecules whereas CARs could be engineered to recognize specific extracellular markers. Traditionally, these modifications have been applied to αβ T cells to target cancer with mixed results, the chief concern being off-target toxicity and ensuing "cytokine storm" that leads to systemic immune activation (van den Berg et al., 2015; Bonifant et al., 2016; Srivastava and Riddell, 2018). Applying these modifications to γδ T cells has shown promise for eliminating these issues while maintaining antigen-specific effector function and therefore constitute an additional advantageous manipulation to consider when targeting HIV-infected cells (**Figure 2**) (Harrer et al., 2017; Capsomidis et al., 2018).

Due to Vδ2 T cells susceptibility to CCR5-mediated cell death and direct infection, the allogeneic adoptive cell therapy would require either finding CCR5Δ32 homozygous healthy individuals or *in vitro* manipulation of the expanded Vδ2 T cells to abrogate CCR5 expression prior to administration into ART-suppressed HIV-seropositive individuals (Li and Pauza, 2011; Soriano-Sarabia et al., 2015). Since learning of the protective effect of the CCR5Δ32/Δ32 mutation, researchers have attempted to manipulate the expression of CCR5 through the use of gene-targeting nucleases (Haworth et al., 2017). Recent studies have shifted to the use of clustered regularly interspaced short palindromic sequences (CRISPR) and CRISPR associated protein 9 (Cas9) which have been shown to successfully confer resistance to transplanted hematopoietic stem cells within a mouse model as well as CD4+ T cells *in vitro* (Xu et al., 2017; Yu et al., 2018). Improvements in this area of research could lead to the development of γδ T cells or other adoptive cellular therapies resistant to infection by CCR5-tropic HIV (**Figure 3**). In addition, co-administration of bispecific or trispecific antibodies to facilitate the lysis of both cancer and HIV-infected cells with a high degree of specificity by binding to a target specific epitope on one arm, and an effector cell receptor, such as CD3 or CD16, on the other arm could also be applied for increased γδ T cell cytotoxic specificity (Ferrari et al., 2016; Schiller et al., 2016). Additionally, co-culturing γδ T cells with NK cells treated with an anti-CD137 Ab could promote their interaction *in vitro* leading to increased expansion and cytotoxic capability (Chu et al., 2019; Vidard et al., 2019). Further boosting of effector function could be achieved by drug-mediated upregulation of cytotoxic receptor ligands on the surface of target cells (Niu et al., 2017). Lessons from these cross-disciplinary studies could be applied to increase the efficacy of strategies utilizing γδ T cells to treat persistent HIV infection. Taken together, γδ T cells are amenable to a variety of *in vitro* modifications that could be optimized for generating tailored cytotoxic effector populations.

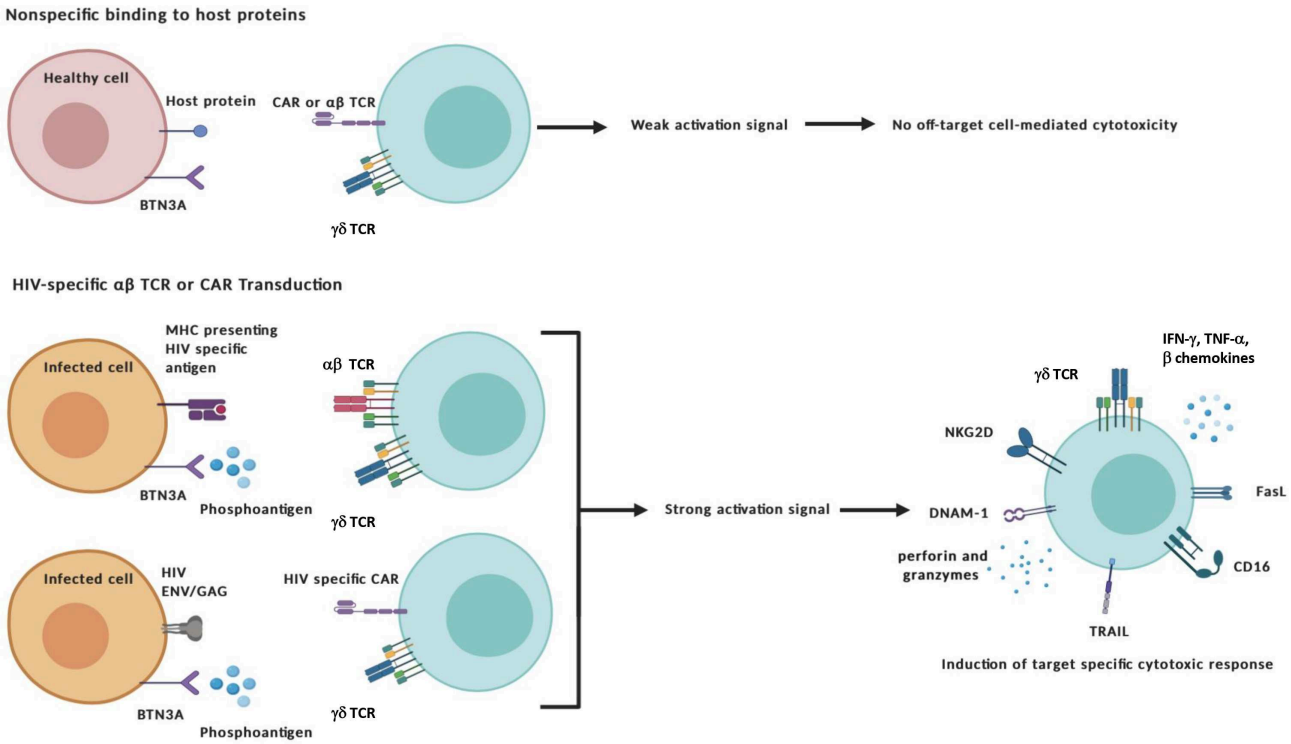

FIGURE 2 | Specific targeting and activation of γδ T cell effectors. The transduction of HIV-specific αβ TCRs or chimeric antigen receptors (CAR) combined with the unique antigen recognition of γδ T cells could reduce off-target toxicity issues. Target-specific activation of γδ T cells can lead to the exertion of cytotoxic functions through a number of different mechanisms: (i) direct cytolysis of malignant or infected cells through the release of cytotoxic granules containing perforin and other granzymes; (ii) Induction of apoptosis by Fas Ligand and TRAIL death receptors; and (iii) antibody-dependent cellular cytotoxicity (ADCC) triggered by binding of the Fcγ receptor CD16 to the constant region of IgG antibody-coated targets. In addition to secreting pro-inflammatory cytokines interferon-γ (IFN-γ) and tumor-necrosis factor-α (TNF-α), γδ cells also produce CC-chemokines that inhibit CCR5-dependent entry of HIV virions into CD4+ T cells. Created with BioRender.com.

THE APPROACH: ALLOGENEIC γδ T CELL IMMUNOTHERAPY

Although the anti-HIV capacity of γδ cells to inhibit viral replication is well-documented (Wallace et al., 1996; Soriano-Sarabia et al., 2015), only recently we have reported the proof of concept of their capacity to specifically kill reactivated latently infected cells (Garrido et al., 2018). This work sets the basis for further investigation toward understanding mechanisms of HIV recognition and specific killing toward an HIV cure. We speculate that an allogeneic strategy that utilizes prescreening of uninfected donors for the CCR5Δ32/Δ32 mutation or gene editing of CCR5 as the only possible way to implement the adoptive transfer of γδ T cells for HIV cure since γδ T cells can be infected by HIV (Poccia et al., 1999; Imlach et al., 2003; Soriano-Sarabia et al., 2015), and their contribution in the tissues, where they are numerically more abundant, remains unknown.

The study of allogeneic γδ T cell transfer to treat chronic disease is still in its infancy but there have been recent developments in the cancer field. Haploidentical hematopoietic stem cell transplantation markedly improved survival rates of patients with hematologic cancers (Godder et al., 2007). Heightened, post-transplantation levels of γδ T cells were determined to be a critical component of the trial's success. The key findings of this study revealed γδ T cells could achieve strong anti-leukemic activity in the absence of graft-vs.-host disease. A follow up study by Wilhelm *et al.* reinforced this observation by transplanting haploidentical γδ T cells and expanding them *in vivo*. With three out of four patients experiencing brief but complete remission and no major side effects, allogeneic transfer shows potential as an immunotherapeutic tool (Wilhelm et al., 2014). Most recently, a regimen of allogeneic *ex vivo* expanded γδ T cells was used in the treatment of an individual with late-stage cholangiocarcinoma. In addition to anti-tumor activity, the authors observed signs of positive immune regulation in autologous αβ T cell and NK cell populations that possibly contributed to the improved clinical outcome (Alnaggar et al., 2019). This suggests that the transfer of γδ T cells may act directly against malignant tissue as well as recruit other immune populations to join in the anti-tumor response. It should be noted that each of these cases include patients that may have been immunosuppressed or exposed to a

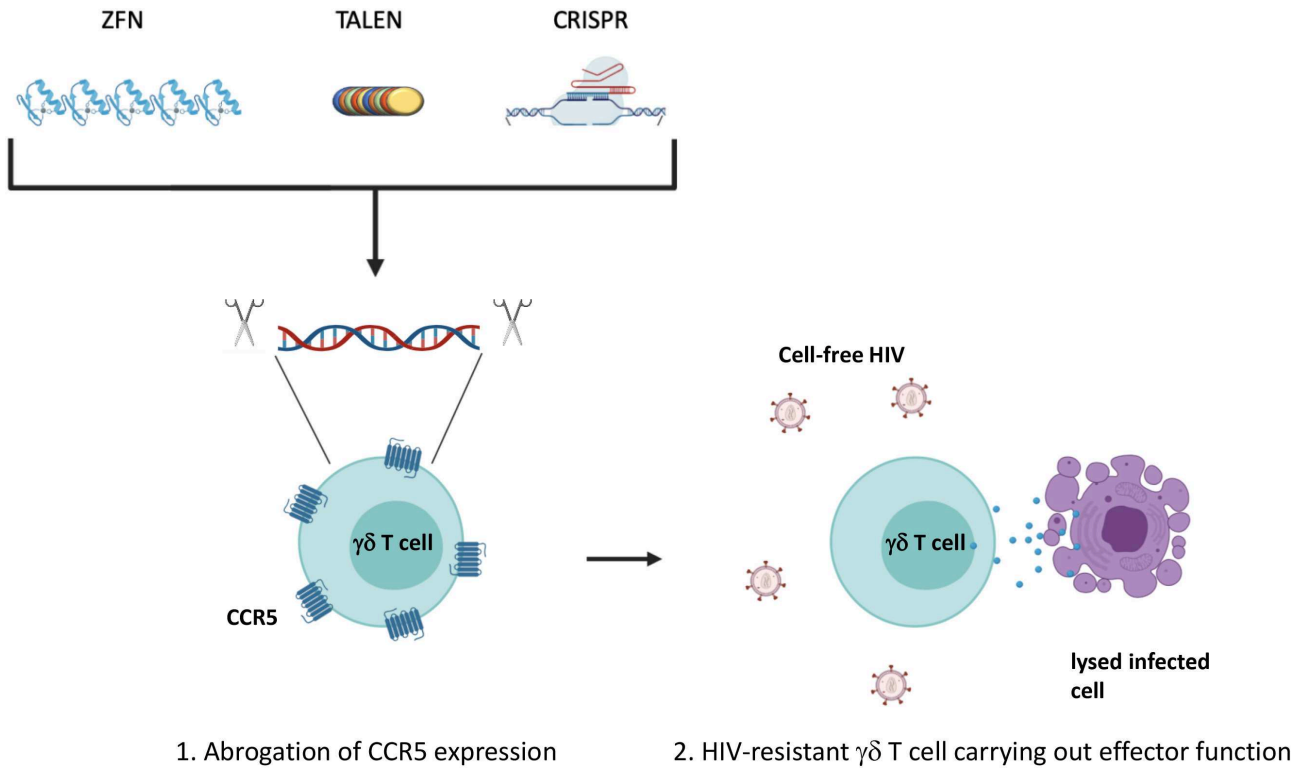

FIGURE 3 | Manipulation of CCR5 expression. The expression of CCR5 leaves Vδ2 T cells susceptible to direct infection as well as activation of the p38-mediated cell death signaling pathway. (1) Ablation of the CCR5 gene could confer a degree of resistance to effector subsets prior to adoptive transfer generating (2) HIV-resistant γδ T cells. Traditional gene editing strategies have utilized zinc-finger nucleases or transcription activator-like effector nucleases (TALENs) to recognize and cleave specific genomic sequences. More recently, focus has shifted to the use of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9) that rely on a small single guide RNA that is complementary to the target gene sequence. Created with BioRender.com.

myriad of previous treatments that could exert an impact on these findings.

These studies provide the possibility that allogeneic transfer may be a safe, feasible treatment for certain cancers. Whether this applies to relatively healthier ART-treated individuals will require direct studies to sufficiently assess safety and efficacy for HIV cure. Nevertheless, as a byproduct of the cancer trials, untethering from strict HLA-matching engenders the possibility of generating an off-the-shelf repository of γδ T cells to treat disease. Improvements to *ex vivo* expansion protocols as a prior step toward generating a powerful cytotoxic γδ T cell population, would bring this prospect closer to reality. Several groups have shown that automation of cell isolation and co-culture with artificial APCs expressing co-stimulatory molecules such as CD40L and CMV antigen pp65 can drastically increase the number, purity, and effector function of the expanded γδ T cells while maintaining compliance with current good manufacturing practices (Lamb et al., 2018; Polito et al., 2019). Despite these improvements, the inherent heterogeneity of γδ T cell populations between potential donors could pose a significant challenge to manufacturing a consistent cellular product barring the development of an efficient pre-clinical screening method (Cairo et al., 2010; Ryan et al., 2016). Although many basic science questions will need adequate attention, allogeneic transfer presents an enticing strategy for utilizing γδ T cells as a multipurpose immunotherapy.

FURTHER CONSIDERATIONS

Effect of LRAs on Immune Cell Function

It is important to determine how these compounds impact the cytotoxic capacity of effector cells in HIV-seropositive individuals. Epigenetic modifiers, belonging to the same categories as current LRAs, have been shown to affect γδ T cell or NK cell functions. Current information about the impact of HDACis on γδ T cells is limited and comes from cancer studies using Valproic acid (Bhat et al., 2019), which was shown to be ineffective at reactivating persistent HIV *in vivo* (Archin et al., 2008). This cancer study showed a downregulation of the NKG2D receptor and redistribution of γδ T cell memory subsets (Bhat et al., 2019). Additional studies have focused on the effect of different LRAs on NK cell function showing mixed results. For example, panobinostat (PNB) had deleterious effects to NK function by reducing cytotoxicity and IFN-γ production (Rasmussen et al., 2013; Garrido et al., 2016). However later *in vivo* observations did not

support *in vitro* findings, highlighting the absolute requirement of *in vivo* validation (Garrido et al., 2019). Furthermore, the PKC agonist prostatin (PROST) may have beneficial impact on NK function by increasing NKG2D expression (Garrido et al., 2016). Conversely, bromodomain inhibitors have been shown to increase NKG2D ligands on the surface of cancer cells with the added benefit of downregulating PD-L1, potentially boosting cytotoxicity while mitigating immune exhaustion (Abruzzese et al., 2016; Zhu et al., 2016). These studies highlight the need to characterize the cellular effects of LRAs and strategically choose LRA types, doses, and administration regimens that optimize latency reversal without negatively impacting the antiviral activity of effector cells.

Potential Use of γδ T Cells for HIV Cure

A number of fundamental questions that require investigation will need to be answered prior to the clinical implementation of γδ T cells in HIV therapy. How do γδ T cells recognize HIV infected cells? Do allogeneic γδ T cells specifically target and kill reactivated latently infected cells similar to autologous cells? Will the Vδ2 subpopulation alone be sufficient or will both subpopulations be required to maximize efficacy of an allogeneic approach? Additionally, what is the ideal cytotoxic phenotype for clearing reactivated latently infected cells and how will this phenotype be produced through *ex vivo* manipulation? Will the heterogeneity between donors preclude scaling up production and implementation? Our initial study only compared the expansion of Vδ2 T cells using PAM + IL-2 to cells expanded with HMB-PP + IL-2 and IL-2 alone (Garrido et al., 2018). Previous studies have shown the positive effects of IL-12, IL-15, IL-18, and IL-21 on producing *ex vivo* expanded Vδ2 T cell cytotoxic phenotypes (Thedrez et al., 2009; Van Acker et al., 2016; Domae et al., 2017). Similarly, we are currently optimizing Vδ2 T cell expansion to consistently produce a highly homogeneous cytotoxic phenotype despite the interindividual variation of Vδ2 T cell populations (Ryan et al., 2016). A *post hoc* comparison of donor phenotypes prior to expansion may also help identify the subset receptor expression profiles most likely to produce our desired cytotoxic effectors. Identification of the activation, cytotoxic, or chemotactic markers expressed in the donors from this comparison study could help us develop a preclinical screening panel for potential future donors.

CONCLUDING REMARKS

Targeted therapy utilizing the potent cytotoxic capabilities of γδ T cells is a compelling avenue of research that has broad implications in treating a variety of diseases. Extensive focus within the field of cancer continues to produce a steady stream of innovations that may boost the efficacy of γδ T cell therapies. Advances in latency reversal combined with the allogeneic transfer of expanded effector γδ T cells could provide a dynamic two-prong strategy to cure persistent HIV infection. While our recent work showed the feasibility of using γδ T cells to specifically target and kill HIV infected cells (Garrido et al., 2018), a more detailed investigation into γδ T cell responses to HIV infection, and specifically persistent HIV infection, is needed before moving into clinical application. Based on the current knowledge summarized in this review, γδ T cells merit further consideration in investigations toward an HIV cure. Allogeneic transfer of *ex vivo* expanded γδ T cells presents an intriguing therapeutic option that may 1 day bring us a step closer to a sustained ART-free HIV remission or complete cure.

AUTHOR CONTRIBUTIONS

All authors have contributed to writing, editing, and reviewing the manuscript.

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A Trispecific Anti-HIV Chimeric Antigen Receptor Containing the CCR5 N-Terminal Region

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Anti-HIV chimeric antigen receptors (CARs) promote direct killing of infected cells, thus offering a therapeutic approach aimed at durable suppression of infection emerging from viral reservoirs. CD4-based CARs represent a favored option, since they target the essential conserved primary receptor binding site on the HIV envelope glycoprotein (Env). We have previously shown that adding a second Env-binding moiety, such as the carbohydrate recognition domain of human mannose-binding lectin (MBL) that recognizes the highly conserved oligomannose patch on gp120, increases CAR potency in an *in vitro* HIV suppression assay; moreover it reduces the undesired capacity for the CD4 of the CAR molecule to act as an entry receptor, thereby rendering CAR-expressing $CD8^+$ T cells susceptible to infection. Here, we further improve the bispecific CD4-MBL CAR by adding a third targeting moiety against a distinct conserved Env determinant, i.e. a polypeptide sequence derived from the N-terminus of the HIV coreceptor CCR5. The trispecific CD4-MBL-R5Nt CAR displays enhanced *in vitro* anti-HIV potency compared to the CD4-MBL CAR, as well as undetectable HIV entry receptor activity. The high anti-HIV potency of the CD4-MBL-R5Nt CAR, coupled with its all-human composition and absence of immunogenic variable regions associated with antibody-based CARs, offer promise for the trispecific construct in therapeutic approaches seeking durable drug-free HIV remission.

Keywords: HIV, HIV functional cure, immunotherapy, cell therapy, chimeric antigen receptor, CD4, mannose binding lectin, CCR5

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INTRODUCTION

The development of antiretroviral therapy (ART) drug regimens represents an exceptional medical achievement that has enabled individuals infected with human immunodeficiency virus (HIV) to remain asymptomatic and lead essentially normal lives (Saag et al., 2018). However, drug toxicities, high costs, adherence difficulties, emergence of viral resistance, and challenges to medication accessibility to communities at greatest need represent major burdens for the global HIV population (Mouton et al., 2016). These factors are fueling extensive efforts to develop HIV curative approaches that either eradicate the infection from the body ("sterilizing cure") or achieve long-term remission by durably suppressing the virus ("functional cure") (Siliciano and Siliciano, 2016; Davenport et al., 2019; Ndung'u et al., 2019; Peterson and Kiem, 2019). Amongst the diverse HIV cure concepts under development, enhancement/engineering of HIV-specific T

cell function is receiving considerable attention (Patel et al., 2016; Yang et al., 2018). A rare subset of individuals, termed "elite controllers," are able to naturally maintain virus loads below the level of detection in the absence of ART, thanks in large part to their strong HIV-specific CD8+ T cell responses (Goulder and Deeks, 2018). This has fueled considerable interest in devising approaches to endow HIV-infected subjects with elite-controller-like T cell responses to enable long-term discontinuation of ART without a viral rebound. In this context of durable remission, anti-HIV chimeric antigen receptors (CARs) represent a particularly active area of research (Kuhlmann et al., 2018; Wagner, 2018; Kim et al., 2019; Liu et al., 2019; Mylvaganam et al., 2019).

CAR technology involves the design of synthetic protein constructs containing a recognition/targeting domain attached via a hinge and transmembrane elements to functional intracellular domains, typically sequences involved in T cell costimulation and activation (Srivastava and Riddell, 2015; June and Sadelain, 2018). When expressed on the T cell surface, the CARs mediate selective killing of the chosen target cell type. In the case of anti-HIV CARs, killing is based on recognition of the intact HIV envelope glycoprotein (Env) on the surface of infected cells. Env is a trimer of gp120/gp41 heterodimers produced by proteolytic cleavage of the gp160 precursor (Sanders and Moore, 2017; Ward and Wilson, 2017; Chen, 2019). Upon interaction with the primary receptor CD4 at the T cell surface, gp120 undergoes a conformational change that exposes the otherwise concealed/unformed binding site for coreceptor (CCR5 or CXCR4). Coreceptor binding in turn triggers further conformation changes leading to exposure of the gp41 subunit and insertion of its hydrophobic fusion peptide into the target cell membrane. The resulting fusion of the viral membrane with the plasma membrane culminates in HIV entry. The bulky surface-exposed gp120 subunit contains highly variable regions that aid the virus in immune evasion, as well as conserved elements involved in critical functions that can be targeted by therapeutic approaches.

From the earliest days (Bitton et al., 2001) until the present (Kuhlmann et al., 2018; Wagner, 2018; Kim et al., 2019; Liu et al., 2019; Mylvaganam et al., 2019), CD4 has been a favored component for the targeting domain of anti-HIV CARs, because it recognizes an essential conserved feature of gp120 that presumably must be retained for viral persistence and pathogenicity. Our group has reported improved bispecific designs of CD4-based CARs containing a second moiety that targets a distinct conserved determinant on gp120 (Liu et al., 2015; Ghanem et al., 2018). The purpose of the second moiety is to enhance the anti-HIV potency of the CAR, and to inhibit the undesired potential for the CD4 to act as an HIV entry receptor on the CAR-expressing T cell. Importantly, to minimize immunogenicity, both moieties derive from human protein sequences. The most promising construct employed the carbohydrate recognition domain of human mannose-binding lectin (MBL), which recognizes the highly conserved oligomannose patch on gp120 (Ghanem et al., 2018).

In contemplating additional motifs for further CAR enhancement, we considered the N-terminal region of the HIV coreceptor CCR5. This region binds to a highly conserved site on HIV-1 gp120, and has been shown to function in various structural contexts including synthetic peptides and recombinant proteins (Choe and Farzan, 2009; Gardner and Farzan, 2017). As a "self" sequence, the CCR5 amino terminal region is likely to be minimally immunogenic as a CAR component. Because the CCR5/gp120 interaction is highly CD4-dependent (Ward and Wilson, 2017; Chen, 2019), we studied its functionality in the context of CD4-based CAR constructs. The data presented herein describe potency and protective enhancements provided by the CCR5 N-terminal region, and suggest its potential value as a component of a trispecific anti-HIV CAR.

RESULTS

CAR Constructs

Figure 1 shows the design of the CAR constructs analyzed in this report. In each case, the indicated targeting domain is attached via a 3 amino acid linker to a segment of human CD28 (truncated extracellular, transmembrane, and intracellular costimulatory domains) followed by the intracellular activation domain of human CD3 zeta. Within each targeting domain, the different recognition moieties are separated by linkers of 5 or 10 amino acids. The full amino acid sequences are shown in **Supplementary Figure 1**.

The monospecific CD4 CAR, the bispecific CD4-MBL CAR, and 139 control CAR (irrelevant scFv) have been reported previously (Liu et al., 2015; Ghanem et al., 2018). The new constructs contain a 25 amino acid segment based on residues 2-26 of human CCR5 (herein designated R5Nt). In keeping with previously reports using synthetic peptides (Farzan et al., 2000, 2002), residue 20 was changed from Cys to Ser to avoid inappropriate disulfide formation. We first generated a bispecific CAR designated CD4-R5Nt, *in order to evaluate R5Nt's potential as CAR component,* even when not located at the N-terminus of the recombinant protein. The native CCR5 N-terminus contains between 2 and 4 sulfated tyrosine residues at positions 2, 10, 14, 15, which have been shown to be necessary for HIV coreceptor function (Choe and Farzan, 2009). Structural studies indicate that it is the sulfate groups themselves, rather than the polypeptide side chains, that mediate the interaction with gp120 (Huang et al., 2007); in fact, the unsulfated CCR5 amino terminal region is inactive. Therefore, we constructed a control CAR, designated CD4-R5Nt(Y/A), wherein the four tyrosine residues were mutated to alanine.

Expression and Env-Mediated Activation of the CD4-R5Nt CAR

Stimulated PBMCs from normal donor blood were transduced with retroviral vectors encoding the previously described CD4 and CD4-MBL CARs, as well at the CD4-R5Nt and CD4-R5Nt(Y/A) CARs. **Figure 2A** shows analysis of CAR surface expression by flow cytometry. Based on detection of surface CD4 on the $CD8^+$ T cell population, CAR expression was found to be comparably high for all the CD4-based constructs (∼50–70% CD4-positive, compared to ∼1% for control cells transduced with the 139 CAR). We next tested whether the CAR-T cells can be

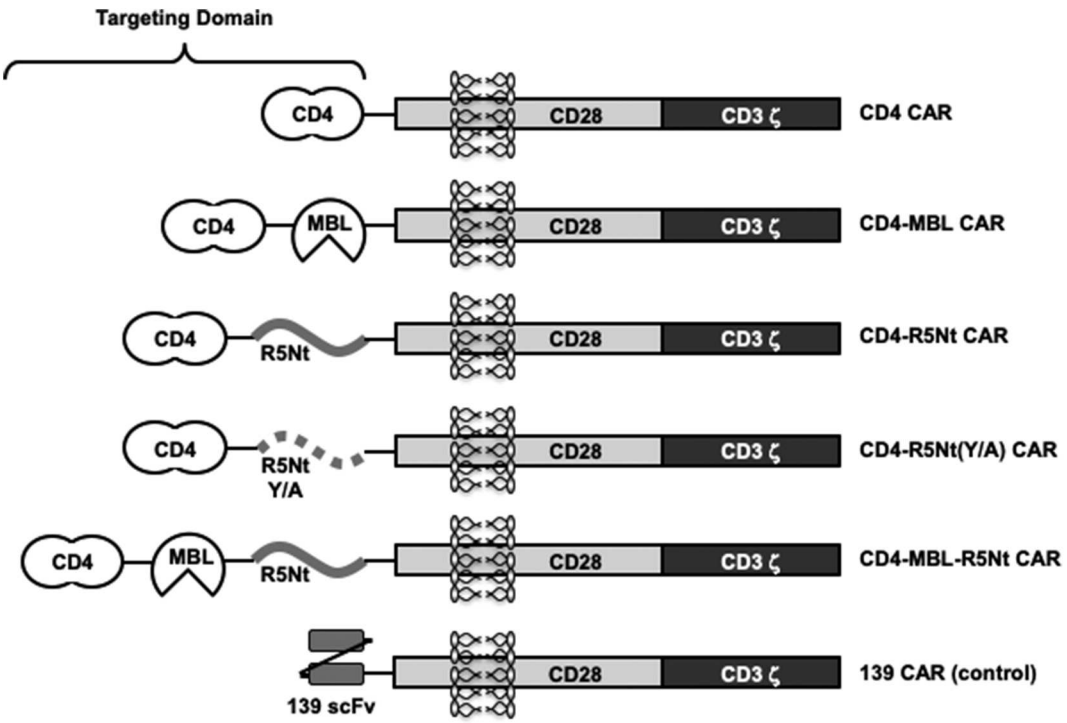

FIGURE 1 | Schematic representation of various CAR constructs used in this study. "CD4" moiety is domains 1 and 2 of human CD4, residues 1-208, including the 25 residue signal peptide. "MBL" is the carbohydrate recognition domain of human mannose-binding lectin (residues 214-330). "R5Nt" represents the N-terminal segment (residues 2-26) of human CCR5, with a Cys to Ser substitution at residue 20. "R5Nt(Y/A)" is a control version of R5Nt containing four tyrosine-to-alanine substitutions that eliminate the sulfation targets required for binding to gp120. The recognition domain of the control 139 CAR is an irrelevant scFV. Solid black lines indicate flexible linkers of various lengths. See **Supplementary Figure 1** for amino acid sequences.

activated by cells expressing surface HIV-1 Env. T cells expressing the various CD4-based CARs were co-cultured for 6 h with either parental CHO cells or CHO-*env* transfectant cells that stably express surface Env; brefeldin A and monensin were included in the co-cultures to enable staining for accumulated intracellular IFN-γ and production of the CD107a degranulation marker. The results shown in **Figure 2B** demonstrate minimal background activation mediated by any of the CARs upon co-culture with CHO cells, but dramatic upregulation of IFN-γ and CD107a in all the CD4-based CAR-T cells upon co-culture with CHO-*env* cells. The antigen-specific activation was somewhat greater with the bispecific CARs compared to the monospecific CD4 CAR.

Effects of the R5Nt Moiety on Anti-HIV Activity in the Context of a Bispecific CD4-Based CAR

To assess the anti-HIV activities of the CARs, we performed *in vitro* spreading infection coculture assays as described previously (Liu et al., 2015; Ghanem et al., 2018). PBMC from the same donor were infected with HIV-1 and incubated overnight to generate "target" cells. The following day, cocultures were established containing a fixed number of infected target cells plus CAR-expressing "effector" cells, at various effector-to-target (E:T) ratios (ranging from 0.008:1 to 1:1). Controls included cultures with no effector cells, or with effectors transduced with the irrelevant 139 control CAR. At 2-day intervals, aliquots of supernatants were collected for analysis of p24 content. Results with the HIV-1 primary isolate BX08 isolate are shown in **Figure 3**. As one form of analysis, CAR potencies were compared at varying E:T ratios (**Figure 3** Top, day 10). At the highest E:T ratio of 1:1, all CD4-containing CARs gave full suppression, with p24 levels below detectable limits. However, significant potency differences were revealed at lower E:T ratios. The bispecific CD4-R5Nt CAR, like the previously described CD4-MBL CAR (Ghanem et al., 2018), displayed higher potency than the monospecific CD4 CAR. A similar pattern emerged from analysis CAR activities over the time course of infection (**Figure 3** Bottom, E:T of 0.04:1); the bispecific CD4-R5Nt CAR was significantly more potent than the CD4 monospecific CAR, approaching the efficacy of the CD4-MBL CAR. In both the varying E:T ratio and the time course analyses, the CD4-R5Nt was more potent than the mutant CD4-R5Nt(Y/A) CAR, presumably reflecting specific interaction of the CCR5 N-terminal moiety with its cognate coreceptor binding site on HIV-1 gp120. The mutant CD4-R5Nt(Y/A) CAR also displayed somewhat higher potency than the CD4 CAR, indicative of effects unrelated to specific binding.

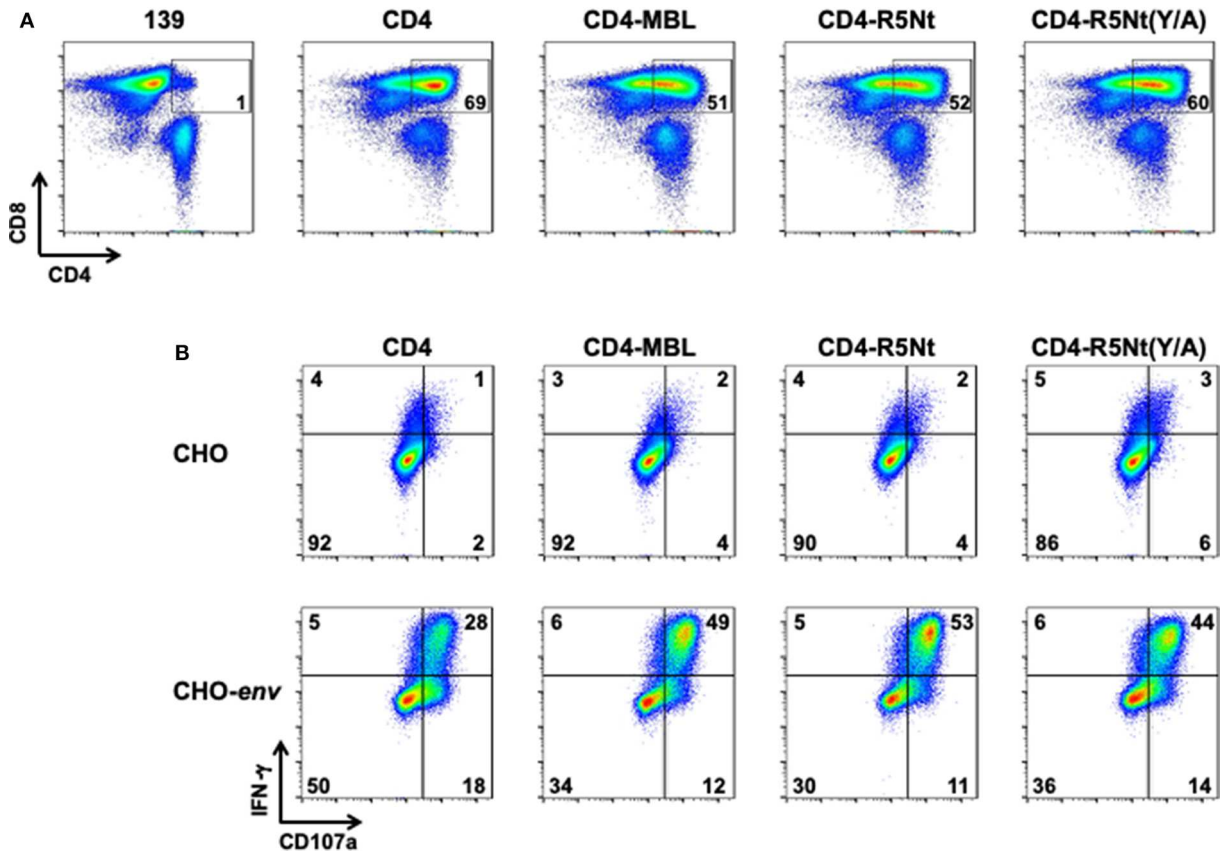

FIGURE 2 | Flow cytometry analysis of surface expression and activation of CARs, analyzed at day 6 following PBMC transduction. **(A)** CAR surface expression. After gating on live T lymphocytes, CAR expression levels were determined by the presence of CD4 on the $CD8^+$ T cell populations; inside boxes indicate % CD4-positive. **(B)** CAR activation by Env-expressing cells. CAR-transduced PBMCs were co-cultured for 6 h with either CHO cells (top), or CHO-env cells (bottom), which express surface HIV-1 Env. Cells were then stained for activation markers IFN–γ and CD107a. The % of cells in each quadrant are indicated.

Expression and Anti-HIV Activity of a Trispecific CD4-Based CAR Containing the R5Nt Moiety

The efficacy of the R5Nt moiety in the context of the bispecific CAR prompted us to analyze it's potential to enhance our previously favored CD4-MBL CAR construct. The trispecific CAR, herein referred to as CD4-MBL-R5Nt, showed high-level expression comparable to the other CD4-based CARs (**Figure 4A**). Anti-HIV-1 activities were analyzed in spreading infection assays. **Figure 4B** shows results with the primary HIV-1 isolates BX08 (left panels) and JR-FL (right panels). When analyzed in the varying E:T ratio mode (top panels) or the time course of infection (bottom panels), the CD4-MBL-R5Nt CAR displayed consistently higher potency than the CD4-MBL CAR.

Full Blockage of CAR-Mediated HIV Entry Receptor Activity in the Trispecific CAR

An important concern regarding CD4-based CARs is the potential for the CD4 moiety to act as an HIV entry receptor. This is particularly true for $CD8^+$ CAR-T cells, which are presumed to be important effectors for CAR-mediated control. The presence of endogenous CCR5 on $CD8^+$ T cells (Brenchley et al., 2004) makes these otherwise HIV-refractory cell types potentially vulnerable to infection via the transduced CD4 moiety, thus compromising their function and viability. We previously reported that the second moiety of a bispecific CAR, whether a single chain antibody variable fragment (Liu et al., 2015) or a carbohydrate recognition domain of a C-type lectin (Ghanem et al., 2018), significantly inhibits this undesired activity. However, some residual activity persists, as shown in the experiment detailed in **Figure 5**. We analyzed whether expression of the CD4-based CARs confers HIV-1 pseudovirus entry susceptibility to HOS.CCR5, a transfectant cell line stably expressing CCR5 (but not CD4). **Figure 5A** demonstrates comparable surface expression of the various CARs upon plasmid transfection of these cells. The pseudovirus entry assays shown in **Figure 5B** (left panels), using pseudoviruses from both primary strains Ba-L (upper panel) and YU2 (lower panel), demonstrate undetectable entry in untransfected HOS.CCR5 cells, but robust entry in cells transfected with the CD4 CAR. The bispecific CD4-MBL CAR conferred much lower entry permissiveness for both strains, as did the bispecific CD4-R5Nt CAR, indicating that the second moiety of each construct significantly impaired the

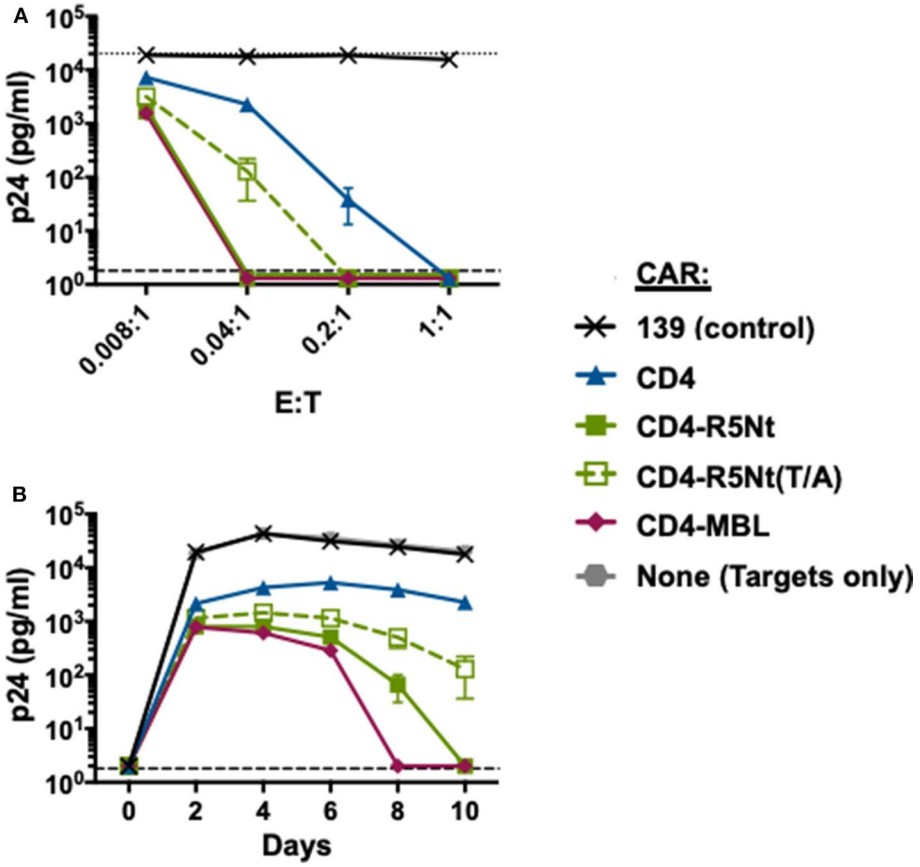

FIGURE 3 | Effects of the R5Nt moiety in the context of bispecific CD4-based CARs. The activities of the various CARs were tested in the HIV-1 spreading infection assay (Ba-L primary isolate). PBMCs were transduced with retroviral vectors and cultured for 6 days to generate T cells expressing the indicated CARs (Effectors). Cocultures were performed with autologous PBMCs infected with HIV-1 (Targets). Cocultures at varying E:T ratios were continued for 10 days, with aliquots taken at 2-day intervals for p24 assay. **(A)** Effects of varying E:T ratios. p24 values are shown for samples collected on Day 10. The dotted line represents Target cells only. **(B)** Time course of infection. p24 values are shown for cocultures at E:T ratio of 0.04:1. In both graphs, the dashed line represents the limit of detection determined from the standard curve. All assay points represent the mean of triplicate samples, with error bars denoting standard deviation.

capacity of the CD4 to serve as an entry receptor. However, for both bispecific CARs, low levels of pseudovirus entry were in fact detected, as best illustrated in the expanded graphs (right panels). Here, the superiority of the trispecific CD4-MBL-R5Nt CAR is clearly revealed, with significantly lower entry activities compared to the CD4-MBL CAR, for pseudovirus from both the Ba-L and YU2 strains; in fact, entry mediated by the trispecific CAR is indistinguishable from the background observed in untransfected cells.

DISCUSSION

CAR technology has potential to promote durable suppression of HIV in the absence of antiretroviral drugs. Because of the lifelong persistence of latently infected cellular reservoirs (Sengupta and Siliciano, 2018), an HIV "functional cure" would presumably require long-term (life-long?) persistence and functional efficacy of the CAR-T cells. To achieve these ends, we believe that anti-HIV CAR design must strive not only to optimize potency, but also to minimize both immunogenicity and potential for viral mutational escape. CD4 thus seems an ideal targeting component: as a "self" protein it is predicted to have low immunogenicity, and as the primary HIV receptor, it presumably cannot be escaped without major losses to viral fitness and pathogenicity. Strict killing specificity for Env-expressing cells stems from previous findings that CD4-based CARs do not recognize or kill cells expressing surface MHC class II (Romeo and Seed, 1991; Liu et al., 2015; Leibman et al., 2017), presumably due to the very weak binding affinities of these molecules, as previously discussed (Ghanem et al., 2018). Our approach has involved design of multispecific CD4-based CARs, with the additional component(s) intended to both enhance anti-HIV potency and minimize entry receptor activity of the CD4 moiety. While we initially achieved success with an scFv against the coreceptor binding site as the second moiety (Liu et al., 2015), we have since diverged from antibody-based motifs because of immunogenicity concerns of the associated variable regions. Indeed, efficacy-impairing anti-idiotypic antibody responses have been reported for therapeutic antibodies (Krishna and Nadler, 2016; Martinez-Navio et al., 2016) and scFv-based

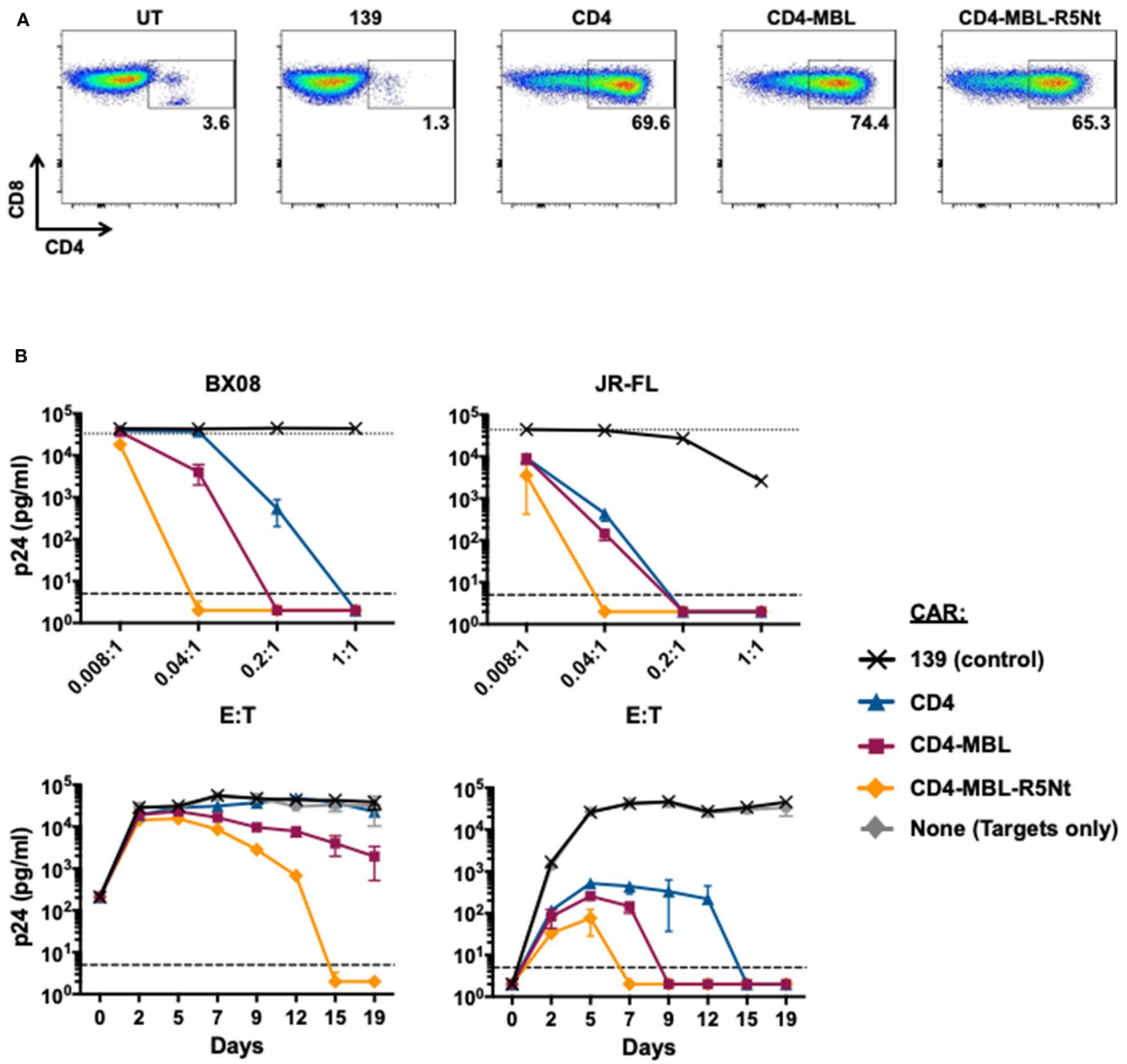

FIGURE 4 | Effects of the R5Nt moiety in the context of the trispecific CD4-MBL-R5Nt CAR. **(A)** Flow cytometry analysis of surface expression of CARs. After gating on live T lymphocytes, the levels of CAR expression were determined by the presence of CD4 on the $CD8^+$ T cell populations (inside box). Cells were analyzed at day 11 post-transduction; inside boxes indicate % CD4-positive. **(B)** Activities of CARs in the HIV-1 spreading infection assay for the BX08 (left panels) and JR-FL (right panels) primary isolates. Cocultures at varying E:T ratios were continued for 19 days, with aliquots taken at 2-3-day intervals for p24 assay. Top panels show the effects of varying E:T ratios. p24 values are shown for BX08 at day 15, and for JR-Fl at day 7. In both graphs, the dotted line represents Target cells only. Bottom panels show the time courses of infection. P24 values are shown for cocultures at E:T ratio of 0.04:1. In all graphs, the dashed line represents the limit of detection determined from the standard curve. All assay points represent the mean of triplicate samples, with error bars denoting standard deviation.

CARs (Lamers et al., 2011). Hence our shift to non-antibody "self" sequences, such as the previously reported carbohydrate recognition domain of human MBL (Ghanem et al., 2018), and the present use of the CCR5 amino terminal region.

The greater potency of the bispecific CD4-R5Nt CAR relative to the CD4-R5Nt(Y/A) variant (**Figure 3**) speaks to the enhancement associated with specific binding of the second moiety to its cognate site on gp120. However, non-specific effects also occurred, as indicated by the modestly increased potency of the CD4-R5Nt(Y/A) variant compared to the monospecific CD4 CAR (**Figure 3**). Multiple factors may contribute to enhancement conferred by the non-binding mutant moiety, including increased CD4 distance from the effector cell surface, changes in chain flexibility, and other variables that might

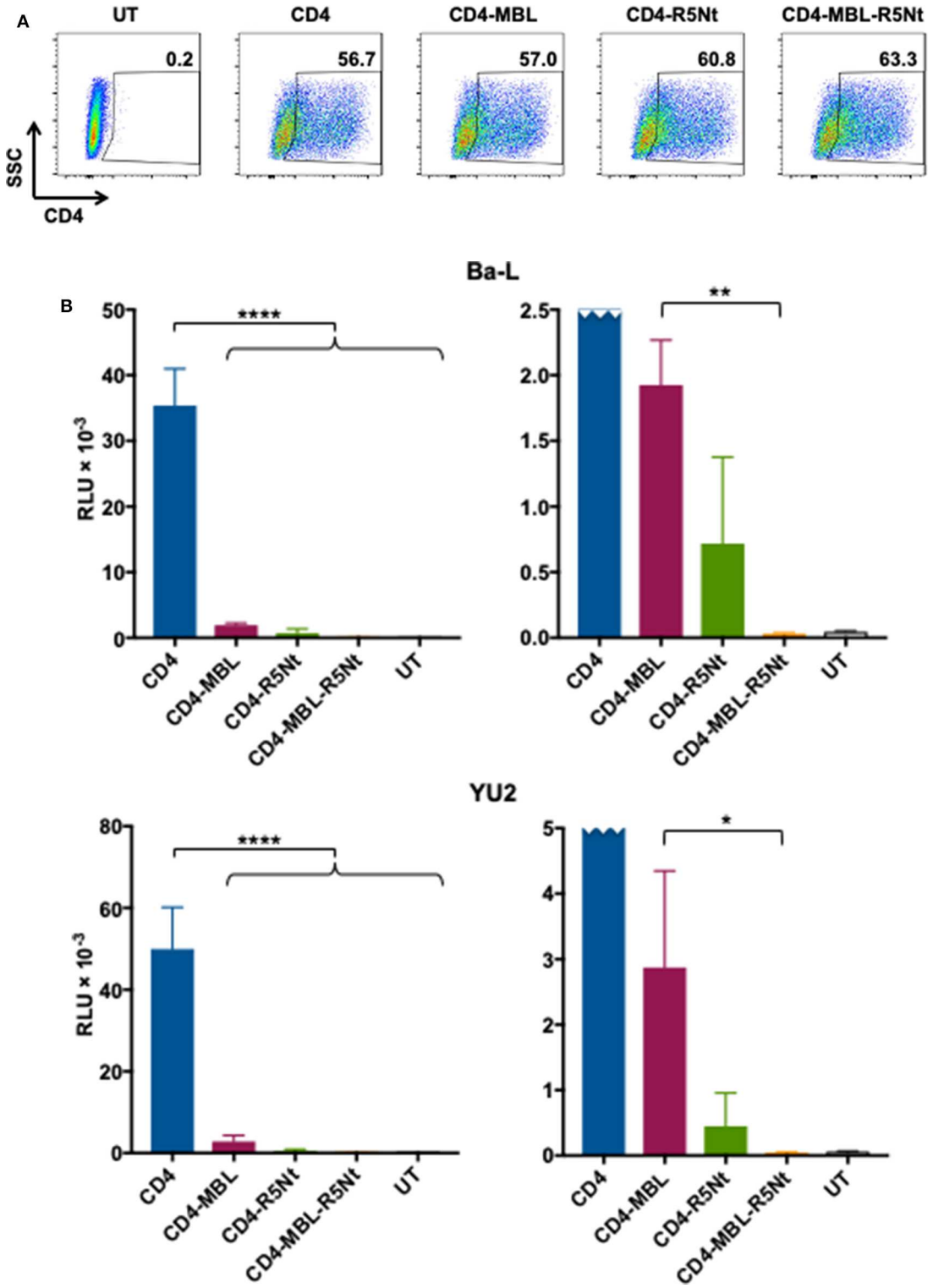

FIGURE 5 | Ability of the CD4 moiety of various CAR molecules to function as an entry receptor for HIV-1 pseudoviruses. **(A)** Flow cytometry analysis of CAR expression on transfected HOS.CCR5 cells. Following gating on live cells, CAR expression was determined based on surface CD4 levels; inside boxes indicate % CD4-positive. **(B)** Pseudovirus entry receptor activity. HOS.CCR5 cells transfected to express the indicated CAR (or untransfected, UT) were analyzed in the HIV-1 pseudovirus entry assay using pseudoviruses bearing Envs from the Ba-L (top) or YU2 (bottom) primary isolates. Pseudovirus entry was determined at 48 h based on luciferase activity. Assay points represent the mean of quadruplicate samples, with error bars denoting standard deviation. For the left panel (full scale), statistics was performed by one-way ANOVA, comparing the CD4 CAR with each of the multispecific CARs; ****$P < 0.0001$. For the right panel (expanded scale, CD4 CAR off-scale), statistics were performed by the paired *t*-test to compare the CD4-MBL bispecific CAR to the CD4-MBL-R5Nt trispecific CAR; **$P = 0.0017$, *$P = 0.0313$.

influence access/binding of the CD4 moiety to gp120 on the target cell surface.

In considering possible mechanisms governing the enhanced potency of the multispecific CARs, we note that increased binding affinity for gp120 is an unlikely explanation. The linkers between the different recognition components are only 5 or 10 amino acid residues, far too short to enable multiple moieties of a single CAR molecule to simultaneously engage their cognate binding sites on a single gp120 subunit. In fact, our previous bispecific CAR studies demonstrated that potency was actually enhanced by deliberately rendering the linker between the two recognition moieties too short for simultaneous binding (Liu et al., 2015). An alternative possibility is that the short linkers enable a single gp120 subunit to simultaneously engage multiple CAR molecules, each by a distinct recognition component. Additional studies will be required to unravel the mechanistic complexities underlying the relationships between CAR multispecificity and potency.

The trispecific CD4-MBL-R5Nt CAR reported herein has significant advantages over the previously favored bispecific CD4-MBL CAR, namely greater anti-HIV potency (**Figure 4B**) and absence of detectable HIV entry receptor activity for the CD4 moiety (**Figure 5B**). This construct is thus a favored candidate for advancing to preclinical studies in relevant murine and simian models. Such studies will help assess the *in vivo* benefits of the trispecific design, with the goal of evaluating its potential in the HIV cure agenda.

MATERIALS AND METHODS

PBMCs and Cell Lines

Buffy coats from healthy donors were obtained with informed consent from the NIH Clinical Center Department of Transfusion Medicine under Protocol 99-CC-0168. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Hypaque grandient. Frozen aliquots were stored in Recovery Cell Culture Freezing Medium (Thermo), and when thawed, grown in an AIM-V-based media (Thermo), containing 5% human AB serum (Valley Biomedical) and recombinant human interleukin-2 (Chiron).

GP2-293 cells (Clontech), used for retroviral vector production, were cultured in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and the cells were grown at 37°C with 5% CO_2.

Chinese hamster ovary (CHO) cells and CHO cells stably expressing HIV Env (CHO-*env*) were grown in DMEM containing 10% FBS, 2 mM glutamine, 1% non-essential amino acids, and 25 mM HEPES buffer. In addition, 250 nM methotrexate (MTX) was added to the growth medium of CHO-*env* cells, which constitutively express the Env protein from HIV-1 isolate III_B (Pitts et al., 1991). All cell culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin and the cells were grown at 37°C with 5% CO_2.

Human osteosarcoma cells expressing CCR5 (HOS.CCR5) cells were obtained from the NIH AIDS Reagent Program and grown DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1:100 dilution of GlutaMAX supplement. On the day of use, 1 μg/ml of puromycin was added. The cells were grown at 37°C with 5% CO_2.

Plasmids

The CAR-encoding retroviral constructs are all based on the pMSGV-1 gammaretroviral vector (Hughes et al., 2005). Each CAR constructs shares the same hinge region, transmembrane domain, cytoplasmic signaling domains of CD28 and CD3 zeta. The extracellular recognition domains are as indicated in the CAR's designations. For the multi-specific CARs the domains are listed N- to C-terminus. All of the CD4-containing CARs contain domains 1 and 2 of human CD4 (residues 1-208, including the 25 residue signal peptide). The negative control CAR (referred to as 139 CAR), contains a single-chain variable fragment (scFv) from human MAb 139 which is specific for a variant of the epidermal growth factor (EGF) receptor only found on glioma cells (Morgan et al., 2012). All sequences are codon optimized for human expression. Gene fragments were synthesized by GenScripts, and the final constructs were stitched together by overlap extension PCR. The CD4-R5Nt(Y/A) mutant was generated from the CD4-R5Nt car using site directed mutagenesis. All primers were obtained from Thermo. Schematic representations of the CAR constructs are shown in **Figure 1**, and the corresponding amino acid sequences are presented in **Supplementary Figure 1**.

Retroviral Vector Production

The retroviral vectors carrying the gene encoding the various CARs were generated by transfection of GP2-293 cells as described previously (Kochenderfer et al., 2009). The day before the transfection the cells were plated out on poly-D (or L)-lysine coated plates in media without penicillin or streptomycin. After a brief wash antibiotic free media, Lipofectamine 2000 (Thermo) was used to transfect the cells with a 2:1 mixture of plasmids encoding the CAR and the RD114 envelope glycoprotein (Porter et al., 1996). The co-transfected cells were replenished with DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated for 48 h at 37°C in 5% CO_2. After the incubation the supernatant was collected and stored at −80°C in aliquots.

Transduction of PBMCs With Retroviral Vectors Encoding the CARs

In preparation for transduction with the CAR-bearing retroviral constructs, the PBMCs were thawed, washed with growth media and resuspending at 2×10^6 cells/ml in AIM-V containing 5% human AB serum, 300 IU/ml interleukin-2 (IL-2; Roche), and 50 ng/ml of OKT3, an anti-CD3 monoclonal antibody (Abcam). The 2 ml of the cell suspension was aliquoted into each well of a 24-well tissue culture coated plate and cultured in 5% CO_2 at 37°C for 2 days. To prepare duplicate sets of transduction plates, RetroNectin reagent (TaKaRa Bio) was diluted to 10 μg/ml in PBS and added to wells of non-tissue culture-treated 6-well plates at 1.5 ml/well and stored overnight at 4°C. The following day, aliquots of the retrovirus containing supernatant was rapidly thawed and diluted 1:1 in AIM-V supplemented with 5% human AB serum. The RetroNectin reagent was removed from the 6-well plate and replaced with 2 ml/well of a 2% bovine serum albumin

(BSA) solution in PBS and incubated at room temperature for 30 min. The wells were washed twice with PBS, 2 ml/well of the retroviral solution was added, and the plates were centrifuged at 2,000 × *g* for 2 h at 32°C.

The activated PBMCs were pooled from the wells of the 24-well plate and adjusted to 0.5 × 10^6 cells/ml in AIM-V supplemented with 5% human AB serum and 300 IU/ml of IL-2. One retrovirus coated plate was used for the first transduction, and the duplicate plate was stored at 4°C until the following day for the second transduction. After aspirating the supernatant from the first retrovirus coated 6-well plate, 3 ml of the cell suspension was added (for a total of 1. 5 × 10^6 cells/ml) and centrifuged for 10 min at 2,000 × *g* at 32°C. The plate was incubated for 24 h 37°C in 5% CO_2. The next day, the cells were pipetted up and down to gently dislodge them from the wells and transferred to the duplicate retrovirus coated plate (prepared as described above). The cells were incubated at 37°C in 5% CO_2 for up to 2 weeks in T25 flasks standing upright. For subculturing, the cells were adjusted to 0.5–1 × 10^6 cells/ml in AIM-V supplemented with 5% human AB serum and 300 IU/ml of IL-2 about every 3 days.

Analysis of CAR Expression by Flow Cytometry

Transduced cells were monitored for CAR expression starting at 3 days after the second transduction. Approximately 1 × 10^6 cells were removed from the culture and washed with 2 ml fluorescence-activated cell sorter (FACS) buffer (0.4% BSA in PBS) and resuspended in 100 ul of antibody solution diluted in FACS buffer (0.4% BSA in PBS). The antibody solution included anti-CD3-PerCP-Cy5.5 (BD Pharm), anti-CD4-APC-Cy7 (BD Pharm), anti-CD8-PE-Cy7 or anti-CD8-Alexa Fluor 700 (both from BD Pharm), and Aqua fluorescent reactive dye (LifeTech). The cells were incubated in the antibody solution at room temperature for 30 min, then washed with 2 ml FACS buffer. The cells were resuspended in 250 μl 3% paraformaldehyde diluted in PBS. The instruments used for flow cytometry acquisition were either BD FACSCalibur or FACSCanto II (BD Biosciences), and data analysis was performed using FlowJo (Treestar).

Env-Specific Activation of CAR T Cells

CHO or CHO-*env* cells were seeded into the wells of a 12-well tissue culture-treated plate at 0.3 × 10^6 cells/wells and grown overnight at 37°C in 5% CO_2. The following day the media was aspirated and 1 × 10^6 CAR T cells were added in 2 ml volume of AIM-V supplemented with 5% human AB serum to each well. Each well also received 5 μg/ml Brefeldin (BioLegend), 2 μM Monensin (BioLegend), and anti-CD107a (LAMP-1)-Brilliant Violet 785 (BioLegend). The plate was centrifuged at 1000 rpm for 2–3 min and incubated for 6 h at 37°C in 5% CO_2. After the incubation, the cells were pipetted up and down to gently resuspend the CAR T cells and collected into FACS tubes. The cells were washed with 2 ml FACS buffer and resuspended in 100 μl of the antibody mixture described previously for analysis of CAR expression. After a 30-min incubation, the cells were washed with 2 ml FACS buffer, and incubated in 200 μl Cytofix solution (BD Scientific) and incubated at room temperature for 20 min. The cells were washed with 2 ml Perm/Wash buffer (BD Scientific) then resuspended in 100 μl anti-interferon gamma-PE (BioLegend) diluted in Perm/Wash buffer. After a 15 min incubation the cells were washed with 2 ml Perm/Wash buffer, and resuspended in 250 μl 3% paraformaldehyde diluted in PBS. Data was acquired using either BD FACSCalibur or FACSCanto II (BD Sciences), and data analysis was performed using FlowJo (Treestar).

Inhibition of Spreading of Primary HIV-1 Infection by CAR-T Cells

To prepare targets cells for the infection, an aliquot of autologous PBMCs were rapidly thawed, washed and resuspended at 2 × 10^6/ml in AIM-V containing 5% human AB serum, 300IU/ml IL-2, and 5 μg/ml phytohemagglutinin (PHA; Sigma). The cells were added to wells of a 24-well tissue culture-treated plate at 2 ml/well and incubated at 37°C in 5% CO_2 for 1 day. Subsequently, the cells were pooled, washed in PBS and resuspended at a density of 0.5–1 × 10^6 cells/ml in AIM-V supplemented with 5% human AB serum and 300 IU/ml of IL-2. The cells were incubated in an upright T25 flask at 37°C in 5% CO_2 for 3 days.

In preparation for infection with HIV, 10 million cells the cells were washed with PBS and resuspended in AIM-V complemented with 5% human AB serum and mixed with 1 ml volume of primary HIV-1 isolate stock (p24 titer of 50–150 ng/ml). The final volume is 4 ml including 30 IU/ml of IL-2 in an upright T25 flask. The cells were incubated at 37°C in 5% CO_2 for 1 day. The following day the infected "target" cells were washed three times with an excess volume of PBS and resuspended at 1 × 10^6/ml in AIM-V containing 5% human AB serum, 30 IU/ml IL-2. The CAR-expressing "effector" cells (see "Transduction of PBMCs with retroviral vectors encoding the CARs" section), were washed with PBS and also resuspended at 1 × 10^6/ml in AIM-V containing 5% human AB serum, 30 IU/ml IL-2. The effector cells were serially diluted in 1:5 dilutions in the wells of a 96-well round bottom tissue culture-treated plate, so that the most concentrated well contained 100,000 cells in 100 μl volume. Subsequently, 100 μl of target cells were added to each well. The resulting co-cultures contain effector-to-target ratios of 1:1, 0.2:1, 0.04:1, and 0.008:1 in a 200 μl volume. The plate was incubated at 37°C in 5% CO_2 for up to 19 days. At 2 or 3 day intervals 180 ul of the supernatant was aspirated and saved for analysis. To monitor the level of infection in the wells, the p24 content of the supernatant was determined by HIV p24 AlphaLISA Detection Kit (Perkin Elmer). The signals were evaluated using an EnSpire Multimode Plate Reader (Perkin Elmer).

Susceptibility of CAR-Expressing HOS.CCR5 Cells to Pseudovirus Entry

HOS cells expressing CCR5 were seeded out in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), and 1:100 dilution of GlutaMAX supplement to be confluent the following day. On the following day, the media was replaced with fresh media and the cells were transfected with the various CAR-expression constructs described above using Lipofectamine 2000 transfection reagent. After transfection the media was replaced with DMEM containing 10% heat-inactivated fetal bovine serum

(FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1:100 dilution of GlutaMAX supplement, as well as 1 μg /ml of puromycin and incubated at 37°C in 5% CO_2 for 48 h. After the incubation the cells were harvested from the transfection plates using CellStripper Dissociation Reagent (Thermo) and washed. About 0.5×10^6 cells were removed and stained with anti-CD4-APC-Cy7 (BD Pharm) and Aqua fluorescent reactive dye (LifeTech) and analyzed on a flow cytometer to monitor expression levels of the CD4-containing CARs. The cells were added to wells of a 96-well flat bottom plate at 3.5×10^6 cells/well. Pseudovirus stocks with Env either from Ba-L or YU2 primary isolates and carrying a luciferase (Luc) gene were diluted 1:5 or 1:25 and added to the cells. The wells also contained 20 μg/ml DEAE-dextran. The plates were incubated at 37°C in 5% CO_2 for 48 h. After the incubation the plates were centrifuged at 1,500 rpm for 5 min and the media was aspirated. The cells were evaluated for luciferase production using the Bright-Glo Luciferase Assay System (Promega), and the plates were read on an Ensight Multimode Plate Reader (Perkin Elmer).

Biosecurity and Institutional Safety Procedures

All experiments and samples with infectious HIV were performed and maintained in the Biosafety Level 3 laboratory with Biosafety Level 2 practices, in accordance with Building 33 NIAID requirements. All other procedures were conducted according to NIH/NIAID standard biosecurity and safety regulations.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AH contributed to design of CAR constructs and experiments, conduct of experimental work including production of CAR constructs and retroviral vectors, HIV/CAR-T cell coculture assays, pseudovirus entry assays, analysis of data, and drafting of manuscript at multiple stages. DD contributed to conduct of experimental work including production of CAR constructs and retroviral vectors and analysis of data. DE contributed to conduct of experiments including production of CAR constructs and retroviral vectors and analysis of data. VB contributed to conduct of experimental work including production of CAR constructs and retroviral vectors, and HIV/CAR-T cell coculture assays and analysis of data. AS contributed to experimental work including production plasmids and corresponding soluble proteins and analysis of data. EB oversaw all aspects of CAR design, characterization of CAR expression and function in coculture assays, obtained funding, and contributed to drafting of this manuscript at multiple stages.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00242/full#supplementary-material

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REVIEW
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The Use of Toll-Like Receptor Agonists in HIV-1 Cure Strategies

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Toll-like receptors (TLRs) are a family of pattern recognition receptors and part of the first line of defense against invading microbes. In humans, we know of 10 different TLRs, which are expressed to varying degrees in immune cell subsets. Engaging TLRs through their specific ligands leads to activation of the innate immune system and secondarily priming of the adaptive immune system. Because of these unique properties, TLR agonists have been investigated as immunotherapy in cancer treatment for many years, but in recent years there has also been growing interest in the use of TLR agonists in the context of human immunodeficiency virus type 1 (HIV-1) cure research. The primary obstacle to curing HIV-1 is the presence of a latent viral reservoir in transcriptionally silent immune cells. Due to the very limited transcription of the integrated HIV-1 proviruses, latently infected cells cannot be targeted and cleared by immune effector mechanisms. TLR agonists are very interesting in this context because of their potential dual effects as latency reverting agents (LRAs) and immune modulatory compounds. Here, we review preclinical and clinical data on the impact of TLR stimulation on HIV-1 latency as well as antiviral and HIV-1-specific immunity. We also focus on the promising role of TLR agonists in combination strategies in HIV-1 cure research. Different combinations of TLR agonists and broadly neutralizing antibodies or TLRs agonists as adjuvants in HIV-1 vaccines have shown very encouraging results in non-human primate experiments and these concepts are now moving into clinical testing.

Keywords: HIV-1, TLR, innate immunity, HIV-1 cure, HIV-1 vaccine, latency reversing agents, immune modulation

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection can today be completely suppressed by antiretroviral therapy (ART). However, during the early phase of primary HIV-1 infection, the virus establishes a reservoir in infected long-lived immune cells, which necessitates life-long ART in order to prevent disease progression (1, 2). This latent HIV-1 reservoir is predominantly found in long-lived memory CD4+ T cells in which the provirus is transcriptionally silent and thus go undetected by the host immune system (3, 4). These proviruses can be (re)activated and go on to transcribe and form infectious particles, leading to re-emergence of active infection if ART is stopped. The HIV-1 reservoir is believed to be maintained through the long-lived nature and homeostatic proliferative capabilities of the latently infected memory T cells (5, 6).

The innate immune system constitutes a vital part of the early defense against infections and in controlling established HIV-1 infection. Therefore, strategies aimed at boosting innate immunity have gained great interest in HIV-1 cure research. Endogenous interferon production induced by activation of Toll-like receptors (TLRs) is one of the innate immune'system's first antiviral

responses upon infection. TLRs are in the family of pattern recognition receptors (PRRs) which detect pathogen-associated molecular patterns (PAMPs) (7, 8). TLRs also respond to signs of host cell damage through ligands called damage-associated molecular patterns (DAMPs) (9).

TLRs are expressed on many different immune cells including natural killer (NK) cells, macrophages, B cells, and to a high degree on dendritic cells (DCs) (10) (**Table 1**). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are located in the membrane of the intracellular endosomes (8, 14, 20). This partition in localization reflects the disparate pathogen-sensing function of these two groups. The cell surface-associated TLRs are mainly responsible for detecting components from extracellular microbes such as bacteria and fungi, whereas the TLRs in the endosomal compartment mainly detect virus and intracellular bacteria (18, 22, 23) (**Table 1**). TLRs are transmembrane proteins consisting of three different domains: an ectodomain consisting of leucine-rich-repeats (LRR) mediating ligand recognition, a transmembrane domain, and the cytosolic domain Toll/IL-1R (TIR), which mediates downstream signaling (18). The cytosolic domain can, upon activation of the TLR, recruit different domain-containing adaptor molecules such as myeloid differentiation primary-response protein 88 (MyD88), TIR-domain containing adaptor protein (TIRAP)] also called MyD88 adaptor-like (MAL), TIR-domain containing adaptor protein inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) (23–25). The MyD88 signaling pathway used by all of the TLRs, except for TLR3, activates nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) leading to the production of pro-inflammatory cytokines. The TRIF–dependent pathway used by TLR3 and TLR4 leads to induction of type 1 interferons (IFNs) in addition to pro-inflammatory cytokines (18, 20, 23). The cytokine induction initiated by TLR activation not only triggers an innate immune response, but also takes part in initiating and shaping the adaptive immune response (22, 26).

The present review focuses on the emerging potential of synthetic TLR agonist treatment in HIV-1 cure research both alone and in combination with other interventions.

TABLE 1 | A schematic outline of TLR localization, expression on immune cells, receptor complex formations, ligands, recruited TIR domain-containing adaptor molecules, and cytokine outcomes.

TLR type	Cell type	Receptor-complex	Ligand	TIR-adaptor	Outcome
(A) TLRs on cell surface					
TLR1	Monocytes DCs T cells	1–2	- Lipopeptides from bacteria and mycobacteria	MyD88	Pro-inflammatory cytokines
TLR2	Monocytes Macrophages DCs	2–1 2–2 2–6 2–10	- Components from the cell wall of gram-positive bacteria - Glycoprotein from virus - Zymosan from fungi	MyD88	Pro-inflammatory cytokines
TLR4	Monocytes DCs	4-MD2	- Lipopolysaccharides from gram-negative bacteria - Envelope components of respiratory syncytial virus	MyD88 TRIF TRAM TIRAP/MAL	Pro-inflammatory cytokines IFNs
TLR5	Monocytes T cells	5–5	- Flagellin from flagellated bacteria	MyD88	Pro-inflammatory cytokines
TLR6	Monocytes Macrophages B cells	6–2	- Lipopeptides from Mycoplasma	MyD88	Pro-inflammatory cytokines
TLR10	B-cells	10–2	- Ligands from Listeria - Ligands from Influenza A		
(B) TLRs in endosomal compartments					
TLR3	DCs	3-3	- Viral double-stranded RNA - Self-RNA from damaged cells	TRIF	Pro-inflammatory cytokines IFNs
TLR7	pDCs B cells	7-7	- Viral single-stranded RNA	MyD88	Pro-inflammatory cytokines IFNs*
TLR8	Monocytes DCs	8-8	- Viral single-stranded RNA - Bacterial RNA	MyD88	Pro-inflammatory cytokines
TLR9	pDCs B cells	9-9	- CpG containing DNA from bacteria and virus - Self-DNA from damaged cells - Hemozoin from Plasmodium Falciparum	MyD88	Pro-inflammatory cytokines IFNs*

**IFNs through MyD88.*

The table is a summary of TLR properties described previously (11–20). It should be noted that some immune cells subsets and ligands have been left out for the sake of overview and relevance to the subjects at hand. TLR expression on epithelial cells have been left out of ***Table 1*** *as well, but have been reviewed elsewhere (21).*

TLR AGONISTS AS LATENCY REVERSING AGENTS (LRA)

The latent HIV-1 reservoir constitutes the main barrier to a cure for HIV-1 infection (27, 28). One of the proposed strategies toward overcoming this obstacle is the "shock and kill" approach (28, 29). The hypothesis behind this strategy is that LRA administration will (re)activate HIV-1 transcription in latently infected cells, leading to expression of viral antigens on their surface. This will in turn expose infected cells to immune-mediated killing and/or apoptosis due to viral-cytopathic effects while concurrent ART prevents released virions from infecting other immune cells (30).

A widely investigated group of LRAs is the histone deacetylase inhibitors (HDACi) including romidepsin, vorinostat, panobinostat, and chidamide (31–33). Inhibition of the histone deacetylase enzymes leads to a more accessible chromatin structure, thus enabling transcription of the latent proviral HIV-1 DNA (34, 35). While HDACi are now being investigated in combination with HIV-1 vaccines (36), broadly neutralizing antibodies (bNAbs) (www.clinicaltrials.gov: NCT03041012), and other LRAs, none of these HDACi alone have so far been capable of inducing a substantial reduction of the HIV-1 reservoir in clinical trials (37).

Protein kinase C (PKC) agonists such as bryostatin and prostratin (38) have shown significant latency reversal activity *ex vivo* but may be too toxic to be dosed at active concentrations in humans (39). However, the latency reverting effects of a natural plant extract containing ingenols, yet another group of PKC agonist, is currently under being tested in a clinical trial in HIV-1 infected individuals (NCT02531295). Disulfiram, a drug used for alcohol cessation, has also been tested in clinical trials as a potential LRA (40). Disulfiram induced increased levels of cell-associated unspliced HIV-1 RNA (usRNA) in study participants of three different dosing groups, but it did not lead to significant changes in either total HIV-1 DNA or plasma levels of HIV-1 RNA. The latency reversing properties of other compounds such as cytokines and other epigenetic modifiers have similarly been investigated (41, 42). However, the discovery of a single therapeutic capable of inducing significant HIV-1 reservoir alterations is still to be made.

The first TLR agonist to draw attention to the potential utilization of TLR agonists as LRAs was that of the antisense oligodeoxynucleotide (ODN) phosphorothioate Gene-Expression Modulator 91 (GEM91). GEM91 was initially shown to inhibit HIV-1 replication *ex vivo* in human peripheral blood mononuclear cells (PBMCs) from HIV-1 infected donors (43). Unexpectedly, a subsequent GEM91 dose-escalation study showed increased viremia following administration in HIV-1 infected individuals contradictory of the *ex vivo* findings (44). It was later discovered that this potential induction of viremia was due to a CpG motif in GEM91 leading to TLR9 stimulation. Thus, it was proposed that the increased viremia was caused by innate immune activation and concomitant HIV-1 (re)activation (45–47).

Several TLR agonists have since been investigated as LRAs because of their ability to induce immune activation, and in doing so, causing (re)activation of silent HIV-1 in latently infected cells and boosting the antiviral immune response. These mechanisms are elaborated in the section "Immunomodulatory properties of TLR agonists."

Ex vivo Experiments

Utilizing the optimal cell model for assessment of latency reversal is of great importance and should consider the type of LRA investigated. The majority of *in vitro/ex vivo* LRA experiments focuses on latently infected T cell lines or primary T cells. These cell models work well when investigating LRAs such as HDACi which (re)activate HIV-1 transcription by a direct impact on the target cell. Yet, most TLRs are not expressed at physiological levels on CD4+ T cells, which is why this lymphocyte subset is often unresponsive to direct TLR stimulation (e.g., by TLR7 or TLR9 agonists) (**Table 1**). Instead, these TLR agonists induce HIV-1 transcription indirectly through activation of innate immune cells such as DCs (**Table 2**) (18, 22, 23, 54).

This issue was highlighted in a study by Novis et al. who tested a broad panel of TLR agonists as LRAs (48). In a central memory CD4+ T cell (T_{CM}) model, they found that only the TLR1/2 agonist Pam3CSK4 was able to significantly increase HIV-1 transcription, measured by intracellular p24 Gag protein expression, after stimulating the cells for 3 days (48). Subsequently, the latency reversing properties of Pam3CSK4 were tested *ex vivo* utilizing T_{CM} isolated from aviremic HIV-1 infected donors. They found that HIV-1 transcription increased in two out of seven donors after Pam3CSK4 stimulation compared to five out of seven after panobinostat stimulation, measured by the level of usRNA. Pam3CSK4 was demonstrated to (re)activate HIV-1 transcription via an NF-κB and NFAT-dependent pathway, but without induction of IFNs, thereby avoiding T cell activation (CD69+ and CD25+) and proliferation (cell proliferation dye assessment). However, the degree of HIV-1 (re)activation induced by Pam3CSK4 was lower than that of panobinostat.

In 2018, Kaur et al. conducted a comprehensive investigation of the LRA properties of agonists of 6 different TLRs(1/2, 3, 4, 5, 7, and 8) on both PBMCs and isolated CD4+ T cells from HIV-1 infected donors on long term ART (49). All TLR agonists were able to induce a modest but statistically significant fold-increase of supernatant HIV-1 RNA from PBMCs compared to vehicle controls. In isolated CD4+ T cells however, only the TLR1/2 agonist Pam3CSK4 was able to significantly increase the supernatant HIV-1 RNA level. These findings are in line with the findings of Novis et al. and indicate TLR1/2 expression on CD4+ T cells.

Thibault et al. tested the direct HIV-1 latency reversing properties of the TLR5 agonist flagellin on T_{CM} (50), which in a previous study showed indications of TLR5 expression (55) (**Table 1**). Resting T_{CM} isolated from healthy donor PBMCs were infected with luciferase-encoding pseudotyped HIV-1 particles. When treated with flagellin, the isolated T_{CM} showed enhanced HIV-1 gene expression compared to mock-treated controls, assessed by fold increase of HIV-1 long terminal repeat-driven luciferase activity. However, when flagellin stimulation

TABLE 2 | Schematic overview of *ex vivo* experiments investigating the utility of TLR agonists as LRAs included in the manuscript.

References	TLR agonist	Cell type	Study design	Endpoint	Results
Novis et al. (48)	1) 1/2, 2/6, 3, 4, 5, 7, 7/8, 9	1) T_{CM} cell model	1) *In vitro* stimulation	1) Intracellular p24 Gag protein expression	1) Only the TLR1/2 agonist induced significant increase (but the T_{CM} cell model does not express most TLRs)
	2) 1/2	2) T_{CM} from aviremic HIV-1 infected donors	2) *Ex vivo* stimulation	2) Intracellular level of usRNA	2) Significant increase in 2 of 7 donor samples (5 of 7 for panobinostat)
Kaur et al. (49)	1) 1/2, 3, 4, 5, 7, 8	1) PBMCs from aviremic HIV-1 infected donors	1) *Ex vivo* stimulation	1) Supernatant HIV-1 RNA level	1) All TLR agonists induced significant increase
	2) 1/2, 3, 4, 5, 7, 8	2) CD4+ T cells from aviremic HIV-1 infected donors	2) *Ex vivo* stimulation	2) Supernatant HIV-1 RNA level	2) Only TLR ½ agonist Pam3CSK4 induced significant increase
Thibailt et al. (50)	1) 5	1) Resting T_{CM} from healthy donor PBMCs, infected with luciferease-encoding pseudotyped HIV-1	1) *Ex vivo* stimulation	1) HIV-1 long terminal repeat-driven luciferase activity	1) Increase
	2) 5	2) resting CD4+ T cells from aviremic HIV-1 infected donors	2) *Ex vivo* stimulation	2) Intracellular p24 Gag protein level	2) No increase
Tsai et al. (51)	1) 7	1) PBMCs from aviremic HIV-1 infected donors	1) *Ex vivo* stimulation	1) Supernatant mean HIV-1 RNA levels	1) Increase
	2) 7	2) PBMCs from aviremic HIV-1 infected donors, treated with antibodies against IFNAR on T cells	2) *Ex vivo* stimulation	2) Supernatant mean HIV-1 RNA levels	2) No increase
	3) 7	3) CD4+ T cells from aviremic HIV-1 infected donors	3) *Ex vivo* stimulation	3) Supernatant mean HIV-1 RNA levels	3) No increase
Bam et al. (52)	1) 7	1) PBMCs from healthy donors	1) Pre-stimulating with GS-9620 for 2 days prior to infection with a luciferase reporter virus containing HIV-1	1) HIV-1 replication	1) Inhibition
	2) 7	2) CD4+ T cells from healthy donors	2) Pre-stimulating with GS-9620 for 2 days prior to infection with a luciferase reporter virus containing HIV-1	2) HIV-1 replication	2) No inhibition
Offersen et al. (53)	1) 9	1) PBMCs from aviremic HIV-1 infected donors	1) *Ex vivo* stimulation	1a) Level of usRNA in CD4+ T-cells extracted from PBMCs post stimulation	1a) Increase
				1b) Level of IFN-α in cell supernatant	1b) Increase

The studies all have several endpoints in their study setup. The most relevant endpoints in the setting of this review have been outlined as (1), (2), and (3) for overview.

was tested on resting CD4+ T-cells from virally suppressed HIV-1 infected donors, no induction of intracellular p24 Gag protein was detectable. The results of Thibault et al. are in accordance with those of Novis et al. and Kaur et al., but raise uncertainty regarding the physiological effects of the previously indicated TLR5 expression on T cells.

Tsai et al. demonstrated that GS-9620, a TLR7 agonist, was capable of (re)activating latent HIV-1 in PBMCs from HIV-1 infected donors after 4 days of stimulation, measured as a 1.6-fold increase in mean HIV-1 RNA levels in the cell supernatant compared to vehicle-treated controls (51). This effect was mediated by activation of plasmacytoid dendritic cells (pDCs) leading to an IFN-driven CD4+ T cell activation (CD69+), which was demonstrated by the lack of HIV-1 RNA production when PBMCs were treated with antibodies against interferon-α receptors (IFNAR) on T cells. No changes in supernatant HIV-1 RNA was found when performing direct stimulating of pure CD4+ T-cells, which is consistent with the above-mentioned inconsiderable TLR7 expression on human T-cells. In corroboration with these findings, Bam et al. demonstrated inhibition of HIV-1 replication in PMBCs pre-stimulated with GS-9620 for 2 days prior to infection with a luciferase reporter virus containing HIV-1 (52). This antiviral effect was not observed when isolated CD4+ T cells or macrophages were equivalently pre-stimulated.

As briefly described above, synthetic ODNs containing CpG motifs were the first version of the TLR9 agonists and were mainly investigated in the context of cancer treatment (56). The structurally different TLR9 agonists such as MGN1703 were later developed to improve the tolerability of the drug class (57). Our group investigated the "shock and kill" properties of the TLR9 agonist MGN1703 on PBMCs from HIV-1 infected individuals on ART (53). MGN1703 stimulation led to increased levels of usRNA in CD4+ T-cells after 16 h. This effect was mediated indirectly by activated pDCs secreting IFNs, inducing T-cell activation (CD69+). MGN1703 did however not increase the usRNA levels to the same extent as panobinostat.

Lastly, other studies have also suggested that agonists of TLR2/7 and TLR8 possess latency reversing properties *ex vivo* and *in vivo* (58, 59). Extensional investigation of latency reversing properties of TLR agonists utilizing cell lines and mouse models have been reviewed elsewhere (25, 60, 61).

The findings of the preclinical studies presented here suggest that while some TLRs are present on T cells, the physiological effect of TLR-mediated (re)activation of latent HIV-1 in transcriptionally quiescent memory T cells occur in large by means of DC activation and subsequent IFN-α stimulation of T cells. Therefore, the full immune stimulating or latency reversing effect of synthetic TLR agonists can often not be evaluated by utilizing single immune cell models (**Table 2**).

Non-Human Primate (NHP) Studies

Evaluating pre-clinical *in vivo* studies investigating the therapeutic potential of TLR agonists in HIV-1 cure research, the present review will be focusing on NHP studies, as the TLR expression on immune cells of NHPs bare the closest resemblance to that of human immune cells, compared to smaller animal models (62, 63).

Of the TLR agonists investigated *ex vivo* as described above, only agonists of TLR3, TLR7, and TLR9 have progressed into clinical testing as potential LRAs in the field of HIV-1 cure research. However, TLR3 agonist testing quickly progressed from small animal models into clinical trials in advanced cancer disease and its LRA potential has not been assessed in NHPs (64–67). The majority of the preclinical work concerning TLR9 agonists have similarly been conducted in the field of cancer treatment and is thus beyond the scope of this review but has been described elsewhere (68).

Two different groups have investigated the potential of TLR7 agonists as LRAs *in vivo* in NHP. Lim et al. showed that the TLR7 agonist GS-986, was capable of inducing transient simian immunodeficiency virus (SIV) plasma RNA blips of up to 1,000 copies/mL, after 24 to 48 h (69). GS-986 was administered in escalating doses (0.1–0.3 mg/kg) by oral gavage and led to SIV RNA blips in 4 out of 4 virally suppressed rhesus macaques on ART, compared to 0 of 6 vehicle-treated controls. The blips were seen after dose 4, 5, 6, and 7, but not after the first 3 doses, which could indicate a priming effect of the TLR agonist. T, NK, and B cells were transiently activated within 24 to 48 h following stimulation and then returned to baseline. In the TLR7 agonist treated NHPs, the viral reservoir was reduced by an average of 75% as measured by total SIV DNA in memory CD4+ T cells [isolated from PBMCs, mononuclear cells from lymph node (LNMCs) and gastrointestinal mucosa (GMMCs)] compared to no change in total SIV DNA among control animals. However, there was no difference in time to viral rebound between the GS-986-treated group and the control group following ART cessation.

In a subsequent experiment, 9 NHPs, received 10 administrations of either GS-986 (0.1 mg/kg) or GS-9620 (0.05 or 0.15 mg/kg) followed by a 3-months resting period and then another 9 doses. 2 NHPs constituted a vehicle-control group. All TLR7 agonist-treated NHPs experienced blips in plasma SIV RNA levels during the first interventional period, but during the second interventional period, only 1 viral blip was measured, even though the activation level of T, NK and B cells was comparable between the two dosing periods. The reason for this variation is not clear but it could indicate increasing immune tolerance to TLR7 activation following repeated stimulation. Total SIV DNA was however again significantly reduced in both PBMCs and GMMCs in a combined analysis of all TLR7-agonists-treated groups compared to controls. In a modified viral outgrowth assay, the majority of TLR7-treated NHPs displayed a reduction in the inducible SIV reservoir. Two of the NHPs had no inducible SIV reservoir and showed durable control for more than 700 days after interruption of ART. Subsequent CD8+ T-cell depletion did not lead to emergence of virus, and neither did adoptive transfer of PBMCs and LMMCs to naïve macaques, suggesting a complete eradication of the viral reservoir.

Surprisingly, Del Prete et al. were unable to reproduce the findings of Lim et al. (70). Their study also included SIV-infected rhesus macaques that received 12 doses (0.15 or 0.5 mg/kg) of GS-9620, administered by oral gavage. No spikes in plasma SIV RNA levels were observed and no significant changes in transcriptional RNA/DNA ratios in PBMCs, LNMCs, or GMMCs were detected at 24 and 48 h post-dosing. All NHPs rebounded within 4 weeks upon ART cessation. CD4+ T cells showed no increased activation but at 24 h post-dosing CD8+ T cells had increased co-expression of CD38 and HLA-DR.

The cause of the difference in LRA effect and outcome in the two comparable NHP studies is unclear. In the study by Lim et al., ART was initiated on day 65 of infection and continued for around 437 days before the intervention, whereas Del Prete et al. initiated ART on day 13 and waited 525 days before intervening. The timing of ART initiation and/or of intervention might affect the viral reservoirs differently which could contribute to the discrepant findings. NHPs were infected intrarectally with SIVmac251 in the study of Lim et al., and intravenously with SIVmac239X in the study of Del Prete et al., which could also lead to varying reservoir properties.

Collectively, these findings indicate that the latency reversing effect of TLR7 agonists in NHPs might be very sensitive to alterations in model conditions such as timing of treatment initiation in relation to the course of infection, immune status, and SIV/SHIV challenge strain. The measured effects of the treatment may also depend greatly on the administered dose, dosing intervals, and timing of sampling.

Clinical Trials

The first clinical trial investigating the effects of a TLR3 agonist in HIV-1 infected individuals on long-term ART was recently published by Saxena et al. (71). The synthetic double-stranded RNA polyinosinic:polycytidylic acid (poly-I:C) and its more stabilized form containing poly-L-lysine (poly-ICLC) have previously been tested in human cancer studies (67, 72). Saxena et al. tested poly-ICLC on 12 HIV-1 infected donors in doses of 1.4 mg administered subcutaneously once daily on two consecutive days. Follow-up measurements were obtained on day 4 and 8 and at week 4, 16, and 48. During the study period, poly-ICLC treatment did not affect the level of usRNA or total HIV-1 DNA in CD4+ T-cells. In addition, no significant changes in activation of DCs (CD40+CD83+CD86^{+}) or T cells (HLA-DR+CD38+) were observed, except for a significant upregulation of CD38 expression on CD8+ T-cells at day 4, which normalized at day 8. The authors speculated that higher poly-ICLC doses, more frequent administration, or combinations with other therapeutics might be needed to achieve a robust immunological impact.

Assessing the safety of the TLR7 agonist GS-9620, Riddler et al. recently reported findings from a phase I dose escalation placebo-controlled study including 48 HIV-1 infected individuals on ART (NCT02858401) (73). In 6 treatment groups, GS-9620 doses ranging from 1 to 12 mg GS-9620 were administered every other week for up to 19 weeks. GS-9620 was generally well-tolerated, even in the 12 mg group, and there were no discontinuations due to adverse events. There was however no evidence of GS-9620 effectively impacting the latent HIV-1 reservoir as no consistent changes were observed in levels of plasma HIV-1 RNA, usRNA or total HIV-1 DNA in CD4+ T cells between the different groups. Another ongoing study with this compound is assessing the safety and efficacy of GS-9620 in ART-treated HIV-1 infected controllers, defined as individuals having a pre-ART viral load between 50 and 5,000 copies/mL (NCT03060447).

Since 2007, our group has been working with TLR9 agonists both as vaccine adjuvant and immune stimulator in HIV-1 infection. We conducted a pilot study in HIV-1 infected individuals whom received 60 mg of TLR9 agonist MGN1703, administered subcutaneously twice weekly over 4 weeks (74). During the 4-week intervention period, 6 of the 15 participants had detectable plasma HIV-1 RNA levels in the range of 21–1,571 copies/mL. This suggested a moderate latency reversing effect of MGN1703, but no reduction was observed in the proviral reservoir size assessed by total and integrated HIV-1 DNA in CD4+ T cells. No changes in levels of replication-competent proviruses were detected either. Both pDCs and T cells were activated as demonstrated by increased expression of the co-stimulatory markers CD40 and CD86 on pDCs and CD38 and HLA-DR on T cells. Surprisingly, the level of transcriptionally active CD4+ T cells, measured as cell-associated usRNA levels, decreased significantly during the 80-days follow-up period.

Based on these favorable immunological findings, an extension of the this study was conducted during which MGN1703 was administered twice weekly for 24 weeks to 12 HIV-1 infected participants (75). The prolonged intervention did, however, not reduce the size of the viral reservoir, assessed by measurements of total HIV-1 DNA or replication competent virus in a viral outgrowth assay. One participant (ID113) was able to control viral replication to undetectable levels for 150 days upon ART cessation. Immunological control in this individual was shown to be mediated in part by a superior level of HIV-1-specific CD8+ effector memory T cells, compared to the other study individuals. Of note, the IgG neutralization capacity of ID113 had increased during the MGN1703 treatment, supporting beneficial effect of the TLR agonist on adaptive immunity and in particular on B cell maturation (76).

Although several *ex vivo* and some *in vivo* studies have demonstrated encouraging results regarding the latency reversing effect of TLR agonists, there are no reproduceable data showing significant impact on the HIV-1 reservoir in clinical trials following TLR agonist treatment. A potential issue of LRAs as part of a HIV-1 cure strategy is that the focus is on latently infected CD4+ T cells as the main target of the latency reversal. While infected memory CD4+ T cells constitutes a long-lived HIV-1 reservoir, other cell types have been shown to harbor replication competent virus such as monocytes, macrophages, and dendritic cells (77, 78). Additionally, infected cells reside in other compartments than the blood including the lymph nodes and gut associated lymphoid tissue, and potentially also reside in the brain, the genital tract, and the lungs (79–84). However, the latter compartments are notoriously difficult to sample in clinical trials.

Collectively, based on the current knowledge we believe there is evidence for latency reversal following TLR (particularly TLR9) agonist treatment in HIV-1-infected individuals. However, TLR agonists' potency as LRAs appear to be relatively modest but as we outline below, certain TLR agonists are potent immune stimulators and it is in this capacity that they may have the biggest role to play in HIV-1 cure strategies.

IMMUNOMODULATORY PROPERTIES OF TLR AGONISTS

Upon TLR activation of DCs, proinflammatory cytokines such as IL-12 and IFNs are secreted, leading to auto- and/or paracrine activation of immune cells including other DCs, macrophages, NK cells, and T cells (85, 86). Activation of the DCs additionally leads to a downregulation of the inflammatory chemokine receptor CCR6 on the DC surface and upregulated expression of the lymphoid-homing receptor CCR7. This triggers DC migration from tissues to lymph nodes where they can present antigen to T and B cells and thus mediate an adaptive immune response (22, 87, 88). IFNα-α, produced mainly by pDCs activates both CD4+ and CD8+ T cells via their interferon alpha receptor (IFNAR) (51, 89, 90). Krieg et al. demonstrated a significantly increased frequency of antigen specific CD8+ T cells (0.07–3.00%) in 8 of 8 melanoma patients receiving a melanoma antigen vaccine adjuvanted with CpG ODNs compared to eight control patients receiving the vaccine alone (91). Accordingly, several clinical studies from our group have demonstrated HIV-1 (re)activation through an IFNα-α induced CD4+ T-cell activation and increased HIV-1-specific polyfunctionality of

CD8+ T cells following TLR9 stimulation (74, 75, 92). Similar effects have been demonstrated *ex vivo* and *in vivo* in TL7TLR7 agonist studies (51, 93, 94).

NK cells can mediate direct killing upon interaction with foreign pathogens or cells exhibiting signs of stress, but they are also the primary effector cells in mediating antibody-dependent cellular cytotoxicity (ADCC) via their Fcγ receptor (CD16) (26, 95). The cytotoxic effect is executed through the release of perforins which allows secreted cytotoxic granzymes to enter the targeted cell (96). The analogous anti-viral effector function of activated macrophages is that of phagocytosis, where the macrophage will devour entire infected cells (96). This effect can, like ADCC, be enhanced by antibodies and is thus called antibody-dependent cellular phagocytosis (ADCP). Both macrophages and NK cells are important in clearing HIV-1 infected cells (97, 98), and TLR agonists can thus work to increase their effector functions both directly through TLRs expressed on macrophages and NK-cells (**Table 1**) and indirectly via activation of B-cells and DCs (**Figure 1**).

Our group demonstrated enhanced NK cell function following *ex vivo* MGN1703-stimulation of PBMCs from HIV-1-infected individuals on long-term ART (53). The cytotoxic ($CD56^{dim}$ CD16+) and cytokine-producing ($CD56^{bright}$ CD16±) NK cells showed a significant 7.5- and 2.2-fold increase in CD69-expression, respectively, after 48 h of stimulation. Compared to unstimulated NK cells, MGN1703-stimulated

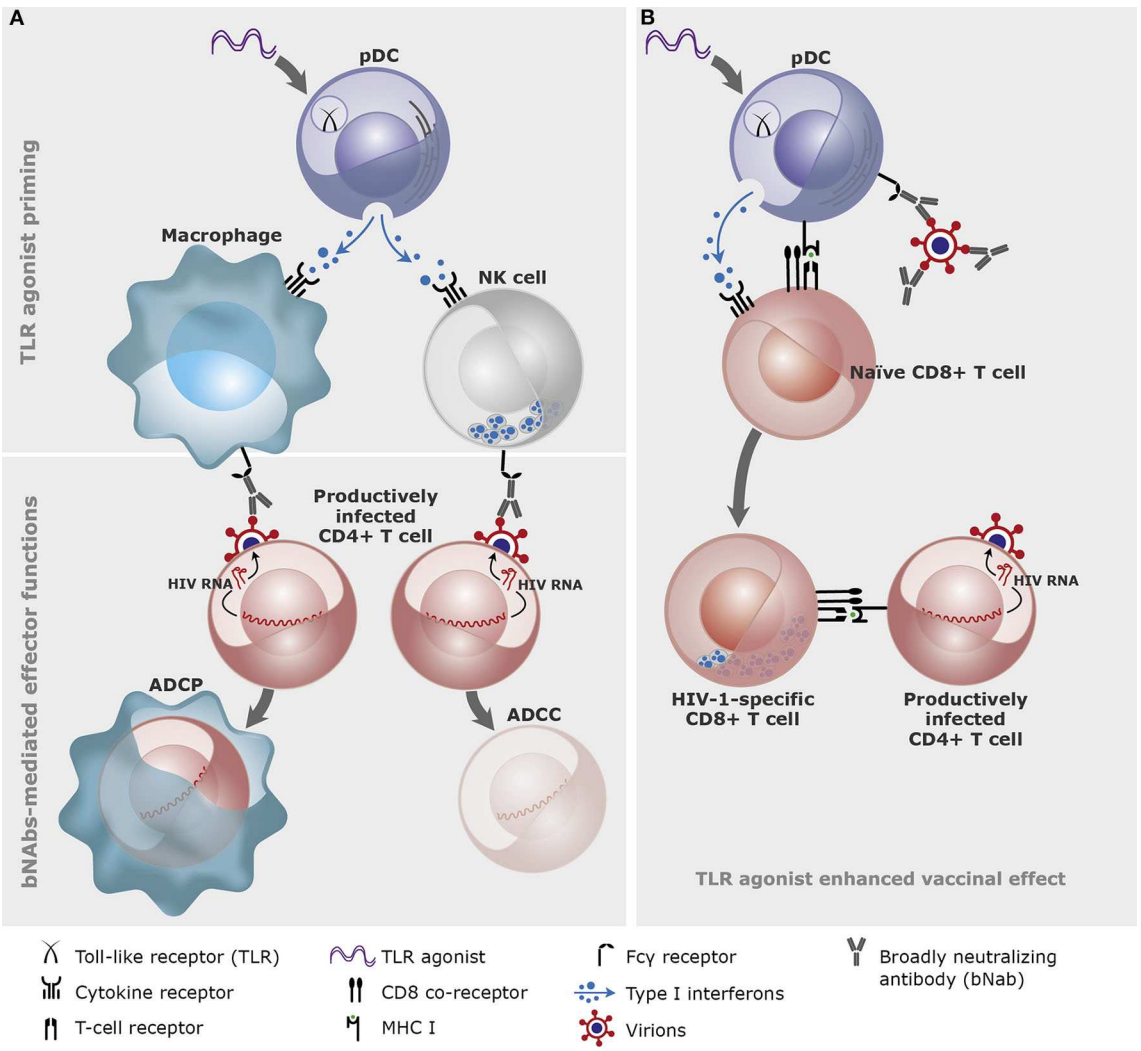

FIGURE 1 | A conceptual illustration of the effects of Toll-like receptor (TLR) agonists and broadly neutralizing antibodies (bNAbs) in combination. **(A)** TLR agonist priming of innate immune cells through plasmacytoid dendritic cells (pDC). The primed innate immune cells (here depicted macrophage and natural killer (NK) cell) bind the broadly neutralizing antibodies via the Fcγ receptors and mediate antibody-dependent cellular phagocytosis (ADCP) or cytotoxicity (ADCC) of the productively infected CD4+ T cells. **(B)** TLR agonists and bNAbs-antigen complexes cross-prime CD8+ T cells. TLR agonists and bNAbs-antigen complexes bind to pDCs which cross-presents viral antigens on the MHC class I molecule to the naïve CD8+ T cells leading to development of HIV-1-specific CD8+ T cells (graphics: Gitte Skovgaard Jensen, AUH).

NK cells additionally showed enhanced inhibition of HIV-1 production from autologous infected CD4+ T cells. Tsai et al. similarly demonstrated increased levels of activated NK cells (both $CD56^{dim}$ CD16+ and CD56+ CD16-) upon GS-9620 stimulation of PBMCs from HIV-1-infected donors on long-term ART (51). Thus, both TLR7 and TLR9 agonists have demonstrated NK cell-activating properties *ex vivo* which have subsequently been confirmed *in vivo* (69, 70, 73–75, 99). Broad activation of antiviral immunity evidenced by enhanced transcription of interferon-stimulated genes (ISGs) have been observed with TLR3, TLR7, and TLR9 agonists *in vivo* (69–71, 74).

Thus, both TLR7 and TLR9 agonists are very potent enhancers of innate immune effector functions and broad stimulators of adaptive immunity. The activation of antigen presenting immune cells in the presence of relevant antigens helps focusing the adaptive immune response to effectively target HIV-1. The parallel activation of immune effector cells could boost the clearance of infected cells and hence the ability to control the infection. These features of TLR agonists could be valuable assets in the development of a functional cure for HIV-1, based on the induction of immune control of the infection.

Tissue Effects

As most TLRs are also expressed on epithelial cells throughout the body, TLR agonists may also affect resident cells in tissue (21). Our group investigated the effect of MGN1703 on gut mucosa epithelial cells, which express TLR9, using biopsies of the sigmoid colon from study participants of the short course MGN1703 treatment trial (74, 92). A global transcriptomic analysis showed that MGN1703 upregulated an ISG signature consistent with potent IFN-α induction. Furthermore, high levels of ISG proteins MX1 and ISG15 were detected by *in-situ* hybridization during MGN1703 stimulation in the epithelial cells. This induction was however not accompanied by excessive inflammation, evident by the lack of IFN-γ mRNA and absence of infiltrating neutrophils in the gut mucosa. These findings indicate that subcutaneously administered MGN1703 may have beneficial effects in the gut of infected individuals, but further investigations are needed to account for the exact mechanisms.

A sub-study to the 24-weeks MGN1703 treatment study (75) investigated the tissue-specific effects on lymph nodes (76). In LNMCs, augmented activation of pDCs, (CD86+ CD40+), NK cells (CD69+), and CD8+ effector memory T cells (HLA-DR+ CD38+) was detected and correlated with increased IFN levels and ISG15 expression. Interestingly, lymph node B cells displayed enhanced expression of activation-induced cytidine deaminase (AID) which is an essential enzyme in regulating B cell differentiation and somatic hypermutation. Consistently, a LNMC gene expression analysis showed markedly increased numbers of plasma cells post-MGN1703-treatment as well as increased levels of total IgG and subtypes IgG1, IgG2, and IgG3 antibodies. The induced antibodies showed specific neutralizing properties toward HIV-1 clade B and C, suggesting a possible autologous vaccinal effect of MGN1703. Finally, when assessing the architecture of the lymph nodes, it was demonstrated that the frequency of secondary follicles increased over the 24-weeks treatment period, suggestive of a restorative effect of the intervention.

In conclusion, these two studies from our group demonstrated broad immune enhancement and tissue restoration in the gut mucosa and lymph nodes following TLR9 agonist treatment. These results are encouraging for further clinical development as induction of potent immune responses in tissue compartments may be essential for the therapeutic success of HIV-1 cure strategies.

TLR-AGONISTS IN COMBINATION WITH OTHER THERAPEUTICS

TLR Agonists as Vaccine Adjuvants

Developing a therapeutic HIV-1 vaccine has long been an approach to boost immune control of HIV-1. Moody et al. tested the adjuvant properties of three different TLR agonists, both alone and in combination, to HIV-1 envelope protein immunogens in rhesus macaques (100). They found that the combination of TLR9 agonist CpG ODN and TLR7/8 agonist R848 mixed with base adjuvant Span85-Tween 80-squalene (STS+oCpG+R848) elicited the most potent antibody response against HIV-1 envelope gp140 and V1V2-gp70 as measured by antibody titers. The elicited antibodies were superior in terms of neutralizing HIV-1 when comparing animals treated with STS+oCpG+R848 to those treated with STS alone. Additionally, STS+oCpG+R848-elicited antibodies had greater capacity for inducing ADCC against HIV-antigen-covered cells compared to antibodies from control animals. However, the elicited antibodies were short-lived with an estimated half-life of 8.5 weeks.

Kasturi et al. attempted to assess the issue of short-lived antibodies by altering the formulation of the adjuvant in a NHP preventive vaccine study (101). The adjuvants R848 and TLR4 agonist monophosphoryl lipid A were packed in nanoparticles (NP) and administered with soluble recombinant SIVmac239-derived envelope gp140 and Gag protein 55, together referred to as the protein-NP vaccine. This protein-NP vaccine elicited a durable antibody response present in both serum and mucosa of the rhesus macaques. Ten NHPs in each group received 1 of 4 combinations of immunogens and adjuvants (virus-like particles or soluble envelope combined with either gag + NP or alum). The vaccines were administered 4 times: at week 0, 8, 16, and 25. The protein-NP group had a significantly greater peak in antibody responses at week 27 with an envelope-specific IgG level of 680.77 μg/mL compared to 104.47, 78.71, and 19.81 μg/mL, in the three other groups. The protein-NP group also showed significantly higher levels of envelope-specific plasma cells in bone marrow and draining lymph nodes. In addition, the protein-NP group exhibited the most potent protection when all study animals were challenged 12 times intravaginally with low-dose SIV once weekly starting at week 41 after the first vaccination. Although the protection against infection was stronger in the protein-NP vaccination group, 5 of 10 NHPs still became infected after 10 challenges, stressing that although the NP administration improved the adjuvating

effect of the TLR agonists, this was not sufficient to elicit complete protection.

Borducchi et al. conducted a study on SIV-infected Indian origin rhesus macaques, testing a novel therapeutic vaccine compound named Ad26/MVA. The vaccine comprised an adenovirus vector (Ad26) expressing SIV gag-pol-env, which was boosted by modified vaccinia Ankara (MVA), also expressing gag-pol-env and adjuvanted by TLR7 agonist GS-986 (93). Levels of IFN-γ were increased and CD4+ and CD8+ T cell activation was evident by increased CD69-expression in the vaccine group, indicating effective immune stimulation. Upon ART discontinuation, the 9 NHPs receiving the Ad26/MVA vaccine adjuvanted with GS-986 showed a 2.5-fold delay in time to viral rebound compared to sham controls. Additionally, three of the nine animals that initially appeared to have rebounded after ART cessation regained durable virologic control throughout 160 days. None of the three components administered alone were able to induce similar levels of control suggesting a synergistic effect of the TLR7 agonist and the Ad26/MVA vaccine.

In 2008, our group investigated the immunostimulatory effect of TLR9 agonist CpG 7909 as adjuvant to pneumococcal vaccines in a randomized double-blinded, placebo-controlled trial among 97 HIV-1 infected individuals (102). All study participants received a 7-valent pneumococcal conjugate vaccine (7vPnC) at 0 and 3 months and a 23 valent pneumococcal polysaccharide vaccine (PPV-23) at 9 months. The experimental group received 1 mg of CPG 7909 as adjuvant with each vaccine dose, while the control group received a placebo adjuvant. At 9 months, the proportion of high vaccine responders, defined as a 2-fold increase in IgG levels for at least 5 of 7 of the 7vPnC serotypes, was 48% in the experimental group ($n = 48$) compared to 25% in the control group ($n = 49$). CpG 7909 significantly enhanced the immunogenicity of the pneumococcal vaccine. Interestingly, 10 individuals in each study group were treatment-naïve. In the experimental group, the treatment-naïve individuals had a slight increase in plasma-HIV-1 RNA compared to the control treatment-naïve individuals which may reflect broad immune activation (102). A *post-hoc* analysis by Winckelmann et al. showed a significant reduction of 12.6% in the level of total HIV-1 DNA in PBMCs among individuals receiving CPG 7909 as adjuvant, compared to those receiving placebo (saline) (103). These findings triggered the further investigation into the TLR9 agonist MGN1703 as immunomodulator and latency reversing agent described in the sections above.

TLR Agonists in Combination With bNAbs

Based on the immunological findings outlined above it has been proposed that administration of a TLR agonist in combination with bNAbs may enhance killing of infected cells by enhancing antibody-dependent effector mechanisms such as ADCC and ADCP (**Figure 1**). bNAbs function through three mechanisms: by direct viral neutralization, by opsonizing infected cells for immune mediated killing, and by activating the adaptive immune system via HIV-1-epitope-antibody-complexes (104–106).

Borducchi et al. investigated the effect of combining a bNAb (PGT121) with a TLR7 agonist (GS-9620) in a study with simian-human immunodeficiency virus (SHIV) infected rhesus macaques (99). The 44 NHPs were infected intrarectally with SHIV and ART was initiated at day 7 post-infection and continued for 96–104 weeks before bNAb and TLR7 agonist administration. ART was continued for another 16 weeks after the last administration of PGT121 before an ATI was initiated at week 130. By day 196 following ART cessation, only 6 of 11 NHPs in the PGT121+GS-9620 group had rebounded compared to 9 of 11 in the PGT121 group, 10 of 11 in the GS-9620 group, and 11 of 11 in the sham group. Of note, the viral reservoir measured as SHIV DNA in PBMCs was undetectable across all groups indicating that all animals hadvery small SHIV reservoirs, presumable due to the early initiation of ART. However, viral DNA was detectable in LNMCs and here, the PGT121+GS-9620 group had lower levels compared to the other groups. Subsequent anti-CD8α mediated CD8+ T-cell depletion in the non-rebounding NHPs failed to induce plasma viremia. Finally, adoptive transfer experiments where performed. PBMCs and LNMCs were first collected from the 2 PGT121+GS-9620 treated NHPs, who showed transcient rebound followed by durable virologic control. Cells were collected during the control phase of the ATI, but when transferred to SHIV-naïve NHPs, the cells induced infection. In contrast, adoptive transfer of cells from the 5 PGT121+GS-9620 treated NHPs who did not rebound upon ATI, did not cause infection in SHIV-naïve NHPs. These findings indicated that the viral SHIV reservoir was significantly reduced, maybe even completely eradicated in some animals by the combination of PGT121 and GS-9620 and have engouraged further investigations of this combination.

Along these lines, our group is performing an ongoing double-blinded randomized placebo controlled phase IIa clinical trial testing the TLR9 agonist MGN1703 and a combination of two bNAbs (3BNC117 and 101074) in HIV-1-infected donors on long-term ART, aiming to reduce the viral reservoir and induce immunological HIV-1 control (NCT03837756).

TLR Agonists and Programmed Death-1 (PD-1) Inhibition

The PD-1 receptor is a marker of T cell exhaustion, which is upregulated following prolonged antigen-stimulation during infection (107) and in cancer (108). By alleviating this T cell exhaustion, immunotherapy with antibodies blocking the PD-1 receptor has dramatically improved the prognosis of many different types of cancers such as malignant melanoma and renal cell carcinoma (109–111). In HIV-1 cure research the hope is that treatment with anti-PD-1 antibodies might lead to a more efficient targeting of the latently infected cells by HIV-specific T cells.

Bekerman et al. tested the immunomodulatory effect of a chimeric anti-PD-1 antibody and the TLR7 agonist GS-9620 in chronically SIV infected rhesus macaques (94). In a four-arm controlled design, NHPs received 0.15 mg/kg GS-9620 by oral gavage every other week for a total of 10 administrations, alone or in combination with 10 mg/kg anti-PD-1 antibody. Upon ART cessation all of the 20 NHPs rebounded within 14 days with no delay compared to the placebo group. Assessment of the viral reservoir measured by total SIV DNA levels in PBMCs

similarly showed no reduction. Proportions of IFN-γ- and IL-2-producing SIV-specific T cells also did not differ between the groups. Previous studies have found beneficial effects of PD-1 receptor inhibition during SIV infection in rhesus macaques, when intervening in viremic animals or shortly after ART initiation (112, 113). Importantly, Bekerman et al. administered the PD-1 receptor antibody and TLR7 agonist after 2 years of suppressive ART. This suggests that the possible therapeutic benefits of PD-1 receptor blockade may depend on timing of administration in relation to ART initiation.

CONCLUDING REMARKS

Emerging preclinical and clinical studies strongly indicate that TLR agonists have great potential as immune boosting and priming agents in HIV-1 cure research. However, not all TLR agonist are created equally and so far, the most promising results have been observed following TLR7 and TLR9 administration. Expression patterns of TLRs in humans and the diverse response of the TLR subtypes are important factors to consider when assessing the potential clinical effect of TLR agonist treatment in HIV-1. Further investigation is needed into the physiological function of TLRs on immune cell subsets, differences in TLR expression in blood and tissue, as well as gender determined differences. TLR agonists should probably only be considered as moderately potent LRAs but while much interest in TLR agonists have evolved around their potential use as LRAs, we believe that pre-clinical and clinical findings demonstrate that the most important aspect of TLR agonists is their ability to enhance innate and adaptive immunity. This is underscored by the durable virologic control in the absence of ART achieved in NHPs by combining TLR agonists with AD26/MVA-vector SIV vaccines or bNAbs. Cross-priming of the CD8+ T cell response could be an important element of the antigen-dependent mechanisms seen with TLR agonist. Novel clinical trials testing these very concepts are now underway and their highly anticipated results will further inform the research field of the potential of TLR agonists as components in HIV-1 cure strategies.

AUTHOR CONTRIBUTIONS

JM, JG, and OS conceived and wrote the manuscript. All authors (+ JH and MT) contributed to manuscript revision and approved the submitted version.

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MINI REVIEW
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Virus-Specific T Cell Therapies for HIV: Lessons Learned From Hematopoietic Stem Cell Transplantation

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Human immunodeficiency virus (HIV) has caused millions of deaths and continues to threaten the health of millions of people worldwide. Despite anti-retroviral therapy (ART) substantially alleviating severity and limiting transmission, HIV has not been eradicated and its persistence can lead to other health concerns such as cancer. The only two cases of HIV cure to date are HIV$^+$ cancer patients receiving an allogeneic hematopoietic stem cell transplantation (allo-HSCT) from a donor with the CCR5 Δ32 mutation. While this approach has not led to such success in other patients and is not applicable to HIV$^+$ individuals without cancer, the encouraging results may point toward a breakthrough in developing a cure strategy for HIV. Adoptive transfer of virus-specific T cells (VSTs) post HSCT has been effectively used to treat and prevent reactivation of latent viral infections such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), making VSTs an attractive therapeutic to control HIV rebound. Here we will discuss the potential of using adoptive T cell therapies in combination with other treatments such as HSCT and latency reversing agents (LRAs) to achieve a functional cure for HIV.

Keywords: HIV, T cells, immunotherapy, hematopoietic stem cell transplantation, latent reservoir

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INTRODUCTION

According to the latest UNAIDS statistics, HIV impacted an estimated 37.9 million individuals worldwide, and resulted in over 770,000 deaths by the end of 2018. Unfortunately, a cure for the immunity-demolishing virus is still lacking. T cells play a key role in the immune response against viral infections, including HIV. Anti-HIV CD8$^+$ T cell responses can be induced upon infection and are associated with a transient reduction in viral load (Walker et al., 1987; Koup et al., 1994). In most cases without any medical intervention, the anti-viral cellular responses fail to clear the virus, leading to a progressive loss of CD4$^+$ T cells and eventually a state with a severely impaired immune system known as acquired immunodeficiency syndrome (AIDS). A combination of drugs targeting different stages of HIV life cycle in anti-retroviral therapy (ART) has been used to treat HIV infection, rendering the virus undetectable and preventing transmission (Arts and Hazuda, 2012). Nevertheless, ART requires life-long adherence to medication and does not eradicate the virus (Wong et al., 1997; Chun et al., 1999).

Even with active viral replication being halted by ART, the quiescent immune cells—especially memory $CD4^+$ T cells—harboring replication-competent provirus can persist and constitute the latent HIV reservoir (Chun et al., 1995; Strain et al., 2005; Archin et al., 2012a). HIV reservoir can evade the immune system due to the lack of viral antigen expression and can cause viral rebound after the cessation of ART, making them the major barrier to an HIV cure (Sengupta and Siliciano, 2018).

Both individuals cured of HIV have received HSCT with CCR5 Δ32 donor cells (Hutter et al., 2009; Gupta et al., 2019). This suggests that eradication of HIV requires a reduction in the viral reservoir by myeloablation (Henrich et al., 2013, 2014) followed by immune reconstitution with HIV-resistant cells to prevent further infection. However, the emergence of viral rebound after ART interruption in other patients undergoing similar procedures (Hutter, 2014; The Lancet, 2019) indicates that additional therapeutics are needed to help control viral spread from the lasting latent reservoir. The ability of adoptive transfer of *ex vivo*-grown, virus-specific T cells to treat and prevent viral infections (e.g., CMV and EBV) in immunocompromised patients (Leen et al., 2006; Saglio et al., 2014) makes adoptive T cell therapy (ACT) a promising strategy to prevent HIV rebound post HSCT. Here we will focus on the clinical efforts of using ACT in treating HIV infection (**Table 1**) and discuss the potential of using ACT in combination with HSCT and LRA to eradicate the latent reservoir.

THE SUCCESS OF USING VSTS TO PREVENT AND TREAT VIRAL INFECTIONS POST HSCT

HSCT is considered a curative therapy for hematological malignancies and certain genetic disorders, but viral infections post HSCT are a major cause of morbidity and mortality (Boeckh et al., 2003; Myers et al., 2005; Brunstein et al., 2006). This is because the recipients are devoid of anti-viral immunity when the T cell compartment slowly recovers and immunosuppression is typically used to prevent and treat graft-vs.-host disease (GVHD). While prophylactic and therapeutic pharmacological treatments are available, they can be expensive and their efficacy is often limited by the toxicity and drug resistance (Tomblyn et al., 2009). ACT is an alternative approach showing clinical benefits for viral disease in immunodeficient transplant recipients. Adoptive transfer of donor-derived CMV-specific cytotoxic T lymphocyte (CTL) clones prevents CMV infection (Riddell et al., 1992). Infusions of unselected leukocytes or EBV-specific CTLs derived from EBV-seropositive HSCT donors effectively treated EBV-associated lymphoproliferative disease (Papadopoulos et al., 1994; Doubrovina et al., 2012). When endowed with a CD19-targeting artificial receptor, VSTs also provided antitumor activity against relapsed B cell malignancies post HSCT (Cruz et al., 2013). These findings demonstrate the potential of using antigen-specific T cells as "living drugs" to quickly reconstitute recipient's T cell responses to treat rapid-progressing and life-threatening viral complications post HSCT.

Tremendous efforts have been made to refine the manufacture of cell products, aiming at conferring durable protection without causing GVHD by reproducibly generating clinical doses of T cells with the desired specificity, long-term *in vivo* persistence, and minimal alloreactivity. While it has yet been adapted to other viruses, ACT has emerged as a safe and efficient therapeutic for combating CMV, EBV, and adenovirus infections (Cruz et al., 2010; Mui et al., 2010; Bollard, 2013; Houghtelin and Bollard, 2017; Kaeuferle et al., 2019). Traditionally, the source of VSTs is limited to the stem cell donor, which may not be a readily available VST donor source to treat immunocompromised patients suffering from devastating viral diseases. In contrast, "off-the-shelf" VSTs that have been generated from "third-party donors" with diverse HLA types and banked can be used to treat high-risk patients even if HLA matching between the third-party donor and the patient is only at a single allele. Hence, the emerging use of third-party VSTs has broadened the applicability of adoptive T cell therapy for viral infections (Haque et al., 2007; Leen et al., 2013). For these reasons, infusion of HIV-specific T cells could be a logical treatment option to restore anti-HIV immunity in infected individuals.

FROM EBV, CMV, ADENOVIRUS TO HIV

ART has effectively decreased the incidence of HIV-infected individuals progressing to AIDS and the subsequent AIDS-defining illnesses, but the deaths by non-AIDS-defining cancers have been escalating (Deeken et al., 2012). The Berlin patient, the very first case of HIV cure, developed acute myeloid leukemia (AML) while on ART and received two HSCTs from an HLA-matched donor with the homozygous CCR5 Δ32 mutation (Hutter et al., 2009). He has remained devoid of detectable virus for >10 years since the discontinuation of ART (Lederman and Pike, 2017). The other case is the London patient, who received a less aggressive conditioning regimen for Hodgkin's lymphoma and has been in HIV remission without ART since September, 2017 (Gupta et al., 2019, 2020). Myeloablative conditioning not only killed cancer cells but substantially reduced the size of the HIV reservoir (Koelsch et al., 2017; Salgado et al., 2018). However, the reduction in the HIV reservoir alone appears insufficient to achieve a cure because viral rebound was observed after stopping ART in patients receiving HSCT from CCR5 wild-type donors (Henrich et al., 2013, 2014; Cummins et al., 2017).

In addition to a substantial reduction in the HIV reservoir, achieving full donor chimerism with allogeneic HIV-resistant donor cells is critical for preventing viral rebound. However, the success of the Berlin and London patients has not been reproduced in other patients transplanted with CCR5 Δ32 donors (even homozygous donors) (Hutter, 2014; The Lancet, 2019). The only possible exception is that of the Düsseldorf patient who has been in remission since the discontinuation of ART in November, 2018 as reported by the IciStem (International Collaboration to guide and investigate the potential for HIV cure by Stem Cell Transplantation) consortium (The Lancet, 2019). The failure to achieve a cure in most patients was likely due

TABLE 1 | Summary of T-cell-based therapies in HIV clinical trials.

Strategy	ART status	Trial status (start date/completion date)	Clinicaltrials.gov identifier	Locations
Autologous T cells transduced with a CD4z CAR in combination with IL-2	ART suppressed	Active and not recruiting (2001-09/N.A.)	NCT01013415 (Scholler et al., 2012)	• University of Pennsylvania, Philadelphia, Pennsylvania, United States
Autologous T cells transduced with a bNAb-based CAR	ART suppressed	Active and recruiting (2017-10/N.A.)	NCT03240328	• Guangzhou 8th People's Hospital, Guangzhou, Guangdong, China
Autologous CD8$^+$ T cells transduced with two gag-specific TCRs	Subject to ART interruption post infusion	Completed (2009-11/2014-01)	NCT00991224	• University of Pennsylvania, Philadelphia, Pennsylvania, United States
Autologous mitogen-expanded CD8$^+$ T cells	ART suppressed	Completed (N.A./2002-06)	NCT00000756 (Lieberman et al., 1997)	• New England Medical Center/Tufts University, Boston, Massachusetts, United States
Autologous HIV peptide-expanded CD8$^+$ T cell clones	ART suppressed	Completed (1998-09/2005-04)	NCT00110578 (Chapuis et al., 2011)	• Fred Hutchinson Cancer Research Center, Seattle, Washington, United States • University of Washington, Seattle, Washington, United States
Autologous HIV peptide-expanded multi-specific T cells	Treated with ART during acute or chronic infection	Completed (2015-04/2017-11)	NCT02208167 (Sung et al., 2018)	• University of North Carolina, Chapel Hill, North Carolina, United States
Autologous HIV peptide-expanded multi-specific T cells	ART suppressed	Active and recruiting (2019-03/N.A.)	NCT03485963	• Whitman Walker Health Research Department, Washington, District of Columbia, United States • Children's National Health System, Washington, District of Columbia, United States • Children's National Medical Center, George Washington University Hospital and Whitman Walker Health Research Department, Washington, District of Columbia, United States
Autologous T cells transduced with a bNAb-based CAR in combination with Chidamide	ART suppressed	Active and recruiting (2017-12/N.A.)	NCT03980691	• Guangzhou 8th People's Hospital, Guangzhou, Guangdong, China
Autologous HIV peptide-expanded multi-specific T cells in combination with Vorinostat	ART suppressed	Active and recruiting (2017-06/N.A.)	NCT03212989	• University of North Carolina, Chapel Hill, North Carolina, United States
Autologous CD4$^+$ T cells transduced with ZFN to disrupt CCR5	Subject to ART interruption post infusion	Completed (2009-01/2013-01)	NCT00842634 (Tebas et al., 2014)	• Jacobi Medical Center, Bronx, New York, United States • University of Pennsylvania, Philadelphia, Pennsylvania, United States
Autologous CD4$^+$ T cells transduced with ZFN to disrupt CCR5	ART suppressed with one cohort being subject to treatment interruption	Completed (2009-12/2014-12)	NCT01044654	• Nine institutes in California, Connecticut, Florida, Missouri, New Mexico, New York, and Texas, United States
Autologous CD4$^+$ T cells transduced with ZFN to disrupt CCR5	Non-ART suppressed (HIV viral load between 1e3 and 1e6 copies/ml)	Completed (2010-11/2015-05)	NCT01252641	• Four institutes in California and Florida, United States
Autologous CD4$^+$ T cells transduced with C34-CXCR4	Subject to ART interruption post infusion	Completed (2017-01/2020-03)	NCT03020524	• University of Pennsylvania, Philadelphia, Pennsylvania, United States
Autologous HSPC and CD4$^+$ T cells transduced with a CCR5-targeting shRNA and C46 with or without preconditioning for transplant	ART suppressed	Completed (2013-04/2017-11)	NCT01734850	• UCLA CARE Center, Los Angeles, California, United States • Quest Clinical Research, San Francisco, California, United States
Autologous T cells transduced with a CD4 CAR and ZFN to disrupt CCR5	Subject to ART interruption post infusion	Active and not recruiting (2019-07/N.A.)	NCT03617198	• University of Pennsylvania, Philadelphia, Pennsylvania, United States
Autologous CD4+ T cells transduced with an *env*-targeting antisense	Subject to ART interruption post infusion	Completed (2006-01/2013-12)	NCT00295477 (Tebas et al., 2013)	• University of Pennsylvania, Philadelphia, Pennsylvania, United States

to the high post-transplant mortality and the emergence of an X4-tropic escape variant (Hutter, 2014; Kordelas et al., 2014; Duarte et al., 2015; Rothenberger et al., 2018). Nevertheless, aside from the rarity of donors with the homozygous mutant alleles (Solloch et al., 2017) and the toxicity of the allograft procedure, these studies suggest that allo-HSCT using donor cells that are resistant to HIV infection along with continuous ART could effectively reduce and maintain the size of the HIV reservoir at low levels. Despite not being curative for most patients, this approach could however open a window for adoptive anti-viral immunity to prevent viral rebound, given the success of infusing VSTs to treat EBV, CMV and adenovirus infections in HSCT recipients.

ADOPTIVE T-CELL THERAPY FOR HIV

ACT is an efficient way to quickly replenish a patient's T cell responses. With advancing culture procedures and genome-editing techniques, autologous or allogeneic T cells can be expanded *ex vivo* and endowed with antigen-specificity and improved persistence and function within the tissue microenvironment. While ACT had little success with HIV treatment in the past (Koenig et al., 1995; Lieberman et al., 1997), infusion of improved T cell products along with efficient ways to reduce the viral reservoir and increase immunogenicity of the viral antigens may offer an opportunity to eradicate the virus in ART-treated patients.

HIV-Specific Chimeric Antigen Receptors

The chimeric antigen receptor (CAR) is an artificial receptor with an extracellular antigen-binding domain connected to an intracellular signaling domain by a hinge region and a transmembrane domain. When expressed on the surface of a T cell, CAR can induce T-cell activation upon ligand binding independently of T cell receptor (TCR)-major histocompatibility complex (MHC) interactions, thereby bypassing MHC restrictions and MHC downregulation mechanisms often used by cancer cells or viruses, including HIV (Dotti et al., 2014; Wagner, 2018). Despite the recent success in treating hematological malignancies, CAR-T-based therapies are limited by obstacles such as antigen loss, poor T cell persistence, and immune-related toxicities (Sun et al., 2018; Shah and Fry, 2019). While HIV-targeting CARs showed limited success in the early clinical trials, primarily due to viral escape variants and susceptibility of CAR-T cells to HIV infection, new CAR-based therapies developed to overcome these hurdles have shown promising results in preclinical studies (reviewed in Kuhlmann et al., 2018; Wagner, 2018; Yang et al., 2018; Kim et al., 2019; Qi et al., 2020). Their clinical efficacy is being actively investigated (**Table 1**).

HIV-Specific TCRs

Naturally-occurring TCRs can also recognize HIV-associated antigens in the context of MHC and therefore can be used to engineer T cell specificity. An artificial TCR specific for the HLA-A*02-restricted p17 *gag* epitope SLYNTVATL (SL9) was derived from a naturally-occurring TCR isolated from an HIV-infected individual and demonstrated a higher affinity to SL9 (Varela-Rohena et al., 2008). $CD8^+$ T cells transduced with this supraphysiologic TCR can better control the infection with wild-type and SL9 escape variants of HIV than the original TCR *in vitro*. A phase 1 trial was initiated to test T cells transduced with these TCRs either before or during ART interruption (NCT00991224), but the results have not been reported. Engineering T cell specificity with TCRs may yield a highly efficient T cell response, but its use is limited by MHC restriction, antigen loss, and off-target toxicities including death as reported for cancer patients infused with MAGE-A3 TCR T cells (Cameron et al., 2013; Linette et al., 2013).

Another option to achieve HIV specificity is to use CTL lines or clones derived from preformed memory T cells, which exist at high frequencies in HIV-infected individuals (Hoffenbach et al., 1989). Mitogen-expanded, autologous $CD8^+$ T cell lines enriched for reactivity against HIV antigens gp120, p17, p24, and Nef were infused to six HIV-infected subjects. Despite increased $CD4^+$ T cell counts and decreased plasma viremia following CTL infusion, the effects were transient (2 weeks) and did not reach statistical significance (NCT00000756 Lieberman et al., 1997). In another study, autologous HIV Gag-specific $CD8^+$ T cell clones obtained by limiting dilutions were infused to three ART-suppressed subjects. Infusion of CTL was accompanied by a reduction in HIV-infected $CD4^+$ T cells in the peripheral blood, but the reduction was transient and the plasma HIV RNA levels were not diminished (Brodie et al., 1999). While utilizing $CD8^+$ T cell lines or clones may be an efficient and low-risk way to obtain clinically relevant numbers of HIV-targeting T cells without any genetic manipulation, their antigen breadth is still limited and may even cause a selective expansion of antigen escape variants (Koenig et al., 1995). Moreover, the lack of $CD4^+$ T cells in the infused cell products may result in an impaired persistence of HIV-specific $CD8^+$ T cells *in vivo*. This will be discussed in more detail below.

HIV-Targeting Multi-Specific T Cells

With the understanding that the mutation rate of HIV *in vivo* may be higher than that originally estimated (Cuevas et al., 2015), targeting multiple epitopes from different antigens simultaneously presents a logical solution to conquer immune escape by increasing T cell target breadth. It was first shown that a polyclonal HIV-specific T cell population can be obtained from HIV seropositive blood samples by three rounds of stimulation with peptide-pulsed antigen-presenting cells (APCs) (Lam et al., 2015). The peptides used for T cell stimulation were pools of ∼150 15-mers spanning the most conserved regions of *gag*, *pol*, and *nef* across all clades of HIV. Due to the concern for propagation of HIV *in vitro* during manufacturing, all T cell products from HIV seropositive blood samples intended for clinical uses were grown in the presence of anti-retroviral drugs. The resultant HIV-specific T cells (HXTCs), a mixture of $CD8^+$ and $CD4^+$ T cells, were able to lyse peptide-pulsed targets and suppress the viral spread of an HIV lab strain *in vitro*. Moreover, HXTCs were also capable of controlling the outgrowth of autologous reservoir-derived HIV and, more

importantly, clearing resting $CD4^+$ T cell reservoir post LRA treatment *in vitro* (Sung et al., 2015). To further increase the ability of infused T cells to target HIV escape variants, a bivalent mosaic of peptides targeting the regions of Gag and Pol antigens known to be functionally conserved, common in escape variants, and associated with natural immune protection (Ondondo et al., 2016) along with the Nef peptide library were used to manufacture T cell products using a similar platform as HXTCs (Patel et al., 2020). These HIV-specific T cells targeting non-escape epitopes (HST-NEETs) demonstrated peptide specificity, but their ability to control viral spread remains to be determined. While a significant proportion of HXTCs or HST-NEETs were likely derived from pre-existing memory T cells in HIV-infected individuals, they can also be generated from seronegative adult or cord blood donors, broadening its applicability in settings such as post-HSCT treatment or third-party VST banks (Patel et al., 2016, 2018, 2020).

The *in vivo* efficacy of HXTCs has been evaluated in a phase 1 clinical trial (NCT02208167). When infused to HIV-infected participants on ART, HXTCs were safe and well-tolerated, but showed little impact on the frequency of cells harboring replication competent HIV, measured by the quantitative viral outgrowth assay (Sung et al., 2018). An ongoing clinical study (NCT03485963) was initiated to test HST-NEETs in ART-suppressed, HIV-infected individuals. While preliminary results suggest that these HIV-specific T cell products are safe, feasible to manufacture, and functional in *in vitro* assays, they were not expected to demonstrate therapeutic effects for several reasons. First, the viral antigen levels in ART-suppressed individuals are likely too low to trigger the activation of multi-specific T cells. Second, the infusion products contain $CD4^+$ T cells, which can benefit the accompanying $CD8^+$ T cells but also become targets of HIV infection. Lastly, during the manufacturing process, cell products may acquire the expression of co-inhibitory receptors and/or lose the expression of proper homing receptors, resulting in poor persistence and homing ability to the latent reservoir.

PROVIDING *IN VIVO* HELP TO INFUSED T CELLS

Strategies have been proposed to assist infused T cells to overcome the immunosuppressive mechanisms deployed by HIV. To increase viral antigen levels for detection by TCRs or CARs, administration of LRAs during ART can potentially reactivate viral replication without causing massive viral rebound as proposed in the "shock and kill" or "kick and kill" approach (Archin et al., 2012b; Deeks, 2012; Lopez, 2013). Although the potency and durability of LRAs on increasing immunogenicity of the viral reservoir and their immunomodulatory effects on infused T cells *in vivo* remain unclear (Spivak and Planelles, 2018), combining LRAs with ACT could in theory reduce the size of the latent reservoir as supported by *in vitro* studies using viral outgrowth assays (Shan et al., 2012; Sung et al., 2015). An ongoing clinical trial is testing the safety and efficacy of a broadly-neutralizing antibody (bNAb)-based CAR-T cell therapy combined with a histone deacetylase inhibitor (HDACi), Chidamide, in ART-suppressed patients without treatment interruption (NCT03980691). Another phase 1 trial is evaluating the effects of HXTCs and another HDACi, Vorinostat, on ART-suppressed participants who show increased cell-associated viral RNAs in response to the initial Vorinostat administration (NCT03212989). These clinical studies will provide valuable information about the feasibility of using LRAs as adjuvants to boost the adoptive T cell responses without the need to interrupt ART.

One of the biggest concerns of using T cells to treat HIV is the risk of infection of the infused $CD4^+$ T-cell compartment by the reactivated virus. One option is to prevent HIV entry by using allogeneic T cells from donors with the homozygous CCR5 Δ32 mutation, but this approach is not applicable to most HIV-infected individuals. Alternative strategies to confer HIV resistance include genome-editing technologies. A clinical study reported that the infusion of autologous $CD4^+$ T cells edited by zinc finger nuclease (ZFN) to delete the *CCR5* gene was largely safe, and that the gene-modified cells engrafted and persisted (NCT00842634 Tebas et al., 2014). In a case report where an HIV-infected patient with acute lymphoblastic leukemia received an allo-HSCT with $CD34^+$ hematopoietic stem and progenitor cells edited by clustered regularly interspaced short palindromic repeats technology to delete the *CCR5* gene, full chimerism was achieved and the resultant *CCR5*-ablated $CD4^+$ T cells persisted for months (NCT03164135 Xu et al., 2019). HIV fusion inhibitors such C34 and C46 have also been used to prevent infused T cells from HIV infection in clinical trials, but the results have not been reported (NCT03020524 and NCT01734850). An ongoing clinical trial is examining the efficacy of infusing autologous T cells modified to deactivate the *CCR5* gene and to express a CD4-based CAR, followed by ART interruption (NCT03617198). Moreover, autologous $CD4^+$ T cells transduced with a lentiviral vector expressing an antisense targeting HIV *env* were infused to 13 ART-treated patients followed by treatment interruption, attempting to block viral replication upon infection (Tebas et al., 2013). Strategies to confer HIV resistance should be considered for any types of cell-based therapy to avoid exacerbation of viral rebound.

Obtaining clinically relevant numbers of T cells for infusion often requires multiple rounds of TCR stimulation, which can result in an effector memory (T_{EM})-enriched cell population lacking CD62L and CCR7 expression (Sung et al., 2018; Patel et al., 2020). T_{EM} cells have been shown to demonstrate inferior persistence and anti-tumor function *in vivo* compared to T stem cell memory (T_{SCM}) or central memory (T_{CM}) cells (Berger et al., 2008; Gattinoni et al., 2011). Not only is the expression of the homing receptors–CD62L and CCR7–linked to a gene signature favoring long-term persistence and better effector function (Klebanoff et al., 2005), but they are also required for T cell trafficking to secondary lymphoid tissues (Weninger et al., 2001), the major site of the viral reservoir (Boritz and Douek, 2017; Dimopoulos et al., 2017). Additional homing receptors, such as α4β7 and CXCR5, are also crucial for T cells to traffic to gut-associated lymphoid tissues and B cell follicles, respectively (Schaerli et al., 2000; Arthos et al., 2018). Ensuring the expression of these homing receptors, either by

gene-engineering approaches or improved culture protocols, may allow efficient accumulation of infused T cells in the viral reservoir. Furthermore, co-inhibitory receptors such as CTLA-4, PD-1, and Tim-3 are induced transiently following TCR stimulation, and remain expressed on exhausted T cells (Kuchroo et al., 2014). Blockade of these pathways *in vivo* by administration of monoclonal antibodies, namely checkpoint inhibitors, has achieved great clinical benefits in the cancer immunotherapy field (Korman et al., 2006; Wei et al., 2018). Ipilimumab, a fully humanized monoclonal antibody targeting CTLA-4 shown to improve the overall survival of patients with metastatic melanoma (Hodi et al., 2010), was used to treat HIV-infected participants resistant to ART (Colston et al., 2018). Ipilimumab was overall well-tolerated but showed little clinical efficacy. Combining checkpoint inhibitors with cell therapies may be an attractive strategy, but potential immune-related adverse effects must be carefully evaluated. Blocking the inhibitory pathways may provide a boost to infused T cells, but it may concurrently cause autoimmunity or awaken the quiescent latent reservoir, as increased viral loads had been observed in nearly 60% of the participants receiving ipilimumab in a prior trial (Colston et al., 2018).

SUMMARY AND FUTURE PERSPECTIVE

The two cases of long-term viral remission without ART may not offer a generalized therapeutic approach for a functional cure, but they have undoubtedly provided valuable insights into the underlying mechanisms and inspired novel research toward the ultimate goal. Modern ART regimens have redefined HIV diagnosis from a death sentence to a chronic disease, and their ability to stop active viral replication may give immunotherapy an opportunity to eradicate the virus altogether. Given the immune evasion mechanisms shared by cancer cells and HIV (Mylvaganam et al., 2019), researchers in the HIV field are hoping to mirror the success enjoyed by cancer immunotherapy experts. The fact that ART-treated, HIV-infected individuals have a higher risk of malignancies further tangles the two disease types and creates more treatment options for HIV.

Replacing the HIV-harboring immune system with an HIV-resistant immune system seems to be essential in curing HIV. The chemo-radiotherapy preparative regimen together with the allodepletion of the recipient's immune system should remove most of the latent reservoir. However, the delayed HIV rebound observed in the Boston patients and Mississippi baby (Ananworanich and Robb, 2014) months after ART interruption indicates that the HIV still present in the reservoir can re-infect the host T cells before HIV-specific immunity can be elicited *in vivo*. Nevertheless, even allo-HSCT with HIV-resistant donors did not cure most patients, raising the question: how was the residual reservoir post HSCT eradicated or contained in those patients who did achieve a cure? While we cannot rule out the possibility that the reactivated virus unable to re-

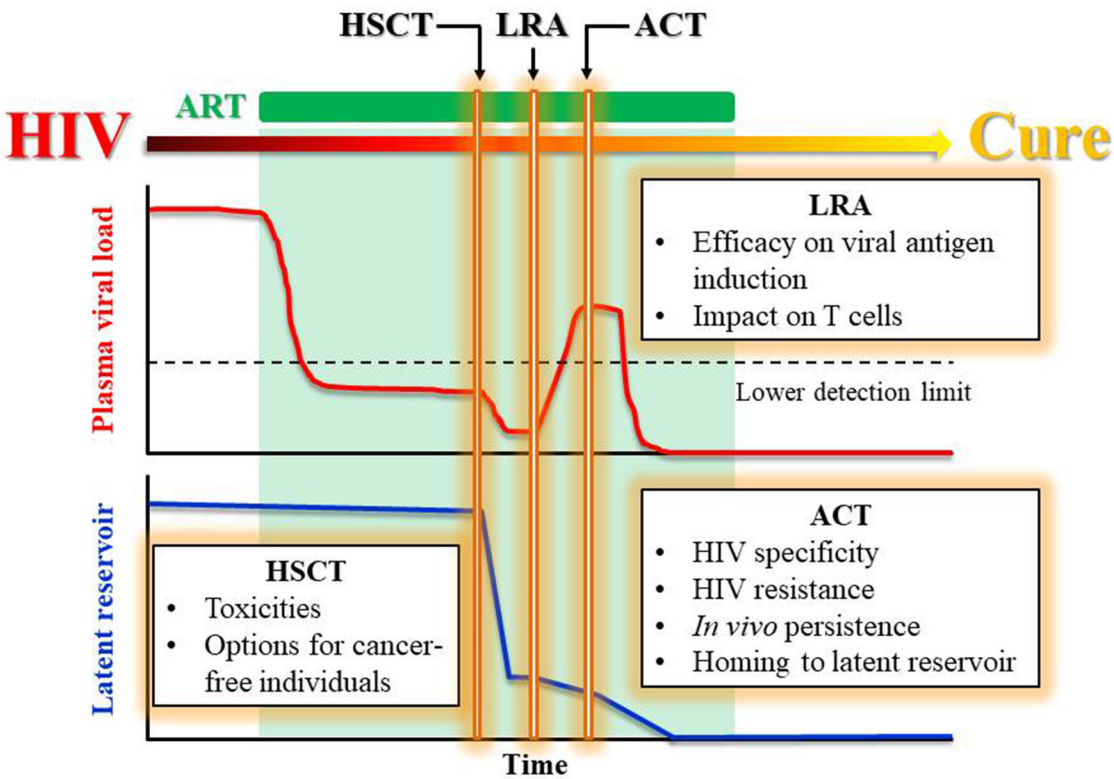

FIGURE 1 | Proposed treatments leading to a functional cure for HIV. Proposed treatment plan to achieve a functional HIV cure by a stepwise reduction in viral load and latent reservoir while maintaining ART. The rectangle boxes list the challenges faced by each therapeutic component including HSCT, LRA, and ACT.

infect a donor cell eventually died off, it is more likely that the cure was achieved only in the cases where the donor immune system eradicated the reservoir via a graft-vs.-HIV reservoir response. Therefore, we believe that, in the post-HSCT settings, infusion of HIV-specific, HIV-resistant T cells with the help from continued ART to suppress new infection and the help from LRAs to reverse viral latency can be a route to a functional cure (**Figure 1**).

In cancer-free HIV-infected individuals, however, efficient and safe alternatives to HSCT are needed to initiate a reduction in the viral reservoir before ACT takes effect. It remains to be seen if the combination of HIV-specific T cells and LRAs can exert any clinical benefits in ART-suppressed patients (NCT03212989), but the infused T cells may be at disadvantages for three reasons: (1) the latent reservoir is expected to be substantially bigger without lymphodepletion, (2) the lymphoreplete host environment is suboptimal for the expansion of infused T cells, and (3) the infused T cells may be vulnerable to HIV infection upon viral reactivation. The occurrence of "elite controller" individuals who can keep the virus in check without ART by maintaining strong HIV-specific T cell responses (Saez-Cirion et al., 2007) support the idea of harnessing the immune system to achieve a functional cure for a broader population. Although $CD8^+$ T cells appear to be the main effectors to kill the virus, other genetic and cellular components may also be required for "elite" anti-HIV immunity (Walker and Yu, 2013). A multifaceted approach will therefore be needed to eliminate the HIV reservoir and provide better curative options to patients.

AUTHOR CONTRIBUTIONS

P-HL wrote the manuscript. MK, PH, and CB reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Safety and Immunological Evaluation of Interleukin-21 Plus Anti-α4β7 mAb Combination Therapy in Rhesus Macaques

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Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections compromise gut immunological barriers, inducing high levels of inflammation and a severe depletion of intestinal $CD4^+$ T cells. Expression of α4β7 integrin promotes homing of activated T cells to intestinal sites where they become preferentially infected; blockade of α4β7 with an anti-α4β7 monoclonal antibody (mAb) prior to infection has been reported to reduce gut SIV viremia in rhesus macaques (RMs). Interleukin-21 (IL-21) administration in antiretroviral therapy-treated, SIV-infected RMs reduces gut inflammation and improves gut integrity. We therefore hypothesized that the combination of IL-21 and anti-α4β7 mAb therapies could synergize to reduce inflammation and HIV persistence. We co-administered two intravenous doses of rhesus anti-α4β7 mAb (50 mg/kg) combined with seven weekly subcutaneous infusions of IL-21–IgFc (100 μg/kg) in four healthy, SIV-uninfected RMs to evaluate the safety and immunological profiles of this intervention in blood and gut. Co-administration of IL-21 and anti-α4β7 mAb showed no toxicity at the given dosages as assessed by multiple hematological and chemical parameters and did not alter the bioavailability of the therapeutics or result in the generation of antibodies against the anti-α4β7 mAb or IL-21–IgFc. Upon treatment, the frequency of CD4 memory T cells expressing β7 increased in blood and decreased in gut, consistent with an inhibition of activated CD4 T-cell homing to the gut. Furthermore, the frequency of T cells expressing proliferation and immune activation markers decreased in blood and, more profoundly, in gut. The combined IL-21 plus anti-α4β7 mAb therapy is well-tolerated in SIV-uninfected RMs and reduces the gut homing of $\alpha4\beta7^+$ CD4 T cells as well as the levels of gut immune activation.

Keywords: anti-α4β7, IL-21, immune activation, T- cell homing, macaques, rhesus macaques, combined immune intervention

INTRODUCTION

Human immunodeficiency virus (HIV) infection induces high and persistent levels of immune activation and inflammation, which are associated with the loss of $CD4^+$ T cells and accelerated disease progression (1, 2). With the advances in antiretroviral therapy (ART), the incidence of HIV infection and transmission has been reduced significantly. However, despite effective viral suppression in plasma, ART does not cure HIV infection, with virus persisting in long-lived $CD4^+$ T cells or macrophages in different tissues and organs (3). Furthermore, ART-treated HIV-infected individuals can still present persistent chronic inflammation, limited $CD4^+$ T-cell reconstitution, and mucosal immune dysfunction (1, 4–6), which have all been linked to increased HIV- and non–HIV-associated comorbidities and mortality. Therefore, new therapeutic strategies aimed at reducing both viral reservoir and chronic immune activation in combination with ART could be beneficial for a potential cure strategy.

Interleukin-21 (IL-21) is a pleiotropic cytokine, member of the common ɤ-chain-signaling family, which includes IL-2, IL-4, IL-7, IL-9, and IL-15, and it is mainly produced by CD4 T helper (T_H) cells (including T_H17 and Tfh), ɤδ T cells, CD8 T, and natural killer (NK) T cells. Interleukin-21 affects multiple pathways of both humoral and cell-mediated immune responses (7). Previously, we showed that in simian immunodeficiency virus (SIV)-infected rhesus macaques (RMs) loss of IL-21–producing $CD4^+$ T cells in the gut is associated with T_H17 cell depletion, loss of gut mucosa integrity, and mucosal immune dysfunction (8). Moreover, we have shown that administration of a rhesus IL-21–IgFc fusion protein in acute (9) or chronic ART-treated SIV-infected RMs (10) resulted in the preservation of intestinal T_H17 cells, improved mucosal immune function, and reduced microbial translocation. Finally, we also showed that IL-21 treatment resulted in a reduction of the replication competent viral reservoir in lymph nodes (10). Importantly, a cross-sectional human study showed that IL-21 production is decreased at the very early stage of HIV infection and that serum IL-21 concentrations correlate with $CD4^+$ T-cell counts (11). In contrast, normal levels of IL-21–producing $CD4^+$ T cells were observed in HIV elite controllers, individuals able to naturally (without ART) control HIV replication to very low levels (11). Furthermore, it has been shown that IL-21 promotes degranulation and effector functions of $CD8^+$ T cells (12, 13) and that IL-21–producing HIV-1–specific $CD8^+$ T cells are more abundant in elite controllers (14).

α4β7 integrin is a key molecule for mucosal homing of lymphocytes (15), and $\alpha4\beta7^+$ CD4 T cells, including T_H17 cells, are the primary targets and thus rapidly depleted during the initial phase of HIV and SIV infection (2, 16, 17). Previous studies in RMs suggest that α4β7 blockade could limit the number of activated and preferentially infected cells to gastrointestinal-associated lymphoid tissues (GALTs), with the potential to reduce both viral loads and chronic inflammation within the gut. Treatment with a primatized anti-α4β7 monoclonal antibody (mAb) initiated prior to SIV infection in RMs has been shown to reduce mucosal transmission and reduce the viral loads within the gut (18).

Collectively, data generated with these single interventions showed that α4β7 blockade limited viremia in mucosal sites of HIV persistence, and IL-21 promoted the reconstitution of mucosal T_H17 cells, critical to maintain mucosal integrity and limit microbial translocation, one key cause of chronic immune activation in HIV and SIV infection. Therefore, we propose that a combined strategy based on administration of IL-21 and anti-α4β7 mAb could have the potential to limit inflammation and, as a consequence, improve antiviral immune responses and reduce viral persistence in ART-suppressed HIV-infected individuals. Although IL-21 and anti-α4β7 mAb administration has been tested individually and found to be safe, co-administration of the two compounds has never been tested or reported. Here, we conducted a pilot study aimed at determining the safety, tolerability, and biological activity of the combined IL-21 and anti-α4β7 mAb treatment in healthy, SIV-uninfected RMs. The data generated from this pilot study will guide future combined interventions in ART-treated SIV-infected non-human primates, aimed at limiting residual inflammation and viral persistence.

MATERIALS AND METHODS

Animal Ethical Consideration and Treatment

All animal experiments were conducted following guidelines established by the Animal Welfare Act and the National Institutes of Health (NIH) for Housing and Care of Laboratory Animals and performed in accordance with institutional regulations after review and approval by the Institutional Animal Care and Usage Committees at the Yerkes National Primate Research Center (YNPRC). Anesthesia was administered prior to performing any procedure, and proper steps were taken to minimize the suffering of the animals in this study. A total of four Indian origin RMs (*Macaca mulatta*) were enrolled in this pilot study (**Supplementary Table 1**). All macaques were housed and maintained at the YNPRC (Atlanta, GA, USA). All animals received two doses of rhesus anti-α4β7 mAb (50 mg/kg, intravenous route) obtained from NIH Non-human Primate Reagent Resource, University of Massachusetts Medical School, Worcester, MA, USA, at 3-week interval (days 0 and 21) and seven weekly doses (days 0, 7, 14, 21, 28, 35, and 42) of recombinant rhesus IL-21–IgFc (IL-21-Fc, 100 μg/kg, subcutaneous route) obtained from Resource for Nonhuman Primate Immune Reagents of the New Iberia Research Center.

Sample Collection and Processing

Blood and rectal biopsies (RBs) were collected at multiple time points before, during, and after the interventions. Blood samples were used for complete blood counts and comprehensive serum chemistry panels. Plasma was separated from EDTA-anticoagulated blood by centrifugation within 1 h of phlebotomy. Density centrifugation was used to isolate peripheral blood mononuclear cells (PBMCs). Up to 20 RBs were collected with biopsy forceps under visual control via an anoscope. Rectal

biopsy–derived lymphocytes were isolated by digestion with 1 mg/mL collagenase for 2 h at 37°C and then passed through a 70-μm cell strainer to remove residual tissue fragments. All samples were processed, stained, fixed (1% paraformaldehyde), and analyzed by flow cytometry within 24 h of collection as described previously (10).

Flow Cytometric Analysis

Flow cytometric analysis was performed on PBMCs and RB-derived cells according to standard procedures using a panel of mAbs that others and we have shown to be cross-reactive with RM immune cells (10, 19) (**Supplementary Table 2**). The following Abs were used: anti–CD4-APCCy7 (clone OKT4), anti–HLA-DR-BV711 (clone L243), and anti-CD20 PerCpCy5.5 (clone 2H7) all from Biolegend, San Diego, CA, USA; anti–CD95-CF594 (clone DX2), anti–beta7-PECy5 (clone FIB504), anti–CCR7-PECy7 (clone 3D12), anti–Ki67-Alexa700 (clone B56), anti–CD3-BUV395 (clone SP34-2), anti–CD8-BUV496 (clone RPA-T8), anti–CD56-BV605 (clone B159), and anti–CD16-BV650 (clone 3G8) all from Becton–Dickinson, BD Biosciences, San Jose, CA, USA; anti–NKG2A-APC (clone Z199), from Beckman Coulter, Brea, CA, USA; Aqua Live/Dead amine dye-AmCyan from ThermoFisher Scientific, Invitrogen, Waltham, MA, USA; anti–CD38-FITC (clone AT-1) from STEMCELL Technologies, Vancouver, British Columbia, Canada; and anti–α4β7-PE (clone Act-1) obtained from the NIH Non-human Primate Reagent Resource, University of Massachusetts Medical School. Flow cytometric acquisition was performed on at least 100,000 $CD3^+$ T cells on a BD LSRII Flow Cytometer driven by BD FACSDiva software. Analyses of the acquired data were performed by FlowJo software, Tree Star, Inc., Ashland, OR, USA.

Measurement of Rhesus Anti-α4β7 mAb in Plasma

Levels of rhesus anti-α4β7 mAb in plasma samples from the four macaques were quantified as previously described (20). Briefly, HuT78 cells were first incubated at 37°C for 3 days in RPMI 1640 media containing 1 μM retinoic acid to increase surface expression of α4β7; 1×10^5 cells/well were dispensed into 96-well plates and incubated with plasma (1:10, diluted in phosphate-buffered saline (PBS)/2% fetal bovine serum) for 30 min at 4°C. Cells in the wells were washed and incubated with biotinylated antirhesus IgG1 kappa (clone 7H11; NIH Non-human Primate Reagent Resource, University of Massachusetts Medical School) for 30 min at 4°C and then washed again and resuspended in neutravidin-PE (A-2660; ThermoFisher Scientific) for 20 min at 4°C. Cells were washed, fixed in 2% paraformaldehyde, and analyzed on a flow cytometer (Attune NxT; ThermoFisher Scientific). Rhesus anti-α4β7 antibody was quantified using a standard curve method by comparing the mean channel fluorescence intensity mean channel fluorescent intensity (MFI) of cells treated with macaque plasma to the mean channel fluorescence intensity MFI of cells treated with serially diluted rhesus anti-α4β7 mAb (clone Act-1, obtained from NIH Non-human Primate Reagent Resource, University of Massachusetts Medical School).

Measurement of Rhesus Anti-rhesus (Anti-drug) Antibodies

To determine whether the rhesus may potentially generate antibodies against the infused recombinant rhesus anti-a4b7 mAb, an enzyme-linked immunosorbent assay (ELISA)–based assay was developed to monitor the detection of such rhesus anti-rhesus Ig antibodies (RARA), also named anti-drug antibodies (ADAs). Detection of antibodies generated against rhesus IgG1 kappa chain in RM plasma was measured by detecting monoclonal anti-lambda light chain bound to immobilized rhesus recombinant anti-α4β7 antibody by ELISA assays. In brief, ELISA plates (ThermoFisher Scientific) were coated with anti-α4β7 antibody (NIH NHP RR–Rhesus Recombinant IgG1 kappa, CDR-g, lot no. 092012G) in coating buffer (1× PBS) at 10 μg/mL. 100 μl were added to individual wells of the 96-well microtiter plate and left overnight at 4°C. Plates were then washed six times with wash buffer (PBS/0.05% Tween 20) and blocked with 300 μL per well of Superblock solution (ThermoFisher Scientific) for 15 min at room temperature (RT) followed by washing six additional times with wash buffer. The test sera from the monkeys to be screened for ADAs were 4-fold diluted (starting at 1:10) in dilution buffer (PBS/2% bovine serum albumin) and dispensed into duplicate wells at 100 μL per well. After 1-h incubation at RT, plates were washed six times with wash buffer followed by the addition of 100 μL of a 1:100 dilution of a monoclonal anti–lambda light chain-biotin (clone IS7-24C7; Miltenyi Biotech, Cologne, Germany) per well. After 1-h incubation at RT, plates were washed six times with wash buffer followed by the addition of 100 μL of a 1:10,000 dilution of streptavidin–horseradish peroxidase (HRP) (Invitrogen) per well. After 1-h incubation at RT, wells were washed six times with wash buffer followed by the addition of TMB substrate (SeraCare, Gaithersburg, MD, USA) at 100 μL per well to develop color and finally halted with stop solution containing H_2SO_4 (KPL). The optical density (OD) was recorded at 450 nm on Spectramax i3x plate reader (Molecular Devices, San Jose, CA, USA). Controls consisted of wells with baseline monkey sera and PBS alone (negative control); sera from a previously titered ADAs containing positive sera served as a positive control. Briefly, the positive control RNo13 was a RM infected intravenously with SIV_{mac239}, which initiated ART at week 5 of infection for a 90-day course. At week 9 of infection, and similarly to our study, this animal received 50 mg/kg of mAb against α4β7 intravenously every 3 weeks (21). The end point was noted as the highest dilution of the test sera with OD >2× pretreatment sample, and this dilution was considered positive for the assay.

Measurement of Rhesus IL-21–Fc in Plasma

Maxisorp 96-well plates were precoated overnight at 4°C with 2 μg/mL purified anti–human IL-21 capture mAb (clone J148-1134; BD Biosciences) in 100 μL bicarbonate buffer pH 9.6 per well. The next morning, the unbound antibody was removed, and the coated plates were blocked for 2 h with 300 μL per well of PBS with 2% bovine serum albumin at 37°C. The plates were then washed four times with PBS supplemented with 0.05%

Tween 20, added serial 2-fold dilutions of test plasma samples in duplicates and a dilution series of a IL-21–Fc standard, and incubated for 2 h at RT. Plates were washed and added 100 μL of anti–IL-21–biotin detection mAb (clone I76-539; BD Biosciences) at a 1:2,000 dilution and incubated for 2 h at RT. After washing, the plates were added HRP-conjugated Avidin D (Vector Laboratories, Burlingame, CA, USA) at a 1:2,000 dilution followed by TMB substrate (KPL) in sequential steps. The reaction was stopped by the addition of 20 μL of 1 M H_2SO_4, and the absorbance read at 450 nm using a Bio-Tek Synergy HT multimode microplate reader. Baseline plasma samples for each test subject collected prior to IL-21 administration were included to determine background values. The lower detection limit of IL-21–Fc was 15.6 pg/mL.

Measurement of Rhesus Anti–IL-21–Fc Antibodies

To determine whether the rhesus may potentially generate antibodies against the infused recombinant rhesus IL-21–Fc, an ELISA-based assay was performed. Maxisorp 96-well plates were precoated overnight at 4°C with 4 μg/mL recombinant rhesus IL-21–Fc in 100 μL of 1× coating solution (KPL) per well. The next morning, plates were washed with PBS 0.05% Tween-20 and blocked by adding 200 μL/well of PBS with 1% bovine serum albumin (blocking buffer) at 4°C overnight. After washing the plates, 100 μL plasma samples diluted (1:100, 1:1,000, and 1:10,000) in blocking buffer were added in duplicate to wells, or blocking buffer was added to wells for negative and positive control wells. Following an incubation at 4°C overnight, the plates were washed, and 100 μL of monkey cross-reactive goat anti–human kappa-Biot (Southern Biotech, Birmingham, AL, USA) diluted 1:1,000 in blocking buffer was added to sample and negative control wells. To positive control wells, 100 μL of goat anti–monkey IgG biotin (Rockland, Limerick, PA, USA) diluted 1:1,000 in blocking buffer was added. The plate was incubated for 2 h at RT. After washing, HRP-conjugated avidin D at a 1:1,000 dilution was added to wells and incubated for 1 h. Plates were washed and developed with TMB substrate and read on a microplate reader as described above for the IL-21–Fc capture ELISA. Baseline plasma samples for each test subject collected prior to IL-21 administration were included to determine background values. The positive control confirmed the correct coating of the wells and consisted of a mouse anti–human IL-21 mAb (clone I76-539, BD #558502) that cross-reacts with the rhesus IL-21–Fc, and it specifically binds to this cytokine. Of note, with this assay, we do not detect the lambda chain antibodies, but across multiple isotypes (IgA, IgG, IgM).

Statistical Analysis

Data analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The results are expressed as the mean ± SD. Statistical significance (*P*-value) of immunophenotyping data between time points was not reported because of the limited number of animals included in the pilot study.

RESULTS

The Combined Administration of IL-21 and Anti-α4β7 mAb Is Safe and Tolerated in RMs

IL-21 and anti-α4β7 mAbs have been previously administered as single interventions in naive or SIV-infected RMs with an acceptable safety profile (10, 18, 21–25). However, combined administration of the two reagents has not yet been tested. To determine the safety and tolerability of the combined IL-21 and anti-α4β7 mAb administration in non-human primates, four healthy, SIV-uninfected RMs were treated with two doses of anti-α4β7 mAb (50 mg/kg, intravenous) at a 3-week interval (days 0 and 21) and seven weekly doses (from days 0 to 42) of recombinant rhesus IL-21–IgFc (IL-21-Fc, 100 μg/kg, subcutaneous) (see section Materials and Methods and **Figure 1A**). First, we measured variations in weight and multiple hematological parameters. All four RMs included in the study showed stable or increased weights up to day 78 post-infusion of the combined treatment, the latest assessed experimental point, when compared with pre-treatment baseline (**Figure 1B**). Multiple hematological parameters were analyzed to determine possible anemias [red blood cell (RBC) count and hemoglobin (HGB)] or kidney dysfunction [blood urea nitrogen (BUN)]. We did not find any significant changes in RBC**,** HGB, and BUN levels, which remained stable over the entire follow-up period (**Figures 1C–E**). Then, we monitored serum chemistry parameters such as creatinine (kidney function), alanine aminotransferase (ALT; liver function), total protein (T-Prot; kidney and liver functions), and aspartate aminotransferase (AST; kidney, liver, and heart function) and found no significant variation from baseline with all measured values for the entire follow-up period (**Figures 1F–I**). Thus, at the doses administered, combined administration of IL-21 and anti-α4β7 mAb is well-tolerated and has no detectable toxic effects.

Co-administration of IL-21 and Anti-α4β7 mAb Does Not Induce ADAs Or Alter the Bioavailability of the Two Compounds

Previous studies have shown that the administration of anti-α4β7 mAb can lead to the development of ADA in a subset of RMs, which resulted in loss of anti-α4β7 mAb biological activity (21, 23, 24). In one of those studies, in which 11 RMs received eight intravenously doses of the anti-α4β7 mAb (50 mg/kg each; at weeks 9, 12, 16, 18, 20, 24, 28, and 32 post-SIV infection), three animals developed ADA responses starting either after two, three, or six doses (21). In order to test whether repeated and combined infusions of IL-21 and anti-α4β7 mAb induced ADA responses, we measured the levels of rhesus ADA against the anti-α4β7 mAb in the plasma of the four treated RMs. The plasma end point titers for all RMs before infusion as well as after infusion and until day 78 remained unchanged (**Figure 2A**). A positive control serum from monkey RNo13 was used as a positive control, which was collected during the aforementioned *in vivo* study (21), with a titer of 1:10,000 (**Figure 2A**). Similarly, we did not find any measurable levels of anti–IL-21–Fc in the plasma of the four RMs

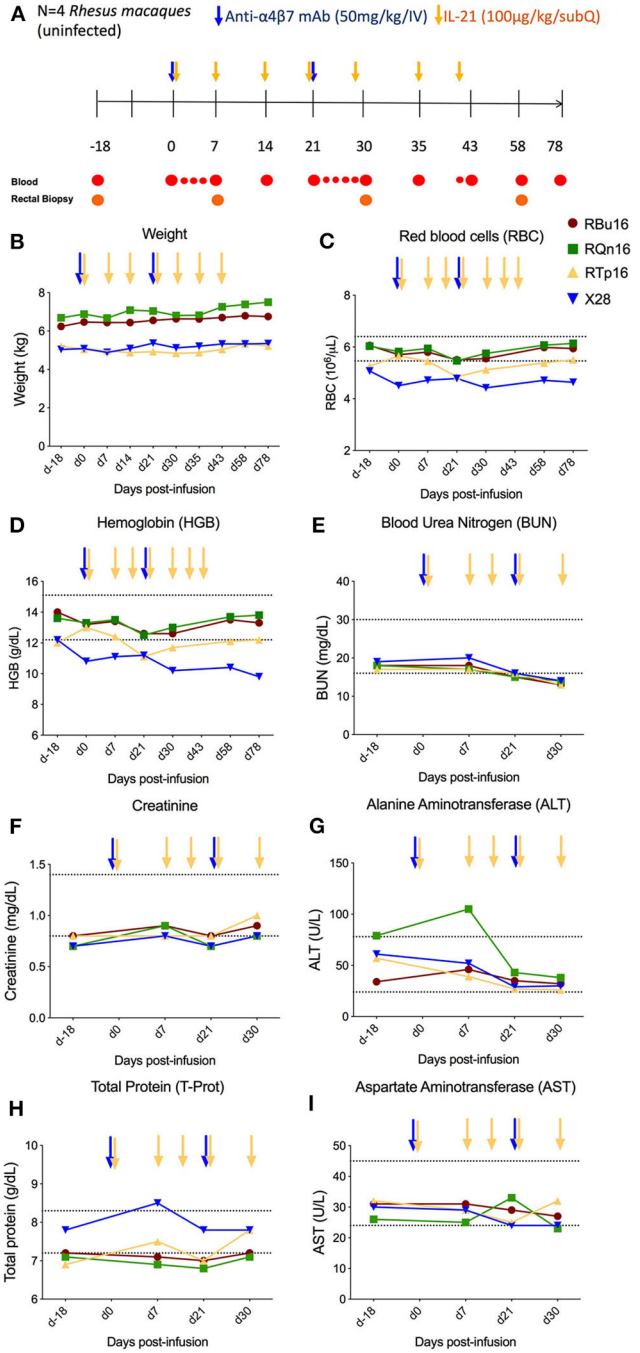

FIGURE 1 | Co-administration of anti-α4β7 mAb and IL-21. **(A)** Schematic study design of co-administration of anti-α4β7 mAb and IL-21 in uninfected RMs ($n = 4$). Prior to treatment, baseline blood and RBs were collected. On day 0, 50 mg/kg of anti-α4β7 mAb was administered intravenously along with 100 μg/kg of IL-21 subcutaneously. IL-21 was given weekly up to 6 weeks (day 42 post-infusion). 50 mg/kg of anti-α4β7 mAb was infused in two doses on day 0, week 3 (day 21 post-infusion). Blood samples were collected at regular intervals as shown in the schema. **(B)** Weight changes were recorded regularly until the end of treatment. **(C)** Red blood cells, **(D)** hemoglobin, **(E)** BUN, **(F)** creatinine, **(G)** ALT, **(H)** total protein, and **(I)** AST were analyzed from the blood collected at regular intervals until the end of the study. Individual

(Continued)

FIGURE 1 | animals are represented with different colors and symbols. Baseline days are indicated as d-18 and d0. Normal range levels of each parameter analyzed are indicated in dashed lines. Blue arrows indicate the anti-α4β7 mAb intravenous infusions, and yellow arrows indicate IL-21 subcutaneous infusions.

neither at any tested time points or at any tested dilutions (1:100, 1:1,000, and 1:10,000) (**Figure 2B**), whereas positive control showed measurable anti–IL-21–Fc levels, confirming the correct coating of the plate (data not shown). These results indicate that, at least under the conditions used in this study, the co-administration of IL-21 and anti-α4β7 mAb did not promote the induction of ADA against either therapeutic agent. Next, we quantified the levels of anti-α4β7 mAb in plasma using flow cytometry as described in section Materials and Methods and as previously published (20). Mean baseline levels of anti-α4β7 mAb in all RMs before anti-α4β7 mAb administration were less than 40 μg/mL (**Figure 2C**); this is likely due to pre-existing antibodies against anti-α4β7 or assay background. The mean plasma levels of anti-α4β7 mAb increased to 95.6 μg/mL and to 213 μg/mL by day 7 after the first and second dose of 50 mg/kg infusion of anti-α4β7 mAb, respectively; the mean plasma levels were maintained at 92 μg/mL until day 42 post-infusion (**Figure 2C**). The stable levels of anti-α4β7 mAb during weekly administration of IL-21 suggest that IL-21 did not markedly influence levels of the anti-α4β7 mAb and that there were no drug–drug interactions. Finally, we quantified IL-21–Fc levels in plasma by ELISA. IL-21–Fc plasma levels increased after IL-21 infusion for all four animals compared with their baseline levels (<15.625 pg/mL in all animals). Its maximum concentration was achieved at day 1 post-infusion for RTp16 (323.24 pg/mL), and day 3 post-infusion for the remaining animals (RBu16: 696.54 pg/mL, RQn16: 228.3 pg/mL, and X28: 116.6 pg/mL) (**Figure 2D**). Increased plasma levels of IL-21 were still evident on day 22, 1 day after the fourth IL-21 infusion (performed on day 21), but attenuated afterward.

The Combined IL-21 and Anti-α4β7 mAb Treatment Reduces Gut Homing of Memory CD4 T Cells Expressing α4β7

To investigate the impact of combined IL-21 and anti-α4β7 mAb therapy impact on T-cell gut homing, we quantified the frequency of memory CD4 ($CD3^+CD4^+CD95^+$) and CD8 ($CD3^+CD8^+CD95^+$) T cells expressing $\alpha4\beta7^{hi}$ or β7 in blood (**Figures 3A–C**, and **Supplementary Figures 1A–C**) and RBs (**Figures 3D–F** and **Supplementary Figures 1D–F**) collected longitudinally during the study. The frequencies of $\alpha4\beta7^{hi}$ CD4 memory T cells decreased by greater than 99% in blood (**Figure 3B**) and 92.5% in RBs (**Figure 3E**) already at 1 week after the first anti-α4β7 mAb infusion; these measured levels remained constant up to day 58 post-infusion, ∼5 weeks after the second dose of anti-α4β7 mAb and gradually increased thereafter, although they remained still below baseline on day 78 post-infusion, the latest time point of the study (**Figures 3B,E**). Similar results were found for CD8 T cells, with the frequencies

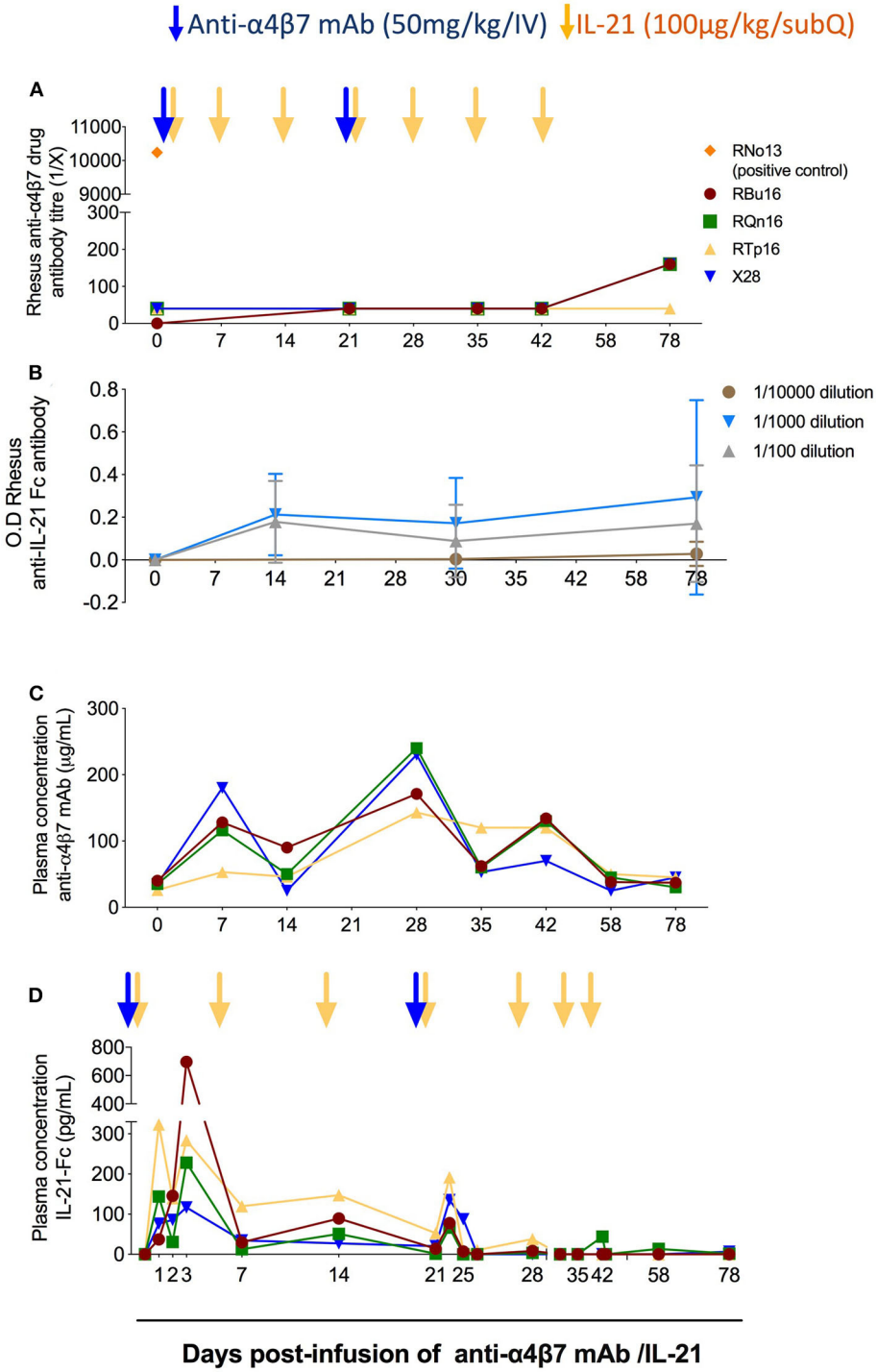

FIGURE 2 | Co-administration of anti-α4β7 mAb and IL-21 does not elicit reactive antibodies**. (A)** Analysis of rhesus antibodies anti-α4β7 mAb in plasma at different time points. The anti-drug antibodies levels were measured by ELISA end point titer method as described in the Materials and Methods section. RNo13, indicated in orange, corresponds to the plasma of an animal from a previous study that developed ADAs (21), and it was used as a positive control. **(B)** Measurement of anti–IL-21–Fc antibodies in plasma at different time points. Anti–IL-21–Fc antibodies were measured by ELISA longitudinally until the end of the study with three different dilutions (1:100, 1:1,000, and 1:10,000). **(C)** Measurement of anti-α4β7 mAb plasma levels (µg/mL) in RMs ($n = 4$). The assay was performed using flow cytometry with HuT 78 cells. The levels of anti-α4β7 mAb were measured employing standard curve method. Mean fluorescence intensity (MFI) of known concentration of anti-α4β7 mAb was obtained, and then the MFI of plasma levels of anti-α4β7 mAb was plotted. **(D)** Measurement of IL-21–Fc plasma levels (pg/mL) in RMs ($n = 4$). ELISA background and plasma baseline values were subtracted from the values analyzed at each time point. Individual animals are represented with different colors and symbols. Blue arrows indicate the anti-α4β7 mAb intravenous infusions, and yellow arrows indicate IL-21 subcutaneous infusions.

of $\alpha4\beta7^{hi}$ CD8 memory T cells decreased by greater than 99% in blood (**Supplementary Figure 1B**) and 97% in RB (**Supplementary Figure 1E**). Because the mAb used for flow cytometry staining recognizes the same epitope as the anti-α4β7 mAb used *in vivo*, these data indicate the biological activity of the anti-α4β7 mAb in targeting α4β7 expressed on CD4 and CD8 T cells in blood and gut. Of note, the very low frequencies of $\alpha4\beta7^{hi}$ CD4 and CD8 memory T cells were maintained during the IL-21 only administrations (days 30, 35, and 43 post-infusion) that followed the last dose of anti-α4β7 mAb (day 21 post-infusion), showing that the administration of IL-21 did not influence the expression of α4β7 or the ability of the anti-α4β7 mAb to target it. Furthermore, PBMCs were monitored for the expression of β7 using an anti-β7 mAb that does not compete for the epitope recognized by the anti-α4β7 mAb administered *in vivo*; as such, this analysis allows for discriminating whether the inability to stain for α4β7 results from *in vivo* depletion of α4β7 expressing cells or masking of the α4β7 molecule on cells, as well as to determine the impact of the treatment on the trafficking of $\beta7^{+}$ cells to the gut. As shown in **Figure 3C**, β7 expression on blood memory CD4 T cells was increased up to 2-fold at day 30 post-infusion as compared to pre-treatment levels (**Figure 3C**; from 30.25 to 61.88%). In RBs, frequencies of $\beta7^{+}$ cells were significantly lower up to day 58 post-infusion as compared to pre-treatment (**Figure 3F**, from 49.7 to 4.74%). Interestingly, β7 expression on memory CD8 T cells differed from that of memory CD4 T cells after anti-α4β7 mAb administration. Specifically, frequencies of memory CD8 T cells expressing β7 were reduced from 38.7 to 10.77% in blood, whereas they remained stable in the gut (**Supplementary Figures 1C, F**). Together, these data indicate that anti-α4β7 mAb treatment combined with IL-21 effectively reduces the homing of $\alpha4\beta7^{hi}$ memory CD4 T cells to the gut mucosa.

Effect of Combined IL-21 and Anti-α4β7 mAb Treatment on NK Cells

We then measured the effect of combined IL-21 and anti-α4β7 mAb treatment on the frequency (in PBMCs and RB) and absolute number (limited to PBMCs) of NK cells and NK cell subsets ($CD56^{-}CD16^{+}$, $CD56^{+}CD16^{-}$, and $CD16^{-}CD56^{-}$). Overall, the levels of NK cells and their subsets remained stable during the treatment both in PBMCs and RB. A slight increase was noted in the frequency (of total lymphocytes) of $CD56^{+}CD16^{-}$ and $CD56^{-}CD16^{-}$ NK cells in PBMCs during the treatment (**Supplementary Figures 2A,B**). In RB, increased frequency (of live cells) of NK cells was observed between days 7 and 30, followed by reduction to baseline levels upon interruption of IL-21 therapy (d58; **Supplementary Figure 2C**). This slight increase of bulk NK cells resulted in an increased frequency of $CD56^{-}CD16^{+}$ from baseline (d-18) to day 30 (**Supplementary Figure 2D**) and of $CD56^{+}CD16^{-}$ from baseline to day 7 (**Supplementary Figure 2E**), which return to baseline levels at day 58. In RB, $CD56^{-}CD16^{-}$ NK cells remained constant across the course of the study and were not affected by withdrawal of IL-21 therapy (not shown).

Combined IL-21 and Anti-α4β7 mAb Treatment Limits Immune Activation and Cell Cycling of Gut Memory CD4 T Cells

We assessed the effects of the combined IL-21 and anti-α4β7 mAb therapy on systemic and gut immune activation. For this aim, we measured the frequency of memory T cells expressing markers of activation (HLA-DR and CD38) and cell cycling (Ki67). The frequency of blood memory CD4 and CD8 T cells with an $HLA\text{-}DR^{+}CD38^{+}$ (**Figures 4A,C**) or $Ki67^{+}$ (**Figures 4B,D**) phenotype remained similar overall, with a slight decrease at specific time points. Specifically, the frequency of $HLA\text{-}DR^{+}CD38^{+}$ T cells was lower as compared to baseline on days 30, 58, and 78 after IL-21 plus anti-α4β7 mAb treatments for memory CD4 and at day 58 post-treatment for memory CD8 T cells. The frequency of $Ki67^{+}$ T cells was lower as compared to baseline only at day 14 post-treatment both for memory CD4 and CD8 T cells. Importantly, differences were more pronounced in gut, with a progressive reduction in the frequency of memory CD4 and CD8 T cells that are $HLA\text{-}DR^{+}CD38^{+}$ (**Figures 4E,G**) or $Ki67^{+}$ (**Figures 4F,H**) from baseline to day 30 post-treatment. The reduction in both immune activation and cell cycling in the gut is consistent with CD4 T cells expressing $\alpha4\beta7^{hi}$ being retained in blood as a result of the combined treatment. Collectively, our data show that a strategy based on the combined administration of IL-21 and anti-α4β7 mAb is effective in blocking the homing of memory $CD4^{+}$ $\alpha4\beta7^{hi}$ T cells to the gut and in reducing mucosal immune activation, even in healthy, SIV-uninfected RMs.

DISCUSSION

The results of the present study indicate that treatment of healthy, SIV-uninfected RMs with a combined IL-21 and anti-α4β7 mAb intervention (1) is safe and well-tolerated (at the tested doses), (2) does not affect bioavailability of both compounds, (3) can effectively bind the α4β7 receptor on both blood and gut mucosa T cells, and (4) reduces cell cycling and immune activation, particularly in gut mucosa. The data generated in this pilot study support future combined interventions in ART-treated, SIV-infected non-human primates, aimed at limiting residual inflammation and viral persistence, particularly in the gut mucosa.

At the doses administered in our study, combined administration of IL-21 and anti-α4β7 mAb was well-tolerated and did not result in any detectable toxicity. These results are aligned with previous studies where IL-21 and anti-α4β7 mAb were tested independently and proved to be safe in SIV-infected RMs (9, 10, 21). IL-21 treatment in RMs can increase the JAK/STAT signaling pathway, implicated to have roles in inflammation processes (10), whereas gene expression studies in vedolizumab-treated patients revealed dysregulated expression of genes related to cell cycle, cell growth, and inflammation (26). In our study, the combined treatment was not associated with any increase in parameters related to inflammation or immune activation.

The production of antibodies against drugs administered *in vivo* not only can reduce the bioavailability and biological

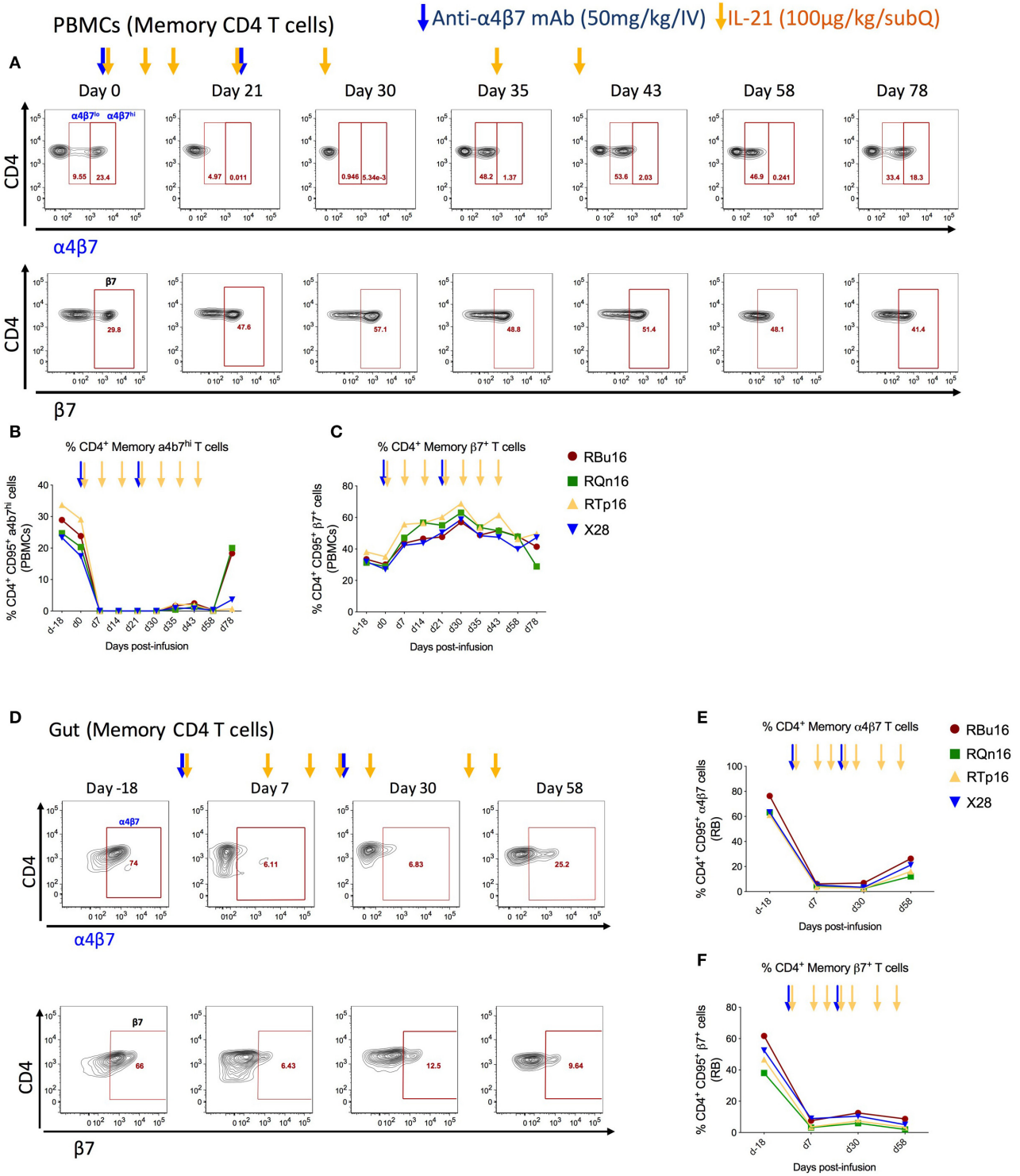

FIGURE 3 | Co-administration of anti-α4β7 mAb and IL-21 decreases gut homing of CD4 α4β7^{hi} to gut. **(A)** Representative flow cytometry staining of memory CD4 T cells expressing α4β7^{hi} (top panel) and β7^{+} (bottom panels) in PBMCs. **(B)** Frequency of α4β7^{hi} and **(C)** β7^{+} memory CD4 T cells in PBMCs. **(D)** Representative flow cytometry staining of memory CD4 T cells expressing α4β7^{+} (top panel) and β7^{+} (bottom panels) in gut. **(E)** Frequency of α4β7^{+} and **(F)** β7^{+} memory CD4 T cells in gut. Individual animals are represented with different colors and symbols. Baseline days are indicated as d-18 and d0. Blue arrows indicate the anti-α4β7 mAb intravenous infusions, and yellow arrows indicate IL-21 subcutaneous infusions.

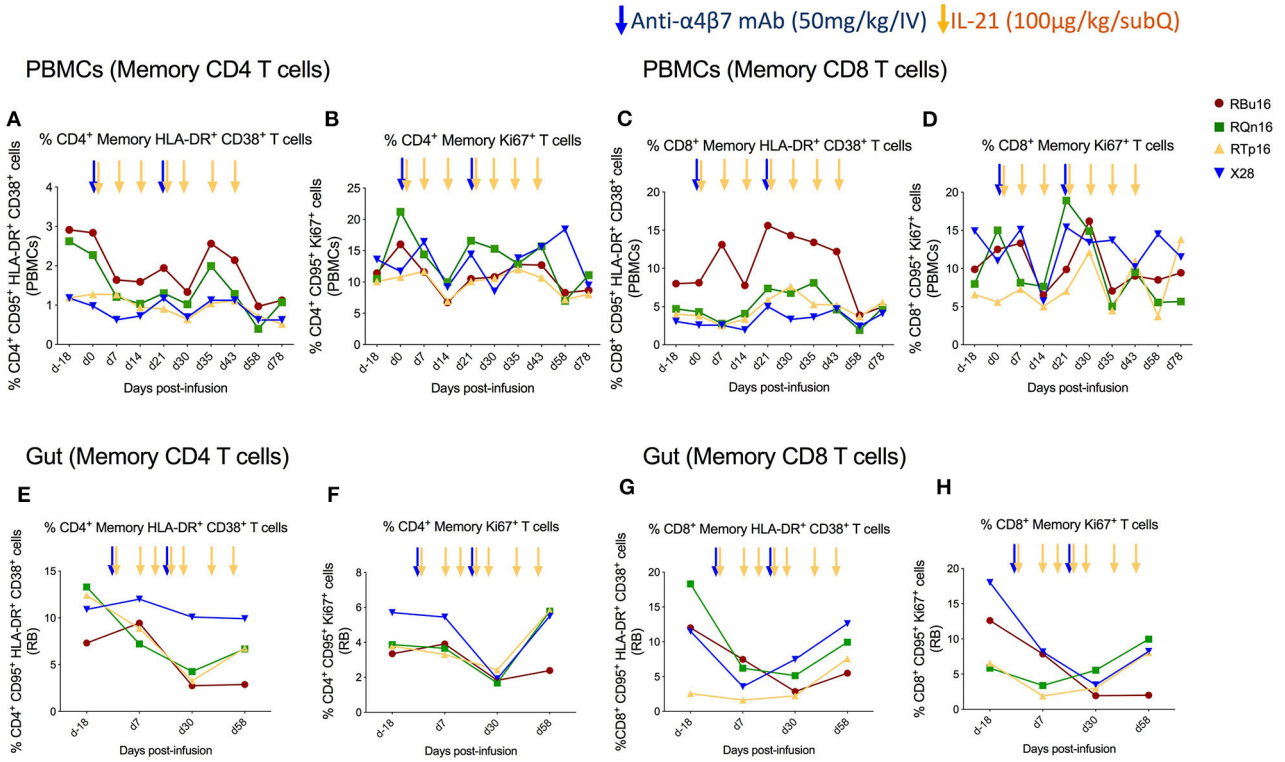

FIGURE 4 | Co-administration of anti-α4β7 mAb and IL-21 limits immune activation and cell cycling of memory CD4 T cells in gut**. (A)** Percentage of memory CD4 T cells expressing HLA-DR$^+$CD38$^+$, **(B)** Ki67$^+$, **(C)** memory CD8 T cells expressing HLA-DR$^+$CD38$^+$, and **(D)** Ki67$^+$ in PBMCs. **(E)** Percentage of memory CD4 T cells expressing HLA-DR$^+$CD38$^+$, **(F)** Ki67$^+$, **(G)** memory CD8 T cells expressing HLA-DR$^+$CD38$^+$, and **(H)** Ki67$^+$ in gut mucosa. Individual animals are represented with different colors and symbols. Baseline days are indicated as d-18 and d0. Blue arrows indicate the anti-α4β7 mAb intravenous infusions, and yellow arrows indicate IL-21 subcutaneous infusions.

activity of the administered compounds, but can also elicit the development of immune-mediated adverse events. In our study, we have not observed development of ADA responses against anti-α4β7 mAb or IL-21. In previous studies, which used anti-α4β7 mAb in SIV-infected RMs at similar doses as our study (500 μg/kg), a fraction of the animals developed ADA starting from the second, third, or sixth infusion (Byrareddy et al., 3 of 11 animals; Di Mascio et al., 1 of 12 animals). Our pilot study suggests that IL-21 does not favor the generation of ADA against anti-α4β7 mAb. Otherwise, we have not been able to detect antibodies against IL-21–Fc, despite we found a reduced plasma concentration of IL-21 after the fourth dose. To date, we have not seen development of ADA in any of the RMs we treated with a similar dose of IL-21 in the past several years (9, 10). It is possible that inhibition of α4β7 using anti-α4β7 mAb contributed to decreased levels of IL-21 and/or that repeated dosage of IL-21 resulted in saturation or decreased expression of the IL-21 receptor, making IL-21 to be freely available for a faster clearance as compared to bound IL-21. The consistent increase of plasma concentration of anti-α4β7 mAb during IL-21 infusions indicates that IL-21 does not negatively affect anti-α4β7 mAb bioavailability. Interestingly, there is an unexpected increase of anti-α4β7 plasma levels at day 42, present in three of the four treated RMs, without any new anti-α4β7 mAb administration. One possibility is that this results from differences in receptor activation (27) and or recycling processes, as observed for β1 integrin receptor in a previous study (28), altering the number of receptors able to bind the administered anti-α4β7 Ab. It is also possible that IL-21 administration contributed to increase plasma levels of anti-α4β7, although this cannot be directly proved in our pilot.

Of note, the anti-α4β7 antibodies used for staining and infusing the animals bind to the same antigen; thus, a lack of α4β7 staining by flow cytometry is interpreted as a measure of targeting engagement, that is, ability of the inoculated antibody to bind α4β7 expressed on cell surface, without discriminating if the lack of staining is due to receptor downregulation, cell lysis, or receptor blockage by the competing antibody. Interestingly, we discovered an increase in the frequency of β7$^+$ CD4 memory T cells in blood, but a decrease of these cells in RB. These findings suggest that the administration of anti-α4β7 mAb reduced the trafficking of α4β7^{hi} T cells to the gut, confirming the mode of action, prevention of trafficking of activated T cells to the gut, of vedoluzimab as adjunctive therapy in inflammatory bowel disease and Crohn disease (IBD/CD) (29–34). As such, a similar strategy is of interest in the context of HIV infection, where CD4 T cells that express CCR5 and α4β7 are the preferred target for HIV infection in the gut, a major site for early HIV infection and replication (2, 35–37).

Combined administration of IL-21 and anti-α4β7 antibody decreased the frequency of T cells expressing immune activation and proliferation markers in the gut of healthy RMs, despite the low baseline level. This result supports the use of this combined treatment in the context of SIV infection in RMs. Our previous studies showed that, by favoring maintenance of T_H17 and T_H22 cells, IL-21 improves mucosal integrity and reduces inflammation when administered in acute (9) or chronic, ART-treated (10) SIV-infected RMs. Similarly, anti-α4β7 antibody reduced SIV infection in the GALT when animals were challenged either intravenously, intrarectally, and intravaginally (18, 38, 39). In another recent study, the combination of primatized anti-α4β7 and VRC01 significantly delayed vaginal SHIV exposure and reduced viral loads in rectal tissues compared to control (40). The effect of anti-α4β7 mAb administered in SIV-infected RMs during ART continues to be a highly debatable issue. While an earlier study showed that this treatment can limit viral rebound after ART interruption (21), more recent pre-clinical (23–25) and clinical (41) studies did not show any significant benefit from anti-α4β7 mAb treatment in ART-suppressed, HIV-infected individuals or SIV-infected RMs in inducing viral remission in the absence of ART (42). Recently, using samples obtained from various gastrointestinal sites from IBD/CD patients, it was found that anti-α4β7 therapy led to a significant reduction of lymphoid aggregates, mostly in the terminal ileum (43). Because lymphoid aggregates serve as important sanctuary sites for maintaining viral reservoirs, the authors proposed that their ablation by anti-α4β7 mAb should be considered in developing novel therapies for HIV remission. These findings highlight that much has yet to be learned about the mechanisms of action and biologic effects of anti-α4β7 therapy, as well as on the combination of anti-α4β7 mAb with additional immunotherapies to provide immunologic and virologic benefits.

This is the first study in non-human primates showing that anti-α4β7 mAb and IL-21 treatment can be administered safely and can reduce cell cycling and immune activation, particularly in gut mucosa. As such, our study provides rationale to explore this combined treatment as a strategy aimed at limiting immune activation and viral persistence in ART-suppressed, SIV-infected RMs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Usage Committees at the Yerkes National Primate Research Center, Emory University.

AUTHOR CONTRIBUTIONS

MPi, MPa, and SB contributed to study design. MPi, SU, KP, CK, KN, IS, and KR contributed to data collection. MPi, SU, KP, KR, and SB contributed to data quality and analysis. MPi, SU, FV, MPa, and SB wrote the manuscript. All authors contributed to manuscript development and have critically reviewed and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01275/full#supplementary-material

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Measuring the Inducible, Replication-Competent HIV Reservoir Using an Ultra-Sensitive p24 Readout, the Digital ELISA Viral Outgrowth Assay

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Quantifying the inducible HIV reservoir provides an estimate of the frequency of quiescent HIV-infected cells in humans as well as in animal models, and can help ascertain the efficacy of latency reversing agents (LRAs). The quantitative viral outgrowth assay (QVOA) is used to measure inducible, replication competent HIV and generate estimations of reservoir size. However, traditional QVOA is time and labor intensive and requires large amounts of lymphocytes. Given the importance of reproducible and accurate assessment of both reservoir size and LRA activity in cure strategies, efforts to streamline the QVOA are of high priority. We developed a modified QVOA, the Digital ELISA Viral Outgrowth or DEVO assay, with ultra-sensitive p24 readout, capable of femtogram detection of HIV p24 protein in contrast to the picogram limitations of traditional ELISA. For each DEVO assay, 8–12 $\times$ 10^6 resting CD4 + T cells from aviremic, ART-treated HIV + participants are plated in limiting dilution and maximally stimulated with PHA, IL-2 and uninfected allogeneic irradiated PBMC. CD8-depleted PHA blasts from an uninfected donor or HIV-permissive cells (e.g., Molt4/CCR5) are added to the cultures and virus allowed to amplify for 8–12 days. HIV p24 from culture supernatant is measured at day 8 by Simoa (single molecule array, ultra-sensitive p24 assay) confirmed at day 12, and infectious units per million CD4 + T cells (IUPM) are calculated using the maximum likelihood method. In all DEVO assays performed, HIV p24 was detected in the supernatant of cultures as early as 8 days post stimulation. Importantly, DEVO IUPM values at day 8 were comparable or higher than traditional QVOA IUPM values obtained at day 15. Interestingly, DEVO IUPM values were similar with or without the addition of allogeneic CD8-depleted target PHA blasts or HIV permissive cells traditionally used

to expand virus. The DEVO assay uses fewer resting CD4 + T cells and provides an assessment of reservoir size in less time than standard QVOA. This assay offers a new platform to quantify replication competent HIV during limited cell availability. Other potential applications include evaluating LRA activity, and measuring clearance of infected cells during latency clearance assays.

Keywords: QVOA, DEVO, HIV, outgrowth, IUPM

INTRODUCTION

With an estimated 40 million people living with HIV (PLWH), and given the health, stigma, and financial burden associated with chronic HIV infection, eliminating the HIV pandemic remains a priority both from a public health and societal perspective. While successful antiretroviral therapy (ART) has significantly reduced the mortality and morbidity associated with HIV infection, the existence of long-lived viral reservoirs capable of reigniting fulminant infection in the absence of ART remains one of the major barriers toward achieving an HIV cure. With two documented functional cures, first the Berlin Patient (1) and more recently the London Patient (2), there is renewed hope and interest in the quest to eliminate persistent HIV infection. Modalities to target HIV persistence are being tested in the clinic. A large proportion of persistent HIV is defective and unable to replicate (3–5). Clinical interventions targeting the HIV reservoir would benefit greatly from assays that can rapidly and precisely quantitate the replication competent HIV reservoir in order to assess the efficacy of therapeutic interventions aimed at depleting the reservoir. Standard PCR-based assays offer a relatively rapid and sensitive method to quantitate persistent HIV infection. However, as most of these assays amplify one conserved genomic region, they do not distinguish between replication-competent and defective provirus (3–5). The recently reported Intact Proviral DNA Assay (IPDA) has the added advantage over standard PCR assays in that by using two sets of primer probes targeting an intact packaging signal (PS) and the Rev-responsive element within *Env*, it increases the probability of amplifying mostly intact proviral genome (6). Although relatively streamlined and amenable to high throughput, 30–40% of virus amplified by this method is likely to be defective, and sequence polymorphism may limit the ability of primers and probes to amplify intact provirus (6, 7). The QVOA is considered the gold standard to measure replication-competent, inducible provirus. The QVOA provides a minimal, but definitive estimate of the inducible HIV reservoir (8–10). However, this assay can be costly and labor intensive. Additionally, as latently infected CD4 + T cells are present at low frequency, large numbers of cells are often required to increase sensitivity. Furthermore for some participants, the QVOA may under-represent the true frequency of latent but replication-competent proviruses due in part to the presence of "non-induced" proviruses unresponsive to a single round of cell stimulation (4, 11). Despite its limitations, the QVOA remains the most reliable method to measure replication competent HIV (12). Thus several modifications of this assay have been made to improve throughput, sensitivity and increased its dynamic range [reviewed in Falcinelli et al. (13)]. We report here a modified QVOA, the Digital ELISA Viral Outgrowth or DEVO assay which takes advantage of the Simoa platform (Quanterix Inc., Billerica, MA, United States) (14, 15). The Simoa or single molecule array is an ultrasensitive, fully automated immune assay platform capable of femtogram detection of HIV p24 protein in contrast to the picogram limitations of traditional ELISA (15–17). During the DEVO assay 8–12 $\times$ 10^6 purified resting CD4 + T cells from aviremic, ART-treated HIV + participants are PHA stimulated in limiting dilution in a 96 well-format and HIV p24 measured by Simoa.

To reduce non-specific signal, we use an optimized Simoa p24 protocol (16) in our assay. We found that virus can be expanded using either the CD4 T cell input alone (i.e., addition of exogenous donor cells is not necessary), PHA blasts from an uninfected donor, or HIV permissive cell lines such as the MOLT4/CCR5. Furthermore, with the DEVO assay, we obtained IUPM comparable or higher than the traditional QVOA at an earlier time point, thus reducing the overall length of the assay (18). While there have been other reports using the Simoa as a p24 readout for other QVOA modifications (19–21), to our knowledge, this is the first study describing a specific Simoa HIV outgrowth assay that has been meticulously and carefully evaluated for demonstrable accuracy and reproducibility.

RESULTS

Participant Characteristics

To develop the DEVO assay we used resting CD4 + T cells isolated from 12 PLWH, stably suppressed (<50 copies of HIV-1 RNA/ml on ART). Participants were 75% male and 25% female, had a mean age of 43.5 years with an average CD4 count of 740 cells/μl, and on ART for an average of 7.3 years, with a mean duration of suppression of 4.9 years (**Supplementary Table S1**). Leukapheresis or whole blood samples were obtained from participants through an ongoing longitudinal collection protocol approved by the University of North Carolina (UNC) biomedical institutional review board. All samples were collected in accordance with UNC guidelines and all participants provided informed consent prior to sample donation.

Simoa Detects HIV *gag* p24 Earlier Than Standard p24 ELISA During QVOA

Our primary goal for developing the DEVO assay was to investigate whether or not using ultra-sensitive p24 measurements by Simoa would shorten the duration of standard QVOA by detecting HIV p24 positive wells earlier than traditional ELISA methods. To that end, we harvested

TABLE 1 | HIV antigen is detected earlier using Simoa compared to standard ELISA in the traditional QVOA[Ψ].

	Number of HIV p24 positive wells/total wells cultured		
	Day 8		Day 15
Resting CD4 + T cells ($\times 10^6$)	**Simoa**	**Standard ELISA**	**Standard ELISA**
2.5	18/18	2/18	18/18
0.5	4/6	2/6	5/6
0.1	0/6	0/6	0/6
IUPM	1.927	0.088	2.49

[Ψ] *Representative QVOA experiment.*

supernatant from a standard QVOA assay on days 8, 15, and 19 post-stimulation and measured HIV p24 by both Simoa (day 8) and standard HIV *gag* p24 ELISA (days 8, 15, and 19). Seventy three percent of the wells were p24 positive by Simoa at day 8 compared to 13% of the wells by standard ELISA (**Table 1**). Importantly, 95% of the wells slated to become positive at day 15 during the traditional QVOA assay were already positive at day 8 by Simoa and the IUPM at day 8 were comparable to the day 15 traditional QVOA IUPM (**Table 1**).

We next performed a pilot experiment to determine whether the sensitivity of the Simoa would be maintained when fewer cells are used for the viral outgrowth assay. We stimulated approximately 8 million resting CD4 + T cells in limiting dilution as described under methods. Culture wells were then targeted with PHA blasts to amplify virus. We subsequently assessed p24 production from culture supernatant at day 8 post-stimulation. We observed that detection of p24 positive wells by Simoa was more sensitive with 10 positive wells detected, as compared to 3 wells by standard ELISA (**Supplementary Figure S1**).

Determination of the Lower Limit of Quantitation of the DEVO Assay

During the development of the DEVO assay, we initially used the lower limit of quantitation (LLOQ) generated from the Simoa assay standard curve, which is approximately 12 fg/ml and 3 times the median absolute deviation (MAD) as the cut off value from which to assign a well as positive. Additionally, to reduce non-specific noise in the assay, we employed an optimized Simoa p24 protocol developed by our colleagues at Merck Pharmaceuticals (16). However, despite these careful approaches, we observed that in some cases, wells that were low-positive for HIV p24 would become negative if cultured for additional days, suggesting further modification of the DEVO assay was necessary to capture true replication competent-HIV and avoid recording poorly adapted virus that are incapable of spreading in culture. To better understand the level of background noise in the assay, we performed a mock viral outgrowth assay using PBMC isolated from an uninfected donor. Approximately 2–6 million PBMC were maximally stimulated in limiting dilution with PHA/IL-2/irradiated PBMC or with survival amount of IL-2 (10–20 U/ml) and cultured over 8 days. Cultures were targeted with PHA-blasts or no targets were added. Supernatant from cultures were harvested on day 8 and assayed by Simoa, using the optimized HIV p24 protocol referenced. Although false p24 signal was detectable in most of the wells, the values were below the limit of quantitation of the assay except for two wells in the PHA-stimulated, no target added culture conditions where values above the LLOQ were registered (**Figure 1**).

To eliminate the influence of low-level false p24 positive signal in the assay, we next employed the methods standardized by The Clinical and Laboratory Standards Institute (CLSI) to determine a new limit of quantitation based on total error of the assay (22). Resting CD4 + T cells were isolated from a normal donor and stimulated with PHA/IL-2/irradiated PBMC for 24 h. Cultures were washed to remove the PHA and targeted with PHA-blasts from an uninfected donor twice over the course of 19 days. On three separate days, supernatant harvested on days 8 and 12 post-stimulation were spiked with different concentrations of HIV p24 (Quanterix, Inc.) and assayed by Simoa. Values obtained were used to calculate assay bias, total error and percent total error as described under methods. The concentration at which there was $<$20% of total error was found to be approximately 50 fg/ml and this value was used as the DEVO assay LLOQ onward (**Figure 2**). In addition, for added rigor and to eliminate recording cryptic p24 signal from wells containing only defective provirus, only wells exhibiting sustained or increase in p24 production over the 2 days of the assay are scored as positive (**Supplementary Tables S2, S3**).

Comparison of the DEVO Assay to the Traditional QVOA

Using the optimizations defined above, we next compared the performance of the DEVO assay to the traditional QVOA in measuring the replication competent HIV reservoir of stably suppressed, ART-treated PLWH. Both assays were set-up in parallel using resting CD4 + T cells isolated from HIV + ART-suppressed donors. The QVOA was performed as described elsewhere (12, 23) using 34–50 million resting CD4 + T cells that are maximally stimulated with PHA/IL2/irradiated PBMC in limiting dilution. PHA-blasts from an uninfected CCR5 high donor are added to the cultures twice to expand virus and supernatant harvested on days 15 and 19 and assayed for HIV p24 by ELISA. For the DEVO assay 8–12 million resting CD4 + T cells are stimulated in limiting dilution as done for QVOA. Wells were either targeted with PHA blasts or in corresponding experiments received no blasts in order to assess whether virus can be expanded without the addition of exogenous cells. Culture supernatant were initially harvested on days 8, 12, 15, and 19 and p24 measured by Simoa. For both assays, the maximum likelihood method (24, 25) was used to calculate IUPM values. At day 8 post-stimulation, the DEVO assay generated IUPM values that were comparable to or in some assays higher than QVOA IUPM obtained at day 15 post-stimulation (**Figure 3A** and **Supplementary Figure S2**). However, the overall difference in IUPM between the DEVO at day 8 and the QVOA at day 15 were not statistically significant. Importantly, IUPM values for the two assays highly correlated (**Figure 3B**). Interestingly,

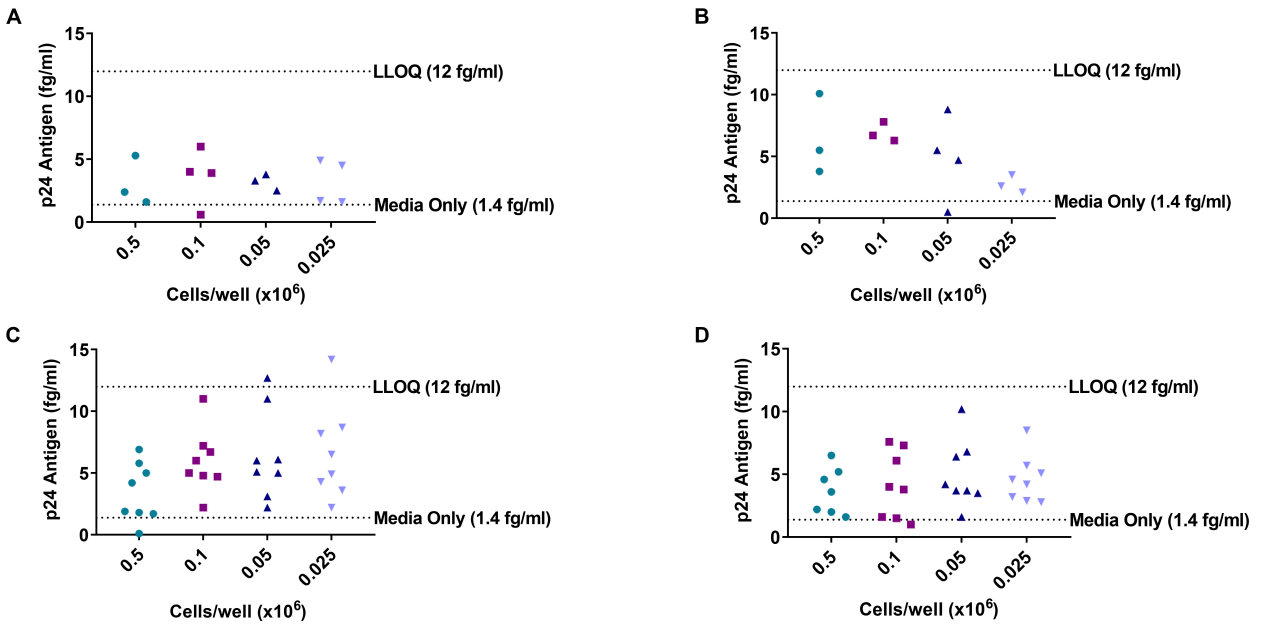

FIGURE 1 | Limiting dilution culture of uninfected donor PBMC reveals background noise of Simoa. PBMC from an uninfected donor were stimulated in limiting dilution with PHA/IL-2 and irradiated PBMC or treated with low amount of IL-2 only. Cultures were either targeted with PHA blasts or no targets were added. Supernatant were harvested at day 8 and HIV p24 assayed by Simoa. **(A)** Low IL-2 treated/no targets added. **(B)** Low IL-2 treated/cultures targeted with PHA blasts. **(C)** PHA stimulated/no targets added. **(D)** PHA stimulated/cultures targeted with PHA blasts. Each dot represents a well at the indicated cell dilution. LLOQ, lower limit of quantitation.

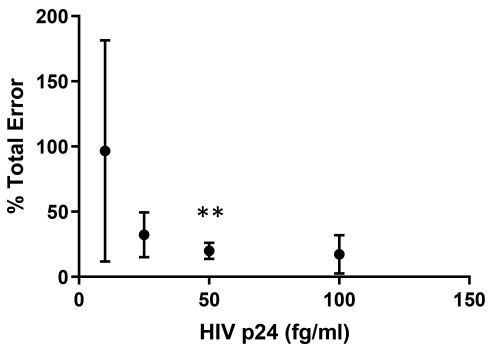

FIGURE 2 | The lower limit of quantitation for the DEVO assay. Freshly isolated resting CD4 + T cells from an uninfected donor were stimulated with PHA and irradiated PBMC, then co-cultured with PHA blasts over the course of 19 days. On three separate, but not consecutive days, supernatant from day 8 and 12 were spiked with HIV p24 protein at different dilutions and assayed by Simoa. Bias, total error and % total error was calculated from replicate well values using the following formula:
Bias = Average − Actual Value; Total Error = |Bias| + 2 × Standard Deviation; % Total Error = Total Error Actual Value × 100. The concentration with <20% total error was determined to be 49.21fg/ml**. This value was used as the DEVO lower limit of quantitation.

similar IUPM values were obtained in the DEVO assay whether or not exogenous cells were added to expand virus, suggesting that the assay can be performed without the addition of target cells (**Figure 4A**). Additional days in culture beyond day 12 post-stimulation increased the number of positive wells in some, but not all assays (**Figure 4B**; not shown). This is similar to the standard QVOA where in most assays, all wells containing HIV outgrowth are p24 positive by day 15 with very few or no additional wells turning positive with longer culture. As one of the goals of the DEVO was to reduce the length of the QVOA assay, and given that by day 8, IUPM values were comparable or higher than the traditional QVOA assay, day 8 p24 assessment with a day 12 confirmation were subsequently selected as the assay endpoint (**Supplementary Tables S2, S3**).

The use of HIV permissive cell lines such as Molt4/CCR5 instead of PHA-blasts to amplify virus outgrowth in co-culture assays could diminish the cost associated with PHA-blast production and eliminate donor-to-donor variability in longitudinal studies (26). We therefore compared the ability of Molt4/CCR5 cells to expand virus in the DEVO assay as compared to PHA blasts. We observed no significant difference in IUPM values whether Molt4/CCR5 or PHA blasts were used in the assay (**Figure 4C**).

Longitudinal Variation of the DEVO Assay

Longitudinal reservoir measurements are important metrics to evaluate the efficacy of therapeutic interventions and therefore depend on reliable and reproducible assays for accurate assessment. We previously reported on the longitudinal reproducibility of the traditional QVOA (12) and therefore, we compared the reproducibility of the DEVO assay with the QVOA. In two participants, the DEVO assay was run in parallel with the QVOA using resting CD4 + T cells collected longitudinally, spanning 6–14 months. In one participant, we compared the

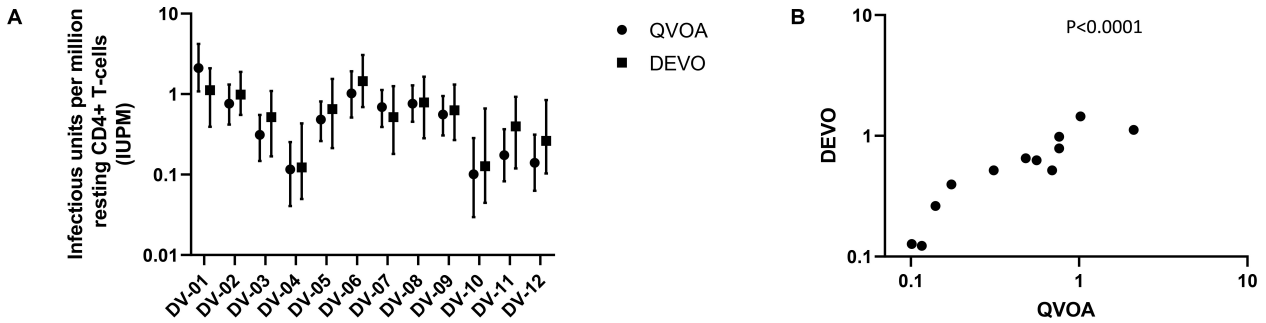

FIGURE 3 | Comparison of IUPM values obtained in the DEVO assay at day 8 post-stimulation versus IUPM values obtained from the QVOA at day 15 post-stimulation. **(A)** The DEVO assay generated IUPM values that were either comparable or in some cases higher than values obtained with the traditional QVOA. However, overall, there was no significant difference in IUPM values between the two assays. $P = 0.1294$, Wilcoxon matched-pairs signed rank test. Twelve independent, paired assays from 12 distinct participants is shown. IUPM + 95% CI (upper and lower limits) is shown. **(B)** Correlation of the DEVO assay at day 8 post-stimulation with the QVOA at day 15 post-stimulation. Spearman $r = 0.9282$.

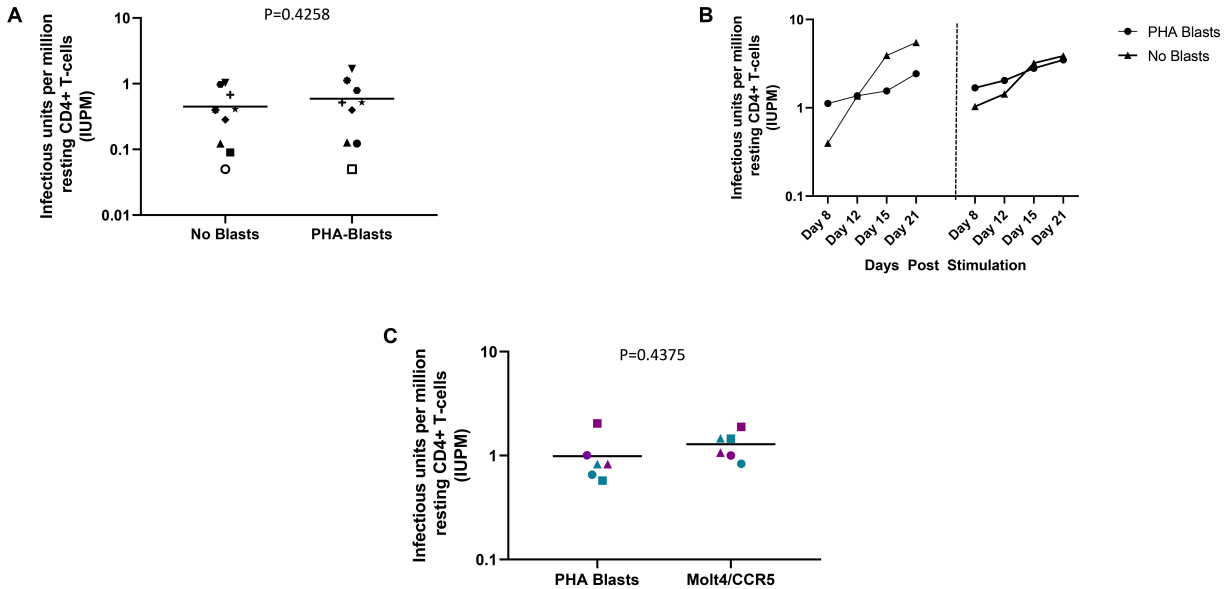

FIGURE 4 | DEVO assay outcome using either no blasts, or PHA blasts or the HIV permissive cell line, Molt4/CCR5 to expand virus. **(A)** Increase in IUPM values over time in cultures receiving either PHA blasts or no exogenous targets (no blasts). Two independent assays using cells from two participants are shown (left and right panel, DV-01 and DV-02). **(B)** Similar IUPM values are obtained in the DEVO assay whether or not exogenous targets to expand virus are used. Each symbol represents an independent assay performed with cells from distinct participants. **(C)** The HIV permissible cell line Molt4/CCR5 expands virus as well as PHA blasts in the DEVO assay. Results from independent assays performed with cells from three different participants (DV-01, DV-05 and DV-06) at day 8 (green) and day 12 (purple) are shown. For each experiment, cultures either received Molt4/CCR5 or PHA blasts to expand virus. Each symbol represents a participant. Open symbols indicate values below the limit of detection. Wilcoxon matched-pairs signed rank test was used to compare statistical differences.

DEVO assay performed using whole blood resting CD4 T cells collected longitudinally (17.5 months between donations), with the QVOA performed using leukapheresis-derived resting CD4 T cells acquired within 3 months of the blood cells (15.2 months between donations). We observed that the DEVO assay tracked with the QVOA within a given participant and across participants over time (**Figure 5**).

The ability to perform the DEVO assay using whole blood-derived instead of leukapheresis-derived resting CD4 + T cells would be beneficial for several reasons. It could reduce the costs associated with obtaining leukapheresis product and the time a participant spends in the clinic. Removing the need for leukapheresis procedures would also allow for the inclusion of additional time points to measure the replication competent reservoir during clinical trials. We therefore compared the performance of the DEVO assay on resting CD4 + T cells isolated from peripheral blood versus cells donated at leukapheresis in three different donors. We observed no significant difference in IUPMs obtained from the DEVO assay performed with the two different sources of resting CD4 T cells suggesting that the DEVO assay could be incorporated as an important tool in clinical trials seeking to deplete the HIV reservoir (**Figure 6**).

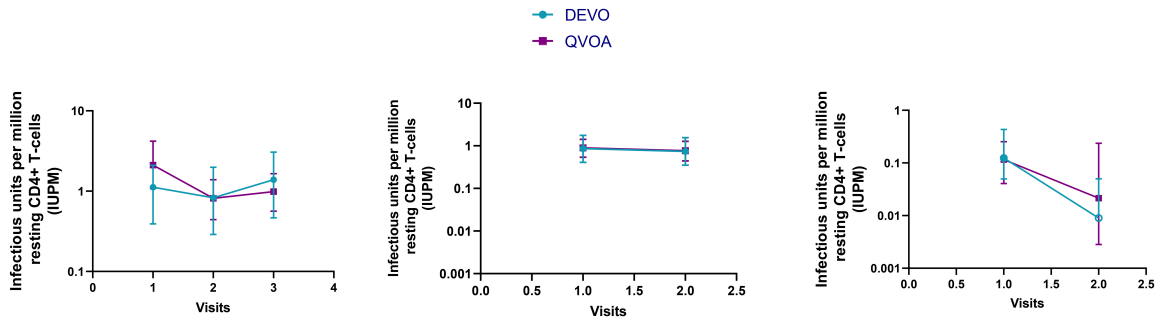

FIGURE 5 | The DEVO assay tracked similarly overtime as the traditional QVOA. The DEVO assay and QVOA were performed with resting CD4 + T cells collected at multiple time points from 3 participants, DV-01 (first panel), DV-09 (second panel), and DV-04 (third panel). All assays were performed using cells from the same leukapheresis donation, except for VV-09 where the DEVO was performed using cells from whole blood donated within 3 months of leukapheresis donations used for the QVOA. The time elapsed between visits 1 and 2, 1 and 3, and 2 and 3 for DV-01 is 7.9, 14.1, and 6.2 months respectively; for DV-09 the time elapsed between visit 1 and 2 is 17.9 months (DEVO) and 14.9 months (QVOA); For DV-04, the time between the first and second donation is 15. 9 months. IUPM + 95% CI (upper and lower limits) is shown.

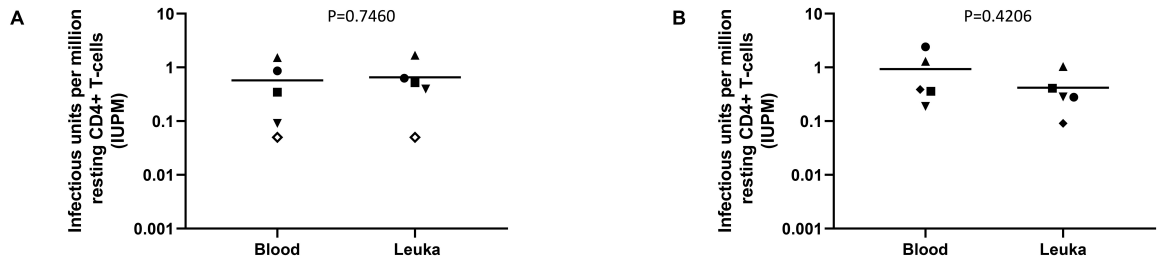

FIGURE 6 | Comparison of the DEVO assay using resting CD4 + T cells isolated from whole blood vs leukapheresis product (Leuka). **(A)** Targets (either PHA blasts or MOLT4/CCR5) added to expand virus. **(B)** No targets added. Whole blood was collected 6–17 months from leukapheresis donation time. Each symbol represents an independent assay performed using cells from 4 distinct participants (DV-02, DV-03, DV-04, DV-09 and DV-11). For each experiment, cultures either received exogenous targets to expand virus or no targets. Open symbols indicate values below the limit of detection. The Mann–Whitney *U* test was used to compare statistical differences.

DISCUSSION

Interventions to deplete the HIV reservoir are being explored in the clinic and would greatly benefit from endpoint assays that can rapidly and reliably measure the replication competent HIV reservoir. While PCR assays to measure DNA and RNA are high-throughput, rapid and relatively streamlined, they do not distinguish between defective proviruses and replication competent virus (3–5). The newly described IPDA offers a significant advantage over traditional PCR methods targeting only a single genomic region of the virus as the IPDA uses primer/probe sets simultaneously targeting multiple conserved regions of the HIV genome to more accurately detect intact provirus. However, the IPDA still over-estimates the frequency of true intact proviruses (6, 7). Near full-length or full genome sequencing may provide better assessment of intact proviruses than standard DNA PCR, however, the inefficiency associated with long distance PCR for DNA sequencing, and the extreme costs and labor involved makes them difficult to apply on a large scale [reviewed in Falcinelli et al. (13)]. The QVOA, though labor intensive, provides a minimal estimate of the frequency of latent HIV infection, and is still the gold standard for measuring true replication competent HIV. We report here a validated, modified version of the QVOA which incorporates a digital p24 ELISA as a readout for sensitive and accelerated detection of HIV. We showed that the DEVO assay can be performed in less time and with fewer cells than the traditional QVOA. Importantly, IUPM values obtained from the DEVO assay were comparable or in some cases higher than the values from the traditional QVOA. Finally, we also show that the assay varies minimally longitudinally and can be performed using resting CD4 + T cells isolated from either whole blood or leukapheresis product. An optimized HIV p24 protocol is used in the DEVO assay to improve sensitivity (16). Additionally, to improve accurate measurement of replication competent virus, we meticulously defined a limit of quantitation for the assay and included an additional day of culture to confirm positive wells. While others have reported using the Simoa to quantitate the inducible reservoir, to our knowledge this the first report with detailed description of the steps taken to validate the assay and demonstrate longitudinal reproducibility and accuracy.

Given the importance of the QVOA in assessing replication competent HIV, it is no surprise that efforts have been devoted to streamlining the assay (13). Increasing sensitivity to detect viral outgrowth while minimizing cell input has been a common goal in the field. Other laboratories have also reported modifications

of the assay to use fewer than 10 million cells while maximizing the ability to record the frequency of latently infected cells (19, 27, 28). One of the challenges of all viral outgrowth assays is the phenomenon of non-induced, replication competent virus (4, 11). Capturing cell free RNA from the supernatant of outgrowth wells using magnetic beads before assaying for HIV gag by PCR, was shown to increase sensitivity to detect inducible HIV (19, 28). However, while RNA measurements may provide more sensitivity, the detection of RNA may not represent a replication-competent viral particle. Further, false positive detection because of PCR contaminants could be a disadvantage. In a more recent report, effector memory T cells were found to contain a higher frequency of inducible HIV, leading to the suggestion that the effector memory state was overall more conducive to HIV latency reversal than other T cell differentiation states (29). This lead to the development of a modified QVOA termed the dQVOA, during which resting CD4 + T cells are first differentiated into effector memory T cells using a cocktail of cytokines before the stimulation and viral outgrowth steps (27). The differentiation step resulted in significantly higher frequencies of reactivated HIV compared to the traditional QVOA (27). Whether or not including this differentiation-to-effector-memory step in the DEVO assay would further increase the frequency of virus detected remains to be determined.

There are some limitations to our assay. Because we are interrogating fewer cells, the confidence interval around our point estimate (IUPM) is wide, especially when the frequency of latently infected cells is low. Thus in stably suppressed PLWH with extremely small inducible reservoirs, or if someday anti-latency interventions are able to significantly deplete the reservoir to very low levels, the applicability of the DEVO assay as described, in such situations will be limited as large numbers of cells will have to be interrogated to make an accurate assessment of the frequency of replication competent HIV. In such cases, either a modified DEVO assay or standard QVOA using large numbers of cells might be preferred. As an alternative, murine viral outgrowth models (mVOA or hmVOA) may provide an attractive, *in vivo* approach to record difficult to detect replication competent virus [(30, 31) reviewed in Schmitt and Akkina (32)]. Another limitation of our assay relates to selecting day 8 as the assay end point. As mentioned previously, in some assays, additional days of culture produce additional p24 positive wells. Thus wells that were p24 negative at day 8 may become positive at a later day but would not be considered positive, and result in a lower IUPM estimate than if the assay was extended to a later timepoint. However, there are obvious costs to extending times in culture, and a negative result (no positive cultures at the end of an assay) simply defines a limit of detection under the conditions employed. Increased sensitivity, at the burden of increased cost, may be obtained by extending time in culture.

In summary, the DEVO assay represents an advance to the available validated toolkit to measure replication competent HIV. The DEVO assay offers a new platform to quantify replication competent HIV for a variety of applications, such as measuring the frequency of infection in situations where the number of cells available may be limited, evaluating LRA activity, and measuring clearance of infected cells following the addition of autologous immune effectors.

MATERIALS AND METHODS

Cell Culture

MOLT4/CCR5 cells were acquired from the NIH AIDS Reagent Bank. Cells were maintained in culture in RPMI supplemented with 1% penicillin/streptomycin (Gibco, ThermoFisher), 10% FBS (Gibco, ThermoFisher) and 1 mg/mL G418 until use.

PHA-blasts were prepared from PBMC obtained from selected HIV seronegative donors screened for adequate CCR5 expression (33). PBMC were CD8-depleted and maintained in culture in IMDM supplemented with 1% penicillin/streptomycin, 10% FBS and 20 U/ml IL-2. Cells were stimulated for 2–3 days with 2 μg/ml PHA prior to usage.

Isolation of Resting CD4 + T Cells

HIV+ participants underwent continuous flow leukapheresis to obtain large amount of white blood cells or 150 ml of whole blood was obtained by venipuncture at a different time point from the leukapheresis from selected participants. PBMCs were isolated by Ficoll-gradient. Resting CD4 + T cells were isolated from PBMC by negative selection as previously described (34). Resting CD4 + T cells were maintained in culture for 1–2 days in the presence of ARV, without IL-2 prior to performing outgrowth assays.

Buffy coats from HIV seronegative donors were obtained from the New York Blood Center (New York, NY, United States) and PBMC isolated by Ficoll-gradient. Resting CD4 + T cells were isolated using the EasySep^{TM} Human Resting CD4 + T Cell Isolation Kit (StemCell Technologies, Vancouver, BC, United States). Cells were cultured overnight in media containing 20 U/ml IL-2 prior to assay set-up.

Traditional QVOA

The QVOA assay was performed as previously described (12, 23). Briefly, 34–50 million resting CD4 + T cells were plated in replicate limiting dilutions of 2.5 million (12–18 cultures), 0.5 million (6 cultures) and 0.1 million (6 cultures) cells per well, activated with PHA (Remel, ThermoFisher) and a fivefold excess of allogeneic irradiated PBMCs from a seronegative donor, and 60 U/ml IL-2 for 24 h. Cultures were washed and co-cultivated with CD8-depleted PHA-blasts. Culture supernatants were harvested on days 15 and 19 and assayed for virus production by p24 antigen capture ELISA (ABL, Rockville, MD, United States). Cultures were scored as positive if p24 was detected at day 15 and was increased in concentration at day 19. A maximum likelihood method was used to estimate the frequency of resting cell infection, reported as infectious units per million CD4 + T cells (24, 25). Unless otherwise indicated, IUPM from day 15 post-stimulation of the QVOA is reported.

DEVO Assay

Resting CD4 + T cells were plated in limiting dilution of a combination of 0.5×10^6, 0.25×10^6, 0.1×10^6, 0.05×10^6 and 0.025×10^6 cells per well at 12 replicates each. Cells were stimulated with 2 μg/mL PHA, 60 U/mL Interleukin-2 (IL-2), and irradiated PBMCs from an HIV-seronegative donor.

After 24 h, the cells were washed to remove the PHA, and MOLT-CCR5 or PHA-blasts were added at 0.05×10^6 or 0.2×10^6 per well respectively to amplify outgrowth of the virus. For experiments testing whether virus outgrowth would occur without the addition of exogenous feeder cells, no targets were added to the wells. Fresh media was added to cultures every 3–4 days. On day 8, the wells were split and targeted with another round of MOLT-CCR5 or PHA blasts. Supernatants were harvested on days 8, 12, 15, and 19 or just days 8 and 12. An optimized protocol was used to quantify HIV p24 antigen in culture supernatant by the Simoa HD-1 Analyzer (Quanterix, Billerica, MA, United States). Only wells exhibiting sustained or increase in p24 expression over the multiple days of harvest were scored as positive. A maximum likelihood method was used to estimate the frequency of resting cell infection, reported as infectious units per million CD4 + T cells (24, 25). Unless otherwise indicated, all DEVO assays were performed with the addition of exogenous targets (PHA blasts or Molt4/CCR5) to amplify virus and IUPM from day 8 post-stimulation is reported.

Determination of DEVO LLOQ

Sero-negative donor resting CD4 + T cells were cultured in triplicate at 1×10^6 cells/ml in cIMDM with 0.2×10^6 irradiated normal donor PBMCs/ml, 3 μg/ml PHA and 100 units/ml IL-2. After 1 day, cells were washed one time to remove PHA. Cells were then co-cultured with target cells with wells receiving 0.4×10^6 normal donor PHA-stimulated, CD8-depleted PBMCs per million resting cells. On day 5, a half of media was removed and replaced with an equivalent volume of media containing IL-2. On day 8, supernatant was collected, aliquoted and stored at −80°C. An equivalent volume of media containing IL-2 and the appropriate number of targets cells were added back to each culture. Half volume of supernatant was collected from each well on days 8,12, 15, and 19, aliquoted and stored at −80°C for later processing.

On three separate, but not consecutive days, Using Calibrator I from Quanterix Simoa HIV p24 kit, day 8 and day 12 supernatants were spiked in triplicate at 100, 50, 25, 10, and 0 fg/ml of p24 and assayed on the Simoa on. For each concentration, supernatant day and assay day, the average and standard deviation of the three replicates were used to determine the bias, total error and% total error using the following formula: Bias = Average − Actual Value; Total Error = |Bias| + 2 × Standard Deviation; % Total Error = Total Error Actual Value × 100 (22). Percent total error versus concentration was plotted and fit using a 4-pl curve fit. From this fit, the concentration at which there is <20% total error was interpolated and found to be approximately 50 fg/ml. This value is used as the DEVO assay lower limit of quantitation.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism version 8.0.0. Wilcoxon matched-pairs signed rank or Mann-Whitney *U* Test were used as appropriated to compare differences between groups. For IUPM below the limit of detection, resting cell infection was estimated assuming that 1 culture at the highest input cells was positive, and one-half of this value was used for the statistical analyses (12). Correlations between the DEVO and QVOA were assessed using the Spearman correlation coefficient. A p value of less or equal to 0.05 was considered to be significant in all analyses performed.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Samples were obtained from participants through an ongoing longitudinal collection protocol approved by the University of North Carolina (UNC) biomedical institutional review board. All samples were collected in accordance with UNC guidelines and all participants provided written informed consent prior to sample donation.

AUTHOR CONTRIBUTIONS

NA and ES conceptually designed the study. ES, KJ, JLK, and BA performed the experiments. NA, ES, and KJ performed the data analysis. NA wrote the manuscript. JDK, CB, DM, and CG provided clinical coordination and support. All authors edited and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01971/full#supplementary-material

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