NOVEL INSIGHTS INTO A FUNCTIONAL HIV CURE

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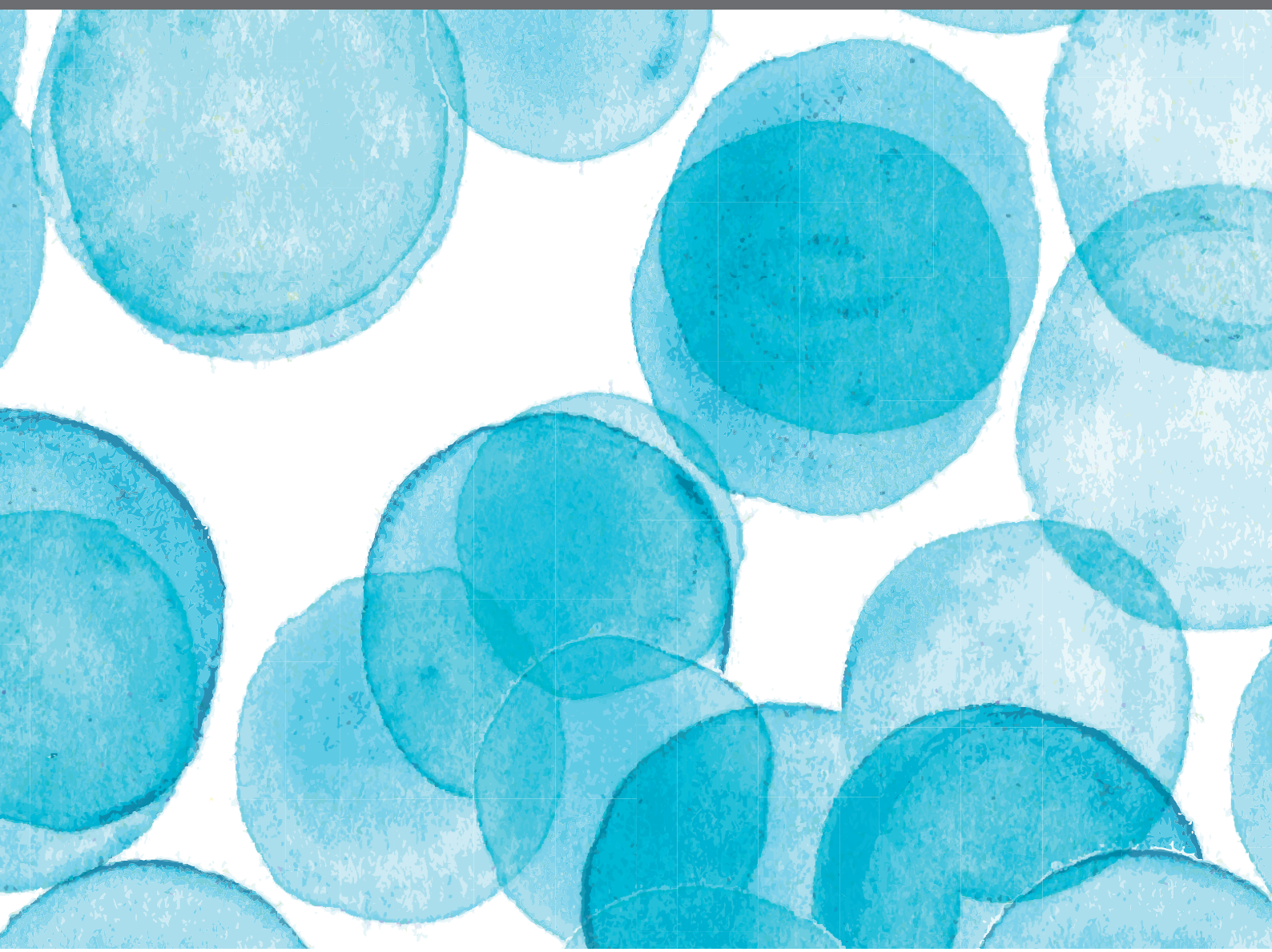

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NOVEL INSIGHTS INTO A FUNCTIONAL HIV CURE

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Table of Contents

04 ***Editorial: Novel Insights Into a Functional HIV Cure***
Luca Sardo, Cristina Parolin, Takeshi Yoshida, Alfredo Garzino-Demo and Taisuke Izumi

07 ***Non-synonymous Substitutions in HIV-1 GAG Are Frequent in Epitopes Outside the Functionally Conserved Regions and Associated With Subtype Differences***
Babatunde A. Olusola, David O. Olaleye and Georgina N. Odaibo

22 ***Predicting Post-treatment HIV Remission: Does Size of the Viral Reservoir Matter?***
Alexander O. Pasternak, Christina K. Psomas and Ben Berkhout

31 ***FRET-Based Detection and Quantification of HIV-1 Virion Maturation***
Anamaria D. Sarca, Luca Sardo, Hirofumi Fukuda, Hiroyuki Matsui, Kotaro Shirakawa, Kazuki Horikawa, Akifumi Takaori-Kondo and Taisuke Izumi

44 ***Improved Detection of HIV Gag p24 Protein Using a Combined Immunoprecipitation and Digital ELISA Method***
Guoxin Wu, Carol Cheney, Qian Huang, Daria J. Hazuda, Bonnie J. Howell and Paul Zuck

56 ***A Therapeutic Strategy to Combat HIV-1 Latently Infected Cells With a Combination of Latency-Reversing Agents Containing DAG-Lactone PKC Activators***
Kouki Matsuda, Takuya Kobayakawa, Ryusho Kariya, Kiyoto Tsuchiya, Shoraku Ryu, Kohei Tsuji, Takahiro Ishii, Hiroyuki Gatanaga, Kazuhisa Yoshimura, Seiji Okada, Akinobu Hamada, Hiroaki Mitsuya, Hirokazu Tamamura and Kenji Maeda

71 ***Towards a Functional Cure of HIV-1: Insight Into the Chromatin Landscape of the Provirus***
Julie Janssens, Anne Bruggemans, Frauke Christ and Zeger Debyser

83 ***The Interplay of HIV-1 and Macrophages in Viral Persistence***
Chynna M. Hendricks, Thaissa Cordeiro, Ana Paula Gomes and Mario Stevenson

98 ***Cleavage of TANK-Binding Kinase 1 by HIV-1 Protease Triggers Viral Innate Immune Evasion***
Sundararaj Stanleyraj Jeremiah, Kei Miyakawa, Satoko Matsunaga, Mayuko Nishi, Ayumi Kudoh, Akinori Takaoka, Tatsuya Sawasaki and Akihide Ryo

109 ***$CD8^+$ T-Cell Mediated Control of HIV-1 in a Unique Cohort With Low Viral Loads***
Amber D. Jones, Svetlana Khakhina, Tara Jaison, Erin Santos, Stephen Smith and Zachary A. Klase

122 ***Potential Utilization of APOBEC3-Mediated Mutagenesis for an HIV-1 Functional Cure***
Terumasa Ikeda, Yuan Yue, Ryo Shimizu and Hesham Nasser

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EDITORIAL
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Editorial: Novel Insights Into a Functional HIV Cure

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Keywords: human immunodeficiency virus (HIV), functional cure, latency, latency reversing agents (LRA), immunotherapy

Editorial on the Research Topic

Novel Insights Into a Functional HIV Cure

INTRODUCTION

Although efficient suppression of HIV replication below the limits of detection is achievable with potent combination antiretroviral therapy (cART), the risks of adverse effects and drug resistance still exist. HIV latently infected cells are in a state of non-productive infection, do not respond to cART and are not cleared by the host immune system. As a consequence, latently infected cells establish reservoirs that persist during cART and become a source of productive infection upon therapy interruption. Various strategies for achieving an HIV functional cure have been proposed, including a kick-and-kill approach using latency-reversing agents (LRA) to reactivate the reservoir, a block-and-lock strategy to permanently silence provirus transcription, and others that aim to induce immune control after cART interruption (Zerbato et al., 2019; Li et al., 2021; Ward et al., 2021). We hereby invited interdisciplinary researchers to bring their scientific knowledge and observations to advance novel treatment paradigms toward achieving cure.

INNATE IMMUNITY AND HIV RESERVOIR CELLS

Acquired resistance to type I IFNs can occur both in HIV actively and latently infected cells, contributing to the maintenance of the reservoir. Sundaraj et al. demonstrated an intriguing connection between the IFN pathway and HIV protease activity. IFN signaling was inhibited via the HIV protease-mediated cleavage of TANK-binding Kinase 1, which prohibited IFN regulatory factor 3 phosphorylation. Hendricks et al. highlighted how HIV evolution generates an immunologic sanctuary that sustains persistent virus infection in macrophages avoiding IFN-mediated antiviral responses.

ACQUIRED IMMUNITY

Jones et al. examined a cohort of people living with HIV (PLWH) who have consistently low viral loads. The authors analyzed various immune cell populations isolated from these individuals' PBMCs before and after cART initiation and revealed that CD8+ Cytotoxic T lymphocytes (CTL) were critical to controlling HIV replication. HIV Gag is widely considered a potent antigen to

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induce robust HIV-specific CTL and is the target for developing an anti-HIV vaccine (Edwards et al., 2002; Zuñiga et al., 2006; Kiepiela et al., 2007). Olusola et al. reported non-synonymous mutations in functionally conserved HIV Gag epitopes in blood isolated from PLWH. Since several escape mutations were found in the epitopes of HIV DNA vaccine design, the authors suggested that slight modifications should be required for further HIV Gag-derived vaccine development. Ikeda et al. reviewed the potential role of the antiviral host protein, APOBEC3, for both innate and adaptive anti-HIV immunities. While APOBEC3G induced premature termination codons, it could also enhance the abundance of HIV-derived epitopes and activation of HIV-specific CTL. The sublethal mutations may contribute to the generation of CTL escape variants due to the changes in the CTL epitopes and flanking regions.

HOST VIRUS INTERACTIONS AND CURE STRATEGIES

A long-standing approach for cure in the cART era is to purge latently infected cells that persist during therapy. Matsuda et al. proposed using the DAG-lactone YSE028 as an LRA and evaluated its ability to synergize with other LRAs to selectively activate mechanisms of cell death in infected cells. Janssens et al. reviewed the state of the art on the importance of proviral DNA integration sites and relative chromatin organization for HIV cure. Viral DNA integration sites are shifted toward the inner nucleus from the nuclear periphery in close association to the nuclear pore by inhibiting Integrase-LEDGF/p75 interaction with inhibitors (LEDGINs), with a consequent larger population of the provirus exhibiting transcriptional silence. Pasternak et al. discussed how the chromatin context of the proviral integration sites and the number of intact proviruses that contribute to the replication competence of the reservoir likely influence latent proviruses reactivation capacity. A small HIV reservoir size is necessary but insufficient for post-treatment remission to achieve a functional HIV cure. Therefore, complementary biomarkers would be required to accurately predict post-treatment HIV remission.

METHODS FOR EXAMINING HIV RESERVOIRS

Quantifying the size of the long-lived reservoir of cells carrying replication-competent HIV is essential to measuring the impact of curative efforts. Wu et al. developed a novel immunoassay combining immunoprecipitation and a digital ELISA method with a single molecule array to improve the measurement of HIV and SIV Gag proteins from biological samples, particularly at low concentrations. Recent intriguing reports (Imamichi et al., 2020) revealed that a portion of the defective reservoir is translationally competent and can generate structural proteins, potentially producing virus-like particles (VLPs). The immunogenicity of VLPs is affected by their maturation status and can be relevant to sustain chronic immune stimulation. Sarca et al. focused on developing a fluorescence-based microscopy tool to rapidly screen virus maturation with high throughput and unbiased computational analysis.

SUMMARY

The HIV field has successfully advanced antiretroviral therapies that allow a near-normal life expectancy for PLWH. However, a strategy to achieve viral control in the absence of cART remains an unmet challenge. The main obstacle in achieving a functional cure is ascribed to the integration of the proviral DNA in the host genome and the formation of reservoirs of infected cells that persist even during cART. Although significant progress has been made, questions remain about the major cell types contributing to persistent reservoirs and their anatomical localization, possibly indicating that HIV persistence is established through multiple mechanisms. Persistent immune stimulation in PLWH on cART remains a health concern as inflammatory cytokines are elevated by HIV infection, likely in response to expression of viral antigens in transcriptionally and translationally active proviruses found in the reservoir. In this Research Topic, we focused on HIV persistence and reached the conclusion that a combination of multiple cure strategies, including kick-and-kill, block-and-lock, and immunotherapies may be required to achieve a functional cure. Recognizing that HIV remains a chronic disease, understanding the events that sustain persistence and developing therapies targeting the elimination of infected cells remain keys toward cure and remissions strategies.

AUTHOR CONTRIBUTIONS

LS and TI contributed to formulating this Research Topic theme, inviting authors, and collecting articles. All authors contributed to acting as handling editors for the submitted manuscripts and writing the editorial article.

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ORIGINAL RESEARCH
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Non-synonymous Substitutions in HIV-1 GAG Are Frequent in Epitopes Outside the Functionally Conserved Regions and Associated With Subtype Differences

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In 2019, 38 million people lived with HIV-1 infection resulting in 690,000 deaths. Over 50% of this infection and its associated deaths occurred in Sub-Saharan Africa. The West African region is a known hotspot of the HIV-1 epidemic. There is a need to develop an HIV-1 vaccine if the HIV epidemic would be effectively controlled. Few protective cytotoxic T Lymphocytes (CTL) epitopes within the HIV-1 GAG (HIV_gagconsv) have been previously identified to be functionally conserved among the HIV-1 M group. These epitopes are currently the focus of universal HIV-1 T cell-based vaccine studies. However, these epitopes' phenotypic and genetic properties have not been observed in natural settings for HIV-1 strains circulating in the West African region. This information is critical as the usefulness of universal HIV-1 vaccines in the West African region depends on these epitopes' occurrence in strains circulating in the area. This study describes non-synonymous substitutions within and without HIV_gagconsv genes isolated from 10 infected Nigerians at the early stages of HIV-1 infection. Furthermore, we analyzed these substitutions longitudinally in five infected individuals from the early stages of infection till after seroconversion. We identified three non-synonymous substitutions within HIV_gagconsv genes isolated from early HIV infected individuals. Fourteen and nineteen mutations outside the HIV_gagconsv were observed before and after seroconversion, respectively, while we found four mutations within the HIV_gagconsv. These substitutions include previously mapped CTL epitope immune escape mutants. CTL immune pressure likely leaves different footprints on HIV-1 GAG epitopes within and outside the HIV_gagconsv. This information is crucial for universal HIV-1 vaccine designs for use in the West African region.

Keywords: non-synonymous substitutions, HIV-1 GAG, MHR, HIV-1 subtypes, HIV cure

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INTRODUCTION

Thirty-eight million people lived with HIV-1 infection in 2019, with 690,000 AIDS-related deaths. Over 50% of HIV infection and about 63% of its associated deaths occurred in Sub-Saharan Africa (UNAIDS, 2020). The rate of new infections also increased in the region despite a 40% global reduction in new infections since 1998 (UNAIDS, 2020; WHO, 2020). About 69% of these

new HIV infections occurred in Western and Central Africa. Nigeria accounts for over 60% of new HIV infections in West and Central Africa. The country also has one of the massive HIV epidemic globally (UNAIDS, 2020; WHO, 2020). Combination antiretroviral therapies (cART) have effectively suppressed viremia to undetectable levels, increasing survival and quality of life. It has also decreased infectiousness in infected individuals (Herout et al., 2016; Hanke, 2019).

However, just about half of infected individuals are on antiretroviral therapy. Sub-Sahara Africa has the lowest access to treatment despite being the highest hit region with the virus (UNAIDS, 2019). Access to treatment is unlikely to increase to optimal levels because of economic, social, and pharmacologic challenges associated with cART use (Hanke, 2019; Ndung'u et al., 2019). Moreover, cART is not curative as persons on treatment have to use drugs to control the emergence of latent reservoir HIV (immune escape and drug-resistant strains) for the rest of their lives (Papuchon et al., 2013; Hanke, 2019). Therefore, to eliminate the HIV epidemic, especially in African countries, there is the need to develop a safe, cost-effective, durable, and accessible HIV-1 vaccine(Ondondo et al., 2016; Ndung'u et al., 2019; Bekker et al., 2020; Lé tourneau et al., 2020).

Induction of broadly neutralizing antibodies is the mainstay of all protective anti-viral vaccines. However, this has been very difficult to generate for HIV-1 because of the virus genes' plasticity (Ondondo et al., 2016; Hanke, 2019; Ndung'u et al., 2019). Studies have shown that broadly neutralizing antibodies develop very late in infection after the latent reservoir landscape is already established (Burton et al., 2004; Muema et al., 2017). Therefore, at present, two arms of universal HIV-1 vaccine research is ongoing, with a group focusing on generating robust neutralizing antibodies and the other on effective CTL immune responses. Findings from these fields will hopefully be merged into one universal HIV vaccine (Ondondo et al., 2016). Cytotoxic T Lymphocytes (CTL), commonly referred to as CD8 + T cells have been extensively shown to control HIV-1 replication, especially during the early stages of infection (Ndhlovu et al., 2015; Leitman et al., 2016; Ndung'u et al., 2019). Previous studies have associated expansion of CTL with the control of acute infections (Borrow et al., 1994; Koup et al., 1994; Ogg et al., 1998; Goulder and Watkins, 2004; Goonetilleke et al., 2009). Initially, several studies associated CTL control of acute HIV infections with HLA protection (Gillespie et al., 2002; Brumme et al., 2008; Honeyborne et al., 2010; Mori et al., 2014); however, recent studies have shown HIV/AIDS outcomes are better predicted by the (i) magnitude and breadth of CTL responses as well as (ii) presentation of functionally conserved CTL epitopes during the early stages of infection (Balla-Jhagjhoorsingh et al., 1999; Lazaro et al., 2011; Matthews et al., 2012; Kløverpris et al., 2014; Ndhlovu et al., 2015; Radebe et al., 2015; Koofhethile et al., 2016).

Recently, the conserved region T cell-based vaccine strategy was developed (Létourneau et al., 2007; Hanke, 2019). This strategy is aimed at eliciting effective CTL responses by (a) using functionally conserved HIV-1 proteins for vaccine constructs, (b) blocking common HIV immune escape paths, and (c) including epitopes associated with low viral load in untreated people (Ondondo et al., 2016; Hanke, 2019). Using proteomic and bioinformatics methods, which included the Shannon entropy algorithm, 14 highly conserved consensus HIV-1 proteins were assembled into a chimeric vaccine construct (Barouch et al., 2010; Santra et al., 2010; Borthwick et al., 2014; Abdul-Jawad et al., 2016). This construct induced high frequencies of HIV-1 specific T cells capable of inhibiting HIV-1 replication *in vitro* and rhesus monkeys (Barouch et al., 2010; Abdul-Jawad et al., 2016). The immune coverage of the construct against diverse HIV strains was also noted (Santra et al., 2010). The construct was further developed into just six functionally conserved regions, which spanned six areas, namely, the whole of Gag p24, one part in Gag p15, and four regions of Pol overlapping with protease, polymerase, and integrase (Ondondo et al., 2016). This second-generation vaccine construct was showed to possess strong immunogenicity in mice (Mothe et al., 2015). The construct also elicited CD8 T cells, which correlated with high CD4 T-cell count in untreated patients (Ondondo et al., 2016).

Three conserved epitopes out of the six, namely Gag 240–249, TSTLQEQIGT; Gag 162–172, KAFSPEVIPMF and Gag 203–212, ETINEEAAEW, were shown to be functionally conserved and possessed high immune coverage among diverse HIV-1 strains. One of these epitopes- Gag 162–172, KAFSPEVIPMF is found within the major homology region (MHR) of the HIV-1 Gag gene. CTL epitopes within the MHR are promiscuously presented by similar HLA alleles (Carlson et al., 2012). The MHR, found within the capsid gene, is a conserved motif among retroviruses (Honeyborne et al., 2010; Tanaka et al., 2016). This region is essential in particle assembly and viral infectivity (Reicin et al., 1995). Furthermore, CTL epitopes within the MHR are also hydrophobic, highly immunogenic, and immuno-dominant (Tenzer et al., 2009; Bennett et al., 2010; Kløverpris et al., 2015; Yang et al., 2015).

It is also hypothesized that escape mutations within the MHR are likely to be deleterious to HIV as they seem to be associated with fitness costs (Martinez-Picado et al., 2006; Troyer et al., 2009; Liu et al., 2014). Ondondo et al. and others identified Gag 240–249, TSTLQEQIGT, and Gag 203–212, ETINEEAAEW as functionally conserved epitopes with high coverage within HIV-1 diverse strains (Fischer et al., 2007; Abdul-Jawad et al., 2016; Ondondo et al., 2016). These epitopes also showed robust T cell immune responses when assembled in vaccine constructs and tested in macaques (Mothe et al., 2015; Ondondo et al., 2016). However, despite ongoing research in this field, insufficient data exists on these CTL epitopes' functionally conserved abilities in early HIV-1 infected individuals' in real-world scenarios. There is also a dearth of information on the rate of non-synonymous substitutions in these epitopes compared to other epitopes of the HIV-1 GAG region in natural settings. This knowledge gap is prominent in Sub-Saharan African countries where diverse HIV strains circulate.

These countries are also of very high priority in the development of HIV-1 vaccines (Ndung'u et al., 2019). When developed, HIV-1 vaccines must be effective against strains circulating in African countries (Ndung'u et al., 2019). Despite the West African epicenter having one of the highest numbers of diverse circulating HIV-1 strains, very few longitudinal studies on HIV-1 have been reported from the region. Escape due to CTL

epitopes, which is driven by frequencies of non-synonymous and synonymous substitutions (Kosakovsky Pond et al., 2008; Garcia-knight et al., 2016), outside the functionally conserved region may be a crucial factor to consider in the design of therapeutic and universal HIV vaccines (Murakoshi et al., 2019). They may also provide opportunities for compensatory mutations on replicative fitness (Crawford et al., 2011).

This study describes non-synonymous substitutions within and without the three functionally conserved epitopes (HIV_gagconsv) of HIV-1 GAG genes isolated from 10 infected Nigerians at the early stage of HIV-1 infection. These individuals were part of a previously described study (Olusola et al., 2017). Furthermore, using phylogenetic tools, programs and databases in the Los Alamos National Laboratory HIV Sequence Database[1], we analyze these substitutions longitudinally in five infected individuals from the early stages of infection until after seroconversion.

METHODS

Study Sites and Patient Population

Twenty-three individuals at the early stages of HIV-1 infection identified and described previously were recruited for this study (see **Table 1**). Ten out of these individuals were studied for early HIV-1 infection. Another five individuals identified in 2017 were followed up until after seroconversion. The profile of the follow-up schedule is shown in **Table 2**. These individuals were screened for HIV-1 infection at every visit using the earlier described protocol (Olusola et al., 2017, 2020b).

Recruitment of Participants, Sample Collection, and Processing

Participants were recruited for this study after obtaining informed consent. Experiments were conducted with the understanding and the consent of participants. Socio-demographic data of participants were collected using a structured questionnaire. Feedback on results was provided within a week of sample collection. Individuals were counseled and encouraged to continue the presentation for testing at the scheduled intervals. Five milliliters of whole blood were collected in EDTA bottles from participants at every visit. Plasma was separated from the samples immediately after collection, stored at −20°C, and transported in a cold chain to a central laboratory for analysis. The samples were then stored at −80°C until analyzed. Blood samples were analyzed for HIV antigen/antibody, serum creatinine, HIV-1 RNA viral load (at baseline), and HIV-1 GAG DNA.

Identification of Early HIV-1 Infection and Detection of Antibodies

The updated CDC algorithm of laboratory testing for the diagnosis of early and chronic HIV-1 infection was used for this study, as previously described (Olusola et al., 2017).

[1]https://www.hiv.lanl.gov

Clinical Chemistry Assay for Serum Creatinine

Plasma samples were analyzed on a Roche cobas® C11 blood chemistry analyzer (Roche Diagnostics, Indianapolis, United States). Each sample was analyzed to determine the level of serum creatinine, according to the manufacturer's instruction. Normal reference ranges for plasma creatinine is 62–133 μmol/L.

HIV-1 RNA Viral Load Testing

Serum samples collected at baseline were tested for Plasma HIV-1 viral load (copies/ml) using the COBAS® Ampliprep/COBAS TaqMan96®HIV-1 Test, v2:0 (Roche Molecular Diagnostics, Branchburg, NJ, United States) according to manufacturer's instruction or by an in house real-time PCR protocol. Briefly, the in-house real-time PCR protocol entails a two-step reaction. First, reverse transcription PCR for cDNA which is a 25 μL reaction utilizing 5 μL of extracted RNA, 12.5 μL of 2X superscript III-RT polymerase reaction mix, and 1 μL superscript III RT/Platinum Taq high fidelity mix (Jena Bioscience, Jena, Germany) as well as random hexamers (1 μL) and RNase free water (5.5 μL). Thermal cycling was performed at 20°C for 10 min, 45°C for 30 min, 70°C for 15 min and an RNase H step of 37°C for 20 min using Applied Biosystem 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, MA, United States). The second stage involved a quantitative real-time PCR targeting a 140bp *Nef- Env* region of HIV-1. The 20 μL reaction utilized 5 μL of cDNA, 10 μL of qPCR SYBR Master UNG (Jena Bioscience, Jena, Germany) as well as 0.6 μL each of Nef8343 (ATGGGTGGCAAGTGGTCAAAAG) (Tcherepanova et al., 2008) and Env3out (TTGCTACTTGTGATTGCTCCATGT) primers (Keele et al., 2008). Inqaba Biotechnology, South Africa synthesized the primers, and thermal cycling was performed at 50°C for 2 min, 95°C for 2 min and then 35cycles of 95°C for 15 s, 55°C for 20 s using Applied Biosystem 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, MA, United States). Quantitation standards were used to interpolate the quantitative values of the HIV-1 RNA viral load for samples.

PCR Amplification and Sequencing of the GAG Gene

Total DNA was extracted from whole blood samples collected at each visit using guanidium thiocyanate in house protocol. A fragment of the gag-pol region (900 base pairs) of the virus was amplified using previously published primers and cycling conditions by Gall 2012 (Gall et al., 2012) with slight modifications. Briefly, PCR was performed using platinum TaqDNA High fidelity polymerase (Jena Bioscience). Each 25 μl reaction mixture contained 12.5 μl reaction mix (2x), 4.5 μl RNase-free water, 1 μl each of each primer (20 pmol/μl), 1 μl Platinum Taq DNA High Fidelity mix, and 5 μl of template DNA. Pan-HIV-1_1R (CCT CCA ATT CCY CCT ATC ATT TT) and Pan-HIV-1_2F (GGG AAG TGA YAT AGC WGG AAC) were used. Cycling conditions were 94°C for 5 min; 35 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 1 min 30 s; and finally, 68°C for 10 min. Positive HIV samples that were undetectable using the above-stated primers were retested

TABLE 1 | Summary of 23 early HIV infected Nigerians.

Sample ID	Gender	Age(years)	High-Risk group	Location	Date of HIV detection	HIV-1 RNA Viral Load	Subtype	GenBank accesssion number
EHIV001	Female	21	RM	Saki	26 Jan 2016	Undetectable	Unassigned	KY786266
EHIV002	Female	45	RM	Ibadan	26 Jan 2016	Undetectable	K	KY786267
EHIV003	Female	31	RM	Saki	26 Jan 2016	4999281	CRF 02-AG	KY786268
EHIV004	Female	32	RM	Saki	26 Jan 2016	5142127	CRF 02-AG	KY786269
EHIV005	Female	38	RM	Saki	26 Jan 2016	5856363	CRF 02-AG	KY786270
EHIV006	Female	28	RM	Saki	26 Jan 2016	1742364	Recombinant GD	KY786271
EHIV007	Male	48	RM	Ibadan	26 Jan 2016	Undetectable	CRF 02-AG	KY786272
EHIV008	Male	38	RM	Saki	26 Jan 2016	Undetectable		Not Sequenced
EHIV009	Female	32	RM	Ibadan	20 Feb 2017	Undetectable		Not Sequenced
EHIV010	Male	31	RM	Ibadan	21 Feb 2017	6492033	A	MN943617
EHIV011	Male	26	RM	Ibadan	21 Feb 2017	5449249	A	MN943616
EHIV012[b]	Female	29	RM	Ibadan	05 Oct 2017	5413537	G	MN943624
EHIV013[b]	Female	32	RM	Ibadan	21 Aug 2017	6906290	A	MN943613
EHIV014	Male	25	RM	Ibadan	21 Jul 2017	10084640	CRF 02-AG	MN943628
EHIV015	Male	42	VBD	Ibadan	26 Jul 2017	5499245	A	MN943615
EHIV016[b]	Male	29	VBD	Ibadan	02 Aug 2017	4013634	G	MN943627
EHIV017	Female	26	RM	Ibadan	11 Nov 2016	5613523	CRF 02-AG	MN943629
EHIV018	Female	29	RM	Ibadan	20 Feb 2017	5642093	CRF 02-AG	MN943633[a]
EHIV019	Male	46	VBD	Ibadan	27 Jul 2017	Undetectable		Not Sequenced
EHIV020	Male	40	VBD	Ibadan	17 Aug 2017	4392180	CRF 02-AG	MN943634[a]
EHIV021	Male	29	VBD	Ibadan	21 Jul 2019	Undetectable	CRF 02-AG	MN943635[a]
EHIV022[b]	Male	33	VBD	Ibadan	24 Jul 2017	6820582	G	MN943625
EHIV023[b]	Male	22	VBD	Ibadan	17 May 2017	[c]Undetectable	A	MN943619

TABLE 2 | Analysis of samples collected during follow- up.

S/N	Sample ID		Follow-up Schedule					
				Early HIV infection stage				Detection of Abs[#]
		Accession Numbers	Baseline	3months	6months	9months	1 year	∼2 years
1	EHIV 012	MN943626; MN943624; MN943630	05/10/2017[a]		✓			✓
2	EHIV 013	MN943613; MN943614; MN943631	21/08/2017[a]		✓		✓	✓
3	EHIV 016	MN943627; MN943623	02/08/2017[a]		✓			✓
4	EHIV 022	MN943625; MN943622	24/07/2017[a]		✓			✓
5	EHIV 023	MN943618; MN943619; MN943620; MN943621	17/05/2017[a]			✓	✓	✓

[a] *HIV-1 GAG gene isolated and sequence.*
[#] *Antibodies detection does not correspond to seroconversion dates as these individuals had seroconverted at an earlier date.*

using another set of GAG primers for nested PCR as described previously (Vidal et al., 2000). Positive PCR reactions were shipped on ice to Macrogen, South Korea, for Big Dye sequencing using the same amplification primers (Pan-HIV-1_1R and Pan-HIV-1_2F; or G60 andG25).

Detection of HIV-1 Subtypes and Phylogenetic Analysis

The sequences were cleaned and edited using Chromas and Bioedit software. Subtyping was performed using a combination of four subtyping tools: The Rega HIV-1 Subtyping Tool, version 3.0[2], Comet, version 2.2[3], National Center for Biotechnology Information, Bethesda, MD[4] and jpHMMM: Improving the reliability of recombination prediction in HIV-1[5]. The first three tools were used simultaneously, while jpHMMM was used to resolve discordant subtypes. Phylogenetic analyses were performed using MEGA software version 10. Alignment of sequences was performed using MAFFTS online software. Genetic distances were inferred using the Tamura-Nei model, and a phylogenetic tree was generated using the maximum likelihood method. The robustness of the tree was evaluated with 1000 bootstrap replicates. All consensus nucleotide sequences obtained in this study were submitted to GenBank database and assigned accession numbers MN943617-635.

[2]http://dbpartners.stanford.edu/RegaSubtyping/
[3]http://comet.retrovirology.lu
[4]http://www.ncbi.nlm.nih.gov/Blast.cgi
[5]http://jphmm.gobics.de/submission_hiv

Non-synonymous Substitutions in Cytotoxic T Lymphocytes (CTL) Epitopes Within HIV_gagconsv of HIV-1 GAG Gene Isolated From 10 Early Infected Individuals

Reference GAG sequences for subtypes G, A, and CRF02-AG were downloaded from the Los Alamos National Laboratory HIV Sequence Database[6]. Deduced amino acid (aa) sequences were translated for both reference and sample sequences with the standard genetic code using Bioedit software. CTL epitope corresponding to the three highly conserved sites (HIV_gagconsv), namely KAFSPEVIPMFSALSEGATPQD, DTINEEAAEWDR, and TSTLQEQIR (Yang et al., 2015; Ondondo et al., 2016; Hanke, 2019; Lé tourneau et al., 2020), were used for comparison and identification of amino acid substitution. HIV-1 GAG sequences identified as subtypes A, G, and CRF02_AG in this study were aligned with Reference A(GenBank accession numbers DQ676872; AB253421 and AB253429), G(GenBank accession numbers AF084936; AF061641; U88826 and AY612637), and CRF02_AG(GenBank accession numbers L39106 and DQ168578) sequences respectively.

Non-synonymous Substitutions in Cytotoxic T Lymphocytes (CTL) Epitopes Outside the HIV_gagconsv of HIV-1 GAG Gene

The Virus Epidemiology Signature Patterns Analysis[7] program was used to identify variations in other sites HIV-1 GAG sequence outside the **HIV_gagconsv** corresponding to CTL epitope regions (Korber and Myers, 1992). Already defined CTL epitope in HIV-1 database can be found in https://www.hiv.lanl.gov/content/immunology/ctl_search. The VESPA program is a user-friendly amino and nucleic acids signature pattern analysis tool. The program can calculate numbers of variations in an amino acid sequence relative to background sequence(s) using bioinformatics algorithms. By selecting positions where the most common character in a query set differs from that in the background set, differences between groups of sequences can quickly be detected. This analysis invariably can also identify conserved sequence signature patterns. The frequencies of distinguishing amino acids in each set can also be determined. The program has previously been used for HIV-1 and Chikungunya sequence analysis (Ou et al., 1992; Salvatierra and Florez, 2017). This program can also distinguish non-synonymous substitutions from synonymous substitutions based on the threshold settings. Similarity scores or thresholds represent Hamming distances (Nowak et al., 1991) or the number of point mutations between two aligned sequences, calculated using the score: (1 — D) X 100% where D is the hamming distance. This algorithm has previously been described in Nowak et al. (1991), Korber and Myers (1992).

With a threshold setting of 100%, only non-synonymous substitutions that are not due to chance were reported in this study. Sequences were aligned using CLC Main Workbench version 6.5, after which VESPA analysis was performed.

Deduced amino acid (aa) sequences were translated for both reference and sample sequences with the standard genetic code using Bioedit. HIV-1 GAG sequences identified as subtypes A, G, and CRF02_AG in this study were aligned with Reference A (GenBank accession numbers DQ676872; AB253421 and AB253429), G (GenBank accession numbers AF084936; AF061641; U88826 and AY612637), and CRF02_AG (GenBank accession numbers L39106 and DQ168578) sequences respectively. Only amino acid replacements with 100% non-synonymous substitution between reference sequences and sample sequences were considered. Percentage substitution rates were calculated by finding the ratio of the number of substitutions to the total possible substitution sites. These mutations were compared with the Los Alamos National Laboratory HIV Immunology Database for CTL/CD8 + Epitope Variants and Escape Mutants[8].

Ethical Approval

This research was conducted following the declaration of Helsinki. Experiments were conducted with the understanding and the consent of each participant. Ethical approvals for this research were obtained from the University of Ibadan/University College Hospital (UI/UCH) Research and Ethics Committee (UI/EC/15/0076) and the Oyo State Ministry of Health Committee on Human Research (AD13/479/951). All results were delinked from patient identifiers and anonymized.

Eligibility/Exclusion Criteria

Only individuals between 18 and 65 years of age were included in the study. Individuals who already knew their HIV status were excluded from the study.

Data Management and Statistical Analysis

Statistical analyses were performed using SPSS version 20. Data are expressed as means ± standard deviations. Statistical significance was estimated using the Kruskal-Wallis test, with SPSS package version 12.0, while Statistical significance was defined as P-values = 0.05.

RESULTS

Participants' Characteristics

Twenty-three individuals were identified to be at the early stages of HIV-1 infection. **Figure 1** shows the phylogeny of HIV-1

[6]https://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html

[7]http://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html

[8]https://www.hiv.lanl.gov/content/immunology/variants/ctl_variant.html

subtypes. Out of the ten early infected persons studied, five were infected with Subtype A, three with subtype G, and the rest CRF02-AG. Five individuals at the early stages of HIV-1 infection were followed up until after seroconversion. However, samples in which antibodies were detected were collected after these individuals had seroconverted. The participants were identified to be at the early stages of infection at different periods in 2017. One individual in October, July, and May each and two in August. Three of the individuals were males and were voluntary blood donors. The remaining two females were identified when referred for malaria antigen test. Three individuals were infected with HIV-1 subtype G, while the other two were infected with subtype A (**Table 1**).

Table 2 shows the analysis of the samples collected from these individuals from baseline until after seroconversion. In four individuals, blood samples were collected at three time points, twice during the early stages of infection and once after seroconversion. Samples were collected four times in the fifth individual, thrice during the early stages of infection and once after seroconversion. HIV-1 GAG sequences of these infected individuals were determined at every time point of blood collection.

Phylogenetic Analysis

Figure 1 shows the estimated phylogeny of HIV-1 subtypes with respect to reference sequences available in the HIV Los Alamos National HIV Sequence database. As shown in the Figure, Subtypes A, G, and CRF02_AG were identified with green, blue, and pink symbols, respectively. HIV-1 subtypes A identified in this study were closely related to Ref A1 DQ676872 (from Nigeria) and subtypes AF457075, KF716486 and AY521631 from Kenya, Uganda and Senegal, respectively. Those identified as subtypes G and CRF02-AG were closely related to the Nigerian subtypes DQ168573 and Ref.02 AG IBNG. L39106 respectively.

Substitutions in HIV_gagconsv of CTL Epitope Regions of Subtype A HIV-1 GAG Gene During Early Infection

We compared intra and inter variations among 10 HIV-1 GAG sequences isolated from persons at the early stage of infection. These sequences were grouped by subtypes and analyzed alongside reference sequences. As shown in **Table 3**, variations occurred mostly in HIV-1 Subtype A at the CTL epitope region of 243-251aa. The conserved epitope of TSTLQEQIR was not found in both the reference subtypes and those from early infected individuals. HIV-1 Subtype A also had the highest variations (50%) for HIV_gagconsv corresponding to the CTL epitope region (203-214aa). Although the HIV_gagconsv for CTL epitope region 162-183aa was the most conserved among the subtypes, a substitution rate of 2.7% was found for HIV-1 subtype A isolates. The CTL escape region (162-183) KAFSPEVIPMFSALSEGATPQD had the lowest frequency of mutations. However, sample MN943615 had two mutations-K162R and A163G, while MN943616 had mutation A163G only.

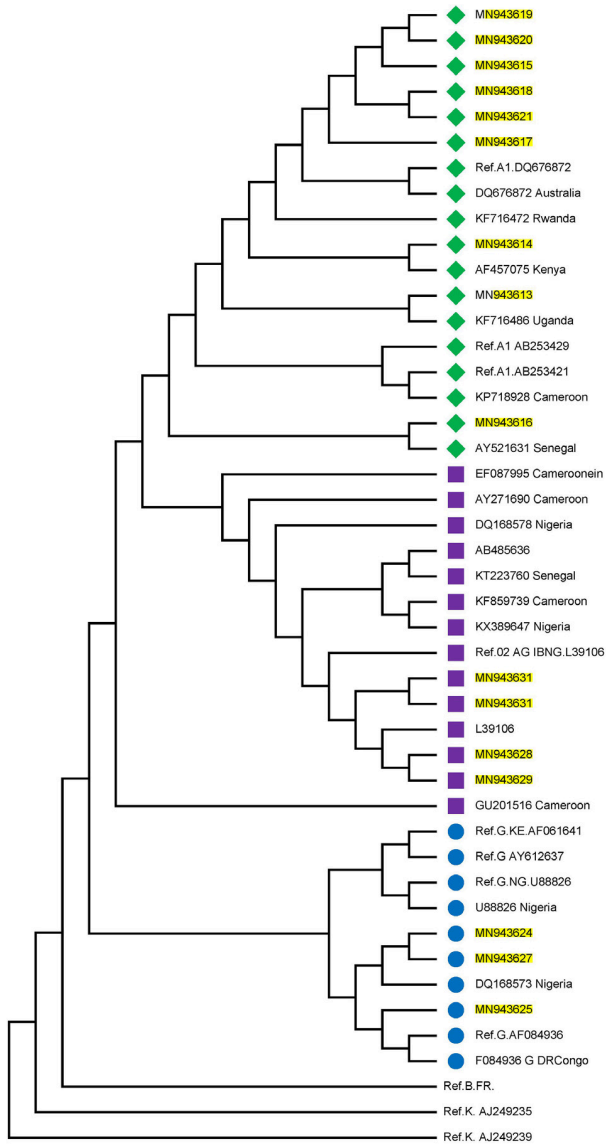

FIGURE 1 | Phylogenetic tree of the P17/P24 regions of the GAG gene of HIV-1. Reference subtypes are indicated with Ref. before their accession numbers. Other sequences are shown with their accession numbers and country of isolation. Subtypes obtained from samples in this study are shown with their accession numbers only. Subtypes A, G, and CRF02_AG were identified with green diamond, blue circle, and pink square symbols respectively. Multiple sequence alignment and phylogenetic tree were constructed using MAFFTS and Maximum Parsimony algorithm in MEGA 6 software. Statistical significance of the tree topology was tested by 1000 bootstrap replication.

Sequences Isolated After Seroconversion Are Associated With a Higher Rate of Substitutions in HIV_gagconsv of CTL Epitope Regions

Substitutions in the HIV_gagconsv of subtype B HIV-1 GAG gene have been shown to come with a fitness cost.

TABLE 3 | Substitutions associated with HIV_gagconsv in HIV-1 GAG gene during early HIV infection.

	EHIV accession no	CTL epitope region (162-183)	CTL epitope region (203-214aa)	CTL epitope region (243-251aa)	P-value
	Ref Seq	KAFSPEVIPMFSALSEGATPQD	DTINEEAAEWDR	TSTLQEQIR	
G	MN943624	Conserved	Conserved	Conserved	ns
	MN943625	Conserved	D203E	Conserved	
	MN943627	Conserved	Conserved	Conserved	
	% Substitution	Nil	2.7	Nil	
	Ref Seq			*TSTPQEQLQWMT	
A	MN943613	Conserved	Conserved	TSTTQEQIAWMT	
	MN943615	K162R; A163G	DTSPTR*PWAGI	*HXQELLVPLK	ns
	MN943616	A163G	DTSMRKLQNGTD	*HXQELLVPLK	
	MN943617	Conserved	Conserved	*HXQELLVPLK	
	MN943619	Conserved	DTIN*G*PXXGQ	*HNRSY*YPSRT	
	% Substitution	2.7	50	65	
CRF-02 AG	MN943628	Conserved	Conserved	Conserved	ns
	MN943629	Conserved	Conserved	Conserved	
	% Substitution	Nil	Nil	Nil	
***P*-Value**		ns	ns	**0.00902**	

P = 0.00902 for comparisons of non-synonymous substitutions in sequences across the CTL epitope TSTLQEQIR group.

These substitutions have shown to be deleterious to HIV-1 strains' eventual survival and transmission. However, minimal information exists on substitutions associated with immune epitopes during early non-subtype B HIV-1 infection. We followed five individuals from the early stage of infection till after seroconversion. Three of these individuals were infected with subtype G, while the remaining two were subtypes A (see **Table 4**). There were no substitutions in CTL epitope regions for EHIV016, while EHIV022 had single aa substitutions before and after seroconversion.

EHIV012 had single aa substitutions each after seroconversion in the three CTL epitope regions studied. These substitutions have not been reported before, to the best of our knowledge. For HIV-1 subtype A samples, EHIV023 had very high substitution rates in the CTL epitope region of 203-214aa (31.2%), although there were reversions after seroconversion. Amino acid substitutions were also observed in the CTL epitope region spanning 243-251aa (8.3%). Significant differences ($P = 0.04929$) in substitution rates before and after seroconversion across two of the three CTL epitope regions were observed for the EHIV023 sample (see **Table 4**). Substitutions were more associated with the HIV-1 GAG gene sequenced after seroconversion for EHIV013 across the three CTL epitope regions studied. The two amino acid substitutions, A166G and L216V, observed in EHIV013, occurred after seroconversion and were not previously reported.

Amino Acid Signature Patterns in Variable Sites of HIV-1 GAG Genes

In this study, only sequences with non-synonymous substitutions compared to the reference sequence were analyzed. In **Table 5**, these substitutions were compared between the reference sequence and sequences isolated from individuals in the early stages of infection. In contrast, in **Tables 6**, **7**, the substitutions were compared within sequences isolated per sample spanning early HIV infection till after seroconversion. As shown in **Table 5**, non-synonymous substitutions were mostly observed in subtype G. However, a substitution, E105K, observed in CRF02_AG had been previously identified as a variant not recognized by the HXB2 epitope (Li et al., 2007). Subtype A had a frameshift mutation at aa85-88. There were no substitutions in sequences isolated from sample EHIV016, while EHIV 012 had the highest substitution within Subtype G sequences (see **Table 6**). EHIV022 and EHIV 012 had substitutions within sequences at aa positions of 106 and 110-113.

Lysine was the commonest aa used at position 106 (3/5), while a single substitution was observed for glutamate and asparagine. KSQ was the commonest aa usage in positions 110-113 (3/5); other aa in these positions were RRE (1/5) and KSK (1/5). In **Table 7**, non-synonymous substitutions associated with HIV-1 subtype A are described. EHIV013 had the highest number of substitutions spanning aa region 15 to 267 in this study. Samples EHIV023 and EHIV013 had substitutions within sequences at aa positions 215, 218, and 228. In position 215, Leucine was the highest aa used (4/7), while glutamine, asparagine, and valine were present in one sequence each. Valine (4/7) and Leucine (3/7) were the only aa used in position 218. Aspartate (3/7) was the highest aa used in position 228, while methionine (2/7), proline (1/7), and isoleucine (1/7) were also present in some isolates.

Non-synonymous Substitutions Associated With Immune Escape Variants Are More Within Epitopes Outside the HIV_gagconsv

Although four substitutions (E105K/CRF02AG; E203D/subtype G; K162R and A163G/subtype A) previously associated with immune escape were observed in HIV_gagconsv, more substitutions were found in regions outside the HIV_gagconsv. Out of the five individuals followed up, two had substitutions previously associated with immune escape strains in GAG gene sites outside the HIV_gagconsv (see **Figures 2**, **3**). Most

mutations associated with previously described immune escape strains were identified after seroconversion in this study. Fourteen mutations in 16 HIV-1 GAG sites outside the HIV_gagconsv were identified before seroconversion, while 21 mutations in 23 HIV-1 GAG sites outside the HIV_gagconsv were identified after seroconversion. As shown in **Figures 2**, **3**, EHIV012 had three mutations before (L31M, L101V, and S172T) and after (V82I, Y86W, and F172S) seroconversion. H28K, M30R, A224P, and A248G mutations were identified pre and post seroconversion, while V82I and Y86W mutations were identified only after seroconversion.

Serum Creatinine Concentration During Longitudinal Follow Up

We had earlier reported high levels of creatinine among HIV-infected Africans and African-Americans during the early stages of infection (Olusola et al., 2017, 2020b). In one of these studies (Olusola et al., 2020b), we also showed that immune activation of CTL was ongoing and correlated positively with high creatinine levels in early HIV-1 infected individuals. However, the effect of antibodies on creatinine levels were not observed in the previous study (Olusola et al., 2020b). As shown in **Figure 4**, there were differences in serum creatinine concentrations between early HIV-1 infection and seroconversion for the three

TABLE 4 | Substitutions associated with HIV_gagconsv in HIV-1 GAG gene isolated from 5 individuals followed from early HIV infection till after seroconversion.

Subtype	Sample ID	EHIV accession no	CTL epitope region (162-183)	CTL epitope region (203-214aa)	CTL epitope region (243-251aa)	
		Ref Seq	KAFSPEVIPMFSALSEGATPQD	DTINEEAAEWDR	TSTLQEQIR	*P*-Value
G	EHIV016	MN943627	Conserved	Conserved	Conserved	ns
		MN943623[a]	Conserved	Conserved	Conserved	
		% Substitution	Nil	Nil	Nil	
	EHIV022	MN943625	Conserved	D203E	Conserved	ns
		MN943622[a]	Conserved	E203D	Conserved	
		% Substitution	Nil	4	Nil	
	EHIV012	MN943626	Conserved	Conserved	Conserved	ns
[1pt]		MN943624	Conserved	Conserved	Conserved	
		MN943630[a]	M175k	D207E	R251G	
		% Substitution	2	2	2	
		Ref Seq			TSTPQEQLQWMT	**0.04929**
A	EHIV023	MN943619	Conserved	DTIN*G*PXXGQ	*HNRSY*YPSRT	
		MN943618	Conserved	Conserved	*HSRSY*YPSRT	
		MN943620	Conserved	DTINEEEFG*SN	*HSRSY*YPSRT	
		MN943621[a]	Conserved	Conserved	*HSRSY*YPSRT	
		% Substitution	Nil	31.2	8.3	
	EHIV013	MN943613	Conserved	Conserved	TSTTQEQIAWMT	ns
		MN943614	Conserved	Conserved	TSTPQEQIGWMT	
		MN943631[a]	A166G	L216V	TSTLQEQIGWMT	
		% Substitution	2.7	2.7	16.6	
	P-Value		ns	ns	ns	

[a]Sequence isolated after seroconversion

There were significant differences (P = 0.04929) in non-synonymous substitutions within EHIV023 sequences for the three functionally conserved epitopes.

TABLE 5 | Frequency of non-synonymous substitutions in sites outside the HIV_gagconsv of HIV-1 GAG gene amino acids.

	aa position in HIV-1 GAG gene	16	21	34	68	70	103	107	%sub
G	aa in Ref seq	L	V	Y	S	X	G	D	
	aa in EHIV samples	X	M	N	G	Y	C	V	2.3
	aa position in HIV-1 GAG gene	92	97	105	116	176			
CRF02 AG	aa in Ref seq	L	T	E	Q	T			
	aa in EHIV samples	Q	K	K	I	K			1.6
	aa position In HIV-1 GAG gene	89							
A*	aa in Ref seq								
	aa in EHIV samples	X							

*These aa replacements were 100% substituted in the EHIV samples compared to Ref seq. *Indel in aa positions 85-88 introduced a frameshift.*

individuals studied among the five persons followed up. EHIV 023 had lowest serum creatinine concentration at baseline (0.9 mg/dl) and after seroconversion (0.8 mg/dl) while EHIV 022 had the highest serum concentration of creatinine at baseline (1.1 mg/dl) and after seroconversion (1.0 mg/dl). Sample EHIV016 had no serum creatinine concentration changes from baseline until after seroconversion (1.0 mg/dl).

DISCUSSION

This study shows that diverse HIV-1 subtypes circulate in Nigeria, as subtypes A, G, and CRF02_AG were identified. Our study identified three non-synonymous substitutions within the HIV_gagconsv of HIV-1 GAG genes isolated from 10 early infected Nigerians. One substitution was, however, observed outside the HIV_gagconsv epitopes. Three (E105K, K162R, and A163G) of these substitutions have been previously related to immune escape (Currier et al., 2005; Li et al., 2007). These substitutions were associated with subtypes A and CRF02_AG. Most subtype G substitutions within the HIV_gagconsv were related to periods after seroconversion, while subtype A with early HIV infection.

Although few, substitutions within the HIV_gagconsv is a significant call for concern. Recent T cell-based vaccine studies have reported the likelihood of a single substitution within

TABLE 6 | Frequency of non-synonymous substitutions in variable sites outside the HIV_gagconsv of HIV-1 Subtype G GAG gene isolated from 3 individuals followed up from early infection till after seroconversion.

Sample ID	Accession no											
EHIV016	MN943627	No variable site										
	MN943623[a]											
EHIV022 (2.3% sub)	aa position	106	107	108	110-113							
	MN943625	N	V	C	RREK							
	MN943622[a]	K	I	Q	KSQE							
EHIV012 (8.6%sub)	aa position	31	62	66	69	82	86	90-96	101	104	106	110-114	121-2
	MN943626	M	T	L	Q	V	Y	QEFWLKG	V	V	K	KSQQE	GN
	MN943624	M	K	P	Q	V	Y	QRMGVKD	V	V	K	KSQQE	GN
	MN943630[a]	L	E	S	R	I	W	QRIDIRD	L	M	E	KSKQK	GN
	aa position	158	170	172	185	189	206	210	214	217	224	248	
	MN943626	V	M	T	T	T	D	E	L	TQ	A	R	
	MN943624	V	M	T	T	I	D	E	T	TQ	A	R	
	MN943630[a]	I	K	S	X	I	E	D	T	VH	P	G	

These aa replacements were 100% substituted in the early HIV-1 infected samples compared to [a]sequences from the same persons isolated after seroconversion.

TABLE 7 | Frequency of non-synonymous substitutions in variable sites outside HIV_gagconsv of HIV-1 Subtype A GAG gene isolated from 2 individuals followed up from early infection till after seroconversion.

Sample ID	Accession no													
EHIV023 (4% sub)	**aa position**	**205**	**215-222**	**228-230**										
	MN943619	I	LHPVHAGX	DKR										
	MN943618	I	QG*LYPRC	PAX										
	MN943620	T	NG*LYPRC	DKX										
	MN943621[a]	I	LHPVHAGX	DKR										
EHIV013 (13.6%sub)	**aa position**	**15**	**28**	**30**	**41**	**49**	**60**	**62-62**	**66**	**69**	**72**	**76**	**82**	**86**
	MN943613	K	T	K	M	G	V	EQ	S	K	T	R	V	Y
	MN943614	S	K	R	L	S	I	DR	P	K	S	R	V	Y
	MN943631[a]	A	K	R	L	G	L	EQ	S	R	S	K	I	W
	aa position	**92-93**	**104**	**107**	**115**	**118**	**121**	**124**	**125-127**	**139**	**143**	**146-7**	**182**	**215**
	MN943613	EV	I	I	E	A	D	N	SKV	Q	V	AV	G	L
	MN943614	DV	I	M	P	A	D	S	SKV	Q	V	TL	Q	L
	MN943631[a]	DI	M	I	V	T	A	S	***	R	T	SM	Q	V
	aa position	**218**	**223-224**	**228**	**243**	**248**	**252**	**260**	**263**	**267**				
	MN943613	L	IA	I	T	A	G	D	R	I				
	MN943614	V	VP	M	P	G	S	D	K	I				
	MN943631[a]	V	IP	M	L	G	S	E	K	V				

These aa replacements were 100% substituted in the EHIV samples compared to [a]Sequence isolated after seroconversion. The red letters indicate sequence reversion after seroconversion.

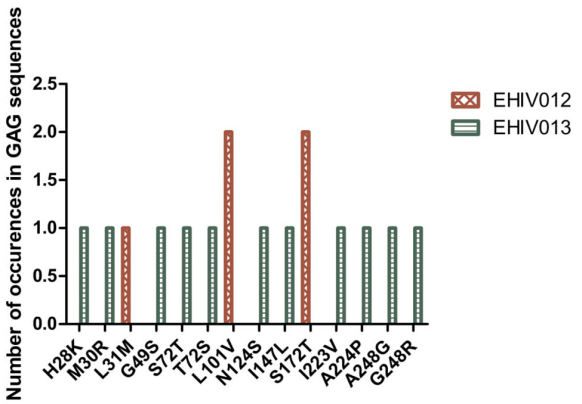

FIGURE 2 | Distribution of mutations associated with escape in EHIV012 and EHIV013 before seroconversion. Three individuals had mutations previously associated with escape, diminished responses, non-susceptible forms, etc. These mutations were compiled from the Los Alamos National Laboratory HIV Immunology Database for CTL/CD8 + Epitope Variants and Escape Mutations[8]. The list of all the identified mutations is presented in **Supplementary Table 1**. Two individuals (EHIV012 and EHIV013) had mutations outside the GAG HIV_GAGCONSV, while EHIV022 had a mutation corresponding to escape (Murakoshi) – E203D. **Figure 3** shows the distribution of mutations associated with escape in EHIV 012(Red Bars) and EHIV013 (Green Bars). The number of occurrences of the mutations in GAG sequences is shown in the Y-axis while the X-axis shows aa mutations.

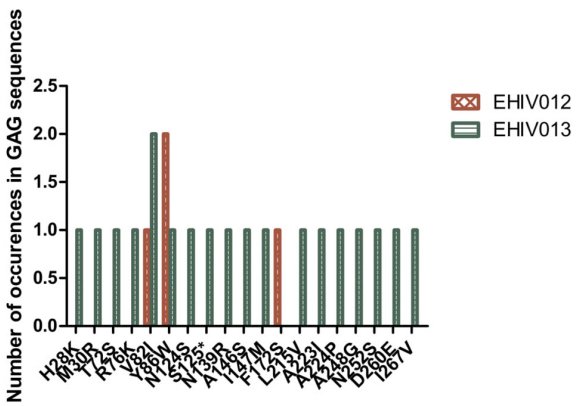

FIGURE 3 | Distribution of mutations associated with escape in EHIV012 and EHIV013 after seroconversion. Three individuals had mutations previously associated with escape, diminished responses, non-susceptible forms, etc. These mutations were compiled from the Los Alamos National Laboratory HIV Immunology Database for CTL/CD8 + Epitope Variants and Escape Mutations[8]. The list of all the identified mutations is presented in **Supplementary Table 1**. Two individuals (EHIV012 and EHIV013) had mutations outside the GAG HIV_GAGCONSV, while EHIV022 had a mutation corresponding to escape (Murakoshi) – E203D. **Figure 4** shows the distribution of mutations associated with escape in EHIV 012(Red Bars) and EHIV013 (Green Bars). The number of occurrences of the mutations in GAG sequences is shown in the Y-axis while the X-axis shows aa mutations.

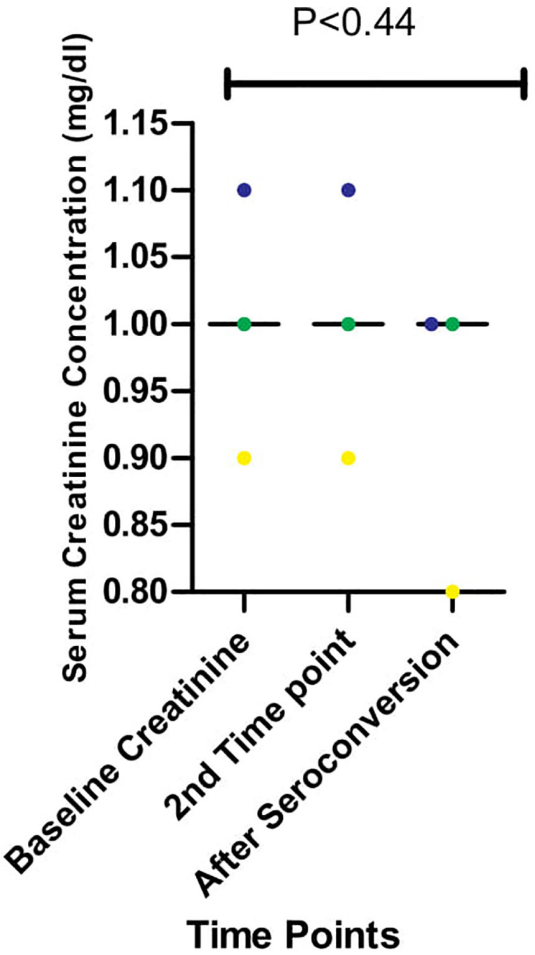

FIGURE 4 | Serum creatinine concentration during longitudinal follow up of three individuals from early infection till after seroconversion. The three individuals followed up are represented in blue, yellow, and green. Serum creatinine levels for these individuals were measured in three-time, Baseline, second-time, and after seroconversion. Significant differences in the levels of serum creatinine (measured in mg/dl) observed across the three-time points ($P < 0.44$) were calculated using 1-way ANOVA.

HIV_gagconsv affecting the immunogenicity vaccine constructs (Ondondo et al., 2016; Hanke, 2019). Immunogenicity of functionally conserved epitopes is a foundational requirement for an effective universal T cell-based HIV-1 vaccine (Létourneau et al., 2007). However, studies have shown that D203E mutations in the ETINEEAAEW epitope do not impact the epitope's function and strain diversity coverage (Ondondo et al., 2016). Mutations after seroconversion may be due to the pressure of viral evasion mechanisms such as NEF mediated evasion of antibodies and HIV-1 subtype differences (Buckheit et al., 2012; Omondi et al., 2019). Studies have shown that these factors may account for replication and increased viremia during HIV-1 infection (Buckheit et al., 2012; Omondi et al., 2019).

We observed fourteen and nineteen mutations previously associated with immune escape outside the HIV_gagconsv before and after seroconversion, respectively, in this study. This finding is a far cry from the four observed within the HIV_gagconsv. It is also in accordance with previous reports that observed minimal non-synonymous substitutions in CTL epitopes within the HIV_gagconsv (Amicosante et al., 2002; Lazaro et al., 2011;

Ondondo et al., 2016). APOBEC-3G has previously been noted as a major cause of hypermutation in the HIV-1 proviral landscape during the early stages of infection (Lee et al., 2019). Identifying these functionally conserved epitopes in our study participants lends credence to their immunodominance and high strain diversity coverage (Ondondo et al., 2016; Shu et al., 2020). However, the high rate of non-synonymous substitutions outside the HIV_gagconsv epitopes implies that escape mutants outside the HIV_gagconsv are more likely to be integrated into the HIV-1 latent cellular reservoir landscape. This is because latent reservoir strains are established during the early stages of infection and are known to compose majorly of CTL immune escape strains (Deng et al., 2014; Gounder et al., 2015; Roberts et al., 2015; Leitman et al., 2017). Therefore, these latent reservoirs may require a broad CTL response for clearance, as previously alluded to Deng et al. (2014). If this is the case, then using functionally conserved HIV-1 DNA vaccines for therapeutic clearance of latent reservoirs (hybrid HIV-1 cure) may be very difficult.

Therapeutic HIV-1 vaccines are intended to be used after cART stoppage, particularly against latent reservoir strains. However, as shown in this study, immune escape strains generated during the early stages of infection, which are likely significant constituents of the latent reservoir, may lead to therapeutic vaccine failures (Daucher et al., 2008; Ondondo et al., 2016; Hanke, 2019; Lé tourneau et al., 2020). Since immune escape occurs during the early stages of infection, cART must commence early to reduce the reservoir size and the incorporation of immune escape variants into the reservoir landscape (Brockman et al., 2015; Takata et al., 2017). Other studies have alluded that post-treatment control may be possible if treatment commences at the early stages of HIV infection since blips observed after cART stoppage are mainly due to immune escape variants incorporated into reservoir cells (Conway and Perelson, 2014). However, rapid and high magnitude CTL responses observed during the early stages of infection (Ndhlovu et al., 2015) may be affected by early treatment (Takata et al., 2017; Lee et al., 2019; Ndhlovu et al., 2019). This treatment can impair subsequent CTL responses during cART stoppage in post-treatment control trials. This study has shown that immune escape variants may be from those arising from CTL epitopes outside the HIV_gagconsv. However, functional and molecular studies on the nature and characteristics of HIV-1 strains in latent reservoirs need to be carried out to ascertain our claims. Furthermore, we cannot fully corroborate this theory in our study because of the relatively low sample size.

Also, universal HIV-1 vaccines are supposed to be broadly effective against all HIV-1 clades. However, immune escape strains encoding CTL epitopes outside the HIV_gagconsv may reduce the sensitivities of these vaccines. Observations of CTL escape mutants after seroconversion in this study suggest that immune pressures by other cells other than CTL may aid the generation of CTL immune escape mutants. Mapping HIV immune epitopes in different regions of the genome will further clarify this hypothesis (Matthews et al., 2012; Adland et al., 2013). As observed previously in other studies, CTL epitope KAFSPEVIPMF was the most conserved (Garcia-knight et al., 2016; Gama Caetano et al., 2018). This epitope has been associated with very low frequencies of CTL response selective pressures and has been a choice for many T cell-based HIV vaccines (Hanke, 2019). However, two substitutions previously associated with immune escape, K162R, and A163G were observed in this epitope for Subtype A during the early stages of infection.

On the other hand, CTL epitope DTINEEAAEWDR was associated with more substitutions, although most of these mutations reverted to wild type after seroconversion. This phenomenon was observable in both subtypes A and G. It seems that although more mutations were observed during the early stages of HIV infection, reversions of these mutations occurred later on in infection. Previous studies have also associated CTL epitopes' reversions with the early stages of HIV infection (Li et al., 2007). This epitope was included in the second generation of functionally conserved HIV DNA vaccines because of its high conservation and coverage of strains diversity (Abdul-Jawad et al., 2016; Hanke, 2019). The immunogenicity of this epitope in the vaccine construct was also observed in macaques and untreated HIV infected individuals (Ondondo et al., 2016). These unique properties of this epitope were also observed in this study. However, a non-synonymous substitution, E203D, was observed in CTL epitope DTINEEAAEWDR. The selection of this epitope for immune escape strains has been previously described (Murakoshi et al., 2019). However, the substitution has also been shown not to impact the epitope's immunogenicity as a vaccine construct (Ondondo et al., 2016). While the substitution may not affect function, it may be integrated into the latent reservoir landscape. Hence, the amino acid position should be excluded in the epitope's design as a DNA vaccine construct.

CTL epitope TSTLQEQIR was conserved for subtype G. However, this epitope does not possess high coverage for subtype A sequences. Previous studies have documented that TSTLQEQIR may have lower HIV-1 strain diversity coverage, may be presented early, and probably associated with elite controllers (O'Connell et al., 2011; Balamurugan et al., 2013; Ondondo et al., 2016). This epitope in T cell-based HIV-1 DNA vaccines has shown moderate coverage but a strong HIV-1 specific CTL (Ondondo et al., 2016). The epitope may not be functional against subtype A and may probably be expressed by rare HLAs since it was identified in only six individuals in this study. This study does not favor using the epitope in a vaccine construct for the West African region where subtype A predominantly circulates. However, the epitope's association with HIV-1 RNA viral load control and NEF gene downregulation (O'Connell et al., 2011; Balamurugan et al., 2013) is a plus for its use in a therapeutic HIV-1 DNA vaccine.

Several previously recognized immune escape substitutions were observed in this study. Majority of these substitutions emanated from epitopes outside the HIV_gagconsv. This is the first longitudinal study from West Africa on the kinetics of previously recognized functionally conserved epitopes of the HIV-1 GAG gene to the best of our knowledge. It is worthy of note that these epitopes have already been used in second-generation T cell HIV-1 DNA vaccines as a proof of concept. Our study provides real-life evidence of the immunodominance, conservative, and highly diverse strain coverage of these epitopes.

These properties form the basis of the strategies employed in the design of conserved region vaccines.

We have also shown that numerous non-synonymous substitutions associated with CTL epitopes outside the HIV_gagconsv occur during the early stages of HIV-1 infection among HIV-1 subtypes and recombinant forms circulating in West Africa. It is essential to state that these substitutions were identified from HIV-1 DNA sequences against plasma RNA used in a similar study (Gounder et al., 2015). Proviral sequences have previously been associated with rare mutations on CTL epitopes (Gama Caetano et al., 2018; Lee et al., 2019). These substitutions may have to be considered in designing universal and therapeutic vaccines for HIV-1 strains circulating in West African countries (Ndung'u et al., 2019; Shu et al., 2020). Recent studies have shown the significant role of poorly recognized CTL epitopes in viral escape (Grossman et al., 2019).

While H28K, M30R, A224P, and A248G non-synonymous substitutions were observed before and after seroconversion, others, namely V82I and Y86W, were consistently identified after seroconversion. V82I has been previously identified with the emergence of higher viral loads in studies among HIV-infected individuals (Arcia et al., 2018; Karlsson et al., 2020). On the other hand, Y86W was associated with HIV-1 clade B and E (Fukada et al., 2002). Besides previous studies, we have also reported the high levels of creatinine among HIV-infected Africans during the early stages of infection(Bruggeman et al., 2000; Marras et al., 2002; Olusola et al., 2017, 2020b). In our study, we also showed that immune activation of CTL was ongoing and correlated positively with high creatinine levels in these early HIV-1 infected individuals (Olusola et al., 2020b). In this present study, we showed that a reduction in creatinine concentrations occurred after seroconversion. CTL immune pressures may be associated with high creatinine levels in Africans. However, this needs further investigation.

In summary, we have shown that there is a high genetic diversity of HIV-1 strains in Nigeria. Also, very high frequencies of non-synonymous substitutions occur in the HIV-1 GAG gene during the early stages of infection up until seroconversion. These substitutions include previously mapped CTL epitope immune escape mutants that are frequent in epitopes outside the HIV_gagconsv. Observation of the immunodominance of functionally conserved epitopes used in current T cell-based HIV-1 DNA vaccines in this study emphasizes the usefulness of these vaccines in a region where it is highly needed (Ndung'u et al., 2019). However, future directions for slight modifications to the use of the epitopes in the West African region are also noted. CTL immune pressure likely leaves different footprints and signature patterns on HIV-1 GAG epitopes within and outside the HIV_gagconsv.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved. This research was conducted in accordance with the declaration of Helsinki. Experiments were conducted with the understanding and the consent of each participant. Ethical approvals for this research were obtained from the University of Ibadan/University College Hospital (UI/UCH) Research and Ethics Committee (UI/EC/15/0076) and the Oyo State Ministry of Health Committee on Human Research (AD13/479/951). All results were delinked from patient identifiers and anonymized. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BO, DO, and GO conceptualized and designed the study. BO preformed the experiments, analysed and interpreted the data as well as wrote the first draft of manuscript. DO and GO supervised the work and reviewed the manuscript. All authors contributed to the article and approved the submitted 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/fmicb.2020.615721/full#supplementary-material

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PERSPECTIVE
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Predicting Post-treatment HIV Remission: Does Size of the Viral Reservoir Matter?

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Combination antiretroviral therapy (ART) suppresses human immunodeficiency virus (HIV) replication and improves immune function. However, due to the persistence of long-lived HIV reservoirs, therapy interruption almost inevitably leads to a fast viral rebound. A small percentage of individuals who are able to control HIV replication for extended periods after therapy interruption are of particular interest because they may represent a model of long-term HIV remission without ART. These individuals are characterized by a limited viral reservoir and low reservoir measures can predict post-treatment HIV remission. However, most individuals with a low reservoir still experience fast viral rebound. In this Perspective, we discuss the possible reasons behind this and propose to develop an integral profile, composed of viral and host biomarkers, that could allow the accurate prediction of post-treatment HIV remission. We also propose to incorporate information on the chromatin context of the proviral integration sites into the characterization of the HIV reservoir, as this likely influences the reactivation capacity of latent proviruses and, together with the actual number of intact proviruses, contributes to the replication competence of the reservoir.

Keywords: HIV, viral reservoir, antiretroviral therapy, post-treatment controllers, predictive marker, biomarker, profile

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INTRODUCTION

Combination antiretroviral therapy (ART) can successfully manage human immunodeficiency virus (HIV) replication but is not curative, due to the persistence of long-lived viral reservoirs (Deeks et al., 2016; Ndung'u et al., 2019). The main reservoir is thought to reside in latently infected resting CD4 + T cells in peripheral blood and lymphoid tissue, although other cell types such as macrophages may contribute as well (Darcis et al., 2019; Ganor et al., 2019). Even after many years of successful treatment, ART interruption typically leads to a viral rebound within 2–4 weeks; however, some individuals, termed "post-treatment controllers," are able to control HIV replication after therapy interruption for extended periods and thus may represent a model of long-term HIV remission without ART or functional cure (Hocqueloux et al., 2010; Steingrover et al., 2010; Salgado et al., 2011; Goujard et al., 2012; Lodi et al., 2012; Van Gulck et al., 2012; Stöhr et al., 2013; Sáez-Cirión et al., 2013; Assoumou et al., 2015; Kinloch-de Loes et al., 2015; Frange et al., 2016; Maggiolo et al., 2018; Namazi et al., 2018; Violari et al., 2019). Therefore, recent years have seen accelerated research into mechanisms of HIV control in these rare individuals and in the macaque models of post-treatment control (PTC) (Strongin et al., 2020). Several excellent reviews on PTC have been published (Cockerham et al., 2016; Goulder and Deeks, 2018; Martin and Frater, 2018;

Etemad et al., 2019), and a mathematical model of the underlying mechanisms has been proposed (Conway and Perelson, 2015). PTC is more frequent after ART initiated during early HIV infection (Namazi et al., 2018), which is not surprising given that a low viral reservoir has been consistently measured in post-treatment controllers (Goujard et al., 2012; Van Gulck et al., 2012; Sáez-Cirión et al., 2013), and early ART initiation is much more efficient in reducing the reservoir than ART initiated during chronic infection (Strain et al., 2005; Jain et al., 2013; Buzon et al., 2014). However, early ART initiation is not sufficient for PTC as most early treated individuals, even those treated very early after infection, still demonstrate fast viral rebound upon therapy interruption (Gianella et al., 2015; Colby et al., 2018). Longer ART duration has also been proposed to increase the chances of PTC (Stöhr et al., 2013; Fidler et al., 2017), but this factor on its own is insufficient to confer this phenotype. Identification of individuals with a higher probability of PTC, in whom it is safer to interrupt ART than in others, is of utmost importance in light of HIV cure research, where every therapeutic intervention necessitates an analytical treatment interruption (ATI) to assess its efficacy. A low risk of viral rebound during an ATI means low risks of reservoir replenishment, selection of drug resistance, disease progression, and HIV transmission (El-Sadr et al., 2006; Julg et al., 2019). The absence of reliable predictive markers of viral rebound complicates clinical decision-making on ATI and therefore hinders HIV cure research (Li et al., 2015). To fill this knowledge gap, a number of studies have been undertaken to identify biomarkers that could predict PTC or the time to viral rebound after ART interruption (Williams et al., 2014; Assoumou et al., 2015; Li et al., 2016; Sharaf et al., 2018; Pasternak et al., 2020). The latter measure may be more inclusive than the former, as PTC is a spectrum (Martin and Frater, 2018), and its definitions differ between studies, in particular in terms of the minimal duration of viral control and of the threshold for the viral rebound (Sáez-Cirión et al., 2013; Williams et al., 2014; Li et al., 2016; Namazi et al., 2018). Variable definitions of PTC also contribute to the variability in the frequency of post-treatment controllers between studies, which ranges from $< 1\%$ to $> 20\%$ and is inversely proportional to the duration of control (Hocqueloux et al., 2010; Sáez-Cirión et al., 2013; Maenza et al., 2015; Namazi et al., 2018). To standardize these definitions, Martin et al. proposed to reserve the term "PTC" for the cases of long-term continuous HIV control (several years) below the lowest possible detection limit of commercial plasma viral load assays (20 copies/mL) and to use the term "virological remission" for the intermediate cases that do not fulfill these strict criteria (Martin and Frater, 2018). Hence, using time to viral rebound as an outcome measure allows the inclusion of all these intermediate cases, increasing statistical power and potentially allowing additional insights into the mechanisms of control. On the other hand, some individuals who demonstrate various degrees of post-treatment HIV remission undergo transient viral rebound shortly after ART interruption before resuppressing the virus (Namazi et al., 2018), and measuring the time from ART interruption to the viral rebound will exclude these individuals. Clearly, improved definitions of post-treatment remission are needed to guide future HIV cure trials.

Predicting Post-treatment HIV Remission: Time for a Comprehensive Approach

A number of candidate predictive biomarkers for the virological remission have been proposed. However, the measured markers, timing of their measurement, thresholds for viral rebound, and the statistical analyses that were performed differ significantly between studies. This, in combination with limited sample sizes, resulted in different and even some contradictory conclusions. Several studies identified total HIV DNA, measured just before ART interruption, as a predictor of time to viral rebound (Goujard et al., 2012; Williams et al., 2014; Assoumou et al., 2015), and this marker even outperformed the number of intact proviruses in distinguishing individuals with post-treatment virological remission from non-controllers in a recent study (Sharaf et al., 2018). On the other hand, three independent groups reported that cell-associated (CA) HIV unspliced RNA, measured at ART interruption, could predict time to viral rebound, while total HIV DNA was not predictive in these studies (Li et al., 2016; Sneller et al., 2017; Pasternak et al., 2020). It must be noted that CA RNA was not measured in most studies that did identify total DNA as a predictor, precluding a direct comparison between these markers. Importantly, we demonstrated that the pre-treatment-interruption level of CA unspliced RNA was predictive not only of the time to viral rebound to both >50 and >400 copies/mL but also of the magnitude of the viral rebound, independently of pre-ART virological biomarkers (Pasternak et al., 2020). This suggests that measurements of the "active reservoir" (Pasternak et al., 2013) or "transcription-competent reservoir" (Baxter et al., 2018) can help support the HIV cure-directed clinical trials (Abdel-Mohsen et al., 2020). However, standardization of assays and the CA RNA transcripts that are measured is warranted in order to obtain meaningful results. Apart from the unspliced RNA, PCR-based assays have been developed to measure levels of total, completed (polyadenylated), or multiply spliced CA HIV RNA transcripts in infected individuals (Pasternak et al., 2008; Shan et al., 2013; Yukl et al., 2018). The assay for total CA RNA uses primers that bind to the HIV TAR region and thus measures the level of transcription initiation. This TAR RNA is more abundant than unspliced RNA, but most of these transcripts are short and do not encode viral proteins (Lassen et al., 2004; Yukl et al., 2018). On the other hand, the presence of multiply spliced RNA may be a more proximal surrogate of productive infection compared with unspliced RNA only (Pasternak and Berkhout, 2018). However, multiply spliced RNA is much less abundant than unspliced (Kaiser et al., 2007; Pasternak et al., 2020), due to both proviral genetic defects (as splicing requires the presence of several intact genomic regions) and latency blocks to completion of transcription and splicing (Yukl et al., 2018; Moron-Lopez et al., 2020). As a consequence, it is challenging to detect multiply spliced RNA in ART-treated individuals without *ex vivo* cellular stimulation, which explains why it has not yet been assessed as a potential predictor of the post-treatment remission.

In addition to CA RNA, plasma HIV RNA blips on ART were also shown to predict shorter time to rebound (Fidler et al., 2017). Further studies in larger cohorts are necessary in order

to establish whether the total number of HIV proviruses, HIV transcriptional activity, or a combination of these markers, can be used to support HIV curative interventions. Although total HIV DNA is mostly composed of replication-defective proviruses (Bruner et al., 2016; Hiener et al., 2017), and a significant proportion of CA RNA molecules might be transcribed from such defective proviruses as well (Pollack et al., 2017; Imamichi et al., 2020), both HIV DNA and CA RNA correlate with the inducible provirus levels (Darcis et al., 2017; Cillo et al., 2018), suggesting their utility as surrogate markers of the replication-competent HIV reservoir (Avettand-Fènoël et al., 2016; Pasternak and Berkhout, 2018). In any case, it is clear that a low viral reservoir is extremely important for HIV remission, and mathematical models have been developed that are based on the assumption that the duration of remission is inversely proportional to the replication-competent HIV reservoir size (Hill et al., 2014, 2016; Conway and Perelson, 2015; Davenport et al., 2019). It would therefore seem logical that ATI performed in a group of ART-treated individuals with low reservoir measures may result in HIV remission in a substantial proportion of cases. This has indeed been attempted by several groups (Chun et al., 2010; Calin et al., 2016; Colby et al., 2018; Pannus et al., 2020), but the absolute majority of cases experienced a quick viral rebound, suggesting that a low reservoir alone is insufficient for HIV remission and that other factors need to be considered. Here it must be noted that our understanding of the HIV reservoirs and their importance for the prediction of the post-treatment remission is still largely limited to the peripheral blood, whereas tissue reservoirs might play an even more important role. Different cellular and anatomical compartments, such as follicular T helper cells in the lymph node germinal centers, may serve as sanctuaries for HIV persistence under ART (Banga et al., 2016; Chaillon et al., 2020) and fuel the viral rebound upon ATI (De Scheerder et al., 2019). Although sampling peripheral blood is obviously easier, better characterization of tissue reservoirs can improve the predictive value of the HIV reservoir for the post-treatment remission.

In addition to virological markers, several host biomarkers have been proposed to predict post-treatment HIV remission. Pre-ART levels of T-cell exhaustion markers (PD-1, Tim-3, and Lag-3) have been shown to predict the time to viral rebound, although their on-ART levels were not predictive (Hurst et al., 2015). Two recent studies identified plasma and antibody glycomic biomarkers, in particular digalactosylated G2 glycans on IgG, as predictive markers of post-treatment remission (Giron et al., 2020; Offersen et al., 2020). Moreover, pre-ATI levels of HIV gp120-specific G2 glycans inversely correlated with CA HIV unspliced RNA levels (Offersen et al., 2020), providing a possible explanation why it was predictive of longer time to rebound. Although the role of cytotoxic T lymphocytes (CTLs) in post-treatment HIV remission is probably not as pronounced as in spontaneous ("elite") HIV control and post-treatment controllers mostly lack protective HLA alleles (Goujard et al., 2012; Sáez-Cirión et al., 2013; Maenza et al., 2015), this does not mean that other components of the host immunity are not important. In fact, ART initiated extremely early, during the "hyperacute" HIV infection (Fiebig stage I), rarely results in prolonged post-treatment remission (Henrich et al., 2017; Colby et al., 2018), which is thought to reflect an insufficient time window for maturation of the adaptive immune responses (Goulder and Deeks, 2018). In contrast, the SPARTAC and Primo-SHM studies where temporary ART was started during primary infection, but not too early, resulted in some participants experiencing various degrees of post-treatment remission (Stöhr et al., 2013; Pasternak et al., 2020).

It therefore appears useful to develop a comprehensive molecular profile, incorporating multiple viral and host biomarkers, that could reliably predict post-treatment HIV remission. Such a profile could be based on the principle of diagnostic multivariate index assays that are already used in other medical fields (Zhang, 2012). The advantage of such a composite molecular profile, compared to single biomarker assays, is that the aggregated information from complementary biomarkers is expected to outperform each of the individual component biomarkers in sensitivity, specificity, and predictive value. Applied to the prediction of post-treatment HIV remission, such a profile may be composed of metabolomic, lipidomic, and proteomic biomarkers, in combination with virological and immunological profiling. In addition, the expression of recently identified cellular markers of the HIV reservoirs, such as CD32a, CD30, CD20, PD-1, and others (Fromentin et al., 2016; Descours et al., 2017; Abdel-Mohsen et al., 2018; Hogan et al., 2018; Serra-Peinado et al., 2019; Darcis et al., 2020; Neidleman et al., 2020; Adams et al., 2021), as well as T-cell phenotypic markers (Hiener et al., 2017), could be incorporated in this profile. Indeed, CD30+ CD4+ T cells, as well as expression of some HIV restriction factors, were shown to increase before viral rebound after ATI (De Scheerder et al., 2020; Prator et al., 2020). Furthermore, Mitchell et al. (2020) recently demonstrated that plasmacytoid dendritic cells can sense HIV replication before detectable viremia following treatment interruption, which was evidenced by a transient loss of IFNα production. Expression of cellular factors that are involved in long-term cell survival and proliferation vs. apoptosis could also play a role (Kuo et al., 2018; Angin et al., 2019). In this regard, as no single molecule has yet been described that marks all reservoir cells, a combinatorial approach will again be beneficial and perhaps even necessary. Ideally, the evolution of such a comprehensive molecular profile could allow the development of a personalized approach to HIV curative interventions. In particular, a gender-specific approach might be necessary, since several (but not all) studies demonstrated lower CA HIV RNA levels in women compared to men (Scully et al., 2019; Falcinelli et al., 2020; Gianella et al., 2020), and estrogen has been shown to repress HIV transcription (Das et al., 2018). Such approach should also include the personal medical history of each individual, namely the level of persistent immune activation despite ART, the history of comorbidities that is often associated with chronic inflammation, as well as current and historical ART regimens, all of which may contribute to the probability and timing of viral rebound.

Post-treatment Remission and the HIV Reservoir Size: Are We Measuring the Right Markers?

Low HIV reservoir is necessary but apparently not sufficient for post-treatment remission, as even individuals with very low levels

of reservoir markers experience fast viral rebound upon ART interruption. As discussed above, one possible solution to this problem is to identify other, complementary biomarkers, thus increasing the predictive power of the resulting profile. However, another possibility is that our current toolkit simply does not allow sufficiently accurate measurement of the HIV reservoir size. The latter is defined as the number of cells carrying replication-competent proviruses, in other words integrated viral genomes capable of reigniting viral spread upon ART interruption (Eisele and Siliciano, 2012; Pasternak and Berkhout, 2016). However, it is difficult to estimate the real HIV reservoir size, as PCR-based methods that measure HIV DNA and RNA overestimate the reservoir because most of proviruses are genetically defective (Bruner et al., 2016). On the other hand, the quantitative viral outgrowth assay (qVOA) will not score defective proviruses, but is thought to underestimate the reservoir as only a small fraction of genetically intact proviruses can be activated *ex vivo* (Ho et al., 2013; Bruner et al., 2016; Kwon et al., 2020; Martin et al., 2020). The most accurate surrogate marker of the reservoir size is currently considered to be the number of intact proviruses, estimated by either full-length proviral sequencing (Bruner et al., 2016; Hiener et al., 2017; Pinzone et al., 2019) or the recently developed digital droplet PCR-based intact proviral DNA assay (IPDA) (Bruner et al., 2019). Not all intact proviruses are replication-competent, as full-length sequencing is only able to identify gross genetic defects, such as large internal deletions, hypermutation, stop codons, frameshift mutations, or defects in the major splice donor site or the packaging signal, and will not identify other genetic changes that may be deleterious for HIV replication. However, most of the intact proviruses demonstrate normal replication kinetics *in vitro* (Ho et al., 2013), suggesting that the majority of proviruses identified as intact by full-length proviral sequencing are replication-competent. In comparison, IPDA overestimates the intact reservoir somewhat, as only ∼70% of proviruses that are identified as intact by IPDA are also intact by full-length proviral sequencing (Bruner et al., 2019).

There might be, however, another level of complexity to the measurement of HIV reservoir. By applying the novel multiple displacement amplification (MDA)-based matched integration site and proviral sequencing (MIPSeq) technique, the Lichterfeld group demonstrated that in individuals on prolonged ART, in comparison to defective proviruses, intact HIV proviruses were enriched for non-genic chromosomal positions and other features of "deep latency" (Einkauf et al., 2019). This bias was subsequently confirmed by another group that also used the MDA method (Patro et al., 2019). More importantly, the same technique, recently applied to the characterization of the HIV reservoir in elite controllers, revealed that this population demonstrates an even more extreme phenotype than ART-treated individuals: 40% of intact proviral clones in elite controllers were integrated into non-genic or pseudogenic regions, compared to 13% in ART-treated individuals (Jiang et al., 2020). Moreover, in contrast to ART-treated individuals, intact proviral sequences from elite controllers were preferentially integrated in centromeric satellite DNA or in other regions associated with heterochromatin, and at an increased distance to transcriptional start sites and accessible chromatin, and were enriched in repressive chromatin marks. As infection of CD4+ T cells from elite controllers *ex vivo* with a laboratory HIV strain led to a normal integration pattern, it is likely that this skewed integration pattern observed *in vivo* is the result of selective elimination of cells infected with transcriptionally competent intact proviruses over time by the immune system, resulting in enrichment for intact proviruses that are in a state of "deep latency" (also referred to as "blocked and locked" state) and are unlikely to be reactivated (Jiang et al., 2020). It was demonstrated 20 years ago that the provirus transcriptional activity is influenced by the integration site (Jordan et al., 2001), and indeed, levels of HIV transcription in elite controllers were shown to be at least 10-fold lower than in ART-treated individuals (Jiang et al., 2020), confirming the results of previous studies (Van Gulck et al., 2012; Hatano et al., 2013). Interestingly, Battivelli et al. (2018) found that HIV reactivation in a primary CD4+ T-cell model of latency occurred in at most 5% of the infected cells and depended on integration in an open chromatin context, which was confirmed by another group that demonstrated that inactive chromatin marks accumulate across the provirus with time (Lindqvist et al., 2020). The proportions of clonally expanded intact proviruses were shown to be larger in elite controllers than in ART-treated individuals (Veenhuis et al., 2018; Jiang et al., 2020), with the same pattern observed in a post-treatment controller (Veenhuis et al., 2018). Moreover, CD8+ T cells from the elite and post-treatment controllers were capable of suppressing replication of their autologous clonally expanded viruses *in vitro* (Veenhuis et al., 2018), suggesting that these intact proviruses can undergo clonal expansion without or with minimal viral gene expression (Hosmane et al., 2017; Musick et al., 2019).

Taken together, these recent insights imply that the integration site-imposed reactivation potential of a provirus could be as important as its genetic intactness (Chomont, 2020). In other words, not only the size of the reservoir, but also its repertoire (not only in terms of chromatin context but also in terms of diversity and clonality of proviral integration sites) matters for the replication competence. Moreover, they bring into doubt the concept that qVOA profoundly underestimates the replication-competent reservoir, providing a possible explanation why only a tiny fraction of intact proviruses can be reactivated *ex vivo*. On the other hand, Ho et al. reported that most non-induced intact proviruses in their study were integrated into active transcription units, suggesting that other factors exist that prevent intact provirus reactivation, at least *ex vivo* (Ho et al., 2013). Although the reactivation abilities *ex vivo* and *in vivo* cannot be directly compared, and there always is a possibility that a provirus that cannot become reactivated *ex vivo* even after multiple rounds of TCR stimulation, still can reignite viral rebound *in vivo* after ART interruption, we might consider the number of intact proviruses as the upper, conservative, limit of the replication-competent reservoir. In most infected individuals, the reservoir is probably much lower than this limit and in order to be able to accurately quantify the reservoir size, it would be necessary to combine the measurement of genetic intactness with that of *in vivo*

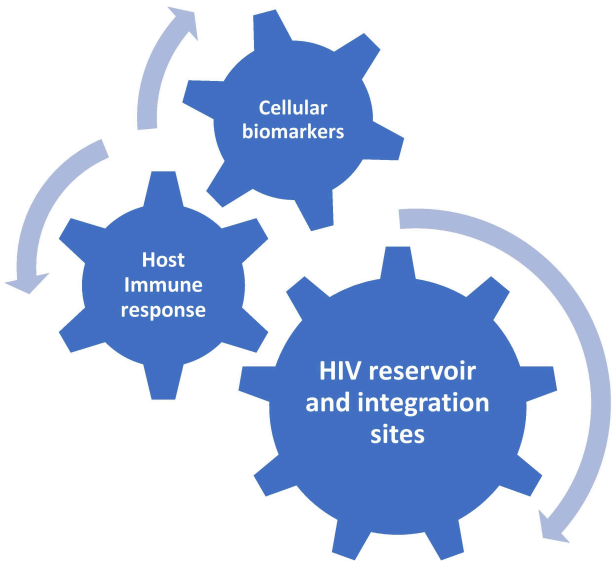

FIGURE 1 | Proposed molecular profile for the prediction of post-treatment HIV remission.

reactivation potential, although measuring the latter is very difficult if not impossible. As a surrogate, a simple score based on the provirus intactness and the chromosomal context of its integration site could be developed, as MDA-based and other similar assays that simultaneously measure the provirus intactness and map the integration site provide the possibility to do this. Importantly, such a combined score may be able to predict time to viral rebound after ATI and/or post-treatment HIV remission better than other reservoir measures. To further improve the predictive power, this score could be incorporated into the molecular profile proposed above (**Figure 1**). Indeed, although the mechanisms of control are different between most elite and post-treatment controllers, studies have identified a subpopulation of elite controllers with both markedly inefficient *ex vivo* HIV reactivation from resting CD4+ T cells and low HIV-specific CD8+ T-cell responses (Noel et al., 2016; Canouï et al., 2017). This subpopulation may in fact resemble post-treatment controllers, in most of which no protective HLA alleles were found and CD8+ responses are also not particularly strong, and the virus replication is probably controlled due to infrequent reactivation from latency. Further research is needed to establish whether the HIV integration site landscape in post-treatment controllers resembles that of elite controllers (Jiang et al., 2020). This should involve longitudinal studies to evaluate immune selection for viral reservoir cells (Wang et al., 2018; Huang et al., 2019).

Finally, if the proviral integration sites are so important for the replication competence of the reservoir, is there something that can be done therapeutically to facilitate HIV integration in regions that are associated with the repression of transcription? HIV preferentially integrates in actively transcribed genes (Han et al., 2004; Marini et al., 2015) but long-term ART selects for transcriptionally silent proviruses (Pinzone et al., 2019). However, ART cannot do wonders and even after decennia of suppressive therapy, most individuals will experience a fast viral rebound upon ART interruption. Therefore, a number of strategies to "block and lock" the provirus in the inactive state are currently under investigation (reviewed in Moranguinho and Valente, 2020; Vansant et al., 2020a). In particular, the Debyser group developed a technique, based on the small-molecule (LEDGIN) inhibition of the interaction between the HIV integrase and its host cofactor LEDGF/p75, that allows retargeting HIV integration from active genes to sites that are less transcriptionally active, indeed resulting in lower HIV transcription (Vranckx et al., 2016; Vansant et al., 2020b). However, it is still unclear how this technique could be applied in infected individuals, as the HIV reservoir is formed very early after infection and once the provirus is integrated, it cannot be retargeted. Interestingly, several groups recently reported that in the untreated infection, the reservoir turns over quickly, and that most proviruses in ART-treated individuals match circulating HIV variants from shortly before ART initiation (Brodin et al., 2016; Abrahams et al., 2019; Pankau et al., 2020). In this case, treatment with LEDGINs or similar compounds shortly before the start of ART could indeed result in a lower transcriptional activity of the reservoir and, as a consequence, a higher frequency of post-treatment HIV remission.

CONCLUSION

In summary, although a number of biomarkers are already identified that can predict post-treatment HIV remission, there are still major gaps in our understanding of its underlying mechanisms. Consequently, ATIs are still the only way to assess the efficacy of new HIV curative interventions, and criteria for the recruitment of clinical trial participants remain unclear. Further research is urgently needed to identify robust and validated predictive biomarkers of post-treatment remission. In this regard, the development of an integral biomarker profile as outlined above should facilitate the efforts to achieve prolonged virological control in the absence of ART.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AP wrote the first draft of the manuscript. CP contributed to the figure. All authors contributed to the writing and approved the final manuscript.

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frontiers in Microbiology

ORIGINAL RESEARCH
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FRET-Based Detection and Quantification of HIV-1 Virion Maturation

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HIV-1 infectivity is achieved through virion maturation. Virus particles undergo structural changes via cleavage of the Gag polyprotein mediated by the viral protease, causing the transition from an uninfectious to an infectious status. The majority of proviruses in people living with HIV-1 treated with combination antiretroviral therapy are defective with large internal deletions. Defective proviral DNA frequently preserves intact sequences capable of expressing viral structural proteins to form virus-like particles whose maturation status is an important factor for chronic antigen-mediated immune stimulation and inflammation. Thus, novel methods to study the maturation capability of defective virus particles are needed to characterize their immunogenicity. To build a quantitative tool to study virion maturation *in vitro*, we developed a novel single virion visualization technique based on fluorescence resonance energy transfer (FRET). We inserted an optimized intramolecular CFP-YPF FRET donor-acceptor pair bridged with an HIV-1 protease cleavage sequence between the Gag MA-CA domains. This system allowed us to microscopically distinguish mature and immature virions via their FRET signal when the FRET donor and acceptor proteins were separated by the viral protease during maturation. We found that approximately 80% of the FRET labeled virus particles were mature with equivalent infectivity to wild type. The proportion of immature virions was increased by treatment of virus producer cells with a protease inhibitor in a dose-dependent manner, which corresponded to a relative decrease in infectivity. Potential areas of application for this tool are assessing maturation efficiency in different cell type settings of intact or deficient proviral DNA integrated cells. We believe that this FRET-based single-virion imaging platform will facilitate estimating the impact on the immune system of both extracellular intact and defective viruses by quantifying the Gag maturation status.

Keywords: HIV-1 Gag maturation, Förster Resonance Energy Transfer, single virion imaging, protease inhibitor, fluorescence microscopy

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INTRODUCTION

While the acquired immunodeficiency syndrome (AIDS) is a deadly disease caused by infection with human immunodeficiency virus type 1 (HIV-1), AIDS-related deaths have been reduced due to the tremendous efforts that have gone into researching the virus itself and ways to counteract it (Centers for Disease Control and Prevention (CDC), 2006). Combination antiretroviral therapies (cART) significantly decrease AIDS mortality and reduce further transmission of HIV-1 (Castilla et al., 2005; Kitahata et al., 2009). However, while cART effectively achieves viral suppression and prevents the progression to AIDS, virus eradication or functional cure strategies have not been established yet, and thus lifelong treatments are still required (Holkmann Olsen et al., 2007; Kousignian et al., 2008). The major obstacle to achieving a cure for HIV-1 is the existence of latently infected reservoir cells within memory CD4 T cells and macrophages that can persist even during cART (Chun et al., 1997; Finzi et al., 1997; Siliciano et al., 2003; Hassan et al., 2016; Wong et al., 2019). Latent HIV-1 persistent reservoirs are established early in the acute phase of infection (Finzi et al., 1997, 1999; Daar et al., 1998; Zhang et al., 2000; Whitney et al., 2014; Henrich et al., 2017; Colby et al., 2018). Defective proviruses with sequence deletions and mutations rapidly accumulate within a few weeks after virus infection and persist for decades during the chronic phase (Bruner et al., 2016). The defective proviruses are generated by error-prone reverse transcription, recombination, and other mutation-inducing events such as APOBEC3G mediated G-to-A mutations (Ho et al., 2013; Bruner et al., 2016). Though it was initially thought to have little involvement in HIV-1 pathogenesis, novel unspliced viral RNA transcription was lately identified in defective proviruses which frequently encoded competent gag or gag-pol open reading frames (Ho et al., 2013; Imamichi et al., 2016). In addition, HIV-1 Gag protein expression in cells harboring defective proviruses was detected by fluorescence microscopy (Imamichi et al., 2020). Since most defective proviruses preserve the 5′ end of intact proviral sequences encoding gag and gag-pol (Ho et al., 2013; Imamichi et al., 2016; Hiener et al., 2017), they may be able to assemble and release virus-like particles into the extracellular space.

Viral maturation is the final step of the HIV-1 life cycle and crucial for the formation of infectious virions (Freed, 2015). The structural Gag polyprotein is cleaved into the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins in a stepwise manner by the viral protease (Mattei et al., 2018). The CA protein assembles to form a mature viral core that houses the viral genome, nucleocapsid, reverse transcriptase, and integrase and stabilizes the lipid bilayer of the virus particle (Davidoff et al., 2012; Pornillos and Ganser-Pornillos, 2019). After viral membrane fusion to enter the target cell, the core protects the viral genome from host sensor proteins such as cGAS, serves as location for reverse transcription, and traffics the pre-integration complex as far as the integration site (Forshey et al., 2002; Gao et al., 2013; Rankovic et al., 2017; Novikova et al., 2019; Siddiqui et al., 2019; Burdick et al., 2020). In addition to its role in the HIV-1 life cycle, virion maturation may also play an important role in the ability of the virus to escape immune responses. In this regard, it has been reported that maturation of defective viral particles induces strong cellular responses, such as IFN-γ production, T cell stimulation, and B cell mediated antibody production through efficient Env presentation (Alvarez-Fernandez et al., 2012; Gonelli et al., 2019).

Fluorescence microscopy techniques in the field of virology have recently evolved to perform quantitative unbiased analysis based on the development of automated image data processing tools. However, the resolution of fluorescence microscopy is not sufficient to determine the morphological transitions of the viral architecture. Förster Resonance Energy Transfer (FRET) is a principle that relies on the partial spectral overlap of fluorescent protein pairs distanced within 10 nm from each other. Excitation of the donor fluorophore leads to an energy transfer to the acceptor fluorophore, and the emission from the excited acceptor fluorophore is detected (Sekar and Periasamy, 2003). The application of FRET in virology enabled us to visualize the cleavage of HIV-1 Gag by the viral protease (De Rocquigny et al., 2014; Muller et al., 2014; Sood et al., 2017). FRET protein pairs have been optimized to achieve maximum energy transfer, photostability, brightness, and low spectral crosstalk (Bajar et al., 2016). Cyan and yellow fluorescent proteins (CFP and YFP, respectively) are common FRET pairs that allowed long-term time-lapse imaging of live cells (Heim and Tsien, 1996; Kremers et al., 2006). ECFPΔC11 and cp173Venus, derived from CFP and YFP respectively, are a pair that has been developed and used specifically for intramolecular high-intensity FRET in live cells and even *in vivo* (Nagai et al., 2004; Chiu and Yang, 2012).

This study developed a molecular tool to detect and quantify the frequency of immature virions by FRET-based fluorescence microscopy. We achieved this by inserting the ECFPΔC11-cp173Venus FRET pair into the Gag polyprotein between the MA and CA domains with viral protease cleavage sites, to label infectious virions based on the HIV Gag-iGFP construct (Hubner et al., 2007). This new fluorescence based system, that we named HIV-1 Gag-iFRET, showed equivalent infectivity to wild-type viruses, and proportions of immature virions comparable to previous and our Electron Microscopy (EM) analyses (Burdick et al., 2020; Link et al., 2020). We also applied this tool to evaluate HIV-1 protease inhibitor activity by assessing virus maturation and infectivity. We believe that this would also be a useful tool to quantify the maturation of extracellular defective virus particles derived from full-length Gag-Pol coding sequences and to estimate their potential immunogenicity.

MATERIALS AND METHODS

Plasmid Construction

Double-stranded DNA of the intra-molecular FRET pair genes ECFPΔC11 and cp173Venus (Nagai et al., 2004), flanked by HIV-1 protease cleavage sites (AA: SQNYPIVQ, NA: TCGCAGAACTATCCAATTGTACAA) and containing the 3′ end of HIV-1 5′ LTR, HIV-1 Gag MA and the 5′ end of HIV-1 Gag CA domain sequences was synthesized (**Supplementary Table 1**) and cloned into the pUC57 plasmid (GenScript). The

synthesized FRET DNA and pHIV Gag-iGFP (Hubner et al., 2007) plasmids were digested with *Bss*HII and *Sph*I restriction enzymes (New England Biolabs, Inc), purified with the QIAquick Gel Extraction Kit (QIAGEN), and ligated by T4 DNA ligase (New England Biolabs, Inc) to obtain the pHIV-1 Gag-iFRET plasmid. The protease defective mutant HIV-1 Gag iFRETΔPro, was generated by replacing the DNA region in pHIV-1 Gag-iFRET digested by *Sph*I and *Sbf*I with the extracted fragment of the previously reported protease defective NL4-3 construct, pNL-Hc (Adachi et al., 1991).

Cell Cultures and Virus Production

Adherent HEK293T and TZM-bl cells were cultured in Dulbecco's Modified Eagle's Medium (Nacalai Tesque) containing 10% Fetal Bovine Serum and 1% Penicillin Streptomycin Glutamine (Invitrogen) (D10) at 37°C with 5% CO_2.

FRET labeled virions were produced by co-transfecting HEK293T cells (3.5×10^6 cells/10 cm dish) with the pHIV-1 Gag-iFRET or iFRETΔPro together with the pNL4-3 or pNL4-3ΔPro parental plasmid respectively at a 1:1, 1:10, or 1:20 ratio using a polyethylenimine transfection reagent (GE Healthcare). The culture medium was replaced with fresh D10 with or without Darunavir (Sigma Aldrich) at a final concentration of 0.1, 1.0, 10, 20, 500, or 1000 nM 3.5 h after transfection. The virus-containing supernatant was harvested 24 h after the medium change, filtered through 0.45 μm pore size sterile polyvinylidene difluoride (PVDF, Millipore) membrane, and concentrated up to 20-fold by ultracentrifugation through a 20% sucrose cushion at 25,000 rpm (112,499 *g*) for 90 min at 4°C (CP65; Hitachi Koki Co., Ltd.). The virus pellet was resuspended in 500 μl Hank's Balanced Salt Solution (HBSS) (–) without phenol red (Wako).

Single-Virion Imaging Analysis

To visualize the HIV-1 Gag-iFRET/iFRETΔPro labeled virions, the concentrated virus supernatant was 800x diluted in 0.22 μm PVDF filtered Hank's Balanced Salt Solution (HBSS) (–) without Phenol Red (Wako) and loaded (360 μl) into non-coated 8-well glass-bottom chamber slides (Matsunami), then incubated overnight at 4°C.

Single-virion images were acquired with an A1R MP+ Multiphoton Confocal Microscope (Nikon). Two sets of 21 images were automatically taken for each sample under perfect focus conditions. The first set of images was taken using a 457.9 nm wavelength laser for cyan fluorescent protein (CFP) excitation and by reading the emission spectrums through 482 nm/35 nm or 540 nm/30 nm filter cubes to detect CFP or yellow fluorescent protein (YFP) signals, respectively (FRET images). The second set of images was taken using the 514.5 nm wavelength laser for Venus excitation and by reading the emission spectrum through the 540 nm/30 nm filter cube to detect the YFP signal. The maturation status was defined as FRET efficiency compared with the signal detected in HIV-1 Gag-iFRETΔPro labeled virions.

All images were captured as RAW ND2 datasets and exported to TIFF format files using NIS-Elements (Nikon). Binary images were generated based on the Venus signal to obtain the XY coordinates of each particle. Based on these coordinates, the FRET signal intensity of each virion was extracted from the raw data, and the FRET ratio was calculated for every particle (YFP/[YFP + CFP]) (Preus and Wilhelmsson, 2012). Histograms of distribution were generated for the FRET ratio values within a 100 bins division. Gaussian distribution and Kernel density estimation curves were plotted against the histograms. The proportion of the total Gaussian distribution or Kernel density estimation area overlapped with the HIV-1 Gag-iFRETΔPro area was determined as the proportion of immature virions. The process of image data analysis was performed using an in-house MATLAB program (Fukuda et al., 2019).

Immunoblotting

Transfected HEK293T cells were lysed using RIPA buffer (Wako) supplemented with 1 mM cOmplete^{TM} protease inhibitor cocktail (Sigma-Aldrich), and the supernatants were used for immunoblotting. Briefly, cells were incubated in the lysis buffer for 15 min at 4°C and then centrifuged at 25,000 *g* for 15 min at 4°C. The pellet was sonicated at 45% output (Ultrasonic Processor, GE50) until completely disrupted (∼10 s), centrifuged again as described above, and then supernatants were collected (cell lysate). The protein concentration was measured by BCA assay (Nacalai Tesque). The SDS-PAGE samples were prepared by mixing the cell lysate with 5x Laemmli buffer [312.5mM Tris-HCl (pH 6.8), 10% Glycerol, 10% SDS] containing 5% β-mercaptoethanol and 4% bromophenol blue, and denaturated at 95°C for 5 min. Virus lysates were also prepared in the same way as cell lysates using virions concentrated as described above. Polyacrylamide gel electrophoresis and protein transfer to PVDF membranes (Immobilon, Millipore) were followed by hybridization with primary antibodies. Blots were probed with either mouse anti-p24 (Abcam, ab9071) or mouse anti-GFP (Thermo Fisher Scientific, MA5-15256) primary antibodies overnight at 4°C. HRP-conjugated anti-mouse IgG antibody (GE Healthcare) was used as a secondary antibody. Immunoblotting images were obtained using the ImageQuant^{TM} LAS 500 system (GE Healthcare). After the initial images were taken, the membranes were incubated for 30 min at 50°C in stripping buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.7% β-mercaptoethanol], re-blocked and re-blotted with mouse anti-β-actin primary antibodies as described above.

Single-Round Infection Assay

The pseudotyped HIV-1 Gag-iFRET labeled virus was produced by co-transfecting HEK293T cells with pHIV-1 Gag-iFRETΔEnv and pNL4-3ΔEnv parental plasmids at three different ratios as described in section "Cell Cultures and Virus Production," together with the HIV-1 envelope expression plasmid, pSVIII-92HT593.1. The pSVIII-92HT593.1 construct was obtained from Dr. Beatrice Hahn through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 92HT593.1 gp160 Expression Vector (cat# 3077) (Gao et al., 1996). HIV-1 Gag-iFRETΔPro labeled virus was produced by co-transfecting HEK293T cells with pHIV-1 Gag-iFRETΔPro and pNL4-3ΔPro parental plasmid at the same ratios as HIV-1 Gag-iFRET. The viral titer was measured by HIV Type 1 p24 Antigen ELISA (ZeptoMetrix). The following day after 5×10^3 TZM-bl cells seeded in 96 well plates, an equal amount of virus (total of 5 ng HIV-1 p24) was added to the TZM-bl target cells, and then

cultured at 37°C for 48 h in a CO_2 incubator. Luciferase activity in the infected cells was measured with the Luciferase Assay System (Promega) on a 2030 ARVO X3 plate reader (Perkin Elmer) to quantify virus infectivity.

Transmission Electron Microscopy Images

HIV-1 Gag-iFRET, -iFRETΔPro, or NL4-3 virions were produced as described in section "Cell Cultures and Virus Production" up to the viral pellet. The pellet was then fixed overnight at 4°C with a 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1M PBS solution. The next day, the pellet was washed twice with 0.1M PBS and post-fixed in 1% Osmium tetroxide (OsO4) for 1 h at room temperature (RT), then dehydrated in a series of graded ethanol solutions. After immersion in propylene oxide (Nacalai Tesque), samples were once again immersed in a mixture (1:1) of propylene oxide and LUVEAK-812 (Nacalai Tesque) overnight, embedded in Epon812 resin according to the inverted beam capsule procedure, and polymerized at 60°C for 2 days. Ultrathin sections were examined with an H-7650 electron microscope (Hitachi).

RESULTS

Construction of FRET Labeled HIV-1 Virus Particles

Previous studies have shown that inserting a fluorescent protein between the matrix (MA) and capsid (CA) domains of HIV-1 Gag, which is eventually cleaved away during Gag processing by the HIV-1 protease, is compatible with successful assembly and release of infectious HIV-1 virions (Hubner and Chen, 2006; Hubner et al., 2007). To microscopically visualize the viral core generation, we designed a novel bifunctional HIV-1 labeling system that consists of a tandem of cyan- and yellow-emitting fluorescent protein pair as FRET donor and acceptor and named it HIV-1 Gag-iFRET (**Figure 1A**). We bridged an optimized intramolecular FRET pair, ECFPΔC11 (CFP) and circularly permutated Venus with a new N-terminus starting at Asp-173 (cp173Venus; YFP) (Nagai et al., 2004) with an HIV-1 protease cleavage site, and inserted them between the MA and CA domains of HIV-1 Gag. We hypothesized there would be an efficient energy transfer from the FRET donor (CFP) to the acceptor (YFP) within uncleaved Gag molecules in immature virions. HIV-1 protease cleaves the Gag polyproteins in newly synthesized progeny virions. Thus, HIV-1 Gag-iFRET was designed so that the FRET pair proteins would also be cleaved from the Gag precursor during the maturation process. As a protease deficient mutant to control our experiments, HIV-1 Gag-iFRETΔPro was constructed to contain the same FRET donor-acceptor sequence but could form only immature particles which were expected to have a high FRET efficiency. HIV-1 Gag-iFRET and -iFRETΔPro labeled viral particles were produced by transfecting HEK293T cells with the pHIV-1 Gag-iFRET or -iFRETΔPro constructs alone or at 1:1, 1:10, or 1:20 ratio with the parental pNL4-3 or pNL4-3ΔPro plasmids, respectively. We detected the FRET-pair-fused Gag polyprotein in both HIV-1 Gag-iFRET and -iFRETΔPro transfected HEK293T cells by immunoblot analyses with anti-p24 or anti-GFP antibodies, respectively (**Figure 1B**). The processed forms of p24 CA and fluorescent proteins (CFP and YFP) were observed in HIV-1 Gag-iFRET transfected cells (**Figure 1B** lanes 3–6). The cleavage products of Gag were not detected in cells transfected with the HIV-1 Gag-iFRETΔPro at any of the tested ratios (**Figure 1B** lanes 7–10). Both pHIV-1 Gag-iFRET and -iFRETΔPro construct transfection without their parental helper plasmids seemed to lead to less efficient viral and fluorescent protein expression in the cells (**Figure 1B** lanes 3 and 7). To evaluate the infectivity of the labeled virus, we performed single-round infection assays using TZM-bl cells with HIV-1 Env-pseudotyped Gag-iFRET viruses. The FRET labeled viruses produced by co-transfection at the 1:10 or 1:20 ratio showed similar infectivity to unlabeled virus (NL4-3), while viruses at the 1:1 ratio dramatically lost their capacity to infect TZM-bl cells (**Figure 1C**). Therefore, FRET labeled viruses produced at the 1:10 ratio were used for further experiments. The processing of HIV-1 Gag and fluorescent proteins in wild-type and labeled viruses was confirmed by immunoblotting assays of virus lysates with anti-p24 or anti-GFP antibodies, respectively (**Figure 1D**). We demonstrated that HIV-1 Gag-iFRET virus particles (produced at the 1:10 ratio) contained a conical-shaped structure of the core similar to that of unlabeled parental NL4-3 virions by using Transmission Electron Microscopy (**Figure 1E**). All protease defective and some of the wild-type virions showed immature morphology (**Figure 1E**, purple border). We analyzed approximately one hundred virus particles per condition and observed a similar proportion (∼18%) of immature virions as the FRET and control NL4-3 viruses (17 out of 96 and 18 out of 99 particles, respectively). To summarize, HIV-1 Gag-iFRET labeled viruses produced with wild-type Gag, maintained infectivity and displayed a Gag processing efficiency similar to the parental NL4-3.

Detection of FRET Labeled HIV-1 Virus Particle Maturation

Since we confirmed efficient HIV-1 Gag-iFRET and -iFRETΔPro viral particle production with similar Gag processing and infectivity as the parental NL4-3, we next visualized single virions to distinguish their maturation status by quantifying FRET in fluorescence microscopy. A set of FRET images was taken with HIV-1 Gag-iFRET and -iFRETΔPro labeled virions produced at the 1:10 ratio (**Figure 2A**, upper and lower panels, respectively). Images taken by YFP (cp173Venus) excitation and emission were used to determine the presence of virus particles and their location coordinates for further analysis (**Figure 2A**, left panels). Representative images taken through the CFP excitation channel and reading the emission of both CFP (FRET Donor) and YFP (FRET Acceptor) are shown (**Figure 2A**, middle left and right panels, respectively). The ratio view images were constructed based on FRET donor and acceptor images, showing the FRET energy transfer efficiency from donor to acceptor [FRET ratio = YFP emission/(YFP emission + CFP emission)] in each particle (**Figure 2A**, right

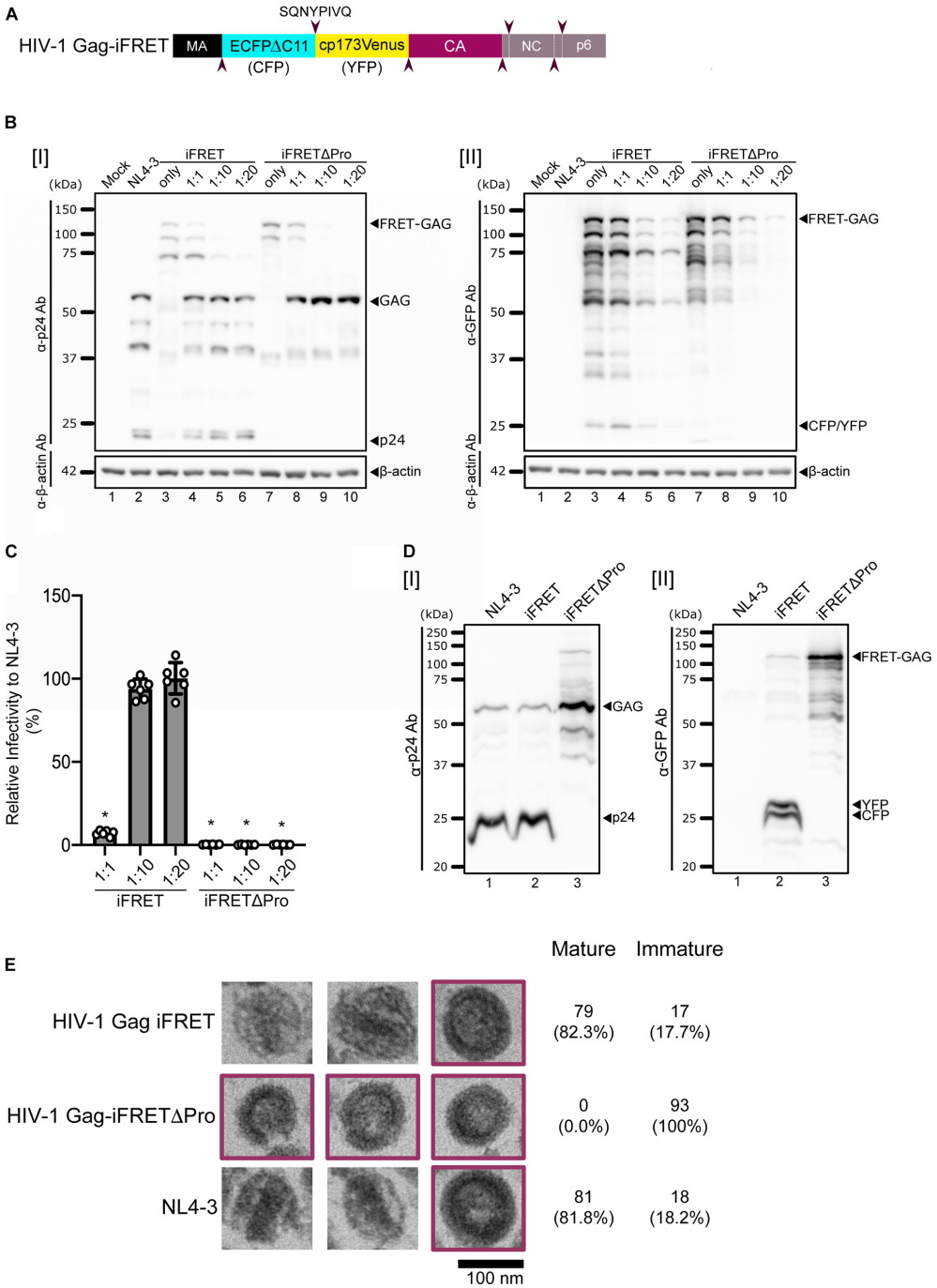


FIGURE 1 | Design and validation of the HIV-1 Gag-iFRET construct. **(A)** Schematic representation of the HIV-1 Gag-iFRET construct in the Gag region. HIV-1 Gag-iFRET was constructed by inserting the efficient single-molecule FRET pair ECFPΔC11-cp173Venus (CFP-YFP) into HIV-1 Gag with HIV-1 protease cleavage sites (SQNYPIVQ, marked by arrowheads). When CFP and YFP are within 10 nm of each other, the excitation energy of the donor CFP transfers to the acceptor YFP and YFP's emission spectra is detected in the immature virion (uncleaved Gag polyprotein). Once Gag is cleaved by the viral protease and rearranged in the mature virion, the energy transfer efficiency drops, and the FRET signal diminishes. **(B)** Immunoblotting results of cell lysates from HEK293T cells transfected with pNL4-3ΔEnv: pHIV-1 Gag-iFRETΔEnv or pNL4-3ΔPro: pHIV-1 Gag-iFRETΔPro at the indicated ratios, blotted with **[I]** anti-p24 or **[II]** anti-GFP antibodies. The membranes were subsequently stripped and re-blotted with anti-β-actin antibodies. **(C)** Single round infectivity assay using HIV-1 Env-pseudotyped HIV-1 Gag-iFRET and -iFRETΔPro labeled virus produced at the same ratios as in **(B)** was performed in TZM-bl cells. Results are shown as relative infectivity (%) compared to parental NL4-3 virus infectivity. Error bars indicate standard deviation of six independent experiments. Statistical significance was calculated by Wilcoxon matched-pairs signed rank test compared to parental NL4-3 virus infectivity (*$p < 0.05$). **(D)** Immunoblotting results of virus lysates produced at the 1:10 ratio blotted with **[I]** anti-p24 or **[II]** anti-GFP antibodies. **(E)** Three representative images of HIV-1 Gag-iFRET (top), -iFRETΔPro (middle), and NL4-3 (bottom) virions taken by Transmission Electron Microscopy (TEM). Images of immature virions are highlighted in purple color frames. The numbers of analyzed immature and mature particles together with proportion (%) in brackets are indicated.

panels). We observed two major groups: virions colored in the green-blue spectrum (low FRET ratio, white arrowheads) and virions colored in the red spectrum (high FRET ratio, yellow arrowheads). Based on our construct design, we hypothesized that CFP and YFP are located next to each other in immature virions and have a high FRET ratio. On the other hand, CFP and YFP are separated and dispersed in mature virions, leading to a reduction of FRET efficiency (low FRET ratio). Consistent with our hypothesis, we observed that most of the HIV-1 Gag-iFRETΔPro labeled virions appeared in the red spectrum (**Figure 2A**, bottom right panel). Accordingly, maturation capable virions labeled by HIV-1 Gag-iFRET appeared mostly in the green-blue spectrum mixed with some particles maintaining a high FRET ratio (**Figure 2A**, top right panel). In other words, based on the FRET ratio view, the HIV-1 Gag-iFRETΔPro virion population comprises solely immature particles, while HIV-1 Gag-iFRET viruses revealed heterogeneous phenotypes of Gag maturation including both mature and immature cores.

We next quantified the proportion of mature and immature virions in the FRET labeled virus carrying the intact HIV-1 protease. The FRET ratio was calculated for each virion from the extracted FRET donor and acceptor signal intensities. FRET efficiencies were then plotted in histograms that reflected sample heterogeneity (**Figure 2B** and **Supplementary Figure 1**). As expected, the Normal Probability plot could fit a Gaussian distribution curve over the histogram plots of the HIV-1 Gag-iFRETΔPro population, which contained only immature virions (**Supplementary Figure 1B**). By comparison, the distribution of HIV-1 Gag-iFRET viruses did not fit a gaussian curve, consistent with the presence of a mixed virion population of mature and immature particles (**Supplementary Figure 1A**). Kernel density estimation is frequently used as a smoothening estimation function for non-normal distributions. Thus, we applied kernel density estimation curves and performed total density calculations in this analysis (**Figure 2B**). The Kernel density estimation curves of HIV-1 Gag-iFRET and -iFRETΔPro were overlapped after the adjustment of total particle counts (**Figure 2C**). We then measured the area occupied by the HIV-1 Gag-iFRET curve that merged with the HIV-1 Gag-iFRETΔPro curve and determined this as the proportion of immature virions out of the total corresponding HIV-1 Gag-iFRET area. We counted over 46,000 particles of HIV-1 Gag-iFRET and over 77,000 particles of HIV-1 Gag-iFRETΔPro labeled virions in three independent experiments. The overall proportion of immature virions in the HIV-1 Gag-iFRET population was 22.4% ± 2.4% calculated based on the 100% immaturity of HIV-1 Gag-iFRETΔPro (**Figure 2D**). We confirmed that these proportions were consistent with the rates determined by electron microscopy analysis in other reports (Burdick et al., 2020; Link et al., 2020) and ours (**Figure 1E**).

Taken together, we were able to visualize the maturation state of virions based on their FRET signal intensity using fluorescence microscopy and to quantify the proportion of immature virions with a rate comparable to that found by electron microscopy-based assays.

Quantitative Assessment of Protease Inhibitor Activity Using the HIV-1 Gag-iFRET Single Virion Visualization System

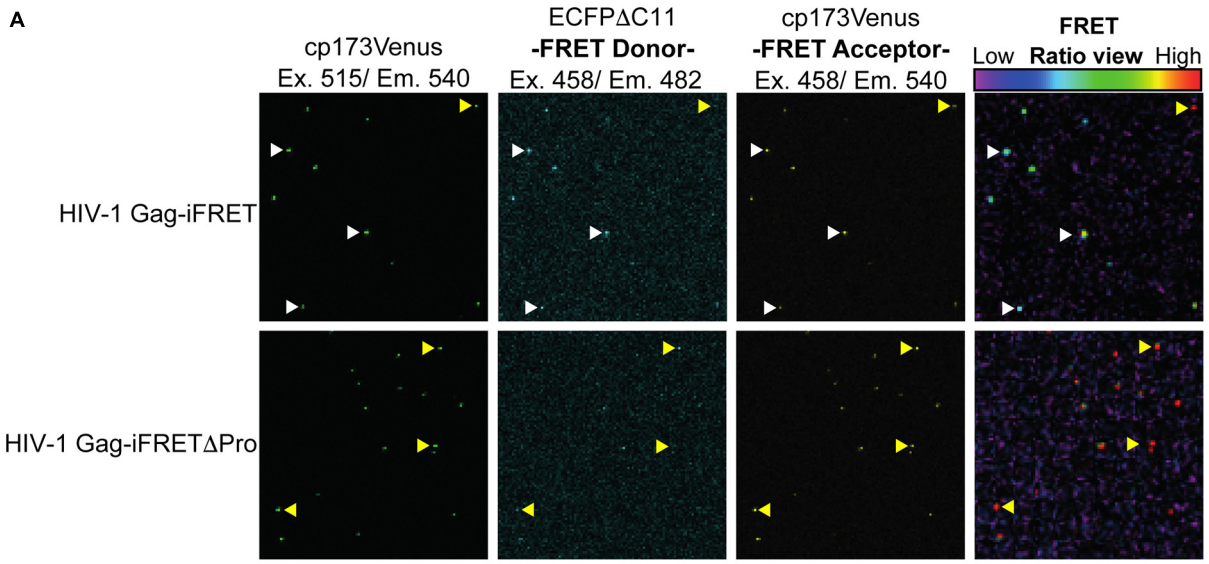
In order to evaluate the applicability of the HIV-1 Gag-iFRET system, we sought to assess the efficacy of a protease inhibitor treatment by measuring the population of immature virions and correlating the results with the associated virus infectivity. For this purpose, we produced HIV-1 Gag-iFRET labeled virions in the absence or presence of Darunavir, a protease inhibitor used in the clinic to treat HIV-1 infection (De Meyer et al., 2005; Spagnuolo et al., 2018), and quantified the proportion of immature virions at four different concentrations. Darunavir treatment shifted the peak of the FRET ratio distribution to the right in a dose-dependent manner (**Figure 3A**). Virions produced by cells treated with the lowest concentration of Darunavir, 0.1 nM, were in the same FRET range as the non-treated control (**Figure 3A**, yellow line), while those treated with 20 nM Darunavir shifted to the iFRETΔPro FRET range (**Figure 3A**, light blue line). The peak of the virion population treated with 10 nM Darunavir was approximately halfway between the non-treated and immature controls (**Figure 3A**, green line).

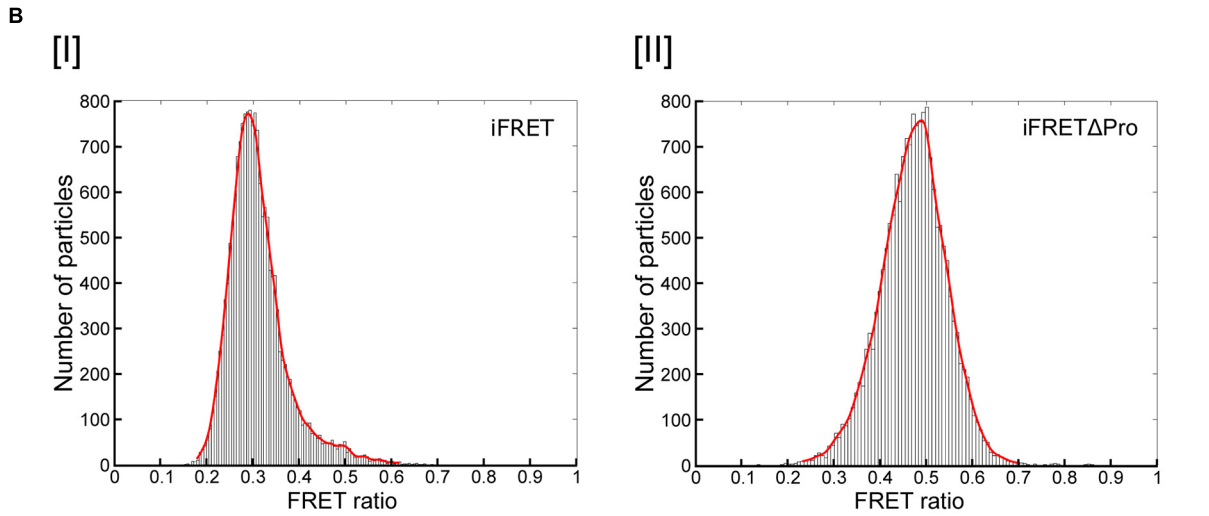
We counted between 17,000 and 33,000 particles in total for each condition and quantified the proportion of immature virions with the same method described in **Figure 2C** (**Figure 3B**). The proportion of immature virions increased dose-dependently with Darunavir treatment from 22.9 to 89.0%. Correspondingly, we assessed the HIV-1 Gag-iFRET virus infectivity produced by cells treated with Darunavir (**Figure 3C**). Virus infectivity was not significantly affected by Darunavir concentrations up to 1.0 nM, whereas a drastic reduction in infectivity was observed at the 10 and 20 nM concentrations. According to the dose-response relationships of Darunavir concentration with virion maturation and virus infectivity (**Figures 3B,C**, respectively), the 50% effective concentration (EC_{50}) against virion maturation was 7.0 nM, and the 50% inhibitory concentration (IC_{50}) of virus infectivity was 2.8 nM (**Figure 3D**). This indicated that the drug concentration required to prevent virus maturation was approximately two-fold higher than that needed for antiviral effect. This suggests that some Darunavir treated viruses that completed maturation also lost infectivity.

In conclusion, the HIV-1 Gag-iFRET labeling strategy we described here was used to quantify the effects of a protease inhibitor on the maturation rate of HIV-1.

DISCUSSION

In this study, we set out to develop a FRET based fluorescence microscopy tool for a large-scale quantitative measurement of morphologically distinct mature and immature HIV-1 virus particles. Electron microscopy (EM) is a technique traditionally used for the structural determination of virion maturation (Lee and Gui, 2016). It remains a powerful method to identify





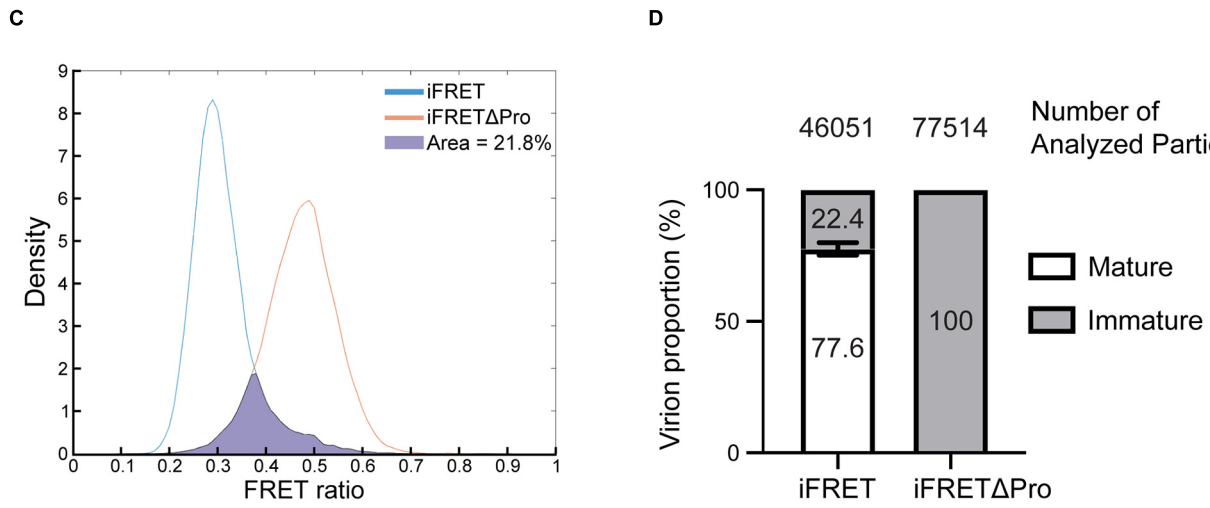


FIGURE 2 | Continued

FIGURE 2 | Differentiation and quantification of mature and immature particles in fluorescence microscopy. **(A)** Representative images of FRET labeled virions. All images show the same field containing either HIV-1 Gag-iFRET (top images) or -iFRETΔPro (bottom images) labeled virions. The left panel shows images taken through the YFP excitation (515 nm) and emission (540 nm) channels. The middle panels show images taken by the CFP (FRET donor) excitation (458 nm) and CFP emission (482 nm, left) or YFP emission (540 nm, right) channels. The right panel shows FRET ratio view images that were computationally constructed based on FRET donor (CFP excitation/CFP emission) and acceptor images (CFP excitation/YFP emission) to show FRET efficiency. The color bar indicates that a high FRET signal appears in red (yellow arrows), and the color shifts toward blue (white arrows) as the FRET signal decreases. **(B)** Representative distribution histograms of FRET intensity from **[I]** HIV-1 Gag-iFRET or **[II]** -iFRETΔPro labeled virions are shown with 100 bins. The *x*- and *y*-axis indicate the range of FRET intensity (from 0 to 1) and the number of particles, respectively. The histograms were fitted with a Kernel density estimation function (red curve). **(C)** The Kernel density estimation curves of HIV-1 Gag-iFRET and HIV-1 Gag-iFRETΔPro virions in **(B)** were adjusted to have the same density. The proportion of the HIV-1 Gag-iFRET area under the curve that overlapped that of HIV-1 Gag-iFRETΔPro was calculated and considered as the proportion of immature virions in the total HIV-1 Gag-iFRET virion population. **(D)** Quantification of the mature and immature virion populations based on the calculation strategy in **(C)**. The stacked bar plot shows the average percentage of mature and immature virions in each HIV-1 Gag-iFRET and HIV-1 Gag-iFRETΔPro population. Error bars indicate the standard deviation of three independent experiments. The total number of analyzed particles for each group is shown above their respective graph bar.

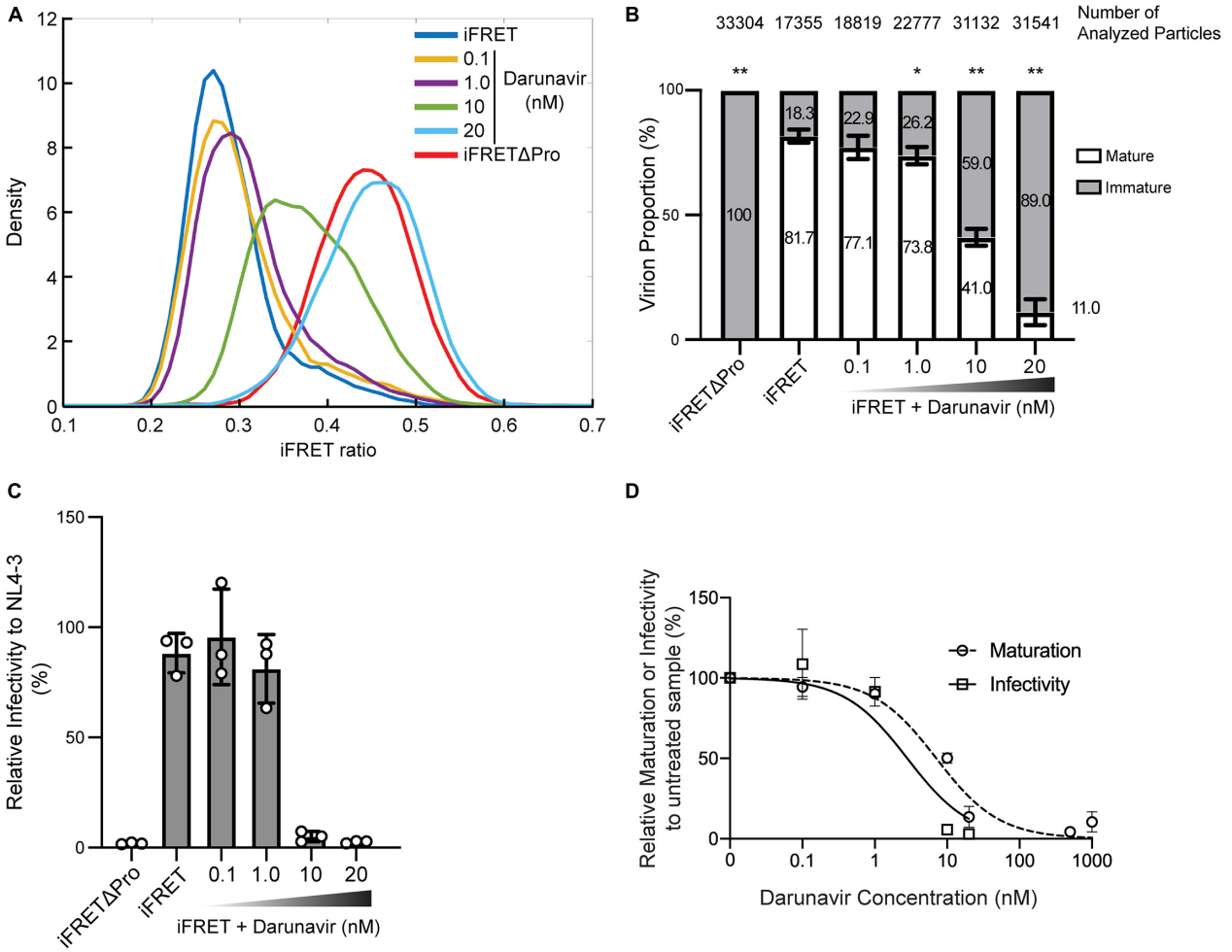


FIGURE 3 | *In vitro* dose-dependent effect of a protease inhibitor detected with the HIV-1 Gag-iFRET system. **(A)** Kernel density estimation curves from a representative experiment of HIV-1 Gag-iFRET virions produced under treatment with a protease inhibitor, Darunavir, at four different concentrations (0.1, 1.0, 10, and 20 nM). **(B)** Quantification of mature and immature virion populations treated with Darunavir, as determined by overlapping kernel density estimation curves as described in **Figure 2C**. The stacked bar plot shows the average percentage of mature and immature virions in each untreated and treated population. Error bars indicate the standard deviation of three independent experiments. The size of the immature virion population in each Darunavir treated sample was compared to that of the untreated HIV-1 Gag-iFRET sample by paired *t*-test and significant differences are marked accordingly (**$p < 0.01$, *$p < 0.05$). **(C)** Single-round infectivity assays with TZM-bl cells were performed to determine the inhibitory activity of Darunavir at each tested concentration. The bar plot shows the infectivity (%) of HIV-1 Gag-iFRET virus with or without Darunavir treatment relative to the infectivity of the parental NL4-3 virus. Error bars indicate the standard deviation of three independent experiments. Statistical significance was calculated by Wilcoxon matched-pairs signed rank test compared to NL4-3 infectivity. **(D)** Dose-response curves for relative inhibition of maturation and infectivity at the tested Darunavir concentrations compared to the untreated sample. The EC_{50} and IC_{50} of Darunavir efficacy were calculated by maturation and infectivity rates at the range of 0.1–1000 nM and 0.1–20 nM concentrations of Darunavir, respectively.

morphological signatures in virions due to its high resolution. However, the proportions of mature virions measured by EM are usually assessed manually which leads to a large variation within the range of 80–99% of the total purified virions (De Marco et al., 2012; Keller et al., 2013; Mattei et al., 2015; Burdick et al., 2020; Link et al., 2020). Fluorescence microscopy on the other hand is expanding to comprise techniques capable of spatiotemporal analysis of the viral life cycle (Campbell and Hope, 2008; Francis and Melikyan, 2018). Hubner et al. (2007) has successfully produced infectious labeled virions by developing a fluorescently tagged HIV-1 construct, HIV Gag-iGFP. HIV Gag-iGFP has been used to track HIV-1 Gag protein through cellular compartments and visualize virological synapses in living cells (Hubner et al., 2009; Wang et al., 2019), but is unable to distinguish immature and mature virions. Although a number of mechanisms in the virus life cycle were elucidated by visualizing virus particles or components in the context of living cells, a fluorescence microscopy technique capable of showing the morphological transition from immature to mature state was still in need. In this work, we created a fluorescently distinguishable system based on the visualization of the Gag maturation status in virus particles. This system based on the FRET principle was achieved by inserting the optimized intracellular CFP-YFP FRET pair proteins (ECFPΔC11 and cp173Venus, respectively) (Nagai et al., 2004) between the MA and CA domains of Gag (HIV-1 Gag-iFRET; **Figure 1A**). The inserted CFP and YFP proteins were flanked by HIV-1 protease cleavage sites to allow the separation of the FRET pair proteins from Gag in the mature virion, which enabled us to differentiate mature and immature virions based on their FRET signal. High FRET intensities were observed in the immature virions generated by the HIV-1 Gag-iFRETΔPro construct due to the vicinity of the FRET donor and acceptor proteins in a single Gag molecule (**Figure 2A**, bottom panels). In our analyses, the histogram plots of FRET signal values derived from the HIV-1 Gag-iFRETΔPro population fitted a normal distribution curve (**Figure 2B** and **Supplementary Figure 1B**), indicating that the virus population consisted of a single phenotype of Gag protein with an immature conformation and also confirmed Gag-iFRETΔPro virions' homogenous immature status. The CFP and YFP proteins inserted in the Gag polyprotein distributed within the viral particle once the two fluorescent proteins were cleaved apart during maturation, followed by FRET signal diminution (**Figure 2A**, upper panels). As not all protease-intact particles seemed to complete the maturation phase, the normal probability plot did not fit a Gaussian distribution (**Supplementary Figure 1A**). The HIV-1 Gag-iFRET virion population was heterogeneous and contained both mature and immature virions as confirmed by our TEM images (**Figure 1E**).

After having confirmed that HIV-1 Gag-iFRET successfully labeled infectious virions and that there was a measurable difference in the FRET signal emitted by mature and immature virions, we proceeded to quantify the proportion of immature virions. We calculated the overlapping area of HIV-1 Gag-iFRET with HIV-1 Gag-iFRETΔPro labeled virions to determine the proportion of immature virions out of the total HIV-1 Gag-iFRET viruses. As a result, nearly 20% of the HIV-1 Gag-iFRET virions were accounted to be immature (**Figure 2**). As we mentioned earlier, it has been previously reported that the frequency of immature virions ranges between 0.1 and 20% of the total HIV-1 particles counted in EM images (De Marco et al., 2012; Keller et al., 2013; Mattei et al., 2015; Burdick et al., 2020; Link et al., 2020). The frequency of the immature state measured through our FRET signal analysis was slightly higher (20%, **Figure 2**), but still in the range of previous EM reports (Burdick et al., 2020; Link et al., 2020) and consistent with our count (**Figure 1E**). Since we are not able to completely exclude false positive counts, as immature virions with a median FRET signal in HIV-1 Gag-iFRET overlapped with those with a lower signal in the protease deficient population, we believe our image analysis scheme has been optimized at this point. Taken together, immature virion quantification using HIV-1 Gag-iFRET yielded reproducible results over multiple experiments with the great advantage of being capable of large-scale virion quantification through semi-automated image processing.

A potential application of the HIV-1 Gag-iFRET system is live-cell imaging to study HIV-1 release at the budding site. Live-cell microscopy using GFP-tagged CA or other fluorescent molecules has provided invaluable information on the behavior of virus components, particularly the localization and various functions of the capsid (Hubner et al., 2009; Burdick et al., 2020; Zurnic Bonisch et al., 2020), and FRET has been used to measure the duration of virion assembly at the plasma membrane (Jouvenet et al., 2008). Hu's group investigated the behavior of viral RNA in fluorescence imaging experiments using RNA-binding proteins that specifically recognize stem-loop sequences engineered into the viral genome (Chen et al., 2009) and revealed that only a portion of the HIV-1 RNAs that reach the plasma membrane became associated with viral protein complexes (Sardo et al., 2015). HIV Gag-iGFP was used in live-cell imaging to show virion trafficking during virological synapses (Hubner et al., 2009). Thus, the combination of RNA labeling techniques with the HIV-1 Gag-iFRET system would provide a unique method in this context to elucidate the dynamics of Gag-viral RNA release from the budding site into progeny virions. Moreover, viral assembly appears to be cell-type dependent (Ono and Freed, 2004), and virions are assembled and released in viral-containing compartments (VCCs) in macrophages, beyond the reach of antivirals or antibodies, and from where cell-to-cell infection can occur unhindered (Pelchen-Matthews et al., 2003; Sharova et al., 2005; Gousset et al., 2008; Groot et al., 2008; Chu et al., 2012; Inlora et al., 2016). HIV-1 Gag-iFRET could be used in live-cell imaging to localize and visualize maturation in various cellular compartments in different cell-type settings. This would circumvent the limitation of some studies in which HIV-1 components are found in VCCs after endocytosis or phagocytosis of the newly released particles (Jouvenet et al., 2006). Together with the large-scale quantification approach to image analysis, our system can provide new

and reliable insights into this fundamental step of the HIV-1 life cycle.

To further evaluate the HIV-1 Gag-iFRET quantitative potential, we tested its applicability to antiretroviral drug assessment. For this purpose, we examined the sensitivity of the HIV-1 Gag-iFRET system in detecting changes in the immature HIV-1 virion population after treatment with the protease inhibitor, Darunavir. A dose-dependent increase of the FRET signals associated with escalation of the immature virion population was observed (**Figure 3A**). According to the single round infectivity assays in TZM-bl cells, the 50% inhibitory concentration (IC_{50}) of Darunavir was 2.8 nM (0.88–8.3 nM in 95% Confidence Interval) (**Figure 3D**), which was in the range of previous reports (1–5 nM) (De Meyer et al., 2005). On the other hand, the 50% effective concentration (EC_{50}) of Darunavir as a protease inhibitor calculated by the frequency of immature virions in our FRET labeling system was 7.0 nM (3.9–12.1 nM in 95% Confidence Interval). We observed that more than double the concentration of IC_{50} is required to inhibit maturation in 50% of the virions (**Figure 3D**). Despite the shift between the two assays being only twofold, this remains an interesting observation showing that using only infectivity assays to determine the specific effect of protease inhibitors on maturation might be insufficient. It has been previously suggested that protease inhibitors including Darunavir also block virus entry, reverse transcription, and integration steps (Rabi et al., 2013). Thus, it stands to reason that Darunavir's IC_{50} is different from its EC_{50} in our calculations. According to this discordance, it could be inferred that approximately half of the particles inactivated by Darunavir still completed maturation. Further studies are needed to elucidate these observations.

It is possible that defective viruses can still produce antigens and virus-like particles that could undergo the maturation process. In this regard, it has been shown that defective viruses play a role in preferentially activating CD4 T cells for productive HIV-1 replication, and in providing a large pool of HIV-1 epitopes that continuously stimulate CD4 T cells with different antigen specificity (Finzi et al., 2006). Our results implied that they might do so in a mature conformation. In support of this idea, studies that looked at the possibility of using defective virions that can only produce virus-like particles for immunization purposes found that the immature morphology enhanced particles' immunogenicity including stimulation of T cell responses, such as IFN-γ production, and eliciting Env targeting antibody production (Alvarez-Fernandez et al., 2012; Gonelli et al., 2019). This further emphasizes the importance of maturation for both treatment and prevention of HIV-1 infection. In addition, more than 95% of proviruses in the peripheral blood are defective in people living with HIV-1 on combination antiretroviral therapy (Ho et al., 2013; Bruner et al., 2016). Defective proviruses have recently been reported to encode novel unspliced forms of HIV-1 RNA transcripts with competent open reading frames and subsequent structural protein expression that may lead to persistent immune activation by triggering both innate and adaptive immunity (Ho et al., 2013; Imamichi et al., 2016). As the majority of defective proviruses have large internal deletions but preserve intact gag or gag-pol sequences, it is possible that defective proviruses form extracellular virus-like particles that activate immune responses. Thus, the maturation status of extracellular defective viruses becomes increasingly important for estimating their potential immunogenicity and our novel FRET labeling system would be suitable for investigating this matter. In addition, the viral integrase has been also shown to be necessary for correct HIV-1 maturation (Fontana et al., 2015; Kessl et al., 2016; Elliott and Kutluay, 2020) and its mechanism of action could also be explored using HIV-1 Gag-iFRET.

CONCLUSION

The HIV-1 Gag-iFRET system, together with the semi-automated, unbiased imaging and analysis strategy provided in this work are a new, powerful addition to the virological and biological molecular tools set. While the current major focus for HIV-1 functional cure strategy is toward reactivating latently infected cells and their elimination, biological activity and pathogenesis of defective proviruses are drawing attention as another potential obstacle to a functional cure. HIV-1 Gag-iFRET can be used to more thoroughly investigate maturation, when viruses acquire their infectivity and immunogenicity. Elucidation of the space-time frame of maturation may reveal therapeutic windows and help broaden our antiviral arsenal.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AS, LS, and TI performed the experiments. KH, KS, AT-K, and TI designed the study. AS, HF, HM, KS, and TI analyzed the data. AS, LS, and TI wrote the manuscript. AS, AT-K, and TI contributed to financial assistance. All the authors contributed to the article and approved the submitted 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/fmicb.2021.647452/full#supplementary-material

Supplementary Figure 1 | Gaussian fitting and normal distribution probability calculation for HIV-1 Gag-iFRET and -iFRETΔPro virion populations. A Gaussian (normal) distribution curve (red line) was fitted to the histograms shown in **(A) Figure 2B** or **(B) Figure 2C**. The normal probability plot assessed the Gaussian distribution of the representative data set.

Supplementary Table 1 | HIV-1 Gag-iFRET insert sequence.

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frontiers in Microbiology

METHODS
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Improved Detection of HIV Gag p24 Protein Using a Combined Immunoprecipitation and Digital ELISA Method

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Greater than 90% of HIV-1 proviruses are thought to be defective and incapable of viral replication. While replication competent proviruses are of primary concern with respect to disease progression or transmission, studies have shown that even defective proviruses are not silent and can produce viral proteins, which may contribute to inflammation and immune responses. Viral protein expression also has implications for immune-based HIV-1 clearance strategies, which rely on antigen recognition. Thus, sensitive assays aimed at quantifying both replication-competent proviruses and defective, yet translationally competent proviruses are needed to understand the contribution of viral protein to HIV-1 pathogenesis and determine the effectiveness of HIV-1 cure interventions. Previously, we reported a modified HIV-1 gag p24 digital enzyme-linked immunosorbent assay with single molecule array (Simoa) detection of cell-associated viral protein. Here we report a novel p24 protein enrichment method coupled with the digital immunoassay to further extend the sensitivity and specificity of viral protein detection. Immunocapture of HIV gag p24 followed by elution in a Simoa-compatible format resulted in higher protein recovery and lower background from various biological matrices and sample volumes. Quantification of as little as 1 fg of p24 protein from cell lysates from cells isolated from peripheral blood or tissues from ART-suppressed HIV participants, as well as simian–human immunodeficiency virus–infected non-human primates (NHPs), with high recovery and reproducibility is demonstrated here. The application of these enhanced methods to patient-derived samples has potential to further the study of the persistent HIV state and examine *in vitro* response to therapies, as well as *ex vivo* study of translationally competent cells from a variety of donors.

Keywords: HIV, p24, SIV, p27, immunoprecipitation, Simoa, rectal biopsy, biomarker

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INTRODUCTION

Antiretroviral therapy (ART) has dramatically improved life quality and survival for people living with HIV (PLWHs). Despite long-term ART, HIV persists and viremia almost invariably rebounds once therapy is withdrawn (Li et al., 2016; Castagna et al., 2019; Cohn et al., 2020). Several HIV cure strategies are being employed to eradicate the virus including latency-reversing agents, broadly

neutralizing antibodies, therapeutic vaccines, and immune-based approaches (Thorlund et al., 2017; Sobieszczyk, 2020). Cure-directed interventions will require well-characterized clinical studies to inform outcomes, as well as appropriate biomarkers to establish proof-of-mechanism and sensitive measurement of both the active and latent HIV reservoir in blood and tissue compartments wherever possible. Biomarkers that can robustly measure viral persistence in PLWHs on ART in a way that reports on proviruses capable of driving rebound off therapy or producing viral protein that influence HIV pathogenesis and immune functions are important tools for more in-depth understanding of HIV persistence and assessment of therapeutic interventions.

Recent studies of viral persistence have relied on differentiating between replication-competent and defective provirus (Bruner et al., 2016; Falcinelli et al., 2019). The quantitative viral outgrowth assay (QVOA) measures the replication-competent HIV reservoir but likely underestimates reservoir size as some genetically intact viruses may not grow in culture after a single round of *ex vivo* stimulation. Limitations also include cost, assay duration, requirement for large cell numbers, throughput, and relying primarily on cells obtained from blood (Crooks et al., 2015; Stone et al., 2020; Stuelke et al., 2020). HIV DNA quantification is a simple, standardized, sensitive, and reproducible assay but lacks specificity for replication-competent proviruses (Avettand-Fènoël et al., 2016). The newer intact proviral DNA assay (Bruner et al., 2019) shows promise and requires relatively fewer cells than QVOA, but the predictive value in measuring changes in reservoir size with cure-directed interventions remains unknown. HIV RNA assays quantify transcriptionally active proviruses but fail to distinguish between intact and truncated proviruses leading to inflated reservoir sizes (Cillo et al., 2014; Pasternak and Berkhout, 2018) and do not inform on whether RNA transcripts produce antigen or virions capable of spreading infection or shaping immune responses. Studying viral protein is becoming increasingly important for cure research as proteins are more likely than nucleic acid to be sensed by the immune system, influence immune functions, and can be produced by some defective proviruses (Imamichi et al., 2020). Although protein-based assays also overestimate replication competent reservoir size, measuring this translationally competent reservoir provides mechanistic insight into HIV persistence, immune response, and evaluation of interventional strategies aimed at clearing these infected cells.

High-sensitivity HIV gag p24 assays have emerged in recent years including the digital enzyme-linked immunosorbent assay (ELISA) or single-molecule array (Simoa). The Simoa approach has shown several logs improvement in p24 detection in plasma and serum over traditional immunoassays (Chang et al., 2013; Passaes et al., 2017). Previously, we first reported use of the Simoa assay for the specific measurement of cell-associated p24 in $CD4^+$ T cells isolated from peripheral blood of ART-suppressed HIV^+ individuals following *ex vivo* or *in vivo* HIV latency reactivation (Wu et al., 2017) and recently extended the methodology to tissues (Wu et al., 2021). Despite these improvements, additional factors may impose challenges in sensitive detection of low-abundant analytes and may lead to underreporting actual analyte concentrations in biophysiological sample types. For example, sample matrix can interfere with assay signal generation due to the concentration and viscosity of the matrix (Wood, 1991). Sample dilution may help to overcome this interference but might not be feasible if high sample concentration is needed for detecting low-abundance proteins. Similar challenges exist for detecting HIV p24-producing cells as these are rare in a population of cells (Graf et al., 2013; Deeks et al., 2016) requiring larger cell numbers to have relatively few p24-producing cells. The challenge in this situation is the balance between concentrated matrix proteins in a lysate of large cell numbers vs. overly dilute analyte. To overcome this, we developed a novel approach to allow for larger cell input numbers while minimizing matrix effects for sensitive and specific measurement of p24 protein from biological samples. A new immunoprecipitation (IP) application was developed for the capture, concentration, and elution of HIV gag p24 protein followed by sensitive measurement using the Simoa p24 assay. We showed greater than 98% recovery of recombinant protein spiked into cell lysates, as well as enhanced p24 measurement from blood-derived $CD4^+$ T cells stimulated *ex vivo* with strong or weak latency-reversing agents to produce p24 and detection of p24 from lymphoid tissue (rectal pinch biopsy) isolated from HIV^+ viremic and aviremic donors. The methods are applicable to HIV p24 as well as SIV p27 proteins and will play a unique role for viral protein detection in cure research beyond the existing nucleic and protein-based assays.

MATERIALS AND METHODS

Participants

Peripheral blood and rectal tissue from HIV-infected subjects were obtained under institutional review board (IRB) approval and patient informed consent through iProcess Global Research (Dallas, TX), University of Pennsylvania (Philadelphia, PA), or internal clinical studies at Merck & Co., Inc. (Kenilworth, NJ). HIV-negative rectal tissue samples were sourced through BioIVT as fresh-frozen tissues taken from healthy adjacent tissues from surgical procedures or postmortem donors. The HIV-negative samples were cut while frozen into small pieces to use in place of HIV-negative pinch biopsies.

Human $CD4^+$ T Cell Separation From Peripheral Blood Mononuclear Cell and Treatment

$CD4^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) with EasySep^{TM} Human $CD4^+$ T Cell Enrichment Kit from StemCell Technologies (Vancouver, BC, NJ) in accordance with the manufacturer's instructions. Cell numbers and viability were determined using a Vi-cell (Beckman Coulter, Brea, CA). To reactivate virus, 4×10^6 $CD4^+$ T cells were incubated with either Dynabeads^{TM} human T-activator anti-CD3/anti-CD28 beads at 25 μL per million $CD4^+$ T cells or final

1 μM histone deacetylase inhibitor (HDACi) vorinostat (VOR) in 24-well plates containing 1 mL cRMPI/10 U/mL interleukin 2 for 3 days at 37°C, 5% CO_2. Cells were collected and lysed at 4 × 10^6 cells/mL with Simoa lysis buffer [1% Triton X-100, 50% Hi-FBS, and 50% casein/phosphate-buffered saline (PBS)] for 30 min with 1 × protease inhibitor cocktail. Culture medium (CM) was also collected and inactivated with 1% Triton-X100 (final concentration). Samples were stored at -80°C until IP or direct p24 measurement. For cell killing experiments, final 1 μM staurosporine was added together with anti-CD3/anti-CD28 beads.

Conjugation of Anti-p24 Antibody to Magnetic Beads

Anti-p24 monoclonal antibodies obtained from Capricorn (Portland, ME), ZeptoMetrix (Buffalo, NY), US Biological (Salem, MA), and R&D systems (Minneapolis, MN) were conjugated to magnetic beads (Thermo Fisher Scientific) according to manufacturer's instructions. In brief, 1 mg/mL antibody (600 μg)/PBS was mixed with 60 mg magnetic beads in kit conjugation buffer for 16 h, 37°C, with rotation. Using magnetic bead capture, beads were washed, blocked, and resuspended in 6-mL storage buffer and stored at 4°C until use. Assuming complete capture of antibody onto the beads, the final antibody concentration is estimated to be 100 μg/mL in the storage solution. Mouse immunoglobulin G (IgG) (mIgG; GenScript) was also used as negative control antibody for conjugation.

IP of HIV p24 Protein

Frozen lysates from $CD4^+$ T cells and rectal biopsies were thawed in 37°C water bath and spun at 20,000 × *g* for 10 min to remove cell debris. Four micrograms antibody-conjugated beads {40 μL beads was washed once with 1 mL of bead washing buffer [PBS + 0.5% bovine serum albumin (BSA)] prior to adding} was added to clarified supernatant. An equal volume of 3% BSA/PBS was added into the inactivated CM and above cell lysate supernatant to yield final 0.5% Triton X-100 in IP mixture. Cell lysate concentration was equivalent to 2 × 10^6/mL. The IP mixture was incubated at 4°C overnight with gentle rocking. The next morning, the supernatant ("flow-through") was removed, and beads were washed twice with 1 mL bead washing buffer in 1.7 mL Eppendorf tubes using a magnetic rack to immobilize the beads. Then the bound p24 protein was eluted with 100 μL of 0.1% trifluoroacetic acid (TFA) elution buffer after mixing in a 37°C water bath for 30 min. The eluate was collected, and the beads were washed once with 100 μL of Simoa lysis buffer containing 1 μL of 1 M, pH 9.0, Tris buffer (1:100 dilution, prepared fresh) and mixed, and residual p24 eluate was combined with the initial eluate (total 200 μL) to maximize recovery, reduce non-specific binding, and neutralize the pH for the subsequent measurement. Samples were spun at 20,000 × *g* for 5 min again before p24 Simoa assay, and the supernatant was collected with magnet to ensure no beads from the IP (2.8 μm in diameter) were carried over into the eluate to minimize potential interference with the Simoa assay. Other elution reagents including 0.1 M glycine pH at 2.5, 0.1 N HCl, 0.1 M citric acid, or heat treatment of the beads after IP were compared for their elution efficiency, recovery, and detection of p24. Additionally, the volume of beads used for IP, sample IP incubation duration, and the impact of protease inhibitor cocktail on p24 IP recovery were also examined (see section "Results") for optimizing IP conditions.

Culture Media and Cell Lysate Precleaning

Frozen CM and lysate were thawed at 37°C and centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatants were collected after spinning and precleaned with 40 μL of Dynabeads™ M-280 Streptavidin beads from Thermo Fisher containing final 10 μg/mL mIgG (this is to preabsorb any proteins that may stick non-specifically to the beads in the Simoa assay). The mixture was incubated at 4°C for 3 h with rotation using a HulaMixer, and then the lysate was spun at 20,000 × *g* for 10 min. The precleaned supernatant was collected for Simoa measurements (no IP).

Direct Lysis of Rectal Biopsy

Rectal pinch biopsy samples from HIV^+ donors or HIV-negative rectal tissue samples (see section "Participants") were lysed directly by putting the tissue into 0.5 mL of 1% Triton X-100 in Simoa lysis buffer containing 30 μL of uncoated Dynabeads™ M-280 streptavidin beads (for precleaning) in 1.7 mL Eppendorf tube overnight at 4°C with rotation on HulaMixer. The lysate was spun at 20,000 × *g* for 10 min and put on the magnet to hold the beads, and the supernatant was collected. In addition, the pellet was washed once with 0.5 mL of 3% BSA/PBS, spun, and put onto the magnet. The supernatant was collected and combined with the above 0.5 mL lysate supernatant to collect any residual p24 protein remaining in the pellet. The combined yields 1 mL lysate with final 0.5% Triton X-100, which is ready for p24 IP.

CD4 Protein Assay

Anti-CD4 monoclonal antibody from R&D systems (Cat# MAB375-500) was used as capture antibody, and anti-CD4 monoclonal antibody OKT4 from Biolegend (Cat# 317424) was used as detection antibody. OKT4 antibody was conjugated with alkaline phosphatase (AP) according to our previous method (Wu et al., 2011) and sandwich ELISA (Wu et al., 2012) to measure CD4 protein. Luminescent CDP−Star with Sapphire−II Enhancer substrate (T−2214; Applied Biosystems, Foster City, CA) was used as AP substrate (Wu et al., 2008). In brief, 100 μL of 2 μg/mL capture anti-CD4 antibody was coated on 96-microwell plate overnight, washed once with 200 μL PBS with Tween (PBST), and blocked with 200 μL of 3% BSA/PBS for 2 h, and then 100 μL of CD4 standard protein or diluted lysate and 50 μL of 1:500 diluted AP-OKT4 detection antibody in 0.3% Tween 20/3%BSA/PBS were added sequentially and incubated at 4°C overnight with gentle shaking at 30 revolutions/min. The next morning, the plate was washed with PBST six times and developed with AP substrate. The luminescent counts were measured

in PerkinElmer EnVision, and the CD4 concentration was calculated based on the CD4 standard curve. Recombinant CD4 protein obtained from R&D systems was used as assay standard protein stored at −20°C at 1 μM stock concentration in 50% glycerol until using.

HIV Gag p24 Protein Simoa

HIV gag p24 protein in samples from either precleaned or IP eluant was measured according to our previously published methods (Wu et al., 2017) on the Quanterix HD-X platform. p24 Simoa kit was obtained from Quanterix (Billerica, MA). p24 concentrations were calculated from raw signal average enzyme per bead (AEB) by the instrument's software with four-parameter logistic regression curve fitting, and p24 levels were reported as pg/million cells or pg/mL in cell lysate and CM (Wu et al., 2017, 2021).

Simian Immunodeficiency Virus Gag p27 Protein Simoa

Simian immunodeficiency virus (SIV) gag p27 protein was immunoprecipitated with Capricorn anti-p24 antibody conjugated beads as for p24 above. The IP procedure for p27 was the same as above p24. For p27 Simoa after IP and elution, the same kit for p24 was used except that the detection antibody was replaced with biotin-labeled anti-p27 antibody from ABL Inc. (Rockville, MD, Cat# ABL-4324) instead of biotin anti-p24 antibody in the p24 kit. EZ-Link^{TM} Sulfo-NHS-LC-Biotinylation Kit from Thermo Fisher was used for biotinylation of the antibody per the manufacturer's instruction. Biotin-ABL-4324 antibody concentration used in p27 Simoa was 3 μg/mL in 3% BSA/PBS buffer (Swanstrom et al., 2018). Recombinant p27 protein used for the assay's standard curve was the gift from Jeff Lifson (Swanstrom et al., 2018). All other assay reagents and parameters were the same as the p24 Simoa conditions. Unknown p27 sample concentrations were calculated based off a p27 assay standard curve.

Data Analysis

In both p24 and p27 IP-Simoa assay, standards and samples were run in duplicate unless noted otherwise, and unknowns were converted to concentrations by the Quanterix software. Graphs and figures were prepared using GraphPad Prism or Kaleidagraph (Synergy). Statistical significance in group comparisons is denoted conventionally by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, using Tukey–Kramer honestly significant difference or Student *t*-test.

RESULTS

Development and Optimization of p24 IP Method

IP conditions were evaluated to select the optimal antibody, bead quantity, incubation times, p24 elution buffer, and compatibility with the Simoa assay. To determine the best antibody for IP, four anti-p24 monoclonal antibodies were identified from commercial sources, conjugated to magnetic beads, and compared for IP efficiency and yield of recovery. Based on these criteria, the Capricorn anti-p24 antibody-conjugated beads were selected and used in all following studies (**Figure 1A**). Using this antibody, we next determined the quantity of antibody-conjugated beads necessary for optimal p24 immunocapture. Antibody-conjugated beads were titrated against a fixed concentration of 1 ng/mL p24. As shown in **Figure 1B**, 10–20 μL of 10 mg/mL antibody bead solution sufficiently captured 1 ng/mL p24 in 1 mL buffer after overnight incubation at 4°C. To avoid variability in the assay, 40 μL conjugated bead (an excess of the minimal needed) was used for all the following experiments. To identify elution conditions for optimal recovery of p24 bound to beads, the following four buffers were evaluated: 0.1% TFA, 0.1 M glycine, 0.1 N HCL, and 0.1 M citric acid. As shown in **Figure 1C**, 0.1% TFA yielded the best recovery of p24. Heat denaturation of the complex at 100°C for 10 min resulted in poor protein recovery (data not shown). Based on these data, 0.1% TFA followed by neutralization with Tris was selected for p24 elution and was used in the subsequent experiments. We evaluated the time needed for immunocapture by studying various incubation times of antibody conjugated beads incubated with 1 ng/mL recombinant p24 spiked into Simoa lysis buffer. As shown in **Figure 1D**, 2- and 4-h incubations resulted in incomplete recovery with 24 and 48 h yielding >95% recovery. Thus, we recommend an overnight incubation (16–24 h) as the optimal time for p24 capture. While no significant impact on p24 recovery was observed following IP overnight with or without protease inhibitor (data not shown), we include it in all experiments to minimize any potential impacts from different samples.

Capture of Low p24 Concentrations and Assay Validation

Previously, we optimized the p24 Simoa for detection of protein from cell lysates and found that the assay limit of detection (LOD) was ∼0.005 pg/mL (Chang et al., 2013; Wu et al., 2017). Because of the rare incidence of HIV^{+} cells in biological samples from ART-suppressed patients, we focused efforts on developing IP Simoa methods to quantify very low levels of viral protein (dynamic range ∼0.001–3 pg/mL) and also because high-level protein detection is not an issue for standard, direct Simoa assay (dynamic range of ∼0.01–30 pg/mL). At the lowest point of the p24 standard curve, the signal was approximately 2-fold over background in assay buffer and cell matrix (Wu et al., 2017). To evaluate whether our new IP procedure could boost assay sensitivity and maintain high recovery even at low p24 concentrations, we performed protein immunocapture at the three lowest concentrations in the standard curve (0.011, 0.024, and 0.085 pg/mL). These three standards were mixed with equal volume of HIV-negative CD4^{+} T-cell lysate at 4×10^{6}/mL in lysis buffer and analyzed in Simoa with or without immunocapture. As shown in **Figure 2A**, p24 measurements were higher after IP compared to non-IP conditions and proportional to the 3-fold volume reduction from the IP procedure, suggesting

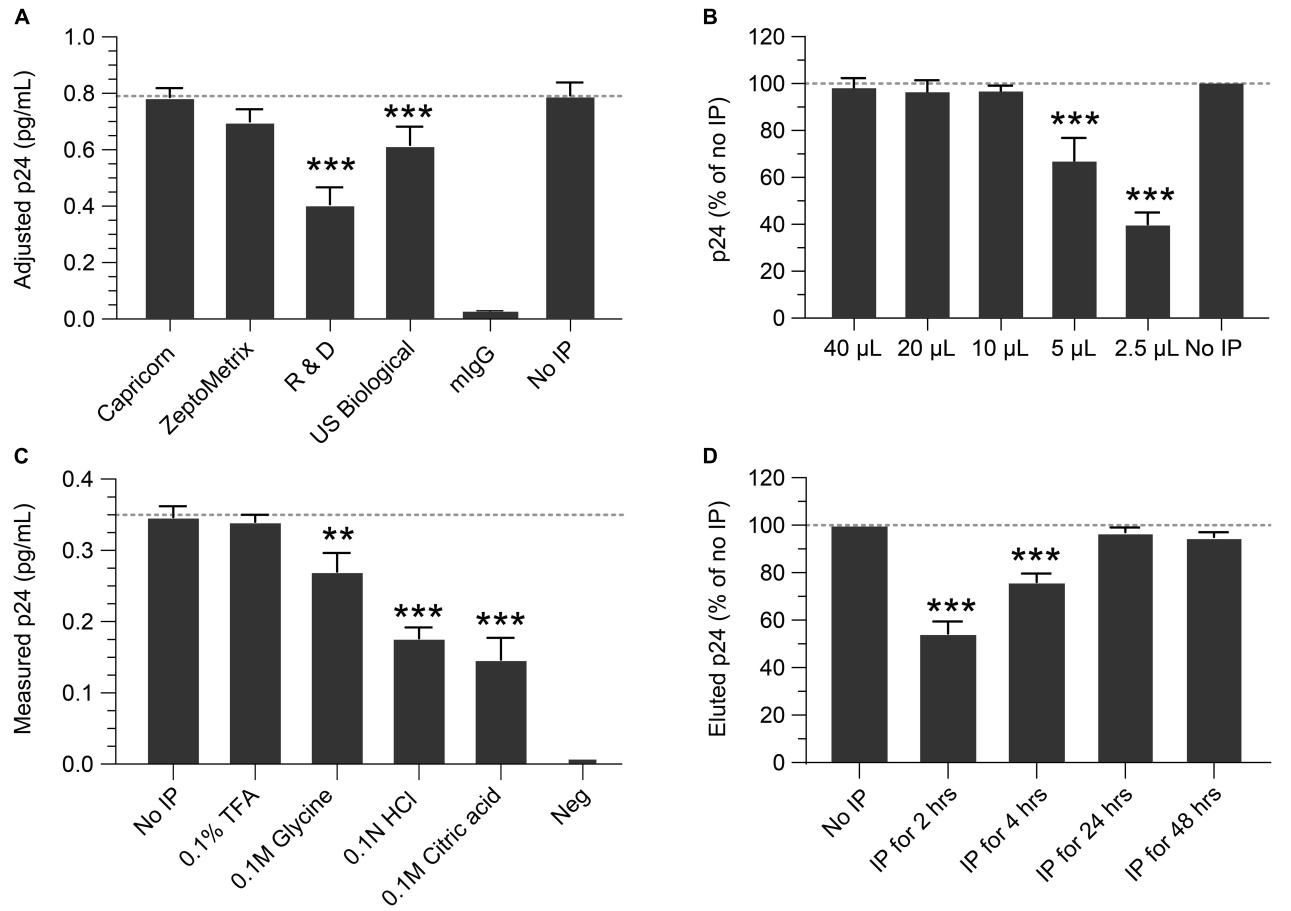

FIGURE 1 | Optimization of the immunoprecipitation (IP) Simoa assay. **(A)** Selection of IP capture antibody. Recovery of p24 using antibodies from various sources, conjugated to magnetic beads, showed the Capricorn antibody to yield the best capture and was similar to the input concentration after release and volume adjustment. **(B)** Optimization of bead concentration for p24 IP. Using a 10 mg/mL stock, adding >10 µL of antibody-conjugated beads to a lysate is sufficient for p24 capture. **(C)** Elution buffer selection. Comparing various elution buffers, 0.1% TFA had no significant difference from input solution after assay, indicating it efficiently releases p24 without destroying crucial epitopes. Other elution buffers resulted in poorer p24 recovery. **(D)** Time required for p24 IP capture. Recovery of p24 with 2- or 4-h incubation with antibody-conjugated beads is markedly lower than that of input solution, whereas recovery with 24 or 48 h gave recovery similar to that of the input solution (no IP). See Materials and Methods for statistical analysis.

near-complete recovery of the p24 from the lysates even at very low analyte concentrations. **Figure 2B** compares measured p24 values between methods, that is, non-IP conditions and post-IP with accounting for adjustments in sample volume following IP concentration. Assay reproducibility was determined by evaluating the coefficient of variation (CV) for protein standards tested in replicate measurements, and they were found to be very consistent (**Figure 2C**). LOD was determined by plotting the known concentration of the standard curve with the calculated concentration for the individual measures (**Figure 2C**). Percent CV was determined for each calculated p24 concentration using the data from the interday runs (n = 3), and the threshold for the LOD was set as the lowest standard with CV less than 20%. Based on this criterion, we determined the LOD to be $\leq$0.005 pg/mL of recombinant p24 standard (**Figure 2C**). Based on these values and the sample volume input into the Quanterix instrument of 200 µL, we estimate the assay could detect 1 fg of protein concentrated from a given lysate volume to the final detection volume of 200 mL. Of importance, LOD may vary slightly across kits (calibrators, buffers) and instrument settings, and thus investigators should validate this value for their specific conditions.

Using validation samples prepared at 0.4 and 0.2 pg/mL, we showed intraday CV of 11.1 and 8.2% for 0.4 and 0.2 pg/mL, respectively, for IP samples, whereas the interday CVs were 10.4 and 7.4%, respectively. The average recoveries of intraday runs were 86 and 90% for 0.4 and 0.2 pg/mL, respectively (n = 3), and the average recoveries from interday runs were 92 and 88% for 0.4 and 0.2 pg/mL, respectively (n = 3). We also compared tissue samples from HIV-negative donors and consistently find AEB values indistinguishable from buffer AEB (**Supplementary Figure S1**). These findings confirm that the immunocapture approach is a robust method for recovering even low amounts of analyte in cell lysates with high reproducibility and shows the potential to enrich p24 from complex matrix, such as cell and tissue lysate.

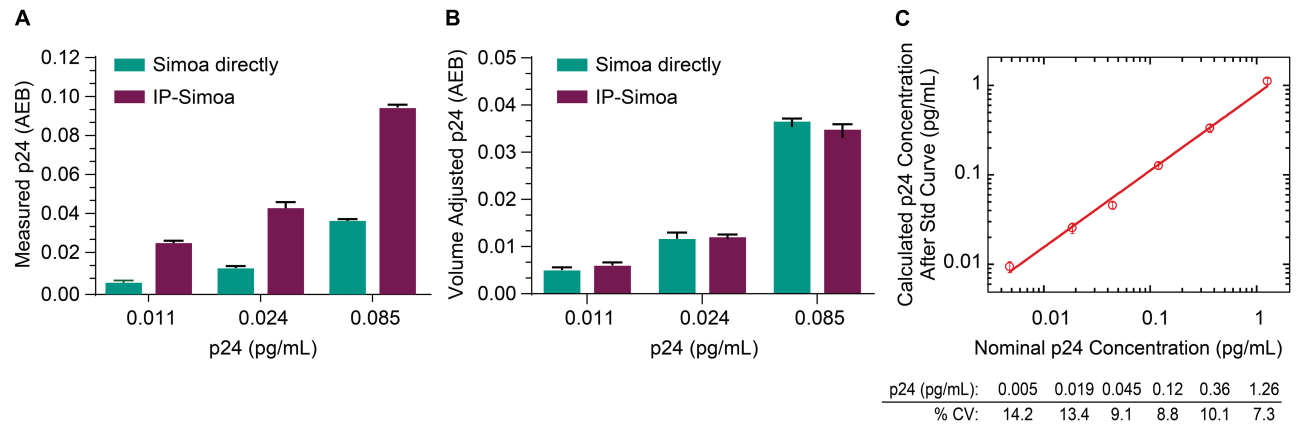

FIGURE 2 | Capture of low p24 concentrations. **(A)** Recombinant p24 at the lowest concentrations of the standard curve was completely recovered after IP, and **(B)** p24 concentration matches between direct Simoa and IP-Simoa after sample volume normalization. **(C)** The IP-Simoa assay's lower limit of detection (LOD) is ≤0.005 pg/mL defined as the coefficient variation (CV) <20% for each p24 standard. This would equate to 1 fg of p24 concentrated from a given volume and released into 200 μL for assay.

Detection of p24 in ART Suppressed HIV$^+$ CD4$^+$ T Cells

The rare incidence of translationally competent HIV proviruses in peripheral blood CD4$^+$ T cells from ART-suppressed HIV$^+$ individuals presents a challenge for quantifying viral protein expression, even with sensitive assays (Falcinelli et al., 2019). To further assess performance of our new approach, we activated peripheral blood CD4$^+$ T cells from ART-suppressed HIV$^+$ individuals and quantified p24 protein production by IP-Simoa. **Figure 3A** represents a single ART-suppressed individual with low-level basal p24 expression in peripheral blood CD4$^+$ T cells in the absence of T-cell stimulation and without IP (mean = 0.023 pg/mL, $n = 3$). *Ex vivo* exposure of CD4$^+$ T cells from this donor with anti-CD3/anti-CD28 conjugated beads for 3 days resulted in a significant 15-fold increase in p24 to an average of 0.34 pg/mL ($n = 3$, $p < 0.05$) even in the absence of IP. IP-Simoa resulted in approximately 5-fold further increase in p24 concentration in the cell lysate (1.65 pg/mL; $n = 3$) from the same donor. This increase in p24 upon 5-fold volume reduction was statistically significant ($p < 0.001$; **Figure 3A**) and indicates that virally produced gag p24, like recombinant p24, was efficiently immunoprecipitated and eluted with this IP procedure. No significant difference was observed between unstimulated p24 levels between non-IP and IP methods with this donor, potentially attributed to the low frequency of transcriptionally and translationally active HIV infected cells in the unstimulated sample and variable expression of p24 across HIV proviral genomes. In our experience, p24 detection in resting cells from ART-suppressed donors is infrequent (data not shown). To extend this work, we evaluated anti-CD3/anti-CD28 bead stimulation of CD4$^+$ T cells isolated from blood of five additional HIV$^+$, ART-suppressed individuals and observed a similar pattern of volume-proportional increases following IP-Simoa vs. without IP Simoa ($p < 0.01$) (**Figure 3B**). p24 values were below assay limits in the flow-through following protein capture on beads, indicating the IP method efficiently captured the expressed p24. Next, we identified a subset of ART-suppressed HIV$^+$ samples lacking measurable p24 by traditional direct, non-IP Simoa methods following anti-CD3/anti-CD28 bead stimulation. We assessed the ability for the IP-Simoa method to enrich and quantify p24 following stimulation and compared results to samples from HIV-negative donors. Following T-cell reactivation and IP-Simoa, low but measurable p24 levels were detected in all HIV$^+$ samples, whereas HIV-negative samples remained below assay limits (**Figure 3C**). Donor cells that have not yielded p24 under any of our assay conditions tested to date have also been encountered (data not shown).

We evaluated inducible p24 levels following treatment with the well-studied latency-reversing agent and HDACi, VOR. Measurable p24 levels were observed in three out of five HIV$^+$, ART-suppressed samples treated with 1 μM VOR for 72 h (**Figure 3D**) and were lower than anti-CD3/CD28 bead-stimulated samples (data not shown), consistent with previous findings (**Figure 3D**; Wu et al., 2017; Falcinelli et al., 2019). Seventy-two hours was chosen to assay for comparison to anti-CD3/anti-CD28 stimulation. Shorter or longer time periods will likely induce different levels of protein and can be explored in future studies, along with the use of different latency-reversing agents. Use of a non-specific kinase inhibitor, staurosporine, to non-selectively kill cells during the stimulation period, prior to protein immunocapture was also evaluated. Under these conditions, p24 decreased dramatically following 1 μM staurosporine treatment of cells prior to cell lysis and immunocapture of p24 (**Figure 3E**), indicating IP-enriched p24 was generated from live cells.

Measurement of p24 From Rectal Pinch Biopsies

As lymphoid tissue is a reservoir for persistently infected HIV cells (Wong and Yukl, 2016; Thornhill et al., 2019), we assessed p24 measurement by IP-Simoa in gut-associated lymphoid tissue (GALT) from ART-suppressed, HIV$^+$ individuals. Our original

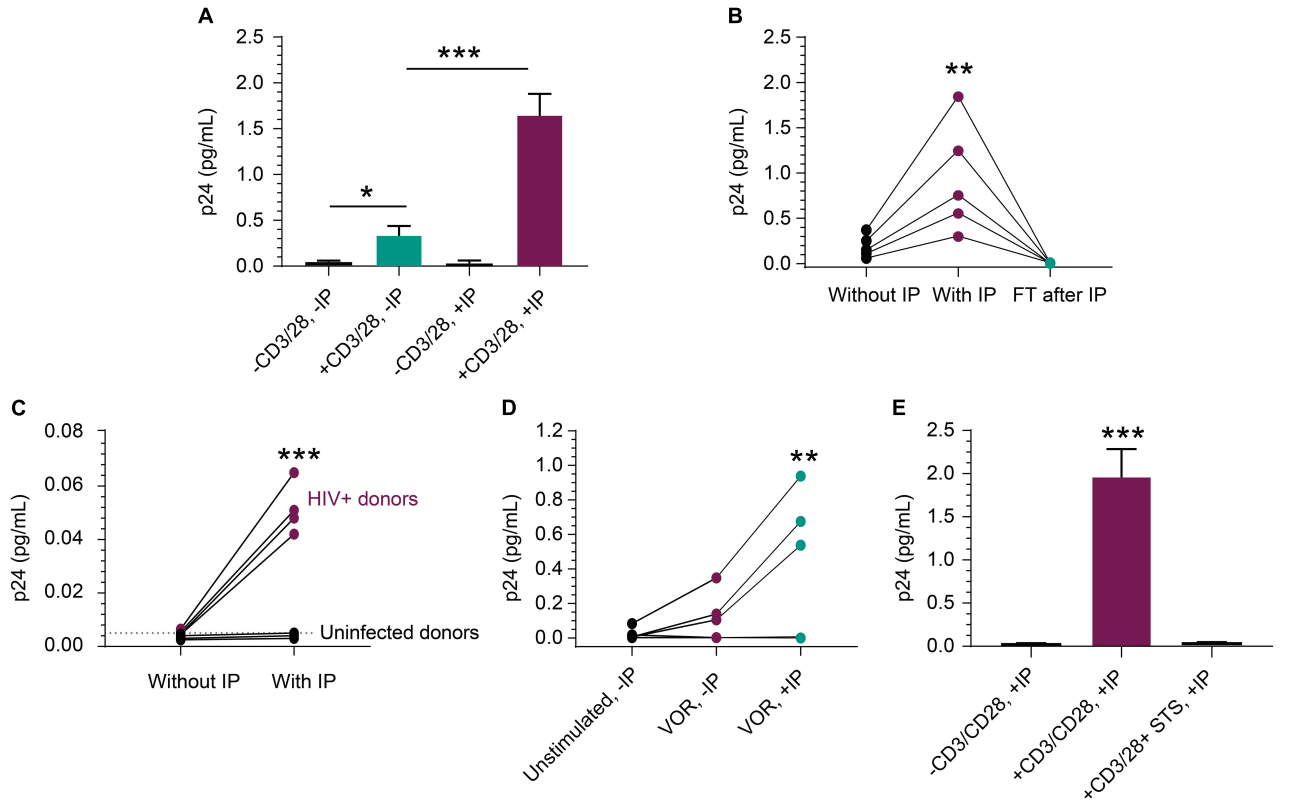

FIGURE 3 | Detection of p24 from HIV$^+$, ART-suppressed peripheral blood CD4$^+$ T cells. **(A)** p24 production was induced in CD4$^+$ T cells following a 3-day stimulation with anti-CD3/anti-CD28 beads ($n = 3$ independent experiments, $p < 0.05$). p24 was enriched 5-fold with a 5-fold volume reduction after IP in treated group ($n = 3$, $p < 0.001$), but not in unstimulated group. **(B)** From samples that respond well to stimulus, p24 was enriched proportionally to volume reduction in five inducible donors ($p < 0.01$), and no detectable p24 was observed in their flow through after IP. **(C)** From samples that do not show robust p24 production after stimulation, IP enrichment was able to demonstrate that p24 is indeed produced but was not at the limit of detection without enrichment. Even with enrichment and bead stimulation, p24 was not detected from HIV-negative CD4$^+$ T cells and shows significant difference ($p < 0.001$) between HIV$^+$ and uninfected donors after IP. **(D)** p24 protein was detected following a 3-day treatment with 1 μM VOR in three of the five donors' CD4$^+$ T cells (4×10^6 cells/condition). The signal was enriched significantly after p24 IP with 4-fold cell lysate volume reduction ($p < 0.01$). No p24 was detected from unstimulated cells. This opens the door for studying even weak LRAs' effects using donor-derived cells. **(E)** Mimicking applications with kill-focused assays, p24 was not detected when including 1 μM staurosporine along with anti-CD3/anti-CD28 bead stimulation for those three inducible donors ($p < 0.001$). See Materials and Methods for statistical analysis.

approach necessitated dissociation and isolation of single-cell suspensions from intact rectal tissue biopsies to overcome high matrix background effect (Wu et al., 2021). This method is quite laborious and low-throughput. To reduce procedure complexity, we evaluated if soaking intact tissue biopsy in 1% Triton X-100/Simoa lysis buffer would sufficiently dissociate tissue and yield high recovery of the cells of interest. As HIV predominantly infects CD4$^+$ T cells, we selected CD4 protein as a surrogate biomarker for the primary target cell population and developed at CD4 ELISA to assess efficiency of tissue cell lysis. A standard curve using recombinant protein was generated and demonstrated robust linearity quantifying CD4 protein in the range of 0–250 pM (**Figure 4A**). The assay was tested with both peripheral CD4$^+$ T cells and normal human rectal tissues. CD4 concentration was measured in five ART-suppressed donor's PBMC and was found to be correlated with CD4 counts in samples using equivalent cell numbers per test ($r = 0.944$, $p < 0.01$, $n = 5$) (Data not shown). CD4 protein was also reliably quantified in HIV-negative rectal biopsy after soaking in 1% Triton X-100/Simoa lysis buffer and decreased linearly upon sample dilution (**Figure 4B**). Time-course studies revealed CD4 levels plateau at some time greater than 4 h of soaking in lysis buffer [signal increases another 10% with overnight soaking with no significant difference between 24 and 48 h (**Figure 4C**)]. No detectable CD4 protein was found in pellet following resuspension in the lysis buffer and homogenization (data not shown). Thus, 24-h soaking was selected as an optimal lysis time for rectal tissue dissociation based on CD4 measurements and use overnight incubation for our procedures.

Next, we evaluated whether p24 could be released from HIV$^+$ biopsies and efficiently immunoprecipitated from the tissue matrix. **Figure 5A** shows IP recovery was comparable between buffer and HIV-negative rectal lysate following the addition of recombinant p24 in the sample at either 0.1 or 0.02 pg/mL. Using p24 isolated from viremic rectal biopsy tissue was recovered at similar levels after inoculation of either buffer or uninfected rectal lysate (**Figure 5B**). These data indicate that rectal tissue lysate matrix has no detrimental impact on capture and detection

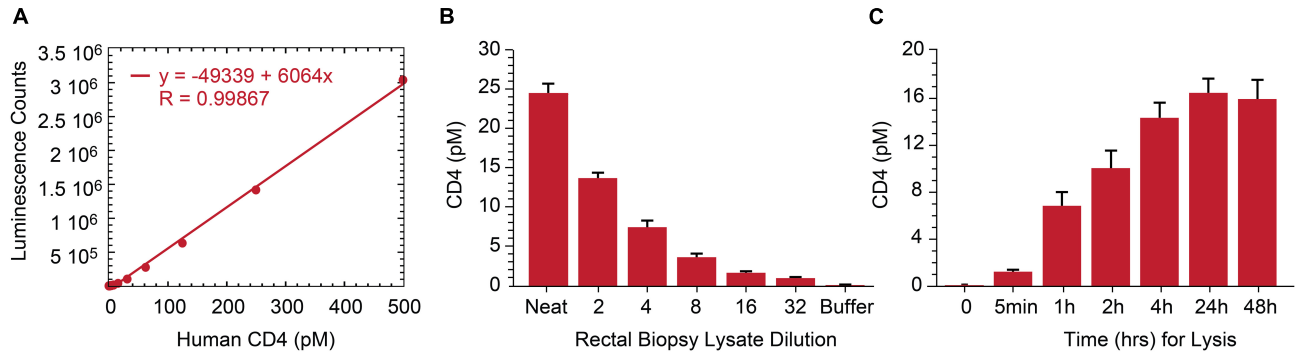

FIGURE 4 | Development of human CD4 protein assay and application to rectal biopsy lysate characterization. **(A)** Signal in the ELISA was proportional to the CD4 protein level over a broad standard curve concentration range from 0 to 250 pM. **(B)** Assay specificity was shown with CD4 signal linearly decreasing following rectal biopsy sample lysate serial dilution. **(C)** Twenty-4 h of biopsy soaking while mixing in lysis buffer yields complete CD4 protein release from rectal biopsy.

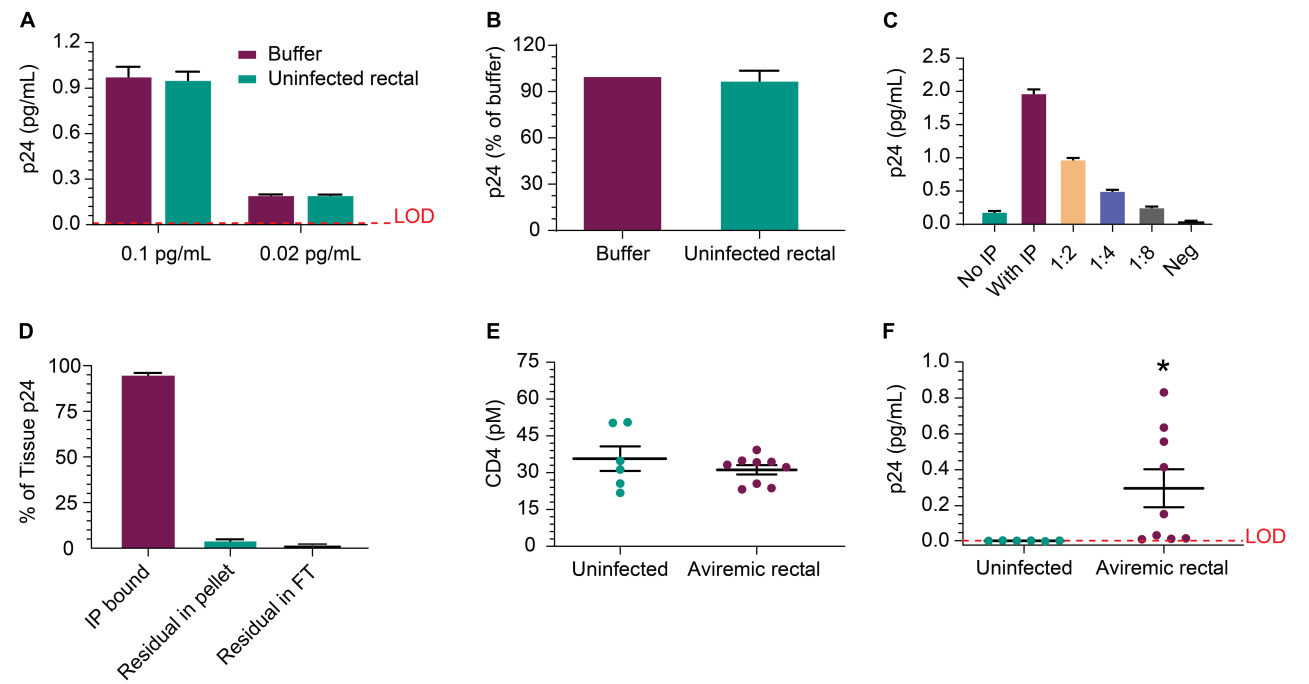

FIGURE 5 | Detection of p24 protein from rectal pinch biopsies. **(A)** Recovery of recombinant p24 spiked into biopsy lysate at 0.02 and 0.1 pg/mL, respectively, is comparable to recovery from buffer as the matrix after IP concentration. **(B)** Recovery was comparable between buffer and uninfected rectal lysate matrix when using p24 protein recovered from a viremic donor-derived sample as the protein source for spike-in. **(C)** Cells isolated from a viremic rectal biopsy were lysed and run in standard p24 Simoa or IP-Simoa. The p24 was enriched by the volume reduction during IP and shows linear signal after dilution supporting signal specificity. **(D)** Reprocessing of rectal biopsies ($n = 3$) for p24 after lysis revealed that a single extraction yielded >96% of the p24 contained within the biopsy. There was no detectable p24 in solution after IP, indicating complete capture of p24 onto the beads. **(E)** Comparing measured CD4 protein from HIV-negative and HIV-positive, ART-suppressed rectal biopsies showed no significant difference in CD4 level, implying that similar numbers of $CD4^+$ cells were present and lysed from each group. **(F)** Using the same lysates as in **(E)**, p24 was measurable in several of the HIV^+ donor biopsy samples (five of nine) after IP-Simoa and not in biopsies from HIV-negative donors ($p < 0.05$, $n = 6$). See Materials and Methods for statistical analysis.

of recombinant p24 and that p24 from donor-derived samples can be detected as well. Using cells dissociated from a rectal pinch biopsy from a viremic donor, p24 was detected well, even without IP and increased ∼10-fold upon 10 × concentration of volume by IP; signal decreased linearly with dilution (**Figure 5C**). This indicates that p24 recovered from a rectal biopsy can be efficiently measured. Using additional viremic biopsies ($n = 3$), we used the direct biopsy lysis method to verify the release of the p24 and quantitate the completeness of p24 release. After soaking and removal of supernatant, we re-ran the assay on the residual tissue and found ∼4% of the original p24 concentration was recovered upon reprocessing, showing high but incomplete recovery by a single cycle. No p24 was detected in the flow-through after IP (**Figure 5D**). To extend these studies, additional

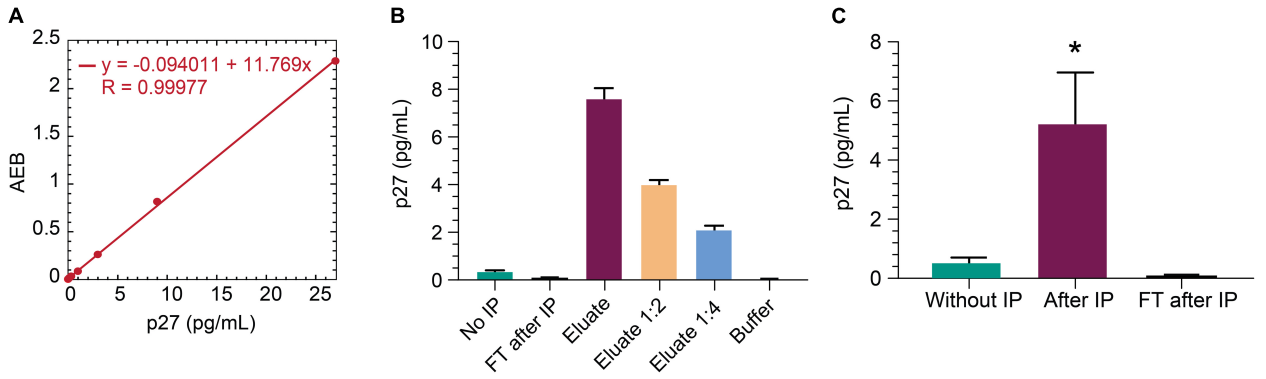

FIGURE 6 | IP-Simoa assay for SIV p27 protein measurement. **(A)** SIV p27 Simoa assay standard curve. Linear signal response for recombinant p27 from 0 to 28 pg/mL. **(B)** Recombinant p27 spiked into uninfected rhesus monkey rectal biopsy (prepared by soaking in lysis buffer) and run with IP Simoa showed enriched p27 signal proportional to the sample volume reduction (20-fold) and expected reduction of signal upon dilution indicating assay specificity. **(C)** p27 was enriched significantly from the lysate of viremic, SIV^+ monkey rectal biopsy ($p < 0.05$, $n = 4$). No detectable p27 was observed in the flow through after IP demonstrating high efficiency of p27 IP from biopsy lysate matrix. See Materials and Methods for statistical analysis.

rectal biopsies were obtained from six uninfected and nine ART-suppressed, HIV^+ individuals with undetectable viral load (<50 copies/mL). CD4 protein level trended higher in uninfected group but not significantly when compared to the HIV-infected group and could be a product of sample preparation (see section "Participants" in "Materials and Methods") (**Figure 5E**). Despite slightly higher CD4 protein level, the HIV^+ samples had significantly ($n = 9$, $p = 0.041$) higher p24 than HIV-negative (**Figure 5F**). We also initiated studies to assess whether CD4 ELISA values correlated with CD4 cell counts. Collectively, p24 was reliably detected after soaking in lysis buffer and IP-Simoa from five of nine samples (∼56%) and near the lower LOD for the other four samples.

IP-Simoa Assay for SIV p27 Protein Measurement

The non-human primate SIV model is an important model for studying HIV cure (Evans and Silvestri, 2013). SIV gag p27 protein is a relevant viral biomarker of SIV persistence, as outlined above for HIV gag p24 protein. To that end, we sought to determine whether the IP-Simoa p24 assay could be adapted for SIV p27. Anti-p24 beads were selected for p27 immunocapture as this p24 antibody efficiently binds both HIV gag p24 and SIV gag p27 (data not shown). An SIV p27-specific antibody was biotinylated and used for detection in the Simoa assay. **Figure 6A** shows the standard curve for recombinant p27 (1:3 dilution; concentration range from 27 to 0.02 pg/mL). Assay LOD was determined to be ∼0.02 pg/mL, similar to HIV gag p24 Simoa assay. To assess if IP-Simoa can enrich for SIV p27, we spiked recombinant p27 into lysates prepared from SIV-negative rhesus rectal biopsy and measured p27 in the bead and flow-through fractions. Only the immunobead fraction had quantifiable levels of p27, and protein concentration decreased linearly upon serial dilution (**Figure 6B**). We extended these studies to rectal biopsies ($n = 4$) obtained from a viremic rhesus monkey (plasma viremia at 9,300 copies/mL) infected with chimeric simian–human immunodeficiency virus (ENV162P3 SHIV; Harouse et al., 2001). As above, most p27 was detected in the immunocaptured fractions with little to no measurable p27 in the flow-through (**Figure 6C**). Thus, the new IP-Simoa methodology is applicable for the sensitive detection of both SIV gag p27 and HIV gag p24 proteins.

DISCUSSION

As shown recently (Wu et al., 2017, 2021), the detection of low-abundant viral proteins from HIV-expressing cells from blood or lymphoid tissue of HIV infected, ART-suppressed individuals is possible but still presents challenges. Various approaches have been described for concentrating cell lysates and enriching analytes (Wood, 1991); however, these approaches can fall short as increasing concentration and sample viscosity may interfere with assay signal. Sample dilution may overcome any matrix interference but is less desirable when high sample concentration is needed for detecting low-abundant analytes. Thus, sample preparation is a fine balance between having the analyte of interest as concentrated as possible without raising matrix and assay background to untenable levels. Furthermore, consideration must be given to the compatibility of the biological sample type and the analyte detection methods.

Here, we apply a novel immunocapture and sensitive ELISA approach (IP-Simoa) for the maximal and specific measurement of HIV and SIV gag proteins from biological samples, particularly at low concentrations. In this study, we have made strides in more clearly delineating between positive and negative samples by immunocapturing the maximal amounts of protein from the sample onto beads, removing non-specific interference from sample matrix through washing, eluting in such a manner as to retain antibody recognition of the analyte, and resuspending in a final volume such that all sample is loaded onto the

instrument for quantitation (**Figure 1**). We experimentally determined that TFA, a common acid used in spectrometry-based proteomics to dissolve cells/tissues and achieve highly efficient protein extraction, was optimal for efficient recovery of viral protein from beads. We then applied the new method to measure both recombinant p24 (or p27) spiked into biological samples at very low concentrations (**Figures 2**, **6**), as well as p24 induced from samples derived from virally suppressed $CD4^+$ T cells (**Figure 3**) or viremic rectal pinch biopsy (**Figure 5**). Our findings suggest a range of protein detection (0.001–3 pg/mL) with recovery efficiency of 85–95%. As the focus of our study was on detecting low levels of p24, we did not extensively investigate the upper range of detection beyond 3 pg/mL; however, we believe this is feasible. Detecting higher concentration of p24 (or p27) can be achieved either by direct Simoa method (ULOD 30 pg/mL; Quanterix, Inc.), or alternatively, conducting independent studies using the IP method to determine optimal bead concentrations for capturing large amounts of protein.

Several tests were carried out to understand the reproducibility of the assay and to ascertain if enrichment reagents might interfere with p24 quantification. In our studies, both intraday and interday CV of spiked-in, low-concentration p24 protein into buffer followed by IP and Simoa is below 15%. The p24 recovery for both intraday and interday is from 85 to 95%. The high recovery is probably due to the saturating amount of high-affinity antibody used during IP, coupled with overnight incubation for capture, and efficient elution conditions. Several studies suggest the lack of interference of the enrichment antibody or IP beads in p24 quantification. For example, we achieved near 100% protein recovery after volume adjustment using the IP-Simoa (**Figure 2**), suggesting the presence of antibody or beads, if released into the eluate, did not interfere with protein measurement on the Quanterix instrument. In addition, there was no measurable signal in blank IP samples, which lacked spiked p24 (data not shown), further strengthening the notion that either IP beads are not retained in the eluate, or the few beads that may be eluted do not interfere in protein quantification.

With optimized conditions, the p24 IP Simoa was successfully employed to measure p24 protein derived from ART-suppressed, HIV-positive donor cells that had been stimulated *ex vivo* to reactivate latent virus. From these, p24 was measured after enrichment, and levels were significantly higher than that of non-enriched lysate and uninfected controls (**Figure 3**). These findings indicate that the assay is specific and works to detect p24 derived from a variety of different donors. This assay has shown value not only for studying p24 from suppressed donor samples that respond robustly to stimulus, but also for weakly inducible donors. As p24 without enrichment is not detected from some donors after stimulation, this limits the number of donors whose cells can be used in *ex vivo* studies that look at protein production. By IP enriching for p24, we were able to expand the number of donors whose cells can be used for such studies. It has been shown that p24 can be detected from a single cell using the digital ELISA format (Passaes et al., 2017). However, the ability to detect p24 in induced samples that were previously undetectable by Simoa with the incorporation of a p24 IP step would suggest that there are p24-producing cells that are still not detectable by standard Simoa procedures. This likely reflects the heterogeneity of the proviral pool with various protein levels inducible from latently infected cells. The method has also been shown to more robustly detect p24 induced by using a weak LRA, such as VOR over no enrichment (**Figure 3**). Starting with cells that respond robustly to strong stimulus, one should to be able to study LRAs of various mechanisms over multiple donor-derived samples. These advances should provide a valuable tool for studying treatment efficacy *ex vivo* with latency-reversing agents or selective kill agents with real-world virus and donor cells. Measuring the kinetics of latency reversal along with washout experiments for various molecules and mechanisms should provide insight into potential dosing schema for *in vivo* testing.

As the gut-associated lymphoid tissues are an important HIV reservoir (Wong and Yukl, 2016; Thornhill et al., 2019), and it is possible to collect rectal tissue pinch biopsies in the context of a clinical program, the IP Simoa has the potential to be used as a pharmacodynamic biomarker for studying the efficacy of HIV latency-reversing agents, therapeutic efficacy of cure-focused agents, rebound after analytical treatment interruption, or to study the possible mechanisms of HIV persistence. The simplified lysis procedure coupled with the enhanced sensitivity and selectivity of the IP Simoa may translate well into a clinical trial setting. Not every suppressed patient's rectal pinch biopsy has shown a positive p24 signal in our preliminary studies, so understanding the factors influencing this and longitudinal sampling reproducibility are important for applications as a biomarker of HIV persistence. Having these enhanced protocols in hand would allow for efficient study of these questions with a proper cohort of donors. Our finding that rectal biopsies lysed directly in Triton X-100 lysis buffer instead of from cells dissociated from rectal biopsy has simplified sample preparation and likely improved protein tissue extraction efficiency. This simplification in the processing procedure makes this combined assay more robust for potential clinical application. While we use CD4 protein as a surrogate marker for cell lysis, the value of this readout as a normalization factor is unknown. Because of the scarcity of p24-producing cells in a suppressed donor, one would need to assume uniform distribution of these cells relative to the $CD4^+$ cells in order to reliably normalize to it. Further insights are needed before employing CD4 measurements as a normalization factor. An additional area of interest would be to assess p24 produced after induction of tissue-derived cells. Understanding the translationally competent reservoir lymphoid tissues (as compared to blood-derived cells) should prove enlightening.

Future directions include use of this method as both a clinical biomarker and a non-clinical tool for studying expression of viral antigen upon *ex vivo* stimulation. In order to facilitate both clinical and research applications, automation of the procedure from the current manual IP done into a 96-well plate-based assay will be very enabling, and efforts are currently underway

in our laboratory. Such an automated assay using patient-derived PBMCs will be powerful for studying latency-reversing agents or selective kill approaches in dose response across multiple donors. The demonstration of the IP Simoa assay to enable detection of p24 from *ex vivo*–stimulated $CD4^+$ T cells from donors who were not previously detectable will enable broader exploration of different donors to help ensure breadth of coverage of novel therapeutics. Further adaption of the assay should also allow for similar studies aimed at effector cell–mediated killing, such as ADCC-type approaches with broadly neutralizing antibodies leveraging patient-derived virus as well as autologous effector cells. We feel that these studies across as many donors as is feasible are necessary to help understand breadth of applicability of different potential therapies. Genetic variation as well as patient-specific variations will undoubtedly be critical to understand as fully as possible prior to initiation of clinical studies. Clinical validation of the IP method will allow the use of viral protein measurements as an endpoint in determining efficacy of novel therapeutics, which rely on the expression of viral proteins. Emerging data are starting to show correlates between p24 expression and other markers of immune status (Wu et al., 2021). Expanding the use of the p24 measurements to further build on these early observations will be critical for understanding how viral protein production impacts pathophysiology and immune status/function in ART-treated patients. For further clinical applications, examining p24 induced from donor samples can be utilized for studies, such as looking at p24 expression magnitude, p24 capable reservoir size, and other potential assays.

In summary, we have developed novel, specific, and sensitive combined IP Simoa p24 and p27 immunoassays. The IP Simoa method has been validated with HIV-negative donors, *ex vivo*–reactivated $CD4^+$ T cells from HIV^+ donors, and rectal biopsies from viremic and aviremic patients and non-human primates. The advantage of combined IP Simoa assay is that all analytes from a given sample will be enriched from the lysate volume, regardless of lysis volume and instrument limitation, and separated from all other cellular proteins that contribute to assay background. We feel that the assay should be amenable to automation and will be beneficial to both clinical and research applications for HIV cure efforts.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRB. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the IACUC. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

GW, DH, BH, and PZ conceptualized and designed the experiments. GW, CC, and QH performed the experiments and generated the data. GW, BH, and PZ wrote the manuscript. All authors edited and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.636703/full#supplementary-material

Supplementary Figure 1 | IP-Simoa assay comparing HIV-negative biopsies to buffer. Tissues from five HIV-negative donors with three replicates each donor were tested alongside buffer-only samples. After the procedure was complete, the buffer only samples were indistinguishable from the tissue samples indicating that there is no background contribution of the matrix to the readout.

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ORIGINAL RESEARCH
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A Therapeutic Strategy to Combat HIV-1 Latently Infected Cells With a Combination of Latency-Reversing Agents Containing DAG-Lactone PKC Activators

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Advances in antiviral therapy have dramatically improved the therapeutic effects on HIV type 1 (HIV-1) infection. However, even with potent combined antiretroviral therapy, HIV-1 latently infected cells cannot be fully eradicated. Latency-reversing agents (LRAs) are considered a potential tool for eliminating such cells; however, recent *in vitro* and *in vivo* studies have raised serious concerns regarding the efficacy and safety of the "shock and kill" strategy using LRAs. In the present study, we examined the activity and safety of a panel of protein kinase C (PKC) activators with a diacylglycerol (DAG)-lactone structure that mimics DAG, an endogenous ligand for PKC isozymes. YSE028, a DAG-lactone derivative, reversed HIV-1 latency *in vitro* when tested using HIV-1 latently infected cells (e.g., ACH2 and J-Lat cells) and primary cells from HIV-1-infected individuals. The activity of YSE028 in reversing HIV-1 latency was synergistically enhanced when combined with JQ1, a bromodomain and extra-terminal inhibitor LRA. DAG-lactone PKC activators also induced caspase-mediated apoptosis, specifically in HIV-1 latently infected cells. In addition, these DAG-lactone PKC activators showed minimal toxicity *in vitro* and *in vivo*. These data suggest that DAG-lactone PKC activators may serve as potential candidates for combination therapy against HIV-1 latently infected cells, especially when combined with other LRAs with a different mechanism, to minimize side effects and achieve maximum efficacy in various reservoir cells of the whole body.

Keywords: HIV-1 reservoirs, HIV-1 latently infected cells, diacylglycerol-lactone, protein kinase C activator, HIV-1

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INTRODUCTION

Although prolonged combination antiretroviral therapy (cART) has succeeded in reducing HIV type 1 (HIV-1) replication, the virus cannot be completely eradicated from the bodies of people living with HIV-1 (PLWH) because of the persistent latently infected cells located in viral reservoirs, or so-called sanctuaries, in the body (Siliciano et al., 2003; Cillo et al., 2014). At present, a novel approach to eradicate these reservoir cells using latency-reversing agents (LRAs), which are small-molecule agents, called "shock and kill" is being considered (Hamer, 2004; Richman et al., 2009). However, recent clinical trials demonstrated no reduction in HIV-1 reservoir cells using LRAs *in vivo*, despite the fact that these drugs are active *in vitro* (Archin et al., 2012; Rasmussen et al., 2014). Furthermore, it is necessary to develop agents with reduced toxicity because most LRA candidates act through host cells rather than viruses.

Recent studies have reported that many small-molecule compounds, including histone deacetylase (HDAC) inhibitors, bromodomain and extra-terminal (BET) inhibitors, and protein kinase C (PKC) agonists, show HIV-1 latency-reversing activity (Contreras et al., 2009; Boehm et al., 2013; Jiang et al., 2015). PKC isozymes are a family of serine-threonine kinases, consisting of several isozymes that play a role in physiological cellular responses (Nishizuka, 1992; Watanabe et al., 1992; Mischak et al., 1993; Li et al., 2003; Martin-Diaz et al., 2007). PKC isozymes are divided into three subfamilies: conventional PKCs (cPKCs: α, β, and γ), novel PKCs (nPKCs: δ, ε, η, and θ), and atypical PKCs (aPKCs: ι and ζ; Griner and Kazanietz, 2007). cPKCs and nPKCs are regulated by ligand binding through their tandem C1 domains (C1a and C1b), with the exception of aPKCs. Additionally, cPKCs require binding of Ca^{2+} to the C2 domain. PKCs are located in the cytosol in the inactive form, and substrate binding is capped by its own pseudosubstrate. This form translocates from the cytosol to the plasma membrane and internal membranes, followed by ligand binding. The endogenous ligand of PKCs is a second messenger, 1,2-diacylglycerol (DAG), which is generated downstream of receptor tyrosine kinases and G-protein coupled receptors (Kishimoto et al., 1980). DAG is produced at the inner face of the plasma membrane, and its binding to the C1 domain induces a conformational change of PKCs into the active form (Ogawa et al., 1981; Sakai et al., 1997). Binding subsequently causes the translocation of PKCs into the plasma membrane, followed by signaling through multiple downstream pathways (Wang, 2006). DAG-lactone derivatives, which function as conformationally constrained DAG analogs, are synthetically tractable and have been structurally optimized to improve PKC binding affinity and selectivity between classes of C1 domain-containing targets (Tamamura et al., 2000; Ohashi et al., 2017).

In this study, we focused on the HIV-1 latency-reversing activity and safety of DAG-lactone derivatives, including YSE028 (Nomura et al., 2011), which exhibited a potent ability to activate latent HIV-1 infected cells without any toxicity *in vitro* and *in vivo*.

MATERIALS AND METHODS

Drugs and Reagents

A panel of DAG-lactone derivatives, including YSE028, was synthesized as described previously (Nacro et al., 2000; Tamamura et al., 2000; Nomura et al., 2011; **Figure 1A**). Prostratin (PKC activator), PEP005 (PKC activator), and JQ-1 (BET inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO, United States), Cyman Chemical (Ann Arbor, MI, United States), and BioVision (Milpitas, CA, United States), respectively. Phorbol 12-myristate 13-acetate (PMA) was purchased from Wako Pure Chemical (Osaka, Japan).

Cells

Two latent HIV-1 infected cell lines, ACH-2 cells derived from the A3.01 cell line, and U1 cells derived from the U937 promonocytic cell line, were used in this study. J-Lat10.6 cells derived from Jurkat T cells were also used. These cell lines were obtained from the National Institutes of Health (NIH) AIDS Reagent Program. Cells were maintained in RPMI1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 50 U/ml penicillin, and 50 μg/ml kanamycin. In experiments (**Supplementary Figure S4**), heat-inactivated (56°C, 30 min) mouse serum from BALB/c mice was used instead of FBS.

HIV-1 Latency Reversal With LRAs

The reactivation of HIV-1 from latently infected cells was determined by intracellular p24 protein expression and quantification of p24 antigen in the supernatant (ACH-2 and U1 cells), or by changes in intracellular green fluorescent protein (GFP) expression (J-Lat 10.6 cells). J-Lat (Jordan et al., 2003), ACH-2, or U1 cells (5×10^5 cells/ml) were seeded in 96-well plates and incubated with different drug concentrations for 24 h to collect the cells. The supernatant was collected after 48 h. The increase in supernatant p24 antigen levels was measured using a Lumipulse G1200 (FUJIREBIO, Tokyo, Japan). J-Lat cells (5×10^5 cells/ml) were placed in 48-well plates and incubated with different drug concentrations for 24 h. Intracellular p24 protein expression and GFP-positive cells were analyzed by flow cytometry.

Cytotoxicity Assays

To determine the cytotoxicity of LRAs, A3.01, and U937 cells (5×10^5 cells/ml), which are the parental cells for ACH-2 and U1 cells, respectively, were cultured in the presence or absence of LRAs. After 7 days, cell viability was examined using the Cell Counting Kit-8 assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The numbers of living cells after drug treatment were measured and compared to those in untreated cells and are presented as a percentage relative to the control.

Primary CD4$^+$ T Cell Isolation From HIV-1$^+$ Individuals and *ex vivo* Reactivation

Primary cells were isolated from seven HIV-1$^+$ individuals, and *ex vivo* reactivation experiments were conducted as previously

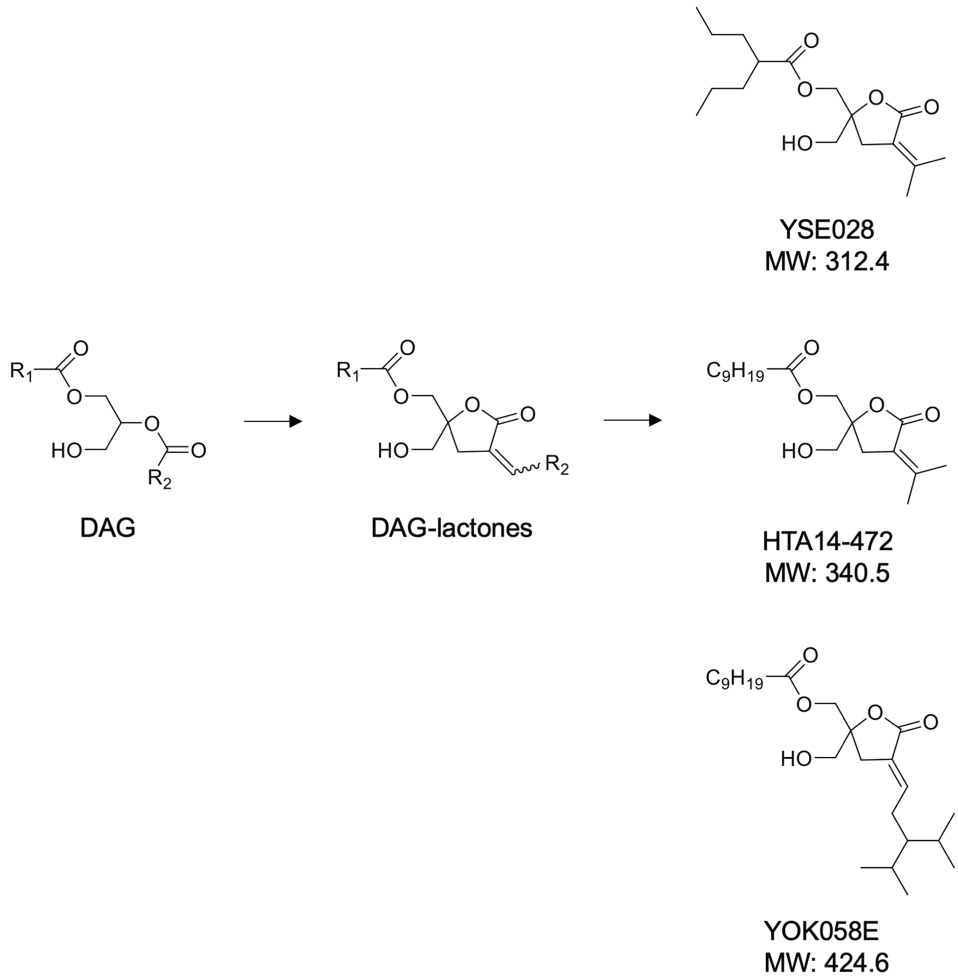

FIGURE 1 | Structures of diacylglycerol (DAG)-lactone derivatives.

described (Matsuda et al., 2019). In brief, peripheral blood samples were collected from HIV-1-infected participants receiving cART for at least 5 years (**Table 1**). All subjects maintained a low viral load (<20 copies/ml, except for occasional "blips") during therapy. $CD4^+$ T cell counts in peripheral blood samples ranged from 477 to 992 cells/mm^3 (average: 631 cells/mm^3), and plasma viral loads were <20 copies/ml (except for one participant whose viral load was 22 copies/ml) as measured by quantitative PCR (qPCR; COBAS AmpliPrep/COBAS TaqMan HIV-1 Test version 2.0; Roche Diagnostics, Basel, Switzerland) at the time of study enrollment. The Ethics Committee at the National Center for Global Health and Medicine approved this study (NCGM-G-002259-00), and each patient provided written informed consent. Whole peripheral blood mononuclear cells were separated by density gradient centrifugation using Ficoll-Paque™ (GE Healthcare, Munich, Germany), and $CD4^+$ T cells were purified using the MojoSort™ Human CD4 T Cell Isolation Kit (BioLegend, San Diego, CA, United States) according to the manufacturer's instructions. Purified $CD4^+$ T cells were plated at a density of $>2.0 \times 10^6$ cells/ml and treated with 100 nM PMA, 2 μM ionomycin, 10 μM YSE028, 1 μM JQ1, or a combination for 24 h, and the cells were collected for RNA purification. For the no-drug control, the same volume of PBS (solvent used for the drugs) was added to the wells. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Real-time qPCR (RT-qPCR) for intracellular HIV-1 RNA was then performed using the One Step PrimeScript III RT-qPCR Mix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The oligonucleotide primers used were as follows: 5'-TGTGTGCCCGTCTGTTGTGT-3' (forward), 5'-GAGTCCTGCGTCGAGAGAGC-3' (reverse), and 5'-FAM-CAGTGGCGCCCGAACAGGGA-BHQ1-3' (probe) for HIV-1 RNA detection. HIV-1 RNA copy numbers were normalized to RNA input (Jiang et al., 2015). In this method, as the reduction of cell numbers due to the toxicity of a drug results in a relatively higher HIV-RNA count, we examined and confirmed that the drug did not induce toxicity in primary cells at the tested concentrations (data not shown). The number of HIV-1 RNA

TABLE 1 | Clinical characteristics of HIV$^+$ participants of this study.

Participant	M/F	Age	VL[a] (copies/ml)	CD4 count[a] (cells/mm^3)	cART[b]	Therapy (years)	Plasma HIV RNA <20 copies/ml for (years)
Participant 1	M	47	<20	992	FTC/TAF/COBI/EVG	22	8
Participant 2	M	54	<20	753	FTC/TAF/COBI/DRV	14	8
Participant 3	M	48	22	531	FTC/TAF/DTG	17	8
Participant 4	M	55	<20	477	FTC/TAF/DTG	22	8
Participant 5	M	59	<20	587	FTC/TAF/RPV	23	8
Participant 6	M	53	<20	536	FTC/TAF/COBI/EVG	23	8
Participant 7	M	51	<20	540	FTC/TAF/COBI/EVG	15	7

[a]*VL and CD4 count: at the time of the study.*
[b]*COBI, cobicistat; DRV, darunavir; EVG, elvitegravir; DTG, dolutegravir; FTC, embricitabine; RPV, rilpivirine; TAF, tenofovir alafenamide fumarate.*

copies was calculated using a standard curve obtained from serially diluted HIV-$1_{pNL4\text{-}3}$ plasmid, and normalized values [HIV-1 RNA copies/input RNA (ng)] for each drug were compared to those without drug treatment. The relative increase in HIV-1 RNA levels in the presence of each drug or combination was then determined.

Flow Cytometry Analysis

The amount of intracellular HIV-1 p24 and the active form of caspase-3 were determined by flow cytometry, as previously described (Matsuda et al., 2015, 2019). In brief, ACH-2 and U1 cells (2.5×10^5 cells/ml) were fixed with 1% paraformaldehyde/PBS for 20 min and permeabilized with Flow Cytometry Perm Buffer (TONBO Biosciences, San Diego, CA, United States). After 5 min of incubation at room temperature, the cells were stained with FITC anti-HIV-1 p24 (24-4) monoclonal antibody (mAb; Santa Cruz Biotechnology, Dallas, TX, United States) or Alexa Fluor 647-conjugated anti-active caspase-3 (C92-605) mAb (BD Pharmingen, San Diego, CA, United States) for 30 min on ice. For T cell activation and exhaustion marker staining, PBMCs from healthy donors separated by the above density gradient centrifugation method were incubated with fixable viability stain Ghost Dye 780 (TONBO Biosciences) for 30 min on ice. The cells were then stained with Brilliant Violet 510 anti-human CD3 (UCHT1) mAb (BioLegend), FITC anti-human CD4 (RPA-T4) mAb (TONBO Biosciences), PE-Cy7 anti-human CD8a (RPA-T8) mAb (TONBO Biosciences), PerCP-Cy5.5 anti-human CD38 (HB-7) mAb (BioLegend), PE anti-human CD69 (FN50) mAb (BioLegend), or Alexa Fluor 647 anti-human CD279/PD-1 (EH12.1) mAb (BD Biosciences, San Jose, CA, United States) for 30 min on ice. Next, the cells were analyzed using BD FACSVerse (BD Biosciences). In the analysis, unstained cells were used as a negative control to set the gating for each experiment. The collected data were analyzed using FlowJo software (Tree Star, San Carlos, CA, United States).

In vivo Toxicity

BALB/c mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Female mice at 5 weeks of age were used for the experiments. The mice were housed and monitored in our animal research facility according to the institutional guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Kumamoto University. PEP005 and YSE028 were dissolved in 1% dimethyl sulfoxide (DMSO) containing RPMI 1640. PEP005 (100, 300, and 1,000 μg/kg), YSE028 (100, 300, 1,000, 3,000, and 10,000 μg/kg), or 1% DMSO containing RPMI 1640 were intraperitoneally injected into BALB/c mice. After 24 h, the survival rate was determined.

Pharmacokinetics in Mice and LC-MS/MS Analysis

Blood was drawn from mice at 0.5, 1, 3, 6, 12, and 24 h after subcutaneous administration of YSE028 at a dose of 10 mg/kg. Plasma samples were prepared on ice. Blood was collected in a heparin tube and then separated at 5,000 rpm for 10 min at 4°C. Two hundred microliter of MeOH was added to 40 μl of mouse plasma immediately after plasma collection to terminate the enzymatic hydrolysis of YSE028 during sample preparation. All samples were vortexed for 10s and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was then separated. Alectinib (10 ng/ml) in MeOH was used as an internal standard (IS) and added to the supernatant for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

A Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) connected to a QTRAP5500 mass spectrometer (AB SCIEX, Framingham, MA, United States) was used for LC-MS/MS analysis. The separation of YSE028 and alectinib (IS) was performed using an XBridge C18 column (2.1 × 50 mm, 3.5 μm, Waters). The mobile phases consisted of 10 mM ammonium formate (A) and MeOH (B). The run time was 6 min with a flow rate of 0.5 ml/min. The oven temperature was set to 40°C, and the autosampler chamber was maintained at 4°C. The gradient elution program was set as follows: 0–3.0 min, 60–80% B; 3.1 min–4.0 min, 95% B; and 4.1 min–6 min, 60% B. The sample injection volume was 5 μl. The electrospray ionization interface in positive mode was used to perform tandem mass spectrometry (MS/MS). The MRM transition for YSE028 was m/z 313.1→187.1. The MS parameters were optimized as follows: desolvation temperature, 500°C; curtain gas (CUR), 40; collision gas (CAD), 7; ionspray voltage (IS), 4500v; ion source gas (GS1), 50 psi; ion source gas (GS2),

80 psi; declustering potential (DP), 110 v; entrance potential (EP), 10v; collision energy (CE), 13v; collision cell exit potential (CXP), 20v.

Statistical Analysis

Differences between groups were analyzed for statistical significance using unpaired *t*-tests. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, United States). CompuSyn software, based on the quantitative analysis of dose-effect relationships between multiple drugs by Chou and Talalay, was utilized to determine the synergism or antagonism of drug combinations (Chou and Talalay, 1984). To confirm synergy, this software calculates combination index (CI) values, which further assist in determining the nature of the combination compared to single drug effects. A CI value <1 indicates synergistic effects.

RESULTS

Ability of DAG-Lactone Derivatives to Reverse HIV-1 Latency

The structures of the DAG-lactone derivatives employed in this study are shown in **Figure 1**. First, we determined the cytotoxicity of these compounds in A3.01 and U937 cells, which are the parental cell lines to HIV-1 latently infected ACH-2 cells and U1 cells, respectively. None of the DAG-lactone derivatives, nor another PKC agonist, prostratin, showed cytotoxicity at a maximum concentration of 100 μM (**Table 2**; **Supplementary Figures S1A,B**). We then examined HIV-1 latency reversal activity. As shown in **Figure 2**, all DAG-lactone derivatives induced an increase in intracellular $p24^+$ cell numbers (**Figure 2A**; **Supplementary Figure S2**) or viral production (**Figure 2B**) in ACH-2 cells and U1 cells. The half-maximal effective concentration (EC_{50}) values were calculated using the maximum viral production level determined in the presence of 10 nM PMA as 100% in ACH-2 and U1 cells (**Table 3**). Notably, YSE028 showed the most potent LRA activity in ACH-2 cells and U1 cells (EC_{50}: 0.87 and 1.81 μM, respectively; **Table 3**).

Previous *in vitro* studies have shown the importance of the combined use of LRAs with different classes to achieve higher levels of HIV-1 reversal activity (Laird et al., 2015). In fact, the combination of a PKC agonist (e.g., PEP005 and benzolactam derivatives) and a BET inhibitor, JQ1, exhibited synergism in HIV-1 reversal activity (Jiang et al., 2015; Matsuda et al., 2019). Thus, we examined the effect of the combination of YSE028 and JQ1 on HIV-1 reversal in J-Lat 10.6 cells. J-Lat 10.6 cells contain a full-length HIV-1 genome with non-functional *Env* due to a frameshift and GFP in the *Nef* region, which enables the detection of HIV-1 reversal as increasing GFP expression levels (Jordan et al., 2003). As shown in **Figure 2C**, treatment with YSE028 or JQ1 (100–400 nM) increased the number of GFP^+ cells to 5.8 and 12.6%, respectively. When cells were treated with the combination, the number of GFP^+ cells increased to 27.4% (**Figure 2C**). The effects of the combination were analyzed using Compusyn software, and it was found that the combination of YSE028 and JQ1 had a synergistic effect at 100, 200, and 400 nM (CI = 0.89, 0.66, and 0.30, respectively; **Figure 2D**).

TABLE 2 | Cytotoxicity of latency-reversing agents (LRAs) in A3.01 and U937 cells.

	CC_{50} (μM)	
Compound	**A3.01**	**U937**
YSE028	>100.0	>100.0
HTA14-472	>100.0	>100.0
YOK058E	>100.0	>100.0
Prostratin	>100.0	>100.0

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay on day 7. CC_{50}, the compound concentration required to reduce the viability of parental cells by 50%.

YSE028 Reverses HIV-1 Latency in Primary $CD4^+$ T Cells From HIV-1-Infected Individuals *ex vivo*

We then examined the effect of YSE028 using primary isolated $CD4^+$ T cells from seven HIV-1-infected individuals undergoing treatment with cART (**Table 1**). $CD4^+$ T cells in the peripheral blood of HIV-1-infected individuals were treated with 10 μM YSE028, 1 μM JQ1, and 100 nM PMA plus 2 μM ionomycin for 24 h, harvested, and HIV-1 mRNA levels in the cells were evaluated. As shown in **Figure 3A**, singular treatment with YSE028 enhanced HIV-1 mRNA transcription in $CD4^+$ T cells in all seven individuals (1.66-fold compared to no drug control; $p = 0.0006$), and JQ1 alone also enhanced HIV-1 mRNA in six out of seven donors (2.25-fold compared to no drug control; $p = 0.0169$). In contrast, a combination of YSE028 and JQ1 significantly enhanced the level of HIV-1 mRNA (6.69-fold compared to no drug control; $p = 0.0006$), and the effect of the combination on LRA activity was significant when compared with YSE028 or JQ1 singular treatment ($p = 0.0023$ and 0.0041, respectively; **Figures 3A,B**). It is possible that the combination of LRAs of different classes causes an unexpected increase in toxicity in primary cells. Thus, we investigated the toxicity of LRAs in combination and found that YSE028 did not cause increased toxicity in combination (**Supplementary Figure S3A**).

DAG-Lactone Derivatives Induce Apoptotic Cell Death in HIV Latently Infected Cells *via* Caspase-3 Activation

In theory, reactivated HIV-1 latently infected cells treated with an LRA are eliminated by host immune systems, such as cytotoxic T lymphocytes (CTL), which is the major mechanism for reducing HIV-1 reservoir cells in the "shock and kill" strategy. However, apoptosis and cell death by the viral cytopathic effect in reactivated cells is regarded as another important mechanism to reduce HIV-1 reservoir cells *in vivo* (Hattori et al., 2018; Kim et al., 2018). Therefore, we determined the effects of DAG-lactone derivative-induced apoptosis in ACH-2

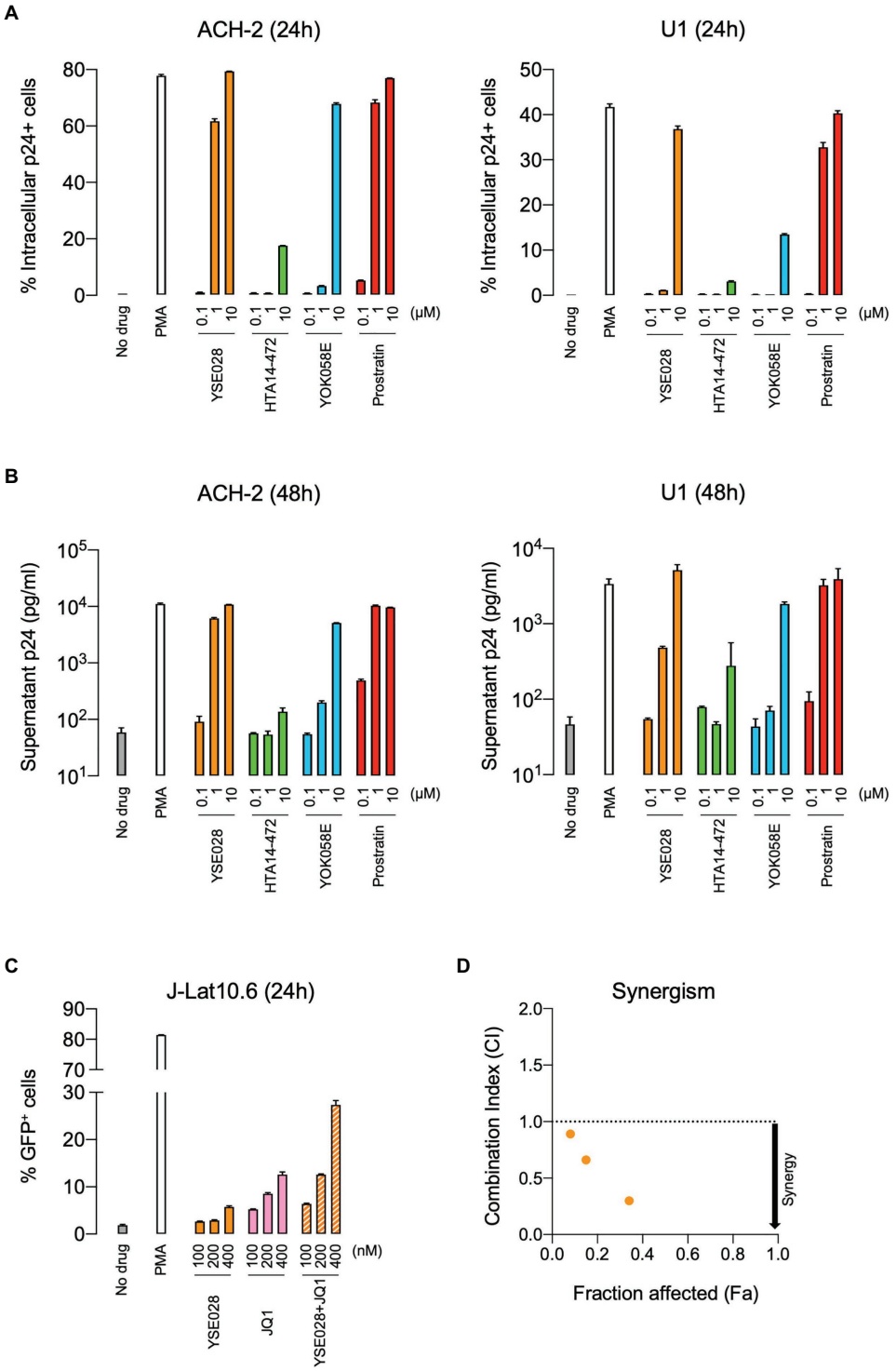

FIGURE 2 | Reversal of HIV-1 latency with DAG-lactone derivatives *in vitro*. ACH-2 and U1 cells were exposed to a DAG-lactone derivative and prostratin. The expression of intracellular HIV-1 p24 protein **(A)** and production of p24 in the supernatant **(B)** were measured after 24 and 48 h of incubation, respectively. **(C)** J-Lat 10.6 cells were exposed to different concentrations of YSE028 or JQ1 or a combination of both, and the change in the number of green fluorescent protein (GFP)-positive cells was analyzed after 24 h by flow cytometry. **(D)** Synergism in drug combinations was examined using CompuSyn software. Combination index (CI) values <1 indicate synergistic effects. Data are shown as means ± standard deviations of three independent experiments.

TABLE 3 | Latency-reversing agent activity of the tested compounds in ACH-2 and U1 cells.

	EC_{50} (μM)	
Compound	**ACH-2**	**U1**
YSE028	0.87	1.81
HTA14-472	>10.0	>10.0
YOK058E	>10.0	8.24
Prostratin	0.33	0.32

The magnitude of reactivation induced by 10 nM PMA was defined as 100% reactivation, and the concentration of each compound resulting in 50% reactivation (viral production) was used to define the EC_{50} values.

cells and U1 cells and compared them with those in their corresponding parental cells. The cells were treated with a compound (100 nM to 10 μM) for 24 h, and active caspase-3 expression levels were measured by flow cytometry. Among these derivatives, YSE028 had the greatest apoptotic effect in HIV-1 latently infected cells (17.85 and 22.7% caspase-3 activation at 10 μM in ACH-2 and U1 cells, respectively). Whereas, it showed minimal caspase-3 induction in their parental cells (5.81 and 6.35% caspase-3 activation at 10 μM in A3.01 and U937 cells, respectively; **Figure 4**). Interestingly, prostratin induced nearly the same levels of caspase-3 activation in U1 and its parental U937 cells (19.1 and 18.8% caspase-3 activation at 10 μM, respectively; **Figures 4C,D**), suggesting that the apoptosis induced in U1 cells by prostratin was not specific to HIV-1 latently infected cells. Taken together, DAG-lactone derivatives, including YSE028, appear to induce greater apoptosis in HIV-1 latently infected cells than in uninfected cells. In the experiment shown in **Figure 4**, we used higher concentrations (e.g., 10 μM) of PKC activators to elicit apoptosis. As shown in **Supplementary Figures S1A,B**, YSE028 showed only moderate toxicity in cell lines; however, it is likely that the immortalized cell lines have different toxicity profiles than human primary cells. Thus, we examined the toxicity of YSE028 at higher concentrations in primary cells and found that PKC activators, including YSE028, did not show acute toxicity up to 100 μM (**Supplementary Figure S1C**).

Because prostratin had a different profile in inducing caspase 3 activation in U937 cells, we performed experiments to investigate the difference in the activation profile of PKC activators. **Figure 5A** shows the changes in the ratio of $CD69^+$ cells in primary cells, which is one of the markers for global T cell activation. While YSE028 had less effect at 1 μM compared to other PKC activators (prostratin and PEP005), all tested compounds at 10 μM or more completely activated $CD4^+$ and $CD8^+$ T cells. The combination of YSE028 and JQ1 also elevated the expression of CD69 in primary T cells (**Supplementary Figure S3B**).

In HIV-1 infected individuals, the increase in $CD8^+$ $PD1^+$ $CD38^+$ T cells is thought to be associated with immune exhaustion and disease progression (Resino et al., 2004; Trautmann et al., 2006). Recently, it has been reported that the administration of certain LRAs (e.g., HDAC inhibitor) impairs CTL-mediated IFN-γ production, which results in dysfunctional immunological responses for clearance of HIV-1 reservoir cells (Jones et al., 2014). In this study, we investigated the effect of PKC activators on $CD8^+$ $PD1^+$ $CD38^+$ T cells, which are indicators of immune exhaustion, and found that a significant increase in CD8, PD1, and CD38 was observed in cells treated with prostratin and PEP005, while the increase with YSE028 was only moderate (**Figure 5B**). The mechanism underlying this difference is still unknown, but the results suggest that the profiles of T cell activation differ depending on the PKC activator.

In vivo Cytotoxicity of YSE028

Finally, we tested the *in vivo* safety of the administration of a DAG-lactone derivative, YSE028, which showed potent LRA activity. PEP005, a PKC activator, was tested as a control. In the acute toxicity tests (for 24 h) with exposure to a single drug in BALB/c mice, groups of five animals were challenged with increasing concentrations of a compound by intraperitoneal injection (**Figures 6A,B**). PEP005 started to show acute toxicity in mice at a concentration of 300 μg/kg, and all tested mice died at 1,000 μg/kg. On the other hand, none of the animals injected with YSE028 (100 μg/kg–10 mg/kg) displayed any abnormalities in their condition as determined by their weight and fur texture (data not shown), and all of them survived (**Figures 6A,B**).

Because YSE028 showed no toxicity in mice at extremely high concentrations, we investigated the pharmacokinetics of YSE028 in mice. YSE028 (10 mg/kg) was administered subcutaneously to each mouse, blood was drawn at each data point, and their concentrations were measured. As shown in **Figure 6C**, the peak concentration was 5.14 ng/ml (median) at 1 h, and the concentration of YSE028 in the blood decreased rapidly (within 3 h; **Figure 6C**). We speculate that YSE028 disappeared from mouse blood because it is metabolized by esterases such as mouse carboxylesterase 1 (CES1). It is known that large amounts of CES1 are present in mouse or rat blood, but not in human blood (Hosokawa, 2008; Di, 2019). Thus, we examined the LRA activities of YSE028 and prostratin in the presence of 10% FBS or 10% mouse serum. The reactivation level of prostratin did not change in either condition, whereas the activity of YSE028 drastically decreased in the presence of mouse serum (**Supplementary Figure S4**). Thus, it is possible that the kinetics of DAG-lactone derivatives in humans may be different from those in mice.

To examine whether LRAs can show activity with such short exposure to HIV-1 latent cells, we conducted an *in vitro* experiment. J-Lat cells were exposed to YSE028 for 0.5 or 1 h, rinsed, and incubated for 24 h, and then the reactivation in cells was determined (**Figure 6D**). We found that exposure to YSE028 for 1 h successfully reactivated HIV-1-latently infected J-Lat cells (**Figure 6E**). Taken together, these results indicate that short-term exposure to LRAs may be a good strategy for reactivating HIV-1-latently infected cells with less toxicity *in vivo*.

DISCUSSION

In this study, we demonstrated the potential for HIV-1 reversal by DAG-lactone derivatives as LRA candidates. Previous studies

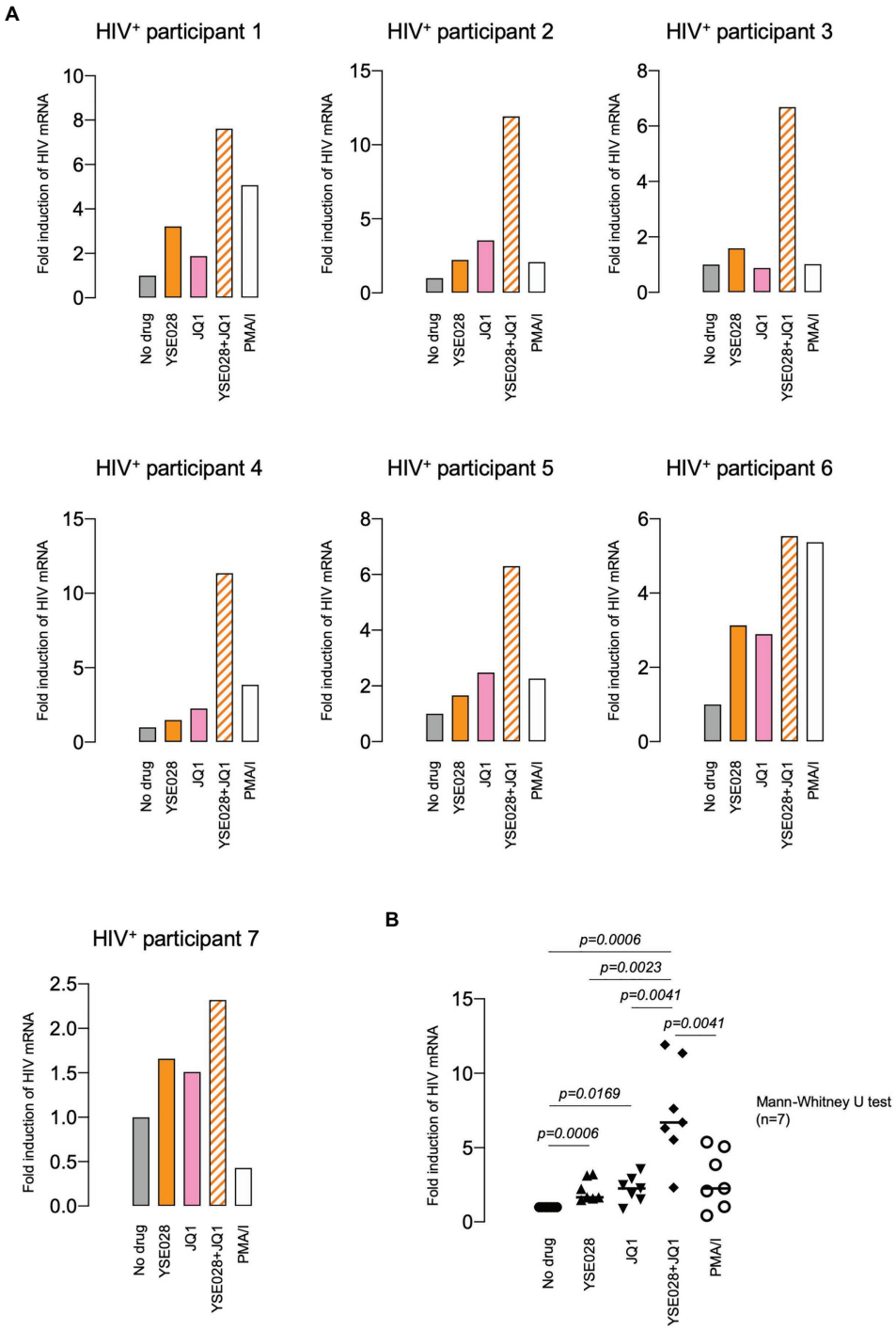

FIGURE 3 | YSE028 reactivates HIV-1 in CD4⁺ T cells from HIV-1-infected individuals. **(A)** Human CD4⁺ T cells purified from seven HIV-1-infected individuals undergoing cART (**Table 1**) were treated with 10 μM YSE028, 1 μM JQ1, a combination of YSE028 and JQ1, or 100 nM PMA plus 2 μM ionomycin for 24 h. Intracellular HIV-1 mRNA levels were detected by quantitative real-time PCR (qRT-PCR) and compared to those in untreated controls. **(B)** Statistical significance was determined using a Mann-Whitney U test, where a value of $p < 0.05$ was considered to be significant.

have reported many candidate small-molecule compounds, including HDAC inhibitors, BET inhibitors, and PKC agonists (Contreras et al., 2009; Boehm et al., 2013; Jiang et al., 2015). Among them, PKC agonists have the most potent activity, but they also exert unexpected side effects. In particular, PKC activation has been reported to lead to global T-cell

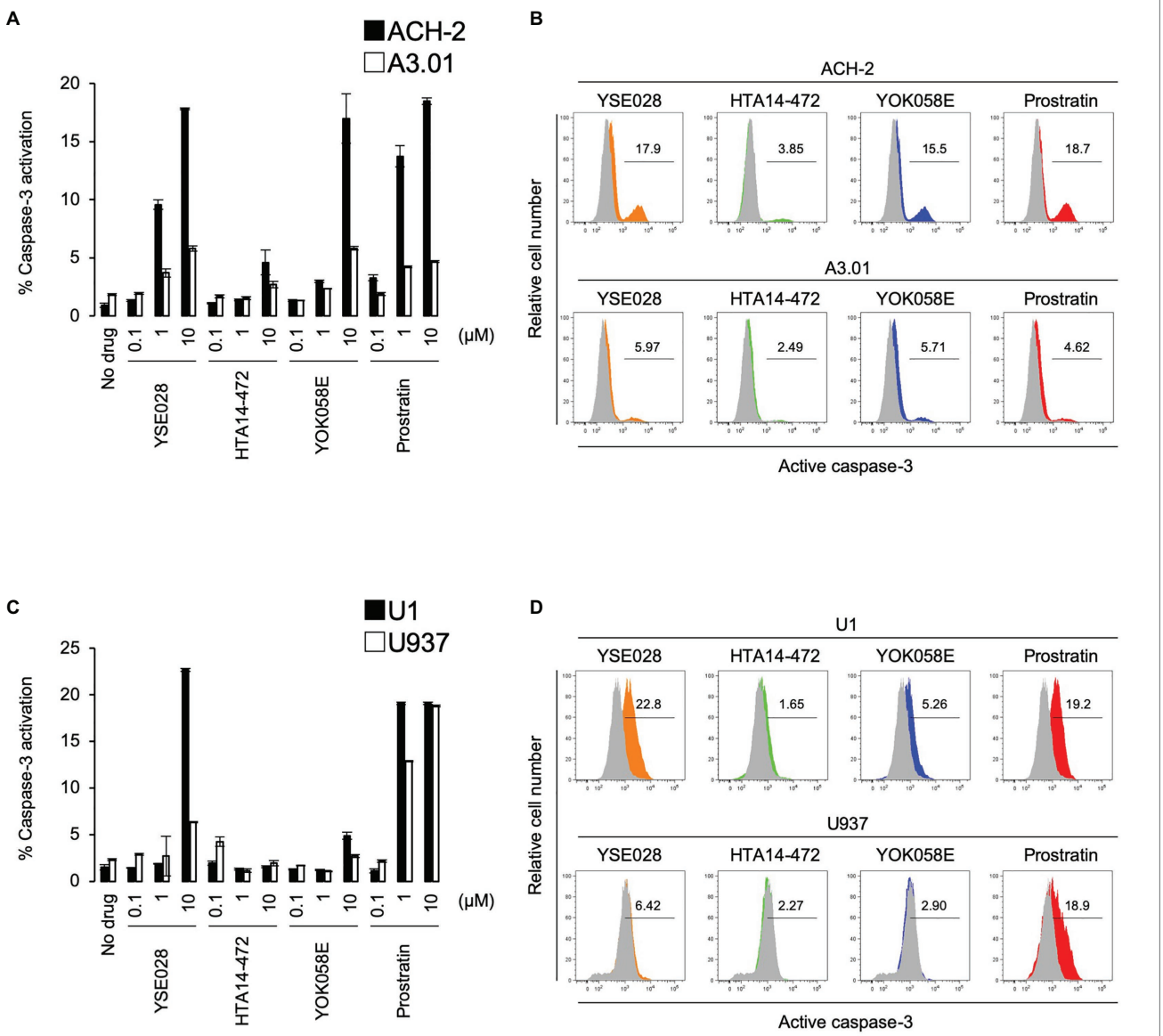

FIGURE 4 | Diacylglycerol-lactone derivatives specifically induce caspase-3 activation in HIV-1 latently infected cells. The active form of caspase-3 was measured by flow cytometry. **(A)** ACH-2 and A3.01 cells were exposed to different concentrations of DAG-lactone derivatives and prostratin for 24 h. **(B)** The histogram shows representative data for caspase-3 activation with exposure to 10 μM reagent in ACH-2 and A3.01 cells. **(C)** U1 and U937 cells were exposed to different concentrations of DAG-lactone derivatives and prostratin for 24 h. **(D)** The histogram shows representative data for caspase-3 activation with exposure to 10 μM reagent in U1 and U937 cells. Data are shown as means ± SDs of three independent experiments.

activation and toxicity. The classic PKC agonist phorbol ester PMA displays tumor-promoting activity that can be of crucial significance to immunodeficient patients. DAG-lactone derivatives mimic the endogenous second messenger DAG of PKC isoforms that play a role in physiological cellular responses (Nishizuka, 1992; Watanabe et al., 1992; Mischak et al., 1993; Li et al., 2003; Martin-Diaz et al., 2007). Therefore, we have a high expectation for the efficacy and safety of DAG-lactone derivatives as novel LRA candidates. As shown in **Figures 2, 3**, all tested derivatives reversed HIV-1 latency in HIV-1 latently infected cell lines and primary $CD4^+$ T cells from HIV-1-infected individuals. Recent studies have highlighted the importance of combining different LRAs from multiple classes (Laird et al., 2015). We also examined the efficacy of DAG-lactone derivatives in combination with a BET inhibitor, JQ1, which reportedly shows synergistic LRA activity with some PKC agonists (Matsuda et al., 2019). Notably, the combination of YSE028 and JQ1 synergistically reactivated HIV-1 latency and exerted potent LRA activity in primary cells from HIV-1^+ individuals.

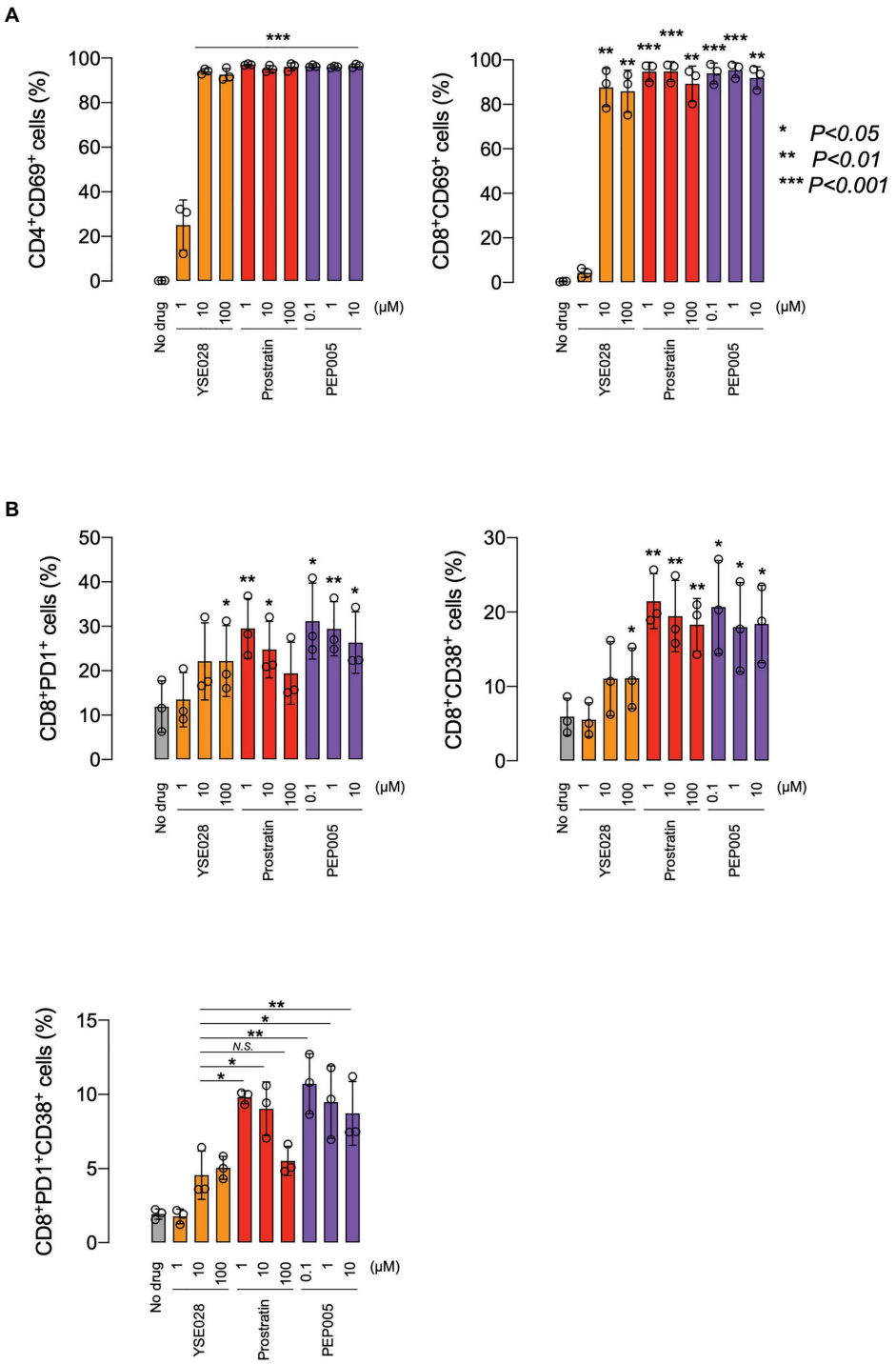

FIGURE 5 | The effect of YSE028 on T cell activation. PBMCs from three healthy donors were exposed to different concentrations of a reagent for 24 h. Changes in CD69 expression on $CD4^+$ or $CD8^+$ primary T cells **(A)**, $CD8^+PD1^+$ primary T cells, $CD8^+CD38^+$ primary T cells, and $CD8^+PD1^+CD38^+$ primary T cells **(B)** were analyzed by flow cytometry. Data are shown as means ± SDs. Statistical significance was determined using a paired T-test, where a value of $p < 0.05$ was considered to be significant.

We observed an increase in HIV-1 mRNA levels in response to LRAs or PMA (**Figure 3**). In this regard, we noticed that some patient cells obtained for the assay did not respond to PMA (data not shown). Possible reasons for this are: (1) we used a relatively smaller number of cells per well compared with previous studies by others and (2) there were a very small

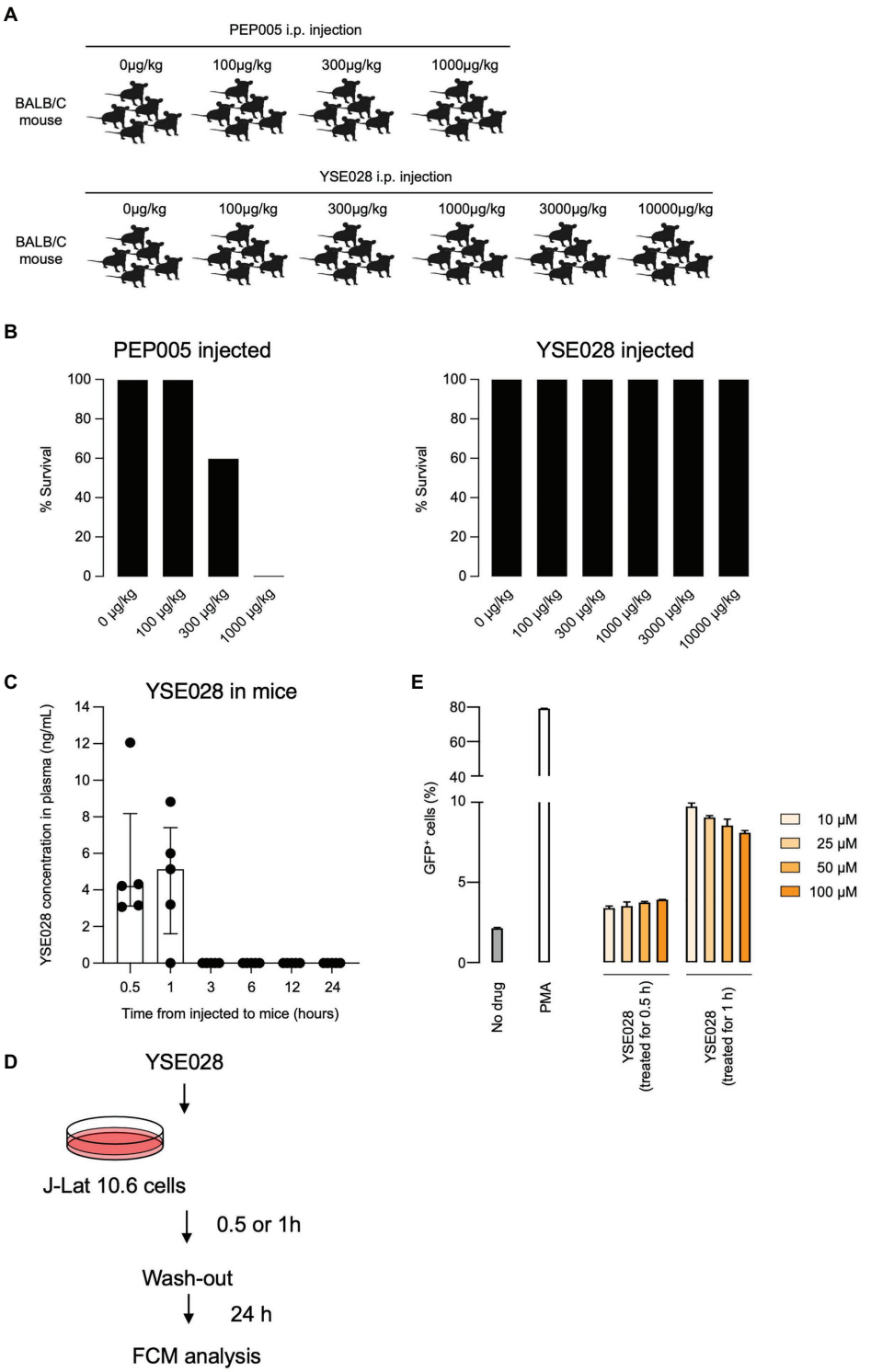

FIGURE 6 | *In vivo* toxicity and pharmacokinetic analyses of YSE028. **(A)** The experimental scheme is illustrated. BALB/c mice were challenged with increasing concentrations of PEP005 or YSE028 by intraperitoneal injection, with five animals in each group. **(B)** The survival rate of PEP005- and YSE028-injected mice. **(C)** Plasma concentration of YSE028 in BALB/c mice. The concentrations were measured by LC-MS/MS at 0.5, 1, 3, 6, 12, and 24 h after administration of YSE028 at a dose of 10 mg/kg. Data are shown as medians with interquartile ranges, $n = 5$. **(D)** The experimental scheme for short-term exposure to YSE028. **(E)** J-Lat 10.6 cells were exposed to YSE028 for 0.5 or 1 h, and then reagent was washed-out. The number of GFP-positive cells was analyzed after 24 h by flow cytometry. Data are shown as means ± SDs of three independent experiments.

number of reservoir cells in patient peripheral blood (all patients were in good condition for a long period of time with cART). In most cases, such primary cells respond not only to PMA, but also to other LRAs. However, in some cases (such as participant 7 in **Figure 3**), we observed an elevation of HIV-1 mRNA with LRAs, even though the cells did not respond to PMA. The mechanism is unknown, but it is thought that if we use a greater number of cells in the assay, they would react to PMA also. We confirmed that exposure to PMA (100 nM with 2 μM ionomycin) for 24 h did not induce cytotoxicity in primary cells (data not shown); however, it is possible that the agents affected cellular homeostasis, resulting in a different response to PMA/ionomycin in patient-derived infected cells.

As shown in **Figure 6B**, YSE028 had no toxicity in mice; however, we found that the concentration of the drug in mice did not reach high levels and disappeared rapidly (**Figure 6C**). In general, the cause(s) of the rapid decline of the compound concentration in the plasma may be due to bioavailability, plasma protein binding, or the specific metabolism of mice. In the present study, we showed that the addition of mouse serum reduced the activity of YSE028 (**Supplementary Figure S4**), indicating the presence of an enzyme(s) that metabolizes the compound. YSE028 has an ester moiety and is thought to be a substrate for CES1, thus it is rapidly metabolized in mouse blood. The distribution pattern of CES1 in organs varies depending on the animal species (Di, 2019). It is thought that the enzyme is more abundant in mouse plasma than human plasma. Thus, it is possible that the kinetics of DAG-lactone derivatives in human blood may be different than they are in mice. The use of other animal models, such as monkeys, that show relatively similar CES1 distribution patterns to humans (Di, 2019), may be suitable for evaluating the PK profiles of this class of molecules. In addition, a plasma esterase-deficient mouse model recently reported could be useful to assess drug PK without the effect of esterase in mouse plasma (Morton et al., 2005). It should be noted that the PK profile of YSE028 presented in this study is limited to the plasma, and it is also important to evaluate drug concentrations in tissues where HIV-1-infected cells exist and replicate.

Protein kinase C consists of several isozymes and is divided into three subfamilies: cPKC, nPKC, and aPKC. Among them, only cPKC and nPKC isozymes require DAG for their activation. Nomura et al. (2011) previously reported that YSE028 works through PKCδ and induces its translocation from the cytoplasm to cell organelles. The potent PKC agonists PEP005, prostratin, and bryostatin-1 also modulate the PKC pathway, but their activation profiles are different (PEP005, PKCδ; prostratin, PKC α; and θ bryostatin-1, and PKC α and δ; Hampson et al., 2005; Trushin et al., 2005; Mehla et al., 2010). Taken together, it is possible that the activation of nPKC isozymes (δ, ε, η, and θ) may contribute to potent and HIV-1-specific activation, which is considered crucial for future LRA candidates. However, in the present study, at higher concentrations, YSE028 induced global T cell activation (**Figure 5A**) *in vitro*. Thus, further evaluation and modification of compounds may be needed to obtain HIV-1-specific LRAs.

We previously reported the mechanism of PKC-induced apoptosis in HIV-1 latently infected cells (Hattori et al., 2018; Matsuda et al., 2019). Activation of PKC induces tumor necrosis factor receptor-mediated nuclear factor-κB activation, which induces viral transcription. In addition, the tumor necrosis factor receptor simultaneously induces activation of the caspase signaling pathway. Thus, tumor necrosis factor receptor-mediated nuclear factor-κB activation not only increases the production of viral proteins in cells but also triggers apoptosis induced by HIV-1-related proteins inside the cells. As shown in **Table 2**; **Supplementary Figure S1**, no cytotoxicity was observed in the tested DAG-lactone derivatives in HIV-1 uninfected cell lines. This reduced toxicity profile of DAG-lactone derivatives was consistent with the minimal caspase-3 induction observed in these cells (**Figure 4**). Given the fact that DAG-lactones strongly activate caspase-3 in HIV-1 latently infected cells, it is likely that DAG-lactone derivatives kill and eliminate HIV-1 latent reservoir cells more specifically than they do HIV-1 uninfected cells.

In the current well-controlled cART era, neuronal disorders are becoming more frequent in aging HIV-infected individuals because of their long lifespans (Thakur et al., 2019). Notably, HIV-1-associated neurocognitive disorders, which also occur in young people in their 20s and 30s, are a serious problem for HIV-1 infected individuals. HIV-1 invades the central nervous system through the migration of infected monocytes and is subsequently transmitted to various types of cells expressing CD4, including microglia, astrocytes, and perivascular macrophages (Cosenza et al., 2002; Rothenaigner et al., 2007). HIV-1-infected cell populations are established and exist as viral reservoirs (or so-called sanctuaries) in the brain (Hellmuth et al., 2015). The infected microglia and astrocytes release neurotoxicity factors, such as cytokines and chemokines, which disrupt the blood-brain barrier (Gonzalez-Scarano and Martin-Garcia, 2005). HIV-1 proteins, such as gp120, Tat, Vpr, and Nef, also induce inflammation and neuronal apoptosis and trigger neurodegenerative disorders (Canet et al., 2018). Aging is a primary risk factor for neurodegenerative disorders, such as Alzheimer's disease, which is believed to be caused by similar cellular pathways as HIV-1-associated neurocognitive disorders. Cho et al. (2017) reported that expression of viral proteins, such as gp120, Nef, and Tat, significantly increased the number and size of amyloid plaques in the cerebral cortex of an HIV-1 transgenic rat model. As a novel therapeutic approach for Alzheimer's disease, PKC activation with PKC agonists (such as benzolactam or bryostatin-1), is thought to be effective in reducing amyloid beta formation *via* the promotion of α-secretase production, which leads to cleavage of the amyloid precursor protein (Etcheberrigaray et al., 2004). Although we did not evaluate the regulation of amyloid beta by PKC agonists in this study, activation by DAG-lactone derivatives (which utilize similar PKC isozymes as bryostatin-1) may also contribute to it. In fact, Lee et al. (2006) reported that DAG-lactone potently

induced α-secretase activation, which resulted in the reduction of amyloid beta peptide deposition. Further investigations are needed to clarify the effect of PKC agonists, including DAG-lactones, on HIV-1-associated neurocognitive disorders and other similar conditions.

In summary, DAG-lactone derivatives, including YSE028, are potential candidates for novel therapeutics to combat HIV-1 reservoirs in HIV-1-infected individuals. However, even though these compounds have been confirmed to be safe in the animal models, more detailed *in vitro* and *in vivo* analyses are necessary to accumulate information regarding the efficacy of these drugs on a wide variety of HIV-1 reservoir cells that hide and exist in many organs *in vivo*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee at the National Center for Global Health and Medicine. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Kumamoto University.

AUTHOR CONTRIBUTIONS

KeM and KoM designed and performed the experiments and wrote the manuscript. TK and HT synthesized the compounds. RK, SR, and SO performed the experiments. KiT, KoT, TI, HG, KY, AH, HM, and HT provided suggestions for the experimental design. All authors contributed to the article and approved the submitted 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/fmicb.2021.636276/full#supplementary-material

Supplementary Figure S1 | Cell viabilities of A3.01 **(A)** and U937 **(B)** cells exposed to different concentrations of diacylglycerol (DAG)-lactone derivatives. Cell viabilities were determined using the 3-(4,5-dimethylthiazol-2-yl)-s,5-diphenyltetrazolium assay on day 7. **(C)** Acute cytotoxicity of protein kinase C (PKC) activators including YSE028 in primary cells. PBMCs from three healthy donors were exposed to different concentrations of a reagent for 24 h. The ratio of living cells was calculated for fixable viability dye negative cell populations by flow cytometry. Data are shown as means ± SDs of three independent experiments.

Supplementary Figure S2 | Expression of intracellular HIV-1 p24 proteins in ACH-2 **(A)** and U1 **(B)** cells. The cells were exposed to different concentrations of DAG-lactone derivatives, and intracellular p24 levels were examined by flow cytometry analysis after 24 h.

Supplementary Figure S3 | **(A)** Toxicity of YSE028 in primary cells. **(B)** Changes in CD69 expression on $CD4^+$ or $CD8^+$ primary T cells after exposure to combined latency-reversing agents (LRAs) for 24 h were analyzed by flow cytometry. Data are shown as means ± SDs.

Supplementary Figure S4 | J-Lat 10.6 cells were exposed to YSE028 or prostratin for 24 h in the presence of 10% fetal bovine serum **(A)** or 10% mouse serum **(B)**. The number of green fluorescent protein (GFP)-positive cells was analyzed after 24 h by flow cytometry. Data are shown as means ± SDs of three independent experiments.

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frontiers in Microbiology

REVIEW
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Towards a Functional Cure of HIV-1: Insight Into the Chromatin Landscape of the Provirus

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Despite potent combination antiretroviral therapy, HIV-1 infection persists due to irreversible integration of the virus in long-living cells of the immune system. The main focus of HIV-1 cure strategies has been on HIV-1 eradication, yet without great success so far. Therefore, HIV-1 remission or a functional cure, whereby the virus is silenced rather than eradicated, is considered as an alternative strategy. Elite controllers, individuals who spontaneously control HIV-1, may point us the way toward a functional HIV-1 cure. In order to achieve such a cure, a profound understanding of the mechanisms controlling HIV-1 expression and silencing is needed. In recent years, evidence has grown that the site of integration as well as the chromatin landscape surrounding the integration site affects the transcriptional state of the provirus. Still, at present, the impact of integration site selection on the establishment and maintenance of the HIV-1 reservoirs remains poorly understood. The discovery of LEDGF/p75 as a binding partner of HIV-1 integrase has led to a better understanding of integration site selection. LEDGF/p75 is one of the important determinants of integration site selection and targets integration toward active genes. In this review, we will provide an overview of the most important determinants of integration site selection. Secondly, we will discuss the chromatin landscape at the integration site and its implications on HIV-1 gene expression and silencing. Finally, we will discuss how interventions that affect integration site selection or modifications of the chromatin could yield a functional cure of HIV-1 infection.

Keywords: HIV-1, latency, integration site selection, chromatin landscape, block-and-lock, LEDGF/p75, HIV-1 reservoir

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INTRODUCTION

Integration of the HIV-1 DNA into the host genome is central in the replication and pathogenesis of HIV-1 infection. Although the majority of cells productively infected with HIV-1 are rapidly eliminated by the host's immune system, some infected cells contain provirus that enters a silent mode. In the absence of viral protein expression, those cells are not recognized by the immune system and thus persist indefinitely (Finzi et al., 1999). Although combination antiretroviral therapy (cART) reduces plasma viremia below detectable levels, the virus inevitably rebounds after cART is discontinued (Chun et al., 1997). As such, the persistence of the latent proviruses in long-living cellular reservoirs remains one of the major hurdles to cure HIV-1 infection (Deeks et al., 2016).

The high complexity of the latent reservoirs fuels ongoing discussions on the mechanisms that contribute to HIV-1 persistence. Latent HIV-1 provirus resides in many cellular and anatomical reservoirs, adding to the unknowns of HIV-1 persistence (Barton et al., 2016). Long-living memory CD4+ T cells are considered as the primary cell types constituting the latent reservoirs (Barton et al., 2016). In addition to memory CD4+ T cells, monocyte-derived macrophages are potential important long-lived HIV-1 reservoirs (Kruize and Kootstra, 2019; Wong et al., 2019). Studies in animal models have shown that macrophages sustain infection and are a source of viral rebound after cART interruption (Honeycutt et al., 2017; Abreu et al., 2019). Replication-competent proviral DNA has been isolated from urethral macrophages (Ganor et al., 2019). Still, macrophages are widely distributed in many different tissues and organs, making them difficult to isolate from patients. Therefore, it remains unknown to which extent they contribute to long-lived HIV-1 reservoirs in patients on treatment.

Persistent viremia in cART-treated individuals can originate from antigen-mediated reactivation of memory cells, initiating new rounds of infection. Although several reports indicate that the latent reservoir is maintained by ongoing viral replication owing to limited drug penetration in some anatomical sites (Fletcher et al., 2014; Lorenzo-Redondo et al., 2016), the absence of viral sequence evolution in some patients questions this hypothesis (van Zyl et al., 2018). Furthermore, attempts to reduce the size of the reservoir by treatment intensification have failed, minimizing the contribution of ongoing replication to the total HIV-1 reservoir (Lafeuillade, 2012). Other than having a long half-life, memory CD4+ T cells are extended through homeostatic proliferation (Chomont et al., 2009), and they have the capacity to undergo clonal proliferation (Murray et al., 2016; Lee et al., 2017). What is not yet clear is the total contribution of the clonally expanded population to the total reservoir. If clonal expansion is a determining factor maintaining the pool of latently infected cells, understanding its underlying mechanisms will be of paramount importance to identify new approaches that aim to purge the latent reservoir.

The functionality of latent reservoirs is in large part determined by the intactness of the proviral sequences. Therefore, efforts were made to develop more sensitive and accurate assays to obtain full sequencing of the HIV-1 genome in patient samples (Lambrechts et al., 2020). Remarkably, only 10% of the proviral sequences exhibit an intact genome and are thus theoretically able to produce replication-competent virus. Still, the frequency of cells harboring intact viral genomes is notably larger than the actual number of cells that can be stimulated to produce replication-competent viral particles (Bruner et al., 2016). What remains unclear is how to distinguish an intact-replication-competent provirus from an intact, but non-inducible provirus. In the context of a functional HIV-1 cure, this remains an extremely important question to answer.

Eradicating the latent reservoirs is the main focus of the most prominent HIV-1 cure strategies (Deeks et al., 2016; Darcis et al., 2017). The first reported case of a complete HIV-1 eradication was Timothy Ray Brown or the so-called Berlin patient (Hutter et al., 2009), who got infected with HIV-1 and was diagnosed with acute myeloid leukemia. The Berlin patient received a complete immune system replacement through an allogenic Hematopoietic Stem Cell Transplantation (HSCT) from a donor carrying a homozygous deletion in the CCR5 co-receptor gene (CCR5Δ32). This deletion renders CD4+ T cells resistant to most HIV-1 strains. However, HSCT is associated with high mortality rates and therefore not widely applicable to cure all HIV-1 patients. In addition, CCR5Δ32 donors are scarce. Alternatively, autologous hematopoietic stem cells have been engineered with gene-editing technologies (e.g., Zinc-finger or CRISPR-Cas) to delete the CCR5 gene (Tebas et al., 2014). Others aim to directly disrupt the provirus using a CRISPR-Cas approach (Liao et al., 2015). Unfortunately, delivery of gene-editing constructs to all reservoir cells *in vivo* remains a formidable hurdle and gene-editing strategies may suffer from unknown off-target risks (Liao et al., 2015; Wang et al., 2016). Additionally, the "shock and kill" HIV-1 cure strategy aims to eradicate the entire latent reservoir by reversing its latent state (Darcis et al., 2017; Abner and Jordan, 2019). Latency-reversing agents (LRAs) are given to deliberately reactivate proviral transcription in latently infected cells. The hypothesis behind this approach is that reactivated cells will express viral proteins, allowing them to be recognized and destroyed by the host immune system or die due to viral cytopathic effects. On the contrary, long-term HIV-1 remission or a functional cure constitutes an alternative strategy that has recently gained more interest (Darcis et al., 2017; Kessing et al., 2017; Debyser et al., 2019; Moranguinho and Valente, 2020; Vansant et al., 2020a). A functional cure could be achieved by durably silencing the latent provirus in infected cells and thereby preventing viral rebound by creating a cellular reservoir resistant to reactivation.

To achieve such a functional cure, however, it will be necessary to broaden our understanding on the molecular mechanisms contributing to HIV-1 silencing. How the chromatin environment affects proviral gene expression is only partially understood. Many studies have shown that epigenetic silencing can contribute to the establishment of HIV-1 latency (Pearson et al., 2008; Tyagi et al., 2010; Gallastegui et al., 2011; Matsuda et al., 2015; Nguyen et al., 2017). Poorly investigated is the impact of HIV-1 integration in the host chromatin and in particular the impact of integration site selection on HIV-1 gene expression. Although no consensus exists about its relative importance, integration defines the 3D nuclear localization, the chromatin landscape, and the gene in which the provirus ends up. HIV-1 integration is not random, but instead controlled by a complex interplay between viral components and the human genome. Integration is biased toward gene-dense regions and active transcription units (Jordan et al., 2001; Schröder et al., 2002; Maxfield et al., 2005; Barr et al., 2006; Albanese et al., 2008; Dieudonné et al., 2009; Marini et al., 2015; Kok et al., 2016) whereby methylated lysine 36 residues in histone 3 (H3K36me2 and H3K36me3) are typical markers associated with actively transcribing genes (Bannister et al., 2005). The chromatin reader LEDGF/p75 is responsible for interaction of the virus with these epigenetic markers (Eidahl et al., 2013).

In this review, we will first give an overview of the most important factors that determine integration site selection. Next,

we will discuss how the chromatin landscape at the integration site affects the establishment of HIV-1 latency. Furthermore, we will discuss how an increased understanding of integration site selection could open new perspectives on the road toward a functional cure for HIV-1 infection.

DETERMINANTS OF INTEGRATION SITE SELECTION

HIV-1 integration is not random. It was shown several years ago that HIV-1 preferentially integrates into active genes (Schröder et al., 2002). The virus has evolved to hijack cellular pathways to target its pre-integration complex (PIC) to active transcription units. Prior to integration of the viral DNA, passage through the nuclear pore is critical to complete the early steps of HIV-1 infection (**Figure 1**). Correct trafficking through the nuclear pore facilitates subsequent integration into the nuclear periphery in active chromatin (Albanese et al., 2008). Interestingly, genes frequently targeted for HIV-1 integration are closely associated with nuclear pore complexes (NPCs; Demeulemeester et al., 2015). These observations provide evidence that the HIV-1 nuclear import pathway is directly coupled to preferential integration site selection. Furthermore, several NPC-associated proteins influence the nuclear localization of integration. Depletion of Nup98, Nup153, Transportin-SR2 (TRN-SR2, TNPO3), or RanBP2 has been shown to hamper integration in gene-dense regions (Ocwieja et al., 2011; Di Nunzio et al., 2013; Lelek et al., 2015). In conclusion, alternative import pathways but also altered chromatin organization after depletion of certain NPC components may lead to reduced integration gene-dense regions (Lee et al., 2010; Liang and Hetzer, 2011; Di Nunzio et al., 2012; Koh et al., 2013).

Secondly, HIV-1 uses cellular cofactors for retroviral integration site selection (**Figure 1**; Debyser et al., 2015). HIV-1 integration is catalyzed by the viral integrase (IN) that employs the cellular chromatin reader lens-epithelium-derived growth factor (LEDGF/p75) to target the virus to active transcription units (Cherepanov et al., 2003; Ciuffi et al., 2005; Llano et al., 2006). The C-terminal region of LEDGF/p75 harbors a protein binding domain responsible for interaction with IN (Cherepanov et al., 2005), while the chromatin reading function is executed by the N-terminal PWWP domain (Eidahl et al., 2013). LEDGF/p75 is known to recognize the H3K36me3 mark on nucleosomes (Pradeepa et al., 2012; Eidahl et al., 2013). Upon knockdown of LEDGF/p75, its paralog HRP-2 can substitute for its function as it is the only other identified human protein that possesses both an IN binding domain and a PWWP domain (Schrijvers et al., 2012a,b). Depletion of chromatin tethering cofactors LEDGF/p75 and HRP-2 shifted integration out of active genes, though the integration pattern was not yet completely random, indicating that other host factors are involved (Schrijvers et al., 2012b). Of note, our lab and others have shown that the 3D nuclear localization of HIV-1 integration is affected by LEDGF/p75 (Marini et al., 2015; Vranckx et al., 2016). Following nuclear translocation of the PIC, HIV-1 integrates preferentially in the nuclear periphery in close association to the nuclear pore (Albanese et al., 2008; Dieudonné et al., 2009; Di Primio et al., 2013; Marini et al., 2015). In 2016, it was reported that depletion of LEDGF/p75 or direct interference of small molecule inhibitors (LEDGINs) with the IN-LEDGF/p75 interaction shifts integration toward the inner nucleus compared to wild-type integration (Vranckx et al., 2016).

Additionally, cleavage and polyadenylation specificity factor 6 (CPSF6) has been put forward as another HIV-1 cellular cofactor important for integration site selection. CPSF6 is known to promote HIV-1 nuclear entry through interaction with the viral capsid (CA; Lee et al., 2010; Price et al., 2012; Matreyek et al., 2013; Bhattacharya et al., 2014). Furthermore, depletion and knockout of CPSF6 decreased integration in active genes (Chin et al., 2015; Rasheedi et al., 2016; Sowd et al., 2016; Achuthan et al., 2018). In agreement, introduction of an HIV-1 CA mutation at the CPSF6 binding site (N74D) resulted in more integration in gene-poor regions (Schaller et al., 2011). It was shown that the CA-CPSF6 interaction is necessary to allow integration in transcriptionally active euchromatic regions of the inner nucleus. Against the more prevailing view (Albanese et al., 2008; Dieudonné et al., 2009; Di Primio et al., 2013; Marini et al., 2015), the study of Achuthan et al. (2018) suggested that HIV-1 does not preferentially integrate in the nuclear periphery. Furthermore, they propose that CPSF6, and not LEDGF/p75, is the major regulator of HIV-1 integration site selection. Moreover, the overall contribution of CPSF6 to HIV-1 replication is still under debate since the depletion of endogenous CPSF6 has no major impact on HIV-1 replication in cell lines (Lee et al., 2010; Fricke et al., 2013), in stark contrast to LEDGF/p75 (Vandekerckhove et al., 2006). Nevertheless, it would be of interest to verify whether depletion of CPSF6 also affects immediate latency and reactivation of the provirus, as discussed in the next section.

Adding to the complexity, the dynamic nuclear architecture contributes to the selection of genomic sequences in which HIV-1 integrates (**Figure 1**). HIV-1 is known to bypass the heterochromatin condensed regions in lamin-associated domains (LADs) and also disfavors centrally located transcriptionally active regions (Lusic and Siliciano, 2016). Earlier work has demonstrated that some genes are more frequently targeted for HIV-1 integration and therefore termed HIV-1 recurrent integration genes (RIGs; Marini et al., 2015). In fact, it was observed that RIGs were prevalently localized at the nuclear periphery (Marini et al., 2015). The nuclear periphery contains open chromatin regions that are associated with NPCs. As mentioned, nuclear trafficking through these complexes affects the integration pattern. Recently, Lucic et al. (2019) reported that certain nuclear sub-compartments were more frequently targeted for HIV-1 integration in activated T lymphocytes. This preferential organization of RIGs was linked to the proximity to super-enhancers (SE). Remarkably, the occupancy of similar 3D compartments by HIV-1 and SE explained the observed phenomenon. Although this strengthens the hypothesis that the 3D nuclear architecture of activated T lymphocytes is a pivotal determinant of the HIV-1 integration, no direct evidence for a mechanism whereby integration is targeted to these sites was given. Furthermore, RIGs only represent a fraction (<30%)

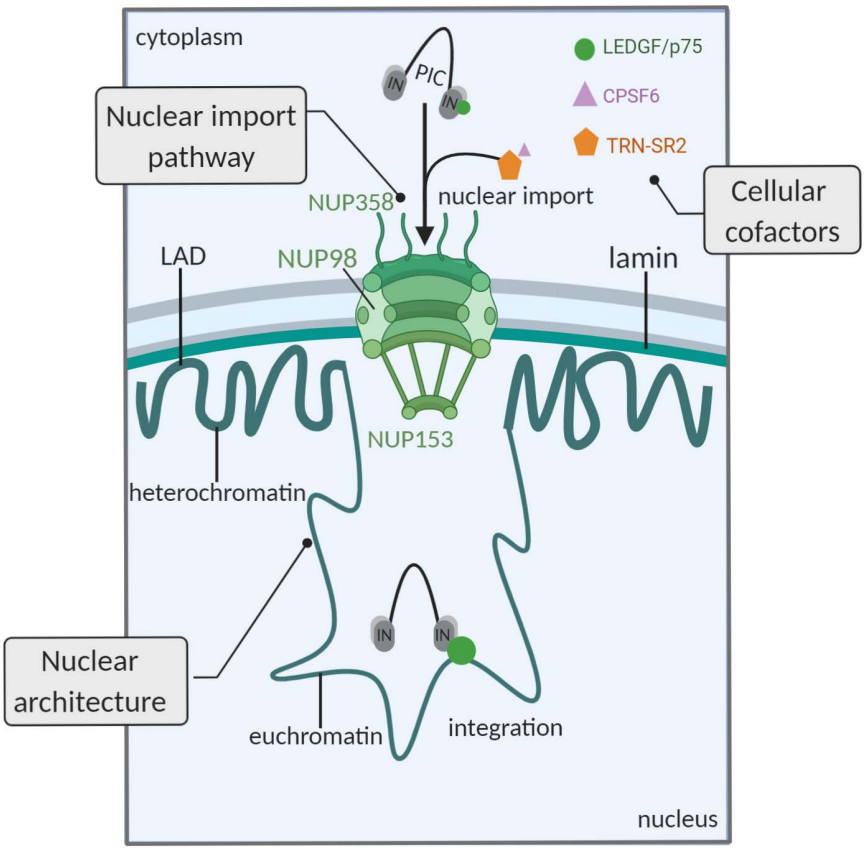

FIGURE 1 | Determinants of HIV-1 integration site selection. The HIV-1 nuclear import pathway is linked to integration. The HIV-1 PIC engages NUPs, favoring integration in close proximity to the NPC (upper left part). Second, cellular cofactors affect the proviral integration site. LEDGF/p75 interacts with HIV-1 IN and targets integration toward active genes. The HIV-1 PIC interacts with other cellular cofactors that affect integration: TRN-SR2 and CPSF6 have been associated with integration in gene-dense regions. TRN-SR2 mediates nuclear import of SR proteins, such as CPSF6, and interacts with the viral IN. The exact mechanism by which these proteins affect nuclear import and integration is not completely understood yet (upper right part). Third, the nuclear architecture affects integration site selection. The heterochromatin condensed regions in the LADs are disfavored, while gene-rich and euchromatin regions in close association with the NPC are frequently targeted by HIV-1 (lower part). PIC, pre-integration complex; NUPs, nucleoporins; LEDGF/p75, lens-epithelium-derived growth factor; IN, integrase; TRN-SR2, transportin-SR2; CPSF6, cleavage and polyadenylation specificity factor 6; LAD, lamina-associated domains; figure created with BioRender.com.

of all integration sites and therefore these findings cannot be generalized to all HIV-1 integration sites.

LEDGINS RETARGET INTEGRATION

In 2010, structure-based drug design targeting the interface between HIV-1 IN and its cellular binding partner LEDGF/p75 resulted in the discovery of 2-(quinolin-3-yl)acetic acid derivatives that impair HIV-1 replication (Christ et al., 2010). Inhibitors of different chemical classes share the identical binding pocket on HIV-1 IN and are therefore called "LEDGINs" (Demeulemeester et al., 2014). In literature, LEDGINs are sometimes termed ALLINIs (allosteric IN inhibitors); however, not all allosteric IN inhibitors target the binding pocket occupied by LEDGF/p75 (Demeulemeester et al., 2014). LEDGINs impede the interaction between HIV-1 and LEDGF/p75 by binding to the IN dimer interface and allosterically inhibit the catalytic activity of IN (the so-called "early effect") (Christ et al., 2012; Kessl et al., 2012). Afterward, it was demonstrated that not only the early steps, but also later steps in the viral replication cycle (the so-called "late effect") (Christ et al., 2012; Balakrishnan et al., 2013; Desimmie et al., 2013; Jurado et al., 2013; Le Rouzic et al., 2013) are hampered by LEDGIN treatment. Viral particles generated in the presence of LEDGINs show aberrant morphology because of LEDGIN-induced IN multimerization. Morphological defects typically include a delocalized ribonucleoprotein outside the capsid core, resulting in crippled viruses that are defective for reverse transcription, nuclear import, and integration of the provirus. This novel class of inhibitors is explored by several pharmaceutical companies actively pursuing HIV-1 treatment or cure strategies [reviewed in Demeulemeester et al. (2014)].

As discussed, LEDGF/p75 is an important determinant of HIV-1 integration site selection, targeting integration toward active regions of the genome (**Figure 2**). As such, LEDGINs could interfere with the LEDGF/p75-mediated integration pattern. In 2016, such studies were first described by Vranckx et al. (2016).

The authors demonstrated that residual HIV-1 integration was clearly shifted out of active genes upon LEDGIN treatment. Furthermore, more provirus was found in the inverse orientation, and the 3D nuclear localization of the provirus was shifted toward the inner nucleus after LEDGIN treatment (Vranckx et al., 2016). Employing a virus with a double reporter designed by the Verdin lab (Battivelli et al., 2018), it was demonstrated that a larger proportion of the provirus exhibited a transcriptionally silent phenotype after LEDGIN-mediated retargeting. Furthermore, this residual reservoir proved less prone to HIV-1 reactivation (Vranckx et al., 2016; **Figure 2**).

Of note, LEDGINs partially retarget proviral integration in cell culture, even though no random integration is observed. Still, at high concentrations of LEDGINs, nearly all residual replication and reactivation of the latent provirus is blocked. This teaches us that LEDGIN-mediated retargeting surpasses mere integration inside or outside of genes. Instead, by interfering with LEDGF/p75-mediated chromatin recognition of specific epigenetic features, LEDGINs likely affect the epigenetic and chromatin landscape in a more profound manner, resulting in a silent viral reservoir. Further research is required to clarify the mechanism whereby LEDGF/p75 mediates optimal integration site selection. Advancing methods, e.g., branched DNA imaging (bDNA; Puray-Chavez et al., 2017), could further corroborate the impact of LEDGIN-mediated retargeting on the transcriptional state of the provirus at the single-cell level. In conclusion, to ensure both a productive and a latent proviral state, HIV-1 may have evolved to use LEDGF/p75 as a molecular tether. In the absence of LEDGF/p75, the provirus may end up in a deep latent population resistant to reactivation.

CHROMATIN LANDSCAPE AT THE HIV-1 INTEGRATION SITE

To date, it is well established that the HIV-1 promotor is governed by the local chromatin environment (Jordan et al., 2003). The chromatin structure is composed of nucleosomes, consisting of eight core histones that are susceptible to

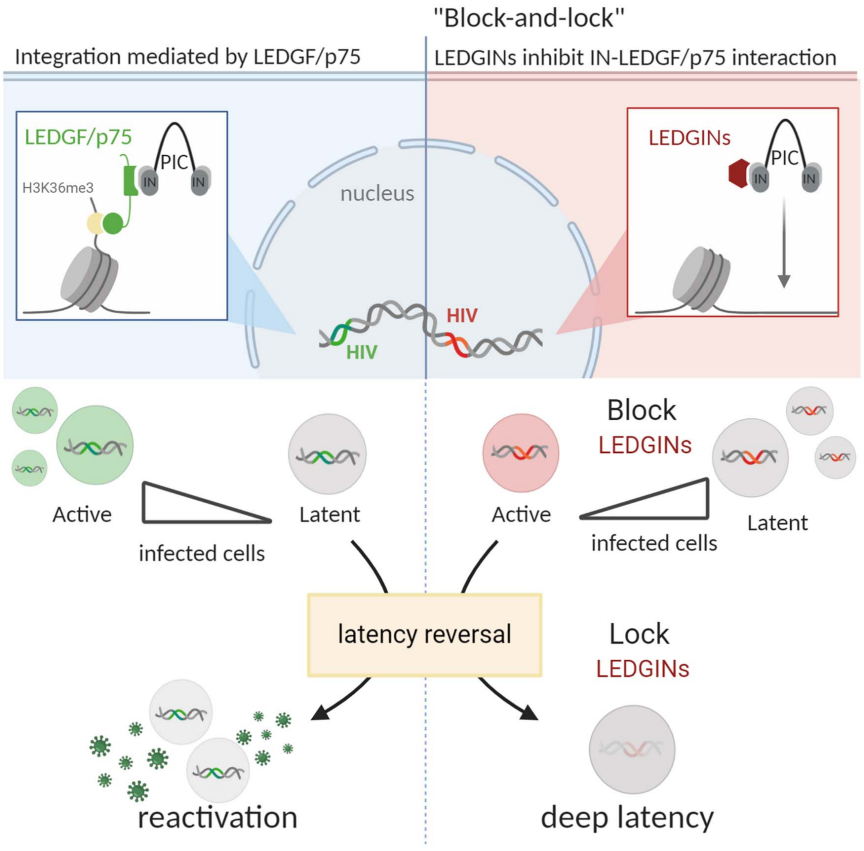

FIGURE 2 | LEDGIN-mediated retargeting provides a "block-and-lock" functional cure. LEDGF/p75 binds to the viral IN and targets the HIV-1 PIC to the chromatin via interaction with H3K36me3 (upper left part). LEDGINs inhibit the LEDGF/p75-IN interaction and retarget integration (upper right part). LEDGF/p75-independent integration in the presence of LEDGINs results in relatively more proviruses that are in a transcriptionally silent or latent state, compared to LEDGF/p75-dependent integration (middle part). After latency reversal, latently infected cells rebound, resulting in reactivation of HIV-1 gene expression (lower left part). In contrast, LEDGIN-mediated retargeting results in a cellular reservoir that is resistant to reactivation after latency reversal. LEDGIN-mediated retargeting provides a potential "block-and-lock" functional cure strategy. LEDGF/p75, lens-epithelium-derived growth factor; IN, integrase; PIC, pre-integration complex; figure created with BioRender.com.

epigenetic modifications. Regardless of the site of integration, two nucleosomes, nuc-0 and nuc-1, are precisely positioned on the viral promotor region downstream of the transcription start site (Verdin, 1991). Active transcription is associated with histone acetylation induced by histone acetyl transferases (HATs). Acetylation loosens the chromatin, rendering the chromatin and in particular promotors more accessible to the transcription factors. In contrast, histone deacetylases (HDACs) are involved in histone deacetylation and usually induce gene silencing. Additionally, DNA methylation at CpG dinucleotides near the viral promoter is associated with HIV-1 latency. CpG methylation has been shown to hamper reactivation in cell lines and patient samples by restricting the accessibility of interacting proteins to the chromatin (Blazkova et al., 2009; Trejbalová et al., 2016). The above findings suggest that HIV-1 gene expression is regulated by the chromatin context surrounding the integration site. Still, at present, studies that have investigated the impact of the chromatin environment on acute or latent HIV-1 infection are scarce.

As mentioned, LEDGINs provide us with an important research tool to study HIV-1 latency. The study by Vranckx et al. (2016) demonstrated that LEDGINs retarget integration toward silent genes that disfavor HIV-1 expression. Still, it was not clear whether the complex nature of the chromatin landscape surrounding the insertion site of those retargeted viruses could explain their silent state. Therefore, B-HIVE technology was employed. By tagging the viral genome with a unique barcode, B-HIVE allows to link individual proviral integration sites with transcriptional levels (Vansant et al., 2020b). As such, expression levels of each single provirus can be correlated to the corresponding integration site. With B-HIVE technology, it was shown that LEDGINs shift the distribution of integration sites out of gene-rich chromosomes, such as chromosomes 16, 17, and 19. In agreement with Vranckx et al. (2016), the residual provirus was retargeted toward silent genes and intergenic regions upon LEDGIN treatment. Furthermore, LEDGINs decreased total RNA expression and enriched the proportion of "no-expression" barcodes, which are silent barcodes without RNA expression. Interestingly, following LEDGIN treatment, these "no-expression" barcodes were located further away from epigenetic markers linked to active transcription (Med1, CBP, H3K4me1, H3K27ac H3K79me2/3, RNAPII, and H3K4me3) (Vansant et al., 2020b). Likewise, the distance of silent provirus to H3K36me3, the epigenetic feature recognized by LEDGF/p75, increased after LEDGINs supporting the direct link between LEDGF/p75-mediated integration and proviral expression. In contrast, the proximity of epigenetic features associated with enhancers (such as H3K27ac, H3K4me1, or Med1) stimulated transcription independent of LEDGIN-mediated retargeting. These data confirm the pivotal role of LEDGF/p75 in integration site selection (Vansant et al., 2020b). However, they also point toward other, LEDGF/p75-independent mechanisms, such as the proximity of integration to genomic enhancers (Chen et al., 2016).

Notably, besides its role in integration site selection, LEDGF/p75 has been proposed to regulate post-integration latency (Gérard et al., 2015; Gao et al., 2020). Recently, Gao et al. published that recruitment of the repressive Pol II-associated factor 1 (PAF-1) by LEDGF/p75 induces Pol II pausing and thereby promotes viral latency. Although somewhat paradoxical, the same study demonstrated distinct effects of LEDGF/p75 during latency formation and latency reactivation. Upon latency reversal, LEDGF/p75 was shown to recruit MLL1, which in turn displaces PAF1 from the viral promotor, and accordingly induces proviral transcription (Gao et al., 2020).

Additionally, polycomb group proteins (PcG) have recently been implicated in epigenetic repression of HIV-1 transcription (Matsuda et al., 2015; Khan et al., 2018). In particular, Polycomb-repressive complex 2 (PRC2) is believed to induce silencing of the HIV-1 promotor by methylation of H3K27, a repressive chromatin mark. Matsuda et al. (2015) published that trimethylation of H3K27 by PRC2 is associated with transcriptional silencing early during the course of infection.

ELITE CONTROL AS MODEL FOR A CURE FOR HIV-1

Although a broadly applicable cure of HIV-1 has not been achieved yet, the identification of a unique population of individuals that spontaneously control the infection provides indispensable information on the mechanisms that allow robust virological control in the absence of treatment (Gonzalo-Gil et al., 2017). Such individuals, referred to as HIV-1 controllers, maintain either undetectable (elite controllers, EC) or low levels (viremic controllers) of viremia, stable $CD4^+$ cell counts, and accordingly, no signs of clinical progression (Gonzalo-Gil et al., 2017).

The molecular mechanisms responsible for robust virological control in EC remain poorly understood. It has been documented that the proviral reservoirs of latently infected cells are smaller in EC (García et al., 2017), yet genomes isolated from this specific group have shown a similar extent of genetic variation and replication compared to individuals on cART (Blankson et al., 2007). The hypothesis that EC restrict viral replication independent of the viral fitness has been supported by several studies (Blankson et al., 2007; Buckheit et al., 2012; Gonzalo-Gil et al., 2017). In fact, the degree of defective HIV-1 was shown to be comparable between EC and individuals on cART. Spontaneous viral control has been linked to host gene polymorphisms, among which the protective HLA class I and the CCR5 delta 32 (CCR5Δ32) alleles are most consistently recognized (Antoni et al., 2013). Within the EC that exhibit protective HLA class I alleles, viremic control is usually linked with a broad CD8+ T cell response (Pereyra et al., 2008). However, such T cell responses are not always necessary for long-term viral control (Emu et al., 2008). Furthermore, a loss of virological control has more often been experienced in EC with protective HLA class I alleles compared to EC without (Koofhethile et al., 2016). This underscores the need to pinpoint alternative, more durable, and T cell-independent pathways that are responsible for virological control.

Recently, an alternative hypothesis underlying sustainable, long-term HIV-1 remission in EC was proposed by

Jiang et al. (2020). They investigated the HIV-1 reservoir in terms of the chromosomal integration site and discovered that individuals naturally controlling HIV-1 (EC) have more proviral DNA in a silent, deep sleep, mode (Chomont, 2020; Jiang et al., 2020). An increased proportion of the integration sites was found in non-coding regions, such as centromeric DNA and dense heterochromatin gene deserts, regions less permissive for active viral transcription (Jiang et al., 2020). Although the absolute number of proviral DNA sequences was lower in EC, they exhibited relatively more genome-intact viral sequences compared to patients on cART. These data strongly suggest that defective HIV-1 cannot explain the clinical status of EC; they rather control HIV-1 through other, still unknown, mechanisms. Interestingly, Jiang and colleagues found no evidence for preferential targeting of repressive chromosomal regions (e.g., centromeric satellite DNA or KRAB-ZNF genes) in EC, which is consistent with the known preferential integration of HIV-1 in active genes and open chromatin (Jordan et al., 2001; Schröder et al., 2002; Maxfield et al., 2005; Barr et al., 2006; Albanese et al., 2008; Dieudonné et al., 2009; Marini et al., 2015; Kok et al., 2016). Therefore, the elite control is probably not the result of an altered integration site preference during reservoir formation in these patients. More likely, proviral sequences integrated in non-silent regions are gradually cleared by the immune system, while a deep latent reservoir is selected over time (Chomont, 2020; Einkauf et al., 2019). This work holds great promises to obtain a functional cure for HIV-1 in which the virus is still present in the latent reservoirs but never reaches high levels of viral replication. EC may provide the translational evidence for the "block-and-lock" induced cure strategy, which aims to permanently lock the virus into a transcriptionally silent or deep latent state, unable to rebound upon cART interruption.

Jiang et al. (2020) also investigated whether epigenetic modifications surrounding the integration site contributed to the long-term transcriptional repression of intact sequences observed in EC. The frequency of CpG methylation at 500- or 1,000-base-pair distance from the integration site was significantly higher for integrants from EC, compared to patients on cART. Genomic regions more sensitive to DNA methyltransferases might be more prone to facilitate deep latency and thus represent preferential hotspots for long-term persistent proviral sequences (Jiang et al., 2020). Furthermore, genome-intact viral sequences of EC were located closer to epigenetic features associated with silent chromatin (H3K9me3) and located further away from the active transcription mark H3K4me1 (Jiang et al., 2020).

In addition, Jiang et al. (2020) frequently observed identical sites derived from clonally expanded cells in EC. They demonstrated that these clonally expanded cells contained intact and replication-competent virus, which is supported by earlier observations (Symons et al., 2018). Remarkably, more integrants originating from a clonal expanded cell population were found in EC compared to cART-treated individuals. So far, the mechanism underlying these large expansions of identical clones remains elusive. Antigen stimulation or homeostatic proliferation are known drivers of clonal expansion (Chomont et al., 2009; Hughes and Coffin, 2016; Kwon and Siliciano, 2017). Clonal expansion of infected cells has also been linked to the proviral integration site (Maldarelli et al., 2014; Wagner et al., 2014; Cohn et al., 2015). Other studies found recurrent HIV-1 integrations in the BACH2, STAT5B, and MKL2 gene of patient samples (Maldarelli et al., 2014; Wagner et al., 2014; Cohn et al., 2015; Cesana et al., 2017). Notably, all three genes code for cellular transcription factors that regulate cell growth and both MKL2 and BACH2 were previously described as oncogenes (Kobayashi et al., 2011; Flucke et al., 2013). These studies support the hypothesis that integration in certain genes promotes clonal expansion of the infected cell and, as such, contributes to HIV-1 persistence. Nevertheless, more investigation is required to prove a causal association between the site of integration and an effect on proliferation or cell survival.

INSIGHT INTO HIV-1 INTEGRATION SITE SELECTION PROVIDES A "BLOCK-AND-LOCK" FUNCTIONAL CURE

Even potent antiretroviral therapy fails to cure HIV-1 infection due to the persistence of long-lived latently infected cellular reservoirs. Viremia inevitably rebounds upon treatment interruption, and therefore, an HIV-1 cure has been extremely difficult to achieve (Chun et al., 1997; Deeks et al., 2016). The well-documented cases of the Berlin and London patients raised hope that curing HIV-1 infection is on the long-term a feasible target (Hutter et al., 2009; Gupta et al., 2020). These patients have obtained complete eradication of all replication-competent HIV-1 and are considered to be cured from HIV-1. Still, such a sterilizing cure is very challenging to achieve and almost impossible to prove with current technologies (Henrich et al., 2014; Abdel-Mohsen et al., 2020). These factors highlight the importance of novel strategies and identification of alternative target routes that can exhaust the latent reservoir and suppress HIV-1 replication in the absence of treatment. The latency-reversing, "shock and kill" strategy has reached considerable attention due to evidence that LRAs may lead to enhanced control of viral load after a treatment interruption (Archin and Margolis, 2014). LRAs enhance HIV-1 transcription *in vivo*, but so far, it has not been conclusively demonstrated that this indeed leads to immune-mediated killing and a sustained reduction in reservoir size (Archin et al., 2012; Søgaard et al., 2015; Darcis et al., 2017). Adding to the complexity, the rebound capacity of latent provirus is determined by the integration site and the nearby chromatin modifications. Hence, a combination of LRAs will be a necessity to reactivate all latent proviruses (Chen et al., 2016; Battivelli et al., 2018). Besides little to no reduction in reservoir size, this strategy is also associated with broad and uncontrolled activation, resulting in undesired off-target immune reactivation (Abner and Jordan, 2019).

Except for the Berlin and London patients, eradication strategies have not resulted into a cure for HIV-1 so far. Therefore, clinicians and scientists had to reevaluate the definition of an HIV-1 cure. Long-term HIV-1 remission or a functional cure whereby the virus is not eradicated from the body but remains suppressed even in the absence of treatment is therefore considered a more feasible outcome. HIV-1 remission

is increasingly used in the field to indicate the goal of long-term undetectable viremia in the absence of treatment. HIV-1 remission is therefore considered as a necessary precursor for the development of an HIV-1 cure. We might argue that EC, which fulfill these criteria, have achieved HIV-1 remission or a functional cure. Hence, the mechanisms responsible for elite control are intensively investigated to gain more insight into the feasibility of such an approach. It is widely accepted, however, that the ability to spontaneously control HIV-1 is the result of a complex interplay between virological and immunological factors. As such, no sole mechanism will explain the phenotype of this heterogeneous group of individuals. Nonetheless, the mechanism proposed by Jiang et al. (2020) holds great promises for the HIV-1 cure field. Chromosomal integration into repressive chromatin is proposed as a crucial factor contributing to HIV-1 remission in EC. This insight is not entirely new; a better comprehension of HIV-1 integration site selection could provide us with new strategies for a functional cure of HIV-1 infection (Debyser et al., 2019).

As discussed, epigenetic regulation of the chromatin structure, an integration site-specific feature, is important in the control and maintenance of HIV-1 latency. Therefore, various LRAs are epigenetic regulators: inhibitors of histone methyl transferase (HMTi), histone deacetylase (HDACi), or DNA methyl transferase (DNMTi) have been tested to reactivate proviral transcription in latently infected cells and patients. However, Battivelli et al. (2018) have reported that no LRA so far could reactivate more than 5% of the total reservoir *in vitro* and hence is potent enough to reduce the reservoir size. Alternatively, durably silencing the reservoir through induction of repressive chromatin modifications could obtain a functional HIV-1 cure. In this regard, PcG proteins could provide promising novel targets for the induction of HIV-1 latency (Khan et al., 2018).

Retargeting HIV-1 integration by LEDGINs creates a deep latent cellular reservoir that is resistant to reactivation. As such, LEDGINs present interesting candidates to be used in a "block-and-lock" functional cure strategy. Although promising, as with all strategies, the "block-and-lock" functional cure strategy faces some challenges. While it is not exactly known when the latent reservoirs are formed, at present, it is clear that they are established early during acute infection. It has been shown that early treatment reduces the size of the reservoir during acute infection (Buzon et al., 2014). Hence, addition of a LEDGIN to early cART regimens could modulate the functionality of the latent reservoirs. If LEDGINs will be tested in clinical trials as antivirals for acute infection, monitoring the reservoir size with quantitative viral outgrowth assay (qVOA) and proviral DNA loads could provide supportive evidence for this hypothesis. In the case of HIV-1 pre-exposure prophylaxis (PrEP), LEDGINs may also be beneficial when added since they may ensure that any residual infection under PrEP treatment results in a deeply latent or non-functional provirus. With regard to chronically infected patients, recent studies now suggest that the majority of the reservoir responsible for HIV-1 rebound might only be established in the year before cART is initiated (Brodin et al., 2016; Abrahams et al., 2020), implying that LEDGINs may also have an effect in a first-line treatment of patients diagnosed years after infection. In the absence of any residual replication when the latent reservoirs have already been established, LEDGINs will fail to modulate the functional reservoir in patients on chronic treatment. Yet, if poor drug penetration by cART at sanctuary sites allows any residual replication, these viruses could be retargeted by LEDGINs, assuming that LEDGIN tissue penetration is optimal. Secondly, repeated treatment interruptions followed by reinitiation of cART including LEDGINs could modulate the functional reservoir in chronically infected patients. Whether enough proviruses can be mobilized upon treatment interruption in a clinical setting remains, of course, to be determined.

The question whether we should shock or lock the latent reservoir has been subject to ongoing debate (Darcis et al., 2017). Likewise, the combination of "lock and shock" presents an alternative, yet unexplored strategy. Addition of LEDGINs to early treatment protocols may already reduce the size of the functional reservoir. Afterward, residual replication-competent virus prone to rebound upon treatment interruption (e.g., integrated close to enhancers) could be eradicated with potent LRAs that induce minimal cellular toxicity. To ensure the establishment of a complete and long-term "deep latency", it may be of interest to target multiple pathways that promote silencing and prevent viral rebound. Besides retargeting integration with LEGDINs, interference with chromatin remodelers and transcription activators such as Tat are interesting approaches to induce long-term suppression of the virus (Li et al., 2019; Vansant et al., 2020a).

In conclusion, several HIV-1 cure strategies have been extensively studied and evaluated in patients. Unfortunately, none of these approaches was successful so far on a large scale in the clinic. All HIV-1 cure approaches face the same obstacle: the persistence of latent reservoirs carrying replication-competent provirus. In this respect, a better understanding of the mechanisms that contribute to silencing of the reservoirs is of paramount importance. Therefore, a persistent effort is required to increase our basic understanding on the virology of HIV-1. As described in this review, increasing our knowledge on the importance of integration site selection in the establishment and maintenance of the reservoir will certainly help us toward a functional cure of HIV-1 infection.

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JJ made the figures. All authors wrote, read, and agreed to the published version of the manuscript.

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REVIEW
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The Interplay of HIV-1 and Macrophages in Viral Persistence

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HIV-1 has evolved mechanisms to evade host cell immune responses and persist for lifelong infection. Latent cellular reservoirs are responsible for this persistence of HIV-1 despite the powerful effects of highly active antiretroviral therapies (HAART) to control circulating viral load. While cellular reservoirs have been extensively studied, much of these studies have focused on peripheral blood and resting memory CD4+ T cells containing latent HIV-1 provirus; however, efforts to eradicate cellular reservoirs have been stunted by reservoirs found in tissues compartments that are not easily accessible. These tissues contain resting memory CD4+ T cells and tissue resident macrophages, another latent cellular reservoir to HIV-1. Tissue resident macrophages have been associated with HIV-1 infection since the 1980s, and evidence has continued to grow regarding their role in HIV-1 persistence. Specific biological characteristics play a vital role as to why macrophages are latent cellular reservoirs for HIV-1, and *in vitro* and *in vivo* studies exhibit how macrophages contribute to viral persistence in individuals and animals on antiretroviral therapies. In this review, we characterize the role and evolutionary advantages of macrophage reservoirs to HIV-1 and their contribution to HIV-1 persistence. In acknowledging the interplay of HIV-1 and macrophages in the host, we identify reasons why current strategies are incapable of eliminating HIV-1 reservoirs and why efforts must focus on eradicating reservoirs to find a future functional cure.

Keywords: HIV, reservoirs, macrophages, latency, functional cure, host factors

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INTRODUCTION

The HIV-1 pandemic persists despite the revolution of suppressive antiretroviral therapy (ART) in controlling HIV-1 replication. Recrudescing viremia emerges from cellular reservoirs once suppressive ART has ceased, as well as from compartments unaffected by ART. Latent cellular reservoirs are the major obstacle in preventing HIV-1 eradication and further characterization and understanding is necessary for cure efforts. Resting memory CD4+ T cells reservoirs are the main focus of HIV-1 reservoir studies, overlooking other CD4-containing cell types, such as myeloid cells. Myeloid cells, specifically macrophages, have been associated with HIV-1 infection since the 1980s (Ho et al., 1986; Koenig et al., 1986). Residual viremia from cART-suppressed patients validates that small populations of viruses are not genetically identical to the proviruses

found in resting CD4+ T cells but are from an unknown source (Bailey and Hutter, 2006; Brennan et al., 2009; Vibholm et al., 2019). Furthermore, a minor population of viruses from macrophage reservoirs appears in rebounding viremia from reservoirs that predate the initiation of cART, which suggests macrophages are infected early on and assist in the persistence of HIV-1 infection (Andrade et al., 2020). Since the 1990s, evidence has implicated macrophages assist in HIV-1 persistence *in vivo* and produce virions *in vitro* (Perelson et al., 1997).

Macrophages, like CD4+ T cells, express CD4 and chemokine coreceptors CCR5 and CXCR4 on their cell surface allowing for HIV-1 susceptibility. Macrophages are susceptible to CCR5-tropic and dual-tropic viral infection, but determinants that assist in tropism are much more complicated than coreceptor usage (Borrajo et al., 2019). Specifically, macrophages express significantly lower levels of CD4, meaning macrophage-tropic viruses must have a high degree of affinity to CD4 to mediate fusion with the macrophage cell membrane (Kazazi et al., 1989; Joseph et al., 2014). Macrophages are permissive to productive infection both *in vivo* and *in vitro*. Furthermore, genetically related Simian Immunodeficiency Virus (SIV) has been shown to infect simian macrophages (Ho et al., 1986; Koenig et al., 1986; Li et al., 2015). These HIV-1 and SIV infected macrophages have been found in various tissue compartments *in vivo*, including the central nervous system (CNS), lymph nodes, gut lymphoid-associated tissue (GALT), and lungs (Albright et al., 2000, 2003; Igarashi et al., 2001; Jambo et al., 2014; Li et al., 2015; Avalos et al., 2016). While HIV-1 infects and sustains infection in macrophages, the contribution of macrophages as a viable reservoir remains in question.

Investigating the role of macrophages in HIV-1 persistence is challenging. Macrophages are heterogenous, non-dividing cells that are influenced by their surroundings, which can make infected macrophages unique to their niche (Gordon and Pluddemann, 2017). Tissue compartments in humans are not readily accessible unless donated from elective and necessary surgeries or post-mortem tissue. Some of these tissue compartments may provide immune sanctuaries, such as lymphoid tissues and the CNS to macrophage reservoirs (Kepler and Perelson, 1998; Smit et al., 2004; Fletcher et al., 2014). Experimental models, such as macrophage quantitative viral outgrowth assays (qVOA) and monocyte-derived macrophages (MDMs) models, have been developed to study macrophage reservoirs *in vitro,* while non-human primate and mice models are used to study macrophages *in vivo*. Yet, none of these models have grasped the complexity of macrophage heterogeneity in their contribution to HIV-1 persistence (Avalos et al., 2016; Honeycutt et al., 2016; Veazey and Lackner, 2017; Abreu et al., 2019).

While growing evidence supports the role of macrophage reservoirs, how they contribute to HIV-1 persistence is still largely unknown and characteristics of the reservoirs are still being understood. This gap in knowledge raises questions as to what extent macrophage reservoirs contribute to infection and if macrophage-tropic viruses truly originate from a macrophage reservoir. In this review, we address the biological characteristics and mechanisms of macrophage reservoirs in controlling HIV-1 latency and the factors that contribute to long term HIV-1 infection in relation to finding a functional cure.

MACROPHAGE BIOLOGY, HIV-1 INFECTION AND CELLULAR RESERVOIRS

Macrophage Heterogeneity Affects HIV-1 Infection

Macrophages are capable of sustaining HIV-1 infection due to a number of biological reasons. Firstly, macrophages are extraordinary plastic cells that exhibit extreme heterogeneity due to sensitivity to the local cytokine microenvironment (Mantovani et al., 2004; Sica and Mantovani, 2012; Gordon and Pluddemann, 2017). Macrophages are primed to install a particular, reversible phenotype and functional response based on cytokine stimuli and signals encountered in their microenvironment (Sica and Mantovani, 2012). This diversity allows macrophages to take on a wide variety of roles in innate immune response, phagocytosis, and tissue repair. Primary macrophages are challenging to investigate *in vivo*, thus *in vitro* models using MDMs have been created (Zalar et al., 2010; Yukl et al., 2013; Cribbs et al., 2015). Monocytes are isolated from peripheral blood and differentiated into MDMs that can be further stimulated in culture to obtain phenotypically distinct macrophages (Kruize and Kootstra, 2019). *In vitro* polarized macrophages can be classified as classically activated or inflammatory macrophages (M1) and alternatively activated or anti-inflammatory (M2) macrophages (Cassetta et al., 2011; Biswas et al., 2012; Mantovani et al., 2013; Schwartz et al., 2014; Sica et al., 2015; Murray, 2017).

These distinct polarization states affect macrophage susceptivity to HIV-1 infection (**Figure 1A**). Macrophage polarization can range in acute and chronic stages of HIV-1 infection (Burdo et al., 2015). During acute infection, macrophages polarize to an M1 inflammatory state that eventually shifts to an M2 anti-inflammatory or immunosuppressive state in chronic infection (Becker, 2004; Li et al., 2009). Unpolarized macrophages are permissive to HIV-1 infection as they are not primed for a certain response. Different cytokine expressions during polarization leads both M1 and M2 macrophages to being refractory to HIV-1 infection, as well as impair HIV-1 viral functions during acute and chronic HIV-1 infection (Cassol et al., 2009, 2010; Galvao-Lima et al., 2017). During *in vitro* studies, IFNγ and TNFα stimulated M1 MDMs inhibited HIV-1 viral DNA synthesis, proviral integration, and transcription in comparison to unpolarized MDMs through an upregulation of APOBEC 3A in M1 MDMs (Cassetta et al., 2013). Furthermore, IFNγ polarized macrophages from the decidua basalis tissue in pregnant woman have been shown to be weakly permissive HIV-1 infection through cyclin-dependent kinase inhibitor p21Cip1/Waf1 and by toll-like receptor (TLR) 7 and TLR8 restriction of HIV-1 replication by further inducing IFNγ to maintain an M1 macrophage phenotype (El Costa et al., 2016). Primary MDMs stimulated IFNγ and TNFα into

an M1 phenotype show viral containment and inhibition of viral replication and integration upon re-stimulation to an M1 phenotype. These M1-double stimulated MDMs have an upregulation in APOBEC3A and APOBEC3G restriction factors as well as negative regulators of proviral transcription, which assisted in keeping the replication-competent virus in a latent state (Graziano et al., 2018).

For M2 polarization, IL-4 and IL-13 polarized M2a macrophages can cause resistance to HIV-1 infection by limiting HIV-1 replication during reverse transcription of HIV-1 RNA (Schuitemaker et al., 1992; Montaner et al., 1997; Wang et al., 1998; Cobos Jimenez et al., 2012). IL-10 polarized M2c and TNFα polarized M1 macrophages control HIV-1 latency at the levels of viral replication at transcription and translation (Kootstra et al., 1994; Montaner and Gordon, 1994; Chang et al., 1996; Perez-Bercoff et al., 2003; David et al., 2006; Cassol et al., 2009; Cobos Jimenez et al., 2012). Although, macrophages in an intermediate polarization state (Mi)—somewhere between M1 and M2—were preferentially infected by HIV-1 in urethral tissue (Ganor et al., 2019). Additionally, macrophage susceptibility has been shown to differ amongst tissues due to mechanisms that are still debated. Heterogeneity of macrophages allows these cells to maintain different roles necessary for the tissue they reside, and the degree to which macrophages differ can be quite significant depending on their niche. Macrophages in the GALT are resistant to infection, whereas macrophages in the rectum and alveolar are readily permissive (King et al., 2013; McElrath et al., 2013; Jambo et al., 2014). Macrophage polarization helps to understand the complexities of macrophage heterogeneity *in vitro*; however, the plasticity of macrophages in different environments contributes to challenges around understanding how heterogeneity affects HIV-1 infection *in vivo*. Macrophage localization has proven to show drastically different susceptibilities to HIV-1 infection and mechanisms that may control latency, which suggests certain macrophage phenotypes provide better reservoirs.

Macrophage Self-Renewal Potential and Latency

Tissue macrophages have the ability to sustain themselves. These macrophages have long been hypothesized as derived from monocytes; however, macrophages can be classified into two broad groups: tissue resident macrophages and infiltrating macrophages. The tissue resident macrophages perform homeostatic functions in their respective tissues in a steady state, while infiltrating macrophages are derived from circulating monocytes that infiltrate tissue and differentiate (**Figure 1B**) (Murray and Wynn, 2011). Early studies show bone marrow derived monocytes replenish tissue resident macrophages and that monocytes contribute to the population of lung alveolar macrophage replacement after they are depleted (van Furth and Cohn, 1968; Virolainen, 1968; Landsman et al., 2007). This has conflicted with evidence that primitive macrophages originate during embryonic stages developing before monocytes and are maintained as long-lived cells in tissue compartments (Takahashi et al., 1989). Microglia in the CNS are derived from these embryonic macrophages and are maintained independently from monocytes through self-renewal in a steady state (Schulz et al., 2012).

Tissue resident macrophages are seeded throughout tissues before birth during the embryonic state (Schulz et al., 2012; Hashimoto et al., 2013; Yona et al., 2013; Mass et al., 2016). Hashimoto et al. (2013) found that monocytes contribute very small portion to the tissue macrophage population to tissue macrophages in a steady state after cell turnover. In the lungs, bone marrow, and CNS, monocytes were found not to be the progenitors for tissue resident macrophages when in a steady state. Even when lung and splenic tissues were depleted of macrophages, they were repopulated by tissue resident macrophages independently of circulating monocytes, which suggests these macrophages are self-maintaining in the steady state and after cell turnover (Hashimoto et al., 2013). Soucie confirmed tissue resident macrophages are able to self-proliferate with minimal contribution from monocytes through a network of genes that control self-renewal potential in these mature cells (Soucie et al., 2016).

Tissue resident macrophages are long-lived innate immune cells that persist from weeks to decades. Infiltrating macrophages with no embryonic origins have estimated half-lives between 4 and 6 weeks, while tissue resident macrophages have a much slower turnover (Scott et al., 2014). Alveolar macrophages have a half-life around 2 months, while microglial cells can last between 4 years to decades (Cassol et al., 2006; Reu et al., 2017). These long-lived macrophages are suitable for maintaining HIV-1 infection in durations comparable to those of resting memory CD4+ T cells. For infected tissue resident macrophages, their ability to self-renew provides a mechanism for sustained HIV-1 infection in a long-lived population of cells throughout different compartments in the host. This provides the scaffolding to protect HIV-1 long term against the effects of ART as the virus goes quiescent and is self-maintained through the tissue macrophage population.

Additionally, acutely infected resting CD4+ T cells have an average half-life of 2 days, whereas infected monocytes and macrophages live significantly longer than their counterparts (Zhou et al., 2005; Koppensteiner et al., 2012). This poses the case that tissue macrophages may be able to maintain infection for a longer duration than CD4+ T cells. Furthermore, HIV-1 infected macrophages have evolved mechanisms to prevent cell death and prolong cell lifespans, thereby allowing macrophages to provide immune sanctuary to HIV-1 in the latent stage (Swingler et al., 2007; Reynoso et al., 2012). Due to the heterogenous nature of macrophages, specifics as to which population of macrophages is preferentially infected still remains unknown. In addition, the long lifespan and self-renewal capacity of macrophages means HIV-1 infection is sustained for lengthy, undisturbed periods of time.

Macrophage Virus-Containing Compartments

As new virus is produced in infected cells, they require fusion, maturation, and assembly. The assembled virus retains the

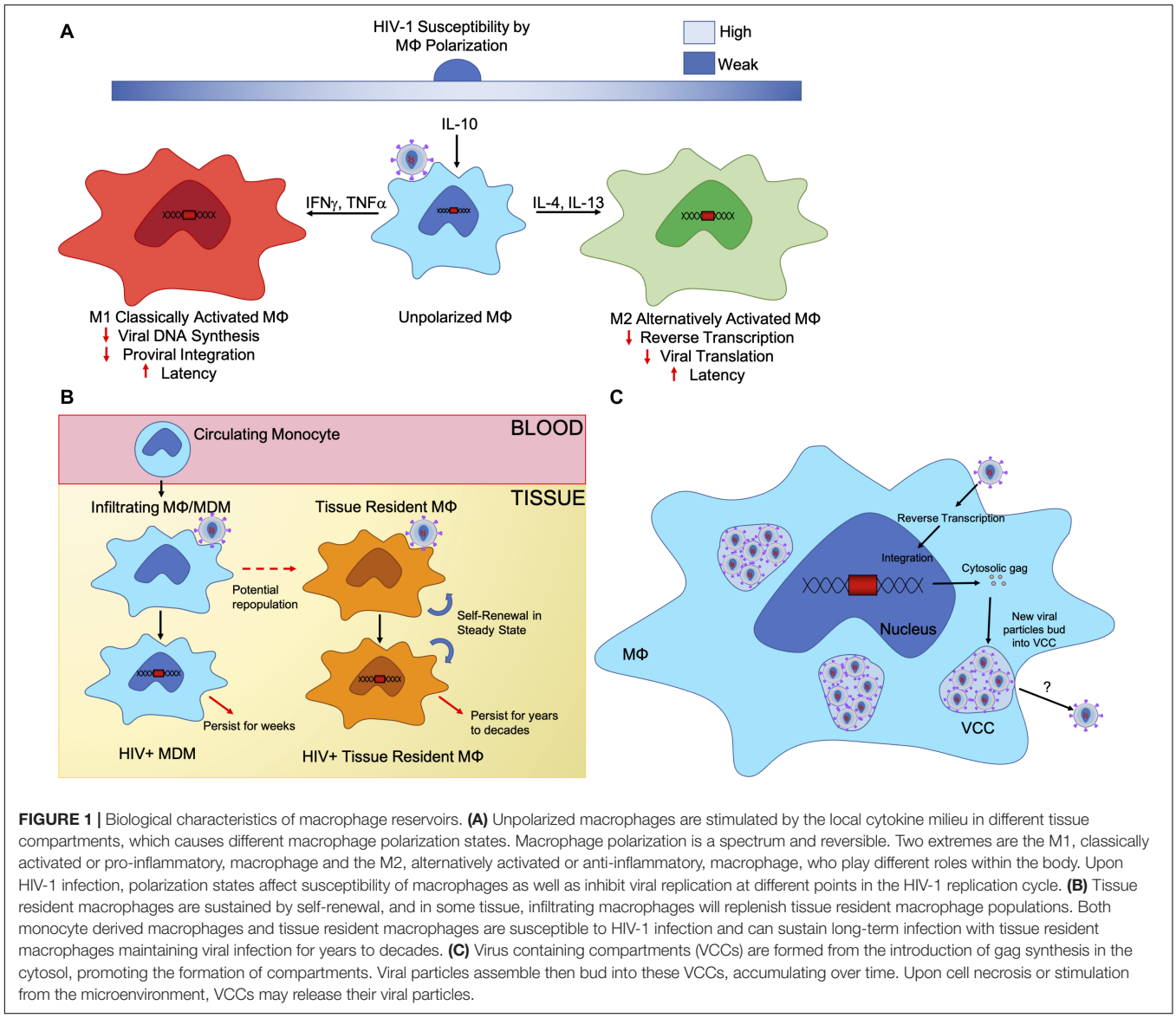

FIGURE 1 | Biological characteristics of macrophage reservoirs. **(A)** Unpolarized macrophages are stimulated by the local cytokine milieu in different tissue compartments, which causes different macrophage polarization states. Macrophage polarization is a spectrum and reversible. Two extremes are the M1, classically activated or pro-inflammatory, macrophage and the M2, alternatively activated or anti-inflammatory, macrophage, who play different roles within the body. Upon HIV-1 infection, polarization states affect susceptibility of macrophages as well as inhibit viral replication at different points in the HIV-1 replication cycle. **(B)** Tissue resident macrophages are sustained by self-renewal, and in some tissue, infiltrating macrophages will replenish tissue resident macrophage populations. Both monocyte derived macrophages and tissue resident macrophages are susceptible to HIV-1 infection and can sustain long-term infection with tissue resident macrophages maintaining viral infection for years to decades. **(C)** Virus containing compartments (VCCs) are formed from the introduction of gag synthesis in the cytosol, promoting the formation of compartments. Viral particles assemble then bud into these VCCs, accumulating over time. Upon cell necrosis or stimulation from the microenvironment, VCCs may release their viral particles.

specific cellular membrane of the host cell, which derives the viral envelope. In infected lymphocytes, and certain cell lines like 293T and HeLa cells, HIV-1 buds straight through the cell plasma membrane (Jouvenet et al., 2006; Finzi et al., 2007). Alternatively, in HIV-1 infected macrophages, budding structures accumulate in subcellular compartments similar to endosomes (Orenstein et al., 1988; Raposo et al., 2002; Finzi et al., 2007). In these infected macrophages, pleomorphic vesicular structures containing viral-like particles have been identified (Ganor et al., 2019). When infected, MDMs produce an accumulation of viral particles in these vesicular structures (Deneka et al., 2007; Jouve et al., 2007). Different from CD4+ T cells, infected macrophages accumulate large internal vacuole containing virus, known as Virus-Containing Compartments (VCCs), that retain their infectious potential for extended periods of time and act as safe storage for infectious particles in a viral reservoir (Gaudin et al., 2013). Gaudin hypothesized that VCCs are formed upon HIV-1 infection. Intracellular gag is synthesized and accumulated in the cytosol to promote the formation of compartments made from the cell membrane. Viral particles are assembled in the cytosol and bud into these compartments, which eventually fill up the lumen (**Figure 1C**) (Gaudin et al., 2013). While HIV-1 gag is responsible for the formation of VCCs in macrophages, Hammond and peers found that cell surface lectin Siglec-1 is capable of attaching to the lipid envelopes of viruses, capturing the viral particles and forming VCCs, and these compartments allowed for direct transfer of virions to other target cells. Depletion of Siglec-1 decreases the production and size of VCCs in macrophages, suggesting the need for Siglec-1 in VCC formation (Hammonds et al., 2017).

The biological production of these structures allows for direct transfer of virus from infected cells in contact with uninfected cells. Macrophages favor retention of accumulated viral particles contained in VCC, triggering release through stimulation or cell

necrosis or apoptosis. Upon stimulation by microenvironmental factors like extracellular ATP, macrophages are triggered to rapidly release infectious virions from VCCs (Graziano et al., 2018). Additionally, bone marrow stromal cell antigen 2 (BST2) has been found contained in VCCs with HIV-1 virions, tethering HIV-1 virions to the cell membrane or these VCCs. HIV-1 Vpu protein downregulates BST2 and removes it from VCCs, allowing for the expansion of VCCs with more viral particles (Leymarie et al., 2019).

The functions of VCCs remains debated. Gaudin noted an increase of density of intracellular viral gag in these compartments post-infection by immuno-EM (Gaudin et al., 2013). Additionally, these experiments highlighted a decrease in secretion of viral particles with their infectivity and transmission rate to CD4+ T cells decreasing overtime, thereby suggesting HIV-1 infected macrophages retain new virions in their compartment lumens (Gaudin et al., 2013). In a study of penile urethral tissue, Ganor et al. (2019) addressed the importance of VCCs in the presence of HIV-1 proteins, in p24, CD68 and CD4 stained urethral tissues on HIV-1/cART individuals. Stained tissue revealed p24 co-localized with macrophages co-expressing CD4 and CD68 in the urethral stroma. In contrast, urethral CD4+ T cells had no co-localization of p24 with high level CD4 expression. Evidence of VCC structures in urethral tissues supports macrophage reservoirs as sustaining HIV-1 in these tissues specifically (Ganor et al., 2019). These results demonstrate macrophages contain and shelter intact HIV-1 virions in VCC-like structures, which may act as an important viral reservoir specifically when viral capsid and virions could not be identified in CD4+ T cells. The inclusion of VCCs in macrophages allows for macrophages to maintain latent infection and to sustain active infection by release sheltered particles safe from ART. These sheltered particles make it possible for direct cell-to-cell infection of uninfected macrophages and CD4+ T cells in the same region as infected macrophages, which provides an evolutionary advantage for macrophages as cellular reservoirs.

MACROPHAGE AND HIV-1 INTERPLAY WITH HOST IMMUNE RESPONSES

Infected Macrophages Resist HIV-1 Cytotoxic Effects

Upon HIV-1 infection, macrophages resist the cytotoxic effects of viral infection. Typically, infected cells undergo apoptosis or cell-mediated killing upon viral infection; however, HIV-1 has evolved mechanisms within infected macrophages to maintain cell health and sustain viral production (**Figure 2A**) (Coiras et al., 2009). In infected macrophages, HIV-1 envelope glycoprotein stimulates macrophage colony-stimulating factor (M-CSF) to downregulate tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) receptor and upregulate anti-apoptotic genes allowing infected macrophages to remain unaffected (Swingler et al., 2007). In acute HIV-1 infection, a small number of infected microglia and macrophages expressed higher levels of Bim, which down regulates pro-apoptotic negative regulator Bcl-2, in the mitochondria both *in vitro* and *in vivo* (Castellano et al., 2017). These mechanisms used to downregulate key players in apoptotic mechanisms allow infected macrophages to persist.

Triggering receptor expressed on myeloid cells 1 (TREM1) has been shown to help mediate the resistance of macrophages to HIV-1 cytotoxicity. HIV-infected macrophages increase expression levels of Bcl-2, TREM1, BCLXL, MFN1 and MFN2, translocating Bim to the mitochondria and decreasing Bcl-2 induced apoptosis. TREM1 silencing leads to significant increase in expressions of BAD and BAX, which results in apoptosis mediated by mitochondrial membrane disruption. TREM1 assist in upregulating BCL2 and mitofusins to avoid apoptosis (Campbell et al., 2019).

In addition, infected macrophages are capable of mediating apoptosis of CD4+ T cells through direct cell-to-cell contact, inducing ligands like FasL and tumor necrosis factor to selectively deplete CD4+ T cells (Badley et al., 1997). This resistance to the cytotoxic effects of HIV-1 enables macrophages to be reservoirs that can produce virus long term. Other mechanisms have been elucidated that may protect infected macrophages from the cytotoxic effects of HIV-1 infection, which suggests protection of macrophages as reservoirs by HIV-1. HIV-1 infected MDMs have been shown to have protection against oxidative stress through increased telomerase activity in comparison to uninfected MDMs. The infected MDMs had significantly less DNA damage when HIV-1 induced telomerase activity, which suggests the evolutionary advantages of sustaining macrophage infection (Reynoso et al., 2012). While mechanisms as to why infected macrophages are resistant to many HIV-1 cytotoxic effects are still under question, this highlights a potential strategy by HIV-1 in protecting macrophages and allowing them to be better suited, immunologically safe sanctuaries for sustained infection.

HIV-1 Induces IFN Resistance in Macrophages

HIV-1 viral fitness can be determined by resistance to type 1 interferon. Transmitted viruses with higher IFNα2 and IFNβ resistance are found to more likely replicate than those not resistant to interferon (Iyer et al., 2017). These viruses are capable of replicating and spreading more efficiently in CD4+ T cells when faced with the innate immune response. Interferon-induced transmembrane proteins (IFITMs) retract viral entry into macrophages and CD4+ T cells, which can be evaded through co-receptor binding by HIV-1 glycoproteins. Transmitted founder viruses are uniquely resistant to the antiviral activities of IFITMs, which suggests the higher fitness of these HIV-1 virions (Foster et al., 2016).

In primary macrophages, HIV-1 has been able to replicate without signaling the innate immune system even though reverse transcription uses genomic RNA that may trigger pattern recognition receptors (PRR) (**Figure 2B**) (Rasaiyaah et al., 2013). Upon entry into macrophages, HIV-1 capsid depletes cofactor cleavage and polyadenylation specificity factor subunit 6 (CPSF6) and cyclophilins to silence innate sensors triggering type 1 IFN response. This allows HIV-1 to remain hidden in primary

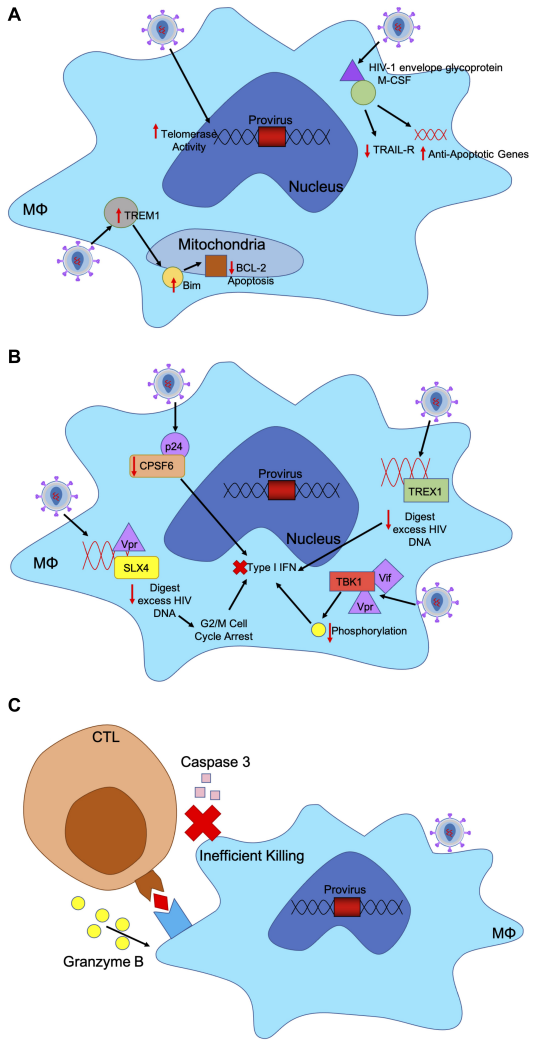

FIGURE 2 | HIV-1 has evolved mechanism to evade host immune responses in macrophages. **(A)** HIV-1 infected macrophages are refractory to the cytopathic effects of viral infection. Infected cells usually undergo apoptosis or cell-mediated killing upon viral infection. HIV-1 upregulates certain factors to evade apoptosis. HIV-1 envelope glycoprotein binds to M-CSF to downregulate TRAIL-receptor and upregulate anti-apoptotic genes. HIV-infected macrophages express higher levels of TREM1, which upregulates and translocates Bim to the mitochondria to downregulate BCL-2 induced apoptosis. Additionally, HIV-infected macrophages have increased telomerase activity, triggering less DNA damage and sustained infection. **(B)** HIV-1 induces mechanisms to evade IFN responses in infected macrophages through accessory proteins. TREX1 binds to excess HIV DNA during reverse transcription to suppress IFN responses. HIV-1 capsid binds to and removes CPSF6 and cyclophilins to silence downstream innate sensors of IFN pathways. HIV-1 Vif and Vpr bind to TBK1, preventing phosphorylation and downstream IFN induction. HIV-1 Vpr also binds to SLX4 complex with reverse transcribed HIV-1 DNA, like TREX1 to degrade nucleic acids and increase cellular replication stress, leading to G2/M cell cycle arrest and prevent IFN response. **(C)** CTLs recognize peptide antigens presented by MHC-I and release cytotoxic particles to kill infected cells. HIV-1 infected macrophages are resistant to CTL killing as CTLs require both granzyme B and caspase-3 to efficiently kill HIV-1 infected macrophages unlike CD4+ T cells which only need granzyme B. CTL binding to MHC-I only triggers release of granzyme B, which renders a much slower and less efficient killing of macrophages.

macrophages (Rasaiyaah et al., 2013). In both macrophages and CD4+ T cells TREX1 was found to also suppress HIV-1 triggered IFN responses by binding to and digesting excess HIV DNA, as well as blocking IFN signaling cascade through STING, TBK1, and IRF3 (Yan et al., 2010).

Moreover, HIV-1 accessory proteins Vpr and Vif are responsible for innate immune evasion in macrophages and dendritic cells. While HIV-1 viral RNA is detected by PRRs, Vif, and Vpr bind to TANK-binding kinase 1 (TBK1) and prevent phosphorylation, thereby blocking IFN induction (Harman et al., 2015). In addition, HIV-2 accessory protein Vpx eliminates the antiviral responses by degrading SAMHD1 in monocytes, macrophages and dendritic cells. SAMHD1 is responsible for restricting dNTPs available in the cytosol, thereby limiting the ability of HIV-1 to replicate in myeloid cells (Plitnik et al., 2018). SAMHD1 has been associated with negatively regulating IFN responses through unknown mechanisms (Rice et al., 2009). Lower levels of dNTPs and reverse transcription in myeloid cells may assist in preventing detection by IFN triggering responses. Consequently, HIV-1 does not have Vpx but Vpr to assist in downregulating antiviral responses. Vpr induces cell cycle arrest at the G2 to M phase transition through various mechanisms, therefore allowing HIV-1 to avoid detect by IFN responses (Andersen and Planelles, 2005; Bregnard et al., 2014; Laguette et al., 2014). Vpr binds to structure-specific endonuclease SLX4 complex to avoid innate immune surveillance. SLX4 complex is necessary for nucleic acid metabolism and repair in DNA lesions. When bound to by Vpr, SLX4 signals downstream cellular replication stress, which leads to G2/M cell cycle arrest (Laguette et al., 2014). SLX4 complex binds directly to newly reverse transcribed HIV-1 DNA and Vpr to inhibit HIV-1 driven type 1 IFN. SLX4 degrades excess nucleic acids derived from HIV-1 reverse transcription that may trigger PRRs and downstream IFN (Bregnard et al., 2014).

While HIV-1 inhibits type I IFN, HIV-1 stimulates interferon-stimulating genes (ISG) in macrophages by reverse transcription of HIV-1 RNA, triggering the RIG-I. HIV-1 Tat stimulates RIG-I, MAVS, IRF1, and IRF7, causing ISG IFN-induced protein with tetratricopeptide repeats (IFIT) to inhibit HIV-1 replication and production. By limiting HIV-1 replication, HIV-1 is rendered quiescent in macrophages, causing replication to persist at low levels and for infected macrophages to evade further immune responses (Nasr et al., 2017). Still, some HIV-1 viruses are highly susceptible to IFN responses, therefore HIV-1 has evolved mechanisms to evade the innate immune and antiviral state during infection. In macrophages specifically, HIV-1 has evolved mechanisms that avoid triggering antiviral responses and provide an immunologic sanctuary to protect HIV-1 against immune responses and sustain infection.

CTL Resistance in Macrophage Reservoirs

HIV-1 infection involves successful evasion of multiple antiviral mechanisms levied by both the innate and adaptive immune systems. While HIV-1 encodes accessory proteins that counteract cellular restriction factors of the innate immune system, HIV-1

must also escape detection from responses triggered by the adaptive immune response. As an intracellular pathogen, HIV-1 must evade attacks from antibodies and cytotoxic cells, such as CD8+ Cytotoxic T lymphocytes (CTLs) (Arcia et al., 2017). CTLs recognized cells expressing peptide antigens through major histocompatibility complex class I (MHC-I) on all nucleated host cells, allowing these cytotoxic cells the ability to identify and attack infected cells. With such a potent adaptive immune response, HIV-1 established infections in CD4+ T cells and macrophages have evolved mechanisms to evade CTL responses (Clayton et al., 2018). HIV-1 specific-CTLs are able to detect HIV-1 viral peptides expressed by MHC-I expressed on infected CD4+ T cells, prompting CTLs to attacked the infected cells. Studies in non-human primates models show that upon antibody depletion of CD8+ T cells, there is a spike in viral replication and viral load found in peripheral blood (Rainho et al., 2015). The critical role of CTLs in controlling HIV-1 infection is seen in HIV-1 elite controllers that maintain very low viral loads without ART. These elite controls have CTLs capable of proliferating more efficiently and producing higher levels of Perforin and Granzyme B (Migueles et al., 2002; Walker-Sperling et al., 2014).

While CTLs are one of the main immune responses involved in controlling HIV-1 infection, high genetic diversity and high mutation rate of the HIV-1 genome assist in evasion of CTL surveillance. CTLs impose one of the strongest selective pressures on HIV-1, thereby being the predominant driving force for HIV-1 evolution (Price et al., 1997; Jones et al., 2004; Allen et al., 2005; Streeck et al., 2006; Liu et al., 2011). Mutations in the HIV-1 genome due to selective pressure of the CTL immune response have been termed CD8 escape mutants. Immunodominant epitopes derived from HIV-1 have been correlated with a strong CTL response, which suggests CTLs with gag-restricted epitopes have higher response activity (Cao et al., 2003). During acute HIV-1 infection, virus-specific CTLs emerge with the ability to target HIV-1 and to secrete inflammatory molecules against the virus. In acute infection, CTLs deplete HIV-1 infected CD4+ T cells, allowing CTLs to partially control the infection (Allen et al., 2005).

Continued CTL responses allow for HIV-1 evolution by selecting for viral escape mutants capable of avoiding the adaptive immune response against HIV-1. The escape mutations are located in critical HIV-1 genome sites presented by MHC-1 as peptides for recognition by CTLs and restricted to those used by MHC-I. In chronic HIV-1 infection, CTL killing targets distinct epitopes in the *gag* region of the HIV-1 genome and structural proteins in *nef*. Over time, mutations in these *gag* epitopes are rapidly selected, allowing the virus to escape CTL immune responses. Cao et al. (2003) have found patients partaking in ongoing ART are protected against the evolution of viral escape mutants due to limited viral replication. For those not on ART, when the viral load decreases due to immune responses, viral strains with CD8 escape mutants comprise the majority of the viral population.

In addition, HIV-1 accessory protein Nef has been shown to assist HIV-1 in successful evasion of CTLs through mislocalization and degradation of MHC-I in the infected cell (Collins et al., 1998). Nef directly acts as an anchor to stabilize the bond between MHC-1 and the clathrin-dependent trafficking through clathrin adaptor protein-1 (AP-1) (Roeth et al., 2004). AP-1 binding of MHC-I directs MHC-I to degradation more rapidly, thereby reducing the amount of MHC-I found on the cell surface (Collins and Collins, 2014). By downregulating MHC-I on CD4+ T cells and macrophages, CTLs are not presented with HIV-1 antigen peptides, which allows HIV-1 infected cells to escape immune surveillance.

Most of the attention on the impact of CTL activity has been on HIV-1 infection of CD4+ T cells while the impact of CTL on HIV-1 infection of macrophages has been understudied. Clayton et al. (2018) found that HIV-infected macrophages are inefficiently killed by CTLs due to an intrinsic resistance of macrophages to CTL killing (**Figure 2C**). CTLs require both granzyme B and caspase-3 to efficiently kill HIV-1 infected macrophages, while granzyme B is not needed in CTL-mediated killing of CD4+ T cells, allowing HIV-1 more resistance to CTLs when hidden in macrophages. For CTLs to effectively kill infected macrophages, longer cell-to-cell contact time and greater interferon responses are needed to trigger macrophage death (Clayton et al., 2018).

Rainho et al. (2015) demonstrated that SIV-specific CTLs are not able to effectively kill SIV-infected macrophages in macaques while CTLs are able to eliminate SIV-infected CD4+ T cells. While Nef is necessary to avoid CTL-mediated killing of CD4+ T cells, macrophages infected with *nef*-deficient HIV-1 variants were still refractory to CTL responses. This suggests macrophages have intrinsic characteristics and other mechanisms that provide resistance to CTLs (Collins and Collins, 2014; Rainho et al., 2015). Through a combination of viral escape mutations, accessory protein inhibition of cellular restriction factors, and capase-3-dependent killing, HIV-1 has been able to overcome the host's adaptive immune responses. These determinants underscore the ability of HIV-1 to persist in the body. While HIV-1 itself has evolved mechanisms to evade CTL-killing, macrophages provide an immune safe haven for HIV-1 without the evolutionary need to evade CTL as macrophages are intrinsically refractory to affects from CTLs.

MACROPHAGE RESERVOIR MODELS, STUDIES AND ERADICATION STRATEGIES

Humanized Mice: Mice Lacking T Cells Have Sustained HIV-1 Replication via Macrophages

To adequately study HIV-1 infection of macrophages *in vivo,* macrophages must be isolated and donated from elective surgeries and post-mortem tissue; however, such analysis does not provide a window for current infection or thorough exploration to outlying factors the host may contribute. Due to challenges in receiving human donor tissue to further study ART and viral reservoirs *in vivo*, humanized mice models have been

designed to simulate HIV-1 infection in humans and study HIV-1 *in vivo* (Hermankova et al., 2003).

In creating these humanized mice models, researchers have trialed various strategies to design accurate models of HIV-1 infection *in vivo*. These strategies have determined suitable immunodeficient mice strains able to maintain HIV-1 infection; which human stem cells or tissue are engrafted; how mice immune cells are irradiated; and how human cells or tissue will be injected or engrafted into the mice. The engraftment of immunodeficient mice with live human cells and tissues has allowed for *in vivo* HIV-1 studies and HIV-1 preclinical research (Weichseldorfer et al., 2020). Scientists have used humanized severe combined immunodeficient (SCID) mice models to analyze HIV-1 infection and replication, as well as the efficacy of ART. These humanized SCID mice are repopulated with human hematopoietic stem cells (HSCs), human peripheral blood lymphocytes (PBLs), or with human tissue (McCune et al., 1988; Mosier et al., 1988; Namikawa et al., 1988). Tissue engraftment is typically from human fetal thymus or liver tissue (McCune et al., 1988). These mice models are capable of sustaining HIV-1 infection, replicating acute HIV-1 infection with limited CD4+ T cell lifespan (Weichseldorfer et al., 2020).

NOD-SCID (NSG) mice have gene mutations to minimize murine cells and better replicate the human immune system (Chen et al., 2009; Rongvaux et al., 2014). These mice models were eventually engrafted with human CD34+ HSCs to fully study HIV-1 persistence and latent reservoirs as the mice supported longer human cell lifespans and hematopoiesis (Brehm et al., 2010; Arainga et al., 2016). Additionally, these HSC engrafted humanized NSG mice developed their own lymphoid system from the HSCs, with lymphoid tissue capable of sustaining latent reservoirs upon HIV-1 infection. This model opened an avenue for in depth reservoir and latency research *in vivo*. Moreover, HIV latency can be duplicated in HIV-infected Human Immune System (HIS) mice under ART, opening a window into assessing HIV latency and distinct treatments that can eradicate HIV reservoirs (Churchill et al., 2016; Olesen et al., 2016; Deruaz and Tager, 2017). To assess these models, novel *in vivo* murine viral outgrowth assays have been developed to detect virus from undetectable viral load or to circumscribe whether elimination approaches are efficient in clinical trials (Metcalf Pate et al., 2015; Charlins et al., 2017; Descours et al., 2017).

Humanized mice models have allowed for the study of myeloid reservoirs in combination with T cells or as a single reservoir in Bone marrow, lymphoid, thyroid (BLT) mice. These mice are transplanted with hematopoietic stem cells and are responsive to ART (Arainga et al., 2017). BLT mice infected with HIV-1 have detectable HIV-1 DNA and RNA from T cells and macrophages (Honeycutt et al., 2016). Recent studies utilizing T-cell-only (TOM) and myeloid-only (MOM) HIS mice have revealed latent CD4+ T cell and myeloid HIV reservoirs can develop independently (Honeycutt et al., 2013; Honeycutt et al., 2017). In a specific mice model, NOD/SCID mice were engrafted with only hematopoietic stem cells but unable to support lymphocytes, which allowed for only myeloid cell growth. Upon infection with macrophage tropic HIV-1, efficient infection and sustained replication of HIV-1 persisted in these Myeloid-Only Mice even in the presence of ART (Honeycutt et al., 2016). Furthermore, HIV disseminated extensively to various tissue compartments in these mice—including the brain—heavily suggesting that myeloid cells can remain a source of HIV composition *in vivo* (Baxter et al., 2014; Calantone et al., 2014; Honeycutt et al., 2017). Unfortunately, these MOM models have a short turnover of around 1 day for human macrophages, greatly underestimating the half-life of macrophages found in the human body. This emphasizes that conditions even in animal models do not fully characterize *in vivo* infection of HIV-1 in humans.

Although there are some limitations shown in murine models, they address the existence of macrophage reservoirs in sustaining persistent HIV-1 infection that is not available from human tissue studies. Further advancement of murine qVOA studies may elucidate detection of HIV-1 in low levels from quiescent macrophage reservoirs in future studies. These murine models are an invaluable resource in the limitation of human *in vivo* studies and allow for further investigation into macrophage reservoirs and their contribution to HIV-1 pathogenesis.

Tissue Macrophages Roles as HIV-1 Reservoirs

Tissue resident macrophages have been implicated in sustaining HIV-1 infection *in vivo* and producing virions *in vitro,* with the earliest evidence of HIV infection of macrophages in 1987 (Gartner et al., 1986; Perelson et al., 1997). The first confirmation highlighting the role of macrophages in HIV-1 persistence was described by Igarashi et al. (2001). When CD4+ T cells were depleted from macaques infected with SHIV, the infection was independently sustained by macrophages. After administration of a potent reverse transcriptase inhibitor, viral production was blocked in circulating CD4+ T cells but not in tissue macrophages, demonstrating that tissue macrophages can sustain HIV-1 infection alone (Igarashi et al., 2001).

Under cART, very low levels of free virus are found in plasma. ART-naïve patients were placed on cART treatment and monitored for HIV-1 RNA concentration in plasma. After 8 weeks of treatment, plasma viremia dropped below detectable levels and no infectious virus was found in PBMCs, yet there was still a secondary source of viremia. The residual viremia from these cART-suppressed patients has shown HIV-1 originates from more than the resting CD4+ T cell population, postulated to be tissue macrophages (Perelson et al., 1997). In a study by Bailey et al. (2006), through intensive sampling plasma and PBMCs from chronically infected patients, a predominant plasma sequence was found that was not related to those proviral sequences found in resting CD4+ T cells. The origin of the small number of clones, while not identified, has evidence of coming from a reservoir different than circulating CD4+ T cells (Bailey et al., 2006). Recrudescing viremia gives an opportunity to study the origin of rebounding viruses as they emerge from their cellular reservoir (Vibholm et al., 2019). While lymph nodes carry the majority of latent virus found in circulation, those that rebound during treatment interruption are not the same

(Vibholm et al., 2019). Residual viremia from cART-suppressed patients is found to have genetically distinct genomes from proviruses found in resting CD4+ T cell, coming from an unidentified source (Brennan et al., 2009). From heavy sampling of residual viremia, some genomes originated from monocytes and unfractionated PBMC.

In a recent study by Andrade et al. (2020), rebound viremia was interrogated from chronically infected patients undergoing analytical treatment interruption (ATI). Single viral genomes were isolated and cloned. While most clones showed T-cell tropism, a small population of highly macrophage tropic clones were identified containing macrophage-specific markers from four patients. The group also enriched those M-tropic viruses from post-ATI plasma with macrophage specific markers CD14 antibody to confirm that M-tropic viruses had a macrophage origin as the viral envelopes contained CD14 from macrophage cell membrane. These results suggest macrophages are a viral reservoir that generate rebound viremia (Andrade et al., 2020).

While it remains unknown whether the source of viral rebound in patients treated during acute infection differs from chronic infection, there is evidence that HIV-1 establishes latent infection early on in both CD4+ T cells and macrophages. For Andrade et al. (2020) using molecular clock analysis, some of macrophage-tropic HIV-1 isolates were found to predate the start of cART and suggest that macrophage reservoirs are established in acute infection and assist in HIV-1 persistence. Furthermore, in many tissues like the lymph nodes, active reservoirs have been found as in quiescent CD4+ T cells and macrophages that have low levels of replication that sustain viral infection even in individuals that have progressed to AIDS (Embretson et al., 1993). For chronic phase infection, the diversity of HIV-1 viruses isolated during viral rebound is incredibly high, with plasma and proviral sequences intermingling and differing in phylogenetic trees (Bailey et al., 2006). This correlates with the differences in sizes of HIV-1 reservoirs as those treated during acute infection have much smaller reservoirs, while those treated during chronic infection have much larger HIV-1 reservoirs and therefore more diversity (Li et al., 2016).

Other studies use distinct strategies to find DNA and RNA of HIV-1 in several tissue compartments of animal models (Orenstein et al., 1997; Igarashi et al., 2001; Swingler et al., 2007; Cribbs et al., 2015). For instance, ART-suppressed humanized mice were infected with macrophage tropic virus to investigate the ability of virally infected cells to build reservoirs. The group detected HIV-1 DNA and RNA in mature macrophages in all treated mice (Arainga et al., 2017). Another study using a SIV/macaque model for HIV-1 encephalitis and AIDS demonstrated infected microglia persisted in the brain in presence of ART (Avalos et al., 2017). In addition, Avalos et al. (2017) exhibited that most suppressed macaques contained latently infected microglial cells and that virus produced by macrophage qVOA was infectious and replication-competent, which suggests microglia are capable of maintaining and reestablishing productive infection upon treatment interruption in macaques.

In human tissue, HIV DNA and RNA was found in the resident macrophages of the lungs, gut and male genital tract from ART-suppressed HIV-1 infected individuals. Cribbs et al. (2015) evaluated the presence of proviral DNA in alveolar macrophages from HIV-1 infected individuals under ART, displaying that alveolar macrophages harbor HIV-1 and may be a potential reservoir. Furthermore, a study has found evidence that human urethral tissue macrophage could constitute a principal HIV-1 reservoir. Ganor et al. (2019) demonstrated urethral penile tissue macrophages have integrated HIV-1 DNA, RNA, proteins, and intact virion. These macrophages were stimulated with lipopolysaccharide on urethral single cells suspension from HIV-1/cART individuals and showed reactivation of HIV-1 through modified qVOA supporting that tissue macrophages have replication-competent virus from integrated HIV-1 DNA, which was not evident in CD4+ T cells stimulated with PHC (Ganor et al., 2019). Thus, macrophages are shown to be the principal reservoir in urethral tissue containing integrated HIV-1 DNA that can induce outgrowth of replication-competent infectious HIV-1. Collectively, these findings have demonstrated that macrophages sustain latent HIV-1 infection and assist in HIV-1 persistence. With this, macrophages are shown to be a viable reservoir that contribute to obstacles in eradicating HIV-1.

Latency Reverse Agents and Challenges of Studying Macrophages Reservoir

While ART is capable of limiting HIV-1 replication, ART alone does not eradicate HIV-1 as these inhibitors are not able to directly attack the latent HIV-1. Several latency reversing agents (LRAs) have been identified and used to reactivate latent HIV-1 from their proviral state in cellular reservoirs *in vitro* and *ex vivo* (Nakamura et al., 2013). Many of these LRAs are designed to reactivate HIV-1 in latent CD4+ T cells, so accessing and reactivating latent virus in macrophages is still largely unknown. Macrophage reservoirs are present in compartmentalized tissues, including the CNS, which may contribute to the lack of efficacy in eliminating infected cells and the failure of reactivating latent proviral DNA as these tissues remain largely unaffected by LRA compounds. The topic of HIV latency within macrophages is debatable, highlighting the need for LRAs on this cell type.

There are six primary groups of LRAs categorized by their mechanism of action within the host cell. These six groups can be categorized as histone post-translational modification modulators, non-histone chromatin modulators, NF-κB stimulators, TLR agonists, extracellular stimulators, and miscellaneous, which is comprised of unique and uncommon compounds (Abner and Jordan, 2019). The histone post-translational modification modulators group includes histones methyltransferase (HMT) and histones deacetylase inhibitors (HDACi). These compounds function by regulating histone tail modulation of nucleosomes with integrated HIV-1 and reversing the latent provirus to active. A few drugs previously approved for cancer treatment are currently being investigated as potential LRAs, such as valproic acid, vorinostat, panobinostat, and romidepsin. Deeks (2012) demonstrated a potential therapeutic approach called "Shock-and-Kill" that accesses latent virus in infected cells by forcing them to become active from a quiescent state, thereby killing active virus and infected cells to eliminate

the viral reservoir (Nakamura et al., 2013). Archin et al. (2012) tested the shock-and-kill approach in a clinical trial using vorinostat that activated viral replication in HIV-1+ individuals (Lanktree et al., 2011; Archin et al., 2017). Activation of HIV-1 genes was validated by a noticeable upregulation of viral RNA synthesis. Subsequently, other clinical trials were conducted using vorinostat for longer periods of time, which confirmed an increase of HIV-1 cell-associated RNA in circulating resting CD4+ T cells and activated the latent CD4+ T cell reservoir (Archin et al., 2014a,b, 2017; Arcia et al., 2017).

Some studies administered HDACi *in vitro* in the monocytic U1 cell line and MDMs, which have shown not only reactivation but decreased HIV-1 release and degradation of viral particles (Rasmussen et al., 2013; Campbell et al., 2015); however, HDACis at higher doses can cause numerous side-effects and are considered weak LRAs. Another particularly important LRA category is NF-κB stimulators, which are Protein Kinase C (PKC) pathway agonists leading to upregulation of transcription factor NF-κB to reactivate HIV-1. Several PKC agonists, like prostratin and ingenols, have also been shown to effectively reactivate latent HIV-1 targeting different pathways in T cells, as well as in monocytic cell lines (Fernandez et al., 2013; Abreu et al., 2014; Darcis et al., 2015; Jiang et al., 2015).

Due to the deficiencies of administration of only one LRA, combinatory "shocks" have taken place in other studies (Margolis and Hazuda, 2013). Combining HDAC inhibitors themselves, or with other classes of LRA drugs, shows increased efficacy at latency reversal since it generates a highly synergic action to reactivate HIV-1. Darcis et al. (2015) combined Bromodomain and Extraterminal (BET) bromodomain inhibitors (BETi) with NF-kB inducing agents, and *ex vivo* models have shown their combination led to synergistic activation of HIV-1 expression at the viral mRNA and protein levels. The combination used *in vitro* of the PKC agonists and P-TEFb-releasing agents administrated together on HIV-1 post-integration latency model cell lines of T-lymphoid and myeloid lineages also have shown the synergistic reactivation of viral components. These results constitute one of the first demonstrations of combined LRA therapies and has shown promising strategies in reducing the size of the total HIV-1 reservoir (Darcis et al., 2015).

Most studies with LRAs evaluate the efficacy of reverse latency in blood compartments, focusing on their effects on resting memory CD4+ T cells with latent provirus (Spivak et al., 2016; Gupta and Dixit, 2018; Spivak and Planelles, 2018). In contrast, the subject regarding macrophages in the CNS is just beginning to be thoroughly explored. The reservoir studies of the CNS are considered particularly challenging due to several characteristics of the environment. First, the CNS is protected by the blood brain barrier and the Choroid plexus, which limits drug access. The CNS is an immune privileged environment, complicating assessment of drug penetration and effectiveness. In considering if an LRA could possibly clear these viruses, there may be an issue with inflammation and neurotoxicity as negative side effects in the brain compartment (Marban et al., 2016). Additionally, the difference in macrophage cell markers and receptors impact drug efficiency. Despite these impediments, there has been evidence that administration of LRA would have beneficial eradication effects on macrophage reservoir.

Campbell et al. (2015) have shown some differences between the effects of HDACis in CD4+ T cells and macrophages, as well as microglia. For macrophages, HDACis could induce autophagy pathways *in vitro* and achieves the inhibition of HIV-1 replication without reaching cell death. In contrast in CD4+ T cells, the administration of HDACis have the ability to reactivate the quiescent transcriptional viruses to be killed. Gama et al. (2017) described a possible effective administration of LRAs on virally suppressed macaques infected with SIV where reactivation of the virus in CNS was observed. The study showed an increase of viral load within the Cerebral Spinal Fluid, which highlights the brain as an important viral reservoir compartment (Gama et al., 2017).

Gray et al. (2016a; 2016b) have studied the toxicity of several commonly used LRA, such as Panobinostat, Romidepsin, and vorinostat, in primary astrocytes and MDMs. The results demonstrated that the administration of therapeutic concentrations was not toxic for these cells. Also, the group noted a greater viral reactivation in primary astrocytes, which could suggest a possibility of activation of latently infected cells in the CNS (Gray et al., 2016a,b). Jiang et al. (2015) utilized combinations of LRAs on well-studied HIV-1 latency lymphocyte and promonocyte cell culture models. The results of the study showed a potential synergism that englobe PEP005 on reactivate latency on J-Lat A1 cells and U1 cells (Jiang et al., 2015). The use of LRAs on macrophages is still in its early stages of research, but initial data and synergism reveal a promising approach that may to advance HIV-1 eradication strategies.

The HIV-1 reservoir field faces several challenges to elucidate mechanisms of HIV-1 persistence. The establishment of cellular reservoir can occur in the early days after individuals are infected. Considering that ART can start as early as a few months after the day of infection, HIV-1 integrates its genome into the host genome early in infection. Host cells will carry the genetic information necessary to produce new infectious virions for the cell's lifetime, and the virus will persist at the individual system. For that reason, reservoir studies have been focused on the development tools to target and measure the cellular reservoir, and specifically latent resting memory CD4+ T cells. The lack of specific markers that could distinguish latently infected from uninfected cells represents a significant impediment in the design of a cure. The study of macrophage reservoirs is even more challenging. Unlike circulating CD4+ T cells, macrophages reside in every tissue in the human body and consequently become a unique population phenotypically distinct from others (Jambo et al., 2014). In addition, the complexity of these reservoir is due to their resistance of cytopathic effect, which leads to a long-lived macrophage and contribute to one of the barriers for elimination. Identification and use of LRAs for eradication of macrophage reservoirs has been limited through sanctuary tissue states. While certain LRAs for macrophages, like lipopolysaccharide, have been identified, their efficiencies are not well understood and limit the advancement of cure strategies in targeting cell specific reservoirs (Ganor et al., 2019).

CONCLUSION

Since the discovery of HIV-1, a cure for HIV/AIDs has eluded researchers. With the introduction of ART to HIV-infected individuals, HIV-1 has become a chronic disease in which focus has shifted from treatment to finding a sterilizing or functional cure. With 38 million individuals currently living with HIV-1, efforts to fully understand cellular reservoirs and mechanisms of viral persistence are vital to reaching this goal. Attention will need to shift to further investigate myeloid reservoirs as literature over the past few decades has shown macrophages are not only susceptible to HIV-1 infection but also assist in sustaining viral persistence, even when CD4+ T cells are not present. A comprehensive understanding as to how HIV-1-infected macrophages are capable of avoiding both innate and adaptive immune responses, and the cytotoxic effects of viral infection, is necessary to discover ways to target and eradicate macrophage reservoirs. While many discoveries as to the mechanisms for why macrophages have been made, much of how macrophage reservoirs contribute to HIV-1 persistence remains unknown. Macrophages heterogeneity and biological characteristics makes it difficult to study macrophages' whole population due to differences in susceptibility and infection based on tissue compartmentalization. Such characteristics affect the outcomes for viral persistence and efficient ways to eradicate macrophage reservoirs. While there has been accumulating evidence supporting macrophage reservoirs contribution to viral persistence, much of their mechanisms and contributions to viral persistence remain unknown. Future studies must focus on ways to eliminate both the latent CD4+ T cell reservoir and macrophage reservoir for there to be a potential cure for HIV-1. This, together with the stigma of HIV-1 infection, drives the rationale for developing a cure.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Cleavage of TANK-Binding Kinase 1 by HIV-1 Protease Triggers Viral Innate Immune Evasion

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Type-I interferons (IFN-I) are the innate immune system's principal defense against viral infections. Human immunodeficiency virus-1 (HIV-1) has evolved several ways to suppress or evade the host's innate immunity in order to survive and replicate to sustain infection. Suppression of IFN-I is one among the multiple escape strategies used by HIV-1 to prevent its clearance. HIV-1 protease which helps in viral maturation has also been observed to cleave host cellular protein kinases. In this study we performed a comprehensive screening of a human kinase library using AlphaScreen assay and identified that TANK binding kinase-1 (TBK1) was cleaved by HIV-1 protease (PR). We demonstrate that PR cleaved TBK1 fails to phosphorylate IFN regulatory factor 3 (IRF3), thereby reducing the IFN-I promoter activity and further reveal that the PR mediated suppression of IFN-I could be counteracted by protease inhibitors (PI) *in vitro*. We have also revealed that mutations of HIV-1 PR that confer drug resistance to PIs reduce the enzyme's ability to cleave TBK1. The findings of this study unearth a direct link between HIV-1 PR activity and evasion of innate immunity by the virus, the possible physiological relevance of which warrants to be determined.

Keywords: HIV-1 protease, type-I interferon, immune evasion, TANK binding kinase-1, pathogenic fitness

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INTRODUCTION

The innate immune system acts as the broad defense against viral infections (Koyama et al., 2008). Viruses being obligate intracellular organisms, the host cells are equipped with specific antiviral restriction proteins and non-specific pattern recognition receptors (PRRs) to fight these invading pathogens. While the former directly and specifically blocks various stages of viral replication, the latter non-specifically recognizes foreign molecules to trigger the downstream gene induction programs such as the production of interferons (IFN) (Colomer-Lluch et al., 2018). Type-I and III IFNs are produced by a wide variety of cells upon exposure to viruses and provide an instant and brisk antiviral effect to limit the incoming pathogen. Type-II IFN on the other hand is produced by natural killer cells and T-cells and helps in macrophage activation and serves as a link between innate and adaptive immunity (Ferreira et al., 2018; Lee and Ashkar, 2018).

The type-I IFNs are the principal first line of defense against viral infections, and failure to mount an effective IFN response leads to widespread systemic infection in the host (Huang et al., 2019). With respect to the human immunodeficiency virus (HIV), innate immunity is of paramount

importance to control HIV-1 infection as it is the principal way not only to prevent widespread establishment of latent reservoirs, but also to deplete them (Soper et al., 2017; Palermo et al., 2019). The success of other strategies to eliminate latent reservoirs for a functional cure could be enhanced if the innate immunity against HIV-1 is strengthened (Altfeld and Gale, 2015).

Type-I and III IFNs are produced through one or more intracellular signaling pathways involving several proteins, which are initiated by the sensing of pathogen-associated molecular patterns (PAMPs) by the PRRs. PAMP recognition by PRRs leads to recruitment of various intracellular adaptor proteins to form a signaling complex which orchestrates the downstream events leading to IFN production. Despite the diverse types of PRRs providing the upstream sensing signals, the TRAF Family Member Associated NFKB Activator protein binding kinase-1 (TBK1) is found to be invariably involved in the signaling complexes of almost all the type-I and III IFN induction pathways currently identified (Beachboard and Horner, 2016; Ni et al., 2018). The key event is the autophosphorylation of TBK1 in the signaling complex. Phosphorylated TBK1 in turn phosphorylates IFN regulatory factor 3 (IRF3) which undergoes dimerization, detaches from the signaling complex and translocates to the nucleus to bind with specific promoters inducing type-I and III IFN production (Hu et al., 2018).

Viruses have evolved along with their hosts and possess mechanisms to overcome or evade innate immunity in order to propagate within the host (Domingo-Calap et al., 2019). Viruses lacking this ability could not infect and replicate inside the host (Iyer et al., 2017). The human immunodeficiency virus (HIV) has developed several ways to thwart the defenses posed by the innate immune system (Yin et al., 2020). These could be broadly classified into two; counteraction of host restriction factors and attenuation of IFN response. The accessory proteins of HIV have shown to counteract the effects of various cellular anti-retroviral restriction factors such as APOBEC3G, SAMHD1, TRIM5α, tetherin and others (Colomer-Lluch et al., 2018). The strategies employed by HIV-1 to dampen the IFN response can be further classified into HIV-1 protease (PR) mediated or HIV-1 PR independent mechanisms. The salient PR independent mechanisms include; evading cytosolic PRR sensing by hijacking host factors to mask its PAMPs (Ringeard et al., 2019), reducing their time of stay in the cytoplasm to minimize the chances of PRR detection (Dharan et al., 2017) and deploying one or more viral accessory proteins to attenuate the functions of cellular molecules involved in signaling pathways of IFN production (Beachboard and Horner, 2016).

Although HIV-1 PR was initially thought to only participate in maturation of viral progeny with their intra viral action in extracellular viruses, recent findings have revealed several mechanisms by which HIV-1 PR interferes with host cell proteins to suppress IFN production and innate immunity (Rajput et al., 2011; Wagner et al., 2015). In the present study, we identify yet another novel, PR dependent immune evasion mechanism used by HIV-1 to suppress IFN production. We demonstrate the HIV-1 PR-mediated proteolytic cleavage of TBK1, the key signaling molecule of type I and III IFN production pathways. We also confirm this finding by demonstrating the inability of the cleaved TBK1 to activate IRF3, thereby diminishing the downstream signaling events of IFN induction pathway and the reversal of this effect by protease inhibitors (PI). Furthermore, we show that drug resistance mutations of HIV-1 PR to PIs reduce the TBK1 cleaving ability of HIV-1 PR which could probably reflect the loss in the pathological fitness with evolution.

MATERIALS AND METHODS

Cell Lines and Transfection

HEK293 cells from ATCC were cultured in DMEM supplemented with 10% (V/V) fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) in a 5% CO_2 at 37°C. To generate T7 cells (THP-1 cells encoding HIV-1 provirus), cells were infected with VSV-G-pseudotyped HIV-1(ΔEnv)-Luc at a multiplicity of infection (MOI) of 0.5 and selected by limiting dilution. T7 cells showed low expression of luciferase in the steady state, but luciferase activity increased when TNF-α was added, suggesting that the viral genome was latent. THP1 and T7 cells were grown in RPMI containing 10% FBS and 1% PS in 5% CO_2 at 37°C. Cell transfection was performed using lipofectamine3000 (Thermo), according to the manufacturer's instructions.

Plasmids

Human kinase library is listed in **Supplementary Table 1** with sequence data of each kinase obtained from HUGO gene nomenclature committee (HNGC), accessible at https://www.genenames.org/. TBK1 cDNA was subcloned into pCMV-HA vector (Clontech) or pEU (CellFree Sciences). TBK1 mutants were generated by PrimeSTAR mutagenesis basal kit (Takara). Plasmids encoding HIV-1 Gag and Gag-Pol were described previously (Kudoh et al., 2014). FLAG-p6*PR was synthesized as explained in previous study (Chiu et al., 2006). pGL3-based IFNβ-promoter plasmid was obtained from addgene (#102597). HIV-1 drug resistant mutants were contributed by Dr. Masashi Tatsumi.

Wheat Germ Cell Free Protein Production

In vitro transcription and cell-free protein synthesis was performed as described previously (Sawasaki et al., 2005). Transcripts were made from each of the DNA templates mentioned above using SP6 RNA polymerase. The synthetic mRNAs were then precipitated with ethanol, collected by centrifugation and washed. Each mRNA (typically 30–35 μg) was added to the translation mixture and the translation reaction was performed in the bilayer mode with slight modifications (Sawasaki et al., 2002). The translation mixture that formed the bottom layer consisted of 60 A260 units of wheat germ extract (CellFree Sciences) and 2 μg creatine kinase (Roche Diagnostics K. K., Tokyo, Japan) in 25 μl SUB-AMIX solution (CellFree Sciences). SUB-AMIX contained (final concentrations) 30 mM Hepes/KOH at pH 8.0, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 4 mM DTT, 0.4 mM spermidine, 0.3 mM each of the 20 amino acids, 2.7 mM magnesium acetate, and 100 mM potassium acetate. SUB-AMIX (125 μl) was placed on the top of the translation mixture, forming the upper

layer. After incubation at 16°C for 16 h, protein synthesis was confirmed by SDS-PAGE. For biotin labeling, 1 μl (50 ng) of crude biotin ligase (BirA) produced by the wheat germ cell-free expression system was added to the bottom layer, and 0.5 μM (final concentration) of D-biotin (Nacalai Tesque, Inc., Kyoto, Japan) was added to both upper and bottom layers, as described previously (Matsuoka et al., 2010).

AlphaScreen Assay

In vitro cleavage activity assays of HIV-1 PR were carried out in a total volume of 15 μl consisting of 100 mM Tris–HCl pH 8.0, 0.01% Tween-20, 1 mg/ml BSA, 1 μl crude recombinant protease (∼ 0.75 μM) and 0.5 μl crude recombinant FLAG-biotin-tagged CA/NC (∼ 0.037 μM) at 37°C for 1 h in a 384-well Optiplate (PerkinElmer, Boston, MA, United States). To assay the effects of HIV-1 PR on various human protein kinases, 3 μl HIV-1 PR and human PK each was incubated at 37°C for 10 min, FLAG-biotin-tagged CA/NC or GST-biotin-tagged p2–p7 was added and the reaction further incubated at 37°C for 1 h in a 384-well Optiplate. In accordance with the AlphaScreen IgG (Protein A) detection kit (PerkinElmer) instruction manual, 10 μl of detection mixture containing 100 mM Tris–HCl pH 8.0, 0.01% Tween-20, 1 mg/ml BSA, 5 μg/ml Anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, United States) or Anti-GST antibody (GE Healthcare, Buckinghamshire, United Kingdom), 0.1 μl streptavidin-coated donor beads and 0.1 μl anti-IgG (Protein A) acceptor beads were added to each well followed by incubation at 26°C for 1 h. Luminescence was analyzed by the AlphaScreen detection program. Each assay was performed in triplicate, and the data represent the means and standard deviations of three independent experiments.

Luciferase Assay

HEK293 cells in 12-well plates transfected with plasmids encoding IFNβ-promoter-Luc (100 ng), HA-TBK1 (100–400 ng), and p6*PR (100 ng) were allowed for 48 h of incubation and were then lysed and added with equivalent volume of Bright-Glo Substrate (Promega). Luciferase activity was measured with GloMax Discover System (Promega).

Immunoblotting and Protein Sequencing

For recombinant protein analysis, 3 μl crude recombinant viral protease (∼ 0.75 μM) and 7 μl crude FLAG-biotin-tagged recombinant proteins were incubated at 37°C for 2 h. To assay the effect of HIV protease inhibitors, 3 μl crude recombinant HIV-1 protease and 1 μl of 10 μM protease inhibitor amprenavir (Sigma-Aldrich) were incubated at 37°C for 10 min followed by addition of 6 μl crude FLAG-biotin-tagged recombinant proteins and incubated at 37°C for 120 min. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore) according to standard procedures. Immunoblot analysis was carried out with anti-FLAG (M2) antibodies (Sigma-Aldrich) or Streptavidin-HRP conjugate (GE Healthcare) according to the procedure described above. For fluorescent imaging, immunoblotted proteins were detected by Alexa592-anti-mouse antibodies (N-cleaved fragments), and Alexa488-streptavidin (C-cleaved fragments). The labeled proteins were visualized using a Typhoon Imager (GE Healthcare). To determine the cleavage site in TBK1 by HIV-1 PR, the cleaved fragments on the PVDF membrane were extracted and washed with methanol and were outsourced to commercial laboratory for sequencing.

For intracellular protein analysis, cells expressing GagPol and TBK1 were harvested with immunoblotting sample buffer. In **Figure 2B**, cells were treated with APV (0.02–2 μM) 16 h before harvesting. After SDS-PAGE and subsequent membrane transfer, the membranes were probed with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). The primary antibodies used were as follows: anti-HA (Roche), FLAG (Sigma-Aldrich), Gag p24 (NIH AIDS Reagent Program), IRF3, IRF3(pS396), TBK1(pS172) (Cell Signaling Technology), and vinculin (Sigma-Aldrich). Detected proteins were visualized using a FluorChem digital imaging system (Alpha Innotech). Band analysis was performed with ImageJ software (National Institutes of Health).

Immunofluorescence Analysis

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100, and then were blocked with Blocking One (Nacalai) at room temperature for 15 min. The cells were incubated with antibodies against HIV-1 p24 antigen (NIH AIDS Reagent Program) and IRF3 (Cell Signaling Technology) at room temperature for 1 h. After incubation, cells were stained with Alexa 568 or 488-labeled anti-IgG antibody (Thermo) for 1 h at room temperature. The nucleus was stained with ProLong Gold Antifade Mountant with DAPI (Thermo). The images were taken by fluorescence microscope BZ-9000 (Keyence).

Protease vs. TBK1 in Infection Model

The HIV-1 latently infected T7 or the uninfected THP1 monocytic cell lines were transfected with 10 ng/ml poly(I:C) (Sigma-Aldrich) in 12 well plates using lipofectamine3000 (Thermo), according to the manufacturer's instructions (Ranganath et al., 2016). Bryostatin-1 (Sigma-Aldrich) at a final concentration of 25 ng/ml was included in the medium of T7 cells immediately after poly(I:C) transfection, followed by the addition of 10 μM of the protease inhibitor indinavir (Sigma-Aldrich) in respective wells. The cells were incubated in 5% CO_2 at 37°C. After 48 h of incubation, mRNA was extracted from cells using RNeasy mini kit (QIAGEN). The amount and purity of the mRNA was assessed using NanoDrop One (Thermo Scientific) and stored at −80°C until further use. Reverse transcription was performed using ReverTra Ace qPCR-RT kit (TOYOBO). PCR reagents were purchased from Takara and the primers for IFNβ1 and ISG15 were obtained from Eurofins with the following sequences; IFNβ1 Fwd: GTCAGAGTGGAAATCCTAAG and IFNβ1 Rev: ACAGCATCTGCTGGTTGAAG (Hu et al., 2010) and ISG15 Fwd: CTCTGAGCATCCTGGTGAGGAA and ISG15 Rev: AAGGTCAGCCAGAACAGGTCGT (Matsunaga et al., 2020). The expression of genes was quantified using CFX96^{TM} Real-time system (Bio-Rad) and normalized against the expression of β-actin in the corresponding cells.

Statistical Analysis

All bar graphs present means and standard deviation obtained from three replicates. The statistical significance of differences between two groups was evaluated by two-tailed unpaired *t*-test. A p value of <0.05 was considered statistically significant.

RESULTS

Screening for Human Kinases Cleaved by HIV-1 Protease

HIV-1 PR is an essential enzyme required for the virus to complete its life cycle. PR gets packed into the immature virions that bud out of the infected cells and cleave the viral precursor proteins into their functional form thereby generating mature extracellular infectious viruses. However, apart from their action within the viral particle, HIV-1 PR has been shown to influence cellular functions by interacting with various host proteins in the infected cell (Yang et al., 2012). Protein kinases (PK) are enzymes which are involved in carrying out biological functions of the cell by phosphorylating proteins. The interaction of HIV-1 PR with host cellular kinases both in the cytoplasm and the nucleus have been reported previously (Devroe et al., 2005; Wagner et al., 2015).

In this context we intended to know if HIV-1 PR could interact and cleave other cellular protein kinases. We constructed a library of 412 human protein kinases and HIV-1 PR using the wheat germ cell free protein production system with the sequences listed in **Supplementary Table 1**. We then studied the interaction of HIV-1 PR with each of these kinases using the versatile AlphaScreen assay. The assay works on the principle of proximity-based luminescence which would be lost if the protein kinase is cleaved by HIV-1 PR (**Figure 1A**). Our screening assay detected the cleavage of previously reported PKs; receptor interacting protein kinases (RIPK), RIPK2 (Wagner et al., 2015), and Nuclear Dbf2-related (NDR) kinases, NDR2 (Devroe et al., 2005) in addition to several new targets (**Figure 1B**). Since these two PKs play direct or indirect roles in regulating innate immunity, we analyzed the other cleaved PKs that maybe involved in similar function. In this context, we selected TBK1 as it is an integral kinase in the IFN gene induction pathway, and we hypothesized that HIV-1 could cleave TBK1 to suppress type-I IFN production in order to evade innate immunity.

HIV-1 Protease Cleaves TBK1

To visually observe the cleavage of TBK1 by HIV-1 PR in the extracellular milieu, we synthesized TBK1 with N-terminal Green (FLAG) and C-terminal red (biotin) tags by wheat cell-free system. Intact TBK1 appeared yellow due to the interference of the colors at either end of the protein. Two-color immunoblot analysis revealed that HIV-1 PR, but not its catalytic inactive mutant PR D25N, could cleave TBK1 protein into C-terminal red and N-terminal green fragments (**Figure 2A**). We then wanted to observe if HIV-1 PR mediated cleavage of TBK1 can occur intracellularly. HIV-1 Gag-Pol (enzymatically active) or Gag (devoid of enzymatic action) was co-transfected with TBK1 into HEK293 cells in the presence or absence of the protease inhibitor (PI) amprenavir. TBK1 was cleaved by Gag-Pol and not Gag, which was inhibited by amprenavir suggesting that HIV-1 PR exerts its proteolytic effect on TBK1 intracellularly (**Figure 2B**).

We further wanted to identify the site at which HIV-1 PR cleaved TBK1. For this, we harvested the TBK1 cleavage bands of proteins obtained upon *in vitro* HIV-1 PR treatment and outsourced them for amino acid sequencing. Sequencing results indicated that full length TBK1 (FL) was 729 amino acids (aa) long and HIV-1 PR cleaved TBK1 between L683 and V684 (direct data provided by the outsourced laboratory) (**Figure 2C**). Based on the sequencing data, we generated different types of TBK1 mutants; three cleavage site specific substitution mutants and one deletion mutant representing the cleaved TBK1 fragment (1–683) (**Figure 2C**). TBK1 wild type (WT) or the mutants were co-transfected with Gag-pol and then western blot was performed to observe the cleaved fragment. Gag-pol cleaved WT TBK1 which was denoted by the presence of a prominent cleavage fragment band. The substitution mutants were not cleaved effectively, and the deletion mutant was not cleaved (**Figure 2C**).

Cleaved TBK1 Does Not Activate IFNβ Promoter

We further wanted to check if the cleaved TBK1 is indeed incapable of activating type I IFN gene induction pathway. For this, we co-transfected HEK293 cells with IFNβ promoter tagged with secreted nanoluciferase (IFNβ-Luc) and full length TBK1 (1–729) or the cleaved TBK1 fragment (1–683) in increasing quantities and checked for luciferase activity. We could observe a dose dependent increase in the luciferase activity in with the full length TBK1 and not the cleaved fragment (**Figure 3A**), suggesting that the latter fails to activate the IFNβ promoter and thereby does not secrete IFNβ. This was further confirmed by triple co-transfection of HEK293 cells with IFNβ-Luc, HIV-1 PR and wild type TBK1 or functionally inactive TBK1 substitution mutant (TBK1 K38A). The wild type TBK1 accentuated the IFNβ promoter activity which was significantly inhibited in the presence of HIV-1 PR (**Figure 3B**).

HIV-1 Protease Inhibits IRF3 Phosphorylation and Regulates Its Localization

TBK1 undergoes autophosphorylation in order to phosphorylate cytoplasmic IRF3 which then translocates to the nucleus to activate transcription that leads to IFN production. We transfected HIV-1 PR and TBK1 into HEK293 cells and harvested the cells after 48 h to perform a western blot in order to observe the role of HIV-1 PR in the phosphorylation of TBK1 and IRF3 (**Figure 4A**). In the absence of HIV-1 PR, we observed prominent bands of both phosphorylated TBK1 (pS172) and IRF3 (pS396). However, both bands were faint in the presence of HIV-1 PR suggesting that TBK1 cleaved by HIV-1 PR does not get phosphorylated and hence does not phosphorylate IRF3.

We then performed an immunofluorescence study to observe the localization of IRF3 in the presence or absence of HIV-1 PR. Cells co-transfected with TBK1 alone or along with

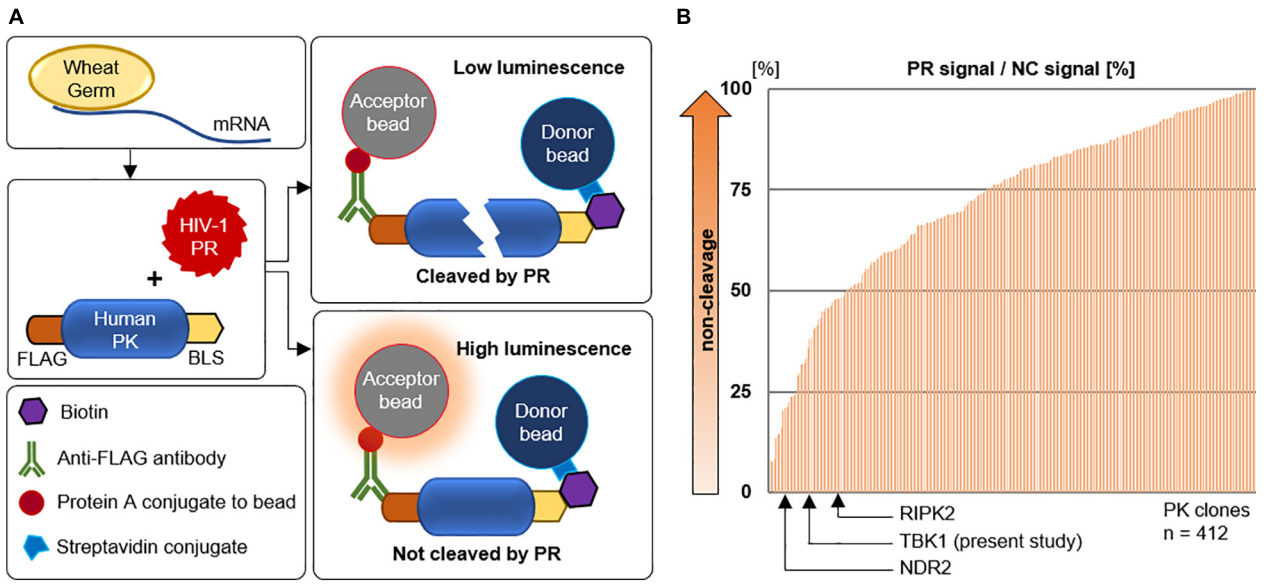

FIGURE 1 | Screening for human kinases cleaved by HIV-1 protease. **(A)** Schematic representation of the AlphaScreen assay performed with HIV-1 protease and various human protein kinases generated by wheat germ cell-free protein synthesis. PR protease, PK protein kinase, GST glutathione S-transferase, BLS biotin linker sequence. **(B)** AlphaScreen assay result of the 412 human protein kinases arranged in order of cleavage to non-cleavage. TANK binding kinase-1 was identified in this study along with two other previously reported protein kinases. PR protease, NC negative control represents dihydrofolate reductase (DHFR), a protein that is not cleavable by HIV-1 protease.

either wild type gag-pol (WT) or catalytically inactive mutant Gag-pol (D25N). WT gag-pol cleaved TBK1 so that IRF3 was not phosphorylated and hence remained in the cytoplasm. Whereas TBK1 was not cleaved by gag-pol D25N, so IRF3 was phosphorylated and translocated into the nucleus in a similar pattern to that seen with TBK1 alone (**Figures 4B,C**).

Drug Resistant Mutant Proteases Have Diminished Efficacy in Cleaving TBK1

The widespread use of protease inhibitors as principal agents for anti-retroviral therapy (ART) has selected out several HIV-1 PR mutants which show cross resistance to several PIs (Rhee et al., 2010). However, the mutations in PR gene are often known to compromise the pathogenic fitness of HIV-1 (de Vera et al., 2013). The principal substrate of HIV-1 PR is the viral precursor polyprotein which it cleaves within the virion to enable maturation (Yang et al., 2012). So, we were intrigued to know if these drug resistance mutations could differentially affect the ability of HIV-1 PR to cleave TBK1 and viral precursor protein. We selected four HIV-1 PR mutants DR1-4 (**Table 1**) to study their ability to cleave TBK1. HIV-1 PR WT or the catalytically inactive mutant D25N or either of the drug resistant mutants DR1-4 were co-transfected along with TBK1 in HEK293 cells and western blot was done after 48 h to check for the presence of TBK1 cleavage fragment band. While the HIV-1 PR WT produced a prominent cleavage band, none of the four drug resistant mutants and D25N cleaved TBK1 effectively (**Figures 5A,B**). Interestingly, the p55 precursor protein was cleaved into p41 and p24 by all the mutants except the replication defective DR1. This finding suggests that mutations in HIV-1 PR alter the enzyme's substrate specificity. Mutant PRs retain their ability to cleave polyproteins into their functional sub-units while losing their ability to cleave TBK1.

Protease Inhibitors Rescue IFN-I in Infected Cells

As we had observed a clear and present effect of HIV-1 PR on TBK1 and its reversal by PIs in transfection studies, we wanted to find if these effects were observable in HIV-1 infected cell lines. We used the uninfected monocytic cell line THP1 as comparison and studied the effects of PIs in the HIV-1 latent infected cell line T7. We transfected the cells with poly(I:C), a robust inducer of IFN-1 to induce its production in both cell types (Ranganath et al., 2016). To activate HIV-1 PR production in T7 cells, we treated them with bryostatin-1, a potent HIV-1 latency reactivating agent known for its superior *in vitro* action on monocytic cell lines (Martínez-Bonet et al., 2015). We observed an increase in IFNβ1 mRNA production in reactivated T7 cells treated with PIs suggesting that PIs relieved the inhibitory effect of HIV-1 PR on IFN-1 secretion pathway (**Figure 6A**). We further wanted to check if this increase in IFNβ1 mRNA caused by PIs translated to downstream effects. The interferon-stimulated gene ISG-15 mRNA was found to be upregulated in PI treated cells, suggesting the effect of IFNβ1 rescued by PIs (**Figure 6B**).

DISCUSSION

PR is an important enzyme of HIV-1 coded by the pol gene along with the other enzymes, reverse transcriptase and integrase.

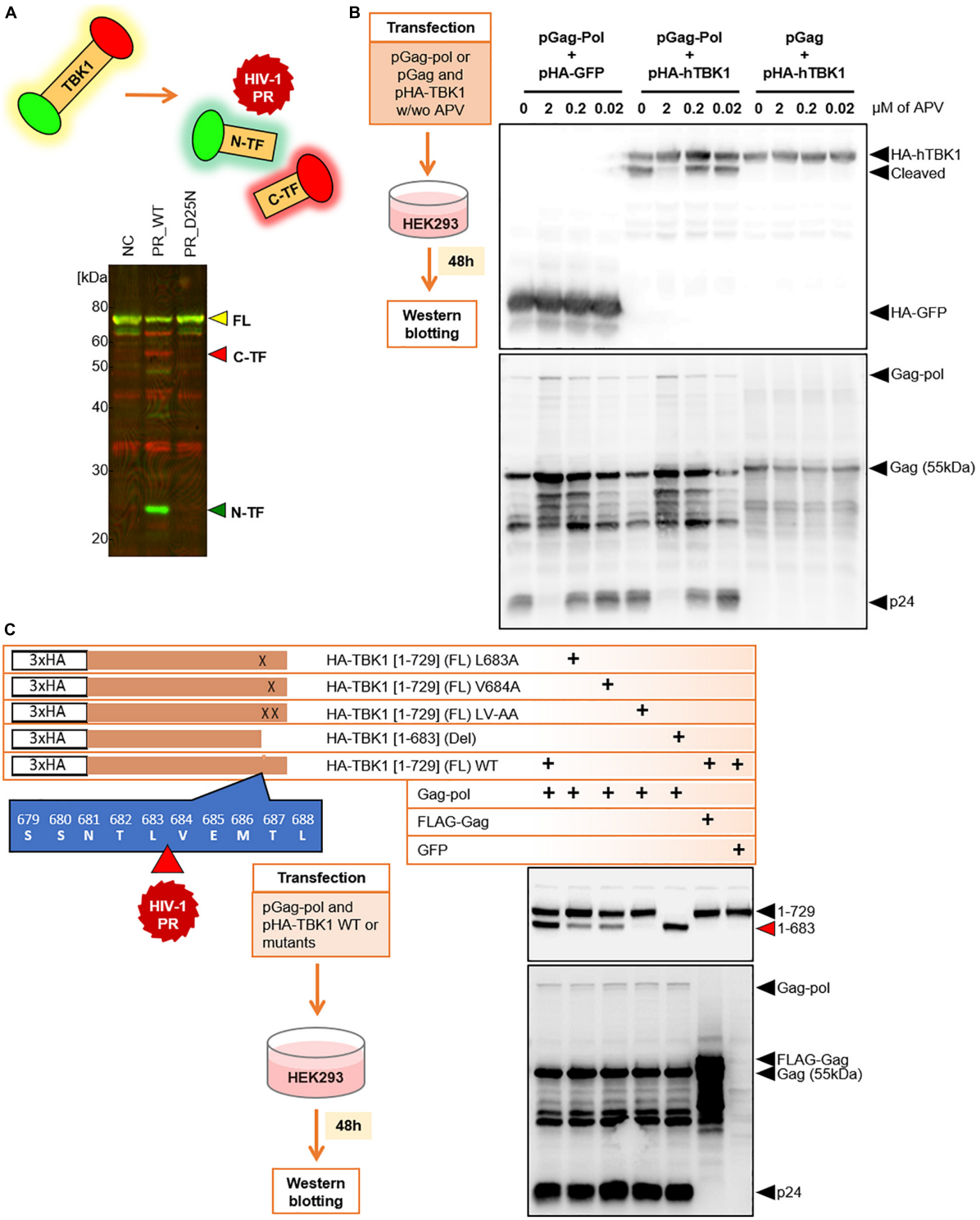

FIGURE 2 | HIV-1 protease cleaves TBK-1. **(A)** Recombinant TBK1 protein with N-terminal green and C-terminal red fluorescence. Full length protein fluoresces yellow when intact. Upon *in vitro* proteolytic cleavage with HIV-1 PR, the N-terminal fragment fluoresces green while the C-terminal fragment fluoresces red detected by distinct bands in western blot. NC Negative control represents recombinant full length fluorescent TBK1 without addition of HIV-1 PR, FL Full length, C-TF C-terminal fragment, N-TF N-terminal fragment. **(B)** Western blot analysis 48 h after transfection of HEK293 cells with Gag-Pol or Gag with HA tagged TBK1 in the presence or absence of serial concentrations of amprenavir. Cleaved and non-cleaved TBK1 bands on the immunoblot were observed with anti-HA antibody. APV amprenavir. **(C)** Full length wild type TBK1, three cleavage site specific substitution mutants and one deletion mutant were generated with HA tag. Western blot analysis done 48 h after transfection of HEK293 cells with either of these TBK1 and pGag-Pol. TBK1 cleavage bands were observed with anti-HA antibody and p55 cleavage bands were visualized with anti-p24 antibody.

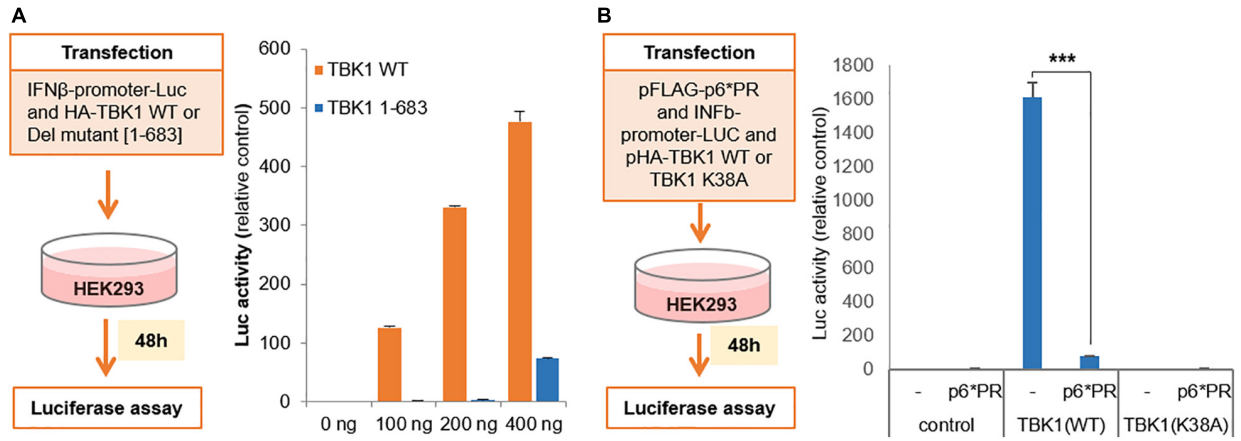

FIGURE 3 | Cleaved TBK-1 does not activate IFNβ promoter. **(A)** Interferon β-promoter-Luc genetic element was co-transfected with serial concentrations of HA tagged wild type TBK1 or its deletion mutant [1–683] in HE293 cells. Relative luciferase activity denoting the interferon β promoter activity was assessed after 48 h of transfection. Bars represent the mean value of three replicates tested. **(B)** pFLAG-p6*PR, interferon β-promoter-Luc and either pHA-TBK1 wild type or non-functional mutant TBK1 K38A were co-transfected in HEK293 cells. Relative luciferase activity denoting the interferon β promoter activity was assessed after 48 h of transfection. Bars represent the mean value of three replicates tested. *** indicates p-value < 0.001.

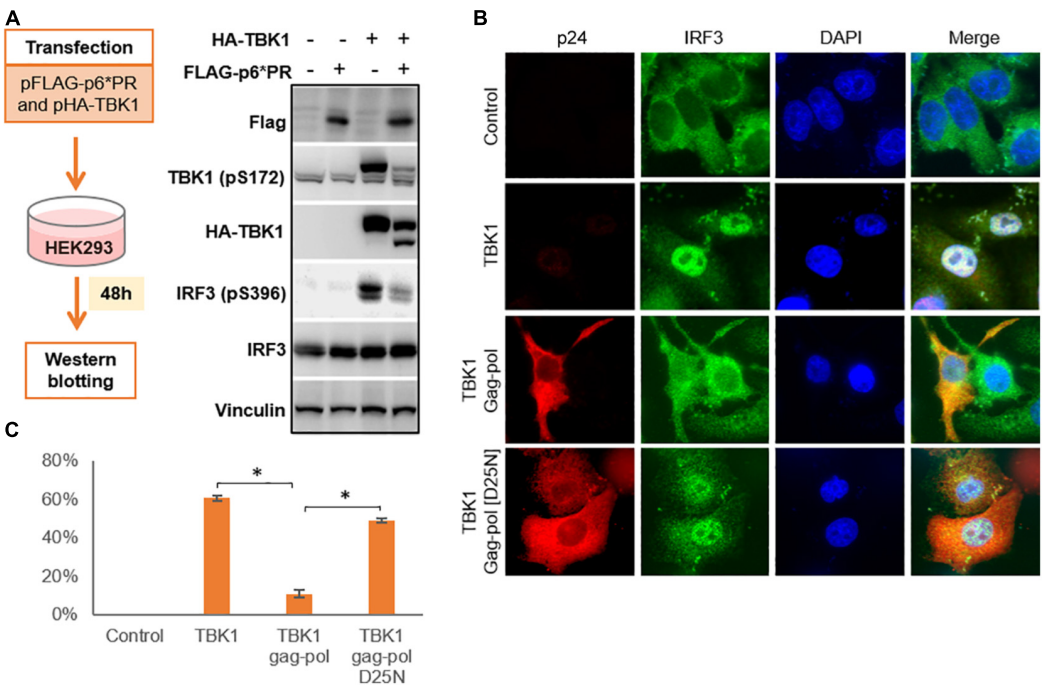

FIGURE 4 | HIV-1 protease inhibits IRF3 phosphorylation and regulates its localization. **(A)** pFLAG-p6*PR and pHA-TBK1 were co-transfected into HEK293 cells and western blot was done after 48 h. Total TBK1, phosphorylated TBK1, total IRF3 and phosphorylated IRF3 were observed with corresponding antibodies. **(B)** Cells co-transfected with TBK1 and wild type Gag-pol or its enzymatically inactive mutant D25N for 48 h after which cells were collected and fixed in paraformaldehyde. Immunofluorescence staining done for gag-pol with AlexaFluor 568 and IRF3 stained with AlexaFluor 488 which emitted red and green fluorescence, respectively. Nucleus stained blue with DAPI. Control represents non-transfected cells. **(C)** Percentage of IRF3 activation was calculated from the IRF3 panel of **Figure 4B** depicting the number of cells containing IRF3 translocated into the nucleus over total cells in the field. Data represents the mean value obtained from counting three fields containing at least 20 cells in each field. * indicates p-value < 0.05.

Unlike the other two which are required for viral replication in the intracellular milieu, PR has long been considered as a late phase enzyme known to act inside the newly budded virions where it cleaves the viral precursor polyproteins into functional subunits to produce mature infectious progeny (Konvalinka et al., 2015). However, there is accumulating evidence that HIV-1 PR plays an accessory role to interfere with host cellular functions by cleaving various intracellular proteins (Wagner et al., 2015).

TABLE 1 | HIV-1 protease mutants resistant to protease inhibitors.

Clone	Major resistance mutations	Minor resistance mutations	Other mutations
DR1	N88S	L10F, T74S, L89V	I13V, K20T, E35D, M36I, R41K, I62V, L63P, H69K
DR2	N88S	L10F, A71T, L89V	I13V, K20T, E35D, M36I, R41K, I62V, L63P, H69K
DR3	N88S	L10F, A71T, L89V	I13V, K20T, E35D, M36I, R41K, L63P, H69K
DR4	G48S, I54V, V82F, L90M	L10I	I13V, G16Q, K20I, E35N, M36I, P39Q, R41K, K45V, I62V, L63P, H69K, L89M

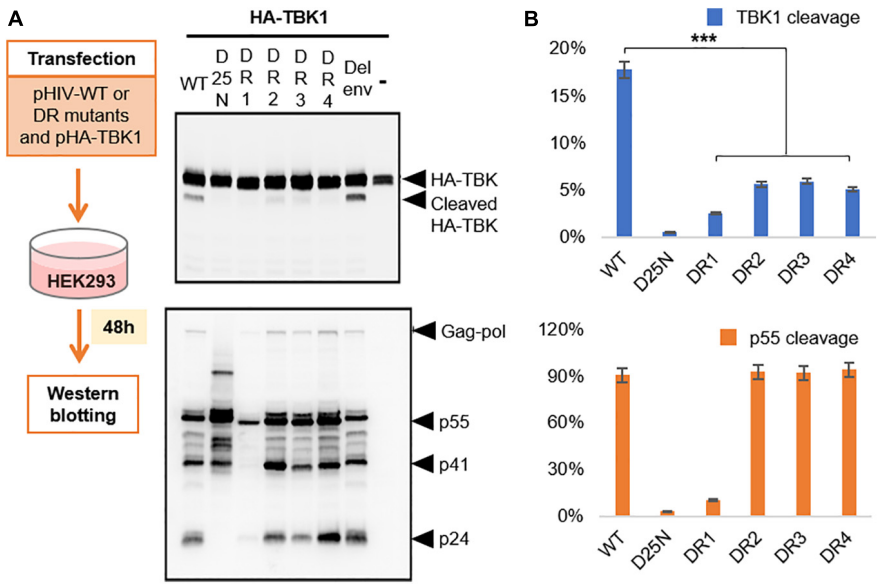

FIGURE 5 | Drug resistant proteases cleave TBK1 ineffectively. **(A)** Wild type HIV or either of the four drug resistant mutants listed in **Table 1** were co-transfected with HA tagged TBK1. Western blot analysis done after 48 h to observe TBK1 cleavage bands with anti-HA antibody and p55 cleavage bands with anti-p24 antibody. **(B)** Quantification of band intensities from **(A)**. Graph on the top indicates cleavage ratio percentage of cleaved TBK1 over un-cleaved TBK1 while the one on the bottom indicates that of p24 over p55. *** indicates p-value < 0.001.

One of the earliest observations in this regard is the induction of apoptosis in infected cells by HIV-1 PR (Ventoso et al., 2005). Although the exact mechanism is unknown, it is presumed to be due to cleavage of different host proteins related to the apoptotic pathway such as Bcl-2 (Strack et al., 1996) and Procaspase 8 (Sainski et al., 2011). Although this action of HIV-1 PR is attributed to the reduction of CD4 T-cells during productive infection and in terminal disease, HIV-1 PR mediated apoptosis makes it deleterious for the virus to replicate.

More recent studies have revealed the role of HIV-1 PR in cleaving the host cellular proteins involved in inflammation and immune response in order to dampen the host defenses and make the intracellular environment conducive for viral replication. The salient observation in this regard is the proteolytic cleavage of host PKs involved in innate immunity by HIV-1 PR. Nuclear Dbf2-related (NDR) kinases, NDR1 and NDR2 are essential PKs instrumental in regulating PRR and cytokines in innate immunity (Ye et al., 2020) and HIV-PR has been shown to cleave both NDR1 and NDR2 (Devroe et al., 2005). Similarly, receptor interacting protein kinases (RIPK) RIPK1 and RIPK3 are involved in eliminating virus infected cells through necroptosis and have also been shown to mediate type 1 IFN signaling for this purpose (Rajput et al., 2011). RIPK1 has been reported to be cleaved by HIV-1 PR during infection (Wagner et al., 2015). In similar lines, our study shows that TBK1 is yet another host PK that is cleaved by HIV-1 PR to inhibit type 1 IFN production possibly to dampen innate immunity and promote viral infection.

Different accessory proteins of HIV-1 have been identified to directly interfere in the IFN-I secretory pathway in different host cells to suppress IFN-I production and the mechanisms vary in productive and latent infections. These viral proteins are expressed in productively infected T-cells where they reduce IFN-I production by degrading IRF3. The proteins Vpr and Vif mediate ubiquitination of IRF3 and cause its subsequent destruction in proteasomes (Okumura et al., 2008) while Vpu interacts with IRF3 to redirect the latter into lysosomes for proteolytic degradation (Doehle et al., 2012). In productively infected dendritic cells and macrophages, Vpr and Vif physically interact with TBK1 to prevent its autophosphorylation thereby inhibiting the subsequent steps of IFN-I production (Harman et al., 2015).

Like productively infected cells, IFN-I production is impaired in cells that are latently infected with HIV-1, but the mechanisms involved in the latter are not as clearly defined as in the former (Ranganath et al., 2016). As expression of accessory proteins are unlikely in latent infection, there must be other mechanisms by

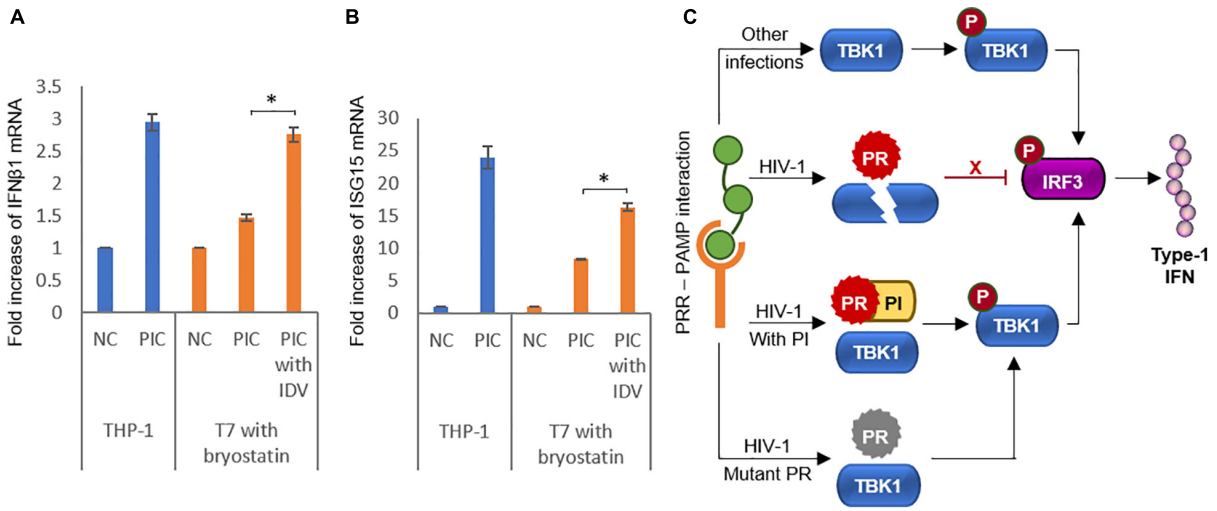

FIGURE 6 | Protease inhibitors rescue interferon activity in infected cells. **(A)** HIV-1 latently infected monocytic cell line T7 was reactivated with 25 ng/ml bryostatin-1 and IFNβ1 mRNA was detected by RT-qPCR in poly(I:C) treated cells in the presence or absence of the protease inhibitor indinavir. Uninfected THP-1 cells were used as positive control. Bars represent the mean value of three replicates. NC Negative control, PIC Poly(I:C), IDV Indinavir. * indicates p-value < 0.05. **(B)** HIV-1 latently infected monocytic cell line T7 was reactivated with 25 ng/ml bryostatin-1 and ISG15 mRNA was detected by RT-PCR in poly(I:C) treated cells in the presence or absence of the protease inhibitor indinavir. Uninfected THP-1 cells were used as positive control. Bars represent the mean value of three replicates. NC Negative control, PIC Poly(I:C), IDV Indinavir. * indicates p-value < 0.05. **(C)** Schematic representation of the findings of the study summarizing the role of HIV-1 protease in the interferon secretion pathway.

which HIV-1 diminishes IFN-I production in these cells. Wagner et al. (2015) have demonstrated that PKs are cleaved by HIV-1 PR expressed by proviral genes after integration and not by the PRs brought in by the incoming viruses during early infection. This could possibly explain that non-PR mediated mechanisms are responsible for IFN suppression in acute productive infection while PR plays a role in IFN suppression in latently infected cells. Rigogliuso et al. (2019) have demonstrated the expression of PR in cells containing proviral genomes of Human endogenous retroviruses (HERVs) and its ability to cleave several cellular proteins. Hence it is interesting to speculate if HIV-1 PR is subtly expressed in latently infected cells to suppress IFN-I production, warranting the need for further studies to identify this phenomenon.

Another interesting observation in western blots was the presence of considerable amount of non-cleaved full length TBK1 bands in addition to the cleavage band (**Figures 2B**, **4A**) denoting that HIV-1 PR cleaves only a fraction of the expressed TBK1. It is intriguing why this non-cleaved TBK1 did not participate in further downstream events to secrete IFN-I despite being present in more copious amounts than their cleaved counterpart. Further studies are needed to detect whether the cleaved fragments possibly hinder the function of the non-cleaved TBK1.

Protease inhibitors are highly effective drugs against HIV-1 and comprise the mainstay agents of ART. With our *in vitro* experiments it is evident that PIs can prevent HIV-1 from cleaving TBK1, suggesting that PIs could improve the innate immune response to facilitate viral clearance. Harman et al. (2015) have shown the effect of HIV-1 PR in cleaving host cellular proteases in an infection model comprising previously uninfected primary CD4 T-cells. We have shown a similar effect of HIV-1 PR in latently infected monocytic cell lines upon reactivation. However, the possibility of this happening *in vivo* remains to be studied.

Due to their widespread use, PIs have selected drug resistant mutants. It has been observed that mutations of protease that enable the enzyme to become resistant to PIs paradoxically reduce the pathogenic fitness of the virus (de Vera et al., 2013). We were able to demonstrate that mutations in PR which confer resistance to PIs make the enzyme less potent to cleave TBK1 while still retaining its ability to cleave the viral polyprotein into functional units to cause viral maturation. Except for one mutant (DR1) which was proliferation defective, all the other three mutants effectively cleaved gag-pol but showed inefficiency to cleave TBK1. This suggest that in order to retain its basic biological function, the evolved virus must pay the price of becoming more vulnerable to innate immune clearance.

Achieving a functional cure to HIV infection depends on successfully eradicating the replication competent viruses from the latent reservoir. However, all attempts till date to shock and kill the proviruses have failed miserably. Palermo et al. (2019) have demonstrated that latency reactivation strategies work better when there is innate immune activation in addition to epigenetic modulation rather than the latter alone. Since HIV-1 uses multiple mechanisms to evade innate immunity, it could be a possible reason for the inefficiency of the shock and kill strategy. We have unraveled a novel mechanism where HIV-1 PR is involved in degradation of TBK1, one of the major proteins responsible in IFN-I secretion. This action could be counteracted by PIs and the drug resistance mutations in PR reduce the enzyme's efficiency to degrade TBK1 (**Figure 6C**). We suggest that the molecular link

between HIV-1 PR and IFN-I secretion could play an important role in the pathophysiology of HIV-1 and highlight the need to study this in further detail in future studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SJ, AK, KM, SM, and MN performed most of the experiments. AK, AT, TS, and AR analyzed the data. SJ, KM, and SM designed the experiments and wrote the manuscript. AT, TS, and AR revised and edited the manuscript. AR conceived the idea and supervised the project. All authors made significant contributions to the article and approved the submitted 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/fmicb.2021.643407/full#supplementary-material

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CD8$^+$ T-Cell Mediated Control of HIV-1 in a Unique Cohort With Low Viral Loads

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A unique population of HIV-1 infected individuals can control infection without antiretroviral therapy. These individuals fall into a myriad of categories based on the degree of control (low or undetectable viral load), the durability of control over time and the underlying mechanism (i.e., possession of protective HLA alleles or the absence of critical cell surface receptors). In this study, we examine a cohort of HIV-1 infected individuals with a documented history of sustained low viral loads in the absence of therapy. Through *in vitro* analyses of cells from these individuals, we have determined that infected individuals with naturally low viral loads are capable of controlling spreading infection *in vitro* in a CD8$^+$ T-cell dependent manner. This control is lost when viral load is suppressed by antiretroviral therapy and correlates with a clinical CD4:CD8 ratio of <1. Our results support the conclusion that HIV-1 controllers with low, but detectable viral loads may be controlling the virus due to an effective CD8$^+$ T-cell response. Understanding the mechanisms of control in these subjects may provide valuable understanding that could be applied to induce a functional cure in standard progressors.

Keywords: HIV-1, HIV controller, Low Viral Load, CD8$^+$ T cell, HLA compatibility, CD4:CD8 ratio

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INTRODUCTION

Human immunodeficiency virus type-1 (HIV-1) infection causes disease by causing immunosuppression (Shearer, 1998; Elfaki, 2014). The degree of immunosuppression is closely and inversely linked to the concentration of CD4$^+$ T-cells circulating in blood (Ingole et al., 2011; Merci et al., 2017). The average CD4$^+$ T cell count is 1,000 cells/μL. HIV-1 infection causes a drop of 60 cells/uL per year on average (Schwartländer et al., 1993; Patrikar et al., 2014; Parsa et al., 2020). When the CD4$^+$ T-cell count falls below 200 cells/uL, a person is at increased risk of opportunistic infections and malignancies (Egger et al., 2002; Institute of Medicine (US) Committee on Social Security HIV Disability Criteria, 2010; Opportunistic Infections Project Team of the Collaboration of Observational Hiv Epidemiological Research in Europe (Cohere) in EuroCoord, Young et al., 2012; Merci et al., 2017). Approximately 3–6 months after

infection, plasma HIV-1 RNA concentration referred to as "viral load" (VL) reaches a steady state with a median of 40,000 copies/ml (c/ml). The level of viremia correlates to the rate of $CD4^+$ T-cell loss (O'Brien W. A. et al., 1996; Mellors et al., 1997; de Wolf et al., 1997). $CD4^+$ T-cells decrease ∼4% per year per log c/ml of HIV-1 RNA. The vast majority of people living with HIV (PLWH) have a VL > 1,000 c/ml of plasma. A very small percentage, < 1%, have a VL below the limit of detection, so-called undetectable.

Initially interested in studying the host factors of PLWH with naturally low viral loads (LVLs), we studied several individuals with VLs < 2,000 c/mL. As in published studies, isolated $CD4^+$ T-cells from all LVLs supported HIV-1 *in vitro* growth. We next tested the ability of PHA-stimulated Peripheral Blood Mononuclear Cells (PBMCs) from LVLs to grow HIV-1. Surprisingly, the results segregated LVLs into two distinct groups, those with consistently undetectable VLs referred to as elite controllers (ECs) and those with VLs between 100–2,000 c/mL, which we refer to as LVLs. PHA-stimulated PBMCs from EC donors supported HIV-1 growth at a level similar to normal donor PBMCs. On the other hand, no growth could be detected in PBMCs from LVLs and we were able to associate this control with $CD8^+$ T-cells.

$CD8^+$ T-cells are known to play a critical role in the control of viral infection and their temporal appearance has been associated with a reduction in plasma viremia following acute HIV infection (Koup et al., 1994). To underscore the importance of $CD8^+$ T-cells in the control of viral replication, previous studies have demonstrated that a robust rebound of plasma viremia results from antibody-mediated depletion of $CD8^+$ T-cells from antiretroviral therapy (ART) treated simian immunodeficiency virus infected rhesus macaques (Cartwright et al., 2016; McBrien et al., 2020a,b). In addition, simian human immunodeficiency virus infected rhesus macaques who gained viral control when treated with combinatorial broadly neutralizing antibodies (bNAbs) became susceptible to infection when depleted of $CD8^+$ T-cells, thus providing further evidence which supports the indispensable role of $CD8^+$ T-cells in the control of viral replication (Nishimura et al., 2017). It was also observed that there was a minimal viral load which persisted in rhesus macaques who gained viral control when treated with bNAbs but their viral loads were so low they required detection via ultrasensitive assays (Nishimura et al., 2017). It was postulated that although negligible, low viral loads in these animals permitted a sustained $CD8^+$ T-cell response sufficient to control infection which is not achieved when viral load levels are further suppressed by treatment with ART such that when ART is interrupted there is an inability of $CD8^+$ T-cells to control viral replication (Nishimura et al., 2017). Therefore, in the presence of a low viral load, we speculate that $CD8^+$ T-cells retain the ability to control viral replication.

In this study, we report on the clinical and cellular factors associated with LVL inhibition of HIV *in vitro* (IHI). IHI is only seen in donors who have a detectable, but low viral load and a "flipped ratio" or CD4:CD8 < 1. IHI is lost when viral load is suppressed by ART and is mediated by $CD8^+$ T-cells in an HLA-restricted fashion. Our results support the conclusion that HIV-1 infected individuals with a low viral load set point controls the virus through a $CD8^+$ T-cell response. This response fades quickly after viremia is suppressed, establishing that this mechanism is different than the mechanism(s) involved in the control of viremia in ECs. Understanding how these subjects are different from standard progressors with detectable CTL response may lead to the development of immunomodulatory therapies that can induce a functional cure.

RESULTS

LVL Donor PBMCs Are Resistant to HIV Infection

For this study, we recruited ART naïve HIV-1 seropositive donors with viral loads below 2,000 copies/mL which we define as low viral load (LVL) donors, donors with a history of high viral loads (HVLs), elite controllers (ECs), or normal donors (**Table 1**). We performed a tissue culture infectious dose 50 (TCID50) assay to determine the relative susceptibility of PBMCs from each donor to infection using a common viral stock (**Figure 1A**). Normal donors and ECs showed the most susceptibility to infection *in vitro*. HVLs were less susceptible to infection, with no difference between HVLs suppressed on therapy and those that are therapy naïve. LVLs had the lowest average susceptibility to *in vitro* infection with HIV-1. However, LVLs were not statistically significant from HVLs off therapy. Blood $CD4^+$ T-cell counts (**Figure 1B**) of LVLs remain high confirming that these donors control viral loads and maintain healthy $CD4^+$ T-cell counts, unlike HVLs. We further analyzed the ability of PBMCs from normal donors, HVL donors on therapy and LVL donors to control spreading infection. PBMCs were activated with PHA and IL-2 for 48 h, washed, infected with NL4-3, and virus accumulation in the supernatant was measured every 2 days by p24 ELISA. Interestingly, PBMCs from LVLs suppressed *de novo* HIV-1 infection (**Figure 2A**). When the same assay was performed on uninfected normal donor and HVL donors PBMCs, we observed robust viral production by day four and six post-infection. As prior studies suggested, $CD4^+$ T-cells from controllers (both LTNPs and ECs) could support viral replication (Cao et al., 1995; Sáez-Cirión et al., 2007; Julg et al., 2010; O'Connell et al., 2011), these data suggest that other cell types present in PBMCs may be mediating suppression in our system.

LVL $CD4^+$ T-Cells Are Susceptible to the HIV Infection

The resistance of PBMCs from LVLs to infection could be due to many factors, including resistance to infection of $CD4^+$ and/or $CD8^+$ T-cell anti-HIV activity. To decipher between a $CD4^+$ and $CD8^+$ T-cell mediated phenotype, we performed an HIV-1 spreading infection assay on an isolated $CD4^+$ T-cell population (**Figure 2B**). $CD4^+$ T-cells were isolated from uninduced PBMCs from HVLs and LVLs using Miltenyi $CD4^+$ T-cell negative isolation kit, purity of > 90% confirmed by flow cytometry

TABLE 1 | Human subject classifications and clinical information.

Classification	Patient ID	VL (copies/ml)	CD4 (cells/mm^3)	CD8 (cells/mm^3)	ART	Therapy
HVL	ADV7140	<20	497	593	**ON**	Truvada/Tivicay
	BRW1143	<20	1,135	828	**ON**	Truvada/Tivicay
	CPU4801	<20	253	851	**ON**	Evotaz/Tivicay
	DER1295	<20	1,178	2,001	**ON**	Descovy/Tivicay
	ESN1170	<20	345	697	**ON**	Truvada/Tivicay
	HSA1084	<20	699	507	**ON**	Atripla
	QMY7270	<20	1,114	1,005	ON	Atripla
	WXM1008	<20	291	1,149	**ON**	Atripla
	XTD8730	30	251	764	**ON**	Tivicay/Reyataz/Norvir
	BPJ1299	9,600	605	1,202	Off	–
	CYJ1314	7,329	631	717	Off	–
	KLU1328	260,140	93	398	Off	–
	KMJ1960	30,000	145	1,367	Off	–
	UNJ7200	1,412,390	27	202	Off	–
	UTF9050	11,390	995	419	Off	–
	WBZ1300	24,000	170	1,206	Off	–
	ZDU1316	15,440	406	494	Off	–
LVL	FJG8070	250	1,070	1,434	Off	–
	FWU1270	100	688	ND	Off	–
	HCQ6670	80	488	506	Off	–
	MPY1313	1,550	473	756	Off	–
	NIM1164	750	723	987	Off	–
	SRS5930	230	699	792	Off	–
	VQY4910	750	723	987	Off	–
	AEM9650	1,630	1,001	2,081	Off	–
Normal donor	BTS1096	–	1,190	358	–	–
	CHT3368	–	360	646	–	–
	HFK1114	–	840	597	–	–
	YXC1164	–	1,061	757	–	–
	ND1	–	–	–	–	–
	ND2	–	–	–	–	–
	ND3	–	–	–	–	–
	ND4	–	–	–	–	–
Elite controller	EXT1011	<20	542	498	Off	–
	PQS6990	<20	1,148	827	Off	–
	RVF1231	<20	726	610	Off	–

and cells were activated and infected as above. All CD4$^+$ T-cells isolated from LVLs were susceptible to HIV-1 infection except one (AEM9650) (**Figure 2B**). HLA typing revealed none of the HLA-B mutations commonly associated with viral resistance (data not shown). Comparing total virus output from infected PBMCs and CD4$^+$ T-cells at days of peak replication, revealed that although CD4$^+$ T-cells from LVLs supported HIV-1 infection, significantly less virus was produced compared to normal donors (**Figure 2C**).

Control of Viral Replication *in vitro* by LVLs Is Mediated by CD8$^+$ T-Cells

We next investigated the role of CD8$^+$ T-cells in the ability of LVLs to control viral replication *in vitro* (**Figure 3**). We depleted CD8$^+$ T-cells from PBMCs from LVLs using a magnetic bead-based method. Depletion was verified by flow cytometry and we confirmed > 90% depletion of CD8$^+$ T-cells. Cells were activated and PBMCs were infected as above. Depletion of CD8$^+$ T-cells from PBMCs of two LVL donors resulted in susceptibility to HIV-1 infection (**Figures 3A,B**). Isolated CD8$^+$ T-cells were also titrated back in to the PBMCs and this addback restored the suppressive phenotype at a ratio of 1:1 and 1:2 PBMCs:CD8s in one donor (**Figure 3A**) and at 1:2 PBMCs:CD8s in another donor (**Figure 3B**). We next tested whether CD8$^+$ T-cells alone were sufficient for suppression of viral replication in CD4$^+$ T-cells from an LVL. Populations of CD4$^+$ and CD8$^+$ T-cells were isolated from PBMCs of an LVL donor and a normal donor, activated and infected as above. As expected, isolated CD4$^+$ T-cells were susceptible to viral replication (**Figures 3C,D**). Titration of CD8$^+$ T-cells back with CD4$^+$ T-cells at all ratios tested mediated suppression of viral replication from the LVL (**Figure 3C**) and not from the normal

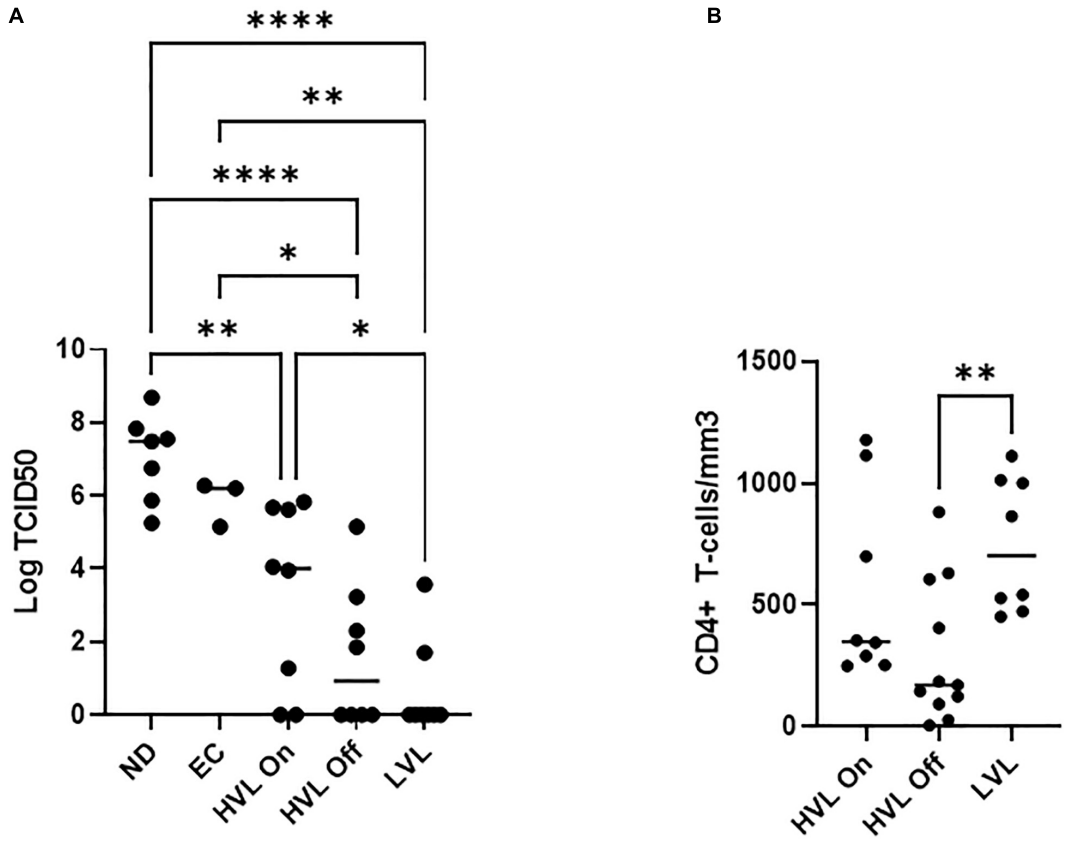

FIGURE 1 | PBMCs from different classes of HIV-1 infected individuals show different susceptibility to *de novo* infection. **(A)** Limiting dilutions of NL4-3 were used to infect arrays of PBMCs from normal donors (ND), elite controllers (EC), high viral load on or off therapy (HVL On, HVL Off), or low viral load (LVL) donors in 96 well plates. p24 ELISA was used to determine productive infection in a given well and TCID50 was calculated. One-way ANOVA with Tukey's multiple comparison was performed. *P*-value: * < 0.05, ** < 0.01, **** < 0.0001. **(B)** $CD4^+$ T-cell counts in the blood of HVL (on/off therapy) and LVL. Mann-Whitney test was used to compare groups $^{**}P < 0.01$.

donor (**Figure 3D**). These results suggest that $CD8^+$ T-cells are both necessary and sufficient to mediate the observed suppression of HIV-1 replication *in vitro*.

HLA Compatibility Is Required for Induced $CD8^+$ T-Cell Anti-HIV Function in Heterologous $CD4^+$ T-Cells

It was previously reported by Killian et al., that an HLA class I match is required for HIV-1 inhibition by uninduced heterologous $CD8^+$ T-cells (Mackewicz et al., 1998; Killian et al., 2018). In our donor cohort, we were able to identify several donors with matching HLA types (**Table 2**). To test the requirement of HLA compatibility for the ability of $CD8^+$ T-cells from LVL donors to suppress HIV replication, we co-cultured activated PBMCs from an LVL donor with HLA-matched and non-HLA-matched isolated, activated heterologous $CD4^+$ T-cells and infected as above (**Figure 4**). Of the LVLs in our study we chose to work with NIM1164 as this donor has matched HLA types with multiple other donors. We performed experiments to examine the ability of PBMCs from NIM1164 to suppress viral replication in isolated $CD4^+$ T-cells from YCX1164, a primary relative containing several HLA matches (**Figure 4A**), BTS1096 a normal donor with matches in HLA-B, HLA-C, and DRB1 (**Figure 4B**), HCQ6670 a LVL with matches in HLA-A, HLA-B, and HLA-C (**Figure 4C**), BRW1143 an HVL with matches in HLA-B, HLA-C, and DRB1 (**Figure 4D**) and HFK1114 a normal donor with no matching HLA alleles (**Figure 4F**). The analysis of HIV-1 spreading infection in $CD4^+$ T-cells and LVL PBMCs co-cultures at all CD4:PBMC ratios demonstrated marked inhibition of HIV-1 replication in cultures with matching HLAs compared to $CD4^+$ T-cells alone (**Figures 4A–E**). Only minimal HIV-1 suppression was observed when LVL PBMCs were mixed with allogeneic $CD4^+$ T-cells without matching HLA (**Figure 4E**). This suggests that HLA-class I compatibility is required for allogeneic suppression of HIV-1 replication by $CD8^+$ T-cells from LVLs.

Concurrent HIV Infection Is Required for $CD8^+$ T-Cell Anti-HIV Function in LVL Donors

During the duration of this study, several donors classified as LVLs were placed on ART (**Table 3**). To determine if the ability to control viral replication *in vitro* was related to viremia, we followed these individuals over time after initiation of therapy.

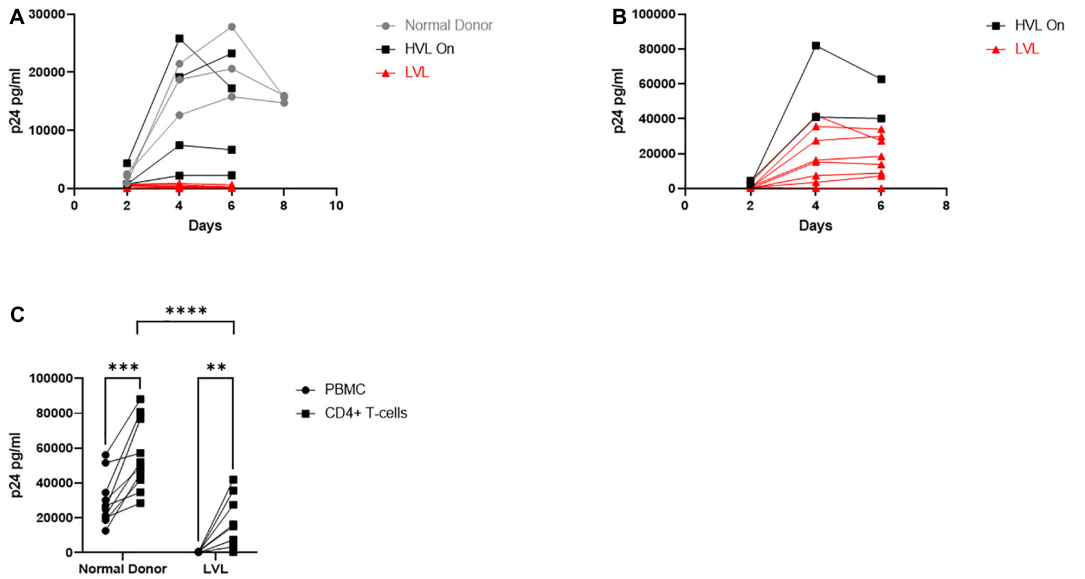

FIGURE 2 | Total PBMCs from low viral load (LVL) donors do not support HIV spreading infection *in vitro*. **(A)** Total PBMCs collected from normal donors (gray, $n = 3$), high viral load donors on ART (HVL, black, $n = 4$) and ART naïve low viral load donors (LVL, red, $n = 8$) were induced with PHA and IL-2 then infected with NL4-3 48 h later. Supernatant was collected every 48 h post infection and virus levels were determined by p24 ELISA. **(B)** $CD4^+$ T-cells were isolated from PBMCs from HVLs (black, $n = 2$) and LVLs (red, $n = 8$) by magnetic bead based isolation. Resulting $CD4^+$ T-cells were induced with PHA and IL-2 then infected with NL4-3 48 h later. Supernatant was collected every 48 h post infection and virus levels were determined by p24 ELISA. **(C)** Measurement of peak p24 production in total PBMCs or isolated $CD4^+$ T-cells from the indicated groups. Two-way ANOVA with Sidak's multiple comparison test was performed. *P*-value: ** < 0.01, *** < 0.001, **** < 0.0001.

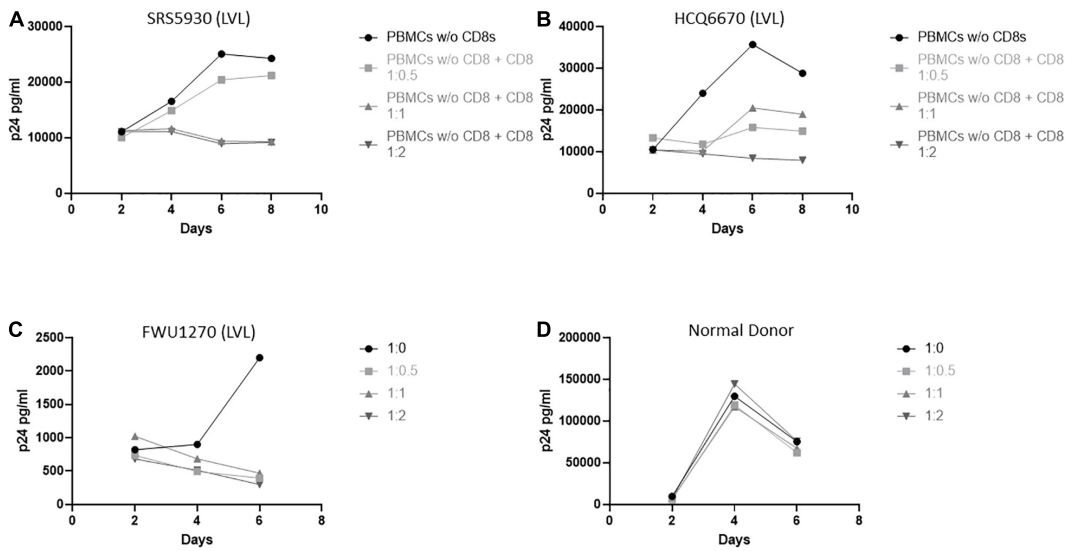

FIGURE 3 | $CD8^+$ T-cells are necessary and sufficient for control of viral replication by LVLs *in vitro*. **(A,B)** $CD8^+$ T-cells were depleted from PBMCs of two LVLs. Depleted PBMCs and $CD8^+$ T-cells were then stimulated for 48 h with PHA and IL-2 before being combined at the indicated ratio of PBMC:CD8 and infected with NL4-3. Supernatant was collected every 48 h post infection and virus levels were determined by p24 ELISA. $CD4^+$ and $CD8^+$ T-cells were isolated from PBMCs from **(C)** an LVL or **(D)** a normal donor. Isolated T-cells were induced with PHA and IL-2, combined as above (CD4:CD8) then infected with NL4-3 48 h later. Supernatant was collected every 48 h post infection and virus levels were determined by p24 ELISA.

PBMCs isolated from pre-therapy (black lines) and post-therapy (blue lines) were evaluated for their ability to support HIV-1 replication *in vitro* (**Figure 5**). For the LVL NIM1164, we observed suppression of viral replication in PBMCs for over a year prior to the start of therapy. ART successfully reduced viral load to undetectable levels (**Table 3**) and with this we observed low levels of viral replication *in vitro* (**Figure 4A**). A similar trend was observed with the LVL MPY1313 wherein therapy

TABLE 2 | HLA typing of subjects.

Patient ID	Class	HLA-A	HLA-A	HLA-B	HLA-B	HLA-C	HLA-C	DRB1	DRB1
NIM1164	LVL	24:02:00	30:01:00	14:02:01	42:01:01	8:02	17:01	1:02	3:02
HCQ6670	LVL	02:ANGA	30:01:00	42:01:01	57:03:01	17:01	18:02	08:AFPMU	13:AHUNK
BRW1143	HVL	32:01:00	33:03:00	42:01:00	44:03:00	4:01	17:01	3:02	13:ASWXB
BTS1096	ND	29:02:00	33:01:00	14:02	44:03:00	8:02	16:01	1:02	07:JDKZ
HFK1114	ND	30:02:00	34:02:00	15:03:01	44:03:01	2:10	4:01	4:05	15:03
YXC1164	ND	24:AVJRX	66:01:00	14:02	58:02:01	6:02	8:02	1:02	13:ASVAC

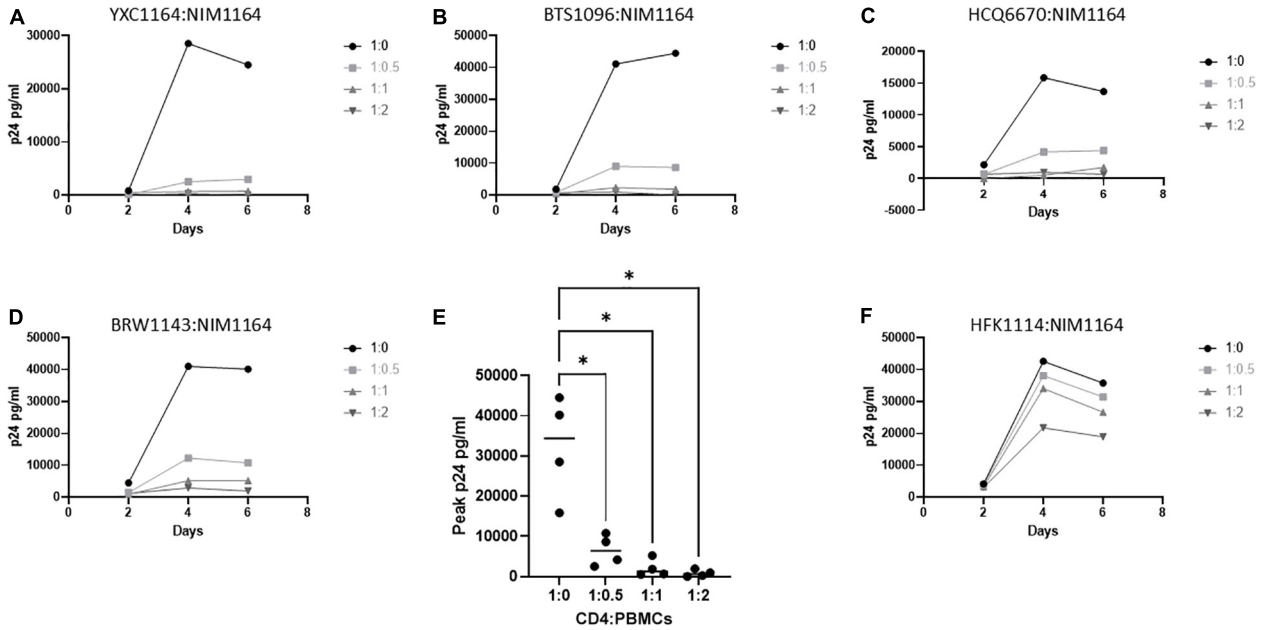

FIGURE 4 | HLA compatibility is a requirement for LVL mediated HIV-1 suppression in heterologous CD4+ T-cells. PBMCs from LVL NIM1164 and isolated CD4+ T-cells from donors with varying degrees of HLA match were induced with PHA and IL-2. Heterologous CD4+ T-cell isolates from HLA-matched and mismatched donors and PBMCs from a LVL (NIM1164) were co-cultured at the indicated ratio of CD4:PBMCs and infected with NL4-3. Supernatant was collected every 48 h post infection and virus levels were determined by p24 ELISA. **(A)** YXC1164 is an HLA-matched normal donor primary relative of NIM1164. **(B)** BTS1096 is an HLA-matched normal donor. **(C)** HCQ6670 is a HLA-matched LVL. **(D)** BRW1143 is a HLA-matched HVL. **(E)** Measurement of peak p24 production from LVL HLA-matched donors at the indicated CD4:PBMC ratio. One-way ANOVA was performed. *P*-value: * < 0.05. **(F)** HFK1114 is a HLA-mismatch normal donor.

quickly suppressed viral load and a subsequent increase in the susceptibility of PBMCs to viral replication was observed. At 273 days post initiation of therapy, robust viral replication was measured *in vitro* (**Figure 5B**). These findings suggest that a detectable viral load is required for the observed control *in vitro*. In LVL VQY4910 a different pattern was observed. Following the start of ART an increase in the susceptibility of PBMCs to viral infection was observed over time (**Figure 5C**). For all three subjects PBMCs from the last time point sampled post-ART were incapable of supporting viral replication (**Figure 5D**). However, several pre-therapy samples for VQY4910 showed the ability to permit low level viral replication *in vitro*. This susceptibility fluctuated over time. Where data was available, we observed that time points that demonstrated viral control had a CD4:CD8 ratio in the blood of far <1 (**Table 3**). The samples with the greatest observed replication had a ratio close to one. Looking back at our other LVL samples that exhibited control, we also saw that these individuals had a CD4:CD8 ratio less than one, despite maintaining healthy CD4+ T-cell counts. This observation suggested that a subject must have both a detectable viral load and a CD4:CD8 ratio <1 in order to exhibit control *in vitro*.

Pre-therapy CD8+ T-Cells From LVLs Are Capable of Suppressing Viral Replication in Post-therapy CD4+ T-Cells

We wanted to determine if CD8+ T-cells from pre-therapy, *in vitro* controlling, LVL donors could suppress viral replication in post-therapy samples that have lost the ability to control HIV-1 replication *in vitro*. To test this we activated and infected mixed PBMC cultures of MPY1313 from pre- and post-therapy time points with NL4-3 (post:pre; **Figure 6A**). As expected, post-therapy PBMC cultures alone supported and pre-therapy cultures alone suppressed viral replication. A mixture of post- and pre-therapy samples at 1:1 and 1:2 showed a complete suppression of viral replication. To confirm that this effect was specific to CD8+ T-cells as described above, we co-cultured activated,

TABLE 3 | LVL subjects placed on ART.

Patient ID	Draw	ART	Days on ART	Viral Load	CD4	CD8	CD4/CD8
NIM1164							
	2	Off	–407				
	3	Off	–379	570	864	2,300	0.38
	4	Off	–330				
	5	Off	–302				
	6	Off	–275				
	7	On	145	<20	914	1,886	0.48
MPY1313							
	1	Off	–12	1,550	473	756	0.63
	2	On	57	<20	427	628	0.68
	3	On	142	<20	747	1,039	0.72
	4	On	184	<20	667	924	0.72
	5	On	273	<20	571	954	0.60
VQY4910							
	3	Off	–411	2,410	543	757	0.72
	4	Off	–383				
	5	Off	–355				
	6	Off	–327	2,410	528	570	0.93
	7	Off	–292				
	8	On	52	<20	895	710	1.26
	9	On	141	<20	728	554	1.31
	10	On	204	<20	1,025	735	1.39

isolated CD4+ T-cells from post-therapy samples with activated, isolated CD8+ T-cells from pre-therapy samples and infected with NL4-3 (Donor MPY1313 and VQY4910; **Figures 6B,C**). CD8+ T-cells from pre-therapy samples successfully suppressed viral replication in CD4+ T-cells from post-therapy samples.

To expand upon these findings and examine the need for cell to cell contact, we performed a similar experiment using a transwell setup to separate the isolated CD4+ and CD8+ T-cells (**Figure 7**). T-cell populations were isolated from MPY1313 and VQY4910 PBMCs. CD4+ T-cells from post-therapy time points were placed in a well and CD8+ T-cells from pre-therapy time points were either co-cultured directly with CD4+ T-cells or placed in transwell. Cultures were infected with NL4-3 and viral replication followed by measuring p24 levels in the supernatant over 6 days. Direct contact of CD8+ and CD4+ T-cells prevented viral replication. CD8+ T-cells in transwell were unable to mediate this effect, suggesting a requirement for cell to cell contact.

Control of Infection Is Associated With Low Expression of the PD-1 Exhaustion Marker on CD8+ T-Cells

We next sought to determine if control in LVLs was the result of measurable changes in CD8+ T-cells. Chronic infection has been associated with alteration of the activation of state of T-cells and the expression of markers of immune exhaustion (Wherry et al., 2007; Wherry and Kurachi, 2015; Saeidi et al., 2018). Increased expression of exhaustion markers such as PD-1 are associated with decreased CD8+ T-cell function (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006) and these markers have been seen in HIV infected individuals (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Sauce et al., 2011). We performed flow cytometry to assess the levels of PD-1 expressed on CD8+ T-cells from normal donors, HVL and LVL donors (**Figure 8**). In keeping with previously published studies, CD8+ T-cells from HIV + subjects with high viral loads had increased levels of PD-1. Our LVL subjects had PD-1 levels indistinguishable from normal donors (**Figures 8B,C**). To determine if expression of

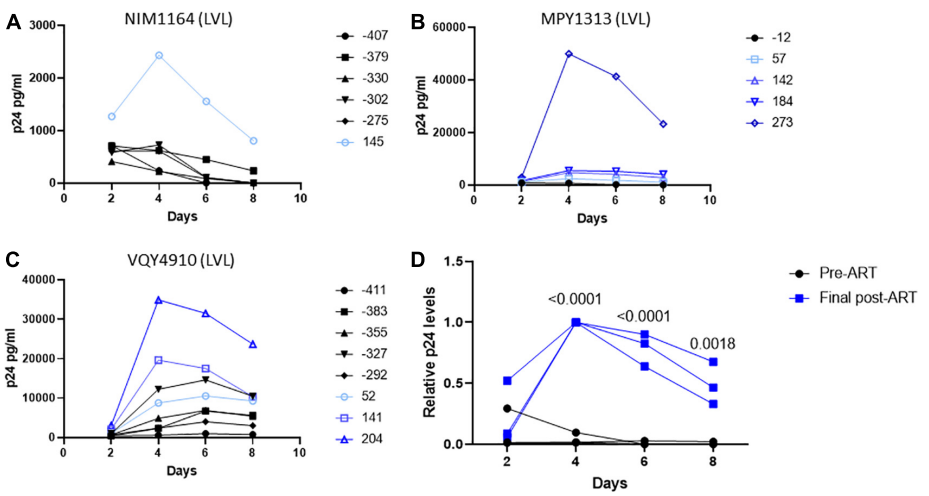

FIGURE 5 | Suppression of viral load in LVLs by ART results in loss of *in vitro* control of viral replication. Longitudinal PBMC samples from LVLs **(A–C)** placed on ART. PBMCs were induced with PHA and IL-2 then infected with NL4-3 48 h later. Supernatant was collected every 48 h post infection and virus levels were determined by p24 ELISA. **(D)** Comparison of relative p24 levels pre- vs final post-ART for LVL donors. One-way ANOVA was performed. **(A–D)** Numbers indicate days post-ART. Black lines are pre-ART PBMC cultures and blue lines are post-ART.

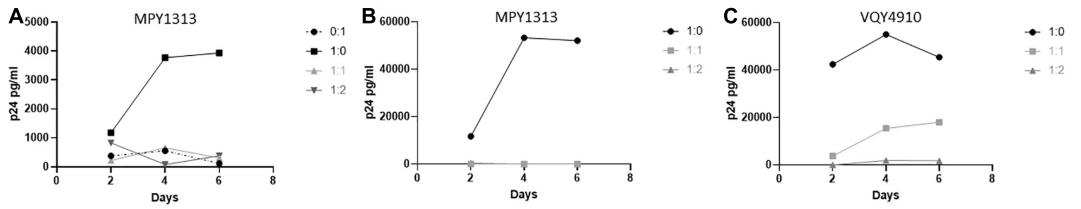

FIGURE 6 | Pre-therapy CD8+ T-cells suppress replication in post-therapy CD4+ T-cells. Samples from before and after initiation of therapy were used to determine if pre-therapy samples could control viral replication in post-therapy samples. **(A)** PBMCs from MPY1313 and isolated CD4+ and CD8+ T-cells from **(B)** MPY1313 and **(C)** VQY4910 pre and post-therapy were mixed at the indicated ratios (post:pre) and infected with NL4-3. Supernatant was collected every 48 h and virus levels were determined by p24 ELISA.

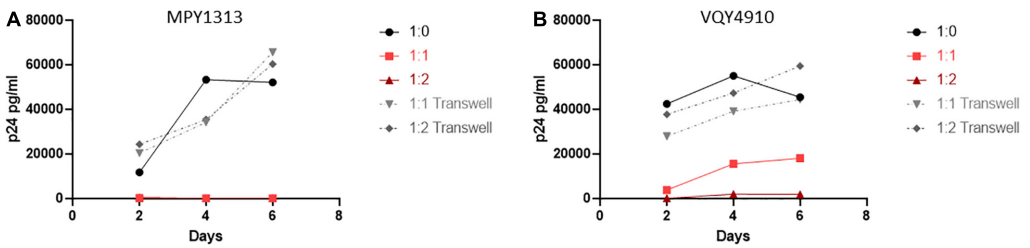

FIGURE 7 | Cell contact is necessary to mediate viral suppression in LVLs. CD4+ T-cells were isolated from post-therapy samples from **(A)** MPY1313 and **(B)** VQY4910 and cultured with isolated CD8+ T-cells from the same donor pre-therapy. CD4+ and CD8+ T-cells were stimulated with PHA and IL-2 for 48 h before being combined in the given ratios (CD4:CD8) in direct contact (red solid lines) or in transwell (gray dashed lines). Cultures were infected with NL4-3, supernatant was collected every 48 h post infection and virus levels were determined by p24 ELISA.

PD-1 correlates with control we examined the level of PD-1 expression on CD8+ T-cells from LVLs before the start of ART and the same subjects after ART when their PBMCs were able to support replication of HIV-1 in culture (**Figure 8D**). PD-1 levels were significantly higher in LVLs after the initiation of ART. We also performed flow cytometry to assess changes in immune activation by measuring the co-expression of HLA-DR and CD38 on CD8+ T cells from normal donors, LVLs, and HVLs (**Figures 8E–G**). Similar to what was observed with exhaustion, CD8+ T cells from HVLs with high viral loads had increased immune activation in comparison to both normal donors and LVLs (**Figure 8F**). To determine if immune activation also correlates with control, we evaluated the longitudinal co-expression of CD38/HLA-DR on CD8+ T cells both pre- and post-ART in our cohort of LVLs and determined that there was no significant difference in immune activation during times of *in vitro* control (pre-ART) in comparison to when PBMCs were susceptible to infection *in vitro* (post-ART) (**Figure 8G**).

DISCUSSION

Despite years of study we still do not completely understand what mediates natural control of HIV-1 infection in some individuals. Although studies have implicated multiple mechanisms that underlie many observed types of control, some mechanisms have not yet been elucidated. Our studies indicate that infected individuals with constantly low viral loads also demonstrate control of viral replication *in vitro* (**Figure 2**). This finding in itself provides another assay that could be performed to help further stratify types of control beyond available clinical observations.

Although absolute CD4+ T-cell count was initially used prognostically to assess progression to AIDS, it was observed that the rate of CD4+ T-cell decline varied significantly. Some individuals lost > 100 cells/μl per year, while others remained immunologically stable with normal CD4+ T-cell counts that sustained little to no loss over several years (Phair et al., 1992; Levy, 1993; Schwartländer et al., 1993; Buchbinder et al., 1994; Keet et al., 1994; Muñoz et al., 1995; Patrikar et al., 2014; Parsa et al., 2020). Before viral loads could be routinely measured, Dr. Ho et al. described a cohort of LTNPs who were HIV positive yet remained clinically asymptomatic without prolonged use of ART (Huang et al., 1995). These LTNPs, who were defined by CD4+ T-cell measurements and clinical outcomes, maintained normal CD4+ T-cell counts and had not developed opportunistic disease over an extended period of time. With the development of new technologies, focus shifted to quantifying plasma HIV-1 RNA as an independent reproducible marker of disease progression (Saksela et al., 1994; Mellors et al., 1995; Mellors et al., 1996). In 1997 Mellors et al., evaluated the rate of HIV disease progression in respect to both CD4+ T-cell count and plasma viremia. It was demonstrated that the rate of CD4+ T-cell loss was directly correlated to the level of HIV-1 viremia and together these parameters constituted the most accurate predictors of HIV-1 progression (O'Brien W. A. et al., 1996; Mellors et al., 1997; de Wolf et al., 1997). Those with high "viral loads" lost CD4+ T-cells more quickly than those with low or undetectable viral loads, who had little to no loss of CD4+ T-cells. Effective HIV-1 therapy

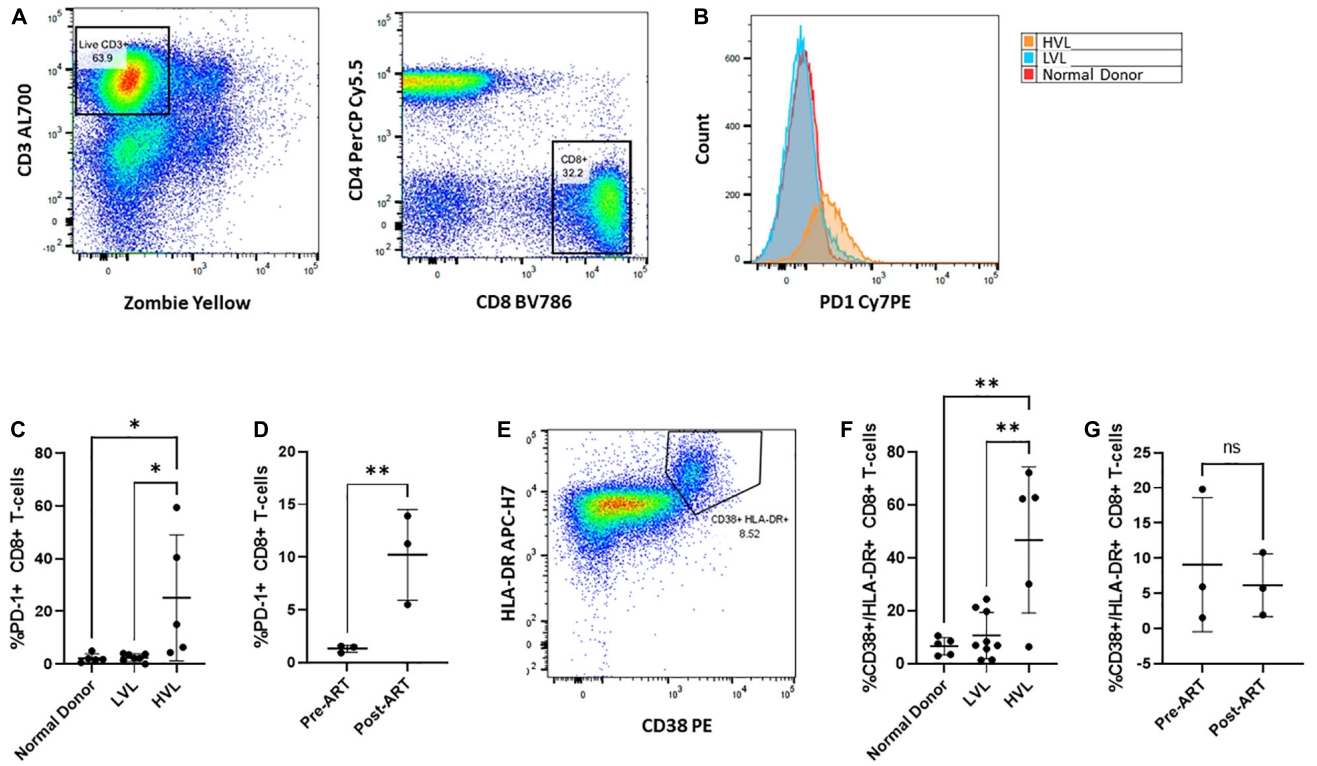

FIGURE 8 | Phenotypic characterization of CD8+ T cells Pre- and Post-therapy. PBMCs from normal, LVL or HVL donors were stained for both T-cell exhaustion using PD-1 and immune activation using HLA-DR and CD38 and analyzed by flow cytometry. **(A)** Gating strategy for determining the exhausted population of CD8+ T-cells. Following gating to exclude debris and doublets, gates were drawn around live CD3 + T-cells and this population used to identify CD4-/ CD8+ T-cell populations. **(B)** Comparison between normal donor (red), ART naïve LVL donor (blue), and ART naïve HVL donor (orange) expression of PD-1. **(C)** Comparison of CD8+ T-cell expression of PD-1 between normal donors (n = 5), ART naïve LVLs (n = 7), and ART naïve HVLs (n = 5) statistical significance was determined by one-way ANOVA, $^{*}P < 0.05$. **(D)** A longitudinal evaluation of CD8+ T-cell exhaustion pre- and post- therapy. A comparison of the expression of PD-1 was evaluated for 3 LVL donors both pre-ART and post-ART, $^{**}P < 0.01$. **(E)** Immune activation was determined by gating CD8+ T-cells that expressed both HLA-DR and CD38. **(F)** Comparison of CD8+ T-cell expression of HLA-DR and CD38 between normal donors (n = 5), ART naïve LVLs (n = 7), and ART naïve HVLs (n = 5) statistical significance was determined by one-way ANOVA, $^{**}P < 0.01$. **(G)** A longitudinal evaluation of CD8+ T-cell activation pre- and post-therapy.

confirmed that control of viremia prevented CD4+ T-cell loss and even allowed some CD4+ T-cell recovery in most cases. In support of this, it was found that the viral loads in LTNP cohorts varied from below average to undetectable (Pinto et al., 1995; García et al., 1997).

The measurement of viral load allowed further delineation of levels of control. Subjects who maintained low, but still detectable viral load remained LTNPs. Infected individuals with undetectable viral loads (<50 RNA copies/ml) began to be appreciated as a separate group (Clerici et al., 1992; Kelker et al., 1992; Cao et al., 1995; Pinto et al., 1995; Rowland-Jones et al., 1995; Fowke et al., 1996; Rowland-Jones et al., 1998; Lefrère et al., 1999; Pereyra et al., 2008). In the 2000's, researchers began focusing on these subjects. This cohort, called elite controllers (EC) by some, has been studied by several groups (Shacklett, 2006; Dyer et al., 2008; Pereyra et al., 2008; Gonzalo-Gil et al., 2017; Pernas et al., 2018; Lopez-Galindez et al., 2019; Nguyen et al., 2019). Each showed that EC CD4+ T-cells supported HIV-1 *in vitro* growth as well as non-EC. Work on understanding the mechanism by which ECs suppress the virus has focused on CD8+ T-cells and the cytotoxic T-lymphocyte (CTL) response. Genetic analysis has found partially "protective" MHC alleles (Migueles et al., 2000). In these studies, isolated CD4+ T-cell were used exclusively (Rabi et al., 2011).

Previous studies, especially those examining ECs, have focused on the role of the CD8+ CTL response in controlling infection (Koup et al., 1994; Borrow et al., 1994; Barker et al., 1998; Wilkinson et al., 1999; Betts et al., 2001; Migueles and Connors, 2001; Sáez-Cirión et al., 2007; Betts et al., 2006; Yan et al., 2013; Cartwright et al., 2016; Nishimura et al., 2017; Nguyen et al., 2019; McBrien et al., 2020a,b). The PBMCs from our LVLs were resistant to infection, but isolated CD4+ T-cells were capable of supporting low level viral replication (**Figure 2**). Further experiments with isolated CD4+ and CD8+ T-cells revealed that CD8+ T-cells were both necessary and sufficient to mediate control of infection *in vitro* (**Figure 3**). The ability of LVL PBMCs to mediate suppression of viral replication in HLA-matched allogeneic CD4+ T-cells (**Figure 4**) and the requirement for cell to cell contact (**Figure 7**) strongly support that the observed control is due to classical CTL function. What remains to be determined is how LVLs maintain effective CD8+ T-cell functionality during HIV infection. That a detectable viral load remains in these individuals and the lack of known protective HLA alleles suggests

this mechanism is different from what has been described for ECs (Migueles et al., 2000; Brener et al., 2015; Adland et al., 2018).

Unique to this study is the realization that the observed control *in vitro* is dependent upon the presence of detectable viral load in the donor and correlates with a low CD4:CD8 ratio (**Figures 4**, **5**). This observation lead us to discover that HVL donors who had these characteristics (detectable plasma viral load and CD4:CD8 ratio < 1) also had PBMCs that were resistant to viral replication *in vitro* (**Figures 5D,E**). Our data does not indicate why this control in tissue culture correlates with successful clinical control in some donors but not others. The observation that LVLs can lose control over time (**Figure 5**) and that $CD8^+$ T-cells from time points before the loss of control can suppress replication in post-control $CD4^+$ T-cells (**Figure 6**) implies a change not in the target T-cell, but the functionality of the $CD8^+$ T-cells.

The elucidation of the $CD8^+$ T-cell driven mechanism of control in our LVL donors strongly supports the examination of viral replication *in vitro* as a way to further delineate types of clinical control. While it is possible that the lack of control in HVLs may be due to CTL escape of the virus, our examination of loss of control in LVLs suggests that the failure to control may be related to the subject's immune system and not the virus. Our studies specifically highlight a role for changes in the level of $CD8^+$ T-cell exhaustion and not immune activation. Although examination of changes over a greater period of time may reveal a role for T-cell activation. Extensive studies in the field have linked chronic immune activation to disease progress (Ascher and Sheppard, 1988; Fuchs et al., 1988; Fahey et al., 1990; Sheppard et al., 1991; Giorgi et al., 1993, 1999; Liu et al., 1997; Zajac et al., 1998; Hazenberg et al., 2003; Deeks et al., 2004; Hunt et al., 2008; Liovat et al., 2009) and more recent studies have examined T-cell exhaustion in chronic HIV infection (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Wherry et al., 2007; Sauce et al., 2011; Wherry and Kurachi, 2015; Saeidi et al., 2018). These two mechanisms go hand in hand and are strong candidates for explaining loss of control in LVLs over time as suggested by our evaluation of PD-1. Understanding this mechanism may lead to the development of interventions that could be used to induce a functional cure in people living with HIV.

MATERIALS AND METHODS

Ethics Statement

The Smith Center for Urban Health and Infectious Disease, East Orange, NJ obtained written informed consent for the collection of blood donations from participating subjects. Samples were collected by trained medical staff under approved University of the Sciences' protocol (IRB protocol 900702-3 and 797649-3).

Human Subjects

All donors were recruited by the Smith Center for Urban Health and Infectious Disease. Two cohorts of HIV-1 infected donors were recruited, Low Viral Load (LVL) and High Viral Load (HVL) (characteristics at recruitment—**Table 1**). For the LVL cohort, 8 HIV-1 seropositive ART naïve donors were recruited, who were able to suppress HIV-1 infection independent of known protective HLA alleles. Four of these LVL donors were placed on combination ART during the course of this study. The HVL cohort included HIV-1 seropositive HVL donors pre- and post-therapy. Additionally, we recruited HIV-1 seronegative healthy control donors, Normal Donors that matched the age and characteristics of the two HIV-1 positive groups. For this study, classification as an HIV-1 controller required viremic control for a duration of at least 12 consecutive months in the absence of ART (O'Brien T. R. et al., 1996; Quinn et al., 2000; Gurdasani et al., 2014). HIV-1 controllers are defined as seropositive donors with viral loads of < 2,000 copies/mL and a $CD4^+$ T-cell count > 500 cells/mm^3 (O'Brien T. R. et al., 1996; Quinn et al., 2000; Gurdasani et al., 2014). HIV-1 seropositive donors that had low $CD4^+$ T-cell counts, below 200 and had high viral load, above 5,000 copies/mL were designated as HVL donors.

PBMC Isolation and Activation

PBMCs were purified from whole blood samples using Ficoll (GE Healthcare) gradient centrifugation and cryopreserved in 90% Fetal Bovine Serum (FBS; HyClone) containing 10% dimethyl sulfoxide (DMSO; Fisher). Frozen PBMCs were thawed and cultured in Roswell Park Memorial Institute (RPMI)-1640 complete media (GenClone) supplemented with 20% heat inactivated FBS, 1x penicillin-streptomycin-glutamine (PSG; Thermo Fisher Scientific), and 5% (5 U/mL) human rIL-2 (NIH AIDS Reagent Program). Cells were induced with 5 μg/mL phytohaemagglutinin-P (PHA-P) (Sigma) for 48 h at 37°C and 5% CO_2.

$CD4^+$ and $CD8^+$ T-Cell Isolation and Activation

$CD4^+$ and $CD8^+$ T-cells were purified from frozen PBMCs using MACS Miltenyi negative isolation kits (cat# 130-096-533 and cat# 130-096-495, respectively) according to manufacturer's protocol. Enriched $CD4^+$ and $CD8^+$ T-cell populations were independently activated in RPMI complete media with 5μg/mL PHA for 48 h at 37°C and 5% CO_2.

HIV-1 Virus Stock

The HIV-1 stock used in this study was generated by transfecting pNL4-3 (NIH AIDS Reagent Program, ARP-2852, contributed by Dr. M. Martin) into HEK293T cells [American Type Culture Collection (ATCC, CRL-11268)] using TransFectin Lipid Reagent (BioRad, Cat# 1703351) following manufacturer's instructions. Transfected cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1x PSG for 48 h at 37°C and 5% CO_2. Virus containing supernatants were aspirated from the cells, filtered, and frozen in 1 ml aliquots. Frozen stocks were quantified by p24 Gag ELISA.

Spreading Infection Assay

Activated total PBMCs, isolated $CD4^+$ T-cells, or mixed $CD4^+$ and $CD8^+$ T-cells were infected with 17 ng/ml NL4-3 virus at 37°C and 5% CO_2. For experiments where isolates were

collected and recombined, cells were co-cultured for 3–6 h prior to infection. After 24 h, the virus containing media was removed and replaced. Virus production was evaluated by measuring the p24 levels in the supernatant using ELISA (Zeptometrix). Time points were collected every 48 h post infection for six to 8 days.

Flow Cytometry

For phenotypic analysis of cell populations, all cells were washed with Phosphate Buffered Saline (PBS) without Ca^{2+} and Mg^{2+} (GenClone) and stained using fluorescently conjugated antibodies against the following cell surface markers following manufacturer's instructions: CD3 (BD Biosciences (BD), Alexa700, clone SP34-2), CD4 (BD, PerCP-Cy5.5), CD8 (BD, BV786, clone RPA-T8) and CD279 (PD-1) (Invitrogen, clone eBioJ105), HLA-DR (BD, APC-H7, clone L243), and CD38 (BD, PE, clone HIT2). Cells were also stained for viability using the live/dead stain Zombie Yellow (BioLegend). Cell enumeration was carried out using Cytek FACSort DxP12 flow cytometer and data analysis using FlowJo v10.6.1 software.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The Smith Center for Urban Health and Infectious Disease, East Orange, NJ obtained written informed consent for the collection of blood donations from participating subjects. Samples were collected by trained medical staff under approved University of the Sciences' protocol (IRB protocol 900702-3 and 797649-3).

AUTHOR CONTRIBUTIONS

ZK and SS conceptualized this project. SK and AJ designed research, performed experiments, and analyzed and interpreted data. TJ assisted in experiments. AJ, SK, SS, and ZK wrote the manuscript. ZK aided in the design of the experiments and the analysis and interpretation of data. ES recruited patients, handled patient education and organization of clinical data. All authors contributed to the article and approved the submitted version.

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Potential Utilization of APOBEC3-Mediated Mutagenesis for an HIV-1 Functional Cure

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The introduction of combination antiretroviral therapy (cART) has managed to control the replication of human immunodeficiency virus type 1 (HIV-1) in infected patients. However, a complete HIV-1 cure, including a functional cure for or eradication of HIV-1, has yet to be achieved because of the persistence of latent HIV-1 reservoirs in adherent patients. The primary source of these viral reservoirs is integrated proviral DNA in $CD4^+$ T cells and other non-T cells. Although a small fraction of this proviral DNA is replication-competent and contributes to viral rebound after the cessation of cART, >90% of latent viral reservoirs are replication-defective and some contain high rates of G-to-A mutations in proviral DNA. At least in part, these high rates of G-to-A mutations arise from the APOBEC3 (A3) family proteins of cytosine deaminases. A general model has shown that the HIV-1 virus infectivity factor (Vif) degrades A3 family proteins by proteasome-mediated pathways and inactivates their antiviral activities. However, Vif does not fully counteract the HIV-1 restriction activity of A3 family proteins *in vivo*, as indicated by observations of A3-mediated G-to-A hypermutation in the proviral DNA of HIV-1-infected patients. The frequency of A3-mediated hypermutation potentially contributes to slower HIV-1/AIDS disease progression and virus evolution including the emergence of cytotoxic T lymphocyte escape mutants. Therefore, combined with other strategies, the manipulation of A3-mediated mutagenesis may contribute to an HIV-1 functional cure aimed at cART-free remission. In this mini-review, we discuss the possibility of an HIV-1 functional cure arising from manipulation of A3 mutagenic activity.

Keywords: APOBEC3-mediated mutagenesis, genetic factors, A3 expression, A3-interacting proteins, adaptive immunity, Vif inhibitors

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) was first discovered in 1983 as the agent that causes acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983). HIV-1 disrupts the immune system by infecting immune cells, such as $CD4^+$ T cells, macrophages, and dendritic cells, which ultimately leads to AIDS and related opportunistic infections. AIDS has become a manageable chronic disease due to the development of combination antiretroviral therapy (cART), which has enabled complete suppression of detectable viremia and controls disease progression in adherent patients. However, cART does not eliminate HIV-1 from these patients because latent reservoirs with the HIV-1 genome integrated into host DNA remain present (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997) and these latently infected

cells clonally expand (Ikeda et al., 2007; Maldarelli et al., 2014; Wagner et al., 2014). When the latent reservoirs harbor replication-competent proviral DNA, they contribute to viral rebound when cART is interrupted (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997).

Efforts have been made to quantify the size of the latent reservoirs [reviewed by Wang et al. (2018); Lambrechts et al. (2020)]; studies have demonstrated that the majority of proviral DNA in the latent reservoirs is replication-defective and does not contribute to viral rebound when cART ceases. Ideally, complete eradication of HIV-1 from infected patients is the goal of a HIV-1 cure; however, because >90% of latent reservoirs harbor defective viruses, the initial goal should be a functional cure for HIV-1, which would comply with detectable proviral DNA and undetectable (or low-level) plasma viremia without cART. Of particular interest are elite controllers and long-term non-progressors, who represent <1% of all patients living with HIV-1 and are natural models for the functional cure [reviewed by Gonzalo-Gil et al. (2017); Promer and Karris (2018)]; however, little is known about the underlying mechanisms by which the immune systems of such patients control the virus.

APOBEC3 (A3) family proteins are cytosine deaminases that play important roles in mammalian innate immune responses. The human genome encodes seven *A3* genes on chromosome 22, which include three single-domain deaminase genes (*A3A*, *A3C*, and *A3H*) and four double-domain deaminase genes (*A3B*, *A3D*, *A3F*, and *A3G*) [reviewed by Koito and Ikeda (2012); Desimmie et al. (2014); Harris and Dudley (2015); **Figure 1A**]. In $CD4^+$ T cells, up to five A3 proteins (A3C-Ile188, A3D, A3F, A3G, and A3H) are involved in HIV-1 restriction (Hultquist et al., 2011; Refsland et al., 2012, 2014; Ooms et al., 2013; Wittkopp et al., 2016; Anderson et al., 2018). These A3s are packaged into nascent viral particles and catalyze the deamination of cytosine-to-uracil in reverse transcription cDNA intermediates (**Figure 1B**) [reviewed by Harris and Dudley (2015); Simon et al. (2015)]. The uracil lesion provides a template for adenine insertion in HIV-1 genomic strands while accumulation of many G-to-A mutations ultimately leads to virus inactivation (**Figure 1B**). HIV-1 viral infectivity factor (Vif) recruits an E3 ubiquitin ligase complex to promote the degradation of A3 proteins through a proteasome-mediated pathway and counteracts their antiviral activity [reviewed by Harris and Dudley (2015); Salamango and Harris (2020); **Figure 1B**]. This model is well established and widely accepted. Nevertheless, reports have shown that some fraction of latent reservoirs harbors high rates of G-to-A mutations in proviral DNA (Kieffer et al., 2005; Ho et al., 2013; Imamichi et al., 2014; Bruner et al., 2016, 2019). Importantly, A3-mediated G-to-A hypermutation in proviral DNA is significantly correlated with disease progression in HIV-1-infected patients including elite controllers and long-term non-progressors (Pace et al., 2006; Land et al., 2008; Kourteva et al., 2012; Eyzaguirre et al., 2013; Cuevas et al., 2015). Additionally, $CD4^+$ T cells from HIV-1 controllers with higher A3G expression levels are less susceptible to *in vitro* HIV-1 infection than are $CD4^+$ T cells from HIV-1 controllers with lower A3G expression levels (Biasin et al., 2007; De Pasquale et al., 2013). Furthermore, A3-mediated mutagenesis seems to influence cytotoxic T lymphocyte (CTL) responses [reviewed by Stavrou and Ross (2015); **Figure 2**]. Taken together, these observations indicate that the mutagenic activity of A3 family proteins is a factor that determines HIV-1/AIDS disease progression. Therefore, the manipulation of A3-mediated mutagenesis to lethal levels may be a potential target for an HIV-1 functional cure. Here, we discuss the possibility of introducing an HIV-1 functional cure mediated by the mutagenesis of A3 family proteins.

CONTRIBUTION OF *APOBEC3* GENE VARIATIONS TO HIV-1 DISEASE PROGRESSION

The *A3H* gene is likely a genetic factor that controls the disease progression of HIV-1 with the particular genotype of the *vif* gene. *A3H* is the most variable *A3* gene in the human population (**Figure 1A**). Four single-nucleotide polymorphisms and one indel in the *A3H* gene determine stable and unstable haplotypes of A3H proteins (OhAinle et al., 2008; Wang et al., 2011; Refsland et al., 2014; Ebrahimi et al., 2018). Stable A3H haplotypes are expressed stably and have the ability to inhibit HIV-1, whereas unstable A3H haplotypes are barely detectable or undetectable at the protein level and lack antiviral activity. Interestingly, not all HIV-1 Vif variants from laboratory and natural isolates can degrade stable A3H haplotypes. Several studies have identified amino acid residues at positions 39, 48, and 60–63 that influence the capability of Vif proteins to degrade stable A3H haplotypes but do not affect counteraction activity against A3D, A3F, and A3G (Binka et al., 2012; Ooms et al., 2013; Refsland et al., 2014). Hereafter, we denote Vif proteins that can degrade stable A3H haplotypes and other HIV-1-restrictive A3 proteins (i.e., A3D, A3F, and A3G) as "hyper-functional," while we term those that fail to degrade stable A3H haplotypes but not other HIV-1 restrictive A3 proteins as "hypo-functional."

Intriguingly, studies have shown that A3H haplotypes are correlated with the global distribution of HIV-1 Vif alleles (Refsland et al., 2014; Nakano et al., 2017b). For example, around 60% of people have stable A3H haplotypes in Sub-Saharan Africa where hyper-functional Vifs are dominant (Refsland et al., 2014; Nakano et al., 2017b). In contrast, hypo-functional Vifs are prevalent in Asia because most people have unstable A3H haplotypes (Refsland et al., 2014; Nakano et al., 2017b). This biogeographical relationship strongly suggests that HIV-1 with hyper-functional, too Vif has evolved to counteract stable A3H haplotypes but that stable A3H haplotypes remain a potential transmission barrier against HIV-1 with hypo-functional Vif outside Africa. This possibility is supported by previous studies showing that HIV-1-infected patients (including long-term non-progressors) with at least one allele of stable A3H haplotypes have slower disease progression to AIDS (Ooms et al., 2013; Sakurai et al., 2015). Therefore, stable A3H haplotypes may be effective for short-term HIV-1 suppression. However, before stable A3H haplotypes can be exploited for long-term HIV-1 suppression, further studies should ascertain the period over which they can control HIV-1 with the hypo-functional *vif* gene in the absence of cART and which variants emerge under the selective pressure of stable A3H haplotypes *in vivo*.

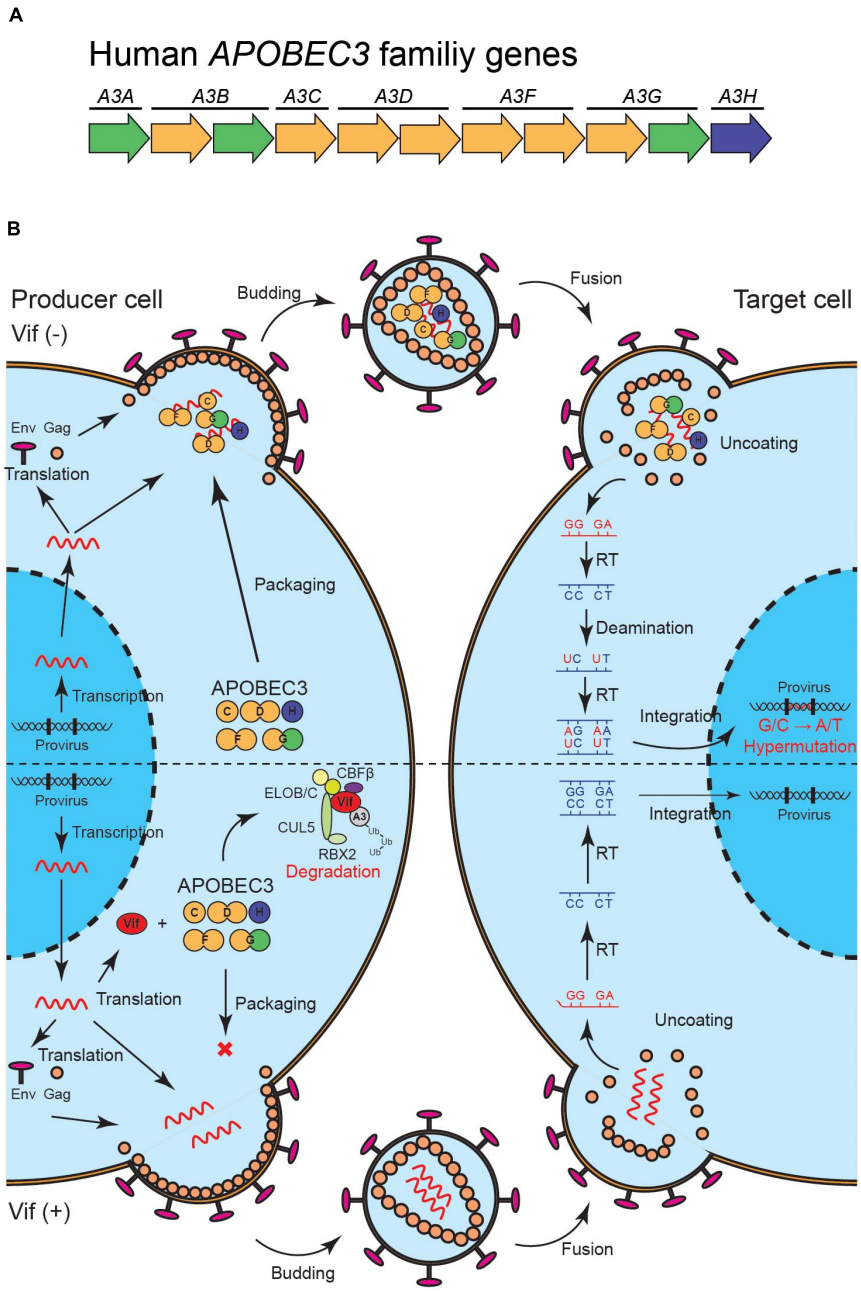

FIGURE 1 | A model of HIV-1 restriction by A3 family proteins and counteraction by HIV-1 Vif. **(A)** Illustration of human *A3* family genes. Human *A3* family genes are composed of seven members with one or two zinc-coordinating domains (single of double-domain deaminases); these belong to three phylogenetically different groups, which are shown in green, yellow, and blue. **(B)** A schematic of HIV-1 restriction by five A3 family proteins and neutralization by HIV-1 Vif. A3C, A3D, A3F, A3G, and A3H are packaged into HIV-1 virions in producer cells and inactivate the virus through cytosine-to-uracil (C-to-U)/guanine-to-adenine (G-to-A) mutations **(top)**. HIV-1 Vif neutralizes the restriction activities of these A3 proteins through proteasome-mediated degradation **(bottom)**. Vif, virus infectivity factor; A3, APOBEC3.

REGULATION OF APOBEC3 FAMILY PROTEINS

Certain populations of proviral DNA in HIV-1-infected patients comprise defective viruses with A3-mediated G-to-A hypermutation (Kieffer et al., 2005; Ho et al., 2013; Imamichi et al., 2014; Bruner et al., 2016, 2019), suggesting that some of the A3 family proteins may avoid Vif-mediated neutralization *in vivo*. A simple explanation for this relates to the abundance of A3 family proteins, which can quantitatively exceed the capacity of Vif proteins (**Figure 1B**). In support of this explanation, $CD4^+$ T cells from HIV-1 controllers with

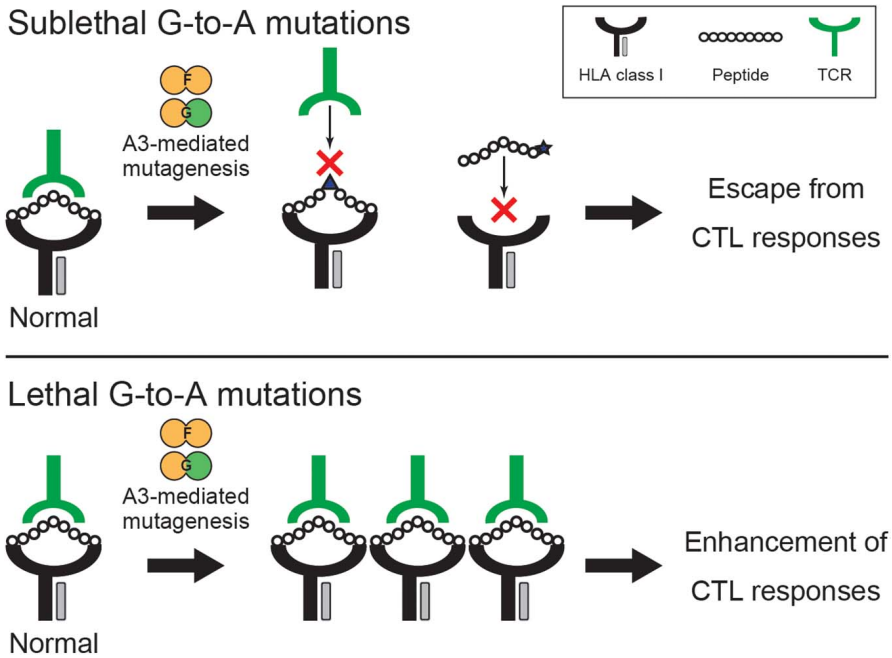

FIGURE 2 | Potential effects of A3-mediated mutagenesis on CTL responses. A3F- and A3G-mediated mutagenesis alters CTL responses through the accumulation of G-to-A mutations on the viral genome, which leads to the modification of epitope sequences and their flanking regions involved in antigen processing/presentation, HLA binding, and TCR recognition. The two examples show that A3-mediated hypermutation on epitope sequences potentially alters HLA binding of the epitopes and TCR recognition. Sublethal A3-mediated mutagenesis is involved in the emergence of CTL escape variants **(top)**, whereas lethal A3-mediated hypermutation likely increases the number of HIV-derived epitopes and consequently enhances HIV-1-specific CTL responses **(bottom)**. A3, APOBEC3; CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen; TCR, T cell receptor.

higher A3G expression levels were found to be more resistant to HIV-1 infection *in vitro* compared with $CD4^+$ T cells from HIV-1 controllers with lower A3G expression levels (Biasin et al., 2007; De Pasquale et al., 2013). Therefore, the upregulation of A3 family proteins seems to increase defective viruses. However, the regulatory mechanisms of A3 family proteins have yet to be fully elucidated, largely because the expression of these proteins is intricately regulated and dependent on multiple determinants such as cell types (Koning et al., 2009, 2011; Refsland et al., 2010; Berger et al., 2011; Burns et al., 2013; Ikeda et al., 2019) and the surrounding environment of development and inflammation, e.g., the interferon (IFN) response (Peng et al., 2006, 2007; Pion et al., 2006; Ellery et al., 2007; Berger et al., 2011; Koning et al., 2011; Land et al., 2013; Mohanram et al., 2013).

Interestingly, most A3 family proteins are incorporated into ribonucleoprotein complexes in human cells (e.g., $CD4^+$ T cells). In these cells, A3G forms enzymatically inactive high molecular mass (HMM) complexes immediately after translation, which are composed of A3G-binding proteins, A3G-binding RNAs, and RNA-binding proteins (Chiu et al., 2006; Soros et al., 2007; Stopak et al., 2007). These complexes are shifted to enzymatically active low molecular mass (LMM) complexes by RNase treatment (Soros et al., 2007). Hence, the formation of A3G HMM complexes in cells is one factor that determines permissiveness to HIV-1 infection (Pion et al., 2006; Ellery et al., 2007), being markedly altered by cytokines such as IFNs and chemokines (Kreisberg et al., 2006; Stopak et al., 2007). Similarly, the formation of HMM and LMM complexes in cells is conserved among other HIV-1 restrictive A3s (at least in A3F and A3H) (Niewiadomska et al., 2007; Ito et al., 2018). Although >100 A3G interactors have been identified (Chiu et al., 2006; Kozak et al., 2006; Gallois-Montbrun et al., 2007; Shirakawa et al., 2008; Sugiyama et al., 2011), *bona fide* interactors that regulate the restrictive capacity of A3s against HIV-1 are yet to be clearly defined. Accordingly, identifying novel A3 interactors that modulate the mutagenic activity of A3 enzymes against HIV-1 and resolving the molecular mechanisms that control these regulatory factors will be important for developing an HIV-1 functional cure that functions *via* A3-mediated mutagenesis.

POTENTIAL UTILIZATION OF VIF-RESISTANT A3 PROTEINS FOR AN HIV-1 FUNCTIONAL CURE

APOBEC3 proteins are examples of host factors that restrict cross-species transmission of lentiviruses [reviewed by Nakano et al. (2017a); Uriu et al. (2021)]. Interactions between mammalian A3 proteins and lentiviral Vifs are largely specific to viruses in their hosts (Mariani et al., 2003; Compton et al., 2012; Etienne et al., 2015; Yamada et al., 2016; Zhang et al., 2016; Nakano et al., 2020). As examples, HIV-1 Vif can degrade A3G protein from humans but not from African green monkeys or rhesus macaques (Bogerd et al., 2004; Schrofelbauer et al., 2004;

Xu et al., 2004). Three laboratories have demonstrated that a single amino acid residue at position 128 of the A3G protein determines this interaction; human A3G D128K proteins, therefore, become resistant to HIV-1 Vif (Bogerd et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004). One potential strategy for controlling HIV-1 under cART-free conditions would be the use of Vif-resistant A3 proteins such as human A3G D128K proteins. Recently, Delviks-Frankenberry et al. (2019) took a novel approach to control HIV-1 using self-activating lentiviral vectors that deliver the human *A3G D128K* gene to target cells. T cell lines in which the human *A3G D128K* gene was transduced by this system were shown to potently inhibit HIV-1 replication and suppress the emergence of resistant viruses against human A3G D128K proteins for >3.5 months. The delivery system was also used to transduce primary $CD4^+$ T cells as well as $CD34^+$ hematopoietic stem and progenitor cells with transduction efficiencies of around 15 and 30%, respectively. Thus, developments in the efficiency of gene-of-interest delivery may improve the feasibility of gene therapy involving Vif-resistant A3 proteins as an HIV-1 functional cure.

Another interesting challenge is the creation of a "super restriction factor" to function as an inhibitor more potent than the original protein (McDonnell et al., 2020). Single-domain deaminase A3C proteins have weak HIV-1 restriction activity compared with that of double-domain deaminases, e.g., A3D, A3F, and A3G proteins (Bishop et al., 2004; Hultquist et al., 2011; Wittkopp et al., 2016; Anderson et al., 2018). Synthetic tandem double deaminase A3C proteins represent one attempt to create HIV-1 restrictive A3C proteins that are more potent than the native proteins (McDonnell et al., 2020). The novel double-domain A3C proteins showed improved HIV-1 restriction activity and were largely resistant to Vif-mediated degradation. Although an efficient delivery system to HIV-1 target cells must be established, a strategy by which single deaminases are genetically connected could generate deaminases that are more potent and Vif-resistant; such proteins could be exploited to develop an HIV-1 functional cure.

ASSOCIATION OF APOBEC3-MEDIATED MUTAGENESIS WITH CTL RESPONSES

Clearly, A3 family proteins contribute to innate immunity against retroviruses including HIV-1. In addition, there is evidence that A3-mediated mutagenesis is involved in adaptive immunity, such as CTL responses, through changes to proviral sequences (**Figure 2**). This can be validated because A3 family protein expression in antigen-presenting cells, e.g., macrophages, varies upon HIV-1 infection or cytokine stimulation with IFN (Peng et al., 2006, 2007; Pion et al., 2006; Biasin et al., 2007; Stopak et al., 2007; Land et al., 2008; Koning et al., 2009, 2011; Berger et al., 2011; Hultquist et al., 2011; Mohanram et al., 2013). Moreover, this process provides an additional layer to a potential HIV-1 functional cure by A3-mediated mutagenesis, i.e., *via* innate and adaptive immune responses.

Human immunodeficiency virus type 1-specific CTL responses and their human leukocyte antigen (HLA) restrictions are likely to be determinants of viral replication control in HIV-1-infected individuals [reviewed by Macatangay and Rinaldo (2015)]. However, mutations accumulate on the HIV-1 genome during infection and escape variants that can avoid HIV-1-specific CTL responses subsequently emerge. HIV-1 sequencing data from patients has indicated that A3-mediated G-to-A mutations (mainly A3F and A3G) are embedded within the predicted CTL epitopes and flanking regions, suggesting that sublethal levels of A3-mediated mutagenesis are involved in the emergence of CTL escape variants [reviewed by Grant and Larijani (2017); Venkatesan et al. (2018); **Figure 2**]. In contrast, one study in which the Vif-null virus was used revealed that A3G-induced mutations enhance HIV-1-specific CTL responses (Casartelli et al., 2010). Interestingly, engineered defective viruses with premature termination codons caused by A3G can enhance the abundance of HIV-1-derived epitopes, resulting in the activation of HIV-1-specific CTLs (Casartelli et al., 2010; **Figure 2**). The degradation of polypeptides from defective mRNAs is efficiently presented on major histocompatibility complex class I molecules (Trentini et al., 2020). Indeed, defective HIV-1 proviruses retain the ability to transcribe mRNAs and produce proteins that can be recognized by HIV-1-specific CTLs (Imamichi et al., 2016, 2020; Pollack et al., 2017). Therefore, lethal A3-mediated hypermutation may contribute to the generation of HIV-1 peptides supplied to the pool of antigens presented on major histocompatibility complex class I molecules and to the enhancement of CTL responses (**Figure 2**). Although effects on CTL responses seem to differ depending on lethal or sublethal levels of A3-mediated mutagenesis, A3-mediated mutagenesis undoubtedly contributes to the quantity and quality of HIV-specific CTL responses. For successful HIV-1 control without cART, A3 family proteins may be required to function alongside factors associated with adaptive immunity such as CTLs. Understanding the potential role of A3 proteins in innate and adaptive immunity would create new avenues of possibility for an HIV-1 functional cure.

TARGETING THE APOBEC3/VIF AXIS TO MANIPULATE APOBEC3-MEDIATED MUTAGENESIS

Proteasomal degradation of HIV-1-restrictive A3s by Vif limits the packaging of these A3 enzymes into viral particles and thereby prevents A3-mediated hypermutation in the subsequent viral infection (**Figure 1B**). Nevertheless, evidence increasingly suggests that sublethal levels of A3-mediated mutagenesis contribute to virus evolution including drug resistance and immune escape [reviewed by Grant and Larijani (2017); Venkatesan et al. (2018)]. One possible therapeutic strategy would involve restoration of the lethal mutagenic activity of A3 family proteins by direct inhibition of Vif. Indeed, this viral protein is an attractive therapeutic target because it is not known to have mammalian homologs. Moreover, it is difficult for Vif-null HIV-1 to adapt to cells expressing full HIV-1 restrictive A3 proteins simultaneously (Haché et al., 2008; Albin et al., 2010; Ikeda et al., 2018).

Many attempts have been made to discover small molecules that target Vif proteins. Of these small molecules, RN-18 and its analogs are particularly well studied. RN-18 was discovered by screening 30,000 small molecules for the effects of Vif on fluorescence-tagged A3G signals (Nathans et al., 2008). RN-18 has been shown to exhibit A3G-dependent anti-HIV-1 activity in three non-permissive T cell lines (IC_{50} = 6 μM in H9); RN-18 treatment reduces the expression of cellular Vif and increases A3G expression levels, leading to the packaging of more A3G in viral particles. In addition, structure–activity relationship studies have revealed RN-18 analogs with improved potency in their antiviral activity relative to that of RN-18 (Ali et al., 2012; Mohammed et al., 2012, 2016; Zhou et al., 2017; Sharkey et al., 2019). For example, the compound 12c enhances the antiviral activity of A3G by >150-fold compared with the effect of RN-18 (Zhou et al., 2017). Furthermore, a recent cell culture study reported the emergence of a resistant virus against the RN-18 analog IMC15 and proposed a docking model of IMC15 bound to the Vif-A3G-E3 ligase complex (Sharkey et al., 2019). Along with RN-18 and its analogs, IMB-26/35, MM-1/2, and VEC-5 have been identified as lead compounds [reviewed by Olson et al. (2018)]. Overall, the development of small molecules that restore the mutagenic activity of A3 enzymes remains at an early stage. However, structural findings, such as the elucidation of the A3F–Vif interface (Hu et al., 2019), help to develop Vif antagonists that are more potent and can improve strategies for an HIV-1 functional cure.

CONCLUSION

HIV-1 has adapted to human cells such as $CD4^+$ T cells; consequently, restriction factors, including A3 family proteins, are less effective against the virus. However, certain populations of HIV-1 are still exposed to lethal or sublethal A3-mediated mutagenesis, suggesting that the manipulation of this process is an attractive target through which to develop a functional cure for HIV-1. Therefore, in combination with several other strategies or therapies, A3-mediated mutagenesis could potentially be applied to functionally cure HIV-1.

AUTHOR CONTRIBUTIONS

TI drafted the manuscript. All authors edited, contributed to the manuscript, and approved the submitted version.

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