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Janus: A New Use of Jak Inhibitors for the Decay of HIV Reservoir

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Janus: A New Use of Jak Inhibitors for the Decay of HIV Reservoir

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Molecular Biology B.S., Western Carolina University, 2019

Biomedical Chemistry B.S., Western Carolina University, 2019

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Abstract

Janus: A New Use of Jak Inhibitors for the Decay of HIV Reservoir

By Monica D. Reece

Antiretrovirals do not target the HIV-1 reservoir in people with HIV (PWH), a major barrier to cure. Infected myeloid and CD4+ T cells exacerbate inflammation despite suppressed viremia. Chronic inflammation drives reservoir maintenance and reactivation in T cells and myeloid derived viral sanctuaries and contributes to non-AIDS comorbidities. We first evaluated the HIV-1 DNA reservoir in peripheral blood mononuclear cells (PBMC) and cerebrospinal fluid (CSF), as a surrogate for the central nervous system (CNS) reservoir, in PWH and associations to cognitive dysfunction. We observed CNS HIV correlated with worse executive function. Next, we evaluated ruxolitinib's impact on the peripheral HIV-1 reservoir and immunomodulatory events driving persistence in PWH enrolled in ACTG A5336, an open-label randomized Phase 2a multi-site clinical trial. Participants (18-75 years old, on antiretroviral therapy (ART) ≥2 years, virologically suppressed, $CD4^+$ count >350 cells/mm^3, and without significant comorbidities except HIV or hypertension) were randomized to Jak inhibitor ruxolitinib plus ART (n=40) or ART alone (n=20) from week 0-5 and observed through week 12. Cellular markers, integrated DNA, and IPDA were measured peripherally at weeks 0, 5, and 12. Reservoir markers decayed in high baseline reservoir (HBR) participants on ruxolitinib by week 12 *versus* controls (p=0.0471). Cellular markers altered by ruxolitinib and associated with decay included pSTAT5+, pSTAT3+, BCL-2+KI67+, CD127+, and CD25+. We predict 99.99% decay in 2.83 years among HBR. These data are foundational for future human trials with Jak 1/2 inhibitors towards HIV-1 elimination. Clinical Trials Registration NCT02475655. Finally, we evaluated baricitinib, an FDA approved, 2nd generation Jak 1/2 inhibitor with a favorable safety, efficacy, and pharmacokinetic profile and once daily dosing. Preliminary *in vitro* data revealed that baricitinib did not impact HIV-GFP DNA or mRNA levels, significantly reduced IL-15-mediated pSTAT5 production, reduced active infection (p24+gp120+) in CD4+ T cells, and significantly reduced BCL-2 expression in both actively and latently (p24+gp120-) infected CD4+ T cells. These findings indicated that anti-viral effects of baricitinib are due to immunomodulation and that baricitinib would likely mirror the reservoir decay effect of ruxolitinib given a therapeutic duration ≥ 5 weeks as evidenced by reduction of reservoir maintenance, reactivation, and reseeding factors.

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Chapter I: Introduction

Overview

The human immunodeficiency virus (HIV) pandemic has been ongoing since 1981. UNAIDs estimated that 39 million people were living with HIV (PWH) as of 2022. The World Health Organization (WHO) estimates a cumulative 40.4 million deaths since the beginning of the pandemic. Many advancements have been made in therapeutics for HIV, including antiretroviral therapy (ART) which can suppress active viral replication and reduce the disease burden of PWH while also limiting transmission potential. HIV is a retrovirus that encodes machinery such as integrase to insert viral DNA into the host cell genome, creating a provirus. HIV provirus, also referred to as the HIV reservoir, is a major barrier to cure efforts. Despite the success of ART, it does not target the HIV reservoir. To date, there are no approved therapies that reduce the HIV reservoir.

The Gavegnano Lab's main focus is drug repurposement. Utilizing Food and Drug Administration (FDA) approved compounds for new indications reduces the timeline from discovery to therapeutic use because approved compouds have already been veted for safety and dosage. In terms of therapeutic discovery, repurposement is much more cost and labor efficient. The primary focus of this dissertation is to study the HIV-1 reservoir, which persists despite viral suppression by ART, and potential therapeutics. The HIV-1 reservoir is maintained by systemic, chronic inflammation, which is observed even in virally suppressed PWH. A primary driver of inflammation is the Jak STAT pathway, which we can target with the Jak inhibitor drug class.

This dissertation ecompases three separate yet related projects: 1) assessment of the HIV-1 reservoir in peripheral blood mononuclear cells (PBMC) versus cerebrospinal fluid (CSF), as a surrogate for the central nervous system (CNS), in humans and identification of associations with neurocognition, 2) evaluating the ability of ruxolitinib, an FDA-approved, first-generation Jak 1/2 inhibitor, to decay the HIV-

1 reservoir in PWH on ART in phase 2a human trial A5336 (NCT02475655), and 3) *in vitro* assessment of baricitinib efficacy, FDA-approved, 2nd generation Jak Inhibitor, in primary human CD4+ T cells and monocyte-derived macrophages.

We found that intact HIV provirus was found in 7/11 PWH assessed, and that defective and total provirus was significantly higher in the CSF compared to PBMC. We observed that higher defective provirus in the CSF was significantly correlated with worse executive function, and that higher defective and total provirus in the CSF was trending towards signifanctly worse learning. Higher total provirus in PBMCs was trending towards significantly worse learning and memory, potentially highlighting a link between peripheral reservoir and cognition.

In the A5336 phase 2a trial, we observed a significant decay in total provirus in a subset of participants with a high baseline reservoir (HBR) from weeks 5-12, and found that cellular markers altered by ruxolitinib and associated with reservoir decay included pSTAT5+, pSTAT3+, BCL-2+KI67+, CD127+, and CD25+. We predict 99.99% decay of the peripheral reservoir in 2.83 years among HBR using the observed decay rate. We found that biomarker expression profiles were opposite between PWH who experienced reservoir growth vs decay and that ruxolitinib was able to reverse the expression profile observed in the control.

Preliminary *in vitro* data revealed that baricitinib did not impact HIV-GFP DNA or mRNA levels, significantly reduced IL-15-mediated pSTAT5 production, reduced active infection (p24+gp120+) in CD4+ T cells, and significantly reduced BCL-2 expression in both actively and latently (p24+gp120-) infected CD4+ T cells. These findings indicated that anti-viral effects of baricitinib are due to immunomodulation and that baricitinib would likely mirror the reservoir decay effect of ruxolitinib given a therapeutic duration $\geq$ 5 weeks as evidenced by reduction of reservoir maintenance, reactivation, and reseeding factors.

These data are foundational for future human trials with Jak 1/2 inhibitors towards HIV-1 elimination, and expand our understanding of the HIV-1 reservoir and inflammation in PWH.

Targeting Macrophage Dysregulation for Viral Infections: Novel Targets for Immunomodulators

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CG and RT conceptualized the review topic. MR performed the literature review, writing, and figure design. CS contributed to figure design. All authors revised and approved the submitted manuscript.

Conflict of Interest

CG is the co-inventor of the internationally issued patent on Jak inhibitors for the treatment or prevention of HIV-1 and other viral infections; CG and Emory University receives royalties from Eli Lilly and Company for the sales of baricitinib for COVID-19. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

A major barrier to human immunodeficiency virus (HIV-1) cure is the latent viral reservoir, which persists despite antiretroviral therapy (ART), including across the non-dividing myeloid reservoir which is found systemically in sanctuary sites across tissues and the central nervous system (CNS). Unlike activated CD4+ T cells that undergo rapid cell death during initial infection (due to rapid viral replication kinetics), viral replication kinetics are delayed in non-dividing myeloid cells, resulting in long-lived survival of infected macrophages and macrophage-like cells. Simultaneously, persistent inflammation in macrophages confers immune dysregulation that is a key driver of co-morbidities including cardiovascular disease (CVD) and neurological deficits in people living with HIV-1 (PLWH). Macrophage activation and dysregulation is also a key driver of disease progression across other viral infections including SARS-CoV-2, influenza, and chikungunya viruses, underscoring the interplay between macrophages and disease progression, pathogenesis, and comorbidity in the viral infection setting. This review discusses the role of macrophages in persistence and pathogenesis of HIV-1 and related comorbidities, SARS-CoV-2 and other viruses. A

special focus is given to novel immunomodulatory targets for key events driving myeloid cell dysregulation and reservoir maintenance across a diverse array of viral infections.

Introduction

In a regulated immune system, macrophages are phagocytic cells that target and break down foreign bodies and regulate lymphocyte activation and recruitment. M1 subtype macrophages are proinflammatory, differentiated through GM-CSF, TNF-α, and IFN-α cytokines, while M2 subtype macrophages are anti-inflammatory macrophages, differentiated through IL-4, IL-13, and IL-10 (1, 2). Macrophage dysregulation results in increased production of pro-inflammatory cytokines leading to accumulation of M1 macrophages and activated immune cells and increased systemic inflammation. Macrophage dysregulation as a result of disease, communicable or otherwise, causes persistent inflammation that leads to comorbidity development. Macrophage dysregulation in viral pathogenesis is an exploitable space for drug development and should be focused on because it presents a broad target as a pathogenic feature shared by multiple viruses (Figure 1).

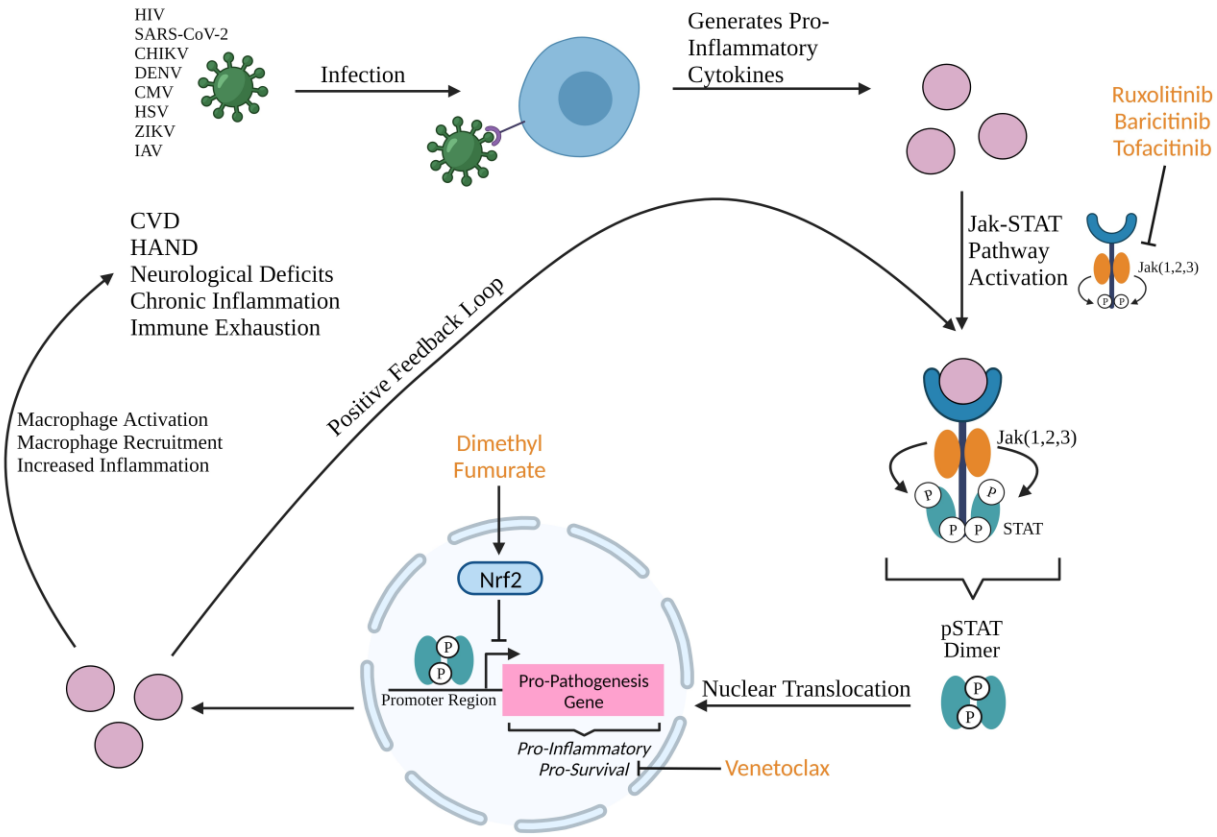

FIGURE 1 | Viral Activation of Jak Stat Pathway and Downstream Effects

Production of pro-inflammatory cytokines upon viral infection can activate the Jak STAT pathway resulting in the formation of a phosphorylated STAT (pSTAT) dimer that binds to the promoter region of pro-inflammatory and pro-survival genes. Upregulation of these genes result in macrophage activation and recruitment, and increased systemic inflammation which all contribute to the development of neurological deficits, chronic inflammation, immune exhaustion, and comorbidities such as HIV-1-associated neurocognitive disorder (HAND) and cardiovascular disease (CVD). Jak 1/2 selective inhibitors ruxolitinib and baricitinib, and Jak 3 selective inhibitor tofacitinib are FDA approved compounds that are candidates to be repurposed in the anti-viral space due to their efficacy in blocking the inflammatory Jak STAT cascade. Baricitinib has been approved (EUA) for the indication of hospitalized COVID-19 patients and tofacitinib has shown significant benefit in treatment of COVID-19 (3–5). Dimethyl fumarate and venetoclax, both FDA approved compounds, are candidates to be repurposed at the transcriptional and post-transcriptional levels. Dimethyl fumarate is an activator of Nrf2 which inhibits the promotor of pro-inflammatory genes during transcription. Venetoclax is a BCL-2 inhibitor that post-transcriptionally sequesters pro-survival protein BCL-2. Created with BioRender.com.

Macrophage Dysregulation in Viral Pathogenesis

Neutrophils interact with macrophages and other immune cell subsets at the beginning of the inflammatory cascade discussed here, often during the innate immune response (Figure 2). Neutrophils, myeloid leukocytes, are the first of the white blood cells to enter traumatized and diseased tissues where the pathogen is residing (6, 7). Neutrophils are constantly generated in the bone marrow and mature via granulocyte colony stimulating factor (G-CSF) (6). G-CSF promotes neutrophil activation, differentiation, and migration through Jak STAT signaling involving STAT3 (8–10). Endothelium activation releases several pro-inflammatory cytokines (IL-1β, IL-6, and IL-8) which results in neutrophil margination (7). Neutrophils are mediators of the acute inflammatory response (11). After microbial challenge, tissue-resident macrophages produce cytokines (TNFα, CXCL1/2, IL-1α, and MCP-1) which recruit neutrophils to the site of infection. These neutrophils then produce azurocidin resulting in the upregulation of E-selectin and vascular cell adhesion molecule-1 (VCAM-1) expression on the endothelium which promotes monocyte recruitment. Infiltrating neutrophils also secrete cytokines (IL-6, IL-12, and IFNg) that contribute to activation of pro-inflammatory macrophages and T helper cell differentiation through Jak STAT signaling (10, 12). GM-CSF production by proinflammatory macrophages elongates neutrophil lifespan and induces monocyte differentiation. Following inflammation resolution, pro-inflammatory macrophages bind to neutrophils via TNF, induce apoptosis, and clear the apoptotic neutrophils (6, 13). A study looking at neutrophils in systemic inflammation response syndrome (SIRS) in a murine model found that mild SIRS induced a pro-inflammatory phenotype (IL-12+CCL3+) while severe SIRS induced an anti-inflammatory phenotype (IL-10+CCL2+) (6, 14). G-CSF production (monocytes, macrophages, fibroblasts, endothelial cells, and bone marrow stromal cells) is regulated by IL-17A which is produced by T cells, natural killer (NK) cells, and macrophages (6, 11, 15). Neutrophils produce CCL2 and CCL20 which are critical for recruitment of T helper cells, a major producer of IL-17A (10, 16). IL-17A production is regulated by IL-23, produced by tissue resident macrophages and dendritic cells (DC) (6). IL-17A is produced through STAT3 activation and recruits monocytes and neutrophils, as well as stimulates astrocytes to produce G-

CSF and CXCL1 in the CNS; this latter mechanism can promote astrogliosis and CNS inflammation, contributing to CNS immune dysfunction and eventual associated cognitive deficits (10, 11, 17). It is clear that neutrophil interaction with macrophages and other immune types in the beginning of the inflammatory cascade is critical and that Jak STAT signaling, and the blockade thereof, could have effects even earlier than the stage at which macrophage function becomes dysregulated.

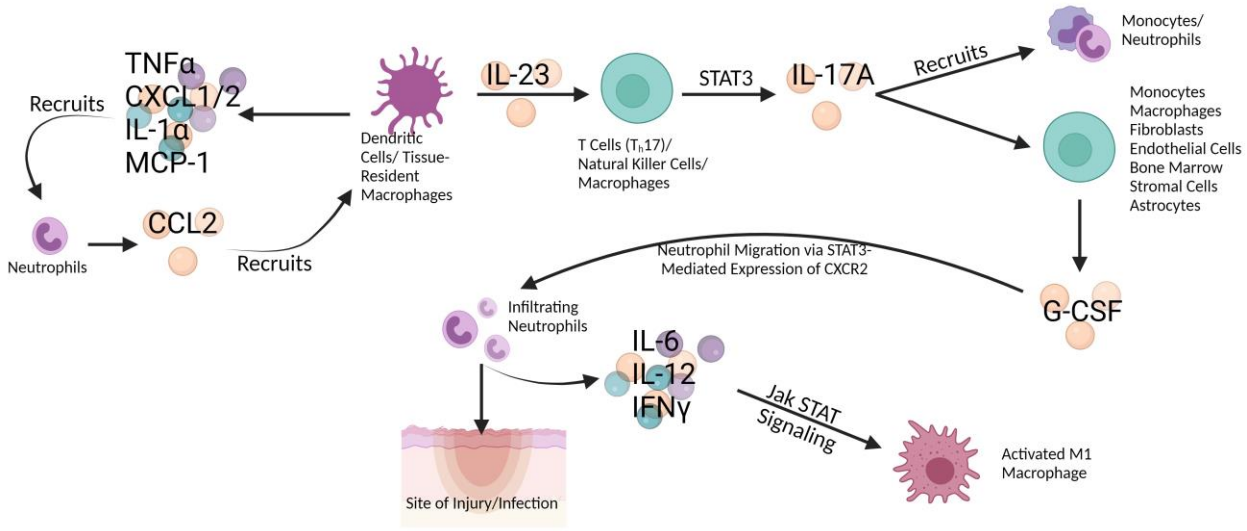

FIGURE 2 | Role of Neutrophils in Inflammatory Cascade

Dendritic cells (DC) and tissue-resident macrophages secrete TNF-α, CXCL1/2, IL-1α, and MCP-1 which recruit neutrophils. In turn, the neutrophils secrete CCL2 which recruits DC and macrophages, effecting a positive feedback loop. DC and macrophages also secrete IL-23 which stimulates T cells like Th17, natural killer (NK) cells, and macrophages to produce IL-17A via STAT3 signaling in the Jak STAT pathway. IL-17A both recruits monocytes and neutrophils and induces G-CSF production in multiple cells types (monocytes, macrophages, fibroblasts, endothelial cells, bone marrow, stromal cells, and astrocytes). G-CSF promotes neutrophil migration to the site of injury or infection via STAT3-mediated expression of CXCR2. Infiltrating neutrophils secrete IL-6, IL-12, and IFN-γ, which activate pro-inflammatory (M1) macrophages through Jak STAT signaling. Created with BioRender.com.

Human Immunodeficiency Virus

Human Immunodeficiency Virus 1 (HIV-1) is a persistent public health problem with an estimated 37.6 million people with HIV-1 (PWH) globally as of 2020 (18). The main barrier to HIV-1 cure is the viral reservoir which is comprised of transcriptionally limited/silent infected cells that are poorly affected by current antiretroviral therapy (ART) due to incomplete penetration across the blood-brain barrier (BBB)

and other tissues as well as differences in effective doses between cell types. ART has been shown to be ineffective in eradicating latently infected cells and blocking reservoir establishment, and there is no approved therapy that directly targets the reservoir (19, 20).

In recent years, the HIV-1 myeloid reservoir has become accepted as a principal reservoir that contributes to viral persistence at the hands of several studies that have shown that macrophages are directly infected, are long-lived, and are a productive source of replication competent virus. An early view of macrophage ontogeny was that macrophages are terminally differentiated and replenished via bone marrow derived monocytes, but this view was expanded by new evidence of long-lived tissue resident macrophages that were derived from yolk sac progenitors and fetal liver derived monocytes (21–24). This long-lived self-renewing phenotype placed macrophages in a new light due to their potential as reservoir cells beyond their known contribution to potentially detrimental bystander effects that can indiscriminately damage tissues. When CD4+ T cells are depleted *in vivo,* viral replication is sustained by infected tissue resident macrophages, underscoring their importance as a viral reservoir (25, 26). Sterile alpha motif and HD domain containing protein 1 (SAMHD1) is a host factor dNTPase that exists in high levels in non-dividing cells such as macrophages and reduce dNTP availability. In HIV-1 infected macrophages, reduced dNTP availability during reverse transcription results in restricted viral replication leading to infected survivor cells that produce low levels of virus. In mitotically active cells, SAMHD1 exists at lower levels resulting in more available dNTPs and thus unrestricted viral replication leading to cell death (27-30). SAMHD1 is a major factor in maintenance of the myeloid reservoir and contributes to the long-lived phenotype of infected cells.

The myeloid HIV-1 reservoir exists in the periphery, tissues, and central nervous system (CNS). Macrophage migration into tissues facilitates spread of infection to the brain, gut, lung, lymph nodes, liver, semen, and urethra (25). HIV-1 can traverse the BBB and establish infection in the CNS which is a sanctuary site because of poor penetration of ART (25). Replication competent virus has been isolated from human urethral macrophages and lymphoid tissues in macaques (22, 25, 31–33). Tissue resident

macrophages were found to be a highly productive source of HIV-1 during opportunistic coinfection in lymph nodes (34).

HIV-1 infection and subsequent viremia and gut microbial translocation triggers acute inflammation. Resulting proinflammatory cytokines recruit macrophages and other innate immune cells, compounding macrophage activation and infection, crossing the line into macrophage dysregulation, and leading to immune exhaustion and persistent inflammation in the host (25).

Severe Acute Respiratory Syndrome Coronavirus 2

Excessive myeloid cell and cytokine storm activation is associated with disease severity in coronavirus disease (COVID-19). Monocytes from infected individuals express angiotensin converting enzyme 2 (ACE2), the receptor for SARS-CoV-2, which suggests that monocytes are infected. Monocyte infection results in immune activation, inflammatory response, and altered gene expression related to immune signaling. M1 macrophages accumulate in the lungs and secrete high levels of IL-6, IL-1β, and TNF-α (summarized in Table 1). Increased IL-6 expression is a hallmark of COVID-19 in association with acute respiratory distress syndrome (ARDS) and respiratory failure (35).

TABLE 1 | Summary of pathogenic features of viral species, associated cytokine profile, and drug targets discussed herein

Virus	Macrophage Pathogenesis	Cytokine Upregulation	Drug Targets and Investigated Therapies
HIV	HAND, CVD	TNF-a, IL-1b, IL-6	
SARS-CoV-2	ARDS, ASCVD, DIC, lymphopenia, encephalopathy, stroke, CNS infection/demyelination, Guillain-Barre syndrome, acute fulminant cerebral edema	IL-6, IL-1β, TNF-a, CCL2, CCL5, IL-8, CXCL9, CXCL10, CXCCL1, TGF-β, ISG, ITAM, TRAM	
CHIKV	Joint pain	IL-12, IFN-a/β, IL-6, IFN-g, CCL2	
DENV	Hemorrhagic fever, shock syndrome	TNF-a, IFN-a, IL-1β, IL-8, IL-12, MIP-1a, RANTES	Jak-STAT Pathway – *Baricitinib, Ruxolitinib, Tofacitinib*
CMV	Microcephaly, mental/motor retardation, epilepsy, progressive vision and auditory deficits	TNF-a, IL-1β, IL-6	
HSV	Encephalitis, meningitis, cerebral palsy, cognitive retardation	CXCL10, TNF-a, CCL5, IL-1β, IL-6	BCL-2 Expression –*Venetoclax*
ZIKV	Microcephalus, cerebral atrophy, intracranial calcifications, hydrocephalus, encephalitis	IL-6, TNF-a, MCP-1, IP-10, IL-8	
IAV	ARDS, pneumonia	CXCL10 / RANTES / TNF-a / MIP-1a / CCL2 / IL-6 / IL-8 / TGF-β3 H5N1: ✓ ✓ ✓ ✗ ✓ ✓ ✓ ✓ H9N2: ✓ ✓ ✓ ✓ ✓ ✗ ✗ ✗ H7N9: ✗ ✗ ✓ ✗ ✗ ✓ ✓ ✗ H1N1: ✓ ✓ ✓ ✓ ✗ ✗ ✓ ✓	Nrf2 Pathway – *Dimethyl Fumurate*

TNF-α, IL-1β, and IL-6 are common pro-pathogenic, pro-inflammatory cytokines upregulated during infection. Upregulation of pro-inflammatory cytokines contribute heavily to viral pathogenesis and leads to development of co-morbidities in chronic infections.

There is a higher risk of severe COVID-19 in patients with CVD, hypertension, and diabetes mellitus (35). Macrophages from diabetics were found to hyperpolarize to a proinflammatory phenotype *in vitro* when exposed to LPS and IFN-γ or TNF (36). M1 macrophage expression of TNF-α and IL-1β drive to renin angiotensin system (RAS)-dependent hypertension through type 1 receptor engagement on macrophages. Angiotensin II (AngII) contributes to hemodynamic injury and monocyte recruitment to the heart, vasculature, and kidneys where differentiation into M1 macrophages occurs (37). In COVID-19, ACE2 receptor engagement by viral spike protein reduces AngII degradation by ACE2, compounding AngII effects on hypertension (38). Atherosclerotic cardiovascular disease (ASCVD) is driven by chronic inflammation and is highly associated M1 macrophage expression of IL-6 and IL-1β. This inflammation is aggravated by risk factors such as smoking, obesity, and chronic viral infection. Macrophage infiltration into adipose tissue releases TNF-α resulting in serine phosphorylation of insulin receptor substrate 1 (IRS-1) which causes insulin resistance through impaired insulin signaling (39). M1 macrophages, the dominant macrophage subtype in ASCVD, stimulated with LPS demonstrate decreased ATP due to impaired oxidative phosphorylation, increased uptake of glucose, ROS production, inflammatory cytokine production, and lipid accumulation which accelerate disease progression in ASCVD. However, M2 macrophages are associated with atherosclerosis regression due to increased fatty acid oxidation and oxidative phosphorylation resulting in increased ATP (40).

Presence of FABP4+ alveolar macrophages and Ficolin1+ monocyte-derived macrophages (MDM)s in lungs of ARDS patients is associated with inflammation (35). FABP4+ macrophages are more dominant in milder disease whereas FCN1+ MDMs become the most dominant in ARDS patients. Lung macrophage populations are dysregulated as COVID-19 progresses in severity, leading to higher levels of macrophages and lower levels of T and natural killer cells in the lungs. This evidence combined with increased macrophage infiltration to lung in autopsy and a murine model indicates that monocyte recruitment to the lung fuels inflammation in severe COVID-19. FCN1+ macrophages have elevated expression of CCL2, CCL5, IL-8, CXCL9, CXCL10, and CXCCL11 which are products of IFN stimulated genes and suggest that FCN1+ macrophages contribute to hyper-inflammation (41). These cytokines are

found to be upregulated in COVID-19 patients along with TGF-β and ISG, ITAM, and TRAM involved in cytokine storm (42). Near abolishment of FABP4+ macrophages in sever COVID-19 also contributes to dysregulated lung function (41).

Autopsy samples revealed macrophages enriched in tissue repair genes which is associated with fibrosis and suggests that macrophages contribute to fibrotic complications observed in COVID-19 patients on mechanical ventilation. It was posited that presence of viral protein in macrophages may be due to uptake of infected cells, and ACE-2 expression was not identified on most macrophages examined. It was suggested that inflammation may be responsible for triggering ACE-2 expression on macrophages. Macrophages containing virus were found to express IL-6 which is associated with lymphopenia. CD68+ macrophages containing viral nucleoprotein were found in kidneys of infected patients and are associated with kidney tubular damage. Macrophage accumulation is linked to damage in the kidneys, lungs, heart, liver, and muscles (42).

In a study of 54 COVID-19 patients, 28 of which had severe infection, all severe patients displayed macrophage activation syndrome (MAS) or low human leukocyte antigen (HLA-DR) expression along with lymphopenia (43). Lymphopenia is a common symptom in COVID-19 leading to reduced regulatory T cells (Treg) which is associated with dysregulated innate and inflammatory immune responses (35).

Interestingly, more than 1 in 10 patients who have cleared SARS-CoV-2 infection still suffer from persistent complications. This phenomenon has been termed long COVID. Up to 87.4% of acute COVID-19 patients reported at least one symptom persisting after several months. Females (23%) are more likely to develop long COVID than males (19%), and the condition is most common in middle aged individuals between 35 and 49 years of age. In children aged 2 to 11 years, it has been reported that 9.8% develop long COVID. Speculation on the source of long COVID suggests a multisystem disorder, prolonged effects of viral fragments, or autoimmune disease, but the actual cause has not yet been determined (44).

Of the current approved and investigated therapies for COVID-19, there are a mix of virus-specific and immune dysregulation targets (Table 2). Therapies such as baricitinib and dimethyl fumarate have direct acting effects on inflammation through the Jak STAT pathway (Figure 1), while inflammation

mediated through the Mitogen-activated protein kinase (MAPK) pathway is targeted through downstream effects of therapies Tocilizumab (an IL-6 receptor inhibitor) and Dexamethasone (a MAPK phosphatase) (45, 46). Antibody-based therapy is a well utilized tool in the treatment of COVID-19. Intravenous immunoglobulin (IVIG) infusion therapy via convalescent plasma has shown efficacy in reducing mortality and disease severity in COVID-19 patients (47–53). The IgG in this therapy is isolated from the sera of convalescent patients with COVID-19 and is composed mostly of IgG1 and IgG2 (54, 55). IVIG therapy has been reported to prevent condition deterioration, recover lymphocyte counts, and prevent the need for mechanical ventilation and supportive care (56). IVIG therapy was first used as a therapeutic tool by Francesco Cenci for measles and was reported as a widely used treatment for the Spanish Influenza A (H1N1) pandemic of 1918 (57). IVIG has been reported as a safe treatment for other diseases including: Guillain-Barre syndrome, thrombotic thrombocytopenia, Goodpasture syndrome, and myeloma (56). There have been no associated adverse events for IVIG therapy and a randomized controlled trial has been initiated to evaluate high-dose IVIG in the management of severe COVID-19 (NCT04261426) (47, 58). IVIG is an immunomodulator of inflammation dysregulation which works to suppress the hyperactive immune response (cytokine storm) elicited by COVID-19, and as such timing of treatment initiation is key (56, 59). One study found that early administration of IVIG (within 3 days of hospital admission) resulted in a significantly shorter hospital stay (median 7 days) while late administration (greater than 7 days post admission) resulted in a significantly longer hospital stay (median 33 days) (60). Administering IVIG within 48 hours of ICU admission reduced the need for mechanical ventilation and boosted the immune response in newly infected patients (58, 61, 62). Another study of 26 patients admitted 10 days post disease onset found that administration of IVIG (5-day course) at an average of 13.2 days post disease onset increased lymphocytes and decreased inflammatory cytokines while reducing the 28-day mortality (IVIG treatment 4%; control 28%) (63). IVIG therapy has a limited EUA for high-titer dosing only and is recommended for early disease course treatment (64). Nonetheless, use of convalescent plasma has not conferred widespread use, as variable results and data demonstrating ineffective results have been reported in additional trials (65). Further, use of commercially produced monoclonal antibodies (summarized in Table 1) provides an

efficacious modality to neutralize early infection in COVID-19 patients, and has become the standardized immunotherapy for first line treatment for mild COVID-19 therapies. Anti-spike protein monoclonal antibodies (Casirivimab, Imdevimab, Sotrovimab, Bamlanivimab, and Etesevimab) aim to block engagement of the ACE2 receptor and thus viral entry, while Remdesivir is a nucleoside analogue that targets viral production as a direct acting antiviral agent. The space for immunomodulatory therapies that target immune dysregulation and inflammation is clearly vital and should be further investigated in the context of viral infections.

TABLE 2 | Current investigated and approved therapies for COVID-19

COVID-19 Therapy	Original Indication	Mechanism of Action	Route of Administration	Disease State Approved For	Approval Status
Baricitinib	Rheumatoid arthritis	Jak 1/2 selective inhibitor	Oral*	Severe	EUA
Dexamethasone Sodium Phosphate	Endocrine, rheumatic, collagen, dermatologic, allergic, ophthalmic, gastrointestinal, respiratory, hematologic, neoplastic, edematous disorders/diseases	Adrenocortical steroid, immune modulator	IV, IM	Severe	FDA Temporary Policy for Compounding of Certain Drugs for Hospitalized Patients by Pharmacy Compounders not Registered as Outsourcing Facilities During the COVID-19 Public Health Emergency
Remdesivir	SARS-CoV-2	Nucleoside analogue, delayed chain termination of viral RNA	IV	Severe	FDA Approved
Tocilizumab	Rheumatoid arthritis, giant cell arteritis, systemic sclerosis-associated interstitial lung disease, polyarticular juvenile idiopathic arthritis, cytokine release syndrome	Anti-IL-6 receptor mAb	IV	Severe	EUA
Dimethyl Fumurate	Multiple sclerosis	Nrf2 pathway activator	Oral	N/A	RECOVERY trial candidate
Casirivimab & Imdevimab	COVID-19	Anti-human IgG1 mAb, bind to non-overlapping epitopes of SARS-CoV-2 spike RBD, blocks binding to ACE2	IV, SI	Mild/Moderate	EUA
Sotrovimab	COVID-19	Anti-human IgG1 mAb, binds to conservative epitope of SARS-CoV-2 spike RBD, non-competitive with ACE2 binding	IV	Mild/Moderate	EUA
Bamlanivimab & Etesevimab	COVID-19	Anti-human IgG1 mAb, binds to different but overlapping epitopes of SARS-CoV-2 spike RBD, blocks attachment to ACE2	IV	Mild/Moderate	EUA
Convalescent Plasma	Measles	Confers passive immunity by infusing naturally produced antibodies against a pathogen	IV	Mild/Moderate	Limited EUA

Therapies are currently under investigation, approved by the Food and Drug Administration (FDA), or have an emergency use authorization (EUA) from the FDA. Administration routes include oral, intravenous (IV), intramuscular (IM), and subcutaneous injection (SI). *Therapy can be alternatively administered when oral is not an option. N/A, not available.

Influenza Virus

Disease severity in influenza (IAV) is associated with increased proinflammatory cytokine production (66, 67). Strains differ in cytokine profiles (Table 1). Infection of MDMs and alteration of cytokine production is a driver of pathogenesis in IAV. H5N1 and H9N2 are highly pathogenic. H9N2 induces CXCL10, RANTES, TNF-α, MIP-1α, and CCL2 expression (68). H5N1 induces TNF-α, CCL2, RANTES, CXCL10, IL-6, IL-8, and TGFb3 (68, 69). H7N9 induces heightened IL-6, TNF-α, and IL-8 expression (69). H1N1 induces elevated TNF-α, IL-8, TGF-β3, CXCL10, RANTES, and MIP-1α expression (68, 69).

In *in vivo* IAV infection, an early increase in IFN-α, TNF-α, IL-1β, and IL-6 was observed in murine lungs. Nasal lavage samples from infected patients revealed heightened IFN-α, TNFα, IL-8, IL-6, MIP-1α/β, and MCP-1 expression. Infected human monocytes and rat and murine macrophages produce IFN-α, TNF-α, IL-1, IL-6, MIP-1α/β, and RANTES. Pro-inflammatory cytokine production during IAV infection peaks 2-3 days post-infection Massive bronchial infiltration of inflammatory cells, dominated by neutrophils, was observed in infected swine 24 hours post-infection. A decrease in viral titer is correlated with a decrease in neutrophil counts (70). Neutrophils cause tissue injury and have been targeted by anti-MIP-2 IgG therapy which decreased neutrophil counts to 37% of control in a murine model (71). Increased IFN-α, CCL7, and CD16-CD14+ monocytes are associated with disease severity in children while monocyte dysregulation also occurs in the elderly with decreased IFN type 1 signaling. Fatal pneumonia is triggered by increased IL-6 and ROS through stimulation of TLR-4 dependent alveolar macrophages by oxidized phospholipids (67). Curcumin has been investigated as a therapy because it inhibits the NF-κβ pathway and has been shown to decrease pro-inflammatory cytokine production in IAV infection (66).

Autopsy samples revealed increased TNF-α and COX2. In a murine model, combined antiviral and COX inhibitor therapy has been shown to decrease lung pathology, pro-inflammatory cytokines, and lymphopenia (72). COX2 expression is induced by IL-1β which opens up a broader upstream target. However, COX inhibitors have been shown to over-suppress the inflammatory response to the host's detriment (73).

Chikungunya Virus

Both Chikungunya Virus (CHIKV) and Ross River Virus (RRV), alphaviruses, infect macrophages and their viral RNA and proteins have been observed in macrophages 18months post-infection (74). Macrophages have been identified as the main reservoir in late stage CHIKV infection (75). Residual viral RNA and proteins may contribute to apoptosis, fibrosis, tissue injury, and inflammation. IL-12 is upregulated in CHIKV infection at time of infection and remains elevated in chronic infection. IL-12 promotes natural killer cell and macrophage activation. Inflammation results in joint pain and arthritis-like pathology (74). In a cynomolgus macaque model, CHIKV was found to target lymphoid tissues, muscles, joints, the liver, and the CNS during acute infection and persist in lymphoid tissues, joints, muscles, and the liver in late-stage infection. In nonhuman primates, major macrophage infiltration was observed in the spleen, liver, and lymph nodes while minor infiltration occurred in joints and muscles. Flow cytometry analysis revealed that 2/3 of CSF macrophages were activated in CHIKV infection. Within 12 days of infection, cytokine expression (Table 1) progressed from continuous macrophage activation (IFN-α/β, CCL2, IFN-γ), to pro-inflammatory (IFN-α/β, IL-6, IFN-γ), and finally to infiltration (CCL2). Peak viremia and pro-inflammatory cytokine expression were found to correlate with reduction in CD14+ cells (75).

Dengue Virus

Dengue Virus (DENV) primary targets Kupffer cells and pulmonary macrophages while infected blood monocytes may disseminate DENV to tissues (76, 77). Macrophages and monocytes infected *in vitro* secreted TNF-α, IFN-α, IL-1β, IL-8, IL-12, MIP-1α, and RANTES (Table 1) (77). In post-mortem samples from 2 infants with primary DENV infection, it was found that DENV antigen was localized to Kupffer cells and splenic, thymic, and pulmonary macrophages. Another autopsy sourced study revealed DENV to be consistently associated with mononuclear phagocytes. Production of monokines or lymphokines resulting from T cell and infected macrophage interactions has been proposed as a source for rash, shock, and hemorrhage in DENV infection. Dengue hemorrhagic fever (DHF)/Dengue shock syndrome (DSS) is associated with DENV antibody being acquired pre-infection (76).

Infantile Effects of Cytomegalovirus, Herpes Simplex Virus, and Zika Virus

In vivo murine microglia with a pro-inflammatory phenotype had elevated IL-6, IL-1β, and TNF-α expression that correlated with increased toxicity to fetal neuronal progenitor cells (NPCs) (Table 1) (78). Hofbauer cells, a target of Zika Virus (ZIKV) have access to fetal blood vessels which has been proposed as a route of viral dissemination to the brain. The main target of ZIKV are NPCs in the brain. Decreased cortical neurons and ultimately a smaller cerebral cortex (microcephaly) result from NPC reduction (79). Induced pluripotent stem cell-derived microglia-like cells have the ability to propagate ZIKV *in vitro* which supports their role as a microglial reservoir in ZIKV. Several cytokines are upregulated during ZIKV infection (IFN-α, IL-6, MCP-1, IP-10, and IL-8) (Table 1), but flaviviruses have been shown to evade the innate immune response by blocking Jak STAT pathway mediated IFN production. Treatment with known Jak inhibitor ruxolitinib elucidated the role of Jak STAT in cell permissiveness to ZIKV infection and as a regulator of mature virion production (80–82). It is important to understand these data in the context of time of addition and that these are *in vitro* studies that cannot model the impact of Jak 1/2 inhibition on disease pathogenesis and spreading. Cytomegalovirus (CMV) can cross the placenta and infect the fetal brain. CMV

infection in the first two trimesters is severe due to interference with placental development. CMV targets microglia, brain macrophages (CAM), and NPCs. An increase in microglia and macrophages due to recruitment within the brain and migration from the meninges was observed in murine CMV infected brains. CMV infected microglia secrete TNF-α, IL-1β, and IL-6 while infected fetal astrocytes secrete CCL2 to recruit microglia. Fetal astrocytes and neurons are infected by Herpes Simplex Virus (HSV) but microglia do not support viral replication. Myeloid dysregulation is actually protective in HSV because infected microglia secrete CXCL10, TNF-α, CCL5, or IL-1β. CXCL10 reduces viral replication in neurons and elevated IL-6 protects against neuronal loss (78).

Neurological Complications of Macrophage-Driven Pro-Viral Events

Myeloid Driven Comorbidity in HIV-1

HIV-1-associated neurocognitive disorders (HAND) and cardiovascular disease (CVD) are two comorbidities that result from persistent basal inflammation associated with the HIV-1 reservoir and chronic immune activation despite ART (83). In cerebrospinal fluid (CSF) from virologically suppressed individuals, gene expression in the myeloid subset was found to overlap that of neurodegenerative disease-associated microglia (84). In another study of 69 virologically suppressed individuals where ART was started during chronic infection, 33 had detectable HIV-1 DNA in the CSF. This supports that the CNS is a site of viral persistence despite ART suppression. The same study also found a significant association between neurocognitive dysfunction and detectable CSF associated HIV-1 DNA (85). In infants, HIV-1 disease progression is significantly faster compared to adults. Before ART was common practice, half of infantile infections resulted in progressive HIV-1 encephalopathy, targeting microglia, leading to microcephaly and developmental delays. Chronic neurological impairment is still observed under ART. Infected microglia release proinflammatory cytokines and reactive oxygen species (ROS) that contribute to neurotoxicity and neural injury (78).

Presence of infected and activated mononuclear phagocytes has been consistently linked to neuronal injury. Mononuclear phagocytes produce pro-inflammatory cytokines and neurotoxic products such as TNFα, IL-1β, IL-6, nitric oxide (NO), and glutamate among others (Table 1). Cathepsin B secretion from MDMs is elevated in response to HIV-1 infection. HIV-1 infected macrophage secreted Cathepsin B is associated with neuronal apoptosis (86). Microglia are the main target of HIV-1 infection in the brain. Infected microglia upregulate glutaminase C, an enzyme responsible for generating glutamate in the CNS, which has been confirmed in post-mortem tissues. HIV-1 infected macrophages have also been suggested to increase glutamate levels through upregulation of glutaminase (87, 88). Glutamate is neurotoxic in high concentration. Glutamate levels in the CSF have been directly correlated with severity of dementia and degree of brain atrophy (89). Glutaminase localizes to the inner mitochondrial membrane and catalyzes deamination of glutamine, thus producing glutamate. HIV-1 infected microglia mediate neurotoxicity through the N-methyl- D-aspartate (NMDA) receptor, for which glutamate engagement has been identified as a critical component of HAND excitotoxicity (89).

Myeloid Driven Complications in COVID-19

About 36% of acute COVID-19 patients develop neurological symptoms regardless of pre-existing neurological disorders. Inflammation-induced dissemination intravascular coagulation (DIC) is a potential outcome of COVID-19 and can cause cerebrovascular ischemia leading to ischemic stroke. At time of discharge, one third of COVID-19 patients were found to have evidence of cognitive and motor impairment. Systemic inflammation is associated with cognitive decline and neurodegenerative disease leading to speculation in the literature that COVID-19 survivors may experience future neurodegeneration. ARDs, a common development in severe COVID-19, is also associated with cognitive decline and executive dysfunction (90). IL-6 is upregulated in patients with neurological symptoms in acute phase of inflammation (91). In a study of previously healthy children with multisystem inflammatory syndrome, 12% developed life-threatening conditions clinically associated with COVID-19 including severe

encephalopathy, stroke, CNS infection/demyelination, Guillain-Barre syndrome, and acute fulminant cerebral edema. Of 43 patients who developed severe COVID-19 neurologic involvement, 40% had accrued new neurological deficits at time of discharge while 26% died (92). In COVID-19 patients with neurologic involvement, 50% were found to be hospitalized compared to 19% of non-neurologic COVID-19 (91).

ApoE4 is associated with heightened risk for development of severe COVID-19. ApoE4 is a genetic risk factor for Alzheimer's disease and neuroinflammation. Neuronal extracellular vesicles (nEVs) have been found to increase inflammation and neurodegenerative proteins in COVID-19 patients. It is unknown if these proteins are transient. It has been suggested that transient neurodegenerative proteins would reflect ongoing neuroinflammation and elimination of toxic proteins for neurons, whereas long-term may indicate chronic neuroinflammation or developing neurodegeneration. nEVs promote neurogenesis, normal signaling in the CNS, and can remove damaged proteins but also spread toxic amyloid beta peptide (Ab) and tau, a microtubule associated protein, between cells (91). Ab accumulation in the brain has been proposed to be an early event in Alzheimer's disease pathogenesis (93). Tau is a main component in intracellular filamentous inclusions in tauopathies like Alzheimer's disease (94).

Effects of Pre- and Post-Natal Infections

Microcephalus cerebral atrophy, intracranial calcifications, hydrocephalus, and encephalitis are potential outcomes of ZIKV and CMV fetal infection while HSV and HIV-1 fetal infection can lead to encephalitis, meningitis, cerebral palsy, and cognitive retardation. CNS macrophages develop early on and activation of these macrophages and inflammation can negatively affect the developing fetal brain. CMV fetal infection has been linked to microcephaly, mental and motor retardation, epilepsy, and progressive vision and auditory deficits (78). Several cases of autism resulting from CMV infection have been documented (95, 96). Elevated pro-inflammatory cytokine levels from pre-natal infection in the mother can compromise placenta barrier integrity and induce heightened cytokine production in the fetus. IL-6, IL-1β, and TNF-α can interfere with neuronal network development. Elevated IL-8 or TNF-α in the mother has

been correlated with schizophrenia in the fetus. HSV infection in the CNS can result in herpes simplex encephalitis (HSE) and aseptic meningitis and can lead to neurological abnormalities in more than 50% of patients despite treatment. Macrophage dysregulation in HSV infection mitigates neurological deficits and is therefore protective (78).

Targeting Macrophage-Driven Pro-Viral Events

Pro-Viral Events

GM-CSF drives macrophage differentiation via activation of the Jak STAT pathway via JAK2 autophosphorylation (97). MDMs are activated and recruited by activated natural killer and T cells via GM-CSF, TNF, and IFN-γ. In COVID-19, accumulation of oxidized phospholipids in the lung activates MDMs through the TLR4-TRAF6-NF-κβ pathway (42). Jak 1 (IL-2, IL-7, IL-15, IL-6, IFN-α/β) and Jak 2 (IL-6, GM-CSF, IFN-γ) are activated by proinflammatory cytokines and in turn phosphorylate STAT 1, 3, and 5 which are then translocated into the nucleus where they bind to the promoter region of pro-inflammatory genes and promote transcription (98–101). Jak STAT activation in HIV-1 results in reduction of CD4+ cell counts, decreased IL-7R expression indicating elevated receptor engagement and ultimately increased homeostatic proliferation, and increased PD1 and CD38 expression indicating immune activation (99).

A downstream effect of Jak STAT activation is BCL-2 expression. BCL-2 is a pro-survival marker that contributes to the longevity of the HIV-1 reservoir. The reservoir is a major source of low-level inflammation despite ART. BCL-2 is upregulated in HIV-1 infection due to an increase in phosphorylated STAT5 that promotes transcription of the BCL-2 gene. Within the BCL-2 protein family, there are pro-survival (BCL-2, Mcl-1, BCL-XL) and pro-apoptotic (BAK, BIM, BAD, BAX) proteins. Pro-survival proteins sequester host BH3 which binds to pro-apoptotic proteins under normal conditions, oligomerize in the mitochondrial outer membrane resulting in permeabilization and release of cell death factors including cytochrome C. Cytochrome C activates caspase-9 to initiate a caspase cascade resulting in apoptosis (102, 103).

The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway is involved in cellular response to oxidative stress [Tecfidera package insert] (104). Nrf2 has anti-inflammatory function that works through inhibition of RNA polymerase II recruitment onto pro-inflammatory genes during transcription. This has been directly demonstrated with IL-6 in a murine model (104, 105).

Macrophage dysregulation in the context of heightened inflammation and factors contributing to reservoir establishment and maintenance should be further explored in HIV-1 and other viral infections.

Immunomodulators

Immunomodulators discussed here particularly target points within the inflammatory cascade leading to macrophage dysregulation, key to pathogenicity of viral diseases discussed herein.

Jak inhibitors have been previously explored in the space of inflammatory disease and have been applied to viral infections in the cases of HIV-1 and COVID-19 in recent years (3, 106). BCL-2 inhibitors were originally developed as an anti-cancer therapy and have recently been evaluated in the context of HIV-1 (107). Using Food and Drug Administration (FDA)-approved compounds can potentially reduce the time of clinical translation due to a wealth of preliminary safety and efficacy profiling.

Tofacitinib is a Jak 3 selective inhibitor that is FDA approved for the indication of rheumatoid arthritis, however its utility in the space of HIV-1 in humans is diminished due to potential for Jak 3 mediated lymphopenia [Xeljanz package insert]. Ruxolitinib, a first-generation Jak 1/2 selective inhibitor, has been evaluated *in vitro*, *ex vivo* and in humans for the indication of HIV-1 and has demonstrated a novel mechanism to block reservoir reseeding and maintenance (99, 108–110). The A5336 ACTG sponsored Phase 2a study demonstrated that ruxolitinib is safe and well tolerated in virally suppressed people living with HIV-1 and conferred a significant reduction in key markers of HIV-1 persistence including HLA-DR/CD38, sCD14, and reservoir lifespan marker Bcl-2. Baricitinib is a second generation Jak 1/2 inhibitor (FDA approved for rheumatoid arthritis and EUA for COVID-19) with an improved safety and efficacy profile versus ruxolitinib, including once per day (qd) dosing, renal clearance that mitigates many drug-drug interactions with hepatically cleared agents, and a markedly improved safety profile including FDA

approval for chronic long-term use and approval in children as young as two years of age [Olumiant package insert]. Baricitinib was first evaluated in the viral infection space for HIV-1 and demonstrated a reversal in HIV-1 associated cognitive deficits along with a reversal of macrophage and microglial activation and key markers of CNS persistence in a murine model of HIV-1 in the CNS (111). Baricitinib was also recently evaluated under compassionate use for the indication of COVID-19 (106), and later found to reduce macrophage activation in a non-human primate model of SARS CoV-2 infection (112). Baricitinib later demonstrated a significant mortality benefit in humans in the NIH sponsored ACTT-2 trial (3), and the Lilly sponsored COVBARRIER trial (Lancet RM, in press), which collectively conferred EUA approvals for baricitinib, and most recently as a monotherapy designation. To date, the class of Jak inhibitors, first crossed over to viral infections in the space of HIV-1, and now COVID-19, demonstrate a promising new class of agents; baricitinib in particular due to its safety and efficacy in humans in the COVID-19 space, coupled with its anti-HIV-1 properties, provides a unique opportunity for additional evaluation for HIV-1 cure studies, and possibly other viral infections with myeloid immune dysregulation.

Other agents have been explored within the repurposed space, which may impact myeloid immune dysregulation or, in the case of HIV-1, confer anti-HIV-1 effects that may down-regulate HIV-1 induced proliferation and expression (77). Venetoclax is currently the only FDA-approved BCL-2 inhibitor and is indicated for acute myeloid leukemia, chronic lymphocytic leukemia, and small lymphocytic leukemia [Venclexta package insert] (113–115). Additional studies are needed with this class of agents to better understand if antiviral properties observed *in vitro* can crosstalk to relevant, safe, human studies for HIV-1 and other viral infections. Dimethyl fumarate is FDA-approved for relapsing multiple sclerosis and has been shown to activate the Nrf2 pathway *in vitro* and *in vivo* [Tecfidera package insert]. Nrf2 inhibits pro-inflammatory cytokine expression, disrupting the inflammatory cascade that exacerbates macrophage dysregulation (105). Dimethyl fumarate induces an antioxidant response and has been shown to suppress HIV-1 replication and macrophage-mediated neurotoxicity through attenuation of NF-κβ and monocyte recruitment to the CNS (116). Dimethyl fumarate has no black box warning and bid oral dosing of 120 mg for 7 days followed by 240 mg thereafter. The half-life of dimethyl fumarate is 1 hours and drug elimination

is primarily through exhalation of carbon dioxide (CO2) with low level renal (16%) and fecal (1%) excretion. Dimethyl fumarate has not been approved in children as the safety and efficacy in this group is unknown [Tecfidera package insert].

Collectively, the field of immunomodulation for viral infections is a relatively new horizon, only recently explored through repurposed efforts, most notably through the Jak 1/2 inhibitor baricitinib, which has been applied for both HIV-1 and COVID-19 successfully. Recent insights across this potential application will provide additional data and understanding about the sentinel interplay between the immune system, macrophages, and treatment or prevention of viral infections.

Conclusions

Macrophage dysregulation, particularly in the M1 subtype, leads to increased immune activation and inflammation that contributes to viral pathogenesis and development of comorbidities. Macrophage dysregulation is a pathogenic feature shared by multiple viruses and presents a broad target for antiviral treatments. In HIV-1, macrophages constitute a principal reservoir that is rarely affected by ART and which exists in tissues and the brain. Constant basal inflammation and immune activation lead to immune exhaustion and development of comorbidities such as CVD and HAND and can compound with other inflammatory diseases like diabetes. Macrophage contribution to viral pathogenesis extends beyond HIV-1 to SARS-CoV-2, Dengue, ZIKV, CMV, HSV, IAV, etc. A common feature of macrophage dysregulation is upregulation of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α, and IFN-α. Because this dysregulation transcends the BBB to reach the CNS, neurological symptoms are sometimes associated with macrophage dysregulation such as HAD and HAND in PWH and brain fog in people with long COVID. We must also consider this problem in the pre and postnatal scope. Macrophage dysregulation can cause physical and intellectual disabilities in infants. Downregulation of inflammation in viral infection should be further explored. Some immunomodulators such as ruxolitinib, baricitinib, venetoclax, and dimethyl fumarate have shown efficacy in this area. Furthermore, repurposing FDA approved compounds for this

use represent an accelerated route from bench to clinic implementation. Inflammation in viral infections presents a broad target shared between multiple viruses. Focusing on this as a drug target, especially with repurposed immunomodulators, is an efficient and effective strategy in terms of cost, resources, availability, applicability, and impact on public health.

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Repurposing BCL-2 and Jak 1/2 Inhibitors: Cure and Treatment of HIV-1 and Other Viral Infections

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Conflict of Interest

Author CG and Emory University receive royalties from Eli Lilly and Company for the sales of Baricitinib for the indication of COVID-19. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

B cell lymphoma 2 (BCL-2) family proteins are involved in the mitochondrial apoptotic pathway and are key modulators of cellular lifespan, which is dysregulated during human immunodeficiency virus type 1 (HIV-1) and other viral infections, thereby increasing the lifespan of cells harboring virus, including the latent HIV-1 reservoir. Long-lived cells harboring integrated HIV-1 DNA is a major barrier to eradication. Strategies reducing the lifespan of reservoir cells could significantly impact the field of cure research, while also providing insight into immunomodulatory strategies that can crosstalk to other viral infections. Venetoclax is a first-in-class orally bioavailable BCL-2 homology 3 (BH3) mimetic that recently received Food and Drug Administration (FDA) approval for treatment in myeloid and lymphocytic leukemia. Venetoclax has been recently investigated in HIV-1 and demonstrated anti-HIV-1 effects including a reduction in reservoir size. Another immunomodulatory strategy towards reduction in the lifespan of the reservoir is Jak 1/2 inhibition. The Jak STAT pathway has been implicated in BCL-2 and interleukin 10 (IL-10) expression, leading to a downstream effect of cellular senescence. Ruxolitinib and

baricitinib are FDA approved, orally bioavailable Jak 1/2 inhibitors that have been shown to indirectly decay the HIV-1 latent reservoir, and down-regulate markers of HIV-1 persistence, immune dysregulation and reservoir lifespan *in vitro* and *ex vivo*. Ruxolitinib recently demonstrated a significant decrease in BCL-2 expression in a human study of virally suppressed people living with HIV (PWH), and baricitinib recently received emergency use approval for the indication of coronavirus disease 2019 (COVID-19), underscoring their safety and efficacy in the viral infection setting. BCL-2 and Jak 1/2 inhibitors could be repurposed as immunomodulators for not only HIV-1 and COVID-19, but other viruses that upregulate BCL-2 anti-apoptotic proteins. This review examines potential routes for BCL-2 and Jak 1/2 inhibitors as immunomodulators for treatment and cure of HIV-1 and other viral infections.

Introduction

B cell lymphoma 2 (BCL-2) family proteins fall into three categories: anti-apoptotic proteins (BCL-2, BCL-XL, BCL-W, MCL-1, BFL-1/A1), pro-apoptotic pore-formers (BAX, BAK, BOK), and pro-apoptotic BH3-only proteins (BAD, BID, BIK, BIM, BMF, HRK, NOXA, PUMA) (1, 2). BCL-2 proteins have a large role in regulating apoptosis, and dysregulation can lead to oncogenesis and senescence. Several intracellular pathogens and/or cancers have developed mechanisms to avoid cellular death to favor its own survival. BCL-2 is a key modulator of cellular lifespan and has been shown to be modulated by pathogen/host interactions and by certain cytokines downstream of viral infection and/or tumor microenvironment. Thus, the development of inhibitors of BCL-2 proteins has been of great interest for the virus infection and cancer fields. Several BCL-2 inhibitor trials have taken place and shown efficacy in inducing apoptosis resulting in better disease outcomes. In this review we will highlight the induction of BCL-2 by different diseases (viral and cancerous), its association with immune senescence and exhaustion, and Food and Drug Administration (FDA) approved drugs that interfere with survival pathways leading to better disease outcomes.

BCL-2 Induction

Jak STAT cascade for BCL-2 expression

The Janus kinase and signal transducer and activator of transcription (Jak STAT) pathway is an upstream regulator of BCL-2 expression. When proinflammatory cytokines engage their cytokine receptor, Janus kinases (Jak) 1, 2, and 3 phosphorylate the receptor which recruits STAT type 5 (STAT5). STAT5 is subsequently phosphorylated (pSTAT5) by Jak 1, 2, and 3 and dimerizes. Dimerized pSTAT5 undergoes nuclear translocation and binds to the promoter region of the BCL-2 gene to enhance transcription (Figure 1) (3–6). Elevated levels of BCL-2, pro-survival factor, are a marker of pathogenesis for many diseases including cancer and human immunodeficiency virus type 1 (HIV-1). Targeting the Jak STAT pathway has thus far proven to decrease BCL-2 expression while also decaying the HIV-1 reservoir and reversing HIV-1-associated neurocognitive dysfunction caused by long-term basal inflammation (3, 7–9). Broadly, pSTAT5 expression has been linked to increased integrated HIV-1 DNA levels *in vivo* , *in vitro*, and *ex vivo*, deregulated homeostatic proliferation, immune activation, reservoir size *in vivo* , and BCL-2 expression *in vitro* and *in vivo* (3, 7). Additionally, STAT5 phosphorylation is involved in homeostatic proliferation (3) and has been reported to have binding sites in the HIV-1 long terminal repeat (LTR) (10).

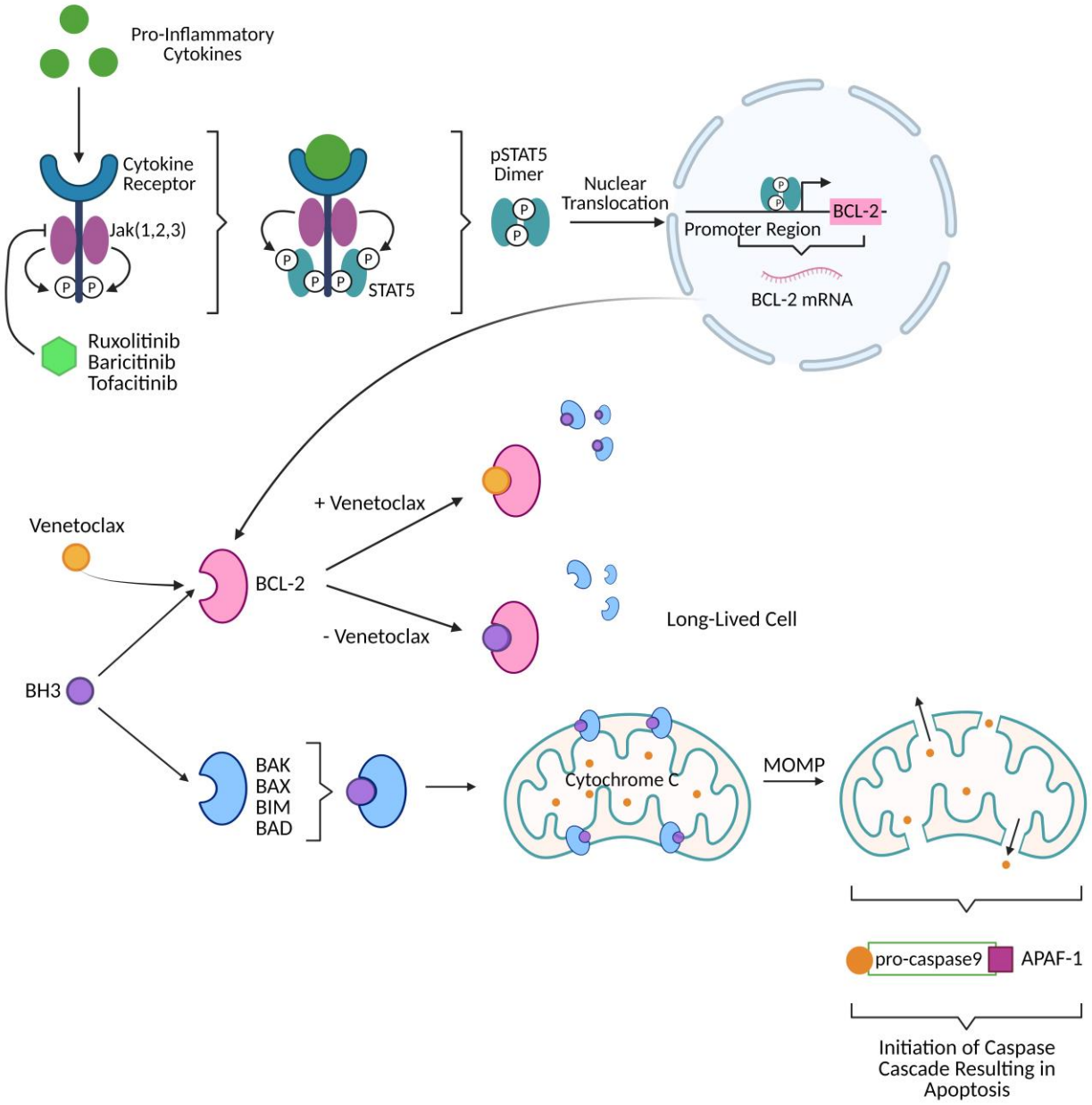

FIGURE 1 | Jak STAT Pathway Activation and Anti-Apoptotic Activity

Activation of the Jak STAT pathway by pro-inflammatory cytokines (like those produced in viral infection) produces dimerized pSTAT5 that directly upregulates BCL-2, a pro-survival factor, resulting in long-lived cells harboring HIV-1 DNA. FDA approved Jak inhibitors (ruxolitinib, baricitinib, tofacitinib) can block the BCL-2 cascade upstream. Venetoclax, FDA approved BCL-2 selective BCL-2 homology 3 (BH3) mimetic, prevents BCL-2 sequestration of BH3. Free BH3 is then able to interact with pro-apoptotic proteins (BAK, BAX, BIM, BAD) to perform mitochondrial outer membrane permeabilization (MOMP) thereby releasing cytochrome C. Cytochrome C interacts with pro-caspase9/apoptotic protease activating factor 1 (APAF-1) to initiate a caspase cascade that culminates in apoptotic cell death. Created with BioRender.

Role of BCL-2 Expression

Cancers

BCL-2 has been extensively studied in cancer, with the original BCL-2 inhibitor intended as a cancer treatment. Within the BCL-2 family of proteins, there are both pro- and anti-apoptotic proteins. The anti-apoptotic proteins contribute to cellular senescence and tumor survival. Senescence is a cellular response to physiological and oncogenic stress that results in the loss of a cell's ability to divide and grow (11). Anti-apoptotic BCL-2 family proteins are upregulated in senescent cells (11). Two major apoptotic pathways that BCL-2 proteins play a critical role in are the intrinsic cell-death pathway, also called the mitochondrial apoptotic pathway, and the extrinsic cell-death pathway involving the FAS and TRAIL receptors that initiate a caspase-8-mediated cascade (12). Within the intrinsic cell-death pathway, anti-apoptotic proteins such as BCL-2, BCL-XL, and MCL-1 possess a C-terminal transmembrane domain anchored in the mitochondrial outer membrane. High expression of BCL-2 or BCL-XL is associated with aggressive malignancies and resistance to chemotherapy (12). BAK and BAX are pro-apoptotic proteins that are blocked by BCL-2, BCL-XL, and MCL-1. The BH3 peptide activates BAK and BAX but is sequestered by BCL-2, BCL-XL, andMCL-1 through binding to a hydrophobic pocket in the protein. When BAK and BAX are successfully activated, they oligomerize in the mitochondrial outer membrane causing mitochondrial outer membrane permeabilization (MOMP). This membrane damage not only causes cellular necrosis but also allows for release of proteins from the mitochondria that contribute to apoptosis. The proteins include caspase activating proteins like cytochrome C, caspase inhibitor neutralizers like SMAC and OMI/Htra2, and intranuclear genome degradation proteins like apoptosis-inducing factor (AIF) (13). Cytochrome C combines with both apoptosis protease-activating factor 1 (APAF-1) and inactive procaspase-9 to form an apoptosome. This formation activates caspase-9 which initiates a caspase cascade involving caspase-3, caspase-6, and caspase-7 (Figure 1) (12). BCL-2 anti-apoptotic proteins' ability to subvert apoptosis can result in a senescent cellular phenotype where the cell is not actively proliferating but in stasis. Cytokines from the tumor microenvironment such as transforming growth factor beta (TGF-β)

and interleukin 10 (IL-10) are known cytokines to promote tumor survival but also to induce immunosenescence by upregulating BCL-2 expression in tumor and immune cells as well as the expression of co-inhibitory receptors (preliminary data from our group DOI: 10.1101/2020.12.15.422949, 10.1101/2021.02.26.432955). While immunosenescence primarily refers to the changes in the immune system associated with age and impacts mainly T lymphocytes; chronic diseases such as cancers and certain infectious diseases (cytomegalovirus (CMV), HIV-1) are also associated with immune dysfunction.

BCL-2 and HIV-1 reservoir persistence

In 2020 in the United States, around 30,69200 people were newly diagnosed with human immunodeficiency virus type 1 (HIV-1), joining a total population of 1.07 million people with HIV (PWH) [CDC 2020 HIV surveillance report] (14). Globally, the UNAIDS report estimates 38.4 million PWH with 1.5 million new infections in 2021 [UNAIDS 2022] (15). Antiretroviral therapy (ART) is the common treatment regimen prescribed to PWH, but several social, geographic, and monetary blockades impact access and adherence to treatment resulting in 66-85% of PWH accessing treatment globally in 2021 [UNAIDS 2022] (15). In 2018, the cost of initial ART in the United States ranged from $36-48,000 annually, reducing accessibility and adherence to ART (16). ART can suppress HIV-1 replication to undetectable levels but is not able to eliminate it entirely due to the virus's ability to form a population of HIV-1-infected cells with a transcriptionally silent LTR, commonly referred to as the HIV-1 latent reservoir. Despite the effectiveness of ART, lifelong adherence to treatment with sustained viral suppression is complicated by the development of drug resistance, intolerance, and interactions, the earlier and more severe onset of diseases associated with aging, and social and psychological stigmas. If ART is interrupted, the HIV-1 reservoir contributes to the rapid rebound of plasma viremia in all but rare HIV-1 controllers (16, 17). Viral rebound not only increases viral load, thus increasing probability of spreading the virus to others, but also contributes to further immune system degradation and eventual progression into acquired immunodeficiency syndrome (AIDS). Eliminating or completely repressing the HIV-1 reservoir is critical for a true or functional cure, respectively. One approach to cure is the shock and kill method,

which involves “shocking” the latent cells out of latency to become transcriptionally active which not only results in viral cytopathic effects but also allows infected cells to be killed by CD8+ T cells. Although this strategy continues to be investigated, there has been no efficacy demonstrated in humans to date. A functional cure refers to viral remission, a similar concept to cancer treatment, in which the viral reservoir is suppressed permanently and monitored for rebound. Jak inhibitors and BH3 mimetics reveal an alternate route in which we may be able to target the HIV reservoir while maintaining viral suppression and targeting downstream effects on the system (chronic inflammation, senescence, etc.), neither reactivating virus nor enforcing remission, but targeting dormant reservoir for decay.

Several mechanisms are in place leading to long term HIV-1 reservoir persistence. We have reported that IL-10 signaling leads to the upregulation of pro-survival pathways resulting in higher HIV-1 reservoir (preliminary data from our group DOI: 10.1101/2021.02.26.432955). *In vitro* blockade of IL-10 signaling led to BCL-2 downregulation and HIV-1 reservoir decay. Importantly, IL-10 also led to the upregulation of coinhibitory receptors such as PD-1, supporting HIV-1 latency, but also CD8 T cells senescence and exhaustion.

STAT type 3 (STAT3) produced via the Jak STAT pathway translocates to the nucleus and binds to the IL-10 promoter to induce expression. IL-10 causes a senescent phenotype by impeding cytokine production, prevention of MHC upregulation, and inhibiting tyrosine phosphorylation in T cells which leads to a decrease in proliferation, differentiation, and IFN-γ and IL-2 production (preliminary data from our group DOI: 10.1101/2021.02.26.432955). Senescent cells in HIV-1 infection are characterized by expression of biomarkers like latency associated peptide (LAP) and glycoprotein A repetitions predominant (GARP) (preliminary data from our group DOI: 10.1101/2020.12.15.422949), the inactive form of TGF- b in the cell surface. TGF-β is a known latency inducer in HIV-1 (18). GARP/LAP cleavage increases active plasma levels of TFG-b which initiates a signal transduction cascade that leads to upregulation of promyelocytic leukemia protein (PML). PML can either establish cellular quiescence by stabilizing forkhead box O3 (FOXO3) and O4 (FOXO4), or it can, in response to cellular stress, associate to protein phosphatase 2A (PP2A). This association inactivates AKT kinase and sequesters DNA damage inducible

transcript 4 (DDIT4) which inhibits the mammalian target of rapamycin (mTOR) pathway which triggers effector differentiation and glycolysis. FOXO4 binds p53 tumor suppressor and downregulates pro-apoptotic machinery (preliminary data from our group DOI: 10.1101/2020.12.15.422949). IL-10 induces activators of STAT3 and promotes STAT3 translocation, ultimately upregulating expression of downstream proteins including p53 (19). FOXO3 upregulates PD-1, a T cell response regulator and marker of disease progression, blocking T cell differentiation (preliminary data from our group DOI: 10.1101/2020.12.15.422949) (20). PD-1 interactions with ligands PD-L1 and PD-L2 tolerize T cells to antigens to defuse T cell effector functions (20–22). Ultimately, downregulation of pro-apoptotic machinery would increase availability of anti-apoptotic machinery and further contribute to HIV-1 reservoir persistence (Figure 2). α-PD-1 monoclonal antibodies reverse T cell exhaustion in cancer patients and restore anti-tumor potential of T cells (20) Further upstream, blocking IL-10 inhibits upregulation of PD-L1 and α-IL-10 decreases frequency of PD1+ cells (preliminary data from our group DOI: 10.1101/2021.02.26.432955) (23). We have shown that TFG-b signaling is associated to heightened inducible HIV-1 reservoir and also to lack of central memory T cell differentiation, associated with increased expression of PD-1 (preliminary data from our group DOI: 10.1101/2020.12.15.422949), confirming the importance of this pathway in HIV-1 persistence and immune senescence. As proposed previously (preliminary data from our group DOI: 10.1101/2020.12.15.422949), combining PD-1 and TGF-β focused therapies with senolytics will improve the therapeutic impact on senescent cells, including CD8+ T cell cytolytic functions, in HIV-1 and cancer.

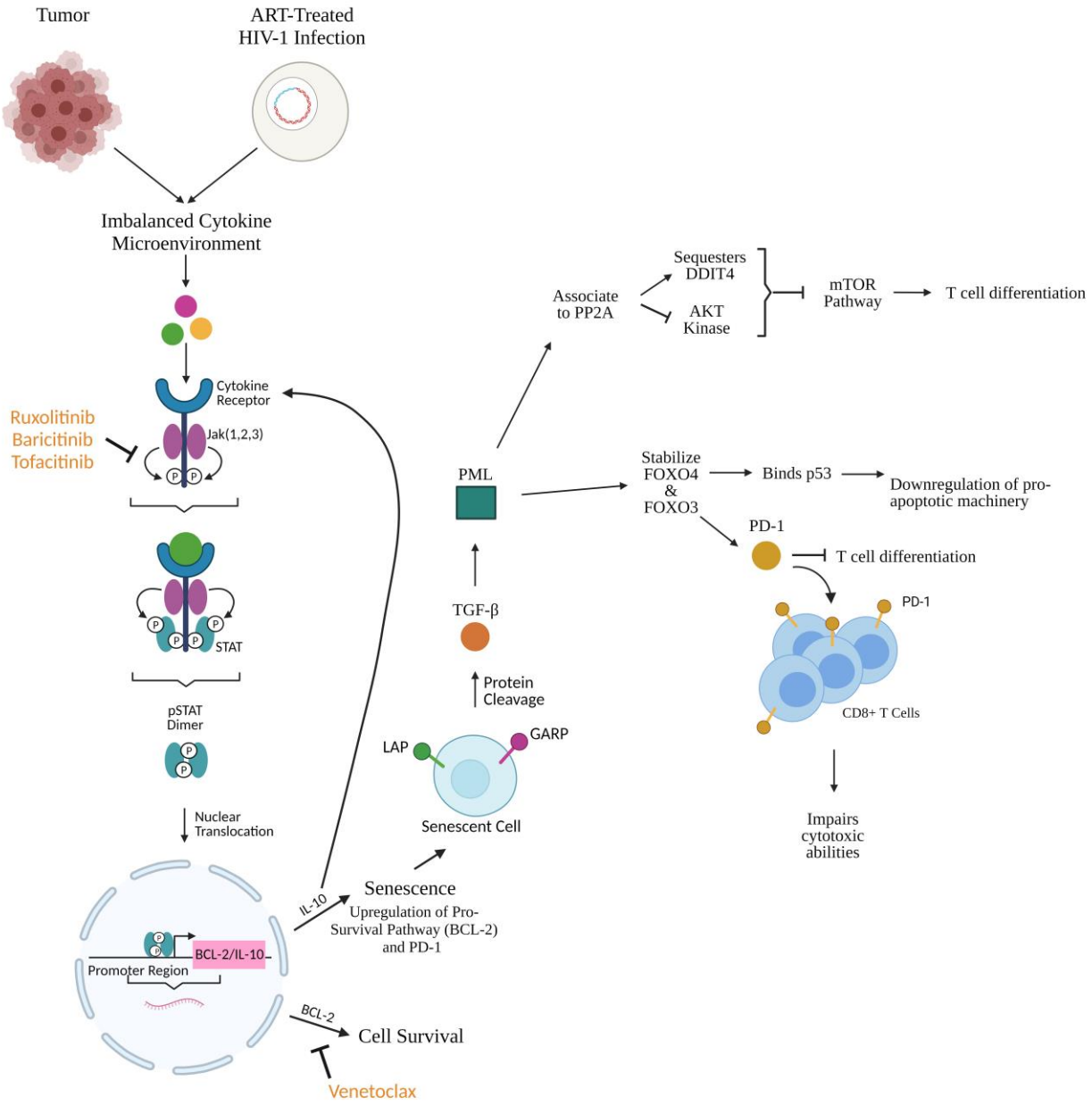

FIGURE 2 | Jak STAT-Mediated Cell Survival, Induction of Senescence, and Impaired T Cell Function

Cytokines produced subsequently from tumors and HIV-1-infected cells initiate the Jak STAT pathway leading to upregulation of pro-survival and pro-senescence factors like BCL-2 and IL-10. Jak inhibitors (ruxolitinib, baricitinib, tofacitinib) prevent upregulation of these factors upstream while BCL-2 homology 3 (BH3) mimetics like venetoclax prevent BCL-2 function downstream, as shown in Figure 1. IL-10 promotes cellular senescence through upregulation of BCL-2 and programmed death protein 1 (PD-1). Senescent cells express latency-associated peptide (LAP) and glycoprotein A repetitions predominant (GARP) which undergo protein cleavage and release bound TGF-β. TGF-β upregulates promyelocytic leukemia protein (PML) which associates to protein phosphatase 2A (PP2A) thereby sequestering DNA damage inducible transcript 4 (DDIT4) and inhibiting Ak strain transforming (AKT) kinase, ultimately

resulting in inhibition of the mammalian target of rapamycin (mTOR) pathway which is responsible for T cell differentiation. In parallel, PML stabilizes forkhead box O4 (FOXO4) and O3 (FOXO3). FOXO4 binds p53 to downregulate pro-apoptotic machinery. FOXO3 upregulates PD-1 expression on CD8+ T cells which blocks T cell differentiation and impairs cytotoxic ability. This creates a phenotype of long-lived senescent cells and T cells with impaired cytotoxic/killing potential further maintaining the HIV-1 reservoir. Created with BioRender.

Furthermore, in a lytic infection, HIV-1 protease cleaves host procaspase-8, generating a fragment called casp8p41. Casp8p41 can either bind to and activate mitochondrial proapoptotic protein BAK or bind to anti-apoptotic BCL-2 (in high concentrations) which sequesters Casp8p41 and prevents apoptosis (24). Venetoclax has been shown to reduce levels of HIV-1 DNA-integrated cells, reduce the reservoir size, and cause preferential killing of HIV-1 expressing cells *in vitro*. In HIV-1 infected cells, casp8p41 binds to BCL-2 and becomes unable to activate pro-apoptotic proteins causing cell survival (25). Venetoclax prevents casp8p41 from binding BCL-2 which allows casp8p41 to bind to BAK and induce apoptosis via the intrinsic cell-death pathway. Reactivation of the HIV-1 reservoir in the presence of high levels of BCL-2 prevented death of the reactivated cells, but the addition of venetoclax circumvented this problem. There was no significant impact on uninfected cell viability or proliferation (24, 26). The addition of venetoclax to *ex vivo* HIV-1 infected samples that were exposed to latency reversal agents (L*Ras*) and co-cultured with HIV-1-specific T cells enabled CD8+ T cell killing of HIV-1 infected cells. Venetoclax did not impair viability or functionality of CD8+ T cells (27). Taken together and considering BCL-2 is necessary for survival of proliferating latently infected cells, this suggests that venetoclax might block persistence and replenishment of the latent reservoir and boost the killing activity in the shock and kill approach to HIV-1 cure (26).

BCL-2, SASP, and INK4α/ARF perpetuated senescence

Senescence is a cellular process that induces stable growth arrest and limits the proliferation of aged cells. Senescence is induced by drivers of damage, and in turn, senescence drives some of the key markers of aging including stem cell exhaustion and chronic inflammation (28). There exists a wide range of triggers of senescence, including oxidative stress, mitochondrial dysfunction, replicative stress,

cytokines, irradiation, and genotoxic agents. As a result of senescence, cells express a phenotype named senescence-associated secretory phenotype (SASP). SASP is comprised of pro-inflammatory cytokines, growth factors, and cytotoxic mediators. The expression of SASP can affect nearby cells via paracrine signaling and consequently convert them to senescence as well (29). There are some benefits to senescence, as the process helps facilitate embryonic morphogenesis and acts to suppress dysfunctional/aged cells. However, this comes at a cost, as senescent cells therefore accumulate in aged tissues, indicating that senescence could itself promote aging (28).

One of the primary factors driving senescence is damage to a cell's telomeres. Telomeres are highly repetitive DNA structures located at the end of chromosomes that act to protect against chromosome degradation. However, with every successive round of cell cycle division, telomeres shorten (28). While cells have DNA repair mechanisms, the damage done to telomeres is hidden by the presence of specialized nucleoprotein complexes called shelterins. As a result of damaged telomeres, cells can no longer divide, thus driving senescence. This phenomenon is better known as telomere attrition (30).

It has been well-established that BCL-2 proteins play an integral role in the apoptotic pathway. However, it has also been revealed that BCL-2 may also play a role in cellular senescence. Specifically, BCL-2 has been seen to accelerate premature senescence in both human lung primary fibroblasts and human foreskin fibroblasts (31). The exact mechanism of how BCL-2 and senescence work in tandem is yet to be completely elucidated, but recent experiments have been able to yield results demonstrating the impact BCL-2 has on senescence.

Senescent cells upregulate BCL-2 and BCL-XL, both of which are anti-apoptotic proteins (32). When senescence in primary human fibroblasts were induced via DNA damage or replicative exhaustion and treated with tumor necrosis factor-a and cycloheximide to induce apoptotic pathway, senescent cells survived at a much higher rate compared to empty vector transduced cells. Furthermore, levels of BCL-2 and BCL-XL were detected to have increased in these senescent cells. When the cells were treated with a known small molecule inhibitor of anti-apoptotic proteins called ABT-737, high levels of cell death were seen in the senescent cells compared to the control (11). In human primary fibroblasts introduced with an

activated *ras* allele, a similar phenomenon was discovered as well. The upregulation of BCL-2 resulting from the introduced *ras* allele increased the rate of appearance of senescence-associated bgalactosidase, a known marker of senescence. Compared to control cells with no *ras* allele, there was a higher percentage of senescence-associated b-galactosidase-positive cells in the cells with the *ras* allele (31).

These discoveries lend to the evidence that BCL-2 levels may decide if cells undergo senescence versus p53-dependent apoptosis. In the presence of high levels of BCL-2, the inhibition of apoptosis may commit cells towards a path of senescence instead (33). As a result, it is plausible to conclude that the expression of anti-apoptotic proteins such as BCL-2 and BCL-XL contribute to the resistance of senescent cells towards programmed cell death.

Senescence in cancer

Cellular senescence plays an important role as a tumor suppressor mechanism. Senescence has antiproliferative power and thus can restrict the progression of tumors (34). Like with aging, the inhibitor of cyclin-dependent kinase 4 alpha/alternate reading frame (INK4α/ARF) locus plays a significant role in cancer. Oncogenic mutations in cells activate the expression of the locus, thereby encoding tumor suppressing proteins such as p15INK4β and p16INK4α. These proteins inhibit and inactivate cyclin-dependent kinase (CDK) 4/6, thus inducing cell cycle arrest and preventing continued division of cancer cells. The ARF protein inhibits mouse double minute homolog 2 (MDM2), a protein that usually inhibits the transcriptional activity of p53. As a result of the inhibition of MDM2, p53 is stabilized and leads to cell-cycle arrest, thus preventing further division of cancer cells as well (35).

Of note is that senescence in tumors is closely tied only to the premalignant states of tumorigenesis. In fact, senescent markers appear absent in tumors once they are in their malignant stages. These findings suggest that senescence acts as a barrier to tumor progression (36). Because of the interconnectedness between cancer pathways and senescence, potential cancer treatments have been targeted towards inducing senescence or blocking the cellular pathways that lead to the proliferation of cancer cells. For example, multiple chemotherapeutic drugs under investigation act to inflict severe DNA damage to cancerous cells,

thus triggering cellular senescence. Further, some small molecule inhibitors are being developed to restore p53 signaling, as p53 function is lost in tumor cells; this could help control the rapid division of tumor cells.

Relationship between senescence and aging

The question of whether senescence and aging are related through causation is still under investigation, but there are components of senescence that do correlate with the process of aging. One of these components is the INK4α/ARF locus where three tumor suppressors reside: p16INK4α, ARF, and p15INK4β (28). At a young age, the INK4α/ARF locus is expressed at low levels in most tissues but slowly becomes derepressed with continued aging (34). Specifically, the p16 protein is of particular interest as it is an important marker of senescence that has been observed to accumulate in aged tissues of mammals (37). P16-mediated senescence works via the retinoblastoma (Rb) pathway. p16 binds to CDK4/6 which thus inactivates the kinases and prevents the phosphorylation of the Rb protein. The lack of phosphorylation in Rb proteins causes them to remain associated with transcription factor E2F1 as an Rb-E2F1 complex. The restriction of the E2F1 transcription factor prevents transcription of E2F1 target genes which are otherwise crucial for the transition from the G1 to S phase in the cell cycle (Figure 3). Consequently, the increase in p16 concentration leads to cellular senescence by inhibiting the CDK pathway (38).

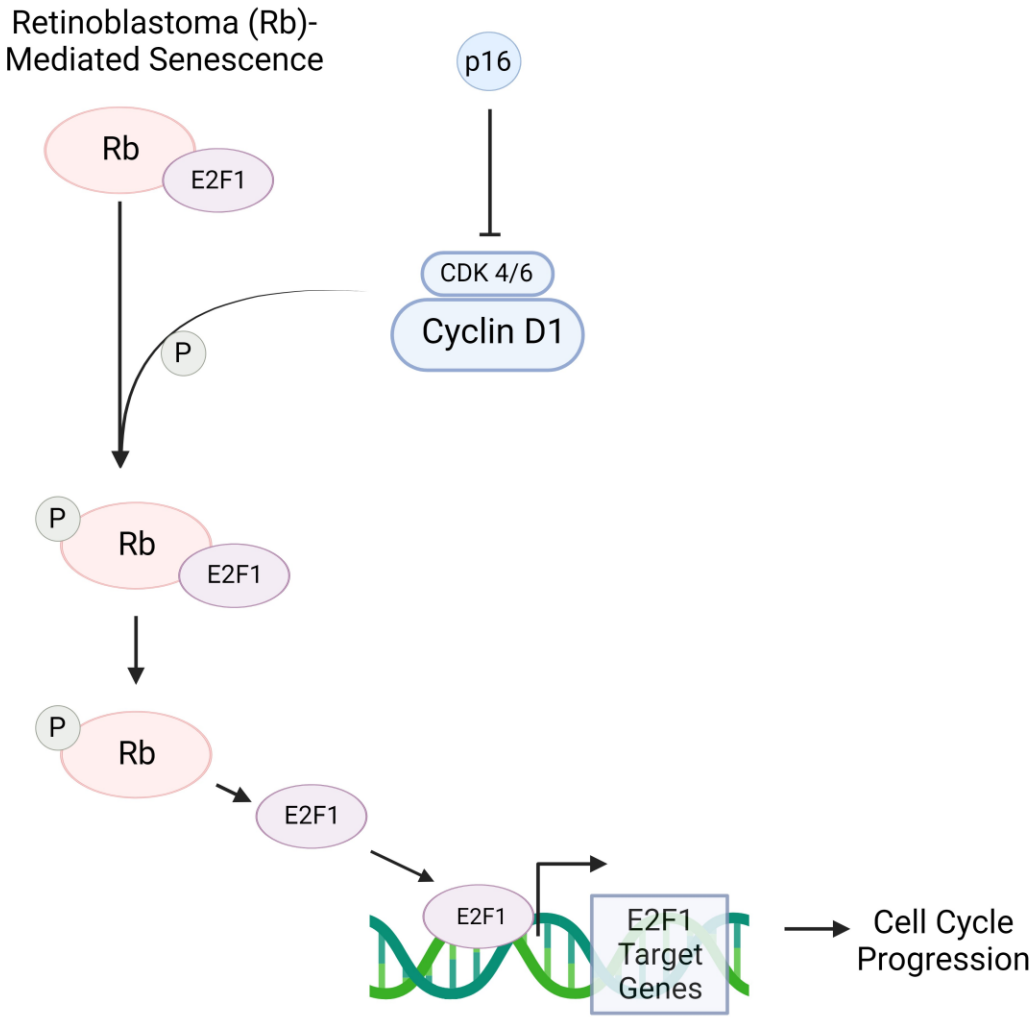

FIGURE 3 | Role of p16 in Retinoblastoma-Mediated Senescence

p16 is a protein that, when in increased concentrations, can induce senescence via the retinoblastoma (RB)-pathway. Rb is a tumor suppressor protein that plays a critical role in regulating the cell cycle. Cyclin D1 activates cyclin-dependent kinase 4/6 (CDK 4/6), allowing the kinase to phosphorylate the Rb-E2F1 complex. This complex consists of the Rb protein and E2F1 transcription factor. When phosphorylated, Rb is inactivated, allowing for the detachment of E2F1; consequently, E2F1 target genes are transcribed. These play a crucial role in the transition of a cell from the G1 to S phase. However, in the presence of p16, CDK 4/6's ability to deactivate Rb through phosphorylation is inhibited. As a result, E2F1 remains bound to Rb, and therefore transcription of E2F1 target genes is prevented. This blocks cell cycle progression, thus causing a cell to move towards senescence. Created with BioRender.

The aforementioned importance of p16 in senescence has been observed in progeroid mice. Usually, these mice show early onset of a few age-associated disorders such as cerebral gliosis and cataracts. In one experiment, the progeroid mice were engineered so cells expressing p16 would be induced to undergo apoptosis. In cont*ra*st to the unaltered mice, these mice displayed delayed onset in the age-

associated disorders. In naturally aging INK-apoptosis through targeted activation of caspase (ATTAC) mice, the same alterations were made; delayed onset of the age-associated disorders was once again observed alongside an increase in lifespan. Altogether, this suggests that senescence plays a role in age-associated pathologies and that senescent cells may limit longevity (37).

SASP as a product of senescence can also contribute to the aging process. The paracrine transmission of the senescence response can contribute to chronic inflammation, or inflammaging. Elimination of senescent cells in aged organs has been seen to reduce levels of IL-6 and IL-1β, both of which are markers of chronic inflammation. Another process called immunosenescence is correlated with aging. The immune system in healthy individuals can usually clear senescent cells (28). However, with age, there is a decline in the production of adaptive immune cells (29). As a result, the clearance of senescent cells becomes compromised, leading to greater inflammation (28).

BCL-2 interactions in SARS-CoV-2

A global pandemic has presented unique challenges in the scientific community. With the technology and means to quickly share data, facilitating collaborative problem-solving, we also see a lag in the peer-review process due to increased submission and importance of information at this time. This has led to some contention on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) interactions with BCL-2 in the field. Ongoing research seeks to characterize SARS-CoV-2 but many predictions and comparisons are drawn based on existing knowledge of Middle East respiratory syndrome (MERS-CoV) and severe acute respiratory syndrome (SARS-CoV) and comparisons with related positive sense single stranded RNA viruses ((+)ssRNA).

It has been posited that because the mitochondria is targeted by related (+)ssRNA viruses, it may also be a target of SARSCoV-2 (39). In observing the human interactome with MERS and SARS-CoV, it was predicted that BCL-2, MCL-1, and BCL-2A1 would be upregulated in SARS-CoV-2 infection (40). Rad9 is a human protein that has a BH3-like region and interacts with BCL-2 and BCL-XL to induce apoptosis (41). Rad9A can purportedly be targeted by SARS-CoV-2 encoded miRNA that suppress the

gene (preliminary data from Xingyin Liu's group at Nanjing Medical University, China, doi: 10.48550/arXiv.2004.04874). This suggests that SARS-CoV-2 relies on BCL-2 for a productive infection. However, there is more evidence to support that SARS-CoV-2 likely downregulates BCL-2 instead.

SARS-CoV induces apoptosis via the intrinsic cell-death pathway and nasopharynx samples from SARS-CoV-2 patients have shown increased apoptosis and expression of caspase-3 (42–48). SARS-CoV infection induces caspase-dependent apoptosis which can be prevented by overexpression of BCL-2 or by caspase inhibitor (42, 48). SARS-CoV produces a 7a protein that induces apoptosis that can be blocked by overexpression of BCL-XL. 7a has been found to interact with BCL-XL, BCL-2, MCL-1, and BCL-A1 (46). SARS-CoV-2 shares the ORF7a gene with SARS-CoV, which makes it likely that SARS-CoV-2 7a protein has a similar interaction with these BCL-2 proteins (48). The SARS-CoV E protein causes apoptosis that can be prevented by BCL-XL (44, 49). SARS-CoV-2 E protein has sequence similarity and highly conserved N-terminal regions with SARS-CoV, and so may share a similar function (50). SARS-CoV-2 infection in the lung increases expression of Noxa, an MCL-1 inhibitor, which increases apoptosis. Younger lung tissue has been found to be more primed for apoptosis because more cytochrome C is released during MOMP than in older lung tissue (51). In Vero cells, BCL-2 expression prevents apoptosis but does not affect viral infection, replication, or release which indicates that although SARS-CoV causes apoptosis, it is not necessary for viral dissemination (42). SARS-CoV-2 induces apoptosis via the extrinsic cell-death pathway through caspase-8 and caspase-9. Caspase-8 cleaves pro-apoptotic BID to tBID (truncated BID) which induces release of cytochrome C. This ultimately results in formation of the apoptosome and activation of caspase-9 which begins the caspase cascade leading to apoptosis (27).

Of note, Jak 1/2 inhibitor baricitinib was granted an emergency use authorization in 2020 for the treatment of severe COVID-19. Combination therapy of baricitinib and remdesivir was shown to reduce recovery time in patients receiving high-flow oxygen or ventilation compared to remdesivir alone. Serious adverse events were reduced from 21% with only remdesivir to 16% with baricitinib (52). The anti-inflammatory effect of baricitinib is especially useful in treating severe COVID-19 and COVID-19 pneumonia which progress due to severe inflammation in the lungs. As the Jak STAT pathway is an

upstream regulator of BCL-2 expression, a potential connection between BCL-2 and severe COVID-19 may exist but needs further investigation.

BCL-2 conservation among other viral species

With BCL-2 inhibitors proving effective against cancer and HIV-1, they could potentially be used as an "antiviral" against other viral species. Its potential efficiency would depend on how the pathogen(s) could lead to BCL-2 upregulation making the infected host-cell a long-lived reservoir.

Upregulation of BCL-2

The following viruses upregulate BCL-2, which make them viable targets for a BCL-2 inhibitor. All gamma herpes viruses and adenoviruses encode viral BCL-2 protein homologs. Adenoviruses encode an anti-apoptotic BCL-2 protein homolog, E1B 19K, that does not have homology with BCL-2 proteins. E1B 19K interacts with BAX and BAK, so BH3 mimetics may also inhibit the homolog (53). Zaire ebolavirus induces lymphocyte apoptosis. Although lymphocytes are not infected, macrophages are and do not undergo apoptosis. BCL-2 upregulation has been documented by day 4 of infection. Because the lymphocyte apoptosis happens at later stages of infection, it is suspected to contribute to suppression of adaptive immunity, which is proposed to play a role in septic shock (54, 55). In human papillomavirus-induced squamous cell carcinoma of the cervix, BCL-2 is overexpressed (56). In picornavirus infection, IFN-b promoter stimulator 1 (IPS-1)-dependent IFN regulatory factor 3 (IRF-3) activation upregulates expression of BCL-2 (57). During infection, mitochondrion-anchored death signaling proteins can activate caspase-8 (1). BCL-2 proteins on the outside of the mitochondrial membrane oligomerize which forms pores that pro-apoptotic factors can access the cytoplasm through (1). Poxviruses encode an N1 anti-apoptotic protein that binds BH3 and prevents apoptosis (58). The crystallographic structure of N1 has revealed similarity to human BCL-2 (58). N1 binds to BH3 with high affinity (58). Similarly, to adenovirus E1B 19K, BH3 mimetics may be effective in inhibiting N1.

Downregulation of BCL-2

The following viruses downregulate BCL-2 and so should not be treated with a BCL-2 inhibitor. Treatment plans should consider this aspect if co-infections are observed. Herpes simplex virus 2 (HSV-2) and Hantaan virus both downregulate BCL-2 for a productive infection as they induce apoptosis in late-stage infection (59). Merkel cell polyomavirus (MCPyV) is associated with deregulated expression of BCL-2 (60). BCL-2 in Merkel cell carcinoma (MCC) patients indicates an earlier clinical stage and longer survival in patients. Downregulation of BCL-2 negative tumors results in local and systemic metastasis (61). Within the Rhabdoviridae family, BCL-2 controls release of cytochrome C and inhibits AIF translocation to the nucleus, preventing DNA damage (62). Within Madin-Darby canine kidney (MDCK) cells, BCL-2 expression has been shown to block orthomyxovirus-induced apoptosis and reduce production of infectious progeny (63). Adherence to seasonal flu vaccine may prevent this from being a problem in treatment plans.

Immunomodulators Affecting BCL-2 Expression

There are both direct and indirect routes to target BCL-2. BH3 mimetics and BCL-2 anti-translational compounds are direct acting whereas compounds like Jak STAT inhibitors have downstream effects on BCL-2 expression. A comprehensive overview of all immunomodulators discussed in this section is provided in Table 1, including indirect and direct acting compounds.

TABLE 1 | Report of drug class, cellular target, clinical trial status (phase I, II, III, approved, terminated), approval in children, oral bioavailability, route of clearance, compound half-life and dosing for Jak and BCL-2 inhibitors

Compound	Class	Target	Clinical Status	Approved in Children	Orally Bioavailable	Clearance	Half-Life	Dosing
Tofacitinib	Jak STAT Inhibitor	Jak 3	FDA approved	No	Yes	Predominantly hepatic	3 Hours	5-10 mg
Ruxolitinib	Jak STAT Inhibitor	Jak 1/2	FDA approved	No	Yes	Predominantly renal	3 Hours	5-25 mg
Baricitinib	Jak STAT Inhibitor	Jak 1/2	FDA approved	Yes (2-17)	Yes	Renal	12 Hours	2-4 mg
Venetoclax	BH3 Mimetic	BCL-2 Specific	FDA approved	No	Yes	Hepatic	26 Hours	10-100 mg
Oblimersen	Phosphorothioate antisense oligonucleotide	BCL-2 mRNA translation	Multiple phase III/ ceased manufacture	No	No	Minimally renal / Predominant not reported	2.5 Hours	3 mg/kg/day
ABT-737	BH3 Mimetic	Pan-BCL-2 inhibitor (BCL-2, BCL-XL, BCL-W)	Pre-clinical	No	No	N/A	N/A	1 μM (sub-cytotoxic)
Navitoclax	BH3 Mimetic/ Senolytic	Pan-BCL-2 inhibitor (BCL-2, BCL-XL, BCL-W)	Multiple phase II/ recruiting multiple phase III	Yes (Birth-17)	No	Not renal/ Predominant not reported	17 Hours	150 mg
Obatoclax	BH3 Mimetic	MCL-1	Multiple phase II/ ceased manufacture	No	No	N/A	7-14 Hours	MTD (28 mg/m2 over 3 hours every 3 weeks)
AT-101	BH3 Mimetic	BCL-2, BCL-XL	Multiple phase II/ ceased manufacture	No	Yes	N/A	N/A	N/A

Some data are undefined for these compounds and are marked as not available (N/A).

BCL-2 inhibitors

The first BCL-2 inhibitor that progressed to clinical trials was an antisense oligodeoxynucleotide that targeted BCL-2 mRNA. This inhibitor was used with chemotherapy to treat chronic lymphocytic leukemia (CLL) (64). BH3 mimetics are a largely researched class of BCL-2 inhibitor. Within the BCL-2 protein family, some proteins have a BH1, BH2, and BH3 domain while some only have the BH3 domain, called BH3-only proteins (65). This homology makes the BH3 domain an ideal target. The BH3 domain on the BCL-2 proteins is where the BH3 peptide binds and thus becomes unable to activate proapoptotic

proteins. The BH3 mimetics can sequester the BCL-2 anti-apoptotic proteins in high concentrations which allows for pro-apoptotic protein activation and subsequent cell death.

Oblimersen

Oblimersen was the first developed BCL-2 inhibitor. Oblimersen is a phosphorothioate antisense oligonucleotide that inhibits BCL-2 mRNA translation. In pre-clinical studies, the dosage was described as 3 mg/kg/day with a half-life of 2.5 hours and with minimal renal clearance (66). There are several completed phase III clinical trials for oblimersen for melanoma, adult myeloid leukemia, multiple myeloma and plasma cell neoplasm and leukemia. However, oblimersen did not show efficacy in increasing patient survival in phase III trials studying lymphoma, which lead to the FDA rejecting the drug twice and subsequent withdraw of the new drug application (NDA) for oblimersen by the pharmaceutical company Genta in 2004 (67, 68).

ABT-737

ABT-737 is a BH3 mimetic pan-BCL-2 inhibitor, targeting BCL-2, BCL-XL, and BCL-W (69). However, if MCL-1 is overexpressed in target cells, ABT-737 will not induce apoptosis (70). MCL-1 can also sequester BAK, and so this drug may be useful in combination with others (71, 72). Preclinical data defines a sub-cytotoxic dosage of 1 μM (73). ABT-737 was found to eliminate senescent cells in mice (11). A preclinical *ex vivo* study of ABT-737 with ovarian cancer (ClinicalTrials.gov Identifier: NCT01440504) is the only clinical trial data available for this compound. Studies with this drug lead to the development of navitoclax (74).

Navitoclax

Navitoclax, previously ABT-263, is an orally bioavailable pan-BCL-2 inhibitor that binds to BCL-2, BCL-XL, and BCL-W. Navitoclax is a BH3 mimetic. Pre-clinical data describes the dosage as 150 mg with a half-life of 17 hours and clearance not occurring renally (75, 76). The European Medicines Agency

(EMA) granted a waiver for use of this drug in pediatrics, ages birth to 17 years, to treat myelofibrosis in 2018 (77). Navitoclax is also considered a senolytic. Other senolytics include dasatinib, quercetin, and fisetin although these are not BCL-2 inhibitors. Interestingly, fisetin is a senolytic flavonoid which has BCL-2 inhibiting activity and was found to reduce mortality in bcoronavirus infected mice (78). This finding has led to an imminent FDA-approved clinical trial (79). There are several clinical trials with Navitoclax currently recruiting: phase II with ruxolitinib for both myelofibrosis and relapsed/refractory myelofibrosis, phase I for myeloproliferative neoplasm, phase III for a tolerability and efficacy combination study with ruxolitinib for myelofibrosis, phase I/II with vistusertib for relapsed small cell lung cancer, phase I/II with trametinib for metastatic, refractory, and unresectable malignant solid neoplasms, and a phase I/II with dabrafenib and trametinib for malignant solid neoplasm, metastatic melanoma, state II (A,B,C) and IV cutaneous melanoma AJCC v7, and unresectable melanoma.

Obatoclax

Obatoclax mesylate, a mesylate salt of obatoclax, is a BH3 mimetic that binds to MCL-1 and has been studied in combination with other drugs against cancer in clinical trials. Pre-clinical data describes the maximum tolerated dose as 28 mg/m (2) over 3 hours every 3 weeks and a half-life of 7-14 hours (80). Several phase II clinical trials have been completed for acute myeloid lymphoma, in combination with rituximab for follicular lymphoma, Hodgkin's lymphoma, myelodysplastic syndromes, myelofibrosis, in combination with caroplatin/etoposide for extensive stage small cell lung cancer, in combination with bortezomib for mantle cell lymphoma, in combination with docetaxel for lung cancer, and in combination with topotecan hydrochloride for recurrent small cell lung cancer. Developers terminated clinical trials in 2012 and discontinued the drug with no updates since (67).

AT-101

AT-101, also referred to as gossypol acetic acid, is an orally bioavailable BH3 mimetic. AT-101 binds to BCL-2 and BCL-XL. It has undergone phase II clinical trials and is currently being investigated

for efficacy against solid tumors (67). AT-101 has been investigated in multiple completed phase II clinical trials for chronic lymphocytic leukemia, in combination with prednisone and docetaxel for hormone refractory prostate cancer, in combination with docetaxel for non-small cell lung cancer, for recurrent and stage III/IV adrenocortical carcinoma, for relapsed/refractory B-cell malignancies, in combination with rituximab for follicular lymphoma, for progressive or recurrent glioblastoma multiforme, for extensive stage and recurrent small cell lung cancer, in combination with bicalutamide for adenocarcinoma of the prostate and stage IV prostate cancer, for prostate cancer, in combination with topotecan for small cell lung cancer, and for hormone refractory prostate cancer. Manufacturing of AT-101 was ceased in 2009.

Venetoclax

Venetoclax, previously ABT-199, is a BH3 mimetic that is BCL-2-specific. Venetoclax was the first BCL-2 inhibitor to be FDA-approved (81). Venetoclax is an approved treatment for acute myeloid lymphoma (MCL), CLL, and small lymphocytic leukemia (SLL) (82, 83). Venetoclax has a dosage of 10-100 mg with a half-life of 26 hours and is orally bioavailable. Venetoclax is contraindicated for strong inhibitors of CYP3A (84). Recently, venetoclax has been experimentally shown to decrease the size of the HIV-1 reservoir (26). Venetoclax has demonstrated ability to penetrate into the CNS compartment, however Venetoclax is highly bound to plasma proteins (> 99%), which may limit its tissue penetration and subsequent free active drug concentrations across two-compartment pharmacokinetic models (84, 85). To date, these data are not defined, however studies are warranted across one and two compartment pharmacokinetic modeling efforts to better define potential for Venetoclax to decay the reservoir systemically. While tissue penetrability would be a desirable trait considering myeloid viral reservoirs and tissular tumor environments, tissue distribution of Venetoclax has not been formally documented but likely has limited tissue distribution to secondary lymphoid organs as it is highly bound to plasma protein (84).

Jak STAT Inhibitors

Tofacitinib

Tofacitinib is a Jak 1/2/3 inhibitor that is FDA approved for the treatment of rheumatoid arthritis and is approved for chronic long-term use. The dosage for tofacitinib is 5-10 mg with a half-life of 3 hours and oral bioavailability of 74%. 70% of clearance occurs hepatically with the remaining 30% being renal. Tofacitinib can cause lymphocytosis, neutropenia, and anemia which diminishes its desirability as a therapy for infection of immune cells. There is a black box warning for observed infections leading to hospitalization or death, lymphoma and other malignancies, and an increased rate of Epstein-Barr virus-associated posttransplant lymphoproliferative disorder in renal transplant patients treated concomitantly with immunosuppressive medication [Venetoclax package insert].

Ruxolitinib

Ruxolitinib is a Jak 1/2 inhibitor that is FDA approved for treatment of myelofibrosis and is approved for chronic long-term use. At a dosage of 5-25 mg, ruxolitinib has a half-life of 3 hours and is 95% orally bioavailable. Renal clearance is the predominant route of excretion at 74% with hepatic clearance at 22% [Jakafi package insert]. A recent human clinical trial with PWH treated for 5 weeks with ruxolitinib demonstrated reduced biomarkers of inflammation, immune dysregulation, T-cell activation, cellular lifespan, and gut microbial translocation while increasing IL-7R expression which is inversely related to homeostatic proliferation (7). In studies with HIV-1, ruxolitinib and tofacitinib inhibit IL-2, IL-7, and IL-15-induced STAT5 phosphorylation and BCL-2 expression. IL-2, IL-7, and IL-15 which all signal through Jak STAT, have integral roles in HIV-1 pathogenesis. IL-2 increases viral reactivation. IL-7 and IL-15 drive homeostatic proliferation and promote survival and proliferation of latently infected cells. pSTAT5 strongly correlates with increased integrated viral DNA levels, directly contributing to HIV-1 persistence (3). Ruxolitinib and tofacitinib have been shown to inhibit virus replication in lymphocytes stimulated (phytohemagglutinin (PHA) + IL-2) for reactivation and inhibit reactivation in central memory

lymphocytes (9). These studies highlight the link between the Jak STAT pathway and HIV-1 reservoir expansion and maintenance.

Baricitinib

Baricitinib is also a Jak 1/2 inhibitor with FDA approval for the treatment of rheumatoid arthritis and an emergency use authorization for COVID-19 (86). Of the three Jak STAT inhibitors discussed herein, baricitinib possess the most favorable safety profile. Baricitinib is administered quaque die orally at 2-4 mg and is blood-brain-barrier penetrant. Baricitinib is 80% orally bioavailable, reaching peak plasma concentrations within an hour of administration, and with an elimination half-life of 12 hours. Baricitinib is approved for chronic long-term use, including in children aged 2-17. Baricitinib is cleared renally, lowering chances of hepatic drug-drug interactions. There is a black box warning for observed infections leading to hospitalization and death, lymphoma and other malignancies, and thrombosis [Olumiant package insert]. More than 50 human trials of baricitinib for inflammatory disorders are currently underway. In the context of HIV-1 infection, basal level inflammation associated with the HIV-1 reservoir leads to long-term complications such as HIV-associated neurocognitive dysfunction (HAND), HIV-associated dementia (HAD), and cardiovascular disease (CVD) (87–89). To date, the tissue penetration profile of Jak 1/2 or BCL-2 inhibitors is not fully elucidated in humans across all tissues and organs. Baricitinib is not fully plasma protein-bound, highlighting its potential to penetrate tissues as an unbound, free agent. We previously proposed baricitinib as a potential therapy to target immune dysregulation resulting from HIV infection in both the lymphocytic and myeloid compartments (90). A recently NIH funded Phase 2a trial with baricitinib to evaluate the ability of this agent to reduce the HIV-1 reservoir in the CNS compartment is now underway (NCT05452564), underscoring potential for baricitinib to not only penetrate across compartments such as, but not limited to the CNS. Further, the data from this forthcoming trial will provide additional pharmacokinetic and pharmacodynamic properties of baricitinib towards reduction of the reservoir not only peripherally but across compartments; these data will be sentinel towards understanding how baricitinib can potentially be utilized in cure-based regimens for PWH.

Overall, baricitinib presents ideal qualities for concomitant use with ART including: a long half-life, no contraindications with ART or other antiretroviral therapies, renal clearance to avoid potential drug-drug interactions with hepatically cleared ART, orally bioavailable for ease of administration, and blood-brain barrier penetrant to target infected cells in the central nervous system (CNS), a notorious sanctuary site in HIV-1 infection.

Conclusion

Several viruses and cancers have evolved to overcome the efficient immunity to promote its own survival. BCL-2 inhibitors have the potential to hinder viremia in viral infections where BCL-2 anti-apoptotic proteins are upregulated, but when these anti-apoptotic proteins are downregulated a BCL-2 inhibitor could aid infection and have d*ras*tic effects on a patient's health. A BCL-2 inhibitor alone may not be effective as an anti-viral, but in combination therapy could be beneficial. Venetoclax, an existing FDA-approved BCL-2 inhibitor, could be repurposed as an antiviral against viruses that upregulate BCL-2 anti-apoptotic proteins in infection. Combination therapy of multiple BCL-2 inhibitors or development of more broadly inhibiting BH3 mimetics would likely be more effective in targeting senescent cells and potentially infected cells and tumors. SARS-CoV-2 is projected to downregulate BCL-2 anti-apoptotic proteins, so a BCL-2 inhibitor could exacerbate cellular damage during infection. Treatment plans should consider co-infections and viral infections in individuals with cancer before incorporating BCL-2 inhibitors so as not to further impair a patient's health. Overall, BCL-2 proteins present a target that could be exploited in some viral infections and BCL-2 inhibitors could further expand to be used as antivirals.

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Chapter II: The Identification of Intact HIV Proviral DNA from Human Cerebrospinal Fluid

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ZZ conducted IPDA measurement and statistical analysis, MDR conducted correlative and statistical analysis, ZZ and MDR contributed equally to manuscript writing, editing, figure creation and literature investigation. SR contributed to manuscript writing, literature investigation, and figure creation. VCM and WT provided conceptual contributions. DRF and SLL conducted assessment of NP tests and computation of NP scores. AMA conducted sample (CSF and PBMC) collection and NP tests with participants, preliminary statistical analysis, manuscript writing, and provided conceptual contribution. CG performed conceptual contribution as well as manuscript writing and editing.

Conflict of Interest

VCM has received investigator-initiated research grants (to the institution) and consultation fees from Eli Lilly, Bayer, Gilead Sciences, and ViiV. The other authors had nothing to declare.

Abstract

We evaluated the HIV-1 DNA reservoir in peripheral blood mononuclear cells (PBMC) and cerebrospinal fluid (CSF) in people with HIV (PWH) and associations to cognitive dysfunction. Using the intact proviral DNA assay (IPDA), an emerging technique to identify provirus that may be the source of viral rebound, we assessed HIV DNA in CSF and PBMC in PWH regardless of antiretroviral therapy (ART). CSF was used as a sampling surrogate for the central nervous system (CNS) as opposed to tissue. IDPA results (3' defective, 5' defective, and intact HIV DNA) were analyzed by compartment (Wilcoxon signed rank; matched and unmatched pairs). Cognitive performance, measured via a battery of nine neuropsychological (NP) tests, were analyzed for correlation to HIV DNA (Spearman's rho). 11 CSF and 8 PBMC samples from PWH were evaluated both unmatched and matched. Total CSF HIV DNA was detectable in all participants and was significantly higher than in matched PBMCs ($p=0.0039$). Intact CSF

HIV DNA was detected in 7/11 participants and correlated closely with those in PBMCs but tended to be higher in CSF than in PBMC. CSF HIV DNA did not correlate with global NP performance, but higher values did correlate with worse executive function (p=0.0440). Intact HIV DNA is frequently present in the CSF of PWH regardless of ART. This further supports the presence of an HIV CNS reservoir and provides a method to study CNS reservoirs during HIV cure studies. Larger studies are needed to evaluate relationships with CNS clinical outcomes.

Introduction

A better understanding of HIV persistence during suppressive combination antiretroviral therapy (ART) is needed given the goal of ending the HIV epidemic. Suppression of HIV to levels of <50 copies/milliliter is achievable in most people with HIV (PWH) using current ART regimens.[1] However, HIV is not eradicated with effective ART.[2] Viral persistence results from the latency that is established shortly after infection, which is known to occur in CD4+ T-lymphocytes.[3, 4] As a result, cure of HIV has been extremely elusive.[5, 6] Furthermore, many antiretrovirals (ARVs) have much lower ARV concentrations in cerebrospinal fluid (CSF) compared to plasma.[7] Dolutegravir has been shown to achieve blood-brain-barrier (BBB) penetration with a CSF concentration comparable to plasma.[8] This trend of poor BBB penetrative ability in ARVs presents an opportunity for active viral replication and reservoir expansion in the brain.

The central nervous system (CNS) has emerged as an important HIV reservoir based on an expanding body of evidence. HIV DNA is commonly found in brain tissue from deceased PWH on suppressive ART.[9-11] Extensive research support that HIV can productively infect microglia,[12] and integrated HIV DNA can be detected in astrocytes from brains of PWH despite virologic suppression.[13] Since many CNS cells such as astrocytes and microglia are long-lived, the HIV reservoir in the CNS may be particularly resistant to decay over time.[14] Viral genetic compartmentalization occurs in the brains of PWH and provides more support for an HIV CNS reservoir.[15] Assessing the HIV CNS reservoir has been

limited to examination of autopsy brain tissues, highlighting a clinical need for a method to characterize HIV impact in the CNS for living PWH.

CSF, which traverses the BBB, provides one window into CNS HIV persistence, and is commonly sampled via lumbar puncture. HIV RNA becomes detectable in the CSF approximately eight days after the estimated date of transmission,[16] indicating early CNS entry. The transactivator of transcription (Tat) HIV protein is detectable in CSF from 36.8% of individuals on suppressive ART.[17] Given its relatively short half-life, the presence of Tat indicates active HIV protein translation in the CNS. HIV persistence in the CNS has also been demonstrated by multiple reports of CSF viral escape despite peripheral viral suppression.[18, 19] HIV DNA in the setting of virologic suppression has been found in CSF cells, and this presence of HIV DNA is associated with neurocognitive impairment, showing the clinical significance of HIV CNS persistence.[20, 21] Innovative new techniques such as HIV Long Terminal Repeat sequencing have yielded detectable HIV DNA and HIV cell-associated RNA in >80% of CSF samples from PWH on ART.[22] This type of HIV CNS persistence is associated with higher neuronal damage by terminal continuation RNA amplification and the presence of cognitive dysfunction.[23]

In a recently published study, the intact proviral DNA assay (IPDA) was successfully used to quantify latent HIV DNA from postmortem brain tissue. Specifically, intact HIV DNA by IPDA was identified from frontal lobe in six of nine individuals who were virologically suppressed on ART.[10] Ideally, the IPDA could be performed with CSF samples given the need to evaluate the CNS HIV reservoir over time as well as in cure studies. We hypothesized that HIV DNA could be detected in the CSF of PWH and would be associated with increased neurocognitive dysfunction. Therefore, we performed an evaluation of the IPDA using cryopreserved CSF cell pellet samples compared to peripheral blood IPDA and then examined associations between IPDA results and neuropsychological (NP) performance. To our knowledge, these data represent the first time that reservoir quantification in the CSF via IPDA has been linked to distinct neurocognitive deficits. These data address a major unmet clinical need towards identifying the role of the persistent HIV-1 reservoir in the CNS and its role in modulation of neurocognitive

deficits and decline. The novelty of these data underscores the importance of conducting larger studies to elucidate the important relationship between the CNS reservoir, neurocognition, and eventual therapeutic interventions that may mitigate or reverse cognitive deficits in PWH.

Methods

PWH were recruited from the Infectious Disease Program (IDP) Ponce de Leon Center, which is a comprehensive HIV clinic within the Grady Health System in Atlanta, Georgia. All PWH were diagnosed with HIV and receiving care at the IDP. Cryopreserved samples from adult PWH, regardless of ART status, enrolled in CNS studies (Emory IRB 00103851 – *Viral immunocapture and isolation of virus from CSF lymphoid and myeloid cells to better understand HIV in the central nervous system[24]*; Emory IRB 00039444 – *Novel tools for the assessment of patients with HIV-associated cognitive impairment: an observational study*) performed at the Emory Center for AIDS Research (CFAR) were used. Exclusion criteria for these studies were: 1) a history of severe neurologic and psychiatric diseases that are known to affect memory (including stroke, malignancy involving the brain, traumatic brain injury, schizophrenia, and AIDS-related opportunistic infections of the central nervous system); 2) active addictive substance use (cocaine, heroin, methamphetamine, or other non-cannabis addictive drug use in the last 30 days; cannabis use was permitted but participants were asked to abstain for 48 hours prior to testing); and 3) heavy alcohol consumption in the last 30 days (defined as >7 drinks per week for women and >14 drinks per week for men). Clinical records were reviewed for substance abuse disorders and other psychiatric and neurological diagnoses. The 30 day abstinence period of non-cannabis addictive substance use was chosen to reduce subacute to chronic changes in the brain during chronic use and after clearance. Substance use abstinence was a historical evaluation. Given participant age range (36-68, median = 53), it is unlikely that neurodegenerative diseases like Alzheimer's would affect the study population, interfering with NP assessments.

A battery of NP tests was administered that included nine tests used commonly in studies of cognition and HIV[25]: 1) Trailmaking Part A, 2) Trailmaking Part B, 3) Hopkins Verbal Learning Test-Revised (HVLT-R) total learning, 4) HVLT-R delayed recall, 5) Grooved Pegboard (dominant), 6) Grooved Pegboard (non-dominant), 7) Stroop Color Naming, 8) Stroop Color-Word interference, and 9) Letter Fluency (Controlled Oral Word Association Test). 7 domains were tested: executive functioning (tests 2,8), memory (test 4), motor (tests 5,6), learning (test 3), processing speed (tests 1,7), attention (test 8), and fluency (test 9).[26]

Scores were adjusted for demographic characteristics (including age, biological sex, race, and education) using published norms.[27-29] A composite global mean T score (NPT-9) was then calculated by averaging individual test T scores (49.76 ± 8.51). Global and domains were examined.

This study was cross-sectional. Blood and CSF samples were obtained via veni and lumbar puncture at the same morning study visit. CSF was processed as per Emory CFAR and HIV Neurobehavioral Research Program (HNRP) protocols. Briefly, 10-25 milliliters (ml) of CSF were centrifuged at 300g for 15 minutes. Peripheral Blood Mononuclear Cells (PBMC) samples were prepared as previously described.[30] After removal of supernatant, fetal bovine serum and dimethyl sulfoxide were added, and the resuspended pellets were then stored in a liquid nitrogen freezer. Cryopreserved cells from CSF and (when available) matched PBMC were thawed in a 37˚C water bath. Next, cells were immediately transferred to pre-warmed sterile Roswell Park Memorial Institute media. After counting and spinning down the cells, genomic DNA was extracted using the Gentra Puregene Cell kit (Qiagen). The frequency of IPDA was determined by duplex droplet digital polymerase chain reaction (ddPCR) (**Figure 1**) as validated and published by Bruner et al.[31] The multiplex PCR was performed on the Bio-Rad QX200 Digital Droplet PCR system. Up to 100 nanograms (ng) of genomic DNA extracted from CSF cell pellets, and 700 ng of genomic DNA extracted from PBMC were added to the master mix containing 2x ddPCR supermix (no dUTP, Bio-rad), primers (final concentration 900 nM), probes (final concentration 250 nM) and nuclease free water. Primer and probe sequences (5’ and 3’) are listed in **Table S1**.[31, 32]

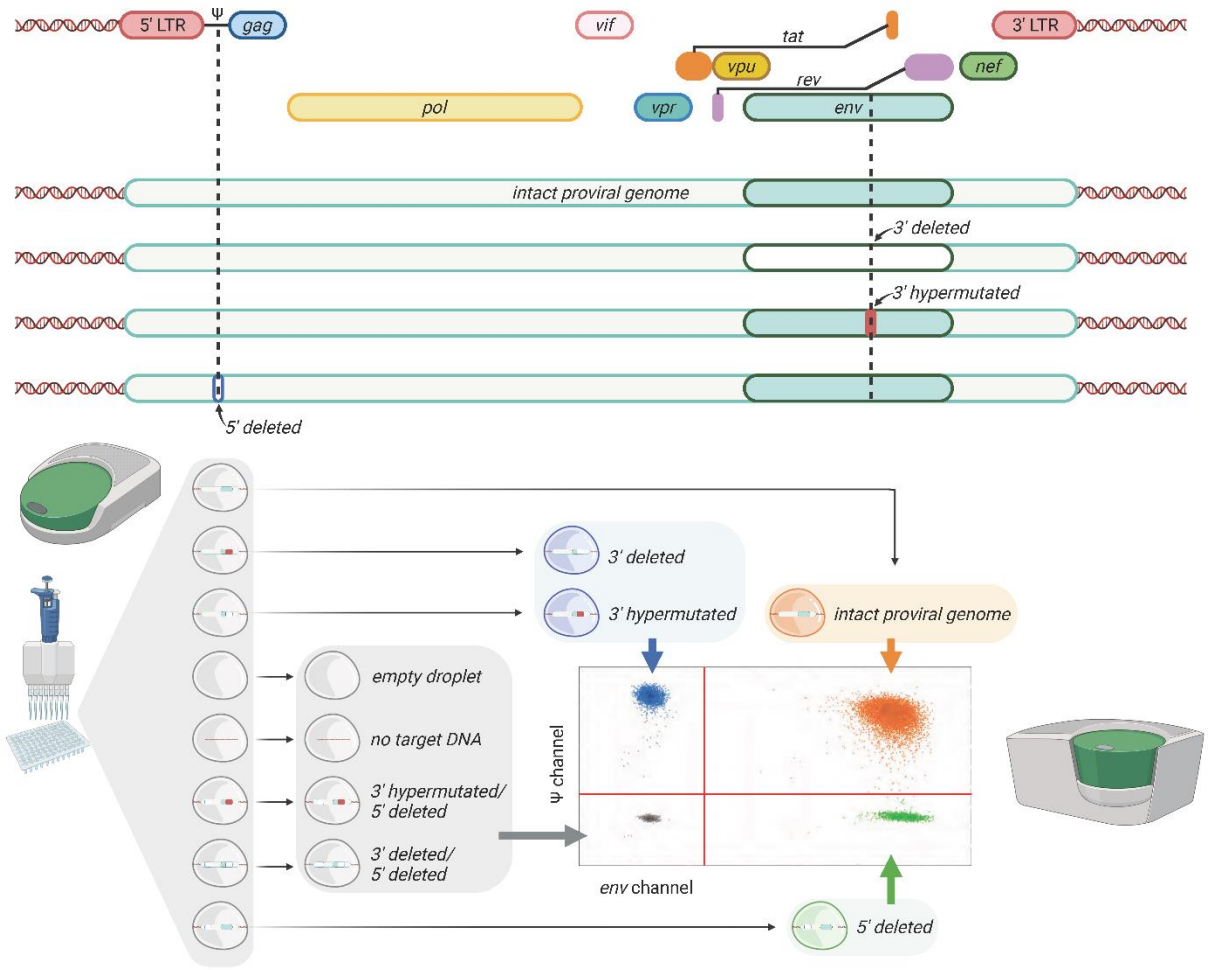

Figure 1 | Schematic Overview of Intact Proviral DNA Assay (IPDA)

Top half: Illustration of IPDA-targeted defective regions of HIV-1 Ψ and Env. Bottom half: Multiplex polymerase chain reaction (PCR)-based 2-dimensional plot allows for discrimination of intact proviral DNA from defective.

Table S1 | Primer and Probe Sequences for Intact Proviral DNA (IPDA) Assay

Primer/Probe Name		Sequence	Fluorophore	Quencher
IPDA Ψ	Ψ F	CAGGACTCGGCTTGCTGAAG	N/A	N/A
	Ψ R	GCACCCATCTCTCTCCTTCTAGC	N/A	N/A
	Ψ intact probe	TTTTGGCGTACTCACCAGT	FAM	MGBNFQ
IPDA env	env F	AGTGGTGCAGAGAGAAAAAAGAGC	N/A	N/A
	env R	GTCTGGCCTGTACCGTCAGC	N/A	N/A
	env intact probe	CCTTGGGTTCTTGGGA	VIC	MGBNFQ
	env hyper probe	CCTTAGGTTCTTAGGAGC	N/A	MGBNFQ

F = forward; R = reverse; N/A = not available.

To ensure consistent PCR results, each sample was quantified in up to six replicates. PCR reaction droplets were prepared with the QX200 Droplet Generator. The thermal cycling program used for ddPCR reactions is in **Table S2**.

Table S2 | Thermal Cycling Program for Droplet Digital Polymerase Chain Reaction (ddPCR) Reactions

Temperature	Time	Cycle
95˚C	10 minutes	X 1
94˚C	30 seconds	
58˚C	1 minute	X 40
98˚C	10 minutes	X 1
12˚C	∞	

The ddPCR plate was stored for at least 4 hours at 4˚C prior to transfer to the QX200 droplet reader, a step which improves the quality metrics of droplets and achieves higher accepted droplet numbers per reaction. To correct for the frequency of total intact proviruses based on the DNA shearing index (DSI)

during intact proviruses quantification, two sets of primers were designed for amplification of the human RPP30 gene (primers and probes listed in **Table S3**). The two regions of RPP30 gene that are targeted during multiplex PCR are spaced at the same distance as the Ψ and *env* amplicons. A previous study verified that DSI was comparable between RPP30 (RPP30-1 and RPP30-2) and HIV (Ψ and env) at all levels of shearing.[31] The Gentra Puregene Cell kit (Qiagen) was also used to limit the sheared DNA rate, and lowest DSI was 0.08.

Table S3 | Primer and Probe Sequences for Human Ribonuclear P Protein Subunit p30 (RPP30) Gene

Prime/Probe Name		Sequence	Fluorophore	Quencher
IPDA RPP30-1	RPP30-1 F	CCATTTGCTGCTCCTTGGG	N/A	N/A
	RPP30-1 R	CATGCAAAGGAGGAAGCCG	N/A	N/A
	RPP30-1 probe	AAGGAGCAAGGTTCTATTGTAG	FAM	MGBNFQ
IPDA RPP30-2	RPP30-2 F	GATTTGGACCTGCGAGCG	N/A	N/A
	RPP30-2 R	GCGGCTGTCTCCACAAGT	N/A	N/A
	RPP30-2 probe	CTGACCTGAAGGCTCT	VIC	MGBNFQ

Used for DNA shearing index [DSI] correction of intact proviral DNA assay (IDPA) results. N/A denotes not available.

Using two amplicons and hypermutations discrimination probes, the intact (Ψ+env+) and defective proviruses (Ψ+env- or Ψ-env+) were distributed into different quadrants (Qs) of the 2-dimensional amplitude ddPCR plots (**Figure 1**). Droplets in Q1 contain proviral genomes including an APOBEC-3G-edited hypermutation or 3' deletion and gave out a fluorescence signal (FAM) arising from an intact Ψ amplicon amplification. Q4, diagonally opposite to Q1, represents an intact *env* region with a deletion towards the 5' encompassing Ψ region. Droplets that contain the intact proviral genome are located in Q2.

Droplets that contain double defective regions or are not occupied with target proviral genomes are distributed in Q3.

For the statistical analyses, HIV DNA was quantified as copies per 10^6 PBMC. Normality was assessed using the Shapiro-Wilk test. Given that the HIV DNA results did not meet assumptions of normality, the comparison between HIV DNA levels by compartment was performed with Wilcoxon signed rank for matched pairs and Wilcoxon rank sum for non-matched pairs. Correlations between HIV DNA and NP scores were performed with Spearman's rho. Statistical analyses were performed with SAS JMP (Cary, NC) as well as GraphPad Prism v10.0.2 (San Diego, CA). The study was approved by the Emory University Institutional Review Board and all participants provided informed consent.

Results

There were 11 PWH (median age = 53 years) with a long history of HIV (median estimated duration = 138 months), see **Table 1**. Eight were men, two were women, and one was transgender man to woman. With respect to comorbidities, five had hypertension, four had depression, and two had chronic hepatitis B virus infection. Seven participants were on stable ART for at least six months with plasma and CSF HIV RNA <40 copies/ml at the time of assessment, while four were not on ART with median 45,708.8 copies/ml at the time of assessment. Median absolute CD4+ T-cell count from blood was 260 cells/microliter while median CD4+ T-cell percentage was 24%. Mean NPT-9 was 49.5 (standard deviation = 8.1).

Table 1 | Demographic/Disease Characteristics of Participants

PID	Age (years)	Sex	Race	ARV Therapy	Nadir CD4	CD4 Count	CSF WBC	CSF RBC	Plasma HIV log10	CSF HIV log10	NPT-9
124	54	F	AA	BIC, TAF, FTC	82	411	2	120	<1.60	<1.60	41.89
127	47	M	AA	FTC/TDF, DRV, COBI	NA	1434	5	1	<1.60	<1.60	42.00
130	58	MtF	AA	DTG, ABC, 3TC	22	364	1	2	<1.60	<1.60	57.67
132	56	M	AA	ATV, COBI, TAF, FTC	18	194	0	0	<1.60	<1.60	55.89
138	47	M	W	BIC, TAF, 3TC	11	730	0	0	<1.60	<1.60	50.22
139	68	M	W	DTG, ABC, 3TC	NA	245	2	2	<1.60	<1.60	42.33
142	40	M	AA	TAF, BIC, FTC*	81	325	0	13	4.68	3.21	56.11
711	36	M	AA	Naïve	13	51	0	0	4.64	1.61	51.77
717	53	F	AA	Naive	89	89	0	0	5.68	4.22	36.99
733	55	M	W	Naïve	153	153	0	0	3.68	3.13	47.44
746	50	M	AA	FTC/TDF, FPV, RTV	9	260	0	0	<1.60	<1.60	62.70

Nadir CD4, CD4 Count, WBC, and RBC are reported in cells/microliter. PID = patient identification; ARV = anti-retroviral; BIC = bictegravir 50mg once daily; TAF = tenofovir alafenamide 10-25mg once daily; FTC = emtricitabine 200mg once daily; FTC/TDF = Truvada (emtricitabine/tenofovir disoproxil fumarate) 1 200-300mg tablet once daily; DRV = darunavir 800mg once daily; COBI = cobicistat 150mg once daily; DTG = dolutegravir 50mg once daily; ABC = abacavir 600mg once daily; 3TC = lamivudine 300mg once daily; ATV = atazanavir 300mg once daily; RTV = ritonavir 100mg once-twice daily; FPV = fosamprenavir 700mg twice daily; M = male; F = female; MtF = transgender male to female; AA = African American; W = White; CSF = cerebrospinal fluid; WBC = white blood cell; RBC = red blood cell; NPT-9 = neuropsychological composite global T score. * denotes PWH recently (<6 months) began ARV therapy and was not virally suppressed at time of draw.

Of the 11 participants, eight had matching PBMC for analysis. Total HIV DNA was detected in all CSF and PBMC specimens (**Table 2**). For the eight matched pairs, median total CSF HIV DNA was $8.29x10^4$ copies per 10^6 cells (Interquartile range [IQR]= $6.75\ x10^3$ – $3.06x10^5$). This was significantly higher (P = 0.0039) than in blood (median= $5.06x10^3$, IQR = $2.00x10^3$ – $1.15x10^4$). This total HIV DNA difference remained significant compared to PBMC (P=0.0006) when all 11 CSF samples were included (median= $1.11x10^5$, IQR= $6.75x10^3$– $3.47x10^5$).

Table 2 | Reservoir (HIV DNA) Measurements in Copies/ 10^6 Cells

Participant ID	CSF Total HIV DNA	CSF Intact HIV DNA	CSF 3' Defective HIV DNA	CSF 5' Defective HIV DNA	CSF% Intact	Plasma Total HIV DNA	Plasma Intact HIV DNA	Plasma 3' Defective HIV DNA	Plasma 5' Defective HIV DNA	Plasma % Intact
124	1.88E+05	4.81E+03	1.04E+05	7.93E+04	2.55%	7.54E+03	1.69E+02	3.48E+03	3.90E+03	2.25%
127	6.75E+03	ND	3.64E+03	3.11E+03	0.00%	2.58E+03	9.16E+01	1.17E+03	1.32E+03	3.55%
130	5.47E+04	1.55E+03	3.08E+04	2.23E+04	2.83%	2.00E+03	1.62E+02	1.06E+03	7.85E+02	8.06%
132	1.69E+05	6.37E+03	9.12E+04	7.12E+04	3.77%	9.15E+03	1.88E+02	7.57E+03	1.39E+03	2.05%
138	1.84E+04	2.87E+02	1.15E+04	6.61E+03	1.56%	9.13E+03	2.12E+02	5.33E+03	3.59E+03	2.32%
139	1.11E+05	1.92E+03	6.59E+04	4.32E+04	1.73%	1.15E+04	5.78E+02	6.46E+03	4.47E+03	5.02%
142	7.67E+03	ND	3.89E+03	3.78E+03	0.00%	2.00E+03	8.13E+00	1.20E+03	7.90E+02	0.41%
711	3.06E+05	ND	3.05E+05	5.58E+02	0.00%	2.23E+03	1.15E+01	1.92E+03	3.02E+02	0.52%
717	3.47E+05	ND	3.45E+05	1.68E+03	0.00%					
733	1.74E+05	3.54E+03	9.74E+04	7.27E+04	2.04%					
746	9.19E+04	1.95E+03	5.32E+04	3.67E+04	2.12%					

ND= not detected (0 copies); CSF = cerebrospinal fluid.

Intact HIV IPDA was detected in seven of 11 CSF samples (median=1950 copies/10^6 cells, IQR= 287 – 6370) (**Figure 2A**) and in eight of eight PBMC samples (median=165.5 copies/10^6 PBMC, IQR= 8.31 – 578) (P=0.1 for comparison of detectability). Overall, the median proportions of intact (PBMC = 2.285%, CSF = 1.73%), Ψ+env- (PBMC = 57.26%, CSF = 56.31%), and Ψ-env+ HIV DNA (PBMC = 39.21%, CSF = 40.86%) were similar between compartments (**Figure 2B**).

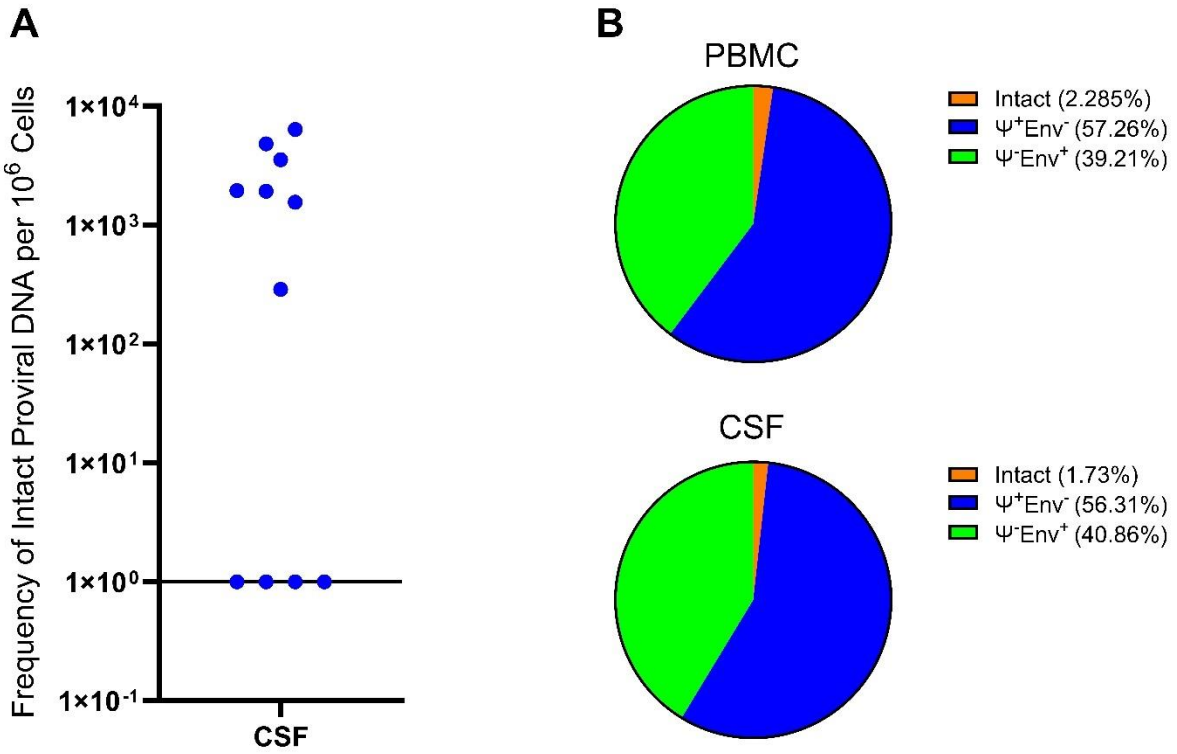

Figure 2 | Detection and Proportion of Intact Provirus

Intact proviral HIV DNA was detected in 7 of 11 people with HIV (PWH) in the cerebrospinal fluid (CSF) with a median of 1950 copies/10^6 cells (IQR = 287 – 6370). **B)** Median percentages of intact (peripheral blood mononuclear cells (PBMC) = 2.285%, CSF = 1.73%), Ψ+env- (PBMC = 57.26%, CSF = 56.31%), and Ψ-env+ HIV DNA (PBMC = 39.21%, CSF = 40.86%) were similar between PBMC from blood (top) and CSF (bottom).

For the eight matched pairs, median CSF intact IPDA was $9.19x10^2$ per 10^6 cells (IQR = 0 – $6.37 x10^3$) while median blood intact IPDA was $1.66x10^2$ per 10^6 cells (IQR= $8.13x10^0$ – $5.78x10^2$), trending higher in CSF (P = 0.0742). However, when all CSF samples were included, the median CSF IPDA was

$1.55x10^3$ (IQR= 0 – 6.37 $x10^3$), resulting in no significant difference from PBMC. In matched pairs, 3' defective (Ψ+env-) (median = 4.84 $x10^4$; IQR = 3.64 $x10^3$ – 3.05 $x10^5$) and 5' defective (Ψ-env+) (median = 4.45 $x10^4$; IQR = 558 – 7.93 $x10^4$) HIV DNA in the CSF was significantly higher than 3' defective (median = 2.70 $x10^3$; IQR = 1.06 $x10^3$ – 7.57 $x10^3$) and 5' defective (median = 1.36 $x10^3$; IQR = 302 – 4.47 $x10^3$) HIV DNA in PBMC (P=0.0039, 0.0039 respectively). This observation also occurred in unmatched pairs where 3' defective (median = 6.59 $x10^4$; IQR = 3.64 $x10^3$ – 3.45 $x10^5$) and 5' defective (median = 2.23 $x10^4$; IQR = 558 – 7.93 $x10^4$) HIV DNA in the CSF was significantly higher than 3' defective (median = 2.70 $x10^3$; IQR = 1.06 $x10^3$ – 7.57 $x10^3$) and 5' defective (median = 1.36 $x10^3$; IQR = 302 – 4.47 $x10^3$) HIV DNA in PBMC (P=0.0004, 0.0079 respectively). Frequency of 3' defective, 5' defective, and total HIV DNA were all significantly higher in CSF than PBMC in both unmatched (Wilcoxon rank sum, one-tailed, α=0.05) and matched pairs (Wilcoxon signed rank for matched pairs, one-tailed, α=0.05) (**Figure 3**).

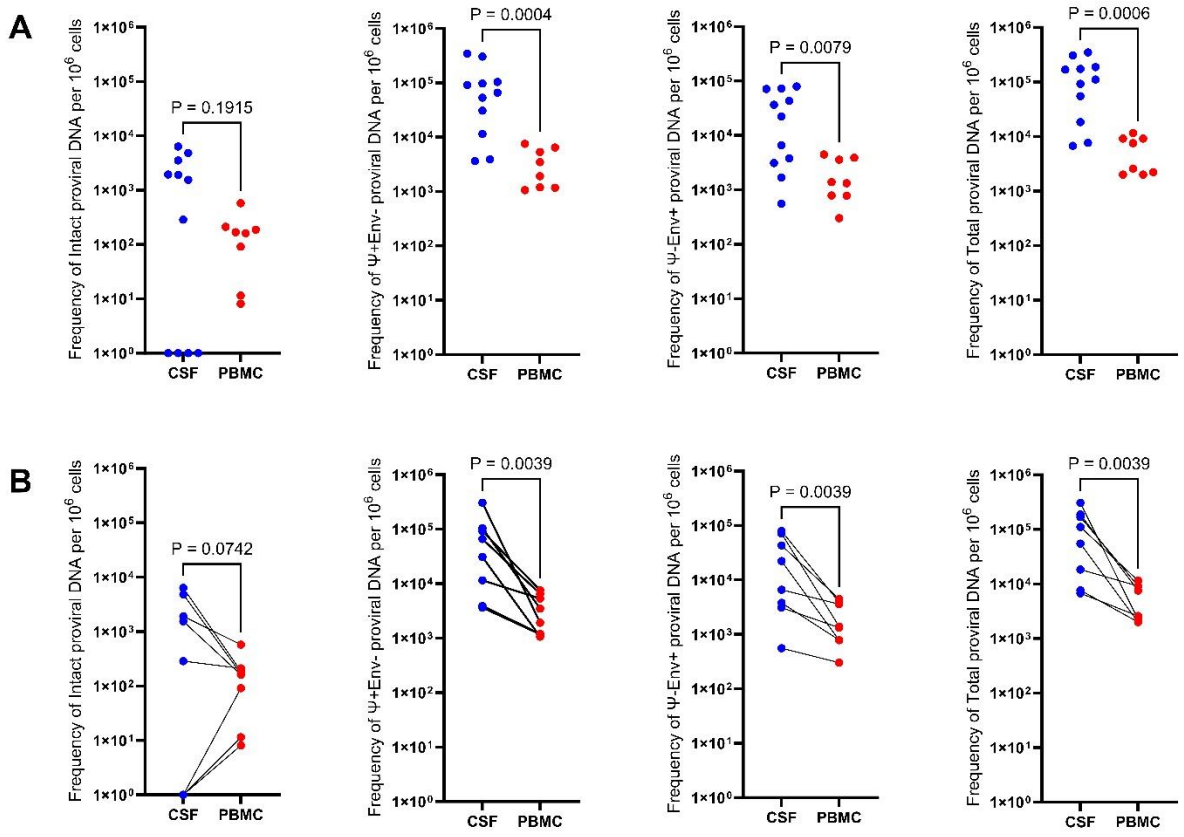

Figure 3 | Frequencies of Total, Defective, and Intact Provirus in the CSF and PBMC

Frequencies of total, 3' defective (Ψ+env-), and 5' defective (Ψ-env+) proviral HIV DNA were all significantly ($p<0.05$) higher in cerebrospinal fluid (CSF) than peripheral blood mononuclear cells (PBMC) in both **A)** unmatched pairs (CSF n=11, PBMC n=8) and **B)** matched pairs (n=8). Intact proviral DNA was trending toward higher in CSF than PBMC in matched pairs ($0.05<p<0.1$). Wilcoxon signed rank for unmatched pairs and Wilcoxon signed rank for matched pairs, one-tailed, comparisons performed with GraphPad Prism v10.0.2, $\alpha=0.05$.

Some but not all HIV DNA results correlated between blood and CSF. Specifically, intact IPDA correlated significantly between the two compartments (rho = 0.7075, P = 0.032), as did 5' defective HIV DNA (rho = 0.6905, P = 0.0347). Meanwhile, neither 3' defective HIV DNA (rho = 0.4286, P = 0.1496) nor total HIV DNA (rho = 0.2395, P = 0.2820) correlated significantly between compartments. With respect to virologic suppression, total HIV DNA tended to be higher (P = 0.0547 for CSF and P = 0.0705 for blood) in those virologically suppressed on ART versus those not receiving ART. 3' defective CSF HIV DNA levels were significantly lower in those on ART versus not receiving ART (P = 0.0284). For

neuropsychological performance, global and domains were examined. There was no significant difference in NPT-9 for those with detectable CSF IPDA versus undetectable CSF IPDA (P=0.5). There was no significant correlation between NPT-9 and levels of CSF HIV DNA (intact or defective, all P >0.2). However, higher CSF total HIV DNA tended to correlate with worse executive function. Specifically, a lower executive function T score was associated with both higher CSF 3' defective HIV DNA (rho = -0.63, P = 0.044) and higher CSF total HIV DNA (rho = -0.63, P = 0.044), (**Figure 4**).

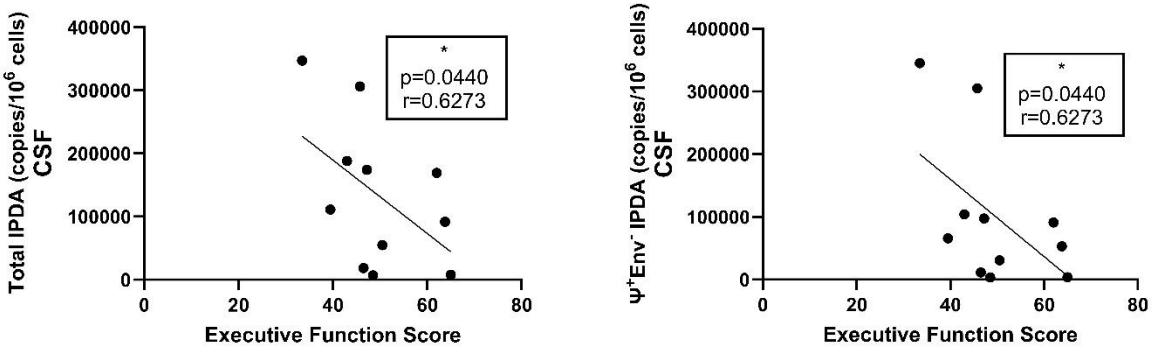

Figure 4 | Provirus in the CSF Correlates with Worse Executive Function

Executive function scores with practice in virally suppressed people with HIV negatively correlate to both total and 3' defective HIV proviral DNA copies per 10^6 cells in cerebrospinal fluid (CSF) (determined via intact proviral DNA assay (IPDA)). Correlations performed via Spearman's rho using Graphpad Prism v10.0.2 (α=0.05).

Discussion

Despite the successes of ART, HIV cure remains extremely difficult to achieve. HIV persists in the CNS, and clinical complications such as HIV-associated neurocognitive disorder (HAND) continue to occur.[33] Therefore, more research is needed to better understand the HIV CNS reservoir. The CSF is the most accessible part of the CNS. Previous studies have shown that HIV DNA is frequently detected from CSF cells and is associated with deleterious consequences such as cognitive impairment and neuronal damage.[20, 22] However, previously published studies did not measure IPDA in CSF. Therefore, it was

previously unclear if HIV DNA in CSF might be intact. The advent of IPDA has advanced HIV persistence research by offering scalability and accessibility advantages over existing approaches. Quantitative Viral Outgrowth Assay (QVOA) measures viral RNA and protein expression in latently infected T cells, but this assay is labor-intensive and requires significant cell numbers.[34] QVOA also may underestimate the size of the replication-competent virus reservoir, as single round T-cell stimulation does not reactivate the majority of latent proviruses *in vitro*.

The intact HIV proviral DNA assay (IPDA) was developed due to these logistical challenges of the QVOA.[31] Briefly, IPDA uses ddPCR and targets two regions of provirus: the Packaging Signal [Ψ] near the 5' end of the viral genome and the Rev Responsive Element (RRE) within the envelope (*env*) gene towards the 3' end. The assay defines intact proviruses by a lack of fatal defects, specifically large deletions in the 50 region of Ψ as well as APOBEC3G-mediated hypermutations or deletions in the RRE. This distinguishes genomically intact proviral sequences from defective ones.

Analysis of near full genome sequencing (nFGS) data in a previous study revealed that strategically positioned amplicons in the packaging signal (Ψ) and env regions collectively identified over 90% of deleted proviruses as defective. Identifying hypermutated proviruses is also crucial. Among these, 73% exhibit mutations in the GG→AG context. Additionally, most show GA→AA mutations. Only 27% of hypermutated proviruses exclusively display GA→AA mutations, with many also featuring deletions. Consequently, attention shifted to GG→AG hypermutation. Researchers pinpointed a conserved region in the Rev-response element (RRE) with neighboring consensus sites (TGGG) for the enzyme APOBEC3G. Among sequences with GG→AG hypermutation, 97% showcase one or more mutations in this region, spanning 13 distinct patterns. By utilizing mutant plasmids carrying each pattern, they developed allelic discrimination probes that accurately identify 95% of hypermutated sequences as defective.[31]

A recent study by Brad Jones's group highlighted that HIV sequence polymorphism might contribute to IPDA detection failure. To overcome this challenge, the group devised a secondary set of primers/probes to accommodate the diversity in HIV sequences. However, incorporating more than two

sets of primers/probes into the current QX200 Droplet Digital PCR system, which only features two fluorescence channels (FAM and HEX), poses a significant challenge due to the complexity of multiplexed digital PCR assays. [32]

The polymorphism results in oligonucleotide-template mismatches, leading to reduced fluorescent amplitude on 2D scatter plots. Nevertheless, in certain instances, specific mismatches can be tolerated. Unlike real-time PCR, which tracks the effectiveness of each amplification cycle, digital droplet PCR primarily focuses on generating quantitative data at the end-point of PCR amplification. Consequently, even lower amplitude signals can still be considered positive, provided they adequately separate from the negative cluster.

We acknowledge the necessity to conduct a comprehensive analysis covering both 5' and 3' hypermutation and deletion. Notably, analysis of nFGS data indicated a notably low frequency (<3.8%) of proviruses carrying defective DNA sequences on both IPDA-targeted amplicons (Ψ-env-).

In multiple studies, IPDA results from blood correlated significantly with QVOA results from blood.[31, 35] In the current study, our group used the IPDA to identify HIV DNA from cryopreserved CSF cell pellets and to differentiate intact versus defective virus. CSF HIV DNA was detectable in all samples. While this yield is higher than some published studies,[20, 21] newer technologies have yielded detectable CSF HIV DNA in upwards of 80% of PWH on ART,[22] meaning that this may become a more common finding with the development and refinement of technology. We acknowledge that some of the participants in the current study were either not on ART or were on ART <2 years. Multiple studies have shown that the HIV reservoir in the blood continues to decay years after ART initiation, including studies identifying slow decay in intact HIV DNA.[35-37] The same may be true for the CNS, such that our results may have been different if all participants were on suppressive ART and for a longer period of time. We found that total HIV DNA levels were higher in CSF compared to blood. This finding is consistent with other studies[20, 22] and may be due to the fact that CSF lymphocytes appear more likely to be positive for CCR5, the major co-receptor for HIV.[22] Therefore, these lymphocytes may be more susceptible to HIV

infection and the establishment of viral latency. In addition, most antiretrovirals have >10-fold less penetration into the brain, resulting in a sanctuary for HIV.[38] Though not statistically significant, we also found that IPDA was slightly less likely to be detected from CSF than from blood, but these levels tended to be higher from CSF than blood when detectable. These findings may be multifactorial. One reason for the lower detectability is the difference in sheer abundance of lymphocytes and other HIV-susceptible mononuclear cells in CSF compared to blood in PWH.[39] Also, the vast majority of lymphocytes in general do not harbor intact HIV.[40] Thus, there are fewer scenarios in which latently infected lymphocytes would be found. Notably, limiting dilution assays have validated that quantitation of HIV DNA may not be substantially distorted by the low numbers of cells analyzed.[10, 20] However, the amount of IPDA per cell might actually be higher in CSF given the enrichment of the CCR5 co-receptor among CSF lymphocytes. CSF lymphocytes in PWH are also more likely to be CXCR3+, especially in viremic individuals, again possibly increasing their susceptibility to HIV infection.[41, 42] CSF lymphocytes (and potentially monocytes and microglia-like cells that have been found in CSF) have many other differences compared to blood lymphocytes based on single cell transcriptomics.[43, 44]

We acknowledge that the current study had a small number of participants, and it is possible that the results may have differed with a larger cohort. We did not find significant associations with overall cognition (NPT-9), but the study was likely underpowered to detect significant associations in this regard. We did find a statistically significant correlation between higher 3' defective HIV DNA in the CSF and worse executive function. While not statistically significant (P=0.0562), we also observed a negative correlation between higher total and 3' defective HIV DNA in the CSF and learning scores. In comparison, we observed non-statistically significant (P=0.0595) negative correlations between learning and memory scores and total HIV DNA copies per million PBMC in the plasma (**Figure 5**), highlighting a potential link between HIV in the periphery and CNS.

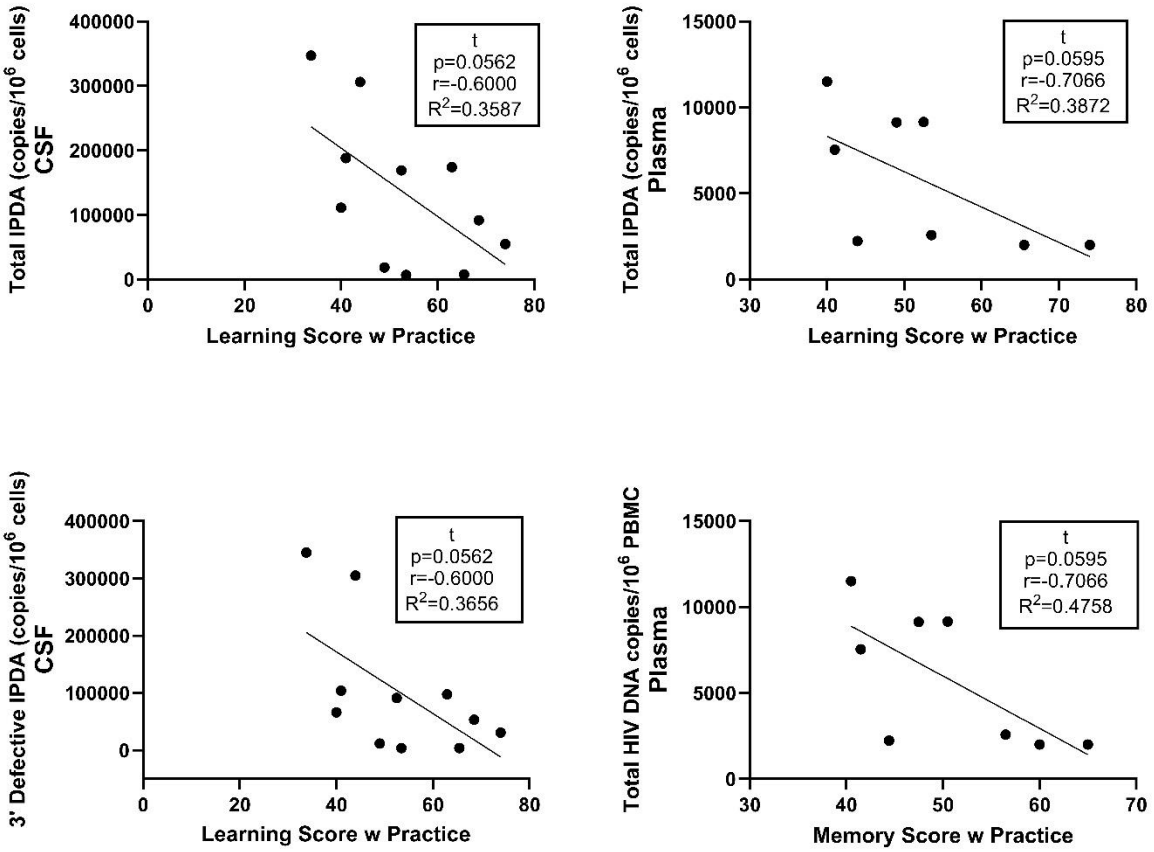

Figure 5 | Trending Correlations Between Provirus and Worse Learning and Memory

Learning scores with practice in virally suppressed people with HIV (PWH) trend (p=0.0562) toward negative correlation to both total and 3' defective HIV proviral DNA copies per 10^6 cells in cerebrospinal fluid (CSF) (determined via intact proviral DNA assay (IPDA)). Learning and memory scores with practice in virally suppressed PWH trend (p=0.0595) toward negative correlation to total HIV proviral DNA copies per 10^6 peripheral blood mononuclear cells (PBMC). Correlations performed via Spearman's rho using GraphPad Prism v10.0.2 (α=0.05).

It is possible that peripherally infected cells traffic into the CNS, infecting resident CNS cells to establish a viral reservoir that contributes to neurocognitive deficits (**Figure 6**). Given that defective HIV DNA can still be the source of viral proteins,[45] CNS HIV DNA may not need to be intact to be detrimental to cognition and other CNS functions. Lastly, the finding that intact and 5' defective CSF HIV DNA levels were higher among participants with virologic suppression is surprising. However, HIV reservoir size varies

widely among PWH.[46] Therefore, the current study may simply reflect that the participants on ART happened to have larger HIV reservoirs. Interestingly, the study of IPDA in human brain tissue showed that levels of intact HIV DNA tended to be higher in individuals on ART.[10]

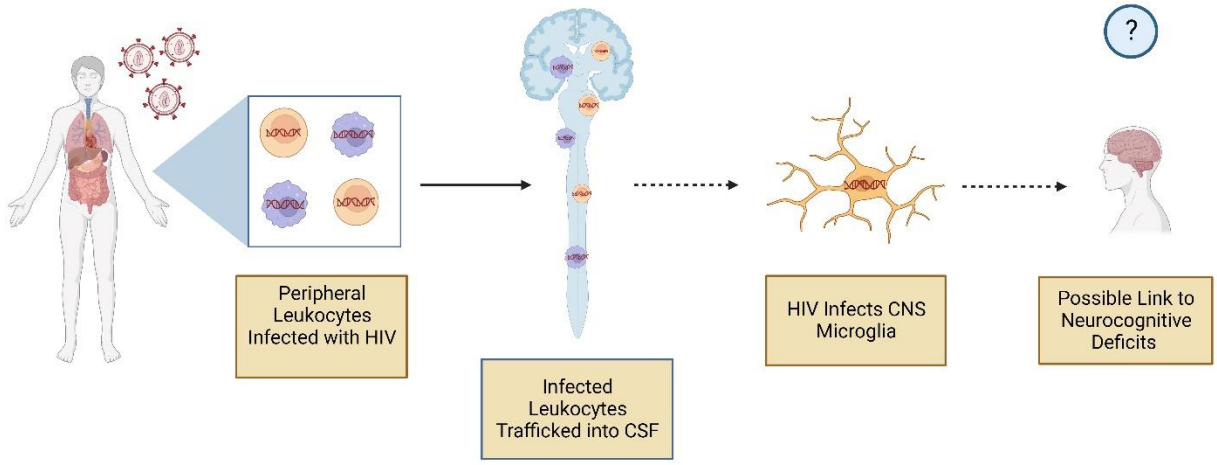

Figure 6 | Potential Route of CNS Reservoir Establishment

Proposed trafficking of peripherally infected cells into the central nervous system (CNS), infecting resident CNS cells and establishing the CNS as a viral reservoir which may lead to the development of neurocognitive deficits. Created with BioRender.

We acknowledge that IPDA has limitations as well. Recent studies have demonstrated that HIV sequence polymorphism may cause IPDA detection failure.[32] To address the detection failure, groups have designed secondary primers/probes to rescue the HIV sequence diversity. Due to the complexity of multiplexed digital PCR assays, however, introducing more than two sets of primers/probes to the current QX200 Droplet Digital PCR system can be challenging since the instrument only contains two fluorescence channels (FAM and HEX). The polymorphism causes oligonucleotides-template mismatch, which leads to reduced fluorescent amplitude on 2D scatter plots. However, in some cases, certain mismatches can be tolerated. Instead of tracking the effectiveness of each amplification cycle from real-time PCR, digital

droplet PCR focuses on generating quantitative data at the endpoint of PCR amplification. Therefore, the lower amplitude signal can still be scored as positive, provided it sufficiently partitions from the negative cluster. Other refinements of the approach may be beneficial. One potential refinement will be the use of high-throughput CSF techniques such as fluorescence-activated cell sorting (FACS).[39, 47] This may allow for the identification of small cellular subsets that harbor the highest frequency of IPDA.

The current study provides further evidence that the CNS is an HIV reservoir and provides a rationale for larger studies comparing replication competent virus across compartments to fully elucidate the role of each compartment and its contribution to neurocognitive impairment in PWH.

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Chapter III: Ruxolitinib-Mediated HIV-1 Reservoir Decay in A5336 Phase 2a Trial

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Author Contributions

CG, VCM, JJL, and CM developed the concept and designed the study. MDR, RMR, SPR, and CM verified data and performed statistical analyses. SPR and RPS provided input on statistical analyses. DAK designed flow cytometry panels. EW and RRT executed and analyzed flow cytometry panels. MDR performed flow cytometry gating. ZZ and JY performed molecular assays including IPDA and Alu PCR. MDR and RMR performed data visualization. MDR wrote the initial draft of the manuscript. All authors provided critical comments and editing, contributed to data interpretation, reviewed analyses of this manuscript, and approved its final version.

Conflict of Interest

VCM has received investigator-initiated research grants (to the institution) and consultation fees from Eli Lilly, Bayer, Gilead Sciences, Merck, and ViiV. VCM has received funding support from the Emory Center for AIDS Research (P30AI050409) for work related to this manuscript. Emory University and Christina Gavegnano are co-inventors for the method of use of Jak inhibitors for retroviral and other viral infections.

Abstract

We evaluated ruxolitinib's impact on the peripheral HIV-1 reservoir and immunomodulatory events driving persistence in people with HIV-1 (PWH) enrolled in ACTG A5336, an open-label randomized Phase 2a multi-site clinical trial. Participants (18-75 years old, on ART ≥2 years, virologically suppressed, $CD4^+$ count >350 cells/mm^3, and without significant comorbidities except HIV or hypertension) were randomized to ruxolitinib plus ART (n=40) or ART alone (n=20) from week 0 through 5, and observed through week 12. Cellular markers, integrated DNA, and IPDA were measured peripherally at weeks 0, 5, and 12. Reservoir markers decayed in high baseline reservoir (HBR) participants on

ruxolitinib by week 12 *versus* controls (p=0.0471). Cellular markers altered by ruxolitinib and associated with decay included pSTAT5+, pSTAT3+, BCL-2+KI67+, CD127+, and CD25+. We predict 99.99% decay in 2.83 years among HBR. These data are foundational for future human trials with Jak 1/2 inhibitors towards HIV-1 elimination. Clinical Trials Registration NCT02475655.

Introduction

Antiretroviral therapy (ART) is the cornerstone of disease management for people with HIV (PWH).[1] ART has been instrumental in HIV treatment and prevention averting 20.8 million AIDS-related deaths as of 2023.[2] However, lifelong adherence to ART has incentivized the search for a cure for HIV. Previous studies found that ART initiation within 30 days of HIV infection improves the magnitude of reservoir decay by limiting reservoir establishment.[3-5] However, ART does not target the HIV reservoir, which is the largest barrier to curing HIV and there are no currently approved interventions that fill this gap. This leaves an unmet need for therapies capable of targeting the HIV reservoir in 39 million PWH globally.[2]

Several Jak inhibitors are FDA approved for various indications. Ruxolitinib is a first-generation Jak 1/2 selective inhibitor that is approved for treatment of primary myelofibrosis as well as post-polycythemia vera and post-essential thrombocythemia myelofibrosis [Jakafi package insert, Incyte]. Prior FDA approval is advantageous when examining a new indication due to the existing foundation of safety and pharmacokinetic data, saving time in advancing a therapy towards clinical use. The Jak STAT pathway is chronically activated in PWH, including those virally suppressed by ART.[6-11] This is due to receptor engagement (virus-host or cytokine interactions) which recruits Jaks and initiates the Jak STAT pathway, ultimately resulting in production of pro-reservoir maintenance factors.[12] Systemic inflammation from Jak STAT activation not only drives reservoir establishment, maintenance, and expansion as reported by our group and others[13-16] but also comorbidities such as cardiovascular disease and neurocognitive dysfunction.[12, 17]

The primary analysis of the A5336 phase 2a, multi-site, randomized clinical trial investigated the impact of ruxolitinib on immune activation and primary safety events.[6] It was determined that ruxolitinib significantly decreased sCD14, as well as reduced activated CD4+ T cells and BCL-2 expression.[6] In this analysis, we sought to examine the effect of ruxolitinib on the HIV reservoir and determine alterations in cellular biomarker expression to elucidate an immunological mechanism.

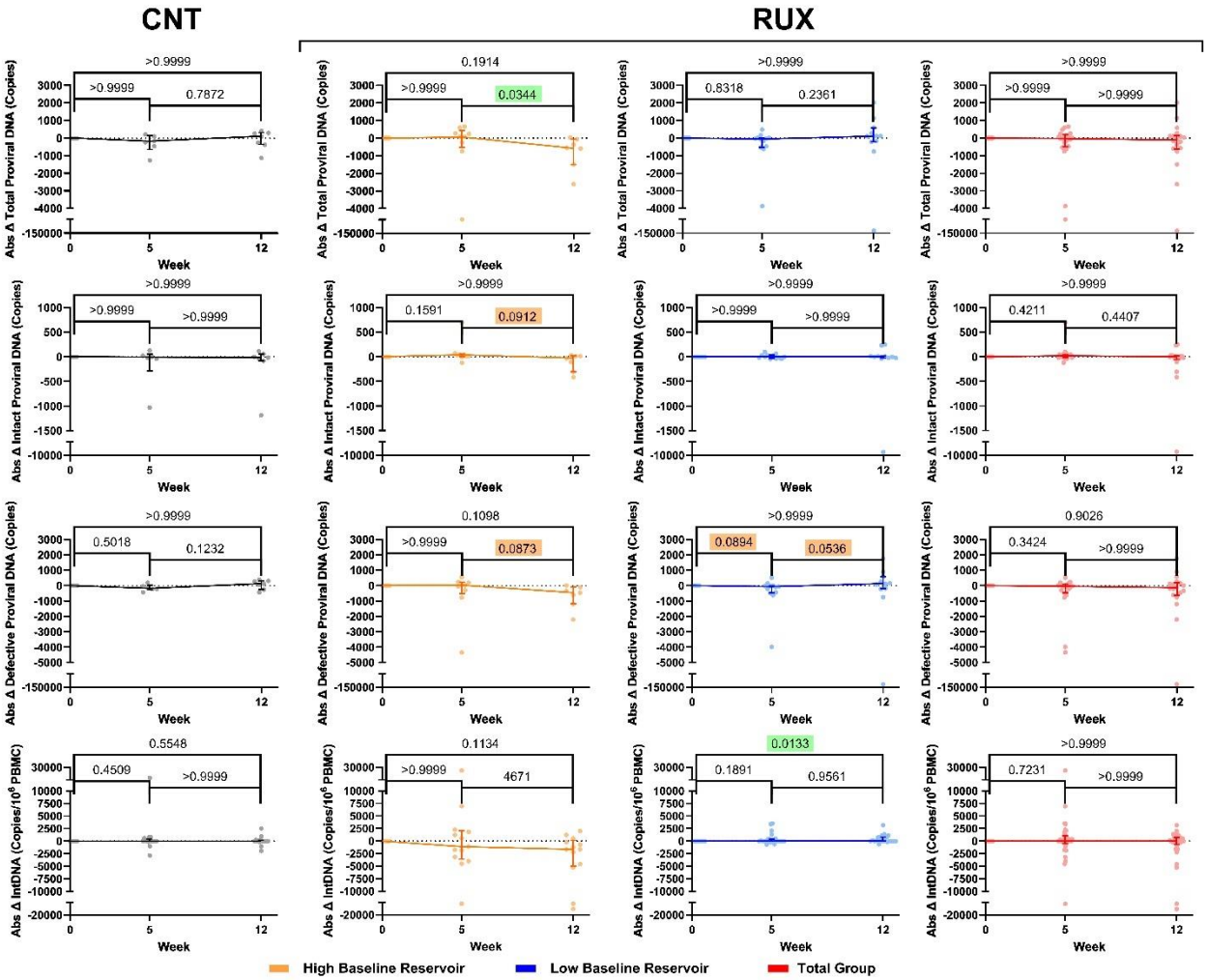
In this study, we discovered that Jak 1/2 selective inhibitor ruxolitinib causes immunomodulation resulting in reduction of systemic inflammation, immune activation, and viral persistence factors resulting ultimately in viral reservoir decay.

Results

Reservoir Modulation

We calculated the absolute change in the HIV reservoir in both methods (Alu PCR and intact proviral DNA assay [IPDA]) and plotted these longitudinally by CNT and RUX groups, with sub-distinctions of total group and high/low baseline reservoir (**Supplemental Fig. 1**). Within RUX, we observed a significant decrease in the absolute change from baseline of total proviral HIV DNA (IPDA) from weeks 5-12 (RUX high reservoir p=0.0344, CNT p=0.7872) and an overall decrease approaching significance from weeks 0-12 (RUX high reservoir p=0.1914, CNT p>0.9999) in participants that had a high baseline reservoir compared to overall CNT. Within the RUX high baseline reservoir subset, we observed a trending decrease in absolute change from weeks 5-12 in intact (p=0.0912) and defective HIV DNA (IPDA) (p=0.0873) (**Supplemental Fig. 1**). In examining potential decay from baseline to week 12, we found that there is not a significant decrease overall for intact HIV DNA (p>0.9999) but there was a trending decrease for defective HIV DNA (p=0.1098). We observed a significant increase in RUX low baseline integrated HIV DNA (Alu PCR) from baseline to week 12 (p=0.0342) compared to CNT which

was paired with a trending increase in defective DNA from baseline to week 5 (p=0.0894) and weeks 5-12 (p=0.0536) but not overall from baseline to week 12 (**Supplemental Fig. 1**).



Supplemental Figure 1 | Changes in HIV-1 Reservoir Over Time

Median absolute change (Abs D) with interquartile range (IQR) in total, intact, and 3'/5' defective proviral DNA copies per million PBMC (quantified via integrated proviral DNA assay [IPDA]) and total integrated HIV DNA (quantified via Alu PCR) between control (CNT) and ruxolitinib (RUX) study arms with subgroups of total or high/low baseline reservoir within the RUX arm longitudinally over the 12-week course of this study. Comparisons were calculated via non-parametric Kruskal-Wallis with Dunn's multiple comparisons test (a=0.05) using GraphPad Prism v9.5.1. RUX data: integrated HIV DNA total (n=31-36), high baseline reservoir (n=11-13), low baseline reservoir (n=20-23); IPDA total (n=22-26), high baseline reservoir (n=8-11), low baseline reservoir (n=14-16). CNT data: integrated HIV DNA (n=17-18), IPDA (n=8-9). There was no significant change in CNT. Abs D in total proviral DNA (IPDA) during weeks 5-12 significantly decreased (p=0.0344) in RUX high baseline reservoir. There was a trending decrease in PWH with a high baseline reservoir from weeks 5-12 in intact (p=0.0912) and defective (p=0.0873) proviral DNA (IPDA). In PWH with a low baseline reservoir, Abs D in integrated HIV DNA (Alu PCR) increased from

baseline to week 12 (p=0.0133), which was coupled with trending increases in defective proviral DNA (IPDA) from baseline to week 5 (p=0.0894) and weeks 5-12 (p=0.0536).

To increase rigor and confirm repeatability, we sought a second perspective and found that, through mixed-model analysis of raw total proviral HIV DNA (IPDA), the participants in RUX with a high baseline reservoir experienced decay compared to CNT from weeks 5-12 (p=0.0185) and weeks 0-12 (p=0.0260) with a non-significant increase from weeks 0-5 (p=0.7100), lending strength to our original observation (**Fig. 1A**). There was a significant difference in integrated DNA (Alu PCR) decay from week 0 to 12 between control and participants on RUX with a high baseline reservoir (p=0.0295), however, changes from weeks 0-5 (p=0.1167) and weeks 5-12 (p=0.5891) were not significant.

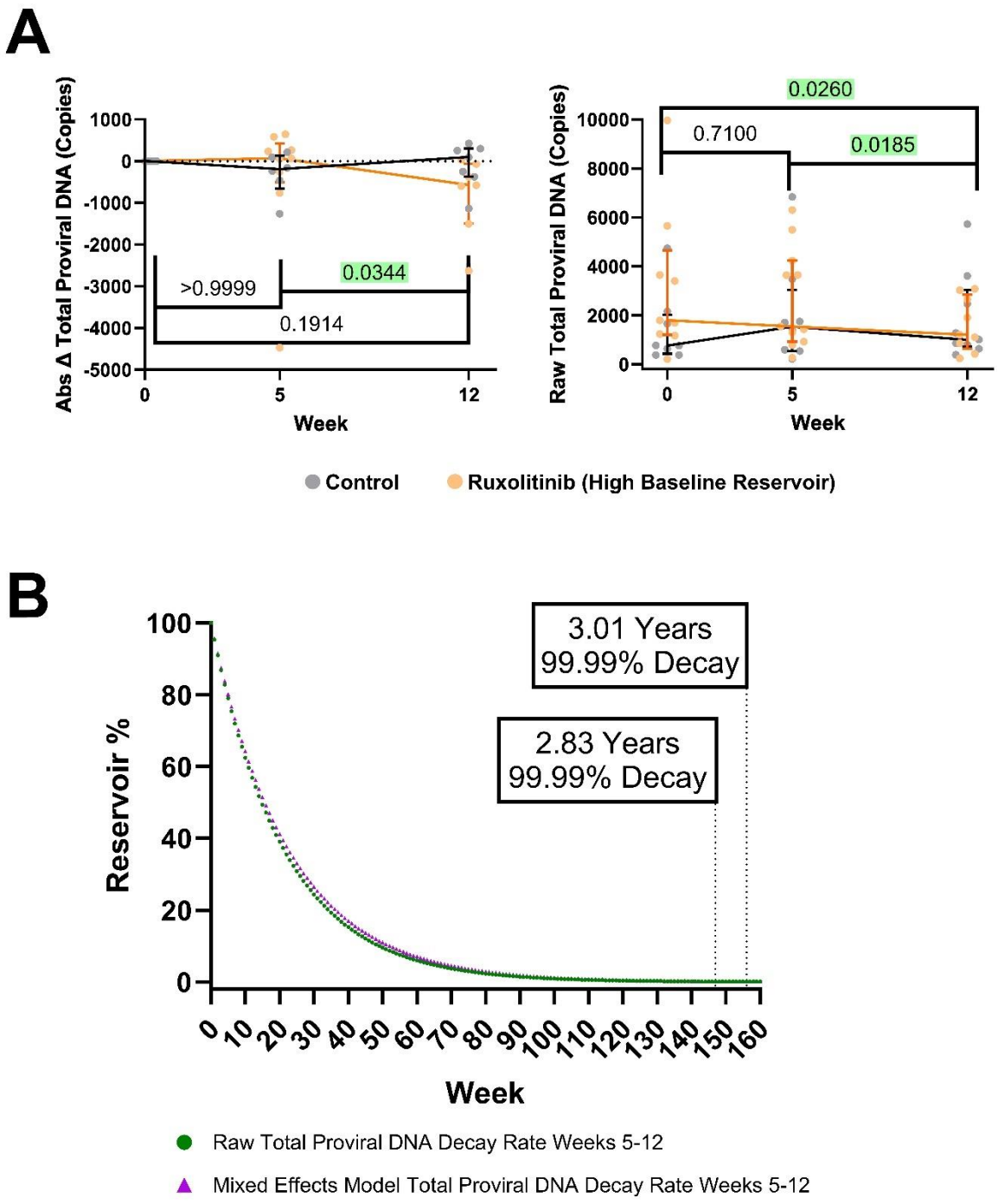
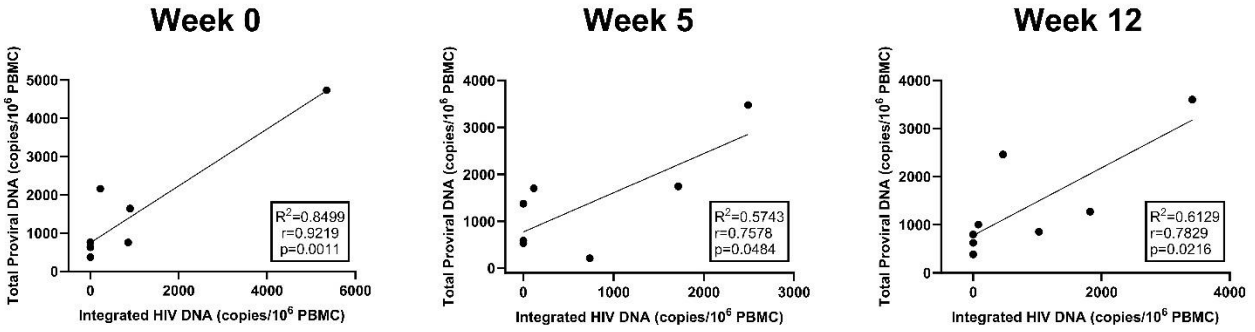


Figure 1 | HIV-1 Reservoir Observed and Predicted Decay

A) Left) Absolute change (Abs D) median with interquartile range (IQR) in total proviral DNA (IPDA) copies per million PBMC between control (CNT, n=17-18) and ruxolitinib (RUX) (high baseline reservoir participants, n=8-11) arms longitudinally over the 12-week course of this study. Comparisons calculated via Kruskal-Wallis with Dunn's multiple comparisons test (GraphPad Prism v9.5.1, a=0.05). A significant reduction in HIV-1 reservoir in participants with high baseline reservoir in response to ruxolitinib treatment

over control was observed from weeks 5-12 (p=0.0344). **Right)** Raw total proviral DNA (IPDA) copies per million PBMC between CNT (n=) and RUX (n=) arms longitudinally. Comparisons were calculated via mixed effects model (lmerTest 3.1-3 with R version 4.2.2 in RStudio v. 2023.12.0.369, a=0.05). A significant reduction in HIV-1 reservoir in participants with high baseline reservoir in response to ruxolitinib treatment over control was observed (weeks 0-12 p=0.0260, weeks 5-12 p=0.0185). Transient T cell demargination in the treatment group at week 5 did not significantly increase measured reservoir. **B)** Model of reservoir decay prediction based on exponential decay rate of total proviral DNA (IPDA) copies per million PBMC, from weeks 5-12, from raw (green circle) and mixed effects model (purple triangle). The reservoir decays sequentially based on decay rate (raw = 4.59% per week, mixed effects model = 4.33%) resulting in a near complete (99.99%) decay of the HIV-1 reservoir in 2.83-3.01 years when treated with ruxolitinib.

We performed Spearman rho correlations (a=0.05) with linear regression trendline generation between reservoir measurements obtained via IPDA (total proviral DNA) and Alu PCR (integrated DNA) within the CNT arm longitudinally across the study (**Supplemental Fig. 2**) (all timepoints, R^2>0.57). We found a strong positive correlation at each study week, indicating that both methods are valid and comparable. We acknowledge a discrepancy in reservoir changes between the two methods. However, a general decay is visible in the high baseline reservoir participants on RUX and this finding is conserved between methods. Furthermore, IPDA is known to be a highly sensitive assay in comparison to Alu PCR and was examined in 10 replicates for this study as opposed to triplicates in Alu PCR quantification. For these reasons, we have identified a significant reduction in HIV-1 reservoir in participants with high baseline reservoir in response to ruxolitinib treatment and continue with total proviral DNA determined by IPDA as the main reservoir quantification in subsequent analyses.



Supplemental Figure 2 | Alu PCR and IPDA Correlation

Spearman rho correlations (a=0.05) with linear regression trendline generation were performed between reservoir quantification methods (IPDA and Alu PCR) within the control arm longitudinally across the study. The two methods strongly correlated at weeks 0 (n=8, p=0.0011), 5 (n=7, p=0.0484), and 12 (n=8, p=0.0216), indicating that the two methods are viable and comparable.

To create a prediction model of decay, the mean percent change per week was calculated (weeks 0-12 -1.8869%, weeks 5-12 -4.5934%). A participant's reservoir baseline at time of treatment would be equivalent to 100% of reservoir. As such, we sequentially subtracted the percent change per week of remaining reservoir over time until 99.99% reservoir decay was reached. According to the week 5-12 decay rate, ruxolitinib would result in near complete reservoir decay in 2.83 years (147 weeks) (**Fig. 1B**). Using the week 0-12 decay rate results in near complete reservoir decay in 6.96 years (362 weeks). This model is based on the decay in total proviral DNA copies (IPDA) observed in the study for participants receiving ruxolitinib who began the trial with a high baseline reservoir. We also plotted the exponential decay from the decay rate (week 5 to 12) estimated with the mixed effects model which predicts near complete reservoir decay in 3.01 years (156 weeks) (**Fig. 1B**).

Cellular Biomarker Modulation

The analysis framework in this study consisted of three rounds of pairwise comparisons to elucidate altered expression from ruxolitinib treatment and the predictive profile of participants who responded to treatment. All pairwise comparisons were calculated via GraphPad Prism v9.5.1 using an unmatched Mann Whitney U Test (a=0.05) to account for biological variation and a Benjamini-Hochberg test (15%) to limit

the false discovery rate. These analyses were overlapped to determine the specific correlates of HIV-1 reservoir decay altered by ruxolitinib treatment.

23 cellular markers were measured as applicable in monocytes, B cells, CD4+ and CD8+ T cellular subsets (naïve (T_N), central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}), and terminally differentiated (T_{TD}).

Absolute change in each cellular marker was determined for each person from their own baseline at week 0 to weeks 5 and 12 of the study (**Supplemental Table 1**). Round 1 of analysis determined which markers were altered from baseline by RUX through comparing the change in marker expression within RUX at baseline and weeks 5 and 12 (**Fig. 2A, Supplemental Table 2**). Round 2 of analysis determined if altered expression in round 1 was significantly ($p<0.05$) or trending ($0.05<p<0.1$) toward differential from CNT via comparing expression change between RUX and CNT (**Fig. 2A, Supplemental Table 3**).

Supplemental Table 1 | Calculated Absolute Change from Baseline (Week 0) in Reservoir and Cellular Markers

https://doi.org/10.15139/S3/8YADL0

Abbreviations: RUX (ruxolitinib arm (n=40)), CNT (control arm (n=20)), IPDA ++ (intact provirus only), IPDA +- (total provirus), IPDA -- (defective provirus only), N (naïve), CM (central memory), TM (transitional memory), EM (effector memory), and TD (terminally differentiated). High and Low reservoir stratification was based on raw week 0 Alu-LTR qPCR reservoir measurements, where the top tertile of each group was defined as high baseline reservoir and the bottom 2/3s were defined as low baseline reservoir. (Hosted by Dataverse v5.9 build750-fb24c87).

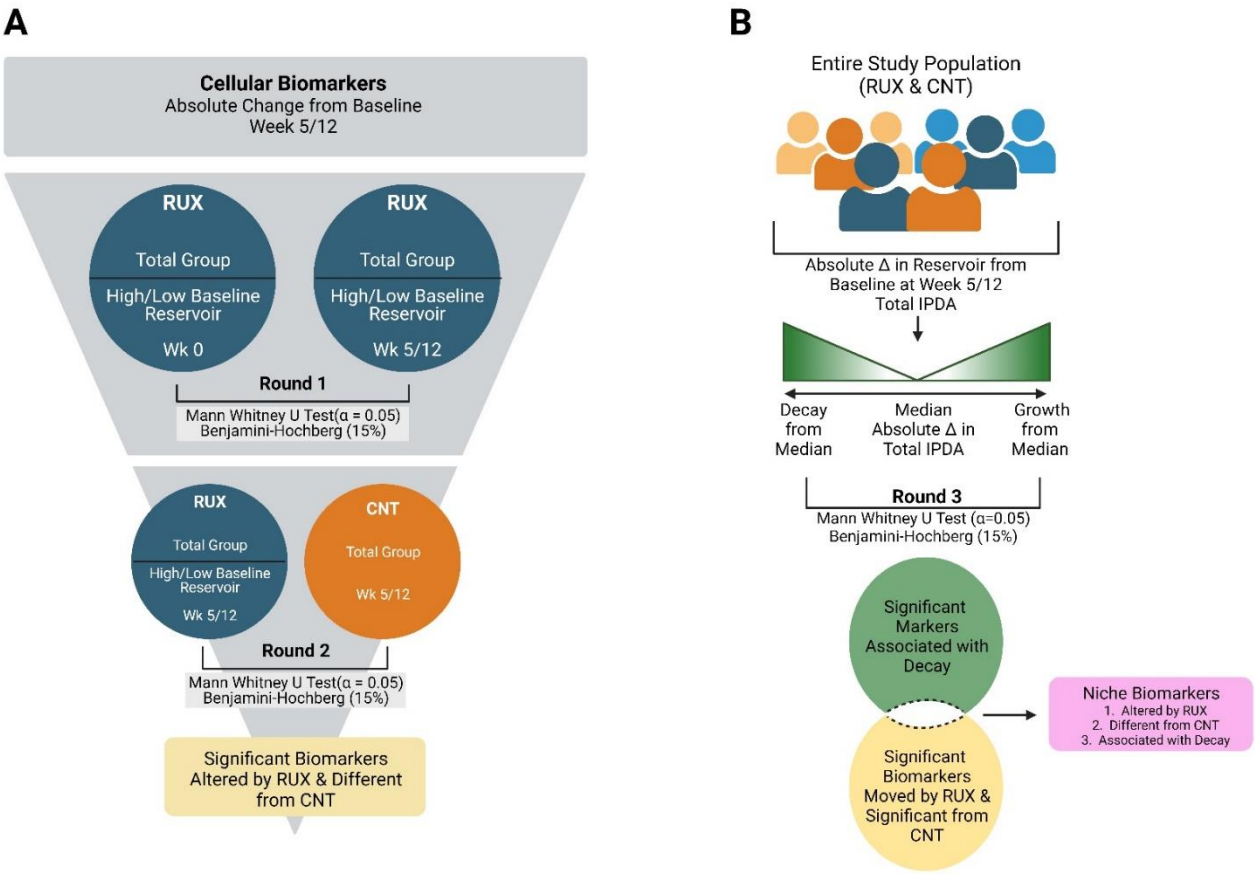


Figure 2 | Framework for Analysis of A5336

A) In round 1 of analysis, absolute change (Abs D) in cellular marker expression from week 0 to weeks 5 and 12 were compared within the ruxolitinib-treated (RUX) group, both as a total group (n=28-30) and with the distinction of high (n=11) or low baseline reservoir (n=17-19), to determine significantly altered biomarkers from treatment. In round 2 of analysis, the absolute change at weeks 5 (RUX n=29, CNT n=15) and 12 (RUX n=28, CNT n=15) (from baseline) was compared between the RUX and control (CNT) groups to determine if altered biomarkers were significantly different from control. **B)** In round 3 of analysis, independent of study arm, the median absolute change in total proviral DNA was determined (n=14) and patients were classified as experiencing either growth or decay from the median. When compared, correlates of reservoir decay, independent of treatment, were determined. Through overlap of markers identified in all 3 rounds, a highly niche group of markers were elucidated that are correlates of decay and are also impacted by ruxolitinib treatment. These pairwise comparisons were calculated using GraphPad Prism v9.5.1 via non-parametric Mann Whitney U test (a=0.05) to account for biological variability and a Benjamini-Hochberg test (15%) to limit the rate of false discovery. Created with BioRender.

Supplemental Table 2 | Cellular Markers Significantly Altered by Ruxolitinib from Baseline

Cell Type	Subset	Reservoir Classification	Week	Biomarker	P-Value
B Cell		Total Group	5	CD39	0.0026
				CD69	0.0066
				LAG3	0.0132
				CD38	0.0274
			12	CD39	0.0003
		High Baseline Reservoir	5	CD38	0.0054
			12	CD39	0.0054
				TIGIT	0.0054
		Low Baseline Reservoir	5	CD69	0.0118
				CD39	0.0268
			12	CD39	0.0219
				CD38	0.0467
Monocyte		Total Group	5	HLA-DR	0.0274
				CD95	0.0274
			12	KI67	0.0001
				PD1	0.0007
				CD69	0.0021
				pSTAT5	0.0089
				CD39	0.0274
				SLAM	0.0359
				BCL-2	0.0438
				CD127	0.0483
		High Baseline Reservoir	12	KI67	0.0054
				PD1	0.0054
				SLAM	0.0404
		Low Baseline Reservoir	12	KI67	0.0025
				CD69	0.0026
				pSTAT5	0.0058
				pSTAT3	0.0433
				TIGIT	0.0467
				PD1	0.0467
CD4+ T Cell	N	Total Group	5	TIGIT	0.0062
				BCL-2/KI67	0.0192
				CD95	0.0274
				PD-L1	0.0407
		High Baseline Reservoir	5	CD127	0.0054
				CD25	0.0095
				TIGIT	0.0351
			12	pSTAT5	0.0351
				SLAM	0.0404
		Low Baseline Reservoir	5	BCL-2/KI67	0.0342
				PD-L1	0.0479
	CM	Total Group	5	HLA-DR	0.0001
				CD127	0.0027
				CD25	0.0131
				CD38	0.0274
				CD39	0.0494
			12	CD25	0.0001

				CTLA-4	0.0081
				HLA-DR	0.0111
				KI67	0.0111
				TIGIT	0.0319
				CD39	0.0438
				CD127	0.0438
				CD28	0.0439
				PD1	0.0440
		High Baseline Reservoir	5	CD25	0.0052
				BTLA	0.0054
				pSTAT3	0.0095
			12	CD62L	0.0054
				CD39	0.0054
				CTLA-4	0.0404
		Low Baseline Reservoir	5	HLA-DR	0.0001
				CD127	0.0037
			12	CD25	0.0001
				KI67	0.0026
				CD95	0.0026
				PD1	0.0026
				HLA-DR	0.0214
	TM	Total Group	5	CD69	0.0001
				HLA-DR	0.0001
				CD127	0.0001
				CD25	0.0066
				BCL-2/KI67	0.0201
				KI67	0.0274
				SLAM	0.0274
			12	BTLA	0.0111
				CD127	0.0439
		High Baseline Reservoir	5	CD69	0.0002
				HLA-DR	0.0002
				CD39	0.0002
				CD25	0.0054
				CD127	0.0054
				pSTAT5	0.0164
			12	CD38	0.0054
				CD62L	0.0054
				TIGIT	0.0404
		Low Baseline Reservoir	5	CD127	0.0037
			12	BTLA	0.0026
	EM	Total Group	5	CD127	0.0026
				CD25	0.0248
				CD69	0.0274
				SLAM	0.0494
			12	LAG3	0.0225
				CD127	0.0438
				HLA-DR	0.0439
		High Baseline Reservoir	5	CD69	0.0054
				CD39	0.0054
				SLAM	0.0054
			12	CD39	0.0002
				CD62L	0.0054

		Low Baseline Reservoir	5	CD127	0.0037
			12	CD39	0.0269
				KI67	0.0390
				TIGIT	0.0393
	TD	Total Group	5	PD1	0.0114
				CD28	0.0221
			12	CD127	0.0217
		High Baseline Reservoir	5	PD1	0.0054
			12	CD62L	0.0164
		Low Baseline Reservoir	12	CD39	0.0048
CD8+ T Cell	N	Total Group	5	CD127	0.0009
				KI67	0.0220
				SLAM	0.0362
			12	PD-L1	0.0219
				PD1	0.0219
		High Baseline Reservoir	5	CD127	0.0054
				PD1	0.0054
				PD-L1	0.0164
			12	PD-L1	0.0002
				PD1	0.0002
		Low Baseline Reservoir	5	SLAM	0.0479
			12	CD38	0.0219
				BTLA	0.0219
	CM	Total Group	5	CD127	0.0001
				HLA-DR	0.0012
				CD69	0.0132
				BCL-2/KI67	0.0407
				CD28	0.0489
			12	CD39	0.0111
				TIGIT	0.0194
				pSTAT3	0.0237
				BCL-2	0.0438
				CD28	0.0438
		High Baseline Reservoir	5	CD127	0.0054
			12	BCL-2	0.0054
		Low Baseline Reservoir	5	CD127	0.0003
				CD28	0.0037
				HLA-DR	0.0121
				PD-L1	0.0224
			12	TIGIT	0.0017
				CD39	0.0215
	TM	Total Group	5	CD38	0.0004
				pSTAT1	0.0060
				HLA-DR	0.0065
				CD25	0.0131
				CD69	0.0274
				PD1	0.0489
				SLAM	0.0494
			12	CTLA-4	0.0051
				HLA-DR	0.0438
				CD38	0.0440
		High Baseline Reservoir	5	CD69	0.0054

				CD25	0.0054
				HLA-DR	0.0054
				pSTAT1	0.0095
			12	CTLA-4	0.0124
				PD-L1	0.0266
		Low Baseline Reservoir	5	CD38	0.0037
				CD28	0.0136
	EM	Total Group	5	CD38	0.0001
				HLA-DR	0.0001
				CD127	0.0001
				CD69	0.0065
				PD1	0.0131
				CD25	0.0431
			12	HLA-DR	0.0021
				CD38	0.0111
				CTLA-4	0.0374
				CD69	0.0438
		High Baseline Reservoir	5	HLA-DR	0.0002
				CD127	0.0002
				CD38	0.0043
			12	CD38	0.0054
				CTLA-4	0.0266
				BCL-2	0.0404
		Low Baseline Reservoir	5	CD38	0.0001
				CD69	0.0121
				CD127	0.0270
			12	HLA-DR	0.0219
				CD62L	0.0467
	TD	Total Group	5	CD127	0.0001
				CD38	0.0012
				HLA-DR	0.0065
				PD1	0.0248
				CD28	0.0489
			12	CD38	0.0003
				HLA-DR	0.0021
				CD28	0.0021
				BTLA	0.0440
		High Baseline Reservoir	5	HLA-DR	0.0054
				CD127	0.0054
			12	HLA-DR	0.0002
				CD28	0.0002
				BTLA	0.0054
		Low Baseline Reservoir	5	CD127	0.0037
				CD38	0.0121
				CD28	0.0270
				KI67	0.0479
			12	CD38	0.0025
				LAG3	0.0219

Complete list of cellular markers that were found to be significantly altered by ruxolitinib treatment from baseline (Round 1) using non-parametric Mann Whitney U tests (a=0.05) with a Benjamini-Hochberg adjustment for a 15% false discovery rate (GraphPad Prism v9.5.1). Subset abbreviations: N (naïve), CM (central memory), TM (transitional memory), EM (effector memory), and TD (terminally differentiated).

Supplemental Table 3 | Pairwise Comparisons of Markers Between Arms

Cell Type	Subset	Reservoir Classification	Week	Biomarker	P-Value
B Cell		Total Group	5	CD69	0.0286
		High Baseline Reservoir	12	PD1	0.0550*
		Low Baseline Reservoir	5	CD69	0.0323
Monocyte		Total Group	5	CD69	0.0657*
				pSTAT5	0.0864*
			12	CD69	0.0263
				CD28	0.0681*
				pSTAT5	0.0937*
		High Baseline Reservoir	5	HLA-DR	0.0274
				CD69	0.0609*
		Low Baseline Reservoir	12	CD127	0.0083
				CD69	0.0177
				pSTAT5	0.0380
				pSTAT3	0.0656*
CD4+ T Cell	N	Total Group	5	CD127	0.0285
				BCL-2/KI67	0.0584*
			12	CTLA-4	0.0412
		Low Baseline Reservoir	5	BCL-2/KI67	0.0741*
	CM	Total Group	5	CD127	0.0097
			12	CTLA-4	0.0720
		Low Baseline Reservoir	5	CD127	0.0247
			12	CTLA-4	0.0832
	TM	Total Group	5	CD127	0.0090
			12	LAG3	0.0121
				CD95	0.0164
				PDL1	0.0949*
		High Baseline Reservoir	5	CD127	0.0229
			12	TIGIT	0.0542*
		Low Baseline Reservoir	5	CD127	0.0297
			12	pSTAT5	0.0575*
	EM	Total Group	5	CD28	0.0684*
				BCL-2/KI67	0.0718*
			12	KI67	0.0672*
				CD95	0.0864*
		High Baseline Reservoir	5	CD28	0.0109
				CD39	0.0130
		Low Baseline Reservoir	12	TIGIT	0.0606*
				CD25	0.0772*
	TD	Total Group	5	CD38	0.0567*
CD8+ T Cell	N	Total Group	5	SLAM	0.0419
				KI67	0.0889*
		High Baseline Reservoir	5	KI67	0.0565*
		Low Baseline Reservoir	5	SLAM	0.0512*
	CM	Total Group	12	pSTAT3	0.0911*
		High Baseline Reservoir	12	CD38	0.0590*
				BCL-2	0.0797*
		Low Baseline Reservoir	12	TIGIT	0.0629*

	TM	Total Group	5	CD39	0.0549*
				CD25	0.0964*
			12	CD38	0.0373
		High Baseline Reservoir	12	CTLA-4	0.0537*
				CD38	0.0609*
	EM	Total Group	5	CD127	0.0085
				HLA-DR	0.0365
				CD25	0.0376
		High Baseline Reservoir	5	HLA-DR	0.0237
		Low Baseline Reservoir	5	CD127	0.0514*
			12	CD62L	0.0743*
	TD	Total Group	5	KI67	0.0088
				CD95	0.0246
				CD127	0.0761*
				CD25	0.0950*
			12	CD127	0.0377
				CD25	0.0973*
		High Baseline Reservoir	5	HLA-DR	0.0973*
			12	HLA-DR	0.0774*
		Low Baseline Reservoir	5	KI67	0.0118
				CD127	0.0830*

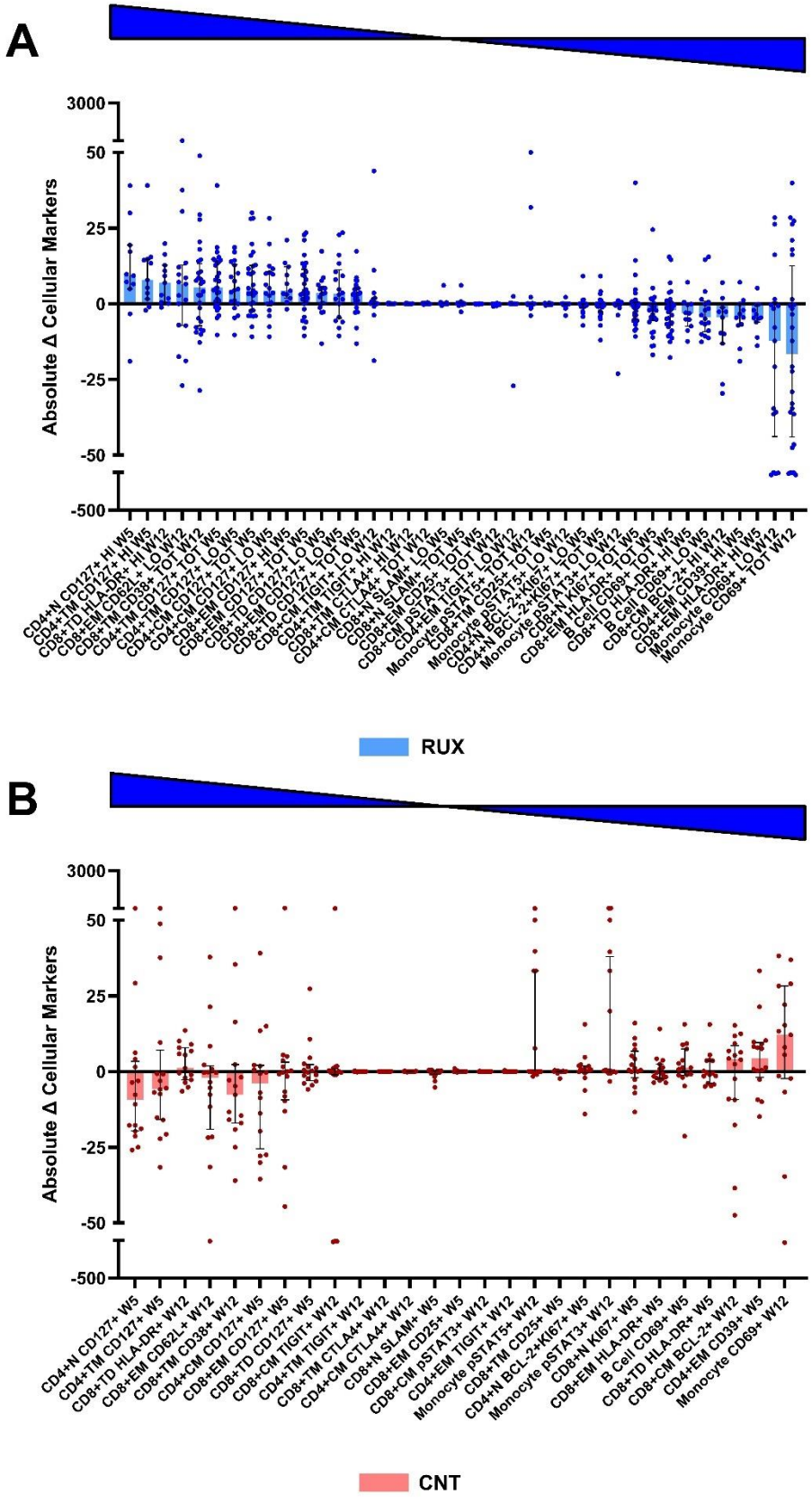
Complete list of significant ($p<0.05$) and trending ($0.05<p<0.10$) cellular biomarkers that were found to have a different expression phenotype from control (Round 2) using non-parametric Mann Whitney U tests ($a=0.05$) with a Benjamini-Hochberg adjustment for a 15% false discovery rate (GraphPad Prism v9.5.1). Subset abbreviations: N (naïve), CM (central memory), TM (transitional memory), EM (effector memory), and TD (terminally differentiated). * denotes trending p-values.

Through overlap of rounds 1 AND 2, markers altered by ruxolitinib treatment compared to control were identified (**Table 1**). The expression profile of identified markers (weeks 5 and 12 markers pooled) was opposite from control when treated with ruxolitinib (**Supplemental Fig. 3**).

Table 1 | Ruxolitinib-Altered Markers over Control

Cell Type	Subset	Reservoir Classification	Week	Biomarker	Round 1 P-Value	Round 2 P-Value	Change from CNT
B Cell		Total Group	5	CD69	0.0066	0.0286	↓
		Low Baseline Reservoir	5	CD69	0.0118	0.0323	↓
Monocyte		Total Group	12	pSTAT5	0.0089	0.0937*	↓
				CD69	0.0021	0.0263	↓
		Low Baseline Reservoir	12	pSTAT5	0.0058	0.0380	↓
				CD69	0.0026	0.0177	↓
				pSTAT3	0.0433	0.0656*	↓
CD4+ T Cell	N	Total Group	5	BCL-2/KI67	0.0192	0.0584*	↓
		Low Baseline Reservoir	5	BCL-2/KI67	0.0342	0.0741*	↓
	CM	Total Group	5	CD127	0.0027	0.0097	↑
			12	CTLA-4	0.0081	0.0720*	↑
		Low Baseline Reservoir	5	CD127	0.0037	0.0247	↑
	TM	Total Group	5	CD127	0.0001	0.0090	↑
		High Baseline Reservoir	5	CD127	0.0054	0.0229	↑
		Low Baseline Reservoir	5	CD127	0.0037	0.0297	↑
	EM	High Baseline Reservoir	5	CD39	0.0054	0.0130	↓
CD8+ T Cell	N	Total Group	5	KI67	0.0220	0.0889*	↓
	CM	Total Group	12	pSTAT3	0.0237	0.0911*	↓
		High Baseline Reservoir	12	BCL-2	0.0054	0.0797*	↓
		Low Baseline Reservoir	12	TIGIT	0.0017	0.0629*	↑
	TM	Total Group	5	CD25	0.0131	0.0964*	↓
		High Baseline Reservoir	12	CTLA-4	0.0124	0.0537*	↑
	EM	Total Group	5	HLA-DR	0.0001	0.0365	↓
				CD127	0.0001	0.0085	↑
		High Baseline Reservoir	5	HLA-DR	0.0002	0.0237	↓
		Low Baseline Reservoir	5	CD127	0.0270	0.0514*	↑
	TD	Total Group	5	CD127	0.0001	0.0761*	↑
		High Baseline Reservoir	5	HLA-DR	0.0054	0.0973*	↓
			12	HLA-DR	0.0002	0.0774*	↑
		Low Baseline Reservoir	5	CD127	0.0037	0.0830*	↑

Identified biomarkers both altered by intervention and differential expression from control with associated p-value per round (Mann Whitney U tests, a=0.05, *denotes $0.05<p<0.1$). Abbreviations: CNT (control group), N (naïve), CM (central memory), TM (transitional memory), EM (effector memory), and TD (terminally differentiated).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50
25
0
-25
-50
-500
CD4+N CD127+ HI W5
CD4+TM CD127+ HI W5
CD8+TD HLA-DR+ HI W5
CD8+EM CD62L+ LO W12
CD8+TM CD38+ TOT W12
CD4+TM CD127+ TOT W12
CD4+CM CD127+ TOT W5
CD4+CM CD127+ LO W5
CD8+EM CD127+ LO W5
CD8+EM CD127+ HI W5
CD8+TEM CD127+ LO W5
CD8+TD CD127+ LO W5
CD8+CM TIGIT+ TOT W5
CD4+TM TIGIT+ HI W12
CD4+CM CTLA4+ TOT W12
CD8+N SLAM+ LO W5
CD8+EM CD25+ TOT W5
CD8+CM pSTAT3+ TOT W12
Monocyte pSTAT5+ LO W12
CD4+EM TIGIT+ TOT W12
CD8+TM CD25+ TOT W5
CD4+N BCL-2+KI67+ LO W5
Monocyte pSTAT3+ LO W12
CD8+N KI67+ TOT W5
CD8+EM HLA-DR+ TOT W5
B Cell CD69+ TOT W5
CD8+TD HLA-DR+ HI W5
CD8+CM BCL-2+ LO W5
CD4+EM CD39+ HI W12
CD8+EM HLA-DR+ HI W5
Monocyte CD69+ LO W12
Monocyte CD69+ TOT W12
RUX
B
Absolute Δ Cellular Markers
3000
50
25
0
-25
-50
-500
CD4+N CD127+ W5
CD4+TM CD127+ W5
CD8+TD HLA-DR+ W12
CD8+EM CD62L+ W12
CD8+TM CD38+ W12
CD4+CM CD127+ W5
CD8+EM CD127+ W5
CD8+TD CD127+ W5
CD8+CM TIGIT+ W12
CD4+TM TIGIT+ W12
CD4+CM CTLA4+ W12
CD8+N SLAM+ W5
CD8+EM CD25+ W5
CD8+CM pSTAT3+ W12
CD4+EM TIGIT+ W12
Monocyte pSTAT5+ W12
CD8+TM CD25+ W5
CD4+N BCL-2+KI67+ W5
Monocyte pSTAT3+ W12
CD8+N KI67+ W5
CD8+EM HLA-DR+ W5
B Cell CD69+ W5
CD8+TD HLA-DR+ W5
CD8+CM BCL-2+ W12
CD4+EM CD39+ W5
Monocyte CD69+ W12
CNT

Supplemental Figure 3 | Ruxolitinib Reverses the Expression Phenotype of Cellular Biomarkers Compared to Control

A) The absolute change (Abs D) from baseline in cellular markers (median with interquartile range) arranged from most positive to most negative change (represented by blue bar at top) in the ruxolitinib-treated arm (blue, n=11-29). **B)** The absolute change from baseline in cellular markers arranged in the same order as A (represented by blue bar at top) in the control arm (red, n=15-16). All markers represented were significantly ($p<0.05$) altered by ruxolitinib (round 1 of analysis, Mann Whitney with 15% Benjamini-Hochberg) and significant or trending ($0.05<p<0.1$) towards differential expression from control (round 2 of analysis, Mann Whitney with 15% Benjamini-Hochberg). Abbreviations: week (W), naïve (N), central memory (CM), effector memory (EM), transitional memory (TM), terminally differentiated (TD), total ruxolitinib-treated group (TOT), high baseline reservoir group (HI), and low baseline reservoir group (LO).

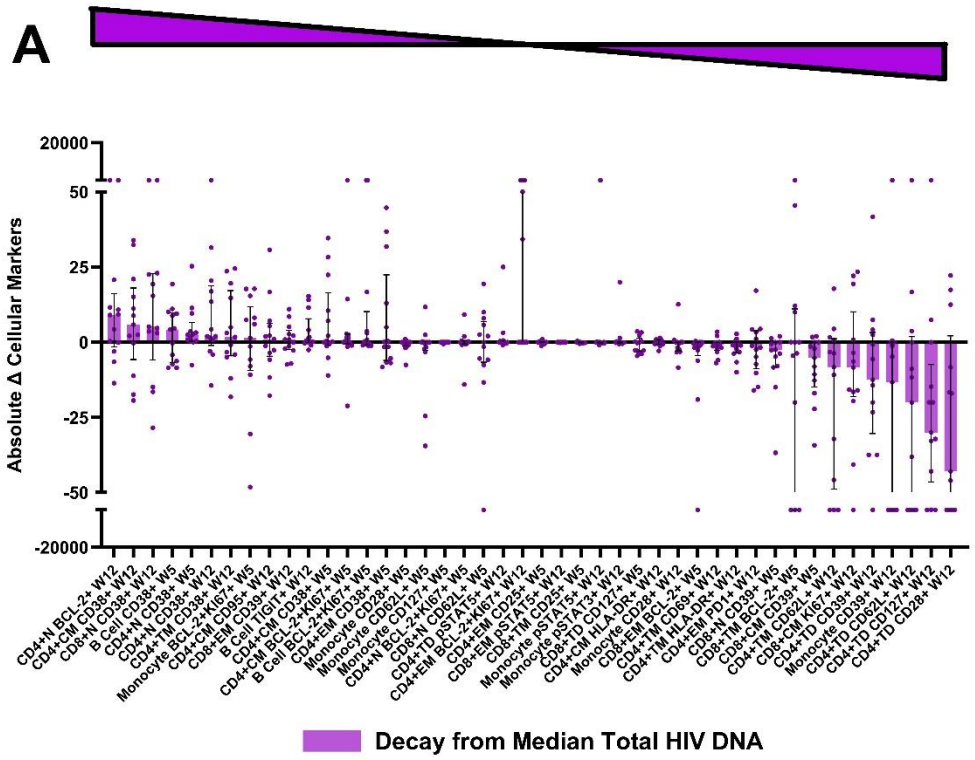
Round 3 of analysis sought to determine cellular marker correlates of reservoir decay independent of treatment. The absolute change in total proviral DNA copies (IPDA) from week 0 to weeks 5/12 were calculated for each individual where matched total proviral DNA (IPDA) measurements were acquired (n=14) (**Supplemental Table 4**). Limited IPDA acquisition was not attributable to samples below the limit of detection but rather limited sample quantity to perform IPDA. Of these 14 participants, the median absolute change in total proviral copies was determined at weeks 5/12 and the group was stratified as positive (reservoir growth, n=7) or negative (reservoir decay, n=7) change from the median. Growth and decay groups were analyzed pairwise, and correlates of reservoir decay were determined (**Fig. 2B, Supplemental Table 2**). The expression profile was opposite between growth and decay groups (**Supplemental Fig. 4**).

Supplemental Table 4 | Cellular Markers Associated with Reservoir Decay

Cell Type	Subset	Week	Biomarker	P-Value
B Cell		5	BCL-2/KI67	0.0350
			CD38	0.0882*
		12	TIGIT	0.0768*
Monocyte		5	CD28	0.0399
			CD127	0.0678*
			BCL-2/KI67	0.0723*
			CD62L	0.0838*

		12	pSTAT5	0.0388
			CD39	0.0523*
			pSTAT3	0.0699*
			CD28	0.0894*
CD4+ T Cell	N	5	CD38	0.0123
			BCL-2/KI67	0.0981*
		12	CD38	0.0153
			BCL-2	0.0523*
	CM	5	CD38	0.0008
			BCL-2/KI67	0.0906*
		12	CD38	0.0080
			CD95	0.0114
			HLA-DR	0.0893*
	TM	12	HLA-DR	0.0166
			CD69	0.0220
			CD38	0.0398
			CD62L	0.0457
	EM	5	CD38	0.0230
			CD25	0.0882*
		12	BCL-2/KI67	0.0127
			PD1	0.0868*
	TD	12	CD39	0.0047
			CD28	0.0188
			CD62L	0.0292
			pSTAT5	0.0400
			CD127	0.0441
CD8+ T Cell	N	5	CD39	0.0115
			CD62L	0.0440
		12	CD38	0.0083
	CM	5	CD39	0.0787*
		12	KI67	0.0474
	TM	5	BCL-2	0.0101
			CD25	0.0568*
	EM	5	BCL-2	0.0072
		12	CD39	0.0597*
			pSTAT5	0.0678*
	TD	5	CD127	0.0019

Complete list of significant ($p<0.05$) and trending ($0.05<p<0.10$) cellular biomarkers that were found to have a different expression phenotype between patients experiencing growth or decay from the median integrated proviral DNA (copies/10^6 PBMC) (Round 3) using non-parametric Mann Whitney U tests (a=0.05) with a Benjamini-Hochberg adjustment for a 15% false discovery rate (GraphPad Prism v9.5.1). Subset abbreviations: N (naïve), CM (central memory), TM (transitional memory), EM (effector memory), and TD (terminally differentiated). * denotes trending p-values.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Absolute Δ Cellular Markers
20000
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25
0
-25
-50
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CD4+N BCL-2+ W12
CD4+CM CD38+ W12
CD8+N CD38+ W12
B Cell CD38+ W12
CD4+N CD38+ W5
CD4+TM CD38+ W12
CD4+TM CD38+ W5
Monocyte BCL-2+KI67+ W5
CD4+CM CD95+ W12
CD8+EM CD95+ W12
B Cell TIGIT+ W12
CD4+CM CD38+ W5
B Cell BCL-2+KI67+ W5
CD4+EM CD38+ W5
Monocyte CD28+ W5
Monocyte CD62L+ W5
Monocyte CD127+ W5
CD4+N BCL-2+KI67+ W5
CD8+N CD62L+ W5
CD4+TD pSTAT5+ W12
CD4+EM BCL-2+KI67+ W12
CD8+EM pSTAT5+ W12
CD8+TM CD25+ W5
Monocyte pSTAT5+ W12
Monocyte pSTAT3+ W12
CD8+TD CD127+ W5
CD4+CM HLA-DR+ W12
Monocyte CD28+ W12
CD8+EM BCL-2+ W5
CD4+TM CD69+ W12
CD4+TM HLA-DR+ W12
CD4+EM PD1+ W12
CD8+N CD39+ W5
CD8+TM BCL-2+ W5
CD4+CM CD39+ W5
CD8+CM CD62L+ W12
CD8+CM KI67+ W12
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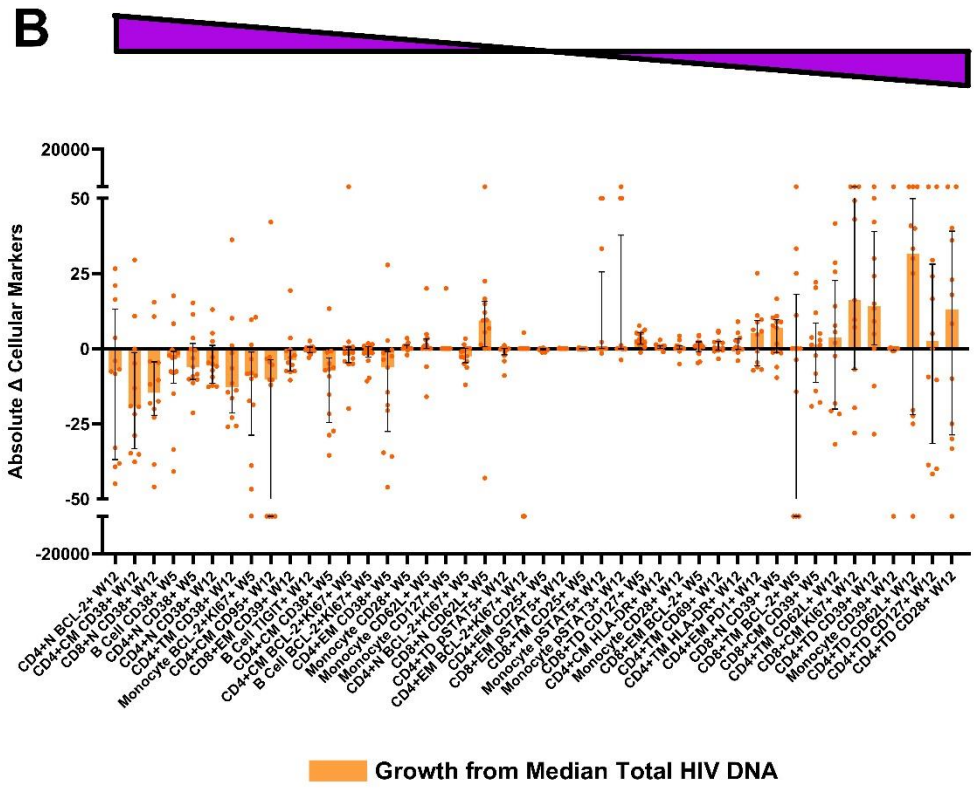
Monocyte CD62L+ W12
CD4+TD CD127+ W12
CD4+TD CD28+ W12
Decay from Median Total HIV DNA


B
Absolute Δ Cellular Markers
20000
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25
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CD4+N BCL-2+ W12
CD4+CM CD38+ W12
CD8+N CD38+ W12
B Cell CD38+ W5
CD4+N CD38+ W5
CD4+TM CD38+ W12
Monocyte BCL-2+KI67+ W5
CD4+TM CD38+ W5
CD4+CM CD95+ W12
CD8+EM CD95+ W12
B Cell TIGIT+ W12
CD4+CM CD38+ W5
B Cell BCL-2+KI67+ W5
CD4+EM CD38+ W5
Monocyte CD28+ W5
Monocyte CD62L+ W5
Monocyte CD127+ W5
CD4+N BCL-2+KI67+ W5
CD8+N CD62L+ W5
CD4+TD pSTAT5+ W12
CD4+EM BCL-2+KI67+ W12
CD8+EM pSTAT5+ W12
CD8+TM CD25+ W5
Monocyte pSTAT5+ W12
Monocyte pSTAT3+ W12
CD8+TD CD127+ W5
CD4+CM HLA-DR+ W12
Monocyte CD28+ W12
CD8+EM BCL-2+ W5
CD4+TM CD69+ W12
CD4+EM PD1+ W12
CD8+N CD39+ W5
CD8+TM BCL-2+ W5
CD8+CM CD39+ W5
CD4+TM CD62L+ W12
CD8+CM KI67+ W12
CD4+TD CD39+ W12
Monocyte CD39+ W12
Monocyte CD62L+ W12
CD4+TD CD127+ W12
CD4+TD CD28+ W12
Growth from Median Total HIV DNA

Supplemental Figure 4 | Cellular Marker Expression of Reservoir Decay is Opposite of Growth

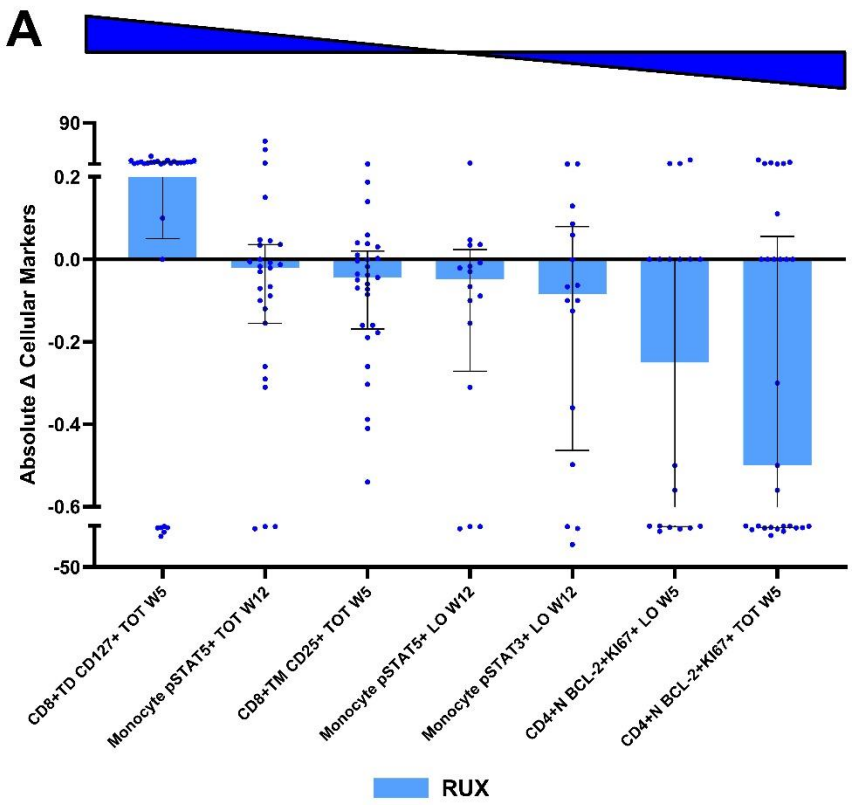
A) The absolute change (Abs D) from baseline in cellular markers (median with interquartile range) arranged from most positive to most negative change (represented by purple bar at top) in participants experiencing reservoir decay (purple, n=13). **B)** The absolute change from baseline in cellular markers arranged in the same order as A (represented by purple bar at top) in participants experiencing reservoir growth (orange, n=11-13). All markers represented were significantly ($p<0.05$) and trending ($0.05<p<0.1$) towards differential expression between the decay and growth groups (round 3 analysis, Mann Whitney with 15% Benjamini-Hochberg). Abbreviations: week (W), naïve (N), central memory (CM), effector memory (EM), transitional memory (TM), and terminally differentiated (TD).

To determine niche biomarkers (ruxolitinib-altered marker expression and association with reservoir decay), we compared biomarkers from each round of analysis and noted overlap between rounds 1 AND 2 AND 3. When assessing significance of these biomarkers, all reported markers in round 1 were statistically significant ($p<0.05$) whereas rounds 2 and 3 accepted biomarkers that were statistically significant and trending ($0.05<p<0.10$). This resulted in a list of 5 niche biomarkers with an opposite expression profile between trial arms (**Table 2, Supplemental Fig. 5**). Longitudinally, ruxolitinib decreased expression of pSTAT3/5 in monocytes, BCL-2/KI67 in CD4+ T_N cells, and CD25 in CD8+ T_{TM} cells, and increased expression of CD127 in CD8+ T_{TD} cells (**Fig. 3**). We examined these niche markers at baseline by reservoir growth or reservoir decay groups as described above. This informed us of the potential to use each marker as a screening tool based on differential baseline expression and we found that PWH having higher expression of pSTAT5 in monocytes and lower expression of BCL-2/KI67 in CD4+ T_N cells were likely to experience reservoir decay (**Fig. 3**).

Table 2 | Niche Ruxolitinib-Altered Markers Associated with Reservoir Decay

Cellular Subset and Biomarker	Round 1 p-Value	Round 2 p-Value	Round 3 p-Value
Monocyte pSTAT5+ (Low Baseline Reservoir Week 12)	0.0058	0.0380	0.0388
Monocyte pSTAT5+ (Total Week 12)	0.0089	0.0937*	0.0388
CD4+ T_N Cell BCL-2+/KI67+ (Total Week 5)	0.0192	0.0584*	0.0981*
CD4+ T_N Cell BCL-2+/KI67+ (Low Baseline Reservoir Week 5)	0.0342	0.0741*	0.0981*
CD8+ T_{TD} Cell CD127+ (Total Week 5)	0.0001	0.0761*	0.0019
CD8+ T_{TM} Cell CD25+ (Total Week 5)	0.0131	0.0964*	0.0568*
Monocyte pSTAT3+ (Low Baseline Reservoir Week 12)	0.0433	0.0656*	0.0699*

Identified niche biomarkers altered by intervention and associated with HIV-1 reservoir day with associated p-value per round (Mann Whitney U tests, a=0.05, *denotes trending $0.05<p<0.1$). Subset abbreviations: N (naïve), CM (central memory), TM (transitional memory), EM (effector memory), and TD (terminally differentiated).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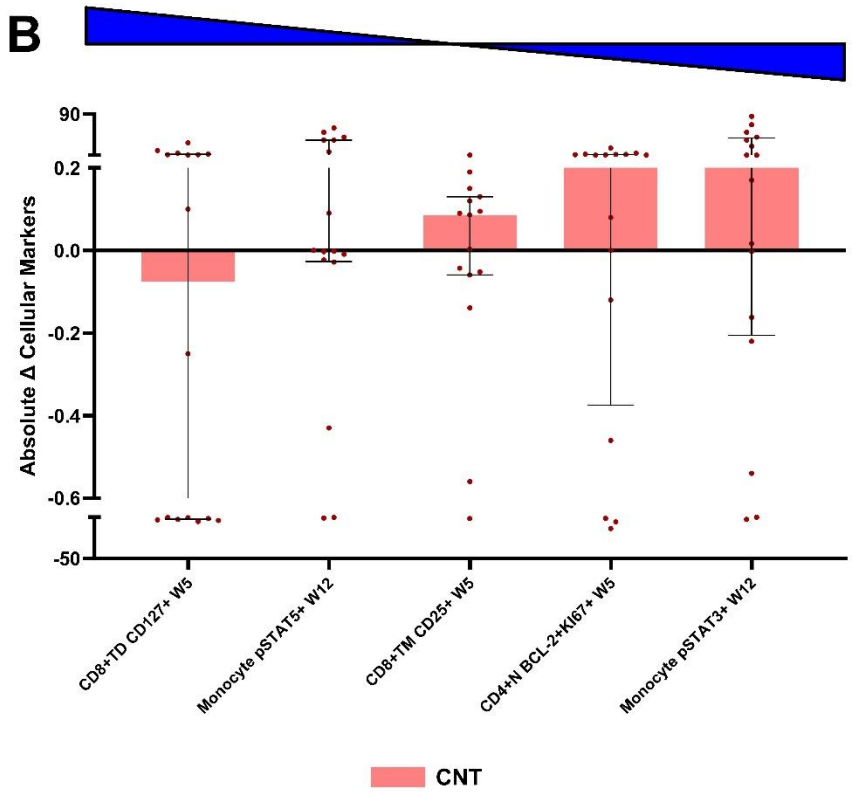
0.2
0.0
-0.2
-0.4
-0.6
-50
Absolute Δ Cellular Markers
CD8+TD CD127+ TOT W5
Monocyte pSTAT5+ TOT W12
CD8+TM CD25+ TOT W5
Monocyte pSTAT5+ LO W12
Monocyte pSTAT3+ LO W12
CD4+N BCL-2+KI67+ LO W5
CD4+N BCL-2+KI67+ TOT W5
RUX


B
90
0.2
0.0
-0.2
-0.4
-0.6
-50
Absolute Δ Cellular Markers
CD8+TD CD127+ W5
Monocyte pSTAT5+ W12
CD8+TM CD25+ W5
CD4+N BCL-2+KI67+ W5
Monocyte pSTAT3+ W12
CNT

Supplemental Figure 5 | Niche Cellular Marker Expression is Reversed with Ruxolitinib Compared to Control

A) The absolute change (Abs D) from baseline in cellular markers (median with interquartile range) arranged from most positive to most negative change (represented by blue bar at top) in the ruxolitinib-treated arm (blue, n=16-29). **B)** The absolute change from baseline in cellular markers arranged in the same order as A (represented by blue bar at top) in the control arm (red, n=15-16). All markers represented were significantly ($p<0.05$) altered by ruxolitinib (round 1 of analysis, Mann Whitney with 15% Benjamini-Hochberg), significant or trending ($0.05<p<0.1$) towards differential expression from control (round 2 of analysis, Mann Whitney with 15% Benjamini-Hochberg), and significant or trending towards differential expression between participants experiencing reservoir decay vs growth (round 3 of analysis, Mann Whitney with 15% Benjamini-Hochberg). Abbreviations: week (W), naïve (N), central memory (CM), effector memory (EM), transitional memory (TM), terminally differentiated (TD), total ruxolitinib-treated group (TOT), high baseline reservoir group (HI), and low baseline reservoir group (LO).

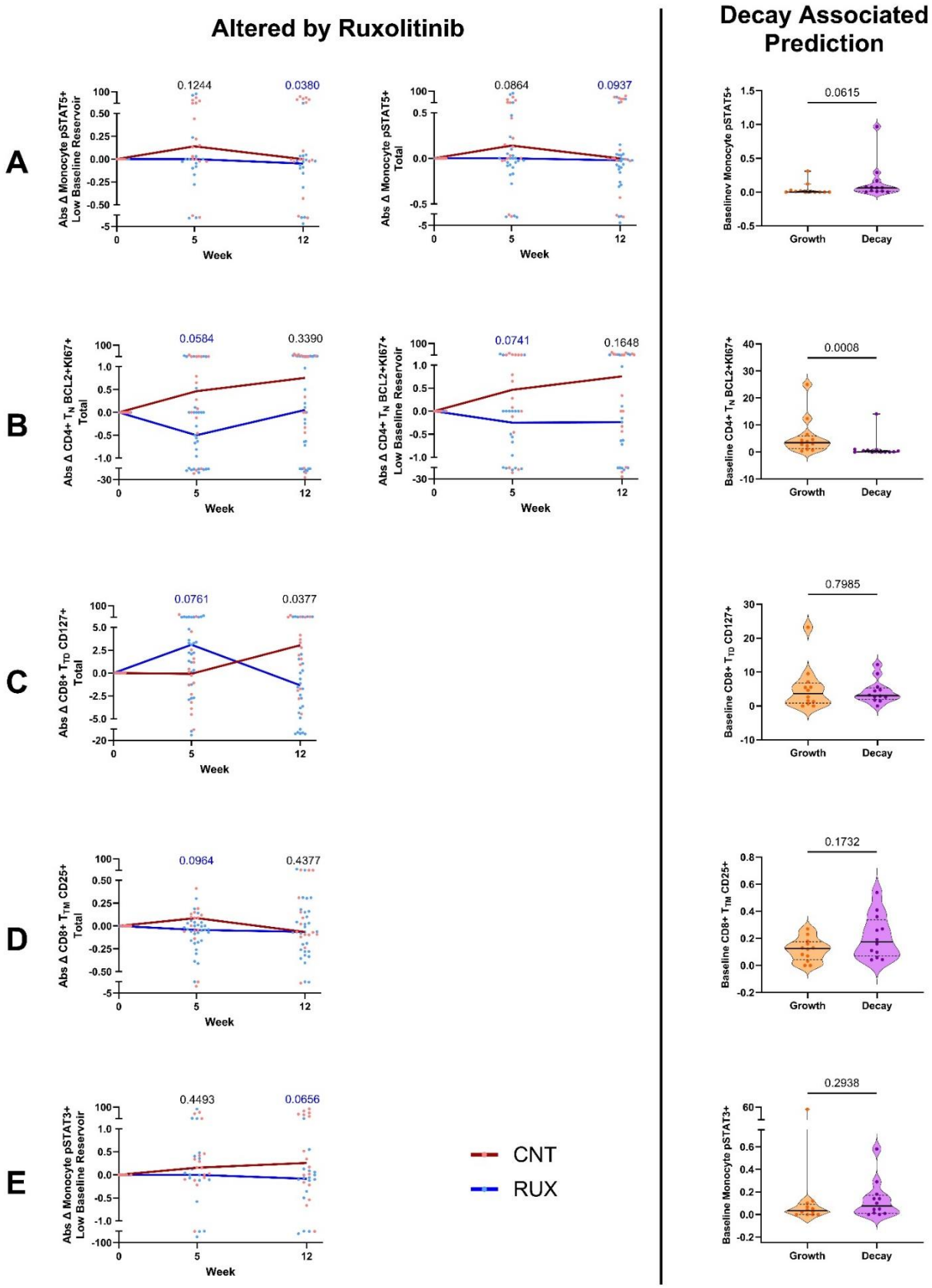

Altered by Ruxolitinib
Decay Associated Prediction
A
B
C
D
E
Abs Δ Monocyte pSTAT5+ Low Baseline Reservoir
Abs Δ Monocyte pSTAT5+ Total
Abs Δ CD4+ T_N BCL2+KI67+ Total
Abs Δ CD4+ T_N BCL2+KI67+ Low Baseline Reservoir
Abs Δ CD8+ T_TD CD127+ Total
Abs Δ CD8+ T_TM CD25+ Total
Abs Δ Monocyte pSTAT3+ Low Baseline Reservoir
Week
0.1244
0.0380
0.0864
0.0937
0.0584
0.3390
0.0741
0.1648
0.0761
0.0377
0.0964
0.4377
0.4493
0.0656
Baselinev Monocyte pSTAT5+
Baseline CD4+ T_N BCL2+KI67+
Baseline CD8+ T_TD CD127+
Baseline CD8+ T_TM CD25+
Baseline Monocyte pSTAT3+
0.0615
0.0008
0.7985
0.1732
0.2938
Growth
Decay
CNT
RUX

Figure 3 | Niche Marker Longitudinal Expression and Predictive Association with Decay

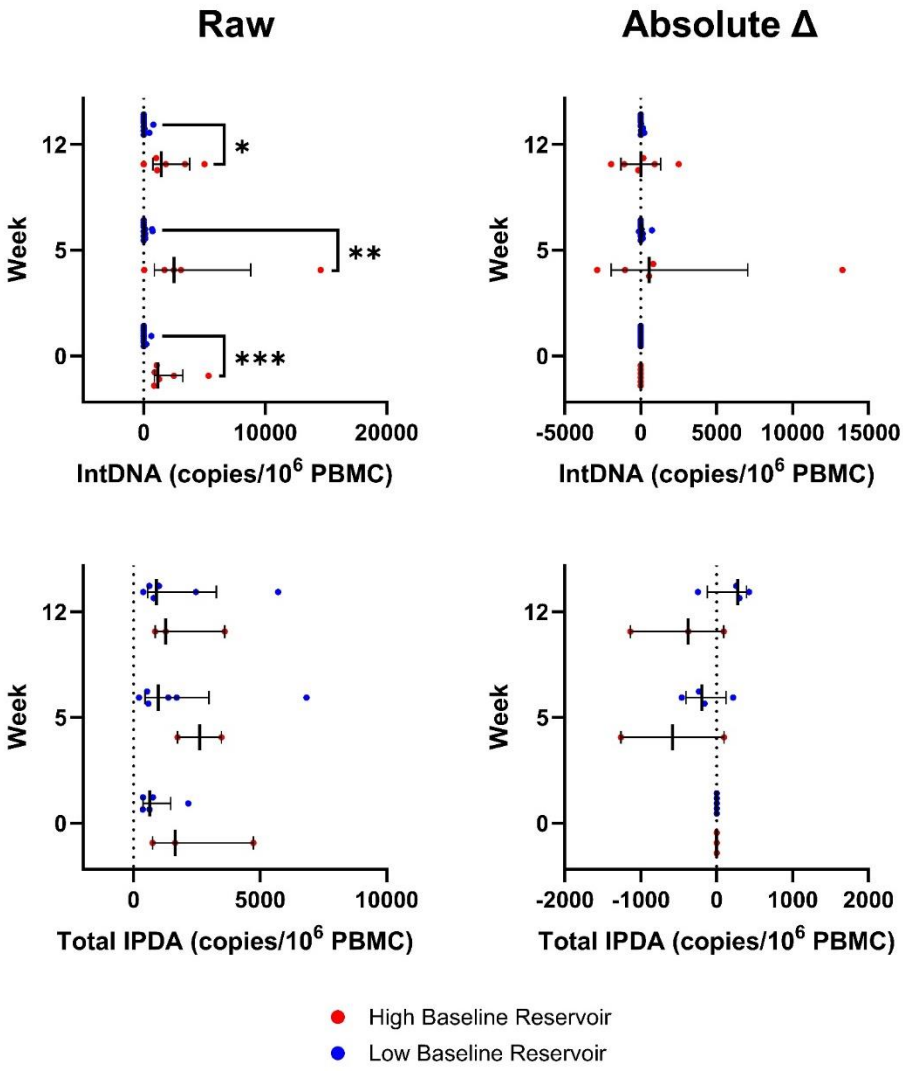
Four niche biomarkers were identified that were altered by ruxolitinib (RUX), different from control (CNT), and associated with reservoir decay. Baseline cellular marker expression in reservoir growth (n=12) vs decay (n=12) groups determined by week of significant ($p<0.05$) or trending ($0.05<p<0.1$) association as a potential predictor/screening tool for patients. Significance was determined via Mann-Whitney comparison between baseline expression in the growth and decay groups (a=0.05). **A)** pSTAT5 expression in monocytes is reduced by week 12 in both the total ruxolitinib-treated group (p=0.0937, RUX n=27-29, CNT n=16-17), and those with low baseline reservoir (p=0.0380, RUX n=16-18, CNT n=16-17). Participants likely to experience decay have a higher baseline expression of pSTAT5 in monocytes (p=0.0615). **B)** BCL-2/KI67 dual expression in naïve CD4+ T cells is reduced by week 5 in both the total ruxolitinib-treated group (p=0.0584, RUX n=28-30, CNT n=16-17) and the low baseline reservoir group (p=0.0741, RUX n=17-19, CNT n=16-17). Participants likely to experience decay have a significantly lower baseline expression of BCL-2/KI67 in CD4+ T_N cells (p=0.0008). **C)** CD127 expression in terminally differentiated CD8+ T cells is increased by ruxolitinib intervention by week 5 (p=0.0761, RUX n=28-30, CNT n=16-17). CD127 expression in CD8+ T_{TD} cells is not different at baseline (p=0.7985). **D)** CD25 expression in transitional memory CD8+ T cells is reduced with intervention by week 5 (p=0.0964, RUX n=28-30, CNT n=15-16). CD25 expression in CD8+ T_{TM} cells is not different at baseline (p=0.1732). **E)** pSTAT3 expression in monocytes is reduced with intervention by week 12 (p=0.0656, RUX n=16-18, CNT n=16-17), but is not a predictor of decay at baseline (p=0.2938). Cellular subset abbreviations: N (naïve), CM (central memory), TM (transitional memory), EM (effector memory), and TD (terminally differentiated).

Discussion

Current clinical trials focus on a plethora of strategies. Antibodies, vaccines, and tyrosine kinase inhibitors do not directly target the HIV reservoir. Gene therapy strategies both target HIV provirus (NCT05144386, NCT02390297), immune response (NCT04648046), and spread (NCT03666871). Systemic inflammation, immune activation, and elevated immune checkpoint expression all contribute to immune exhaustion.[18] Immune checkpoint inhibitors can potentially reverse HIV latency (NCT05330143, NCT05187429). BCL-2 agonists can directly target the viral reservoir via cell death (NCT05668026). Tyrosine kinase inhibitors can target homeostatic proliferation and reduce bystander infections via SAMHD1 phosphorylation blockade.[19] Finally, immunomodulators (such as Janus kinase (Jak) inhibitors as analyzed here) modify the immunological landscape which can have effects on the viral reservoir and immune-mediated clearance (NCT05598580, NCT05452564).[6, 11, 12, 20] Combination therapy is the most diverse area of cure efforts encompassing vaccine, antibody, immunotherapy (NCT05129189) and shock and kill methods (NCT02471430).

We estimate 99.99% total proviral HIV DNA (IPDA) reservoir clearance in 2.83-3.01 years with twice daily treatment of 10mg ruxolitinib in conjunction with peripherally suppressive ART for individuals with elevated levels at baseline. While the decay rate from week 0 to 12 predicts 99.99% decay in 6.96 years, it is also in context of the transient T cell demargination from Jak inhibitor treatment. For this reason, it is more likely that the decay rate from week 5 to 12 is a more accurate reflection of the effect of ruxolitinib post-T cell demargination. Transient T cell demargination into the periphery has been observed during Jak 1/2 inhibitor treatment (baricitinib). In a 12-week study, total lymphocytes increased by week 4 and returned to baseline by week 12.[21] We observed the same effect here, which could account for the transient, non-significant increase in total proviral DNA (IPDA) from week 0 to 5 that resolves by week 12 of therapy [21-23] as opposed to true expansion of the reservoir. Our decay estimate is based on the observed decay rate from week 5 to 12. The reservoir decay post-intervention indicates that ruxolitinib requires time to have an observable physiological effect, and that it may be a lasting effect. We acknowledge that this model cannot reflect changes occurring at time of treatment, but rather view the model as a prediction of long-term reservoir changes in response to ruxolitinib. Our estimate is subject to change based on treatment duration. A longer intervention would reveal potential stagnation or exponential decay effects. We observed a significant decrease in total proviral DNA in ruxolitinib-treated participants with a high baseline reservoir. We also observed trending reduction in intact and defective proviral DNA in those on RUX with a high baseline reservoir from weeks 5-12. When examining absolute change in integrated HIV DNA (Alu PCR), we qualitatively see a decrease from baseline maintained through week 12 in the RUX high baseline reservoir subset compared to control, but the difference is not significant (**Supplemental Fig. 1**), likely due to many more extreme measures from the median in the Alu PCR measures compared to IPDA due to a greater n (Alu PCR mean n=47, IPDA mean n=28). We observed a significant increase from baseline to week 12 in integrated HIV DNA (Alu PCR) of PWH possessing a low baseline reservoir. However, this was paired with a trending increase in defective proviral DNA (IPDA) in those with a low baseline reservoir on RUX, indicating that defective HIV copies are increasing in the RUX low baseline subset as Alu PCR measures both defective and intact HIV DNA copies. We previously demonstrated *in vitro* and *ex vivo* that

Jak inhibitors reduced the frequency of cells with integrated HIV DNA, inhibited viral replication, and reduced activation and lifespan markers.[11] We believe the observed effect is not regression to the mean based on demonstrated non-significant differences of random change in the control group regardless of stratification (**Supplemental Fig. 6**). Further mechanistic studies are required to explore the process of changes in the immune microenvironment when reversing immune dysregulation.



Supplemental Figure 6 | Random Change by Control Group Stratification

(Top) Integrated HIV-1 DNA measured by Alu PCR raw data (median with interquartile range) differs significantly between high (orange, n=5-6) and low (blue, n=12) baseline reservoir stratification in the control group, but there is no significant difference in absolute change over time between subgroups

(Kruskal-Wallis with Dunn's multiple comparisons test, a=0.05, week 0 p=0.0005, 5 p=0.0094, 12 p=0.0167). (Bottom) There are no significant differences between subgroups of total integrated HIV-1 DNA measured by IPDA (high n=2-3, low n=5-6) concerning both raw data and absolute change over time.

Cellular markers that were significantly altered by ruxolitinib and different from control (**Table 1**) are all markers associated with immune activation, Jak STAT activation, proliferation, cell survival, homeostatic proliferation, and immune exhaustion. With a longer duration of treatment, it is possible that these markers may be measurably affected in multiple cellular subsets. Five niche markers were elucidated that likely contributed to reservoir decay.

First, we examined marker downmodulation in this study. Concerning niche markers associated with reservoir decay, pSTAT3/5 in monocytes indicates Jak STAT activation. pSTAT5 is associated with CD25 regulation, BCL-2 production, and T cell proliferation[12, 24, 25] while pSTAT3 is an associated downstream product of IFN-a and IL-6 production.[26] CD25 (IL-2R) expression in CD8+ T cells is indicative of cellular activation and proliferation[27] and depends on IL-2 signaling and pSTAT5.[28] CD25 is upregulated by IL-2 in conjunction with T cell receptor (TCR) stimulation and positively correlates with clinical severity.[29, 30] One study found that chronic inflammation driven by the Jak STAT pathway may be responsible for an increase in CD25+ CD8+ T cells.[31] Another study found that CD25+ CD8+ T cells inhibit CD4+ response to both monoclonal antibodies and antigen.[32] BCL-2/KI67 dual expression in CD4+ T cells is representative of long-lived proliferating cells. KI67 is a proliferative marker and BCL-2 is an anti-apoptotic marker, both upregulated in PWH.[12, 33-36]

In B cells, CD69 is engaged by antibodies resulting in cytokine production and cellular proliferation.[37] In monocytes, CD69 expression is considered a stimulant for lymphoid cell activation.[38, 39] In CD8+ T cells, Ki67 is representative of an activated and proliferating subset.[40] Inflamed tissues can upregulate Ki67 expression driven by inflammatory cytokines.[40] BCL-2 is downregulated on CD8+ T cells in PWH as a method of immune evasion through limiting the antigen-specific response via susceptibility to apoptosis.[41] In CD4+ T cells, CD39 positively correlates with HIV DNA.[42] CD39 subverts ATP-mediated

inflammatory effects by converting ATP to AMP, which is hydrolyzed to adenosine that induces cAMP signaling resulting in an immunosuppressive effect.[42] CD39 expression has been negatively correlated with CD4+ T cell count and positively with T cell activation and viral load.[43] HLA-DR stimulates expansion of CD8+ T cells, which becomes upregulated in PWH.[44] HLA-DR was positively correlated with HIV DNA in one study over 8 years of suppressive ART.[45] Here, HLA-DR expression decreased by week 5 with rebound expression by week 12.

Downmodulation of these markers observed in this analysis indicate an altered physiological environment in which inflammation-driven reservoir maintenance, immune activation, reservoir cell survival, and cellular proliferation are mitigated. These phenomena are all associated with HIV reservoir and tissue pathology as well as development of comorbidities such as immune exhaustion, immunosenesence, and inflammaging.[12, 46]

Next, we examined marker upregulation. CD8+ T cell expression of CD127 was identified as a niche, decay-associated marker. In both CD4+ and CD8+ T cells, CD127 (IL-7R) expression is downregulated in PWH. IL-7 is upregulated in PWH and is a key driver of homeostatic proliferation, an important factor in HIV reservoir maintenance.[16, 47] High IL-7 and low CD127 expression is inversely correlated with CD4+ T cell counts, increased viral replication, and immune activation.[47, 48] CD127 is a receptor of IL-7 as a method of clearance, and increasing CD127 results in CD4+ and CD8+ T cell recovery.[47, 49] Specifically, in CD8+ T cells, downregulation of CD127 correlates with immune activation and CD8+ T cell exhaustion.[48] In the initial analysis of this trial, examining immune activation and safety endpoints, CD127 was also determined to be upregulated in participants receiving ruxolitinib.[6]

TIGIT expression in CD8+ T cells has historically been considered a marker for immune exhaustion and reservoir maintenance, especially when co-expressed with PD-1.[50] However, TIGIT alone on CD8+ T cells demonstrated impaired cytokine production and cytotoxicity in the absence of viral replication.[51] In PWH, CTLA-4 is typically upregulated in activated T cells and serves to reduce IL-2 production and arrest proliferation.[52] CTLA-4 binds CD80/86 ligands which are responsible for T cell

activation via CD28 resulting in IL-2 production, CD25 upregulation, proliferation, and BCL-XL upregulation.[53-55] CTLA-4+PD-1- memory CD4+ T cells are predictive of the SIV reservoir size during ART and are a significant contributor to SIV reservoir persistence.[56] CTLA-4 expression in CD4+ T cells has been shown to contribute to the HIV reservoir.[50] In COVID-19, another disease where Jak 1/2 inhibitors have proven effective, one group hypothesized that CD80/86 signaling is critical in cytokine storm leading to severe disease state.[57] In a CLL model, decreased CTLA-4 resulted in increased pSTAT1, KI67, and BCL2.[58] CTLA-4 is a negative regulator of T cell activation.[59] In CD8+ T cells, engagement of SLAM regulates proliferation and IFN-γ production.[60] Engagement by an antibody promotes CD8+ cytotoxicity and DC and macrophage production of anti-inflammatory molecules.[61] Increased CD62L expression indicates reduced viral load and activation.[62] Increased CD38 in CD8+ T cells contributes to chronic inflammation and activation but is likely a compensatory effect here as observed at week 12.[63, 64]

Upmodulation of CTLA-4 and TIGIT can be interpreted in two ways due to the singly expressed marker framework used here, a limitation of this study, and debate in the literature on the immunomodulatory impact of these markers in PWH. Thus, upregulation here could either indicate improved immune function (singly expressed) or may be a result of rebound to baseline post-treatment as upregulation was observed at week 12. Upregulated CD127 at week 5 is an indication that ruxolitinib limited homeostatic proliferation of the HIV reservoir.

We appraised the feasibility of clinical translation of ruxolitinib for PWH. PWH expressing low levels of BCL-2/KI67 and high levels of pSTAT5 at baseline were more likely to experience reservoir decay. These predictive markers could allow for rapid screening of future trial candidates; however, a longer treatment duration may reveal others.

In considering a standard of care therapy capable of targeting the HIV reservoir, it is important to account for the entire HIV reservoir. In this analysis, we only investigated the effect of ruxolitinib on the peripheral reservoir. Additional phase 2a human trials of second generation Jak 1/2 inhibitor baricitinib are underway (shared investigational new drug application NCT05849038 *Inflammation and Depression in*

People with HIV (n=65; 2:1 baricitinib to placebo) and *Baricitinib for Reduction of HIV-CNS* (n=65; 1:1 baricitinib to placebo)) and will provide additional information about longer duration treatment (10 weeks) and the impact of this class of agents on myeloid reservoir decay and targeting of sanctuary sites. However, Jak 1/2 inhibitors have been demonstrated to inhibit HIV replication in macrophages *in vitro*[10], as well as reduce activated p24+ macrophages and block PMA-induced viral reactivation in macrophages *in vivo* (murine).[65] These data have shown that Jak 1/2 inhibitors affect the myeloid reservoir.

Soluble cytokine data were not examined in this analysis but have been previously studied with Jak 1/2 inhibitors. The observed alteration of pSTAT5, BCL-2, and immune activation in this analysis are supported by historical findings of Jak inhibitor-mediated reduction of IL-6, IL-1a/b, TNFa[6, 66-69] and IL-2, IL-15, and IL-7-mediated pSTAT5 and BCL-2 production.[11] Jak inhibitors serve to reset the immunological milieu associated with chronic immune dysregulation and eventual Immunosenescence in PWH, allowing the immune system a clean slate in which to target the HIV reservoir.

A case study at the Pasteur Institute in France, presented at the 12th International AIDs Society Conference on HIV Science[70], reported ongoing absence of viral rebound and undetectable viral RNA and DNA in multiple tissue compartments (2.16 years to date) in a PWH who received an allogenic hematopoietic stem cell transplant, recently named as the "Geneva Patient". Historically, these wildtype transplants do not prevent viral rebound after analytic treatment interruption (ATI), but in this study ruxolitinib was administered as a treatment for graft-vs-host disease 8 months post-transplant, and was the sole immunomodulator maintained post-ATI. As ruxolitinib has been the sole agent maintained in this person during long-term viral remission, coupled with our data, it is conceivable that ruxolitinib is a potential factor contributing to the reported decrease in proviral HIV DNA and the absence of viral rebound in this person, consistent with our calculated decay prediction model.

Jak 1/2 inhibitors target the HIV-1 reservoir, reduce systemic inflammation driving reservoir maintenance and comorbidities, and reinvigorate the immune response from exhaustion to efficiently target the reservoir. Several Jak inhibitors are FDA approved for various indications, posing an accelerated route

to useable therapy. For these reasons, Jak 1/2 inhibitors provide a foundation for effective combination therapy towards HIV cure.

Methods

Trial Design and Exclusion Criteria

The A5336 phase 2a multicenter randomized (2:1), open label, parallel-arm trial was sponsored by the AIDS Clinical Trials Group (ACTG) and conducted at 14 academic sites across the US. The two study arms were the intervention group who received ruxolitinib (RUX) and the control group that received no intervention (CNT). People with HIV were randomized 2:1 (n=60 total, n=40 in RUX, n=20 in CNT) via a centralized computer at the ACTG Data Management Center in an open-label fashion using permuted blocks stratified by efavirenz status at baseline. In the RUX group, 10 mg of ruxolitinib was administered orally twice-daily. No study drug was administered to CNT participants. All participants continued their previously prescribed ART throughout the study. The RUX group was treated with ruxolitinib for 5 weeks post-entry and followed off-treatment for 7 weeks thereafter, while the CNT group was followed for the entire 12 weeks of the study without study treatment (**Fig. 4**).

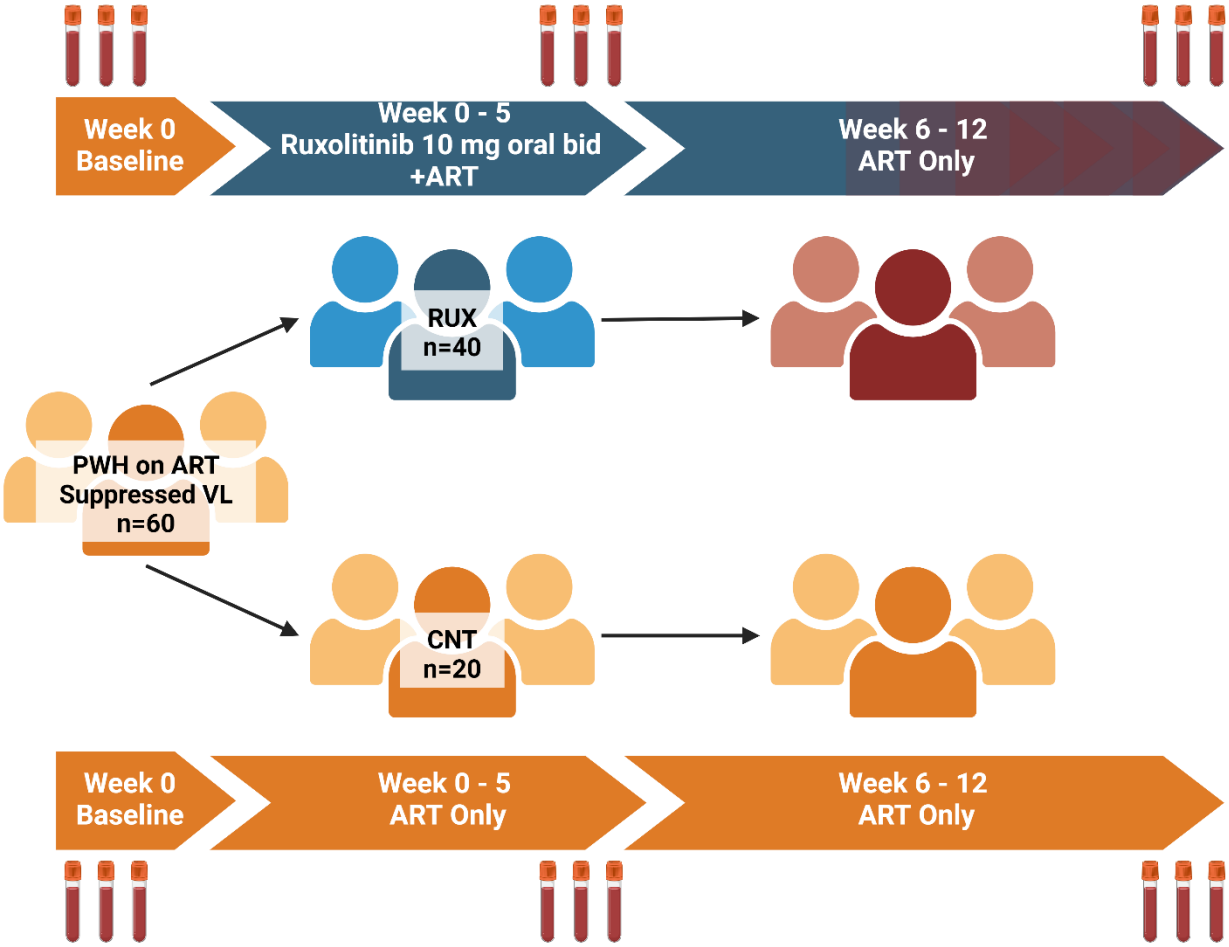


Figure 4 | Trial Design Schematic

60 people with HIV (PWH), virally suppressed ≥ 2 years on antiretroviral therapy (ART) regimen excluding cobicistat, randomized into two trial arms: control (CNT), n=20, and ruxolitinib-treated (RUX), n=40. The CNT arm maintained their ART regimen for the full 12 weeks of the trial with no interventions. The RUX arm was administered ruxolitinib from study weeks 0-5 at 10 mg oral dose bid (twice daily) in addition to patients' ART regimen. Ruxolitinib was discontinued at week 5, ART regimen sustained, and patients were monitored through week 12. Peripheral blood samples were collected at weeks 0, 5, and 12. Created with BioRender.

Peripheral blood samples were obtained at pre-entry, entry (week 0), and weeks 4, 5, 10, and 12. In this analysis, we had access to samples from entry, week 5, and week 12. All participants were aged 18-74 years, virologically suppressed ≥ 2 years (plasma HIV-1 RNA below the limit of assay quantification), receiving an ART regimen ≥ 12 weeks pre-entry containing an NNRTI or INSTI without cobicistat to avoid

potential drug interactions with ruxolitinib via CYP34A metabolism, had no significant comorbidities besides controlled hypertension or HIV, and had a CD4+ T cell count > 350 cells/μL (**Supplemental Table 5**). As previously reported for this clinical trial[6], there was no difference in primary safety events between arms. The study was reviewed by an independent study monitoring committee at 6-month intervals and was registered with ClinicalTrials.gov (NCT02475655).

Supplemental Table 5 | Patient Baseline Characteristics by Study Arm

Characteristic		CNT (n=20)	RUX (n=40)	Entire Population (n=60)
Age	Median (IQR)	43.5 (31-54)	49 (45-54)	49 (36.5-54)
Sex	Male	16	32	48
	Female	4	8	12
Race/ Ethnicity	White non-Hispanic	7	14	21
	Black non-Hispanic	10	19	29
	Hispanic (any race)	2	4	6
	>1 race	2	1	1
Baseline Cell Counts (Median (IQR))	CD4+ T Cell	737 (610-930)	798 (628-973)	791 (622-972)
	CD8+ T Cell	629 (496-852)	704 (483-842)	644 (490-852)
	CD4/8 Ratio	1.3 (0.8-1.6)	1.3 (0.9-1.6)	1.3 (0.9-1.6)
Nadir CD4 Count (cells/mL)	≤50	0	3	3
	51-100	1	2	3
	101-200	3	7	10
	201-500	10	21	31
	>500	5	5	10
HIV-1 RNA (copies/mL)	<40	20	39	59
	114	0	1	1
ART Regimen	TDF/FTC/EFV	6	14	20
	ABC/3TC/DTG	5	8	13
	TAF/FTC/RPV	4	3	7
	TAF/FTC+DTG	1	4	5
	TDF/FTC+RAL	1	4	5
	TAF/FTC+RAL	2	2	4
	TDF/FTC+DTG	0	3	3
	TDF/FTC/RPV	1	1	2
	ABC/3TC+DTG	0	1	1
IV Drug Use	Never	19	33	52
	Previously	1	7	8

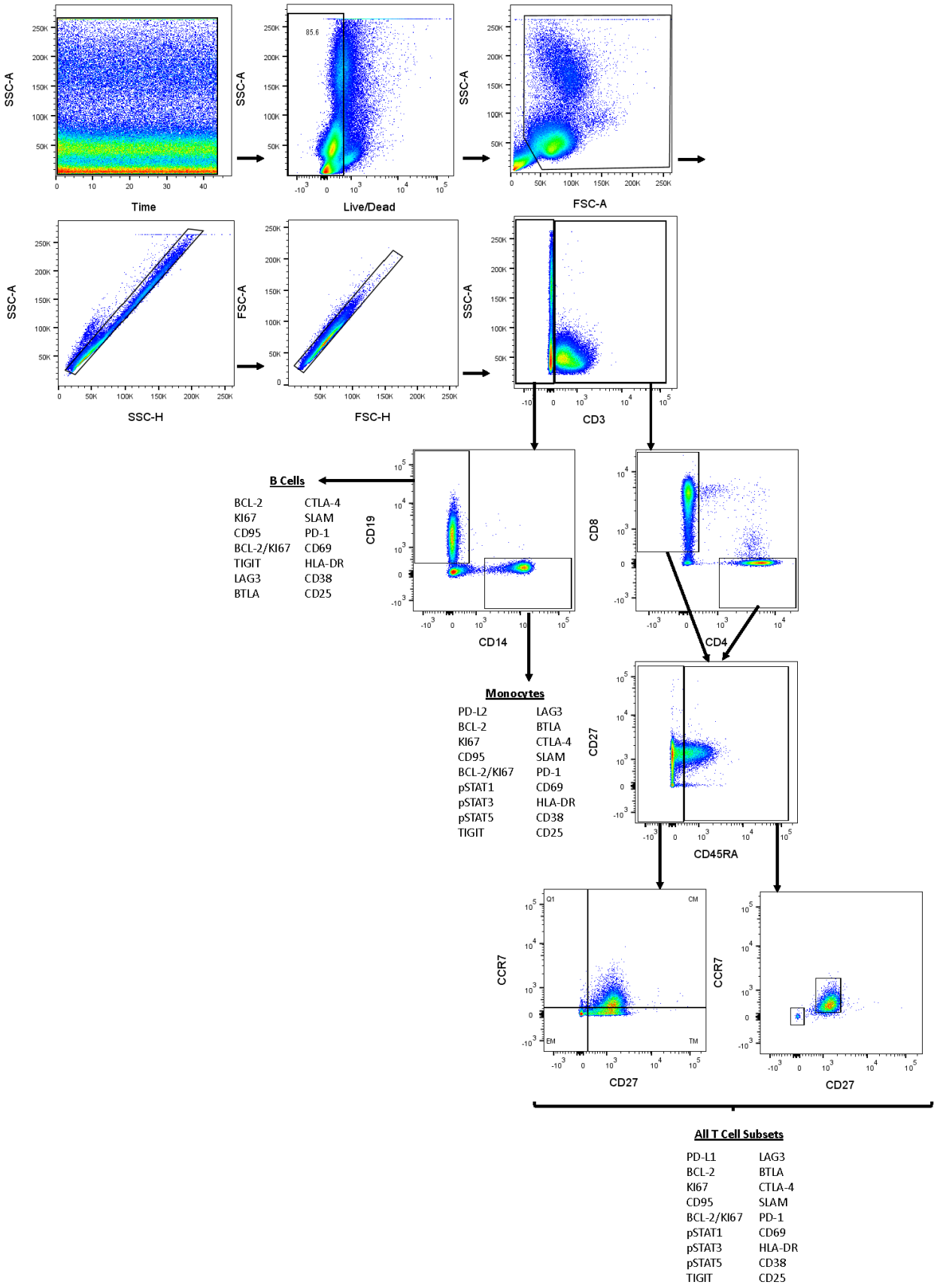
Antiretroviral therapy (ART) regimen abbreviations: TDF (tenofovir disoproxil fumarate), FTC (emtricitabine), EFV (efavirenz), ABC (abacavir), 3TC (lamivudine), DTG (dolutegravir), TAF (tenofovir alafenamide), RPV (rilpivirine), RAL (raltegravir). Median with interquartile range (IQR) reported where indicated; all others are reported number of patients per characteristic. Abbreviations: CNT (control arm) and RUX (ruxolitinib arm).

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by Ficoll density gradient centrifugation and labeled with fluorochrome-conjugated monoclonal antibodies against cellular markers listed in **Supplemental Table 6**. Total events (100,000 targeted) were collected using an LSRII (Becton Dickinson) cytometer and analyzed via FlowJo v10.9 software by forward scatter (FSC) and side scatter (SSC), dual doublet discrimination (FSC-area v FSC-height, and SSC-area v SSC-height), and specific gating for cellular subtypes and cellular markers (**Supplemental Fig. 7**). Negative gates were defined by unstained control samples. Fluorescence-minus-one (FMO) controls were used to set compensation parameters using titrated antibodies. Gates were primarily drawn to examine mono expression with one case of dual expression.

Supplemental Table 6 | Flow Cytometry Cellular Marker Panels

Cellular Marker Panels					
Ligand	**Memory/Proliferation /Apoptosis**	**Phospho-STAT**	**Negative Regulator**	**Memory/Activation**	**Differentiation**
PD-L1	BCL-2	pSTAT1	TIGIT	CD69	CD28
PD-L2	KI67	pSTAT3	LAG3	HLA-DR	CD127
	BCL-2/KI67	pSTAT5	BTLA	CD38	CD39
	CD95		CTLA-4	CD25	CD62L
			SLAM		
			PD-1		


SSC-A
Time
85.6
Live/Dead
FSC-A
SSC-H
FSC-H
CD3
B Cells
BCL-2
KI67
CD95
BCL-2/KI67
TIGIT
LAG3
CTLA-4
SLAM
PD-1
CD69
HLA-DR
CD38
CD25
CD19
CD14
CD8
CD4
Monocytes
PD-L2
BCL-2
KI67
CD95
BCL-2/KI67
pSTAT1
pSTAT3
pSTAT5
TIGIT
LAG3
BTLA
CTLA-4
SLAM
PD-1
CD69
HLA-DR
CD38
CD25
CD27
CD45RA
CCR7
Q1
CM
EM
TM
All T Cell Subsets
PD-L1
BCL-2
KI67
CD95
BCL-2/KI67
pSTAT1
pSTAT3
pSTAT5
TIGIT
LAG3
BTLA
CTLA-4
SLAM
PD-1
CD69
HLA-DR
CD38
CD25

Supplemental Figure 7 | Gating Scheme for Flow Cytometry

Gating was based on live/dead discrimination, dual-doublet discrimination, and lineage marker sub-gating. Single and double channel gates listed for each cell subpopulation.

Reservoir Quantification

Genomic DNA was extracted from PBMC using the Gentra Puregene Cell kit (Qiagen) using RNase. Integrated HIV DNA (as copies per million PBMC) was quantified via a 2-amplicon system in nested Alu qPCR (Roche Light Cycler 480) in triplicate with an ACH-2 standard as previously described.[11, 16, 71] IPDA was used to detect the total, intact, and 3' and 5' defective proviral HIV-1 DNA (as copies per million PBMC) in 10 replicates as previously described via digital droplet PCR (ddPCR) (Bio-Rad QX200 Droplet Generator).[72]

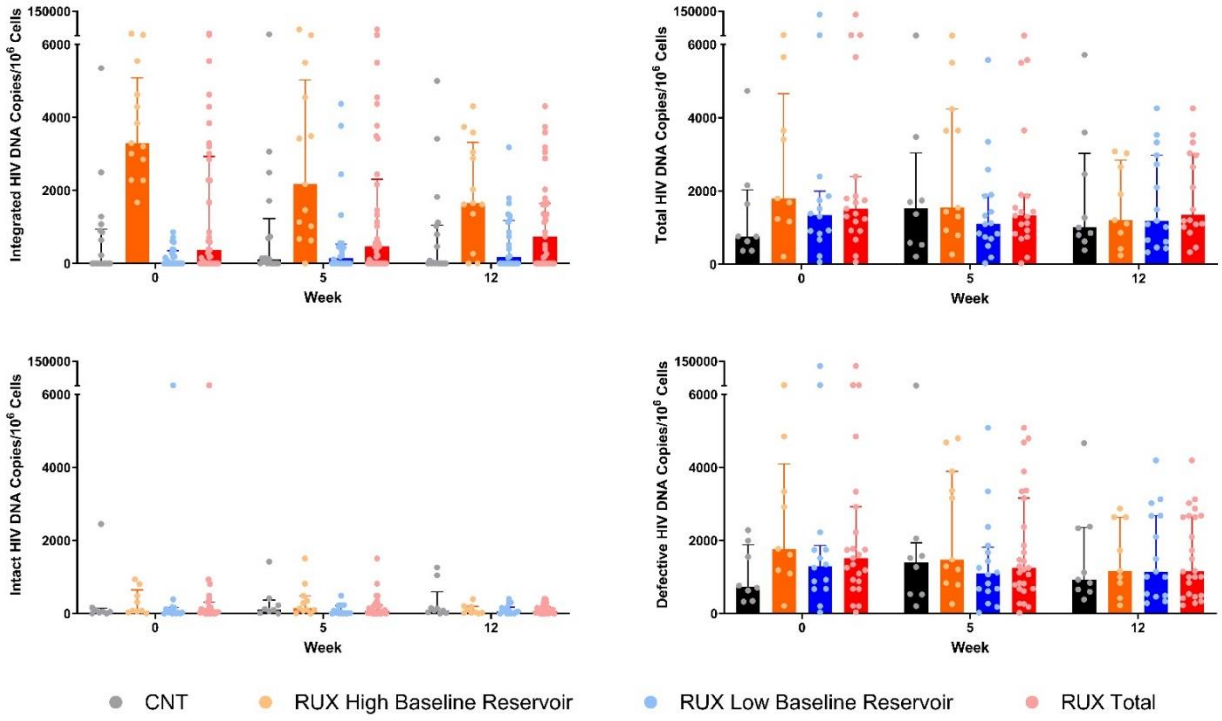
Statistical Analysis

We began with a raw data set of integrated DNA, IPDA (total, intact, 3' defective, and 5' defective), and cellular marker expression for each person (**Supplemental Table 7, Supplemental Fig. 8**). We assessed the baseline reservoir for people on RUX using integrated HIV DNA (Alu PCR) and assigned a status of high (highest 1/3 measures) and low (lowest 2/3 measures) baseline reservoir. Although this classification was arbitrary, it assisted in determining the effect of ruxolitinib stratified by baseline reservoir size. Therefore, within RUX, three groups were analyzed (high baseline reservoir, low baseline reservoir, and total RUX group). Stratification of the control group into high and low baseline reservoir would result in underpowered analyses due to the limited number of participants. For these reasons, the entire control group was used in comparisons. Due to biological variability, absolute change for reservoir measurements and cellular marker expression from baseline to weeks 5 and 12 was determined for each participant to normalize the data. We performed Spearman rho correlations (a=0.05) with linear regression trendline generation between reservoir measurements obtained via IPDA (total proviral DNA) and Alu PCR (integrated DNA) within the CNT arm longitudinally across the study to confirm validity and cohesiveness between methods.

Supplemental Table 7 | Raw HIV-1 Reservoir and Cellular Biomarker Longitudinal Data

https://doi.org/10.15139/S3/8YADL0

Abbreviations: RUX (ruxolitinib arm (n=40)), CNT (control arm (n=20)), IPDA ++ (intact provirus only), IPDA +- (total provirus), IPDA -- (defective provirus only), N (naïve), CM (central memory), TM (transitional memory), EM (effector memory), and TD (terminally differentiated). High and Low reservoir stratification was based on week 0 Alu-LTR qPCR reservoir measurements, where the top tertile of each group was defined as high baseline reservoir and the bottom 2/3s were defined as low baseline reservoir. (Hosted on Dataverse v5.9 build750-fb24c87).



Supplemental Figure 8 | Range of Raw Reservoir Data

Range of raw reservoir data by Integrated HIV DNA (Alu PCR) in control (CNT, n=17-18), total PWH on ruxolitinib (RUX, n=37), and PWH with high (n=13) or low (n=24) baseline reservoir and by IPDA (total, intact, 3'/5' defective) in CNT (n=8-9), total PWH on RUX (n=23-27) and PWH with high (n=9-11) or low (n=14-16) baseline reservoir. Medians with interquartile range shown.

Viral reservoir longitudinal comparisons (IPDA and Alu PCR) between within groups (RUX and CNT) were made using a non-parametric Kruskal-Wallis test with a Dunn's multiple comparisons test via GraphPad Prism v9.5.1 (a=0.05). To analyze trends, we also used linear mixed effects models (lmerTest 3.1-3 with R version 4.2.2 in RStudio v. 2023.12.0.369). In this approach, we fit the full data set being

analyzed at the same time, accounting for repeated measurements within individual. Significant differences between the trends in the different groups were analyzed with an interaction term for the decay in time with ruxolitinib treatment. Significance was assessed at the alpha=0.05 level.

To create a prediction model of decay, the rate of decay from both week 0 to 12 and week 5 to 12 was calculated by percent change of raw total proviral copies (IPDA) per participant and divided by the 7 or 12-week period resulting in a mean percent change per week. We sequentially subtracted the mean percent change per week of remaining reservoir over time until 99.99% reservoir decay was reached. We also created an exponential decay model from the total proviral DNA (IPDA) rate of decay from week 5 to 12 estimated with the mixed effects model.

To assess cellular marker modulation and association with reservoir decay, three rounds of pairwise unmatched Mann-Whitney U tests (a=0.05, GraphPad Prism v9.5.1) with Benjamini-Hochberg tests (15%) to limit the false discovery rate were used. Total proviral HIV DNA (IPDA) was used due to a strong observance of reservoir decay with this measure. However, several participants were missing either week 5 or 12 total proviral DNA (IPDA) data which limited the use of a Wilcoxon matched ranks test for round one (assessment of marker modulation within study arms). For this reason, we decided to use an unmatched Mann-Whitney U Test instead in round one to conserve the number of participants. Cellular markers identified in each round of analysis were overlapped to determine a niche list of markers that were altered by ruxolitinib treatment and were associated with reservoir decay.

Acknowledgements

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Infectious Diseases (NIAID), National Institutes of Health (NIH): Anti-Human α4-β7 integrin monoclonal (Act-1) (catalog number 11718) from Dr A. A. Ansari.

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Chapter IV: Effect of Baricitinib on HIV-1 Reservoir Maintenance *In Vitro*

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Introduction

The HIV reservoir is a major barrier to the cure for HIV-1 and persists despite peripheral viral suppression with direct acting antiretroviral therapy (ART). HIV-1 can establish a reservoir in CD4+ T cells and myeloid cells.[1-4] Elevated inflammation persists despite well-controlled peripheral viremia, and contributes to HIV-1 persistence, including in the central nervous system (CNS) compartment, and specifically the myeloid reservoir.[5] HIV-1 infected macrophages contribute to immune dysfunction that is associated with HIV-1 persistence *in vivo*, including induction of cytokines such as IL-1b, IL-6, IL-7, IL-10, and IL-15. Persistent inflammation and immune dysregulation in macrophages exacerbates comorbidities such as HIV-associated neurocognitive disorder (HAND) and CNS persistence.[5-7] The central nervous system CNS has been identified as a reservoir site.[5, 8-10] HIV can traverse the blood-brain barrier (BBB) and establish a reservoir in tissue resident macrophages in the CNS, and replication competent virus has been isolated from urethral macrophages and lymphoid tissues in macaques.[9, 11] When CD4+ T cells are depleted *in vivo*, viral replication was sustained by tissue resident macrophages suggesting an important role in HIV reservoir and viral persistence.[9, 12] Tissue resident macrophages were found to be a highly productive source of HIV during opportunistic co-infection in lymph nodes.[13] To date, there are no interventions specifically targeting the myeloid derived viral sanctuary, which is a critical unmet need that can be explored as an eventual add on to cure-based regimens or structured treatment interruptions, including CNS eradication strategies. We previously repurposed baricitinib, a novel repurposed immunomodulatory agent (rheumatoid arthritis), for the indication of COVID-19, and seek to do the same for HIV-1.[14-16]

Baricitinib is a second-generation, FDA-approved Jak 1/2 inhibitor with the indication for rheumatoid arthritis, COVID-19, and alopecia areata (Olumiant Package Insert). Baricitinib is given quaque die (qd; once daily) orally, is detectable in the cerebrospinal fluid of Rhesus Macaques up to 24 hours after dosing, and preliminary data from our group demonstrates that baricitinib accumulates in the CSF of humans (n=2) to sufficient concentrations to confer anti-HIV properties.[17] Baricitinib is 80% orally

bioavailable, renally cleared (reducing potential for drug-drug interactions with other hepatically cleared ART or other co-administered agents), reaches peak plasma concentrations within an hour of administration, has an elimination half-life of 12 hours, and is approved for chronic long-term use including in children as young as two years of age (Olumiant package insert). For these reasons, baricitinib represents a second-generation Jak 1/2 selective inhibitor with the most favorable safety and efficacy profile of all approved agents in the Jak inhibitor class and provides a robust foundation for additional studies towards the indication of HIV-1, which our group has extensively studied, including *in vitro, ex vivo*, and human studies.[18-21]

The Janus Kinase Signal Transducer and Activator of Transcription (Jak STAT) pathway is a key part of the inflammatory immune response and becomes dysregulated during chronic infection and immune activation such as in HIV-1 infection. Activation of Jak STAT drives establishment, maintenance, expansion, and reseeding of the HIV reservoir (**Figure 1**).[20, 22-30]

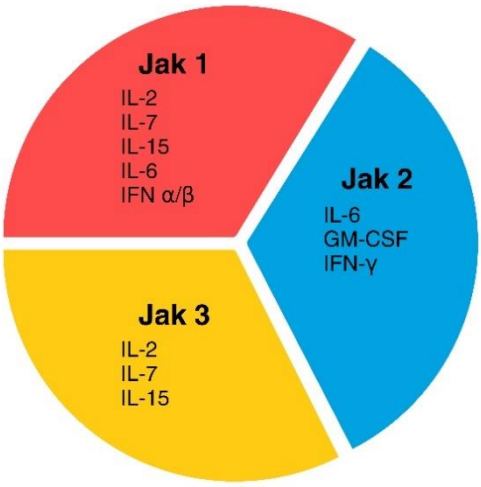

Figure 1 | Jak-Mediated Inflammatory Cytokine Secretion Products

Activated Jak 1/2 signaling drives reservoir lifespan through IL-15 induced pSTAT expression leading to BCL-2 production, expansion through IL-7-mediated homeostatic proliferation, and reactivation and expansion through IL-15-mediated reactivation and expansion of latent HIV and reseeding. Dysregulated inflammatory markers produced via Jak STAT contribute to inflammatory-induced pathologies such as

HAND and CVD. Jak (1/2) STAT activation drives HIV-1 reservoir lifespan, expansion, and reactivation. Made with BioRender.

Our group demonstrated that ruxolitinib and tofacitinib individually and synergistically inhibit viral replication and reactivation in lymphocytes and macrophages, including in HIV-1 strains with polymerase substitutions.[31] Our team also demonstrated that baricitinib is BBB penetrant in a murine model of HIV in the CNS, effective in neuroinflammation reduction, reversal in HAND related behavioral abnormalities, and reduction of reservoir as measured by p24+ cells.[21] We have also shown that baricitinib blocks IL-15 induced activation. IL-15 production confers reactivation of latent HIV-1.[3, 32-35] We have previously shown that ruxolitinib inhibits reactivation of latent HIV-1, and observed that baricitinib demonstrated a similar mechanistic functionality.[20, 31] Ruxolitinib and tofacitinib treatment resulted in higher IL-7R expression suggesting a lack of receptor engagement resulting in less homeostatic proliferation and decreased integrated HIV DNA. We have also previously demonstrated that baricitinib can reduce the frequency of latent cells in a primary tonsillar T cell model, as well as reverse key markers of CNS persistence and directly reduce viral burden in a murine model.[18, 21] We hypothesized that baricitinib inhibition of Jak STAT activation, associated with HIV persistence, increased reservoir size and viral load, will result in a decreased peripheral and myeloid reservoir.

Pro-inflammatory cytokines produced by receptor engagement activate Jak 1/2, which phosphorylates STAT5 (pSTAT5). pSTAT5 is then translocated into the nucleus where it binds to the promoter region of the B cell lymphoma 2 (BCL-2) pro-survival gene (among other pro-reservoir factors) and promotes transcription. BCL-2 is an anti-apoptotic, pro-survival protein that has commonly been studied in cancer but also plays a role in HIV reservoir maintenance. BCL-2 sequesters host BH3 to prevent BH3 from binding to pro-apoptotic proteins such as Bak, Bax, Bim, and Bad. If these pro-apoptotic proteins are engaged, they will oligomerize in the mitochondrial outer membrane inducing mitochondrial outer membrane permeabilization (MOMP) which releases several cell death factors including cytochrome C. Cytochrome C activates procaspase-9 which in turn initiates a caspase cascade that results in apoptosis.[36,

[37] pSTAT5 expression has been linked to increased integrated HIV DNA levels *in vivo*, *in vitro*, and immune activation, reservoir size *in vivo*, and BCL-2 expression *in vitro* and *in vivo*.[20, 38] Ruxolitinib and tofacitinib reduced IFN-α stimulated STAT5 phosphorylation, IL-2, IL-7, and IL-15 induced pSTAT5 expression and consequently IL-2, IL-7, and IL-15 induced BCL-2 levels.[20] For the A5336 human Phase 2a trial with ruxolitinib, a significant reduction in CD4+/BCL-2+ cells was also observed, demonstrating that ruxolitinib can also confer decrease in this key reservoir lifespan marker in a human study.[38] STAT5 transcriptionally targets BCL-2 resulting in prolonged cell survival.[20] The Jak/STAT signaling pathway is linked to HIV latency through IL-7. IL-7 controls latency and signals through STAT5.

Assessment of Direct Acting Antiviral Activity

Baricitinib inhibits viral production, but it is unknown if this happens via direct-acting antiviral activity or through immunomodulation alone.[20, 21, 31, 38, 39] To test the antiviral activity of baricitinib, we first isolated primary human CD4+ T cells and macrophages. Peripheral blood mononuclear cells (PBMCs) were isolated from human leukapheresis via density gradient centrifugation. CD4+ T cells and CD14+ monocytes were isolated from PBMCs via magnetic bead selection (Miltenyi Biotec, Gaithersburg, Maryland, USA). CD4+ T cells were activated via IL-2 (0.06 μg/mL or 50 IU/mL, Miltenyi Biotec, Gaithersburg, Maryland, USA) and PHA (0.006 mg/mL, MP Biomedicals, Santa Ana, California, USA) exposure for 72 hours, and maintained in Roswell Park Memorial Institute medium (RPMI) with 10% heat-inactivated fetal bovine serum US origin (FBS, MedSupply Partners, Atlanta, Georgia, USA) and 1x penicillin/streptomycin (Millipore Sigma, Burlington, Massachusetts, USA). Monocytes were differentiated to macrophages using granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/mL, Miltenyi Biotec, Gaithersburg, Maryland, USA) for 72 hours and maintained in RPMI with 10% heat-inactivated FBS and 1x penicillin/streptomycin. A single-cycle HIV-GFP reporter strain (NL4-3 backbone with an eGFP Nef

insertion) was used to identify viral infection/replication (**Figure 2**) (received from Baek Kim Lab, Emory University).[40, 41]

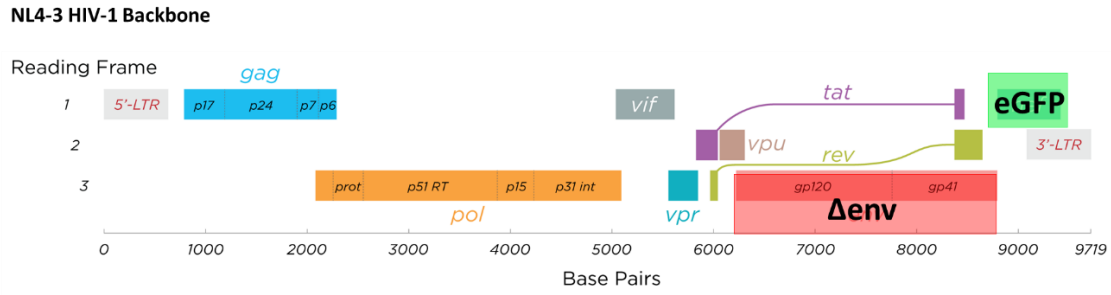

Figure 2 | HIV-GFP Reporter Strain

An eGFP gene was inserted into the Nef gene of a NL4-3 HIV-1 backbone. The strain is a single-cycle reporter which produces GFP upon replication.

CD4+ T cells and macrophages were transduced with the HIV-GFP reporter and incubated with baricitinib at various concentrations for 3 days (T cells) or 5 days (macrophages). Flow cytometry was performed to measure the percentage of GFP+ cells and the mean fluorescence intensity (MFI). The IC_{50} was calculated from GFP MFI over various concentrations of baricitinib via GraphPad Prism (**Figure 3**). Data were normalized to HIV-GFP naïve cells. A one-way ANOVA was performed to compare treatment conditions to an HIV-GFP transduced untreated control (GraphPad Prism, α=0.05). Emtricitabine at 1µM successfully reduced GFP expression in CD4+ T cells and macrophages. In comparison, Baricitinib was able to significantly reduce GFP expression (percent positive) and MFI in CD4+ T cells at 0.1µM. Baricitinib did not appear to reduce GFP expression or MFI in macrophages. The IC_{50} of baricitinib was calculated to be 0.0209µM in CD4+ T cells and >100µM in macrophages. These preliminary data show that baricitinib has an antiviral effect within T cells but not macrophages. Previously, Gavegnano et al. reported that %p24+ macrophages were reduced by baricitinib at 50 mg/kg *in vivo* in a murine model. Lack of antiviral effect observed here does not directly relate to or contradict p24+ macrophage counts in mice

as we assessed active viral replication in **Figure 3**.[21] HIV p24 can be produced without complete active replication.[42]

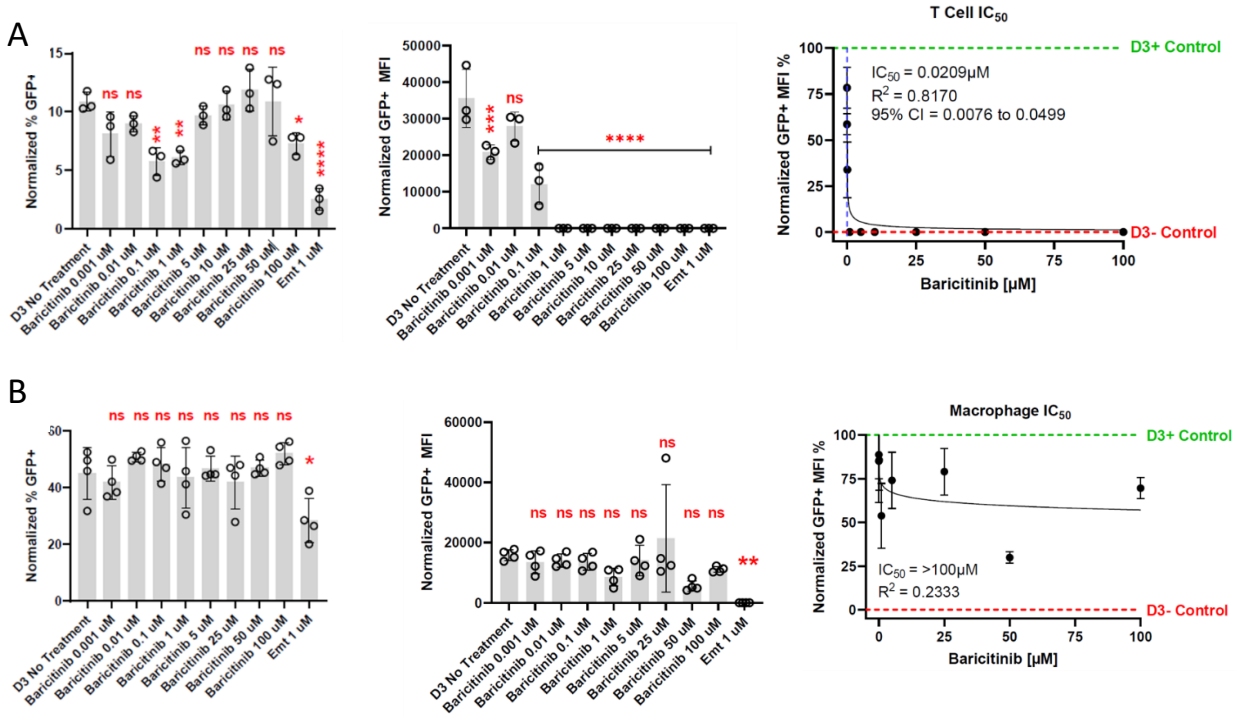

Figure 3 | HIV-GFP Indication of Antiviral Activity in Primary Human Cells

A) HIV-GFP (D3) transduced CD4+ T cells were treated with baricitinib (0-100µM) and emtricitabine (1µM). Flow cytometric analysis showed a significant decrease in the percentage of GFP+ cells and in GFP MFI with 0.1µM baricitinib compared to untreated. The IC_{50} of baricitinib in CD4+ T cells was calculated to be 0.0209µM. **B**) HIV-GFP (D3) transduced macrophages were treated with baricitinib (0-100µM) and emtricitabine (1µM). Flow cytometric analysis showed no significant decrease in the percentage of GFP+ cells or GFP MFI compared to untreated. The IC_{50} of baricitinib in macrophages was calculated to be >100µM.

To determine if baricitinib was able to directly alter replication and transcription, we measured the effect of baricitinib on DNA and mRNA copies of HIV-GFP via qPCR. Cultures were prepared as stated above. This experiment was done in collaboration with Lindsey Ramirez in the Kim Lab at Emory University. We found that the cycle threshold (Ct) value of baricitinib-treated cells was not significantly different compared to untreated HIV-GFP transduced CD4+ T cells and macrophages for both DNA and

mRNA copies (**Figure 4**). Baricitinib does not appear to affect viral replication or transcription. Further studies are required to assess if baricitinib has any impact on translation or post-translational modifications.

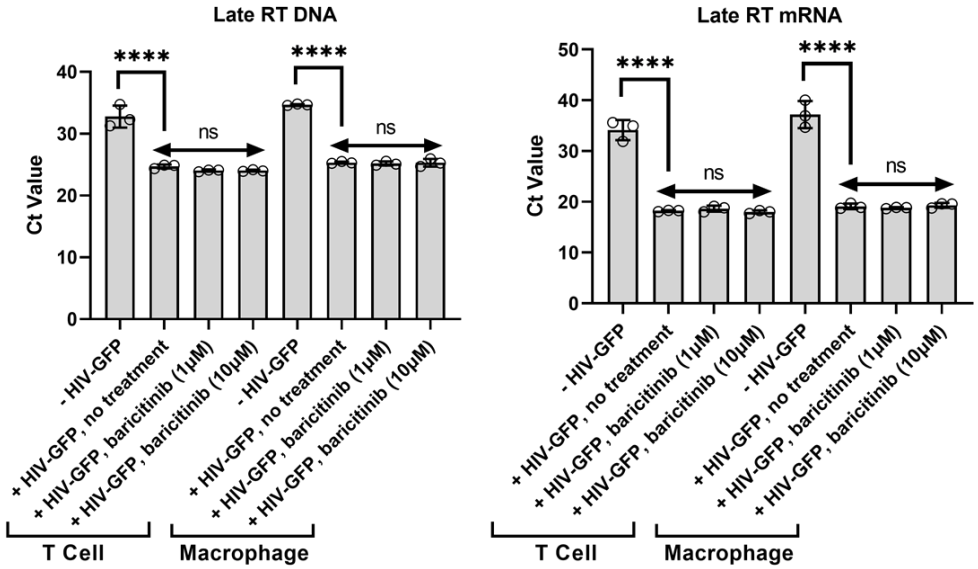

Figure 4 | Baricitinib Effect on HIV-GFP DNA and mRNA

DNA and mRNA copies were measured via qPCR for HIV-GFP transduced CD4+ T cells and macrophages treated with baricitinib (0-10μM). No statistically significant differences in cycle threshold (Ct) value were observed for DNA or mRNA copies compared to untreated HIV-GFP transduced cells in both CD4+ T cells and macrophages.

Baricitinib-Mediated Immunomodulation

Given the lack of apparent direct-acting antiviral effects, we assessed several markers to elucidate the immunomodulation activity of baricitinib. We first examined the ability of baricitinib to block IL-15 mediated pSTAT5 production. IL-15 is produced through Jak1 activation and promotes increased reservoir lifespan and reservoir reactivation from latency. We have previously shown that baricitinib blocks IL-15 mediated reactivation, ruxolitinib and tofacitinib block IL-15 mediated pSTAT5 production, and that ruxolitinib reduces BCL-2 expression, but the impact of baricitinib on IL-15 mediated pSTAT5 expression has not been shown.[3, 20, 32-35, 38] CEMx174 cells (ATCC NIH AIDS Reagent Program, ARP-272, Manassas, Virginia, USA) were pre-treated with baricitinib (1μM, BioVision, Milpitas, CA, USA) or media only for 2 hours, and then IL-15 (10 ng/mL, Miltenyi Biotec, Gaithersburg, Maryland, USA) was added.

Emtricitabine (1 μM, TCI Chemicals, Tokyo, Japan) was used as a control. Cells were cultured for 72 hours and stained for pSTAT5 production via flow cytometry. We found that baricitinib (1μM) was able to significantly reduce IL-15 mediated pSTAT5 production to below that of the media only control (**Figure 5**). Ideally, IL-2 and IL-7 mediated pSTAT5 production should also be assessed.

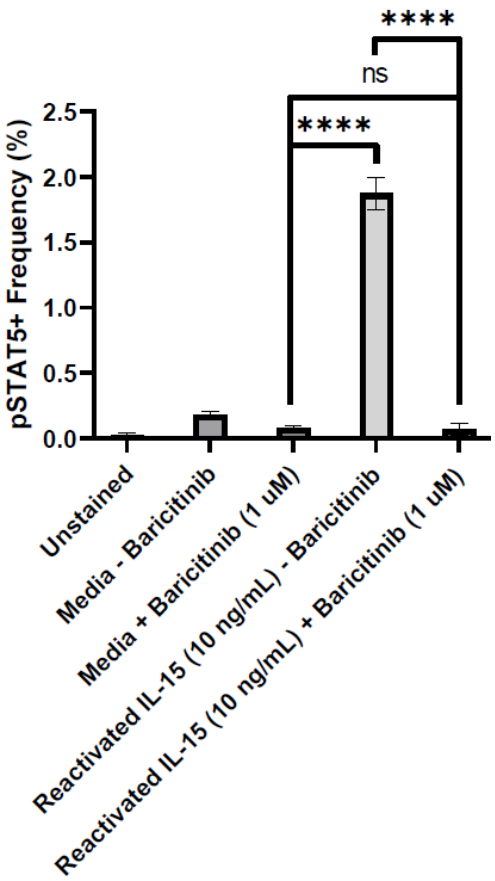

Figure 5 | Baricitinib Reduced IL-15 Mediated pSTAT5 Production

CEMx174 cells were pre-treated with baricitinib (1μM) or media only for 2 hours and then IL-15 (10 ng/mL) was added. The cells were cultured for 3 days. Compared to unstained and media alone controls, IL-15 significantly increased pSTAT5 production. Baricitinib and media alone did not appear to have a major effect on pSTAT5 production. When baricitinib-treated cells were exposed to IL-15, pSTAT5 production was significantly abrogated to below that of the media alone control.

HIV-1 capsid (p24) and envelope (gp120) proteins were measured via flow cytometry in PHA/IL-2-activated (72 hours pre-expsoure) CD4+ T cells exposed to emtricitabine (1μM) or baricitinib (0-1μM) for 21 days *in vitro*. Frequencies of cells undergoing active replication (p24+gp120+) and latently infected

cells (p24+gp120-) were assessed (**Figure 6**). We found that baricitinib significantly (ordinary two-way ANOVA with Tukey's multiple comparisons test and a single pooled variance, GraphPad Prism v10.0.3, α=0.05) reduced active replication in CD4+ T cells by day 5 in a dose-dependent manner, and the impact was sustained through day 21. Baricitinib did not have an apparent effect on latently infected CD4+ T cells (not shown). Macrophages must be examined in this same way. Our preliminary macrophage studies did not show any significant reduction due to baricitinib, but the data were also variable, indicating a need for troubleshooting and repitition. These data indicate that short term immunomodulation severely impacts active viral infection whereas, given the effect we observed with ruxolitinib on reservoir reduction in the phase 2a A5336 study (chapter III), 3 weeks may be too short term to see an impact on latent infection.

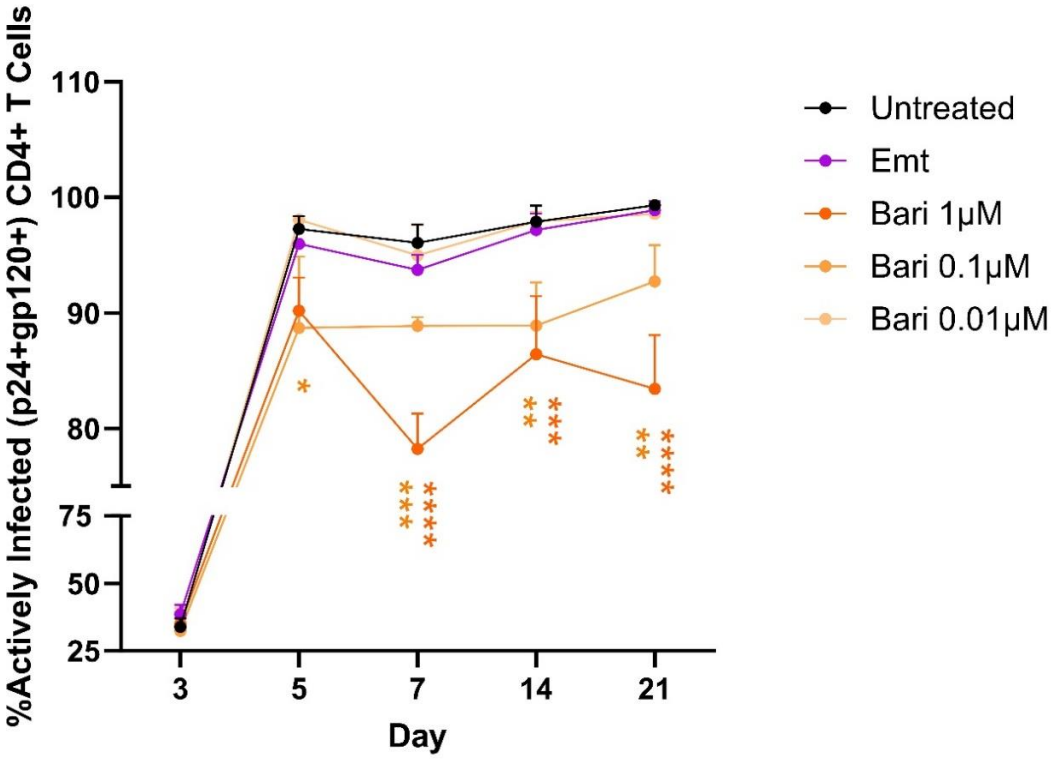

Figure 6 | Baricitinib Reduced Active Viral Replication in CD4+ T Cells

Baricitinib (0.1μM) significantly reduced active viral infection (p24+gp120+) in CD4+ T cells from day 5 through 21 compared to an HIV-1 89.6+ untreated control (day 5 p=0.0113, day 7 p=0.0154, day 14 p=0.0010, day 21 p=0.0340). Baricitinib (1μM) significantly reduced active viral infection in CD4+ T cells from day 7 through 21 compared to an infected, untreated control (day 7 p<0.0001, day 14 p<0.0001, day 21 p<0.0001).

We then examined BCL-2 expression within active infection and latent infection CD4+ T cells. We found that baricitinib (0.01-1μM) significantly reduced BCL-2 expression in active infection CD4+ T cells compared to an HIV-1 89.6+ untreated control, and that BCL-2 expression was significantly reduced by 1μM baricitinib by day 21 in latently infected CD4+ T cells (**Figure 7**) (ordinary one-way ANOVA with Dunnett's multiple comparisons test and a single pooled variance per week, GraphPad Prism v10.0.3, α=0.05). We also found that baricitinib reduced BCL-2 expression compared with a standard antiretroviral therapy, emtricitabine (1μM). Once again, these data indicate that short term immunomodulation severely impacts active viral infection whereas 3 weeks may be too short-term to see an impact on the lifespan of latently infected CD4+ T cells.

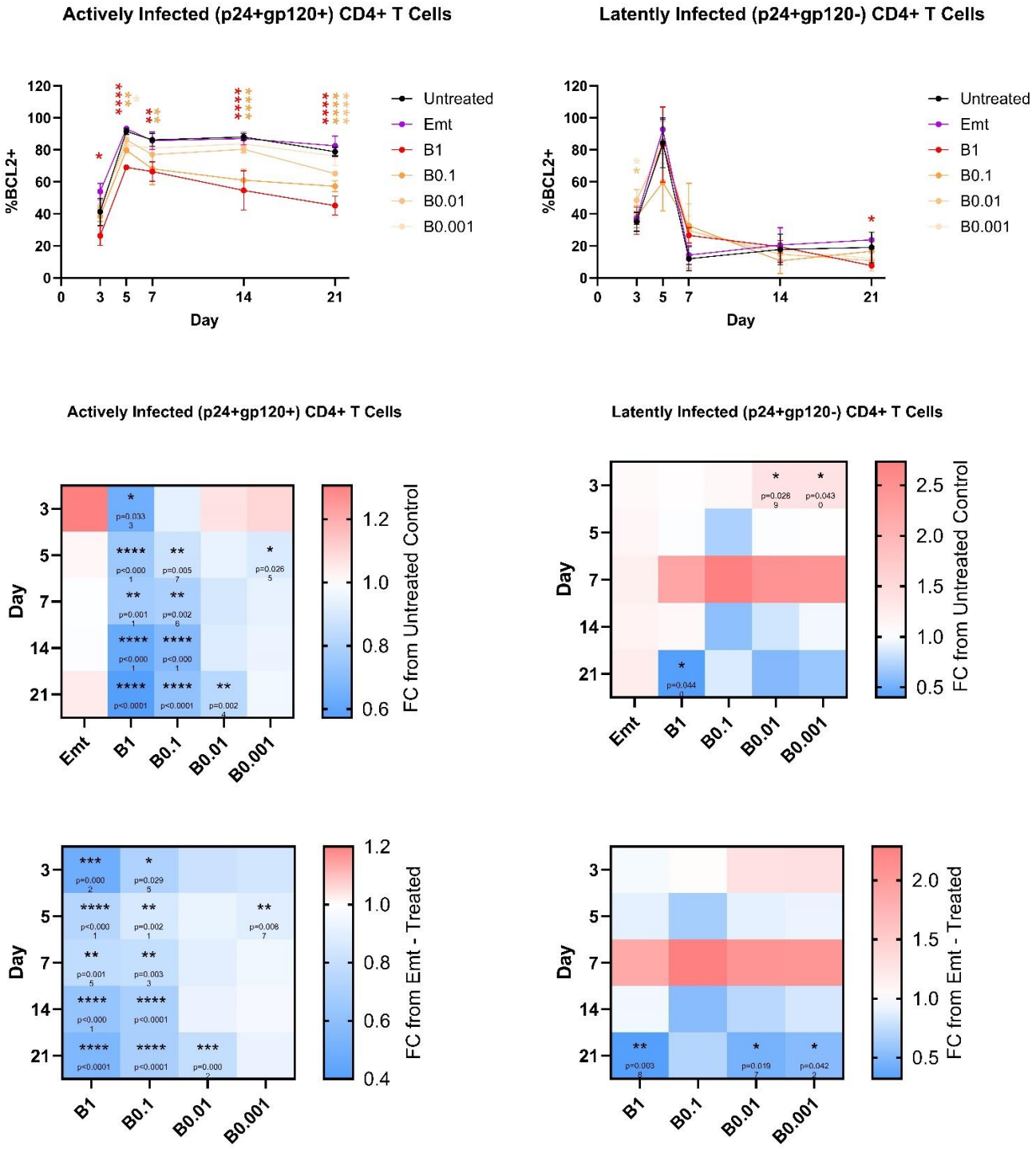

Figure 7 | Baricitinib Reduced BCL-2 in Active and Latently Infected CD4+ T Cells

Baricitinib significantly reduced BCL-2 expression in active infection (p24+gp120+) CD4+ T cells in a dose-dependent manner from day 3 of infection. Baricitinib (1μM) was able to reduce BCL-2 expression in latent infection (p24+gp120-) CD4+ T cells by day 21 of infection. Compared to emtricitabine (1μM), a

common antiretroviral, baricitinib was more effective at reducing BCL-2 expression in both active and latent infected CD4+ T cells.

We have partially assessed down-stream effects of baricitinib on soluble cytokine levels to construct a profile of factors that drive reservoir decay in response to baricitinib. The complete panel of soluble cytokines identified for assessment were CD163, IFN-α, TGF-β, IL-10, IL0-15, IL-18, IL-1α/β, IL-2, IL-6, IL-7, TNF-α, D-dimer, and CRP. We used cryopreserved supernatant from 21-day cultures of CD4+ T cells and macrophages (separately) treated with baricitinib at various concentrations (0.001-1 μM). CD4+ T cells were activated for 72 hours prior to experiment with PHA and IL-2 as stated above. We performed a meso scale multiplex assay, a type of sandwich immunoassay, in which soluble cytokines are detected using a 10-spot U-PLEX plate coated with cytokine specific biotinylated capture reagents coupled to U-PLEX linkers that are specific to 1 of 10 spots on the plate and electro chemiluminescent labels. The plates were analyzed via an MSD Meso Sector S 600 instrument and emission intensity was measured (Emory Multiplexed Immunoassay Core). Baricitinib did not impact IFN-α2a, IL-15, or IL-7 expression in CD4+ T cells. IL-15 expression is not reduced by baricitinib, so the ability of baricitinib to negate IL-15-mediated pSTAT5 expression is likely only due to Jak1 blockade. Baricitinib significantly reduced IL-6 and TNF-α expression over 21 days in a dose dependent manner within CD4+ T cells (**Figure 8**) (Mixed-effects model with Geisser-Greenhouse correction and Dunnett's multiple comparisons test, GraphPad Prism v10.0.3, α=0.05). All other cytokines must be analyzed, and experiments should be repeated with macrophages. Preliminary results in baricitinib-treated macrophages *in vitro* were variable (not shown).

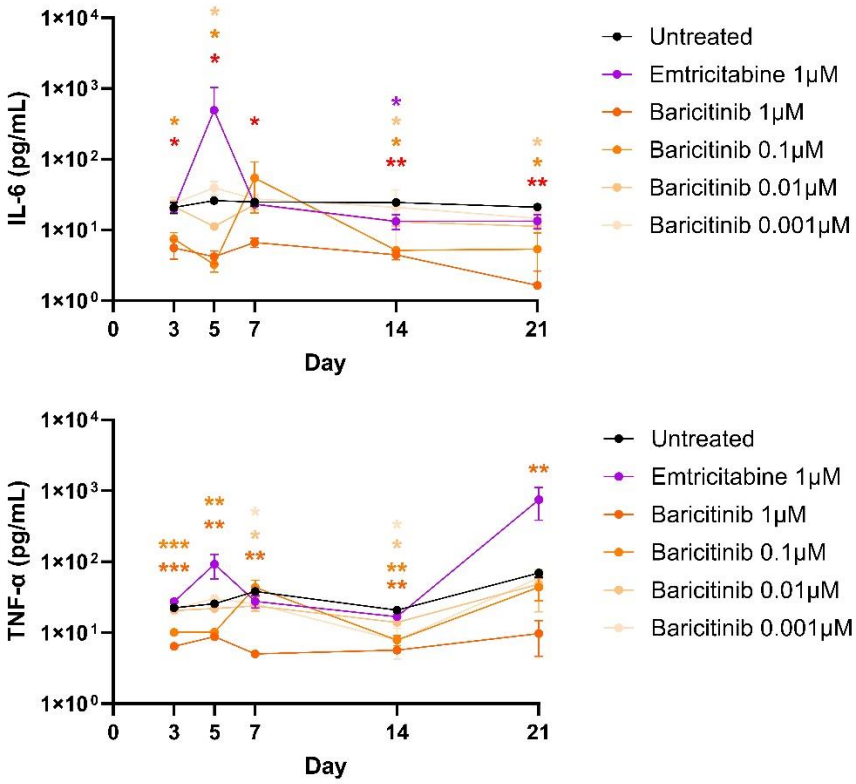

Figure 8 | Baricitinib Reduced IL-6 and TNF-α in CD4+ T Cells

Baricitinib (1μM) consistently reduced IL-6 (day 3 p=0.0270, day 5 p=0.0130, day 7 p=0.0162, day 14 p=0.0099, day 21 p=0.0045) and TNF-α (day 3 p=0.0003, day 5 p=0.0085, day 7 p=0.0035, day 14 p=0.0068, day 21 p=0.0042) compared to an HIV-1 89.6+ untreated control. Baricitinib (0.1μM) reduced IL-6 (day 3 p=0.0372, day 5 p=0.0134, day 14 p=0.0153, day 21 p=0.0176) and TNF-α (day 3 p=0.0007, day 5 p=0.0025, day 14 p=0.0014) compared to an untreated control. Baricitinib (0.01μM) reduced IL-6 (day 5 p=0.0301, day 14 p=0.0170, day 21 p=0.0210) and TNF-α (day 7 p=0.0245, day 14 p=0.03) compared to an untreated control. Baricitinib (0.001μM) reduced TNF-α (day 7 p=0.0171, day 14 p=0.0396) compared to an untreated control.

Conclusions and Future Directions

These data support a mainly immunomodulatory mechanism for baricitinib as opposed to direct acting antiviral activity. We observed that baricitinib seems to have a strong immediate effect on actively infected (p24+gp120+) CD4+ T cells and a lesser effect on latently infected cells. We hypothesize that a 21-day *in vitro* assay in primary may be too short to have a significant impact on the latent HIV reservoir. A stably infected cell line such as ACH-2 may be a viable *in vitro* alternative for long-term reservoir assays.

Likewise, an *in vivo* humanized murine or non-human primate model may be an appropriate option for long-term reservoir quantification. Reservoir quantification assays are planned to fully assess the impact on latently infected cells. Soluble cytokines IL-6 and TNF-α significantly decreased from baricitinib treatment. IL-6 and TNF-α are major pro-inflammatory cytokines that activate Jak STAT. We observed that baricitinib was able to block IL-15 mediated pSTAT5 production and that, downstream, BCL-2 expression was also reduced in actively and latently infected CD4+ T cells. Baricitinib-mediated BCL-2 reduction was also significantly more effective than emtricitabine. The size of the total reservoir (Alu-LTR qPCR), the size of the replication competent reservoir (integrated proviral DNA assay (IPDA)), and the lifespan of the transcriptionally silent and transcriptionally active reservoir should be measured over time in the presence of baricitinib (flow cytometry for BCL-2+/gp120+/-/p24+/- within primary CD4+ T cells and macrophages) to better assess the impact of baricitinib on the HIV reservoir.[42] Bystander assays should be performed to assess the effectiveness of baricitinib in blocking cell-to-cell transmission. Findings in the A5336 study (chapter III) suggest that Jak inhibitors reduce homeostatic proliferation, and so it would be noteworthy to assess the effect of baricitinib on IL-7 and CD127 expression in CD4+ T cells and macrophages. In total, these preliminary data provide a foundation for future studies that will support ongoing clinical trials with baricitinib for inflammatory diseases and in PWH in the Gavegnano Lab at Emory University (NCT05452564, NCT05849038, NCT05858515).

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Chapter V: Discussion

Persistent inflammation from chronic viral infection results in ongoing immune activation and eventual immune dysregulation. Chronic immune activation, of which Jak STAT is a key driver, induces expression of cellular markers and production of cytokines that 1) maintain and expand the HIV-1 reservoir and 2) exacerbate and contribute to inflammatory co-morbidities such as heart disease and neurocognitive dysfunction.[1-11]

There are no approved therapies at present that target the HIV-1 reservoir. When designing cure strategies for HIV-1, it is paramount to consider the entirety of the HIV-1 reservoir, ability of therapies to penetrate sanctuary sites, and feasibility in clinical translation in terms of cost, adherence, and access to PWH.

BCL-2, an anti-apoptotic protein expressed on HIV-1 reservoir cells, is a key factor in enhancing the lifespan of the HIV-1 reservoir. We proposed that treatment with Jak inhibitors would reduce BCL-2 expression and result in reservoir decay because BCL-2 expression is promoted by pSTAT5, a product of Jak STAT.

Our goal in analyzing the phase 2a trial (A5336) was to assess the potential of Jak inhibitors to decay the HIV-1 reservoir. Previous work *in vitro* and *in vivo* showed that Jak inhibitors reduce immune activation but had not addressed the quantifiable effect on HIV provirus.[4, 5, 7, 12] From a wider scope, this analysis was also to determine the suitability of continued assessment of Jak inhibitors for HIV cure in human clinical trials.

We observed a significant decrease in total proviral HIV DNA (IPDA) ($p=0.0344$) from weeks 5-12 in a subset of PWH with a high baseline reservoir. Using the reservoir decay rate from weeks 5-12, we estimate 99.99% reservoir reduction in 2.83 years of ruxolitinib treatment. Ruxolitinib decreased BCL-2+Ki-67+ CD4+ T_N, pSTAT5+ monocytes, pSTAT3+ monocytes, and CD25+ CD8+ T_{TM} and increased CD127+ CD8+ T_{TD}, all of which were associated with HIV-1 reservoir decay. From these identified

markers, we propose that Jak inhibitor ruxolitinib reduced the HIV-1 reservoir via reduction of reservoir lifespan, homeostatic proliferation, and reseeding capabilities.

In assessing the viral reservoir and neurocognitive scores of PWH, our goal was to determine if HIV DNA in the CSF correlated with global or domains of neurocognitive function. Previous work showed HIV DNA detection in the CSF and associated it with lower cognition; however, the specific domains of cognition were not explored.[13] Total, 3' and 5' defective HIV proviral DNA (IPDA) were significantly higher in the CSF vs PBMC. Higher total and 3' HIV DNA (IPDA) in the CSF correlated with worse executive function (both p=0.044). We examined a trend of higher total and 3' defective HIV DNA (IPDA) in CSF correlated with worse learning scores (both p=0.0562). We also observed a trend of higher total HIV DNA (IPDA) correlated with worse learning and memory scores in PBMC (both p=0.0595). This highlights a potential link between HIV in the periphery and CNS.

This study provided supporting evidence of the HIV reservoir in the CNS and novel insights on HIV reservoir correlations to specific neurocognitive domain performance. As CSF is a relatively non-invasive procedure compared to previously used cadaver brains, this study also serves as a proof of concept for CSF as a clinical tool in neurotherapeutics for PWH.

HIV-1 and subsequent inflammatory effect on cognition may depend on level of exposure to foreign bodies and cytokines and cellular composition in the different brain regions. In PWH, while ART blocks active replication, inflammatory cytokines and viral proteins transcribed from both defective and intact proviruses disrupt blood-brain-barrier (BBB) integrity and pass into the CNS compartment.[14-17] This would also allow quiescent intact reservoir cells to be increasingly trafficked to the CNS, ultimately resulting in microglial and astrocyte activation and dendritic synaptic shortening (**Figure 1**).[18-21] Passive trafficking of reservoir cells with intact virus from the periphery to the CNS would only increase inflammation as the reservoir cells would reactivate and produce replication-competent virus to spread infection and neuroinflammation. Microglial percentage in different parts of the brain may play a role in

how severely HIV is able to affect associated cognitive functioning, but further research is required to investigate this possibility.

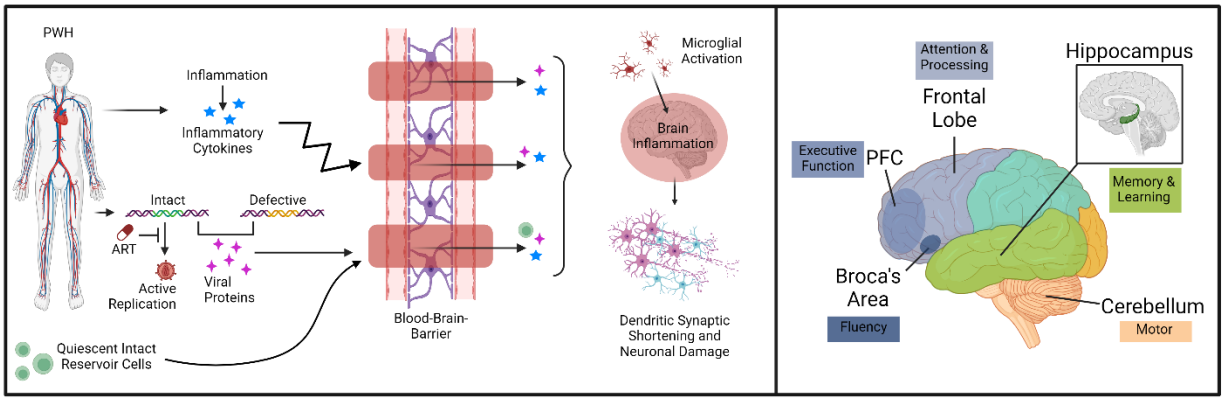

Figure 1 | Potential Route of HIV-Driven Neuroinflammation and Map of Cognitive Function

An in-progress project in the Gavegnano lab is the characterization of baricitinib's ability to decay the HIV-1 reservoir *in vitro.* Preliminary data was collected in the context of ruxolitinib's function *in vivo*, to assess if baricitinib may have the same effect. Baricitinib is a second-generation Jak inhibitor with a more favorable safety profile, renal clearance, and once daily dosing compared to first-generation ruxolitinib. In terms of potential drug interactions and adherence, baricitinib is a better candidate for future studies.

We found that baricitinib (1μM) reduced IL-15 mediated pSTAT5 production in CEMx174 cells. Baricitinib had an IC50 of 0.0209μM in CD4+ T cells. Baricitinib (0.1 and 1μM) significantly reduced the percent of HIV-GFP transduced CD4+ T cells compared to untreated cells. Baricitinib (0.1-100μM) significantly reduced the MFI of HIV-GFP transduced CD4+ T cells compared to untreated cells. Baricitinib did not affect late RT copy number of HIV-GFP DNA or mRNA in CD4+ T cells or macrophages. Baricitinib (0.1 and 1μM) significantly decreases active viral replication (p24+ gp120+) in CD4+ T cells, and furthermore, significantly decreases BCL-2 expression within this subset in a dose dependent manner from 0.01-1μM.-Model is based on decay rate occurring post-treatment in A5336.

There are limitations to our model of reservoir decay. The model only accounts for the peripheral reservoir. The overall decay rate (weeks 0-12) estimated 99.99% decay in 6.96 years. While this is markedly improved over previous decay estimates (44 months in resting CD4+ T cells; 73.4 years for eradication only considering the CD4+ T cell reservoir), it may reflect T cell demargination and so the estimate may be skewed. (PMID: 10229227, 12754504) A longer duration of treatment and data from the gut and CNS reservoirs are required to know the true reservoir decay rate of ruxolitinib.

In July 2023, Drs Asier Sáez-Cirión and Alexandra Calmy from the Pasteur Institute (Paris, France) presented a case study at the International Conference on HIV in Brisbane Australia (IAS2023) highlighting an anonymous PWH that received a wild-type allogenic stem cell transplant and was 20-months in remission following analytical treatment interruption (ATI).[22] Sáez-Cirión et al. describe a PWH living with HIV since 1990 who received a wild-type allogenic stem cell transplant. Within 1 month, the person developed Graft Versus Host Disease (GVHD) and was administered an immunosuppressant cocktail including methotrexate, cyclosporine, and ruxolitinib (10 mg, twice daily). After 1 month, only ruxolitinib was administered and maintained for 1.6 years, at which point it was removed. Upon removal, the person experienced an increase in immune activation markers, prompting the continuation of ruxolitinib about 1.5 months after. This continuation resulted in a reversal of immune activation markers. 1 month later, antiretroviral therapy (ART) was withdrawn but ruxolitinib administration remained consistent. As of June 2024, the person is 2.75 years in remission (ongoing) with no viral rebound or detectable viral RNA or DNA.

This patient was dubbed the ‘Geneva Patient’ and tentatively joined a small cohort of HIV cure patients worldwide including Timothy Ray Brown (the Berlin Patient), Adam Castillejo (the London Patient), Paul Edmonds (the City of Hope Patient), the Dusseldorf Patient, and the New York Patient. The existing cure patients all received a CCR5Δ32 allogenic stem cell or haplo-cord transplant to treat various forms of leukemia and lymphoma.[23-27] This is critical as HIV-1 depends on engagement with a co-receptor (CCR5/CXCR4) to expose gp41 and trigger membrane fusion for entry into the cell.[28, 29] Historically, PWH

who have received wild type allogenic stem cells transplants experience viral rebound upon ATI.[30] Given our findings that ruxolitinib reduced the HIV-1 reservoir within 12 weeks of treatment (10mg, twice daily), it is likely that ruxolitinib administered for GVHD in the Geneva Patient is a major factor in their ongoing viral remission post-ATI.

In A5336, PWH on an ART regimen containing cobicistat were excluded. Cobicistat and other protease inhibitors are metabolized via CYP3A4 in the liver, which is also responsible for clearance of ruxolitinib. PWH on an ART regimen containing efavirenz were stratified between groups. Efavirenz is an inducer of CYP3A4. Post-conclusion of A5336, it was determined that co-administration with efavirenz resulted in accelerated metabolism of ruxolitinib.[31] For these reasons, it is not recommended that ruxolitinib be co-administered with an ART regimen containing protease inhibitors or efavirenz. However, baricitinib does not have these limitations as it is cleared renally and avoids interaction with CYP3A4, which makes it a more favorable candidate moving forward with Jak inhibitor studies.

Baricitinib treatment can result in an increased risk of infection and should not be given to patients with active tuberculosis. The baricitinib package insert states that the following risks were found with a different Jak inhibitor: higher rates of all-cause mortality, lymphomas and lung cancers, cardiovascular death, myocardial infarction, stroke, pulmonary embolism, and venous and arterial thrombosis in rheumatoid arthritis patients (Olumiant package insert, reference ID: 4981738). Ruxolitinib treatment may result in thrombocytopenia, anemia, neutropenia, and higher risk of infection (Jakafi package insert, reference ID: 4860976). To offset the risk of infection, patients should be monitored for signs of opportunistic infection and treated promptly. Changes in lymphocyte, neutrophil, and hemoglobin counts should be monitored and treatment should be adjusted, interrupted, or an infusion should be administered to correct for effects of thrombocytopenia, anemia, and neutropenia. Overall, the direct potential side effects of baricitinib and ruxolitinib can be identified by medical professionals through patient observation and would likely be treatable.

Our findings suggest that a relatively short-term treatment plan with Jak 1/2 inhibitors may be enough to substantially decay the HIV-1 reservoir and reverse inflammation and immune activation in PWH. Ruxolitinib is approved for chronic long-term use but is only recommended to maintain treatment in patients for whom the benefits outweigh potential risk. Baricitinib is also approved for chronic long-term use with no secondary recommendations. Caution should be exercised when prescribing Jak 1/2 inhibitors to patients with renal or hepatic impairment. Baricitinib interacts with strong organic anion transporter 3 (OAT3) inhibitors including HIV integrase inhibitor cabotegravir.[32] Ruxolitinib interacts with fluconazole and strong CYP3A4 inhibitors including antibiotics clarithromycin, erythromycin, and isoniazid, cancer treatments tamoxifen and irinotecan, several hypertension medications, and HIV-1 therapies ritonavir and delavirdine.[33] These interactions should be considered in potential patients with therapy cocktails. Jak inhibitor therapy has been historically well -maintained in a long-term setting without development of opportunistic infections.

Further studies are required to assess the impact of Jak inhibitors on inflammation-driven comorbidities and immunosenescence and inflammaging in PWH. Given findings in the literature and presented here, it is likely that Jak inhibitors would have a positive effect on the incidence rate of inflammatory comorbidities such as CVD and neurocognitive dysfunction as well as accelerated aging and immune dysfunction due to chronic immune activation.

The ultimate impact of this work in the field is the identification of cellular markers that are associated with HIV-1 reservoir decay which progress the overarching goal to develop treatment strategies that lead to elimination of HIV. More focused questions can be pursued concerning these markers in the context of HIV cure and drug development against these identified targets. These data provide a foundation for the analyses of ongoing jak inhibitor trials against HIV in humans (NCT05452564, NCT05849038). Viral reservoir decay, either complete amelioration or severe depletion, would have significant positive impacts on eradication and control of the HIV epidemic. Chronic immune activation and subsequent inflammation, sans contributing factors, would be significantly reduced. In the case of minimal remaining

reservoir, improved cytotoxic function from reduced inflammation may clear remaining infected cells in the event of ART interruption.

Additionally, our findings of BCL-2, pSTAT5, and pSTAT3 reduction *in vitro* and *in vivo* have promising implications for cancer therapy to reduce tumorigenic cells expressing BCL-2 and also for use in combination therapy with senolytics to reduce immune senescence.[34-41] In the landscape of viral infections, BCL-2 is upregulated by gamma herpes virus, adenovirus, Zaire ebolavirus, human papillomavirus-induced squamous cell carcinoma of the cervix, picornavirus, and poxvirus, and presents a potential target for Jak inhibitor therapy.

HIV-1 infection does not exist in a vacuum, and so co-infections must be considered in potential therapeutic regimens. There are several common co-infections in PWH: hepatitis C virus (HCV), hepatitis B virus (HBV), human T-lymphocytic virus 1 (HTLV-1), human herpes virus 8 (HHV-8, Kaposi's sarcoma-associated herpesvirus), human papillomavirus (HPV), *Mycobacterium tuberculosis*, *Cryptococcus neoformans*, *Plasmodium falciparum*, Epstein-Barr virus (EBV, human herpesvirus 4), cytomegalovirus (CMV, human herpesvirus 5), and emerging long COVID-19.[42-45] It is expected that these individuals would be able to tolerate Jak inhibitor therapy, but the multifaceting impact of Jak inhibitors on the pathogenesis of other infections should also be considered.

In immunocompromised individuals, like PWH, latent EBV and CMV are likely to reactivate.[46, 47] One study of ruxolitinib in PWH examined effect on CMV co-infection and found that while ruxolitinib did not reduce CMV DNA levels, CMV was positively correlated with inflammation.[48] Co-infection with HTLV-1 can result in the development of adult T-cell leukemia (ATLL).[49] It's been determined that PWH were more likely to be seropositive for HHV-8, which was reactivated by inflammatory cytokine production by HIV-1-infected T cells.[50, 51] One study found that HPV is more likely to reactivate in women with HIV, while Georgescu et al. purported that chronic inflammation from HPV can induce oxidative stress, promoting carcinogenesis.[52, 53] *P. falciparum* can induce a strong inflammatory response in the host, and in vivo observations of people with related *P. vivax* have shown *Plasmodium*-induced immune modulation

resulting in activated and exhausted T cells. In the setting of co-infection, with a basally inflammation environment, the proinflammatory response to *P. falciparum* may be exaggerated and lead to severe malaria.[54]

Many co-infections can contribute to systemic inflammation and exacerbate pathogenesis of both pathogens. In inflammation-driven co-infections, Jak inhibitors would likely have a bleed over therapeutic effect. However, there are some co-infection pathogens that would be aided by Jak inhibition. During *M. tuberculosis* infection, bacteria are internalized by alveolar macrophages which causes an inflammatory response. In late infection, *M. tuberculosis* can alter the immune response to a more anti-inflammatory response which allows for sustained infection.[55] In PWH and *M. tuberculosis*, Jak inhibitors would likely enhance *M. tuberculosis* infection.

Many co-infection pathogens can cause neuroinflammation, which not only promotes the HIV CNS reservoir, but can also lead to neurologic deficits. EBV is neurotropic and has been found to infect glial and blood-brain barrier endothelial cells.[56] Tiwari et al. report on a hypothesis that EBV-induced oxidative cellular stress and cell cycle malfunctioning result in Alzheimer's disease neurodegeneration.[56-58] ART serves to suppress HIV replication but exacerbates CMV, and CMV contributes to immunosenescence, which can support HIV reservoir maintenance.[59] CMV can cause neurological disease in PWH, commonly manifesting as retinitis, encephalitis, polyradiculopathy, and multifocal neuropathy.[60] HTLV-1 viral protein Tax affects the function of astrocytes and neuronal cells, resulting in HTLV-1-associated myelopathy.[49] *C. neoformans* can be internalized by microglia and cause neuroinflammation which may result in cryptococcal meningitis that causes neurologic deficits.[61] Much is still cryptic about the phenomenon of long COVID-19. It is hypothesized that prolonged inflammation from COVID-19 infection may cause lingering effects. At the 2024 Conference on Retroviruses and Opportunistic Infections (CROI 2024) one study by Horberg et al. revealed that the incidence rate of long COVID-19 in 19% higher in PWH, and another study by Yang X et al. showed that long COVID-19 presents heavily as circulatory disease and mental and behavioral deficits in PWH.[43, 62] Disruption of the blood-brain barrier in conjunction with

systemic inflammation has been implicated in long COVID-19-associated cognitive impairment.[63] Jak inhibitors may play a protective role for neurologic function in many co-infections of PWH. Overall, Jak inhibitor therapy in PWH with co-infections may have more numerous positive impacts beyond HIV decay and reduced systemic inflammation described herein.

It is not likely that Jak inhibitors would be used for prevention of viral transmission. The utility of Jak inhibitors lies in immune modulation which can mitigate disease severity, reset the immunologic milieu, and in the case of HIV, reduce the viral reservoir. Transmission hindrance would still be reliant on pre-exposure prophylaxis (PrEP), treatment as prevention (TasP), condoms, safe needle practices and disposal, and post-exposure prophylaxis (PEP).

The future of this work is multivariate and can extend to many cross disciplinary domains. It would be informative to examine combination therapy using Jak inhibitors and BH3 mimetics to elucidate potential synergistic effects on BCL-2 reduction. Especially considering the prevalence of BCL-2 inhibitors and their success in HIV cure studies at present.[64-70] Within future human trials of Jak inhibitors in PWH with a longer treatment duration, reservoir reduction should be observed and the rate compared to what we have proposed herein. It would be enlightening to longitudinally observe PWH with concurrent inflammatory disorders prescribed Jak inhibitors such as PWH with primary myelofibrosis, post-polycythemia vera myelofibrosis, and post-essential thrombocythemia myelofibrosis taking ruxolitinib, as well as PWH with severe COVID-19, alopecia areata, rheumatoid arthritis taking baricitinib.

There are several clinical trials that are recruiting or completed awaiting reporting that will better inform the nuances and direction of the work presented here (Table 1). These trials will reveal information on the CNS HIV reservoir in children and young adults with HIV and the effect baricitinib may have on the CNS HIV reservoir, which will be critical in understanding how Jak inhibitors may alter the CNS HIV reservoir and the impact it may have on neurocognition. Identifying how baricitinib affects inflammation and depression in PWH will likely reveal important data that will shape our understanding of how neurocognitive dysfunction may be targeted in PWH. We identified BCL-2 reduction as a driver of HIV

reservoir reduction, and a trial examining if Venetoclax will reduce the HIV reservoir will be insightful into how prominent BCL-2 reduction is in HIV reservoir reduction.

Beyond direct treatment for HIV, there are many trials examining Jak inhibitors for other viral infections like COVID-19 and long COVID-19 as well as inflammatory-driven or exacerbated diseases such as community-acquired pneumonia, Alzheimer's disease, amyotrophic lateral sclerosis, neuromyelitis optica spectrum disorders, premalignant breast disease, and T and natural killer (NK) cell lymphoma. Inflammatory-driven comorbidities are common in PWH, even those who are virally suppressed, due to basal chronic inflammation from the HIV reservoir. Not only will these studies uncover the impact of blocking Jak STAT on these diseases but will also provide further insight into how the quality of life may be improved for PWH.

Table 1 | Current Relevant Clinical Trials for Advancement of HIV Reservoir Reduction and Other Applications for Jak Inhibitors in Viral Infections

ClinicalTrials.gov ID	Phase	Indication	Therapy
NCT05452564*	II	CNS HIV Reservoir Reduction	Baricitinib
NCT05849038*	II	Inflammation and Depression in PWH	Baricitinib
NCT05668026	I/II	HIV Reservoir Reduction in PWH on ART	Venetoclax
NCT02735707	III	Community-Acquired Pneumonia	Baricitinib
NCT05189106	I/II	Alzheimer's Disease and Amyotrophic Lateral Sclerosis	Baricitinib
NCT05074420	III	Children with COVID-19	Baricitinib
NCT05792462	I/II	Neuromyelitis Optica Spectrum Disorders	Baricitinib
NCT02928978	II	Premalignant Breast Disease	Ruxolitinib
NCT02974647	II	Relapsed or Refractory T or NK Cell Lymphoma	Ruxolitinib
NCT03416790	Observational	Evaluation of CNS HIV Reservoir in Perinatally-Infected Youth and Young Adults	NA
NCT05858515*	III	Long COVID-19	Baricitinib

* Denotes clinical trials that will be conducted by, or in collaboration with, the Gavegnano Lab at Emory University. CNS = central nervous system, HIV = human immunodeficiency virus, PWH = people with HIV, ART = antiretroviral therapy, NK = natural killer, NA = not applicable.

The exploration of baricitinib to treat severe pneumonia, first related to severe COVID-19 has evolved to a study of severe influenza pneumonia conducted by Dutch researchers at the University Medical Center Utrecht through the IMPRINT study (immune modulation platform for influenza treatment) funded by ZonMw, an independent government body partnership between ZorgOnderzoek Nederland (care research Netherlands) and the medical sciences domain of the Dutch research council.[71] Pathogenesis of many viral infections is tangled with inflammation, and Jak inhibitors may be an emerging therapeutic to target disease severity if not the viruses directly.

Repurposing FDA approved therapies to target the HIV-1 reservoir is a valid approach to cure-based therapy. We have provided empirical evidence that rufinamide and Jak inhibitors baricitinib and ruxolitinib affect factors maintaining the HIV-1 reservoir in macrophages and T cells, including reservoir lifespan, immune activation, homeostatic proliferation, and immunosenescence.

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Appendices

Appendix I: *Ex Vivo* Differentiation of Resting CD4+ T Lymphocytes Enhances Detection of Replication Competent HIV-1 in Viral Outgrowth Assays

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Abstract

Quantifying the number of cells harboring inducible and replication competent HIV-1 provirus is critical to evaluating HIV-1 cure interventions, but precise quantification of the latent reservoir has proven to be technically challenging. Existing protocols to quantify the frequency of replication-competent HIV-1 in resting CD4+ T cells from long-term ART treated individuals have helped to investigate the dynamics of reservoir stability, however these approaches have significant barriers to the induction of HIV-1 expression required to effectively evaluate the intact reservoir. Differentiation of CD4+ T cells to an effector memory phenotype is a successful strategy for promoting latency reversal in vitro, and significantly enhances the performance and sensitivity of viral outgrowth assays.

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Appendix II: A Rationale and Approach to the Development of Specific Treatments for HIV Associated Neurocognitive Impairment

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Abstract

Neurocognitive impairment (NCI) associated with HIV infection of the brain impacts a large proportion of people with HIV (PWH) regardless of antiretroviral therapy (ART). While the number of PWH and severe NCI has dropped considerably with the introduction of ART, the sole use of ART is not sufficient to prevent or arrest NCI in many PWH. As the HIV field continues to investigate cure strategies, adjunctive therapies are greatly needed. HIV imaging, cerebrospinal fluid, and pathological studies point to the presence of continual inflammation, and the presence of HIV RNA, DNA, and proteins in the brain despite ART. Clinical trials exploring potential adjunctive therapeutics for the treatment of HIV NCI over the last few decades have had limited success. Ideally, future research and development of novel compounds need to address both the HIV replication and neuroinflammation associated with HIV infection in the brain. Brain mononuclear phagocytes (MPs) are the primary instigators of inflammation and HIV protein expression; therefore, adjunctive treatments that act on MPs, such as immunomodulating agents, look promising. In this review, we will highlight recent developments of innovative therapies and discuss future approaches for HIV NCI treatment.

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Appendix III: Honokiol Hexafluoro Confers Reversal of Neuropathological Markers of HIV Infection in a Murine SCID Model

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Abstract

Cognitive impairment remains a persistent challenge in people living with HIV (PWLH) despite antiretroviral therapy (ART) due to ART's inability to eliminate brain HIV. HIV-induced cognitive dysfunction results from immune dysregulation, ongoing neuroinflammation, and the continuous virus presence, collectively contributing to cognitive deficits. Therefore, adjunctive therapies are needed to reduce cerebral HIV reservoirs, mitigate neuroinflammation, and impede cognitive dysfunction progression. Our study focused on Honokiol, known for its anti-inflammatory and neuroprotective properties, in an experimental mouse model simulating HIV-induced cognitive dysfunction. Using Honokiol Hexafluoro (HH), a synthetic analogue, we comprehensively evaluated its potential to ameliorate cognitive dysfunction and cerebral pathology in HIV-associated cognitive dysfunction. Our findings showed that HH treatment effectively reversed HIV-induced cognitive dysfunction, concurrently suppressing astrocyte activation, restoring neuronal dendritic arborization, and reducing microglial activation. Furthermore, HH remodeled the metabolic profile of HIV-infected human monocyte-derived macrophages, resulting in decreased activation and the promotion of a quiescent state *in vitro*.

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