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Single cell RNA transcriptome response to fentanyl use in persons with HIV infection

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The US is experiencing a major drug epidemic largely attributed to synthetic opioids including fentanyl. We considered how illicit fentanyl use in persons living with HIV impacted gene expression profiles in the peripheral blood. Whole blood was collected from 10 HIV-positive adults with a diagnosis of opioid use disorder (OUD) and 7 HIV-positive non-opioid using controls. Mean HIV viral loads were higher for the OUD$^+$ group, although this difference did not reach statistical significance (2.44 versus 1.00 $\log_{10}$ copies/mL; $p = 0.12$; two-sample T test). 216,641 cells were evaluated by single cell RNAseq. Cell frequencies were not different by opioid status except for NK cells (lower for opioid use; $p = 0.0045$). For CD4$^+$ T lymphocytes, 10 differentially expressed genes (DEGs) were higher and 8 were lower in opioid-positive persons. In CD8$^+$ T lymphocytes, there were 15 higher and 13 lower DEGs for opioid-positive study participants. In monocytes, 72 DEGs were higher and 37 DEGs were lower in opioid-positive participants. In B lymphocytes, 17 DEGs were higher and 10 were lower in opioid-positive versus opioid-negative participants. These findings highlight multiple pathways by which opioid use may contribute to HIV pathogenesis. Rigorous characterization of the interactions among HIV, opioids, and host cells can improve clinical management paradigms, facilitate rational public health policies, and reveal additional pathways for novel target-specific therapeutic interventions.

Keywords Opioid, Fentanyl, Drug use, HIV, HIV/HCV coinfection, Single cell transcriptome

There are more than 20 million persons in the United States (US) with substance use disorder (SUD)[1]. As of 2018, there were an estimated 3.69 million people who inject drugs (PWID) in the US (1.46% of the adult population)[2]. In recent years, the number of drug overdose deaths has increased significantly[3,4]. From July 2016 through September 2017, more than 142,000 emergency department visits involved suspected opioid-involved overdoses[5]. Rates increased across demographic groups and all regions of the US. Among over 70,000 drug overdose deaths that occurred in 2017, 67.8% involved an opioid[6]. By 2019, 70.6% of drug overdose deaths involved opioids, and 51.5% involved synthetic opioids[7]. Contributing to these increased deaths is the dramatic increase in the availability of heroin and illicitly manufactured fentanyl and fentanyl analogs[8–13]. In Ohio, > 90% of unintentional overdose fatalities were positive for fentanyl[14]. Increased drug use has led to significant increases in incident HIV and hepatitis C virus (HCV) infections as exemplified by the outbreak of HIV and HCV in Scott County, Indiana in 2015, as well as across Appalachia and parts of New England[9,15–23].

Opioids exert their pharmacological effects as analgesics and regulators of immune function. Several drugs of abuse suppress immune responses[24,25]. Possible mechanisms include impaired function of natural killer

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cells, T cells, B cells, neutrophils, dendritic cells, and/or macrophages, altered expression of cytokines and chemokines, and the weakened integrity of the intestinal barrier, all of which contribute to decreased ability to control pathogens and limit their subsequent clearance. Opioid receptors are expressed on various immune cells, such as lymphocytes, macrophages, neutrophils, and monocytes[25,26]. Underappreciated is the fact that many commonly abused substances such as cocaine, methamphetamine, heroin, and alcohol promote HIV replication and virus-mediated pathology. For instance, morphine increased CCR5 receptor expression, inhibited β chemokine production, and enhanced HIV replication in blood-derived macrophages[27,28]. Methadone enhances HIV infection of fetal microglia, blood monocyte-derived macrophages, and latently infected peripheral blood mononuclear cells (PBMCs) from HIV-infected patients[29]. Morphine and heroin both trigger HIV reactivation in latently infected T lymphoblasts[30]. Endogenous opioid peptides also enhance HIV replication[31,32]. We and others recently reported that fentanyl resulted in significantly elevated levels of HIV replication and chemokine co-receptor expression in cell types that are relevant to HIV pathogenesis[33–37]. These data demonstrate that the synthetic opioid fentanyl can promote viral replication in vitro.

While fentanyl is commonly detected in persons experiencing opioid overdose, its effects on viral replication in vivo are poorly characterized. Thus, we evaluated the effects of illicit opioid use on cellular gene expression using single cell transcriptomics.

Methods

Patient population

From March 2019 to December 2024, a non-interventional, observational cohort study of persons with fentanyl use/exposure was conducted in the greater Cincinnati area. The inclusion criteria were adults > 18 years of age who presented to the UC Emergency Department (ED) for unintentional overdose. Exclusion criteria include intentional overdose (suicide attempt) with non-opioids and inability or not willing to provide informed consent. HIV and HCV status were based on patient self-report and review of electronic medical records. Opioid use disorder (OUD) was defined as OUD listed in the electronic medical record and/or scoring positive for opioids on the NIDA-Modified ASSIST tool.

Whole blood was collected from 17 HIV-positive adults, including 10 with a diagnosis of OUD and 7 without OUD. Participants provided written informed consent prior to any study procedures and were compensated $20 for their participation. The University of Cincinnati Institutional Review Board approved this study as 2019_0584. All research involving human research participants was performed in accordance with the Declaration of Helsinki.

Sample processing

PBMCs were isolated from ~ 40 mLs of whole blood collected in BD Vacutainer Cell Prep Tubes according to the manufacturer's protocol and stored in fetal bovine serum (FBS) with 10% dimethyl sulfoxide in liquid nitrogen. To minimize RNA degradation, all samples were processed from collection to freezing within 4 h (mean of 2.4 h; range: 1.7–3.1 h). To prepare PBMCs for 10 × single cell labeling, cells were thawed and counted. Cell viability was confirmed by trypan blue staining as greater than 90%, and the concentration was adjusted to 1×10^6 cells/mL in Dulbecco's phosphate-buffered saline.

Drug use screening

Plasma samples were screened by a targeted Liquid Chromatography Mass Spectrometry (LC–MS/MS) approach for 95 commonly used drugs and volatile compounds such as ethanol by Gas Chromatography Mass Spectrometry (GC/MS). For LC–MS/MS analysis, 0.5 mL of the sample was diluted with buffer and subjected to solid-phase extraction using United Chemical Technologies Clean Screen extraction columns. For GC/MS analysis, 0.1 mL of sample was placed into a headspace vial for analysis. The LC–MS/MS analysis used targeted transitions while the GC/MS was operated in full-scan. This screening process can detect drugs from the following classes: opioids, medications for the treatment of OUD, stimulants, benzodiazepines, barbiturates, antidepressants, hallucinogens, over-the-counter drugs, marijuana, and anti-epileptics. Confirmation and quantitation were performed by LC–MS/MS when the sample volume was sufficient. For samples with multiple positive results, stimulants and opioids (including fentanyl and fentanyl analogs) were prioritized for confirmation testing.

Single cell RNAseq analysis

Single cell barcoding and complementary DNA (cDNA) and V(D)J library preparation were performed at the Gene Expression Core facility (Cincinnati Children's Hospital Medical Center) using Chromium Next GEM Single Cell 5' Reagent Kits v2 (Dual index) according to the manufacturer's protocol. Briefly, cell suspensions, beads, master mix, and partitioning oil were loaded onto a "K" chip for a targeted output of 10,000 cells per library and run on the Chromium X. Reverse transcription was performed at 53 °C for 45 min and cDNA was amplified for 14 cycles using a Bio-Rad C1000 Touch thermocycler followed by cDNA size selection using SpriSelect beads (Beckman Coulter, USA). cDNA quality was confirmed with an Agilent Bioanalyzer High Sensitivity chip. DNA fragmentation, end-repair, A-tailing, and ligation of sequencing adapters were performed per the manufacturer's protocol (10× Genomics, USA). Libraries were run on a NovaSeq 6000 S1 or S4 flow cell (depending on the number of samples) at the DNA sequencing Core Facility at the Cincinnati Children's Hospital Medical Center.

Raw base call files were de-multiplexed with Cell Ranger[38] v6.0.0 mkfastq. Reads were aligned to the human reference genome GRCh38, and gene expression was quantified using Cell Ranger count. Further data analysis was carried out with Seurat[39,40] v4.0.5 in R v4.1.2[41]. Cells displaying more than 20% mitochondrial gene expression—or fewer than 100 total expressed genes—were excluded from analysis. Gene expression counts were normalized with the NormalizeData function in Seurat, which uses a logarithmic normalization

method whereby gene counts for each cell are divided by its total counts and natural log-transformed using log1p and multiplied by a scale factor of 10,000. Samples were integrated using FindIntegrationAnchors and IntegrateData functions from Seurat. This integrated dataset was used for principal component analysis, variable gene identification, Shared Nearest Neighbor (SNN) clustering analysis, and Uniform Manifold Approximation and Projection (UMAP). Cell types and clusters were annotated using a high-quality PBMC dataset. Briefly, cell annotations were transferred from a multi-model PBMC reference dataset using FindTransferAnchors and MapQuery in Seurat[42].

Differentially expressed genes (DEGs) were identified using the Wilcoxon rank sum test with the following inclusion criteria: 1) absolute $\log_2$ fold change ≥ 0.25, 2) minimum of 10% of cells expressing the gene in both compared groups of cells, and 3) Bonferroni-adjusted p-value < 0.01. For DEG analysis, mitochondrial genes and T cell / B cell V(D)J segments as annotated by Ensembl release 112[43] were removed. The effects of HCV infection and gender were regressed out to mitigate the effects of these covariates using the SCTransform function of Seurat. Plots were generated using R ggplot2 v3.3.5[44], ggpubr v0.4.0[45], and Seurat. Enrichment analysis was performed using Enrichr with terms set to 10 each for GO Biological Processes 2021, MGI Mammalian Phenotype Level 4 2021, and KEGG 2021 Human[46–49].

Results

Patient characteristics

Blood samples were collected from 17 HIV-positive individuals including 12 with HCV co-infection (Table 1). The mean age was 39.5 years (range: 20–65). Fourteen participants (82%) were male. Eleven participants were white/non-Hispanic, 5 black/non-Hispanic, and 1 white/Hispanic. For all study participants, the mean $CD4^+$ T cell count was 471.8 cells/mm^3 (range: 194–873). The mean HIV viral load was 1.85 $\log_{10}$ copies/mL (range: 0.00–5.20). Among those with HCV co-infection, the mean HCV viral load was 5.09 $\log_{10}$ copies/mL (range: 0.00–6.74). The mean alanine aminotransferase (ALT) level was 42.2 IU/L (range: 6–177), and the mean aspartate aminotransferase (AST) level was 46.8 IU/L (range: 12–288). Ten individuals (59%) had a diagnosis of OUD, while 7 did not.

Fifteen individuals were receiving highly active antiretroviral therapy—including 8 of 10 in the OUD^+ group. When comparing the OUD^+ and OUD^- groups, mean HIV viral loads were higher for the OUD^+ group (2.44 versus 1.00 $\log_{10}$ copies/mL); however, this difference did not reach statistical significance ($p = 0.12$; two-sample T test). Mean ALT, AST, age, HCV RNA, and CD4 cell count were not significantly different between the OUD^+ and OUD^- groups.

Drug use was evaluated at the time of sample collection by LC–MS/MS, and drug screen results are provided in Supplementary table 1. Fentanyl was detected in 8 individuals, including 7 in the OUD^+ group. Fentanyl analogs and metabolites were also detected, including norfentanyl (n = 7), and acetyl fentanyl (n = 1). Other drugs of abuse that were detected included cocaine (n = 1), 11-carboxy-tetrahydrocannabinol (n = 6), and methamphetamine (n = 2). Buprenorphine was detected in 2 individuals in the OUD^+ group.

A total of 216,641 cells were evaluated by single cell RNAseq with 215,221 (99.3%) cells expressing at least 200 genes and these genes being expressed in at least 3 cells (Supplementary table 2). Cells with greater than 20% mitochondrial content were removed. The median number of cells per individual that passed this filter was 12,660. The mitochondrial content was low for all study samples (Supplementary Fig. 1). Cell clusters within PBMCs from all study participants were identified by transferring annotations from a high-quality reference

Variable	OUD + N = 10	OUD- N = 7
Mean age	35.1	45.9
Gender		
Female	3	0
Male	7	7
Race/ethnicity		
White, non-Hispanic	10	1
Black, non-Hispanic	0	5
White, Hispanic	0	1
HIV seropositive	10	7
Mean HIV viral load, $\log_{10}$ copies/mL	2.40	1.00
Mean CD4 cell count / mm^3	479.5	460.7
Currently receiving HAART	8	7
HCV seropositive	8	4
Mean HCV viral load, $\log_{10}$ copies/mL	5.80	4.00
Mean ALT, IU/L	30.9	58.3
Mean AST IU/L	28.7	72.6

Table 1. Baseline sociodemographic and clinic characteristics for 17 study participants. OUD – opioid use disorder; HAART – highly active antiretroviral therapy; ALT – alanine aminotransferase; AST – aspartate aminotransferase.

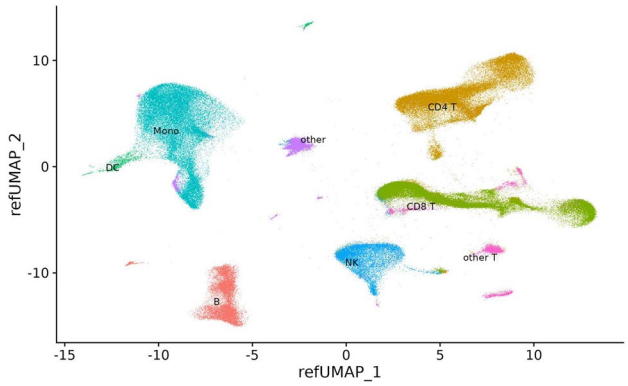


Fig. 1. UMAP visualization of PBMC data from all study participants. Each circle represents an individual cell colored by cell type.

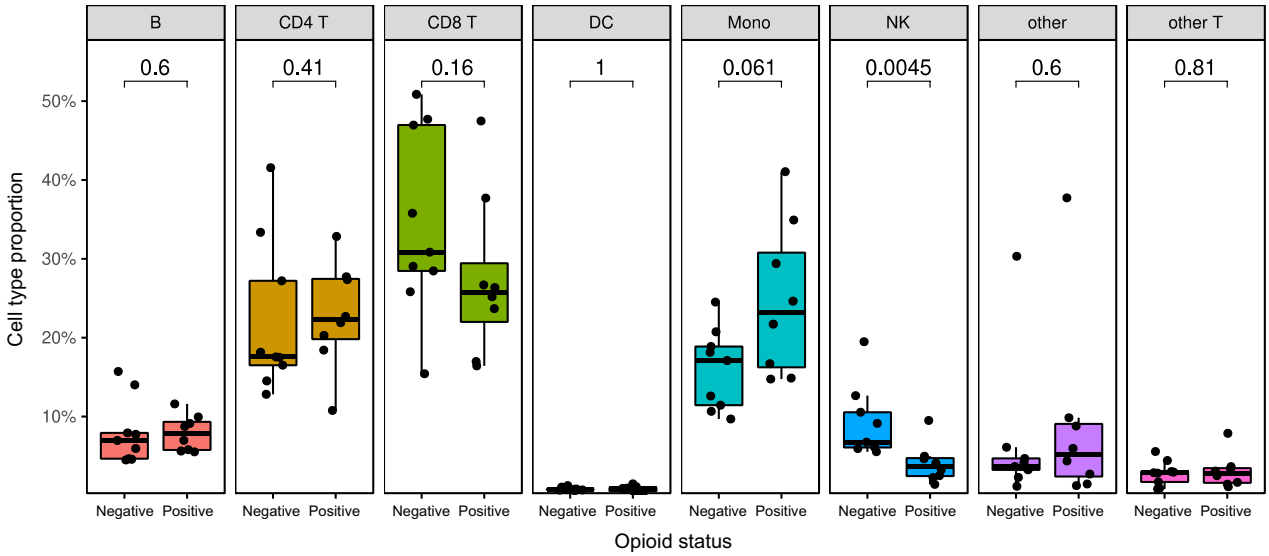


Fig. 2. PBMC cell proportions by opioid status. Each circle represents the cell proportion for a particular cell type from one individual. P values for differences in proportions were calculated using a two-sided Wilcoxon rank-sum test.

dataset (Fig. 1). Data visualizations for individual study participants are included in Supplementary Fig. 2. For cell types including B cells, $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells, monocytes, and other T cells and immune cells, there was no difference in frequency based on opioid status (Fig. 2). However, for NK cells, there was a statistically significant decrease in cell type frequency in opioid-positive versus opioid-negative individuals ($p = 0.0045$). Given the high number of individuals with HCV co-infection, we also analyzed cell type frequencies by HCV status. As shown in Supplementary Fig. 3, there were no statistically significant differences in cell type frequency in opioid-positive versus opioid-negative individuals based on HCV status, except for the NK cell frequency which was lower in opioid-positive versus opioid-negative individuals ($p = 0.051$).

Differential gene analysis (DGA) was performed for multiple PBMC cell types including $CD4^+$ T lymphocytes, $CD8^+$ T cells lymphocytes, monocytes, B lymphocytes, dendritic cells, and NK cells after controlling for HCV status and gender. There were 18 differentially expressed genes (DEGs) in $CD4^+$ T lymphocytes with an adjusted p value less than 0.05 and an absolute $\log_2$ fold change greater than 0.25 for opioid positive versus opioid-negative study participants (Fig. 3 and supplementary table 3). This included 10 DEGs that were higher in opioid-positive persons, while 8 DEGs were lower in opioid-positive persons. In $CD8^+$ T lymphocytes, there were 28 DEGs—15 higher and 13 lower—with an adjusted p value < 0.05 and an absolute $\log_2$ fold change > 0.25 for opioid positive versus opioid-negative study participants (Fig. 4 and supplementary table 4). In monocytes, 72 DEGs were higher and 37 DEGs were lower in opioid-positive versus opioid-negative study participants (Fig. 5 and supplementary table 5). In B lymphocytes, 17 DEGs were higher and 10 DEGs were lower in opioid-positive versus opioid-negative participants (Fig. 6 and supplementary table 6). In dendritic cells, 74 DEGs were higher and 29 DEGs were lower in opioid-positive versus opioid-negative study participants (Fig. 7 and

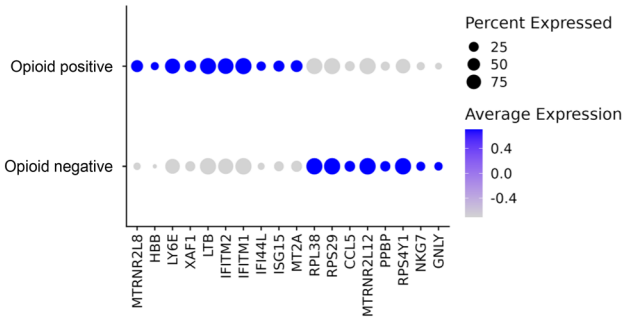


Fig. 3. Dot plot of differentially expressed genes in CD4$^+$ T lymphocytes for study participants with and without opioid use.

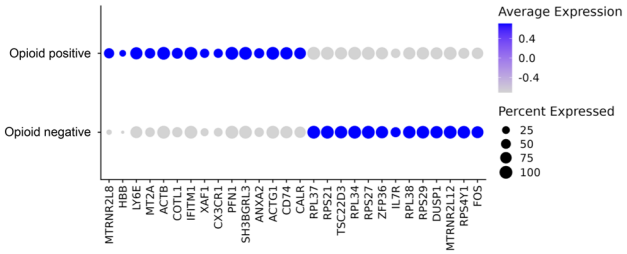


Fig. 4. Dot plot of differentially expressed genes in CD8$^+$ T lymphocytes for study participants with and without opioid use.

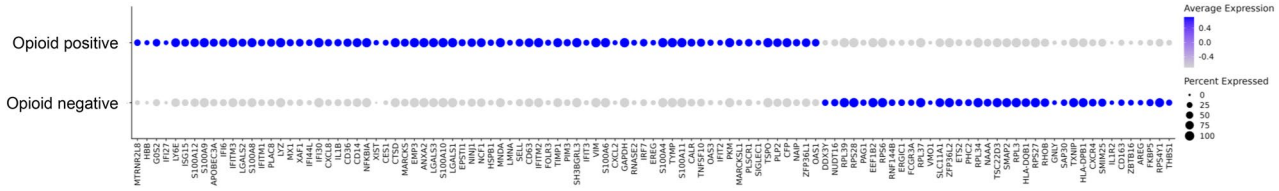


Fig. 5. Dot plot of differentially expressed genes in monocytes for study participants with and without opioid use.

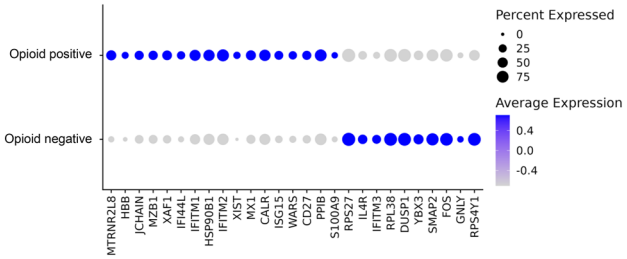


Fig. 6. Dot plot of differentially expressed genes in B lymphocytes for study participants with and without opioid use.

supplementary table 7). In NK cells, 31 DEGs were higher and 30 DEGs were lower in opioid-positive versus opioid-negative study participants (Fig. 8 and supplementary table 8).

Enrichment analysis of differentially expressed genes was performed for each major cell type. In CD4$^+$ T lymphocytes, genes were enriched in cytokine-mediated signaling, regulation of viral genome replication, type I interferon signaling, cellular responses to type I interferon, regulation of viral genome regulation, regulation of viral entry, regulation of the viral life cycle, and response to interferon β (Fig. 9 and Supplementary table 9). In CD8$^+$ T lymphocytes, genes were enriched in genes were enriched in protein targeting to the membrane,

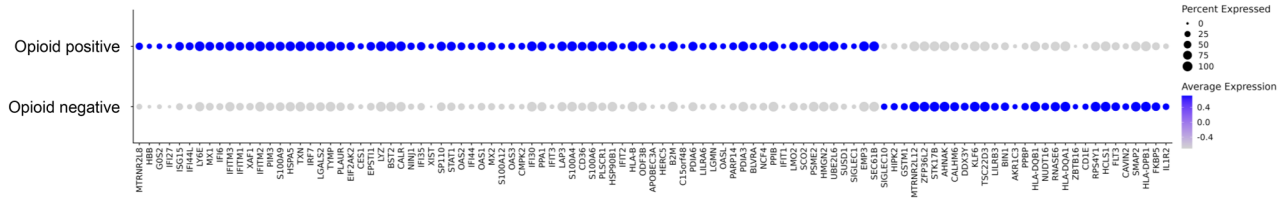


Fig. 7. Dot plot of differentially expressed genes in dendritic cells for study participants with and without opioid use.

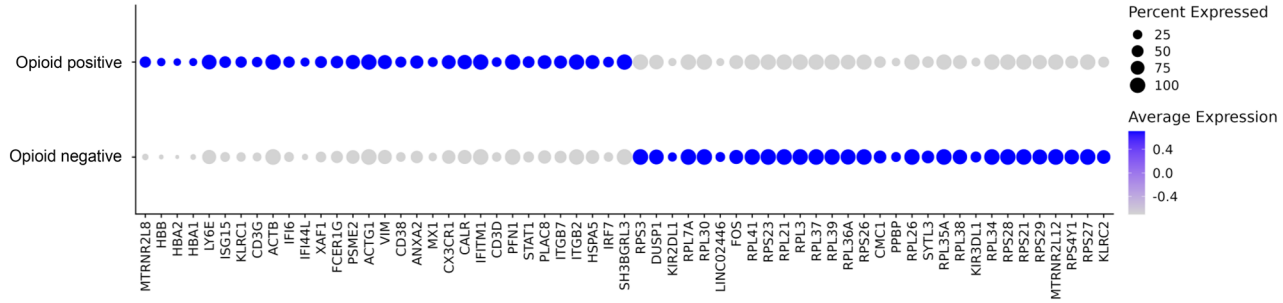


Fig. 8. Dot plot of differentially expressed genes in NK cells for study participants with and without opioid use.

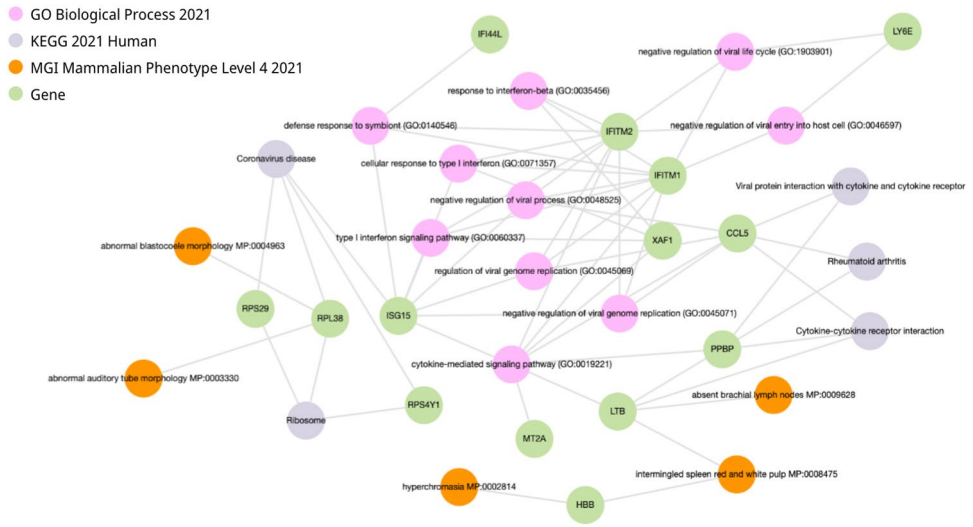


Fig. 9. Enrichment analysis of differentially expressed genes in CD4⁺ T lymphocytes. Pink – GO biological processes; grey – KEGG human cell signaling and metabolic pathways; orange – mammalian phenotypes; green – genes.

nuclear-transcribed mRNA catabolic process, protein targeting to the ER, coronavirus disease, and translation (Fig. 10 and Supplementary table 10). In monocytes, genes were enriched in the defense response to viruses, type I interferon signaling, cellular responses to type I interferon, cytokine-mediated signaling, coronavirus disease, regulation of viral genome replication, and the innate immune response (Fig. 11 and Supplementary table 11). In B lymphocytes, genes were enriched in type I interferon signaling, the cellular response to type I interferon, cytokine-mediated signaling, regulation of viral genome replication, the defense response to viruses, the regulation of viral processes, the response to interferon β and coronavirus disease (Fig. 12 and Supplementary table 12). In dendritic cells, genes were enriched in type I interferon signaling, cellular responses to type I interferon, the defense response to viruses, cytokine-mediated signaling, the regulation of viral processes, regulation of viral genome replication, interferon γ-mediated signaling, antigen processing and presentation, and the response to cytokines (Fig. 13 and Supplementary table 13). In NK cells, genes were enriched in protein targeting to the

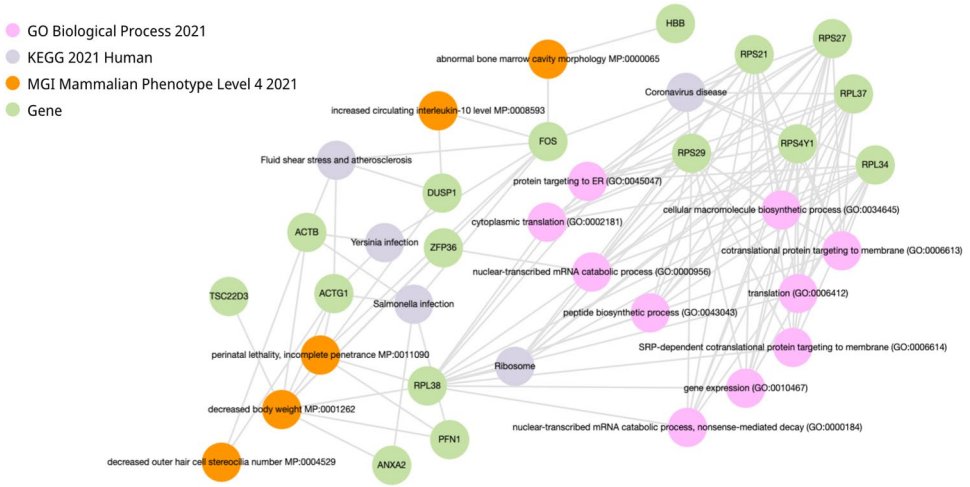


Fig. 10. Enrichment analysis of differentially expressed genes in CD8⁺ T lymphocytes. Pink – GO biological processes; grey – KEGG human cell signaling and metabolic pathways; orange – mammalian phenotypes; green – genes.

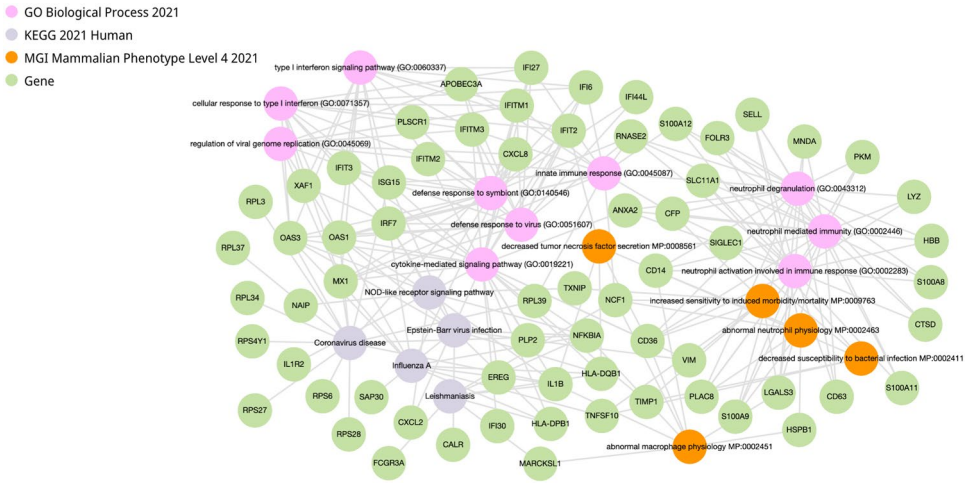


Fig. 11. Enrichment analysis of differentially expressed genes in monocytes. Pink – GO biological processes; grey – KEGG human cell signaling and metabolic pathways; orange – mammalian phenotypes; green – genes.

membrane, protein targeting to the ER, coronavirus disease, nuclear-transcribed mRNA catabolic process, and translation (Fig. 14 and Supplementary table 14).

Several differentially expressed genes were shared among CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, and/or B lymphocytes (Fig. 15). Six DEGS were shared by CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, and B lymphocytes, including RPS4Y1, HBB, MTRNR2L8, XAF1, RPL38, and IFITM1. Four DEGs—LY6E, MT2A, RPS29, and MTRNR2L12—were shared by CD4⁺ T lymphocytes and CD8⁺ T lymphocytes. There were 4 DEGs shared by CD8⁺ T lymphocytes and B lymphocytes, including RPS27, DUSP1, FOS, and CALR. There were 4 DEGs—IFI44L, IFITM2, ISG15, and GNLY—shared by CD4⁺ T lymphocytes and B lymphocytes.

Discussion

Multiple illicit drugs are known to promote viral replication and virus-mediated pathology[25]. The majority of unintentional overdoses in the United States currently involve fentanyl and/or fentanyl analogs[14,50–52]. Nonetheless, little is known regarding how synthetic opioids may impact viral replication and pathogenesis.

We previously demonstrated fentanyl increased replication of HBV and HCV in hepatocytes in vitro[53]. Bulk RNAseq identified multiple hepatocyte genes that were differentially regulated by fentanyl, including those related to apoptosis, the antiviral / interferon response, chemokine signaling, and NFκB signaling. We have also found that fentanyl exposure resulted in a dose-dependent increase in HIV replication and enhanced expression of the HIV chemokine co-receptors CXCR4 and CCR5 in several HIV-susceptible or HIV-infected

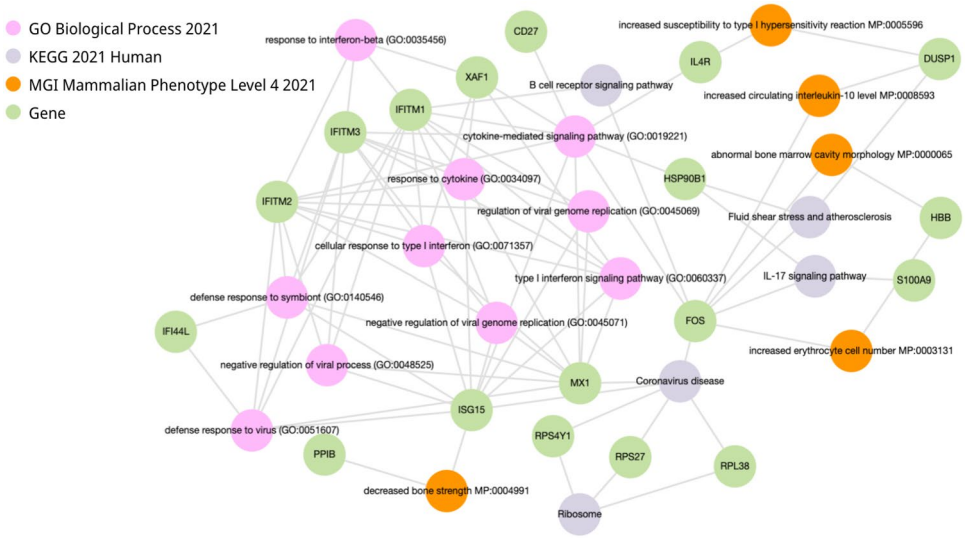


Fig. 12. Enrichment analysis of differentially expressed genes in B lymphocytes. Pink – GO biological processes; grey – KEGG human cell signaling and metabolic pathways; orange – mammalian phenotypes; green – genes.

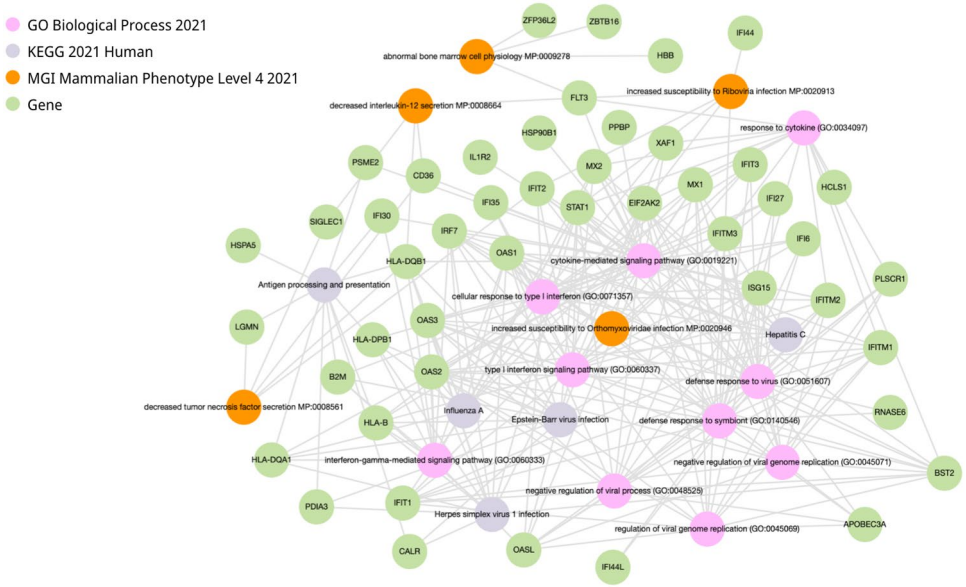


Fig. 13. Enrichment analysis of differentially expressed genes in dendritic cells. Pink – GO biological processes; grey – KEGG human cell signaling and metabolic pathways; orange – mammalian phenotypes; green – genes.

cell types[33–37]. Increased HIV protein synthesis and proviral DNA expression were also observed in CD4$^+$ T cells derived from healthy control PBMCs exposed to HIV and fentanyl ex vivo[35].

Previous studies in non-human animal models and cell types that are not exposed to virus have shown that fentanyl regulates apoptosis, alters NFκB expression, upregulates inflammatory genes, and/or influences the survival of CD4$^+$ T lymphocytes[54–60]. While these pathways were not evaluated explicitly in the current study, they highlight additional mechanisms by which fentanyl may influence HIV pathogenesis. Moreover, fentanyl is a common recreational synthetic opioid in use; yet, over 50 fentanyl analogs have been reported to date[61]. Currently, there are no data on the impact of fentanyl analogs on HIV replication, and it is possible that distinct fentanyl analogs may impact viral replication differently.

A limited number of studies have used scRNAseq to assess the effects of opioids on viral pathogenesis and/or cellular function to date. Avey et al. utilized scRNAseq to examine differential gene expression in oligodendrocytes and astrocytes of mice following acute drug exposure[62]. Unbiased profiling of cell-type-specific transcriptional responses to morphine revealed many alterations in gene expression that are unique to

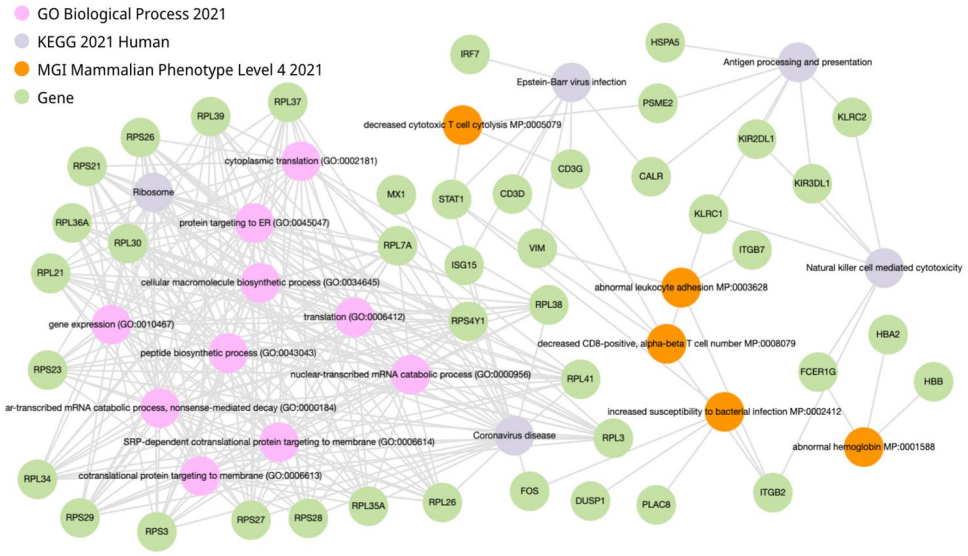
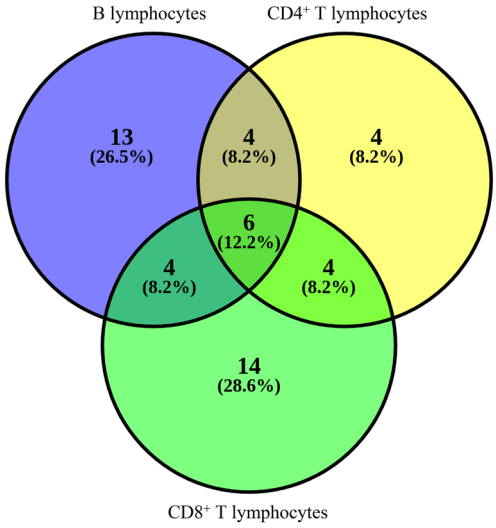


Fig. 14. Enrichment analysis of differentially expressed genes in NK cells. Pink – GO biological processes; grey – KEGG human cell signaling and metabolic pathways; orange – mammalian phenotypes; green – genes.



B and CD4⁺ T	B and CD8⁺ T
IFI44L	RPS27
IFITM2	DUSP1
ISG15	FOS
GNLY	CALR

CD4⁺ T and CD8⁺ T	B, CD4⁺ T, and CD8⁺ T
LY6E	RPS4Y1
MT2A	HBB
RPS29	MTRNR2L8
MTRNR2L12	XAF1
	RPL38
	IFITM1

Fig. 15. Number (left) and list (right) of differentially expressed genes that are shared among CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, and/or B lymphocytes. Shown are those genes with an adjusted p value < 0.05 and an absolute $\log_2$ fold change > 0.25.

oligodendrocytes and suggest a role for opioid signaling in oligodendrocyte maturation and myelination. Reiner et al. used single nuclear RNAseq to identify 1329 differentially expressed genes in the nucleus accumbens of rats that self-administered morphine[63]. Karagiannis et al. performed scRNAseq of PBMCs from opioid-dependent individuals and controls[64]. Their results showed a widespread suppression of interferon-stimulated genes and antiviral genes in multiple innate and adaptive peripheral immune subpopulations, suggesting an adverse effect of opioid usage on the defense response to viral infection. Fox et al. evaluated the effects of morphine in SIV-infected macaques using scRNAseq[65]. Of note, morphine exposure led to an immunosuppressive environment and blunting initial responses to infection which persisted during antiretroviral treatment. Ho et al. performed single nucleus RNA-seq of induced pluripotent stem cell (iPSC)-derived forebrain organoids from three individuals with OUD in response to oxycodone or buprenorphine[66]. Buprenorphine had a significant influence on transcription regulation in glial cells, while oxycodone induced type I interferon signaling in many cell types, including neural cells in brain organoids. Moreover, oxycodone – but not buprenorphine – activated signal transducer and activator of transcription 1 (STAT1) and induced type I interferon signaling in patients with OUD.

Opioids are known to modulate a variety of immune functions (reviewed in[24,67]). The distribution of specific cell types that comprise the peripheral blood may be altered by opioid use as well. For instance, in simian

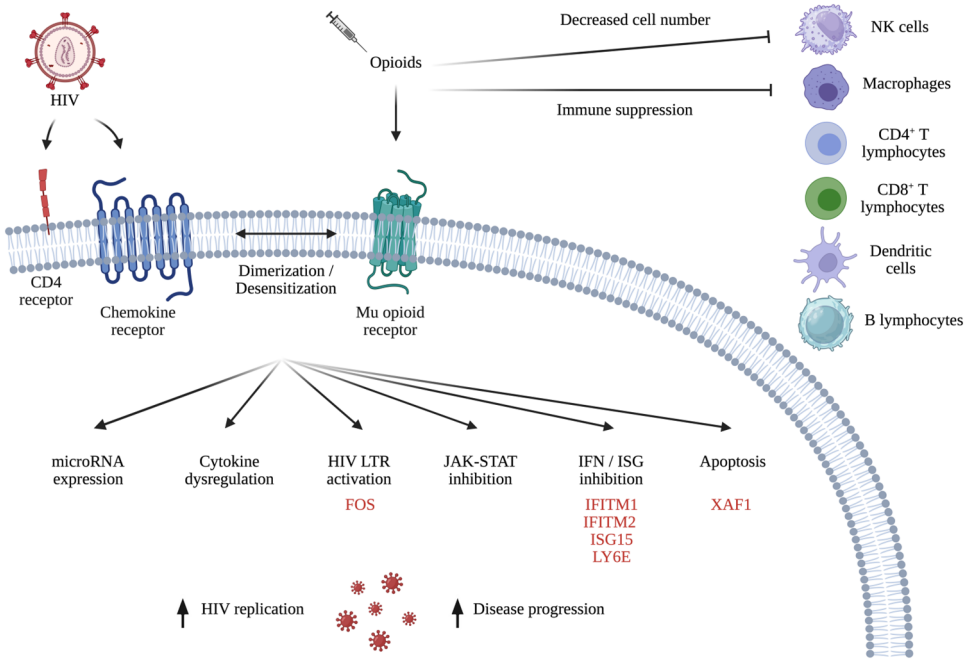


Fig. 16. Cellular processes linking viral infections with opioid use, including genes that were differentially expressed in distinct cell types in this study population. Genes in red are those identified in the current study. Created in BioRender. Blackard, J. (2025) https://BioRender.com/xqnuevn.

immunodeficiency virus-infected rhesus macaques, the number of $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and B cells was lower in morphine-treated animals compared to untreated animals[65]. Monocytes were also less frequent in the morphine group. NK cells are a critical component of HIV pathogenesis given their cytotoxic ability to eliminate virus-infected cells and their ability to modulate immune responses through cytokine production[68]. In our study, opioid-exposed humans showed a statistically significant decrease in NK cell number. A similar decrease in NK cell number and/or activity has been noted in opioid-exposed humans and/or animal models[69–75]. Opioids may suppress NK cell activity through reduction of interferon levels[76], which is known to augment NK cell activity and lead to reduced NK cytotoxicity. Additionally, opioids may influence cytokine production that regulate immune cell function, as well as HIV pathogenesis[69,73,77–79]. Interestingly, freshly isolated NK cells from peripheral blood respond differently to distinct opioids ex vivo[77] suggesting that NK cells may respond differently to distinct opioids in vivo as well.

The cellular processes that link viral infections with opioid use have been reviewed elsewhere (reviewed in[80]). As well, the current study—based on robust gene expression profiling in distinct cell types—reveals transcriptional patterns that can guide future exploration of key regulatory mechanisms (Fig. 16). For instance, the interferon-induced transmembrane proteins (IFITMs) represent antiviral factors that inhibit the entry of several viral pathogens (reviewed in[81]). IFITM1, IFITM2, and/or IFITM3 are potent inhibitors of HIV[82–86]. Raposo et al. reported that IFITM1 targets HIV latently infected cells for antibody-dependent cytolysis[87]. Regulation of IFITMs in the context of illicit drug use is unknown; however, data from the current study suggest that IFITMs are upregulated in several cell types in opioid-using HIV-positive individuals. Interferon-stimulated gene 15 (ISG15) is a ubiquitin-like protein that is induced by type I interferon (reviewed in[88,89]). ISG15 inhibits HIV particle release in vitro[90,91]. In HIV-positive individuals, ISG15 levels are correlated with higher HIV viral loads and lower $CD4^+$ T cell counts[92]. ISG15 levels were also reduced in patients with ART-induced viral suppression. Mackelprang et al. showed that ISG15 gene expression is upregulated during acute and chronic HIV infection and is strongly associated with plasma HIV-1 RNA levels[93]. X-linked inhibitor of apoptosis-associated factor 1 (XAF1) is a pro-apoptotic tumor suppressor that is frequently inactivated in multiple human cancers (reviewed in[94]. Its role in viral pathogenesis is less clear, although in combination with other genes, higher expression of XAF1 is protective from viral rebound after antiretroviral treatment interruption, suggesting its role in maintaining viral suppression[95]. While data on XAF1 expression during illicit drug use are limited, our data suggest that XAF1 is upregulated in multiple peripheral blood cell types in opioid-using HIV-positive individuals. The c-Fos protein is a component of the AP1 transcription factor complex and is part of a multigene family, that includes *fos*-related and *jun*-related genes. AP1 plays a central role in HIV gene transcription[96], but may also contribute to reactivation from latency[97,98]. Previous studies have shown that drugs of abuse such as cocaine, morphine, and methamphetamine induce the expression of AP1 / AP1 complex genes[99–106]. Thus, opioid-induced expression of transcription factors may also contribute to HIV pathogenesis, although this requires additional study. Lymphocyte Ag 6 complex (LY6E) is an interferon-stimulated gene that is overexpressed in HIV progressors[107]. In $CD14^+$ monocytes purified from persons with chronic HIV infection, LY6E levels were inversely correlated with CD4 T cell count but not with viral load. Viral suppression decreased

LY6E expression in monocytes. Others have reported that LY6E knockdown increases HIV infection, while its overexpression inhibits viral entry and replication[108]. In the current study, LY6E expression was elevated in $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, monocytes, and dendritic cells from HIV-positive persons using opioids compared to those that were opioid negative.

Enrichment analysis identified pathways and processes involved in coronavirus disease and host-coronavirus interactions. This is not surprising given that most study participants were enrolled during the COVID-19 pandemic. OUD is associated with respiratory depression, immune modulation, microbial dysbiosis, and bacterial translocation, which can influence COVID-19 severity and disease progression (reviewed in[109]), and a recent diagnosis of OUD is associated with a significantly increased risk of COVID-19 and worse outcomes[110]. Enrichment analysis also identified pathways related to type I interferon signaling, the cellular response to type I interferon, the defense response to viruses, and the regulation of viral genome replication in several cell types including $CD4^+$ T lymphocytes, monocytes, B lymphocytes, and dendritic cells. The roles of interferons and interferon-stimulated genes in regulating HIV replication and disease pathogenesis have been reviewed elsewhere[111].

Polysubstance use is common[8,50,112–114], including in Ohio where this study was conducted. For instance, in high-burden counties in Ohio, 56% of fentanyl deaths tested positive for heroin or cocaine in 2014[50]. In a study conducted by the Montgomery County Coroner's Office / Miami Valley Regional Crime Laboratory that identified drugs present in unintentional overdose fatalities in early 2017, approximately 90% involved fentanyl, fentanyl analogs, or both. Other drugs detected in those testing positive for fentanyl included heroin (4.7%), any pharmaceutical opioid (20.2%), benzodiazepines (25.7%), cocaine (30.8%), methamphetamine (12.6%), marijuana (36.0%), and alcohol (18.2%)[14]. Thus, additional research into the complex interactions between illicit drugs and viral pathogenesis must simultaneously occur at the level of basic research in vitro, as well as in vivo in cohort studies. Rigorous characterization of the interactions among HIV, opioids, and host cells will improve clinical management paradigms for difficult-to-treat populations, facilitate rational public health policies given severely strained resources, and reveal additional pathways for novel target-specific therapeutic interventions.

Our study has several limitations of note. First, this was a single-center study with a modest sample size that may diminish the statistical power to detect minor differences. The sample size reflects significant challenges in recruiting HIV-positive participants with ongoing substance use, as well as the high cost per sample of single cell RNAseq analysis. However, other studies that focused on HIV or illicit drugs typically included fewer patients and/or utilized non-human animal models[62,64–66]. Second, the frequency and duration of opioid exposure are unknown. It is possible that acute versus chronic opioid exposure has distinct effects on HIV pathogenesis. In this study, drug use was determined at the time of sample collection; however, data on drug use before sample collection are not available. Third, polysubstance use is quite common in persons with OUD, including in this cohort. The impact of polysubstance use compared to single illicit drugs is not commonly considered in well-characterized clinical cohorts with viral infections, and we were unable to evaluate drug-drug interactions or potential synergistic effects between multiple illicit drugs. Similarly, while our differential gene analysis controlled for HCV infection and gender, other unmeasured modifiers of gene expression are possible and may have impacted our findings. Gender-specific differences in gene expression were identified in the initial analysis (data not shown). Thus, we controlled for gender in the all subsequent analyses. However, larger studies that allow gender-based analyses and comparisons are needed. Fourth, while HIV infects multiple cell types, other reservoirs of HIV replication exist throughout the body and may be more or less sensitive to the effects of opioids, including fentanyl. Finally, as with other studies that utilize single cell RNAseq, functional studies are needed to fully characterize the significance of the gene alterations identified here.

Collectively, these findings presented here highlight multiple distinct pathways by which opioid use contributes to HIV pathogenesis. Defining the changes in the peripheral blood transcriptome is necessary to better understand virus-cell-opioid interactions and may ultimately lead to improved clinical management paradigms for difficult-to-treat populations and identify new pathways for novel target-specific therapeutic interventions.

Data availability

The single cell RNAseq datasets generated and analyzed during the current study are available in BioProject (ID 976266) at https://www.ncbi.nlm.nih.gov/bioproject/976266.

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Author contributions
KMR performed single cell RNAseq analysis and edited the manuscript. HLM, JMK, and MJ performed lab work and edited the manuscript. JMC, JLB, CF, MSL, and KES enrolled study participants and edited the manuscript. JTB secured study funding, supervised laboratory personnel, and wrote the initial manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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