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An infectious murine model for studying the systemic effects of opioids on early HIV pathogenesis in the gut

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Abstract

Opioids are known to exacerbate HIV pathogenesis, however current studies have been limited by models of HIV infection. Given that HIV causes many systemic effects via direct infection of host cells as well as indirect bystander effects, it is important to establish a systemic infection model in a small animal so that genetic tools can be utilized to elucidate the mechanisms of action. In this study, we describe the systemic effects of EcoHIV infection, a modified HIV which can infect

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Conflicts of Interest

The authors declare no conflicts of interest.

mouse cells, in conjunction with morphine. We observed that EcoHIV infection with opioid treatment induces bacterial translocation from the lumen of the gut into systemic compartments such as liver, which is similar to observations in human patients with LPS. Bacterial translocation corresponds with alterations in gut morphology, disorganization of the tight junction protein occludin, and a concurrent increase in systemic inflammation in both IL-6 and TNF α . Long term infection also had increased expression of inflammatory cytokines in the CNS when co-treated with morphine. Overall, we conclude that EcoHIV is an appropriate model to study the effects of opioids on HIV pathogenesis, including the HIV-induced pathology at early stages of pathogenesis in the gut.

Introduction

HIV infection is highly prevalent in injection drug users (IDU) with upwards of 40% in various regions globally (Beyrer et al. 2010). While IDU was recognized early in the history of HIV as a potential means of transmitting the disease by sharing needles with blood remnants, IDU has also been observed to correlate with a more severe HIV pathogenesis than non-users including a much faster development of AIDS and higher rates of neurocognitive deficiencies (Donahoe and Vlahov 1998; Kapadia et al. 2005). The mechanism for this is currently unknown, however it likely occurs via a combination of the ability of the drugs to modulate systemic changes in the patient along with decreased access to adequate healthcare (Wolfe et al. 2010).

Clinically, it is nearly impossible to delineate how IDU may enhance HIV due to poly-drug use which is estimated to occur in over 70% of IDU patients (Kedia et al. 2007). Opioids, the most common IDU agent in HIV cases, have been shown in mouse models to have drastic systemic effects including the immune system. As a consequence, there are significant interactions between HIV and opioid use in the immune compartment. Understanding these interactions is important, especially at the early stages of disease, in order to develop interventions and prevent the severity of HIV pathogenesis observed in IDU-using infected patients.

Early stages of pathogenesis in HIV infection has been difficult to study in clinical populations since the disease does not make itself obvious until years later when opportunistic infections develop. Nonhuman primates infected with Simian Immunodeficiency Virus (SIV) have been an invaluable tool to overcome this limitation, especially since there are both pathogenic and nonpathogenic species which allows for differences between the two to be mapped (Gordon et al. 2007; Pandrea et al. 2007). Initial stages of HIV pathogenesis are now strongly believed to be reliant on microbial translocation from the gut which drives the systemic inflammation needed for HIV/SIV (Brenchley et al. 2006; Nazli et al. 2010). Despite these findings, studies of mechanisms in nonhuman primates, as in humans, are still very restricted by the lack of genetic tools and increased cost compared to small animal models like mice.

Developing HIV models for mice has been limited by the host specificity of HIV. Most infectious models have focused on “humanizing” the mice either by creating transgenic mice with human HIV co-receptors or generating chimeric mice with human immune cell grafts

(Denton and Garcia 2009; Zhang and Su 2012). The latter method has shown a remarkable ability to recapitulate HIV pathogenesis in the human immune cells within a mouse (Zhang and Su 2012). However, studies using chimeric mice cannot adequately make conclusions about physiological effects of HIV infection on non-immune cells, such as neurons or epithelial cells, because the interactions between human and mouse cells are likely not conserved. Humanized mice are also susceptible to Graft Versus Host Disease, which can further complicate interpretation of results (Shultz et al. 2012).

For this reason, Potash et al created the EcoHIV model, which is a genetically modified HIV virus which uses murine leukemia virus gp80 for cell entry in place of gp120 from HIV-1 (Potash et al. 2005). While previous studies from this group characterized the brain compartment in response to infection (Kelschenbach et al. 2011; He et al. 2014), we hypothesized that EcoHIV would recapitulate early consequences of HIV-1 at the systemic level, such as bacterial translocation, and inquired whether combination of infection with opiates would worsen these symptoms.

Materials and Methods

Mice

All studies using mice in Dr. Roy's laboratory were approved by the University of Minnesota Animal Care and Use Committee and were conducted in full compliance with NIH guidelines. Male or female C57Bl/6 and BALB/c mice were purchased from the Jackson Laboratory, while Mu Opioid Receptor Knock Out (MORKO) and Toll-Like Receptor 4 Knock Out (TLR4KO) mice were bred in house. All animals were between 10–16 weeks of age at the beginning of each experiment.

Morphine treatment

Mice were implanted with either a placebo or 25mg slow-release morphine pellet at 24 hours prior to infection with EcoHIV. Pellets were generously provided by NIH/NIDA. For long term experiments (>7 days), mice were implanted with either 25mg or 75mg morphine pellets using a protocol that would increase released morphine every 7 days to avoid tolerance. Escalating doses in the order used were 1x 25mg, 1x 75mg, 2x 25mg, and 2x 75mg based on a study that showed 2x 25mg morphine pellets resulted in higher morphine release than 1x 75mg pellet, likely due to increased surface area with having two pellets (Dighe et al. 2009). The first dose given (implant at day 0, exposed 0–7 days) was 25 mg slow-release morphine pellet as in short term experiments. The second week (implant at day 7, exposed 7–14 days), mice were pelleted with a 75 mg slow-release morphine pellet. The third week (implant at day 14, exposed 14–21 days) mice were pelleted with 2x 25 mg pellets and the final week (implant at day 21, exposed 21–28 days) with 2x 75 mg pellet. At the end of final week, mice were sacrificed and PBS perfused brains were aseptically removed for the quantification of proinflammatory cytokines. *In vitro* work performed in macrophage cell lines (J774 or RAW) were treated with 1uM morphine.

EcoHIV infection

The EcoHIV constructs used in the present work were EcoHIV/NL4-3 which was constructed on the backbone of HIV-1/NL4-3 (Potash) and EcoHIV/NL4-3-GFP which expresses enhanced green fluorescence protein (EGFP) as a marker protein (He-14). For brevity, the viruses are called EcoHIV and EcoHIV-GFP. The viruses were propagated in HEK293TN cells as previously described (Potash et al. 2005). Mice were injected in the intraperitoneal cavity with 1mL of either saline or 1×10^6 pg p24 units as measured by p24 ELISA following manufacturer's instructions (ZeptoMetrix Corporation). *In vitro* work performed in macrophage cell lines (J774 or RAW) were treated with 1×10^3 pg of p24 units per 1×10^6 cells.

Bacterial Translocation

Tissue was harvested and homogenized in sterile PBS with 100 μ M cell strainers (BD) following aseptic techniques. Homogenized tissue was then plated on blood agar plates and incubated at 37C overnight in aerobic conditions. Colonies were counted and normalized for varying protein concentrations in the tissue homogenate.

RNA isolation and processing

RNA isolation was performed on homogenized tissue using the Qiagen RNeasy prep and cDNA was generated using the Promega Reverse Transcription System, each according to the manufacturer's protocol. RT-PCR was run and quantified with Sybr Green (Roche) for 40 cycles on an Applied Biosystems 7500 machine. Samples which crossed the threshold above 32 Ct were considered to be non-specific amplification and discarded. Primers are listed in Table 1.

Histology

Tissues were preserved in 10% formalin for 24hr and transferred to 70% ethanol. Samples were then embedded in paraffin, sectioned, and stained using a combination of hematoxylin (nuclei, dark brown), alcian blue (at pH 2.5; goblet cells and mucin, blue), and metanil yellow (nonspecific tissue and connective tissue, yellow) (Edgett 2004). Stained slides were imaged on a Nikon light microscope at 100X.

FACS

Lamina propria immune cells were collected from small, cecum, or large intestine following a previously published method (Denning et al. 2007). Briefly, intestine was washed with HBSS with 5% FBS and .5M EDTA to remove epithelial cells. Remaining tissue was digested using HBSS with 5% FBS and .4 mg/mL Collagenase (Sigma-Aldrich). Cells were harvested via centrifugation at 500g and fixed in IC fixation buffer (Ebiosciences) for 20 minutes on ice and stained using I-A, I-E (AF488, Biolegend), CD4 (PE, BD), CD8 (APC, eBioscience), CD19 (Alexa 700, BD), CD11B (PE-cy7, Biolegend), CD11C (PE-cy5, Biolegend), and CD45 (V500, BD) and acquired by a FACS Canto II flow cytometer. Analysis was done using Flowjo software (Treestar Inc).

Phagocytosis

Peritoneal macrophages were harvested by putting 1mL sterile saline inside the omentum sac and removing to a sterile tube, which were then cultured for 30 min in serum-free media and washed to select for adherent cells. Peritoneal, RAW, or J774 macrophages were cultured with enriched DMEM with and without 1uM morphine, 10, 000/well in black 96-well plate or cover slip and incubated with either FITC-dextran or *E. coli* or *S. aureus* opsonized particles (1:20, cell:bacteria/bead ratio) for 30 minutes. Phagocytosis was stopped by addition of trypan blue, which quenches fluorescence of noninternalized particles. Cells were either analyzed by fluorometry in black 96-well plates after staining with DAPI (D9542 Sigma) or fixed for confocal microscopy on sterile glass cover slips, where they were co-stained with rhodamine phalloidin and Prolong Gold with DAPI (Life Technologies) and imaged by confocal microscopy. Fluorescence was recorded at an excitation of 485 nm and an emission of 520 nm for opsonized particles (FITC) and an excitation of 355 nm and an emission of 460 nm for DAPI. Phagocytosis per cell was quantified as phagocytic Index = FITC/DAPI [both given in relative fluorescence units (RFUs)], indicative of particle fluorescence per cell.

ELISA

Liver and lung homogenate were prepared as described above. TNF α and IL-6 Ready-Set-Go kits (Ebiosciences) were performed following the manufacturer's instructions.

Statistics

Statistics were analyzed within Prism software (Graphpad Inc) using two-way ANOVA using Bonferroni post-tests to compare each treatment with the others. Groups were considered to be significant if the p-value from the Bonferroni test was $p < .05$ or less.

Results

Knowing that EcoHIV can infect CD4+ and macrophages in mice (Potash et al. 2005), we first verified the systemic nature of the infection and whether morphine influences the amplitude of infection. We began by validating that EcoHIV can infect macrophage cell lines *in vitro* by examining the RNA expression of LTR-gag in both J774 and RAW cells. In these cell lines, we observed expression of HIV LTR-gag RNA which was undetected in uninfected samples (Figure 1A). We also validated infection *in vivo*, where again we saw expression levels of LTR-gag RNA in the spleen of infected mice 2 days, 5 days, and 7 days post-infection, but not in uninfected mice (Figure 1B). Using EcoHIV which expresses GFP within infected cells (He et al. 2014), we examined expression of GFP in both F4/80+ macrophages and CD4+ lymphocytes isolated from peritoneal lavage and spleen at 5 days post-infection (Figure 1C). Male and female animals manifested similar levels of infection in all cell types with the possible exception of CD4+ peritoneal cells. Peritoneal macrophages were also visually examined using confocal microscopy to verify positive GFP expression in EcoHIV infected animals (Figure 1D). We also measured EcoHIV infection results by looking at systemic p24 levels observed in liver (Figure 1E), lung (Figure 1F), and brain (Figure 1G) on day 5, day 10, and day 21 post infection. There was little difference between mice implanted with either placebo or 25mg pellets of morphine aside from at day

21 in liver (Figure 1E). Together, these results established successful infection of EcoHIV of CD4⁺ T-cells and macrophages in animals when EcoHIV was administered systemically thus recapitulating observations seen in humans with HIV-1 infections.

Translocation of bacterial products have been shown to play a key role in the early pathogenesis of HIV (Brenchley et al. 2006). Similarly, sepsis and bacterial translocation have been observed when animals are implanted with morphine pellets (Hilburger et al. 1997; Meng et al. 2013). In combination, IDU has been shown to increase the translocation induced by HIV (Ancuta et al. 2008), therefore we asked the question if bacterial translocation occurs in the EcoHIV-infected animals and will morphine exacerbate the effects. Significant translocation of colony forming units (CFU) occurred at day 2 post infection (Figure 2A) but not day 5 post infection (Figure 2B). The highest amount of CFU was observed in the liver when compared to the lung and spleen in animals that were infected with EcoHIV and in morphine treated EcoHIV-infected animals. We next incubated liver homogenates with RAW Blue cells to look for the presence of PAMPs such as LPS from gram-negative bacteria or LTA from gram-positive bacteria. Similarly to the CFU translocation observed, day 2 post infection showed significantly higher NF κ B activation in morphine, EcoHIV infected, and EcoHIV infected + morphine treated animals compared to placebo (Figure 2C), whereas day 5 post infection showed no change (Figure 2D). Finally, animals were gavaged with Rhodamine Dextran (Sigma) 4hr prior to Xenogen visualization on day 2 post infection, where noticeable translocation was observed especially in the EcoHIV infected + morphine treated animals (Figure 2E). To determine if morphine was altering this process through the mu-opioid receptor, we examined translocation using the mu-opioid receptor knock out (MORKO) mice at 2 days post-infection. As was expected, no translocation was observed in the MORKO animals following morphine treatment, but surprisingly very little translocation was observed in the EcoHIV or EcoHIV + morphine groups as well (Figure 2F). Similarly, we also examined TLR4 knockout mice, and saw a reduction in the translocation of animals treated singly with morphine or EcoHIV (Figure 2G). However, animals infected with EcoHIV+morphine were found to have translocation at levels similar to the wildtype genotype with the same treatment (Figure 2G). Overall, the induction of bacterial translocation in wild type animals infected with EcoHIV is similar to HIV-1 in humans and is abolished in MORKO and TLR4KO animals, which suggests that both genotypes play a role in the pathogenesis within the gut. We did not find translocation in the assays performed at longer than 2 days post-infection.

Since translocation was observed, we next examined gut morphology to see if any changes were observed in treatment groups. Grossly, placebo animals had characteristic smooth and long lamina propria (LP) while treated animals had shorter LP with inversions and perceived gaps in the epithelial cells (highlighted by black arrows, Figure 3A). Large intestine was similarly compared, but changes did not appear as obvious as in small intestine (Figure 3B). Ratios of small intestine LP length to width (Figure 3C) and LP length to crypt depth (Figure 3D) were measured, which showed that all three treatments had significantly lower ratios than placebo animals ($p < .05$). Large intestine LP depth was measured and EcoHIV + morphine was found to be significantly decreased compared to placebo (Figure 3E, $p < .05$). Together, the pathology of both small and large intestines is negatively impacted within EcoHIV and morphine treated animals, and especially in EcoHIV+morphine where the

greatest translocation was observed, suggesting that bacterial translocation induces the changes observed via resulting inflammation.

Inflammatory pathology in the gut is likely mediated by local immune cells, so we investigated if EcoHIV with morphine caused any changes to immune cell populations within the gut. Within CD4+ and CD8+ T-cell populations, no changes were observed in the small intestine (Figure 4A), cecum (Figure 4B), and large intestine (Figure 4C). In the large intestine, EcoHIV + morphine treated animals had significantly higher percentages of double positive CD4+CD8+ T-cells ($p<.05$) which have been shown to be highly activated, cytokine secreting cells within the gut (Pahar et al. 2006). B-cell percentage, as measured by CD19, was unchanged in the small intestine (Figure 4D), cecum (Figure 4E), or large intestine (Figure 4F). Monocytes populations were examined in terms of CD11B+, CD11C+, or double positive populations. Similarly to T-cells, monocytes has no changes in the small intestine (Figure 4G) or cecum (Figure 4H), but had a higher percentage of CD11B+CD11C+ double positive cells in the large intestine (Figure 4I, $p<.05$), which favors inflammatory cytokines secretion when increased in ratio over CD11B+ CD11C- macrophages (Denning et al. 2007). Supporting this environment of pro-activation in the gut, we also observed higher levels of monocytes which expressed CCR5, a proinflammatory marker which also is a receptor for HIV entry into cells in gut-isolated cells as well as systemically in blood (Figure 4J). At the time points examined, the gross loss of CD4 cells observed in HIV do not appear to occur in EcoHIV and there is no loss of macrophages to account for bacterial translocation. However, changes in CD4+CD8+ T-cells and CD11B+CD11C+ monocytes support a promotion of proinflammatory cytokines which has been shown to drive replication of HIV/EcoHIV.

As shown in Figure 4, systemic translocation of bacteria with morphine alone and EcoHIV + morphine occurred despite unchanged levels of macrophages and T cell subpopulations. Thus, we decided to see if there were any functional differences in the macrophages phagocytic activity. Studies have previously shown that morphine alone can cause deficiency in the phagocytic activity of macrophages (Tubaro et al. 1985; Rojavin et al. 1993; Ninkovi and Roy 2012), so we compared changes by EcoHIV and EcoHIV + morphine. As compared to placebo, morphine, EcoHIV, and EcoHIV + morphine all showed a reduced uptake of FITC dextran beads indicative of compromised phagocytic ability of isolated peritoneal macrophages (Figure 5a). This phagocytic deficiency was verified via confocal imaging of peritoneal macrophages (Figure 5b). Quantification of multiple fields showing a similar trend toward reduction in phagocytosis in the three treatment groups, however significance was not achieved (Figure 5c). This effect can be recapitulated *in vitro* using the RAW 264.7 macrophage cell line, which showed a significant reduction in all three treatment groups (Figure 5D) and a significant difference between EcoHIV and EcoHIV + morphine treated cells. We next examined whether the diminished phagocytosis was biased towards either gram-positive or gram-negative bacteria. We saw similar results as with the FITC dextran beads, with both gram-positive *S. aureus*-opsonized particles (Figure 5E) and gram-negative *E. coli*-opsonized particles (Figure 5F) showing diminished phagocytosis in the three treatment groups. Overall, phagocytosis is reduced by morphine and EcoHIV for either *in vitro* or *in vivo* macrophages, which may

contribute to lack of bacterial clearance and sustained systemic bacteremia. The reduction in phagocytosis is universal and not selective for bacterial phenotype which stimulate TLR2 (gram positive) or TLR4 (gram negative).

Given the observed increase in systemic bacterial translocation combined with increase in activation markers and the lack of phagocytosis, we hypothesized that inflammation would be apparent in systemic compartments. Indeed, at 5 days post infection we observed a significant increase in IL-6 within the liver of EcoHIV and EcoHIV+morphine treated animals, and EcoHIV+morphine animals had a significant increase over EcoHIV alone (Figure 6A). TNF α also showed significantly higher levels at this time point in EcoHIV infected and EcoHIV+morphine animals in liver homogenate above placebo treated animals (Figure 6B). Lung IL-6 levels trended to be higher but did not reach significance (Figure 6C) whereas TNF α levels were significantly higher in EcoHIV+morphine animals and a significant increase in EcoHIV+morphine compared to EcoHIV infection alone (Figure 6D). We examined supernatant from RAW 264.7 cells infected with EcoHIV as in the phagocytosis assay and found that IL-6 (Figure 6E) and TNF α (Figure 6F) were both significantly increased in EcoHIV infection and EcoHIV+morphine. Systemic inflammation, with elevated TNF α and IL-6 observed in the EcoHIV and morphine treated animals corroborates with observations demonstrated in HIV and IDU patients. Sustained systemic Inflammation has been shown to correlate with bacterial translocation and systemic replication of EcoHIV. Morphine in conjunction with EcoHIV infection shows higher levels of inflammation in liver and lung than EcoHIV infection alone.

Finally, we also examined the long-term systemic effects by EcoHIV infection in the presence of morphine. In brain at 30 days post infection, we observed that EcoHIV +morphine increased expression of IL-6 (Figure 7A). We also examined IFN γ (Figure 7B) and IL-1 β (Figure 7C) which had similar trends as IL-6 but did not achieve significance. Therefore, inflammation can be observed in the brain at time points well beyond 2–5 days in animals with EcoHIV+morphine.

Discussion

Our results suggest that a combination of EcoHIV infection and morphine leads to an exacerbated inflammation state compared to EcoHIV infection alone, likely by influencing bacterial translocation and increasing inflammatory cytokines (Figure 8). Bacterial translocation from the gut has been shown to directly correlate with inflammation that drives HIV replication in long term nonprogressive HIV-infected humans despite lower amounts of LPS in their serum at levels virtually identical control patients (Brenchley et al. 2006). LPS as a biomarker has also been highlighted as a vital factor in what drives SIV to be pathogenic in Rhesus Macaques as compared to natural hosts who do not develop AIDS (Pandrea et al. 2008).

Given this tight link between bacterial translocation and HIV replication, it should come as no surprise that increasing LPS further would worsen HIV pathogenesis. Previous studies have shown that morphine alone not only disrupts the physical barrier in the gut provided by epithelial cells, but also alters the function of immune cells including reducing the clearance

of bacterial products by macrophages (Hilburger et al. 1997; Roy et al. 2011; Ninkovi and Roy 2012; Meng et al. 2013), leading to an overt increase in the amount of systemic bacterial translocation. This effect by opioids has been suggested in limited HIV studies in heroin abusers (Ancuta et al. 2008). While research has long suggested that HIV has a faster progression to AIDS with substance abuse, it has been difficult for epidemiological studies to validate this on a population level (Kapadia et al. 2005). This deficiency highlights the major difficulties with studying drug abuse in a human population, especially among HIV patients, and the lack of animal models to study these interactions has limited the progression to understanding the interactions between HIV and morphine. Although there have been a number of studies on effects of morphine on SIV infection in macaques (Rivera-Amill et al. 2010; Bokhari et al. 2011; Spikes et al. 2012; Rivera et al. 2013; Hollenbach et al. 2014), to our knowledge early pathogenesis within the gut has not been investigated. In order to take advantage of genetic tools and low cost of mice, we pursued an infectious model of HIV to combine with our established model of opioid abuse to overcome these limitations in the field.

Our data show that translocation occurred at 2 days post-infection, and EcoHIV+morphine showed the highest amount of bacterial translocation. We utilized a live CFU assay along with the RAW blue assay to account for both gram positive and gram negative bacteria, as opposed to serum LPS which only accounts for gram negative. Translocation occurred highest in the liver, which suggests the bacteria is traversing via the portal circulation. MORKO mice treated with morphine, EcoHIV, or EcoHIV+morphine showed a reduced amount of translocation at 2 days post-infection indicating that opioids, both endogenous or exogenous, likely plays a direct role in exacerbating this pathology. While endogenous opioids have not been examined directly, the stress from infection may increase endogenous opioids enough to mimic the bacterial translocation observed in exogenous opioids such as morphine. Interestingly, TLR4 knockout mice, which lack a functional receptor that recognizes LPS, also had drastically reduced translocation at 2 days post-infection. This supports the notion that LPS is playing a direct role in the pathogenesis, likely due to sustained activation in the presence of morphine (Banerjee et al. 2013). However, TLR4 is not the only player in this process. EcoHIV+morphine had translocation similarly to wildtype animals, which likely reflects an abundance of stress on gut homeostasis even in the absence of TLR4. TLR2 has also been shown to be increased by morphine alone and in the presence of HIV proteins (El-Hage et al. 2011; Kwok et al. 2012; Meng et al. 2013), and very likely plays a role in the bacterial translocation we observed.

The clearance we observed contrasts from HIV and SIV, where LPS is observed in all stages of uncontrolled infection including early, late, and AIDS phases (Brenchley et al. 2006; Estes et al. 2010; Nowroozalizadeh et al. 2010). Gut CD4 loss has been suggested to contribute to microbial translocation in SIV (Brenchley et al. 2008), which we did not observe at the time points examined in this model and could be a reason for this conflict. Given that this model allows us to look at very early stages of disease, we believe EcoHIV is beneficial for studying the mechanisms of early HIV pathogenesis of HIV in the context of drugs of abuse, which has been nearly impossible to study due to the unknown infection status of individuals. This model may also give a resolution to mechanisms at early time points which have been unable to be studied in HIV. Future studies will examine the gut

associated lymphoid tissue (GALT) to understand if EcoHIV infection can disrupt functional immunity at later time points as is observed in HIV and SIV.

Unexpectedly, bacterial translocation was not observed at 5 days post-infection. One explanation is that systemic translocation may be prevented at this point by a recovery of the GALT. While we observed a decrease in phagocytosis at this time point of infection in primary macrophages *ex vivo*, the microenvironment *in vivo* may support a greater clearance of bacteria preventing its translocation to the liver and other systemic tissues. Additionally, other normal gut homeostasis mechanisms such as the increase of mucin or defensin secretion, which would protect the epithelial barrier, may be increased due to inflammation (Clark and Coopersmith 2007). Both mechanisms need be examined in future studies to elucidate how they change given exposure to morphine and EcoHIV infection.

Despite the lack of detectable bacterial translocation at this time point, pathology and inflammation were both still apparent which suggests that the translocation had a persistent effect and could play a role in HIV pathogenesis even after if cleared. However, timepoints this early in HIV pathogenesis have not been examined which could account for the difference and it is unknown whether disruption of gut homeostasis at early stages happens stably or in phases with the ongoing pathogenesis of HIV.

In addition to pathology in the gut, our studies show that EcoHIV and morphine have a negative effect on the ability of peritoneal macrophages to perform phagocytosis, which plays a major role in the clearance of translocated bacteria. This coincides with previous data that showed morphine alone causes a deficiency in phagocytosis (Tubaro et al. 1985; Casellas et al. 1991; Rojavin et al. 1993; Ninkovi and Roy 2012). A breach in epithelial barrier is not enough for translocation to become systemic, so a decrease in phagocytosis likely contributes to the bacterial translocation we observed to liver.

One major difference between this model and HIV pathogenesis is the lack of early CD4+ T-cell depletion within the gut which have been observed at acute stages of HIV infection (Brenchley et al. 2004; Gordon et al. 2010). Prolonged time points in this model are needed to resolve this difference, as our study included time points that were very early relative to what has been examined in HIV or SIV studies. Thus, this model provides flexibility to study time points which are unattainable or impractical to study in HIV or SIV infections. Examining CD4 cells at a deeper resolution, depletion of Th17 cells have been suggested to be the predominant cause for the observed microbial translocation following SIV infection in Rhesus Macaques since Th17 cell depletion is not observed in nonprogressors (Brenchley et al. 2008). Recent data from our lab suggests that morphine alone can induce a deficiency in the Th17 response (communicated), which supports our results in this study. Future work will examine the effect of morphine on CD4 skewing in EcoHIV infection with an emphasis on the role of Th17 cell depletion and microbial translocation.

Despite clearance of bacteria, we observed cytokines TNF α and IL-6 at high levels in EcoHIV treated animals in combination with morphine in both liver and lung at day 5 post-infection. This inflammation can act to drive systemic HIV replication and drive HIV into long-lived cellular reservoirs such as macrophages, where it can continue promoting

secretion of inflammatory molecules in the absence of further translocation. HIV and opioid abuse have been shown to cause more severe neurocognitive deficits due to immune dysfunction (Hauser et al. 2007; Hollenbach et al. 2014), so we investigated if morphine treatment in this model could alter inflammation in the brain. Indeed, we saw higher expression of inflammatory cytokines in the brains of EcoHIV infected mice in combination with morphine 30 days post-infection consistent with inflammation playing a significant role in long-term neurocognitive defects. While neurocognitive defects are commonly observed in IDU HIV patients, it is impossible to study the direct effects of opioids and how they contribute to neurological symptoms in people. Comorbidities in human IDU, such as nutritional deficiencies, stress, or lack of medical care, make an animal model to study the mechanism leading to neurocognitive defects extremely desirable. HIV patients who abuse substances have much lower adherence to medications so, despite reasonable therapeutics being available, they are more susceptible to negative consequences of uncontrolled infection (Hinkin et al. 2004). EcoHIV as an animal model provides a great opportunity to study HIV with drugs of abuse in combination with antiretroviral therapies to finally tease apart the mechanisms of substance abuse with the comorbidities associated with aberrant lifestyles. Future work with EcoHIV will examine whether the inflammation observed in the brain is instigated by the original translocation events observed or is an independent event induced by morphine and EcoHIV infection.

Overall, despite some limitations, we believe that EcoHIV in combination with morphine is a model for early pathogenesis of HIV which recapitulates pathology observed HIV patients such as induction of bacterial translocation, gut pathology, and inflammation. As we are concerned with HIV and how infection influences gut homeostasis, including immune and epithelial cells, this model provides a major advantage over humanized mice where crosstalk between cells from human and mice may be compromised. While the entry into cells by EcoHIV differs from HIV infection (MCAT-1 versus CD4/co-receptors), all HIV proteins are present aside from gp120. Though gp120 may contribute to pathology, we believe that this murine model of HIV offers the best compromise to study the gut as a system between humanized mice infected with HIV and transgenic mice expressing HIV proteins.

Our experimental design examines a very early time point of HIV pathogenesis that has not been examined with other models. For instance it is not known how early translocation occurs in HIV infection, and once established whether it is constant or occurs in cycles. Any differences between the present work and other studies may be resolved with experimental manipulation such as utilizing different lengths of EcoHIV infection.

This model is particularly useful for studying the interactions of opioids with HIV as it provides flexibility for looking at different opioid paradigms in relation to HIV infection, in addition to having the ability to utilize different genetic backgrounds such as MORKO mice which are not available when using humanized mice. The present study pretreated animals with a slow release morphine pellet that limits the mice exposure to withdrawal effects. Shifting the treatment to different dosing patterns of morphine such as giving morphine after EcoHIV infection rather than before or using an opioid dosing paradigm which includes withdrawal may better reflect human exposure to drugs in HIV infection, however this needs to be optimized in future studies.

Given the results presented, we believe this model provides a useful avenue for studying HIV and drugs of abuse where other models have fallen short. Future studies will also investigate whether changes in microbiota within the gut can contribute to translocation, which was not addressed in this study. We believe this model can be used to elucidate the mechanisms underlying the interactions between HIV and drugs of abuse at early stages of disease.

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Fig. 1.

EcoHIV infects both *in vitro* and *in vivo*, but morphine does not alter viral burden. QPCR was performed on EcoHIV infected RAW/J774 cells (A) or spleens from C57BL/6 mice (B) to look for HIV LTR-gag, where we saw expression except in untreated animals. Peritoneal or spleen cells were harvested from Eco-GFP infected mice and analyzed for infected cells by FACS (C), showing a positive shift in peak from uninfected, or microscopy (D), which show positive green staining in infected cells. P24 ELISA was performed on liver (E), lung (F), or brain (G) of infected mice, which show detectable levels at all timepoints and a significant difference between EcoHIV+morphine and EcoHIV at D21 in the liver. $P < .05$

Fig. 2.

Bacterial translocation occurs early in EcoHIV infection and is exacerbated by morphine. Liver, lung, or spleen was homogenized and cultured on blood agar plates in aerobic conditions to count live CFUs. Mice were infected either 2 days (A), which showed significant translocation to liver and lung in EcoHIV+ morphine and to liver in EcoHIV, or 5 days (B), which showed no significant translocation. Homogenate from liver was tested, using RAW blue cells (Invitrogen) which express SEAP on NF κ B promoter upon stimulation by PAMPs, at 2 days (C) and 5 days (D) which validated the translocation results in (A) and (B) for liver. (E) Mice infected for 2 days were gavaged with Rhodamine-dextran 4 hours prior to Xenogen imaging. Finally, MORKO (F) or TLR4KO (G) mice were assessed for CFU growth after 2 days EcoHIV infection as above. *P<.05

Fig. 3.

Gut epithelial tight junctions and gross morphology are altered in EcoHIV infection and worsened in combination with morphine. Representative figures from distal small intestine (A) or colon (B) was stained with Hematoxylin (nuclei, brown), Alcian Blue (goblet cells and mucin, blue), and Metanil Yellow (tissue, yellow). Intact LP from Small intestine sections were measured for length, width, and crypt depth. Morphine, EcoHIV, and EcoHIV+morphine showed a decrease when compared by ratios of LP length:width (E) or LP length:crypt depth (F). Colon was measured for depth (G), which is presented as an average of 6 measurements per section, and found EcoHIV+morphine to have a significantly reduced depth compared with control. Small Intestine n: Placebo, 10; Morphine, 10; EcoHIV, 16; Eco+Ms, 7. Large Intestine n: Placebo, 7; Morphine, 5; EcoHIV, 8; Eco+Ms, 5. * $p < .05$, ** $p < .01$

Fig. 4.

EcoHIV with morphine does not greatly alter proportions of immune cells within the gut. Gut cells were collected from homogenized distal SI (A,D,G), Cecum (B,E,H), or colon (C,F,I) and stained for FACS analysis. CD3+ T-cells were gated and values of CD4, CD8, or CD4/CD8 double positive were examined (A,B,C), with the only significant change in DP cells being higher in morphine, EcoHIV, and EcoHIV+morphine. CD19+ B-cells were examined as a percentage of live cells (D,E,F) and no significant change was observed. CD45+ monocyte cells were gated and values of CD11B, CD11C, or CD11B/CD11C double positive were examined (G,H,I), with the only significant change in large intestine DP cells in morphine, EcoHIV, and EcoHIV+morphine. Cells were gated for CD45+ and MHCII high to see CCR5 expression in macrophages, which saw trending increases at all locations, most notably in EcoHIV+morphine. N: Placebo, 4; morphine, 6; EcoHIV, 5; EcoHIV+Ms, 8. * $p < .05$

Fig. 5.

Macrophage phagocytosis is decreased by EcoHIV infection and worsened in combination with morphine. Peritoneal macrophages were harvested from EcoHIV infected mice and cultured with FITC-dextran and the phagocytic index was calculated from fluorescence as a percentage of control (A). Peritoneal macrophages were incubated with FITC-dextran (green) and stained with phalloidin (Red) and DAPI (blue); shown are representative images (B) and quantification of multiple fields (C). RAW 264.7 macrophage cell line was incubated with FITC-dextran as in part A (D). This approach was repeated with *S. aureus* (E) or *E. coli* (F) opsonized particles to examine any bias between TLR2- or TLR4-mediated phagocytosis. N: all groups=6. *p<.05, **p<.01, ***p<.001

Fig. 6.

EcoHIV increases IL-6 and TNFα systemically, which is exacerbated by morphine. (A) IL-6 ELISA was performed on liver supernatant which had significantly higher levels in EcoHIV and EcoHIV+morphine treatments compared with placebo, and significantly higher levels in EcoHIV+morphine compared with EcoHIV or morphine alone. (B) TNFα in the liver showed similar results, with EcoHIV and EcoHIV+morphine having significantly higher levels than placebo, and EcoHIV+morphine having significantly higher levels than morphine alone. We also examined lung IL-6 (C) and TNFα (D) levels, which showed EcoHIV+morphine had significantly higher levels of TNFα compared to all other groups and IL-6 trended that way but did not achieve significance. To elucidate the potential source of cytokines, supernatant from RAW264.7 cells were examined and shown to have both higher IL-6 (E) and TNFα (F) levels in EcoHIV and EcoHIV+morphine treatments. N: Placebo=4, Morphine=6, EcoHIV=6, EcoHIV+Morphine=6. *p<.05, **p<.01, ***p<.001

Fig. 7. Long term (30 day) EcoHIV infection combined with morphine increases cytokine expression in brain over infection alone. RNA was isolated from brain and analyzed via qPCR for IL-6 (A), IFN- γ (B), and IL-1 β (C). N: Placebo=3, Morphine=5, EcoHIV=3, EcoHIV+morphine=5. * $p < .05$

Fig. 8.

Model of EcoHIV and morphine induced bacterial translocation. Bacterial translocation is induced likely by a combination of morphine altering tight junction expression (Meng et al. 2013) and EcoHIV activating an inflammatory response. (1) Bacteria is able to translocate across the epithelial barrier which alters gut morphology (2), likely by activating basolateral TLRs, (3) Phagocytosis of bacteria by macrophages is diminished, which allows the spread of bacteria systemically to tissues such as liver. (4) We observed continued inflammation in both the liver and brain tissues, despite the clearance of translocated bacteria. This may result from prolonged TLR4 activation, as TLR4KO mice had decreased translocation in our studies. TLR4 typically undergoes tolerance following activation however this has been shown to be disrupted with morphine leading to long term activation (Banerjee et al. 2013).

Table 1

Primers for RT-PCR

Primer	Forward (5' to 3')	Reverse (5' to 3')
IL18	GCCTCAAACCTTCCAAATCA	TGGATCCATTTCTCTCAAAGG
IFN γ	TGAACGCTACACACTGCATCTTGG	CGACTCCTTTTCCGCTTCCTGAG
IL1b	TTGACGGACCCCAAAGATG	AGAAGGTGCTCATGTCCTCA
IL6	GCCTTCTTGGGACTGATGCTGG	GTGTAATTAAGCCTCCGACTTGTGAAGTGG
18S	TTGACGGAAGGGCACCACCAG	CTCCTTAATGTCACGCACGATTC
LTR-gag	TCTCTAGCAGTGGCGCCCGAACA	TCTCCTTCTAGCCTCCGCTAGTC