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Persistent EcoHIV infection induces nigral degeneration in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-intoxicated mice

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Abstract

The widespread use of antiretroviral therapy for treatment of human immunodeficiency virus (HIV) infections has dramatically improved the quality and duration of life of HIV positive individuals. Despite this success, HIV persists for the life of an infected person in tissue reservoirs including the nervous system. Thus, whether HIV exacerbates age-related brain disorders such as Parkinson's disease (PD) is of concern. In support of this idea, HIV infection can be associated with motor and gait abnormalities that parallel late stage manifestations of PD including dopaminergic neuronal loss. With these findings in hand, we investigated whether viral infection could affect nigrostriatal degeneration or exacerbate chemically induced nigral degeneration. We now demonstrate an additive effect of EcoHIV on dopaminergic neuronal loss and neuroinflammation induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication. HIV-1-infected humanized mice failed to recapitulate these EcoHIV results suggesting species-specific neural signaling. The results demonstrate a previously undefined EcoHIV-associated neurodegenerative response that may be used to model pathobiological aspects of PD.

Keywords

Parkinson's disease; central nervous system; EcoHIV; human immunodeficiency virus; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Introduction

Following the introduction of effective antiretroviral therapy (ART) for the treatment of human immunodeficiency virus type one (HIV-1) infection individuals living with disease live life with limited morbidities (Cardoso *et al*, 2013). Indeed, numbers of HIV-infected people over the age of 50 increased dramatically in recent years (Wing, 2016).

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Consequently, as the HIV-infected ART-treated population ages, patients will be prone to age-related neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). Added to this likelihood are known similarities between HIV-associated brain disease and AD or PD (Cardoso *et al*, 2013; Mirsattari *et al*, 1998). PD is characterized by motor dysfunction linked to the loss of dopaminergic neurons along the nigrostriatal system paralleled with the formation of Lewy bodies and neuroinflammation. The latter results from the accumulation of misfolded or modified alpha-synuclein in affected neurons (Kalia and Lang, 2015). HIV infection can also affect the nigrostriatum and lead to motor impairments (Cardoso, 2002). Thus, a major, yet recurrent question is whether HIV infection affects the development of PD-linked neurodegeneration (Moulignier *et al*, 2015).

Past studies indicate that the central nervous system is highly susceptible to HIV infection and related disease (Davis *et al*, 1992; Williams *et al*, 2012; Williams and Hickey, 2002). Such infection, interestingly, affects dopamine-rich brain subregions that parallel, in measure, those regions affected in PD (Ellis *et al*, 2009; Kumar *et al*, 2009). Raising concern for such disease linkages are post-mortem brain tissue examinations. These showed HIV-infected people have increased levels of alpha-synuclein within the substantia nigra pars compacta (Khanlou *et al*, 2009). HIV-infected brain tissues also show signs of neuroinflammation, lymphocyte brain infiltration, impaired mitochondrial integrity, and increased evidence for oxidative stress (Hong and Banks, 2015; Jones *et al*, 1998; Moulignier *et al*, 2015). HIV patients also display similar neurological impairments observed in advanced PD. These include bradykinesia and impaired dexterity, gait, and cognitive functions (Navia *et al*, 1986; Sheppard *et al*, 2015) (Rosso *et al*, 2009). HIV-infected patients have also shown reduced beta oscillations known to affect movement function (Wilson *et al*, 2013). These reflect a loss in dopaminergic neuronal function in the basal ganglia, substantia nigra and striatum (Berger and Arendt, 2000) linked to loss of dopaminergic neurons and decreased availability of dopamine (Groger *et al*, 2014). Indeed, HIV-infected patients display up to 53% decreased dopamine availability that changes little following ART (Kumar *et al*, 2011). Similarly, a decrease in the dopamine metabolite, homovanillic acid (HVA), is common (Kumar *et al*, 2009; Kumar *et al*, 2011).

Mutual links between viral infections and PD are known, but can affect disease by divergent mechanisms. *First*, influenza virus, herpes simplex virus, Epstein-Barr virus, cytomegalovirus, polio virus, HIV, West Nile virus, and Japanese encephalitis B viruses are known to be associated with PD (Jang *et al*, 2009). *Second*, each of these viruses is neurotropic, easily enters the brain, results in neural cell death, and elevates local pro-inflammatory cytokine production (Karim *et al*, 2014). *Third*, and opposing this view, are disease signs and symptoms which differ between HIV and PD (Boyd *et al*, 2016; Pakpoor *et al*, 2017). In attempts to reconcile these views together with the potential that HIV-1 infection could affect neurodegenerative processes, we employed two mouse models of infection in association with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication known to induce nigrostriatal degeneration to assess each influence alone or together on PD-associated neuronal demise. We found that the EcoHIV, a chimeric HIV that can persistently infect conventional mice, enter the brain, and replicate in murine host microglial cells, was selectively neurotoxic to nigral dopaminergic neurons with notable synergy during MPTP exposure (Potash *et al*, 2005). These results provide credible

evidence, for the first time, that in the correct microenvironment, HIV infection affects the onset or progression of PD.

Results

HIV-1 infection does not alter nigrostriatal degeneration in MPTP-intoxicated humanized mice

To assess a potential interplay between HIV infection and PD-associated pathologies, humanized NSG mice were used in experimental assays. These mice were either HIV-1 infected or uninfected then MPTP-intoxicated using a low (14 mg/kg) concentration dose so that potential synergy, if present, could be recorded between virus and chemically induced brain tissue damage. Infected mice were sacrificed at indicated times and neuron numbers were assessed by stereological analysis. TH+Nissl+ dopaminergic neuron counts were decreased with MPTP intoxication, as expected from 9012 ± 679 to 6596 ± 580 (Figure 1A and 1B). Combination of HIV infection and MPTP intoxication did not increase the degree of degeneration observed, suggesting no effect on speed or amount of neurodegeneration associated with MPTP intoxication in this animal model. However, with MPTP alone or HIV infection and MPTP, TH density within the striatal termini decreased ~50%, respectively, when compared to PBS controls (Figure 1C). Likewise, upon analysis of microglial reactivity, no significant differences were observed between treatment groups. Mac-1+ counts remained similar between groups (Figure 2A and 2B), indicating that HIV infection did not enhance microglial reactivity above that observed in MPTP-intoxication alone. Taken together, these results show that chronic and active HIV infection did not enhance nigrostriatal degeneration in MPTP-treated humanized mice. These findings indicate either the lack of a synergistic effect causing neuronal loss, a lack of interaction between the reconstituted human lymphocytes and murine microglia within the humanized system, or the inability of HIV to infect mouse microglial cells. The limited loss of dopaminergic neurons is most likely due to the initial neuronal toxicity of the toxic MPTP metabolite, MPP^+, rather than the ensuing inflammatory cascade associated with immune activation. Based on these findings, we began to explore the use of EcoHIV infection in conventional mice (He *et al*, 2014; Potash *et al*, 2005).

EcoHIV infection and MPTP intoxication affect nigrostriatal degeneration

To determine a time-course evaluation of EcoHIV infection in conventional mice, male, C57BL/6 mice were inoculated with EcoHIV for 2, 3, or 4 weeks, followed by MPTP. Surviving neuron numbers were decreased with all treatments when compared to the control group (Figure 3A-C). EcoHIV infection alone resulted in a significant decrease in neuron counts relative to uninfected controls at all time points, dropping neuron counts from 9556 ± 326 for PBS to 7450 ± 350 at 2 weeks of infection, 7359 ± 364 at 3 weeks of infection, and 6836 ± 999 at 4 weeks of infection, respectively. Addition of MPTP intoxication led to a further decrease in neuronal numbers at all time points, resulting in 5404 ± 671 at 2 weeks of infection + MPTP, 6238 ± 743 at 3 weeks of infection, and 4103 ± 103 at 4 weeks of infection and MPTP. Findings demonstrate that at 4 weeks of infection and MPTP intoxication, numbers of surviving dopaminergic neurons were decreased within the

substantia nigra compared to mice treated with PBS, MPTP, or 3 weeks EcoHIV infection and MPTP, indicating an effect occurs with longer periods of viral infection.

Based on these findings, we selected 4 weeks after infection to evaluate potential interactions between HIV infection and MPTP in affecting nigral neurodegeneration. Given that microglial activation and increased reactivity are associated with neuronal cell death in the MPTP mouse model of PD (Kurkowska-Jastrzebska *et al*, 1999), Mac-1+ reactive microglia were quantified within the ventral midbrain of mice treated with HIV ± MPTP (Figure 4). Numbers of reactive microglia observed were significantly increased from PBS controls in MPTP without infection and at 4 weeks of infection followed by MPTP (Figure 4B). The other experimental groups remained similar to controls. Notably, 4 weeks of infection followed by MPTP intoxication increased reactive microglial counts nearly 10-fold, increasing to 29 ± 4.1 cells/mm^2 compared to 3 ± 0.3 cells/mm^2 in PBS-treated controls and 3-fold compared to mice treated with MPTP alone. These findings support the notion that EcoHIV infection affects MPTP mediated nigral degeneration and enhances the neuroinflammatory response.

Studies show that T cells interact with microglial populations to elicit active immune responses (Brochard *et al*, 2009). To test this possibility in the present system, mice were bled over the indicated time course and peripheral blood collected for analysis of changes in lymphocyte populations (Figure 5). Flow cytometric analysis indicated that CD3, CD4, CD25, and CD8 populations remained relatively unchanged over the course of infection and remained stable after MPTP intoxication, revealing the lack of a shift in number or frequency of T cell populations. These findings confirm that EcoHIV infection does not cause a diminution of peripheral CD4+ T cells, consistent with previous observations (Geraghty *et al*, 2017) and indicate a potential analogy with HIV positive patients on effective HIV-suppressive cART (Wright *et al*, 2011). Also, these results indicate that sufficient levels of CD4+ T cells exist to support the necessary immune reactions for successful neurodegeneration following MPTP intoxication.

Next, we investigated possible mechanisms for increased neuronal loss and microglial reactivity observed at 4 weeks co-insult. Four weeks after EcoHIV infection, we isolated the ventral midbrain of mice following MPTP intoxication alone or at 4 weeks EcoHIV infection alone or at 4 weeks EcoHIV infection followed by MPTP intoxication. Total RNA was isolated to assess changes in inflammatory response gene expression (Figure 6). Although the results did not reach statistical significance, we observed marked trends in gene expression changes suggesting enhanced inflammatory responses upon co-insult. Comparison to EcoHIV alone, co-insult results in increased gene expression levels of the genes encoding for CCR7, CCL2, IFNγ, IL-10, IL-9, and NF-κB and decreased levels of TLR4 and IL-5 (Figure 6A and 6C). When compared to MPTP alone, co-insult leads to upregulation of, CCL2, IFNγ, IL-10, IL-9, NFκB, TLR4, and IL-5 (Figure 6B and 6C). These results suggest a synergistic effect of two insults causing dysregulation in the inflammatory response pathway within the ventral midbrain.

EcoHIV infection does not affect MPTP-induced neuronal degeneration in immunodeficient mice

Next, to evaluate the role of T cell-mediated neurodegeneration in EcoHIV infection, we utilized immunodeficient, non-humanized NSG mice (Figure 7) and C57BL/6 scid mice (Figure 8). Each strain of mice was infected with EcoHIV for 4 weeks, followed by MPTP-intoxication. On day 7 post-MPTP, animals were sacrificed and brains harvested. Neuronal survival and microglial reactivity were assessed and quantified using stereological analysis. MPTP intoxication lead to a 13% and 6% loss in TH+ dopaminergic neurons, respectively, indicating that this strain may not be readily susceptible to MPTP intoxication (Figure 7A and 7B). This is most likely due to the lack of lymphocytes needed for MPTP-induced neurodegeneration susceptibility (Benner *et al*, 2008; Brochard *et al*, 2009). However, EcoHIV infection alone significantly decreased neuronal numbers by 26% compared to PBS, suggesting EcoHIV may have a direct effect on dopamine–producing neurons, which is similar to the results observed in the C57BL/6 strain of mice. Likewise, in humanized NSG mice, dopaminergic neuron numbers diminished after infection with EcoHIV alone (Figure 7B), further supporting the idea that EcoHIV may be directly neurotoxic. However, even though the neuronal cell bodies remained intact following MPTP intoxication, striatal termini were not spared (Figure 7C). MPTP intoxication significantly deceased the relative TH+ density within the striatum compared to PBS controls (Figure 7C), but EcoHIV infection alone did not result in a significant loss of termini. These findings support the idea that striatal termini are more susceptible to MPTP-intoxication and degeneration may occur in cell termini prior to the degeneration of neuronal bodies (Burke and O'Malley, 2013). To assess the presence of an inflammatory response, microglial populations were also stained and quantified in the same tissues, as previously performed. Reactive microglial numbers were not significantly increased with MPTP intoxication when compared to PBS controls (Figure 7D and 7E).

In studies using immunodeficient, C57BL/6 scid mice, the results were similar to non-humanized NSG mice. TH+ neuron counts indicate no significant decrease in cell number after MPTP intoxication (Figure 8A and 8C). However, MPTP intoxication still resulted in an 8% and a 24% loss of dopaminergic neurons within the substantia nigra (Figure 8C). EcoHIV infection alone also led to a 7% loss in cell number, further supporting the concept that the virus itself causes dopaminergic cell death. Similarly, striatal termini in all treatment groups were significantly diminished (Figure 8B and 8D). MPTP intoxication alone or EcoHIV infection alone resulted in the 39% and 20% respective loss of striatal termini, while EcoHIV infection plus MPTP-intoxication displayed a 52% loss, thus indicating increased susceptibility of striatal termini (Figure 8D). Microglial reponses remained unreactive (Figure 8E and 8F) as no significant changes in reactive microglia numbers were discernible among treatment groups (Figure 8F). In fact, little to no inflammatory microglial response could be detected.

Discussion

HIV-1 infection is associated with a spectrum of behavioral, motor, and cognitive disorders commonly called HIV-associated neurocognitive disorders (HAND) (Clifford and Ances,

2013; Ungvarski and Trzcianowska, 2000). While disease severity has decreased during the ART era neurocognitive signs and symptoms remain (Clifford and Ances, 2013). A critical question for understanding HAND in the ART era is whether low-level restricted viral infection affects the onset, severity, or progression of neurodegenerative disease (Deleidi and Isacson, 2012). Post-mortem studies in brain tissue from HAND patients demonstrate increased alpha-synuclein and decreased dopamine levels in infected areas. (Kalia and Lang, 2015; Khanlou *et al*, 2009). However, there are few published reports that investigate the interplay between low-level HIV infection and development of neurodegenerative disease in animal and cellular model systems (Chai *et al*, 2017; Dickens *et al*, 2017; Jones *et al*, 2007; Meeker and Hudson, 2017). Therefore, we investigated the potential interactions between virus and chemically induced neurodegeneration. HIV-1-infected humanized mice were challenged with MPTP. Neither HIV infection alone nor HIV and MPTP showed any virus-specific effect on nigrostriatal degeneration. These results suggested that either interactions between virus and MPTP were lacking or that resident microglia and neurons failed to effectively react with an operative human immune system. The latter possibility reflects our own studies of animals with an operative humanized brain and immune system model of neuroHIV (Li *et al*, 2017).

A second possibility centers on how neurodegeneration occurs following MPTP intoxication. To initiate a neurodegenerative cascade, MPTP must be metabolized into its neurotoxic form, MPP^+. The resulting neurotoxicity accounts for approximately 10% of neuronal cell death (Jackson-Lewis and Przedborski, 2007), and further neuronal loss requires an induced pro-inflammatory microenvironment resulting in reactive microglia. Studies also demonstrate that mice lacking CD4+ T cells are not susceptible to MPTP intoxication (Brochard *et al*, 2009), (Benner *et al*, 2008). Thus, we investigated if the lack of neurodegenerative response may be due to insufficient CD4+ T cell and microglial interactions using scid and NSG mice. In these immunocompromised systems, MPTP intoxication resulted in a minor loss in dopaminergic neuron numbers. This loss was likely due to the effect of MPTP metabolism to MPP+ (Jackson-Lewis and Przedborski, 2007). Without functional CD4+ T cells, the secondary inflammatory cascade does not occur. This finding was also observed in the humanized system. Taken together, it appeared prudent to use species-specific substitutes for studies of innate and adaptive immunity. Thus, EcoHIV infection of conventional immunocompetent mice was used to address this question (Hadas *et al*, 2007; He *et al*, 2014; Jones *et al*, 2016; Potash *et al*, 2005).

EcoHIV/NDK is a chimeric virus on the backbone of HIV-1 clade D, NDK, containing the gp80 envelope gene of murine leukemia virus in place of HIV gp120 (Potash *et al*, 2005). EcoHIV was shown to infect immunocompetent mice by targeting CD4+ T cells, macrophages, and myeloid cells in the brain, induce anti-viral immune responses, invade the CNS, and increase inflammatory mediators in the brain. EcoHIV infection mimics HIV and offers a testing paradigm for studies of virus-induced neurodegeneration (Hadas *et al*, 2007; He *et al*, 2014; Potash *et al*, 2005). Notably, EcoHIV enters and establishes an infection in the mouse brain as early as 3 weeks after viral exposure and can alter BBB permeability by 2 weeks (Jones *et al*, 2016). Infected brains express inflammatory factors and mediators such as C3a, IL-1B, STAT-1, and IL-6 indicating an active infection and anti-viral response (Potash *et al*, 2005). Similarly, we observed a significant decrease in dopaminergic neuron

loss by 2 weeks of EcoHIV infection. These findings highlight the strengths and weaknesses of operative neuroHIV model systems for studies of disease pathobiology.

Indeed, with MPTP and EcoHIV co-insult, microglial reactivity was significantly increased 4 weeks after infection, potentially accounting for the increased timed-responses for the neurodegeneration observed. This may occur by induction of microglial inflammation as EcoHIV is known to readily infect macrophages and microglia leading to their activation (He *et al*, 2014; Potash *et al*, 2005), a finding confirmed in the current studies. While EcoHIV lacks gp120, virus expression in mouse microglia and the consequent microglial activation (He *et al*, 2014) likely results in the production of Tat (Jin *et al*, 2012; Lu *et al*, 2011; Sheng *et al*, 2000). Tat exposure induces prolonged microglial activation when compared to PBS-treated controls and lasts for 28 days post-injection, a similar time-course to our studies (Lu *et al*, 2011). When exposed to Tat, human microglial cells increased production of proinflammatory cytokines and chemokines, TNF-α, IL-1B, IL6, RANTES, and MIP-1α (Sheng *et al*, 2000). Other possibilities for the observed microglial response include the presence of direct neuronal death, causing resident microglia to become reactive as they sample the microenvironment (Kreutzberg, 1996), or the infiltration of infected peripheral macrophages across the leaky BBB to areas of degeneration (McArthur *et al*, 2005).

Nonetheless, with significant increases in both neuronal death and microglial activity by 4 weeks infection followed by MPTP, potential changes in the inflammatory response cascade were assessed. Although no significance was observed, these changes may be important in determining a mechanism of action. Changes in gene expression of both pro- and anti-inflammatory mediators were observed with EcoHIV infection followed by MPTP, indicating a general dysregulation of the inflammatory response. Increases in genes that encode pro-inflammatory mediators including CCL2, IFNγ, NFκB, TLR4, and IL-5 were observed as well as the anti-inflammatory mediators, IL-9 and IL-10. The observed increase in *Ccl2* here may be linked to BBB permeability. Studies show that CCL2 mediates the transmigration of HIV-infected leukocytes across the BBB, resulting in viral neuroinvasiveness (Eugenin *et al*, 2006). This may indicate a potential mechanism of CNS invasion with EcoHIV infection. HIV infection also activates NFkB and TLR4, which correlates with increased expression observed in this EcoHIV study (Fiume *et al*, 2012; Hernandez *et al*, 2012). Increased expression and activation of the NFkB pathway would ultimately lead to increases in overall pro-inflammatory gene levels (Fiume *et al*, 2012). Changes in TLR4 levels could affect the pro-inflammatory response by promoting viral replication and disease progression (Hernandez *et al*, 2012). Overall, changes in protein levels based on these gene changes may give rise to the enhanced microglial reactivity, as well as lead to an enhanced inflammatory microenvironment, further exacerbating neurodegeneration. On the other hand, upregulation of anti-apoptotic and anti-inflammatory interleukins, IL-9 and IL-10, may result from counteracting the neuroinflammatory response in order to maintain a homeostatic balance within the brain (Kwon and Kaufmann, 2010; Renauld *et al*, 1995).

EcoHIV viral infection alone yielded neuronal death in both immunocompetent and immunodeficient mice, indicating a direct effect on the dopaminergic system that does not

require cell-mediated immune responses. To our knowledge, no study has investigated the effect of EcoHIV infection on neurons within the substantia nigra or ventral midbrain, specifically. This brain region exhibits increased susceptibility to neuronal cell death itself, so the presence of virus or viral particles may lead to increased neuronal cell death that has not yet been observed in other regions of the brain (Haddad and Nakamura, 2015; Naoi and Maruyama, 1999). Tat has been implicated in many studies as having a neurotoxic effect on the dopamine transmission system (Gaskill *et al*, 2017). Tat may directly impact dopaminergic neurotransmission through modulation of the dopamine transporter and dysregulating Ca^{2+} needed for baseline activity and cell signaling, or by specifically damaging the areas of the brain that are dopamine-rich, including the substantia nigra and striatum (Gaskill *et al*, 2017; Hu, 2016; Silvers *et al*, 2007). Tat also inhibits the expression of tyrosine hydroxylase (TH), an enzyme needed for dopamine production, ultimately affecting dopamine transmission (Zauli *et al*, 2000). Dopaminergic neuron susceptibility to viral factors could lead to the likelihood of developing PD or Parkinson's-like symptoms later. It is likely that EcoHIV is affecting the dopaminergic system, similarly to HIV infection. Additional studies should be carried out to confirm changes in dopamine synthesis, neurotransmission, and TH gene expression. EcoHIV itself has a cooperative effect in other disease studies as well, including induction of chronic obstructive pulmonary disease (COPD) and increased inflammation within the gut following opioid use (Geraghty *et al*, 2017; Sindberg *et al*, 2015). These studies, along with ours, support the idea that EcoHIV infection may reproduce pathological conditions that are necessary for initiating mild, yet chronic diseases commonly observed in immunocompetent HIV-infected individuals.

MPTP treatment generates a neuroinflammatory response, similar to the one observed in human PD (Hirsch *et al*, 2012; Kurkowska-Jastrzebska *et al*, 1999). It mimics dopaminergic cell loss associated with PD (Jackson-Lewis and Przedborski, 2007). However, it does not encompass all aspects of PD pathology. Thus, the dual effect observed in this report may not be recapitulated in chronic MPTP intoxication or alpha-synuclein overexpression models (Mochizuki *et al*, 2006; Munoz-Manchado *et al*, 2016). Nonetheless, our findings clearly support an effect of EcoHIV infection along the nigrostriatal axis. This observed neurotoxicity is linked to microglial reactivity and changes in the inflammatory microenvironment. If viral infection can elicit neurodegeneration, then it may also affect the onset or progression in PD. Yet, a definitive answer awaits future epidemiological studies.

Materials and Methods

For HIV infection in humanized mouse studies, new-born NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NOD/scid-IL-2Rγ_c^{null} or NSG) mice (stock 005557, Jackson Laboratories, Bar Harbor, ME) were transplanted with human CD34+ stem cells obtained from umbilical cord blood, as previously described (Knibbe-Hollinger *et al*, 2015). Balb/cJ mice (stock 000651, Jackson Laboratories) served as non-humanized controls. After 15 weeks of reconstitution, humanized NSG mice were selected for study based on human CD45+ cells numbers within peripheral blood. Animals with the highest levels of CD45+ populations were selected for infection, whereas those with lower levels were placed into the no infection control groups. The selected animals were infected with HIV-1_{ADA} via intraperitoneal injection at 10^4 tissue

culture infective dose 50 ($TCID_{50}$)/mouse. After 4 weeks of infection, mice received 4 subcutaneous injections of vehicle (DPBS, 10 ml/kg body weight) or MPTP-HCl (Sigma-Aldrich) at 14 mg MPTP (free base)/kg body weight in DPBS; each dose given at 2 hour intervals. Seven days following MPTP intoxication, mice were sacrificed, brains harvested, and tissues processed for analysis. MPTP safety precautions were followed in accordance with determined safety and handling protocol (Jackson-Lewis and Przedborski, 2007). All animal procedures were also in agreement with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Six- to eight-week old, male C57BL/6J mice (stock 000664) or B6.CB17-Prkdc^{scid}/SzJ (C57BL/6 scid) mice (stock 001913) (The Jackson Laboratory) were used in all EcoHIV studies using previously described protocols (Jones *et al*, 2016; Potash *et al*, 2005). Mice were infected by intraperitoneal inoculation of EcoHIV at a dose of 3×10^6 pg p24/mouse. EcoHIV stock was prepared as previously described (Potash *et al*, 2005). After 4 weeks of infection, animals were MPTP intoxicated in the same manner as those performed for the HIV studies.

Plasma collection, flow cytometric assessment and T cell Profiling

Peripheral blood was isolated during the course of infection using a maxillary venipuncture. Plasma was collected for analysis of viral load, and whole blood was stained with fluorescently-conjugated monoclonal antibodies to human CD45, CD3, CD4, CD25, CD8, and FoxP3 to quantify T cell frequencies. For intracellular staining, samples were permeabilized using the FoxP3 staining buffer set kit (eBioscience). Fluorescent staining was analyzed using a FACSCalibur flow cytometer.

Perfusions and immunohistochemistry

Following administration of terminal anesthesia (Fatal Plus, pentobarbital), mice were transcardially perfused with DPBS followed by 4% paraformaldehyde/DPBS (Sigma-Aldrich). On day 7 following MPTP intoxication, numbers of dopaminergic neurons in the substantia nigra (SN) and the integrity of termini in the striatum were investigated. Whole brains were isolated and cryosectioned. Frozen midbrain sections (30 μm) were immunostained for tyrosine hydroxylase (TH) (anti-TH, 1:2000, EMD Millipore) and counterstained for Nissl substance, as previously described (Benner *et al*, 2008). To determine the microglial reactivity, midbrain sections (30 μm) were immunostained for Mac-1 (anti-CD11b, 1:1000, AbD Serotech), as previously described (Kosloski *et al*, 2013). Total numbers of Mac-1+ cells/mm^2, TH+Nissl+ (dopaminergic neurons), and TH-Nissl+ (non-dopaminergic neurons) within the SN were determined by stereological analyses using StereoInvestigator software (MBF Bioscicence). Density of dopaminergic neuron termini in the striatum was calculated by digital densitometry using Image J software (National Institutes of Health).

RNA isolations and polymerase chain reaction

Following 4 weeks of infection and MPTP intoxication, fresh ventral midbrain tissue was isolated, and total RNA was harvested using an RNeasy Mini Kit (Qiagen), using RNAse-

free conditions. cDNA was generated from RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) and pre-amplification was performed using appropriate primer mixes for RT^2 PCR arrays for mouse Inflammatory Response array (Qiagen). Quantitative RT-PCR was performed on an Eppendorf Mastercycler realplex ep as per the manufacturer's instructions (Eppendorf). Data analysis was performed using RT^2 Profiler PCR Array web-based data analysis software, version 3.5 (Qiagen), and gene networks were generated using Ingenuity Pathway Analysis (IPA, Qiagen). The resulting pathway was designed using the Pathway Designer Tool.

Statistical Analysis

All values are expressed as mean ± SEM. Differences in between-group means were analyzed using ANOVA followed by Fisher's least significant difference (LSD) *post hoc* test (GraphPad Software, Inc.).

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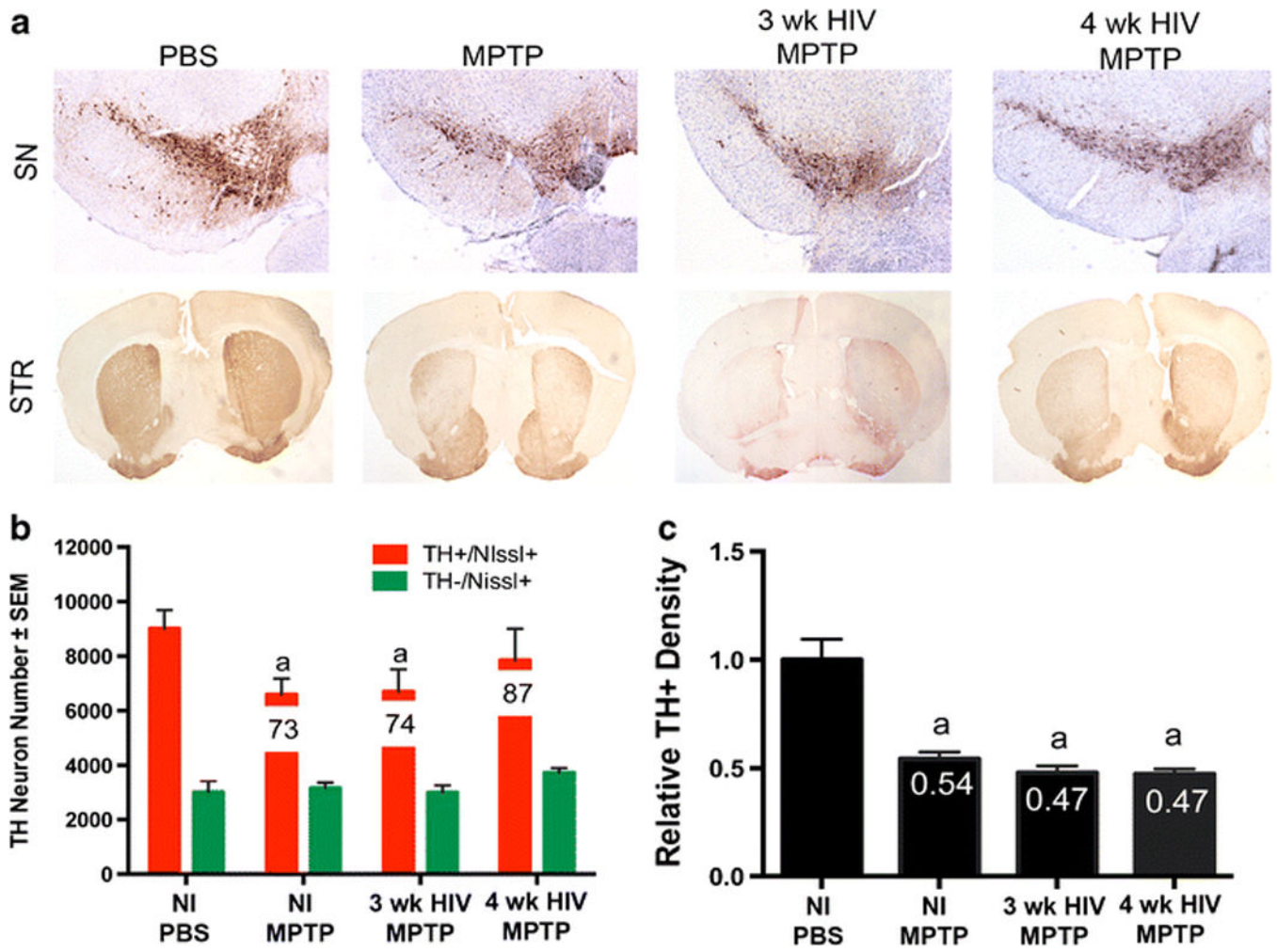


Figure 1. Combination of HIV infection and MPTP intoxication does not affect neurodegeneration in humanized mice

A. Representative images of the substantia nigra (SN) and striatum (STR) of humanized NOD/*scid*-IL-2Rγc null mice treated with MPTP alone or inoculated with HIV for 3 weeks or 4 weeks followed by MPTP intoxication. **B.** Total numbers of surviving dopaminergic neurons (TH+Nissl+) and non-dopaminergic neurons (TH-Nissl+) in the SN following MPTP intoxication, or HIV infection followed by MPTP intoxication. Percentages of spared dopaminergic neurons are displayed on the representative bar for each treatment. **C**. Relative TH+ density within striatal termini of dopaminergic neurons. Differences in means (± SEM, n = 3-6) were determined where $P < 0.05$ compared to PBS (a).

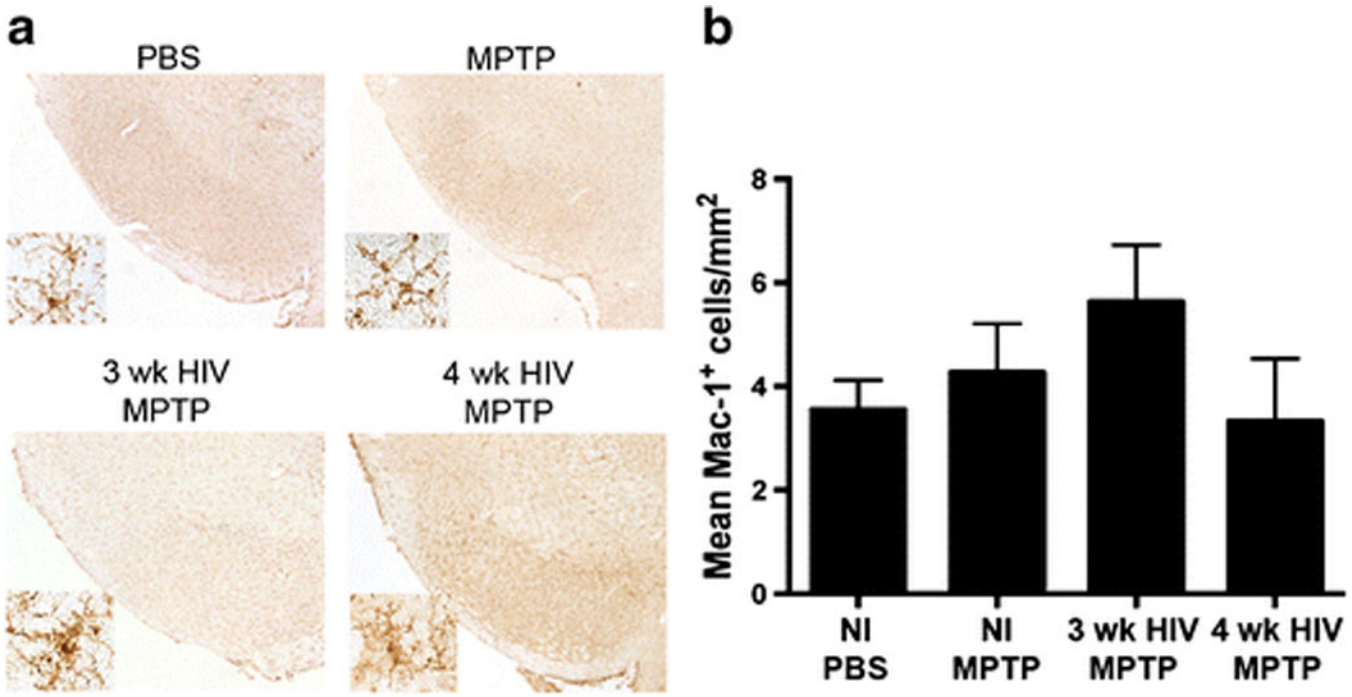


Figure 2. HIV infection does not increase microglial reactivity after MPTP intoxication in humanized mice

A. Representative images Mac-1+ microglia within the ventral midbrain of humanized NOD/*scid*/IL-2Rγc null mice treated with MPTP alone or inoculated with HIV for 3 weeks or 4 weeks followed by MPTP intoxication. **B.** Quantification of reactive microglia cells/mm^2 located within the substantia nigra.

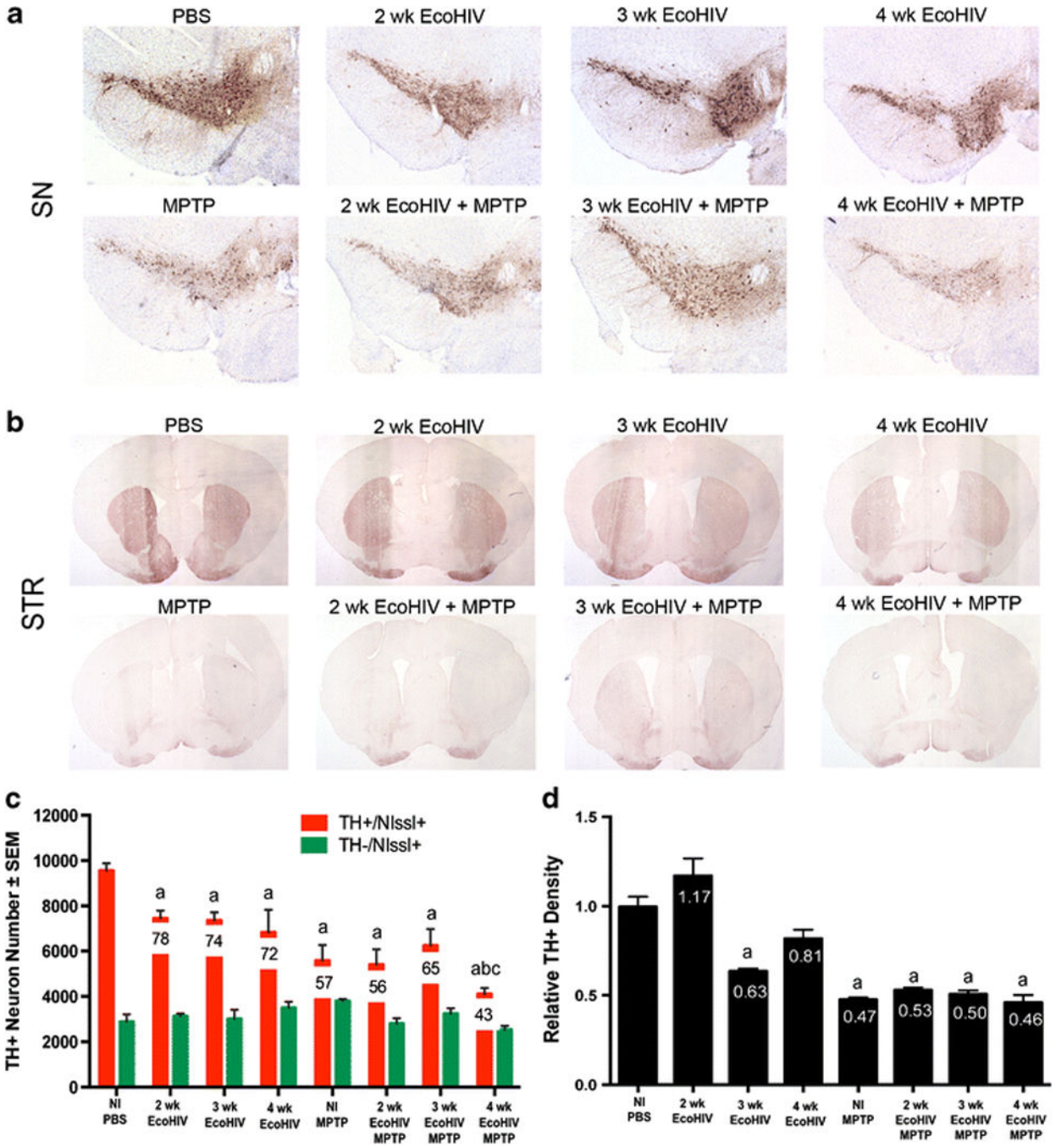


Figure 3. EcoHIV co-infection exacerbates MPTP-induced neurodegeneration

A. Photomicrographs of TH+Nissl+ neurons in the substantia nigra (SN) in C57/Bl6 mice treated with PBS, MPTP, or EcoHIV infection over time. Sections were immunostained with anti-TH and HRP-conjugated secondary antibody, and visualized with DAB. **B.** Photomicrographs of TH+ striatal termini (STR) following PBS, MPTP or EcoHIV coinfection. **C.** Total numbers of surviving dopaminergic neurons (TH+Nissl+) and non-dopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment alone, EcoHIV infection alone, or EcoHIV infection followed by MPTP intoxication. Percentages of spared

dopaminergic neurons are displayed on the representative bar for each treatment. Differences in means (± SEM, n=8) were determined where P<0.05 compared to PBS (a), MPTP (b), and 3 wk EcoHIV + MPTP (c). **D**. Relative TH density of striatal dopaminergic termini following infection. Differences in means (± SEM, n = 8) were determined where P < 0.05 compared to PBS (a), 2 wk EcoHIV (b), and 4 wk EcoHIV(c).

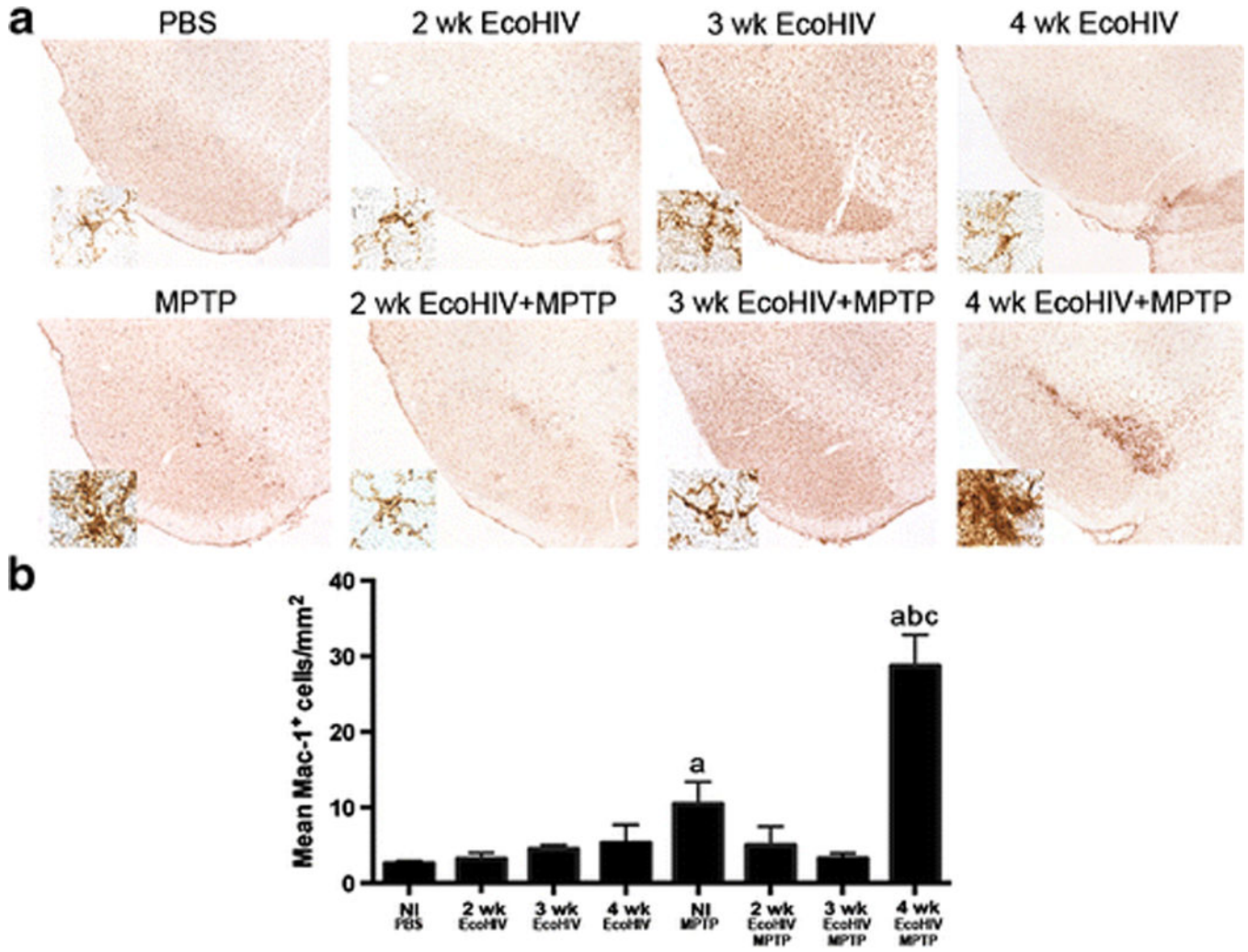


Figure 4. EcoHIV infection leads to increased microglial reactivity

A. Representative photomicrographs of Mac-1+ microglia with the SN of C57/Bl6 mice treated with PBS, MPTP, EcoHIV, or EcoHIV + MPTP over time (4× image, inset = 40× image). **B.** Quantification of reactive microglia within the SN 7 days post MPTP intoxication. Sections were stained using an anti-Mac-1 antibody, followed by an HRP-conjugated secondary antibody and DAB. Numbers of amoeboid microglia were assessed using stereological analysis. Differences in means (± SEM, n = 8) were determined where $P < 0.05$ compared with groups treated with PBS (a), MPTP (b), and 4 wk EcoHIV (c).

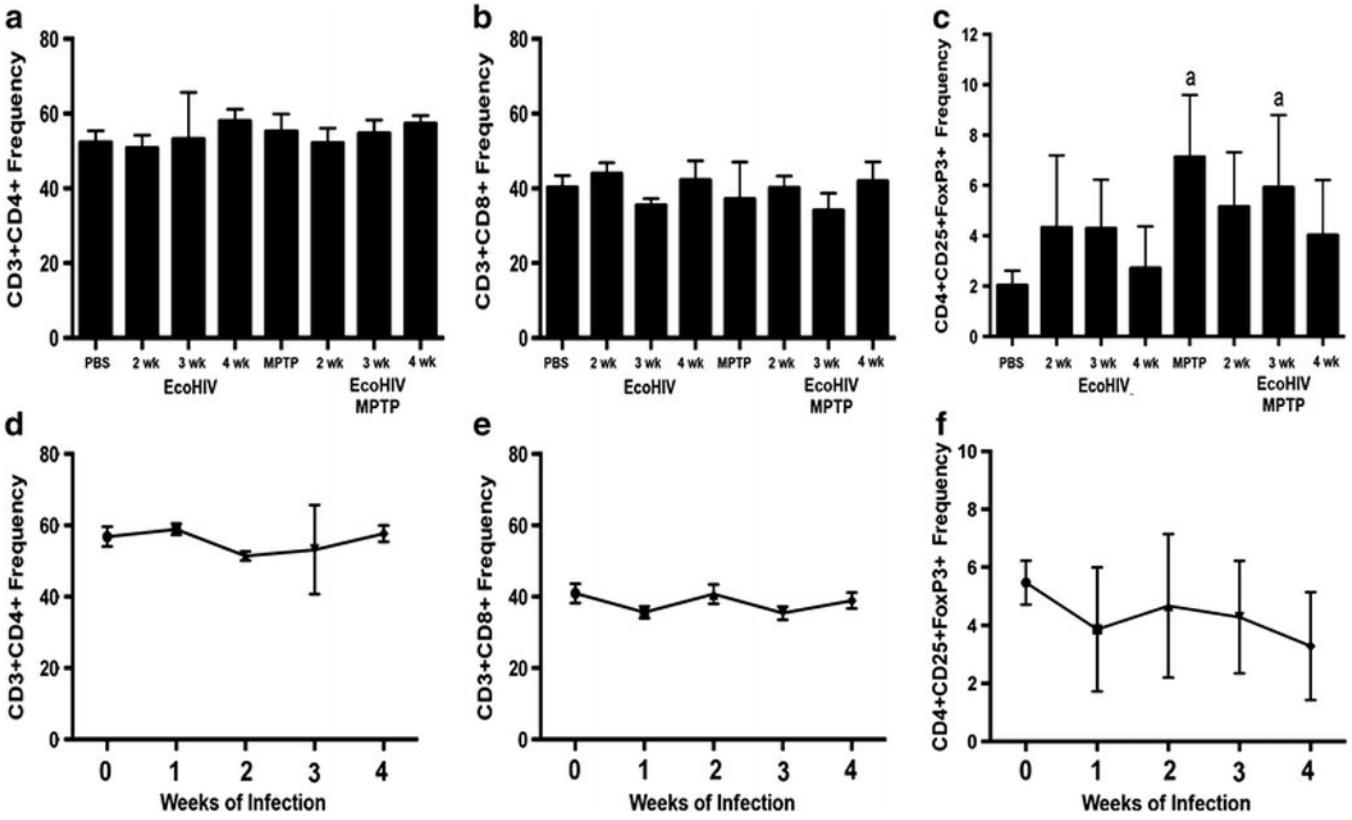
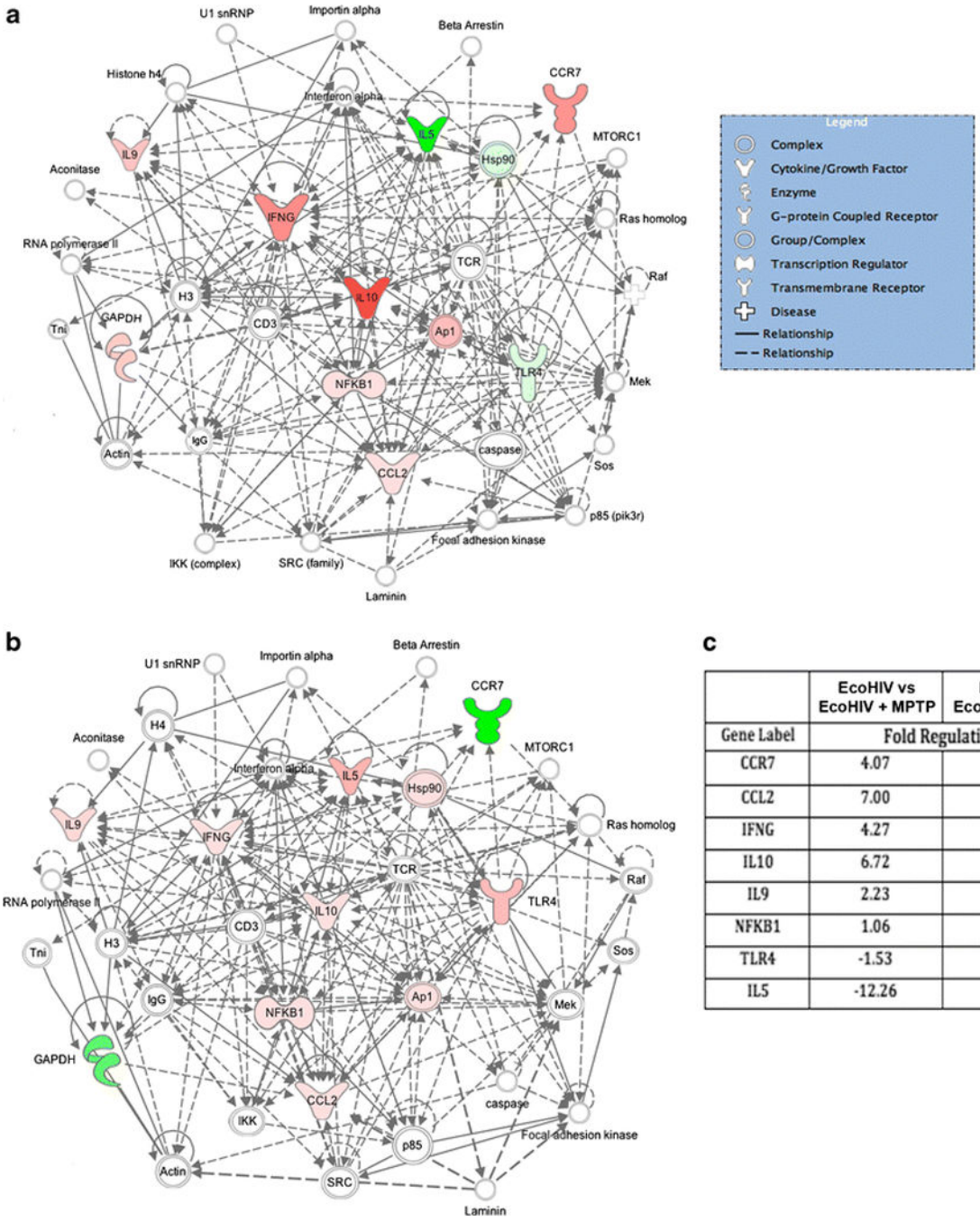


Figure 5. EcoHIV infection does not affect peripheral T cell populations

C57/Bl6 mice were inoculated with EcoHIV for 2–4 weeks and then intoxicated with MPTP. Mice were assessed for frequencies of CD3+CD4+ (**A**), CD3+CD8+ (**B**), and CD4+CD25+FoxP3+ (**C**). Differences in means (± SEM, n = 8) were determined where $P < 0.05$ compared with groups treated with PBS (a). **D–F.** Changes in T cell populations over time following EcoHIV infection.



c

	EcoHIV vs EcoHIV + MPTP	MPTP vs EcoHIV + MPTP
Gene Label	Fold Regulation	
CCR7	4.07	-2.73
CCL2	7.00	15.62
IFNG	4.27	14.95
IL10	6.72	18.89
IL9	2.23	24.07
NFKB1	1.06	3.09
TLR4	-1.53	41.96
IL5	-12.26	46.24

Figure 6. EcoHIV co-infection leads to changes in the inflammatory response pathway

RT-PCR data shows gene expression changes in the ventral midbrains of C57BL/6 mice inoculated with EcoHIV followed by MPTP intoxication (n = 3–4). Fold changes were determined using SABioscience RT^2 Profiler PCR Array Data Analysis software, version 3.5. **A.** Changes in the inflammatory response pathway associated with EcoHIV infection alone vs EcoHIV + MPTP co-treatment. **B.** Changes in the inflammatory response pathway associated with MPTP intoxication alone vs EcoHIV + MPTP co-treatment. Resulting gene networks from each treatment group were analyzed using Qiagen Ingenuity Pathway

Analysis. Pink coloration indicates a modest increase in expression and red indicates a profound increase in expression. Green coloration indicates a decrease in expression. Nodes lacking color indicate a molecule involved in the pathway, but not identified in the PCR data set. **C.** Fold changes for differentially regulated mRNA levels with EcoHIV + MPTP treatment normalized to either EcoHIV treatment alone or MPTP intoxication alone.

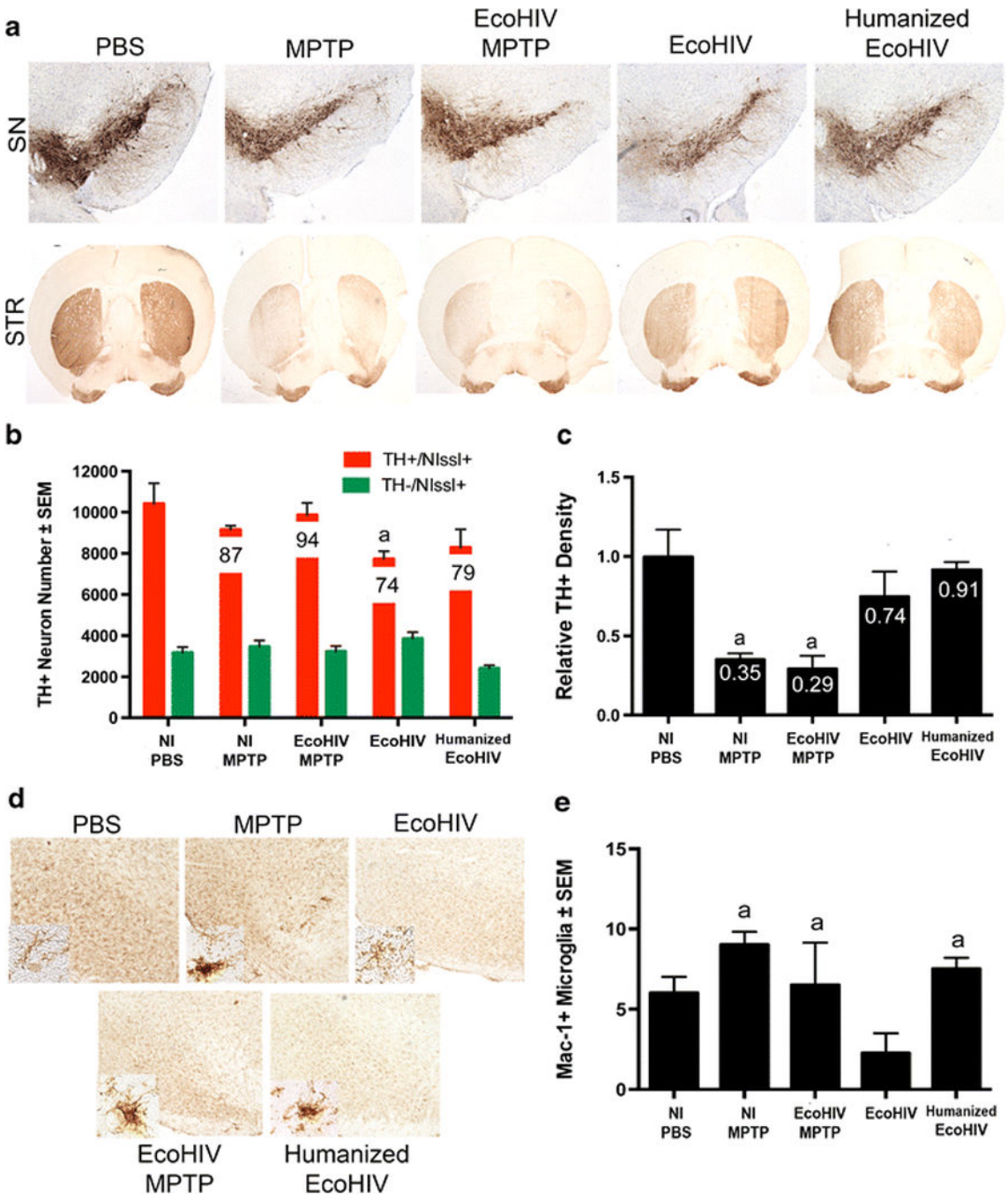


Figure 7. EcoHIV and MPTP co-administration does not exacerbate neuronal degeneration in non-humanized NSG mice

A. Photomicrographs of TH+Nissl+ neurons in the substantia nigra (SN) and striatum (STR) in non-humanized or humanized NOD/*scid*-IL-2Rγc null mice treated with PBS, MPTP, EcoHIV infection, or EcoHIV + MPTP co-administration. Animals were infected for 3 weeks prior to MPTP intoxication. **B.** Total numbers of surviving dopaminergic neurons (TH +Nissl+) and non-dopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment alone, EcoHIV infection alone, or EcoHIV infection followed by MPTP intoxication.

Percentages of spared dopaminergic neurons are displayed on the representative bar for each treatment. Differences in means (± SEM, n=3–4) were determined where P<0.05 compared to PBS (a). **C**. Relative TH density of striatal dopaminergic termini following infection and MPTP intoxication. Differences in means (± SEM, n = 3–4) were determined where P < 0.05 compared to PBS (a). **D.** Representative photomicrographs of Mac-1+ microglia within the midbrain following EcoHIV infection, MPTP intoxication, or EcoHIV infection and MPTP intoxication (4× image, inset = 40× image). **E.** Quantification of reactive microglia within the SN 7 days post MPTP intoxication. Differences in means (± SEM, n = 3–4) were determined where P < 0.05 compared with groups treated with EcoHIV (a).

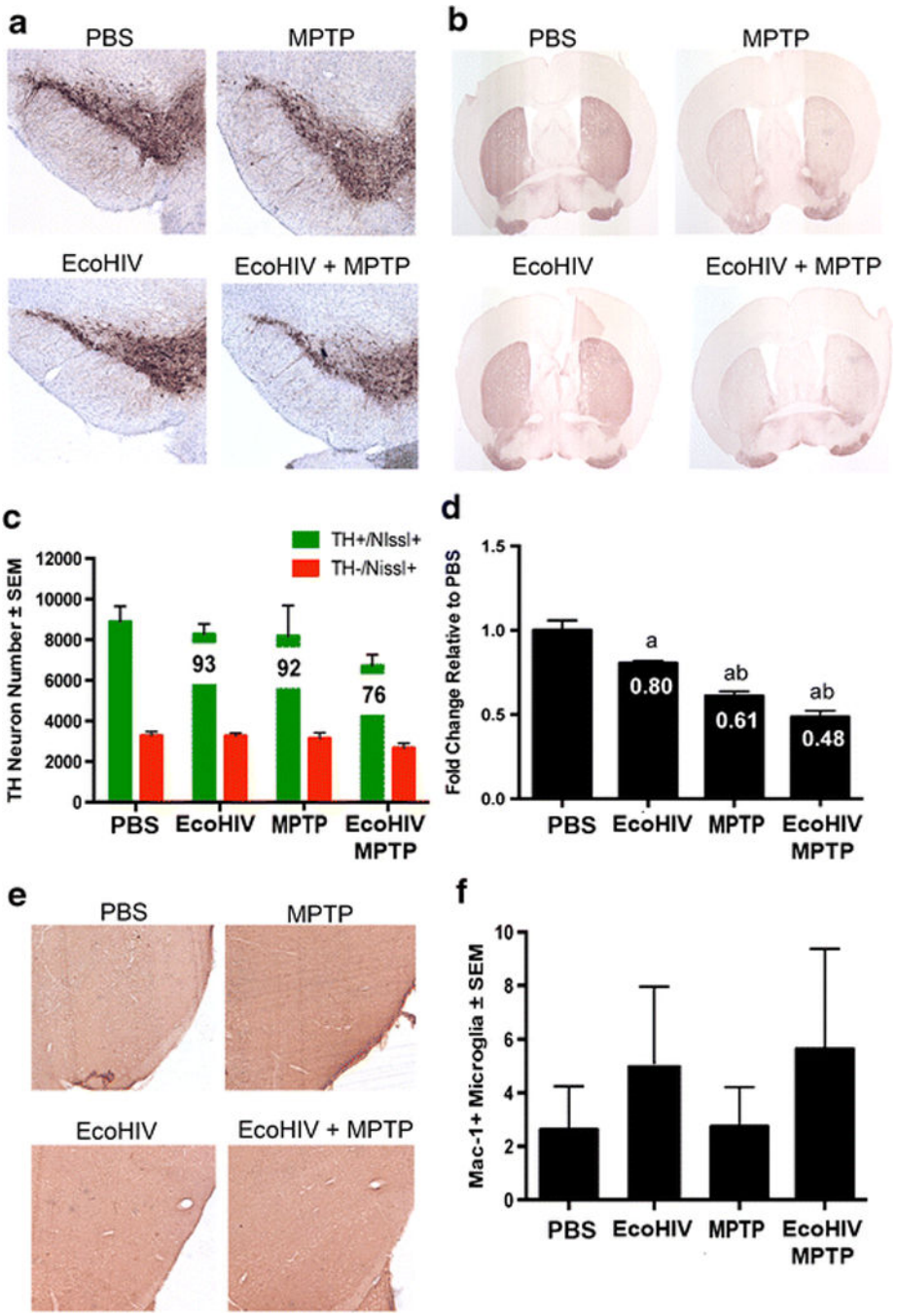


Figure 8. Immunodeficient C57BL/6 scid mice are not susceptible to MPTP intoxication and do not exhibit an inflammatory response

C57BL/6 scid mice were treated with PBS, MPTP, infected with EcoHIV, or infected 3 weeks prior to infection (EcoHIV + MPTP) and tissues examined one week after MPTP. Photomicrographs of (**A**) TH+Nissl+ neurons in the substantia nigra (SN) and (**B**) TH+ termini in the striatum (STR). **C.** Total numbers of surviving dopaminergic neurons (TH+Nissl+) and non-dopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment alone, EcoHIV infection alone, or EcoHIV infection followed by MPTP intoxication

(EcoHIV + MPTP). **D.** Relative TH density of striatal dopaminergic termini following infection and MPTP intoxication. Differences in means (± SEM, n = 5) were determined where $P < 0.05$ compared to PBS (a) or EcoHIV alone (b). **E.** Representative midbrain images stained with Mac-1 following PBS, MPTP, EcoHIV + MPTP, or EcoHIV treatment. **F.** Quantification of Mac-1+ microglial cells/mm^2 within the substantia nigra. No significant differences were observed.