A Unique *in vitro* Neuropathic Phenotype of Clade D Transmitted/Founder Viruses

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ABSTRACT

Background: Expression of the anti-inflammatory, antioxidative phase II detoxifying enzyme heme oxygenase-1 (HO-1) is decreased in the brain prefrontal cortex of HIV+ individuals in association with increased neuroinflammation and neurocognitive impairment. In vitro HIV infection of monocyte-derived macrophages (MDM) induces HO-1 deficiency and increases release of neurotoxic levels of glutamate. We recently showed that HO-1 deficiency is consistently seen with HIV MDM infection by a broad panel of macrophage-tropic clade B HIV-1 strains (n=13). This suggests that induction of HO-1 deficiency is a highly conserved macrophage-tropic HIV phenotype that could be particularly relevant for neuropathogenesis driven by infection within the CNS myeloid reservoir.

Methods: Human MDM (n=4 donors) were infected with viruses derived from infectious molecular clones of each of 5 clade D transmitted/founder (T/F) strains or the clade B 89.6 strain. Viral replication, glutamate concentration, and neurotoxicity of MDM culture supernatants were determined via reverse transcriptase (RT) activity, glutamate assay, and neuron-based MAP2 ELISA, respectively. Data were analyzed by ANOVA with Holm-Sidak post hoc test or Pearson's correlation.

Results: In contrast with MDM infection by 89.6, infection by macrophage-tropic clade D T/F viruses did not significantly decrease HO-1 expression or increase glutamate release, despite similar replication levels as 89.6. Unexpectedly, infection by clade D T/F viruses significantly increased MDM culture supernatant neurotoxicity despite no induction of glutamate release (n=4 donors, p<0.001 for equivalent replication to 89.6). This suggests that macrophage-tropic clade D T/F viruses can activate pathways of neurotoxin production independent of HO-1 and associated effects on glutamate metabolism.

Conclusions: Our results suggest that macrophage-tropic clade D T/F viruses differ from clade B viruses in several potentially critical determinants of neurovirulence; differential ability to i) reduce expression of MDM HO-1 expression and to ii) induce glutamate release from MDM. Nonetheless, macrophage-tropic clade D T/F viruses can induce release of neurotoxins other than glutamate (as yet unidentified) from MDM without altering HO-1 expression, and this suggests potential clade- or T/F virus-specific mechanisms of HIV neurotoxin production. Such differences could contribute to variance in severity of neurological dysfunction among populations infected with different HIV clades.

BACKGROUND

Heme oxygenase-1 (HO-1) is a sentinel, detoxifying enzyme that is induced in response to numerous insults including inflammation and oxidative stress. HO-1 is regulated through the NRF2-dependent antioxidant response element (ARE), which drives the induction of a coalition of antioxidant and detoxifying effector proteins in response to cellular stress and injury. Protective functions of HO-1 have been linked to the enzyme's degradation of heme, a strong pro-oxidant, and the subsequent generation of the anti-inflammatory and anti-oxidative products, carbon monoxide, biliverdin, and bilirubin. The potent anti-inflammatory and cytoprotective actions of HO-1 have identified it as a potential therapeutic target in CNS inflammatory and neurodegenerative diseases. Among these diseases, HIV infection is associated with neurodegeneration that is thought to result from effects of persistent inflammation and oxidative stress in both systemic and CNS compartments, which persists in individuals on antiretroviral therapy (ART). Within the CNS, such effects are driven by HIV infection of macrophages and microglia and contribute to the continued prevalence of cognitive, motor, and behavioral deficits collectively known as HIV-associated neurocognitive disorders (HAND), which affect up to 40% of HIV-infected ART-treated individuals. This high prevalence of HAND strongly underscores the need for adjunctive therapies that target the neuropathological processes associated with persistent inflammation and oxidative stress in HIV-infected individuals, which we believe involve HO-1 dysregulation.

HO-1 expression is significantly reduced in the brain prefrontal cortex of HIV-positive individuals with HIV-associated neurocognitive disorders (HAND) compared to those without HAND. This HO-1 deficiency correlates with brain viral load, markers of macrophage activation, and type I interferon responses. Our previous in vitro studies with HIV-1 subtype B strains isolated from chronically infected individuals demonstrated that HIV replication in monocyte-derived macrophages (HIV-MDM) selectively reduces HO-1 protein and RNA expression. This HO-1 reduction drives the production of neurotoxic levels of glutamate, which is known to activate NMDA subtype glutamate receptors and cause neuronal injury. Pre-treating neurons with NMDA receptor antagonists prior to exposure to supernatants from HIV-MDM blocks this supernatant neurotoxicity. Correction of the HO-1 deficiency in HIV-MDM reduces their neurotoxic glutamate production without affecting HIV replication. We have shown that these HIV effects in MDM are widely conserved across HIV-1 subtype B strains.

Different subtypes of HIV-1 are reported to vary in neurovirulence; for example, HIV-1 subtype D infection associates with higher prevalence of neurocognitive impairment than subtype B, based upon previous reports. We hypothesized that HIV-1 subtype D strains differ from subtype B strains in their neurovirulence phenotype. In this preliminary study, we used available molecularly cloned macrophage-tropic HIV-1 subtype D strains isolated from acutely infected individuals (transmitted/founder viruses; T/F). Analysis of these subtype D T/F viruses could help us begin to elucidate mechanistic differences in HAND neuropathogenesis depending on virus subtype or stage of infection at isolation. We found that while subtype D T/F infection in macrophages produces neurotoxicity, the subtype D T/F neurovirulence phenotype is significantly less robust than that of subtype B at similar levels of replication. This different neurovirulence phenotype could be an effect of HIV-1 subtype or an effect of transmitted/founder strains, each of which we will attempt to distinguish in future studies.

RESULTS

Subtype D T/F viruses replicate similarly to subtype B viruses

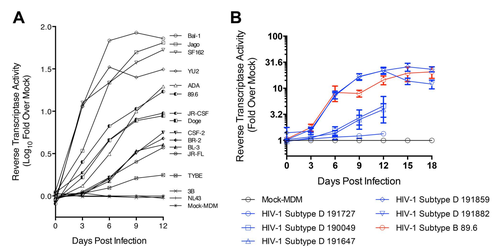


Figure 1: Replication of HIV-1 strains in monocyte-derived macrophages (MDM) isolated from healthy donors was assessed every 3 days over 12-18 days of infection by reverse transcriptase (RT) activity in the supernatant. A) Replication of 15 HIV-1 subtype B strains isolated from chronically infected individuals in macrophages from 5 different donors. B) Replication of 5 HIV-1 subtype D T/F strains and 1 HIV-1 Subtype B strain in macrophages from 3-8 donors. HIV replication is shown as measured by HIV-MDM supernatant RT activity normalized to same-day Mock-MDM. Error bars indicate mean ± SEM.

HO-1 expression in subtype D HIV-MDM associates with replication, but not neurotoxicity or extracellular glutamate

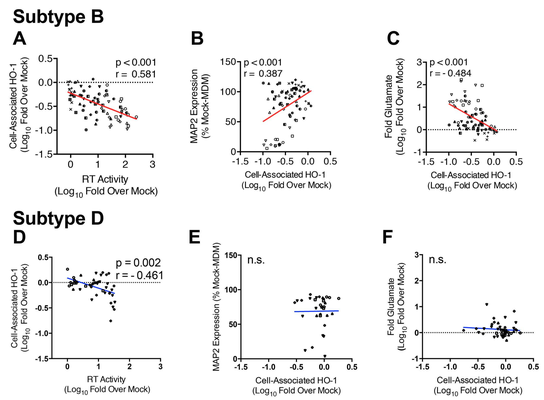


Figure 2: Correlation between HO-1 protein expression in subtype B HIV-MDM (A, B, C) or subtype D HIV-MDM (D, E, F) and A,D) supernatant RT activity, B,E) neurotoxicity, C,F) supernatant glutamate. Neurotoxicity was measured by MAP2 ELISA. Glutamate was measured with Amplex Red glutamate assay. Correlations were assessed by Pearson's correlation.

Donor				HIV-1 Subtype B Strain					HIV-1 Subtype D Strain	
● A	● E		□ Jago	◇ YU2	△ ADA	× 3B	○ 191727	△ 191647
■ B	× F		○ Bal-1	⊗ JR-FL	◐ 89.6	⊠ TYBE	□ 1910049	▽ 191882
▲ C	+ G		▽ SF162	◈ BR-2	◧ Doge	+ NL43	◇ 191859	
◆ D	○ H		▼ CSF-2	▲ BL-3	● JR-CSF			

Key: Symbols to demarcate individual macrophage donors and HIV-1 viral strains

Subtype D T/F viruses reduce HO-1 protein expression less robustly than subtype B strain 89.6

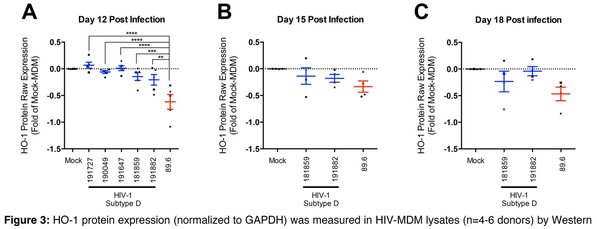


Figure 3: HO-1 protein expression (normalized to GAPDH) was measured in HIV-MDM lysates (n=4-6 donors) by Western blot on A) day 12 post HIV infection, B) day 15 post HIV infection, C) day 18 post HIV infection. Mock-MDM expression was set to 0 on a log scale (represented by dotted line). Error bars indicate mean ± SEM. Statistical comparisons to 89.6 were made by one-way ANOVA with Holm-Sidak post hoc test. * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001

Subtype D T/F HIV-MDM supernatants are less neurotoxic than subtype B strain 89.6

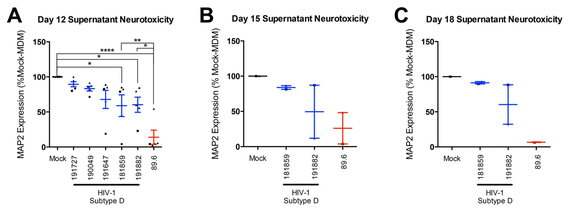


Figure 4: MDM from healthy donors (n=2-5) were infected with 2-5 HIV-1 subtype D strains and 1 HIV-1 subtype B strain (89.6). Supernatants from A) day 12, B) day 15, C) day 18 post infection were analyzed for neurotoxicity as measured by quantification of total microtubule associated protein-2 (MAP2) expression in a cell-based ELISA where primary rat cortical neurons are exposed to HIV-MDM supernatants. Error bars indicate mean ± SEM. Statistical comparisons to Mock-MDM were made by one-way ANOVA with Holm-Sidak post hoc test. * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001

Subtype D T/F infection does not increase HIV-MDM supernatant glutamate

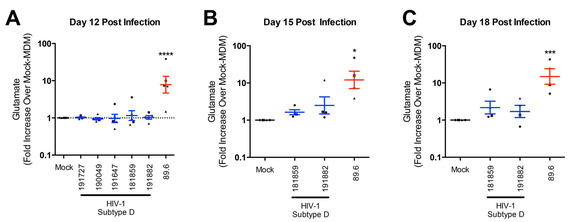


Figure 5: HIV-MDM supernatants (n=4-6 donors) from A) day 12, B) day 15, C) day 18 post infection were analyzed for glutamate content by Amplex Red Glutamate Assay. Error bars indicate mean ± SEM. Statistical comparisons to Mock-MDM were made by one-way ANOVA with Holm-Sidak post hoc test. * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001

Subtype D T/F HIV-MDM supernatant neurotoxicity is NMDA receptor-mediated

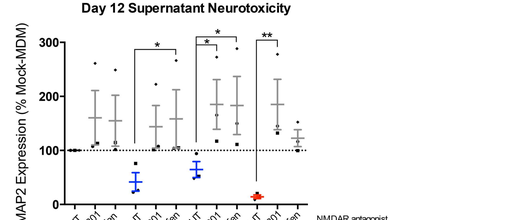


Figure 6: DIV 14 primary rat cortical neurons were pre-treated with NMDA receptor antagonist MK801 or Ifenprodil (Ifen) at 10uM or left untreated (UT) for 1 hour before being exposed to HIV-MDM supernatants (n=3 donors). Supernatants were analyzed for neurotoxicity as measured by MAP2 ELISA. Error bars indicate mean ± SEM. Statistical comparisons to Mock-MDM were made by one-way ANOVA with Holm-Sidak post hoc test. * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001

SUMMARY & CONCLUSIONS

Summary

- Replication of HIV subtype D T/F strains in MDM significantly correlates with reduction of HO-1 protein expression.
- Some HIV-1 subtype D T/F strains can replicate similarly to HIV-1 subtype B strains in MDM.
- In cases of similar replication, subtype D T/F infection of MDM does not significantly reduce HO-1 protein expression compared to subtype B infection.
- In cases of similar replication, subtype D T/F HIV-MDM supernatants are less neurotoxic than subtype B HIV-MDM supernatants.
- In cases of similar replication, subtype D T/F infection does not increase MDM glutamate release, in contrast to subtype B infection.

Conclusions

Our results indicate that HIV-1 subtype D T/F strains have a significantly less robust neuropathic phenotype when compared to a subtype B strain isolated from a chronically infected individual. Whether this difference in phenotype can be attributed to virus subtype or is a T/F remains undetermined. Determining the etiology of this phenotype will help us better understand HAND pathogenesis. If subtype is responsible for this discrepancy, this and future studies will help us better understand how diversity of HIV contributes to HAND pathogenesis in geographically distinct populations. If this less neuropathic phenotype is a T/F effect, our results would suggest T/F HIV strains with macrophage-tropism might acquire a more neuropathic phenotype over the course of infection, leading to HAND.

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