Full Length Article

Prevention and treatment of HIV infection and cognitive disease in mice by innate immune responses

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ABSTRACT

HIV associated neurocognitive impairment affects roughly half of infected individuals on antiretroviral therapy. This disease currently has no treatment. We have previously shown that type I interferon is induced by and partially controls infection and neuropathogenesis in mice infected by chimeric HIV, EcoHIV. Here we investigate the intentional ligation of the pattern recognition receptor Toll-like receptor 3 (TLR3) by polyinosinic-polycytidylic acid (poly I:C) for its ability to prevent or control infection and associated cognitive disease in EcoHIV infected mice. We tested topical, injection, and intranasal application of poly I:C in mice during primary infection through injection or sexual transmission or in established infection. We measured different forms of HIV DNA and RNA in tissues by real-time PCR and the development of HIV-associated cognitive disease by the radial arm water maze behavioral test. Our results indicate that poly I:C blocks primary EcoHIV infection of mice prior to reverse transcription and reduces established EcoHIV infection. Prevention or control of viral replication by poly I:C prevents or reverses HIV associated cognitive disease in mice. These findings indicate that poly I:C or other innate immune agonists may be useful in control of HIV cognitive disease.

1. Introduction

HIV infection can largely be controlled by existing drugs that target the pol proteins: reverse transcriptase, protease, and integrase (Broder, 2010; Volberding and Deeks, 2010). This generally effective therapy does not affect virus present in macrophages that survive productive infection or latently infected T cells (Richman et al., 2009). Sadly, about half of those infected people on combination antiretrovirals develop cognitive disease that persists for life and worsens with age (Harezlak et al., 2011; Robertson et al., 2007). The continued expression of HIV Tat and gp120, among other viral proteins, in chronically infected cells in the brain is considered to be one driver of neuropathogenesis (Carroll and Brew, 2017; Saylor et al., 2016). Interventions that target HIV expression itself are sorely needed.

Host antiviral responses control and can eliminate many viral infections by targeting cells that express viral proteins or nucleic acid. This has been demonstrated in HIV infection or exposure in humans at the level of adaptive immune responses, chiefly T cell responses (Perreau et al., 2013; Rowland-Jones et al., 1999). In addition, the major innate immune effector, Type I interferon (IFN), appears to pose a partially effective block to HIV replication as illustrated in the evolution of viral genes that antagonize IFN-stimulated proteins, APOBEC3G, tetherin, and Serin3/5 (Dubé et al., 2010; Malim, 2009; Usami et al., 2015). The transmitted-founder HIV species that establish infection after sexual transmission are selected, in part, for IFN-resistance (Fenton-May et al., 2013; Parrish et al., 2013). Type I IFN induced early in SIV infection arrests viral infection and transcription in the brain (Barber et al., 2006; Carnathan et al., 2018) and in analogous studies we have shown that knockout of the Type I interferon receptor in mice permits greater neuropathogenesis caused by the chimeric HIV, EcoHIV, than that observed in wildtype mice (He et al., 2014).

Innate immune is initiated by ligation of any of several pattern recognition receptors, the first family identified being Toll-like receptors (TLR) mimicking Drosophila receptors that mount responses against bacteria and virus components and limit infection (Valanne et al., 2011). A synthetic TLR3 ligand, polyinosinic-polycytidylic acid (poly I:C), can prevent some viral infections including hepatitis B and herpes simplex virus type 1 in human beings (Boivin et al., 2008; Martinez et al., 1980; Wu et al., 2014). Multiple studies, including our own, have shown that activation of various TLR on human cells in culture and the resulting
Type I IFN responses can prevent HIV infection (Tsang et al., 2009; Wang et al., 2011; Zhou et al., 2010). Such antiviral responses span the viral life cycle through DNA synthesis, integration, transcription, and virion export (Urbano VDe Crignis and Re, 2018). Clinical trials indicate that treatment with Type I IFN arrests HIV transcription in T cells in infected people (Morón-López et al., 2016). These findings raise the possibility that induction of innate immune responses can silence HIV expression and its associated cognitive disease, as we test here.

To carry out these studies we exploited our system of EcoHIV infection of conventional mice. This HIV has a replacement of gp120 by ecotropic murine leukemia virus envelope to enter mouse cells but otherwise employs all HIV genes (Potash et al., 2005). EcoHIV infects CD4 positive T cells and F4/80 positive macrophages systemically as well as brain microglia. After an early peak its replication is controlled, at least in part, by adaptive immune responses but chronically infected mice maintain some virus expression in macrophages and demonstrate impaired learning analogous to HIV infection in vivo (Potash et al., 2005; Gu et al., 2018; Kelschenbach et al., 2019). The continued expression of HIV proteins in EcoHIV infected mice has allowed evaluation of vaccines to prevent infection and drugs to treat infection (Hadas et al., 2007; Im et al., 2011; Liu et al., 2018; Roshorm et al., 2009, 2012; Saini et al., 2007; Tomusange et al., 2016a, 2016b) and the persistence of HIV-associated neurocognitive impairment (HIV–NCI) in this model has allowed studies of some of the routes to pathogenesis and NCI treatment (He et al., 2014; Kelschenbach et al., 2019; Bertrand et al., 2019; Jones et al., 2016; Kim et al., 2019; Nedelcovych et al., 2017, 2019; Olson et al., 2018). Here we employ this experimental infection to test the activity of poly I:C in different administration formats on HIV infection by various routes and its ability to reverse established infection and cognitive disease.

2. Results

Previous studies indicate that transmitted-founder HIV clones that establish infection in humans after sexual transmission are selectively Type I IFN-resistant (Fenton-May et al., 2013; Parrish et al., 2013). Using EcoHIV infected male mice mated to uninfected females, we developed a model of genuine HIV sexual transmission (Hadas et al., 2013). In other studies we found that intracranial injection of EcoHIV in Type I IFN receptor knock-out (IFNR-KO) mice permits greater virus expression than in wildtype mice (He et al., 2014). Taking these findings together we reasoned that establishment of EcoHIV infection in mice by sexual transmission may be sensitive to Type I IFN signaling. To test this prediction, C57BL/6 females and isogenic IFNR-KO females were allowed to...

Fig. 1. Innate immune responses prevent EcoHIV sexual transmission. A-C. EcoHIV burdens were measured in IFNR-KO mice one week after virus sexual transmission. A. EcoHIV gag DNA in inguinal lymph nodes. B. EcoHIV gag DNA in spleen. C. EcoHIV gag RNA in peritoneal cells. D. EcoHIV gag RNA in peritoneal cells was measured in C57BL/6 mice treated topically with poly I:C or PBS one week after virus sexual transmission. Each symbol represents a single mouse and the horizontal bars indicate the means. *p < 0.05, **p < 0.01.
mate with EcoHIV-infected male nude mice; one week later all females were euthanized with inguinal lymph nodes, spleen, and peritoneal macrophages (PM) collected to measure virus burden (Fig. 1). Fig. 1 shows that Type I IFN signaling clearly reduces virus EcoHIV sexual transmission; in each compartment assayed IFNR-KO mice had significantly more viral DNA or RNA. These observations, like our previous study in IFNR-KO mice (He et al., 2014), indicate that EcoHIV itself induces a protective IFN response analogous to the response observed in humans for selection of IFN-resistant virus (Fenton-May et al., 2013; Parrish et al., 2013). To learn if an exogenous ligand activating innate responses also prevents HIV transmission, female C57BL/6 mice were treated on vaginal surfaces with the TLR3 ligand, poly I:C, or phosphate buffered saline (PBS) prior to mating with EcoHIV-infected nude males. Females were euthanized after one week and EcoHIV gag RNA was measured in PM (Fig. 1D). In this pilot study a single topical application of poly I:C largely prevented EcoHIV sexual transmission.

To probe the efficacy of induced systemic innate responses to prevent HIV infection, male mice were poly I:C treated and EcoHIV infected by IP injection, with poly I:C preceding infection by 12 h. Groups of mice were euthanized 2 or 19 days later for measurement of tissue virus burden (Fig. 2). Injection of poly I:C potently inhibited EcoHIV infection in mice preventing both DNA and RNA synthesis. PBS-treated mice show a 40-fold increase in genomic RNA in macrophages over time but virus spread was completely blocked in the poly I:C group. The findings in Figs. 1D and 2 indicate that the protective innate antiviral response induced by HIV can be augmented by the response to a TLR3 ligand.

We reported that prophylaxis with antiretrovirals prevented EcoHIV infection and the development of HIV–NCI in mice (Gu et al., 2018). To test whether virus inhibited by poly I:C retains the ability to cause disease, the experiment shown in Fig. 2 was repeated with additional poly I:C injections weekly after EcoHIV infection. Three weeks after infection, mice were tested for learning and memory in the radial arm water maze (RAWM) test (Fig. 3). EcoHIV-infected mice showed a highly significant defect in learning as indicated in the persistence of 3–4 errors and roughly 50 s in locating the hidden platform at the point when uninfected mice displayed fewer than 1 error and only 15 s for the task (Fig. 3A-B). In distinct contrast, EcoHIV-infected mice treated with poly I:C were indistinguishable from uninfected mice in learning. Poly I:C itself had no effect in the RAWM. Mice in all groups were similarly able to find and swim to a visible platform indicating no defects in motor function, vision, or intention (Fig. 3C). This study illustrates that prevention of EcoHIV infection by induction of an innate immune response likewise prevents HIV brain disease in mice.

The value and the complexity of innate responses are their breadth. To begin to identify phases in the EcoHIV life cycle affected by Type I IFN responses, we first tested splenic virus burden 24 h after EcoHIV infection of wildtype and IFNR-KO mice, a timepoint in which DNA synthesis and integration are the major ongoing processes (Fig. 4). EcoHIV DNA is abundant in spleen in both mouse strains but is significantly greater in IFNR-KO mice (Fig. 4A). Likewise, viral RNA is increased in the absence of IFN responses, a natural consequence of the greater amount of viral DNA available for transcription. This observation indicates that the HIV-induced-IFN response acts at early phases of replication. To extend this analysis to intentional induction of innate responses, BALB/c mice were

![Fig. 2. Systemic poly I:C prevents EcoHIV infection. EcoHIV burdens in mice were measured 2 (panel A–B) or 19 days (panel C) after infection and poly I:C treatment by injection. A. EcoHIV gag DNA in spleen. B–C. EcoHIV vif RNA in peritoneal cells. **p < 0.01; ***p < 0.001.](image-url)
treated with poly I:C or PBS 6 h prior to EcoHIV infection by intravenous injection; this infection route was chosen to minimize carryover from the viral stock into the peritoneal cavity and allow better analysis of the responses of macrophages, a major innate immune effector cell (Fig. 4). The innate response to poly I:C coupled with the response to EcoHIV itself blocked reverse transcription, and viral DNA burden was significantly reduced in peritoneal cells (Fig. 4B). Transcription was reduced below the limit of detection in spleen (Fig. 4C). To complete the early phase life cycle analysis, C57BL/6 mice were pretreated with poly I:C, infected by EcoHIV, and then treated daily with poly I:C for five days prior to euthanasia and collection of spleen and peritoneal cells. We then performed a two-step QPCR method as described (Gu et al., 2018) to quantify integrated viral DNA. As expected from its effects on reverse transcription, poly I:C significantly reduced integrants in peritoneal cells and reduced EcoHIV integrants in spleen below the level of detection, roughly 20 copies per 10^6 cells (Fig. 4D-E). At a minimum, poly I:C prevents reverse transcription by EcoHIV and also impairs nuclear entry of viral DNA or its integration; unambiguous quantitation of its effects upon transcription requires other analysis.

Innate immune activation results in rapid and often short-lived responses. To parse the kinetics of induction of antiviral genes by EcoHIV and poly I:C we employed mouse bone marrow-derived macrophages in culture and measured relevant transcripts by real-time PCR. The values shown represent fold induction compared to control cultures assayed at 0 time (Fig. 5). Most of the transcripts monitored were induced by both EcoHIV and poly I:C but it is notable that IFN-β preceded other factors with poly I:C and virus exposure. In addition, the host restriction factor, Apobec3, was induced to higher levels in infected cells with poly I:C. The inflammatory cytokines, TNF-α and IL-1β, and the chemokine MCP-1 were highly induced in all systems compared to controls.

Our findings show that poly I:C blocks HIV replication prior to reverse transcription but its reported activity later in infection depends upon the system tested (Tsang et al., 2009; Swaminathan et al., 2012; Trapp et al., 2009). To determine whether poly I:C affects virus expression or activity in established infection, mice were treated with poly I:C by IP injection thrice weekly from 21-35 days after EcoHIV infection; RAWM tests were performed day 28–34 and euthanasia and tissue collection day 35 after infection (Fig. 6). Poly I:C treatment significantly reduced EcoHIV DNA and RNA burden in lymphocytes and viral RNA in macrophages. Having demonstrated the inhibition of chronic EcoHIV infection by poly I:C, we probed whether it also affects ongoing cognitive disease. Fig. 6 illustrates that five weeks after EcoHIV infection, mice suffer impaired learning and memory and that this defect was reversed by poly I:C treatment. Poly I:C itself had no effects upon performance in the RAWM. Our findings suggest that inhibition of chronic HIV expression and spread allows recovery of cognitive function.

A general concern in the use of TLR ligands for HIV treatment is the risk of immune activation as observed in advanced HIV disease when the gut mucosa leaks, allowing bacterial translocation and systemic endotoxin contamination (Klatt et al., 2013). Our working protocol of intermittent poly I:C administration was designed to limit systemic immune activation. We tested the outcome of this approach by repeating the experiment shown in Fig. 6, omitting RAWM tests, and harvested peritoneal macrophages 35 days after infection to measure intracellular chemokines and inflammatory cytokines. Of 45 proteins tested only 15 were detected and expression of none of them was altered by poly I:C treatment. These were CCL5, CCL11, CCL12, CCL21, CX3CL1, CXCL1, CXCL2, CXCL9, IFN-β1, IL-2, IL-9, IL-10, IL-15, IL-16, IP-10, and KC. The
concentrations detected in macrophage lysates and a list of the undetected proteins are found in Supplemental Information.

IP injection is not a preferred route for drug administration to human beings. However, pharmaceuticals can be preferentially targeted to the brain and mucosal immune system through intranasal (IN) application and this route is used for drug delivery in people (Calzas and Chevalier, 2019; Hanson and Frey, 2007). Moreover, we have shown that IN administration of insulin, as used for treatment of some cognitive disorders (Freiherr et al., 2013; Novak et al., 2014) reversed NCI in EcoHIV-infected mice (Kim et al., 2019). To determine whether administration of poly I:C through a route relevant to clinical use would also effectively treat EcoHIV infection and disease in mice we repeated the experiment shown in Fig. 6 but replaced IP injection by IN application of poly I:C to mice while awake (Fig. 7). Poly I:C significantly inhibited
EcoHIV replication in mouse macrophages and lymphocytes. The compound itself administered IN did not affect behavior but it restored cognitive function to infected mice assayed in the RAWM (Fig. 7). The results presented here recommend further evaluation of TLR ligands for Fig. 5.

Heatmap visualization of gene expression. Quantitative PCR was performed to evaluate expression of antiviral genes. Expression was normalized by the housekeeping gene GAPDH. Relative expression or fold changes were calculated versus a control 0h time point and the direction and extent of the regulation is represented in the heatmap by different colors. Gray regions show no significant differences from control. Other levels of statistical significance: *p < 0.05, #p < 0.005, γ < 0.0005, &p < 0.00005, $p < 0.000005, NS not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 6. Poly I:C treatment of mice chronically infected by EcoHIV reduces virus burden and reverses cognitive disease. A-E Mice were treated by IP injection of poly I:C, tested in RAWM, and euthanized for tissue collection. A-B. gag burden in spleen A: DNA, B: RNA. C. vif RNA burden in peritoneal cells. *p < 0.05, **p < 0.01, ***p < 0.001 D. Errors in finding the hidden platform. E. Time to find the hidden platform. EcoHIV vs uninfected * p < 0.05. EcoHIV vs EcoHIV + poly I:C p < 0.05.
eventual treatment of established HIV infection and mild NCI in patients.

3. Discussion

Our findings indicate that innate immune responses to poly I:C can both prevent and control established HIV infection and the cognitive disease it causes in mice. Among our new observations that may have particular relevance to HIV infection of humans are the ability of topical poly I:C to prevent EcoHIV sexual transmission and the efficacy of IN administration of poly I:C to reverse cognitive defects in chronically infected mice.

EcoHIV infection of wildtype mice compared to IFNR-KO mice was employed to illustrate two points: virus infection itself induces an immediate, innate response and this antiviral response is partially protective. When competent for IFN signaling, mice restrict EcoHIV infection by sexual transmission with higher virus burdens obtained in IFNR-KO mice; this mechanism is imposed on the first day of virus exposure (Fig. 1). Having demonstrated the scope of the endogenous response, we amplified it by adding a TLR agonist yielding a highly effective block (Figs. 1-2). It is noteworthy that infection by both injection and sexual transmission was affected suggesting that cells in the peritoneal cavity and the reproductive mucosa mount protective antiviral responses (Figs. 1-2). Innate antiviral responses can act throughout virus replication (Urbano VDe Crignis and Re, 2018) but the first phase blocked by poly I:C in mice precedes reverse transcription (Fig. 4). This finding echoes our report of HIV infection of human cells in culture, where the TLR ligand response was seen after efficient virus entry and before DNA synthesis (Wang et al., 2011).

EcoHIV infection of mice not only reproduces much of the life cycle of HIV in vivo but it also provides a model of HIV–NCI. The virus replicates in macrophages and microglia as well as T cells and establishes an active reservoir in macrophages refractory to antiretroviral treatment (Potash et al., 2005; Gu et al., 2018; Kelschenbach et al., 2019). Infection in the brain reduces expression of certain synaptic function genes, reduces synaptic activity in generating long term potentiation in hippocampal slices tested ex vivo, and drives dendritic dearborization in the hippocampus but not neuronal death (Kelschenbach et al., 2019; Kim et al., 2019). As shown in RAWM and fear conditioning behavioral tests, EcoHIV impairs visuospatial learning and working, contextual, and

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**Fig. 7.** Intranasal poly I:C treatment of mice chronically infected by EcoHIV reduces virus burden and reverses cognitive disease. A-F Mice were treated by intranasal application of poly I:C, tested in RAWM, and euthanized for tissue collection. A-B. gag burden in spleen A: DNA, B: RNA. C-D. RNA burden in peritoneal cells. C: vif, D: gag. *p < 0.05, **p < 0.01, ***p < 0.001. E. Errors in finding the hidden platform. F. Time to find the hidden platform. EcoHIV vs uninfected * p < 0.05. EcoHIV vs EcoHIV + poly I:C + p < 0.05.
Conducted neuroprotective function (Fig. 7). A safety evaluation of injected poly I:C other innate immune triggers to enhance this response (Fig. 1) and responses in reproductive mucosa limit sexual transmission of HIV (Nazli et al., 2013) does not occur, possibly because exposure to the TLR agonist was limited. The question arises how this experimental treatment in mice can be translated to potential clinical applications. It is fascinating to note that the poly I:C induced genes that function to block infection.

Cognitive disease accompanying established EcoHIV infection is not affected by reverse transcription or integration inhibitors (Gu et al., 2018) but the ability of poly I:C to inhibit late phases of virus replication (Sang et al., 2014) suggested that it can treat HIV–NCI. As shown in Fig. 6, intermittent poly I:C injection reduced virus expression and reversed existing HIV–NCI in mice. We also limited systemic exposure to poly I:C by IN administration and found that it preserved its antiviral and neuroprotective function (Fig. 7). A safety evaluation of injected poly I:C conducted five weeks after infection revealed that it did not affect inflammatory cytokine or chemokine proteins expressed by peritoneal macrophages indicating that excessive immune activation (Klatt et al., 2013) does not occur, possibly because exposure to the TLR agonist was limited.

The question arises how this experimental treatment in mice can be translated to potential clinical applications. It is fascinating to note that HIV infection in human beings is already subject to control by TLR3 agonists, presumably present in HIV, as revealed in genetic studies. A polymorphism in the TLR3 gene is associated with a reduced frequency of HIV transmission during intravenous drug use in multiple cohorts (Huik et al., 2013; Sironi et al., 2012). Other human studies indicate that IFN responses in reproductive mucosa limit sexual transmission of HIV (Nazli et al., 2019; Shey et al., 2016) recommending evaluation of poly I:C or other innate immune triggers to elicit this response (Fig. 1) and reduce or prevent this major route of infection. Poly I:C is already investigated clinically for various purposes. A modified poly I:C has been shown to be safe in Phase I trials in HIV-infected people on antiretroviral therapy (Saxena et al., 2019). Also in clinical trial, a proprietary poly I:C vaccine administered IN was found to be effective in prevention of infection by rhinovirus and influenza A (Malcolm et al., 2018). IN administration is preferred for some vaccines, eliciting better responses than intramuscular injection in some cases (Hoft et al., 2017). The IN route is also employed to target interventions to the brain (Hanson and Frey, 2007). IN insulin is used for treatment of certain cognitive disorders (Freiherr et al., 2013; Novak et al., 2014) and we found it effective against HIV–NCI in mice (Kim et al., 2019), an approach currently in trials in humans in NCT03081117.

In conclusion, poly I:C can prevent experimental EcoHIV transmission and control established infection in mice. Once EcoHIV infection is silenced, its impairment of cognitive function is reversed. Interventions that control HIV expression in chronically infected cells, like poly I:C, may help to alleviate progression of HIV–NCI with age and address the growing public health concern in aging HIV-infected individuals on antiretroviral therapy (Brown et al., 2014).

4. Materials and methods

Poly I:C source and administration. Poly I:C was purchased from Sigma-Aldrich, catalogue number P1530. It was prepared as a solution in PBS for all uses with a final dose of 50 μg per mouse administration or 5 μg per ml in culture. For topical administration, it was applied to outer vaginal surfaces of lightly sedated supine females in a volume of 10–20 μl. Mouse hindquarters were slightly elevated for 15–30 min to allow liquid to enter the vaginal cavity. For intranasal administration it was applied to both nostrils of awake mice as described (Kim et al., 2019). Otherwise it was injected IP.

Mice. All animal studies were conducted with the approval of the Icahn School of Medicine Institutional Animal Care and Use Committee, protocol IACUC-2014-0124, and in full compliance with NIH guidelines. Adult male and female C57BL/6 mice, male BALB/c and nude C57BL/6 mice, and male and female IFNR-KO mice on a C57BL/6 background were purchased from Jackson Labs. Except where specified in the text, studies were conducted in male C57BL/6 mice.

Virus preparation and infection. EcoHIV/NDK was propagated and administered by IP or IV injection to mice as described (Potash et al., 2005). For EcoHIV sexual transmission, individual female mice were housed overnight with an individual EcoHIV-infected male nude mouse and then housed separately as described (Hadas et al., 2013). Cells. Bone marrow was obtained from adult mouse femurs and BMM were differentiated and infected as described (Gu et al., 2018).

Tissue isolation and measurement of virus burden. At times indicated in the text, mice were euthanized by carbon dioxide asphyxiation and spleen, peritoneal cells, and inguinal lymph nodes collected. Tissues and cells were prepared and subjected to quantitative PCR for measurement of EcoHIV gag DNA and RNA and vif RNA as described (Hadas et al., 2007). Measurement of integrated EcoHIV DNA was conducted as described (Gu et al., 2018).

Cellular gene expression. Peritoneal cells or BMM were harvested at the times indicated in the text and subjected to quantitative PCR as described (He et al., 2014; Kim et al., 2019).

RAWM. At times indicated in the text, the RAWM was conducted as described (Gu et al., 2018) with the exception that C57BL/6 and not 129X1 mice were used here and uninfected C57BL/6 mice achieved 1 or no errors after 5–7 days of testing at which point the assay was completed.

Declaration of competing interest

The authors of the manuscript, “Prevention and treatment of HIV infection and cognitive disease in mice by innate immune responses”, Dong, Borjabad, Kelschenbach, Chao, Volsky, and Potash declare they have no conflicts of interest regarding any element of this manuscript.

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