HHS Public Access

Author manuscript

Cell Host Microbe. Author manuscript; available in PMC 2017 April 13.

Published in final edited form as:

Cell Host Microbe. 2016 April 13; 19(4): 515–528. doi:10.1016/j.chom.2016.03.001.

NLRX1 Sequesters STING to Negatively Regulate the Interferon Response, Thereby Facilitating the Replication of HIV-1 and DNA Viruses

Haitao Guo[#1], **Renate König**[#2,3], **Meng Deng**[1,4], **Maximilian Riess**[3], **Jinyao Mo**[5], **Lu Zhang**[1], **Alex Petrucelli**[1], **Sunnie M. Yoh**[2], **Brice Barefoot**[7], **Melissa Samo**[7], **Gregory D. Sempowski**[7], **Aiping Zhang**[8], **Anamaris M. Colberg-Poley**[8,9], **Hui Feng**[1], **Stanley M. Lemon**[1,5,10], **Yong Liu**[1,11], **Yanping Zhang**[1,11], **Haitao Wen**[1], **Zhigang Zhang**[1], **Blossom Damania**[1], **Li-Chung Tsao**[1], **Qi Wang**[1], **Lishan Su**[1,10], **Joseph A. Duncan**[1,5,6], **Sumit K. Chanda**[2,*], and **Jenny P.-Y. Ting**[1,10,12,*]

[1]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[2]Immunity and Pathogenesis Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, USA

[3]Host-Pathogen-Interactions, Paul-Ehrlich-Institut, German Center for Infection Research (DZIF), 63225 Langen, Germany.

[4]Department of Oral Biology, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[5]Division of Infectious Diseases, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[6]Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[7]Duke Human Vaccine Institute, Duke University Medical Center, Durham, NC 27710, USA

[8]Research Center for Genetic Medicine, Children's Research Institute, Children's National Medical Center, Washington, DC 20010, USA

[*]**Correspondence:** Dr. Jenny P.-Y. Ting, 450 West Drive, CB#7295, Chapel Hill, NC 27599-7295, Telephone: 919-966-5538, Fax: 919-966-8212, jenny_ting@med.unc.edu, Dr. Sumit K. Chanda, 10901 North Torrey Pine Road; La Jolla, CA 92037, Telephone: 858-795-5241, Fax: 858-795-5225, schanda@sanfordburham.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Other Methods See supplemental Experimental Procedures.

AUTHOR CONTRIBUTIONS

H.G., R.K., J.P.-Y.T., and S.K.C. designed the experiments; M.D., M.R., J.M., J.A.D., L.Z., A.P., S.M.Y., B.B., M.S., G.D.S., H.F., S.M.L., Y.L., Y.Z., H.W., and Q.W. assisted with the experiments and provided intellectual input; A.Z., A.M.C.-P., Z.Z., B.D., L.-C.T., and L.S. provided critical reagents and intellectual input; J.P.-Y.T. and S.K.C. supervised the study; H.G., J.P.-Y.T., R.K., and S.K.C. interpreted the data and wrote the manuscript.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[9]Departments of Integrative Systems Biology, Biochemistry and Molecular Biology, George Washington University School of Medicine and Health Sciences, Washington DC 20037, USA

[10]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[11]Department of Radiation Oncology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[12]Departments of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

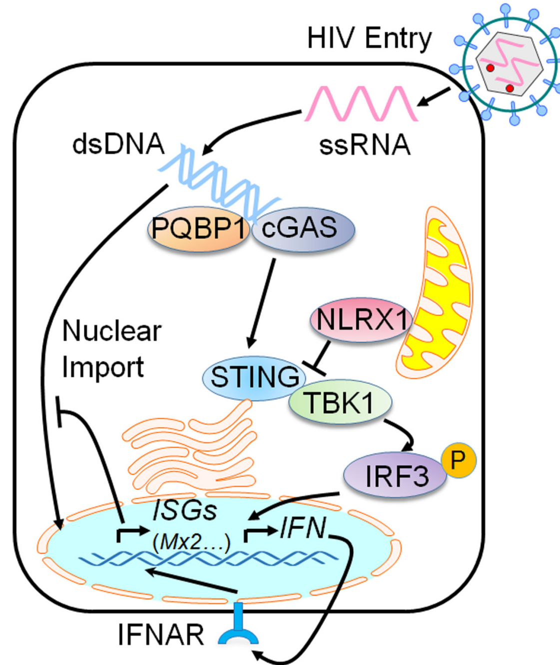
# These authors contributed equally to this work.

SUMMARY

Understanding the negative regulators of anti-viral immune responses will be critical for advancing immune-modulated antiviral strategies. NLRX1, an NLR protein that negatively regulates innate immunity, was previously identified in an unbiased siRNA screen as required for HIV infection. We find that NLRX1 depletion results in impaired nuclear import of HIV-1 DNA in human monocytic cells. Additionally, NLRX1 was observed to reduce type-I interferon (IFN-I) and cytokines in response to HIV-1 reverse-transcribed DNA. NLRX1 sequesters the DNA sensing adaptor STING from interaction with TANK-binding kinase 1 (TBK1), which is a requisite for IFN-1 induction in response to DNA. NLRX1-deficient cells generate an amplified STING-dependent host response to cytosolic DNA, c-di-GMP, cGAMP, HIV-1 and DNA viruses. Accordingly, *Nlrx1*$^{-/-}$ mice infected with DNA viruses exhibit enhanced innate immunity and reduced viral load. Thus, NLRX1 is a negative regulator of the host innate immune response to HIV-1 and DNA viruses.

Abstract

Author Manuscript Author Manuscript Author Manuscript Author Manuscript



INTRODUCTION

A fundamental question in human immunodeficiency virus 1 (HIV-1) research is: Why is the immune system unable to contain the virus. Unlocking this key would provide a significant advance in the advancement of immune-modulated HIV antivirals and the direct strategies for the development of efficacious vaccines/adjuvants. Human Immunodeficiency Virus 1 (HIV-1) is known to utilize host-encoded proteins to facilitate its replication. On the other hand, innate immune response including type-I interferon production triggered by HIV-1 infection has been reported to restrict virus replication and promote systematic inflammation, although innate immunity can also lead to T cell exhaustion, and CD4 T cell depletion (Doyle et al., 2015). It is of great significance to understand the roles of host proteins in viral replication and innate immune response induction by HIV-1 infection. One approach to advance our knowledge of these complex issues is the use of unbiased screening to identify pivotal players in HIV infection (Konig and Stertz, 2015). Previous work from one of our groups used unbiased RNAi screening to identify host factors that are important during the early step of HIV infection that resulted in the promotion of viral infection (Konig et al., 2008). The present work describes the importance of a host protein, NLRX1, in promoting HIV infection. Different innate immune sensors and mechanisms have been identified for HIV-1 components recognition (Altfeld and Gale, 2015). Recent advances show that HIV-1 complementary DNA but not its genomic RNA induces IRF3 activation and type-I interferon production dependent on cyclic GAMP synthase (cGAS)-STING pathway in human macrophages (Gao et al., 2013). Importantly, the polyglutamine binding protein 1 (PQBP1) is recently identified as the co-receptor of reverse-transcribed HIV-1 DNA and associates with cGAS (Yoh et al., 2015). HIV-1 DNA triggers cGAS to synthesize cyclic

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

GMP-AMP (cGAMP), which is the activator of adaptor protein STING to induce IRF3 and NF-κB activation, thereafter innate immune response (Gao et al., 2013).

The nucleotide-binding domain, leucine-rich repeat containing proteins (also known as NOD-like receptors, NLRs) represent a large family of innate immune receptors or regulators which have been primarily associated with inflammasome function (Guo et al., 2015). However recently they are shown to play important roles in modulating type-I interferon production through different mechanisms (Allen, 2014). For example, NLRX1 was the first NLR found to attenuate IFN-I signaling (Allen et al., 2011; Moore et al., 2008; Xia et al., 2011). NLRC3 has been recently identified as an inhibitor of STING function by interfering with STING translocation and STING-TBK1 association (Zhang et al., 2014). NLRP4 negatively regulates type-I interferon signaling by promoting TBK1 degradation (Cui et al., 2012). Nlrp6 binds viral RNA via RNA helicase Dhx15 and interacts with MAVS (mitochondrial anti-viral signaling) to induce type-I/III interferons (Wang et al., 2015). Although the role of NLRP3 inflammasome in HIV-infected human monocytes is known to trigger IL-1β (Chattergoon et al., 2014; Guo et al., 2014), the role of NLRs in modulating HIV-1 induced type-I interferon and HIV-1 infection remains unexplored.

Host factors required for HIV-1 infection were previously screened by an unbiased siRNA strategy, and NLRX1 was identified as one of 295 hits, although its significance was not highlighted nor studied (Konig et al., 2008). NLRX1 is unique in that it resides in the mitochondria and mediates a host of functions, including ROS induction (Abdul-Sater et al., 2010; Tattoli et al., 2008), attenuation of the RIG-I-MAVS and TRAF-IKK signaling (Allen et al., 2011; Moore et al., 2008; Xia et al., 2011), and induction of autophagy (Lei et al., 2012). However, the mechanism by which NLRX1 promotes HIV-1 infection is completely unexplored. In this study, we validated that NLRX1 is required for HIV-1 infection in multiple cell types including primary human macrophages and dendritic cells. Strikingly, the reduction of HIV-1 infection in NLRX1 depletion cells is due to an impaired nuclear import of HIV-1 DNA. Furthermore, we found that NLRX1 promotes HIV-1 infection by attenuating reverse transcribed HIV-1 DNA induced type-I interferon, interferon stimulatory genes (ISGs) and proinflammatory cytokines. Notably, NLRX1 associates with STING to reduce TBK1 activation. We also extended the negative regulatory function of NLRX1 to immunity to DNA viruses, such as HSV-1 and vaccinia virus.

RESULTS

NLRX1 Enhances HIV-1 Infection

A recent report showed that expression of the mitochondrial localized NLR, NLRX1, is decreased in HIV-1 patient (Nasi et al., 2015). From an unbiased siRNA screening for host factors required for HIV-1 infection described previously (Konig et al., 2008), we identified NLRX1 as a validated hit (Figure 1A). To test the function of NLRX1 in HIV-1 infection in more relevant cell types, an HIV-1 permissive, human macrophages cell line, THP-1, was used. *NLRX1* was knocked down in THP-1 by two different shRNAs (Figure 1B, left panel). Notably, HIV-VSV exhibited reduced infection in THP-1 cells containing shRNA for *NLRX1* over time, compared to cells containing the scrambled shRNA (Figure 1C, left). CRISPR/Cas9 strategy was also used to target *NLRX1* by two different single guide-RNAs

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

in THP-1 cells (Figure 1B, right panel). In congruent with the shRNA strategy, THP-1 cells with attenuated NLRX1 expression showed reduced HIV-1 infection (Figure 1C, right). In addition to measurement of luciferase activity for HIV-1 infection, THP-1 cells with attenuated NLRX1 expression displayed a lower level of HIV-1 Gag protein p55 compared to control cells (Figure 1D and 1E). Finally, *NLRX1* was knocked down in human monocytes derived macrophages (MDM) and dendritic cells (MDDC) by two to three different siRNAs (Figure S1A and 1B, upper panels). HIV-VSV infection was reduced in human MDM and MDDC containing siRNA for *NLRX1* from three to four different donors (Figure S1A and 1B, lower panels). Data pooled from different donors were shown in Figure 1F and 1G. Importantly, a CCR5/CXCR4 dual tropic HIV-1 strain R3A also showed reduced replication in cells with attenuated NLRX1 expression (Figure 1H and Figure S1C) (Meissner et al., 2004). To examine the point at which NLRX1 promoted HIV infection, we identified that NLRX1 deficiency reduced nuclear import of HIV-1 DNA as measured by the nuclear imported 2-LTR circles, but did not affect early, minus strand transfer and intermediate stages of HIV-1 reverse transcription (Figure 1I). Taken together, these data indicate that NLRX1 is a host factor that enhances HIV-1 infection by increased HIV-1 DNA import, a process that is partly controlled by ISGs (Kane et al., 2013).

Removal of NLRX1 Enhances Innate Immune Response to HIV-1 Infection

It was previously shown that NLRX1 functions as a negative regulator of innate immune response by inhibiting interferon and cytokines responses (Allen et al., 2011; Moore et al., 2008; Xia et al., 2011). Thus, we tested if NLRX1 inhibits HIV-1 triggered innate immune response which may account for reduced HIV-1 infection. Upon HIV-VSV infection, THP-1 cells with attenuated NLRX1 expression produced more *IFNB* mRNA and IFNβ protein than control cells (Figure 2A). MX2, a key ISG which inhibits HIV-1 cDNA nuclear import (Kane et al., 2013), was also increased in NLRX1 deficient THP-1 cells, consistent with decreased HIV-1 DNA nuclear import in the absence of NLRX1 (Figure 1I). Mouse cells can be infected with HIV-VSV to interrogate the relevance of a specific gene. Bone marrow derived macrophages (BMDM) from *Nlrx1*$^{-/-}$ mice showed higher induction of IFNβ, proinflammatory cytokine genes *Il6* and *Rantes*, and the interferon stimulated gene 15 (*Isg15*) than wildtype control cells (Figure 2B). MEFs from *Nlrx1*$^{-/-}$ mice and littermate WT mice were also subjected to HIV-VSV infection (Gao et al., 2013). Similarly, enhanced innate immune response was observed in *Nlrx1*$^{-/-}$ MEFs over time (Figure 2C). To test whether enhanced type I IFN alone caused the difference in HIV-1 infection between WT and NLRX1 deficient cells, antibody against the interferon-α/β receptor (IFNAR) was used. Anti-IFNAR antibody abolished the difference in HIV-1 infection of WT and NLRX1 deficient cells (Figure 2D), suggesting that increased IFN production by NLRX1 deficient cells accounted for reduced HIV-1 infection. In addition, THP-1 cells with shRNA for *NLRX1* and scrambled shRNA control were infected with HIV-VSV and cultured in transwells to allow the diffusion of soluble products across these wells. As a result the secretory products in the media should reach equilibrium in these two wells. In this configuration, WT and NLRX1 deficient cells exhibited comparable levels of HIV-1 infection, suggesting that the difference in diffusible secreted factors from WT and NLRX1 deficient cells accounted for the difference in HIV-1 infection between these two samples (Figure 2E). We next investigated key signaling innate immune pathways upon HIV-1

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

infection. HIV-1 infection is known to cause phosphorylation of TBK1 and IKKε, which leads to IRF3 activation (Gao et al., 2013; Yoh et al., 2015). The phosphorylation of TBK1, IKKε, and IRF3 was significantly enhanced in THP-1 cells with attenuated NLRX1 expression relative to control cells upon HIV-VSV infection, and this is followed by enhanced STAT1 phosphorylation which is known to be activated by type I IFN (Figure 2F). In summary, removal of NLRX1 enhanced anti-HIV-1 innate immune response and signaling known to affect IFN and cytokine production and this enhancement accounted for reduced HIV-1 infection.

NLRX1 Inhibits HIV-1 reverse transcripts induced innate immune response

Previous reports showed that HIV-1 reverse-transcribed DNA, but not RNA, induces innate immune response such as IFN and cytokine responses (Gao et al., 2013; Yan et al., 2010). We next tested whether NLRX1 inhibits HIV-1 DNA induced innate immune response. As described by the previous papers, an HIV-1 reverse transcriptase inhibitor, nevirapine, and an integrase inhibitor, raltegravir, were used to inhibit HIV-VSV infection in MEFs (Figure S2A). Nevirapine blocks HIV-1 complementary DNA synthesis while raltegravir blocks HIV-1 DNA integration into host genome after HIV-1 complementary DNA synthesis (Figure S2B). As previous reported in MEF, nevirapine abrogated IFNβ, *Isg15*, *Il6*, and *Rantes* induction upon HIV-VSV infection, but raltegravir had no effect. Interestingly, enhanced IFNβ, *Isg15*, *Il6*, and *Rantes* induction from HIV-VSV infected *Nlrx1*$^{-/-}$ MEFs was also abrogated when cells were treated with nevirapine while raltegravir had no effect (Figure S2C-S2F). These data indicate that NLRX1 inhibits HIV-1 reverse transcribed DNA induced innate immune response to promote HIV-1 infection.

NLRX1 Inhibits STING-Dependent DNA Sensing Pathway

In HIV-1 infected cells, PQBP1/cGAS recognizes HIV-1 DNA and generates cyclic cGAMP to activate STING (Gao et al., 2013; Yoh et al., 2015). To investigate the role of NLRX1 in STING-dependent innate immune signal transduction, we first examined the impact of NLRX1 on STING-induced activation of interferon-stimulated responsive element (ISRE)-luciferase reporter, which is known to be activated by STING, TBK1, and IRF3 (Ishikawa and Barber, 2008; Zhong et al., 2008). NLRX1 dramatically reduced ISRE activation by STING, but not downstream molecule TBK1 and IRF3, suggesting that NLRX1 interferes with STING function (Figure 3A-3C). Even though we previously found that NLRC3 inhibited STING signaling (Zhang et al., 2014), this has not been a common feature among NLRs as several different NLRs including NLRP3, NLRP11, NLRC5, and NOD2 did not reduce STING-induced ISRE activation (Figure 3D). STING can be activated by cytosolic DNA and cyclic dinucleotides (Burdette et al., 2011; Ouyang et al., 2012; Sun et al., 2013; Wu et al., 2013; Zhang et al., 2013), thus we evaluated the role of NLRX1 in cytosolic DNA and cyclic dinucleotides induced innate immune signaling. Upon intracellular delivery of interferon stimulatory DNA (ISD) (Stetson and Medzhitov, 2006), cyclic di-GMP (c-di-GMP), or cGAMP, *Nlrx1*$^{-/-}$ MEFs exhibited enhanced induction of *Ifnb*, *Il6* and *Tnf*, when compared to control MEFs (Figure 3E-3G). Phosphorylation of TBK1 is required for STING-dependent interferon and proinflammatory cytokine production. Consistent with the cytokine induction data, cGAMP induced greater phosphorylation of TBK1 with a more rapid kinetics in *Nlrx1*$^{-/-}$ than that in control MEFs (Figure 3H). The effect of NLRX1 was

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

further studied in BMDM and peritoneal macrophage. Intracellular delivery of ISD or another commonly used DNA stimulus for STING, VACV 70-mer (Unterholzner et al., 2010), induced more IFNβ in *Nlrx1*$^{-/-}$ BMDMs than controls (Figure 3I). cGAMP also induced more IFNβ cytokine and *Ifna4* mRNA in *Nlrx1*$^{-/-}$ BMDMs (Figure 3I). Similarly in peritoneal macrophages, intracellular ISD, VACV 70-mer, or c-di-GMP induced more IFNβ in *Nlrx1*$^{-/-}$ cells than controls (Figure 3J). Taken together, these data indicate that NLRX1 attenuates STING-dependent innate signaling pathway.

NLRX1 Associates with STING to Disrupt STING-TBK1 Interaction

To explore the mechanism by which NLRX1 interferes with STING-dependent pathway, we tested the interaction of NLRX1 with STING as a mechanism by which NLRX1 inhibited STING function. In an overexpression system, bi-directional co-immunoprecipitation with anti-FLAG or anti-HA beads was followed by immunoblot to show that FLAG-NLRX1 associated with HA-STING (Figure 4A, left panel). As a specificity control, NLRX1 did not interact with cGAS (Figure S3). Recruitment of TBK1 to STING is a critical step that is required for IRF3 activation and the induction of interferon and cytokines. Overexpressed FLAG-TBK1 and HASTING also showed interaction as expected (Figure 4A, right panel), which made it possible to test if NLRX1 interfered with STING-TBK1 association in a later experiment. Since overexpressed protein interaction analysis could produce artifacts, we tested the association of endogenous NLRX1 with STING. NLRX1-STING association was detected by immunoprecipitation using anti-NLRX1 antibody in THP-1 cells and immunoblotting with the reciprocal anti-STING antibody. Association was detected in controls cells, but not in cells where NLRX1 was eliminated with sg-NLRX1 (Figure 4B, left). Reciprocal immunoprecipitation with anti-STING antibody followed by immunoblotting with anti-NLRX1 also confirmed endogenous NLRX1-STING association (Figure 4B, right). Endogenous NLRX1-STING association was also detected by immunoprecipitation using anti-NLRX1 antibody in MEF (Figure 4C). This NLRX1-STING association was increased during HSV-1 infection of THP-1 derived macrophages and BMDMs (Figure S4). Furthermore, we also determined that the association between NLRX1 and STING is direct by reciprocal pull-down assay using recombinant full-length NLRX1 prepared from insect cells and truncated STING protein (amino acid 139-379 and 139-344) used previously (Figure S5A, Figure 4D and 4E) (Zhang et al., 2014). To rule out the possibility that NLRX1 associates with STING non-specifically, we showed that recombinant ASC prepared similarly to NLRX1 did not interact with recombinant STING (Figure S5B). To better understand how NLRX1 interacted with STING, we mapped the domains of NLRX1 required for this interaction. The results showed that constructs containing NBD alone or NBD in combination with other domains interacted with STING. Constructs lacking the X (NBD+LRR) or LRR (X+NBD) domain also interacted with STING, while construct encoding LRR alone did not interact, suggesting that NBD is the key domain of NLRX1 that associated with STING (Figure 4F). Another approach to examine for NLRX1 interaction with STING is by microscopy. The antibody for NLRX1 was found to be unsuitable for immunofluorescence analysis, thus we overexpressed FLAG-NLRX1 and HASTING to assess their co-localization. Previous reports showed that NLRX1 is a mitochondrial membrane protein and STING mainly localizes to the endoplasmic reticulum (ER). We re-investigated the localization of NLRX1 and found by proteinase K

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

digestion that NLRX1 showed a protease-sensitivity pattern that is consistent with its localization to the mitochondrial outer membrane but not in mitochondrial matrix in HEK293T cells (Figure S6). Co-localization of NLRX1 and STING was observed by confocal microscopy, which could occur on the mitochondria associated membrane (MAM) (Figure 4G). We performed co-immunoprecipitation by using isolated MAMs prepared as described and confirmed that NLRX1 and STING interacted within this fraction (Figure S7 and Figure 4H) (Zhang et al., 2011). Finally, we assessed if NLRX1 could interfere with STING-TBK1 interaction by assaying for STING-TBK1 interaction in the presence of increasing quantities of NLRX1. Increasing NLRX1 reduced STING-TBK1 association in a dose-dependent fashion (Figure 4I). These data suggest that NLRX1 associates with STING to disrupt STING-TBK1 interaction, thus providing a mechanism by which NLRX1 inhibits STING-dependent DNA sensing.

NLRX1 Deficiency Causes Enhanced Innate Immune Response to DNA Viral Infection

We next investigated the broader role of NLRX1 during DNA viruses infection, we infected *Nlrx1*$^{-/-}$ or WT MEFs with DNA viruses vaccinia virus (VACA) or herpes simplex virus 1 (HSV-1). *Ifnb* and various innate immune cytokines were induced 6 hr post infection. VACA infection induced more *Ifnb* mRNA and MCP-1 protein compared to control MEFs (Figure 5A and 5B). *Nlrx1*$^{-/-}$ MEFs infected with HSV-1 also produced more *Ifnb* mRNA and IL-6 protein, as well as MCP-1 and IP-10 than control MEFs (Figure 5C-5F). Furthermore, the extent of TBK1 and NF-κB p65 phosphorylation was higher in *Nlrx1*$^{-/-}$ than that in control MEFs (Figure 5G). More phosphorylation of STAT1, which is activated by IFNβ, was also induced in *Nlrx1*$^{-/-}$ than control MEFs, consistent with the observation that *Nlrx1*$^{-/-}$ cells produced more type I IFN compared to control MEFs. Consistent with enhanced innate immune response to HSV-1 infection in *Nlrx1*$^{-/-}$ cells, *Nlrx1*$^{-/-}$ BMDM contained less HSV-1 genomic DNA than WT cells (Figure 5H). These data suggest that NLRX1 is a negative regulator of innate immune signals induced by DNA virus *in vitro*.

STING Deficiency in THP-1 Abrogates the Enhancement of HIV-1 infection by NLRX1

To demonstrate that the impact of NLRX1 on HIV-1 infection occurred through a STING-dependent pathway, we generated THP-1 cells with diminished NLRX1 and STING expression (Figure 6A). The former was reduced by shRNA while the latter was deleted by sgRNA. Compared to control cells, HIV-VSV infection was enhanced by 4.91 and 2.41 fold 2 days post-infection and 3.93 and 2.12 fold 3 days post-infection in two NLRX1 deficient cell lines. In the absence of STING, this enhancement of HIV-1 infection in the absence of NLRX1 was attenuated at 2 days post- infection and completely abolished at 3 days post-infection (Figure 6B). Similarly, knocking down NLRX1 expression by shRNA amplified HIV-VSV induced *IFNB1* and ISG *MX2* mRNA levels compared to control cells (Figure 6C and 6D). However, induction of *IFNB1* and *MX2* mRNA by HIV-VSV infection was completely abrogated in the absence of STING, regardless of the presence or absence of NLRX1 (Figure 6C and 6D). These data provide direct evidences that NLRX1 enhances HIV-1 infection and reduces *IFNB1* and *MX2* expression by negatively regulating STING-dependent innate immune response. In the absence of STING, NLRX1 does not exert its function.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

NLRX1 Negatively Regulate Host Immunity against HSV-1 Infection in Mice

Next, we evaluated the *in vivo* role of NLRX1 in host innate immune defense against DNA viral infection. *Nlrx1*$^{-/-}$ or WT mice were infected intraperitoneally with HSV-1, and their body weight were monitored. Both WT and *Nlrx1*$^{-/-}$ mice exhibited a transient body weight loss during the first two days after infection and then started to recover at day 3 after infection (Figure 7A). WT mice showed a 10% body weight loss at day 2, while *Nlrx1*$^{-/-}$ mice only had a 5% weight loss and this difference was maintained through the monitored period. As intraperitoneal delivery of HSV-1 did not cause any mortality, we next infected WT and *Nlrx1*$^{-/-}$ mice with HSV-1 intravenously (Zhang et al., 2014). With a lower dose of HSV-1 infection by this route (2×10^7 PFU/mouse), WT mice showed more body weight loss than *Nlrx1*$^{-/-}$ mice (Figure 7B). At a higher dose of HSV-1 infection (5×10^7 PFU/mouse), 40% of WT mice died by day 5 post-infection while only 9% of *Nlrx1*$^{-/-}$ mice died at this time-point (Figure 7C). Consistently, HSV-1 genome copies and live viruses were more than a log higher in the brains of WT mice harvested at day 5 after intravenous infection than those in *Nlrx1*$^{-/-}$ mice (Figure 7D and E). *Nlrx1*$^{-/-}$ mice also displayed a moderate higher production of IFNβ and IL-6 in the serum upon HSV-1 infection than WT mice (Figure 7F and 7G). Taken together, these data indicate that NLRX1 negatively impacts the ability of the host to contain a DNA viral infection.

DISCUSSION

The identification of negative regulators of the adaptive immune system such as PD-1 and CTLA-4 has revolutionized therapeutic approaches to manipulate T cell immunity in infection and cancer (Pardoll, 2012). By comparison, checkpoints or negative regulators of innate immunity have been limited. This is a particularly critical issue in HIV research, since the host immunity is ineffective in eliminating the virus. Previous work has shown that HIV-1 exploits the exonuclease TREX1 to degrade viral DNA and subvert its sensing by host cells (Yan et al., 2010). In this study, we show that NLRX1 serves as a negative regulator of innate immunity resulting in the promotion of HIV-1 infection. Recent findings have demonstrated that reverse transcribed HIV-1 DNA induces type-I interferon and pro-inflammatory cytokines through initiation of the PQBP1/cGAS innate response pathway. Our study shows that NLRX1 inhibits IFN induced by antagonizing this pathway. This is achieved by the association of NLRX1 with STING which results in a block of STING-TBK1 interaction thereby inhibiting TBK1 activation required for type I IFN production. From a broad perspective, the negative regulatory impact of NLRX1 is not limited to innate immunity elicited by HIV-1 DNA, but is extended to those caused by inflammatory DNA, cyclic dinucleotide, and DNA viruses that are reliant on the cGAS-STING-TBK1 pathway. Importantly, NLRX1 also attenuates DNA virus-induced innate immune response in mouse models of infection, supporting the physiologic importance of these findings.

NLRX1 has been previously found to inhibit RIG-I-MAVS and TRAF-IKK signaling (Allen et al., 2011; Moore et al., 2008; Xia et al., 2011), but promote ROS induction (Abdul-Sater et al., 2010; Tattoli et al., 2008), and autophagy (Lei et al., 2012). However, its impact on IFN caused by RNA viruses is not uniformly observed (Soares et al., 2013). In this study, we found that depletion of NLRX1 inhibits HIV-1 infection. This may be due to two

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

possibilities: by a direct alteration of anti-viral IFNs or cytokines that are secreted in the milieu or by a cell-intrinsic mechanism such as altered autophagy. Autophagy has been shown to intersect with HIV-1 biosynthesis and regulates viral yield in macrophages (Kyei et al., 2009). The experiment employing a transwell system favors the former possibility and supports the importance of secreted soluble factors that are negatively regulated by NLRX1 in HIV-1 infection. The targeted usage of anti-IFNR antibody directly points to IFN-I as a soluble factor that is negatively impacted by NLRX1, resulting in increased HIV-1 infection.

This report shows the association of NLRX1 with STING which disrupts STING-TBK1 interaction. NLRX1 resides in mitochondrial outer membrane and STING preferentially localizes to the ER membrane (Ishikawa and Barber, 2008; Moore et al., 2008). The interaction between mitochondrial proteins with ER proteins to regulate inflammation, calcium transfer, and autophagy has been described by others (Marchi et al., 2014). For example, STING is known to interact with the mitochondrial outer membrane protein MAVS at mitochondria-associated membranes, contributing to RNA triggered RIG-I-MAVS signaling pathway (Ishikawa and Barber, 2008; Zhong et al., 2008). Our finding shows that NLRX1 directly interacts with STING as demonstrated by using recombinant proteins. A further analysis of MAM-enriched fractions suggests that NLRX1 associates with STING at MAM.

In addition to NLRX1, we previously showed that another NLR protein, NLRC3, binds to both STING and TBK1 thus blocking their association and subsequent activation (Zhang et al., 2014). In this regard, NLRX1 functions similarly to NLRC3 by regulating STING-dependent pathways. It is possible that NLRC3 and NLRX1 function synergistically in regulating cytosolic DNA triggered innate immune response delivered by HIV-1 or DNA viruses. It will be worthwhile to generate mice lacking both *Nlrc3* and *Nlrx1* or human cells missing both genes to test this hypothesis. Similar to NLRC3, NLRX1 interacts with STING through its NBD domain. Thus NBD is emerging as a common protein interacting domain since the inflammasome NLRC4 also relies on the NBD domain for its binding to NAIP2 (Hu et al., 2015; Zhang et al., 2015).

NLRX1 inhibits HIV-1 mediated innate immune response and promotes HIV-1 infection in myeloid cells, however future studies will be necessary to assess if NLRX1 promotes HIV-1 transmission or pathogenesis in a chronic infection. However, a recent study showed that HIV-1 elite controllers possess an enhanced cGAS-dependent innate responses to HIV-1, and a corresponding proliferation of CD8+ T cells (Martin-Gayo et al., 2015). HIV-1 triggered systematic inflammation is known to cause T cell exhaustion, CD4 T cells depletion, and immune deficiency, while adequately inhibiting IFN-I improves anti-viral T cell function during chronic infection (Li et al., 2014; Wilson et al., 2013). Thus NLRX1 may benefit the host by preventing T cell exhaustion and deficiency in chronic infection. Furthermore, activation of NF-κB has been reported to result in viral reactivation while NLRX1 is known to inhibit NF-κB activity (Allen et al., 2011; Kauder et al., 2009; Xia et al., 2011). Thus, it is possible that NLRX1 may restrict reactivation of HIV from latency. However, in the case of HSV-1 acute infection in mice, our study clearly shows that blocking NLRX1 expression can be beneficial to the host.

Author Manuscript

In summary, NLRX1 is found to serve as a checkpoint of STING activation by HIV-1, DNA viruses, cGAMP and dsDNA. As a consequence, it acts as a host protein that is adopted by HIV-1 and HSV-1 to restrict innate immunity. Taken together, targeting NLRX1 may represent an approach to enhance early host immunity mediated by IFN to restrict these viruses, as well as an adjuvant strategy to enhance the efficacy of vaccines (Towers and Noursadeghi, 2014).

EXPERIMENTAL PROCEDURES

Experimental animals and *in vivo* virus infection

The C57BL/6 *Nlrx1*$^{-/-}$ mice have been described (Allen et al., 2011). Age and sex matched C57BL/6 littermates were produced and used in all the experiments. Mice were treated in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Eight weeks old mice were infected with HSV with 2×10^7 or 5×10^7 pfu of viruses per mouse by intravenous injection or 2×10^7 pfu of viruses per mouse by intraperitoneal injection. The body weight of the mice was monitored accordingly. For cytokine studies, mice were sacrificed 6 hours post-infection and sera were collected through cardiac puncture. For HSV-1 genome copy number and viral titer measurement, brains were harvested 5 days post-infection.

Cell stimulation and virus infection

MEFs, BMDM, or peritoneal macrophages were seeded in 24-well plates and transfected with 2 g/ml of ISD, VACV 70-mer, c-di-GMP, or cGAMP by lipofectamine 2000 (ThermoFisher Scientific). BMDMs were infected with HSV-1 (KOS strain) at the indicated multiplicity of infection (MOI) at 37 °C for 1 hr. MEFs were infected with HSV-1 or vaccinia virus at MOI of 1 or 5 at 37 °C for 1 hr. Cells were then washed with warm PBS and cultured in complete DMEM.

siRNA transfection

Transient knockdown of human *NLRX1* was achieved by transfection of specific siRNAs (si-NLRX1-1; Qiagen (siNOD9_4; SI00659960), si-NLRX1-2; Qiagen (siNLRX1_2; SI04231255), si-NLRX1-3; Qiagen (siNOD9_5; SI03143952)) or unspecific negative control siRNA (si-scramble, target sequence: 5′-AAGGTAATTGCGCGTGCAACT-3′). siRNAs were transfected using Lipofectamine RNAiMAX Reagent (Invitrogen) for HEK293T cells or Stemfect RNA Transfection Kit (Stemgent) for MDMs and MDDCs following the manufacturer’s instructions. Briefly, 293T cells were reverse transfected using 1.5 pmol siRNA and 0.15 μl RNAiMAX reagent per 96-well. MDMs and MDDCs were transfected on day 5 of differentiation after batch lift and seeding in 96-well format using 2.5 pmol siRNA and 0.136 μl stemfect reagent.

HIV-1 propagation and infection

The single round virus pNL4.3-Luc E-R+ (HIV-1 wild-type deltaEnv, encoding firefly luciferase GL3, kind gift of Nathaniel Landau) was pseudotyped with VSV-G (HIV-VSV).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Viruses were produced as described previously (Konig et al., 2008). HIV-1 R3A virus was amplified in SupT1 cells as described previously (Guo et al., 2014).

MDMs and MDDCs were infected with HIV-VSV at a MOI of 0.25 or 0.5. MEFs and BMDMs were infected with HIV-VSV at a MOI of 1 for 6 hr followed by culture in complete DMEM. THP-1 cells were infected with HIV-VSV at a MOI of 1 by spinoculation (1,000 × g, 25 °C) for 3 hr and cultured in completed RPMI medium. Infection readout was performed 24, 48, and 72 hr post-infection by luciferase assay.

Real time PCR analysis of HIV-1 reverse transcripts

THP-1 cells containing scrambled shRNA or shRNA for *NLRX1* were infected with HIV-VSV at a MOI of 1 by spinoculation (1,000 × g, 25 °C) for 3 hr and cultured in completed RPMI medium. Total DNA was extracted 3, 6, 9, 12, 24 hpi and HIV-1 reverse transcripts were quantified by real time PCR as previous described (Mbisa et al., 2009).

Statistical Analysis

Statistical analysis was carried out with Prism 5.0. All data are shown as or mean ± standard error of the mean (SEM). The mean values for biochemical data from each group were compared by Student's t-test or Mann-Whitney U test. Comparisons between multiple time points were analyzed by repeated-measurements analysis of variance (ANOVA) with Bonferroni post-tests. In all tests, P-values of less than 0.05 were considered statistically significant. *P< 0.05, **P<0.01, ***P<0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by NIH grants U19AI109965 (J.A.D., B.D., S.M.L., and J.P.-Y.T.), R37-AI029564 (J.P.-Y.T.), and P01 AI090935 (S.K.C.); the Burroughs Welcome Fund Career Award for Medical Scientists (J.A.D.); and DZIF TTU 01.802 (R.K.), TTU 04.802 (R.K.) and TTU 01.906 (R.K.). We thank Manja Burggraf for scientific advice. We thank the Lineberger (LCCC) imaging core facility for technical supports.

REFERENCES

Abdul-Sater AA, Said-Sadier N, Lam VM, Singh B, Pettengill MA, Soares F, Tattoli I, Lipinski S, Girardin SE, Rosenstiel P, et al. Enhancement of reactive oxygen species production and chlamydial infection by the mitochondrial Nod-like family member NLRX1. The Journal of biological chemistry. 2010; 285:41637–41645. [PubMed: 20959452]

Allen IC. Non-Inflammasome Forming NLRs in Inflammation and Tumorigenesis. Frontiers in immunology. 2014; 5:169. [PubMed: 24795716]

Allen IC, Moore CB, Schneider M, Lei Y, Davis BK, Scull MA, Gris D, Roney KE, Zimmermann AG, Bowzard JB, et al. NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRAF6-NF-kappaB signaling pathways. Immunity. 2011; 34:854–865. [PubMed: 21703540]

Altfeld M, Gale M Jr. Innate immunity against HIV-1 infection. Nature immunology. 2015; 16:554–562. [PubMed: 25988887]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Burdette DL, Monroe KM, Sotelo-Troha K, Iwig JS, Eckert B, Hyodo M, Hayakawa Y, Vance RE. STING is a direct innate immune sensor of cyclic di-GMP. Nature. 2011; 478:515–518. [PubMed: 21947006]

Chattergoon MA, Latanich R, Quinn J, Winter ME, Buckheit RW 3rd, Blankson JN, Pardoll D, Cox AL. HIV and HCV activate the inflammasome in monocytes and macrophages via endosomal Toll-like receptors without induction of type 1 interferon. PLoS pathogens. 2014; 10:e1004082. [PubMed: 24788318]

Cui J, Li Y, Zhu L, Liu D, Songyang Z, Wang HY, Wang RF. NLRP4 negatively regulates type I interferon signaling by targeting the kinase TBK1 for degradation via the ubiquitin ligase DTX4. Nature immunology. 2012; 13:387–395. [PubMed: 22388039]

Doyle T, Goujon C, Malim MH. HIV-1 and interferons: who's interfering with whom? Nature reviews Microbiology. 2015; 13:403–413. [PubMed: 25915633]

Gao D, Wu J, Wu YT, Du F, Aroh C, Yan N, Sun L, Chen ZJ. Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses. Science. 2013; 341:903–906. [PubMed: 23929945]

Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nature medicine. 2015; 21:677–687.

Guo H, Gao J, Taxman DJ, Ting JP, Su L. HIV-1 infection induces interleukin-1beta production via TLR8 protein-dependent and NLRP3 inflammasome mechanisms in human monocytes. The Journal of biological chemistry. 2014; 289:21716–21726. [PubMed: 24939850]

Hu Z, Zhou Q, Zhang C, Fan S, Cheng W, Zhao Y, Shao F, Wang HW, Sui SF, Chai J. Structural and biochemical basis for induced self-propagation of NLRC4. Science. 2015; 350:399–404. [PubMed: 26449475]

Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature. 2008; 455:674–678. [PubMed: 18724357]

Kane M, Yadav SS, Bitzegeio J, Kutluay SB, Zang T, Wilson SJ, Schoggins JW, Rice CM, Yamashita M, Hatziioannou T, et al. MX2 is an interferon-induced inhibitor of HIV-1 infection. Nature. 2013; 502:563–566. [PubMed: 24121441]

Kauder SE, Bosque A, Lindqvist A, Planelles V, Verdin E. Epigenetic regulation of HIV-1 latency by cytosine methylation. PLoS pathogens. 2009; 5:e1000495. [PubMed: 19557157]

Konig R, Stertz S. Recent strategies and progress in identifying host factors involved in virus replication. Current opinion in microbiology. 2015; 26:79–88. [PubMed: 26112615]

Konig R, Zhou Y, Elleder D, Diamond TL, Bonamy GM, Irelan JT, Chiang CY, Tu BP, De Jesus PD, Lilley CE, et al. Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell. 2008; 135:49–60. [PubMed: 18854154]

Kyei GB, Dinkins C, Davis AS, Roberts E, Singh SB, Dong C, Wu L, Kominami E, Ueno T, Yamamoto A, et al. Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages. The Journal of cell biology. 2009; 186:255–268. [PubMed: 19635843]

Lei Y, Wen H, Yu Y, Taxman DJ, Zhang L, Widman DG, Swanson KV, Wen KW, Damania B, Moore CB, et al. The mitochondrial proteins NLRX1 and TUFM form a complex that regulates type I interferon and autophagy. Immunity. 2012; 36:933–946. [PubMed: 22749352]

Li G, Cheng M, Nunoya J, Cheng L, Guo H, Yu H, Liu YJ, Su L, Zhang L. Plasmacytoid dendritic cells suppress HIV-1 replication but contribute to HIV-1 induced immunopathogenesis in humanized mice. PLoS pathogens. 2014; 10:e1004291. [PubMed: 25077616]

Marchi S, Patergnani S, Pinton P. The endoplasmic reticulum-mitochondria connection: one touch, multiple functions. Biochimica et biophysica acta. 2014; 1837:461–469. [PubMed: 24211533]

Martin-Gayo E, Buzon MJ, Ouyang Z, Hickman T, Cronin J, Pimenova D, Walker BD, Lichterfeld M, Yu XG. Potent Cell-Intrinsic Immune Responses in Dendritic Cells Facilitate HIV-1-Specific T Cell Immunity in HIV-1 Elite Controllers. PLoS pathogens. 2015; 11:e1004930. [PubMed: 26067651]

Mbisa JL, Delviks-Frankenberry KA, Thomas JA, Gorelick RJ, Pathak VK. Real-time PCR analysis of HIV-1 replication post-entry events. Methods Mol Biol. 2009; 485:55–72. [PubMed: 19020818]

Meissner EG, Duus KM, Gao F, Yu XF, Su L. Characterization of a thymus-tropic HIV-1 isolate from a rapid progressor: role of the envelope. Virology. 2004; 328:74–88. [PubMed: 15380360]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Moore CB, Bergstralh DT, Duncan JA, Lei Y, Morrison TE, Zimmermann AG, Accavitti-Loper MA, Madden VJ, Sun L, Ye Z, et al. NLRX1 is a regulator of mitochondrial antiviral immunity. Nature. 2008; 451:573–577. [PubMed: 18200010]

Nasi M, De Biasi S, Bianchini E, Digaetano M, Pinti M, Gibellini L, Pecorini S, Carnevale G, Guaraldi G, Borghi V, et al. Analysis of inflammasomes and antiviral sensing components reveals decreased expression of NLRX1 in HIV-positive patients assuming efficient antiretroviral therapy. AIDS. 2015; 29:1937–1941. [PubMed: 26237098]

Ouyang S, Song X, Wang Y, Ru H, Shaw N, Jiang Y, Niu F, Zhu Y, Qiu W, Parvatiyar K, et al. Structural analysis of the STING adaptor protein reveals a hydrophobic dimer interface and mode of cyclic di-GMP binding. Immunity. 2012; 36:1073–1086. [PubMed: 22579474]

Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nature reviews Cancer. 2012; 12:252–264. [PubMed: 22437870]

Soares F, Tattoli I, Wortzman ME, Arnoult D, Philpott DJ, Girardin SE. NLRX1 does not inhibit MAVS-dependent antiviral signalling. Innate immunity. 2013; 19:438–448. [PubMed: 23212541]

Stetson DB, Medzhitov R. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity. 2006; 24:93–103. [PubMed: 16413926]

Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science. 2013; 339:786–791. [PubMed: 23258413]

Tattoli I, Carneiro LA, Jehanno M, Magalhaes JG, Shu Y, Philpott DJ, Arnoult D, Girardin SE. NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-kappaB and JNK pathways by inducing reactive oxygen species production. EMBO reports. 2008; 9:293–300. [PubMed: 18219313]

Towers GJ, Noursadeghi M. Interactions between HIV-1 and the cell-autonomous innate immune system. Cell host & microbe. 2014; 16:10–18. [PubMed: 25011104]

Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, et al. IFI16 is an innate immune sensor for intracellular DNA. Nature immunology. 2010; 11:997–1004. [PubMed: 20890285]

Wang P, Zhu S, Yang L, Cui S, Pan W, Jackson R, Zheng Y, Rongvaux A, Sun Q, Yang G, et al. Nlrp6 regulates intestinal antiviral innate immunity. Science. 2015

Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, Aronow BJ, Karp CL, Brooks DG. Blockade of chronic type I interferon signaling to control persistent LCMV infection. Science. 2013; 340:202–207. [PubMed: 23580528]

Wu J, Sun L, Chen X, Du F, Shi H, Chen C, Chen ZJ. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science. 2013; 339:826–830. [PubMed: 23258412]

Xia X, Cui J, Wang HY, Zhu L, Matsueda S, Wang Q, Yang X, Hong J, Songyang Z, Chen ZJ, et al. NLRX1 negatively regulates TLR-induced NF-kappaB signaling by targeting TRAF6 and IKK. Immunity. 2011; 34:843–853. [PubMed: 21703539]

Yan N, Regalado-Magdos AD, Stiggelbout B, Lee-Kirsch MA, Lieberman J. The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. Nature immunology. 2010; 11:1005–1013. [PubMed: 20871604]

Yoh SM, Schneider M, Seifried J, Soonthornvacharin S, Akleh RE, Olivieri KC, De Jesus PD, Ruan C, de Castro E, Ruiz PA, et al. PQBP1 Is a Proximal Sensor of the cGAS-Dependent Innate Response to HIV-1. Cell. 2015; 161:1293–1305. [PubMed: 26046437]

Zhang A, Williamson CD, Wong DS, Bullough MD, Brown KJ, Hathout Y, Colberg-Poley AM. Quantitative proteomic analyses of human cytomegalovirus-induced restructuring of endoplasmic reticulum-mitochondrial contacts at late times of infection. Molecular & cellular proteomics : MCP 10. 2011 M111 009936.

Zhang L, Chen S, Ruan J, Wu J, Tong AB, Yin Q, Li Y, David L, Lu A, Wang WL, et al. Cryo-EM structure of the activated NAIP2-NLRC4 inflammasome reveals nucleated polymerization. Science. 2015; 350:404–409. [PubMed: 26449474]

Zhang L, Mo J, Swanson KV, Wen H, Petrucelli A, Gregory SM, Zhang Z, Schneider M, Jiang Y, Fitzgerald KA, et al. NLRC3, a member of the NLR family of proteins, is a negative regulator of

innate immune signaling induced by the DNA sensor STING. Immunity. 2014; 40:329–341. [PubMed: 24560620]

Zhang X, Shi H, Wu J, Sun L, Chen C, Chen ZJ. Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for STING. Molecular cell. 2013; 51:226–235. [PubMed: 23747010]

Zhong B, Yang Y, Li S, Wang YY, Li Y, Diao F, Lei C, He X, Zhang L, Tien P, et al. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. Immunity. 2008; 29:538–550. [PubMed: 18818105]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Highlights

- NLRX1 inhibits HIV-1 cDNA-induced innate immune response and enhances HIV-1 infection
- NLRX1 interacts with the DNA sensing adaptor STING to disrupt STING-TBK1 signaling
- STING deficiency abrogates the enhancement of HIV-1 infection by NLRX1
- *Nlrx1*$^{-/-}$ mice show enhanced innate immunity and are more resistant to DNA viruses

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

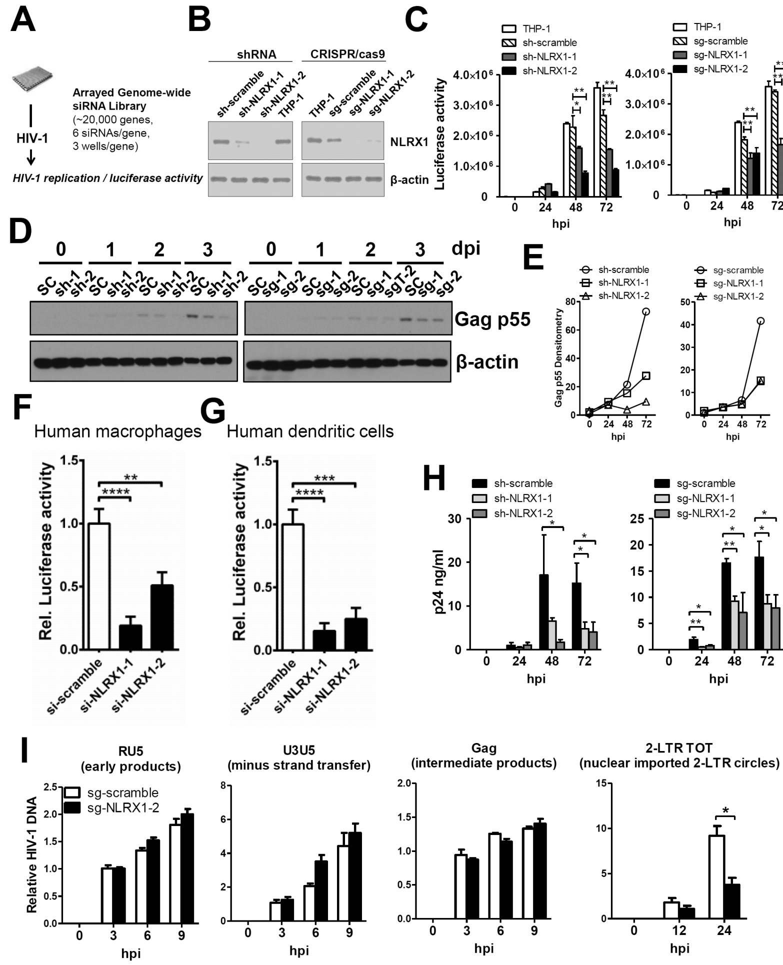


Figure 1. NLRX1 is required for HIV-1 infection

(A) Scheme of unbiased screening of host factors required for HIV-1 infection which identified that siRNA targeting NLRX1 reduced HIV-1 infection.

(B) Knockdown of NLRX1 in THP-1 cells by shRNA (left) or CRISPR-Cas9/sgRNA (right).

(C-E) THP-1 cells with scrambled or shRNA or sgRNA targeting *NLRX1* were infected with a single-cycle luciferase reporter virus pseudo-typed with a pH-dependent envelope VSV-G (HIV-VSV). After 24, 48, and 72 hours, luciferase activity (C) and the expression of HIV-1 Gag p55 protein (D) were determined. Densitometry analysis was performed for Gag p55 levels shown in panel D (E). SC: scrambled control, sh-1: sh-NLRX1-1, sh-2, sh-NLRX1-2, sg-1: sg-NLRX1-1, sg-2: sg-NLRX1-2.

(F and G) Knockdown of NLRX1 decreases HIV-1 infection in human primary macrophages (F) and dendritic cells (G). Monocyte derived macrophage and monocyte derived dendritic cells were transfected with scrambled siRNA or siRNA targeting NLRX1 followed by infection with HIV-VSV. HIV-1 infection as reflected by luciferase activity is shown. Data pooled from three or four different donors.

(H) Same as C except that the infection was done by using a CCR5/CXCR4 dual tropic HIV-1 strain R3A. HIV-1 particle release was quantified by p24 ELISA.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(I) Real-Time PCR analysis of HIV-1 reverse transcription and cDNA nuclear import as previously described (Mbisa et al., 2009). THP-1 cells with scrambled or shRNA or sgRNA targeting *NLRX1* were infected with a HIV-VSV. At indicated time points, total DNA was extracted and HIV-1 DNA was quantified by amplifying indicated target sequences.

Data are presented as the mean ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (Student's t test). All data are representative of two or three independent experiments.

See also Figure S1.

Author Manuscript

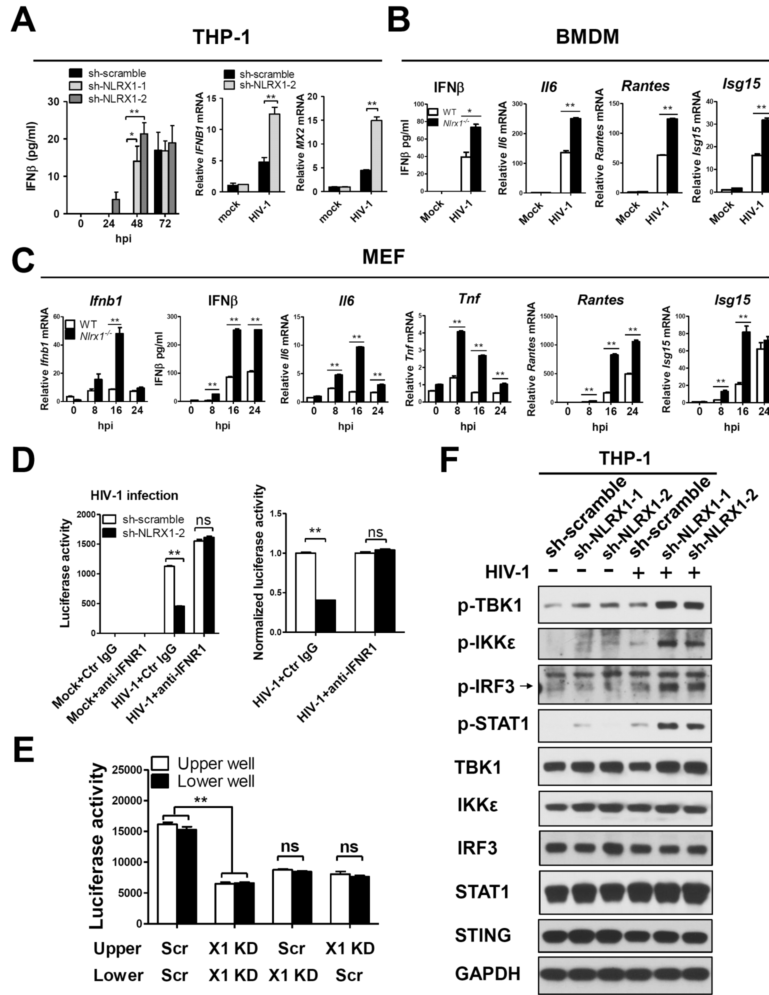


Figure 2. Removal of NLRX1 enhances innate immune response upon HIV-1 infection

(A) THP-1 cells with shNLRX1 or scrambled control shRNA were infected with HIV-VSV (MOI=1) and the induction of IFNβ was determined over time. IFNB1 and MX2 mRNA levels were determined by qPCR 18 hpi.

(B) WT or *Nlrx1*$^{-/-}$ BMDM were infected with HIV-VSV (MOI=1), at different time points, IFNβ was measured by ELISA and *Il6*, *Isg15* and *Rantes* were measured by qPCR.

(C) WT or *Nlrx1*$^{-/-}$ MEF were infected with HIV-VSV, MOI=1, at different time points. *Ifnb* was measured by qPCR and ELISA and *Il6*, *Tnf*, *Isg15* and *Rantes* were measured by qPCR.

(D) THP-1 cells with shNLRX1 or scrambled control shRNA were infected with HIV-VSV (MOI=1) in the presence of anti-IFNR1 or control IgG. At 72 hpi, luciferase activity (normalized by total protein) was determined to measure virus infection (left panel). Luciferase data were normalized by setting scrambled control as 1 (right panel).

(E) THP-1 cells with shNLRX1 (X1 KD) or scrambled control (Scr) shRNA were infected with HIV-VSV, MOI=1, and seeded in the plate with transwell as indicated, and luciferase activity from upper and lower wells was determined for virus infection 72 hpi. Luciferase activity was normalized by total protein.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(F) THP-1 cells with shNLRX1 or scrambled control shRNA were infected with HIV-VSV, MOI=1. 1 day after infection, cells were collected and p-TBK1, p-IKKε, p-IRF3, p-STAT1, TBK-1, IKKε, IRF3, STAT1, and STING were determined by western blot. GAPDH was used as the loading control.

Data are presented as the mean ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ (Student's t test). All data are representative of at least three independent experiments.

See also Figure S2.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

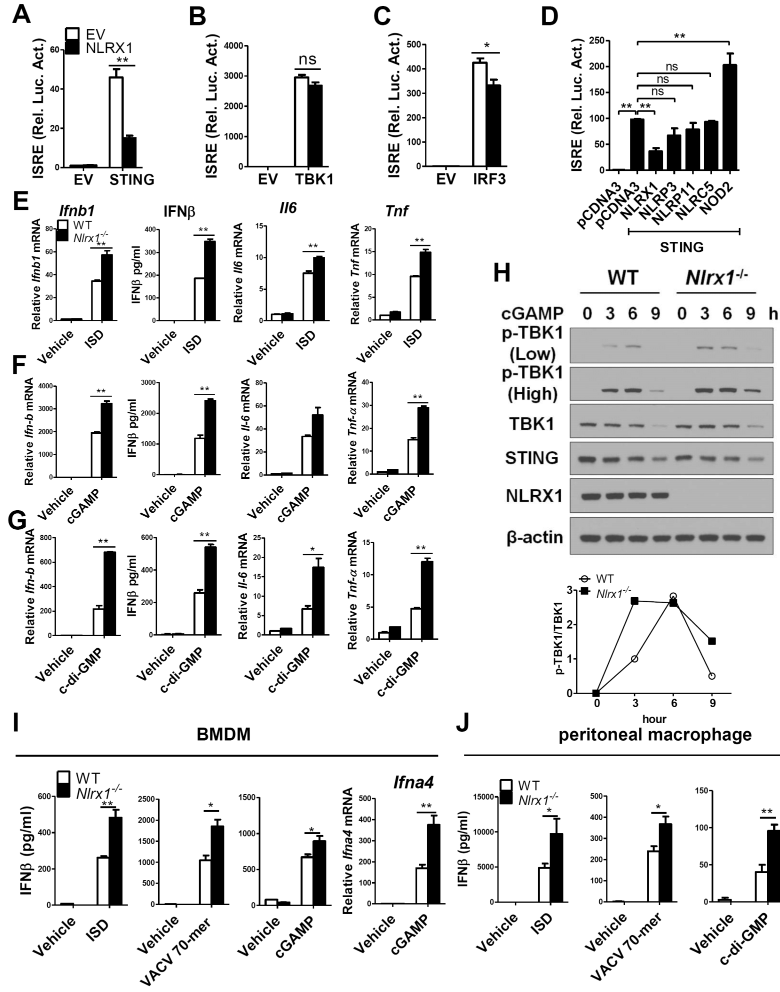


Figure 3. NLRX1 inhibits STING-dependent DNA sensing pathway

(A-C) HEK293T cells were transfected with the ISRE promoter reporter with the internal control Renilla luciferase reporter pLR-TK. Either empty vector or NLRX1 expression plasmid was transfected. STING (A), TBK1 (B) or IRF3 (C) was used as activator of ISRE reporter as indicated. Luciferase assays were performed 24 hr after transfection.

(D) Same as (A), except cells were transfected with either an empty plasmid or with NLRX1, NLRP3, NLRP11, NLRC5 or NOD2 expression plasmid.

(E-G) WT and *Nlrx1*$^{-/-}$ MEFs were transfected with ISD (E), cGAMP (F), or c-di-GMP (G) respectively. *Ifnb*, *Il-6*, and *Tnf-α* transcripts and IFNβ were measured 6 hr after transfection as indicated.

(H) Immunoblot of phosphorylated (p-) TBK1, TBK1, STING, and NLRX1 in lysates of WT and *Nlrx1*$^{-/-}$ MEFs transfected with cGAMP for indicated time points. β-actin was used as the loading control. Densitometry analysis was performed for (p-) TBK1 levels by normalizing to TBK1.

(I) WT and *Nlrx1*$^{-/-}$ BMDMs were transfected by ISD, VACV 70-mer, or cGAMP. IFNβ and *Ifna4* transcript were measured 3 hr after transfection.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

(J) Peritoneal macrophages isolated from WT and *NLRX1*$^{-/-}$ mice were transfected with ISD, VACV 70-mer, or c-di-GMP, respectively. IFNβ was assessed 4 hr after transfection. Data are presented as the mean ± standard error of the mean (SEM). $*p < 0.05$, $**p < 0.01$ (Student's t test). All data are representative of at least three independent experiments.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

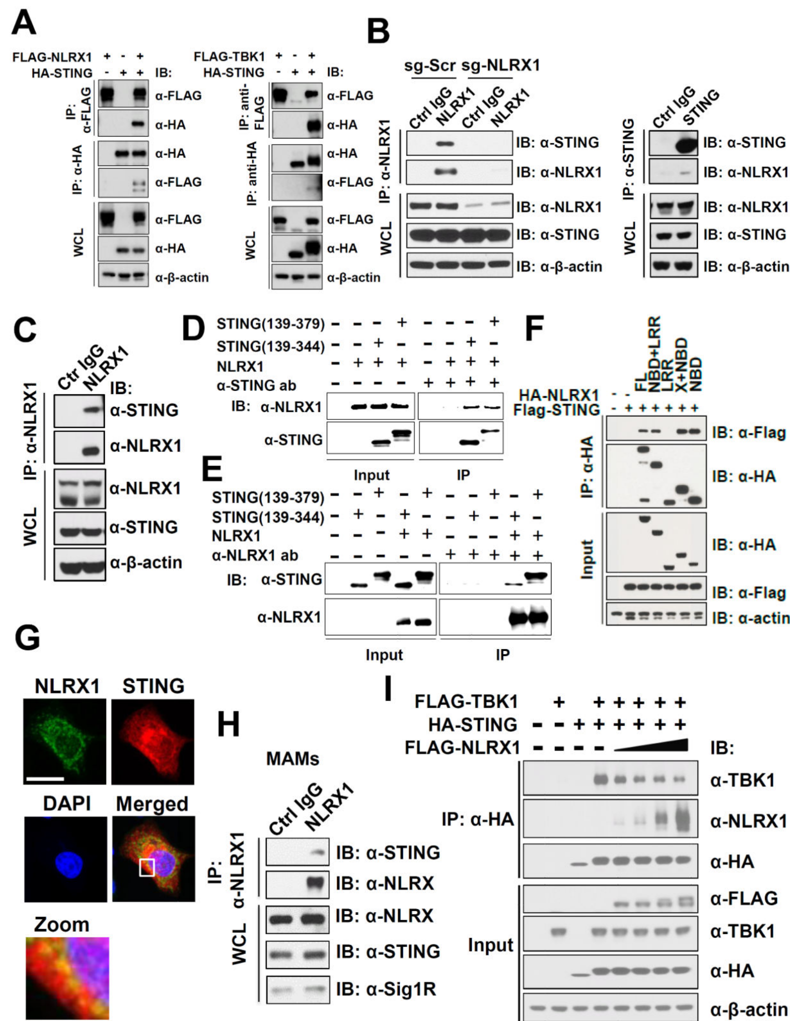


Figure 4. NLRX1 disrupts STING-TBK1 interaction

(A) Association of over-expressed NLRX1 and STING (left) and of overexpressed STING and TBK1 (right) in HEK293T cells. FLAG-NLRX1 and HA-STING was singly expressed or co-expressed in HEK293T cells. Co-immunoprecipitaion was performed with anti-FLAG beads or anti-HA beads respectively. Similarly, FLAG-TBK1 and HA-STING was singly expressed or co-expressed in HEK293T cells. Co-immunoprecipitaion was performed with anti-FLAG beads or anti-HA beads respectively.

(B) Determination of endogenous NLRX1-STING association in THP-1 cells with scrambled sgRNA (sg-Scr) or sgRNA for NLRX1 (sg-NLRX1). Immunoprecipitation was performed by using anti-NLRX1, anti-STING or control IgG.

(C) Determination of endogenous NLRX1-STING association in primary MEF. Immunoprecipitation was performed by using anti-NLRX1 or control IgG.

(D and E) Recombinant NLRX1 and STING in vitro binding assay was performed by using antibodies against STING (D) and NLRX1 (E). Immunoblots were performed with the antibodies indicated.

(F) Mapping of NLRX1 domains involved in interaction with STING. FLAG tagged full length NLRX1, NLRX1 lacking X domain (NBD+LRR), NLRX1 LRR, NLRX1 lacking

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

LRR domain (X+NBD), and NLRX1 NBD was expressed in the absence or presence of HA-STING in HEK293T cells. Immunoprecipitation was performed by anti-HA beads.
(G) Partial co-localization of NLRX1 and STING. FLAG-NLRX1 and HA-STING was co-expressed in HEK293T cells and immunofluorescence staining was performed. Pictures were taken using confocal microscopy. Scale bar equals to 20 μm.
(H) Determination of endogenous NLRX1-STING association in MAMs isolated from human foreskin fibroblast (HFF) cells. Immunoprecipitation was performed by using anti-NLRX1 or control IgG. Sig1R was used as the MAMs marker.
(I) NLRX1 attenuates STING-TBK1 interaction. STING and TBK1 were co-expressed in HEK293T cells with increasing dose of NLRX1. Immunoprecipitation was performed with anti-HA beads.
All data are representative of at least three independent experiments.
See also Figure S3-S7.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

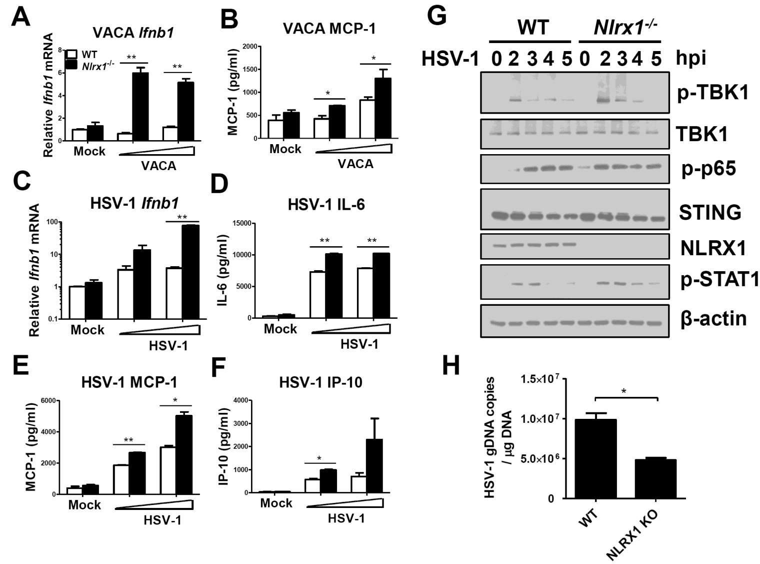


Figure 5. *Nlrx1* deficiency causes enhanced innate immune response to DNA virus infection

(A and B) WT and *Nlrx1*$^{-/-}$ MEFs were infected with vaccinia virus (VACA) (MOI=1 or 5), *Ifnb* transcript (E) and MCP-1 (F) were assessed.

(C-F) WT and *Nlrx1*$^{-/-}$ MEFs were infected with HSV-1 (MOI=0.1 or 1), *Ifnb* transcript (C), IL-6 (D), MCP-1 (E), and IP-10 (F) were assessed.

(G) Immunoblot of phosphorylated (p-) TBK1, TBK1, (p-) STAT1, (p-) p65, STING, and NLRX1 in lysates of WT and *Nlrx1*$^{-/-}$ BMDMs infected with HSV-1 (MOI=1) for indicated time points. β-actin was used as the loading control.

(H) WT or *Nlrx1*$^{-/-}$ BMDMs were infected with HSV-1 (MOI=0.1) and HSV-1 genome copies were quantified by qPCR 24 hr post infection.

Data are presented as the mean ± standard error of the mean (SEM). *p < 0.05, **p < 0.01 (Student's t test).

Data in A-F are from one experiment and data in G and H are representative of at least three independent experiments.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

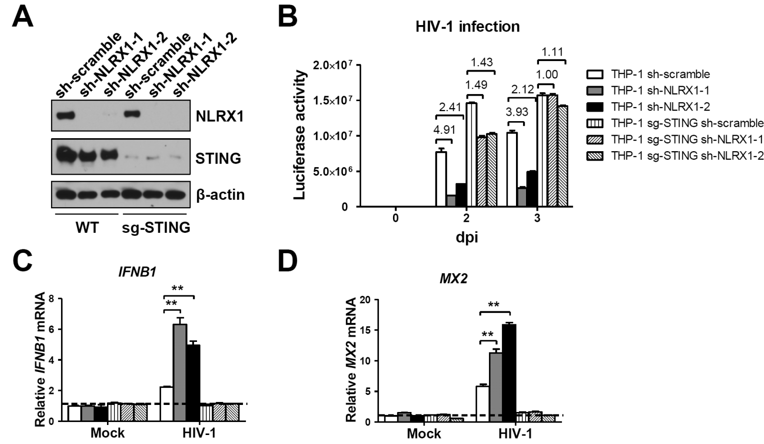


Figure 6. NLRX1 promotes HIV-1 infection in a STING-dependent manner

(A) NLRX1 and STING expression were attenuated by transducing THP-1 cells with lentivirus expressing shRNA for *NLRX1* and Cas9 nuclease plus sgRNA targeting *STING*. Attenuation of NLRX1 and STING expression were confirmed by immunoblot.

(B) THP-1 cells containing scrambled shRNA, shRNA for *NLRX1*, or both shRNA for *NLRX1* and sgRNA for *STING* were infected with HIV-VSV, MOI=1, luciferase activity was determined 2 and 3 dpi. Fold differences between indicated groups were labeled on top of each group.

(C and D). Same as (B) except that *IFNB1* (C) and *MX2* (D) mRNA levels were determined by qPCR 16 hpi.

Data for luciferase and qPCR are presented as the mean ± standard error of the mean (SEM). *p < 0.05, **p < 0.01 (Student's t test).

Data are representative of at least three independent experiments.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

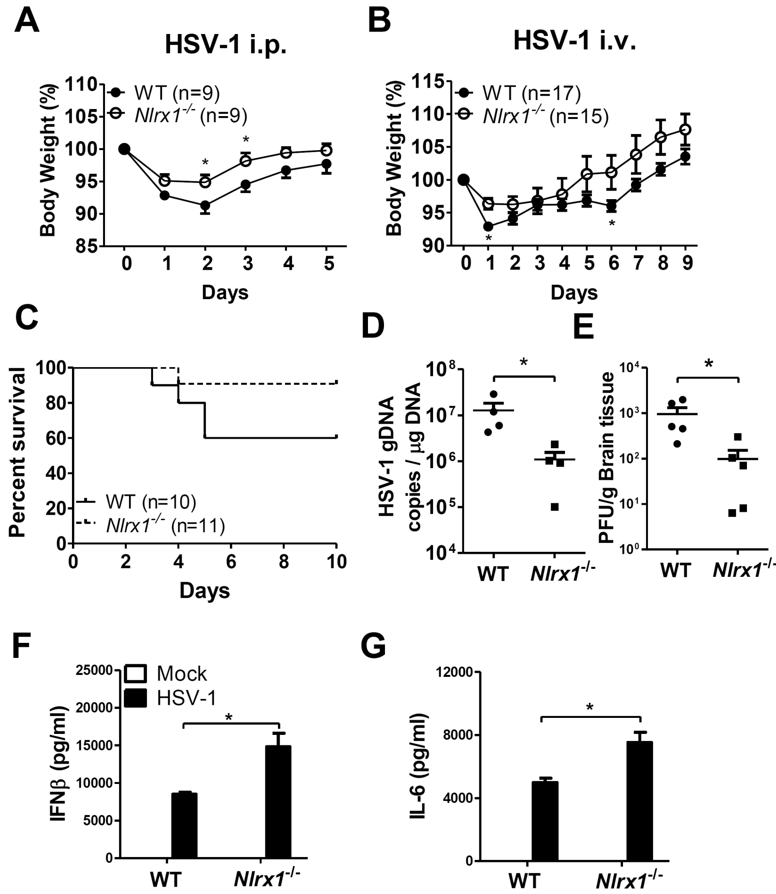


Figure 7. *Nlrx1* deficient mice are more resistant to HSV-1 infection

(A) WT and *Nlrx1*$^{-/-}$ mice were infected i.p. with HSV-1 (2×10^7 pfu) and body weight was monitored.

(B-G) WT and *Nlrx1*$^{-/-}$ mice were infected i.v. with HSV-1. Body weight (B), percentage of survival (C), HSV-1 genomic DNA copies (D), viral titers in the brain (E), IFNβ (F) and IL-6 (G) in the serum were assessed. In B, D, E, F and G, the dose of infection is 2×10^7 pfu/mouse and in C, the dose of infection is 5×10^7 pfu/mouse. *$p < 0.05$ (Student's t test or Mann-Whitney U test). Data are representative of at least two independent experiments.