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Toll-Like Receptor 3 Signaling Inhibits Simian Immunodeficiency Virus Replication in Macrophages from Rhesus Macaques

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Abstract

Toll-like receptor 3 (TLR3) recognizes double-stranded RNA and induces multiple intracellular events responsible for innate antiviral immunity against viral infections. Here we demonstrate that TLR3 signaling of monocyte-derived macrophages (MDM) from rhesus monkeys by poly I:C inhibited simian immunodeficiency virus (SIV) infection and replication. Investigation of the mechanisms showed that TLR3 activation resulted in the induction of type I and type III interferons (IFNs) and IFN-inducible antiviral factors, including APOBEC3G (A3G), tetherin and SAMHD1. In addition, poly I:C-treated macaque macrophages expressed increased levels of CC chemokines including CCL3, CCL4 and CCL5, the ligands for HIV or SIV coreceptor CCR5. Furthermore, TLR3 signaling of macaque macrophages induced the expression of cellular microRNAs (miR-29a, -29b, -146a and -9), the newly identified intracellular SIV restriction factors. TLR3 activation-mediated anti-SIV effect could be compromised by the knockdown of IRF3 and IRF7. These findings indicate that TLR3-mediated induction of multiple viral restriction factors contribute to the inhibition of SIV infection in macaque macrophages, which support future preclinical studies using rhesus macaques to determine whether *in vivo* TLR3 activation is safe and beneficial for treating people infected with HIV.

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Authorship

Contribution: M.S. and WZ.H. conceived and designed the experiments; M.S., JB.L., and M.D. performed the experiments; M.S., JG.W. and WZ.H. analyzed the data; JB.L. and M.D. contributed to reagents, materials and analysis tools; and M.S., JG.W. and WZ.H. wrote the paper.

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Keywords

Rhesus macaques; Toll-like receptor 3 (TLR3); Simian immunodeficiency virus (SIV); CC chemokine; Poly I:C; Tetherin; Interferon; microRNA

1. Introduction

Innate immunity plays a crucial role in the control of viral infections, including HIV (Deeks and Walker, 2007). Macrophages are a major components of innate immune system (Gordon and Taylor, 2005). The importance of macrophages in the pathogenesis of HIV infection is highlighted by their dual roles in HIV infection, particularly as mediators of the host anti-HIV immune responses and as targets for HIV. As HIV can persist in macrophages, it is believed that macrophages are an important virus reservoir and contribute to viral latency (Aquaro et al., 2002; Koppensteiner et al., 2012). Macrophages are the most important target of HIV in the central nervous system. Circulating cells from the monocyte/macrophage lineage become infected and traffic to the brain where they induce cytokine signaling and infect other macrophage lineage cells and astrocytes (Barber et al., 2004; Roberts et al., 2003). Macrophages mount broad antiviral responses through producing CC chemokines and antiviral cytokines, including type I interferons (IFNs). Several studies (Baca-Regen et al., 1994; Barr et al., 2008; Liu et al., 2012) have shown that exposure of macrophages to IFNs results in HIV inhibition at several steps of the viral replication cycle. However, the precise mechanisms of IFN-mediated intracellular anti-HIV response in macrophages remain to be determined.

In order to suppress and eliminate HIV in its reservoirs, such as macrophages, it is crucial to identify and activate innate immune factors within virus reservoirs. Induction of the intracellular antiviral innate immunity depends on a family of innate immune receptors, such as Toll-Like Receptors (TLRs) (Delneste et al., 2007; Iwasaki, 2012; Janeway and Medzhitov, 2002). Engagement of TLRs activates signaling cascades that culminate in inflammatory and immune defense responses (Akira et al., 2006). Among the 11 identified mammalia TLRs (Kabelitz and Medzhitov, 2007), TLR3 has been recognized as a major receptor in virus-mediated innate immune responses (Takeuchi and Akira, 2009). The principal TLR3-expressing cells include peripheral leukocytes, such as dendritic cells (Kadowaki et al., 2001), CD8+ T cells (Tabiasco et al., 2006) and NK cells (Schmidt et al., 2004), and monocyte-macrophage (Chattergoon et al., 2014; Zhou et al., 2013; Zhou et al., 2010). TLR3 specifically senses double-stranded RNA (dsRNA) (Alexopoulou et al., 2001), a common intermediate of viral replication. TLR3 activation induces production of IFN- $\alpha/\beta/\lambda$ and the cellular antiviral factors (Abrahams et al., 2006; Trapp et al., 2009; Wang et al., 2009b). Activation of TLR3 has been shown to inhibit a number of viral infections, including herpes simplex virus-1 (Zhou et al., 2009), West Nile virus (Daffis et al., 2008), hepatitis C virus (Broering et al., 2008), influenza virus (Lau et al., 2009) and HIV (Zhou et al., 2010). TLR3 has also been studied in SIV infection, showing increase of TLR3 expression in lymph nodes (Sanghavi and Reinhart, 2005). In light of extensive use of SIV infection of rhesus macaques as a non-human primate (NHP) model for HIV/AIDS research, we examined whether TLR3 signaling by poly I:C can inhibit SIV infection of macaque

macrophages. We also examined the mechanisms involved in TLR3-mediated anti-SIV activity in macaque macrophages.

2. Materials and methods

2.1. Macaque macrophage preparation

Peripheral blood was obtained in heparinized vacutainer collection tubes from healthy rhesus macaques of Chinese origin at the Center for Animal Experiment (Wuhan University School of Medicine) under animal care procedures according to Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of rhesus macaques by Ficoll gradient centrifugation. Macaque PBMCs were plated in 48-well-plate (3×10^6 cells/well) or 96-well-plate (0.8×10^6 cells/well) Cell BIND culture dishes (Corning, USA) with RPMI1640 containing 2% autologous serum, 100 U/ml penicillin (Gibco), 100 μ g/ml streptomycin (Gibco) and 1% non-essential amino acids (Gibco) for 24h. After the removal of non-adherent cells from the cultures, the monocytes were cultured with RPMI1640 containing 2% autologous serum for 7 days when monocytes differentiated into macrophages. Macaque monocytes cultured with RPMI1640 containing 2% autologous serum for 4 days (Fig. 1A) or 7 days (Fig. 1B) showed typical macrophage morphology, which was confirmed by CD14 staining with purity of day 4 (Fig. 1C) and day 7 (Fig. 1D) were 91.3 % and 96.5 %, respectively. Viability of the cells cultured for 4 and 7 days exceed 96.3 % and 95.8 % respectively. Poly I:C treatment had little effect on cell viability (supplemental Fig. 1).

2.2. SIV infection of macaque macrophages and poly I:C treatment

The SIV and simian-human immunodeficiency virus (SHIV) strains (SIVmac251, SHIV KU-1) were obtained from the AIDS Research and Reference Program of the National Institutes of Health (Bethesda, MD). High molecular weight poly I:C was purchased from InvivoGen (Diego, CA). Cultured macaque macrophages were incubated with or without poly I:C (1 or 10 μ g/ml), either 12 hr before or 72 hr after SIV or SHIV infection. The cells were infected with an equal amount (10^3 TCID₅₀) of cell-free SIV strains (SIVmac251) or SHIV strain (SHIV KU-1) for 2 hr at 37°C. The cells were washed three times with RPMI-1640 to remove input viruses, and fresh medium without poly I:C was added to the cultures. The final wash was tested for detection of SIV GAG by real-time RT-PCR and shown to be free of residual viruses. The cells were incubated for 7 days, and culture supernatant was harvested for SIV or SHIV RNA measurement by the real-time RT-PCR.

2.3. Real-time RT-PCR

Total cellular or viral RNA was extracted from cells or supernatant using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Total RNA (1 μ g) was subjected to the reverse transcription using reagents obtained from Promega (Madison, WI). The real-time RT-PCR for the quantification of mRNAs for IFN- α , IFN- β , IFN- λ_1 , apolipoprotein B mRNA-editing catalytic polypeptide-like 3 G (A3G), tetherin, sterile alpha motif and histidine-aspartic domain containing protein 1 (SAMHD1), tripartite motif 5a (TRIM5 α), myxovirus resistance protein A (MxA), the interferon-stimulated gene 56 (ISG56), 2'-5'-oligoadenylate synthetase 1 (OAS-1), viperin, CCL3, CCL4, CCL5, IFN regulatory factor 1 (IRF1), IRF3,

IRF5, IRF7, IRF9, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were performed with the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) as described previously (Guo et al., 2004). The real-time RT-PCR for the quantification of SIV GAG was carried out with Script SYBR Green PCR Kit (Qiagen, Germany). The specific oligonucleotide primers used in this study are listed in supplemental Table 1 and Table 2. The oligonucleotide primers were synthesized by Integrated DNA Technologies Inc. (AnyGene, Wuhan). The levels of GAPDH mRNA were used as an endogenous reference to normalize the quantities of target mRNA. Target gene mRNA expression levels were calculated from delta cycle threshold (ΔC_t) values and reported as the fold increase ($FI = 2^{-\Delta C_t}$) of the mRNA levels in treated samples compared to those untreated.

2.4. Western Blot

Total cell lysates of macaque macrophages treated with poly I:C 24 hr were prepared by using the cell extraction buffer (Invitrogen) with 1% protease inhibitor cocktail (Sigma, MO) according to the manufacturer's instructions. Equal amounts of protein lysates (20 μ g) were separated on 4% to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis precast gels and transferred to a polyvinylidene difluoride membranes (Millipore, Germany). The blots were incubated with primary antibodies in 2% BSA in phosphate-buffered saline with 0.05% Tween 20 (PBST) overnight at 4°C (IRF1, 1:3000; IRF3, 1:2000; IRF5, 1:2000; IRF7, 1:2000; IRF9, 1:2000; MxA, 1:3000; ISG56, 1:3000; tetherin, 1:1000; A3G, 1:2000; GAPDH, 1:5000). Antibodies against IRF1, 3, 5 were purchased from Cell Signaling Technology (Danvers, MA), IRF7 from Santa Cruz Biotechnology (Dallas, TX), IRF9, MxA, A3G and GAPDH from Abcam (Cambridge, MA), tetherin from Acris (San Diego, CA). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG), anti-goat IgG and anti-mouse IgG were diluted at 1:5000 to 1:8000 in 2% nonfat milk PBST. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

2.5. Flow Cytometry

For detection of cell surface marker CD14 expression, four- or seven-day cultured monocyte/macrophage were detached from the plate bottom by incubated with 0.25% trypsin for 5 min, the cells were harvested and washed twice with phosphate-buffered saline containing 1% fetal bovine serum, incubated with Percp-cy5.5-conjugated anti-rhesus CD14 (BD, Biosciences, CA) on ice for 20 min after fixation and permeabilization. For detection of intracellular expression of CCL3, CCL4 and CCL5, cultured macrophages (5×10^5 cells/well in 48-well plates) were treated with or without poly I:C (10 μ g/ml) for 12 hr. Cells were then harvested and washed twice with phosphate-buffered saline containing 1% fetal bovine serum, incubated with PE-conjugated anti-rhesus CCL3 (BD, Biosciences, CA), PE-CyTM7-conjugated anti-human CCL4 (BD, Biosciences, CA) and Alexa Fluor 647-conjugated anti-rhesus CCL5 (BioLegend, San Diego) on ice for 20 min after fixation and permeabilization. Unstained or isotype-matched immunoglobulin G-stained cells were included in parallel as a negative control. Stained cells were analyzed using an (FACS Verse; BD Bioscience, CA). The data was analyzed using FLOW-JO software (Tree Star Inc., Ashland, OR).

2.6. MicroRNA detection

To conduct microRNA (miRNA) detection, total cellular RNA, including miRNA, was extracted from cells using the miRNeasy Mini Kit from Qiagen (Valencia, CA). Total RNA (1 µg) was reverse-transcribed with a miScript Reverse Transcription Kit from Qiagen. The real-time RT-PCR for the quantification of a subset of miRNAs (miR-29a, -29b, -146a and -9) was carried out with miScript Primer Assays and miScript SYBR Green PCR Kit from Qiagen. The levels of GAPDH mRNA were used as an endogenous reference to normalize the quantities of miRNAs.

2.7. Knockdown of IRF3 and IRF7

Lentiviral plasmids with sequence-verified shRNA for gene silencing of IRF3 and IRF7 were gifts from Dr. Rongtuan Lin (McGill University). Macaque macrophages were cultured in 48-well plates at 5×10^5 per well. The cells were then transfected with the plasmids using lipofectamine 2000 (Invitrogen) at a ratio 1:3 (µg:µl) prepared in Opti-Mem. After 48 hr, cells were used for poly I:C treatment or subsequent experiments. For negative knockdown, cells were transfected with the nontarget shRNA control vector pLKO.1 (SHC002, Sigma).

2.8. Cell viability assay

The cell viability of macaque macrophages cultured in 96-well-plate treated with or without poly I:C (1, 10, 40, 100 µg/ml) for 72h were measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Measurements were corrected for background (culture medium + MTS reagent) and scaled to positive control wells (untreated cells + MTS).

2.9. Statistical analysis

Where appropriate, data were expressed as mean $\pm$ standard deviation of the mean (mean $\pm$ SD) of triplicate samples. Data presented in the figures represent the average values from triplicate wells. All experiments were repeated for at least three times using monocytes from different animals. For comparison of the mean of two groups (treated versus untreated), statistical significance was assessed by Student's t-test. If there were more than two groups, one-way repeated measures of analysis of variance were used. Statistical analyses were performed with GRAPHPAD INSTAT STATISTICAL SOFTWARE (GraphPad Software Inc., San Diego, CA). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. TLR expression and regulation in macaque macrophages

Although it has been reported that human immune cells, including macrophages, express TLR-1 to -10 (Akira et al., 2006; Zhou et al., 2010), we know little about TLR expression in macaque macrophages, particularly their functionality. Thus, we investigated whether poly I:C could regulate the expression of TLRs in macaque macrophages. Poly I:C-treated macaque macrophages showed the up-regulation of TLR2, TLR3 and TLR7 by three-, six- and two-fold, respectively (Fig. 1E).

3.2. TLR3 signaling of macaque macrophages inhibits SIV or SHIV replication

To investigate the effect of TLR3 activation on SIV or SHIV replication in macaque macrophages, the cells were treated with or without poly I:C before or after infection with SIV strain (SIVmac251) or SHIV strain (SHIV KU-1). The cells were then incubated for 7 days, and cultured supernatants were harvested for SIV or SHIV GAG gene expression by the real-time RT-PCR. As shown in Fig. 2, a single pre-treatment of macaque macrophages with poly I:C at a dose of 1 or 10 µg/ml for 12 hr resulted in significant suppression of infection with either SIVmac251 (Fig. 2A) or SHIV KU-1 (Fig. 2B). The duration of this protective effect on macrophages was up to 7 days (Fig. 2). In addition, poly I:C treatment of macaque macrophages after SIV or SHIV infection also significantly suppressed viral replication (Fig. 2).

3.3. TLR3 signaling of macaque macrophages induces type I and III IFN expression

Since TLR3 activation triggers intracellular signaling, resulting in the production of chemokines and antiviral cytokines, including type I and type III IFNs (Akira et al., 2006; Alsharifi et al., 2008), we next examined whether endogenous IFN- $\alpha/\beta/\lambda$ expression was induced in poly I:C-treated macaque macrophages. We found that poly I:C treatment dose-dependently induced the expression of IFN- α/β (Fig. 3A) and IFN- λ (Fig. 3B) in macaque macrophages. To determine the mechanism of the effect of TLR3 activation on IFN- $\alpha/\beta/\lambda$ expression, we then examined whether the TLR3 activation could induce the expression of IFN regulatory factors (IRFs). We showed that poly I:C-treated macaque macrophages expressed higher mRNA levels of IRFs (IRF1, 3, 7 and 9) (Fig. 3C) and protein levels of IRF1, IRF3 and IRF7 (Fig. 3D) than untreated macaque macrophages.

3.4. Poly I:C induces the expression of IFN inducible antiviral factors

To understand the molecular mechanism(s) by which TLR3 signaling of macaque macrophages inhibits SIV infection, we examined the expression of several type I IFNs stimulated genes (ISGs) and important intracellular HIV/SIV restriction factors (MxA, ISG56, tetherin, A3G, OAS-1, viperin, SAMHD1 and TRIM5 α) in poly I:C-treated macaque macrophages. These factors are known to have the ability to inhibit viral replication within cells. We observed that poly I:C-treated macaque macrophages had higher levels of MxA, ISG56, tetherin and A3G) at both mRNA (Fig. 4A) and protein (Fig. 4B) levels. This effect of poly I:C on those antiviral factors was dose-dependent (Fig. 4A, 4B). The expression of OAS-1, viperin, SAMHD1 and TRIM5 α was also dose-dependently induced in macaque macrophages at mRNA levels by poly I:C (Fig. 4C).

3.5. TLR3 activation induces CC chemokine expression

Unlike HIV, both M-tropic and T-tropic SIV strains exclusively use CCR5 but not CXCR4 as the entry coreceptor (Hill et al., 1997; Riddick et al., 2010). The natural ligands for CCR5, including the CC chemokines CCL3, CCL4 and CCL5, are known to play a contributing role in TLR3-mediated inhibition of HIV infection of human macrophages (Proost and Schols, 2002). We therefore investigated whether poly I:C treatment of macaque macrophages has a positive impact on the expression of these CC chemokines. As shown in

Fig. 5, poly I:C treatment of macaque macrophages enhanced the expression of CCL3, CCL4 and CCL5 at both mRNA (Fig. 5A) and protein (Fig. 5B) levels.

3.6. TLR3 activation induces the expression of intracellular anti-SIV miRNAs

Recently, four anti-SIV miRNAs (miR-29a, -29b, -146a and -9) were documented to have the ability to decrease SIV replication in primary macaque macrophages (Sisk et al., 2013), as they could bind to the SIV Nef/U3 and R regions. We therefore examined whether these miRNAs contribute to TLR3 signaling-mediated inhibition of SIV replication in macaque macrophages. As shown in Fig. 6, out of these four anti-SIV miRNAs, miR-29a, -29b, -146a and -9 were up-regulated in poly I:C-treated macaque macrophages.

3.7 TLR3-Mediated anti-SIV effect is partially regulated by IRF3 and IRF7

The IFN regulatory factors (IRFs), especially IRF3 and IRF7, have been implicated in the control of type I IFN expression. To determine the role of IRF3 and IRF7 in the mechanism for the anti-SIV effect of TLR3 activation, we demonstrated a cell model of shRNA-mediated knockdown of IRF3 and IRF7 in macaque macrophage. Seven-day-cultured macaque macrophages were transfected with control vector, IRF3 shRNA, or IRF7 shRNA. IRF3 and IRF7 were knockdown at 48 hr post-transfection (Supplemental Fig. 2). As above-mentioned, poly I:C treatment of macaque macrophage induced the expression of IRF3 and IRF7 in a dose-dependent fashion (Fig. 3C, 3D). To determine the role of IRF3 and IRF7 in TLR3-activation-mediated IFN- $\alpha/\beta/\lambda$ expression, we examined whether the knockdown of IRF3 and IRF7 could compromise the action of poly I:C treatment on the activation of IFN- $\alpha/\beta/\lambda$. Interestingly, either IRF3 or IRF7 knockdown attenuated IFN- α/β (Fig. 7A) and IFN- λ (Fig. 7B) expression. Furthermore, knockdown of IRF3 or IRF7 compromised anti-SIV effect of TLR3 activation in either a single pre-treatment (Fig. 7C) or post-treatment (Fig. 7D) with poly I:C.

4. Discussion

TLR3 recognizes various forms of dsRNA, including viral dsRNA. Therefore, TLR3 is an important intracellular nucleic acid sensor and initiates antiviral signaling pathways in the host, including macrophages (Akira and Takeda, 2004; Alexopoulou et al., 2001). In this study, we show that TLR3 activation by a dsRNA analogue, poly I:C, resulted in the inhibition of SIV or SHIV infection of macaque macrophages. This poly I:C-mediated antiviral effect is highly potent, as SIV or SHIV infection was almost abolished by poly I:C either pre-treatment or post-treatment of macaque macrophages (Fig. 2). Investigation of the mechanisms showed that the TLR3 signaling of macaque macrophages triggers potent antiviral activities against SIV through the induction of multiple antiviral cellular factors. Type I IFNs (IFN- α/β) are the first line of the TLR3 activation-mediated antiviral response (Akira and Takeda, 2004). IFN- α/β not only have the ability to inhibit HIV but also SIV replication in macrophages (Abel et al., 2002). A strong type I IFN response to TLR3 activation is critical for the production of down-stream antiviral mediators including IFN-inducible genes, such as MxA, ISG56 and OAS-1 (Fig. 4A, 4B, 4C). Therefore, poly I:C-mediated induction of IFN- α/β should be accounted for the anti-SIV effect of TLR3 action in macaque macrophages. In addition to IFN- α/β , IFN- λ was also induced by poly I:C in

macaque macrophages. IFN- λ has been demonstrated to have the ability to inhibit a number of viruses, including HIV (Hou et al., 2009; Liu et al., 2012). The induction of IFN- $\alpha/\beta/\lambda$ could be due to the result of TLR3 activation-mediated IRF induction in macaque macrophages. We showed that several IRFs, particularly IRF7, were induced in poly I:C-treated macaque macrophages (Fig. 3C, 3D). IRF7 plays a key role in activating type I IFNs (Honda et al., 2005). We demonstrated that IRF3 or IRF7 knockdown attenuated the expression of IFN- α/β (Fig. 7A) and IFN- λ (Fig. 7B). In addition, the knockdown of IRF3 or IRF7 compromised TLR3 activation-mediated anti-SIV effect (Fig. 7C and 7D). These findings indicate that IRF3 and IRF7 contribute to TLR3 activation-mediated SIV inhibition in macaque macrophages.

Our further investigation demonstrated that several newly identified HIV or SIV restriction factors, including tetherin (BST2), TRIM5 α , A3G and SAMHD1, were induced in poly I:C-treated macaque macrophages (Fig. 4A, 4B, 4C). Tetherin is a trans-membrane protein that specifically inhibits HIV or SIV infection by preventing virus release from infected cells (Neil et al., 2008; Ruiz et al., 2010). TRIM5 α is a potent retrovirus inhibitor that targets the viral capsid and disrupts the structure of assembled HIV capsid complexes (Black and Aiken, 2010). SAMHD1 has the ability to block the infection of HIV before their reverse transcription in resting CD4⁺ T cells (Baldauf et al., 2012), dendritic cells and macrophages (Hrecka et al., 2011; Laguette et al., 2011). In addition to intracellular factors, we also examined the expression of CC chemokines (CCL3, CCL4, CCL5) in poly I:C-treated macaque macrophages, as SIV also uses CCR5 coreceptor together with CD4 for entry into target cells. The CC chemokines hamper the entrance of SIV strains that use CCR5 coreceptor by competitive blocking. We found that TLR3 activation facilitated the expression of CC chemokines (Fig. 5).

Among a growing list of innate cellular factors that impair various steps of HIV life cycle, increasing evidence indicates that many cellular miRNAs participate in host innate immunity against viral infections. Several cellular miRNAs have been identified to potentially target a set of accessory genes of HIV (Kumar, 2007; Lecellier et al., 2005). It was reported that miRNAs (miR-28, -125b, -150, -223 and -382) could target the 3'-untranslated region of HIV-1 transcripts that are responsible for viral latency in resting CD4⁺ T cells (Huang et al., 2007). We (Wang et al., 2009a) showed that the differential levels of these anti-HIV miRNAs in human monocytes and macrophages contribute to the susceptibility of cells to HIV infection. These miRNAs play a crucial role in TLR3-mediated suppression of HIV replication in human macrophages (Zhou et al., 2010). More recently, it was reported (Sisk et al., 2013) that four cellular miRNAs (miR-29a, -29b, -146a and -9) could directly bind to the U3 region of the SIV RNA, suppressing SIV replication in infected primary macaque macrophages. Therefore, we investigated whether TLR3 activation could modulate the expression of the anti-SIV miRNAs in macaque macrophages. We found that poly I:C treatment induced the expression of anti-SIV miRNAs, which provides an additional mechanism for TLR3 activation-induced anti-SIV action in macaque macrophages. This poly I:C-mediated induction of anti-SIV miRNAs could be the result of IFN activation, as both IFN- α and IFN- β are the potent inducers of anti-HIV miRNAs (Zhou et al., 2010).

Collectively, the observations described above indicate that TLR3 signaling of macaque macrophages inhibits SIV replication through multiple antiviral mechanisms at both entry and transcription levels. Although additional mechanism(s) might also be involved, the induction of multiple cellular restriction factors against HIV or SIV should account for much of the TLR3-mediated anti-SIV activity. There are both extracellular and intracellular factors involved in TLR3 signaling-mediated anti-SIV activities: the induction of extracellular factors, CC chemokines that block SIV entry into macrophages; and the activation of intracellular viral restriction factors, such as A3G, tetherin, MxA, ISGs and anti-SIV miRNAs. These antiviral mechanisms of TLR3 activation offer an attractive alternative for HIV treatment, as it would be extremely difficult for HIV to develop resistance to the actions that directly inhibit the virus at different steps of its replication cycle. Therefore, these findings are clinically important, indicating that the activation of the TLR3 signaling pathway may represent a promising novel strategy for treatment of people with HIV infection. Currently, the therapeutic TLR agonists are being developed for the treatment of viral infections. Agonists for TLRs, particularly TLR3, TLR7 and TLR9, have been shown to offer promising treatment against infectious diseases, especially viral infections, including HIV (Averett et al., 2007; Buitendijk et al., 2014; Kanzler et al., 2007). Because of its ability to induce innate antiviral immunity, poly I:C has been used in clinical or preclinical trials of HIV therapy (Kanzler et al., 2007). However, TLR3 activation can induce the production of pro-inflammatory cytokines, which may attenuate antiviral effects. Because of this concern, significant efforts have been taken to develop therapeutic TLR agonist for treatment of viral infections, including HIV (Kanzler et al., 2007). Obviously, the findings of this study support the notion for further developing a TLR3 agonist-based treatment against HIV disease, in which host cell innate immune responses are significantly compromised by the virus. However, *in vivo* studies using SIV-infected macaques are necessary in order to validate the potential of TLR3 agonists for the treatment of people infected with HIV. The challenge has been to focus these inflammatory actions for optimal clinical benefit, with minimal unwanted inflammation and toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

A3G, APOBEC3G	apolipoprotein B mRNA-editing enzyme-catalytic polypeptide 3 G
ART	anti-retroviral therapy
CCL	CC chemokine

CCR5	CC chemokine receptor type 5
CNS	central nervous system
dsRNA	double-strand RNA
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IFN	interferon
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
miRNA	microRNA
MxA	myxovirus resistance A
NHP	non-human primate
OAS	2'-5'-oligoadenylate synthetase
PBMC	peripheral blood mononuclear cell
SAMHD1	sterile alpha motif and histidine-aspartic domain containing protein 1
SHIV	simian-human immunodeficiency virus
SIV	simian Immunodeficiency Virus
TCID50	Tissue Culture Infectious Dose 50%
TLR	Toll-like receptor
TRIM5α	tripartite motif containing 5 alpha

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Highlight

1. TLR3 signaling of macaque macrophages inhibited SIV replication.
2. TLR3 activation induced expression of multiple antiviral factors, including newly identified SIV restriction miRNAs.
3. TLR3 signaling of macrophages induced the expression of CC-chemokines, the entry inhibitors of HIV.
4. These findings support future preclinical studies on host innate immunity-based HIV therapy using rhesus macaques.

Fig. 1. Phenotype of macaque macrophage and effect of poly I:C on TLRs expression in macaque macrophages. (A, B) Macaque PBMCs were plated in 48-well-plate (3×10^6 cells/well) for 24h. After the removal of non-adherent cells from the cultures, the cells were cultured in RPMI 1640 supplemented with 2% autologous serum for four (A) or seven (B) days. (C, D) Flow analysis of cell surface marker CD14 expression of four- (C) or seven- (D) day-cultured macaque macrophage. Data (A, B, C, D) represent one representative experiment out of three independent experiments. (E) Seven-day-cultured macaque macrophages were treated with or without poly I:C (1μg/ml) for 12 hr. Total RNA extracted from cells were

then subjected to the real-time RT-PCR for the mRNA levels of TLR1 to TLR10 and GAPDH. The data are expressed as mRNA levels for TLR1 to TLR10 relative (fold) to the control (without poly I:C treatment, which is defined as 1). The results shown are the mean $\pm$ SD of triplicates in one representative experiment out of three independent experiments. Significant differences compared with cells treated without Poly I:C are denoted by *($P<0.05$) and **($P<0.01$).

Fig. 2.

TLR3 activation suppresses SIV or SHIV infection of macaque macrophages. Seven-day-cultured macaque macrophages were treated with or without poly I:C at the indicated doses (1 or 10 µg/ml), either 12 hr before or 72 hr after infection with (A) SIVmac251 or (B) SHIV KU-1. Culture supernatant collected at day 7 after SIV or SHIV infection was subjected to the real-time RT-PCR for SIV or SHIV GAG RNA copies number. The results shown are the mean $\pm$ SD of triplicate samples in one representative experiment out of three independent experiments. Significant differences compared with cells treated without Poly I:C are denoted by *($P < 0.05$) and **($P < 0.01$).

Fig. 3.

Effect of TLR3 activation on the expression of IFNs and IRFs in macaque macrophages. Seven-day-cultured macaque macrophages were treated with or without poly I:C at the indicated concentrations for 12 hr. Total RNA extracted from cells was then subjected to the real-time RT-PCR for the mRNA levels of IFN- α/β (A), IFN- λ_1 (B), IRF1, IRF3, IRF5, IRF7, IRF9 (C) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data are expressed as mRNA levels relative (fold) to the control (without poly I:C treatment, which is defined as 1). The results shown are the mean $\pm$ SD of triplicate samples in one representative experiment out of three independent experiments. Significant differences compared with cells treated without Poly I:C are denoted by * ($P < 0.05$) and ** ($P < 0.01$). (D) IRF1, IRF3, IRF5, IRF7 and IRF9 protein expression. Total proteins extracted from macaque macrophages cultured in the presence or absence of poly I:C at indicated dose for 24 hr were subjected to Western blot assay using antibodies against IRF1, IRF3, IRF5, IRF7, IRF9 and GAPDH. GAPDH was used as the loading control.

Fig. 4.

Effect of TLR3 activation on ISGs and HIV/SIV restriction factors. Seven-day-cultured macaque macrophages were treated with poly I:C at the indicated doses for 12 hr. Total RNA extracted from cells was then subjected to the real-time RT-PCR for mRNA levels of antiviral factors (MxA, ISG56, tetherin and A3G) (A) and (OAS-1, viperin, SAMHD1 and TRIM5α) (C) expression and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data are expressed as target genes mRNA levels relative (fold) to the control (without poly I:C treatment, which is defined as 1). The results shown are the mean $\pm$ SD of triplicate samples in one representative experiment out of three independent experiments. Significant differences compared with cells treated without Poly I:C are denoted by * ($P < 0.05$) and ** ($P < 0.01$). (B) MxA, ISG56, tetherin and A3G protein expression. Total proteins extracted from macrophages cultured in the presence or absence of poly I:C at indicated doses for 24

hr were subjected to Western blot assay using antibodies against MxA, ISG56, tetherin, A3G and GAPDH. GAPDH was used as the loading control.

Fig. 5.

Effect of TLR3 activation on CC chemokines. Seven-day-cultured macaque macrophages were treated with or without poly I:C at the indicated concentrations for 12 hr. (A) Total RNA extracted from cells was then subjected to the real-time RT-PCR for CCL3, CCL4, CCL5 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data are expressed as CCL3, CCL4 or CCL5 mRNA levels relative (fold) to the control (without poly I:C treatment, which is defined as 1). The results shown are the mean $\pm$ SD of triplicate samples in one representative experiment out of three independent experiments. Significant differences compared with cells treated without Poly I:C are denoted by * ($P < 0.05$) and ** ($P < 0.01$). (B) Macaque macrophages were treated with Poly I:C (10 μ g/ml) for 12hr, and

then stained with the fluorescence-conjugated antibodies and analyzed for intracellular CCL3, CCL4 and CCL5 by flow cytometry. Control (Shaded histogram): cell were stained with isotype-matched antibodies (immunoglobulin G); Poly I:C (Open histogram): treated cells were stained with antibodies against CCL3, CCL4 and CCL5. Histogram graph represent one representative experiment out of three independent experiments.

Fig. 6.

Effect of TLR3 activation on anti-SIV miRNAs expression. Seven-day-cultured macaque macrophages were treated with or without poly I:C at the indicated doses for 4 hr. Total RNA extracted from cells was subjected to the real-time RT-PCR for the cellular miRNAs (miR-29a, miR-29b, miR-146a, miR-9) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data are miRNAs expression levels relative (fold) to the control (without poly I:C treatment, which is defined as 1). The results shown are the mean $\pm$ SD of triplicate samples in one representative experiment out of three independent experiments. Significant differences compared with cells treated without Poly I:C are denoted by * ($P < 0.05$).

Fig. 7.

Effect of knockdown of IRF3 and IRF7 on poly I:C-mediated IFNs expression and anti-SIV activity in macaque macrophages. (A, B) ShRNA-mediated knockdown of IRF3 and IRF7 impaired IFN- $\alpha/\beta/\lambda$ expression induced by poly I:C treatment. Seven-day-cultured macaque macrophages were transfected with control vector, IRF3 shRNA, or IRF7 shRNA. Forty-eight hours post-transfection, cells were treated or not with poly I:C (1 $\mu\text{g}/\text{ml}$) for an additional 12 hr. Total RNA was extracted, and IFN- $\alpha/\beta/\lambda$ and GAPDH mRNA levels were determined by real-time RT-PCR. Data are expressed as the fold compared with the untreated control. The results shown are the mean $\pm$ SD of triplicate samples in one representative experiment out of three independent experiments. Significant differences compared with cells treated without poly I:C are denoted by * ($P < 0.05$) and ** ($P < 0.01$). (C, D) Knockdown of IRF3 and IRF7 compromised poly I:C-mediated anti-SIV activity in macaque macrophages. Seven-day-cultured macaque macrophages were transfected with control vector, IRF3 shRNA, or IRF7 shRNA. Forty-eight hours post-transfection, cells were then treated with or without poly I:C at the dose 1 $\mu\text{g}/\text{ml}$, either 12 hr before (C) or 72 hr after (D) infection with SIVmac251. Culture supernatant collected at day 5 after SIV infection was subjected to the real-time RT-PCR for SIV GAG RNA copies number. The results shown are the mean $\pm$ SD of triplicate samples in one representative experiment out of three independent experiments. Significant differences compared with cells treated without Poly I:C are denoted by * ($P < 0.05$) and ** ($P < 0.01$).