UNIVERSITY OF CALIFORNIA SAN DIEGO

Tat-Vpu Peptide Inducing a Shock Effect on Human Immunodeficiency Virus Type-1 Infected Primary Macrophage Cells and Understanding a Novel NLRP3 Inflammasome Activation Induced by GU-rich Human Immunodeficiency Virus Type-1 ssRNA

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Jonathan Hanna is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Tat-Vpu Peptide Inducing a Shock Effect on Human Immunodeficiency Virus Type-1 Infected Primary Macrophage Cells and Understanding a Novel NLRP3 Inflammasome Activation Induced by GU-rich Human Immunodeficiency Virus Type-1 ssRNA

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Human immunodeficiency virus type-1 (HIV-1) is a global pandemic that has infected millions across the globe and is the precursor to acquired immunodeficiency syndrome (AIDS). Antiretroviral therapy (ART) allows HIV-1 infected individuals to live a full life; however, ART does not remove HIV-1 infected cells, allowing infected cells to become latent. These latent HIV-1 reservoirs cannot only reactivate once ART administration ends, but these latent HIV-1 reservoirs despite producing no replication

competent virus can produce chronic inflammation in an individual leading to an assortment of other health conditions.

A strategy to eradicate latent reservoirs is designed to reactivate HIV-1 from latently infected cells and kill the virus prior to it infecting uninfected bystander cells (often called “shock and kill”). Here, we identify Tat-Vpu peptide as a potential “shock” in the “shock and kill” cure strategy. We show that Tat-Vpu is able to increase HIV-1 viral release in infected macrophages through the LVEM[66] amino acid sequence that is not observed in the tat-scramble.

To analyze the inflammatory pathway induced by HIV-1, we used a GU-rich single stranded RNA (RNA40) derived from the long terminal repeat (LTR) region of HIV-1. We identified that TLR8 is critical in causing “bystander” $CD4^+$ T cells to elicit IL-1β production. We also show a lack of cell death in RNA40 stimulated macrophages, and identify that caspase-8 and RIPK3 are involved in efficient IL-1β release suggesting the involvement of an alternative inflammasome. We also show that potassium efflux is essential in producing an inflammatory response in RNA40 stimulated macrophages. The involvement of TLR8, no pyroptosis, potassium efflux, and involvement of proteins in the alternative inflammasome indicate that a novel inflammatory pathway is involved with the induction of IL-1β by RNA40.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a major global health issue that currently infects ~38 million people across the world [1]. HIV-1 infects human $CD4^+$ T cells which include: $CD4^+$ T lymphocytes, macrophages, dendrocytes, and microglia [2]. After infection with HIV-1, the infection will cause depletion and degradation of mature $CD4^+$ T cells through a number of mechanisms, but most notably through infected $CD4^+$ T cells undergoing apoptosis [3]. Eventually, when the $CD4^+$ T cell total number drops below 200 cells per µL, an individual is considered to have acquired immunodeficiency syndrome (AIDS) [4]. AIDS itself is the major fear of acquiring HIV-1 and does not cause death directly; however, it compromises an individual's immune system and makes the individual more susceptible to death from opportunistic infections [5].

HIV-1 consists of nine genes divided into three subgroups: structural proteins, essential regulatory elements, and accessory proteins. The structural proteins consist of gag, pol, and env. Gag produces the inner structural proteins for viral shape including the caspid protein and matrix protein [6]. Pol produces viral enzymes including: protease, reverse transcriptase, and integrase [7]. Env produces gp160 which is cleaved into gp120 and gp41 which are essential for viral attachment and fusion to a target cell [6]. Essential regulatory elements are important for transcription and translation of HIV-1 which include tat and rev. Tat is a transcription factor that allows RNA polymerase II to generate full HIV-1 mRNAs [8]. Rev remains in the nucleus of the host cell and directs transport of RNA and viral proteins and provide RNA for the newly completed budding HIV-1 virus [9]. These structural proteins and essential regulatory elements are essential for HIV-1 replication but there are also 4 other accessory

proteins that help enhance viral replication. Vif counters the host APOBEC3 innate immune proteins that usually inhibit replication by causing mutations in the virus [10]. Vpu enhances viral replication through removing BST2 and CD4 from the cell surface [11]. Vpr assists in the integration process of HIV-1 RNA into an uninfected cell and arrests the cell cycle in infected cells [12, 13]. Lastly, Nef is produced early in the HIV-1 infection of a cell and downregulates many cell surface receptors including CD4, MHC-I, and BST2 [14]. All these genes have an important function and are used in the HIV-1 life cycle.

HIV-1, a retrovirus within the lentiviral genus, has a complex and efficient life cycle. HIV-1 infects $CD4^+$ cells through a series of steps. Initially HIV-1's envelope consisting of transmembrane protein gp41, and external glycoprotein gp120 binds to a $CD4^+$ receptor on a cell as well as a coreceptor: CXCR4 or CCR5 [15]. Then the virus fuses with the membrane of the cell and releases its viral RNA, reverse transcriptase, integrase, and protease into the cell. HIV-1 RNA undergoes reverse transcription by its own reverse transcriptase enzyme to make viral DNA [16]. The virus then uses integrase to bind to binding factor p75 to allow for HIV-1 DNA to integrate into the cell [17]. Then, HIV-1 transcription is performed with the assistance of HIV-1 Tat protein using the host cells P-TEFb to promote transcription of the virus [18]. After the virus is transcribed and translated, the virus is sent to the cell membrane through the host cells ESCRT pathway to bud out of the cell and infect other $CD4^+$ T cells [19].

Current treatment to stop HIV-1 from completing its life cycle and releasing more viruses into an individual is through the use of combination antiretroviral therapy (ART). The first ART drugs approved were nucleoside reverse transcriptase inhibitors (NRTIs)

due to their ability to reduce viral load and increase survival in HIV-1 infected individuals [20]. Since then, many drugs have been discovered and approved by the FDA to halt the many parts of the HIV-1 life cycle including CCR5 antagonist, fusion inhibitor, non-nucleoside reverse transcriptase inhibitors (NNRTIs), integration inhibitors, and protease inhibitors [21]. Most ART treatments involve two NRTIs as well as a drug from another class to inhibit HIV-1 production from multiple points in its life cycle.

Viral protein U (Vpu) is an HIV-1 accessory protein that is not required in the process of infection but helps enhance viral release from the cell. Vpu is an 81 amino acid transmembrane protein that is translated along with the envelope protein by a Rev-regulated bicistronic mRNA late during infection [22, 23]. Vpu has two main roles: downregulating the CD4 receptor from the cell surface and enhancing the release of virus from the cell [24, 25].

CD4 is a glycoprotein that is the main receptor of HIV and is expressed on the cell surface of $CD4^+$ T lymphocytes and other myeloid cells like macrophages and dendritic cells [26]. CD4 is downregulated upon HIV-1 infection by viral envelope, Nef, and Vpu [27]. Vpu initially binds to the cytoplasmic domain of CD4 in the endoplasmic reticulum of the cell [28]. Then, Vpu's serine 52 and 56 is phosphorylated which leads to Vpu binding to beta-transducin repeat-containing (β-TrCP) E3 ubiquitin protein ligases [29]. The CD4-Vpu-β-TrCP bindings leads to ubiquitination of CD4 which marks it for proteasomal degradation while Vpu avoids degradation and is able to repeat the cycle [30, 31]. Proteasomal degradation of CD4 follows a non-canonical endoplasmic reticulum-associated protein (ERAD) dependent pathway. Vpu blocking of CD4 results in the prevention of "superinfections", enhancement of viral release, reducing HIV-1

infected cell apoptosis, and freeing gp160 from CD4 binding [24, 32]. Although Vpu degrades CD4, Vpu also retains CD4 in the ER through formation of a complex with Env even without the presence of ERAD [32, 33]. Overall, Vpu is seen to downregulate CD4 surface expression via degradation and localization in the ER.

Vpu also antagonizes bone marrow stromal cell antigen 2 (BST2; formerly known as tetherin). BST2 is used as a defense against viral infections. Specifically, BST2 inhibits the release of HIV-1 by BST2 and anchoring itself to the viral envelope to inhibit virus budding and release from the cell [34]. Many viruses have their own proteins to counteract BST2 including HIV-2 envelope and SIV Nef [35]. HIV-1 Vpu can downregulate BST2's antiviral activity by decreasing surface expression of HIV-1 infected cells [36]. This downregulation is almost exclusively seen in HIV-1 group M strain [37]. Vpu degradation pathway of BST2 is very similar to the CD4 degradation pathway. BST2 binds to Vpu which then binds to F-box/WD repeat-containing protein 1A in the trans-golgi network which then undergoes degradation via an endosome-lysosome degradation pathway [38]. Vpu also interacts with LC3C to induce a non-canonical autophagy pathway to remove more BST2 from the cell surface [39]. Though the current method of BST2 removal by Vpu is still yet to be fully understood as it has been seen to occur in many different pathways, Vpu overall decreases the BST2 profile on the cell surface to induce viral release from an infected cell.

Vpu also alters the ion channels within a cell by forming homo-oligomers at the transmembrane domain [40]. Vpu forms ion channels that select for monovalent K^+ cations to lead to the degradation of CD4 in a cell [41]. The ion channels mostly seen by Vpu are K^+ channels that help with cellular depolarization, degradation of potassium

channel subfamily K member 3 (KCNK3; a mammalian K^+ channel), and induce viral release [42, 43]. The interactions of Vpu with KCNK3 causes formations of dysfunctional hetero-multimers to reduce KCNK3 [44]. KCNK3 reduces viral release by removing KCNK3, Vpu allows for increased viral-release during the infection.

Cells with Vpu expression have higher rates of apoptosis following HIV-1 infection. Vpu through the localization and manipulations of β-TrCP also leads to the downregulation of IkB which leads to a decreased activity of NF-κB. NF-κB is known to increase cell proliferation and induce apoptosis [45]. Vpu inhibition of NF-κB induces apoptosis in infected cells [46]. When Vpu is inhibited, HIV-1 infected cells undergo half the amount of apoptosis compared to the Vpu control through lowering the induction of caspase-3 apoptosis [47].

Despite improved treatment, ART does not remove integrated HIV-1 DNA from already infected cells, thus allowing the formation of HIV-1 latent reservoirs that can virally rebound when ART treatment is discontinued [48, 49]. The goal of many researchers has been to combat the latent reservoirs and find a cure for HIV-1. Several cure strategies have shown progress including: bone marrow transplantation, genetic editing, and shock and kill strategies [50-52]. In 2007, Timothy Brown, also known as the “Berlin patient,” underwent a bone marrow transplant with cells that had a mutated CCR5Δ32, which completely removed all HIV-1 RNA with no viral rebound [53]. Since then only one other person has been cured of HIV-1, the “London Patient", who underwent a similar bone marrow transplant with cells having a CCR5Δ32 gene [54]. Although there are two successful cases of bone marrow transplants curing HIV-1, there are potentially serious complications of doing such treatment such as: graft-versus-host

disease, and graft rejections, as well as being impractical for large scale application to the millions of people already infected with HIV-1 [55].Thus a safer and more reliable and reproducible therapy is sought after.

The shock and kill therapy has gained increased attention. This strategy to eliminate the latent viral reservoirs involves “shocking” the cells with latency reversing agents to cause a production of virus being made from the cell, then killing the HIV-1 infected cells through pro-apoptosis compounds or virus-mediated cytopathic events [56]. Many options have been explored to find a latency reversing agent including the use of: epigenetic modifiers, TLR agonists, NF-κB agonists, and protein kinase C agonists. Some HDACis like vorinostat, panobinostat, and romidepsin have made it to clinical trials and had seen an increase in HIV RNA transcription [57-59].

HIV-1 infected cells, even in the presence of ART, produce defective viruses that can still present HIV antigens to neighboring cells. The HIV-1 antigens present themselves onto neighboring uninfected “bystander” cells such as $CD4^+$ T cells, macrophages, and other immune cells. These bystander cells, although not infected, will still induce depletion of $CD4^+$ T cells through apoptosis and induce chronic inflammation [60, 61]. These immune cells will not get infected; however, they will produce inflammatory markers that cause chronic inflammation in the infected individual [62]. This chronic inflammation is linked to other kinds of diseases such as lymphoid fibrosis, neurocognitive impairment, cardiovascular disease, immunosenescence, and premature aging [63].

Interleukin-1 beta (IL-1β) is a known proinflammatory cytokine produced by "bystander" uninfected cells. IL-1β is produced through the activation of the inflammasome. IL-1β production is controlled by its precursor pro-IL-1β induction and by caspase-1 mediated pro-IL-1β cleavage which leads to caspase-1 activation [64]. Several inflammasome complexes can also be used to induce IL-1β in the NLR family; however, NLRP3 is the most well-studied inflammasome. IL-1β production through the NLRP3 inflammasome is associated with many diseases such as: gout, type 2 diabetes, heart failure, recurrent pericarditis, rheumatoid arthritis, and smoldering myeloma [65]. Also, many viruses activate IL-1β through NLRP3 such as influenza A, hepatitis C, HSV-1, adenovirus, myxoma virus, and many other viruses [66-68]. HIV-1 induces *NLRP3* mRNA expression and HIV-1 Tat protein induces IL-1β secretion in human monocytes [69, 70]. The NLRP3 inflammasome is known to be a part of multiple different diseases and can be activated through a canonical and noncanonical pathway [71].

Canonically, the NLRP3 inflammasome goes through a two-step activation process where a stimulus primes and causes transcription of inflammatory genes and a secondary stimulus causes the inflammasome to assemble [72]. Classically, this occurs when PAMPs activate toll-like receptors which then activate MYD88 and NF-κB to trigger the production of pro-IL-1β which then elicits the release of IL-1β [73]. This process can also be enhanced by other factors such as K^+ efflux, Ca^{2+} influx, and mitochondrial damage [71]. The activation of the inflammasome brings together many inflammasome inducers such as caspase-1, to induce pyroptosis [74]. However, the non-canonically NLRP3 inflammasome is activated by a primary stimulus that activates

the inflammasome and assembles the inflammasome complex through different caspases such as caspase-5, caspase-11, and caspase-4 [75].

Recently, there has been increased interest in an alternative inflammasome pathway where a signal is triggered through LPS to activate TRIF that brings together RIPK1, FADD, and caspase-8 to trigger NLRP3 inflammasome formation to cause IL-1β release without causing pyroptosis [76]. It has been shown that pyroptosis complexes are uniformly formed around the activation of caspase-1 suggesting that pyroptosis can be reduced by inactivating caspase-1 activation [77]. The mechanism(s) associated with the alternative pathway have yet to be fully described.

My thesis is divided into two parts. The first part is a discussion of the potential of a novel Tat-Vpu peptide to be used to facilitate latency reversal and viral release in a potential shock and kill cure strategy. The Tat peptide is a cell penetrating peptide that can be used to transport cargo into the cell by using its highly positive charge from the guanidinium to cross the negatively charged cell membrane [78]. We use a Tat peptide in conjunction to Vpu peptide, including the segments found to induce viral release, to make a “Tat-Vpu” peptide to show increased HIV-1 release in HIV-infected monocyte-derived-macrophages.

The second part of my thesis is the exploration of the mechanism of how HIV-1 causes chronic inflammation in bystander cells. The IL-1β production pathway in monocyte-derived macrophages “bystander” cells is studied by using RNA40, a GU-rich RNA sequence derived from the LTR of HIV-1. We show that non-canonically, TLR8 stimulation and potassium efflux induce the IL-1β release. Also, we have observed that

these bystander cells do not experience cell death when treated with RNA40 leading us to explore the alternative inflammasome. We have shown that caspase-8 and RIPK3 are also involved in the production of IL-1β in RNA40 stimulated cells indicating that alternative inflammatory pathway markers have a role in these HIV-1 “bystander” cells. Since the inflammatory activation includes non-canonical elements without causing pyroptosis, with potassium efflux, and members of the alternative inflammasome, this leads us to think that we have identified a new novel inflammatory pathway.

MATERIALS AND METHODS

Ethics statement

Venous blood was obtained from HIV-seronegative subjects through an approved protocol by the Human Research Protections Program of the University of California, San Diego which are in accordance with the requirements of the Code of Federal Regulations on the Protection of Human Subjects (45 CFR 46 and 21 CFR 50 and 56). Each donor has provided written consent prior to their participation.

Preparation of Tat-Vpu and Tat-Vpu scramble and reagents

Identification of essential Tat and Vpu sequences were acquired and were formulated by (LifeTein). Tat-Vpu (RRRQRRKKRGY-GG-EDSGNESEGEVSALVEMGVEMG) and Tat-scrambled (RRRQRRKKRGY- GG-EDSGNESEGAVSAAAEMGVEMG) were prepared using DMSO (Sigma) and were stored at -80°C until use. RNA40 (Invivogen) and ssRNA41 (Invivogen) were resuspended in endotoxin-free water as instructed by the manufacturer and used at 5µg/mL. Z-IETD-FMK (Selleck Chemicals) and GSK’872 (Selleck chemicals), were diluted both diluted in DMSO at concentrations of 10µg/mL. Kcl (Fisher) was diluted to 100mM and 50mM in water.

Isolating primary macrophage and culture

Whole blood was drawn from human volunteers and PBMCs were isolated from whole blood by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare). PBMCs were then plated and incubated for 24 hours with MDM media (RPMI 1640

[Gibco] supplemented with 10% [vol/vol] heat-inactivated FBS [Sigma], 2 mM L-glutamine, 0·1 mg/mL streptomycin, 100 U/mL penicillin [all Gibco], and 10 ng/mL CSF1 [Peprotech]) for 24 hours. The cells then receive a phosphate buffer saline to remove non-adherent cells and the adherent monocytes were cultured for 10 days with 5% CO_2, and 37°C in the MDM media. Media changes occurred every three days.

Cytotoxicity

Cell death was estimated using lactate dehydrogenase cytotoxicity detection kit (TaKaRa). 100ul of the cell cultures supernatant were mixed with 100ul LDH reaction solution for 30 minutes. The report signal was measured by an absorbance at wavelength 490 nm wavelength.

Viral isolation and infection

$HIV_{Ba\text{-}L}$ was obtained through the AIDS Research and Reference Reagent program from Dr. Suzanne Gartner and Dr. Robert Gallo. Virus stocks were prepared by infecting PHA (Sigma) and IL-2 (Roche) activated PBMC grown for 3 days. Then the virus titer was purified using Vivaspin 20 columns (Sartorius). The 50% tissue culture infective dose $TCID_{50}$ was determined using serial dilutions and the Spearman-Karber method as described previously [79]. The HIV-1 p24 antigen concentration in cell culture supernatant was determined using HIV-1 Gag p24 DuoSet ELISA kit (R&D Systems).

Macrophages were infected with a multiplicity of infection of 0.004 for 24 hours. Cells were then washed three times using phosphate buffer saline and fresh MDM

media was replaced. Infected cells' media was taken on day 3, 5, 7, and 10 days post-infection. Afterwards, the cells were washed with phosphate buffer saline and MDM media with Tat-Vpu and Tat-scramble were administered to the media. After incubation for 72 hours, the cell supernatants were taken and used in HIV-1 Gag p24 DuoSet ELISA kit (R&D Systems).

siRNA

Monocyte-derived macrophages were grown then transfected with siNS control and si*TLR8* (both Invitrogen) The scramble peptide (RNA41) that replaces U nucleotides with adenosines was used as a scramble control. siRNA was incubated with the monocyte-derived macrophages for 48 hours before being treated with RNA40 or RNA41 for 24 hours. The cell supernatant was collected and processed in Human IL-1 beta/IL-1F2 DuoSet ELISA (R&D) and the cells were lysed and taken for immunoblotting.

Immunoblotting

Cell lysates were prepared using a solution of 20 mM HEPES, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA supplemented with 1% (v:v) 4-(1,1,3,3-tetramethylbutyl) phenyl-polyethylene glycol (Sigma) and 1% (v:v) Halt protease and phosphatase inhibitor mixture (Thermo Scientific). Lysates were run on 2-[bis(2-hyxroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol buffered 12% polyacrylamide gels (Novex) and then transferred to 0.2 µm pore-size nitrocellulose membranes (Thermo Scientific). The nitrocellulose was then probed with primary antibody TLR8 (Cell Signaling) and β-Actin (Sigma) overnight followed by detection with alkaline

phosphatase-tagged secondary antibodies (Invitrogen) and 0.25 mm disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2′-(5-chlorotricyclo[3.3.1.13.7]decan])-4-yl)-1-phenyl phosphate supplemented with 5% (v/v) Nitro-Block II (Applied Biosystems). Densitometric analysis was performed on the primary antibody band to β-Actin band using ImageJ (National Institutes of Health).

Statistical analysis

Data are shown as mean values. Comparisons between groups were performed using the Student's t-test, with differences considered to be statistically significant when $*p < 0.05$.

RESULTS

Tat-Vpu induces HIV replication in human primary macrophages.

HIV-1 rapidly produces virus during the acute infection phase. However, when HIV-1 infected individuals undergo ART, the infectious virus is dramatically reduced but a reservoir of latent virus persists. The goal of shock therapies is to induce HIV-1 transcription and budding from the cell while infected individuals are undergoing suppressive ART therapy. Thus, we decided to test the ability of Tat-Vpu to increase the release of p24 antigen from HIV-1 infected primary macrophages.

Human primary macrophages were infected for 10 days to ensure all cells were infected. Cells were then washed with PBS and Tat-Vpu and Tat-scramble were introduced in increasing concentrations (1µm, 5µm, 10µm). The infected human primary macrophages were grown for 3 days then supernatant was taken to perform a p24 antigen test. Tat-Vpu peptide was seen to have a dose-dependent effect on HIV-1 virus release (Figure 1A). Tat-Vpu at 10µm was seen to have a 116% increase in p24 production than the control (Figure 1A). Tat-scramble peptide showed no increase in viral release (Figure 1B). Overall, The Tat-Vpu peptide seems to act in a dose-dependent manner due to its $LVEM^{66}$ amino acid sequence ($p = 0.02$) while the Tat-scramble has no significant effect.

Tat-Vpu and Tat-scramble do not induce cell death in human primary macrophages

Since we observed that Tat-Vpu induced an increase in HIV-1 p24 release, we wanted to determine if it had any effect on cell survival [80]. To assess cell death, macrophages were infected with $HIV_{Ba\text{-}L}$ for 10 days followed by the addition of Tat-Vpu

peptide and Tat-scramble peptide for three days. The supernatants were analyzed using the LDH cytotoxicity. The LDH did not show a significant difference in extracellular LDH release after the addition of Tat-Vpu or Tat-scramble (Figure 2A and 2B). These data indicate that Tat-Vpu does not induce cell death and is not cytotoxic to human primary HIV-infected macrophages. Thus, the increase in p24 release is not due to cells dying and releasing viral particles. These findings suggest that Tat-Vpu is inhibiting BST2 in primary macrophages while not causing death in these cells.

TLR8 is critical to induce IL-1β *release*

TLR8 is a member of the toll-like receptor family that recognizes single stranded RNA to recruit MYD88 and lead to activation of NF-kB response [81]. TLR8 has been known to be activated by bacterial RNA in human blood mononuclear cells [82]. TLR8 is primarily expressed in macrophages and myeloid dendritic cells [83]. TLR8 activation causes an increase in IL-1β levels and increased inflammation in patients with arthritis [84]. TLR8 has also been seen to be induced in HIV-1 infected human monocytic cells through an NLRP3 inflammasome pathway [85]. However, the mechanism(s) driving NLRP3 activation by ssRNA in human macrophages has not been clearly delineated.

To address the question of NLRP3 activation, differentiated macrophages were grown in culture and TLR8 was silenced using si*TLR8* and siNS was used as a negative scramble control. TLR8 silenced cells and controls were then exposed to RNA40 for 48 hours, supernatants were then collected and run on western blots and ELISA. As seen in the western blot when compared to the control, there was successful silencing of *TLR8* (Figure 3A and 3B). When exposed to RNA40, cells with TLR8 silenced showed a

significant decrease in release of IL-1β (Figure 3C and 3D). Thus, TLR8 is at least in part associated with the induction of IL-1β in “bystander” cells.

Potassium efflux is essential for eliciting IL-1β release

Potassium efflux has been known to induce NLRP3 production of IL-1β in the canonical pathway [86]. In primary human monocytes during toxoplasma gondii infection, extracellular potassium has been seen to significantly reduce IL-1β production [87]. Studies have found that enhancing potassium efflux in cells was also seen to upregulate IL-1β production [88]. Since we have already seen that endosomal TLR8 is involved in RNA40 -mediated NLRP3 inflammasome activation, we next examined if potassium efflux is involved in IL-1β production as it is downstream of TLR8 stimulation and upstream of NLRP3 inflammasome formation

Monocyte-derived macrophages were grown to maturity (10 days), then treated with media containing increasing potassium chloride concentrations for one hour. This addition of potassium chloride to the media will inhibit potassium efflux from the cell as the amount of potassium outside the cell exceeds the potassium within. After one hour of inhibiting potassium efflux, the cells were then stimulated with RNA40 for 24 hours after which cell supernatants were collected for IL-1β ELISA and LDH.

Inhibition of potassium efflux showed no significant increase in cell cytotoxicity when compared to the control (Figure 4A). The macrophages not stimulated with RNA40 did not elicit any IL-1β release (Figure 4B and 4C). The dose-dependent increase in potassium efflux inhibition with stimulation of RNA40 led to a significant decrease in IL-1β production (Figure 4B and 4C). Thus, potassium efflux appears to be

important for IL-1β production and does not require a secondary stimulus, implying that potassium efflux can non-canonically trigger IL-1β production in RNA40 stimulated macrophages

Caspase-8 and RIPK3 are involved in RNA40 -mediated IL-1β release

In addition to apoptosis, HIV-1 infection can lead to cell death of “bystander” cells through pyroptosis resulting from persistent inflammation caused by the release of non-infectious viral particles from infected cells [80]. The observed lack of increased death of cells exposed to RNA40 (Figure 4A) suggested to us that a novel pathway might be driving the induction of the NLRP3 inflammasome by RNA40. One of the defining features of the alternative pathway is that the induction of IL-1β occurs without pyroptosis and is dependent on a TLR4-TRIF-RIPK-CASP8 pathway [76].

Having shown that the induction of IL-1β by HIV-1 RNA40 was likely going through at alternative pathway, our next set of experiments were designed to determine if elements of this alternative inflammasome might have a role in HIV-1 mediated inflammation. To test this possibility, macrophages were treated with Z-IETD-FMK (a caspase-8 inhibitor) and GSK’ 872 (a RIPK3 inhibitor) for 24 hours and then treated with RNA40 for 24 hours. After RNA40 exposure, cell supernatants were removed for LDH and IL-1β ELISA. Here, we observed that there was a decrease in cell death between the cells exposed and not exposed to RNA40 (Figure 5A) suggesting that a novel pathway of inflammasome activation was possible. Overall, there was a decrease in IL-1β release in the presence of the caspase-8 and RIPK3 inhibitors in RNA40 stimulated macrophages (Figure 5B and 5C). Also, the caspase-8 and RIPK3 inhibitors combined

demonstrated the greatest decrease in IL-1β production (Figure 5B and 5C). This indicates that elements of the alternative inflammasome may be part of the pathway to produce IL-1β in “bystander” cells.

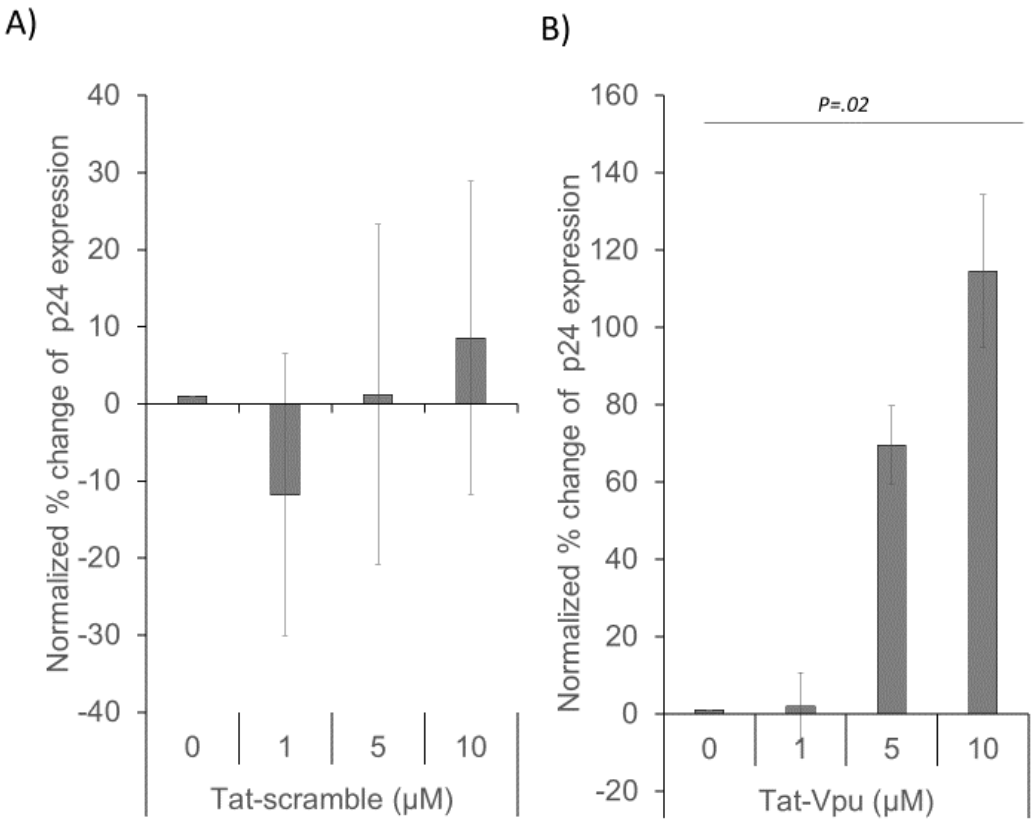

Figure 1. Tat-Vpu peptide induces an increase in p24 release in human monocyte-derived macrophages. Monocyte-derived macrophages infected with HIV_{bal} at MOI 0.004. These infected cells were grown for 10 days then treated with Tat-Vpu peptide for 72 hours (A) and Tat-scramble peptide for 72 hours (B) then supernatant was taken and p24 was detected by ELISA. Tat-Vpu dose-dependently increased HIV release into the cell supernatant. Data are presented as a mean of three independent donors (n=3) ± s.e.m.

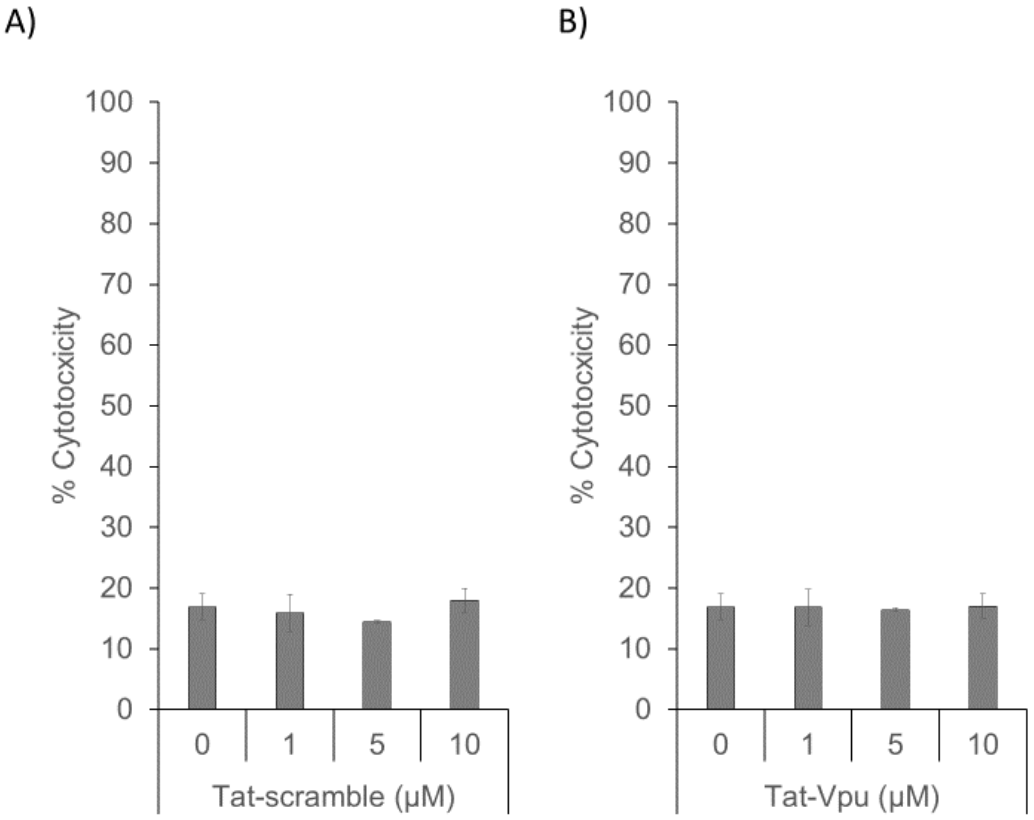

Figure 2. Tat-Vpu peptide does not induce cell death in HIV-1 infected monocyte-derived macrophages. Monocyte-derived macrophages infected with HIV_{bal} at MOI 0.004. These infected cells were grown for 10 days then treated with Tat-Vpu peptide for 72 hours (A) and Tat-scramble peptide for 72 hours (B) then supernatant was taken and LDH was detected by ELISA. Tat-Vpu did not cause a significant increase in cell death. Data are presented as a mean of three independent donors (n=3) ± s.e.m.

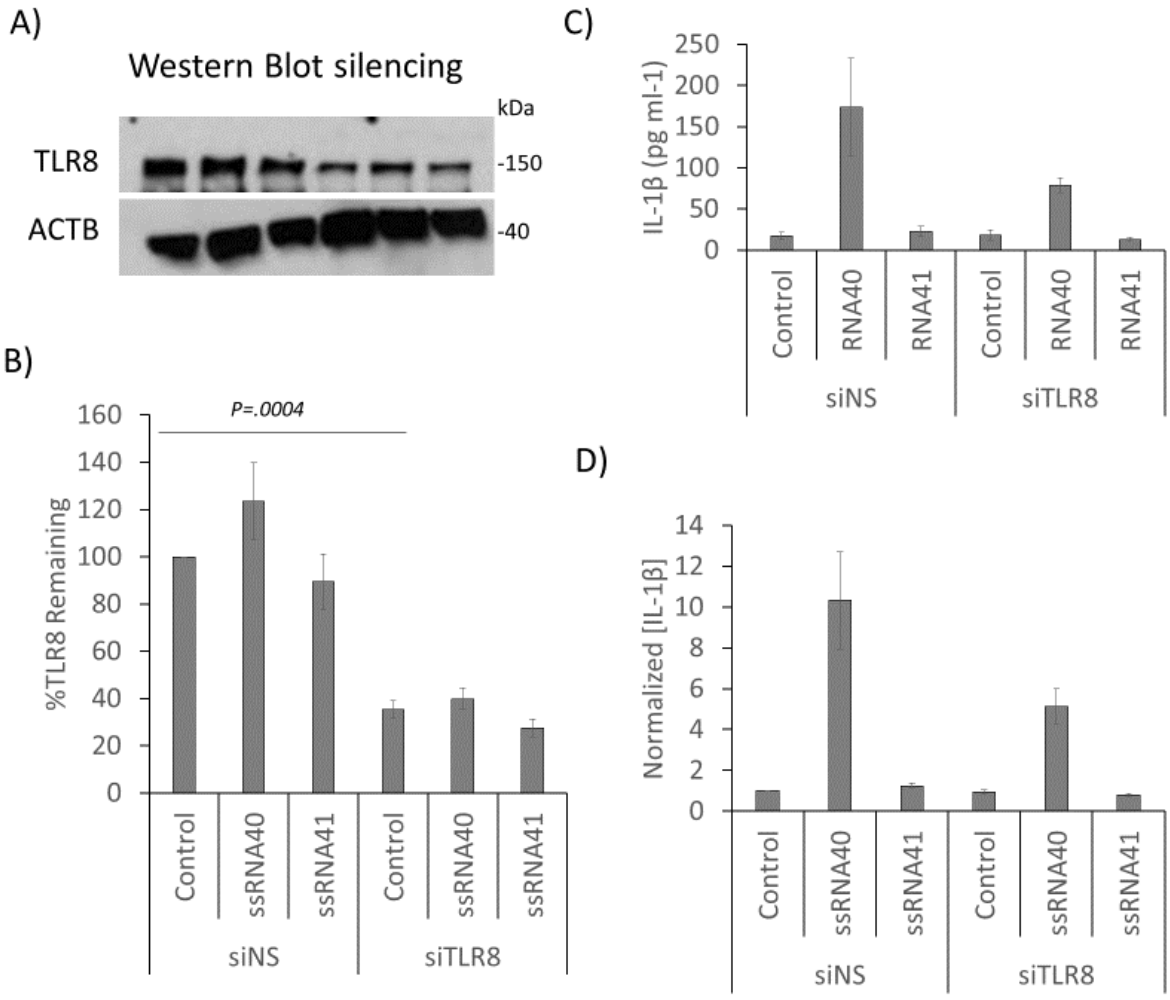

Figure 3. TLR8 is critical for IL-1β release in RNA40 activated human monocyte-derived macrophages. Macrophages were treated with siTLR8 for 48 hours prior to treatment with ssRNA40 5μg/mL. siNS treated macrophages were used as a scrambled siRNA control. (A) A western blot representation from one donor to show the knockdown of TLR8 protein expression. (B) A densitometric analysis showing TLR8 knockdown. (C) IL-1β protein detection using ELISA in pg/mL and (D) normalized IL-1β to account for donor variety. All data is a representation of 3 individual donors (n=3) ± s.e.m.

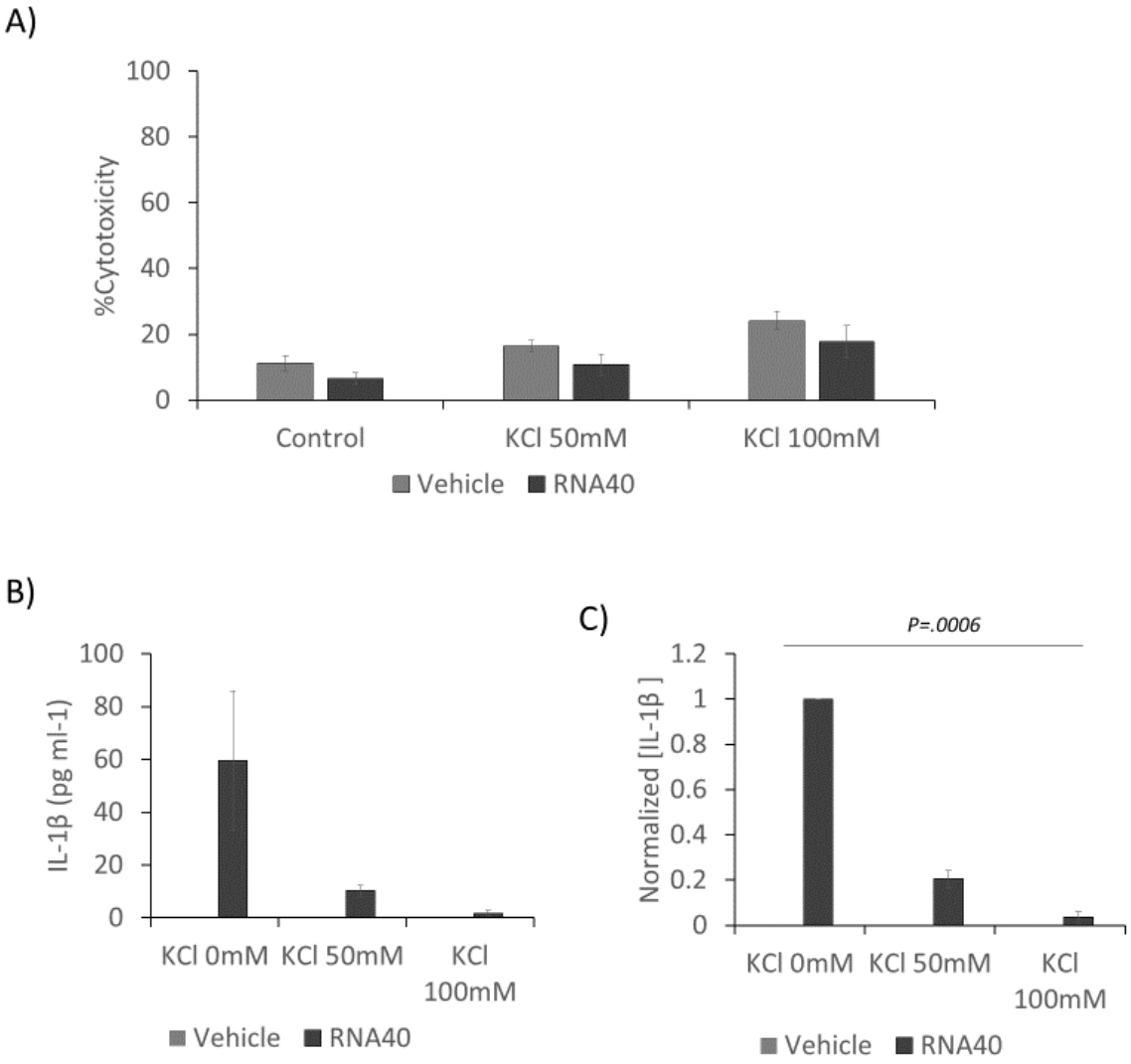

Figure 4. Potassium efflux is essential in the non-canonical inflammasome for RNA40 stimulated macrophages. Macrophages were pretreated with KCl for 1 hour before being treated with RNA40 for an additional 24 hours. (A) a LDH ELISA demonstration of cell cytotoxicity. (B) Cell supernatants were collected for IL-1β release measured through ELISA and (C) normalized for donor variety. All data is a representation of 3 individual donors (n=3) ± s.e.m.

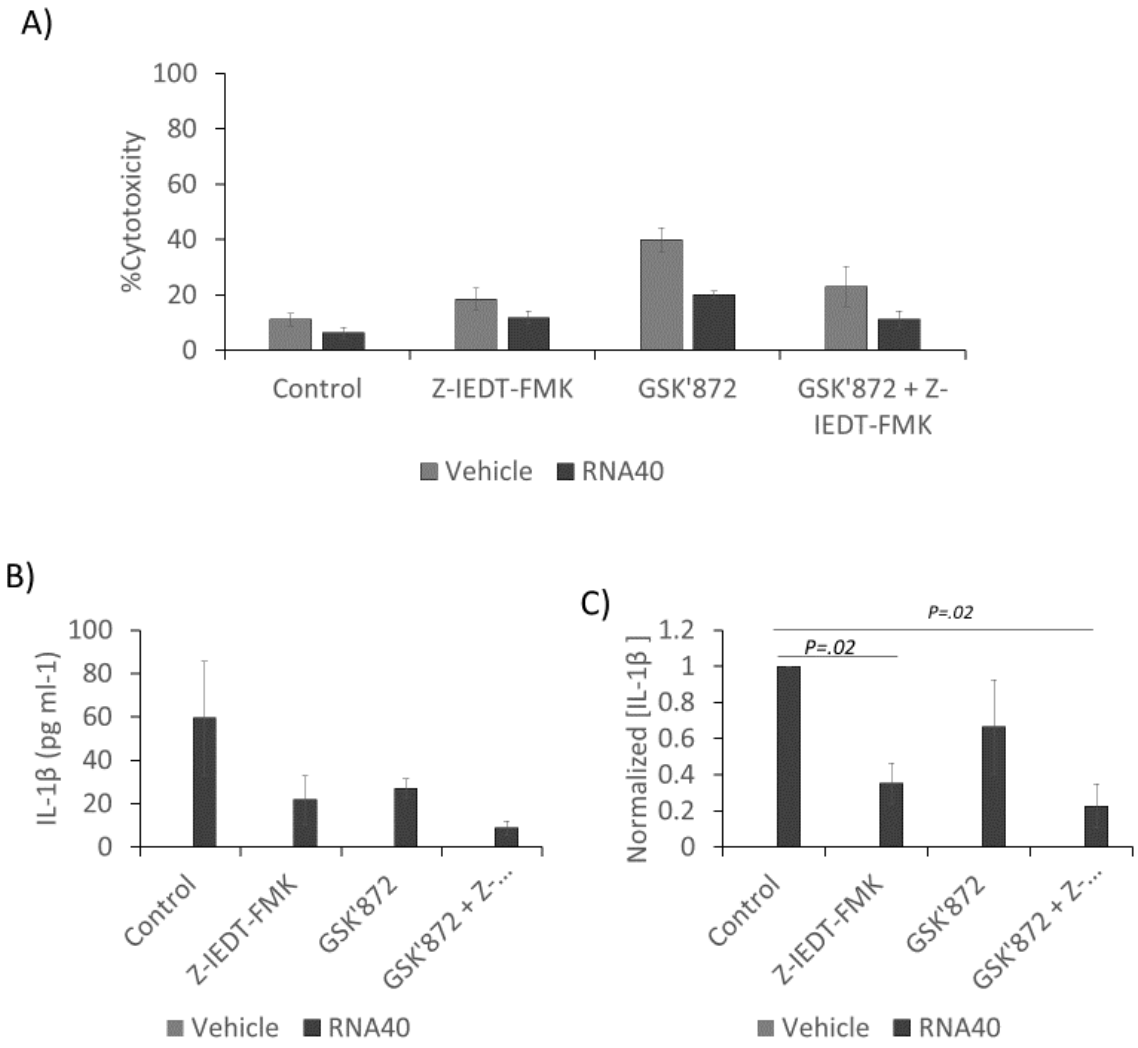

Figure 5. The alternative inflammasome is involved in the production of IL-1β. Macrophages were grown then treated with inhibitors for 1 hour then stimulated with ssRNA40 for an additional 24 hours. After, the cell supernatants were taken for LDH cytotoxicity (A) and IL-1β ELISA (B). Donor's IL-1β expression were normalized to account for donor variability (C). All data is a representation of 3 individual donors (n=3) ± s.e.m.

DISCUSSION

HIV-1 still currently infects 1.2 million people in the United States and as of now there is still no cure [89]. ART has greatly improved the life expectancy and quality for infected individuals; however, ART is not a complete cure as it leaves latent HIV reservoirs [90]. Thus, it is important to seek a potential cure for HIV and understand its harmful inflammatory effects on currently suppressed patients.

The “shock and kill” method focuses on a unique strategy of reversing the latent cell reservoir to produce HIV-1 so those cells can be detected and extinguished. Many latency reversing agents, or “shock” drugs, have little success in activating all of the latent reservoir cells and can only activate a small percentage of them [91]. Thus, research for a better latency reversing agent is sough for.

Traditionally, Vpu is an HIV-1 accessory protein that greatly reduces the amount of BST2 on the cell surface [92]. Since BST2 normally tethers and inhibits HIV-1 budding from an infected cell, removing this restriction factor greatly increases the amount of released virus from a cell [93]. Here we use a Tat-Vpu peptide as a potential adjunct to latency reversing agents to enhance the expression of viral proteins on cells with reactivated virus to enhance killing of cells undergoing viral reactivation. We have shown that Tat-Vpu can induce an increase in HIV-1 viral release from already infected cells through a $LVEM^{66}$ amino acid sequence. This sequence is associated with LC3C Vpu-mediated reduction of BST2 in HIV-1 infected cells as it acts through a non-canonical autophagic pathway [39].

In the future, it would be critical to observe if the increase viral release by Tat-Vpu can work on ART silenced macrophages and $CD4^+$ latent T cells, the major reservoirs of HIV-1 in ART patients. These studies can be performed using Tat-Vpu alone and to enhance the effects when combined with other drugs currently being studied as latency reactivating agents. Eventually, the Tat-Vpu peptide may proof useful in strategies designed to provide an HIV-1 cure.

HIV-1 activates the immune system to induce chronic inflammation through releasing viral antigens that cause an increase in IL-1β release [62]. This chronic inflammation by individuals with HIV-1 even with ART can promote and accelerate numerous inflammatory diseases [62]. Thus, understanding the cell pathway for HIV-1 induced IL-1β release is important to understand. We have shown that HIV-1 GU-rich RNA40 activates the non-canonical NLRP3 inflammasome through potassium efflux and endogenous TLR8 activation. We have also observed a lack of cell death indicating no pyroptosis with these RNA40 activated cells and observed that markers in the alternative inflammasome, RIPK3 and caspase-8, contribute to the production of IL-1β.

Our silencing data on TLR8 showing an inhibition of IL-1β release indicates a role for TLR8 in IL-1β activation. This indicates that the non-canonical inflammasome is being activated rather than the canonical inflammasome as TLR4 is not the primary TLR activator and TLR8 alone without a secondary stimulus result in inflammasome activation. Although we have shown the importance of TLR8 in inflammasome activation in human primary macrophages, the role of TLR7 in the activation of the non-canonical NLRP3 pathway is unclear. TLR8 has been seen to induce a MYD88 response through TLR7/8 activation in PBMCs and now we have shown that this response likely leads to

a non-canonical inflammasome activation rather than a canonical NLRP3 inflammasome activation [94].

Interestingly K^+ efflux inhibition resulted in a decrease in IL-1β release. Traditionally, PAMPs initiate NLRP3 activation resulting in eventual lower intracellular potassium through K^+ efflux using TWIK2 [95]. Thus, it is not surprising that K^+ efflux in our RNA40 stimulated macrophages is also required for efficient NLRP3 inflammasome activation. This is supported by NLRP3 inflammasome activation occurring in bone marrow-derived macrophage with low- K+ medium, which indicates that K^+ efflux alone can activate the NLRP3 inflammasome [96].

When NLRP3 inflammasome activation occurs, there is generally an increase in caspase-mediated cell death through pyroptosis. This occurs through caspase-1, caspase-4, or caspase-11 binding to gasdermin D (GSDMD) inducing pyroptosis [97]. However, RNA40 stimulated macrophages show no increase in cell cytotoxicity even with an increase in IL-1β release through K^+ efflux inhibition and TLR8 silencing. However, NLRP3 inflammasome activation occurs through the alternative inflammasome that relies on TLR4, RIPK3, RIPK1, FADD, and caspase-8 signaling without the presence of K^+ efflux [76]. Furthermore, our research indicates that RIPK3 and caspase-8 silencing decreases IL-1β release suggesting their importance in NLRP3 inflammasome activation. To indicate if the alternative inflammasome is actually taking an effect in RNA40 exposed macrophages, additional experiments with RIPK1 inhibitors and IL-6 ELISA should be performed.

Since we have observed that TLR8, K^+ efflux, caspase-8, and RIPK3 are essential for NLRP3 inflammasome activation in RNA40 stimulated macrophages and the process of NLRP3 activation does not drive pyroptosis in these cells, we hypothesize that these cells are causing NLRP3 inflammasome activation through a novel pathway. Understand this novel pathway has the potential to lead to novel strategies to achieve a cure for HIV-1 and its associated chronic inflammation. Reduction in this chronic inflammation would allow ART patients to live a more disease-free and healthier life.

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