UNIVERSITY OF CALIFORNIA SAN DIEGO

Human Immunodeficiency Virus Type-1 GU-rich ssRNA Activates the NLRP3 Inflammasome in Macrophages through a Non-canonical Pathway

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 Rachel Kim-Chie To is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2019

DEDICATION

In loving memory of my beloved father, whose legacy inspires me to live daily with courage, humility, and passion. To my wonderful mother, whose home-cooked meals, prayers, and selfless understanding has made this all possible. To my big brother, who is my role model and teacher for everything in life. I love you – family is forever.

And above all, I give thanks to God, who has graciously provided me with more than I could ever ask or imagine. All glory to Him.

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ABSTRACT OF THE THESIS

Human Immunodeficiency Virus Type-1 GU-rich ssRNA Activates the NLRP3 Inflammasome in Macrophages through a Non-canonical Pathway

by

Rachel Kim-Chie To

Master of Science in Biology

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Human immunodeficiency virus type-1 (HIV) pathogenesis is associated with immune activation and chronic inflammation during active viral replication as well as during antiretroviral viral suppression. Even in cases where levels of replication competent virus are undetectable due to effective antiretroviral therapy, non-replicative virus is still produced with release of non-infectious viral RNA. This RNA is sensed by

surveying innate immune cells, such as macrophages, which then induce pro-inflammatory cytokines such as interleukin-1 beta (IL-1β). Processing of IL-1β is associated with nod-like receptor protein-3 (NLRP3) inflammasome and caspase activation in many inflammatory diseases; however, the mechanism of IL-1β induction in human primary macrophages exposed to HIV RNA is unknown. To elucidate the mechanism of IL-1β activation by HIV RNA, we exposed human primary macrophages to RNA40 (a GU-rich ssRNA derived from the HIV long-terminal repeat region) and observed a significant induction of IL-1β. Processing of IL-1β in macrophages exposed to RNA40 required activation of the NLRP3 inflammasome independent of potassium efflux or reactive oxygen species production. RNA40 also triggered caspase-4 and -5 activation to mediate IL-1β release in a one-step event that occurs shortly after exposure to RNA40.

In total, these findings indicate that following exposure of human primary macrophages to GU-rich ssRNA from the HIV LTR region, the NLRP3 inflammasome is activated through a non-canonical pathway involving caspase-4 and caspase-5 to process IL-1β. This mechanism provides further insight into HIV pathogenesis, and suggests that targeting caspase-4 and caspase-5 could be useful in controlling HIV-associated chronic inflammation.

INTRODUCTION

Currently, approximately 37 million people in the world are living with human immunodeficiency virus type-1 (HIV), an infectious disease characterized by immune dysfunction and susceptibility to opportunistic infections. More than 50% of these HIV-infected individuals are on antiretroviral therapy (ART) that preserves healthy CD4+ T lymphocyte cells from depletion (UNAIDS Data Report, 2018). Although the advent of ART has dramatically improved life expectancy mostly due to a decline in AIDS-related deaths, HIV-infected individuals still experience higher morbidity and mortality rates than healthy individuals (van Sighem *et al.* 2005). Cohort studies investigating the cause of death in HIV-infected individuals on ART suggest that diseases associated with hyperimmune activation and inflammation are predictors of non-AIDS mortality in HIV infection (Palella *et al.* 2006; Collaboration TATC, 2008; Antiretroviral Therapy Cohort Collaboration, 2010). Specifically, an increased incidence for cardiovascular disease was reported in HIV-infected individuals on ART as a result of elevated immune activation and altered lipid profiles (Rose *et al.* 2008; Triant *et al.* 2007). HIV-infected individuals experience other inflammatory, non-AIDS related diseases, including neurocognitive degeneration, renal failure, liver disease, and kidney disease (Deeks *et al.* 2013). These diseases are often associated with an aging immune system, or “immunosenescence”, and chronic, systemic inflammation (Meir Shafrir *et al.* 2012).

One model for HIV chronic inflammation suggests that HIV infection destroys gut mucosa and leads to microbial translocation that leaks into the periphery and causes a systemic, inflammatory response (Deeks *et al.* 2013). Inflammation then promotes co-morbidity with inflammatory diseases in HIV infected individuals (Deeks *et al.* 2013). In

contrast, HIV produces both replication competent and defective (replication incompetent) virus that present HIV antigens during infection despite effective ART (Sanchez *et al.* 1997; Nath *et al.* 1999; Sereti *et al.* 2017). These HIV antigens can elicit an immune response in neighboring, uninfected cells such as CD4+ T cells and tissue-resident macrophages (Sanchez *et al.* 1997; Van Lint *et al.* 2013). Immune surveying cells, such as monocytes and macrophages, sense these viral antigens and become activated, producing proinflammatory biomarkers and contributing to chronic inflammation (Nath *et al.* 1999; Deeks *et al.* 2013; Sereti *et al*. 2017).

One of the proinflammatory biomarkers associated with HIV-associated chronic inflammation is interleukin-1 beta (IL-1β). Numerous cell types produce IL-1β, and its overexpression is involved in a plethora of inflammatory and pathological diseases, including gouty arthritis, inflammatory bowel disease, and neuropathy (Ren *et al.*, 2009). IL-1β also plays an important role in HIV-associated neurocognitive disorders and neuroinflammation (Chivero *et al.* 2017; Mamik *et al.* 2017). HIV gp120 and envelope pseudotype particles activate microglia cells *in vitro* to induce IL-1β, paralleling the elevated levels of IL-1β observed in the brains of HIV infected individuals (Walsh *et al.* 2014). HIV proteins including Tat and gp120 also upregulate proinflammatory cytokines and trigger monocyte invasion to the brain. Activated monocytes and macrophages increase cytokine levels in the periphery and are associated with on-going HIV-pathogenesis (Pu *et al.* 2003; Le Saout *et al.* 2012).

On a molecular level, IL-1β production is initiated by toll-like receptor (TLR) signaling and is transcribed in an NFκβ -dependent pathway to produce an inactive, pre-cursor form known as pro-IL-1β (Hazuda *et al.* 1991). Intracellular pattern recognition

receptors known as inflammasomes and pro-inflammatory caspases regulate IL-1β processing. Inflammasomes are multimeric protein complexes made up of adaptor proteins that oligomerize upon activation and play a crucial role in regulating inflammation (Stutz *et al.* 2009). Of the adaptor proteins that bind and form these complexes are nod-like receptor (NLR) proteins, which make up the NLRP1, NLRP3, NALC4, and the AIM2 inflammasomes (Stutz *et al.* 2009). The most studied inflammasome is the NLRP3 as it is known to have the broadest variety of stimuli (Guo et al., 2015). Overactivation or dysfunction of the NLRP3 inflammasome is characterized in cryopyrin-associated periodic syndromes, gout, type II diabetes, cancer, and various other inflammatory diseases (Menu *et al.* 2011). Both HIV Tat and RNA40 induce an inflammatory response in microglia in a NLRP3-dependent manner indicating a role for the NLRP3 inflammasome in HIV-associated neurocognitive disorders and neuroinflammation (Chivero *et al.* 2017; Rawat *et al.* 2018). To date, evidence for NLRP3's involvement in HIV inflammation report of its activation through the conventional NLRP3 inflammasome pathway.

The conventional NLRP3 inflammasome is characterized by a two-step activation in which 1) a primary stimulus primes and signals transcription of inflammasome genes, while 2) a secondary stimulus initiates inflammasome complex assembly and caspase-1 activation (Schroder *et al.* 2010). Priming stimuli include bacteria cell-wall components (i.e. LPS) or other toll-like receptor ligands (Stutz *et al.* 2009). The initial priming step is required for inflammasome activity as transcriptionally upregulated levels of NLRP3 are necessary to sense molecular changes associated with tissue damage (Stutz *et al.* 2009). These molecular changes can be induced by damage associated molecular patterns

(DAMPs), pathogen associated molecular patterns (PAMPs), crystal particulates, pore-forming molecules, and ATP (Jo *et al.* 2015; Schroder *et al.* 2010). These activators trigger NLRP3 inflammasome activation by inducing ion flux or reactive oxygen species (ROS) production (Munoz-Planillo *et al.* 2013; Abais *et al.* 2015). The activation by these secondary signals initiates NLRP3 oligomerization and recruitment of the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC) to induce proteolytic activity of caspase-1 (Stutz *et al.* 2009). Active caspase-1 is then able to process pro-IL-1β into its mature form, IL-1β (Martinon *et al.* 2002).

In contrast, the non-canonical NLRP3 inflammasome does not require a secondary signal as its stimuli both prime and activate the inflammasome complex to process cytokines through caspase-4 and caspase-5 (Broz *et al.* 2013). In the mouse model, intracellular LPS activates the non-canonical inflammasome by directly binding to caspase-11, the murine homolog to caspase-4 and caspas-5, inducing NLRP3 complex assembly and activation (Kayagaki *et al.* 2013). Although the exact mechanism of how caspase-11 activates the NLRP3 inflammasome is unknown, evidence indicates that auto-proteolysis of caspase-11 is necessary for non-canonical activity (Lee *et al.* 2018). Vigano *et al.* (2013) proposed that caspase-11 activates caspase-1 to induce processing of pro-IL-1β and inflammatory cell death through pyroptosis. Similarly, caspase-4 functions upstream of caspase-1 activation to induce pyroptosis through the non-canonical NLRP3 inflammasome during dengue virus infection (Cheung *et al.* 2018). In contrast, caspase-5 is likened to caspase-11 as it also undergoes auto-proteolysis at early exposure to LPS (Vigano *et al.* 2015). While the function and effector mechanism

of murine caspase-11 and human caspase-4 and caspase-5 require further investigation, they remain characteristic to the non-canonical inflammasome.

The induction of pro-inflammatory cytokines from macrophages exposed to low levels of non-replication competent HIV antigens contributes to the chronic inflammation present in patients despite viral suppression with antiretroviral therapy (Nath *et al.* 1999; Le Saout *et al.* 2012; Sereti *et al.* 2017). Previous reports from our lab indicate that HIV RNA40, a 20-mer single-stranded GU-rich RNA sequence (RNA40) encoded within the HIV long terminal repeat, induces IL-1β production in human microglia in a NLRP3-dependent pathway (Rawat *et al.* 2018). However, the mechanism for IL-1β production by human primary macrophages exposed to RNA40 remains unknown. In this research, we hypothesized that RNA40 would induce IL-1β in human primary macrophages. Our findings indicate that RNA40 not only induces IL-1β, but also activates a non-canonical NLRP3 inflammasome that is dependent on caspase-4 and caspase-5 but independent of potassium efflux and ROS production.

MATERIALS AND METHODS

Ethics statement

Venous blood was drawn from HIV seronegative subjects using a protocol that was reviewed and approved by the Human Research Protections Program of the University of California, San Diego (Project 08-1613) 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). Written informed consent was obtained from all blood donors prior to their participation.

Cell cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from anonymous, healthy human blood donors using Ficoll-Paque PLUS (GE Healthcare) density centrifugation. PBMCs were plated at a concentration of 5×10^6 cells mL^{-1} in macrophage media (RPMI 1640 [Gibco] supplemented with 10% [vol/vol] heat-inactivated fetal bovine serum [Sigma], 2 mM L-glutamine, 0.1 mg mL^{-1} streptomycin and 100 U mL^{-1} penicillin [all Gibco]) for 24 hr in 5% CO_2 at 37°C. Monocytes were selected by plastic adherence and non-adherent cells were removed by aspiration. Remaining primary human monocytes were differentiated into macrophages using macrophage media supplemented with 10 ng mL^{-1} colony stimulating factor 1 (CSF1; Peprotech) for 8-10 days at 37°C, 5% CO_2 with media changes every 2 days before use. Donors with monocyte-derived macrophages that displayed elongation and healthy morphology through microscopy were used in experiments.

Chemicals

RNA40 (tlrl-lrna40) and RNA41 (tlrl-lrna41) were obtained from Invivogen. RNAs were complexed with the cationic lipid Lyovec and reconstituted with endotoxin-free water, per the manufacturer's instructions. Inhibitors were obtained from the following manufacturers: Z-YVAD-FMK (S7023) and Glyburide (S1716) from Selleck Chemicals, AC-YVAD-CMK (SML0429) from Sigma, Z-YVAD-FMK (FMK005) from R&D Systems, Z-LEVD-FMK (ALX-260-142-R100) from Enzo Life Sciences, YCG063 (557354) from Millipore Sigma, and MCC950 (AG-CR1-3615-M001) from AdipoGen.

Western blotting

Cell lysates were collected using lysis buffer consisting of 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (Gibco), 150 mM NaCl (Fisher), 1 mM 2,2',2'',2'''-(ethane-1,2-diyldinitrilo)tetraacetic acid supplemented (Sigma) with 1% (vol/vol) Triton X-100 (Sigma) and 1% (vol/vol) Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). 50 µg of protein was loaded onto 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol buffered 12% polyacrylamide gels (Invitrogen) and ran through gel electrophoresis. 0.2 μm nitrocellulose membranes (Thermo Scientific) were used for protein transfer and incubated with blocking buffer (Dulbecco's phosphate buffered saline supplemented with 50 g L^{-1} nonfat milk and 1 g L^{-1} polyoxyethylene (20) sorbitan monolaurate [Sigma]) prior to immunoblotting with primary antibodies. Incubation with alkaline phosphatase tagged secondary antibodies (Novex) was followed by immunodetection using 0.25 mM CDP-Star supplemented with 5 % (vol/vol) Nitro-Block II (both Applied Biosystems). Densitometric analysis was performed

on the bands obtained and normalized to the reference ACTB bands using ImageJ (NIH). Antibodies for pro-IL-1β (D3U3E), NLRP3 (D2P5E), CASP4 (4450S), and CASP5 (D3G4W) were obtained from Cell Signaling Technology. ACTB (A2228) was obtained from Sigma Aldrich.

LDH Assay

Cytotoxicity was measured using a lactate dehydrogenase assay kit obtained from Takara Biotech. Cell culture supernatants were collected and analyzed as per manufacturer's instructions immediately after each experiment.

siRNA-mediated knockdown

Macrophages were transfected with Silencer Select small-interfering RNA obtained from ThermoFisher Scientific for *NRLP3* (s534396), *CASP4* (s2414), or *CASP5* (s2417). Stealth RNAi was used for the scrambled negative control (12935200) and obtained from Invitrogen. siRNAs were incubated with cell cultures for 48 hr prior to treatment with RNA40 for 24 hr. Protocol was performed as per manufacturer's instructions. Silencing efficacy was measured using western blot analysis.

ELISA

Mature IL-1β was measured in cell culture supernatants using enzyme-linked immunosorbent assay kits obtained from R&D Systems (DLB50). Protocol was performed as per manufacturer's instructions.

Statistical analysis

Statistical difference between the means of two normally distributed groups was measured using two-tailed student t-tests, in which the *p-value < 0.05 was statistically significant.

RESULTS

NLRP3 inflammasome mediates IL-1β production in human primary macrophages exposed to RNA40.

HIV derived RNA40 induces IL-1β production from the human monocytic cell line, THP-1 cells, in a NLRP3-dependent manner (Guo et al. 2014); however, whether this mechanism occurs in human primary macrophages remains unknown. To determine if RNA40 can induce pro-IL-1β expression in human primary macrophages, we exposed monocyte-derived macrophages (macrophages) to RNA40 for 24 hr and measured protein levels by western blot. We observed a 4.5-fold increase in pro-IL-1β expression in macrophages exposed to RNA40 compared with vehicle treated and control RNA41 treated cells (Figure 1A). We then examined if RNA40 induces mature IL-1β release by measuring secreted cytokine levels by ELISA. RNA40 induced a significant increase in secreted mature IL-1β compared with untreated cells and RNA41-treated cells (Figure 1B). Importantly, we failed to observe significant RNA40-mediated cell death at the concentrations used in this study, as released levels of lactate dehydrogenase (LDH) were comparable to both the vehicle and RNA41 (Figure 1C). This suggests that the mechanism of IL-1β production in macrophages exposed to RNA40 is not associated with pyroptosis and that RNA40 alone is sufficient to upregulate IL-1β production and processing.

Although inflammasomes are conventionally associated with pyroptosis and a two-step signaling activation, they are involved in the processing of pro-inflammatory cytokines such as IL-1β and IL-18. Therefore, despite the lack of pyroptosis, we investigated if the NLRP3 inflammasome is involved in the production of mature IL-1β in

RNA40-treated macrophages. Following silencing of *NLRP3* using siRNA, we observed a decrease in IL-1β release, demonstrating a role for NLRP3 in the release of IL-1β in macrophages exposed to RNA40 (Figure 2A-D). However, since RNA40, unlike LPS, is independent of pyroptosis and does not require an additional secondary stimulus, our findings suggested that RNA40 must work through an unconventional NLRP3 pathway to induce the expression, processing and release of IL-1β from macrophages.

Conventional NLPR3 inhibitors do not reduce levels of IL-1β in macrophages exposed to RNA40

ATP, a known secondary stimulus of the canonical NLRP3 inflammasome, activates ion channels to reduce intracellular potassium concentrations by inducing potassium efflux (Parham 2015). To determine if RNA40 activates the NLRP3 inflammasome by inducing ATP release, we pre-treated macrophages with glyburide, a known inhibitor of potassium efflux. RNA40 does not reduce IL-1β levels with glyburide, suggesting that potassium efflux is not required to activate the NLRP3 inflammasome (Figure 3A). This also suggests that RNA40 does not elicit ATP production to activate the NLRP3. Next, we investigated if RNA40 induces reactive oxygen species (ROS) production, another known stimulator of the NLRP3 inflammasome, by pre-treating macrophages with a ROS inhibitor, YCG063, prior to RNA40 exposure. Again, we observed no reduction in IL-1β with the ROS inhibitor, indicating that RNA40 does not induce the production of ROS to signal inflammasome complex formation (Figure 3B). These data suggest that two common activating stimuli for the conventional NLRP3

inflammasome, potassium efflux and ROS production, are not involved in RNA40-induced IL-1β production.

We further investigated the mechanism of NLRP3 inflammasome activation using an inhibitor specific for NLRP3, MCC950. Although the mechanism of action for MCC950 remains elusive, its inhibition has been associated with pathways involving the conventional NLRP3 inflammasome activation in humans. To our surprise, MCC950 did not affect levels of IL-1β in macrophages exposed to RNA40 in comparison to the LPS primed-ATP stimulated control (Figure 3C and D). Increasing the concentration of MCC950 still failed to have an effect on IL-1β production in macrophages treated with RNA40 (Figure 3E). These findings support the notion that RNA40 does not activate a canonical inflammasome pathway but instead stimulates a non-canonical inflammasome pathway as glyburide, ROS inhibition, and MCC950 failed to reduce IL-1β levels in macrophages.

RNA40 induces IL-1β in a caspase-dependent manner.

Caspases play a key role in the processing of cytokines after inflammasome activation (Stutz *et al.* 2009). Hence, we sought to investigate if caspases are involved in NLRP3 activation following exposure of macrophages to RNA40. In order to determine a general involvement of caspases, we pre-treated macrophages with a pan-caspase inhibitor, Z-YVAD-FMK, for 1 hr prior to exposure to RNA40. Inhibition of caspases significantly reduced IL-1β, indicating that caspases are involved in this mechanism of pro-IL-1β processing (Figure 4A). We then sought to identify the specific caspases associated with this mechanism by using inhibitors for pro-inflammatory caspases,

caspase-1, caspase-4, and caspase-5. Inhibiting caspase-1, the primary caspase commonly reported to be involved in most inflammasome mechanisms, led to a significant decrease in IL-1β levels in macrophages and further confirms that pro-IL-1β is being processed through an inflammasome-associated pathway (Figure 4B). Interestingly, pre-treatment with inhibitors for caspase-4 and caspase-5, caspases that are associated with non-canonical NLRP3 activation, significantly reduced IL-1β levels in macrophages (Figures 4C and 4D). However, since the caspase inhibitors for caspase-4 and caspase-5 are not mutually exclusive, we sought to identify if caspase-4 and caspase-5 play separate and independent roles in the processing of IL-1β by silencing caspase protein expression using siRNA. Silencing of *CASP4* and *CASP5* led to a reduction in IL-1β levels, confirming the role of these caspases and suggesting an unconventional NLRP3 pathway for IL-1β processing (Figure 5A-D and Figure 6A-D).

Caspase-5 is activated rapidly after exposure to RNA 40 initiating NLRP3 inflammasome activation

After confirming the involvement of caspase-4 and caspase-5 in the induction of IL-1β from macrophages exposed to RNA40, we next examined caspase activity by analyzing their cleaved products as proteolytic cleavage is indicative of caspase activation. A previous study regarding the involvement of caspase-4 and caspase-5 in the non-canonical inflammasome found that caspase-5 was cleaved in a time-dependent manner (Vigano et al. 2015). For caspase-4 and caspase-5 activity, we measured cleaved products at early exposure to RNA40. We observed an increase in caspase-5 cleavage as early as 5 and 8 hr after RNA40 treatment, while caspase-4 did not show cleavage at

these early time points (Figures 7A and 7B). Although caspase-4 does not appear to be activated at early time points, we did note an overall increase in both caspase-4 and caspase-5 protein expression at 24 hr post-RNA40 exposure, confirming that both caspases are upregulated during IL-1β processing (Figure 8A and 8B). Taken together, these results suggest that caspase-5, but not caspase-4, is activated during the early stages of RNA40 exposure and that the effector role of the non-canonical inflammasome may be initiated early after priming with stimuli.

TLR7/8 stimulation induces IL-1β release in human primary macrophages.

Our observation that RNA40 is able to induce IL-1β led us to ask if non-canonical inflammasome activation and IL-1β production in human macrophages was specific to only RNA40, or if it could also be induced upon general TLR7/8 stimulation. To elucidate this, we treated macrophages for 24 hours with CL097, a known TLR7/8 agonist that is derived from the imidazoquinoline compound, R848. We observed a significant induction of IL-1β without the addition of a secondary, exogenous signal, suggesting that general stimulation of TLR7/8 may only be needed to activate the non-canonical inflammasome in human primary macrophages (Figure 9).

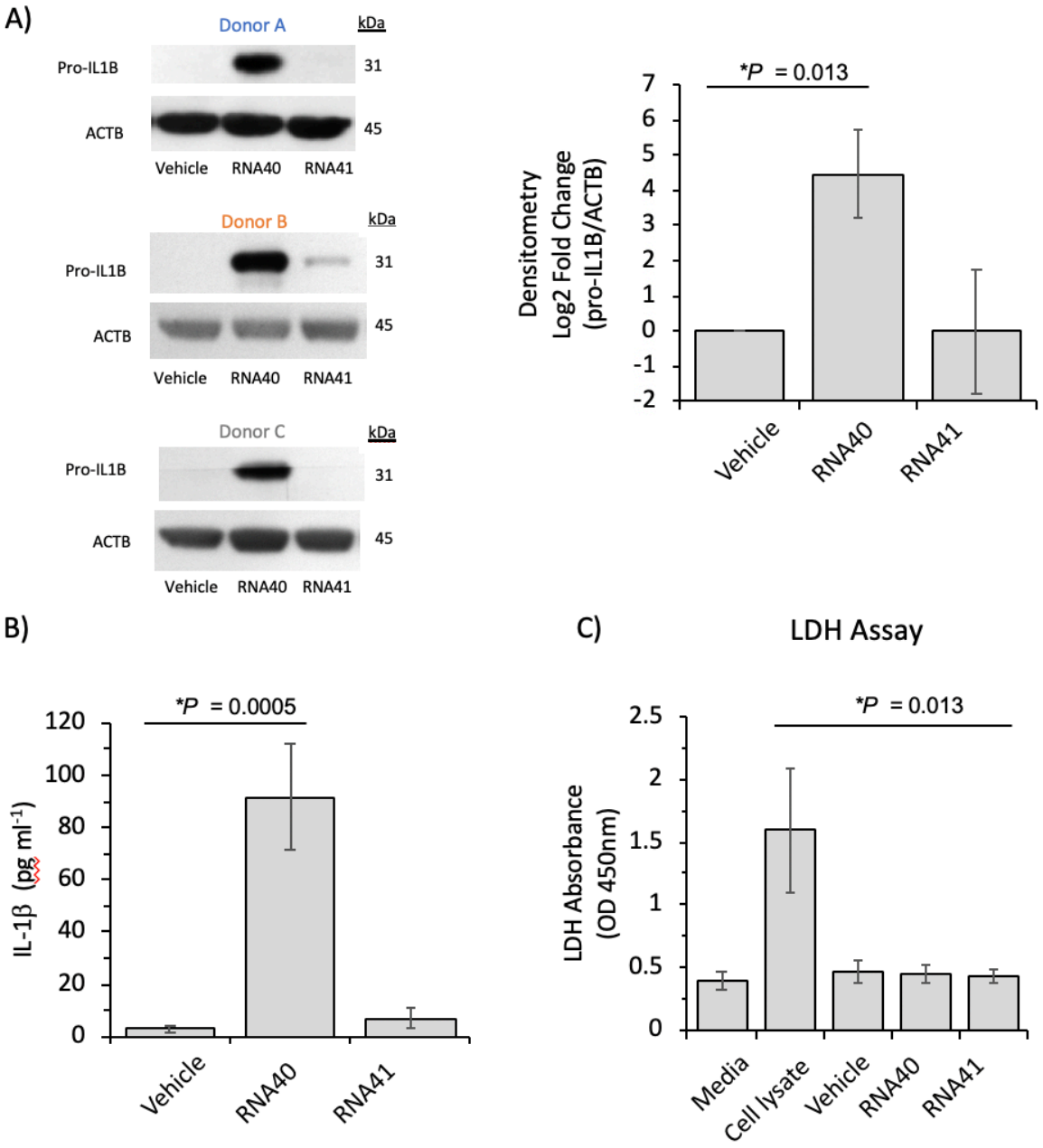

Figure 1. RNA40 induces pro-IL-β production and IL-1β maturation in human primary macrophages without causing cell death. A) Macrophages were treated with 5μg/mL of RNA40 for 24hr. Culture supernatants were collected and mature IL-1β was determined by ELISA (n = 4). B) Macrophages were exposed to RNA40 for 24hr before culture supernatants were collected and cell death was measured using an LDH assay (n = 4). C) Levels of pro-IL1B were measured using western blot analysis and assessed using densitometric analysis (n = 3). Statistical significance was measured using student's t-test. Error bars are representative of the standard error.

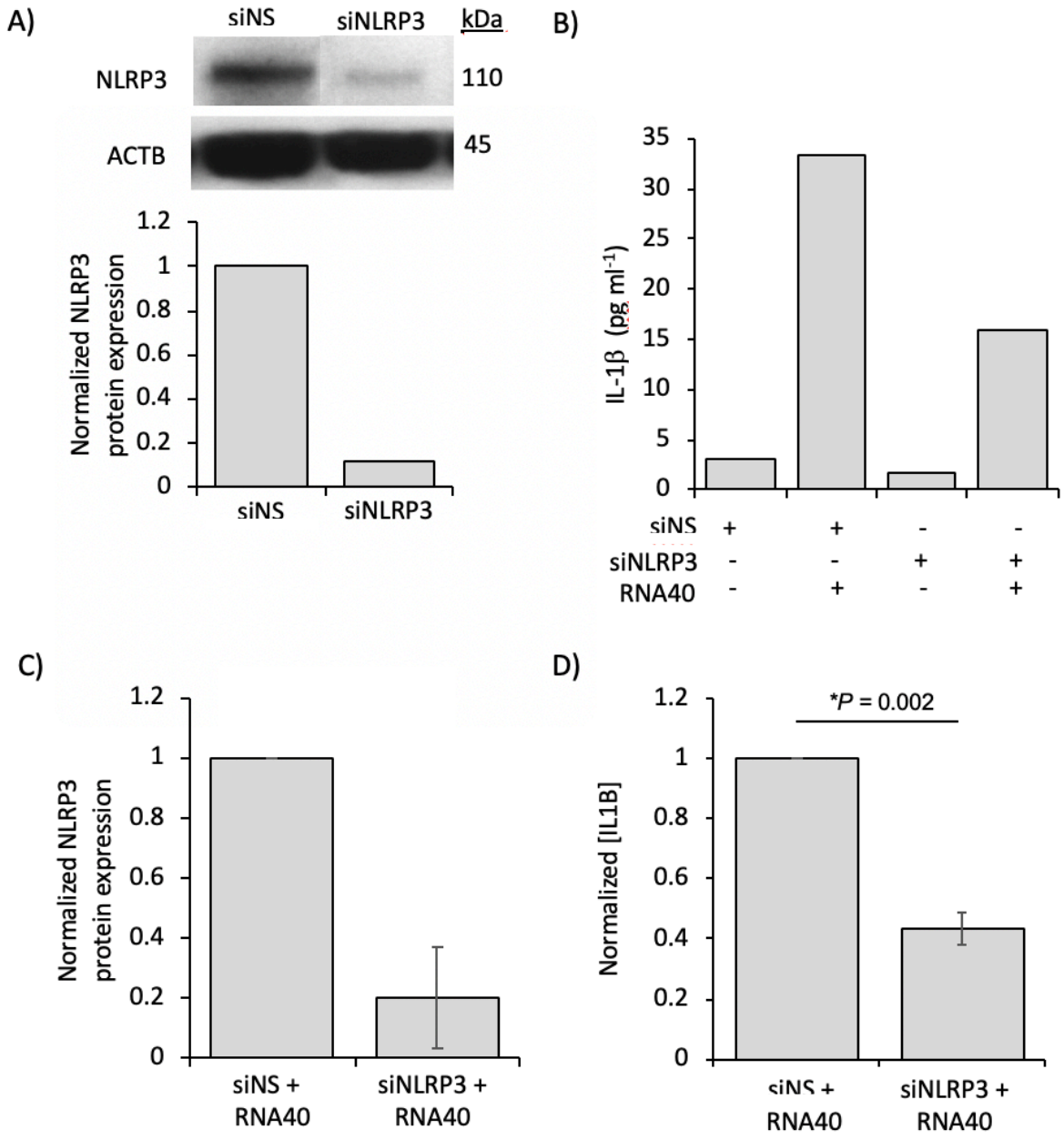

Figure 2. RNA40 utilizes the NLRP3 inflammasome to induce IL-1β in human primary macrophages. Macrophages were treated with NLRP3 siRNA for 48hr prior to exposure with 5μg/mL of RNA40 for 24hr. A) A representative western blot from one donor was chosen to show the knockdown of NLRP3 protein expression (n = 1). B) The culture supernatant from that donor was collected and levels of IL-1β were measured using ELISA (n = 1). The experiment was repeated, and C) the silencing of NLRP3 protein expression along with D) the levels of IL-1β were normalized to the vehicle in each donor (n=4). Statistical significance was measured using student's t-test. Error bars are representative of the standard error.

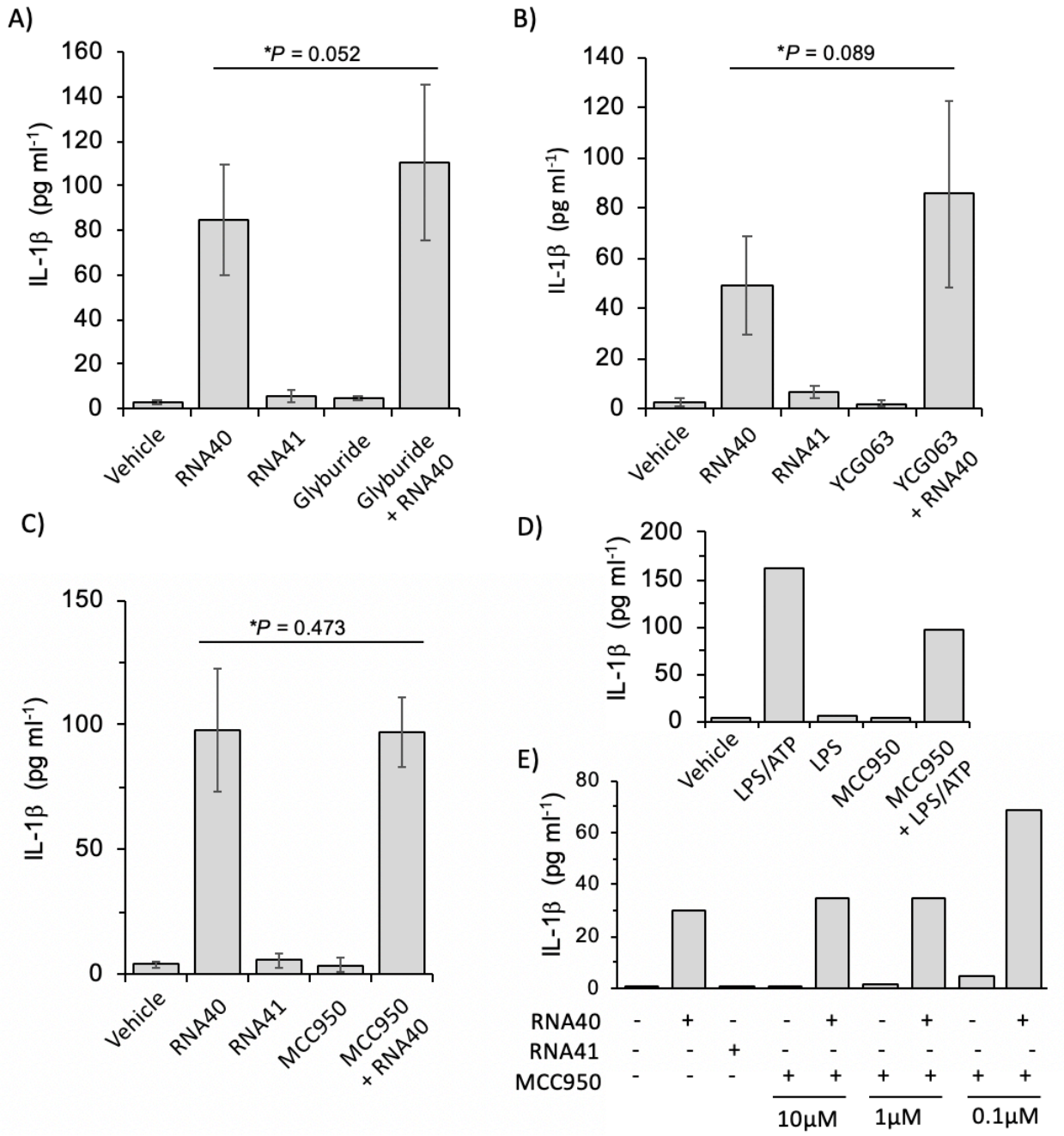

Figure 3. Conventional NLRP3 inhibitors, Glyburide, ROS inhibitor YCG063, and MCC950 do not reduce levels of IL-1β in macrophages stimulated with RNA40. Macrophages were pre-treated with A) Glyburide (100μM), a potassium efflux inhibitor, B) YCG063 (20μM), a ROS inhibitor, and C) MCC950 (10μM), a common NLRP3 inhibitor, for 1hr prior to 5μg/mL of RNA40 exposure for 24 hr (n = 4). D) Macrophages were treated with MCC950 for 1hr followed by LPS (10ng/mL) for 3hr and ATP (5mM) for 24hr (n=1). E) Increasing concentrations of MCC950 were added to macrophages prior to 5μg/mL of RNA40 treatment for 24hr (n =1). Cell culture supernatants were collected and IL-1β was measured using ELISA. Statistical significance was measured using student's t-test. Error bars are representative of the standard error.

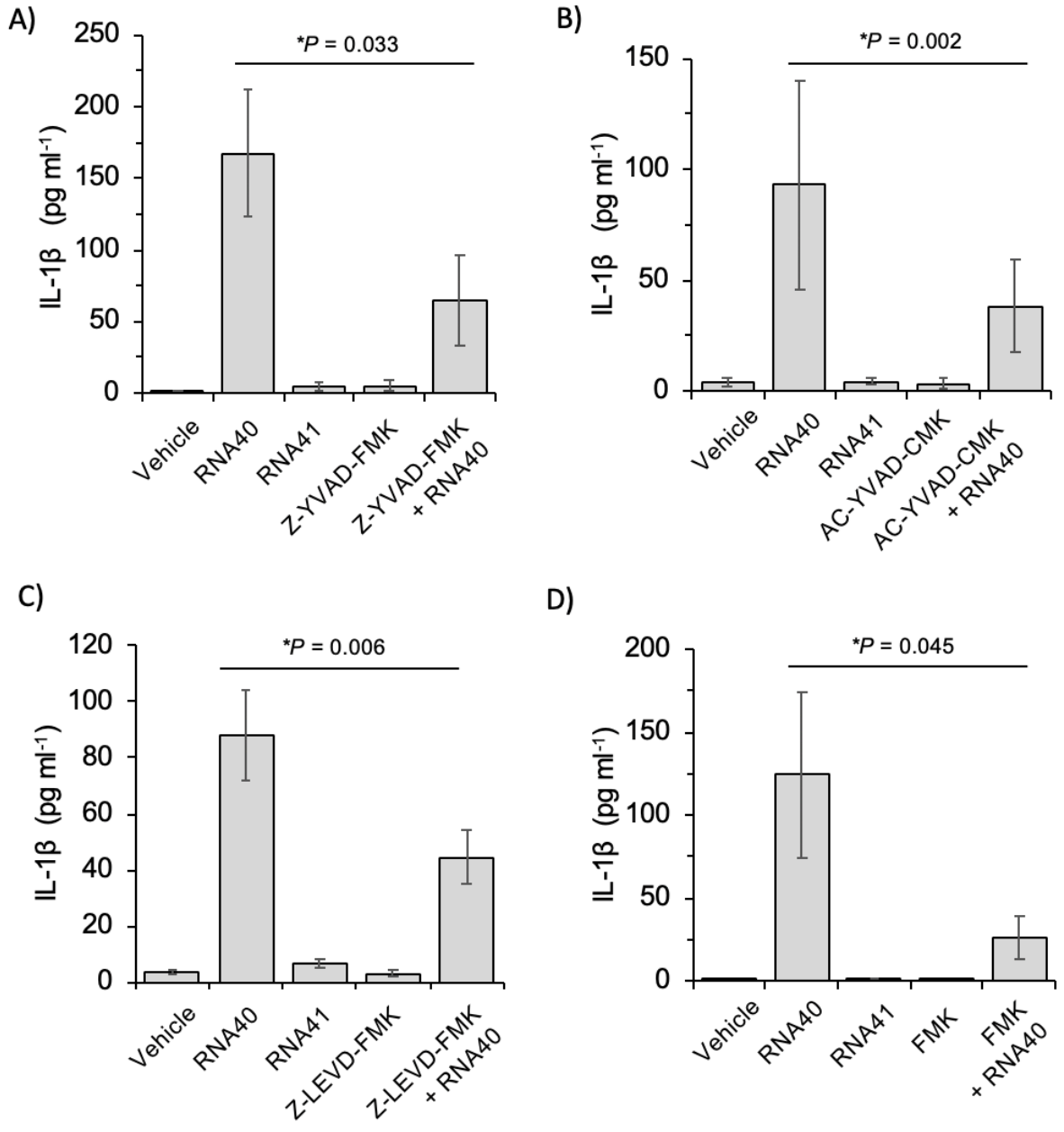

Figure 4. Human macrophages exposed to RNA40 induce IL-1β in a caspase-dependent manner. Macrophages were treated with A) a pan-caspase inhibitor, Z-YVAD-FMK (100μM), or B) a caspase-1 inhibitor, AC-YVAD-CMK (100μM), for 1hr prior to stimulation with RNA40 (n=3 and 5, respectively). Macrophages were also treated with C) a caspase-4 and caspase-5 inhibitor, LEVD (10μM), or D) a caspase-4 and caspase-1 inhibitor, FMK (10μM), for 1hr prior to stimulation with 5μg/mL of RNA40 (n=5 and 4, respectively). Culture supernatants were collected and mature IL-1β was determined by ELISA. Statistical significance was measured using student's t-test. Error bars are representative of the standard error.

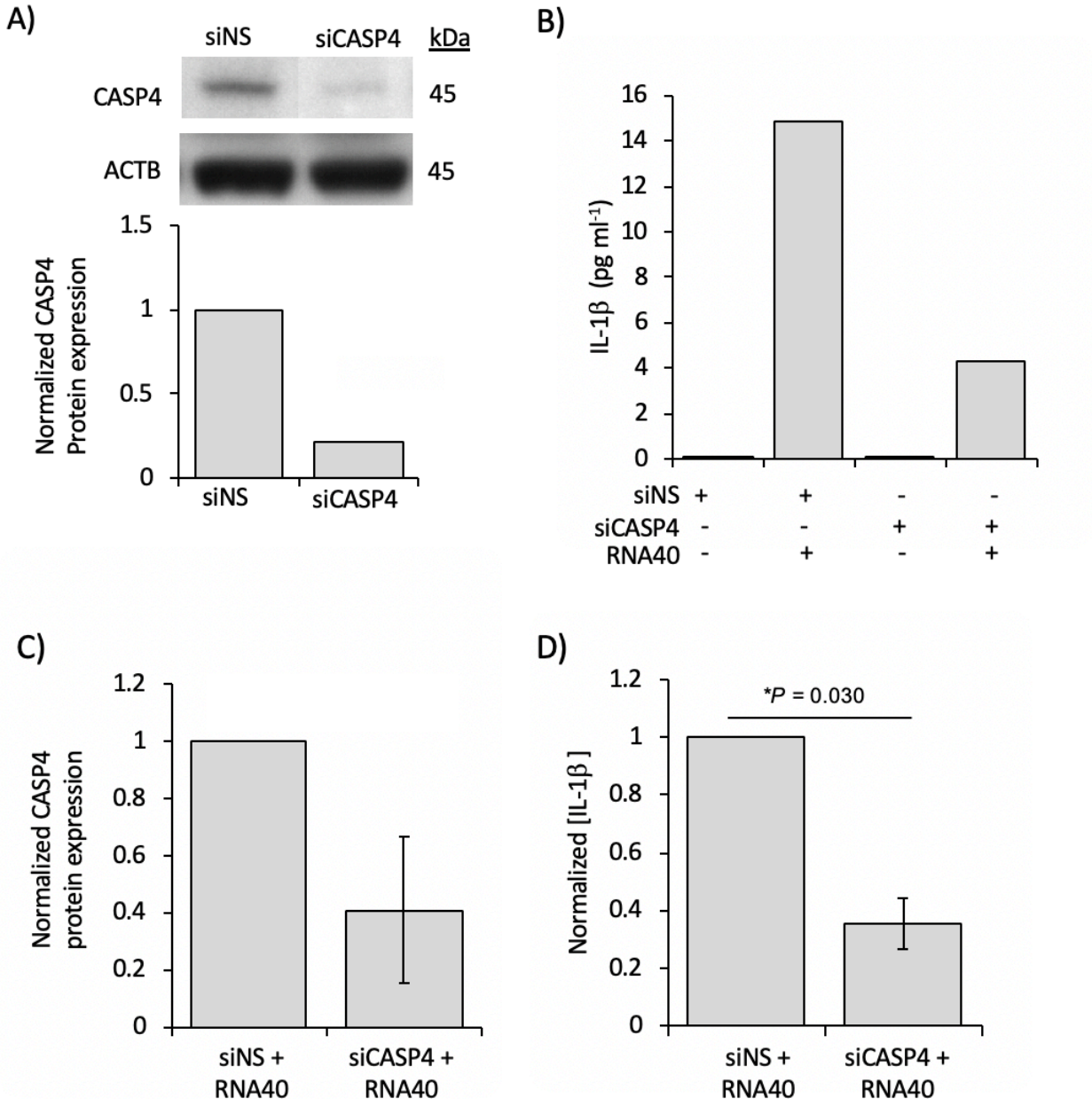

Figure 5. Caspase-4 is involved in an unconventional NLRP3 mediated IL-1β production. Macrophages were treated with siRNA for 48hr prior to treatment with 5μg/mL of RNA40 for 24hr. siNS treated cells were used as a scrambled siRNA control. A) A representative western blot from one donor was chosen to show the knockdown of CASP4 protein expression (n = 1). B) The culture supernatant from that donor was collected and levels of IL-1β were measured using ELISA (n = 1). The experiment was repeated, and C) the silencing of CASP4 protein expression along with D) the levels of IL-1β were normalized to the vehicle in each donor (n=2). Statistical significance was measured using student's t-test. Error bars are representative of the standard error.

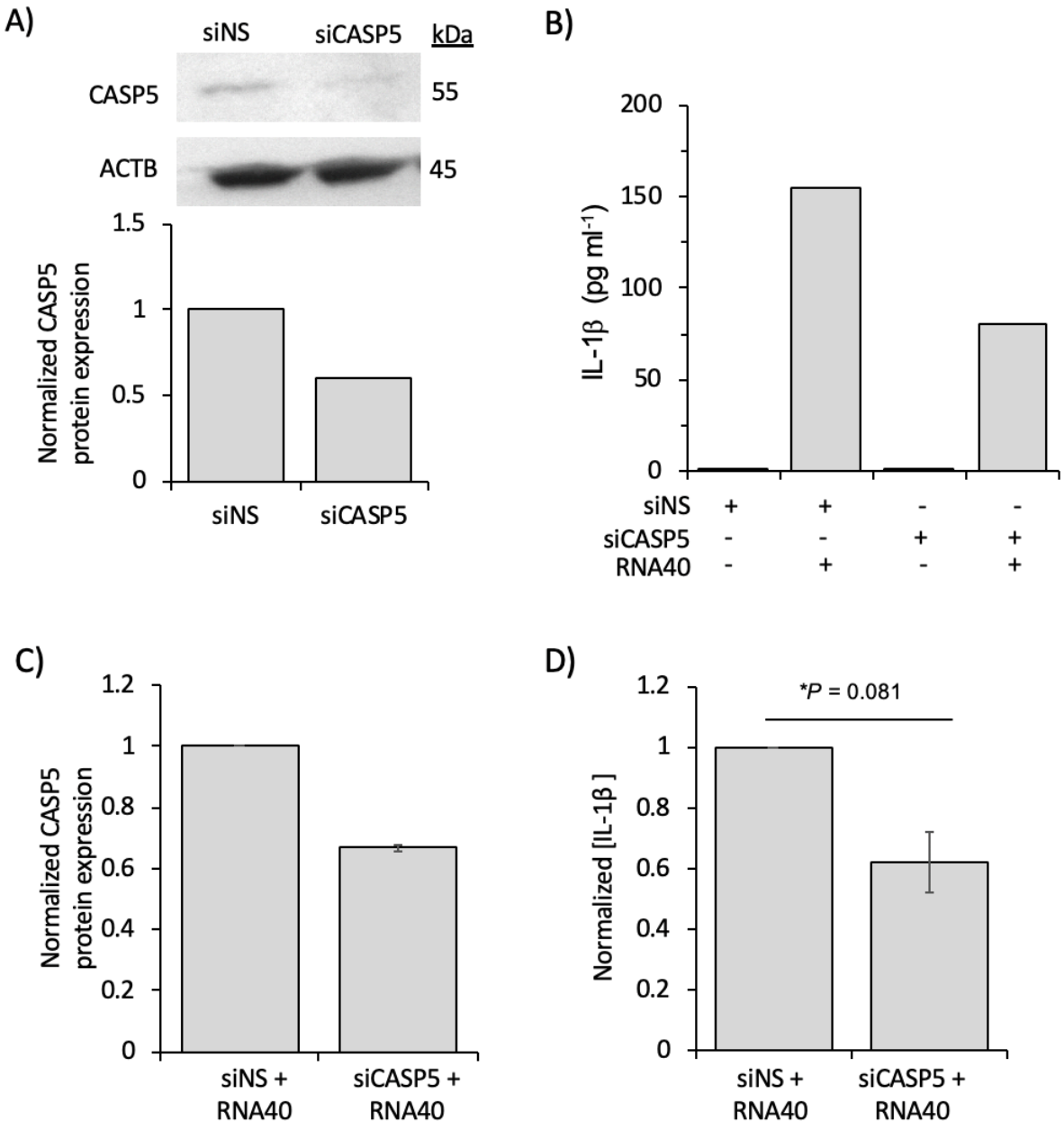

Figure 6. Caspase-5 is involved in an unconventional NLRP3-mediated IL-1β production. Macrophages were treated with siRNA for 48hr prior to treatment with 5μg/mL of RNA40 for 24hr. siNS treated cells were used as a scrambled siRNA control. A) A representative western blot from one donor was chosen to show the knockdown of CASP5 protein expression (n = 1). B) The culture supernatant from that donor was collected and levels of IL-1β were measured using ELISA (n = 1). The experiment was repeated, and C) the silencing of CASP5 protein expression along with D) the levels of IL-1β were normalized to the vehicle in each donor (n=2). Statistical significance was measured using student's t-test. Error bars are representative of the standard error.

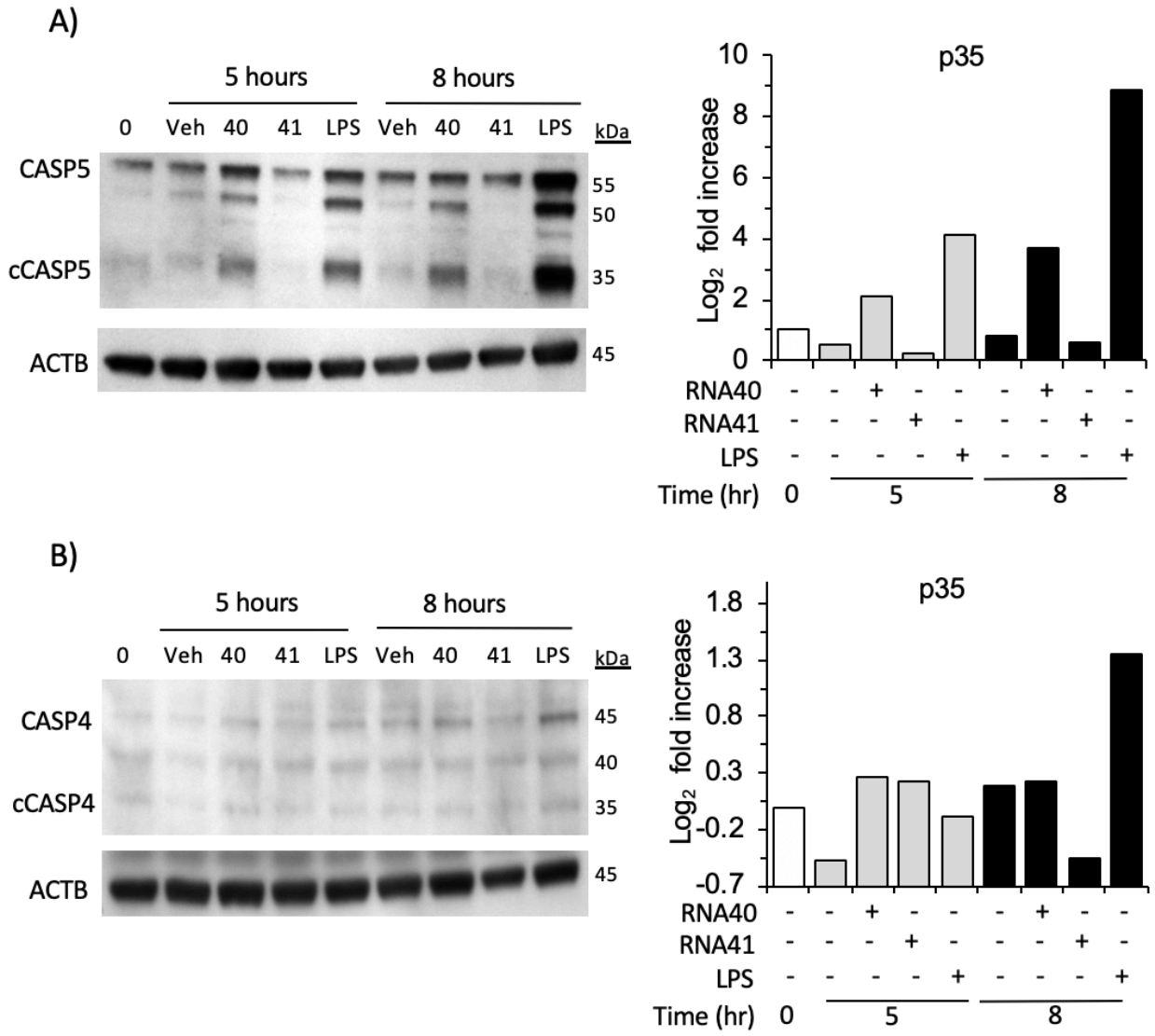

Figure 7. RNA40 triggers processing of caspase-5, but not caspase-4, in human macrophages. Prior to collecting the cell lysates, macrophages were treated with 5µg/mL of RNA40 for 5hr and 8hr. Western blot analysis was performed and A) caspase-5 and B) caspase-4 protein cleavage was analyzed using densitometric analysis. Blots are a representative of blot of 2 independent experiments.

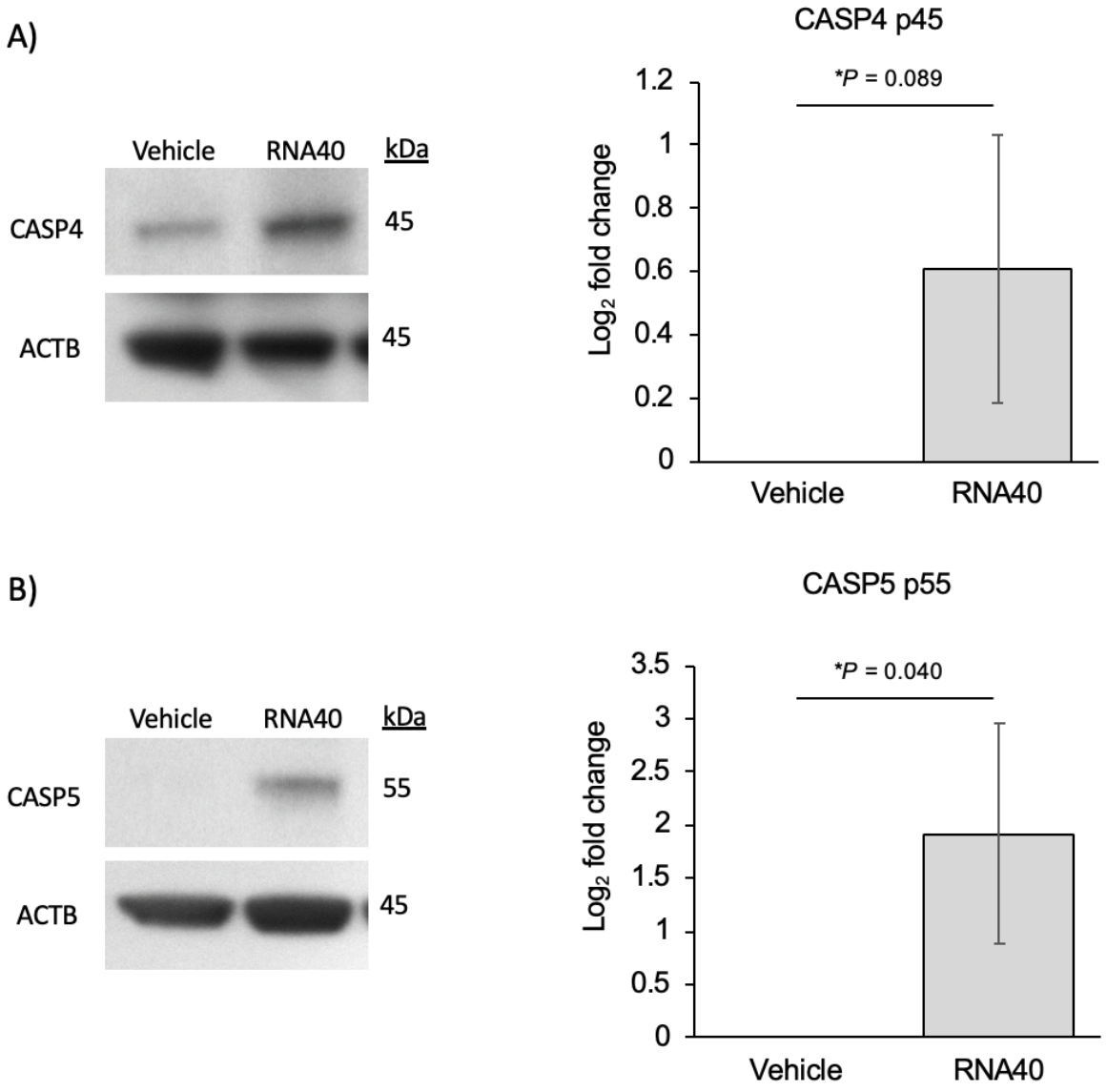

Figure 8. RNA40 induces protein expression of caspase-4 and caspase-5. Macrophages were treated with 5μg/mL of RNA40 for 24hr. Cell lysates were collected and western blotting was performed for A) caspase-4 and B) caspase-5 protein expression (n=3). Error bars are representative of the standard error.

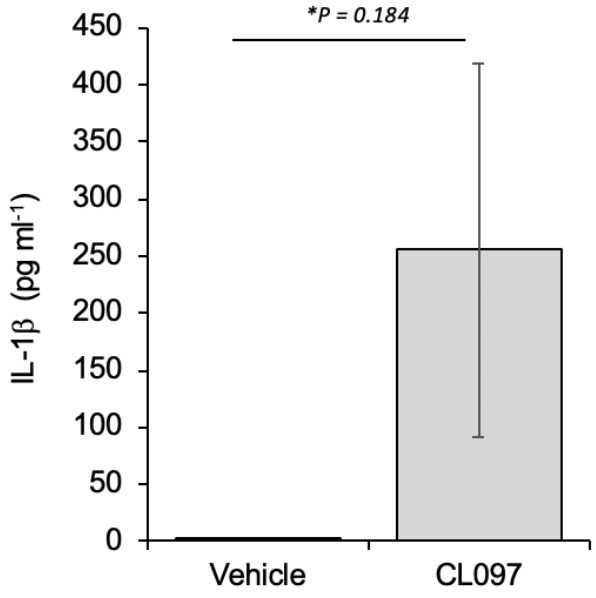

Figure 9. TLR8 agonist, CL097, induces IL-1β in human macrophages. Macrophages were treated with 5μg/mL of CL097 for 24hr. Cell supernatants were collected and IL-1β release was measured through ELISA (n = 2). Error bars are representative of the standard error. Statistical significance was measured using student's t-test.

DISCUSSION

HIV pathogenesis is associated with immune activation and chronic inflammation despite effective viral suppression from antiretroviral treatment (Palella *et al.* 2006; Collaboration TATC, 2008; Gill *et al.* 2010). HIV-infected cells release HIV antigens, including RNA, and can induce inflammatory responses in neighboring cells and increase levels of proinflammatory cytokines, such as IL-1β (Nath *et al.* 1999; Deeks *et al.* 2013; Sereti *et al*. 2017). Elevated levels of pro-inflammatory cytokines contribute to non-AIDS related inflammatory diseases and are associated with inflammasome activity (Pu *et al.* 2003; Le Saout *et al.* 2012). Although there is evidence that HIV Tat induces NLRP3 activity in the brain, the mechanism of the inflammatory response in human macrophages to HIV RNA requires further investigation (Walsh *et al.* 2014). Here, we report that HIV-derived GU-rich RNA40 from the HIV LTR region activates the non-canonical NLRP3 inflammasome pathway, involving caspase-4 and caspase-5 to process IL-1β in macrophages independently of potassium efflux and ROS production.

While the canonical NLRP3 inflammasome pathway requires a two-step activation process, the non-canonical inflammasome does not require a secondary signal as its stimuli both prime and activate the NLRP3 (Schroder *et al.* 2010). To date, cytosolic LPS is the most widely discussed activator of the non-canonical NLRP3 inflammasome while few other stimuli have been reported (Diamond *et al.* 2015). Our observation that RNA40 is able to activate the NLRP3 inflammasome to induce IL-1β in the absence of a secondary, exogenous stimulus provides evidence that RNA40 could be an activator of the non-canonical inflammasome. Our findings with CL097 further support that RNA40 is activating the non-canonical inflammasome through TLR7/8, suggesting that non-

canonical inflammasome activation in human macrophages is not restricted to RNA40 but is rather indicative of a broad TLR7/8 stimulation. However, further investigation is required to provide insight into TLR7/8's role in the unconventional NLRP3 pathway. There may also be limitations to the specificity of cell type for RNA40 to act as an activator of the non-canonical inflammasome, as RNA40 was able to induce the non-canonical inflammasome in human primary macrophages, but induced the canonical inflammasome in THP-1 cells (Guo *et al.* 2014). It is likely that THP-1 cells, being an immortalized cell line, differs genetically and phenotypically from human primary macrophages, and further emphasizes the need to use primary cells when evaluating the pathogenesis of pathogens *in vitro*. A

NLRP3 inflammasome activation has also been associated with caspase-mediated cell death through pyroptosis. In canonical inflammasome activation, caspase-1 directly cleaves pore-forming protein gasdermin D (GSDMD) to become active, subsequently inducing lysis due to differences in osmotic pressure (Shi *et al.* 2015). In non-canonical inflammasome activation, caspase-4 and caspase-11 directly bind and activate GDSMD to induce pyroptosis in human and murine cells, respectively (Kayagaki *et al.* 2015). However, not all NLRP3 inflammasome activity is associated with cell death. This is demonstrated by an alternative inflammasome activation that relies on TLR4-TRIF-RIPK1-FADD-CASP8 signaling to activate the NLRP3 inflammasome without causing pyroptosis (Gaidt *et al.* 2016). Although caspase-4 is involved in RNA40-associated inflammasome activation as shown through our *CASP4* silencing experiments, caspase-4 does not appear to play a role in cell death when macrophages are exposed to HIV RNA40 as we observed no pyroptosis. Macrophages may also be

intrinsically refractory to HIV RNA-induced cell-death, a phenomenon consistent with macrophage survival despite HIV-infection in contrast to the massive cell death observed in HIV-infected CD4 T-cells (Van Lint *et al*. 2013). Moreover, macrophage survival in spite of HIV RNA detection may also contribute to a continuous secretion of pro-inflammatory cytokines and ultimately, systemic chronic inflammation in HIV infected individuals despite effective antiretroviral therapy.

Many studies reporting on the non-canonical inflammasome have been performed in murine models and focus on murine caspase-11, while only a few studies have looked at human caspase-4/-5 in human primary cells (Kayagaki *et al.* 2013; Broz *et al.* 2013; Yang *et al.* 2015). Although caspase-4/-5 are considered to be ancestrally derived from the murine caspase-11 gene, distinct functions and features between the murine and human non-canonical caspases exist (Vigano *et al.* 2013). One such difference is that caspase-4 is constitutively expressed in humans while caspase-11 must be induced by a stimulus to be expressed in mouse cells (Shi *et al.* 2014). Another is that the activation of caspase-5 in human monocytes has been reported to occur in the first 5 hours of exposure to intracellular LPS, while no such activation of caspase-11 at early time points has been found (Vigano *et al.* 2015). Our findings are consistent with an early effector mechanism for caspase-5 as we demonstrate that RNA40 is able to induce proteolytic cleavage of caspase-5 as early as 5 hours post-treatment in human macrophages. This is specific to caspase-5 as RNA40 does not induce proteolytic cleavage of caspase-4 at early exposure to RNA40, a phenomenon again consistent in human monocytes treated with intracellular LPS (Vigano *et al.* 2015). However, both caspase-4 and caspase-5 activity are required for IL-1β production in macrophages exposed to RNA40 as caspase-

4 and caspase-5 inhibitors and silencing of their expression led to a reduction in IL-1β levels. Our data indicating that caspase-5 is cleaved at early exposure to RNA40 suggests that caspase-5 becomes functional upon TLR7/8-stimulation even before caspase-4 is activated. This can be further elucidated with follow-up studies that focus on caspase-5 cleavage after TLR7/8 stimulation with CL097 and other TLR7/8 agonists. Moreover, since little has been discovered regarding the role and function of caspase-5, investigating the activity of caspase-5 upstream of IL-1β transcription may elucidate its role in TLR signaling and non-canonical inflammasome activity.

As previously mentioned, a defining characteristic of the canonical NLRP3 inflammasome pathway is its reliance on a secondary signal to activate inflammasome complex assembly. In bacterial infections, LPS is sensed by TLR4 to induce the transcription of inflammasome genes, while PAMPs or DAMPs, such as nigericin or ATP, serve as the secondary stimulus (Stutz *et al.* 2009). PAMPs/DAMPs initiate NLRP3 oligomerization by disrupting cell membrane integrity or by inducing mitochondrial dysfunction (Parham, 2015). The resulting downstream effects, ion flux and (ROS production, respectively, then lead to inflammasome assembly (Munoz-Planillo *et al.* 2013; Abais *et al.* 2015). In contrast, the signaling for inflammasome oligomerization in the unconventional NLRP3 inflammasome pathway is not fully understood since it does not require a secondary stimulus. Our findings with RNA40 indicate that IL-1β processing does not rely on potassium efflux or ROS production in our non-canonical inflammasome pathway. This may indicate that RNA40 signaling through TLR7/8 is sufficient to induce inflammasome assembly. Moreover, RNA40-induced non-canonical inflammasome activity may share similar mechanistic activities to the “alternative inflammasome”, as

described by Gaidt *et al.* 2016, which does not require potassium efflux for NLRP3 inflammasome activation. It is worthwhile to note that use of glyburide to inhibit potassium efflux and YCG063 to inhibit ROS production did lead to a slight overall increase in IL-1β production in macrophages exposed to RNA40. It is possible that inhibiting potassium efflux and ROS production exacerbated the NLRP3 inflammasome and caused an increase in IL-1β production. However, further experiments to investigate this hypothesis are needed.

In recent years, a potent and selective inhibitor for the NLRP3 inflammasome has been discovered and used to inhibit inflammasome activity in many studies. Although it has been shown that this inhibitor, which is known as MCC950, does not inhibit other inflammasomes such as AIM2, NLRC4, and NLRP1, its mechanism of action is still not fully known (Coll *et al.* 2015). Some studies have suggested that MCC950 may block caspase-1 activity and subsequent IL-1β production by inhibiting the cleavage of caspase-1 and IL-1β (Ismael *et al.* 2018). However, it is still uncertain if MCC950 inhibits further upstream of caspase-1 activation and targets inflammasome oligomerization or events downstream of secondary signaling in conventional inflammasome activation. Interestingly, our findings indicate that MCC950 did not inhibit RNA40-induced non-canonical inflammasome activity in human macrophages despite NLRP3-dependency. To our knowledge, we are the first to report that MCC950 does not inhibit NLRP3 inflammasome activation. It could be possible that RNA40 not only induces the NLRP3 inflammasome but also other inflammasomes, leading MCC950 to be less effective in reducing IL-1β production. Even then, however, MCC950 would theoretically still have an inhibitory effect on at least NLRP3's contribution to IL-1β maturation and release. On the

other hand, MCC950 may work to inhibit a process that is present in other canonical and non-canonical pathways but is not present in our system of RNA40-induced NLRP3 inflammasome activation. If this were the case, RNA40-induced NLRP3 inflammasome activation may provide critical insight into the mechanism of action for MCC950. Moreover, MCC950 failing to inhibit IL-1β production by RNA40-induced non-canonical NLRP3 inflammasome activation may also be cell-specific. A study using RNA40 in human primary microglial cells demonstrated effective reduction in IL-1β levels with MCC950 when RNA40 activated the canonical NLRP3 inflammasome (Rawat *et al.* 2018). This could indicate that MCC950 preferentially works in certain cells over others. However, it may also be that RNA40 induced the conventional NLRP3 pathway in the human microglial cells while it activated the unconventional NLRP3 mechanism in human macrophages; thus, it may be differences in the conventional versus unconventional NLRP3 activity that render MCC950's function variable. Nonetheless, further investigation into MCC950's failure to reduce IL-1β levels in macrophages exposed to RNA40 may provide relevant insight into the mechanism of action for MCC950.

Considering inflammation and hyperimmune activation are markers for HIV pathogenesis, the study of inflammasomes is still a relatively new field of study in HIV inflammation (Paiardini *et al.* 2013; Sereti *et al.* 2016). However, there is emerging evidence for an involvement of NLRP3 in HIV pathogenesis. Specifically, NLRP3 may promote HIV disease progression during gut-associated lymphoid tissue (GALT) destruction as its overexpression is speculated to favor HIV replication and cell death (Feria *et al.* 2018). Dysfunctional inflammasomes may also promote HIV pathogenesis as NLRP3 polymorphisms may increase susceptibility to HIV infection (Pontillo *et al.* 2012;

Zhang *et al.* 2015). Although HIV infection predisposes infected persons to inflammatory diseases associated with NLRP3 activation such as irritable bowel syndrome, cardiovascular disease, and neurological diseases, further investigation into the role of NLRP3 in HIV inflammation is required to elucidate therapeutic targets that may mitigate or decrease incidence of non-AIDS related diseases (Lazaridis *et al.* 2017; Zhou *et al.* 2018; Song *et al.* 2017). Moreover, studying the NLRP3 inflammasome in HIV drug abuse may also elucidate therapeutic targets for HIV co-morbidity and neuroinflammation (Xu *et al.* 2017). NLRP3 and IL-1β expression is upregulated in human macrophages infected with HIV in the presence of cocaine as compared to HIV infected macrophages alone (Alturi *et al.* 2016). This suggests an enhanced inflammatory response via the NLRP3 inflammasome in HIV drug users that may contribute to accelerated HIV pathogenesis. Hence, investigating inflammasomes in HIV infection, pathogenesis, and co-morbidity is essential to understanding HIV-associated chronic inflammation.

Concluding remarks

Although the non-canonical inflammasome is mechanistically different from the canonical inflammasome in its activation pathway, it is not fully understood. To our knowledge, we are the first to show that HIV RNA40 activates the non-canonical inflammasome through a caspase-4 and caspase-5 dependent pathway. Our findings not only elucidate a novel inflammatory mechanism associated with HIV inflammation and pathogenesis, but also provide insights into a caspase-4 and caspase-5 mediated non-canonical NLRP3 inflammasome that is independent of pyroptosis, potassium efflux, and ROS production. Inhibiting the unconventional inflammasome may be a therapeutic target to ameliorate HIV-associated chronic inflammation.

REFERENCES

1. Abais, J. M., Xia, M., Zhang, Y., Boini, K. M., & Li, P. L. (2015). Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector?. *Antioxidants & redox signaling*, *22*(13), 1111-29.

2. Atluri, V. S., Pilakka-Kanthikeel, S., Garcia, G., Jayant, R. D., Sagar, V., Samikkannu, T., Yndart, A., … Nair, M. (2016). Effect of Cocaine on HIV Infection and Inflammasome Gene Expression Profile in HIV Infected Macrophages. *Scientific reports*, *6*, 27864.

3. Antiretroviral Therapy Cohort Collaboration (2010). Causes of death in HIV-1-infected patients treated with antiretroviral therapy, 1996-2006: collaborative analysis of 13 HIV cohort studies. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, *50*(10), 1387-96.

4. Broz, P., & Monack, D. M. (2013). Noncanonical inflammasomes: caspase-11 activation and effector mechanisms. *PLoS pathogens*, *9*(2), e1003144.

5. Cheung, K. T., Sze, D. Myuen, Chan, K. H. & Leung, P. H. (2017). Involvement of caspase-4 in IL-1 beta production and pyroptosis in human macrophages during dengue virus infection. *Immunobiology,* 223, 356–364.

6. Chivero, E. T., Guo, M. L., Periyasamy, P., Liao, K., Callen, S. E., & Buch, S. (2017). HIV-1 Tat Primes and Activates Microglial NLRP3 Inflammasome-Mediated Neuroinflammation. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *37*(13), 3599-3609.

7. Coll, R. C., Robertson, A. A., Chae, J. J., Higgins, S. C., Muñoz-Planillo, R., Inserra, M. C., Vetter, I., Dungan, L. S., Monks, B. G., Stutz, A., Croker, D. E., Butler, M. S., Haneklaus, M., Sutton, C. E., Núñez, G., Latz, E., Kastner, D. L., Mills, K. H., Masters, S. L., Schroder, K., Cooper, M. A., … O'Neill, L. A. (2015). A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nature medicine*, *21*(3), 248-55.

8. Antiretroviral Therapy Cohort Collaboration (2008). Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *Lancet (London, England)*, *372*(9635), 293-9.

9. Deeks, S. G., Tracy, R., & Douek, D. C. (2013). Systemic Effects of Inflammation on Health during Chronic HIV Infection. *Immunity*, *39*(4), 633–645.

10. Diamond, C. E., Khameneh, H. J., Brough, D., & Mortellaro, A. (2015). Novel perspectives on non-canonical inflammasome activation. *ImmunoTargets and therapy*, *4*, 131-41.

11. Feria MG, Taborda NA, Hernandez JC, Rugeles MT. HIV replication is associated to inflammasomes activation, IL-1beta, IL-18 and caspase-1 expression in GALT and peripheral blood. *PLoS One, 13*(4), e0192845.

12. Gaidt MM, Ebert TS, Chauhan D, Schmidt T, Schmid-Burgk JL, Rapino F, Robertson AA, Cooper MA, Graf T, Hornung V. (2016). Human monocytes engage an alternative inflammasome pathway. *Immunity, 44*(4), 833–846.

13. Guo, H., Gao, J., Taxman, D. J., Ting, J. P. Y., & Su, L. (2014). HIV-1 Infection Induces Interleukin-1β Production via TLR8 Protein-dependent and NLRP3 Inflammasome Mechanisms in Human Monocytes. *The Journal of Biological Chemistry*, 289(31), 21716–21726.

14. Hazuda, D.J., Strickler, J., Simon, P., and Young, P.R. (1991). Structure-function mapping of interleukin 1 precursors. Cleavage leads to a conformational change in the mature protein. *The Journal of Biological Chemistry*, 266, 7081–7086.

15. Ismael S., Nasoohi S., Ishrat T. (2018). MCC950, the Selective Inhibitor of Nucleotide Oligomerization Domain-Like Receptor Protein-3 Inflammasome, Protects Mice against Traumatic Brain Injury. *Journal of Neurotrauma, 1;35*(11), 1294-1303.

16. Jo, E. K., Kim, J. K., Shin, D. M., & Sasakawa, C. (2015). Molecular mechanisms regulating NLRP3 inflammasome activation. *Cellular & molecular immunology*, *13*(2), 148-59.

17. Kayagaki, N., Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, Miyake K, Zhang J, Lee WP, Muszyński A, Forsberg LS, Carlson RW, Dixit VM. (2013). Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science*, 341, 1246–1249.

18. Lazaridis, N., & Germanidis, G. (2018). Current insights into the innate immune system dysfunction in irritable bowel syndrome. *Annals of gastroenterology*, *31*(2), 171-187.

19. Lee, B. L., Stowe, I. B., Gupta, A., Kornfeld, O. S., Roose-Girma, M., Anderson, K., Warming, S., Zhang, J., Lee, W. P., … Kayagaki, N. (2018). Caspase-11 auto-proteolysis is crucial for noncanonical inflammasome activation. *The Journal of experimental medicine*, *215*(9), 2279-2288.

20. Catalfamo, M., Le Saout, C., & Lane, H. C. (2012). The role of cytokines in the pathogenesis and treatment of HIV infection. *Cytokine & growth factor reviews*, *23*(4-5), 207-14.

21. Martinon, F., Burns, K., Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of pro-IL-1β. *Molecular Cell, 10*(2), 417-26.

22. Mamik M.K., Hui E., Branton W.G., McKenzie B.A., Chisholm J., Cohen E.A., Power C. (2017). HIV-1 Viral Protein R Activates NLRP3 Inflammasome in Microglia: implications for HIV-1 Associated Neuroinflammation. *Journal of Neuroimmune Pharmacology, 12*(2), 233-248.

23. Meir-Shafrir, K., & Pollack, S. (2012). Accelerated Aging in HIV Patients. *Rambam Maimonides Medical Journal*, *3*(4), e0025.

24. Menu, P., & Vince, J. E. (2011). The NLRP3 inflammasome in health and disease: the good, the bad and the ugly. *Clinical and experimental immunology*, *166*(1), 1-15.

25. Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., & Núñez, G. (2013). K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity*, *38*(6), 1142-53.

26. Nath A., Conant K., Chen P., Scott C., Major E.O. (1999). Transient Exposure to HIV-1 Tat Protein Results in Cytokine Production in Macrophages and Astrocytes*. Journal of Biological Chemistry*, 274, 17098–17102.

27. Paiardini, M., & Müller-Trutwin, M. (2013). HIV-associated chronic immune activation. *Immunological reviews*, *254*(1), 78-101.

28. Palella F.J., Jr, Baker R.K., Moorman A.C., Chmiel J.S., Wood K.C., Brooks J.T., Holmberg S. (2006). Mortality in the highly active antiretroviral therapy era: changing causes of death and disease in the HIV outpatient study. *Journal of Acquired Immune Deficiency Syndromes, 43(*1), 27–34.

29. Parham, Peter. (2015). The Immune System. 4th edition. 55.

30. Pontillo A., Oshiro TM, Girardelli M, Kamada AJ, Crovella S, Duarte AJ. (2012). Polymorphisms in inflammasome' genes and susceptibility to HIV-1 infection. *Journal of Acquired Immune Deficiency Syndromes, 59*(2), 121-5.

31. Pu H., Tian J., Flora G., Lee Y.W., Nath A., Hennig B., Toborek M. (2003). HIV-1 Tat protein upregulates inflammatory mediators and induces monocyte invasion into the brain*. Molecular and Cellular Neuroscience., 24*, 224-237.

32. Ren, K., & Torres, R. (2009). Role of interleukin-1β during pain and inflammation. *Brain Research Reviews*, *60*(1), 57–64.

33. Rawat P., Teodorof-Diedrich C., Spector SA. (2018). Human immunodeficiency virus Type-1 single-stranded RNA activates the NLRP3 inflammasome and impairs

autophagic clearance of damaged mitochondria in human microglia. *Glia,* Epub ahead of print.

34. Rose, H., Hoy, J., Woolley, I., Tchoua, U., Bukrinsky, M., Dart, A., & Sviridov, D. (2007). HIV infection and high density lipoprotein metabolism. *Atherosclerosis*, *199*(1), 79-86.

35. Sanchez, G., Xu, X., Chermann, J. C., & Hirsch, I. (1997). Accumulation of defective viral genomes in peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected individuals. *Journal of Virology*, *71*(3), 2233-40.

36. Schroder K., Tschopp J. (2010). The inflammasomes. *Cell,* 140:821–832.

37. Sereti I, Altfeld M. (2016). Immune activation and HIV: an enduring relationship. *Current Opinions in HIV and AIDS*, *11*(2), 129-30.

38. Sereti, I., Krebs, S. J., Phanuphak, N., Fletcher, J. L., Slike, B., Pinyakorn, S., … Utay, N. S. (2017). Persistent, Albeit Reduced, Chronic Inflammation in Persons Starting Antiretroviral Therapy in Acute HIV Infection. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *64*(2), 124–131.

39. Shi J., Zhao Y., Wang Y., Gao W., Ding J., Li P., Hu L., Shao F. (2014). Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature,* 514(7521), 187-192

40. Shi J., Zhao Y., Wang Y., Shi X., Wang Y., Huang H., Zhuang Y., Cai T., Wang F., Shao F. (2015). Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature, 526*(7575), 660-665.

41. Song, L., Pei, L., Yao, S., Wu, Y., & Shang, Y. (2017). NLRP3 Inflammasome in Neurological Diseases, from Functions to Therapies. *Frontiers in Cellular Neuroscience*, *11*, 63.

42. Stutz, A., Golenbock, D. T., & Latz, E. (2009). Inflammasomes: too big to miss. *The Journal of Clinical Investigation*, 119(12), 3502–3511.

43. Triant, V. A., Lee, H., Hadigan, C., & Grinspoon, S. K. (2007). Increased acute myocardial infarction rates and cardiovascular risk factors among patients with human immunodeficiency virus disease. *The Journal of clinical endocrinology and metabolism*, *92*(7), 2506-12.

44. UNAIDS Data Tables | 2018. Published by the Joint United Nations Programme on HIV/AIDS (UNAIDS). Accessed February 2018.

45. Van Lint, C., Bouchat, S., & Marcello, A. (2013). HIV-1 transcription and latency: an update. *Retrovirology*, *10*, 67.

46. van Sighem A., Danner S., Ghani A.C., Gras L., Anderson R.M., de Wolf F. (2005). Mortality in patients with successful initial response to highly active antiretroviral therapy is still higher than in non-HIV-infected individuals. *Journal Acquired Immune Deficiency Syndrome, 40*(2), 212–218.

47. Viganò E., Diamond C., Spreafico R., Balachander A., Sobota R., Mortellaro A. (2015). Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nature Communications*, *6*, 8761.

48. Viganò E., Mortellaro A. (2013). Caspase-11: The driving factor for noncanonical inflammasome. *European Journal of Immunology, 43*(9), 240-245.

49. Walsh, J. G., Reinke, S. N., Mamik, M. K., McKenzie, B. A., Maingat, F., Branton, W. G., Broadhurst, D. I., … Power, C. (2014). Rapid inflammasome activation in microglia contributes to brain disease in HIV/AIDS. *Retrovirology*, *11*, 35.

50. Xu, E., Liu, J., Wang, X., & Xiong, H. (2017). Inflammasome in drug abuse. *International journal of physiology, pathophysiology and pharmacology*, *9*(6), 165-177.

51. Yang J., Zhao Y., Shao F. (2015). Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity. *Current Opinion in Immunology, 32,* 78-83.

52. Zhang Q., Fan H.W., Zhang J.Z., Wang Y.M., Xing H.J. (2015). NLRP3 rs35829419 polymorphism is associated with increased susceptibility to multiple diseasese in human. *Genetics and Molecular Research, 14*(4), 13968-13980.

53. Zhou, W., Chen, C., Chen, Z., Liu, L., Jiang, J., Wu, Z., Zhao, M., … Chen, Y. (2018). NLRP3: A Novel Mediator in Cardiovascular Disease. *Journal of immunology research*, *2018*, 5702103.