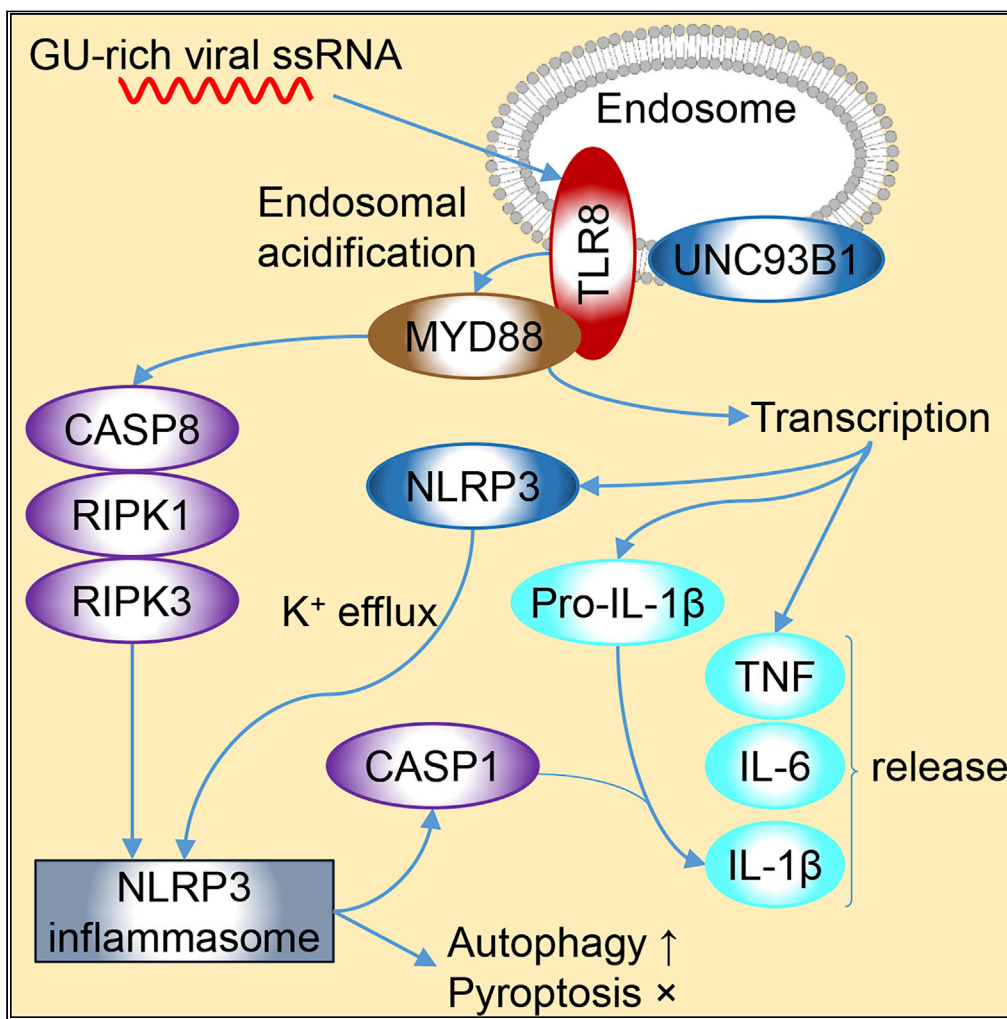


Article

SARS-CoV-2, SARS-CoV-1, and HIV-1 derived ssRNA sequences activate the NLRP3 inflammasome in human macrophages through a non-classical pathway



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Highlights

GU-rich ssRNA induces IL-1β, TNF, and IL-6 from human macrophages through TLR8

SARS-CoV-2 RNA elicits a greater inflammatory response than SARS-CoV-1 or HIV-1 RNA

SARS-CoV-2 RNA elicits TLR8-mediated IL-1β production in the absence of pyroptosis

TLR8-mediated IL-1β release is dependent on CASP8, K+ efflux, NLRP3, and autophagy



Article

SARS-CoV-2, SARS-CoV-1, and HIV-1 derived ssRNA sequences activate the NLRP3 inflammasome in human macrophages through a non-classical pathway

Grant R. Campbell,^{1,4,*} Rachel K. To,^{1,3} Jonathan Hanna,¹ and Stephen A. Spector^{1,2,*}

SUMMARY

Macrophages promote an early host response to infection by releasing pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), TNF, and IL-6. The bioactivity of IL-1 β is classically dependent on NLRP3 inflammasome activation, which culminates in caspase-1 activation and pyroptosis. Recent studies suggest a role for NLRP3 inflammasome activation in lung inflammation and fibrosis in both COVID-19 and SARS, and there is evidence of NLRP3 involvement in HIV-1 disease. Here, we show that GU-rich single-stranded RNA (GU-rich RNA) derived from SARS-CoV-2, SARS-CoV-1, and HIV-1 trigger a TLR8-dependent pro-inflammatory cytokine response from human macrophages in the absence of pyroptosis, with GU-rich RNA from the SARS-CoV-2 spike protein triggering the greatest inflammatory response. Using genetic and pharmacological inhibition, we show that the induction of mature IL-1 β is through a non-classical pathway dependent on caspase-1, caspase-8, the NLRP3 inflammasome, potassium efflux, and autophagy while being independent of TRIF (TICAM1), vitamin D3, and pyroptosis.

INTRODUCTION

Coronavirus-19 disease (COVID-19) is a highly contagious and sometimes fatal respiratory illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a betacoronavirus closely related to SARS-CoV-1, which causes severe acute respiratory syndrome (SARS). COVID-19 symptoms can include fever and pneumonia, and in a small percentage of cases an acute respiratory distress syndrome (ARDS) reminiscent of the cytokine release syndrome (CRS)-induced ARDS and secondary hemophagocytic lymphohistiocytosis previously observed in both SARS and Middle East respiratory syndrome (Huang et al., 2020). High concentrations of pro-inflammatory cytokines, including interleukin (IL) 1 β , IL-6, and tumor necrosis factor (TNF), characterize this "cytokine storm," which can lead to alveolar damage, pulmonary fibrinolysis, and pulmonary injury. Increased levels of IL-6 correlate with increased mortality, and IL-6, IL-10, and TNF concentrations inversely correlate with lymphocyte count, suggesting that SARS-CoV-2-mediated CRS potentially hampers the adaptive immune response (Pedersen and Ho, 2020). Human immunodeficiency virus type 1 (HIV-1) pathogenesis is also associated with immune activation and chronic inflammation despite effective viral suppression from antiretroviral treatment (Antiretroviral Therapy Cohort Collaboration, 2008), which contributes to non-AIDS related inflammatory diseases activity (Catalano et al., 2012).

Virally infected cells release antigens, including RNA (collectively termed pathogen-associated molecular patterns [PAMPs]), which can induce inflammatory responses in neighboring cells such as the transcription and release of proinflammatory cytokines. A variety of pattern recognition receptors, including toll-like receptors (TLR) recognize these PAMPs. There are 10 human TLRs, each of which recognizes a distinct group of PAMPs. TLRs 1, 2, 4, 5, 6, and 10 are located at the plasma membrane and recognize bacterial membrane components, whereas TLRs 3, 7, 8, and 9 are predominantly located within endosomes and recognize microbial nucleic acids. TLR8 is phylogenetically and structurally related to TLR7, is expressed as a homodimer in macrophages and myeloid dendritic cells, and is activated by viral or bacterial uridine-rich short single-stranded RNA during infection or through phagocytosis (Cervantes et al., 2013; Heil et al., 2004; Moen et al., 2019; Vierbuchen et al., 2017). Upon ligand-induced conformational change that is required for its activation (Zhu et al., 2009), TLR8 associates and interacts with the adapter protein MYD88, which recruits the IL-1 receptor-associated kinases, leading to the nuclear factor- κ B-dependent transcription of

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numerous proinflammatory mediators including IL-6, IL-12, IL-27, TNF, interferon (IFN) γ , and IL-1 β (Akira et al., 2006; Ghosh et al., 2006; Vierbuchen et al., 2017).

IL-1 β is synthesized as an inactive pro-protein that must be proteolytically cleaved by caspase (CASP)-1 to generate the active pro-inflammatory cytokine (Sutterwala et al., 2006). One of the complexes that participates in this process is the NLRP3 (NLR family pyrin domain containing 3) inflammasome. This multimeric cytosolic protein complex canonically requires two distinct steps for activation (Elliott and Sutterwala, 2015). The first is a priming step resulting in the production of pro-IL-1 β , and transcription and posttranslational modification of NLRP3. The second remains elusive; a common denominator of its activation is the cytosolic efflux of K⁺ (Muñoz-Planillo et al., 2013), which appears to be necessary and sufficient to trigger NLRP3 to interact with and recruit PYCARD (pyrin domain [PYD] and C-terminal caspase-recruitment domain [CARD] containing protein; umquhile ASC). The CARD of PYCARD then recruits the pro-CASP1 zymogen via its CARD to form an NLRP3-PYCARD-pro-CASP1 complex. The pro-CASP1 zymogen then self-activates through proteolytic cleavage. The enzymatically active heterodimer of CASP1 then cleaves pro-interleukin-1 β into mature IL-1 β . Upon activation, the NLRP3 inflammasome classically promotes pyroptosis, an inflammatory form of cell death. Little is known regarding the role of TLR8 in the innate immune response against infection with SARS-CoV-2, SARS-CoV-1, and HIV-1. In this study, we investigated the mechanism through which GU-rich single-stranded RNA sequences (GU-rich RNA) derived from SARS-CoV-2, SARS-CoV-1, and HIV-1 trigger pro-inflammatory responses from human macrophages.

RESULTS

SARS-CoV-2-, SARS-CoV-1-, and HIV-1-derived GU-rich RNA treatment of macrophages results in IL-1 β release through a TLR8-dependent mechanism

The importance of identifying the activity and mechanism(s) by which innate immunity is activated by viral single-stranded RNA has become magnified as serious COVID-19, SARS, and AIDS are all associated with a dramatic increase in inflammatory cytokines. To address this, we identified a GU-rich RNA from the spike protein of SARS-CoV-2 (RNA649 [5'-GUCAGAGUGUGUACUUG-3'; position 24649–24665 nt; accession number: NC_045512.2]) (Wu et al., 2020), and together with RNA524 (also derived from the spike protein of SARS-CoV-1) (Li et al., 2013) and RNA40 (Heil et al., 2004), we evaluated their ability to induce IL-1 β production from primary human macrophages (see transparent methods). All three GU-rich RNAs induced the expression of pro-IL-1 β and the release of mature IL-1 β in a dose-dependent manner (Figures 1A and 1B). Of note, macrophages exposed to RNA from SARS-CoV-2 (RNA649) released significantly greater levels of IL-1 β than RNAs from SARS-CoV-1 ($p = 0.03$) and HIV-1 ($p = 0.04$) (RNA649 > RNA524 > RNA40). Additionally, GU-rich RNA-mediated IL-1 β secretion was evident in the absence of an additional exogenously added stimulus for the NLRP3 inflammasome such as what is required for lipopolysaccharide (LPS)-mediated IL-1 β production (Gaidt et al., 2016). We observed a similar pattern for the release of TNF and IL-6, with RNA649 inducing the greatest amount of both cytokines ($p < 0.05$; Figure 1A). Importantly, neither RNA41 nor RNA649A (inactive derivatives of RNA40 and RNA524/649, respectively, in which adenosine replaces all uracil nucleotides) had any effect on either the expression or secretion of IL-1 β , TNF, or IL-6. Moreover, in agreement with our previous studies, we observed no increase in cell death with any of the GU-rich RNAs (Figure 1C) (Campbell and Spector, 2012; Campbell et al., 2019).

As all human nucleic acid-specific TLRs (TLR3, 7, 8, and 9) are located in the endosomal compartment and rely upon UNC93B1 for their trafficking from the endoplasmic reticulum (ER) to the endosomes (Lee and Barton, 2014), we used RNAi for *UNC93B1* (Figure S1) to confirm that viral GU-rich RNAs are recognized by endosomal TLRs. *UNC93B1* silencing abolished GU-rich RNA-mediated production of IL-1 β , TNF, and IL-6 (Figure 1D). We next investigated whether either of TLR7 or TLR8 was required for GU-rich RNA-mediated production of IL-1 β , TNF, and IL-6 using RNAi for *TLR7* and *TLR8* (Figure S1). *TLR8* silencing abolished GU-rich RNA-induced IL-1 β , TNF, and IL-6 production from macrophages, whereas *TLR7* silencing had no effect (Figure 1D). Additionally, RNAi for *MYD88* (Figure S1) also attenuated IL-1 β , TNF, and IL-6 production (Figure 1D). Conversely, RNAi for *TICAM1* (toll-like receptor adaptor molecule 1; TRIF) had no effect on IL-1 β , TNF, and IL-6 production, indicating that TLR3 is not involved in GU-rich RNA signaling. Thus the *TLR8/MYD88* pathway is required for viral GU-rich RNA-induced IL-1 β , TNF, and IL-6 expression from human macrophages similar to what was previously observed for RNA derived from the archaeon *Methanosphaera stadtmanae* in transdifferentiated BLaER1 cells (Vierbuchen et al., 2017).

Preventing endosomal acidification inhibits endosomal TLR activation and signaling (Hart et al., 2005; Rutz et al., 2004). Therefore, we blocked endosomal acidification using chloroquine and observed that this

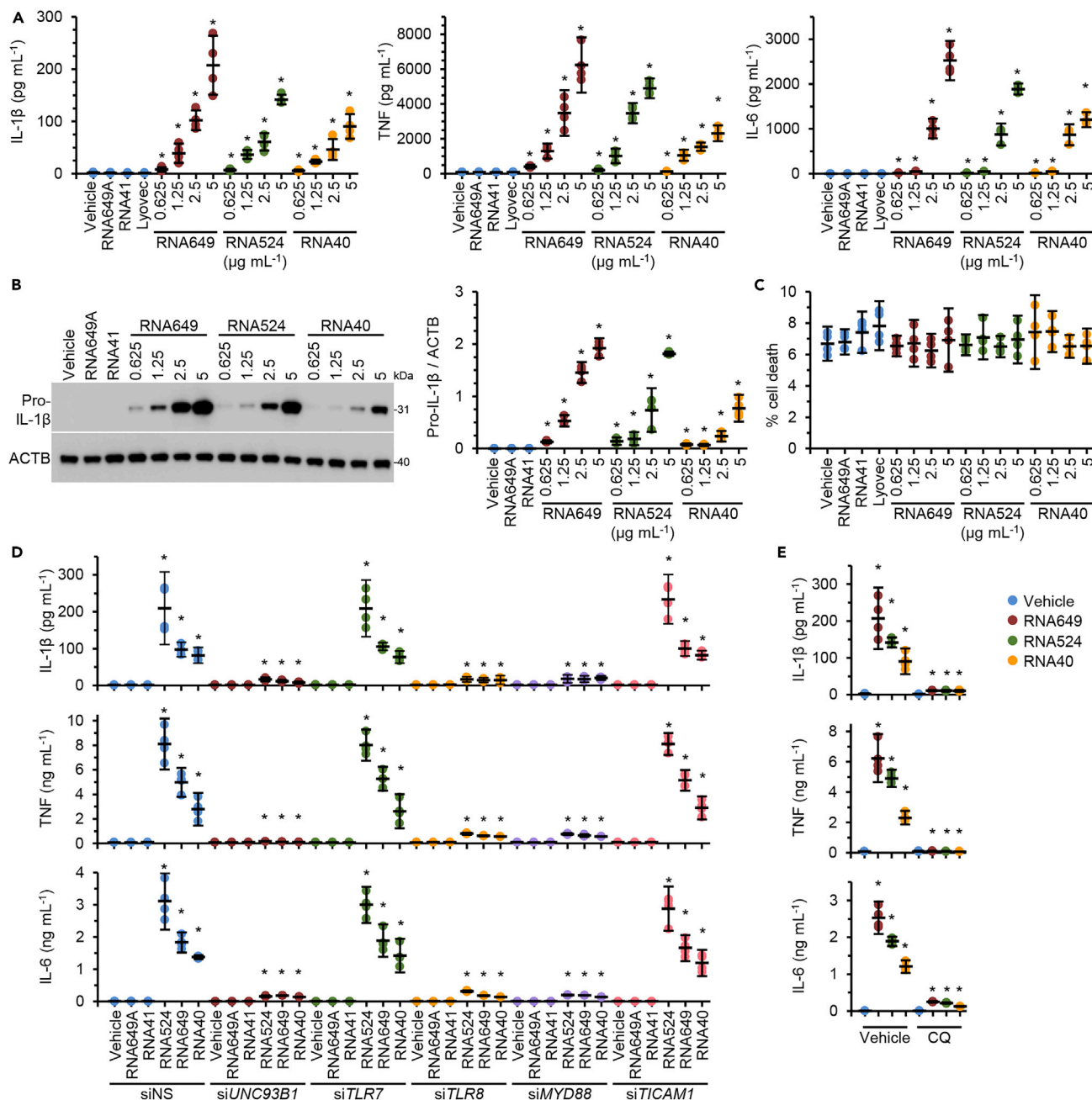


Figure 1. GU-rich RNAs induce the release of proinflammatory cytokines in the absence of pyroptosis through TLR8 signaling

(A–C) Macrophages were treated for 24 h with GU-rich RNA. GU-rich RNA sequences are shown in Table S1. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. $*p < 0.05$. (A) Cell supernatants were collected and analyzed for cytokines. (B) Left, representative western blots of pro-IL-1 β and ACTB. Right, densitometric analysis of blots. (C) Aliquots of supernatants were spectrophotometrically tested for LDH.

(D) Macrophages were transfected with *UNC93B1* small interfering RNA (siRNA) (siUNC93B1), *TLR7* siRNA (siTLR7), *TLR8* (siTLR8), *MYD88* (siMYD88), *TICAM1* (siTICAM1), or scrambled siRNA (siNS) for 48 h then treated with 5 μ g mL⁻¹ GU-rich RNA or controls for 24 h. Supernatants were collected and analyzed for cytokine secretion. Silencing is shown in Figure S1. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. $*p < 0.05$. (E) Macrophages were pre-treated with 10 μ M chloroquine (CQ) or vehicle control (vehicle) for 2 h and then treated for 24 h with 5 μ g mL⁻¹ GU-rich RNA. Cell supernatants were collected and analyzed for cytokines. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. $*p < 0.05$.

inhibited GU-rich RNA-induced IL-1 β , TNF, and IL-6 production (Figure 1E). However, chloroquine also blocks autophagic flux, and previous studies have demonstrated that autophagy is involved in both the AIM2-inflammasome- (Wang et al., 2014) and NLRP3-inflammasome-induced secretion of IL-1 β (Dupont

et al., 2011). Moreover, as both RNA40 and the imidazoquinoline CL097 promote autophagic responses in human macrophages through a TLR8-, beclin-1 (BECN1)-, and vitamin D3-dependent mechanism (Campbell and Spector, 2012), we investigated the role for autophagy in GU-rich RNA-mediated IL-1 β release. Although we observed the suppression of GU-rich RNA-mediated autophagy under vitamin D3-deficient conditions in accordance with our previous data (Campbell and Spector, 2012) (Figure 2A), we observed no significant decrease in secreted IL-1 β , TNF, and IL-6 from those cells indicating that cytokine secretion is not dependent on induced autophagy (Figure 2B). To address whether constitutive autophagic flux is required for IL-1 β secretion, we silenced ATG5, which inhibits not only GU-rich RNA-mediated autophagy (Figure 2C) but also constitutive autophagy (Ye et al., 2018). Interestingly, ATG5 silencing markedly reduced the secretion of IL-1 β induced by GU-rich RNA, but it did not impact pro-IL-1 β synthesis or the release of TNF or IL-6 (Figures 2C and 2D), indicating that constitutive autophagy is required for IL-1 β release from macrophages.

SARS-CoV-2-, SARS-CoV-1-, and HIV-1-derived GU-rich RNA treatment of macrophages results in IL-1 β release through the NLRP3 inflammasome

We detected CASP1 p20 and IL-1 β p17 release in the supernatant of primary human macrophages after 24-h treatment with GU-rich RNA indicative of inflammasome activation and IL-1 β processing and release (Figure 3A). To confirm a role for CASP1, we used Ac-YVAD-cmk, a specific inhibitor of CASP1. We show that Ac-YVAD-cmk completely suppressed IL-1 β release induced by GU-rich RNA but had no effect on IL-6 release (Figure 3B). In addition to cleaving IL-1 family cytokines, active CASP1 cleaves the cytosolic protein gasdermin D (GSDMD). Upon cleavage, the N-terminal 30-kDa fragment of GSDMD forms a pore in the plasma membrane, which leads to lysis of the cell through pyroptosis (Shi et al., 2015). As lactate dehydrogenase (LDH) release is a marker of pyroptosis and we observed no increase in macrophage LDH release post-GU-rich RNA exposure (Figure 1C), we examined whether GU-rich RNA treatment of macrophages resulted in GSDMD processing. In contrast to LPS treatment, which triggers GSDMD-dependent release of IL-1 β from human macrophages, GU-rich RNA exposure did not induce the processing of GSDMD to its 30-kDa fragment (Figure 3A).

To identify the type of inflammasome activated by GU-rich RNA, we used RNAi to silence *NLRP3*. *NLRP3* silencing abolished IL-1 β , but not IL-6, release from GU-rich RNA-treated macrophages (Figure 3C), indicating that that GU-rich RNA activates the NLRP3 inflammasome in macrophages. As potassium efflux is a common feature of NLRP3 inflammasome activation (Muñoz-Planillo et al., 2013), we next applied glibenclamide, a sulfonylurea drug that acts upstream of NLRP3 and downstream of the P2X7 receptor, a purinergic type 2 ligand-gated ion channel that allows the passage of molecules of up to 900 Da, Na⁺ and Ca²⁺ influx and K⁺ efflux, and is used to inhibit LPS-induced NLRP3 inflammasome-mediated CASP1 activation, secretion of IL-1 β , and pyroptosis in human macrophages (Lamkanfi et al., 2009). Glibenclamide had no effect on reducing RNA40-mediated IL-1 β secretion despite severely attenuating the LPS-stimulated release of IL-1 β (Figure 3D). We next applied an extracellular high potassium solution (KCl), which directly inhibits K⁺ efflux (Muñoz-Planillo et al., 2013). We observed a dose-dependent inhibition of GU-rich RNA-mediated IL-1 β release and CASP1 cleavage in macrophages pre-treated with KCl (Figure 3E). These data indicate that K⁺ efflux is necessary and sufficient for GU-rich RNA-mediated CASP1 activation and IL-1 β release from macrophages, and that it is independent of the glibenclamide-sensitive K⁺ efflux.

The alternative inflammasome pathway is also involved in GU-rich RNA NLRP3-mediated release of IL-1 β from macrophages

As GU-rich RNA-induced IL-1 β processing and secretion is devoid of the classic NLRP3 inflammasome characteristic of pyroptosis and is released from living cells, and as LPS triggers an alternative NLRP3 inflammasome activation pathway in human monocytes in the absence of a secondary stimulus of ATP K⁺ efflux (Gaidt et al., 2016), we investigated the possibility that the alternative pathway using necroptotic mediators may play a role in GU-rich RNA-induced IL-1 β secretion from human macrophages using inhibitors for CASP8 (Z-IETD-FMK), RIPK1 (necrostatin-1) and RIPK3 (GSK'872). Inhibition of CASP8 decreased GU-rich RNA-induced IL-1 β without inducing cell death (Figure 4). Inhibition of RIPK1 had no significant reduction of GU-rich RNA-induced IL-1 β , whereas inhibition of RIPK3 increased cell death and inhibited GU-rich RNA-induced IL-1 β . When we co-treated macrophages with Z-IETD-FMK and GSK'872, we observed no change in cell death in the vehicle-treated macrophages, but in the GU-rich RNA-treated cells, we observed a significant reduction in both cell death and IL-1 β production. Of note, no combination of these inhibitors perturbed GU-rich RNA-stimulated IL-6 production (Figure 4). Collectively, these data indicate that, unlike LPS

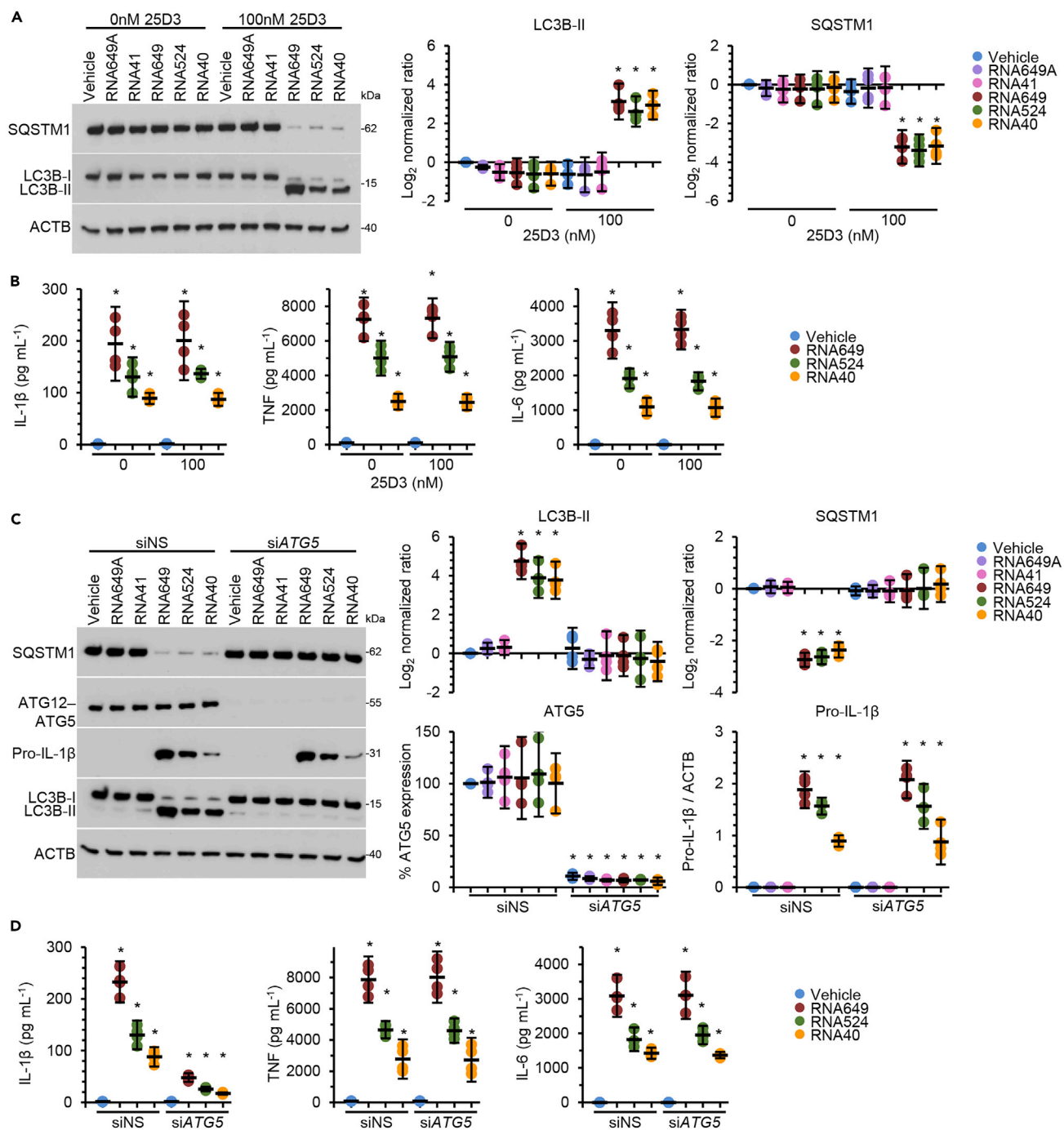


Figure 2. GU-rich RNA-induced IL-1 β secretion is dependent on autophagy

(A) Macrophages were treated for 24 h with 5 $\mu\text{g mL}^{-1}$ GU-rich RNA in the presence or absence of vitamin D3 (calcifediol; 25-hydroxycholecalciferol [25D3]). Left, representative western blots of LC3B isoforms and SQSTM1. Right, densitometric analysis of blots. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. * $p < 0.05$.

(B) Supernatants from cells in (A) were collected and analyzed for cytokines. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. * $p < 0.05$.

(C) Macrophages transfected with ATG5 siRNA (siATG5) or scrambled siRNA (siNS) were treated for 24 h with 5 $\mu\text{g mL}^{-1}$ GU-rich RNA in macrophage media. Left, representative western blots of LC3B isoforms, SQSTM1, pro-IL-1 β , and ATG5. Right, densitometric analysis of blots. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. * $p < 0.05$.

(D) Supernatants from cells in (C) were collected and analyzed for cytokines. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. * $p < 0.05$.

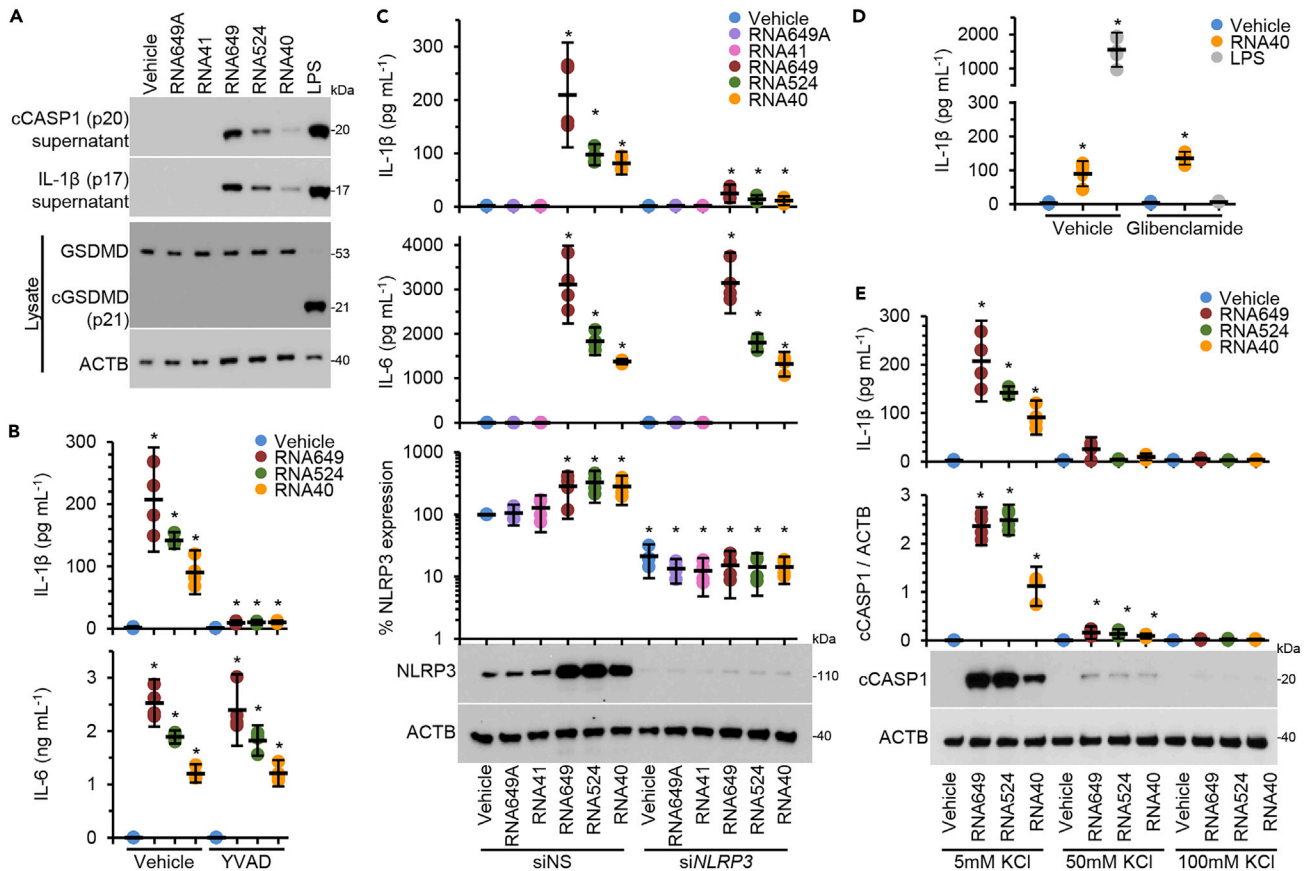


Figure 3. GU-rich RNAs activate the NLRP3 inflammasome

(A) Macrophages were treated for 24 h with 5 $\mu\text{g mL}^{-1}$ GU-rich RNA. Cell supernatant was collected and cells lysed. Top, a representative western blot of extracellular cleaved CASP1 (cCASP1) and IL-1 β . Bottom, a representative western blot of cleaved GSDMD (cGSDMD) and ACTB. $n = 4$.

(B) Macrophages were pre-treated with a CASP1 inhibitor (100 μM Ac-YVAD-cmk; YVAD) or vehicle control (vehicle) for 2 h. Macrophages were then treated for 24 h with 5 $\mu\text{g mL}^{-1}$ GU-rich RNA. Cell supernatants were collected and analyzed for cytokines. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. $*p < 0.05$.

(C) Macrophages were transfected with NLRP3 siRNA (siNLRP3) or scrambled siRNA (siNS). Macrophages were then treated for 24 h with GU-rich RNA and supernatants were collected and analyzed for cytokine secretion. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. $*p < 0.05$.

(D) Macrophages were pre-treated with a 100 μM glibenclamide or vehicle control (vehicle) for 2 h. Macrophages were then treated for 24 h with 5 $\mu\text{g mL}^{-1}$ GU-rich RNA or 10 ng mL^{-1} lipopolysaccharide (LPS) + 2.5 mM ATP. Cell supernatants were collected and analyzed for cytokines. Data are shown as a scatter plot with means \pm 95% confidence interval. $n = 6$. $*p < 0.05$.

(E) Macrophages were cultured under different concentrations of KCl for 2 h. Macrophages were then treated for 24 h with 5 $\mu\text{g mL}^{-1}$ GU-rich RNA under these same conditions. Top, cell supernatants were collected and analyzed for IL-1 β . Bottom, cells were lysed and analyzed for cleaved CASP1 (cCASP1) by western blot. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. $*p < 0.05$.

in monocytes, GU-rich RNA-stimulated macrophages do not respond with necroptotic protein-driven inflammasome activation upon CASP8 perturbation.

DISCUSSION

Classical modes of LPS-mediated NLRP3 activation (canonical and non-canonical) in murine macrophages comprise a priming step that enables NLRP3 activation (signal 1) followed by a K^+ efflux-dependent activation signal (signal 2) that triggers lytic cell death in the form of pyroptosis, necessary for IL-1 β release (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015), whereas in human monocytes, LPS can induce the transcription and release of IL-1 β in the absence of the K^+ efflux signal 2. Named the "alternative inflammasome" (Gaidt et al., 2016), it relies upon TLR4-TICAM1-RIPK1-FADD-CASP8 to activate NLRP3 and secrete IL-1 β in the absence of pyroptosis. In the present study, we demonstrate that in addition to inducing the transcription and release of pro-inflammatory cytokines IL-6 and TNF, GU-rich RNA from

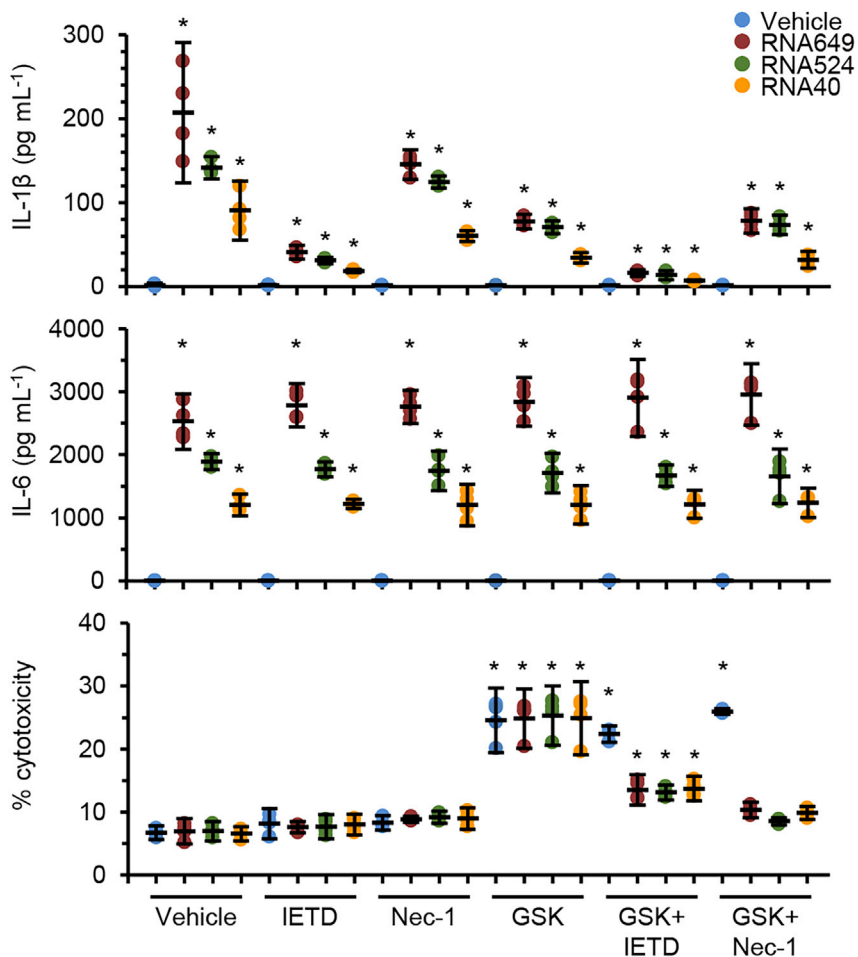


Figure 4. GU-rich RNA activation of the NLRP3 inflammasome involves the alternative pathway

Macrophages were pre-treated with inhibitors for CASP8 (10 μM Z-IETD-FMK; IETD), RIPK1 (10 μM necrostatin-1; Nec-1), or RIPK3 (5 μM GSK'872) or vehicle control (vehicle) for 2 h. Macrophages were then treated for 24 h with 5 $\mu\text{g mL}^{-1}$ GU-rich RNA. Cell supernatants were collected and analyzed for cytokines and LDH. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. * $p < 0.05$.

SARS-CoV-2, SARS-CoV-1, and HIV-1 all activate the NLRP3 inflammasome and induce the transcription and release of IL-1 β . Moreover, RNA derived from the spike protein of SARS-CoV-2 induces the greatest inflammatory response. Although we cannot rule out the contribution of other inflammasomes, GU-rich RNA-mediated secretion of IL-1 β is completely lost in the NLRP3-silenced cells, indicating an NLRP3-dependent pathway. With the use of inhibitors and RNAi, we also demonstrate that activation of the NLRP3 inflammasome by GU-rich RNA is specifically TLR8-dependent and RNA-mediated, leading to CASP1 activation and the conversion of pro-IL-1 β to its active form. Furthermore, we demonstrate that this mechanism proceeds in a CASP8- and K⁺ efflux-dependent mechanism that is independent of TICAM1 and pyroptosis. Thus we have identified that components of both the non-canonical pathway and the alternative activation pathway are involved in GU-rich RNA-mediated IL-1 β release from human macrophages.

Demonstrating the species- and ligand-specific role and impact on IL-1 β secretion, the TLR7/8 agonists imiquimod and CL097 both activate the NLRP3 inflammasome in LPS-primed murine bone-marrow-derived dendritic cells independent of K⁺ efflux and induce all the classical downstream effects including PYCARD oligomerization, GSDMD-driven pyroptosis, and secretion of IL-1 β (Groß et al., 2016). This specificity extends to the virus species and strain from whence we obtain the GU-rich RNA. Specifically, we observed that both betacoronavirus-derived GU-rich RNA induced significantly more IL-1 β , IL-6, and TNF than that induced by the well-studied RNA40 derived from HIV-1. More interestingly, we also observed that within the species of severe acute respiratory syndrome-related coronavirus, the GU-rich RNA derived from SARS-CoV-2 was more inflammatory than that derived from

SARS-CoV-1. There are a number of variables to consider when comparing these single-stranded RNAs, including the difference in length (the coronavirus-derived RNAs are 17-mers, whereas the HIV-1-derived RNA is a 20-mer) and sequence, but the differences are, nonetheless, interesting considering the different pathologies. In both COVID-19 and SARS, there is associated lung damage believed to be the result of a fulminant and sustained virally triggered inflammatory reaction mediated by a host of cytokines including IL-1 β , IL-6, and TNF leading to alveolar infiltration by macrophages and monocytes (Hsueh et al., 2004; Ye et al., 2020; Zhang et al., 2020). In sicker patients, the levels of these pro-inflammatory cytokines are higher and lead to a potent inflammatory response, respiratory decompensation, and multi-organ failure that can lead to death (Herold et al., 2020; Ng et al., 2004). The overwhelming inflammatory response has led investigators to examine the potential beneficial immunomodulatory effects of vitamin D and the cholinergic pathway (Farsalinos et al., 2020; Turrubiates-Hernández et al., 2021).

It was previously thought that IL-1 β release and pyroptosis could not be uncoupled (Cullen et al., 2015; Evavold et al., 2018). However, we observe IL-1 β release from live, metabolically active cells following CASP1 activation, similar to previous studies (Conos et al., 2016; Gaidt et al., 2016). In response to both GU-rich RNA and HIV-1 infection, human macrophages upregulate their expression of TREM1, which is essential for macrophage survival under these conditions (Campbell et al., 2019; Yuan et al., 2017). It is thus possible that human macrophages are intrinsically refractory to GU-rich RNA-induced cell death due to TREM1 upregulation, a phenomenon consistent with the abundance of monocyte-derived macrophages in the bronchoalveolar lavage of patients with severe COVID-19 (Liao et al., 2020) and macrophage survival during HIV-1-infection, in contrast to the massive cell death observed in HIV-1-infected CD4⁺ T cells (Van Lint et al., 2013).

Considering that inflammation and hyper-immune activation are markers for HIV-1 pathogenesis, the study of inflammasomes is still a relatively new field of study in HIV-1 research (Paiardini and Müller-Trutwin, 2013; Sereti and Altfield, 2016). Despite this, there is emerging evidence for NLRP3 involvement in HIV-1 pathogenesis as (1) NLRP3 may promote HIV-1 disease progression during gut-associated lymphoid tissue (GALT) destruction as its overexpression is speculated to favor HIV-1 replication and cell death (Feria et al., 2018); (2) single-nucleotide polymorphisms in NLRP3 increase susceptibility to HIV-1 infection (Zhang et al., 2015); (3) the expression of NLRP3 inflammasome-associated genes are upregulated in the brains of HIV-1-infected patients (Walsh et al., 2014); and (4) the NLRP3 inflammasome is a driver of HIV-1-mediated cardiovascular disease (Kearns et al., 2019). Moreover, productive HIV-1 infection is required for NLRP3 inflammasome activation as antiretroviral therapy decreases the transcription and release of IL-1 β (Guo et al., 2014).

In summary, GU-rich RNA derived from SARS-CoV-2, SARS-CoV-1, and HIV-1 induce the rapid induction of IL-6, TNF, and IL-1 β from human primary macrophages in the absence of viral infection. The IL-1 β release in response to these GU-rich RNA is dependent upon NLRP3 with K⁺ efflux acting as signal 2. These data suggest that TLR8 and and/or NLRP3 may prove to be excellent therapeutic targets to ameliorate SARS-CoV-2- and HIV-1-associated chronic inflammation.

Limitations of the study

In this study, we demonstrate that viral GU-rich RNA oligonucleotides elicit the expression and maturation of IL-1 β from human macrophages through the TLR8-dependent activation of the NLRP3 inflammasome in the absence of pyroptosis. The major limitation of the research presented is that we have not demonstrated that TLR8 is triggered during active viral infection. However, we have previously shown that exposure of human macrophages to purified live, heat-inactivated, or RNase/DNase I/2,2'-dithiodipyridine-treated (which inactivates the infectivity of retroviruses by covalently modifying the nucleocapsid zinc finger motifs) HIV-1 induces TLR8-dependent transcription factor EB nuclear translocation and autophagy similar to what we observe using LyoVec-complexed RNA40 (Campbell et al., 2015). Thus, it is likely that SARS-CoV-2 and SARS-CoV-1 trigger TLR8 signaling and NLRP3 inflammasome activation during active infection.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Grant Campbell (gcampbell@ucsd.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

METHODS

All methods can be found in the accompanying [transparent methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2021.102295>.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.R.C., R.K.T., and S.A.S.; methodology, G.R.C., R.K.T., and S.A.S.; investigation, G.R.C., R.K.T., and J.H.; validation, G.R.C., R.K.T., and J.H.; formal analysis, G.R.C., R.K.T., J.H., and S.A.S.; writing – original draft, G.R.C., R.K.T., and S.A.S.; writing – review & editing, G.R.C. and S.A.S.; visualization, G.R.C.; funding acquisition, S.A.S.; resources, S.A.S.; supervision, G.R.C. and S.A.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

**SARS-CoV-2, SARS-CoV-1, and HIV-1 derived ssRNA
sequences activate the NLRP3 inflammasome in human
macrophages through a non-classical pathway**

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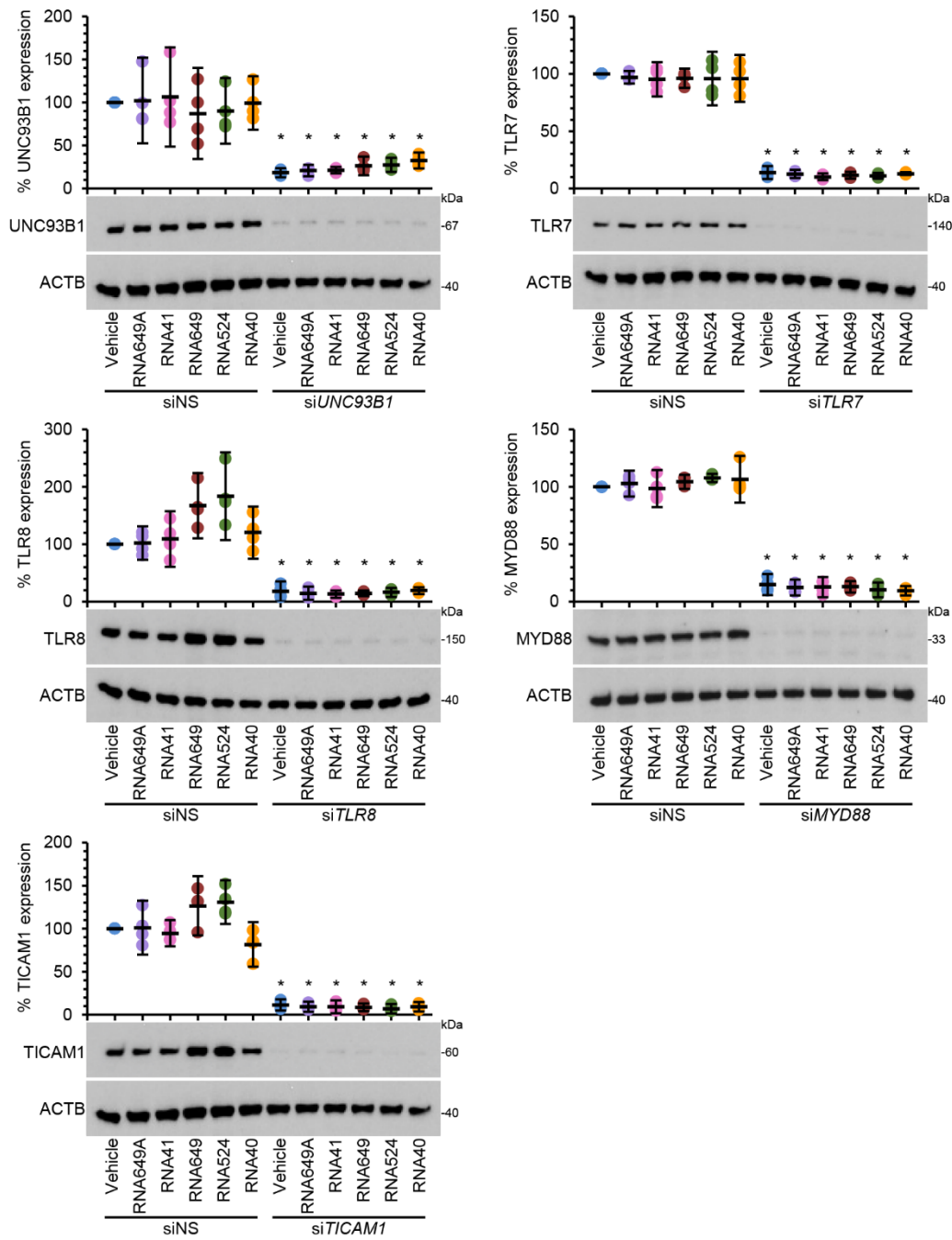


Figure S1. GU-rich RNA induce IL-1 β through TLR8-signaling.

Related to Figure 1D

Macrophages were transfected with *UNC93B1* siRNA (si*UNC93B1*), *TLR7* siRNA (si*TLR7*), *TLR8* (si*TLR8*), *MYD88* (si*MYD88*), *TICAM1* (si*TICAM1*), or scrambled siRNA (siNS). Macrophages were then treated for 24 h with 5 $\mu\text{g mL}^{-1}$ GU-rich RNA, supernatants collected and analyzed for cytokine secretion and cells lysed and analyzed for silencing. Cytokine expression is shown in Fig. 1D. Shown are representative western blots for each silencing experiment with corresponding densitometric analysis of blots. Data are shown as scatter plots with means \pm 95% confidence interval. $n = 4$. * $p < 0.05$.

**Table S1. Chemical and reagent list.
Related to Transparent Methods and Figure 1A.**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α -human ACTB	Sigma	Cat#A2228, RRID:AB_476697
α -human ATG5	Cell Signaling Technology	Cat#2630, RRID:AB_2062340
α -human CASP1	Cell Signaling Technology	Cat#3866, RRID:AB_2069051
α -human GASDM2	Cell Signaling Technology	Cat#97558, RRID:AB_2864253
α -human IL-1 β	Cell Signaling Technology	Cat#12703, RRID:AB_2737350
α -human cleaved IL-1 β	Cell Signaling Technology	Cat#83186, RRID:AB_2800010
α -human MAP1LC3B	Novus Biologicals	Cat# NB100-2220, RRID:AB_10003146
α -human MYD88	Cell Signaling Technology	Cat#4283, RRID:AB_10547882
α -human NLRP3	Cell Signaling Technology	Cat#13158, RRID:AB_2798134
α -human SQSTM1	Abcam	Cat#ab56416, RRID:AB_945626
α -human TICAM1	Cell Signaling Technology	Cat#4596, RRID:AB_2256555
α -human TLR7	Cell Signaling Technology	Cat#5632, RRID:AB_10692895
α -human TLR8	Novus Biologicals	Cat# NBP2-24917, RRID:AB_284789
Biological Samples		
Human peripheral blood mononuclear cells (PBMC)	UC San Diego Health Sciences, San Diego, CA, USA	N/A
Chemicals, Peptides, and Recombinant Proteins		
25-hydroxycholecalciferol	Sigma	Cat# H4014
BLOCK-iT Alexa Fluor red fluorescent control	Invitrogen	Cat# 14750100
Chloroquine diphosphate salt	Sigma	Cat# C6628
Fetal bovine serum	Sigma	Cat# F4135
Fetal bovine serum - charcoal stripped	Sigma	Cat# F6765
Ficoll-Paque PLUS	GE Healthcare	Cat# 17-1440-03
Glibenclamide	Selleck Chemicals	Cat# S1716
GSK2399872A	Selleck Chemicals	Cat# S8465
Lipofectamine RNAiMAX transfection reagent	Invitrogen	Cat# 13778030
LPS	Invivogen	Cat# tlrl-eklps
Lyovect	Invivogen	Cat# lyec-3
Necrostatin-1	Selleck Chemicals	Cat# S8037
Recombinant human CSF1	Peptotech	Cat# 300-25
ssRNA40	Invivogen	Cat# tlrl-lrna40

ssRNA41	Invivogen	Cat# tlr-lrna41
Z-IETD-FMK	Selleck Chemicals	Cat# S7314
Ac-YVAD-cmk	Sigma	Cat# SML0429
Critical Commercial Assays		
Cytotoxicity detection KitPLUS (LDH)	Roche	Cat# 4744934001
Human IL-1 beta/IL-1F2 Quantikine ELISA Kit	R&D Systems	Cat# DLB50
Human IL-1 beta/IL-1F2 DuoSet ELISA	R&D Systems	Cat# DY201-5
Human IL-6 DuoSet ELISA	R&D Systems	Cat# DY206-5
Human TNF-alpha DuoSet ELISA	R&D Systems	Cat# DY210-05
Oligonucleotides		
ATG5 RNAi	Invitrogen	ID# s18159
MYD88 RNAi	Invitrogen	ID# HSS181395
NLRP3 RNAi	Invitrogen	ID# s534396
TLR7 RNAi	Invitrogen	ID# HSS121963
TLR8 RNAi	Invitrogen	ID# HSS1299001
TICAM1 RNAi	Invitrogen	ID# HSS152364
RNA40: 5'-GCCCGUCUGUUGUGACUC-3'	Heil et al., 2004	N/A
RNA41: 5'-GCCCGACAGAAGAGAGACAC-3'	Heil et al., 2004	N/A
RNA524: 5'-GUCUGAGUGUGUUCUUG-3'	Li et al., 2013	N/A
RNA649: 5'-GUCUGAGUGUGUACUUG-3'	This paper	N/A
RNA649A: 5'-GACAGAGAGAGAACAAG-3'	This paper	N/A
Silencer Select Negative Control No. 2 siRNA	Invitrogen	Cat# 4390846
Stealth RNAi siRNA Negative Control	Invitrogen	Cat# 12935300
Software and Algorithms		
Adobe Acrobat DC	Adobe	https://acrobat.adobe.com/
Adobe Photoshop 2021	Adobe	https://www.adobe.com/products/photoshop.html
ImageJ	Fiji	https://fiji.sc/ RRID:SCR_002285
Microsoft Office 365	Microsoft	https://www.office.com/

TRANSPARENT METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Primary human cells

Venous blood was drawn from HIV-seronegative healthy volunteers, ages between 18 and 65 years, at UC San Diego Health Sciences. In accordance with the Human Research Protections Program of the University of California, San Diego, all samples were de-identified and donors remained anonymous. Thus, the authors did not obtain personal identifying information and cannot report on their sex, gender identity, or age. Samples were assigned to experimental protocols through simple random sampling. Peripheral blood mononuclear cell (PBMC) were isolated from whole blood by density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare). Macrophages were prepared by incubating 6×10^6 PBMC mL^{-1} in macrophage media (RPMI 1640 [Gibco] supplemented with 10% [vol/vol] heat-inactivated fetal bovine serum [FBS; Sigma], 2 mM L-glutamine, 0.1 mg mL^{-1} streptomycin, 100 U mL^{-1} penicillin [all Gibco], and 10 ng mL^{-1} colony stimulating factor 1 [Peprotech]), after which non-adherent cells were removed by aspiration and washed with Dulbecco's phosphate buffered saline (Gibco). Adherent cells were further incubated in macrophage media for 10 d at 37°C, 5% CO₂ with media changes every 2 days before use. For vitamin D3 experiments, cells were cultured using 10% [vol/vol] charcoal/dextran treated, heat-inactivated FBS (Sigma) in place of regular FBS with or without calcifediol (25-hydroxycholecalciferol; Sigma #H4014) (Table S1).

Ethical statement

Venous blood was drawn from human subjects using protocols that were reviewed and approved by the Human Research Protections Program of the University of California, San Diego 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) and were fully compliant with the principles expressed in the Declaration of Helsinki. All volunteers gave written informed consent prior to their participation.

GU-rich RNA

RNA649 (5'-GUCAGAGUGUGUACUUG-3'; position 24649-24665 nt in the SARS-CoV-2 genome [S2 spike protein] [accession number: NC_045512.2]) (Wu et al., 2020), RNA524 (5'-GUCUGAGUGUGUUCUUG-3'; position 24524-24540 in the SARS-CoV-1 genome [S2 spike protein] [accession number: NC_004718.3] (also known as RNA120; Li et al., 2013)), RNA649A (a derivative of RNA524 and RNA649 in which adenosine replaces all uracil nucleotides), RNA40 (5'-GCCCGUCUGUUGUGUGACUC-3'; at U5 region 108-127 nt of HIV-1 genome [accession number: NC_001802.1]) (Heil et al., 2004), and RNA41 (a derivative of RNA40 in which adenosine replaces all uracil nucleotides) were synthesized by Integrated DNA Technologies. LyoVec (InvivoGen), a cationic lipid-based transfection reagent was used to complex GU-rich RNA in a 2:1 (LyoVec:RNA) ratio according to the manufacturer's instructions. When not being compared to RNA649 or RNA524, LyoVec pre-complexed RNA40 (ttrl-Irna40), and LyoVec pre-complexed RNA41 (ttrl-Irna41; both InvivoGen) were used.

METHOD DETAILS

Chemicals

Z-IETD-FMK (S7314), necrostatin-1 (S8037), GSK2399872A (GSK'872; S8465), and glibenclamide (S1716) were from Selleck Chemicals, Ac-YVAD-cmk (SML0429) was from Sigma, and lipopolysaccharide (LPS) from *Escherichia coli* K12 (ttrl-eklps) was from InvivoGen.

Lactate dehydrogenase activity

To assess the extent of necrotic cell death, lactate dehydrogenase (LDH) activity of supernatants was measured using a mixture of diaphorase/NAD⁺ and iodonitrotetrazolium chloride/sodium 2-hydroxypropanoate according to the manufacturer's protocol (Roche). Percent cytotoxicity was calculated per the manufacturer's instructions.

ELISA

IL-1 β (Cat# DLB50 and DY201), IL-6 (Cat# DY206), and TNF (Cat# DY207) were measured in cell culture supernatants using enzyme-linked immunosorbent assay kits obtained from R&D Systems according to the manufacturer's instructions.

RNA interference

Macrophages were transfected with Thermo Fisher *ATG5* (ID# s18159), *MYD88* (ID# HSS181395), *NLRP3* (ID# s534396), *TLR7* (ID# HSS121963), *TLR8* (ID# HSS1299001), *TICAM1* (ID# HSS152364), or control (siNS; Cat# 4390846 and 12935300) siRNA for 48 h using lipofectamine RNAiMAX transfection reagent (Thermo Fisher) in Opti-MEM (Gibco) according to the manufacturer's instructions. Transfection efficiency was assessed with BLOCK-iT Alexa Fluor Red Fluorescent Control (Thermo Fisher) using flow cytometry (Campbell et al., 2019).

Western blotting

The following antibodies were used: anti-SQSTM1 (Cat# ab56416, RRID:AB_945626) from Abcam, anti-ATG5 (Cat# 2630, RRID:AB_2062340), anti-CASP1 (Cat# 3866, RRID:AB_2069051), anti-GSDMD (Cat# 97558, RRID:AB_2864253), anti-IL-1 β (Cat# 12703, RRID:AB_2737350), anti-cleaved-IL-1 β (Cat# 83186, RRID:AB_2800010), anti-MYD88 (Cat# 4283, RRID:AB_10547882), anti-NLRP3 (Cat# 13158, RRID:AB_2798134), anti-TICAM1 (Cat# 4596, RRID:AB_2256555), and anti-TLR7 (Cat# 5632, RRID:AB_10692895) from Cell Signaling Technology, anti-ACTB (Cat# A2228, RRID:AB_476697) from Sigma, and anti-MAP1LC3B (#NB100-2220, RRID:AB_10003146) and anti-TLR8 (Cat# NBP2-24917, RRID:AB_2847894) from Novus Biologicals. Cell lysates were prepared using 20 mM HEPES (Gibco), 150 mM NaCl (Fisher Scientific), 1 mM EDTA (Sigma) supplemented with 1% (vol/vol) Triton X-100 (Sigma) and 1% (vol/vol) Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Cell lysates were resolved using 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol buffered 12% polyacrylamide gels (Thermo Scientific) and transferred to 0.2 μ m polyvinylidene difluoride membranes (Thermo Scientific), followed by detection with primary antibodies followed by alkaline phosphatase tagged secondary antibodies (Invitrogen) and 0.25 mM disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-(5-chlorotricyclo[3.3.1.1^{3,7}]decan])-4-yl]-1-phenyl phosphate supplemented with 5% (vol/vol) Nitro-Block II (both Applied Biosystems). Relative densities of the target bands were compared to the reference ACTB bands and were calculated using Fiji (RRID:SCR_002285). Each data point was normalized to the vehicle then log₂ transformed.

Statistics

Samples were assigned to experimental groups through simple random sampling. Sample size (n) was determined using a 2-sample 2-sided equality test with power $(1-\beta) = 0.8$, $\alpha = 0.05$ and preliminary data where the minimum difference in outcome was at least 70%. Data were assessed for symmetry, or skewness, using Pearson's skewness coefficient. Normalized ratiometric data were log₂ transformed. Comparisons between groups were performed using the paired, two-tailed, Student's t test. In all experiments, differences were considered significant when $p < 0.05$. Data are represented as scatter plots with arithmetic means \pm 95% confidence interval.

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