iScience, Volume *24*

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

Grant R. Campbell, Rachel K. To, Jonathan Hanna, and Stephen A. Spector

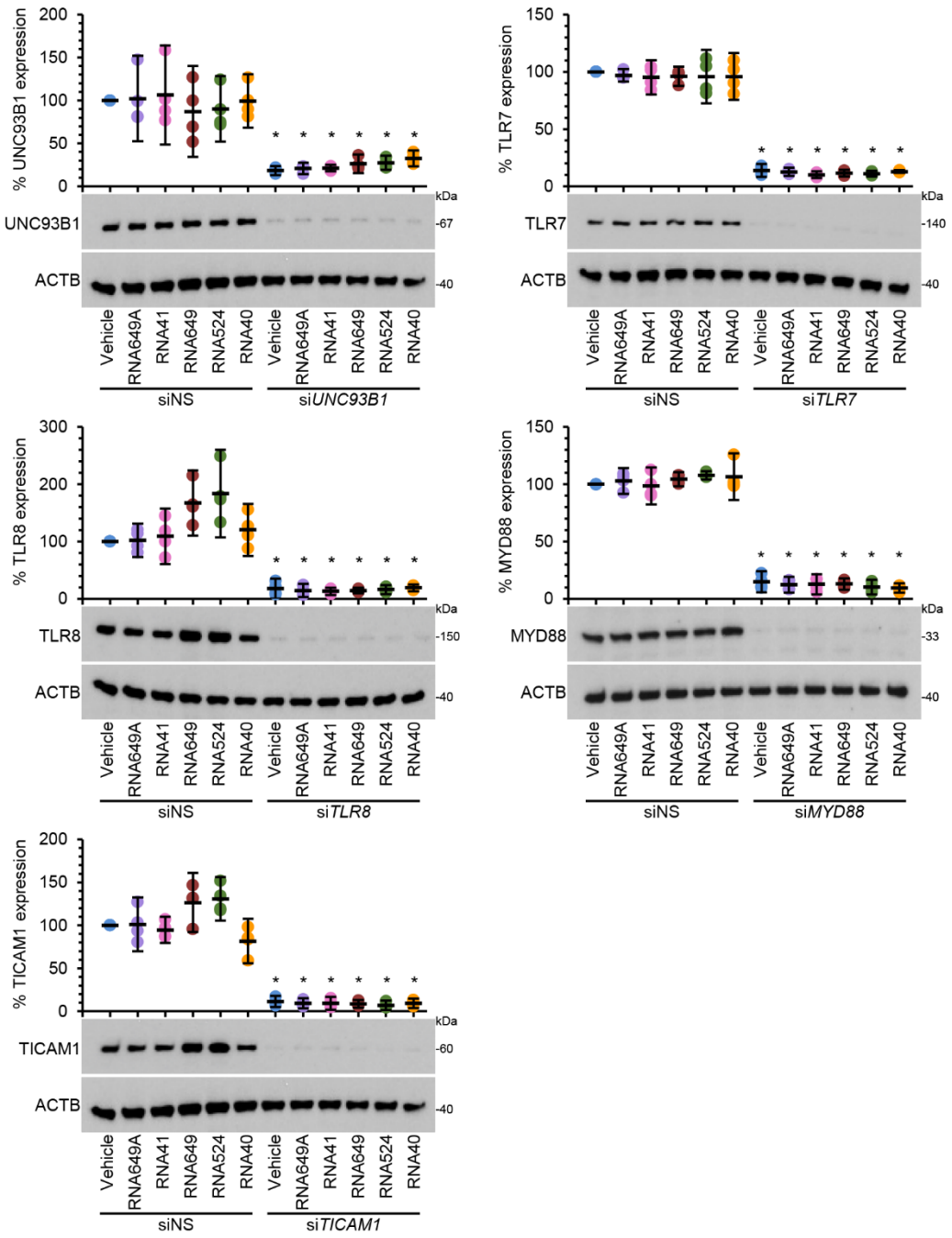

Figure S1. GU-rich RNA induce IL-1β through TLR8-signaling.
Related to Figure 1D

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 μg 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 ± 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 GASDMD	Cell Signaling Technology	Cat#97558, RRID:AB_2864253
α-human IL-1β	Cell Signaling Technology	Cat#12703, RRID:AB_2737350
α-human cleaved IL-1β	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
Lyovec	Invivogen	Cat# lyec-3
Necrostatin-1	Selleck Chemicals	Cat# S8037
Recombinant human CSF1	Peprotech	Cat# 300-25
ssRNA40	Invivogen	Cat# tlrl-lrna40

ssRNA41	Invivogen	Cat# tlrl-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′-GCCCGUCUGUUGUGUGACUC-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% CO_2 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 (tlrl-lrna40), and LyoVec pre-complexed RNA41 (tlrl-lrna41; 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 (tlrl-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-1β (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), *NRLP3* (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-1β (Cat# 12703, RRID:AB_2737350), anti-cleaved-IL-1β (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 μm 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 $\log_2$ 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, α = 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 $\log_2$ 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 ± 95% confidence interval.

SUPPLEMENTAL REFERENCES

Campbell, G.R., To, R.K., and Spector, S.A. (2019). TREM-1 protects HIV-1-infected macrophages from apoptosis through maintenance of mitochondrial function. mBio *10*, e02638-02619.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science *303*, 1526-1529.

Li, Y., Chen, M., Cao, H., Zhu, Y., Zheng, J., and Zhou, H. (2013). Extraordinary GU-rich single-strand RNA identified from SARS coronavirus contributes an excessive innate immune response. Microbes Infect *15*, 88-95.

Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y.*, et al.* (2020). A new coronavirus associated with human respiratory disease in China. Nature *579*, 265-269.