PNAS

Supplementary Information for

Activation of the NLRP1 inflammasome in human keratinocytes by the dsDNA mimetic poly(dA:dT)

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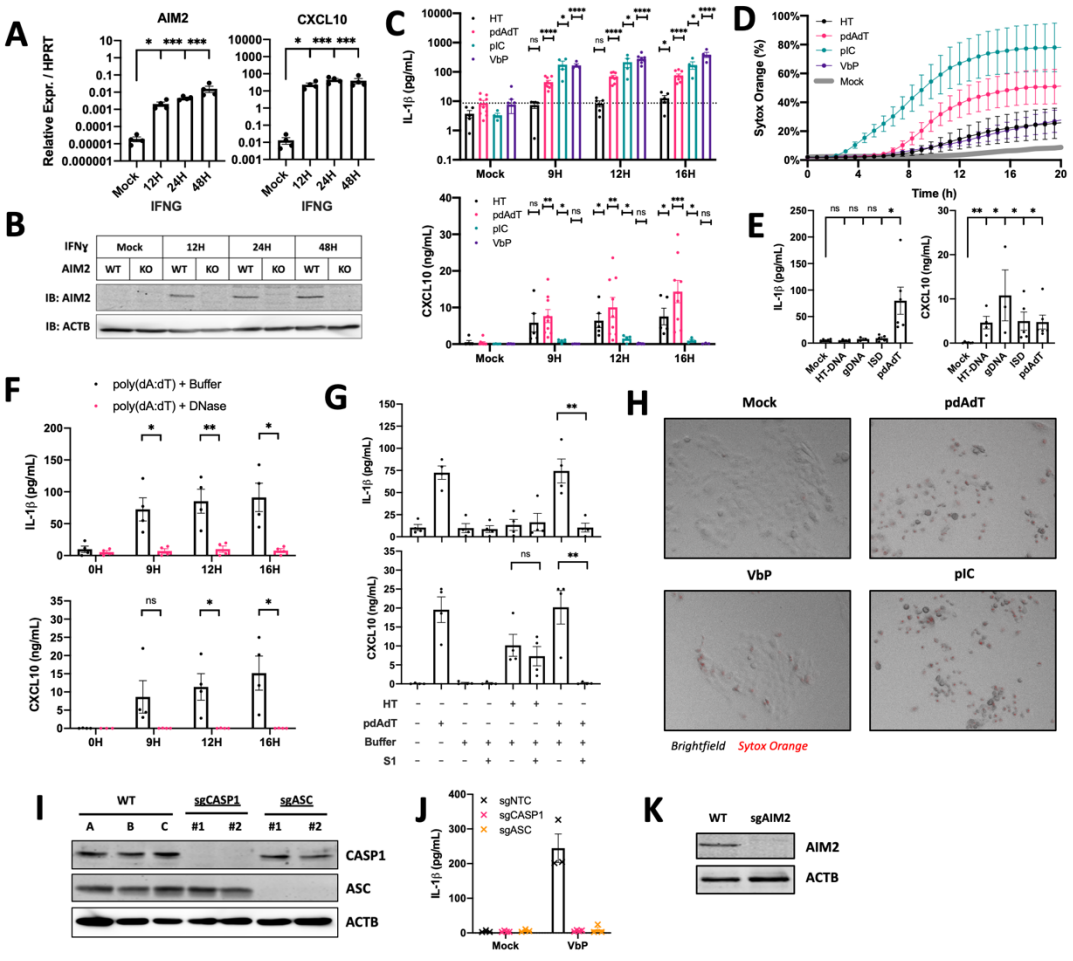

Figure S1. The complex structure of poly(dA:dT) distinguishes it from linear dsDNA. (**A-B**) N/TERT2G wild type or monoclonal knockout keratinocytes were treated with 100 ng/mL IFNγ for indicated time points and collected for qPCR or western blot (n=3). (**C**) N/TERT2G were transfected with 1 µg/mL HT-DNA, poly(dA:dT), poly(I:C), or stimulated with 5 µM VbP and analyzed by ELISA (n=5-8) or (**D**) sytox orange time lapse imaging (n=4-6) with a (**H**) representative image at 12H. (**E**) N/TERT2G were transfected with 1 µg/mL of the indicated nucleic acid and collected at 12H for ELISA (n=3-5). (**F-G**) 1 µg/mL poly(dA:dT) or HT-DNA was digested with DNase I, S1 nuclease, or buffer without enzyme and transfected into N/TERT2G for 12H. Supernatants were analyzed by ELISA (n=4). (**I, K**) Western blots were performed on monoclonal N/TERT2G knockout cell lines. (**J**) Monoclonal N/TERT2G knockout cell lines were stimulated with 5 µM VbP (each data point is an independent clone) and ELISA was performed on supernatants. Data represents mean ± SEM (**A, C-G, J**) or a representative image (**B, H**) from at least three independent experiments. If not otherwise indicated, statistical comparisons were made with respect to the mock-treated control. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student's t-test.

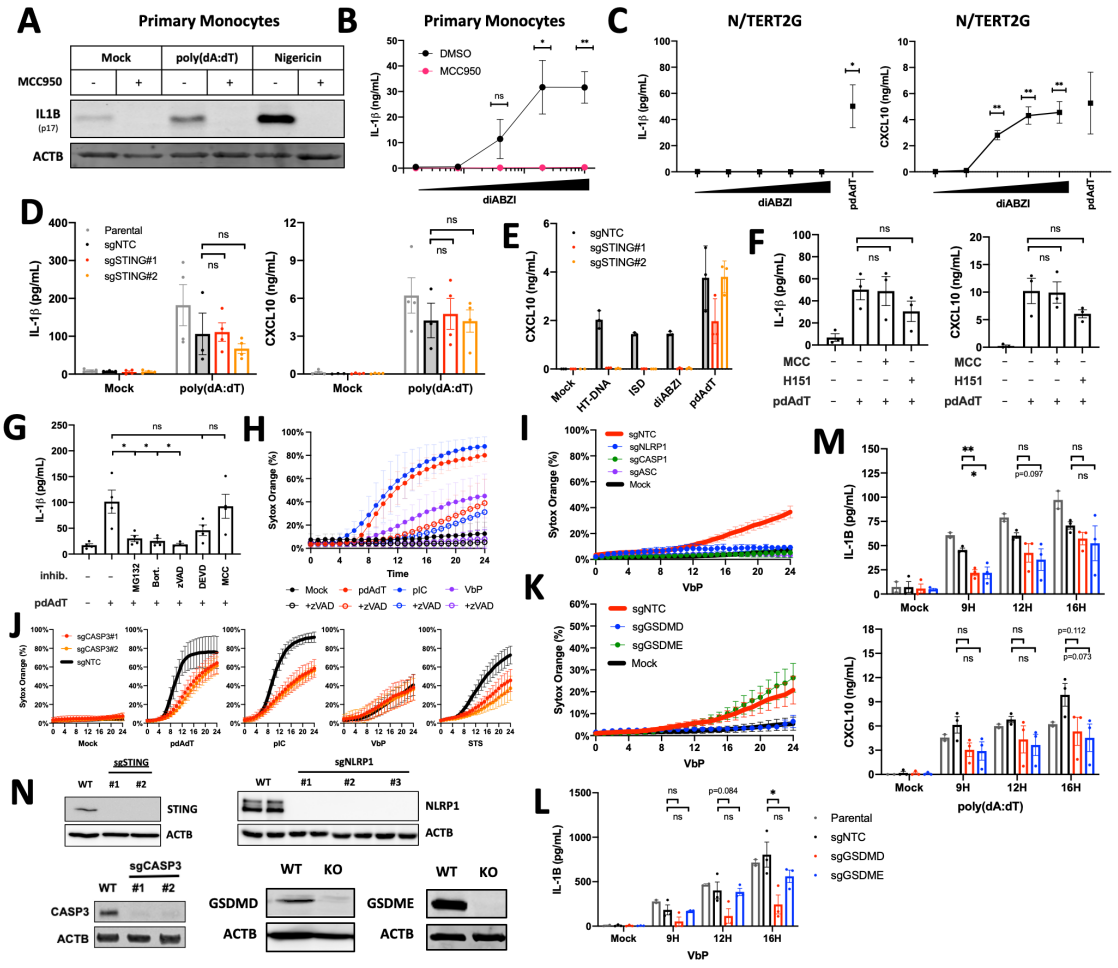

Figure S2. STING activation propels the poly(dA:dT)-induced inflammasome in human monocytes but not keratinocytes. (**A**) Human monocytes were primed with 2 μg/mL PAM3CSK4 for 3H, pretreated with 1H 10 μM MCC950, then transfected with 1 μg/mL poly(dA:dT) for 9H or stimulated with 5 μM nigericin for 6H in serum-free conditions. Supernatants were precipitated for Western blot. (**B, C**) PAM3CSK4-primed human monocytes (n=4) or N/TERT2G keratinocytes (n=3) were pretreated with 10 μM MCC950 and stimulated with 0.6 nM to 10 μM diABZI for 9H. As a control, N/TERT2G were transfected with 1 μg/mL poly(dA:dT) for 9H and supernatants were analyzed by ELISA. (**D-E**) Monoclonal N/TERT2G knockout cell lines were transfected with 1 μg/mL poly(dA:dT), 1 μg/mL HT-DNA, 1 μg/mL ISD, or stimulated with 1 μM diABZI for 12H and ELISA was performed on supernatants (n=2-4). (**F**) N/TERT2G were pretreated with 10 μM MCC950 or 10 μM H-151 and transfected with 1 μg/mL poly(dA:dT) for 12H, then analyzed by ELISA (n=4). (**G**) N/TERT2G were pretreated 2 μM MG-132, 0.1 μM Bortezomib, 25 μM z-VAD-FMK, 25 μM z-DEVD-FMK, or 10 μM MCC950 and transfected with 1 μg/mL poly(dA:dT) for 12H for analysis by ELISA (n=4). (**H**) N/TERT2G keratinocytes were incubated with 25 uM z-VAD-FMK for 1H, then transfected with 1 μg/mL poly(dA:dT), 1 μg/mL poly(I:C), or stimulated with 5 uM VbP and monitored with sytox orange live cell imaging (n=2). (**I, K-M**) Monoclonal N/TERT2G knockouts were stimulated with 5 μM VbP or transfected with 1 μg/mL poly(dA:dT) for indicated timepoints and subject to sytox orange live cell imaging (n=2-3) or ELISA (n=3). (**J**) Polyclonal N/TERT2G CASP3 knockouts were transfected or stimulated with 1 μg/mL poly(dA:dT), 1 μg/mL poly(I:C), 5 μM VbP, and 1 μM STS and monitored with sytox orange live cell imaging (n=3). (**N**) Western blots were performed on N/TERT2G knockout cell lines. Data represents mean ± SEM (**B-D, F-G, J-M**) from at least three independent experiments. Otherwise, mean ± SD (**E, H-I**) or a representative blot (**A**) of at least

two independent experiments is shown. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student's t-test.

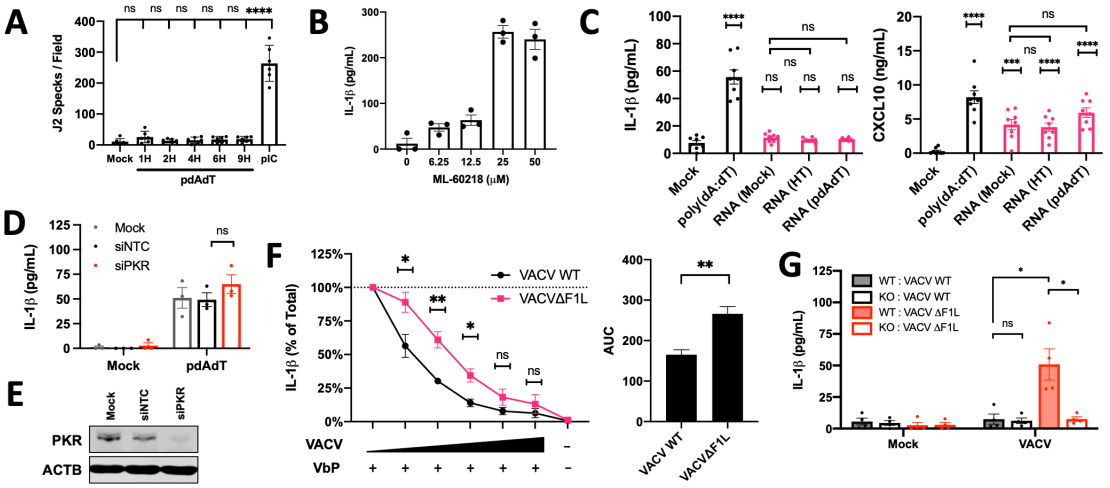

Figure S3. VACV F1L antagonizes the NLRP1 inflammasome. (**A**) N/TERT2G cells were transfected with 1 μg/mL poly(dA:dT) for indicated time points or poly(I:C) for 4H. Cells were fixed, stained, and J2 positive specks were quantified by immunofluorescence (n=6). (**B**) N/TERT2G cells were treated with RNA polymerase III inhibitor ML-60218 at indicated concentrations for 9H and ELISA was performed on supernatants (n=2). (**C**) 293T cells were transfected with 2 μg/mL HT-DNA or poly(dA:dT) for 12H and total RNA was isolated with trizol or silica-based columns. Then, 1 μg/mL of isolated RNA was transfected into N/TERT2G cells for 9H and supernatants were analyzed by ELISA (n=8). (**D-E**) N/TERT2G keratinocytes were transfected with 25 nM DharmaFECT siRNA for 48 hours. Then, cells were transfected with 1 ug/mL poly(dA:dT) or stimulated with 2 uM anisomycin for 6 hours. Supernatants were analyzed by ELISA and lysates by Western Blot (n=3). (**F**) N/TERT2G were infected with increasing multiplicity of infection (MOI) of VACV COP WT or ΔF1L (0.003 < MOI < 2) then co-stimulated with 5 μM VbP for 16H. Supernatants were assessed by ELISA and IL-1β released was normalized as a percentage of total (n=3). (**G**) N/TERT2G wild type or NLRP1 knockout cells were infected with VACV COP WT or ΔF1L (MOI 10) for 9H and ELISA was performed on the supernatant (n=4). Data represents mean ± SEM (**C-D, F-G**) from at least three independent experiments, mean ± SD (**A**) of combined data from two independent experiments performed in triplicate, mean ± SD (**B**) from a representative experiment of two independent experiments performed in triplicate, or a representative blot (**E**) from three independent experiments. If not otherwise indicated, statistical comparisons were made with respect to the mock-treated control. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student's t-test.

Figure S4

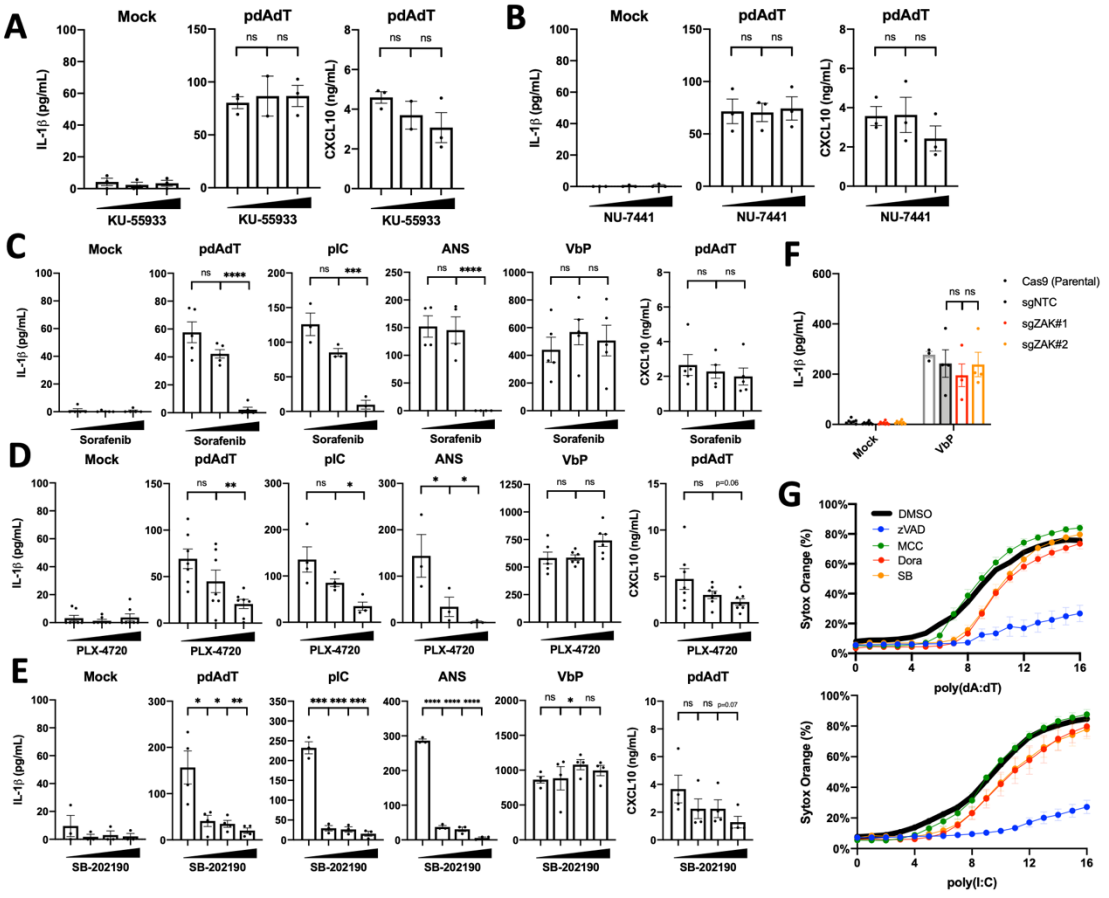

Figure S4. Second generation p38 inhibitors but not DNA damage pathway kinase inhibitors antagonize poly(dA:dT) IL-1β release. (**A-E**) N/TERT2G were pretreated with 0.1/1 μM KU-55933, 0.1/1 μM NU-7441, 0.2/2 μM Sorafenib, 0.1/1 μM PLX-4720, or 0.1/1/10 μM SB-202190, then transfected with 9H 1 μg/mL poly(dA:dT), 9H 1 μg/mL poly(I:C), or stimulated with 6H 2 μM anisomycin and 16H 5 μM VbP. Supernatants were analyzed by ELISA (n=3-6). (**F**) Polyclonal ZAK knockout N/TERT2G keratinocytes were stimulated with 12H 5 μM VbP and supernatants were used for ELISA (n=4). (**G**) Wild-type N/TERT2G were pretreated with 25 μM z-VAD-FMK, 10 μM MCC950, 100 nM doramapimod, or 100 nM SB-202190 and transfected with 1 μg/mL poly(dA:dT) or poly(I:C) and monitored with sytox orange live cell imaging (n=4). Data represents mean ± SEM (**A-G**) from at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student's t-test.

Dataset	N	Reference
GSE171170	3	Gupta et al. (2021)[1]
GSE124939	5	Tsoi et al. (2019)[2]
GSE107871	4	Swindell et al. (2017)[3]
GSE173902	2	Murai-Yamamura et al. (2021)[4]
GSE109182	3	Swindell et al. (2018)[5]
GSE101216	4	Bandiera et al. (2021)[6]
GSE120784	3	Witte-Handel et al. (2019)[7]
GSE119317	4	Anderson et al. (2018)[8]
GSE185309	8	Unpublished

Table S1. RNA-seq studies utilized in meta-analysis

sgRNA	Target Sequence
sgGFP	GGTGAACCGCATCGAGCTGA
sgAIM2	GATACTCTTGCTAACAGGCC
sgNLRP1	GCTCCCCATACTGAGCCACC
sgCASP1#1	TAATGAGAGCAAGACGTGTG
sgCASP1#2	ATGTCTCATGGTATTCGGGA
sgCASP3#1	GAAGCGAATCAATGGACTC
sgCASP3#2	ATTATACATAAACCCATCTC
sgPYCARD#1	CTGGAGAACCTGACCGCCG
sgPYCARD#2	AACTTCTTGAGCTCCTCGG
sgTMEM173	AGAGCACACTCTCCGGTACC
sgGSDMD	GCATGGGGTCGGCCTTTGAG
sgGSDME	GTCGGACTTTGTGAAATACG
sgMAP3K20#1	TGTATGGTTATGGAACCGAG
sgMAP3K20#2	TGCATGGACGGAAGACGATG

Table S2. CRISPR/Cas9 Target Sequences

Gene	Forward Primer	Reverse Primer
AIM2	GCC ACT AAG TCA AGC TGA AAT G	CAG GCT TAA CAT GAG GAG AGA C
CXCL10	GTG GCA TTC AAG GAG TAC CTC	TGA TGG CCT TCG ATT CTG GAT T
HPRT	ATC AGA CTG AAG AGC TAT TGT AAT GA	TGG CTT ATA TCC AAC ACT TCG TG
IL1B	TCC CCA GCC CTT TTG TTG A	TTA GAA CCA AAT GTC GCC GTG

Table S3. qPCR Oligos

Target	Clone	Type	Genotype
sgNLRP1	#1	Homozygous KO	-4/-4
sgNLRP1	#2	Homozygous KO	-1/-1
sgNLRP1	#3	Compound Heterozygous KO	-1/+1
sgCARD8	#1	Compound Heterozygous KO	-2/-3
sgTMEM173	#1	Homozygous KO	-4/-4
sgTMEM173	#2	Homozygous KO	-8/-8
sgGSDMD	#1	Homozygous KO	+1/+1
sgGSDME	#1	Homozygous KO	+1/+1

Table S4. Genotyping results for monoclonal CRISPR/Cas9 cell lines

Dataset S1 (separate file).

Public RNA-seq data of healthy human keratinocytes (n=38) were downloaded and a meta-analysis was performed to re-quantify gene expression based on a uniform reference transcriptome. Transcripts per million (TPM) of each sample and gene was computed. Columns represent samples and rows represent genes.

Movie S1 (separate file).

N/TERT2G keratinocytes were transfected with 1 µg/mL poly(dA:dT) with 2 uL/mL lipofectamine 2000, or stimulated with 5 µM VbP. Cells were monitored by live-cell imaging using sytox orange dye with pictures taken at 1-hour intervals for 24 hours.

SI References

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2. Tsoi, L. C. *et al.* Hypersensitive IFN Responses in Lupus Keratinocytes Reveal Key Mechanistic Determinants in Cutaneous Lupus. *J. Immunol.* **202**, 2121–2130 (2019).
3. Swindell, W. R. *et al.* RNA-seq identifies a diminished differentiation gene signature in primary monolayer keratinocytes grown from lesional and uninvolved psoriatic skin. *Sci. Reports 2017 71* **7**, 1–13 (2017).
4. Murai-Yamamura, M. *et al.* TH2 cytokines and Staphylococcus aureus cooperatively induce atopic dermatitis-like transcriptomes. *Allergy* **76**, 3534–3537 (2021).
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6. Bandiera, R. *et al.* RN7SK small nuclear RNA controls bidirectional transcription of highly expressed gene pairs in skin. *Nat. Commun. 2021 121* **12**, 1–14 (2021).
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8. Anderson, E. D. *et al.* Prolonging culture of primary human keratinocytes isolated from suction blisters with the Rho kinase inhibitor Y-27632. *PLoS One* **13**, e0198862 (2018).