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Supplemental information

Self-assembling short immunostimulatory duplex RNAs with broad-spectrum antiviral activity

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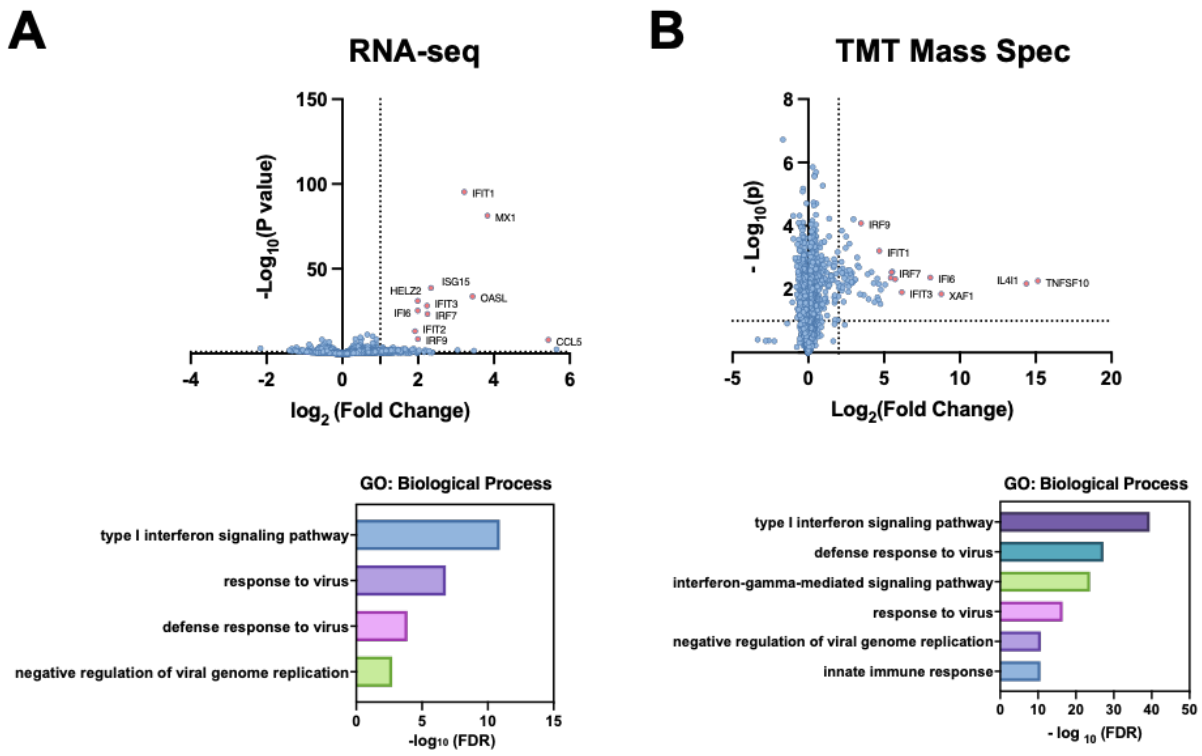


Figure S1. Profiling the effects of RNA-2 by RNA-seq and TMT mass spectrometry. A549 cells were transfected with RNA-2 or scrambled RNA control, cell lysates were collected at 48 h, and analyzed by RNA-seq (left) or TMT Mass Spec (right). Differentially expressed genes (DEGs) or proteins are shown in volcano plots (top) and GO Enrichment analysis was performed for the DEGs (bottom) (N = 3). Plot (top) and GO Enrichment analysis was performed for the differentially expressed proteins (bottom) (N = 3).

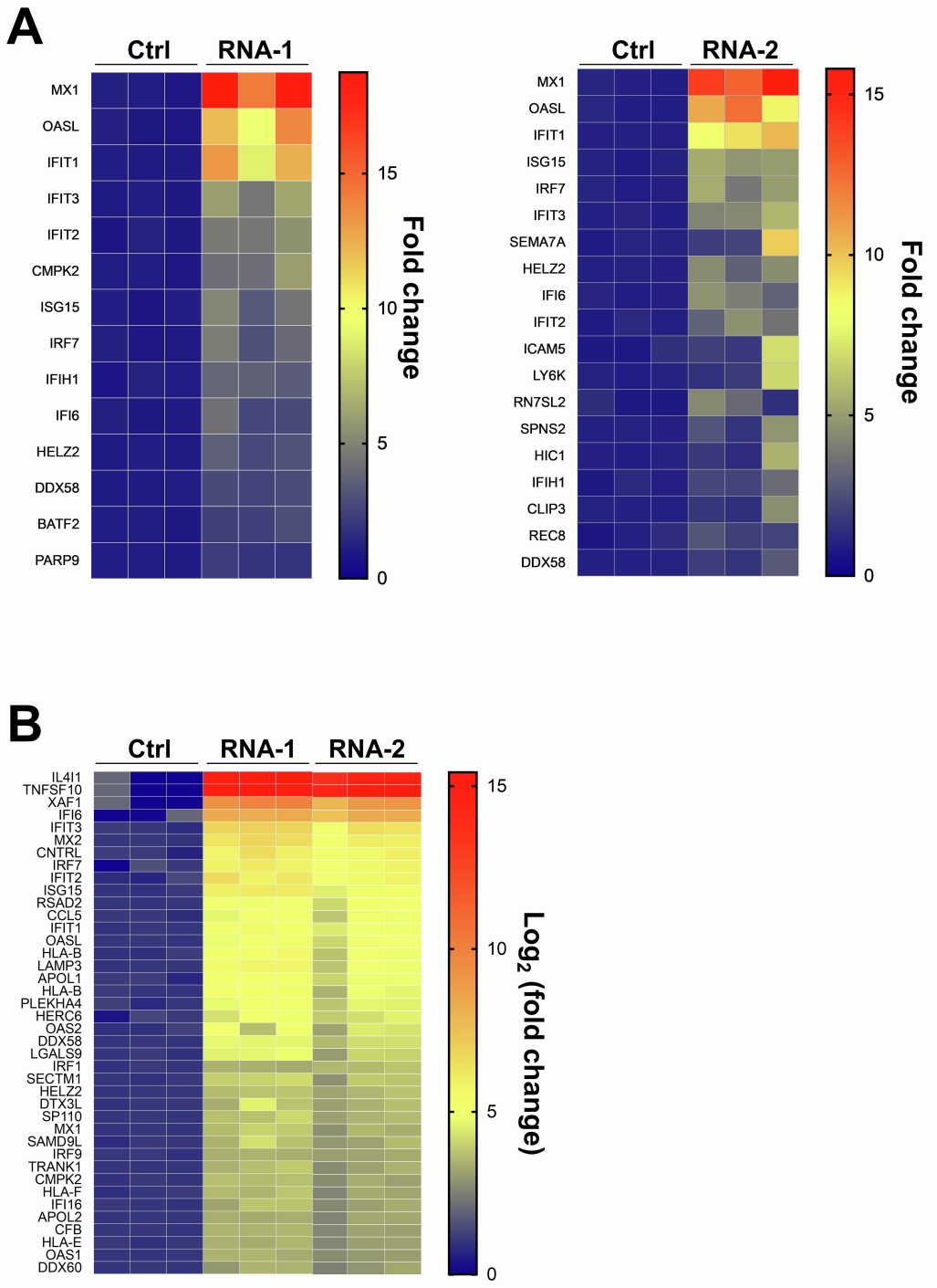


Figure S2. Heat maps showing the effects of immunostimulatory RNAs on IFN pathway-relevant gene levels. DEGs from RNA-seq (**A**) and differentially expressed proteins from TMT Mass Spec analyses (**B**) shown in Fig. 1B and fig. S1 are presented here as heat maps (gene levels of the scrambled RNA control were set as 1; N = 3).

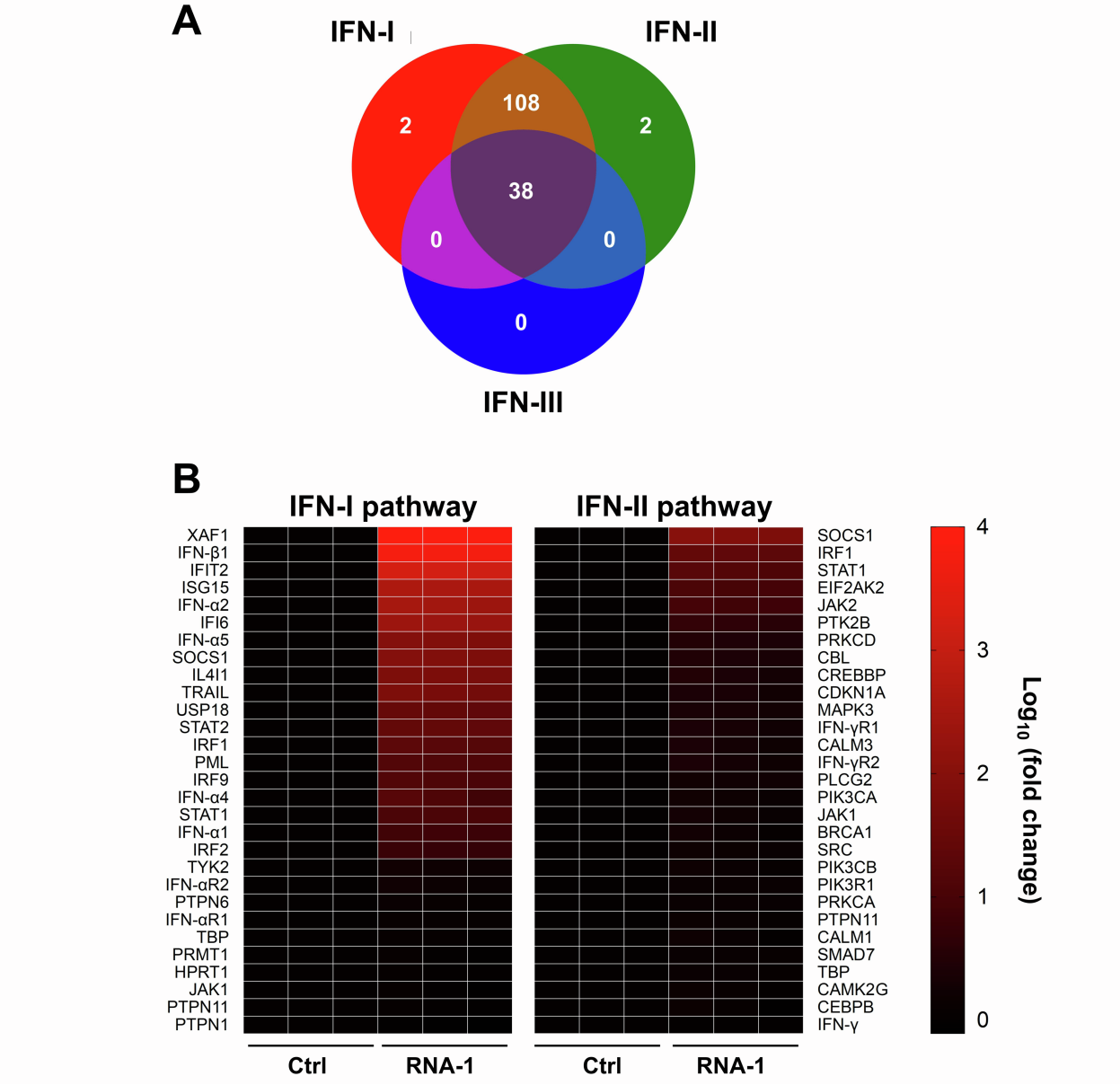


Figure S3. RNA-induced gene expression associated with type I interferon pathway. (**A**) Venn diagram showing differentially expressed ISGs from TMT Mass Spec by RNA-1 belong to type I or type II interferon stimulated genes. (**B**) Heat map of qPCR results showing RNA-I preferentially activates type I interferon pathway. A549 cells were transfected with RNA-1 or scrambled dsRNA control, collected at 48 hr and analyzed by qPCR (expression levels were normalized to GAPDH; gene levels induced by the RNA control were set as 1; N = 3).

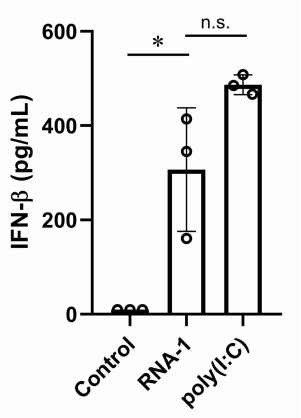


Figure S4. The levels of IFN-β protein induced by RNA-1 and poly(I:C). A549 cells were transfected with RNA-1 or poly(I:C) (34 nM) for 48 h, and then supernatants were collected for detection of IFN-β using ELISA. Scrambled RNA control NC-1 is used as negative (N = 3). *, $P < 0.05$; n.s., not significant.

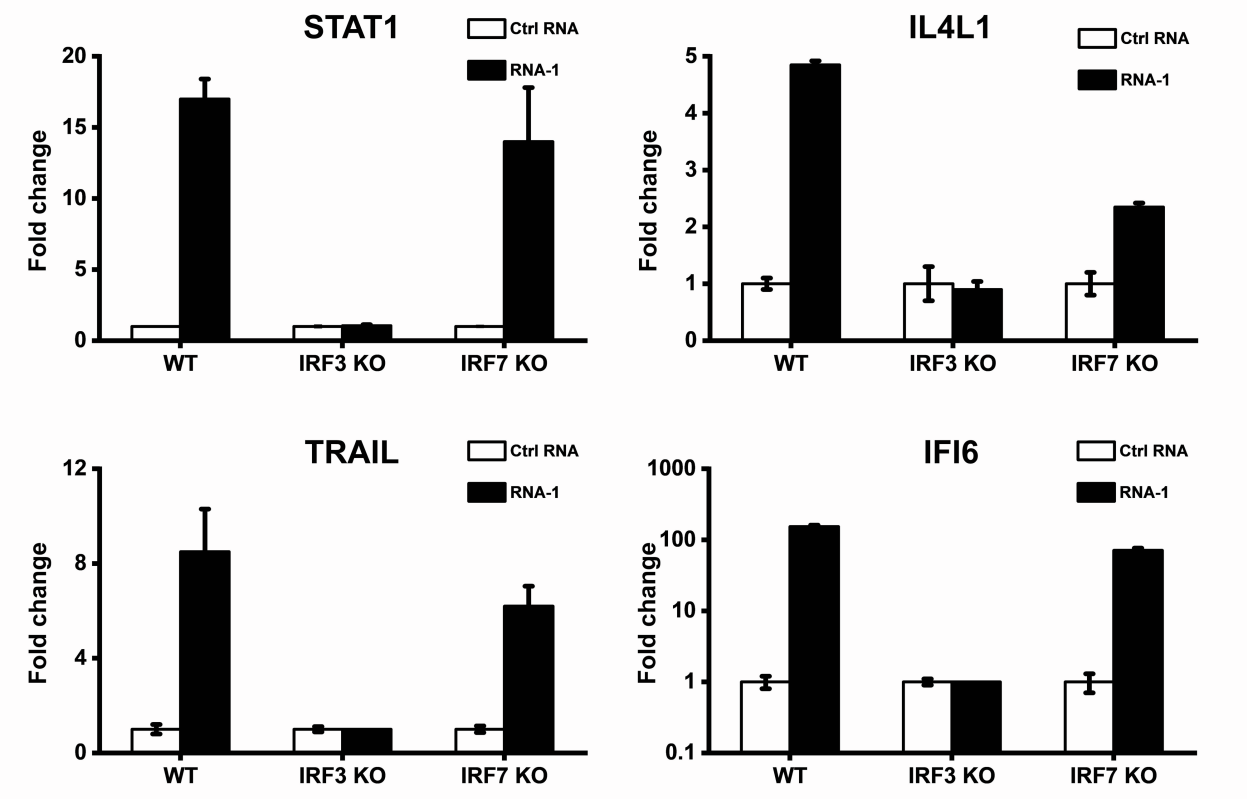


Figure S5. IRF3 knockout abolished the ability of immunostimulatory RNAs to induce IFN-I pathway associated genes. Wild-type (WT) HAP1 cells, IRF3 knockout HAP1 cells, or IRF7 knockout HAP1 cells were transfected with RNA-1 or a scrambled RNA control and STAT1, IL4L1, TRAIL, and IFI6 mRNA levels were quantified by qPCR at 48 h post transfection. Data are presented as fold change relative to RNA control (N = 3).

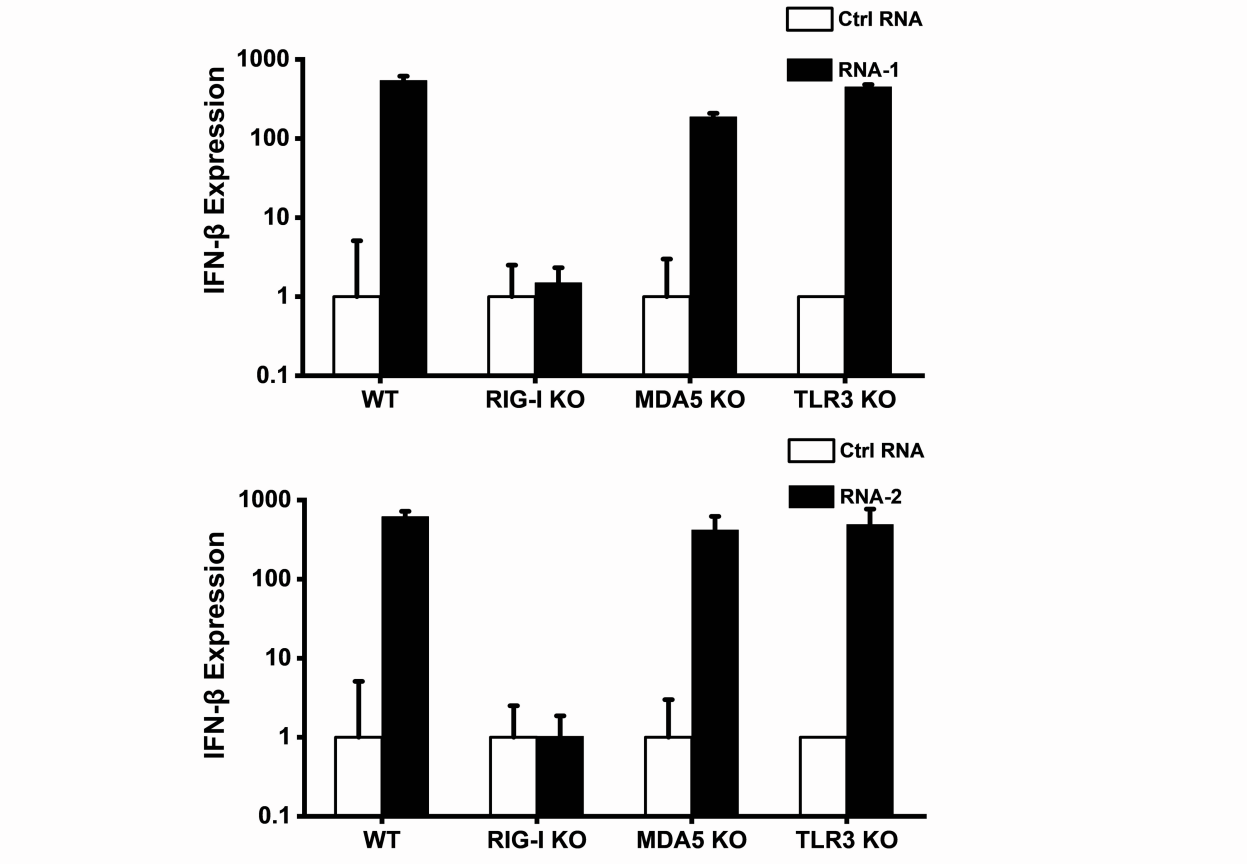


Figure S6. RIG-I knockout abolished the induction effects of the immunostimulatory RNAs on IFN-β. Wild-type (WT) A549-Dual cells, RIG-I knockout A549-Dual cells, MDA5 knockout A549-Dual cells, or TLR3 knockout A549 cells were transfected with RNA-1, RNA-2, or a scramble RNA control and IFN-β mRNA levels were detected by Quanti-Luc assay in WT, RIG-I KO, and MDA5 KO A549-Dual cells or qPCR in TLR3 KO A549 cells at 48 h post transfection. Data are shown as fold change relative to the scrambled RNA control (N = 6).

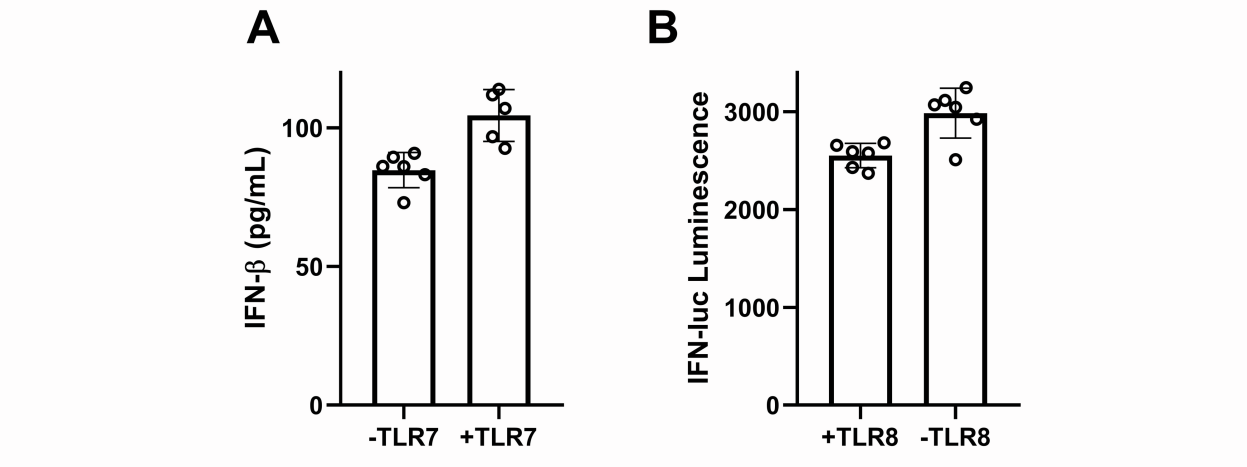


Figure S7. TLR7/8 knockout or overexpression did not have effect on the immunostimulatory activity of RNA-1. (**A**) Graph showing that the overexpression of TLR7 in HEK cells had no effect on production of IFN-β induced by RNA-1. (**B**) Graph showing that the knockout of TLR8 in THP1 cells had no effect on IFN production induced by RNA-1. These cell lines are commercial and could be purchased from InvivoGen.

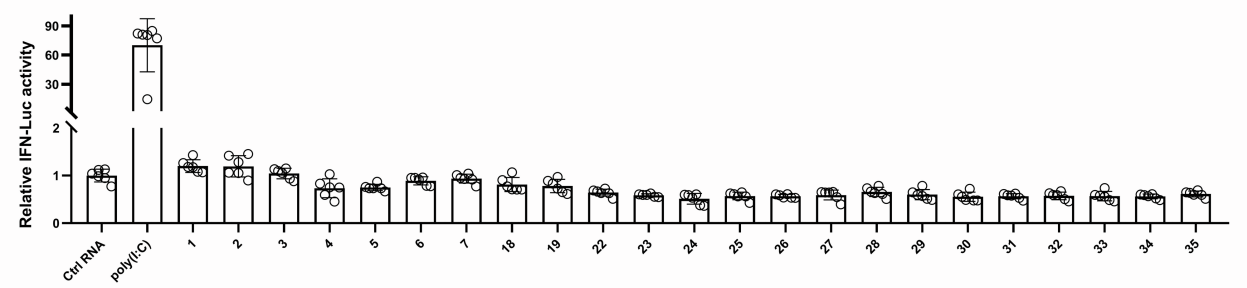
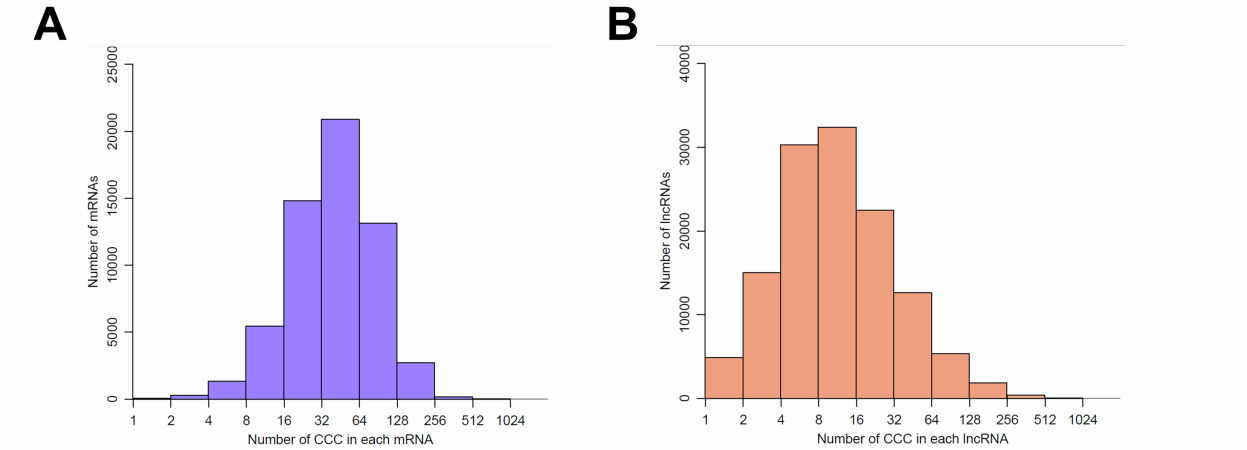


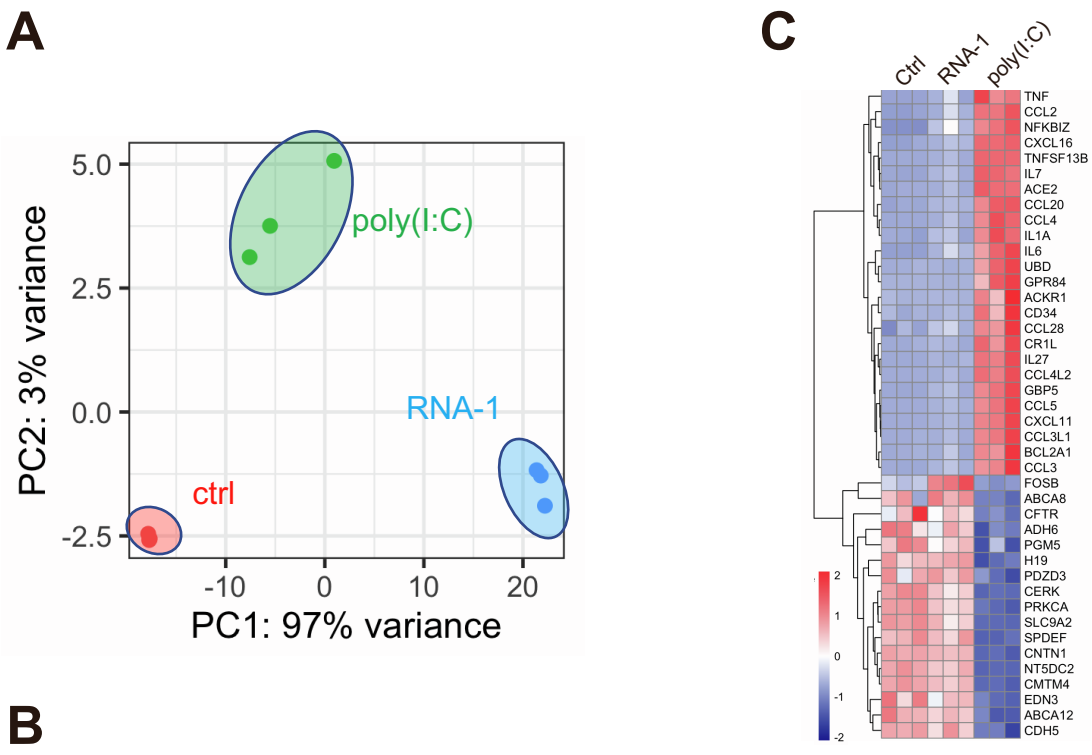
Figure S8. RIG-I knockout completely abolished the immunostimulatory activity of RNAs. RIG-I knockout A549-Dual cells were transfected with poly (I:C), immunostimulatory RNAs or a scrambles RNA control (34 nM) and 48 h later, activation of the IFN pathway was measured by quantifying luciferase reporter activity. Data are shown as fold change relative to the scrambled RNA control (N = 6). Note that poly (I:C) induced potent production of IFN in RIG-I knockout A549-Dual cells, while the immunostimulatory RNAs did not induce IFN in RIG-I knockout A549-Dual cells.



C

	Total number of RNAs analyzed	Number of RNAs containing 'CCC'	Percentage [Number of RNAs containing 'CCC'/ Total number of RNAs analyzed]	Average distance of 'CCC' in RNA [sum(RNA length)/ sum(CCC_number)]
mRNA	58852	58831	99.96%	75.45 bp
lncRNA	127802	125353	98.08%	75.93 bp

Figure S9. 'CCC' motif is widely distributed in human genome. (**A**) Graph showing the distribution of the number of CCC sequences in human mRNAs (retrieved from UCSC hg38 refGene with prefix NM). (**B**) Graph showing the distribution of the number of CCC sequences in human lncRNAs (retrieved from lncipedia). (**C**) Table showing the percentage of human mRNAs and lncRNAs containing the CCC motif and their average density.



B

IFN Type	Gene	Log2(Fold Change)	P_{adj}
Type I	IFNB1	8.0	3.6E-08
Type III	IFNL1	7.9	1.3E-16
	IFNL2	8.3	2.9E-17
	IFNL3	7.9	9.4E-21
	IFNL4	5.7	3.4E-06

Figure S10. RNA-seq analysis to characterize host responses induced by RNA-1 and poly(I:C). (A) Principal component analysis of A549 cells transcriptomes when transfected with scrambled dsRNA (ctrl), RNA-1 (isRNA) or poly(I:C) for 48 hours. N=3. (**B**) Table showing induction of Type I and III IFN genes based on RNAseq data shown in **Fig. 5A**. (**C**) Heat map showing top upregulated inflammatory genes and top downregulated genes involved in ion transport and cell-cell adhesion in the poly(I:C) transfected but not in the isRNA (RNA-1) transfected A549 cells.

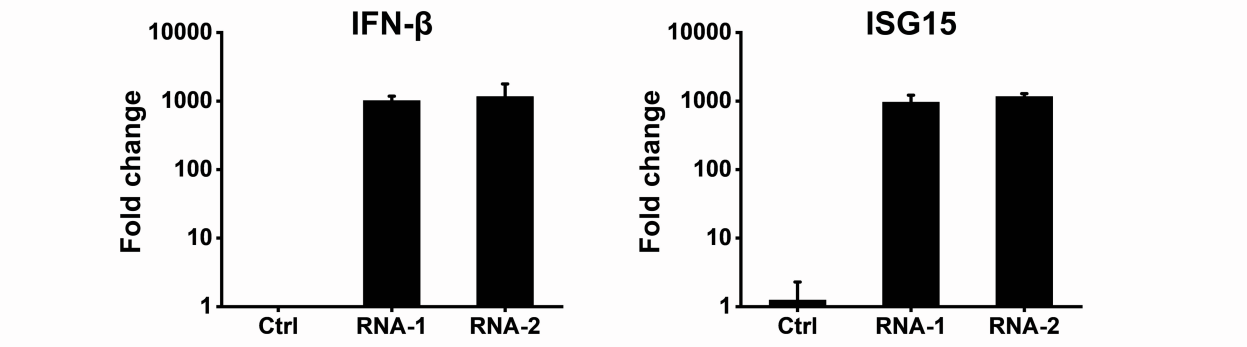


Figure S11. Immunostimulatory RNA-mediated production of IFN in ACE2-overexpressing A549 cells. IFN-β and ISG15 levels were detected in cells transfected with RNA-1, RNA-2, or scramble dsRNA control by qPCR at 48 h post-transfection. The IFN-β or ISG15 level induced by the scramble dsRNA control was set as 1. Data are shown as fold change relative to the control (N = 3).

Table S1. Summary of characteristics of reported immunostimulatory RNAs.

Characteristic	Signaling pathway	Cytokines
5'-UGUGU-3' motif	Toll-like receptor (TLR)8	IFN-alfa
5'-GUCCUUCAA-3' motif	TLR7/8	IFN-alfa
GU or AU rich	TLR7/8	IFN-alfa, TNF-alfa
Uracil repeats	TLR7	IFN-alfa, IL-6, TNF-alfa
Blunt ended dsRNA	RIG-I	Type I IFN, p56
5'-triphosphate; 5'-diphosphate	RIG-I	IFN-alfa, IFN-beta
MicroRNA-like siRNA	TLR7/8	IFN-alfa, TNF-alfa
Long dsRNA	MDA5	Type I IFN
Long dsRNA	TLR3	Type I IFN
Single stranded (ss)RNA	TLR7	Type I IFN