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Supplementary Materials for

A minimal RNA ligand for potent RIG-I activation in living mice

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
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Supplementary Materials

RNA Characterization: SLRs are palindromic sequences terminated by a stabilizing UUCG tetraloop, and are therefore most stable as monomeric stemloop (Fig. 1A). Although the monomeric nature of the SLRs has been confirmed by crystallography, ultracentrifugation (*10*), and fluorescence anisotropy experiments (*38*), at high SLR concentrations, we used native-gel electrophoresis to examine their propensity to form self-complementary duplexes (dimers) and to assess the purity of the monomeric stemloop form under the conditions used in this study (including the lipofectamine formulation). Non-palindromic triphosphorylated oligonucleotides of identical internal duplex stability were synthesized as dimerization controls. Under all conditions SLRs migrate exclusively as monomeric hairpin RNA molecules, without any trace of dimerized product (Supplementary Fig. 1), confirming that SLR RNA is monomeric when it is introduced into cells and animals. We cannot rule out the possibility of disproportionation once SLRs enter cells, but given the increased dilution upon transfection and the high entropic cost of dimerization, it is highly unlikely.

ppp-NS	5′***ppp***–GAAGCAAUCUCCACUUACUAGAAA–OH3'
OH-SLR10	5′OH–GGACGUACGU U ||||||||||| U 3′OH–CCUGCAUGCA G C
ppp-SLR10	5′***ppp***–GGACGUACGU U ||||||||||| U 3′OH–CCUGCAUGCA G C
OH-SLR14	5′OH–GGAUCGAUCGAUCG U |||||||||||||| U 3′OH–CCUAGCUAGCUAGC G C
pp-SLR14	5′***pp***–GGAUCGAUCGAUCG U |||||||||||||| U 3′OH–CCUAGCUAGCUAGC G C
ppp-SLR14	5′***ppp***–GGAUCGAUCGAUCG U |||||||||||||| U 3′OH–CCUAGCUAGCUAGC G C
19mer dS-ppp	5′***ppp***–GCAUGCGACCUCUGUUUGA–OH3′ ||||||||||||||||||| 3′OH–CGUACGCUGGAGACAAACU–OH5′
21mer dS-ppp	5′***ppp***–AACACACACACACACACACACACUUU–OH3′ ||||||||||||||||||||||| 3′OH–UUGUGUGUGUGUGUGUGUGUG–OH5′
23mer dS-ppp	5′***ppp***–AACACACACACACACACACACACUUU–OH3′ |||||||||||||||||||||||| 3′OH–UUGUGUGUGUGUGUGUGUGUGAA–OH5′
24mer dS-OH	5′OH–GGACGUACGUUUCGCGACUGUAGA–OH3′ |||||||||||||||||||||||| 3′OH–CCUGCAUGCAAAGCGCUGACAUCU–OH5′
24mer dS-ppp	5′***ppp***–GGACGUACGUUUCGCGACUGUAGA–OH3′ |||||||||||||||||||||||| 3′OH–CCUGCAUGCAAAGCGCUGACAUCU–OH5′

fig. S1. RNA ligands for RIG-I activation. All oligonucleotides were synthesized as described in Methods. The dsRNAs used in this experiment vary in length and stability, and were tested to facilitate comparisons with published studies on annealed duplexes as RIG-I ligands. The 24mer dsRNA duplex (43.9 kcal/mol stability) has a terminal sequence analogous to SLR10 and SLR14, and approximately twice the duplex length. The 19mer dsRNA duplex (32.7 kcal/mol) is the same sequence and reported composition as an RNA that is marketed as a RIG-I ligand (Invivogen), but it was synthesized in-house, in parallel with all other RNAs used in this study. The 21mer dsRNA (35.9 kcal/mol) & 23mer dsRNA (37.4 kcal/mol) duplexes are identical in sequence and reported composition to those reported previously in studies of RIG-I activation (*9*). Importantly, these have a variable 3'-overhang on the non-triphosphorylated duplex end. Free energies of all duplexes, including overhanging ends, were calculated as described (*72*).

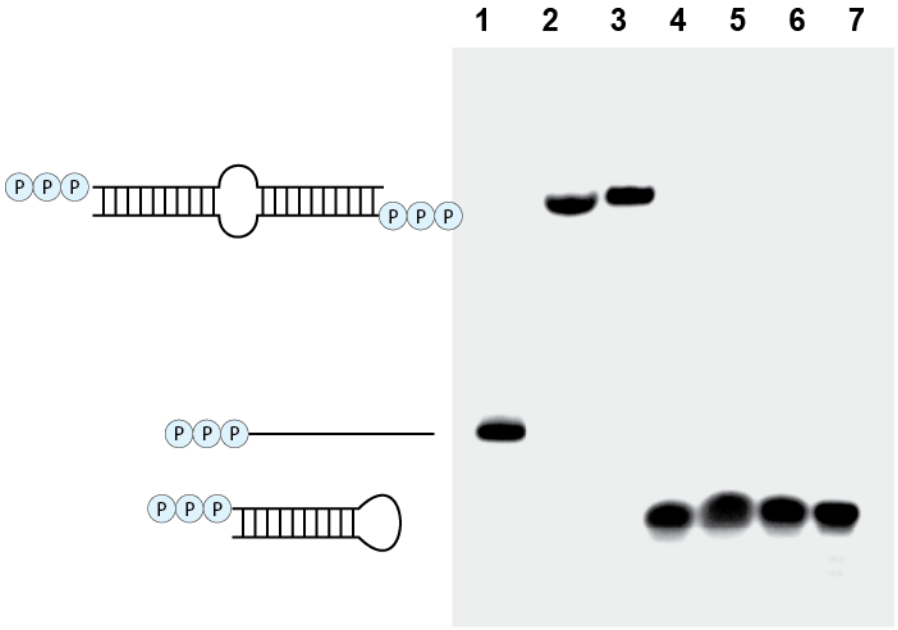

fig. S2. SLR10 is monomeric in conditions that mimic cell culture and in vivo experiments. Monomeric and dimeric RNA species are separated on a native gel. **1)** 24-mer single stranded RNA marker (5' - pppUCUACAGUCGUUCGACGUACGUCC - 3'); **2)** 24-mer duplex marker corresponding to a putative SLR10 dimer control (5' - pppGGACGUACGUUUCGCGACUGUAGA – 3' / 5' – pppUCUACAGUCGUUCGACGUGCAUCC – 3'). 0.5 mM RNA is in ME buffer (10 mM Na-MOPS pH 6.0, 1 mM trisodium EDTA); **3)** 24-mer duplex marker in cell culture reagents: 0.001 mM RNA in Opti-MEM + lipofectamine; **4)** 0.5 mM SLR10 in ME buffer, heated to 95 °C and snap cooled immediately prior to running to control for freezing/thawing effect on HP folding; **5)** 1 mM SLR10 in ME buffer, thawed from -80 °C and loaded without refolding; **6)** 0.5 mM SLR10 in ME buffer, thawed from -80 °C; **7)** SLR10 in cell culture reagents: 0.001 mM RNA in Opti-MEM + lipofectamine

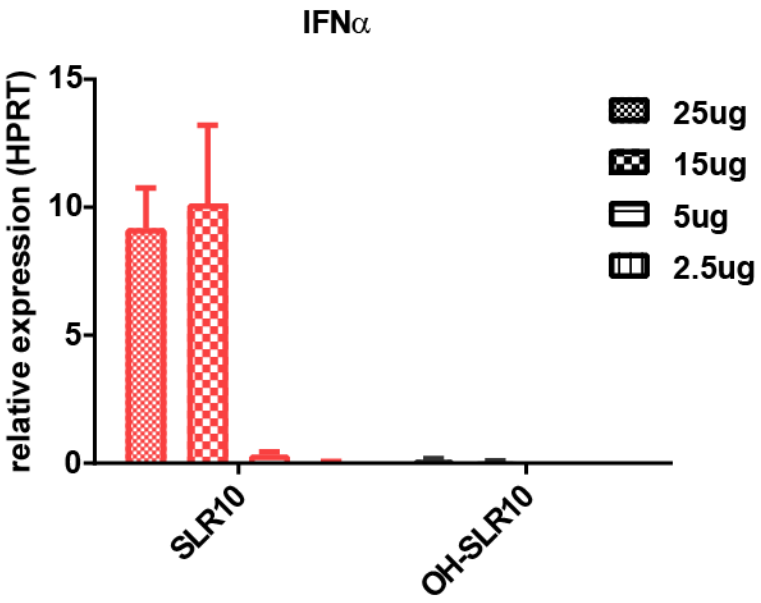

fig. S3. Dose response of SLRs. Mice were injected i.v. with SLR10 or OH-SLR10 and spleens harvested after 3 hours for RNA isolation. IFNα was measured by qRT-PCR. 25 μg was chosen as the optimal dose for all subsequent experiment.

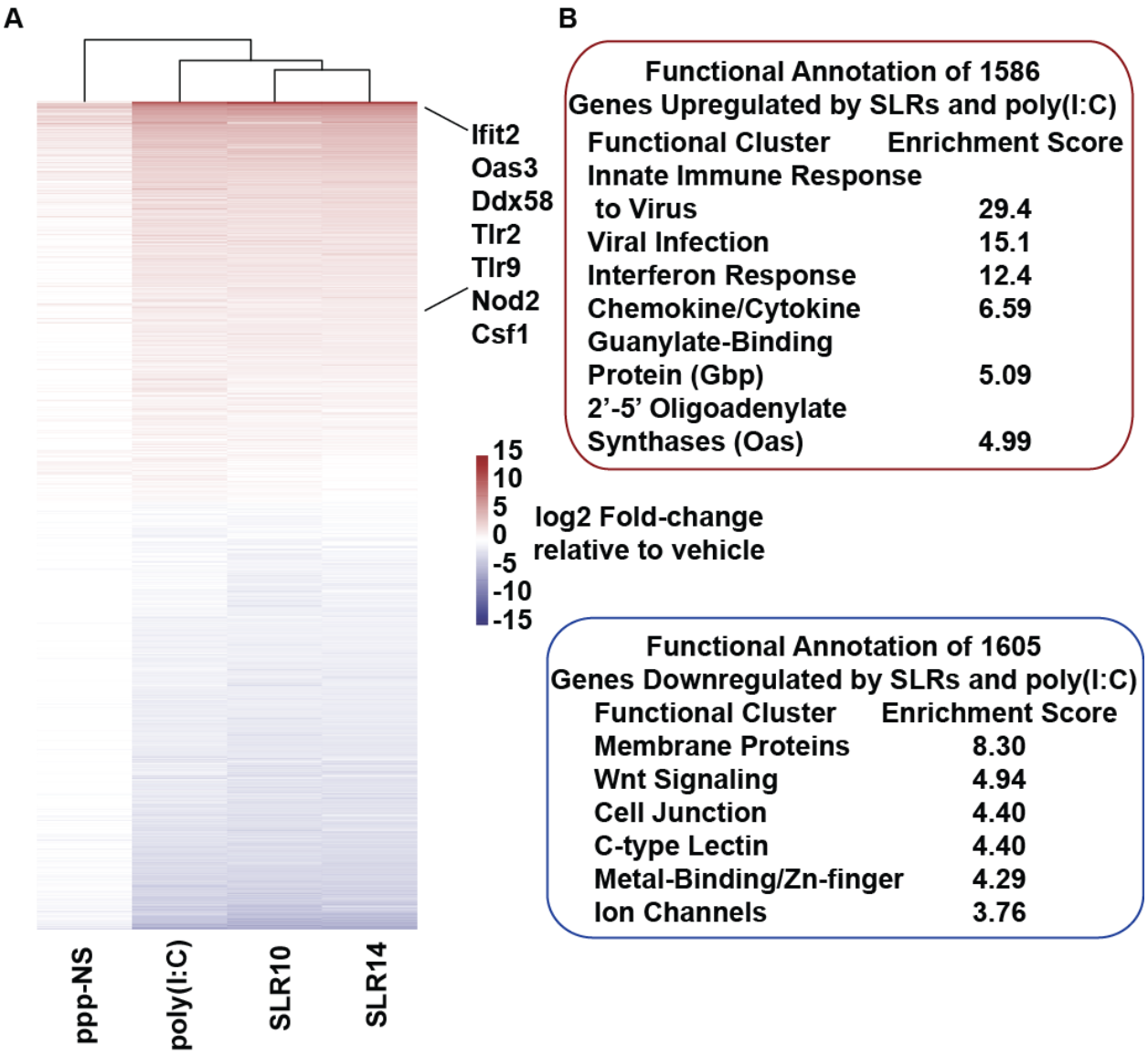

fig. S4. SLRs and poly(I:C) regulate a shared set of genes. Heatmaps were generated for all genes that were differentially expressed relative to vehicle upon treatment with RNA (**A**). Many genes associated with innate immunity and antiviral response were upregulated by SLR and poly(I:C) to similar extents. Several examples are indicated to the right of the heatmap. DAVID pathway analysis was performed on the annotated functions of genes mutually up/downregulated by treatment with SLR and poly(I:C) (**B**). Genes selected for analysis were differentially expressed by SLR14 or poly(I:C) relative to vehicle (>2 fold-change, FDR < 0.05) but were not differentially expressed between the two treatments (< 2 fold-change or FDR > 0.05).

table S1. List of genes mutually regulated by SLRs and poly(I:C). A list and statistical metrics for all genes that are differentially expressed by SLR14 or poly(I:C) relative to vehicle (>2 fold-change and FDR < 0.05) but not differentially expressed between SLR and poly(I:C) (< 2 fold-change or FDR > 0.05). Complete results from the DAVID analysis in Supplementary Figure 4B are also included.

table S2. List of genes preferentially regulated by SLRs or poly(I:C). A list and statistical metrics for all genes that are differentially expressed by SLR14 or poly(I:C) relative to vehicle (>2 fold-change and FDR < 0.05) and differentially expressed between SLR and poly(I:C) (> 2 fold-change and FDR < 0.05). These data correspond to Figure 5.