Appendix 2. Supplemental methods

I. Details of vector construction

The TLR3/TLR4 chimera was constructed by introducing a novel AseI site between the TLR3ecd and the TLR4 transmembrane and intracellular domains by PCR. Note that this method mutates the first amino acid of TLR4 from isoleucine (I) to leucine (L), which is expected to largely conserve local structure. Three products were ligated together to make pUNO hTLR3/4: 1) AseI/NheI-digested PCR of hTLR4 sequences from pUNO hTLR4 (Invivogen), 2) ClaI/AseI-digested PCR of a portion of hTLR3 from pUNO hTLR3 (Invivogen) and 3) NheI/ClaI-digested pUNO vector. PCR primers used were:

Oligo name	Sequence (5' to 3')
TLR3_ClaI(+)	TCCAACGGCTTTGACG
TLR3_AseI(-)	ATTAATAGTTCAAAGGGGGCACTGTC
TLR4_AseI(+)	ATTAATTGGTGTGTCGGTCC
TLR4_NheI(-)	GCTAGCTCAGATAGATGTTGCTTCC

The NF-κB-responsive NEE (NF-κB-ELAM-EGFP) reporter cassette was constructed by excising the luciferase gene from the pNifty2-Luc reporter plasmid (Invivogen) using NheI/NcoI and replacing it with EGFP from pEGFP-N3 (BD Biosciences) cut with NcoI/XbaI. The NEE cassette was cut with EcoRI/NotI and subcloned into the lentivector pLenti6.2/V5-Dest (Invitrogen), which was cut with PspOMI and EcoRI. The extraneous CMV promoter was removed by digestion with BamHI and ClaI, blunting with Klenow, and self-ligation to yield pLenti6.2/NEE. The lentiviral vector HIV CS CN, which contains a CMV-Neo^R cassette inserted into the HIV CS vector (1), was used for transgene introduction. The hEF1-HTLV TLR3 expression cassette was cut from pUNO hTLR3 with PvuI/NheI, HIV CS CN was cut with SacII/BamHI, and both products were blunted with Klenow and ligated to yield HIV CS TLR3 CN. Similarly, HIV CS TLR3/4 CN was constructed from pUNO hTLR3/4.

II. Synthesis of dsRNA oligonucleotides

Sense and antisense templates were constructed for each dsRNA ligand. Templates for dsRNA 35 bp and shorter were created by synthesizing a DNA oligonucleotide containing the T7 promoter followed by the RNA-encoding sequence and annealing it to a complementary DNA oligonucleotide. For larger ligands, each template was constructed by amplifying a portion of the pFastBac-CPrME plasmid (2) by PCR, ligating the PCR product into the pGEM-T Easy plasmid (Promega) in each orientation, and linearizing the resulting plasmids with NdeI. Oligonucleotides used for annealing and as PCR primers were:

Oligo name	Sequence (5' to 3')
PCR primers for amplifying dsRNA templates from pFastBac-CPrME	
Universal(+)	CTGCGGATTATTTGGC
62bp(-)	GAGCAGGCAAATTTGG
90bp(-)	CAAGATGGTTCTTCC
139bp(-)	TAGTTGGTCCATGGAC
532bp(-)	CAACTTGACAGTGTTGC
Oligos for annealing as dsRNA templates, to be ligated into pGEM-T Easy	
39bp_template(+)	CTGCGGATTATTTGGCAAAGGAAGCATTGAC
48bp_template(+)	CTGCGGATTATTTGGCAAAGGAAGCATTGACACATGCGCC
Oligos for direct dsRNA synthesis (contain T7 promoter and initial 'G')	
T7_25bp_sense(+)	GGATCCTAATACGACTCACTATAGCTGCGGATTATTTGGCAAAGGAAGC
T7_25bp_antisense(+)	GGATCCTAATACGACTCACTATAGGCTTCCTTTGCCAAATAATCCGCAG
T7_29bp_sense(+)	GGATCCTAATACGACTCACTATAGTGCGGATTATTTGGCAAAGGAAGCATTGA
T7_29bp_antisense(+)	GGATCCTAATACGACTCACTATAGTCAATGCTTCCTTTGCCAAATAATCCGCA
T7_35bp_sense(+)	GGATCCTAATACGACTCACTATAGCTGCGGATTATTTGGCAAAGGAAGCATTGACACAT
T7_35bp_antisense(+)	GGATCCTAATACGACTCACTATAGATGTGTCAATGCTTCCTTTGCCAAATAATCCGCAG

All PCR reactions utilized the same forward primer, Universal(+). Each of the oligonucleotides listed under "Oligos for annealing as dsRNA templates..." and "Oligos for direct dsRNA synthesis..." was annealed to an exactly complementary partner DNA oligonucleotide (not shown here). dsRNA synthesis was carried out using the T7 Ribomax kit (Promega), as per the manufacturer's instructions. Note that all templates to be cloned into the pGEM-T Easy plasmid are 8 bp shorter than the final dsRNA product. In this vector, four self-complementary plasmid-derived bases flank the dsRNA template (GATT-Template-AATC), and these bases (but not other plasmid sequences) pair during sense/antisense ssRNA annealing and, therefore, resist RNAse A digestion (which is part of the synthesis procedure) and remain as part of the final dsRNA product. Mfold software (version 3.2) (3, 4) did not predict any stable alternative secondary structures for any of these sequences (data not shown).

References

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3. Mathews D H, Sabina J, Zuker M, Turner D H (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* 288**:** 911-40.
4. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31**:** 3406-15.