Materials and Methods

**Reagents.** R-848 was commercially synthesized by the Pharmaceutical and Biotechnology Laboratory, Japan Energy Cooperation or by GLSynthesis Inc., Worcester, MA, USA and provided by Coley Pharmaceuticals GmbH (Langenfeld, Germany), respectively. *E. coli* LPS 055:B5, poly (I:C), guanosine and uridine (cell culture grade) were from Sigma-Aldrich (Taufkirchen, Germany). The sequences of CpG DNAs are 1668 (5’-TsCsCsAsTsGsAsCsGsTsTsCsTsGsAsTsGsCsTs), 2006 (5’-TsCsGsTsCsGsTsTsTsGsTsGsTsGsTsGsTsGsTs), 2216 (5’-GsGsGGGACGATCGTGsGsGsGsGsGsGsG) and D19 (5’-GsGsTGCATCGATGCAsGsGsGsGsGsG) where ‘s’ depicts a phosphothioate linkage. Cell culture grade phosphothioate protected RNA oligonucleotides, RNA33 (5’-GUAGUGUGUsG), RNA34 (5’ GUCUGUUGUGUsG), RNA40 (5’-GsCsCsGsUsCsUsGsUsGsUsGsUsGsAsCsUsC), RNA41 (5’-GsCsCsGsAsCsAsGsAsGsAsGsAsGsAsCsAsC), RNA42 (5’-AsCsCsCsUsCsUsAsUsAsUsAsUsAsUsAsUsAsCsAsC) and corresponding DNA40-42 sequences were synthesized by IBA GmbH (Göttingen, Germany) (phosphothioate protected positions are indicated by ‘s’). Expression plasmid for human TLR2 and TLR3 were kindly provided by Tularik Inc. (South San Francisco, USA). Expression plasmids for human and murine TLR7, 8 and 9 have been described previously (1,2). APC-conjugated streptavidin, biotinylated α-human TNF-α, α-human CD11c-PE, CD123-PE, α-murine CD40-FITC, CD69-FITC and CD86-FITC antibodies were from BD PharMingen, San Diego, CA and α-BDCA4-PE antibody from Milteny, Bergisch Gladbach, Germany.
**Cells.** Human PBMC were generated by Ficoll gradient centrifugation and seeded at $3 \times 10^5$ or $1 \times 10^6$ cells/well for cytokine induction or intracellular staining, respectively. Human plasmacytoid DC were depleted from PBMC and enriched by incubation with PE-labelled $\alpha$-CD123 or $\alpha$-BDCA-4 followed by anti-PE beads from Miltenyi (Bergisch Gladbach, Germany) according to the manufacturers recommendation. PDC depleted PBMC or enriched pDC were seeded at $3 \times 10^5$ or $2 \times 10^4$ cells/well, respectively. FLT-3 ligand induced mixed cultures of murine myeloid and plasmacytoid dendritic cells were grown as described (3). Plasmacytoid DC from FLT-3 ligand induced bone marrow cultures were sorted on the basis of high expression for CD11c and CD45RA and lack of expression for CD11b in the presence of propidium iodide to exclude dead cells using a MoFlo instrument (Cytomation Inc., Fort Collins, CO). Murine macrophages were grown with 10 ng/ml MCS-F for 6 days. Murine DC and macrophages were seeded at $2 \times 10^5$ or $2 \times 10^4$ cells/well, respectively. Human and murine primary cells were cultivated in RPMI (PAN, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS), 4mM L-glutamine and $10^{-5}$ M mercaptoethanol. Human embryonic kidney 293 (HEK293) cells were obtained from ATCC and cultivated in Dulbecco's modified Eagle's medium (PAN, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS) and 4 mM L-glutamine.

**Cell stimulation and staining.** For extracellular staining and cytokine induction, human or murine cells were incubated for 14-16 hours with $1 \mu$M CpG-ODN 1668, 2216 or $3 \mu$M CpG-ODN D19, 1-10 $\mu$M or 100 nM R-848 (depending on the source), 100 ng/ml LPS, 50 $\mu$g/ml poly (I:C) or 10 $\mu$g/ml RNA40-42. Complexation of RNA with DOTAP (Roche, Mannheim, Germany) was performed according to the manufacturers recommendation. Shortly, RNA in 50 $\mu$l DOTAP-buffer was combined with 50 $\mu$l DOTAP solution (10 $\mu$g)
and incubated for 15 min. 100 µl complete RPMI medium were added and the solution was mixed. 100 µl were used for stimulation of a 96-well that contained primary cells in 100 µl complete medium. For intracellular staining human PBMC were stimulated with 50 µg/ml poly (I:C), 1 µM R-848 and 10 µg/ml RNA40-42 and incubated with 5 µg/ml brefeldin A for 4 hours. Cells were stained with α-CD3-PE and after permeabilization incubated with biotinylated anti human TNF-α and APC-conjugated streptavidin according to the intracellular staining protocol provided by BD Pharmingen.

Cytokine measurement. Concentrations of human and murine TNF-α, IL-12 p40 or IL-6 in the culture supernatants were determined by ELISA according to the manufacture’s instructions (BD Pharmingen, San Diego, CA). Murine IFN-α was analysed using a PBL Biomedical Laboratories (New Brunswick, NJ) ELISA kit. The IFN-α standard used was recombinant mouse IFN-α from HyCult Biotechnology, Uden, Netherlands or from PBL Biomedical Laboratories. Elisa for human IFN-α was performed with module set from Biozol Diagnostics (Eching, Germany) according to the manufacturers protocol.

Mice. MyD88, TLR3, TLR7 and TLR9 deficient mice were established as described (4-7). TLR2 deficient mice (8) were crossed with TLR4 deficient mice (9) to yield double TLR2 and TLR4 deficient mice. C57BL/6 wild type mice were from Harlan (Borchen, Germany) or from CLEA Japan (Tokyo, Japan).
Transfection and reporter assay. For monitoring transient NF-kB activation, 3x10^6 HEK293 cells were electroporated at 200 volt and 960 µF with 1 µg of TLR expression plasmid and 20 ng NF-κB luciferase reporter-plasmid. The overall amount of plasmid DNA was held constant at 15 µg per electroporation by addition of the appropriate empty expression vector. Cells were seeded at 10^5 cells per well and after over night culture stimulated with 25 µg/ml RNA40-42 complexed to DOTAP, 1 µM CpG-ODN 2006, 10 µM R-848, 50 µg/ml poly (I:C) or 1 µg/ml Pam3Cys for a further 7 to 10 hours. Stimulated cells were lysed using reporter lysis buffer (Promega, Mannheim, Germany) and lysate was assayed for luciferase activity using a Berthold luminometer (Wildbad, Germany) according to the manufacturer’s instruction.
Supplemental figure legends

**Fig. S1.** RNA and not DNA sequences are stimulatory for immune cells. Human PBMC were stimulated with 1 µM R-848, 50 µg/ml poly (I:C), 7.5 µg/ml RNA40-42, corresponding DNA sequences DNA40-42, free or complexed to DOTAP and TNF-α secretion determined. One representative experiment of at least two independent experiments with different donors is shown (n=2, ± SD).

**Fig. S2.** Stimulation of GU-rich ssRNA is MyD88 dependent and independent of TLR2 and TLR4. FLT3-ligand induced murine DC from wild-type, MyD88 deficient and TLR2/4 double deficient mice were stimulated for 14-16 h with DOTAP, 10 µg/ml RNA40 complexed to DOTAP, 100 ng/ml LPS, 50 µg/ml poly (I:C) or 1 µM CpG-ODN 1668 and stained for CD40 (black line). CD40 expression without stimulation is depicted as grey shaded region. One representative experiment of at least two independent experiments is shown.
Heil et al., Fig. S1
Supplemental References


