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Supplementary Information for

Polymerase III transcription is necessary for T cell priming by dendritic cells

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This PDF file includes:

Supplementary Material and Methods
Figs. S1 to S4
Tables S1 to S4

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Supplemental Material and Methods

Reagents

Reagents and used concentrations were as follows: HMW Poly(I:C) (10 μg/mL)) was from InvivoGen; LPS (Escherichia coli O55:B5) (100 ng/ml), Rapamycin (100 nM) and Sodium Arsenite (500 μM, 30min) were from Sigma-Aldrich. RNA Polymerase III Inhibitor ML-60218 (50μM) was from Merck. CX-4945 (Silmitasertib)(10 μM) was from Abcam. ISRIB (750 nM) was a gift from Peter Walter, UCSF, San Francisco.

Mice

Wild-type (WT) female C57BL/6 mice were purchased from Janvier, France; Ifnar1-/- mice (B6.129S2-Ifnar1^{tm1agt}) were a kind gift of Elena Tomasello (Dalod Lab, CIML); animals were maintained in the animal facility of Centre d'Immunologie de Marseille-Luminy (CIML). OT-II mice (12) on a RAG-2 deficient background were kept in CIPHE and were a kind gift of Sandrine Henri (Malissen Lab, CIML). Animals were kept under specific pathogen–free conditions accredited by the French Ministry of Agriculture to perform experiments on live mice. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the French Ministry of Agriculture and of the European Union. All experiments were approved by the Comité d'éthique de Marseille and the Direction Départementale des Services Vétérinaires des Bouches du Rhône (approval number A13-543). All efforts were made to minimize animal suffering.

Molecular Biology and gene expression analysis

4×10^6 BM-DCs were transfected with 4 ug of origene pCMV6 mouse Maf1 plasmid and pCMV6 empty plasmid using the Amaxa Mouse Dendritic Cell Nucleofector™ Kit following the provider´s instructions. Cells were activated 12 h post transfection and analysed. FlexiTube siRNA targeting *Maf1* and FlexiTube siRNA scramble as control (QIAGEN) were used for RNA silencing experiments. 4×10^6 BM-DCs were transfected with 1 μg of siRNA using the Amaxa Mouse Dendritic Cell Nucleofector™ Kit. Cells were activated 16 h post transfection and analysed. Total RNA was isolated using Trizol® (Invitrogen) and the Direct-zol™ kit (Zymo Research). cDNA was prepared with random hexamers and SuperScript III reverse transcriptase (Invitrogen). Real-time qPCR analysis was performed using SYBR Green PCR master mix (Takara) with Applied Biosystems PRISM 7700 Sequence Detection System. Primers are listed in Table S1.

Translation intensity measurement - Puromycin labelling

Puromycin labelling for measuring translation levels was done as previously described (Schmidt et al., 2009). Puromycin (Sigma-Aldrich) was added at 12,5µg/ml to the culture medium and the cells were incubated for 15 min at 37°C and 5% CO2. After puromycin incorporation the cells were harvested, washed in PBS and processed with Cytofix/Cytoperm buffer (BD Biosciences), stained with the anti-puromycin antibody (12D10) directly coupled with Alexa Fluor 488, and diluted in Perm/Wash buffer (BD Biosciences). Samples were acquired on a FACS Canto II (BD Biosciences), and data were analysed using FlowJo (Tree Star).

Immunofluorescence

Cells were harvested and dropped on a 12-mm coverslip covered with alcian blue. The coverslips were then incubated for 10 min at 37°C and fixed with 3,7% paraformaldehyde for 10min at room temperature. Cells were permeabilized with 0,1% Triton X-100 in 5% FCS PBS with 100 mM glycine, for 15 min at room temperature and stained over night at 4ºC with indicated primary antibodies, MAF1 (Abgent), G3BP1 and DCP1A (Santa Cruz). Coverslips were mounted using Prolong Gold and images taken with a laser-scanning confocal microscope (LSM 780; Carl Zeiss MicroImaging) using a 63× objective and accompanying imaging software. Nuclear and cytosolic quantification was performed using the isolateCells.ijm Image J pluggin developed by Jan Valečka (CIML and Labex INFORM).

Confocal immunofluorescence microscopy combined with fluorescent mRNA *in situ* hybridization (FISH) or immuno-proximity ligation assay.

Cells on coverslips were fixed with 3.7% formaldehyde for 15 min, then permeabilized in 70% EtOH overnight and blocked with 5% bovine serum albumin (Sigma) containing ribonucleoside vanadyl complex (2 mM). Cells were stained for G3BP1 and RNase inhibitor (Rnasin, Promega) was added to the staining buffer. After this, cells were washed with 10% formamide in 2× SSC. A fluorescent probe (Quasar 570) against CD86 mRNA (Stellaris, Biosearch Technologies) was diluted in hybridization buffer containing dextran sulfate 10 mg/ml and 10% formamide in 2× SSC, according to the manufacturer's instructions. Probe was incubated with cells for 4 h at 37°C. Coverslips were mounted with Prolong Gold containing DAPI (Invitrogen). For immuno-proximity ligation assay (iPLA), cells were layered on a 12-mm coverslip covered with alcian blue. The coverslips were incubated for 10 min at 37°C and fixed with 3,7% paraformaldehyde for 10min at room temperature. iPLAs were done using the Duolink Kit according to the manufacturer’s instructions (Sigma-Aldrich). Specifically, the primary antibodies were: mTOR and CK2a from Santa Cruz and BRF1 was form Abcam. Duolink iPLA Probe anti-rabbit minus and anti-mouse plus were used. Samples were incubated in the ligation solution consisting of Duolink Ligation Stock and Duolink Ligase. Detection of the amplified probe was done with the Duolink Detection Kit Orange. All confocal microscopy images acquisition was performed with an LSM 780 laser-scanning microscope (Carl Zeiss MicroImaging) using a 63× objective.

Microarrays analysis.

tRNA microarrays were performed as previously described (14). Shortly, total RNA (5-10 µg) was extracted from cells at basic conditions using TRIzol® (Invitrogen) which leads to complete deacylation of tRNAs (14). Cy3- or Atto647-labeled stem-loop RNA/DNA oligonucleotide was ligated overnight at 16°C with T4 DNA ligase (NEB) to all deacetylated tRNAs. The tRNAs isolated from sample at the onset of experiment (zero-time point) were labeled with Atto647-labeled stem-loop oligonucleotide, while the samples representing other time points with Cy3-labeled one and hybridized together on one microarray for 16 h at 60°C in. The arrays were normalized to spike-in standards (14) and quantified with in-house python and R scripts.

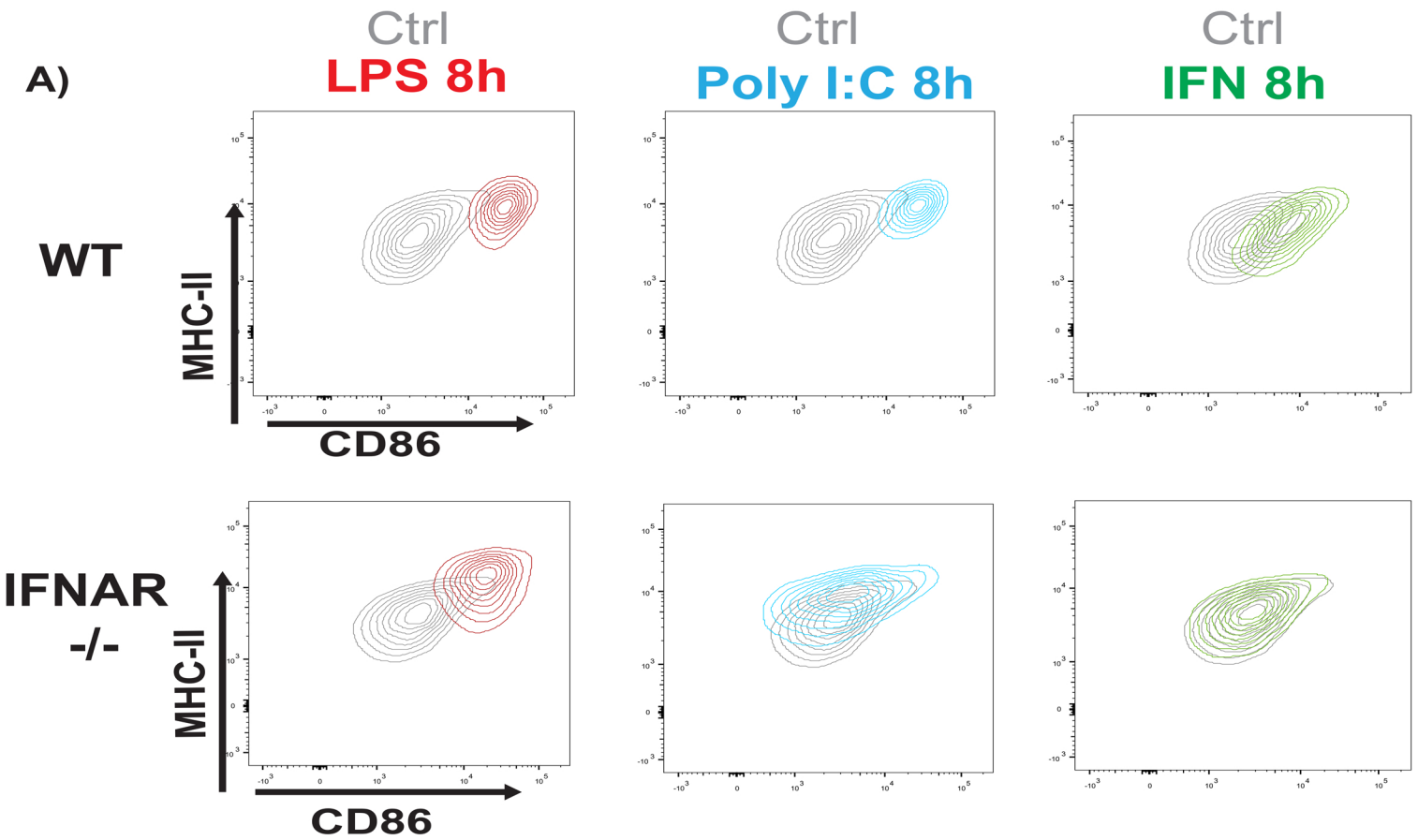
For gene expression analysis the total RNA was isolated with RNeasy kit (Qiagen). cDNA was synthesized with random hexamers and superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed with Applied Biosystems PRISM 7700 Sequence Detection System. The list of primers used is included in supplementary table 1. For Affymetrix microarray analysis, GM-CSF DCs were cultured in RPMI supplemented with 5 % FCS, 50µM beta-mercaptoethanol and GM-CSF. Cells differentiated for 6 days were treated for 8h with microbial stimuli and harvested before lysis. Control and Proteus mirabilis treated DCs were incubated with the bacteriostatic chloramphenicol to avoid bacterial growth. Guanabenz was used at 50 µM. Hybridization to arrays (Affymetrix GeneChip Mouse Gene 1.0ST) and image scanning were performed according to the Affymetrix Expression Analysis Technical Manual. Gene Expression microarray raw data were normalized using limmaGUI software (R/Bioconductor, Boston, MA, USA). Data are deposited in the GEO repository (GSE90831).

T cell proliferation

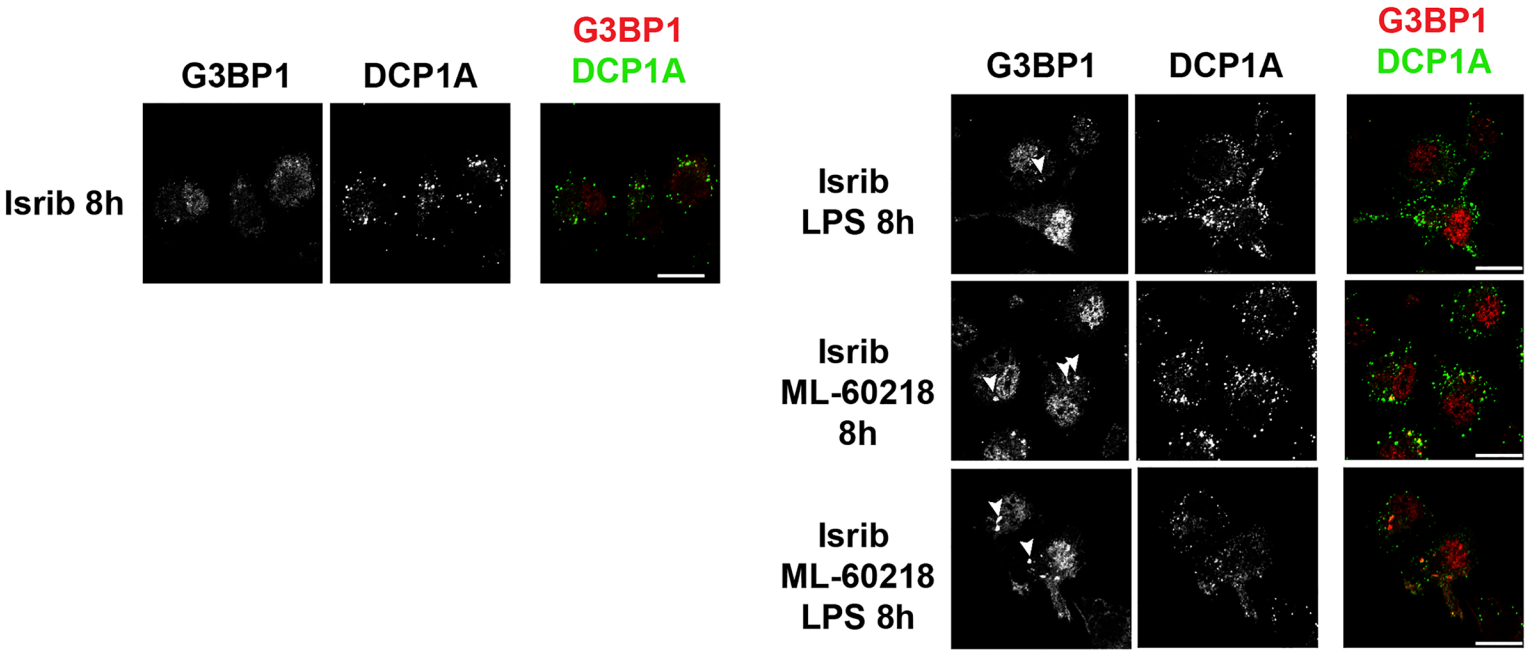
T cell proliferation assays were performed as described in (16). Briefly, the spleens and lymph nodes of OT-II *Rag-2*$^{-/-}$ mice were used, cells were isolated using a CD4 untouched Dynabeads® kit (Invitrogen). Purified OT-II Rag-2−/− T cells were labelled with CellTrace ™ Violet (Invitrogen). A total of 3 × 10^3 DCs were cocultured with 2 × 10^4 CTV-labeled OT-II *Rag-2*$^{-/-}$ T cells in 150 µL in the presence of ovalbumin (323-339) peptide (0.03 µg/mL). After 4 days of culture, proliferation (a loss of CTV staining) and Foxp3 expression were measured by flow cytometry. Foxp3 expression levels were analysed using the Foxp3 staining set (eBioscience) following the manufacturer's protocol.

Statistical analysis

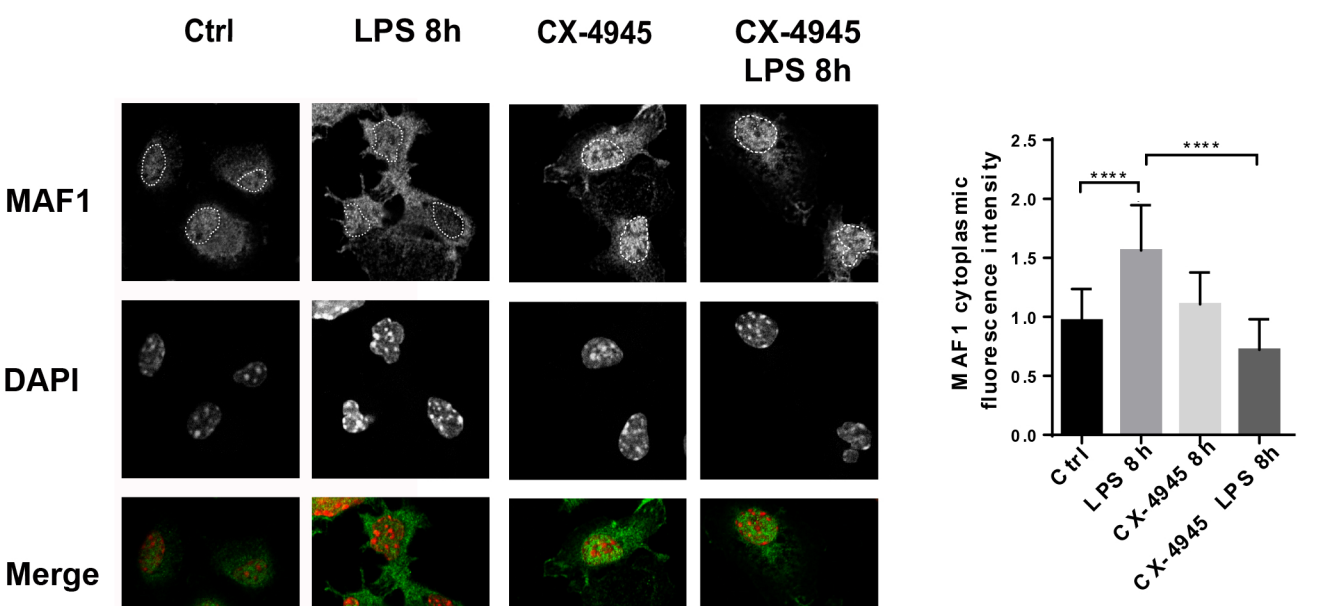
Statistical analysis was performed using GraphPad Prism Software. * P<0.5, ** P<0.01, *** P< 0.001, ****P<0.0001. Mainly unpaired Student's *t*-test was used. Additionally, multiple comparisons analysis with the Holm-Sidak correction was used when comparing multiple treatments and covariance analysis was performed on the tRNA microarray data.



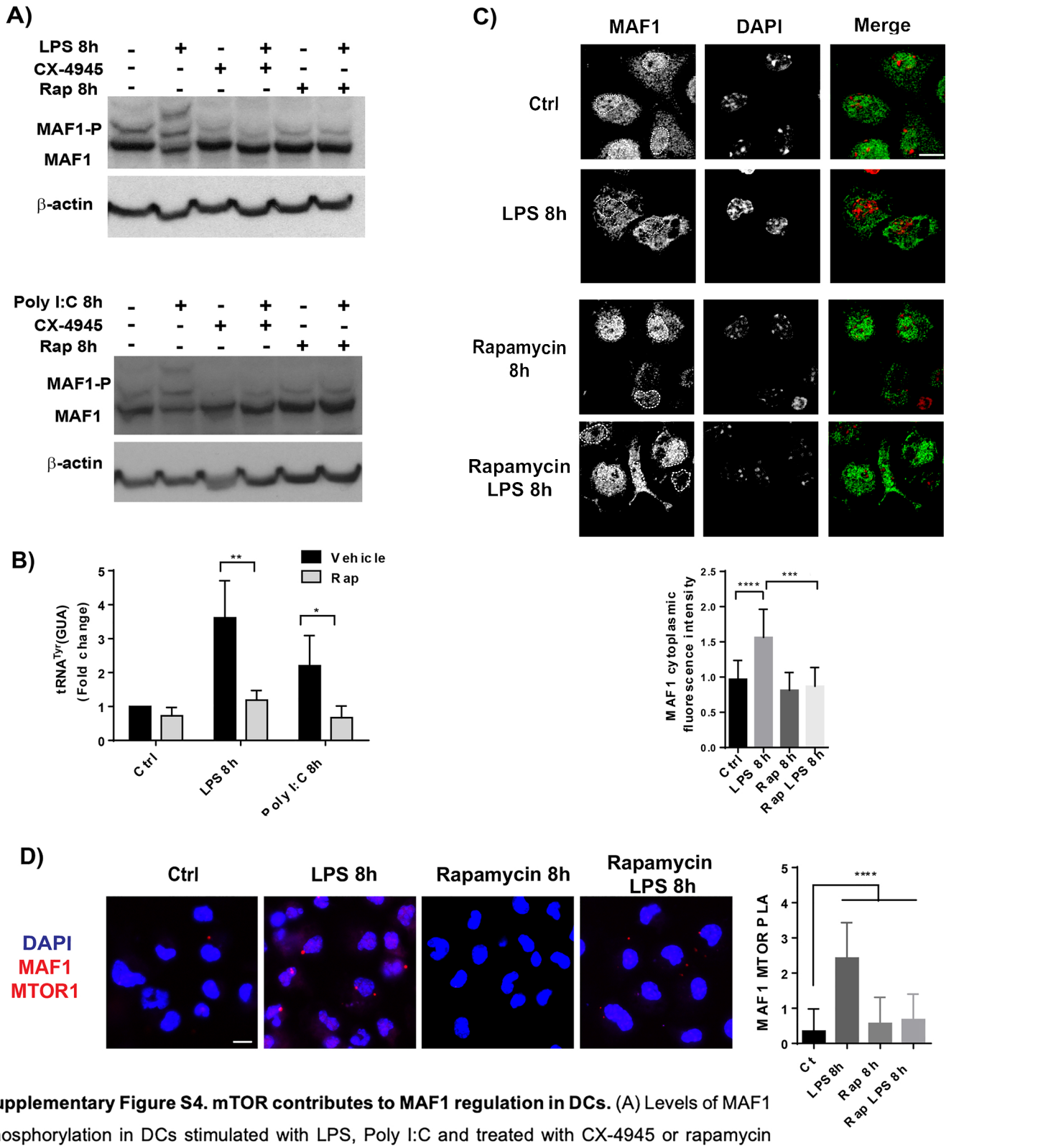
Supplementary Figure S1. DC maturation is delayed in absence of IFNAR. WT and IFNAR -/- DCs were stimulated with LPS, Poly I:C and Interferon β for 8h. Surface upregulation of co-stimulatory molecules CD86 and MHCII was measured by flow cytometry. Data are representative of three independent biological experiments.



Supplementary Figure S2. SG formation is not inhibited in presence of ISRIB. Immunofluorescence microscopy for G3BP1 and DCP1A of DCs stimulated with LPS, Poly I:C and treated with ML-60218 and ISRIB for 8h. Data is representative of three independent experiments G3BP1-positive SGs are marked with arrowheads. Scale bar, 10 μm.



Supplementary Figure S3. CK2 inhibition prevents MAF1 export from the nucleus of LPS activated DCs. Immunofluorescence microscopy for MAF1 in DCs stimulated with LPS and treated with CX-4945 for 8h. Images are representative of three independent experiments. Nuclei were counterstained with DAPI and nuclear area is marked with a dashed line. Scale bar, 10 μm. Quantification is shown as MAF1 relative fluorescence intensity in the cytosol (n>25) ****P < 0.0001.



Supplementary Figure S4. mTOR contributes to MAF1 regulation in DCs. (A) Levels of MAF1 phosphorylation in DCs stimulated with LPS, Poly I:C and treated with CX-4945 or rapamycin (rap) for 8h analysed using Phos-tag™ immunoblotting, β-actin served as loading control. Data is representative of three independent experiments with similar results. (B) Levels of tRNA^{Tyr} (GUA) measured by RT-qPCR. Data are mean ± SD (n = 3 independent samples). *P < 0.05, **P < 0.01 by unpaired student's t-test. (C) Immunofluorescence microscopy for MAF1 in DCs stimulated with LPS and treated with Rapamycin for 8h. Images are representative of three independent experiments. Nuclei were counterstained with DAPI and nuclear area is marked with a dashed line. Scale bar, 10 μm. Quantification is shown as MAF1 relative fluorescence intensity in the cytosol (n>25), ***P < 0.001, ****P < 0.0001. D) Immunofluorescence proximity ligation assay (iPLA) of DCs stimulated with LPS for indicated times and stained for MAF1 and mTOR. Confocal images are representative of three independent experiments. Quantification of the iPLA foci per cell is shown on the right of the panel. Data are mean ± SD (n = 3). ****P < 0.0001 by multiple comparison with Holm–Sidak correction.

Supplementary Table 1

Primer sequences used for RT-qPCR.

Target	**Sequence**
POLR3CFw	GGTAAGAGGAGGAGATCATCTG
POLR3CRev	GGTTGACTTGCCAATAAATCCC
Maf1Fw	TTGCCAAGCCAACCCCACACT
Maf1Rev	TGCTTGCTCATCGAGGGAGGT
IL6Fw	AGTCCTTCCTACCCCAATTTCC
IL6Rev	GTCTTGGTCTGCTGCCACTCC
TnfαFw	GATATTGTTGCGAGGGCTGCC
TnfαRev	GAGGATTGCTATCACACTATTC
Internal control	CAATTGCAGATGAGATGGATCA
RPS16Fw	ATTTGCTGGTGTGGATATTCG
RPS16Rev	CTTGGAGGCTTCATCCACATA

Supplementary Table 2

tRNAs expression levels in WT and IFNAR -/- DCs stimulated with LPS for 8h. tRNAs were quantified by tRNA-tailored microarrays. tRNAs are represented by the anticodon and cognate amino acid. Meti-CAU denotes initiator tRNA pairing to AUG start codon. Data are means of two biological replicates which were highly reproducible similar 0.76, 0.85, 0.88 and 0.87 (Kolmogorov-Smirnov test) for WT LPS 4h, WT LPS 8h, IFNAR-/- LPS 4h and IFNAR-/- 8h, respectively. Bold indicates a considerably higher expression of the tRNA in IFNAR-/- DC stimulated for 8h with LPS than in WT cells.

tRNA	WT LPS 4h	WT LPS 8h	IFNAR-/- LPS 4h	IFNAR-/- LPS 8h
Arg-ICG	1,61	0,83	0,72	1,65
Arg-C/UCG	1,56	0,80	0,67	1,61
Arg-CCU	**1,55**	**0,95**	**0,99**	**2,01**
Arg-UCU	1,67	0,88	0,83	1,50
His-GUG	1,40	1,06	1,19	0,96
Lys-CUU	1,38	0,93	0,81	0,79
Lys-UUU1	**1,44**	**0,87**	**0,92**	**3,38**
Lys-UUU2	1,33	1,04	0,81	0,73
Lys-UUU3	1,57	0,95	1,15	1,42
Asp-GUC	1,13	0,75	0,59	0,70
Glu-UUC	1,30	0,79	0,78	1,25
Glu-C/UUC	1,22	0,79	0,83	1,12
Asn-GUU	1,40	0,83	0,80	1,07
Cys-UGU/C	1,35	0,98	0,94	1,50
Gln-C/UUU	**1,30**	**1,01**	**0,99**	**2,01**
Ser-CGA	1,23	1,04	0,89	0,89
Ser-A/G/UGA	1,30	1,06	0,80	0,69
Ser-GCU	1,15	0,97	0,82	0,72
Thr-A/CGU	1,37	1,16	1,04	1,15
Thr-UGU	1,46	1,05	0,99	1,44
Thr-CGU	1,91	1,47	0,91	1,32
Ala-A/C/UGC	**1,31**	**1,10**	**0,98**	**2,76**
Gly-G/CCC	1,54	1,17	0,58	1,57
Gly-UCC	1,06	1,09	1,10	1,35
Ile-IAU	1,32	1,35	0,92	1,70
Ile-UAU	**2,13**	**1,07**	**0,94**	**3,34**
Leu-A/UAG	**1,54**	**1,05**	**0,92**	**2,31**
Leu-CAG	**1,45**	**0,94**	**0,89**	**2,14**
Leu-CAA	1,43	1,06	0,99	1,43
Leu-UAA1	1,76	0,95	0,78	1,88
Leu-UAA2	**1,26**	**0,76**	**0,94**	**3,52**
Meti-CAU	1,02	0,90	1,13	1,18
Met-CAU	1,32	1,11	0,95	1,59
Phe-GAA	1,25	1,18	0,99	1,35
Pro-A/C/UGG	1,39	0,85	0,71	0,66
Sec-UCA2	1,18	1,04	0,85	0,64
Trp-CCA	1,33	1,02	0,99	1,39
Tyr-GUA	1,13	0,89	0,78	0,71
Val-mAC	1,08	1,06	0,92	0,89
Val-UAC	1,50	1,24	0,97	1,04

Supplementary Table 3

Covariance analysis of the tRNAs expression levels in DCs stimulated with LPS for 4 and 8h. The values for WT (A) and IFNAR -/- (B) DCs stimulated for 4 and 8h were compared. tRNAs are represented by the anticodon and cognate amino acid. Meti-CAU denotes initiator tRNA pairing to AUG start codon.