EXPRESSION VECTORS

Full-length mouse NOD5 cDNA was amplified from expressed sequence tag clone (IMAGE clone ID: 30012053) by standard PCR with Pwo Superyield polymerase (Roche). PCR products were cloned into a derivative of pCR3 (Invitrogen), in frame with a C-terminal Flag tag. Retroviral pMSCV plasmid expressing NOD5 was generated by subcloning from pCR3. Packaging plasmids for pMSCV, pCG (encoding VSV G envelope glycoprotein) and pHit60 (encoding gag and pol retroviral genes), were kind gifts of C.A. Benedict (San Diego, USA).

MICE

NOD5-deficient mice were generated by Ingenius, New York. NOD5 mice were on a mix background C57BL/6-129/SvJ: all experiments were therefore performed with NOD5 WT and KO littermate mice obtained from crossing of NOD5 +/- mice. All animal procedures were conducted in compliance with Swiss federal legislation for animal experimentation.

CELLS AND CULTURE CONDITIONS

The human embryonic kidney (HEK) 293T were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% heat inactivated FCS. Wild-type and knockout mouse embryonic fibroblasts (MEFs) were generated from E14 embryos in accordance with standard procedures. MEFs were grown in DMEM supplemented with 10% heat inactivated FCS and 100 μM β-mercaptohetanol (Invitrogen). Primary MEF were used until passage 6. Primary bone marrow cells were differentiated by culture for 6 days in DMEM supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin (Invitrogen), 10%(vol/vol) fetal calf serum (PAA) and 20% (vol/vol) supernatant from L929 cells that contains the macrophage growth and differentiation factor, CSF-1.

ANTIBODIES

Antibodies used for immunoblot analysis were: NLRX1/NOD5 (17215-1-AP, Proteintech), Flag (Sigma), MAVS (ALX-804-847, Alexis), CoxIV (4844, Cell Signaling), caspase-3 (C31720, Transduction Lab), UQCRC2 (13G12AF12BB11, Mitoscience) and beta actin (ab8227, Abcam). Secondary peroxidase-conjugated antibodies were from Jackson ImmunoResearch.

ELISA

Cell culture supernatants were assayed for mouse IFN-β (PBL Interferon Source) according to manufacturer's instructions.

QUANTITATIVE PCR

Cellular RNA was extracted using RNeasy kits (Qiagen). Contaminating genomic DNA was removed using RNase-Free DNase (Qiagen), and cDNA was synthesized using M-MLV RT, RNase H(–) point mutant (Promega) and Random Primers (Invitrogen). Gene expression was quantified using the LightCycler® 480 SYBR Green I Master (Roche Diagnostics) on a LightCycler® 480 machine (Roche Diagnostics). Standard cycling was used (45 cycles of 95°C-, 60°C- and 72°C-steps of 10 seconds each). Expression was determined relative to the abundance of the housekeeping gene hypoxanthine guanine phosphoribosyl-transferase (Hprt). Data were analyzed and SD calculated using the LightCycler® 480 software release 1.5.0 SP3. Primers used were as follows (5'-3'): Hprt F: GCAGTACAGCCCCAAAATGG; Hprt R:

AACAAAGTCTGGCCTGTATCCAA; Ifnβ F: GGAGATGACGGAGAAGATGC Ifnβ R: CCCAGTGCTGGAGAAATTGT

IN VITRO STIMULATION EXPERIMENTS

Primary MEFs were stimulated with the indicated doses of poly(I:C) (tlrl-pic, Invivogen) or poly(dA:dT) poly(dT:dA) (Sigma) after complex formation with Lipofectamine 2000 (Invitrogen) (ratio of 1 μl Lipofectamine to 1 μg nucleotides) according to manufacturer's instructions. For Sendai infection, BMDMs were incubated with virus (strain Z, 20 MOIs) for 1 hr in serum-free mediun before replacement with the complete media containing 10% FCS.

IN VIVO STIMULATION EXPERIMENTS

Mice were injected intravenously with 200 μg/body of poly(I:C) (tlrl-pic, Invivogen). Sera was collected at the indicated times for ELISA measurements.

STABLE GENE EXPRESSION

For generation of stable cell populations of HEK293T cells expressing NOD5, HEK293T cells in 10 cm dishes were transfected with a mixture containing pMSCV NOD5 construct or pMSCV empty vector, in combination with pCG and pHIT60 packaging plasmids. 8 h after transfection the cells were washed and cultured O/N in the presence of 10 mM Na-butyrate (Sigma). 24 h post-transfection Na-butyrate was removed from the cells, which were cultured for another day. 48 h post-transfection cell supernatants were harvested, filtered and used for O/N infections of HEK293T cells, in the presence of 6 mg/ml polybrene (Sigma). 48 h post-infection 2.5 mg/ml puromycin (Sigma) was added onto the media for selection.

IMMUNOPRECIPITATION

mNOD5-stably expressing HEK293T cells were resuspended in Nonidet P (NP)-40 lysis buffer (1 % NP-40, 50 mM Hepes pH 7.4, 250 mM NaCl, 5 mM EDTA, 10 mM NaF, 20 mM b-glycerophosphate, 1 mM NaVO4, complete protease inhibitor cocktail (Roche)) for 15 min on ice, and lysis was completed by three cycles of freeze and thaw. Lysates were incubated for 1 h at 4°C with 20 ml sepharose6B (Sigma) on a rotating wheel for a pre-clearing step. After centrifugation, 1/10 supernatant (SN) was frozen (loaded as a cell extract control) and 9/10 SN was incubated overnight at 4 C on a rotating wheel with anti-Flag (M2) beads (Sigma). Beads were recovered and washed four times with lysis buffer before analysis by SDS-PAGE and immunoblotting.

PROTEOMIC ANALYSIS

The proteomic screen and the identification of NOD5 interaction partners were performed by the Protein Analysis Facility (PAF) of the Department of Biochemistry, University of Lausanne.