Supporting Information

Heinhuis *et al*. 10.1073/pnas.0710445105

SI Text

RNA Isolation and PCR Amplification. Immediately after cervical dislocation synovial tissue was isolated from the inflamed knee joints. The synovium samples were immediately stored in N_2 until total RNA isolation. Using the Magnalyzer system (Roche), the synovium samples were ground, and total RNA was extracted in 1 ml of TRIzol reagent (Life Technologies). Subsequently, 200 μl of chloroform and 500 μl of 2-propanol (Merck) were used to separate the RNA from DNA and proteins. Finally, after a wash step with 75% ethanol (Merck), the dry RNA was dissolved in 30 μl of water. To obtain ds cDNA, standard RT-PCR was performed using oligo dT primers. Subsequently quantitative PCR was performed using ABI/PRISM 7000 Sequence Detection System (Applied Biosystems). PCRs of GAPDH, IL-1β, IL-6, IL-10, TNF-α, COX2, Nod1, and Nod2 were performed with Sybr Green PCR Master Mix (Applied Biosystems). Quantification of the PCR signals of each sample was performed by comparing the cycle threshold values (C_t), in duplicate, of the gene of interest with the C_t values of the GAPDH housekeeping gene.

Stimulation of HEK293-Nod1-, HEK293-Nod2- Reporter Cells, and Peritoneal Macrophages by SCW Fragments. HEK293 cells were cultured in 24-well plates with DMEM (Invitrogen) supplemented with 10% FCS. Subconfluent HEK293T cells were cotransfected using Fugene6 (Roche) with 75 μg of NF-κB–luciferase reporter (Stratagene) and 3 ng of Nod1 or Nod2 expression plasmid. Expression plasmids for Nod1 and Nod2 were kindly provided by Gabriel Nuňez (University of Michigan Medical School, Ann Arbor, MI). Transfected HEK293 cells were exposed for 24 h to a dose-range of SCW fragments or a positive control (50 nM FK156 or 50 nM MDP). Luciferase activity was measured using the Luciferase Reporter-Gene Assay (Promega), as described (1). The luminescence values were normalized to total protein and expressed as fold activation relative to unstimulated, mock-transfected cells. Murine macrophages were isolated from naïve C57Black/6, Nod1-/-, and Nod2-/- mice by the lavage of the peritoneal cavity using 10 ml of cold medium (DMEM + 10% FCS). Adherent cells were harvested and cultured for 4 days before use. For cytokine production, cells were incubated with 1 μg/ml SCW fragments for 24 h. Thereafter, cytokine concentrations were determined.

Isolation of Mononuclear Cells and Stimulation of Cytokine Production. Isolation of peripheral blood mononuclear cells (PBMC) was performed as described (2), with minor modifications. 5×10^5 MNC in a 100-μl volume were added to round-bottom 96-well plates (Greiner) and incubated with either 100 μl of culture medium, or the various stimuli: MDP (10 nM), MTP (10 nM) and a dose range of streptococcal cell wall fragments (1, 5, and 25 μg/ml). The stimuli were checked for the contamination with LPS in the LAL assay and found to be negative. After 24 h, the supernatants were collected and stored at -70°C until assayed.

1. Girardin SE, *et al*. (2001) CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. *EMBO Rep* 2:736–742.
2. Endres S, Ghorbani R, Lonnemann G, van der Meer JW, Dinarello CA (1988) Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. *Clin Immunol Immunopathol* 49:424–438.

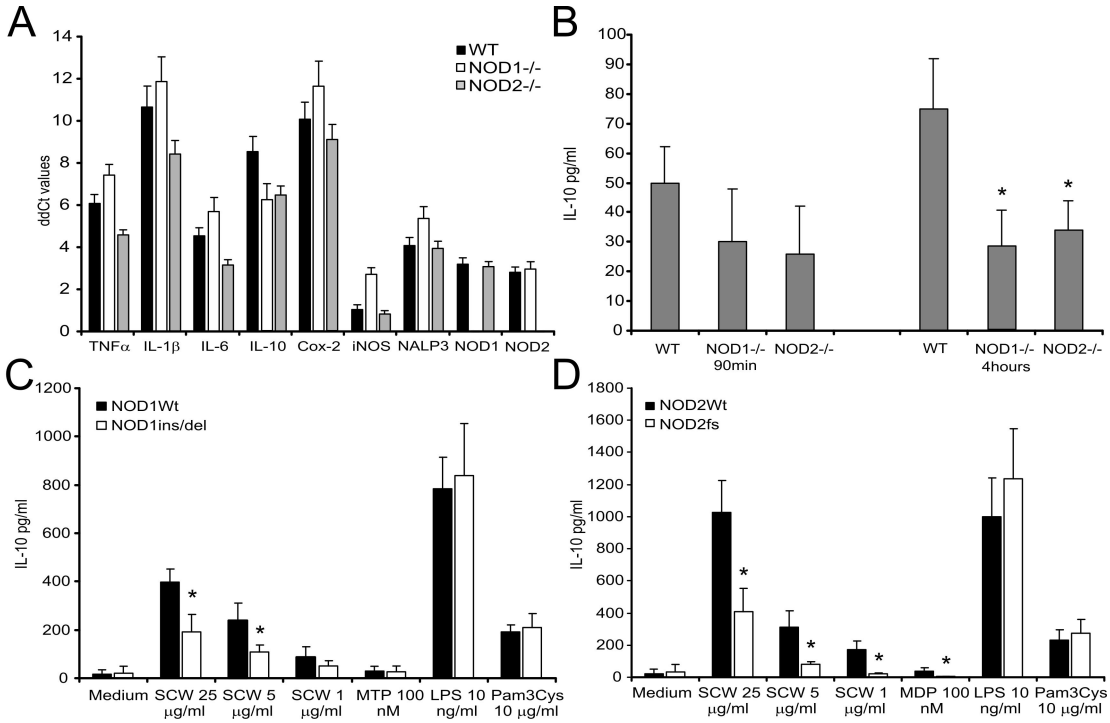

Fig. S1. Synovial mRNA expression of Nod1 and Nod2 gene-deficient mice and IL-10 production in human PBMCs carrying Nod1 or Nod2 frameshift. Synovial mRNA expression and IL-10 production in Nod1 or Nod2 gene-deficient mice (*A* and *B*) and IL-10 production in human PBMCs carrying Nod1ins/del or Nod2fs. (*A*) mRNA of synovium biopsies at day 2 of SCW-induced arthritis (quantitative PCR technology). All genes examined, except IL-10, were up-regulated in Nod1 gene-deficient and down-regulated in Nod2 gene-deficient mice. RNA was extracted from 2 × 3 knee joints per group. Data expressed as mean ± SD ddCt values, thus corrected for expression in naïve synovium and GAPDH. (*B*) Local IL-10 concentrations in wild-type, Nod1, and Nod2 gene-deficient mice at 90 min and 4 h after induction of SCW arthritis. (*C* and *D*) IL-10 production in human PBMC with either Nod1 or Nod2 mutation. Both mutations are associated with decreased IL-10 production after exposure to SCW fragments. *$P < 0.05$, Mann–Whitney *U* test, compared to wild-type control.