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Ncf1 Provides a Reactive Oxygen Species-Independent Negative Feedback Regulation of TLR9-Induced IL-12p70 in Murine Dendritic Cells[1]

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Permanent exposure to pathogens requires decisions toward tolerance or immunity as a prime task of dendritic cells. The molecular mechanisms preventing uncontrolled immune responses are not completely clear. We investigated the regulatory function of Ncf1, an organizing protein of NADPH oxidase, in the signaling cascade of Toll-like receptors. TLR9-stimulated spleen cells from both *Ncf1*-deficient and B10.Q mice with a point mutation in exon 8 of *Ncf1* exhibited increased IL-12p70 secretion compared with controls. This finding was restricted to stimulatory CpG2216 and not induced by CpG2088. Because only CpG/TLR9-induced IL-12p70 was regulated by *Ncf1*, we used TRIF$^{-/-}$ and MyD88$^{-/-}$ cells to show that TLR9/MyD88 was primarily affected. Interestingly, additional experiments revealed that spleen cells from NOX2/gp91^{phox}-deficient mice and the blocking of electron transfer by diphenylene iodonium had no influence on CpG-induced IL-12p70, confirming an NADPH oxidase-independent function of *Ncf1*. Finally, proving the in vivo relevance CpG adjuvant-guided OVA immunization resulted in a strong augmentation of IL-12p70-dependent Th1 IFN-γ response only in Ncf1-deficient mice. These data suggest for the first time an important role for *Ncf1* in the fine tuning of the TLR9/MyD88 pathway in vitro and in vivo that is independent of its role as an activator of NOX2. *The Journal of Immunology,* **2009, 182: 4183–4191.**

Autoimmune diseases such as rheumatoid arthritis are difficult to understand due to their complex genetic and environmental causes. Reactive oxygen species (ROS),[3] controlled by the NADPH oxidative complex are believed to be an important inflammatory mediator. Recent positional cloning identified *Ncf1* polymorphism as associated with autoimmune arthritis and, surprisingly, the allele mediating low oxidative burst capacity predisposed rats to more severe arthritis (1). Subsequently, a connection between a lack of ROS production by NADPH oxidase and increased autoimmunity and severity of rheumatoid arthritis in mice and rats was proposed (1, 2).

A similar effect on arthritis was demonstrated in a mouse strain with a mutation affecting splicing of the *Ncf1* gene, leading to a defect in the oxidative burst response mediated by the NADPH oxidase complex (3). The first mouse model for the human chronic granulomatous disease, which is characterized by a defective bacterial and fungal defense, was a Ncf1 knockout mouse targeted using a 129 ES cell and backcrossed to the C57BL/6 strain (4, 5). During microbial infections, activation of the multisubunit complex NADPH oxidase is an important innate function for killing invading pathogens. The activated NADPH oxidase catalyzes a reaction using coenzyme NADPH to transfer a single electron on molecular oxygen to produce superoxide anion (O^{2-}) (6, 7). In further enzymatic processes O^{2-} is used for the production of ROS supporting phagocytic reactions to defend pathogenic components. The NADPH oxidase complex consists of membrane (Cybb/gp91/NOX2, Cyba, and small G protein Rap1A) and cytosolic (Ncf4, Ncf1, Ncf2, and small G proteins Rac2 and Cdc42) components (8). The cytosolic subunit *Ncf1*/p47^{phox} is necessary for the activation of the NADPH complex, to which end it is phosphorylated by serine and threonine kinases (9–11). Phosphorylation of Ncf1 unmasks a PX domain and allows the transport of *Ncf2* and interaction with the membrane-located cytochrome b_{558}/*cybb* (8).

This study focuses on a NADPH oxidase-independent role of *Ncf1* and its potential regulatory function in TLR signaling to control the immune response. TLRs are expressed on different cell types and recognize a variety of pathogen-derived molecules.

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C.R. performed most of the experiments, collected and analyzed the data, performed statistics, designed figures, and wrote the basic manuscript. M.H.S.J. performed part of the experiments. J.W. performed part of the experiments. R.P.B. provided the p47^{phox}- and gp91^{phox}-deficient mice and critically helped with ROS measurements and with the manuscript. M.H. supported mouse experiments with *Ncf1* mutants. R.H. provided the *Ncf1* mutated mice and was greatly involved in writing the manuscript. U.K. supported experiments with the TRIF- and MyD88-deficient mice. S.A. provided the MyD88$^{-/-}$ mice initially. J.M.P. supplied basic laboratory equipment. H.H.R. had the idea, designed and closely supervised all experiments, checked all data in detail, and wrote and finalized the manuscript.

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[3] Abbreviations used in this paper: ROS, reactive oxygen species; DC, dendritic cell; DPI, diphenylene iodonium chloride; IRAK, IL-1R-associated kinase; IRF, IFN regulatory factor; Ncf, neutrophil cytosolic factor (*Ncf*, gene; Ncf, protein); pDC, plasmacytoid DC; PMA, phorbol myristate acetate; TRAF, TNFR-associated factor; TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β; WT, wild type.

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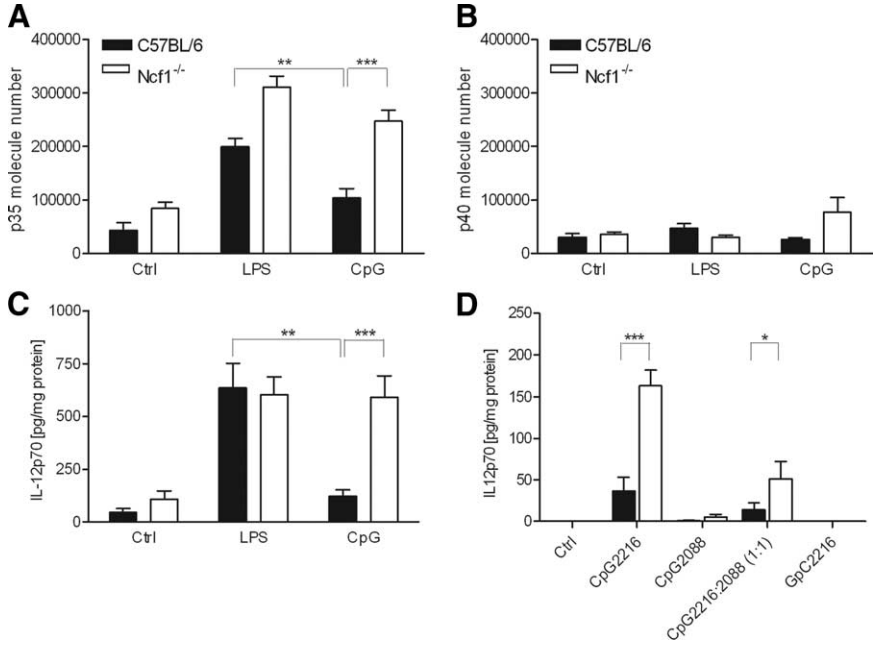

FIGURE 1. Regulation of IL-12p70 at mRNA and protein levels in spleen cells from Ncf1$^{-/-}$ and C57BL/6 (WT) mice. *A* and *B*, The mRNA expression of the IL-12p70 subunits p35 and p40 was determined with real-time PCR. *A*, Enhanced mRNA expression of the IL-12p70 subunit p35 was established after TLR9 stimulation with CpG2216 (2.5 μM) in Ncf1$^{-/-}$ cells in comparison to WT cells. In addition, in WT (C57BL/6) cells mRNA expression of p35 is decreased after TLR9 stimulation compared with TLR4 stimulation. Statistical significance was calculated by Student's *t* test ($n = 6$; ***, $p < 0.001$; **, $p < 0.01$). *B*, Expression of p40 mRNA is not significantly altered. *C*, Secretion of IL-12p70 protein, measured in duplicate from the six experiments by specific ELISA, was also increased in CpG2216 stimulated Ncf1$^{-/-}$ cells. TLR4 stimulation with LPS (1 μg/ml) revealed no significant differences between Ncf1$^{-/-}$ and WT cells. Data represent five independent experiments (***, $p < 0.001$; **, $p < 0.01$). *D*, Combination of agonistic CpG2216 (2.5 μM) with antagonistic CpG2088 (2.5 μM) resulted in diminished IL-12p70 secretion, but CpG2088 on its own had no influence on the negative feedback in WT ($n = 4$; ***, $p < 0.001$; *, $p < 0.05$) (*D*). Ctrl, Control.

Ligand binding results in the activation and dimerization of TLRs followed by conformational changes required for the recruitment of downstream signaling molecules (12, 13). In this context, our aim was to clarify the function of Ncf1 in the regulation and modulation of these signaling pathways. Recently, it was shown that IL-1R-associated kinase (IRAK) 4 is able to phosphorylate Ncf1. Interestingly, IRAK4 not only phosphorylates serine residues like protein kinase C but also threonine residues of the Ncf1 protein (14). Because IRAK4 is strongly involved in the signal cascades of TLRs, we wondered whether alternative phosphorylation of Ncf1, different from that of protein kinase C, may point to an important role for Ncf1 (15). This could have an effect not only for NADPH oxidase activation, but also on the modulation of TLR downstream signaling. Another hint toward a link between Ncf1 and the TLR pathways was suggested by Takeshita et al. as they discovered an interaction between TNFR-associated factor (TRAF) 4 and the TLR signaling molecules TRAF6, Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF), and IRAK1 (16). Results from yeast two-hybrid screening indicated that TRAF4 was able to physically form a complex with TRAF6 and Ncf1. Apart from a cell membrane and an intracellular location of TLR4 and TLR9, respectively, their downstream signaling components additionally differ regarding adaptor molecules and subsequent kinase cascades. The TLR9 signals are transmitted via a MyD88-TRAF6-dependent pathway, resulting in NF-κB activation as well as in the phosphorylation of IFN regulatory factor (IRF)-7 by IRAK1. The TLR4 signaling, in contrast, can act via the same MyD88-TRAF6 pathway but alternatively via a TRIF-mediated pathway that results in the induction of IFN-β through NF-κB and the activation of IRF-3 (16). Activation of TLR signaling cascades resulted in the induction of different cytokines, e.g., the proinflammatory IL-12p70. IL-12p70, which is critical for the differentiation of Th1 cells and the initiation of cell-mediated immune responses, consists of two subunits, p35 and p40, which are regulated differentially (17). Whereas p40 expression is regulated predominantly at the transcription level via NF-κB, AP-1, IRF-1, and other transcription factors, p35 regulation occurs at both the transcriptional and the posttranscriptional level (18, 19). Because the role of IL-12p70 in autoimmune diseases and chronic inflammation has not been completely clarified, in particular with regard to the role of Ncf1 (19–21), our main objective was to identify and characterize the regulatory mechanisms and to investigate whether Ncf1 might have additional functions than operating as a functional component of the NADPH oxidase complex. With this investigation into the proinflammatory and anti-inflammatory responses following TLR stimulation, we suggest an important regulatory function of Ncf1 within TLR signaling that is independent of NADPH oxidase activation and ROS production.

Materials and Methods

Mice and spleen cells

Wild-type (WT) C57BL/6 mice were purchased from Harlan Winkelmann; Ncf1 knockout (Ncf1$^{-/-}$) and *Cybb* knockout (gp91$^{phox-/-}$) mice, backcrossed to the C57BL/6 background, were obtained from The Jackson Laboratory and bred at the local animal facility under specific pathogen-free conditions. Ncf1$^{-/-}$ mice (produced by J. Roes, University College London, London, U.K.) for the in vivo experiments (provided by A. M. Shah, King's College London, London, U.K.) were backcrossed to C57BL/6 for 10 generations (22, 23). MyD88$^{-/-}$ mice were provided by S. Akira (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) (24) and TRIF$^{-/-}$ mice by B. Beutler (Scripps Research Institute, La Jolla, CA) (25). *Ncf1* mutated mice (*Ncf1*$^{*/*}$) with a point mutation in the splice site of exon 8 on a B10.Q background and B10.Q WT mice have previously been described (3). Whole spleens were dissected from mice, gently

homogenized with a glass tissue homogenizer, and cultured in Iscove's medium supplemented with 5% FCS (PAA Laboratories), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 100 μM nonessential amino acids, and 50 μM 2-ME in an humidified incubator (5% CO_2 and 37°C).

Positive selection of CD11c$^+$ dendritic cell (DC)

Single-cell suspensions from whole spleens were prepared by enzymatic disaggregation using collagenase D (Roche Diagnostics). Subsequently, spleen cells were magnetically labeled with CD11c micro beads (Miltenyi Biotec), incubated, and separated using a magnetic field according to the manufacturer's protocol.

FACS analyses

Original spleen cells, magnetically separated CD11c positive and negative cells, were stained for CD11c surface expression with an anti-CD11c Ab labeled with the fluorochrome allophycocyanin (Miltenyi Biotec). The cells were subsequently washed twice with PBS/FCS buffer and fixed with 1% paraformaldehyde. Expression of CD11c was detected with a FACSCalibur flow cytometer (BD Biosciences) and evaluated with CellFlow software.

Stimulation and cytokine measurement

Spleen cells were seeded at 3×10^6 cells/ml in T25 cell culture flasks with Iscove's medium without FCS and were left untreated or stimulated with 1 μg/ml LPS (Sigma-Aldrich) or 2.5 μM CpG ODN2216, CpG ODN2088, and GpC ODN2216 (InvivoGen). ROS production of cells was stimulated with 100 nM phorbol myristate acetate (PMA; Alexis Biochemicals) and inhibited using 5 μM diphenylene iodonium chloride (DPI) from Sigma-Aldrich. After an incubation time of 20 h the cells were scraped and centrifuged and the cell-free supernatants were collected and concentrated 6-fold with 10-kDa Centriprep columns (Millipore). Cytokines were measured in the supernatant. Therefore, mouse-specific ELISAs for the detection of IL-12p70 (eBioscience), IL-10 (R&D Systems), and IFN-α (PBL Biomedical Laboratories) were used. Results were standardized with standard curves and normalized to whole cell protein as measured by a bicinchoninic acid protein assay (Pierce).

RNA isolation and cDNA synthesis

Cellular mRNA was isolated with the RNeasy mini kit (Qiagen) and the concentration of RNA was determined photometrically by using an UV spectrometer (260 nm). First-strand cDNA synthesis was performed using the ThermoScript RT-PCR system (Invitrogen).

Quantitative real-time PCR

Primers (1 μM) and fluorogenic probes (1 μM; labeled with 5′ FAM and 3′ TAMRA) for murine p35 and p40 were purchased from Thermo Fisher Scientific. Real-time PCRs were performed in duplicate with 2.5 μl of cDNA and 0.5 μl of forward primer, 0.5 μl of reverse primer, 0.5 μl of probe, 8.5 μl of distilled water, and 12.5 μl of Platinum quantitative PCR SuperMix-UDG (Invitrogen) with the following cycling program: 1) activation of *Taq* polymerase for 2 min at 50° and 2 min at 95°; and 2) amplification with 45 cycles at 95° for 15 s and at 60° for 45 s. Primer and probes sequences for real-time PCR are as follows: p35 forward, 5′-CCA CCCTTGCCCTCCTAAAC-3′; p35 reverse, 5′-GTTTTTCTCTGGCCGT CTTCA-3′; p35 probe, 5′-ACCTCAGTTTGGCCAGGGTCATTCCA-3′; p40 forward, 5′-GGAAGCACGGCAGCAGAATA-3′; p40 reverse, 5′-AACTTGAGGGAGAAGTAGGAATGG-3′; and p40 probe, 5′-CATCAT CAAACCAGACCCGCCCAA-3′.

Detection of ROS

H_2O_2 release of spleen cells was measured fluorometrically with Amplex Red reagent purchased from Molecular Probes. The Amplex Red reagent in combination with HRP (Sigma-Aldrich) reacts with H_2O_2 in a 1:1 stoichiometric ratio to produce resorufin (an oxidation product). For experiments measuring ROS production, spleen cells were isolated and stimulated with LPS, CpG2216, or left untreated for a 20-h period as described above. Subsequently, 5×10^5 spleen cells were seeded in 96-well plates in a reaction buffer (200 μl/well) containing Amplex Red and HRP. ROS release of seeded cells was measured continuously from the start and, after the addition of PMA 35 min later, was performed for a total period of 75 min.

Adjuvant-guided immunization

Referring to classical protocols described for eliciting delayed-type hypersensitivity, three groups of five Ncf1$^{-/-}$ and C57BL/6 mice, respectively, were injected s.c. at the base of the tail at 7–9 wk of age. OVA (Sigma-Aldrich) alone or with ODN2216 (InvivoGen) and PBS alone as a control, respectively, were mixed with IFA (Sigma-Aldrich) at a 1:1 ratio and emulsified. Ncf1-deficient and WT mice each received injections of a 200-μl emulsion with either 50 μg of OVA per mouse, a combination of 50 μg of OVA and 50 μg of ODN2216 per mouse, or PBS in IFA, and were sacrificed after 10 days. Inguinal and axillary lymph nodes were isolated and single lymphocyte suspensions were used for ELISPOT analysis.

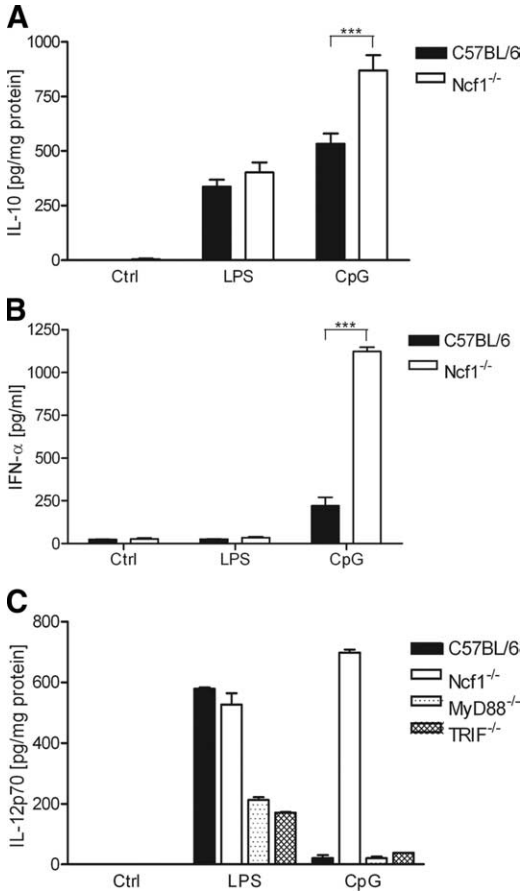

FIGURE 2. Regulation of IL-10 and IFN-α and the involvement of TRIF and MyD88. *A*, IL-10 secretion into the supernatant of spleen cells is enhanced in Ncf1$^{-/-}$ cells following TLR9 stimulation in comparison to WT, whereas no significant difference in TLR4-induced IL-10 secretion between WT and Ncf1$^{-/-}$ cells was observed. *B*, IFN-α was measured in supernatants from four independent experiments in duplicate by mouse-specific ELISA. Exclusive TLR9 stimulation with CpG2216 (2.5 μM) resulted in secretion of IFN-α. In Ncf1$^{-/-}$ cells IFN-α is significantly increased in comparison to WT cells. *A* and *B*, Statistical significance was calculated by Student's *t* test (for IL-10 $n = 7$, for IFN-α $n = 4$; *** = $p <$ 0.001). *C*, Secretion of IL-12p70 was measured in MyD88$^{-/-}$ and TRIF$^{-/-}$ spleen cells. After LPS stimulation, decreased IL-12p70 was observed in both MyD88$^{-/-}$ and TRIF$^{-/-}$ cells compared with WT (C57BL/6) and Ncf1$^{-/-}$ cells. After TLR9 stimulation with CpG2216 (2.5 μM) in Ncf1$^{-/-}$ cells, IL-12p70 was clearly increased whereas in all other cells IL-12p70 remained at low levels. One representative experiment of two is shown. Ctrl, Control.

ELISPOT assay to determine IFN-γ frequency

ELISPOT assays were performed as described (26). In brief, ELISPOT plates (Millipore) were coated with capture Ab for murine IFN-γ (BD Biosciences) overnight at 4°C. Plates were then washed and blocked with 1% cell culture-grade BSA/PBS for 1 h at room temperature. After washing, freshly isolated lymph node cells were seeded at 3×10^5/well in RPMI 1640 medium (Invitrogen) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μM nonessential amino acids, and 10% FCS.

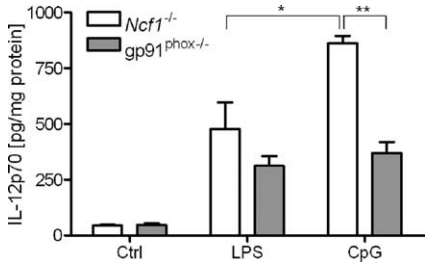

FIGURE 3. Different regulation of IL-12p70 in Ncf1$^{-/-}$ and gp91$^{phox-/-}$ cells. Both Ncf1$^{-/-}$ and gp91$^{phox-/-}$ spleen cells are characterized by a defective NADPH oxidase complex leading to a lack of O^{2-} release. IL-12p70 secretion was measured in the supernatants of Ncf1$^{-/-}$ and gp91$^{phox-/-}$ spleen cells after incubation with LPS (1 μg/ml) and CpG2216 (2.5 μM) for 20 h. In contrast to gp91$^{phox-/-}$ spleen cells, IL-12p70 secretion in Ncf1$^{-/-}$ cells was significantly enhanced after TLR9 stimulation ($n = 5$; *, $p < 0.05$; **, $p < 0.01$). Ctrl, Control.

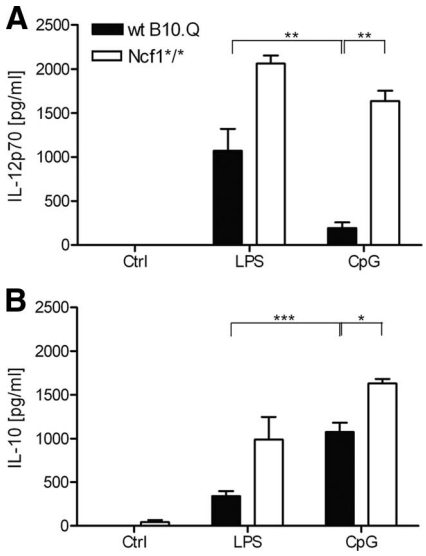

FIGURE 5. IL-12p70 and IL-10 regulation in *Ncf1*$^{*/*}$ mutant B10.Q mice. *A*, In *Ncf1*$^{*/*}$ spleen cells characterized by a single point mutation in exon 8 of *Ncf1*, the CpG2216 (2.5 μM) stimulated secretion of IL-12p70 protein was increased compared with WT cells. After TLR4 stimulation with LPS (1 μg/ml) no significant difference between *Ncf1*$^{*/*}$ and WT cells was observed. *B*, IL-10 secretion was measured in the supernatant of stimulated spleen cells with specific IL-10 ELISA. IL-10 secretion in TLR9-stimulated *Ncf1* mutated cells was significantly increased compared with WT cells. After TLR4 stimulation there is no significant difference in IL-10 secretion detectable ($n = 4$. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$). Ctrl, Control.

Cells were stimulated with or without 13.5 μg/ml OVA and cultured for 24 h at 37°C. Thereafter, cells were removed and plates were washed with PBS and PBS plus Tween 20 (PBST). The detection Ab for murine IFN-γ (BD Biosciences) was added in 1% BSA plus PBST and incubated overnight. Plates were washed with PBST and PBS before developing the colorimetric assay by the addition of 3-amino-9-ethylencarbazole and *N*,*N*-dimethylformamide in 0.1 M sodium acetate buffer (1/30). Colorimetric reaction was stopped with distilled water and air dried. ELISPOT plates were subjected to the computerized ELISPOT reader system A.EL.VIS (ELI.Scan) and enumerated with the ELI.Analyze software version 5.0 (A.EL.VIS).

Statistics

The Prism software package (Graph Pad Prism 4.03 for Windows) was used for data collection and presentation. Data from three and up to seven separate experiments are presented as mean ± SD. Where appropriate, paired and unpaired Student's *t* tests were applied to evaluate statistical significance (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

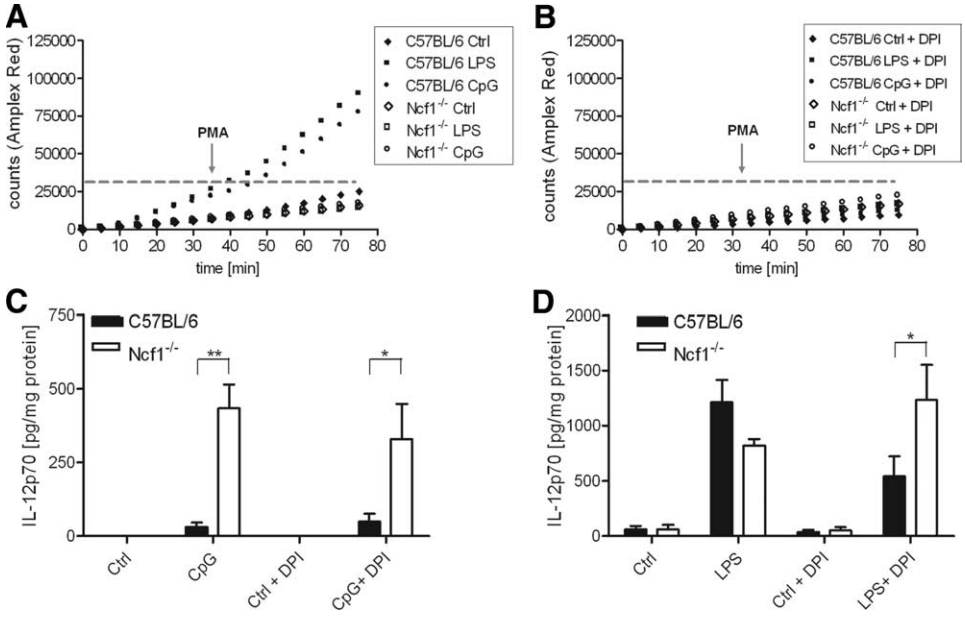

FIGURE 4. Measurement of ROS and ROS-independent regulation of IL-12p70. *A* and *B*, Increased ROS production detected via Amplex Red following PMA treatment (100 nM) was only determined in WT cells prestimulated with LPS (1 μg/ml) or CpG2216 (2.5 μM), whereas ROS production in Ncf1$^{-/-}$ cells was absent (*A*). After blocking electron transfer of NADPH oxidase by DPI (5 μM), ROS production was completely abolished (*B*). The dashed line indicates the basal production of ROS. One representative experiment of four in duplicate is shown. *C* and *D*, IL-12p70 secretion was measured in the supernatants of spleen cells after incubation with LPS (1 μg/ml) and CpG2216 (2.5 μM) or in combination with DPI (5 μM) after 20 h. Secretion of IL-12p70 protein was increased in Ncf1$^{-/-}$ cells after TLR9 stimulation alone as well as in combination with the inhibitor DPI compared with WT cells (*C*). DPI treatment resulted also in decreased levels of IL-12p70 in LPS/TLR4 stimulated WT cells. This effect of negative feedback regulation was not observable in WT cells treated with LPS (1 μg/ml) alone (*D*). Statistical significance was calculated by Student's *t* test ($n = 3$; *, $p < 0.05$; **, $p < 0.01$). Ctrl, Control.

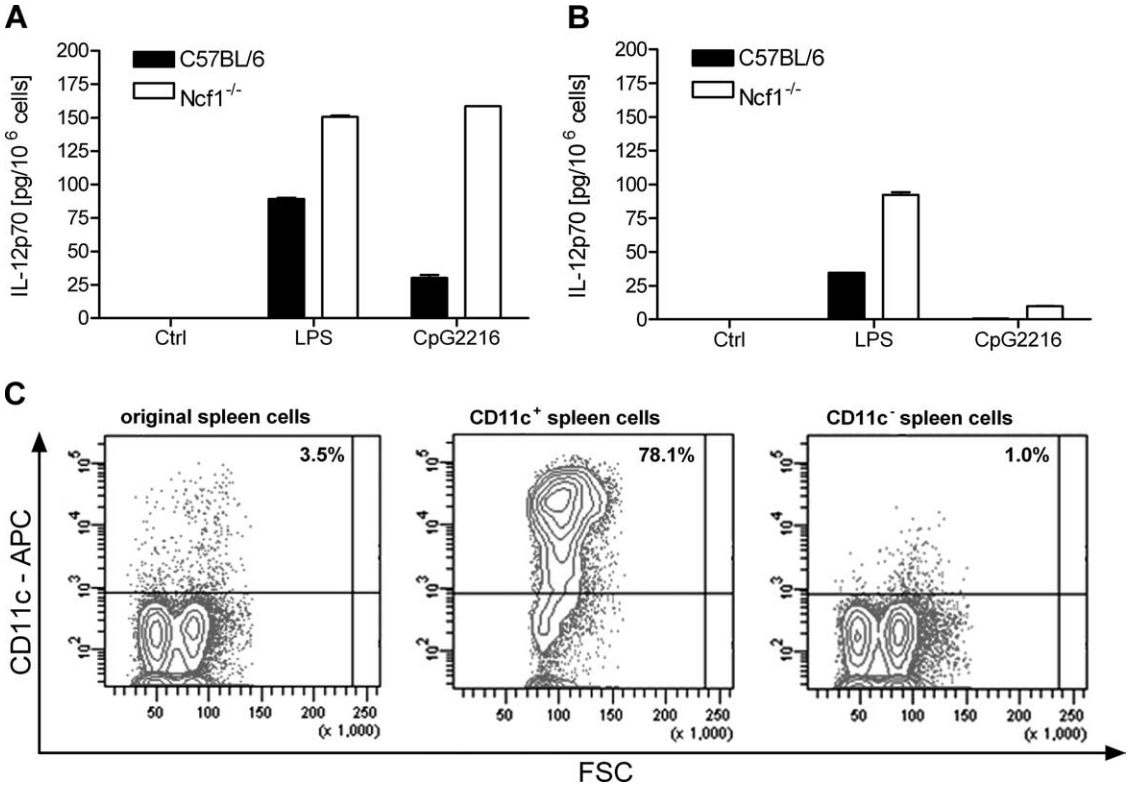

FIGURE 6. Negative feedback regulation of IL-12p70 is defective in Ncf1$^{-/-}$ CD11c$^+$ DCs. *A*, CD11c$^+$ cells were positively selected from spleen cell suspensions of Ncf1$^{-/-}$ and WT (C57BL/6) mice. CD11c$^+$ and CD11c$^-$ cell fractions were stimulated with LPS (1 μg/ml), CpG2216 (2.5 μM), or left untreated (Ctrl, Control). After 20 h, IL-12p70 secretion was measured by ELISA. In CD11c$^+$ cells of Ncf1$^{-/-}$ mice IL-12p70 was enhanced after TLR4 and TLR9 stimulation, whereas in WT CD11c$^+$ cells IL-12p70 was slightly diminished after TLR4 but clearly decreased after TLR9 stimulation. *B*, In comparison to CD11c$^+$ cells, IL-12p70 secretion of both Ncf1$^{-/-}$ and WT CD11c$^-$ cells was generally reduced and hardly detectable after TLR9 stimulation. One representative experiment of three is shown. *C*, In FACS analysis, whole spleen cells and CD11c$^+$ and CD11c$^-$ cells were characterized regarding CD11c expression. In whole spleen cells 3.5% CD11c$^+$ cells were detected, whereas in the CD11c purified fraction ~78% CD11c positive cells were found. In the CD11c negative fraction, 1% CD11c$^+$ cells are still remaining. One representative FACS analysis of two is shown.

Results

Negative feedback regulation of IL-12p70 is absent in Ncf1-deficient cells following TLR9 stimulation

Our initial investigations were targeted at the regulation of IL-12p70 in spleen cells from Ncf1 knockout and WT C57BL/6 mice. We found in Ncf1$^{-/-}$ spleen cells an enhanced mRNA expression of IL-12p35 compared with that in WT mice (Fig. 1*A*). In addition, a difference between TLR4 and TLR9 signaling was discernible, because in WT cells CpG2216 stimulation resulted in a lower expression of p35 compared with LPS (TLR4) stimulation (Fig. 1*A*). IL-12p40 mRNA expression was not significantly different between WT and Ncf1$^{-/-}$ or between TLR4 and TLR9 stimulatory conditions (Fig. 1*B*). Corresponding to mRNA data, protein data revealed enhanced TLR9-induced IL-12p70 protein secretion in Ncf1$^{-/-}$ cells compared with WT cells (Fig. 1*C*). To prove the specificity of the agonistic TLR9 ligand CpG2216 with respect to the negative feedback regulation, we performed additional experiments with antagonistic and control TLR9 ligands. Antagonistic CpG2088 and control CpG alone did not stimulate IL-12p70. Although increasing concentrations of CpG2088 added to CpG2216 resulted in reduced IL-12p70 secretion as expected, we observed no substantial change in the TLR9-induced negative feedback of IL-12p70 in WT cells (Fig. 1*D*).

Negative feedback regulation of IL-10 and IFN-α response

To follow up our hypothesis regarding Ncf1 regulation in TLR signaling, we extended our investigations toward additional immune regulatory cytokines. To this end we stimulated spleen cells of Ncf1$^{-/-}$ and WT mice with LPS and CpG2216 and measured IL-10 secretion after a 20-h incubation period. IL-10 protein levels were significantly elevated in Ncf1$^{-/-}$ cells after TLR9 stimulation compared with WT conditions (Fig. 2*A*). In contrast, a TLR4-mediated response through stimulation with LPS led to IL-10 secretion with no difference between Ncf1$^{-/-}$ and WT cells (Fig. 2*A*). To confirm the differences between TLR4 and TLR9 signaling, our interest focused also on IFN-α as an important factor in antiviral immune response. For this purpose we stimulated spleen cells of Ncf1$^{-/-}$ and WT mice with LPS and CpG2216 and measured IFN-α secretion in the supernatant. We found that IFN-α was exclusively induced after stimulation with the TLR9 ligand CpG2216, but not after LPS, in both groups of mice (Fig. 2*B*). Additionally, we observed significantly higher levels of IFN-α secretion in spleen cells of Ncf1$^{-/-}$ mice compared with WT (Fig. 2*B*).

To evaluate the relative contribution of different TLR downstream signaling adaptor modules, we performed additional experiments with MyD88$^{-/-}$ and TRIF$^{-/-}$ mice. In MyD88$^{-/-}$ and TRIF$^{-/-}$ spleen cells stimulated with the TLR4 ligand LPS, IL-12p70 secretion was markedly reduced due to lack of either the

MyD88- or the TRIF-dependent signaling pathway. When cells were stimulated with the TLR9 agonist CpG2216, IL-12p70 secretion was completely abolished in both MyD88$^{-/-}$ and TRIF$^{-/-}$ cells (Fig. 2*C*).

Negative feedback regulation of IL-12p70 by Ncf1 is independent of ROS production

To investigate whether the observed unrestrained TLR9-mediated IL-12p70 response in cells lacking *Ncf1* is independent of the lack of ROS production we performed control experiments with spleen cells from gp91$^{phox-/-}$ mice. Like Ncf1-deficient cells, functionally these cells are also unable to assemble an active NADPH oxidase. Stimulation of spleen cells from gp91$^{phox-/-}$ mice with the TLR9 ligand resulted in a decreased IL-12p70 secretion as shown in WT cells previously, whereas again Ncf1$^{-/-}$ mice secrete significantly more IL-12p70 after CpG2216 stimulation (Fig. 3).

Additional experiments to confirm a ROS-independent role of Ncf1 were performed with an inhibitor of ROS production, DPI. Spleen cells were investigated regarding their inducible ROS production by prestimulation with LPS or CpG2216. Additional samples were stimulated with PMA to induce ROS production and measured at different time points. In the process we detected higher levels of ROS in WT cells pretreated with LPS and CpG2216, whereas ROS production in Ncf1$^{-/-}$ cells, as expected, was not detectable (Fig. 4*A*). Parallel to ROS measurement, IL-12p70 secretion was determined in Ncf1$^{-/-}$ and WT cells. Although ROS formation was undetectable after DPI treatment (Fig. 4*B*) no impact on feedback regulation by Ncf1 in TLR9 signaling was observed (Fig. 4*C*). Surprisingly, after blockade of ROS production, feedback regulation is now clearly observable also in TLR4 signaling (Fig. 4*D*).

Negative feedback regulation of IL-12p70 and IL-10 in Ncf1-mutant (Ncf1/*) mice*

A problem with the Ncf1 knockout mouse is that it does not only differ by the defective *Ncf1* gene in comparison with the WT C57BL/6 mouse, but also with a large and unknown linked chromosomal fragment derived from the 129 strain. To exclude the possibility of such a linked genetic polymorphism, as well as differences in the remaining background of the poorly defined C57BL/6 strains, we used a genetically better controlled strain with a mutation in the *Ncf1* gene leading to ROS deficiency on the B10.Q background (B10.Q.*Ncf1*/** mice). To confirm our hypothesis, we investigated spleen cells of B10.Q.*Ncf1*/** and B10.Q WT mice regarding TLR4- and TLR9-induced IL-12p70 and IL-10 secretion. We indeed observed a negative feedback regulation in TLR9-induced IL-12p70 in WT cells, whereas in *Ncf1*/** cells IL-12p70 was elevated (Fig. 5*A*). In parallel, we measured IL-10 secretion and detected significantly lower levels after TLR9 stimulation in WT compared with *Ncf1* mutants (Fig. 5*B*).

Negative feedback regulation of IL-12p70 is defective in Ncf1$^{-/-}$ CD11c^{+} DCs

Because all experiments so far had been performed in whole spleen cells, we wondered which specific cell type might be responsible for the negative feedback regulation of IL-12p70. In the spleen, DCs are one of the major IL-12p70 producers. To investigate the role of DCs, we isolated CD11c positive cells from the spleen as described previously and stimulated CD11c^{+} and CD11c^{-} cells with TLR4 and TLR9 ligands. IL-12p70 was enhanced after TLR4 stimulation in both Ncf1$^{-/-}$ and WT CD11c^{+} cells (Fig. 6*A*). Stimulation with the TLR9 ligand CpG2216 only resulted in elevated IL-12p70 secretion in Ncf1$^{-/-}$ CD11c^{+} cells compared with WT CD11c^{+} cells (Fig. 6*A*). In comparison with CD11c^{+} spleen cells, CD11c^{-} cells clearly showed lower IL-12p70 secretion after TLR4 stimulation and hardly detectable IL-12p70 after TLR9 stimulation (Fig. 6*B*). In parallel, as a control we monitored the isolation and purification of spleen cells by FACS. Results show that we were able to enrich CD11c^{+} cells from 3.5% in whole spleen cell suspension to ~78% of CD11c^{+} positive cells (Fig. 6*C*).

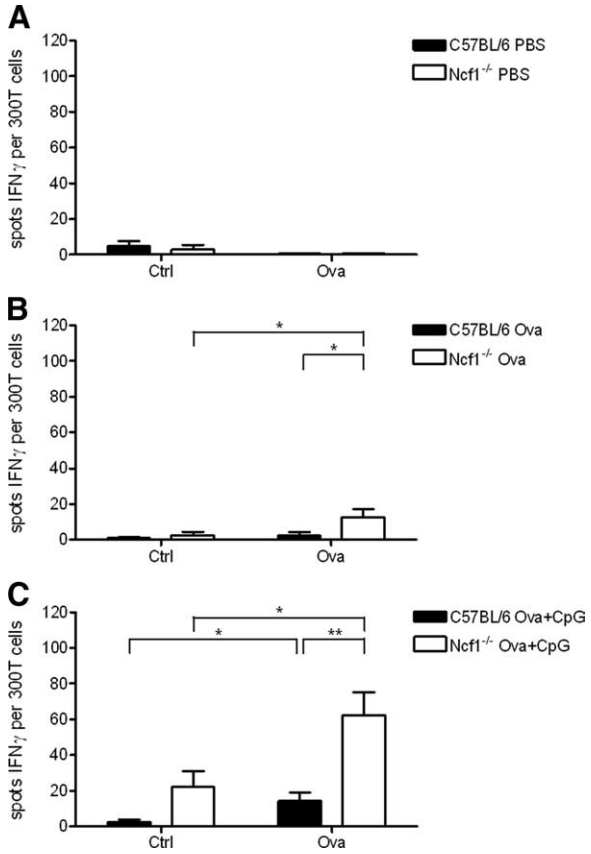

FIGURE 7. Defective negative feedback regulation of CpG2216 adjuvant-guided immunization influences Th1 response in Ncf1$^{-/-}$ mice in vivo. Ncf1$^{-/-}$ and WT (C57BL/6) mice were immunized with OVA alone (Ova) or OVA in combination with CpG2216 (Ova+CpG). Lymph node cells were isolated 10 days postimmunization and incubated in IFN-γ Ab-coated spot ELISA plates with or without OVA (13.5 μg/ml). After 24 h, plates were developed and IFN-γ specific spots were counted by A.EL. VIS. *A*, In control (Ctrl) animals (PBS), no IFN-γ is detectable. *B*, In cells from Ncf1$^{-/-}$ mice injected with OVA alone, IFN-γ spots were found upon restimulation of cells, but not in WT cells. *C*, Combined OVA and CpG2216 injection of mice resulted in an induction of IFN-γ upon OVA treatment in the ELISA spot assay. In Ncf1$^{-/-}$ cells, the rise of IFN-γ frequency was already detectable without OVA, but strongly enhanced following in vitro restimulation. Statistical significance was calculated by Student's *t* test ($n = 10$; *, $p < 0.05$; **, $p < 0.01$).

Lack of negative feedback regulation in Ncf1$^{-/-}$ mice strongly enhances CpG2216 adjuvant-guided, Ag-specific Th1 response in vivo

Reasoning that the induction of the third signal, i.e., IL-12p70 elicited by the TLR9 ligand CpG2216 in DCs, should strongly enhance a Th1-polarized, Ag-specific response in Ncf1$^{-/-}$ mice in vivo also, we performed immunization experiments closely resembling the classical delayed-type hypersensitivity-like protocols. Mice received either PBS, OVA alone, or OVA mixed with CpG2216 in IFA s.c. to activate and polarize naive T cells toward

IFN-γ producing Th1 cells. After 10 days, isolated lymph node cells from immunized Ncf1$^{-/-}$ and WT mice were incubated in a spot ELISA with or without OVA and the frequency of IFN-γ producing T cells was quantified. Lymph node cells from both mouse strains treated with PBS in vivo exhibited no IFN-γ production upon restimulation with OVA (Fig. 7*A*). In vitro recall of OVA immunization of WT and Ncf1$^{-/-}$ mice resulted in an increased IFN-γ frequency in the Ncf1$^{-/-}$ lymph node cells (Fig. 7*B*). Providing a polarization signal, in our case IL-12p70, induced by TLR9 activation with CpG2216 enhanced the frequency of IFN-γ-producing Th1 in WT mice significantly (Fig. 7*C*). However, in Ncf1$^{-/-}$ lymph node cells the IFN-γ response, already detectable without in vitro OVA restimulation, was strongly augmented upon OVA restimulation (Fig. 7*C*).

Discussion

In this study we have addressed the hypothesis that Ncf1, independently of its role for NADPH oxidase activation, is involved in the regulation of TLR signaling. Such a role may be essential for maintaining the balance during a TLR9/MyD88-dependent immune response. Spleen cells of Ncf1-deficient mice showed an increased IL-12p70 secretion after stimulation of the intracellular TLR9 with CpG2216 compared with the responses of WT cells. Consequently, we found that low secretion of IL-12p70 observed after TLR9 stimulation in WT cells is controlled by a negative feedback mechanism. This may prevent excessive proinflammatory immune responses against foreign molecular structures derived from invading pathogens. Stressing the relevance of these in vitro findings with a delayed-type hypersensitivity-like immunization protocol, we could demonstrate that the CpG/TLR9 adjuvant-guided Th1 response was indeed strongly augmented in Ncf1$^{-/-}$ mice in vivo.

It has been suggested that type A CpGs, including CpG2216, do not induce IL-12p70 in human PBMCs (27), whereas type B CpGs can also activate B cells in humans and induce the secretion of IL-12p70 in GM-CSF-differentiated bone marrow cells derived from C57BL/6 mice (28). Because it has been described for plasmacytoid DCs (pDCs) that type A CpGs are weak inducers of TLR9-dependent NF-κB signaling, resulting in IL-12p70 expression, but strong inducers of IFN-α (29–31), we wanted to show with this study that it is *Ncf1* that is involved in the down-regulation of TLR9-mediated IL-12p70 secretion in WT cells. Indeed, our data confirm that IL-12p70 secretion is clearly enhanced in Ncf1-deficient mice. To test whether only proinflammatory IL12p70 is affected by Ncf1 activity, we also investigated IL-10 secretion after stimulation with LPS and CpG2216. We found significantly lower IL-10 levels after TLR9 stimulation of WT compared with Ncf1$^{-/-}$ splenocytes, whereas after TLR4 stimulation no difference between WT and Ncf1 knockout cells was discernible. The mechanism of IL-10 regulation via different TLR pathways and the role of Ncf1 is unclear, and published data regarding a connection between IL-10 and IL-12p70 regulation and the respective cellular sources of either cytokine are manifold and partly contradictory (32–34). Our spleen cell system consists of a variety of immune cells so that the producers of IL-12p70 may differ from the IL-10-producing cells. Because the sequence of TLR9 with a subsequent IL-12p70 secretion is particularly affected by Ncf1 modulation, we suggest that pDC or another specific TLR9-positive subset of DC plays a prominent role. Indeed, in all experiments after stimulation with CpG2216, we determined there were high amounts of secreted IFN-α in Ncf1$^{-/-}$ cells and moderate levels in WT cells. Thus, the negative feedback regulation by Ncf1 of the IFN-α response seems to support our hypothesis that pDCs, which are the major producers of IFN-α, may be specifically sensitive to Ncf1 feedback also regarding IL-12p70. In addition, the type of CpG being agonistic and the respective control motifs are in line with the deduction of a prime pDC involvement (35, 36). Because we additionally detected a negative feedback regulation by Ncf1 of TLR9-induced IL-12p70 secretion in spleen cells of TRIF$^{-/-}$ mice, we suppose that the feedback is restricted to the TLR-MyD88 pathway specifically. In addition to the IFN-α data, the primary effect on TLR9 signaling strongly suggests an involvement of pDCs. In ongoing studies we are currently testing this hypothesis.

To exclude any influence of a lack of ROS production due to a nonfunctional NADPH oxidase, we compared the effect of Cybb/gp91^{phox} deficiency with *Ncf1* deficiency. These results showed that the negative effect on TLR9-mediated IL-12p70 secretion was dependent on Ncf1 rather than a functional NADPH oxidase complex. In subsequent inhibition experiments we could demonstrate that the negative feedback regulation of TLR9-mediated IL-12p70 secretion is not influenced by DPI, a flavoprotein inhibitor (37, 38), which confirms a ROS-independent mechanism. Surprisingly, in this experimental series we discovered a negative feedback regarding IL-12p70 secretion also after stimulation of LPS in combination with DPI. According to our understanding, these data suggest that the TRIF and MyD88 pathways exhibit a different sensitivity toward ROS inhibition, i.e., blocking a ROS-dependent TRIF signaling by DPI uncovered the MyD88 part of LPS/TLR4 signaling, which again is regulated by Ncf1. Given the diverse cell types of an entire spleen, not only DCs but also macrophages and neutrophils could be involved in ROS production. However there is a major difference between these cells and DCs, especially with regard to the time course and level of ROS production and the acidification in the phagosomal compartments. Whereas DCs, with their main function being to present processed peptides as Ags to naive T lymphocytes, are characterized by a continuous and sustained production of ROS, macrophages and neutrophils produce high amounts of ROS very rapidly within a few minutes ("burst") (39). In addition, the function of ROS during Ag presentation by DCs may be limited by the secretion of catalases (40). Our data demonstrating ROS production following TLR stimulation alone suggest that ROS in DCs might have a special function independent of phagocytosis. It has been shown that in DCs the NADPH oxidase is cryptic, which means that oxidase activation and ROS production, respectively, are only recovered upon TLR activation (41). With respect to our confirmed ROS-independent results regarding IL-12p70 regulation by Ncf1, we conclude that the signaling is TLR-MyD88 dependent.

It has been published that IL-12p70 production in DCs is abolished when MyD88 is absent (42, 43), but in a more recent publication it has been described that only IL-12p70 was absent, whereas intracellular IL-12p40 was still detectable (28). This finding reinforces the idea that Ncf1 influences primarily IL-12p35 expression via the TLR-MyD88 pathway and, to a lesser extent, IL-12p40 expression. Although it is not yet clarified whether IL-12p70 or its subunits p35 and p40 are the most important regulators in autoimmune diseases (20, 44, 45), we consider that the regulation of IL-12p35 and IL-12p70, respectively, by Ncf1 particularly in DCs via the TLR/MyD88 pathway influences the development of autoimmune diseases. Because mice lacking TLR9 or its adaptor molecule MyD88 are protected from experimental autoimmune encephalitis (46), it is accepted that the regulation and fine tuning of the TLR9/MyD88 pathway resulting in IL-12p70 production plays a major role in autoimmune diseases.

Finally, in our study we have proven that within the spleen the CD11c positive cells are the major carriers of the observed Ncf1-dependent feedback of TLR9-induced IL12p70. Therefore, we

suppose that the role of *Ncf1*, particularly in DCs, is crucial for the regulation of chronic inflammation and autoimmune diseases. Along these lines we have earlier shown that *Ncf1* polymorphism is associated with arthritis and that *Ncf1* deficiency leads to more severe inflammation. In fact, B10.Q *Ncf1* mutant mice developed spontaneous arthritis as well as more severe and chronic collagen-induced arthritis (3). The Ncf1-mediated mechanism for this effect, however, is not clarified, and currently several different possibilities have been suggested. First, release of ROS into endosomes may affect the processing of Ags and thereby change the priming capacity of DCs (39); second, Ncf1 has been proposed to be critical for tryptophan metabolism in DC and thereby makes them less proinflammatory (47); and third, macrophages may be more efficient Ag presenters and proinflammatory if they are deficient in ROS production (48). These mechanisms are all dependent on the ROS-inducing function of Ncf1 as a part of a NADPH oxidase complex. With our present investigation we are now suggesting an additional mechanism that is independent of oxidation, as Ncf1 may negatively regulate TLR9-mediated IL-12p70 secretion.

Although our initial short-term in vivo experiments point toward an increased Th1 response, the consequence of this type of regulation is not completely clear. If type I IFNs are activated together with an increased IL12p70 secretion, it might operate to both drive and protect against chronic inflammation (49, 50). *Ncf1* may also have different roles in different cells, because the function of ROS as protective transmitters on T cell activation operates with macrophages as APCs, whereas the mechanism suggested here is more likely to operate via plasmacytoid DCs. In fact, the molecular role of *Ncf1* may mediate complex and opposing functions. Observations in the experimental autoimmune encephalitis model may visualize such phenomena as induction of the disease with native myelin oligodendrocyte glycoprotein (MOG) as compared with the MOG peptide having completely different effects in *Ncf1*-deficient mice (3). However, the results of our initial short-term in vivo experiments clearly point toward a prominent role of a missing negative feedback regulation by Ncf1 for the development of an IL-12p70-dependent Th1 polarized response. As described before immunization with Ag in the absence of a polarizing adjuvant, i.e., in IFA only, usually leads to a Th2-type response (51). Classically, immunization with "complete Freund's adjuvant" (IFA containing *Mycobacterium tuberculosis*) or IFA with inflammatory class A CpGs and Ag will evoke a Th1 response (51, 52). Given our data in the in vivo experiment with Ncf1$^{-/-}$, we now have identified a strong regulator of the adjuvant effect, which indeed may be exploited for both the guidance of an anti-inflammatory therapy in autoimmunity or vice versa to elicit a strong antitumor response.

In conclusion, our investigation revealed that *Ncf1* may be a major regulator of the polarization of the DC response in both chronic inflammation and cancer.

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Disclosures

The authors have no financial conflict of interest.

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